Production and Characterization of an Extensive Rapeseed Protein Hydrolysate

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ABSTRACT: Rapeseed protein isolate has been used as starting material for the generation of an extensive protein hydrolysate. Protein hydrolysis was produced by using sequentially an endopeptidase (Alcalase) and an exopeptidase (Flavourzyme). The final hydrolysate has a 60% degree of hydrolysis and was completely soluble between pH values 2.5 and 7. Molecular weight profile of the protein hydrolysate was characterized by gel filtration chromatography. A reduction in protein size was observed during the hydrolysis process with accumulation of small peptides and free amino acids after Flavourzyme digestion. Amino acid composition of fractions with different molecular weights of the final hydrolysate was analyzed. Some of these fractions, enriched or poor in certain amino acids, could be used for supplementation or treatment of determined clinical syndromes.

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KEY WORDS: Alcalase, enzymatic hydrolysis, Flavourzyme, rapeseed.

The increasing growth population in the Third World with the reduction in food resources and the excessive intake of animal proteins makes the search for new sources of proteins from nonconventional raw materials of interest. Oilseeds are becoming of increasing interest as a source of edible proteins (1). Rapeseed is one of the most important oilseed crops cultivated in the world, ranking fifth with respect to oil production after soybean, cottonseed, peanut, and sunflower. Rapeseed is rich in protein (between 30 and 45%), and hence defatted rapeseed meal may constitute a good source of proteins for humans (2). Amino acid composition is well-balanced in regard to FAO requirements and is rich in sulfur-containing amino acids and lysine, generally limiting in legumes and cereals, respectively (3).

The presence of antinutritive substances, such as glucosinolates, phenols or phytic acid, was a drawback for the utilization of rapeseed meal in human nutrition. Fiber, a main component of the defatted meal, must also be eliminated to enrich the final product in protein. Protein isolates obtained from defatted rapeseed meal, free of these antinutritional components, can be used for the fortification of foods (4–7). But, the use of proteins from new raw materials has also been limited by other undesirable properties such as low solubility. In rapeseed this problem is higher because during the industrial extraction of the oil, the proteins suffer a denaturation process that reduces even more their solubility. To improve solubility and increase the fields of application of these byproducts, proteins may be partially hydrolyzed. This digestion will also enhance other functional properties such as foaming (8).

The improved properties of the protein hydrolysates make them a good protein source in human nutrition, e.g., for special medical uses in patients with reduced absorption surface or reduced digestive capacity (9). Protein hydrolysates may undergo other modifications, such as fractionation by molecular weight or removal of hydrophobic amino acids to obtain tailor-made protein hydrolysates for the fortification of specific clinical diets for specific treatments.

Rapeseed proteins were hydrolyzed in the past using different proteases, such as Alcalase, Pronase, or Neutrase (10,11). In the present paper we describe the generation and physicochemical characterization of an extensive rapeseed protein hydrolysate using as starting material a protein isolate. The hydrolysis is carried out sequentially with an endopeptidase (Alcalase) and an exopeptidase (Flavourzyme). The combined action of these proteases, with different catalytic activities, produced an extensive hydrolysate of high quality that can used directly in the supplementation of liquid foods and can widen the field of application of rapeseed proteins.

MATERIALS AND METHODS

Raw material. Rapeseed meal (*Brassica campestris* L.), industrially produced by Koipesol (Sevilla, Spain) and obtained by solvent extraction, was used as a protein source. Trinitrobenzenesulfonic acid (TNBS) was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

Proteolytic enzymes. The enzymatic complexes used were Alcalase 2.4 L and Flavourzyme 1000 MG (Novo Nordisk,

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Bagsvaerd, Denmark). Alcalase 2.4 L is a microbial protease of *Bacillus licheniformis* with endopeptidase activity. A main component of the commercial preparation is the serine protease subtilisin A. The specific activity of Alcalase 2.4 L is 2.4 Anson Unit (AU) per gram. One AU is the amount of enzyme, which under standards conditions, digests hemoglobin at an initial rate that produces an amount of trichloroacetic acid-soluble product which gives the same color with the Folin reagent as one milliequivalent of tyrosine released per minute. Flavourzyme 1000 MG is a protease complex of *Aspergillus oryzae* that contains both endo- and exoprotease activities. It has an activity of 1.0 Leucine aminopeptidase unit (LAPU)/g. One LAPU is the amount of enzyme which hydrolyzes 1 µmole of leucine-*p*-nitroanilide per minute.

Total nitrogen determination. Samples (0.1 g) were used for the determination of total nitrogen by the micro-Kjeldahl method (12). Crude protein content was calculated using a conversion factor of 6.25.

Preparation of protein isolate. Rapeseed defatted flour (100 g) was suspended in 1,000 mL of 0.25% Na_2SO_3 at pH 10.5. Na_2SO_3 was used to prevent oxidation of polyphenols and to avoid the darkening of the final product. The suspension was extracted by stirring for 1 h at room temperature. After centrifugation at 8,000 × *g*, two additional extractions were carried out with half of the volume of alkaline solution. The supernatants were pooled, and the pH of the soluble proteins was adjusted to the isoelectric point (pH 5.0) and the precipitate formed recovered by centrifugation as above. The precipitate was washed with distilled water adjusted to pH 5.0 and freeze-dried.

Hydrolysis. The protein isolate (50 g resuspended in 1,000 mL of water) was hydrolyzed batchwise by treatment sequentially with Alcalase and Flavourzyme in a pH-stat. Alcalase was added first to achieve a predigestion, and after 60 min, Flavourzyme was added to the reactor and incubated for 2 h. The hydrolysis curve was obtained by the pH-stat technique using the following hydrolysis parameters: substrate concentration (S), 5%; enzyme/substrate ratio (E/S), 0.3 AU/g for Alcalase and 50 LAPU/g for Flavourzyme; pH 8 for Alcalase and 7 for Flavourzyme; temperature, 50°C. The hydrolysis was conducted in a reaction vessel, equipped with a stirrer, thermometer and pH electrode. Hydrolysis was stopped by pH drop to pH 5. Hydrolysates were clarified by filtration to remove insoluble substrate fragments, and the filtrate was lyophilized and freeze-dried for further use.

Degree of hydrolysis. The degree of hydrolysis, defined as the percentage of peptide bonds cleaved, was calculated by the determination of free amino groups by reaction with TNBS according to Adler-Nissen (13). Total number of amino groups was determined in a sample 100% hydrolyzed at 110°C for 24 h in 6 N HCl (10 mg sample in 4 mL HCl).

Solubility curve determination. Protein isolate and hydrolysates (10 g) were extracted twice with 200 mL of 1 N NaOH stirring for 2 h at room temperature. Aliquots were taken for precipitation of the proteins at different pH values adjusted with HCl. The samples were centrifuged at 4,000 × *g* for 30 min, and the nitrogen content determined in the supernatant. With these values the percentage solubility was calculated as the percentage distribution of protein in the soluble and insoluble fractions.

Gel filtration chromatography. Samples were passed through a PD-10 column (Amersham Pharmacia, Uppsala, Sweden) to remove nonprotein components. Gel filtration was carried out in a fast protein liquid chromatography system equipped with a Superose 12 HR 10/30 column from Amersham Pharmacia. Volume injection was 200 µL. The eluent was 20 mM phosphate buffer, 0.5 M sodium chloride buffer pH 8.3 at a flow rate of 0.4 mL/min. Elution was monitored at 214 nm to detect small peptides lacking aromatic residues. The molecular masses were determined using a calibration curve made with blue dextran 2000 (2000 kDa), catalase (240 kDa), α-amylase (200 kDa), bovine serum albumin (67 kDa), ovoalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa) as molecular weight standards.

Amino acid analysis. Samples (10 mg) were hydrolyzed with 4 mL of 6 N HCl. The solutions were sealed in tubes under nitrogen and incubated in an oven at 110°C for 24 h. Amino acids were determined in the acid hydrolysis, after derivatization with diethyl ethoxymethylenemalonate, by high-performance liquid chromatography (HPLC), according to the method of Alaiz *et al*. (14), with D,L-α-aminobutyric acid as internal standard. The HPLC system consisted of a Model 600E multi-system with a 484 UV-Vis detector (Waters, Milford, MA). Separations were attained with a 300 × 3.9 mm i.d. reversed-phase column (Novapack C18, 4 m; Waters) using a binary gradient system. The solvents used were (A) 25 mM sodium acetate containing 0.02% sodium azide (pH 6.0) and (B) acetonitrile. The solvent was delivered to the column at a flow rate of 0.9 mL/min as follows: time 0.0–3.0 min, linear gradient from A/B (91:9) to A/B (86:14); 3.0–13.0 min, elution with A/B (86:14); 13.0–30.0 min, linear gradient from A/B (86:14) to A/B (69:31); 30.0–35.0 min, elution with A/B (69:31). The column was maintained at 18°C with a temperature controller.

RESULTS AND DISCUSSION

Generation of rapeseed protein hydrolysates. Protein isolate used for the generation of hydrolysates was obtained by alkaline extraction and acid precipitation of proteins, as described in the Material and Methods section. This protein isolate is a good substrate, with more than 95% protein content (Table 1), to be used as starting material for the hydrolysis.

For obtaining an extensive hydrolysate, a mixture of exo- and endopeptidases is often used. In general, single enzymes cannot provide an extensive hydrolysate in a reasonable period of time (15). The rapeseed protein isolate was hydrolyzed batchwise in a pH-stat using Alcalase and Flavourzyme sequentially. Alcalase is a well-known nonspecific endoprotease broadly used in food research for the generation of protein hydrolysates (16). By starting the hydrolysis with this enzyme, predigestion is achieved, increasing the number of

TABLE 1
Chemical Composition of Rapeseed Protein Isolate Calculated on a Dry Weight Basis

Component	Composition (%)
Ash	0.52
Fiber	0.05
Protein content	97.7
Lipids	1.14
Soluble sugars	0.35
Dissolved solids	0.12
Polyphenols	0.12

N-terminal sites for the action of the exopeptidase Flavourzyme. This sequential hydrolysis will reduce costs since a lesser amount of Flavourzyme is needed to obtain the same degree of hydrolysis. With this sequential process, an extensive hydrolysate with a 60% degree of hydrolysis was obtained. This degree of hydrolysis is higher than using Alcalase or Flavourzyme alone during the same time (data not shown).

The rate of hydrolysis with Alcalase is very fast in the initial minutes, reaching a steady state after 20–30 min (Fig. 1). In this sense, the time of incubation with Alcalase could be reduced to improve the yield of the process. On the other hand, Flavourzyme shows a kinetic of hydrolysis with a smaller slope, but a constant increment of the hydrolysis even after 3 h incubation, probably because of the exopeptidase activity of the enzyme (Fig. 1).

A problem in the generation of protein hydrolysates is the production of bitter peptides that limits the use of the hydrolysates. Bitterness seems to be produced by the exposure of hydrophobic residues as a consequence of protein hydrolysis (17). Free amino acids are less bitter than the corresponding peptides, and bitterness is highest when the hydrophobic amino acids are nonterminal (16). These factors favor the use of an exopeptidase to reduce bitterness of hydrolysates. In this sense, the hydrolysate produced by the individual action of Alcalase showed a high degree of bitterness. On the other hand, the final hydrolysate, after the action of Flavourzyme, when tested in a panel test, completely lacked bitterness.

Molecular weight characterization of hydrolysates. The most evident change observed in hydrolysates with respect to the protein isolates is the reduction in the molecular weight of proteins as a direct consequence of protease activity. Gel filtration profile of the protein isolate shows a main peak that corresponds probably to the 12S globulin "cruciferin," the major protein component of protein isolates (18) (Fig. 2). During the digestion with Alcalase, the relative amount of this peak decreased, with increasing proportions of others of lower molecular weight. Thus, after 1 h digestion with Alcalase, the main peptides range from 20 to 45 kDa. Digestion with Flavourzyme produced the appearance of a peak of free amino acids, due to the exopeptidase activity of this enzyme.

Solubility of hydrolysates. Solubility is probably the main functional characteristic of proteins, giving useful information about other properties, such as foaming, emulsion, and gelling (19). Increased protein solubility with enzymatic hydrolysis, particularly at the isoelectric point of the native protein, is well-documented (8,20–22). This increase in solubility is due to the smaller size of peptides and higher hydrophilicity of the hydrolisates (23). The increased solubility may be useful for the supplementation of drinks (with acidic pH) to improve their nutritional quality.

Rapeseed protein hydrolysates obtained with Alcalase and Flavourzyme increased their solubility drastically with respect to the protein isolate (Fig. 3). Thus, after 60 min hydrolysis with Alcalase, the proteins were more than 90% soluble at the isoelectric point (pH 5) and over 70% in the pH ranges tested. The final protein hydrolysate obtained after the treatment with Flavourzyme was almost 100% soluble at pH tested, making it a suitable component for the supplementation of liquid beverages at different pH.

Amino acid composition of hydrolysates. Amino acid com-

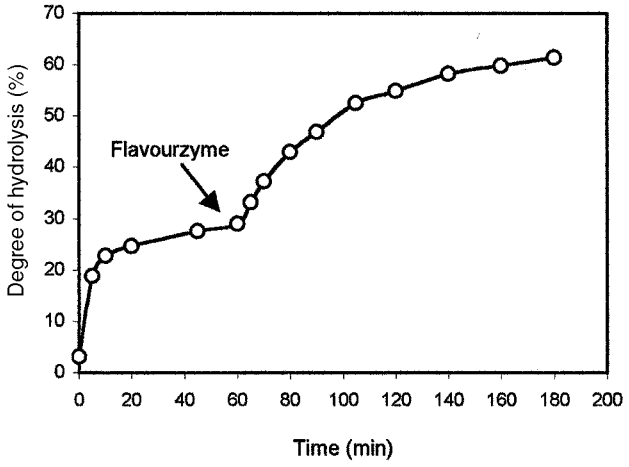

FIG. 1. Enzymatic hydrolysis of rapeseed protein isolate with Alcalase and Flavourzyme (added at 60 min).

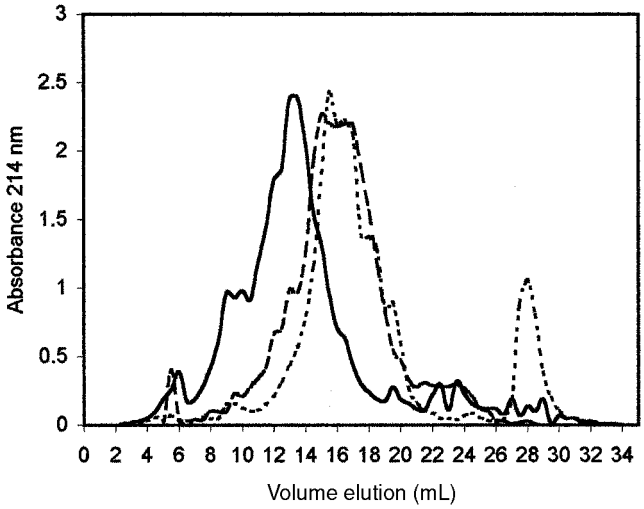

FIG. 2. Gel filtration chromatography of rapeseed protein isolate (—), protein hydrolysate after Alcalase treatment (– – –), and final protein hydrolysate (Alcalase + Flavourzyme) (- - - -).

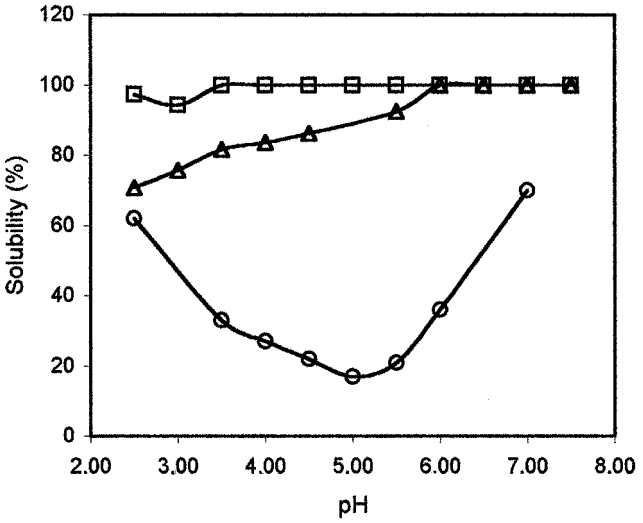

FIG. 3. Solubility at different pH values of protein isolate (○), protein hydrolysate after Alcalase treatment (△), and final protein hydrolysate (Alcalase + Flavourzyme) (□).

position of rapeseed protein isolate is well-balanced. Also, there were no apparent differences between the protein isolate and protein hydrolysate amino acid compositions, indicating that the process of enzymatic hydrolysis is a gentle procedure that does not affect the amino acid profile.

Amino acid composition in different fractions of the final protein hydrolysate is given in Table 2. Significant differences are observed between the amino acid profile of those fractions and total hydrolysate. Thus, fraction of peptides between 45 and 150 kDa shows lower amounts of aromatic and sulfur amino acids. On the other hand, the contents in branched-chain amino acids and Aspartic (Asp + Asn) are above the observed in the protein hydrolysates. This low content of aromatic amino acids could be useful for the treatment of congenital illness such as phenylketonuria or tyrosinamie, in which diets low in these amino acids are recommended. Also, this fraction may be of special interest for clinical use in the treatment of patients with liver diseases. This is due to the high Fischer ratio (ratio of Val + Leu + Ile to Tyr + Phe) observed, 7.3 in comparison to 2.5 in the protein isolate and total hydrolysate. Also, this fraction is suitable for the treatment of encephalopathies because of the low content of aromatic amino acids (1.5%), that should not exceed 2% (24).

The fraction corresponding to peptides between 20 and 45 kDa also shows a high Fischer ratio (5.8).

Finally, the fraction corresponding to peptides between 9 and 20 kDa, with reduced quantities of sulfur-containing amino acids could be helpful in the fortification of foods for patients with problems related to the metabolism of these amino acids that require diets with low percentages of them.

Thus, the protein hydrolysate obtained not only could have application by itself in the supplementation of different kinds of foods, but also it could be fractionated by means of membrane technology, such as by ultrafiltration, to obtain fractions with particular amino acid composition, that could be useful for the modification of clinical diets for specific treatments or congenital illnesses.

In conclusion, we generated an extensive hydrolysate using as starting material a rapeseed protein isolate and combining sequentially the action of two different proteases with different catalytic activities. The high solubility of the final hydrolysate make it a suitable material for the supplementation of liquid foods or high-energy beverages. The differences observed in amino acid composition depending on the fraction of the final hydrolysate could be useful in the fortification of foods for clinical diets enriched or free of certain amino acids. With the process described, a raw material may be processed in a high-quality product with an added value.

TABLE 2
Amino Acid Composition (g/100 g protein) of Rapeseed Protein Isolate, Total Protein Hydrolysate, and Fractions of Different Molecular Weights Collected from the Final Protein Hydrolysate

	Protein isolate	Protein hydrolysate	45–150 kDa	20–45 kDa	9–20 kDa	Free amino acids
Asp + Asn	10.2	10.0	21.6	10.8	4.9	13.4
Glu + Gln	20.8	19.5	23.3	20.2	12.6	14.8
Ser	7.2	7.0	6.7	10.1	9.7	11.0
His	2.6	2.5	2.8	1.9	4.8	2.3
Gly	10.3	9.7	12.5	15.7	12.9	13.3
Thr	4.7	4.7	5.5	6.5	6.7	5.6
Ala	7.1	7.2	4.5	5.8	5.8	9.5
Arg	6.2	6.1	4.4	5.4	9.6	4.1
Tyr	2.1	2.8	Trace	0.6	1.1	Trace
Val	5.1	6.2	3.9	5.1	8.1	4.6
Met	2.0	2.1	1.8	2.2	0.9	1.0
Cys	1.3	1.1	Trace	1.1	0.2	Trace
Ile	3.8	4.5	2.7	3.6	5.7	3.1
Leu	8.2	8.0	4.3	5.3	10.3	7.5
Phe	4.5	4.5	1.5	1.8	4.1	4.8
Lys	4.1	4.2	4.6	3.8	2.6	5.0

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REFERENCES

1. Altschul, A.M., in *New Protein Foods,* Vol. IA, Academic Press, New York, 1974.
2. Finlayson , A.J., The Seed Protein Contents of Some Cruciferae, in *The Biology and Chemistry of the Cruciferae,* edited by J.G. Vaughan, A.J. McLeod, and B.M.G. Jones, Academic Press, London, 1976.
3. Cheftel, J.C., in *Proteines Alimentaires: Biochemie propietés functionelle. Valeur nutritionelle. Modificationes chimiques,* edited by Techniques et Documentation, Lavosier, Paris, 1985, p. 192.
4. El Nockrashy, A.S., K.D. Mukherjee, and H.K. Mangold, Rapeseed Protein Isolates by Countercurrent Extraction and Isoelectric Precipitation, *J. Agric. Food Chem. 25*:193–197 (1977).
5. Dev, D.K., and K.D. Mukherjee, Functional Properties of Rapeseed Protein Products with Varying Phytic Acid Contents, *Ibid. 34*:775–780 (1986).

6. Zhou, B., Z.-Q. He, H.-M. Yu, and Mukherjee, K.D., Proteins from Double-Zero Rapeseed, *Ibid.* 38:690–694 (1990).
7. Gonçalves, N., J. Vioque, A. Clemente, R. Sánchez-Vioque, J. Bautista, and F. Millán, Obtention and Characterization of Rapeseed Protein Isolates, *Grasas Aceites 48*:282–289 (1997).
8. Chobert, J.M., L. Briand, J. Guéguen, Y. Popineau, C. Larré, and T. Haertlé, Recent Advances in Enzymatic Modifications of Food Proteins for Improving Their Functional Properties, *Nahrung 40*:177–182 (1996).
9. Frokjaer, S., Use of Hydrolysates for Protein Supplementation, *Food Technol. 48*:86–88 (1994).
10. Kim, C.H., H.S. Kim, Y.H. Jung, and Y.J. Kang, Conditions for Hydrolysis of Rapeseed Proteins by Pronase, *J. Korean Soc. Food Nutr. 21*:513–518 (1992).
11. Kim, C.H., H.S. Kim, J.S. Lee, and Y.J. Kang, Functionality Changes of Rapeseed Protein upon Proteolysis, *Ibid. 21*:519–524 (1992).
12. AOAC (1975) Association of Official Analytical Chemists, *Official Methods of Analysis,* 12 edn., Washington, DC.
13. Adler-Nissen, J., Determination of the Degree of Hydrolysis of Food Protein Hydrolysates by Trinitrobenzenesulfonic Acid, *J. Agric. Food Chem. 27*:1256–1262 (1979).
14. Alaiz, M., J.L. Navarro, J. Girón, and E. Vioque, Amino Acid Analysis by High-Performance Liquid Chromatography After Derivatization with Diethyl Ethoxymethylenemalonate, *J. Chromatogr. 591*:181–186 (1992).
15. Lahl, W.J., and S.D. Braun, Enzymatic Production of Protein Hydrolysates for Food Use, *Food Technol. 48*:68–71 (1994).
16. Adler-Nissen, J., *Enzymic Hydrolysis of Food Proteins,* Elsevier, London, 1986.
17. Pedersen, B., Removing Bitterness from Protein Hydrolysates, *Food Technol. 48*:96–99 (1994).
18. Schwenke, K.D., Rapeseed Proteins, in *New and Developing Sources of Food Proteins,* edited by B.J.F. Hudson, Chapman & Hall, London, 1994, pp. 281–306.
19. Zayas, J.F., *Functionality of Proteins in Food,* Springer-Verlag, Berlin, Germany, 1997.
20. Chobert, J.-M., C. Bertrand-Harb, and M.-G. Nicolas, Solubility and Emulsifying Properties of Caseins and Whey Proteins Modified Enzymatically by Trypsin, *J. Agric. Food Chem. 36*:883–892 (1988).
21. Chobert, J.-M., M.Z. Sitohy, and J. Whitaker, Solubility and Emulsifying Properties of Caseins Modified Enzymatically by *Staphylococcus aureus* V8 Protease, *Ibid. 36*:220–224 (1988).
22. Turgeon, S.L., S.F. Gauthier, and P. Paquin, Emulsifying Property of Whey Peptide Fractions as a Function of pH and Ionic Strength, *J. Food Sci. 57*:601–604 (1992).
23. Mahmoud, M.I., Physicochemical and Functional Properties of Protein Hydrolysates in Nutritional Products, *Food. Technol. 48*:89–95 (1994).
24. Okita, M., A. Watanabe, and H. Nagashima, Nutritional Treatment of Liver Cirrhosis by Branched-Chain Amino Acids-Enriched Nutrient Mixture, *J. Nutr. Sci. Vitaminol. 31*:291–303 (1985).

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