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HYDROLYSATE ANTIMICROBIAL ACTIVITY RELEASED FROM BOVINE WHEY PROTEIN CONCENTRATE BY THE ASPARTYL PROTEASE Eap1 OF *Sporisorium reilianum*

HIDROLIZADOS CON ACTIVIDAD ANTIMICROBIANA LIBERADOS POR LA ACCIÓN DE LA ASPARTIL PROTEASA Eap1 DE *Sporisorium reilianum* A PARTIR DE UN CONCENTRADO PROTEICO DEL SUERO LÁCTEO BOVINO

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Abstract

Whey, a by-product of cheese processing, has important nutritional qualities, as it is a rich source of proteins balanced with amino acids. As such, it has a broad range of functional and biological properties that can be exploited biotechnologically for diverse applications. Several studies have demonstrated that the enzymatic hydrolysis of whey proteins releases peptides that can perform biological activities, depending on the precise enzymatic process applied and the specific protein used. The present study examines the antimicrobial activity obtained from hydrolysates of protein concentrates of bovine whey by means of the aspartyl protease Eap1. It demonstrates antimicrobial activity against the following human non-opportunistic (*Salmonella* sp.) and opportunistic pathogens (*Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*), and then compares the findings obtained to the action of two commercial enzymes: trypsin (TP) and chymotrypsin (CMTP). Results indicate that the antimicrobial activity of the hydrolysates produced with Eap1 was higher than that generated by TP and CMTP. Also, the study determined that the Eap1 products were the only ones that inhibited growth of the yeast *C. albicans*.

Keywords: antimicrobial protein hydrolysates, protein concentrate from bovine whey, aspartyl protease of *Sporisorium reilianum*, trypsin, chymotrypsin.

Resumen

El suero lácteo es un subproducto de la elaboración de queso con importantes cualidades nutrimentales, ya que representa una fuente rica en proteínas y equilibrada en aminoácidos que poseen amplio rango de propiedades funcionales y biológicas que pueden ser aprovechadas biotecnológicamente para diversas aplicaciones. A través de diversos estudios se ha podido comprobar que la hidrólisis enzimática de las proteínas séricas conduce la liberación de péptidos que pueden presentar actividad biológica, dicha actividad depende del proceso enzimático utilizado y la proteína de origen. El presente trabajo muestra la actividad antimicrobiana obtenida de hidrolizados de concentrados proteicos de suero lácteo bovino, mediante una aspartil proteasa (Eap1). La actividad antimicrobiana fue probada contra patógenos humanos no oportunistas (*Salmonella* sp.) y oportunistas (*Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* y *Candida albicans*) y comparada con la obtenida por acción de enzimas comerciales, tripsina (TP) y quimotripsina (QMTP). Los resultados indicaron que Eap1 produjo hidrolizados con mayor actividad antimicrobiana comparada con los generados por TP y QMTP. Además, se encontró que los productos provenientes de Eap1 fueron los únicos en inhibir el crecimiento de la levadura *C. albicans*.

Palabras clave: hidrolizados proteicos antimicrobianos, concentrado proteico del suero lácteo bovino, aspartil proteasa de *Sporisorium reilianum*, tripsina, quimotripsina.

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1 Introduction

Today, the food sciences are promoting the use of functional foods that have the potential to improve health by decreasing the risks of illness in humans. For this reason, some food industries have incorporated functional ingredients like peptides extracted from animal and vegetable sources into their products (Chatterjee *et al*., 2015). The benefits of these products reside, primarily, in their capacity to reduce and/or prevent gastrointestinal illnesses.

One potential source for obtaining functional ingredients are whey proteins, thanks not only to their nutritional effects due to their amino acid content, but also because, in many cases, they show biological and physiological effects *in vivo* (Baró *et al*., 2001). Specifically, 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), promises possible applications after performing hydrolysis to obtain bioactive peptides (Muro-Urista *et al*., 2011; Sinha *et al*., 2007) that can be incorporated into foods as functional ingredients (Torruco-Uco *et al*., 2008). Bioactive peptides are defined as specific fragments of proteins of animal or vegetable origin that have a positive impact on bodily functions or conditions that can affect human health beyond areas of basic nutrition (Alvarado-Carrasco and Guerra 2010; González-Olivares *et al*., 2011). Among the biological activities that have been studied in relation to these compounds are their antimicrobial, antioxidant and antihypertensive properties. These peptides are usually obtained from proteins using chemical or enzymatic hydrolysis (López-Exposito *et al*., 2006; Huang *et al*., 2010; Szwajkowska *et al*., 2011).

The peptides that possess antimicrobial activity are characterized by their size: from 3 to 20 amino acids. Research has demonstrated that fragments of LF and the isoenzymes, LP and Ig, present in whey exert inhibitory properties on microbial growth, apparently related to the net positive charge of these peptides. A high percentage of basic amino acids exert antimicrobial effects by forming an α-helix-shaped loop at the carboxyl terminus that leads to the formation of ionic channels in a microorganism's membrane, thus modifying its permeability and triggering cell death (Figueroa-González *et al*., 2010).

Earlier work has shown that the peptides released by proteolytic enzymes like pepsin from porcine gastric mucosa, alcalase from *Bacillus licheniformis*, chymotrypsin (CMTP), chymosin or trypsin (TP) of bovine origin, all inhibit both gram-positive (*Staphylococcus*, *Bacillus*, *Listeria*, *Streptococcus*) and gram-negative (*Escherichia*, *Klebsiella*, *Pseudomonas*, *Salmonella*, etc.) bacteria, as well as pathogenic yeasts such as *Candida albicans* (McCann *et al*., 2006; Szwajkowska *et al*., 2011; Elbarbary *et al*., 2012). However, few studies have used aspartyl proteases, which acquire affinity by hydrolyzing in the adjacent carbon where the amino acids phenylalanine, proline, tyrosine and leucine are found (Narayanan *et al*., 2007). These amino acids are proximal to positively-charged amino acids in proteins present in whey, like LF and lactalbumin (Muro-Urista *et al*., 2011).

The present study set out to determine the antimicrobial activity of peptides contained in the hydrolysates of protein concentrates from bovine whey against important pathogens that affect humans: *Salmonella* sp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*. The enzymatic hydrolysates were obtained by the action of the purified enzyme Eap1 from *S. reilianum*, which has recently shown high proteolytic capacity (Mandujano-González *et al*., 2013). The peptides obtained were then compared to hydrolysates obtained from the commercial proteases most often used for this purpose: TP and CMTP from bovine pancreases.

2 Materials and methods

2.1 Obtaining protein concentrates from whey

Sweet whey was provided by a dairy company in Pachuca de Soto, Hidalgo, México. The proteins were concentrated by precipitation with ammonium sulphate to obtain the whey protein concentrate (WPC). The concentration was determined following the procedure described by Tovar-Jiménez *et al*., (2012). The WPC obtained had an initial concentration of 99.80 ± 0.1 μg/mL. This was later concentrated 12 times to reach a final value of 1219.5 ± 0.6 μg/mL.

2.2 Microorganisms

The *S. reilianum* strain was donated by Dr. Santos Gerardo Leyva of the Universidad Autónoma

Chapingo (México). 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. The bacterial strains *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, *Salmonella* sp., and *E. coli*, as well as the *C. albicans* yeast were donated by the Hidalgo State Public Health Laboratory. Short-term growth was conducted in plates with nutritive agar (Bioxon) incubated at 37 °C for 24 h and stored at 4 °C; while they were preserved in glycerol for long-term storage (J. T. Baker) at 25 % and -20 °C.

2.3 *Purification of the aspartyl protease Eap1*

Purification of the enzyme of interest, Eap1, was performed as indicated by Mandujano-González *et al*., (2013).

2.4 *Determination of proteolytic activity*

The method employed to perform enzymatic hydrolysis kinetics used different units of proteolytic activity (2.01, 4.6 and 7.19 U/mg protein). For this purpose, the purified enzyme Eap1 from *S. reilianum* and two commercial enzymes of animal origin, trypsin (TP) from bovine pancreas (Sigma) and chymotrypsin (CMTP) from pancreas bovine (Sigma), were used, considering the optimum pH and temperature for each one. The procedure used 100 μL of purified enzyme at the afore mentioned units of enzymatic activity and 200 μL of the protein solution (875 μg WPC) at different hydrolysis times (0, 30, 60, 90 and 120 min). The non-hydrolyzed proteins were precipitated with 500 μL of 10 % TCA. The reaction mixture was centrifuged at 13000 rpm 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, considering the following controls to avoid false positives: WPC+TCA+Enzyme (stopped reaction at time zero), WPC+Sterile distilled water+Enzyme (To determine the effect of TCA), and Sterile distilled water+TCA+Enzyme (To determine the effect of WPC).

2.5 *Determination of antimicrobial activity by the disk-diffusion method in agar*

The peptides released after enzymatic hydrolysis were used to determine antimicrobial activity according to the methodology described by Tovar-Jiménez *et al*., (2012), using gram-positive bacteria *S. aureus*, gram-negative bacteria *K. pneumoniae*, *P. aeruginosa*, *Salmonella* sp and *E. coli*, and *C. albicans* yeast. For this purpose, a pre-inoculum in nutritive broth (Bioxon) was prepared and each microorganism was grown at 37 °C for 18 h with agitation at 200 rpm. Then, plates with nutritive agar (Bioxon) were inoculated with 200 μL of the pre-inoculum adjusted to an optimum density of 0.2 (λ = 600 nm) were added and dispersed homogeneously using a Digralsky handle. The samples (34 μg of released peptides/disk) were impregnated in 6-mm diameter disks previously sterilized, which were deposited symmetrically in the Petri dishes using sterilized tweezers, and incubated for 24 h at 37 °C to determine the inhibition halo. The halo was measured and interpreted following the recommendations of the National Committee for Clinical Laboratory Standards (1997) (NCCLS). The positive control used for the bacteria was nalidixic acid in suspension, while for the yeast it was Nizoral in suspension, at concentrations of 30 and 15 μg/disk, respectively. Results are reported as percentages of inhibition compared to the positive control, which was considered as 100 % inhibition against the strain studied. All determinations were performed in sextuplicate, considering as controls: the active and inactive enzymes (inactivated enzymes by heat at 80 ºC for 5 min) to the units of activity studied, WPC, sterile distilled water and TCA at 10 %.

2.6 *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 response variable (antimicrobial 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.

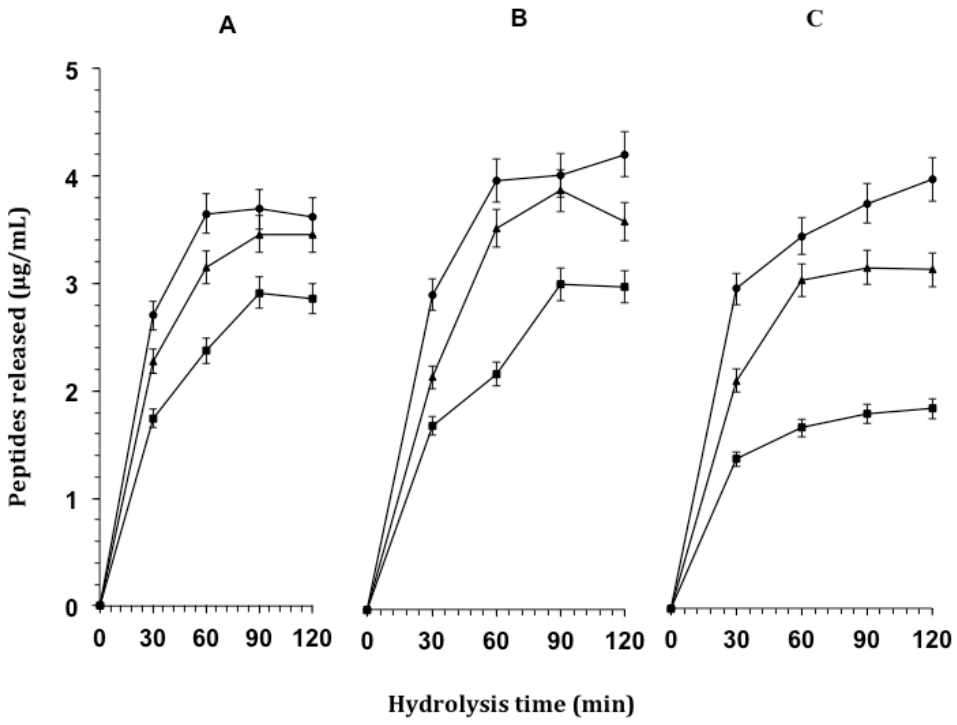

Fig. 1. Peptides released by hydrolysis of WPC with A: Trypsin, B: Chymotrypsin and C: Eap1 to different units of proteolytic enzymatic activity (■ 2.01 U/mg protein, ▲ 4.6 U/mg protein and •7.19 U/mg protein) and hydrolysis time.

3 Results and discussion

3.1 Enzymatic hydrolysis of WPC

The peptides released during hydrolysis of whey proteins depends on the amount of the proteolytic enzyme and time (Figure 1); that is, larger quantities of these compounds are released after 60 min of hydrolysis with 7.19 U/mg protein. This study observed that the protease Eap1 has the ability to hydrolyze whey proteins with results similar to those obtained with commercial enzymes. The statistical analysis conducted showed that there are statistically significant differences (p <0.05; $R^2_{adjusted}$: 0.9709) between the units of enzymatic activity and the hydrolysis times evaluated.

3.2 Antimicrobial activity of the peptides released after enzymatic hydrolysis

Of all the treatments performed, antimicrobial activity was observed only in the peptides obtained under the condition of 2.01 U/mg protein with proteolytic activity. It is important to note that none of the controls used in any treatment had antimicrobial effects. We only present the results where the greatest microbial inhibition was observed; that is, the enzymatic activity of 2.01 U/mg protein (Figure 2).

The presence of peptides with antimicrobial activity against *Salmonella* sp. (Figure 2A) was detected when chymotrypsin (CMTP) and the protease Eap1 were used. In the case of the peptides generated by hydrolysis of the WPC using trypsin (TP), no antimicrobial activity was observed. The highest inhibition was observed in the hydrolysates of Eap1 after hydrolysis for 90 and 120 min, but no significant differences were observed between these two treatments (LSD contrast test).

With respect to *S. aureus* (Figure 2B), all the peptides obtained under the different hydrolysis conditions presented antimicrobial activity. The best results were with the enzyme Eap1 after 90 and 120 min of hydrolysis. As above, the LSD contrast test found no significant difference between these two treatments.

The peptides obtained from the purified enzymes inhibited *K. pneumoniae* (Figure 2C). The greatest activity was observed with the hydrolysates obtained

with CMTP and no statistically significant difference was noted after 30 min of hydrolysis. Regarding the inhibition of *P. aeruginosa* (Figure 2D), the best results were obtained with Eap1 after 30 min of hydrolysis, as no statistically significant difference was detected after that time. Only the peptides obtained by hydrolysis with Eap1 inhibited growth of *C. albicans* (Figure 2E) after 30 min of hydrolysis; however, all the hydrolysates obtained with the different enzymes presented antimicrobial activity against *E. coli* (Figure 2F). In all cases, analyses indicated statistically significant differences between the hydrolysis time and the type of enzyme used (p <0.05: $R^2_{adjusted}$: 0.9569).

4 Discussion

In general, Eap1 released antimicrobial peptides with greater activity since the aspartyl proteases had a preference for the carbon adjacent to where the amino acids phenylalanine, proline, leucine and tyrosine are found (Narayanan *et al*., 2007). In contrast, chymotrypsin (CMTP) has greater affinity in the carbon adjacent to the amino acids tyrosine, phenylalanine and tryptophan (Zakharova *et al*., 2009). Trypsin (TP), meanwhile, showed a predilection for the amino acids lysine and arginine (Perona and Craik 2008), which are proximal to the positively-charged amino acids in lactoferrin, thus allowing us to attribute antimicrobial activity to them (Drago-Serrano 2006; Muro-Urista *et al*., 2011; Balcão *et al*., 2013).

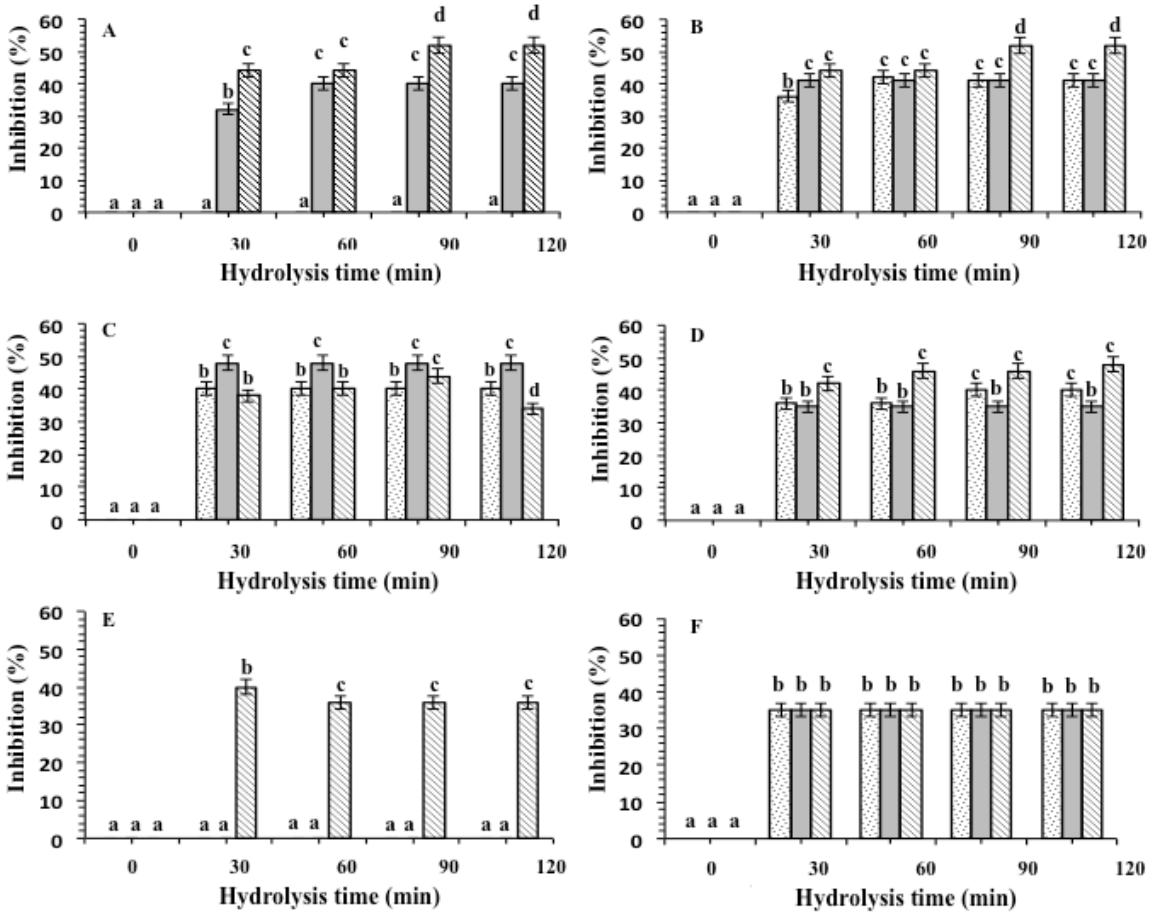

Fig. 2. Antimicrobial activity vs A: *Salmonella* sp., B: *S. aureus*, C: *K. pneumonae*, D: *P. aeruginosa*, E: *C. albicans*, F: *E. coli* from peptides released with: trypsin ⬚, chymotrypsin ▨ and Eap1 ■ at 2.01 U/mg protein.

In this sense, the study's success in obtaining peptides with greater mayor antimicrobial activity when accompanied by Eap1 may be due to the fact that the β-Lg and α-La are composed of 15.5% and 14.1 % of leucine, 4.7 % and 4 % of proline, 3.7 % and 4.1 % of phenylalanine, and 3.7 % and 4 % of tyrosine, respectively. This property means that they have a greater potential to release peptides with antimicrobial activity than the commercial enzymes evaluated. Similarly, for the sequence of β-Lg and α-La, the study found 1.9 and 2.1 % of tryptophan, 9.2 % and 10 % of lysine, and 1.8 % and 9 % of arginine, respectively (Drago-Serrano 2006; Muro-Urista *et al*., 2011; Tovar-Jiménez *et al*., 2012; Balcão *et al*., 2013); that is, the amino acids where commercial enzymes can also be hydrolyzed.

Given the type of inhibition observed, it is highly likely that during hydrolysis of the LF by the protease Eap1 peptides with elevated antimicrobial activity were released since, as mentioned above, diverse experimental evidence shows that the antimicrobial activity of peptides derives primarily from the hydrolysis of lactoferrin, which is made up of a simple polypeptide chain folded in two symmetric globular lobes (N and C) connected by a hinge region. Specifically, performing hydrolysis in the N lobe allows the release of cationic peptides like lactoferricin (Gifford *et al*., 2005). Descriptions suggest that this peptide damages the external membrane of gram-negative pathogenic bacteria, causing cellular lysis, as in the cases of *Salmonella* sp., enterohemorrhagic *E. coli* O157:H737, and *E. coli* O111.38. Other antimicrobial properties attributed to lactoferricin include its capacity to block bacterial adherence, and to inhibit DNA and RNA synthesis in both gram-positive and gram-negative bacteria (Gifford *et al*., 2005; Drago-Serrano 2006). Gifford *et al*., (2005) mention that the peptide lactoferricin has greater antimicrobial properties than intact lactoferrin, perhaps because its smaller size facilitates access to target sites on the microorganism's surface.

In addition to the hydrolysis of lactoferrin, there are reports of antimicrobial peptides derived from different proteins present in whey, such as lactoglobulin. Hydrolysis of this protein releases the peptides f15-20, f25-40, f78-83 and f92-100, which have antimicrobial activity against *Salmonella typhimurium*, *Streptococcus pneumonia* and *E. coli* (Pellegrini 2003). Similarly, we have descriptions of peptides derived through the hydrolysis of other whey proteins or oligopeptides. One of these is kappacin, a fragment of the glycomacropeptide identified by Rizzello *et al*., (2005). It is characterized by a phosphorylated residue of serine (Ser) and by the fact that it accumulates on the cell membrane where it forms an anionic pore. This makes it a powerful antibacterial peptide against gram-positive (*Streptococcus mutans*) and gram-negative (*Pseudomonas* gingivalis and *E. coli*) bacteria. Chatterton *et al*., (2006), meanwhile, mention that digestion of β-Lg and α-La with TP generates, respectively, four and two peptides with antibacterial effects against gram-positive bacteria. Also, McCann *et al*., (2006) hydrolyzed αs1-caseina with pepsin to isolate and identify positively-charged peptides (f99-109) that showed antimicrobial activity against *S. typhimurium*, *E. coli*, *S. enteritidis*, *C. freundii*, *B. subtilis* and *L. innocua*.

The peptides with antifungal activity, specifically against *C. albicans* (Figure 2E), were obtained only when the aspartyl protease of *S. reilianum* (Eap1) was used, as shown above. This may be due to the preference for hydrolysis in certain amino acids. In this regard, it is possible to release cationic peptides that could trigger a reduction in the mitochondrial function of *C. albicans*, as suggested by Lupetti *et al*., (2002). Also, the interaction of these cationic peptides with the cell membrane can form ionic channels that increase permeability, leading to cell death (Drago-Serrano 2006). This is an especially interesting result, since *C. albicans* is a pathogenic yeast that is difficult to control and can cause severe systemic infections in children and adults (Kumar and Singhi 2013; McManus and Coleman 2014). The fact that the same antimicrobial activity against the *E. coli* (Figure 2F) with the three enzymes evaluated may be due to that there are two characteristics common to most antimicrobial peptides, regardless of their structure or size. First, they have a positive charge due to the presence of a large number of basic amino acids (mostly lysine and arginine), and second, approximately 30% of the amino acids that constitute them are hydrophobic as mentioned by Hale and Hancock, (2007). Also, these amino acids are present in the structures of β-Lg, α-La and lactoferrin, mainly (Drago-Serrano 2006; Muro-Urista *et al*., 2011; Tovar-Jiménez *et al*., 2012; Balcão *et al*., 2013).

Conclusions

Analysis of these results clearly indicates that Eap1 was capable of releasing peptides that have better antimicrobial activity than the commercial enzymes

tested at 2.01 U/mg protein against the pathogens analyzed under these study conditions. 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.

We can also conclude that the hydrolysis of whey proteins using purified aspartyl protease (Eap1) of *S. reilianum* produces peptides with greater biological activity against the human pathogens studied, compared to commercial proteases. The use of this fungal protease has distinct advantages over the commercial enzymes most-widely used for this type of study (TP and CMTP), because in addition to making it possible to obtain peptides with greater inhibition capacity, the hydrolysates are active against *C. albicans*, thus offering an option for the generation of compounds potentially useful for treating and/or preventing the illnesses caused by this yeast.

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