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Purification and Some Properties of a-amylase from Indian Major Carp *Catla catla*

K.J. ROYCHAN and A. CHAUDHARI*

Division of Fish Genetics and Biotechnology
Central Institute of Fisheries Education
Versova, Mumbai 400 061
India

Abstract

The major amylolytic enzyme of a freshwater fish *Catla catla* was purified from the hepatopancreas and characterized as an α–amylase. The α–amylase was purified using ammonium sulphate fractionation and size exclusion chromatography on Sephadex G-100 column.

The α–amylase of *C. catla* was found to have an optimum pH of 6.5. The presence of calcium and chloride ions activated the amylase, while EDTA was found to inhibit it. The amylase was determined to be endo-acting by observing the rate of loss of iodine staining power against that of reducing sugar production. The maximum percentage of starch hydrolysis was 60%. The electrophoretic analysis of Sephadex G-100 purified amylase showed only one band on activity staining. The corresponding band obtained in SDS-PAGE was found to have a molecular weight of approximately 86 kD.

Introduction

Interest in the digestive enzymes of aquatic animals was generated by the development of controlled culture techniques in marine and freshwater species and farming of fish for commercial purposes. It has been observed that in fish, proteins together with lipids are the major sources of energy (Walton and Cowey 1982). Although poorly utilized by fish, carbohydrates do have a wide range of digesting enzymes (Phillips 1969). The nature of relative activity of digestive enzymes in fish correlates with the nature of the fish's normal diet. This is demonstrated by the observation that in predominantly herbivores tilapia, amylase activity is distributed throughout the gastrointestinal tract, but in carnivores perch, pancreas is the only source of amylase activity (Fish 1962). In carp, acinous cells of pancreas are believed to be the major center of amylase production.

Yamada et al. (1991) isolated and characterized two different amylases from the intestine of *Tilapia nilotica*, while amylases from several crustacean species have also been characterized (Robson 1979, Stark and Walker 1983, King 1967, Wigglesworth and Griffith 1994).

*Corresponding author

The purification and characterization of the carbohydrate digesting enzymes may provide an insight into the reasons of inefficient utilization of carbohydrates by fishes. Such a study also gains significance in the era of custom-made artificial diets, especially since knowledge of natural activators and inhibitors of these enzymes can help in deciding on the appropriate sources and levels of carbohydrate in feeds. *C. catla* is an Indian major carp and an important commercial species. The present paper describes the localization, purification from hepatopancreas and some properties of an α-amylase from *C. catla* for the first time. The presence of two other amylolytic enzymes is also indicated.

Materials and Methods

Experimental animal

Ten specimens of *C. catla* weighing between 500 to 700 g were procured from Shivaji Tank, Thane and brought to the laboratory under oxygen packing within two hours.

Preparation of extract

Specimens were dissected and the alimentary canal and hepatopancreas were carefully removed. Stomach and proximal intestine contents, tissues of stomach, proximal intestine, distal intestine (with contents) and hepatopancreas were separated. The tissues were weighed and homogenized with 10 volumes of chilled 0.1M phosphate buffer, pH 7.2. The homogenates were centrifuged at 10,000 xg for 30 min at 4°C. The stomach and proximal intestine contents were diluted 10 times with phosphate buffer. The preparations were kept in ice until the next procedure.

Amylase assay, protein estimation

Enzymatic extract (20 ml), 1% w/v Lintner's starch solution (0.5 ml) and 0.1 M phosphate buffer, pH 6.9 (1.48 ml) was incubated at 37°C, for 30 min (Stark and Walker 1983). Reducing sugars produced were estimated following the dinitrosalicylic acid method (Somogyi 1945). Appropriate enzyme blanks and substrate blanks were incubated along with the reaction mixture. A calibration curve of maltose was also prepared. Units of amylase activity were expressed as μmoles of maltose produced per min at 37°C. Protein was estimated following the method of Peterson (1977).

Purification

AMMONIUM SULPHATE FRACTIONATION

The extract was fractionated by introducing 25, 40, 60, 80 and 100% ammonium sulphate cuts as described by Englard and Seifter 1990. Precipi-

tate from each cut was suspended in 20 mM phosphate buffer, pH 7.2 and tested for amylase activity and protein concentration.

SIZE EXCLUSION CHROMATOGRAPHY

A Sephadex G-100 column (ht = 25 cm; d = 1 cm) was prepared in a glass column in the laboratory as described by Stellwagen 1990. The column was equilibrated with 20 mM phosphate buffer, pH 6.9 and 60F fraction was loaded on it. Ten mg protein was loaded per run. Flow rate was kept at 1 ml·min and 19 fractions of 3 ml each were collected. The entire separation was carried out in a cold chamber at 4°C and the fractions were immediately stored in ice. Protein estimation and amylase assay were done for each fraction.

ELECTROPHORESIS

Native vertical polyacrylamide slab gel electrophoresis, native-PAGE (Garfin 1990), was used to visualize amylase. A 7.5% separating gel and 4% stacking gel were used. The gel was stained as described by Robson 1979. Immediately upon completion of the run, the gel was washed thrice for 10 min each with 50 mM phosphate buffer, pH 6.5 immersed in starch (2% w/v in phosphate buffer) and kept in shaking water bath at 37°C for 2 hr. The gel was then stained with I/KI reagent (2% KI and 0.2% I_2). Upon destaining using distilled water, the bands appear as clearings against a dark blue background. The gels were immediately photographed.

SDS-PAGE separation was also done as previously described (Garfin 1990). A 10% separating gel was used and the sample buffer contained both SDS and mercaptoethanol. Molecular weight markers (185-29 kD, Bangalore Genei, India) were included in the gel. The lane loaded with purified sample was silver stained using the standard protocol, while molecular weight markers were stained with Coomassie blue.

Effect of pH on amylase activity

Effect of pH on amylase was studied by assaying the activity in 60F and purified fraction at different pH values of 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. Citrate phosphate buffer was used between pH 3.5 to 6.0, phosphate buffer was used between pH 6.5 to 8.0 and Tris-HCl buffer was used at pH 8.5 and 9.0. Amylase activity was assayed as given above.

Activity pattern of amylase hydrolysis of starch

A reaction mixture was set up containing 1% Lintner's starch solution (0.5 ml), 0.1 M phosphate buffer, pH 6.9 (0.25 ml) and purified enzyme fraction, F6 (0.25 ml). The reaction mixture and a substrate blank (0.5 ml starch solution and 0.5 ml phosphate buffer) were incubated at 37°C. Samples (0.2 ml) were withdrawn at 30 min. intervals and 0.1 ml each

were used to estimate sugar reduction and to determine the iodine staining capacity according to the method given by Wigglesworth and Griffith 1994.

Inhibition of amylase activity by EDTA

The inhibitory effect of EDTA on amylase activity was studied at zero, 0.5, 1, 2, 3 and 4 mM concentrations of EDTA. Amylase was assayed after adding nil, 10, 20, 40, 60 and 80 ml of 0.1M EDTA to the reaction mixture given above and adjusting the volume of the buffer accordingly. Per cent inhibition of amylase activity at various concentrations of EDTA were calculated.

Effect of activators on amylase activity

To study the effect of activators on amylase activity, reaction mixtures containing 1% Lintner's starch solution (0.2 ml), phosphate buffer, pH 6.9 (0.2 ml), DMW or 0.2 M sodium chloride or 0.1 M calcium sulphate (0.4 ml) and purified fraction, F6 (0.2 ml) were incubated at 37ºC for 30 min. Appropriate substrate and enzyme blanks were also included simultaneously and amylase activity was assayed as given above.

Results and Discussion

Localization of amylase activity

Stomach and proximal intestine contents and tissue extracts of stomach, proximal intestine, distal intestine (with contents) and hepatopancreas were assayed for amylase activity. While stomach, proximal and distal intestine tissue extracts showed no activity, stomach contents had the highest activity of 2.94 µmole maltose·min·mg. Proximal intestine contents and hepatopancreas extract had 0.71 and 0.2 µmol maltose·min·mg. The complete absence of amylase activity in the stomach and intestine walls is an indication that hepatopancreas is the only source of amylase present in the contents of stomach and proximal intestine. This is further supported by the identical bands of amylase visualized on native-PAGE (Fig. 1). Small pieces of hepatic and pancreatic tissues were separately homogenized and the extracts were separated on native-PAGE. It was established that amylase activity was localized only in the pancreatic tissue. Amylase was purified from the hepatopancreas extract with the view

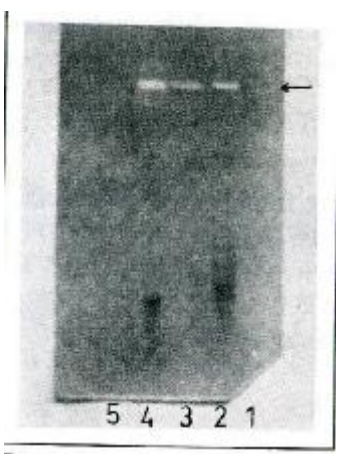

Fig. 1. Native-PAGE of amylase in various portions of the alimentary canal. Lane1- stomach, lane 2 – stomach contents, lane 3 – hepatopancreas, lane 4 – proximal intestine contents, lane 5 – proximal intestine.

that unlike stomach and intestine contents it would have less interference from active proteases and extraneous materials.

Purification

AMMONIUM SULPHATE PRECIPITATION

The extract containing 350 units of amylase was fractionated by introducing cuts of 25, 40, 60, 80 and 100% saturation of ammonium sulphate. Amylase was found only in the fraction obtained from the 40 to 60% cut (60F). The 60F contained 90 units of amylase, amounting to 25.7% of the initial activity and 3-fold purification of the enzyme (Table 1).

SIZE EXCLUSION CHROMATOGRAPHY

Fraction 60F from above was further separated on Sephadex G-100 column. Ten mg protein was loaded per run and 3 ml fractions were eluted in 20 mM phosphate buffer, pH 6.9. The elution profile is depicted in figure 2. Amylase activity peaked in fractions 5 and 6, with 1.5 and 1.2 units respectively. Specific activity was, however, two times higher in fraction 6 and it was used for further analyses. Taking into account only the amylase recovered in fraction 6, 27- fold purification was finally achieved, while recovery was only 6.6%. (Table 1).

Yamada et al. 1991, purified two different isoamylases from the intestine of *Tilapia nilotica* and Robson 1979, separated an amylase fraction from *Asellus aquaticus* using similar methods.

Table 1. Purification of amylase from hepatopancreas of *C. catla.*

Sample	Protein (mg)	Amylase	Specific units·mg	Recovery (%)	Fold purification
Extract	230	350	0.15	100.0	1
$(NH_4)_2SO_4$ cut (40 to 60%)	190	90	0.47	25.7	3
Sephadex G-100	5.7	23	4.00	6.6	27

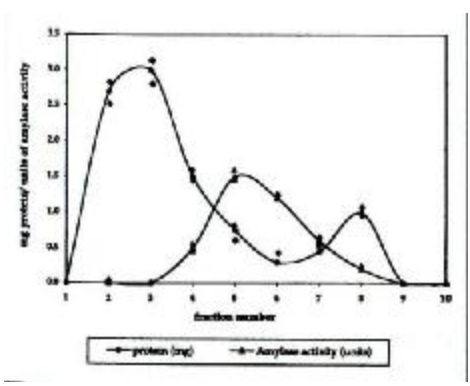

Fig. 2. Purification of amylase (60F) on Sephadex G-100 column.

Effect of pH on amylase activity

Purified amylase (F6) and 60F fraction were assayed at various pH values between 3.5 to 9.0. In both cases maximum activity was observed at pH 6.5 (Fig. 3). In the 60F fraction, however, two minor peaks were found at pH 4.5 and 8.5, that were absent in the F6 fraction. The two minor peaks may indicate the presence of two other amylolytic enzymes, not necessarily α-amylases. However, since the F6 fraction obtained from Sephadex G-100 column showed only one pH optimum of 6.5, further efforts were devoted to characterization of only this major amylase.

The pH optimum for the catla α-amylase is in the same range as that reported for α-amylase from other sources. Wigglesworth and Griffith 1994, working on carbohydrate digestion in *P. monodon*, found starch hydrolysis to be optimal at pH 7.0, with a second smaller peak at pH 9.0. The optimum pH range for mammalian α-amylase from human saliva and porcine pancreas is between 6.0 to 7.0 (Kulp 1975), while amylase from *Tilapia nilotica* showed highest activity at pH 6.0 (Yamada et al. 1991). The optimum pH of crustacean amylase is acidic (Takakashi et al. 1964, Robson 1979, Stark and Walker 1983).

Electrophoresis

Hepatopancreas extract, 60F and amylase containing fractions from size exclusion chromatography were visualized on native-PAGE by KI/I_2 staining. All samples showed only one amylase band as a clearing against a blue background. Since amylase activity had exhibited multiple pH optima in the 60F fraction, the extract and 60F were expected to contain more than a single amylase band. Even when the gels were incubated in 2% starch made in 50 mM citrate-phosphate buffer, pH 4.5, or in 2% starch in 50 mM Tris-HCl buffer, pH 8.5 for 3 hours before staining with KI/I_2 stain, no bands could be seen.

In SDS-PAGE analysis of the purified F6 fraction, only one band could be seen on the silver stained gel. Molecular weight of the band was found to be 86,000 (Fig 4). These results further support our conviction that there

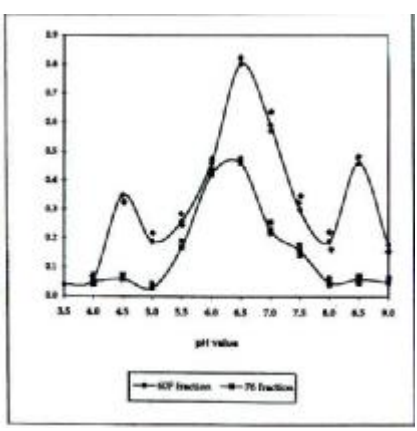

Fig. 3. Effect of pH on amylase activity in 60F and F6 fractions.

is a single α-amylase in *C. catla* with an optimal activity at pH 6.5. Robson 1979 reported a molecular weight of 70,000 for an amylase purified from the mid-gut of the crustacean, *Asellus aquaticus*.

Activity pattern of amylase hydrolysis of starch

The reduction in iodine staining capacity plotted against the production of reducing sugars is used as a method to distinguish between exo and endo hydrolysis of starch (Manners and Stark 1974). By plotting the iodine staining power against production of reducing sugars expressed as percentage conversion of starch (Fig. 5), it was observed that the hydrolysis stops at 60% hydrolysis of starch with no further increase in reducing sugar levels. The data suggests an endo-type of hydrolysis, this is typical of α-amylase since there is a rapid reduction in iodine stain with increased reducing power (Whitaker 1994).

It is possible that the two other amylases, indicated by the pH optima 4.5 and 8.5, have an exo-acting nature, and could not be seen as bands in native-PAGE since the iodine staining capacity of starch is retained for a prolonged duration in that case.

Effect of activators on amylase activity

Purified amylase fraction F6 was assayed in the presence of sodium chloride and calcium sulphate. The presence of 80 mM sodium chloride and 40 mM calcium sulphate was observed to cause 1.7 and 1.6-fold increase in amylase activity, respectively.

It has been reported that presence of calcium ions is necessary to maintain the secondary and tertiary stability of the α-amylase molecule (Fischer and Stein 1960) and the chloride ions are required for its maximum activity (Blandamer and Beechey 1964, Telford 1970, Hori 1972). The observations in this study indicate that the same may be true for the α-amylase of *C. catla*.

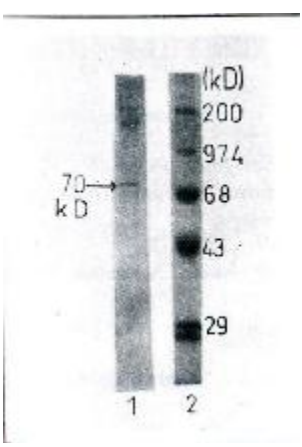

Fig. 4. SDS - PAGE of purified fraction (F_6). Lane 1 - F_6, lane 2 - molecular weight markers

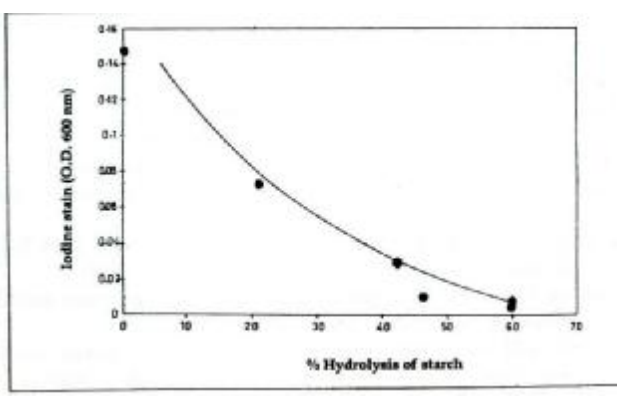

Fig. 5. Hydrolysis of starch and reduction in iodine stain by purified amylase.

Inhibition of amylase activity by EDTA

EDTA (5 mM) specifically inhibits α-amylase activity (Vallee 1959, Brosmer and Rutter 1961) by chelating Ca^{2+} ions required for its stability and activity (Telegdi and Strub 1973). The presence of 3 mM EDTA in the reaction mixture inhibited 96% of the amylase activity present in the purified enzyme fraction F6. The presence of 4 mM EDTA did not result in any further inhibition.

Enhanced activity in the presence of calcium sulphate and sodium chloride, and inhibition by EDTA of the enzyme purified from the hepatopancreas of *C. catla* further establishes that it is α-amylase.

Conclusion

It can be concluded that the major starch hydrolysing enzyme of the Indian Major Carp *C. catla* is an α-amylase, produced in the pancreas. The activity is found to be less than that reported for tilapia by Yamada et al. (1991). Two other amylolytic enzymes, possibly exo-acting are also indicated with pH optima 4.5 and 8.5. It may perhaps be that the inability of *catla* to utilize high starch diets is not from want of starch hydrolysing enzymes.

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