Digestive proteases of three carps *Catla catla*, *Labeo rohita* and *Hypophthalmichthys molitrix*: partial characterization and protein hydrolysis efficiency

S. KUMAR[1], F.L. GARCIA-CARREÑO[2], R. CHAKRABARTI[1], M.A.N. TORO[2] & J.H. CÓRDOVA-MURUETA[2]

[1] *Department of Zoology, Aqua Research Lab, University of Delhi, Delhi, India;* [2] *Centro de Investigaciones Biologicas del Noroeste (CIBNOR), La Paz, Mexico*

Abstract

Characteristics and functional efficacy of digestive proteases of *Catla catla*, catla, *Labeo rohita*, rohu and *Hypophthalmichthys molitrix*, silver carp were studied. Total protease activity was significantly ($P < 0.05$) higher in rohu (1.219 ± 0.059 U mg protein^{-1} min^{-1}) followed by silver carp (1.084 ± 0.061 U mg protein^{-1} min^{-1}), and catla (0.193 ± 0.006 U mg protein^{-1} min^{-1}). Trypsin activity of silver carp and rohu was 89–91% higher than catla. Chymotrypsin activity was significantly ($P < 0.05$) higher in silver carp compared with rohu and catla. The protease activity of rohu and silver carp displayed bell-shaped curves with maximum activity at pH 9; whereas in catla, maximum activity was found between pH 8 and 11. Inhibition of protease activity with soybean trypsin inhibitor (SBTI), phenylmethylsulfonyl fluoride (PMSF) revealed the presence of serine proteases and inhibition of activity with N-α-p-tosyl-L-lysine-chloromethyl ketone (TLCK) and N-tosyl-L-phenylalanychloromethane (TPCK) indicated the presence of trypsin-like and chymotrypsin-like enzymes in all these three carps. SDS-PAGE showed the presence of several protein bands ranging from 15.3 to 121.9 kDa in enzyme extracts of catla, rohu and silver carp. The substrate SDS-PAGE evidenced the presence of various protease activity bands ranging from 21.6–93.7, 21.6–63.8 and 26.7–98.5 kDa for catla, rohu and silver carp respectively. In pH-stat hydrolysis of Chilean fishmeal showed significantly ($P < 0.05$) higher degree of hydrolysis compared with soybean meal, silver cup (a commercial fish feed of Mexico) and wheat flour, with enzyme preparations of three fishes. The rate of hydrolysis was significantly ($P < 0.05$) higher in silver carp compared with others.

KEY WORDS: carps, chymotrypsin, pH-stat hydrolysis, protease, substrate SDS-PAGE, trypsin

Received 26 May 2006, accepted 13 February 2007

Correspondence: R. Chakrabarti, Department of Zoology, Aqua Research Lab, University of Delhi, Delhi 110 007, India. E-mail: aquaresearch-lab@yahoo.co.in and http://www.aquaresearchlab.org

Introduction

The ability of any fish to digest a given diet and absorption of its nutrients depends on the presence and the quality of digestive enzymes. Although several on-farm feeds are in use in aquaculture, commercial fish feeds manufactured on the basis of proper understanding of digestive physiology of fish are yet to be established for most of the cultured species (Seenappa & Devaraj 1995). As protein utilization is fundamental to growth, proteases have an important role to play in the larval fish as in the adult. The digestive proteases of different species showed variations (Chakrabarti & Sharma 2005), which may influence their digestive capability and feeding habits. Carps are used in polyculture because they occupy different feeding niches in the pond *viz*. some species are phytoplankton feeders, others are omnivorous or zooplankton feeders. Indian major carp, catla *Catla catla*, rohu *Labeo rohita* and the silver carp *Hypophthalmichthys molitrix*, are extensively used in commercial fish culture throughout India. In adult stage, catla is a zooplanktivore, surface feeder, rohu is a predominantly mid column feeder, planktophage (phytoplankton and zooplankton feeder) (Jhingran 1991) and silver carp is a typical pelagic filter feeder, feeding mainly on phytoplankton and detritus (Opuszynski & Shireman 1995; Xie 1999).

Intensification of pond culture of carps has necessitated the supply of nutritious and easily digestible supplementary feed.

In choosing a protein source, fish meal is an option. But fish meal is expensive and is in high demand. As a result, there is a tendency to produce alternative plant-based feed. However, plant protein has some disadvantages which are important to consider before including it in feed. Plant protein may require processing to eliminate or reduce anti-nutritional compounds (Alarcón *et al.* 1999). These anti-nutritional compounds have to be evaluated on the target species to avoid affecting physiological functions in the species, such as reducing digestive enzyme activities. Some well-documented anti-nutritional factors for digestive proteases are the soybean trypsin inhibitor and tannins (Maitra & Ray 2003). *In vitro* methods of evaluating protein digestibility are important as they are rapid, less expensive and they allow close observation of the dynamics of the breakdown of protein by using only small amounts of raw materials (Grabner 1985). Thus, characterization of digestive proteases is essential along with the quantitative estimations for the better understanding of digestive capability of the cultured species and to assess protein ingredients in feed formulations (Moyano *et al.* 1996). Efforts have been made to characterize the digestive protease of three species of carps, and also to study the efficacy of their digestive proteases to hydrolyse the protein present in different types of commercially available fish diets.

Materials and methods

Sample preparation

Catla (364 ± 18 g), rohu (347 ± 15 g) and silver carp (366 ± 8 g) were obtained from a local fish farm in Delhi, India. Fishes were acclimated in plastic pools (450 L) under controlled conditions (photoperiod: 12D : 12L, 28 ± 2 °C) and were kept without food for 24 h. Then fish were dissected in a cold room (temperature 4 °C). Whole digestive tract, including hepatopancreas was taken out from each fish, cut open, washed and were homogenized with ice-cold distilled water. The homogenate was centrifuged at 12 500 ***g*** for 30 min at 4 °C. Three replicates were used for each species. Supernatant was lyophilized and kept in refrigerator at −80 °C until use. Lyophilized powder of each sample was dissolved in cold distilled water (w/v 1 : 10) and centrifuged at 6500 ***g*** for 10 min at 4 °C. Supernatants were kept at −80 °C until analysis. The soluble protein of enzyme extracts was measured according to Bradford (1976).

Enzyme assays

Total protease activity was estimated with azocasein (García-Carreño & Haard 1993). Approximately 10 μL sample was incubated with 0.5 mL Tris-HCl buffer and 0.5 mL of 0.5% azocasein (in 50 mM Tris-HCl buffer, pH 7.5) for 10 min at 37 °C. Reaction was stopped with 0.5 mL of 5% TCA. Sample was centrifuged at 14 300 ***g***. Absorbance of supernatant was recorded at 366 nm. Enzyme activity was expressed as Units mg protein^{-1}min^{-1}. Trypsin and chymotrypsin activities were assayed using 1.0 mM *N*-α-benzoyl-DL-arginine-*p*-nitroanalidine (BAPNA, Sigma B-4875) and 0.1 mM succinyl-Ala$_2$-Pro-Phe-*p*-nitroanilide (SAPNA, Sigma S-7388) in 50 mM Tris-HCl buffer, pH 7.5 containing 20 mM $CaCl_2$ respectively (Erlanger *et al.* 1961). The optimum pH and presence of calcium ions and substrates in the reaction mixture helps in the rapid activation of trypsin and chymotrypsin from their respective zymogen forms. The absorbance was recorded at 410 nm for 3 min (Erlanger *et al.* 1961). Activity unit was calculated by the following equation:

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

Extinction coefficient of *p*-nitroaniline liberated from chromogens of BAPNA and SAPNA is 8800.

Effect of pH on enzyme activity

The effect of pH on protease activity was measured using Universal buffer (Stauffer 1989), pH ranging from 5.0 to 12.0 at 25 °C. Substrate, 0.5% azocasein was dissolved in each pH buffer solution and the pH value was established. Substrate was dissolved in each pH buffer solution prior to enzymatic reaction. Then 10 μL enzyme extracts (supernatants obtained from digestive tissue extracts) were mixed with 500 μL of each pH buffer and 500 μL of respective pH buffered-substrate. Reaction was stopped after 10 min with 500 μL of 20% TCA. Samples were centrifuged at 14 000 ***g*** for 5 min and supernatants were separated from undigested substrate. Absorbance of supernatants was recorded at 366 nm.

Enzyme inhibition

Protease inhibition assay was performed using different specific inhibitors (García-Carreño 1992). Soybean trypsin inhibitor (SBTI, 250 μM in distilled water) and Phenyl methyl sulfonyl fluoride (PMSF, 100 mM in 2-propanol) were used as

inhibitors for proteases belonging to the serine class. *N*-α-*p*-tosyl-L-lysine chloromethylketone (TLCK, 10 mM in 1.0 mM HCl, pH 3.0) and *N*-tosyl-L-phenylalanine chloromethylketone (TPCK, 5 mM in methanol) were used as specific inhibitors for trypsin and chymotrypsin respectively (García-Carreño 1992). Commercial porcine trypsin and bovine chymotrypsin were used as enzyme controls. 10 μL enzyme preparations were incubated with different inhibitors (v/v 1 : 1 ratio) for 1 h at 25 °C. The assay was performed for total protease activity. Sample without inhibitors served as control. Reduction in enzyme activity in treated samples was recorded as % inhibition. Each assay was carried out in triplicate.

SDS-PAGE and substrate SDS-PAGE

SDS-PAGE was performed according to Laemmli (1970) for electrophoretic separation of protein in crude enzyme extracts. Enzyme extracts were diluted (1 : 1) in sample buffer containing SDS but without reducing agent. Samples were not boiled before loading onto gels. Protease compositions of enzyme extracts were recorded after separation of proteases by substrate SDS-PAGE as described in García-Carreño *et al*. (1993). Electrophoresis was performed at 4 °C with a constant current of 20 mA. Samples containing 5 mU enzyme activity was loaded onto gel for each enzyme extract. After electrophoresis, gel was washed and immersed in 30 mg g^{-1} casein (in 50 mM Tris-HCl buffer, pH 7.5) for 30 min at 5 °C, and then temperature was raised to 25 °C for 90 min. The protein was fixed and stained. Clear zones in blue background were observed as enzyme activity bands.

The protease class in the enzyme extracts of three species was evaluated using specific protease inhibitors (García-Carreño & Haard 1993). Samples containing 5 mU activities were incubated with different inhibitors *viz*. SBTI (250 μM), PMSF (100 mM), TLCK (10 mM) for 1 h at 25 °C prior to substrate SDS-PAGE. After incubation samples were diluted (1 : 1) with sample buffer and subjected to substrate SDS-PAGE. After electrophoresis, gels were treated as described in legend of Fig. 3b. A conspicuous reduction in intensity of the activity bands was recorded as inhibition and compared with the activity bands of untreated samples.

In vitro digestibility study

The degree of hydrolysis of casein, soybean meal, a commercial fish feed available in Mexico and Chilean fish meal were evaluated by the pH-stat method (Pedersen & Eggum 1983) modified by Dimes & Haard (1994), using enzyme extracts of catla, rohu and silver carp. pH-stat analysis was carried out with a 718 stat Titrino (Metrohom Ion Analysis, Switzerland) using Metrodata Menu computer programme. Enzyme extract (200 μL) previously adjusted at pH 8.0, containing 0.25 U protease activity (Units activity of enzyme extracts were adjusted by dilution with distilled water for the comparison of hydrolytic efficiency of different species) was added to the substrate containing 0.08 g protein. The reaction mixture was maintained at 27 °C, using a jacketed reaction vessel and temperature controlled circulating water bath. The reaction was monitored for 1 h. Each assay was carried out in triplicate. The degree of hydrolysis (DH%) was calculated using the following equation:

$$\mathrm{DH\%} = B \times N_B \times 1/1.4 \times 1/0.08 \times 1/8 \times 100,$$

Where B is the amount of solution (mL) of NaOH consumed to maintain the reaction mixture at pH 8.0; N_B is the normality of the NaOH solution; 1.4 is the average degree of dissociation of the α-amino groups related with the pK of the amino groups at pH 8.0 and 27 °C; 0.08 is the amount of crude protein (g) in the reaction mixture, and 8 is the milliequivalents of peptide bonds per g protein.

Statistical analysis

All data were statistically analysed by one-way ANOVA (using SPSS programme) and Duncan's Multiple Range test (Montgomery 1984) to assess differences in enzyme activities. Level of significance was $P < 0.05$.

Results

Total protease activity

Among these three carps, total protease activity was significantly ($P < 0.05$) higher in planktophage rohu (1.219 ± 0.059 U mg $protein^{-1}$ min^{-1}), followed by herbivore silver carp (1.084 ± 0.061 U mg $protein^{-1}$ min^{-1}) and zooplanktivore catla (0.193 ± 0.006 U mg $protein^{-1}$ min^{-1}) (Fig. 1).

Trypsin and chymotrypsin activity

Trypsin activity of silver carp (0.297 ± 0.019 U mg $protein^{-1}$ min^{-1}) and rohu (0.354 ± 0.016 U mg $protein^{-1}$ min^{-1}) were 89–91% higher than catla (0.032 ± 0.007 U mg $protein^{-1}$ min^{-1}). Chymotrypsin activity was significantly ($P < 0.05$) higher in silver carp (0.594 ± 0.042 U mg $protein^{-1}$ min^{-1}) than rohu (0.498 ± 0.007 U mg $protein^{-1}$ min^{-1}) and catla (0.253 ± 0.009 U mg $protein^{-1}$ min^{-1}) (Fig. 1).

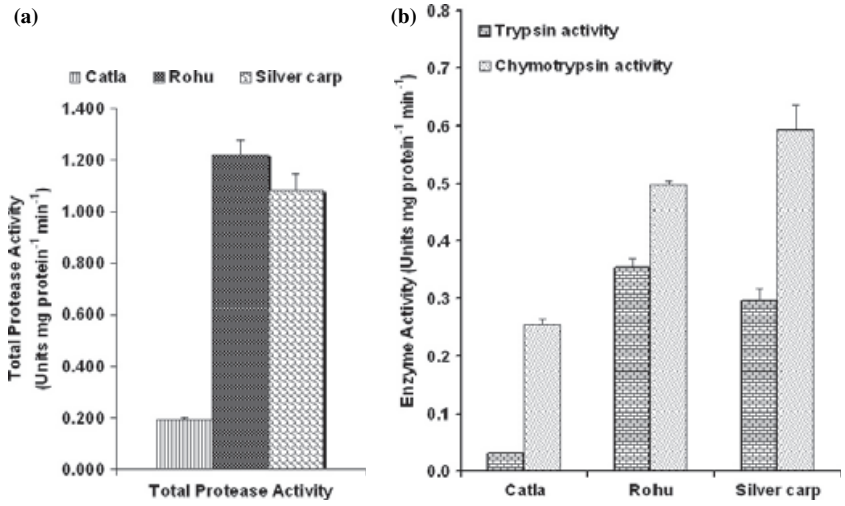

Figure 1 Total protease, trypsin and chymotrypsin activities (mean ± SE) of catla, rohu and silver carp ($n = 3$).

Effect of pH on enzyme activity

Digestive protease activity of rohu and silver carp displayed bell-shaped pH curve and showed maximum activities at pH 9.0. In contrast, catla digestive protease activity showed a broad pH range, maximum activity being in the pH range 8.0–11.0 (Fig. 2). In acidic pH range, e.g. at pH 6.0 enzymes of catla, rohu and silver carp retained 61.0%, 74.2% and 81.8% of their maximum activities. In these three species of carps, proteolytic enzyme activity was maximum in alkaline pH range.

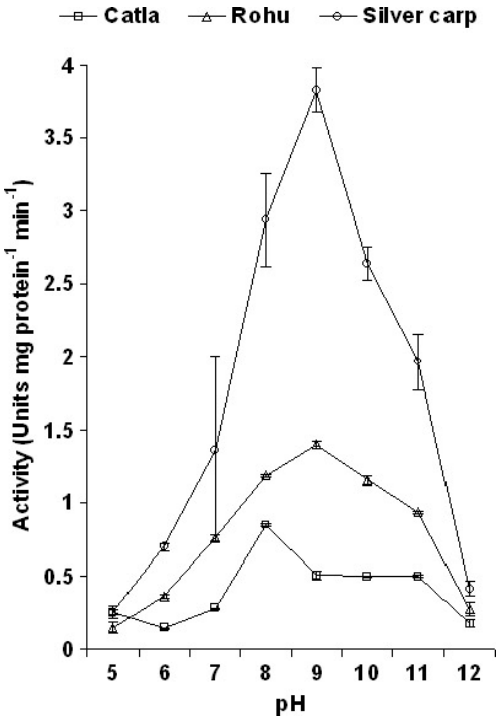

Figure 2 Effect of pH on protease activity of catla, rohu and silver carp.

Inhibition of enzyme activity

Inhibition of enzyme activity was evaluated with different specific inhibitors (Table 1). A total of about 67.4–77.9% protease activity was inhibited by SBTI in these three species evaluated. PMSF reduced the enzyme activity by 59.1–79.6%. The significant reduction in total protease activities by SBTI and PMSF revealed the presence of protease belonging to serine group in all these three species. TLCK, a specific inhibitor for trypsin, reduced azocasein hydrolysis by 29.7–40.2%. TPCK, the specific inhibitor for chymotrypsin, showed 29.3–35% inhibition of enzyme activity.

SDS-PAGE and substrate SDS-PAGE of protein and protease

The protein composition of all enzyme extract was evaluated by SDS-PAGE. Several protein bands were found in enzyme extracts of these three species (Fig. 3a). Molecular weight of protein bands was species-specific and ranged from 15.3 to

Table 1 Inhibition (%) of protease activity in three different species of carp with specific inhibitors

Inhibitors	Catla	Rohu	Silver carp
SBTI	67.4 ± 2.27 b	78.1 ± 2.05 a	77.9 ± 0.93 a
PMSF	59.1 ± 4.71 c	79.6 ± 0.70 a	65.3 ± 5.81 b
TLCK	29.7 ± 6.53 c	34.5 ± 5.40 b	40.2 ± 1.82 a
TPCK	35.0 ± 2.89 a	29.3 ± 4.36 c	32.4 ± 3.83 b

Mean values within the row not sharing a common letter are significantly ($P < 0.05$) different.

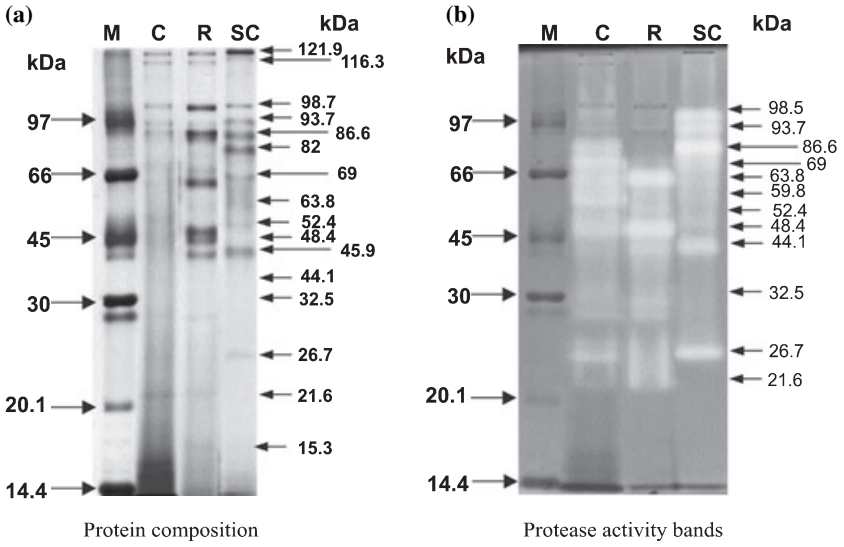

Figure 3 (a) SDS-PAGE of enzyme extract of catla, rohu and silver carp. Samples were diluted (1 : 1) with sample buffer. Lane 1: (M) molecular weight markers comprising Phosphorylase b (97 000), Bovine serum albumin (66 000), Ovalbumin (45 000), Carbonic anhydrase (30 000), Trypsin inhibitor, soybean (20 100) and α-Lactalbumin (14 400); Lane 2: (C) catla; Lane 3: (R) rohu; Lane 4: (SC) silver carp. 20 μg protein was loaded onto gel for each enzyme extract. After electrophoresis, gel was stained with Coommassie Brilliant Blue for 3 h and washed. (b) Substrate SDS-PAGE. Gel was prepared according to Laemmli (1970).

121.9, 21.6 to 121.9 and 26.7 to 121.9 for catla, rohu and silver carp respectively.

Protease activity zones of enzyme extracts of catla, rohu and silver carp by substrate SDS-PAGE are shown in Fig. 3b. Some protease activity zones were similar in catla and rohu. But species-specific protease activity bands with different electromobility in each species were also visible. The numbers of protease activity bands were 10 in catla (26.7–93.7 kDa), eight in rohu (21.6–69 kDa) and eight in silver carp (26.7–98.5 kDa).

Substrate SDS-PAGE showed the protease activity bands were inhibited by SBTI, PMSF and TLCK (Fig. 4). In catla high MW (93.7, 86.6 and 69 kDa) activity bands were inhibited by PMSF. Activity bands of 63.8 and 59.8 kDa were inhibited by SBTI and TLCK, whereas the activity bands of 52.4, 48.4 and 32.5 kDa were inhibited by SBTI, PMSF and TLCK. The activity band of 26.7 kDa was inhibited by TLCK only (Table 2).

In rohu, the activity bands of 69 and 63.8 kDa were inhibited by PMSF and TLCK, on the other hand the activity band of 59.8 kDa was inhibited only by PMSF. The activity bands of 44.1, 32.5 and 21.6 were inhibited by all inhibitors. The activity band of 48.4 was inhibited by SBTI and TLCK whereas the activity band of 26.7 kDa was inhibited by SBTI and PMSF (Table 2).

In silver carp, PMSF inhibited almost all the activity bands. The activity bands of 86.6, 69, 32.5 and 26.6 kDa were inhibited by SBTI. Whereas the activity bands of 86.6, 69, 59.8, 48.4 and 32.5 kDa were inhibited by TLCK (Table 2).

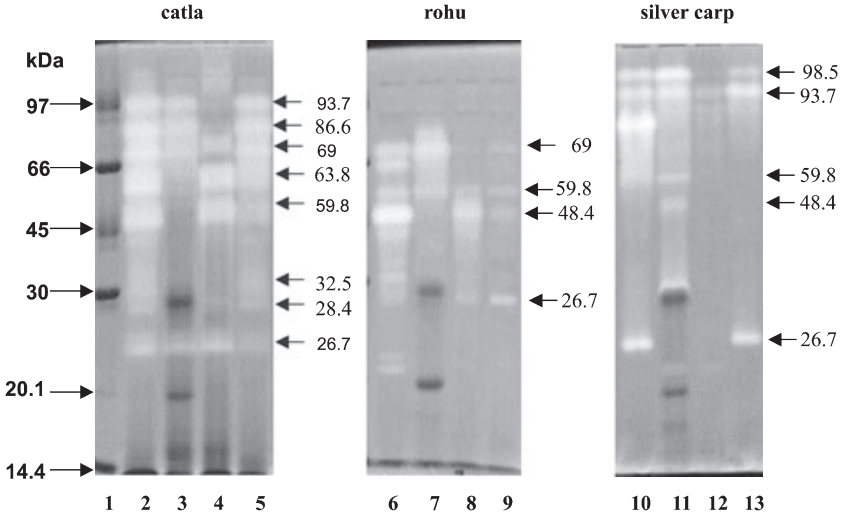

Figure 4 Zymogram of enzyme extracts of catla, rohu and silver carp. Samples containing 5 mU activity were incubated with different inhibitors for 1 h before loading onto gel. Lane 1: (M) molecular weight markers as in Fig. 3; Lanes 2, 6 and 10: control, enzyme extract without inhibitor; Lanes 3, 7 and 11: enzyme extract incubated with SBTI; Lanes 4, 8 and 12: enzyme extract incubated with PMSF; Lanes 5, 9 and 13: enzyme extract incubated with TLCK. After electrophoresis, gels were treated as described in Materials and methods section.

Table 2 Molecular weight of protease activity bands inhibited by SBTI, PMSF and TLCK in three species of carps

Protease activity bands molecular weight (kDa)	Catla	Rohu	Silver carp
98.5	A	A	I^P
93.7	I^P	A	I^P
86.6	I^P	A	$I^SI^PI^T$
69	I^P	I^PI^T	$I^SI^PI^T$
63.8	I^SI^T	I^PI^T	A
59.8	I^SI^T	I^P	I^PI^T
52.4	$I^SI^PI^T$	A	A
48.4	$I^SI^PI^T$	I^SI^T	I^PI^T
44.1	A	$I^SI^PI^T$	A
32.5	$I^SI^PI^T$	$I^SI^PI^T$	$I^SI^PI^T$
28.4	I^SI^P	A	A
26.7	I^T	I^SI^P	I^SI^P
21.6	A	$I^SI^PI^T$	A

A, absent; I^S, activity band inhibited by SBTI; I^P, activity band inhibited by PMSF; I^T, activity band inhibited by TLCK.

The activity bands of trypsin-like enzyme were observed in enzyme extracts. The molecular weights of observed trypsin-like enzymes were 26.7, 32.5, 48.4, 59.8 and 63.8 kDa for catla, as evidenced by inhibition with SBTI and TLCK (Table 2). In rohu, the MW of trypsin-like enzymes were 69, 63.8, 48.4, 44.1, 32.5 and 21.6 kDa. In silver carp, the MW of observed trypsin-like enzymes was 86.6, 69, 59.8, 48.4 and 32.5 kDa (Fig. 4).

In vitro *hydrolysis with pH-stat*

Among various protein sources, casein showed significantly ($P < 0.05$) higher degree of hydrolysis with different digestive enzymes (2.9–6.2%), except rohu (1.2%). The hydrolysis of Chilean fish meal with different enzyme preparations was significantly ($P < 0.05$) higher than silver cup and soybean meal. Among these three fish species, Chilean fish meal showed significantly ($P < 0.05$) higher degree of hydrolysis with the enzyme extract of rohu (Table 3). The degree of hydrolysis of silver cup ranged from 0.9 to 1.6% with different enzyme extracts. Silver carp showed significantly ($P < 0.05$) higher degree of hydrolysis (DH%) than catla and rohu regardless of protein sources, except soybean meal.

Discussion

In the present study, total protease activity was significantly higher in planktophage rohu followed by phytoplanktivore silver carp and zooplanktivore catla. Herbivorous fish try to compensate the lower amount of available proteins in their diet by increasing consumption rate and enzyme production (Hofer 1982). Sabapathy & Teo (1995) also observed similar results with rabbitfish *Siganus canaliculatus*. Protease activity has been described in catla (Phadatae & Srikar 1988;Kumar *et al*. 2000), but the knowledge of enzyme classes is limited particularly for catla and rohu. In the present study, chymotrypsin activity was always higher (1.5–8 folds) than trypsin activity.

The pH values for maximum protease activity in different enzyme extracts had been observed in alkaline range. The maximum protease activity observed at pH 9 for rohu and silver carp and pH 8–11 for catla. This study indicates the alkaline nature of digestive proteases present in these species. This may be due to absence of functional stomach in these three species. Several researchers also showed maximum protease activity at pH range of 8.0–10.0 in the intestine of carps (Jonas *et al*. 1983), rainbow trout and Atlantic salmon (Torrissen 1984), halibut and turbot (Glass *et al*. 1989). Erzsebet *et al*. (1983) found that in silver carp, enzyme activity increased slightly between pH 2 and pH 6, followed by a smaller plateau-like ranged at pH 7–8 and above pH 8, the activity increased again. Bitterlich (1985) observed that trypsin and amylase of silver carp had pH optima of 8.3. Carp and tench had proteolytic activity at neutral and alkaline pHs, whereas only very low activity was observed at acid pHs (Hidalgo *et al*. 1999).

Use of protease inhibitors with known specificity is helpful in the characterization of proteases class. Reduction in intensity of protease activity bands in samples treated with SBTI and TLCK was evidence of trypsin-like, and by SBTI and PMSF was evidence of chymotrypsin-like enzymes (Lemos *et al*. 2000). TLCK and TPCK are the specific inhibitors of trypsin and chymotrypsin, respectively, binds to the histidine residue within the active site (Shaw *et al*. 1965). Inhibition study of digestive proteases with SBTI, PMSF, TLCK and

Table 3 Degree of hydrolysis (DH%) of different protein sources was performed with a pH-stat method using commercial and carp digestive enzymes

Source of protein	Commercial enzymes	Catla	Rohu	Silver carp
Casein	3.9 ± 0.14 b	2.9 ± 0.08 c	1.2 ± 0.08 d	6.2 ± 0.08 a
Soybean meal	0.9 ± 0.14 b	0.5 ± 0.00 c	1.7 ± 0.16 a	1.8 ± 0.08 a
Silver cup	0.9 ± 0.08 b	1.0 ± 0.08 b	1.1 ± 0.08 b	1.6 ± 0.00 a
Chilean fish meal	2.3 ± 0.14 b	1.0 ± 0.08 c	2.1 ± 0.13 b	3.1 ± 0.08 a

Mean values within the row not sharing a common letter are significantly ($P < 0.05$) different.

TPCK had been determined in the present study. Significant inhibition of protease activity with SBTI and PMSF showed the abundance of serine proteases in these three species.

In the present study, several proteases having high molecular weight were found. Presence of several trypsin-like and chymotrypsin-like enzymes showed the variation in the isomeric form of these enzymes in different species. The MW of observed trypsin-like and chymotrypsin-like enzymes varied in these three fishes, which may highlight the species-specificity of proteases. In the present study high MW trypsin-like enzymes were observed in catla (26.7–63.8 kDa), rohu (21.6–69 kDa) and silver carp (32.5–86.6 kDa). Rathore *et al*. (2005) also observed high molecular weight trypsin-like enzymes (16.6–58.8 kDa) during early development of catla. The activity bands of trypsin-like enzymes (24 and 58.1 kDa) were also observed in hybrid of silver carp × bighead carp (Chakrabarti *et al*. 2006). Moyano *et al*. (1996) also identified bands of high molecular weight (23.5–95 kDa) trypsin-like enzymes in the digestive system of gilthead seabream. Different feeding habits of catla, rohu and silver carp may influence the composition of digestive protease. In silver carp, highly intense band of 26.7 kDa, the expected chymotrypsin was observed, which may be the reason for better performance of enzyme extract in *in vitro* hydrolysis of all protein substrates than other species (Table 3).

In *in vitro* hydrolysis study, casein showed higher degree of hydrolysis than other protein sources. The reason may be, the casein is a pure, highly digestible protein, free of anti-nutritive factors. In fact, it contains all the essential amino acids, and the basis of its use as a digestion protein standard. Among other commercial feedstuffs, Chilean fishmeal showed higher DH% compared with soybean meal and Silver cup. In an *in vitro* digestibility study, Eid & Matty (1989) had incubated different protein sources with intestinal extract of common carp *Cyprinus carpio*. They found that fish meal diets containing 140 and 270 g kg^{-1} protein had apparent digestibility of 90.2% and 93%; casein (180 and 360 g kg^{-1} protein), 91.5 and 83.2; soybean (100 and 200 g kg^{-1} protein), 84.2% and 85.3%; sunflower (80 and 160 g kg^{-1} protein), 64.2% and 66.1% and fish meal plus soybean meal (182 g kg^{-1}), 86.5%. When studying digestibility of protein feed sources by carp digestive enzymes, Chilean fish meal was better digested than Silver Cup and soybean meal, due to the presence of inhibitors in soybean meal. Protease inhibitors present in soybean meal were found responsible for the retardation of growth of grass carp (Dabrowski & Kozak 1979). Dabrowski *et al*. (1989) suggested that the increase in secretion of pancreatic proteases caused by the soybean protease inhibitors does not compensate for the decrease in the efficiency of dietary protein digestion. Apparent absorption of amino acid in carp fed soybean meal was much lower than rainbow trout, catfish and grass carp (Dabrowski 1983). Enzyme preparation from silver carp, when compared with the other carps, digested all feedstuff more adequately. Ronaldo *et al*. (1986) found that gelatin was the most digestible protein for milkfish *Chanos chanos*; casein, defatted soybean meal and fish meal were moderately digestible (50–90%).

Conclusion

This study provides insight to understand the protein digestion capabilities of three species of carps. Regardless of feeding habits, they possess some similar type of digestive proteases, but differ in digestive capability. Difference in the types of protease may reflect their efficiency to hydrolyse the protein used as food source. Further purification of proteases is required to understand the specific properties and their efficacies to hydrolyse protein rich diets to be used for a potential aquaculture. The information of digestive protease may help in the formulation of ideal artificial diet of the cultured species.

Acknowledgements

Authors are thankful to the Department of Science and Technology (DST), New Delhi, India and Consejo Nacional de Cienciay Tecnologia (CONACYT) Mexico, for grants that allowed travelling of researchers between La Paz and New Delhi.

References

Alarcón, F.J., Moyano, F.J. & Díaz, M. (1999) Effect of inhibitors present in protein sources on digestive proteases of juvenile sea bream (*Sparus aurata*). *Aquat. Living Resour*., **12,** 233–238.

Bitterlich, G. (1985) Digestive enzyme pattern of two stomachless filter feeders, silver carp, *Hypophthalmichthys molitrix* Val., and bighead carp, *Aristichthys nobilis*Rich. *J. Fish Biol*., **27,** 103–112.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of micro gram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem*., **72,** 248–254.

Chakrabarti, R. & Sharma, J.G. (2005) Digestive physiology of fish larvae during ontogenic development: a brief overview. *Indian J. Anim. Sci*., **75,** 1337–1347.

Chakrabarti, R., Rathore, R.M., Mittal, P. & Kumar, S. (2006) Functional changes in digestive enzymes and characterization of proteases of silver carp (♂) and bighead carp (♀) hybrid, during early ontogeny. *Aquaculture*, **253,** 694–702.

Dabrowski, K. (1983) Digestion of protein and amino acid absorption in stomachless fish, common carp (*Cyprinus carpio* L). *Comp. Biochem. Physiol*., **74A,** 409–415.

Dabrowski, K. & Kozak, B. (1979) The use of fish meal and soybean meal as protein source in the diet of grass carp. *Aquaculture*, **18,** 107–114.

Dabrowski, K., Poczyczynski, P., Kock, G. & Berger, B. (1989) Effect of partial or totally replacing fish meal protein by soybean meal protein on growth, food utilization and proteolytic enzyme activities in rainbow trout (*Salmo gairdneri*). New *in vivo* test for exocrine pancreatic secretion. *Aquaculture*, **77,** 29–49.

Dimes, L.E. & Haard, N.F. (1994) Estimation of protein digestibility-I. Development of an *in vitro* method for estimating protein digestibility in salmonids (*Salmo gairdneri*). *Comp. Biochem. Physiol.*, **108A,** 349–362.

Eid, A.E. & Matty, A.J. (1989) A simple *in vitro* method for measuring protein digestibility. *Aquaculture*, **79,** 111–119.

Erlanger, B.F., Kokowsky, N. & Cohen, W. (1961) The preparation and properties of two chromogenic substances of trypsin. *Arch. Biochem. Biophys.*, **95,** 271–278.

Erzsebet, J., Ragyanszki, M., Olah, J. & Boross, L. (1983) Proteolytic digestive enzymes of carnivorous (*Silurus glanis* L.), herbivorous (*Hypophthalmichthys molitrix* Val.) and omnivorous (*Cyprinus carpio* L.) fishes. *Aquaculture*, **30,** 145–154.

García-Carreño, F.L. (1992) Protease inhibition in theory and practice. *Biotech. Educ.*, **3,** 145–150.

García-Carreño, F.L. & Haard, N.F. (1993) Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. *J. Food. Biochem.*, **17,** 97–113.

García-Carreño, F.L., Dimes, L.E. & Haard, N.F. (1993) Substrate gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Anal. Biochem.*, **214,** 65–69.

Glass, H.D., McDonald, N.L., Moran, R.M. & Stark, J.R. (1989) Digestion of protein in different marine species. *Comp. Biochem. Physiol.*, **94B,** 607–611.

Grabner, M. (1985) An *in vitro* method for measuring protein digestibility of fish feed component. *Aquaculture*, **48,** 97–110.

Hidalgo, M.C., Urea, E. & Sanz, A. (1999) Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. *Aquaculture*, **170,** 267–283.

Hofer, R. (1982) Protein digestion and proteolytic activity in the digestive tract of an omnivorous cyprinid. *Comp. Biochem. Physiol.*, **72A,** 55–63.

Jhingran, V.G. (1991) *Fish and Fisheries of India, 3rd edn*, p. 727. Hindustan Publishing Corporation, New Delhi, India.

Jonas, E., Razyanszki, M., Olah, J. & Bross, L. (1983) Proteolytic digestive enzymes of carnivorous (*Silurus lanis* L.), herbivorous (*Hypophthalmicthys molitrix*) and omnivorous (*Cyprinus carpio*). *Aquaculture*, **30,** 145–154.

Kumar, S., Sharma, J.G. & Chakrabarti, R. (2000) Quantitative estimation of proteolytic enzyme and ultrastructure study of anterior part of intestine of Indian major carp (*Catla catla*) larvae during ontogenesis. *Curr. Sci.*, **79,** 1007–1011.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature*, **277,** 680–685.

Lemos, D., Esquerra-Maurer, J.M. & García-Carreño, F.L. (2000) Studies on protein digestion in penaeid shrimps: digestive proteinase inhibitor and feed digestibility. *Aquaculture*, **186,** 89–105.

Maitra, S. & Ray, A.K. (2003) Inhibition of digestive enzymes in rohu, *Labeo rohita* (Hamilton), fingerlings by tannin: an *in vitro* study. *Aquac. Res.*, **34,** 93–95

Montgomery, D.C. (1984) *Design and Analysis of Experiments*, p. 533. John Wiley, New York, NY.

Moyano, F.J., Diaz, M., Alarcon, F.J. & Sarasquete, M.C. (1996) Characterization of digestive enzyme activity during larval development gilthead seabream (*Sparus aurata*). *Fish Physiol. Biochem.*, **15,** 121–130.

Opuszynski, K. & Shireman, J.V. (1995) *Herbivorous Fishes. Culture and Use for Weed Management*, p. 223. CRC Press, London, Tokyo.

Pedersen, B. & Eggum, B.O. (1983) Prediction of protein digestibility-an in vitro enzymatic pH-stat procedure. *Tierphysiol Tieternahrg u Futtermittelkde*, **49,** 277–286.

Phadatae, S.V. & Srikar, L.N. (1988) *Effect of Formulated Feeds on Protease Activity and Growth of Three Species of Carps*. The First Indian Fisheries Forum, Proceedings, December 4–8, 1987, Mangalore, Karnataka, 1988, p. 85–88.

Rathore, R.M., Kumar, S. & Chakrabarti, R. (2005) Digestive enzyme patterns and evaluation of protease classes in *Catla catla* (Family: Cyprinidae) during early developmental stages. *Comp. Biochem. Physiol.*, **142B,** 98–106.

Ronaldo, P.F., Mae, R.C., Rey, L.M. & Adan, P.J. (1986) Digestibility in milkfish, *Chanos chanos* (Forsskal): effects of protein source, fish size and salinity. *Aquaculture*, **59,** 93–105.

Sabapathy, U. & Teo, L. (1995) Some properties of the intestinal proteases of the rabbitfish, *Siganus canaliculatus* (Park). *Fish Physiol. Biochem.*, **14,** 215–221.

Seenappa, D. & Devaraj, K.V. (1995) Effect of different levels of protein, fat and carbohydrate on growth, feed utilization and body carcass composition of fingerlings in *Catla catla* (Ham.). *Aquaculture*, **129,** 243–249.

Shaw, E., Mares-Guia, M. & Cohen, W. (1965) Evidence for an active center histidine in trypsin through the use of a specific reagent 1-chloro-3-tosylamido-7-amino-2-heptaone, the chloromethyl ketone derived from *N*-tosyl-L-lysine. *Biochemistry*, **4,** 2219–2224.

Stauffer, C. (1989) *Enzyme Assays for Food Scientists*, p. 317. Van Nostrand, New York.

Torrissen, K.R. (1984) Characterization of proteases in the digestive tract of Atlantic salmon (*Salmo salar*) in comparison with rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.*, **77B,** 669–674.

Xie, P. (1999) Gut contents of silver carp, *Hypophthalmicthys molitrix*, and the disruption of a centric diatom, *Cyclotella*, on passage through the esophagus and intestine. *Aquaculture*, **180,** 295–305.