Plant Protein Hydrolysates: Preparation of Defined Peptide Fractions Promoting Growth and Production in Animal Cells Cultures

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A new approach was applied with the aim at producing plant protein hydrolysates less heterogeneous and less contaminated with nonpeptide substances than are the presently available digests. A significant reduction of nonprotein contaminants was achieved by extraction of the plant material, soy flour or wheat flour, with acetone prior to isolation of the protein. Enzymes of nonanimal origin, papain or Pronase, were used for protein hydrolysis. The components of the hydrolysates were resolved by low-pressure liquid chromatography. Separation of peptide fractions and of remaining nonpeptide contaminants was achieved using small-pore size-exclusion chromatography matrices, Sephadex G-15 or Biogel P-2. Individual peptide fractions, both from soy protein and from wheat gluten, varied substantially in their growth-promoting and production-enhancing activities when tested on a mouse hybridoma culture in protein-free medium. The highest enhancement of viable cell density in batch cultures was 180% of control, and the highest enhancement of final immunoglobulin concentration was more than 230% of control. The existence of marked differences in activity of individual peptide fractions leads to a suggestion that the hydrolysates may provide peptides exerting specific positive effects on cultured animal cells.

Introduction

The beneficial effect of protein hydrolysates on the growth of animal cell cultures has been known for more than two decades (1). Protein hydrolysates are generally thought to act as concentrated balanced nutrient mixtures that may partly or fully replace serum (2, 3). Antiapoptotic activity of protein hydrolysates has been also reported (3). The low cost of these preparations makes them attractive for application in large-scale technologies.

The choice of the protein substrate is influenced by the present trend of avoiding ill-defined animal-derived materials in biopharmaceutical processes. This trend provokes heightened interest in plant proteins.

The present knowledge cannot answer unequivocally whether all of the beneficial activity of the hydrolysates resides in the small peptide fraction or whether the larger peptides play a positive role as well. Very small peptides, as well as liberated free amino acids, evidently contribute to the nutritional value of the medium (4, 5). However, larger peptides may possibly act as signals mimicking growth factors or survival factors.

Distinct biological activities of peptides obtained by enzymatic hydrolysis of both plant and animal proteins have been reported. Peptides as short as tetra- or pentapeptides obtained from wheat gluten hydrolysate, exorphins, are endowed with opioid activity (6). Peptidic angiotensin I-converting enzyme inhibitors and peptidic α-glucosidase inhibitors are present in sardine muscle hydrolysate (7, 8). Thus, peptides exerting specific effects on the growth and metabolism of cultured animal cells are undoubtedly present in protein hydrolysates. The present state of knowledge does not allow estimation of whether peptides of this character appear exceptionally or frequently.

A substantial drawback of most commercially available crude protein digests is the presence of nonpeptide components of mostly unknown nature. These ballast substances may possess unfavorable biological activities. Drying at elevated temperature, commonly used in the preparation of final hydrolysate products, may result in formation of peptide conjugates with other organic substances. Thus, low-cost crude protein digests can hardly meet severe criteria applied on media components employed in technologies yielding products destined to human therapy.

The aim of this work was to achieve advancement in the quality and safety of peptide mixtures applied in animal cell technology. Our approach was based on the purification of plant proteins to a higher degree prior to digestion by enzymes and on fractionation of the digests by liquid chromatography. Results with peptide fractions, obtained in laboratory-scale experiments, are reported.

Materials and Methods

Materials. Sephacryl S 200 HR and Sephadex G-15 were products of Pharmacia Biotech. Biogel P-2 superfine was purchased from Biorad. All solvents were of analytical grade. Cell culture media and supplements were from Life Technologies.
Soy Protein Hydrolysate (SO-PA). Finely ground soy powder was prepared under special conditions avoiding elevated temperature. Oil was extracted by hexane in a Soxhlet apparatus at a temperature not exceeding 30 °C. The defatted powder was freed of remaining hexane by free evaporation at room temperature. The powder was then extracted by acetone at −5 °C and subsequently dried at room temperature. A 2% (w/v) suspension of powder in deionized water was stirred at 50 °C for 2 h. The bulk protein was isolated from the clarified water extract by isoelectric precipitation at pH 4.2. The wet paste was suspended in minimum water at pH 5.0, papain and cysteine (1/100 extracted powder mass each) were added, and the slurry was stirred at 35 °C overnight. The insoluble portion was removed by centrifugation, and the supernatant was subjected to ultrafiltration through membrane PM-30 (Amicon). The ultrafiltrate was freeze-dried.

Wheat Gluten Hydrolysate (HY-TRIT). Wheat flour was extracted by acetone at −5 °C. The remaining acetone was removed from the flour by free evaporation at room temperature. The extracted flour, swollen with addition of a minimum volume of water, was used for preparation of gluten by washing under tap water. The water-insoluble protein was further washed in deionized water, minced, and suspended in deionized water. Hydrolysis by Pronase (1/1000 original flour mass) was carried out under stirring for 2 h at 37 °C. The remaining insoluble portion was removed by centrifugation, and the supernatant was subjected to ultrafiltration through membrane PM-30 (Amicon). The ultrafiltrate was freeze-dried.

Liquid Chromatography. The resolution of the protein hydrolysates was carried out by low-pressure liquid chromatography in 50 mM acetic acid. The columns were run at flow rates in the range of 5–10 mL cm⁻² h⁻¹.

Analyses of Peptide Fractions. Peptide fractions were subjected to total hydrolysis in 6 M hydrochloric acid for 20 h at 110 °C. The amino acid composition of the total hydrolysates was determined in duplicate on an automatic analyzer Biochrom 20 (Pharmacia Biotech). The experimental error involved in amino acid analyses was ±10%. Total nitrogen was determined on a CHNS/O analyzer 2400 (Perkin-Elmer).

Cell Culture. Mouse hybridoma ME-750 was cultured in DMEM/F12/RPMI 1640 (2:1:1) medium supplemented with BME amino acids, 2.0 mM glutamine, 0.4 mM each of alanine, serine, asparagine, and proline (9), 15 mM HEPES, 2.0 g L⁻¹ sodium bicarbonate, and with the iron-rich protein-free growth-promoting mixture containing 400 μM ferric citrate (10). The cultures in 25 cm² T-flasks were kept at 37 °C in a humidified atmosphere containing 5% CO₂.

Assay of Hydrolysate Fraction Activity. For testing the growth-promoting activity of peptide fractions the cultures were inoculated at a viable cell density of (300 ± 50) × 10⁵ mL⁻¹ in the total volume of 6 mL and incubated for 6 days. The standard concentration of the tested peptide preparation was 0.2% (w/v). The assays were conducted at least in duplicate. Viable cells and dead cells were counted in a hemocytometer using the trypan blue exclusion test. The concentration of immunoglobulin (lg) in the culture medium was determined by immunoturbidimetry (11). The experimental error involved in the estimation of cell count, viability, and immunoglobulin concentration was ±10%.

Results

Soy Protein-Derived Peptide Fractions. The hydrolysate SO-PA was resolved with employment of two steps of chromatography. The chromatography on the broad-range matrix Sephacryl S 200 HR yielded several peaks (Figure 1). The largest peptides, eluted near the break-through volume (i.e., 2–3 L), were discarded, as well as the ascending part of the highest peak (5–6 L effluent volume) that was slightly opalescent. The descending part, designated a 2, was supposed to contain small peptides. This part was collected and freeze-dried. The material of the peaks eluted at the volume of 7 L and higher was evidently retarded on the Sephacryl matrix by adsorption. Amino acid analysis did not reveal any significant amount of peptidic substances in the retarded fractions.

Fine resolution of SO-PA a 2 was achieved on Sephadex G-15 (Figure 2). The cultures supplemented with the fraction SO-PA a 24 showed significantly higher viable cell density and immunoglobulin yield (Table 1). This finding supports the view that some of the peptides arising through enzymic cleavage of plant proteins do not serve as a supply of amino acids solely but possess specific growth-promoting activities.

The amino acid composition of SO-PA a 24 was found to be rather balanced (Table 2). Even repeated amino acid analyses of SO-PA a 24 from various batches did not result in obtaining whole numbers of individual amino acids. The fraction SO-PA a 24 has to be considered as a mixture of several peptides. The total nitrogen content is higher than the average nitrogen content in commercial protein hydrolysates (Table 2) (manufacturer’s data, Quest International).

Wheat Gluten-Derived Peptide Fractions. The hydrolysate HY-TRIT was applied to a column of Biogel P-2. The elution profile was characterized by multiple overlapping peaks (Figure 3). This character of the elution profile could be expected, because cleavage of gluten by a relatively nonspecific protease yielded necessarily tens of different peptides. The chromatographic profile, as depicted in Figure 3, was reproducible between
individual chromatography runs, as well as between batches of the hydrolysate, if the conditions of enzymic hydrolysis were kept constant.

The break-through portion consisting of the largest peptides was not collected. The fractions HY-TRIT a 1 and a 21 to a 23 were collected, freeze-dried and tested for their growth-promoting activity (Table 3). The frac-

### Table 1. Growth-Promoting and Product-Enhancing Activity of SO-PA Fractions

<table>
<thead>
<tr>
<th>additive</th>
<th>viable cell density</th>
<th>immunoglobulin concn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\times 10^3$ cells mL$^{-1}$</td>
<td>% control</td>
</tr>
<tr>
<td>none (control)</td>
<td>1190</td>
<td>100</td>
</tr>
<tr>
<td>SO-PA (unfrac)</td>
<td>1270</td>
<td>107</td>
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<tr>
<td>SO-PA a 2</td>
<td>1490</td>
<td>125</td>
</tr>
<tr>
<td>SO-PA a 23</td>
<td>1120</td>
<td>94</td>
</tr>
<tr>
<td>SO-PA a 24</td>
<td>1790</td>
<td>150</td>
</tr>
<tr>
<td>SO-PA a 25</td>
<td>1070</td>
<td>90</td>
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</table>

$^a$ Values on day 6 of the culture are given.

### Table 2. Amino Acid Composition of Peptide Fractions

<table>
<thead>
<tr>
<th>amino acid</th>
<th>SO-PA a 24</th>
<th>HY-TRIT a 1</th>
<th>HY-TRIT a 21</th>
<th>HY-TRIT a 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp + Asn</td>
<td>6.3</td>
<td>2.4</td>
<td>3.1</td>
<td>3.7</td>
</tr>
<tr>
<td>Met</td>
<td>3.9</td>
<td>0.4</td>
<td>1.2</td>
<td>3.9</td>
</tr>
<tr>
<td>Thr</td>
<td>4.2</td>
<td>3.4</td>
<td>4.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Ser</td>
<td>5.5</td>
<td>8.4</td>
<td>8.8</td>
<td>8.6</td>
</tr>
<tr>
<td>Glu + Gln</td>
<td>7.6</td>
<td>30.1</td>
<td>24.8</td>
<td>21.2</td>
</tr>
<tr>
<td>Gly</td>
<td>10.3</td>
<td>13.8</td>
<td>7.2</td>
<td>3.8</td>
</tr>
<tr>
<td>Ala</td>
<td>5.7</td>
<td>4.4</td>
<td>7.5</td>
<td>3.8</td>
</tr>
<tr>
<td>Val</td>
<td>4.3</td>
<td>9.9</td>
<td>7.6</td>
<td>4.2</td>
</tr>
<tr>
<td>Ile</td>
<td>4.3</td>
<td>3.6</td>
<td>6.3</td>
<td>5.7</td>
</tr>
<tr>
<td>Leu</td>
<td>10.9</td>
<td>4.7</td>
<td>10.7</td>
<td>17.6</td>
</tr>
<tr>
<td>Tyr</td>
<td>8.4</td>
<td>1.0</td>
<td>0.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Phe</td>
<td>9.5</td>
<td>1.4</td>
<td>1.6</td>
<td>11.5</td>
</tr>
<tr>
<td>His</td>
<td>5.5</td>
<td>1.1</td>
<td>2.9</td>
<td>1.8</td>
</tr>
<tr>
<td>Lys</td>
<td>2.8</td>
<td>0.7</td>
<td>2.8</td>
<td>6.1</td>
</tr>
<tr>
<td>Arg</td>
<td>5.4</td>
<td>0.8</td>
<td>2.8</td>
<td>6.1</td>
</tr>
<tr>
<td>Pro</td>
<td>5.4</td>
<td>13.9</td>
<td>8.2</td>
<td>3.3</td>
</tr>
<tr>
<td>total N, %</td>
<td>13.9</td>
<td>16.7</td>
<td>15.6</td>
<td>11.2</td>
</tr>
</tbody>
</table>

$^a$ Relative quantities (molar percent) obtained upon total hydrolysis of the fractions are given.

individual chromatography runs, as well as between batches of the hydrolysate, if the conditions of enzymic hydrolysis were kept constant.

The break-through portion consisting of the largest peptides was not collected. The fractions HY-TRIT a 1 and a 21 to a 23 were collected, freeze-dried and tested for their growth-promoting activity (Table 3). The frac-

### Table 3. Growth-Promoting and Product-Enhancing Activity of HY-TRIT Fractions

<table>
<thead>
<tr>
<th>additive</th>
<th>viable cell density</th>
<th>immunoglobulin concn</th>
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</tr>
<tr>
<td>none (control)</td>
<td>1220</td>
<td>100</td>
</tr>
<tr>
<td>HY-TRIT (unfrac)</td>
<td>1810</td>
<td>148</td>
</tr>
<tr>
<td>HY-TRIT a 1</td>
<td>1860</td>
<td>152</td>
</tr>
<tr>
<td>HY-TRIT a 21</td>
<td>2200</td>
<td>180</td>
</tr>
<tr>
<td>HY-TRIT a 22</td>
<td>1700</td>
<td>139</td>
</tr>
<tr>
<td>HY-TRIT a 23</td>
<td>1590</td>
<td>130</td>
</tr>
</tbody>
</table>

$^a$ Values on day 6 of the culture are given.

Addition of the most potent fraction, HY-TRIT a 21, at 0.1% concentration to a hybridoma batch culture had a profound effect on the growth profile of viable and total cells (Figure 4). The final immunoglobulin yield was higher by a factor of 2.27, relative to control.

The elution volume of glycine standard (Figure 3) shows that nonadsorbed molecules of the size of amino acids emerge from the Biogel P-2 column at volumes corresponding to the fraction HY-TRIT a 22. This fact indicates that the substances constituting the fraction HY-TRIT a 21 are most likely small oligopeptides. The retarded peaks HY-TRIT b and HY-TRIT c were found to contain largely nonpeptide material and colored sub-

In contrast to SO-PA a 24, characterized by a balanced amino acid composition, all of the analyzed HY-TRIT fractions are characterized by a strikingly high sum of glutamic acid and glutamine. Because upon total hydrolysis by hydrochloric acid original glutamine residue cannot be distinguished from original glutamic acid residue, we can make only a guess, at present, that a high content of glutamine-containing peptides is responsible for the highly beneficial effect of HY-TRIT fractions.
The total nitrogen content of HY-TRIT a 21 exceeds that of available protein hydrolysates (manufacturer’s data, Quest International).

**Discussion**

Crude protein hydrolysates contain usually a significant fraction, up to tens of percent, of ballast nonpeptide substances of ill-defined character. Some plant protein hydrolysates are intensely colored. Selective separation of low-molecular contaminants from peptides is difficult as a result of similar molecular size. We have found that the ballasts may be markedly reduced if the starting plant material, i.e., powdered grain or beans, is extracted by organic solvents prior to isolation of the protein. The extraction by hexane, which is a common procedure in soy technology, undoubtedly leaves in the defatted flour some undesirable substances that stick to protein molecules. In our experience, further ballast substances may be removed both from soy and from wheat by more polar acetone. Other authors have used 70% acetone for pretreatment of soy flour (12).

Ultrafiltration is the method of first choice when larger and smaller molecules are to be separated in large scale. However, because of the relatively broad distribution of membrane pore diameters, fractionation of peptides by ultrafiltration is not sharp enough. Therefore, we have used ultrafiltration as a preliminary purification step only, and we developed chromatography procedures for resolution of hydrolysate components. Our choice was low-pressure liquid chromatography based on the size-exclusion principle, because it requires relatively inexpensive equipment and can be easily scaled up. Moreover, chromatography on small-pore matrices combines size-exclusion and adsorption and is thus suitable for fine resolution of complex mixtures. It is unlikely, however, that a peptide mixture composed of tens of peptides, differing in size, composition, and electric charge, will yield nonoverlapping individual peptides when resolved on this kind of matrix.

Soy protein-derived peptides were fractionated in two chromatography steps. In both steps some nonpeptide components were retarded by adsorption to the matrix and were eluted at volumes higher than was the total volume of the column. The same phenomenon could be observed in single-step chromatography of wheat gluten peptides. This finding justifies the application of chromatography when removal of nonprotein ballast is one of the goals of the procedure. Even though the hydrolysates prepared from acetone-extracted flour were less colored than the hydrolysates prepared from crude flour, nonprotein ballast components could not be evidently removed completely by the extraction. The purification efficiency of chromatography is documented by the high total nitrogen content of the peptide fractions, as well as by the absence of colored substances in the final freeze-dried preparations.

Individual peptide fractions of the hydrolysate obtained by chromatography, both from soy protein and from wheat gluten, varied significantly in their growth-promoting and production-enhancing activities, some of them exceeding the activity of unfraccionated digests (see Tables 1 and 3). This finding corroborates the notion that some of the peptides may exert specific effects on cultured cells. At present, we can only make a rough estimate of the size of the most active peptides. The peptides of the HY-TRIT a 21 fraction are undoubtedly retarded on Biogel P-2 by size exclusion (exclusion limit 1800 Da according to manufacturer’s data) but not retarded that much as free glycine. For the expected size of the peptides constituting the fraction a 21, between 2 and 10 amino acid residues would be a realistic guess.

It remains to be analyzed what fraction of the enhanced final immunoglobulin yield is a result of higher viable cell density in the batch cultures supplemented with the peptides (see Figure 3) and what is the result of enhanced cell specific productivity. More data are needed to clarify this aspect of the beneficial effect of peptide mixtures.

Any progress in protein hydrolysate processing represents a step toward revealing the true active principles of the hydrolysates and toward obtaining them in a pure state. Attempts at replacing crude protein hydrolysate in nutrient media by defined peptide(s) have been reported several decades ago. Isolation of casein peptides promoting growth of Lactobacillus casei has been achieved by chromatography on cellulose and by extraction in a phenol/water system (13).

Several authors who have analyzed the activities of commercially available peptones (4, 5) have concluded that the beneficial effect of hydrolysates is of nutritional character. Our present findings with peptide fractions, prepared under very mild conditions avoiding elevated temperature, do not support the view that the effect of protein hydrolysates is solely nutritional.

The present state of the development of hydrolysates of higher quality allows the preparation of peptide fractions virtually free of nonpeptide contaminants and substantially less heterogeneous than is the whole digest. Fractionation by chromatography allows the identification of the most potent peptide mixtures and the definition of them in terms of effluent volumes at which they emerge in the preparative chromatography runs. It is intended to identify the most active peptides by employing further separation techniques, such as ion-exchange chromatography. We believe that availability of pure growth-promoting peptides would represent a challenge...
for novel media formulations meeting the most severe
criteria of biological safety.

Conclusions
Resolution of the enzymic hydrolysates of plant pro-
teins by low-pressure liquid chromatography on small-
pore size-exclusion chromatography matrices is a crucial
step toward obtaining peptide fractions of substantially
higher quality than are the presently available crude
digests. Extraction of the starting plant material with
organic solvents prior to isolation of the protein contrib-
utes to the reduction of nonpeptide components contami-
nating the hydrolysates.

Individual peptide fractions obtained by chromatogra-
phy vary substantially in their growth-promoting activi-
ties, as well as in their beneficial effect on the yield of
monoclonal immunoglobulin by a mouse hybridoma. This
is true both for a papain digest of soy proteins and for a
Pronase digest of wheat gluten. The findings suggest that
protein hydrolyzates not only serve as a source of
utilizable amino acids but also may provide peptides
exerting specific effects, e.g., peptides mimicking growth
factors or survival factors.

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ing amino acid analyses is highly appreciated.

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