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Antioxidant and antihypertensive activity of bovine whey protein concentrate enzymatic hydrolysates

Hidrolizados enzimáticos con actividad antioxidante y antihipertensiva obtenidos del concentrado proteico del suero lácteo bovino

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ABSTRACT

Whey is a polluting by-product of cheese processing. However, it has valuable nutritional properties that can be exploited for diverse applications. Research has shown how whey proteins enzymatic hydrolysis releases bioactive peptides. In the present study, the whey protein concentrate (WCP) hydrolysis was performed using purified *Sporisorium reilianum* aspartyl protease (Eap1), with commercial enzymes chymotrypsin (C) and trypsin (T), as well as different enzymatic combinations in order to determine which enzyme or combination allowed for the release of peptides presenting the highest antioxidant and antihypertensive activity levels. Our results indicated that hydrolysis with Eap1 releases the best-performing peptides (1467.30 mM AAEAA/100g WCP; 835.20 mM $FeSO_4$/100g WCP and 83.36 % ACE inhibition) in comparison with individual enzymes (Q: 1164.60 mM AAEAA/100g WCP; 305.35 mM $FeSO_4$/100g WCP; 92.35 % ACE inhibition and T: 354.87 mM AAEAA/100g WCP; 205.82 mM $FeSO_4$/100g WCP; 83.8 % ACE inhibition) and their combinations (Eap1-C: 1050.23 mM AAEAA/100g WCP; 399.09 mM $FeSO_4$/100g WCP; 85.58 % ACE inhibition, Eap1-T: 882.5 mM AAEAA/100g WCP; 220.63 mM $FeSO_4$/100g WCP; 85.40 % ACE inhibition, C-T: 994.67 mM AAEAA/100g WCP; 310.43 mM $FeSO_4$/100g WCP; 61.27 % ACE inhibition and C-Eap1-T: 93.23 mM AAEAA/100g WCP; 23.3 mM $FeSO_4$/100g WCP; 38.35 % ACE inhibition).

Keywords: antioxidant peptides; antihypertensive peptides; protein concentrate from bovine whey; aspartyl protease of *Sporisorium reilianum*; commercial enzymes.

RESUMEN

El lactosuero es considerado un subproducto contaminante de la industria quesera, sin embargo, cuenta con importantes propiedades nutrimentales que pueden ser aprovechadas. Estudios indican que la hidrólisis enzimática de sus proteínas permite liberar péptidos bioactivos. En este estudio, se realizó la hidrólisis del concentrado proteico del lactosuero (WCP) por acción de la aspartil proteasa purificada de *Sporisorium reilianum* (Eap1), con las enzimas comerciales quimotripsina (C), tripsina (T) y de su combinación enzimática, con la finalidad de determinar que enzima permite liberar péptidos con mejor actividad antioxidante y antihipertensiva. Los resultados indicaron que la Eap1 libera péptidos con mejor actividad (1467.30 mM AAEAA/100g WCP; 835.20 mM $FeSO_4$/100g WCP; 83.36 % inhibición ACE) en comparación con las enzimas individuales (Q: 1164.60 mM AAEAA/100g WCP; 305.35 mM $FeSO_4$/100g WCP; 92.35 % inhibición ACE y T: 354.87 mM AAEAA/100g WCP; 205.82 mM $FeSO_4$/100g WCP; 83.8 % inhibición ACE) y sus combinaciones (Eap1-C: 1050.23 mM AAEAA/100g WCP; 399.09 mM $FeSO_4$/100g WCP; 85.58 % inhibición ACE, Eap1-T: 882.5 mM AAEAA/100g WCP; 220.63 mM $FeSO_4$/100g WCP; 85.40 % inhibición ACE, C-T: 994.67 mM AAEAA/100g WCP; 310.43 mM $FeSO_4$/100g WCP; 61.27 % inhibición ACE y C-Eap1-T: 93.23 mM AAEAA/100g WCP; 23.3 mM $FeSO_4$/100g WCP; 38.35 % inhibición ACE).

Palabras clave: péptidos antioxidantes, péptidos antihipertensivos, concentrado proteico del suero lácteo bovino, aspartil proteasa de *Sporisorium reilianum*, enzimas comerciales.

INTRODUCTION

Whey represents a rich and balanced protein source of amino acids, which allow for a wide range of functional properties. Specifically, whey proteins make up to 20 % of cow milk protein content. Besides their nutritional value (due to their high biological value indexes), in many cases, they also exert biological and physiological effects on the organism (Baró *et al.*, 2001). The presence of leucine, isoleucine, tyrosine, methionine, proline, and valine in the structure of α-Lactalbumin (α-La), β-Lactoglobulin (β-Lg), immunoglobulins (Ig), albumin (BSA), lactoferrin (LF), and lactoperoxidase (LP), has given rise to possible applications for the hydrolysis of these proteins to obtain peptides with different biological properties (Muro-Urista *et al.*, 2011; Tovar-Jiménez *et al.*, 2017). Biological properties are partially attributed to peptides encoded in proteins, which are released after proteolytic digestion (Clare and Swaisgood, 2000; Gobbetti *et al.*, 2002). Whey peptides have multifunctional properties such

as antioxidant, antihypertensive, opioid, and immunomodulatory, among others, and these bioactive peptides are used in products such as infant foods, energy drinks, and hypoallergenic foods to offer added value and improve consumer health (Mensink, 2006; Pfeuffer and Schrezenmeir, 2006).

The release of bioactive peptides from whey proteins has been studied over the past decade, but many of the studies have used only digestive enzymes chymotrypsin (C) and trypsin (T) (Lagrange and Clark, 2018; Maoa *et al*., 2018; Kamal *et al*., 2018; Atacan *et al*., 2016; Rocha *et al*., 2011; Bayram *et al*., 2008). Studies on the hydrolysis of these proteins have not yet been carried out using the enzyme Eap1, produced by the phytopathogen *S. reilianum*, which has 41 kDa molecular weight and it is an aspartyl protease stable at a wide range of temperatures (4 to 45 °C) and pH (3 to 5) values, but 45 °C and pH 3 are optimal points (Mandujano-González *et al*., 2013) and shows high proteolytic capacity, because it allows us to obtain hydrolysates with antimicrobial activity higher that that generated by C and T, and it was also shown that Eap1 hydrolysates were the only ones that inhibited growth of the yeast *C. albicans* (Tovar-Jiménez *et al*., 2017). This suggests it could be used like an important biotechnological alternative in the food industry, in the improvement of bio-functional properties or modulating the techno-functional properties of foods due to its high stability at temperature and pH. Since in recent years much of the scientific interest has focused on finding functional foods or ingredients that contribute to the treatment and prevention of degenerative diseases, the rise of bioactive peptides, as a health issue, still remains open, since peptides obtained and their activity depend on the source of the protein and enzymes used.

Likewise, the enzymatic hydrolysis has not been carried out with the combination of Eap1 with enzymes C and T. Thus, the purpose of the present study was to determine whether the action of Eap1-released peptides presents better biological activity than those released by commercial enzymes C and T; in addition, enzymatic cocktails were prepared to determine whether the enzymatic activities of these three enzymes are summative and whether they result in higher levels of antioxidant and antihypertensive activity in the obtained peptides.

MATERIALS AND METHODS

Ten liters of bovine cheese whey from a cheese industry (México country) were used to obtain whey protein concentrate. Samples of 500 mL (511.5 g) of whey were stored at 5 °C before use. Bovine whey proteins were precipitated with $(NH_4)_2SO_4$, then filtered using nitrocellulose filter paper (Millipore, pore size: 0.45 µm, and dialyzed using a membrane (Thermo Scientific, pore size: KDa). Dialysis was performed until samples reached conductivity <1 µS cm^{-1} (Tovar-Jiménez *et al*., 2012).

Microorganism

The *S. reilianum* strain was donated by Dr. Santos Gerardo Leyva, of the Universidad Autónoma Chapingo (Mé-xico). It was grown by periodic cultures in YPD-Agar medium (yeast extract 1%, bacto™ peptone 2%, glucose 2%, and agar 1.5%), incubated at 28 °C for 3 days.

Enzymatic hydrolysis

Purification of the Eap1enzyme, was performed as indicated by Mandujano-González *et al*. (2013), using anion exchange chromatography with a Bio-Scale Mini UNO sphere Q Cartridge column, coupled to an FPLC system (Bio-Rad). A lineal NaCl gradient was applied (J. T. Baker®), 1 M in Tris (Sigma®), 20 mM at pH 8, and the eluted fraction was taken at a NaCl concentration (J. T. Baker®) between 0.45 and 0.55 mol/L, and commercial enzymes of animal origin, trypsin (T) from bovine pancreas (Sigma®) and chymotrypsin (C) from bovine pancreas (Sigma®) were used to perform hydrolysis at the enzymatic activity levels of 1.37, 3.13, and 3.7 mUA/mL; hydrolysis was also carried out using four enzyme combinations (Eap1-C, Eap1-T, CT, and C-Eap1-T) at an enzymatic activity level of 1.37 mUA/mL.

Hydrolysis kinetics was carried out from 0 to 120 min at 37 °C and optimum pH for each individual enzymes and pH 6. The substrate was adjusted to a concentration of 179.4 µg WCP protein. The non-hydrolyzed proteins were precipitated with 500 µL of 10% trichloroacetic acid. The reaction mixture was centrifuged at g for 5 min. Finally, the supernatant was used to quantify the peptides released after hydrolysis following the micro-Lowry method as reported by Figueroa-Hernández et al. (2012). Calculation of the peptides released was done on the basis of curve pattern of tyrosine at a concentration of 0-23 µg/mL. All determinations were performed in sextuplicate with the following controls: WCP+TCA+Enzyme, WCP+Sterile distilled water+Enzyme, and Sterile distilled water+TCA+Enzyme. Finally, the biological activity of the hydrolysis product was determined.

Determination of biological activity of released peptides

The antioxidant and antihypertensive activities of the peptides released after the enzymatic hydrolysis were analyzed.

Determination of antioxidant activity

A solution of 7 µM ABTS (AMRESCO ultra-pure grade) was mixed with 4.9 µM ammonium persulfate (Sigma Aldrich brand) at a 1:1 ratio. The radical ABTS$^+$ was formed after 16 h. ABTS solution was diluted until reaching absorbance between 0.7 and 754 nm, which was achieved by mixing approximately 40 µL of the ABTS$^+$ solution with 960 µL of absolute ethanol (v/v). Twenty µL of the sample (released peptides) were added to 980 µL of the dilution, and the change in absorbance was measured 7 min after the reaction. The percentage inhibition of ABTS$^+$ radical was calculated using the pattern curve prepared with ascorbic acid [2mM] (MP Biomedicals), in concentrations from 0 to 0.8 mM. Data are reported as mM of antioxidant activity equivalent to ascorbic acid per 100 g WCP (mM AAEAA/100 g WCP) (Tovar-Jiménez *et al*., 2012; Mendoza-Jiménez *et al*., 2018).

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed according to the method described by Luo *et al*. (2012). In the assay kit with FRAP method, stock solutions include detective buffer, 2,4,6-tripyridyl-s-triazine (TPTZ) solution, and TPTZ solution. The working solution was prepared before use by mixing TPTZ dilution, detective buffer, and TPTZ solution at a ratio of 10:1:1 (v/v/v), respectively, then the working solution was incubated at 37 °C. Fourteen µL of hydrolyzed sample (0.1 mg peptides/mL in PBS) were added to the TPTZ solution (504 µL) in a eppendorf tube. After incubation for 30 min at 25 °C, the absorbance of the reaction mixture was measured at 593 nm with a spectrophotometer. The ferric reducing activity was calculated using the $FeSO_4$ solution (200 mM–1200 mM) calibration curve and was converted to the Fe^{2+} antioxidant capacity value, which was expressed as $FeSO_4$ mM/100 g WCP.

Determination of antihypertensive activity

The present study used a method described by Nakamura *et al*. (1995). Eighty µL of water or 80 µL of the sample adjusted to a pH of 8.3, were added to 200 µL of the substrate solution, consisting of Hipuryl-Histidyl-Leucine at a concentration of 5 mM, dissolved in 0.1 M sodium borate buffer and 0.3 M NaCl, at pH 8.3. Mixtures were pre-incubated at 37 °C. Twenty µL of ACE (0.1 U/mL, Sigma-Aldrich) were added to initiate the reaction, and the mixture was incubated at 37 °C for 30 min, and then decreasing pH with 250 µL of 1 M HCl to inactivate the enzyme. The hippuric acid formed in the reaction was extracted by vigorous stirring for 10 s after adding 1 mL of ethyl acetate. It was then centrifuged at 4000 g for 10 min at 25 °C, and 500 µL of the organic phase were withdrawn. Ethyl acetate was removed by heating at 95 °C for 10 min. The hippuric acid residue was re-dissolved in 1 mL distilled water, and after shaking, the absorbance at 228 nm was measured by the spectrophotometer. A reaction target was prepared by adding HCl prior to the addition of ACE and Captopril was used as the positive control in this study. The activity of each sample was determined in triplicate, and the results were expressed as percentages of ACE inhibition.

Statistical analysis

For data analysis, the multifactorial analysis of variance methodology was used to determine the individual and joint effects of the factors (enzymatic activity, hydrolysis time and enzyme) on the responses variables (antioxidant and antihypertensive peptides released). Later, the LSD contrast test (minimum significant difference) was performed at a 95% confidence level. The Statistica 7.0 for Windows statistical program was used. Graphs are expressed in means ± LSD interval.

RESULTS AND DISCUSSION

Kinetics of WCP hydrolysis with Eap1, C and T enzymes

Peptides released during hydrolysis of whey proteins depends on the proteolytic activity and time (Figure 1); that is, larger quantities of these compounds are released with
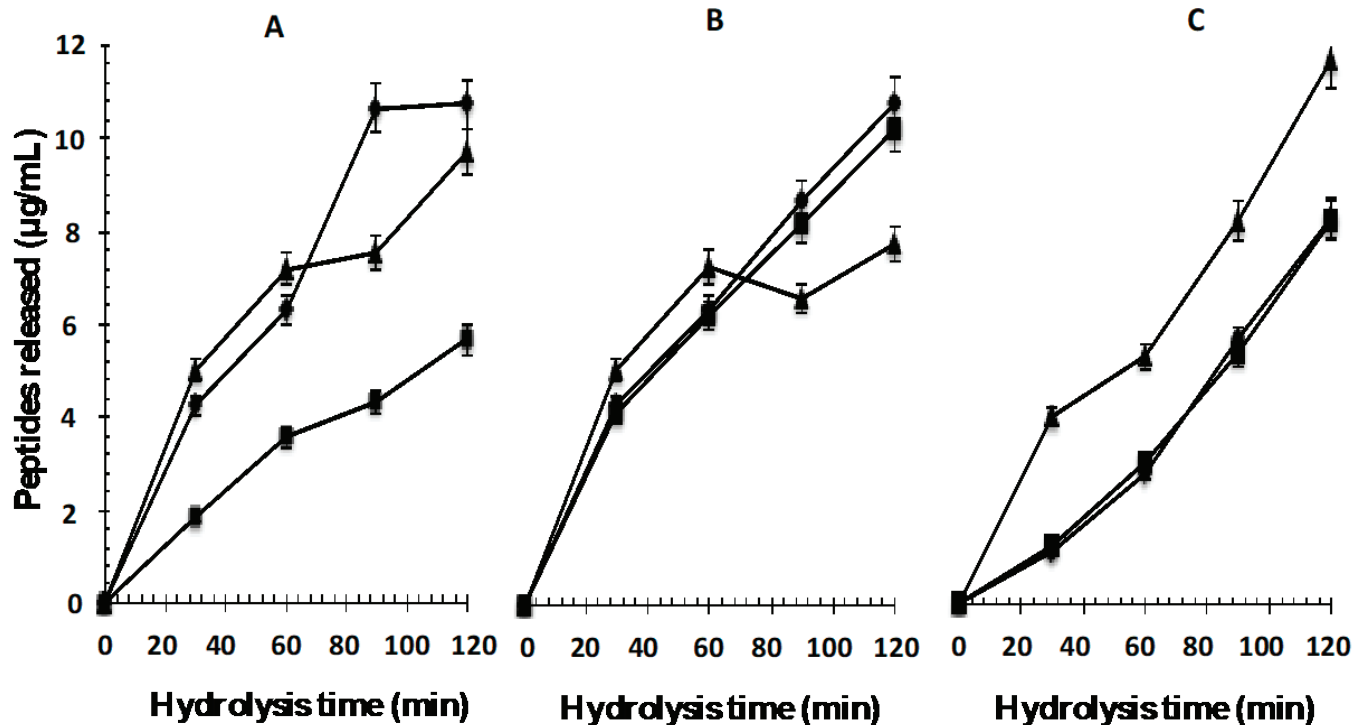

Figure 1: Peptides released from the whey protein concentrate by enzymatic hydrolysis with enzymes A: Eap1, B: chymotrypsin and C: trypsin at different units of enzyme activity (▲ 1.37 mAU/mL, ■ 3.13 mAU/mL, ◆ 3.71 mAU/mL) and hydrolysis time.
Figura 1: Péptidos liberados del concentrado proteico del suero lácteo por hidrólisis enzimática empleando las enzimas A: Eap1, B: Quimotripsina y C: Tripsina a diferentes unidades de actividad enzimática (▲ 1.37 mUA/mL, ■ 3.13 mUA/mL, ◆ 3.71 mUA/mL) y tiempo de hidrólisis.

Eap1 and C to 3.71 mUA/mL without statistically significant differences (p >0.05; $R^2_{adjusted}$: 0.9056) with the T enzyme to 1.37 mUA/mL to 120 min hydrolysis time. The statistical analysis showed that there are significant differences (p <0.05; $R^2_{adjusted}$: 0.9902) between the units of enzymatic activity and the hydrolysis times evaluated. More extensive hydrolysis of WPC with these enzymes may be attributed to their broader specificities to cleave various peptide bonds. In this sense, the aspartyl protease Eap1 prefers to hydrolyze the adjacent carbon, where amino acids phenylalanine, proline, tyrosine, and leucine, while C enzyme hydrolyze the adjacent carbon of amino acids tyrosine, phenylalanine, and tryptophan, and T enzyme hydrolyze preferentially in the adjacent carbon of amino acids arginine and lysine. Likewise, the reaction time only determines the final degree of hydrolysis but the enzyme/substrate ratio directly influences the degree of hydrolysis and composition of the released peptides (Lagrange and Clark, 2018; Maoa *et al*., 2018; Kamal *et al*., 2018; Muro-Urista *et al*., 2011; Zakharova *et al*., 2009; Peng *et al*., 2009; Erdmann *et al*., 2008).

Determination of biological activity of released peptides

The antioxidant and antihypertensive activities of the peptides obtained from the hydrolysis were measured to determine which enzyme or combination resulted in the most substantial peptide-releasing activity. Our observations show a positive association between the release of bioactive peptides and enzyme activity units (Figure 2, 3 and 4).

Peptides with antioxidant activity released by the action of Eap1, and enzymes C and T

Figure 2 shows the antioxidant activity of peptides released at different times (0, 30, 60, 90, and 120 min). Eap1-released peptides present better antioxidant activity (1467.30 ± 23.86 mM AAEAA/100 g WCP) at an enzymatic activity of 1.37 mUA/mL in comparison with enzymes C (1164.60 ± 31.25 mM AAEAA/100 g WCP) and T (354.87 ± 7.16 mM AAEAA/100 g WCP) at the different activity levels studied. Likewise, Figure 3 shows the ferric reducing capacity of peptides released at different hydrolysis time. At an activity of 1.37 mUA/mL, Eap1-released peptides showed better ferric reducing capacity (835.20 ± 12 FeSO$_4$ mM/100 g WCP) in comparison with enzymes C and T at the different activity levels studied. The statistical analysis showed significant differences (p <0.05; $R^2_{adjusted}$: 0.9864 and $R^2_{adjusted}$: 0.9824, respectively) between hydrolysis times and enzymatic activities among the different enzymes studied for both studies.

The results obtained in the present study may be due to the fact that Eap1 prefers to hydrolyze the adjacent carbon, where amino acids phenylalanine, proline, tyrosine, and leucine are found (Hedstrom, 2002), which results in the generation of peptides with antioxidant activity, since these peptides require having amino acids such as tyrosine, methionine, tryptophan, and histidine at its terminal carbon as indicated Erdmann *et al*. (2008). In this sense, The WCP used contained β-Lg and α-La that present 3.7 and 4 % of the amino acid tyrosine in their structures, respectively, which
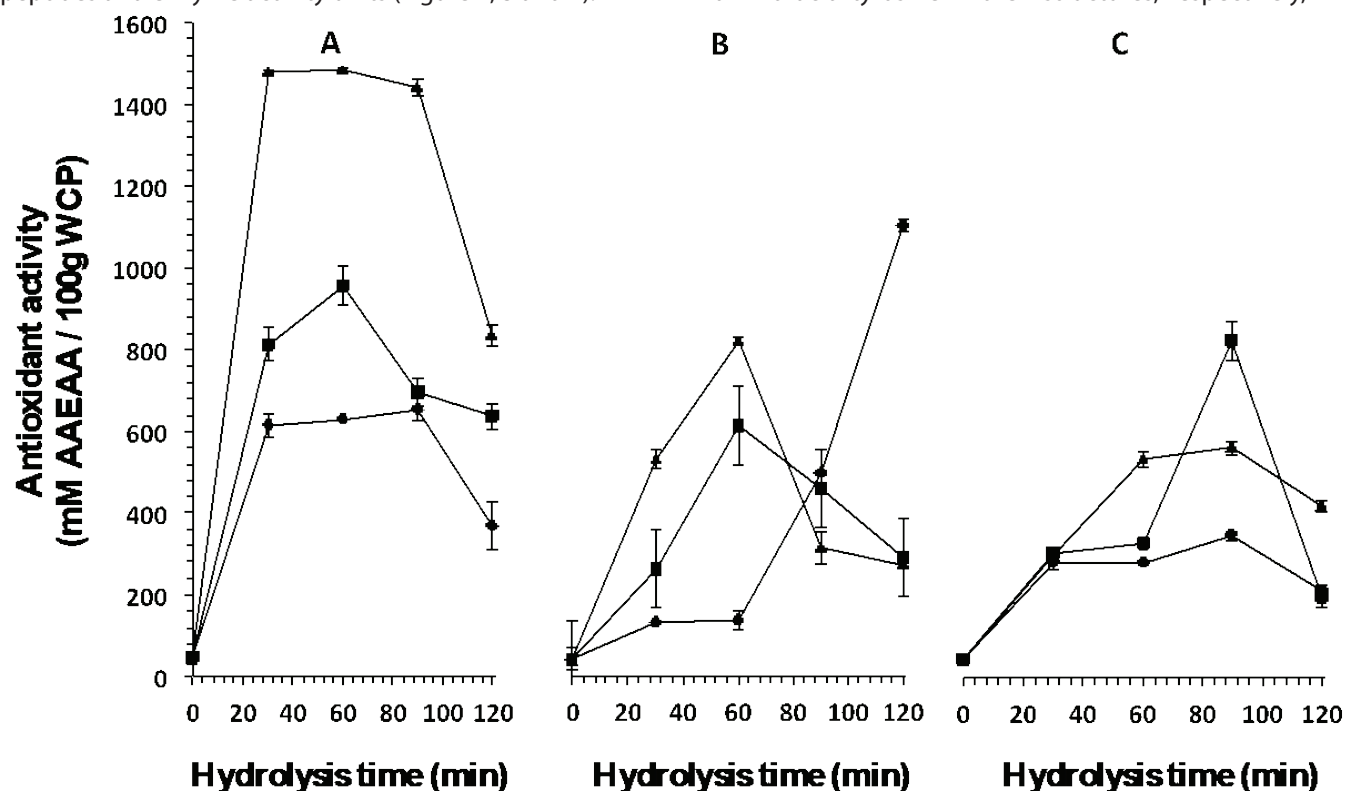

Figure 2: Antioxidant activity of peptides released from the whey protein concentrate by enzymatic hydrolysis with enzymes A: Eap1, B: chymotrypsin and C: trypsin at different units of enzyme activity (▲1.37 mAU/mL, ■ 3.13 mAU/mL, ◆ 3.71 mAU/mL) and hydrolysis time.
Figura 2: Actividad antioxidante de los péptidos liberados del concentrado proteico del suero lácteo por hidrólisis enzimática empleando las enzimas A: Eap1, B: Quimotripsina y C: Tripsina a diferentes unidades de actividad enzimática (▲1.37 mUA/mL, ■ 3.13 mUA/mL, ◆ 3.71 mUA/mL) y tiempo de hidrólisis.
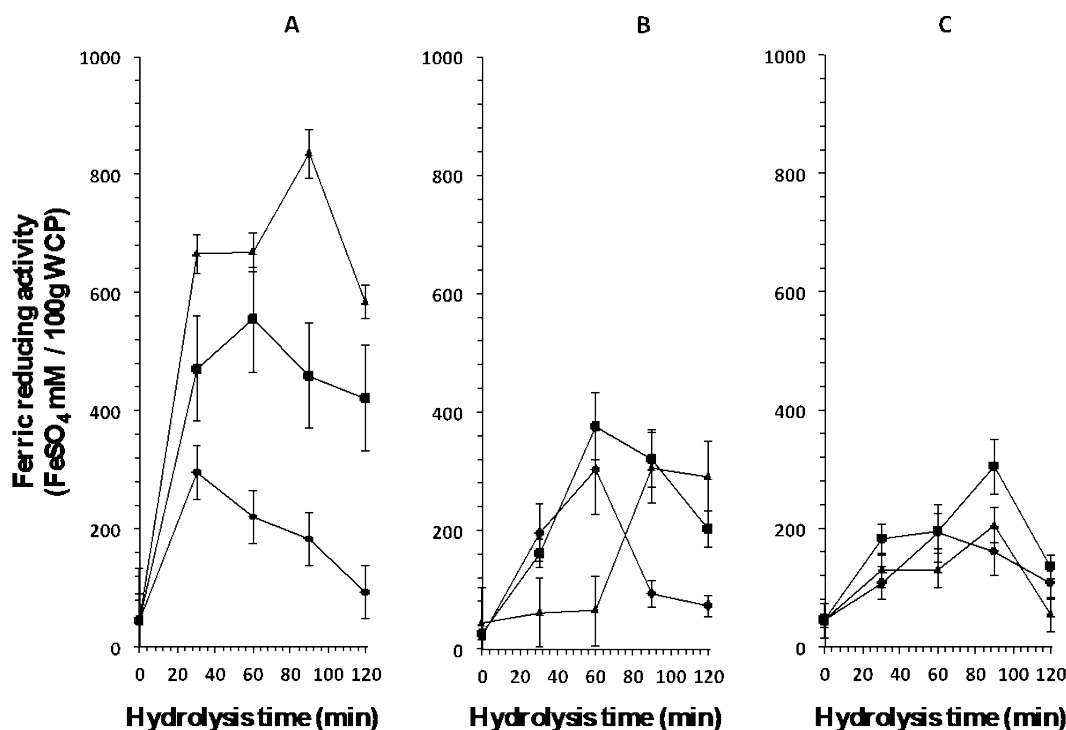
Figure 3: Ferric reducing activity of peptides released from the whey protein concentrate by enzymatic hydrolysis with enzymes A: Eap1, B: chymotrypsin and C: trypsin at different units of enzyme activity (▲ 1.37 mAU/mL, ■ 3.13 mAU/mL, ◆ 3.71 mAU/mL) and hydrolysis time.

Figura 3: Potencial reductor férrico de los péptidos liberados del concentrado proteíco del suero lácteo por medio de hidrólisis enzimática con las enzimas A: Eap1, B: quimotripsina y C: tripsina a diferentes unidades de actividad enzimática (▲ 1.37 mAU/mL, ■ 3.13 mAU/mL, ◆ 3.71 mAU/mL) y tiempo de hidrólisis.
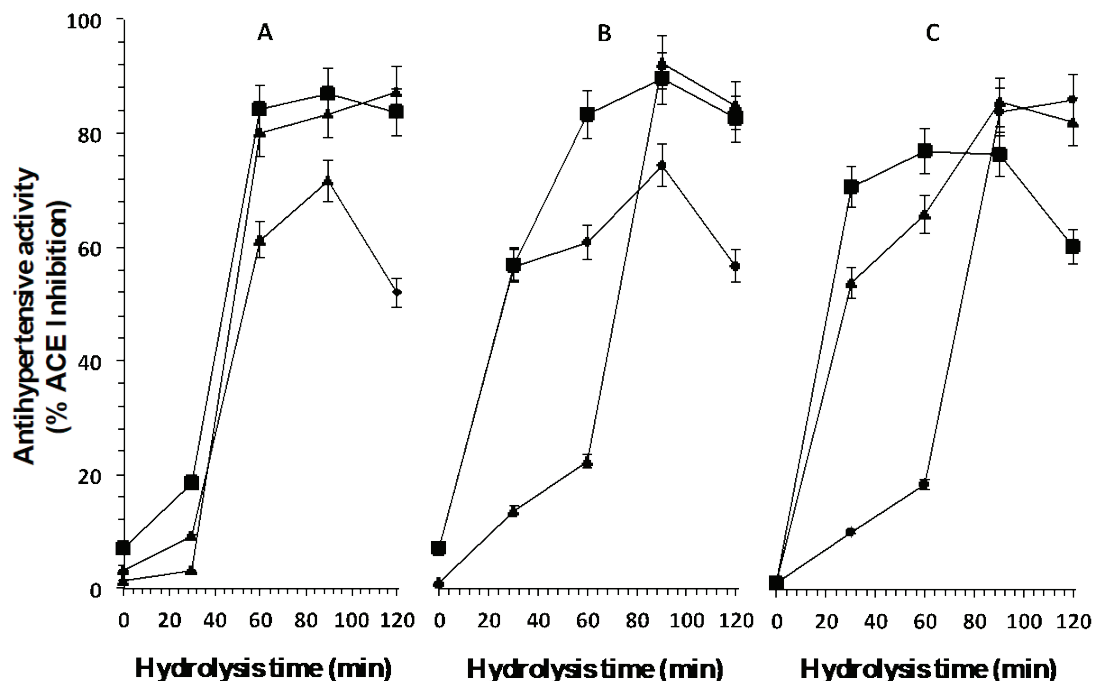

Figure 4: ACE inhibition percentage of peptides released from the whey protein concentrate by enzymatic hydrolysis with enzymes A: Eap1, B: chymotrypsin and C: trypsin at different units of enzyme activity (▲ 1.37 mAU/mL, ■ 3.13 mAU/mL, ◆ 3.71 mAU/mL) and hydrolysis time.

Figura 4: Actividad antihipertensiva de los péptidos liberados del concentrado proteico del suero lácteo por hidrólisis enzimática empleando las enzimas A: Eap1, B: Quimotripsina y C: Tripsina a diferentes unidades de actividad enzimática (▲ 1.37 mUA/mL, ■ 3.13 mUA/mL, ◆ 3.71 mUA/mL) y tiempo de hidrólisis.

can be released by the action of Eap1. The fact that peptides presenting lower activity were obtained from hydrolysis with C could be explained because, in this case, the enzyme prefers to hydrolyze the adjacent carbon of amino acids tyrosine, phenylalanine, and tryptophan (Zakharova *et al*., 2009), and even in the presence of amino acid tryptophan the antioxidant activity is lower than that obtained by Eap1, because the structures of β-Lg and α-La contain approximately 1 % of this amino acid, and this biological activity also depends on the amino acid sequence and the size of the released peptides (Peña-Ramos and Xiong, 2001; Erdmann *et al*., 2008; Peng *et al*., 2009; Muro-Urista *et al*., 2011). As for the T enzyme, the low activity of the peptides obtained can be attributed to the fact that β-Lg, BSA, Lf, and Ig are constituted by lysine and arginine (30 and 6 %, 59 and 23 %, 54 and 37 %, and 82 and 84 %, respectively) (Muro-Urista *et al*., 2011), which may indicate a higher degree of hydrolysis because enzyme T hydrolyzes preferentially in the adjacent carbon of amino acids arginine and lysine (Zakharova *et al*., 2009) (Figure 1), and as reported by Peña-Ramos and Xiong (2001) and Peng *et al*. (2009) who indicate that the effect of the antioxidant activity depends on the composition of amino acids, the sequence, and the configuration of the peptides obtained. Moreover, results presented by Peña-Ramos and Xiong (2001) suggest that the degree of hydrolysis exerted by T on β-Lg is higher compared to the degree exerted by T on α-La, generating peptides with lower antioxidant activity. According to Bayram *et al*. (2008), antioxidant activity is inherent to β-Lg, BSA, and Lf.

Peptides with antihypertensive activity released by the action of Eap1, C and T.

Figure 4 shows antihypertensive activity (ACE inhibition percentage) and hydrolysis time. As can be observed, when WCP hydrolysis was carried out using the three enzymes, with different activity units, peptides with antihypertensive activity were released. Higher levels of biological activity and shorter reaction times were obtained in the present study in comparison with those reported by Hernández-Ledesma *et al*. (2006), who hydrolyzed β-Lg from sheep and goat whey using enzymes of animal origin (T and C) and reported that, after hydrolysis for 36 h with T, the peptides obtained from the β-Lg of ovine origin presented 74.6 % of ACE inhibition, and those obtained from β- Lg of caprine origin presented 73.9 % inhibition after hydrolysis for 24 h. The same authors reported that, when the hydrolysis of β-Lg of ovine and caprine origin is carried out using C, 73.6 % and 64.9 % of ACE inhibition are obtained, respectively, after hydrolysis for 24 h, whereas the released peptides in the present study showed an activity of 83 % of ACE inhibition after hydrolysis for 90 min with Eap1 and T. The statistical analysis showed a significant difference (p <0.05, R^2_{adjusted}: 0.97) between the time of hydrolysis, enzyme and enzymatic activity evaluated.

These results can be due to the fact that β-Lg, BSA, Lf, and α-La constitute the WCP used, and this activity is inherent to these proteins (Maneva *et al*., 2003; Prioult *et al*., 2004). Also, as reported in the literature, peptides that show

ACE inhibitory activity possess hydrophobic residues, such as tryptophan, tyrosine, or phenylalanine, in at least one of the three C-terminal positions where they bind to the ACE active sites, blocking its activity (Aleixandre *et al*., 2008). It has also been reported that sequences of short chain peptides carrying proline residues, in combination or not with hydrophobic residues, inhibit ACE (Hartmann and Meisel, 2007; Murakami *et al*., 2004). Additionally, the inhibitory potency can be increased with the presence of positive charges of lysine and arginine in the C-terminal, because they represent 30% of WCP (Muro-Urista *et al*., 2011; Hernández-Ledesma *et al*., 2008; Dziuba *et al*., 1999).

When analyzing the biological activity results obtained in connection with the three studied activities (1.37, 3.13, and 3.71 mUA/mL) of the evaluated enzymes (Eap1, C, and T), it was decided to use the enzymatic activity of 1.37 mUA/mL, since it presented an acceptable level of biological activity and the process requires a lower amount of enzyme, which could result in lower costs for bioactive peptide production. In this regard, studying an enzymatic cocktail would make it possible to determine whether the enzymatic activities of these three enzymes are summative and their combination results in higher biological activity in comparison with peptides obtained using individual enzymes.

Hydrolysis Kinetics of WCP with the enzymatic combination.

Four enzyme combinations, aspartyl protease-chymotrypsin (Eap1-C), aspartyl protease-trypsin (Eap1-T), trypsin-chymotrypsin (TC), and aspartyl protease-chymotrypsin-trypsin (Eap1-CT), were evaluated to determine their ability to release bioactive peptides at different times (30, 60, 90, and 120 min). Figure 5 show that peptides released during hydrolysis of whey proteins, depends on hydrolysis time and enzyme or enzyme combinations; likewise, that the larger quantities of these peptides are released with enzyme combinations, which suggests a higher degree of hydrolysis, however, biological activity peptides also depends on the composition of amino acids, the sequence, and the configuration of the peptides obtained (Tovar-Jiménez *et al*., 2017). The statistical analysis showed significant differences (p <0.05; R^2_{adjusted}: 0.9593) between hydrolysis times and enzyme used.

Antioxidant activity peptides released by enzymatic combination

Figure 6 shows antioxidant activity (mM AAEAA/100g WCP) with respect to hydrolysis time. As can be observed, enzyme Eap1 (1467.30 ± 23.86 mM AAEAA/100 g WCP) and combinations C-T (994.67 ± 14.55 mM AAEAA/100 g WCP) and Eap1-C (1050.23 ± 13.99 mM AAEAA/100 g WCP) released peptides with better antioxidant activity; with no significant differences observed. However, Eap1 released the antioxidant peptides in a shorter time than the combinations. Figure 7 shows the ferric reducing capacity of peptides released at different hydrolysis time, Eap1-released
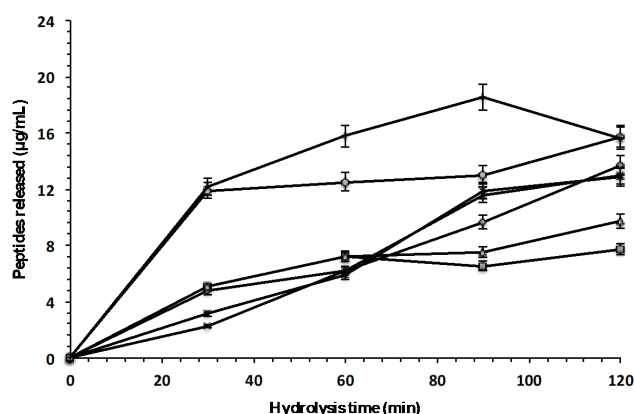
Figure 5: Peptides released from the whey protein concentrate by enzymatic hydrolysis with individual enzymes (◆ Eap1, ■ chymotrypsin and ▲ trypsin) and with enzymatic cocktail (× Chymotrypsin-Eap1, ✳ Trypsin-Eap1, ● Chymotrypsin-Trypsin and ｜ Chymotrypsin-Eap1-Trypsin).
Figura 5: Péptidos liberados del concentrado proteico del suero lácteo empleando las enzimas individuales (◆ Eap1, ■ Quimotripsina ▲ Tripsina) y las combinaciones enzimáticas (× Quimotripsina-Eap1, ✳ Tripsina-Eap1, ● Quimotripsina-Tripsina y ｜ Quimotripsina-Eap1-Tripsina).
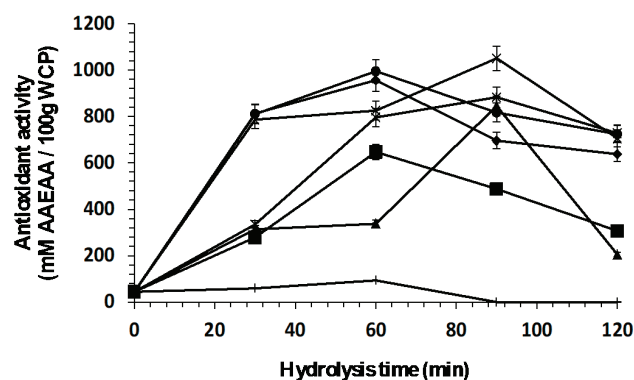

Figure 6: Antioxidant activity of peptides released from the whey protein concentrate by enzymatic hydrolysis with individual enzymes (◆ Eap1, ■ chymotrypsin and ▲ trypsin) and with enzymatic cocktail (× Chymotrypsin-Eap1, ✳ Trypsin-Eap1, ● Chymotrypsin-Trypsin and ｜ Chymotrypsin-Eap1-Trypsin).
Figura 6: Actividad antioxidante de los péptidos liberados del concentrado proteico del suero lácteo empleando las enzimas individuales (◆ Eap1, ■ Quimotripsina ▲ Tripsina) y las combinaciones enzimáticas (× Quimotripsina-Eap1, ✳ Tripsina-Eap1, ● Quimotripsina-Tripsina y ｜ Quimotripsina-Eap1-Tripsina).

peptides present better ferric reducing capacity (835.20 ± 12 $FeSO_4$ mM/100g WCP) in comparison with enzymes C (305.35 ± 7.1 $FeSO_4$ mM/100g WCP) and T (205.82 ± 3.2 $FeSO_4$ mM/100 WCP) and enzymatic combinations (Chymotrypsin-Eap1 [399.09 ± 5.3 $FeSO_4$ mM/100g WCP], Trypsin-Eap1 [220.63 ± 2.8 $FeSO_4$ mM/100g WCP], Chymotrypsin-Trypsin and Chymotrypsin-Eap1-Trypsin [23.3 ± 1.5 $FeSO_4$ mM/100g WCP]). The statistical analysis showed significant differences (p<0.05, R^2 adjusted: 0.9307; R^2 adjusted: 0.9543) in hydrolysis times depending on the enzymatic mixture.
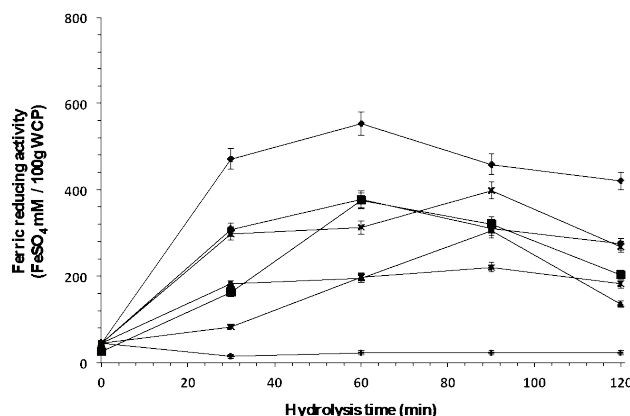

Figure 7: Ferric reducing activity of peptides released from the whey protein concentrate by enzymatic hydrolysis with individual enzymes (◆ Eap1, ■ chymotrypsin and ▲ trypsin) and with enzymatic cocktail (× Chymotrypsin-Eap1, ✳ Trypsin-Eap1, ● Chymotrypsin-Trypsin and ｜ Chymotrypsin-Eap1-Trypsin).
Figura 7: Potencial reductor férrico de los péptidos liberados del concentrado proteíco del suero lácteo por medio de hidrólisis enzimática con las enzimas individuales (◆ Eap1, ■ quimotripsina y ▲ tripsina) y con el cóctel enzimático (× Quimotripsina-Eap1, ✳ Tripsina-Eap1, ● Quimotripsina-Tripsina y ｜ Quimotripsina-Eap1-Tripsina).

Antihypertensive activity peptides released by the action of the enzymatic combination

Figure 8 shows the percentage inhibition of ACE with respect to hydrolysis time. The enzymatic combination releases peptides with better antihypertensive activity after hydrolysis for 120 min (96 % ACE inhibition). Similarly, the peptides obtained with enzymes Eap1, C, and the enzymatic combination Eap1-C presented no significant differences; however, Eap1 released these peptides in less time. The statistical analysis revealed that hydrolysis time depends on the enzymatic combination used (p <0.05, R^2 adjusted: 0.9991).
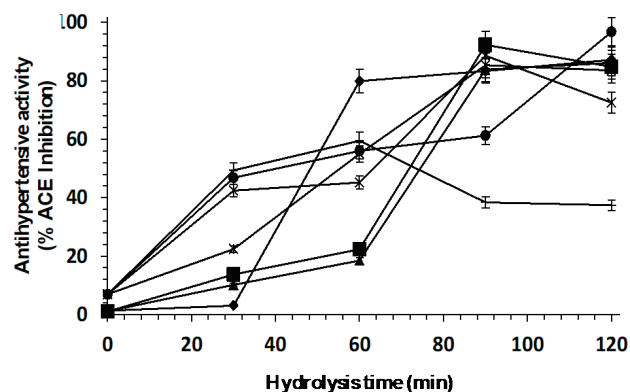

Figure 8: ACE inhibition percentage of peptides released from the whey protein concentrate by enzymatic hydrolysis with individual enzymes (◆ Eap1, ■ chymotrypsin and ▲ trypsin) and with enzymatic cocktail (× Chymotrypsin-Eap1, ✳ Trypsin-Eap1, ● Chymotrypsin-Trypsin and ｜ Chymotrypsin-Eap1-Trypsin).
Figura 8: Actividad antihipertensiva de los péptidos liberados del concentrado proteico del suero lácteo empleando las enzimas individuales (◆ Eap1, ■ Quimotripsina ▲ Tripsina) y las combinaciones enzimáticas (× Quimotripsina-Eap1, ✳ Tripsina-Eap1, ● Quimotripsina-Tripsina y ｜ Quimotripsina-Eap1-Tripsina).

The peptides obtained with the different enzymatic combinations, suggest their possible uses in the elaboration of protein hydrolysates for applications requiring higher degrees of protein hydrolysis, than what is possible using individual enzymes (Figure 5). This experimental evidence can be interpreted starting from the fact that proteolytic enzymes act only on peptide bonds containing certain amino acids, for this reason, more substantial hydrolysis is obtained when using enzyme mixtures to maximize hydrolytic action. Our results differ from those reported by Ubalde and Cantera (2002), who used a mixture of *Bacillus subtilis* protease and obtain whey hydrolysates presenting a low degree of hydrolysis; however, the authors failed to determine the biological activity of such hydrolysates. Nevertheless, the results of our study coincide with those reported by Morris-Quevedo *et al*. (2001), who evaluated the effect of the use of five enzymatic combinations (bromelain-trypsin, bromelain-pancreatin, papain-bromelain, papain-trypsin, and pancreatin-papain) in obtaining protein hydrolysates from the biomass of the microalgae *Chlorella vulgaris* previously treated with ethanol; the best results were obtained with the papain-trypsin and papain-pancreatin mixtures. They also state that the use of enzymatic mixtures has a positive effect on the production of hydrolysates presenting a high degree of hydrolysis in comparison with individual enzymes, as shown by the comparison of their hydrolytic actions.

In general, the antioxidant and antihypertensive biological activity results obtained by the hydrolysis of milk serum proteins using individual enzymes (Eap1, C, and T) and enzymatic combinations (Eap1-C, Eap1-T, T-C, and C- Eap1-T) show that the best results are obtained when using Eap1 enzyme individually.

CONCLUSIONS

The aspartyl protease obtained from *S. reilianum* (Eap1) allows for the obtention of peptides with better antioxidant activity and similar antihypertensive activity in comparison with those obtained using commercial enzymes trypsin and chymotrypsin at 1.37 mUA/mL under these study conditions.

The peptides obtained using the enzymatic combinations evaluated suggest that their hydrolysates present a higher degree of hydrolysis in comparison with those of individual enzymes.

These findings are interesting because obtaining active peptides by hydrolysis using proteases of fungal origin offers an important biotechnological alternative for the use of an agroindustrial by-product like whey, since Eap1 allows obtention bioactive peptides in less time and with less enzymatic quantity.

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