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High pressure and the enzymatic hydrolysis of soybean whey proteins

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Abstract

The effect of high-pressure (HP) treatment on the hydrolysis of soybean whey proteins by trypsin, chymotrypsin and pepsin was studied. The experiments were carried out at atmospheric pressure (0.1 MPa, for 30 min, at 37 °C) and at HP (100 and 200 MPa for 15 min at 37 °C) before or during the reaction of hydrolysis. The extent of hydrolysis was measured by OPA method and in the extracts from TCA. The results showed that HP treatments increased the hydrolysis in the three enzymes used and 100 MPa was the better pressure to enhance the hydrolysis. Polyacrylamide gel electrophoreses (SDS–PAGE), showed five peptides lower than 14 kDa after hydrolysis by chymotrypsin and trypsin, and 11 peptides by pepsin. Soybean whey proteins, which are industrially discarded, could be used as a source of peptides, with applications as base in some diets.

Keywords: Soybean whey proteins; High-pressure; Hydrolysis; Enzymes; Trypsin; Pepsin; Chymotrypsin

1. Introduction

Soybeans are rich in nutrients and bioactive substances, but has anti-nutritional factors (ANFs), such as trypsin inhibitors, lectins and phytic acid. These factors are generally inactivated or decreased by heat treatment during food processing (Friedman, Brandon, Bates, & Hymowitz, 1991). However, an excess of heating decreases the sensorial parameters and nutritive value of products.

Emerging technologies such as high pressure treatments have been used in the last decade as excellent non thermal techniques for food preservation. They can compete with the traditional heating treatment (Arroyo, Sanz, & Préstamo, 1997, 1999; Cheftel 1995; Préstamo, Arabas, Fonberg-Broczek, & Arroyo, 2001), by decreasing the microbial population, maintaining the sensorial and nutritional properties of the foods (Préstamo & Arroyo 2000; Préstamo, Sanz, & Arroyo, 2000).

The consumption of soy foods is increasing due to the benefits reported on nutrition and health. In this way, soybean is associated with the reduction of several diseases, such as certain cancers (Adlercreutz et al., 1995; Fotsis, Pepper M., Adlercreutz, Hase, Montesano, & Schweigerer, 1995; Messina and Loprinzi 2001; Wang et al., 1999) and heart illness (Honoré, Williams, & Anthony, Clarson, 1997; Lovati et al., 2000). On the other hand, the role played by soybeans in the reduction of cholesterol (Tovar-Palacio, Potter, Hafermann, & Sahy, 1998) or in the prevention of obesity (Jurowska, Grondin, Masse, & Morisset, 1992; Mori, Aoyama, Fukui, Kurokawa, Komiya, & Ikeda, 1998; Aoyama, Fukui, Takamatsu, Hashimoto, & Yamamoto, 2000) is firmly established. Studies performed on Chinese athletes showed that soy proteins in the diet produced an increase of lean body mass, reducing body fat and increasing resistance to fatigue (Stroescu, Dragan, & Georgescu, 1994). These effects were also found in animals (Fushiki, Matsumoto, Uohashi, & Inoue, 1994). Consequently, soybean proteins offer special benefits not found in other foods and are being used to replace some dairy foods, or in diet.

Protein hydrolysates have properties that make them attractive as a source of protein in human nutrition.

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They are physiologically better than intact proteins because their intestinal absorption appears to be more effective due to the increase of solubility and peptide content (Ziegler, Nitenberg, Coudray-Lucas, Lasser, Giboudeau, & Cynober, 1998). Soybean whey hydrolysates could also be useful, because of their high solubility and their high pressure treatment could be a good tool in the process of their hydrolysis.

Soy proteins which consist of two major components 7S and 11S globulins (Damelson, 1949), are dissociated under pressure (Kajiyama, Isobe, Eumura, & Noguchi, 1995) creating new free thiol residues, some of them recombine to give –S–S– exchange reactions or new –S–S– binding by oxidation (Swanson, Yang, Powers, & Dunker, 2002). Soybean "whey" includes 2S and 7S fractions with good foaming capacity and high solubility. Pressure induced denaturation is a complex phenomenon due to the disruption of both hydrophobic bonds and salt bridges. It depends on some factors such as temperature, pH, solvent and ionic strength as well as the protein structure and the applied pressure (Kajiyama et al., 1995).

During the process of making tofu (soy curd) the "whey" is usually discarded, but could be used as a good source of proteins. The aim of this research is to observe the effect of high-pressure treatment on soybean whey proteins when they are hydrolysed under or after the pressure treatment to improve the hydrolysis.

2. Materials and methods

2.1. Plant material

Soybean whey was obtained from overnight soaked soybean, then homogenised (soybean: water, 1:3 v/v) and drained through a cheesecloth, according to the scheme presented in Fig. 1. The white solution obtained is called soymilk and the insoluble residue is the okara. Soymilk is used to make tofu, which is the curd resulting from the coagulation by nigary (calcium sulphate and magnesium chlorate). The supernatant obtained ("whey") was lyophilised. The lyophilised powder (WP) was dissolved in 50 mM sodium phosphate buffer, pH 8.0, at the concentration of 50 mg/ml, and dialysed against the same phosphate buffer. The WP was used as substrate (S) for the hydrolysis.

2.2. High-pressure treatment

The samples (hydrolysis reactions) were subjected to the pressures of 100 and 200 MPa for 15 min at 37 °C in a high-pressure machine (ACB GEC, Alsthom, Nantes, France) with a hydrostatic pump. The capacity of the vessel was 2.35 l (steel container 100 mm in diameter and 300 mm in height), and water was used as a fluid of low compressibility. In each experiment, the indicated pressure was achieved within 1–2 min, held for 15 min and release to atmospheric pressure within 1–2 min. Each sample was replicated three times.

2.3. Hydrolysis experiments

Hydrolysis was performed at atmospheric pressure (0.1 MPa) for 30 min at 37 °C using untreated substrate (S) and substrate treated by HP. Hydrolysis was also performed during the HP treatment with the untreated substrate. The HP conditions were 100 and 200 MPa at 37 °C for 15 min.

An amount of 2.5 ml of substrate (50 mg/ml) was subjected to pressure. After the pressure was released, the HP treated substrate was utilised for proteolysis at atmospheric pressure. The proteolysis was carried out separately with three different enzymes, pepsin from porcine stomach (EC. 3.4.23.1, Sigma), trypsin from porcine pancreas (EC. 3.4.21.4, Sigma), and α-chymotrypsin from bovine pancreas (EC. 3.4.21.1, Sigma, Alcobendas, Madrid, Spain) .A total volume of 1.250 ml was used for the reaction of hydrolysis: 1.1 ml of specific buffer for each enzyme was used (50 mM sodium phosphate buffer pH 8 for trypsin and chymotrypsin, and 50 mM sodium citrate pH 4 for pepsin), 25 μl enzyme (0.5 mg/mL) and 125 μL of substrate. Blank was performed in the same conditions in the absence of enzyme.

The hydrolysis was also carried out during the HP treatment. Twice amount (2.5 ml) of the reactants of hydrolysis as described previously was used. Incubation without enzyme (used as blank) was also performed.

As control and to compare with the other experiments, the hydrolysis was performed at atmospheric pressure (0.1 MPa) with untreated substrate.

Hydrolysis was performed in triplicate, and the hydrolysates were stored at −20 °C until analysis.

The extent of hydrolysis was studied using two different procedures. First, the degree of hydrolysis was assayed directly by quantification of cleaved peptide bonds as assessed by the o-phthaldialdehyde (OPA) spectrophotometric assay described by Church, Swaisgood, Porter, and Catignani (1983). The α-amino groups released by hydrolysis react with OPA and β-mercaptoethanol to form an adduct that absorbs strongly at 340 nm. The OPA reagent also reacts with ε-amino groups, and the resulting absorbance represents the blank for proteolytic assays. The OPA solution was prepared by combining 25 ml of 100 mM sodium tetraborate, 2.5 ml 20% SDS, 40 mg of OPA in 1 ml methanol and 100 μl of β-mercaptoethanol to a total volume of 50 ml. To assay proteolysis, 50 μl of the hydrolysates was directly added to 1 ml of OPA solution. The solution was swirled by inversion, incubated for 2 min at

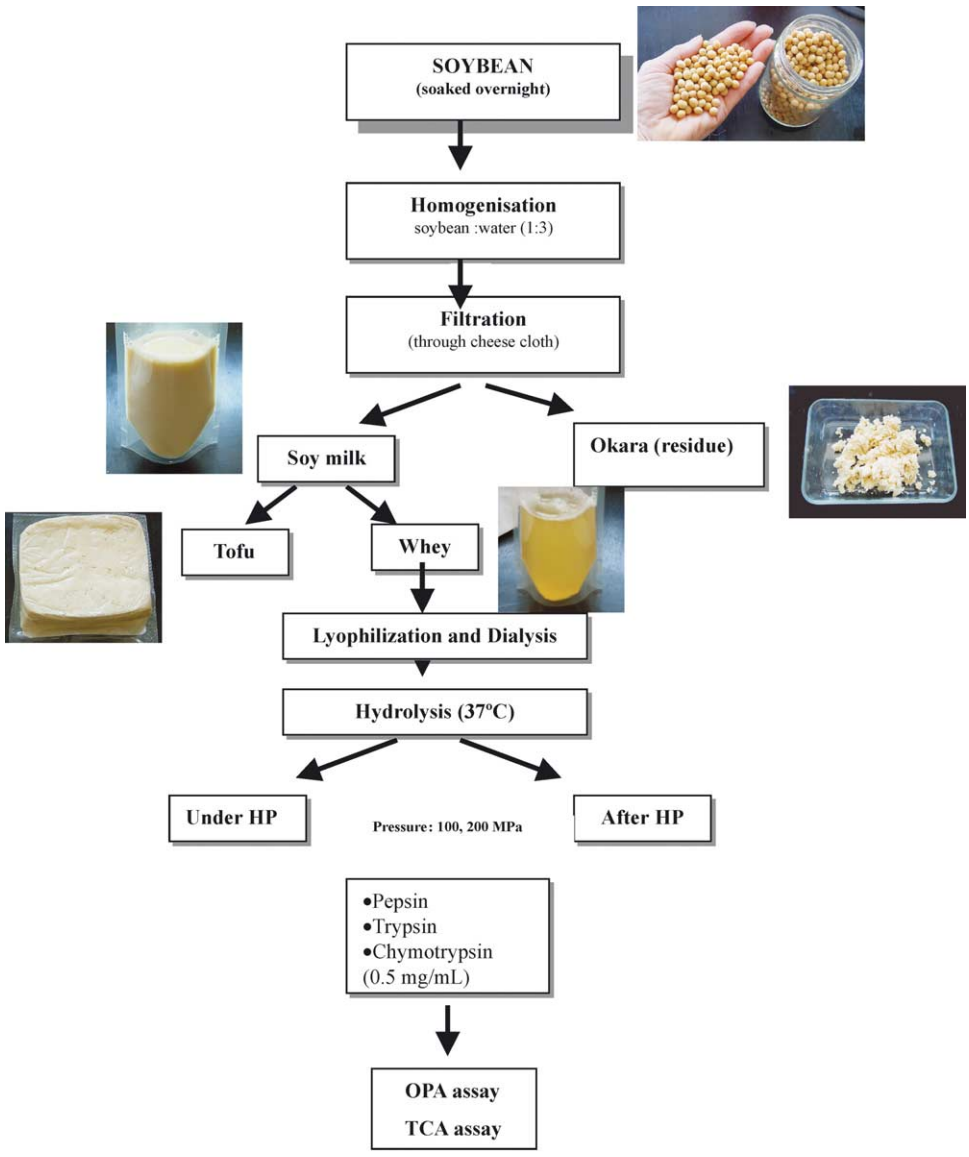

Fig. 1. Scheme of tofu process. Description of soybean whey used in the experiment.

room temperature, and the absorbance at 340 nm was measured in a spectrophotometer (Shimadzu, UV-1601). Second, the degree of hydrolysis was determined in the extract from 10% trichloroacetic acid (TCA) by measuring the absorbance at 280 nm, in the supernatant obtained after centrifugation (8000 *g*, for 10 min), according to the method described by Hayashi, Kawamura, and Kunugi (1987) and Okamoto, Hayashi, Enomoto, Kaminogawa, and Yamaguchi (1991). Degree of hydrolysis was expressed as the percentage ratio of the absorbance of the enzymatic digest to that of the original solution. The TCA assay is complementary to the OPA assay.

The enzymatic digest from soybean whey proteins was also analysed by electrophoresis (SDS–PAGE), according to Laemmli (1979), using pre-cast gels of 16.5% Acrylamide-Tris-Tricine/peptide (ready gels, Bio Rad), 0.75 mm thick. The bands were stained with Coomassie Brillant blue G 250 (Merck). The samples were lyophilised and 7.5 μl of sample (10X concentrated) were loaded (3 μg of protein without enzyme and 70 μg of protein with enzyme).

2.4. Statistical analysis

Data were analysed with the SPSS program, following one way lineal model, using as post-hoc the Duncan test with significance $P \geqslant 0.05$.

3. Results and discussion

Since the extent of enzymatic hydrolysis of proteins is increased by high pressure treatments as previously

reported on the case of soy and dairy proteins (Hayashi et al., 1987; Okamoto et al., 1991; Stapelfeldt, Petersen, Kristiansen, Qvist, & Skibsted, 1996), most favourable pressure treatments for soybean whey proteins were studied. Pressure treatments of 100 and 200 MPa were chosen on the basis of prior studies (Peñas & Préstamo, 2002) on enzymatic hydrolysis of soybean whey proteins subjected to pressures of 100, 200, 300 and 400 MPa. It has been reported that a sharp decrease of hydrolysis occurred when the proteins were treated at 300 or 400 MPa, which could be related to aggregation of proteins at these conditions. These effects were also reported using β-lactoglobulin subjected to high pressure (Dumay, Kalichevsky, & Cheftel, 1994; Iametti et al., 1997).

The degree of hydrolysis obtained at 25, 30, 37 and 40 °C under atmospheric pressure (0.1 MPa) of soybean whey proteins by pepsin, trypsin and chymotrypsin enzymes was determined by measuring the α-amino groups released in the enzymatic reaction by the OPA reaction. The highest level of hydrolysis was found at 37 °C for the three enzymes.

The extent of hydrolysis was measured using two different spectrophotometric assays, by the o-phthaldialdehyde (OPA) method, measuring the absorbance at 340 nm, and in the extract from 10% trichloroacetic acid (TCA) at 280 nm. Fig. 2a shows the degree of hydrolysis of soybean whey proteins treated by HP prior to enzymatic hydrolysis by OPA assay. Trypsin shows the greatest hydrolysis after 100 MPa treatment,

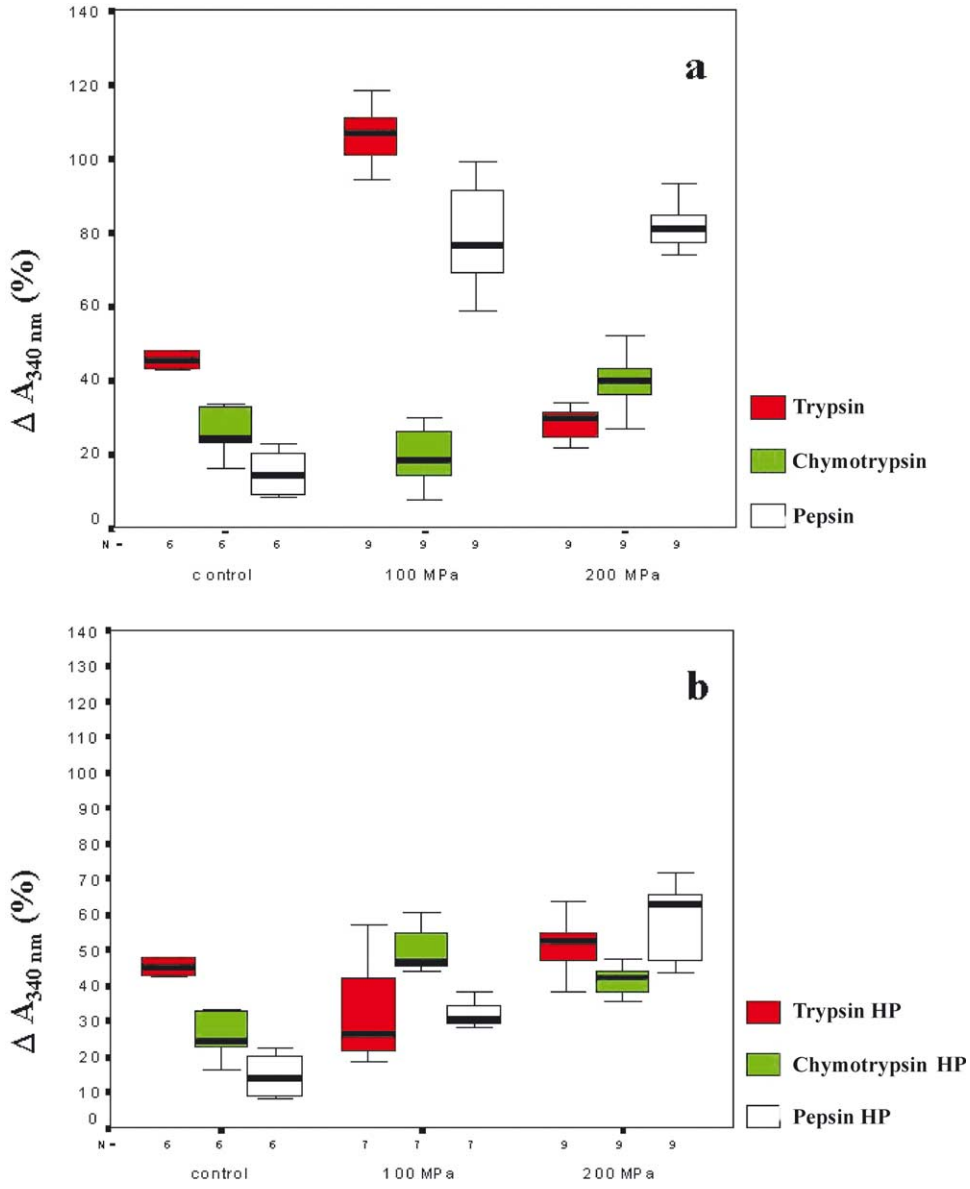

Fig. 2. Increase of absorbance at 340 nm determined by OPA method during hydrolysis of soybean whey proteins. (a) Untreated (control) and treated by high pressure (100 or 200 MPa, for 15 min at 37 °C) prior hydrolysis with pepsin, chymotrypsin and trypsin. (b) Hydrolysis performed during the high-pressure treatment (100 or 200 MPa, for 15 min at 37 °C).

followed by pepsin. An enhancement of hydrolysis with a significant differences ($P \leqslant 0.05$) was observed for both enzymes when compared with control. There were no significant differences between the hydrolysis by pepsin at 100 and 200 MPa. However, a considerable diminution of hydrolysis was observed for trypsin at 200 MPa with a significant difference ($P \leqslant 0.05$) as compared with the untreated or pressurised proteins after 100 MPa. Pepsin presents the lowest at atmospheric pressure (0.1 MPa).

Significant increases of hydrolysis by chymotrypsin was found when the reaction took place under high pressure (100 MPa), with significant differences ($P \leqslant 0.05$) in comparison with control and 200 MPa (Fig. 2b); and a sharp decrease was obtained when it was performed at 200 MPa. Pepsin presented the highest values at 200 MPa with significant differences as compared to control and 100 MPa. However, no significant differences ($P \geqslant 0.05$) were observed for trypsin when hydrolysis was performed at atmospheric pressure (control) and compared with high pressure of 100 and 200 MPa.

The extent of hydrolysis was also determined in the extract from 10% TCA protein precipitation of soybean whey proteins treated by HP prior enzymatic hydrolysis. Fig. 3a presents that the maximum hydrolysis by trypsin and chymotrypsin was obtained in the case of proteins treated at 100 and 200 MPa, respectively. Pepsin presented the highest hydrolysis at 100 MPa. Fig. 3b shows that the highest hydrolysis under high pressure was at 100 MPa, for pepsin, trypsin and chymotrypsin. Lowest levels of hydrolysis by pepsin were observed for soy proteins treated at 200 MPa before or during the HP treatment. However, greater hydrolysis was observed at pressures of 100 and 200 MPa in the reaction mixture by OPA method.

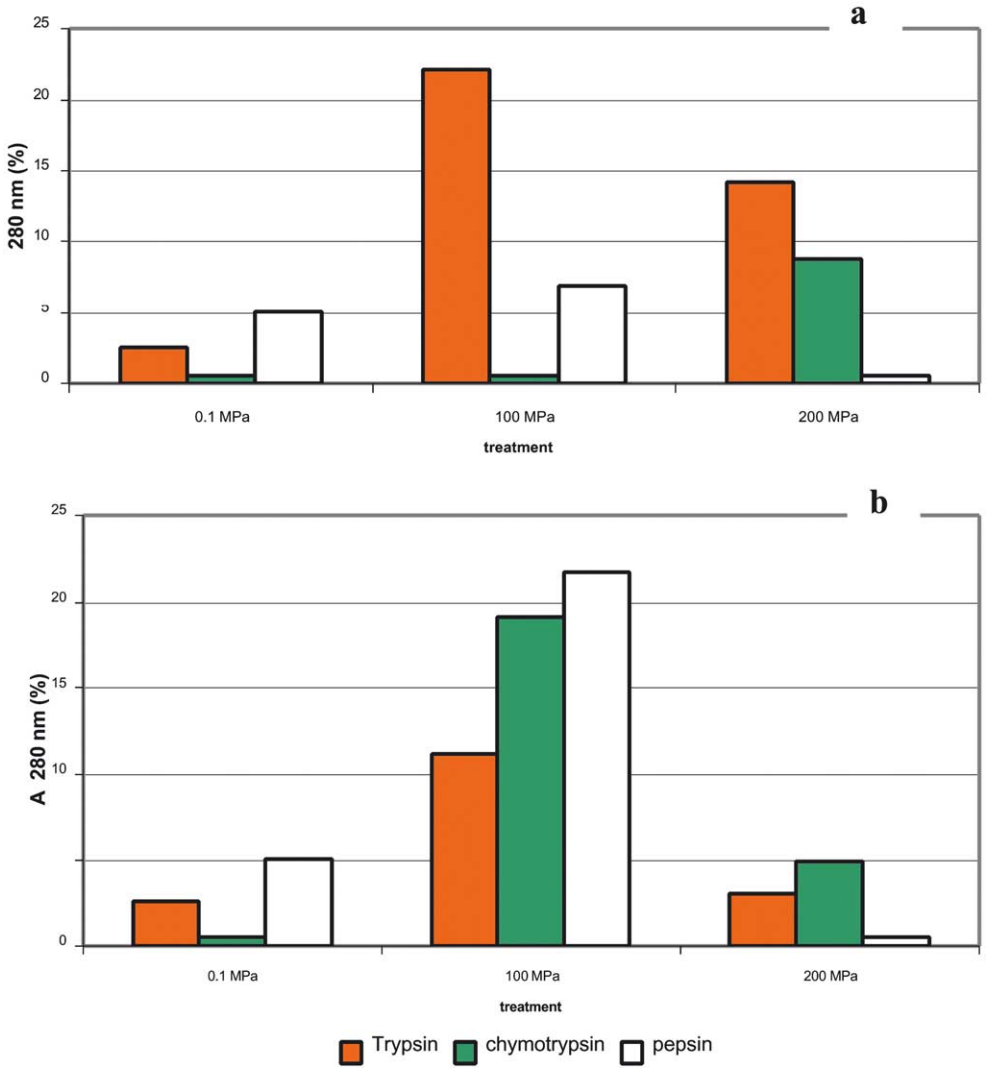

Fig. 3. Increase of absorbance at 280 nm measured in the 10% TCA extract after the hydrolysis of soybean whey proteins. (a) Untreated (control) and treated by high pressure (100 or 200 MPa, for 15 min at 37 °C) prior the hydrolysis with pepsin, chymotrypsin and trypsin. (b) Hydrolysis performed during the high-pressure treatment (100 or 200 MPa, for 15 min at 37 °C).

In general, an increase of hydrolysis by pepsin, trypsin and chymotrypsin was observed on soybean whey proteins treated before or during the HP treatment. According to Kajiyama et al. (1995) the HP treatment dissociates the proteins and as a result, the hydrolysis increases.

The discrepancy between the proteolysis observed by both procedures could be related to modification of substrate or binding of the substrate to the enzyme induced by pressure, which could affect the size of peptides produced in the enzymatic reaction. Peptides of large or intermediate size insoluble in TCA could be formed under 200 MPa, which contribute to digestibility measured in the whole reaction mixture but not to that measured in the soluble extract in TCA.

There is a correlation between both methods for trypsin and chymotrypsin, when the proteins were pressurised before the enzymatic hydrolysis, and also for chymotrypsin in the reactions performed under pressure.

These results are in accordance with those of Stapelfeldt et al. (1996) for β-lactoglobulin, showing a three times increase in the degree of hydrolysis by trypsin for proteins treated at 200 MPa. However a pressure of 100 MPa found in the present research as optimal for the increase of hydrolysis of soybean proteins by trypsin was lower than that determined by Stapelfeldt et al. (1996) for β-lactoglobulin, which may result from the different pressure resistance of the proteins used in each study. The pH could be playing an important role in the hydrolysis, because it changes under pressure treatment as Neuman, Kauzmann, and Zipp (1993) reported.

Consequently, the best hydrolysis after the HP treatment of the substrate was at 100 MPa for trypsin and pepsin and at 200 MPa for chymotrypsin. In addition, when the hydrolysis was performed during the HP treatment, the higher values of hydrolysis were at 100 MPa for chymotrypsin.

Different patterns of peptides were observed by SDS-PAGE for the three enzymes studied (Fig. 4). Chymo-

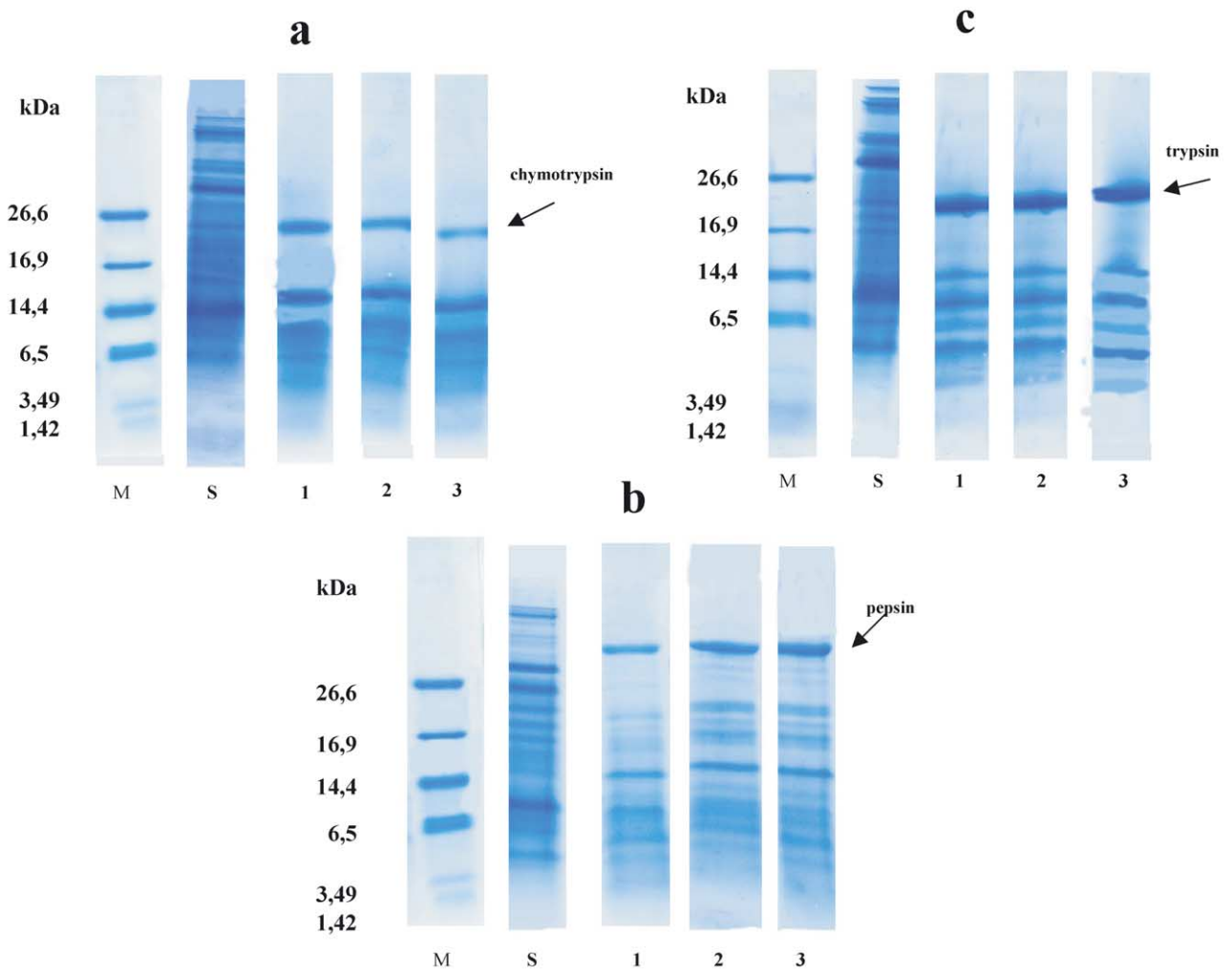

Fig. 4. SDS–PAGE on 16.5% Acrylamide-Tris-Tricine/peptide (ready gels, Bio Rad). Standard marker (M), unhydrolysed soybean whey (S), peptides obtained after hydrolysis with chymotrypsin (a), pepsin (b) and trypsin (c). (1) control, (2) treated by HP at 100 MPa during hydrolysis, (3) treated by HP at 100 MPa prior hydrolysis.

trypsin and trypsin were the enzymes that presented better hydrolysis with five bands well resolved of about 14,4- 10,7 - 5,4- 4,5- 1 kDa (Fig. 4 a) and 14- 11,5- 6-5,3 and 3,7 kDa (Fig. 4c) respectively and the same pattern was observed in the samples treated by HP at 100 MPa and control. Most of the peptides were smaller than 14.4 kDa and according to Calderón de la Barca, Salazar-Ruíz, and Jara-Marini (2000), soybean hydrolysates below 10 Kda present 100% solubility at all pH values. Pepsin presented 11 bands of about 27-26—20,4- 18,9-16,5-15-13-9-6,5-6- and 5 kDa (Fig. 4b) after enzymatic hydrolysis and the same pattern was obtained for the samples treated by HP at 100 MPa and control.

The spectrophometric methods and electrophoresis give important information about hydrolysis, however to observe the differences between peptides with the same molecular weight should be necessary the use of 2D electrophoresis, where they can be separated by the IP (isoelectric point).

In conclusion, the spectrophotometric results indicate that high pressure increases hydrolysis and a pressure of 100 MPa presented the best condition for the three enzymes. The visualisation by SDS–PAGE shows different pattern for each enzyme used for hydrolysis but was not enough to observe the differences between peptides treated by HP. Chymotrypsin and trypsin presented five visible peptides lower than 14 kDa after hydrolysis and pepsin about 11 peptides.

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