

Influence of Enzymatic Hydrolysis on the Allergenicity of Roasted Peanut Protein Extract

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Key Words

Enzymatic hydrolysis • Food allergy • Legumes • Roasted peanut

Abstract

Background: Peanut allergy is recognized as one of the most severe food allergies. Some studies have investigated the effects of enzymatic treatments on the in vitro immunological reactivity of members of the Leguminosae family, such as the soybean, chickpea and lentil. Nevertheless, there are only a few studies carried out with sera from patients with a well-documented allergy. **Methods:** Roasted peanut protein extract was hydrolyzed by the sequential and individual action of 2 food-grade enzymes, an endoprotease (Alcalase) and an exoprotease (Flavourzyme). Immunoreactivity to roasted peanut extract and hydrolyzed samples was evaluated by means of IgE immunoblot, ELISA and 2-dimensional electrophoresis using sera from 5 patients with a clinical allergy to peanuts and anti-Ara h 1, anti-Ara h 2 and anti-Ara h 3 immunoblots. **Results:** Immunoblot and ELISA assays showed an important decrease of IgE reactivity and Ara h 1, Ara h 2 and Ara h 3 levels in the first 30 min of hydrolyzation with Alcalase. In contrast, individual treatment with Fla-

avourzyme caused an increase in IgE reactivity detected by ELISA at 30 min and led to a 65% inhibition of IgE reactivity at the end of the assay (300 min). Ara h 1 and the basic subunit of Ara h 3 were still recognized after treatment with Flavourzyme for 300 min. **Conclusion:** Hydrolysis with the endoprotease Alcalase decreases IgE reactivity in the soluble protein fraction of roasted peanut better than hydrolysis with the exoprotease Flavourzyme.

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Introduction

Peanut allergy is recognized as one of the most severe food allergies due to its persistency and its often life-threatening nature [1]. The prevalence of peanut allergy appears to have increased in the western world during the last decades. An estimate of the prevalence of peanut allergy in children was 1.4% in 2008 compared with 0.8% in 2002 and 0.4% in 1997 in a self-reported population survey [2]. Currently, the only effective treatment for peanut allergy is avoidance of this nut in any form. However, total avoidance is difficult due to the widespread use of peanuts in the diet as an economical protein source.

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Therefore, it is necessary to explore strategies designed to decrease the ability of peanuts to elicit dangerous allergic responses.

Several studies have evaluated the effects of enzymatic hydrolysis on the allergenicity and digestibility of food proteins [3–6]. Enzymatic hydrolysis is an efficient process for disrupting sequential and conformational epitopes [7]; therefore, protein hydrolysates could be an alternative to intact proteins in the development of special formulations for food-allergic patients [8]. However, depending on the type of enzymes used and the conditions of hydrolysis, peptides of different length may be obtained carrying more or less allergenicity [7, 9, 10]. Porcine trypsin/chymotrypsin is frequently used for producing hypoallergenic formulas, but proteases extracted from bacteria or of fungal origin are also increasingly used [7]. Two studies have analyzed the effects of the sequential action of an endoprotease from *Bacillus licheniformis* (Alcalase) and an exoprotease from *Aspergillus oryzae* (Flavourzyme) in chickpea [5] and lentil allergenicity [6]. These enzymes produce protein hydrolysates with a significantly higher degree of hydrolysis than papain, trypsin, and α -chymotrypsin [11]. In the chickpea and the lentil the sequential hydrolyzation with Alcalase and Flavourzyme produces a significant decrease in IgE recognition as shown with in vitro assays. Although further studies are needed to characterize the clinical relevance of these findings, this enzymatic procedure could be a suitable method to obtain less allergenic protein hydrolysates [5, 6].

The objective of this study was to investigate the effect of the individual and sequential action of Alcalase and Flavourzyme in the IgE-binding properties of the soluble protein fraction of roasted peanut (RP), using sera from patients with a clinical allergy to peanuts.

Material and Methods

Patients and Sera

Sera from 5 patients with a peanut allergy, confirmed on the basis of a positive double-blind placebo-controlled food challenge with peanut, were used in this study (table 1). An informed consent, approved by the Ethics Committee of our institution, was signed by the patients to carry out the study (Permission No. 0312150129). Subjects had a specific serum IgE level to peanuts ranging from 0.9 to 7.4 kU/l (median = 3.5 kU/l) as quantified by the fluorescent enzyme immunoassay (CAP-FEIA system, Phadia, Uppsala, Sweden).

A serum pool made with sera from the 5 peanut-allergic patients, and individual sera from patients 1, 2 and 4 were used in IgE immunodetection and ELISA assays.

Table 1. Clinical and immunological findings of 5 peanut-allergic patients

Patient No.	SPT, mm	CAP-FEIA, kU/l	Symptoms
1	4.5	3.5	A; DS; P; R; U
2	7	3.5	P
3	0	0.9	AE; DS; E; P; U
4	15.5	6.7	OAS
5	10	7.4	A; OAS; P; U

SPT = Skin prick testing; A = asthma; AE = angioedema; DS = difficulty swallowing; E = erythema; OAS = oral allergy syndrome; P = pruritus; R = rhinitis; U = urticaria.

Plant Material and Enzymatic Treatments

RPs (*Arachis hypogaea*, Virginia variety) obtained from Apeitivos Medina SL (Spain) were used in the study. Cleaned and free from foreign materials, they were milled to pass through a 1-mm sieve (Tecator, Cyclotec 1093, Sweden) and the resulting meal was defatted with n-hexane (34 ml/g of flour) for 4 h, shaken and air-dried after filtration.

Peanut flour was extracted according to the method reported by Cuadrado et al. [12] but using 0.05 M Tris-HCl (pH 8.0) plus 0.5 N NaCl as an extraction buffer. 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 g for 20 min at 4°C, and the combined supernatants were dialyzed against distilled water for 48 h at 4°C using a dialysis membrane (Spectra/Por, Serva, Heidelberg, Germany) with a cutoff of 3.5 kDa, and then freeze-dried until use. The soluble protein content of the extract was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, Calif., USA) using bovine serum albumin (Sigma, Mo., USA) as a standard.

Peanut protein extract was hydrolyzed according to Clemente et al. [5] with modifications. The food-grade enzymatic complexes used were Alcalase 2.4 L and Flavourzyme 1000 L (Novozyme A/S, Bagsvaerd, Denmark). Alcalase (2.4 L) is an endoprotease from *B. 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 a trichloroacetic acid-soluble product which produces the same color with the Folin reagent as 1 mEq of tyrosine released per min. Flavourzyme (1000 L) is an exoprotease and endoprotease complex from *A. oryzae*, 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 min. According to the technical bulletin of Novozymes, Flavourzyme is mainly an exoprotease, the endoprotease activity being almost residual.

The hydrolysis was conducted in a 100-ml reaction vessel, equipped with a stirrer, a thermometer and a pH electrode. The protein extract was hydrolyzed batchwise with Alcalase and Flavourzyme by sequential and individual treatment. (1) Sequential treatment (480 min) was carried out with the initial hydrolysis (180 min) using Alcalase as endopeptidase and the second one

(300 min) using Flavourzyme as exopeptidase. Hydrolysis parameters for Alcalase were as follows: protein concentration (S) = 2%, enzyme to substrate ratio (E/S) = 0.4 AU/g of protein, temperature (T) = 50°C, pH 8.0. Flavourzyme hydrolysis parameters were: S = 2%, E/S = 100 LAPU/g of protein, T = 50°C, pH 7.0. (2) Individual treatment using Alcalase was developed during 150 min. Hydrolysis parameters for Alcalase were: S = 2%, E/S = 0.2 AU/g of protein, T = 50°C, pH 8.0. (3) Individual treatment using Flavourzyme was developed during 300 min. Flavourzyme hydrolysis parameters were: S = 2%, E/S = 100 LAPU/g of protein, T = 50°C, 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 the free amino groups by reaction with trinitrobenzene sulfonic acid (TNBS; Sigma) using leucine as the standard, according to the method of Adler-Nissen [13]. The total number of amino groups was determined by acid hydrolysis with 6 N HCl at 120°C for 24 h.

Immunodetection Assays

Protein Electrophoresis and IgE Immunoblot Experiments

SDS-PAGE was performed according to Laemmli [14]. 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 R-250 staining. Western blot was performed by electrophoretic transfer to nitrocellulose membranes (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 (1:40 dilution) or individual sera (1:10 dilution), washed, and then treated with mouse anti-human IgE mAb HE-2 ascitic fluid (1:5,000 dilution for 1 h) [15]. After washing, a goat anti-mouse IgG peroxidase-conjugated antibody (1:2,500 dilution for 1 h; Pierce, Ill., USA) was added. Detection of IgE-binding components was achieved by means of enhanced chemiluminescence using an ECL substrate according to the manufacturer's instructions (Amersham, UK). Membranes incubated directly with mouse anti-human IgE and goat anti-mouse IgG were used as negative controls.

Two-Dimensional Analysis

Two-dimensional analysis was carried out in the Proteomic Facility of the Instituto de Investigación Hospital 12 de Octubre (i+12). Lyophilized peanut extracts were dissolved in an appropriate volume of lysis buffer with 7 M urea, 2 M thiourea, and 2% CHAPS until completely resuspended. Samples were cleaned and desalted with the 2-D Clean-Up Kit (Amersham). Proteins were dissolved for at least 5 min in loading buffer containing 7 M urea, 2 M thiourea, 2% w/v CHAPS, 2% v/v IPG buffer, pH 3–11 NL, and 65 mM DTT. Immobililine DryStrip gel, pH 3–11 NL, 7 cm, was rehydrated in DeStreak Rehydration Solution (Amersham) and 2% IPG Buffer, pH 3–11. Seventy micrograms of protein were applied by cup loading and focused using the IPGphor 3 electrophoresis unit (GE Healthcare) at 20°C to reach 14 kV-h, with a maximum voltage of 1,200 V. For the second dimension, the IPG strips were reduced for 15 min in SDS equilibration buffer (6 M urea, 50

mM Tris-HCl, pH 8.8, 20% glycerol, 10% SDS, 1% w/v DTT, and a trace of bromophenol blue), and proteins were alkylated in the same buffer containing 2.5% w/v iodoacetamide instead of DTT for 15 min. Subsequent SDS-PAGE separation was performed using a mini PROTEAN II cell (Bio-Rad, Munich, Germany). Two-dimensional gels were Coomassie blue stained following the manufacturer's instructions (Brilliant Blue G-Colloidal Concentrate, Electrophoresis Reagent, B2025-1EA, Sigma-Aldrich). Proteomic MALDI-TOF/TOF analysis of Coomassie blue-stained spots of interest was carried out in the UCM-PCM Proteomic Facility, a member of the ProteoRed network. Spots were washed, reduced, alkylated and digested as previously described [16]. Mass spectrometry analyses were performed in a MALDI-TOF spectrometer 4800 Proteomics Analyzer (PerSeptive Biosystems, Framingham, Mass., USA) [16]. Proteins for which peptide mass fingerprints provided an ambiguous identification were subjected to MS/MS sequencing analyses [16]. For protein identification, the nonredundant National Center for Biotechnology Information database was searched using MASCOT 2.1 (www.matrixscience.com) through the Global Protein Server v3.6 from Applied Biosystems. Identifications were accepted as positive when at least 5 matching peptides and at least 20% of the peptide coverage of theoretical sequences matched, setting the probability score at $p < 0.05$.

ELISA

Specific IgE binding to RP extract and hydrolyzed samples was assessed by means of indirect ELISA with the serum pool from the 5 patients with a clinical allergy to peanuts. Polystyrene microtiter plates (Costar 3590, Corning, N.Y., USA) were coated with 100 µl/well of extract (30 µg/ml in 0.01 M PBS) and incubated at 4°C overnight. Wells were washed with 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, 1:10 dilution) and binding of IgE was detected by incubation for 1 h with mouse anti-human IgE mAb HE-2 ascitic fluid (100 µl/well, 1:5,000 dilution) [15] followed by goat anti-mouse IgG peroxidase-conjugated (100 µl/well, 1:2,500 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 4 N H₂SO₄, and the optical density (OD) was measured at 492 nm. The following negative controls were included: (1) wells were coated with nonfat milk (instead of peanut samples) and incubated with the serum pool from the 5 patients following the process described above and (2) wells were coated with peanut samples and incubated directly with mouse anti-human IgE and goat anti-mouse IgG.

All 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 cutoff point for positivity.

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

Anti-Ara h 1, Anti-Ara h 2 and Anti-Ara h 3 Immunoblots

RP before and after sequential and individual treatment with Alcalase and Flavourzyme was assessed for Ara h 1, Ara h 2 and Ara h 3 profiles. Samples (20 µg protein per lane) underwent elec-

trophoresis and were transferred. Membranes were preblocked for 1 h at room temperature (RT) in 5% w/v nonfat milk, 0.05% v/v Tween 20 in PBS. Chicken anti-Ara h 1 (1:10,000), chicken anti-Ara h 2 (1:8,000) and chicken anti-Ara h 3 (1:5,000) (custom-synthesized by Sigma Immunosys, The Woodlands, Tex., USA) were diluted in 5% w/v nonfat milk, 0.05% v/v Tween 20 in PBS and incubated with the membrane for 1 h at RT. The HRP-labeled antichick IgY (1:100,000; Sigma Immunosys) was diluted in 2% w/v nonfat milk, 0.05% v/v Tween 20 in PBS and incubation time was 30 min at RT. Detection was achieved as described above.

Results

Protein Extract Hydrolysis

Peanut protein hydrolysates were obtained by: (1) sequential treatment with the endoprotease Alcalase (E/S = 0.4 AU/g) and the exoprotease Flavourzyme (E/S = 100 LAPU/g), (2) individual treatment with Alcalase (E/S = 0.2 AU/g) and (3) individual treatment with Flavourzyme (E/S = 100 LAPU/g). Figure 1 shows the hydrolysis curve of peanut protein extract over the indicated times. The x-axis shows the time of reaction and the y-axis shows the DH. Protein hydrolysis took place rapidly in the first 30 min; further, progression was slower. The combination of both enzymes yielded a DH of 69% at the end of the process (480 min). Individual treatment with Alcalase (0.2 AU/g) reached 17% DH after 150 min and individual treatment with Flavourzyme reached 29% DH after 300 min.

Electrophoretic Characterization of Protein Hydrolysates

Figure 2a shows the SDS-PAGE protein patterns of RP before and after sequential treatment with Alcalase and Flavourzyme (fig. 2a1), and individual treatment with Alcalase (fig. 2a2) and Flavourzyme (fig. 2a3). Multiple bands can be seen in the RP sample with molecular weights between 10 and 63 kDa. Less stained bands but an increase of low molecular weight smears were observed after treatment with Alcalase 0.4 AU/g (fig. 2a1) and 0.2 AU/g (fig. 2a2) for 15 s. Hydrolysates with DH >16 and 11% (Alcalase 0.4 AU/g and 0.2 AU/g >30 min) showed a decrease of high-molecular-weight proteins. RP after individual treatment with Flavourzyme (fig. 2a3) showed fewer effects over the stained bands.

All samples were analyzed with IgE immunoblot using a serum pool from the 5 patients with a clinical allergy to peanuts. Figure 2b shows the IgE-binding protein patterns of RP before and after sequential treatment with Alcalase and Flavourzyme (fig. 2b1), and individual treat-

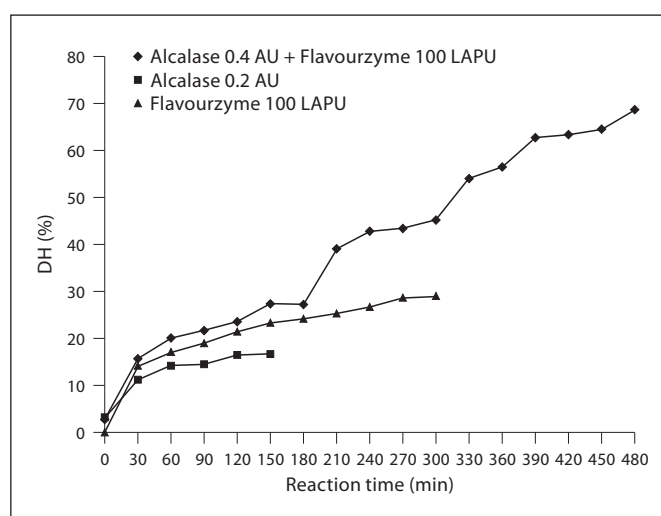


Fig. 1. Hydrolysis curves of peanut protein obtained by sequential and individual treatments with Alcalase and Flavourzyme. DH is defined as the percentage of peptide bonds cleaved.

ment with Alcalase (fig. 2b2) and Flavourzyme (fig. 2b3). RP showed a pattern of IgE-binding proteins in the range of 12–16 to 55 kDa. The overall IgE immunoreactivity was reduced after sequential endo- and exoprotease hydrolysis (480 min) and individual hydrolysis with Alcalase 0.2 AU/g (150 min). However, RP after treatment with Alcalase 0.2 AU/g (fig. 2b2) showed more IgE-binding proteins after hydrolysis during 15 s than Alcalase 0.4 AU/g (fig. 2b1). Individual treatment with Flavourzyme caused less reduction in IgE-binding proteins (fig. 2b3). A band of 22 kDa was still strongly recognized after treatment with Flavourzyme for 300 min. No bands were found in the negative controls (data not shown).

IgE reactivity to RP and the selected times of sequential and individual hydrolysis of RP with Alcalase and Flavourzyme were screened using 3 individual sera (patients 1, 2 and 4) (fig. 3). None of the sera recognized any RP proteins after sequential endo- and exoprotease hydrolysis (480 min). However, after individual treatment with Flavourzyme, the 3 sera recognized multiple bands. Proteins of 22 and 10 kDa were recognized by the 3 sera after hydrolysis during 300 min, and sera from patients 1 and 4 detected a 63- and a 30-kDa protein at this time of hydrolysis.

Two-Dimensional Analysis

In order to study the proteins still recognized by the sera after 5 h of hydrolysis with Flavourzyme, a 2-dimen-

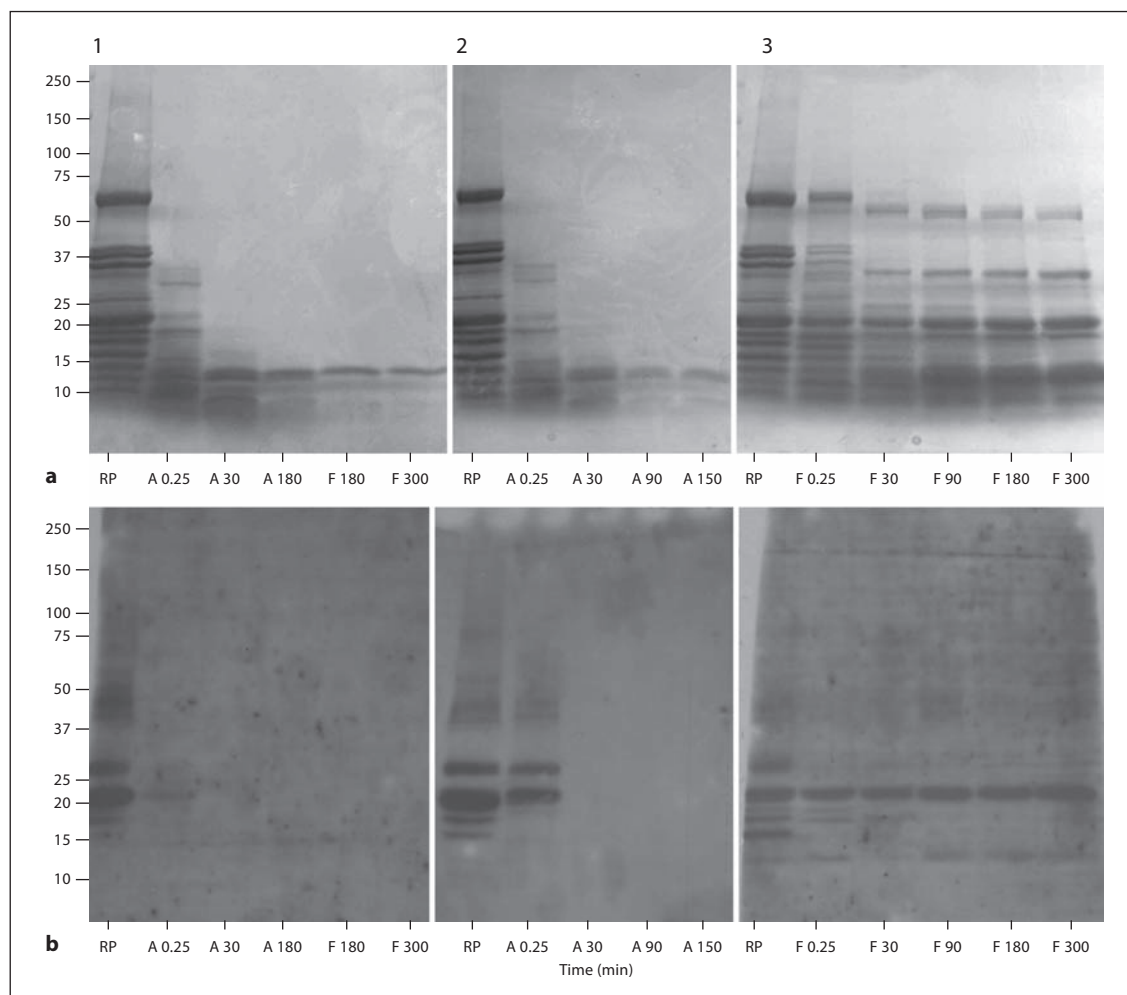


Fig. 2. SDS-PAGE (**a**) and Western blotting (**b**) of RP before and after sequential treatment with Alcalase 0.4 AU/g and Flavourzyme 100 LAPU/g (lane 1), individual treatment with Alcalase 0.2 AU/g (lane 2) and individual treatment with Flavourzyme 100 LAPU/g (lane 3), at the times indicated at the bottom of each figure. Western blotting was carried out with a serum pool from 5 patients with a clinical allergy to peanuts. A = Alcalase; F = Flavourzyme.

sional analysis of RP and RP after individual treatment with Flavourzyme during 300 min was carried out. Figure 4 shows the 2-dimensional protein staining of both samples and the Western blot of RP after individual treatment with Flavourzyme during 300 min, using the serum pool from the 5 peanut-allergic patients. Two-dimensional protein staining of RP showed several protein spots, whereas RP after individual treatment with Flavourzyme during 300 min showed spots of approximately 22 kDa and an isoelectric point (pI) ranging from 6 to 9.5 and spots of 15 kDa and pI between 3 and 9. Three spots of 22 kDa and pI ranging from 5 to 7 reacted with IgE antibod-

ies. The spots were tryptic-digested in order to carry out the analysis by MALDI-TOF. The study of MALDI-TOF/MS using Mascot enabled us to match the 3 spots with the basic subunit of Ara h 3 (data not shown).

ELISA

In order to assess the IgE reactivity of RP hydrolysates, an ELISA was carried out using the serum pool from the 5 patients with a clinical allergy to peanuts. The positive cutoff point was 0.085 OD units, which was the highest value after applying the formula: mean (OD) + 3 × SD for each negative control. The ELISA results are summa-

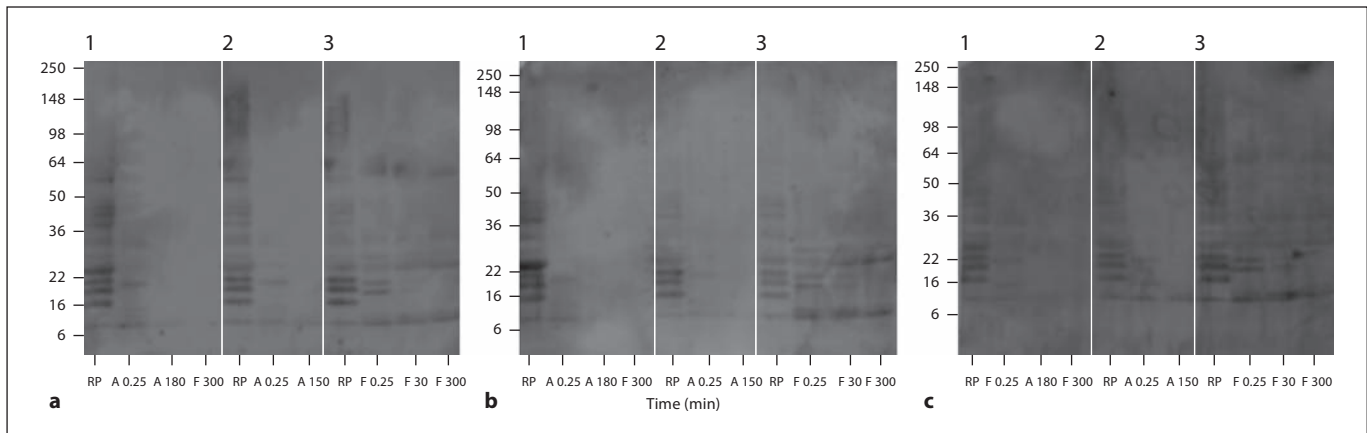


Fig. 3. Western blotting of RP before and after sequential treatment with Alcalase 0.4 AU/g and Flavourzyme 100 LAPU/g (lane 1), individual treatment with Alcalase 0.2 AU/g (lane 2) and individual treatment with Flavourzyme 100 LAPU/g (lane 3), at the times indicated at the bottom of each figure. Membranes were incubated with individual sera from 3 patients with a clinical allergy to peanuts: patient 1 (a), patient 2 (b) and patient 4 (c). A = Alcalase; F = Flavourzyme.

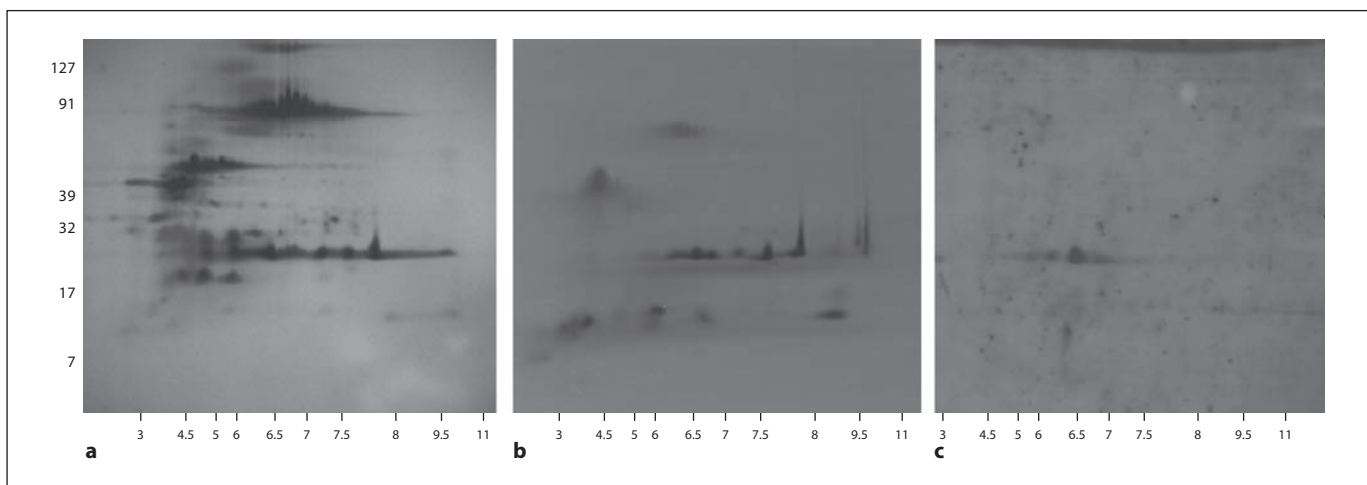
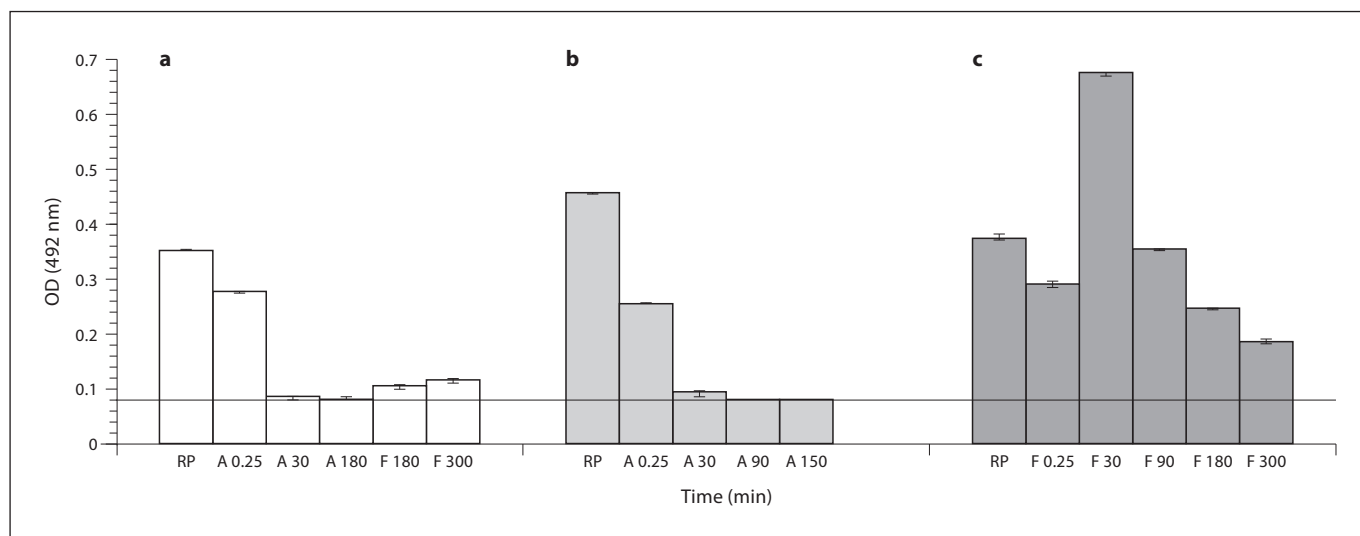


Fig. 4. Two-dimensional protein staining of RP (a), RP after individual treatment with Flavourzyme during 300 min (b), and Western blot of RP after individual treatment with Flavourzyme during 300 min (c), using a serum pool from 5 peanut-allergic patients.

rized in figure 5. Percentages of reduction in IgE reactivity are shown in the table annexed to figure 5. Hydrolysis with Alcalase/Flavourzyme sequentially and with Alcalase individually caused a higher loss of IgE reactivity in RP than hydrolysis with Flavourzyme individually. Alcalase was very effective in reducing the IgE reactivity of RP proteins since the enzyme at 0.4 and 0.2 AU/g led to a 100 and 98% reduction in IgE reactivity, respectively, in the

first 30 min (DH 16 and 11%, respectively); the addition of Flavourzyme in the sequential assay caused a slight increase in IgE reactivity above the positive cutoff point. Individual treatment with Flavourzyme during 30 min caused an increase in IgE reactivity. Nevertheless, Flavourzyme led to a 65% reduction in IgE reactivity at the end of the assay (300 min).



Alcalase (0.4 AU) and Flavourzyme (100 LAPU)		
Time, min	reduction, %	DH, %
A 0.25	29	3
A 30	100	16
A 180	100	27
F 180	92	56
F 300	88	69

Alcalase (0.2 AU)		
Time, min	reduction, %	DH, %
A 0.25	54	3
A 30	98	11
A 90	100	14
F 150	100	17

Flavourzyme (100 LAPU)		
Time, min	reduction, %	DH, %
F 0.25	29	0
F 30	0	14
F 90	7	19
F 180	44	24
F 300	64	29

Fig. 5. Specific IgE to RP before and after sequential treatment with Alcalase 0.4 AU/g and Flavourzyme 100 LAPU/g (a), individual treatment with Alcalase 0.2 AU/g (b), and individual treatment with Flavourzyme 100 LAPU/g (c), at the times indicated at the bottom of each figure, using a serum pool from 5 peanut-allergic patients. The horizontal line indicates the cutoff point for positivity (0.085). Percentages of reduction in IgE reactivity and DHs are also shown. A = Alcalase; F = Flavourzyme.

Anti-Ara h 1, Anti-Ara h 2 and Anti-Ara h 3 Immunoblots

Specific anti-Ara h 1, anti-Ara h 2 and anti-Ara h 3 antibodies were used to identify Ara h 1, Ara h 2 and Ara h 3 molecules in RP before and after sequential and individual treatment with Alcalase and Flavourzyme. In figure 6, the Ara h 1 (63 kDa), Ara h 2 doublet bands (19 and 21 kDa), Ara h 3 (40 kDa) acidic subunit and Ara h 3 (23 kDa) basic subunit are indicated with arrows. There was a marked decrease in recognition of Ara h 1 (fig. 6a), Ara h 2 (fig. 6b) and Ara h 3 (fig. 6c) in RP after sequential endo- and exoprotease hydrolysis and individual hydrolysis with Alcalase. Individual treatment with Flavourzyme caused a decrease of Ara h 2 and the acidic subunit of Ara h 3 levels in the first 30 min of hydrolysis; after this time, both allergens were undetected. Ara h 1

levels decreased with increased hydrolysis time with Flavourzyme; however, it was still detected after 300 min of hydrolysis. The basic subunit of Ara h 3 was not affected by Flavourzyme hydrolysis.

Discussion

In this study an endoprotease (Alcalase) and an exoprotease (Flavourzyme) were used, both individually and sequentially to evaluate their effects in the IgE antibody reactivity to RP protein extract. RP was selected in this study because it has been recognized that it is more allergenic than raw peanut [17–22]. RP extract binds IgE from patients with peanut allergy at approximately 90-fold higher levels than that of raw peanuts, and the protein

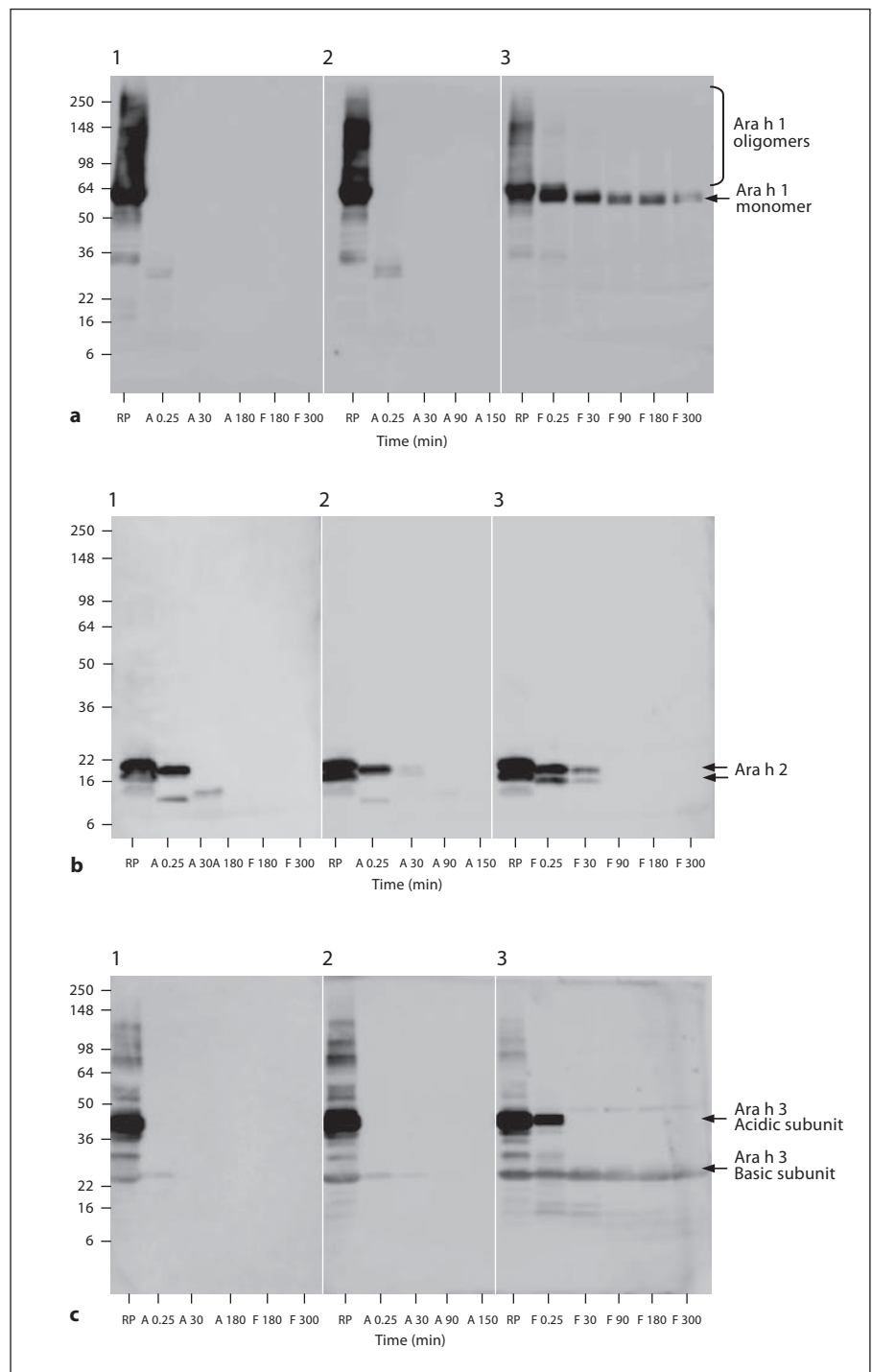


Fig. 6. Western blot analysis with anti-Ara h 1 (**a**), anti-Ara h 2 (**b**) and anti-Ara h 3 (**c**) antibodies. The Ara h 1, Ara h 2 and Ara h 3 profiles were assessed in RP before and after sequential treatment with Alcalase 0.4 AU/g and Flavourzyme 100 LAPU/g (lane 1), individual treatment with Alcalase 0.2 AU/g (lane 2), and individual treatment with Flavourzyme 100 LAPU/g (lane 3), at the times indicated at the bottom of each figure. A = Alcalase; F = Flavourzyme.

modifications induced by the Maillard reaction contribute to the observed effect [17, 20, 21].

Our results showed that individual treatment with Flavourzyme at 300 min reached a higher DH (29%) than individual treatment with Alcalase 0.2 AU/g (17%) at 150

min and similar to Alcalase 0.4 AU/g (27%) at 180 min; however, SDS-PAGE protein patterns were very different in both cases. In contrast to Flavourzyme hydrolysates, Alcalase hydrolysates showed no detectable stained proteins in SDS-PAGE. Similar results have been previously

reported for chickpeas [5]. Individual hydrolysis of chickpeas with Flavourzyme or Alcalase reached a similar DH (27%); however, Alcalase hydrolysates did not show visible electrophoretic bands in contrast to those obtained with Flavourzyme. An important decrease in stained proteins in SDS-PAGE during hydrolysis with Alcalase has been also reported for lentils [6].

Immunoblot and ELISA assays carried out with the serum pool from the 5 patients with a clinical allergy to peanuts showed a decrease of IgE reactivity in the first minute of hydrolyzation with Alcalase 0.4 AU/g (sequential treatment) and at 30 min with Alcalase 0.2 AU/g (individual treatment). After 30 min of hydrolysis, no bands were detected in immunoblotting and a 100% reduction in IgE reactivity was observed in ELISA. These results were confirmed by Western blot with individual sera. None of the sera recognized any RP proteins after sequential endo- and exoprotease hydrolysis. In contrast, individual treatment with Flavourzyme caused an increase in IgE reactivity detected by ELISA at 30 min. However, Flavourzyme led to a 65% decrease in IgE reactivity at the end of the assay (300 min). Clemente et al. [5] found that partially hydrolyzed chickpea proteins produced by individual treatment with Flavourzyme increased the IgE reactivity compared to the protein isolate. It has been hypothesized that new antigenic determinants could be found after the exposure of this legume to the exoprotease Flavourzyme, resulting in an increase of allergenicity. The most effective reduction of chickpea protein antigenicity was obtained by sequential treatment with Alcalase and Flavourzyme. In a study on lentils, sequential hydrolysis with Alcalase and Flavourzyme produced a decrease in IgE recognition when evaluated by *in vitro* assays using sera from patients with a clinical allergy to lentils [6].

Three spots of 22 kDa and pI ranging from 5 to 7 were still recognized after individual treatment with Flavourzyme for 300 min in Western blot using the serum pool. The spots reacting with IgE were identified as the basic subunits of Ara h 3 (11S globulin) by MALDI-TOF analysis. Although the basic subunit of Ara h 3 is considered a minor allergen, Restani et al. [23] suggested that it may have been a major allergen in a group of children allergic to peanuts in Italy. This finding was not in agreement with previous studies [24] indicating that allergenic epitopes occurred only in the acidic Ara h 3 subunit. However, other studies [25–27] have shown that Ara h 3 basic subunits may be important allergenic peptides. The resistance of the 11S globulin basic subunit to enzymatic hydrolysis has been reported previously in soybeans [28].

Three of the individual sera tested in Western blot recognized the basic subunit of Ara h 3 and also a 10-kDa band after individual treatment with Flavourzyme for 300 min. Sera from patients 1 and 4 detected a 63-kDa (Ara h 1) and a 30-kDa protein at this time point. These results highlight the importance of testing individual sera, since individual allergen recognition of specific sera might be diluted in a pool [6].

Specific anti-Ara h 1-, anti-Ara h 2- and anti-Ara h 3-binding experiments revealed that sequential endo- and exoprotease hydrolysis and individual hydrolysis with Alcalase decreased Ara h 1, Ara h 2 and Ara h 3 levels from the first minutes of hydrolysis. However, Ara h 1 and the basic subunit of Ara h 3 were not affected by Flavourzyme hydrolysis, confirming the results obtained in Western blot with pooled and individual sera.

Our results show that hydrolysis with Alcalase produces a decrease in IgE recognition in RP protein extract due to a decrease in the main peanut allergens. These enzymatically treated protein hydrolysates could constitute an alternative to intact proteins in the development of different products. Peanut protein isolates are used where bland and highly concentrated forms of peanut protein are desired, e.g. in bread and bakery goods [29]. Fortification of cereals with legumes, such as peanuts, has resulted in improving the nutritional quality of human dietary proteins [30]. Further studies are needed to evaluate the effects of these enzymes in the insoluble fraction of RP. Roasting can cause large changes in the biochemical characteristics of proteins due to the Maillard reaction. Proteins could form oligomers, become denatured, degraded, aggregated, crosslinked, fragmented and reassembled and these changes most often cause a reduction in solubility [31, 32].

In conclusion, although *in vivo* and *ex vivo* experiments will be necessary to evaluate the allergenicity of hydrolyzed peanut protein extract, the results in the present study show that hydrolysis with the endoprotease Alcalase decreases IgE reactivity in the soluble protein fraction of RP better than hydrolysis with the exoprotease Flavourzyme.

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