Trypsin Purification by *p*-Aminobenzamidine Immobilized on Macroporous Chitosan Membranes

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Membranes with suitable mechanical properties, chemical stability, and macroporosity which have a selectivity for trypsin were prepared by coupling a ligand (*p*-aminobenzamidine) to cross-linked chitosan membranes via a spacer (succinic acid). The chitosan membranes were cross-linked with ethylene glycol diglycidyl ether in order to avoid their dissolution in acidic media. The cross-linked chitosan membranes were then covalently coupled with succinic anhydride, at ambient temperature, to obtain hydrophilic spacers with carboxyl terminals. The amino groups of the chitosan membranes which have not reacted with the succinic anhydride were blocked with acetic anhydride to avoid the nonspecific interactions. Finally the ligand *p*-aminobenzamidine (PAB) was incorporated via the coupling between the carboxyl groups of the succinic acid and the amino groups of PAB, using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC) as the activating agent. A PAB density of 2.7×10^{-3} mol/g of chitosan was achieved. Since the PAB−chitosan membranes have a much higher affinity toward trypsin than toward chymotrypsin, they have been employed for the purification of crude trypsin solutions. A purification factor > 7, a specific activity > 9500 BAEE units/mg, and activity yields between 60 and 80% were obtained. These results suggest that the PAB−chitosan affinity membrane can be efficient for trypsin purification.

Introduction

Affinity membrane chromatography has attracted attention in the last years (Brandt et al., 1988; Huang et al., 1988; Gerstner et al., 1992; Suen and Etzel, 1992; Suen et al., 1993; Liu and Fried, 1994; Serafica et al., 1994; Klein et al., 1994a,b; Thömmes and Kula,1995; Roper and Lightfoot, 1995; Charcosset et al., 1995; Schisla et al., 1995; Zeng and Ruckenstein, 1996a,b; Ruckenstein and Zeng, 1997a,b). Compared to column chromatography, membrane chromatography brings solute into close proximity to bound ligands through convective transport. The resulting reduction in mass transport resistance enables processing advantages, such as lower pressure drops and higher flow rates. Different kinds of biospecific ligands [protein A or G (Klein et al., 1994a,b; Charcosset et al., 1995; Kochan et al., 1996), trypsin inhibitors (Carter and Howell, 1987; Huang et al., 1988)] as well as group-specific ligands [dyes (Liu and Fried, 1994; Schisla et al., 1995; Zeng and Ruckenstein, 1996a; Denizli et al., 1997) and metal chelates (Iwata et al., 1991; Serafica et al., 1994; Rodemann and Staude, 1994)] have been incorporated in micro- or macroporous membranes [cellulose (Manganaro and Goldberg, 1993), polyethylene (Iwata et al., 1991), polysulfone (Klein et al., 1994a,b), poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) (Tennikova et al., 1991), poly(ethylene-*co*-vinyl alcohol) (Bueno et al., 1995), chitosan (Zeng and Ruckenstein, 1996a), and poly(2-hydroxyethyl methacrylate) (Denizli et al., 1997).

Suitable materials for affinity membranes should be hydrophilic, should have low nonspecific adsorption, a large number of reactive groups available for ligand coupling, chemical stability, and good mechanical properties, and should allow the easy formation of a micro- or a macroporous structure. In view of these criteria, chitosan and chitin are expected to be most suitable materials. Recently, macroporous chitin and chitosan membranes with controlled porosity and good mechanical properties have been successfully prepared using a novel method (Zeng and Ruckenstein, 1996b; Ruckenstein and Zeng, 1997a). The method consisted of casting an acidic chitosan solution that contained silica particles of selected size, removing the solvent by evaporation, and dissolving the silica particles by immersing the membranes into alkaline solutions (chitosan is soluble in acidic solutions and insoluble in the alkaline ones). Chitosan and chitin membranes are hydrophilic, highly reactive chemically (since they contain a large number of OH and/or NH_2 groups), and chemically resistant (chitin and cross-linked chitosan are insoluble in both acidic and basic solutions). The macroporous chitin membranes contain a ligand (*N*-acetyl-D-glucosamine) with a high affinity for lysozyme; for this reason, they were employed for the affinity separation of lysozyme from egg white. High purity (>98%) and high adsorption capacity (50 mg/ml membrane) were achieved (Ruckenstein and Zeng, 1997b).

Trypsin is a digestive enzyme whose recovery and purification from the pancreas has industrial significance. While it has a different enzymatic function, its structure, molecular weight, and other properties are very similar to those of chymotrypsin. Therefore, the separation of trypsin constitutes a difficult process. Several methods have been developed for trypsin separation, such as the following: (1) Affinity column chromatography (Feinstein, 1970a,b; Robinson et al., 1971; Hixson and Nishikawa, 1973; Jameson and Elmore, 1974) in which trypsin inhibitors [soybean trypsin inhibitor (STI), ovomucoid, and aminobenzamidine] were covalently coupled to various kinds of beads (dextran, agarose, sepharose, cellulose, poly(acryla-

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mide), and polystyrene). However, this method is laborious, time consuming due to the low flow rate, and susceptible to fouling and plugging. (2) The affinity precipitation which employed alginate as the carrier and STI as the ligand (Linné et al., 1992). (3) The affinity ultrafiltration technique, which combined the high-volume processing capacity of the ultrafiltration membrane with the high selectivity of the affinity carrier and could operate continuously. STI or *m*- or *p*-aminobenzamidine coupled to beads of dextran, water-soluble polymers, or liposomes (Adamski-Medda et al., 1981; Choe et al., 1986; Male et al., 1987; Luong et al., 1988a,b; Powers et al., 1990) were used as affinity carriers. (4) Affinity membrane chromatography in which the ligand *p*-aminobenzamidine was covalently coupled to the internal pores of a Sartorius SM 16278 membrane (Carter and Howell, 1987). *p*-Aminobenzamidine (PAB) can serve as a ligand for trypsin and has been widely used for the purification of trypsin and trypsin-like enzymes (such as urokinases, plasminogen activators, and serine proteases). The trypsin commercially available is frequently contaminated with its autolytic products, which must be removed before it is used in analytical assays.

So far chitosan and chitin powders or beads have been employed in the food, medicine, chemical, and pharmaceutical industries (Zikakis, 1984; Muzzarelli et al., 1986). For instance, β-galactosidase (Carrara and Rubiolo, 1994), lipoprotein lipase (Itoyama et al., 1994), and pepsin (González and Alea, 1995) were immobilized on chitosan beads. However, the chitosan membranes have rarely been used for protein separation (Ruckenstein and Zeng, 1997b). In this paper we report about a new kind of trypsin affinity membrane in which macroporous chitosan membranes are used as supports and PAB is used as a trypsin ligand. The succinic anhydride was first coupled to the chitosan membrane to obtain succinic carboxyl groups as spacers. The succinylated chitosan membranes were then coupled with PAB, using 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDAC) as the activating agent. Finally, the PAB-containing membranes were employed for the purification of trypsin from crude trypsin solutions.

Experimental Section

Materials. *Chemicals and Buffers.* Chitosan (molecular weight 400 000) and sodium hydroxide were obtained from Fluka. Trypsin (type IX), α-chymotrypsin (type II), crude trypsin (type II-S, from porcine pancreas), Trizma Base (SigmaUltra), calcium chloride dihydrate (SigmaUltra), sodium chloride (SigmaUltra), 2-*N*-morpholinoethanesulfonic acid (MES), sodium azide (SigmaUltra), *p*-aminobenzamidine dihydrochloride, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (SigmaUltra), and succinic anhydride were purchased from Sigma. Methanol, ethylene glycol diglycidyl ether, and sodium borohydride were obtained from Aldrich. A 0.05 M Tris solution (pH 8), containing 0.02 M $CaCl_2$, 0.1 M NaCl, and 0.02 wt % NaN_3, was used as the binding buffer for trypsin and chymotrypsin. The eluant for both proteins was a 0.1 M aqueous acetic acid solution, containing 0.02 M $CaCl_2$ and 0.02 wt % NaN_3. Both the buffer and eluant were filtered through a 0.2 μm membrane (Supor-200, Gelman Science, Inc.) prior to their use.

Methods. *1. Immobilization of p-Aminobenzamidine on Macroporous Chitosan Membranes.* Macroporous chitosan membranes were first prepared using the method developed in this laboratory (Zeng and Ruckenstein, 1996b; Ruckenstein and Zeng, 1997a). *p*-Aminobenzamidine was immobilized on the membranes as follows (Figure 1): First, the chitosan membranes were cross-linked at 50 °C, using a mixture containing 5 vol % ethylene glycol diglycidyl ether and a 95 vol % aqueous solution of 1 wt % NaOH and 0.2 wt % $NaBH_4$. This was followed by washing with water and methanol. Carboxyl groups were then incorporated in the cross-linked chitosan membranes by immersing the membranes in 1 wt % succinic anhydride in methanol, at room temperature for 16 h. The unreacted amino groups were blocked to avoid nonspecific interactions, using 5 vol % acetic anhydride in methanol at 50 °C for 2 h. The residual acetic anhydride and succinic anhydride were removed by immersing the membranes in 1 N NaOH for 1 h. Finally, PAB was coupled to the succinylated chitosan membranes, using an excess amount of activating agent (EDAC), in a 0.1 M MES aqueous solution (pH 4.75), at room temperature for 16 h.

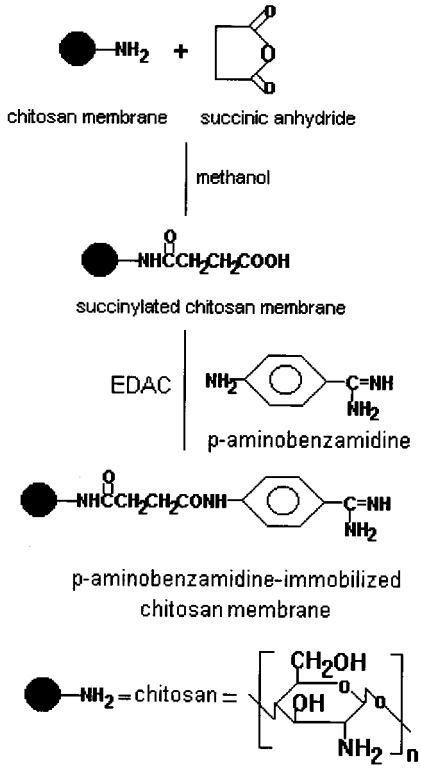

Figure 1. Scheme of the covalent coupling of *p*-aminobenzamidine to macroporous chitosan membranes.

The carboxyl density can be calculated using the expression:

$$\text{carboxyl density (mol/g of chitosan)} = \frac{(w_2 - w_1)/100}{w_1}$$

where w_1 and w_2 are the weights of the chitosan membrane before and after the introduction of succinic acid, respectively, and 100 is the molecular weight of the succinic acid.

The density of PAB was calculated using the expression:

$$\text{PAB density (mol/g of chitosan)} = \frac{(w_4 - w_3)/117}{w_1}$$

where w_3 and w_4 are the weights of the membranes before and after the coupling of PAB, respectively, and 117 is the molecular weight of PAB minus the weight of a molecule of water.

Ten PAB−chitosan flat membranes (total 1 g, 3 mm high, 4.1 mL volume) were sandwiched in a cartridge, identical with that used in a previous paper (Ruckenstein and Zeng, 1997b), which has four inlet ports, one bubble relief port, one outlet port, and two flow distributors. A gasket was inserted between two succesive membranes to avoid leakage. The membranes were compressed with an O-ring to seal the fluids. This was followed by successive washing with large volumes of ethanol, water, MES buffer, water, a 1 N NaCl aqueous solution, water, Tris buffer, and 0.1 M acetic acid to remove any residuals. The protein solution, buffer, and eluant were loaded to the cartridge with a peristaltic pump (Masterflex, Model 7520−00; Cole-Parmer Instrument Co.).

The permeability of the cartridge was determined by forcing via pressurized nitrogen the Tris buffer to flow through the cartridge, which was connected to a pressure gauge.

2. Protein Determination. The protein concentration was assayed spectrophotometrically with Bradford's method (1976) at 595 nm, using the Coomassie plus protein assay reagent (Pierce) and trypsin as standard.

3. Assay of the Trypsin Activity. The trypsin activity was determined with Schwert and Takenaka's method (1955), using N^{α}-benzoyl-L-arginine ethyl ester (BAEE) as the substrate, at pH 7.6 and 25 °C. The spectrophotometrical measurements were carried out at 253 nm with a UV/vis spectrophotometer (DU-650; Beckman Instruments Inc.) using a 1 cm light path cell. A 0.2 mL enzyme solution was added to a 3.0 mL aqueous solution containing 0.5 mM BAEE, 50 mM Tris buffer, and 10 mM $CaCl_2$, at pH 7.6, followed by immediate mixing by inversion. The initial slope, $\Delta A_{253}/\Delta t$, where ΔA_{253} is the increase of A_{253} (absorbance at 253 nm) and the time Δt is expressed in minutes, was determined from experiment. One BAEE unit of trypsin is defined as the increase of A_{253} by 0.001/min in a 3.2 mL mixture at pH 7.6 and 25 °C. The number of BAEE units of trypsin can be calculated using the expression:

$$\text{BAEE units/mL of enzyme} = \frac{[(\Delta A_{253}/\text{min})\ \text{test} - (\Delta A_{253}/\text{min})\ \text{blank}](d_f)}{(0.001)(0.2)}$$

where d_f is the dilution factor, 0.001 is the change in A_{253}/min for one trypsin unit, and 0.2 is mL of enzyme (trypsin) used.

The specific activity is defined as

$$\text{specific activity} = \text{units/mg of protein} = \frac{\text{units/mL of enzyme}}{\text{mg of protein/mL of enzyme}}$$

4. Specific and Nonspecific Adsorption by PAB−Chitosan Membranes of Trypsin and Chymotrypsin. A trypsin or chymotrypsin solution (1 mg/mL) was loaded to the PAB−chitosan cartridge at a flow rate of 5 mL/min until saturation was reached. The Tris buffer was then employed to remove the weakly adsorbed protein from the cartridge at a flow rate of 10 mL/min. Finally, the strong bound protein was eluted with a 0.1 M acetic acid aqueous solution at a flow rate of 5 mL/min, the effluent collected, and the protein concentration determined.

In order to investigate the nonspecific adsorption of trypsin or chymotrypsin by succinylated chitosan membranes, the above experiment was performed using the succinylated chitosan membrane instead of the PAB−chitosan membrane.

5. Adsorption Isotherm of Trypsin on PAB−Chitosan Membranes. The adsorption isotherm of trypsin was determined in batch experiments. PAB−chitosan membranes were cut into small pieces and introduced in test tubes containing 10 mL trypsin solutions of different initial concentrations. These suspensions were gently shaken at room temperature for at least 5 h to approach saturation. The concentrations of the supernatants were determined by the Bradford method. The membrane adsorption capacities were obtained from the mass balance and the amount adsorbed per gram of chitosan plotted against the final concentration in solution.

6. Effect of the Flow Rate on the Elution Profiles. A total of 20 mL of a 0.1 mg/mL trypsin solution was loaded at 2 mL/min, followed by washing with Tris buffer at 2 mL/min until the absorbance returned to the baseline. Then a 0.1 M acetic acid aqueous solution was passed through the cartridge at flow rates of 2, 5, and 10 mL/min. The elution profiles were recorded, and the amount of eluted trypsin was determined.

7. Comparison of Eluted Trypsin at Different Loadings and Concentrations. In order to investigate the effect of the loading of trypsin on the yield (defined as the ratio of eluted trypsin to loaded trypsin), 1, 2, 4, 6, and 8 mL of a 1 mg/mL trypsin solution were loaded at a flow rate of 0.5 mL/min, followed by washing with Tris buffer at 2 mL/min until the absorbance returned to the baseline and elution with 0.1 M acetic acid at a flow rate of 5 mL/min. The eluted effluent was collected, and the concentration was determined.

The effect of trypsin concentration on the yield was also investigated by loading 2 mg of trypsin of different concentrations (0.1, 0.25, 0.5, and 1 mg/mL) into the cartridge. The other conditions were the same as above.

8. Purification of Trypsin from Crude Trypsin Solutions. A crude trypsin solution was prepared by dissolving crude trypsin powders (1300 BAEE units/mg of solid) in Tris buffer. This solution was loaded to the cartridge at a flow rate of 0.5 mL/min, washed with Tris buffer at 2 mL/min until the absorbance returned to the baseline, and eluted with 0.1 M acetic acid at 5 mL/min. Fractions of 2 mL were collected with a Retriever II 500 fraction collector (ISCO Inc.), and the concentrations and activities were determined.

The stability of the PAB−chitosan membrane was investigated by performing four cycles of adsorption/washing/elution/regeneration. A total of 5 mL of a 5 mg/mL crude trypsin solution was loaded at 0.5 mL/min, washed with 0.05 M Tris buffer at 2 mL/min until the absorbance returned to the baseline, and eluted with 0.1 M acetic acid at 5 mL/min. In order to remove any traces of proteins, the membrane was washed after each cycle with a 1 M NaCl solution and regenerated with Tris buffer.

Table 1. Characteristics of the PAB−Chitosan Membrane Cartridge

membrane material	chitosan
configuration	a stack of 10 flat membranes
average pore size of the chitosan membrane in a dry state (μm)	18
porosity of the chitosan membrane in a wet state (distilled water) (%)	62
total thickness (mm)	3
effective membrane area (cm^2)	13.8
membrane volume (cm^3)	4.1
specific surface area of the chitosan membrane[a] (m^2/g)	1.6
density of succinic acid coupled to chitosan (mol/g of chitosan)	3.2×10^{-3}
density of *p*-aminobenzamidine on chitosan (mol/g of chitosan)	2.7×10^{-3}
hydraulic permeability of 0.05 M tris buffer (cm/min/psi)	0.35

[a] Measured by the BET method.

Results and Discussion

1. PAB−Chitosan Membrane Characteristics. Since the chitosan molecules contain reactive amino groups, the carboxyl groups of succinic anhydride can be easily coupled to them, even at room temperature. The coupling of the succinic acid groups to the chitosan membrane increases, however, its hydrophilicity, hence the swelling; the system becomes even soluble in water at high degrees of substitution. To avoid this swelling, the chitosan membranes were cross-linked with ethylene glycol diglycidyl ether before succinylation. After succinylation, the remaining amino groups (which have not reacted with the succinic anhydride) were blocked with acetic anhydride (by converting NH_2 to $NHCOCH_3$) in order to eliminate the possible nonspecific interactions between the $-NH_2$ groups and proteins. The succinic acid groups on the membrane surface not only serve as reactive groups for coupling but also constitute spacer arms which alleviate the hindrance to the adsorption of the protein molecules if the PAB should have been located directly on the chitosan surface. The PAB density on the membranes is an important parameter in protein adsorption. The higher the density, the larger the adsorption capacity. However, very high densities of PAB can lead to multipoint adsorption of trypsin and thus to a decrease of the specificity of the ligand. In addition, a high density of PAB makes the membrane brittle. For this reason, in this paper, the density of PAB was at most 2.7×10^{-3} mol of PAB/g of chitosan. At about 5×10^{-3} mol of PAB/g of chitosan the membrane became brittle. One can evaluate the coupling efficiency of PAB to the carboxyl groups by dividing the density of PAB to that of succinic acid. The data in Table 1 lead to a coupling efficiency of 85%, which indicates that most of the negatively charged carboxyl groups have been coupled.

The characteristics of PAB-chitosan membranes are listed in Table 1. Figure 2 presents the relationship between the pressure drop and the flow rate of the Tris buffer solution through 10 PAB−chitosan membranes stacked together and shows that high flow rates can be achieved at relatively low pressure drops. The relationship is almost linear for pressure drops below 10 psi, which indicates that the membranes are almost incompressible.

In order to investigate if the distribution of protein solution is uniform, the cartridge was first saturated with buffer, and then a trypsin solution in the eluant (1 mg/mL) was loaded to the cartridge. The breakthrough curve is presented in Figure 3, which shows that the first protein peak appears when the effluent volume reaches about 3 mL and then rapidly approaches saturation. The dimensionless concentration C/C_0 was about 0.8, 0.95, and 1 at effluent volumes of 4, 6, and 12 mL, respectively. Consequently, the void volume of the cartridge can be estimated to be about 3−4 mL, and the flow pattern can be considered to be approximately a plug flow.

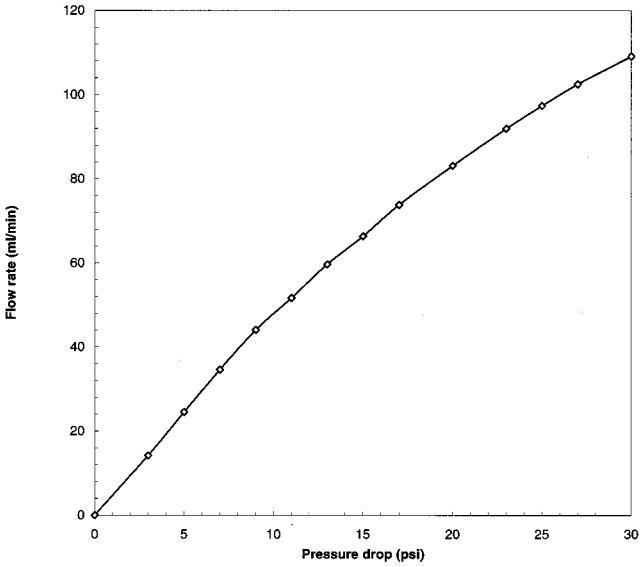

Figure 2. Relation between flow rates (0.05 M Tris buffer, containing 0.1 N NaCl and 0.02 M $CaCl_2$, pH 8) and pressure drops for a *p*-aminobenzamidine−chitosan membrane cartridge (3 mm thick, 13.8 cm^2 surface area).

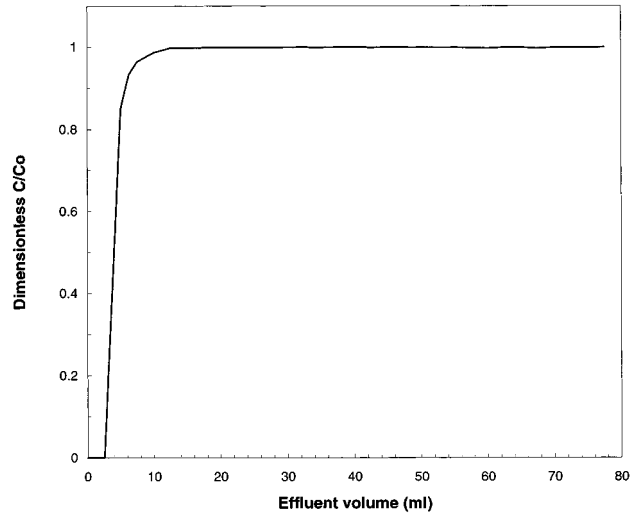

Figure 3. Dispersion of a PAB−chitosan membrane cartridge filled initially with buffer, using 1 mg/ml trypsin dissolved in a 0.1 M acetic acid aqueous solution (eluant) at a flow rate of 5 mL/min.

2. The Affinity of PAB−Chitosan Membrane for Trypsin or Chymotrypsin. Parts a and b of Figure 4 present the breakthrough curves for trypsin and chymotrypsin (1 mg/mL protein solution at a loading flow rate of 5 mL/min), respectively. A total of 36.4 mg of trypsin and only 6.1 mg of chymotryspin are eluted by the acetic acid solution from the membranes. This indicates that the PAB−chitosan membranes have a higher affinity for trypsin than chymotrypsin and that

Table 2. Purification of Trypsin by PAB−Chitosan Membranes from a Crude Trypsin Solution

crude solution					purified solution				
conc. (mg of crude/mL)	vol. (mL)	total crude (mg)	total activity (units)	units/mg of crude	product (mg)	total activity (units)	units/mg of product	activity yield (%)	purification factor
1	10	10	13 000	1300	1.09	10 514	9646	80.9	7.4
5	5	25	32 500	1300	2.08	20 678	10340	63.6	8.0
10	3	30	39 000	1300	2.39	23 306	9751	59.8	7.5

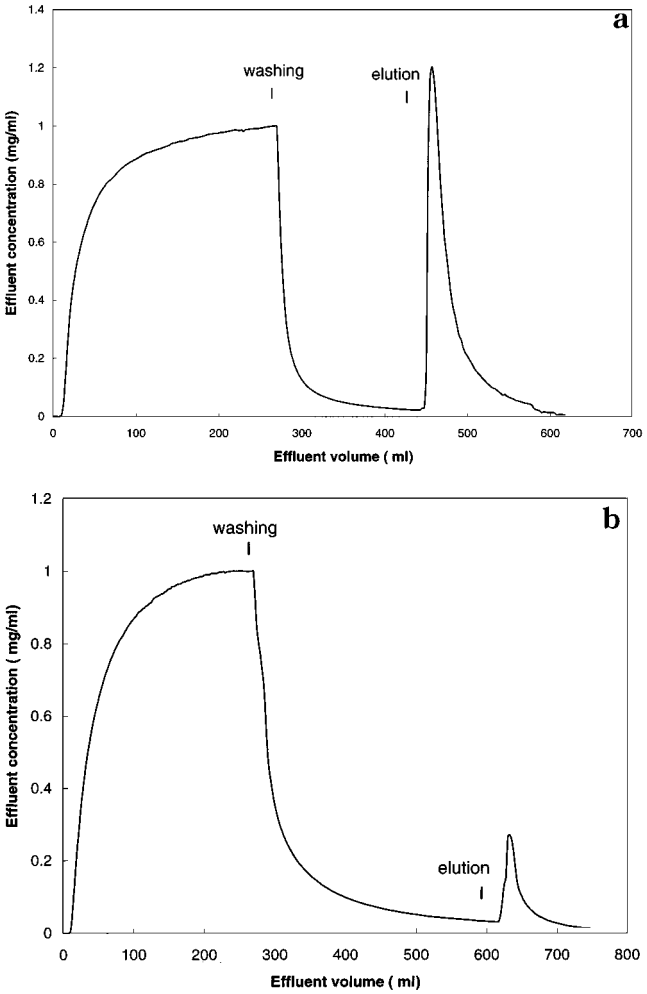

Figure 4. Breakthough curves for a trypsin or chymotrypsin solution of 1 mg/mL: (a) trypsin; (b) chymotrypsin. Flow rate: 5 mL/min for loading; 10 mL/min for washing; 5 mL/min for elution.

Table 3. Stability of PAB−Chitosan Membranes for Purification of Trypsin from a Crude Trypsin Solution (5 mL of 5 mg of crude/mL, 1300 units/mg of crude)

	crude trypsin loaded (mg)	trypsin eluted (mg)	total activity (units)	specific activity (units/mg)	activity yield (%)	purification factor
1.	25	2.40	23 414	9 756	72.0	7.5
2.	25	2.20	21 001	9 546	64.6	7.3
3.	25	2.31	23 196	10 085	71.4	7.8
4.	25	2.08	20 678	10 340	63.6	8.0

a high purification of trypsin from a crude trypsin solution can be achieved. Of course, the interactions between the other impurities in the crude trypsin and PAB can decrease the extent of purification. The nonspecific adsorption by the uncoupled succinylated chitosan for trypsin or chymotrypsin was also studied, but only 0.9 mg of trypsin and 0.4 mg of chymotrypsin could be eluted from the membranes, which represent only 3 and 6% of the amounts of trypsin and chymotrypsin eluted from the PAB−chitosan membranes. This

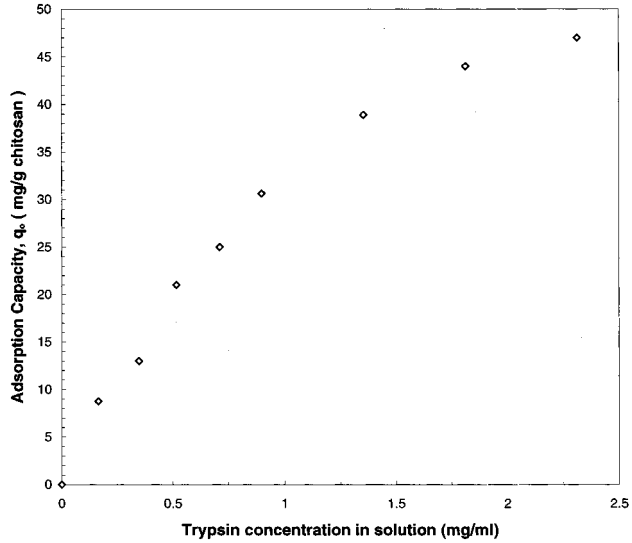

Figure 5. Adsorption isotherm of trypsin on PAB−chitosan membranes at 20 °C.

indicates that the negatively charged succinic acid groups uncoupled to the PAB−chitosan membranes have a small contribution to the protein adsorption.

3. Adsorption Isotherm. The adsorption isotherm of trypsin on the PAB−chitosan membranes was determined in batch experiments, and the data are presented in Figure 5 and fitted to a Langmuir isotherm. By nonlinear least-squares regression of the Langmuir equation

$$q = \frac{q_m C}{K_d + C}$$

one obtains, for the maximum adsorption capacity, the value $q_m = 78.4 \pm 4.2$ mg/g of chitosan and, for the constant K_d, the value $K_d = 1.46 \pm 0.15$ mg/mL solution. This maximum adsorption capacity is much greater than that of a PAB− Sartorius SM 16278 membrane (16 mg/g) (Carter and Howell, 1987).

4. Elution of Trypsin from PAB−Chitosan Membranes at Different Flow Rates. Figure 6 presents the elution profiles of trypsin from PAB−chitosan membranes at three different elution flow rates (2, 5, and 10 mL/min). A total of 2 mg of a trypsin solution (0.1 mg/mL) was loaded at 2 mL/min for each flow rate. The highest effluent concentrations are 0.19, 0.16, and 0.12 mg/mL for elution flow rates of 2, 5, and 10 mL/min, respectively. The sharpest elution peak occurs for the lowest elution flow rate (2 mL/min), and the broadest peak, for the fastest elution flow rate (10 mL/min). By integrating the area under the elution peak, the eluted trypsin was estimated to be 1.72 ± 0.02 mg. One can also evaluate the eluant volume (or time) needed for a 95% recovery of trypsin from the membranes; one obtains 24 (12), 29 (5.8), and 33 (3.3) mL(min) for elution flow rates of 2, 5, and 10 mL/min, respectively.

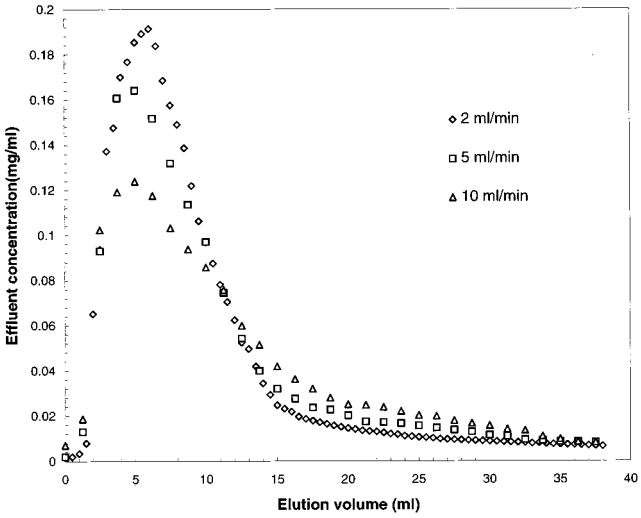

Figure 6. Elution of trypsin from PAB−chitosan membranes with a 0.1 M acetic acid aqueous solution at three flow rates. 20 mL of 0.1 mg/mL trypsin loaded at 2 mL/min; washed with buffer at 2 mL/min.

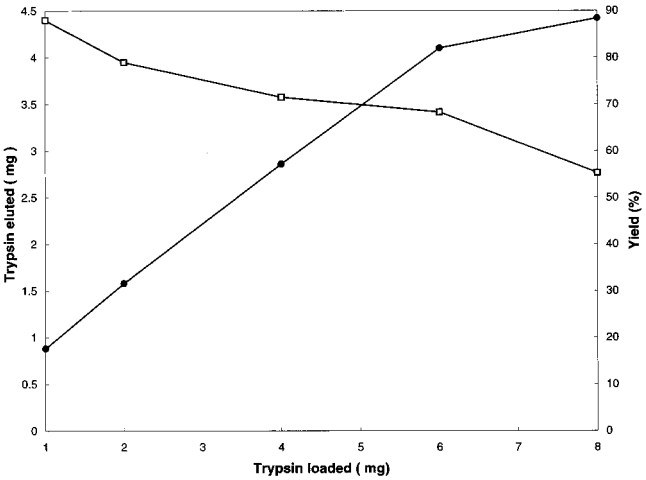

Figure 7. Relationship between eluted trypsin and yield versus the loading amount of a pure trypsin solution (1 mg/mL) at a flow rate of 0.5 mL/min. Washing with buffer at 2 mL/min and elution at 5 mL/min. (●) Trypsin eluted. (□) Trypsin yield.

5. Adsorption Capacity of PAB−Chitosan Membranes for a Pure Trypsin Solution under Various Conditions. Figure 7 presents the relationship between the amount of trypsin eluted from the membranes and the amount of trypsin loaded at a flow rate of 0.5 mL/min. While the amount of eluted trypsin increases, the yield of trypsin decreases with increasing loading. For a loading below 4 mg, the yield is above 70%. For higher loadings (larger than 8 mg), the yield is below 60%.

Figure 8 presents the effect of the concentration of a trypsin solution on the yield. A total of 2 mg of trypsin was loaded at 0.5 mL/min for each concentration. High yield (about 80%) is achieved, even for a dilute solution. This suggests that the PAB−chitosan membrane can recover trypsin from a large volume of a dilute solution.

6. Purification of Trypsin from a Crude Trypsin Solution. Crude trypsin contains a large amount of impurities (71.2 wt % impurities; information provided by Sigma). Figure 9 presents the purification of trypsin by PAB−chitosan membranes from a crude trypsin solution (5 mL of 5 mg of solid/mL, total protein 7.2 mg). One can see from this figure that the first 17 fractions do not exhibit trypsin BAEE activity. They may contain

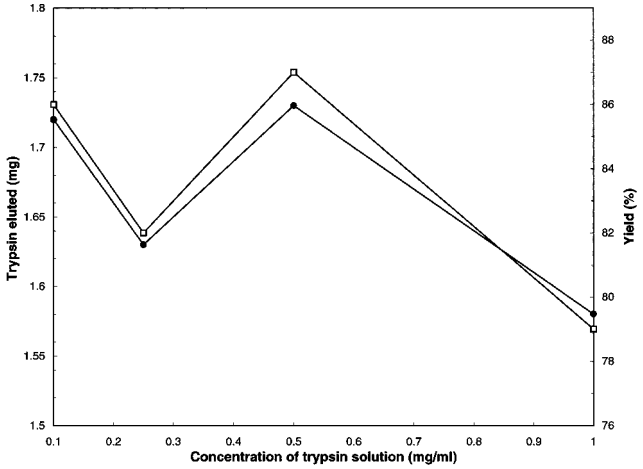

Figure 8. Relationship between the eluted trypsin and yield versus the concentration of a trypsin solution. Total trypsin loading: 2 mg at a flow rate of 0.5 mL/min; washing at 2 mL/min; elution at 5 mL/min. (●) Trypsin eluted. (□) Trypsin yield.

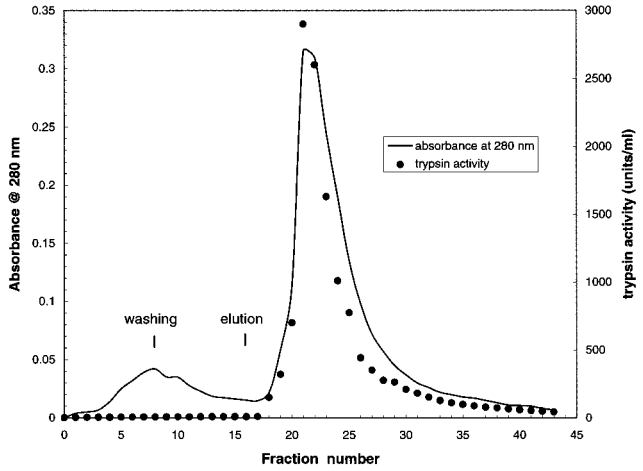

Figure 9. Purification of trypsin from a crude trypsin solution by a PAB−chitosan membrane cartridge. A total of 5 mL of a 5 mg/mL crude trypsin solution (6500 BAEE units/mL) was loaded at 0.5 mL/min, followed by washing with 0.05 Tris buffer at 2 mL/min and elution with 0.1 M acetic acid at 5 mL/min. Size of the effluent fraction: 2 mL.

digested trypsin, chymotrypsin, and other impurities. However, when the eluant was loaded, elution peaks with trypsin BAEE activity appeared. High trypsin activities were exhibited by the fractions 20−26. By mixing the fractions 20−43, the total product was determined to be about 2.1 mg, with the high specific activity of 10 340 BAEE units/mg. The purification factor, defined as the ratio between the activity/mg of product and initial activity/mg of crude trypsin, was about 8, indicating an advanced purification of trypsin from crude trypsin.

Table 2 lists the results of purification of trypsin from crude solutions under different loading conditions. High specific activities (>9500 BAEE units/mg) were obtained, and advanced purification was achieved (purification factor > 7) for these three cases.

The stability of PAB−chitosan membranes for trypsin purification was also investigated. Four cycles of adsorption/washing/elution/regeneration under identical conditions were performed. One can see From Table 3 that the PAB−chitosan membranes exhibit stability for the trypsin purification from crude solutions and that the purification factor remains high.

Conclusions

Trypsin adsorbents have been prepared, using macroporous chitosan membranes as the matrix and *p*-aminobenzamidines as trypsin ligands. The obtained affinity membranes have high permeability, good mechanical properties, chemical stability, and high ligand density. These membranes allow an advanced purification of trypsin from crude trypsin solutions.

Literature Cited

Adamski-Medda, D.; Nguyen, W. T.; Dellacherie, E. Biospecific ultrafiltration: A promising purification technique for proteins? *J. Membr. Sci.* **1981**, *9*, 337−342.

Bradford, M. A rapid and sensitive method for the quantitation of microgram of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Brandt, S.; Goffe, R.; Kessler, S.; O'Connor, J.; Zale, S. Membrane-based affinity technology for commercial scale purifications. *Bio/Technology* **1988**, *6*, 779−782.

Bueno, S. M. A.; Haupt, K.; Vijayalakshmi, M. A. In vitro removal of human IgG by pseudobiospecific affinity membrane filtration on a large scale. A preliminary report. *Int. J. Artif. Organs* **1995**, *18*, 392−398.

Carrara, C. R.; Rubiolo, A. C. Immobilization of β-galactosidase on chitosan. *Biotechnol. Prog.* **1994**, *10*, 220−224.

Carter, A.; Howell, J. A. Adsorption of commercially prepared trypsin using a membrane support material. *Biotechnol. Tech.* **1987**, *1*, 115−116.

Charcosset, C.; Su, Z. G.; Karoor, S.; Daun, G.; Colton, C. K. Protein A immunoaffinity hollow fiber membranes for immunoglobulin G purification: experimental characterization. *Biotechnol. Bioeng.* **1995**, *48*, 415−427.

Choe, T. B.; Massé, P.; Verdier, A. Separation of trypsin from trypsin−α- chymotrypsin mixture by affinity−ultrafiltration. *Biotechnol. Lett.* **1986**, *8*, 163−168.

Denizli, A.; Salih, B.; Arica, M. Y.; Kesenci, K.; Hasirci, V.; Piskin, E. Cibacron Blue F3GA-incorporated macroporous poly(2-hydroxyethyl methacrylate) affinity membranes for heavy metal removal. *J. Chromatogr. A* **1997**, *758*, 217−226.

Feinstein, G. Purification of trypsin by affinity chromatography on ovomucoid−sepharose resin. *FEBS Lett.* **1970a**, *7*, 353−355.

Feinstein, G. Interaction of insoluble protein inhibitors with protease. *Biochim. Biophys. Acta* **1970b**, *214*, 224−227.

Gerstner, J. A.; Hamilton, R.; Cramer, S. M. Membrane chromatographic systems for high-throughput protein separations. *J. Chromatogr.* **1992**, *596*, 173−180.

González, G.; Alea, J. Pepsin immobilized by covalent binding to a chitosan derivative. *J. Chem. Technol. Biotechnol.* **1995**, *63*, 247−248.

Hixson, H. F.; Nishikawa, A. H. Affinity chromatography: purification of bovine trypsin and thrombin. *Arch. Biochem. Biophys.* **1973**, *154*, 501−509.

Huang, S. H.; Roy, S.; Hou, K. C.; Tsao, G. T. Scaling-up of affinity chromatography by radial-flow cartridges. *Biotechnol. Prog.* **1988**, *4*, 159−165.

Itoyama, K.; Tokura, S.; Hayashi, T. Lipoprotein lipase immobilization onto porous chitosan beads. *Biotechnol. Prog.* **1994**, *10*, 225−229.

Iwata, H.; Saito, K.; Furusaki, S. Adsorption characteristics of an immobilized metal affinity membrane. *Biotechnol. Prog.* **1991**, *7*, 412−418.

Jameson, G. W.; Elmore, D. T. Affinity chromatography of bovine trypsin−A rapid separation of bovine α- and β-trypsin. *Biochem. J.* **1974**, *141*, 555−565.

Klein, E.; Eichholz, E.; Yeager, D. H. Affinity membranes prepared from hydrophilic coatings on microporous polysulfone hollow fibers. *J. Membr. Sci.* **1994a**, *90*, 69−80.

Klein, E.; Eichholz, E.; Theimer, F.; Yeager, D. Chitosan modified sulfonated poly(ether sulfone) as a support for affinity separation. *J. Membr. Sci.* **1994b**, *95*, 199−204.

Kochan, J. E.; Wu, Y. J.; Etzel, M. R. Purification of bovine immunoglobulin G via protein G affinity membranes. *Ind. Eng. Chem. Res.* **1996**, *35*, 1150−1155.

Linné, E.; Garg, N.; Kaul, R.; Mattiasson, B. Evaluation of alginate as a ligand carrier in affinity precipitation. *Biotechnol. Appl. Biochem.* **1992**, *16*, 48−56.

Liu, H.-C.; Fried, J. R. Breakthrough of lysozyme through an affinity membrane of cellulose−cibacron blue. *AIChE J.* **1994**, *40*, 40−49.

Luong, J. H. T.; Male, K. B.; Nguyen, A. L. A continuous affinity ultrafiltration process for trypsin purification. *Biotechnol. Bioeng.* **1988a**, *31*, 516−520.

Luong, J. H. T.; Male, K. B.; Nguyen, A. L. Synthesis and characterization of a water-soluble affinity polymer for trypsin purification. *Biotechnol. Bioeng.* **1988b**, *31*, 439−446.

Male, K. B.; Luong, J. H. T.; Nguyen, A. L. Studies on the application of a newly synthesized polymer for trypsin purification. *Enzyme Microb. Technol.* **1987**, *9*, 374−398.

Manganaro, J. L.; Goldberg, B. S. Protein purification with novel porous sheets containing derivatized cellulose. *Biotechnol. Prog.* **1993**, *9*, 285−290.

Muzzarelli, R. A. A.; Jeuniax, C.; Gooday, G. W. *Chitin in Nature and Technology*; Plenum Press: New York, 1986.

Powers, J. D.; Kilpatrick, P. K.; Carbonell, R. G. Trypsin purification by affinity binding to small unilamellar liposome. *Biotechnol. Bioeng.* **1990**, *36*, 506−519.

Robinson, N. C.; Tye, R. W.; Neurath, H.; Walsh, K. A. Isolation of trypsins by affinity chromatography. *Biochemistry* **1971**, *10*, 2743−2747.

Rodemann, K.; Staude, E. Synthesis and characterization of affinity membranes made from polysulfone. *J. Membr. Sci.* **1994**, *88*, 271−278.

Roper, D.; Lightfoot, E. Separation of biomolecules using adsorptive membranes. *J. Chromatogr. A* **1995**, *702*, 3−26.

Ruckenstein, E.; Zeng, X. F. Microporous or macroporous filtration membranes: preparation and use. U.S. Patent Application, 1997a.

Ruckenstein, E.; Zeng, X. F. Macroporous chitin affinity membranes for lysozyme separation. *Biotechnol. Bioeng.* **1997b**, *56*, in press.

Schisla, D. K.; Carr, P. W.; Cussler, E. L. Hollow fiber array affinity chromatography. *Biotechnol. Prog.* **1995**, *11*, 651−658.

Schwert, G.; Takenaka, Y. A spectrophotometric determination of trypsin and chymotrypsin. *Bichim. Biophys. Acta* **1955**, *16*, 570−575.

Serafica, G. C.; Pimbley, J.; Belfort, G. Protein fractionation using fast flow immobilized metal chelate affinity membranes. *Biotechnol. Bioeng.* **1994**, *43*, 21−36.

Suen, S.-Y.; Etzel, M. R. A mathematical analysis of affinity membrane bioseparation. *Chem. Eng. Sci.* **1992**, *47*, 1355−1364.

Suen, S.-Y.; Caracotsios, M.; Etzel, M. R. Sorption kinetics and axial diffusion in binary solute affinity membrane bioseparations. *Chem. Eng. Sci.* **1993**, *48*, 1801−1812.

Tennikova, T. B.; Bleha, M.; Svec, F.; Almazova, T. V.; Belenkkii, B. G. High-performance membrane chromatography of proteins, a novel method of protein separation. *J. Chromatogr. A* **1991**, *555*, 97−107.

Thömmes, J.; Kula, M. Membrane chromatography−An integrative concept in the downstream processing of proteins. *Biotechnol. Prog.* **1995**, *11*, 357−367.

Zeng, X. F.; Ruckenstein, E. Supported chitosan-dye affinity membranes and their adsorption of protein. *J. Membr. Sci.* **1996a**, *117*, 271−278.

Zeng, X. F.; Ruckenstein, E. Control of pore sizes in macroporous chitosan and chitin membranes. *Ind. Eng. Chem. Res.* **1996b**, *11*, 4169−4175.

Zikakis, J. P. *Chitin, chitosan and related enzymes*; Academic Press: New York, 1984.

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