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EXPLORING THE MILK-CLOTTING PROPERTIES OF EXTRACTS FROM *Bromelia pinguin* FRUIT

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ABSTRACT

Plants represent an attractive source of milk-clotting proteases with potential use for chymosin substitution in cheesemaking process. A crude enzymatic extract from *B. pinguin* fruit showed capacity to clot milk efficiently in a short time. It showed a maximum milk-clotting activity around 4 U/mL in the range of 70-80 ºC. This value indicated that one mL of the extract was capable to clot around 400 mL of milk in 40 min at the specified condition. *B. pinguin* extract presented only 25% of its maximum activity under standard temperature (30-35 ºC) for cheese-making. Proteases inhibitors indicated that cysteine and serine proteases were present in the extract and could be responsible for the milk-clotting activity found. SDS-PAGE analysis of casein hydrolysis indicated that proteases in *B. pinguin* extract were more proteolytic than chymosin. The high presence of milk-clotting proteases in *B. pinguin* fruit offers an alternative new source of proteases for biotechnological processes.

Keywords: *Bromelia pinguin*, milk-clotting activity, proteolytic activity, chymosin, cheesemaking

INTRODUCTION

Rennet or milk coagulants are enzymatic preparations employed in cheesemaking process for thousand years. Rennet is an enzymatic preparation traditionally extracted from the abomasum (fourth stomach) of young ruminants, mainly from calves (**Richardson and Chaudhari, 1970**). Calf rennet contains a high concentration of chymosin (EC 3.4.23.4), which can represent up to 95% of total proteases in young calves abomasum extracts. However, this proportion decreases with animal age, becoming pepsin the predominant enzyme in adult cattle abomasum extracts (**Chitpinityol and Crabbe 1998**). Chymosin is the preferred enzyme in cheesemaking process due to its high specificity over the Phe_{105}-Met_{106} peptide bond of κ-casein (first step in enzymatic milk coagulation), causing casein micelle destabilization, aggregation and clot formation (**Hyslop, 2003**). The worldwide increase in cheese consumption and the reduction in natural rennet supply has induced to look for new alternative sources of milk coagulants such as those obtained from microbial fermentation or genetically modified microorganisms. Some specific genera such as *Mucor baciliformis* and *Rhizomucor miehei* can produce, by fermentation, chymosin-like enzymes with similar milk-clotting properties (**Chitpinityol and Crabbe, 1998; Machalinski *et al.* 2006**). On the other hand, genetically modified organisms (e.g., *Aspergillus nigger, Kluyveromyces lactis and E. coli* carrying the calf chymosin gene) have been used to obtain recombinant chymosin (**Barbano and Rasmussen, 1992; Kappeler *et al.* 2006; Mazorra-Manzano *et al.* 2013a**). Regardless, the use of animal derived or genetically engineered products are limited in some countries due to religious reasons (e.g. Judaism and Islam), diet or legal regulations (**Roseiro *et al.* 2003**). Recently, emphasis has been placed on the screening of plants as a source of proteases with potential biotechnological applications such as meat tenderness, beer clarification and natural rennet substitutes for cheese production (**Feijoo-Siota and Villa, 2011**). A high concentration of proteases has been found naturally in some plants such as in melon (*Cucumis melo*) and kiwi (*Actinidia deliciosa*) fruits, ginger (*Zingiber officinale*) rhizome, and moringa (*Moringa oleifera*) flowers (**Pontual *et al.* 2012; Mazorra-Manzano *et al.* 2013b**). Roots, leaves, stems, fruits, flowers and some plant secretions (e.g. latex) have been commonly researched for protease characterization, exploring their proteolytic and milk-clotting properties.

Cynara cardunculus flowers extract, containing high concentration of chymosin-like proteases (cynarases, cyprosines and cardosines), is the most representative example of phytoproteases use in cheesemaking (**Esteves *et al.* 2003**). This extract has been traditionally used in the production of Mediterranean cheeses such as Serra da Estrella and La Serena (**Roseiro *et al.* 2003; Galán *et al.* 2012**). Their unique texture and flavor characteristics have made possible their Protected Designation of Origin status (**Jacob *et al.* 2011**).
Bromelia pinguin is a plant species from the Bromeliaceae family broadly distributed in some regions of Mexico and tropical America. In some Mexican regions, its fruit, known as "aguama" or "guamaras", is collected from wild cultivars and commercialized in regional markets. It has been reported that possesses medicinal properties against respiratory diseases (cough, asthma or bronchitis) and antiparasitic properties (**Camacho-Hernández *et al.* 2002; Payrol *et al.* 2005a;**). Previous studies have evidenced the presence of proteases in fruits of this plant (**Toro-Goyco *et al.* 1968; Payrol *et al.* 2005b; Moreno-Hernandez et al., 2017**). To the best of our knowledge, there are not studies related with the evaluation of its milk coagulation properties. Therefore, the objective of this research was to evaluate the milk-clotting properties of *B. pinguin* fruit as a new source of proteases.

MATERIALS AND METHODS

Vegetable material and regents

Ripe fruit (approx. 1500 g) from *Bromelia pinguin* were collected from wild plants in the valley of Culiacan, Sinaloa, México. The washed fruit, packed in plastic zipper bags, was stored at -30ºC until use. All chemicals used were analytical grade supplied from Sigma (Sigma-Aldrich Co., St. Louis, MO), unless otherwise specified.

Enzymatic extract preparation

Enzymatic crude extracts (CE) were prepared from *B. pinguin* fruit pulp obtained by peel and seeds removal. One part of fruit pulp was mixed with five parts (w/v) of cold buffer (20 mM Tris-HCl, pH 7) and homogenized in a blender for 2 min

(Osterizer Mexicana, S.A, México). The homogenate was filtered using cheesecloth and centrifuged at 20,000×*g* for 25 min at 4ºC in a Sorvall RC-6 centrifuge (Thermo Scientific, Waltham, MA, USA). Supernatant, named CE, was used the same day of preparation and kept cold at all times.

Protein quantification

CE protein concentration was determined by the **Bradford (1976)** method. Briefly, CE was mixed with Bradford reagent (0.1:1 v/v) and incubated at room temperature during 10 min for color development. Absorbance at 595 nm was registered. A standard curve of bovine serum albumin (0.05-0.5 mg/mL) was used for protein quantification.

Milk-clotting activity

Milk-clotting activity (MCA) was determined according to **Arima *et al.* (1970)** with some modifications. Briefly, 10 mL of rehydrated low fat milk (10% w/v, containing 0.022% of $CaCl_2$) was mixed with 500 µL of CE and incubated at different temperatures in the range of 25-90 ºC. Milk-clotting time (*t*) was registered as the time elapsed from the addition of enzymatic solution until milk clot formation was observed. MCA for commercial chymosin (diluted 1:9, v/v, Cuamix®, CUAMEX, México) was determined at 35 °C and used as reference. One milk-clotting unit was defined as the amount of CE (mL) required for clotting 100 mL of milk in 40 min (2400 sec), under the assay conditions. The MCA was calculated by using the following equation and expressed in Soxhlet units per mL of coagulant (SU/mL).

$$\text{MCA (SU/mL)} = \frac{2400}{t} \times \frac{S}{E} \qquad (1)$$

where *t*=clotting time (sec); *S*= volume of milk (mL); E= volume of enzymatic solution

Proteolytic activity assay

This activity was assayed as reported previously by **Mazorra-Manzano *et al.* (2013a)**. Briefly, a volume (450 µL) of 1% (w/v) substrate casein or bovine serum albumin (BSA) solution (100 mM sodium phosphate buffer, pH 6.8) was mixed with 50 µL of CE or chymosin and incubated at 35 ºC for 60 min. Enzymatic reaction was stopped by the addition of 500 µL of 5% (w/v) trichloroacetic acid (TCA). The mixture was incubated on an ice bath for 10 min and then centrifuged at 10,000 ×*g* for 20 min at 4 °C, then the absorbance of supernatant was registered at 280 nm. Control sample was prepared following the same protocol but adding TCA solution immediately after mixing enzyme and substrate. One unit of proteolytic activity (PA) was defined as the amount of protein (mg) that increased one unit of absorbance under the assay conditions. PA was determined in triplicate and the determination repeated three times using independents extracts.

SDS-PAGE analysis of casein hydrolysis by *B. pinguin* extract and its inhibition

Casein hydrolysis profile obtained by the action of B. pinguin proteases was evaluated by incubating a mixture of 0.45 mL of 1 % (w/v) of casein solution (100 mM sodium phosphate, pH 6.8) and 50 µL of enzymatic extract at 35 ºC for 10, 20, 30, 40 and 50 min. The enzymatic reaction was terminated by the addition of 500 µL of 2XSDS sample buffer and heated at 100 ºC for 5 min. SDS-PAGE analysis was performed using 15% acrylamide gels under reducing conditions, according to **Laemmli (1970)**. Protein separation was performed using an electrophoresis unit SE300 mini VE (Hoefer, Holliston, MA, USA) at 120 V for 90 min and gels stained with Coomassie Brilliant Blue R250. Inhibition studies were assayed by incubating CE with the following inhibitors: pepstatin A (1.5 mM), soybean trypsin inhibitors (SBTI; 5 µM), tosyl phenylalanyl chloromethyl ketone (TPCK; 5 mM), phenylmethylsulfonyl fluoride (PMSF; 100 mM), $HgCl_2$ (5 mM) and dithiothreitol (DTT; 5 mM). After 15 min in contact with the inhibitors, the proteolytic activity was assayed using casein (1%) as substrate at 35 ºC for 60 min. Samples were prepared and analyzed by SDS-PAGE as described above.

RESULTS AND DISCUSSION

Milk-clotting activity in *B. pinguin* fruit extract

Milk coagulation, by proteolytic enzymes, is a crucial basic step in the manufacture of most cheeses. The amount and type of protease used for milk coagulation are important factors since could affect the final characteristics of cheeses produced. In this study, the concentration of proteolytic enzymes in *B. pinguin* extract was adequate to clot milk in a reasonable time comparable to commercial rennet. Milk-clotting activity in CE was highly temperature dependent (Figure 1).The CE showed maximum MCA in the temperature range of 75-80 ºC, where 3.99 SU/mL was determined. This value indicates that around one mL of CE is necessary to clot 400 mL of milk in 40 min at that temperature range. However, in conventional cheesemaking process employing chymosin or other common rennets, milk coagulation occurs around 32-37 ºC (**Chitpinityol and Crabbe, 1998**). At this temperature range, the CE showed approximately 25% (1 SU/mL) of maximum milk-clotting activity observed, indicating that 1 mL of CE would clot 100 mL of milk at 35 ºC (Figure 1). It was reported that a cardoon extract (15% w/v) was able to coagulate a similar volume of milk (123 mL at 37 ºC in 20 min) (**Low *et al.* 2006**). On the other hand, 1 mL of *Cynara scolymus* flowers extract was able to clot around 400 mL of milk in just 10 min. This high capacity to clot milk was attributed to the high concentration of clotting aspartic proteases in the extract (**Chazarra *et al.* 2007**). Other plant sources contain high amount of other types of proteases such as the serine proteases found in *Solanum dubium* seeds. It was reported that 1 mL of this extract was enough for clotting 880 mL of milk at 37 ºC, supporting its successful application for cheesemaking (**Mohamed *et al.* 2009a**). Several plant preparations have been reported that contain proteases with high milk-clotting activity in a broad temperature range, mostly from 35 to 60 °C (**Pontual *et al.* 2012; Mazorra-Manzano *et al.* 2013b**). However, some plant sources such as *Solanum dubium* (**Mohamed *et al.* 2009b**), *Euphorbia nivulia* (**Badgujar and Mahajan, 2014**), *Streblus asper* (**Tripathi *et al.* 2011**) and *Ficus benghalensis* (**Sharma *et al.* 2009**) and *B. pinguin* extract, here reported, have showed more activity at high temperature (70-90 °C) indicating the thermotolerance of proteases found in these extracts.

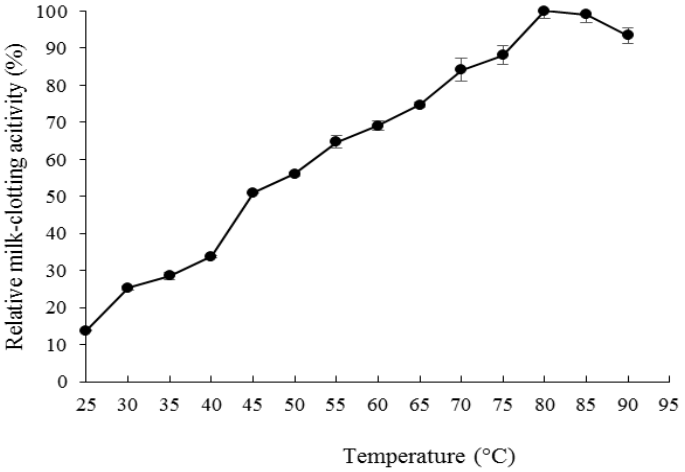

Figure 1 Effect of temperature on the milk-clotting activity (MCA) of extract from *B. pinguin* fruit.

Milk-clotting activity is reported as a % relative to the maximum observed at the different temperatures. Data are averages of three independent fresh extracts samples performed in triplicates. Bars represent mean standard error.

Casein hydrolysis by *B. pinguin* proteases

The milk-clotting characteristics of proteases are related with their activity and hydrolytic preference on kappa-casein (κ-CN) over others milk proteins during early stages of milk coagulation. Therefore, in order to increase the knowledge of milk coagulation mechanisms, the hydrolytic effect of *B. pinguin* proteases over milk caseins was explored by SDS-PAGE. Figure 2 shows the profile of casein hydrolysis in the early stages of coagulation. It can be observed that κ-casein was preferentially hydrolyzed. The release of a peptide fragment around 14 kDa could correspond to the para-κ-casein fragment ($f_{1\text{-}105}$) resulting from κ-CN hydrolysis at the Phe_{105}-Met_{106} position, as commonly occurs when chymosin is used as the coagulant (**Drohse and Foltmann, 1989; Hyslop, 2003**). After 30 min of incubation, an extended hydrolysis of κ-CN occurred as noted by the increase in para-κ-casein band intensity as well as other peptide fragments with molecular weight below 6 kDa. Longer incubation times (40-50 min) caused a complete hydrolysis of the primary substrate κ-CN and the partial hydrolysis of α-CN and β-CN.

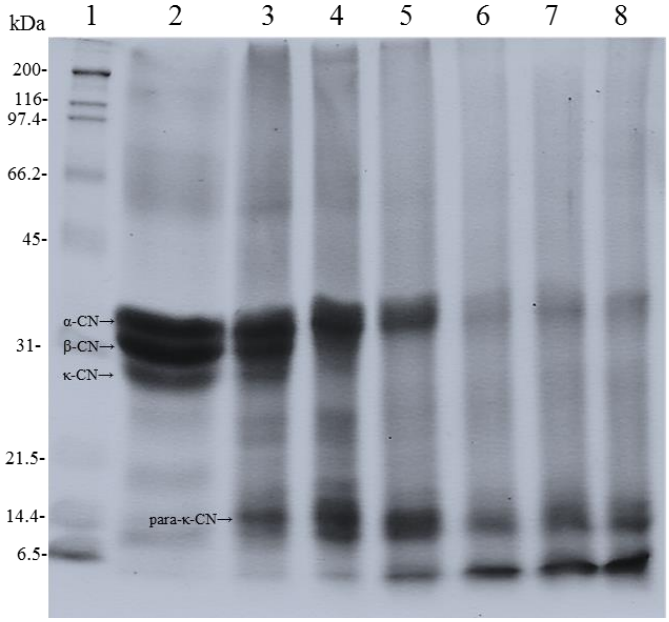

Figure 2 SDS-PAGE patterns for bovine casein hydrolysis by *B. pinguin* proteases.
Lane 1, molecular weight marker; lane 2, bovine caseins; line 3-8, bovine casein incubated with *B. pinguin* extract at pH 6.8/35ºC for 10, 20, 30, 40 and 50 min, respectively. Eighty micrograms of protein were loaded per well.

In order to elucidate the type of proteases present in *B. pinguin* extract, the effect of specific proteinase inhibitors on caseins hydrolysis was evaluated (Figure 3). Proteases in the crude extract were sensible to mercury chloride ($HgCl_2$, 5 mM), tosyl phenylalanyl chloromethyl ketone (TPCK, 5 mM) and phenylmethylsulfonyl fluoride (PMFS, 100 mM) indicating that cysteine and serine proteases were present. It can be observed that proteases inhibition with these specific inhibitors reduced the band intensity of para-κ-casein (black arrow) and low MW peptides (<6 kDa). On the other hand, it has been reported that soybean trypsin inhibitor (SBTI) has the capacity to inhibit microbial or animal serine proteases (**Sharma *et al*. 2009; Kumari *et al*. 2012**); however, these type of proteases were not inhibited by SBTI in the present study, as reported for other plant sources as well (**Verissimo *et al*. 1995; Esteves *et al*. 2003**). Further studies on the purification of these proteinases in order to explore the biochemical and biotechnological properties of individual milk-clotting enzymes in *B. pinguin* are recommended.

Proteolytic activity in *B. pinguin* fruit extract

The potential use of new sources of milk-clotting proteases in cheesemaking process requires the determination of the proteolytic activity (PA) in the extract. This property, together with milk-clotting activity (MCA), give an indication of quality of coagulants for use in cheesemaking process. Coagulants with a high MCA/PA ratio form better curds, high cheese yield and impart less bitter flavor during cheese ripening (**Arima *et al*. 1970**). In this study, *B. pinguin* crude extract presented MCA/PA ratios of 1.29 and 2.98 using casein and bovine serum albumin as substrate for proteolytic activity determination (at pH 6.8/37 °C), respectively (Table 1). In comparison with chymosin (with 209 and 2434 ratios, respectively), *B. pinguin* enzymatic extract presented ratios 162 and 816 times lower. **Pardo *et al*. (2010),** using casein as substrate, found that extracts form *Asclepias fruticosa* seeds exhibited a lower MCA/PA ratio (0.68) than the value reported in this study. In addition, proteases found in *Jacaratia curumbensis* (**Arruda *et al*. 2012**), *Lactuca sativa* (**Lo Piero *et al*. 2002**) and *Asclepias fruticosa* (**Trejo *et al*. 2001)** and *Onopordon acanthium* (**Brutti *et al*. 2012**), *Cucumis melo* (**Uchikoba and Kaneda, 1996) and** *Zingiber officinale* cv. Laiwu Shandong (**Hashim *et al*. 2011)** possessed high caseinolytic activity in comparison with calf chymosin limiting their application for cheesemaking. In the present study, the low MCA/PA ratio for *B. pinguin* extract could be due to the presence of different active proteases with different specificity, since a poor specificity increases the proteolytic activity, having a negative impact in this value and limiting its application in cheesemaking. However, other plant proteases such as serine protease religiosin C from *Ficus religiosa* latex (**Sharma *et al*. 2012**) and the cysteine proteinase actinidin form *Actinidia deliciosa* (**Grozdanovic *et al*. 2013**) had ratios only 5.25 and 2.17 times lower than chymosin, respectively. Using casein as substrate, extracts from *Bromelia hieronymi* **(Bruno *et al*. 2010),** *Bromelia balansae* **(Pardo *et al*., 2001)** and *Philiberta gilliesii* **(Sequeiros *et al*., 2005)** also showed high MCA/PA ratios (4.18, 5.19 and 4.82, respectively). The diversity and abundance of proteases in some plant sources make them attractive for multiple biotechnological applications. The low potential of some plant proteases for the cheesemaking process, do not limit their use. Thus, further studies exploring their properties will determine their applications in food processing (e.g. meat tenderization, hydrolysates production) and other industries (e.g. biological detergents) (**Moreno-Hernandez *et al*. 2016**).

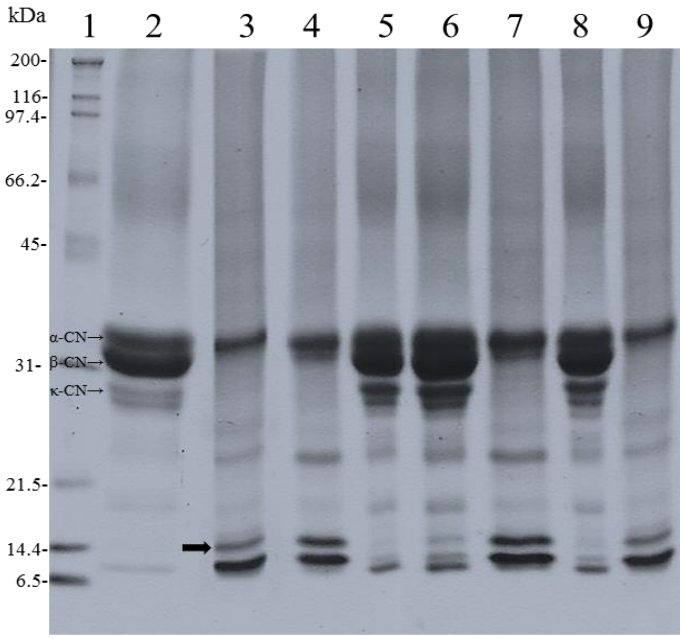

Figure 3 Effect of protease inhibitors on the activity of *B. pinguin* fruits extracts on caseins
Lanes: (1) molecular weight markers, (2) bovine caseins, (3) Casein hydrolysis by CE proteases at pH 6.8/35ºC for 60 min and its inhibition by (4-9) pepstatin A (1.5 mM), mercury chloride (5 mM), tosyl phenylalanyl chloromethyl ketone (5 mM), soybean trypsin inhibitors (5 µM), phenylmethylsulfonyl fluoride (100 mM), and dithiothreitol (5 mM). Arrow markers: α-CN for alpha-caseins, β-CN for beta-casein, κ-CN for kappa-casein and bold arrow for para-κ-CN hydrolysis product. Eighty micrograms of protein were loaded per well.

Table 1 Milk-clotting and proteolytic activity of *B. pinguin* extract and chymosin.

Coagulant	Milk-clotting activity (U mg^{-1})	Proteolytic activity (U mg^{-1})		MCA/PA ratio	
		Substrate		Substrate	
		BSA	Casein	BSA	Casein
Chymosin	164.75±0.14	0.068±0.002	0.791±0.05	2434	209
B. pinguin	2.59±0.65	0.869±0.196	2.0±0.12	2.98	1.29

Values represent the mean ± standard deviation of three independent determinations. Milk-clotting (MCA) and proteolytic (PA) activities were evaluated at 35 ºC and pH 6.8.

CONCLUSION

B. pinguin proteases possess milk-clotting activity in a broad temperature range with milk coagulation times comparable with commercial chymosin. Enzymatic extract showed a maximum milk-clotting activity at high temperature which could be related with its thermostability. Cysteine and serine proteases were responsible for the major milk-clotting activity and showed excessive casein proteolysis after prolonged incubation time. The high presence of milk-clotting proteases in *B. pinguin* fruit offers an alternative new source of proteases for biotechnological processes. However, further studies exploring their properties will determine their applications in food processing (e.g. meat tenderization, hydrolysates production) and other industries (e.g. biological detergents)

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