ISSN 0021-9673

VOL. 587 NO. 1 NOVEMBER 29, 1991

Period.

6th FPLC Seminar
Titisee, February 27–March 1, 1991

JOURNAL OF

CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

SYMPOSIUM VOLUMES

EDITORS
E. Heftmann (Orinda, CA)
Z. Deyl (Prague)

EDITORIAL BOARD
E. Bayer (Tübingen)
S. R. Binder (Hercules, CA)
S. C. Churms (Rondebosch)
J. C. Fetzer (Richmond, CA)
E. Gelpí (Barcelona)
K. M. Gooding (Lafayette, IN)
S. Hara (Tokyo)
P. Helboe (Brønshøj)
W. Lindner (Graz)
T. M. Phillips (Washington, DC)
S. Terabe (Hyogo)
H. F. Walton (Boulder, CO)
M. Wilchek (Rehovot)

ELSEVIER

JOURNAL OF CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

Scope. The *Journal of Chromatography* publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications. The section *Biomedical Applications*, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis or alterations during medical treatment; screening and profiling of body fluids or tissues with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; drug level monitoring and pharmacokinetic studies; clinical toxicology; analytical studies in occupational medicine.

Submission of Papers. Manuscripts (in English; *four* copies are required) should be submitted to: Editorial Office of *Journal of Chromatography*, P.O. Box 681, 1000 AR Amsterdam, Netherlands, Telefax (+31-20) 5862 304, or to: The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, 1000 AR Amsterdam, Netherlands. Review articles are invited or proposed by letter to the Editors. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation. Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere. For copyright regulations, see below.

Publication. The *Journal of Chromatography* (incl. *Biomedical Applications*) has 39 volumes in 1992. The subscription prices for 1992 are:

J. Chromatogr. (incl. *Cum. Indexes, Vols. 551–600*) + *Biomed. Appl.* (Vols. 573–611):
Dfl. 7722.00 plus Dfl. 1209.00 (p.p.h.) (total *ca.* US$ 4421.25)

J. Chromatogr. (incl. *Cum. Indexes, Vols. 551–600*) only (Vols. 585–611):
Dfl. 6210.00 plus Dfl. 837.00 (p.p.h.) (total *ca.* US$ 3488.50)

Biomed. Appl. only (Vols. 573–584):
Dfl. 2760.00 plus Dfl. 372.00 (p.p.h.) (total *ca.* US$ 1550.50).

Subscription Orders. The Dutch guilder price is definitive. The US$ price is subject to exchange-rate fluctuations and is given as a guide. Subscriptions are accepted on a prepaid basis only, unless different terms have been previously agreed upon. Subscriptions orders can be entered only by calender year (Jan.–Dec.) and should be sent to Elsevier Science Publishers, Journal Department, P.O. Box 211, 1000 AE Amsterdam, Netherlands, Tel. (+31-20) 5803 642, Telefax (+31-20) 5803 598, or to your usual subscription agent. Postage and handling charges include surface delivery except to the following countries where air delivery via SAL (Surface Air Lift) mail is ensured: Argentina, Australia, Brazil, Canada, Hong Kong, India, Israel, Japan*, Malaysia, Mexico, New Zealand, Pakistan, PR China, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. *For Japan air delivery (SAL) requires 25% additional charge of the normal postage and handling charge. For all other countries airmail rates are available upon request. Claims for missing issues must be made within three months of our publication (mailing) date, otherwise such claims cannot be honoured free of charge. Back volumes of the *Journal of Chromatography* (Vols. 1–572) are available at Dfl. 208.00 (plus postage). Customers in the USA and Canada wishing information on this and other Elsevier journals, please contact Journal Information Center, Elsevier Science Publishing Co. Inc., 655 Avenue of the Americas, New York, NY 10010, USA, Tel. (+1-212) 633 3750, Telefax (+1-212) 633 3990.

Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Chromatography Abstracts, Clinical Chemistry Lookout, Current Contents/Life Sciences, Current Contents/ Physical, Chemical & Earth Sciences, Deep-Sea Research/Part B: Oceanographic Literature Review, Excerpta Medica, Index Medicus, Mass Spectrometry Bulletin, PASCAL-CNRS, Pharmaceutical Abstracts, Referativnyi Zhurnal, Research Alert, Science Citation Index and Trends in Biotechnology.

See inside back cover for Publication Schedule, Information for Authors and information on Advertisements.

© ELSEVIER SCIENCE PUBLISHERS B.V. — 1991

0021-9673/91/$03.50

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system or transmitted in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without the prior written permission of the publisher, Elsevier Science Publishers B.V., Permissions Department, P.O. Box 521, 1000 AN Amsterdam, Netherlands.

Upon acceptance of an article by the journal, the author(s) will be asked to transfer copyright of the article to the publisher. The transfer will ensure the widest possible dissemination of information.

Submission of an article for publication entails the authors' irrevocable and exclusive authorization of the publisher to collect any sums or considerations for copying or reproduction payable by third parties (as mentioned in article 17 paragraph 2 of the Dutch Copyright Act of 1912 and the Royal Decree of June 20, 1974 (S. 351) pursuant to article 16 b of the Dutch Copyright Act of 1912) and/or to act in or out of Court in connection therewith.

Special regulations for readers in the USA. This journal has been registered with the Copyright Clearance Center, Inc. Consent is given for copying of articles for personal or internal use, or for the personal use of specific clients. This consent is given on the condition that the copier pays through the Center the per-copy fee stated in the code on the first page of each article for copying beyond that permitted by Sections 107 or 108 of the US Copyright Law. The appropriate fee should be forwarded with a copy of the first page of the article to the Copyright Clearance Center, Inc., 27 Congress Street, Salem, MA 01970, USA. If no code appears in an article, the author has not given broad consent to copy and permission to copy must be obtained directly from the author. All articles published prior to 1980 may be copied for a per-copy fee of US$ 2.25, also payable through the Center. This consent does not extend to other kinds of copying, such as for general distribution, resale, advertising and promotion purposes, or for creating new collective works. Special written permission must be obtained from the publisher for such copying.

No responsibility is assumed by the Publisher for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions or ideas contained in the materials herein. Because of rapid advances in the medical sciences, the Publisher recommends that independent verification of diagnoses and drug dosages should be made.

Although all advertising material is expected to conform to ethical (medical) standards, inclusion in this publication does not constitute a guarantee or endorsement of the quality or value of such product or of the claims made of it by its manufacturer.

This issue is printed on acid-free paper.

Printed in the Netherlands

JOURNAL OF CHROMATOGRAPHY

VOL. 587 (1991)

JOURNAL of CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

SYMPOSIUM VOLUMES

EDITORS

E. HEFTMANN (Orinda, CA), Z. DEYL (Prague)

EDITORIAL BOARD

E. Bayer (Tübingen), S. R. Binder (Hercules, CA), S. C. Churms (Rondebosch), J. C. Fetzer (Richmond, CA), E. Gelpí (Barcelona), K. M. Gooding (Lafayette, IN), S. Hara (Tokyo), P. Helboe (Brønshøj), W. Linder (Graz), T. M. Phillips (Washington, DC), S. Terabe (Hyogo), H. F. Walton (Boulder, CO), M. Wilchek (Rehovot)

ELSEVIER
AMSTERDAM — LONDON — NEW YORK — TOKYO

J. Chromatogr., Vol. 587 (1991)

Titisee, ca. 1835

© ELSEVIER SCIENCE PUBLISHERS B.V. — 1991

0021-9673/91/$03.50

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system or transmitted in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without the prior written permission of the publisher, Elsevier Science Publishers B.V., Permissions Department, P.O. Box 521, 1000 AN Amsterdam, Netherlands.

Upon acceptance of an article by the journal, the author(s) will be asked to transfer copyright of the article to the publisher. The transfer will ensure the widest possible dissemination of information.

Submission of an article for publication entails the authors' irrevocable and exclusive authorization of the publisher to collect any sums or considerations for copying or reproduction payable by third parties (as mentioned in article 17 paragraph 2 of the Dutch Copyright Act of 1912 and the Royal Decree of June 20, 1974 (S. 351) pursuant to article 16 b of the Dutch Copyright Act of 1912) and/or to act in or out of Court in connection therewith.

Special regulations for readers in the USA. This journal has been registered with the Copyright Clearance Center, Inc. Consent is given for copying of articles for personal or internal use, or for the personal use of specific clients. This consent is given on the condition that the copier pays through the Center the per-copy fee stated in the code on the first page of each article for copying beyond that permitted by Sections 107 or 108 of the US Copyright Law. The appropriate fee should be forwarded with a copy of the first page of the article to the Copyright Clearance Center, Inc., 27 Congress Street, Salem, MA 01970, USA. If no code appears in an article, the author has not given broad consent to copy and permission to copy must be obtained directly from the author. All articles published prior to 1980 may be copied for a per-copy fee of US$ 2.25, also payable through the Center. This consent does not extend to other kinds of copying, such as for general distribution, resale, advertising and promotion purposes, or for creating new collective works. Special written permission must be obtained from the publisher for such copying.

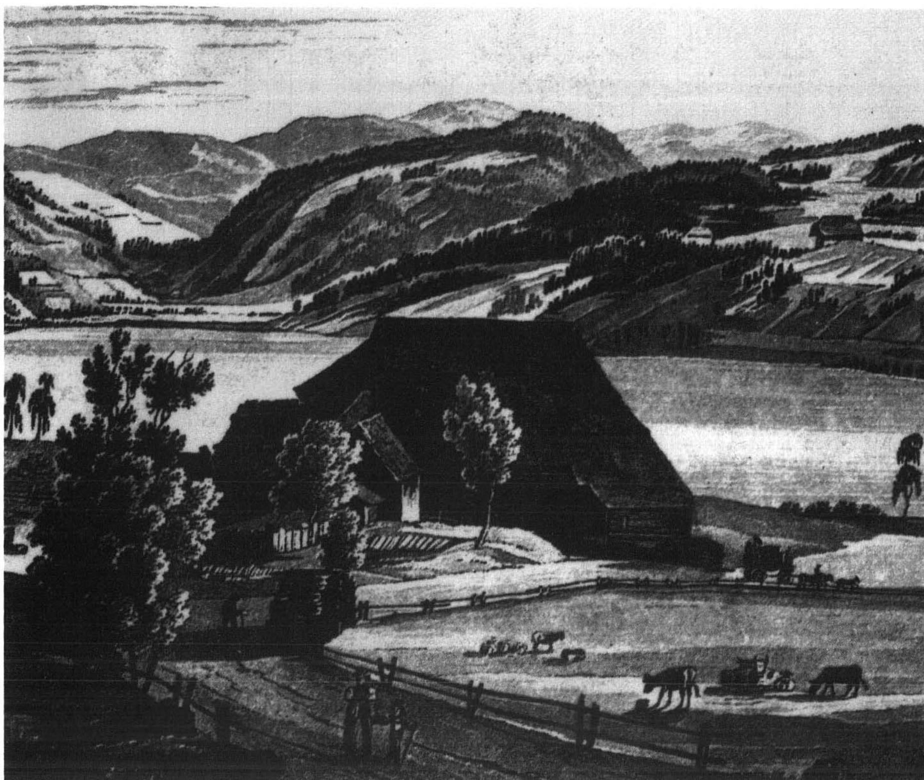
No responsibility is assumed by the Publisher for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions or ideas contained in the materials herein. Because of rapid advances in the medical sciences, the Publisher recommends that independent verification of diagnoses and drug dosages should be made.

Although all advertising material is expected to conform to ethical (medical) standards, inclusion in this publication does not constitute a guarantee or endorsement of the quality or value of such product or of the claims made of it by its manufacturer.

This issue is printed on acid-free paper.

Printed in the Netherlands

SYMPOSIUM ISSUE



SIXTH FAST PROTEIN LIQUID CHROMATORGRAPHY SEMINAR

Titisee (Germany), February 27–March 1, 1991

Guest Editors

RUDOLF DERNICK
(Hamburg, Germany)

ULRICH STAUDER
(Freiburg, Germany)

Participants of the seminar.

CONTENTS

6TH FAST PROTEIN LIQUID CHROMATOGRAPHY SEMINAR, TITISEE, FEBRUARY 27–MARCH 1, 1991

Foreword
by R. Dernick (Hamburg, Germany) and U. Stauder (Freiburg, Germany) 1

Use of anion-exchange chromatography and chromatofocusing to reveal the structural and functional heterogeneity of topoisomerase II in a HL-60 cell line resistant to multi-drug treatment
by F. Boege, F. Gieseler, H. Biersack and M. Clark (Würzburg, Germany) 3

High-resolution gel filtration of the ecdysteroid receptor–DNA complex — an alternative to the electrophoretic mobility shift assay
by A. Ożyhar and H.-H. Kiltz (Bochum, Germany) 11

Hydrophobic interaction chromatography for the purification of a mycobacterial heat shock protein of relative molecular mass 60 000
by B. Schoel and S. H. E. Kaufmann (Ulm, Germany) 19

Isolation of multiple protein factors involved in ribosomal DNA transcription
by G. Heilgenthal and I. Grummt (Heidelberg, Germany) 25

Immobilized metal affinity chromatography as a means of fractionating microsomal cytochrome P-450 isozymes
by P. H. Roos (Bochum, Germany) 33

High-performance metal chelate affinity chromatography of cytochromes P-450 using Chelating Superose
by M. Kastner and D. Neubert (Berlin, Germany) 43

Purification and characterization of transglutaminases from the genital tract of the male rat
by J. Seitz, C. Keppler and S. B. Hüntemann (Marburg, Germany) 55

Development and optimization of a single-step procedure using protein A affinity chromatography to isolate murine IgG_1 monoclonal antibodies from hybridoma supernatants
by G. Schuler and M. Reinacher (Giessen, Germany) 61

Application of reversed-phase medium-pressure liquid chromatography to the isolation, separation and amino acid analysis of two closely related peptide toxins of the cyanobacterium *Microcystis aeruginosa* strain PCC 7806
by J. Cremer and K. Henning (Munich, Germany) 71

The cytyoskeletal protein villin as a parameter for early detection of tubular damage in the human kidney
by L. B. Zimmerhackl, B. Leuk and H. Hoschützky (Freiburg, Germany) 81

Purification and crystallization of yeast hexokinase isoenzymes. Characterization of different forms by chromatofocusing
by L. Jacob, V. Beecken and L. J. Bartunik (Hamburg, Germany), M. Rose (Frankfurt/M, Germany) and H. D. Bartunik (Hamburg, Germany) 85

Purification of the coenzyme B_{12}-containing 2-methyleneglutarate mutase from *Clostridium barkeri* by high-performance liquid chromatography
by C. Michel and W. Buckel (Marburg, Germany) and D. Linder (Giessen, Germany) 93

Analysis of alanine and aspartate aminotransferase isoforms in mustard (*Sinapis alba* L.) cotyledons
by J. M. Penther (Freiburg i. Br., Germany) 101

Separation of the complete thioredoxin pattern of soybean leaves (*Glycine max*) by high-performance anion-exchange chromatography on Mono Q
by I. Häberlein (Kassel, Germany) 109

Isolation of cytochrome P-450 components from marmoset liver microsomes by high-performance liquid chromatography
by M. Kastner and D. Neubert (Berlin, Germany) 117

In articles with more than one author, the name of the author to whom correspondence should be addressed is indicated in the article heading by a 6-pointed asterisk (*)

Journal of Chromatography, 587 (1991) 1
Elsevier Science Publishers B.V., Amsterdam

Foreword

This issue of the *Journal of Chromatography* contains lectures presented at the *6th FPLC Seminar in Titisee*, held from February 27 to March 1, 1991. The FPLC Seminars in Titisee, organized by Pharmacia Biosystems, have been held since 1984 in this beautiful mountain resort of the Black Forest in the south west of Germany. Whereas the last seminar dealt especially with membrane proteins, this year's seminar was devoted to different protein groups.

The lectures presented were grouped roughly into four topics: (I) Protein purification in molecular biology; (II) Membrane proteins and glycoproteins; (III) Antibodies, peptides and special cellular proteins; and (IV) Enzymes and thioredoxin. Further lectures were devoted to proteins reacting with nucleic acids. A number of lectures were presented on purification methods at the nanogram to microgram level, whereas others described preparative separations at the miligram to gram level. Some lectures contained detailed information on separation strategies and on sophisticated automated techniques. The separated proteins were derived from a variety of organisms ranging from bacteria, through plants and animals, to men.

The trends in methodology demonstrated an increasing use of "soft gels", which have been much improved in recent years. The frequent use of high-performance chromatographic systems in many steps of the purification procedures for proteins was also observed.

The contributions were presented by experts in the field coming mainly from Germany, representing clinical and biochemical laboratories from universities and research institutes dealing with chromatographic methods for protein separation and purification. The strong orientation towards techniques and apparatus led to intense and fruitful discussions among the 150 participants of this pleasant seminar.

Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie, Universität Hamburg, Hamburg (Germany)

Rudolf Dernick

Pharmacia Biosystems GmbH, Freiburg (Germany)

Ulrich Stauder

Journal of Chromatography, 587 (1991) 3–9
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2374

Use of anion-exchange chromatography and chromatofocusing to reveal the structural and functional heterogeneity of topoisomerase II in a HL-60 cell line resistant to multi-drug treatment

Fritz Boege*, Frank Gieseler, Harald Biersack and Michael Clark

Medizinische Poliklinik der Universität Würzburg, Klinikstrasse 6–8, 8700 Würzburg (Germany)

ABSTRACT

Fractionation of nuclear extracts from a multi-drug-resistant subclone of the human promyelocytic subline HL-60 by anion-exchange chromatography and chromatofocusing resolves at least two different subtypes of topoisomerase II, which are not identical to the known α- and β-forms of the enzyme because both forms are contained in each subtype. The two subtypes are present in about equal proportions and differ remarkably with respect to the optimum of reaction and sensitivity to *m*-amsacrine and orthovanadate. Both subtypes are highly insensitive to etoposide inhibition *in vitro*.

INTRODUCTION

DNA topoisomerases can relax supercoiled DNA and their action is vital in cell division and gene transcription [1]. Topoisomerase I (Topo I) is independent of 5′-triphosphate (ATP) and acts via transient DNA single-stranded breaks, whereas topoisomerase II (Topo II) is dependent on ATP and acts via tansient DNA double-stranded breaks [2]. Two forms of Topo II with relative molecular masses of 180 000 and 170 000 have been purified from mamalian cell lines by Pharmacia fast protein liquid chromatography (FPLC). The two forms are encoded by separate genes and differ biochemically and pharmacologically [3].

A number of cytostatic agents are inhibitors of Topo II. Some of these substances, for example, anthracyclines, epipodophyllotoxines and *m*-amsacrine (mAMSA) are important compounds in therapeutic protocols used in the treatment leukaemia and malignant lymphoma. These substances inhibit Topo II after binding to the DNA. This results in DNA double-stranded breaks which cannot be repaired by the cell because of the covalently bound topoisomerase subunits ("cleavable complex"). The number of double-stranded breaks correlates to the cytostatic potency of the substances [4]. The resistance of malignant cells to these cytostatic drugs is a major problem in the treatment of these diseases. Several molecular mechanisms of cellular resistance have been described, one of which is an alteration of the drug target Topo II [5,6]. Tumour cells with this form of "atypical" (AT) resistance display a broad cross-resistance to many anti-cancer drugs that interact with Topo II, but are not altered in drug accumulation, do not over-express P-glycoprotein (Pgp), are unaffected by Pgp modulators such as verapamil, and show alterations of Topo II activity [7,8]. It has been shown in one instance that AT resistance can be associated with a point mutation in the Topo II gene [9].

In this work topoisomerases were studied in a subclone of the human promyleocytic cell line HL-60, which exhibits a typical AT resistance. Topoisomerase subtypes were separated by anion-exchange chromatography and chromatofocusing to evaluate the contribution of each subtype to the resistance pattern. This kind of analysis may be a possible route to pre-treatment screening of haematopoietic malignancies.

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V. All rights reserved

EXPERIMENTAL

Cells

Cells were grown in liquid culture medium (RPMI 1640 + 5% fetal calf serum, 1% Pen/Strep, all from Gibco) in a humidified atmosphere containing 5% carbon dioxide. The medium was changed twice weekly. Only cells in the logarithmic growth phase ($1-5 \cdot 10^6$ cells per ml) were harvested. To determine the cellular resistance to various cytostatic drugs the half-lethal concentrations (LD_{50}) of the cytotoxic substances studied were determined for each individual cell line and the factor of resistance between two indicated cell lines was expressed as the LD_{50} quotient. Cell viability was evaluated by determining the cellular trypan blue exclusion rate after growing the cell for 48 h in the presence of various concentrations of the indicated substance. This study was carried out in a subclone of the human promylelocytic cell line HL-60 resistant to multi-drug treatment (American tissue culture collection CCL240, Rockville, MD, USA), which developed by spontaneous mutation. Compared with the parent cell line, the subclone (HL-60 R) exhibited a markedly increased resistance to typical Topo II inhibitors such as etoposide (sixty-fold) and *m*-amsacrine (twenty-fold). The resistance could not be modulated by verapamil, proving an AT-type of resistance, which was expressed in a stable manner without selection and was routinely monitored by LD_{50} (etoposide) measurements.

Preparations

Isolation of nuclei. All steps were performed on ice. A total of 10^6–10^8 cells were suspended in 7.5 ml of isolation buffer (0.3 *M* sucrose, 0.5 m*M* ethyleneglycol tetraacetate (EGTA), 60 m*M* potassium chloride, 15 m*M* potassium phosphate, 15 m*M* sodium chloride, 0.15 m*M* spermine, 0.05 m*M* spermidine, 15 m*M* 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES), pH 7.5, and 14 m*M* 2-mercaptoethanol) and 0.5 ml of isolation buffer containing 40 μl of Triton X-100 (mixed at 37°C) was added. After 15 min the nuclei were sedimented (1000 *g*, 10 min, 4°C), resuspended in 100–400 μl of isolation buffer and resedimented through 1 ml of 50% sucrose (3500 U/min, 10 min, 4°C). To avoid the contamination of nuclear proteins with cytosolic proteases it was important to use only preparations which contained more than 95% intact nuclei (the integrity of the nuclear membrane was controlled by trypan blue exclusion). The overall yield (nuclei per cells) was greater than 90%, indicating that this step did not select for the mechanical stability of the nuclei.

Extraction of nuclear proteins. Nuclei were resuspended in extraction buffer (5 m*M* potassium phosphate, pH 7.5, 100 m*M* sodium chloride, 10 m*M* 2-mercaptoethanol and 0.05 m*M* phenylmethylsulphonyl fluoride) at a concentration of $3 \cdot 10^7$ per 780 μl, and 220 μl of 2 *M* sodium chloride were added dropwise to a final concentration of 0.45 *M*. After 10 min the nuclear membranes and debris were sedimented (10 000 U/min, 4°C, 10 min). The supernatant, containing high activities of topoisomerase, but also large amounts of genomic DNA fragments, was bound to a one tenth volume of hydroxyapatite (Bio-Rad Labs.). DNA fragments were removed by washing with twenty bed volumes of 500 m*M* potassium phosphate, pH 7.5, and the DNA-free topoisomerase activity was eluted with two bed volumes of 800 m*M* potassium phosphate, pH 7.8. The nuclear extracts were stable at 4°C for at least 96 h and could be stored at −70°C at least 4 weeks without loss of activity after the addition of 25% glycerol.

Anion-exchange chromatography (Mono Q). Nuclear extracts were desalted using a Pharmacia FPLC fast desalting column equilibrated with Q-buffer (20 m*M* Tris–HCl, pH 7.8, 10% glycerol, 10 m*M* β-mercaptoethanol and 0.5 m*M* EDTA) and loaded onto a Mono Q HR 5/5 column (Pharmacia) from a superloop at a flow-rate of 0.5 ml/min. The column was washed with 8–10 ml of Q-buffer and then eluted with a 20-ml linear gradient from 0 to 400 m*M* sodium chloride. Each 1-ml fraction was collected for the DNA relaxation activity assay.

Chromatofocusing (Mono P). Active fractions from Mono Q runs were diluted three-fold with Q-buffer and loaded onto a Mono P HR 5/5 column (Pharmacia) equilibrated with the same buffer at a flow-rate of 0.5 ml/min. The column was eluted isocratically with 25 ml of a mixture of 1.5% Polybuffer 96, 3.4% Polybuffer 74, pH 5.0 (hydrochloric adid), 10% glycerol, 10 m*M* β-mercaptoethanol and 0.5 m*M* EDTA from a superloop. This created a pH gradient from 8 to 5 as measured by a flow-through pH electrode (Pharmacia). Fractions (1 ml)

were collected and tested for topoisomerase activity.

Assays

Topoisomerase catalytic assays. The relaxation of 500 ng of pBR322 plasmid DNA by 4-μl aliquots of the eluate fractions was assessed in assay buffer [70 m*M* Bis–Tris propane buffer, pH adjusted with hydrochloric acid to 8.0 or as indicated, which contained 140 m*M* potassium chloride, 0.7 m*M* EDTA and 0.2 mg/ml of bovine serum albumin, (BSA). For Topo II activation, 10 m*M* magnesium chloride and 2 m*M* ATP (Boehringer Mannheim, vanadate-free) were freshly added to a final volume of 20 μl. After incubation at 37°C for 30 min, the DNA was electrophoresed overnight at 20 V in a 1% agarose submarine gel. DNA was stained for photography with ethidium bromide. The electrophoretic mobility of native twelve-fold supercoiled pBR322 was typically reduced stepwise by topoisomerase action. The activity was quantified from the disappearance of supercoiled pBR322 substrate by densitometric scanning of the photographic negatives [10] and expressed as units/μl (one unit being the amount of topoisomerase that will give a 90% relaxation of 500 ng of pBR322 plasmid DNA under these conditions). For a fast control of the assay results, 0.1 μl (25 ng of pBR322) was applied to 0.7% agarose gels (0.5 mm) cast on GelBond films (pharmacia) and electrophoresed in a PhastSystem at 100 V, 24 mA for 20 min at 13°C. Topo II specific catalytic activity was assayed by the unknotting of bacteriophage P4 DNA [11]. Briefly, 500 ng of P4 DNA (Biotechnology Centre, University of Oslo, Oslo, Norway) were incubated with 4-μl Topo II fractions in a final volume of 20 μl of assay buffer containing 10 m*M* magnesium chloride and 2 m*M* ATP (freshly added) at 37°C for 30 min. P4 DNA was electrophoresed in 0.7% agarose submarine gels (100 V, 2 h) and detected with ethidium bromide. The enzyme converts the knotted P4 DNA, which forms a smear to its unknotted closed circular form which migrates as a band.

Immunoblot analysis of Topo II. Active Mono Q eluate fractions were concentrated (Centricon, Amicon Grace, incubated with a one tenth volume of ten-fold concentrated sample buffer (1.25 *M* Tris–HCl, pH 6.8, 10% sodium dodecyl sulphate, 10 mg/ml bromophenol blue) for 1 h at room temperature and the proteins were separated in 12.5% homogeneous PhastGels (Pharmacia) and transferred to nitrocellulose sheets using a PhastSystem and standard conditions. Immunostaining of Topo II was carried out at room temperature. Nitrocellulose sheets were blocked with phosphate-buffered saline (PBS) containing 5% fat-free dried milk (Toepfer) for 1 h, washed with PBS containing 0.1% BSA (PBSA) and incubated overnight with a 1:500 dilution (PBSA) of rabbit anti-human Topo II antiserum (a kind gift of Dr. E. Schneider from Professor L. F. Liu's Laboratory), which recognizes both forms of human Topo II. After washing again with PBSA, the sheets were incubated with gold-labelled goat anti-rabbit IgG (Auroprobe, Amersham, 1:100 dilution in PBSA containing 5% gelatin) for 4 h, again washed with PBSA and finally developed with InteseBL (Amersham) silver enhancement for 30–120 min. Marker proteins (Pharmacia) included in each run contained rabbit muscle myosin, α_2-macroglobulin, β-galactosidase, transferrin and glutamic dehydrogenase and were stained with gold after electrophoretic transfer using Aurodye (Amersham).

RESULTS AND DISCUSSION

As shown in Fig. 1, the pBR322 DNA relaxation activities present in nuclear extracts of HL-60 R cells eluted from a Mono Q column over a relatively wide concentration range (50–250 m*M* sodium chloride). Three distinctive maxima of activity showing complete pBR322 relaxation (marked activity peaks 1–3) are separated by less active fractions showing only partial pBR322 relaxation (fractions 6, 7 and 9). The relaxation activity in the first peak (eluting at 50–120 m*M* sodium chloride) was independent of ATP. This is typical of Topo I. Activity peaks eluting at 170 and 200 m*M* sodium chloride showed ATP-dependent relaxation activity, typical for the type II enzyme. Titrating the activity in each peak by serial dilution gave only 3% of the total acitvity in peak 1 (independent of ATP), 53% in peak 2 and 44% in peak 3 (both dependent on ATP). The activity maxima could not clearly and reproducibly be related to UV-absorption peaks.

A further biochemical characterization of the three activity peaks is summarized in Table I. In

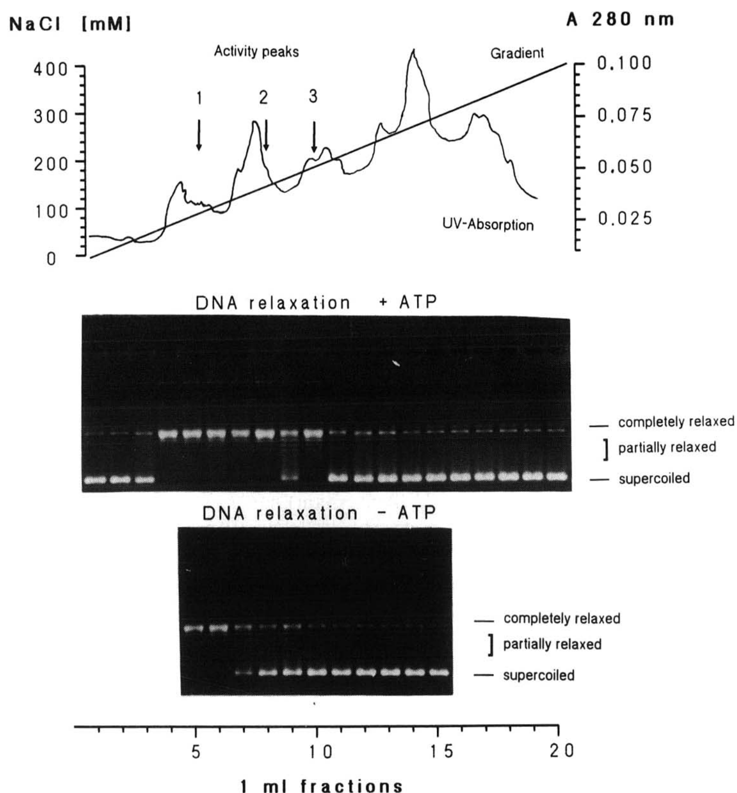


Fig. 1. Separation of topoisomerase subforms by anion-exchange chromatography using Mono Q. A 10-ml volume of nuclear extract (hydroxyapatite eluate) was desalted, loaded onto a Mono Q HR 5/5 column at pH 7.9 and eluted with a 20-ml linear gradient from 0 to 400 m*M* sodium chloride. Fractions (1 ml) were assayed for pBR322 plasmid DNA relaxation activity in the presence and absence of ATP (2 m*M*). This is one of at least five similar experiments.

Western blots a double band of relative molecular mass 170 000/180 000, typical of human Topo II [3], could be immunostained with anti-human Topo II antibodies in both peaks 2 and 3 but not in peak 1, indicating that the ATP-dependent pBR322 relaxation observed in peaks 2 and 3 was due to the presence of both α- and β-forms of the human type II enzyme. This is further confirmed by the observation that Topo II-specific P4-DNA unknotting activity [11] could only be measured in these two peaks.

Studies of the *in vitro* inhibition of pBR322 DNA relaxation by Topo II inhibitors is also summarized in Table I. Both activity peaks containing Topo II (peaks 2 and 3) and peak 1 were completely resistant to *in vitro* inhibition by etoposide (up to 100 μg/ml), which was to be expected as the cells are 100-fold resistant to etoposide. For *m*-amsacrine marked differences were found. Peak 1 (presumably Topo I) and peak 2 activities were both relatively insensitive. The IC_{50} (concentration of drug at which a 50% inhibition of enzyme activity was achieved *in vitro*) of 3 mg/ml observed most probably reflects non-specific steric inhibition of DNA binding, due to the DNA-intercalating properties of the drug. In contrast, peak 3 was inhibited at 300-fold lower doses, the IC_{50} (0.01 mg/ml) observed being in accordance with Topo II-specific inhibition [4].

Orthovanadate, an inhibitor of certain types of

TABLE I

BIOCHEMICAL CHARACTERISTICS OF HL-60-R TOPOISOMERASE ISO-ACTIVITIES

Parameter	Mono-Q fractions		
	Peak 1	Peak 2	Peak 3
Eluate concentration (m*M*)	50–100	170	200
Nuclear extract[a] (units/ml)	25	400	333
IC_{50}			
Etoposide	–	–	–
m-Amsacrine (mg/l)	3	3	0.01
Orthovanadate (μM)	–	0.2	30
Anti-Topo II immunoblot (relative molecular mass)	–	170 000/180 000	170 000/180 000
ATP/Mg^{2+} dependency	–	+	+
P4 unknotting activity	–	+	+
Iso-form	I	IIα,β	IIα,β
Optimum pH	7.9	8.3	7.9/9.0

[a] One unit is defined as the amount of topoisomerase that catalyses 90% relaxation of 500 ng of supercoiled pBR322 plasmid DNA at 37°C, pH 8.3, in 30 min.

ATPases, most notably ion-translocating enzymes such as the NA/K ATPase [12] has also been shown to be a potent inhibitor [IC_{50} 2 μM] of both forms of ATP-dependent human Topo II [3]. The dose-dependent inhibition of pBR322 relaxation activity by orthovanadate was studied in all three activity peaks of HL-60 R topoisomerase (Table I). As expected, the ATP-independent enzyme present in peak 1 was completely insensitive to orthovanadate (tested up to 100 μM), whereas the two ATP-dependent peaks differed in sensitivity: the peak 3 activity (IC_{50} 30 μM) was 150-fold more resistant to orthovanadate than the peak 2 activity (IC_{50} 0.2 μM). This is of particular interest as recent findings [7,9] suggest that changes in the Topo II ATPase activity are involved in AT resistance.

It was recently observed [13] that the distinct iso-activities of topoisomerases can be discriminated on the basis of pH profiles in nuclear extracts of wild-type HL-60 cells and that a reduced topoisomerase activity at pH 7.9 is particularly involved in etoposide cytotoxity in these cells. In this work the pH profile of fractionated topoisomerase from etoposide resistant HL-60 R cells was studied. In peak fractions 1 and 2 the pBR322 relaxation activity had a single narrow maximum at pH 7.9 and 8.3, respectively, whereas peak 3 showed two narrow and distinctive maxima at pH 7.9 and 9.0 (Table I). This finding suggested that the activity of peak 3 might contain two functionally different enzyme types. To subfractionate peak 3, high-resolution chromatofocusing was performed. As shown in Fig. 2, this technique allowed the resolution of at least three distinct activity peaks: the activity maxima in fracitons 6/7, 11/12 and 19 showed complete pBR322 relaxation and were separated by fractions of lesser activity, which only partially relaxed supercoiled pBR322. The three activity peaks were not reproducibly related to UV-absorption peaks, which are caused by major contaminant proteins.

Taken together, the data presented here suggest that 0.45 *M* sodium chloride nuclear extracts from HL-60 R cells contain mainly Topo II in addition to minor contaminations of Topo I (about 3% of the total activity). Anion-exchange chromatography allows the separation of at least two (a further resolution into three or four might be obtained by chromatofocusing) functionally different subtypes of Topo II which are not identical to the two known molecular forms α and β, as both are contained in each subtype in about equal proportions. The Topo II subtypes, which are present in about equal proportions, differ with respect to the optimum-pH of reaction and sensitivity to orthovanadate and *m*-amsacrine. Neither subtype can be inhibited by etoposide *in vitro* and therefore they most probably both contribute to the high etoposide resistance of HL-60 R cells.

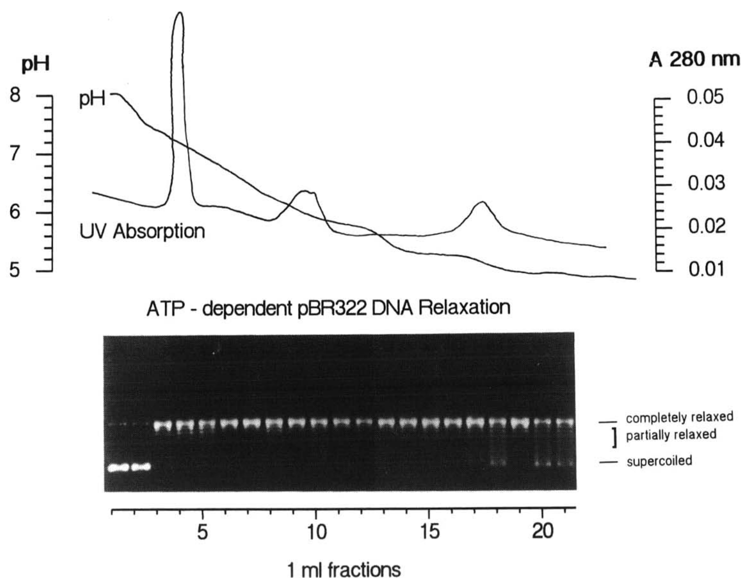


Fig. 2. Separation of topoisomerase isoactivities by chromatofocusing using Mono P. Fraction 10 from the Mono-Q run shown in Fig. 1 was diluted and loaded onto a Mono P HR 5/5 column at pH 7.9. The column was isocratically eluted with 25 ml of Polybuffer, pH 5.0 (for details, see text). Fractions (1 ml) were assayed for pBR322 plasmid DNA relaxation activity in the presence of ATP (2 m*M*). This is one of three similar experiments.

It is temping to speculate about the structural feature that causes the observed differences in function and protein net charge. Whereas isoenzymes in the classical sense can be virtually excluded on the basis of the immunoblot results, epigenetic modifications such as phosphorylation, acylation and isoprenylation are the most probable structures. Little is known about the *in vivo* regulation of Topo II, but it has been reported that purified mammalian Topo II is a substrate for protein kinase C [14,15] and that *in vitro* phosphorylation by protein kinase C alters the DNA catalytic activity in addition to the ATPase activity of the enzyme [14]. This is of particular interest as the induction of cell differentiation by dimethylsulphoxide involves both the stimulation of protein kinase C and alterations of Topo II activity and drug sensitivity [13,14,16]. It may therefore be speculated that phosphorylation–dephosphorylation could be a possible regulatory mechanism to modulate Topo II activity and as by-product to produce target protein resistance to Topo II inhibitors.

ACKNOWLEDGEMENTS

This work was supported in part by the Wilhelm-Sander Stiftung, Grant 90.038.1, to F.B. and the Deutsche Forschungsgemeinschaft, SFB 172, C9. (F.G.). The authors are indebted to Professor L. F. Liu for the gift of the rabbit anti-human topoisomerase II antiserum.

REFERENCES

1 C. A. Kafiani, I. B. Bronstein, A. V. Timofeev, I. Gromova and V. Terskikh, *Adv. Enzyme Regul.*, 25 (1986) 439–457.
2 N. Osheroff, *Pharmacol. Ther.*, 41 (1989) 1–2.
3 F. H. Drake, G. A. Hofmann, H. F. Bartus, M. R. Mattern, S. T. Crooke and C. K. Mirabelli, *Biochemistry*, 28 (1989) 8154–8160.
4 L. F. Liu, *Ann. Rev. Biochem.*, 58 (1989) 351–375.
5 L. A. Zwelling, L. Silbermann and E. Estey, *Int. J. Radiat. Oncol. Biol. Phys.*, 12 (1986) 1041–1047.
6 A. M. Deffie, J. K. Batra and G. J. Goldemberg, *Cancer Res.*, 49 (1989) 58–62.
7 W. T. Beck, M. K. Danks and D. P. Suttle, *Cancer Res. Clin. Oncol.*, 116 (1990) 1145.

8 F. Gieseler, F. Boege, H. Biersack, M. Clark and R. Erttmann, *J. Cancer Res. Clin. Oncol.*, 116 (1990) 418.
9 D. P. Suttle, B. Y. Bugg, M. K. Danks and W. T. Beck, *Proc. Am. Assoc. Cancer Res.*, 31 (1990) 358.
10 N. Osheroff, E. R. Shelton and D. L. Brutlag, *J. Biol. Chem.*, 258 (1983) 9536–9543.
11 L. F. Liu, J. L. Davis and R. Calendar, *Nucleic Acids Res.*, 9 (1981) 3979–3989.
12 B. R. Nechay, L. B. Nanninga, P. S. E. Nechay, R. L. Post, J. J. Grantham, I. G. Macara, L. F. Kubena, T. D. Philips and F. H. Nielsen, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, 45 (1986) 123–132.
13 F. Gieseler, F. Boege, B. Spohn, H. Biersack and M. Clark, *Leukemia Lymphoma*, in press.
14 N. Sahyoun, M. Wolf, J. Besterman, T.-S. Hsieh, M. Sander, H. LeVine III, K.-J. Chang and P. Cuatrecasas, *Proc. Natl. Acad. Sci. USA*, 83 (1986) 1603–1607.
15 M. Rottmann, H. C. Schröder, M. Gramzow, K. Renneisen, B. Kurelec, A. Dorn, U. Friese and W. Müller, *EMBO J.*, 6 (1987) 3939–3944.
16 F. Gieseler, F. Boege and M. Clark, *Environ Health Perspect.*, 88 (1990) 183–185.

Journal of Chromatography, 587 (1991) 11–17
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2380

High-resolution gel filtration of the ecdysteroid receptor–DNA complex —an alternative to the electrophoretic mobility shift assay

Andrzej Ożyhar* and Hans-Hermann Kiltz

Lehrstuhl für Biochemie, Fakultät Chemie, Ruhr Universität Bochum, W-4630 Bochum 1 (Germany)

ABSTRACT

The mobility shift assay is a well established method for proving binding of protein to DNA. However, this method depends on the stability of the protein–DNA complex during the electrophoretic process. Ecdysteroid receptor shows a strong tendency to aggregate under low-salt conditions of electrophoresis to a non DNA-binding form. We have developed a high-resolution gel filtration method which allows the interaction of ecdysteroid receptor with specific DNA sequences to be studied. The method seems to be generally applicable. It does not depend on the availability of a purified protein. Crude preparations could be used to characterize the stoichiometry and the molecular parameters of the complexes formed between DNA and DNA-binding proteins.

INTRODUCTION

Protein–DNA interactions are essential steps in many cellular functions. To study the binding of proteins to DNA the electrophoretic mobility shift assay is frequently used [1,2]. In this assay, protein–DNA complexes can be separated from free DNA by electrophoresis. The method depends on the ability of the electrophoretic system to resolve the reaction components and on the stability of the complexes during the separation process [2]. To obtain clearly interpretable results, highly purified protein samples are necessary for most applications. In the case of vertebrate steroid hormone receptors the method has been used very successfully to identify and characterize DNA sequences which specifically bind the hormone receptor [3] and are called hormone-responsive elements (HREs).

20-Hydroxy-ecdysone, found in invertebrates, is involved in the regulation of moults and the pupation of insects [4]. Owing to the lack of purified ecdysteroid receptor (EcdR) it was not yet possible to define clearly DNA sequences responsible for specific binding of EcdR (ecdysteroid responsive elements; EcdREs) [5,6]. In addition, EcdR aggregates under the low-salt condition of electrophoresis to a high-molecular-mass form which disturbs the mobility shift assay [7]. Gel filtration on Superose 6HR offers and alternative as the elution buffer can be freely chosen independent of the reaction conditions.

Based on the advantages of high-resolution gel chromatography, we have developed a method with which the specific interaction between EcdR and EcdREs can be studied in crude extracts. The method allows the characterization in terms of stoichiometry and molecular parameters of the protein–DNA complex and can be applied to other DNA binding proteins.

EXPERIMENTAL

Preparation of nuclear extracts and labelling with [^{3}H]ponasterone A

Nuclear extracts were prepared from embryos of an Oregon R stock of *Drosophila melanogaster* as described previously [8]. [^{3}H]Ponasterone A ([^{3}H]PonA) labelling of nuclear extract was routinely done by 30-min incubation with 40 n*M* [^{3}H]PonA at 25°C. The [^{3}H]PonA–EcdR concentration was determined using PD 10 column chromatography [8].

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V. All rights reserved

Oligonucleotides

Double-stranded (ds) oligonucleotides were obtained by annealing of appropriate strands synthesized with a Gene Assembler (Pharmacia) and purified as recommended by the manufacturer. Oligonucleotide concentrations were determined spectrophotometrically assuming 37 μg of DNA per A_{260} unit for single-stranded oligonucleotides and 50 μg for double-stranded oligonucleotides [9]. The hsp27 ds-oligonucleotide (see Fig. 1), which according to Riddihough and Pelham [11] should contain an EcdRE, was ^{32}P labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase [13] and unincorporated [γ-^{32}P]ATP was removed using an NAP10 column (Pharmacia).

Gel filtration of [^{3}H]PonA–EcdR complexes

The nuclear extract was labelled with [^{3}H]PonA as described above. Excess of [^{3}H]PonA was removed using PD10 columns (Pharmacia) equilibrated with buffer A [20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)–KOH, 100 m*M* KCl–1 m*M* dithiothreitol–2 m*M* KH_2PO_4–1 m*M* EDTA, 10% (v/v) glycerol, pH 7.6 at 20°C]. Samples of 0.5 ml containing 0.42 ± 0.05 pmol of [^{3}H]PonA–EcdR were incubated for 30 min with 0.0–25 μg of poly(dI–dC) and subsequently for 30 min with 0.0–0.9 pmol of ^{32}P-labelled hsp27 ds-oligonucleotide on ice as indicated in the legends.

Gel filtration of the samples was carried out in a Pharmacia FPLC apparatus equipped with a Superose 6HR column equilibrated with buffer A. The flow-rate was 0.15 ml/min and 0.25-ml fractions were collected. In each fraction ^{3}H and ^{32}P radioactivities were determined as described below. Except where stated otherwise, the column was washed after each run with 3 *M* KCl solution, re-equilibrated with buffer A and coated by chromatography of 500 μl of a sample containing coating proteins as described in Fig. 2.

The column was calibrated with bovine thyroglobulin (Sigma) (Stokes radius, R_s = 8.6 nm; molecular mass, m = 670 000 dalton), bovine serum albumin (Sigma) (R_s = 3.6 nm, m = 67 000 dalton), ovalbumin (Sigma) (R_s = 2.9 nm, m = 45 000 dalton) and myoglobin (Serva) (R_s = 2.0 nm, m = 17 800 dalton). The void volume was determined by filtration of dextran blue (Pharmacia).

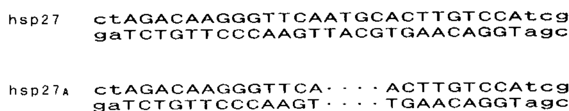


Fig. 1. Structure of the synthetic ds-oligonucleotides used to study the specific interaction of [^{3}H]PonA-labelled EcdR with DNA. hsp27 represents a DNA fragment derived from the upstream region of the hsp27 gene [10], which was sufficient to confer 20-hydroxyecdysone inducibility on a heterologous gene [11]. Small letters represent nucleotides added to the original hsp27 sequence for cloning purposes. hsp27$_A$ represents the sequence in which four bases from the hsp27 central palindrome were deleted [12].

Radioactivity measurement

Samples were mixed with 7 ml of Quickszint 2000 (Zinsser). Radioactivity was measured in a LKB liquid scintillation spectrometer with an efficiency of 40% for ^{3}H and 100% for ^{32}P. Both radioactivities were corrected for ^{32}P spillover and for radioactive decay of the labelled DNA assuming a ^{32}P half-life of 14.3 days.

RESULTS

Pretreatment of the chromatographic system

Handling of picomolar amounts of proteins or nucleic acids in chromatographic systems is sometimes difficult. Wall and resin surface effects can adversely affect the elution behaviour of the solutes. Also, low recoveries are not unusual. Such problems can only be overcome if the status of the column is carefully controlled. After each experiment the column has to be cleaned and active adsorptive centres must be covered with substances that show a minimum interaction with the chromatographic substrates. Such pretreatment of the column is a prerequisite for reproducible elution results.

The conditioning of the Superose 6HR column was started in all instances by applying 2 ml of 3 *M* NaCl solution and washing with one column volume of buffer A. To find the optimum conditions, a constant amount of preformed [^{3}H]PonA–EcdR–[^{32}P]hsp27 ds-oligonucleotide complexes were chromatographed (Fig. 2). Free [^{3}H]PonA eluted behind the column volume (V_t), indicating an interaction of the hormone with the gel matrix. Unbound [^{32}P]hsp27 ds-oligonucleotide appeared around

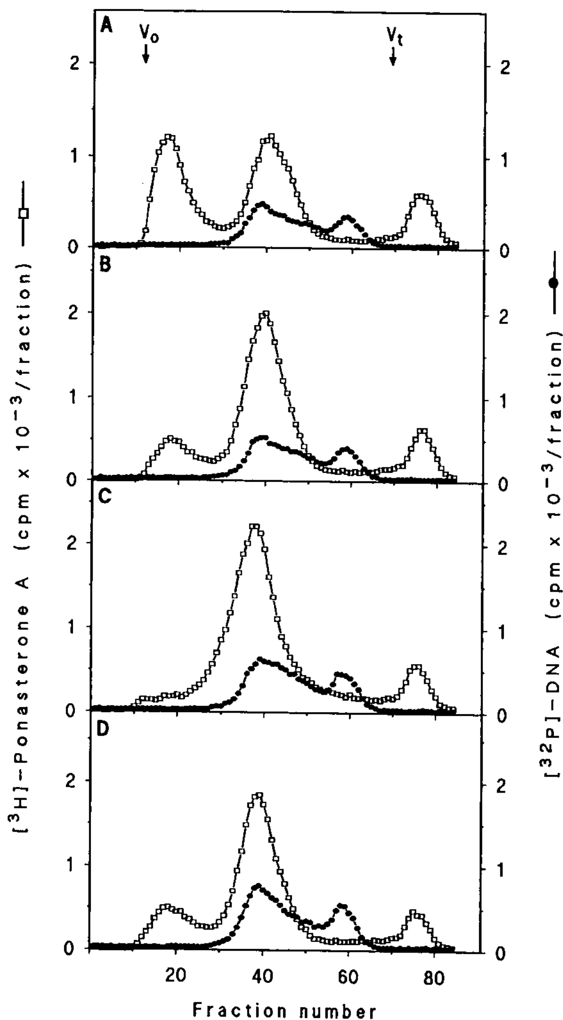


Fig. 2. Gel filtration analysis of specific [^{3}H]PonA–EcdR complex with ^{32}P-labelled hsp27 DNA. Effect of primary treatment of the Superose 6HR column. [^{3}H]PonA–EcdR was separated from excess of [^{3}H]PonA as described under Experimental. Samples of 0.5 ml containing *ca.* 0.42-pmol of [^{3}H]PonA–EcdR were incubated for 30 min with 0.25 μg of poly(dI–dC), followed by a 30-min incubation with 0.09 pmol of [^{32}P]hsp27 DNA. EcdR–DNA complexes were analysed on the Superose 6HR column which was prepared as follows: (A) the column was washed with 3 *M* KCl and equilibrated with buffer A; (B) the column was washed with 3 *M* KCl and after equilibration with buffer A coated with 2 mg of ovalbumin at a flow-rate of 0.15 ml/min; (C) the column prepared as described in (B) was used for chromatography of a nuclear extract sample containing [^{3}H]PonA–EcdR–DNA complexes and without additional preparation used for chromatography of a new sample; (D) the column was washed with 3 *M* KCl, equilibrated with buffer A and coated by chromatography of a 500-μl probe containing 5 mg of ovalbumin and 5 mg of γ-globulin; the flow-rate was 0.06 ml/min. V_0 = void volume of the column; V_t = total volume of the column.

fraction 59. Both elution positions were independent of the pretreatment of the column.

After only cleaning the column with NaCl the [^{3}H]PonA–EcdR eluted around fraction 41 (Fig. 2A). The peaks of the [^{3}H]PonA–EcdR and of the [^{32}P]hsp27 ds-oligonucleotide did not match and both peaks were strongly tailing, which may suggest an interaction between the constituents of the just-formed EcdR–DNA complexes and the column matrix. High-molecular-mass aggregates of [^{3}H]-PonA–EcdR which did not bind [^{32}P]hsp27 ds-oligonucleotide eluted just behind the void volume.

When the column was coated with a solution containing 2 mg of ovalbumin using a flow-rate of 0.15 ml/min (Fig. 2B), the yield of the [^{3}H]PonA–EcdR was drastically increased. However, the [^{3}H]PonA–EcdR and the [^{32}P]hsp27 peaks were still broad and tailing. The column was then used without a further 3 *M* NaCl wash, which means *de facto* a pretreatment with nuclear extract (Fig. 2C). Again, the yield of [^{3}H]PonA–EcdR was increased and the high-molecular-mass material brought a minimum, but now the peak of the protein-bound [^{32}P]hsp27 ds-nucleotide was strongly tailing and was significantly retarded.

It is known in the gel chromatography of large biomolecules that decreasing the column flow frequently results in better resolution. However, at the same time the yield of the chromatographic substrate is sometimes drastically decreased. This effect was used for a more efficient coating of the Superose 6HR column. During pretreatment with a solution of 5 mg of ovalbumin and 5 mg of γ-globulin the flow-rate was decreased to 0.06 ml/min (Fig. 2D), allowing the coating run to be finished overnight. Only under these conditions could good chromatographic yield of [^{3}H]PonA–EcdR and [^{32}P]hsp27 ds-oligonucleotide of over 80% be achieved and the [^{3}H]PonA–EcdR peak had symmetrical appearance. For all further experiments the column was conditioned according to Fig. 2D.

Effect of general competitor DNA

In these experiments crude nuclear extracts which contain many DNA-binding proteins in addition to the EcdR were used [12]. In the electrophoretic mobility shift assay, poly(dI–dC) is commonly used as a competitor to suppress the binding of such proteins [1,2]. The optimum amount of competitor

DNA had to be determined by titrating [^{3}H]PonA–EcdR–[^{32}P]hsp27 ds-oligonucleotide complexes with poly(dI–dC). [^{3}H]PonA–EcdR–[^{32}P]hsp27 ds-oligonucleotide complexes were chromatographed in the presence of 0, 0.25, 2.5 and 25 μg of poly(dI–dC) (Fig. 3). With 25 μg most of the [^{3}H]PonA–EcdR co-elutes with the poly(dI–dC) in the void volume of the column (Fig. 3D). Obviously in the presence of such a large excess of competitor the [^{3}H]PonA–EcdR binds to poly(dI–dC). With 0.25 μg of poly(dI–dC) the [^{32}P]hsp27 ds-oligonucleotide peak co-eluting with the [^{3}H]PonA–EcdR is still very asymmetric (Fig. 3B). This indicates that other nuclear proteins still bind to the [^{32}P]hsp27 ds-oligonucleotide and that such complexes are eluting in the region between peaks 2 and 3. A 2.5-μg amount seems to be optimum, resulting in symmetrical peaks of both the [^{3}H]PonA–EcdR and the [^{32}P]hsp27 ds-oligonucleotide.

All further experiments were done in the presence of 2.5 μg of poly(dI–dC).

Can DNA binding of EcdR be saturated?

To answer this question, increasing amounts of [^{32}P]hsp27 ds-oligonucleotide were added to the nuclear extracts containing EcdR. Molar ratios of [^{32}P]hsp27 ds-oligonucleotide to [^{3}H]PonA–EcdR of 0.20:1, 0.74:1, 1.5:1 and 2.1:1 were used. The elution diagrams are shown in Fig. 4. For information purposes the A_{280} profile is also given in Fig. 4A. This shows where the main protein material of the nuclear extract is eluted. DNA-binding protein(s) eluting under peak 3 in Fig. 4A–D cannot be saturated within the limits of [^{32}P]hsp27 ds-oligonucleotide applied. Integration of the ^{32}P peak 3 yields a linear dependence on the amount of [^{32}P]hsp27 ds-oligonucleotide added. In contrast, the [^{3}H]PonA–EcdR peak 2 is clearly saturable with [^{32}P]hsp27 ds-oligonucleotide. By integration of the ^{32}P and ^{3}H radioactivities, a maximum binding at a molar ratio of [^{32}P]hsp27 ds-oligonucleotide to [^{3}H]PonA–EcdR of 1:2 can be calculated [12].

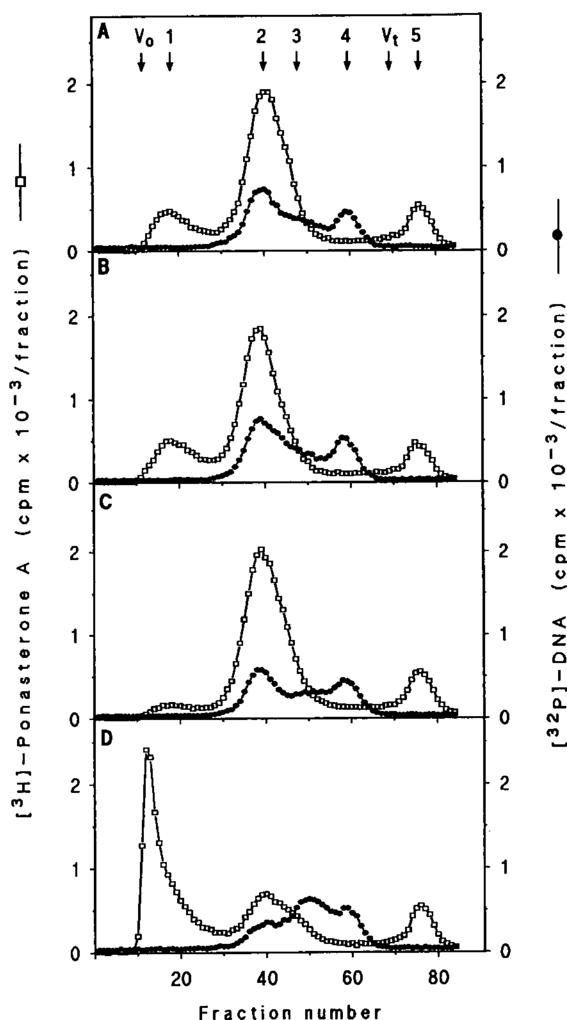


Fig. 3. Effect of poly(dI–dC) concentration of specific complex formation between [^{3}H]PonA–EcdR and ^{32}P-labelled hsp27 DNA. Samples of 0.5 ml containing *ca.* 0.42 pmol of [^{3}H]PonA–EcdR were incubated for 30 min wiht (A) 0, (B) 0,25, (C) 2.5 or (D) 25 μg of poly(dI–dC), followed by incubation with 0.09 pmol of [^{32}P]hsp27 DNA. EcdR–DNA complexes were analysed with the Superose 6HR column prepared as in Fig. 2D. Peak 1 corresponds to a high-molecular-mass form of [^{3}H]PonA–EcdR, 2 to the [^{3}H]PonA–EcdR–[^{32}P]hsp27 complexes, 3 to unspecific protein–[^{32}P]hsp27 DNA complexes, 4 to unbound [^{32}P]hsp27 DNA and 5 to unbound [^{3}H]PonA. Other symbols as in Fig. 2.

Molecular mass determination

Free [^{3}H]PonA–EcdR elutes as a broad peak tailing to the high-molecular-mass side (Fig. 5). From the peak maximum a molecular mass of 130 000 dalton can be calculated, as indicated in the inset in Fig. 5. This is in excellent agreement with earlier reports where the determination was based on sodium dodecyl sulphate–polyacrylamide gel electrophoresis [14]. In the presence of poly(dI–dC), the peak becomes more symmetrical and seems to be very slightly shifted to higher molecular mass. This

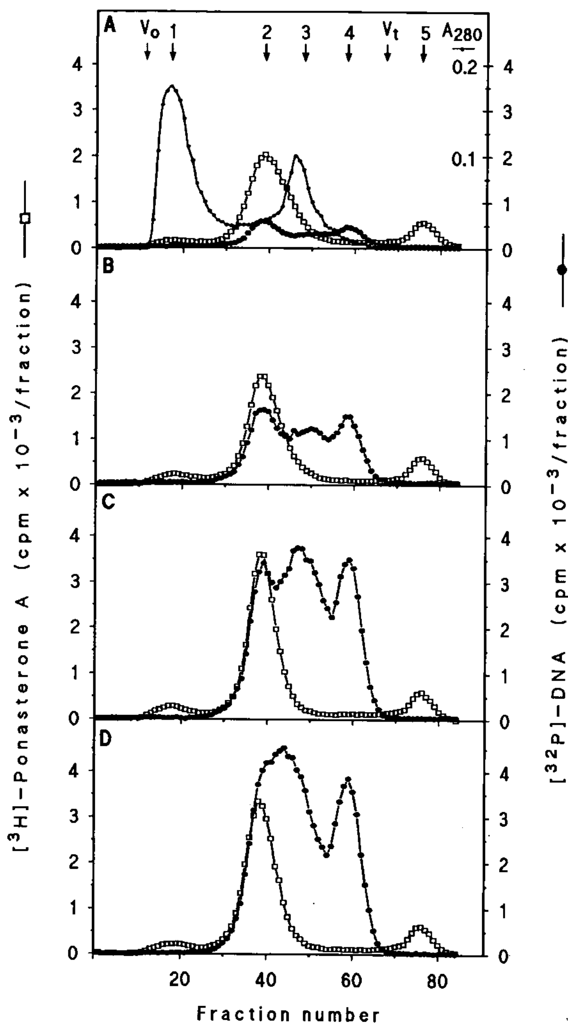


Fig. 4. Saturation of EcdR with hsp27 DNA. Samples of 0.5 ml containing *ca.* 0.42 pmol of [^{3}H]PonA–EcdR were incubated with 2.5 μg of poly(dI–dC) and then with (A) 0.09, (B) 0.31, (C) 0.61 or (D) 0.90 pmol of [^{32}P]hsp27 ds-oligonucleotide. Samples were analysed with the Superose 6HR column prepared as in Fig. 2D. Symbols as in Fig. 3.

indicates that under the influence of DNA, in this instance poly(dI–dC), the EcdR has some increased tendency to aggregate. During saturation with an excess of [^{32}P]hsp27 ds-oligonucleotide the appearance of the [^{30}H]PonA–EcdR is dramatically changed to a sharp peak. At the same time, the peak is shifted and the maximum now appears at a molecular mass of 290 000 dalton, demonstrating a

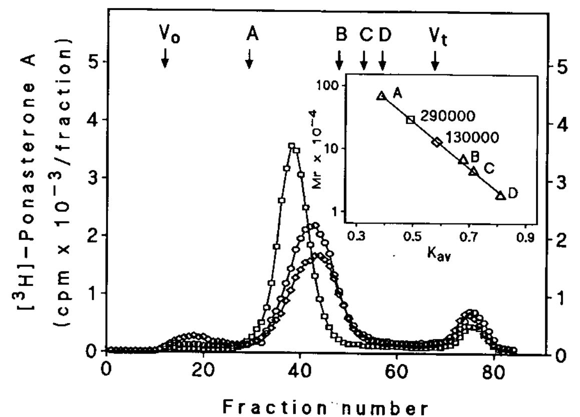


Fig. 5. Changes in the apparent molecular mass of [^{3}H]PonA–EcdR complexes on interaction with DNA. [^{3}H]PonA–EcdR complexes (0.42 pmol) were chromatographed on the Superose 6HR column (◇) after 60-min incubation, (○) after 60-min incubation with 2.5 μg of poly(dI–dC) or (□) after 30-min incubation with 2.5 μg of poly(dI–dC) followed by 30-min incubation with 0.90 pmol of hsp27 DNA. Arrows mark the elution of standard proteins (see Experimental). The column was prepared as in Fig. 2D. Inset: distribution coefficients (K_{av}) were calculated from elution volumes (V_e) according to Laurent and Killander [15]. Apparent molecular mass values for [^{3}H]PonA–EcdR (130 000 ± 15 000 dalton) and for [^{3}H]PonA–EcdR saturated with hsp27 ds-oligonucleotide (290 000 ± 30 000 dalton) were calculated by reference to a calibration graph.

complex of two molecules of EcdR and one molecule of hsp27 ds-oligonucleotide [12].

Competition by specific DNA

Assuming specific binding of the [^{32}P]hsp27 ds-oligonucleotide to the [^{3}H]PonA–EcdR, competition is provided by an excess of non-radioactive ds-oligonucleotide. This experiment is shown in Fig. 6A. The conditions are as in Fig. 4B except that a 6.7–fold excess of non-radioactive over [^{32}P]hsp27 ds-oligonucleotide was used. This excess is sufficient to move the main ^{32}P material away from the [^{3}H]PonA–EcdR peak. The [^{3}H]PonA–EcdR peak shows the typical sharp appearance of the nucleotide-saturated EcdR (see Fig. 5).

Such competition experiments can also be used to determine the binding of modified ds-oligonucleotides to the EcdR. An example is demonstrated in Fig. 6B. Again the conditions as in Fig. 4B were used. To preformed [^{3}H]PonA–EcdR–[^{32}P]hsp27 ds-oligonucleotide complexes a 6.7-fold excess of hsp27A ds-oligonucleotide (see Fig. 1) was added.

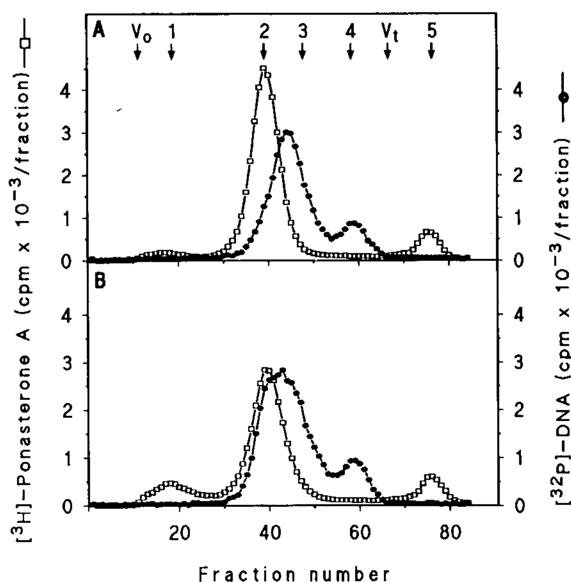


Fig. 6. Gel filtration analysis of the specificity of interaction between [^{3}H]PonA-EcdR and [^{32}P]hsp27 DNA fragment. (A) A 0.5-ml sample containing *ca.* 0.42 pnmol of [^{3}H]PonA-EcdR complexes was incubated for 30 min on ice with 2 pmol of unlabelled hsp27 ds-oligonucleotide. After additional incubation with 0.30 pmol of [^{32}P]hsp27 DNA the gel filtration analysis of the competition between ^{32}P-labelled and unlabelled hsp27 DNA for binding of [^{3}H]PonA-EcdR was done using the Superose 6HR column pretreated as in Fig. 2D. (B) The same as in (A), but 2 pmol of the unlabelled hsp27$_A$ ds-oligonucleotide (see Fig. 1) were used. Symbols as in Fig. 3.

Obviously the modified ds-oligonucleotide can only partially compete with the original.

DISCUSSION

The basic conditions of a gel filtration assay have been worked out which can be used for the identification of DNA sequences tightly bound by proteins.

If a specific detection system for the protein is available, crude extracts can be studied. In our case a ^{3}H-labelled ligand of the protein together with the [^{32}P]hsp27 ds-oligonucleotide made the fractions of the column effluent directly amenable to liquid scintillation counting, but all specific detection systems usually used in column chromatography can in principle be used.

The DNA binding assay depends on the ability of the gel filtration column to resolve the DNA–protein complex from free protein and from the unbound ds-oligonucleotide. Therefore, the size of the ds-oligonucleotide must be carefully chosen so that a separation of the complex within the limits of gel filtration is possible.

The assay also is dependent on the stability of the protein–DNA complex during the chromatographic separation process. Here the gel filtration system shows great advantages as the buffers can be chosen fairly freely. All kinds of additives which stabilize the reaction products or cofactors can be incorporated into the solvents. The addition of proteinase or DNase inhibitors may be crucial if crude extracts are used which frequently contain degrading enzymes. In contrast to electrophoresis, the buffer constituents do not influence the temperature during separation and optimum temperature can be easily maintained.

High-performance gel filtration columns, like the Superose 6HR, can be operated fairly fast without losing resolution. In the experiments discussed here the EcdR–hsp27 ds-oligonucleotide complex was eluted after *ca.* 90 min. This might be important for such systems where the protein–DNA or the protein–ligand complex has a short half-life.

Additional information on the protein–DNA complex is directly available from the elution diagram. With the help of saturation experiments the stoichiometry and specificity of the complex can be studied. As the elution positions in a modern chromatographic system are extremely reproducible, the molecular mass of the complex and any molecular mass changes resulting from interaction of a protein with specific DNA can be determined.

ACKNOWLEDGEMENTS

We gratefully acknowledge the continuous interest and the helpful advice of Professor Dr. O. Pongs. This work was supported by a grant from the Deutsche Forschungsgemeinschaft to O. Pongs. A. O. was a recipient of a Heinrich-Hertz Fellowship. The permanent address of A. O. is Institute of Organic and Physical Chemistry, Division of Biochemistry, Technical University of Wrocław, 50 370 Wrocław, Poland.

REFERENCES

1 M. G. Fried and D. M. Crothers, *Nucleic Acids Res.*, 9 (1981) 6505–6525.
2 M. G. Fried, *Electrophoresis*, 10 (1990) 366–376.
3 M. Beato, *Cell*, 56 (1989) 335–334.
4 J. Koolman, *Ecdysone. From Chemistry to Mode of Action.* Georg Thieme, Stuttgart, New York, 1989.
5 W. A. Segraves and G. Richards, *Inv. Reprod. Devel.*, 18 (1990) 67–76.
6 E. Martinez and W. Wahli, in M. G. Parker (Editor), *Nuclear Hormone Receptors*, Academic Press, London, 1990, pp. 125–153.
7 F. Kreklau, *Ph.D. Thesis*, Fakultät Chemie, Ruhr-Universität Bochum, 1989.
8 A. Ożyhar, H.-H. Kiltz and O. Pongs, *Eur. J. Biochem.*, 192 (1990) 167–174.
9 S. R. Gallanger, in F. M. Asubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl (Editors), *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York, 1987, pp. A.3.9–A.3.8.
10 C. M. Morganelli, E. M. Berger and H. R. B. Pelham, *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 5865–5869.
11 G. Riddihough and H. R. B. Pelham, *EMBO J.*, 6 (1987) 3729–3734.
12 A. Ożyhar, M. Strangmann-Diekmann, H.-H. Kiltz and O. Pongs, *Eur. J. Biochem.*, 200 (1991) 329–335.
13 T. Maniatis, E. F. Fritsch and J. Sambrook, in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1982, pp. 122–127.
14 K. Schaltmann and O. Pongs, *Hoppe-Seyler's Z. Physiol. Chem.*, 361 (1980) 207–210.
15 T. C. Laurent and J. Killander, *J. Chromatogr.*, 14 (1964) 317.

Journal of Chromatography, 587 (1991) 19–23
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2373

Hydrophobic interaction chromatography for the purification of a mycobacterial heat shock protein of relative molecular mass 60 000

Bernd Schoel* and Stefan H. E. Kaufmann

Department of Immunology, University of Ulm, Albert-Einstein-Allee 11, W-7900 Ulm (Germany)

ABSTRACT

A recombinant mycobacterial heat shock protein of relative molecular mass 60 000 was purified by hydrophobic interaction chromatography. Chromatographic media with ligands of medium hydrophobicity, such as phenyl-Sepharose, bound too strongly to be used for the purification of this heat shock protein. Butyl-Sepharose, with weak hydrophobicity, allowed binding and elution with decreasing concentrations of ammonium sulphate, but only alkyl-Superose allowed the separation of two similar proteins from the *Escherichia coli* clone expressing the recombinant heat shock protein (relative molecular mass 60 000) of *Mycobacterium bovis* BCG. The binding parameters of recombinant human heat shock proteins of relative molecular mass 60 000 and 70 000 indicate that phenyl-Sepharose also binds too strongly for the separation of these two heat shock proteins.

INTRODUCTION

Heat shock proteins (hsp) are induced by different types of stress in eukaryotic and prokaryotic cells. Different families of hsp exist which are grouped according to their molecular size. The hsp 60 family is ubiquitous among all species and its members have a very high degree of sequence homology [1]. The biological functions of hsp 60 cognates are now known. Hsp 60 and hsp 70 both act as chaperons, *i.e.* they are involved in the assembly of protein subunits to form multimers, the unfolding of partially denatured proteins, and in keeping the proteins in an unfolded state prior to translocation across the membrane of the endoplasmic reticulum. In mammalian cells, hsp 60 cognates are located in mitochondria and are responsible for the assembly of intruded proteins [1].

The mycobacterial hsp of relative molecular mass 60 000 is a major T-cell antigen of *Mycobacterium tuberculosis* immune mice, tuberculosis patients and normal healthy subjects [2,3]. It is identical in *M. tuberculosis* and *M. bovis* BCG, shares significant homology with mammalian hsp 60 and has been linked with autoimmunity [4,5]. T-cells from arthritic rats cross-react with hsp 60 of *M. bovis* BCG (hsp 60 BCG) [5] and cytotoxic CD8 T-cells generated *in vitro* with a tryptic digest of hsp 60 BCG lyse stressed target cells [6]. Reactivity against mycobacterial hsp of relative molecular mass 60 000 has been described for a subgroup of T-cells bearing the γ/δ T-cell receptor [7]. It has been speculated that γ/δ T-cells perform scavenger functions.

Large amounts of purified hsp are needed for immunological studies. Recombinant (r) hsp 60 BCG is routinely semi-purified by anion-exchange chromatography according to Thole *et al.* [8]. The eluted material is pure except for a double band visible on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Both bands are recognized by antibodies against hsp 60 BCG, their difference in molecular mass being 3000.

This paper describes a hydrophobic interaction chromatography (HIC) technique for the efficient separation of these highly similar proteins. The binding properties of human r-hsp 60 (r-hsp 60hu) and human r-hsp 70 (r-hsp 70hu) towards media of different hydrophobicity are also discussed.

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V. All rights reserved

EXPERIMENTAL

Anion-exchange chromatography of hsp 60 BCG

The protein was purified from 10-l fermenter cultures of *Escherichia coli* M1546 with plasmid pRIB1300, kindly provided by J. van Embden [8]. After sonication of the bacteria, the 20–55% ammonium sulphate fraction was dialysed against 50 m*M* piperazine–hydrochloric acid (pH 6.0) and subjected to anion-exchange chromatography on Q-Sepharose Fast Flow (Pharmacia). The hsp 60 BCG eluted within a linear gradient of sodium chloride at 0.1–0.12 *M* in 50 m*M* piperazine–hydrochloric acid (pH 6.0).

Hydrophobic interaction chromatography of hsp 60 BCG

Alkyl-Superose, butyl-Sepharose and phenyl-Sepharose were from Pharmacia. Samples were adjusted to 35% ammonium sulphate in 20 m*M* sodium phosphate (pH 6.5). Up to 5 mg of prepurified protein from the anion exchanger were loaded onto alkyl-Superose HR5/5. Elution was performed with a decreasing linear gradient of ammonium sulphate in 20 m*M* sodium phosphate (pH 6.5).

Identification of hsp

Samples (1 μl) were dotted on reinforced nitrocellulose (Schleicher and Schuell) and developed as Western blots using monoclonal antibodies (mab) IIC8 for hsp 60 BCG, ML30 for hsp 60hu and N27 for hsp 70hu. Hsp 60hu and hsp 70hu were kindly supplied by Dr. R. A. Young, mab IIC8 by Dr. T. Gillis, ML30 by Dr. J. Ivanyi and N27 by Dr. W. J. Welch.

SDS-PAGE and Western blotting

Electrophoresis was performed under reducing conditions in a 10% polyacrylamide gel according to standard procedures [9] in a mini-electrophoresis system (Bio-Rad) and the blots were silver-stained [10]. For the immunological detection of specific bands, proteins were transferred to nitrocellulose in a semi-dry blotting system. After blocking the membrane in 1% casein–20 m*M* Tris (pH 8.0), mab IIC8 against hsp 60 BCG was used as the first antibody (1 μg/ml). Further incubation steps were performed with goat anti-mouse $(Fab')_2$ (H + L) biotin conjugate (1:20 000) and streptavidin alkaline phosphatase conjugate (1:10 000) (both from Dianova). The blots were then developed with 0.8 μg/ml 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and 1.6 μg/ml 4-nitrotetrazolium blue chloride (both Fluka) in 1 *M* diethanolamine, 0.5 m*M* magnesium chloride (pH 9.8) [11].

RESULTS AND DISCUSSION

Lysates of a recombinant *E. coli* clone expressing r-hsp 60 BCG were first purified by anion-exchange chromatography. The material obtained contained two similar proteins, both recognized by mab against hsp 60 BCG, and differing by 3000 in molecular mass. Previous attempts to separate these two similar proteins on metal chelate chromatographic columns had failed, although r-hsp 60 BCG could be bound to copper ions in a sodium phosphate buffer at pH 8.0. Elution occurred as a single peak at 20 m*M* glycine. Other methods were not feasible because r-hsp 60 BCG has a p*I* of 4.6, which excludes cation-exchange chromatography and chromatofocusing, and both proteins are too similar in size to allow their separation by gel permeation chromatography. Therefore, HIC appeared to be a promising means to further purify r-hsp 60 BCG.

The binding and elution properties of proteins from HIC resins are essentially controlled by four parameters: (1) the hydrophobic strength of the ligands bound to the matrix; (2) the general solvent effects of ions in the solvent system; (3) pH; and (4)

TABLE I

CONDITIONS USED FOR THE PRECIPITATION OF HSP

Aliquots of hsp were incubated for 1 h under different conditions and, after centrifugation, the supernatants were tested for residual, soluble hsp with a dot-blot assay.

Condition	r-hsp 60 BCG	r-hsp 60hu	r-hsp 70hu
pH[a]	5	6	< 4
AS at pH 8 (%)[b]	40	25	30
AS at pH 6 (%)[b]	30	20	25

[a] Highest pH at which the hsp precipitated when assayed at pH 7, 6, 5 and 4 using 100 m*M* citric acid–sodium hydroxide.

[b] Lowest concentration of ammonium sulphate (AS) at pH 8 or 6 at which the hsp precipitated. Assayed at concentrations from 15 to 50% in steps of 5% in 50 m*M* Tris–hydrochloric acid (pH 8) or 100 m*M* citric acid–sodium hydroxide (pH 6).

temperature. In an attempt to establish the conditions for the chromatographic separation of three different hsp, the pH and ammonium sulphate concentration at which these proteins begin to precipitate were determined. A semi-purified fraction of r-hsp 60 BCG after anion-exchange chromatography and crude extracts of r-hsp 60hu and r-hsp 70hu were subjected to different pH conditions ranging from pH 7 to 4 at intervals of one pH unit. Different concentrations of ammonium sulphate ranging from 15 to 50% in 5% steps at pH 8 and 6, respectively, were used. After high-speed centrifugation, the supernatants were assayed by blotting onto nitrocellulose and development with specific mab.

Table I summarizes the results. With decreasing pH, r-hsp 60 BCG remained soluble until pH 5, r-hsp 60hu until pH 6 and r-hsp 70hu until pH 4. Precipitation with ammonium sulphate started at 40% for r-hsp 60 BCG, 25% for r-hsp 60hu and 30% for r-hsp 70hu, when tested at pH 8. At pH 6, precipitation occurred at lower concentrations of ammonium sulphate.

The binding properties of these proteins to two different HIC media, butyl-Sepharose and phenyl-Sepharose, were assessed. Aliquots of each hsp were added to 100 μl of an equilibrated suspension containing one volume of packed HIC resin and one volume of buffer. The supernatants were analysed by dotblot assay for residual, non-bound hsp. The results shown in Table II indicate that all the hsp bound to phenyl-Sepharose at 20% ammonium sulphate. At a 0% concentration of ammonium sulphate, phenyl-Sepharose still bound r-hsp 60hu and r-hsp 70hu. r-hsp 60 BCG did not bind at pH 8, but binding occurred at pH 6. Butyl-Sepharose bound r-hsp 60 BCG at a concentration of 30%, and r-hsp 70hu at 20% ammonium sulphate. At 0% ammonium sulphate butyl-Sepharose did not bind any of these hsp; butyl-Sepharose is therefore a suitable chromatographic medium for the HIC of hsp. Although it was valuable for prepurification and concentration, the resolving power of butyl-Sepharose was not sufficient to separate prepurified r-hsp 60 BCG. To improve the separation alkyl-Superose was used, which has a similar hydrophobicity as butyl-Sepharose but a higher resolving power.

Attempts to separate these hsp were finally successful using alkyl-Superose. Fig. 1 shows an elution profile and Fig. 2 the purity of the separated proteins. The protein with the higher molecular mass of 65 000 eluted at higher concentration of ammonium sulphate. By comparing the binding and elution properties of r-hsp 60 BCG on butyl-Sepharose and alkyl-Superose it is concluded that alkyl-Superose has a less hydrophobic character. At 16% ammonium sulphate and pH 8.0 the protein could not be bound to alkyl-Superose, whereas under the same conditions it just eluted from butyl-Sepharose. The binding of the protein to alkyl-Superose was enhanced at higher concentrations of ammonium sulphate (35%) and pH 6.5. Elution then occurred at 26% ammonium sulphate.

The hsp 60 cognates from different species could have similar chromatographic properties because of

TABLE II

BINDING PROPERTIES OF hsp TO HIC MEDIA

Medium	pH	AS (%)[a]	r-hsp 60 BCG	r-hsp 60hu	r-hsp 70hu
Butyl-Sepharose	8	None	–[b]	–	–
		High	+	–	+
	6	None	–	–	–
		High	+	–	+
Phenyl-Sepharose	8	None	–	+	+
		High	+	+	+
	6	None	+	+	+
		High	+	+	+

[a] Concentration of ammonium sulphate (AS) at which binding of hsp was assayed. For r-hsp 60 BCG, 30% was used as high concentration and for r-hsp 60hu and r-hsp 70hu 20% AS was used.

[b] (–) No binding; (+) binding to HIC medium.

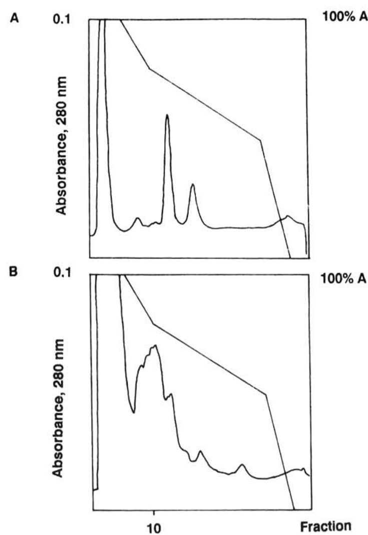


Fig. 1. Separation of r-hsp 60 BCG on alkyl-Superose HR5/5. (A) Prepurified r-hsp 60 BCG (200 μg) was loaded onto the column in 50 m*M* sodium phosphate–35% ammonium sulphate (pH 6.5) and eluted with a linear gradient of ammonium sulphate down to 0%. Each fraction contained 1 ml of eluent. (B) *E. coli* lysate (500 μg) separated as in (A).

the high sequence homology. Another hsp of the relative molecular mass 60 000 family and one of the 70 000 family were included. Hsp of the 70 000 family are usually purified by ion exchangers and affinity chromatography on columns with adenosine 5′-triphosphate as the ligand [12]. Owing to their similar biological function and involvement in the folding and unfolding of proteins, these two families might have similar properties on HIC columns. r-hsp 60hu could not be bound to HIC media of low hydrophobicity owing to the precipitation of this protein at too low concentrations of ammonium sulphate, but r-hsp 70hu could be bound and eluted from alkyl-Superose. Phenyl-Sepharose was too strongly hydrophobic for these two hsp.

Only a few proteins of *E. coli*, the host of these recombinant genes, bound to alkyl-Superose (Fig. 2A). If a hsp can be bound to a weak hydrophobic ligand, then this kind of media is valuable as an early hsp purification step, because few non-membrane-bound proteins bind to HIC media of low hydrophobicity.

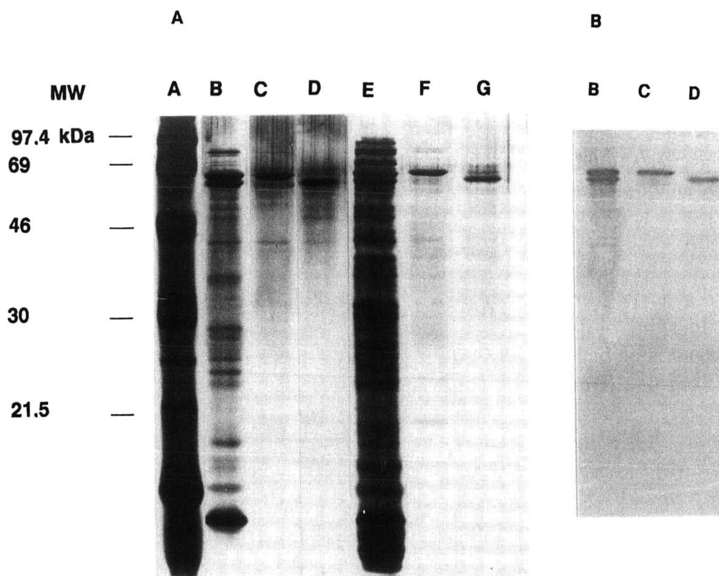


Fig. 2. SDS-PAGE of hsp: (A) silver-stained and (B) Western blot. (A) Molecular weight markers; (B) partially purified r-hsp 60 BCG of an *E. coli* lysate expressing r-hsp 60 BCG, after chromatographic separation on the anion-exchanger Q-Sepharose Fast Flow (1 μg of protein); (C) and (D) first and second peaks of eluted proteins from alkyl-Superose HR5/5 loaded with partially purified r-hsp 60 BCG from (B) (0.1 and 0.05 μg of protein, respectively); (E) 2 μg of protein from a crude lysate of *E. coli* expressing r-hsp 60 BCG; (F) and (G) similar peaks to (C) and (D), from alkyl-Superose HR5/5 loaded with crude *E. coli* lysate from (E) (0.2 and 0.05 μg of protein, respectively).

ACKNOWLEDGEMENTS

The authors thank Mrs. R. Mahmoudi for typing the manuscript. The work described was supported by the following grants to S.H.E.K: UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases, EC-India Science and Technology Cooperation Program, A. Krupp Award for Young Professors, and the SFB 322. We thank T. Gillis, J. Ivanyi and W. J. Welch for mab, J.D.A. v. Embden and R. van der Zee for recombinant *E. coli* clones, and R. A. Young and S. Jindal for recombinant hsp. K. Schilling and T. Schechinger provided much helpful technical information for the establishment of the FPLC system.

NOTE ADDED IN PROOF

Sequence analysis of the separated proteins confirmed that the larger protein (Fig. 2, lane C) was r-hsp 60 BCG. This protein was sequenced up to 30 amino acids resulting in an identical sequence. The smaller protein (Fig. 2, lane D) was sequenced up to 20 amino acids and identified as the truncated r-hsp 60 BCG, the aminoterminus starting with WGAPT (amino acid single letter code) at position 42 from the whole protein. Sequence analysis was done by Dr. Thomas Ruppert at the Department of Virology, University of Ulm, with an Applied Biosystems 471A Protein Sequencer.

REFERENCES

1 T. Langer and W. Neupert, *Curr. Top. Microbiol. Immunol.*, 167 (1991) 3.
2 F. Emmerich, J. Thole, J. van Embden and S. H. E. Kaufmann, *J. Exp. Med.*, 163 (1986) 1024.
3 S. H. E. Kaufmann, U. Väth, J. E. R. Thole, J. D. A. van Embden and F. Emmrich, *Eur. J. Immunol.*, 17 (1987) 351.
4 D. Elias, D. Markovits, T. Reshef, R. van der Zee and I. R. Cohen, *Proc. Natl. Acad. Sci. USA*, 87 (1990) 1576.
5 W. van Eden, J. E. Thole, R. van der Zee, A. Noordzij, J. D. van Embden, E. J. Hensen and I. R. Cohen, *Nature (London)*, 331 (1988) 171.
6 T. Koga, A. Wand-Württenberger, J. DeBruyn, M. E. Munk, B. Schoel and S. H. E. Kaufmann, *Science*, 245 (1989) 1112.
7 S. H. E. Kaufmann and D. Kabelitz, *Curr. Top. Microbiol. Immunol.*, 167 (1991) 191.
8 J. E. R. Thole, W. J. Keulen, A. H. J. Kolk, D. G. Groothuis, L. G. Berwald, R. H. Tiesjema and J. D. A. van Embden, *Infect. Immun.*, 55 (1987) 1466.
9 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
10 C. R. Merril, D. Goldmann and M. L. van Keuven, *Methods Enzymol.*, 104 (1984) 441.
11 D. A. Knecht and R. L. Dimond, *Anal. Biochem.*, 136 (1984) 180.
12 W. J. Welch and J. R. Feramisco, *Mol. Cell. Biol.*, 5 (1985) 1229.

Journal of Chromatography, 587 (1991) 25–32
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2397

Isolation of multiple protein factors involved in ribosomal DNA transcription

Gisela Heilgenthal* and Ingrid Grummt

Institute of Cell and Tumor Biology, German Cancer Research Center, Im Neuenheimer Feld 280, W-6900 Heidelberg (Germany)

ABSTRACT

Studies were made of the molecular mechanisms which regulate ribosomal gene transcription in response to changes in the growth rate of cells. Extracts prepared from exponentially growing Ehrlich ascites cells faithfully and efficiently transcribe cloned mouse rDNA, whereas extracts from growth-arrested cells are virtually inactive. In an attempt to identify and characterize functionally the proteins that mediate the accuracy and the control of transcription initiation, a fractionation procedure was developed which allows the purification of RNA polymerase I and four accessory factors that are required for transcription initiation at the ribosomal gene promoter. Starting from about 300 ml of cell extract, each of the individual factors and the polymerase was purified on at least four different chromatographic columns, including ion-exchange chromatography on DEAE-Sepharose, heparin-Ultrogel, Mono Q and Mono S, gel filtration and specific affinity chromatography. The resulting protein fractions are functionally active, as shown by reconstitution of specific rDNA transcription in the presence of purified polymerase and the additional factors.

INTRODUCTION

During the last few years, detailed analysis of many eukaryotic genes has revealed at least two types of *cis*-acting regulatory elements: promoters and enhancers. These elements are recognized by *trans*-acting proteins via either protein–DNA or protein–protein interactions. The primary goal of current research is to identify and characterize these proteins and to determine their precise role in cellular physiology. A useful model for such studies is provided by the ribosomal RNA genes, which belong to the most actively transcribed genes in growing cells. Further, rDNA transcription is regulated in response to a variety of extracellular stimuli including growth factors, steroid and peptide hormones and nutrient supply [1–4], and therefore represent an excellent experimental system to study the molecular mechanisms which couple gene expression and cell proliferation.

A major step towards understanding rDNA transcription regulation at the molecular level was the development of cell-free systems which faithfully transcribe cloned rDNA sequences [5]. The availability of such *in vitro* systems facilitates the identification and functional analysis of both the *cis*-acting DNA sequences and the *trans*-acting proteins involved in rDNA transcription initiation. To our knowledge, rDNA transcription initiation is mediated by a complex interplay between RNA polymerase I (pol I) and at least four additional transcription initiation factors, termed TIF-IA, TIF-IB, TIF-IC and UBF [6].

TIF-IB and UBF are specific DNA-binding proteins which form a strong cooperative complex on the rDNA gene promoter. The binding of TIF-IB (the protein that confers promoter specifity to pol I) to the rDNA promoter is an early step in the formation of an active transcription complex [7,8]. Binding of TIP-IB and UBF to the rDNA promoter enables the polymerase and also the two accessory factors TIF-IA and TIF-IC to assemble into a productive initiation complex. Both TIF-IA and TIF-IC do not bind to DNA but rather associate with pol I. Whereas TIF-IC may serve a function in selection of the correct start site [9], TIF-IA is a positive-acting factor whose level or activity fluctuates in response to the physiological state of cells [4,10].

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V. All rights reserved

EXPERIMENTAL

Materials

All chemicals were of analytical-reagent grade and were purchased from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Sigma (Deisenhofen, Germany).

DEAE-Sepharose CL-6B, CM-Sephadex, heparin-Ultrogel A4/R, Q-Sepharose and the prepacked fast protein liquid chromatographic (FPLC) columns Mono Q HR 5/5, Mono S HR 5/5, S-Sepharose High Performance 16/100 and HiLoad 26/60 Superdex 200 were obtained from Pharmacia–LKB (Freiburg, Germany). A polyethyleneimine (PEI) high-performance liquid chromatographic (HPLC) column was obtained from Baker (Gross-Gerau, Germany).

Cell culture and extract preparations

Ehrlich ascites cells were cultured in RPMI medium containing 5% newborn calf serum for 20–40 h. Transcriptionally active extracts were obtained from a logarithmically growing cell culture ($9 \cdot 10^5$ cells/ml). S 100 extracts were prepared according to Weil *et al.* [11] and nuclear extracts according to Dignam *et al.* [12].

In vitro transcription assays

The soluble cell-free transcription system and the analysis of the RNA synthesized has been described previously [5]. Usually, 50–100 ng of plasmid pMr600 truncated with Eco RI were incubated with 15 μl of a mixture of S-100 and nuclear extracts in a total volume of 25 μl containing 12 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 7.9), 0.1 m*M* EDTA, 0.5 m*M* dithioerythriol (DTE), 5 m*M* $MgCl_2$, 75 m*M* KCl, 10 m*M* creatine phosphate, 12% (v/v) glycerol, 0.66 m*M* each of ATP, CTP and UTP, 0.01 m*M* GTP and 1.5 μCi of [α-^{32}P]GTP (400 Ci/mmol). After incubation for 60 min at 30°C, the nucleic acids were extracted, precipitated and analysed on non-denaturing 5% polyacrylamide gels.

Total pol I activity was assayed in a 25-μl reaction mixture containing 6 m*M* Tris–HCl (pH 7.9), 0.1 m*M* EDTA, 5 m*M* $MgCl_2$, 80 m*M* KCl, 6% glycerol, 0.5 m*M* DTE, 0.66 m*M* each of ATP, CTP and GTP, 3.6 μM [^{3}H]UTP (5.5 Ci/mmol), 7.5 μg of calf thymus DNA and 5 μg of α-amanitin. After incubation for 30 min at 30°C, the reaction was stopped by the addition of 0.2 ml of saturated $Na_4P_2O_7$ containing 50 μg of carrier DNA and precipitated with 5% trichloroacetic acid. The precipitates were collected on glass-fibre filters and quantified by scintillation counting.

One unit of RNA polymerase I activity represents the incorporation of 1 pmol of [^{3}H]UMP during 10 min of incubation under these conditions.

Purification of transcription factors

A typical factor preparation was started from 300 ml (1.8 g of protein) of a mixture of nuclear and cytoplasmatic extracts prepared from cultured Ehrlich ascites cells. All buffers contained 0.5 m*M* DTE and 0.5 m*M* phenylmethylsulphonylfluoride (PMSF), added immediately prior to use. Extract proteins were applied to a DEAE-Sepharose CL-6B column, washed with buffer A [20 m*M* Tris–HCl (pH 7.9)–0.1 m*M* EDTA–20% glycerol] containing 100 m*M* KCl and step eluted at 280 m*M* KCl. This fraction, which contains pol I and the four transcription factors, was loaded (at 200 m*M* KCl) on a heparin-Ultrogel A4-R column and eluted with buffer AM (buffer A with 5 m*M* $MgCl_2$) containing 400, 600 and 1000 m*M* KCl, yielding fractions H-200 (flowthrough fraction), H-400, H-600 and H-1000, respectively.

TIF-IA and TIF-IC activities present in the H-200 fraction were concentrated on a Q-Sepharose column. Separation of TIF-IA and TIF-IC was achieved by HPLC on a polyethylenimine column using a linear gradient from 0.1 to 1 *M* KCl. TIF-IC and TIF-IA eluted at 300 and 700 m*M* KCl, respectively. As a next step TIF-IA, was applied to a Mono Q column and eluted at 230 m*M* KCl in a gradient from 200 to 300 m*M* KCl. TIF IC was purified to near homogeneity on an Orange A dye affinity matrix from which it elutes at 230 m*M* KCl.

RNA polymerase I present in the H-400 fraction was further purified on S-Sepharose High Performance, HiLoad 26/60 and Mono Q HR5/5 columns. TIF-IB was recovered in the H-600 fraction and purified further by chromatography on CM-Sepharose, Mono S (FPLC) and affinity chromatography on calf thymus DNA cellulose [8].

UBF activity was present in the H-1000 fraction and purified to homogeneity by chromatography on a Mono Q FPLC column followed by a specific oligonucleotide affinity column [13].

Conventional chromatography

In the first step of the purification procedure 300 ml of cell extracts (1.8 g of protein) were loaded on a DEAE-Sepharose CL 6B column (80 ml), equilibrated with buffer A at a flow-rate of 3 ml/min; the fraction size was 8 ml.

Chromatography on heparin-Ultrogen A4/R, CM-Sephadex and Q-Sepharose was performed in buffer AM 100 at the appropriate flow-rates; fraction sizes were normally one tenth of the column volume. Protein absorbance was monitored at 280 nm (Uvicord SD). The individual column fractions were dialysed against buffer AM-100 before they were assayed in the reconstituted transcription system.

High-performance liquid chromatography

An HPLC system manufactured by Pharmacia–LKB was used. Buffers were degassed and filtered through a 0.22-μm membrane (Sartorius) prior to use. Ion-exchange HPLC was performed on a PEI column (4 ml) using AM buffer with a KCl gradient from 0.1 to 1 *M*. The flow-rate was 0.5 ml/min and the fraction size was 1.0 ml.

Fast protein liquid chromatography

An FPLC system (Pharmacia) consisting of an LCC-Controller-500 Plus (gradient programmer), two P-500 pumps, a UV-M monitor (280 nm), an REC 102 recorder, an MV-7 multi-position motor valve and a FRAC 100 fraction collector was used. For all columns buffer AM [20 m*M* HEPES (pH 7.9)–0.1 m*M* EDTA–5 m*M* $MgCl_2$–20% glycerol] was used. All buffers were degassed and ultrafiltered (Sartorius 0.22-μm filter). Ion-exchange FPLC was performed with Mono Q HR 5/5 and Mono S HR 5/5 columns at a flow-rate of 0.5 ml/min, fraction size 0.5 ml and pressure 2 MPa. Cation-exchange FPLC was carried out on an S-Sepharose High-Performance column at a flow-rate of 2 ml/min, pressure 0.3 MPa and fraction size 4 ml. Gel filtration FPLC was performed with HiLoad 26/60 (Superdex 200) at a flow-rate of 2 ml/min and a pressure of 0.3 MPa; 3-ml fractions were collected.

DNA affinity chromatography

UBF was purified to apparent homogeneity on a sequence-specific DNA affinity column as described by Bell and co-workers [14,15]. Affinity chromatography on calf-thymus DNA cellulose was carried out according to Schnapp *et al.* [8].

Protein analysis

Proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [16]. Silver staining was carried out according to the method of Blum *et al.* [17]. Protein was determined by the method of Bradford [18].

RESULTS AND DISCUSSION

Four factors besides RNA polymerase I are required for mouse rDNA transcription initiation

To study the mechanisms directing faithful transcription initiation of the ribosomal genes, the purification of the various factors involved in this process is required. We have shown previously that rDNA transcription *in vitro* reflects the rRNA synthetic capacity of the cells from which the extracts have been prepared. Extracts derived from cultured cells support transcription of cloned rDNA whereas extracts from tissues or slowly growing cells are virtually inactive [4,10]. This finding implies that the activity of one or several proteins involved in ribosomal gene transcription is growth controlled and, therefore, the individual proteins should be prepared from cultured cells. The concentration of the rDNA transcription initiation factors within the cell is known to be extremely low and it is laborious and expensive to obtain enough cultured cells as starting material. For this reason it was necessary to develop a fractionation procedure which allows the purification of both the polymerase and the additional essential factors from the same starting material. The procedure established involves a combination of several chromatographic steps, including ion-exchange chromatography on DEAE-Sepharose, Mono Q and Mono S (FPLC), gel filtration and specific affinity chromatography.

Using the fractionation strategy shown in Fig. 1A, we isolated four factors (termed TIF-IA, TIF-IB, TIF-IC and UBF) that in addition to pol I are required for efficient transcription initiation from the mouse rDNA promoter. The complete reconstituted system contains five fractions including RNA polymerase I and four auxiliary factors. The re-

quirement of the various factors for transcription initiation from the mouse rDNA promotor is shown in Fig. 1B. When all fractions are present at optium concentrations 297 nucleotide (nt) run-off transcripts are synthesized (lane 1). In the absence of pol I or any of the other factors, no specific transcripts are generated (lanes 2–6), indicating that each of these factors plays an indispensable role in transcription initiation. The labelled bands on the top of the gel represent non-specific transcripts. Importantly, although most of the factor preparations used are not yet homogeneous, they show no cross-contamination, as the full complement of all four factors was necessary for efficient transcription.

Purification of RNA polymerase I

We shall focus here on the isolation of pol I, but all purification problems and working conditions can be transferred to the four essential pol I specific transcription initiation factors. The purification of these factors is described under Experimental. Eukaryotic RNA polymerase I is notoriously unstable and thermolabile and therefore all operations were carried out at 4°C. To stabilize the enzyme, 20% glycerol is included in all buffers, which results in low flow-rates and high back-pressures for Mono Q and Mono S FPLC columns (flow-rate 0.5 ml/min, pressure 2 MPa). In addition, dilution of the enzyme preparations frequently resulted in severe losses of activity. During all chromatographic steps steep salt gradients resulted in greater recoveries of activity than did shallow gradients, although the resolution and the degree of purification were better in the latter instance.

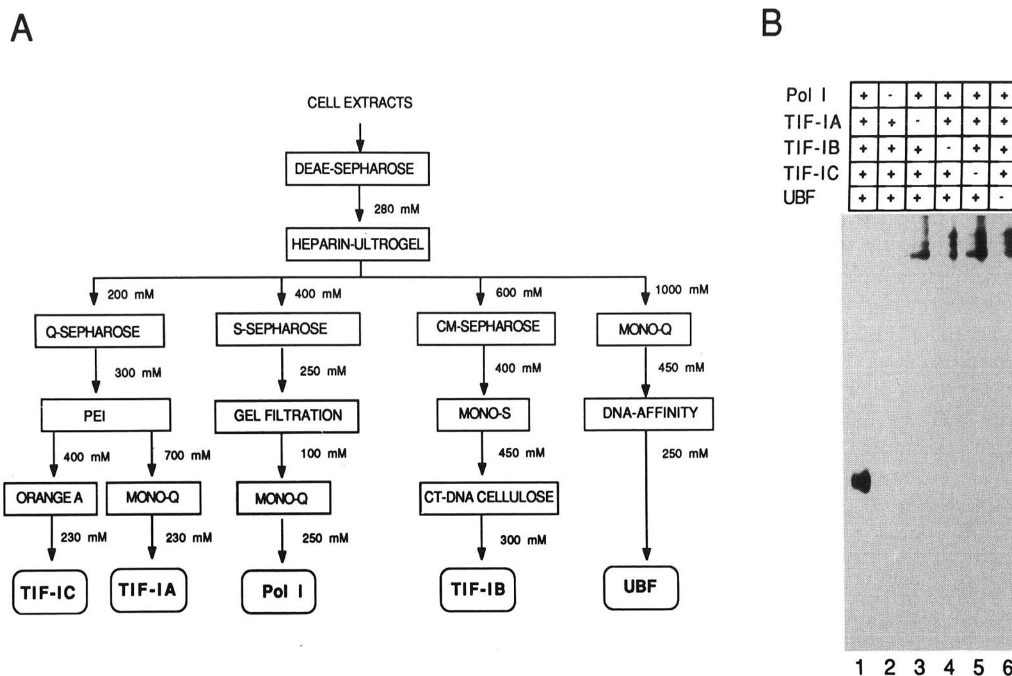


Fig. 1. Separation and functional identification of multiple factors required for faithful rDNA transcription initiation. (A) Schematic representation of the chromatographic separation of murine pol I transcription factors. The samples were loaded on the individual columns and eluted either stepwise or with gradients. The numbers represent the concentration of KCl (m*M*). For details, see Experimental. (B) Factors required for transcription from the mouse rDNA promoter. The complete reconstituted transcription assay contained 60 ng of plasmid pMr600 truncated with EcoRI, 5 μl of partially purified pol I, 3 μl of TIF-IA, 2 μl of TIF-IB, 3 μl of TIF-IC and 2 μl of UBF (lane 1). In lanes 2–6 one of the individual fractions (as indicated above the lanes) was omitted from the reactions. The band represents the specific 297 nt run-off transcript.

Ion-exchange chromatography on S-Sepharose

As a first step in the purification protocol, extract proteins were applied to a DEAE-Sepharose column and step eluted at 280 m*M* KCl. This fraction, which contains pol I and the four transcription factors, was loaded directly on a heparin-Ultrogel column and eluted with 200, 400, 600 and 1000 m*M* KCl, yielding fractions H-200, H-400, H-600 and H-1000, respectively. Chromatography on the heparin column turned out to be the most essential part of the purification scheme, because it separates pol I and the individual transcription initiation factors. In addition, this step results in a 15-fold purification of pol I with a 10-fold increase in specific activity.

An efficient purification of pol I-containing H-400 fraction was achieved on an S-Sepharose High-Performance column, as shown in Fig. 2. On this column 80% of the protein was removed from the H-400 eluate, resulting in a 3.5-fold increase in specific activity. Hence S-Sepharose HP is a good alternative to Mono-S columns in large-scale preparations, because the resolution on S-Sepharose was nearly as good as that on a Mono S FPLC column.

Gel filtration on HiLoad 26/60

Eukaryotic pol I has a molecular weight of *ca.* 500 000 dalton [19,20]. Because of the large size of pol I, gel filtration or glycerol gradients should be included in the purification procedure. A disadvantage of glycerol gradients is the small amount (about 200–500 μl) of sample which can be applied in the gradient. To overcome this problem a preparative gel filtration column (HiLoad 26/60) was used, which allows the applicaton of up to 13 ml of sample. Prior to application of pol I, the column was calibrated under identical conditions using marker proteins as indicated in Fig. 3.

A HiLoad 26/60 column was equilibrated with buffer AM-100 and 10 ml (3 mg of protein) of active S-Sepharose fractions were applied. As shown in Fig. 3, pol I activity elutes at 132 ml, which corresponds to a molecular weight of about 500 000 dalton. Gel filtration resulted in an 8-fold purification of the enzyme. However, a large decrease in pol I activity (about 50%) was observed, which is probably due to dilution of the enzyme, which results in partial denaturation and inactivation of enzyme activity (after 2 days of storage at -80°C the pol I activity was completely lost). Nevertheless, this step

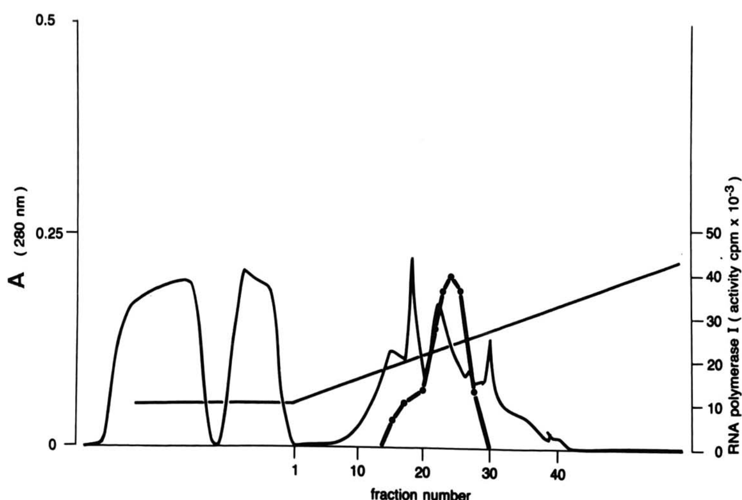


Fig. 2. Purification of RNA polymerase I on S-Sepharose HP. A 90-ml heparin 400 fraction (75 mg of protein) was applied to S-Sepharose High Performance 16/100 (20 ml), equilibrated with three bed volumes of buffer AM 100. Pol I activity was eluted with a gradient from 100 to 450 m*M* KCl in 250 ml of buffer AM. The flow-rate was 2 ml/min. Fractions of 4 ml were collected and dialysed against buffer AM 100.

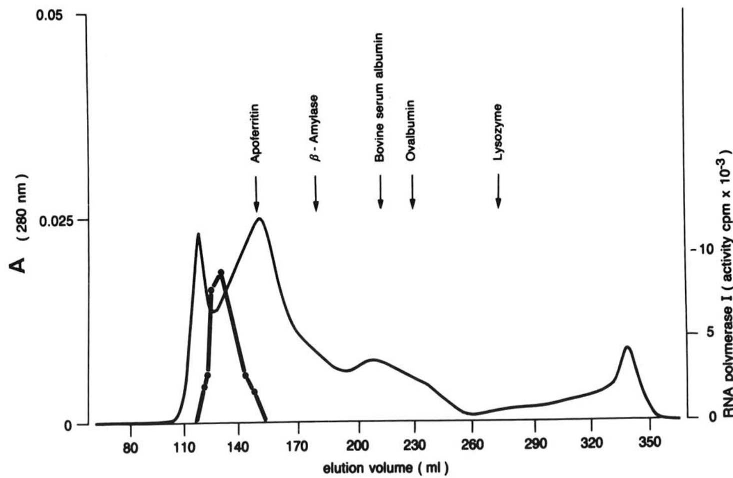


Fig. 3. Gel filtration chromatography of pol I using a HiLoad 26/60 (Superdex 200) column. Fractions of 10 ml of active S-Sepharose were applied to the column, pre-equilibrated with buffer AM-100. The flow-rate was 2 ml/min, pressure 0.3 MPa and fraction size 3 ml. Pol I activity appears at an elution volume of 132 ml. The following standard proteins were used to calibrate the column: apoferritin (443 000 dalton), β-amylase (200 000 dalton), bovine serum albumin (66 000 dalton), ovalbumin (43 000 dalton) lysozyme (14 000 dalton).

turned out to be very important in our purification scheme, because cross-contamination of the DNA binding factors UBF and TIF-IB could be avoided. To stabilize the enzymatic activity, pol I-containing fractions were concentrated on a Mono Q column.

Ion-exchange chromatography on Mono Q

The pool of pol I-containing fractions from the HiLoad 26/60 column was loaded immediately on a Mono Q column, which was equilibrated with buffer AM-100, and the bound proteins were eluted

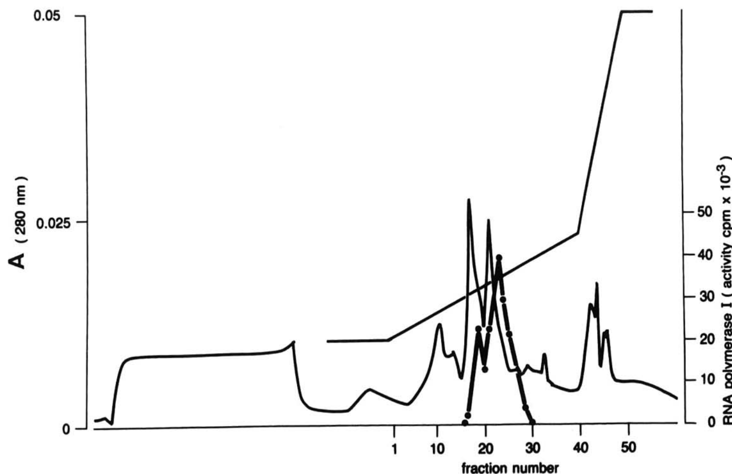


Fig. 4. Anion-exchange chromatography of RNA polymerase I on a Mono Q HR 5/5 column. About 30 ml of pol I-containing fractions size-fractionated on HiLoad 26/60 were loaded on a Mono Q HR 5/5 column. Pol I activity was eluted with a salt gradient from 150 to 450 m*M* KCl in 20 ml of buffer AM. The flow-rate was 0.5 ml/min, pressure 2 MPa and fraction size 500 μl.

TABLE I

PURIFICATION DATA FOR RNA POLYMERASE I

The data indicate the recovery of RNA polymerase I from 300-ml cell extracts. The purification procedure and the preparation of the cell extracts are described under Experimental.

Fraction	Volume (ml)	Protein (mg)	Activity (units)	Recovery (%)	Specific activity (units/mg)	Purification (-fold)
Extract	300	1800	55 000	100	30	1
DEAE	150	450	44 000	79	98	3.1
Heparin	30	25	27 000	49	1092	35
S-Sepharose	10	3	11 000	19	3666	120
HiLoad	30	0.7	5500	10	7800	252
Mono Q	2.5	0.18	2300	4	12 670	410

with a gradient from 150 to 450 mM KCl in 20 ml of buffer. A typical elution profile on Mono Q is shown in Fig. 4. Purification of pol I on Mono Q yielded a 4 fold purification and a 2-fold increase in specific activity. Chromatography of pol I on Mono Q is a very effective step to concentrate and therefore to stabilize pol I. After this step, pol I-containing fractions could be stored for several weeks at −80°C without a severe loss of activity.

CONCLUSION

The state of purification of pol I in each step is summarized in Table I. Starting from about 300 ml

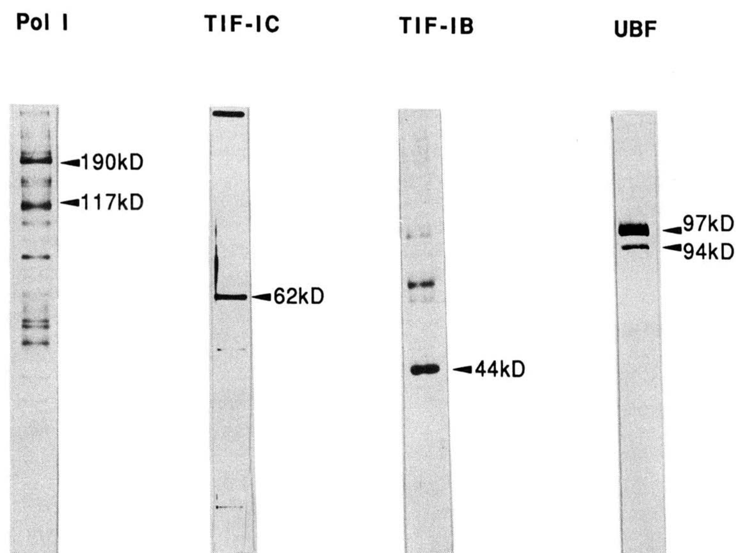


Fig. 5. SDS-PAGE of factors purified according to Fig. 1A. RNA polymerase I: 20 μl of a Mono Q peak fraction were loaded on a 7.5–15% discontinous SDS polyacrylamide gel. The sizes of the two largest subunits of pol I are indicated. TIF-IC: 25 μl of an Orange A fraction were electrophoresed on an 8% SDS polyacrylamide gel. TIF-B: 25 μl of a calf thymus DNA cellulose fraction were electrophoresed on an 11% SDS polyacrylamide gel. UBF: 40 μl of the DNA affinity column fraction were loaded on a 7% SDS polyacrylamide gel. All SDS polyacrylamide gels were silver stained and the molecular weights of the proteins are indicated in kilodalton (kD).

of cell extract (1.8 g of protein), which contained a total pol I activity of 55 000 units, 4% of the total enzyme activity was recovered. The degree of purification obtained was more than 400-fold. This level of purification, however, is not sufficient to obtain a homogeneous pol I preparation. As shown in Fig. 5, a number of discrete protein bands are visible on a silver-stained SDS gel. Nevertheless, the two largest subunits of pol I are clearly visible and their sizes (190 000 and 117 000 dalton) are in agreement with published data [19,20]. In addition, the second largest subunit was identified by the Western blot technique using antibodies directed against the second largest subunit of *Drosophila melanogaster* pol I (data not shown). Fig. 5 also shows the proteins present in the most highly purified UBF, TIF-IB and TIF-IC fractions which were prepared according to the fractionation scheme shown in Fig. 1. UBF, which has been reported to consist of two 97 000- and 94 000-dalton polypeptides [14,15], has been purified to apparent homogeneity. The TIF-IB activity correlates with a 44 000-dalton polypeptide and TIF-IC activity appears to reside within a 62 000-dalton protein. The most purified TIF-IA preparations still show a complex protein pattern (data not shown) and therefore it is not yet possible to attribute TIF-IA activity to a defined polypeptide.

Using the proposed purification scheme, we are now able to use a highly purified *in vitro* system, which makes it feasible to study the transcription initiation process at the rDNA promoter in a detailed manner and to clone individual factors and to determine their precise role in cellular physiology.

ACKNOWLEDGEMENTS

We thank H. Rosenbauer and A. Schnapp for their help in factor purification and all members of the group for valuable discussions. This work was supported by the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie and by a grant from the Science Programme of the Commission of the European Community (SCI*-0259-C).

REFERENCES

1 E. Surmacz, O. Ronning, L. Kaczmarek and R. Baserga, *Mol. Cell. Biol.*, 7 (1987) 657–663.
2 A. H. Cavanaugh and E. A. Thompson, *J. Biol. Chem.*, 25 (1983) 9768–9772.
3 M. L. Hammond and L. H. Bowman, *J. Biol. Chem.*, 263 (1988) 17785–17791.
4 D. Buttgereit, G. Pflugfelder and I. Grummt, *Nucl. Acids Res.*, 13 (1985) 8165–8180.
5 I. Grummt, *Proc. Natl. Acad. Sci. U.S.A.*, 78 (1981) 727–731.
6 A. Schnapp, H. Rosenbauer and I. Grummt, *Mol. Cell. Biochem.*, 104 (1991) 137–147.
7 J. Clos, D. Buttgereit and I. Grummt, *Proc. Natl. Acad. Sci. U.S.A.*, 83 (1985) 604–608.
8 A. Schnapp, J. Clos, R. Schreck, A. Cvekl and I. Grummt, *Nucl. Acids Res.*, 18 (1990) 1385–1393.
9 H. Rosenbauer, A. Schnapp and I. Grummt, in preparation.
10 A. Schnapp, C. Pfleiderer, H. Rosenbauer and I. Grummt, *EMBO J.*, 9 (1990) 2857–2863.
11 P. A. Weil, D. S. Luse, J. Segall and R. G. Roeder., *Cell*, 18 (1979) 469–484.
12 J. D. Dignam, R. M. Lobowitz and R. G. Roeder, *Nucl. Acids. Res.*, 11 (1983) 1475–1489.
13 H. Rosenbauer, *Ph.D. Thesis*, University of Würzburg, 1991.
14 S. P. Bell, H. M. Jantzen and R. Tijan, *Genes Dev.*, 4 (1990) 943–954.
15 S. P. Bell, C. S. Pikaard, R. H. Reeder and R. Tijan, *Cell*, 59 (1989) 489–497.
16 U. K. Laemmli, *Nature (London)*, 227 (1970) 680–685.
17 H. Blum, H. Beier and H. Gross, *Electrophoresis*, 8 (1987) 93–99.
18 M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
19 A. Sentenac, *Crit. Rev. Biochem.*, 18 (1985) 31–91.
20 K. M. Rose, D. A. Stetler and S. T. Jacob, *Proc. Natl. Acad. Sci. U.S.A.*, 78 (1981) 2833–2837.

Journal of Chromatography, 587 (1991) 33–42
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2371

Immobilized metal affinity chromatography as a means of fractionating microsomal cytochrome P-450 isozymes

Peter H. Roos

Institut für Physiologische Chemie I, Abteilung Bioenergetik, Geb. MA 2/136, Ruhr-Universität Bochum, W-4630 Bochum (Germany)

ABSTRACT

Fractionation of microsomal cytochrome P-450s is usually done by chromatography on ion-exchange resins and hydroxyapatite. The resolution of the great number of similar P-450 isozymes, however, requires additional methods based on different separation parameters. For this purpose immobilized-metal affinity chromatography (IMAC) was applied to the separation of P-450 isozymes. The method in its application to rat liver microsomes is described in detail. For method optimization and for the reproducibility of analytical fractionations a completely automatic fast protein liquid chromatographic system especially designed for IMAC is presented. Optimization is done with respect to the choice of the immobilized metal ion and the elution conditions. The chromatographic resolution is markedly enhanced by using segmented *vs.* linear gradients. The efficiency of P-450 resolution is demonstrated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting, verifying the different retention behaviours of the isozymes. However, for all the isozymes analysed so far, reactivity with one particular polyclonal antibody is observed with more than two IMAC fractions of a single run. This may be explained in part by the occurrence of isozymic forms distinguishable by the pattern of chymotryptic peptides. Hence IMAC appears to be suitable for the separation of closely related isozyme forms.

INTRODUCTION

The microsomal monooxygenase system responsible for the metabolism of numerous exogenous and endogenous compounds, such as drugs, xenobiotica, steroids and eicosanoids, includes four protein components: cytochromes P-450, NADPH–cytochrome P-450 reductase, cytochrome b_5 and NADPH–cytochrome b_5 reductase. The broad spectrum of enzymatic activities catalysed by this system is due to the multiplicity of P-450 isozymes [1,2]. According to recent estimates, more than 60 different P-450 isozymes are potentially present in mammalian species [3]. Only a fraction of them is expressed constitutively [4–6], others are induced xenobiotically [7] or as a consequence of pathological alterations [8–10]. Further, the level of individual isozymes is modulated by developmental processes [5,11,12] or hormonal regulation [6,13,14] and varies with respect to sex [4]. Hence the metabolic capacity of the hepatic monooxygenase system for different substrates, such as drugs, carcinogens and endogenous steroids, is variable. A knowledge of the P-450 pattern is therefore of significance for therapeutic measures and the establishment of xenobiotic effects on an organism.

Analysis of the microsomal P-450 pattern is complex, however, because of the large number of structurally and catalytically similar isozymes [15–17]. Therefore, a comprehensive analytical procedure needs to use a combination of different methods. As discussed earlier [18], the application of high-performance liquid chromatography for analytical fractionations of P-450 isozymes has been the subject of only a few studies based on separation by ion-exchange chromatography [19–23]. We have shown that microsomal cytochrome P-450s can be separated by successive anion- and cation-exchange fast protein liquid chromatography (FPLC) into eleven P-450-containing fractions [18]. Several of these fractions still contain a mixture of different P-450 species.

Methods based on additional separation principles might be advantageous for the further chromatographic resolution of these fractions. In this paper we demonstrate the suitability of immobilized-met-

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V. All rights reserved

al affinity chromatography (IMAC) for this purpose. The principle of this method is based on the property of proteins to bind to metal ions that are immobilized on a chelating gel matrix. This binding occurs essentially by histidine residues [24]. To our knowledge, IMAC has not yet been used for the fractionation of P-450 isoenzymes. In a previous paper [18] we reported preliminary experiments demonstrating its suitability for P-450 separation. This method has been further optimized using a completely automatic FPLC system to obtain highly reproducible elution profiles.

EXPERIMENTAL

Animals and animal treatment

Sprague Dawley rats (200–250 g) (Lippische Versuchstierzucht, Extertal, Germany) were treated with P-450 inducers as described by Guengerich and Martin [25] for phenobarbital and β-naphthoflavone, by Ryan *et al.* [26] for isonicotinic acid hydrazide and by Arlotto *et al.* [27] for troleandomycin.

Preparation and solubilization of rat liver microsomes

Liver microsomes of male rats were prepared as described by Guengerich [28] and solubilized in 0.8% Lubrol PX [18]. Insoluble material was removed by centrifugation at 105 000 *g* for 1 h at 4°C. The clear supernatant was passed through a 0.2-μm filter (Minisart NML, SM 16534, Sartorius) and diluted tenfold in equilibration buffer A (see below) prior to chromatographic fractionation.

Immobilized-metal affinity chromatography

For analytical IMAC runs, an automatic FPLC system (Pharmacia, Uppsala, Sweden) was designed, including two high-precision FPLC pumps, one peristaltic pump, a gradient mixer, a gradient controller, four motor valves, two solenoid valves, four sample loops and a fraction collector (Fig. 1). Protein was detected by continuous monitoring at 280 nm. Monitoring at 417 nm is impeded by a strong and variable drift in absorption which cannot be corrected by baseline subtraction. Data storage and processing were performed with a personal computer (Atari PC 3) equipped with a PC Integration Pack (Softron, Gräfeling, Germany). To obtain reproducible elution profiles and identical starting conditions, the followign obligatory washing, equilibration and elution steps were controlled by a program: (1) columns wash with water, (2) charge with appropriate metal ion, (3) column wash with water, (4) columns wash with equilibration buffer, (5) column wash with elution buffer, (6) column wash with equilibration buffer, (7) partial metal discharge by pumping first 1 ml of water and then a defined volume of 200 mM EDTA under reversed-flow conditions, (8) column wash with equilibration buffer from the top, (9) sample injection, (10) column wash with equilibration buffer, (11) elution from either the top or bottom (12) column regneration with 200 mM EDTA and (13) column wash with water.

Because of the relatively low capacity of the metal-charged chelating Sepharose for cytochrome P-450s (*ca.* 50 μg/ml gel), a column with a volume of

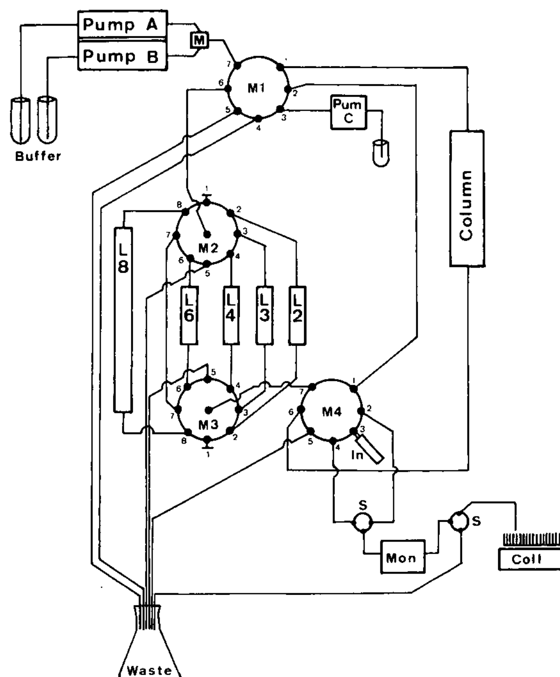


Fig. 1. FPLC system for automatically controlled protein fractionation by IMAC. Coll = fraction collector; In = input for syringe injection; L with figures for the port sets = loops for sample (2), 200 mM EDTA (3), metal chloride solution (4) and water (8); M = gradient mixer; Mon = monitor for UV–VIS detection; M1, M4 = motor valves (type MV7); M2, M3 = motor valves (type MV8); S = solenoid valves.

8 ml (Pharmacia, HR 10/10) was used for the analytical runs. Metal loading was done by passing 9 ml of 200 m*M* metal chloride solution ($NiCl_2$, $ZnCl_2$ or $CuCl_2$ in water) through the column followed by the steps given above. The composition of the equilibration buffer was 50 m*M* potassium phosphate (pH' 7.2)–20% glycerol–0.2% Lubrol PX–0.5 *M* NaCl (buffer A). The elution buffer contained (B) 100 m*M* imidazole or (C) 2 *M* NH_4Cl in addition.

Analytical methods

Solubilized microsomes and column fractions were analysed by the following methods:

(i) Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) with an acrylamide concentration of 8% was carried out according to Laemmli [29] with subsequent silver staining of the protein bands [30] or transfer of the peptides to nitrocellulose membranes by transblotting for immunostaining [31]. To prevent non-specific antibody binding, the membranes were indubated with 10% bovine serum albumin for 2 h prior to addition of the antiserum.

(ii) Quantitative determination of total P-450 was done according to Omura and Sato [32].

(iii) The acitivity of the NADPH–cytochrome P-450 reductase was measured photometrically by cytochrome *c* reduction [33].

(iv) Protein content was determined by the method of Lowry *et al.* [34].

Enzyme purification and antibody production

P-450IA1 (= P-450c), P-450IIB1 (= P-450b), P-450IIE1 (= P-450j), P-450IIIA1 (= P-450p) and NADPH–P-450 reductase were purified as described [25,26,35–37]. Detergent was removed from the samples by chromatography on hydroxyapatite [38] prior to immunization. Rabbits were immunized by subcutaneous injections of purified enzymes emulsified in Freund's complete adjuvant for the first and incomplete adjuvant for the subsequent injections. Blood was drawn from the ear vein and unfractionated serum was used for immunoblotting.

Chemicals

Chemicals were of analytical-reagent grade and purchased from Merck (Darmstadt, Germany), except for Lubrol PX, β-naphthoflavone (Sigma, Deisenhofen, Germany), goat anti-rabbit IgG–peroxidase conjugate (Nordic, Bochum, Germany), acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine, phenobarbital (Serva, Heidelberg, Germany), hydroxyapatite (Bio-Rad Labs., Munich, Germany) and chelating Sepharose Fast Flow (Pharmacia). Troleandomycin was a kind gift from Pfizer (Karlsruhe, Germany).

RESULTS AND DISCUSSION

Automatic control of the chromatographic procedure

Analytical IMAC, as presented here, is a complex chromatographic procedure involving several consecutive equilibration, washing and fractionation steps. Automatic control of all these steps, already helpful for optimization, is essential for the reproducibility of analytical elution profiles. For this purpose we designed a completely automatic FPLC system (Fig. 1).

Depending on the composition of the samples and buffers used, the immobilized metal ion can be dissociated from the resin. For this reason, post-column rebinding of liberated ions by an uncharged gel section is necessary to avoid contamination of the protein fractions. A metal ion-free portion of the column with a defined volume is obtained by EDTA washing from the bottom. In the automatic chromatographic system reported here, all components, *i.e.*, metal ions, EDTA and sample, may be applied from either the top or the bottom of the column, thus allowing great flexibility in the design of chromatographic procedures.

Choice of the immobilized metal

A decisive parameter for P-450 fractionation by IMAC is the type of the immobilized metal ion. Of the ions Zn^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+} and La^{3+}, only the first three were studied in detail and are dealt with here. Their suitability was determined by two parameters: (i) the ability to bind P-450 and (ii) the ability to release active P-450 using standard buffers (see Experimental) and a simple gradient (Fig. 2). The results are summarized in Table I. Judged by these criteria, Ni^{2+}, was found to be the most appropriate ion for our purposes because more than 80% of applied P-450 is usually retained on the column and about 50% recovery is obtained, whereas the amount of unretained P-450 is greater

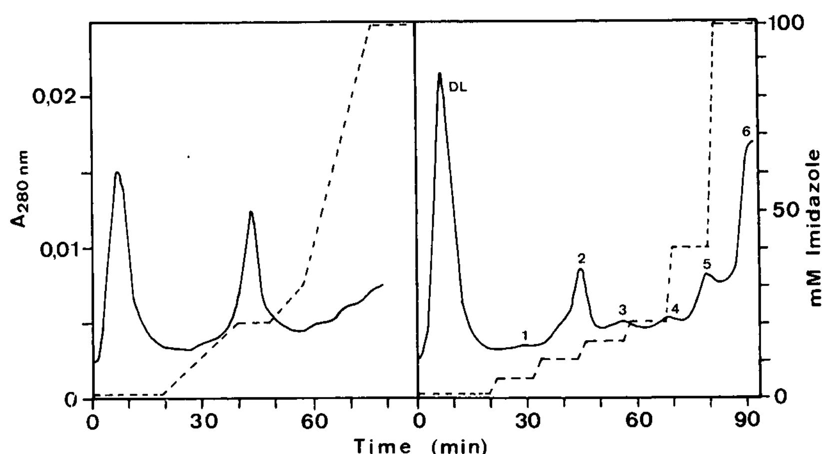


Fig. 2. Effect of the gradient form on the chromatographic resolution of microsomal proteins by IMAC. Gel: chelating Sepharose Fast Flow charged with nickel. Column dimensions: 1 cm diameter, 5.7 ml metal ion-charged gel, 1.2 ml uncharged gel section. Equilibration buffer (A): 50 m*M* potassium phosphate (pH 7.2)–20% glycerol–0.2% Lubrol PX–0.5 *M* NaCl. Elution buffer (B): buffer A + 100 m*M* imidazole. Sample: Lubrol-solubilized liver microsomes of phenobarbital-treated rats, P-450 content 0.86 nmol, protein content 0.51 mg. DL = pass-through fraction. Numbers (1–6): designation of peak fractions corresponding to those given in Fig. 5–7. The gradient forms are shown by the broken lines.

than 50% with immobilized Zn^{2+}. The superiority of Ni^{2+} over Zn^{2+} cannot be derived from the comparison of elution profiles, which appear similar (Fig. 3). In addition, spectroscopic determination of P-450 has to be included for evaluation (Table I).

The observed low capacity of the zinc-charged gel confirms previous results [18]. In contrast to our earlier study [18], however, nickel, which was deemed to be unsuitable for P-450 fractionation by IMAC, proved to be the most appropriate ion in the optimized chromatography system described here. Chelating Sepharose Fast Flow charged with Cu^{2+}

TABLE I

BINDING AND RECOVERY OF CYTOCHROME P-450s FRACTIONATED BY IMAC

Samples: Lubrol-solubilized liver microsomes of rats treated with the inducers indicated (β-NF = β-naphthoflavone; PB = phenobarbital; INH = isonicotinic acid hydrazide). Amount of P-450 applied: 1 nmol. Amounts of protein applied: 1.4 mg (β-NF), 0.6 mg (PB) and 1.8 mg (INH). Chromatographic procedure as described under Experimental. The P-450 contents of the pass-through and eluted fractions were determined spectroscopically [32].

Ion	Eluent	Recovery of applied cytochrome P-450 (%)			Sample
		Pass through fraction	Eluted fraction	Total	
Zn^{2+}	NH_4Cl	56.5	⩽1	57	β-NF
Zn^{2+}	Imidazole	58.4	5.8	64.2	β-NF
Ni^{2+}	NH_4Cl	15.5	12.5	28.0	β-NF
Ni^{2+}	Imidazole	13.1	35.4	48.5	β-NF
Cu^{2+}	NH_4Cl	0	0	0	PB
Ni^{2+}	Imidazole	12.3	30.1	42.4	β-NF
Ni^{2+}	Imidazole	15.0	40.2	45.2	INH
Ni^{2+}	Imidazole	21.4	47.6	68.7	PB

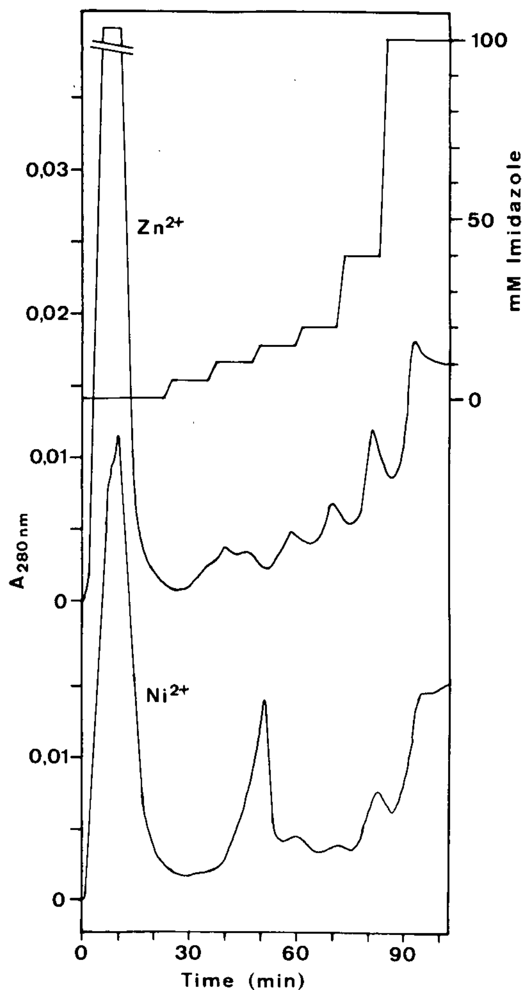


Fig. 3. Binding and elution of cytochrome P-450s of phenobarbital-treated rats to chelating Sepharose charged with nickel or zinc. Conditions as in Fig. 2. Sample: Lubrol-solubilized liver microsomes of phenobarbital-treated rats, P-450 content 0.5 nmol, protein content 0.29 mg. The shape of the gradient is given by the stepped line at the top.

leads to strong and apparently irreversible binding of P-450.

Optimization of P-450 fractionation by IMAC

Binding and recovery of cytochrome P-450s from the nickel-charged gel are dependent on several different parameters such as type of buffering component, pH, type of eluting agent and presence of detergent. For the following studies we used potassium phosphate in equilibration and elution buffers. Some organic buffer substances, such as tris(hydroxymethyl)aminomethane (Tris), are less suitable because they weaken the metal–chelate complex. In this instance dissociated metal is observed by accumulation of coloured ions in the uncharged gel section. As shown in a previous paper [18], P-450 elution by decreasing pH results in a very low recovery of spectroscopically detectable P-450, *i.e.*, about 8% of the applied cytochrome. Elution with a competitive ligand leads to higher yields (Fig. 4, Table I).

With respect to the recovery of spectroscopically detectable P-450, imidazole proved to be more advantageous than ammonium chloride as an eluting agent (Table I). This is probably due to the high concentrations of up to 2 *M* ammonium chloride necessary for P450 elution. Early studies by Imai and Sato [39] demonstrated rapid destruction of P-450 in the presence of high salt concentrations. Therefore, we used the nickel–imidazole system for further studies. Routinely, imidazole elution leads to about 50% recovery of spectroscopically detectable P-450 (Fig. 4, Table I). The exact value might be even higher, because we found that imidazole interferes with the spectroscopic determination of P-450 by inhibiting the formation of the $P\text{-}450_{reduced}$–CO complex. It has long been known that imidazole is a ligand for P-450s [39,40]. Preliminary studies showed that the extent of this inhibition is dependent on the isozyme composition of the sample, because liver microsomal P450s of rats treated with various inducers exhibit different susceptibilities for imidazole-dependent inhibition (not shown).

Successful P-450 desorption from the column requires the presence of detergent. We routinely supplement the equilibration and elution buffers with 0.2% of Lubrol PX. If detergent is ommitted from the buffers the yield of spectroscopically detectable P-450 decreases to about 35%. Simple linear gradients of the competitive ligands result in low resolution of cytochrome P-450 isozymes (Fig. 2). One or two P-450-containing peaks are obtained. Similar to ion-exchange FPLC [18,41], resolution is decisively increased by application of stepwise or segmented gradients leading to five well separated P-450-containing peaks (Fig. 2).

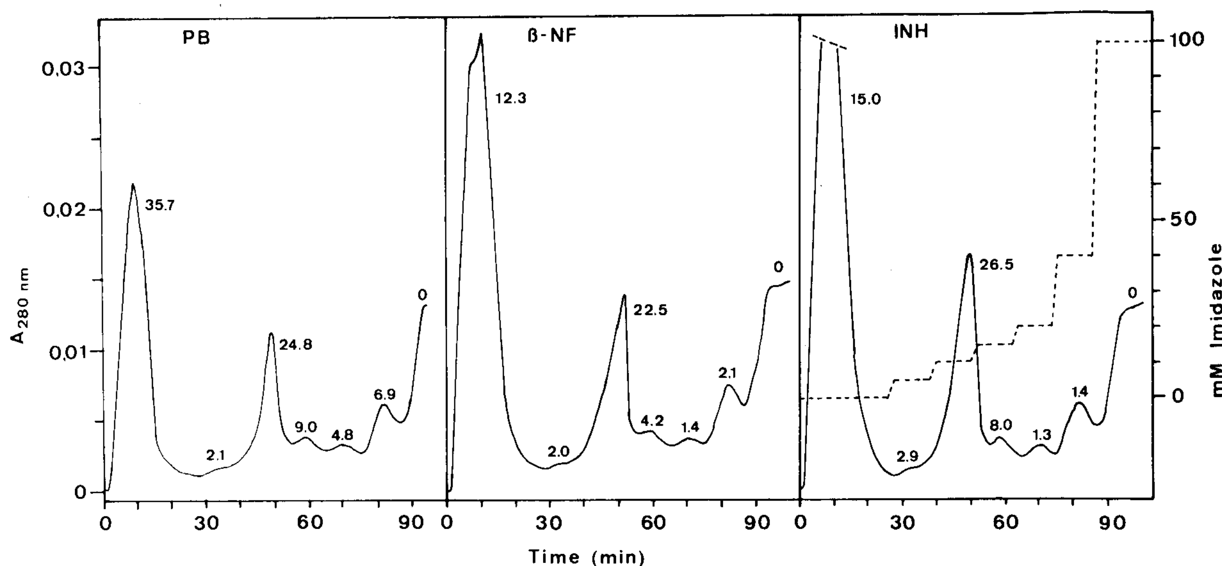


Fig. 4. IMAC fractionation of detergent-solubilized liver microsomes of rats treated with various P-450 inducers. Conditions as in Fig. 2. Flow-rate: 1 ml/min. Samples: Lubrol-solubilized liver microsomes of rats treated with various P-450 inducers. P-450 and protein content of the samples (in nmol and mg, respectively): PB-induced, 1.15 nmol, 0.67 mg; β-NF-induced, 2.3 nmol, 3.29 mg; INH-induced, 1.33 nmol, 2.42 mg. Figures give the percentage of applied cytochrome P-450 in the pooled peak fractions. Gradient form is indicated by the broken line in the chromatogram shown in the right-hand panel.

Chromatographic resolution of microsomal P-450s by IMAC

The efficiency of P-450 fractionation by IMAC was analysed by a combination of different methods, *i.e.*, UV–VIS spectrophotometry of P-450–ligand complexes, SDS-PAGE and immunoblotting. Elution profiles obtained by fractionation of liver microsomal P-450s of rats treated with various inducers are shown in Fig. 4. Although their overall shapes and their quantitative P-450 distributions in the peak fractions (Fig. 4) are very similar, the different P-450 compositions become apparent if the protein patterns of these fractions after SDS-PAGE are compared (Figs. 5–7).

Because of the large number of different isozymes with similar molecular weights, distinct protein bands cannot be assigned to definite P-450 species. Identification of individual P-450 isozymes after fractionation is achieved by immunoblotting using specific or group-specific antibodies. Some of the results of the immunochemical analyses are shown in Figs. 6 and 7 and are summarized in Table II. The differential elution of the analysed isozymes is clearly seen. Treatment of rats with phenobarbital leads to induction of cytochromes P-450 of the IIB family [36] and to a lesser extent of the IIIA family. The immunoblot analyses show that the cytochromes P450IIB1 and -IIB2 are mainly eluted with 40 m*M* imidazole, whereas P450IIIA is either found in the pass-through fraction or is mainly eluted with lower imidazole concentrations (Figs. 6 and 7).

The detection of two proteins with different electrophoretic mobilities reacting with the antibody against P450IIB isozymes (Fig. 6) can be explained by the occurrence of two very closely related isozymes (IIB1 and IIB2). Cross-reactivity of the antibody is very probably because the homology of the amino acid sequences of these two proteins is 97% [42]. Microsomes of rats pretreated with β-naphthoflavone lack isoenzymes of the IIB family [43] (Fig. 5A) but contain P450IA species [35,43] which are concentrated in peak 3 of the IMAC fractionation eluted with 15 m*M* imidazole (Table II, Fig. 5A). Similarly to P-450IIIA, P450IIE1, which can be induced by isonicotinic acid hydrazide [26], shows weak or no binding to the nickel ion-charged column (Table II). The protein band marked with 52.5 kilodalton in Fig. 5B represents the P450IIE isoenzyme as determined by immunoblotting (not shown). The 46.5-kilodalton protein shown in Figs.

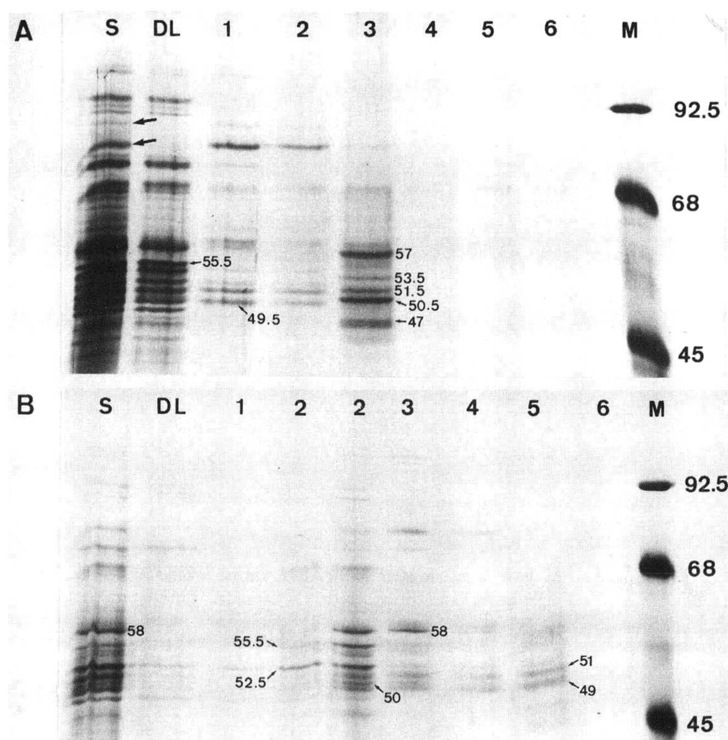


Fig. 5. SDS-PAGE analysis of P-450 fractions obtained by IMAC. Samples for the IMAC runs: Lubrol-solubilized liver microsomes of rats treated with (A) β-naphthoflavone (P-450 content 1.5 nmol, protein content 2.1 mg) and (B) isonicotinic acid hydrazide (P-450 content 1.3 nmol, protein content 2.4 mg). Fractionation: optimized IMAC as described under Experimental using the nickel–imidazole system. Column dimensions and buffers as in Fig. 2. For designation of the fractions and form of the gradient, see Fig. 2, right-hand panel. S = unfractionated sample; DL = pass-through fraction; M = molecular weight markers, with the indicated values in kilodalton. Apparent molecular weights (kilodalton) are assigned to individual protein bands. Large arrows (top left) point to protein bands which are not found in the pass-through fraction but are present in imidazole-eluted fractions showing that the column capacity was not exhausted.

6 and 7 is probably the constitutively expressed and slightly phenobarbital-inducible P450IIA1, which has the lowest apparent molecular weight of all known microsomal P-450 species. NADPH–cytochrome P-450 reductase is bound by the nickel-charghed column and can be eluted in an enzymatically active form with about 15 m*M* imidazole (Table II).

The immunoblot analyses show that isozymes detectable with a single antiserum are present in more than two peak fractions of an IMAC run. An explanation for this may be the existence and chromatographic separation of closely related isozyme forms. Such forms have been described, for example, for the P-450IIB and -IIIA protein families [44–46]. Preliminary results obtained with immunoblotting of IMAC-fractionated, chymotryptic digested P450IIIA species of rats treated with triacetyloleandomycin support this view [47]. Hence IMAC may also be suitable for the separation of closely related P-450 isozyme forms.

In addition to its suitability for analytical P-450 fractionation, IMAC may also provide a useful tool for preparative isoenzyme purification. Starting with a crude extract, *i.e.*, solubilized microsomes, fractions containing only a few proteins are obtained with a single IMAC run (Fig. 6, fractions 1 and 5).

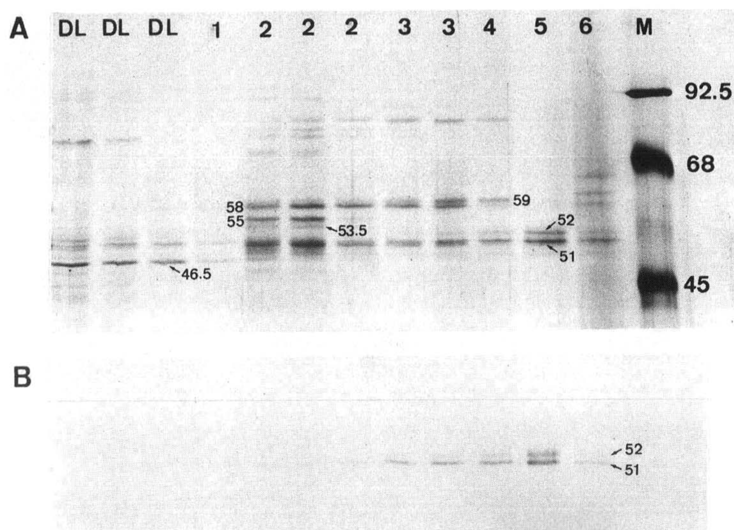


Fig. 6. Identification of P-450 isozymes in IMAC fractions by immunoblotting after SDS-PAGE. Sample for the IMAC-run: Lubrol-solubilized liver microsomes of phenobarbital-treated rats containing 0.53 nmol of cytochrome P-450 and 0.31 mg of protein. Fractionation: optimized IMAC as described under Experimental using the nickel–imidazole system. Column dimensions and buffers as in Fig. 2. For designation of the fractions and form of the gradient, see Fig. 2, right-hand panel. S = unfractionated sample; DL = pass-through fraction; M = molecular weight markers, with the indicated values in kilodalton. (A) Analysis of SDS-PAGE with (B) subsequent immunoblotting using an antibody against cytochromes P-450 IIB1 and IIB2. Apparent molecular weights (kilodalton) are assigned to individual protein bands.

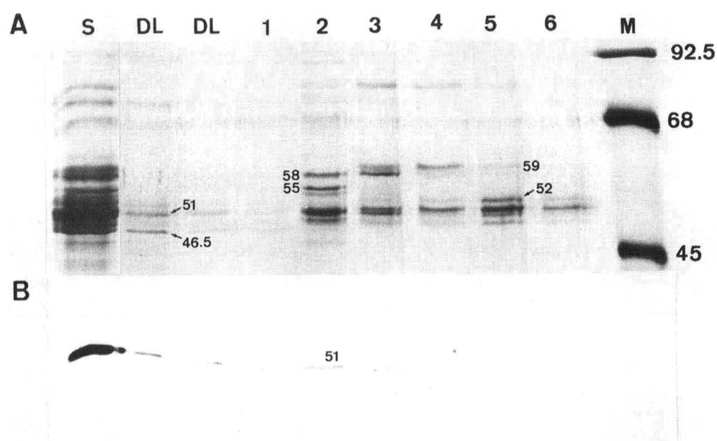


Fig. 7. Identification of P-450 isozymes in IMAC fractions by immunoblotting after SDS-PAGE. Sample for the IMAC-run: Lubrol-solubilized liver microsomes of phenobarbital-treated rats containing 1.15 nmol of cytochrome P-450 and 0.67 mg of protein. Fractionation: optimized IMAC as described under Experimental using the nickel–imidazole system. Column dimensions and buffers as in Fig. 2. For designation of the fractions and form of the gradient, see Fig. 2, right-hand panel. S = unfractionated sample; DL = pass-through fraction; M = molecular weight markers with the indicated values in kilodalton. Apparent molecular weights (kilodalton) are assigned to individual protein bands. (A) Analysis by SDS-PAGE with (B) subsequent immunoblotting using an antibody against cytochrome P-450 IIIA.

TABLE II

DISTRIBUTION OF P-450 ISOZYMES AND NADPH–P-450 REDUCTASE IN IMAC FRACTIONS AS ANALYSED BY IMMUNOBLOTTING AFTER SDS-PAGE OR ENZYMATIC ACTIVITY

Fractionation of microsomal cytochrome P-450s by IMAC was done with the optimized method as described under Experimental. Samples: Lubrol-solubilized liver microsomes of rats treated with various inducers (β-NF = β-naphthoflavone; PB = phenobarbital; INH = isonicotinic acid hydrazide; TAO = troleandomycin). Amount of P-450 applied: *ca.* 1.5 nmol. Amounts of protein applied: 0.88 mg (PB), 0.32 mg (TAO), 2.72 mg (INH) and 2.14 mg (β-NF). Peak designation: DL = pass-through fraction; numbers correspond to the peak fractions as indicated in Fig. 2. The activity of the NADPH–P-450 reductase was determined by reduction of cytochrome *c* [33]. Symbols indicate relative enzymatic activity or staining intensity after peroxidase reaction with chloronaphthol: −, (+), +, ++ and +++ = not visible, faint, slight, medium and strong staining, respectively.

Sample	Antibody to	Peak						
		DL	1	2	3	4	5	6
		Imidazole (m*M*)						
		0	5	10	15	20	40	100
PB	IIB1/IIB2	−	−	(+)	+	+	++	+
PB	IIIA1	++	+	++	+	+	+	+
TAO	IIIA1	++	(+)	+++	++	+	+	+
INH	IIE1	+	+	+	−	−	−	−
β-NF	IA1/IA2	−	−	(+)	+	−	−	−
Sample	**Activity**							
PB	Reductase	−	−	+	++	(+)	−	−

CONCLUSIONS

Our aim is to use IMAC for analytical fractionations in combination with ion-exchange FPLC to elaborate P-450 patterns in microsomal samples. The above results show the suitabiliy of IMAC for this purpose, as closely related forms can be separated and subsequently identified by immunochemical methods. The optimized chromatographic procedures presented here are part of an analytical strategy combining the application of different methods [48]. Fractionation of the cytochromes with high-resolution techniques in combination with immunochemical and spectroscopic methods should allow the detection and determination of very similar known and unknown forms in a complex mixture of P-450 isozymes. Comprehensive analyses of P-450 patterns should help in the study of P-450 induction processes, the evaluation of chemotherapeutic measures and pathologically altered P-450 patterns.

ACKNOWLEDGEMENTS

The author thanks Mrs. Georgia Sommer for expert technical assistance and her untiring enthusiasm and Professor Dr. W. G. Hanstein for fruitful discussions on the subject and the final draft of the manuscript. Thanks are also due to Pharmacia LKB (Freiburg, Germany) for loan of FPLC accessories which were necessary for the automatic IMAC system. This work was supported by the Bundesministerium für Forschung und Technologie (FKZ 07048591).

REFERENCES

1 F. P. Guengerich, *ISI Atlas of Science: Pharmacology*, Institute for Scientific Information, Philadelphia, PA, 1987, p. 205.
2 F. P. Guengerich, *Trends Pharmacol. Sci.*, 10 (1989) 107.
3 F. J. Gonzalez and D. W. Nebert, *Trends Genet.*, 6 (1990) 182.
4 S. Bandiera, *Can. J. Physiol. Pharmacol.*, 68 (1990) 762.

5 D. J. Waxman, G. A. Dannan and F. P. Guengerich, *Biochemistry*, 24 (1985) 4409.
6 S. Bandiera, D. E. Ryan, W. Levin and P. E. Thomas, *Arch. Biochem. Biophys.*, 248 (1986) 658.
7 A. J. Conney, *Life Sci.*, 39 (1986) 2493.
8 L. V. Favreau and J. B. Schenkman, *Biochem. Pharmacol.*, 37 (1988) 3505.
9 K. Sano, Y. Fujioka, K. Nagashima, T. Hashizume, M. Komori, M. Kitada, T. Kamataki and T. Miyazaki, *Hum. Pathol.*, 20 (1989) 1015.
10 M. D. Ferrari, E. A. J. Peeters, J. Haan, R. A. C. Roos, P. Vermey, F. A. de Wolff and O. J. S. Buruma, *J. Neurol. Sci.*, 96 (1990) 153.
11 M. Barroso, O. Dargouge and M. C. Lechner, *Eur. J. Biochem.*, 172 (1988) 363.
12 S. Fujita, R. Morimoto, M. Chiba, K. Kitani and T. Suzuki, *Biochem. Pharmacol.*, 38 (1989) 3925.
13 G. A. Dannan, F. P. Guengerich and D. J. Waxman, *J. Biol. Chem.*, 261 (1986) 10728.
14 A. Mode, E. Wiersma-Larsson and J.-A. Gustafsson, *Mol. Endocrinol.*, 3 (1989) 1142.
15 D. C. Swinney, D. E. Ryan, P. E. Thomas and W. Levin, *Biochemistry*, 26 (1987) 7073.
16 R. E. Thomas, S. Bandiera, L. M. Reik, S. L. Meines, D. E. Ryan and W. Levin, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, 46 (1987) 2563.
17 A. W. Wood, D. E. Ryan, P. E. Thomas and W. Levin, *J. Biol. Chem.*, 258 (1983) 8839.
18 P. H. Roos, *J. Chromatogr.*, 521 (1990) 251.
19 A. N. Kotake and Y. Funae, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 6473.
20 S. K. Bansal, J. H. Love and H. L. Gurtoo, *Eur. J. Biochem.*, 146 (1985) 23.
21 T. Sakaki, A. Soga, Y. Yabusaki and H. Ohkawa, *J. Biochem.*, 96 (1984) 117.
22 M. Kastner and T. Schulz, *J. Chromatogr.*, 397 (1987) 153.
23 J. F. Sinclair, S. Wood, L. Lambrecht, N. Gorman, L. Mende-Mueller, L. Smith, J. Hunt and P. Sinclair, *Biochem. J.*, 269 (1990) 85.
24 J. Porath and B. Olin *Biochemistry*, 22 (1983) 1621.
25 F. P. Guengerich and M. V. Martin, *Arch. Biochem. Biophys.*, 205 (1980) 365.
26 D. E. Ryan, L. Ramanathan, S. Iida, P. E. Thomas, M. Haniu, J. E. Shively, C. S. Lieber and W. Levin, *J. Biol. Chem.*, 260 (1985) 6385.
27 M. P. Arlotto, A. J. Sonderfan, C. D. Klaassen and A. Parkinson, *Biochem. Pharmacol.*, 36 (1987) 3859.
28 F. P. Guengerich, *J. Biol. Chem.*, 272 (1977) 3970.
29 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
30 C. R. Merril, D. Goldman, S. A. Sedman and M. H. Ebert, *Science*, 211 (1981) 1437.
31 H. T. Towbin, T. Staehelin and J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 4350.
32 T. Omura and R. Sato, *J. Biol. Chem.*, 239 (1964) 2370.
33 Y. Yasukochi and B. S. S. Masters, *J. Biol. Chem.*, 251 (1976) 5337.
34 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
35 D. E. Ryan, P. E. Thomas and W. Levin, *J. Biol. Chem.*, 255 (1980) 7941.
36 D. E. Ryan, P. E. Thomas, D. Korzeniowski and W. Levin, *J. Biol. Chem.*, 254 (1979) 1365.
37 S. A. Wrighton, P. Maurel, E. G. Schuetz, P. B. Watkins, B. Young and P. S. Guzelian, *Biochemistry*, 24 (1985) 2171.
38 Y. Funae and S. Imaoka, *Biochim. Biophys. Acta*, 842 (1985) 119.
39 Y. Imai and R. Sato, *J. Biochem.*, 62 (1967) 239.
40 C. R. E. Jefcoate, J. L. Gaylor and R. L. Calabrese, *Biochemistry*, 8 (1969) 3455.
41 P. H. Roos, B. Golub-Ciosk and W. G. Hanstein, in E. Schuster (Editor), *Cytochrome P-450: Biochemistry and Biophysics*, Taylor and Francis, London, 1989, p. 53.
42 Y. Suwa, Y. Mizukami, K. Sogawa and Y. Fujii-Kuriyama, *J. Biol. Chem.*, 260 (1985) 7980.
43 P. E. Thomas, L. M. Reik, D. E. Ryan and W. Levin, *J. Biol. Chem.*, 258 (1983) 4590.
44 G. P. Vlasuk, J. Ghrayeb, D. E. Ryan, L. Reik, P. E. Thomas, W. Levin and F. G. Walz, *Biochemistry*, 21 (1982) 789.
45 M. Oertle, D. Filipovic, G. Vergeres, K. H. Winterhalter, C. Richter and E. E. Di Iorio, in E. Schuster (Editor), *Cytochrome P-450: Biochemistry and Biophysics*, Taylor and Francis, London, 1989, p. 57.
46 J. R. Halpert, *Arch. Biochem. Biophys.*, 263 (1988) 59.
47 P. H. Roos and W. G. Hanstein, in preparation.
48 P. H. Roos, B. Golub-Ciosk, P. Kallweit and W. G. Hanstein, *Biol. Chem. Hoppe-Seyler*, 371 (1990) 747.

Journal of Chromatography, 587 (1991) 43–54
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2377

High-performance metal chelate affinity chromatography of cytochromes P-450 using Chelating Superose

Michael Kastner* and Diether Neubert

Institut für Toxikologie und Embryopharmakologie, Garystrasse 5, 1000 Berlin 33 (Germany)

ABSTRACT

High-performance metal chelate affinity chromatography [immobilized metal ion affinity chromatography (IMAC)] using Chelating Superose (iminodiacetic acid adsorbent) was investigated for its suitability in purifying phenobarbital-induced rat liver microsomal cytochrome P-450 isozymes (P450) and optimized for preparative purposes. Starting with an 8-aminooctyl-Sepharose fraction of partially purified P450, it was found that only Ni^{2+}- and Cu^{2+}-charged columns could bind P450. No binding was ever observed when Zn^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+}, Fe^{3+}, Fe^{2+} or Tl^{3+} ions were employed. Of eight commonly used elution buffers, imidazole and tryptamine were found to cause some denaturation of P450. For desorption of proteins bound to Ni^{2+}-charged columns, the following order of decreasing elution buffer strength was determined: cysteine $\approx$ histidine $>$ glycine $>$ histamine $>$ tryptophan $>$ ammonium chloride. During protein desorption with some of these buffers, metal ions were found to bleed from the gel, resulting in P450 denaturation. This could be eliminated by prebleeding the charged columns prior to sample application and had an effect on product recovery and homogeneity. Ni^{2+} and glycine were chosen as a standard for further optimization involving sample adsorption conditions as influenced by equilibration buffer, detergent, load capacity and flow, gradient and temperature conditions. In this way, potassium phosphate (pH 7.75) and 0.4% Emulgen 911 were used to equilibrate a 1.6-ml column and purify 20–50 nmol of P450 (5–15 mg of protein) within 15 min. One gradient fraction consisted of a single sodium dodecyl sulphate–polyacrylamide gel electrophoresis band as judged by silver staining and represented about 25% of the total P450 applied to the column; total recoveries were usually more than 80%. Comparison with the molecular weights and spectral, catalytic and immunological properties of P450 forms isolated according to established procedures indicated that the form isolated here using Chelating Superose comprises mainly P450 2B1 (PB-B). A method is described for fully automated, programmable column regeneration and sample runs.

INTRODUCTION

The history and principles of high-performance metal chelate affinity chromatography [immobilized metal ion affinity chromatography (IMAC)] have been dealt with in recent reviews [1–3] and will not be discussed here. To our knowledge, the use of this type of chromatography in separating forms of cytochrome P-450 isozymes (P450) for preparative purposes has not been reported. IMAC has, however, recently been examined by Roos [4,5], who employed Chelating Sepharose Fast Flow on an analytical basis for fractionating these enzymes. Today, endeavours to isolate preparative amounts of P450 may still be encountered, however, for example in *in vitro* testing of drugs [6] and in characterizing P450 forms derived from hitherto unstudied animal species [7]. More often than not, classical purifications involving, for example, ion-exchange and hydroxyapatite gels, even in fast protein liquid chromatographic (FPLC) or high-performance liquid chromatographic (HPLC) systems, result in poor yields, inhomogeneous products or both. For this reason, we considered IMAC as a possible approach to this task, and attempted to optimize systematically a number of parameters affecting this method. For a new protein, we expect that, as in any affinity chromatography, a certain amount of empiricism will be unavoidable.

In view of the fact that each run necessitated a column regeneration involving sequential treatment with up to seven different solutions, an increased number of FPLC pumps and valves were utilized, and the automation of this is described.

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V. All rights reserved

EXPERIMENTAL

Sample preparation

An 8-aminooctyl-Sepharose pool of combined P450 forms derived from phenobarbital-induced male Wistar rats was prepared as described [7] and kept at −80°C until further use. Prior to IMAC, salt removal and detergent exchange were effected by gel filtration on HR 10/10 columns of Sephadex G-25 Superfine or disposable NAP-25 columns (Pharmacia, Uppsala, Sweden). The buffer used for gel filtration elution was always the same as the start buffer described below. Samples for IMAC usually consisted of 2–5 nmol of P450. The specific content of this particular sample lot was 3.4 nmol of P450 per milligram of total protein.

Chemicals

All substances were of the highest purity commercially available. Imidazole was recrystallized once from toluene and then once from ethanol. Tryptamine was recrystallized twice from ethanol.

FPLC

All parts of the equipment necessary for all runs were purchased from Pharmacia. Chelating Superose, consisting of the chelating group iminodiacetic acid (IDA) covalently linked to a Superose 12 base matrix of 13-μm particle size, was used as supplied in a prepacked HR 10/2 (20 mm × 10 mm I.D., 1.6 ml) glass column. Chromatography was carried out as described for this column by the manufacturer and necessitated the following steps for each single run.

(a) Regeneration by successive washings with 6 ml of 40% acetic acid, 6 ml of 500 m*M* NaCl in water (pH 5–6), 4 ml of 200 m*M* metal chloride (usually nickel chloride, see below) in water (pH 5–6) and then 8 ml of water (pH 5–6). When a different metal ion was to be charged, the regeneration procedure was preceded by a wash with 16 ml of 50 m*M* EDTA and 500 m*M* NaCl in water (pH 5–6).

(b) Equilibration with 6 ml of buffer A: 50 m*M* start buffer [usually potassium phosphate (pH 7.5)], 20% glycerol and 500 m*M* NaCl. Detergents were always present and consisted in most instances of 0.4% (w/v) Lubrol PX (Sigma, St. Louis, MO, USA). Prebleeding (see below) consisted of a wash with usually 2 ml of buffer B (elution buffer as given in Table I) at a concentration of 50–500 m*M* in buffer A, and then 6 ml of buffer A alone.

(c) Sample load and injection via motor valve and 2-ml sample loop.

(d) Sample run. After the pass-through fraction had eluted, a gradient of buffer B in buffer A was applied over a volume of 20 ml. Detection was at 280 and 405 nm simultaneously and the flow-rate was (in most instances) 2.0 ml/min. The temperature was 25°C and fractions of 1 ml were collected in a rack filled with ice.

Automation

Full automation of all steps outlined above from the beginning of column regeneration to the end of fraction collection was facilitated by integrating the following accessories: a third high-pressure pump (P-500), a 15-pole 24-V relay switch-box, a peristaltic pump (P-1) and three motor valves (MV-8). Illustrations of similar assemblies have been presented in detail by the manufacturer in an FPLC Technical Note on automation under the headings Multidimensional Chromatography and Column Cleaning and will be reiterated here only briefly. The third high-pressure pump, filled with water, allows continuous column regeneration without having to empty and refill the buffer solution originally present in pump A or B between each run. A novelty in this work is the relay switch-box which allows programmable automation of the third pump, including solvent exchange via a port (number 3 or 4) from the controller. The peristaltic pump and one motor valve allow successive washings with each of the column regeneration solutions described above. Finally, addition of one more motor valve (MV-8) between the peristaltic pump and the original motor valve (MV-7) makes it possible to alternate between load via peristaltic pump (for regeneration solutions) and load via syringe (for sample solution). For chromatographic runs, variation of options such as prebleeding or charging with a new metal ion, and also evaluation, was carried out by combining in one program any number of sub-programs controlled by the software package FPLC-manager.

Optimization strategy

A list of parameters noticeably affecting any IMAC separation will include several items not en-

countered in more conventional types of chromatography, such as ion exchange, hydrophobic interaction, hydroxyapatite or gel filtration [1]. As the optimum results for most parameters will also depend on the order of selection of these, the following sequence was followed: choice of metal ion, elution buffer, bleeding and prebleeding, suitable metal ion–elution buffer pairs, variation in further chemical parameters such as pH, and lastly physical parameters such as flow-rate.

Assays

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 7.5% homogeneous and 4–15% gradient gels with silver staining in an automated PHAST system (Pharmacia) as described previously [7]. Use of the word homogeneity in this work refers in SDS electrophoreses to the area of the main band relative to the total area of all bands in the same lane, as computed by a laser densitometer (Elscript 400, Hirschmann, Unterhaching, Germany). Assays concerning spectral, magnetic and catalytic properties [7] and immunological characterizations [8] were also performed as described.

RESULTS AND DISCUSSION

Chelating ligands

The chelating ligand investigated towards its affinity chromatographic properties was iminodiacetic acid (IDA). Coupling of tris(carboxymethyl)ethylenediamine (TED) yields a metal-chelating gel which, in relation to IDA, has been shown elsewhere to result in stronger retention of metal ion and thus weaker retention of protein [1,9]. Use of this chelating ligand for the purification of P450 was not investigated here.

Metal ions

When Chelating Superose was charged with Zn^{2+}, Co^{2+}, Mn^{2+}, Cd^{2+}, Fe^{3+}, Fe^{2+} or Tl^{3+} ions and equilibrated using the standard conditions described above [with start buffer 50 m*M* potassium phosphate (pH 7.5), 500 m*M* NaCl, 20% glycerol and 0.4% Lubrol) and then loaded with 5 nmol of P450, no binding of this protein was ever observed. Using Chelating Sepharose Fast Flow, Roos [4] found that only Zn^{2+}-charged columns were capable of binding a similar preparation of P450, but this may possibly reflect a difference between properties of the gel matrices. The recoveries of P450 (in the pass-through fractions) as judged spectroscopically [10] were quantitative for all metal ions except Cd^{2+} and Fe^{2+}. Prebleeding (see below) Cd^{2+}- and Fe^{2+}-charged columns increased the total recovery of spectroscopically intact P450 but did not result in binding of this protein.

Cu^{2+}- and Ni^{2+}-charged columns were reproducibly found to be capable of binding P450, and the degrees of this were dependent on both the choice of elution buffer and the use of prebleeding. In all instances, Cu^{2+}-charged columns permitted tighter binding, in agreement with Porath *et al.* [11]. This was evidenced in this work by the finding that the total yields for elutions from Cu^{2+}-charged columns were consistently less than those from Ni^{2+}-charged columns. Remaining bound protein in such instances was nonetheless retrieved quantitatively and spectroscopically intact with a stepwise gradient of 50 m*M* EDTA or, with substantial denaturation, using a descending pH gradient as described below.

Elution buffers

Commonly used buffers in IMAC for the desorption of bound P450 were first inspected for their ability to retain enzyme activity. Concentrations in m*M* were chosen for gradients found appropriate for the elution of P450. In addition, the buffer solutions contained 50 m*M* potassium phosphate (pH 7.5), 500 m*M* NaCl, 20% glycerol and 0.4% Lubrol PX. Judgment of intact P450 and P420 (denatured P450) contents recovered in the presence of these buffer solutions prior to any chromatography was based on spectrophotometric measurements [10] after contact for 15 min at 25°C. Assessment of real catalytic activities based on substrate reactions is considered below. The recoveries of P450 and P420 shown in Table I are each given as a percentage of the total P450 originally present. Imidazole and tryptamine each gave in our opinion unacceptable degrees of denaturation even after having been recrystallized twice before use. Loss of P450 seen here appeared to be over and above that seen to cause the type II spectral changes which are known for these kinds of compounds [12]. These two buffers will not be considered further here.

TABLE I

EFFECT OF ELUTION BUFFERS ON P450 STABILITY

Contents of intact P450 recovered in the presence of various elution buffers were determined spectrophotometrically [10]. The recoveries of P450 and P420 are each given as a percentage of the total P450 originally present. Values shown represent means of triplicate measurements at 25°C. Sample buffer includes 50 mM potassium phosphate and 0.4% Lubrol. For further details, see text.

Elution buffer	Concentration (mM)	Recovery (%)		
		P450	P420	Total
None		100	0	100
Glycine	200	100	0	100
Ammonium chloride	500	99	0	99
Cysteine	50	97	3	100
Tryptophan	50	93	0	93
Histidine	50	92	3	95
Histamine	50	83	17	100
Imidazole[a]	50	43	20	63
Tryptamine[a]	50	28	0	28
EDTA	50	100	0	100
pH	pH 4.5	34	18	52

[a] Recrystallized as described under Experimental.

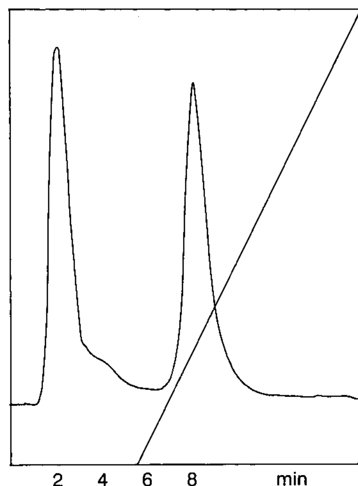


Fig. 1. Elution profile of FPLC purification of P450 on Chelating Superose. Sample, 3.9 nmol of P450 (1.13 mg of protein); column size, 20 mm × 10 mm I.D. (1.6 ml); metal ion, Ni^{2+}; flow-rate, 2.0 ml/min; sample buffer, 50 mM potassium phosphate (pH 7.5)–20% glycerol–500 mM sodium chloride–0.4% Emulgen 911; gradient, 0–200 mM glycine over 20 ml; detection, 405 nm at 0.05 a.u.f.s. Prior to application of the sample, the column was prebled with 2 ml of 200 mM glycine.

EDTA and decreased pH values were also found to be capable of eluting P450 bound to IMAC columns. However, as EDTA resulted in complete stripping of coordinated metal ion, and as low pH values resulted in considerable denaturation, these two modes of elution were not investigated further. Interestingly, the non-ionic detergent Emulgen 911 in the start buffer allowed more elution of spectroscopically intact P450 and at a lower pH than did Lubrol PX. After a pH gradient of 7.7–3.0, the highest recovery of P450 in the gradient using 0.4% Emulgen 911 was 55% (at pH 4.5), whereas using 0.4% Lubrol PX the highest recovery was only 34% (at pH 5.5).

In all instances after the application of a continuous gradient of any elution buffer, there appeared only a single, narrow, symmetrical peak. A typical chromatogram is shown in Fig. 1. This appearance of the chromatographic profile has also been reported for the IMAC purifications of α_1-antitrypsin and α_1-macroglobulin [13] and platelet-derived growth factor [14].

Bleeding and prebleeding

Bleeding (the loss or leakage of metal ions from the gel's complexing groups during application of the sample and/or elution buffer gradient) is common for many metal ion–elution buffer pairs and often of no significance [1] other than the fact that the eluents containing the proteins of interest may also contain metal ions. In such instances, this can be obviated by deliberately charging the column under non-saturating concentrations of metal ion, by subsequent batch treatment of the eluents with uncharged gel (such as Chelating Sepharose Fast Flow) or by attaching a second, uncharged, column in tandem as employed by Roos [4].

Bleeding during initial application of elution buffer may, however, adversely affect the adsorption and stability of the applied protein owing to a type of metal contact denaturation. This minimum, "threshold" concentration of elutable metal ion causing protein denaturation can be removed by prebleeding the charged column with elution buffer before applying the sample.

We found that using the six elution buffers described above, metal ions were routinely bled from both Ni^{2+}- and Cu^{2+}-charged columns (see Table II, where the values given represent contents of

P450 recovered after chromatography as a percentage of the applied load). The effects of this can now be summarized into three categories.

First, bleeding from Ni^{2+}-charged columns in most instances had no great effect on the total yield (Table II, except for elution buffers cysteine and histidine which are discussed below). That is, for elution buffers such as glycine or histamine and to a lesser extent tryptophan and ammonium chloride with Ni^{2+}-charged columns, there is no dramatic difference between the total recoveries of P450 for "non-prebled" and "prebled" columns. Prebleeding these columns therefore appears to be unnecessary. It appears, in fact, only to lead to less binding and more pass-through material. This finding is in accordance with that of Porath and Olin [9], who omitted prebleeding in such a system. The amount of charged Ni^{2+} ion left on the column as a function of the volume of prebleeding with the elution buffer glycine is discussed below. Ni^{2+} ions in the eluents can be eliminated with uncharged gel as described above or by subsequent hydroxyapatite chromatography, *e.g.*, in conjunction with removal of excess detergent.

Second, prebleeding Cu^{2+}-charged columns appeared to be necessary. If these were not prebled, almost no P450 protein was recovered spectrophotometrically intact (Table II). Note that this denaturation also applies to the pass-through fraction (*i.e.*, before the application of any elution buffer). In this instance, subsequent treatment with uncharged gel was of no use. Prebleeding Cu^{2+}-charged col-

TABLE II

EFFECTS OF BLEEDING AND PREBLEEDING ON TOTAL RECOVERY

Recoveries of P450 contents as a percentage of the applied load were detected spectrophotometrically [10] in pass-through and gradient fractions subsequent to chromatography on Cu^{2+}- or Ni^{2+}-charged columns, each with six different elution buffers. The start buffer included 0.4% Lubrol PX (pH 7.5) (see also text). In each instance, values are given for non-prebled and prebled columns. For Ni^{2+}-charged columns, values in parentheses indicate recoveries of P450 plus P420. It is these values for the gradient fractions in parentheses which were used to deduce a relative order of elution buffer strength.

Elution buffer	Fraction	Metal ion				
		Ni^{2+}-charged columns			Cu^{2+}-charged columns	
		Non-bled	(P450 + P420)	Prebled	Non-bled	Prebled
Cysteine	Pass-through	16	(14)	94	0	95
	Gradient	49	(84)	3	0	0
	Total	65	(98)	97	0	95
Histidine	Pass-through	16	(13)	89	0	91
	Gradient	71	(82)	4	7	0
	Total	87	(95)	93	7	91
Glycine	Pass-through	14	(15)	46	0	6
	Gradient	78	(78)	50	0	13
	Total	92	(93)	96	0	19
Histamine	Pass-through	16	(15)	45	0	5
	Gradient	57	(69)	36	3	40
	Total	73	(84)	81	3	45
Tryptophan	Pass-through	15	(16)	33	0	4
	Gradient	34	(45)	39	0	23
	Total	49	(61)	72	0	27
Ammonium chloride	Pass-through	16	(15)	32	0	5
	Gradient	13	(13)	13	0	2
	Total	29	(28)	45	0	7

umns, however, markedly and reproducibly increased the yield of intact P450, especially in the gradient fractions where the elution buffers glycine, histamine and tryptophan were used.

Third, with Ni^{2+}- or Cu^{2+}-charged columns and elution buffers cysteine or histidine, prebleeding appeared to be impossible as these buffers not only desorbed protein (from non-prebled columns) but also removed all of the charged metal ion from the gel matrix prior to loading of any protein. This was evidenced by the bluish colours of the gradient eluents and the subsequent quantitative yields of (spectroscopically still intact) P450 in the pass-through eluents.

In Table II, the values given in parentheses for Ni^{2+}-charged, non-prebled columns represent the total amount of P450 and P420 recovered as a percentage of the total P450 applied to the column. Using these values, and given the premise that cysteine and histidine are judged as "stronger" buffers where P450 and P420 are taken together, the decreasing order of elution strength is then cysteine $\approx$ histidine $>$ glycine $>$ histamine $>$ tryptophan $>$ ammonium chloride. Judging qualitatively, of course, glycine is a "better" elution buffer for high recoveries of intact P450.

An attempt to summarize qualitatively the results on bleeding and prebleeding according to the three categories described above is presented in Table III.

Table IV illustrates the effect of the degree of prebleeding on sample binding and homogeneity of the gradient fraction. As might be expected, it was found that more prebleeding results in less capacity (for any or all forms of P450). At the same time, however, more prebleeding also seems to result in less apparent electrophoretic homogeneity of bound P450, suggesting that the binding of Ni^{2+}-charged columns is specific for only one (or a few) forms of P450. With regard to Table IV, the gradient recovery (of up to 86%) and apparent homogeneity (of up to 85%) actually imply a yield of homogeneous P450 of up to 70%. Evidence for the presence of more than one P450 form in this fraction is discussed below under *Characterization of the purified P450 form(s)*.

Suitable metal ion–elution buffer pairs

Regarding the protein sample and elution conditions present, it is obvious from Table II that some pairs are more suitable than others for preparative chromatographic separation. Cysteine and histidine are less suited because they strip too much metal ion. On the other hand, ammonium chloride was found to elute very little bound P450. Increasing the concentration of this elution buffer to 2 *M* had no further effect. Up to 100% of the P450 load was, however, retrieved spectroscopically intact after elution with 50 m*M* EDTA. The final criterion thus involves (a) a good overall yield and (b) a reasonable amount of binding for subsequent elution.

TABLE III

BLEEDING AND PREBLEEDING IN IMAC: QUALITATIVE SUMMARY OF RESULTS

Chelating Superose columns with various metal ion–elution buffer combinations were used to purify P450. For chromatographic conditions, see Table II.

Bleeding occurs during application of	Result	Example	Remedy	Prebleeding category
Elution buffer	Metal ions in eluent	Ni^{2+}–glycine	Metal ions capture methods (see text)	Unnecessary
Sample	Denaturation of pass-through and gradient	Cu^{2+}–NH_4Cl, glycine, tryptophan, histamine	Prebleeding	Necessary
Elution buffer (Ni^{2+}), sample and elution buffer (Cu^{2+})	Total stripping of metal ions	Ni^{2+}, Cu^{2+}–cysteine, histidine	None	Impossible

TABLE IV

EFFECT OF DEGREE OF PREBLEEDING ON SAMPLE BINDING AND HOMOGENEITY OF THE GRADIENT FRACTION

A Ni^{2+}-charged column of Chelating Superose was prebled with various volumes of the elution buffer (200 m*M* glycine) and then equilibrated further with start buffer which included 0.4% Lubrol PX (for other constituents, see Experimental) prior to sample loading. The sample load in each instance was 6.8 nmol of P450. Values depict the recoveries of spectroscopically intact P450 as a percentage of the load. Values for homogeneity indicate the apparent homogeneity of the protein(s) eluted in the gradient peak and are in percent as defined under Experimental

Parameter	Volume (ml) of elution buffer used for prebleeding					
	0	1	2	4	8	15
Recovery of pass-through (%)	14	34	46	72	86	99
Recovery of gradient (%)	86	61	50	23	5	0
Total recovery (%)	100	95	96	95	91	99
Homogeneity of gradient fraction (%)	85	79	74	70	65	n.d.[a]

[a] Not determined.

Four pairs which meet these requirements are Ni^{2+} and glycine, histamine or tryptophan, and Cu^{2+} and histamine.

For the sake of simplicity of comparison, only Ni^{2+}–glycine, Ni^{2+}–histamine and Cu^{2+}–histamine systems were investigated further. The yields in the gradient portions of such chromatographic separations using prebleeding (in Table II 50, 36 and 40%, respectively), are considered acceptable for preparative purposes and were reproducible to within ±10% of those mean values from three runs each.

Judging by the overall yields and apparent electrophoretic homogeneity of the gradients in these three systems (Fig. 2) the Ni^{2+}–glycine system seemed to be the most suitable, and was chosen for further optimization.

As among such combinations of metal ions and elution buffers the elution strengths of the buffers necessary to elute substantial amounts of bound P450 are also high enough to elute some amounts of metal ion, this means that metal ions must be recharged prior to each run. This is in contrast, for example, to a combination involving the separation of standard marker proteins using Cu^{2+} (a "strong binder") and ammonium chloride (a "weak eluter") [1]. In this instance, the proteins could be loaded and eluted more than twenty times without having to recharge the column with Cu^{2+} ions.

Start buffers

The use of potassium or sodium phosphate as start (or "equilibration" or "sample") buffer resulted in very narrow, symmetrical gradient peaks. Tris–HCl as start buffer gave similar profiles where the gradient peaks were much smaller and contained up to 70% less P450, indicative of a reduction in binding as reported elsewhere for this buffer [15]. Sodium acetate gave even smaller, very round and tailing peaks.

pH values

When runs were made at pH 7.0–8.0, higher values resulted in gradient peaks being narrower and having decreased retention volumes and lower yields. This is summarized in Table V, where it is noticeable that recoveries in the gradient fraction decreased by 30%. This is in agreement with the results of Kato *et al.* [16], who used a Zn^{2+}-charged column and glycine to separate a mixture of transferrin and carbonic anhydrase. Here, too, proteins were more strongly retained at lower pH, although as originally postulated by Porath *et al.* [11] for IMAC, the affinity of proteins for these metal ions at alkaline pH should be more effective but less specific. We observed with increasing pH, however, more electrophoretic homogeneity (Table V). A reasonable pH optimum for the sample in this work therefore seems to be between 7.75 and 8.0.

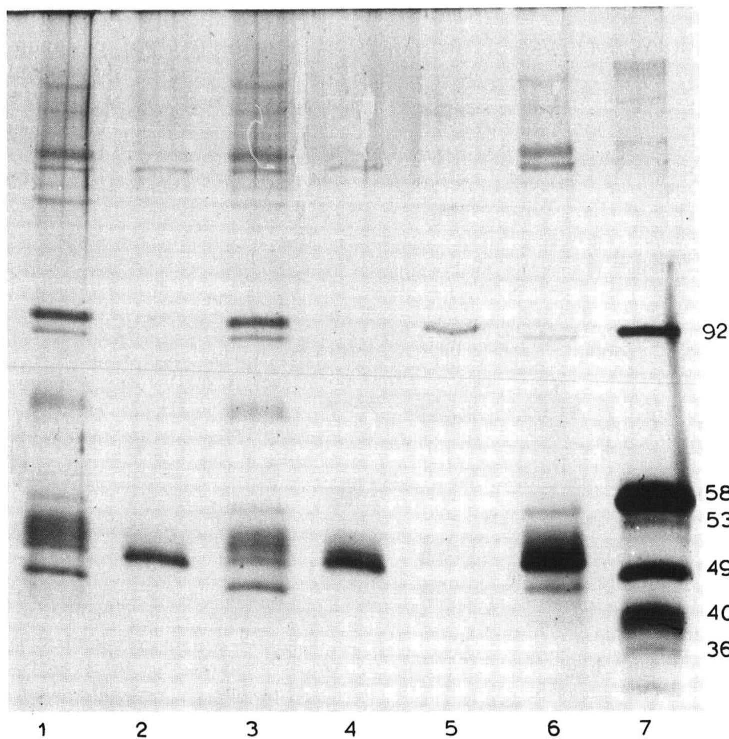


Fig. 2. SDS-PAGE of P450 as purified using Chelating Superose. Start buffer includes 0.4% Lubrol PX (pH 7.5) (see also Experimental). Metal ion and elution buffer, Ni^{2+} or Cu^{2+} and glycine or histamine as depicted. Load in each instance, 8-aminooctyl-Sepharose pool of solubilized phenobarbital-induced rat liver microsomes. The homogeneous 7.5% gel was stained with silver [7] and the anode is at the bottom. Lanes: 1 and 2 = pass-through and gradient fraction of a Ni^{2+}-charged column eluted with glycine; 3 and 4 = pass-through and gradient fraction of a Ni^{2+}-charged column eluted with histamine; 5 and 6 = pass-through and gradient fraction of a Cu^{2+}-charged column eluted with histamine; 7 = marker proteins (with molecular weights in kilodalton): phosphorylase *a* (92), catalase (58), glutamate dehydrogenase (53), fumarase (49), aldolase (40) and lactate dehydrogenase (36).

TABLE V

EFFECT OF pH ON RETENTION TIME, PEAK WIDTH, RELATIVE P450 RECOVERY, AND HOMOGENEITY OF THE GRADIENT FRACTIONS

The relative P450 recoveries in the pass-through fractions were not determined here; total P450 recoveries of gradient and pass-through fractions together for all pH values were consistently 85–95%. For definitions, see Experimental. Metal ion, Ni^{2+}; elution buffer, glycine; detergent, 0.4% Lubrol; sample load, 3.6 nmol.

Parameter	pH				
	7.0	7.25	7.5	7.75	8.0
Retention time (min)	3.2	4.0	2.7	2.6	2.7
Peak width (ml)	9.8	9.4	7.3	6.8	5.3
Relative P450 recovery (%)	63	64	55	44	33
Homogeneity (%)	79	83	84	88	91

Detergents

Chromatographic separations incorporating 0.4% (w/v) concentrations of five of the most commonly used non-ionic detergents in P450 purification schemes were compared: Emulgen 911, Emulgen 913, Lubrol PX, Nonidet P40 and Renex 690 [17,18].

As far as apparent SDS-PAGE homogeneity, P450 recovery, peak volume (area under the curve, AUC), peak width and retention time were concerned, Emulgen 911, Emulgen 913, Lubrol PX and Renex 690 all gave similar results. Emulgen 911 was slightly more advantageous with regard to P450 recovery and peak width. As mentioned above, during descending pH gradients, Emulgen 911 also had a higher stabilizing effect on P450 than did Lubrol PX.

Nonidet P40, on the other hand, gave very flat profiles and poor yields, and it was noticed that during chromatography the column back-pressure increased strongly. This was also found, albeit to a lesser extent, when the anionic detergent sodium cholate was used, presumably owing to detergent binding to the gel matrix, as has been shown elsewhere for the strong anion exchanger Mono Q [17].

In contrast to Kato *et al.* [19], who used the detergent octaethylene glycol dodecyl ether (polyoxyethylene 8 lauryl ether, $C_{12}E_8$) with IMAC (where the adsorbent was also IDA) to fractionate human placenta mitochondrial membranes, we could not demonstrate that this detergent was in any way superior to Emulgen 911. It should be pointed out that for the chromatogram illustrated there, the gradient was started concomitant with sample application.

Flow-rates

A flow-rate of 2.0 ml/min (2.53 cm/min) was used for routine screenings, allowing a complete run with a continuous gradient (see below) in 15 min. Higher values were avoided as this resulted in more than the 1.0 MPa back-pressure reported by the manufacturer to be deleterious to this gel matrix. Flow-rates of down to 0.5 ml/min did not resolve gradient peak profiles into more peaks or shoulders as judged optically and did not have any effect on the separation of proteins as judged by SDS-PAGE of individual fractions. There was also no noticeable negative effect on the total recovery with prolonged contact with the gel at 25°C.

Gradients

Fast chromatographic runs incorporated continuous gradients over 20 ml. Using a Ni^{2+}-charged column with 0–200 m*M* glycine at a flow-rate of 2 ml/min, fractionation of this P450 sample routinely yielded, as mentioned above, a single symmetrical gradient peak between 20 and 60 m*M* glycine. Increasing the gradient size to 30 ml or more brought about no apparent improvement in resolution. Using a flow-rate of 0.5 ml/min reduced the concentration at which P450 eluted with this buffer; a stepwise gradient of 6–10 m*M* (in place of 20–60 m*M*) glycine at this flow-rate resulted in a gradient peak shoulder. Again, however, this caused no apparent heterogeneity with regard to molecular weight as revealed by SDS-PAGE. Use of such segmented gradients may, of course, be helpful in analytical fractionations of P450 derived from a variety of other inducing agents, as was recently reported by Roos [5].

Temperature

Chromatographic runs at 25°C using the conditions described above routinely yielded 80–100% recoveries of P450. Maintaining the column in a 4°C ice-bath during chromatography in fact reduced the recovery and resolution, as has been reported in ion-exchange chromatography [20]. In the present work, the basic chromatographic profile of separations at 0–4°C remained unaltered, but the peak width of the gradient fraction was more than doubled and the total recovery here was decreased by 40%.

Sample size

Varying the sample load from 5 to 100 nmol demonstrated that for this column size of 1.6 ml, 20–50 nmol of this type of P450 (5–15 mg of protein) could be accommodated for fractionation. Hence, this column appears to be suitable for both preparative and analytical needs. When more than 20–50 nmol of the P450 sample under study were loaded, lower relative protein recoveries and apparent homogeneity of bound material were obtained (Table VI).

Characterization of the purified P450 form(s)

The specific contents of the gradient fractions derived from optimized conditions (Tables IV–VI)

TABLE VI

EFFECTS OF SAMPLE LOAD (IN NMOL P450) ON RELATIVE P450 RECOVERY, RELATIVE RECOVERY OF PROTEIN (AUC) AND HOMOGENEITY OF THE GRADIENT FRACTIONS

For definitions see Experimental. Metal ion, Ni^{2+}; elution buffer, glycine; pH, 7.75; detergent, 0.4% Lubrol.

Parameter	Sample load (nmol)				
	5	10	20	50	100
Relative P450 recovery (%)	46	44	48	46	32
Relative protein recovery (%)	38	37	35	28	24
Homogeneity (%)	85	86	80	79	63

ranged from 14.3 to 15.1 nmol of P450 per milligram of protein. Hence, in comparison with the values given for homogeneity in Tables IV–VI, the loss of haeme from P450 may possibly amount to as much as 10%.

Guengerich *et al.* [21] have described in detail the purification and characterization of several P450 forms present in liver microsomes of rats induced with phenobarbital (and other substances). Using this procedure in our laboratory, we obtained these forms for use as authentic samples for comparison purposes. P450 isolated in the gradient fraction of the Chelating Superose column was freed from residual non-ionic detergent as described [7], and preliminary analyses may now allow us to make a tentative allocation.

The apparent molecular weight of 50–51 kilodalton as judged by SDS-PAGE is consistent with those found for P450 forms 2B1 [22] (Guengerich *et al.*'s designation PB-B), 2B2 (PB-D) and 2C11 (UT-A). Fig. 3 illustrates that in SDS-PAGE the Chelating Superose fraction co-migrates with 2B1. The iron(II)–CO difference spectrum maximum of 450 nm is consistent with those seen for 2B1, 2B2 and 2C6 (PB-C).

More important, however, is the finding that the present P450 form gave a very strong ELISA reaction with monoclonal antibodies prepared against 2B1, some reaction with anti-2B2 (known to cross-react to some extent with 2B1 [21]), only minimum reaction with anti-2C6 and no reaction with anti-2C11. Finally, a reconstituted system of P450 taken from the Chelating Superose gradient fraction was also capable of dealkylating pentoxyresorufin at the relatively high rate of 1.9 nmol of product per minute per nanomole of P450, which is similar to that found by us previously for 2B1 [7]. As has been shown elsewhere [23] for seven different forms of P450 induced either mainly or marginally by phenobarbital, only 2B1 demonstrated such high specificity towards this substrate.

Taken together, these results suggest that the Chelating Superose fraction of P450 may contain large amounts of 2B1. Roos [5] has pointed out recently that phenobarbital-induced P450 fractions eluting in IMAC gradients may also contain certain amounts of 3A1 and/or 3A2 (PB/PCN-E). At present, we have no evidence for or against this finding.

CONCLUSION

The aim of this work was to investigate the general usefulness of IMAC in purifying P450 for preparative purposes. Under suitably chosen conditions, and with respect to the P450 sample load used here, this type of chromatography is apparently capable of yielding high recoveries of virtually homogeneous protein in one step. In light of the fact that several different P450 forms have virtually identical mobilities as judged by SDS-PAGE, we cannot yet assume that the P450 form encountered here is indeed only a single form.

The number of steps in preparing each IMAC run is unusually large, as is the number of parameters affecting this chromatographic method itself, but this can be mitigated by taking advantage of fully programmable automation. Consequent care in cleaning and recharging the column as described under Experimental ensured excellent reproducibility of chromatography, with no increase in back-

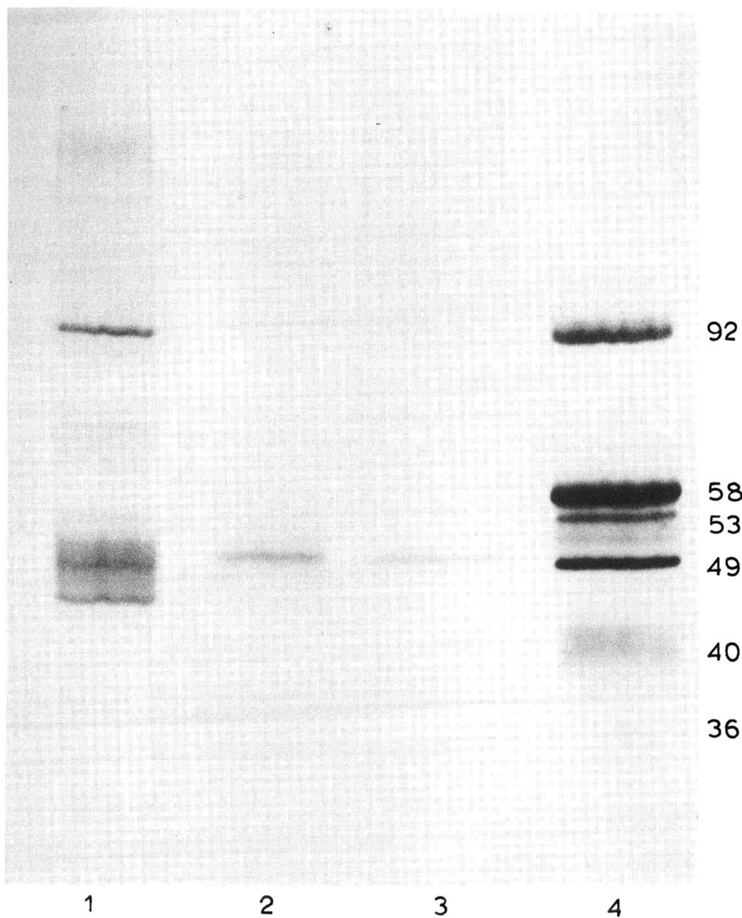


Fig. 3. SDS-PAGE of P450: purification by IMAC and co-migration of an authentic sample. Start buffer includes 0.4% Emulgen 911 (pH 7.75). Metal ion and elution buffer, Ni^{2+} and glycine. The 4–15% gradient gel was stained with silver. For details, see Fig. 2. Lanes: 1 = load; 2 = gradient fraction; 3 = P450 2B1 [22] (P450 PB-B) prepared according to Guengerich *et al.* [21]; 4 = marker proteins as in Fig. 2.

pressure, even after 200 runs. The type of set-up incorporated here also lends itself well, of course, to general routine column hygiene of any other types of columns.

IMAC may prove to be a useful method in supplementing conventional types of chromatography such as ion-exchange and hydroxyapatite for the purification of a larger number of constitutive and inducible P450 forms.

ACKNOWLEDGEMENTS

We are indebted to Ilse Lass for carrying out the ELISA tests and to Michael Schwiede (Pharmacia, Freiburg, Germany) for constructing the 15-pole relay switch-box.

REFERENCES

1 L. Kågedal, in J.-C. Janson and L. Rydén (Editors), *Protein Purification: Principles, High Resolution Methods, and Applications*, VCH, New York, 1989, pp. 227–251.
2 S. Ostrove and S. Weiss, *Methods Enzymol.*, 182 (1990) 373–376.
3 R. M. Chicz and F. E. Regnier, *Methods Enzymol.*, 182 (1990) 417–421.
4 P. H. Roos, *J. Chromatogr.*, 521 (1990) 251–265.
5 P. H. Roos, *J. Chromatogr.*, 587 (1991) 33.
6 M. Kastner, G. Blankenburg, T. Schulz, G. Schack and D. Neubert, *Arch. Toxicol.*, 61 (1988) 426–432.
7 M. Kastner, T. Schulz-Schalge and D. Neubert, *Toxicol. Lett.*, 45 (1989) 261–270.
8 I. Lass, N. Hinz, M. Kastner, D. Xu, T. Schulz, H.-J. Merker and D. Neubert, in D. Neubert, H.-J. Merker and A. Hendrickx (Editors), *Non-Human Primates —Developmental Biology and Toxicology*, Ueberreuter Wissenschaft, Vienna, Berlin, 1988, pp. 373–389.
9 J. Porath and B. Olin, *Biochemistry*, 22 (1983) 1621–1630.
10 T. Omura and R. Sato, *J. Biol. Chem.*, 239 (1964) 2370–2378.
11 J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature (London)*, 258 (1975) 598–599.
12 Y. Imai and R. Sato, *J. Biochem.*, 62 (1967) 239–249.
13 T. Kurecki, L. F. Kress and M. Laskowski, *Anal. Biochem.*, 99 (1979) 415–420.
14 A. Hammacher, U. Hellman, A. Johnsson, A. Östman, K. Gunnarsson, B. Westermark, Å. Wasteson and C.-H. Helding, *J. Biol. Chem.*, 263 (1988) 16493–16498.
15 C. A. K. Borrebaeck, B. Lönnerdal and M. E. Etzler, *FEBS Lett.*, 130 (1981) 194–196.
16 Y. Kato, K. Nakamura and T. Hashimoto, *J. Chromatogr.*, 354 (1986) 511–517.
17 M. Kastner and T. Schulz, *J. Chromatogr.*, 397 (1987) 153–163.
18 D. E. Ryan and W. Levin, *Pharmacol. Ther.*, 45 (1990) 153–239.
19 Y. Kato, T. Kitamura, K. Nakamura, A. Mitsui, Y. Yamasaki and T. Hashimoto, *J. Chromatogr.*, 391 (1987) 395–407.
20 M. Warner, M. V. LaMarca and A. H. Neims, *Drug Metab. Dispos.*, 6 (1978) 353–362.
21 F. P. Guengerich, G. A. Dannan, S. T. Wright, M. V. Martin and L. S. Kaminsky, *Biochemistry*, 21 (1982) 6019–6030.
22 D. W. Nebert, D. R. Nelson, M. J. Coon, R. W. Estabrook, R. Feyereisen, Y. Fujii-Kuriyama, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, J. C. Loper, R. Sato, M. R. Waterman and D. J. Waxman, *DNA Cell Biol.*, 10 (1991) 1–14.
23 C. R. Wolf, S. Seilman, F. Oesch, R. T. Mayer and M. D. Burke, *Biochem. J.*, 240 (1986) 27–33.

Journal of Chromatography, 587 (1991) 55–60
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2366

Purification and characterization of transglutaminases from the genital tract of the male rat

Jürgen Seitz*, Claudia Keppler and Susanne B. Hüntemann

Department of Anatomy and Cell Biology, Robert Koch Strasse 6, W-3550 Marburg (Germany)

ABSTRACT

In the genital tract of the male rat two different forms of the enzyme transglutaminase (TGase) could be identified and characterized. The coagulating gland and the dorsal prostate secrete a glycosylated and acylated TGase with a molecular weight of 65 000 dalton and p*I* value of 8.7.

This secretory form was purified to homogeneity using preparative isoelectric focusing and gel filtration on a Superdex 200 column. Running fast protein liquid chromatographic gel filtration on a Superose 12 column in the presence of calcium ions, high-molecular-weight aggregates were physically formed which could only be eluted using drastic conditions (0.1 *M* sodium hydroxide). In the presence of 10 m*M* EDTA this tendency to aggregate was greatly diminished. Utilizing a Superdex 200 column for gel filtration, the secretory TGase was even eluted as a monomeric protein. Testicular TGase was isolated by ion-exchange fast protein liquid chromatography on a Mono Q and by gel filtration on a Superdex 200 column. This enzyme represents a tissue-type TGase with a molecular weight of 82 000 dalton and p*I* value of 5.25. Hydrophobic interaction chromatography on a phenyl-Superose column showed no further enrichment of the GTP-binding form of transglutaminase.

INTRODUCTION

Transglutaminases (TGase, EC 2.3.2.13, *R*-glutaminyl-peptide: amine-γ-glutamyl-transferase) are a class of enzymes widely distributed in animal tissues and body fluids. They catalyze an acyl transfer reaction between the γ-carboxamide group of peptide bound glutaminyl residue and a primary amino group of various acceptor substrates, with a concomitant release of ammonia [1,2]. Although different transglutaminases appear similar in their substrate specificity, several distinct forms of the enzyme have been identified [2]: two forms —transglutaminases such as the tissue-type form, purified from liver and erythrocytes [3,4] and epidermal TGase, which is also present in hair follicle [5,6], are found exclusively inside cells, whereas plasma factor XIII and the TGase of rodent seminal plasma are extracellular enzymes. Plasma factor XIII occurs as a zymogen in plasma, platelets and histiocytes [7] and is activated through a thrombin cleavage near the N-terminus. Coagulation of semen in rodents results from polymerization of seminal vesicle secretion proteins by transglutaminases of prostatic origin [8].

Studies in primary sequences as well as immunological comparisons revealed differences between the transglutaminases. All forms of the enzyme are calcium dependent, they need at least 0.1–1 m*M* Ca^{2+} for catalytic activity [9], and different concentrations of the cation are necessary for optimal activation. The active site of TGases contains an essential SH-group which is completely inactivated after alkylation [10].

Two forms of transglutaminases, which we identified in the genital tract of the male rat, were enriched utilizing different steps of purification procedures. Tissue-type TGase is expressed by stromal cells of the accessory sex glands as well as by myoid peritubular cells of the testis [11] and the secretory enzyme is present in epithelial cells of the coagulating gland and the dorsal prostate [12]. Because they tend to form physical aggregates and autocatalytic cross-linking even under physiological conditions, isolation of these TGases has been difficult. However, by using conventional and fast protein liquid

chromatography (FPLC) techniques we were able to purify the secretory TGase to homogeneity. The testicular enzyme, however, was still contaminated with lower-molecular-weight (32 000 and 24 000 dalton) proteins.

EXPERIMENTAL

Transglutaminase assay: estimation of purity

Enzymatic activity was measured by incorporation of [14C]putrescine (Amersham, Braunschweig, Germany) into N,N'dimethylated caseine (Sigma, Munich, Germany) [2,13]. Purity of the fractions was estimated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), analytical isoelectric focusing (IEF) and by immunoprint analysis, using either a specific antibody to prostatic secretory TGase [12] or a monoclonal antibody to guinea pig liver TGase (generous gift of Dr. P. J. Birckbichler, Ardmore, OK, USA).

Purification of secretory TGase

Mature male Wistar rats (Ivanovas, Kisslegg, Germany) weighing 250–300 g were anesthetized and sacrificed by cervical dislocation. After dissection of the coagulating glands and the testes, the expressed residual secretions of the coagulating glands were extracted in physiological saline containing 5 m*M* Ca^{2+}. After centrifugation at 15 000 *g* for 20 min, TGase present in the supernatant was enriched by fractionated ammonium sulphate precipitation (25–50% saturation). Preparative IEF in a granulated Sephadex G-75 gel bed (Pharmacia/LKB, Freiburg, Germany) (pH gradient 3–8.5) eliminated contaminating secretory proteins. Pharmalytes (Pharmacia/LKB) were removed by gel filtration on a Superdex 200 column (Pharmacia/LKB). The apparent molecular weight of the native enzyme was estimated by FPLC gel filtration on a Superdex 200 or a Superose 12 (Pharmacia/LKB) column and the effect of 5 m*M* Ca^{2+} and 10 m*M* EDTA upon the elution profile of this TGase was tested.

Isolation of testicular tissue-type TGase

After removal of the testes the tunica albuginea and the testicular artery were discarded. The tissue was minced and subsequently homogenized in a pH 8.5, 50 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) 0.33 *M* sucrose, 5 m*M* DTE, 50 m*M* KSCN, 1 m*M* EDTA (10% w/v) mixture. After centrifugation at 150 000 *g* for 45 min, the TGase present in the cytosol was pelleted by fractionated ammonium sulphate precipitation (50–60% saturation). The pellet was dissolved in distilled water, dialyzed overnight against the starting buffer (50 m*M* Tris–HCl, 1 m*M* EDTA pH 7.5, and subsequently applied to anion-exchange FPLC (Mono Q, Pharmacia/LKB). Fractions containing enzyme activity were applied to a Phenyl-Superose column (Pharmacia/LKB) which had been equilibrated with a pH 7.5, 50 m*M* Tris–HCl, 1 m*M* EDTA and 0.3 *M* NaCl mixture. Elution of the fractions was carried out in the presence of 1 m*M* GTP [14]. Samples containing enzyme activity were pooled, concentrated in a vacuum concentrator (Bachofer, Reutlingen, Germany) and separated by FPLC gel filtration on a Superdex 200 column.

RESULTS

Secretory transglutaminase

Secretory transglutaminase from the rat coagulating gland was identified by SDS-PAGE after preparative IEF for fractions 2–6 (Fig. 1a). Estimation of the enrichment of the enzyme and yield was affected by Pharmalyte contamination, which also impaired the determination of protein concentration. Therefore, Pharmalytes were removed by gel filtration on a Superdex 200 column. Compared with crude organ extracts, the enrichment factor was 4.5, although a yield of little more than 1% was obtained. SDS-PAGE revealed a single band of 65 000 dalton (Fig. 1b)) which was also seen after analytical IEF at p*I* 8.7 (not shown here). The influence of calcium ions upon gel filtration under native conditions was also investigated by applying crude TGase extract to a Superose 12 column. If the column was run in the presence of calcium ions (5 m*M*), only 8% of the TGase activity applied could be eluted in the void volume of the column (>300 000 dalton). Immunoprint analysis of this fraction revealed an autocatalytic cross-linking as well as a small degree of physical association of the enzyme. An additional 50% of the initial activity could be eluted if relatively strong conditions (0.1 *M* sodium hydroxide) were used. Activity appeared at an elution volume corresponding to 60 000–

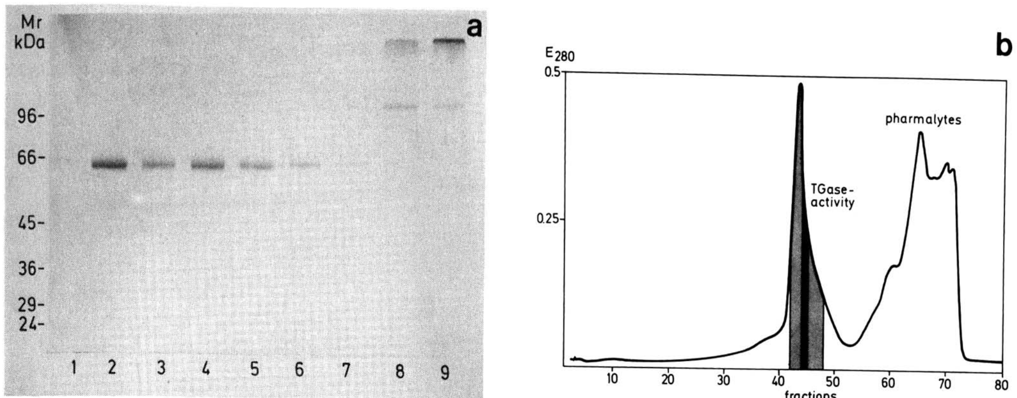


Fig. 1. (a) Analysis of fractions 1–9 from preparative IEF by SDS-PAGE (7.5–20% acrylamide) stained with Serva Blue G: identification of secretory TGase (65 000 dalton) in fractions 2–6. Molecular weight (M_r) indicated in kilodalton (kDa). (b) Gel filtration of pooled fractions 2–6 on Superdex 200 column and removal of Pharmalytes. Hatched area indicates fractions containing TGase activity (fractions 42–47).

70 000 dalton. If the column was run with calcium-free Tris buffer containing 10 m*M* EDTA, then about 38% of the applied TGase eluted at a volume corresponding to 200 000 dalton and an additional 20% eluted at a volume corresponding to a molecular weight of 60 000–70 000 dalton (Fig. 2a). A much different elution profile was obtained utilizing a column of Superdex 200 instead of Superose 12. Running the column with a buffer containing EDTA, TGase eluted in a separate peak (Fig. 2b) in a monomeric form, indicating the prevention of aggregation. Using FPLC gel filtration on a Superdex 200 column subsequent to ammonium sulfate precipitation, we obtained a highly purified enzyme with an enrichment factor of 5 and a yield of more than 30% (Fig. 3a). As a side effect, an additional

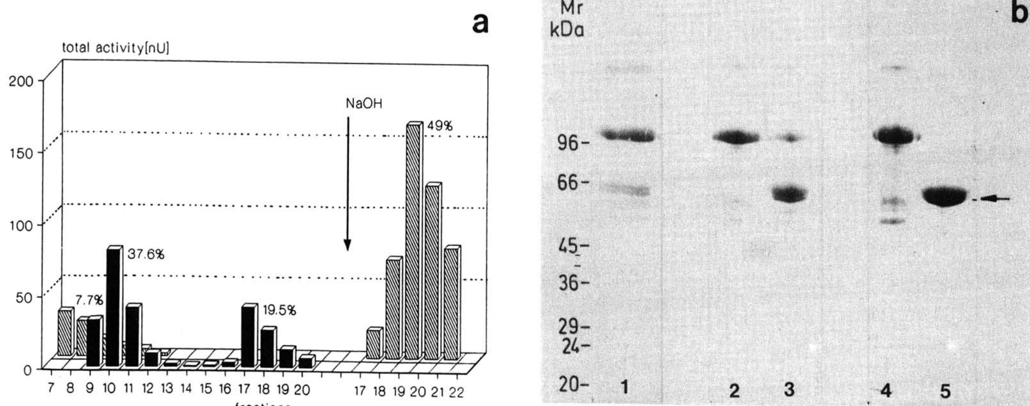


Fig. 2. (a) FPLC gel filtration of crude TGase extract on a Superose 12 column. Hatched areas represent elution with Tris buffer containing 5 m*M* Ca^{2+}. The major part of TGase is only eluted by 0.1 *M* sodium hydroxide (arrow). Black areas indicate elution of TGase as oligomers and monomers utilizing Tris buffer containing 10 m*M* EDTA. (b) Analysis of fractions in (a) containing high enzyme activity by SDS-PAGE. Lanes: 1 = crude coagulating gland extract; 2 = fraction 8 after elution with Tris/Ca^{2+}; 3 = fraction 19 after elution with 0.1 *M* sodium hydroxide; 4 and 5 = fractions 10 and 17 after gel filtration with Tris/EDTA.

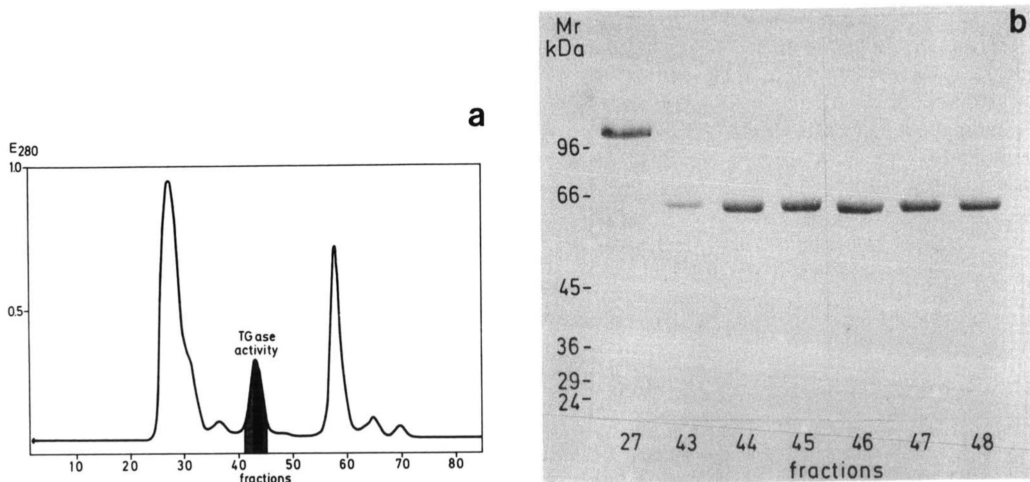


Fig. 3. (a) Gel filtration of secretory TGase on a Superdex 200 column after ammonium sulphate precipitation. Hatched fractions 43–48 contain enzyme activity. (b) SDS-PAGE of selected fractions in (a): in fraction 27, a secretroy protein of 100 000 dalton is highly enriched. Fractions 43–48 contain purified TGase.

secretory protein was isolated in a different fraction, corresponding to a molecular weight of more than 100 000 dalton (Fig. 3b, fraction 27).

Testicular transglutaminase

Using anion-exchange FPLC on a Mono Q column, a highly enriched enzyme eluted at a single peak corresponding to a concentration of 0.3 *M* sodium chloride. Immunoprint analysis revealed a prominent band in the range of 82 000 dalton as well as smaller bands due to proteolysis of the enzyme (Fig. 4b). Nevertheless, an enrichment factor of 30 and a yield of more than 70% were obtained. All enzyme activity applied to the phenyl-Superose column appeared in the void volume and no activity was eluted by GTP. After application of concentrated fractions to a Superdex 200 column which was run in the presence of 1 m*M* EDTA, no enzyme

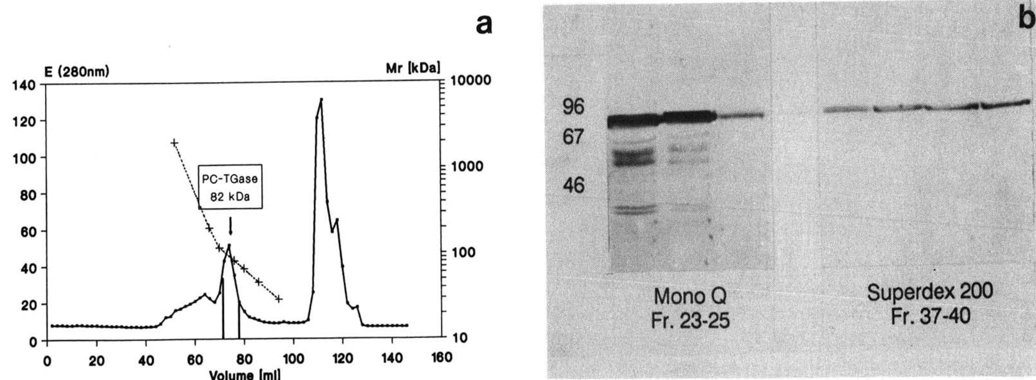


Fig. 4. (a) Gel filtration of testicular TGase on a Superdex 200 column after ion-exchange chromatography. Marked area represents enzyme activity and immunoreactivity. (b) Immunoprint analysis of fraction (Fr.) containing testicular TGase (82 000 dalton) activity after ion-exchange chromatography and gel filtration.

activity or immunoreactivity was detected in the void volume, indicating the absence of aggregates. Enzyme acitivity as well as immunoreactivity appeared as a single peak in a monomeric form corresponding to a molecular weight of 82 000 dalton as estimated by gel filtration (Fig. 4a) and immunoprint analysis (Fig. 4b). Although enzyme activity was low (1.5% yield), maybe due to the phenyl-Superose step, only two major contaminating protein bands with low molecular weights (about 32 000 and 24 000 dalton) were present as revealed by SDS-PAGE (not shown).

DISCUSSION

Purification of transglutaminase is known to be difficult because of its tendency to form aggregates even under native conditions, and especially in the presence of calcium ions. The enzymatic function of TGases consists of covalent cross-linking of glutaminyl and lysyl residues [2]. Since transglutaminases themselves contain a sufficient number of these amino acids, an autocatalytic cross-linking instead of a physical aggregation is conceivable. For further elucidation of this problem, two different TGases were isolated from the genital tract of the male rat: (i) an extracellular protein secreted by the coagulating gland and (ii) a cytosolic enzyme expressed by myoid peritubular cells of the seminiferous epithelium [15]. Although both forms share structural and functional properties, such as calcium dependence in the millimolar range and presence of an essential SH-group at the active site, significant differences are observed at the molecular and immunological level. The prostatic enzyme which is exported by the unusual pathway of apocrine secretion [12] has a molecular weight of 65 000 dalton and a p*I* value of 8.7. Furthermore it is membrane-bound by a phosphoinositol anchor which prevents autocatalytic cross-linking and obstruction of the excretory ducts [16]. The testicular enzyme, in comparison has a higher molecular weight (82 000 dalton) and is anionic (p*I* 5.25). Moreover, it is closely related to the tissue-type form expressed by rat and guinea pig livers as revealed by immunohistochemistry and immunoprint analysis. Like other tissue-type TGases, its activity is inhibited by micromolar concentrations of GTP [17] and it binds to GTP–agarose columns [18]. As described above the secretory enzyme was isolated to homogeneity. Gel filtration in presence of calcium ions resulted in a great loss of enzyme and therefore the yield of purification was low. When using FPLC (Superose 12 column, buffer containing calcium ions) more than 40% of applied enzyme were cross-linked autocatalytically. Approximately 49% aggregated physically and could only be eluted using drastic conditions (0.1 *M* sodium hydoxide), whereas 8% of enzyme activity appeared in the void volume. Using a Tris buffer containing 10 m*M* EDTA to bind endogenous calcium, 38% of aggregated prostatic TGase eluted at a volume corresponding to 200 000 dalton and 20% at a volume corresponding to 60 000–70 000 dalton, the latter indicating the presence of monomers. A significant improvement concerning the elution profile of the enzyme was obtained by changing the matrix for gel filtration. Using FPLC on a Superdex 200 column in the presence of EDTA, aggregation of the protein was significantly dimished and it eluted as a monomeric pure enzyme with a high specific activity. We therefore presume that, in contrast to Superose 12, Superdex 200 gel matrix does not induce physical aggregation of the secretory TGase.

TGase activity present in testicular homogenates and Sertoli cell cultures has been reported previously [19] and an effect of transglutaminase substrates on the cellular sequestration and processing of follicle-stimulating hormone (FSH) has been proposed [20]. Furthermore, TGase activity in bovine calf testicular membranes is thought to participate in the interaction of FSH with its receptor [21]. Utilizing a monoclonal antibody to tissue-type TGase we have demonstrated that in the testis only interstitial cells and myoid peritubular cells express the tissue-type form [15]. We therefore conclude that TGase activity present in Sertoli cell cultures is due to contamination by peritubular or interstitial cells. For further characterization of the enzyme, tissue-type TGase was isolated from testicular homogenates using ion-exchange FPLC on a Mono Q column, and gel filtration on a Superdex 200 column in the presence of 1 m*M* EDTA. Neither on Mono Q nor on Superdex 200 column did enzyme activity or immunoreactivity appear in the void volume —indicating the absence of covalently linked or physically aggregated TGase. Using ion-exchange FPLC subsequent to ammonium sulphate precipitation, the enzyme eluted as a single peak corresponding to 0.3

M sodium chloride. An enrichment factor of 30 and a yield of more than 70% were achieved. Running reversed-phase FPLC on a phenyl-Superose column showed no further purification of the GTP-binding enzyme. After gel filtration on a Superdex 200 column, testicular TGase eluted as a single peak with low specific activity corresponding to a native molecular weight of about 80 000 dalton. Using immunoprint analysis, the enzyme was shown to consist of a monomeric form of 82 000 dalton, which is antigenically unrelated to the prostatic secretory TGase.

REFERENCES

1 L. Lorand and S. M. Conrad, *Mol. Cell. Biochem.*, 58 (1984) 9–35.
2 J. E. Folk, *Ann. Rev. Biochem.*, 49 (1980) 517–531.
3 P. J. Birckbichler, G. R. Orr and M. K. Patterson, *Cancer Res.*, 36 (1976) 2911–2914.
4 G. E. Siefring, A. B. Apostol, P. T. Velasco and L. Lorand, *Biochemistry*, 17 (1978) 2598–2604.
5 L. A. Goldsmith, H. P. Baden, S. I. Roth, R. Colman, L. Lee and B. Fleming, *Biochim. Biophys. Acta*, 351 (1974) 113–125.
6 M. M. Buxman and K. D. Wuepper, *J. Invest. Dermatol.*, 65 (1975) 107–112.
7 S. J. Chung, in H. Boyer (Editor) *Isoenzymes 1*, Academic Press, New York, 1975, pp. 259–273.
8 H. G. Williams-Ashman, *Mol. Cell. Biochem.*, 58 (1984) 51–61.
9 L. Fésüs and K. Laki, *Biochemistry*, 16 (1977) 4061–4066.
10 J. E. Folk and P. W. Cole, *J. Biol. Chem.*, 241 (1966) 3238–3240.
11 S. B. Hüntemann, J. Seitz and G. Aumüller, *Eur. J. Cell Biol.*, 54 (1991) 17.
12 J. Seitz, C. Keppler, U. Rausch and G. Aumüller, *Histochemistry*, 93 (1990) 525–530.
13 R. Egbring, W. Schmid and K. Havemann, *Blut*, 27 (1973) 6–19.
14 C. M. Bergamini, Ferrara, Personal communication.
15 S. B. Hüntemann, J. Seitz and G. Aumüller, presented at the *2nd International Conference on Transglutaminases and Protein Cross-Linking REactions, Cannes, France, June 24–28, 1990.*
16 J. Seitz, C. Keppler, S. B. Hüntemann, U. Rausch and G. Aumüller, *Biochim. Biophys. Acta*, 1078 (1991) 139–146.
17 C. M. Bergamini, *FEBS Lett.*, 239 (1988) 255–258.
18 K. N. Lee, P. J. Birckbichler and M. K. Patterson Jr., *Biochem. Biophys. Res. Commun.*, 162 (1989) 1370–1375.
19 J. A. Dias, *Biol. Reprod.*, 33 (1985) 835–843.
20 J. A. Dias, *Biol. Reprod.*, 35 (1986) 49–58.
21 P. Grasso, B. Dattatreyamurty, J. A. Dias and L. E. Reichert, Jr., *Endocrinology*, 121 (1987) 459–465.

Journal of Chromatography, 587 (1991) 61–70
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2370

Development and optimization of a single-step procedure using protein A affinity chromatography to isolate murine IgG_1 monoclonal antibodies from hybridoma supernatants

Gerhard Schuler and Manfred Reinacher*

Institut für Veterinärpathologie der Justus-Liebig-Universität, Frankfurter Strasse 96, W-6300 Giessen (Germany)

ABSTRACT

Protein A affinity chromatography is a standard method of purifying murine monoclonal antibodies (mabs), primarily because it can be performed easily and achieves high-purity levels. Because of its high concentration capacity, it lends itself particularly well to the isolation of mabs from the supernatants of hybridoma cultures. Unfortunately, murine immunoglobulin (Ig) G_1 antibodies, a subclass which occurs frequently in the IgG mabs of mice, binds very poorly to protein A, leading to problems in this isolation procedure. For this reason an attempt was made to increase the effectiveness of protein A affinity chromatography in purifying mabs of this IgG subclass by optimizing the binding conditions. The influence of ionic strength, pH and temperature on the binding capacity of a protein A column was studied. The results show the significance of temperature in the binding of the murine IgG_1 mab tested to protein A. Further investigations were carried out to optimize the elution conditions and to study the contamination of mab preparations obtained with non-specific bovine protein A reactive Igs originating from culture medium supplement (10% foetal calf serum). An optimized, automatic single-step procedure to obtain highly purified murine IgG_1 mabs from hybridoma culture supernatants was developed.

INTRODUCTION

Parallel to the significance assumed by monoclonal antibodies (mabs) in almost all areas of medicine, biology and related disciplines, there has been an increase in the number of purification methods. This reflects the heterogeneity of this group of molecules and the various demands made on purity [1]. Today the amounts of mabs required may lie in the range of grams or even kilograms [2–4]. To produce amounts of this kind in laboratory animals, it is necessary to run a large-scale colony. This is not practicable and cannot be justified on ethical grounds [2,5–7]. For this reason large amounts of mabs are now produced in bioreactors [3,7,8]. In contrast, many research institutes and universities still produce small amounts of mabs in the "ascites mouse" [8]. However, even with laboratory-scale production, there is pressure from animal protection groups and some researchers to abandon the production of mabs in the peritoneal cavities of laboratory animals and to seek alternative sources. For this reason the production of mabs to be used in small- and medium-scale cell culture systems will assume increasing significance and therefore the development of purification methods is of particular interest.

The procedures used to purify proteins consist in various stages which are interspersed by intermediate conditioning steps such as concentration or the adjustment of pH and ionic strength [9]. However, the protocols often consist of an unnecessary number of stages [10]. The greater the number of purification steps required the lower the yield, hence single-step procedures are desirable. Such procedures are also less time-consuming and less labour-intensive.

The greatest drawback in isolating mabs from hybridoma culture supernatants is the large volume of the supernatants; compared with ascites, the concentration of the target molecule is lower by a factor of 100–1000 [11]. The concentrations which can be obtained by classical *in vitro* culture methods are between 10 and 100 μg/ml [12]. The major contami-

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V. All rights reserved

nant in the hybridoma supernatants is water. This must be eliminated via reduction using filtration systems [3,8] or by the initial application of a purification method which has a high concentration capacity, such as cation-exchange or affinity chromatography [2,3,13]. A reduction in volume is required at the outset of the purification process as a large number of purification methods have only a very limited volume capacity.

The composition of the medium plays a significant role in the purification process [2–6,12,14,15]. The growth factors of mammalian cells are frequently not clearly defined and 10% volume of animal serum, usually foetal calf serum (FCS), is often added to the media as a source of growth factors. At concentrations most frequently used, the protein concentration in the supernatants is between 0.5 and 5 mg/ml, *i.e.* the concentration of the contaminating proteins may be greater than that of the mabs [12]. Therefore a single-step procedure for the isolation of mabs from hybridoma culture supernatants containing serum must be capable of both concentrating and isolating the mabs required.

Protein A affinity chromatography is a standard method of purifying murine immunoglobulin G (IgG) mabs. A prerequisite for its concentration capacity and thus for the effectiveness of the technique is that there is an effective binding affinity between the Fc fragment of the mab and the immobilized protein A of the column [16,17].

Most mabs are produced in mice, the most common being the IgG subclasses, 1, 2a, 2b and 3. The IgG_1 subclass occurs particularly frequently in the IgG mabs of mice [3,18]. However, although the affinity of the IgG subclasses 2a, 2b and 3 to bind with protein A is high, the purification of IgG_1 mabs from hybridoma culture supernatants by protein A affinity chromatography generally proves problematic. The reason for this is that the protein A affinity chromatography column has a very restricted binding capacity as a result of low affinity constants. This why many workers regard the purification of murine IgG_1 mabs by protein A affinity chromatography as ineffective and use alternative methods. However, compared with protein A affinity chromatography, the alternative methods have a number of drawbacks.

As a result of the frequent occurrence of the IgG_1 subclass among murine IgG mabs and the diverse advantages offered by protein A affinity chromatography, an attempt was made to increase the effectiveness of protein A affinity chromatography in purifying mabs of this IgG subclass by optimizing the binding conditions. An optimized, automatic single-step procedure was developed to obtain highly purified murine IgG_1 mabs from hybridoma culture supernatants.

EXPERIMENTAL

Monoclonal antibody DF4B7A6

The murine mab studied is designated as DF4B7A6 and was produced and characterized in the Institute of Veterinary Pathology of the Justus-Liebig-University (Giessen, Germany). In indirect enzyme-linked immunosorbent assay (ELISA) it recognizes the tumour-specific pyruvate kinase isoenzyme M_2 (M_2PK), but does not react with M_1PK from skeletal muscle, with pulmonary-specific M_2PK nor with L-PK taken from the liver [19]. DF4B7A6 belongs to the IgG_1 subclass; the light chains are of the kappa type.

Production and pretreatment of hybridoma supernatant

The hybridoma supernatants were produced with a commercially available cell culture system (Dynacell-Millipore, Eschborn, Germany). RPMI with added 10% FCS was used as the culture medium.

Immediately after harvesting, the supernatants were frozen at −30°C. Indirect ELISA was used to examine samples of the individual charges and to establish their anti-M_2PK titre. The charges with the highest anti-M_2PK titres were selected, pooled and mixed with 0.2 g/l sodium azide for the stemming of microbial growth. After sterile filtration through a 0.2-μm filter, the sample was split into portions and the supernatant frozen and stored for future use at −30°C. The protein content of the culture supernatant pooled was 5.1 mg/ml. The concentration of mabs was 51.6 μg/ml as determined by purifying a small volume of supernatant and estimating the protein concentration of the eluted mabs.

Immediately prior to chromatography, the supernatant was thawed in water at 38°C and degassed with a vacuum pump while being stirred gently with a magnetic stirrer for at least 30 min.

Equipment

A high-performance liquid chromatographic (HPLC) system (MAPS 700, Bio-Rad, Munich, Germany) equipped with two dual-piston pumps, a UV detector absorbing at 280 nm, a conductivity monitor, a programmable fraction collector and a HPLC gradient mixer was used. Each of the two dual-piston pumps could be connected alternately with two solvent reservoirs by a programmable motor valve. A personal computer was used to control the apparatus and to collect and process the data acquired. With the help of the standard HPLC software it was possible to load and wash the column automatically.

Although HPLC equipment was available, a traditional gel column was used for the chromatographic separation. Protein A Sepharose CL 4B (11 ml, Pharmacia, Freiburg, Germany) was used as the column material. The gel bed was 5.5 cm deep and the column was 1.6 cm I.D.

To assess the temperature dependence of the binding, the separations were conducted both at room temperature (about 22°C) and using a fitted cooling jacket. A thermostatically controlled waterbath was used to maintain the cooling water at a constant temperature of 2°C. A regulated tubular pump pumped the cooling water through the cooling jacket of the column and through a reverse-current cooler around the steel capillary leading to the column. This allowed the temperature inside the column to be reduced to 3.5°C.

Binding buffers

To investigate the effect of pH on the binding of the mabs onto the column, the binding capacity of the column was compared using buffers of various pH values. A 1.5 *M* glycine–sodium hydroxide buffer and a 1.5 *M* glycine–sodium hydroxide buffer with 3 *M* sodium chloride were used. A mix of one part binding buffer and two parts supernatants resulted in pH values given in Table I.

To clarify the effect of ionic strength on the binding between the mabs and the protein A of the column, binding buffers of various ionic strength were studied. The three buffers used had low (0.15 *M* glycine–sodium hydroxide), medium (1.5 *M* glycine–sodium hydroxide) and high (1.5 *M* glycine–sodium hydroxide plus 3 *M* sodium chloride) ionic strengths. Mixing the binding buffer in a ratio of 1:2 with the culture supernatant during loading and alternatively with phosphate-buffered saline (PBS) during equilibration and washing immediately prior to application to the column, gave a reduction in the saline concentration in the mix. A list of the binding buffers tested is given in Table I.

Chromatographic procedure

To optimize the binding of the mabs to the protein A of the column, the influence of pH, ionic strength and temperature during loading and washing on the binding capacity of the column was studied. Pooled supernatants (160 ml) were separated at three different ionic strengths, two different pH values and two different temperatures during loading and washing.

The column was first equilibrated for 40 min at a flow-rate of 1.5 ml/min, the mix consisting of two parts of PBS (pH 7.45) and one part of the respective binding buffer. This mixture was used to simulate closely the conditions present in the supernatant–binding buffer solution added later. The column was loaded continuously with a mix consisting

TABLE I

BINDING BUFFERS TESTED FOR THEIR CAPACITY TO ENHANCE THE BINDING OF MURINE IgG_1 MABS TO PROTEIN A

Values in parentheses indicate pH of buffer mixed with supernatant or PBS washing solution. ND. = not determined.

Binding buffer	pH I	pH II
1.5 *M* Glycine–sodium hydroxide plus 3 *M* sodium chloride	8.3 (8.2)	8.9 (8.7)
1.5 *M* Glycine–sodium hydroxide	8.3 (8.2)	8.9 (8.7)
0.15 *M* Glycine–sodium hydroxide	8.5 (8.2)	N.D.

of two parts supernatant and one part binding buffer. The HPLC preparative sample loop was not used to apply the supernatant–binding buffer mix. Instead, the supernatant and binding buffer were channelled along different lines, each to one of the two dual-piston pumps. The supernatant and the respective binding buffer were mixed immediately before being applied to the column in the HPLC gradient mixer. The flow-rate was kept constant throughout loading at a continuous rate of 1.2 ml/min. In this way, 160 ml of the pooled supernatant were applied to the column for each separation.

For the washing, the proportion of supernatant was again substituted by PBS (pH 7.45). After loading, the flow-rate was kept at 1.2 ml/min for the next 40 min until the UV detector signal had reached the baseline. It was then increased to 1.5 ml/min. During the 100-min washing procedure, the column was washed with 138 ml, *i.e.* with 12.5 times the volume of the column.

The mabs bound on the column were eluted with 100 m*M* citric acid, adjusted to pH 4.5 with 40% sodium hydroxide solution. Earlier experiments had shown that, under these conditions, a complete elution of the mabs could be achieved in an acceptable elution volume and that below this pH no additional protein peaks were visible on the chromatogram. To attain a greater concentration effect, the flow-rate was reduced to 0.5 ml/min while the elution was in progress.

The entire mab peak was collected without fractionation. The sample was immediately neutralized with a saturated sodium phosphate solution. Following elution, the column was regenerated with 100 m*M* citric acid adjusted to pH 2.8 with 40% sodium hydroxide solution to remove completely any small amounts of bound material. PBS (pH 7.45) with 0.05% sodium azide was used as a storage buffer for the column. Between separations the column was stored at 4°C.

Quantification of antibodies eluted

To compare the column capacity under various conditions, the amount of protein in the peak was calculated photometrically (Protein-Assay, Bio-Rad, Munich) and by integration of the chromatogram. The results were identical. If not indicated otherwise, values from the integration of the chromatogram are presented.

To determine the amount of mabs eluted using chromatogram integration, the mab peaks were integrated after their buffer gradients had been subtracted and this value was multiplied by a proportionality factor that had been established beforehand. The subtraction of the respective buffer gradient was necessary because, during elution, the change of buffers led to changes in the baseline. This made a correct evaluation of the chromatogram more difficult. For this reason, each of the binding buffers had a specific elution buffer gradient. This was achieved by beginning elution after the column had been equilibrated with a mix consisting of the binding buffer and PBS (pH 7.45) at a ratio of 1:2.

Determination of the specific activity of the mab in the post-column flow by indirect ELISA

Samples of post-column flow were taken automatically at 30 min intervals starting from the 20th minute after loading had commenced so that their anti-M_2PK activity could be determined later by indirect ELISA. This achieved with a programmable fraction collector and allowed the saturation kinetics of the column to be traced throughout the separations.

Investigations of contamination with non-specific bovine Ig

To determine the maximum contamination of the antibody preparations with bovine Ig contained in the FCS added to the culture media, 160 ml of unconditioned culture medium were applied to the column and, after elution, the amount of bovine antibodies bound on the column was determined. The 1.5 *M* glycine–sodium hydroxide buffer (pH 8.3) was used as the binding buffer. Chromatography was performed at 3.5°C. This experiment was performed twice, with the FCS used to prepare the culture medium from different charges from the same manufacturer. The amount of protein in the eluted material was determined by the corresponding integral. The purity of the mab preparations was determined in a silver-stained gel after sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The samples were run on a 10% gel with a total monomer concentration of 10% under denaturating conditions.

RESULTS

Influence of pH on the binding capacity of the column

A comparison of the amount of protein eluted (*cf.* Table II) shows that raising the pH to 8.7, from 8.2, gives no improvement yield of mabs. In fact, the amount of protein in the separations performed at higher pH are slightly lower. Hence the influence of the pH in the pH span studied is only minor.

Influence of ionic strength on the binding capacity of the column

For the separations of 160 ml of supernatant with the cooled column the saline concentration did not significantly affect the mab yield (Table II). In contrast, when the separations were performed at room temperature, the mab yield increased as the saline concentration of the binding buffer increased. However, even when a binding buffer with the highest saline concentration (1.5 *M* glycine plus 3 *M* sodium chloride) was used, the yield was lower than that obtained using the cooled column. At room temperature, an increase in the glycine concentration and the addition of sodium chloride both increase the mab yield.

Influence of temperature on the binding capacity of the column

A decrease in the temperature to 3.5°C during loading and washing markedly increases the binding capacity (Table II). For separations with the cooled column, the other two parameters tested did not affect the yield. To estimate the effect of the ionic strength of the binding buffer on the mab yield during cooling, the volume of the supernatant was increased to 400 ml to saturate the column. These separations were performed only at a pH of 8.2 as no improvement in the yield is be expected when the pH value is raised to 8.7.

The results of these experiments show the great effect that temperature has on the binding of the mab to protein A. In contrast to separation at room temperature, the increase in post-column activity during the separations performed with the cooled column are slower. Therefore the corresponding activities measured in the post-column flow are achieved much later during loading (Fig. 1). Under the conditions tested, cooling the column leads to a stronger increase in the binding capacity than increasing the ionic strength. The curves for the three buffers tested are almost identical.

The protein values determined in the antibody peaks are almost identical in all three buffers tested (Table III). Therefore, with the cooled column, no further increase in column capacity can be achieved by increasing the saline concentration.

Performing the separations at 3.5°C resulted in a markedly improved efficiency of the purification procedure. A total of 19 mg of a murine IgG_1 mab in a 9.8-ml volume could be purified from 400 ml of supernatant without using a high salt concentration for binding. The concentration factor was 36.7. The

TABLE II

INFLUENCE OF pH AND IONIC STRENGTH OF THE BINDING BUFFER AND TEMPERATURE ON THE BINDING CAPACITY OF THE COLUMN

Volume of loaded hybridoma supernatant is 160 ml. N.D. = not determined.

Binding buffer	Antibody yield (mg)		Antibody yield (%)	
	pH 8.2	pH 8.7	pH 8.2	pH 8.7
Chromatography at room temperature				
0.15 *M* Glycine–sodium hydroxide	4.3	N.D.	51.8	N.D.
1.50 *M* Glycine–sodium hydroxide	5.6	4.9	68.2	59.3
1.50 *M* Glycine–sodium hydroxide plus 3 *M* sodium chloride	6.4	6.2	77.6	75.2
Chromatography at 3.5°C				
0.15 *M* Glycine–sodium hydroxide	8.3	N.D.	100.1	N.D.
1.50 *M* Glycine–sodium hydroxide	8.3	8.2	100.1	99.4
1.50 *M* Glycine–sodium hydroxide plus 3 *M* sodium chloride	8.5	8.1	103.0	98.2

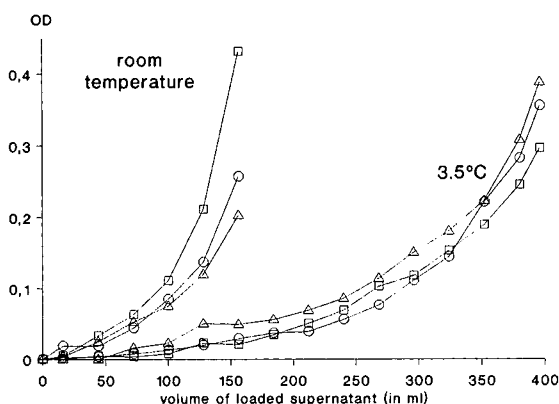


Fig. 1. Breakthrough curves as a function of ionic strength and temperature during loading and washing. Specific mab activity in the post-column flow during loading was measured by indirect ELISA. Curves of corresponding separations performed at room temperature and at 3.5°C have the same symbols. △ = 1.50 *M* glycine–sodium hydroxide plus 3 *M* sodium chloride; ○ = 1.5 *M* glycine–sodium hydroxide; □ = 0.15 *M* glycine–sodium hydroxide.

whole procedure takes about 12 h for equilibration, loading, washing and elution. The final concentration of mabs bound to the column after the throughput of 400 ml of supernatant was 1.73 mg/ml gel bed. The overall antibody yield under these conditions was more than 85%.

Optimization of elution conditions

The aim of an optimum elution procedure for mabs bound to the column is to obtain them in as concentrated a form as possible, under conditions that are as mild as possible. To optimize the elution conditions, the influence of temperature and the reversal of flow direction was investigated.

The curves given in Fig. 2 show the elution profiles of separations in which the column was loaded and washed under identical conditions. For peak A, cooling was maintained throughout the separation, including the elution. For peak B, the cooling apparatus was switched off after washing had been completed and the cooling jacket emptied. Peak C represents the elution profile of a separation in which the flow direction was reversed 20 min before washing was complete. At the start of the elution, the cooling was switched off and the cooling jacket emptied.

Although cooling throughout the elution only negligibly increases the retention time, the resulting peak is broader and flatter than the peak obtained without cooling. In addition, cooling throughout the elution produces peak tailing. This means that cooling not only increases the binding during loading, but also intensifies the weak interaction between the mab and protein A as the pH changes between binding and complete elution.

The reversal of the flow direction prior to elution significantly affects the form and position of the mab peak on the chromatogram. The emerging peak is sharper and appears earlier on the chromatogram than the peak seen without flow direction reversal.

Contamination with non-specific bovine Ig

When the column is loaded with 160 ml of culture medium, significant amounts of protein are bound (Fig. 3). The area under the chromatogram corre-

TABLE III

INFLUENCE OF IONIC STRENGTH OF THE BINDING BUFFER ON THE BINDING CAPACITY OF THE COLUMN

Volume of loaded hybridoma supernatant, 400 ml; pH of binding buffer–supernatant, 8.2; chromatography performed at 3.5°C.

Binding buffer	Antibody yield (mg)	Antibody yield (%)
0.15 *M* Glycine–sodium hydroxide	19.0	92.3
1.50 *M* Glycine–sodium hydroxide	18.0	87.2
1.50 *M* Glycine–sodium hydroxide plus 3 *M* sodium chloride	18.3[a]	81.3[a]

[a] In this separation a slight decrease of the flow-rate was noticed during the late elution procedure due to an air bubble in one of the inlet check valves of the pump head. The corresponding chromatogram could not therefore be used for quantitative purposes. The corresponding values from photometric estimation are presented.

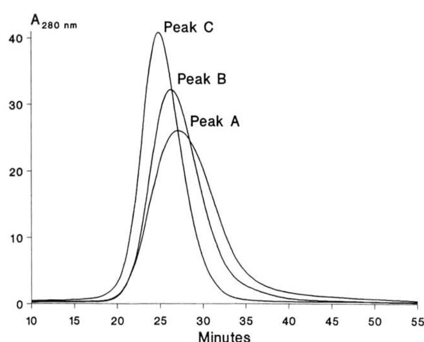


Fig. 2. Optimization of elution conditions. Peak A: elution at 3.5°C; retention time, 27.1 min; relative peak height (rph), 1.0; amount of bound protein, 8.3 mg; relative volume for collecting 95% of eluted Ig (vol. 95%), 1.6. Peak B: elution at room temperature; retention time, 26.6 min; rph, 1.3; amount of bound protein, 8.3 mg; vol. 95%, 1.3. Peak C: elution at room temperature; reversal of flow direction before elution; retention time, 24.8 min; rph, 1.7; amount of bound protein, 8.2 mg; vol. 95%, 1.0.

sponding to the amount of bovine Igs is about one sixth of the area of a comparable peak after the separation of an identical volume of culture supernatants. Two peaks can be seen which partially overlap (Fig. 4). A comparison of the chromatogram with the elution profile obtained after the column was loaded with an equally large volume of

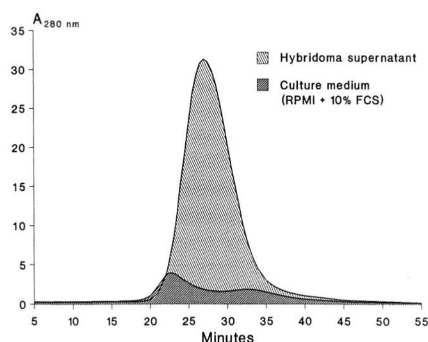


Fig. 3. Binding of non-specific bovine Ig originating from medium supplement (10% FCS) to the protein A column after loading of 160 ml of unconditioned culture medium. A corresponding peak from a separation of an equal volume of hybridoma supernatant performed under identical conditions (binding buffer 1.50 *M* glycine–sodium hydroxide; pH of binding buffer–supernatant mix, 8.2; chromatography performed at 3.5°C) is given for comparison.

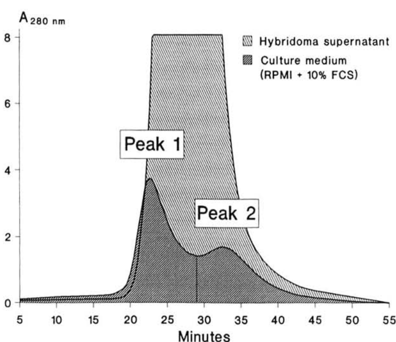


Fig. 4. Elution of non-specific bovine Ig. The chromatogram shows two overlapping peaks, suggesting the existence of two distinct Ig populations with different affinities towards protein A in FCS.

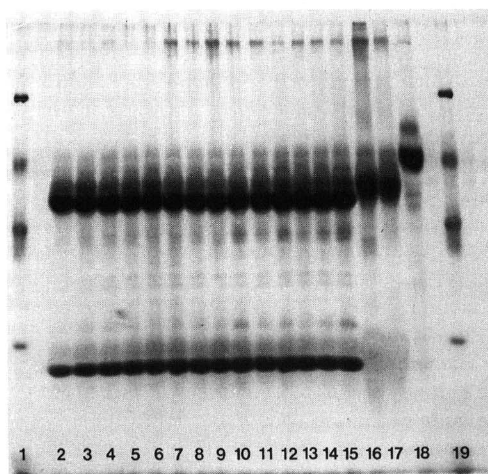


Fig. 5. Electrophoretic evaluation of mabs deriving from separations performed under different loading and washing conditions, non-specific bovine Ig originating from culture medium supplement (FCS) and crude supernatant. Lanes 1 and 19, molecular weight markers (94, 67, 43, 30 kDa); lanes 2–15, eluted mabs from separations of hybridoma culture supernatant performed under different conditions of ionic strength, pH, temperature and sample volume; lane 16, non-specific, protein A-reactive Ig from FCS (peak 1); lane 17, non-specific, protein A-reactive Ig from FCS (peak 2); lane 18, crude hybridoma culture supernatant. Purified mabs from different separations do not show significant differences with respect to contaminants and degree of contamination. Purified mabs are almost free of contamination with the exception of protein A-reactive bovine Ig, which can be seen as weak bands slightly above the light and heavy chains of murine mabs. Although eluting in two different peaks from the protein A column, non-specific bovine Ig from FCS cannot be distinguished by SDS-PAGE.

hybridoma supernatant under identical test conditions reveals that the first, taller peak for the bovine protein has a shorter retention time than the murine mabs. In contrast, the second, smaller peak has a longer retention time. No essential differences can be seen between the two media samples which were prepared using FCS from differing charges from the same manufacturer.

The visual evaluation of a silver-stained gel after SDS-PAGE reveals neither qualitative nor quantitative differences with respect to contaminants between the mab preparations obtained under differing conditions. The bovine Ig of both peaks obtained by chromatographic separation of unconditioned culture medium gives identical results on the silver-stained gel (Fig. 5). Compared with murine mabs, the heavy and light chains of the bovine protein A-reactive Ig have been shifted slightly towards heavier molecules. At each of the peaks obtained by purifying the hybridoma supernatants, an indistinct weak band is seen immediately above the light and heavy chains of the murine mabs. These bands represent the light and heavy chains of the contaminating bovine antibodies. With the exception of the Igs from the FCS supplement, there are no contaminants of any quantitative significance.

DISCUSSION

The aim of this work was to develop a simple, optimized single-step procedure for the preparative isolation of murine IgG_1 mabs. HPLC equipment was available which made it possible to perform the procedure almost entirely automatically, to store and process the data collected and to determine the amount of eluted mabs rapidly and smoothly. The supernatant was not applied with the integrated sample loop, but with one of the four buffer lines. This allowed the binding buffer to be mixed during loading via the gradient mixer in the ratio required. There are several advantages to this form of loading compared with the traditional sample loop method. The preparation of the raw material is reduced to a minimum; if the culture supernatants are sterily filtered and then frozen immediately after preparation, they only need to be thawed in water and degassed just before the chromatographic separation. The pH values and the saline concentration are fixed by mixing in binding buffer. If potentially denaturing conditions are required to achieve optimum binding, this form of loading allows the mabs to remain in a physiological environment until immediately before application to the column. One further advantage is that volumes of any size can be loaded continuously onto the column.

Binding occurring between the ligate and ligand is influenced by temperature, ionic strength, pH and the dielectric constants of the mobile phase [20]. The influence of the first three of these parameters on the binding strength of murine IgG_1 mabs to protein A was studied. It has been known for some time that the binding of murine IgG_1 to protein A is dependent on the pH [21]. In contrast to the other murine IgG subclasses, complete binding cannot be achieved if the pH is less than 8.0. Recommendations have been made that binding is performed at pH values considerably higher than 8.0 [18]. In this work, raising the pH value from 8.2 to 8.7 did not give an improvement in yield at any of the saline concentrations and temperatures tested.

Buffers with very high concentrations of saline are often recommended to strengthen the binding between protein A and murine IgG, in particular, IgG_1. Examples of these concentrations are 3 *M* sodium chloride plus 1.5 *M* glycine [22], 2 *M* sodium chloride plus 2 *M* glycine [23], 0.5 *M* phosphate [24] or 1 *M* Tris [18]. Improved binding is achieved by the intensification of hydrophobic interaction [17].

The work reported here confirmed the positive correlation between the saline concentration of the binding buffer and the binding capacity of the column. However, the increase in yield achieved with the highest saline concentrations tested (a mix of 1 *M* sodium chloride and 0.5 *M* glycine) was clearly lower than that from separations with the cooled column. In addition, applying such high concentrations of saline causes several problems. For example, parts of a liquid chromatographic system that are movable cannot be completely sealed. The result is that the mobile phase leaks between the moving and fixed parts, especially around the pump head pistons. The evaporation of the leaking mobile phase causes a crystalline residue to form, which causes wear and tear in these sections [25]. This problem is worse at high saline concentrations. Another problem is that at high saline concentrations there is a danger of aggregation formation and loss

of activity due to denaturing. The volumes that are then required mean that a lot of material is used.

Temperature has the most significant effect on the strength of binding. The strengthening of binding through cooling may occur as a result of the effects of two mechanisms. Low temperatures weaken hydrophobic interactions [17]. With a weakening of the intramolecular hydrophobic bonds, slight changes may be induced in conformation in either one or both binding partners which result in an overall increase in affinity. Alternatively, this effect could be achieved thermodynamically.

As the reaction that DF4B7A6 shows towards protein A is that of a "typical" murine IgG_1 mab, and since other workers [26] also showed that the binding of murine IgG_1 mab to protein A is sensitive to temperature, it can be assumed that despite the reported heterogeneity of this murine IgG subclass [27], binding to protein A depends greatly on temperature, at least in some mabs of the IgG_1 subclass.

Reversing the flow direction has already been described in the elution of mabs from an ion-exchanger column [2]. This procedure can also be used to advantage in affinity chromatography. The elution volume is smaller and the material obtained more concentrated. "Tailing", which is frequently lengthy, does not occur and the peak appears earlier on the chromatogram, *i.e.* the pH of the eluate obtained is higher and the volume required for neutralization is smaller. For these reasons flow reversal is greatly recommended with soft-gel columns that allow the flow to be reversed easily.

The use of medium supplements containing serum as the source of growth factors leads to the contamination of the hybridoma supernatants with unspecific bovine Ig. To reduce this contamination, FCS is most frequently used as it contains a relatively low level of Ig, which is often assumed to be negligible. However, FCS also contains a considerable concentration of non-specific Ig [16]. Of the bovine Igs it is only the IgG_2 subclass that possesses pronounced protein A reactivity. IgG_1 binds very poorly; no protein A reactivity is observed in the other bovine Ig classes [28].

According to previously reported information the protein A-reactive Ig contained in commercially-available FCS shows evidence of considerable fluctuations (between 0.03 and 0.26 mg/ml) between the individual charges. In the 10% supplement of FCS usually used, concentrations of non-specific bovine Ig were measured in culture media that correspond to the mab content. There is therefore a great potential risk of contamination with bovine protein A-reactive Ig for mabs purified with protein A from culture supernatants containing serum [16].

It is difficult to separate bovine IgG contained in FCS from murine IgG as a result of the conserved structure of the IgG. The most common methods of purifying mabs, such as ammonium sulphate precipitation, protein A affinity chromatography and ion-exchange chromatography, result in a "co-purifying" of non-specific Ig. When the concentration of bovine protein A-reactive Ig was determined in samples of differing murine mabs purified by protein A affinity chromatography of culture supernatants, contamination rates of up to 50% were found [16].

As is shown in the evaluation of the gels after SDS-PAGE and silver-staining, bovine protein A-reactive Ig is the only contaminant of quantitative significance. In the chromatographic separation of 160 ml of unconditioned medium, *i.e.* under conditions in which the binding sites on the column are available only to bovine Ig, the yield is about one sixth of the amount of protein obtained by purifying a corresponding volume of hybridoma culture supernatant. In the chromatographic separation of culture supernatant, a reduction in contaminants is expected, as a result of competition from superfluous murine mabs. Therefore, in the visual evaluation of samples of "purified" mabs on a silver-stained gel, in which it was possible to make a limited distinction between bovine and murine Ig, only relatively weak bands were seen at the position of the heavy and light chains of the bovine Ig. The actual rate of contamination is probably far below 17%, the maximum contamination rate possible. This degree of purity in the mabs obtained is adequate for most laboratory requirements.

It is therefore possible, with a minimum amount of effort and in a single-step procedure, to effectively purify and simultaneously concentrate murine IgG_1 mabs from culture supernatants using protein A affinity chromatography with a cooled column. As a result of the generally acknowledged longevity of protein A Sepharose, this procedure is economical, despite the relatively high initial outlay for the

column material. Relatively mild conditions are adequate for loading and elution and murine IgG_1 mabs can be obtained in a highly purified form using protein A affinity chromatography. This is important as a large proportion of murine mabs belongs to this subclass. The procedure developed is particularly well suited to laboratory-scale application and can also be applied to the purification of much larger volumes if correspondingly larger columns are used. Even on a laboratory scale, it is possible to obtain highly purified murine IgG mabs from mass cell cultures using a simple technique.

REFERENCES

1 A. Danielsson, A. Ljunglöf and H. Lindbloom, *J. Immunol. Methods*, 115 (1988) 79–88.
2 B. Malm, *J. Immunol. Methods*, 104 (1987) 103–109.
3 R. W. Scott, S. A. Duffy, B. J. Moellering and C. Prior, *Biotechnol. Prog.*, 3 (1987) 49–55.
4 Y.-J. Schneider and A. Lavoix, *J. Immunol. Methods*, 129 (1990) 251–268.
5 D. Velez, S. Reuveny, L. Miller and J. D. Macmillan, *J. Immunol. Methods*, 86 (1986) 45–52.
6 J. P. A. M. Klerx, C. Jansen Verplanke, C. G. Blonk and L. C. Twaalfhoven, *J. Immunol. Methods*, 111 (1988) 179–188.
7 H. Baumgarten and R. Franze, in J. H. Peters and H. Baumgarten (Editors), *Monoklonale Antikörper: Herstellung und Charakterisierung*, Springer Verlag, Berlin, 2nd ed., 1990, pp. 223–225.
8 I. Kuhlmann, W. Kurth and I. Ruhdel, *Altern. Tierver.*, 11 (1989) 12–26.
9 E. L. V. Harris, in E. L. V. Harris and S. Angal (Editors), *Protein Purification Methods: A Practical Approach*, IRL, Oxford, 1989, pp. 51–66.
10 J. A. Asenjo and I. Patrick, in E. L. V. Harris and S. Angal (Editors), *Protein Purification Applications: A Practical Approach*, IRL, Oxford, 1990, pp. 1–28.
11 M. Oppermann, in J. H. Peters and H. Baumgarten (Editors), *Monoklonale Antikörper: Herstellung und Charakterisierung*, Springer Verlag, Berlin, 2nd ed., 1990, pp. 259–262.
12 Y.-J. Schneider, *J. Immunol. Methods*, 116 (1989) 65–77.
13 M. Carlsson, A. Hedin, M. Inganäs, B. Härfast and F. Blomberg, *J. Immunol. Methods*, 79 (1985) 89–98.
14 W. L. Cleveland, I. Wood and B. F. Erlanger, *J. Immunol. Methods*, 56 (1983) 221–234.
15 E. Shacter, *J. Immunol. Methods*, 99 (1987) 259–270.
16 P. A. Underwood, J. F. Kelly, D. F. Harmann and H. M. Macmillan, *J. Immunol. Methods*, 60 (1983) 33–45.
17 S. Angal and P. D. G. Dean, in E. L. V. Harris and S. Angal (Editors), *Protein Purification Methods: A Practical Approach*, IRL, Oxford, 1989, pp. 245–292.
18 H. Baumgarten, in J. H. Peters and H. Baumgarten (Editors), *Monoklonale Antikörper: Herstellung und Charakterisierung*, Springer Verlag, Berlin, 2nd ed., 1990, 265–270.
19 E. Eigenbrodt, S. Leib, W. Krämer, R. R. Fries and W. Schoner, *Biomed. Biochim. Acta*, 42 (1983) 278–282.
20 H. A. Chase, *J. Chromatogr.*, 297 (1984) 179–202.
21 P. L. Ey, S. J. Prowse and C. R. Jenkin, *Immunochemistry*, 15 (1978) 429–436.
22 *Separation News*, Vol. 13, No. 5, Pharmacia, Freiburg, 1986, pp. 1–3.
23 S. Ohlson and J. Wieslander, *J. Chromatogr.*, 397 (1987) 207–212.
24 M. Perry and H. Kirby, in E. L. V. Harris and S. Angal (Editors), *Protein Purification Applications*, IRL, Oxford, 1990, pp. 147–166.
25 J. W. Dolan, *LC · GC Int.*, 2 No. 9 (1989) 16–18.
26 Y. Y. Tu, F. J. Primus and D. M. Goldenberg, *J. Immunol. Methods*, 109 (1988) 43–47.
27 C. L. Villemez, M. A. Russel and P. L. Carlo, *Mol. Immunol.*, 21 (1984) 993–998.
28 J. Goudswaard, J. A. van der Donk, A. Noordzij, R. H. van Dam and J.-P. Vaerman, *Scand. J. Immunol.*, 8 (1978) 21–28.

Journal of Chromatography, 587 (1991) 71–80
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2368

Application of reversed-phase medium-pressure liquid chromatography to the isolation, separation and amino acid analysis of two closely related peptide toxins of the cyanobacterium *Microcystis aeruginosa* strain PCC 7806

Josef Cremer* and Klaus Henning

Institute of Microbiology, Federal Armed Forces Medical Academy, Neuherbergstrasse 11, W-8000 Munich 45 (Germany)

ABSTRACT

A mixture of two compounds, related to cyanoginosin-LR, was isolated from the cyanobacterium *Microcystis aeruginosa* strain PCC 7806. Using an optimized reversed-phase fast protein liquid chromatography (RP-FPLC) method, up to 1.5 mg of this toxin mixture could be separated into two distinct compounds by a single preparative scale run. To perform an amino acid analysis of the two components, the hydrolysates derivatized with phenylisothiocyanate were analysed separately by RP-FPLC. A method was developed which could be used for the automatic sequential analysis of up to fourteen samples. The amino acid analysis by RP-FPLC was linear in the range 1–5 nmol. On the basis of this analysis, the two compounds differ by only a methylated *versus* a demethylated aspartate. Only six of the seven amino acids known for cyanoginosin-LR could be reliably detected.

INTRODUCTION

Microcystis sp., which produce highly active hepatotoxins, are algal organisms, most frequently isolated from toxic algal blooms in water [11]. The organisms occur in supplies of drinking water, and, as a result of their toxicity and high stability, are a threat for drinking water hygiene [2–4].

The most important toxic substances of *Microcystis* have been identified as cyclic heptapeptides with a molecular weight of about 1000. These are composed of three D-amino acids, one dehydroamino acid, one atypical β-amino acid and two strain-dependent variable L-amino acids. *Microcystis* toxins are generally termed cyanoginosin-XY. The variables X,Y describe the two variable L-amino acids [5].

It is assumed that the toxin produced by *Microcystis aeruginosa* strain PCC 7806 and cyanoginosin-LR are identical [6], with a structure of cyclo-(D-Ala-L-Leu-D-*erythro*-β-methylaspartate-L-Arg-Adda-D-Glu-N-methyldehydro-Ala-). The atypical amino acid Adda is a 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid [7].

Reversed-phase high-performance liquid chromatographic (RP-HPLC) studies imply that the toxic fraction of PCC 7806 is not only one substance [6,8]. This is in accordance with previous findings using thin-layer chromatography (TLC) (unpublished data). It has been shown that this *Microcystis* strain produces two very similar components which have the properties of the cyanoginosins. Considering the possible implications for further toxicological studies, it was intended to isolate, separate and analyse these two substances on a preparative scale. Until now, the cyanoginosins have been purified and analysed by HPLC [8–11]. In this work the preparative and analytical capacity of the fast protein liquid chromatography (FPLC) technique was investigated. The chromatographic conditions were optimized by systematic variation and methods were developed to perform amino acid analysis by FPLC.

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V. All rights reserved

EXPERIMENTAL

Algal strain and cultivation

Microcystis aeruginosa strain PCC 7806 was kindly supplied by Professor Weckesser, Institute for Microbiology of the University of Freiburg, Germany. Cyanobacteria were harvested from 10 l of fermentation cultures, continuously supplied with BG-11 medium [12] and illuminated with 2000-lux fluorescent light.

Isolation of the toxin

Lyophilized cyanobacteria (5 g) were extracted in 500 ml of doubly distilled water by exposure to ultrasound and centrifugation at 30 000 *g* for 30 min. For solid-phase extraction the supernatant was applied to a column packed with 5 g of C_{18} (J. T. Baker, Gross-Gerau, Germany), previously activated by flushing with 50 ml of 100% methanol (Uvasol Merck, Darmstadt, Germany) and subsequently with 50 ml of water. The toxic fraction was desorbed by elution with 100% methanol. After evaporation to dryness, the residue was dissolved in 200 ml of methanol–water (30:70, v/v). The precipitated lipids were removed by centrifugation at 30 000 *g* and subsequent passage of the supernatant through a 0.22-μm filter (Acrodisc, Gelman Sciences, Frankfurt, Germany).

To perform anion-exchange chromatography, an XK 16/10 column (Pharmacia, Freiburg, Germany), packed with 5 g of QMA (Waters Millipore, Eschborn, Germany) was used [13]. Prior to loading of the C_{18} eluate (200 ml), the QMA column was equilibrated with 100 ml of 0.1 *M* ammonium hydrogencarbonate in methanol–water (30:70, v/v) and washed by rinsing with 100 ml of the salt-free solvent. The toxic fraction was almost selectively detached from the matrix by elution with 0.02 *M* ammonium hydrogencarbonate (methanol–water, 30:70, v/v; flow-rate 2 ml/min; monitored at 240 nm). Finally, the toxic QMA eluate was evaporated to dryness and redissolved in methanol–water (30:70, v/v) and photometrically adjusted to the desired concentration using a standard calibration graph (Cyanoginosin-LR, molecular weight 994, Medor, Hersching, Germany).

Preparative RP-FPLC

The components contained in the QMA eluate were separated on a preparative scale using an FPLC system (Pharmacia) with the following configuration: LCC-500 plus gradient controller, two P-500 pumps, MV7 motor injection valve, 500-μl sample loop, VWM 2141 variable-wavelength monitor and FRAC-100 fraction collector. FPLC software (FPLC Manager, Pharmacia) facilitated the operation, data processing and documentation. A preparative PepRPC HR 16/10 column (pre-packed with 15-μm silica particles with covalently bonded C_2/C_{18}, average pore size 100 Å) was used as the stationary phase. The solvents were acetonitrile (LiChrosolv, Merck) and Milli-Q water (Millipore). For the optimum separation the application of very shallow gradients was necessary and therefore the eluents were premixed: solvent A, acetonitrile–water (24:76, v/v); solvent B, acetonitrile–water (50:50, v/v). Depending on the experiment trifluoroacetic acid (TFA) or pentafluoropropanate (PFPA, Merck) were added in different amounts to both solvents. The solutions were freshly prepared and degassed for 15 min before use. After separation the toxin fractions were desalted by the HR 16/10 column. For evaluation, the resolution (R_s) of the separated compounds was calculated as follows [14]:

$$\alpha = r_2/r_1 \quad (1)$$

$$N = (r_1/w_{0.5})^2 \quad (2)$$

$$k' = (r_1 + r_2)/2 \quad (3)$$

$$R_s = 0.25\,[(\alpha - 1)/\alpha]\,N^{1/2}[k'/(1+k')] \quad (4)$$

where r_1 = retention time of peak 1, r_2 = retention time of peak 2, $w_{0.5}$ = peak width at half height, α = selectivity, N = efficiency and k' = capacity factor. A baseline separation is achieved, if R_s exceeds 1.5.

UV spectroscopy

The UV spectra of toxin samples were recorded on a MQ-3 spectrophotometer (Zeiss, Oberkochen, Germany). The samples were evaporated, dissolved in methanol–water (30:70, v/v) and adjusted to 0.6 absorption units at 240 nm ($A.U._{240\ nm}$).

TLC

UV_{254nm}-C_{18} RP-HPTLC plates, 10 × 20 cm (Merck) were used. The tank was equilibrated with

100 ml of methanol–water–acetic acid (70:28:2, v/v/v) 1 h before inserting the plates. Sample volumes of 10–20 μl dissolved in methanol–water (50:50, v/v) were spotted on the plates. After developing, bands were visualized by UV light (254 nm).

Amino acid analysis

Automatic FPLC amino acid analyser. The FPLC system could be programmed and automated for amino acid analysis. For automatic sample application, two MV8 valves (MV8-2 and MV8-3), each with eight inlets and one outlet, and the peristaltic pump C were connected in series between the solvent reservoir A and the motor injection valve MV7. As position .1 of the MV8 valves is reserved for wash functions, the valve positions .2 to .8 are available for sample loading. Programming methods for the LCC-500 gradient controller, sample loading by defined MV8 settings and the sample injection, gradient control and washing of the sample application system could be performed automatically (Table I). Fourteen LCC-500 methods (MV8.1 to MV8.14) were provided for the different valve positions and the FPLC Manager program was used for control.The program automatically analysed each of the fourteen samples successively by activating the LCC-500 methods in addition to recording, evaluating and printing the corresponding chromatograms.

Sample preparation. Standard L-amino acids (Asp, Glu, Ser, Gly, Thr, Ala, Arg, Tyr, Met, Leu) were purchased from Merck. D-Glu, D-Ala, DL-*threo*-β-methylaspartate and methylamine were obtained from Sigma (Deisenhofen, Germany). Defined aliquots of standards and purified toxin samples were suspended in 4 *M* hydrochloric acid (Titrisol, Merck) and placed in glass tubes. After evacuating and flushing with nitrogen, the tubes were sealed and incubated at 110°C for 18 h. To eliminate residues of hydrochloric acid, the samples were evaporated to dryness by vacuum centrifugation. The samples were then dissolved in 10 μl of an ethanol–water–triethylamine solution (2:2:1, v/v/v) (Pierce, Oud Beijerland, Netherlands). After redry-

TABLE I

FUNCTIONS OF THE LCC-500 METHODS MV8.%1 CONTROLLING THE MV8- VALVE SETTING AND THE GRADIENT

Programmed methods are MV8.1 to MV8.7 for valve MV8-2 and MV8.8 to MV8.14 for valve MV8-3. The valve position is controlled by the the FPLC Manager program calling up the appropriate method by the value for the variable %1, corresponding to the numeral behind the point in the method name.

Time (min)	Processing of		Setting	Description
	Sample	Gradient		
0		Conc. %B	0	
0		ml/min	1	Adjust flow-rate
0	VALVE.POS		2.X or 3.X[a]	Set valve to sample
0	FLOW C		0.5	Load sample (pump C)
1		cm/min	0.5	Activate recorder
1	FLOW C		0	Loop filled
1	VALVE.POS		1.2	Inject (valve 1 = MV7)
2	VALVE.POS		1.1	Stop injection
2	VALVE.POS		2.1 or 3.1	Reset active valve
2	FLOW C		1	Wash loop
3		Conc. %B	0	Start gradient
5	FLOW C		0	Stop washing
34		Conc. %B	32	Start column washing
49		Conc. %B	100	and reequilibrate to
53		Conc. %B	100	initial conditions
55		Conc. %B	0	
60		Conc. %B	0	END METHOD

[a] To determine the valve position depending on the method activated, fixed X-values from 2 to 8 are programmed for the methods MV8.1 to MV8.7 and MV8 to MV8.14.

ing for 1 h, 20 μl of the coupling solution [ethanol–water–triethylamine–phenylisothiocyanate (PITC), 70:19:10:1, v/v/v/v] were added, followed by mixing and incubation for 20 min at room temperature. By reaction with free amino acids, phenylthiocarbamyl (PTC) derivatives are obtained. Any excess PITC was removed by vacuum centrifugation. The samples were dissolved in eluent A prior to chromatographic separation.

Column chromatography. For eluent A, 0.9 l of water (HPLC grade, Merck) plus 0.8 ml of phosphoric acid (85%) were adjusted to pH 6.4 with sodium hydroxide solution 30%. The volume was adjusted to 1 l with HPLC-grade water and 5 ml of acetonitrile were added. Eluent B consisted of 300 ml of eluent A and 700 ml of acetonitrile. Before use, the freshly prepared solutions were degassed for 15 min. An HR 5/5 column (prepacked with C_2/C_8 silica, average particle diameter 5 μm, pore size 100 Å) was used as the stationary phase. The separation was carried out with 0% B for the first 2 min and then a linear gradient of 1% B per min for 32 min. Washing and re-equilibration was controlled by the program (Table I).

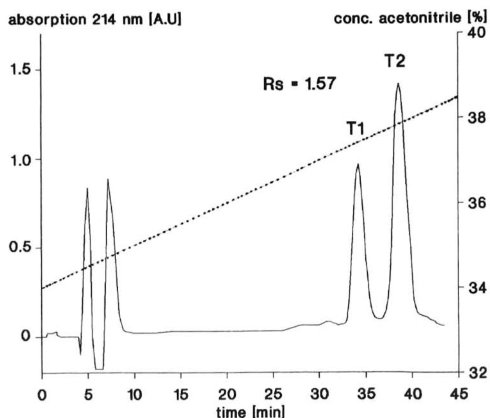


Fig. 1. Separation of the cyanoginosin homologues T1 and T2, isolated from strain PCC 7806, on the PepRPC HR16/10 column. The resolution R_s was calculated by eqn. 4. Chromatographic conditions: gradient start, 34% acetonitrile; slope, 0.1% min; additive, 0.3% PFPA; flow-rate, 3 ml/min; temperature, 4.3°C; sample, 1.4 mg per 500 μl (methanol–water, 30:70, v/v). The first signals (< 10 min) are solvent peaks.

RESULTS

Toxin preparation

Characteristic UV absorption spectra and the typical double-banded spot on HPTLC indicate the presence of cyanoginosin in the QMA eluate. The isolation procedure to the stage of the QMA eluate seems to give an almost pure toxin preparation; the RP-FPLC elution profiles of the QMA eluate recorded at 214 nm (Fig. 1), TLC (Fig. 2) and UV absorption spectra (Fig. 3) displayed no significant contamination.

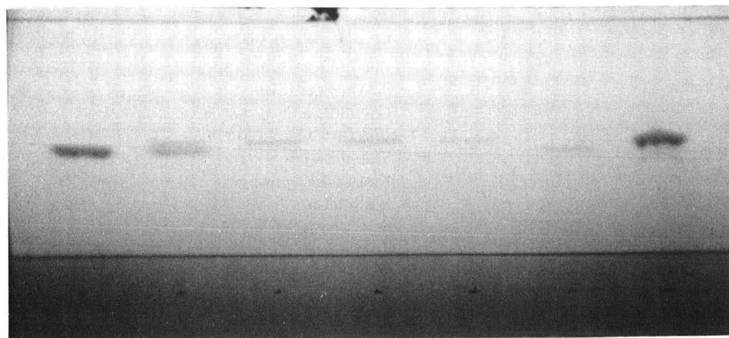


Fig. 2. HPTLC separation (chromatographic conditions as described in the text). The lanes are numbered from left to right. The arrangement of the samples was: lane 2, mixture of T1 and T2 (QMA eluate) prior to RP-FPLC; lanes 1 and 6, T1 after separation; lanes 3, 5 and 7, T2 after separation; lane 4, cyanoginosin-LR standard (Medor). The amounts spotted were about 20 μg. Lanes 1 and 7 were overloaded to visualize possible contaminations.

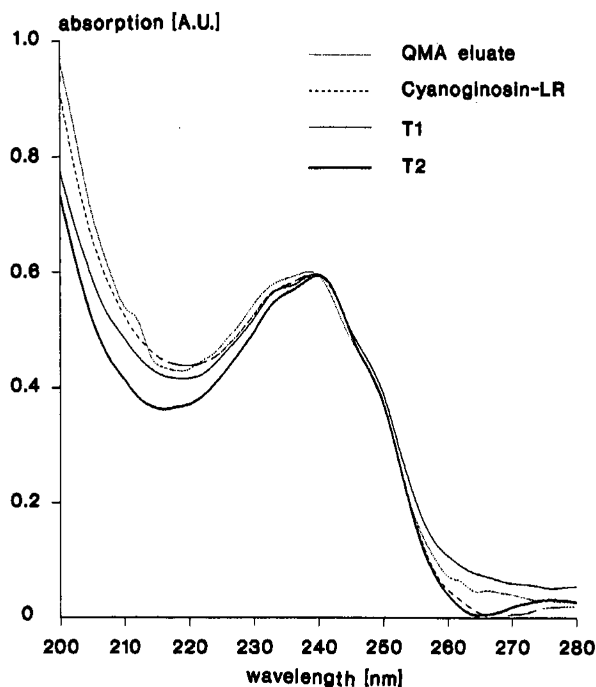


Fig. 3. UV absorption spectra of QMA eluate, T1, T2 and cyanoginosin-LR standard. Solvent was methanol–water (30:70, v/v).

Optimization of separation of the peptides by FPLC

The organic solvent concentration at which the desired components were eluted from the HR 16/10 PepRPC was in the range 35–42% acetonitrile. First 0.1% TFA was used as the ion-pairing agent, but under these conditions the resolution was not satisfactory. Therefore the more hydrophic PFPA was introduced. By increasing the retention time and selectivity by the addition of at least 0.3% PFPA, the separation was significantly improved. For further optimization the influence of different gradient slopes and different flow-rates was investigated. Starting at a concentration of 32% acetonitrile, the gradient slope was decreased stepwise from 0.32 to 0.08% per min and the flow-rate from 8 to 2 ml/min. The resolution was increased by lowering the flow-rate and gradient slope at the expense of time taken for the analysis and peak sharpness, which affected the efficiency (Table II). Isocratic elution in the concentration range 32–38% acetonitrile was not of advantage.

The following conditions provided an acceptable separation within 60 min: gradient start, 34% acetonitrile; gradient slope, 0.1% per min; flow-rate, 3 ml/min.

The influence of temperature in the range 0–30°C was investigated. Below 10°C almost baseline separation could be achieved (Fig. 1). Mathematically, the two components (T1 and T2) are separated completely. As a result of the slightly raised baseline, the fraction between the peaks T1 and T2 below the threshold of 0.15 A.U. was ignored to ensure physical separation. Up to 1.5 mg of the T1–T2 mixture can be separated by this method.

Analysis of separated peaks T1 and T2

The separation results were verified by repeating the chromatographic separation and by HPTLC (Fig. 2). The separation of T1 and T2 was complete and the absorption at 214, 239 and 254 nm suggests that the purity of the substances approaches 100%. The R_F values of the toxin standard (Cyanoginosin-

TABLE II

INFLUENCE OF GRADIENT SLOPE AND FLOW-RATE ON THE RESOLUTION AND RETENTION TIME

Conditions: temperature, 20°C, 0.3% PFPA to solvent A and B.

Gradient slope (% min)	Flow-rate (ml/min)					
	8		4		2	
	R_s	Time (min)	R_s	Time (min)	R_s	Time (min)
0.33	0.88	18.60	1.10	28.00	0.83	40.91
0.17	0.93	23.53	1.15	37.06	1.20	55.79
0.08	0.96	28.04	1.14	47.03	1.26	74.19

LR, molecular weight 994, Medor) was 0.48, whereas the components T1 and T2 had R_F values of 0.44 and 0.48, respectively.

A further indication of the structural relationship of the isolates to cyanoginosin-LR was obtained by the UV absorption spectra from 200 to 280 nm (Fig. 3). Apart from slight differences, the QMA eluate, T1 and T2 showed similar UV absorption behaviour to the cyanoginosin-LR standard with an absorption maximum at 240 nm.

Amino acid analysis by RP-FPLC

Standards were chosen according to known amino acid composition of cyanoginosin-LR. The sequence of the standards eluted from the HR 5/5 PepRpc column could be determined reproducibly. Comparing the standard chromatograms from different series ($n \leqslant 5$), each performed with fresh eluent preparations, the maximum standard deviation of the retention times was about 0.6 min. As the cyanoginosins contain D-Glu and D-Ala, the corresponding D- and L-stereoisomers were tested. Significant differences in retention times were not detectable (Table III).

TABLE III

ELUTION SEQUENCE OF SINGLE PTC STANDARD AMINO ACIDS ON THE HR 5/5 PepRPC COLUMN

See also Fig. 4C for Asp, Glu, *threo-β*-MeAsp, Ala, Arg, MeAmine and Leu.

PTC derivative[a]	Time (min)	Standard deviation (min)	n
L-Asp	3.05	0,.37	4
DL-*threo-β*-MeAsp	3.76	0.08	5
D-Glu	3.89	0.18	2
L-Glu	3.93	0.15	5
L-Ser	7.77	0.41	2
Gly	8.57	–	1
L-Thr	11.03	0.44	2
D-Ala	11.73	0.31	2
L-Ala	11.89	0.55	5
L-Arg	13.84	0.62	3
MeAmine	16.78	0.38	3
L-Tyr	20.59	–	1
L-Met	21.43	–	1
L-Leu	24.98	0.29	3

[a] Known components of cyanoginosin-LR are printed in bold.

Almost idential chromatograms were recorded for the PITC-derivatized T1 and T2 hydrolysates. The only difference was the exchange of one amino acid (Fig. 4A, peak 1; Fig. 4B, peak 2). Asp, Glu, Ala, Arg and Leu could be identified directly by the standards (Tables III and IV; for discrepancies of the retention times, see Discussion). The cyanoginosin component N-methyldehydroalanine (N-Medha) and the atypical amino acid Adda are destroyed by the hydrolysis procedure [6,15,16], but N-Medha is known to liberate methylamine (MeAmine) during hydrolysis. The presence of MeAmine was proved by a PTC derivative of a MeAmine standard (Fig. 4C, Tables III and IV). The commercially available standard for *β*-MeAsp is a *threo*-diastereomer, which co-eluted with Glu. On the basis that the racemization of the *β*-MeAsp standard to an equimolar mixture of *threo* and *erythro* configurations can be effected by exposure to acid and heat (see Discussion) [15,17], this standard was also submitted to the hydrolysis procedure (4 *M* hydrochloric acid, 110°C, 18 h) and then derivatized with PITC (Fig. 4D). Only after this treatment the analysis of the *β*-MeAsp standard did reveal an additional peak at 6 min. A fraction of 15–20% of *threo-β*-MeAsp was converted to another substance, which is assumed to be *erythro-β*-MeAsp (quantification by peak-area distribution). This compound had exactly the same retention time as the second peak of the T2 hydrolysate (Fig. 4B, Table IV).

The elution pattern of T1 and T2 hydrolysates could be qualitatively reproduced by a mixture of amino acids which are assumed to be components of the cyanoginosin-LR (Fig. 4C). This standard mixture was also exposed to the acid hydrolysis conditions (for discrepancies of the retention times, see Discussion).

The characteristic elution behaviour of the T1- and T2-derived PTC amino acids could be confirmed by repetitive determinations of the retention times and amounts of amino acids. To test the reproducibility, toxin samples from different preparations were hydrolysed, derivatized separately and chromatographed in one series. Approximately equimolar amounts of the detected amino acid components were calculated for T1 and T2 (Table V).

Determining the range for the analysis of PITC-derivatized cyanoginosin hydrolysates by RP-FPLC, the quantification of amino acids is linear in

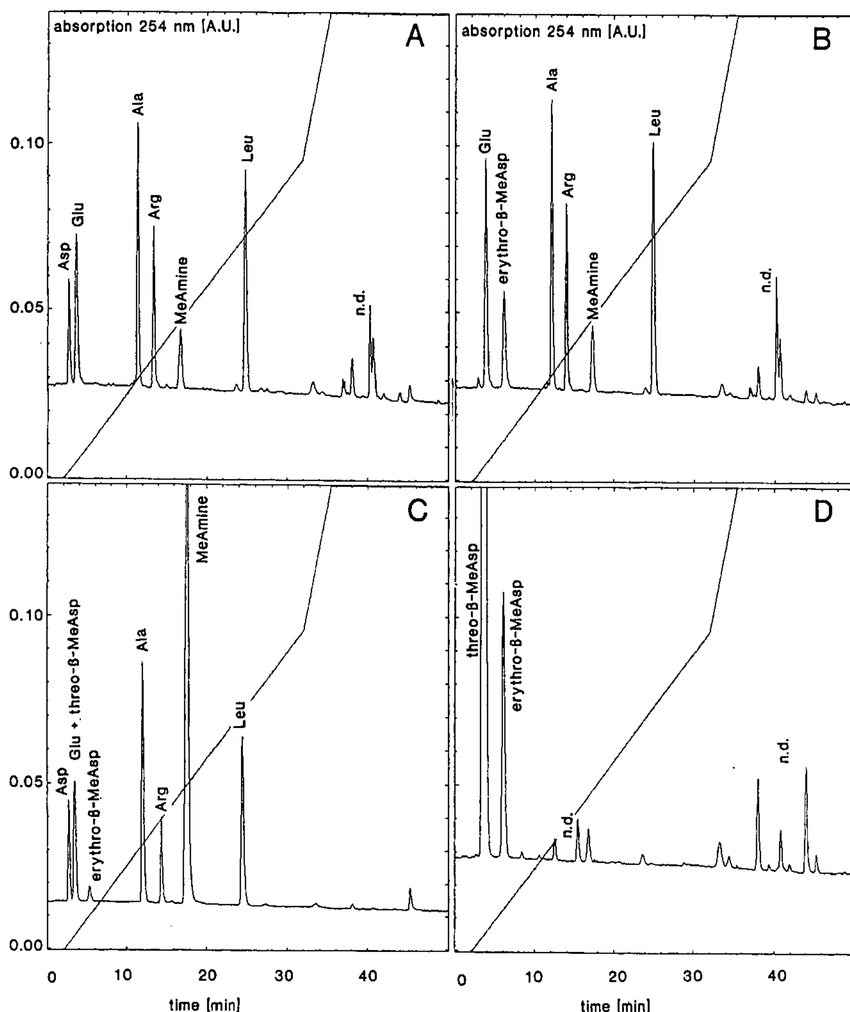


Fig. 4. (A) Amino acid analysis of 3 nmol PITC-derivatized T1 hyrolysate; (B) 3 nmol T2 hydrolysate; (C) Elution sequence of PTC standard amino acids exposed to acid hydrolysis prior to the derivatization (concentration range about 1–3 nmol of each amino acid and about 10 nmol MeAmine). Glu and *threo-β*-MeAsp are not resolved; (D) Chromatogram of *threo-β*-MeAsp standard (34 nmol) exposed to acid hydrolysis prior to the derivatization. About 4.8 nmol were converted to *erythro-β*-MeAsp (for discrepancies, comparing the retention times of C to A, B and D, see Table IV and the comments in the discussion). MeAmine = methylamine, a hydrolysis product of N-methyldehydroalanine; n.d. = unidentified PITC derivatives produced during sample derivatization.

the range 1–5 nmol. The detection limit was less than 1 nmol.

DISCUSSION

RP-HPLC is a powerful tool for separating different peptide toxins of *Microcystis aeruginosa* sp. at the analytical and semi-preparative scales [8–11]. The data presented here verify that RP-FPLC offers a practical alternative. The method allows the separation of 1.5 mg of a T1–T2 mixture, two very similar cyanoginosin-LR homologues produced by *Microcystis aeruginosa* PCC 7806, in a single run within 45 min. Although the similar UV absorption spectra and HPTLC analysis confirm purity of the two isolates, non-UV-absorbing impurities might still be present [18]. However, the amino acid analysis detected no significant contamination.

TABLE IV

ELUTION BEHAVIOUR OF THE TOXIN-DERIVED PTC-AMINO ACIDS COMPARED WITH THE PTC DERIVATIVES OF A STANDARD MIXTURE AND *threo*-β-METHYL-ASPARATE ON THE HR 5/5 PepRPC COLUMN

All standards were submitted to the hydrolysis procedure prior to derivatization. The data correspond to the chromatograms in Fig. 4. Glu and Ala in the mixture were of L configuration.

PTC derivative	Hydrolysate		Standard mixture	*Threo*-β-MeAsp
	T1	T2		
L-Asp	2.65	–	2.62	–
DL-β-MeAsp	–	–	3.41[a]	3.27[b]
D-Glu/L-Glu	3.58	3.71	3.41[a]	–
DL-*erythro*-β-MeAsp	–	6.03	5.32	6.03[b]
D-Ala/L-Ala	11.37	12.07	11.97	–
L-Arg	13.38	13.98	14.42	
MeAmine	16.69	17.27	17.56	–
L-Leu	24.70	24.91	24.42	–

[a] *Threo*-β-MeAsp and Glu are not resolved by the PepRPC column.
[b] *Threo*-β-MeAsp is chromatographed in two peaks only when exposed to acid hydrolysis; it is assumed that a conformational change from the *threo* to the *erythro* diastereomer occurs (see also Discussion).

The close relationship of the isolates to cyanoginosin-LR is illustrated by comparing the QMA eluate, T1 and T2 to the standard cyanoginosin-LR by UV absorption spectra and HPTLC. Almost equivalent UV spectra with absorption maxima at 240 nm, which are typical for the cyanoginosins [8], were recorded. By TLC the T2 compound had the same R_F value as the standard; T1 displayed only a slightly lower mobility. For further chemical characterization an RP-FPLC method for the analysis of amino acids in the isolates was developed.

Compared with the commonly used RP-HPLC methods for amino acid analysis, the analytical capacity of FPLC, which is predominantly used at a preparative scale, may have some technical limitations. Factors influencing the reproducibility and

TABLE V

COMPARISON OF THE RETENTION TIMES AND THE MOLAR RATIOS OF THE T1- AND T2-DERIVED PTC-AMINO ACIDS

Compound	T1 ($n=6$)			T2 ($n=6$)		
	Time (mean ± S.D.) (min)	Q[a] (nmol	M.R.[a]	Time (mean ± S.D.) (min)	Q (nmol)	M.R.
Asp	2.71 ± 0.10	3.82	1.12(1)	–	–	–
Glu	3.68 ± 0.12	3.45	1.01(1)	3.68 ± 0.15	3.20	1.18(1)
β-MeAsp[b]				6.06 ± 0.27	1.78	0.66(1)
Ala	11.47 ± 0.16	3.41	1.00(1)	11.71 ± 0.33	2.70	1.00(1)
Arg	13.36 ± 0.12	3.04	0.89(1)	13.55 ± 0.33	2.97	1.10(1)
MeAmine[c]	16.68 ± 0.11	1.91	0.56(1)	16.86 ± 0.30	1.93	0.71(1)
Leu	24.81 ± 0.37	2.54	0.74(1)	24.90 ± 0.17	2.46	0.91(1)

[a] Q = quantity and M.R. = molar ratio of the amino acids present in the peptides with respect to molarity of Ala. The value in brackets is the rounded M.R. value.
[b] The distinct lower molarity of *erythro*-β-MeAsp might be due to racemization to *threo*-β-MeAsp which co-elutes with Glu.
[c] It is unknown whether methyldehydroalanine liberates MeAmine quantitatively.

sensitivity are, for example, higher dead volumes, slight fluctuations in gradient steering, flow-rate and pressure, and column parameters. To account for the reported discrepancies in retention times, it should be noted that FPLC requires 1 h for the chromatographic separation of one sample. As an autosampler was not available and sampling had to be performed by two FPLC MV-8 valves, only fourteen samples could be analysed in one series. Therefore some deviations may be due to variations in preparing fresh eluents for new analytical series. For instance, the slightly different retention times of the *erythro*-β-MeAsp in the standard mixture (Fig. 4C, 5.32 min) to the hydrolysate (Fig. 4B, 6.03 min) and the single standard (Fig. 4D, 6.03 min) is most probably due to variations in the eluents, because chromatogram C was recorded within a different series to A, B and D. The maximum standard deviation of the retention times obtained from different series was 0.6 min. Despite this relatively high scattering, in most instances a definite identification of single peaks is still possible if the wide spread elution profile and the selectivity of the peaks are considered. However, signals of PITC-derived toxin components and standard amino acids could be measured with sufficient reproducibility to less than 1 nmol (Tables III and V). Thus the analytical capability of this method is sufficient for a realiable amino acid analysis.

The major problem occurred in identifying unusual amino acids (Adda, N-methyldehydroalanine, *erythro*-β-methylaspartate) in the toxin hydrolysates. It is known that not all amino acid components of the cyanoginosins can be detected by the commonly used derivatization procedures. A reference substance for *erythro*-β-MeAsp is not available. The atypical β-amino acids Adda and N-Medha are destroyed by the hydrolysis procedure [6,15,16]. However, N-Medha could be detected by its hydrolysis product, methylamine, which reacts with PITC [10,11] (peak 5 in Fig. 4A and B). Another difficulty was identifying peak 2 of the T2 hydrolysate by a standard substance. With respect to the basic structure of cyanoginosin-LR, the most likely amino acid responsible for this peak is the *erythro*-β-methylaspartate. Against this, the commercially available β-MeAsp standard has to be of almost 100% D,L-*threo* configuration (an *erythro* standard does not exist). The standard *threo*-β-MeAsp was co-eluted with glutamate (Tables III and IV; Fig. 4C). For this reason a definite assignment of this peak was not possible. Nevertheless, *erythro*-β-MeAsp is one of the five D-amino acids of cyanoginosin-LR (molecular weight 994) [5]. This has been confirmed electrophoretically and by stereospecific gas chromatography [15,19]. On HPTLC the T2 had the same R_F value as the standard cyanoginosin-LR (Fig. 2), for which a molecular weight of 994 is documented. This indicates chemical identity and consequently the presence of *erythro*-MeAsp in the T2 hydrolysate. The solution of this problem was racemization of the *threo*-β-MeAsp to the *erythro* diastereomer to produce an "*erythro*-β-MeAsp standard". A significant peak at 6 min appeared only if the standard β-MeAsp was exposed to acid hydrolysis (4 *M* hydrochloric-acid for 18 h). This behaviour is probably a result of a configuration change (Table IV, Fig. 4C and D). The molar ratio of the *threo* to *erythro* diastereomer after 18 h of acid hydrolysis was about 6:1. After 72 h of hydrolysis in 5.7 *M* hydrochloric acid, the racemization proceeds to equimolar amounts of the diastereomers [15,17]. These findings agree with other RP-HPLC studies in which the PTC-derivatives of *threo*- and *erythro*-β-MeAsp were chromatographed to give two separate peaks with a time difference of 4 min [10]. Additionally, the HPLC elution patterns of PTC derivatives of cyanoginosin-LR, originated by other cyanobacteria [11], are very similar to those obtained by FPLC; even the elution behaviour of *erythro*-β-MeAsp is comparable. In consideration of these findings, peak 2 of the T2 hydrolysate is assigned to *erythro*-β-MeAsp. Obviously, the configuration considerably infuences the polarity of the PTC-β-MeAsp derivatives.

Amino acid analysis in association with the UV absorption spectra and TLC relate the T1 and T2 isolates very closely to cyanoginosin-LR. According to the chromatographic separation of the PTC derivatives, the molar ratio of the T1 and T2 amino acids was equal to 1. As D- and L-configurations cannot be separated, no further specification of the compounds is possible. The atypical amino Adda cannot be detected by amino acid analysis. However, considering that the dien conformation in Adda is regarded to be responsible for the characteristic UV spectra with an absorption maximum at 240 nm [10,16], it should be a component of T1 and T2.

It is concluded that the presented data give some evidence that *Microcystis aeruginosa* PCC 7806 produces cyanoginosin-LR and a demethylated homologue. The two peptides were separated on a preparative scale and a partial amino acid analysis was performed by RP-FPLC. Of the seven amino acids known for cyanoginosin-LR, six could be reliably detected.

The possibility of separating both cyanoginosin homologues may have some relevance for further toxicological studies as the demethylation is accompanied by a distinct loss of toxicity [11].

RP-FPLC is a valuable supplement to the current chromatographic techniques for the isolation and analysis of small amphiphilic peptides. In particular, the possibility of connecting analytical and preparative applications is of advantage. Even complex problems such as amino acid analysis can be resolved in a reasonable time using automation.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the very productive discussions with Dr. H. Meyer.

REFERENCES

1 G. A. Codd and W. W. Carmichael, *FEMS Microbiol. Lett.*, 13 (1982) 409–411.
2 I. R. Falconer, A. M. Beresford and M. T. C. Runnegar, *Med. J. Aust.*, 1 (1983) 511–514.
3 C. M. Meyer, *S. Afr. J. Sci.*, 83 (1987) 517–518.
4 O. M. Skulberg, G. A. Codd and W. W. Carmichael, *Ambio*, 13 (1984) 244–247.
5 D. P. Botes, A. A. Tuinman, P. L. Wessels, C. Viljoen, H. Kruger, D. Williams, S. Santikarn, R. J. Smith and S. J. Hammond, *J. Chem. Soc. Perkin Trans.*, 1 (1984) 2311–2318.
6 I. M. Birk, R. Dierstein, I. Kaiser, W. A. König, R. Krebber and J. Weckesser, *Arch Microbiol.*, 151 (1989) 411–415.
7 K. Harada, K. Ogawa, K. Matsuura, H. Murata and M. Suzuki, *Chem. Res. Toxicol.*, 3 (1990) 473–481.
8 R. Dierstein, I. Kaiser and J. Weckesser, *FEMS Microbiol. Lett.*, 49 (1988) 143–147.
9 W. P. Brooks and G. A. Codd, *Lett. Appl. Microbiol.*, 2 (1986) 1–3.
10 D. P. Botes, H. Kruger and C. Viljoen, *Toxicon*, 20 (1982) 945–954.
11 K. Sivonnen, W. W. Carmichael, M. Namikoshi, K. L. Rinehart, A. M. Dahlem and S. I. Niemelä, *Appl. Environ. Microbiol.*, 56 (1990) 2650–2670.
12 R. Rippka, J. Deruelles, J. B. Waterbury, M. Herdman and R. Y. Stanier, *J. Gen. Microbiol.*, 111 (1979) 1–61.
13 C. Martin, K. Sivonnen, U. Matern, R. Dierstein and J. Weckesser, *FEMS Microbiol. Lett.*, 68 (1990) 1–6.
14 O. Mikes, *High-Performance Liquid Chromatography of Biopolymers and Biooligomers, Part A*, Elsevier, Amsterdam, 1988, Ch. 2, p. A39.
15 D. P. Botes, C. C. Viljoen, H. Kruger, P. L. Wessels and D. H. Williams, *Toxicon*, 20 (1982) 1037–1042.
16 I. M. Birk, U. Matern, I. Kaiser, C. Martin and J. Weckesser, *J. Chromatogr.*, 449 (1988) 423–431.
17 M. Bochenska and J. F. Biernat, *Ann. Soc. Chim. Polonorum*, 46 (1972) 1429–1448.
18 K. Harada, M. Suzuki, A. M. Dahlem, V. R. Beasly, W. W. Carmichael and K. L. Rinehart, Jr., *Toxicon*, 26 (1988) 433–439.
19 P. Painuly, R. Perez, T. Fukai and Y. Shimizu, *Tetrahedron Lett.*, 29 (1988) 11–14.

Journal of Chromatography, 587 (1991) 81–84
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2404

The cytoskeletal protein villin as a parameter for early detection of tubular damage in the human kidney

L. B. Zimmerhackl* and B. Leuk

Department of Pediatrics, University of Freiburg, Mathildenstrasse 1, W-7800 Freiburg (Germany)

H. Hoschützky

Max-Planck Institute for Immunobiology, W-7800 Freiburg (Germany)

ABSTRACT

Villin is a cytoskeletal protein of brush borders in the kidney and gut. After renal tubular cell injury the brushborder fragments are shedded into the tubular lumen and excreted with urine indicating renal tubular damage (so called "renal antigen" shedding). In urine villin appears as intact molecule (95 000 dalton) and as fragment with 70 000, 45 000 and 22 000 dalton. The major villin fragment (70 000 dalton) was purified after ammonium sulphate precipitation from urine of human renal transplant recipients. Final purification of the villin 70 000 dalton fragment was achieved by gel filtration with TSK 3000 SWG preparative grade. Purification was varified by sodium dodecyl sulphate–polyacrylamide gel eletrophoresis and western blotting.

INTRODUCTION

Kidney damage often leads to renal failure with the consecutive hazard of kidney replacement therapy [1,2]. As kidney failure at early onset does not demonstrate early subjective recognizable warning signs, it is important to develop markers of renal tubular and glomerular function that are able to detect early on any dysfunction with a view to preventing renal damage. A new approach in this direction was the determination of "renal antigens" in urine. The idea was that any cell damage will be followed by a shedding of tubular fragments into urine, which can be detected by enzyme immunoassay [3,4].

Villin is a cytoskeletal protein of brush borders with a molecular weight of 95 000 dalton as the intact molecule [5]. It is linked to actin and stabilizes the actin filaments that anchor the individual microvilli [5–8]. This is its role under normal Ca^{2+} concentrations of $<10^{-7}$ *M*. However, in conditions of high Ca^{2+} concentrations of $>10^{-6}$ *M*, which are present during cellular damage such as hypoxia or direct toxic agents, villin acts as an F-actin severing protein [9]. This causes a release of brush-border bundles which are shed into the lumen of the tubule and excreted with urine [3]. The appearance of villin in the urine is therefore an indicator of renal tubular damage. In urine villin appears as the intact protein and also as fragments of 70 000, 45 000 and 22 000 dalton. The most abundant fragment is the 70 000-dalton fragment. Brush borders in the kidney are present in the proximal tubule only. Shedding of villin would therefore indicate damage to the proximal tubular cells.

In order to develop an assay to detect human villin in urine, we stratified our approach as follows: (1) development of a polyclonal antibody against villin, (2) development of a monoclonal antibody against villin and (3) purification of human villin 70 000-dalton fragment.

The production of antibodies has partially been reported elsewhere [3]. In this paper we focus on the purification of a 70 000-dalton villin fragment from urine of patients with routine techniques.

EXPERIMENTAL

Fast protein liquid chromatography (FPLC)

For gel filtration under low-pressure conditions (FPLC), an HPLC pump and controller (LKB–Pharmacia, Freiburg, Germany) was used. The detection of the effluent was performed with a monochromatic UV detector at 280 nm and recorded on strip-chart recorder or computer (Shimadzu CR3-A). The columns used were TSK 3000 SWG (Pharmacia–LKB) and Superdex 200 16/60 (Pharmacia–LKB). The buffer used was 20 m*M* Tris–HCl (pH with 8 *M* urea.

Flow conditions

The typical flow-rate was between 0.5 and 1.0 ml/min in all instances. The effluent was collected at 2–8-min intervals and further processed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE

SDS-PAGE was performed on 13% slab gels with Coomassie Brilliant Blue staining as routinely used in our laboratory or by automated SDS-PAGE (Phast system; Pharmacia–LKB) with automated silver staining (Pharmicia product information).

Samples

Urine from two children following renal cadaver transplantation was collected daily without the addition of any preservative and immediately stored in a refrigerator. Urine from one child with acute renal failure following haemolytic uraemic syndrome (HUS) was collected similarly. The procedure was approved by our local ethics committee.

RESULTS

To 500-ml aliquots of urine, ammonium sulphate was added to achieve a final concentration of 50%. After centrifugation the pellet was further processed. After dissolution in sterile distilled water the solution was freeze-dried and stored until processing.

Fig. 1 depicts SDS-PAGE with silver staining of the original urine. The gel filtration of the crude solution on a Superdex 200 60/16 preparative column is shown in Fig. 2. According to the SDS-PAGE and the gel filtration patterns, the greatest amount of protein is in the range 45 000–75 000 dalton. Although the higher-molecular-weight proteins are separated very effectively, the lower-molecular-weight proteins are not cut off. In addition, with this column a final separation of albumin with a

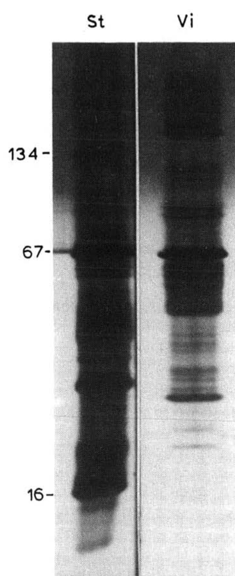


Fig. 1. SDS-PAGE with Phast system with automated silver staining. Discontinuous gel with 8–25% PA. St = standard (molecular weights in kilodalton); Vi = crude villin preparation.

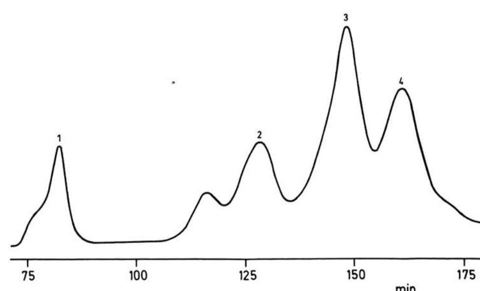


Fig. 2. Separation of crude villin preparation by gel filtration on Superdex 200 16/60 together with two marker proteins, aldolase (158 000 dalton) and ovalbumin (43 000 dalton) in the presence of 8 *M* urea. Flow-rate, 0.5 ml/min; absorbance, 0.64; attenuation, 8. Peaks: 1 = elution peaks; 2 = aldolase; 3 = villin (+albumin); 4 = ovalbumin.

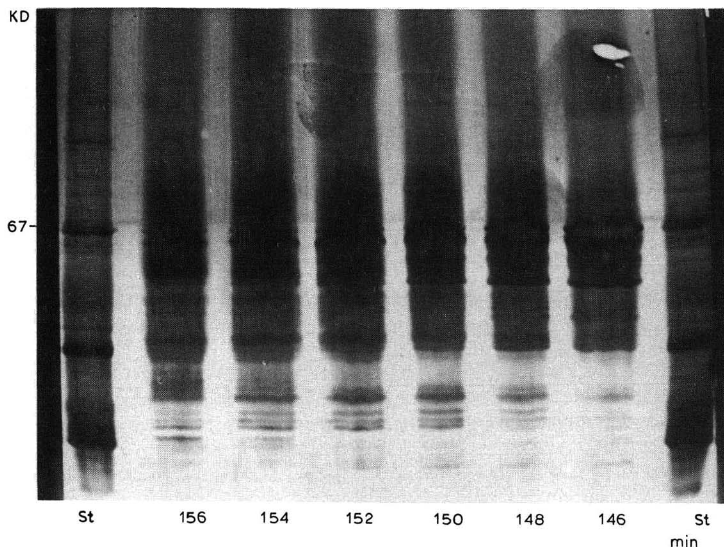


Fig. 3. SDS-PAGE with Phast system (see also Fig. 1) from effluent of the gel filtration separation shown in Fig. 2. The time corresponds to the time scale of the gel filtration tracing. St = standard proteins (molecular weights in kilodalton, kD).

molecular weight of 67 000 dalton and the villin fragment with a molecular weight of 70 000 dalton could not be achieved (Fig. 3).

A better resolution of the villin preparation was achieved with another gel filtration column, TSK 3000 SWG. As can be seen in Fig. 4., the main villin peak as demonstrated by Fig. 2 is separated into two clearly distinguishable peaks. The two peaks correspond to the different molecular weights of villin and albumin, as shown in the corresponding SDS-PAGE patterns in Fig. 5.

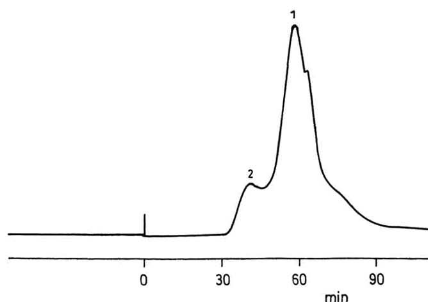


Fig. 4. Separation of a 70-kilodalton villin fragment by gel filtration on a TSK 3000 SWG collumn in the presence of 8 *M* urea. Flow-rate, 0.5 ml/min; absorbance, 0.1; injection volume, 500 μl. Peaks: 1 = albumin; 2 = villin.

DISCUSSION

The original proposal to purify villin derived from brush-border vesicles of chicken intestine was reported by Bretscher and Weber [10], but the technique is time consuming, difficult and yields protein of low purity. In addition, from fifteen chicken intestines a maximum of 5 mg of villin can be isolated. In the urine of patients with severe renal damage, *e.g.*, those who have undergone kidney transplantation, which in experimental terms is nothing but a recovery from acute renal failure with brush-border shedding, we observed villin concentrations up to 20 mg/l [3].

With the present approach it was possible to highly enrich villin substantially by gel filtration in the presence of 8 *M* urea. We used two different gel filtration media that were not equally suitable for the present problem. The advantage of Superdex 200 is the very clear separation between high- and low-molecular-weight proteins in the range 200 000–20 000 dalton. The separation of human

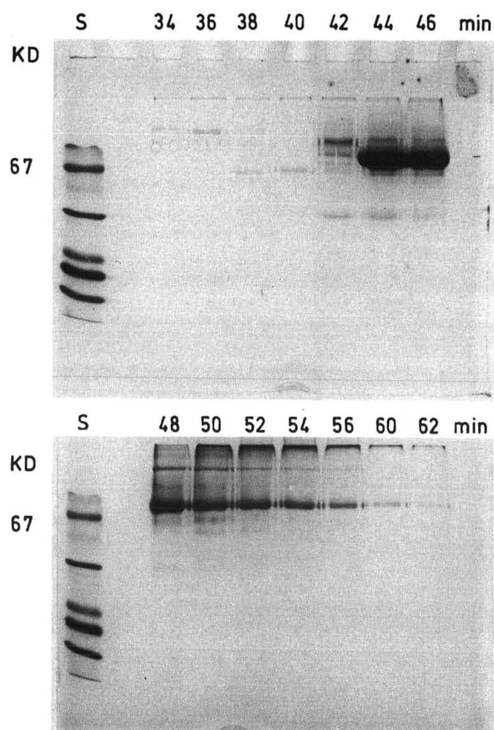


Fig. 5. SDS-PAGE with continuous slab gel with 13% PA of the gel filtration given in Fig. 4. The time corresponds to the time scale of the gel filtration tracing. S = standard proteins (molecular weights in kilodalton, KD).

serum albumin (67 000 dalton) and the villin fragment (70 000 dalton) was not possible on Superdex 200. In contrast, the TSK 3000 SWG column resolved these two proteins. This approach is simple, straightforward and inexpensive.

Owing to the detection of the gene locus and the exact sequence of villin, further developments such as tumour markers are coming into the field [11,12].

In conclusion, renal antigens, that is, proteins which are released by the kidney under conditions of damage, are new parameters of tubular cell injury. With classical biochemical methods and the development of improved separation media, the purification of proteins can be resolved by simple and fast techniques.

ACKNOWLEDGEMENT

This study was supported by the German Research Foundation (Ho 1164/2-1 and Zi 314/1-2).

REFERENCES

1 M. Steinhausen, N. Parekh and B. Zimmerhackl, in D. Seybold and U. Gessler (Editors), *Acute Renal Failure*, Karger, Basle, 1982, pp. 9–22.
2 L. B. Zimmerhackl, C. R. Robertson and R. L. Jamison, *Circ. Res.*, 57 (1985) 657–667.
3 L. B. Zimmerhackl, G. Weber, D. Höfer, T. Fabricius, D. M. Scott, M. Brandis, D. Drenckhahn and R. Kinne, in P. H. Bach, N. J. Gregg, M. F. Wilks and L. Delacruz (Editors), *Nephrotoxicity, Proceedings of the IVth International Symposium on Nephrotoxicity*, Marcel Dekker, New York, 1991, pp. 549–554.
4 P. Tijsen, *Practice and Theory of Enzyme Immunoassays, (Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 15), Elsevier, Amsterdam, 1985.
5 D. Drenckhahn and R. Dermietzel, *J. Cell. Biol.*, 107 (1988) 1037–1048.
6 H. A. Alicea and M. S. Mooseker, *Cell. Motil. Cytoskeleton*, 9 (1988) 60–72.
7 E. Coudrier, D. Kerjaschki and D. Louvard, *Kidney Int.*, 34 (1988) 309–320.
8 M. Arpin, E. Pringault, J. Findori, A. Garcia, J. M. Jeltsch, J. Vanderkerckhove and D. Louvard, *J. Cell. Biol.*, 107 (1988) 1759–1766.
9 A. Bretscher and K. Weber, *Cell*, 20 (1980) 839–847.
10 A. Bretscher and K. Weber, *Exp. Cell. Res.*, 116 (1978) 379–407.
11 H. J. Gröne, K. Weber, U. Helmchen and M. Osborn, *Am. J. Pathol.*, 124 (1986) 294–302.
12 M. F. Rousseau-Merck, D. Simon-Chazottes, M. Arpin, E. Pringault, D. Louvard, J. L. Guenet and R. Berger, *Hum. Genet.*, 78 (1988) 130–133.

Journal of Chromatography, 587 (1991) 85–92
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2369

Purification and crystallization of yeast hexokinase isoenzymes

Characterization of different forms by chromatofocusing

L. Jacob*, V. Beecken and L. J. Bartunik

Max-Planck-Society, Research Unit for Structural Molecular Biology, c/o DESY, Notkestrasse 85, W-2000 Hamburg 52 (Germany)

M. Rose

Institut für Mikrobiologie, Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, Haus 75A, W-6000 Frankfurt/M (Germany)

H. D. Bartunik

Max-Planck-Society, Research Unit for Structural Molecular Biology, c/o DESY, Notkestrasse 85, W-2000 Hamburg 52 (Germany)

ABSTRACT

The yeast hexokinase isoenzymes PI and PII have been purified in large amounts (20 mg) from overproducing yeast strains. The purification procedures of hexokinase PI and PII include anion-exchange chromatography on DEAE-Sephacel and chromatofocusing on PBE 94, hydrophobic interaction chromatography on phenyl-Sepharose (necessary for the isolation of the isoenzyme PI); in the final step either a Mono Q HR 5/5 or a Fractogel EMD TMAE 650(S) column was used. Hexokinase preparations were characterized before cyrstallization by chromatofocusing on a Mono P HR 5/20 FPLC column, where different forms of hexokinase can be rapidly distinguished by their elution behaviour. From both purified hexokinase PI and PII, large crystals were grown that diffract X-rays to high resolution.

INTRODUCTION

A number of investigations of structure–function relationships of the hexose phosphorylating yeast enzyme hexokinase were based on X-ray diffraction analyses of both the PI and PII isoenzymes. The structure of native hexokinase PII in monomeric form was determined at 2.1 Å resolution [1] and that of the PI isoenzyme with bound glucose at a medium resolution of 3.5 Å [2]. Unfortunately, it was then not possible to crystallize the same isoenzyme with and without bound sugar. Therefore, direct evidence of a conformational change induced by the binding of the substrate could only be derived from results of small-angle X-ray scattering from hexokinase in solution [3]. Further, the correct sequences of the isoenzymes were not known until later [4,5]. A detailed knowledge of the sugar and nucleotide binding sites is of interest for a better understanding of the role of conformational changes in the functioning of hexokinase and of the different specificities of the isoenzymes towards fructose and glucose. It is further of interest for a comparison with other proteins with similar conformations to hexokinase such as actin [6] and the N-terminal ATPase fragment of the 70 000 dalton bovine heat shock cognate protein [7]. In order to provide a basis for detailed crystal structure analyses of the substrate binding sites, we developed an improved scheme for the purification of both yeast hexoki-

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V. All rights reserved

nase isoenzymes on a semi-preparative scale and grew crystals in the native and complexed form which were suitable for X-ray diffraction measurements at high resolution.

Numerous proteolytic degradation products of hexokinase were described in the past, when protein purification methods were less sophisticated [8,9]. Most of the large-scale purification procedures published in the 1960s and 1970s were modifications of the method of Darrow and Colowick [10]. Depending on the elution conditions, different hexose phosphorylating fractions were eluted from DEAE-cellulose columns [8,11–14]. Among these, two predominant proteolysed forms, SI and SII, which are both still active, occur during purification [9]. It has been shown recently by cloning the *Saccharomyces cerevisiae* hexokinase genes that there are two isoenzymes, PI and PII, with overall homologies in their amino acid sequence of only about 76% [4,5]. New attempts to better separate the isoenzymes and eliminate the proteolytic forms have been made successfully by Womack *et al.* [15] using hydroxyapatite column chromatography as the final step and by Kopetzki and Entian [16] using affinity chromatography followed by chromatofocusing. The latter procedure was developed for the small-scale purification of hexokinase.

The preparation described in this paper involves the construction of genetically manipulated yeast strains which facilitate the protein purification of milligram amounts of hexokinase. This work is part of a project aimed at providing information about the kinetics of the hexokinase molecule at a structural level.

EXPERIMENTAL

All chemicals were of pro analysi or puriss grade. Enzymes, substrates and cosubstrates were obtained from Boehringer Mannheim and hexokinase type C301 from Sigma.

Yeast strains and overexpression

A DNA fragment harbouring the complete HXK1 gene of *Saccharomyces cerevisiae* [4] was ligated into the yeast episomal plasmid YEp24 [17]. In a similar approach, the complete HXK2 gene [5] was cloned into the plasmid YEp24. The *in vitro* recombination of DNA was performed by standard techniques [18]. The resulting plasmids pMR22 and pMR47 contained the HXK1 and the HXK2 gene, respectively. Both hexokinase genes were under the control of their native promotors. Transformation marker genes and sequences necessary for replication in *Escherichia coli* and yeast were derived from the plasmid YEp24. The origin of plasmid replication in yeast was derived from the 2-μm circle DNA via YEp24. The use of this origin results in a high plasmid copy number [19]. Plasmids pMR22 or pMR47 were transformed in *Saccharomyces cerevisiae* strain WAY.10-1C according to the method of Ito *et al.* [20]. The chromosomal loci of the HXK1 and HXK2 genes were deleted in this strain [21]. After transformation, hexokinase was only obtained by expression of the plasmid encoded HXK1 and HXK2 genes. The transformed yeasts were grown under selective conditions on synthetic media in order to prevent plasmid loss in cultures up to a volume of 100 ml. The preculture so obtained was transferred to a maximum of 10 l of rich medium (1:20) and incubation was stopped when the wet weight reached 15–20 g/l. After centrifugation, the pellet was resuspended in water. The cells were pelleted again by centrifugation and stored at -20°C.

Enzyme assays

Hexokinase activity was determined spectrophotometrically as described by Bergmeyer [22] by coupling the formation of glucose-6-phosphate from glucose to the reduction of NAD with glucose-6-phosphate dehydrogenase. Unless indicated otherwise, standard assays were performed at 20°C in a total volume of 1 ml containing 50 m*M* triethanolamine–HCl (pH 7.5), 11 m*M* glucose, 1.5 m*M* NAD, 8.9 m*M* magnesium chloride, 0.66 m*M* ATP and 1 U glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Boehringer Mannheim). Isoenzymes were distinguished by their *F/G* ratio, *i.e.*, their relative fructose to glucose phosphorylating activities [11]. With fructose as substrate (11 m*M*), 1 unit/ml of phosphoglucose isomerase was added.

Protein determination

The protein peaks of all chromatographic separation steps were recorded at 280 nm (Uvicord SD 2158; LKB). Protein concentrations in hexokinase-

containing fractions were determined with Coomassie protein assay reagent (Pierce). Additionally, pooled fractions and purified samples of hexokinase were assayed with biuret reagent (Sigma).

Preparation of yeast cell extracts

Up to 35 g of freshly thawed cells of the PI-producing strain were suspended in one volume of the above buffer; 2 g of glass beads (0.5 mm) were added to 1 ml of yeast suspension. Purification of the isoenzyme PII started from 10 g of overproducing yeast cells (yielding 15 mg of pure enzyme). An IMA (Zeppelinheim, Germany) disintegrator operating at 4000 rpm for 20 min was used for disruption of yeast cells. Immediately after centrifugation at 20 000 *g* for 30 min at 4°C the supernatant was removed and the cell pellet was resuspended in about 10 ml of piperazine–HCl buffer (0.025 *M*, pH 6.5). After centrifugation, (20 000 *g* for 30 min at 4°C) both supernatants were combined.

Chromatographic procedures

All chromatographic steps were performed in rapid succession at 4°C in order to avoid loss of hexokinase activity. The columns used were connected to a Model 2249 high-performance liquid chromatographic gradient pump (Pharmacia LKB). The cell-free crude extract was rapidly loaded onto a DEAE-Sephacel (Pharmacia LKB) column (5 × 5 cm I.D.) that had been equilibrated with 0.025 *M* piperazine–HCl buffer (pH 6.5). After washing with the equilibration buffer, elution was performed using 0.025 *M* piperazine–HCl buffer (pH 3.5) at a flow-rate of 2 ml/min. The pooled fractions containing hexokinase activity were adjusted to pH 5.5 by the slow addition of 0.025 *M* piperazine (pH 10.5). Large-scale chromatofocusing was performed using Polybuffer Exchanger 94 (Pharmacia LKB) filled into a chromatographic column (46 × 1 cm I.D.). After the equilibration with piperazine buffer (0.025 *M*, pH 5.5), samples which consisted of the pool of active fractions from the DEAE-Sephacel column were loaded at flow-rates of 0.35 ml/min. The columns were washed with equilibration buffer and eluted with decreasing pH gradient developed with 250 ml of Polybuffer 74 (pH 4.0) (diluted 1:10 from stock solution).

The pooled fractions containing hexokinase isoenzyme PI were brought to 40% saturation with solid ammonium sulphate with gentle stirring; no precipitation was visible. The protein solution was applied to a column (20 × 2.6 cm I.D.) filled with 85 ml of phenyl-Sepharose CL-4B (Pharmacia LKB) that had been equilibrated with 40% saturated ammonium sulphate–piperazine buffer (0.025 *M*; pH 6.5) at a flow-rate of 0.5 ml/min. The column was washed with 150 ml of the same buffer and hexokinase was eluted with a 500-ml linear gradient of decreasing ammonium sulphate saturation (40–0%) in combination with an increasing linear gradient of ethylene glycol concentration (0–80%), both in 0.025 *M* piperazine buffer (pH 6.5).

Fractions containing hexokinase PI were desalted using PD 10 columns and then loaded with a 50-ml superloop (1 ml/min) on a Mono Q HR 5/5 FPLC column (Pharmacia LKB) or a Fractogel EMD TMAE 650(S) column (Merck) which had been equilibrated with degassed 0.025 *M* piperazine–HCl buffer (pH 6.5). With the Mono Q column aliquots of 17 mg of protein were applied. The TMAE 650(S) column had a capacity of about 80 mg and the pooled fractions could be loaded in one step. After washing with equilibration buffer, the column was eluted with a linear gradient of 0–1 *M* sodium chloride [in 0.025 *M* piperazine–HCl buffer (pH 6.5)] in 60 min. The protein solution was concentrated by ultrafiltration using Centricon 30 micro-concentrators (Amicon) to final concentrations of about 20–30 mg (5000–7000 rpm, 4°C, 1–2 h).

Purified hexokinase (0.1 mg per run) was applied to a Mono P HR 5/20 FPLC column (Pharmacia LKB) that had been equilibrated with 0.025 *M* piperazine–HCl buffer (pH 5.5). The column was developed with Polybuffer 74 (pH 4.0) (1:10 dilution) and fractions of 1 ml were collected. Gel-permeation experiments were done on a Superose 6 HR 10/30 FPLC column (Pharmacia LKB) equilibrated with 0.05 *M* sodium phosphate buffer (pH 7.2) containing 150 m*M* potassium chloride. For each run, 100 μl of purified isoenzymes PI and PII were loaded onto the column. The flow-rate was 0.5 ml/min. The molecular mass of hexokinase PI and PII was re-examined using protein standards (low- and high-molecular-weight gel filtration kit; Pharmacia LKB) for calibration. The void volume was determined with blue dextran.

Polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl sulphate (SDS)-PAGE was performed with the GE 2/4-LS electrophoresis apparatus (Pharmacia LKB) using the system of Laemmli [23]. PAGE under non-denaturing conditions was carried out according to the method of Davis [24] using 7.5% polyacrylamide gels (stacking gel 4.5%).

RESULTS

Purification of hexokinase isoenzyme PI and PII

The purification of *Saccharomyces cerevisiae* hexokinase isoenzymes PI and PII was achieved from overproducing yeast cells with yields of about 23% for the isoenzyme PI and about 42% for hexokinase PII. In order to circumvent co-purification of both isoenzymes, we used a mutant of *Saccharomyces cerevisiae* lacking the HXK2 gene for the purification of isoenzyme PI; the corresponding procedure was employed for PII. We found specific acitivities of about 8.3 U/mg protein in the crude extract of the recombinant PI-producing cells and about 48 U/mg protein for the PII-producing yeast strain.

The first step in the purification of both isoenzymes was anion-exchange chromatography on DEAE-Sephacel using high flow-rates. This chromatographic step resulted in a very good separation of each hexokinase isoenzyme from the bulk protein; a peak of hexokinase PI with a ratio of fructose to glucose phosphorylating acitivity of 2.8 appeared between pH 5.5 and 4.8. Hexokinase PII could be eluted in the same range. The recovery of hexokinase activity from DEAE-Sephacel was about 65% for PI and 70% for PII (Table I). The specific acitivities increased from 8.3 U/mg in the crude extract to 36.7 U/mg in the pooled hexokinase PI fractions. With isoenzyme PII the pooled fractions contained 140 units/mg protein (Table I).

For efficient binding to the PBE 94 polybuffer exchanger column, it was necessary to adjust the pH of the pooled fractions to 5.5. This also prevented a loss of acitvity. All chromatofocusing columns developed with Polybuffer 74 resulted in elution profiles comparable to that shown in Fig. 1. A peak of hexokinase PI was eluted at about pH 4.8 (Fig. 1A). Hexokinase isoenzyme PII was also obtained as a homogenous peak at about pH 4.65 (Fig. 1B). For each isoenzyme, the activities found in the pooled fractions corresponded to 70–80% of the activities applied to the column. Owing to the concentrating effect of chromatofocusing, the volumes of the pooled fractions were about 50 ml, independent of the sample volume (up to 200 ml) that was applied. With hexokinase PII, the peak fractions were nearly free from contaminating proteins. For further purification, therefore, it was sufficient to remove the remaining proteins with a TMAE 650(S) or a Mono Q column. In contrast, the purification of isoenzyme PI required an additional hydrophobic interaction chromatographic step following the chromatofocusing. Hexokinase could be detected at about 25% ammonium sulphate saturation (Fig. 2).

TABLE I

PURIFICATION OF YEAST HEXOKINASE ISOENZYMES

Data are from a representative purification starting with 26 g of hexokinase isoenzyme PI overproducing yeast cells and 7 g PII producing cells. The yield was calculated as a percentage of the amount of hexokinase present in the crude extract.

Purification step	Hexokinase PI				Hexokinase PII			
	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	1140.0	9509	8.3	100	192.3	9176	47.8	100
DEAE-Sephacel	170.0	6208	36.7	65	46.4	6438	140.0	70
Chromatofocusing	76.3	4964	65.0	52	18.8	4465	226.0	49
Phenyl-Sepharose	36.0	3423	94.8	36	–	–	–	–
Mono Q	23.0	2135	95.0	23	12.4	3853	310.2	42

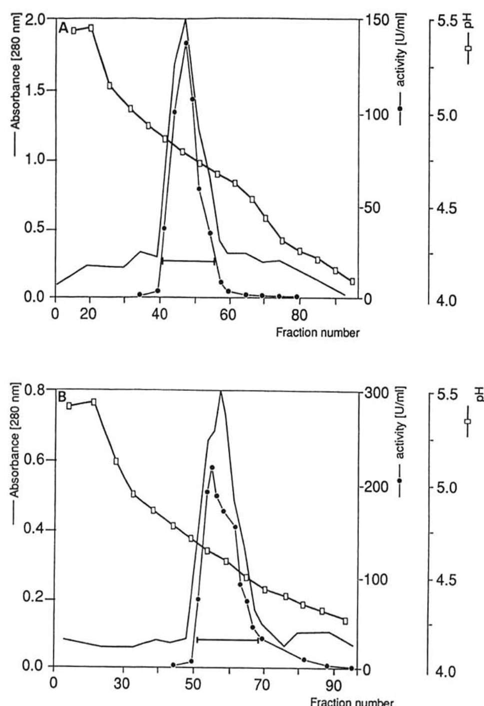


Fig. 1. Chromatofocusing elution profile of the pooled DEAE fractions containing (A) hexokinase PI and (B) isoenzyme PII on a PBE 94 column. Each column was developed using Polybuffer 74 (1:10) (pH 4.0). The pH gradient in the effluent is indicated. and the bars represent the pooled fractions.

About 36% of hexokinase PI could be recovered after the hydrophobic interaction chromatography, and the specific activity increased to 95 U/mg. The last separation to homogeneity was done either on a Fractogel EMD TMAE 650(S) column or on a Mono Q HR 5/5 FPLC column. As a high ionic strength had to be avoided, the remaining ammonium sulphate was removed by rapid desalting on PD 10 columns instead of dialysis. Both anion-exchange chromatographic steps were highly reproducible.

The hexokinase isoenzyme PI was eluted in five fractions (each 1 ml) in the range between 0.28 and 0.35 M potassium chloride depending on the total amount of protein that was applied to the column (Fig. 3A). Isoenzyme PII could be recovered under identical conditions (Fig. 3B). As evidenced by protein staining, fractions 14 and 15 contained a single

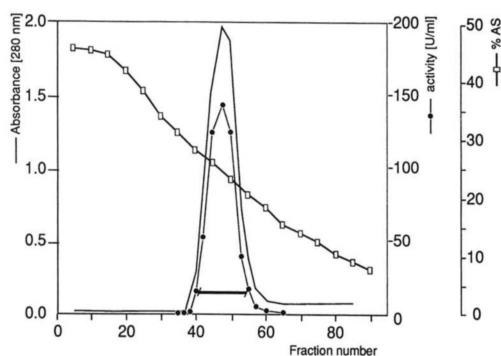


Fig. 2. Hydrophobic interaction chromatography of the pooled fractions from Fig. 1A on phenyl-Sepharose CL-4B. Elution was performed using a linear gradient of decreasing ammonium sulphate saturation (40–0%) in combination with an increasing linear gradient of ethylene glycol concentration (0–80%), both in 0.025 M piperazine buffer (pH 6.5). Hexokinase PI eluted at about 25% saturation ammonium sulphate. Fractions containing more than 20 U/ml (marked with a bar) were pooled.

protein in the molecular mass range of hexokinase. In fractions 17 and 18, a smaller protein (40 000 dalton) was visible. Each of the purified isoenzymes could be stored at −20°C in 0.025 M piperazine–HCl (pH 6.5) containing 50% glycerol for at least several months with only a minor loss of activity.

Prior to crystallization trials, glycerol was removed by PD 10 columns, and the samples were concentrated to 20–30 mg/ml of protein using Centricon 30 microconcentrators. From this material, it has been possible to obtain for the first time large crystals of hexokinase PI in the presence and absence of glucose, and also crystals of hexokinase PII; the characterization of the crystals by means of X-ray diffraction will be described elsewhere [25].

Criteria of purity

The purities of the different preparations described above and of commercial hexokinase samples which were also used for protein crystallization were determined by a number of electrophoretic and chromatographic methods including SDS-PAGE, non-denaturing PAGE, rechromatography on an anion exchanger, gel permeation and chromatofocusing. Both hexokinase isoenzymes purified from overproducing strains were apparently homogeneous when enzyme preparations were analysed by SDS-PAGE. Both isoenzymes yielded only one

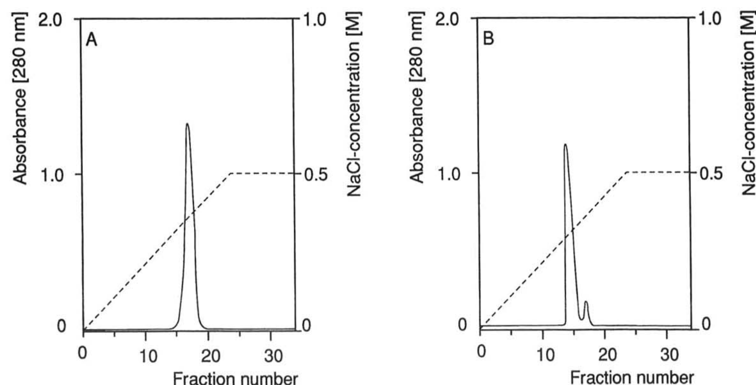


Fig. 3. Anion-exchange chromatography on a Mono Q HR5/5 column of hexokinase isoenzyme (A) PI and (B) PII. With isoenzyme PII a minor peak was separated by a salt gradient. The major peak contains hexokinase PII activity (ratios of fructose to glucose phosphorylating activity 1.4).

protein staining band (10 μg per lane) corresponding to a molecular mass of about 54 000 dalton. On electrophoresis without SDS, isoenzyme PI shows a single band that migrates with nearly the same mobility as the isoenzyme PII (Fig. 4).

In the hexokinase preparation obtained from Sigma, one protein band was visible on the SDS gel, whereas under non-denaturing conditions two major bands could be detected on the gel (Fig. 4). These two proteins could not be separated by anion-exchange chromatography.

During rechromatography of both isoenzymes purified from yeast cells on a Mono Q or TMAE Fractogel column, only one peak was observed which coincided exactly with that of hexokinase activity; this is shown in Fig. 5 for the example of isoenzyme PII. Gel permeation of purified isoenzymes PI and PII on a Superose 6 FPLC column also produced one peak. For each isoenzyme, the hexokinase activity emerged at positions corresponding to a globular protein of molecular weight about 55 000–60 000 dalton.

In order to characterize the protein samples before crystallization, we carried out chromatofocusing using a Mono P HR5/20 column. In our preparation, a distinct species of isoenzyme PI eluted at pH 4.87 (Fig. 6A). With purified hexokinase PII, a peak occurred at pH 4.74 (Fig. 6B). Two species of hexokinase PII could be distinguished (Fig. 6C) for the commercially obtained hexokinase (Type C301) which we also crystallized. The first minor peak occurs at pH 4.53 and corresponds to the slowly migrating band in the native PAGE shown in Fig. 4. A second peak eluted at pH 4.4; it corresponds to the fast band in the non-denaturing polyacrylamide gel. Both proteins separated on the Mono P column had

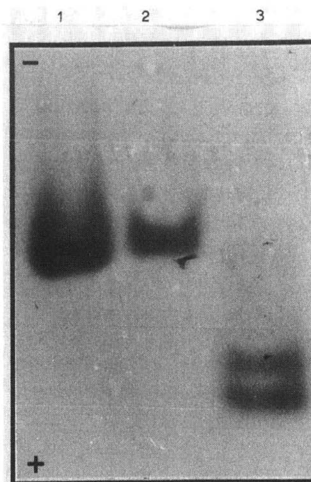


Fig. 4. PAGE at pH 6.8 of hexokinase under non-denaturing conditions. Single band of isoenzyme PI (lane 1), isoenzyme PII (lane 2) and the two bands of commercially obtained hexokinase (lane 3; Sigma Type 301) are shown. The direction of migration is from top to the bottom of the gel.

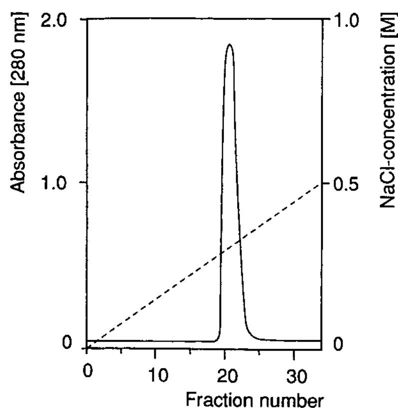


Fig. 5. Anion-exchange chromatography of hexokinase PII on a Fractogel EMD TMAE 650(S) column. Purified isoenzyme PII was applied to the column and eluted as a homogeneous peak. Elution was performed using a linear gradient of increasing sodium chloride concentration.

ratios of fructose to glucose phosphorylating activity of about 1.2, indicating the presence of isoenzyme PII.

DISCUSSION

As shown here, the molecular cloning of each of the *Saccharomyces cerevisiae* hexokinase isoenzymes provides attractive conditions for the purification and subsequent crystallization of the enzyme. From this approach, we obtained crude extracts containing large amounts of either isoenzyme PI or PII at high specific acitivities. Growing sufficiently large crystals for structure determination requires milligram amounts of protein. The procedure described here for the preparation of hexokinase indeed yields large amounts of highly purified isoenzyme PI or PII. As neither the use of protease inhibitors [11] nor rapid cell rupture [13] seems to protect against proteolytic alterations of hexokinase, a combination of rapid cell disruption immediately followed by DEAE-Sephacel chromatography was preferred. During the separation of hexokinase from the bulk protein, most of the proteases were also removed. Chromatofocusing proved to be a convenient method for purifying hexokinase, even if only one isoenzyme is present. The final specific activities were 95 U/mg for isoenzyme PI and 310 U/mg for hexokinase PII. These relatively low values are explained by the assay temperature of 20°C. When the activity of purified isoenzymes was determined at 30°C, specific acitivities of 152 U/mg for hexokinase PI and of 580 U/mg for isoenzyme PII were obtained; these values are comparable to those described by Kopetzki and Entian [16]. The purified isoenzymes were shown to be homogeneous by PAGE both under denaturing and non-denaturing conditions. In contrast to the data reported by Colowick [9], the differences in the mobilities in gel electrophoresis at pH 6.8 are less significant. Both purified yeast hexokinase isoenzymes had a molecular mass of about 54 000 dalton in SDS-PAGE and migrated as a single protein-staining band with the same mobility. For both hexokinase isoenzymes, gel filtration on Superose 6 also showed the same single peak corresponding to the molecular mass of the monomeric form. Similar results for hexokinase PII were described by Furman and Neet [26].

The existence of only one hexokinase isoenzyme in the strains constructed here ensured a rapid isolation, as a distinction between PI and PII during the

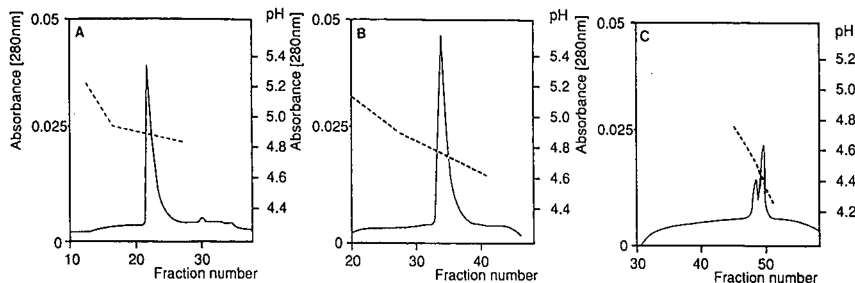


Fig. 6. Chromatofocusing of the purified isoenzyme PI and PII on a Mono P HR5/20 column. Hexokinase PI eluted as a homogenous peak at pH 4.87 (A), whereas a peak containing isoenzyme PII was obtained at pH 4.74 (B). With commercially obtained hexokinase (Sigma, Type C301) two peaks eluting at about pH 4.53 and pH 4.4 could be distinguished (C).

purification was not necessary. The glucose phosphorylating enzyme glucokinase was not detected during the different purification steps. The overall recovery of the hexokinase isoenzymes PI and PII was considerably increased by the cloning and overexpression of the inserted genes. Comparing the final amount of isoenzyme PI and PII obtained here with the results of previous purifications [16], a 10-fold increase for PI and a 40-fold increase for PII was achieved. Hence only small amounts of yeast cells are needed as starting material for the purification. Owing to the small sample volumes, all chromatographic steps could be performed rapidly. The reproducibility and high resolution of the Mono P column make this column particularly suitable for molecular characterization before protein crystallization. As demonstrated in Fig. 5, both purified isoenzymes PI and PII eluted as homogeneous peaks. The pH values at which each isoenzyme eluted differ only minimally, and they are in the same range as those of the chromatofocusing on PBE 94. It should be noted that the precise pH value of elution varied slightly with the total amount of protein that was applied to the columns. In the presence of 50% glycerol, no loss of activity was observed after storage at −20°C for several months; the protein could still be used for crystallization assays.

As the succes of protein crystallization is strongly dependent on the homogeneity and the total amount of the starting material, the combination of genetic engineering techniques with methods of protein crystallization is very useful. Both isoenzymes produced from the cloned genes were suitable for protein crystal growth, and the crystals which were grown diffracted to high resolution [25]. We observed different crystallization behaviours for different commercial preparations which showed homogeneity in SDS-PAGE but exhibited different electrophoretic protein patterns on native gels. Chromatofocusing is a high-resolution method for the rapid characterization of proteins, even in the case of very similar proteins.

ACKNOWLEDGEMENTS

We thank Professor K. D. Entian for many stimulating discussions. This work was funded (to H. D. Bartunik) by the German Federal Minister for Research and Technology (BMFT) under contract number 05 180MP B O. Part of this work was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

REFERENCES

1 C. M. Anderson, R. E. Stenkamp and T. A. Steitz, *J. Mol. Biol.*, 123 (1978) 15.
2 W. S. Bennett and T. A. Steitz, *J. Mol. Biol.*, 140 (1980) 211.
3 R. C. McDonald, D. M. Engelman and T. A. Steitz, *J. Biol. Chem.*, 254 (1979) 2942.
4 E. Kopetzki, K. D. Entian and D. Mecke, *Gene*, 39 (1985) 95.
5 K. U. Froehlich, K. D. Entian and D. Mecke, *Gene*, 36 (1985) 105.
6 W. Kabsch, H. G. Mannherz, D. Suck, E. F. Pai and K. C. Holmes, *Nature (London)*, 347 (1990) 37.
7 K. M. Flaherty, C. DeLuca-Flaherty and D. B. McKay, *Nature (London)*, 346 (1990) 623.
8 A. Kaji, K. A. Trayser and S. P. Colowick, *Ann. N.Y. Acad. Sci.*, 94 (1962) 798.
9 S. P. Colowick, in P.D. Boyer (Editor), *The Enzymes*, Vol. 9, Academic press, New York, London, 3rd ed., 1973, p. 1.
10 R. A. Darrow and S. P. Colowick, *Methods Enzymol.*, 5 (1962) 226.
11 N. R. Lazarus, A. H. Ramel, Y. M. Rustum and E. A. Barnard, *Biochemistry*, 5 (1966) 4003.
12 I. T. Schulze and S. P. Colowick, *J. Biol. Chem.*, 244 (1969) 2306.
13 A. H. Ramel, Y. M. Rustim, J. G. Jones and E. A. Barnard, *Biochemistry*, 10 (1971) 3499.
14 J. S. Easterby and M. A. Rosemeyer, *Eur. J. Biochem.*, 28 (1972) 241.
15 F. C. Womack, M. K. Welch, J. Nielsen and S. P. Colowick, *Arch. Biochem. Biophys.*, 158 (1973) 451.
16 E. Kopetzki and K. D. Entian, *Anal. Biochem.*, 121 (1982) 181.
17 D. Botstein, S. L. Falco, S. E. Stewart, M. Brennan, S. Scherer, D. T. Stinchcomb. K. Struhl and R. W. Davis, *Gene*, 8 (1979) 17.
18 T. Maniatis, E. F. Fritsch and J. Sambrook, *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
19 J. R. Broach, *Methods Enzymol.* 101 (1983) 307.
20 H. Ito, Y. Fukuda, K. Murata and A. Kimura, *J. Bacteriol.*, 153 (1983) 163.
21 M. Rose, W. Albig and K. D. Entian, *Eur. J. Biochem.*, 199 (1991) 511.
22 H. U. Bergmeyer, *Methoden der Enzymatischen Analyse*, Verlag Chemie, Weinheim, 1970, p. 432.
23 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
24 B. J. Davis, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
25 L. J. Bartunik, V. Beecken, L. Jacob, M. Rose, K. D. Entian and H. D. Bartunik, in preparation.
26 T. C. Furman and K. E. Neet, *J. Biol. Chem.*, 258 (1983) 4930.

Journal of Chromatography, 587 (1991) 93–99
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2400

Purification of the coenzyme B_{12}-containing 2-methyleneglutarate mutase from *Clostridium barkeri* by high-performance liquid chromatography

Christoph Michel and Wolfgang Buckel*

Laboratorium für Mikrobiologie, Fachbereich Biologie, Philipps Universität, Karl-von-Frisch-Strasse, W-3550 Marburg (Germany)

Dietmar Linder

Fachbereich Humanmedizin, Biochemisches Institut, Justus-Liebig-Universität Giessen, W-6300 Giessen (Germany)

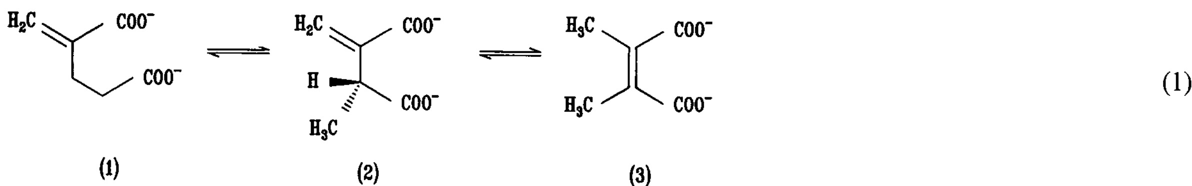
ABSTRACT

Two methods are described by which the enzymes 2-methyleneglutarate mutase and 3-methylitaconate Δ-isomerase from *Clostridium barkeri* have been separated by high-performance liquid chromatography on a much larger scale than reported previously. First, the mutase eluted before the Δ-isomerase after incubation with the mild detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate (CHAPS) followed by high-performance anion-exchange chromatography on Mono Q in the presence of the same detergent. Second, an even better separation, although with a lower yield of mutase, was obtained by hydrophobic interaction chromatography on phenyl-Sepharose HiLoad, whereby the enzymes were eluted in the reverse order. Final high-performance anion-exchange chromatography of the latter preparation on Mono Q at pH 8 gave highly purified 2-methyleneglutarate mutase (> 95% purity) which had a pink-orange colour (λ_{max} 280, 375, 470 and 532 nm). The enzyme was active in the absence of coenzyme B_{12} (adenosylcobalamin) and contained 2.1 mol of this coenzyme per homotetramer (molecular mass, m = 300 kilodalton).

INTRODUCTION

2-Methyleneglutarate mutase (E.C. 5.4.99.4) and 3-methylitaconate Δ-isomerase (E.C. 5.3.3.6) are involved in two consecutive steps in the fermentation of nicotinate to ammonia, propionate, acetate and carbon dioxide by *Clostridium barkeri* [1,2]; for a review of the pathway, see ref. 3. The former enzyme catalyses the reversible, coenzyme B_{12}-dependent rearrangement of 2-methyleneglutarate (**1**, eqn. 1) to (*R*)-3-methylitaconate (**2**) with inversion of configuration at the methylene carbon (C-4 of **1**), which becomes the methine carbon of **2** [4]. The second enzyme, 3-methylitaconate Δ-isomerase, catalyses the reversible shift of the *exo* double bond of (*R*)-3-methylitaconate to 2,3-dimethylmaleate (**3**).

In order to study the mechanism of the unusual rearrangement of 2-methyleneglutarate to (*R*)-3-methylitaconate, a pure mutase free of Δ-isomerase is required. In addition, the Δ-isomerase serves as an auxiliary enzyme for the spectrophotometric assay [5]. However, purification of both cytosolic enzymes was impeded by the fact that they could not



(1)

be separated by conventional techniques or by fast protein liquid chromatography (FPLC) on the anion exchanger Mono Q and on the molecular sieve Superose 6. Although the enzymes were recently resolved by non-denaturing polyacrylamide gel electrophoresis (PAGE), the scale was very small [5]. This paper describes two methods by which 2-methyleneglutarate mutase free of Δ-isomerase is obtained in larger amounts using FPLC. A short account of one method was published elsewhere [6].

EXPERIMENTAL

Materials

All chemicals were of analytical-reagent grade or higher quality and were purchased from Merck (Darmstadt, Germany), Sigma (Deisenhofen, Germany) and Biomol (Hamburg, Germany) if not stated otherwise. The syntheses of 2-methyleneglutarate and (*R*,*S*)-3-methylitaconate and the growth of *C. barkeri* were described previously [4,5].

Spectrophotometric enzyme assays

The activities of 2-methyleneglutarate mutase and 3-methylitaconate Δ-isomerase were determined in a continuous spectrophotometric assay at 256 nm under aerobic conditions as described recently [5] and modified according to ref. 6. The assay is based on the higher absorption of dimethyl maleate (**3**, eqn. 1) compared with 2-methyleneglutarate (**1**) and 3-methylitaconate (**2**), $\Delta\varepsilon$ = 0.66 l $mmol^{-1}$ cm^{-1}.

Protein determination

Protein concentrations were determined by the bicinchonic acid method [7] with bovine serum albumin as a standard. The reagent kit was purchased from Pierce (Rockford, IL, USA).

Gel electrophoresis

PAGE in the presence of sodium dodecyl sulphate (SDS) was performed in 12% gels by the method of Laemmli [8] using a Mini Protean II equipment from Bio-Rad Labs. (Munich, Germany). Protein bands were stained with Coomassie Brilliant Blue R-250 from Serva (Heidelberg, Germany).

Fast protein liquid chromatography (FPLC)

All chromatographic procedures were performed with an FPLC system on the following chromatographic media or prepacked columns (Pharmacia, Freiburg, Germany). Anion-exchange chromatography was carried out on Q-Sepharose fast flow or on a Mono Q HR 10/10 column. For hydrophobic interaction chromatography and gel filtration a phenyl-Sepharose HiLoad 26/10 column and a Superdex 200 HiLoad 26/60 column, respectively, were used. All buffers were degassed and filtered through 0.45-μm membrane filters (Sartorius, Göttingen, Germany). Prior to loading the columns by a super loop, samples were passed through 0.2-μm sterile filters (Schleicher & Schüll, Dassel, Germany).

Enzyme separation and purification

The purification procedures were carried out in a cold room (4°C) under red light. Potassium phosphate buffer (20 m*M*, pH 7.4) containing 2 m*M* dithiothreitol (buffer A) was used in all purification steps if not stated otherwise. According to the desired amount and quality of the enzyme preparations, two different purification methods were applied.

Fast separation protocol. Frozen *C. barkeri* cells (15 g) were suspended in 25 ml of 50 m*M* potassium phosphate (pH 7.4)–5 m*M* magnesium chloride–5 m*M* dithiothreitol–deoxyribonuclease I (10 μg ml^{-1}, Boehringer, Mannheim, Germany). The cells were sonicated in three 10-min intervals at 0°C with a Branson sonifier (Branson Ultrasonics, Danbury, CT, USA) at a power of 80 W. Cell debris and membranes were removed by centrifugation at 130 000 *g* for 90 min.

The cell-free extract was diluted with two volumes of buffer A and pumped on a Q-Sepharose column (60-ml bed volume) which had previously been equilibrated with buffer A at a flow-rate of 2 ml min^{-1}. After washing with 100 ml of buffer A a linear NaCl gradient (0–1 *M* NaCl in 500 ml of buffer A) was applied, whereby both enzymes were eluted in one peak between 350 and 380 m*M* NaCl. The active fractions were combined (35 ml) and desalted by repeated ultrafiltration through a YM 100 membrane (Amicon, Witten, Germany) with buffer A.

Solid CHAPS was added to this solution to a concentration of 6 m*M* and incubated for 30 min.

This mixture was applied to a Mono Q column equilibrated with buffer A containing 2 m*M* CHAPS at a flow-rate of 2 ml min^{-1}. After washing with 50 ml of the same buffer, the enzymes were eluted by a linear NaCl gradient (see Fig. 1B). Fractions containing either mutase or isomerase were pooled, concentrated by ultrafiltration and stored at −80°C.

Large-scale purification protocol. The same procedure as in the fast separation protocol was carried out with 45 g of frozen cells in 80 ml of buffer yielding 105 ml crude extract. Solid ammonium sulphate (313 mg ml^{-1}) was added to the cell-free extract while stirring at 0°C to achieve 50% saturation. After 30 min the suspension was centrifuged at 25 000 *g* for 30 min and the precipitate was dissolved in 70 ml of buffer A. This solution was dialysed overnight against buffer A. Anion-exchange chromatograhpy on Q-Sepharose was performed, applying the same conditions as described in the fast separation protocol.

The mutase pool from Q-Sepharose was concentrated to 14 ml by ultrafiltration (YM 100 membrane) and applied to the Superdex 200 column which had been pre-equilibrated with buffer A containing 0.1 *M* NaCl at a flow rate of 3 ml min^{-1}. Mutase and Δ-isomerase appeared together in an elution volume from 160 to 180 ml. These fractions were concentrated to 7 ml by ultrafiltration.

Solid ammonium sulphate was added to the active Superdex fractions to a final concentration of 0.85 *M*. After incubation for 30 min this solution was pumped on a phenyl-Sepharose HiLoad column which had already been equilibrated with 0.85 *M* ammonium sulphate in buffer A at a flow-rate of 3 ml min^{-1}. After washing with 50 ml of this buffer the enzymes were eluted by a linear descending ammonium sulphate gradient from 0.85 to 0 *M* (see Fig. 3). The peaks containing mutase and Δ-isomerase were each concentrated by ultrafiltration in Centricon 30 microconcentrators (Amicon). The Δ-isomerase was not further purified and was stored at −80°C. The mutase was dialysed overnight against 20 m*M* potassium phosphate (pH 8.0).

Unlike the Mono Q step in the fast separation protocol, this run was performed at pH 8.0 in the absence of CHAPS. The Mono Q column was equilibrated with 20 m*M* potassium phosphate (pH 8.0) containing 2 m*M* dithiothreitol. The running conditions and the gradient were the same as in the above-described Mono Q step (Fig. 1). The mutase, eluting at 360 m*M* NaCl, was concentrated in a Centricon 30 microconcentrator and stored at −80°C.

Determination of the corrinoid content

Pure 2-methyleneglutarate mutase was incubated in 1 *M* KCN (pH 12) for 15 h at ambient temperature. During this time dicyanocobalamin was formed, which was indicated by its typical UV–VIS spectrum. The amount of the corrinoid was calculated from its absorbance at 369 nm (ε = 30.4 l $mmol^{-1}$ cm^{-1}) and 543 nm (ε = 8.6 l $mmol^{-1}cm^{-1}$) [9].

Determination of the N-terminal amino acid sequence

2-Methyleneglutarate mutase samples (ten lanes, 10 μg of protein each) for N-terminal sequence determination were obtained from SDS-PAGE (12% acrylamide). The protein was blotted onto siliconized glass-fibre membranes (Glassybond; Biometra, Heidelberg, Germany) using the procedure supplied by the manufacturer. Electrotransfer was performed with a constant current of 6 mA cm^{-2} for 10 h at 4°C in a Mini Trans-Blot Cell (Bio-Rad Labs.). The transferred protein was stained with 0.1% Coomassie Brilliant Blue dissolved in 10% methanol for 3 min.

Destaining was performed in an aqueous solution of 45% methanol in 10% acetic acid for 20 min with two changes of the solution. The membrane was thoroughly washed overnight in distilled water. The stained bands were then excised, dried under air and subjected to amino acid sequence analysis [10]. The bands were placed on top of a Biobrene Plus pretreated glass-fibre disc (Applied Biosystems, Weiterstadt, Germany) and mounted in the cartridge of a Model 477A Protein/Peptide Sequenator (Applied Biosystems). Sequence determination was performed by the Edman procedure using the standard protocol given by the manufacturer (normal-1). The identification of the phenylthiohydantion (PTH) amino acid derivatives was performed with a Model 120A on-line PTH analyser (Applied Biosystems).

RESULTS AND DISCUSSION

Fig. 1A shows that 2-methyleneglutarate mutase and 3-methylitaconate isomerase eluted almost together by FPLC on the anion exchanger Mono Q at pH 7.4. The enzymes were also not separated by running the same chromatography at pH 8.0 (data not shown). However, when the enzymes were preincubated with 6 m*M* CHAPS and the FPLC was repeated in the presence of 2 m*M* of the same non-denaturating detergent at pH 7.4, complete separation was achieved (Fig. 1B). CHAPS influenced neither the activity nor the stability of 2-methyleneglutarate mutase. This separation was apparently specific, as the general elution profile of the protein as measured at 280 nm was not changed much by CHAPS. Notably, 2-methyleneglutarate mutase and 3-methylitaconate isomerase are both soluble cytosolic proteins, whereas CHAPS is usually applied for solubilization of membrane proteins [11]. At this stage the mutase was not pure (about 27% purity), as revealed by its specific activity (Tables I and II) and by SDS-PAGE (Fig. 2), whereby this enzyme represented the lower of the double band at 67 000 dalton. However, this preparation could be used for studies on the sensitivity of the mutase towards light and oxygen [6]. The whole purification protocol is summarized in Table I. Further but not complete purification was achieved by FPLC on Phenyl-Superose HR 10/10 (data not shown). By the same procedure (Table I) 3-methylitaconate Δ-isomerase was purified 24-fold up to a specific activity on 108 U mg^{-1} and 46% yield, but the preparation was also not homogeneous (about 10% purity).

In order to obtain pure 2-methyleneglutarate mutase, another protocol involving additional steps was developed (Table II). Starting from a larger amount of cell-free extract, precipitation with ammonium sulphate was introduced in order to remove much of the inactive protein. Thus, the same Q-Sepharose column as in the former method could be used. Subsequently the enzyme was subjected to FPLC on the molecular sieve Superdex 200. By each of the two chromatographic steps the mutase was purified threefold with recoveries of 82% and 95%, respectively. The separation of the mutase from the Δ-isomerase was achieved on a phenyl-Sepharose column in the absence of CHAPS (Fig. 3). Although this step resulted in a loss of 85% of the mutase, it could not be replaced by a milder

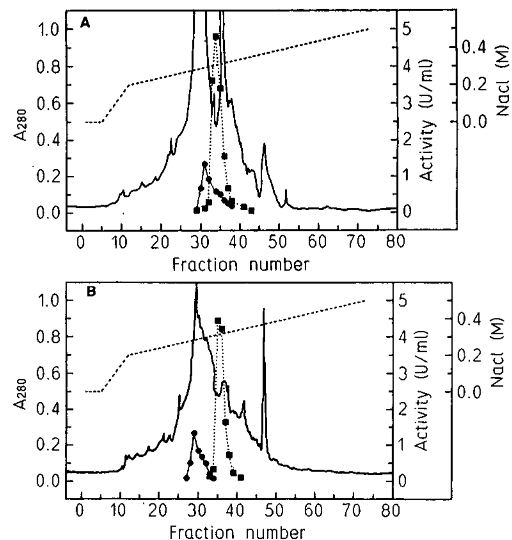


Fig. 1. FPLC on the anion exchanger Mono Q 10/10 (fast separation protocol). Solid line, absorbance at 280 nm; dashed line, NaCl gradient; dotted lines, enzymatic activity: ● = 2-methyleneglutarate mutase; ■ = 3-methylitaconate isomerase (the activity of the isomerase is 15 times higher than indicated on the scale "activity"). (A) In the absence of CHAPS; (B) in the presence of CHAPS.

TABLE I

FAST SEPARATION PROTOCOL FOR 2-METHYLENEGLUTARATE MUTASE

Step	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	19	917	64	0.07	100
Q-Sepharose	9	146	52	0.36	81
Mono Q/CHAPS	6	25	37	1.47	58

TABLE II

LARGE-SCALE PURIFICATION PROTOCOL FOR 2-METHYLENEGLUTARATE MUTASE

Step	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	105	4935	444	0.09	100
Ammonium sulphate	70	2450	337	0.14	76
Q-Sepharose	14	616	276	0.45	62
Superdex 200	7	172	262	1.53	59
Phenyl-Sepharose	5	10	38	3.8	9
Mono Q at pH 8.0	6	5	27	5.4	6

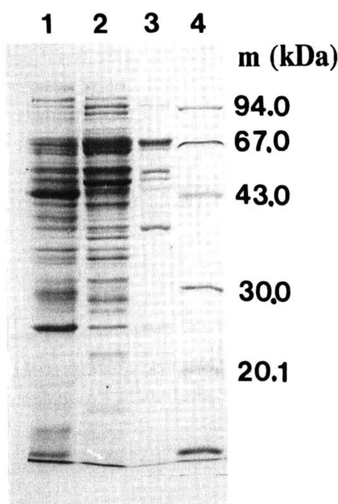


Fig. 2. SDS-PAGE of the steps of the fast separation protocol of 2-methyleneglutarate mutase. Lanes: 1 = cell-free extract (19 μg); 2 = Q-Sepharose (6.5 μg); 3 = Mono Q (4 μg); 4 = marker proteins (m = molecular mass; kDa = kilodalton).

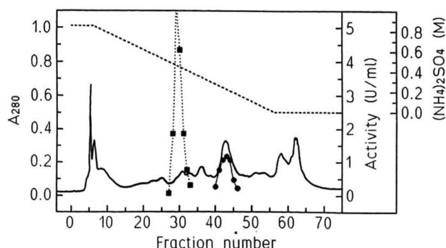


Fig. 3. FPLC on phenyl-Sepharose HiLoad (large-scale protocol). Solid line, absorbance at 280 nm; dashed line, ammonium sulphate gradient; dotted lines, enzymatic activity: ● = 2-methyleneglutarate mutase; ■ = 3-methylitaconate isomerase (the activity of the isomerase is 15 times higher than indicated on the scale "activity").

procedure. On a smaller scale, phenyl-Superose HR 10/10 instead of phenyl-Sepharose was used with almost identical results. Nearly homogeneous mutase was obtained by the final purification on Mono Q at pH 8 (Fig. 4, lane 6). When ten times as much mutase was applied to the gel, an impurity (67 000 dalton) of less then 5% became visible. The specific activity of the highly purified enzyme (5.4 U mg^{-1}, Table 2) was 2.4 times higher than that obtained earlier by preparative PAGE (2.4 U mg^{-1}) [5]. The 3-methylitaconate Δ-isomerase preparation which

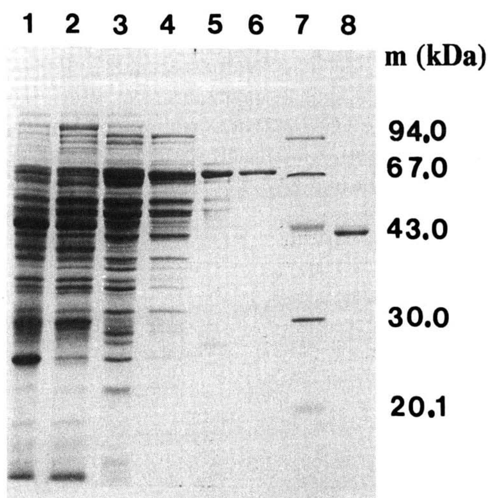


Fig. 4. SDS-PAGE of the steps of the large-scale purification protocol of 2-methyleneglutarate mutase. Lanes: 1 = cell-free extract (19 μg); 2 = ammonium sulphate fraction (21 μg); 3 = Q-Sepharose (18 μg); 4 = Superdex 200 (12 μg); 5 = phenyl-Sepharose HiLoad (2 μg); 6 = Mono Q (1 μg); 7 = marker proteins; 8 = phenyl-Sepharose fraction of the 3-methylitaconate isomerase (2 μg).

was obtained from the phenyl-Sepharose was not as active (44 U mg^{-1}) as that obtained with the CHAPS procedure (108 U mg^{-1}). The enzyme probably represented the less intensively stained upper band of the SDS-PAGE (Fig. 4, lane 8).

The successful separation of 2-methyleneglutarate mutase and 3-methylitaconate isomerase by a detergent or by chromatography on phenyl-Sepharose suggested a possible association of both enzymes by hydrophobic interactions. In addition to solubilization of membrane proteins, the application of mild detergents should be considered in related separation problems in order to improve the resolution of chromatographic steps. Hydrophobic interaction chromatography was also very efficient in purifying other enzymes, *e.g.*, 2-hydroxyglutaryl-CoA dehydratase from *Fusobacterium nucleatum* [12].

If the purification of 2-methyleneglutarate mutase was performed in the dark, the enzyme was active without added coenzyme B_{12} (adenosylcobalamin). The pink-orange colour of the mutase and the UV–VIS spectrum resembling that of adenosylcobalamin (Fig. 5) indicated that the coenzyme remained bound to the enzyme during purification. Using the molar absorption coefficient of adenosylcobalamin (ε_{522} = 8.0 l $mmol^{-1}$ cm^{-1}), the content of this coenzyme was calculated as 2.15 mol mol^{-1} homotetramer (mol. mass 300 000 dalton [5]). When the coenzyme was converted into the dicyano form [9], similar values were obtained (two different preparations gave 2.11 and 2.08 mol mol^{-1}). The protein

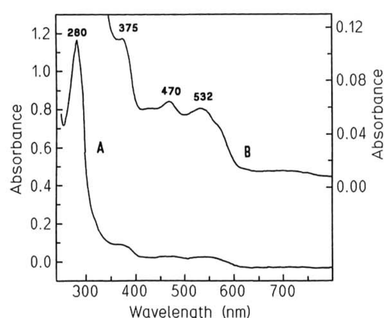


Fig. 5. UV–VIS spectrum of highly purified 2-methyleneglutarate mutase (0.8 mg ml^{-1}, purified through the Mono Q step, Table II) in 20 m*M* potassium phosphate (pH 7.4). Line A, left-hand scale; line B, right-hand scale.

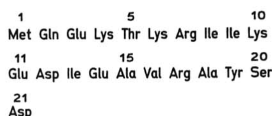


Fig. 6. N-Terminus of 2-methyleneglutarate mutase.

content of a sample was thereby taken from the average of the values estimated by three different methods [7,13,14]. Notably, the mutase preparation obtained earlier by non-denaturing preparative PAGE without protection from light was also pink but its enzymatic activity was completely dependent on added adenosylcobalamin [5].

The purity of the 2-methyleneglutarate mutase was also checked by the determination of the N-terminal amino acid sequence of the polypeptide. Therefore, an SDS-PAGE of the mutase was blotted on glass-fibre membranes and the protein band was subjected to Edman degradation by a gas-phase sequenator. The sequence obtained (Fig. 6) demonstrated the homogeneity of the enzyme but had no similarity to those of the N-termini of other adenosylcobalamin-dependent enzymes, *e.g.*, glutamate mutase from *Clostridium cochlearium* (E.C. 5.4.99.1) [15] or methylmalonyl-CoA mutase (E.C. 5.4.99.2) from mice [16] or *Propionibacterium shermanii* [17].

ACKNOWLEDGEMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. The authors thank Oskar Zelder (Philipps Universität Marburg) for cultivation of the bacteria.

REFERENCES

1 H.-F. Kung, S. Cederbaum, L. Tsai and T.C. Stadtman, *Proc. Natl. Acad. Sci. U.S.A.*, 65 (1970) 978.
2 H.-F. Kung and L. Tsai, *J. Biol. Chem.*, 246 (1971) 6436.
3 H. Eggerer, *Current Topics in Cellular Regulation*, 26 (1985) 411.
4 G. Hartrampf and W. Buckel, *Eur. J. Biochem.*, 156 (1986) 301.
5 C. Michel, G. Hartrampf and W. Buckel, *Eur. J. Biochem.*, 184 (1989) 103.
6 C. Michel and W. Buckel, *FEBS Lett.*, 281 (1991) 108.
7 P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M.

Goeke, B. J. Olson and D. C. Klenk, *Anal. Biochem.*, 150 (1985) 76.
8 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
9 W. Friedrich, in R. Ammon and W. Dirscherl (Editors), *Fermente, Hormone, Vitamine, Vol. III/2, Vitamin B_{12} und Verwandte Corrinoide*, Georg Thieme, Stuttgart, 1975, p. 50.
10 R. M. Hewick, M. W. Hunkapiller, L. E. Hood and W. J. Dreyer, *J. Biol. Chem.*, 256 (1981) 7990.
11 A. J. Binonti, J. Moss, L. Hjemeland and M. Vaughan, *Biochemistry*, 21 (1982) 3650.
12 A.-G. Klees, D. Linder and W. Buckel, unpublished results.
13 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
14 O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
15 U. Leutbecher, D. Linder and W. Buckel, unpublished results.
16 M. F. Wilkemeyer, A. M. Crane and F. D. Ledley, *Biochem. J.*, 271 (1990) 449.
17 N. Marsh, N. McKie, N.K. Davis and P. F. Leadlay, *Biochem. J.*, 260 (1989) 345.

Journal of Chromatography, 587 (1991) 101–108
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2372

Analysis of alanine and aspartate aminotransferase isoforms in mustard (*Sinapis alba* L.) cotyledons

Jürgen Michael Penther

Institut für Biologie II, Botanik, Schänzlestr. 1, W-7800 Freiburg i. Br. (Germany)

ABSTRACT

Two aminotransferases present in crude extracts of mustard seedling cotyledons were separated into isoforms using a Mono Q ion-exchange column. Four isoenzymes of aspartate aminotransferase were found, three of which were constitutively expressed. Only the predominant isoform exhibited changes in enzyme level that depended on plastidic factor and the nitrogen source. In comparison, alanine aminotransferase appeared to be regulated by light and the plastidic factor. After chromatographic separation, four isoforms of the enzyme were detected. The subcellular localization of these isoforms and their regulation are discussed.

INTRODUCTION

Higher plants are capable of reducing inorganic nitrate (NO_3^-), via nitrite (NO_2^-) to ammonium (NH_4^+), which is ultimately assimilated into organic substances. Enzymes required for this reduction are nitrate reductase (EC 1.6.6.1; NR) and nitrite reductase (EC 1.7.7.1; NiR). In the following assimilatory steps ammonium is first transferred to L-glutamate (Glu), an amino acid which acts as the primary acceptor molecule, and a molecule of L-glutamine (Gln) is generated. The reaction is catalysed by glutamine synthetase (EC 6.3.1.2). The following transamidation reaction between glutamine and the final acceptor 2-oxoglutaric acid (2OG), is catalysed by glutamate synthase (EC 1.4.7.1) [1]. The transfer of ammonium from glutamate to other 2-oxo acids is catalysed by different aminotransferases (EC 2.6.1.X) [2]. During the reaction of one of these enzymes the α-amino moiety of glutamate is transferred to a 2-oxo acid, *e.g.*, pyruvate or oxaloacetate [3]. The 2-oxoglutaric acid is recovered and reutilized [4]. In plants aspartate aminotransferase (EC 2.6.6.1; AsAT) and alanine aminotransferase (EC 2.6.1.2; AlAT), which are also known as glutamate oxaloacetate transaminase and glutamate pyruvate transaminase, respectively, are widely distributed. Both reactions are fully reversible. Aminotransferases are also involved in other processes, such as in the synthesis of most of other amino acids, in the hydrogen shuttle and in photorespiration [3].

At the level of aminotransferases, nitrogen metabolism, which has now become the metabolism of amino acids, diverges into several metabolic pathways.

The regulation of the levels of AsAT and AlAT in plants has not been clarified, although the temporal pattern of the appearance of these enzymes during germination has been investigated in some systems [5]. A systematic analysis of the influence of light, which acts in the mustard seedling exclusively through phytochrome (P_{FR}), has not as yet been investigated at the level of aminotransferases.

Thomas [6] reported two or three isoforms of AlAT in preparations from leaves of *Lolium tenulentum* that had been separated by DEAE-cellulose chromatography. In similar studies, Biekmann and Feierabend [7] found two isoforms of AlAT in the leaves of rye. One of these forms, which represented 90% of the total activity, was found to be located inside peroxisomes, whereas the other isoform was localized within mitochondria. In contrast, alanine synthesis from [2-^{14}C]pyruvate has been demonstrated in purified chloroplasts [8]. Other studies

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V. All rights reserved

have led to the conclusion that mitochondrial and soluble isoforms are actually the same protein [9]. Collectively, these results suggest that appropriate aminotransferases are widely distributed within plant cells [8].

The separation of AsAT isoforms is much easier: after gel electrophoresis coloured bands, representing isoforms, could be detected after staining [10,11]. By this technique up to five isoforms were observed [4]. The methods used allowed the determination of the number of isoenzymes present, but not their determination. AsAT isoforms can be found in different compartments, *e.g.*, cytosol, mitochondria, microbodies and plastids [4,5].

In *Sinapis alba* L. cotyledons, AsAT enzyme activity reaches a maximum at 60 h after sowing. Only small changes in enzyme activity are observed to result from exposure to light or alteration of the nitrogen content of the growth medium (data not shown). In contrast, the AlAT level is regulated and exhibits a strong light-mediated rise. A knowledge of AsAT and AlAT levels during seedling development is necessary in order that seedlings of an appropriate age can be analysed for their isoenzyme patterns.

This paper reports the separation of AlAT and AsAT isoforms from crude plant extracts using a Mono Q anion-exchange column.

EXPERIMENTAL

Growth conditions

Seeds of white mustard (*Sinapis alba* L., harvest 1986) were produced by a commercial grower from our original seed stock [12]. They were selected and grown at 25 $\pm$ 0.5°C with 3.5 ml of water or 15 m*M* solutions of KNO_3 and NH_4Cl on four layers of paper as described previously [13]. Where accumulation of coloured carotenoids should be prevented, the herbicide Norflurazon (HF, SAN 9789) was added to the medium from sowing onwards [14].

Light treatment

Standardized light fields were used as described by Mohr and Drumm-Herrel [15]: red light (R, 6.8 W m^{-2}, $\varphi_R = 0.8$) and far-red light (FR, 3.5 W m^{-2}, $\varphi_{FR} = 0.03$). P_{FR} is the physiologically active species of the phytochrome system. A gauge for the state of the phytochrome system is given by the φ value, the wavelength-dependent photoequilibrium of the phytochrome system, which is defined as follows: $\varphi = P_{FR}/P_{tot}$ = far-red absorbing form of phytochrome/total phytochrome.

Extraction conditions

For the extraction of AlAT, twenty pairs of cotyledons were homogenized on ice with a mortar and pestle in 2 ml of extraction buffer [100 m*M* tris(hydroxymethyl)aminomethane (Tris)–HCl (pH 7.1)–6 m*M* EDTA–5 m*M* $MgCl_2$–10 m*M* DTE–63 μM pyridoxal-5-phosphate (PLP)]. Homogenization was performed in the presence of 1 g of quartz sand and 0.1 g of Dowex 1-X2 to remove phenolics and polyphenolics [16]. When Triton X-100, bovine serum albumin (BSA) (purchased from Boehringer, Mannheim, Germany; fraction V, lyophilizate, protein content >95%) or betaine was added to the extraction buffer, they were used at the following concentrations: 1.5% (v/v) Triton X-100, 0.4% (w/v) BSA and 0.4, 1.0 and 7.5% (w/v) betaine. After addition of another 4 ml of extraction buffer, the homogenate was centrifuged in the cold for 20 min at 39 000 *g*. For sonication experiments, homogenates standing on ice were sonicated three times for 15 s with ultrasonic waves of 20-kHz frequency and a power of 30 W using a Branson (Danbury, CT, USA) Model B 15 cell disruptor connected with the standard microtip, and finally centrifuged. Supernatants contained 3.5–8 mg ml^{-1} of protein and were used as crude extracts.

AsAT (EC 2.6.1.1) extractions were performed as above, except that the extraction buffer was 100 m*M* Tris–HCl (pH 7.8) and 0.15 g of Dowex 1-X2 was added prior to each homogenization.

Enzyme assay

AlAT activity was measured spectrophotometrically at 25°C by the decrease in NADH in a coupled assay using lactate dehydrogenase (EC 1.1.1.27) (LDH) as auxiliary enzyme by a modification of the method of Hørder and Rej [17]. All solutions were prepared in 100 m*M* Tris–HCl buffer (pH 7.8). The final concentrations were 100 m*M* L-alanine (Ala), 16.7 m*M* 2OG, 0.27 m*M* NADH, 0.11 μM PLP and 239 nkat of LDH.

AsAT activity was measured according to Rej and Hørder [18], but L-aspartate (Asp) was substituted for Ala and malate dehydrogenase (EC 1.1.1.37) (MDH) for LDH. The final concentrations were

67 m*M* Asp, 10 m*M* 2OG, 0.27 m*M* NADH, 0.11 μM PLP and 160 nkat of MDH.

Ion-exchange chromatography

Separation of AlAT isoforms. Extracts were either prepared as described above with detergent or by sonicating. Low-molecular-weight substances were removed and buffers exchanged by gel filtration on Sephadex G-25 according to Neal and Florini [19] followed by filtration (Millipore, Type GVWP, pore size 0.22 μm). Separations were performed with a Mono Q HR 5/5 anion-exchange column attached to a fast protein liquid chromatography (FPLC) system (Pharmacia-LKB, Uppsala, Sweden). A 4-ml volume of extract, diluted with buffer A [20 m*M* Tris–HCl (pH 8.0) containing 5 m*M* DTE] to 10 ml, was loaded onto the column. The column was equilibrated with 30 ml of buffer A and pre-eluted with 22.5 ml of buffer A. The different peaks of AlAT activity were eluted with a linear gradient of 0–100% buffer B (= buffer A plus 0.4 *M* NaCl), in a volume of 20 ml. Fractions of 0.5 ml were collected at a flow-rate of 1 ml min^{-1}.

In order to minimize aggregation effects, the zwitterion betaine was used in some experiments as described in the figure legends.

Separation of AsAT isoforms. To separate the AsAT isoforms, the Mono Q HR 5/5 column was equilibrated with 20 ml of buffer A [20 m*M* Tris–HCl (pH 8.5)]. A 1.5-ml sample containing up to 2.5 mg of protein was diluted with buffer A to 10 ml. The column was pre-eluted with 22.5 ml of buffer A. The different peaks of AsAT activity were eluted with a linear gradient of 0–40% buffer B (= buffer A plus 1 *M* NaCl) in a volume of 20 ml, followed by a step to 100% buffer B. Fractions of 0.5 ml were collected at a flow-rate of 1 ml min^{-1}. Into each of the first thirty test-tubes 0.5 ml of a linear but opposite salt gradient from 30 to 0% B was filled. This leads to an identical salt concentration of 300 m*M* NaCl in the samples.

Recovery of enzyme activity compared with the total activity applied to the column was >95% activity for AlAT and >98% for AsAT. All steps were carried out at 4°C, except some ion-exchange experiments, which were prepared at room temperature (see figure legends). All buffers were prepared at 25°C.

RESULTS AND DISCUSSION

The time course of AsAT activity reaches its maximum 2.5 days after sowing. The activity then declines and after an additional 24 h reaches a steady state. There is only a slight difference between enzyme activities of dark- and light-grown seedlings, and induction by NO_3^-, which was previously found to induce a number of nitrate assimilatory enzymes [20–22], does not occur. Rather, the level of AsAT is lower if NO_3^- is applied to seeds. The influence of NH_4^+ is also very small, although it increases the enzyme level (unpublished data). It is concluded that the appearance of AsAT is constitutive. Light, which acts via phytochrome, or different nitrogen forms (NO_3^- or NH_4^+) can only slightly alter the time course. Hence the possibility that different isoforms of the enzyme exist was investigated. After separation on an anion-exchange column, four peaks of AsAT activity were detected (Fig. 1). The first isoform, called $AsAT_1$, eluted at an approximate salt concentration of 75 m*M*. Isoform $AsAT_2$ eluted at 145 m*M*, $AsAT_3$ at 195 m*M* and $AsAT_4$ at 235 m*M*. Isoforms 1 and 3 were the most prominent enzyme components. Polyacrylamide gel electrophoresis [11] of the crude extracts demonstrated the presence of five bands of AsAT activity: three strong bands, a small band and a very small band (data not shown).

In the case of AsAT the yield of activity recovered was >120% of that activity applied to the column. This is probably the result of the influence of salt on enzyme activity. Therefore, a gradient from 0.3 *M* NaCl to buffer A (volume 15 ml, flow-rate 1 ml min^{-1}) was collected in the fraction vials before the chromatographic separation was initiated. Consequently, the salt concentration was 0.3 *M* NaCl in all fractions containing AsAT activity and the recovery was about 98%.

The elution profile of AsAT showed distinct differences only for isoform 3 (Fig. 1). $AsAT_3$ was decreased by NO_3^- but increased by NH_4^+. In herbicide-treated plants grown under continuous red light, cotyledons did not contain normal chloroplasts and only small chlorophyll-free rudiments were present. Protein, especially enzymes, and mRNAs normally contained inside the chloroplast are no longer detectable under these treatment conditions [14,23]. If the extractable enzyme level is

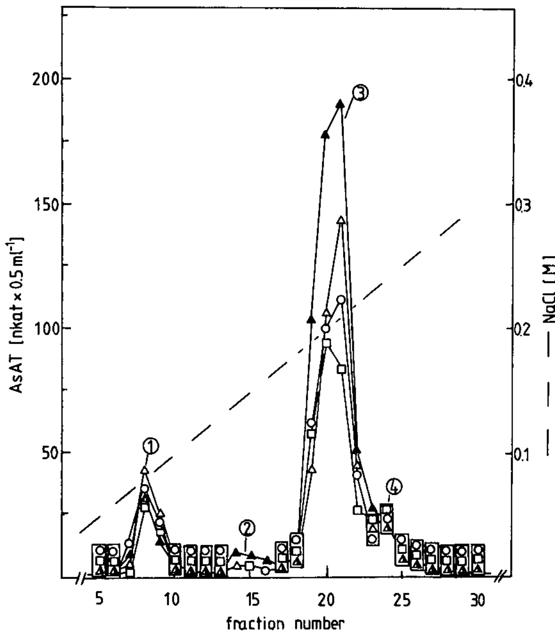


Fig. 1. Mono Q (HR 5/5) chromatography of AsAT isoforms from crude extracts of *Sinapis alba* L. cotyledons prepared with 100 m*M* Tris–HCl (pH 7.8). Extracts were centrifuged through Sephadex G-25 to change buffers. A 2.5-mg amount of protein diluted to a final volume of 10 ml with buffer A, corresponding to 1.5 ml of crude extract, was applied to the column using the Superloop. Starting buffer (A): 20 m*M* Tris–HCl (pH 8.5); gradient, 0–0.4 *M* NaCl in a volume of 20 ml, followed by a step to 1.0 *M* NaCl in a volume of 3 ml; flow-rate, 1 ml min^{-1}. No AsAT activity was detected beyond fraction 30. The data for AsAT chromatography are based on five independent experiments each. Plants were grown on (○) water, (□) 15 m*M* KNO_3, (△) 15 m*M* NH_4Cl or (▲) 15 m*M* NH_4Cl in the presence of Norflurazon ($1 \cdot 10^{-5}$ *M*) for 60 h in continuous red light (cR).

not affected by this treatment, the localization of AsAT inside chloroplasts can be excluded. Growth of plants in the presence of NH_4^+ resulted in a substantial increase in $AsAT_3$, while other isoforms are not affected (Fig. 1).

The second aminotransferase which is widely distributed among plants is AlAT. Whereas the level of AlAT in dark-grown seedling increases only slightly, a strong increase is seen in light-grown seedlings. Far-red light (cFR) is more effective than cR (unpublished data). Hence AlAT activity is regulated by light, which acts via the high irradiance reaction of the phytochrome system.

When plants are grown under photooxidative conditions (NF and cR), the AlAT level reaches a level between cD and cR (unpublished data), which indicates that the presence of intact plastids, which are capable of sending off the plastidic signal [23–25], is essential to give the full expression of AlAT activity. These data suggest a localization of an AlAT isoform inside the plastidic compartment or inside a compartment which is related to the presence of intact plastids [23].

To test this, a further experiment was necessary. Plants grown in far-red light show no effect of photooxidation. If such plants are transferred to photooxidative red light, the plastidic compartment is quickly destroyed, including most of the contained compounds (proteins, mRNAs), whereas DNA is not. Far-red light shows no effect on the AlAT level in the presence of NF (unpublished data), but the level of AlAT is not negatively affected in plants transferred to red light. Hence a plastidic localization can be excluded. These findings are in accordance with former findings that intact plastids are necessary for the appearance of peroxisomal enzymes [23] and the localization of an AlAT isoform [7,26]. Under conditions where intact chloroplasts could not develop, peroxisomal isoforms of AlAT do not appear and the enzyme level is reduced. AlAT is not affected, however, if already developed chloroplasts are photooxidized. Therefore, a localization inside peroxisomes is suggested. Surprisingly, the AlAT level increases above the normal physiological range, a phenomenon termed "superinduction". These results are not easy to explain, and an analysis of the isoform pattern was needed.

AlAT activity is not fully soluble and a detergent is often used for complete extraction. Extraction using the non-ionic detergent Triton X-100, which was optimized for the measurement of the AlAT level in crude extracts [27], leads to severe problems with protein separation, owing to lipid contamination of the column. The application of the detergent can be substituted by sonicating the homogenates before centrifugation.

If extracts were prepared by sonication rather than detergent treatment to disrupt compartments such as microbodies, the same AlAT activity was measured in crude extracts. To minimize aggregation effects between proteins themselves and other compounds of the crude extract, betaine was added to all buffers at a concentration of 1.0% or 7.5% (w/v). The result of anion-exchange chromatogra-

phy using a Mono Q (HR 5/5) column is shown in Fig. 2. With 1% betaine broadened peaks eluted over the whole gradient; 7.5% betaine did not lead to a better solution.

The separation of crude extracts prepared with detergent (Fig. 3a) but without addition of zwitterions showed nearly the same result. The major peak of AlAT activity eluted within a volume of 5 ml, a quarter of the gradient volume. Under both experimental conditions (Figs. 2 and 3a) the distribution of AlAT was very similar with one predominant "peak", followed by two smaller peaks.

When the extraction method was optimized for crude extracts, neither the use of common protease inhibitors, such as phenylmethylsulphonyl fluoride (PMSF) or leupeptin [28], nor the addition of BSA influenced the level of detectable AlAT activity. BSA only affected the stability of the extracts. Therefore, the addition of protein was chosen to protect AlAT from proteolytic digestion. Where separation into isoforms was necessary for a better

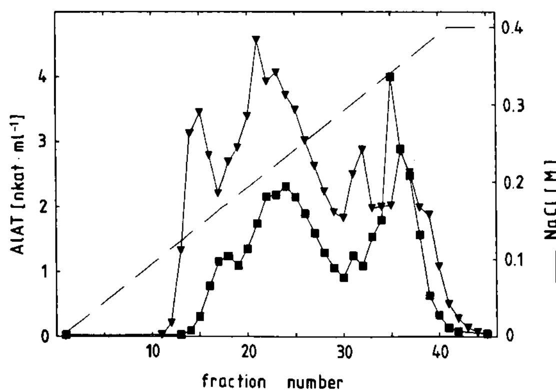


Fig. 2. Anion-exchange chromatography of AlAT isoforms present in crude extracts of cotyledons of *Sinapis alba* L. on a Mono Q (HR 5/5) column. In order to minimize aggregation effects, betaine was added to all buffers at concentrations of (■) 1 and (▼) 7.5% (w/v). Extracts were sonicated (for details, see Experimental). After gel filtration through Sephadex G-25, 4 ml of extract (3.5 mg ml^{-1} of protein) were diluted with buffer A to a final volume of 10 ml and were loaded onto the column. After elution of non-specific bindings with 22.5 ml of buffer A [20 mM Tris–HCl (pH 8.0)–5 mM DTE], the AlAT activity was eluted with a linear gradient of 20 ml rising to 0.4 M NaCl. The flow-rate was 1 ml min^{-1}. The data for AlAT chromatography are based on two independent experiments. Plants were grown (■) on water for 79 h in continuous red light (cR) or (▼) on 15 mM KNO_3 in the presence of Norflurazon ($1 \cdot 10^{-5}$ M) for 96 h in continuous far-red light (cFR).

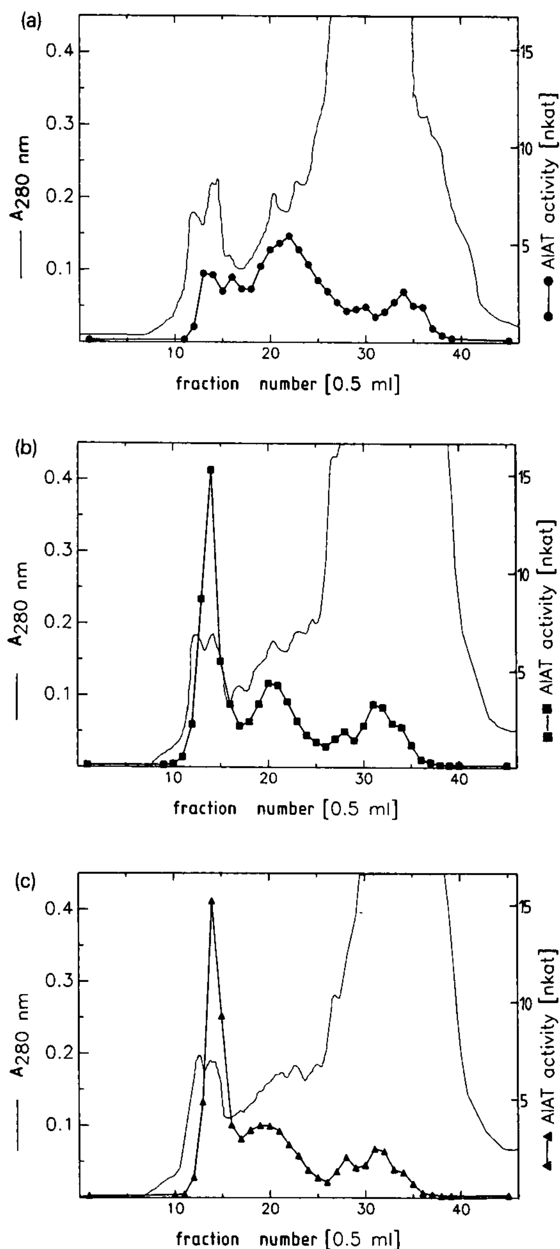


Fig. 3. Elution profile of AlAT activity present in extracts of mustard seedling cotyledons after anion-exchange chromatography on a Mono Q (HR 5/5) column. Extracts were prepared with extraction buffer containing (a) 1.5% Triton X-100, (b) 1.5% Triton X-100 plus 0.4% BSA or (c) 0.4% BSA plus sonication of the extract (for details, see Experimental). Chromatographic conditions as in Fig. 2. The data for AlAT chromatography are based on two independent experiments each. Seedlings were grown on 15 mM KNO_3 in the presence of Norflurazon ($1 \cdot 10^{-5}$ M) for 4 days in continuous far-red light (cFR).

understanding of AlAT regulation, the BSA concentration was halved, from 0.8 to 0.4%, to avoid high protein concentrations during the chromatographic step. The binding capacity of the column was not exceeded and no AlAT activity could be detected in the pre-elution volume.

The presence of a protective protein in the extraction medium caused the pattern of AlAT activity to change (Fig. 3a and b). Both extraction methods lead to the same distribution of AlAT (Fig. 3b and c): four peaks of enzyme activity were detected, a major peak followed by three minor peaks. Whereas the AlAT distribution changed significantly after protein addition, the protein absorbance at 280 nm did not (Fig. 3a and c). This indicates that chromatographic resolution is not negatively affected by addition of BSA. AlAT seems to be particularly sensitive to proteolytic digestion, which could be prevented in this way.

In order to exclude the possibility that BSA might affect non-specific binding or aggregation, the separation was repeated in the presence of 0.4% betaine (Fig. 4). As shown above (Fig. 2), higher concentrations of the zwitterionic compound betaine did not improve the resolution, so only a small amount was used for this experiment. The major peak eluted was closely followed by three peaks of enzyme activity. Differences between the curves are caused by different ages of plant material.

Both extraction methods (Figs. 3b and c and 4) lead to four peaks of AlAT activity with nearly the same position and the same distribution of activity. As the extraction procedure in the presence of a detergent leads to problems with dissolved lipids, the sonication procedure without betaine addition was chosen for extract preparation. In this instance more than 95% of AlAT activity, as compared with the total activity applied to the column, was recovered. The isoforms of AlAT (Fig. 5) are named $AlAT_1$ for the first-eluting form, at *ca.* 135 m*M* NaCl, $AlAT_2$ at 195 m*M*, $AlAT_3$ at 275 m*M* and $AlAT_4$ at 305 m*M*.

To study the regulation of the enzyme, plants were grown under different conditions. In dark-grown seedlings $AlAT_4$ is the main component and $AlAT_{1-3}$ are hardly detectable. Plants grown in the light (cR) show a dramatic increase in $AlAT_1$, a less dramatic increase in $AlAT_{2+3}$ and only a slightly increase in $AlAT_4$. Under photooxidative conditions (NF and cR) the pattern of distribution is completely different. $AlAT_{1+2}$ show levels comparable to dark-grown plants. $AlAT_3$ increases strongly and $AlAT_4$ doubles its activity compared with dark conditions. As indicated above, a plastidic localization was suggested by the results obtained with crude extracts from continuous light (cR + NF), but the "superinduction" experiment (where plants were transferred from cFR to cR) leads to different results. $AlAT_{1+2}$ shows an increase compared with plants grown in continuous red light, but this increase is normal, if compared with far-red-grown plants (data not shown). This shows that the expression of the affected isoforms is under the control of phytochrome and depends on the presence of the plastidic factor during the first 3 days of seedling development. $AlAT_4$ showed an increase that is slightly more than the detectable far-red level. Only $AlAT_3$ rises distinctly and reaches nearly the same extent as in the former experiment.

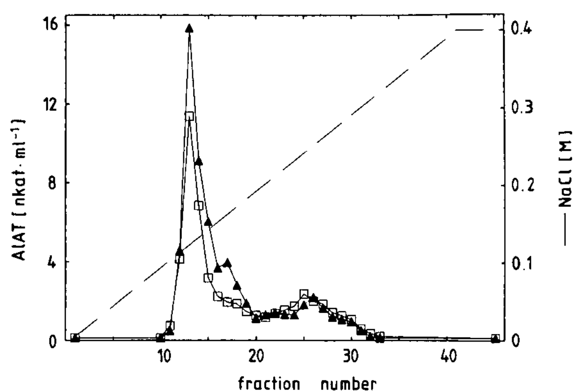


Fig. 4. Mono Q (HR 5/5) chromatography of AlAT from cotyledons of mustard. Extracts were prepared with extraction buffer containing 1.5% Triton X-100 and 0.4% (w/v) BSA. Where betaine was used (closed symbol) to minimize aggregation effects, it was present in all buffers in a concentration of 0.4%. Extracts contained up to 8 mg ml^{-1} of protein; 4 ml of extract were used in each experiment. Chromatographic conditions as in Fig. 2, except that separations were carried out at room temperature. Pre-elution showed no AlAT activity. The data for AlAT chromatography are based on two independent experiments each. The plants were grown on 15 m*M* KNO_3 for either (□) 72 h or (▲) 77 h in continuous far-red light (cFR).

It appears that if plastidic factor is lacking, the level of $AlAT_3$ increases. This supports a recent finding, first described for the isoform 1 of NiR by Schuster and Mohr [29], at the level of mRNA

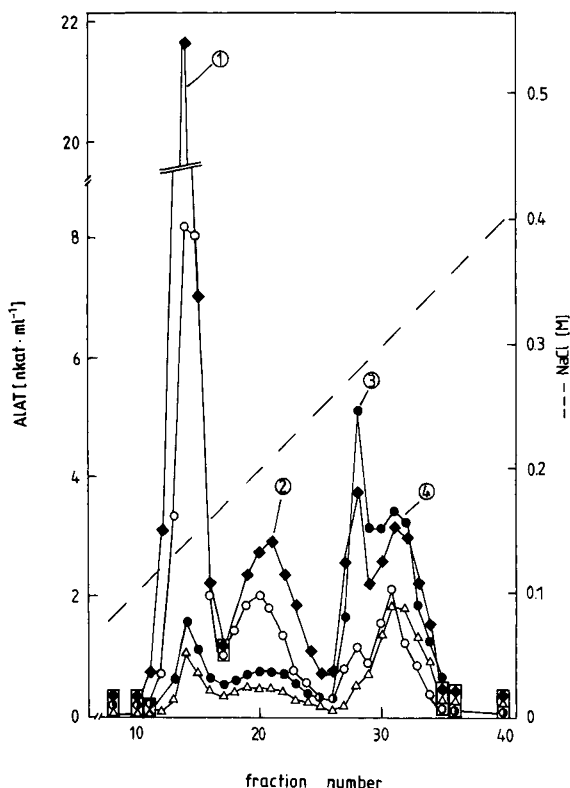


Fig. 5. Separation of AlAT isoforms from cotyledons of *Sinapis alba* L. carried out by anion-exchange chromatography (FPLC, Mono Q HR 5/5). The extraction buffer contained 0.4% (w/v) of BSA. After homogenization the extracts were sonicated (for details, see Experimental). Up to 20 mg of protein, diluted in buffer A to a final volume of 10 ml, corresponding to 4 ml of crude extract, were applied to the column using the Superloop. Starting buffer (A): 20 m*M* Tris–HCl (pH 8.0)–5 m*M* DTE; gradient, 0–0.4 *M* NaCl in a volume of 20 ml; flow-rate, 1 ml min^{-1}. Four peaks of activity were eluted ($AlAT_1$ at *ca.* 135 m*M*, $AlAT_2$ at 195 m*M*, $AlAT_3$ at 275 m*M* and $AlAT_4$ at 305 m*M* NaCl). No AlAT activity was detected below fraction 10 or beyond fraction 36. The data for AlAT chromatography are based on 3–10 independent experiments each. Seedlings were grown on 15 m*M* KNO_3 for 4 days in (△) continuous darkness (cD) or (○) continuous red light (cR) or (●) on 15 m*M* KNO_3 for 4 days in the presence of Norflurazon ($1 \cdot 10^{-5}$ *M*) under photooxidative conditions in continuous red light (cR). In order to investigate "superinduction" effects, the seedlings were grown in FR from sowing onwards for 3 days and then transferred to photooxidative R (◆).

translatable *in vitro*. It is concluded that lack of the plastidic factor can lead to an increase in proteins which are not located inside chloroplasts. Expressed differently, if the plastidic factor is present it leads to a suppression of gene expression. It appears that the selected proteins are located in the cytoplasm. $AsAT_3$ also showed this kind of dependence, but only to a small extent.

Two isoforms $AlAT_{1+2}$, were affected by the plastidic signal. If it is missing from the beginning of seedling development an increase in enzyme level does not take place. Apparently there is a positive correlation between the presence of the plastidic factor and the rise in the enzyme level. As a plastidic localization can be excluded, no decrease in $AlAT_1$ or $AlAT_2$ is detectable in the transition experiment; a localization outside the chloroplast but inside an organelle with a close relation to the plastid is suggested. A localization inside the peroxisomal compartment appears probable, which is in agreement with previous findings [7,23,26].

The separation of aminotransferases, AsAT and AlAT, into their isoforms shows their different regulation. AsAT is regulated only slightly with small modulations of activity, which could be lead back to isoform 3. This showed a certain dependence on negative control by the plastidic factor. On the other hand, the AlAT level is strongly regulated by light and the plastidic factor. The latter affects different isoforms differently, namely negatively in the case of $AlAT_3$, which represents a new phenomenon, and positively in the case of $AlAT_{1+2}$.

The complex relationships described can only be understood if separation into isoforms with a high recovery becomes possible. An important goal for the future is to confirm the "double character" of the plastidic factor, which was discovered recently by Schuster and Mohr [29], in the case of the two NiR isoforms in mustard cotyledons. The present results support these findings.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 206). The author is greatly indebted to the Studienstiftung des Deutschen Volkes for a scholarship, and thanks Professor H. Mohr for his steady support during this work and Professor R. L. Nicholson (Purdue University, West Lafayette, IN, USA) for assistance with the English.

REFERENCES

1 T. ap Rees, in D. D. Davies (Editor), *The Biochemistry of Plants*, Vol. 12, Academic Press, New York, 1987, Ch. 3, p. 87.
2 H. Mohr, *Der Stickstoff*, Springer, Berlin, 1990, p. 305.
3 R. J. Ireland and K. W. Joy, in P. Christen and D. E. Metzler (Editors), *Transaminases*, Wiley, New York, 1985, Ch. 6B, p. 376.
4 C. V. Givan, in B. J. Miflin (Editor), *The Biochemistry of Plants*, Vol. 5, Academic Press, New York, 1980, Ch. 8, p. 329.
5 F. Wightman and J. C. Forest, *Phytochemistry*, 17 (1978) 1455.
6 H. Thomas, *Z. Pflanzenphys.*, 74 (1975) 208.
7 S. Biekmann and J. Feierabend, *Biochim. Biophys. Acta*, 721 (1982) 268.
8 J. K. Bryan, in B. J. Miflin and P. J. Lea (Editors), *The Biochemistry of Plants*, Vol. 16, Academic Press, New York, 1990, Ch. 5, p. 161.
9 M. Gazeu-Reyjal and J. Crouzet, *Phytochemistry*, 15 (1976) 1619.
10 H. Thomas and J. L. Stoddart, *Annu. Rev. Plant Physiol.*, 31 (1980) 83.
11 F. J. Vences, F. Vaquero, A. M. Vazquez and P. De La Vega, *J. Plant Physiol.*, 132 (1986) 31.
12 H. Mohr, *Planta*, 49 (1957) 389.
13 H. Mohr, *Z. Pflanzenphysiol.*, 54 (1966) 63.
14 T. Reiss, R. Bergfeld, G. Link, W. Thien and H. Mohr, *Planta*, 159 (1983) 518.
15 H. Mohr and H. Drumm-Herrel, in H. Smith (Editor), *Plants and the Daylight Spectrum*, Academic Press, New York, 1981, p. 423.
16 W. D. Loomis, J. D. Lile, R. P. Sandstrom and A. J. Burbott, *Phytochemistry*, 18 (1979) 1049.
17 M. Hørder and R. Rej, in J. Bergmeyer and M. Grassl (Editors), *Methods of Enzymatic Analysis*, Vol. 3, Verlag Chemie, Weinheim, 3rd ed., 1983, Ch. 6.2, p. 444.
18 R. Rej and M. Hørder, in J. Bergmeyer and M. Grassl (Editors), *Methods of Enzymatic Analysis*, Vol. 3, Verlag Chemie, Weinheim, 3rd ed., 1983, Ch. 6.1, p. 416.
19 M. W. Neal and J. R. Florini, *Anal. Biochem.*, 55 (1973) 328.
20 U. Hecht, R. Oelmüller, S. Schmidt and H. Mohr, *Planta*, 175 (1988) 130.
21 C. Schuster, S. Schmidt and H. Mohr, *Planta*, 177 (1989) 74.
22 S. Schmidt and H. Mohr, *Planta*, 177 (1989) 526.
23 R. Oelmüller, *Phytochem. Phytobiol.*, 49 (1989) 229.
24 R. Oelmüller, I. Levitan, R. Beryfeld and H. Mohr, *Planta*, 168 (1986) 482.
25 A. Batschauer, E. Mösinger, K. Kreuz, J. Dörr and K. Apel, *J. Biochem.*, 154 (1986) 625.
26 T. Noguchi and S. Hayashi, *Biohem. J.*, 195 (1981) 235.
27 R. Weimar and G. M. Rothe, *Physiol. Plant.*, 69 (1986) 692.
28 P. Gegenheimer, *Methods Enzymol.*, 182 (1990) 174.
29 C. Schuster and H. Mohr, *Planta*, 181 (1990) 327.

Journal of Chromatography, 587 (1991) 109–115
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2381

Separation of the complete thioredoxin pattern of soybean leaves (*Glycine max*) by high-performance anion-exchange chromatography on Mono Q

Ingo Häberlein

Fachbereich Biologie-Chemie der Universität Kassel, Heinrich-Plett-Strasse 40, W-3500 Kassel (Germany)

ABSTRACT

Chromatographic studies are essential for assessing the physiological roles of the multiple thioredoxins (TRs) found in plants. Green leaves of the soybean (*Glycine max*) were shown to contain six different thioredoxins, which could be separated by anion-exchange chromatography. Three different thioredoxin activities were obtained by DEAE-cellulose chromatography (TRI, TRII and TRIII), which were further resolved by high-performance anion-exchange chromatography on a Mono Q column into TRIa, TRIb, TRIIa, TRIIb and TRIIc; TRIII could not be subdivided into additional thioredoxin species. In isolated chloroplasts of soybean leaves only TRIa and TRIb have been detected.

INTRODUCTION

Thioredoxins are ubiquitous, heat- and acid-stable proteins composed of about 110 amino acids. Their active centre contains a tetrapeptide, –Cys–Gly–Pro–Cys–, which enables the proteins to transfer reducing equivalents through reversible cysteine–cystine changes in a wide variety of biochemical reactions [1]. In plants, thioredoxins participate as protein disulphide reductase in he light–dark regulation of carbon dioxide fixation. For example, fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase and ribulose 5-phosphate kinase are activated in the light via reductive cleavage of regulatory disulphide bridges by reduced thioredoxin [2]. On the other hand, it has been demonstrated recently that thioredoxins can be inactivated by sulphitolysis of their own disulphide bond [3]. Based on this observation, a biochemical hypothesis of sulphur dioxide toxicity in plants has been developed [4]. All these properties make the analysis of plant thioredoxins a continuing and challenging task.

Since the discovery of multiple thioredoxins in spinach [5–7], it has been assumed that only the chloroplasts of a plant cell contain thioredoxins, in particular as the cytosolic thioredoxins originally described in spinach [6] were later recognized as artefacts [8]. Thus, all the many thioredoxins fractionated from total leaf extracts of other plants (*e.g.*, six proteins in maize [8] have usually been associated with chloroplasts. However, Langlotz *et al.* [9] described clearly non-chloroplastic thioredoxins in green algae (*Scenedesmus obliquus*), and Bodenstein-Lang *et al.* [10] also found thioredoxins in mammalian and plant mitochondria.

A knowledge of the complete thioredoxin pattern of an organism and of the intracellular distribution of these proteins is obviously required in order to define the specifities and numerous physiological functions of thioredoxins in plant cells. Here we describe a systematic analysis of the thioredoxins present in soybean leaves by high-performance anion-exchange chromatography [fast protein liquid chromatography (FPLC)].

EXPERIMENTAL

All chemicals and reagents were of the highest available purity and were obtained from Merck,

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V. All rights reserved

Biomol or Serva. Standard chromatographic steps were carried out at 5–10°C, using a Uvicord SII (Pharmacia–LKB) UV detector operating at 280 nm. The FPLC system used (Pharmacia–LKB) was equipped with a UV 1 optical unit and was operated at ambient temperature. Soybean (Maple Arrow variety) plants were grown in a greenhouse under standard conditions.

Thioredoxin assay

The assay is based on thioredoxin-dependent activation of NADP–malate dehydrogenase (EC 1.1.1.82) of spinach. The assay conditions and enzyme preparation have been described in detail previously [9,11]. A Uvikon 930 spectrophotometer (Kontron) was used in the enzyme assays.

Preparation of thioredoxins from soybean leaves

Unless noted otherwise, all steps were carried out at 4°C. About 100 g of leaves from 8–12 week-old plants were extracted with 250 ml of extraction buffer (100 m*M* Tris–HCl–1 m*M* EDTA, pH 7.5) in a Waring blender and the homogenate was filtered through three layers of Miracloth. Heat precipitation (70°C, 3 min) denatured or inactivated endogenous enzymes which could react with thioredoxin. Precipitated proteins were removed by centrifugation (20 min at 38 700 *g*). Ammonium sulphate (90% saturation) was then added to the supernatant to precipitate the thioredoxins, which were collected by centrifugation (20 min at 38 700 *g*). The pellet was redissolved in 20 m*M* ammonium acetate buffer–2 m*M* EDTA (pH 8.6) and dialysed against 2 × 3 l of the same buffer.

DEAE-Cellulose chromatography. A DEAE-cellulose column (Whatman DE-52; 8 × 2.8 cm I.D.) equilibrated with 20 m*M* ammonium acetate buffer–2 m*M* EDTA (pH 8.6) separated three different thioredoxin activities, TRI, TRII and TRIII. TRI was found in the void volume, whereas TRII and TRIII were resolved in a linear salt gradient of 0–300 m*M* NaCl in 400 ml of column buffer (flow-rate, 50 ml/h). Each thioredoxin fraction was further purified separately by the following chromatographic steps.

CM-Cellulose chromatography. A CM-cellulose column (Whatman CM-52; 5 × 2.8 cm I.D.) was equilibrated with 10 m*M* sodium acetate buffer (pH 4.6). Bound thioredoxins were eluted in a linear salt gradient of 50–250 m*M* NaCl in 400 ml of column buffer (flow-rate, 30 ml/h). TRI, TRII and TRIII all eluted at 200 m*M* NaCl in independent runs. Active fractions were collected, neutralized with 1 *M* NaOH and concentrated by ultrafiltration on an Amicon YM 5 membrane.

Gel filtration. The individual thioredoxin pools (TRI, TRII and TRIII) were chromatographed on a Sephadex G-50 column (140 × 1.5 cm I.D.) equilibrated with 50 m*M* Tris–HCl buffer–100 m*M* NaCl (pH 7.5) at a flow-rate of 10 ml/h. Active fractions were collected and dialysed against 10 l of 20 m*M* Tris–HCl buffer (pH 8.3) to prepare the different thioredoxins for the final chromatographic step.

Mono Q chromatography. FPLC on a Mono Q HR 5/5 column equilibrated with 20 m*M* Tris–HCl buffer (pH 8.3) was performed in a standard gradient of 0–200 m*M* NaCl in 20 ml of column buffer at a flow-rate of 0.5 ml/min and room temperature. Fractions of 0.5 ml were collected.

Thioredoxins in extracts of soybean chloroplasts

Isolation of chloroplasts from soybean leaves was done by analogy with the method of Mourioux and Douce [12]. The pelleted chloroplasts were suspended in Mono Q column buffer, disrupted by freezing at −70°C. The lysate was heat treated (3 min, 70°C) and centrifuged for 15 min at 38 700 *g*. The supernatant was dialysed against 5 l of 20 m*M* Tris–HCl buffer (pH 8.3) and analysed by FPLC on a Mono Q column as described above.

RESULTS AND DISCUSSION

Thioredoxins of soybean leaves

Plants possess a complex thioredoxin system with up to six different isoproteins; in contrast, non-photosynthetic organisms contain only one or two thioredoxins [1,2]. All these proteins exhibit a molecular weight of about 12 000 and isoelectric points between 4 and 6. Although the heat and acid stability of the thioredoxins can be exploited in their purification, steps which also remove target enzymes, thus faciliating thioredoxin assays, further purification and characterization of the individual thioredoxins are difficult owing to their close similarity. Chromatography of the heat-stable soybean leaf proteins on DEAE-cellulose resulted in the separation of three different thioredoxin acitivities, which

were numbered by their sequence of elution (Fig. 1). (We advocate that the functional thioredoxin classification f or m [6–8,13] is not used as long as the cellular functions of proteins are unknown). TRI remained unbound and was found in the void volume, whereas TRII and TRIII eluted at 70 and 200 m*M* NaCl, respectively, in a linear salt gradient. A DEAE-cellulose column has also been used successfully in analysing thioredoxins from spinach leaves [6], barley leaves [13], wheat flour [14] and soybean seeds [15]. It can be regarded as the most general initial separation step for thioredoxin fractionation, but not sufficient, however, to resolve all isothioredoxins reliably.

Before taking advantage of the high resolving power of FPLC on Mono Q, each individual thioredoxin separated by DEAE-cellulose chromatography had to be further purified. This could be achieved by conventional CM-cellulose chromatography followed by a Sephadex G-50 gel filtration as described under Experimental. No differences between the chromatographic behaviours of TRI, TRII and TRIII were observed. In separate runs all thioredoxins eluted at 200 m*M* NaCl in a linear salt gradient from CM-cellulose and the gel filtration indicated a molecular weight of about 12 000 for all of them (not shown).

The number and pattern of thioredoxins in soybean leaves could be extended and completed by FPLC on Mono Q (Figs. 2 and 3). The elution characteristics are summarized in Table I. In these experiments, the TRI and TRII fractions were subdivided into two (TRIa and b) and three (TRIIa, b and c) protein species, respectively. TRIII eluted as a single peak without evidence of additional TRIII species (Fig. 3). The quality of separation decreased substantially at other pH values (*e.g.*, pH 7.5 or 9.0) and it turned out that less than 10 mg of total protein had to be applied to achieve complete separation of the individual thioredoxin species. Virtually the same chromatographic profiles were obtained when the preparation of leaf thioredoxins was carried out in the presence of protease inhibitors [0.5 m*M* phenylmethylsulphonyl fluoride (PMSF), 2

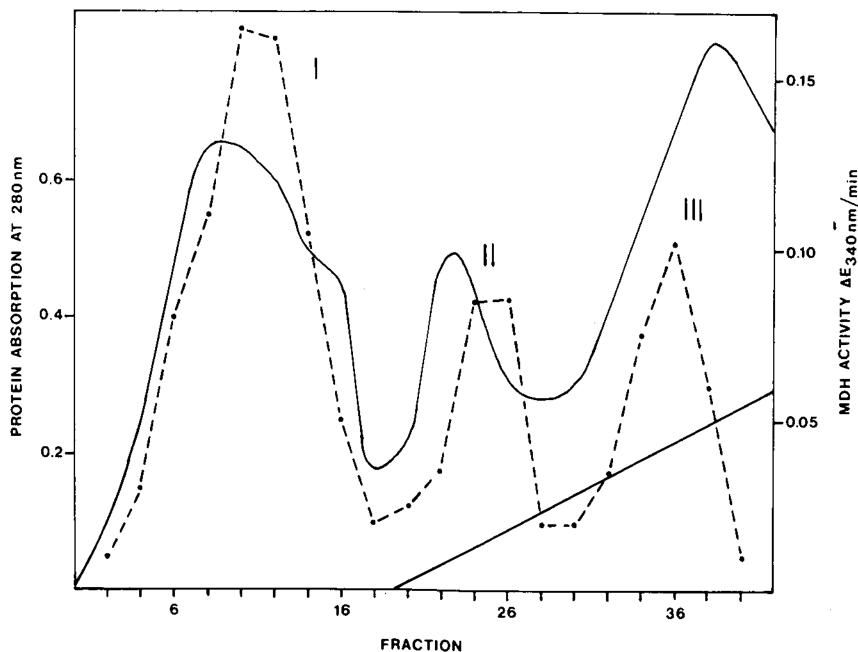


Fig. 1. DEAE-cellulose chromatography of thioredoxins in a heat-stable soybean leaf extract. A linear salt gradient (straight line) of 0–300 m*M* NaCl was applied within 400 ml of column buffer (20 m*M* ammonium acetate–2 m*M* EDTA, pH 8.6). Thioredoxin activity (dashed line) was measured by the thioredoxin-dependent activation of NADP–malate dehydrogenase (MDH). Solid line, protein absorption at 280 nm.

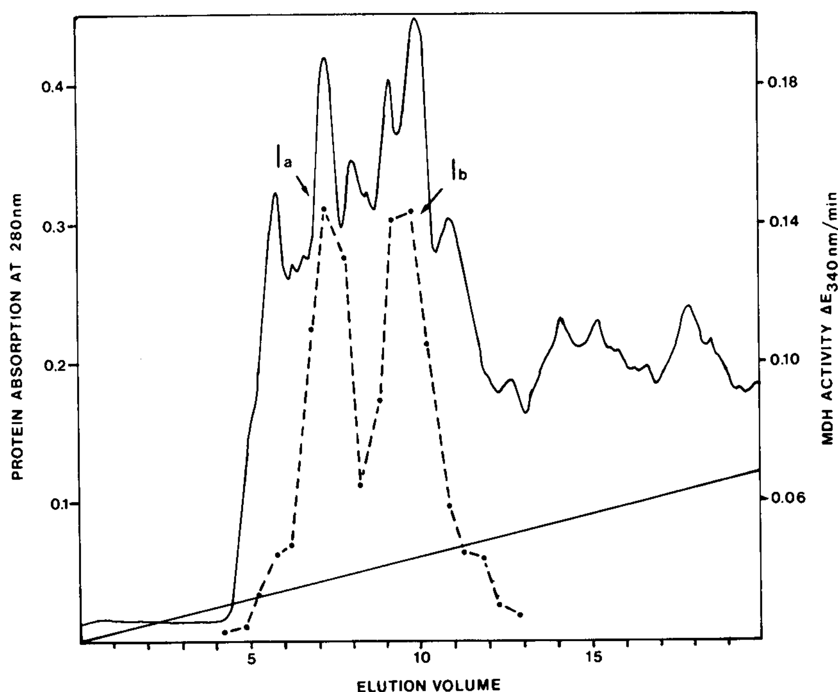


Fig. 2. Subdivision of TRI by Mono Q FPLC. A linear gradient (straight line) of 0–200 m*M* NaCl in 20 ml of 20 m*M* Tris–HCl column buffer (pH 8.3) was used for the separation. Solid line, protein absorption at 280 nm. Thioredoxin activity (dashed line) was measured by the thioredoxin-dependent increase of NADP–malate dehydrogenase (MDH) activity.

m*M* aminocaproic acid and 2 m*M* benzamidine–HCl], minimizing the risk that any of the above thioredoxins arise by artefactual, proteolytic degradation. Subsequently, each soybean leaf thioredoxin (TRIa and b, TRIIa, b and c and TRIII) was further analysed by Mono S FPLC. No indication of any additional thioredoxin species was observed in these cation-exchange analyses, in which every thioredoxin was chromatographed separately under identical conditions [50 m*M* sodium acetate buffer (pH 4.0); data not shown].

The present results indicate a total of six thioredoxins in green soybean leaves. A variety of other chromatographic techniques have been applied to the problem of resolving these six isoproteins, including chromatography on Blue Sepharose, hydroxyapatite and Fractogel TSK DEAE-650 S. None of these materials reached the quality of protein fractionation given by FPLC, nor could any further protein fraction be resolved (unpublished data). It therefore appears that FPLC is currently the superior, and probably the only, method for analysing thioredoxin patterns in plants. Other workers have also described six different thioredoxins in maize by the combined use of Mono Q and Mono S FPLC [8]. However, we recommend that thioredoxin patterns, previously described in higher plants without using FPLC techniques, should certainly be reinvestigated for their completeness.

Thioredoxins in isolated soybean cell organelles

Thioredoxins in plants have mostly been assigned to chloroplasts [5–7]. Non-chloroplastic thioredoxins have not been considered, although a likely cytosolic function of thioredoxin, ribonucleotide reduction, has been demonstrated in soybean cells [16]. A knowledge of the complete thioredoxin pattern in soybean leaves should now allow the individual cellular species to be classified. Two thioredoxins have been observed in isolated soybean chloroplasts (Fig. 4). The chromatographic behaviours of these two chloroplastic thioredoxin were identical with the behaviour of TRIa and TRIb in the standard Mono Q chromatography, described

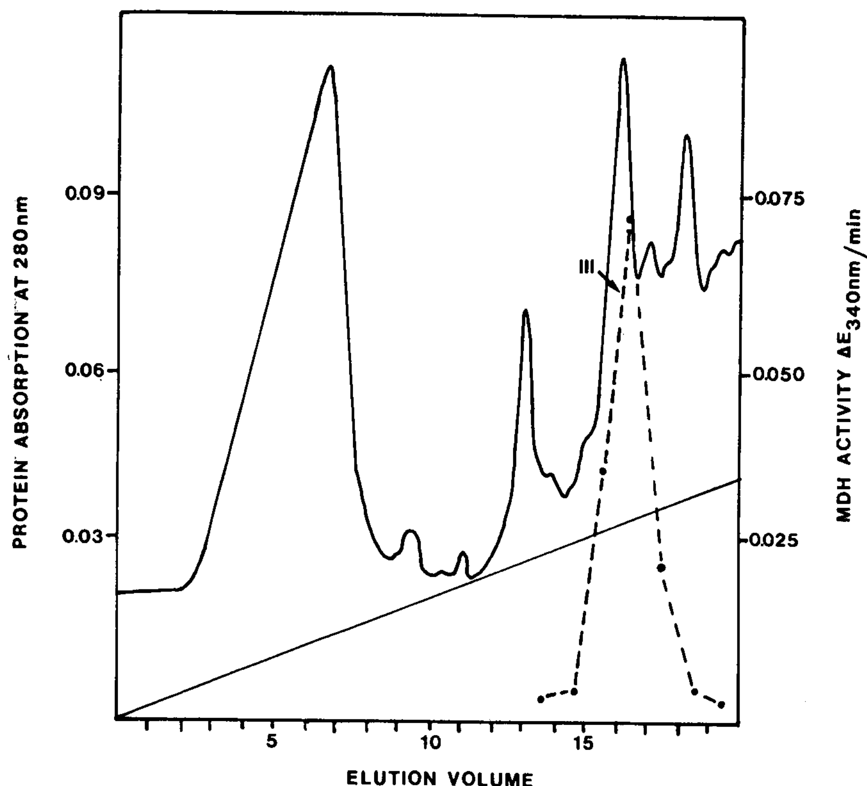


Fig. 3. Analysis of TRIII by Mono Q FPLC after preceding purification by DEAE-cellulose and Sephadex G-50 gel filtration chromatography. A linear gradient (straight line) of 0–200 m*M* NaCl in 20 ml of Tris–HCl column buffer (pH 8.3) was applied. Solid line, protein absorption at 280 nm. Thioredoxin activity (dashed line) was measured by the thioredoxin-dependent increase of NADP-malate dehydrogenase (MDH) activity.

above. This allows the specification of TRIa and TRIb as the chloroplastic species in a soybean leaf cell. Spinach chloropasts also possess two thioredoxins [6,17,18]; chloroplasts of other plants have not been analysed for thioredoxin so far.

TABLE I

ANION-EXCHANGE CHROMATOGRAPHY OF SOYBEAN LEAF THIOREDOXINS ON DEAE-CELLULOSE AND BY FPLC ON MONO Q

DEAE-cellulose		Mono Q	
TR fraction	NaCl molarity for elution	TR fraction	NaCl molarity for elution
TRI	(Unbound)	TRIa	75 m*M*
		TRIb	95 m*M*
TRII	70 m*M*	TRIIa	85 m*M*
		TRIIb	115–120 m*M*
		TRIIc	135–140 m*M*
TRIII	200 m*M*	TRIII	170 m*M*

A preliminary analysis of mitochondria from soybean leaves indicates that TRIIb and TRIIc are located in these cell organelles [19]; one mitochondrial thioredoxin had been observed in potato tuber mitochondria [10]. The intracellular localization of TRIIc and TRIII remains to be established. We expect that at least one of these thioredoxins is of cytosolic origin, to function as the essential hydrogen donor for soybean ribonucleotide reduction [16] as it does in green algae [20].

Interestingly, soybean leaves and seeds contain the same number of thioredoxins, which are very similar but not identical in their chromatographic behaviour. Changes in the thioredoxin pattern should indeed be expected during morphogenesis from resting and germinating seeds to light-regulated green plants. Further, thioredoxin profiles

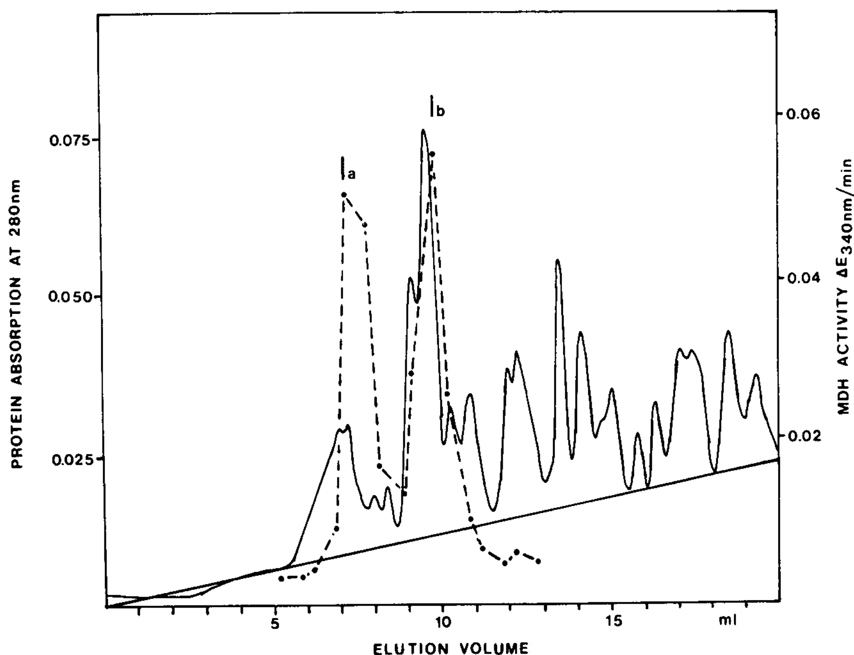


Fig. 4. Mono Q chromatography of a heat-treated protein extract from soybean chloroplasts. Only proteins separated within the standard linear salt gradient (straight line) are illustrated. Thioredoxin activity (dashed line) was observed by the thioredoxin-dependent activation of NADP–malate dehydrogenase (MDH). Solid line, protein absorption at 280 nm.

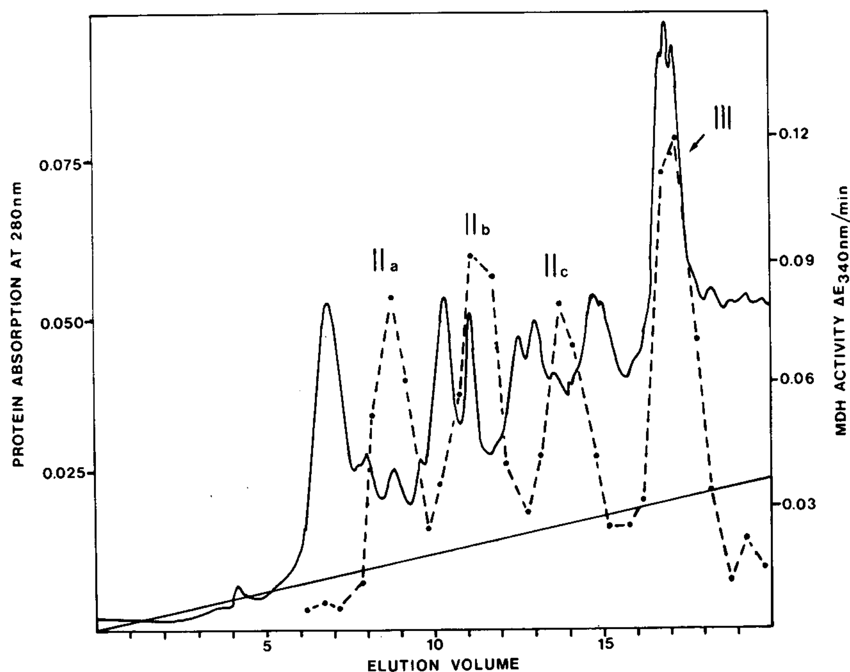


Fig. 5. Separation of a TRII–TRIII mixture by Mono Q FPLC. The TRII–TRIII mixture was eluted together from DEAE-cellulose at a 300 mM NaCl concentration and further enriched by CM-cellulose and gel filtration chromatography. A linear salt gradient (straight line) of 0–200 mM NaCl in 20 ml of 20 mM Tris–HCl column buffer (pH 8.3) was used to resolve the mixture. Thioredoxin activity (dashed line) was measured by the thioredoxin-dependent activation of NADP–malate dehydrogenase (MDH). Solid line, protein absorption at 280 nm.

may be subject to environmental influences, *e.g.* sulphur dioxide cause sulphitolysis and inhibition [3,4]. Studies of the thioredoxin profiles at different stages of development or under different environmental conditions demand a facile and reproducible analytical scheme. To this end, DEAE-cellulose chromatography of soybean thioredoxins was further simplified by first isolating TRI, which is present in the void volume, and then TRII and TRIII together by a one-step elution with 300 m*M* NaCl. The TRII–TRIII pool has been similarly prepared for Mono Q FPLC by a CM-cellulose and gel filtration step as already described for the individual TRI, TRII and TRIII fractions (see Experimental). TRIIa, TRIIb, TRIIc and TRIII could all be separated by one standard Mono Q FPLC (Fig. 5). The two chloroplastic thioredoxins have to be analysed separately in this scheme because TRIb and TRIIa cannot be resolved reproducibly enough by one standard Mono Q run (see Table I). Optimum conditions of Mono Q chromatography are currently being investigated to achieve the analysis of all soybean thioredoxins in one run with a minimum of preceding purification steps.

ACKNOWLEDGEMENTS

The excellent technical assistance of Martina Würfel and Verena Reinhardt is gratefully acknowledged. This study was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 305 (Ökophysiologie).

REFERENCES

1 A. Holmgren, *Annu. Rev. Biochem.*, 54 (1985) 237–271.
2 C. Cseke and B. B. Buchanan, *Biochim. Biophys. Acta*, 853 (1986) 43–63.
3 M. Würfel, I. Häberlein and H. Follmann, *FEBS Lett.*, 268 (1990) 146–148.
4 I. Häberlein and H. Follmann, *Naturwissenschaften*, 78 (1991) 259–265.
5 R. A. Wolosiuk and B. B. Buchanan, *Nature (London)*, 266 (1977) 565–567.
6 R. A. Wolosiuk, N. A. Crawford, B. C. Yee and B. B. Buchanan, *J. Biol. Chem.*, 254 (1979) 1627–1632.
7 P. Schürmann, K. Maeda and A. Tsugita, *Eur. J. Biochem.*, 116 (1981) 37–45.
8 N. A. Crawford, B. C. Yee, S. W. Hutcheson, R. A. Wolosiuk and B. B. Buchanan, *Arch. Biochem. Biophys.*, 244 (1986) 1–15.
9 P. Langlotz, W. Wagner and H. Follmann, *Z. Naturforsch. C*, 41 (1986) 979–987.
10 J. Bodenstein-Lang, A. Buch and H. Follmann, *FEBS Lett.*, 258 (1989) 22–26.
11 R. A. Wolosiuk, B. B. Buchanan and N. A. Crawford, *FEBS Lett.*, 81 (1977) 253–258.
12 G. Mourioux, and R. Douce, *Plant Physiol.*, 67 (1981) 470–473.
13 N. A. Crawford, B. C. Yee and B. B. Buchanan, *Plant Sci. Lett.*, 22 (1981) 317–326.
14 K. Vogt and H. Follmann, *Biochim. Biophys. Acta*, 873 (1986) 415–418.
15 A. Bertsermann, K. Vogt and H. Follmann, *Eur. J. Biochem.*, 131 (1983) 339–394.
16 B. Hovemann and H. Follmann, *Biochim. Biophys. Acta*, 561 (1979) 42–52.
17 K. Maeda, A. Tsugita, D. Dalzoppo, F. Vilbois and P. Schürmann, *Eur. J. Biochem.*, 154 (1986) 197–203.
18 M. Komo, A. Tsugita, C. Wiessner, N. Wedel, D. Bartling, R. G. Herrmann, F. Aquilar, L. Gordet-Salvi and P. Schürmann, *Eur. J. Biochem.*, 182 (1989) 315–322.
19 A. Konrad, unpublished results.
20 R. Hoffmann, W. Feller, M. Pries and H. Follmann, *Biochim. Biophys. Acta*, 832 (1985) 98–112.

Journal of Chromatography, 587 (1991) 117–126
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2391

Isolation of cytochrome P-450 components from marmoset liver microsomes by high-performance liquid chromatography☆

Michael Kastner* and Diether Neubert

Institut für Toxikologie und Embryopharmakologie, Garystrasse 5, 1000 Berlin 33 (Germany)

ABSTRACT

A fast protein liquid chromatographic (FPLC) system with pre-packed and laboratory-packed columns was used for the analytical and preparative isolation of marmoset monkey cytochrome P-450 (P450) and NADPH–P450-reductase. Chromatographic separations also allowed the recovery of cytochrome b_5, NADH–b_5-reductase and epoxide hydratase. Cholate-solubilized liver microsomes from phenobarbital-induced marmosets were crudely purified on 8-aminooctyl-Sepharose or 6-aminohexyl-Sepharose and then fractionated into several isoenzyme groups using hydroxyapatite. Further purification on Mono S or CM-Sepharose and finally on phenyl-Superose, phenyl-Sepharose or octyl-Sepharose yielded a P450 fraction which was apparently homogeneous as judged by sodium dodecyl sulphate–polyacrylamide gel electrophoresis in the automated Phast system using silver staining. Removal of excess of non-ionic detergent was effected by hydroxyapatite columns, and this was compared with other methods. For the isolation of P450 isoenzymes from untreated marmosets, Mono Q columns were employed and yielded at least two highly purified forms. NADPH–P450-reductase was recovered from the 8-aminooctyl-Sepharose column or crudely fractionated on DEAE-Sepharose Fast Flow. Subsequent purification via 2′,5′-ADP-Sepharose and Superose 12 chromatography resulted in a homogeneous preparation.

INTRODUCTION

Cytochrome P-450 (P450) is a collective term for a wide variety of chromatographic closely related monooxygenases which, depending on animal species and induction type, cover an increasing number of different proteins [1]. NADPH–P450-reductase, on the other hand, seems to exist in only one form per species. Purification of monooxygenases is, in general, difficult as most of these membrane proteins have molecular weights between 47 000 and 55 000 dalton and isoelectric points of about 5.5–6.5, a range shared by many other proteins [2].

P450 has been examined mainly and exhaustively using rat liver microsomal tissue [2,3]. Additional difficulties arising with P450 from primate as against rodent sources almost invariably include lower specific contents, lower metabolic activities and genetic polymorphisms. Studies involving primate P450 forms usually involve human tissue, but the use of non-human primates as a model has the advantage that these can be induced in a controlled way.

The purpose of this work was twofold. First, marmoset P450 and reductase were to be isolated and characterized for subsequent comparison with corresponding rodent forms [4,5], and second, sufficient amounts were to be prepared that would permit the establishment of a metabolizing system in organ culture [6,7]. A wide range of commercially available, prepacked fast protein liquid chromatographic (FPLC) columns and laboratory-packed columns were employed. This paper describes their usefulness and applicability for such purification.

☆ Parts of this paper were presented in a lecture at the *4th FPLC Seminar, Titisee, February 15–17, 1989.*

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V. All rights reserved

EXPERIMENTAL

Chemicals and chromatographic gels

Sodium cholate was purchased from Serva (Heidelberg, Germany), sodium deoxycholate from Merck (Darmstadt, Germany), 2′-AMP, NADPH, polyethylene glycol and Amberlite XAD-2 from Sigma (St. Louis, MO, USA), Emulgen 911 from Kao-Atlas (Tokyo, Japan) and Renex 690 from Atlas-Chemie (Essen, Germany). 8-Aminooctyl-Sepharose 4B was synthesized as described [8]. HA-Ultrogel hydroxyapatite was obtained from Serva (Munich, Germany) and Bio-Beads SM-2 from Bio-Rad Labs. (Munich, Germany). Hydroxyapatite prepared according to Mazin *et al.* [9] was also employed. AH-Sepharose (6-aminohexyl-Sepharose), 2′,5′-ADP-Sepharose, phenyl-Superose, phenyl-Sepharose, octyl-Sepharose, Superose 12, Mono Q, Mono S, DEAE-Sepharose Fast Flow and CM-Sepharose Fast Flow were obtained from Pharmacia (Uppsala, Sweden). The prepacked FPLC-grade gels each had a consistent particle size of 10 μm.

General conditions

Liver microsomes from phenobarbital-induced and from untreated male marmoset monkeys (*Callithrix jacchus*) were obtained and solubilized as described [5]. Buffer solutions usually contained 20% glycerol, 0.1 m*M* EDTA and 0.2 m*M* phenylmethylsulphonyl fluoride (PMSF). The temperature for all chromatographic steps was 18°C except for affinity, hydroxyapatite and hydrophobic interaction chromatography, which were carried out at 4°C. In this instance, the columns were maintained in a refrigerator or held in an ice-bath. Absorbances were recorded at 280 and 405 nm, and fractions were collected in a rack filled with ice.

Except where noted otherwise, all chromatographic equipment was purchased from Pharmacia. Laboratory packing of gels in high-resolution (HR) glass columns was carried out as follows: the initial packing was done in the customary manner [10] using *ca.* 1 m hydrostatic pressure. The column was then attached to the FPLC system and washed over "load/inject" with 5–10 gel bed volumes of 50% glycerol and increasing flow-rates from 0.1 to 1 or 2 ml/min until the back-pressure began to exceed 4.0 MPa. Laboratory packed columns were equilibrated and developed using flow-rates corresponding to 0.1–0.2 MPa.

Purification of P450

Laboratory-packed 8-aminooctyl-Sepharose 4B or 6-aminohexyl-Sepharose in an HR 16/10 column (10 cm × 16 mm I.D.) were equilibrated with 100 m*M* potassium phosphate (pH 7.4) and 0.6% sodium cholate, loaded with a centrifuged solution of solubilized microsomes, and then washed with 0.4% cholate in the same buffer [8]. Elution with 0.33% cholate and 0.06% Emulgen 911 yielded a single pool consisting of all P450 forms originally present. Further washing with 0.33% cholate and 0.35% sodium deoxycholate resulted in a broad peak of epoxide hydratase followed by a narrow peak of NADPH–P450-reductase.

The P450 fraction derived from phenobarbital-treated marmosets was then diluted with 20 volumes of 20% glycerol (pH 7.2) and loaded onto an HR 10/10 column (10 cm × 10 mm I.D.) packed with HA-Ultrogel hydroxyapatite which was equilibrated with 5 m*M* phosphate and 0.5% Emulgen 911 (pH 7.2). Successive washings with 20, 50, 100, 200 and 500 m*M* phosphate and 0.5% Emulgen 911 were collected. The 200 m*M* phosphate fraction was concentrated on an Amicon PM 30 ultrafiltration chamber, desalted on pre-packed or laboratory-packed HR 10/10 columns of Sephadex G-25 Superfine using 10 m*M* phosphate and 0.5% Emulgen 911 (pH 6.8) as running buffer and then loaded onto HR 5/5 (5 cm × 5 mm I.D.) pre-packed Mono S or laboratory-packed CM-Sepharose Fast Flow columns. The flow-rates were 1.0 and 0.2 ml/min, respectively. After application of a 20-ml continuous gradient of 0–250 m*M* NaCl in the above buffer, fractions were collected between 100 and 150 m*M* NaCl and freed from residual detergent on hydroxyapatite as described below. The Mono S or CM-Sepharose Fast Flow fraction (now in 200 m*M* phosphate buffer) was then loaded onto HR 5/5 phenyl-Superose, phenyl-Sepharose or octyl-Sepharose columns. For phenyl-Superose the flow-rate was 1.0 ml/min and for the Sepharose columns 0.3 ml/min. Subsequent to washing with 100 m*M* phosphate and 0.4% cholate, proteins were eluted with a linear or step gradient of 0–1.0% Emulgen 911 in this buffer. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) indicated that a 0.05–0.1% Emulgen 911 pool was homogeneous.

In contrast to an 8-aminooctyl-Sepharose frac-

tion of phenobarbital-induced microsomes, an 8-aminooctyl-Sepharose fraction of untreated marmoset microsomes was purified by a further chromatographic step on an HR 5/5 Mono Q column. A flow-rate of 1.0 ml/min and a 20-ml 0–200 m*M* NaCl gradient in 10 m*M* Tris buffer containing 0.5% Renex 690 resulted in highly purified forms.

Purification of NADPH–P450-reductase

8-Aminooctyl-Sepharose chromatography as described above yielded a crude fraction of reductase. In place of this, a large-scale fractionation of solubilized microsomes from phenobarbital-treated rats or rabbits on a 50 cm × 5 cm I.D. column of DEAE-Sepharose Fast Flow equilibrated with 0.6% sodium cholate and 0.8% Renex 690 was also used for elution with a step gradient between 120 and 360 m*M* KCl [11]. In either case, the reductase-containing fractions were then loaded directly onto a laboratory packed HR 10/10 column of 2′,5′-ADP-Sepharose which had been equilibrated with 300 m*M* phosphate and 0.1% Renex 690 or Emulgen 911. Using a flow-rate of 1.0 ml/min, the column was washed with this buffer and with 30 m*M* phosphate containing 0.15% deoxycholate, and then the flow direction was reversed. After application of 5 m*M* 2′-AMP in the same buffer, the reductase eluted in a sharp band. The eluent was concentrated over Amicon PM-30, divided into small aliquots suitable for gel filtration, frozen in liquid nitrogen and stored at −80°C. Finally, contaminating proteins, AMP, detergent and PMSF were removed via Superose 12 where the running buffer was devoid of detergent, EDTA and PMSF. An HR 10/30 pre-packed column (30 cm × 10 mm I.D.) accommodated a 200-μl loop load; a laboratory-packed HR 16/50 column (50 cm × 16 mm I.D.) of Superose Prep grade allowed 2-ml loads. In order to eliminate as much as possible the dead volume between the load valve and the column, the column prefilter was removed.

Removal of detergents using hydroxyapatite

An HR 10/2 column (20 mm × 10 mm I.D. was packed with hydroxyapatite and equilibrated with 20 m*M* phosphate (pH 7.2). The P450 fraction to be freed from excess of non-ionic detergent was diluted with 20% glycerol (pH 7.2) to the same phosphate concentration. After loading at a reduced flow-rate of 0.1–0.2 ml/min and if necessary recycling the pass-through fraction, the column was washed with 20 m*M* phosphate until excess of detergent was removed. This could be evidenced for detergents such as Emulgen or Renex by following the decrease in absorbance at 280 nm. A wash with 15–20 bed volumes was sufficient and, after reversal of the flow direction and elution with 300 m*M* phosphate, the P450 was recovered in a sharp peak.

Assays

P450 contents, NADPH–P450-reductase activities, reconstitution and substrate activities and protein assays correcting for non-ionic detergents were carried out as reported previously [4–5]. SDS-PAGE analyses were performed with an automated Phast system (Pharmacia) using 10–15% gradient gels and silver staining.

RESULTS AND DISCUSSION

Choice of detergent

For the general reader, a comment on the detergents used may be of value. Anionic cholate and deoxycholate are powerful membrane solubilizers and tend to dissociate interactions between proteins more effectivley than non-ionic detergents. Deoxycholate is more capable of complete dissociation. For most of the conventional column chromatographic separations of P450 forms, a combination of cholate and a non-ionic detergent such as the polyoxyethylene alkyl aryl ethers Renex 690 or Emulgen 911 has been preferred [2,3]. Protocols for the purification of many different P450 forms using several conventional anion-exchange resins may also involve various concentrations of both ionic and non-ionic detergents together [2,3]. For FPLC- and HPLC-quality anion-exchange gels, however, only non-ionic detergents were used in this work (see below). Renex 690 and Emulgen 911 were found to give similar results as regards stability and resolution of these enzymes. The fact that these two detergents absorb at 280 nm (in contrast to *e.g.*, Lubrol PX) was considered advantageous in that this property allowed for monitoring during their removal.

Other types of chromatographic gels, notably aminoalkyl-Sepharose, which exploit the amphipathic character of such proteins, have also utilized

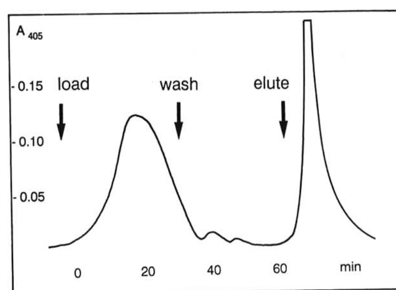


Fig. 1. Affinity chromatography using 2′,5′-ADP-Sepharose 4B. Sample: 143 μmol (cytochrome *c* reduced) per minute of phenobarbital-treated marmoset liver microsomal NADPH–P450-reductase prefractionated on 8-aminooctyl-Sepharose. Load: in 300 m*M* phosphate (pH 7.7)–0.1% Renex 690–20% glycerol–0.4% PMSF–0.1 m*M* EDTA. Wash: 30 m*M* phosphate–0.1% deoxycholate–20% glycerol. Elution: 5 m*M* 2′-AMP. Column: HR 10/10. Flow-rate: 1.0 ml/min. Detection: 405 nm with 0.2 a.u.f.s. Yield: 85–90%.

several concentrations and combinations of detergents [2,3,8]. In almost all instances, this was determined empirically.

8-Aminooctyl- and 6-aminohexyl-Sepharose chromatography

This was found to be a good choice as a first step in reducing to a minimum especially low-molecular-weight, non-monooxygenase proteins as has been reported for rat P450 [8]. This type of chromatography is apparently based more on a combination of hydrophobic interaction and ion exchange than on haeme–ligand affinity [3]. Successive elutions as described under Experimental then yielded a pool of collected P450 forms in 65–70% yield and the reductase in 80–90% yield. Gels used and washed even three or four times were routinely found to bind more than 20 nmol/ml of marmoset P450 and to increase the specific content about threefold. Commercially available 6-aminohexyl-Sepharose has also been used [12,13] and gave basically similar results here, except that the yield and capacity were both about 20% lower.

Polyethylene glycol (6000 or 8000 dalton) was also employed as used for rat material in an initial step prior to hydroxyapatite or ion-exchange chromatography [14]. This was found to increase the

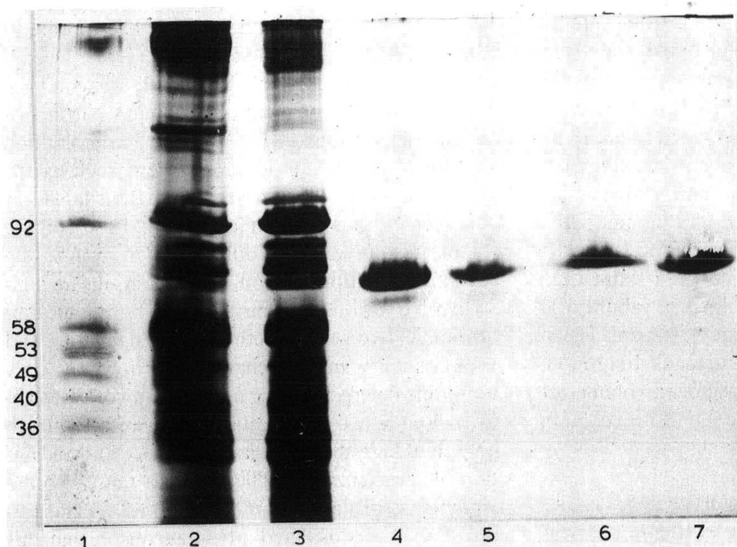


Fig. 2. SDS-PAGE gel of phenobarbital-treated marmoset NADPH–P450-reductase in various steps of purification. The anode is at the bottom, and the 10–15% gradient gel was stained with silver. Lanes: 1 = marker proteins phosphorylase *a* (molecular weight 92 kilodalton), catalase (58), glutamate dehydrogenase (53), fumarase (49), aldolase (40) and lactate dehydrogenase (36); 2 = solubilized microsomes; 3 = 8-aminooctyl-Sepharose fraction; 4 = ADP-Sepharose fraction; 5 = Superose 12 fraction as described in the text; 6 = as in lane 5 but from rat; 7 = as in lane 5 but from rabbit.

P450 specific content slightly more than as was described above, but in consideration of the poorer yields was not used further.

2′,5′-ADP-Sepharose chromatography

In contrast to 8-aminooctyl-Sepharose, 2′,5′-ADP-Sepharose chromatography required as sample load a certain degree of prefractionation of solubilized microsomes. This was achieved by using the 8-aminooctyl-Sepharose pool described above or a DEAE-Sepharose pool as described under Experimental [11]. It is likely that these two types of chromatography may thereby eliminate amounts of phosphatases which are capable of destroying the ADP groups bound to Sepharose [15]. A 1-ml volume of this resin was capable of binding at least 150 μmol (cytochrome *c* reduced) per minute. Fig. 1 shows a chromatographic profile of this affinity chromatography. The great increase in purification during this step is also evident in the SDS-PAGE gel (Fig. 2, lane 4). Instead of NADPH as recommended for elution by the manufacturer of this gel, 2′-AMP was used as subsequent substrate reactions with reconstituted enzyme systems required the addition of NADPH for initiation.

Superose 12 chromatography

In the SDS-PAGE gel (Fig. 2), it can be seen that a very slight degree of contaminating protein is still present in the ADP-Sepharose fraction (lane 4). Originally, for rat or pig reductases, this lower molecular weight protein was removed by Ultrogel AcA 34 gel filtration [11], which has a separation range of 20 000–350 000 dalton. FPLC-quality Superose 12 (separation range 1000–300 000 dalton) in a pre-packed HR 10/30 column was utilized in this work and yielded an almost quantitative recovery of this enzyme from the ADP load in much less time. This constituted the first, main peak shown in Fig. 3. At higher elution values, lower molecular weight protein, AMP and PMSF then appeared. As for our embryotoxicological studies [6,7] not only P450 but also excess stoichiometric amounts of reductase are required, this chromatographic step is considered to be especially useful. In contrast to an earlier report [11], the present description of separating minimal protein contaminants resulted in a great increase in the specific activity of the reductase.

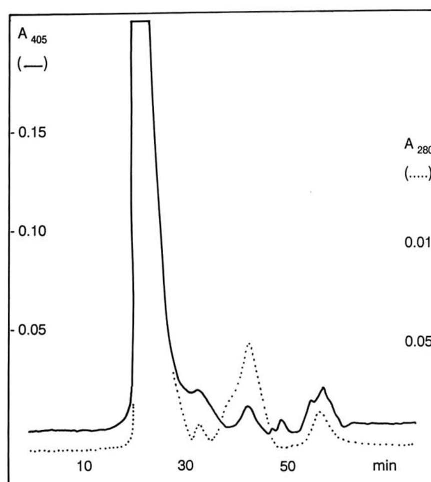


Fig. 3. Gel filtration chromatography using Superose 12. Sample: 48 μmol (cytochrome *c* reduced) per minute of phenobarbital-treated marmoset NADPH–P450-reductase prefractionated on ADP-Sepharose. Column: HR 10/30. Buffer: 50 m*M* phosphate (pH 7.7)–20% glycerol. Flow-rate: 0.4 ml/min. Detection: solid line, 405 nm with 0.02 a.u.f.s.; and dotted line, 280 nm with 0.2 nm. Yield: 80–90%.

In addition to purification of marmoset reductase as described above, we have also prepared with the same scheme the corresponding reductases from rat and rabbit. Judging from the preformed Phast 10–15% gradient gels (Fig. 2, lanes 5–7), the relative molecular weights of these are all about 77 000 dalton.

As indicated above for our work with organ culture, the conditions are very much dependent on highly purified, highly active reductase. To this end we have found it convenient to maintain aliquot portions of 200 μl of ADP-Sepharose reductase at −80°C, which can then be fractionated on Superose 12 at a flow rate of 0.4–0.5 ml/min in about 30 min just prior to use in culture. Tenfold larger sample volumes were loaded onto an HR 16/50 column filled with the bulk gel Superose 12 Prep-Grade, in which case the elution time was fivefold.

Sephadex G-25 chromatography

Desalting of sample loads for ion-exchange chromatography was accomplished by gel filtration on laboratory-packed or prepacked HR 10/10 columns of Sephadex G-25 Superfine. Load volumes of 0.5–

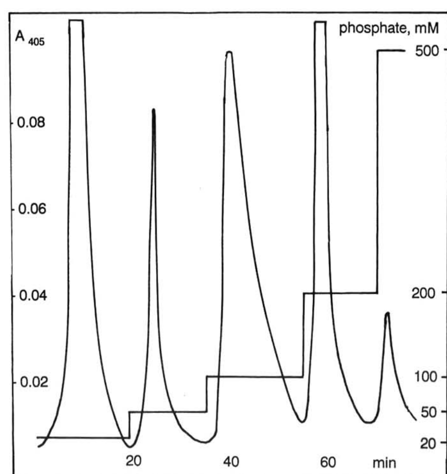


Fig. 4. Hydroxyapatite chromatography. Sample: 51 nmol of phenobarbital-treated marmoset P450 prefractionated on 8-aminooctyl-Sepharose. Buffer: 5 m*M* phosphate (pH 7.2)–20% glycerol–0.5% Emulgen 911. Gradient: 20–500 m*M* phosphate. Column: HR 10/10. Flow-rate: 0.5 ml/min. Detection: 405 nm with 0.1 a.u.f.s. Yield of P450 in the 200 m*M* fraction, 35%; in all fractions, 65–75%.

1.0 ml could be desalted with 90% protein recovery in 5 min. As ascertained by conductivity measurements, at least 95% of the salt was removed. Up to 2.5-ml loads were conveniently desalted on disposable PD-10 or NAP-25 cartridges (Pharmacia) at atmospheric pressure, and gave the same recovery but required 40 min each.

Hydroxyapatite chromatography

This type of purification was found to be useful for an initial separation into different P450 groups as was shown earlier for rat P450 [16]. At the same time, NADH–cytochrome b_5-reductase could be removed during washes with up to 50 m*M* phosphate (Fig. 4). Application of various gradient steps between 50 and 500 m*M* resulted in several fractions characterized by varying yields, electrophoretic purities, specific contents and metabolic activities [5]. Of all hydroxyapatite fractions, the 100–200 m*M* step apparently had the highest purity and yield and, as at this time the main aim was to isolate only one or the "main" P450 form present in phenobarbital-induced marmosets, this fraction alone was purified further. In comparison with the 8-aminooctyl-Sepharose fraction itself, this hydroxyapatite fraction displayed a considerable degree of electrophoretic purity (Fig. 5, lanes 2 and 3).

HA Ultragel gel could be used for more runs and at higher flow-rates than could granulated hydroxyapatite [9], but (at the same flow-rate) the latter was seen to bind P450 samples more strongly. Both types of gel were each capable of binding more than 25 nmol/ml of P450, and were not seen to cause any loss of haeme.

Hydroxyapatite gels of the Bio-Gel HT or HTP type were not used routinely as they were repeatedly found to compress on chromatography, resulting in greatly elevated back-pressures, even at reduced flow-rates.

Detergent removal

Excess amounts of non-ionic detergents not only interfere with protein determinations and substrate assays, but are also toxic towards tissue and organ culture [6,7]. Detergents present in fractions of marmoset P450 were removed with Bio-Beads or Amberlite [14,16] in 2–3 h but for the above-mentioned biological purposes, detergent removal by these resins was found to be too incomplete. Polyethylene glycol precipitation [17] offered the advantages of simultaneous desalting and concentration of the enzymes but was too cumbersome and gave too little recovery of enzyme. The same applied with calcium phosphate extraction [14], which had the advantage, however, of batchwise application. As already implemented for rat P450 [13,18], we found for marmoset enzymes that hydroxyapatite column chromatography was the most suitable method for removal of excess detergent, especially for *in vitro* assays.

Mono Q chromatography

When a sample of 8-aminooctyl-Sepharose pool was desalted as described above and loaded onto a Mono Q column (strong anion exchanger) and then washed and eluted as described under Experimental, P450 was recovered in both the pass-through fraction and the gradient fraction. The usefulness of this column at this stage of purification was found to be dependent on the type of marmoset induction: when phenobarbital was used as inducing agent and an 8-aminooctyl-Sepharose pool was loaded, only a modest resolution was found (Fig. 6a). This is in

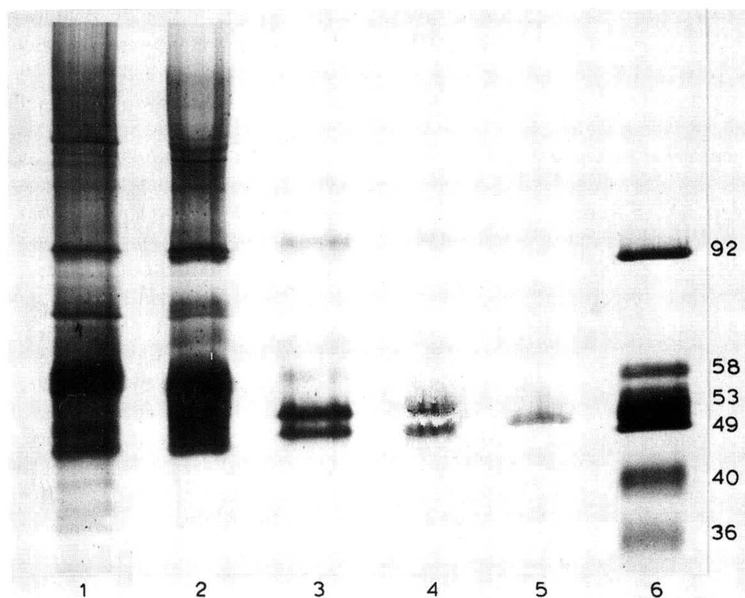


Fig. 5. SDS-PAGE gel of phenobarbital-treated marmoset P450 in various steps of purification. Lanes: 1 = solubilized microsomes; 2 = 8-aminooctyl-Sepharose fraction; 3 = hydroxyapatite fraction; 4 = Mono S fraction; 5 = phenyl-Superose fraction; 6 = marker proteins in kilodalton as described in Fig. 2.

accordance with that found for Mono Q fractionations of solubilized marmoset microsomes [4] and, recently, solubilized rat microsomes [19]. In SDS-PAGE analyses of the fractions obtained by such separations, the protein in the pass-through fraction was more homogeneous than that recovered in the gradient fraction (results not shown). We therefore conclude that this type of gel is more suitable for the simultaneous isolation of several P450 forms when the starting material is already more highly purified [5]. On the other hand, Roos [19] has recently shown that detailed segmented gradients on Mono Q columns are capable of yielding more discrete fractionations of variously induced P450 samples.

When non-induced marmoset 8-aminooctyl-Se-

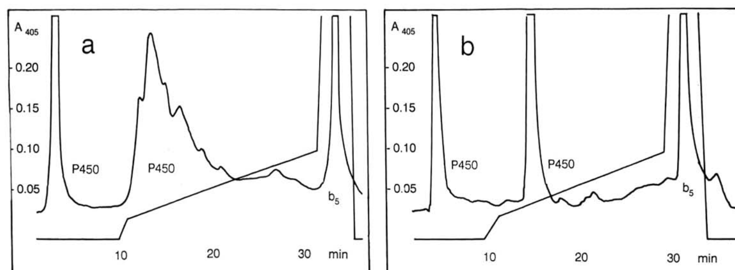


Fig. 6. Anion-exchange chromatography on Mono Q of (a) 6.3 nmol of phenobarbital-treated and (b) 6.1 nmol of untreated marmoset P450, each prefractionated on 8-aminooctyl-Sepharose. Column: HR 5/5. Buffer A: 10 m*M* Tris (pH 7.8)–20% glycerol–0.5% Renex 690. Buffer B: 1 *M* NaCl in buffer A. Gradient: 0–40–200–1000–0 m*M* NaCl. Flow-rate: 1.0 ml/min. Detection: 405 nm with 0.25 a.u.f.s. Yield: 70–90%.

pharose samples where fractionated on Mono Q columns, a good separation of P450 into two well defined, more homogeneous fractions was obtained (Fig. 6b). In this instance, in comparison with phenobarbital-induced P450, a smaller number of effective proteins were to be separated, so that this column may be considered adequate for this sample.

Mono Q fractionations of P450 derived from induced or non-induced marmoset material led to the isolation of cytochrome b_5 by NaCl gradients between 230 and 250 m*M*. P420 was recovered, if at all, mainly in the pass-through fraction as reported by us earlier [4] and by Roos [19].

DEAE chromatography

For phenobarbital-induced marmoset material, DEAE-Sepharose Fast Flow columns revealed a greater resolution of P450-containing peaks than did Mono Q columns. As this difference was also apparent between DEAE-Sepharose Fast Flow and Q Sepharose Fast Flow columns, we assume that this is indeed due to the different anion-exchange moieties, and not to any differences in gel matrix composition. It has often been reported [2,3] that for separations involving conventional anion-exchange of P450 on several types of DEAE matrices, a combination of anionic and non-ionic detergents results in better resolution than when using non-ionic detergents alone. In contrast to DEAE-Sepharose, DEAE-Sephacel or Whatman DE-52 [2,3], however, use of the anionic detergent sodium cholate with Mono Q is not possible, as this combination can cause substantial detergent-to-matrix binding which leads to a serious increase in back-pressure [4]. We have recently found that use of cholate with HPLC-quality DEAE gels also results in increased back-pressure and lower resolution, the latter of which has been reported earlier [13].

Mono S and CM-Sepharose chromatography

Mono S or CM-Sepharose chromatography of the 8-aminooctyl-Sepharose fraction (of phenobarbital-induced marmoset P450) using an NaCl gradient also resulted in a poorly defined profile. In contrast to Mono Q, Mono S yielded more homogeneity in the gradient fractions. For Mono S also, however, better separation of P450 forms alongside each other is to be expected when the load material is already more purified. The classical example, of course, in recovering a wide variety of P450 forms present is to load the pass-through fraction of an anion exchanger onto a cation exchanger and then utilize the gradient portion [13,16,19]. Again, Roos [19] has found that the use of segmented gradient can greatly enhance the resolution of Mono S separations.

Fig. 7a and b illustrate profiles of linear and step gradients in Mono S separations incorporating as the load a 100–200 m*M* phosphate pool derived from hydroxyapatite chromatography. Division of the gradient into the steps incorporated was determined by SDS-PAGE. The 100–150 m*M* fraction was the most homogeneous and, as seen in Fig. 5, lane 4, consists of a clear double band.

Phenyl-Superose, phenyl-Sepharose and octyl-Sepharose

After none of the above-discussed types of chro-

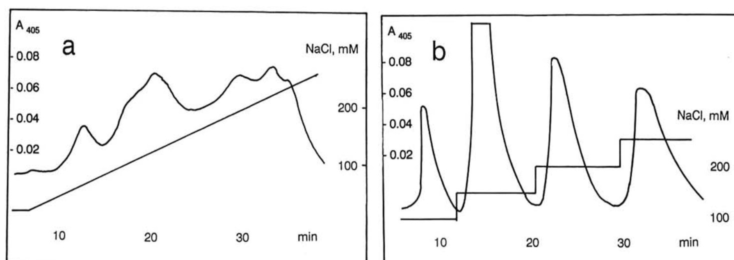


Fig. 7. Cation-exchange chromatography on Mono S using (a) linear and (b) step gradients. Sample: 5.6 nmol of phenobarbital-treated marmoset P450 prefractionated on hydroxyapatite (200 m*M* pool). Column: HR 5/5. Buffer A: 10 m*M* phosphate (pH 6.8)–20% glycerol–0.5% Emulgen 911. Buffer B: 1 *M* NaCl in buffer A. Gradient: 10–300 m*M* NaCl. Flow-rate: 1.0 ml/min. Detection: 405 nm with 0.1 a.u.f.s. Yield of P450 in the 100–150 m*M* fraction, 65–75%; in all fractions, 85–95%.

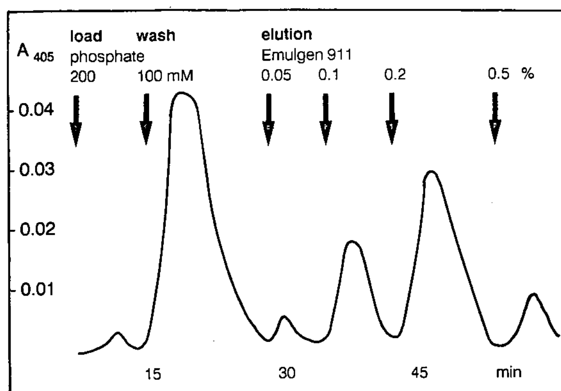


Fig. 8. Hydrophobic interaction chromatography on phenyl-Superose. Sample: 7 nmol of phenobarbital-treated marmoset P450 prefractionated on hydroxyapatite (200 mm$M$ pool). Column: HR 5/5. Load and wash: phosphate (pH 7.2)–20% glycerol. Elution: increasing concentrations of Emulgen 911. Flow-rate: 1.0 ml/min. Detection: 405 nm with 0.05 a.u.f.s. Yield in the 0.1% Emulgen fraction, 20%; in all fractions, 80%.

matographic gel types either alone or in combination had led to homogeneous fractions of marmoset P450, it seemed of interest to exploit differences in hydrophobicity. Starting from the high phosphate and low detergent concentration of a 100–200 mM phosphate gradient on hydroxyapatite (after dilution) or from a Mono S gradient (after detergent removal on hydroxyapatite), phenyl-Superose proved to be capable of further significant separation of this sample. After loading the sample, decreasing the salt concentration to 100 mM phosphate and then applying a gradient of Emulgen 911, several different P450 fractions were obtained (Fig. 8). The 0.1% Emulgen fraction was homogeneous as judged by SDS-PAGE (Fig. 5, lane 5), but did not represent the largest recovery among the different fractions of P450 from this column. The prepacked phenyl-Superose column, which had a bed volume of 1.0 ml, bound at least 30 nmol of P450.

Laboratory-packed phenyl-Sepharose and octyl-Superose columns gave essentially similar chromatographic profiles and SDS-PAGE patterns; separations with these gels, however, took three to four times longer and yielded less sharply defined peaks. The finding that these latter two gels themselves behaved so similarly with regard to the applied sample may indicate that the individual P450 forms fractionated here are not especially hydrophobic. If this were indeed the case, then separations on octyl-Sepharose (in comparison with phenyl-Sepharose) should have resulted in the elution peaks being more retarded. In support of this is the finding that among various membrane proteins, only averagely high LIVM values (percentage of leucine, isoleucine, valine and methionine as an expression of hydrophobicity) were registered for P450 (forms) [20].

Relationships between various results and strategies for column use

In FPLC, as in conventional chromatography, ion exchange is an integral step, applicable at almost any stage of purification, particularly at the onset and where large volumes are to be applied. The Mono Q and Mono S columns used here probably represent the most useful steps, and showed good reproducibility. Hydrophobic interaction was found to complement ion-exchange chromatography, and can also be used as a first step. On the other hand, P450 forms differing mainly in hydrophobicity can be easily separated, for example with phenyl-Superose, in a last step subsequent to hydroxyapatite as after this latter method the salt (phosphate) concentration is already elevated. Gel filtration was found to be much faster for desalting than, say, dialysis but is limited to small sample volumes. Hydroxyapatite was found to be useful for general purification, especially as a last step where non-ionic detergents can be removed and the sample is concentrated.

CONCLUSIONS

The FPLC system utilized here was found to be well suited to the rapid isolation of marmoset monooxygenase components for which little or no chromatographic information was available. Also, the sizes of the columns employed seemed to represent a good choice in that they were small enough to enable a good number of analytical and optimization runs in one day, yet were large enough to yield amounts sufficient for use in organ culture or immunological studies.

In the marmoset, as in many other animal species after induction with different agents, a large number of P450 forms probably also exist, and FPLC may prove useful in accelerating their isolation. To this end, we consider that over and above the tradi-

tional anion- and cation-exchange fractionations, a wider variety of gel types in prepacked and laboratory-packed columns should be exploited.

ACKNOWLEDGEMENTS

These studies were supported financially by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 174) and the Bundesministerium für Technologie (Projekt CMT 35).

REFERENCES

1 J. B. Schenkman and D. Kupfer (Editors), *Hepatic Cytochrome P-450 Monooxygenase System,* Pergamon Press, New York, 1982.
2 F. P. Guengerich, *Pharmacol. Ther.*, 6 (1979) 99–121.
3 D. E. Ryan and W. Levin, *Pharmacol. Ther.*, 45 (1990) 153–239.
4 M. Kastner and T. Schulz, *J. Chromatogr.*, 397 (1987) 153–163.
5 M. Kastner, T. Schulz and D. Neubert, *Toxicol. Lett.*, 45 (1989) 261–270.
6 M. Kastner, G. Blankenburg and T. Schulz, in H. Nau and W. J. Scott, Jr. (Editors), *Pharmacokinetics in Teratogenesis,* Vol. II, CRC Press, Boca Raton, FL, 1987, pp. 145–152.
7 M. Kastner, G. Blankenburg, T. Schulz, G. Schack and D. Neubert, *Arch. Toxicol.*, 61 (1988) 426–432.
8 F. P. Guengerich and M. V. Martin, *Arch. Biochem. Biophys.*, 205 (1980) 365–379.
9 A. L. Mazin, G. E. Sulimova and B. F. Vanyushin, *Anal. Biochem.*, 61 (1974) 62–71.
10 *Ion Exchange Chromatography: Principles and Methods,* Pharmacia, Uppsala, 1983, pp. 40–41.
11 Y. Yasukochi and B. S. S. Masters, *J. Biol. Chem.*, 251 (1976) 5337–5344.
12 K. Ogita, E. Kusunose, K. Ichihara and M. Kusunose, *J. Biochem.*, 92 (1982) 921–928.
13 Y. Imaoka and S. Funae, *Biochim. Biophys. Acta,* 842 (1985) 119–132.
14 T. A. van der Hoeven and M. J. Coon, *J. Biol. Chem.*, 249 (1974) 6302–6310.
15 D. B. Craven, M. J. Harvey, C. R. Lowe and P. D. G. Dean, *Eur. J. Biochem.*, 41 (1974) 329–333.
16 F. P. Guengerich, G. A. Dannan, S. T. Wright, M. V. Martin and L. S. Kaminsky, *Biochemistry,* 21 (1982) 6019–6030.
17 D. Ryan, A. Y. H. Lu and W. Levin, *Methods Enzymol.*, 52 (1978) 117–123.
18 W. L. Backes, I. Jansson, J. E. Mole, G. G. Gibson and J. B. Schenkman, *Pharmacology,* 31 (1985) 155–169.
19 P. H. Roos, *J. Chromatogr.*, 521 (1990) 251–265.
20 G. W. Welling, K. Slopsema and S. Welling-Wester, *J. Chromatogr.*, 397 (1987) 165–174.

END OF SPECIAL ISSUE

PUBLICATION SCHEDULE FOR 1992

Journal of Chromatography and *Journal of Chromatography, Biomedical Applications*

MONTH	O 1991	N 1991	D 1991	J	
Journal of Chromatography	585/1	585/2 586/1 586/2 587/1	587/2 588/1 + 2	589/1 + 2 590/1 590/2	The publication schedule for further issues will be published later
Cumulative Indexes, Vols. 551–600					
Bibliography Section					
Biomedical Applications				573/1 573/2	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 558, pp. 469–472. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Review articles and Short Communications. Short Communications are usually descriptions of short investigations, or they can report minor technical improvements of previously published procedures; they reflect the same quality of research as Full-length papers, but should preferably not exceed five printed pages. For Review articles, see inside front cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he/she is submitting the paper for publication in the *Journal of Chromatography*.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Abstract, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Abstract. All articles should have an abstract of 50–100 words which clearly and briefly indicates what is new, different and significant.

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press" (journal should be specified), "submitted for publication" (journal should be specified), "in preparation" or "personal communication".

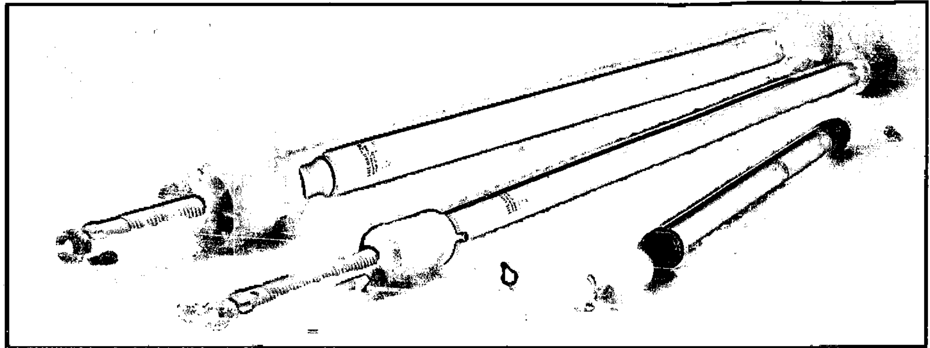
Dispatch. Before sending the manuscript to the Editor please check that the envelope contains four copies of the paper complete with references, legends and figures. One of the sets of figures must be the originals suitable for direct reproduction. Please also ensure that permission to publish has been obtained from your institute.

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers and Short Communications will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

Advertisements. The Editors of the journal accept no responsibility for the contents of the advertisements. Advertisement rates are available on request. Advertising orders and enquiries can be sent to the Advertising Manager, Elsevier Science Publishers B.V., Advertising Department, P.O. Box 211, 1000 AE Amsterdam, Netherlands; courier shipments to: Van de Sande Bakhuyzenstraat 4, 1061 AG Amsterdam, Netherlands; Tel. (+31-20) 515 3220/515 3222, Telefax (+31-20) 6833 041, Telex 16479 els vi nl. *UK*: T. G. Scott & Son Ltd., Tim Blake, Portland House, 21 Narborough Road, Cosby, Leics. LE9 5TA, UK; Tel. (+44-533) 753 333, Telefax (+44-533) 750 522. *USA and Canada*: Weston Media Associates, Daniel S. Lipner, P.O. Box 1110, Greens Farms, CT 06436-1110, USA; Tel. (+1-203) 261 2500, Telefax (+1-203) 261 0101.

P H A R M A C I A · L K B · B I O T E C H N O L O G Y



Superdex 75 HR 10/30

Gel medium:	Superdex 75 (13 µm mean bead diameter)
Bed dimensions:	10 mm i.d., 30 cm height
Sample:	25 µl Fab (approx. 3 mg/ml)
System:	FPLC
Buffer:	PBS, pH 7.4
Flow rate:	1.5 ml/min (110 cm/h)
Run time:	Approx. 15 min

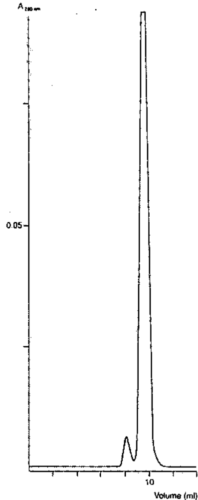


Fig. 1. Analysis of dimer content in a Fab preparation (~50 kDa). (Kindly supplied by David L. Foster, Senior Research Scientist, Immunotherapeutic Development, Corvas Inc., San Diego, CA, USA.)

HiLoad 16/60 Superdex 75 prep grade

Gel medium:	Superdex 75 prep grade (34 µm mean bead diameter)
Bed dimensions:	16 mm i.d., 60 cm height
Sample:	1 ml partially purified ZZ-Brain IGF (fusion protein) solution
Buffer:	0.3 M ammonium acetate, pH 6.0
Flow rate:	0.5 ml/min (15 cm/h)

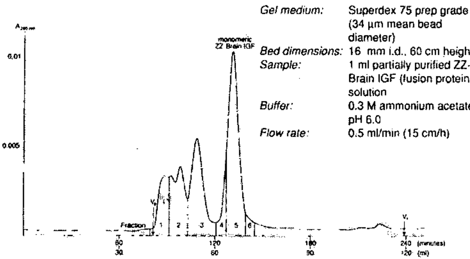


Fig. 2. Separation of monomeric ZZ-Brain IGF (22 kDa) from dimers and multimers in a fusion protein solution.

HiLoad 26/60 Superdex 200 prep grade

Gel medium:	Superdex 200 prep grade (34 µm mean bead diameter)
Bed dimensions:	26 mm i.d., 60 cm height
Sample:	18 ml culture supernatant containing mouse IgG_1 anti-gp 120, 0.61 mg/ml
System:	BioPilot
Buffer:	Sterile PBS
Flow rate:	2.65 ml/min (30 cm/h)

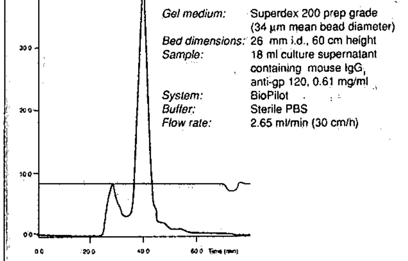


Fig. 3. Purification of mouse monoclonal IgG_1, anti-gp 120 for intravenous use in clinical studies on the clinical indication AIDS. (Purification developed in collaboration with B. Wahren et al. of the National Bacteriological Laboratory (SBL), Dept. of Virology, Sweden.)

Superdex™ gel filtration columns - outstanding resolution whatever your system or application

Outstanding resolution in gel filtration is within easy reach. Just pick up a pre-packed column of Superdex.

It doesn't matter whether you run FPLC®, HPLC, or even standard LC. If you're doing liquid chromatography of biomolecules, the steep selectivity and high physicochemical stability of the dextran/agarose composite matrix hand you the best gel filtration performance available today - straight from the box.

Check which Superdex column is best for you.

• Superdex 75 HR 10/30

This high performance column, which separates in the mol. wt. range 3 000-70 000 daltons, combines high resolution with high flow rates. Within minutes, Superdex 75 HR 10/30 separates monomeric and dimeric forms of proteins and peptides - usually down to the baseline. In the analysis shown in Fig. 1, the dimer and monomer eluted in 10 minutes.

• HiLoad™ Superdex 75 prep grade

The prep grade version of Superdex 75 brings high resolution to preparative purification and process development. With the same selectivity as Superdex 75 HR 10/30, pre-packed HiLoad Superdex 75 prep grade separates proteins and peptides from dimers, multimers, and other contaminants (Fig. 2).

• HiLoad Superdex 200 prep grade

This column shares all the characteristics of HiLoad Superdex 75 prep grade, but selects in the mol. wt. range 10 000-600 000 daltons. It fractionates best between 30 000 and 250 000, separating monoclonal antibodies from critical contaminants and aggregates, often down to the baseline. As the final step in a purification, HiLoad Superdex 200 prep grade gives well-resolved peaks and very low levels of contaminants (Fig. 3).

Test the outstanding resolution of pre-packed Superdex gel filtration columns - there's one for your application. Ask for more details.

Pharmacia LKB Biotechnology

Head office Sweden Tel 46 (0)18 16 30 00 **Australia** Tel 61 (0)2 888 36 22 **Austria** Tel (0222) 68 66 25 0 **Belgium** Tel (02) 242 46 60 **Brazil** Tel 011-288 9122 **Canada** Tel (514) 457 6661 **Denmark** Tel 48 14 10 00 **Federal Republic of Germany** Tel 49 (0)761 490 30 **Finland** Tel (90) 5021 077 **France** Tel 1-30 64 34 00 **Great Britain** Tel (0908) 661101 **Hong Kong** Tel 852 814 84 21 **Holland** Tel 03480 77911 **India** Tel (0812) 269631 **Italy** Tel 02 273221 **Japan** Tel (03) 3492-9488 **Norway** Tel (02) 18 10 00 **People's Republic of China** Tel 861 256 5603, Ext.1202/1204 **Portugal** 417 24 72 **Republic of Korea** Tel 02 5110801 **Spain** Tel 582 17 17 **Sweden** Tel (08) 623 85 00 **Switzerland** Tel (01) 821 18 16 **Taiwan** (02) 831 60 21 **United States** Tel (908) 457 8000 **Eastern Europe** Tel 43 (222) 982 38 26 **Far East** Tel 852 814 84 21 **Middle East** Tel +30 -1 96 27 396 **Other countries** Tel 46 (0)18 16 30 00 (9105)