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Potential antioxidant peptides produced from whey hydrolysis with an immobilized aspartic protease from *Salpichroa origanifolia* fruits

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ABSTRACT

An aspartic protease from *Salpichroa origanifolia* fruits was successfully immobilized onto an activated support of glutaraldehyde agarose. The immobilized enzyme presented higher thermal stability than the free enzyme from 40 °C to 50 °C and high reusability, retaining 54% of the initial activity after ten cycles of the process. Whey protein concentrates (WPC) were hydrolyzed with both free and immobilized enzyme, reaching a similar degree of hydrolysis of approximately 6–8% after 20 h. In addition, the immobilized derivate hydrolyzed α-lactalbumin protein with a higher affinity than β-lactoglobulin. The hydrolysate was ultra-filtrated, and the fractions were evaluated for antioxidant activities with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity method. The fraction containing peptides with a molecular mass below 3 kDa demonstrated a strong radical quenching effect (IC_{50}: 0.48 mg/ml). These results suggest that hydrolyzed WPC could be considered as a promising source of natural food antioxidants for the development of functional food.

1. Introduction

Whey is the most important by-product of the dairy industry; it is obtained during cheese manufacturing from milk, specifically during the coagulation process. Due to the huge production of whey and its high organic content, this liquid is commonly regarded as an environmental problem whose disposal causes great difficulty for the dairy industry (Lamas, Barros, Balcão, & Malcata, 2001). Whey by-products contain more than half of the solids present in the original milk (20% protein), and can therefore be exploited as a resource for a number of valuable end products rather than as a waste stream (Tavares et al., 2011). This is especially critical in small factories, which require economically feasible alternative ways to reutilize whey proteins. Recently, the development of biotechnological processes to isolate or concentrate added-value proteins and peptides has acquired a special relevance. Several studies have reported that these milk proteins constitute an important source of biologically active peptides. Such peptides are inactive within the sequence of the original protein and can be released in three ways: (a) through hydrolysis by digestive enzymes, (b) through hydrolysis by proteolytic microorganisms and (c) through the action of proteolytic enzymes derived from microorganisms or plants (Korhonen, 2006; Madureira, Tavares, Gomes, Pintado, & Malcata, 2010). Biopeptides are defined as specific protein fragments with a beneficial impact on body functions or conditions that influence human health (Kitts & Weiler, 2003). These peptides can affect a large variety of activities, such as antimicrobial, antithrombotic, antihypertensive, immunomodulatory and antioxidative activities (Capriotti, Cavaliere, Piovesana, Samperi, & Laganà, 2016; Hernández-Ledesma, Recio, & Amigo, 2008; Urista, Fernández, Rodriguez, Cuenca, & Jurado, 2011; Wada & Lönnerdal, 2014).

Whey proteins have been determined to confer antioxidant effects, due to bioactive peptides. Recent studies have shown that antioxidative peptides scavenge free radicals and protect the body from the onslaught of stressors (Elias, Kellerby, & Decker, 2008; Lollo et al., 2014). The link between oxidative stress and metabolic/neurodegenerative diseases has been consistently demonstrated (Patel, 2015). Antioxidants can protect against damage from reactive oxygen species that alter membrane lipids, proteins and DNA and play important roles in cardiovascular, diabetes mellitus, cancer and Alzheimer diseases (Valko et al., 2007). In addition, antioxidant peptides are gaining interest because the use of synthetic antioxidants is under strict regulation due to the potential negative health effects caused by such compounds. Moreover, lipid peroxidation is a great concern in the food industry, as deterioration of food quality has been identified due

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to oxidation of lipids and the formation of secondary lipid peroxidation products (Shahidi & Zhong, 2010).

Recently, we isolated and characterized an aspartic protease (AP) from *Salpichroa origanifolia* fruits, named salpichroin, that hydrolyzes natural substrates, including casein, gelatin and hemoglobin (Rocha et al., 2015). We also reported that casein hydrolysates demonstrated antimicrobial activity (Rocha, Kise, Rosso, & Parisi, 2013).

The use of enzymes from plants in industrial processes is limited by the availability of the plant material. Therefore, the immobilization techniques, despite the cost, constitutes an economically viable alternative for these processes because they can be recycled and reused (del Mar Yust, Pedroche, del Carmen Millán-Linares, Alcaide-Hidalgo, & Millán, 2010). The immobilization of enzymes on solid supports, such as glutaraldehyde agarose, has been used to increase the stability and enable the reuse of these enzymes (Betancor et al., 2006; Lamas et al., 2001; Pessato et al., 2016; Tavano, Fernandez-Lafuente, Goulart, & Monti, 2013).

The aim of this study was to immobilize an AP from *Salpichroa origanifolia* fruits on glutaraldehyde agarose supports and to evaluate the effect of enzymatic hydrolysis of whey proteins on their potential antioxidant properties.

2. Materials and methods

2.1. Materials

Commercial WPC Lacprodan80™ with a total protein content of 80% was provided by ARLA FOODS INGREDIENTS S.A. (Buenos Aires, Argentina). Casein from bovine milk, agarose 6 BCL, glutaraldehyde, sodium tetrahydroborate, o-phthalaldehyde, L-methionine, β-mercaptoethanol and 2,2-diphenyl-1-picrylhydrazyl were purchased from Sigma-Aldrich Chemical Co. Ltd (Saint Louis, USA). All other chemicals were of analytical grade.

2.2. Plant material

Ripe fruits from *S. origanifolia* were collected from plants grown near Luján city (Buenos Aires, Argentina). The mature fruits were approximately 2 cm long and looked like small white eggs. The fruits were thoroughly washed with deionized water after they were collected and were then stored at 4 °C until use.

2.3. Crude extract

Fresh and mature fruit were ground in 50 mM phosphate buffer, pH 7.0, and centrifuged at 20,000*g* for 20 min at 4 °C (SS-34 Sorvall rotor). The supernatant was diluted with two volumes of cold ethanol at −20 °C and kept for 2 h with gentle stirring for complete precipitation before vacuum filtration. The precipitate was dissolved in the same buffer and then centrifuged at 20,000*g* for 20 min at 4 °C. The partially purified supernatant called 'crude extract' was stored at −20 °C for further analysis (Rocha et al., 2015).

Protein concentration was determined by the Bradford method (1976) using bovine serum albumin (BSA) as a standard and expressed as mg/ml.

2.4. Activity assay

Proteolytic activities with hemoglobin and casein as protein substrates were assayed on the following enzyme preparations: crude extract, pure enzyme, supernatant of the immobilization mixture and immobilized enzyme.

Proteolytic activity against hemoglobin as a natural substrate was determined according to Anson (1938) with slight modifications. The enzyme preparation (0.2 ml) was incubated for 10 min (40 °C) with 0.2 ml 1.0% (w/v) acid-denatured bovine hemoglobin in 100 mM citrate buffer, pH 4.0. The reaction was stopped by adding 1.0 ml of 5% (w/v) trichloroacetic acid (TCA). The reaction mixture was allowed to settle for 15 min, and then, it was centrifuged at 16,000*g* for 10 min to remove the precipitate. The acid soluble material (0.5 ml) was added to Folin reagent (0.3 ml) in 0.5 N NaOH, and the absorbance at 750 nm was spectrophotometrically estimated. An arbitrary enzyme unit (U_{Hem}) was defined as the increase in absorbance of 0.001 per minute at 750 nm across a 1 cm path length, under the assay conditions.

Caseinolytic activity was measured with casein as protein substrate, as previously performed (Parisi, Moreno, & Fernández, 2008). The enzyme preparation (0.1 ml) in 50 mM acetate buffer, pH 6.0, was added to 0.9 ml of 0.5% (w/v) casein. After a 30 min incubation at 40 °C, the reaction was stopped with the addition of 1 ml of 5.0% (w/v) TCA. The reaction mixture was settled for 15 min and then centrifuged at 16,000*g* for 10 min to remove the precipitate. Soluble products were determined in 1.0 ml of the supernatant by measuring the absorbance at 280 nm. An arbitrary enzyme unit (Ucas) was defined as the increase in absorbance of 0.001 per minute at 280 nm across a 1 cm path length, under the assay conditions.

2.5. Preparation of glutaraldehyde agarose support

Agarose 6 BLC was esterified with glycidol. The resulting glyceryl-agarose gels were further oxidized with sodium periodate to obtain glyoxyl-agarose gel. Then, MANAE-agarose supports (monoaminoethyl-*N*-aminoethyl-agarose) were prepared by amination of the glyoxyl-agarose beads with ethylenediamine. For each gel, 1 g of glyoxyl-agarose was added to 4 ml of a 2 M ethylenediamine solution, pH 10.0 (previously cold prepared). The suspension was gently stirred for 2 h at room temperature. Sodium borohydride (57.1 mg) was added to the suspension, and it was stirred for another 2 h. The MANAE-agarose beads were washed with 100 mM acetate buffer, pH 4.0, and then washed with 100 mM borate buffer, pH 9.0, and deionized water (Lamas et al., 2001).

Glutaraldehyde agarose was prepared by mixing 10 ml of MANAE-agarose support (Barros et al., 2003) with 20 ml of 25% (v/v) glutaraldehyde in 200 mM phosphate buffer, pH 7.0. The resulting suspension was kept under mild stirring at 25 °C for 12 h. Then, the supports were filtered and washed exhaustively with 25 mM phosphate buffer, pH 7.0, and then with deionized water. The modified support was stored at 4 °C until use (Betancor et al., 2006).

2.6. Enzyme immobilization

Crude extracts were diluted with 25 mM phosphate buffer, pH 7.0, to a final volume of 20 ml, containing 2000 Ucas. Glutaraldehyde agarose support (10 g) was added to the crude extract solution. The resulting suspension was gently stirred at 25 °C. To discontinue the immobilization procedure (when the activity of the supernatant was sufficiently low), the derivative was reduced with the addition of 1 mg of $NaBH_4$ per milliliter of gel (to transform the Schiff's bases obtained into covalent bonds, therefore strengthening the bonds between enzyme and support), followed by mild paddle-agitation for 30 min. After this period, the derivative was washed with 100 ml of deionized water. The modified support was then stored at 4 °C (Barros et al., 2003).

The immobilization procedure of salpichroin on glutaraldehyde agarose supports at pH 7 was monitored by the decrease of the proteolytic activity remaining in supernatant, compared with the activity of the blank sample (which consisted of the crude extract

without the glutaraldehyde agarose support). Samples of the supernatant were periodically withdrawn and assayed for activity, together with the blank.

The covalent immobilization was confirmed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to Bezbradica, Mateo, & Guisan (2014). The derivatives with the immobilized enzyme were boiled in the disruption buffer (which contains mercaptoethanol and SDS) with 1 M ethylenediamine, thus releasing any reversibly bound protein from the support. Gel electrophoresis was performed with disrupted glutaraldehyde agarose derivatives, crude extract, pure salpichroin and the selected low molecular weight standards. Samples were analyzed in 12% polyacrylamide PAGE gels using silver staining.

2.7. Biochemical characterization of the immobilized enzyme

The effect of pH, thermal stability and reuse capacity of the immobilized enzyme were studied. Equal units of proteolytic activity against casein and hemoglobin units (Ucas or Uhem) of free and immobilized enzymes were used in all the assays.

The effect of different pH values on the proteolytic activity of the immobilized enzyme was studied using both casein and hemoglobin as protein substrates. The pH of the reaction mixtures were adjusted with the following buffers: 50 mM glycine-HCl buffer (pH 2.0–2.5), 50 mM citrate buffer (pH 3.0–6.0) and 50 mM phosphate buffer (pH 6.5–8.0).

Thermal stability was determined incubating the immobilized enzyme (50 mM phosphate buffer, pH 6.0) at 40 °C and 50 °C for 15, 30, 60, 90 and 120 min. The residual caseinolytic activity was determined and compared with the initial activity at each assayed temperature.

The reuse capacity of the immobilized enzyme was analyzed by measuring the residual activity after ten operational cycles, using casein as the substrate. In each cycle, the substrate was hydrolyzed for 2 h at 40 °C. To estimate the residual activity in the other cycles, the initial activity was considered to be 100%.

2.8. Preparation of pre-heated WPC

Stanciuc, Hintoui, Stanciu, and Rapeanu (2010) demonstrated that WPC is more heat-sensitive for hydrolysis at 75 °C, confirming that the substrate nature is changing from dimmer to monomer when the pH is adjusted at neutral and alkaline values, with the exposure of hydrophobic residues. This result was confirmed with salpichroin (data not shown).

WPC at a final concentration of 0.25% (w/v) in 50 mM phosphate buffer, pH 8.0, was heated at 75 °C for 15 min. After heating, the suspension was allowed to cool down to room temperature and the pH was adjusted to 4.0 with 0.5 M HCl. The solution was made fresh for each use.

2.9. Enzymatic hydrolysis

WPC substrate was equilibrated at 40 °C for 30 min. Free and immobilized enzymes were then added at a final enzyme/substrate ratio of 1 Ucas mg^{-1}. The reaction mixture was incubated at 40 °C and 100 rpm for 20 h. Samples were withdrawn in duplicate at the selected time intervals (0, 1, 2, 4 and 20 h) and then freeze dried and kept at −20 °C for further analysis.

The degree of hydrolysis (DH) was determined by the method described by Nielsen, Petersen, and Dambmann (2001).

2.10. Size-exclusion chromatography

Protein profiles of WPC samples (undigested and pre-heated) and the hydrolysates were analyzed by size-exclusion chromatography on an FPLC system (SEC-FPLC). The data processing was completed with Unicorn™ software (GE). Lyophilized samples were dissolved in deionized water to a final concentration of 5 mg/ml and filtered through 0.20 μm Minisart SFCA sterile filters (Sartorius Stedim Biotech GmbG, Goettingen, Germany). Samples (0.1 ml) were loaded onto a Superdex™ 75 column 10 × G 300 mm (GE Healthcare Uppsala, Sweden), connected to an FPLC system (ÄKTAPurifier 100/10; GE Healthcare Life Science, Baied'Urfé, QC, Canada), and eluted with 50 mM phosphate buffer, pH 7.0, with 0.15 M NaCl at a flow rate of 0.5 ml min^{-1} at 20 °C. Protein and peptides were detected by measuring the absorbance at 280 nm.

2.11. Hydrolysate separation by ultrafiltration

The hydrolysates obtained after 20 h of hydrolysis were filtered sequentially through two ultrafiltration membranes with molecular mass cut-off of 10 and 3 kDa using an ultrafiltration cell (Model 8050, Amicon, USA). Three fractions were separated: fraction I with molecular mass higher than 10 kDa, fraction II with molecular mass from 3 to 10 kDa and fraction III with molecular mass lower than 3 kDa. The fractions were lyophilized and stored at −20 °C until further analysis. The peptide concentration was determined by the Lowry, Rosebrough, Farr, and Randall (1951) method using BSA as the standard. All assays were performed in triplicate.

2.12. In vitro radical scavenging activity of the hydrolysates

The ability to scavenge DPPH free radicals was determined based on the method of Brand-Williams, Cuvelier, and Berset (1995) with minor modifications. Potential antioxidant solutions of the different hydrolysate fractions (0.5 ml) were added to a 0.2 ml DPPH solution. A control containing the same volume of solvent instead of the sample was used to measure the maximum DPPH absorbance. The mixture was vigorously shaken and incubated in darkness at room temperature for 30 min. After incubation, the concentration of the remaining DPPH was recorded by measuring the absorbance at 517 nm.

The DPPH radical scavenging (S%) was calculated from the following equation:

$$S\ \% = \frac{(\text{Acontrol} - \text{Asample})^{*}}{\text{Acontrol}}\,100,$$

where Acontrol was the absorbance of the control and Asample was the absorbance of each individual sample (Ştef et al., 2009).

Fractions I, II and III were suspended in deionized water at a final concentration from 1.0 to 10.0 mg/ml. The IC_{50} values were determined by plotting the percentage inhibition as a function of the fractions concentration expressed in mg/ml. Each sample was analyzed in triplicate.

The IC_{50} value was defined as the antioxidant compound concentration required to scavenge 50% of the DPPH radical.

2.13. Statistical analysis

Statistical analyses (ANOVA) were performed with GraphPad Prism v .01 (http://www.graphpad.com/scientific-software/prism/). Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Immobilization on glutaraldehyde agarose support

Partially purified crude extracts from ripe fruits of *Salpichroa origanifolia* were obtained by precipitation with cold ethanol (−20 °C). Only one proteolytic enzyme belonging to the aspartic

protease family was extracted by this method. The enzyme was called salpichroin. In previous works, it was found that the purified enzyme showed a low thermal stability at moderate temperatures (Rocha et al., 2015). In this work, immobilization techniques were developed to produce an increase in the stability of salpichroin.

General conditions for protease immobilization were selected in relation to the physicochemical characteristics of the enzyme. Crude extract preparations were used to avoid possible enzyme denaturation, as the AP salpichroin was inactivated at alkaline pH (Rocha et al., 2015). For this reason, glyoxyl-agarose could not be used as supports (Fernández-Lorente et al., 2015). The immobilization on glutaraldehyde agarose beads at neutral pH was selected.

Enzyme preparation (crude extract) was fully stable in the immobilization conditions, that way the immobilization rate may be directly related to the decrease of the activity in the supernatant. For these reasons, the immobilization of the AP salpichroin was monitored by comparing the proteolytic activity of the supernatant of the immobilization mixture (containing the free unbound enzyme) and the blank. According to these results, thirty minutes were sufficient to achieve the maximum amount of enzyme immobilization (66%); however, an additional time of 20 h was used to increase the covalent attachment between the support and the enzyme (data not shown). When the environment had low ionic strength, the enzyme was first adsorbed onto the support through the negatively charged residues of the surface and then reacted via the terminal amino group (Barros et al., 2003). The immobilization course was similar to that reported by Bezbradica et al. (2014) for trypsin immobilization on the same support. Alternatively, the immobilization of cardosin on glutaraldehyde agarose was slower (Lamas et al., 2001).

The formation of covalent bonds between the solid support and the enzyme at the end of the immobilization was confirmed by SDS-PAGE. No bands whatsoever could be found in the gel lane corresponding to the derivative, thus providing evidence for the irreversibility of the immobilization procedure.

3.2. Biochemical characterization of the immobilized enzyme

The effect of pH on the activity of free and immobilized salpichroin was analyzed with hemoglobin in the pH range from 2.0 to 8.0, and casein for the pH range from 6.0 to 8.0 (Fig. 1). The profiles of free and immobilized enzyme with both substrates were similar. The optimum pH observed for hemoglobin was pH 4.0, while pH 6.0 was optimum for casein.

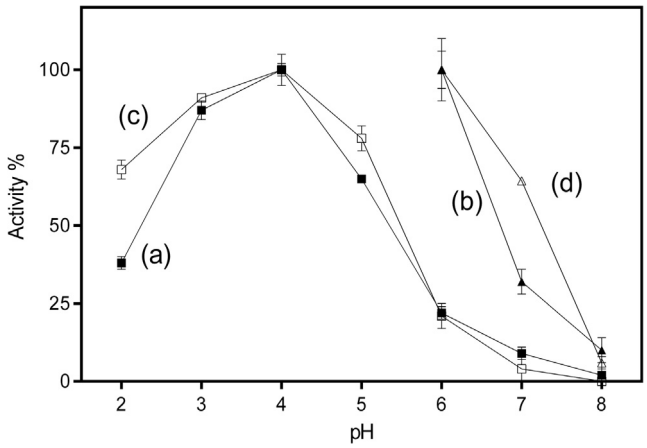

Fig. 1. Effect of pH on proteolytic activity of free enzyme (a) with hemoglobin and (b) with casein and the immobilized enzyme (c) with hemoglobin and (d) with casein as substrates (error bars represent ± standard deviations, n = 3).

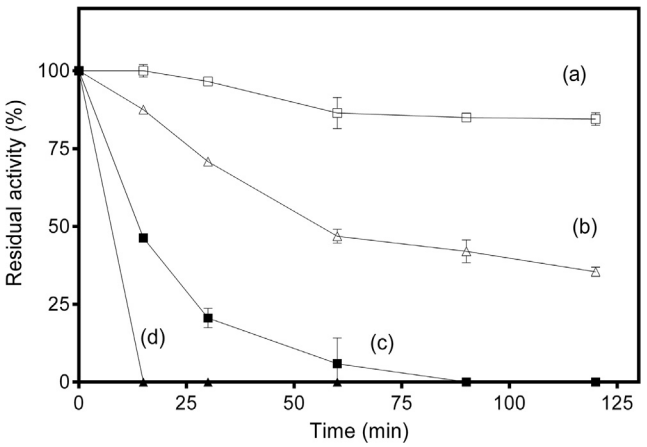

Fig. 2. Thermal stability of immobilized enzyme at (a) 40 °C and (b) 50 °C and free enzyme at (c) 40 °C and (d) 50 °C in 50 mM phosphate buffer, pH 6.0. Activity was measured with casein as the substrate (error bars represent ± standard deviations, n = 3).

The thermal stability of both the free and immobilized salpichroin was also evaluated. Fig. 2 demonstrates that the immobilization process significantly increased the stability of the free enzyme. After 15 min at 50 °C, the free enzyme was completely inactivated, while the immobilized enzyme retained 88% of the initial activity. Similarly, after a 60 min incubation at 40 °C, the free salpichroin only retained 6% of the initial activity, while the immobilized derivate retained 96% of the initial enzymatic activity. Rigidification of the enzyme structure via multipoint attachment and reduction of autolysis are the likeliest explanations for these results (Rocha el al., 2015; Tavano et al., 2013 and Lamas et al., 2001).

The application of biocatalysts in industry requires a high operational stability of the immobilized enzymes. To evaluate the operational stability of the immobilized salpichroin, the capacity of reuse was determined for ten cycles. Fig. 3 demonstrates that the immobilized enzyme retained 54.0 of the initial activity after ten consecutive cycles of use. Thus, the immobilized enzyme could be reused for several cycles without substantial loss of activity. A decrease in the enzyme activity during repeated use might be due to the frailty associated to agarose structure and the behaviour of these gels under stirring (Mateo et al., 2006).

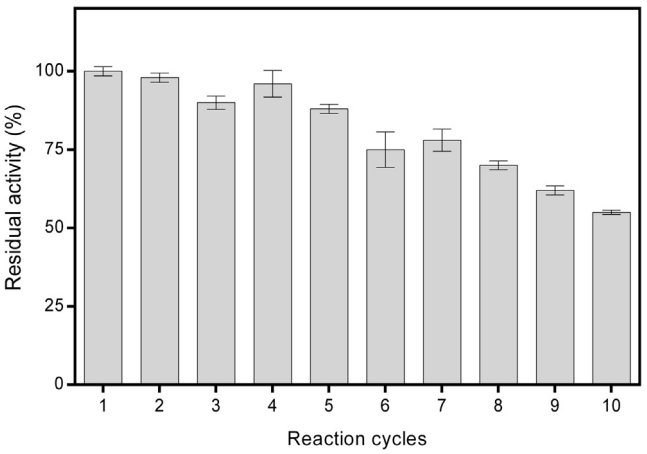

Fig. 3. Reusability of immobilized enzyme, using casein as substrate (error bars represent ± standard deviations, n = 3).

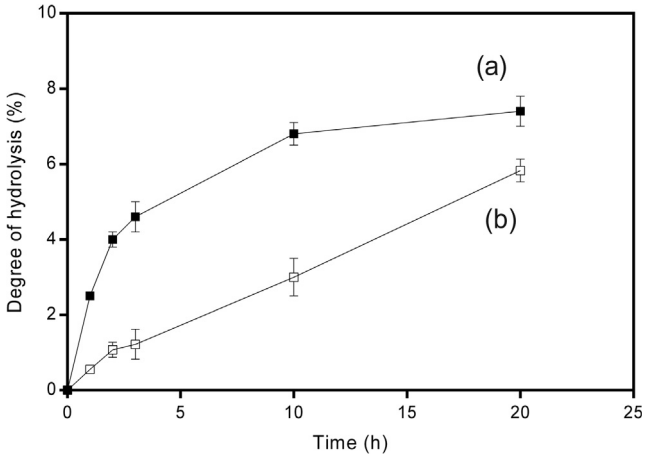

Fig. 4. Degree of hydrolysis of Whey protein concentrate with (a) free and (b) immobilized enzyme (error bars represents ± standard deviations, n = 3).

3.3. WPC hydrolysis

WPC hydrolysis with free and immobilized enzyme was compared. The DH achieved with free enzyme increased rapidly during the first 3 h and then remained constant between 10 and 20 h (Fig. 4). In contrast, when the immobilized enzyme was used, the course of hydrolysis increased progressively over 20 h. DH differences arising from free and immobilized enzyme during WPC hydrolysis were statistically significant ($p \leq 0{,}05$) in favour of the free enzyme. However, these differences were much less pronounced at the end of the hydrolysis (20 h). In this case, the DH values obtained with free and immobilized enzyme were 7.4 0.4 and 5.8 ± 0.3, respectively.

One possible explanation for these differences is based on the diffusional limitations of the substrate. A decrease of the catalytic activity of the immobilized enzymes when substrates with high molecular weight are used has been widely studied. The activity of immobilized Alcalase™ (Pessato et al., 2016), Flavourzyme™ (del Mar Yust, del Carmen Millán-Linares, Alcaide-Hidalgo, Millán, & Pedroche, 2013), and trypsin (Rocha, Gonçalves, & Teixeira, 2011) onto glyoxyl-agarose supports also decreased compared with the free enzymes.

Fig. 5 demonstrates the protein profiles obtained by size-exclusion chromatography FPLC (SEC-FPLC) of WPC and the hydrolysates with the immobilized enzyme. After 20 h, α-La protein was completely hydrolyzed, while the peak corresponding to β-Lg was not significantly reduced. These results demonstrated that the immobilized enzyme exhibits higher cleavage affinity for α-La than β-Lg. Lamas et al. (2001) reported similar results with immobilized APs from *Cynara cardunculus*. Alternatively, the serine protease Alcalase™ produce the same hydrolysis profile on both proteins (Pessato et al., 2016).

3.4. DPPH radical scavenging

Radical quenching is the primary mechanism of antioxidants to inhibit oxidative processes. The DPPH radical scavenging activities of WPC hydrolysate and the corresponding ultrafiltrate fractions are depicted in Table 1.

The DPPH radical scavenging activity of the WPC was significantly lower than that of the hydrolysate ($p < 0.05$) (data not shown). The IC_{50} value of the hydrolysate was 3.36 ± 0.07 mg/ml, which was on the same order as in previous studies; Zhang, Wu, Ling, and Lu (2013) reported an IC_{50} of 4.21 ± 0.12 mg/ml for WPC hydrolysate produced with Alcalase 2.4L™. Fraction III, with the lowest molecular mass peptides (<3 kDa), demonstrated the highest antiradical scavenging activity. These results indicate that protein hydrolysis is effective at significantly increasing the antioxidant activity, and short peptides had the strongest radical quenching effect. This result is consistent with the results obtained by Peng, Xiong, and Kong (2009) with the commercial protease Alcalase™. Further research will be needed to isolate and identify the individual peptides responsible of the antioxidant activity of WPC hydrolysates. Amino acid sequences would enable a better

Table 1
IC_{50} values of the different fractions of the Whey protein concentrate hydrolysate with immobilized salpichroin.

Fraction	IC_{50} DPPH radical scavenging activity (mg/ml)
Hydrolysate	3.36 ± 0.07
Fraction I (>10 kDa)	6.01 ± 0.13
Fraction II (3–10 kDa)	0.94 ± 0.02
Fraction III (<3 kDa)	0.48 ± 0.02

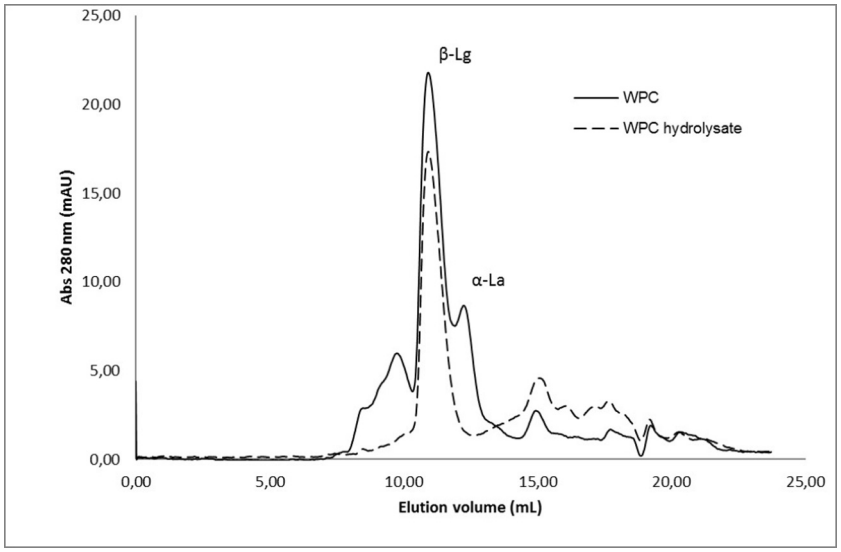

Fig. 5. Size-exclusion chromatography (SEC-FPLC) profile of Whey protein concentrate and its hydrolysates with immobilized enzyme obtained at 40 °C for 20 h.

understanding of the peptides structure–function relationship. Additionally, *in vitro* results could be complemented with *in vivo* studies.

4. Conclusions

In the present study, an AP from *Salpichroa origanifolia* fruits was successfully immobilized onto an activated support of glutaraldehyde agarose. The derivate obtained presented higher thermal stability than the free enzyme between 40 °C and 50 °C and a high reusability, retaining 54% of the initial activity after ten cycles. The immobilized enzyme was used to hydrolyze whey proteins, and it exhibited higher affinity for α-La than for β-Lg. Protein hydrolysis increased the antioxidant activity as the hydrolysate demonstrated higher DPPH radical scavenging activity than the WPC. In addition, peptides with the lowest molecular mass demonstrated the strongest radical quenching effect. Though further studies are still needed, these findings are important, as the potential antioxidant peptides obtained could be used as nutraceutical ingredients in functional foods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.05.112.

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