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Solvent-Stable Digestive Alkaline Proteinases from Striped Seabream (*Lithognathus mormyrus*) Viscera: Characteristics, Application in the Deproteinization of Shrimp Waste, and Evaluation in Laundry Commercial Detergents

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Abstract Alkaline proteases from the viscera of the striped seabream (*Lithognathus mormyrus*) were extracted and characterized. Interestingly, the crude enzyme was active over a wide range of pH from 6.0 to 11.0, with an optimum pH at the range of 8.0–10.0. In addition, the crude protease was stable over a broad pH range (5.0–12.0). The optimum temperature for enzyme activity was 50 °C. The crude alkaline proteases showed stability towards various surfactants and bleach agents and compatibility with some commercial detergents. It was stable towards several organic solvents and retained more than 50% of its original activity after 30 days of incubation at 30 °C in the presence of 25% (*v*/*v*) dimethyl sulfoxide, *N*,*N*-dimethylformamide, diethyl ether, and hexane. The crude enzyme extract was also tested for shrimp waste deproteinization in the preparation of chitin. The protein removal with a ratio enzyme/substrate of 10 was about 79%.

Keywords *Lithognathus mormyrus* · Alkaline proteases · Biochemical characterization · Detergent · Organic solvent-stable proteases · Enzymatic deproteinization

Introduction

During fish processing, solid wastes including heads and viscera are generated and can be seen as 30% of the original material [1] and constitute an important source of proteins and enzymes. Traditionally, fish processing by-products have been converted to powdered fish flour for animal feed [2], fertilizer, and fish silage. However, most of these products possess low economic values.

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The question remains on what to do with the interesting amounts of fish by-products. One alternative to convert the fish processing wastes into more marketable products is to isolate and purify proteolytic enzymes which are abundant in fish viscera. Proteases account for nearly 60% of the total worldwide enzyme sales and then represent one of the most important enzymes from an industrial point of view [3]. The use of alkaline proteases has been increased remarkably in a various industrial processes such as detergent, animal feed, leather processing, food processing, silk degumming, decomposition of gelatin on X-ray films, and peptide synthesis.

The detergent industry has now emerged as a major consumer of several hydrolytic enzymes acting at alkaline pH. Detergent proteases account for at least a quarter of all protease sales throughout the world [4–7]. They are primarily used as detergent additives since they are biodegradable and increase performance/cost ratios [5, 7].

Moreover, although the enzymes selected for detergent composition have been subtilisins, they are not the ideal enzymes for detergents due to their low thermal stability, the presence of detergents, and also because of their short shelf life [8]. Thus, it is relevant to search for proteases from new sources presenting high thermal stability, alkaline activity, and more compatible with washing systems [9]. These properties have already been observed in trypsin-like enzymes.

Shrimp by-products have been identified as an animal protein source of a great potential and also as an important source of chitin and astaxanthin [10]. Only 65% of the shrimp is edible. The remainder is discarded as inedible waste (cephalothorax and exoskeleton). Over the years, techniques have been developed for the recovery and exploitation of these by-products in valuable biopolymers such as chitin and chitosan [11–13]. These biomolecules are widely used in the food industry, pharmacy, textiles, chemical industries, etc. [14]. Conventionally, to extract chitin from crustacean shells, chemical processing for demineralization and deproteinization has been applied by treatment with strong acids and bases to remove calcium carbonate and proteins, respectively [15]. However, the use of these chemicals may destroy the chitin. To overcome the hazards from chemical treatments, alternative methods on use of micro-organisms [16–18] or proteolytic enzymes [19, 20] for the deproteinization of crustacean wastes have been reported.

A variety of digestive proteolytic enzymes has been isolated from the internal organs of fish. The most important digestive enzymes from fish viscera are pepsin, secreted from gastric mucosa, and serine proteases trypsin and chymotrypsin, secreted from the pancreas, pyloric caeca, and intestine [21]. Acidic proteases from fish stomachs display high activity between pH 2.0 and 4.0, while alkaline digestive proteases, such as trypsin, are most active between pH 8.0 and 10.0.

Biotechnology is able to provide the means for transforming such raw material into valuable products such as enzymes [22]. However, little information is available in the literature regarding the use of active proteases from fish for detergent compositions [23]. The striped seabream (*Lithognathus mormyrus*) is a marine fish belonging to the Sparidae family and frequent along Mediterranean coasts. In Tunisia, striped seabream (*L. mormyrus*) catches were about 637 tons in 2008, and it has been exploited for human consumption. During processing, large quantities of waste, including heads and viscera, are generated and discarded. They can represent about 30% of the original raw material.

Until now, no studies are reported on organic solvent-stable proteases from fish. The present paper describes the extraction and characterization of alkaline proteases from *L. mormyrus* viscera, as well as their compatibility with commercial laundry detergents,

oxidants, surfactants agents, and organic solvents. The application in the deproteinization of shrimp wastes was also investigated.

Materials and Methods

Striped Seabream Viscera

Striped seabream (*L. mormyrus*) was purchased from the fish market of Sfax City, Tunisia. The samples were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (*w*/*w*), and transported to the research laboratory within 30 min. After the fish was washed with water, their viscera were separated and then stored in sealed plastic bags at −20 °C until used for enzyme extraction.

Preparation of Crude Alkaline Enzyme Extract

Viscera from *L. mormyrus* (100 g) were rinsed with distilled water and homogenized for 60 s with 200 ml of extraction buffer (10 mM Tris–HCl, pH 8.0). The homogenate was centrifuged at 8,500×*g* for 30 min at 4 °C. The pellet was discarded, and the supernatant was collected and used as crude protease extract.

Biochemical Characterization of the Crude Enzyme Extract

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli [24], using 5% (*w*/*v*) stacking and 15% (*w*/*v*) separating gels. Samples were prepared by mixing the crude enzyme extract at 1:5 (*v*/*v*) ratio with distilled water containing 10 mM Tris–HCl (pH 8.0), 2.5% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.002% bromophenol blue. Samples were heated at 100 °C for 5 min before loading in the gel. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R-250 in 45% ethanol–10% acetic acid and destained with 5% ethanol and 7.5% acetic acid. The molecular mass markers (Sigma) used were bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), glyceraldehyde-3-dehydrogenase (36,000 Da), bovine trypsinogen (24,000 Da), soybean trypsin inhibitor (20,100 Da), and bovine α-lactoalbumin (14,200 Da).

Detection of Protease Activity by Zymography

Protease activity staining was performed on SDS-PAGE according to the method of Garcia-Carreno et al. [25] with a slight modification. The sample was not heated before electrophoresis. After electrophoresis, the gel was submerged in 100 mM glycine–NaOH buffer (pH 10.0) containing 2.5% Triton X-100, with shaking for 30 min to remove SDS. Triton X-100 was removed by washing the gel three times with 100 mM glycine–NaOH buffer (pH 10.0). The gel was then incubated with 1% (*w*/*v*) casein in 100 mM glycine–NaOH buffer (pH 10.0) for 20 min at 50 °C. Finally, the gel was stained with 0.25% Coomassie Brilliant Blue R250 in 45% ethanol–10% acetic acid and destained with 5% ethanol–7.5% acetic acid. The development of clear zones on the blue background of the gel indicated the presence of protease activity.

Effect of pH on Activity and Stability of Alkaline Proteases

Protease activity of the alkaline crude extract was assayed over the pH range of 5.0 to 13.0 using casein as a substrate at 50 °C.

For the measurement of pH stability, the crude enzyme extract was incubated at 25 °C for 60 min in different buffers and then the residual proteolytic activity was determined under standard assay conditions. The non-incubated enzyme was considered as control (100%). The following buffer systems were used: 100 mM sodium acetate, pH 5.0–6.0; 100 mM phosphate buffer, pH 7.0; 100 mM Tris–HCl buffer, pH 8.0; 100 mM glycine–NaOH buffer, pH 9.0–11.0; and 100 mM KCl–NaOH buffer, pH 12.0–13.0.

Optimum Temperature and Thermal Stability of Alkaline Proteases

The effect of temperature on protease activity was studied from 30 to 70 °C for 15 min at pH 10.0. Thermal stability was determined by incubating the crude enzyme extract 60 min at different temperatures, and the remaining activity was determined at standard conditions. The non-heated enzyme was considered as control (100%).

Effects of Metal Ions on the Alkaline Proteases Activity

The effects of various metal ions (5 mM) on alkaline proteases activity were investigated, using casein as a substrate, by adding the monovalent (Na^+ or K^+) or divalent (Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ba^{2+}, or Mg^{2+}) metal ions to the reaction mixture. The activity of the enzyme in the absence of metal ions was taken as control.

Effects of Enzyme Inhibitors and Denaturing Reagents on Alkaline Proteases

The effects of enzyme inhibitors on protease activity were studied using phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol, and ethylenediaminetetraacetic acid (EDTA). The alkaline crude enzyme extract was preincubated with each inhibitor for 30 min at 25 °C, and then the remaining protease activity was tested using casein as a substrate. The activity of the enzyme assayed in the absence of inhibitors was taken as control.

The effects of some surfactants (Triton X-100, Tween 20, Tween 80, and SDS) and oxidizing agent (sodium perborate) on enzyme stability were studied by preincubating the alkaline proteases for 1 h at 30 °C. The residual activity was measured at pH 10.0 and 50 °C. The activity of the enzyme without any additive was taken as 100%.

Detergent Compatibility

The compatibility of the alkaline crude protease extract with commercial solid laundry detergents was studied using Dixan (Henkel, Spain), Ariel (Procter & Gamble, Switzerland), New Det (Sodet, Tunisia), and Axion (Colgate-Palmolive, France). Commercial detergents were diluted in tap water to give a final concentration of 7 mg/ml to simulate washing conditions. The endogenous proteases contained in these detergents were inactivated by heating the diluted detergents for 1 h at 65 °C prior to the addition of the enzyme preparation. The crude protease was incubated with different detergents for 1 h at 30 and 40 °C, and then the remaining activities were determined under the standard assay conditions. Commercial protease Purafect® 2000E (Genencor International®, Leiden, The

Netherlands) was used for comparative study. The enzyme activity of a control, without detergent, incubated under the similar conditions, was taken as 100%.

Organic Solvent-Stability Assay

The organic solvent stability of the enzyme was studied by incubating the crude enzyme with various organic solvents (25%; *v*/*v*; methanol, *N*,*N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), diethyl ether, hexane, acetone, and isopropanol) at 30 °C with shaking (150 rpm) for 30 days. Aliquots were withdrawn at desired time intervals to test the remaining protease activity. Crude enzyme tested in the absence of organic solvent and diluted using buffer was considered as control [20].

Deproteinization of Shrimp Wastes by Striped Seabream Proteases

Shell wastes were mixed with water at a ratio of 1:2 (*w*/*v*) and minced then cooked for 20 min at 90 °C to inactivate endogenous enzymes. The cooked sample was then homogenized in a Moulinex blender for about 2 min. The pH of the mixture was adjusted to 8.0, and then the shrimp waste proteins were digested with the crude protease extract of *L. mormyrus* using different enzyme/substrate (E/S) ratio (unit of enzyme per milligram of protein). After incubation for 3 h at 40 °C, the reaction was stopped by heating the solution at 90 °C during 20 min to inactivate enzymes. The shrimp waste protein hydrolysates were then centrifuged at 5,000×*g* for 20 min to separate insoluble and soluble fractions. The solid phase was washed and then dried for 1 h at 60 °C.

Deproteinization (DP) was expressed as percentages and computed by the following equation as described by Rao et al. [26]:

$$\%\ \mathrm{DP} = \frac{[(P_{\mathrm{O}} \times O) - (P_{\mathrm{R}} \times R)] \times 100}{P_{\mathrm{O}} \times O},$$

where P_{O} and P_{R} are the protein concentrations (percent) before and after hydrolysis, while O and R represent the mass (grams) of original sample and hydrolyzed residue in dry weight basis, respectively.

Protease Activity Assay

Protease activity in the alkaline crude extract was measured by the method of Kembhavi et al. [27] using casein as a substrate. A 0.5-ml aliquot of the crude enzyme extract, suitably diluted, was mixed with 0.5 ml of 100 mM glycine–NaOH (pH 10.0) containing 1% (*w*/*v*) casein, and incubated for 15 min at 50 °C. The reaction was stopped by addition of 0.5 ml of 20% (*w*/*v*) trichloroacetic acid. The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000×*g* for 15 min to remove the precipitate. The acid soluble material was estimated spectrophotometrically at 280 nm. A standard curve was generated using solutions of 0–50 mg/l tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg tyrosine per minute under the experimental conditions used.

Chymotrypsin and Trypsin Activities Assay

Chymotrypsin activity assay was evaluated according to Tsai et al. [28] with slight modifications, using SAAPNA as a substrate [29]. Trypsin activity assay was measured

according to the method of Erlanger et al. [30], modified by Benjakul et al. [31], using Nα-benozyl-DL-arginine-p-nitroanilide (BAPNA) as a substrate.

Results and Discussion

Characterization of Viscera Enzyme Extract

SDS-PAGE and Zymography of Crude Alkaline Proteases

A preliminary study on the characterization of the crude enzyme extract was carried out. In order to estimate the number of proteases in the alkaline crude enzyme extract, sample was separated by SDS-PAGE, and then the activity was revealed by casein zymogram activity staining. Figure 1 shows the separation of various protease bands. The crude enzyme extract showed at least three clear zones of protease activity with different molecular weights. This result suggests that at least three major proteinases were present in striped seabream viscera. Specific activities of 0.482 and 3.62 U/mg were obtained using BAPNA and SAPNA as a substrate, respectively.

Effects of Enzyme Inhibitors on Protease Activity

Proteases can be classified by their sensitivity to various inhibitors [32]. In order to determine the nature of proteases in the crude protease extract, the effects of different enzyme inhibitors, such as chelating agent and a specific group reagent on the protease activity, were investigated (Table 1).

Proteases from *L. mormyrus* were affected by PMSF, a serine protease inhibitor. The enzyme retained 70% of its initial activity after 30 min of incubation in the presence of 10 mM PMSF. On the other hand, metalloprotease inhibitor EDTA and β-mercaptoethanol inhibited the enzyme activity by 15% (Table 1).

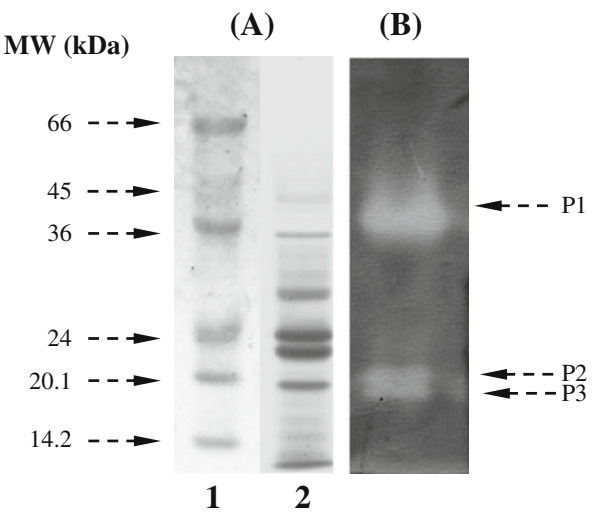

Fig. 1 **a** SDS-PAGE of alkaline proteases from the viscera of striped seabream. *Lane 1* standard proteins marker of different molecular weights, *lane 2* crude enzyme extract; **b** zymogram of the crude extract

Table 1 Effects of various enzyme inhibitors and metal ions on the activity of the alkaline crude enzyme extract from *L. mormyrus*

Chemicals	Concentration (mM)	Relative activity (%)
None	–	100
PMSF	5	100
	10	70
β-mercaptoethanol	5	87
	10	85
EDTA	5	87
	10	86
Ca^{2+}	5	100
Ba^{2+}	5	100
Zn^{2+}	5	123
Cu^{2+}	5	82
Mg^{2+}	5	100
Mn^{2+}	5	69
K^{+}	5	100
Na^{+}	5	100

The alkaline crude enzyme was preincubated with various enzyme inhibitors for 30 min at 25 °C, and the remaining activity was determined at pH 10.0 and 50 °C. Enzyme activity measured in the absence of any inhibitor was taken as 100%. The effect of metal ions on the activity of the alkaline crude enzyme was determined by incubating the enzyme in the presence of various metal ions for 15 min at 50 °C and pH 10.0

The effect of enzyme inhibitors on protease activity was also analyzed by zymogram. As shown in Fig. 2, only one clear zone of protease activity was detected after treatment with PMSF; thus, P2 and P3 inhibited by PMSF are serine proteases. However, only P3 was inhibited by EDTA, this suggests that this low molecular weight enzyme is a metalloprotease.

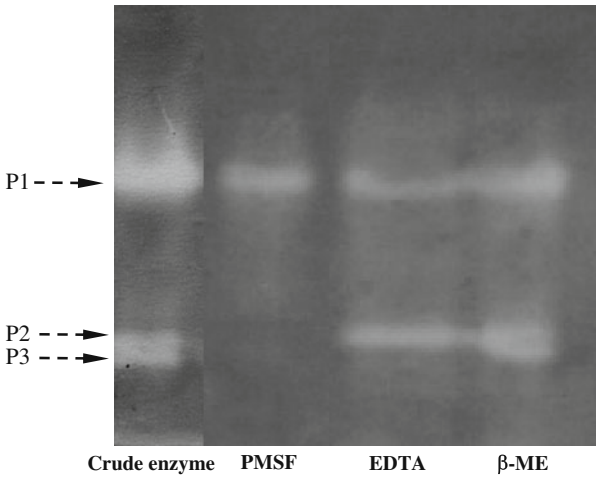

Fig. 2 Zymogram of alkaline proteases from the viscera of striped seabream in the presence of various inhibitors. *Lane 1* crude extract, *lane 2* crude extract with PMSF (10 mM), *lane 3* crude extract with EDTA (10 mM), *lane 4* crude extract with β-mercaptoethanol (10 mM)

Effect of pH on Activity and Stability of Alkaline Enzyme Extract

The activity of proteolytic enzymes was determined at different pH values (5.0–13.0). The pH activity profile of alkaline proteases is shown in Fig. 3a. The crude enzyme was highly active between pH 8.0 and 10.0, with an optimum at pH 10.0. The relative activities at pH 7.0, 8.0, and 11.0 were about 83%, 98.5%, and 56%, respectively, of that at pH 10.0. The visceral protease activity pattern was similar to those of other fish species such as intestine proteases of Nile tilapia (*Oreochromis niloticus*), proteases from the intestine and pyloric caeca of crevalle jack (*Caranx hippos*), spotted goatfish (*Pseudopeneus maculates*), parrotfish (*Sparisoma* sp.), and traira (*Hoplias malabaricus*) [33], although, alkaline proteases from tambaqui (*Colossoma macropomum*) and common carp (*Cyprinus carpio*) intestine were reported to be active at higher pH values (10.0–12.0) [23, 34].

As displayed in Fig. 3b, crude enzyme extract is highly stable over a wide broad pH range, maintaining more than 96% of its original activity between pH 5.0 and 11.0 and 88% at pH 12 after 1 h incubation at 25 °C. These results suggest that the viscera of *L. mormyrus* would be a potential source of proteases for certain industrial applications that require high alkaline conditions.

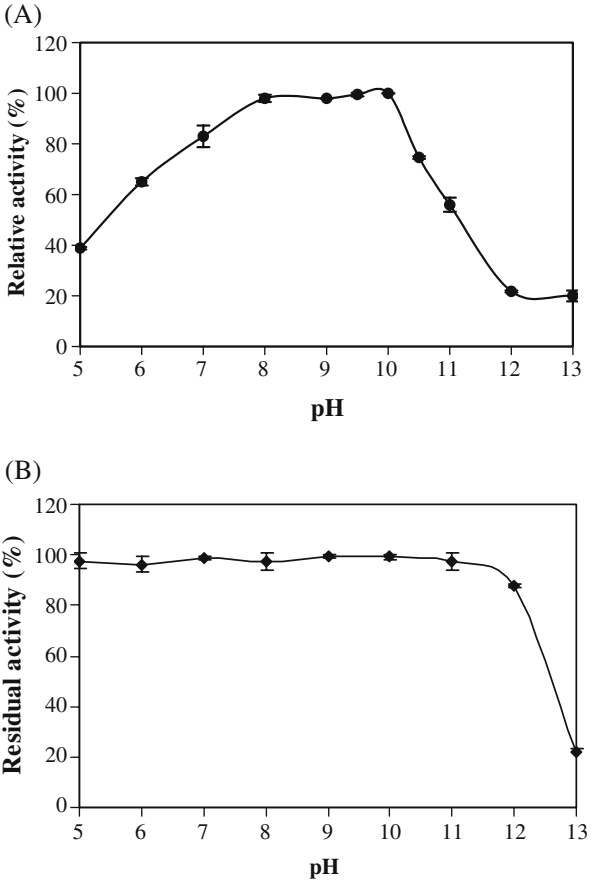

Fig. 3 pH profile (**a**) and pH stability (**b**) of the alkaline crude protease from viscera of the striped seabream (*L. mormyrus*). Protease activity was assayed in the pH range from 5.0 to 13.0 at 50 °C. The maximum activity obtained at pH 10.0 was considered as 100% activity. The pH stability was determined by incubating the enzyme in different buffers for 60 min at 25 °C, and the residual enzyme activity was determined at pH 10.0 and 50 °C using casein as a substrate. The activity of the enzyme before incubation was taken as 100%. Buffer solutions used for pH activity and stability are presented in "Materials and Methods"

Effect of Temperature on the Activity and Stability of the Visceral Crude Enzyme Extract

Optimum temperature for activity of alkaline proteases was determined in order to assess their suitability for biotechnological applications. Figure 4a shows the enzyme activity of the crude extract as a function of temperatures. Alkaline proteases were active at temperatures from 30 to 70 °C with an optimum around 50 °C. The relative activities at 40 and 60 °C were about 76% and 37.5%, respectively, of that at 50 °C. The optimal temperature of *L. mormyrus* proteases was similar to that of common carp (*Cyprinus carpio* L.) [34] and lower than that of Nile tilapia and tambaqui proteases [23, 35] which have optimal temperatures in the range of 55 and 60 °C, respectively.

The thermal stability profile showed that alkaline enzyme extract is fully active for at least 60 min at 30 and 40 °C, which is desirable for laundry purposes and from the ecological and economical point of view, mainly, because of saving of energy. The alkaline crude extract retained more than 81% and 38% of its initial activity after 60 min incubation at 50 and 60 °C, respectively (Fig. 4b).

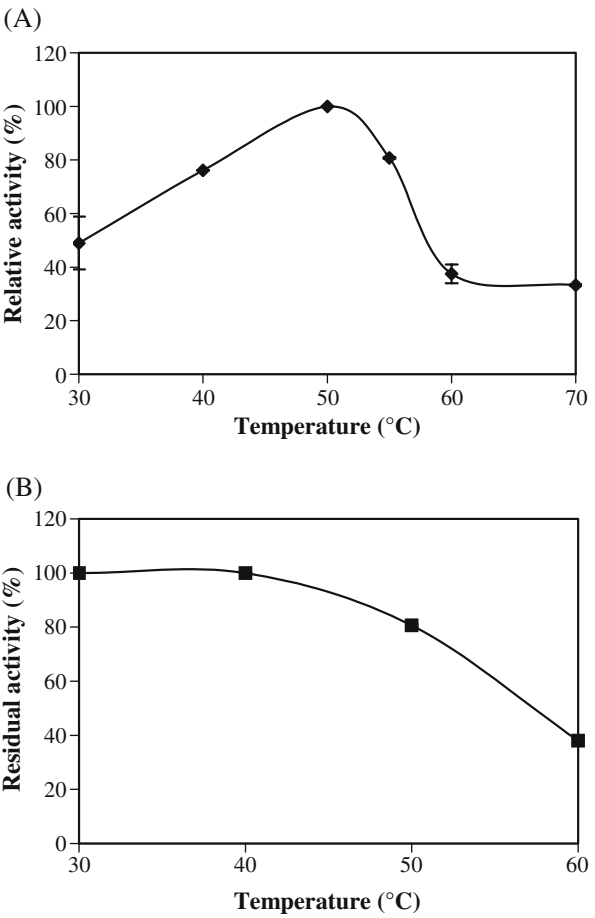

Fig. 4 Temperature profile (**a**) and thermal stability (**b**) of the alkaline crude enzyme from the viscera of the striped seabream (*L. mormyrus*). Enzyme activity was assayed at different temperatures ranging from 30 to 70 °C at pH 10.0, using casein as a substrate. The activity of the enzyme at 50 °C was taken as 100%. For thermal stability, the enzyme was incubated at different temperatures for 60 min. The residual enzyme activity was assayed at pH 10.0 and 50 °C. The non-heated enzyme was considered as control (100%)

Effects of Metal Ions

The effects of some metal ions, at a concentration of 5 mM, on the activity of *L. mormyrus* visceral crude enzyme extract were studied at pH 10.0 and 50 °C by the addition of metal ions to the reaction mixture. As shown in Table 1, Ca^{2+}, Ba^{2+}, Mg^{2+}, Na^{+}, and K^{+} did not affect protease activity, whereas Zn^{2+} increased the protease activity to 123%. However, Cu^{2+} and Mn^{2+} affect the enzyme activity, with 18 and 31% inhibition, respectively.

El Hadj Ali et al. [29] showed that Ca^{2+}, Cu^{2+}, and Zn^{2+} improved protease activity of trypsin of *L. mormyrus* by 114%, 110%, and 117%, respectively. Thermostable dipeptidase from common carp (*Cyprinus carpio*) intestine was activated by Mg^{2+}, Mn^{2+}, and Sr^{2+} at 1 mM with a relative activity of 194%, 548%, and 113%, respectively [36]. Li^{+}, Na^{+}, K^{+}, Mg^{2+}, and Ba^{2+} ions showed no influence on enzyme activity of trypsin from the intestine of hybrid Tilapia (*O. niloticus*×*Oreochromis aureus*), but Ca^{2+} slightly improved enzyme activity with a relative activity of 125% [37].

Effect of Oxidizing Agents and Surfactants on Protease Stability

In order to be effective during washing, a good detergent protease must be compatible and stable with all commonly used detergent compounds such as surfactants, oxidizing agents, and other additives, which might be present in the formulation [7, 38]. Alkaline proteases from high-yielding strains have been studied extensively. However, very few published reports are available on the compatibility of the alkaline proteases with detergents ingredients [39]. Important commercial detergent proteases like subtilisin Carlsberg, subtilisin BPN, Alcalase, Esperase, and Savinase are stable in the presence of various detergent components; however, most of them are unstable in the presence of oxidant agents [38]. Thus, it is desirable to search for new proteases with novel properties from many different sources as possible.

The suitability of the *L. mormyrus* proteases as detergent additive was determined by testing the stability in oxidants and surfactants. As shown in Table 2, the alkaline crude enzyme extract is highly stable in the presence of the non-ionic surfactants like Tween 20, Tween 80, and Triton X-100, retaining 100% of its activity. However, the *L. mormyrus* proteases were less stable against the strong anionic surfactant (SDS) and retained 62.5% and 33% of its activity in the presence of 0.5% and 1% SDS, respectively.

Interestingly, *L. mormyrus* protease activity was little influenced by oxidizing agent and retained about 82.5% and 78.5% of its activity after incubation 1 h at 30 °C in the presence of 1% and 2% sodium perborate, respectively. This is a relevant property because bleach stability has been attained only by site-directed mutagenesis [40, 41] or by protein engineering [42] of bacterial enzymes. The high stability of the alkaline proteases towards oxidizing agents is a very important characteristic for its eventual use in detergent formulations.

Stability of the Alkaline Crude Enzyme Extract with Commercial Solid Detergents

Alkaline proteases added to laundry solid and liquid detergents play a catalytic role in the hydrolysis of protein stains such as blood, milk, etc. The high activity and stability of the alkaline crude enzyme extract in the pH range from 8.0 to 11.0 and its stability towards surfactants and oxidizing agents are very useful for its eventual application as detergent additive. To check the compatibility of the alkaline crude extract with solid detergents, the

Table 2 Stability of *L. mormyrus* alkaline crude enzyme extract in the presence of various surfactants and oxidizing agents

Additives	Concentration	Residual activity (%)
None		100
Tween 20	5% (*v*/*v*)	100
Tween 80	5% (*v*/*v*)	100
Triton X-100	5% (*v*/*v*)	100
SDS	0.5 (*w*/v)	52.5
SDS	1% (*v*/*v*)	33
Sodium perborate	1% (*w*/*v*)	82.5
Sodium perborate	2% (*w*/*v*)	78.5

The alkaline crude enzyme extract was preincubated with surfactants and oxidizing agents for 1 h at 30 °C, and the remaining activity was measured at pH 10.0 and 50 °C. The activity is expressed as a percentage of the activity level in the absence of additives

crude enzyme was preincubated in the presence of various solid commercial detergents for 1 h at 30 and 40 °C. The data presented in Fig. 5 show that the alkaline proteases are highly stable at 30 °C and relatively stable at 40 °C. The alkaline proteases exhibited higher stability with Axion and Ariel than Dixan, Nadhif, and New Det. The crude protease retained 100% of its activity in the presence of Axion and Ariel after 1 h incubation at 30 °C, while 81% and 57% of activity were retained at 40 °C, respectively. At 30 °C, stability of striped seabream crude proteases was similar to commercial protease (Purafect® 2000E). Since the proteolytic activity varied with each laundry detergent, the obtained results clearly indicated that the performance of enzymes in detergents depends on number of factors, including the detergents' compounds.

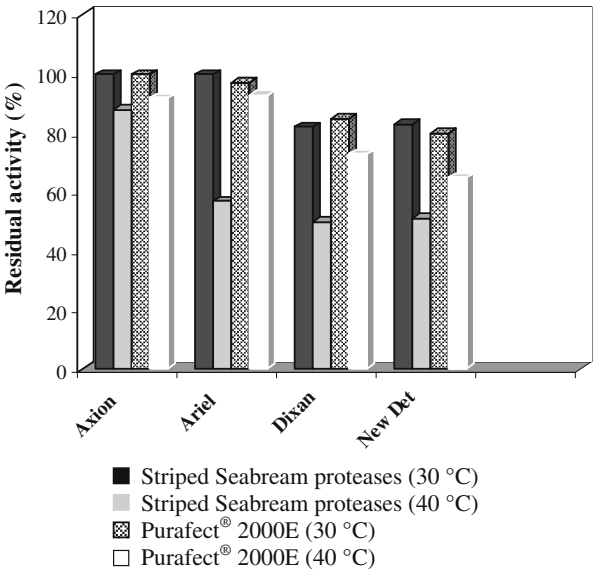

Fig. 5 Stability of the alkaline crude protease extract from *L. mormyrus* in the presence of various commercial solid detergents. The crude enzyme at 385 U/ml was incubated 1 h at 30 or 40 °C and pH 10.0 in the presence of solid detergents at a final concentration of 7 mg/ml, and the remaining activities were determined at pH 10.0 and 50 °C using casein as a substrate. Commercial protease (Purafect® 2000E) was tested under the same conditions as *L. mormyrus* proteases. Enzyme activity of control sample without any detergent, incubated under the similar conditions, was taken as 100%

Espósito et al. [23] and Mendes et al. [35] reported also the stability of tambaqui (*Colossoma macropomum*) and Nile tilapia (*O. niloticus*) proteases in the presence of several commercial detergents. 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, and maximum stability was achieved with Surf®, since the enzyme retained 73.70% of its activity.

Considering its promising properties, *L. mormyrus* crude protease may be considered a potential candidate for future use in detergent processing industries.Further research is needed to use the crude extract at industrial process and avoid some disadvantages such as smell of fish, the possibility of formation of spots, and the seasonal effect on the enzymatic activity.

Effect of Organic Solvents on Enzyme Stability

To achieve a high yield in protease-catalyzed peptide synthesis, the stability of protease is very important because enzymes are usually denatured or inactivated in the presence of organic solvents before completing the reaction. Therefore, proteases, which are naturally stable in the presence of organic solvents, are very useful for synthetic reactions and peptide synthesis.

The solvent stability of reported solvent-stable proteases is generally tested at the concentration of 25% [43–45]. In this study, the effects of various organic solvents on the stability of the crude protease were investigated. Half-lives of the activity of the crude protease are shown in Table 3. In the absence of organic solvents, the half-life of *L. mormyrus* proteases was approximately 8 days. In the presence of methanol and acetone, the half-lives of *L. mormyrus* proteases were about 18 and 17 days, respectively. Interestingly, the stability of the enzyme was highly enhanced by DMSO, DMF, diethyl ether, and hexane; the half-lives of the enzyme in the presence of these solvents were more than 30 days.

These results were similar to earlier reports showing increased protease stability in the presence of organic solvent. In fact, the half-life of the *Bacillus cereus* SV1 protease was approximately 34 days in the absence of organic solvent. However, in the presence of ethyl acetate and DMF, the half-lives were 51 and 48 days, respectively [20]. Jellouli et al. [46]

Table 3 Half-lives of the activity of the alkaline crude enzyme extract from *L. mormyrus* in the presence of 25% (*v*/*v*) organic solvents

Organic solvent	Half-life (days)
None	8
Methanol	18
DMSO	>30
Isopropanol	8
DMF	>30
Hexane	>30
Acetone	17
Diethyl ether	>30

The alkaline crude enzyme was preincubated with various organic solvents (25%; *v*/*v*; methanol, DMSO, isopropanol, DMF, hexane, acetone, and diethyl ether) at 30 °C with shaking (150 rpm) for 30 days. Aliquots were withdrawn at desired time intervals to test the remaining activity

showed that the purified elastase of *Pseudomonas aeruginosa* MN7 was stable in the presence of 25% methanol and DMSO after 60 days of incubation. This enhanced stability may be due to conformational rigidity of the proteins in the dehydrated state and its resistance to deleterious process causing irreversible inactivation [47]. These results are in line with many studies showing that protease stability might be improved or disproved in the presence of different organic solvents [48, 49].

Enzymatic Deproteinization of Shrimp Wastes by *L. mormyrus* Proteases

Chitin, a polysaccharide found in abundance in the shell of crustaceans, is closely associated with proteins. Therefore, deproteinization in chitin extraction process is crucial. Chemical treatment requires the use of HCl and NaOH, which can cause chitin deacetylation and depolymerization. Many reports have demonstrated the application of proteolytic microorganisms for the deproteinization of marine crustacean wastes to produce chitin [17, 18, 50, 51]. However, few studies on the use of proteolytic enzymes for the deproteinization of crustacean wastes have been reported [20]. Further, to the best of our knowledge, there are no available reports on the enzymatic deproteinization of shrimp wastes by fish proteases.

In this study, the crude enzyme from *L. mormyrus* was also tested for the deproteinization of shrimp waste to produce chitin. Different E/S ratios between 0 and 20 were used to compare the deproteinization efficiency. As shown in Fig. 6, the deproteinization rate with a ratio of 0.1 was only 38%. The percentage of protein removal increased with increasing E/S ratio and reached about 79% with E/S ratio of 10 after incubation for 3 h at 40 °C. Beyond a ratio of 10, no significant increase in the deproteinization rate was observed.

The deproteinization activity of *L. mormyrus* crude protease was better than many proteases reported in previous studies. Protein removal from natural shrimp waste was 78% after a 7-day incubation at 37 °C with the culture supernatant from *Pseudomonas aeruginosa* K-187 [19]. Bustos and Healy [16] compared the effects of microbial and enzymatic deproteinization. A maximum value of 82% was achieved with *Pseudomonas maltophilia* after 6 days and 64% with purified microbial protease under the same condition. Protein removal reached 88% with E/S ratio of 20 in the presence of solvent-

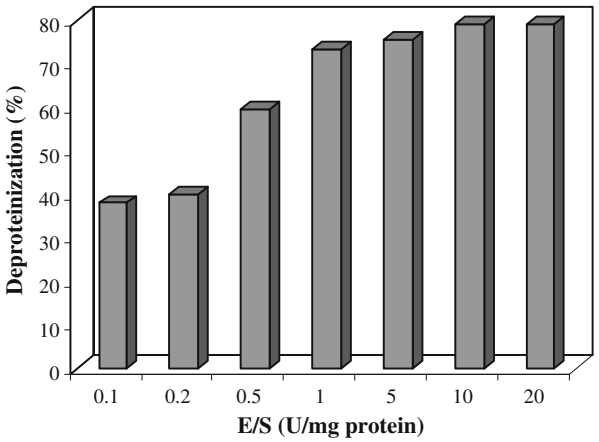

Fig. 6 Effect of the E/S ratio on the deproteinization of shrimp waste

stable metalloprotease of *B. cereus* SV1 [20]. The fact that deproteinization cannot reach 100% may be explained by the non-accessibility of enzymes to some proteins protected by chitin.

The obtained results demonstrated that proteases from *L. mormyrus* viscera could be used effectively in the deproteinization of shrimp wastes. Furthermore, the application of proteases or proteolytic bacteria for the deproteinization would be a good solution for the environmental problems associated with crustacean processing.

Conclusion

The present study reports the extraction, characterization, and evaluation of digestive proteases from *L. mormyrus* as detergent additive and shrimp waste deproteinization. The alkaline crude proteases showed efficient activity within the recommended pH and temperature parameters for enzymes as laundry detergent additives. Interestingly, *L. mormyrus* proteases activity remained stable against oxidizing agent (sodium perborate) and in the presence of commercial detergents (Axion, Ariel, Dixan, and New Det).

Protease activity was enhanced by DMSO, DMF, diethyl ether, and hexane, and the half-life of the crude enzyme in the presence of these solvents were more than 30 days.

Further, alkaline digestive proteases from *L. mormyrus* were found to be effective in the deproteinization of shrimp wastes, and a high level of deproteinization (79%) was recorded with an E/S ratio of 10 (10 U/mg).

Further research is needed to purify all alkaline proteases and to determine their properties as a possible biotechnological tool in the fish processing and detergent processing industries.

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References

1. Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., Simpson, B. K., & Saeki, H. (2006). *Comparative Biochemistry and Physiology, 144*, 47–56.
2. Ström, T., & Eggum, B. O. (1981). *Journal of the Science of Food and Agriculture, 32*, 115–120.
3. Joo, H. S., & Chang, C. S. (2006). *Enzyme and Microbial Technology, 38*, 176–183.
4. Anwar, A., & Saleemuddin, M. (1998). *Bioresource Technology, 64*, 175–183.
5. Gupta, R., Beg, Q. K., & Lorenz, P. (2002). *Applied Microbiology and Biotechnology, 59*, 15–32.
6. Horikosh, K. (1996). *FEMS Microbiology Reviews, 18*, 259–270.
7. Kumar, C. G., & Takagi, H. (1999). *Biotechnology Advances, 17*, 561–594.
8. Samal, B. B., Kara, B., & Stabinsky, Y. (1990). *Biotechnology and Bioengineering, 35*, 650–652.
9. Banerjee, U. C., Sani, R. K., Azmi, W., & Soni, R. (1999). *Process Biochemistry, 35*, 213–219.
10. Shahidi, F., & Synowiecki, J. (1991). *Journal of Agricultural and Food Chemistry, 39*, 1527–1532.
11. Bhaskar, N., Suresh, P. V., Sakhare, P. Z., & Sachindra, N. M. (2007). *Enzyme and Microbial Technology, 40*, 1427–1434.
12. Sini, T. K., Santhosh, S., & Mathew, P. T. (2007). *Carbohydrate Research, 342*, 2423–2429.
13. Du, Y., Zhao, Y., Dai, S., & Yang, B. (2009). *Innovative Food Science & Emerging Technologies, 10*, 103–107.
14. Rinaudo, M. (2006). *Progress in Polymer Science, 31*, 603–632.
15. Roberts, G. A. F. (1992). Chitin Chemistry. London: Macmillan.

16. Bustos, R.O. & Healy, M.G. (1994). Institution of Chemical Engineers Symposium Series, Institution of Chemical Engineers, England: Rugby pp. 13–15.
17. Oh, K. T., Kim, Y. J., Nguyen, V. N., Jung, W. J., & Park, R. D. (2007). *Process Biochemistry, 42*, 1069–1074.
18. Jo, G. H., Jung, W. J., Kuk, J. H., Oh, K. T., Kim, Y. J., & Park, R. D. (2008). *Carbohydrate Polymers, 74*, 504–508.
19. Oh, Y. S., Shih, I. L., Tzeng, Y. M., & Wang, S. L. (2000). *Enzyme and Microbial Technology, 27*, 3–10.
20. Manni, L., Jellouli, K., Ghorbel-Bellaaj, O., Agrebi, R., Haddar, A., Sellami-Kamoun, A., et al. (2010). *Applied Biochemistry and Biotechnology, 160*, 2308–2321.
21. Simpson, B. K. (2000). Digestive proteases from marine animals. In N. F. Haard & B. K. Simpson (Eds.), *Seafood enzymes* (pp. 191–213). New York: Marcel Dekker.
22. Castillo-Yanez, F. J., Pacheco-Aguilar, R., Garcia-Carreno, F. L., & Navarrete-Del Toro, M. A. (2005). *Comparative Biochemistry and Physiology, 140B*, 91–98.
23. Espósito, T. S., Amaral, I. P. G., Buarque, D. S., Oliveira, G. B., Carvalho, L. B., Jr., & Bezerra, R. S. (2009). *Food Chemistry, 112*, 125–130.
24. Laemmli, U. K. (1970). *Nature, 227*, 680–685.
25. Garcia-Carreno, F. L., Dimes, L. E., & Haard, N. F. (1993). *Analytical Biochemistry, 214*, 65–69.
26. Rao, M. S., Muñoz, J., & Stevens, W. F. (2000). *Applied Microbiology and Biotechnology, 54*, 808–813.
27. Kembhavi, A. A., Kulkarni, A., & Pant, A. (1993). *Applied Biochemistry and Biotechnology, 38*, 83–92.
28. Tsai, I. H., Chuano, K. L., & Chuang, J. L. (1986). *Comparative Biochemistry and Physiology. Part B: Biochemistry & Molecular Biology, 85*, 235–239.
29. El Hadj Ali, N., Hmidet, N., Bougatef, A., Nasri, R., & Nasri, M. (2009). *Journal of Agricultural and Food Chemistry, 57*, 10943–10950.
30. Erlanger, B. F., Kokowsky, N., & Cohen, W. (1961). *Archives of Biochemistry and Biophysics, 95*, 271–278.
31. Benjakul, S., Visessanguan, W., & Thummaratwasik, P. (2000). *Journal of Food Biochemistry, 24*, 107–127.
32. North, M. J. (1982). *Microbiology Review, 46*, 308–340.
33. Alencar, R. B., Biondi, M. M., Paiva, P. M. G., Vieira, V. L. A., Carvalho, L. B., Jr., Bezerra, R., et al. (2003). *Brazilian Journal of Food Technology, 6*, 279–284.
34. Espósito, T. S., Amaral, I. P. G., Marcuschi, M., Carvalho, L. B., Jr., & Bezerra, R. S. (2009). *Journal of Food Biochemistry, 33*, 821–834.
35. Mendes, C. M., Brito, M. A., Porto, T. S., Porto, A. L. F., Bezerra, R. S., Carvalho, L. B., Jr., et al. (2009). *Chemical Papers, 63*, 662–669.
36. Aranishi, F., Watanabe, T., Osatomi, K., Cao, M., Hara, K., & Ishihara, T. (1998). *Journal of Marine Biotechnology, 6*, 116–123.
37. Wang, Q., Gao, Z. X., Zhang, N., Shi, Y., Xie, X. L., & Chen, Q. X. (2010). *Journal of Agriculture and Food Chemistry, 58*, 655–659.
38. Gupta, R., Gupta, K., Saxena, R. K., & Khan, S. (1999). *Biotechnological Letters, 21*, 135–138.
39. Haddar, A., Bougatef, A., Agrebi, R., Sellami-Kamoun, A., & Nasri, M. (2009). *Process Biochemistry, 44*, 29–35.
40. Outtrup, H., Dambmann, C. & Aaslyng D.A. (1993). Patent number WO/1993/024623.
41. Outtrup, H., Dambmann, C., Christiansen, M. & Aaslyng, D.A. (1995). US patent number 5,466,594.
42. Boguslawski, G. & Shultz, J.W. (1992). US Patent number 5, 118,623.
43. Wang, S. L., Hsu, W. T., Liang, T. W., Yen, Y. H., & Wang, C. L. (2008). *Bioresource Technology, 99*, 5679–5686.
44. Wang, S. L., & Yeh, P. Y. (2006). *Process Biochemistry, 41*, 1545–1552.
45. Fang, Y., Liu, S., Wang, S., & Lv, M. (2009). *Biochemistry Engineering Journal, 43*, 212–215.
46. Jellouli, K., Bayoudh, A., Manni, L., Agrebi, R., & Nasri, M. (2008). *Applied Microbiology and Biotechnology, 79*, 989–999.
47. Volkin, D., Staubli, A., Langer, R., & Klibanov, A. (1991). *Biotechnology and Bioengineering, 37*, 843–853.
48. Gupta, A., Roy, I., Khare, S. K., & Gupta, M. N. (2005). *Journal of Chromatography A, 1069*, 155–165.
49. Ghorbel, B., Sellami-Kamoun, A., & Nasri, M. (2003). *Enzyme and Microbial Technology, 32*, 513–518.
50. Yang, J. K., Shih, I. L., Tzeng, Y. M., & Wang, S. L. (2000). *Enzyme and Microbial Technology, 26*, 406–413.
51. Jung, W. J., Jo, G. H., Kuk, J. H., Kim, Y. J., Oh, K. T., & Park, R. D. (2007). *Carbohydrate Polymers, 68*, 746–750.