Production of Extensive Chickpea (*Cicer arietinum* L.) Protein Hydrolysates with Reduced Antigenic Activity

Alfonso Clemente, Javier Vioque, Raul Sanchez-Vioque, Justo Pedroche, and Francisco Millán*

Departamento de Fisiología y Tecnología de Productos Vegetales, Instituto de la Grasa (CSIC), Avenida Padre García Tejero 4, 41012 Sevilla, Spain

Chickpea protein isolate was used as starting material for the production of hypoallergenic protein hydrolysates. Western blotting of the protein isolate showed that IgE in sensitized patient sera strongly bound to the basic polypeptidic chains and recognized the acidic ones of 11S globulin. During the hydrolysis process by the individual and/or sequential action of endo- and exoproteases, a high reduction of antigenic activity was observed. Results suggest that the presence of intact or partially hydrolyzed basic polypeptide chains of 11S globulin are responsible for the formation of IgE complexes in protein hydrolysates obtained by exoprotease treatment; however, the digestion of these polypeptide chains by individual action of endoprotease caused a high loss of antigenic activity. The most effective reduction of antigenicity, >90%, was observed in extensive hydrolyzed chickpea proteins obtained by sequential treatment with endo- and exopeptidases. This chickpea protein hydrolysate could be useful for the elaboration of specialized hypoallergenic food products.

Keywords: *Chickpea; Cicer arietinum L.; enzymatic protein hydrolysate; antigenic activity*

INTRODUCTION

Plant proteins are increasingly being used as an alternative to proteins from animal sources in human nutrition. Among plants, legume seeds represent a rich source of proteins and carbohydrates. Soybean is the legume most used for plant protein products with added value (Waggle et al., 1989). Recently, other legumes such as common beans, lentils, lupins, or chickpeas have been recognized as valuable protein sources (Friedman, 1996). In this sense, chickpea seeds have been considered a suitable source of dietary protein due to their good amino acid composition balance, high bioavailability, and low level of antinutritional factors (Newman et al., 1987).

The main use of chickpea seeds is for direct human consumption following different processing methods (Chavan et al., 1986). However, due to different reasons such as size, the hard-to-cook phenomenon, or breakage of the seeds, >20% of total production is considered as byproduct and used for livestock feeding. Recently, the use of this product as a source of high-quality protein preparations has been investigated. Ulloa et al. (1988) reported the use of chickpea protein concentrates as potential ingredients in an infant formula. Sánchez-Vioque et al. (1998) reported the characterization of a chickpea protein isolate and its potential use for the preparation of cheese or bakery and meat products because of its high water and fat absorption. Also, a good balance of essential amino acids and high in vitro protein digestibility (95%) in chickpea protein isolates have been shown (Paredes-Lopez et al., 1991). Besides, George et al. (1997) reported the production of methionine-rich fractions from chickpea protein hydrolysate as a potential peptide fortifier in food products.

* Corresponding author (telephone 34-5-4611550; fax 34-5-4616790; e-mail frmillan@cica.es).

Over the past few years, the use of protein hydrolysates in the food industry has broadly expanded (Faergeman, 1994). In this sense, plant protein hydrolysates are widely used for obtaining added value products from dietary proteins because of the improvement in nutritional and functional characteristics, retardation of deterioration, and removal of toxic or inhibitory ingredients (Lahl and Braun, 1994). They are often used in the preparation of different nutritional formulations, such as supplementation of drinks to enhance their nutritional and functional properties, or special medical diets (Schmidl et al., 1994).

Against what appears to be an increasing incidence of many allergic disorders, the production of safe and effective hypoallergenic diets at a commercial scale has become of increasing interest. Although elemental diets with free amino acids are allergen free, and can be used with success in therapy, protein hydrolysates are applied in hypoallergenic formulas for feeding food-allergic or allergy-prone infants (Siemmensma et al., 1993; Mahmoud, 1994). They show a reduced or null antigenic activity and because peptides are less hypertonic than free amino acid mixtures, their use as a nitrogen source in enteral feeding improves absorption efficiency and reduces osmotic problems (Minami et al., 1992). Several investigators have evaluated the immunogenicity and/or allergenicity of enzymatically hydrolyzed proteins for potential use in formulas for feeding food-allergic individuals (Cordle, 1994). Most commercial hypoallergenic hydrolysates are assayed for antigenicity according to the method of enzyme-linked immunoabsorbent assay (ELISA) because of its sensitivity and relative simplicity. Thus, the effect of the degree of hydrolysis (DH) on antigenicity has been studied in whey proteins (Asselin et al., 1989) and casein (Cordle et al., 1991). Asselin et al. (1989) reported that the most effective reduction of antigenicity in whey proteins was obtained by treatment with pepsin and chymotrypsin successively. Although

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studies in legumes, such as soybean and peanuts (Burks et al., 1992) have been reported, few references are available regarding the potential use of chickpea proteins for the obtention of hypoallergenic hydrolysates. Hindi-Tamelikecht et al. (1997) reported a partial reduction in the antigenic activity of the starting material after enzymatic hydrolysis. However, the reaction of rabbit anti-chickpea protein antibodies with native chickpea proteins was reduced by only 58% with α-chymotrypsin and by only 45% with bromelain.

Our objective was to evaluate the reduction of antigenic activity during extensive chickpea protein hydrolysis generated by the sequential action of an endoprotease (Alcalase) and an exoprotease (Flavourzyme). Results are considered in relation with the potential use of these hydrolysates as hypoallergenic food ingredients or hydrolyzed formulas in human nutrition.

MATERIALS AND METHODS

Materials. Kabuli chickpea (cv. Athenas) was used for the study. Seeds, cleaned and freed from dust and other foreign materials, were ground, and the flour was defatted for 9 h with boiling hexane in a Soxhlet system. Coomassie Brilliant Blue G 250 was purchased from Serva (Heidelberg, Germany). Trinitrobenzenesulfonic acid (TNBS), Tween 20, alkaline phosphatase-conjugate mouse antihuman IgE and nitrocellulose membranes were purchased from Sigma Chemical Co. (St. Louis, MO). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were supplied by Boehringer Mannheim GmbH (Mannheim, Germany). A 96-well plate of polyethylene terephthalate glycol (PETG) was purchased from Corning (Corning Costar Corp., Cambridge, MA). Diethanolamine and *p*-nitrophenyl phosphate (PNPP) were supplied by Pasteur (Sanofi Diagnostics Pasteur, Chaska, MN). All other chemicals were of analytical grade.

The peptidases 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 that under standard conditions digests hemoglobin at an initial rate that produces an amount of thrichloroacetic acid-soluble product which gives the same color with the Folin reagent as 1 mequiv of tyrosine released per minute. Optimal endopeptidase activity was obtained by application trials to pH 8.0. Flavourzyme 1000 MG is an exopeptidase and endoprotease complex with an activity of 1.0 leucine aminopeptidase unit (LAPU) per gram. One LAPU is the amount of enzyme that hydrolyzes 1 mmol of leucine-*p*-nitroanilide per minute. Optimal exopeptidase activity was obtained by application trials at pH 7.0.

Chickpea Protein Isolate Preparation. The protein isolate was obtained according to the method of Sanchez-Vioque et al. (1998). Chickpea protein isolate was produced by dispersing defatted chickpea flour in 0.25% Na_2SO_3 at pH 10.5 to avoid the darkening of the final product (Gheyasuddin et al., 1970) and extracting by shaking for 1 h at room temperature. After centrifugation at 8000*g* for 15 min, two additional extractions were carried out with half the volume of alkaline solution. Supernatants were pooled, and the pH was adjusted to the isoelectric point (pH 4.3). The precipitate obtained was successively washed with distilled water adjusted to pH 4.3, ethanol, and acetone and dried at room temperature.

Chickpea Protein Hydrolysis. The protein isolate was hydrolyzed batchwise with Alcalase and Flavourzyme by individual or sequential treatment. Individual hydrolyses were developed during 3 h while sequential treatment was carried out with an initial hydrolysis (3 h) using Alcalase as endopeptidase and a second one (5 h) using Flavourzyme as exopeptidase. 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 of protein; temperature T = 50 °C; pH 8.0; (B) Flavourzyme hydrolysis, S = 2%; E/S = 100 LAPU/g of protein, T = 50 °C, pH 7.0. The hydrolysis was conducted in a 1000 mL reaction vessel, equipped with a stirrer, thermometer, and pH electrode. Hydrolysis was stopped by heat treatment at 85 °C for 10 min after the hydrolysis.

Degree of Hydrolysis (DH). The DH, defined as the percentage of peptide bonds cleaved, was measured by determination of free amino groups by reaction with TNBS according to the method of Adler-Nissen (1979). The total number of amino groups was determined by acid hydrolysis with 6 N HCl at 110 °C for 24 h.

Patient Sera. Sera of six allergic patients, who reported a history of adverse reactions after ingestion of chickpea, were used. Negative control sera from healthy nonallergic individuals were used. All sera were stored at −20 °C until use.

Electrophoresis and Immunoblotting. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to the method of Laemmli (1970). The gel system, containing 0.2% (w/v) SDS, consisted of a 20% polyacrylamide resolving gel (pH 8.8) and a 4% stacking gel (pH 6.8). Samples for electrophoresis were prepared as follows: Lyophylized samples (1 g) were dissolved in 10 mL of 0.1 M sodium borate and 0.2 M sodium chloride buffer (pH 8.3) and eluted with the same buffer in a Sephadex G-25 column from Pharmacia Biotechnology (Uppsala, Sweden) as above. Purified extracts were diluted to 2 mg of protein/mL and mixed (1:1 v/v) with a solubilization buffer of 80 mM Tris-HCl, 0.57% EDTA, 0.26% DTT, 3.3% SDS, 0.008% blue bromophenol, and 20% sucrose (pH 6.8) and reduced with 2-mercaptoethanol in boiling water. The running buffer was 0.1 M Tricine and 0.1% SDS (pH 8.45). The electrophoresis was run at 20 mA, and molecular masses were determined using the low molecular weight and peptide standards from Pharmacia Biotechnology.

For immunoblotting, the electrophoretically resolved proteins of the protein isolate were electroblotted onto 45 mm nitrocellulose membranes using a transblot apparatus (Bio-Rad Laboratories, Richmond, CA). The transfer was performed for 3 h at 400 mA at 4 °C using as transfer buffer of 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.3). To evaluate whether the electrophoretic transfer had been performed correctly, several membranes were stained with Coomassie Brilliant Blue G 250. The remaining membrane active sites were blocked by incubation with 5% defatted milk in TBS (20 mM Tris-HCl, 0.05 M NaCl, pH 7.5) for 2 h. The membranes were then washed three times with TTBS (0.05% Tween 20 in TBS) for 15 min each and incubated with the serum of sensitive individuals (1:1000 v/v dilution) overnight at 4 °C. The nitrocellulose blots were then washed three times with TTBS for 15 min each, and alkaline phosphatase−conjugate mouse antihuman IgE (1:1000 v/v in TTBS) was added and incubated at room temperature for 2 h. The membranes were then washed two times with TTBS and once with 1 M borate buffer (pH 8.3) for 15 min each. The blot was developed with 50 mL of NBT and 37.5 mL of BCIP in 10 mL of 1 M borate buffer at room temperature for 10 min. The reaction was stopped by washing the nitrocellulose membranes for 15 min with distilled water.

Quantitative Determination of Antigenic Activity of Chickpea Protein Hydrolysates. An antibody-capture assay format was developed to quantify the reduction of antigenic activity of the protein hydrolysates with respect to the protein isolates. Two rows of a polyethylene terephthalate glycol assay plate were filled with 100 mL of a solution of protein isolates using a dilution series in the concentration range of 10−1000 mg/mL diluted in phosphate-buffered saline (PBS, pH 7.2), being considered as appropriate a concentration of 200 mg of protein/mL for the evaluation of antigenic activity.

The remaining wells were filled with 100 mL of protein hydrolysate samples (200 mg of protein/mL) and incubated for 12 h at 4 °C and then washed three times for 15 min each with PBS containing 0.05% Tween 20 (TPBS). Remaining sites were blocked by incubating for 1 h with 100 mL/well of 0.7%

bovine serum albumin in PBS. Wells were then rewashed two times for 15 min each with TPBS and filled with 100 mL of a solution of patient sera sensitized against chickpea proteins diluted 1:200 (v/v) in PBS. After incubation at 37 °C for 1 h in a microplate incubator (Sanofi Diagnostics Pasteur), the plate was washed three times for 15 min each with TPBS. A solution of alkaline phosphatase−conjugate mouse antihuman IgE, diluted 1:1000 (v/v) in PBS, was added to the plate at 100 mL per well. After 30 min of incubation at 37 °C followed by three washes for 15 min each with TPBS, 100 mL of a solution of 14 mg of PNPP in 8 mL of 10.5% diethanolamine and 0.02% $MgCl_2$ (pH 9.8) was added to each well. After 1 h, 100 mL of 1 M K_3PO_4 (pH 12) was added as stopping solution. The intensity of the resulting color developing at room temperature was read in a microplate spectrophotometer LP400 (Sanofi Diagnostic Pasteur) at 405 nm using a 620 nm reference filter. To ensure the validity of the results in every assay, we included a control blank, negative (nonallergic patient serum) and positive controls (allergic patient serum) being processed similarly to samples. All samples were run in triplicate.

The results were expressed as percentage loss of antigenic activity, calculated with the formula

$$(1 - A_{CPH}/A_{CPI}) \times 100$$

were A_{CPH} is the absorbance value obtained by hydrolyzed samples and A_{CPI} is the absorbance value of chickpea protein isolate sample.

RESULTS AND DISCUSSION

Chickpea Protein Isolate Composition and Antigenicity. In a previous paper, we reported the chemical composition of the protein isolate (Sanchez-Vioque et al., 1998). It was characterized by a high protein content (88.1%) and low amounts of nonprotein compounds, such as soluble carbohydrates and polyphenols. Because the protein isolate showed a low ratio of soluble carbohydrates to protein (0.022) and polyphenols to protein (0.011), browning problems associated with Maillard reactions between free amino groups of proteins and carbohydrates, as well as the oxidation of polyphenols, were minimized (Lusas, 1985; Parrado et al., 1991), and this protein isolate was considered a good starting material for the obtention of a protein hydrolysate.

SDS−PAGE of the protein isolate exhibited a complex protein profile characterized by the presence of electrophoretic bands corresponding to 11S and 7S globulins (Figure 1a). The acidic (a) and basic (b) peptides of 11S globulin were observed as a cluster of bands between 46.5 and 39.8 kDa and between 25.3 and 24.3 kDa, respectively. The 7S proteins showed typical electrophoretic bands between 65 and 60 kDa.

The detection of IgE-staining bands in the protein isolate was carried out by Western blot analysis under dissociating conditions. Because allergens are multivalent and the IgE antibody response to them is probably polyclonal, there is a good chance that sufficient determinants will survive the denaturating conditions to allow the detection of each allergen under dissociating conditions (Tovey and Baldo, 1987; Ortolani et al., 1993). Although the chickpea protein isolate is subjected to different processing conditions (i.e., boiling hexane extraction, a pH 10.5 $NaSO_3$ extraction, isoelectric precipitation, ethanol and acetone extractions), a typical pattern against 11S globulin, the major protein constituent in chickpea protein isolate (Sánchez-Vioque et al., 1998), was observed in all samples. IgE was strongly bound to the basic polypeptidic chains and recognized

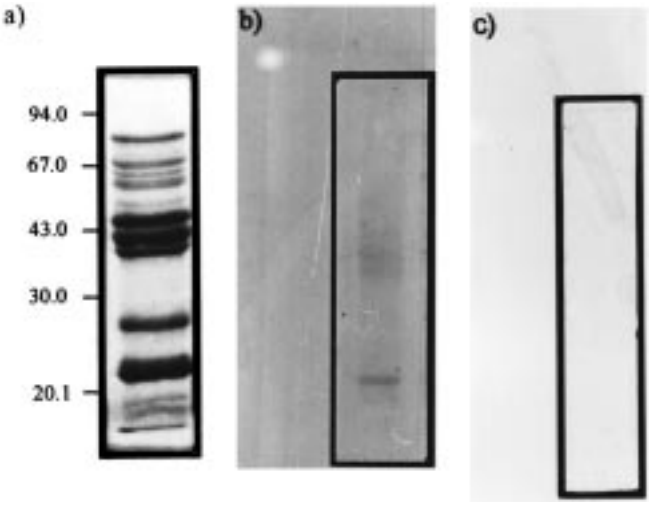

Figure 1. (a) SDS−PAGE analysis of the chickpea protein isolate. Molecular weight markers are indicated on the left. (b) IgE-specific immunoblot of the chickpea protein isolate with the serum from a patient allergic to chickpea proteins.

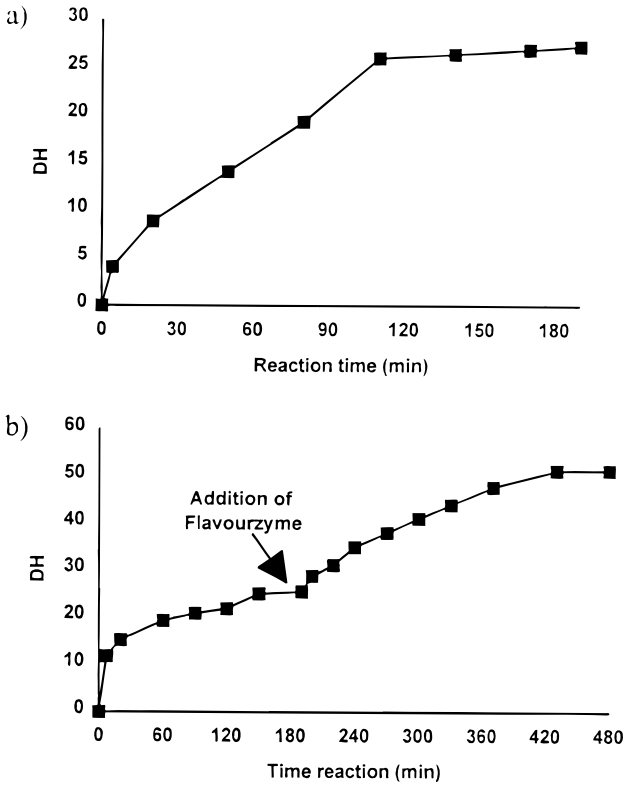

Figure 2. Hydrolysis curves of chickpea protein isolate: (a) individual treatment with Flavourzyme; (b) sequential treatment with Alcalase and Flavourzyme.

the acidic ones of 11S globulin (Figure 1b). In other legumes, such as peanut or soybean, the 11S protein has been also identified as a major allergenic protein (Herian et al., 1990), although no differences in the IgE-binding capacities to a and b polypeptidic chains were reported. No IgE-reactive electrophoretic bands in the negative control were found (Figure 1c).

Chickpea Protein Isolate Hydrolysis. The protein isolate was hydrolyzed batchwise by individual or sequential treatment with Alcalase (endopeptidase) or Flavourzyme (exopeptidase) in a pH-stat (Figure 2). Individual treatments with Flavourzyme or Alcalase were continued until a maximun solubilization of in-

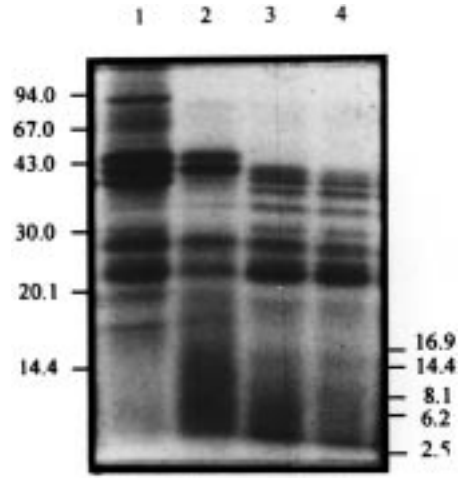

Figure 3. SDS−PAGE pattern of chickpea protein isolate and hydrolysates with different DH values after treatment with Flavourzyme: (lane 1) chickpea protein isolate; (lane 2) protein hydrolysate (DH = 9%); (lane 3) protein hydrolysate (DH = 14%); (lane 4) protein hydrolysate (DH = 27%). Low molecular weight and peptide markers are on the left and right, respectively.

soluble chickpea proteins was obtained. In both cases, however, the enzymatic hydrolysis of the protein isolate was very fast in the initial stages and a decrease of reaction rate in the final stages was observed, reaching a 27% DH after 3 h of hydrolysis with Alcalase or Flavourzyme. Marquez and Fernandez (1993) reported that the downward curvature of the hydrolysis curves could be attributed to substrate inhibition and second-order enzymatic deactivation with respect to enzyme concentration and depended upon the substrate concentration.

The combination of both enzymes in a two-step process gave a DH >50% at the end of the hydrolytic process. Although the globular structure of globulins in the protein isolates is per se an important limitation to the action of single proteolytic enzymes, the use of a sequential hydrolysis with an endoprotease and an exoprotease seems to solve this problem. The cleavage of peptide bonds by the endopeptidase Alcalase increases the number of end peptide sites for the action of the exoprotease Flavourzyme.

Electrophoretic Characterization of Chickpea Protein Hydrolysates. Molecular weight distribution of chickpea proteins and peptides after hydrolysis was determined by SDS−PAGE. Hydrolysis with Flavourzyme led to the progressive and total disappearance of several bands (Figure 3). Although the Flavourzyme hydrolysate with a DH of 9% showed a partial digestion of acidic polypeptidic chains of 11S globulins, intact forms of basic polypeptidic chains were also observed. Flavourzyme hydrolysate with a DH >9% showed a residual presence of acidic polypeptidic chains of 11S globulin and absence of electrophoretic bands between 60 and 45 kDa corresponding to the 7S proteins. Besides, a diffuse electrophoretic pattern composed of proteins with molecular weight <37 kDa and enriched in low molecular weight proteins and peptides as a result of the digestive process was observed. However, even in the hydrolysates with a DH of 27%, the basic polypeptidic chains of 11S globulin remain unaltered. The high resistance to proteolysis of basic polypeptidic chains has been also observed by us in helianthin, the 11S storage protein from sunflower (*Helianthus annuus* L.) (unpublished data).

Although individual treatments with Flavourzyme or Alcalase reached a similar DH (27%), the electrophoretic patterns were very different in both cases. In contrast to that obtained with Flavourzyme, Alcalase and Alcalase−Flavourzyme hydrolysates did not show visible electrophoretic bands, even after sample overloading of protein hydrolysates with DH <9% (data not shown). This fact could be due to the high proportion of small peptides and free amino acids with respect to the low molecular weight proteins.

Antigenicity of Chickpea Protein Hydrolysates. Loss of antigenicity was defined as the decrease in the capacity of antigenic determinants to interact with immunologically specific antibodies. Because the ELISA test measures antigenicity, it detects and quantifies fragmented as well as intact antigen remaining in the protein hydrolysates which retain the capacity to bind to IgE antibodies from individual serum of chickpea-allergic patients. The direct ELISA format used could not detect the ability of short peptides to bind at active epitope sites and at the same time bind with high affinity to the ELISA plate. However, because peptide molecules with only one epitope do not induce allergic reactions in vivo, the evaluation method could be adequate to evaluate the residual reactivity, which requires two or more epitopes on the same protein fragment bound to IgE on the surface of mast cells to promote mediator release (Aas, 1978). Therefore, hydrolysis, which destroys the cross-linking ability of the allergen, may be sufficient to eliminate its allergenic activity. This might be achieved without the complete hydrolysis of all antigenic epitopes (Taylor, 1992).

A typical pattern of the binding inhibition of IgE antibodies to different protein hydrolysates, obtained by individual and sequential treatment with Flavourzyme and Alcalase, is shown in Figure 4. Compared to the protein isolate, the antigenic activity of the hydrolysates was significantly reduced by enzymatic hydrolysis. Antiserum from healthy patients used as control did not show detectable reactivity to the protein isolate or hydrolysate.

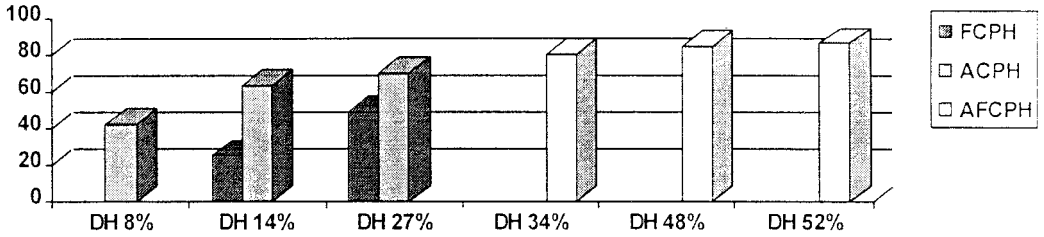

Figure 4. Inhibition of antigenic activity of protein hydrolysates with respect to protein isolates. Chickpea protein hydrolysates were obtained by individual treatment with Flavourzyme (FCPH), Alcalase (ACPH), and Alcalase plus Flavourzyme sequentially (AFCPH).

The loss of antigenic activity of protein hydrolysates obtained with Alcalase was different from the one obtained with Flavourzyme. Treatment with Alcalase was more effective in reducing the antigenic activity of partially hydrolyzed proteins (DH = 9%). In the initial stage of hydrolysis, a rapid loss of chickpea antigenic activity, above 60%, was observed. This fact might be due to immediate destruction of highly active surface epitopes. On the contrary, partially hydrolyzed proteins produced with Flavourzyme increased the antigenic activity compared to the protein isolate. This could be explained in part by an increased solubility of the resulting protein hydrolysates with antigenic determinants unhydrolyzed. Besides, new antigenic determinants could apparently be found after the exposure of chickpea proteins to the exoprotease Flavourzyme. Several investigators have also reported the presence of new antigens generated by pepsin hydrolysis of bovine milk proteins (Spies et al., 1970; Schwartz et al., 1980). As hydrolysis continued, more stable epitopes were destroyed. However, although the Alcalase hydrolysate of DH = 14 and 27% reached loss of antigenic activity >80%, the Flavourzyme hydrolysate did not reach 70% inhibition. Differences might be attributed to the presence in the Flavourzyme hydrolysate of intact or partial degraded basic polypeptidic chains of 11S proteins, with a high IgE-binding capacity. The proteolytic accessibility to the acidic chains and inaccessibility to the basic ones support the belief that the basic chains are buried within the interior of the protein molecule and the acidic chains are more exposed (Lambert and Yarwood, 1992). The most effective reduction of antigenicity was observed in extensively hydrolyzed chickpea proteins obtained by sequential treatment with Alcalase and Flavourzyme. Thus, the Alcalase−Flavourzyme hydrolysate with DH >34% decreased the antigenicity activity by >90%. The residual antigenicity was due probably to the presence of monovalent peptide fragments.

In conclusion, the sequential hydrolysis of an allergenic chickpea protein isolate with Alcalase and/or Flavourzyme is a helpful strategy to obtain hypoantigenic chickpea protein hydrolysates. This product could be used together with other nonsensitizing ingredients to elaborate specialized hypoallergenic products that will meet essential nutritional requirements, improving nutritional quality and safety.

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