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High hydrostatic pressure assisted enzymatic hydrolysis of whey proteins

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ABSTRACT

Whey proteins, due to their high nutritional value, are generally hydrolyzed to reduce the allergenicity and used as ingredients in many special products, such as infant formulae, geriatric products, highly energetic supplements or dietetic foods or in foods produced to prevent nutrition related diseases, like food intolerances and allergies. The aim of this work was to assess the applicability of innovative technologies, such as high hydrostatic pressure (HHP) processes, to assist the enzymatic hydrolysis of target proteins, namely whey protein concentrate (WPC-80), in order to modify their antigenicity. Experiments were carried out to verify the effectiveness of HHP technology to accelerate whey protein hydrolysis reaction with selected enzymes (α-chymotrypsin, bromelain), and to affect the protein allergenic power. To this purpose, different HHP treatments were carried out at several pressure levels (100, 200, 300 and 400 MPa) and the untreated whey protein samples were used as control. A defined enzyme/substrate ratio of 1/10 w/w was used in the experiments, while the treatment time was changed from 0 to 30 min (0, 5, 15, or 30 min).

The experimental data demonstrated that High Hydrostatic Pressure (HHP) induced WPC-80 unfolding at the highest value of the pressure applied (400 MPa) as indicated by the higher exposure of free sulfhydryl groups. When HHP was used in combination with enzymatic hydrolysis, the degree of hydrolysis increased not only with the pressure level applied but also with the processing time. These results suggested that, even if the exposure of hidden epitopes upon protein unfolding increased the antigenicity of whey proteins, further peptide bonds cleavage also took place after hydrolysis. This effect could modify whey proteins antigenic sequences, and thus their antigenic power.

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1. Introduction

Whey, a by-product of the dairy industry, represents an excellent source of functional and nutritional compounds (proteins and peptides, lipids, vitamins, minerals and lactose). Sweet whey (pH > 6.4) derives from cheese manufacturing and casein production by the rennet coagulation of milk, while acidic whey results from processes based on destabilization of milk casein colloids by acidification at pH level below 5.0 (Carvalho & Maubois, 2010; Tamime, 2009). Whey proteins recovered from whey are generally characterized by a high biological value mainly due to the high concentration of essential amino acids (isoleucine, leucine, threonine, tryptophan and valine), which play an important role as metabolic regulators in protein and glucose homeostasis and lipid metabolism (De Wit, 1998).

Whey proteins consist mainly of β-lactoglobulin (55–60%) and α-lactalbumin (15–20%), but also other minor proteins are present, such as bovine serum albumin (5–10%), immunoglobulins, lactoferrin, phospholipoproteins, as well as bioactives and enzymes (Heine, Klein, & Reeds, 1991; Peñas, Prestamo, Luisa Baeza, Martinez-Molero & Gomez, 2006; Peñas, Restani, et al., 2006; Peñas, Snel, Floris, Prestamo & Gomez, 2006). β-lactoglobulin (β-lg), which represents approximately 50% of total whey proteins, is a globular protein extremely stable in acidic environment and produced in the mammary gland and secreted in milk. The primary structure of β-lactoglobulin contains 162 amino acids, one free thiol group and two disulfide bridges and has a molecular weight of 18.3 kDa. α-Lactalbumin is a globular protein found in the milk of all mammals. Its primary structure contains 123 amino acids, has a molecular weight of 14.2 kDa and plays an important role in lactose biosynthesis. The primary structure of Bovine Serum Albumin (BSA), a residual protein found in both blood serum and in milk of all mammals, contains 582 amino acids and has a molecular weight of 66 kDa. BSA is the only whey protein that is not produced by the mammary gland but enters in the milk by passive diffusion from blood streams (Heine et al., 1991; Peñas, Prestamo, et al., 2006; Peñas, Restani, et al., 2006; Peñas, Snel, et al., 2006).

Whey protein concentrate (WPC) and whey protein isolates (WPI) are used in the manufacturing of yogurt, processed cheese, infant

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formulae, products for athletes and weight management products and in various bakery applications, since it is combining the effects of protein, lactose and minerals, as well as ingredients in the cosmetic and pharmaceutical sectors.

However, notwithstanding the extensive utilization of whey protein in many industrial applications, there are many concerns in their uncontrolled diffusion mainly related to their allergenicity.

β-lactoglobulin, α-lactalbumin and caseins are the main allergens in cow's milk and whey proteins, while other proteins, namely BSA and even lactoferrin (present in traces) are also potential allergens (Bu, Luo, Chen, Liu, & Zhu, 2013; Castro, Peryronel, & Cantera, 1996; Fritsche, 2003; Sharma, Kumar, Betzel, & Singh, 2001). Some processing technologies (glycation, enzymatic hydrolysis and lactic acid fermentation) have been investigated with the aim of reducing the allergenicity of milk proteins by controlling and optimizing the processing conditions (Bu et al., 2013).

Among these processes, protein hydrolysis represents a well-known method to reduce the allergenicity, to improve functional properties (foaming, solubility, etc.) and to preserve the nutritional value of whey proteins (Castro et al., 1996). Different processes were developed to carry out protein hydrolysis, including enzymatic hydrolysis, which is widely used to produce high quality protein hydrolysates at the industrial scale (Clemente, 2000). Proteolytic enzymes, extracted from animal sources (pancreatin, trypsin, pepsin) and plant sources (bromelain, papain), allow to carry out the hydrolysis reactions in milder conditions and to control the hydrolysis degree and the fragmentations in peptides to higher extent (Clemente, 2000; Clemente et al., 1999). Protein hydrolysates have enhanced nutritional, functional and biological properties with respect to the original proteins due to their smaller size and structural rearrangements, which cause the exposure of some hydrophobic regions, originally buried within the protein molecule, to the contact with the aqueous phase.

The extent of the enzymatic hydrolysis mainly depends on the accessibility of the peptide bonds, which stabilize the protein structure and control the processing time and the composition of the mixture of peptides produced. Protein unfolding, which increases the number of the binding sites exposed to the enzymatic attack, may be used as a strategy to fasten the hydrolysis reactions. To this purpose, in industrial practice enzymatic treatments are carried out at temperatures able to induce the modification of the protein structure, in particular the unfolding.

Among novel methods to induce protein unfolding, high hydrostatic pressure (HHP) has been specially focused, since this technology brings about structural changes in milk proteins able to modify epitopes, such as denaturation and formation of aggregates (Iametti et al., 1996). Pressure denaturation is a complex phenomenon that depends on protein structure, pressure range, temperature, pH, and solvent composition, and where electrostatic and hydrophobic interactions in protein molecules can be modified (Palou, Lopez-Mato, Barbosa-Canovas, & Swanson, 1999). High pressure causes deprotonation of charged groups and disruption of salt bridges and hydrophobic interactions, thereby resulting in conformational and structural changes of proteins (Martin, Barbosa-Canovas, & Swanson, 2002), as well as aggregation and gelation. Most of the whey proteins have a globular conformation and are susceptible to denaturation and aggregation induced by heat as well as by HHP. Among the whey proteins, β-lactoglobulin is the most sensitive to high pressure. Exposure to pressure levels higher than 300 MPa causes irreversible changes to the tertiary and quaternary structure of β-lactoglobulin, resulting in the formation of homopolymeric aggregates, in contrast to heat induced unfolding where heteropolymers may be detected.

α-lactalbumin is resistant to denaturation at pressures up to 500 MPa while it undergoes thermal unfolding at a lower temperature with respect to β-lactoglobulin. Differences in pressure stability of these whey proteins is due to the more rigid structure of the former, caused partially by the number of intramolecular disulfide bonds present in both proteins and the lack of free sulfhydryl groups in α-lactalbumin (Hinrichs, Rademacher, & Kessler, 1996; Hinrichs and Rademacher 2004; Huppertz, Fox, & Kelly, 2004; Huppertz, Smiddy, Upadhyay, & Kelly, 2006). According to these observations, HHP treatments induce irreversible structural and functional changes in α-lactalbumin. Reversible unfolding begins at 200 MPa and the loss of native conformation becomes irreversible beyond 400 MPa. The extent of HP-induced denaturation of α-lactalbumin and β- lactoglobulin increases with treatment time and temperature (Huppertz et al., 2004, 2006). BSA is relatively stable to high pressures (800 MPa) despite the presence of a free thiol group (De Maria, Maresca, & Ferrari, 2015, 2016). BSA undergoes substantial secondary structure changes but, differently from β-lactoglobulin, the changes are reversible, apparently due to the protection of the hydrophobic core of the protein by the large number of disulfide bonds (Huppertz et al., 2004, 2006).

When applied to whey proteins, HHP treatments were found to enhance their antigenicity, which was associated to the exposure of epitopes buried in the native protein becoming accessible for the antibodies (Kleber, Maier, & Hinrichs, 2007). Although HHP-induced unfolding may have a negative effect on the allergenicity of the proteins, the conformational changes may improve the efficiency of the enzymatic digestion, by allowing the access of the enzymes to previously hidden sites (Chicón, Belloque, Recio, & López-Fandiño, 2006). Thus, HHP technology can be proposed, alternatively to thermal treatments, to assist the reduction of food allergenicity.

The aim of the present work was to assess the applicability of HHP treatments to assist the enzymatic hydrolysis of whey protein concentrate solutions (WPC-80), in order to decrease their antigenicity. Therefore, experiments were carried out to verify the effectiveness of HHP technology to accelerate the hydrolysis reaction of whey protein concentrates with α-chymotrypsin and bromelain, and to affect the proteins allergenic power.

2. Materials and methods

2.1. Preparation of the samples

Whey protein concentrate solutions (WPC-80, Lacprodan 80, Arla foods®) were prepared by adding the proteins in Sodium phosphate buffer (100 mM, pH = 7.5) at a concentration of 1% (w/v) under gentle mixing until complete solubilization at 25 °C. The pH of the protein solutions was measured with a pH-meter (S400 SevenExcellence, Mettler Toledo International Inc.) and adjusted to the final value of 7.5. The protein concentration in the prepared solution, determined by Kjeldahl method, was 8 mg/mL.

Two different enzymes, α-chymotrypsin and bromelain (Sigma-Aldrich, Italy), were used in the experimental design. The enzymatic solutions were prepared by dissolving the enzymes (50 mg/mL) at a temperature of 25 °C in Sodium Phosphate Buffer (100 mM and pH = 7.5 for α-chymotrypsin; 100 mM and pH = 6.5 for bromelain) and stored in refrigerated conditions at 4 °C before utilization.

2.2. Experimental apparatus

The HHP system U22 (Institute of High Pressure Physics, Polish Academy of Science, Unipress Equipment Division, Poland), which is a laboratory scale unit provided with a vessel with a maximum processing volume of 50 mL, was used during the experiments. The system can be operated in a wide pressure range (0–1400 MPa) under controlled thermal conditions (25–120 °C). Operating pressure, ramp rate and processing time are set up on a control panel, which, in turn, allows the opening and closure of the HHP vessel. A portable Temperature Power and Control Unit (TCU), connected to the main unit with electrical cables and thermocouples (K-type), permits the set up and control of the operating temperature in the HHP vessel. The vessel can be heated with electrical heaters and cooled with compressed air. The pressurizing

medium is Plexol (Bis (2-ethylhexyl) sebacate from Sigma-Aldrich, Italy) and the estimated temperature increase due to pressure build-up is 2–3 °C/100 MPa.

2.3. Experimental plan

2.3.1. Experimental protocols

Preliminary experiments were carried out to detect the effect of HHP treatments on whey protein unfolding. In this experimental campaign samples of 2 mL were packed in thermo-sealed bags (OPP30-A19-LDPE70) and processed in HHP treatments (100, 200, 300 and 400 MPa for 5, 15 and 30 min) at controlled temperature (25 °C). Untreated samples (0.1 MPa) were used as control. Free sulfhydryl groups of unprocessed and processed samples were determined immediately after the treatments and after a fixed time of incubation at ambient temperature (60 min).

Tests of enzymatic hydrolysis at ambient pressure were also performed on the WPC-80 samples. In all experiments, carried out in duplicate, the enzymatic solution (α-chymotrypsin or bromelain) was added (1:10 (w/w)) to 2 mL of protein solution. Samples were hydrolyzed at the optimal temperature for the enzyme (37 °C for α-chymotrypsin; 45 °C for bromelain) at atmospheric pressure for 5, 15, or 30 min. After treatments, enzyme's inactivation was carried out by heating the samples up to 100 °C, holding them at this temperature for 5 min, and immediately cooling them in an ice bath. Untreated samples were used as control. Treated samples were stored at 4 °C before undergoing physicochemical characterization, evaluating hydrolysis degree and SDS-PAGE pattern.

In HHP assisted enzymatic hydrolysis experiments, the enzymatic solution (α-chymotrypsin or bromelain) was added (1:10 (w/w)) to 2 mL of protein solution. Samples were packed in thermosealed bags (OPP30-A19-LDPE70; 2,5 cm × 5 cm) and processed in HHP treatments at 100, 200, 300 and 400 MPa for 5, 15 or 30 min at the optimal temperature for the enzyme (37 °C for α-chymotrypsin; 45 °C for bromelain). After treatments, the enzyme's inactivation was obtained by heating the samples up to 100 °C, holding them at this temperature for 5 min, and immediately cooling them in an ice bath. Untreated samples were used as control for zero enzyme activity, while samples hydrolyzed at atmospheric pressure were used as reference samples to detect the variation in the hydrolysis kinetics. Treated samples were stored at 4 °C before undergoing physicochemical characterization, evaluating hydrolysis degree and SDS-PAGE pattern.

2.3.2. Determination of free SH groups

Free sulfhydryl (SH) groups of WPC-80 samples were evaluated according to the protocols reported by Beveridge et al. (1974) and by Hardham (1981). Ellman's reagent was prepared by dissolving 4 mg of 5,5-dithio-bis 2-nitrobenzoic acid (DTNB) (Sigma-Aldrich, Italy) in 1 mL of Tris–Glycine buffer (0.1 M Tris-(hydroxymethyl)-aminomethane (Tris), 0.1 M glycine, and 4 mM ethylenediamine-tetraacetic acid disodium salt, pH = 8.0; Sigma-Aldrich, Italy)).

The sample, dissolved in 5 mL of 8 M urea in Tris–Glycine buffer, was added to the Ellman's reagent (40 μL) and incubated for 30 min at room temperature (25 °C). The absorbance was measured at 412 nm with a UV/VIS spectrophotometer (V-650, Jasco Europe Srl, Italy), using Tris–Glycine buffer as blank. Free sulfhydryl groups were evaluated according to Eq. (1):

$$SH(\mu M/g) = 73.53 * A_{412} * D/C \quad (1)$$

being A_{412} the absorbance at 412 nm, C the sample concentration in mg solid/mL and D the dilution factor. Results were expressed as the mean of three measurements.

2.3.3. Determination of hydrolysis degree

The evaluation of the degree of hydrolysis (DH) of WPC-80 samples was performed by the OPA method, as described by Spellman, McEvoy, O'Cuinn, and Fitzgerald (2003). The method is based on the reaction of primary amino nitrogen with ortho-phthalaldehyde (OPA), which form a compound detectable at 340 nm in a UV–Vis spectrophotometer (V-650, Jasco Europe, Italy). OPA reagent was prepared by dissolving Natetraborate decahydrate, Na-dodecyl-sulfate (SDS), o-phthaldialdehyde 97% (OPA) and dithiothreitol 99% (DTT) in deionized water solution. A Serin solution (0.1 g/L) in deionized water was used as standard. In each measurement, 3 mL of OPA reagent were added to 400 μL of deionized water (blank), serine solution (standard) or sample. All measurements, performed in triplicate, were carried out at 25 °C using deionized water as a control after 2 min of reaction. The measurements of the absorbance were worked out in order to determine the hydrolysis degree (HD%).

2.3.4. SDS-PAGE electrophoresis

Whey protein samples were analyzed using a TV100 Twin-Plate Mini Cell (Hercules, CA). Polyacrylamide gel electrophoresis (6–15%) in presence of sodium dodecyl sulfate (SDS-PAGE). Thirty μL of control and treated samples were diluted with 30 μL of 50 mM Tris–HCl (pH 6.8), containing 50% glycerol, 0.02% bromophenol blue, 2% SDS, and 5% Β-mercaptoethanol. A solution of β-lactoglobulin (18.0 kDa), and a mix of α-lactoglobulin (18.0 kDa) and BSA (66 kDa) were prepared as samples and used as protein markers to calibrate the gels. Prior to the analysis, solutions were heated for 5 min in a water bath set at 100 °C, and then immediately cooled in icy water. Electrophoresis was run at ambient temperature for 15 min at 100 V and 60 min at 150 V. The gels were first fixed with a solution containing 50% methanol and 10% acetic acid for 30 min, and then stained with a Coomassie blue solution containing 20% isopropanol, 10% acetic acid, and 0.1% Coomassie Blue R-250 for 60 min. The destaining step was performed in three steps with a solution containing 30% methanol and 10% acetic acid. Gels were digitalized for further analysis.

2.4. Statistical analysis

The experiments were carried out in triplicate (3 independent runs) and the standard deviations of the results were calculated and reported.

The analysis of variance test for significant effects of treatments and assay samples were determined using the SPSS Statistics software. Experimental data were statistically analyzed performing an analysis of variance (two-way ANOVA). Main effect differences were considered significant at the $p < 0.05$ level. The Pearson product-moment correlation coefficient was used to assess the strength of the linear relationship between two variables.

3. Results and discussion

3.1. Protein unfolding upon high pressure processing

The analysis of thiol groups and disulfides provides important information on the conformational structure of the proteins, given the relevant role that these groups play in their stability.

The concentration of free SH groups in the WPC-80 samples (8 mg/mL) treated at pressure levels from 100 to 400 MPa for 5, 15 and 30 min was estimated.

Fig. 1 shows the content of free sulfhydryl groups measured for the WPC-80 samples according to the protocol based on Ellman's reagents.

Results clearly evidence that the SH-groups exposure increases with both the pressure levels, if higher than 200 MPa ($p < 0.05$), and the treatment time ($p < 0.05$). The negative values of free SH - groups variation, ΔSH, observed for the samples processed at pressure level below 300 MPa may be attributed to the reversible unfolding and refolding of the protein occurring at after pressure release. According to Huppertz

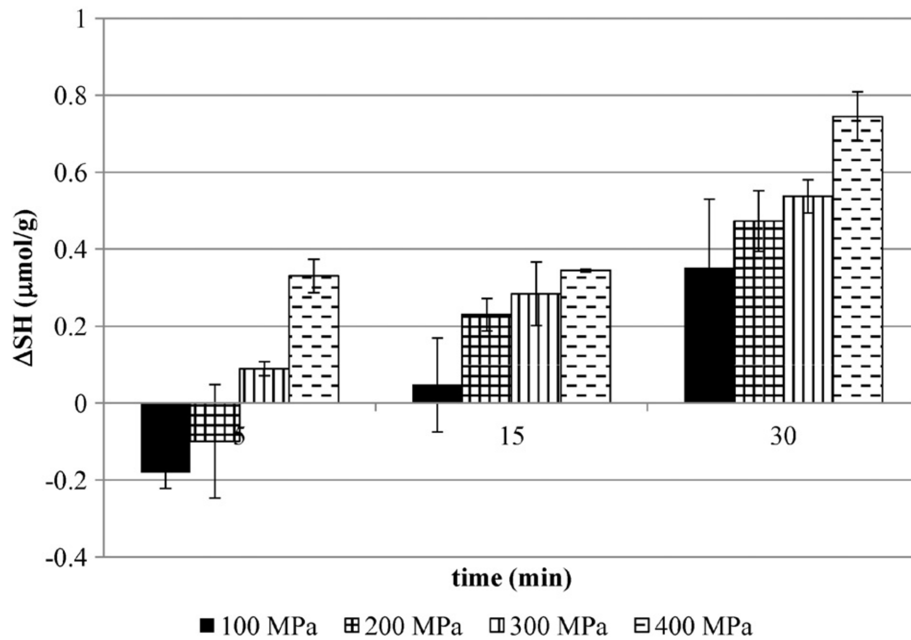

Fig. 1. Free SH-groups in WPC-80 samples processed in HHP treatments at different pressure levels (100–400 MPa) and processing times (5, 15 and 30 min). (Free SH-groups are expressed as difference, ΔSH, with respect to native proteins).

et al. (2004), the increase in the number of reactive SH-groups at pressure level above 200 MPa confirms that, as a consequence of the exposure to the HHP, the unfolding of proteins occurred. After the HHP treatments, when pressure is released, unfolded molecules that have not interacted with other proteins may refold to a state close to their native state. This process has been shown to be highly dependent on the level of pressure applied. Belloque et al. (2007) showed a faster refolding rate of the proteins treated at 200 MPa with respect to those treated at 400 MPa, suggesting that at pressures higher than a certain threshold level, further structural changes may take place.

Experimental data demonstrate that the effect of the pressure on the denaturation of the individual whey protein fractions depends not only on the pressure level, but also on the duration of the treatment, in agreement with previous studies (Hinrichs & Rademacher, 2004).

It is well-known that covalent bonds are unaffected by high pressures and therefore, when applied as the sole treatment, only the quaternary and tertiary structures of proteins will be particularly affected (Goyal, Sharma, Upadhyay, Sihag, & Kaushik, 2013), while the primary protein structure will remain intact. It has been shown that oligomeric proteins are dissociated at around 200 MPa, while monomeric proteins require pressures greater than 300 MPa to undergo the same effect (Cheftel, 1995).

To evaluate the effect of HHP on the refolding process, WPC-80 solutions were exposed to different pressure levels (0.1, 100, 200, 300, 400 MPa) for a holding time of 15 min. After HHP treatments, samples were stored at ambient temperature and the content of free SH groups was measured during the storage time (every 10 min for 60 min) to determine the refolding curves under the described conditions. Fig. 2

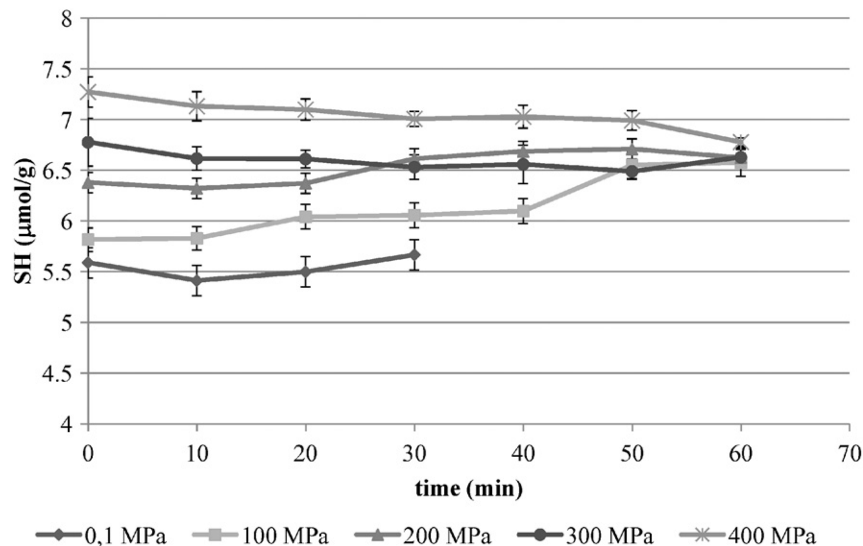

Fig. 2. Variation of Free SH-groups (refolding) in WPC-80 samples processed in HHP treatments at different pressure levels (100–400 MPa) for 15 min as a function of residence time (0–60 min) at ambient temperature (25 °C).

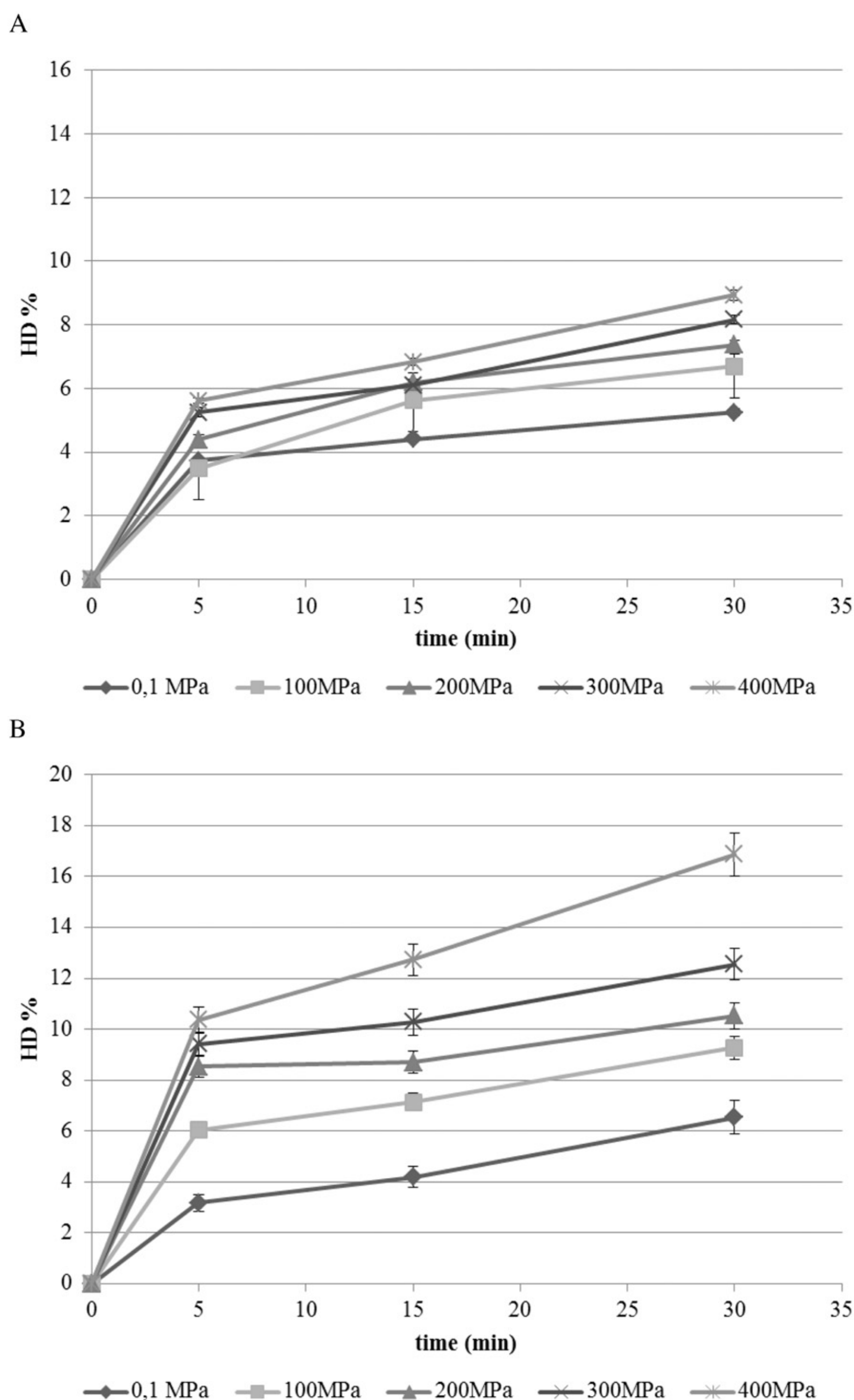

Fig. 3. Hydrolysis degree (HD%) of WPC-80 hydrolyzed at ambient pressure (untreated samples) and in HHP treatments at different pressure levels (100, 200, 300, 400 MPa) and processing time (0, 5, 15, 30 min) with two different enzymes. A: α-chymotrypsin, processing temperature: 37 °C B: bromelain, processing temperature: 45 °C).

shows the concentration of free SH groups in WPC-80 samples processed at the different pressures as a function of the storage time.

Results demonstrate that the highest pressure levels (300 and 400 MPa) also provoked the highest exposure of thiol groups. It can be speculated that the number of these groups detected after the application of pressure would increase in parallel with the number of hidden linear epitopes exposed during the unfolding process. This fact can be linked to the increase in protein antigenicity brought about by high pressure (Kleber et al., 2007). From the technological point of view, particular advantage of this phenomenon can be taken to develop a combined strategy to modify protein antigenicity, namely applying an enzymatic hydrolysis assisted by HHP. This strategy has been proved successful by different authors (Bonomi et al., 2003; Dufour, Herve, Cedex, & Haertle, 1995; Knudsen, Otte, Olsen, & Skibsted, 2002; Lopez-Exposito, Chicon, Belloque, Lopez-Fandifio, & Berin, 2012; Peñas, Prestamo, et al., 2006; Peñas, Restani, et al., 2006; Peñas, Snel, et al., 2006).

Interestingly, Fig. 2 shows that after pressure release, the variation of the number of thiol groups with the exposure time depended on the processing pressure. The values of the free SH groups measured for the samples hydrolyzed at ambient pressure (0.1 MPa) were not significantly different ($p = 0.08$), this demonstrating the stability of the

samples. The samples processed at 100 and 200 MPa showed a different behavior, being the values of free SH-group significantly increased with the time ($p < 0.05$) up to 50 min and 30 min of observation, respectively. It is worth noting that longer exposure time did not significantly modify ($p > 0.05$) the number of free SH-groups. According to these observations, it can be hypothesized that, after pressure release, the protein conformational structure changed in order to reach a stable configuration. In the samples treated at 300 and 400 MPa the number of thiol groups decreased with time, this suggesting the occurrence of proteins refolding. According to Somkuti and Smeller (2013), there would be a protein core able to resist high pressure, although this structure might be modified under pressures higher than 400 MPa. Kleber et al. (2007) found a similar pressure threshold, this suggesting that the protein unfolding process can be reversible for pressure treatments up to 400 MPa. Therefore, pressure levels higher than this value would prevent the occurrence of protein refolding.

3.2. HHP assisted enzymatic hydrolysis

Fig. 3A and B show the extent of the hydrolysis upon HHP assisted enzymatic hydrolysis reaction with α-chymotrypsin and bromelain, respectively, in comparison with the process carried out at atmospheric pressure (0.1 MPa). In these Figures, the HD% of the WPC-80 samples hydrolyzed at ambient pressure and at different pressure levels (100–400 MPa) for several processing times are reported.

The results of hydrolysis experiments carried out at ambient pressure demonstrated that the hydrolysis degree of WPC-80 samples did

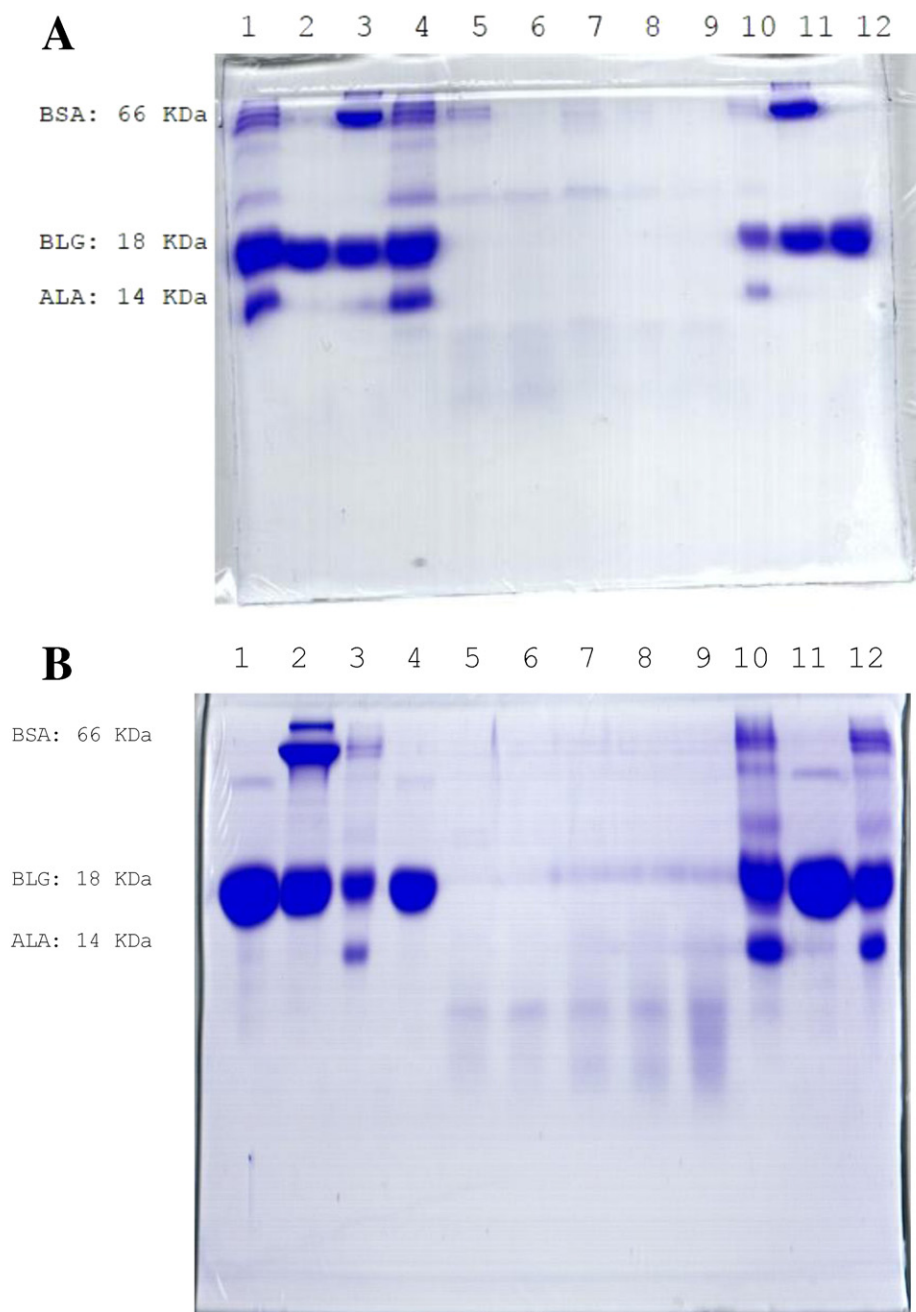

Fig. 4. SDS-PAGE Electrophoresis of WPC-80 samples (protein concentration = 1%) treated in HHP assisted hydrolysis with two different enzymes. 1: WPC; 2: 3-lactoclubulin (18 KDa); 3: 13-lactoclubulin (18 KDa) + BSA (66 KDa); 4: CT WPC + Ei; 5: 0,1 MPa, 30 min; 6: 100 MPa, 30 min.; 7: 200 MPa, 30 min.; 8: 300 MPa, 30 min; 9: 400 MPa, 30 min; 10: WPC 11: 13-lactoglubulin + BSA A: α-chymotrypsin, processing temperature: 37 °C B: bromelain, processing temperature: 45 °C.

not change significantly with increasing the reaction time ($p = 0.06$), indicating that hydrolysis proceeded very slowly in the first 30 min.

On the contrary, when high pressure assisted hydrolysis was carried out in the pressure range between 100 and 400 MPa, the reaction rate increased with pressure and very relevant differences between ambient pressure and high pressure treated hydrolysates were observed already after 5 min ($p < 0.05$). The values of HD% measured in the samples treated at 300 MPa for 5 min are comparable to those measured for samples hydrolyzed at ambient pressure for 30 min ($p > 0.05$). In the case of the most extensive treatment (400 MPa for 30 min), two times the level of HD was achieved by comparison to the control treatment, reaching a value of around 10% for the samples hydrolyzed with α-chymotrypsin. By comparing the results reported in Fig. 3A and B, it can be concluded that in HHP assisted hydrolysis bromelain was more effective than α-chymotrypsin and allowed to obtain samples with higher hydrolysis degree. The maximum values of HD% measured in the samples treated at 400 MPa for 30 min were about 17%.

The results of the SDS-Page analysis also confirm the higher efficiency of the HHP assisted hydrolysis and the high extent of hydrolysis achieved with both proteolytic enzymes. In Fig. 4 the electrophoretic pattern of WPC-80 samples treated at different pressure levels (0.1–400 MPa) for the longest holding time (30 min) and hydrolyzed with α-chymotrypsin (Fig. 4A) and bromelain (Fig. 4B) are shown. The electrophoretic patterns of WPC-80 samples clearly demonstrate that at pressure levels higher than 300 MPa (lines 8–300 MPa and line 9–400 MPa), all protein bands vanished, proving the high extent of hydrolysis obtained, whatever was the proteolytic enzyme used in the hydrolysis reactions.

In the literature, several studies showed that hydrolysis assisted by HHP occurs more rapidly than hydrolysis per se. It has been demonstrated that the combined use of proteolytic enzymes and HHP is able to enhance the effects of hydrolysis (Lopez-Exposito et al., 2012; Peñas, Prestamo, et al., 2006; Peñas, Snel, et al., 2006; Somkuti & Smeller, 2013). To support this conclusion, Dufour et al. (1995) reported that the degree of hydrolysis induced by HHP treatment was the result of pressure-induced enzyme's activation and partial unfolding of proteins by compression.

The analysis of the correlation between the HD% values and the content of free SH-groups of WPC-80 samples, summarized in Table 1, confirms these observations. The parameters analyzed for both samples hydrolyzed with α-chymotrypsin (Table 1A) and bromelain (Table 1B) have a strong positive correlation. Moreover, when processing time at high pressure is inreased, the values of Pearson's correlations coefficient increase, thus demonstrating a stronger positive correlation between the parameters analyzed. For prolonged processing time (30 min), the Pearson's correlations coefficient is close to one, and, accordingly, a linear relationship between the content of free SH-groups and the HD% exists. The results of this statistical analysis demonstrate that the efficiency of the HHP assisted hydrolysis can be correlated to the unfolding of the proteins occurring under pressure and that the structural modification induced by HHP may contribute to accelerate the reaction kinetics during high pressure assisted hydrolysis.

HHP assisted hydrolysis can be proposed as a novel strategy especially focused on the production of hydrolysates. The experimental results confirm the observations reported in the literature. Belloque et al. (2007) observed that although in the range of 200 to 300 MPa a faster proteolysis can be achieved, and that this process was significantly improved at a pressure level of 400 MPa. Peñas, Snel, et al. (2006) reported an important reduction in the antigenicity of hydrolysates especially for two of the enzymes tested, namely Corolase PN-L and Neutrase, when hydrolysis is combined with HHP, applied either prior or during the hydrolytic process. In another study with trypsin, α-chymotrypsin, and pepsin, Peñas, Prestamo, et al. (2006) reported that α-chymotrypsin had the best performance at 100 and 200 MPa, followed by pepsin at 300 MPa. Interestingly, the different whey proteins showed different levels of hydrolysis, and even some of them, such as α-lactalbumin, could not be digested by α-chymotrypsin, regardless of the pressure applied. In addition to the protease performance, this behavior could be also linked to the different sensitivity of whey proteins to pressure. In this regards, β-lactoglobulin was reported to be the most pressure-sensitive, while α-lactalbumin the most resistant (Goyal et al., 2013).

Concerning the kinetics of hydrolysis, the results obtained in the present study demonstrate that high pressure can accelerate the reaction kinetics up to two or three times in the conditions investigated. The improvement in the effectiveness of the enzymes under pressure to reduce the immunoreactivity would be associated to their ability to hydrolyze hydrophobic regions transiently exposed during pressurization, which are not accessible in the native protein, as observed also by Bonomi et al. (2003).

In spite of the breakthrough attained in the different studies, there is still more research needed to improve and optimize other relevant aspects related to the quality of whey hydrolysates. In this regards, Chicón, Belloque, Alonso, Martín-Álvarez, and López-Fandiño (2008) warn that the hydrolysates obtained under pressure would retain higher levels of residual IgE-binding properties in comparison to those obtained under normal pressure. It was found that HHP-assisted hydrolysis might render, in some cases, longer and more hydrophobic peptides by comparison to the process carried out at atmospheric pressure. Therefore, it was speculated that HHP probably accelerates the first step of proteolysis through dimer dissociation, although some portions of the protein could remain more resistant to the attack of proteases (Chicón et al., 2006).

Table 1
Pearson's correlation coefficients between hydrolysis degree (HD%) and free SH-groups content of WPC-80 samples processed with HHP treatments at different pressure levels (100–400 MPa) and processing times (5 min, 15 min, 30 min) with α-chymotrypsin and bromelain.

		HD%		
Enzyme		5 min	15 min	30 min
α-Chymotrypsin		0.877	0.908	0.988
Bromelain	SH	0.584	0.943	0.951

4. Conclusion

The experimental data obtained in the present study demonstrated that a great exposure of free SH groups occurred after the application of pressure treatments of high intensity with further tertiary structure disruption at the longest holding times. Under these conditions, HPP treatments would not have an important impact on protein antigenicity, which mainly depends on the modifications of the primary structure of proteins. Instead, when the treatment was applied in combination with enzymatic hydrolysis a significant increase of HD% was observed, which could be linked to whey proteins antigenicity reduction.

HHP process increased the values of the hydrolysis degree in treatment conditions, namely pressure level and processing time, which were maximizing the protein unfolding and, consequently, the protein exposure to the attack of proteolytic enzymes. This represented the key-factor controlling the efficiency of the HHP assisted hydrolysis treatments.

In conclusion, although an increased antigenicity may be apparent, due to the exposure of hidden linear epitopes upon HHP application, protein denaturation could induce further peptide bonds cleavage when a combined treatment (HHP assisted hydrolysis) is applied, having a potential effect on whey proteins antigens, and thus on their antigenic power.

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