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Immunoreactivity and digestibility of high-pressure-treated whey proteins

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Abstract

The effect of high-pressure (HP) treatments on the IgG- and immunoglobulin E (IgE)-binding properties and digestibility of whey proteins was investigated. The formation of protein aggregates was important in whey protein isolate (WPI) and β-lactoglobulin (β-Lg) treated at 200 and 400 MPa at pH 6.8, but was not detected at pH 2.5. Treatment of β-Lg and WPI at 200 and 400 MPa increased binding to β-Lg-specific rabbit IgG and did not affect binding to IgE from allergic patients, but there was no apparent relationship between these responses and the degree of protein aggregation. HP treatment at 400 MPa promoted the hydrolysis of β-Lg by pepsin, but this increased susceptibility of β-Lg to proteolysis was progressively lost during the refrigerated storage of the HP-treated WPI. The higher digestibility of the HP-treated WPI with pepsin did not have an impact on the IgE-binding of the proteolysis products, as compared to non-HP-treated WPI, probably because of the release, in both cases, of specific IgE-binding peptides. Some of these immunoreactive fragments remained after hydrolysis with Corolase PP and probably accounted for a low, but detectable, IgE-binding response.
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1. Introduction

The dominant whey protein, β-lactoglobulin (β-Lg), is largely responsible for the physicochemical properties and the functional behaviour of whey protein products used as food ingredients (Foegeding, Davis, Doucet, & McGuffey, 2002). Some characteristics of β-Lg are its absence from human milk, its resistance to gastric acid and to proteolytic attack and its ability to cross the intestinal mucosa and reach mother's milk (Reddy, Kella, & Kinsella, 1988; Sorva, Makinen-Kiljunen, & Juntunen-Backman, 1994). It has been considered to be the most important milk allergen for a long time, although it is now recognised that most allergic patients are sensitised to several milk proteins at a time (Wal, 2004).

Food processing can alter the allergenic properties of proteins by hiding, destroying or disclosing allergenic epitopes through conformational changes, or by changing digestibility of proteins (Guo, Fox, Flynn, & Kindstedt, 1995; Peyron, Mouécoucou, Frémont, Sanchez, & Gontard, 2006; Takagi, Teshima, Okunuki, & Sawada, 2003). In the case of β-Lg, denaturation per se does not appear to be sufficient to reduce its allergenicity; rather, the main functional consequence of denaturation is to increase its digestibility and decrease the ability of the protein to elicit an allergic response (Kleber, Krause, Illgner, & Hinrichs, 2004; Svenning, Brynhildsvold, Molland, Langsrud, & Vegarud, 2000). Heat-induced denaturation makes β-Lg more susceptible to hydrolysis by pepsin, and pectic digestion has a negative correlation with the allergenicity of proteins in several models (Moreno, 2007). In addition, heat-induced denaturation also modifies the immunogenic properties of β-Lg (Rytkönen et al., 2002).

High-pressure (HP) treatment of β-Lg leads to protein unfolding, which favours SH/SS exchange interactions, leading to the formation of disulphide-bonded aggregates (Funtenberger, Dumay, & Cheftel, 1995); unfolding also appears to unmask buried hydrophobic groups. At high pressures, hydrophobic bonding is hampered, but hydrophobic interactions may lead to protein aggregation during or after pressure release (Hosseini-nia, Ismail, & Kubow,

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1999; Pittia, Wilde, Husband, & Clark, 1996). The structural changes undergone by β-Lg under HP are partially reversible (see López-Fandiño, 2006 for review). The refolding ability of β-Lg depends on the pressure, temperature and pH of the treatment, being more favoured at 200 than 400 MPa, at 37 than 20 °C, and at acid than neutral pH values (Belloque, Chicón, & López-Fandiño, 2007; Ikeuchi et al., 2001). In general terms, the situation is similar to what occurs during thermal denaturation of β-Lg; however, there are important differences between the effects of both treatments that lead to different structures and product characteristics (see Considine, Patel, Anema, Singh, & Creamer, 2007 for review).

Therefore, like many other physical and chemical procedures that alter protein structure, HP can be used to modify immunoglobulin E (IgE)-binding properties (Ehn, Ekstrand, Bengtsson, & Ahlstedt, 2004). On the other hand, HP-induced changes in the conformation of the proteins may make enzymatic digestion easier, and HP treatment has been reported to enhance the susceptibility of β-Lg to subsequent proteolysis at atmospheric pressure (Bonomi et al., 2003; Chicón, Belloque, Recio, & López-Fandiño, 2006a; Chicón, López-Fandiño, Quirós, & Belloque, 2006b; Knudsen, Otte, Olsen, & Skibsted, 2002; Otte, Zakora, Qvist, Olsen, & Barkholt, 1997).

The increased application of HP to the manufacture of new products and the extensive use of whey proteins as ingredients in many food commodities makes it necessary to have a better understanding of the effects of HP on whey proteins, by evaluating the effect of this process on their allergenic potential. This study assessed the effect of HP treatment on the IgG- and IgE-binding properties of whey proteins, especially β-Lg. The influence of this process on the digestibility of whey proteins and IgE-binding properties of the resulting digestion products were also examined.

2. Materials and methods

2.1. High-pressure treatments

Genetic variant A of β-Lg was isolated from the pH 4.6—soluble fraction of a milk sample obtained from a homozygotic cow. The protein content of the purified preparation, as determined by the Kjeldhal method, was 82%. Whey protein isolate (WPI, minimum 92% protein on dry basis, 5% maximum humidity, with low lactose-content) was from Carbery Food Ingredients (Cork, Ireland).

Solutions of β-Lg (2.5 mg mL^{-1}) or WPI (6 mg mL^{-1}) were prepared either in 50 mM Tris–HCl buffer, pH 6.8, 50 mM citrate buffer, pH 2.5, or water, poured into Eppendorf vials (500 μL) avoiding headspace and HP-treated at 200 or 400 MPa using a 900 HP apparatus (Eurotherm Automation, Lyon, France). The pressure was increased at a rate of 2.5 MPa s^{-1}, maintained for 10 or 30 min, and released at the same rate. The temperature of the hydrostatic fluid medium was controlled by circulating water through a jacket surrounding the pressure vessel and, before pressure processing, was set to either to 30 or 50 °C. The increase in temperature as a result of the pressure treatments was approximately 2 °C per 100 MPa. HP treatments were performed in duplicate. After removal from the HP unit, samples were kept refrigerated at 5 °C and, within the same day, the HP-treated proteins were incubated in microtiter plates at appropriate dilutions for the IgE- and IgG-binding analysis.

2.2. Simulation of gastrointestinal digestion

For the digestion experiments, solutions of WPI in water (6 mg mL^{-1}), sealed in plastic bags, were treated at 400 MPa and 25 °C for 30 min. After HP treatment, the bags were stored at 5 °C for different periods (0, 1, 2, 8, 19 and 30 days) and afterwards subjected to a process that simulated gastrointestinal digestion, as explained below. Non-HP-treated WPI solutions were used as controls.

Hydrolysis was carried out according to the method of Alting, Meijer, and Van Beresteijn (1997). Samples were first hydrolysed with porcine pepsin (EC 3.4.23.1, 570 units mg^{-1}, 20 mg g^{-1} of protein, Sigma, St. Louis, MO, USA) at pH 2.0 and at 37 °C for 90 min with a stirring speed of 150 rev min^{-1}. After hydrolysis with pepsin the pH was raised to 7–8 with 1 M NaOH and aliquots were withdrawn. Corolase PP, a proteolytic enzyme preparation from pig pancreas glands that, as well as trypsin and chymotrypsin, contains numerous amino- and carboxylpeptidase activities (Röhm, Darmstadt, Germany), was added at a concentration of 40 mg g^{-1} of protein and samples were incubated for 150 min at 37 °C. The enzyme was inactivated by heating at 95 °C for 10 min, followed by cooling to room temperature. Hydrolyses were performed in quadruplicate. Each sample was stored at −20 °C until further analysis by RP-HPLC.

2.3. SDS–PAGE analysis

HP-treated samples were dissolved in 10 mM Tris–HCl buffer, pH 8.0, containing 2.5% SDS and 10 mM EDTA (non-reducing conditions), and heated at 100 °C for 10 min. Analysis by SDS–PAGE used the PhastSystem Electrophoresis apparatus, precast Homogeneous Gels 20% and PhastGel SDS buffer strips (Amersham Biosciences, Uppsala, Sweden), following the electrophoretic and silver staining conditions of the manufacturer.

2.4. RP-HPLC–MS/MS analysis

RP-HPLC with UV detection, on-line with electrospray ionisation and quadrupole ion trap instrument (ESI-MS/MS) analyses were performed as described by Chicón et al. (2006a). An Agilent 1100 Series HPLC equipment (Agilent Technologies, Waldbronn, Germany) with a Hi-Pore® Reversed Phase RP-318 Column (250 × 4 mm i.d.; Bio-Rad Laboratories, Richmond, CA, USA) and an Esquire 3000

mass spectrometer (Bruker Daltonik, Bremen, Germany) were used. The peak areas of β-Lg and five peptide groups (F1–F5), eluting at different times in the RP-HPLC-UV chromatograms (Fig. 2), were obtained by setting manually the integration limits and automatically integrating the areas, using an Agilent Chemstation software (revision A.05.01, Agilent Technologies). The extent of proteolysis in the different samples was compared by calculating the peptide content as the sum of the area of all peptide fractions (PF = F1 + F2 + F3 + F4 + F5) relative to the total peptide–protein content, i.e., PF/total = PF/(PF + β-Lg). The extent of accumulation of peptides at different eluting time ranges was compared among samples by calculating the area of the fractions relative to the total peptide content, i.e., F1/PF, etc.

2.5. Indirect ELISA

Individual serum samples from 30 children with clinically allergic symptoms to bovine α-lactalbumin (α-La) and β-Lg were collected from the Gregorio Marañón Hospital (Madrid, Spain). All patients had specific IgE antibodies towards milk proteins as determined by the FEIA-CAP System (Pharmacia Diagnostics, Uppsala, Sweden). A normal serum, consisting of the pooled sera of five healthy non-allergic people, was used as negative control (NCS).

For the estimation of the IgE-binding, high-binding polystyrene microtiter plates (Corning, Cambridge, MA, USA) were used as solid support. Single wells were coated with antigen (either 0.125 μg of β-Lg or 0.3 μg of WPI or digested WPI) in 0.01 M phosphate-buffered saline solution (PBS), pH 7.4, and incubated overnight at 6 °C. Plates were washed with PBS containing 0.05% of Tween 20 (PBST) using a Microplate Washer (Nunc, Roskilde, Denmark). This washing system was used after each incubation step. Residual free binding sites were blocked with PBS containing 2.5% of Tween 20 for 1 h at room temperature. Then, plates were incubated with 50 μL per well of human serum diluted 1:20 or 1:50 in PBST and incubated overnight at 6 °C. The same dilution of NCS was used as negative control. A solution of rabbit anti-human IgE conjugated with horseradish peroxidase (HRP) (DakoCytomation, Glostrup, Denmark) diluted 1:1000 in PBST was added (50 μL per well) and incubated for 1 h at room temperature. Before the addition of the enzyme substrate, a signal amplification system based on the subsequent addition of tyramide-biotin and streptavidin-HRP was introduced following the instructions of the manufacturer (ELAST ELISA amplification system, Perkin-Elmer Life Sciences, Waltham, MA, USA). A solution of freshly prepared *o*-phenylene-diamine dihydrochloride (OPD, from DakoCytomation), containing H_2O_2, was added following the conditions of the manufacturer. Plates were incubated for 30 min at room temperature and the reaction was stopped by adding 50 μL per well of 0.5 M H_2SO_4. Optical densities were read at 492 nm on an automated ELISA plate reader Multiskan Ascent (Labsystems, Helsinki, Finland). ELISA determinations were carried out in triplicate and measurements averaged. A blank without antigen (PBS), the negative control (NCS) and positive controls (different concentrations of β-Lg or WPI) were included in each plate.

Residual IgG-binding of the samples was evaluated against anti-bovine β-Lg IgG, following the ELISA described above with slight modifications. Briefly, wells were coated with antigen (either 0.025 μg of β-Lg or 0.06 μg of WPI or digested WPI) as described above. After the blocking step, plates were incubated for 1 h at room temperature with 50 μL per well of HRP-conjugated rabbit anti-bovine β-Lg (Bethyl Laboratories, Montgomery, TX, USA) diluted 1:20,000 in PBS. Finally, 50 μL per well of the OPD solution was added and incubated for 30 min. The reaction was then stopped with H_2SO_4 and the plate was read.

2.6. Statistical analyses

A two sample *t*-test was used to compare RP-HPLC peak areas arising from WPI, with or without previous HP treatment, after incubation with pepsin and Corolase PP. One-way analysis of variance (ANOVA), applying the Student–Newman–Keuls test for means comparison, was used for the statistical evaluation of the RP-HPLC areas and IgE-binding results of the digested WPI samples. A one-way ANOVA was also applied to estimate the effect of pressure on the IgG- and IgE-binding of β-Lg or WPI. In this case, to establish a comparison between HP-treated samples and controls, the Dunnett test was applied. The SPSS program (version 14.0, SPSS Inc., Chicago, IL, USA) was used for data processing.

3. Results and discussion

3.1. Structural changes and IgG- and IgE-binding properties of HP-treated whey proteins

As an example, Fig. 1 shows the SDS–PAGE patterns under non-reducing conditions of β-Lg and WPI treated at 400 MPa at two pH values and temperatures. The electrophoretic patterns of the isolated β-Lg and the WPI changed as a result of HP treatment at pH 6.8, but not at pH 2.5, thus confirming that the stability of proteins under pressure largely depends on pH. Unfolding of β-Lg under pressure is more extensive and irreversible at pH 7.0 than at pH ⩽ 3.0 and refolding is achieved more slowly when the protein is treated at neutral than at acidic pH (Belloque et al., 2007; Ikeuchi et al., 2001). In addition, the formation of disulfide-linked oligomers of β-Lg is promoted at neutral pH, due to the enhanced reactivity of the SH groups (Funtenberger et al., 1995).

As a result of HP treatment at 200 MPa (not shown) and 400 MPa (Fig. 1) at neutral pH, new bands corresponding to protein aggregates appeared that increased with the pressure level. HP treatment of β-Lg at 400 MPa decreased

the band corresponding to the monomeric protein and increased the band for the β-Lg dimer. In addition, a band corresponding to β-Lg trimer was detectable with the sensitive silver staining. These changes were more noticeable at the higher treatment temperature, probably because of an increased flexibility of the molecule (Belloque et al., 2007). Furthermore, the structural changes in β-Lg induced by the combined application of pressure and temperature are more extensive at pH 7.0 than at pH 5.6 (Tedford & Schaschke, 2000).

HP-treatment of WPI at pH 6.8 at 200 MPa (not shown) and 400 MPa (Fig. 1) gave rise to the formation of a new band with a molecular mass similar to that of the β-Lg dimer. In addition, the electropherogram of the HP-treated WPI also revealed bands corresponding to larger aggregates, possibly trimers and tetramers, as well as aggregates at the boundary between the stacking and resolving gel or in the sample loading well. The presence of other whey proteins can favour the formation of high-order disulfide-bonded β-Lg polymers (Considine et al., 2007; Patel, Singh, Havea, Considine, & Creamer, 2005). Whey proteins were also pressure-treated in water, which led to less marked changes in the patterns as compared with Tris, a pressure-resistant buffer, at pH 6.8 (Fig. 1). In water, high pressure favours the separation of ionic species, leading to a lower pH and a less extensive polymerisation of β-Lg (Funtenberger et al., 1995).

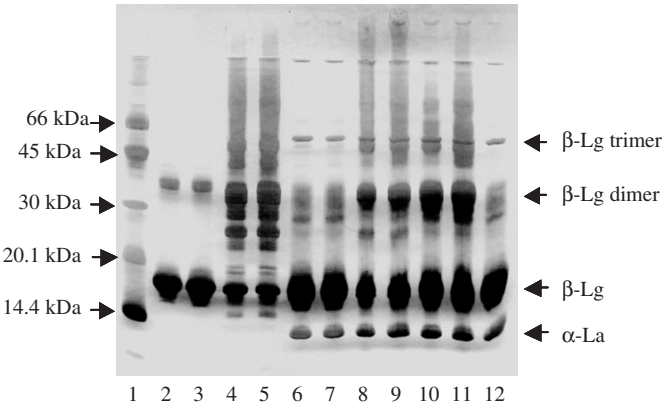

Fig. 1. SDS–PAGE patterns (non-reducing, silver staining) of: (1) molecular mass markers and β-Lg treated at 400 MPa for 30 min at (2) pH 2.5 and 30 °C, (3) pH 2.5 and 50 °C, (4) pH 6.8 and 30 °C, (5) pH 6.8 and 50 °C and WPI treated at 400 MPa for 30 min at (6) pH 2.5 and 30 °C, (7) pH 2.5 and 50 °C, (8) pH 6.8 and 30 °C, (9) pH 6.8 and 50 °C, (10) in water at 30 °C, (11) in water at 50 °C and (12) control WPI.

Indirect ELISA was used to determine IgG- and IgE-binding properties of HP-treated β-Lg and WPI (Table 1). In general, HP treatment of β-Lg and WPI resulted in a greater IgG-binding response to bovine β-Lg ($P<0.05$). However, there was not an apparent relationship between the IgG-binding and the degree of protein aggregation as observed in the HP-treated samples by SDS–PAGE under non-reducing conditions (Fig. 1), because the pressure, pH and temperature of the treatments did not lead to noticeable variations in the reactivity of the samples towards IgG. Kleber, Maier, and Hinrichs (2007) found that the reactivity of β-Lg against polyclonal IgY of chicken egg yolk increased significantly after treatment of WPI at 400 or 600 MPa for 10–30 min at 25 °C and, at 600 MPa, there was a synergistic effect with heat treatment up to 68 °C. However, according to these authors, the HP-induced changes in the antigenicity of β-Lg was not correlated with the degree of denaturation measured as the loss of solubility at pH 4.6.

In general, the IgE-binding response of β-Lg and WPI increased on HP treatment, but differences were only significant ($P<0.05$) for treatments at 200 MPa, pH 6.8 and 30 °C (Table 1). The lack of a significant effect when other combinations of pressure, pH and temperature were used could not be explained by differences in the degree of

Table 1
Effect of HP treatments of β-Lg and WPI at 200 or 400 MPa for 30 min, at different temperatures and pH values, on the ELISA response against IgG and IgE[a]

Absorbance at 492 nm							
Control		pH	T (°C)	200 MPa		400 MPa	
IgG	IgE			IgG	IgE	IgG	IgE
β-Lg							
1.04±0.02	0.62±0.09	2.5	30	2.08±0.12*	1.00±0.06	2.06±0.11*	0.81±0.23
		2.5	50	1.66±0.20*	1.10±0.14	2.11±0.07*	0.89±0.03
		6.8	30	1.83±0.08*	1.24±0.16*	1.43±0.01*	1.03±0.02
		6.8	50	1.67±0.09*	0.71±0.39	1.52±0.03*	0.86±0.12
WPI							
0.93±0.07	1.52±0.04	2.5	30	1.24±0.06*	1.55±0.07	1.28±0.03*	1.74±0.20
		2.5	50	1.45±0.03*	1.39±0.14	1.00±0.09*	1.57±0.29
		6.8	30	1.24±0.03*	1.96±0.09*	1.23±0.04*	1.80±0.16
		6.8	50	1.10±0.03*	1.51±0.08	1.13±0.04*	1.73±0.28

[a]Specific IgE titers: 16.3 kU L^{-1} (β-Lg) for β-Lg samples and 10.8 kU L^{-1} (β-Lg) and 23.5 kU L^{-1} (α-La) for WPI samples. Values given are means±S.D. ($n=3$). An asterisk (*) indicates a significant difference between the sample and the corresponding control ($P<0.05$).

protein aggregation. Whereas it should be noted that allergenic epitopes of β-Lg only partially overlap with antigenic determinants (Bernasconi, Fritsche, & Corthésy, 2006), the significant increases in IgG-binding following HP treatment at 200 or 400 MPa under similar conditions suggest that hidden epitopes may have been exposed by these treatments.

3.2. In vitro digestion of HP-treated WPI by pepsin and Corolase PP, and IgE-binding properties of the digestion products

The RP-HPLC chromatograms of the control and HP-treated WPI digested with pepsin and Corolase PP, under conditions mimicking gastrointestinal digestion, are

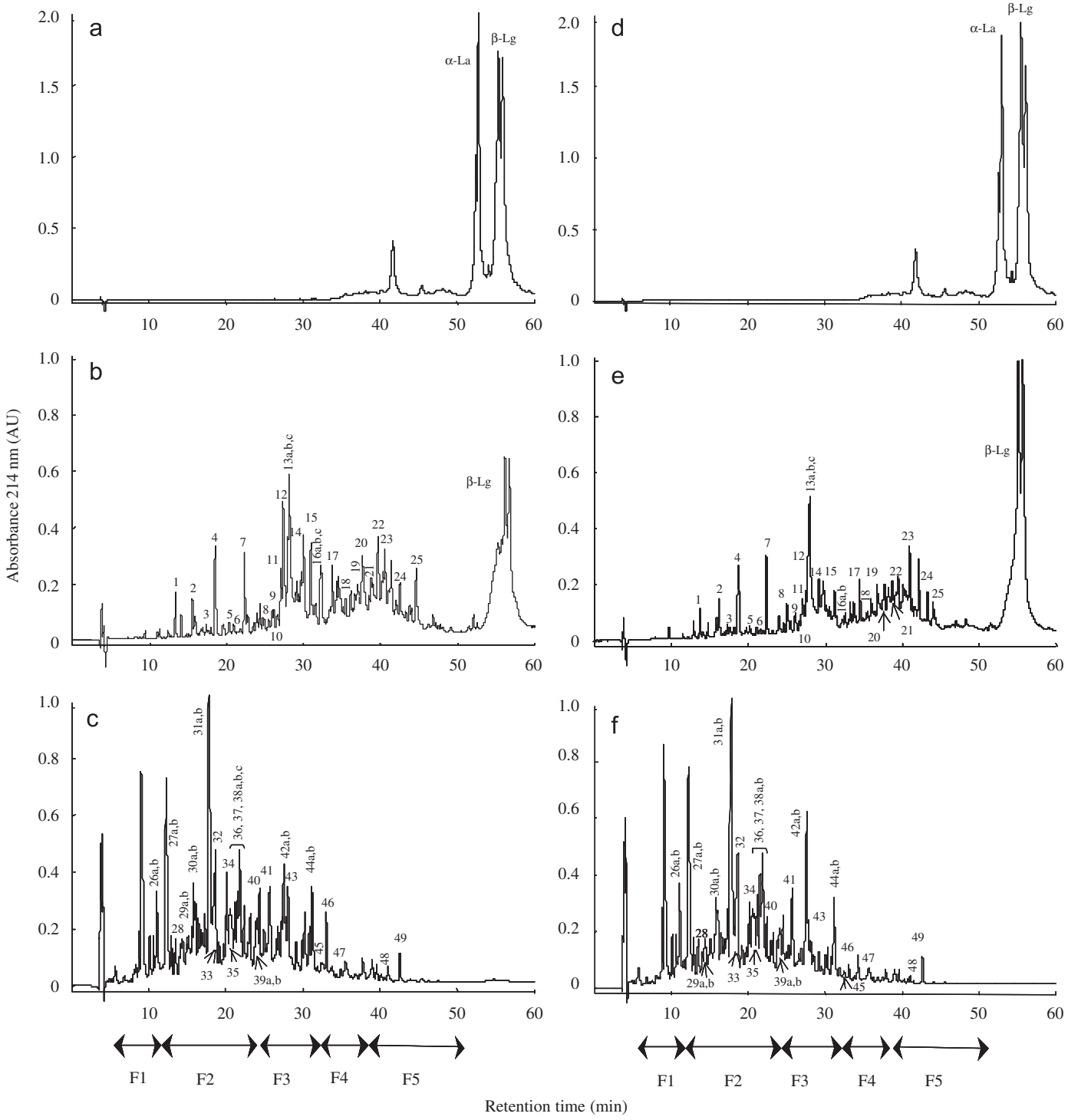

Fig. 2. RP-HPLC patterns of: (a) HP-treated WPI (400 MPa, 25 °C, 30 min), (b) freshly HP-treated WPI hydrolysed with pepsin, (c) freshly HP-treated WPI hydrolysed with pepsin and Corolase PP, (d) control WPI, (e) control WPI hydrolysed with pepsin, (f) control WPI hydrolysed with pepsin and Corolase PP (f). Peak numbers refer to the peptide sequences indicated in Tables 2 and 3.

illustrated in Fig. 2. In general, β-Lg was more resistant to the action of pepsin than α-La and BSA, which were completely hydrolysed after 90 min of pepsin digestion. Compared to the control WPI, HP-treatment at 400 MPa promoted the proteolysis of β-Lg by pepsin (Fig. 2b and e). A two sample *t*-test was used to compare the RP-HPLC peak areas of the pepsin hydrolysates of non-HP-treated WPI samples with WPI samples digested immediately after HP treatment (Table 2). In freshly HP-treated samples the disappearance of the substrate was enhanced (as revealed by a higher quotient PF/total) and this was accompanied by a significantly higher accumulation of the intermediate degradation products (mainly F3 and F4) ($P<0.05$).

It is assumed that the resistance of native β-Lg to digestion by pepsin reflects its stable conformation at pH 2.0 and the poor accessibility of the enzyme to target residues (Guo et al., 1995). HP treatment of β-Lg at 400 MPa and neutral pH can enhance subsequent proteolysis, probably because the misfolded protein produced under those conditions, as revealed by NMR analysis, is more susceptible to enzymatic attack (Belloque et al., 2007). In fact, Vilela, Lands, Chan, Azadi, and Kubow (2006) reported that pressure improved the in vitro digestibility of WPI. However, HP-induced changes in protein structure, which made it more susceptible to proteolysis, were slowly reversed throughout storage under refrigeration. Thus, digestibility of HP-treated WPI stored up to 30 days prior to incubation with pepsin indicated increased resistance of β-Lg to degradation with storage time (Table 2).

Subsequent hydrolysis by Corolase PP degraded the remnant β-Lg and the largest or more hydrophobic peptide products of pepsin (Fig. 2c and f). No significant differences were found in the profile of peptides between control and HP-treated WPI when incubations were performed with both pepsin and Corolase PP (results not shown).

Table 2
Comparative analysis of RP-HPLC peptide fractions among control, freshly HP-treated WPI and HP-treated WPI stored at 5 °C for different periods after digestion with pepsin[a]

WPI pressurization (MPa)	Storage time (days)	Relative area					
		PF/total	F1/PF	F2/PF	F3/PF	F4/PF	F5/PF
0.1	None	0.65*±0.02	0.02±0.01	0.11*±0.01	0.33*±0.01	0.20*±0.01	0.34*±0.03
400	None	0.74b±0.02	0.03a±0.01	0.10a±0.01	0.35a,b±0.00	0.24b±0.01	0.29a±0.01
400	1	0.71a±0.01	0.02a±0.01	0.10a±0.00	0.36b±0.01	0.21a±0.01	0.31b±0.01
400	2	0.71a±0.01	0.02a±0.00	0.10a±0.01	0.35a,b±0.01	0.24b±0.02	0.30a±0.01
400	8	0.70a±0.02	0.02a±0.01	0.10a±0.01	0.35a,b±0.01	0.23a,b±0.01	0.31b±0.01
400	19	0.70a±0.02	0.02a±0.00	0.10a±0.01	0.35a,b±0.01	0.22a,b±0.01	0.31b±0.01
400	30	0.69a±0.01	0.02a±0.01	0.10a±0.01	0.34a±0.01	0.21a±0.01	0.34c±0.00

[a]Data are mean values±S.D. ($n=4$). Selected peptide fractions F1, F2, F3, F4 and F5 are indicated in the RP-HPLC chromatograms of Fig. 2; PF = F1+F2+F3+F4+F5; total = F1+F2+F3+F4+F5+β-Lg. Different letters (a,b,c) within the same column indicate significant differences ($P<0.05$) among the HP-treated samples digested with pepsin. An asterisk (*) denotes a significant difference between control WPI and freshly HP-treated WPI digested with pepsin ($P<0.05$).

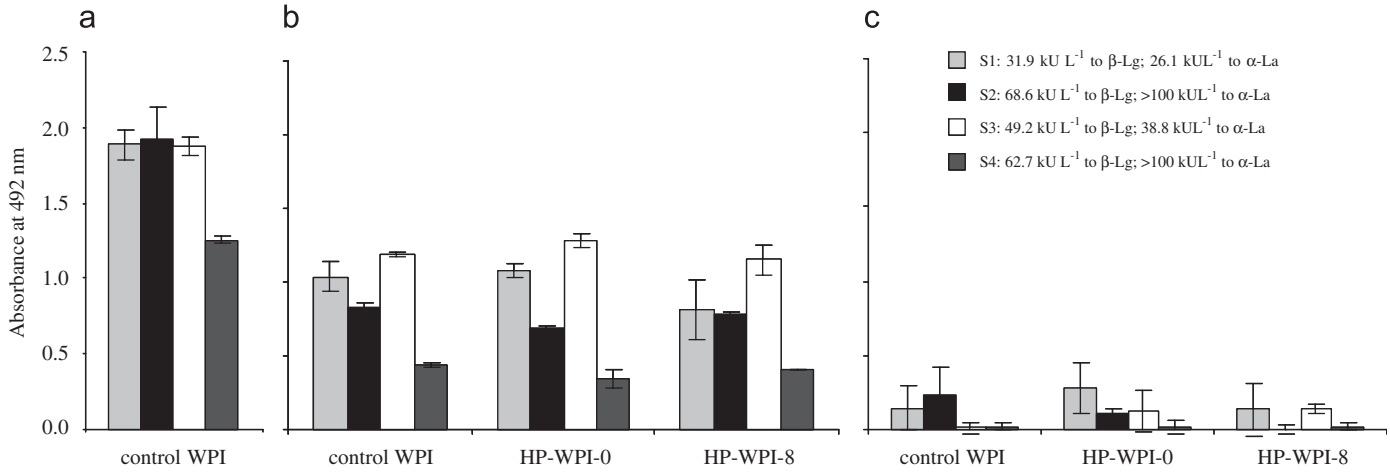

Fig. 3. ELISA response against IgE from individual sera of allergic patients (S1 to S4) of: (a) control WPI, (b) control and HP-treated WPI hydrolysed with pepsin, (c) control and HP-treated WPI hydrolysed with pepsin and Corolase PP. HP-WPI-0, freshly HP-treated WPI. HP-WPI-8, HP-treated WPI stored for 8 days at 5 °C. Results are presented as mean values±S.D. ($n=3$).

Fig. 3 shows the binding of IgE from individual sera of patients allergic to β-Lg to the peptic hydrolysates. No differences in IgE-binding were found after peptic hydrolysis of the HP-treated WPI, either digested immediately after HP-treatment or after 8 days of refrigerated storage, as compared with the control WPI substrate ($P>0.05$). In addition, the higher digestibility of the HP-treated WPI with pepsin did not have an impact on the IgE reactivity of the Corolase PP digestion products, which exhibited a low IgE-binding activity, similar to that of the digestion products of the control WPI ($P>0.05$). Our results agree with earlier reports that indicate that, even if β-Lg is barely hydrolysed by pepsin, the subsequent hydrolysis by pancreatic enzymes is very efficient (Takagi et al., 2003), leading to hydrolysates with low residual IgE and IgG-binding properties (Asselin, Hébert, & Amiot, 1989; Peyron et al., 2006; Schmidt, Meijer, Slangen, & Van Beresteijn, 1995).

Nevertheless, after hydrolysis of WPC in a system mimicking gastrointestinal digestion, Ena, Van Beresteijn, Robben, and Schmidt (1995) reported that intact epitopes of α-La and β-Lg remained. To test the possibility that the released peptides included allergenic epitopes, the identification by RP-HPLC–MS/MS of the main peptide peaks arising from the hydrolysis of WPI by pepsin and Corolase PP was attempted. The results are shown in Tables 3 and 4, respectively, where the peptide sequences identified refer to the peak numbers shown in Fig. 2.

In addition to peptides arising from hydrolysis of α-La and β-Lg, some of the fragments identified derived from κ-casein (region 106–169, corresponding to caseinmacropeptide) and β-casein (region 1–107, corresponding to the soluble polypeptides released by plasmin cleavage, i.e., proteose peptones) (Farrell et al., 2004). Digestion of WPI with pepsin released peptides related to β-Lg fragments that were identified by Sélo et al. (1999) as major (41–60, 102–124, 149–162), intermediate (1–8, 25–40, 92–100) and minor (9–14, 84–91, 125–135, 78–83) IgE-binding epitopes, in view of the frequency and intensity of detection, some of which remained after digestion with Corolase PP. In particular, peptides from the same major allergenic epitope region of β-Lg f(Val_{41}-Lys_{60}) were predominant in the RP-HPLC separation of both control and HP-treated WPI digested by pepsin (peaks 13b and 14 in Figs. 2b and 3) and were similarly affected by the combined digestion with pepsin and Corolase PP (peaks 40, 42b, 43

Table 3
Peptide sequences arising from the digestion of WPI with pepsin, as determined by RP-HPLC–MS/MS

Peak[a]	Calculated mass[b]	m/z[c]	Protein fragment	Related allergenic epitopes (immunoreactivity)
1	1018.46	1019.5(1)	α-La f(41–49)	
2	978.40	979.5(1)	α-La f(32–40)	
3	674.32	675.5(1)	β-CN f(1–5)	
4	1289.60	1290.6(1)	κ-CN f(125–137)	
5	929.45	930.5(1)	β-Lg f(123–130)	β-Lg f(125–135) (low)[d], β-Lg f(127-146) (high)[e]
6	1058.45	1059.5(1)	β-Lg f(123–131)	β-Lg f(125–135) (low)[d], β-Lg f(127-146) (high)[e]
7	801.42	802.5(1)	β-Lg f(83–89)	β-Lg f(84–91) (low)[d]
8	769.44	770.5(1)	β-Lg f(35–41)	β-Lg f(25–40) (intermediate)[d]
9	955.51	956.5(1)	β-Lg f(33–41)	β-Lg f(25–40) (intermediate)[d]
10	1104.58	1105.5(1)	β-Lg f(2–11)	β-Lg f(1–8) (intermediate)[d]
11	1351.63	1352.7(1)	α-La f(41–52)	
12	901.50	902.6(1)	β-Lg f(12–19)	β-Lg f(9–14) (low)[d]
13a	1734.72	1735.9(1)	κ-CNa f(147–161)	
13b	1325.67	1326.7(1)	β-Lg f(43–54)	β-Lg f(41–60) (high)[d]
13c	1068.59	1069.7(1)	β-Lg f(32–41)	β-Lg f(25–40) (intermediate)[d]
14	1488.73	1489.5(1)	β-Lg f(42–54)	β-Lg f(41–60) (high)[d]
15	805.40	806.5(1)	β-Lg f(150–156)	β-Lg f(150–162) (high)[d]
16a	1217.67	1218.7(1)	β-Lg f(1–11)	β-Lg f(1–8) (intermediate)[d]
16b	1157.60	1158.6(1)	β-Lg f(96–104)	β-Lg f(92–100) (intermediate)[d]
17	1624.83	1626.0(1)	β-CN f(1–14)	
18	1129.61	1130.6(1)	β-Lg f(10–19)	β-Lg f(9–14) (low)[d]
19	1369.75	1370.8(1)	β-Lg f(94–104)	β-Lg f(92–100) (intermediate)[d]
20	1467.87	1468.7(1)	β-Lg f(83–95)	β-Lg f(84–91) (intermediate)[d]
21	1909.10	1910.3(1)	β-Lg f(134–149)	β-Lg f(127–146) (high)[e]
22	1304.67	1305.8(1)	β-Lg f(96–105)	β-Lg f(92–100) (intermediate)[d]
23	2093.22	1047.7(2)	β-Lg f(132–149)	β-Lg f(127–146) (high)[e]
24	2222.26	1112.1(2)	β-Lg f(131–149)	β-Lg f(127–146) (high)[e]
25	1450.79	1451.9(1)	β-CN f(81–93)	

[a]Peak numbers refer to Fig. 2. Peaks with the same number but different letters are coeluting peptides.
[b]Monoisotopic mass for the neutral molecule, calculated from the amino acid sequence.
[c]Molecular ion (m/z) selected for MS/MS.
[d]Taken from Sélo et al. (1999).
[e]Taken from Järvinen et al. (2001).

Table 4
Peptide sequences arising from the digestion of WPI with pepsin and Corolase PP, as determined by RP-HPLC–MS/MS

Peak[a]	Calculated mass[b]	m/z[c]	Protein fragment	Related allergenic epitopes (immunoreactivity)
26a	505.24	506.2(1)	κ-CN f(138–142)	
26b	747.31	747.3(1)	β-Lg f(110–116)	β-Lg f(102–124) (high)[d]
27a	889.41	890.5(1)	α-La f(41–48)	
27b	807.28	808.3(1)	α-La f(82–88)	
28	1018.46	1019.5(1)	α-La f(41–49)	
29a	779.31	780.4(1)	α-La f(32–38)	
29b	645.32	646.5(1)	β-CN f(100–105)	
30a	978.40	979.5(1)	α-La f(32–40)	
30b	445.25	446.2(1)	κ-CN f(162–165)	
30c	674.32	675.4(1)	β-CN f(1–5)	
31a	803.32	804.5(1)	β-Lg f(125–131)	β-Lg f(125–135) (low)[d], β-Lg f(127–146) (high)[e]
31b	673.30, 673.40	674.5(1)	β-Lg f(1–6) or β-Lg f(71–76)	
32	528.33	529.3(1)	β-Lg f(76–80)	β-Lg f(75–86) (high)[e]
33	874.36	875.5(1)	β-Lg f(125–132)	β-Lg f(125–135) (low)[d], β-Lg f(127–146) (high)[e]
34	1057.65	1058.6(1)	β-Lg f(68–76)	
35	700.34	701.4(1)	β-Lg f(33–39)	β-Lg f(25–40) (intermediate)[d]
36	1044.51	1045.6(1)	κ-CN f(113–121)	
37	655.37	656.6(1)	κ-CN f(106–111)	
38a	1040.54	1041.5(1)	β-Lg f(43–51)	β-Lg f(41–60) (high)[d]
38b	885.41	886.6(1)	κ-CN f(154–161)	
38c	669.30	670.5(1)	κ-CN f(156–161)	
39a	673.30, 673.40	674.5(1)	β-Lg f(1–6) or β–Lg f(71–76)	
39b	544.29, 544.33	545.3(1)	β-Lg f(9–13) or α-La f(10–13)	
40	1203.60	1204.7(1)	β-Lg f(42–51)	β-Lg f(41–60) (high)[d]
41	1116.48	1117.2(1)	β-Lg f(125–134)	β-Lg f(125–135) (low)[d], β-Lg f(127–146) (high)[e]
42a	1500.78	1501.9(1)	κ-CN f(112–124)	
42b	1454.71	1455.8(1)	β-Lg f(43–55)	β-Lg f(41–60) (high)[d]
43	1302.67	1303.8(1)	β-Lg f(41–51)	β-Lg f(41–60) (high)[d]
44a	1067.43	1068.6(1)	α-La f(80–88)	
44b	1488.73	1489.9(1)	β-Lg f(42–54)	β-Lg f(41–60) (high)[d]
45	1307.71	1308.9(1)	β-CN f(69–80)	
46	1139.58	1140.7(1)	β-CN f(59–68)	
47	1564.80	1565.9(1)	β-CN f(59–72)	
48	1162.57	1163.6(1)	α-La f(17–27)	
49	1319.75	1320.9(1)	β-CN f(81–92)	

[a]Peak numbers refer to Fig. 2. Peaks with the same number but different letters are coeluting peptides.
[b]Monoisotopic mass for the neutral molecule, calculated from the amino acid sequence.
[c]Molecular ion (m/z) selected for MS/MS.
[d]Taken from Sélo et al. (1999).
[e]Taken from Järvinen et al. (2001).

and 44b in Fig. 2c and f). This could partially explain why digestion of HP-treated WPI may not completely eliminate its ability to cause allergic reactions. Järvinen, Chatchatee, Bardina, Beyer, and Sampson (2001) also described the β-Lg sequences 75–86 and 127–146 as the most frequently IgE-bound epitopes. It should be noted that not all of these peptides might be responsible for the IgE binding signal found in the ELISA (Fig. 3), as the small size of some of them may prevent their binding to the polystyrene plate. On the other hand, none of the α-La digestion products found coincided with previously described IgE-binding epitopes (1–18, 13–26, 47–58 and 93–102) (Järvinen et al., 2001).

4. Conclusion

HP treatment of β-Lg and WPI at 200 and 400 MPa increased IgG-binding and did not change IgE-binding. The antibody-binding response of the HP-treated proteins was not related with the degree of HP-induced protein aggregation, which was important in β-Lg and WPI treated at 200 and 400 MPa at pH 6.8, but was not detected at pH 2.5. HP treatment of WPI at 400 MPa promoted the hydrolysis of β-Lg by pepsin, but this increased susceptibility of β-Lg to proteolysis was progressively lost during the refrigerated storage of the HP-treated WPI. The enhanced in vitro digestibility of the HP-treated WPI did not result in a decreased reactivity of the hydrolysates against IgE from human sera of allergic patients as compared to non HP-treated WPI. In the peptic digests of HP-treated and control WPI specific IgE-binding peptides were identified that could contribute to the IgE response to different extents. Some of these immunoreactive fragments remained after hydrolysis with Corolase PP and probably accounted for the low, but detectable, IgE

binding response. Therefore, while HP treatment at up to 400 MPa induced changes in protein structure and increased the in vitro digestibility of β-Lg, it did not reduce its IgE-binding properties and thus may not affect its allergenic potential.

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