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Effects of high pressure and microwave on pronase and α-chymotrypsin hydrolysis of β-lactoglobulin

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Abstract

The effects of hydrostatic high pressure, microwave irradiation, and conventional heating on hydrolysis of bovine β-lactoglobulin AB by pronase and α-chymotrypsin were studied. The high pressure treatments were performed between 100 and 300 MPa, the microwave irradiation was applied at different power conditions, pronase (30 W) and α-chymotrypsin (15 W). All enzymatic reactions were carried out during 10 and 20 min, at 40 °C. The initial rate of pronase and α-chymotrypsin hydrolysis of protein was increased by using either physical treatment during the enzymatic digestion. The microwave-assisted digestion by pronase was more effective than those performed under conventional heating. The only apparent microwave induced difference on protein digestion by α-chymotrypsin was in a lower relative concentration of peak eluting at 24.5 min, and the conversion of this fraction into final peaks eluting from 24.5 to 28.5 min (F1 fraction) after 20 min of incubation. Under high pressure, the β-lactoglobulin AB is completely hydrolyzed by pronase and α-chymotrypsin.
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1. Introduction

Milk proteins are used extensively because of their nutritional and functional properties, such as foam and emulsion forming and stabilizing properties. Enzymatic hydrolysis is used to alter the functional properties of the proteins (Caessens, Visser, Gruppen, & Voragen, 1999). Peptides originating from the enzymatic hydrolysis of milk proteins having a diverse range of biological activities have been described (Mullally, Meisel, & Fitzgerald, 1997). Enzymatic hydrolysis of whey proteins is considered desirable to reduce their antigenicity (Asselin, Amiot, Gauthier, Mourad, & Herbert, 1988; Asselin, Hebert, & Amiot, 1989; Nakamura, Sado, & Syukunobe, 1993; Okamoto, Hayashi, Enamoto, Kaminogawa, & Yamauchi, 1991). However, there is discrepancy in the optimal extent of hydrolysis for non antigenic response (Calvo & Gómez, 2002).

Milk proteins in general and β-lactoglobulin (β-Lg) in particular, have a high content of essential amino acids. β-Lg is resistant to gastric digestion and apparently remains intact after it passes through the stomach, in experimental animals, and thus its component amino acids may nutritionally unavailable (Reddy, Kella, & Kinsella, 1988).

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β-Lg is the major whey protein found in bovine milk and comprises approximately half the total whey proteins. It is a globular protein with a monomer molecular weight of 18,362 for genetic variant A and 18,276 for the B, corresponding to 162 amino acids. β-Lg exists naturally as a noncovalently linked dimer (Pittia, Wilde, Husband, & Clark, 1996).

Hydrolysis of proteins by enzymes at elevated hydrostatic pressure is a new method for enzymatic hydrolysis of substrates that can not be hydrolyzed without difficulty at ambient pressure conditions. The treatment of samples in different pressure conditions has been investigated as an alternative to heat treatment of foods (Stapelfeldt, Petersen, Kristiansen, Qvist, & Skibsted, 1996). High pressures act by altering the balance of intramolecular and solvent-protein interactions (Balny, Masson, & Travers, 1989; Heremans, 1982; Weber & Drickamer, 1983). Pressure-induced denaturation is a complex phenomenon, principally ensuing from the disruption of both hydrophobic bonds and salt bridges. The extent of pressure-induced changes in proteins depends on the native protein structure, as well as on factors such as ionic strength, temperature, solvent, pH and on the levels of applied pressure (Dumay, Kalichevsky, & Cheftel, 1994; Iametti et al., 1997).

High pressures (>200 MPa) induces protein unfolding of monomeric proteins and aggregation (Iametti et al., 1997; Weber & Drickamer, 1983). Dumay et al. (1994) has reported that pressure-induced unfolding of β-Lg is partially reversible with storage time after pressurization. The pressure affects proteolysis and it has been demonstrated that the hydrolysis of β-Lg by pepsin increased considerably with pressure up to 300 MPa, when only negligible digestion occurred at 0.1 MPa (Chobert et al., 1997; Dufour, Hervè, & Haertle, 1995; Stapelfeldt et al., 1996). The increased enzymatic digestion under high pressure conditions were also observed during chymotryptic (Van Willige & Fitzgerald, 1995) and thermolysin hydrolysis of β-Lg (Dufour et al., 1995). For thermolysin the effect showed to be produced by a combined influence of pressure-induced thermolysin activation and partial unfolding of β-Lg by compression (Maynard, Weingand, Hau, & Jost, 1998). Also it seems that the selective hydrolysis of β-Lg by thermolysin in whey protein concentrates occurs under pressure, while the α-lactoalbumin is resistant to the treatment (Hayashi, Kawamura, & Kunigi, 1987; Okamoto et al., 1991). Peñas, Préstamo, and Gómez (2004) reported significant increase of hydrolysis of soybean whey proteins by pepsin, trypsin and chymotrypsin when the enzymatic reactions took place under 100 MPa.

Another treatment that it is known to affect protein structure is the microwave irradiation. Microwaves (MW) are electromagnetic waves and the heating of proteins by microwave energy is accomplished both by the absorption of microwave energy by rotation of the bipolar water molecules and translation of the ionic components of the proteins. This energy is converted into heat (Ohlsson & Bengtsson, 2001). Numerous studies have dealt with the application of microwave as an alternative method to conventional heat treatments of foods. One aspect that has not been much addressed is to what extent external radiation affects enzymatic hydrolysis. Microwave irradiation has been used to accelerate protein hydrolysis for preparing samples for amino acids analysis (Chen, Chiou, Chu, & Wang, 1987; Chiou & Wang, 1989; Marconi, Panfili, Bruschi, Vivanti, & Pizzoferrato, 1995), and transesterification reactions of *N*-acetyl-L-phenyl alanine ethyl ester with propanol by subtilisin and esterification reaction of *N*-acetyl-L-phenyl-alanine with ethanol by α-chymotrypsin (Roy & Gupta, 2003). Pramanik et al. (2002) also reported the efficacy of this technique for protein mapping by their trypsin digestion. According these authors, the digestion of proteins occurs in minutes using this technique, in contrast to the hours required by conventional methods. The authors of the present work do not know works related to use of MW in enzymatic digestion of dairy proteins, for their further use as ingredients of foods for special diets.

In the present study we have investigated comparatively the influence of pressurization, and microwave irradiation on β-Lg AB hydrolysis by pronase and α-chymotrypsin, in the aim to accelerate the enzymatic reactions and also to establish whether the application of these physical treatments would affect the nature of end product from enzymatic digestions.

2. Materials and methods

2.1. Materials

β-Lactoglobulin AB from bovine milk, pronase (protease from *Streptomyces griseus*), α-chymotrypsin type I-S (from bovine pancreas), and pepsin (pepsin A, from porcine stomach mucosa) were obtained from Sigma Chemical Company, (St. Louis, MO, USA). *o*-Phthaldialdehyde (OPA) was obtained from Aldrich Chemical co (Milwaukee, WI, USA).

2.2. Hydrolysis experiments

β-lactoglobulin AB hydrolysis was performed by α-chymotrypsin and pronase at atmospheric pressure (0.1 MPa) and conventional heating for 20 and 30 min, respectively. Hydrolysis was also performed under high pressure (HP) and microwave (MW) irradiation. Blanks were performed in the same conditions in the absence of enzyme.

Enzymatic hydrolysis were carried out at 40 °C in 0.05 M phosphate buffer (pH 8.0) with an E:S ratio of 1:5 (w/w). A total volume of 500 μl was used for each reaction. Hydrolysis was performed in triplicate. Pronase or α-chymotrypsin dissolved in phosphate buffer was added to 0.5% (w/v) solution of β-Lg AB, which was pre-equilibrated at 40 °C in a water bath for 5 min. After desired periods of incubation, 500 μl of TCA (10% w/v) or of urea (6 M) were added to stop the reactions, and samples stored at 4 °C until they were analyzed.

2.3. Microwave irradiation treatments

MW-irradiation during the enzymatic hydrolysis was carried out using a non contact infrared continuous feedback temperature system, single beam microwave applicator Synthewave 402 (Prolabo, Fontenay Sous-Bois, France), with a maximum power of 300 W; only 5% (15 W) and 10% (30 W) of the available power was used for chymotrypsin and pronase reactions, respectively. The sample vial containing the substrate dissolved in buffer was preheated at 40 °C, and immediatly after adding the enzyme the vial was placed in a one-hole Teflon insertion rack, which in turn was lowered into the irradiation chamber. The temperature of the apparatus was programmed at fixed instrumental temperature setting of 40 °C.

2.4. High pressure treatments

The samples (hydrolysis reactions) were subjected to the pressures of 100, 200 and 300 MPa for 10 and 20 min at 40 °C in a high pressure machine (Autoclave Engineers, IP42260, Columbus, OH, USA) with a hydrostatic pump and a cylindrical chamber of 10 cm (diameter) × 55 cm (height). Water containing 2% water-soluble oil (Autoclave Engineers, Part No. 5019) was used as a fluid of low compressibility. The temperature was controlled by a thermostatized bath (±0.2 °C). In each experiment, the indicated pressure was achieved within 1–2 min held for 10–20 min and release to atmospheric pressure within 1–2 min. Each sample was replicated three times.

2.5. Analytical determinations

2.5.1. Proteolysis

The extent of hydrolysis was assayed directly by quantification of cleaved peptide bonds as assessed by the *o*-phthaldialdehyde (OPA) spectrophotometric assay described by Peñas et al. (2004). A standard curve was prepared using L-leucine, such that assay system contains [NH_2] ranging from 0 to 20 mM. The [–NH_2] groups in hydrolysates were determined in their soluble fraction in 5% (w/v) TCA. 100 μl of supernatant was added directly to 3.0 ml of OPA reagent in a 3.5 ml quartz cuvette; the solution was mixed briefly by inversion and after 2 min, the absorbance (340 nm) was measured using a 4049 LKB Biochrom spectrophothometer (Biochrom Ltd, Cambridge, UK), and [–NH_2] groups were determined from the standard curve.

2.5.2. RP-HPLC

Reversed-phase HPLC analysis of hydrolysed products from β-Lg AB was performed using automated HPLC System consisting of a Consta metric 4100 pump, Spectra System AS 1000 autosampler, and Spectromonitor 5000 diode array detector (thermo Separation products, Riviera Beach, FL, USA.) fitted with a Purospher Star column (5 μm, 250 × 4.0 mm; Merck, Darmstadt, Germany). The samples in a ratio 1:1 (v/v) with urea (6 M) were eluted with two solvents; solvent A, 0.1% (v/v) trifluoroacetic acid (TFA, sequential grade, Sigma, St. Louis, MO, USA) in acetonitrile (Scharlau, Barcelona, Spain) – MilliQ (Millipore, Saint-Quentin-Yvelines, France) (1:9); while solvent B consisted of 0.07% (v/v) trifluoroacetic acid in acetonitrile-MilliQ (9:1). Twenty microliters of samples were applied to the column and eluted at a flow rate of 0.8 ml/min with 100% A for 5 min followed by a linear gradient of 0–50% B over 45 min, 70% B over the next 5 min, and maintained at 70% B for 5 min. Column temperature was 25 °C throughout the experiment. The column was stabilised at initial conditions, and washed with pure acetonitrile for 45 min before the next injection. Peak detection was at 214 nm.

2.5.3. Native-PAGE

Native-PAGE electrophoresis of insoluble fractions in TCA (5%) was carried out on slab gels by method of Davis (1964), using Tris-HCl polyacrylamide slabs (T, 12%, w/v; C, 3.4%, w/v, pH 8.8) with stacking gel (T, 4%, w/v; C, 3.4%, w/v, pH 6.8). Electrophoresis was performed at constant current (15 mA/gel) for 1.5–2 h. The gels were stained with 0.1% PhastGel Blue R (Pharmacia Biotech, Sweden) in methanol–water–acetic acid (20:70:10, v/v).

2.5.4. Quantitative densitometry

Digital images of native-PAGE electrophoresis gels were analyzed by the Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MA, USA). The band intensities were recorded as peaks on a densitogram. The calibration was performed using known amounts of standard β-Lg AB. The amount of each band was measured as percent of the total amount.

2.5.5. Statistical analysis

Data were analysed with GraphPad Prism (GraphPad sofware, Inc.), following one way ANOVA lineal model, using Bonferroni test.

3. Results and discussion

3.1. Microwave and high pressure-assisted enzymatic digestion of β-Lg AB

Figs. 1 and 2 compare the proteolysis of β-Lg AB by pronase and chymotrypsin, respectively, from CH digestion and those performed under high pressure (HP) (100, 200, and 300 MPa) and MW irradiation (10% power, 30 W). The protein contains similar proportion of both A and B genetic variants as checked by PAGE-electrophoresis and HPLC analysis. The pronase hydrolysis for 10 min results in significant ($p \leqslant 0.001$) increasing release of [–NH2] groups with HP and MW irradiation in comparison with CH digestion. There were no significant differences between the proteolysis by pronase under 200 and 300 MPa or MW irradiation (Fig. 1(a)). Higher levels of proteolysis were obtained in the CH and 100 MPa digestions after 20 min of digestion, showing again those performed under 100 MPa the highest levels (Fig. 1(b)). However, no significant ($p \geqslant 0.05$) enhancement of [–NH2] groups was obtained at this period in the MW-assisted digestion and those performed under 200 and 300 MPa with respect to 10 min of incubation, which is probably because of inactivation of pronase at these conditions.

Fig. 2 shows that the digestions of β-lactoglobulin by α-chymotrypsin performed under 100 and 200 MPa were most effective, showing those performed under MW irradiation significant lower hydrolysis grade than the control digestion.

Increase of enzymatic hydrolysis by HP has also been observed on dairy proteins (Chobert et al., 1997; Maynard et al., 1998; Stapelfeldt et al., 1996) and soybean whey proteins (Peñas et al., 2004). Enhanced chemical/enzymatic hydrolysis of proteins by MW irradiation has been reported on the case of preparing samples for amino acids analysis (Chen et al., 1987; Chiou & Wang, 1989; Marconi et al., 1995), transesterification and esterification reactions (Roy & Gupta, 2003), and protein mapping by their trypsin digestion (Pramanik et al., 2002). This researcher group described that the MW-assisted digestion by endoproteases trypsin or lysine C of several biologically active proteins, including

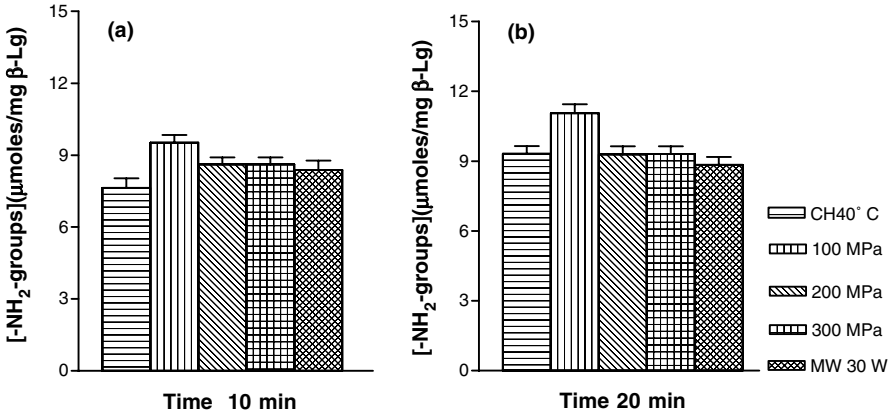

Fig. 1. Release of [–NH2] groups during hydrolysis of β-Lg AB by pronase at 40 °C, at various pressures and microwave irradiation, after (a) 10 and (b) 20 min of incubation. CH: conventional heating at atmospheric pressure (0.1 MPa).

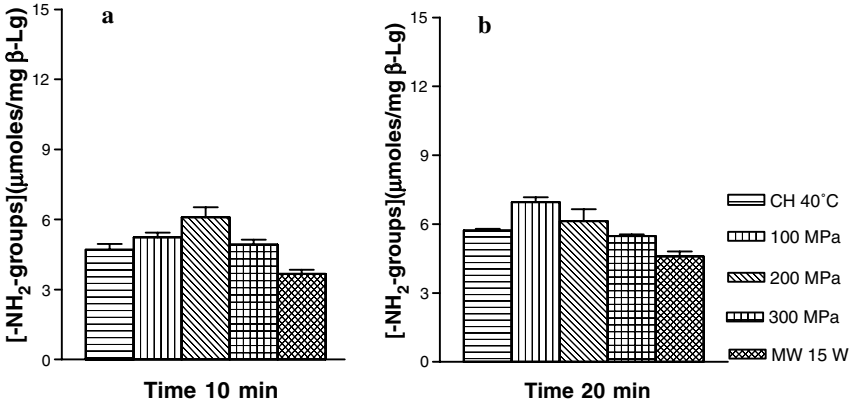

Fig. 2. Release of [–NH2] groups during hydrolysis of β-Lg AB by chymotrypsin at 40 °C, various pressures and microwave irradiation, after (a) 10 and (b) 20 min of incubation. CH: conventional heating at atmospheric pressure (0.1 MPa).

cytochrome c, ubiquitin, lysozyme, myoglobin and interferon-2b led to smaller peptides in minutes, in contrast to the hours required for the peptide fragmentation by conventional methods.

The native-electrophoretograms of the insoluble fractions in TCA (5%, w/v) from CH, MW and HP-assisted β-Lg digestions by pronase and α-chymotrypsin, confirmed the OPA data obtained, and in addition they allow to distinguish the effect of physical treatments on A and B genetic variants of β-lactoglobulin. No significant amount of β-Lg A and 15–20% of undigested β-Lg B remained in the CH digestions by both enzymes after 10 min. In contrast, insignificant amount of both genetic variants was found in the digested obtained under 100, 200 or 300 MPa, and also in the MW-assisted pronase digestion. However, a 30% undigested B genetic variant was found in the MW-assisted chymotrypsin digestion. These results suggest that the MW effect is enzyme dependent, while the HP seems to favour the hydrolysis of B genetic variant by both enzymes.

The results of the present work are consistent in qualitative terms with those of Nakamura et al. (1993); Schmidt and Van Markwijk (1993), and Van Willige and Fitzgerald (1995) for trypsin and papain, who also found that the enzymes hydrolysed native β-Lg A more rapidly than the B variant at ambient pressure. The difference in rate of hydrolysis of β-Lg A may be ascribed to the substitution of Asp_{64} in this protein with Gly_{64} in β-Lg B. This substitution, resulting in a decrease in the overall negative electrical charge in β-Lg B in comparison to β-Lg A, might considerably influence the binding of the enzyme to its substrate (Van Willige & Fitzgerald, 1995). These authors also found that the genetic variant associated differences in β-Lg hydrolysis rates disappeared under high pressures, results attributed by the similar extent of A and B genetic variants unfolding due to applied pressure and consequently similar affinities of enzyme for each substrate.

Similar reversed-phase HPLC peptide profiles were generated by pronase and also α-chymotrypsin digestions under CH (control), HP and MW irradiation. There are several peaks that appeared in all pronase chromatograms at retention times between 3 and 6 min, 11, 19, 21, 21.5 and 23 min. Some quantitative differences were observed in α-chymotrypsin chromatograms (Fig. 3). Thus, the relative concentration of

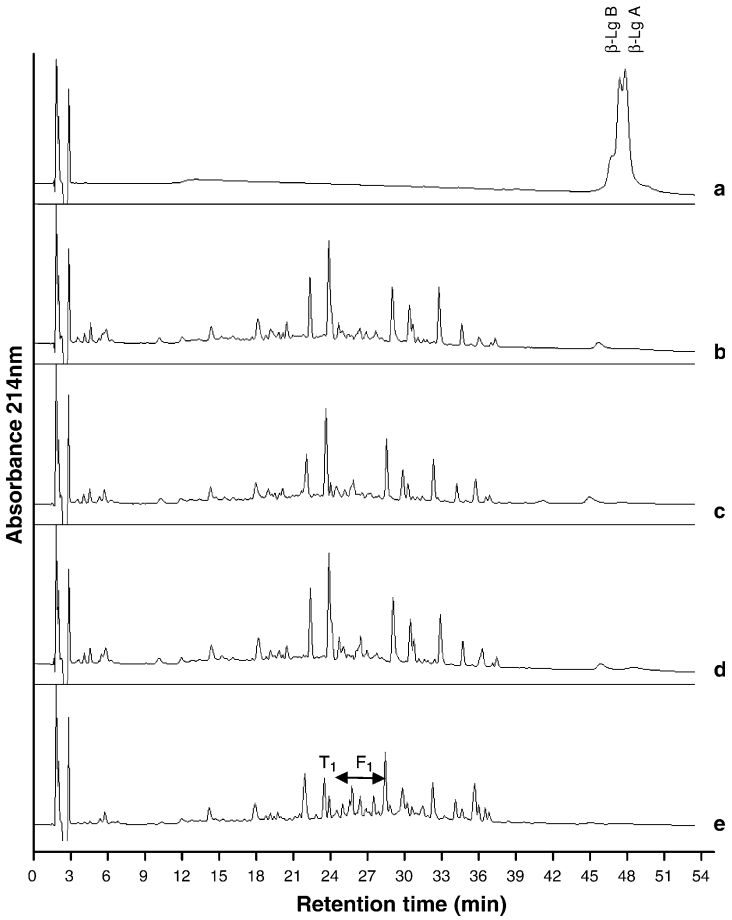

Fig. 3. Reverse-phase-HPLC chromatograms from β-Lg AB digested by chymotrypsin for 20 min under several conditions. (a) undigested protein, (b) conventional heating, at 40 °C and 0.1 MPa, (c) 100 MPa, (d) 200 MPa, (e) microwave irradiation, 15 W.

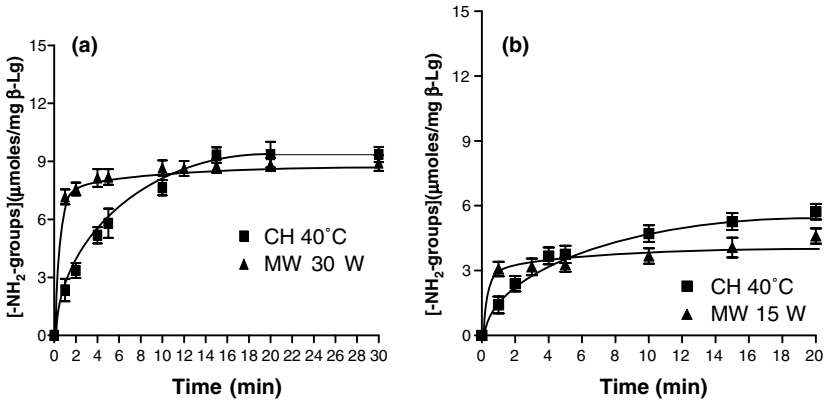

Fig. 4. Time course for enzymatic hydrolysis of β-Lg AB at 40 °C under conventional heating (CH) and microwave irradiation (MW) by (a) pronase and (b) chymotrypsin.

peak eluting at retention time of 24.5 min (T1) was lower in the MW-assisted digestion than in the CH digestion or in those performed under HP. The hydrolysates obtained under MW also show higher amount of peaks eluting from 24.5 to 29.5 min (F1) than those obtained under CH.

The results of the present work suggest that the specificity of the pronase and α-chymotrypsin remained unchanged upon the physical treatments applied. Their main effects seems to be related to the rate at which primary hydrolysis products were further hydrolyzed to end-products, the nature of them being the same. Dufour et al. (1995) reported that HP induced denaturation on β-Lg, which seems to facilitate the earliest stage of proteolysis

3.2. Time course for β-Lg hydrolysis under conventional heating and microwave irradiation

Fig. 4 shows reaction kinetics for β-lactoglobulin digestion by pronase and α-chymotrypsin under conventional heating (CH) and MW irradiation. This last no conventional heating results in an initial increase of the reaction rate. The MW irradiation improved pronase properties as regards to the digestion of β-Lg AB. The levels of [–NH2] groups released under MW in 1 and 4 min were similar to those released after 7 and 10 min of CH digestion, respectively. No significant differences ($p \geqslant 0.05$) in proteolisis were found between CH or MW digestion after 10 min.

The advantage of MW irradiation when compared to CH is less evident for chymotrypsin as described above for pronase, since significant ($p \leqslant 0.001$) higher releasing of [–NH2] groups under MW irradiation than in CH digestion was only observed the first minute of reaction, whereas the CH digestion was most effective after 10 min.

The results of reaction kinetics are consistent in qualitative terms with those described by Roy, Mondal, and Gupta (2003) and Roy and Gupta (2003) for MW-assisted enzymatic reactions. The first researcher group described that the treatment of chitin by MW works successfully for more efficient enzymatic hydrolysis of the macromolecule. According to authors this may be due to greater accesibility of the susceptible bonds in the microwave-irradiated chitin. Roy and Gupta (2003) reported that MW-irradiation increases by 2.1–4.7 times the initial reaction rates of transesterification of *N*-acetyl-L-phenyl alanine ethyl ester with propanol by subtilisin and esterification of *N*-acetyl-L-phenyl-alanine with etanol by α-chymotrypsin, carried out in six solvents of differing polarities and at three different temperatures.

In conclusion, the proteolysis of β-Lg AB by pronase was higher than by α-chymotrypsin, independently of physical treatment applied during the enzymatic digestion. MW and HP treatment may be effective in accelerating enzymatic hydrolysis of β.Lg, although the effect seems to be enzyme dependent and limited under the conditions tested. The specificity of the used enzymes has not been changed upon the physical treatments applied. The only apparent MW induced difference on protein digestion by chymotrypsin was in a lower relative concentration of peak eluting at 24.5 min, and conversion of this fraction into final peaks eluting from 24.5 to 29.5 min (F1 fraction).

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