Available online at www.sciencedirect.com

SCIENCE DIRECT

Comparative Biochemistry and Physiology, Part B 142 (2005) 98 – 106

CBP

www.elsevier.com/locate/cbpb

Digestive enzyme patterns and evaluation of protease classes in *Catla catla* (Family: Cyprinidae) during early developmental stages

R.M. Rathore, S. Kumar, R. Chakrabarti *

Aqua Research Lab, Department of Zoology, University of Delhi, Delhi-110007, India

Received 18 March 2005; received in revised form 13 June 2005; accepted 14 June 2005

Abstract

Digestive enzymes of *Catla catla* were studied during ontogenic development. Specific amylase activity was 0.12 ± 0.01 mg maltose mg protein^{-1} h^{-1} in fish 4 days after hatching (DAH) and reached a maximum on (0.41 ± 0.12 mg maltose mg protein^{-1} h^{-1}) 34 DAH. Total protease activity was minimum (123.2 ± 16.5 mU mg protein^{-1} min^{-1}) on day-8 and reached its highest level (2713 ± 147.2 mU mg protein^{-1} min^{-1}) on day-32. Trypsin activity showed constant increasing trend from day-16 onwards and was maximum on day-34 (118.1 ± 7.09 mU mg protein^{-1} min^{-1}). Highest chymotrypsin activity was found on day-32 (1789.0 ± 111.7 mU mg protein^{-1} min^{-1}). Lipase activity was detected in 4 DAH catla. Lipase activity increased steadily from day-22 onwards. SDS-PAGE of crude enzyme extracts showed that high molecular mass bands (41.8–127.8 kDa) appeared during the early stages followed by low molecular mass bands (17.8–37.2 kDa). The number of protease activity bands in substrate SDS-PAGE increased with age of fish. During ontogenesis of carp, soybean trypsin inhibitor (SBTI), PMSF and TLCK inhibited 75.5 ± 1.19% to 92.8 ± 0.85%, 53.3 ± 9.47% to 90.5 ± 2.6% and 39.8 ± 3.8% to 84.7 ± 1.54% of total protease activity, respectively. There was only 2.58 ± 0.66% to 10.21 ± 0.09% inhibition of protease activity with EDTA. SBTI and PMSF inhibited 8 and 4 activity bands, respectively. TLCK, a specific trypsin inhibitor, inhibited four trypsin-like enzymes in carp during ontogenesis.

Keywords: *Catla catla*; Digestive enzymes; Inhibitors; Ontogenesis; Substrate SDS-PAGE

1. Introduction

Fishes experience evolutionary adaptations in the morphogenesis of their digestive system during early developmental stages due to the changing nutritional requirement and shifting from endogenous to exogenous feeding. This fact is reflected in the ontogeny of digestive enzyme patterns. A comprehensive analysis of the ontogenic changes occurring during the early life stages of fish is essential for the design of adequate larval rearing and feeding strategies and for formulation of dry diets (Verreth and Segner, 1995). Many workers have described that the digestive enzyme activity in fish is influenced by age and/or stage of development (Kuz'mina, 1980; Lauff and Hofer, 1984; Hofer and NasirUddin, 1985; Buddington and Doroshev, 1986; Cousin et al., 1987). The knowledge of temporal appearance of key enzymes in the gut of cultivable species in aquaculture is essential to understand age specific formulation of feeds that contributes to rapid and efficient growth rates (Tengjaroenkul et al., 2002). Indian major carp *Catla catla* (catla) is planktivorous and is one of the most economically important species in India. Very little is known about the pattern of digestive enzymes of catla during early stages of development (Chakrabarti and Sharma, 1997; Kumar and Chakrabarti, 1998). Information on the intestinal enzyme activities during ontogeny is essential to know the digestive efficiency of the cultured species at particular stages of development. Besides that, it is equally important to characterize and know the classes of the digestive enzymes for a better understanding of their course of action in the digestive physiology. The present investigation focused on the qualitative and quantitative study of digestive enzymes during early ontogeny of catla.

* Corresponding author. Tel.: +91 11 27666496.
E-mail address: rina@ndf.vsnl.net.in (R. Chakrabarti).

1096-4959/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2005.06.007

2. Materials and methods

2.1. Animals and culture conditions

Larvae of Indian major carp *C. catla* (catla) were procured from Chatterjee Brothers' Fish Farm, West Bengal 72 h after hatching (length 5.50±0.22 mm; mass 0.96±0.06 mg). Fish were reared in a recirculating system at a density of 45,000 larvae m^{-3} in each tank (450 L). The photoperiod was maintained on a 12 L : 12 D cycle. Three replicates were used for the experiment. Larvae were fed with live food (*Brachionus* spp., *Ceriodaphnia* spp., *Mesocyclops* spp.) ad libitum up to 19 days after hatching (DAH). Mixed feeding with artificial diet (40% protein) was started from day-20 onwards at 4% of body weight. Ten fish were collected randomly from each tank at weekly intervals in order to estimate the food requirement of the growing larvae. The duration of the experiment was 30 days. Temperature and pH ranged from 27 to 30 °C and 7.3 to 8.4, respectively throughout the experiment. Dissolved oxygen was maintained above 5 mg/L by constant aeration.

2.2. Sample preparation

Larvae were collected randomly from three tanks at a fixed time (9 A.M.) before morning feeding every other day. Fish were washed properly through a sieve and were immediately frozen at −20 °C. The digestive systems of individual fish were removed using a glass plate maintained at 0 °C under a dissecting microscope. The examination of digestive system under microscope confirmed the absence of live food in the gut of the fish. The numbers of fish sampled for 100 mg tissue were initially 211±3.17 on day-4 and subsequently reduced to 24±1.15 on day-34 as fish grew. 100 mg of gut tissue was collected in a microcentrifuge tube and homogenized with 1 mL of chilled distilled water. The homogenate was centrifuged at l0,000 ×*g* at 4 °C for 10 min and the supernatant (pH 5.5) was collected and stored at −20 °C. Three replicates were used for each sample. Total soluble protein was measured by the method of Bradford (1976).

2.3. Analysis of digestive enzymes

2.3.1. Amylase

Amylase was determined by measuring the increase in reducing power of buffered starch solution with 3,5-dinitrosalicyclic acid (DNS) at 540 nm (Bernfeld, 1955). The enzyme preparations were incubated with 1% starch solution (pH 7.0) at 37 °C for 1 h and the reaction was stopped by adding DNS. Specific amylase activity was expressed by milligram of maltose released per milligram of protein per hour at 37 °C.

2.3.2. Protease

Total protease activity was assayed by using 1% azocasein in 50 mM Tris–HCl, pH 7.5 (Garcia-Carreno, 1992). 10 µl of enzyme extract was mixed with 0.5 mL of buffer (pH 7.5), 0.5 mL of substrate solution and incubated for 10 min at 25 °C. The reaction was stopped by addition of 0.5 mL 20% TCA and then centrifuged for 5 min at 14,000 ×*g*. The absorbance of the supernatant at 366 nm was recorded.

Evaluation of the protease class was performed according to Garcia-Carreno and Haard (1993). Enzyme extract was incubated with different specific inhibitors. Phenylmethylsulfonylfluoride (PMSF) and soybean trypsin inhibitor (SBTI) were used as specific inhibitors of protease belonging to the serine class. *N*-α-*p*-tosyl-L-lysine chloromethylketone (TLCK) was used as specific inhibitor of trypsin. Ethylenediamine tetraaceticacid (EDTA) was used for inhibiting metalloproteases. All inhibitors were pre-incubated at a 1 : 1 ratio for l h at 25 °C.

2.3.3. Trypsin and chymotrypsin

Trypsin activity was measured with *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). BAPNA (1 mM in 50 mM Tris–HCl, pH 7.5, 20 mM $CaCl_2$) was incubated with enzyme extract at 37 °C. A change of absorbance was recorded continuously for 3 min at 410 nm (Erlanger et al., 1961). Chymotrypsin activity was measured by using 0.1 mM Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAPNA) in 50 mM Tris–HCl, pH 7.5, 20 mM $CaCl_2$. The enzyme preparation was incubated with substrate at 37 °C and monitored at 410 nm for 3 min. Trypsin and chymotrypsin activity units were expressed as change in absorbance per minute per milligram protein. Activity units were calculated by the following equation:

$$\text{Activity units} = \frac{(Abs_{410}/\text{min}) \times 1000 \times \text{mL of reaction mixture}}{\text{Extinction coefficient of chromagen} \times \text{mg protein in reaction mixture.}}$$

The molar extinction coefficient of *p*-nitroanilide is 8800.

2.3.4. Lipase

Lipase was assayed according to the method of Winkler and Stuckman (1979), following the release of *p*-nitrophenol (*p*NP) from *p*-nitrophenyl palmitate (*p*NPP). The substrate was prepared by dissolving 30 mg *p*NPP in 10 mL of isopropanol mixed with 90 mL of 0.05 M Sorensen phosphate buffer (pH 8.0) containing 207 mg of sodium deoxycholate and 100 mg of gum arabic. A 2.4 mL aliquot of the freshly prepared substrate was pre-warmed at 37 °C and incubated with the enzyme extract for 15 min, at 37 °C. Then the absorbance was recorded at 410 nm against enzyme free control. One enzyme unit is defined as 1 nmol of *p*-nitrophenol released from the substrate per milliliter per minute. The extinction coefficient of *p*-nitrophenol is 15,000 cm^2 mg^{-1}.

2.4. SDS-PAGE and substrate SDS-PAGE

Separation of proteins in the enzyme extracts was done by 12% SDS-PAGE according to Laemmli (1970). Enzyme