Analytic and Immunologic Characterization of Chickpea *(Cicer arietinum)* Protein Hydrolysates Obtained by Bromelain and α-Chymotrypsin

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A water soluble concentrate of chickpea *(Cicer arietinum)* proteins was hydrolyzed by bromelain and α-chymotrypsin. Hydrolysis was verified by polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate. The use of ELISA in an inhibition system, conducted with chickpea protein antiserum raised in rabbits, showed that enzymatic hydrolysis resulted in a considerable reduction in the antigenic character of the proteins. Thus, the percentage inhibition by the α-chymotrypsin of hydrolysate was 58 ± 2.3% and that by the bromelain hydrolysate was 45 ± 4.5%. The peptides were measured by size exclusion chromatography. Peptides with molar mass lower than 1000 Da could be fractionated into fraction F1 containing peptides with molar mass higher than 500 Da, fraction F2 containing peptides with molar mass included between 200 and 500 Da, essentially small peptides containing 2, 3, or 4 amino acids, and fraction F3 containing free amino acids. The purified fractions were quantified with the TNBS method (2,4,6-trinitrobenzenesulfonic acid). The small peptides in fraction F2 were separated by reverse phase HPLC and were sequenced.

Keywords: *Chickpea; hydrolysates; bromelain; α-chymotrypsin; antigenicity; ELISA; HP SEC*

INTRODUCTION

Enzymatic hydrolysis methods are the most widely used technological processes for obtaining added value from dietary proteins, increasing their functional and nutritional properties. Proteolysate-based diets are broadly applied in artificial nutrition and pediatrics. In addition to their hypoallergenic properties, their richness in oligopeptides confers on them a higher nutritional value than native proteins or their equivalent in free amino acids (Meguid et al., 1984; Silk et al., 1985). Work by Grimble et al. (1986) showed that di- and tripeptides can easily traverse the intestinal barrier without prior hydrolysis. Also, some dietary peptides have biological activities (Mahe et al., 1990). These bioactive peptides can change cellular metabolism and act as growth factors, immunomodulators, or neurotransmitters (Robert and Zaloga, 1994).

The principal aim of this study was to characterize chickpea protein hydrolysates obtained by the action of bromelain and α-chymotrypsin. The value of the chickpea arises from its high protein content of 20%, which is even higher than that of wheat (13%) (FAO, 1970; Fernandez and Berry, 1988). Chickpea proteins also have excellent nutritional value. Thus, Sotelo et al. (1987) showed that the protein nutritional quality is higher than other leguminous seeds and the protein efficiency ratio is similar to that of milk casein (Valencia et al., 1988), in spite of the presence of antinutritional factors in chickpea, such as phytates, trypsin, and chymotrypsin inhibitors, and its deficit in sulfur amino acids (Khan et al., 1988). The present work was thus directed toward obtaining added value from these proteins via enzymatic hydrolysis and thus to suggest their use as a more bioethical protein source for human nutrition.

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MATERIALS AND METHODS

Extraction of Chickpea Proteins. The chickpea protein concentrate (CpPC) was extracted as described below: the flour was suspended in distilled water at a concentration of about 10% (w/v). Following manual mixing and centrifugation (2500*g*, 30 min, 20 °C), the supernatant was recovered. The pellet obtained was resuspended and washed. This operation was repeated twice.

All the supernatants were pooled, an equal volume of 35% (w/v) ammonium sulfate was added and the resulting suspension was centrifuged (2500*g*, 30 min, 20 °C). The protein pellet recovered in a minimum volume of distilled water was the CpPC, which was dialyzed against 0.1 M NaCl pH 7, against distilled water, and lyophilized and stored at 4 °C until use.

Enzymatic Digestion. Fifty μL of an enzymatic solution of bromelain (EC 3.4.22.4) (Böhringer Mannheim) or α-chymotrypsin (EC.3.4.21.1) (Böhringer Mannheim), both at 0.1% (m/v) in 0.2 M sodium phosphate buffer (pH 8) were added to 2 mL of a solution of chickpea proteins at 2 mg/mL in the same buffer. The solution of chickpea proteins had previously been heated for 10 min at 90 °C (Bansal et al., 1988) and cooled to room temperature. The mixture was incubated for 2 h at 37 °C. The enzyme/substrate ratio was 1/80, and hydrolysis was stopped by immediately freezing the samples.

The protein concentrations were determined according to the method of Bradford (1976) in all of this work.

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate (SDS). The lyophilizates of soluble chickpea proteins (CpPC) and their respective hydrolysates (HCpPC−Br and HCpPC−Chym) were dissolved in 10 mM Tris-HCl (pH 8) containing 0.5% (m/v) SDS, 1 mM EDTA, and 0.01% (v/v) bromphenol blue (Prolabo). The samples were then reduced by adding 1% (v/v) dithiothreitol (Prolabo) according to the method of Laemmli (1970) and heated for 10 min at 90 °C, followed by centrifugation for 5 min at 10000*g*.

Ten microliters of the solution (0.10 μg of protein) was deposited on 1 mm thick, 10−20% gradient polyacrylamide gels (Novex, Prolabo). Each gel was placed in a vertical electrophoresis tank (Novex, Prolabo), and migration was run at a constant voltage of 150 V and an intensity of 80 mA. Gels

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were developed by staining with silver nitrate according to the method of Blum et al. (1987).

Antigenic Activity of the Water Soluble Protein Hydrolysates. *Production of Chickpea Protein Antiserum.* Antiserum to chickpea proteins a was obtained by immunizing a rabbit with 15 mg of a crude extract of chickpea flour by intraperitoneal injection. A booster was administered after 3 weeks, and the animal was bled 7 days later.

Inhibition Reaction of the Antigen−Antibody Reaction by Hydrolysates. Two rows of a 96-well microplate (Maxisorps, Nunk, Denmark) were filled with 100 μL of a solution of chickpea proteins in the concentration range 0−1000 μg/mL, as serial 3-fold dilutions in phosphate-buffered saline (PBS, pH 7.4). The rest of the plate was filled with the same protein solution at 3 μg/mL. The plate was incubated for 2 h at 37 °C. After the contents of the plate were eliminated and washed four times with rinse solution [0.9% NaCl, 0.1% Tween 20 (v/v)], 200 μL of a saturation solution [1% (m/v) BSA (Sigma) and 0.1% (v/v) Tween 20 in PBS] was added to all wells in the plate, which was then incubated for 1 h at room temperature. After the contents were eliminated and washed four times as above, the plate was filled again with a solution of rabbit antiserum against chickpea protein diluted 1/2000 in saturation buffer at 100 μL per well, at the same time as 100 μL of the solution of inhibitors. The inhibitors were the two types of hydrolysates and native proteins: two rows received the bromelain hydrolysates and the last two rows received the α-chymotrypsin hydrolysate. The protein concentration of the three inhibitors varied from 0 to 1000 μg/mL. The plate was incubated overnight at 37 °C, the contents were eliminated, and the plate was washed four times. A solution of peroxidase-labeled anti-rabbit IgG antibodies raised in goats (Sigma), diluted 1/1000 in saturation buffer, was added to the plate at 100 μL per well. After 1 h of incubation followed by four washes, 100 μL of a solution of 10 mg of *o*-phenylenediamine (Sigma) in 10 mL of 0.1 M citrate buffer (pH 5) was added to each well. The intensity of the resulting color developing at room temperature was read in a reader (Dynateck, M_r 5000) after 25 min at 492 nm.

The results were expressed as percentage inhibition calculated with the following formula:

$$\frac{A_{max} - A_{min}}{A_{max}}$$

where A_{max} is the maximal absorbance value obtained in the absence of inhibitor and A_{min} is the absorbance value in the presence of each concentration of inhibitor.

Statistical comparisons were carried out with Student's test. Each point was run in duplicate, and there were 10 determinations per range point.

Size Exclusion Chromatography (HP SEC). The hydrolysates were fractionated by size exclusion chromatography (HP SEC) on a column of silica bonded with 2-hydroxyethylpolyaspartamide (PHEA), (250 mm × 9.4 mm i.d., 200 Å, Poly-LC Inc., Columbia, MD). The chromatographic system was composed of a pump (Shimadzu LC-9A), an automatic sample injector (Marathon, Varain), a UV detector (Applied Biosystems 738 A) at the wavelength of 230 nm, and an integrator (Chromatopac C-R6A). The mobile phase (formic acid (0.05 M/L) flow rate was set at 0.5 mL/min. The lyophilizate fraction F2 was analyzed by reversed phase liquid chromatography (RPC) on a column of nucleosyl [C18, Visac (2.1 × 250) mm, 218 T.P. 52] using a water/acetonitrile gradient (98/2 to 80/20) in the presence of 0.1% (v/v) trifluoroacetate (TFA) for 25 min. The chromatographic system was composed of a syringe pump (Brownlee MPLC micropump), a diode array detector (Applied Biosystems 100 S., 214 nm), a strip chart recorder (Kipp & Zonen), and an integrator (Chromatopac C-R6A). Samples were diluted in a solution of 0.1% (v/v) TFA at 20 μg/μL. A 20 μL amount of this solution was injected on the column. Fractions composing each peak were recovered and sequenced with a microsequencer (Applied Biosystems 470 or 473).

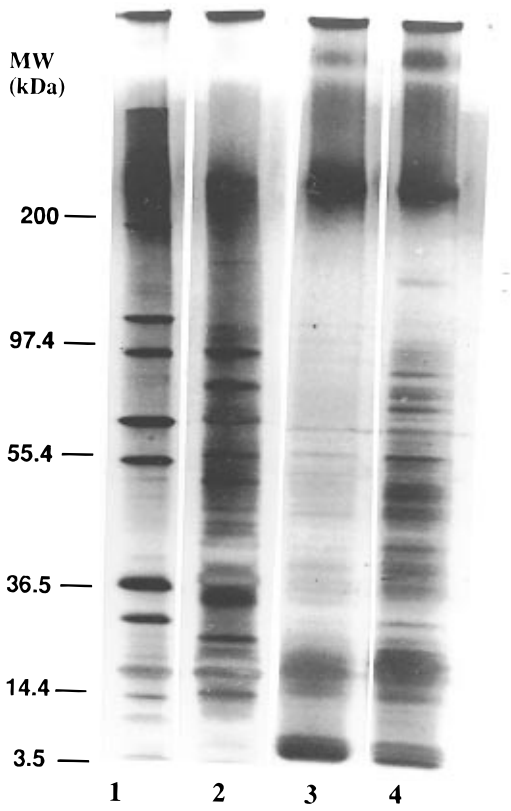

Figure 1. SDS−PAGE of chickpea proteins and their hydrolysates on a 10−20% gradient polyacrylamide gel. (Line 1) Reference proteins: myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylases *b* (97.4 kDa), bovine serum albumin (66.3 kDa), glutamine dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), aprotinin (6 kDa), insulin B chain (3.5 kDa), insulin A chain (2.5 kDa). (Line 2) CpPC. (Line 3) HCpPC-Br. (Line 4) HCpPC−Chym. Protein concentration, 10 μg/mL.

Reagents used for HPLC were all analytical grade and purchased from Merck, all solutions were degassed for 10 min with ultrasound before use, and ultra-pure water was purified with a Milli-Q system (Millipore, Belfort, MA). The peptides used to calibrate the columns were provided by Sigma, and all solutions used for mobile phases were analytical grade (Merck).

Assay of Peptide Fractions with the Method Using 2,4,6-Trinitrobenzenesulfonic Acid (TNBS). The fractions recovered after HP SEC were subjected to total acid hydrolysis (6 N HCl, 110 °C, 24 h). The amino acids were assayed with the TNBS method as described by Adler-Nissen (1979).

RESULTS AND DISCUSSION

Electrophoretic Profile of Hydrolysates. Figure 1 shows the SDS−PAGE separations of native chickpea proteins and the same proteins after the action of each of the two proteases for 2 h. Comparing the profiles of the hydrolysates to that of the native proteins (line 2) shows that enzymatic digestion led to the disappearance of a number of bands. Hydrolysis by bromelain (line 3) led to the total disappearance of all proteins with molar masses included between 56 and 150 kDa, while α-chymotrypsin (line 4) resulted in only partial digestion. The majority of the proteins was hydrolyzed to proteins of lower molar mass, which enriched the hydrolysates in small peptides with molecular weights included between 2 and 20 kDa. These proteases of plant (bromelain) and animal origin (α-chymotrypsin) are widely used industrially for the production of food proteolysates (Driou

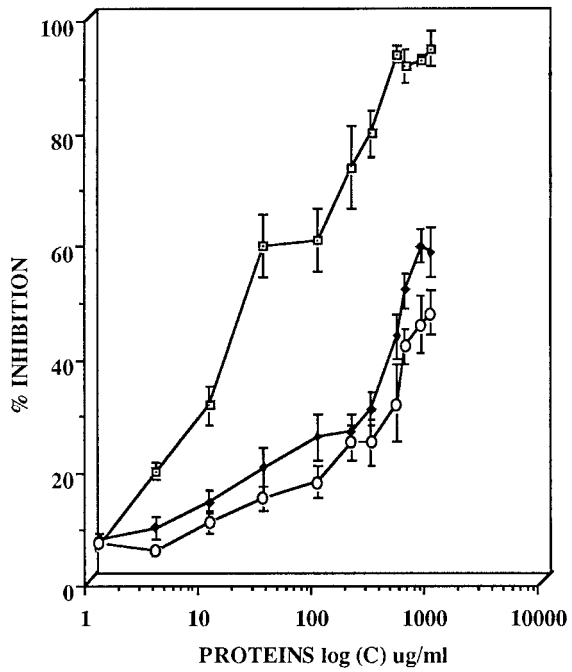

Figure 2. Inhibition of the reaction between anti-chickpea protein antibodies from rabbit serum and native chickpea proteins by their respective bromelain (HCpPC−Br) and α-chymotrypsin (HCpPC−Chym) hydrolysates. (⊡) Chickpea proteins; (◆) HCpPC−Chym; (○) HCpPC−Br.

et al., 1985). They act preferentially at the carboxyl bond of aromatic amino acids. Nevertheless, the electrophoretic profiles between the two were very different, showing that the action of bromelain was much more effective than that of α-chymotrypsin. This difference could be explained by the presence of selective antiprotease factors in chickpeas, as is the case for most leguminous plants (Bansal et al., 1988); chickpeas are especially rich in trypsin and chymotrypsin inhibitors. The purpose of heating the proteins before hydrolysis was to denature these inhibitors (Bansal et al., 1988), and this result showed that the temperature had little effect on their inactivation, since a large proportion of the proteins resisted the action of α-chymotrypsin. In addition, bromelain used in the form of a pineapple juice extract could attenuate the bitterness arising from the presence of hydrophobic peptides formed by hydrolysis.

Hypoantigenicity of Hydrolysates. Figure 2 shows ELISA results in an inhibition system. This test was used to evaluate the inhibition reaction of rabbit anti-chickpea protein IgG antibody with the native antigen and with that modified by enzymatic hydrolysis. There was a significant variation of percentage inhibition in this reaction. The reaction of rabbit anti-chickpea protein antibodies with native chickpea proteins was reduced by 58 ± 2.3% with α-chymotrypsin hydrolysates and by 45 ± 4.5% with bromelain hydrolysates, whereas maximum inhibition was obtained with intact native chickpea proteins (94 ± 3%). The inter-test coefficient of variation (CV) was 12.5% for the lowest point and 9% for the highest point in the concentration range of chickpea proteins. It was 12% for the highest point of bromelain and α-chymotrypsin hydrolysates and was 38% and 10% for the lowest points of the two hydrolysates. Thus, enzymatic proteolysis clearly led to an approximately 50% decrease in recognition of the antigen−antibody reaction and caused the destruction of a portion of antigenic epitopes of chickpea proteins, thereby producing hypoantigenic proteolysates.

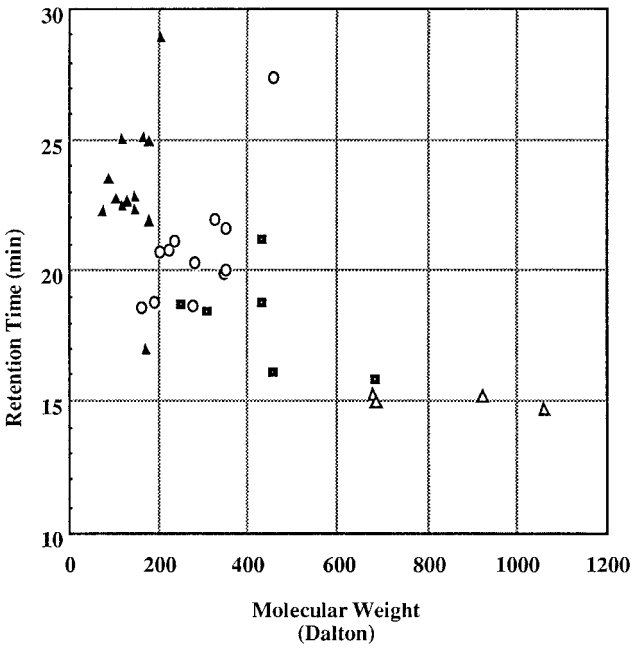

Figure 3. Variation of retention times vs molar masses of the standard peptides used to calibrate the PHEA column. (▲) AA; (○) 2−3 AA; (■) 4−5 AA; (△) 6−7−8 AA.

A number of studies on the effect of enzymatic hydrolyses on the antigenicity and allergenicity of dietary proteins have been carried out on proteins of cow milk (Semant et al., 1977; Asselin et al., 1989), soybeans and peanuts (Burks et al., 1992). To our knowledge, no similar study has been conducted on chickpeas. Even so, most authors agree that enzymatic hydrolysis attenuates the antigenic and allergenic character of dietary proteins (Jost et al., 1987; Pahud et al., 1985).

Fractionation of Hydrolysates of Chickpea Proteins by HP SEC. Size exclusion chromatography was carried out on a column of silica bonded to 2-hydroxyethylpolyaspartamide and calibrated with a range of peptides and amino acids. The curve of retention times versus molar masses of reference peptides is shown in Figure 3. Peptides with molar masses lower than 1000 Da were separated as a function of their size and molar mass into three fractions: fraction F1 contained the "large and medium peptides" with molar masses higher than 500 Da eluting at a retention time less than 16 min, fraction F2 contained "small peptides" composed primarily of di-, tri-, and tetrapeptides eluting at retention times between 16 and 22 min; most free amino acids and several oligopeptides were eluted beyond 22 min in fraction F3. The two types of hydrolysates of chickpea proteins could be separated on the same PHEA column. Figures 4a (HPCpC−Chym) and 4b (HPCpC−Br) show the chromatographic profiles obtained.

Size exclusion chromatography enables the separation of peptides and amino acids as a function of their molar mass and size. Nevertheless, the rapid elution of some basic amino acids such as lysine (t_r = 18 min, M_r 146) and the considerable retention of the dipeptide Glu−Gly (t_r = 28 min, M_r 204) by the column are explained by interference of the polarity of these molecules with the mechanism of separation. PHEA is in fact a highly polar support with the possibility of hydrogen bond formation between the NH2 group of amino acid side chains and the hydroxyl groups of the support. According to Alpert (1990), formic acid penetrates the polymer layer and forms a network via hydrogen bonds. Molec-

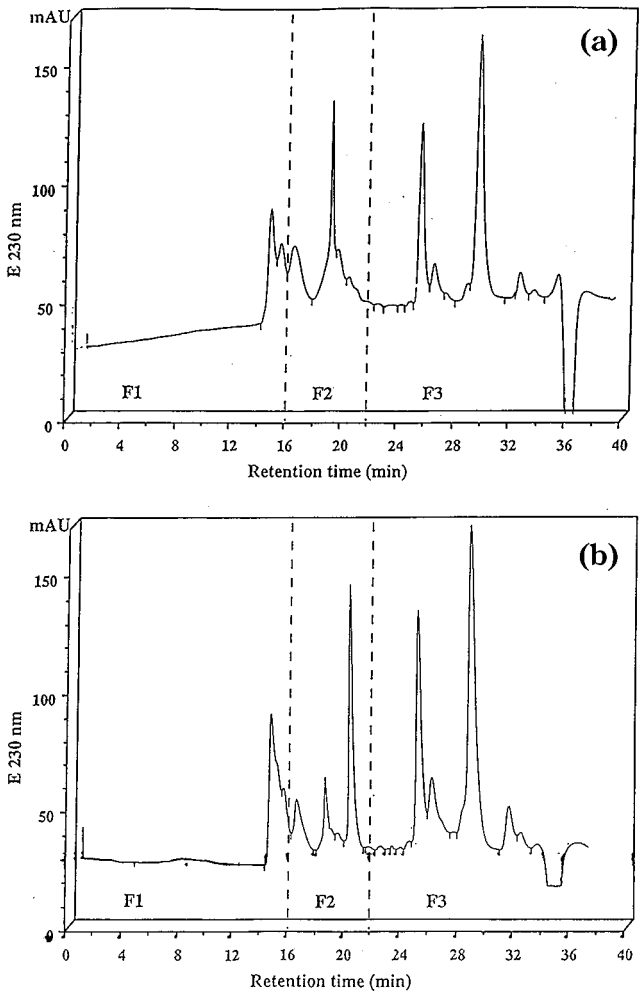

Figure 4. Chromatographic profiles of chickpea protein hydrolysates separated by HP SEC on a column of polyhydroxyethylaspartamide. (a) HCpPC−Chym. (b) HCpPC−Br. Mobile phase: 50 mM formic acid, pH 2, λ 230 nm, flow rate 0.5 mL/min.

ular sieving now occurs in the meshes of the polymer network formed. Fractionation covers the molar mass range of about 100 to 1000 Da with better resolution than that obtained with TSK G-2000 columns (Lemieux et al., 1991).

This separation method thus leads to the fractionation of peptides and to the purification of small peptides (di-, tri-, and tetrapeptides) by separating them from oligopeptides and from amino acids. This type of separation is not applicable to linear polypeptide chains such as polyglycines, which all elute with the same retention time (t_r 18.7 min) or to tryptophan and its derivatives that all elute with a t_r around 28 min. The results we obtained were similar to those reported by Silvestre et al. (1992) with casein hydrolysates and confirm the caracteristics of a separation by the PHEA column.

Table 1. Quantification of the Fraction Eluted by HP SEC Using the TNBS Method[a]

hydrolysates	HCpPC−Br (μg of protein)	HCpPC−Chym (μg of protein)
F1	(0.224 ± 0.03) 3.5%	(1.32 ± 0.14) 14.5%
F2	(2 ± 0.13) 30%	(1.62 ± 0.35) 18%
F3	(4.56 ± 0.26) 67%	(5.04 ± 0.85) 56%
total protein content (μg)	6.8	7.9

[a] Five determinations for each point, r = 0.998, CV = 3% HCpPC−Br, CV = 6% HCpPC−Chym.

Peptides in the different fractions were quantified by assaying amino functions α to a carboxyl (α-NH_2) and total amines (NH_2) with the TNBS method. The results (Table 1) confirm the conclusions reached with electrophoresis results, i.e. hydrolysis by bromelain was more effective than that by α-chymotrypsin. Fraction F2 of the bromelain hydrolysate was richer in small peptides than fraction F2 of the α-chymotrypsin hydrolysate. Similarly, fraction F1 of the α-chymotrypsin preparation was richer in large and medium size peptides and in slightly hydrolyzed proteins. This method may be proposed for determining the quantity of peptides in dietary protein hydrolysates used for enteral nutrition

Analysis of Small Peptides in Fraction F2 by HP RPC. The nutritional value of small peptides from dietary protein hydrolysates explains why we continued the analysis of fraction F2, rich in di-, tri-, and tetrapeptides. They were separated by reverse phase chromatography (HP RPC) on a column of nucleosyl C18 resulting in the separation profile shown in Figure 5. Sequencing the peptides in fraction F2 of the bromelain hydrolysate thus separated showed that each peak contained 5−6 peptides composed of 2−5 amino acids each (Table 2). With this technique, separation is on the basis of groups of peptides with similar hydropho-

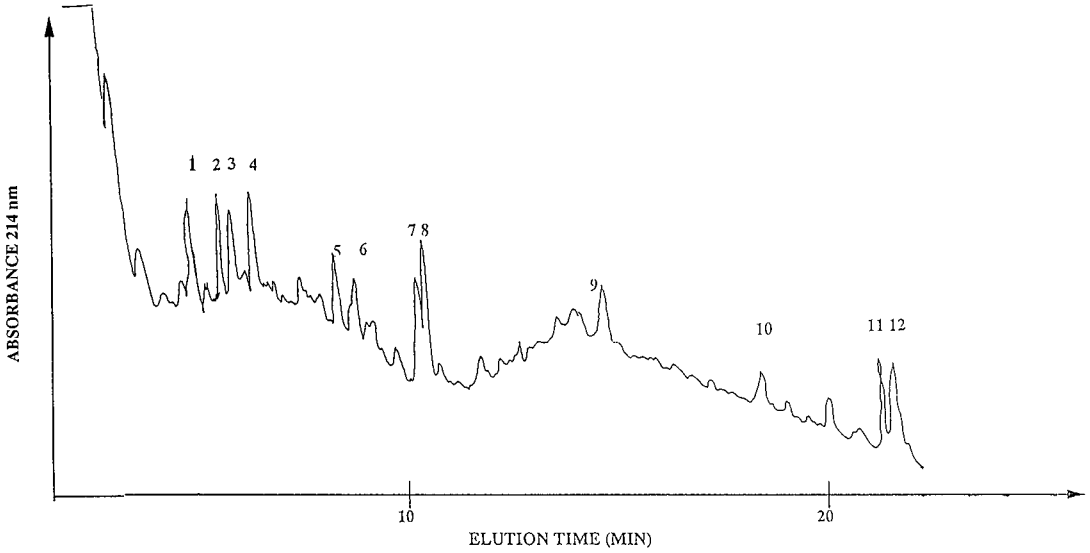

Figure 5. Separation profile of fraction F2 of HCpPC−Br by HP RPC.

Table 2. Sequences of the Different Peptides in Fraction F2 of HCpPC−Br

peak no.	sequence	peak no.	sequence
1	K	7	Y-V-G-Y-T
2	L-V		V-D-T-G-G
3	X-V-G		I-N-Q-E
	L-V		L-I-Y
	G-I		X-E
	V-E		X-P
	A		
4	G-I-G-G-M		L-G-E-X
	V-Y-V-E	8	
	A-P-Y		SV-Y
	Q-A		X-P-F
	F		not detected
	L	9, 10	A-G-I-D
5	F-I-G-G-M	11	V-D-L-Q
	V-Y-V-E		L-P-F
	M-P-Y		W-P
	N-A		V-D-V-I
	G-T	12	W-P-N
6	A-V-T-Y		G-L-Y
	T-E-L		S-F
	Q-P-Y		
	I-N-P		

bicity. In light of these results, it is thus seen that the principle of polarity is insufficient for the complete separation of peptides with molecular weight lower than 1000 Da; however, chikpea proteins are very glycosylated, and their structures can explain some interactions which interfere in these two chromatographic separations. Nevertheless, fraction F2 was very rich in di-, tri-, and tetrapeptides. These results are consistent with those of Lemieux et al. (1991) obtained with a peptide mixture obtained from a casein hydrolysate.

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