Characterization of Protein Hydrolysates Prepared for Enteral Nutrition

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A method is described for the comprehensive characterization of protein hydrolysates for enteral nutrition. It includes (1) gel filtration on a 0.9 × 250 cm column of Sephadex G-25F eluted with 5% acetic acid, to prepare pools; (2) amino acid analysis with and without acid hydrolysis to determine the amounts of peptide bound and free amino acids; (3) reaction with TNBS to determine the amount of NH_2-terminal groups; and (4) calculation of number-average peptide size of pools from TNBS and amino acid data. The preparation of three hydrolysates of casein with pancreatic enzymes on the industrial scale (5000 L) is described. The number-average peptide size distribution of hydrolysate B, Hydrocase, was 18% free amino acids, most of which were essential; 62% peptides containing 2–9 residues; and 20% peptides containing 10–40 residues. The number- and weight-average peptide size distribution of Hydrocase is similar to that reported by the manufacturer of Peptamen, and both differ from the casein hydrolysate in Pregestimil and Nutramigen reported by the manufacturer to contain 50% free amino acids by amino acid analysis.

INTRODUCTION

The use of partially hydrolyzed proteins for the clinical treatment of patients with specific disorders of digestion (Milla et al., 1983), absorption (Silk, 1987), and amino acid metabolism (Arai et al., 1986) has been extended to patients with malnutrition associated with cancer, burns, and trauma (Milla et al., 1983; Meredith et al., 1990) as well as for the nutritional support of children with chronic and acute diarrhea or allergies to milk proteins (Knights, 1985; Buzinco et al., 1989). The rationale for the use of protein hydrolysates to facilitate absorption is based on independent uptake systems for amino acids and peptides (Matthews, 1977). The peptide systems are size specific and favor the rate of uptake of di- and tripeptides (Silk et al., 1980; Grimble et al., 1987) and their transport (Adibi, 1989). Enteral administration of hydrolysates has some advantages over parenteral administration of amino acid mixtures because the continuous utilization of the digestive tract prevents hypotrophy (Silk, 1986a,b) and because of lower cost and relative ease of administration. The efficacy of enterally administered protein hydrolysates and peptide fractions of defined molecular size has been reviewed in Silk (1986a,b), Webb (1990), and Mobarhan and Trumbore (1991).

Commercially available hydrolysates are usually based on proteins prepared from milk such as casein, lactalbumin, and whey because of their high nutritional value, commercial availability in large quantities, and moderate cost. There are few published studies documenting the characterization of the size distribution of peptides in a total hydrolysate (Crampton et al., 1971; Hernandez and Asenjo, 1982) or the effect of hydrolysis conditions on the peptide size distribution of hydrolysates prepared on the laboratory or industrial scale (Clegg et al., 1974; Hernandez and Asenjo, 1982; Savoie et al., 1988).

The objective of the present paper is to describe a comprehensive method for the characterization of hydrolysates in terms of free and peptide-bound amino acids, as well as the relative size distribution of peptides. We also describe the results of a systematic study of conditions for the industrial-scale preparation of casein hydrolysates with pancreatic enzymes designed to maximize the content of small peptides without significantly increasing the relative amount of free amino acids. The size distributions of three representative hydrolysates prepared on the industrial scale by us are reported, and one, hydrolysate B (Hydrocase), is compared to that reported by the manufacturers of Peptamen, Pregestimil, and Nutramigen.

MATERIALS AND METHODS

Sodium caseinate was purchased from Barbosa and Marques (Governador Valadares, MG, Brazil). The pancreatic proteolytic enzymes used, porcine pancreatin (4NF), Proteomix (2000 USP units/mg of mixture of bovine and porcine trypsin and 400 USP units/mg of mixture of bovine and porcine chymotrypsin), and bovine chymotrypsin (1100 USP units/mg), were products of Biobrás SA (Montes Claros, MG, Brazil). Sephadex G-25F was purchased from Pharmacia (Uppsala, Sweden). Reagents for amino acid analysis, the amino acid mixture Standard H, and individual amino acids were obtained from Pierce Chemical Co. (Rockford, IL) as was trinitrobenzenesulfonic acid (TNBS). Bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, MO), and T-kinin was synthesized by Dr. A. C. M. Paiva, Escola Paulista de Medicina, São Paulo, Brazil. All other chemicals were of reagent grade or equivalent.

Preparation of Casein Hydrolysates. Hydrolysates of casein dispersed in water (16%, w/v) were prepared in a 5000-L reactor at 37–40 °C at pH 7.6–7.8 using ammonium hydroxide to maintain pH. The reaction was stopped by heating, and the

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dispersion was clarified and spray-dried. Thirteen hydrolysates were prepared under different conditions and characterized. Three representative ones documented here were prepared as follows. The quantities of enzymes reported as percent (w/w) relative to casein are given in parentheses, followed by incubation time. Hydrolysate A: pancreatin (0.6) and Proteomix (0.05) for 9 h. Hydrolysate B: pancreatin (1.8) and Proteomix (0.15) for 27 h. Hydrolysate C: pancreatin (3.6), Proteomix (0.6), and chymotrypsin (3.0) for 27 h. One-third of the total amount of enzymes was added to hydrolysates B and C at 0, 9, and 18 h. Note that there are differences of 11-fold in the amount of pancreatic enzymes and of 3-fold in incubation time when A is compared to C, with hydrolysate B occupying an intermediate position in terms of the intensity of hydrolysis conditions.

Characterization of the Hydrolysates. *Amino Acid Analysis.* Total and free amino acids were determined by automatic amino acid analysis (Alonzo and Hirs, 1968) using the method of Spackman et al. (1958). Total amino acids were obtained by analysis after hydrolysis of 3–5 mg for 22 h at 110 °C in 0.500 mL of constant-boiling HCl containing 0.01% phenol in an evacuated sealed tube. Methionine and cysteine were determined as methionine sulfone and cysteic acid, respectively, after performic acid oxidation of 3–5 mg of protein or hydrolysate (Moore, 1963). Tryptophan was determined by amino acid analysis after hydrolysis of 3–5 mg of protein or hydrolysate with lithium hydroxide (Lucas and Sotelo, 1980). No corrections were made for losses of serine, threonine, or tyrosine during acid hydrolysis when the data were reported.

Free amino acids were measured in samples not submitted to acid hydrolysis by the same method. Peak width and the ratio of absorbance at 570 and 440 nm of the amino acid analyzer elution profiles were used to distinguish free amino acids from small peptides. Data for free amino acids are reported as serine plus asparagine and threonine plus glutamine because these pairs of amino acids were not separated by the sodium citrate buffer system used. Since these pairs of amino acids were present in much lower concentrations than other free amino acids, no corrections were made when the data were reported.

Gel Filtration. The hydrolysates were separated into pools after gel filtration on a 0.9 × 250 cm Sephadex G-25F column developed with 5% acetic acid (v/v) at 10 mL/h flow rate of 22 °C, and fractions of 1.5 mL were collected. The sample, 100 mg of hydrolysate, was applied in 2.5 mL of 5% acetic acid containing 1 M NaCl. NaCl was included in the sample solution to prevent electrostatic interaction of positively charged peptides or amino acids with small amounts of carboxyl groups which may have been present due to the oxidation of the resin and to provide a fixed elution volume to compare elution profiles. Solutes were detected in the effluent by (1) absorbance at 280 nm, (2) reaction of 0.100 mL of effluent with ninhydrin before and after alkaline hydrolysis (Hirs, 1967), and (3) conductivity at 22 °C of a 1:50 dilution of the effluent in distilled water.

The Sephadex G-25 column was standardized with bovine albumin, T-kinin (SLRPPGFSPFR), a mixture of amino acids (Standard H, Pierce), 1 M NaCl, and tryptophan (Figure 1, top). The column effluent of the hydrolysates (Figure 1, middle and bottom) was combined into pools indicated below the elution profiles on the basis of the elution position of the standards [albumin (pool 1) and tryptophan (A_{280}, pool 7), tyrosine (A_{280}, pool 5), sodium chloride (conductivity, pool 4)] and on the basis of free and peptide-bound amino acid profiles of the hydrolysates (A_{570}, ninhydrin ± alkaline hydrolysis, pools 2 and 3). The recovery of peptides and free amino acids reported for the gel filtration columns was calculated on the basis of acid hydrolysis of an aliquot of the sample applied to the column and the sum of amino acids present in acid hydrolysates of the pools. The recoveries for 13 columns were 91–106%. Unless otherwise identified, all data are reported in terms of moles.

Peptide Size. Number-average peptide size was determined by dividing the total molar concentration of amino acids after acid hydrolysis by the molar concentration of α-amino-terminal residues present in the same solution measured spectrophotometrically at 420 nm after reaction with TNBS (Spadaro et al., 1979). The average molar absorbtivity of (2.04 ± 0.16) × 10^{-4} M^{-1} cm^{-1} was determined from concentration-response curves of glycine, lysine, Phe-Gly-Gly, Ala-Ala-Ala-Ala, bradykinin, and insulin. Proline, imidazole nitrogens, and the guanidino group of arginine do not react with TNBS (Spadaro et al., 1979; Fields, 1971). The ε-amino groups of lysine do not react with TNBS under the condition employed (data not shown). This procedure does not require the control of reaction time necessary for the TNBS method described by Fields (1971) because the reagent blank (0.060 absorbance at 420 nm after 2 h) was extremely small and essentially constant in relation to the absorbance of the test solution (Spadaro et al., 1979).

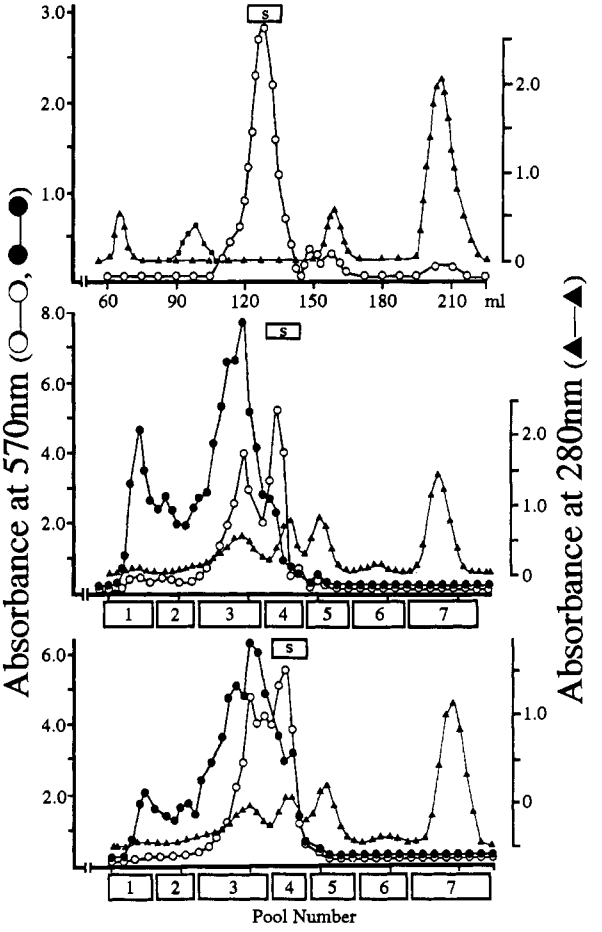

Figure 1. Sephadex G-25 gel filtration of casein hydrolysates. The G-25F column (0.9 × 250 cm) was eluted with 5% (v/v) acetic acid, pH 2.5, at 10 mL/h, 22 °C, and fractions of 1.5 mL were collected. The samples were applied in 3 mL of 5% acetic acid containing 1 M NaCl. (Top panel) The diagram is a composite of two runs: (1) 6 mg of albumin, 0.1 μmol of Ile-Ser-bradykinin, 1.6 μmol of tyrosine, and 1.5 μmol of tryptophan; (2) a Pierce Standard H containing 2.14 μmol of each amino acid. (Middle panel) 100 mg of hydrolysate A. (Bottom panel) 100 mg of hydrolysate B. The boxes containing the letter S above the elution diagram in each panel indicate the elution position of NaCl added to the sample. Pools were formed by combining the effluent indicated by the boxes below each elution diagram. (●) Absorbance at 280 nm; (○) absorbance at 570 nm (ninhydrin reaction without prior alkaline hydrolysis); (◐) absorbance at 570 nm (ninhydrin reaction after alkaline hydrolysis).

Samples containing 5–100 μmol of α-amino groups in 400 μL of H_2O were mixed with 400 μL of 1 M potassium borate buffer, pH 9.2; 200 μL of TNBS, 1.65 mg/mL, was added and allowed to react at room temperature for 30 min. Sodium sulfite (200 μL; 2 M) was then added and the absorbance read at 420 nm within the next 2 h (Spadaro et al., 1979). Data are reported in Table II as number-average peptide size of the pools (amino acid/peptide) corrected for free amino acids. When the correction for free amino acids was employed, the method provided reliable results for pools containing moderate amounts of amino acids (pool 3) but gave higher results than expected for pools containing large amounts of free amino acids (pool 4), presumably because

Table I. Amino Acid Composition of Casein, Casein Hydrolysates, and Free Amino Acids Present in the Hydrolysates[a]

	casein, mol/100 mol		casein hydrolysates: total amino acids, % of col 3			casein hydrolysates: free amino acids, mol/100 mol of specific amino acid		
amino acid	FAO	present study	A	B	C	A	B	C
Lys	6.58	7.22	96	93	103	50	45	59
Leu	8.75	9.13	103	100	103	28	54	53
Ile	6.58	4.52	98	115	108	3	10	12
Met	2.21	2.58	109	111	97	c	27	37
Phe	3.69	4.19	99	96	92	38	43	52
Val	7.06	6.42	90	99	108	3	14	16
Thr	4.78	4.13	107	107	109	c	c	11
Trp	b	1.23	109	106	125	71	64	94
His	2.20	2.29	93	97	101	4	14	19
Arg	2.46	3.18	93	86	101	75	81	74
Asx	5.73	6.51	105	104	110	c	c	c
Ala	4.59	4.22	98	105	109	4	11	17
Ser	7.19	6.18	113	111	108	3	8	8
Glx	19.87	19.38	96	94	95	c	c	2
Pro	12.37	11.17	109	117	98	c	c	c
Gly	3.59	3.19	109	122	117	4	8	9
half-Cys	0.36	0.30	133	173	127	c	c	c
Tyr	3.70	4.05	79	58	35	36	38	34
total free amino acids, %, mol						14	18	21
moisture, % (w/w)		7.67	6.6	6.3	7.9			
ash, % (w/w)		2.57	4.5	4.6	5.6			
protein, % (w/w)		85	82	74	76			

[a] The literature values for casein (column 2) are based on FAO data (FAO, 1970). The data for intact casein used in the present study (column 3) are average values for four lots purchased from 1984 to 1989 and hydrolyzed for 22 h with HCl except for the determination of Trp as described under Materials and Methods. Data for hydrolysates (total amino acids, columns 4, 5, and 6) were obtained in the same manner and are reported as percent of the average amino acid composition (column 3) of the casein from which they were prepared. The data for free amino acids are percent referred to the total amount of each amino acid in each hydrolysate. The data for Thr include Gln, and the value for Ser includes Asn (see Materials and Methods). The values for total free amino acids given in columns 7, 8, and 9 refer to the total hydrolysate as follows: 100 × total free amino acids/total amino acids after acid hydrolysis for each hydrolysate. Note that all data are reported in terms of mol, *not weight*. [b] Not reported by FAO. [c] ≤1.0.

the denominator of the final value depended on the difference between two numbers of similar size.

The number-average peptide size distribution (18%, 62%, and 20%) reported in Table III, column 4, for Hydrocase (hydrolysate B) was obtained from the data in Table II in the following manner. Pools 1 and 2, which contained peptides with number-average sizes of 23 and 21 residues/mol, respectively (Table II, column 5), were assigned to the peptide size range 10–40 residues, and together they account for 20% (mol) of the hydrolysate (Table II, column 4). Pools 3–7, which represent the remaining 80% (mol) of the hydrolysate, contain both peptides and free amino acids which were not completely separated from each other during gel filtration. However, from the data in Table I, column 8, we obtain 18% (mol) for free amino acids in hydrolysate B. A similar result was obtained by measuring free amino acids in pools 3–7. Thus, the peptides in pools 3–7 correspond to 62% (mol) of Hydrocase. The assignment of the peptides in pools 3–7 in the range 2–9 was based on this bracketing approach, their elution position from G-25, and their number-average peptide size (Table II, column 5).

The weight-average peptide size distribution for Hydrocase (hydrolysate B) given in Table III, column 6 (2%, 51%, and 47% for peptides of 1, 2–9, and 10–40 residues/mol, respectively), was calculated from the number-average peptide size distribution [Table III, column 4; 18%, 62%, and 20% (obtained as described in the preceding paragraph)] together with the estimated average molecular weights of 133, 892, and 2548 for the same peptide size groups. The calculation for the weight-average percent of free amino acids in the hydrolysate is

$$100 \times (0.18 \times 133)/[(0.18 \times 133) + (0.62 \times 892) + (0.20 \times 2548)] = 2.2\%$$

The molecular weights were assigned on the following basis: free "average" hydrated amino acid = 133; peptides having 2–9 residues/mol, 7.6 (Table II, column 5) × 115 + 18 = 892; and peptides having 10–40 residues/mol, (21 + 23)/2 (Table III, column 5) × 115 + 18 = 2548, where 115 is the molecular weight of the average anhydro amino acid and 18 corresponds to the molecular weight of 1 H_2O/peptide located at the amino- and carboxyl-terminal positions of the peptide consisting of anhydro amino acids.

The number- and weight-average peptide size distributions of Peptamen in Table III were provided by the manufacturer (Clintec Nutrition Co., 1991). The manufacturer of Pregestimil and Nutramigen has informed us that these products are prepared from the same hydrolysate which contains 50% free amino acids by amino acid analysis (personal communication from Dr. Y-H. Lee and Mr. D. Manes of the Mead Johnson Research Center, Bristol Meyers Squibb Co., Evansville, IN).

RESULTS

Characterization of the Hydrolysate Mixture. The average amino acid compositions of four commercial preparations of intact casein purchased in Brazil over a period of 5 years are reported as moles per 100 mol recovered amino acids in Table I, column 3. The coefficients of variation were within an acceptable range (≤8%) for all amino acids except Leu, Arg, Glx, and Pro, which were ≤16%. Note that the mean values for each amino acid were similar to that reported in the literature (FAO, 1970; compare column 3 with column 2 in Table I) except for Ile, which was somewhat low (4.52 vs 6.58).

The amino acid composition of the hydrolysates (Table I, columns 4–6) did not differ significantly from the average values for the intact casein from which they were prepared, except for a reduction of tyrosine, presumably because of its solubility and removal during clarification, and a systematic overrecovery of half-cystine, glycine, and, in some cases, proline and tryptophan. Note that the data for the hydrolysates are reported as percent of the average value for the intact casein preparations from which they were prepared (column 3, Table I). The hydrolysates

Table II. Distribution of Peptides and Their Number-Average Size in Pools Prepared by Gel Filtration of Casein Hydrolysates[a]

	casein hydrolysates					
	A		B		C	
pool	rel amt, mol %	number-av peptide size, r/mol	rel amt, mol %	number-av peptide size, r/mol	rel amt, mol %	number-av peptide size, r/mol
1	21	20	9	23	8	18
2	17	14	11	21	10	15
3	50	6.5	70	7.6	66	5.8
4	9	2.0	8	1.5	14	1.9
5	1.9	2.0	1.1	1.2	1.3	1.8
7	0.8	2.1	0.8	1.2	0.8	2.0

[a] The pools of hydrolysates A and B are identified in Figure 1, and the pools of hydrolysate C were similar. The data for the relative amount in each pool after acid hydrolysis are reported as percent of the total moles recovered in all pools after hydrolysis (mol %). Number-average peptide size data, reported as residues/peptide (r/mol), were determined by TNBS and amino acid analyses and corrected for free amino acids as described under Materials and Methods. Pool 6 contained <0.1 mol %.

contained 74–82% protein and similar amounts of moisture (3.6–7.9%) and ash (4.0–5.8%). The material not accounted for in the hydrolysates (5–13%) probably consists of carbohydrate (Fiat and Jollès, 1989) and lipids. These data demonstrate that the hydrolysates prepared at the industrial level were not significantly different from the FAO standard (FAO, 1970) or casein available in Brazil used for their preparation (Table I, column 3).

The free amino acid contents (percent mole) of hydrolysates A, B, and C were 14%, 18%, and 21%, respectively. The amount released was related to the quantity of enzyme and duration of hydrolysis (cf. columns 7–9, Table I) for most of the individual amino acids and for the total amount released. The large amounts of basic, aromatic, and hydrophobic amino acids released are due to the specificity (Kasper, 1975; Ambler, 1972) of endo/exopeptidase pairs: trypsin/carboxypeptidase B and chymotrypsin/carboxypeptidase A present in bovine and porcine pancreatic digestive enzymes. Note that for hydrolysate B the essential amino acids Lys, Leu, Met, Phe, Trp, and His are released to the extent of 14–64% (average = 41%), whereas Thr+Gln, Asx, Ser+Asn, Glx, Pro, and Gly are released to a much lesser extent (0–8%, average <3%).

Preparation and Characterization of Pools. The use of gel filtration to separate mixtures into pools provides accurate and detailed information about the size distribution of peptides in the mixtures, not available in data describing the entire mixture. Figure 1 shows the elution diagrams for the separation of standards (top panel), hydrolysate A (middle panel), and hydrolysate B (bottom panel). The pools obtained by combining the effluent are indicated by the blocks below the hydrolysate elution profiles. Albumin and the undecapeptide T-kinin (Ser-Ile-bradykinin) were eluted in pools 1 and 2–3, respectively, whereas most free amino acids were eluted with NaCl in pool 4. The aromatic amino acids tyrosine (pool 5) and tryptophan (pool 7) were adsorbed (Ferreira et al., 1970) to the resin and eluted from this 250-cm column after salt and free amino acids. Phenylalanine was almost completely separated from tyrosine (>89% enrichment of each) by this system (data not shown).

The difference in height between the solid circles (ninhydrin + alkaline hydrolysis) and open circles (direct ninhydrin) is a semiquantitative (Hirs, 1967) indication of peptide size. The ninhydrin-positive peak in the salt position probably corresponds to ammonia added to control pH during preparation of the hydrolysate, since it disappeared after alkaline hydrolysis. The differences between ninhydrin + and − alkaline hydrolysis, taken together with elution position, demonstrate that hydrolysate A (middle panel) contains greater amounts of larger peptides than hydrolysate B (bottom panel). The A_{280} elution diagrams () were similar, with more than half of the UV-absorbing material at 280 nm being due to free tyrosine (pool 5) and free tryptophan (pool 7). Data from the ninhydrin profiles (± hydrolysis) permitted us to combine the effluent into pools on the basis of the distribution of quantities of the peptides and of estimated molecular weight and to identify the elution position of free amino acids. Note that the A_{280} elution profile is not proportional to the distribution of the amount of peptides, especially small peptides, as indicated by ninhydrin + hydrolysis (•) for pools 3 and 4. The A_{280} can be used for detecting proteins in column effluents because proteins have similar contents of aromatic amino acids. Absorbance at A_{280} is not appropriate for mixtures of peptides which, because of their size, may not contain any aromatic amino acids and thus not be detectable at 280 nm.

Distribution of Peptides and Amino Acids in Pools. The data in Table II (columns 2, 4, and 5) provide the distribution of the amounts of peptides and amino acids in each pool. The data are reported as mole percent amino acids after acid hydrolysis in each pool compared to total amino acids recovered (sum of all pools). The G-25 column recoveries were 97%, 102%, and 98% for hydrolysates A, B, and C, respectively. Pools 1–4 account for >96% of the material. Note that the size of the peptides decreases with increasing pool number (read column 3, 5, or 7 from top to bottom). In the series A, B, and C, the amount of larger peptides (pools 1 and 2) decreased from 38% to 20% to 18% and the amount of smaller peptides and free amino acids (pools 3, 4, 5, and 7) increased from 62% to 80% and 82%, respectively, as hydrolysis conditions became more severe (i.e., increased hydrolysis time and quantities of enzymes for A compared to B). However, increased amounts of enzymes (compare B to C) did not significantly improve peptide hydrolysis (pool 3, Table II) or the extent of liberation of free amino acids.

Number-Average Peptide Size of Pools. The data given in Table II (columns 3, 5, and 7) provide number-average peptide size for each pool, for each hydrolysate. Pools 1 and 2, as expected, contained the largest number-average peptide size, which ranged from 14 to 23 residues/peptide, and these peptides corresponded to 18–38% of the total amount (mole) of peptides and amino acids in the hydrolysates. Pool 3, which contained most of the material present in the hydrolysate (50–70%), contained peptides with a number-average size of 6–8 amino acids/mol. Pools 4 and 5 contained mostly free amino acids and some small peptides containing aromatic amino acids which were eluted in this position because of the absorption

Table III. Number- and Weight-Average Distributions of Peptide Size and Free Amino Acids in Peptamen (Pe) and Hydrocase (H) Casein Hydrolysates Used for Enteral Nutrition[a]

		distribution, %			
		by number		by weight	
peptide size	approx MW	Pe	H	Pe	H
1	133	12	18	1	2
2–4	>250 <500	17	62	3	51
5–9	>600 <1100	38	62	18	51
10–40	>1200 <4600	20	20	50	47
>40	>4600	5	0	28	0

[a] Data for Peptamen were furnished by Clintec Nutrition Co. (1991). Number-average peptide size distribution data for Hydrocase, calculated from amino acid analysis and TNBS data in Tables I and II as described under Materials and Methods, are reported as % mol. The weight-average peptide size distribution was calculated from the number-average peptide size distribution as described under Materials and Methods.

of aromatic amino acids to the Sephadex G-25 matrix. Pool 7 contained primarily free tryptophan, which was completely separated from other amino acids and NaCl.

The data in Table II indicate that the hydrolysates B and C were similar in terms of the relative amounts of smaller peptides and that they contained about 50% less of the larger peptides than hydrolsate A. Since hydrolysate C was prepared with 3.7 times more enzyme than hydrolysate B, we conclude that B and C represent limit products of casein. The condition used to prepare hydrolysate B was chosen for the production of Hydrocase, a casein hydrolysate for enteral nutrition.

Comparison of the Size Distribution of Peptides in Hydrocase with Data Provided by the Manufacturers of Hydrocase, Peptamen, Pregestimil, and Nutramigen. The data in Table III permit the comparison of the peptide size distribution of Hydrocase with Peptamen, and indirectly with Pregestimil and Nutramigen, three casein hydrolysate formulations that are commercially available in the United States. Although not directly comparable in detail, the overall distributions are probably reasonably accurate. Peptamen and Hydrocase have similar number-average size distributions containing primarily peptides of 2–9 residues/(55% vs 62%), followed by larger peptides of 10–40 residues/(33% vs 20%) and with 12% vs 18% free amino acids. The weight-average size distributions are less similar. Pregestimil and Nutramigen, in contrast to Hydrocase and Peptamen, contain 50% amino acids, with the remainder being small peptides.

DISCUSSION

The present paper describes a method for the characterization of protein hydrolysates, its application to hydrolysates prepared in a systematic study of the industrial-scale hydrolysis of casein with pancreatic enzymes, and the comparison of one hydrolysate, Hydrocase, with data reported by manufacturers of Peptamen, Pregestimil, and Nutramigen in terms of the relative distribution of peptides by size and the amount of free amino acids.

The proposed method is a combination of several standard techniques of protein chemistry. The method includes (1) gel filtration on Sephadex G-25 to prepare pools, (2) amino acid analysis to determine the amount of peptides and free amino acids, (3) the TNBS reaction to determine the amount of amino terminal end groups, and (4) the number-average peptide sizes calculated from TNBS and amino acid analysis data.

The direct measurement of number-average peptide size provides more accurate data than the use of elution position from conventional gel filtration gels (Silk et al., 1980; Hernandez and Asenjo, 1982; Keohane et al., 1985) or HPLC gel permeation chromatography (Moughan et al., 1990) because aromatic amino acids (free or in peptide bond) adsorb to conventional gels (Ferreira et al., 1970; Figure 1, top) and gel permeation resins (Swegold and Rubin, 1983). Chelation with Cu^{2+} using Chelex 100 (CuII) (Silk et al., 1980) or Sephadex (CuII) (Grimble et al., 1987) has been used to separate small peptides (di-, tri-, and tetrapeptides) by size, but large peptides are not resolved effectively, thus providing only partial characterization of a total hydrolysate mixture. We have found only one study in the literature in which the TNBS reaction and amino acid analysis have been used to determine number-average peptide size, but this was applied to a prefractionated mixture of peptides obtained by ultrafiltration, which was then subjected to gel filtration on Sephadex (CuII) which separated peptides in five groups (free amino acids, di-, tri-, and tetrapeptides, and > tetrapeptides) (Grimble et al., 1986).

UV absorbance at 280 nm does not detect peptides and amino acids that do not contain aromatic amino acids, although this method has been used by several investigators (Hernandez and Asenjo, 1982; Keohane et al., 1985). The limitation of this measurement is illustrated in Figure 1, middle and bottom panels (compare UV absorbance with ninhydrin after alkaline hydrolysis absorbance or compare UV absorbance with amino acid analysis data for each pool given in Table II). The use of lower wavelengths such as 206 or 220 nm does not overcome this limitation because the aromatic residue as well as the peptide bond absorb in the lower UV. The use of the Lowry method (Lowry et al., 1951) by Hernandez and Asenjo (1982) has similar disadvantages because the method measures both peptide bonds and tyrosine content. For these reasons the measurement of peptide concentration by absorbance at 206, 220, or 280 nm or by the Lowry method would overestimate the quantities of peptides containing aromatic residues in comparison with peptides of the same number of residues that do not contain aromatic amino acids. Thus, the direct measurement of quantities of peptides by amino acid analysis and of average peptide size by TNBS and amino acid analysis data in pools prepared by gel filtration is the most accurate method available today to characterize hydrolysates short of separating and enumerating all of the constituents. We are not aware of any study in the literature which uses all of the methods cited here for the complete characterization of a hydrolysate. The proposed method could be made more accurate by increasing the number of pools to obtain narrower molecular weight ranges.

The three hydrolysates documented in this paper illustrate representative industrial-scale conditions used in an attempt to force hydrolysis to provide high yields of small peptides without forming unusually large amounts of free amino acids, to be able to make use of the independent systems available for peptide and amino acid uptake and utilization during enteral nutrition (Matthews, 1977). Increased amounts of enzymes (3×) and incubation time (3×) made a significant difference in the size

distribution of peptides when B is compared to A (Table II). However, the attempt to force the reaction (compare C to B) by increasing enzyme content 3.7-fold did not significantly alter the distribution of the size of the products or the amount of free amino acids liberated. This apparent "limit" mixture of casein products is due, in part, to the amino acid sequence of the casein chains (Fiat and Jollès, 1989) because hydrolysis is limited by the specificity of the pancreatic enzymes used. For example, pancreatic enzymes do not contain peptidases capable of hydrolyzing the peptide bonds adjacent to proline (Greene et al., 1963). A second important factor leading to formation of a limit mixture is transpeptidation (i.e., resynthesis of peptide bonds by proteolytic enzymes) due to the high concentration of the substrate dispersion, for which 16% casein (w/v) corresponds to 7 mM for casein forms of molecular mass of about 22 kDa (Fiat and Jollès, 1989), if all were soluble. Both peptide concentration and transpeptidation product insolubility would favor resynthesis of peptide bonds by transpeptidation (Northrop, 1948; Adler-Nissen, 1986). Note that the formation of peptides with new sequences could be favored by relative insolubility and that parts of these peptides could be solubilized by subsequent hydrolysis.

Since hydrolysate C was not significantly different from hydrolysate B, especially with respect to the amount of free amino acids and larger peptides, the use of less enzyme is more economical and leads to lower amounts of pancreatic enzymes and their autolysis/hydrolysis products being included in the hydrolysate, since they may be antigenic. For these reasons process B is used to prepare Hydrocase.

Increased enzyme concentration did, however, increase the release of free amino acids (Table I, compare A with B or C), and it is important to point out that there is selectivity for the rapid release of relatively large amounts of many essential amino acids compared to nonessential amino acids. This is due to the specificity of the proteolytic enzymes. If we may present a teleological argument, it makes sense to release free essential amino acids first during the duodenal phase of digestion after food intake.

A final consideration concerns the amino acid composition of the hydrolysates. The data in Table I (compare column 4, 5, or 6 with column 3) indicate that the hydrolysates closely resemble the amino acid composition of the protein from which they were prepared, except for tyrosine, which probably reflects the low solubility of this amino acid. If a large number of insoluble peptides had been generated as products, and lost during the clarification step, the amino acid composition of the product would not have coincided with casein.

The number- and weight-average size distribution of hydrolysate B (Hydrocase) calculated from the data in Table II as described under Materials and Methods may be compared to that reported by the manufacturers of Peptamen, Pregestimil, and Nutramigen (Table III). Note that this is an overall comparison, because different methods were used to characterize hydrolysates. The number- and weight-average amino acid and peptide size distributions of Hydrocase resemble that reported by the manufacturer of Peptamen, except that the former contains smaller amounts of larger peptides which is reflected in the weight-average size distribution. In contrast, Pregestimil and Nutramigen contain about 50% free amino acids by amino acid analysis and small peptides, compared to 12% and 18% by number and 1% and 2% by weight for Peptamen and Hydrocase, respectively.

It would be useful for making comparisons if all manufacturers used the same methods to characterize these complex mixtures of peptides and amino acids.

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LITERATURE CITED

Adibi, S. A. Glycyl dipeptides: new substrates for protein nutrition. *J. Lab. Clin. Med.* **1989**, *113* (6), 665–673.

Adler-Nissen, J. *Enzymatic hydrolysis of food proteins*; Novo Industri S/A, Elsevier Applied Science Publishers: London, 1986; pp 9–10.

Alonzo, N.; Hirs, C. H. W. Automation of sample application in amino acid analyzers. *Anal. Biochem.* **1968**, *23*, 272–288.

Ambler, R. P. Enzymatic hydrolysis with carboxypeptidases. *Methods Enzymol.* **1972**, *25*, 143–154.

Arai, S.; Maeda, A.; Matsumura, M.; Hirao, N.; Watanabe, M. Enlarged-scale production of a low-phenylalanine peptide substance as a foodstuff for patients with phenylketonuria. *Agric. Biol. Chem.* **1986**, *50* (11), 2929–2931.

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

Clegg, K. M.; Smith, G.; Walker, A. L. Production of an enzymic hydrolysate of casein on a kilogram scale. *J. Food Technol.* **1974**, *9*, 425–431.

Clintec Nutrition Co. "Carnation Peptamen Liquid, Isotonic, Complete Elemental Diet: Clinical Rationale"; Company bulletin, 1991; Clintec Technologies Inc., Three Parkway North, Suite 200, P.O. Box 760, Deerfield, IL 60015-0760.

Cramptom, R. F.; Gangolli, S. D.; Simson, P.; Matthews, D. M. Rate of absorption by rat intestine of pancreatic hydrolysates of protein and their corresponding amino acid mixtures. *Clin. Sci.* **1971**, *41*, 409–417.

FAO. *Amino acid content of foods and biological data on proteins*; Nutritional Studies 24; Food Policy and Food Science Service, Nutrition Division FAO, FAO: Rome, 1970; 283 pp.

Ferreira, S. H.; Bartelt, D. C.; Greene, L. J. Isolation of bradykinin potentiating peptides from *Bothrops jararaca* venom. *Biochemistry* **1970**, *9*, 2583–2593.

Fiat, A. M.; Jollès, P. Casein of various origins and biologically active casein peptides and oligosaccharides. Structural and physiological aspects. *Mol. Cell. Biochem.* **1989**, *87*, 5–30.

Fields, R. The measurement of amino groups in protein and peptides. *Biochem. J.* **1971**, *124*, 581–590.

Greene, L. J.; Hirs, C. H. W.; Palade, G. On the protein composition of bovine pancreatic zymogen granules. *J. Biol. Chem.* **1963**, *238*, 2054–2070.

Grimble, G. K.; Keohane, P. P.; Higgins, B. E.; Kaminski, M. V., Jr.; Silk, D. B. A. Effect of peptide chain length on amino acid and nitrogen absorption from two lactalbumin hydrolysates in the normal human jejunum. *Clin. Sci.* **1986**, *71*, 65–69.

Grimble, G. K.; Rees, R. G.; Keohane, P. P.; Cartwright, T.; Desreumaux, M.; Silk, D. B. A. Effect of peptide chain length on absorption of egg protein hydrolysates, in the normal human jejunum. *Gastroenterology* **1987**, *92*, 136–142.

Hernandez, R.; Asenjo, J. A. Production and characterization of an enzymatic hydrolysate of skim milk lactose and proteins. *J. Food Sci.* **1982**, *47*, 1895–1899.

Hirs, C. H. W. Detection of peptides by chemical methods. *Methods Enzymol.* **1967**, *11*, 325–329.

Kasper, C. B. Fragmentation of protein for sequence studies and separation of peptides mixture. In *Protein sequence determination*; Needleman, S. B., Ed.; Springer-Verlag: Berlin, 1975; Chapter 5, pp 114–161.

Keohane, P. P.; Grimble, G. K.; Brown, B.; Spiller, R. C.; Silk, D. B. A. Influence of protein composition and hydrolysis method on intestinal absorption of protein in man. *Gut* **1985**, *26*, 907–913.

Knights, R. J. Processing and evaluation of the antigenicity of protein hydrolysates. In *Nutrition for special needs in infancy*; Lifeshitz, F., Ed.; Dekker: New York, 1985; pp 105–118.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

Lucas, B.; Sotelo, A. Effect of different alkalies, temperature and hydrolysis times on tryptophan determination of pure proteins and of foods. *Anal. Biochem.* **1980**, *109*, 192–197.

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* (7), 825–828.

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

Mobarhan, S.; Trumbore, L. S. Enteral tube feeding: a clinical perspective on recent advances. *Nutr. Rev.* **1991**, *49* (5), 129–140.

Moore, S. On the determination of cystine as cysteic acid. *J. Biol. Chem.* **1963**, *238*, 235–237.

Moughan, P. J.; Pedraza, M.; Smith, W. C.; Williamsm, M.; Wilson, M. N. An evaluation with piglets of bovine milk, hydrolyzed bovine milk, and isolated soybean proteins included in infant milk formulas. I. Effect on organ development, digestive enzyme activities, and digestibility. *J. Pediatr. Gastroenterol. Nutr.* **1990**, *10*, 385–394.

Northrop, J. H. Enzymes and the synthesis of proteins. In *Crystalline enzymes*; Dunn, L. C., Ed.; Columbia University Press: New York, 1948; pp 222–251.

Savoie, L.; Galibois, I.; Parent, R.; Charbonneau, R. Sequential release of amino acid and peptide during in vitro digestion of casein and rapeseed proteins. *Nutr. Res.* **1988**, *8*, 1319–1326.

Silk, D. B. A. Diet formulation and choice of enteral diet. *Gut* **1986a**, *27* (Supp. 1), 40–46.

Silk, D. B. A. Future of enteral nutrition. *Gut* **1986b**, *27* (Suppl. 1), 116–122.

Silk, D. B. A. Towards the optimization of enteral nutrition. *Clin. Nutr.* **1987**, *6*, 61–74.

Silk, D. B. A.; Fairclough, P. D.; Clark, M. L.; et al. Use of a peptide rather than a free amino acid nitrogen source in chemically defined "elemental diet". *J. Parenter. Enteral Nutr.* **1980**, *4*, 548–553.

Spackman, D. H.; Stein, W. H.; Moore, S. Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.* **1958**, *30*, 1190–1206.

Spadaro, A. C. C.; Draghetta, W.; Del Lama S. N.; Camargo, A. C. M.; Greene, L. J. A convenient manual trinitrobenzenesulfonic acid method for monitoring amino acids and peptides in chromatography column effluent. *Anal. Biochem.* **1979**, *96*, 317–321.

Swegold, G. D.; Rubin, C. S. High performance gel permeation chromatography of polypeptides in a volatile solvent: rapid resolution and molecular weight estimation of proteins and peptides on a column of TSK-G3000-PW. *Anal. Biochem.* **1983**, *131*, 295–300.

Webb, K. E., Jr. Intestinal absorption of protein hydrolysis products. A review. *J. Anim. Sci.* **1990**, *68*, 3011–3022.

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