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A Laundry Detergent-Stable Alkaline Trypsin from Striped Seabream (*Lithognathus mormyrus*) Viscera: Purification and Characterization

Nedra El Hadj Ali, Noomen Hmidet, Ali Bougatef,* Rim Nasri, and Moncef Nasri

Laboratoire de Génie Enzymatique et de Microbiologie, Ecole Nationale d'Ingénieurs de Sfax, B.P. "W", 3038 Sfax, Tunisia

An alkaline trypsin from the intestine of striped seabream (*Lithognathus mormyrus*) was purified and characterized. The enzyme was purified to homogeneity by precipitation with ammonium sulfate, Sephadex G-100 gel filtration and CM-Sephadex cation-exchange chromatography, with a 24.9-fold increase in specific activity and 13% recovery. The molecular weight of the purified alkaline trypsin was estimated to be 27.5 kDa by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and size exclusion chromatography. The purified trypsin appeared as a single band on native PAGE. Interestingly, the enzyme was highly active over a wide range of pH from 8.0 to 11.0, with an optimum at pH 10.0 using *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as a substrate. The relative activities at pH 8.0, 11.0, and 12.0 were 73%, 67% and 50.4%, respectively. The enzyme was extremely stable over a broad pH range (5.0−12.0). The optimum temperature for enzyme activity was 50 °C. The purified enzyme was strongly inhibited by soybean trypsin inhibitor (SBTI). In addition, the enzyme showed excellent stability toward various surfactants and bleache agents and compatibility with some commercial solid and liquid detergents. The trypsin kinetic constants, K_m and k_{cat} of the enzyme for BAPNA, were 0.29 mM and 1.36 s^{-1}, respectively, while the catalytic efficiency k_{cat}/K_m was 4.68 s^{-1} mM^{-1}.

KEYWORDS: Alkaline trypsin; purification; biochemical characterization; striped seabream; *Lithognathus mormyrus*; viscera

INTRODUCTION

Proteases constitute the most important group of industrial enzymes used in the world today, accounting for about 50% of the total industrial enzyme market (*1*). They have diverse applications in a wide variety of industries such as detergent, food, agrochemical and pharmaceutical industries (*2*, *3*). Proteases are mainly derived from animal, plant and microbial sources.

Today, there is an increasing demand for fish proteolytic enzymes in food processing. Viscera, one of the most important byproducts of the fishing industry, have wide biotechnological potential as a source of digestive enzymes, especially proteases that have high activity over a wide range of pH and temperature conditions (*4*, *5*) and exhibit high catalytic activity at relatively low concentration (*6*). These characteristics of fish proteases have made them suitable for different applications in many food-processing operations. Considering the specific characteristics of these enzymes, fish processing byproducts are currently used for enzyme extraction.

The most important digestive enzymes from fish and aquatic invertebrate viscera are the aspartic protease pepsin and the serine proteases, trypsin, chymotrypsin, collagenase and elastase. 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.

One of the main digestive proteinases detected in the pyloric ceca and intestine of fish is trypsin (EC 3.4.21.4). Trypsin is a member of a large family of serine proteinases which specifically cleave proteins and peptides at the carboxyl group of arginine and lysine residues and play the major roles in biological processes including digestion and activation of zymogens of chymotrypsin and other enzymes (*7*,*8*). Trypsins are digestive enzymes that have many biochemical and industrial applications due to their high specificity allowing a controlled proteolysis. Fish trypsins are of immense interest because they exhibit higher catalytic activity than their mammalian counterparts, making them more suitable for a number of biotechnological and food processing applications (*9*−*11*). Trypsins have been isolated and characterized thoroughly based on their physiochemical and enzymatic properties from several species of fish, e.g. grey triggerfish (*Balistes capriscus*) (*12*), cuttlefish hepatopancreas (*Sepia officinalis*) (*13*) and crayfish (*Astacus fluviatilis*) (*14*), the spleen of skipjack tuna (*Katsuwonus pelamis*) (*15*) yellowfin tuna (*Thunnus albacores*) (*16*); the pyloric ceca of Chinook salmon (*Oncorhynchus tshawytscha*) (*17*), tambaqui (*Colossoma macropomum*) (*18*), Monterey sardine (*Sardinops sagax caerulea*) (*19*)

*Corresponding author. Tel: 216 74-274-088. Fax: 216 74-275-595. E-mail: bougatefali@yahoo.fr.

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and the entire viscera of true sardine (*S. melanostictus*) (*20*), Japanese anchovy (*Engraulis japonica*) (*21*) and sardine (*Sardina pilchardus*) (*22*).

The striped seabream *Lithognathus mormyrus* is a marine fish belonging to the Sparidae family. It is a demersal species living in groups over various types of sea bottoms, especially rocky, sand and seagrass beds, at depths ranging from 0 to 150 m. This species is distributed in the eastern Atlantic and in the western Indian Ocean. It is also present in the Mediterranean, Black, Azov and Red seas (*23*). In Tunisia, striped seabream is utilized for human consumption. Similar to other fish species, deterioration is also very rapid in muscle and especially in the digestive tract, which suggests that striped seabream viscera might be a good source of enzymes. So far, no information regarding trypsins or trypsin-like enzymes from byproducts of *L. mormyrus* has been documented.

In the present study, we describe the purification of an alkaline trypsin from striped seabream (*L. mormyrus*), and provide basic information about its main biochemical and kinetic characteristics.

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 were 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 A (10 mM Tris-HCl, pH 8.0). The homogenate was centrifuged at 8500*g* for 30 min at 4 °C. The pellet was discarded, and the supernatant was collected and used as crude protease extract.

Enzyme Purification. *Ammonium Sulfate Precipitation*. The crude enzyme extract was first subjected to ammonium sulfate fractionation. Ammonium sulfate fractions of 0−30%, 30−60% and 60−80% (w/v) were collected by centrifugation at 10000*g*, and the precipitate obtained in each fraction was suspended in a minimal volume of buffer B (25 mM Tris-HCl, pH 8.0). The precipitates were dialyzed for 24 h at 4 °C against repeated changes in buffer B.

Sephadex G-100 Gel Filtration. The 30−60% (w/v) ammonium sulfate fraction was subjected to gel filtration on a Sephadex G-100 column (2.5 cm × 80 cm) pre-equilibrated with buffer C (25 mM Tris-HCl, pH 8.0 containing 0.5‰ Triton X-100). Fractions of 5 mL were eluted at a flow rate of 25 mL/h with the same buffer. Protein content (Abs 280 nm) and protease activity were determined. Fractions showing trypsin activity were pooled.

CM-Sephadex Cation Exchange Chromatography. The active fractions pooled from Sephadex G-100 (peak 2) were applied to a CM-Sephadex column (2 cm × 25 cm) pre-equilibrated with buffer B. After being washed with the same buffer, bound proteins were eluted with a linear gradient of sodium chloride in the range of 0−0.4 M in the equilibrating buffer. Fractions of 5 mL were collected at a flow rate of 80 mL/h and analyzed for trypsin activity and protein concentration. The fractions with high trypsin activity were pooled, concentrated by lyophilization and subsequently used for characterization.

All the purification steps were conducted at temperatures not exceeding 4 °C.

Trypsin Activity Assay. Amidase activity was measured according to the method of Erlanger et al. (*24*), modified by Benjakul et al. (*25*), using BAPNA as a substrate. Values are the means of three independent experiments.

Polyacrylamide Gel Electrophoresis. SDS−PAGE was carried out for the control of the purity and determination of molecular weight of the purified enzyme as described by Laemmli (*26*), using 5% (w/v) stacking and 15% (w/v) separating gels. The molecular weight of the enzyme was estimated using a low molecular weight calibration kit as markers, consisting of 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).

Native PAGE was performed according to the procedure of Laemmli (*26*), except that the sample was not heated and SDS and reducing agent were left out.

Detection of Trypsin Activity by Zymography. Zymography was performed on native PAGE according to the method of Garcia-Carreno et al. (*27*). Briefly, after electrophoresis, the gel was submerged in 1% (w/v) casein in 100 mM glycine−NaOH buffer, pH 10.0, and incubated at 50 °C for 20 min. After washing, the gel was stained with Coomassie Brilliant Blue R-250 for zymography analysis. Development of a clear zone on the blue background of the gel indicated the presence of protease activity.

Protein Determination. Protein concentration was determined by the method of Bradford (*28*), using bovine serum albumin as a standard, and during the course of enzyme purification by measuring the absorbance at 280 nm.

Biochemical Properties. *Effect of pH on Trypsin Activity and Stability*. Trypsin activity was assayed over the pH range of 5.0 to 13.0 at 50 °C for 10 min using BAPNA as a substrate. The effect of pH on enzyme stability was evaluated by measuring the residual enzyme activity after incubation at various pHs (pH 5.0−13.0) for 60 min at 25 °C. The following buffer systems were used: 100 mM sodium acetate buffer, 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; 100 mM KCl−NaOH buffer, pH 12.0−13.0.

Effect of Temperature on Trypsin Activity and Stability. To investigate the effect of temperature, trypsin activity was tested at different temperatures ranging from 30 to 70 °C, using BAPNA, as a substrate for 10 min at pH 10.0. For thermal stability, the enzyme was incubated at different temperatures for 60 min in the absence and presence of 5 mM $CaCl_2$. Aliquots were withdrawn at the desired time intervals to test the remaining activity at standard conditions. The nonheated enzyme was considered to be the control (100% activity).

Effects of Enzyme Inhibitors and Denaturing Reagents on Trypsin Activity. The effects of enzyme inhibitors on trypsin activity were studied using phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), β-mercaptoethanol and ethylene-diaminetetraacetic acid (EDTA). The purified enzyme was preincubated with each inhibitor for 30 min at 25 °C, and then the remaining enzyme activity was tested using BAPNA 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 purified trypsin 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%.

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

Kinetic Studies. The activity of the purified trypsin was evaluated at 25 °C with different final concentrations of BAPNA, ranging from 0 to 1000 μM. The determinations were repeated twice, and the respective kinetic parameters, including the apparent Michaelis−Menten constant (K_m) and the maximum velocity (V_{max}), were calculated from Lineweaver−Burk plots (*29*).

The value of the turnover number (k_{cat}) was calculated from the equation $k_{cat} = V_{max}/[E]$, where [E] is the active enzyme concentration.

Detergent Compatibility. The compatibility of the purified alkaline trypsin with commercial solid laundry detergents was studied using Dixan (Henkel, Spain), Nadhif (Henkel-Alki, Tunisia), Ariel (Procter and 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 compatibility of the purified enzyme with commercial liquid detergents

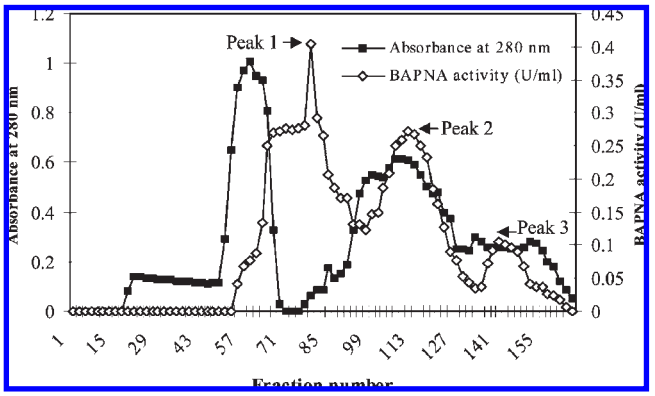

Figure 1. Purification profile of *L. mormyrus* trypsin by gel filtration on a Sephadex G-100 column. The enzyme preparation (30−60% (w/v) saturation with ammonium sulfate) was applied to a 2.5 cm × 80 cm column, equilibrated and eluted with buffer C at a flow rate of 25 mL/h. Fractions (5 mL each) collected from the column were assayed for proteins content at 280 nm and trypsin activity.

was also investigated using Dixan (Henkel, Spain), Nadhif (Henkel-Alki, Tunisia) and Lav^{+} (Best LAV, Tunisia). The liquid detergents were diluted 100-fold in tap water (1/100) 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 purified alkaline trypsin was incubated with different detergents for 1 h at 30 °C, and then the remaining activities were determined under the standard assay conditions. The enzyme activity of a control, without detergent, incubated under the similar conditions, was taken as 100%.

RESULTS AND DISCUSSION

Characterization of the Alkaline Enzyme Extract. A preliminary study on the characterization of the alkaline protease extract from the viscera of striped seabream was carried out. The optimal pH and temperature for proteolytic activity were pH 10.0 and 50 °C, respectively, using casein as a substrate. In order to estimate the number of proteases in the alkaline enzyme extract, the sample was separated by SDS−PAGE and then the activity was revealed by zymogram. The crude enzyme extract showed three clear bands on casein zymography indicating the presence of at least three major proteases (result not shown).

Purification of the *L. mormyrus* Trypsin. Trypsin from the viscera of *L. mormyrus* was purified successively by the three-step procedure described in Materials and Methods. In the first step, the crude enzyme extract was fractionated with ammonium sulfate. The fraction F2 (30−60% w/v saturation) showed higher specific activity (1.308 U/mg of protein) than fraction F1 (0−30%; 0.121 U/mg of protein) and fraction F3 (60−80%; 0.106 U/mg of protein). No activity was detected in the final supernatant. The 30−60% ammonium sulfate precipitate, which gave the highest specific activity, was then subjected to Sephadex G-100 gel filtration column. This procedure yielded at least three peaks of protease activity (**Figure 1**). Active fractions of peak 2 were pooled and then loaded on CM-Sephadex column that had been equilibrated with buffer B. Binding proteins were eluted with a linear gradient of NaCl (0−0.4 M). Protease activity appeared in a single peak together with adsorbed fractions (**Figure 2**). After the final purification step, the enzyme was purified 24.9-fold with a recovery of 13% and a specific activity of 12.01 U/mg protein, using BAPNA as a substrate. The results of the purification procedure are summarized in **Table 1**.

Purity and Molecular Weight. The purified enzyme gave a single band on SDS−PAGE, and its molecular weight was estimated to

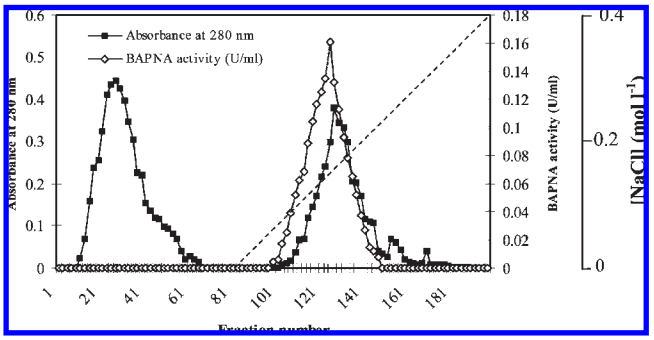

Figure 2. Elution profile of *L. mormyrus* trypsin from a CM-Sephadex column. Active fractions from gel filtration G-100 (peak 2) were collected and then applied to a CM-Sephadex column (2 cm × 25 cm), equilibrated with buffer B. The enzyme was eluted with a linear gradient of NaCl (0−0.4 M) in buffer B at a flow rate of 80 mL/h.

Table 1. A Summary of the Purification of Trypsin from *L. mormyrus*[a]

purification steps	total activity (U)	total protein (mg)	sp act. (U/mg)	recovery (%)	purification fold
crude enzyme	157.25	326	0.482	100	1
ammonium sulfate precipitation (30−60% w/v)	112.5	86	1.308	71	2.93
Sephadex G-100	28.12	3.45	8.152	18	16.9
CM-Sephadex	20.43	1.7	12.01	13	24.9

[a] All operations were carried out at 4 °C.

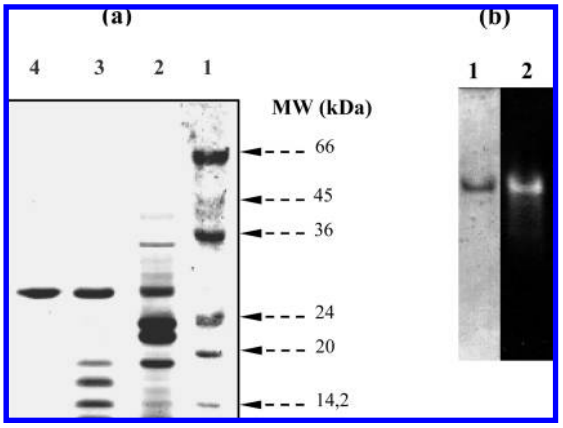

Figure 3. (**a**) SDS−PAGE of the purified trypsin from striped seabream. Lane 1: standard proteins marker of different molecular weights. Lane 2: crude enzyme extract. Lane 3: Sephadex G-100. Lane 4: purified trypsin. (**b**) Native PAGE (lane 1) and zymogram detection of proteolytic activity (lane 2) of the purified trypsin from *L. mormyrus* viscera.

be 27.5 kDa, corresponding to that determined by gel filtration. Fish trypsins have been reported to have molecular weights in the range of 23 to 28 kDa. The molecular weight of *L. mormyrus* trypsin was similar to those from other fish species, such as bluefish (*Pomatomus saltatrix*) (*7*) and carp hepatopancreas (*8*), and was higher than those of trypsins from grey triggerfish (*B. capriscus*) (*12*), *S. officinalis* (*13*), skipjack tuna (*K. pelamis*) (*15*), yellowfin tuna (*T. albacores*) (*16*), sardine (*S. pilchardus*) (*22*), and jacopever (*Sebastes schlegelii*) and elkhorn sculpin (*Alcichthys alcicornis*) (*30*),

The purity of the purified trypsin was also evaluated by using native gel electrophoresis. As shown in **Figure 3b**, *L. mormyrus* trypsin migrated as a single protein band in the native PAGE,

Table 2. Effects of Various Enzyme Inhibitors and Metal Ions on the Activity of the Purified Trypsin from *L. mormyrus*[a]

chemicals	concn	relative activity (%)
none		100
PMSF	1 mM	73
	5 mM	34
β-mercaptoethanol	5 mM	100
EDTA	1 mM	38
	5 mM	34
SBTI	1 mg/mL	0
Ca^{2+}	5 mM	114
Ba^{2+}	5 mM	60
Zn^{2+}	5 mM	117
Cu^{2+}	5 mM	110
Mg^{2+}	5 mM	100
Mn^{2+}	5 mM	100
Hg^{2+}	5 mM	43
K^{+}	5 mM	100
Na^{+}	5 mM	100

[a] Purified 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 using BAPNA as a substrate. Enzyme activity measured in the absence of any inhibitor was taken as 100%. The effects of metal ions on the activity of the purified trypsin were determined by incubating the enzyme in the presence of various metal ions for 10 min at 50 °C and pH 10.0.

confirming the homogeneity of the enzyme. The proteolytic activity of this protein band was confirmed by zymogram activity staining. A unique clear band of casein hydrolysis was observed in the gel, indicating the homogeneity of the purified trypsin (**Figure 3b**).

Effects of Enzyme Inhibitors on Trypsin Activity. Proteases can be classified by their sensitivity to various inhibitors (*31*). In order to determine the nature of the purified protease, the effects of different enzyme inhibitors, such as chelating agent and specific group reagents on the protease activity were investigated (**Table 2**).

Protease from *L. mormyrus* was strongly inhibited by the well-known trypsin inhibitor investigated, namely, SBTI (1 mg/mL). Further, the enzyme activity was highly affected by PMSF, a serine protease inhibitor. The enzyme retained 73 and 34% of its initial activity after 1 h incubation in the presence of 1 and 5 mM PMSF, respectively. These results indicate that the *L. mormyrus* enzyme is a serine-protease and belongs to the trypsin family. On the other hand, β-mercaptoethanol was without influence on the activity of the purified trypsin. However, metalloprotease inhibitor EDTA (5 mM) inhibited the enzyme activity by 66%, indicating the importance of Ca^{2+} in enzyme stabilization. Similar results were observed with trypsins from other fish species such as skipjack tuna spleen (*K. pelamis*) (*15*), yellowfin tuna (*T. albacores*) (*16*), and sardine (*S. pilchardus*) (*22*).

Effect of pH on the Activity and Stability of *L. mormyrus* Trypsin. The pH activity profile of the purified striped seabream trypsin is shown in **Figure 4a**. The purified enzyme was highly active between pH 7.0 and 12.0, with an optimum between pH 9.0 and 10.5. The relative activities at pH 7.0, 9.0, and 12.0 were about 66%, 96% and 51.5%, respectively, of that at pH 10.0. The optimum pH of *L. mormyrus* trypsin was higher than those of most described trypsins, which showed maximum activity at pH 8.0−9.0. However, the optimum pH was lower than that of *B. capriscus* trypsin which exhibited maximum activity at pH 10.5 and retained about 91% activity at pH 11.0 (*12*).

The pH stability of trypsin from *L. mormyrus* is shown in **Figure 4b**. The enzyme is highly stable over a wide broad pH range, maintaining more than 90% of its original activity between pH 5.0 and 12.0 (**Figure 4b**). The pH stability of *L. mormyrus* protease is higher than monterey sardine and sardinelle

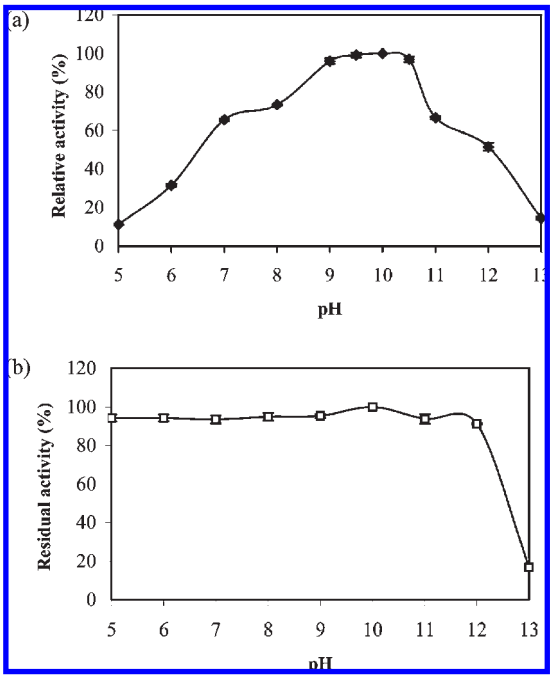

Figure 4. pH profile (**a**) and pH stability (**b**) of the purified trypsin from viscera of the striped seabream (*L. mormyrus*). Trypsin activity was assayed in the pH range from 5.0 to 13.0 at 50 °C. The maximum activity obtained at pH 10.0 was considered as 100% activity. The pH stability was determined 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 BAPNA 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.

(*Sardinella aurita*) trypsins, which were stable in the pH range of 7.0−9.0 and 8.0−9.0, respectively (*19*, *32*). These results suggest that the viscera of *L. mormyrus* would be a potential source of trypsin for certain food processing operations that require high alkaline conditions.

Effect of Temperature on the Activity and Stability of *L. mormyrus* Trypsin. Temperature profile of trypsin from *L. mormyrus* is depicted in **Figure 5a**. The enzyme was active at temperatures from 30 to 60 °C with an optimum around 50 °C. The relative activities at 30 and 60 °C were about 48% and 35.4%, respectively, of that at 50 °C. The optimal temperature of *L. mormyrus* trypsin was similar to those from walleye pollock (*Theragra chalcogramma*) (*33*) and jacopever (*S. schlegelii*) (*30*), higher than those of trypsins from cold-water fish, which had optimal temperatures in the range of 40−45 °C (*11*, *34*) and lower than that of cuttlefish trypsin (*13*).

The thermal stability profile of the purified trypsin showed that the enzyme is highly stable at temperatures below 40 °C but was inactivated at higher temperatures (**Figure 5b**). The thermostability of the purified enzyme was also examined by incubating the enzyme at 50 °C in the presence of 5 mM $CaCl_2$. As shown in **Figure 5b**, the stability of the enzyme was considerably enhanced by the addition of $CaCl_2$, and the enzyme retained 89% of its initial activity after 60 min incubation at 50 °C in the presence of 5 mM $CaCl_2$; however, in the absence of $CaCl_2$, the enzyme retained only 14.5% of its initial activity. These results indicated that trypsin from *L. mormyrus* was most likely stabilized by calcium ion. The presence of calcium ions activates trypsinogen to trypsin and increases the thermal stability of the enzyme. This stabilizing effect is accomplished by a conformational change in the trypsin molecule, resulting in a more compact structure

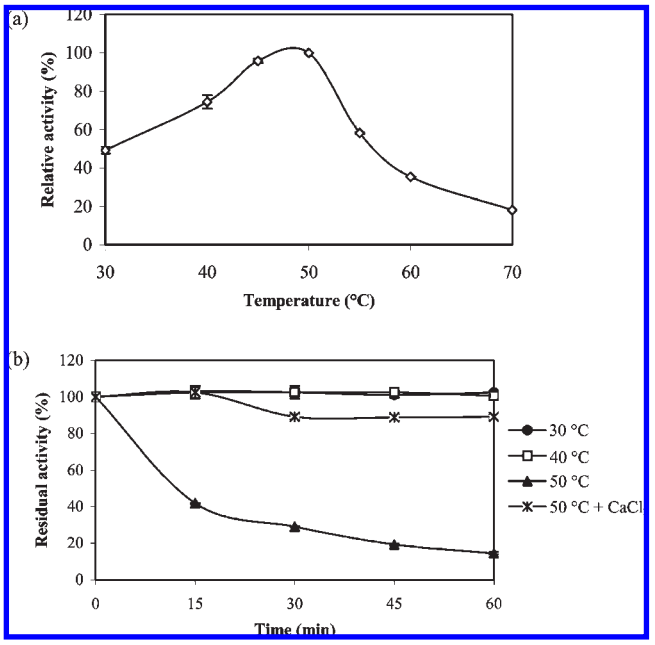

Figure 5. Temperature profile (**a**) and thermal stability (**b**) of the purified trypsin from the intestine of the striped seabream (*L. mormyrus*). Enzyme activity was assayed at different temperatures ranging from 30 to 70 °C at pH 10.0, using BAPNA as a substrate. The activity of the enzyme at 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 nonheated enzyme was considered as control (100%).

(*35*, *36*). Stabilization against thermal inactivation by calcium was also reported for the trypsins from true sardine (*20*), eel (*37*) and rainbow trout (*Oncorhynchus mykiss*) (*38*). Nevertheless, calcium ion did not enhance the stability of trypsins from sardine (*39*), Arctic fish capelin (*Mallotus villosus*) (*40*) and Nile tilapia (*Oreochromis niloticus*) (*41*). These findings suggest a difference in the structure of the primary calcium binding site among different marine fish trypsins.

Effects of Metal Ions. The effects of some metal ions, at a concentration of 5 mM, on the activity of *L. mormyrus* trypsin were studied at pH 10.0 and 50 °C by the addition of metal ions to the reaction mixture. As shown in **Table 2**, Na^+, K^+, Mg^{2+} and Mn^{2+} did not affect trypsin activity, whereas Ca^{2+}, Cu^{2+} and Zn^{2+} increased the protease activity to 114%, 110% and 117%, respectively. However, Ba^{2+} and Hg^{2+} affect greatly the enzyme activity, with more than 40% inhibition. Similar results regarding the effect of calcium on trypsins were identified in common carp (*8*), mandarin fish (*Siniperca chuatsi*) (*34*), true sardine (*Sardinops melanostictus*) and arabesque greenling (*Pleuroprammus azonus*) (*20*), tuna (*36*). It is known that calcium ions promote the formation of active trypsin from trypsinogen and stabilize trypsin against autolysis (*42*). Bode and Schwager (*43*) reported that calcium not only protected trypsin against self-digestion but also slightly increased its proteolytic activity.

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 (*44*, *45*). 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 detergent ingredients (*46*). Important commercial detergent

Table 3. Stability of *L. mormyrus* in the Presence of Various Surfactants and Bleaches[a]

tensioactives/oxidizing agents	concn	residual activity (%)
none		100
SDS	0.1 (w/v)	66
	0.5 (w/v)	23.5
Triton X-100	5 (v/v)	125.8
Tween 20	5 (v/v)	100
Tween 80	5 (v/v)	100
sodium perborate	0.2 (w/v)	72.5
	1 (w/v)	40

[a] The enzyme was incubated with different surfactants and oxidizing agents for 1 h at 30 °C, and then the remaining activity was measured under standard conditions. The activity is expressed as a percentage of the activity level in the absence of additives.

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 (*45*). Thus it is desirable to search for new proteases with novel properties from many different sources as possible.

The suitability of the *L. mormyrus* trypsin as a detergent additive was determined by testing the stability in oxidants and surfactants. As shown in **Table 3**, the alkaline trypsin is stable in the presence of the nonionic surfactants like Tween 20 and Tween 80 and activity was increased in the presence of Triton X-100. However, the *L. mormyrus* trypsin was less stable against the strong anionic surfactant (SDS) and retained 66% and 23.5% of its activity in the presence of 0.1% and 0.5% SDS, respectively.

Interestingly, *L. mormyrus* trypsin activity was little influenced by oxidizing agent, and retained about 72% and 40% of its activity after incubation for 1 h at 30 °C in the presence of 0.2% and 1% sodium perborate, respectively. The relative stability of the enzyme in the presence of oxidizing agents is a very important characteristic for its eventual use in detergent formulations. The stability of *L. mormyrus* trypsin against sodium perborate was higher than A21 protease from *Bacillus mojavensis* which retained 35% of its initial activity in the presence of 1% oxidizing agent after incubation for 1 h at 30 °C (*46*), and lower than grey triggerfish trypsin which retained 87% of its activity after incubation for 1 h at 40 °C in the presence of 1% sodium perborate (*12*).

Stability of the Alkaline Trypsin with Commercial Liquid and Solid Detergents. Alkaline proteases added to laundry liquid and solid detergents play a catalytic role in the hydrolysis of protein stains such as blood, milk, etc. The high activity and stability of the purified alkaline trypsin in the pH range from 8.0 to 12.0 and its relative stability toward surfactants and oxidizing agents are very useful for its eventual application as detergent additive. To check the compatibility of the alkaline trypsin with liquid and solid detergents, the purified enzyme was preincubated in the presence of various commercial laundry detergents for 1 h at 30 °C. The data presented in **Figure 6a** show that the alkaline protease is extremely stable in the presence of all liquid detergents tested. The enzyme retained 100% of its activity in the presence of Dixan and Nadhif, and 91.5% in Lav^+ after 1 h incubation at 30 °C. A similar result was observed by Haddar et al. (*46*) where both BM1 and BM2 proteases from *B. mojavensis* A21 showed excellent stability with a wide range of commercial liquid detergents when incubated under the same conditions.

The data presented in **Figure 6b** show that the enzyme is also stable in the presence of solid detergents, retaining 87.5%, 84%, 77%, 73.4% and 69% of its initial activity after 1 h incubation at 30 °C in the presence of Axion, New Det, Dixan, Ariel and

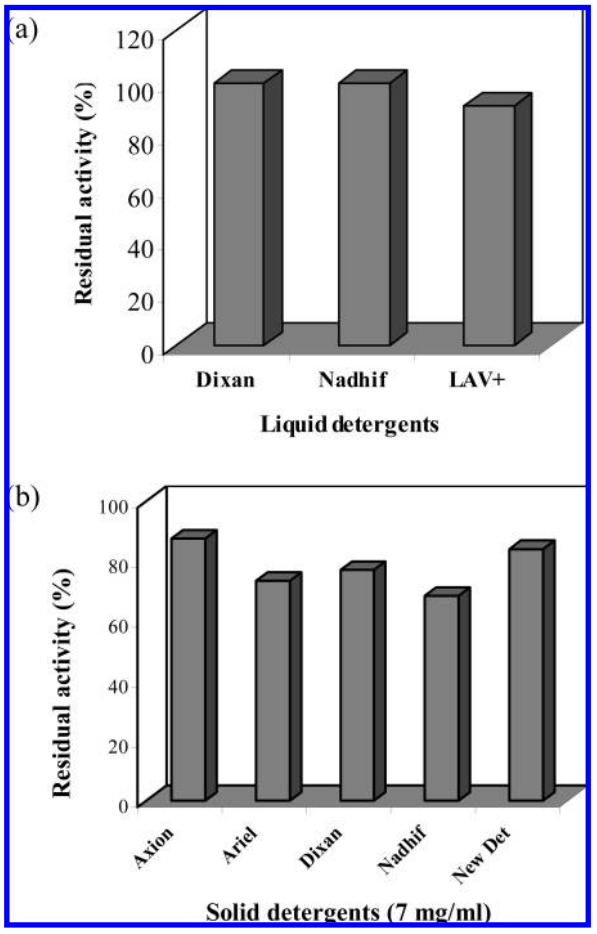

Figure 6. Stability of trypsin from *L. mormyrus* in the presence of various commercial laundry detergents. The enzyme at 150 U/mL was incubated 1 h at 30 °C and pH 10.0 in the presence of (**a**) liquid detergents diluted 100-fold in tap water (1/100) and (**b**) solid detergents at a final concentration of 7 mg/mL, and the remaining activities were determined at pH 10.0 and 50 °C using casein as a substrate. Enzyme activity of control sample without any detergent, incubated under the similar conditions, was taken as 100%.

Nadhif, respectively. These findings were similar to those for Grey triggerfish trypsin (*B. capriscus*) (*12*). Espósito et al. (*47*) reported also the stability of tambaqui proteases (*Colossoma macropomum*) in the presence of several commercial detergents.

Since the proteolytic activity varied with each laundry detergent, the obtained results clearly indicated that the performance of enzymes in detergents depends on a number of factors, including the detergents' compounds.

Kinetic Properties. Kinetic constants K_m and k_{cat} of the purified *L. mormyrus* trypsin were determined using Lineweaver−Burk plots (*29*) (**Table 4**). The K_m and k_{cat} of the purified enzyme using BAPNA were 0.29 mM and 1.36 s^{-1}, respectively, and were close to those reported for trypsins from bigeye snapper (*Priacanthus macracanthus*) (*48*) and anchovy (*E. encrasicholus*) A (*49*). The catalytic efficiency (k_{cat}/K_m) of *L. mormyrus* trypsin, 4.68 s^{-1} mM^{-1}, was close to those of trypsins from anchovy (*E. encrasicholus*) (*49*) and bigeye snapper (*Priacanthus macracanthus*) (*48*).

Conclusion. A novel trypsin active and stable over a broad pH range was purified to apparent homogeneity from *L. mormyrus*. The purification to homogeneity of the protease was achieved by ammonium sulfate precipitation (30−60%), gel filtration through Sephadex G-100 and CM-Sephadex column. After the final purification step, the enzyme was purified 24.9-fold with a specific activity of 12.017 U/mg and 13% recovery. The purified protease was homogeneous on SDS−PAGE and its molecular weight was estimated to be 27.5 kDa. The optimal pH and temperature for enzyme activity were pH 10.0 and 50 °C, respectively. Interestingly, the trypsin was highly stable at high pH (10.0−12.0). *L. mormyrus* trypsin was more active and stable at alkaline pH than the most described trypsins. In addition, the *L. mormyrus* trypsin showed excellent stability and compatibility with some commercial liquid and solid detergents tested. The enzyme retained between 91.5% and 100% of its initial activity with liquid detergents, and 69% and 87.5% with solid detergents, even after 1 h incubation at 30 °C.

The obtained results suggest that the viscera of *L. mormyrus* would be a potential source of trypsin for certain food processing operations that require high alkaline conditions. In addition, considering the high activity and stability in high alkaline pH, relative stability in the presence of surfactants and oxidizing agents, *L. mormyrus* trypsin may find application in laundry detergents. Further studies will be necessary to improve the stability of *L. mormyrus* trypsin.

Table 4. Kinetic Constants of *L. mormyrus* and Other Trypsins

trypsins	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	refs
striped seabream (*L. mormyrus*)[a]	0.29	1.36	4.68	this study
grey triggerfish (*B. capriscus*)[a]	0.068	2.76	40.58	*12*
cuttlefish (*S. officinalis*)[a]	0.064	2.32	36.25	*13*
sardinelle (*S. aurita*)[a]	1.67	3.87	2.31	*32*
anchovy (*E. japonica*)[a]	0.049	1.55	31.0	*50*
salmon (*O. keta*)[a]	0.029	2.29	79.0	*51*
carp (*C. carpio*)[a]	0.039	3.10	79.5	*52*
bigeye snapper (*Priacanthus macracanthus*)	0.312	1.06	3.4	*48*
anchovy (*E. encrasicholus*) A[a]	0.830	1.55	1.86	*49*
anchovy (*E. encrasicholus*) B[a]	0.660	3.2	4.84	*49*
Monterey sardine (*S. sagax caerula*)[a]	0.051	2.12	41.0	*19*

[a] Substrate: *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA).

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