Analysis of Protein Hydrolysates. 2. Characterization of Casein Hydrolysates by a Rapid Peptide Quantification Method

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Casein hydrolysates prepared in the laboratory or purchased from a manufacturer were characterized for the proportion of amino acids in free form, in short-chain (di- and tripeptides) or larger peptides. The hydrolysates were fractionated by size exclusion chromatography on a poly(2-hydroxyethylaspartamide)–silica column (PHEA), and the fractions were analyzed by amino acid analysis. This led to an accurate characterization of hydrolysates which allowed a classification of the hydrolysates according to the extent of hydrolysis and their nutritional quality. A rapid method for quantifying peptides in SE-HPLC fractions was also proposed. It was based on UV absorbance measurement at 230 nm, with a correction for the absorbance of aromatic amino acid measured at three wavelengths (230, 280, and 300 nm). A good correlation was obtained between corrected areas and the amino acid analysis. Because of nonideal size exclusion behavior on the PHEA column, further analysis of free amino acids would be required to correct the data obtained from the chromatographic patterns. The quantitative technique described here proved to be a valuable tool for characterizing unknown protein hydrolysates.

Keywords: *Chromatography; peptide quantification; UV spectrophotometry; casein hydrolysates*

INTRODUCTION

Interest in protein hydrolysates has increased over the past few years, since it has been shown that preparations containing a predominance of short-chain peptides from partially hydrolyzed proteins could be utilized more efficiently and have a higher nutritive value than an equivalent mixture of free amino acids (Grimble et al., 1986). Moreover, free tyrosine and free cystine are poorly soluble, while free glutamine and free cysteine are unstable during sterilization procedures and storage (Furst et al., 1990). Thus, it is important to substitute these free amino acids with soluble and stable short-chain peptides containing glutamine, cystine, and tyrosine (Adibi, 1987). The advantage of protein hydrolysates having low free amino acid and high short-chain peptide content is also related to the fact that a lower number of small molecules in the same volume leads to a decrease in osmolality. This avoids harmful effects, such as diarrhea and dehydration, particularly in children (Deardoff, 1980; Lanssade and Hamon, 1983). Therefore, the nutritional quality of protein hydrolysates depends on the initial protein, which determines their amino acid composition, and also on their small peptide content. There is also firm evidence that hydrolysate formulas are useful for feeding patients with clinically manifest milk intolerance, since the allergenic potential of milk-based formula is also related to peptide chain length (Seban et al., 1977; Jost et al., 1991).

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Different techniques have been used to characterize protein hydrolysates. Some of them are based on the direct determination of the α-amino nitrogen/total nitrogen ratio, α-amino nitrogen being measured by different official methods (AOAC, 1980; *French Pharmacopoeia*, 1989). Others consist in estimating the proportion of free amino acids in the hydrolysates (Armstead and Ling, 1991) or the free amino group proportions (Silvestre et al., 1993). However, all of these "direct" techniques give only a partial picture of the composition of these complex mixtures. In a different way, many techniques of characterization are based on the separation of hydrolysate constituents followed by their quantitative measurement. Size exclusion chromatography (SEC), especially when applied in high-performance mode, has been reported as an attractive procedure for investigating peptide profiles in protein hydrolysates (Amiot and Brisson, 1980; Barth, 1982; Pellerin et al., 1985; Vijayalakshmi et al., 1986; Lemieux et al., 1991; Visser et al., 1992). Silvestre et al. (1994) showed that a poly(2-hydroxyethylaspartamide)–silica column (PHEA) used in size exclusion high-performance liquid chromatography mode (SE-HPLC) is efficient for separating peptides in casein hydrolysates by size, especially when the peptides have a molecular mass lower than 1000 Da. However, even when a suitable separation of free amino acids from peptides has been achieved, the peptide quantification commonly made by using methods based on amino acid analysis (Verneuil et al., 1990; Aubry et al., 1992) or on the determination of nitrogenous compounds (Amiot and Brisson, 1980) remains a long and arduous step in the analytical process. Faster techniques, based on UV absorbance

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measurements, are quantitatively inaccurate owing to the higher absorbance of aromatic amino acids (Hernandez and Asenjo, 1982; Keohane et al., 1985).

In the present study, we characterized eight casein hydrolysates, either prepared in the laboratory or purchased from a manufacturer. From these data we designed a rapid method for quantifying peptides in SE-HPLC fractions of casein hydrolysates eluted from a PHEA column. The method was based on UV absorbance measurements at 230 nm that had also incorporated a correction for the higher absorbance of aromatic amino acids.

MATERIALS AND METHODS

Materials. Amino acid analyses were performed with a Biotronik LC 3000 analyser (Biotronik, Maintal, Germany). The HPLC system consisted of two M 510 pumps, a Wisp 710 injector, and a Lambda Max M 481 spectrophotometer (Waters, Milford, MA). The system was coupled to a computer equipped with Baseline 810 software (Waters). A PHEA column, 250 × 9.4 mm, 5 μm, 200 Å pore size (PolylC, Columbia, MD), was used for HPLC.

Hydrochloric and formic acids (98–100%, analytical grade) were obtained from Merck (Darmstadt, Germany). For HPLC, water was purified by passing through a Milli-Q water purification system (Millipore, Belford, MA). All solvents used for HPLC were carefully degassed by sonication for 10 min prior to use. Commercial casein hydrolysates (H1, H2, and H3) were provided by Nutripharm-Gallia (Steenvoorde, France).

Methods. *Preparation of Casein Hydrolysates.* Five hydrolysates were prepared from bovine whole casein as described elsewhere (Silvestre et al., 1994). A solution of whole casein 1% (w/v) in 0.01 M phosphate buffer (pH 7.5) was subjected to proteolysis by pancreatin (P1, P2, P3) or trypsin (T1, T2) at 37 °C as described by Silvestre et al. (1994). These five preparations will hereafter be referred to as standard hydrolysates.

Fractionation of Casein Hydrolysates by High-Performance Size Exclusion Chromatography (SE-HPLC). The fractionation of casein hydrolysates was carried out on a PHEA column, as described elsewhere (Silvestre et al., 1994) using 0.05 M formic acid as the mobile phase at a flow rate of 0.5 mL/min. Fifty microliters of 0.4% (standard hydrolysates) or 0.8% (commercial hydrolysates) solutions was injected on the column. Peptides were detected at three wavelengths: 230, 280, and 300 nm. Four fractions (F1–F4) were collected after each run: F1, from 13.5 to 18 min; F2, from 18 to 21.5 min; F3, from 21.5 to 22.5 min; and F4, from 22.5 to 32 min.

Amino Acid Analysis. An aliquot of SE-HPLC fractions was evaporated in a Speed-Vac concentrator (Savant, Hicksville, NY) and hydrolyzed (5.7 N HCl, 110 °C, 24 h). Another aliquot was only evaporated. Both hydrolyzed and nonhydrolyzed fractions were dissolved in 0.1 M citrate buffer (pH 2.2) before analysis. Tryptophan concentrations were determined in nonhydrolyzed fractions only.

Estimation of the Corrected Fraction Area. Absorbances of tryptophanyl and tyrosyl residues at 230 nm are higher than those of other amino acid residues. Thus, to quantify peptides in the eluate using the absorbance measurement at 230 nm, the removal of the contribution of these amino acids from each fraction is required.

Peptide absorbance was further measured at 300 and 280 nm (at 300 nm only tryptophanyl residues absorb; at 280 nm both residues absorb). Therefore, for all hydrolysates, the area of all fractions (F1–F4) was integrated at three wavelengths. The well-resolved tyrosine and tryptophan peaks were also integrated at three wavelengths. This is illustrated for the P1 hydrolysate in Figure 1. The ratios of peak area (PA) of free tyrosine (fTyr) and free tryptophan (fTrp) at these wavelengths can be calculated as follows:

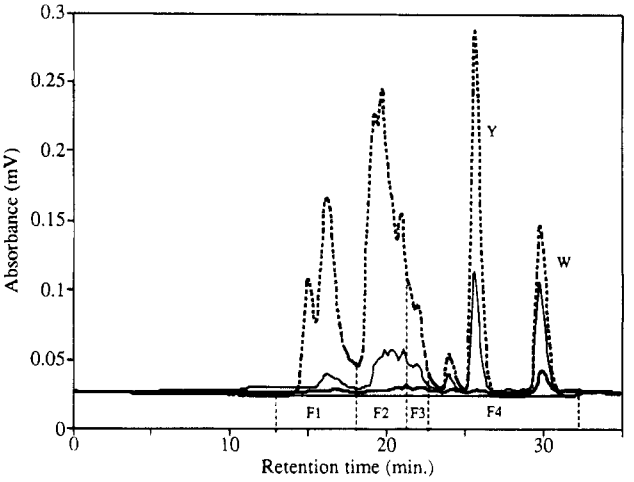

Figure 1. SEC of pancreatin casein hydrolysate P1 at 230 (broken line), 280 (thin solid line), and 300 nm (heavy solid line): Y, tyrosine peak; W, tryptophan peak. PHEA column: mobile phase, 0.05 M formic acid; flow rate, 0.5 mL/min; collected fractions, F1 (13.5–18 min), F2 (18–21.5 min), F3 (21.5–22.5 min), and F4 (22.5–32 min).

$$XY = (PA\ fTyr\ 230\ nm/PA\ fTyr\ 280\ nm)$$

$$XW = (PA\ fTrp\ 230\ nm/PA\ fTrp\ 300\ nm)$$

$$YW = (PA\ fTrp\ 280\ nm/PA\ fTrp\ 300\ nm)$$

Then the peak area (PA) due to tryptophanyl (Trp) and tyrosyl (Tyr) residues in each fraction at 230 nm can be calculated as follows:

1. PA Trp 230 nm = FA 300 nm (XW)

2. PA Tyr 230 nm calculation requires three steps

a. PA Trp 280 nm = FA 300 nm (YW)

b. PA Tyr 280 nm = FA 280 nm − PA Trp 280 nm

c. PA Tyr 230 nm = PA Tyr 280 nm (XY)

FA is the area of each fraction at the specified wavelength.

The corrected fraction area (CFA) is finally calculated by subtracting the sum of PA Trp 230 and PA Tyr 230 from the area of each fraction at 230 nm (FA 230):

$$CFA = FA\ 230\ nm - (PA\ Trp\ 230\ nm + PA\ Tyr\ 230\ nm)$$

RESULTS AND DISCUSSION

Characterization of Casein Hydrolysates. *Fractionation by SE-HPLC.* The chromatographic patterns of standard and commercial casein hydrolysates are shown in Figures 2 and 3, respectively. As described elsewhere (Silvestre et al., 1994), the casein hydrolysates were resolved in four fractions (F1–F4). The mean number of amino acid residues of peptides determined by the trinitrobenzenesulfonic acid (TNBS) assay was shown (Silvestre et al., 1994) to vary in fraction 1 from 7.5 to 9.2 (large peptides), in fraction 2 from 3.7 to 7.7 (medium peptides), in fraction 3 from 1.7 to 3.4 (small peptides), and in fraction 4 from 1.0 to 1.7 (free amino acids).

The last two peaks in the elution pattern of fraction 4 of pancreatin hydrolysates (Figure 2a–c) correspond to tyrosine (peak Y) and tryptophan (peak W). These two peaks were not observed in the tryptic hydrolysates (Figure 2d,e). The chromatographic patterns of com-

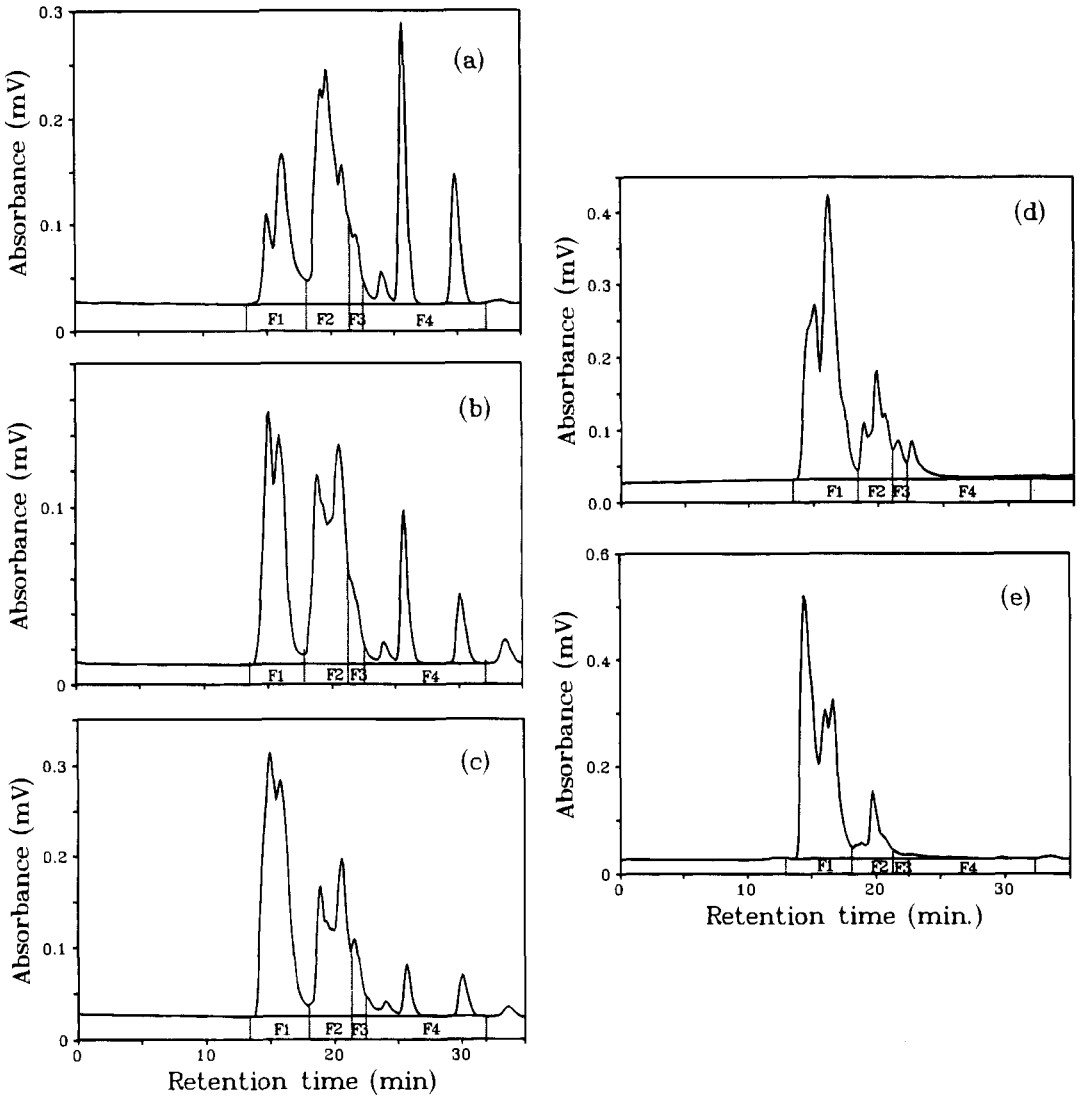

Figure 2. SEC of pancreatin casein hydrolysate P1 (a), P2 (b), and P3 (c) and of tryptic casein hydrolysates T1 (d) and T2 (e). PHEA column: mobile phase, 0.05 M formic acid; flow rate, 0.5 mL/min; detection at 230 nm; collected fractions, same as in Figure 1.

mercial hydrolysates (Figure 3) looked like those of standard pancreatin hydrolysates. However, an inversion of the relative abundance of the tyrosine and tryptophan is observed. Considering that the tyrosine/tryptophan ratio in whole casein is around 5.7 and that absorbance of tyrosine at 230 nm is about 1.2 times greater than that of tryptophan, the higher level of tryptophan in commercial hydrolysate suggests a tryptophan supplementation. This supplementation could increase the nutritional quality of casein hydrolysates which need to be enriched with tryptophan to compensate for its low content in the protein molecule as well as its losses during processing. Moreover, a tryptophan addition would be important for preparing products capable of reducing infant sleep latency, *i.e.*, the time taken to fall asleep (Steinberg et al., 1992).

Amino Acid Analysis of SE-HPLC Fractions. The amino acid analyses of the fractions collected from casein hydrolysates are displayed in Table 1. No free amino acids were detected in fractions 2 and 3. Therefore, although the di- and tripeptides were not separated into a sharp peak (F3 in Figures 2 and 3), the small peptide fractions were not contaminated by free amino acids. The amino acids of the nonhydrolyzed F1 fraction correspond to the free basic amino acids (Lys, Arg, and His), and the difference observed between the composition of hydrolyzed and nonhydrolyzed F4 fractions is due to the occurrence of small acidic peptides. In fact, we have shown previously (Silvestre et al., 1994) that when PHEA support is used, the basic amino acids elute earlier (in fraction 1) and the small acidic peptides, *e.g.* Asp-Glu, elute later (in fraction 4) than expected.

Considering this nonideal size exclusion behavior, additional calculations are required to obtain a true size-sorted peptide composition of casein hydrolysates (Table 2). Thus, basic amino acids (nonhydrolyzed F1 frac-

Table 1. Amino Acid Content of Hydrolyzed (F1h–F4h) and Nonhydrolyzed (F1–F4) Fractions from Casein Hydrolysates[a]

hydrolysate	fraction							
	F1	F1h	F2	F2h	F3	F3h	F4	F4h
P1	10.3	39.7		44.6		2.6	13.3	12.9
P2	7.5	59.6		28.1		2.0	9.4	10.1
P3	1.8	73.4		18.4		2.1	5.7	6.0
T1	2.5	80.6		12.5		1.3	5.2	5.5
T2	1.8	87.4		9.1		0.7	2.5	2.7
H1	0.7	56.7		37.2		2.9	1.3	3.0
H2	3.4	61.1		33.7		2.3	2.3	2.9
H3	7.8	35.6		38.9		3.4	17.5	22.0

[a] The data are reported as percent of total number of amino acid moles found in the four hydrolyzed fractions.

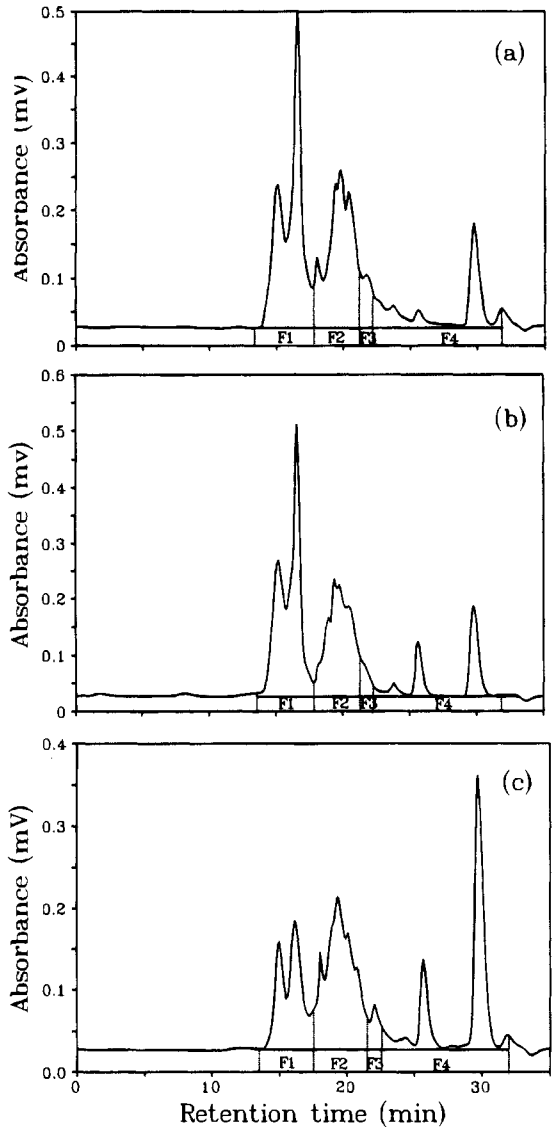

Figure 3. SEC of commercial casein hydrolysate H1 (a), H2 (b), and H3 (c). PHEA column: mobile phase, 0.05 M formic acid; flow rate, 0.5 mL/min; detection at 230 nm; collected fractions, same as in Figure 1.

Table 2. True Peptide Composition of Casein Hydrolysates[a]

hydrolysate	large peptides (>7 residues)	medium peptides (4–7 residues)	small peptides (2–3 residues)	free amino acids
P1	29.4	44.6	2.6	23.7
P2	52.1	28.1	2.8	16.9
P3	71.6	18.4	2.4	7.6
T1	78.2	12.5	1.6	7.6
T2	85.6	9.1	0.9	4.3
H1	56.1	37.2	4.6	2.0
H2	57.7	33.7	2.9	5.6
H3	27.7	38.9	7.8	25.4

[a] The data are reported as percentage of total number of amino acid moles found in the casein hydrolysate fractions, after suitable corrections for the nonideal size exclusion behavior on the PHEA support (for more details, see Amino Acid Analysis of SE-HPLC Fractions).

tions) should be subtracted from the amino acids of the hydrolyzed F1 fractions to give the true proportion of large peptide. As the amino acids of the hydrolyzed F4 fractions are the sum of free amino acids and amino acids from small acidic peptides, the concentrations of the latter are obtained by subtracting the values of

Table 3. Relative Proportion of Tyrosyl and Tryptophanyl Residues in Casein Hydrolysate Fractions[a]

		hydrolysate							
fraction		P1	P2	P3	T1	T2	H1	H2	H3
F1	Tyr	4.6	50.6	40.0	91.9	96.9	47.9	8.6	3.6
	Trp	7.8	14.4	35.7	52.0	70.9	16.1	19.8	2.6
F2	Tyr	29.6	18.0	37.8	2.1	0.85	21.9	13.9	21.7
	Trp	23.6	47.7	30.6	34.1	25.7	27.3	22.1	4.7
F3	Tyr	1.0	0.0	7.0	6.0	0.7	3.2	0.1	53.5
	Trp	12.5	7.1	7.1	5.2	2.6	4.1	4.4	0.5
F4	Tyr	64.9	31.4	15.2	0.0	1.6	27.0	77.4	21.1
	Trp	56.1	30.7	26.5	8.7	0.9	52.5	53.8	92.2

[a] The data are reported as percentage of the total area of the four fractions measured at 230 nm, corresponding to tyrosyl (Tyr) or tryptophanyl (Trp) residues.

nonhydrolyzed F4 fractions from those of hydrolyzed F4 fractions. Subsequently, the results obtained for small acidic peptides have to be added to those of hydrolyzed F3 fractions to give the total of small peptide content. Finally, the proportion of free amino acids is obtained by adding the basic amino acids (nonhydrolyzed F1 fraction) to the amino acids of the nonhydrolyzed F4 fraction (*i.e.* for P2 large peptides = 59.6 − 7.5 = 52.1, medium peptides = 28.1, small peptides = 2.0 + (10.1 − 9.4) = 2.7, and free amino acids = 7.5 + 9.4 = 16.9).

As previously reported (Gauthier et al., 1986), the conditions used to hydrolyze the protein molecule influence the size of the peptides released. In fact, an increase in the E/S ratio (P1 in comparison to P2) led to higher medium peptide and free amino acid content and lower large peptide content. The use of pancreatin produced a hydrolysate (P3) which had higher proportions of almost all constituents, except large peptides, than that prepared by using trypsin (T2). This stronger hydrolysis is due to the mixed-enzyme composition of pancreatin. Also, the longest hydrolysis time produced the strongest hydrolysate (P2), having higher contents than P3 in almost all constituents, except for large peptides. These results show that the reaction conditions of protein hydrolysis (number of enzymes, E/S, and time) should be controlled to obtain a molecular weight distribution compatible with the desired end use of the product.

According to the nutritional quality of casein hydrolysates, H3 seems to be the most attractive. Thus, less than 30% of its amino acid content is in the form of large peptides (number of amino acid residues greater than seven). Around 40% is in the form of medium peptides (number of amino acid residues varying from four to seven). These peptides only need an ultimate degradation by the brush border peptidases before they can be absorbed either as free amino acid or as di- or tripeptides. Almost 8% is in directly absorbable di- or tripeptide form and 25% is in free form. The similar peptide composition of P1 might suggest a nutritional quality close to that of H3, but one must note its disadvantageous di- and tripeptide content, 3-fold lower than that of H3. The high level of small peptides of H3 is probably due to the addition of di- or tripeptidylpeptidases in the enzyme mixture used.

Distribution of Tyrosine and Tryptophan in Different Fractions of Casein Hydrolysates. The distribution of tyrosyl and tryptophanyl residues in the different fractions was estimated from the relative areas at 230 nm due to these residues (Table 3). For the pancreatin hydrolysates, the distribution changes with the extent

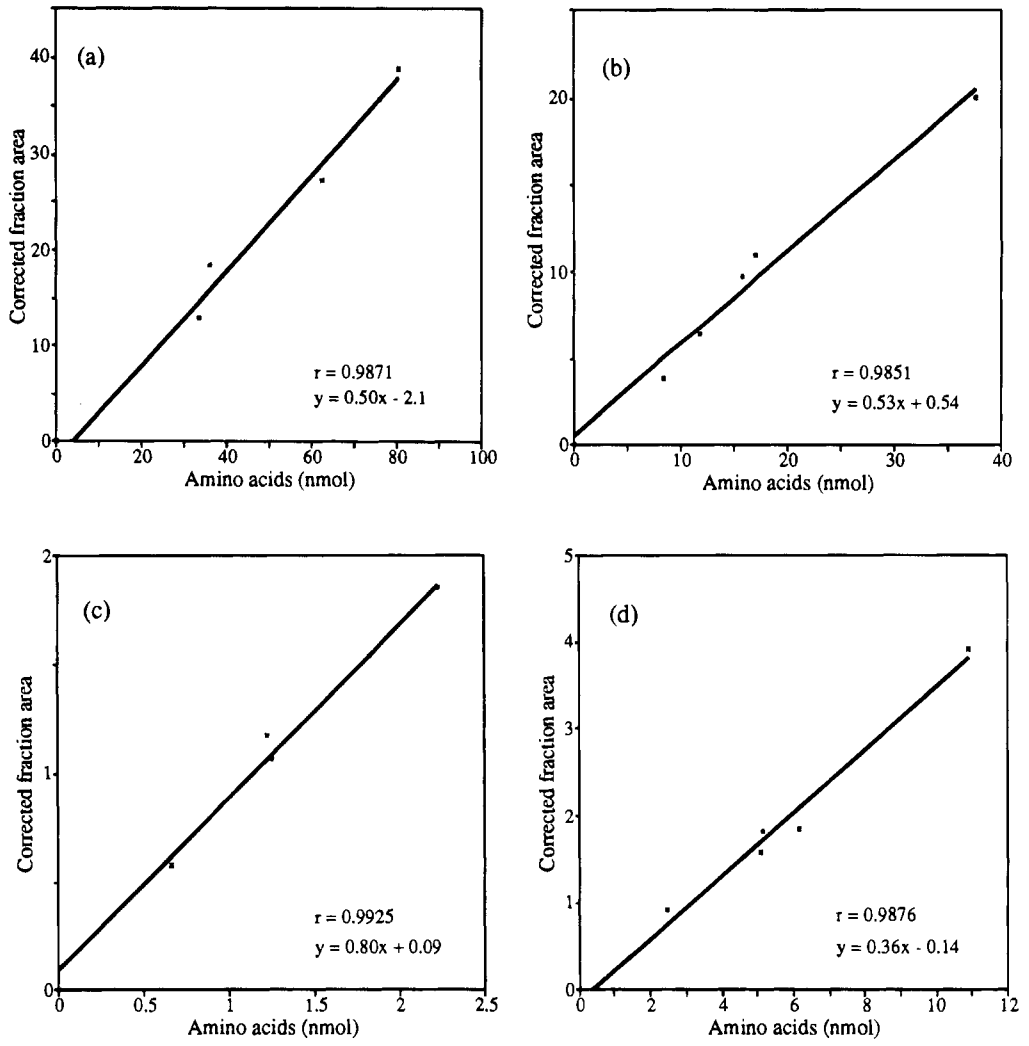

Figure 4. Plots of corrected fraction area *vs* amino acids (nanomoles) of (a) large peptides (F1), (b) medium peptides (F2), (c) small peptides (F3), and (d) free amino acids (F4).

of hydrolysis. Thus, most tyrosine and tryptophan residues are in free form in P1, while in P3 they are mainly in the form of large and medium peptides. In P2, the highest content of tyrosine is in large peptide form and that of tryptophan is in medium peptide form. For the tryptic hydrolysates, increasing both the E/S ratio and the hydrolysis time simultaneously had no effect on the distribution of aromatic amino acids. Most tyrosine and tryptophan were found in the form of large peptides. For the commercial hydrolysates most tyrosine in H1 is in large peptide form, while tryptophan is mainly in free form. In H2 most of these amino acids are in free form, and in H3 tyrosine is mainly in short-chain peptide form, while tryptophan is predominantly in free form.

Considering the poor solubility of free tyrosine, it would be interesting to substitute it with the highly soluble tyrosine-containing short-chain peptides in dietary preparations (Fürst et al., 1990). Note that the proportion of tyrosine contained in small peptides is quite different for all hydrolysates. Within the standard preparations, P3 has the higher value, but H3 is the richest of all products analyzed, with more than 50% of its tyrosine content in the highly soluble small peptide form.

Quantification of Peptides in Casein Hydrolysates: CFA Method. The quantitative method proposed here is based on a correlation between absorbance (chromatographic area) and amino acid content, the determination of amino acids being recognized as the most reliable method for protein and peptide assay.

Correlation between CFA of Standard Hydrolysates and Peptide Content. Owing to the occurrence of various amounts of aromatic amino acid residues in the different chromatographic fractions of the hydrolysates, the raw areas of the fractions at 230 nm poorly correlate with the amino acid determination. Using multidetection at three different wavelengths (230, 280, and 300 nm), it was possible to estimate in each chromatographic fraction, at 230 nm, the area due to the occurrence of tyrosyl and tryptophanyl residues and thereafter to eliminate their interference.

The ratios of peak areas of tyrosine and tryptophan in the pancreatic hydrolysates (P1, P2, P3) measured at three wavelengths were rather similar. The means ± standard deviations for XY, YW, and XW were 3.5 ± 0.1, 5.3 ± 0.1, and 8.9 ± 0.5, respectively. Therefore, these means were used for calculating the CFA of all standard and commercial preparations.

The CFA of standard casein hydrolysates (P1, P2, P3, T1, T2) were plotted against the amino acid content of the hydrolyzed fractions (Figures 4 and 5). A rather good linear relationship was achieved using either the values obtained for each fraction separately (Figure 4) or those found when the four fractions were combined together (Figure 5).

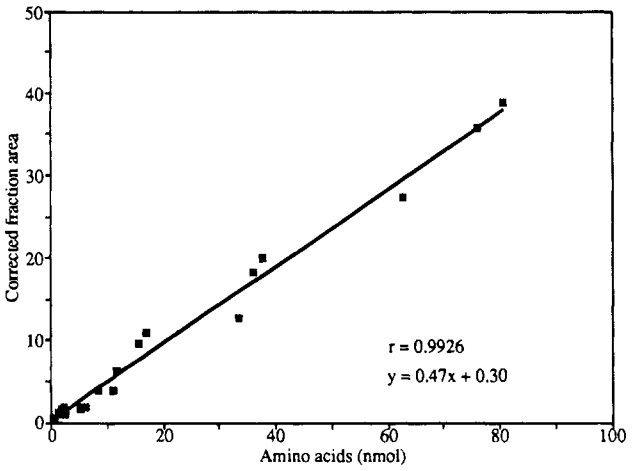

Figure 5. Plot of corrected fraction area *vs* amino acids (nanomoles) of all four fractions (F1–F4).

Table 4. Amino Acid Content of Commercial Hydrolysate Fractions (F1–F4) As Obtained by Amino Acid Analysis and by Corrected Fraction Area (CFA) Method

hydrolysate		amino acid analysis[a]	CFA method[b]	
			each fraction	all 4 fractions
H1	F1	56.7	57.6	52.4
	F2	37.2	37.7	41.9
	F3	2.9	2.8	4.6
	F4	3.0	1.8	1.0
H2	F1	61.1	60.3	55.7
	F2	33.7	34.7	39.1
	F3	2.3	2.9	3.4
	F4	2.9	1.9	1.6
H3	F1	35.6	41.4	37.4
	F2	38.9	45.4	50.2
	F3	3.4	3.6	5.9
	F4	22.0	9.5	6.4

[a] The data are reported as percentage of total number of amino acid moles found in the four hydrolyzed fractions. [b] The results of CFA method were obtained using the standard curves made separately for each fraction or made with the data of all four fractions.

Determination of Peptides in Commercial Hydrolysates. The standard curves developed by the CFA method (Figures 4 and 5) were used to determine the amino acid content in the SEC fractions of commercial hydrolysates. As shown in Table 4, these results are very close to those obtained by the amino acid analysis, notably by using the standard curves made for each separate fraction. Only the content of the free amino acid fraction was underestimated by the standard curves, especially for the H3 hydrolysate. This is probably related to the fact that tyrosine and tryptophan were not taken into account, since the areas due to these residues were removed from the CFA calculation. Moreover, the estimation of small peptides and free amino acids was more accurate when using the standard curves made for each separate fraction rather than that made with all of the data together. In fact, the slopes of the curves obtained with small peptides and free amino acids are different from the others, which are all similar to each other. The degree of polymerization of amino acids probably has some influence on the UV absorbance at 230 nm because of the absorbance of peptide bonds at this wavelength, but the specific amino acid composition of each fraction could also be responsible for this behavior.

Although corrected areas of fractions were well related to amino acid content, the results obtained from the chromatographic patterns did not give the exact peptide composition of the hydrolysates, because of nonideal size exclusion behavior on the PHEA column. Thus, depending on the amount of basic amino acids released during casein hydrolysis, the amount of large peptides is more or less overestimated while that of free amino acids is underestimated. This difference can reach 10% of the total amino acids as in the P1 hydrolysate. This artifact can be corrected by a direct determination of free amino acids in the hydrolysates. The free basic amino acids could then be subtracted from the large peptide fraction. This direct determination of free amino acids would also be more accurate than the estimation obtained by the CFA method.

One problem remains because of the small acidic peptides, which are eluted later than expected from the PHEA column. In the H3 hydrolysate, which contains the highest amount of small acidic peptides, the di- and tripeptide proportion was underestimated by 4.5%. It is difficult to correct for that without determining the amino acid content in the hydrolyzed and nonhydrolyzed F4 fractions. However, when the problems related to the nonideal size exclusion behavior are resolved, the quantitative analysis described here could be used as a valuable alternative to amino acid analysis for characterizing protein hydrolysates. This approach already allows a rapid and good estimation of peptide composition of an unknown protein hydrolysate.

The technique described here could also be used for quantifying tyrosine in free form since it is eluted from the PHEA column as a sharp isolated peak just after the other amino acids. Similarly, the free tryptophan content could easily be estimated because of its delayed elution from the PHEA column. The adsorption of tryptophan on other chromatographic columns was previously reported as an advantageous tool for determining its content in various feedstuffs and in protein hydrolysates (Kowalska, 1969; Landry et al., 1988; Iliev and Tchorbanov, 1992). By calculating the relative areas at 230 nm due to these residues in each fraction, as described under Materials and Methods, it was possible to estimate the distribution of tyrosine and tryptophan in the different fractions of casein hydrolysates.

The hydrolysates used to establish standard curves were prepared from the same protein (casein) as that used for commercial hydrolysates. We did not study hydrolysates of other protein. Because of the specific amino acid composition of each protein, it seems safer that standard and "unknown" hydrolysates should come from the same protein.

Only limited equipment is required for this quantitative method. An HPLC system connected to a variable-wavelength UV detector is sufficient. However, a diode array detector, allowing the simultaneous recording at three wavelengths, and software for data processing are preferable.

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