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Effects of Heat Treatment and Pectin Addition on β-Lactoglobulin Allergenicity

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The specific effects of heat treatment and/or addition of low/high-methylated pectin (LMP/HMP) on the allergenicity of β-lactoglobulin (β-Lg) and its hydrolysis products were investigated through a two-step in vitro digestion approach. β-Lg was first hydrolyzed by pepsin and then by a trypsin/chymotrypsin (T/C) mixture done in a dialysis bag with a molecular weight cutoff of 1000. The protein digestion was followed by SDS-PAGE electrophoresis performed on each digestion product, and their in vitro allergenicity was analyzed by immunoblotting. Such procedure was applied on β-Lg samples mixed with the two kinds of pectin before or after heating (80 °C, 25 min) to determine the respective impact of heat treatment and pectin addition. Heat denaturation improved significantly the susceptibility of β-Lg against the pepsin and the T/C. This effect, which was coupled to a reduction in immunoreactivity of the digested β-Lg, appeared to be distinctively modulated by LMP and HMP. Through nonspecific interaction with the β-Lg, pectin could reduce the accessibility of cleavage sites and/or epitope sequences. This mechanism of action is discussed in relation to the intra- and intermolecular interactions between β-Lg and pectin initiated under the experimental conditions.

KEYWORDS: β-Lactoglobulin; pectin; heat treatment; protein−polysaccharide interactions; immunoreactivity; food allergy

INTRODUCTION

Changes in eating habits and in the environment are thought to be connected to the recent increase in food allergies. As a consequence, prevention of food allergies becomes indeed a clinical public health concern and constitutes a major challenge for food industries (*1*). Among identified food allergens, milk exhibits a relevant allergenic potential related to the multiplicity and diversity of the involved proteins. Although sensitivity to milk proteins is widely distributed and polysensitization to several proteins most often occurs, β-lactoglobulin (β-Lg) is usually considered to be the major cow's milk allergen, that is, the protein most frequently and intensively recognized by human IgE (*2*). The antigenicity of β-Lg has been extensively studied, and its epitopes are now clearly identified (*3*−*6*). Immunoreactive structures are widely spread along the 162 amino acid molecule. Some of them are short linear sequences, whereas others are large fragments constituting conformational epitopes (*7*). Therefore, the allergenic potential of milk products depends in part on the integrity of these epitopes. Considering the technological treatments performed in the dairy industry, product formulation and heating processes can be considered to be the most significant factors suspected to induce severe structural modifications of β-Lg. Many investigations have been performed about the thermal stability of the β-Lg allergen structures, and significant reduction in β-Lg allergenicity was observed according to the thermal procedure (*8*−*10*). In particular, the IgE binding capacities of β-Lg are decreased by heating at 80−100 °C for 15 min, but residual allergenicity remains even after severe heating (*11*, *12*). Depending on the heating process (blanching, baking, cooking, grilling, roasting), possible structural and chemical changes of proteins are denaturation/aggregation, side-chain amino acid alteration, and Maillard reaction with other molecules such as sugars (*9*, *13*, *14*). In addition to the possible alteration of heat-labile conformational epitopes (*8*), heat treatment can thus modify the accessibility of the cleavage site of digestive enzymes and modulate the allergenic potential. The allergic reaction results from the interaction of allergenic peptide issued from the digestion of food protein with mast cell or basophil-bound IgE antibodies

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after crossing mucosal membrane barriers. According to this statement, the allergenic potential of proteins such as β-Lg was related to its susceptibility to gastric and pancreatic enzymes and, as a consequence, to the possible subsequent cleavage of epitope sequences.

In addition to the thermal denaturation induced by heat treatment, the possible interactions of allergen protein with other food ingredients can be considered as a further element influencing the susceptibility of protein to proteolysis. On this point, recent work demonstrated the significant effect of hydrocolloid addition on the β-Lg digestibility. Polyanionic and neutral polysaccharide proved to induce distinct effects with, in particular, an increase in protein digestion induced by the addition of low-methylated pectin (LMP) (*15*). Non-covalent interactions involving polysaccharides appear as a determining factor of the β-Lg digestibility in complex food matrices (*16*, *17*) and, as a possible consequence, of the allergenicity of food product. A study carried out on peanut protein isolate confirmed the influence of polysaccharide addition on the IgE/IgG binding activities of peptides issued from an in vitro digestion process (*18*).

Heating conditions and formulation both appear accordingly to be able to modulate the susceptibility of allergen to proteolysis and the allergenicity of food products. However, if their individual impacts were now demonstrated, their combined effect has never been investigated. This study aimed to determine the respective and combined influence of heat treatment and polysaccharide addition on the allergenicity of β-Lg. The investigation was carried out on a model food system simulating a dairy dessert, which consisted of mixtures of β-Lg and pectins, polysaccharides widely used in dairy industry for their gelling, thickening, and stabilizing properties in milk products such as yogurt beverages. Evaluation of β-Lg digestibility was followed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and N release of product issued from peptic followed by trypsin/chymotrypsin (T/C) digestion models. Resulting in vitro allergenicity and immunogenicity of protein and digestion products was investigated at each hydrolysis step by immunoblotting.

MATERIALS AND METHODS

Materials. Acid-processed bovine β-Lg isolate (batch 838) was provided by Lactalis (Retiers, France) and contained 90% dry matter (DM) protein consisting of 91% DM β-Lg and 9% DM of α-lactalbumin, bovine serum albumin, and β-casein. LMP (batch 0B800; 87% polysaccharide) and high-methylated pectin (HMP) (batch HD250; 87% polysaccharide) were provided by Degussa Texturant Systems (Boulogne-Billancourt, France).

Porcine pepsin (activity = 3800 units/mg of protein, EC 3.4.23.1), bovine trypsin (13800 units/mg, EC 3.4.21.4), porcine chymotrypsin (51 units/mg, EC 3.4.4.5), and thimerosal were purchased from Sigma. Bio-Rad broad molecular range markers were used as standard markers.

IgG and IgE Sera. Rabbit antiserum raised against cow's milk, obtained after incubation for 63 days, and rabbit preimmune serum were used (Agrobio). Sera from patients with known cow's milk sensitivity were pooled and used for our experiments. The cow's milk-specific IgE level determined by RAST was 53 kilounits/L, as measured by the Pharmacia CAP system. Normal serum from patients without a cow's milk hypersensitivity reaction was used as an IgE negative control.

Methods. *Preparation of β-Lg/Pectin Mixtures.* Powdered β-Lg containing 40 mg of nitrogen (1.7% w/v of protein) was taken and dissolved in 15 mL of 0.17 M sodium phosphate, pH 7, buffer. Pectins were dissolved in the same buffer at the same concentration (1.7% w/v). The dispersions were left overnight at 4 °C to allow complete hydration of the macromolecules. The stock β-Lg and pectin dispersions were blended so as to obtain a 0 (control β-Lg) and 50% (w/w) β-Lg/pectin mixed dispersion, this proportion having been used in a previous study (*17*). Thimerosal (50 mg/L) was added to all solutions to prevent bacterial and fungal contamination.

Heat Treatment. β-Lg solution and β-Lg/pectin mixtures were poured into 100 mL blocked flasks and then immersed in an 80 °C water bath during 25 min under 500 rpm stirring. By this procedure, solution temperature was controlled at 80 °C during 15 min. Treatment was stopped by the cooling solutions in an ice bath.

Enzymatic Hydrolysis. (i) Peptic Digestion. One milliliter of pepsin in 0.02 N HCl (1 mg/mL) was added to 15 mL of β-Lg dispersions or β-Lg/pectin mixtures (E/S = 1/250). To simulate in vivo gastric digestion, the pH of dispersions was progressively reduced from 7 to 2 over 2 h by adding 0.02 N HCl with a peristaltic pump (Ecoline, Ismatec, Zurich, Switzerland) (flow rate = 80 μL/min) at 37 °C. The digestion was stopped when the pH reached a value of 2 by cooling the samples and freeze-drying.

(ii) T/C Digestion in Dialysis Bags. The in vitro total digestion of β-Lg dispersions or β-Lg/pectin mixtures was carried out at 37 °C in a dialysis cell (Serna, Laval, Quebec, Canada) using the two-step hydrolysis method developed by Savoie and Gauthier (*19*) with minor modifications of the first step. In the first step, peptic digestion of β-Lg dispersions and β-Lg/pectin mixtures was done as described in the previous section. Peptic digestion was stopped by raising the pH to 8 with 2 N NaOH. In the second step, the samples were transferred to dialysis bags with molecular weight cutoffs (MWCO) of 1000 (SpectraPor 6, Interchim, Montluçon, France). One milliliter of a T/C mixture (1/2.3, w/w) at a weight concentration of 2.5 mg/mL (E/S = 1/50) was then added. Digestion products that diffused through the dialysis bag were collected for 6 h by circulating (1.6 mL/min) a 0.01 M sodium phosphate buffer at pH 8. The experiments were done in duplicate. Permeates and retentates were freeze-dried and stored at −18 °C until analysis.

Protein Assays. To perform the electrophoresis with a fixed and constant amount of protein, the protein content of the different samples was determined using the bicinchoninic acid assay (commercial kit, Interchim, Montluçon, France).

Electrophoresis and Immunoblotting. The β-Lg samples after peptic hydrolysis and the retentates after T/C hydrolysis were characterized by SDS-PAGE, according to the method of Laemmli and Favre (*20*). Two hundred micrograms of protein from each sample was 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 15% 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). A prestained broad-range protein marker was run with each gel to estimate the MW of the electrophoresed protein and hydrolysis products.

The in vitro IgG/IgE binding of undigested β-Lg and its hydrolysis products was analyzed by immunoblotting, using the sera containing anti-cow's milk IgG from rabbits or IgE from patients. Samples were loaded onto a gel for SDS-PAGE as described above. After electrophoresis, the proteins were transferred onto polyvinyl difluor membranes (Millipore) by applying a constant current of 300 mA for 60 min with a semidry blotting (Trans-Blot, Bio-Rad). Cut strips (4 mm) were used for immunodetection. All of the patient or rabbit sera were diluted with 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. The strips were washed three times for 15 min with 0.1% TBS−Tween between successive incubation steps. The strips were incubated with primary antibodies (rabbit anti-cow's milk or rabbit preimmune, 1:10000; allergenic or nonallergenic patient sera 1:8) for 12 h at 4 °C. Biotinylated anti-rabbit IgG or biotinylated anti-human IgE (Biovaley) was 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-indolyl phosphate using a commercial kit (Sigma).

Quantitative Image Analysis of Electrophoresis and Immunoblotting Patterns. The staining intensity of restriction bands was quantified using

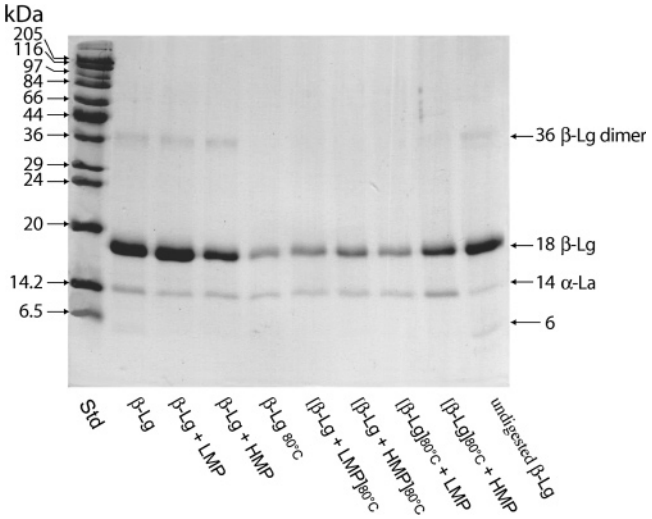

Figure 1. SDS-PAGE patterns of pepsin-digested β-Lg after hydrolysis with or without pectins (LMP, HMP) mixed before or after heat treatment (80 °C/25 min). MM (kDa) markers (Std) are indicated on the left-hand side.

the NIH image v3.01b software (Scion Corp.). The optical density of all the pixels in the area of each band was quantified and adjusted by a background subtraction of density in an adjacent blank area of the same size.

Nitrogen Analysis. The total nitrogen content in permeates was analyzed by using the Kjeldahl method. Protein digestibility estimated by the nitrogen (N) release was calculated as follows:

$$\text{N release (\%)} = \frac{\text{N in permeate (mg)}}{\text{N in protein sample (mg)}} \times 100$$

Size Distribution Analysis. Particle size distribution of heat-treated samples was determined by laser light scattering using a Mastersizer S granulometer equipped with a 5 mW He/Ne laser emitting at 632.8 nm (Malvern Instruments, Orsay, France). The optical system was composed by a 300 reverse Fourier lens covering a particle size range from 0.05 to 880 μm. The concentrated aliquots were introduced in the measuring sample unit (120 mL) containing distilled water to obtain an obscuration value of ≈15% at a stirring rate of 1000 rpm. A low obscuration value is needed to avoid multiple light scattering effects. Measurements were triplicated (two assays per experiment). The size distributions of particles were calculated on a volume basis after treatment of the scattered intensity data with a polydispersed model provided by the Malvern Mastersizer-S v2.17 software.

RESULTS

Peptic Digestion. *SDS-PAGE Pattern of Peptic Digestion Products.* β-Lg is known to be a poor substrate for pepsin with generally <10% of the protein digested for 20 h of incubation (*21*, *22*). The digestion of β-Lg by pepsin assessed using SDS-PAGE confirmed this general observation (**Figure 1**). Undigested β-Lg exhibited four major bands with apparent molecular masses (MM) of about 6, 14, 18, and 36 kDa. The band with MM of 14 kDa characterized the α-lactalbumin, which occurred in minor amount in the protein isolate. The two other bands (18 and 36 kDa) corresponded to the monomer and dimer forms of β-Lg, respectively. The last band revealed the occurrence of coextracted small peptides of 6 kDa. A similar electrophoresis pattern was obtained with sample digested for 2 h, which confirmed the strong resistance of β-Lg to peptic hydrolysis. The addition of pectin did not modify the protein profile of the untreated sample excepted for the removal of small peptides of 6 kDa. However, if LMP addition did not influence the band intensity, HMP induced a decrease in intensity of the band corresponding to the monomer form of β-Lg, suggesting an improvement of its digestibility.

Heat treatment caused the most significant effect on the β-Lg digestibility with a significant reduction in intensity of monomer band (38% compared to the untreated β-Lg) and a removal of the dimer. This result, observed on each heat-treated sample regardless the presence of pectin, was in agreement with previous studies performed in similar heating conditions (*23*). It was established that heat treatments (80−100 °C) led to significant proteolysis of β-Lg on subsequent incubation with pepsin due to measurable changes in the conformation of the protein (*24*).

Although the increase of β-Lg digestibility was observed with all of the heat-treated samples, LMP and HMP addition modulates in different ways the effect of heat treatment. If LMP did not significantly modify the digestibility of heat-treated sample, the band corresponding to the monomer β-Lg was ≈20% more intense in the presence of HMP whatever the sequence of mixture showing an increase in protein digestibility.

IgG and IgE Immunoblottings of Peptic Digestion Products. **Figure 2** reports the immunoblotting obtained from the studied samples. The staining of specific bands showed the large IgG spectra of serum pool against the major milk proteins (**Figure 2a**). The occurrence of a band located at a MM of 14 kDa confirmed the presence of α-lactalbumin in the protein isolate, which exhibited a high IgG binding activity and evidenced the polysensitivity of used rabbit serum. The large band with a MM of ≈18 kDa revealed the strong β-Lg immunogenicity. Regarding the immunoblotting pattern obtained with the different samples, each band was detected but exhibited various staining intensities. Focusing on the β-Lg immunogenicity, pectin addition did not modify the IgG binding activity of the unheated β-Lg. On the other hand, LMP addition induced a significant decrease of staining on the heated β-Lg (≈44% compared to the control). The effect of LMP was evenly observed on every peptide beyond the sequence of experimental design, that is, added before or after the heat treatment. Because no effect of LMP on the heat-treated β-Lg digestibility was observed in the electrophoresis pattern, LMP decreased the IgG binding activity of the native protein and the peptides issued from its peptic digestion. In contrast, HMP addition did not influence the staining intensity of the 18 kDa band. In good agreement with the electrophoresis pattern, HMP seemed to influence the β-Lg digestibility without significant influence on the immunoreactivity.

Regarding the immunoblotting performed with IgE serum, only the band corresponding to 18 kDa exhibited an immunoreactivity (**Figure 2b**) that demonstrated a specific sensitivity of the pooled serum against the monomeric β-Lg. The in vitro β-Lg allergenicity was evidenced on each unheated sample with no significant influence of pectin addition. The heat treatment induced a loss of β-Lg antigenicity, which could be explained by the increase of its digestibility. Because the band with an apparent MM of 18 kDa was detected on the SDS-PAGE pattern, this result suggested that native β-Lg was not concentrated enough to produce visible immunodetection. The effect of pectins after β-Lg heating was contrasted depending on their methylation degree. Addition of LMP to the β-Lg dispersion before or after the heating step produced a result similar to the heated control sample. On the other hand, the presence of HMP was associated with a detectable IgE binding activity. Because HMP addition induced a reduction of heat-treated β-Lg susceptibility against pepsin, native protein concentration of these samples exceeds threshold of immuno-blotting response.

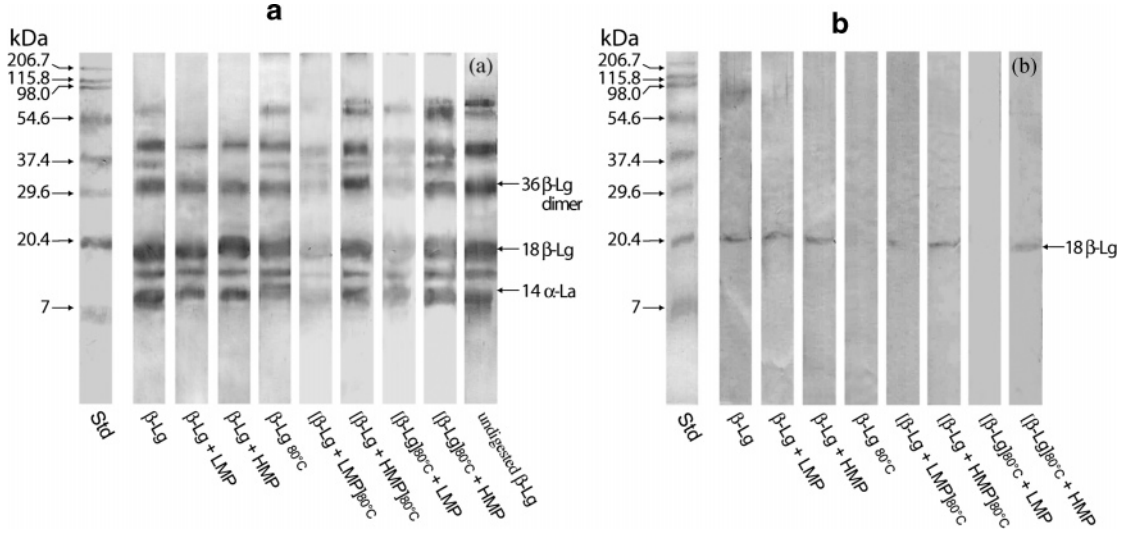

Figure 2. Rabbit IgG (**a**) and human IgE (**b**) immunobloting of pepsin-digested β-Lg after hydrolysis with or without pectins (LMP, HMP) mixed before or after heat treatment (80 °C/25 min). MM (kDa) markers (Std) are indicated on the left-hand side.

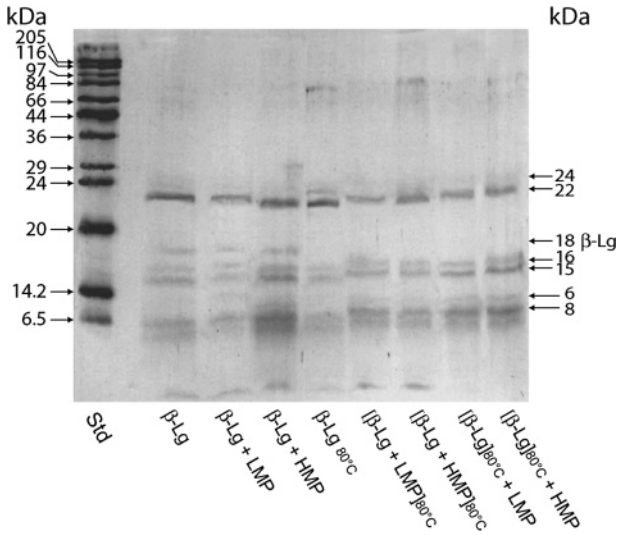

Figure 3. SDS-PAGE patterns of β-Lg retentates after T/C hydrolysis with or without pectins (LMP, HMP) mixed before or after heat treatment (80 °C/25 min). MM (kDa) markers (Std) are indicated on the left-hand side.

Trypsic and Chymotrypsic Digestion. *SDS-PAGE Pattern of Retentates.* Trypsic and chymotrypsic digestion was carried out in dialysis bags with a MWCO of 1000 to roughly mimic the intestinal mucosal barrier (*25*). At pH 7, β-Lg is known to be quite resistant to T/C hydrolysis (*23*) as <10% of the native protein was degraded by enzyme in the absence of a heat denaturation step (*26*). In opposition to this statement, the occurrence of bands corresponding to peptides or residual proteins observed in the electrophoresis pattern of untreated β-Lg demonstrated that native proteins were hydrolyzed to a large extent in our conditions (**Figure 3**). In agreement with previous experiments carried out in similar conditions (*15*), the retentate from the β-Lg isolate without pectin showed slight and intense bands with apparent MM of 24 and 22 kDa, respectively, which corresponded probably to the partly digested dimeric β-Lg. Three bands with apparent MM of 18, 16, and 15 kDa revealed the occurrence of both residual undigested and partially hydrolyzed β-Lg, respectively. Two bands with MM of about 8 and 6 kDa revealed released peptides generated during this digestion.

The protein isolate digested in the presence of pectins did not show changes in the electrophoresis pattern. However, significant variations in signal intensity were produced by LMP and HMP addition. In the presence of LMP, the total band intensity was weaker than the control sample, and a 20% reduction of intensity was measured for the 18 kDa band compared to the control β-Lg. In contrast, the presence of HMP induced a significant increase ($\approx$16%) in signal intensity for the band corresponding to an apparent MM of 18, 16, or 15 kDa. In agreement with previous results (*15*), HMP and LMP produced antagonist effects on the proportion of digested β-Lg.

Susceptibility of β-Lg to T/C hydrolysis is known to be significantly increased at a heating temperature of 80 °C (*24*). In the present study, the major modification induced by heating was the absence of the band with MM of 18 kDa, demonstrating a total digestion of the monomeric β-Lg. Considering the heat-treated samples containing pectins, the complete digestion of β-Lg occurred in the presence of the two classes of pectin (HMP/LMP) when the mixture was carried out either before or after heat treatment. However, increases of 19 and 37% in grayscale values were observed on released peptides (MM of 6, 8, 15, and 16 kDa) when LMP and HMP were added after β-Lg heat denaturation as compared to their respective controls.

IgG and IgE Immunoblottings of Retentate. The IgG blotting of the same samples emphasized the previous observations deduced from electrophoresis analysis (**Figure 4**). On the digested β-Lg sample, monomeric β-Lg (18 kDa) and hydrolysis products identified on the electrophoresis pattern (MM of 8, 6, 15, and 16 kDa) exhibited an IgG binding activity (**Figure 4a**), which suggested that all released peptides included one or several immunoreactive epitopes. The immunoblots obtained with β-Lg/pectin mixture exhibited the same bands with difference of signal intensity. In agreement with the electrophoresis results, LMP addition induced a decrease of 34% of total band intensity, whereas HMP addition did not produce significant variation. This result suggested that variation in band intensity of IgG blotting was related to the band intensity observed on the electrophoresis pattern. As a consequence, antigenicity expressed through the immunoblotting was more particularly related to the amount of residual peptides material than to their IgE binding activity.

The heat treatment of the different samples caused the loss of the 18 kDa band. Because this result was previously noted

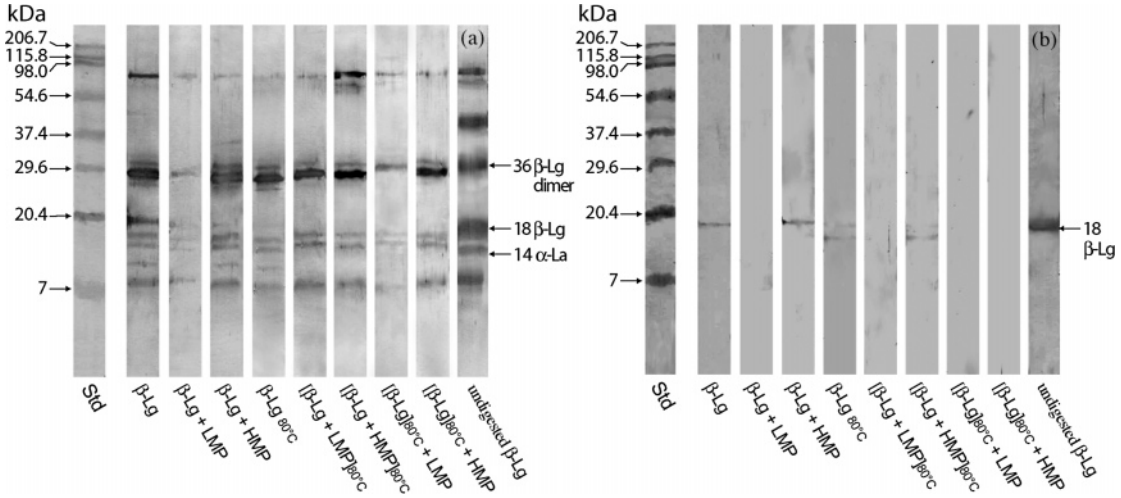

Figure 4. Rabbit IgG (**a**) and human IgE (**b**) immunoblotting of β-Lg retentates after T/C hydrolysis with or without pectins (LMP, HMP) mixed before or after heat treatment (80 °C/25 min). MM (kDa) markers (Std) are indicated on the left-hand side.

on the electrophoresis pattern, the absence of IgG binding activity of native β-Lg in heated samples was due to its digestion. However, in contrast with previous observations carried out from the electrophoresis pattern, relevant differences of immunoreactivity were detected when LMP was adjoined distinctively before and after β-Lg heat denaturation. A 22% reduction in staining of bands with MM of 15 and 16 kDa was measured when the addition of LMP was carried out before heat treatment. This decrease in band intensity became more marked (≈60% for 15 and 16 kDa bands) when LMP was added after the heat treatment.

Immunoblotting performed with IgE serum allowed further progress in the identification of respective impacts of pectin addition and heat treatment on the allergenicity of β-Lg (**Figure 4b**). The blotting issued from digested β-Lg revealed the presence of intact monomer (18 kDa) only, which suggested that digestion products did not show IgE binding activity or were not concentrated enough to be detectable. In good agreement with the electrophoresis pattern, the low amount of native β-Lg in the presence of LMP was associated with a loss of IgE binding activity, whereas the HMP addition did not affect the blotting of the band of 18 kDa.

Considering the impact of heat treatment, the β-Lg sample exhibited two thin bands with apparent MM of 18 and 16 kDa corresponding to the native protein and one hydrolysis product, respectively. The other heat-treated samples were characterized by the absence of compounds showing IgE binding activity, except for the β-Lg heat-treated with HMP, which exhibited a pattern similar to that of the heat-treated sample. Once more, LMP moderated the β-Lg allergenicity and, referring to the electrophoresis pattern, this result appeared to be more related to an action on the β-Lg digestibility than on its IgE binding ability. Concurrently, the residual IgE binding activity observed in the presence of HMP tends to confirm that HMP affected to a lesser extent the β-Lg degradation and demonstrated the determining influence of the charge density of pectin on the β-Lg digestion.

Permeates Quantification. **Table 1** reports N release (percent) through dialysis bags with a MWCO of 1000 after peptic digestion and then 6 h of β-Lg T/C hydrolysis. Significantly lower than values reported in previous studies performed under similar conditions (*15*), the fraction of β-Lg collected in the permeate after digestion accounted for 7−9% of the total protein amount. With regard to the influence of pectin addition, LMP did not change the N content in permeates, whereas HMP induced a 50% increase of N-released fraction. The value obtained after heat treatment of β-Lg was similar to that obtained for the untreated sample. However, pectins produced significant changes in the fraction of peptide material in permeates depending on the order of addition. With regard to the impact of LMP, an addition performed before the heat treatment produced a slight but significant increase in permeates content, whereas ≈40% reduction was obtained when pectin was added after β-Lg heat treatment. On the other hand, the HMP addition induced a 45% increase of permeate N only when the mixture was performed before heating. The results could appear in opposition to the SDS-PAGE because retentate analysis suggested an improvement of β-Lg digestion in the presence of LMP. As previously suggested (*18*), possible interactions between LMP and β-Lg hydrolysis products would cause the partial retention of removed peptide inside the dialysis bag. Trypsin is a serine protease with substrate specificity based upon positively charged lysine and arginine side chains. Therefore, some peptide removed during trypsin digestion could exhibit local positive charges, allowing electrostatic bindings with pectins (*4*, *27*). Thus, the interaction degrees of pectin with charged peptides were weaker for HMP than for LMP, which could explain the different values of permeate content observed in the two samples. N release in permeate material did not represent an appropriate measure of protein digestibility and must be coupled to an analysis and quantification of released peptide in retentate material.

Table 1. N Release through Dialysis Bags with a MWCO of 1000 after 6 h of β-Lg T/C Hydrolysis

	% N released[a]
β-Lg control	7.96 ± 1.33
β-Lg + LMP	8.13 ± 0,82^{NS}
β-Lg + HMP	11.93 ± 1.03**
β-Lg at 80 °C	8.82 ± 0.27*
[β-Lg + LMP] at 80 °C	9.60 ± 0.54*
[β-Lg + HMP] at 80 °C	11.28 ± 1.57**
[β-Lg] at 80 °C + LMP	5.04 ± 0.09***
[β-Lg] at 80 °C + HMP	8.93 ± 0.43*

[a] *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.001$; ^{NS}, nonsignificant (ANOVA, $n = 4$).

Size Distribution of Heat-Induced β-Lg Aggregates. The influence of the two classes of pectin on the heat-induced β-Lg aggregation was assessed through measurements of particle size

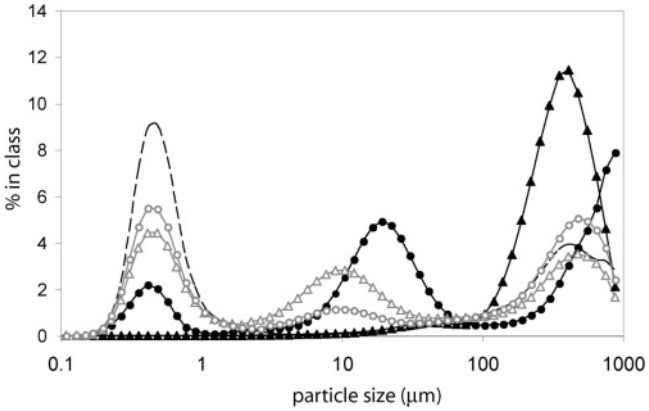

Figure 5. Size distribution of β-Lg solution (1.7% w/v) heat-treated at 80 °C during 25 min (- - -), of β-Lg mixed with LMP (50:50, v/v) before (▲) and after (△) heat treatment, and of β-Lg mixed with HMP (50:50, v/v) before (●) and after (○) heat treatment.

distributions (**Figure 5**). Large variations in size of β-Lg aggregates were observed according to the sample. The size distribution obtained on β-Lg alone showed two aggregate populations. The first and major class exhibited an average particle diameter of $\approx$0.7 μm, which corresponded to a low level of protein aggregation. The second population exhibited an average particle diameter of $\approx$600 μm, which corresponded to visible clusters.

The postheating addition of HMP and LMP produced similar impacts on the particle size distribution with a most marked effect with LMP. Whereas the amount of large aggregates (600 μm diameter) did not change to a significant extent, a decrease in proportion of small aggregates occurred with a concomitant formation of an intermediate class of aggregates with an average diameter of 10 μm.

The point in time of pectin addition had much influence on the particle size distribution. Although HMP and LMP both promoted the β-Lg aggregation when they were added after the heat treatment of protein, the two classes of pectin produced contrasting effects. In presence of HMP, a decrease in proportion of the fraction of 0.7 μm was associated with the formation of a class of 20 μm particle diameter and a strong increase in the amount of large-size aggregates. The presence of LMP induced β-Lg association under the exclusive form of large clusters, which was coupled to a removal of the other populations of aggregates exhibiting lower diameters. In this configuration where polymers were mixed before heat treatment, the considerable increase in sizes of aggregates as compared to the heat-treated β-Lg control could be alternatively explained by the association of protein aggregates (i.e., clusters formation) due to charge repulsions between the two polymers or by the interaction of the two polymers leading to β-Lg/pectin complexes.

DISCUSSION

Susceptibility against proteolysis is a determining factor for allergenicity of food proteins because it controls in part the sensitization of the immune system after transport across the intestinal barrier. Prior to a clinical investigation, the in vitro digestion method using two-step hydrolysis allowed the impact of technological process and formulation on the digestibility of specific allergens to be assessed. Coupled to an immunoblotting test, this approach evaluates the allergenic potential.

β-Lg was an allergen model mainly studied in previous work. Its high resistance to pepsin attack at pH 2 was previously observed (*28*, *29*), and the increase of its susceptibility by heat treatment was also established (*22*). Addition of polysaccharides including pectins previously proved to increase the degree of β-Lg hydrolysis (*17*). In our study, this observation was especially confirmed by the addition of HMP. Because the two classes of pectins induce a similar decrease (−20%) in enzyme activity (*17*), this increase of β-Lg hydrolysis, already observed in similar conditions, was rather due to an increase of protein solubility resulting from protein/polysaccharides interactions (*16*).

A previous heat treatment of β-Lg induced a high increase of peptic digestion. Heating at 80 °C causes a dissociation of dimeric β-Lg and a following partial unfolding of β-Lg backbone. The possible exposure of cleavage sites resulting from the thermal denaturation can explain the increase of the susceptibility to proteolysis by pepsin. This effect appeared to be modulated by the addition of pectins with, in particular, a decrease of β-Lg degradation in the presence of HMP. This effect cannot be explained by pepsin inhibition because no decrease in pepsin activities was observed on unheated samples. Consequently, HMP action was probably related to its ability to interact with β-Lg, and the lower digestibility of β-Lg observed in the presence of HMP could be then induced by local protein/pectin interactions, which decrease the accessibility of cleavage sites to protease.

In addition to their distinct effect on β-Lg digestibility, HMP and LMP modified in different ways the immunoreactivity. The immunoblottings pointed out a significant decrease of the IgG binding activity of heated β-Lg and its pectic digestion products in the presence of LMP. In agreement with previous study performed on peanut protein protein (*18*), the action of LMP was unrelated to the digestibility but most probably related to the decrease of epitope accessibility due to the conformational change of peptides or the IgG epitope masking.

Distinct influences of HMP and LMP were observed during T/C digestion once again with antagonist effects on the unheated β-Lg. Because a previous study demonstrated no influence of the two classes of pectin on the T/C activities (*15*), their action was consequently also related to their ability to interact with the β-Lg without specific inhibition of the enzyme. Whereas HMP probably prevented the enzymatic action by masking the cleavage site, LMP addition induced an enhancement of the β-Lg hydrolysis, which resulted in a reduction of its immunoreactivity. The action of LMP during T/C hydrolysis, already observed on peanut allergens (*18*), occurred also on the heat-treated β-Lg, but the mechanism of action was not identified. Besides its influence on β-Lg susceptibility against T/C, LMP appeared to induce an additional decrease of β-Lg immunoreactivity with a higher effect when the addition was carried out after heat treatment. This result suggested a potential impact of order of unit operations of food processing on product allergenicity.

On the other hand, the charge density of mixed pectins appeared as a determining factor of the hydrolysis degree of the β-Lg. Through nonspecific interactions, the two classes of pectins produced masking effects both on the cleavage site of protease and on the antibody binding region. However, although local positive patches on the β-Lg allowed possible electrostatic interactions with pectins, it was probable that the HMP and LMP addition led to distinct structure formation of biopolymer suspension at macroscopic scale. The different interactions between the two polymers have been investigated in detail through several studies (*30*−*33*), and it was established that thermodynamic compatibility between β-Lg and pectin arises

if attractive interactions are greater than repulsive forces. In our experimental conditions (pH 7, 25 °C) (*30*), β-Lg was mainly negatively charged and the occurrence of polyanionic biopolymers such as pectin resulted in phase separation (*34*). This polymer organization was induced by repulsion force between the two negatively charged polymers and would be accordingly favored by the LMP, which exhibited a higher charge density than HMP. Considering the electrophoresis results, it could be thus suggested that the distinct effects of HMP/LMP on proteolysis progress resulted from their different influences on a such a demixed-phase system.

Furthermore, HMP and LMP proved to distinctively influence the heat-induced aggregation process of β-Lg. Particle size analysis showed that the pectin addition promoted the formation of large aggregates, which could result from β-Lg self-association or interpolymer complexing. LMP improved to a larger extent the aggregation with a higher effect if pectin addition occurred after the heat treatment. The supramolecular assembly, which was partially controlled by the charge density of pectins and the sequence of mixture, appeared to influence both the digestibility and the immunoreactivity of the β-Lg.

According to this statement, this work pointed out the possibility of modulating food allergy through optimized process and formulation. If heat treatment was known to influence the allergenic potential of food product, this effect appeared to be variably influenced by the addition of polyelectrolyte such as pectin.

ABBREVIATIONS USED

β-Lg, β-lactoglobulin; HMP, high-methylated pectin; LMP, low-methylated pectin, MM, molecular mass; MWCO, molecular weight cutoff.

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