 DOI 10.1002/mnfr.200900249 *Mol. Nutr. Food Res.* 2010, *54*, 1266–1272

RESEARCH ARTICLE

Effects of enzymatic hydrolysis on lentil allergenicity

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Received: May 29, 2009
Revised: September 11, 2009
Accepted: September 14, 2009

Enzymatic hydrolysis and further processing are commonly used to produce hypoallergenic dietary products derived from different protein sources, such as cow's milk. Lentils and chickpeas seem to be an important cause of IgE-mediated hypersensitivity in the Mediterranean area and India. Some studies have investigated the effects of enzymatic treatments on the *in vitro* immunological reactivity of members of the *Leguminosae* family, such as soybean, chickpea, lentil, and lupine. Nevertheless, there are only a few studies carried out to evaluate the effect on IgE reactivity of these food-hydrolysis products with sera from patients with well-documented allergy to these foods. In this study, lentil protein extract was hydrolyzed by sequential action of an endoprotease (Alcalase) and an exoprotease (Flavourzyme). Immunoreactivity to raw and hydrolyzed lentil extract was evaluated by means of IgE immunoblotting and ELISA using sera from five patients with clinical allergy to lentil. The results indicated that sequential hydrolysis of lentil results in an important proteolytic destruction of IgE-binding epitopes shown by *in vitro* experiments. However, some allergenic proteins were still detected by sera from four out of five patients in the last step of sequential hydrolyzation.

Keywords:
Allergens / Enzymatic hydrolysis / Food allergy / Legumes / Lentil

1 Introduction

In recent years, legume seed proteins are gaining importance as beneficial food ingredients and their consumption is recommended by health organizations and dieticians [1] Among plant proteins, soybean has been the most widely used source, but other legumes are also important [2]. Lentil consumption is increasing, mainly in developing countries as a vehicle for mineral biofortification [3].

Legumes are among the most common allergenic foods causing allergic reactions in children in the Mediterranean area [4–6]. A previous study showed that lentils ranked fourth, after hen eggs, fish, and cow's milk, as a cause of food allergy in children from Spain [7]. Two lentil allergens have been described so far. A major lentil allergen Len c 1 has been isolated and identified as a 48 kDa vicilin; its processing fragments, corresponding to subunits 12–16 and 26 kDa are also relevant lentil IgE-binding proteins [8, 9]. Len c 2 has been isolated and identified as a 66 kDa seed-specific biotinylated protein [8].

A number of studies have evaluated the effects of enzymatic hydrolysis on the allergenicity and digestibility of food proteins. Cow's milk hydrolysate formulae have been developed to decrease or eliminate its allergenicity [10, 11]. Changes in allergenicity of enzymatically hydrolyzed legumes have been evaluated mainly in soybean [12, 13] and peanut [13]. Studies with other members of the *Leguminosae* family are scarce. Clemente *et al.* [14] evaluated the reduction of immunoreactivity to chickpea allergens during extensive hydrolysis generated by the sequential action of an endoprotease (Alcalase) and an exoprotease (Flavourzyme). A decrease in IgE-binding capacity by more than 90% was detected using sera from six patients who reported reactions after chickpea ingestion. Moreover, this sequential

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Abbreviations: AU, Anson units; **DH,** degree of hydrolysis; ***E/S,*** enzyme substrate ratio; **LAPU,** leucine aminopeptidase unit

hydrolysate is considered an interesting material as food additive due to its high protein quality [15]. Sormus de Castro Pinto *et al.* [16] investigated the effects of pepsin and trypsin on the *in vitro* reactivity of 11S globulin of lupine, chickpea, and lentil using rabbit antiserum for these proteins. They obtained a large amount of short peptides and free amino acids after enzymatic treatment. The assessment of different enzyme activities to decrease allergenicity seems to be important; moreover, these studies should be carried out with sera from patients with documented clinical allergy to the source food [17].

Enzymatic protein hydrolysates constitute an alternative to intact proteins in the development of special formulations designed to provide nutritional support to specific population groups with different needs, such as infants, elderly, and food-allergic patients. In addition, protein hydrolysates show technological advantages. The combination of extensive enzymatic treatment coupled with post hydrolysis food processing procedures, such as heat treatment, ultrafiltration, *etc.*, is considered the most effective way to obtain protein products with an added high value for human nutrition and reduced allergenicity [18].

The objective of this study was to investigate the effect of sequential action of Alcalase (endoprotease) and Flavourzyme (exoprotease) in lentil allergenicity using sera from patients with well-documented lentil allergy. The potential utility of these lentil protein hydrolysates as hypoallergenic ingredients in food formulae is considered.

2 Materials and methods

2.1 Patients and sera

Sera from five patients with a convincing history of recent severe systemic anaphylaxis after isolated lentil ingestion were used in this study (Table 1). An informed consent, approved by the Ethic Committee of our Institution (Permission No. 0312150129) was signed by the patients to carry out the study. All subjects had a positive skin prick test response and a specific serum IgE level to lentil ranging from 1.24 to 30.7 kU/L (median=7.78 kU/L), as quantified by the CAP-fluorescent enzyme immunoassay System (Phadia, Uppsala, Sweden). A serum from a patient with a specific IgE to *Anisakis* spp. (9.09 kU/L), a specific IgE $<$ 0.35 kU/L to lentil, and a total serum IgE value of 53.4 kU/L was used as a negative control.

A serum pool made with sera from the five lentil-allergic patients and individual sera from all patients were used in the IgE immunodetection and ELISA assays.

Table 1. Clinical and immunologic findings of five patients with a convincing history of severe systemic anaphylaxis to lentil

Patient no.	Lentil		
	SPT (mm)	CAP-FEIA (kU/L)	Symptoms
1	13.00	1.24	A;G;H;P
2	8.50	20.20	A;AE;DS;G;GI;T;OAS;P;U
3	5.00	6.13	A;DY;P
4	16.50	30.70	A;O;OAS;P;R;U
5	12.00	7.78	A;AE;DS;G;GI;OAS;T

SPT, skin prick testing; A, asthma; AE, angioedema; DS, difficulty swallowing; DY, dysphonia; G, general malaise; GI, gastrointestinal symptoms; H, hypotension; O, ocular symptoms T, tongue swelling, OAS, oral allergy syndrome; P, pruritus; R, rhinitis; U, urticaria.

2.2 Plant material and enzymatic treatments

Lentil seeds (*Lens culinaris* cv Guareña) obtained from the *Servicio de Investigación y Tecnología Agraria* (Valladolid, Spain) were used for the study. Seeds, cleaned and free from foreign materials, were ground to pass through a 1-mm sieve (Cyclotec 1093,Tecator, Sweden), and the flour was defatted with *n*-hexane (34 mL/g of flour) for 4 h, shaken and air-dried after filtration.

The defatted lentil flour was extracted according to the method reported by Cuadrado *et al.* [19],] but using 0.1 M PBS (pH 7.4) buffer plus 0.15 M NaCl containing a 1% PVP (Calbiochem, Darmstadt, Germany) as extraction buffer. The flour was extracted twice at a 1:10 w/v ratio for 1 h at 4°C by stirring. The extract was centrifuged at 27 000 $\times$ *g* for 20 min at 4°C, and the combined supernatants were dialysed against distilled water for 48 h at 4°C and freeze-dried until use. The soluble protein content of the extract was determined by Bradford dye-binding assay (Bio-Rad, CA, USA), using bovine serum albumin (Sigma, MO, USA) as a standard.

The lentil protein extract was hydrolyzed according to the method reported by Clemente *et al.* [14] with modifications. The enzymatic complexes used were Alcalase 2.4 L and Flavourzyme 1000 L (Novozyme A/S, Bagsvaerd, Denmark). Alcalase (2.4 L) is an endopeptidase from *Bacillus licheniformis*, with Subtilisin Carlsberg as the major enzymatic component, having a specific activity of 2.4 Anson units (AU) *per* gram. One AU is the amount of enzyme that digests hemoglobin under standard conditions at an initial rate that produces an amount of trichloroacetic acid-soluble product which gives the same colour with the Folin reagent as one mequiv of tyrosine released *per* minute. Flavourzyme (1000 L) 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 leucine-*p*-nitroanilide *per* minute.

The hydrolysis was conducted in a 100-mL reaction vessel, equipped with a stirrer, thermometer, and pH electrode. The protein extract was hydrolyzed batchwise with Alcalase and Flavourzyme by sequential treatment

(480 min), which was carried out with an initial hydrolysis (180 min) using Alcalase as an endopeptidase and a second one (300 min) using Flavourzyme as an exopeptidase. Hydrolysis parameters for Alcalase were as follows: protein concentration (S) = 2%; enzyme substrate ratio (E/S) = 0.2 AU/g of protein; temperature (T) = 50°C; and pH 8.0. Flavourzyme hydrolysis parameters were: (S) = 2%; E/S = 50 LAPU/g of protein, T = 50°C; and pH 7.0. Samples were withdrawn at certain time intervals and proteases in the aliquots were inactivated by heating at 80°C for 20 min.

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 (Sigma) using leucine as the standard, according to the method of Adler-Nissen [20]. The total number of amino groups was determined by acid hydrolysis with 6 N HCl at 120°C for 24 h.

2.3 Immunodetection assays

2.3.1 Protein electrophoresis and IgE immunoblot experiments

SDS-PAGE was performed according to Laemmli [21]. Samples (20 μg protein *per* lane) were mixed with Laemmli sample buffer (Bio-Rad) and 2-mercaptoethanol (Bio-Rad) heated at 90°C for 10 min, and electrophoresed in 4–20% Tris-HCl linear gradient precast gel (Bio-Rad). Proteins were visualized with Coomassie Brilliant Blue R250 staining. Western blotting was performed by electrophoretic transfer to nitrocellulose membrane (Whatman, Dassel, Germany). After blocking with 3% w/v nonfat milk, 0.1% v/v Tween-20 in PBS (pH 7.4; blocking buffer), the membranes were incubated overnight with the serum pool or individual sera (1:20 dilution), washed, and then treated with mouse anti-human IgE mAb HE-2 ascitic fluid (1:5000 dilution for 1 h) [22]. After washing, a goat anti-mouse IgG peroxidase-conjugated antibody (Pierce, IL, USA) (1:2500 dilution for 1 h) was added. Detection of IgE-binding components was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (Amersham, Buckinghamshire, UK). A serum (1:10 dilution) from a patient with a positive specific IgE to *Anisakis* spp. was tested as a negative control.

2.3.2 ELISA

Specific IgE binding to raw lentil extract and hydrolyzed samples was assessed by means of ELISA with the serum pool from the five patients with clinical allergy to lentil. Polystyrene microtiter plates (Costar 3590, Corning, NY, USA) were coated with 100 μL/well of extract (30 μg/mL in PBS) and incubated at 4°C overnight. The wells were washed with 0.01M PBS containing 0.5% Tween-20 v/v and blocked with PBS containing 3% nonfat milk w/v and 0.1% Tween-20 (200 μL/well). Plates were incubated overnight with the serum pool (100 μL/well, 1:20 dilution), and the binding of IgE was detected by incubation for 1 h with mouse anti-human IgE mAb HE-2 ascitic fluid (100 μL/well, 1:5000 dilution) [22] followed by goat anti-mouse IgG peroxidase-conjugated (100 μL/well, 1:2500 dilution for 1 h) (Pierce). Finally, the peroxidase reaction was developed with 50 μL of peroxidase substrate buffer (Dako, Glostrup, Denmark). After 30 min, the reaction was stopped with 50 μL of 4N H_2SO_4, and the OD was measured at 492 nm. The following three negative controls were used: (i) wells coated with non-fat milk (instead of lentil samples) and incubated with the serum pool from the five patients following the process described above; (ii) wells coated with lentil samples and incubated with the negative control serum (instead of the lentil-allergic serum pool) following the process already described; and (iii) wells coated with lentil samples and incubated directly with mouse anti-human IgE and goat anti-mouse IgG.

All the tests were performed in triplicate. The formula: mean [OD] + 3 × SD was calculated for each negative control and the highest value was considered as a cut-off point for positivity.

The percentage of the decrease in antigenic activity was calculated with the formula: $(1-A_H/A_N)\,100$ where A_H is the absorbance value obtained from the hydrolyzed samples and A_N is the absorbance value of the protein extract sample.

3 Results

3.1 Protein extract hydrolysis

Lentil protein hydrolysates were obtained by the sequential action of the endoprotease (Alcalase) (E/S = 0.2 AU/g) and the exoprotease (Flavourzyme) (E/S = 50 LAPU/g). Figure 1 shows the hydrolysis curve of the lentil protein extract using the sequential treatment with Alcalase and Flavourzyme. Protein hydrolysis took place rapidly in the first 30 min; further, progression developed more slowly, reaching 24% DH after 180 min of Alcalase treatment. As hydrolysis of

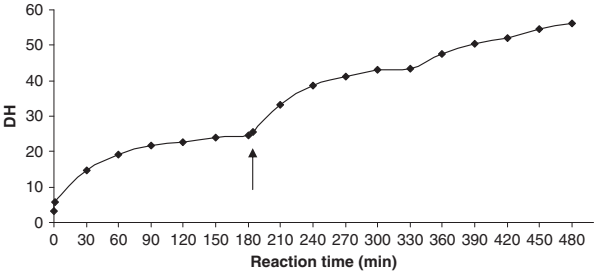


Figure 1. Hydrolysis curves of lentil protein by sequential treatment with Alcalase and Flavourzyme. The arrow indicates the moment Flavourzyme is added. DH: degree of hydrolysis.

protein isolates continued, the DH increased. The combination of both enzymes yielded a DH of 56% at the end of the process (480 min).

3.2 Electrophoretic characterization of protein hydrolysates

Figure 2A shows the SDS-PAGE protein patterns of raw and enzymatically processed lentil extracts. The raw sample showed multiple bands with molecular weights between 14 and 91 kDa. Protein hydrolysates with DH=6% (Alcalase 15 s) showed less stained bands and an increase of low-molecular-weight proteins. Hydrolysates with DH>15% (Alcalase>30 min) did not show any visible electrophoretic bands.

All the samples were analyzed with IgE-immunoblot using the serum pool from the five patients with clinical allergy to lentil (Fig. 2B). Raw lentil showed a complex pattern of IgE-binding proteins in the range of 12–76 kDa. The overall IgE immunoreactivity of lentil was strongly reduced at the end of the sequential endo- and exoprotease hydrolysis (480 min), although bands of 48, 24 and 60 kDa (putative Len c 1, its processing fragments and putative Len c 2, respectively) were still recognized after treatment with Alcalase for 15 s and two tenuous bands of 48 and 60 kDa (putative Len c 1 and Len c 2, respectively) were still present after treatment with Alcalase for 180 min. A spot was recognized after treatment with Flavourzyme during 300 min. This spot could be an artifact since a further immunoblot experiment with raw lentil and with lentil after Flavourzyme treatment for 300 min using the serum pool, showed several IgE-binding proteins in raw lentil and no IgE-binding bands in the hydrolyzed sample (this figure is shown in Supporting Information).

IgE antibody reactivity to raw and hydrolyzed lentil with Alcalase for 180 min and Flavourzyme for 300 min was screened using the five individual sera (Fig. 3). All the sera recognized a wide range of IgE-binding proteins of raw lentil. Four out of five sera recognized fewer IgE-binding proteins combined with a decreased band intensity after Alcalase treatment for 180 min. The IgE reactivity was strongly reduced after Flavourzyme treatment for 300 min. With this treatment, sera from patient 5 did not recognize any proteins; sera from patients 1 and 3 recognized an 18 kDa band (patient 1) and putative Len c 1 (patient 3), both with a decreased intensity. Sera from patients 2 and 4 still detected allergenic proteins of 12 and 14 kDa and a protein between 45 and 48 kDa.

3.3 ELISA

The ELISA test was carried out to assess the reduction of antigenic activity of the protein hydrolysates, using the serum pool from the five patients with clinical allergy to lentil. Specific serum IgE levels > 0.17 OD units (mean [OD] $+3 \times$ SD to raw lentil incubated directly with mouse anti-human IgE and goat anti-mouse IgG: $0.093 + 3 \times 0.024 = 0.17$) were considered as positive. The results are shown in Fig. 4. The IgE reactivity of the hydrolysates was reduced by the sequential action of Alcalase and Flavourzyme. Most proteins from lentil did not maintain their IgE-binding properties during digestion with Alcalase–Flavourzyme. Alcalase was very effective in reducing the antigenic activity of lentil proteins since the enzyme led to a 95% inhibition of antigenicity in the first few seconds (DH 6%); the sequential addition of Flavourzyme caused the destruction of all antigenic epitopes (DH = 56%).

4 Discussion

Enzymatic protein hydrolysates have been reported as a suitable source of protein for human nutrition, as their gastrointestinal absorption seems to be more effective than that of intact proteins [23, 24]. Protein hydrolysates from legumes have been widely used in specific formulations to improve their nutritional and functional properties. These

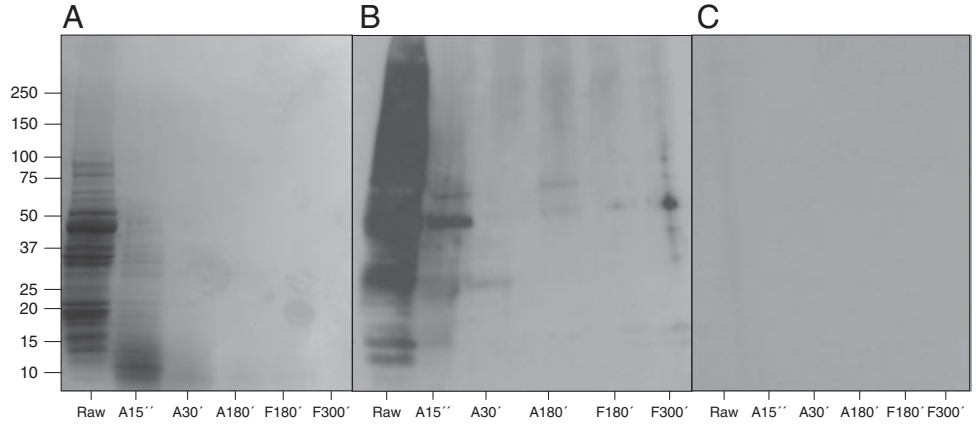


Figure 2. SDS-PAGE (A) and immunodetection with a serum pool from five patients with anaphylactic reaction to lentil (B) or a negative control serum (C) of the following samples: extract from raw lentil (Raw), hydrolysates after sequential treatment with Alcalase during 15 s (A15′′), 30 min (A30′), 180 min (A180′), and Flavourzyme treatment during 180 min (F180′) and 300 min (F300′).

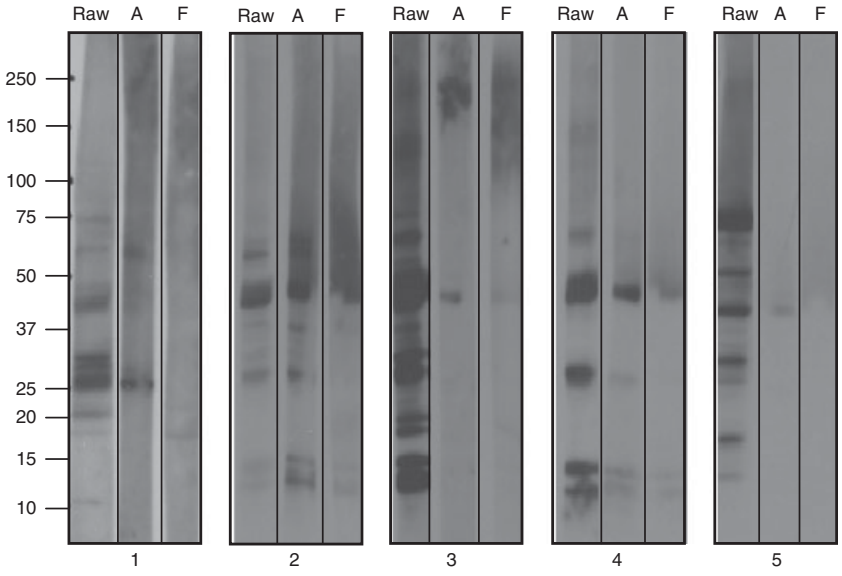
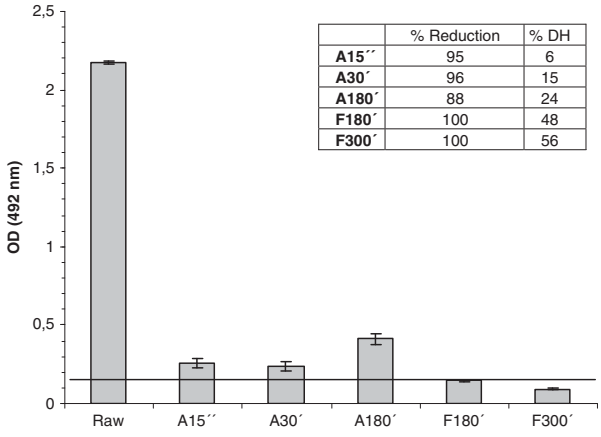


Figure 3. Western blotting of raw lentil and hydrolysates after sequential treatment with Alcalase during 180 min (A) and Flavourzyme during 300 min (F), incubated with individual sera from patients with anaphylactic reaction to lentil.



	% Reduction	% DH
A15´´	95	6
A30´	96	15
A180´	88	24
F180´	100	48
F300´	100	56

Figure 4. Specific IgE to raw lentil and hydrolysates after sequential treatment with Alcalase (15 s, 30 and 180 min) and Flavourzyme (180 and 300 min), using a serum pool from five lentil-allergic patients. Percentages of reduction in antigenic activity and DHs are shown in the table annexed to Fig. 4.

uses include clinical applications, such as geriatric products and therapeutic diets [18].

Food hydrolysate formulae, such as cow's milk or soybean, have been developed with the aim to produce hypoallergenic food with markedly reduced sensitizing capacity. However, residual antigenicity and allergenicity have been reported even in extensively hydrolyzed formulae [25, 26]. Reduction of allergenicity of dietary products may be assessed *in vitro* using various immunological methods. ELISA has been chosen to detect antigenicity in most commercial hypoallergenic hydrolysates because of its sensitivity and relative simplicity [14].

In this study, a sequential hydrolyzation of lentil extract with Alcalase and Flavourzyme was carried out. Clemente *et al.* [14] obtained the most effective reduction of antigenicity in chickpea by a sequential treatment with Alcalase and Flavourzyme compared with both enzymes tested individually. This hydrolysate presented a high protein quality [15].

The present results showed in sequential lentil hydrolysis, a 24% DH at 180 min produced by Alcalase; the addition of Flavourzyme, increased the DH to 56% in the final reaction time (480 min). These data seem to indicate that endoprotease Alcalase produced an initial digestion that increased the number of target sites for the action of the exoprotease Flavourzyme. These values are similar to those obtained for pea [27, 28] and soy [29] protein hydrolysates. Clemente *et al.* [14] obtained a similar DH for chickpea but using also a double *E*/*S*.

The ELISA results showed that Alcalase elicited a 95% decrease of IgE reactivity before the first minute of hydrolyzation. The immunoblot assay carried out with the serum pool from the five patients with clinical allergy to lentil showed recognition of only three proteins at this time: putative Len c 1 (48 kDa protein), its processing fragment (24 kDa protein), and putative Len c 2 (60 kDa protein); these proteins are probably more resistant to digestion than other immunoreactive proteins. Food allergens are usually resistant to proteases, heat and denaturants allowing them to avoid degradation during food preparation and digestion [30]. The differential thermostability of several lentil IgE-binding proteins recently reported [19] could support the higher resistance to enzymatic digestion of some IgE-binding proteins in comparison with others. After Flavourzyme hydrolyzation, a 100% inhibition was obtained in ELISA and no bands were detected in immunoblotting. However, immunoblot with individual sera showed that two sera detected IgE-binding proteins (12, 14 and 45–48 kDa) in Flavourzyme hydrolyzation during 300 min; the other two

sera exhibited a tenuous recognition of proteins of 18 kDa and putative Len c 1 in the same sample. Sormus de Castro Pinto *et al.* [16] using rabbit antiserum for lentil 11S globulin found weakly immunogenic bands in immunoblotting only after 1 min of treatment of lentil with pepsin and trypsin. The ELISA showed a high reduction of antigenicity with both enzymes. Clemente *et al.* [14] obtained similar results in chickpea, when sequential hydrolysis of Alcalase and Flavourzyme was tested. Humiski and Aluko [27] used Alcalase and Flavourzyme, among other enzymes, to study the effect of proteolytic treatments on pea. They found that both the enzymes produced protein hydrolysates with a significantly higher DH when compared with other proteases (papain, trypsin, and α-chymotrypsin); moreover, the amino acid profile analysis using HPLC system showed that the higher number of peptide fractions obtained for Alcalase and Flavourzyme was probably a reflection of the higher DH of their hydrolysate, which could indicate that more peptide bonds were broken and hence a greater number of peptides were produced.

In conclusion, the results of this study seem to indicate that sequential hydrolysis of lentil with Alcalase and Flavourzyme, produces an important proteolytic destruction of epitopes as shown with *in vitro* assays using sera from patients with clinical allergy to lentil. Although further studies are needed to characterize the biological activity of the residual allergens and to assess the clinical relevance of our findings, this enzymatic procedure seems to be a promising method to obtain hypoallergenic protein hydrolysates. Given the good nutritional value of lentil proteins, this sequential hydrolysis product could be useful to elaborate hypoallergenic food formulae for the population groups with specific nutritional requirements.

This work was supported by the AGL 2004-07971 research project.

The authors have declared no conflict of interest.

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