Low Molecular Weight Sunflower Protein Hydrolysate with Low Concentration in Aromatic Amino Acids

Juan Bautista,*,† Inmaculada Hernandez-Pinzon,† Manuel Alaiz,‡ Juan Parrado,† and Francisco Millan‡

Departamento de Bioquímica, Bromatología y Toxicología, Facultad de Farmacia, Universidad de Sevilla, 41012 Sevilla, Spain, and Instituto de la Grasa y sus Derivados, CSIC, Sevilla, Spain

A method to obtain low molecular weight hydrolysates with a high Fischer ratio (molar ratio of Val + Leu + Ile to Tyr + Phe) is described. Sunflower protein concentrate, with a low concentration in polyphenols, was hydrolyzed with kerase in a pH-stat to a 16.5% degree of hydrolysis. The hydrolysate was fractionated by ultrafiltration using membranes with cutoffs of 30 000 and 5000. The obtained fractions, UC-30, UC-5, and UF-5, were characterized for molecular weight distribution and amino acid composition. From a quantitative point of view, the two main fractions were UC-5 and UF-5. Fraction UC-5 contained a high percentage of proteins and peptides with molecular weights above 5000 and below 30 000 (61.9%), with a considerable content of peptides and free amino acids (37.2%). Fraction UF-5 was composed only of peptides having molecular weights below 5000 and free amino acids (99.8%). The main difference in total amino acid composition of the fractions UC-5 and UF-5 was a higher value of Fischer ratio for fraction UF-5. The treatment of this fraction with actinase followed by Sephadex G-15 chromatography (fraction A) led to a marked reduction in the content of aromatic amino acid, the Fischer ratio increasing from a value of 5.52 in UF-5 to 20.47 in fraction A.

Keywords: *Sunflower protein hydrolysates; low molecular weight hydrolysates; kerase; ultrafiltration*

INTRODUCTION

Short peptides with characteristic amino acid composition and defined molecular weight are highly desirable in nutrition and food science for both functional and nutritional purposes (Siemensman et al., 1993; Adachi et al., 1991). For food (clinical/dietetic) applications this type of peptide is superior to free amino acids in two main features: (i) peptides are absorbed in the digestive tract at a higher rate (Matthews, 1977), and (ii) the osmotic pressure of peptides is lower than that of the corresponding free amino acids (Adibi, 1989; Parrado et al., 1991). In this context, preparation of peptides with defined characteristics promises to be useful in producing physiologically functional foods for some specific needs, such as patients with malnutrition associated with cancer, burns, and trauma (Milla et al., 1983; Meredith et al., 1990) and hepatic encephalopathies (Adachi et al., 1991; Fischer et al., 1976), as well as for nutritional support of children with chronic and acute diarrhea or milk protein allergies (Knights, 1985; Buzinco et al., 1989).

There are two ways to obtain such peptides. One is an enzymatic or chemical synthesis of peptides from their constituent amino acids (Nakanishi et al., 1986; Kimura et al., 1990; Hermann et al., 1991). The other, which is the subject of our work, is a separation of characteristic peptide groups from an enzymatic protein hydrolysate (Tanimoto et al., 1991). The latter may be more economical than the former when the peptides are to be produced at larger scale as foodstuffs.

The objective of this work was to prepare a sunflower protein hydrolysate of low molecular weight, enriched in branched-chain amino acids (BCAA), and with a low content in aromatic amino acid (AAA), which can be considered as starting material for the formulation of specific parenteral diets for patients with liver diseases, using membrane technology (ultrafiltration).

* Author to whom correspondence should be addressed (fax 34-5-423.37.65).

† Universidad de Sevilla.

‡ CSIC.

MATERIALS AND METHODS

Enzymatic Hydrolysis of Sunflower Protein Concentrate (SFPC). SFPC, low in polyphenols (Parrado et al., 1991), was hydrolyzed batchwise with kerase (CEPA S.A., Madrid) in a pH-stat at constant pH of 7.5 until a degree of hydrolysis (DH) of 16.5 was achieved, as described in a previous work (Parrado et al., 1993). Kerase is a technical food-grade enzyme mixture with endopeptidase activities (trypsin- and chymotrypsin-like activities) (Parrado, 1991), obtained from *Streptomyces fradiae*.

Fractionation by Ultrafiltration. The hydrolysates were clarified by tangential flow microfiltration through an open channel cassette equipped with a 0.3 mm membrane (Filtron). The filtrate was fractionated by tangential flow ultrafiltration through a screen channel cassette equipped with a 30 000 membrane (Filtron) into two fractions: ultraconcentrate (UC-30), composed of peptides with molecular weights >30 000, and ultrafiltrate (UF-30), composed of peptides with molecular weights <30 000. The UF-30 was subjected to ultrafiltration using a 5000 membrane (Filtron), and fractionated into two fractions: ultraconcentrate (UC-5), composed of peptides of molecular weight >5000 and <30 000, and ultrafiltrate (UF-5), composed of peptides of molecular weight <5000 (see Figure 1). Retentate (microfiltration) and concentrates (ultrafiltration) were diafiltered three times with a volume equal to that of the retentate or concentrate, respectively.

Protein Nitrogen. All nitrogen derived from proteins, peptides, and free amino acid was considered protein nitrogen. Protein nitrogen was determined by amino acid analysis after total hydrolysis of the samples with 6 N HCl at 110 °C for 24 h under vacuum. Results were expressed as milligrams of amino acids per 100 mg of sample.

Molecular Weight Distribution. Molecular weight distribution of proteins and peptides in the different fractions was

0021-8561/96/1444-0967$12.00/0

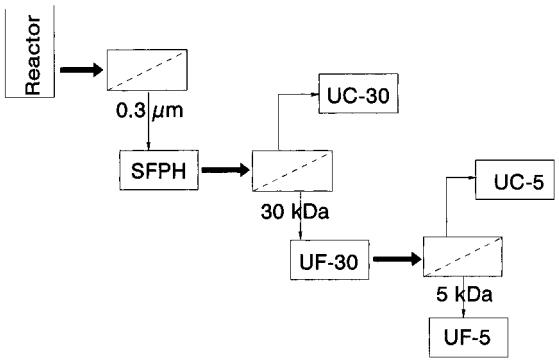

Figure 1. Scheme of process used for the production of low molecular weight sunflower protein hydrolysates.

determined by gel permeation chromatography on a Superose 12-HR 10/30 column (Pharmacia/LKB, Uppsala, Sweden) according to the procedure described by Visser et al. (1992), and by SDS−high-density polyacrylamide gel electrophoresis (SDS−PAGE) using the Trizine method of Schägger and von Jagow (1987). Proteins and peptides were stained according to a procedure based on a modification of Coomassie blue staining, using Cu ions, which improves the staining of low molecular weight peptides (5000 to 1000) (Parrado, 1991). Two protein standard mixtures were used: a low molecular weight standard to cover the range 16 950 to 2510 and a high molecular weight standard to cover the range 92 000 to 14 400.

Amino Acid Analysis. Amino acid composition and content of free amino acids were determined by HPLC using the method of Bidlingmeyer et al. (1984). Briefly, samples were hydrolyzed using 6 N HCl/1% (w/v) phenol vapor at 110 °C for 24 h *in vacuo*. Protein hydrolysates were treated with phenyl isothiocyanate to form phenylthiocarbamyl derivatives of the amino acids, which were then analyzed using a Water HPLC system (Millipore Ltd.) fitted with a reversed-phase C_{18} column. For cysteine estimation, aliquots were first oxidized with performic acid and then analyzed as above. For determination of free amino acids, the samples were deproteinized with 10% (w/v) trichloroacetic acid, oxidized with performic acid, and then analyzed as above.

Actinase Treatment. Actinase treatment was basically as described by Yamashita et al. (1979). Briefly, this comprised the following conditions: substrate concentration, 2%; solvent, 10% ethanol in water (pH 6.5); enzyme−substrate ratio, 1:200; temperature, 37 °C; reaction time of 6 h.

Sephadex G-15 Chromatography. Sephadex G-15 chromatography was basically as described by Tanimoto et al. (1991). Lyophilized UF-5 (1.5 g) was dissolved in 30 mL of 10% ethanol, and the solution was poured onto a Sephadex G-15 column (5 × 70 cm). Elution was performed with the same solvent at 200 mL/h flux. Absorbance was measured at 220 nm.

RESULTS AND DISCUSSION

SFPCs, with a low concentration in polyphenols, were hydrolyzed batchwise by treatment with kerase in a pH-stat, using the following hydrolysis parameters: substrate concentration (S) = 10%; enzyme−substrate ratio (E/S × 100) = 2%; pH, 7.5; temperature (T) = 55 °C; and 0.15% $CaCl_2$ as stabilizer of the enzyme. The reaction was continued until a 16.5% degree of hydrolysis was achieved. Under these conditions, we are confident that maximum solubilization of insoluble sunflower proteins is reached (Parrado et al., 1991). The hydrolysis curve obtained by kerase hydrolysis of SFPC low in polyphenols is shown in Figure 2, and the chemical composition of the resulting product, sunflower protein hydrolysate (SFPH), is shown in Table 1.

Size exclusion chromatograms of SFPHs at different DHs are shown in Figure 3a. From the chromatographic data we observed that hydrolysates with a DH

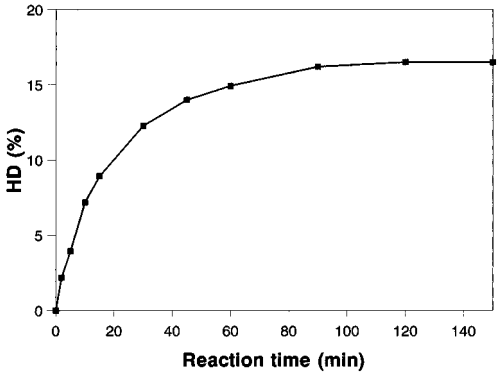

Figure 2. Hydrolysis curve of SFPC with kerase (substrate, 10%; enzyme−substrate ratio, 2%; pH, 7.5; temperature, 55 °C).

Table 1. Chemical Composition of SFPC, SFPH, UC-5, and UF-5[a]

	protein (%)	ash (%)
SFPC	58.3 ± 4.1	3.69 ± 0.4
SFPH (16.5% DH)	78.9 ± 3.5	12.63 ± 1.0
UC-5	93.1 ± 1.4	3.82 ± 0.3
UF-5	95.5 ± 1.1	3.66 ± 0.3

[a] Each value represents the mean ± SD of three experiments. Results are expressed on dry matter basis.

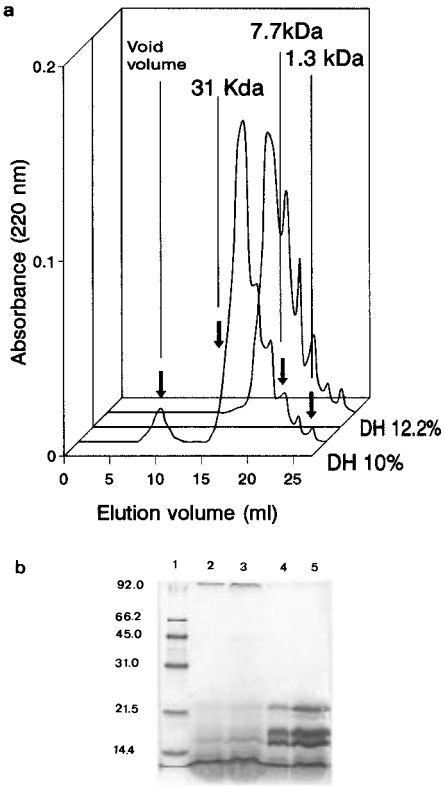

Figure 3. (a) Size exclusion chromatography of SFPHs of 10% and 12.2% DH on a Superose 12-HR 10/30 column. Protein standards: carbonic anhydrase (31 000), aprotinin (7 700), vitamin B_{12} (1350). (b) SDS−PAGE of SFPHs of different DHs in 10−18% linear gradient gel. (Lane 1) Protein standards: phosphorylase *b* (92 000), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400). (Lane 2) 3.6% DH. (Lane 3) 10% DH. (Lane 4) 12.2% DH. (Lane 5) 16.5% DH.

≤ 10% are composed of (i) large proteins with an approximate relative molecular weight (M_r) of 300 000 (probably helianthin) that eluted with the void volume, (ii) small proteins of molecular weight below 30 000, and

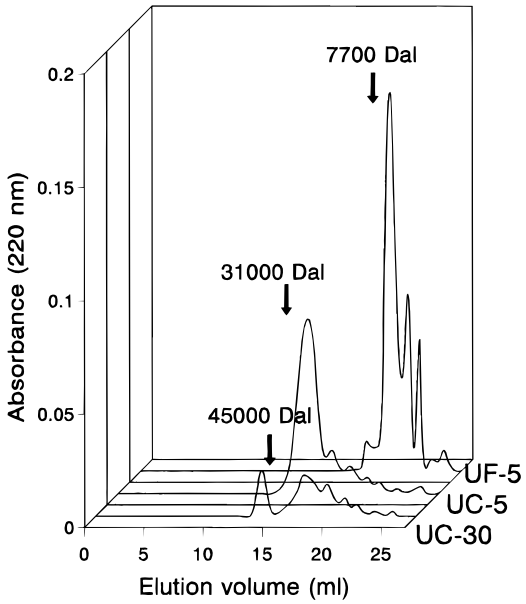

Figure 4. Size exclusion chromatography of ultrafiltration fractions UC-30, UC-5, and UF-5 on Superose 12-HR 10/30 column. Protein standards: ovalbumin (45 000), carbonic anhydrase (31 000), aprotinin (7700).

Table 2. Molecular Weight Distribution (Percent of Protein Nitrogen)

A. SFPH (16.5% DH), UC-30, UC-5, UF-5, and Fraction A	MW > 30	30 > MW > 5	MW < 5
SFPH (16.5% DH)	0.6	11.5	87.9
UC-30	25.0	7.3	67.7
UC-5	0.9	61.9	37.2
UF-5		0.2	99.8
fraction A			100

B. UF-5 and Fraction A	5 > MW > 1	MW < 1
UF-5	51.8	48
fraction A	36	64

(iii) peptides of molecular weight <5000. At higher DH, ≥12.2% (particularly at 16.5% DH), only the low molecular weight proteins and peptides are present, whereas the large proteins of 300 000 are not detected. Electrophoretic analysis by SDS−PAGE confirmed these results (Figure 3b).

Chromatographic and electrophoretic data show that SFPHs with a DH ≥ 12.2, e.g., 16.5% DH, are composed only of low molecular weight proteins and peptides. These data suggest that it is possible to obtain, by ultrafiltration, different hydrolysates with defined molecular weight ranges. Fractionation of SFPH of 16.5% DH with a UF membrane of cutoff 30 000 followed by a second fractionation with a UF membrane of cutoff 5000 (Figure 1) yielded three fractions, UC-30, UC-5, and UF-5, which represent 0.6%, 11.5%, and 87.9% (see Table 2A) of the total protein nitrogen, respectively. The size exclusion chromatography profiles of the three UF fractions obtained are shown in Figure 4. The molecular weight distribution of the different fractions was calculated by assuming an exponential relationship between molecular weight and elution volume (Visser et al., 1992). The results are summarized in Table 2.

From the results, and from a practical and quantitative point of view, the two most interesting fractions are UC-5 and UF-5. Fraction UC-5 was characterized by a very high percentage of proteins with a molecular weigh above 5000 and below 30 000 (61.9%), and with an appreciable content of peptides (<5000) and free amino acids (37.2%). Fraction UF-5 was composed practically only of peptide (<5000) and free amino acids (99.8%). A detailed analysis of this fraction, by FPLC, shows three main peptide peaks, with relative molecular weight 4950, 2100, and 780, and two minor peaks that correspond to free amino acids (see Figure 4).

Table 3. Amino Acid Composition of SFPC, SFPH, UC-5, UF-5, and Fraction A[a]

	SFPC	SFPH[b]	UC-5	UF-5	fraction A
Asp + Asn	9.70	9.95	9.28	9.83	9.92
Thr	3.70	4.20	4.53	6.13	5.96
Ser	5.15	5.25	4.91	5.31	5.53
Glu + Gln	16.70	17.35	17.85	16.86	17.04
Gly	8.60	8.40	9.02	7.86	8.72
Ala	9.70	9.30	10.00	8.67	9.34
Val	6.15	5.85	3.80	6.43	6.94
Met	3.15	2.40	1.45	3.05	4.65
Ile	4.45	4.10	4.39	5.85	5.61
Leu	8.50	7.15	7.02	7.95	8.13
Tyr	2.50	2.30	2.51	1.56	0.55
Phe	3.75	3.60	3.72	2.05	0.46
His	2.30	2.50	2.10	2.43	2.30
Pro	4.95	5.39	4.80	4.98	4.62
1/2 Cys	0.60	0.30	0.41	0.46	0.42
Lys	3.65	4.05	4.73	3.23	2.62
Arg	7.60	7.30	8.56	5.72	4.45
Trp	0.65	0.59	0.65	0.36	0.30
BCAA	19.10	17.15	15.21	19.93	20.68
AAA (Tyr + Phe)	6.25	5.90	6.23	3.61	1.01
Fischer ratio[b]	3.06	2.91	2.44	5.52	20.47

[a] Results are expressed as grams per 100 g of protein. [b] 16.5 DH. [c] Molar ratio of (Val + Leu + Ile) to (Tyr + Phe).

The data of the molecular weight distribution, presented in Table 2, should be considered carefully. The peak area in the chromatogram was divided by vertical lines, which is somewhat questionable. Furthermore, peak detection was at 220 nm, at which peptide bonds strongly absorb. The approach used could result in underestimation of the small peptides and the free amino acids (Visser et al., 1992). Therefore, this method cannot be applied to absolute determinations of molecular weight distribution but is well suited to the comparative purpose of the present study. A more precise method, based on estimation of peptide lengths and their relative abundance by the use of mathematical algorithms applied to quantitative data obtained from automated Edman degradation of the samples, was proposed by Simensman et al. (1993).

The electrophoretic pattern shown in Figure 3b confirms the results obtained by chromatography. Proteins and peptides with molecular weights ranging from 22 246 to 12 259 were detected in SFPHs by SDS−PAGE. Shorter peptides and free amino acids could be undetectable, assuming that they run with the electrophoretic front. The use of SDS−high-density PAGE also reveals the presence in SFPH of short peptides. Therefore, we used SDS−high-density PAGE for the analysis of ultrafiltration fractions. The data obtained with this technique are similar to those obtained with size exclusion chromatography (data not shown).

Amino acid composition (see Table 3) and molecular weight distribution (see Table 2) of the fractions obtained by ultrafiltration of SFPHs with a DH of 16.5% demonstrate that the fraction UF-5 is of special interest for clinical use in the treatment of patients with liver diseases. This observation is based on two important features: (i) the composition in short peptides and (ii) the high Fischer ratio (molar ratio of Val + Leu + Ile to Tyr + Phe). The content in free amino acids was analogous in all three fractions (data not shown).

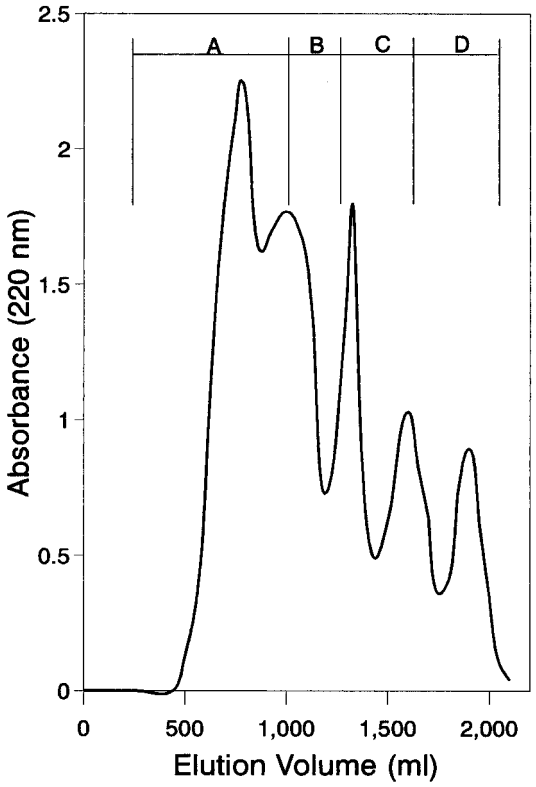

Figure 5. Sephadex G-15 chromatography of SFPH-I.

As can be observed from the data shown in Table 3, the main difference in total amino acid composition of the fractions UC-5 and UF-5 is a higher value of Fischer ratio for the fraction UF-5. This high value is due to a high abundance of peptides rich in BCAA and a low concentration of peptides rich in AAA. The treatment of this fraction with actinase, which has both aminopeptidase and carboxypeptidase activities (Yamashita et al., 1979; Tanimoto et al., 1991), leads to a considerable liberation of basic and aromatic amino acids. Therefore, these amino acids were probably localized at the C terminus of the peptides due to the trypsin- and chymotrypsin-like activities of kerase (Parrado, 1991). Optimal treatment time, for the liberation of AAA, was estimated as 6 h. Prolonged incubation times lead to a marked loss of BCAA (results not shown). The hydrolysate obtained under these conditions was designated SFPH-I. For final purification of the product we used Sephadex G-15 chromatography (Yamashita et al., 1979; Tanimoto et al., 1991), a hydrophobic-like chromatography (Janson, 1967). Due to the hydrophobic nature of some parts of the Sephadex molecule, the hydrophobic molecules of the sample are retarded in their elution with respect to similar MW hydrophilic molecules (Janson, 1967). The elution profile of SFPH-I on a Sephadex G-15 column is shown in Figure 5. As can be observed, the eluate was divided into four fractions (A−D). Free and bound amino acid analysis of each fraction demonstrated that fraction A is composed of peptides with molecular weight <5000, fraction B is composed mainly of free amino acids, and fractions C and D are exclusively free phenylalanine and tyrosine, respectively.

These results show that the final treatment of SFPH-I by chromatography on Sephadex G-15 leads to a marked reduction in the content of aromatic amino acid in fraction A. In this sense the Fischer ratio increased considerably from a value of 5.52 in the UF-5 to a final value of 20.47 in fraction A (Table 3). For patients with hepatic diseases, mainly encephalopathies, it is important that the total AAA content is below 2% by weight and that the Fischer ratio (BCAA/AAA) exceeds 20 (Okita et al., 1985). Both conditions are satisfied by fraction A.

Table 4. Overall Mass Balance of the Process

	wt (g)	vol (mL)	protein (g)	yield (%)
SFPC	100		58.3	100
SFPH		5160	37.3	64.0
UF-5		7980	32.8	56.3
fraction A		8250	14.4	24.8

Finally, Table 4 shows the overall mass balance for the process. These data show that with this method, based on enzymatic treatments followed by membrane separation and specific adsorption processes, it is possible to obtain a low molecular weight hydrolysate with a high Fischer ratio (20.47) at a yield of 24.8% on a nitrogen basis. Such a high Fischer ratio and reasonable yield would allow a practical application of this method to obtain such hydrolysates on an industrial scale.

LITERATURE CITED

Adachi, S.; Kimura, Y.; Murakami, K.; Matsuno, R.; Yokogoshi, H. Separation of peptide groups with definite characteristics from enzymatic protein hydrolysate. *Agric. Biol. Chem.* **1991**, *55*, 925−932.

Adibi, S. A. Glycyl dipeptides: new substrates for protein nutrition. *J. Lab. Clin. Med.* **1989**, *113*, 665−673.

Bidlingmeyer, B. A.; Cohen, S. A.; Tarvin, T. L. Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr.* **1984**, *336*, 93−104.

Buzinco, L.; Cantani, A.; Longhi, M. A.; Giampietro, P. G. Anaphylactic reactions to cow's milk whey protein hydrolysate (Alpha-Ré, Nestlé) in infant with cow's milk allergy. *Ann. Allergy*, **1989**, *62*, 333−335.

Fisher, J. E.; Rosen, H. M.; Ebeid, A. M.; James, J. H.; Keane J. M.; Soeters, P. B. The effect of normalization of plasma amino acids on hepatic encephalopathy in man. *Surgery* **1976**, *80*, 77−91.

Hermann, G.; Schwarz, A.; Wandrey, Ch.; Kula, M-R.; Knaup, G.; Drauz, K. H.; Berndt, H. Scale-up of enzymatic peptide synthesis in an enzyme membrane reactor. *Biotechnol. Appl. Biochem.* **1991**, *31*, 346−353.

Janson, J.-C. Adsorption phenomena on Sephadex. *J. Chromatogr.* **1967**, *28*, 12−20.

Kimura, Y.; Nakanishi, K.; and Matsuno, R. Enzymatic synthesis of the precursor of Leu-enkephalin in water-immiscible organic solvent systems. *Enzyme Microb. Technol.* **1990**, *12*, 272−276.

Knights, R. J. Processing and evaluation of antigenicity of protein hydrolysates. In *Nutrition for Special Use in Infancy*; Lifeshitz, F., Ed.; Dekker: New York, 1985; pp 105−118.

Matthews, M. D. Protein absorption—then and now. *Gastroenterology* **1977**, *73*, 1267−1279.

Meredith, J. W.; Ditesheim, J. A.; Zaloga, G. Visceral protein levels in trauma patients are greater with peptide diet than with intact protein diet. *J. Trauma* **1990,** *30*, 825−828.

Milla, P. J.; Kilby, A.; Rassan, U. B.; Ersser, M. L.; Harrires, J. T. Small intestinal absorption of amino acids and a dipeptides in pancreatic insufficiency. *Gut* **1983**, *24*, 818−824.

Nakanishi, K.; Kimura, Y.; Matsuno, R. Kinetics and equilibrium of enzymatic synthesis of peptides in aqueous/organic biphasic systems—thermolysin-catalysed synthesis of *N*-(benzyloxy-carbonyl)-L-aspartyl-L-phenylalanine methyl ester. *Eur. J. Biochem.* **1986**, *161*, 541−545.

Okita, M.; Watanabe, A.; Nagashima, H. Nutritional treatment of liver cirrhosis by branched-chain amino acids-enriched nutrient mixture. *J. Nutr. Sci. Vitaminol.* **1985**, *31*, 291−303.

Parrado, J. Development of an enzymatic process for the use of sunflower meal proteins. Ph.D. Thesis, Universidad de Sevilla, Sevilla, 1991.

Parrado, J.; Bautista J.; Machado, A. Production of soluble enzymatic protein hydrolysate from industrially defatted undehulled sunflower meal. *J. Agric. Food Chem.* **1991**, *39*, 447−450.

Parrado, J.; Millan, F.; Hernandez-Pinzon, I.; Bautista, J.; Machado, A. Characterization of enzymatic sunflower protein hydrolysates. *J. Agric. Food Chem.* **1993**, *41*, 1821−1825.

Schäger, H.; von Jagow, G. Tricine-sodium dedecylsulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **1987**, *166*, 368−379.

Siemensman, A.; Weijer, W. J.; Bak, H. J. The importance of peptide lengths in hypoallergenic infant formulae. *Trends Food Sci. Technol.* **1993**, *4*, 16−21.

Tanimoto, S.; Tanabe, S.; Watanabe, M.; Arai, S. Enzymatic modification of zein to produce a non-bitter peptide fraction with a very high Fisher ratio for patients with hepatic encephalopathy. *Agric. Biol. Chem.* **1991**, *55*, 1119−1123.

Visser, S.; Slangen, Ch. J.; Robben, A. J. P. M. Determination of molecular mass distribution of whey protein hydrolysates by high-performance size-exclusion chromatography. *J. Chromatogr.* **1992**, *599*, 205−209.

Yamashita, M.; Arai, S.; Fujimaki, M. A low-phenyalanine, high-tyrosine plastein as an acceptable dietetic food. *J. Food Sci.* **1979**, *41*, 1029−1032.

Received for review December 5, 1995. Accepted January 2, 1996.[⊗] This work was supported by a grant from the Plan Nacional I+D CICYT (ALI-93/0492) to J.B.

JF940726C

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1996.