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Fish processing waste as a source of alkaline proteases for laundry detergent

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ABSTRACT

Proteases were extracted from the viscera of *Colossoma macropomum* and precipitated with ethanol (30–70%, v/v). The enzymatic extract was partially purified with a yield of 75% (2926 U/g of tissue); at least five caseinolytic proteases bands were observed in zymogram. The optimum pH of the preparation was in the alkaline pH range (10–12). The optimum temperature of activity was 60 °C and only about 15% of the initial activity was lost after an incubation period of 30 min at the above mentioned temperature. Both trypsin and chymotrypsin-like enzymes were detected in the proteases, but with a stronger prevalence for the former. These proteolytic enzymes remained stable in the presence of non-ionic (Tween 20 and Tween 80) and ionic surfactants (saponin and sodium choleate). They also revealed high resistance (60% residual activity) when incubated with 10% H_2O_2 for 75 min. Furthermore, the preparation retained approximately 80% of its proteolytic activity after incubation for 1 h at 40 °C with the commercial detergent.

1. Introduction

Proteases constitute one of the most important groups of industrial enzymes, and account for at least 60% of all global enzyme sales (Gupta, Beg, & Larenz, 2002; Johnvesly & Naik, 2001; Rao, Tanksale, Ghatge, & Deshpande, 1998). The detergent industry has now emerged as a major consumer of several hydrolytic enzymes acting at alkaline pH. The major industrial use of detergent-compatible proteases is found in the composition of laundry detergent. These enzymes account for at least a quarter of all protease sales throughout the world (Anwar & Saleemuddin, 1998; Gupta et al., 2002; Horikosh, 1996; Kumar & Takagi, 1999). They are primarily used as detergent additives since they are biodegradable and increase performance/cost ratios (Gupta et al., 2002; Kumar & Takagi, 1999).

Alkaline proteases from bacteria, fungi or insect origin can be exploited commercially (Anwar & Saleemuddin, 1998). Bacterial proteases have long been used in detergents, but their main drawback is that they require cost-intensive technologies to obtain a microrganism-free enzyme preparation. On the other hand, fungal proteases have proved to be advantageous because mycelium can easily be removed by filtration. However, to obtain high, commercially viable yields of proteases, it is essential to optimize the fermentation medium for the growth and production of proteases (Phadatare, Deshpande, & Srinivasan, 1993; Samal, Kara, & Stabinsky, 1990).

Moreover, although the enzymes selected for detergent composition have been subtilisins (US patent nos. 1240058, 374971, 370482 and 4266031 and UK patent no. 13155937) they are not the ideal enzymes for detergents due to their low thermal stability in the presence of detergents and also because of their short shelf life (Samal et al., 1990). Thus, it is relevant to search for proteases from different sources presenting properties such high thermal stability and alkaline activity that are more compatible with washing systems (Banerjee, Sani, Azmi, & Soni, 1999). These properties have already been observed in trypsin-like enzymes in tropical fish (Alencar et al., 2003; Bezerra, Santos, Lino, Vieira, & Carvalho, 2000; Bezerra et al., 2001; Bezerra et al., 2005; Souza, Amaral, Espírito Santo, Carvalho, & Bezerra, 2007).

Acid proteases in the stomach and alkaline proteases in the intestine are the most important digestive proteolytic enzymes of fish viscera, accounting for about 5% of the total body mass (Gildberg & Overbo, 1991; Simpson, 2000; Simpson & Haard, 1987). As a rule, alkaline proteases from tropical fish have thermal stability, a long shelf life and high activity over a wide range of pH levels (Alencar et al., 2003; Bezerra et al., 2000; Bezerra et al. 2001; Bezerra et al., 2005; Souza et al., 2007).

Contamination caused by the residues from fish processing has created an imperative challenge that needs to be efficiently dealt.

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For instance, every year in Mexico, tons of Monterey sardines are processed, generating vast amounts of residue, mainly stick-water, viscera, heads and processing effluents and are directly discarded into the sea (Castillo-Yáñes, Pacheco-Aguiar, García-Carreño, & Toro, 2005). Therefore, alternatives, including the commercial use of the by-products and waste, are urgently required.

Tambaqui (*Colossoma macropomum*) is the most eminent native fish species of Brazilian aquaculture, with an estimated production of 25,000 tons in 2004 (IBAMA, 2005). Proteases from this tropical fish have already been subjected to study. Those from the stomach as well as crude extracts of pyloric caeca have both been characterized (Bezerra et al., 2000). A trypsin-like enzyme has also been identified (Bezerra et al., 2001). It is possible to recover large amounts of this trypsin-like enzyme from tambaqui residue since previous information indicates that 1 g of the enzyme is encountered per 1 kg of pyloric caeca (Bezerra et al., 2001). Earlier reports have revealed that 1 kg of cod intestine produces 1 g of trypsin-like enzyme (Asgeirsson, Fox, & Bjarnason, 1989; Gildberg & Overbo, 1991). Thus, by estimating that the digestive-tract portion corresponds to 5% of the total body weight, one can conclude that high quantities of digestive enzymes may be produced by the appropriate use of fish residue.

Biotechnology is able to provide the means for transforming such raw material into valuable products such as enzymes (Castillo-Yáñes et al., 2005). However, little information is available in the literature regarding the use of active proteases from fish for detergent compositions (Espósito, Amaral, Marcuschi, Carvalho Jr., & Bezerra, 2009). The present paper describes the extraction, partial purification and characterization of alkaline proteases from *C. macropomum* pyloric caeca and intestine, as well as their compatibility with commercial laundry detergents, oxidants and surfactants agents.

2. Materials and methods

2.1. Materials

C. macropomum specimens were obtained from the Departamento de Pesca e Aquicultura at the Universidade Federal Rural de Pernambuco, Recife-PE, Brazil. All reagents used in enzymatic assays were from an analytical grade purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

2.2. Enzyme extraction

The fishes were transported alive to the Laboratório de Enzimologia (LABENZ) at the Universidade Federal de Pernambuco. Pyloric caeca and intestines of *C. macropomum* (immediately dissected after being killed in an ice bath) were collected and 40 mg (w/v) of tissue/mL in 0.9% (w/v) NaCl were homogenized with the use of a tissue homogenizer (Bodine Electric Company – Chicago, USA). The resulting preparation was centrifuged (Sorvall RC-6 Superspeed Centrifuge – North Carolina, USA) at 10,000*g* for 10 min at 10 °C to remove cell debris and nuclei. The supernatant (crude extract) was frozen at −20 °C and used for purification steps (Bezerra et al., 2005).

2.3. Enzyme partial purification

The crude extract was incubated for 30 min at 45 °C and centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant (300 mL) obtained was then fractionated with iced ethanol by adapted methodology of Cortez and Pessoa (1999). The ethanol concentration values employed in the precipitation experiments were 0–30% (Fraction F_1) and 30–70% (Fraction F_2). For each fraction, the ethanol was slowly added to the extract under agitation (200 rpm) at 4 °C with a burette in a cold chamber. After the addition of ethanol, the agitation was stopped for 2 h and the mixture was centrifuged at 10,000*g* for 15 min at 4 °C. The pellets (Fraction F_1 and F_2) were dissolved in 75 mL 0.1 M Tris–HCl buffer (pH 8.0) at 25 °C and dialyzed against 4 L of 0.05 M Tris–HCl buffer (pH 8.0) for 2 h. After this period, the buffer was renewed and dialysis was repeated for a further 2 h. Finally, after a third renewal of the buffer, dialysis was allowed to proceed overnight.

2.4. Enzymatic assay

Proteolytic activities were determined in the crude extract and the fractions using 1% azocasein as substrate, according to Alencar et al. (2003) and Bezerra et al. (2005). Triplicate samples of each enzyme extract (30 μL) were incubated with 1% azocasein (50 μL) dissolved in 0.1 M Tris–HCl pH 8.0, for 60 min at 25 °C. Following, 10% trichloroacetic acid (120 μL) was added to stop the reaction and the mixture was centrifuged at 8000*g*, for 5 min. The supernatant (70 μL) was mixed with 1 M NaOH (130 μL) and absorbance was measured in a microtiter plate reader (Bio-Rad 680) at 450 nm against a similarly prepared blank, except that 0.9% (w/v) NaCl replaced the crude extract sample. Previous experiments showed that for the first 60 min the reaction carried out under the above mentioned conditions follows first order kinetics. A unit (U) of enzymatic activity was defined as the amount of enzyme capable of hydrolyzing azocasein to produce a 0.001 change in absorbance per minute. Protein content was estimated by measuring sample absorbance at 280 and 260 nm and using the following equation: [protein] mg $mL^{-1} = A_{280\ nm} \times 1.5 - A_{260\ nm} \times 0.75$ (Warburg & Christian, 1941). Specific enzyme activity was calculated by dividing the enzyme activity (U) per amount of protein in the sample (mg/mL) and expressed as U/mg of protein. The fraction that presented the highest specific activity (obtained by Fraction 2 with 30–70% ethanol) was chosen for compatibility and characterization studies.

2.5. Electrophoresis

The dialyzed enzyme (100 μg of protein) was concentrated by lyophilization and used for electrophoresis (Bio-Rad) according to Laemmli (1970), using 4% (w/v) stacking gel and 12.5% (w/v) separating gel. The gel was stained with 0.1% (w/v) Coomasie Blue for 2 h and destained in 10% (v/v) acetic acid and 25% (v/v) methanol. Molecular weight markers were used. The zymogram was adapted from Garcia-Carreño, Dimes, and Haard (1993) and carried out as follows: after electrophoresis, the gel was immersed in 50 mL of 3% casein in 0.1 M Tris–HCl buffer, pH 8.0, for 30 min at 5 °C. The temperature was then increased to 25 °C and incubated for further 90 min to allow for the digestion of the protein substrate (casein) by the active fractions. The gels were then stained with 0.1% (w/v) Coomasie Blue for 2 h and destained in 10% (v/v) acetic acid and 25% (v/v) methanol.

2.6. Effect of inhibitors

Inhibition was measured according to Alencar et al. (2003) and Bezerra et al. (2005). Enzyme extracts were incubated during 30 min with different specific protease inhibitors (4 mM): PMSF (phenyl-methyl-sulphonyl-fluoride), TPCK (tosyl-L-phenylalanyl-chloromethyl ketone), TLCK (tosyl-L-lysine chloromethyl ketone) and Benzamidine. After incubation time, 4 mM trypsin and chymotrypsin specific substrates were added: BApNA (benzoyl arginine *p*-nitroanilide) and Suc-Phe-p-Nan (succinyl phenylalanine *p*-nitroanilide), respectively. The release of *p*-nitroaniline was followed by an increase in absorbance at 405 nm. The enzyme and

substrate blanks were similarly assayed without enzyme and substrate solution, respectively. The 100% values of activities were established without inhibitors.

2.7. Effect of pH

Protease activity was measured at different pH values under standard assay conditions, with azocasein as a substrate. The enzymatic activity was assayed at pH 6.5–12.5 with 0.1 M phosphate buffer (pH 6.5–7.5), 0.1 M Tris–HCl buffer (pH 7.2–9.0) and 0.1 M NaOH/glycine buffer (pH 8.6–12.5). The effect of pH on the stability of the enzyme preparation was studied by incubating the enzyme at 25 °C for 30 min with the above buffers and the enzymatic activities were measured as described above, at 25 °C, using the 0.1 M NaOH/glycine buffer (pH 11.0).

2.8. Effect of temperature

The protease activity was assayed at various temperatures (25–80 °C) to determine the optimum temperature, at pH 11.0. In order to establish the thermal stability, prior to enzymatic activity, the enzyme preparation was incubated for 30 min at temperatures ranging from 25 °C to 80 °C. The incubated temperature of the preparation was lowered to 25 °C and its proteolytic activity assayed.

2.9. Effect of oxidizing agent and surfactants

The hydrogen peroxide stability of the proteases from tambaqui was investigated by incubating samples (600 μL) with H_2O_2 (600 μL) at concentrations of 5%, 10% and 15% at 40 °C. Samples (150 μL) were withdrawn at 15, 30 and 75 min intervals to establish their activities (duplicates) on azocasein, and to compare them to the non-treated sample. Stability with regard to ionic (saponin and sodium choleate) and non-ionic surfactants (SDS, Tween 20 and Tween 80) was investigated by incubation in 1% solution concentrations of surfactant (w/v) for 30 and 60 min at 40 °C, after which enzyme activity was assayed (Moreira, Albuquerque, Teixeira, Porto, & Lima Filho, 2002).

2.10. Compatibility with commercial detergents

The protease at a concentration of 0.20 mg mL^{-1} was incubated at 40 °C with commercially available detergents: Ala® (Protec & Gamble); Bem-te-vi® (Alimonda); Omo Multi-Ação® (UniLever) and Surf® (UniLever) to a final concentration of 7 mg of detergent mL^{-1}. Samples (150 μL) were removed at 10 min intervals (total period of 60 min). The residual proteolytic activity in each sample was determined at 25 °C, assayed and compared with the control sample incubated at 40 °C without detergent pH 11.0 (Moreira et al., 2002).

3. Results and discussion

In the present study alkaline proteases were precipitated with ethanol at concentrations of 0–30% (v/v) and 30–70% (v/v) to partial purification. The highest enzymatic recovery was observed in Fraction F_2 (30–70%, v/v, of ethanol concentration), presenting purification and yield of 2.6-fold and 74.9%, respectively. The result of the purification procedure was a clear preparation, which allowed the concentration of the enzyme, with no trace of the typical fish smell. Furthermore, the 30–70% ethanol precipitate (Fraction 2) contained about 75% of specific activity (2926 U/g of tissue) when compared to the crude extract (3906 U/g of tissue). The ethanol is known as an important protein industrial precipitant (Cortez & Pessoa, 1999) and when used in this way its residue may be easily industrially recovery by simple techniques such as flash evaporator (Gosh & Ghose, 2003).

The electrophoresis and zymogram of the crude extract and Fraction F_2 are presented in Fig. 1. The standard protein markers of different molecular weights and the proteins of the crude extract and Fraction F_2 are displayed in lane 1, 2 and 3, respectively. Their proteolytic activities on casein are also shown (zymogram) in lane 4 (crude extract) and 5 (Fraction F_2). As can be observed in this figure, the ethanol precipitation protocol was partially able to purify and concentrate three major proteins with proteolytic activity from the crude extract. They presented molecular weights of 40.8, 35.5 and 22.7 kDa and two other smaller proteases presented molecular weights of between 14 kDa and 22.7 kDa. In fact, a mixture of proteases rather than purified preparations is usually employed in the food and detergent industries (Rao et al., 1998).

The effect of pH on the proteolytic enzyme is shown in Fig. 2a. The proteases of the ethanol Fraction F_2 obtained from the tambaqui pyloric caeca and intestine displayed maximum activities at a pH range of 10 to 12. The tambaqui proteases were also stable over a pH range of 10–12.5 (Fig. 2b). One of the most important parameters for selecting proteases for detergents is the optimum pH. The desired pH of a detergent solution in which proteases work should be approximately the same as the optimum pH for the enzyme (Gupta et al., 2002). Since the pH of laundry detergents is commonly alkaline (Banerjee et al., 1999), protease and other enzymes currently used in detergent compositions should be alkaline in nature with a high optimum pH. These properties were displayed by the tambaqui proteases purified by ethanol precipitation.

Fig. 3a shows the effect of temperature on the tambaqui protease activities. An optimum activity was encountered at 60 °C. Furthermore, they remained thermally stable as they retained about 86% of the activity after being incubated at the optimum temperature (60 °C), for 30 min (Fig. 3b). This result is similar to that described for proteases from *Bacillus brevis* (Banerjee et al., 1999). Moreover, about 60% of the maximum activity was retained after incubation for 30 min at 30 °C which is desirable for laundry purposes and from the ecological and economical point of view, mainly, because saving of energy.

All detergent-compatible enzymes are alkaline with a high optimum pH and are thermally stable. These are important characteristics because the pH of laundry detergent generally ranges

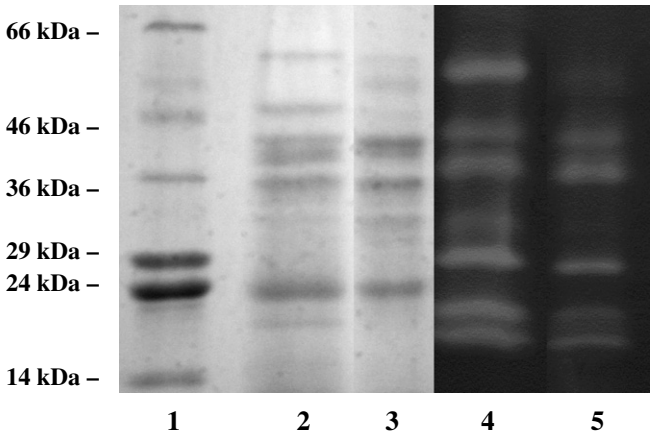

Fig. 1. SDS-PAGE of alkaline protease from the viscera of *C. macropomum*. Lane 1: molecular weights of standard protein markers (bovine serum albumin 66 KDa, ovoalbumin 45 KDa, glyceraldehydes 3-phosphate dehydrogenase 36 KDa, carbonic anhydrase 29 KDa, trypsinogen 24 KDa, and α-lactoalbumin 14,2 KDa); lane 2: crude extract; lane 3: precipitate with 30–70% ethanol; lane 4: zymogram of the crude extract and lane 5: zymogram of the precipitate with 30–70% ethanol.

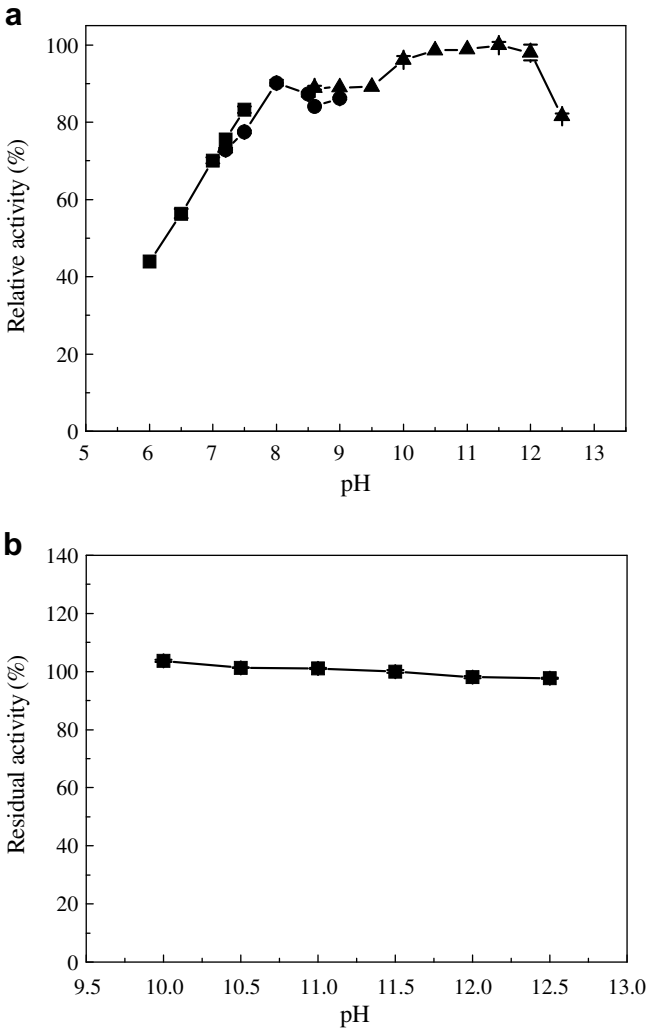

Fig. 2. Effect of pH on the activity (a) and alkaline stability (b) of proteases from *C. macropomum* pyloric caeca and intestine, precipitated with 30–70% ethanol. The enzyme activity on azocasein was established at different pH levels provided by the following buffer solutions: 0.1 M phosphate (■), Tris–HCl (●) and NaOH/glycine (▲). The specific enzyme activity of control sample (100%) was 142.0 U/mg using azocasein as substrate.

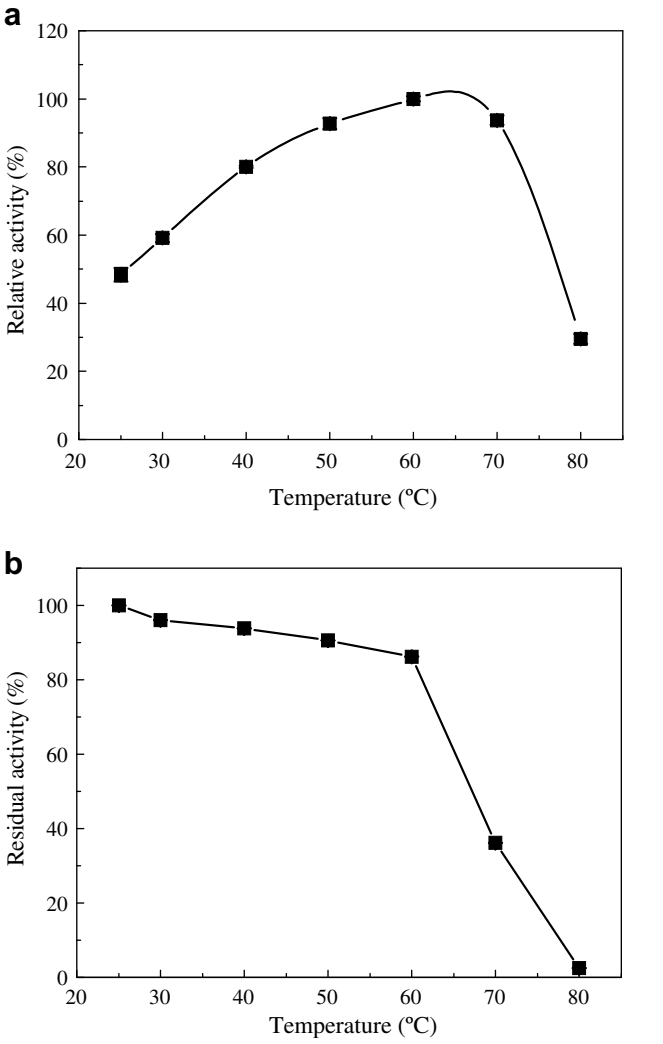

Fig. 3. Temperature profile (a) and thermal stability (b) of proteases from *C. macropomum* pyloric caeca and intestine precipitated by 30–70% ethanol. (a) The protease activity was assayed at indicated temperatures, 0.1 M NaOH/glycine, pH 11.0, and (b) the enzyme preparation was incubated for 30 min. at the indicated temperatures, and after the preparation had reached 25 °C their proteolytic activities were assayed. The specific enzyme activity of the control sample (100%) was 142.0 U/mg using azocasein as substrate.

between 9.0 and 12.0 and the thermal stability of these alkaline enzymes usually varies between 50 and 60 °C (Moreira et al., 2002).

Specific substrate and protease inhibitors were employed to identify the presence of trypsin-like and chymotrypsin-like enzymes in the ethanol-precipitated proteases from the tambaqui pyloric caeca and intestines. Proteases acting on BApNA (1.59 mU mL^{-1}) were strongly inhibited by Benzamidine (100%) and TLCK (98%), classical trypsin-like inhibitors, whereas those hydrolyzing Suc-Phe-p-Nan (0.04 mU mL^{-1}) were strongly inhibited by TPCK (100%), a typical chymotrypsin inhibitor. However, the results suggest that the trypsin-like enzyme is the major protease in the preparation (about 40 times higher). The BApNA and Suc-Phe-p-Nan proteolysis were, respectively, inhibited 54% and 96% by PMSF, a serine protease inhibitor. Similar results are frequently observed for other tropical fish proteases (Alencar et al., 2003; Bezerra et al., 2005).

Many parameters are involved in selecting a protease for detergents, such as compatibility with detergent components, e.g., surfactants, perfumes and bleaches (Gupta, Gupta, Saxena, & Khan, 1999; Kumar, Malik, & Tiwari, 1998). Tambaqui proteases were assayed in the presence of non-ionic (Tween 20 and Tween 80) and ionic surfactants (saponin and sodium choleate) using azocasein as substrate. Activities were not lost after being incubated for 60 min with these surfactants (Table 1). Sodium dodecyl sulfate (SDS) was the only detergent capable of inactivating them.

Table 1
Effect of surfactants on proteases of *C. macropomum* pyloric caeca and intestine purified by ethanol precipitation

Surfactants (1% w/v)	Residual activity[a] (%)	
	After 30 min	After 60 min
Saponin	117.5 ± 0.3	118.4 ± 2.1
Sodium choleate	94.2 ± 7.3	107.3 ± 4.4
Tween 20	117.3 ± 5.4	108.2 ± 0.5
Tween 80	112.0 ± 8.1	107.8 ± 4.7
SDS	15.1 ± 1.3	7.3 ± 1.0

[a] Values are expressed in ± standard deviation. $n = 4$. The specific enzyme activity of control sample (100%) was 142.0 U/mg using azocasein as substrate.

Important commercial detergent proteases like Subtilisin Carlsberg, Subtilisin BPN, Alcalase, Esparase and Savirase are stable in

the presence of various detergent components. However, most are unstable in the presence of oxidant agents, such as hydrogen peroxide (Gupta et al., 1999). Fig. 4 presents the oxidant stability of the tambaqui proteases in the presence of hydrogen peroxide. The peroxide inactivation curve indicates that the tambaqui digestive proteases are stable even at high concentrations of H_2O_2 (10% v/v). After incubation for 75 min they retained 53.33% of the initial activity. This is a relevant property because bleach stability has been attained only by site directed mutagenesis (Outtrup, Dambmann, & Aaslyng D.A., 1993; Outtrup, Dambmann, Christiansen, & Aaslyng, 1995) or by protein engineering (Boguslawski & Shultz, 1992) of bacterial enzymes.

An ideal detergent enzyme should be stable and active in the detergent solution for a long period of time. It should also maintain adequate temperature stability in order to be effective throughout a wide range of washing temperatures (Banik & Prakash, 2004; Moreira et al., 2002). The tambaqui proteases retained more than 50% of their activities when incubated with the detergents Ala®, Bem-te-vi® and Omo® for 1 h at 40 °C (Fig. 5). Maximum stability was achieved with Surf®, since the enzyme retained 73.70% of its activity. Protease stability in the presence of detergents varies according to the source of the enzyme. For example, enzymes from the fungi *Conidiobolus coronatus* and *Nocardiopsis* sp. retained 64% and 90% of their activities, respectively (Moreira et al., 2002), whereas protease from the bacteria *Bacillus cereus* (Banik & Prakash, 2004) and *Bacillus brevis* (Banerjee et al., 1999) retained more than 70% and 60%, respectively. Proteases should be effective at low enzyme levels. The tambaqui proteases were effective, that is, they retained their activities and were able to degrade proteins even at a lower concentration of 0.2 mg mL^{-1}, and in the presence of detergent solutions.

The high costs involved in purification procedures have been a limiting factor for the use of fish proteases (Bezerra et al., 2001; Bezerra et al., 2005). Ethanol precipitation is a technique that can be applied to proteins on an industrial scale due to its favourable physicochemical properties, such as complete miscibility with water, good freezing-point depression, absence of explosive mixtures, high volatility, chemical inertness, low toxicity and low cost (Cortez & Pessoa, 1999). Moreover, this method has proved efficient in removing the characteristic smell of fish viscera and in clarifying the crude extract. Ethanol precipitation has also proved

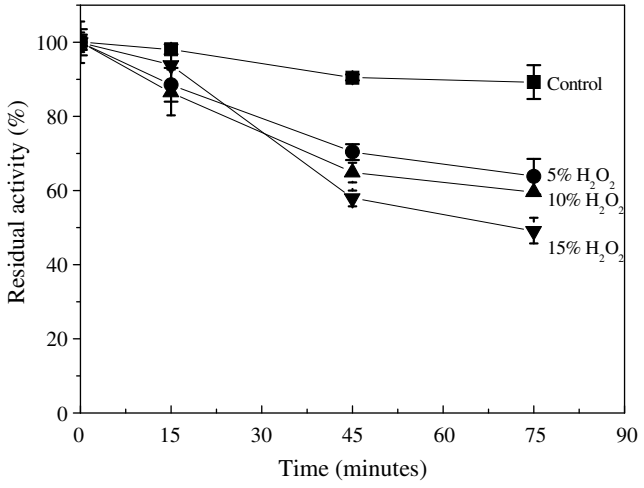

Fig. 4. The inactivation curve of the H_2O_2 of proteases from the *C. macropomum* pyloric caeca and intestine precipitated by 30–70% ethanol. Enzyme preparations were incubated at pH 11.0 and 40 °C with H_2O_2 at the concentrations of 5% (●), 10% (▲), 15% (▼). Samples were withdrawn at time intervals, their activities (duplicates) were established using azocasein as substrate and compared to the non-treated sample (■). The specific enzyme activity of the control sample (100%) was 146.0 U/mg using azocasein as substrate.

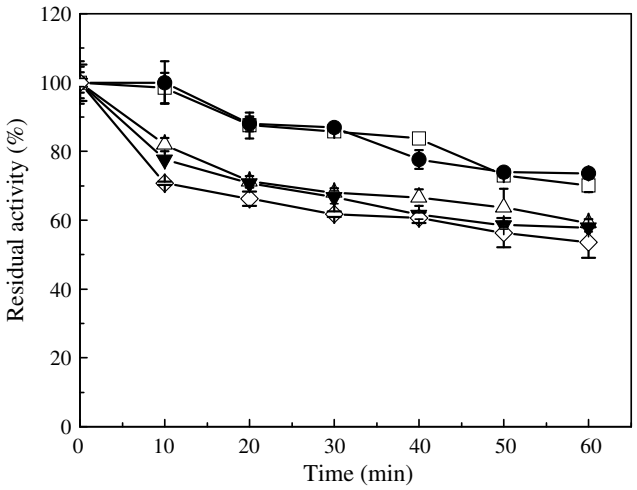

Fig. 5. The stability of protease in commercially available detergents. Protease (0.2 mg mL^{-1}) was incubated at 40 °C in the presence of detergents at 7 mg mL^{-1}. Activity of the control sample devoid of any detergent incubated under similar conditions (●), Surf® (□), Ala® (△), Bem-te-vi® (▼), Omo Multi-Ação® (◇). The specific enzyme activity of the control sample (100%) was 146.0 U/mg using azocasein as substrate.

itself to be a useful, fast, low cost procedure for obtaining proteolytic extract from the residue of fish processing with the potential aim of using it as an additive for laundry detergents (Espósito et al., 2009). Furthermore, the industrial use of this solvent for enzymes concentration and purification has some advantages such as: the process does not need interruption, requires simple equipment, and can be easily scaled up (Cortez & Pessoa, 1999).

To minimize the costs of viscera transport an operational unit for the waste processing could be set up as near as possible to the unit of processing fish. In a short description, the ethanol precipitation of 1 ton/day viscera may be carried out using about 30 simple reactors (1000L) with temperature control. The use of an industrial centrifuge and dialysis system would fulfill the enzymatic precipitation step. Finally, the used ethanol could be recovered using an industrial flash evaporation and reused (Gosh & Ghose, 2003).

4. Conclusions

Ethanol precipitation was carried out to obtain alkaline proteases (trypsin-like enzyme mostly) from *C. macropomum* pyloric caeca and intestines. These proteases acted efficiently within the recommended pH and temperature parameters for enzymes as laundry detergent additives. They also presented stability at these recommended pH and temperature levels as well as in the presence of non-ionic and ionic surfactants, except SDS. Moreover, they remained stable at high H_2O_2 concentrations and in the presence of several commercial detergents (Ala®, Bem-te-vi®, Omo® and Surf®).

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