Biochemical characterization of a semi-purified aspartic protease from sea catfish *Bagre panamensis* with milk-clotting activity

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Abstract Pepsin from stomach of *Bagre panamensis* was semi-purified and biochemically characterized. The acid proteolytic activity and purification fold were 3875 U/mg protein and 91.85, respectively, after purification process. The optimum pH and temperature for semi-purified protease were 2–3 and 65 °C, respectively. The enzyme activity was stable after heating proteases at 50 °C for 120 min, but only 30% residual activity was detected after heating at 65 °C for 30 min. SDS-PAGE analysis showed two proteins bands after dialysis (26.1 and 38.6 kDa). Only the band of 38.6 kDa had proteolytic activity, which was inhibited using pepstatin A. Organic solvents, surfactants and reducing agents affect the proteolytic activity at different extent; however, metal ions or EDTA have no impact on protease activity. The semi-purified protease exhibited milk coagulant activity, with a maximum activity

at 45 °C. The obtained results highlight the potential biotechnological use of *B. panamensis* pepsin.

Keywords Proteases · Milk clotting activity · Catfish · Pepsin · Protein purification · By-products

Introduction

The processing of fishery products generates significant amounts of biological by-products including viscera, skin, fins, bone, heads, blood, among others. Commonly, these by-products are converted into meal and oil with low commercial value and, occasionally, are disposed of into the environment, where these become pollution sources (Arvanitoyannis and Kassaveti, 2008) and foster the

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proliferation of pests. However, such biological wastes and by-products contain valuable protein and lipid fractions with high nutritional quality as well as are rich in bioactive chemicals such as vitamins, peptides, collagen, fatty acids, hydrolytic enzymes (mainly proteases), and pigments, among other compounds, all of which are characterized by a high added value (Olsen et al., 2014).

Proteases from marine organisms have interesting catalytic properties, such as functioning efficiently at low reaction temperatures and being readily denatured at relatively low temperatures (Homaei et al., 2016). These properties make them suitable for use in various applications in the food-processing industry, since low-temperature processes are associated to low thermal costs, protection of substrates or products from thermal degradation, and reduced incidental reactions (Olsen et al., 2014). The main and most studied digestive proteolytic enzymes from fish viscera are the aspartic protease pepsin secreted by stomach (if it is present), and the serine proteases, trypsin and chymotrypsin secreted by pyloric caeca (if it is present), pancreas and/or intestines. Both proteolytic enzymes have been used with biotechnological purposes (Homaei et al., 2016). Pepsins (EC 3.4.23.1) from marine fish have been used in the preparation of protein hydrolysates (Nalinanon et al., 2011), as well as in the extraction of collagen (Nalinanon et al., 2007) and gelatin (Nalinanon et al., 2008a). To date, there are some reports about the purification and characterization of gastric proteases, mainly pepsins, from marine fish (Bkhairia et al., 2016; Bougatef et al., 2008; Klomklao et al., 2007; Nalinanon et al., 2010). These and others studies report a wide variety of physical, chemical and catalytic properties for purified fish pepsins compared to homologous enzymes from mammals (Zhao et al., 2011).

On the other hand, proteases from marine organisms might be excellent alternatives to replace conventional milk-clotting enzymes such as chymosin. This is of great importance, taking in account the current demand for milk-clotting enzymes (Jacob et al., 2011) and the restriction of commercial imports of bovine products due to health issues such as bovine spongiform encephalopathy (Simmons et al., 2018). However, scarce reports are currently available on the application of marine enzymes to cheesemaking; these include the use of enzymes from crustacean (Pereira and Fernández-Gimenez, 2017) and fishes (Tavares et al., 1997) where results shown their potential use as natural rennet substitutes.

The chihuil sea catfish (*Bagre panamensis*) is a demersal species endemic to the eastern Pacific. It is one of the most commercially important species in the Gulf of California because of the large sizes attained, local abundance and quality of meat. *B. panamensis* is captured in estuaries and coastal areas by small-scale fisheries, as well as in the open

sea by industrial shrimp fisheries. Catfish processing generates by-products (head, skin, skeletons and viscera) representing about 55% of total wet weight (Sathivel et al., 2009). Viscera accounts for approximately 10% of live weight, being a potential source of high-quality protein, oil and mainly proteolytic enzymes. In addition to the best of our knowledge, little is known about the characterization and application of sea catfish proteases. Therefore, the aim of the present study was the biochemical characterization of acidic proteases isolated from the stomach of the sea catfish *Bagre panamensis* and the evaluation of its stability in the presence of chemical denaturants and its potential use as a new source of milk clotting proteases. Moreover, present research serves as a baseline for further studies which will optimize the use of stomach proteases obtained from *B. panamensis* for cheese making.

Materials and methods

Biological samples

A sample of nine stomachs of juvenile Chihuil sea catfish *Bagre panamensis* (corporal weight of 445 ± 180 g; total longitude of 37 ± 5 cm) were obtained from artisanal fisheries from Mazatlán, Sinaloa, Mexico. Organisms were transported in ice to the laboratory and eviscerated immediately, after that, viscera were frozen at -80 °C until use.

Enzyme extracts and semi-purification of stomach proteolytic enzymes

Enzyme crude extracts (ECE) were prepared from whole stomach (mean weight of 17.3 ± 6.5 g) and homogenized with cold distilled water using a mass/water ratio of 1:3 (w/w). The homogenate was centrifuged at $8000 \times g$ for 20 min at 3 °C. The fat layer on the top was removed and the supernatant was recovered. Then, supernatant (ECE) was precipitated with ammonium sulfate using a method described by Burgess (2009). Chilled ECE was placed in a beaker immersed in ice and solid ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$ was added to reach 20% saturation. After 30 min, the suspension was centrifuged at $8000 \times g$ at 4 °C for 30 min. The supernatant was recovered and saturated to 70% with solid ammonium sulfate, mixed 30 min in ice, and centrifuged at $8000 \times g$ at 4 °C for 30 min. The precipitate was recovered, resuspended with 10 mM Gly-HCl buffer at pH 3.0, and dialyzed for 24 h against two changes of 2 L of resuspension buffer at 4 °C and using a dialysis tubing of 12–14 kDa MWCO (Fisherbrand™, Fisher Scientific). Dialyzed protein fraction (semi-purified acidic

enzyme) was stored at $-20\text{ }^{\circ}\text{C}$ until use for enzyme analysis.

Protein quantification and enzyme assay

Soluble protein concentration was determined according to Bradford (1976), using bovine serum albumin as protein standard.

Acid proteolytic activity was determined using 1% (w/v) bovine hemoglobin in 0.2 M glycine-HCl pH 3.0 as substrate (Anson, 1938). Both enzyme source and substrate were incubated at $25\text{ }^{\circ}\text{C}$ and the absorbance of reaction products was measured at 280 nm. The specific activity in semi-purified extracts was determined using the following relation: Activity units (U/mg protein) = $[(\text{Abs} \times \text{mL reactions mix} \times \text{dilution factor})/(\text{MEC} \times \text{time (min)} \times \text{mL extract volume} \times \text{mg soluble protein in enzyme source})]$; where MEC is a molar extinction coefficient for the reaction product (tyrosine) of $0.005\text{ mL}/\mu\text{g}/\text{mL}$. One unit of enzyme activity was defined as 1 μg tyrosine released per minute.

SDS-PAGE and zymograms

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a stacking gel with 5% polyacrylamide (PAA) and a resolving gel with 10% SDS and 12% PAA (Laemmli, 1970).

To identify proteolytic activity present on protein fractions contained in enzyme crude extracts and semi-purified enzymes, a zymogram was performed; an electrophoresis was run under non-denaturing native conditions (Native-PAGE) using a continuous PAA gel (12%) in buffer 25 mM Tris and 192 mM glycine at pH 8.3. After native-PAGE electrophoresis, the gels were treated to reveal proteases bands with activity (zymogram). The gels were soaked in 100 mM Tris-HCl pH 2.0. After 15 min, gels were submerged for 60 min in a solution containing 0.25% hemoglobin (100 mM glycine-HCl buffer, pH 2.0, $4\text{ }^{\circ}\text{C}$), and then was change to same solution at $37\text{ }^{\circ}\text{C}$ for 30 min as reaction time. At the end of reaction, the gels were washed with distilled water and fixed in trichloroacetic acid (12%) solution for 15 min. The native-PAGE was stained using a 0.1% Coomassie brilliant blue R-250 solution, while destaining was carried out in a 35:10:55 solution of methanol-acetic acid-water. Then, the clear zones revealed the activity of proteases. Native-PAGE electrophoresis was complemented with the use of specific inhibitors, pre-incubating enzyme source for 1 h with pepstatin A (1 mM).

Inhibition assays

Semi-purified acidic protease was incubated with specific inhibitors to determine the class of protease contained. Thus, 1 mM EDTA dissolved in distilled water was used to inhibit metalloproteases. To inhibit aspartic protease activity, pepstatin A (Sigma-Aldrich Co., St. Louis, MO) dissolved at 1 mg/mL in 10% acetic acid in methanol was used. Briefly, 10 μL of acidic protease was mixed with 0.5 mL 0.2 M glycine-HCl buffer pH 3.0 and 10 μL of a specific inhibitor, and incubated for 60 min at $25\text{ }^{\circ}\text{C}$ (Nalinanon et al., 2010). Then, the mixture was assayed for acid protease activities using 1% hemoglobin, as above described. The residual enzyme activity was reported as the loss of enzyme activity compared to a control with no inhibitor (as 100%).

Effect of pH and temperature and thermal stability

The effect of pH and temperature on enzyme proteolytic activity from semi-purified acidic proteases was evaluated. The effect of pH (2–7) on proteases was evaluated using 1% (w/v) hemoglobin dissolved in universal buffer (Stauffer, 1989). Then, the effect of temperature on proteolytic activity was determined by incubating at different temperatures ($25\text{--}75\text{ }^{\circ}\text{C}$) at pH 3.0.

To determine the thermal stability of enzyme activity on semi-purified acidic proteases, aliquots were previously incubated at different temperatures (5, 25, 50 and $65\text{ }^{\circ}\text{C}$) for 0, 30, 60, 90, 120 and 150 min prior to the enzyme reaction assays. The residual proteolytic activity of semi-purified acidic proteases was measured at a temperature of $25\text{ }^{\circ}\text{C}$ and pH 3.0. Residual enzyme activity was calculated as percentage (%) of peak activity measured at time zero with no incubation (Saborowski et al., 2004).

Effect of metal ions and sodium chloride

To evaluate the effect of metal ions on acidic proteolytic activity, aliquots of semi-purified protease enzymes were mixed with 0.2 M glycine-HCl buffer at pH 3.0 containing 5 mM KCl, CaCl_2 , MgCl_2 , FeSO_4 and CuSO_4 , respectively, and incubated for 60 min at $25\text{ }^{\circ}\text{C}$ (Bkhairia et al., 2016). Immediately after incubation, acidic enzyme activity was measured as described above. A control treatment was considered incubating enzymes in buffer without salts. Similarly, effect of sodium chloride was determined using different concentrations of a NaCl (0, 2.5, 5, 7.5, 10, 12.5 and 15%) dissolved in 0.2 M glycine-HCl buffer at pH 3.0 (Klomklao et al., 2007).

Table 1 Purification steps of the acidic protease from *B. panamensis*

Purification steps	Total volume (mL)	Soluble protein (mg/mL)	Specific activity (U/mg protein)	Total activity (U)	Purity (fold)	Recovery (%)
Crude extract	31.9	14.54	42.1	19,568	1	100
Ammonium sulfate fractionation (20–70%)	4.2	2.69	950	10,836	22.5	57
Dialyzed	7	0.59	3875	15,990	91.85	81.7

Effect of surfactants and reducing agents

To determine the effect of surfactants (SDS and Tween 80TM) and reducing (2-mercaptoethanol and dithiothreitol) agents, semi-purified acidic protease was separately incubated for 60 min at 25 °C with such agents at different concentrations. SDS was assayed at 0.1, 0.5 and 1% (Nalinanon et al., 2008b), Tween 80TM at 1, 2 and 5%, 2-mercaptoethanol was assayed at 5, 10 and 15%, and dithiothreitol (DTT) at 5 mM concentration. After incubation, enzyme activity was determined. Residual activity was compared with a control treatment without an agent.

Effect of organic solvents

To determine the effect of solvents on acidic proteolytic activity, semi-purified acidic proteases were incubated for 60 min at 25 °C with acetone, ethanol, methanol and propanol. Solvents were assayed at 10, 20, 40 and 80% concentration. After incubation, enzyme activity was measured as above described. Residual activity was compared with a control treatment without solvents (Saborowski et al., 2004).

Milk clotting activity of semi-purified extract enzyme

Milk-clotting activity (MCA) was determined according to modified method described by Mazorra-Manzano et al. (2013). Briefly, 10 mL of pasteurized low-fat milk (< 1% total fat) containing 0.022% of CaCl₂ was mixed with 500 µL of semi-purified acidic proteases and incubated at different temperatures (25–60 °C). 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 (CuamixTM, CUAMEX, Mexico) was also determined as reference at same range of temperature. A control treatment was assayed where 10 mM Gly-HCl buffer at pH 3.0 was used instead of enzymatic source. One milk-clotting unit (Soxhlet unit) was defined as the amount of enzyme source (mL) required for clotting 100 mL of milk in 40 min (2400 s), under the assay conditions. The MCA was calculated by using the

following equation and expressed in Soxhlet units per mL of coagulant (SU/mL). $MCA (SU/mL) = (2400/t) \times (S/E)$. Where t = clotting time (s); S = volume of used milk (mL); and, E = volume of enzymatic solution.

Statistical analyses

The results of all assays are reported as mean $\pm$ standard deviation. Comparisons were analyzed by one-way ANOVA. Tukey's test was used for multiple comparisons at $p < 0.05$ level. All statistical analyses were performed using the statistical program SigmaPlot version 11.0 (2018 Systat Software, Inc.; Erkrath, Germany).

Results and discussions

Semi-purification process

The semi-purification process of stomach acid proteases from *B. panamensis* through precipitation with ammonium sulfate followed by dialysis is summarized in Table 1. The stomach enzyme crude extract (ECE) yielded a specific acid proteolytic activity of 42.1 U/mg protein. When the ECE was fractionally precipitated, bringing the supernatant to 20% saturation followed by 70%, the specific acid enzyme activity rose to 950 U/mg protein, equivalent to 22.5-fold purification. The dialysis stage increased the acid proteolytic activity 91.85-fold relative to the activity recorded in the ECE, with a specific activity of 3875 U/mg protein in the dialyzed fraction. At the end of the purification process, 81.7% of the total acid proteolytic activity in the crude extract was recovered. Thus, the method here used to concentrate and semi-purify gastric proteases from chihuila catfish shown a good efficacy to perform a protein recovery avoiding a loss of enzyme activity. This is interesting due to large scale of pepsin recovery is still in demand in the industry at present.

The SDS-PAGE analysis of the semi-purified enzyme showed two intensely stained protein bands with molecular weights of 26.1 and 38.6 kDa (Fig. 1a, line 4). On the other hand, the native-PAGE zymogram revealed a single band with proteolytic activity at acid pH (pH 2.0) for the semi-

Fig. 1 SDS-PAGE (A) and zymogram showing proteolytic activity at pH 2.0 (B) of semi-purified stomach proteases of *B. panamensis*. (1) Molecular weight marker; (2) Enzyme crude extract; (3) supernatant from 20% saturation; (4) semi-purified acidic enzyme after 70% saturation and dialysis processes; (5) Semi-purified acidic proteases; (6) Semi-purified acidic proteases mixed with pepstatin A

purified acidic proteases, which showed a relative electrophoretic mobility (Rf) of 0.79 (Fig. 1b, lines 5) corresponding to the protein band of 38.6 kDa. Those protein band with a molecular weight of 26.1 kDa has not shown acidic proteolytic activity in zymogram analysis. Gastric proteases with similar (0.79) and higher Rf values (0.92 and 0.93), as well as similar molecular weight (in the range of 36–38 kDa), have been reported for the Greenland cod *Gadus ogac* using native conditions (Squires et al., 1986). The band with proteolytic activity evidenced in zymograms corresponds to a protein of low molecular weight that was inhibited by pepstatin A (Fig. 1b, lines 6); therefore, the gastric protease semi-purified in present study is a pepsin-type aspartic endopeptidase. These results suggest that chihuil pepsin is a monomeric protein whose molecular weight is higher to that of pepsin from the *Anguilla anguilla* (30 kDa; Wu et al., 2009), *C. pectoralis* (31 kDa; Klomklao et al., 2007), and *T. alalunga* (32.7 kDa; Nalinanon et al., 2010); but almost similar than those reported for various species of marine fish such as *Mustelus mustelus* (35 kDa; Bougatef et al., 2008), and sardine (33.4 kDa; Noda and Murakami, 1981).

Optimum pH

In teleost fish, four isoforms of pepsins or pepsinogens (PG) have been found, classified according their activity, stability, kinetics and structural properties (Zhao et al., 2011). The optimal pH lies between 3.0 and 4.0 for pepsin I, and between 2.0 and 3.0 for pepsin II (Gildberg, 1988). The acid protease of *B. panamensis* showed the highest specific activity at pH of 2 and 3, with values of 3601 and

3979 U/mg protein, respectively, with no significant differences between them. Similar optimum pH values were recorded for pepsins from *Thunnus alalunga* (at pH of 1.5–3; Nalinanon et al., 2010) and *Liza aurata* (at pH of 3; Bkhairia et al., 2016). In contrast, at higher pH levels, the enzyme activity trends to decrease (to pH 5 and 6, remained 34 and 1% of maximum relative activity, respectively), indicating that an alkaline medium brings unfavorable conformational changes in pepsin. However, the broad pH range in which the partially purified acid protease was active (pH of 2–5) is characteristic of fish pepsins, which contrasts to mammal pepsins whose peak activity occurs at pH 2 and disappears at pH 4 (Gildberg, 1988).

Optimum temperature and thermal stability

Temperature significantly affected the proteolytic activity of *B. panamensis* pepsin. An increase in acid proteolytic enzyme activity was observed concomitant with reaction temperature, peaking at 65 °C (11,670 U/mg protein). Previous studies have shown that pepsins extracted from fish living in warm waters, such as *B. panamensis*, have higher optimum temperatures (40 °C) versus cold-water fish (near to 30 °C); however, the optimum temperature for the pepsin studied here is higher compared to the peak temperatures recorded (50–55 °C) for other fish species (Miura et al., 2015; Nalinanon et al., 2010; Noda and Murakami, 1981) and similar to the one recorded for porcine pepsin (65 °C; Gildberg, 1988).

Thermal stability tests showed that approximately 85% of the baseline acid enzyme activity in the partially purified protease remained at 50 °C within 90 min of incubation, with no significant differences in the activity recorded when enzymes are kept at 25 °C. However, when partially purified acid proteases are kept at an incubation temperature of 65 °C, about 90% of the activity was lost within 30 min (Fig. 2). Other fish species that produce pepsins with a similar thermostability profile are *C. pectoralis* (Klomklao et al., 2007) and *M. mustelus* (Bougatef et al., 2008), which retain more than 70% of baseline activity at an incubation temperature of 50 °C. Therefore, at temperatures higher than 60 °C pepsins of chihuil catfish underwent denaturation and lost its activity. In this regard, the high proteolytic activity exhibited by chihuil acid enzymes after incubation in the range of 5–50 °C and its fast inactivation at 65 °C are important characteristics for the potential application of these proteases in food processes where low temperatures and rapid inactivation of proteases are needed (e.g., milk clotting in cheese making processes).

Fig. 2 Thermal stability of the semi-purified gastric enzymes of *B. panamensis*. Activity was normalized to 100% for absorbance recorded at 0 min

Effect of inhibitors

When the partially purified acid protease is incubated with pepstatin A, only 6.4% of initial enzyme activity remained, indicating that this semi-purified protease belongs to aspartic endopeptidase, as previously mentioned. On the other hand, EDTA slightly inhibited the acid proteolytic activity by 2.6%. The almost zero inhibition by the metalloprotease inhibitor EDTA indicates that the pepsin studied here is not a metalloprotease requiring metal ions as activation cofactors, contrary to some purified pepsins from other marine fish species (Klomklao et al., 2007).

Effect of metals

There was no effect of monovalent (KCl, NaCl), or divalent (CaCl_2 , MgCl_2 , CuSO_4 and FeSO_4) metallic ions on the acid proteolytic activity of the partially purified protease. This further confirms the results of the EDTA inhibition test, thus demonstrating that purified pepsin is not an enzyme activated by metallic cofactors. The effect of NaCl on the enzymatic activity of marine fish pepsins is highly variable. For example, the addition of NaCl (5 mM) at low concentrations did not affect the activity of pepsins from *T. alalunga* (Nalinanon et al., 2010); moreover, it has been reported that the addition of concentrations of 20–70 mM NaCl increases the activity of pepsins from Greenland cod *Gadus ogac* (Squires et al., 1986), but high NaCl concentrations decrease the activity of pepsins from sardine (3.4 M NaCl; Noda and Murakami, 1981), and from *C. pectoralis* (> 0.85 M NaCl; Klomklao et al., 2007). In contrast, the activity of the pepsins that were partially purified in this study was unaffected when using concentrations up to 2.55 M NaCl (15% NaCl). Due to the

stability of these enzymes at high salt concentration, proteases from the stomach of *B. panamensis* could be used in food processing applications, such as fish sauce production and meat tenderizing.

Effect of surfactants and reducing agents

The effect of surfactants and reducing/oxidizing agents on the activity of serine proteases from aquatic organisms has been extensively studied (Nasri et al., 2011), resulting some enzymes with potential to be included in detergent formulations. In contrast, few studies have evaluated the effect of surfactants on the proteolytic stability of aspartic proteases. In this study, acid enzyme activity decreased significantly only by diluting SDS concentrations of 0.5% or higher in the reaction buffer (Fig. 3). A 0.1% SDS solution did not affect enzyme activity in this study, contrary to the findings reported by Nalinanon et al. (2008b) for pepsins from skipjack tuna (*Katsuwonus pelamis*), *T. alalunga* and longtail tuna (*Thunnus tonggol*), in which the activity of pepsins disappeared when SDS was diluted to 0.05%. The concentrations of Tween 80TM in the reaction buffer (1, 2, and 5%) used in this study did not affect acid enzyme activity. In contrast, the concentrations of 2-mercaptoethanol used here favored acid enzyme activity, with up to an 80% increase in enzyme activity by using 15% 2-mercaptoethanol, relative to the control treatment. Contrary results were observed with acidic proteases from grey mullet (*Liza aurata*) which are partially inhibited in the presence of mercaptoethanol (Bkhairia et al., 2016). The addition of 5 mM DTT did not affect acid enzyme activity.

Fig. 3 Effect of surfactants (SDS and Tween 80TM) and reducing agents (2-mercaptoethanol and DTT) at different concentrations on the activity of gastric enzymes of *B. panamensis*. 2-mercaptoethanol: Merc. Values with different letter are significantly different ($p < 0.05$)

Thus, it would be interesting to study the structural configuration of pepsins from *B. panamensis* which gives it stability at different physical and chemical conditions compared to others marine fish pepsins.

Effect of organic solvents

Specific industrial applications require enzyme stability under harsh conditions, such as reactions in the presence of organic solvents to increase the solubility of nonpolar substrates, suppress water-dependent side reactions, or avoid microbial contamination (Ogino and Ishikawa, 2001). There was no significant effect on enzyme activity when methanol and ethanol were added at up to 80% to the reaction buffer (Fig. 4), relative to the control treatment (no solvent). However, propanol and acetone did negatively affect acid enzyme activity at concentrations of 10% and 20% in the reaction buffer, respectively. These results contrast with the effect of solvents such as methanol and 1-propanol on alkaline proteases, mainly trypsin, from fish such as European seabass *Dicentrarchus labrax* (Harpaz et al., 1994), in which solvents significantly increased enzymatic activity. This effect of solvents on the activity of serine proteases has been associated with an increased hydrophobicity of the medium, which may lead to the disruption of hydrogen bonds and hydrophobic interactions in the active site (Barberis et al., 2006), leading to changes in the protein structural conformation and affecting either positively or negatively the catalytic properties of the enzyme. In the case of the partially purified aspartic protease in this study, the decreased proteolytic activity at high solvent concentrations might be due to several factors, mainly structural changes in the protein, i.e. structural dysfunction and stripping of the essential water layer from

the enzyme molecule (Shankar and Laxman, 2015). Another interesting feature for proteolytic enzyme solvent resistance is that, in organic media, proteases synthesize peptide bonds by shifting the thermodynamic equilibria favoring the synthesis (Kumar and Bhallaor pepsins from skipjack tu, 2005). Therefore, acidic proteases from *B. panamensis* offer a potential use as enzymatic biocatalysts for peptide synthesis.

Milk clotting activity

When chihuil acidic proteases were applied to the substrate, milk clotting occurred. The effect of temperature on milk clotting activity (MCA) and clotting time (CT) is shown in Fig. 5. The peak MCA (18.3 ± 1.7 SU/mL) was recorded at 45 °C. At this same temperature, the lowest CT (11.0 ± 1.0 min) was achieved. MCA increased with temperature within 30–45 °C, but temperatures above 55 °C decreased the MCA, probably due to enzyme denaturation (Fig. 5). MCA of chihuil acidic proteases resulted thermolabile compared to chymosin which at 55–60 °C showed its highest MCA with a CT of 9 and 7.3 min, respectively. Moreover, clotting was not observed when acid solution was used instead of enzyme (Control treatment). The use of chihuil acidic proteases could be interesting if bioactivity or biochemical properties of thermolabile compounds contained in milk want to be maintained during a cheese making process. According to Mazorra-Manzano et al. (2013) an acceptable clotting time is less than 90 min. Therefore, the extent of proteolytic activity in the chihuil extract was sufficient to clot milk within the acceptable clotting time. Interestingly, compared to plant proteases, only a few digestive enzymes from marine species have been considered for their chymosin-

Fig. 4 Effect of different concentrations (0, 10, 20, 40 and 80%) of methanol, ethanol, propanol and acetone on the enzyme activity (mean $\pm$ SD) of semi-purified gastric enzymes of *B. panamensis*

Fig. 5 Effect of temperature (°C) on the milk clotting activity (SU/mL) and time for clotting (min; black circles) of gastric enzymes of *B. panamensis*. Also, time for clotting (min) for chymosin (white circles) is shown. Values with different letter are significantly different ($p < 0.05$)

like characteristics as potential rennet substitutes (Pereira and Fernández-Gimenez or pepsins from skipjack tu, 2017). In line with our results, Tavares et al. (1997) reported that the peak MCA of gastric proteases isolated from the stomach of bigeye tuna (*Thunnus obesus*) was observed at temperatures around 30–38 °C. Recently, Pereira and Fernández-Gimenez (2017) found a high MCA of a crude extract from Argentine red shrimp (*Pleoticus muelleri*). To the best of our knowledge, this is the first study that supports the presence of milk-clotting proteases in a tropical fish.

Thus, the semi-purified enzyme of stomach extracts from the catfish *B. panamensis* is an aspartic protease with a molecular weight of 38.6 kDa, thermally stable at 50 °C and with a peak proteolytic activity at acidic pH levels of 2–3. It is not a metalloprotease, as the presence of EDTA or ionic metals produced no effect on its proteolytic activity. The aspartic protease was adversely affected by low and high organic solvent concentrations. The partially purified stomach enzyme extract exhibited milk-clotting activity, with a peak at 45 °C. In conclusion, information here obtained would be useful for the development of a cost-effective process to recovery and obtain pepsin from a marine fish potentially suitable for use in cheesemaking and other biotechnological or food applications.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Human and animal rights statement This article does not contain studies with animals subjects performed by the any of the authors.

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