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Enzymatic proteolysis, under high pressure of soybean whey: Analysis of peptides and the allergen Gly m 1 in the hydrolysates

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Abstract

Soybean (*Glycine max*) whey was hydrolyzed with Alcalase, Neutrase, Corolase 7089 and Corolase PNL during high pressure (HP) treatment at 100, 200 and 300 MPa and at atmospheric pressure for 15 min. The protein content and the degree of hydrolysis were determined. Furthermore, the allergen Gly m 1 in the treated soybean whey and the hydrolysates was detected. The results showed that HP treatments increased the hydrolysis by the four proteases used. Pressure at 200 and 300 MPa proved to be better pressures to enhance the proteolysis. The immunochemical response of soybean whey to anti-Gly m 1 monoclonal antibodies decreased after the HP treatments and this decrease was greater after the combined treatment of high pressure and enzymatic hydrolysis. Soybean whey proteins hydrolysed at high pressure could be used as sources of peptides with low antigenicity when incorporated as food ingredients.

Keywords: Allergy; *Glycine max*; Soy-whey; Gly m 1; High pressure; Hydrolysis

1. Introduction

Soybean whey, a by-product from the manufacturing of tofu, has been considered, for a long time to be a waste product by the food industry and its disposal actually constitutes an environmental and the industrial problem. However, soy-whey is a good source of proteins (Peñas, Préstamo, & Gomez, 2004), polyunsaturated fat and bioactive substances, such as isoflavones and oligosaccharides, and thus should not be considered a waste. Nowadays, many researchers are developing new ideas of minimizing vegetable residues by making them useful.

The intake of soy products has increased in western countries due to their nutritional and health benefits (Anderson, Anthony, Cline, Washburn, & Garner, 1999; Steinke, 1992). Despite all these advantages of soy products, they can unfortunately cause allergies in some people. Allergies are abnormal inflammatory responses to the immune system and the intolerance of soy protein may cause different clinical syndromes, such as rhinitis, urticaria, asthma, atopic dermatitis, anaphylatic shock or even death. Soy has substances that can produce allergic reactions but, in this aspect it is far behind other foods such as peanuts, tree nuts, milk, eggs, shellfish, fish and wheat.

The industrial process of high hydrostatic pressure (HP) is one of the most promising techniques for food preservations. It is called "cold treatment" and is an alternative to heat treatment. Most often, this technique produces a decrease in the microbial populations, and normally maintains the nutritional and sensorial properties of the food as the raw product (Préstamo & Arroyo, 2000; Préstamo, Lesmes, Otero, & Arroyo, 2000). Recently, HP treatment has been used in the enzymatic hydrolysis of proteins by enhancing the proteolysis, as has been reported by several

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authors (Dufour, Hervé, & Haertle, 1995; Maynard, Weingand, Hau, & lost, 1998; Stapelfeldt, Petersen, Kristiansen, Qvist, & Skibsted, 1996; Van Willige & Fitzgerald, 1995).

What we envisaged in the present study was the detection of the residual allergenicity of soy-whey and their hydrolysates after high-pressure treatment.

2. Materials and methods

2.1. Source of material

The soybean-whey was provided from Tofu-Ya company and it was normally obtained from overnight soaked soybean, drained and then homogenized (soyabean:water, 1:3 w/v). The white solution obtained, called soy-milk, was coagulated with nigari (calcium sulphate and magnesium-chlorate). From the results of this process, two fractions were separated, the curd, which is the tofu, and the supernatant, that is, the ''whey''. The soybean-whey was lyophilized (WP) and dissolved in MilliQ-water at a concentration of 50 mg/ml, to be used as substrate for the hydrolysis.

2.2. Gly m 1 allergen

There are several allergens detected in soybean that can produce allergies, e.g., in food, by inhalation and occupational hazard. In the case of food we can find Gly m 3, Gly m Bd 68 K, Gly m Bd 30 K, Gly m Bd 28 K and β-conglycinin allergens, by inhalation Gly m 1 and Gly m 2 allergens and in occupational hazard, the Kunitz-trypsin-inhibitor is the allergen.

We have chosen the Gly m l allergen to analyze the effect of hydrolysis when it was performed at atmospheric pressure and under high pressure.

Gly m l is a glycoprotein, rich in cysteine, that is located in the hull of the soybean seed. According to González, Varela, Carreira, and Polo (1995), Gly m l serves as valuable model for studying the mechanism of the human allergic response.

2.3. Immunodetection of Gly m 1 by ELISA test

The concentration of Gly m l in samples was measured by a monoclonal antibody (Mab)-based two-site ELISA method, as described by González et al. (2000). Briefly, microtitre 96-well plates (EIA/RIA plates, Costar, Cambridge, Massachusetts) were coated overnight at 4 °C with 100 μl of anti-Gly m 1 Mab 6G1 at 5 μg/ml in PBS. The coated wells were washed with PBS containing 0.1% Tween 20 and blocked with PBS containing 1% BSA, and 0.1% Tween 20. Then, wells were washed and sequentially incubated with samples and references, biotin-labelled anti-Gly m 1 Mab 1G10, and streptavidin/peroxidase conjugate (Amersham, Buckinghamshire, UK). Samples, controls and reagents were conveniently diluted in PBS containing 1% BSA and 0.1% Tween 20 and all the incubations were carried out for 1 h at room temperature with intermediate washes after each step. Finally, wells were incubated for 30 min at room temperature in the dark with a solution of *ortho*-phenylenediamine (OPD, DAKO, Glostrup, Denmark), and the colour reaction was stopped by adding 2 M HC1. The optical density was then read at 490 nm with a 650 nm reference filter. Samples were assayed in triplicate in several two-fold dilutions. The Gly m 1 content of samples was obtained by interpolating in a standard curve from serial two-fold dilutions of a reference extract, previously calibrated against a preparation of purified Gly m l; the protein concentration was determined by quantitative amino acid analysis.

2.4. Hydrolysis

The hydrolysis was carried out in the soybean-whey at concentration of 50 mg/ml and four enzymes Alcalasa, Neutrase Corolase 7089 and Corolase PN-L 100, were used. Each enzyme has a specific temperature: 50 °C was for Alcalasa, Neutrase and Corolase 7089 and 40 °C for Corolase PN-L 100. The HP treatment was performed at 100, 200 and 300 MPa for 15 min and hydrolysis was also carried out at atmospheric pressure (0.1 MPa). Five ml of substrate and 12.5 mg of enzyme were used for the proteolysis. The same reactions were performed in the absence of enzyme and were considered as blanks for the experiment. Hydrolysis was performed in triplicate, and the hydrolysates were stored at −20 °C prior to analysis.

The proteolysis was performed separately with the four enzymes: Alcalase (E.C. 3.4.21.62, Novozymes, Bagsvaerd, Denmark), Neutrase (E.C. 3.4.24.28, Novozymes, Bagsvaerd, Denmark), Corolase 7089 (B.C. 3.4.24.28, AB Enzymes, Darmstadt, Germany) and Corolase PN-L 100 (E.C. 3.4.21.63, Darmstadt, Germany).

2.5. High-pressure treatment

A high-pressure machine (ACB GEC, Alsthom, Nantes, France) with a steel-vessel of 2.35 l capacity (100 mm in diameter and 300 mm in height) was used for the HP treatment at pressures of 100, 200 and 300 MPa. The vessel was filled with water as a fluid of low compressibility. During the treatment, the temperature was held constant by using a water bath. In each experiment, the indicated pressure was achieved within 1–2 min, held for 15 min and released to atmospheric pressure within 1–2 min. Each sample was done in triplicate.

2.6. Protein content

The protein content was determined, following the method described by Bradford (1976), using the Bio-Rad Proteins Assay (Bio-Rad, Madrid, Spain). The results were expressed as mg of protein/g of WP. A protein standard curve was obtained as BSA (bovine serum albumin, Sigma Chemical Co, St. Louis, USA). The absorbance at 595 nm was recorded in a Shimadzu UV-1601 spectrophotometer (Shimadzu Corporation, Germany).

2.7. Determination the degree of hydrolysis

The degree of hydrolysis was determined by quantification of α-aminogroups released in the hydrolysis reaction by the *o*-phthaldialdehyde (OPA) spectrophotometric assay, as described by Church, Swaisgood, Porter, and Catignani (1983). The OPA solution was prepared by combining 25 ml of 100 mM sodium tetraborate, 2.5 ml of 20% SDS, 40 mg of OPA in 1 ml methanol and 100 μl of β-mercaptoethanol to a total volume of 50 ml. An aliquot of 50 μl of the hydrolysates was added to 1 ml OPA solution. The solution was mixed by inversion, incubated for 2 min at room temperature, and the absorbance at 340 mn measured in a spectrophotometer (Shimadzu, UV-1601).

2.8. One-dimensional electrophoresis (SDS-PAGE)

Sodium Dodecylsulfate, polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1979) on 12% acrylamide gels, using the Bio-Rad Miniprotean II system (Bio-Rad, Richmond, CA). Two hundred microliters of the samples were precipitated in 1 ml of ammonium acetate:methanol (1:74 v/v) solution. The precipitate obtained after centrifugation for 10 min at 10,000 rpm was washed with acetone:water (80:20 v/v) and dissolved in the hydration buffer, (7 M urea, 2 M thiourea, 50 mM dithiothreitol, 0.2% Bio-Lyte™ 3–10 amholite and 2% CHAPS). Two microliter of the extracted sample, with 7 μl of loading buffer (0.5 M Tris–HCl pH 6.8, 2% SDS, 20% glycerol, 5% mercaptoethanol, 0.5% bromophenol blue) were used. After running, the gels were stained with Coomassie Brillant Blue G 250 (Merck, Darmstadt, Germany). Urea and dithiothreitol were bought from Sigma Aldrich (St. Louis, USA), thiourea from Merck (Darmstadt, Geraiany), and CHAPS from Pharmacia (Sweden).

3. Results and discussion

Fig. 1 shows the protein content of the untreated soy-whey and that after the HP treatment. The results turned out to be lower at 100 and 300 MPa than at 0.1 MPa, the proteins underwent a protein denaturation due to the partial hydrolysis that the HP produced. In the case of 200 MPa, the values were the same as the control and this could be explained by the formation of some aggregates as Smeller (2002) reported, due to the pressure applied. Through these results, we have found out that HP affects the proteins producing denaturation.

The protein contents of the hydrolysates, obtained from hydrolysis with the four industrial enzymes used, are also shown in Fig. 1. The lowest protein content for the Neutrase was at 200 MPa. In the case of Alcalase, as the pressure increased, the protein content diminished and the lowest content was at 300 MPa. On the other hand the Corolase 7089 was more effective at atmospheric pressure

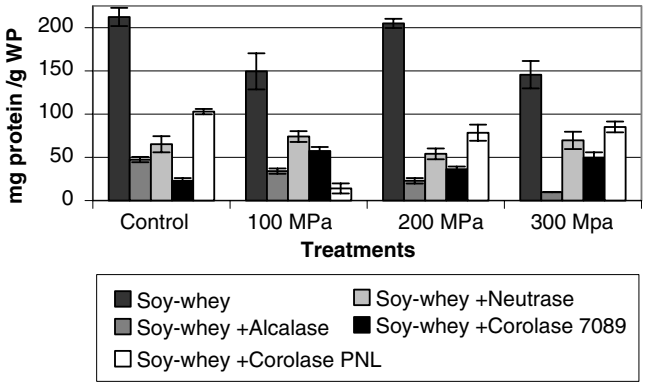

Fig. 1. Protein contents of soybean-whey and their hydrolyzates obtained by hydrolysis with Alcalase, Neutrase, Corolase 7089 and corolase PNL at atmospheric pressure and under high pressure at 100,200 and 300 MPa.

(0.1 MPa) than at HP. But the Corolase PNL presented the lowest content of proteins at 100 MPa.

The degree of hydrolysis of soybean whey proteins digested under HP was measured using the *o*-phthaldialdehyde (OPA) spectrophotometric assay. The results are shown in Fig. 2, where an enhancement of proteolysis at 200 and 300 MPa, with significant differences for Alcalase, Corolase 7089 and Corolase PNL, was observed compared to control (0.1 MPa). These enzymes presented the highest extent of hydrolysis at 200 MPa. There were no significant differences between the hydrolyses by Corolase PNL at 200 and 300 MPa. However, the differences between 200 and 300 MPa were significant for Alcalase. Significant enhancement of hydrolysis by Neutrase was obtained when the digestion was performed at 300 MPa as compared with control (0.1 MPa), 100 and 200 MPa. A considerable decrease of hydrolysis was found for Alcalase and Neutrase when the reaction took place at 100 MPa in comparison with control, 200 and 300 MPa.

These results are in accordance with those obtained by Stapelfeldt et al. (1996), Chobert et al. (1997) for β-lactoglobulin, who reported an enhancement of proteolysis of

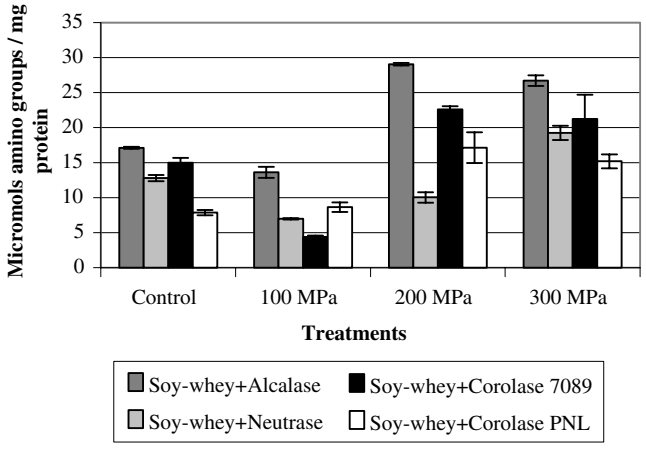

Fig. 2. Degree of hydrolysis, by the OPA method, of soybean-whey proteins hydrolyzed at atmospheric pressure or under high pressure at 100, 200 and 300 MPa by Alcalase, Neutrase, Corolase 7089 and corolase PNL.

this protein under pressures up to 200 MPa. The increase could be related to the denaturation of the proteins and dissociation into monomers at the moment when the protease was present, as Kajiyama, Isobe, Eumura, and Noguchi (1995) reported.

In the case of Corolase 7089, a sharp diminution of hydrolysis was found at 100 MPa, with significant differences when compared with control at 200 and 300 MPa. Thus, the pressure of 100 MPa could affect negatively the binding between the substrate and the enzyme, decreasing the proteolysis at this pressure.

The gels (SDS-PAGE) confirmed that the hydrolysis at atmospheric pressure was less effective than at HP (data no shown). The results of soy-whey at 200 MPa turned out to be similar to those at 100 and 300 MPa (data not shown). The effect of hydrolysis for the four enzymes are visualized, on the gels, in Fig. 3. Most of peptides obtained were lower in MW than 20 kDa for Alcalase, Corolase 7089 and Corolase PNL (Fig. 3, lines 1, 3 and 4). Regarding Neutrase, one band of around 70 kDa was also observed. These results are in accordance with those from the OPA-assayed (Fig. 2), where Alcalase presented the best hydrolysis, as is shown in Fig. 3 line 1, and the lowest was for Neutrase (Fig. 3, line 2).

The results of the detection of the allergen Gly m 1 in soybean whey by the ELISA test are shown Table 1. We have found that the soy-whey was immunoreactive to the antibodies against Gly m 1. However, the response of these antibodies decreased after HP treatment, reaching the lowest value at 200 MPa. Moreover, as the epitope, defined by the anti-Gly m 1 Mab 6gl, is also an immunodominant IgE-binding epitope (González et al. unpublished results), it can be concluded that the allergenicity of Gly m 1 is reduced as well. Several authors (Balny & Mason, 1993; Silva & Weber, 1993) have reported the modification of secondary, tertiary and quaternary structures of proteins under high pressure. Messens, Van Camp, and Huyghebaert (1997) reported the cleavage of non-covalent peptide bonds which stabilize the structures of the proteins. These modifications probably lead to the removal of the conformational antigenie epitopes of the allergens.

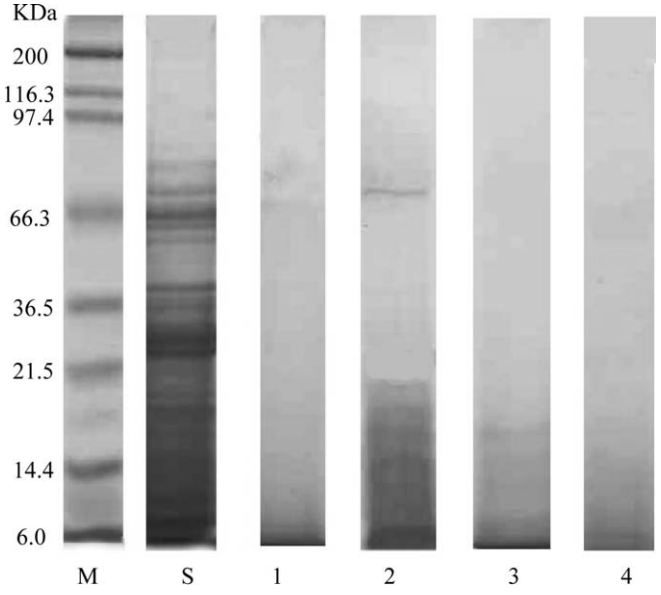

Fig. 3. SDS-PAGE on 12% acrylamide–Tris–glycine gels. (M) Standard marker, (S) unhydrolyzed soybean whey, (1) peptides obtained after hydrolysis with Alcalase at 200 MPa, (2) Neutrase at 200 MPa, (3) Corolase 7089 at 200 MPa, (4) Corolase PNL at 200 MPa.

Table 1
Concentration of Gly m 1 allergen (μg/ml) in soy-whey untreated (0.1 MPa) and treated by HP at 100, 200 and 300 MPa, determined by ELISA as indicated in Section 2

	Soybean whey
	Gly m 1 (μg/ml)
0.1 MPa	14.2
100 MPa	12.5
200 MPa	11.0
300 MPa	13.0

A marked decrease in immunoreactivity of anti-Gly m 1 antibodies was observed in the soybean whey samples hydrolyzed at atmospheric pressure by the four proteases studied, as Table 2 shows in comparison to soy-whey (Table 1). The immunoreactivity in most of the hydrolysates, obtained after the combined treatment of high pressure and enzymatic hydrolysis, was lower than in untreated soy-whey (14.2 μg Gly m 1/ ml), except for Corolase 7089 and Corolase PN-L, in which the values were higher at 100 MPa (20.2 and 19.5 μg/ml, respectively). The results for both corolases at 100 MPa could be explained by the denaturation of proteins under high pressure (Smeller, 2002). As a result of this, the antigenic epitopes could be more accessible to the antibodies. Besides, the HP treatment could be responsible for breaking aggregates between Gly m 1 and other proteins, allowing the binding of Gly m 1 to the antibodies. On the other hand, the HP produced the highest reduction of immunological response on the Corolase PN-L hydrolysates at 300 MPa, followed by the Alcalase hydrolysates at 200 MPa, and Neutrase-hydrolysates at 100 MPa and, in the case of the hydrolysis by Corolase 7089, it was at atmospheric pressure (0.1 MPa).

What remains clear is that the allergen went down in the hydrolysates when the hydrolysis was performed under HP conditions. But, the immunological responce to Gly m 1 antibodies was different, depending on the protease used and the high-pressure treatment applied.

Table 2
Concentration of Gly m 1 allergen (μ/ml) in soy-whey hydrolyzed by Alcalase, Neutrase, Corolase 7089 and Corolase PNL at atmospheric pressure (0.1 MPa) or under high pressure at 100, 200 and 300 MPa, determined by ELISA, as indicated in Section 2

	Gly m 1 (μg/ml)			
	Alcalase	Neutrase	Corolase 7089	Corolase PN-L
0.1 MPa	8.53	9.48	8.08	8.01
100 MPa	8.89	8.29	20.2	19.5
200 MPa	7.63	14.4	10.6	10.2
300 MPa	8.52	10.8	10.7	6.54

The best pressure conditions for reducing the antigenicity of Gly m l in soybean whey hydrolysates are thus a compromise between the optimal pressure to destabilize the native structure of the protein, allowing the exposure of antigenic epitopes to the enzyme, and the pressure compatible with the retention of protease activity. Gly m 1 has a globular structure, stabilized by disulphide bonds (González et al., 1995), and its antigenicity decreases after the hydrolysis under high pressure. Probably, other proteins will lose the immunoreactivity too.

Allergies to soybean are considered one of the most common allergies among children but, in the case of adults, the prevalence has been estimated at 1%. On the other hand, the use of hydrolysis and industrial processes, such as HP, could make an important reduction in terms of allergenicity of soy food.

In conclusion, the results of the detection of the allergen Gly m 1 in the soy-whey indicated that the combination of both enzymatic hydrolysis and HP treatment reduced its allergenicity. Besides that, the HP treatment enhanced the proteolysis of soy-whey proteins in the four proteases assayed. On the other hand, the best condition for the proteolysis and allergenicity depends on the type of enzyme used. These results may be valuable not only to soy-whey, but also to soymilk, which is actually widely consumed in Western countries. Thus, the combination of HP treatment and hydrolysis could be an important tool for reducing or removing the immunoreactivity of soybean whey proteins. Above all, their hydrolysates might be used as base ingredients of food.

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