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Alkaline Chymotrypsin from Striped Seabream (*Lithognathus mormyrus*) Viscera: Purification and Characterization

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An alkaline chymotrypsin from the intestine of striped seabream (*Lithognathus mormyrus*) was purified by precipitation with ammonium sulfate, Sephadex G-100 gel filtration, Mono Q-Sepharose anion-exchange chromatography, ultrafiltration, second Sephadex G-100 gel filtration, and a second Mono Q-Sepharose anion-exchange chromatography with a 80-fold increase in specific activity. The molecular weight of the purified alkaline chymotrypsin was estimated to be 27 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography. The enzyme was highly active over a wide range of pH from 7.0 to 12.0, with an optimum at pH 10.0−11.0 using succinyl-L-ala-ala-pro-L-phenylalanine-*p*-nitroanilide (SAAPNA) as a substrate. The relative activities at pH 7.0 and 12.0 were about 66% and 45.5%, respectively. Further, the enzyme was extremely stable over a broad pH range (6.0−12.0). The optimum temperature for enzyme activity was 50 °C, and the enzyme displayed higher enzyme activity at low temperatures when compared to other enzymes. The purified enzyme was strongly inhibited by soybean trypsin inhibitor (SBTI) and phenylmethylsulfonyl-fluoride (PMSF), a serine protein inhibitor, and *N*-toluenesulfonyl_L_lysine chloromethyl ketone (TLCK), a chymotrypsin specific inhibitor. The N-terminal amino acid sequence of the first nine amino acids was IVNGEEAVP. The chymotrypsin kinetic constants, K_m and k_{cat} on SAAPNA as a substrate, were 30.7 μM and 14.35 s^{-1}, respectively, while the catalytic efficiency k_{cat}/K_m was 0.465 μM^{-1} s^{-1}. The high activity at high alkaline pH and low temperatures make this protease a potential candidate for future use in detergent processing industries.

KEYWORDS: Alkaline chymotrypsin; purification; biochemical characterization; striped seabream; viscera

INTRODUCTION

Today, there is an increasing demand for fish proteolytic enzymes in food processing. Viscera, one of the most important by-product of fishing industry, have wide biotechnological potential as a source of digestive enzymes, especially proteases having high activity over a wide range of pH and temperature conditions (*1*, *2*).

Various digestive proteolytic enzymes have been isolated from the internal organs of fish. The most important digestive enzymes from fish and aquatic invertebrates' viscera are the aspartic acid pepsin and the serine protease trypsin. Acid proteases from fish stomachs display high activity between pH 2.0 and 4.0 (*3*−*5*), while alkaline digestive proteases, such as trypsin, are most active between pH 8.0 and 10.0 (*6*).

Among the digestive enzymes, trypsin and trypsin-like proteases have received much interest and are well documented (*7*−*10*). However, information about chymotrypsins are less available (*11*).

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The common feature of these enzymes is that they preferentially cleave polypeptide chains at the carboxyl sides of bulky hydrophobic amino acid residues such as phenylalanine, tyrosine, tryptophan, and leucine residues and also synthetic substrates, such as SAAPNA and BTEE (benzoyl-L-tyrosine-ethyl-ester). Fish chymotrypsins have molecular weights in the range of 22−30 kDa and display optimum activity between pH 7.5 and 9.0 and 45 and 55 °C (*12*).

Chymotrypsins have been isolated and characterized from several species of fish, including dogfish (*Squalus acanthias*) (*13*, *14*), carp (*Cyprinus carpio*) (*15*, *16*), Atlantic cod (*Gadus morhua*) (*17*, *18*), rainbow trout (*Oncorhynchus mykiss*) (*11*), and anchovy (*Engraulis japonica*) (*5*). These studies revealed that fish chymotrypsins have similar molecular weight to bovine and porcine chymotrypsins. However, some important differences have been detected in fish chymotrypsin, including higher catalytic activity, lower thermostability, and differences in polypeptide amino acid composition (*14*, *15*, *19*).

Chymotrypsins from higher vertebrates exist in three different isotype forms A, B, and C. The activated A and B enzymes consist of three polypeptides chains interconnected by disulfides bridges,

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while the C isotype consists of two polypeptide chains (*20*). However, active fish chymotrypsin consists of a single-chain monomer (*16*, *18*, *21*).

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 (*22*).

In Tunisia, striped seabream (*Lithognathus mormyrus*) catches were about 637 T in 2008 and it has been exploited for human consumption. Similar to other fish species, deterioration is also very rapid in muscle and especially in the digestive tract, suggesting that striped seabream viscera might be a good source of digestive enzymes. So far, no information regarding chymotrypsin or chymotrypsin-like enzymes from *L. mormyrus* has been documented.

In a previous study, we reported the purification and characterization of a novel trypsin which is highly active over a wide range of pH from 8.0 to 11.0 with an optimum at pH 10.0 using BAPNA as a substrate (*10*). In the present study, we describe the purification of an alkaline chymotrypsin, with high activity at low temperatures, from striped seabream (*L. mormyrus*) and provide basic information about its main biochemical and kinetic characteristics.

MATERIALS AND METHODS

Reagents. TLCK, ethylenediaminetetra-acetic acid (EDTA), PMSF, benzoyl-L-tyrosine ethyl-ester (BTEE), succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (SAAPNA), *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), glycine, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO).

SBTI was obtained from Fluka Biochemica (USA). Sodium dodecyl sulfate (SDS), acrylamide, ammonium persulphate, *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (TEMED) and Coomassie Brilliant Blue R-250 were from Bio-Rad Laboratories (Mexico). Sephadex G-100 and Mono Q-Sepharose were from Pharmacia Biotech (Uppsala, Sweden). Tris (hydroxymethyl) aminomethane was procured from Panreac Quimica SA (Spain). All other reagents were of analytical grade.

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 the sample/ice ratio of approximately 1:3 (w/w), and transported to the research laboratory within 30 min. Fish was washed with water, and viscera were separated and then used immediately for the extraction of digestives enzymes.

Preparation of Crude Alkaline Protease Extract. Viscera from *L. mormyrus* (100 g) were rinsed with distilled water, and then homogenized for 60 s with 200 mL of extraction buffer A (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 the crude protease extract.

Enzyme Purification. The crude protease extract was first subjected to ammonium sulfate fractionation. 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 4 mL of buffer B (25 mM Tris-HCl, pH 8.0). The precipitates were dialyzed for 24 h at 4 °C against repeated changes in the same buffer. The 30−60% (w/v) fraction was then 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 each were collected with the same buffer at a flow rate of 25 mL/h and analyzed for protein content and chymotrypsin activity. Active fractions were pooled and then applied to a Mono Q-Sepharose column (2 cm ×10 cm) pre-equilibrated with buffer B. The column was washed with the same buffer, and then bound proteins were eluted with a linear gradient of sodium chloride (0−0.4 M). Fractions of 5 mL were collected at a flow rate of 80 mL/h and analyzed for chymotrypsin activity and protein concentration. Fractions with high chymotrypsin activity were pooled, concentrated by ultrafiltration using a 10 kDa membrane, and then fractionated on a second Sephadex G-100 gel filtration as described previously. Fractions (103−117) showing chymotrypsin activity were pooled and then applied to a second Mono Q-Sepharose column (2 cm × 10 cm) pre-equilibrated with buffer B and eluted as described previously. The fractions with high chymotrypsin activity were pooled and stored at −20 °C for further analysis. All the purification steps were conducted at temperatures not exceeding 4 °C.

Chymotrypsin Activity Assay. Amidase activity was evaluated according to Tsai et al. (*23*) with slight modifications, using SAAPNA as a substrate. The reaction mixture contained 10 μL of enzyme solution suitably diluted and 990 μL of 0.1 mM SAAPNA in 50 mM glycine−NaOH pH 10.0. The assays were carried out for 10 min at 50 °C. Chymotrypsin activity was measured by the absorbance at 410 nm due to *p*-nitroaniline released. Amidase activity was calculated with the following equation: activity (U/mL) = $[A_{(410)}/\text{min} \times 1000 \times 1]/8800$, where 8800 = *p*-nitroaniline molar extinction coefficient in $M^{-1}\,cm^{-1}$, 1000 is the factor to convert M to μmoles in 1 mL, which is the volume of the reaction mixture.

Esterase activity was evaluated according to Hummel (*24*), using BTEE as a substrate. A 20 μL of enzyme solution was mixed with 980 μL BTEE (1 mM) in 50 mM Tris−HCl buffer pH 8.0 and incubated at 25 °C. The increase in absorbance at 256 nm was measured every 30 s for 10 min. Esterase activity was calculated with the following equation: $[A_{(256)}/\text{time} \times 1000 \times 1]/964$, where 964 = benzoyl-tyrosine molar extinction coefficient in $M^{-1}\,cm^{-1}$, and 1 is the volume of the reaction mixture.

Values are the means of three independent experiments.

Polyacrylamide Gel Electrophoresis and Detection of Protease Activity by Zymography. SDS-PAGE was carried out as described by Laemmli (*25*), using a 5% (w/v) stacking and a 15% (w/v) separating gels. The molecular weight of the enzyme was estimated using a low molecular weight calibration kit as markers (Pharmacia): phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Zymography, a sensitive and rapid assay method for analyzing protease activity, was performed according to the method of Garcia-Carreno et al. (*26*). 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 buffer (glycine−NaOH, pH 10.0). The gel was then incubated with 1% (w/v) casein in 100 mM glycine buffer (glycine−NaOH, pH 10.0) for 40 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.

Determination of the N-Terminal Amino Acid Sequence of *L. mormyrus* Chymotrypsin. The purified enzyme was transferred from SDS-PAGE to a polyvinylidene difluoride membrane (PVDF). The PVDF band was excised and the N-terminal amino acid sequence was determined by the Edman degradation method on an ABI Procise 494 protein sequencer (Applied Biosystems).

Protein Determination. Protein concentration was determined by the method of Bradford (*27*), using bovine serum albumin as standard, and during the course of enzyme purification by measuring the absorbance at 280 nm.

Biochemical Properties. *Effect of pH and Temperature on Chymotrypsin Activity and Stability*. Chymotrypsin activity was assayed over the pH range of 5.0−12.0 at 50 °C, using SAAPNA as a substrate. The effect of pH on enzyme stability was evaluated by measuring the residual enzyme activity after incubation at various pHs (pH 5.0−12.0) 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.

To investigate the effect of temperature, chymotrypsin activity was tested at different temperatures ranging from 20 to 70 °C at pH 10.0 using SAAPNA as a substrate. For thermal stability, the enzyme was incubated at different temperatures for 60 min. Aliquots were withdrawn at the desired

time intervals to test the remaining activity under standard conditions. The nonheated enzyme was considered as control (100% activity).

Effects of Enzyme Inhibitors and Metal Ions on Chymotrypsin Activity. The effects of enzyme inhibitors on chymotrypsin activity were studied using PMSF, SBTI, TLCK, β-mercaptoethanol, and EDTA at final concentration of 5 mM. The purified enzyme was preincubated with each inhibitor for 30 min at 25 °C, and then the remaining enzyme activity was tested using SAAPNA as a substrate. The activity of the enzyme assayed in the absence of inhibitors was taken as control.

The effects of various metal ions (5 mM) on chymotrypsin activity were investigated, 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.

Kinetic Studies. The activity of the purified chymotrypsin was evaluated at 25 °C with different final concentrations of SAAPNA, ranging from 0 to 100 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 (*28*).

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

RESULTS AND DISCUSSION

Purification of *L. mormyrus* Chymotrypsin. Chymotrypsin from the viscera of *L. mormyrus* was purified successively by the five-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) which gave the highest specific activity (12 U/mg protein) was successively subjected to Sephadex G-100 gel filtration, Mono Q-Sepharose anion-exchange chromatography, a second Sephadex G-100 gel filtration, and finally to a second Mono Q-Sepharose anion-exchange chromatography. The elution profiles of the chymotrypsin activity and proteins of the final step are shown in **Figure.1**. The enzyme was purified 80-fold with a recovery of 2% and a specific activity of 289.5 U/mg protein, using SAAPNA as a substrate. The results of the purification procedure are summarized in **Table 1**.

The purified enzyme was analyzed by SDS-PAGE under reducing conditions. As shown in **Figure 2a**, the purified enzyme gave a single band, and its molecular weight was estimated to be 27 kDa, corresponding to that determined by gel filtration indicating that the active enzyme is a single-chain monomer. The molecular weight of the purified *L. mormyrus* chymotrypsin was close to those from other fish species such as cod (*18*), grass carp (*29*), crucian carp (*21*), Atlantic cod (*17*), rainbow trout (*11*), anchovy (*12*), Monterey sardine (*30*), and carp (*15*).

L. mormyrus chymotrypsin migrated as a single protein band in the native PAGE, confirming the homogeneity of the enzyme (result not shown). The obtained result showed that there is one dominant isoform of chymotrypsin in the viscera of *L. mormyrus*. However, Yang et al. (*21*) reported the presence of two chymotrypsins from crucian carp. The proteolytic activity of this protein band was confirmed by zymogram activity staining. As shown in **Figure 2b**, a unique clear band of casein hydrolysis was observed in the gel, indicating the homogeneity of the purified chymotrypsin, whereas the crude enzyme extract showed three clear bands indicating the presence of at least three proteases.

N-Terminal Amino Acid Sequence of *L. mormyrus* Chymotrypsin. To further characterize the *L. mormyrus* chymotrypsin, we investigate its N-terminal amino acid sequence. The N-terminal sequence of the purified chymotrypsin determined by automated Edman degradation was found to be IVNGEEAVP. The N-terminal amino acid sequence of *L. mormyrus* chymotrypsin showed uniformity, indicating that it was isolated in a pure form.

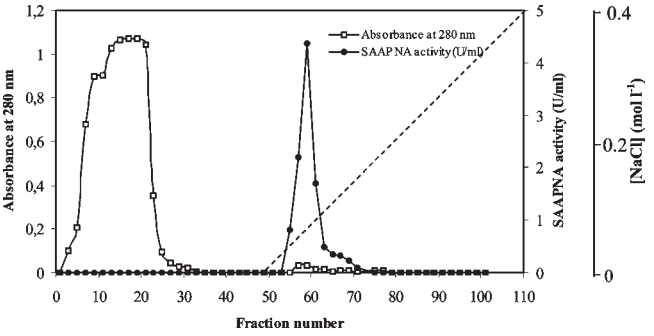

Figure 1. Elution profile of *L. mormyrus* chymotrypsin from a Mono Q-Sepharose column. Active fractions from the second Sephadex G-100 gel filtration were collected and then applied to a second Mono Q-Sepharose column (2 cm × 10 cm), equilibrated with buffer B. The enzyme was eluted with a linear gradient of NaCl (0−0.4 M) in buffer B at a flow rate of 80 mL/h.

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

purification steps	total activity (U)	total rotein (mg)	specific activity (U/mg)	recovery (%)	purification fold
crude enzyme	2701	746	3.62	100	1
ammonium sulfate precipitation (30−60% w/v)	2269	188	12	84	3.3
Sephadex G-100	1248	34.5	36.2	46.2	10
Mono Q-Sepharose	836	18	46.4	30.9	12.8
ultrafiltration	516	9.51	54.2	19.1	15
Sephadex G-100	268	1.4	191.4	10	53
Mono Q-Sepharose	55	0.19	289.5	2	80

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

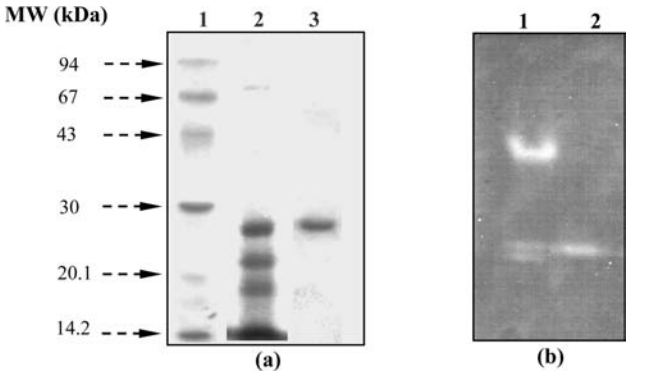

Figure 2. (**a**) SDS-PAGE of the purified chymotrypsin from striped seabream. Lane 1, standard proteins marker of different molecular weights; lane 2, crude enzyme extract; lane 3, purified chymotrypsin. (**b**) Zymogram detection on SDS-PAGE of proteolytic activity of the crude extract (lane 1) and the purified chymotrypsin (lane 2) from *L. mormyrus* viscera.

The amino terminal sequence of *L. mormyrus* chymotrypsin was aligned with the sequences of other known chymotrypsins (**Figure 3**). The sequence of *L. mormyrus* chymotrypsin (IVNGE) was identical to the conserved region with all other animal chymotrypsins except that of shrimp. The sequence of *L. mormyrus* chymotrypsin was identical to those of Atlantic cod and cow (*20*, *31*) and showed high homology with those from human and rat (*32*, *33*). However, *L. mormyrus* chymotrypsin differs from that of shrimp (*34*), with three amino acids in the N-terminal amino acid sequence.

Substrate Specificity. Striped seabream chymotrypsin showed activity on amide (SAAPNA) and ester (BTEE) specific substrates

containing phenylalanine and tyrosine, respectively, but did not display any activity against trypsin substrates such as BAPNA. This indicated that chymotrypsin was not contaminated by trypsin-like enzymes. Striped seabream chymotrypsin showed a specific activity of 470.2 U/mg enzyme on the ester substrate, and 289.5 U/mg enzyme on the amide substrate, indicating a 1.65 times faster hydrolysis of BTEE than SAAPNA. These results are in agreement with those of chymotrypsins from anchovy and Monterey sardine, which showed an esterase specific activity 3.4 and 3.2 times higher than amidase specific activity (*12*, *30*).

Effect of pH on the Activity and Stability of *L. mormyrus* Chymotrypsin. The pH activity profile of the purified striped seabream chymotrypsin is shown in **Figure 4**. The purified enzyme was highly active between pH 8.0 and 11.0, with an optimum at pH 10.0−11.0. The relative activities at pH 7.0, 8.0, and 12.0 were about 66%, 94% and 45.5%, respectively, of that at pH 10.0. The optimum pH of *L. mormyrus* chymotrypsin was higher than those of most described chymotrypsins, which showed optimum activity at pH 7.5−9.0.

Chymotrypsins A and B from the hepatopancreas of crucian carp (*Carassius auratus*) exhibited maximal activity toward Suc-Leu-Leu-Val-Tyr-AMC at pH 7.5 and 8.0, respectively (*21*).

The pH stability profile of *L. mormyrus* chymotrypsin is shown in **Figure 4**. Interestingly, the enzyme is highly stable over a wide broad pH range, maintaining more than 92% of its original activity between pH 7.0 and 12.0. However, chymotrypsin was unstable at pH below 6.0, retaining only 10% activity at pH 5.0.

The high activity and stability in high alkaline pH conditions make this enzyme a potential candidate for applications for certain food processing operations that requires high alkaline conditions.

Effect of Temperature on the Activity and Stability of *L. Mormyrus* Chymotrypsin. The temperature activity profile of *L. mormyrus* chymotrypsin is depicted in **Figure 5a**. The enzyme was active at temperatures from 20 to 70 °C, with an optimum around 50 °C, and displayed a high activity at low temperatures. The relative activities at 20, 30, 40, and 70 °C were about 69%, 78.5%, 94%, and 49%, respectively, of that at 50 °C. This result is

Striped seabream (*L. mormyrus*)	IVNGEEAVP--
Shrimp	IVGGVEATP--
Atlantic cod ChT1	IVNGEEAVPPS
Atlantic cod ChT2	IVNGEEAVPI-
Human	IVNGEDAVP--
Rat (*Rattus norvegicus*)	IVNGENAVP--

Figure 3. Alignment of the N-terminal amino acid sequence of the purified chymotrypsin from striped seabream (*L. mormyrus*) with the sequences of other chymotrypsins: Atlantic cod (*20*), Shrimp (*34*), rat (*32*) and human (*32*).

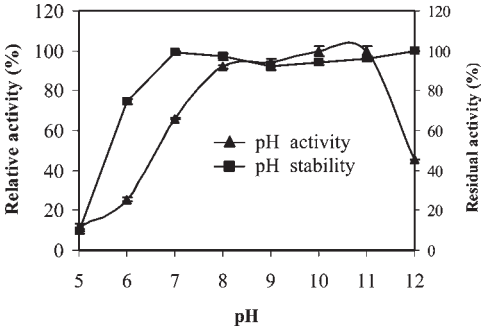

Figure 4. Effect of pH on the activity and stability of the purified chymotrypsin from the viscera of striped seabream (*L. mormyrus*). Chymotrypsin activity was assayed in the pH range from 5.0 to 12.0 at 50 °C using SAAPNA. The maximum activity obtained at pH 10.0 was considered as 100% activity. The 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 SAAPNA 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.

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

chemicals	concentration (mM)	relative activity (%)
none		100
SBTI	1 mg/mL	0
PMSF	5	2
β-mercaptoethanol	5	100
EDTA	5	90
TLCK	5	0
Ca^{2+}	5	114
Na^{+}	5	100
K^{+}	5	105
Ba^{2+}	5	82
Zn^{2+}	5	65
Cu^{2+}	5	20
Mg^{2+}	5	120
Mn^{2+}	5	100

[a] The purified enzyme was pre-incubated with various enzyme inhibitors for 30 min at 25 °C, and the remaining activity was determined at pH 10.0 and 50 °C using SAAPNA 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 chymotrypsin were determined by incubating the enzyme in the presence of various metal ions for 10 min at 50 °C and pH 10.0.

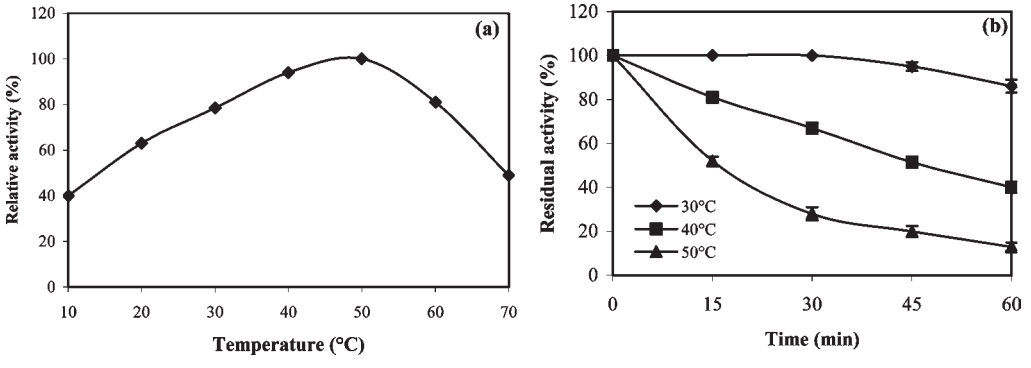

Figure 5. Effect of temperature on activity (**a**) and stability (**b**) of the purified chymotrypsin from the viscera of the striped seabream (*L. mormyrus*). Enzyme activity was assayed at different temperatures ranging from 10 to 70 °C at pH 10.0, using SAAPNA as a substrate. The activity of the enzyme at 50 °C was taken as 100%. For thermal stability, the enzyme was incubated at 30, 40, and 50 °C, and residual enzyme activity was determined from 0 to 60 min at regular intervals at pH 10.0 and 50 °C. The nonheated enzyme was considered as control (100%).

Table 3. Kinetic Constants of *L. mormyrus* Chymotrypsin and Other Chymotrypsins

chymotrypsins	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	refs
striped seabream (*L. Mormyrus*)[a]	30.7	14.35	0.465	this study
Monterey sardine (*S. sagax* C.)[a]	74	18.6	0.251	Castillo-Yanez et al. (2006) (*30*)
anchovy (*E. japonica*)[a]	89	14.7	0.165	Heu et al. (1995) (*12*)
rainbow trout (*O. mykiss*)[a]	35	2.2	0.0628	Kristjansson and Nielsen (1992) (*11*)
carp (*C. carpio*)[a]	300	4.4	0.0146	Cohen et al. (1981a (*15*), 1981b (*16*))

[a] Substrate: succinyl-L-ala-ala-pro-L-phenylalanine-*p*-nitroanilide (SAAPNA).

in line with several earlier reports showing high activity at low temperatures of chymotrypsins from anchovy, Monterey sardine, and crucian carp, which showed about 50%, 60%, and more than 60%, respectively (*12*, *21*, *30*). However, *L. mormyrus* chymotrypsin was more higly active at low temperatures than that of rainbow trout, which retained only 20% activity at the same temperature (*11*).

The optimal temperature of *L. mormyrus* chymotrypsin was similar to those from Monterey sardine (*30*) and crucian carp (chymotrypsin B) (*21*). It was higher than those of chymotrypsins from crucian carp (chymotrypsin A (40 °C)) (*21*) and anchovy (45 °C) (*12*) but lower than that of rainbow trout chymotrypsin (55 °C) (*11*).

The thermal stability profiles showed that the purified chymotrypsin is highly stable at 30 °C for 1 h but was labile at relatively high temperatures (>40 °C) (**Figure 5b**). The enzyme retained more than 86%, 40%, and 22% of its initial activity after 60 min incubation at 30, 40, and 50 °C, respectively.

Effects of Enzyme Inhibitors and Metal Ions on Chymotrypsin Activity. Proteases can be classified by their sensitivity to various inhibitors (*35*). To confirm the nature of the purified protease, the effects of different enzyme inhibitors, such as chelating agent and by specific group reagents on the protease activity were investigated (**Table 2**). Protease from *L. mormyrus* was completely inhibited by the serine-protease inhibitors PMSF, SBTI, and by TLCK, a chymotrypsin specific inhibitor. Further, metalloproteinase inhibitor, EDTA, showed minimal inhibitory effects.

However, β-mercaptoethanol was without influence on the activity of the purified chymotrypsin. Similar inhibition patterns were reported for chymotrypsins from Atlantic cod (*17*), anchovy (*12*), rainbow trout (*11*), Monterey sardine (*30*), and crucian carp (*21*). These results suggest that the purified enzyme is chymotrypsin type serine proteinases.

The effects of some metal ions, at a concentration of 5 mM, on the activity of *L. mormyrus* chymotrypsin 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^+ and Mn^{2+} did not affect chymotrypsin activity, whereas Ca^{2+} and Mg^{2+} increased the protease activity to 114% and 120%, respectively. However, the purified enzyme was partially inhibited by Ba^{2+}, Zn^{2+} and strongly affected by Cu^{2+}, suggesting the adverse effect of heavy metal ions on chymotrypsins. El Hadj Ali et al. (*10*) showed that Ca^{2+}, Cu^{2+}, and Zn^{2+} improved protease activity of trypsin of *L. mormyrus* by 114%, 110%, and 117%, respectively.

Kinetic Properties. Kinetic constants K_m and k_{cat} of the purified *L. mormyrus* chymotrypsin were determined using Lineweaver–Burk plots (*28*) (**Table 3**, **Figure 6**). The K_m and k_{cat} of the purified enzyme using SAAPNA were 30.7 μM and 14.35 s^{-1}, respectively. *L. mormyrus* chymotrypsin K_m was similar to that reported for chymotrypsin from rainbow trout (*11*). The K_m value of *L. mormyrus* chymotrypsin was lower than those of chymotrypsins from Monterey sardine (*30*), anchovy (*E. japonica*) (*12*), and Carp (*C. carpio*) (*15*, *16*). This suggests that it has higher affinity.

k_{cat} value was close to those reported for chymotrypsins from Monterey sardine (*30*) and anchovy (*E. japonica*) (*12*).

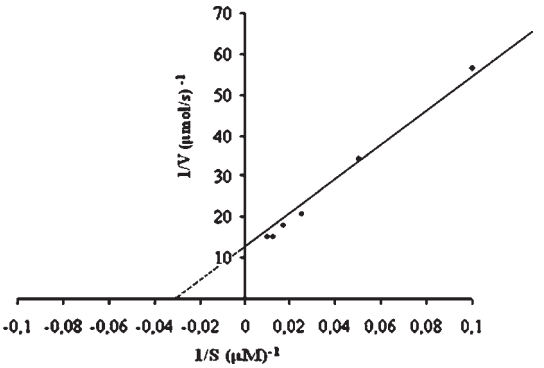

Figure 6. Lineweaver–Burk plot of the purified chymotrypsin from the viscera of the striped seabream (*L. mormyrus*). 1/*V* and 1/*S* represent the reciprocal of velocity and substrate, respectively. Each point represents the mean value of three experiments.

The catalytic efficiency (k_{cat}/K_m) of striped seabream chymotrypsin (465.6 s^{-1} mM^{-1}) was higher than other previously cited fish chymotrypsins (**Table 3**).

In conclusion, a novel chymotrypsin highly active and stable over a broad pH range was purified to homogeneity from *L. mormyrus*. After the final purification step, the enzyme was purified 80-fold with a specific activity of 289.5 U/mg. The purified protease was homogeneous on SDS-PAGE with a molecular weight of 27 kDa. The optimal pH and temperature for enzyme activity were pH 10.0–11.0 and 50 °C, respectively. Interestingly, *L. mormyrus* chymotrypsin was more active and stable at alkaline pH than the most described chymotrypsins. In addition, the purified enzyme exhibited high activity between 20 and 40 °C. These characteristics suggested the potential application of the chymotrypsin where low processing temperatures and higher enzymatic activity are needed. Further, as the enzyme was highly active and stable at high alkaline conditions, it may find application in laundry detergents.

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