

***In vitro* allergenicity of peanut after hydrolysis in the presence of polysaccharides**

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Summary

Background Peanut is a major allergenic product. Manufacturing processes used in food industries to improve the physicochemical properties of food-based peanut (stabilization, texturization), could cause a modification of the digestibility of peanut proteins and, consequently, their allergenicity.

Objective This study aimed at examining the influence of polysaccharides, i.e., gum arabic, low methylated pectin (LMP) and xylan, on the *in vitro* hydrolysis of peanut protein isolate (PPI) and the *in vitro* allergenicity of the digestion products.

Methods PPI was hydrolysed during a two-step *in vitro* hydrolysis by pepsin, followed by a trypsin/chymotrypsin (T/C) mixture performed in dialysis bags with molecular weight cut-offs (MWCO) of 1000 or 8000 Da. SDS-PAGE electrophoresis and immunoblotting were assessed on the peptic and T/C digestion products in (retentates) and out of the dialysis bags (dialysates).

Results Hydrolysis by all of the digestive enzymes showed retention of some proteins in the dialysis bags in the presence of gum arabic and xylan. The retentates were recognized by IgG and IgE, particularly peptides <20 kDa. The IgE binding with peptides of retentate containing xylan from the dialysis bag with an MWCO of 1000 Da was reduced. The immunoreactivity of hydrolysis products in dialysates was considerably reduced by polysaccharides, regardless of the dialysis bag.

Conclusion Reduction of PPI hydrolysis was probably due to non-specific interactions between polysaccharides and peptides. In retentates, IgE-binding epitopes were reduced by digestion and the presence of xylan. In dialysates, they were reduced by all of the polysaccharides. This work highlights the possibility of modulating this food allergy through optimized formulation.

Keywords dialysis bag, enzymatic hydrolysis, food allergy, IgG/IgE epitopes, immunoreactivity, peanut protein isolate, protein–polysaccharide interactions

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Introduction

Among the food allergens, peanut (*Arachis hypogaea* L.) is a major allergenic product [1–3]. Consumption of peanut products is on the rise throughout the world, particularly in developing countries where they replace animal protein [4–6]. In the USA, the entire production is essentially reserved for human consumption [4–6]. Sensitivity to peanuts occurs early in life and, once established, usually persists indefinitely [7]. Allergy to peanuts is constantly increasing, particularly in the USA, and it has become common over the last 10 years in Europe where it is increasing on a regular basis [1, 2]. Peanut allergenicity is due to the presence of several allergens. Ara h 1

(vicilin, 63.5 kDa), Ara h 2 (conglutin, 17 kDa) and Ara h 3 (glycinin, 14 kDa) are the major peanut protein allergens [8]. Some studies consider Ara h 1 and Ara h 2 as major allergens since they are recognized by over 90% of peanut-allergic patients [8, 9]. Other studies showed that Ara h 1 is recognized only by 35% [10], 65% [11] or 70% [12] of the patients in three European populations. These differences in relative recognition were not found for Ara h 2 [13]. Minor allergens are also present in peanuts. They are either a fragment or an isoform of Ara h 1, Ara h 2 or Ara h 3 [3, 8]. These allergens are glycoproteins containing particular binding sites [8] that are not hydrolysed by digestive enzymes as in the case of Ara h 1 and Ara h 2 [8, 14]. Consequently, intact proteins or peptides containing epitopes can be absorbed across the intestinal barrier, causing allergic sensitization [8, 15, 16].

The different processing methods used in formulated foods can modify the allergenic properties of proteins [17]. In the case of peanut, some cooking methods produce fewer allergenic products in comparison with roasting. The apparent

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amount of IgE binding to Ara h 1, Ara h 2 and Ara h 3 was lower in raw, fried and boiled peanuts compared with that found in roasted peanuts [18–21]. Maleki et al. [22] showed that the enhancement of the allergic properties of roasted peanuts is caused by the chemical modifications resulting from the Maillard reaction involving covalent linkage between reducing sugar and the primary amine group of proteins.

Due to their functional and nutritional properties and their pleasant flavour, peanuts are found in a number of manufactured foods such as milk, ice cream, beverages, candies, bakery products, etc. [4, 5, 23, 24]. Plant polysaccharides are generally added to these preparations. This is the case for pectins, carrageenan used as a stabilizing agent, gum arabic used as an emulsifying agent or as a coating agent in candies, etc. [25, 26]. The main objective of this study was to determine the effects of plant polysaccharides, widely used in food processing for their emulsifying, stabilizing and texturing properties, on the *in vitro* digestibility of peanut protein isolate (PPI) and on the *in vitro* immunogenicity (IgG binding) and the *in vitro* allergenicity (IgE binding) of the hydrolysis products. Gum arabic (acacia gum) and low methylated pectin (LMP) are both used in the food industry. The presence of uronic acids in their structure is responsible for their polyanionic character [27, 28]. Xylan extracted from *Palmaria palmata*, a red seaweed acceptable for human nutrition, is a low molecular weight (MW) linear oligomer composed of xylose.

PPI was hydrolysed during a two-step *in vitro* hydrolysis by digestive enzymes (pepsin, followed by a trypsin/chymotrypsin (T/C) mixture). Hydrolysis by the T/C mixture was carried out in dialysis bags with two different molecular weight cut-offs (MWCO). The aim of using these dialysis bags was to assess the effects of polysaccharides on PPI digestibility in a physiologic situation (bag with an MWCO of 1000 Da) [29], and in a non-physiologic situation where high MW peptides passed through the intestinal barrier as in the case of increased intestinal permeability often observed in food allergies (bag with an MWCO of 8000 Da). Evaluation of PPI digestibility was performed by SDS-PAGE electrophoresis. Immunoblotting was used in order to evaluate the influence of the nature of polysaccharides on the *in vitro* IgG/IgE binding of peptic digestion and T/C digestion products in (retentates) and out of the dialysis bags (dialysates).

Materials and methods

Isolation of PPI

Crude peanut meal (Huilerie Beaujolaise, Beaujeu, France) was defatted for 4 h by petroleum ether in a Soxhlet. One hundred grams of defatted powdered peanut were then macerated with 800 mL of high-salt phosphate buffer (20 mM Na₂HPO₄, 2 mM KH₂PO₄, 5.4 mM KCl, 1 M NaCl, pH 7.4). The mixture was centrifuged for 30 min at 15 000g and the supernatant was recovered. The pellet was recovered and remixed as previously described with 800 mL of high-salt phosphate buffer. After centrifugation, supernatants were mixed and proteins were precipitated by supplementing the supernatant with (NH₄)₂SO₄ to 90% saturation. After centrifugation at 15 000g for 30 min, the pellet was dialysed with distilled water overnight at 4 °C using a dialysis bag with

an MWCO of 3500 Da. The retentate was then freeze-dried. Total protein determined by the Kjeldhal method was 91% (N × 5.46) and referred to as PPI.

Polysaccharides and enzymes

Powdered gum arabic (lot no. 97 J 716) was provided by the Colloides Naturels International Company (CNI, Rouen, France). It contained 89.95% polysaccharide. Xylan (lot no. PP28-4-00) was extracted from a Rodophyceae, *P. palmata* or dulce by the Laboratoire de Pathologie Cellulaire et Moléculaire en Nutrition (Faculté de Médecine, Vandoeuvre-lès-Nancy, France). Its composition was (per g/100 g): 80.4% pentose, 13.2% hexose. LMP (lot no. 0B800) with a 37% degree of esterification. It was provided by Degussa Texturant Systems (Boulogne-Billancourt, France). The powder contained 87% polysaccharide.

Porcine pepsin (3800 U/mg protein, 1 : 60 000, EC 3.4.23.1), bovine trypsin (13 800 U/mg, EC 3.4.21.4), porcine chymotrypsin (51 U/mg, EC 3.4.4.5) and thimerosal, an antibacterial agent, were purchased from Sigma (Saint-Quentin Fallavier, France). Pre-stained Marker, Broad Range (Bio-Rad, Marnes la Coquette, France) was used as a standard marker.

Serum IgG and IgE

Rabbit antiserum against PPI, obtained according to a classical protocol after incubation for 181 days, and rabbit pre-immune serum were used (Agrobio, Villeny, France). Serum from 11 patients with identified peanut sensitivity was pooled and used for our experiments. The peanut-specific IgE level determined by RAST was 93.3 kU/L as measured by the Pharmacia CAP system (Pharmacia, Uppsala, Sweden). Normal serum from patients without a peanut hypersensitivity reaction was used as a negative IgE control.

Preparation of the PPI/polysaccharide mixtures

Powdered PPI containing 46 mg of nitrogen (250 mg of protein, N × 5.46) was taken in triplicate and dissolved in 15 mL (1.7%, w/v) of sodium phosphate buffer (0.17 M, pH 7). Polysaccharides were dissolved in the same buffer and at the same concentration (1.7%, w/v). Solutions were left overnight at 4 °C to allow complete hydration of the macromolecules. The PPI and polysaccharide stock solutions were blended so as to obtain solutions with 0 (PPI without polysaccharide), or 50% (w/w) of relative polysaccharide concentrations (as compared with the PPI concentration). Thimerosal was added to all of the solutions at 50 mg/L.

Enzymatic hydrolysis

The samples described above were hydrolysed in the first case by pepsin and in the second case by pepsin followed by the T/C mixture.

Peptic hydrolysis

Samples were hydrolysed by pepsin as follows: 1 mL of pepsin in 0.02 N HCl was added to 15 mL of PPI or the PPI/polysaccharide mixtures (E/S: 1/250). For simulating *in vivo* gastric digestion, the pH of the mixture was progressively

reduced from pH 7 to pH 2, by adding HCl 0.02 N with a peristaltic pump (flow rate: 80 µL/min) for 2 h at 37 °C. Digestion was stopped at pH 2 by cooling samples and freeze-drying.

Peptic and T/C hydrolysis

The peptic and T/C hydrolysis of samples was carried out at 37 °C in a dialysis cell (Serna, Laval, Quebec, Canada), according to the two-step hydrolysis method developed by Savoie and Gauthier [30], with minor modifications of the first step. In the first step, peptic digestion, carried out as previously described, was stopped by raising the pH to 8 with NaOH 2 N. Samples were then transferred to dialysis bags with an MWCO of 1000 or 8000 Da (SpectraPor 6, Interchim, Montluçon, France). One millilitre of a mixture of T/C (1/2.3, w/w) 2.5 mg/mL (E/S: 1/50) was then added. Digestion products that passed through the dialysis bags were collected over a period of 6 h by the circulation (1.6 mL/min) of a sodium phosphate buffer (0.01 M pH 8). After 6 h of hydrolysis, the pooled dialysates and retentates in the dialysis bags were collected, freeze-dried and stored until analysis.

Protein assay

The protein content of all of the samples was estimated using the bicinchoninic acid assay (Commercial kit, Interchim).

Electrophoresis and immunoblotting

PPI after peptic hydrolysis, retentates and pooled dialysates were characterized by SDS-PAGE, according to the method of Laemmli and Favre [31]. One hundred or 200 µg of protein from each sample were loaded into each well. Using 0.1% (w/v) SDS and 5% (v/v) 2-mercaptoethanol, SDS-PAGE was performed with 4.5% polyacrylamide concentration gel in 0.125 M Tris (pH 6.8) and with 20% polyacrylamide separation gel in 0.38 M Tris (pH 8.8). Proteins were stained by Coomassie blue (R 250 0.1%) (w/v) in 50% ethanol (v/v) and 10% acetic acid (v/v) or by silver nitrate (Bio-Rad kit). Pre-stained Protein Marker, Broad Range, was run with each gel to estimate the MW of the electrophoresed proteins.

The allergenicity of undigested PPI and hydrolysis products was analysed by immunoblotting using the sera containing anti-PPI IgG from rabbits or IgE from patients. Retentates and dialysates (100 µg) were loaded onto a gel for SDS-PAGE, as described above. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Saint Quentin en Yvelines, France) by applying a constant current of 300 mA for 60 min with a semi-dry blotting (Trans-Blot, Bio-Rad). Cut strips (4 mm) were used for immunodetection. All patient and rabbit sera were diluted in Tris buffer saline (TBS, 50 mM, pH 7.5) containing 0.05% (v/v) Tween-20 (TBS-Tween). The total incubation volume was 2 mL per strip. Strips were washed three times for 15 min with 0.1% TBS-Tween between all incubation steps. The strips were incubated with primary antibodies (rabbit anti-peanut or rabbit pre-immune, 1:10 000; patient allergenic or non-allergenic sera 1:5) for 12 h at 4 °C. Biotinylated anti-rabbit IgG or biotinylated anti-human IgE (Biovaley, Marnes la Vallée, France) were subsequently incubated (1:2000) for 2 h at room temperature. Antigen-antibody conjugates were

visualized by their reaction to streptavidin alkaline phosphatase (1:1000) (Biovaley) for 30 min. Enzymatic staining was performed with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate using a commercial kit (Sigma).

Results

SDS-PAGE electrophoresis patterns

Electrophoresis of PPI after peptic hydrolysis (Fig. 1) SDS-PAGE electrophoresis of undigested PPI (lane 6) presented several and distinct proteins with apparent MW ranging from 64 to 6 kDa, as estimated from standard MW (lane 1). PPI allergens were identified only by their MW. The band with an apparent MW of 64 kDa corresponds to Ara h 1; 22 and 20 kDa proteins represent Ara h 2 doublet; Ara h 3 corresponds to bands with MW 46, 44 and 28 kDa that form a triplet and a band with an MW of 15 kDa. All protein bands were intact after PPI peptic digestion (lane 2). The presence of gum arabic (lane 3) and xylan (lane 5) did not change the PPI peptic hydrolysis. In contrast with LMP (lane 4), some bands disappeared or were reduced, particularly bands with apparent MW 64 (Ara h 1 protein), 46, 44, 28 (triplet Ara h 3) and 24 kDa.

Electrophoresis of PPI retentates (Fig. 2a) PPI hydrolysis by pepsin followed by the T/C mixture in dialysis bags led to the disappearance of some proteins and the appearance of peptides, depending on the polysaccharide and the MWCO of the dialysis bag used.

The electrophoresis pattern of retentates of PPI without polysaccharides from dialysis bags with an MWCO of 1000 Da (lane 2) showed the disappearance of the protein bands corresponding to apparent MW from 64 to >29 kDa and bands with apparent MW from 24 to 20 kDa. Bands corresponding to proteins or peptides with an apparent MW of 29, 28 and 17 kDa, and peptides with apparent MW <12 kDa were observed. The addition of polysaccharides modified the PPI electrophoresis pattern of retentates. With gum arabic (lane 3), protein bands with apparent MW >29 kDa, bands with 27, 24, 23 and 15 kDa and a small peptide <6 kDa were present, indicating a reduction of PPI degradation. In the PPI-LMP retentate (lane 4), all bands were less intense than the ones observed with the retentate of the PPI-gum arabic mixture. There were no proteins or peptides with an apparent MW between 17 and 28 kDa and >29 kDa; a 12-kDa band was present. The addition of xylan in the PPI (lane 5) led to an electrophoresis pattern of the retentate similar to the one obtained with a gum arabic-PPI mixture (presence of bands >29 kDa and bands with MW 27, 24 and 23 kDa). An intensification of the band with an apparent MW of 12 kDa and a disappearance of the band with an apparent MW of 17 kDa were observed.

The electrophoresis pattern of retentate without polysaccharides in a dialysis bag with an MWCO of 8000 Da (lane 6) was slightly different from the one with a membrane with an MWCO of 1000 Da, with an additional band with an apparent MW of 12 kDa observed and an intensification of the 9-kDa band. In the presence of polysaccharides, the electrophoresis patterns of retentates were similar to the one obtained with the membrane with an MWCO of 1000 Da, in

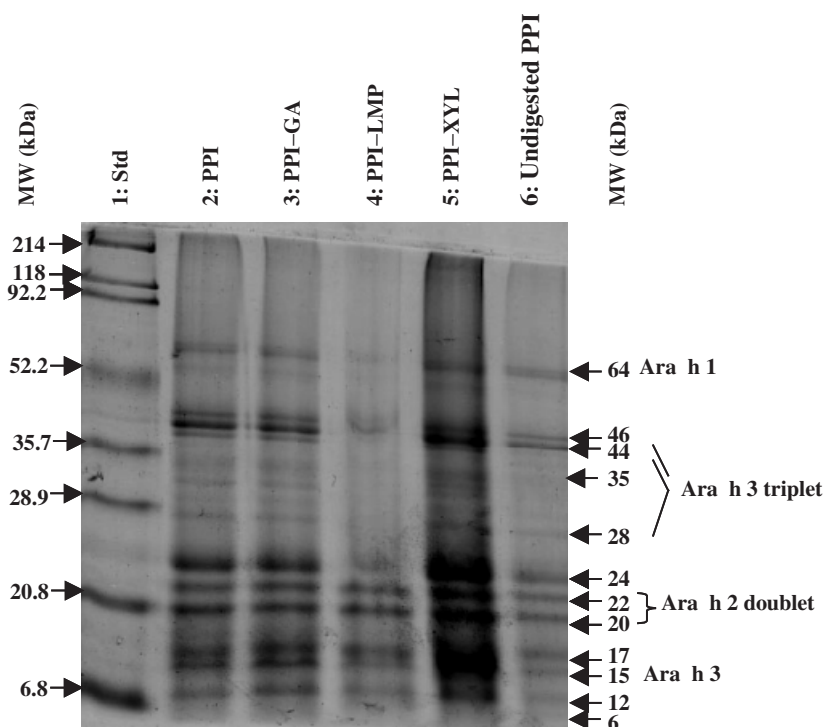


Fig. 1. SDS-PAGE electrophoresis of peanut protein isolate (PPI) after peptic digestion. Lane 1: Std (standard molecular weight (MW)); lane 2: PPI without polysaccharide; lane 3: PPI-gum arabic (GA); lane 4: PPI-low methylated pectin (LMP); lane 5: PPI-xylan (XYL); lane 6: undigested PPI. Each lane was loaded with 100 µg of proteins; proteins were stained with Coomassie blue.

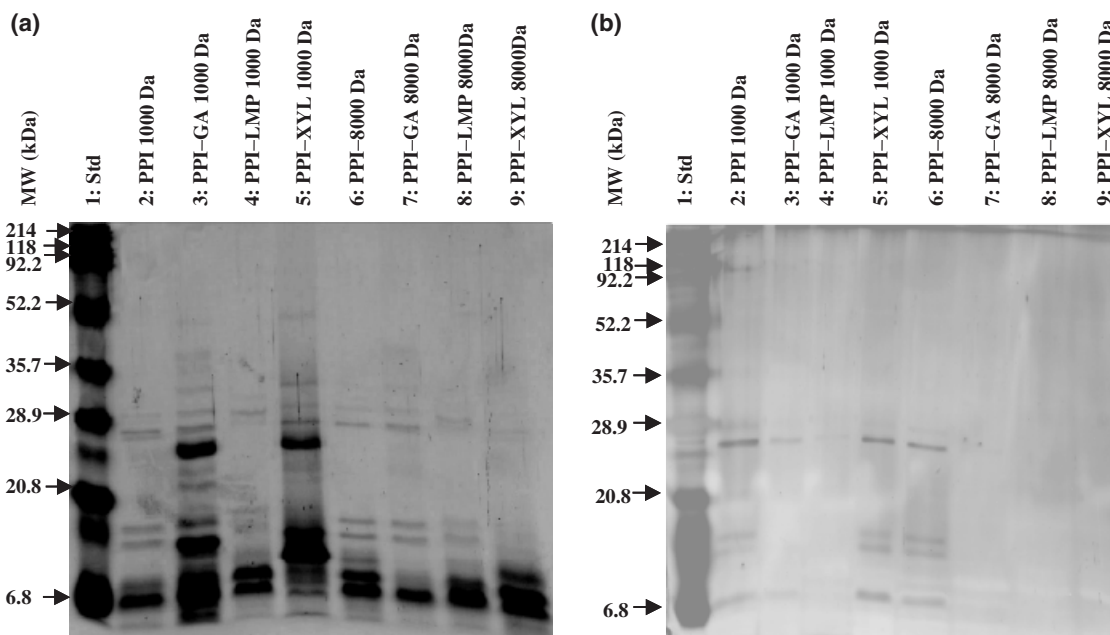


Fig. 2. SDS-PAGE electrophoresis of peanut protein isolate (PPI) digestion products in (retentates) and out of (dialysates) the dialysis bag, after PPI peptic digestion followed by trypsin/chymotrypsin (T/C) digestion. (a) retentates; (b) dialysates. (a, b) PPI T/C digestion in dialysis bag with a molecular weight cut-offs (MWCO) of 1000 Da; lane 2: PPI without polysaccharide; lane 3: PPI-gum arabic (GA); lane 4: PPI-low methylated pectin (LMP); lane 5: PPI-xylan (XYL). (a, b) PPI digestion in dialysis bag with an MWCO of 8000 Da; lane 6: PPI without polysaccharide; lane 7: PPI-GA; lane 8: PPI-LMP; lane 9: PPI-XYL. Lane 1: Std (standard MW). (a) Each lane was loaded with 100 µg of proteins; proteins were stained with Coomassie blue. (b) Each lane was loaded with 200 µg of proteins; proteins were stained with silver nitrate.

the presence of gum arabic, with slightly less intense bands (lane 7) and LMP (lane 8). The addition of xylan (lane 9) resulted in an intensification of the bands <12 kDa and a disappearance of the bands with an MW between 15–28 and >28 kDa.

Electrophoresis of dialysates (Fig. 2b) Bands with apparent MW 29, 28, 17, 15 and 7 kDa were clearly observed in the dialysate electrophoresis pattern from the dialysis bag with an MWCO of 1000 Da for the PPI control (lane 2). Some bands were less intense or disappeared when hydrolysis was

performed in the presence of gum arabic (lane 3) and LMP (lane 4). In the presence of xylan (lane 5), the electrophoresis pattern was comparable to the one of the PPI control. In the dialysis bag with an MWCO of 8000 Da, bands with apparent MW 29, 28, 17, 15 and 7 kDa were observed in the PPI control (lane 6). All bands disappeared in the presence of polysaccharides (lanes 7–9).

Immunoblotting

IgG immunoblotting The immunoblot of undigested PPI presented several proteins that were recognized by PPI rabbit antibodies (Fig. 3a). The most important recognition was of the proteins with apparent MW 64, 46 and 44 and all proteins with apparent MW $\leq$ 24 kDa. All major peanut allergens, Ara h 1 (64 kDa), Ara h 2 doublet (22 and 20 kDa) and Ara h 3 (46, 44 and 15 kDa), were detected. The incubation of the same sample with a pre-immune rabbit serum did not present IgG-binding peanut proteins (results not shown).

The peptic hydrolysis products (Fig. 3b) showed a pattern of IgG-binding protein similar to the ones obtained with undigested PPI, except in the case of the PPI–LMP mixture where some proteins had disappeared (64 and 24 kDa).

The immunoblot of retentates showed several peptides recognized by the PPI rabbit antibodies (Fig. 3c). The most significant reaction was observed in all cases with peptides with an MW of 28 and $\leq$ 17 kDa, particularly with two peptides with an apparent MW of 9 and 7 kDa. This was true whether or not hydrolysis was carried out with polysaccharides and regardless of the MWCO of the dialysis bag used. The IgG binding of bands with an MW $>$ 35 kDa and bands with an MW of 17, 15 and 12 kDa were weak in the retentate of the PPI control (lane 2). In the PPI–gum arabic retentate (lane 3), an intensification of the 15-kDa band and the IgG binding of the 27- and 24-kDa bands were observed. In the PPI–LMP retentate (lane 4), there was no IgG binding with 17-kDa band. In the presence of xylan (lane 5), the IgG binding displayed the same pattern as the one of PPI–gum arabic. The 12-kDa band, very intense in the SDS-PAGE electrophoresis pattern (Fig. 2, lane 5), displayed a weak IgG binding. When hydrolysis was performed in the dialysis bag with an MWCO of 8000 Da, there was no IgG binding with peptides between 28 and 15 kDa in any of the cases. An intensification of the 12-kDa band IgG binding was observed in the retentate of the PPI–control (lane 6) and the PPI–xylan (lane 9).

The immunoblots of dialysates (Fig. 3d) present the greatest degree of IgG binding with peptides with an MW of 28, 9 (not detected in the electrophoresis pattern) and 7 kDa, rather than with peptides with an MW of 29, 15 and 12 kDa in the dialysate of the PPI control (lane 2) from the dialysis bag with an MWCO of 1000 Da. Adding polysaccharides during the PPI hydrolysis reduced the IgG binding. This reduction was not as great in the presence of gum arabic (bands with an MW of 28, 9 and 7 kDa), than with xylan (bands with an MW of 28, 9 and 7) and with LMP (bands with an MW of 28 and 9 kDa). Dialysates of the PPI control from the membrane with an MWCO of 8000 Da showed only two bands (28 and 7 kDa) binding with PPI rabbit antibodies. No IgG binding was observed in the presence of polysaccharides.

IgE immunoblotting The immunoblot of samples with IgE from pooled allergenic patient sera showed the immunoreactive effect of the proteins of undigested PPI (Fig. 4a). IgE binding with protein with apparent MW 64, 46 and 44 kDa was weaker than with IgG. In contrast to the IgG binding, the protein with an apparent MW of 24 kDa was not recognized by IgE. Bands with MW 22, 20, 17, 15 and $<$ 15 kDa displayed high IgE-binding intensity. Peptic hydrolysis (Fig. 4b) slightly modified the IgE binding with the 24 kDa-band recognized by human antibodies in the case of the PPI–control (lane 2), PPI–gum arabic (lane 3) and PPI–xylan (lane 5). Proteins with an MW $>$ 22 kDa did not display IgE binding in the case of PPI–LMP (lane 4). In retentates (Fig. 4c), as in the case of IgG binding, IgE mainly recognized the peptides with an apparent MW of 9 and 7 kDa. Except in the case where digestion took place in the presence of xylan (lane 5) in the membrane with an MWCO of 1000 Da, the IgE binding was not as great (only with the 9-kDa band). The IgE binding with the 27-kDa band was observed in the PPI–gum arabic (lane 3) and the PPI–LMP (lane 4) retentates only. In retentates from the dialysis bag with an MWCO of 8000 Da, the IgE binding with the 12-kDa band was observed in the PPI–gum arabic (lane 7) and the PPI–xylan (lane 9) retentates. A weak IgE binding with the 27-kDa band was observed in the PPI–gum arabic only (lane 6). The incubation of undigested PPI with serum from patients without peanut sensitivity did not have IgE-binding proteins (results not shown).

The IgE binding in dialysates (Fig. 4d) was observed only with the 7-kDa band when hydrolysis was performed without polysaccharides. The 7-kDa IgE binding was greater when it came from the membrane with an MWCO of 8000 Da than from the membrane of an MWCO of 1000 Da.

Discussion

Stability under digestion is thought to be an important requirement for the allergenicity of food proteins [15, 17, 32, 33]. This stability may be important for both sensitization of the immune system after penetration of the intestinal mucosa and for the elicitation of gastrointestinal symptoms of food allergy [33]. However, some studies have demonstrated that indigestibility of food protein is not a prerequisite for inducing allergenic symptoms [34, 35]. Astwood et al. [15] treated purified proteins with simulated gastric fluid for 60 min and monitored degradation by multiple sampling during this period. The use of single enzymes may underestimate digestibility. As a result, the *in vitro* digestion method used in this study simulates the *in vivo* situation by using a two-step hydrolysis [30, 36]. The protein source is first hydrolysed by pepsin, the pH being progressively reduced from pH 7 to pH 2 in 2 h [29]. Pepsin did not hydrolyse PPI in low MW peptides under our experimental conditions. Pepsin is a pre-digestive enzyme that cleaves bonds between aromatic amino acids (tyrosine and phenylalanine) and facilitates digestion with pancreatic enzymes [30, 37, 38].

In the second step, where hydrolysis by the T/C mixture took place in dialysis bags after peptic hydrolysis, retention of some proteins or peptides indicates a reduction of PPI hydrolysis as observed in *in vitro* and *in vivo* studies [39–41].

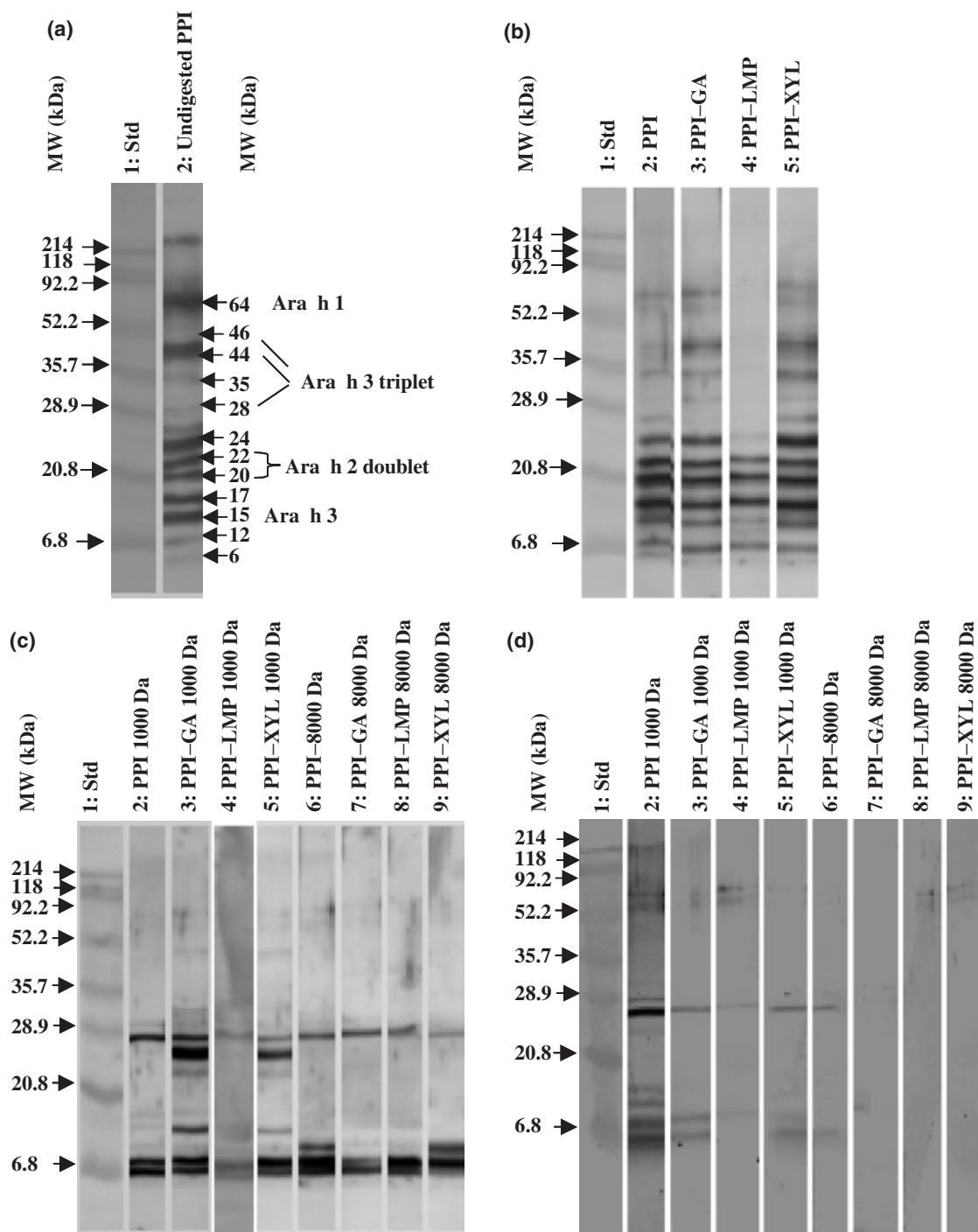


Fig. 3. Immunoblot of IgG-binding proteins of undigested peanut protein isolate (PPI), (a) PPI after peptic hydrolysis, (b) PPI products digestion after peptic followed by trypsin/chymotrypsin (T/C) digestion in (retentates) and out of (dialysates) the dialysis bags. (c) retentates; (d) dialysates. PPI after peptic hydrolysis (b): lane 1: Std (standard molecular weight (MW)); lane 2: PPI without polysaccharide; lane 3: PPI-gum arabic (GA); lane 4: PPI-low methylated pectin (LMP); lane 5: PPI-xylan (XYL). (c, d) PPI T/C digestion in dialysis bag with a molecular weight cut-offs (MWCO) of 1000 Da: lane 2: PPI without polysaccharide; lane 3: PPI-GA; lane 4: PPI-LMP; lane 5: PPI-XYL. (c, d) PPI T/C digestion in dialysis bag with an MWCO of 8000 Da: lane 6: PPI without polysaccharide; lane 7: PPI-GA; lane 8: PPI-LMP; lane 9: PPI-XYL. Lane 1: Std (standard MW). (a–c) Each lane was loaded with 100 µg of proteins; (d) each lane was loaded with 200 µg of proteins.

In a previous study, we showed that the polysaccharides used here had no effect on the T/C enzymatic activities (results not shown). According to previous studies, it is unlikely that the observed effects could be induced by an increased viscosity of the mixture at the low polysaccharide concentration used [41, 42]. The reduction of PPI hydrolysis could therefore be due to

the formation of complexes between the hydrolysis products (rather than undigested proteins) and polysaccharides, as suggested by electrophoresis patterns, by non-specific interactions such as electrostatic interactions or hydrogen bonding. Electrostatic interactions between negative carboxylic polysaccharides such as LMP and gum arabic and peptides

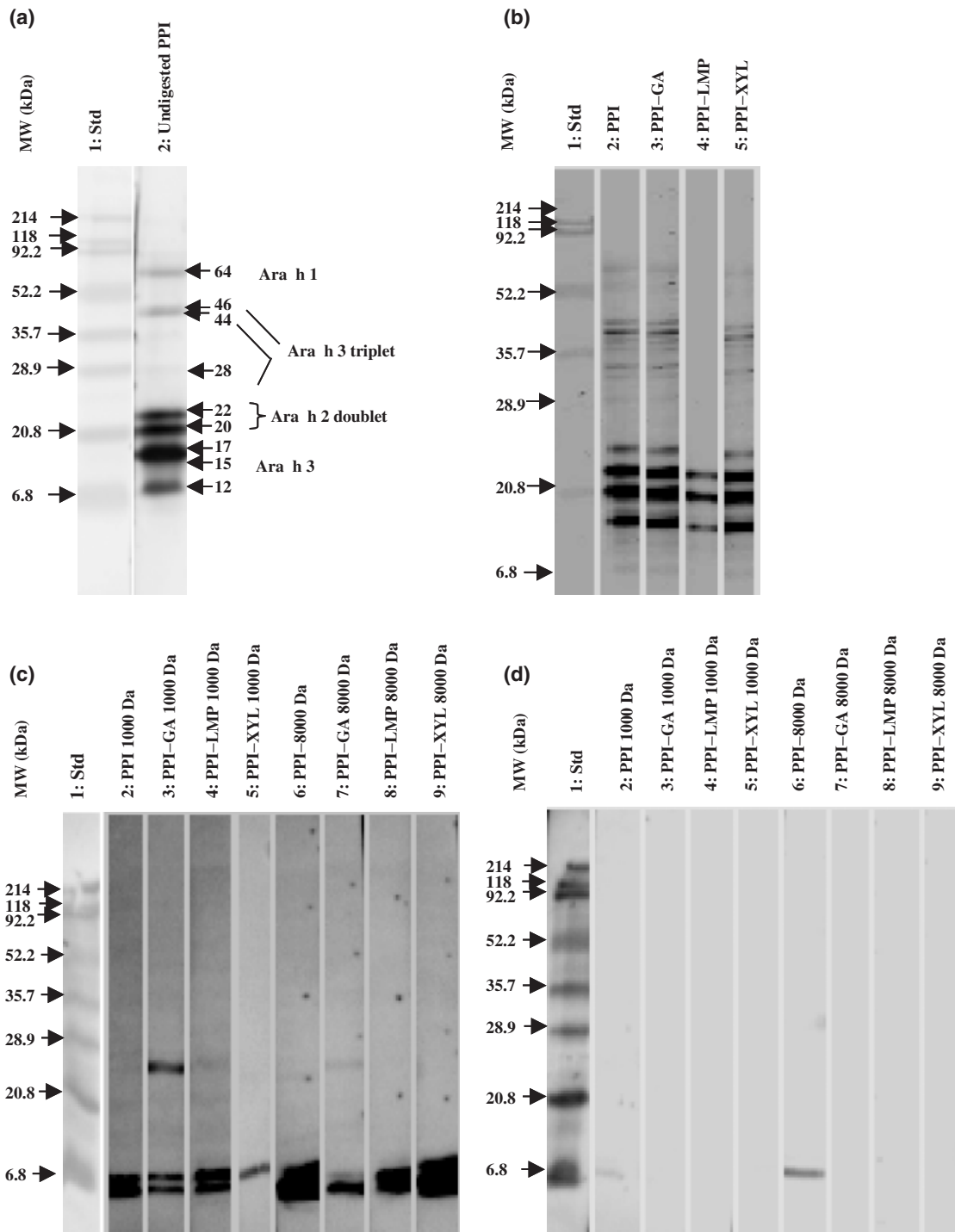


Fig. 4. Immunoblot of IgE-binding proteins of undigested peanut protein isolate (PPI) (a), PPI after peptic hydrolysis (b), PPI digestion products in (retentates) and out of (dialysates) the dialysis bags after peptic followed by trypsin/chymotrypsin (T/C) digestion in dialysis bags. (c) retentates; (d) dialysates. PPI after peptic hydrolysis (b): Lane 1: Std (standard molecular weight (MW)); lane 2: PPI without polysaccharide; lane 3: PPI-gum arabic (GA); lane 4: PPI-low methylated pectin (LMP); lane 5: PPI-xylan (XYL). (c, d) PPI T/C digestion in dialysis bag with a molecular weight cut-offs (MWCO) of 1000 Da: lane 2: PPI without polysaccharide; lane 3: PPI-GA; lane 4: PPI-LMP; lane 5: PPI-XYL. PPI T/C digestion in dialysis bag with an MWCO of 8000 Da: lane 6: PPI without polysaccharide; lane 7: PPI-gum arabic (GA); lane 8: PPI-LMP; lane 9: PPI-XYL. Lane 1: Std (standard MW). (a–c) Each lane was loaded with 100 µg of proteins; (d) each lane was loaded with 200 µg of proteins.

can exist at alkaline pH during T/C digestion, particularly with peptides containing positive amino acids such as arginine and lysine released from cleavage by trypsin. Non-ionic interactions are also possible between these polysaccharides [43–45], particularly xylan, a neutral oligomer, and peptides.

In the case of LMP, we are unable to explain the disappearance of some bands during PPI peptic digestion and the increase of T/C hydrolysis at this stage of our study.

The slight difference between the electrophoresis patterns of retentates from the two dialysis bags is due to the porosity of

the latter. The dialysis cell allows continuous separation of the final products that cross through the dialysis membrane into the outer compartment [36], leading to a reduction of the substrate in the dialysis bags, depending on the MWCO of the bags. The smaller the pores, the less hydrolysed products that are released, as demonstrated by the value of N release in dialysates [46], increasing the probability of complex formation between polysaccharides and peptides. Polysaccharides consequently influence the nature of the peptides produced. The type of polysaccharide influences protein hydrolysis as well.

In this study, Ara h 1 (64 kDa), Ara h 2 (doublet 22 and 20 kDa) and Ara h 3 (triplet 46, 44 and 28 kDa) were hydrolysed only after peptic followed by T/C hydrolysis, independent of the presence of polysaccharides and regardless of the dialysis bag used. The hydrolysis of peanut allergens (Ara h 1 and Ara h 2) has already been observed by other researchers [14, 33, 47]. In previous studies [47], Ara h 1 was digested by pepsin, trypsin or chymotrypsin and various protease-resistant fragments containing IgE-binding sites were identified. Sen et al. [14], demonstrated the existence of a 10-kDa protease-resistant peptide of Ara h 2 that contains multiple IgE-binding epitopes. In contrast, the Ara h 2 that was first reduced before digestion by trypsin did not produce any enzyme-resistant protein fragment and appeared to be much more sensitive to the action of protease. This observation shows the role of the structural conformation of these allergens on their ability to be hydrolysed by digestive enzymes. The hydrolysis of Ara h 3 has never been carried out and our results showed the Ara h 3 triplet digestion by pepsin followed by T/C. According to some authors, IgE/IgG-binding epitopes are located throughout the length of Ara h 1, Ara h 2 and Ara h 3 [8, 14, 48], explaining why peptides from PPI hydrolysis activate the response to antibodies.

Despite the possibility of peptide-polysaccharide complex formation in the dialysis bags, IgG epitopes of the peptides of the retentates were not destroyed or masked and were able to recognize rabbit antibodies. The IgG binding with the peptides that crossed the dialysis bag was slightly reduced in the presence of polysaccharides. The IgE antibodies recognized only one part of the peptides in and out of the dialysis bags. This observation confirms that IgE epitopes significantly differ from IgG epitopes. After total digestion (pepsin followed by T/C) in the presence of xylan, the considerable reduction of IgE binding with the peptides of retentate suggests that xylan modulated the T/C hydrolysis in a way that reduced the IgE binding to the generated peptides. The reduction of antibody binding, particularly significant in the case of the IgE binding with peptides that crossed the dialysis bags in the presence of all of the polysaccharides, once again suggests that these peptides do not contain IgE epitopes accessible to the antibodies. This could be due to the conformational change of these peptides or their IgE epitopes masked in the presence of the polysaccharides.

The size of peptides did not influence the response to antibodies because small peptides such as those obtained after PPI hydrolysis (<10 kDa) were recognized by antibodies. According to some authors, there seems to be a size limitation on the ability of peptides to react with the IgE of already sensitized individuals. Van Beresteijn et al. [49] tested the immunological properties of hydrolysates of whey protein

concentrate and found that the minimal molecular mass necessary to elicit immunogenicity and allergenicity appeared to be between 3 and 5 kDa. In dialysates, the peptide with an apparent MW of 7 kDa that displays IgE binding could be compared with the 10-kDa fragment obtained by Sen et al. [14] after Ara h 2 peptic, trypsin or chymotrypsin hydrolysis and that contains multiple IgE-binding epitopes.

In our experiment, the non-specific interactions between PPI and polysaccharides produce a reduction of antibodies binding with digestion products. That is not the case in other studies [18–21] that showed the enhancement of the allergenic properties of roasted peanuts (particularly Ara h 1 and Ara h 2) in the presence of various monosaccharides caused by the chemical modifications resulting from the Maillard reaction, involving covalent linkage that may create novel IgE-binding sites and enhance other allergenic properties such as resistance to heat, degradation and digestion by gastric secretion.

In the present study, we have shown that the presence of polysaccharides modifies PPI digestibility as a result of interactions between the two macromolecules and, consequently, influences the nature of the peptides released. Hydrolysis products in the dialysis bags reacted with IgG and IgE antibodies, whereas the immunoreactivity of peptides that crossed through the dialysis bags were considerably reduced when hydrolysis was performed in the presence of polysaccharides. The reaction between the hydrolysis products in the dialysis bags and IgG antibodies indicates that their epitopes are not masked by the polysaccharides. Although this study shows that the polysaccharides investigated have an effect at the chosen ratio, the influence at ratios found in existing foods may be different and requires further clarification.

In this study, xylan proved to be the most efficient polysaccharide for reducing the *in vitro* allergenicity of PPI hydrolysis products in the dialysis bag. However, all of the polysaccharides used reduced the *in vitro* allergenicity of the hydrolysis products that crossed the dialysis bags, regardless of the MWCO. This work highlights the possibility of modulating this food allergy through optimized formulation.

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