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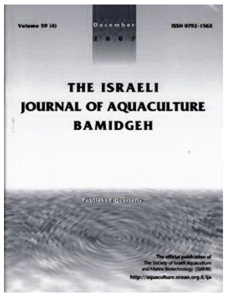

Partial Purification and Characterization of Amylases from the Digestive Tract of the Indian Medium Carp *Labeo fimbriatus* (Bloch, 1797)

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Abstract

Partial purification of amylases from the digestive tract of the Indian medium carp *Labeo fimbriatus* through acetone fractionation and Sephadex G-75 gel filtration resulted in 5-fold purification with 29% recovery. Characterization of amylase activity revealed two pH optima at 4.5 and 6.5-7.0. Activity was stable over a wide pH range of 4.0-10.0. Optimum incubation temperature was 25°C. The enzyme lost 80% activity at 50°C within 30 min and was inhibited by 1 mM p-chloromercuri benzoate, ethylene diamine tetra-acetate, and phenyl methyl sulphonyl fluoride. The heavy metal ions Hg^{++}, Cd^{++}, Cu^{++}, Zn^{++}, Fe^{++}, Pb^{++}, Bi^{++}, and Ag^{+} strongly inhibited enzyme activity whereas Ca^{++} activated it. Native polyacrylamide gel electrophoresis of the purified amylase fractions revealed four bands, with corresponding molecular weights of 72, 68, 66, and 65 kDa. Amylase activity from *L. fimbriatus* exhibited linear hydrolysis of starch up to 7% concentration.

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Introduction

The fish species *Labeo fimbriatus*, also known as fringe-lipped carp, is distributed in Pakistan, India, Nepal, Myanmar (Talwar and Jhingran, 1991), and Bangladesh (Menon, 1999). The fish is herbivorous and feeds on diatoms, blue-green and green algae, higher aquatic plants, insects, and detritus (Talwar and Jhingran, 1991). The species has been successfully bred at CIFA and efforts are underway to induct the species into culture systems.

The protein-sparing action of carbohydrates is well known in fishes (Wilson, 1994). Starch, the predominant carbohydrate in fish feed, is made available to fish by the action of amylases on starch. Introduction of a new species into a culture system requires information regarding its food, feeding habits, and digestive physiology, which governs nutrient utilization. *Labeo fimbriatus* (family Cyprinidae) is an indigenous medium carp of central and peninsular India (Hora and Pillay, 1962). As an herbivore, this fish has vast potential for aquaculture due to its amenable nature for captive breeding. Knowledge of the characteristics of herbivore amylases will be helpful in formulating its feed, especially in selecting carbohydrate sources for enhancing growth performance. This work reports on the partial purification and characterization of amylase from *L. fimbriatus.*

Materials and Methods

Enzyme extracts. Five specimens of *Labeo fimbriatus* (average length 30 cm; 300 g) were obtained from the culture ponds of the CIFA research center. The digestive tract and hepatopancreas of the specimens were dissected under ice-cooled conditions and washed repeatedly with ice-cooled distilled water. The tissues were homogenized individually with distilled water (4 ml/g; 15 g tissue in 60 ml) and centrifuged at 15,000 rpm for 20 min at 4°C. The supernatants (crude enzyme extract) were frozen and stored at -20°C in 20-ml aliquots for use in purification studies.

Enzyme estimation. Amylase activity was estimated by using a 1% starch solution in Tris-HCl buffer (0.1 M, pH 7.0) as the substrate. The assay mixture contained 0.05 ml crude enzyme extract plus 1.0 ml substrate and was incubated at 25°C for 1 h. The resulting reducing sugars were determined by the method of Nelson (1944) and Somogyi (1952) using glucose as the standard. Enzyme activity was expressed as µg glucose liberated/mg protein/h. Protein in the crude enzyme extract and other enzyme fractions was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard. All assays were carried out in triplicate.

Acetone fractionation (AF). The crude enzyme extract obtained from the digestive tract was subjected to solvent fractionation by the addition of chilled acetone (-20°C) equal to V/v (100% saturation) followed by centrifugation after 2 h at 15,000 rpm for 20 min at 4°C. The resulting supernatant was again treated with chilled acetone (250% saturation-V/v) and stored at 4°C overnight, followed by centrifugation as above. The precipitate obtained in each step was suspended in chilled distilled water and subjected to amylase and protein assay as described above. The fold purification and enzyme recovery were calculated in each case.

Gel filtration chromatography (GF). The amylase fraction obtained from the acetone fractionation step (equivalent to 12 mg protein) was layered on a Sephadex G-75 column (column length = 38 cm, r = 0.5 cm), equilibrated with Tris-HCl (0.1M, pH 7.0). The flow rate was maintained at 0.2 ml/min and 1.0 ml fractions were collected. Active major enzyme fractions GF1 (8-14) were pooled (designated as partially purified amylase), and fold purification and enzyme recovery were calculated. The pooled fractions were dispensed into aliquots of 0.5 ml in 1% glycerol and stored at -20°C for use in characterization studies. An aliquot of 25 µl from this pool was used in each characterization study and assay, conducted as above in triplicate.

Characterization of amylase. The partially purified amylase was incubated with substrate for periods ranging 15-180 min and enzyme activity was estimated. The reaction mixture containing the partially purified amylase and substrate was incubated at temperatures ranging 10-70°C for 45 min and enzyme activity was determined. The partially purified amylase was exposed to temperatures ranging 20-70°C for 10 min and residual activity estimated. The partially purified amylase was exposed to 30°C, 40°C, 50°C, and 60°C for 15-120 min, cooled in an ice bath, and thermostability was assayed for residual activity.

Amylase activity was assayed at 2-12 pH using the following buffers: 0.1 M KCl-HCl for pH 2, 0.2 M glycine-HCl for pH 3, 0.1 M phosphate buffer for pH 4-7, 0.1 M Tris-HCl for pH 7.0-9.0, and 0.1 M glycine-NaOH for pH 10-12. The partially purified amylase was exposed to the buffers for 30 min and residual activity was determined.

The partially purified amylase was incubated with solutions of the following metal ions: $HgCl_2$, $CaCl_2$, $CdCl_2$, $ZnSO_4$, $CoSO_4$, $FeSO_4$, $CuSO_4$, Li_2SO_4, $PbNO_3$, $Bi(NO_3)_2$, and $AgNO_3$ at concentrations of 0.1 mM and 1 mM for 30 min, then assayed for enzyme activity. Relative activity was calculated in relation to activity in enzymes not exposed to metal ions. The effect of inhibitory compounds on amylase activity was studied by incubating partially purified amylase in p-chloromercuric benzoate (PCMB), ethylene diamine tetra-acetate (EDTA), thiomersol, N-ethylmaleimide, and phenylmethyl-sulphonyl fluoride (PMSF) at various concentrations, then assaying enzyme activity. Relative activity was calculated against activity in partially purified amylase not exposed to inhibitors.

Using starch as the substrate at concentrations of 1-10%, the rate of hydrolysis was measured for 45 min in terms of increase in hydrolytic product.

Polyacrylamide gel electrophoresis. Partially purified AF, GF1-(8-14), GF2-(16-21), and crude enzyme extract of the digestive tract and the hepatopancreas were subjected to native slab polyacrylamide gel electrophoresis (PAGE) at 8% gel concentration (Garfin, 1990). Fresh gel containing the enzyme was soaked in 2% starch solution in Tris-HCl buffer (pH 7.0) for 30 min, followed by staining with iodine solution (I/KI-5mM I_2 in 2% KI solution). Active amylase fractions appeared as colorless bands.

Molecular weight determination by non-denatured protein electrophoresis. Relative mobility (R_f) of partially purified enzymes and standard proteins such as α-lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), albumin (chicken egg white; 45 kDa), albumin (bovine serum) monomer (66 kDa), and dimer (132 kDa) were determined at gel concentrations of 4.5%, 6%, 8%, and 10% by native PAGE (Davis, 1964). The ordinates of R_f values (100[Log (R_f x 100)]) were plotted against the % gel concentrations to obtain the retardation coefficient (Kr) as the slope of the plot for each standard protein. Logarithms of negative slopes were plotted against logarithms of standard molecular weights to obtain liner plots using the equation Y = 0.4628x - 0.0455. Molecular weights of the amylase isomers were determined using this plot, after determining the retardation coefficient as above.

Results

Purification. A two-fold purification with 43% recovery of amylase activity was obtained in the 250% acetone fraction. The fold purification increased to five with 29% recovery (Table 1) by using Sephadex G-75 gel filtration chromatography (Fig. 1).

Characterization of amylase (EC 3.2.1.1). The incubation time for optimum hydrolysis was 45 min (Fig. 2). The optimum incu-

Table 1. Purification of amylase from the digestive tract of *Labeo fimbriatus.*

Sample fraction	*Fraction volume*	*Protein (mg)*	*Enzyme activity (glucose µg)*	*Specific activity*	*Recovery (%)*	*Fold purification*
CEE	20	166.18	1,357,850	8171	100	1.0
AF	4.5	35.84	580,084	16,185	42.72	2.0
GF	1.5	11.946	193,361	16,185		
GF*	7.0	3.14	133,317	42,458	9.82	5.2

CEE = crude enzyme extract, AF = acetone fractionation, GF = gel filtration chromatography
*****Correspond to one run of the column 1.5 ml (11.9 mg).

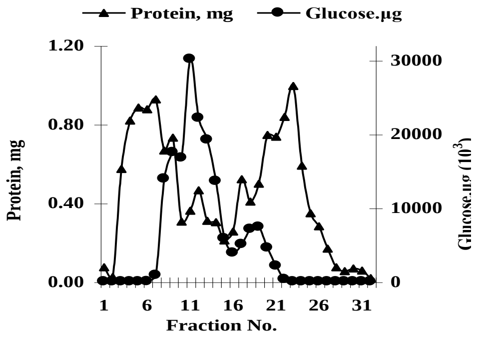

Fig. 1. Purification of amylase by Sephadex G-75 gel column.

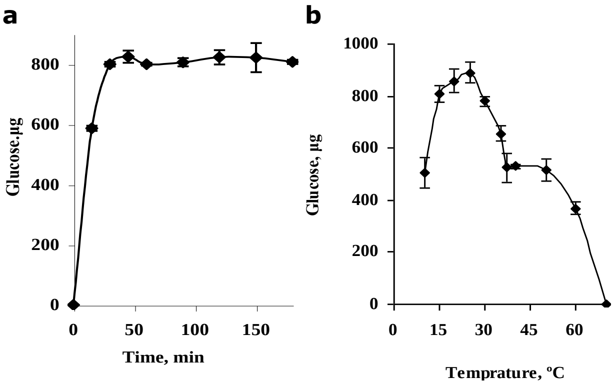

Fig. 2. Effect of incubation (a) time and (b) temperature on amylase activity.

bation temperature was 25°C. Activity in the partially purified amylase was heat stable up to 30°C and decreased beyond 30°C. Activity was 85% at 40°C, 25% at 50°C, and 10% at 60°C. The enzyme became completely inactive at 70°C (Fig. 3). The enzyme was stable up to 25°C; beyond this temperature, activity declined. The enzyme retained 85% activity at 40°C after 30 min, then decreased to 20% at 50°C. At 60°C the decrease in activity was 90% after 15 min.

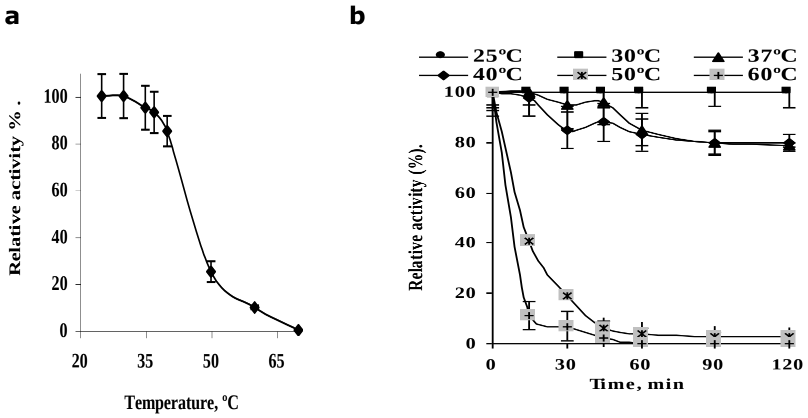

Fig. 3. Relative activity of amylases due to (a) heat treatment and (b) thermostability.

The amylase enzyme in the partially purified amylase had two pH optima: a sharp optima at 4.5 and broad activity optima at 6.5-7.5 (Fig. 4). It was stable over a wide range of pH (4.0-10). Starch hydrolysis was linear up to 7% starch concentration; beyond this, the increase in starch concentration resulted in a saturation plateau (Fig. 5).

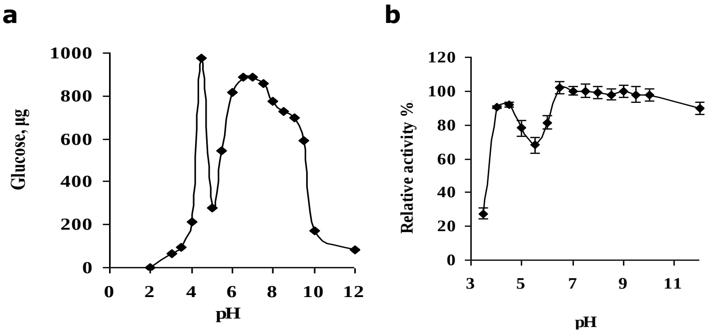

Fig. 4. Effect of pH of incubation on amylase (a) activity and (b) stability.

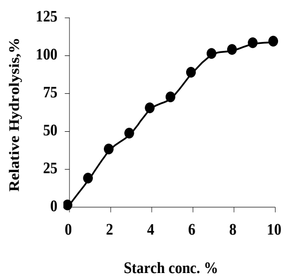

Fig. 5. Effect of starch concentration in the substrate on amylase activity.

Most heavy metal ions strongly inhibited the amylase at both concentrations (Table 2). Group specific agents strongly inhibited (95-100%) the enzyme while inhibition was 19% and 5% with N-ethylmeleimide (Table 3).

Table 2. Relative activity (%) of metal ions on amylase activity.

Concentration	Hg^{++}	Ca^{++}	Cd^{++}	Zn^{++}	Co^{++}	Fe^{++}	Cu^{++}	Li^{+}	Pb^{+}	Bi^{++}	Ag^{+}
1 mM	0	110	0	0	10	15	0	44	8	0	4
0.1 mM	33	100	16	24	47	19	26	60	52	43	21

Table 3. Relative activity (%) of enzyme modulators on amylase activity.

Concentration	*PCMB*	*EDTA*	*N-ethyl maleimide*	*Thiomersol*	*PMSF*
1 mM	0	5	81	0	0*
0.1 mM	10	51	95	15	5.0**

PCMB = p-chloromercuri benzoate, EDTA = ethylene diamine tetra-acetate, PMSF = phenyl-methyl sulphonylfluoride
Carried out at a concentration of *5 mM or **10 mM.

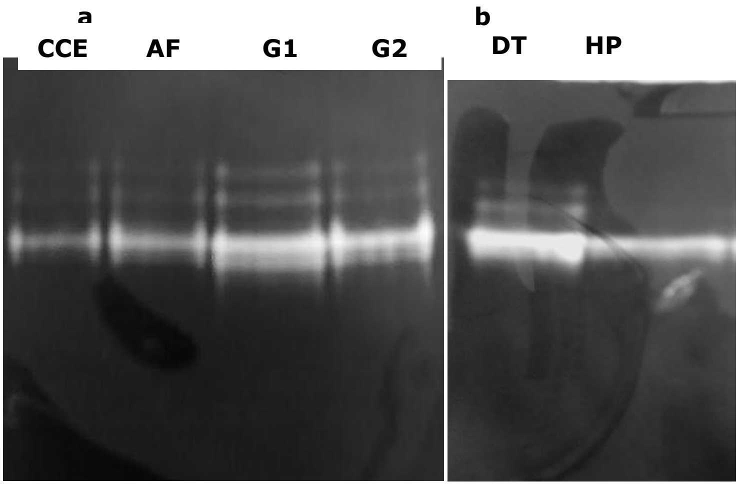

Fig. 6. Amylase activity in (a) substrate-stained crude enzyme extract (CEE), acetone fraction (AF), fractions 8-14 (G1), fractions 16-21 (G2), and (b) the digestive tract (DT) and hepatopancreas (HP).

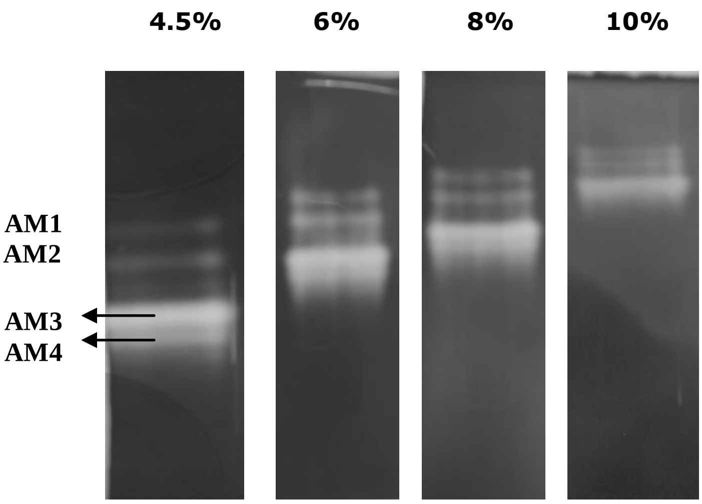

Fig. 7. Substrate-stained amylases by native-PAGE (four gel concentrations.

All digestive tract fractions had four amylase activity bands (Fig. 6) in native-PAGE, while crude enzyme extract from the hepatopancreas had only two.

Retardation coefficients (Kr) of the standard proteins were 3.45, 4.11, 4.95, 7.28, and 8.47 respectively (Fig. 7). Kr of the four isozymes (AM1-4) were 6.51, 6.34, 6.26, and 6.21. By extrapolating these values in the equation $Y = 0.4628x - 0.0455$, molecular weights were 72, 68, 66, and 65 kDa, respectively.

Discussion

The low recovery of 29% during amylase purification reveals the labile nature of the enzyme to the purification procedure. The obtained 5-fold purification shows the dominant nature of the enzyme, in agreement with higher amylase activities reported in omnivorous and herbivorous fishes (Philips, 1969; Hofer et al., 1982; Hidalgo et al., 1999). Higher fold purification (27-fold) with very low recovery (6.6%) was reported in the omnivorous Indian major carp, *Catla catla* (Roychan and Chaudhari, 2001). The amylases were found to be α-amylases from starch hydrolysis and the substrate staining patterns were similar to other observations (Blandamer and Beechey, 1964; Wigglesworth and Griffith, 1994, Roychan and Chaudhari, 2001).

The *L. fimbriatus* amylases were unique in that they were active even at 10°C. Enzyme activity (relative to maximum activity) at the low temperatures that correlate with the natural environment in which a species lives was reported for boreal fish (Kuzmina et al., 2003). The temperature in the habitat of *L. fimbriatus* ranges 15-27°C (Jhingran, 1991). Amylase activity for *L. fimbriatus* was optimum at 25°C. From 25 to 30°C, the enzymes retained complete activity for 10 min and thereafter steadily lost activity. Within 10 min, activity was 26% at 40°C and within 15 min, activity was 60% at 50°C and 90% activity at 60°C, indicating that the amylases are temperature sensitive. However, optimum temperatures of fish digestive enzymes are usually higher than habitat temperatures (Hai-ying et al., 2006).

The sharp optimum pH at 4.5 and broader pH at 6.5-7.0 agrees with observations in *Catla catla* (Roychan and Chaudhari, 2001) and in other freshwater and marine species (Ugwumba, 1993; Munila-Morán and Saborido-Rey, 1996; Hidalgo et al., 1999). Exogenous amylase production has been reported from distinct microbial sources apart from the endogenous sources in fish digestive tracts in freshwater teleosts with different feeding habits: *Labeo rohita*, *C. catla*, *Cirrhinus mrigala, Labeo bata,* and *Labeo calbasu* (Mondal et al., 2008). Our finding of an acidic pH optimum for amylases conforms with the presence of extracellular enzyme-producing aerobic bacteria in the digestive tract of *L. rohita* that have amylase producing ability at pH 5 (Kar et al., 2008). Acidic pH optima for amylase activity were also reported in crustaceans (Robson, 1979; Stark and Walker, 1983) and *Tilapia nilotica* (Yamada et al., 1991). In contrast, pH optima for amylase activity was alkaline in sparids: *Pagrus pagrus, Pagellus erythrinus, P. bogaraveo, Boops boops*, and *Diplodus annularis* (Fernández et al., 2001), and *Penaeus monodon* (Wigglesworth and Griffith, 1994). Corroborating our findings, specific activity for amylases was high in habitat temperatures with two pH optima peaks at 4.5 and 6.5 pHs in rabbitfish (*Siganus canaliculatus*) and sea bass (*Lates calcarifer*) suggesting that the neutral and acidic forms of amylases are more important than the alkaline form (Sabapathy and Teo, 1993). Amylase activity in *L. fimbriatus* was less stable at pH 3.5, overall stability increased from pH 4.5 to 10, while stability fell sharply at pH 5-6. Such multiple pH optima coupled with varied stability suggests the presence of more than one enzyme. Similarly, a broad range of pH stability (2-11) of the enzyme was reported in sparids (Fernández et al., 2001).

Calcium ions are required to maintain configuration and stability of α-amylase (Fischer and Stein, 1960) and chloride ions are required for maximum activity (Blandamer and Beechey, 1964; Telford, 1970; Hori, 1972; Ghosh et al., 2001). Our results agree in that amylases of *L. fimbriatus* were activated by 1 mM calcium chloride. EDTA inhibited amylase activity in *L. fimbriatus* by the chelation of calcium ions, as reported by Vallee et al. (1959), Brosemer and Rutter (1961), and Telegdi and Strub (1973). Inhibition of amylase activity by thiomersol, PMSF, and PCMB indicate the involvement of the sulfhydryl group in the enzyme activity.

The electrophoretic pattern of the crude enzyme extract from the digestive tract reveals, in addition to the two bands (66 and 65 kDa) found in both the hepatopancreas and digestive tract, the presence of two high molecular weight amylase activity bands (72 and 68 kDa) that could be of microbial origin from the intestine or synthesized by the digestive tract in *L. fimbriatus*. The possible existence of amylases produced not only by the pancreas but also by intestinal microflora has been demonstrated (Sugita et al., 1997). Similarly, two and five isozymes of amylases were reported in *T. nilotica* (Yamada et al., 1991) and sparids (Fernández et al., 2001), suggesting functional diversity of amylases regulated according to variations in environmental conditions, food supply, or the site of origin. The presence of different isozymes is closely related to the ability of fish to digest different kinds of foods, showing activity in a wide range of pH and temperature in addition to sensitivity to inhibitors (Natarajan et al., 1992), which may represent an ecological advantage (Fernández et al., 2001).

A dominant enzyme in *L. fimbriatus*, α-amylase was purified 5-fold with 29% recovery; it was active at the low temperature of 10°C and optimum at 25°C. Acidic and neutral forms of amylases and their stability in a wide range of pH shows the importance of carbohydrates in herbivores such as *L. fimbriatus*. The amylases of *L. fimbriatus* were sensitive to heavy metal ions, which might be a cause for the dwindling numbers of the fish in the polluted rivers of the Indian peninsular. The four isomers of amylases could be a gift of nature for functional diversity, to regulate the digestive physiology of the animal during variations in environmental conditions and food availability. In this study, we aimed to better understand carbohydrate utilization in the medium carps so as to incorporate them into aquaculture. Future investigations on deciphering the properties of individual isozymes might reveal their significance in this predominantly herbivorous species and help in meeting their energy needs.

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