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The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin

Ana Quirós, Rosa Chichón, Isidra Recio, Rosina López-Fandiño *

Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

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Abstract

Enzymatic hydrolysis of food proteins can release peptides able to exert different biological activities. Among the bioactive peptides known so far, those with angiotensin converting enzyme (ACE)-inhibitory properties are receiving special attention due to their potential beneficial effects in the treatment of hypertension. In a previous work, we identified active peptide sequences that derived from proteolysis of ovalbumin. We have now explored the possibility of using high hydrostatic pressure to promote the release of bioactive peptides. Treatment of ovalbumin under high pressures, up to 400 MPa, with chymotrypsin, trypsin and pepsin, enhanced its hydrolysis and changed the proteolytic pattern. However, under the conditions assayed, the *in vitro* ACE inhibitory activity of the hydrolysates did not improve as compared with those obtained at atmospheric pressure. Nevertheless, proteolysis under pressures of 200–400 MPa accelerated the release of the peptides YAEERYPIL, FRADHPFL and RADHPFL, with demonstrated antihypertensive effects *in vivo*.

Keywords: Ovalbumin peptides; Angiotensin I-converting enzyme; Antihypertensive activity; High pressure; Enzymatic treatment

1. Introduction

Bioactive peptides with angiotensin converting enzyme (ACE)-inhibitory properties can be enzymatically released from food proteins during food processing and/or gastrointestinal digestion (Li, Le, Shi, & Shrestah, 2004). ACE controls blood pressure because it is responsible for the generation of the vasoconstrictor agent angiotensin II and for the inactivation of the vasodilator agent bradykinin and thus, ACE inhibitors are effective in the prevention and treatment of essential hypertension (López-Fandiño, Otte, & van Camp, 2006).

The hydrolysis of crude egg white with pepsin, trypsin and chymotrypsin was shown to produce peptides with ACE-inhibitory activity *in vitro* that mainly derive from proteolysis of ovalbumin (Miguel, Recio, Gómez-Ruiz, Ramos, & López-Fandiño, 2004). Further experiments demonstrated that the hydrolysate of egg white (with pepsin) reduces blood pressure in spontaneously hypertensive rats (SHR) (Miguel, López-Fandiño, Ramos, & Aleixandre, 2005). This hydrolysate contains the potent ACE-inhibitors, YAEERYPIL, FRADHPFL and RADHPFL, that exhibit significant antihypertensive effects in SHR (Fujita, Sasaki, & Yoshikawa, 1995; Miguel, Aleixandre, Ramos, & López-Fandiño, 2006; Miguel et al., 2005; Miguel et al., 2004). Moreover, the long term intake of the hydrolysate of egg white with pepsin attenuates the development of hypertension in SHR, which suggests that it could be used as a functional food ingredient with antihypertensive activity (Miguel, López-Fandiño, Ramos, & Aleixandre, 2006).

Some proteins have been reported to exhibit an improved proteolytic digestibility when submitted to high hydrostatic pressure. This can be due to conformational changes in the protein that make it vulnerable to proteolysis because, under high pressure conditions, protein unfolding can expose new cleavage sites to enzymatic hydrolysis (Bonomi et al., 2003). In addition, enhancement of proteol-

* Corresponding author. Tel.: +34 91 5622900; fax: +34 91 5644853.
E-mail address: rosina@ifi.csic.es (R. López-Fandiño).

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ysis under pressure has also been attributed to pressure effects on the enzyme and/or to effects on the substrate–enzyme interaction (Stapelfeldt, Petersen, Kristiansen, Qvist, & Skibsted, 1996). The aim of this work was to elucidate whether the use of high pressure changes the proteolytic pattern of ovalbumin and to evaluate the impact of this treatment on the release of peptides with ACE-inhibitory and/or antihypertensive properties.

2. Materials and methods

2.1. Preparation of hydrolyzates from egg white proteins

Ovalbumin grade VI (99% purity), pepsin (EC 3.4.23.1, 570 U/mg, from pork stomach), trypsin (EC 3.4.21.4, TPCK treated 11,100 U/mg, from bovine pancreas) and chymotrypsin (EC 3.4.21.1, TLCK treated, 55 U/mg, from bovine pancreas) were purchased from Sigma Chemicals (St. Louis, MO, USA).

The substrate was dissolved at a concentration of 3.2 mg/ml in 50 mM citrate buffer, pH 2.5, for pepsin hydrolysis (E/S 1/100, wt/wt), or in 50 mM Tris–HCl buffer, pH 8.0, for trypsin and chymotrypsin hydrolyses (E/S 1/16, wt/wt). For pressure experiments, substrate and enzyme mixtures were immediately submitted to 100–400 MPa and 37 °C for different periods (5–60 min), using a 900 HP equipment (Eurotherm Automation, Lyon, France). Ovalbumin was also pressurized at both pHs, without enzyme, for 20 min. Control hydrolysis experiments were carried out at atmospheric pressure and 37 °C for different times up to 24 h. Pepsin was inactivated by increasing the pH to 7.0 with 2 M NaOH. Trypsin and chymotrypsin were inactivated by lowering the pH to 3.0 with 1 M HCl. Intact ovalbumin was separated from the hydrolysates by ultrafiltration through a hydrophilic 30,000 Da cut-off membrane (Centricon, Amicon Inc, Beverly, MA, USA) by centrifugation at 5000*g* for 30 min at 5 °C.

2.2. SDS–PAGE analyses

Ovalbumin samples, pressurized without enzyme, were immediately frozen and freeze-dried. The lyophilized protein was dissolved either in 10 mM Tris–HCl buffer, pH 8.0 (non-reducing conditions) or in the same buffer containing 2.5% SDS, 10 mM EDTA, and 5.0% β-mercaptoethanol (β-ME) (reducing conditions), and heated at 100 °C for 10 min. SDS–PAGE was performed in a PhastSystem Electrophoresis apparatus, with pre-cast High Density Gels and PhastGel SDS buffer strips (Amersham Biosciences, Uppsala, Sweden), following the electrophoretic and silver-staining conditions of the manufacturer.

2.3. Analysis by on-line RP-HPLC-MS/MS

RP-HPLC analysis of the fraction of the hydrolysates with molecular weight lower than 30,000 and peptide identification were performed on an Agilent HPLC system (Agilent Technologies, Waldbronn, Germany) connected on-line to a variable wavelength absorbance detector set at 220 nm, and to an Esquire-LC quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany), as described by Gómez-Ruiz, Ramos, and Recio (2004). The column was a 250 mm × 4.6 mm Widepore C_{18} (Bio-Rad, Richmond, CA, USA). Solvent A was 0.37 ml/l of TFA in milli-Q water and solvent B, 0.27 ml/l of TFA in acetonitrile. A linear gradient of solvent B in A, from 0% to 50% in 60 min, followed by 70% B for 5 min was used. Using Data Analysis™ (version 3.0; Bruker Daltoniks), the *m*/*z* spectral data were processed and transformed to spectra representing mass values. BioTools (version 2.1; Bruker Daltoniks) was used to process the MS(n) spectra and to perform peptide sequencing.

2.4. ACE-inhibitory activity

ACE-inhibitory activity was measured *in vitro* in the fraction of the hydrolysates with molecular weight lower than 30,000, following the spectrophotometric assay described by Cushman and Cheung (1971) with some modifications, as explained by Miguel et al. (2004). The substrate, hippuryl-histidyl-leucine and angiotensin converting enzyme (ACE) from rabbit lung (EC 3.4.15.1) were purchased from Sigma. Triplicate tests were performed for each sample. Inhibitory activity was expressed as the concentration needed to inhibit 50% of ACE activity (IC_{50}). For this purpose, protein concentration was determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) using bovine serum albumin as standard.

3. Results and discussion

3.1. High pressure-induced changes in ovalbumin

SDS–PAGE in the absence of reducing agents showed that pressurization of ovalbumin at pH 8.0 led to the formation of protein aggregates corresponding to dimers, with apparent molecular mass of, approximately, 90 kDa, and to higher polymers too large to enter the resolving gel (Fig. 1, lanes 5–7). Protein dimers were already present in the unpressurized ovalbumin, although at a lower level (Fig. 1, lane 1). Upon addition of the reducing agent, the high molecular mass bands almost disappeared, with an increase in the bands migrating in the area of monomeric ovalbumin and the appearance of a lower molecular mass component (between 20 and 30 kDa) (Fig. 1, lanes 12–14). The observation that the aggregates were effectively reduced in the sample buffer containing 2-mercaptoethanol indicates that they were stabilized by disulfide bonds. Native ovalbumin possesses one disulfide bridge and four free sulfhydryl groups, whose reactivity increases upon denaturation induced by temperature, and pH (Mine, 1996). Pressurization of ovalbumin at pH 2.5 did not give rise to protein polymers, probably as a result of the low reactivity of the sulphydryl groups at acidic pH (Fig. 1, lanes 2–4).

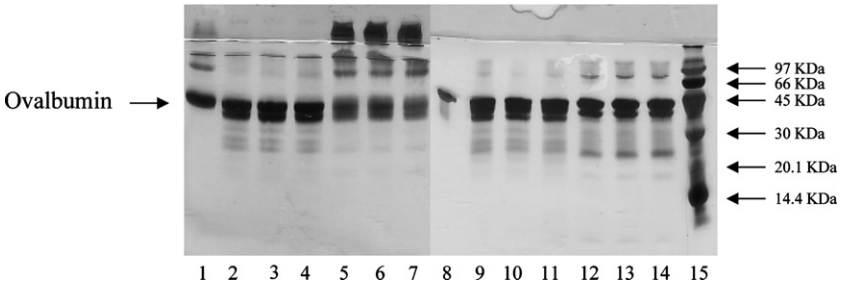

Fig. 1. SDS–PAGE patterns under non-reducing conditions of: native ovalbumin (1), ovalbumin pressurized for 20 min at 37 °C at 100 (2), 200 (3) and 300 (4) MPa and pH 2.5 and at 100 (5), 200 (6) and 300 (7) MPa and pH 8.0, and patterns under reducing conditions of: native ovalbumin (8), ovalbumin pressurized for 20 min at 37 °C at 100 (9), 200 (10) and 300 (11) MPa and pH 2.5 and at 100 (12), 200 (13) and 300 (14) MPa and pH 8.0. (1%). Low molecular mass calibration kit: α-lactalbumin, 14.4 kDa; trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 45 kDa; bovine serum albumin, 66 kDa; phosphorylase B, 97 kDa.

We did not observe precipitation of ovalbumin (3.2 mg/ml) during the pressure treatments up to 400 MPa at pH 2.5 or 8.0. Similarly, Iametti et al. (1998) did not find any decrease in the solubility of ovalbumin in 50 mM phosphate buffer pH 6.8, at pressures of 450 MPa and protein concentrations below 5 mg/ml. However, these authors reported the formation of insoluble aggregates at increasing pressure levels and protein concentrations. The insolubilization was prevented by blocking the accessible free sulphydryl groups of the protein before the high pressure treatments, which indicates that a disulfide exchange mechanism is involved in pressure-induced denaturation of ovalbumin (Iametti et al., 1998). Van der Plancken, Van Loey, and Hendricks (2005a, 2005b) also reported the formation of intermolecular disulfide bonds during pressurization of egg white solutions at pH 7.6 that resulted in the formation of aggregates. It should be mentioned that several ingredients, such as sucrose and NaCl, were found to protect against pressure-induced modifications of ovalbumin (Iametti et al., 1998). Thus, pressurization, at 400 and 600 MPa, of albumen samples in the presence of 1.7 M NaCl prevented the formation of protein aggregates stabilized by disulfide interactions, giving rise to SDS–PAGE patterns identical to that of the fresh albumen (Iametti et al., 1999).

On the other hand, in our study we observed other deep changes in the SDS–PAGE pattern of pressurized ovalbumin. After pressurization, at both pH 8.0 and pH 2.5, in the separation under non-reducing conditions, a second band was observed with lower apparent molecular mass than monomeric ovalbumin (Fig. 1, lanes 2–7). This altered band pattern was maintained under reducing conditions (Fig. 1, lanes 9–14), which suggests that, in addition to disulfide bonding, high pressure promotes other structural changes in ovalbumin that change its electrophoretic mobility.

3.2. Effects of high pressure on the susceptibility of ovalbumin to the proteolytic attack

Pressurization of ovalbumin during the enzyme treatments considerably accelerated the hydrolysis with the three enzymes tested. In particular, the susceptibility to proteolysis by chymotrypsin (Fig. 2a and b) and trypsin (results not shown), which attack this protein very slowly at atmospheric pressure, was considerably enhanced by the application of pressures of 300–400 MPa for 60 min. The comparison between the RP-HPLC chromatograms of ovalbumin treated with trypsin and chymotrypsin at atmospheric and high pressure revealed mainly quantitative differences, with an increased level of all the proteolysis products when the hydrolyses were conducted under high pressure. High pressure also accelerated ovalbumin hydrolysis by pepsin, leading to changes in the peptide pattern, since hydrophobic, late eluting peptides, were predominant in the chromatograms, particularly at the initial stages of hydrolysis under high pressure conditions (Fig. 2c and d). These peptides were further cleaved as the hydrolysis proceeded (results not shown).

Pressure-induced inactivation of trypsin and pepsin has been reported to occur at 400 MPa (Dufour, Hervé, & Haertle, 1994; Maynard, Weingand, Hau, & Jost, 1998), while the activity of chymotrypsin is greatly enhanced at 360 MPa (Mozhaev, Lange, Kudrashova, & Balny, 1996). However, despite the different specificities and pressure sensitivities of the enzymes used, it is likely that, as has been found for other substrates, such as β-lactoglobulin, the effects of high pressure mainly result from changes in ovalbumin conformation (Bonomi et al., 2003; Stapelfeldt et al., 1996). High pressure may promote structural events that could favour proteolysis. In fact, it has been found that pressurization increases the surface hydrophobicity of egg-white proteins (Van der Plancken et al., 2005a). High pressure treatment of ovalbumin increases the solvent exposure of aromatic residues. Furthermore, when the protein is subjected to treatments at pH 3.0, it is less stable to pressure than at neutral pH, so that disclosure of the hydrophobic core is observed at much lower pressures (Smith, Galazka, Wellner, & Summer, 2000).

Several studies have dealt with the proteolysis at atmospheric pressure of ovalbumin that was previously pressure-treated. Iametti et al. (1998) found that treatments, at 600 and 800 MPa, of ovalbumin did not modify the sus-

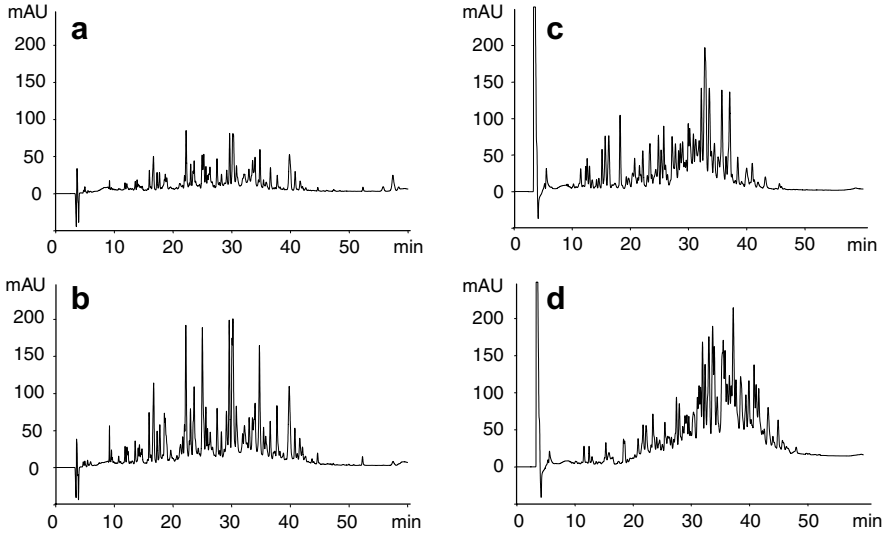

Fig. 2. RP-HPLC analyses of the fractions with molecular weight lower than 30,000 corresponding to ovalbumin hydrolysed with: (a) chymotrypsin for 1 h at 0.1 MPa; (b) chymotrypsin for 1 h at 400 MPa; (c) pepsin for 8 h at 0.1 MPa and (d) pepsin for 20 min at 200 MPa.

ceptibility of the pressurized protein to trypsin, probably due to the pressure-induced insolubilization of the protein. However, when the pressure treatments were conducted in the presence sucrose or NaCl, that made the protein lose its compactness but remain in a soluble form, the sensitivity to trypsin of pre-pressurized ovalbumin and diluted egg white was greatly increased (Iametti et al., 1999; Iametti et al., 1998). Similarly, Van der Plancken, Delattre, Indrawati, Van Loey, and Hendricks (2004) and Van der Plancken et al. (2005a) reported a pressure- and temperature-dependent increase in the susceptibility of pre-pressurized egg white solutions, containing 0.15 M NaCl, to enzymatic hydrolysis by trypsin and chymotrypsin, but mainly at pressures above 400 MPa at 25 °C. In comparison with those experiments, our results at basic pH indicate that simultaneous high pressure and enzyme treatments up to 400 MPa are more effective than pre-pressurization for the proteolysis of ovalbumin, as was found for other proteins, such as β-lactoglobulin. It is hypothesized that, when the enzyme treatments are conduced under pressure, unfolding of the hydrophobic core exposes new cleavage sites to enzymatic hydrolysis, so that proteolysis precedes protein aggregation by disulfide bonds that would otherwise diminish the accessibility to the hydrolytic enzymes (Chicón, Belloque, Recio, & López-Fandiño, 2006; Chicón, López-Fandiño, Quirós, & Belloque, 2006).

3.3. ACE-inhibitory activity of the hydrolysates and formation of antihypertensive peptides

An increase in the ACE-inhibitory activity was observed when hydrolysis of ovalbumin was conducted with chymotrypsin under high pressure, as compared with atmospheric pressure (Table 1), while none of the hydrolysates with

Table 1
ACE-inhibition, expressed as the protein concentration needed to inhibit 50% of the enzyme activity (IC_{50}) and protein concentration, determined by the BCA assay, corresponding to the fractions with molecular weight lower than 30,000 of the hydrolysates of ovalbumin with pepsin and chymotrypsin

Enzyme	Hydrolysis conditions	IC_{50} (μg/ml)	Protein (mg/ml)
Chymotrypsin	0.1 MPa, 1 h	84.5	0.35
Chymotrypsin	200 MPa, 1 h	63.5	0.55
Chymotrypsin	300 MPa, 1 h	62.5	0.74
Chymotrypsin	400 MPa, 1 h	84.5	0.69
Pepsin	0.1 MPa, 20 min	36.8	0.33
Pepsin	0.1 MPa, 1 h	30.0	0.39
Pepsin	0.1 MPa, 3 h	17.0	0.52
Pepsin	0.1 MPa, 5 h	14.9	0.60
Pepsin	0.1 MPa, 8 h	9.00	0.70
Pepsin	0.1 MPa, 24 h	22.5	0.88
Pepsin	100 MPa, 20 min	26.7	0.42
Pepsin	200 MPa, 20 min	38.5	0.67
Pepsin	200 MPa, 1 h	37.0	1.05
Pepsin	300 MPa, 20 min	50.0	1.10
Pepsin	300 MPa, 1 h	45.3	1.23
Pepsin	400 MPa, 1 h	44.0	1.26

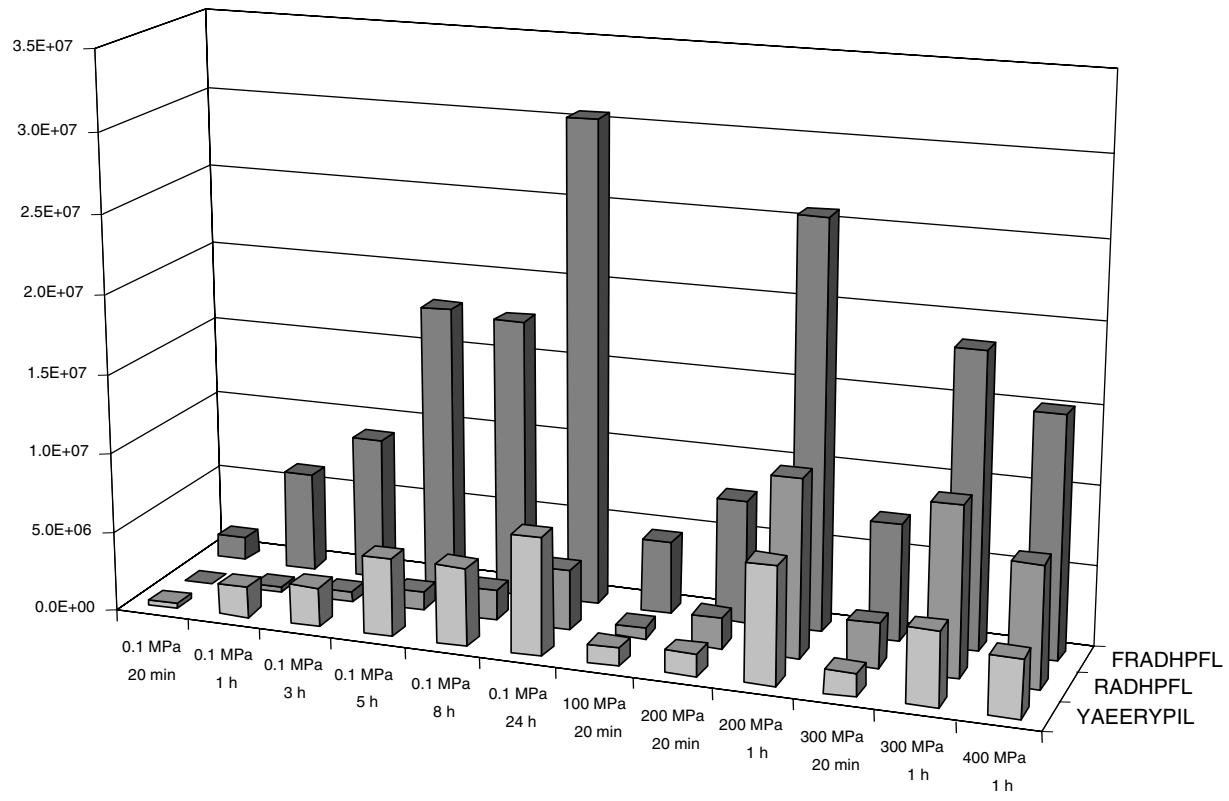

Fig. 3. Estimated amount of peptide (in arbitrary units) after ion extraction of their corresponding characteristic ions (molecular ion and doubly charged ion, when present) in the fractions with molecular weight lower than 30,000, corresponding to ovalbumin hydrolysed with pepsin at 0.1 MPa and at 100, 200, 300 and 400 MPa for different times.

trypsin exerted a relevant inhibition (results not shown). The IC_{50} of ovalbumin hydrolysed with pepsin at atmospheric pressure decreased with the incubation time up to 8 h (Table 1). Even though simultaneous pressurization enhanced proteolytic degradation by pepsin, as explained above, it did not significantly increase the ACE-inhibitory activity. Furthermore, looking at the IC_{50} of ovalbumin hydrolysed with pepsin at different pressure levels and time periods, it can be seen that there was not a direct relationship between the degree of proteolysis of ovalbumin (as shown by the release of protein products with molecular weight lower than 30,000) and the ACE inhibitory activity of the hydrolysates. These results illustrate that the specificity of the proteinase used to release the peptides plays a very important role and that there is an optimum degree of proteolysis that leads to hydrolysates with high ACE inhibition.

Previous studies have highlighted a lack of correlation between the ACE inhibitory activity *in vitro* and the antihypertensive action, because the measurements *in vitro* do not take into consideration the physiological transformations that determine the bioavailability of the peptides and because there might be other mechanisms of action different from ACE inhibition (López-Fandiño et al., 2006). For these reasons, we followed, in the hydrolysates with pepsin, the formation of previously identified peptides with antihypertensive activity demonstrated in SHR, such as YAEERYPIL, FRADHPFL and RADHPFL (Fujita et al., 1995; Miguel et al., 2004). For this purpose, we analysed the fractions with molecular weight lower than 30,000 by HPLC-MS/MS and extracted the characteristic ions of the peptides of interest. Quantification of the area of the extracted ions gave us an estimation of the amount of these peptides present in the 30,000 Da permeates.

Fig. 3 shows the estimated amount of the peptides FRADHPFL, RADHPFL and YAEERYPIL in the hydrolysates of ovalbumin with pepsin obtained after different time periods at atmospheric pressure and at different pressure levels. In general terms, proteolysis under high pressure promoted the release of these sequences. Higher amounts of FRADHPFL, RADHPFL and YAEERYPIL were produced after 1 h of hydrolysis under 200–400 MPa than after 8 h of hydrolysis at atmospheric pressure. The amounts of these selected peptides, shown in Fig. 3, did not correlate with the IC_{50} values of the corresponding hydrolysates (Table 1), which indicates that, in addition to these sequences, other peptides contribute to the *in vitro* ACE-inhibitory activity of the hydrolysates.

4. Conclusions

Simultaneous high pressure and enzymatic treatment of ovalbumin up to 400 MPa, with pepsin, chymotrypsin and trypsin, considerably promoted its hydrolysis. We found

quantitative differences in the proteolysis pattern, particularly at acidic pH, that were consistent with a higher accessibility of the enzyme to hydrophobic regions of the substrate exposed during pressurization. At basic pH, ovalbumin also exhibited an enhanced susceptibility to the hydrolytic enzymes under high pressure, despite the fact that these treatments, in the absence of enzyme, induce protein aggregation by disulfide bonds. Even if ovalbumin hydrolysis with the three enzymes tested was accelerated under the conditions assayed, the *in vitro* ACE inhibitory activity of the hydrolysates did not improve as compared with those obtained at atmospheric pressure, pointing that there is an optimum degree of hydrolysis that determines a high concentration of peptides with ACE-inhibitory properties. However, the hydrolysates obtained are complex mixtures of peptides, many of which may not exhibit effects *in vivo*. In this respect it should be indicating that proteolysis with pepsin under pressures of 200–400 MPa accelerated the release of the peptides YAEERYPIL, FRADHPFL and RADHPFL, with demonstrated antihypertensive effects in SHR. This suggests that hydrolysis under high pressure can be used to promote the proteolysis of ovalbumin for the quick production of specific active sequences.

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