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New peptides obtained by hydrolysis of caseins from bovine milk by protease extracted from the latex *Jacaratia corumbensis*

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ABSTRACT

The casein and its hydrolysates have many desirable functional properties, so there is great interest in its use in food and pharmaceutical industries. The hydrolysis of casein by a new protease obtained from latex *Jacaratia corumbensis* was performed to obtain antimicrobial peptides. Four proteins were visualized by electrophoresis of the enzyme extract of *J. corumbensis*, a band of molecular weight of approximately 30 kDa with the presence of other bands of low density. The fractions αs-CN, β-CN and κ-CN, were subjected to hydrolysis for a period of 1 min 24 h, where aliquots were collected and analyzed by SDS-PAGE to the characterization of peptides. The hydrolysates were evaluated for antimicrobial activity by determining the Minimum Inhibitory Concentration (MIC). The hydrolysates obtained at time of 2 h showed antimicrobial activity against microorganisms *Enterococcus faecalis*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*, indicating that the peptides obtained by hydrolysis of bovine casein by latex extract *J. corumbensis* showed activity antimicrobial. This fraction was subjected to reverse phase chromatography for purification of peptides and subsequent mass spectrometric evaluation. The peptides were sequenced and evaluated for their antimicrobial potential.

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1. Introduction

The *Jacaratia corumbensis* O. Kuntze is a native plant of the semiarid, a region of the Brazil's northeastern, and a member of the *Caricaceae* family. Due to its ability to survive long periods of drought, the plant is widespread in the semiarid zone and it grows in red and yellow soil in a region where the average annual rainfall is between 400 and 800 mm (Albuquerque, Soares, & Araújo Filho, 1982).

Proteases presenting coagulant activity in milk were identified in the latex obtained from the *Jacaratia* root, and were measured the biochemical properties of latex extract and the best conditions for hydrolysis of milk proteins (Duarte et al., 2009).

Antimicrobial peptides (AMPs) have been identified in proteins from different sources such as proteins derived from spinach, royal jelly (Fontana et al., 2004), marine fish (Rajanbabu & Chen, 2011) and flowers (Tavares et al., 2008) but most antimicrobial peptides are derived from food proteins in milk (Floris, Recio, Berkhout, & Visser, 2003).

AMPs are generated by proteolytic cleavage from pro-proteins antimicrobial. Most AMPs contain between 12 and 50 residues, including two or more positively charged residues and a large proportion (generally > 50%) of hydrophobic residues (Dürr, Sudheendra, & Ramamoorthy, 2006). The cationic side chains of arginine, lysine and histidine are used to mediate peptide interactions with negatively charged membranes and/or cell walls of bacteria, including lipopolysaccharide. The ability to associate with membranes is a characteristic of AMPs (Barzyk, Campagna, Więcław, Korchowiec, & Rogalska, 2009). It has been shown that some AMPs with longer chains are more effective in killing both Gram-negative and Gram-positive bacteria when compared to smaller peptides (Liu et al., 2007).

Although powerful antibiotics are available to treat microbial infections, antimicrobial peptides from casein have the benefit of

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killing target cells rapidly and with a broad spectrum of action (Salami et al., 2008).

Herein we described the identification of peptides formed by the hydrolysis of caseins from bovine milk by protease extracted latex *J. corumbensis* and evaluated its antimicrobial activity.

2. Materials and methods

2.1. Enzyme extraction

Fresh biological material (*J. corumbensis* O. Kuntze root) was washed several times with distilled water and disinfected with sodium hypochloride (100 mL L^{-1}). Next, the latex obtained from the root was diluted (1 g) in 100 mL in 9 g L^{-1} NaCl solution. The solution was then stirred at room temperature (25 °C) for 1 h and filtered through a filter paper and maintained at 4 °C. This solution was used to determine total protein, proteolytic and milk clotting activities, according to Duarte et al., 2009.

2.2. Protease assay

Total protease activity was assayed at 25 °C as described by Ginther (1979), using azocasein (10 g L^{-1}, Sigma) in 0.1 mol L^{-1} Tris–HCl (pH 7.6) containing 1.7 g L^{-1} $CaCl_2$ as a substrate. One unit of activity was defined as the amount of enzyme that produces a 1.0 absorbance increment in 1 h at 440 nm. Total protein was determined by the method described by Bradford (1976) using bovine serum albumin as standard.

2.3. Milk clotting protease assay

The clotting activities of plant extracts were determined according to the method of Berridge (1952).

2.4. Preparation of bovine whole casein

Raw milk was obtained from a local dairy herd of Dutch cows (Garanhuns city, Pernambuco state, Brazil) and immediately stored at 20 °C until used. The milk was skimmed by centrifugation (2100 *g* at 32 °C for 30 min) and the whole casein was prepared by isoelectric precipitation at pH 4.6 with 1 mol L^{-1} HCl. The precipitate was washed three times with pure water, solubilized at pH 7.0 by addition of 1 mol L^{-1} NaOH, and the precipitation–solubilization cycle was repeated twice. Finally, the whole casein was solubilized at pH 7.0 with 1 mol L^{-1} NaOH, dialyzed against pure water at 4 °C and freeze-dried (Egito et al., 2006).

2.5. Hydrolysis of casein by root latex of J. corumbensis O. Kuntze

Whole casein was dissolved to 2 mg mL^{-1} in 0.069 g L^{-1} sodium phosphate buffer pH 6.5 and incubated with 5.1 μg of extract *J. corumbensis*. During a 24 h incubation period, samples were removed at the following times since the beginning of incubation: 1 min, 5 min, 10 min, 15 min, 20 min, 40 min, 1 h, 2 h, 4 h, 6 h, and 24 h.

Hydrolysis was performed at 37 °C and reaction stopped by heating for 5 min at 100 °C for SDS-PAGE analysis, or for 10 min for HPLC or mass spectrometry. HPLC samples were directly stored at −20 °C whereas mass spec samples were first freeze-dried and then stored at −20 °C for future analysis (Egito et al., 2007).

2.6. SDS-Page analysis

For SDS-PAGE analysis 2.5×10^{-5} U mL^{-1} were used of *J. corumbensis* protein extract to allow visualization of the electrophoretic bands of the breakdown products. For the electrophoretic analysis, 300 mL of 0.125 mol L^{-1} Tris–HCl buffer, pH 6.8, containing 0.28 g L^{-1} SDS, 50 mL L^{-1} 2-mercaptoethanol, 100 mL L^{-1} glycerol, and 0.1 mL L^{-1} bromophenol blue were added for 100 mL of each hydrolysate solution. The latter was then boiled for 3 min before electrophoretic analysis (Laemmli, 1970).

2.7. Evaluation of antimicrobial activity

Antimicrobial activity of potential bioactive peptides contained in bovine milk caseins hydrolysates generated by the proteases from *J. corumbensis* extracts were tested by the MIC method (Minimum Inhibitory Concentration) as described by Standard Clinical and Laboratory Standards Institute (CLSI) (2003). The microorganisms used were *Enterococcus faecalis* ATCC 6057, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 29665 and *Staphylococcus aureus* ATCC 6538. The amount of the casein hydrolysate and peptides tested against these microorganisms was 250 mg, 125 mg, 62.5 mg, 50 mg, 40 mg, 30 mg, 20 mg and 10 mg mL^{-1}. The antimicrobial activity was performed with hydrolysates of all times analyzed.

2.8. High-performance liquid chromatography (HPLC)

The identification of peptides was performed using a C18 column analytical Shim-Pack CLC-ODS (M) 25 cm (250 × 4 mm, 5 μm size particle, 10 nm porosity, Shimadzu, Japan) connected to HPLC model LC20A Prominence, Shimadzu, Japan. The casein used as standard and the hydrolysate solutions were dissolved to 20 mg mL^{-1} in 0.1 mol L^{-1} phosphate buffer, pH 6,5. 100 μL of this solution was injected into the column C18. The column was eluted in a binary gradient from 300 mL L^{-1} and 500 mL L^{-1} acetonitrile in the presence of 1 mL L^{-1} trifluoroacetic acid. The wavelength for detection was at 215 nm with photodiode array detector model SPD-M20A (Shimadzu) and flow of 3.0 mL min^{-1}.

2.9. Identification of peptides by electrospray ionization tandem mass spectrometry

Active peptide fractions from HPLC were analyzed by Matrix-Assisted Laser Desorption/Ionization–Time of Fight Mass Spectrometry (MALDI–TOF MS) by dried droplet method (Karas & Hillenkamp, 1988) with modifications, using an Autoflex III mass spectrometer (Bruker Daltonics, Billerica, USA), equipped with a 355 nm Nd:YAG laser. One microliter of the sample was mixed with 3 μL of 10 mg mL^{-1} matrix solution (α-cyano-4-hydroxycinnamic acid in 500 mL L^{-1} acetonitrile with 3 mL L^{-1} trifluoroacetic acid). Then 0.5 μL of this mixture was spotted onto a MALDI target plate (MTP 384 ground steel, Bruker Daltonics) and left to dry at room temperature for 10 min. Mass spectra were acquired in positive reflector mode with an acceleration voltage of 19 kV and laser frequency of 100 Hz. The detection range was m/z 1000–4480, and usually 200 laser shots were accumulated for each spectrum. After that, the selected parent ions were fragmented using LIFT mode. External calibration was performed using standard mixture of peptides (Sigma). Data were acquired using the Flex Control software (Version 3.0, Bruker Daltonics), and spectra were processed using Flex Analysis software (Version 3.0, Bruker Daltonics). The obtained amino acid sequences were compared with sequences in the protein database using the NCBI BLAST program (www.ncbi.nlm.nih.gov).

3. Results

3.1. Latex of J. corumbensis has protease activity and hydrolyses casein

The latex of *J. corumbensis* had 0.68 mg of protein with proteolytic activity of 11.43 U mL^{-1}. The extract showed high specific milk coagulation activity (2.5 × 10^{-4} U mg^{-1}). Four proteins of the *J. corumbensis*'s extract were visualized by SDS-PAGE (Fig. 1). A strong band of approximately 30 kDa was seen along with other bands of low molecular weight.

Fig. 2 shows a progressive increase in hydrolysis of caseins by the enzyme extract. A wide range of larger, medium and smaller peptides were generated, depending on enzyme specificity. Aliquots were removed from 1 min up to 24 h of hydrolysis of peptides for further evaluation. The β- and κ-casein were pooled in the used standard.

3.2. Evaluation of peptides against microorganisms

To evaluate the antimicrobial activity of the obtained hydrolysate by treatment with crude enzyme extract from *J. corumbensis*, we assessed the Minimum Inhibitory Concentration (MIC) (Table 1) with all the times of hydrolysis. The time of 2 h of hydrolysis was only which showed activity.

The concentrations of peptides in the time of 2 h that inhibited microbial growth of all strains tested were 250 mg, 125 mg and 62.5 mg. The concentration of 50 mg showed activity only for *S. aureus*.

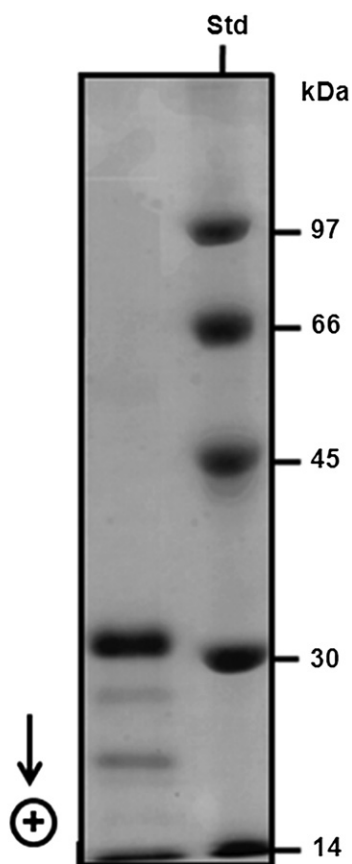

Fig. 1. Analysis of protein crude extract of the latex *Jacaratia corumbensis* (0.68 mg mL^{-1}) performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Std: molecular mass standards.

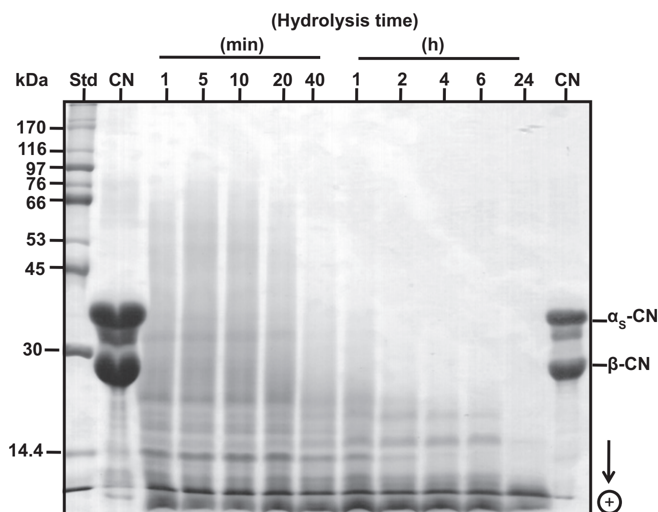

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of bovine sodium caseinate (10 mg mL^{-1}) hydrolyzed as a function of time at pH 6.5 and 37 °C by crude extract latex *Jacaratia corumbensis* (2.5 × 10^{-4} U mg^{-1}). Std: molecular mass standards; CN: bovine sodium caseinate; α_s-CN: αs1 + αs1-caseins; β-CN: β-casein. CN on the left (10 μg mL^{-1}) and CN on the right (5 μg mL^{-1}).

3.3. Purification of antimicrobial peptides

The antimicrobial activity was performed with hydrolysates of all time, but only antimicrobial activity was detected in the hydrolyzed of 2 h. From these data, this hydrolyzed was analyzed by HPLC and the profile is shown in the (Fig. 3). The chromatogram obtained at 215 nm wavelength showed a number of peaks. These six peaks obtained with greater intensity of absorbance were collected and analyzed for their antimicrobial activity; except for peak 1 and peak 4, all showed antimicrobial activity. All peaks showed the same degree of antimicrobial activity, as shown in Table 1. These six peaks were further analyzed by mass spectrometry. Next, MS analysis was then performed to better determine the molecular weight of AMPS. The peptides that showed no antimicrobial activity were also subjected to sequencing for the knowledge of its structure, as well as for better understanding of their lack of antimicrobial activity.

The first HPLC peak corresponded to a 1252.61 Da molecule. MS/MS fragmentation revealed a sequence of 10 residues with a modified N-terminus (pyroglutamic) Pyro-glu-E-Q-N-Q-E-QP-I-R (Fig. 4). A BLAST search identified the sequence as a precursor of bovine κ-casein, and rich in positive amino acids. Despite the

Table 1
Minimum inhibitory concentration (MIC) of peptides generated during hydrolysis of casein by 2 h from *Jacaratia corumbensis* latex and the same results were presented with the peaks isolated by HPLC except for peak 1 and peak 4 that showed no activity. Column 1 shows the microorganisms, as well as their respective concentration in Colony Forming Units (CFU) and the other columns represent the concentration of peptide 250 mg, 125 mg, 62.5 mg, 50 mg, 40 mg, 30 mg, 20 mg and 10 mg mL^{-1} respectively. The symbols (+) and (−) represents the presence or absence of growth of microorganisms.

Microorganism (10^7 CFU mL^{-1})	MIC (mg mL^{-1})							
	250	125	62.5	50	40	30	20	10
E. faecalis	−	−	−	+	+	+	+	+
B. subtilis	−	−	−	+	+	+	+	+
E. coli	−	−	−	+	+	+	+	+
P. aeruginosa	−	−	−	+	+	+	+	+
K. pneumonia	−	−	−	+	+	+	+	+
S. aureus	−	−	−	−	+	+	+	+

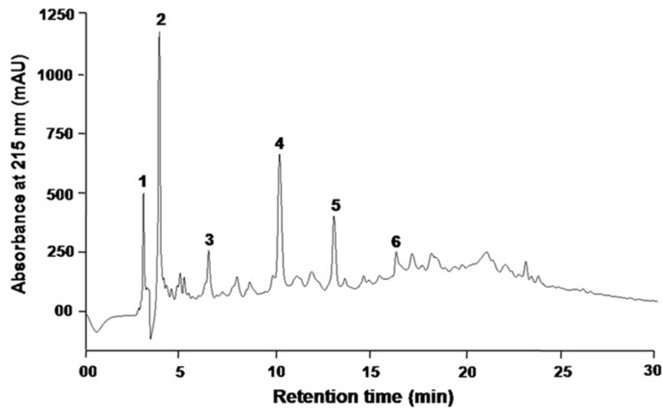

Fig. 3. Reversed-phase high-performance liquid chromatography (C18 column) antimicrobial peptides generated for 2 h casein hydrolysis by crude extract latex *Jacaratia corumbensis* (2.5×10^{-4} U mg^{-1}) at pH 6.5 and 37 °C, of 30 min and absorbance at 215 nm. The peaks numbered 1 to 6 were presented the antimicrobial activity.

presence of several positively charged residues, this peptide had no antimicrobial activity and does not show similarity to some bioactive peptides known.

The second peak represented a molecule of 2616.54 Da with a positively charged sequence of 21 residues corresponding to R-P-K-H-P-I-K-H-Q-G-L-P-Q-E-V-L-N-E-N-L-L (Fig. 5). The sequence shared 71% identity with a αs1-casein precursor. The third peak (1494.82 Da) represented a sequence of the following 13 residues: G-L-P-Q-E-V-L-N-E-N-L-L-R (Fig. 6). Of these, 7 were positively charged. This sequence is also a precursor of αs1-casein. The fourth peak corresponded to a molecular weight of 1601.77 Da that contained the A-F-L-L-Y-E-Q-P-V-L-G-P-V-R sequence of 14 residues, which is a precursor of β-casein with a predominance of non-polar amino acids. This sequence does not have antimicrobial activity, despite the presence of non-polar amino acids that could facilitate the antimicrobial. The fifth peak indicated a peptide of 2910.98 Da corresponding to 23 residues R-P-K-H-P-I-K-H-Q-G-L-P-Q-E-V-L-N-E-N-L-L-R-F (Fig. 7) of which 13 were positively charged having αs1-casein as a precursor. The last peak (Fig. 8) indicated a peptide of 2053.75 Da corresponding to 15 residues H-Q-G-L-P-Q-E-V-L-N-E-N-L-L-R from αs1-casein. Of these, seven were positively charged. Note that most sequences derive from αs1-casein and share great similarity with the amino acid sequences here described.

4. Discussion

Proteolytic enzymes from plant sources are well suited to the pharmaceutical and food industries, as they are active over a wide range of temperature and pH, and possess broad substrate specificity and high stability under extreme conditions (Tripathi, Tomar, & Jagannadham, 2011). The uses of enzymes extracted from plants to obtain peptides by hydrolysis of bovine caseins have intensified greatly in recent years. Results found by Egito et al. (2007) where proteases extracted from the seeds of *Albizia lebbeck* and *Helianthus annus* clotted milk as a function of time, are similar to enzymatic extract latex *J. corumbensis* which showed the ability to hydrolyze bovine milk caseins exhibiting activity coagulant. These results confirm data obtained by Duarte et al. (2009), suggesting that this plant has one or more enzymes with rennet-like. Usually, plants with latex have several proteases, as is the case of *Euphorbia dupifera*, *Euphorbia milli* (Moro et al., 2008) and others.

In Fig. 1 we visualize four bands, where we can interpret as four proteins or one that had its sulfide bridges cleaved by denaturing electrophoresis conditions. The extract showed a high activity milk coagulation (2.5×10^{-4} U mg^{-1}) (Fig. 1) as compared to that found in the seed extract of *H. annus* (5.8×10^{-3} U mg^{-1}) and *A. lebbeck* (153×10^{-3} U mg^{-1}) as previously reported (Egito et al., 2007).

Proteolysis of caseins performed by enzyme extract latex *J. corumbensis* during incubation was assessed by SDS-PAGE and was presented in Fig. 2. The presence of several prolyl residues in the primary structures of CNs interrupts the secondary structure and also results in an open (as opposed to a compact) tertiary structure, with greater conformational flexibility than compact globular proteins, and making these proteins more susceptible to digestion (Salami et al., 2008). The degree of casein hydrolysis by the *J. corumbensis* enzymatic extract increased with time and at

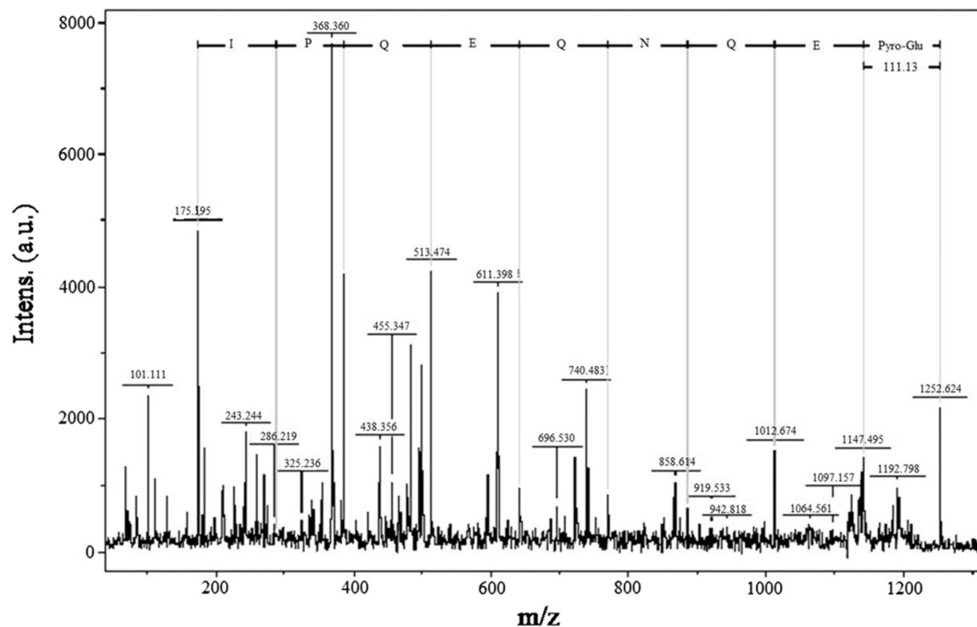

Fig. 4. Reconstructed mass from Matrix-assisted laser desorption/ionization–time of flight MS/MS (MALDI–TOF MS/MS) of the main breakdown products generated from bovine casein hydrolyzed for 2 h by crude extract latex *Jacaratia corumbensis* (2.5×10^{-4} U mg^{-1}), which yielded first peak of the sequence with 10 residues with a modification N-terminal pyroglutamic, but not presented antimicrobial activity.

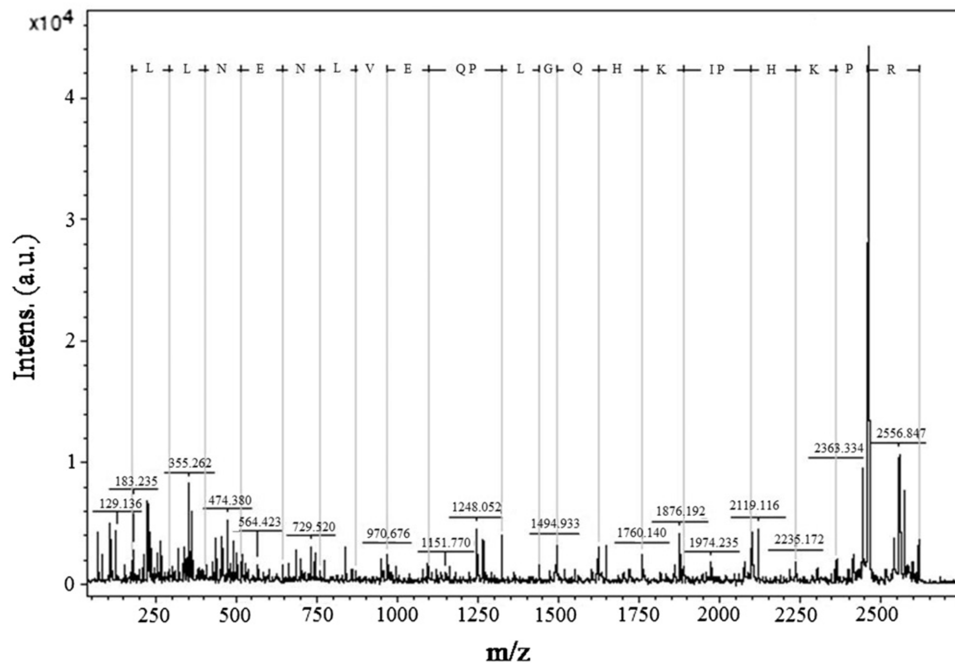

Fig. 5. Reconstructed mass from Matrix-assisted laser desorption/ionization–time of flight MS/MS (MALDI–TOF MS/MS) of the main breakdown products generated from bovine casein hydrolyzed for 2 h by crude extract latex *Jacaratia corumbensis* (2.5×10^{-4} U mg^{-1}), which yielded second peak of the sequence with 19 residues and presented antimicrobial activity.

a higher rate when compared to those described by Egito et al. (2007) and Bruno et al. (2010).

Antimicrobial peptides encrypted in milk proteins may be found in lactoferrin (LF), casein subunits (αs1, αs1) as well as α-lactalbumin and β-lactoglobulin (Jenssen & Hancock, 2009). The most studied peptide domain is the 25mer lactoferricin of LF (f 17–41), which is released by pepsin digestion and displays a broad range of antimicrobial activity (Wakabayashi, Takase, & Tomita, 2003). Casein-encrypted 39-amino acid casocidin (αS2-CN f [165–203]), which is obtained by pepsin digestion and is active against *Staphylococci* and *E. coli*, and the 23-amino acid isracidin (Somkuti & Paul, 2010) are examples of less studied peptides.

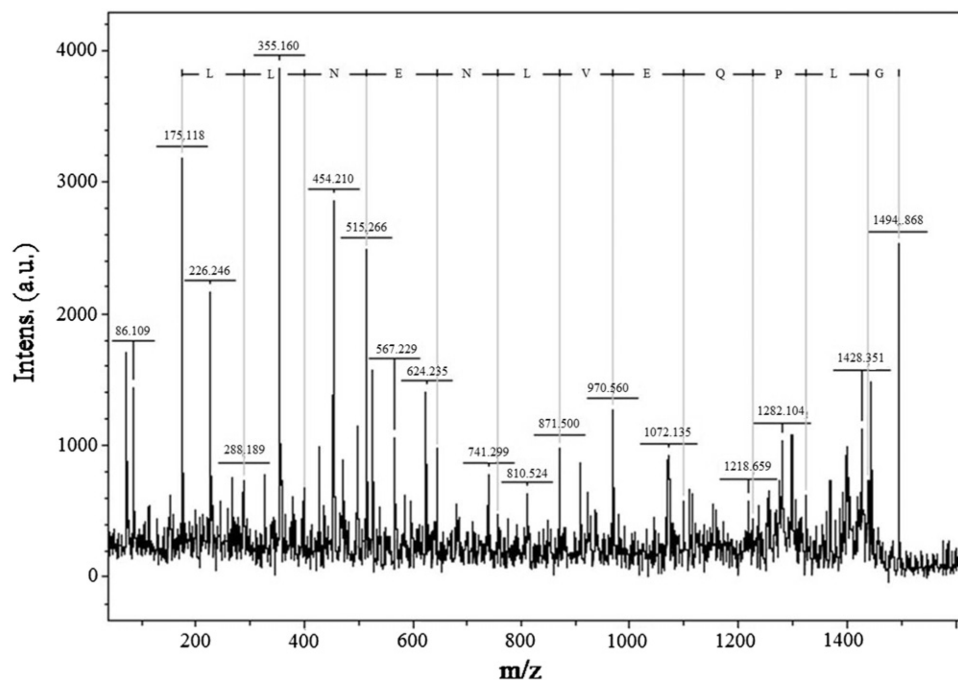

Fig. 6. Reconstructed mass from Matrix-assisted laser desorption/ionization–time of flight MS/MS (MALDI–TOF MS/MS) of the main breakdown products generated from bovine casein hydrolyzed for 2 h by crude extract latex *Jacaratia corumbensis* (2.5×10^{-4} U mg^{-1}), which yielded third peak of the sequence with 12 residues and presented antimicrobial activity.

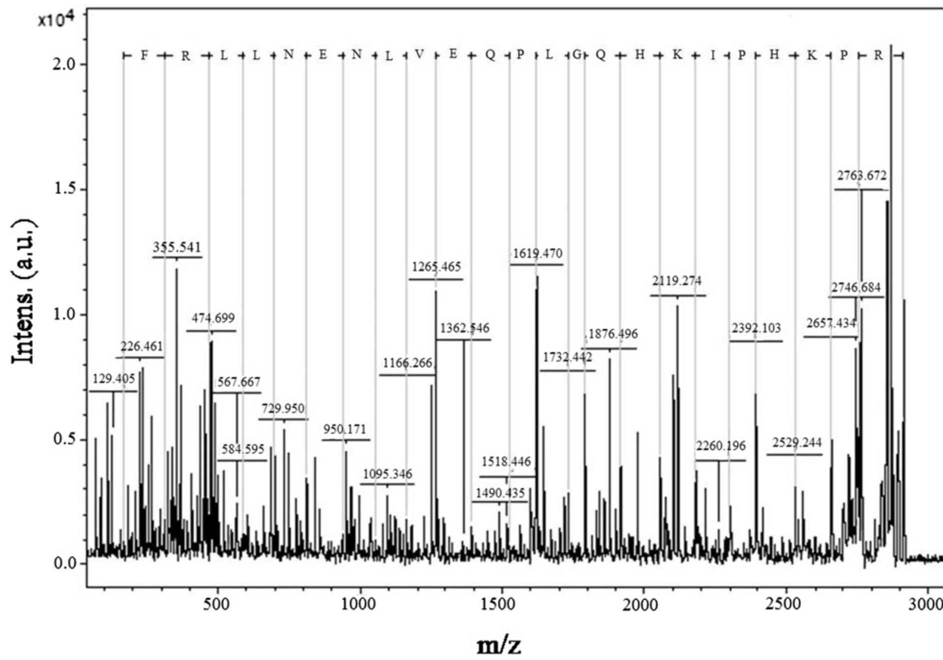

Fig. 7. Reconstructed mass from Matrix-assisted laser desorption/ionization–time of flight MS/MS (MALDI–TOF MS/MS) of the main breakdown products generated from bovine casein hydrolyzed for 2 h by crude extract latex *Jacaratia corumbensis* (2.5×10^{-4} U mg^{-1}), which yielded fifth peak of the sequence with 23 residues and presented antimicrobial activity.

Herein we investigated whether the hydrolysis of bovine milk caseins by proteases from the root latex of *J. corumbensis* could generate peptides presenting antimicrobial activity. We found that the concentrations of casein hydrolysate with time 2 h the 250 mg, 125 mg and 62.5 mg had antimicrobial activity against all the strains and the concentration of 50 mg only eliminate *S. aureus*. This result is very interesting because *S. aureus* is one of the major pathogens in hospital infections and is responsible for inactivating the action of various antibiotics, making the multidrug resistance, a major problem public health.

The antimicrobial activity of the 2 h casein hydrolysates may have resulted from a high concentration of AMPs, which is

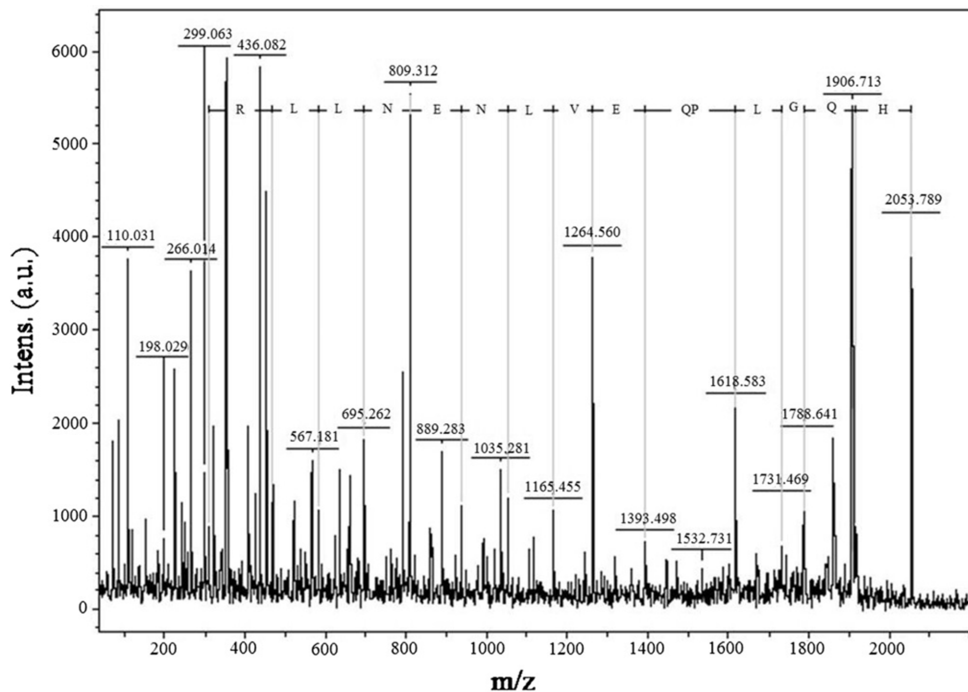

Fig. 8. Reconstructed mass from Matrix-assisted laser desorption/ionization–time of flight MS/MS (MALDI–TOF MS/MS) of the main breakdown products generated from bovine casein hydrolyzed for 2 h by crude extract latex *Jacaratia corumbensis* (2.5×10^{-4} U mg^{-1}), which yielded sixth peak of the sequence with 14 residues and presented antimicrobial activity.

evidenced by the great number of bands seen in the gel (Fig. 2). According to Melo, Ferre, and Castanho (2009), the average ratio required for microbial cell death is one AMP molecule for every six phospholipid molecules. Antibiotics have been shown to act in two different ways, by targeting the physiology of the pathogen or by disrupting the cellular structure of the bacterial cells. Antibiotics that act according to the latter mechanism rarely encounter resistant strains (Andrushchenko, Aarabi, Nguyen, Prenner, & Vogel, 2008). Therefore, antibiotics that disrupt the plasma membrane via pore or ion channel formation have been widely used. Small bioactive peptides that act against bacteria, yeast, and even certain enveloped viruses, are among the most potent antibiotics (Jeong et al., 2010).

The sequence of antimicrobial peptides obtained by mass spectrometry was analyzed and the first peak shows the formation of pyroglutamic acid, a known post-translational change that occurs in proteins carrying a glutamine in the N-terminus. This results from an internal nucleophile attack in which a molecule of ammonia is expelled. This occurs in an acidic environment similar to that of our experiments (Baldwin et al., 1990). According to the BLAST search, the corresponding sequence of the first peak of the chromatogram was a precursor of κ-casein with 33.7%, but showed no antimicrobial activity, probably due to its modification N-terminal or the sequence showed no similarity to the peptides described in the literature.

The fourth peak chromatogram showed no antimicrobial activity against any of the microorganisms used and showed no similarity with most antimicrobial peptides in the literature. According to the BLAST search, the corresponding sequence of the chromatogram was a precursor of β-casein, but does not show similarity to the peptides derived from β-casein in the literature.

The second, third, fifth, and sixth peaks of the chromatogram obtained at 2 h of reaction were also precursors of the α-s1-casein. These peptides shared high similarity with an antimicrobial peptide of 23 amino acids known as isracidin (αs1-CN f[1–23]). The latter is generated by the digestion of chymosin and chymotrypsin is known by its activity against *Staphylococci* and *E. coli* (Hayes, Ross, Fitzgerald, Hill, & Stanton, 2006; Somkuti & Paul, 2010). The fifth peak also had 23 amino acids, similar to what is found in isracidin. However, our sequence presented a phenylalanine in the N-terminus region instead of an arginine. The peptides corresponding to the third and sixth peaks presented a VLNENLLR domain also found in isracidin and caseicin B. The latter is also highly active against *E. coli* and *Enterobacter. sakazakii* and is also released from casein by *Lactobacillus acidophilus* DPC6026 (Hayes et al., 2006). In terms of specific plant protease used, the sites of cleavage of substrate were all next amino acids residues of positively charged and apolar, predominantly arginine.

This study has shown that proteases found in the latex of *J. corumbensis* may be used for the proteolysis of the milk casein, as well as for generating bioactive peptides. We found that with 2 h of hydrolysis obtained antimicrobial peptides that were capable of inhibiting the growth of all strains tested and the lowest concentration was effective against *S. aureus.* More studies are needed to verify the action of these peptides on the organism animal for future application as an antimicrobial.

Acknowledgments

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