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Protein quality of chickpea (*Cicer arietinum* L.) protein hydrolysates

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Abstract

Chickpea protein isolate (CPI) was used as the starting material in the production of chickpea protein hydrolysates (CPHs). To obtain a highly extensive hydrolysate with a degree of hydrolysis higher than 50%, a sequential utilisation of endoprotease (Alcalase) and exoprotease (Flavourzyme) was necessary. Molecular weight patterns of CPHs were determined by gel filtration chromatography. As a result of the enzymatic activity, differences in the chromatographic pattern of CPHs were observed. Although significant ($P \leqslant 0.05$) decreases of Phe and Arg were observed after hydrolysis, adequate amounts of essential amino acids in relation to the reference pattern of FAO (FAO/WHO/ONU, 1985. Energy and requirements. Technical report series No. 724. Geneva) were found. In vitro protein digestibility of CPHs (95%) were similar to that of the starting material (CPI), and TIA was not detected in any case. A high increase of solubility in CPHs, with respect to CPI, was observed, one CPH being totally soluble over a wide pH range (2–10) when the enzymes were added sequentially. Due to their high protein quality and solubility, CPHs might be considered as potential ingredients in the food industry.

Keywords: Chickpea; Protein isolate; Protein hydrolysate; Enzymic hydrolysis

1. Introduction

Proteins are essential components of the diet for human nutrition as sources of energy and amino acids. Their nutritional quality depends on their amino acid content and on the physiological utilization of specific amino acids after digestion, absorption, and minimal obligatory rates of oxidation (Friedman, 1996). Also, availability of amino acids varies with protein source, processing treatment and interaction with other components of the diet.

Enzymatic protein hydrolysates have been reported as suitable sources of protein for human nutrition because of their gastrointestinal absorption, especially di- and tripeptides, which seem to be more effective than both intact protein or free amino acids (Siemensma, Weijer & Bak, 1992; Ziegler et al., 1990). Therefore, protein hydrolysates have been widely used in specific formulations, in order to improve nutritional and functional properties (Mahmoud, 1994). These uses include clinical applications, such as geriatric products, high-energy supplements, weight-control and therapeutic or enteric diets (Frokjaer, 1994). Because of their reduced antigenic activity, the development of hydrolysates, for use in peptide-based hypoallergenic infant formulas, has been reported (Cordle, 1994).

The protein sources most commonly used in nutritional products are casein and whey proteins. However, plant proteins are finding commercial application in a number of formulated foods as an alternative to proteins from animal sources. Among plant proteins, soybean is the source most widely used to obtain protein hydrolysates, but other sources such as peas (Periago et al., 1998) and chickpeas (George, Sivasankar, Jayaraman & Vijayalakshni, 1997) have been successfully used.

In recent years, chickpea seeds have been considered a suitable source of dietary protein, due to their good balance of amino acids, their high protein bioavailability and their relatively low levels of antinutritional factors (Friedman, 1996; Newman, Roth & Lockerman, 1987). Chickpea seeds have been used to obtain protein products with added value, including concentrates and

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protein isolates. Ulloa, Valencia, and García (1988) reported the use of chickpea protein concentrates as potential ingredients in an infant formula. The potential utilisation of chickpea protein isolates (CPI) in the preparation of cheese, bread and meat products has been recognised (Sánchez-Vioque, Clemente, Vioque, Bautista & Millán, 1999). No data were available to us about the use of CPI to obtain new and improved protein products, including chickpea protein hydrolysates (CPH), with well-defined nutritional and functional characteristics. An extended knowledge of the protein quality in different CPHs would be useful in understanding their use as potential additives for food and dietary items.

2. Material and methods

2.1. Material

The starting material (CPI) was obtained according to Sánchez-Vioque et al. (1999). Diethyl-ethoxymethylenemalonate was purchased from Fluka (Buchs, Switzerland). The enzymes, trypsin (porcine pancreatic trypsin tipe IX, 17,700 BAEE U mg^{-1}), chymotrypsin (bovine pancreatic chymotrypsin tipe II, 43 U mg^{-1}) and peptidase (porcine intestinal peptidase grade III, 50 U g^{-1}1), amino acid kit, D,L-α-aminobutyric acid and chlorogenic acid were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were of analytical grade.

The enzymic complexes used were Alcalase 2.4 L and flavourzyme 1000 MG (Novo Nordisk, Bagsvaerd, Denmark). Alcalase 2.4 L is an endopeptidase from *Bacillus licheniformis*, with subtilisin carlsberg as the major enzymic component, having a specific activity of 2.4 Anson Units (AU) per gram. One AU is the amount of enzyme which, under standard conditions, digests haemoglobin at an initial rate that produces an amount of trichloroacetic acid-soluble product which gives the same colour with the Folin reagent as one milliequivalent of tyrosine released per minute. Flavourzyme 1000 MG is an exopeptidase and endoprotease complex with an activity of 1 LAPU g^{-1}. One LAPU (Leucine aminopeptidase unit) is the amount of enzyme which hydrolyzes 1 μmol of leucine-*p*-nitroanilide per minute. Optimal endoprotease and exopeptidase activities were obtained by application trials at pH 8 and 7, respectively.

2.2. Chemical analysis

Total nitrogen and ash were analyzed according to AOAC (1990) approved methods. Crude protein content was calculated using a conversion factor of 6.25. Soluble sugars were estimated colorimetrically by the phenol-sulphuric acid method using a standard curve of glucose (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Total fibre was determined according to an enzymatic-gravimetric method (Lee, Prosky & De Vries, 1992). Amounts of phenolic compounds were estimated as chlorogenic acid equivalents according to Moores, McDermott, and Wood (1948). In vitro protein digestibility (IVPD) was evaluated using a multienzyme solution of trypsin, chymotrypsin and peptidase (Hsu, Vavak, Satterlee & Miller, 1977). Amino acids were determined in the acid hydrolysate after derivatisation with diethylethoxymethylene-malonate by reverse phase-HPLC, according to the method of Alaiz, Navarro, Girón, and Vioque (1992), with D,L-α-aminobutyric acid as internal standard.

2.3. Trypsin inhibitor activity (TIA)

TIA was determined by assaying trypsin activity in the presence and absence of inhibitor extract, using casein as the substrate for trypsin at a ratio of 160:1 (w/w). In order to observe maximal inhibition, it was necessary to pre-incubate the enzyme and putative solutions together during 30 min at 37°C before addition of the substrate (Richardson, 1991). The assay mixture containing 3 ml of 0.1 M phosphate buffer, pH 7.6, 0.25 ml trypsin solution (1 mg ml^{-1} 1 mN HCl), 0.5 ml HCl, 1 mN, 2 ml casein solution (2% in phosphate buffer, 0.1 M pH 7.6) and 1 ml inhibitor extract (5 mg protein ml^{-1}) was incubated for 30 min in a water bath at 37°C. The reaction was stopped by addition of 5 ml of 5% trichloroacetic acid. A blank sample was prepared by adding trichloroacetic acid before the addition of trypsin solution. A control sample was also prepared in the absence of the inhibitor extract. After incubation, samples were filtered through Whatman No. 1 filter paper. The decrease in the hydrolysis of casein was determined measuring free amino groups by the trinitrobenzenesulfonic acid (TNBS) method, according to Adler-Nissen (1979). Inhibition percentage (%I) of a given extract was calculated by comparing the reduction in enzymatic activity on the addition of the extract (S) by the same enzyme concentration in the absence of any inhibitor (C), according to the following equation:

$$\%I = (1 - S/C) \times 100$$

2.4. Enzymatic hydrolysis

CPI was hydrolyzed batchwise with Alcalase as endoprotease and Flavourzyme 1000 MG as exopeptidase, by individual or sequential treatment, in a pH stat. The hydrolysis was carried out using the following hydrolysis parameters: (a) Alcalase hydrolysis: substrate concentration (S) = 2%, enzyme-substrate ratio (E/S) = 0.4 AU g^{-1} protein; temperature (T) = 50°C, pH 8; (b) Flavourzyme hydrolysis: substrate concentration (S) = 2%, enzyme-substrate ratio (E/S) = 100 LAPU g^{-1} protein, temperature

(T) = 50°C, pH 7. The hydrolysis was conducted in a 1000 ml reaction vessel, equipped with a stirrer, thermometer and pH electrode.

The degree of hydrolysis (DH), defined as the percentage of peptide bonds cleaved, was measured by determination of free amino groups by reaction with trinitrobenzenesulphonic acid (TNBS) (Adler-Nissen, 1979). Total numbers of amino groups were determined by acid hydrolyses (HCl 6 N) at 110°C for 24 h. Individual and sequential hydrolyses were carried out until DHs of 27 and 52% were reached, respectively. Hydrolyses were stopped by heat treatment at 85°C for 10 min.

2.5. Gel filtration

Lyophilised samples (0.1 g) were dissolved in 10 ml of 0.1 M sodium borate buffer, 0.2 M sodium chloride, pH 8.3. Gel filtration was carried out in a FPLC system equipped with a Superose 12 HR 10/30 column from Pharmacia Biotechnology (Uppsala, Sweden). Volume injection and sample concentration were 200 µl and 1.6 mg of protein per ml. The eluent was the above cited buffer at a flow rate of 0.4 ml min^{-1}. Elution was monitored at 214 nm and the approximate molecular masses were determined using the following molecular weights standards from Pharmacia: blue dextran (2000 kDa), catalase (240 kDa), γ-globulin (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and ribonuclease A (13.7 kDa).

2.6. Solubility curve

CPI and CPH (10 g) were extracted twice with 200 ml of 0.1 N NaOH, stirring for 2 h at room temperature. Aliquots were taken for precipitation of the proteins at different pHs adjusted with HCl by stirring at room temperature for 1 h. The samples were centrifuged at 4000 $\times g$ for 30 min and the nitrogen content determined in the soluble and insoluble fractions. Solubility was expressed as the percentage of the total nitrogen of the original sample that was present in the soluble fraction.

2.7. Statistical analysis

Data were subjected to analysis of variance, and LSD value (0.05 significance) was calculated to identify pairs of means that were significantly different using the Tukey test.

3. Results and discussion

3.1. Hydrolysis of chickpea protein isolate

The use of single enzymes, Alcalase and Flavourzyme led to the production of protein hydrolysates (ACPH and FCPH, respectively) with no more than 27% of DH. The combination of both enzymes in a two-step process gave a final protein hydrolysate (AFCPH) with a DH higher than 50% at the end of the hydrolysis. Although the globular structure of the major proteins of CPI is per se an important limitation on the action of proteolytic enzymes, the use of Alcalase facilitated the action of Flavourzyme in the second step to obtaining an extensive hydrolysate. The hydrolysis process was accompanied by a release of H^+. However, no correlation between base consumption and DH could be established. The consumption of NaOH was highly reduced (90%) when the hydrolysis reaction was carried out with Flavourzyme. This could be due to the different relationship between equivalent peptide bonds cleaved and equivalent base consumed as a result of the pKa values of the amino and carboxyl group (Adler-Nissen, 1986).

3.2. Chemical composition of chickpea protein hydrolysates

Chemical composition of CPH is shown in Table 1. The main feature of ACPH, FCPH and AFCPH was their high protein content (91%). The ash content (5.5–7.2%), mainly Na^+ and Cl^- ions, came from CPI and the pH adjustment during hydrolysis. Because the high levels of these ions might cause diarrhoea, their contents must be reduced by diafiltration (Olsen & Adler-Nissen, 1981).

Molecular weight patterns of CPI and the different CPH were determined by gel filtration chromatography (Fig. 1). CPI profile on gel filtration was characterized by the presence of major protein fractions, with molecular masses of 60 and 21.5 kDa, corresponding to those of the αβ and β-subunits of the 11S protein, respectively. This fact, and the minor presence of a protein fraction of 360 kDa that correponds to the 11S protein, confirms the reported breakdown of 11S proteins due to the basic

Table 1
Chemical composition of chickpea protein isolate (CPI) and different chickpea protein hydrolysates (CPH)[a]

Constituent	CPI	FCPH[b]	ACPH[b]	AFCPH[c]
Protein (N×25)	90.3 ± 2.6	91.8 ± 2.4	91.2 ± 2.8	91.2 ± 2.6
Total fibre	2.1 ± 0.1	< 0.5	< 0.5	< 0.5
Fat	1.0 ± 0.1	< 0.1	< 0.1	< 0.1
Total soluble sugars	0.2 ± 0.01	< 0.1	< 0.1	< 0.1
Ash	4.23 ± 0.2	5.5 ± 0.2	7.2 ± 0.2	6.6 ± 0.3
Polyphenols	< 0.1	< 0.1	< 0.1	< 0.1
IVPD	96.1 ± 3.2	96.4 ± 4.8	97.4 ± 5.1	96.9 ± 3.6
TIA	10.3 ± 2.1	< 1	< 1	< 1

[a] Data, expressed as g 100 g^{-1} of dry matter, represent the mean ± SD of three independent determinations.
[b] 27% HD.
[c] 52% HD.

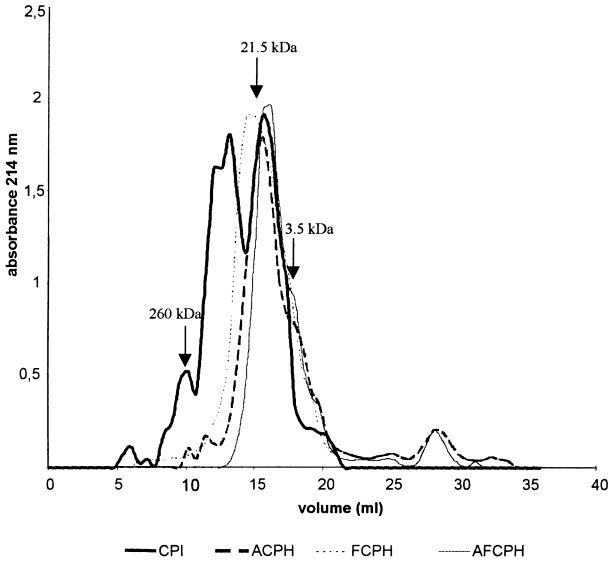

Fig. 1. Gel filtration chromatography of chickpea protein isolate (CPI), and final protein hydrolysates obtained after individual Flavourzyme (FCPH) and Alcalase (ACPH) as well as sequential (AFCPH) treatment.

protein extraction during the preparation of the protein isolate (Guéguen, Chevalier, Barbot & Schaeffer, 1988).

As a result of the hydrolysis process, FCPH showed a major chromatographic peak of intermediate molecular masses between 60 and 21.5 kDa. This was possibly due to the partial proteolysis of acidic polypeptidic chains of the 11S proteins. ACPH and AFCPH showed total disappearance of the protein fraction with molecular mass about 60 kDa. In both CPHs, a similar chromatographic profile, with molecular masses ranging from 20 to 2 kDa, was observed. Since measurements were carried out at 214 nm, at which peptide bonds are strongly absorbent, small peptides and most free amino acids, mainly in AFCPH, could not be detected (Visser, Slangen & Robben, 1992). Although this underestimation must be taken into account, these results might be considered suitable for the comparative purpose of the present paper.

The amino acid composition of CPI and the different CPHs, in comparison to the FAO/WHO reference pattern of essential amino acids (FAO/WHO/ONU, 1985), is shown in Table 2. Amino acid data indicate that CPI contains adequate amounts of essential amino acids in relation to the reference pattern and constitutes a suitable protein source for the elaboration of chickpea protein hydrolysates.

Enzymatic hydrolysis was strongly preferred over chemical methods for producing chickpea protein hydrolysates. It was carried out under mild biological conditions so that the overall amino acid composition and IVPD (higher than 95%) of CPH were similar to that of the starting material (CPI), maintaining its nutritional value. CPH showed a significant ($P \leqslant 0.05$) decrease in TIA values, which were found to be 80% of the initial activity. However, it is still not known what residual level of TIA is necessary to provoke a physiological response in humans and what residual level may be accepted as safe for the human population (Liener, 1994).

The different CPHs showed highest values for aspartic and glutamic acid, arginine, leucine, serine, phenylalanine and lysine. The lowest values were obtained for histidine, tyrosine, methionine and cysteine. After hydrolysis, significant ($P \leqslant 0.05$) decreases of phenylalanine and arginine were observed. These losses could be due to the enzymatic activity and/or conformational

Table 2
Amino acid composition[a] of chickpea protein isolate (CPI) and different chickpea protein hydrolysates (CPH)

Amino acid	CPI	FCPH	ACPH	AFCPH	FAO pattern[b]
Aspartic acid	13.3 ± 0.33	12.2 ± 0.23	12.3 ± 0.26	12.0 ± 0.17	
Glutamic acid	19.2 ± 0.31	17.2 ± 0.25	16.5 ± 0.31	16.2 ± 0.21	
Serine	6.53 ± 0.24	6.42 ± 0.16	6.32 ± 0.13	6.32 ± 0.14	
Histidine	2.94 ± 0.10	2.96 ± 0.12	2.91 ± 0.09	2.97 ± 0.09	
Glycine	3.63 ± 0.10	3.84 ± 0.09	3.55 ± 0.12	3.65 ± 0.11	
Threonine	3.76 ± 0.19	3.78 ± 0.10	3.64 ± 0.11	3.64 ± 0.08	3.4
Arginine	12.2 ± 0.38	12.3 ± 0.21	11.0 ± 0.23[c]	11.0 ± 0.21[c]	
Alanine	3.57 ± 0.24	3.7 ± 0.15	3.62 ± 0.09	3.75 ± 0.15	
Tyrosine	2.82 ± 0.12	3.02 ± 0.14	3.24 ± 0.10	3.36 ± 0.09	
Valine	3.77 ± 0.24	3.93 ± 0.20	3.72 ± 0.12	3.97 ± 0.16	3.5
Methionine	1.59 ± 0.18	1.52 ± 0.11	1.61 ± 0.03	1.53 ± 0.09	2.5[d]
1/2 Cystine	1.23 ± 0.17	1.32 ± 0.04	1.28 ± 0/.02	1.33 ± 0.05	
Isoleucine	4.06 ± 0.16	4.18 ± 0.06	3.95 ± 0.04	4.08 ± 0.10	2.8
Leucine	8.24 ± 0.35	8.49 ± 0.12	8.03 ± 0.17	8.04 ± 0.10	6.6
Phenylalanine	6.95 ± 0.24	5.82 ± 0.18[c]	5.44 ± 0.12[c]	5.63 ± 0.12[c]	6.3[e]
Lysine	6.67 ± 0.33	6.52 ± 0.13	6.45 ± 0.21	6.57 ± 0.16	5.8

[a] Each value is the mean ± SD of three determinations. Grammes of amino acid/100 g protein protein.
[b] FAO/WHO/ONU (1985).
[c] Significantly different ($P < 0.05$) from their respective controls in CPI.
[d] Met + Cys.
[e] Tyr + Phe.

aspects that limit the enzymatic action during the hydrolysis process. However, the different CPHs satisfied the amino acid requirements in comparison with the reported FAO pattern (FAO/WHO/ONU, 1985). With respect to the essential amino acid to total amino acid ratio (E/T%), CPH did not show significantly ($P \leqslant 0.05$) different values with respect to CPI. These values are clearly above the third of the total amino acid content, which is an index of the nitrogenous equilibrium according to the nutritional recommendations (FAO/WHO/ONU, 1985).

Proteolytic modification has special importance for the improvement of solubility of protein, e.g. from legumes that are poorly soluble in aqueous media. The solubility of chickpea proteins increased with the enzymatic treatment; however, individual treatment with Flavourzyme did not reach 100% solubility even at DH 27% at pH 8 (Fig. 2). The sequential use of Alcalase and Flavourzyme changed the pH solubility curve from the U-shape of CPI to a flat curve over a wide pH range (2–10). The improved solubility of CPHs compared to CPI was due to its smaller molecular size and a corresponding increase in the number of exposed ionizable amino and carboxyl groups that increase the hydrolysate hydrophilicity (Turgeon, Gauthier & Paquin, 1992).

In conclusion, protein quality of extensive hydrolysates obtained by individual or sequential treatment of an exo- (Flavourzyme) and endopeptidase (Alcalase), using a chickpea protein isolate as a starting material, have been reported. The different CPHs were characterized by their high protein quality, mainly balanced amino acid content and a high IVPD. The high solubility over a wide pH range makes them a suitable material for the elaboration of liquid foods, or high-energy beverages, in which apperance of an insoluble sediment is undesirable. In this sense, AFCPH is an interesting material for possible use as a food additive.

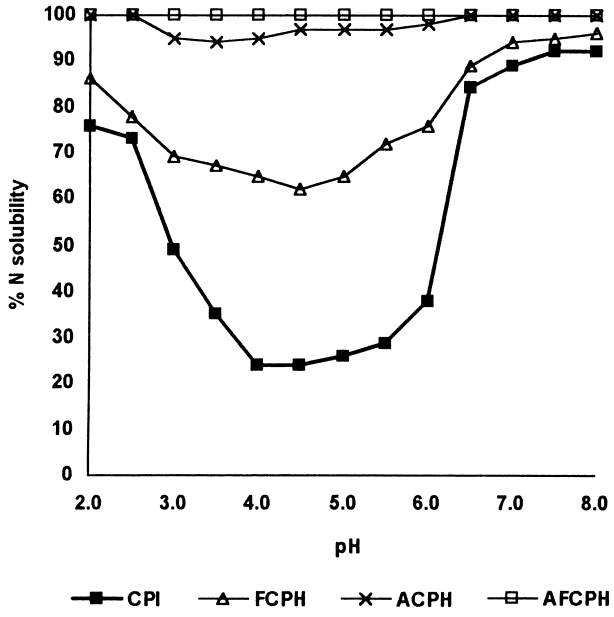

Fig. 2. Solubility profile at different pH values of chickpea protein isolate (CPI), and final protein hydrolysates obtained after individual Flavourzyme (FCPH) and Alcalase (ACPH) as well as sequential (AFCPH) treatment.

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