ELSEVIER

Aquaculture 203 (2002) 321–333

Aquaculture

www.elsevier.com/locate/aqua-online

# Partial characterization and activities of proteases from the digestive tract of discus fish (*Symphysodon aequifasciata*)

Alexander S.C. Chong *, Roshada Hashim, Lee Chow-Yang, Ahyaudin B. Ali

*School of Biological Sciences, Universiti Sains Malaysia, 11800, Penang, Malaysia*

Received 18 January 2001; received in revised form 26 March 2001; accepted 26 March 2001

**Abstract**

A series of studies based on biochemical assays and electrophoretical observations was conducted in order to partially characterise various types of proteases present in the digestive tract of discus (*Symphysodon aequifasciata*), a highly valuable ornamental species. Casein digestion assays revealed the presence of acidic protease from the stomach region with optimum activity at pH 2.0 and alkaline proteases from the intestinal section with optimum activities observed at pH ranging from 7.5 to 9.0 and 11.5 to 12.5, respectively. Further assays with specific substrates and also pre-incubation with specific protease inhibitors showed the importance of trypsin and chymotrypsin in protein digestion while also revealing the presence of serine-proteases and metallo-proteases, respectively. SDS-PAGE technique using casein as substrate and specific protease inhibitors also showed the presence of eight distinct proteases based on molecular weights ranging from 19.2 to 76.5 kDa. 

*Keywords:* Proteases; Discus (*Symphysodon aequifasciata*); Digestive tract

## 1. Introduction

Discus (*Symphysodon aequifasciata*) is an important ornamental fish cultured in Asia with a high and consistent demand for export. Discus farming in recent years has shifted from cultivation of wild strains to various types of cultivated varieties generated through strain selection and interbreeding programmes (Koh et al., 1999). Most discus farmers rely on freshly prepared wet feed based on several ingredients such as beef heart, shrimp

* Corresponding author. Tel.: +604-6577-888 ext. 4006; fax: +604-6565-125.
*E-mail address:* sendalex@hotmail.com (A.S.C. Chong).

0044-8486/02/$ - see front matter 
PII: S0044-8486(01)00630-5

and cockles as protein sources. These feeds are inconsistent in terms of nutrient content and disintegrate easily in water. The development of a dry, economical and highly digestible formulated feed with adequate nutrient content is therefore desirable for intensive culture of this fish. However, ingredient digestibility in formulated feeds is an important issue and the development of these feeds involves the screening and evaluation of alternative plant and non-fish meal-based ingredients as protein sources.

The ability of the fish to utilise ingested nutrients depends on the activities of digestive enzymes present in various locations along the digestive tract. Investigations on proteases activities of several marine species have been carried out in order to develop an effective diet for intensive farming of these species through proper understanding of their digestive capabilities towards various feed ingredients (Clark et al., 1985; Alarcon et al., 1998). Glass et al. (1989) for instance suggested that understanding of the properties, function and optimised conditions for protein hydrolysis of digestive proteases in fish will enable a more accurate measurement of protein digestibility by a particular species. Rate of digestion and adsorption of essential amino acids during proteolysis could also be determined with proper knowledge of functional activities of proteases (Eshel et al., 1993). Krogdahl et al. (1994) have reported a positive relationship between trout intestinal trypsin secretion rate with digestibility coefficient values of feed by fish. In chinook salmon, weight gain was found to be positively correlated with the ability of the digestive enzymes to hydrolyse diets (Haard et al., 1996). Functional and characterization studies were also carried out on digestive proteases of crustaceans with commercial aquaculture value for similar purposes mentioned above (Galgani et al., 1984; Galgani and Nagayama, 1987; Jiang et al., 1991; Garcia-Carreno, 1992; Vega-Villasante et al., 1995; Ribeiro and Jones, 2000). Hence, this study was designed to determine the presence of several major proteases in the digestive tract of this species.

## 2. Material and methods

### *2.1. Fish and preparation of crude enzyme extract*

Juvenile discus (*S. aequifasciata*) were obtained from stock originating from a breeding programme initiated at the Aquaculture Research Complex, Universiti Sains Malaysia. Ten-week-old fish with a mean initial weight of $5.2 \pm 0.8$ g were selected for enzyme extraction. The fish were previously maintained on *Artemia* nauplii for the first 3 weeks after hatching followed by dry pellets (Tetrabits®) at a rate of 5% body weight per day.

The fish were starved for approximately 12 h prior to sampling and subsequently killed and dissected immediately. Stomach and intestines were separated, the tissue contents removed and rinsed with cold distilled water. Tissues from 10 fish were pooled and homogenised in cold Tris–HCl 50 mM buffer (pH 7.5) at 1 g tissue per ml buffer using a hand-held glass homogeniser. The homogenate was then centrifuged at 4°C at $10{,}000 \times g$ for 15 min. The supernatant containing the enzymes was stored at $-70$°C before analysis. The soluble protein content of enzyme extract was measured according to Lowry et al. (1951) using a protein assay kit by Sigma®.

## 2.2. Enzyme assay

### 2.2.1. Effect of pH on total protease activity

The effect of different pH incubation on the proteolytic activities of crude enzyme extract was determined based on the casein hydrolysis assay of Kunitz (1947) as modified by Walter (1984). A series of different buffers was used for different pH conditions: 0.1 M KCl–HCl (pH 1.5–2.5), 0.2 M glycine–HCl (pH 3.0–4.0), 0.2 M phosphate buffer (pH 5.0–6.0), 0.1 M Tris–HCl (pH 7.0–9.0) and glycine–NaOH (pH 10.0–13.0) (Glass et al., 1989; Sabapathy and Teo, 1993; Munilla-Moran and Rey, 1996; Hidalgo et al., 1999). The enzyme-substrate mixture consisted of 0.3 ml 1% (w/v) casein in water, 0.5 ml selected buffer and 0.3 ml crude enzyme extract incubated in a water bath for 1 h at 37°C. A total of 0.5 ml trichloroacetic acid (TCA, 12% w/v) was then added to the reaction mixture to stop the reaction. This mixture was then allowed to stand for 1 h at 4°C before centrifuging at $8000 \times g$ for 15 min. Absorbance of the supernatant was recorded at 280 nm to measure the amount of tyrosine produced. The blank used for this assay was prepared by incubating a mixture of the crude enzyme extract, buffer and water for 1 h at 37°C, followed by the addition of TCA and casein. One unit of specific activity was defined as the amount of enzyme needed to produce 1 μg tyrosine per min per mg soluble protein of enzyme extract (U mg protein$^{-1}$).

### 2.2.2. Trypsin and chymotrypsin activities

Trypsin activity of the enzyme extract was assayed using benzoyl-DL-arginin-*p*-nitroanilide (BAPNA) as substrate according to Erlanger et al. (1961). The 43.5 mg BAPNA (Fluka Chemicals®) was dissolved in 1 ml of dimethylsulfoxide (DMSO) and made up to 100 ml with 0.05 M Tris–HCl buffer containing 0.02 M of $CaCl_2 \cdot 2H_2O$, pH 7.5. Twenty-five microliters of enzyme extract was mixed with 1.25 ml of freshly prepared BAPNA substrate solution and left for 10 min at 37°C before adding 30% acetic acid to stop the reaction. The absorbance of the resulting mixture was then determined at 410 nm followed by calculation of trypsin amidase activity (BAPNA U mg protein$^{-1}$) using the following formula (Erlanger et al., 1961):

$$\frac{(\text{Absorbance value at 410 nm/min} \times 1000 \times \text{volume of reaction mixture})}{(8800 \times \text{mg protein in the assay})}$$

where 8800 is the extinction coefficient of *p*-nitroaniline.

Chymotrypsin activity was assayed according to Erlanger et al. (1961) using succinyl-$(Ala)_2$-Pro-phe-*p*-nitroanilide (SAPNA) as substrate. Freshly prepared substrate comprising of 0.1 mM SAPNA in 50 mM Tris–HCl and 20 mN $CaCl_2$ at pH 8.5 was used for the assay. A 0.59-ml aliquot of the substrate solution was mixed with 10 μl enzyme extract with reaction temperature of 25°C. The increase of absorbance value (410 nm) was recorded every min for 5 min. Chymotrypsin activity was then expressed as SAPNA units/mg as

$$\frac{(\text{Absorbance value at 410 nm/min} \times 1000 \times \text{volume of reaction mixture})}{(8800 \times \text{mg protein in the assay})}$$

*2.2.3. Effect of inhibitors on protease activity*

The major classes of proteases present in the discus enzyme extract were characterised using selected specific protease inhibitors (Garcia-Carreno, 1992; Munilla-Moran and Rey, 1996). The list of inhibitors were as follows: 100 mM phenylmethylsulfonyl fluoride (PMSF; Fluka® Chemicals) in ethanol for serine proteases inhibition, 10 mM soybean trypsin inhibitor (SBTI; Sigma®) for trypsin inhibition, 10 mM tosyl-lysine chloromethyl ketone (TLCK; Sigma®) in 1 mM HCl for trypsin inhibition, 5 mM tosyl-phenylalanine chloromethyl ketone (TPCK; Sigma®) in ethanol for chymotrypsin inhibition and 0.5 M ethylenediaminetetraacetic acid (EDTA) for metalloprotease inhibition.

An aliquot of the enzyme extract was incubated with the respective inhibitor at a ratio (v/v) of 1:1 for 60 min at 26°C. This enzyme-inhibitor mixture was then assayed using casein as the substrate and at the pH determined previously. The enzyme extract incubated with a solution reagent of inhibitors prior to casein assay was the control. Percentage of inhibition was calculated as

$$\frac{(\text{Enzyme activity of control} - \text{enzyme activity in the presence of inhibitors})}{\text{Enzyme activity of control}} \times 100$$

*2.3. Classification of proteases by SDS-PAGE*

SDS-PAGE electrophoresis (Laemmli, 1970; Bollag and Edelstein, 1991) was also used to characterise the proteases present in the crude enzyme extract. This technique involved the use of zymograms for substrate SDS-PAGE electrophoresis, the application of molecular weight markers and specific protease inhibitors as described by Garcia-Carreno and Haard (1993). Crude enzyme extract was mixed with sample buffer (Tris–HCl 1 M pH 6.8, glycerol, SDS, bromophenol blue) at a ratio (v/v) of 2:1. Five-microliter extract/sample buffer mixture was loaded into SDS-PAGE gels ($6.0 \times 8.0$ cm) with thickness of 0.5 mm. The gel consisted of 5% of stacking gel and a 12% separating gel according to Bollag and Edelstein (1991). Electrophoresis was conducted at 120 V using the Mini Protean III® electrophoresis system (BIORAD® Laboratories, California) for approximately 90 min at 4–6°C with an electrophoresis buffer of Tris–glycine–sodium dodecyl sulfate. The gel was then immersed in casein solution (3% in 50 mM Tris–HCl at pH of 7.5) at 4°C for 30 min to allow absorption of casein into gel. The gel with the absorbed casein was then removed and placed in a waterbath at 27°C for an additional 90 min to allow proteases in gel to digest the casein. This was followed by staining using Coomassie Brillant Blue R-250 (BIORAD® Laboratories) dissolved in a solution containing acetic acid, methanol and distilled water (1 g Brillant Blue in 450 ml methanol, 450 ml distilled water and 100 ml of acetic acid) for 90 min. The whole gel was stained blue due to the presence of casein except in areas containing protease activity which had digested the substrate. The clear bands, indicating the presence of protease, were more apparent upon destaining in methanol-acetic-acid-distilled water solution for an additional 60 min. Molecular weight markers (SDS-PAGE Standards, Broad Range, BIORAD® Laboratories) with molecular weight range of 7–205 kDa

were also used for protease molecular weight determination. Electrophoretic protease characterization was then carried out using the specific inhibitors mentioned above. Here, the enzyme extract was incubated together with the various inhibitors for 60 min at 27°C prior to electrophoresis.

## 3. Results

Fig. 1 shows the pH dependence of the proteolytic activities in the stomach and intestine of discus, respectively. Stomach proteases showed a maximum activity ($0.64 \pm 0.09$ U mg protein$^{-1}$) at pH 2.0, indicating the presence of pepsin-like proteases. Proteolytic activities decreased as pH increased to near zero activity in alkaline solutions. The intestinal extract showed two peak activity regions between pH 7.5–9.0 and at a higher pH of 11.5–12.5 indicating the existence of two groups of alkaline proteases functioning in the intestinal regions of the discus. The BAPNA and SAPNA assays both showed the presence of chymotrypsin ($0.61 \pm 0.04$ U mg protein$^{-1}$) and trypsin ($0.79 \pm 0.05$ U mg protein$^{-1}$) in the intestine but significantly lower levels in the stomach (Fig. 2).

The effect of different protease inhibitors on the proteolytic ability of stomach and intestinal extracts is shown in Fig. 3. The results show that the inhibitors tested did not cause a significant reduction in the activity of stomach proteases as compared to those from the intestine. Percentage inhibition of the intestine proteases was highest with SBTI ($69.03 \pm 6.90\%$), followed by PMSF ($58.92 \pm 4.89\%$), TLCK ($46.43 \pm 5.31\%$), EDTA ($45.90 \pm 4.21\%$) and TPCK ($39.71 \pm 6.78\%$). This indicates the presence of serine

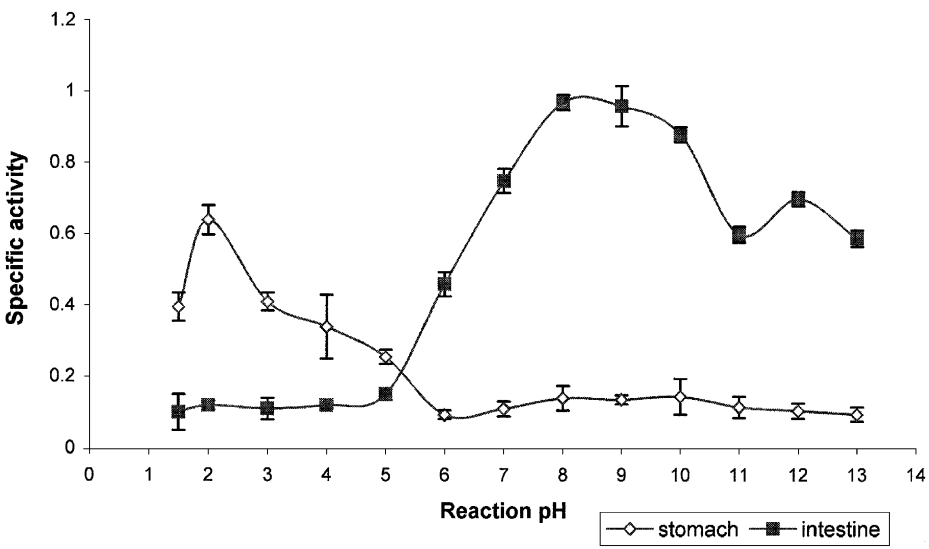


Fig. 1. Effect of incubation pH on the proteolytic activity of extract from discus stomach and intestine based on casein assay. Results are means from triplicate assays.

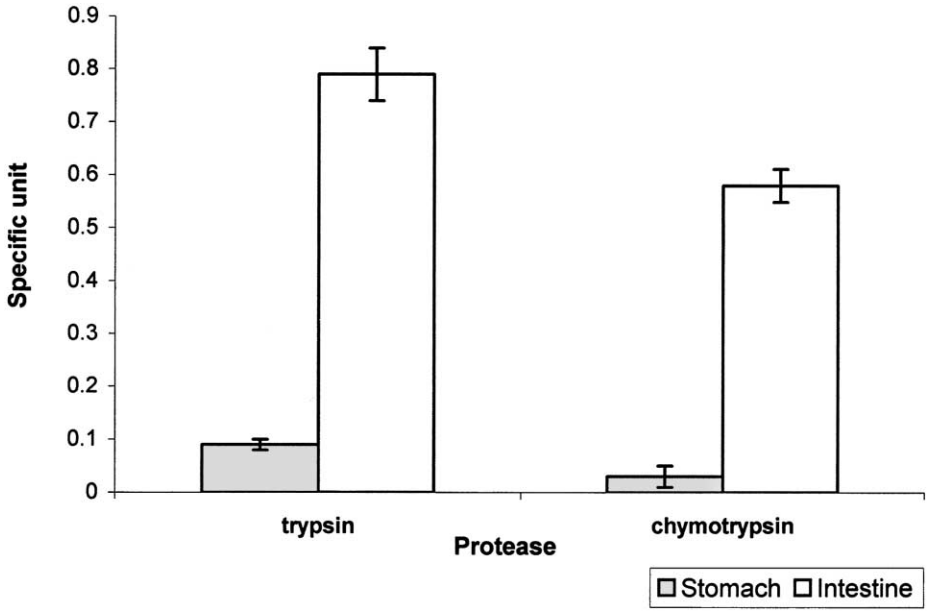


Fig. 2. Trypsin and chymotrypsin activity based on BAPNA and SAPNA assays from discus stomach and intestine. Results are means from triplicate assays.

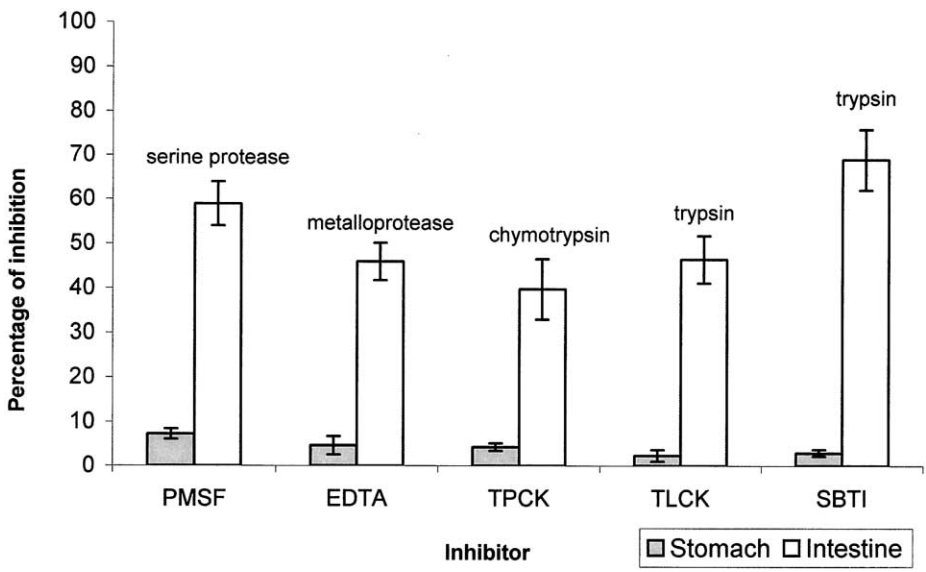


Fig. 3. Effect of selected inhibitors on the protease activity of discus stomach and intestine based on casein assay. Results are means from triplicate assays.

proteases, in particular trypsin and chymotrypsin, together with metalloproteases as the major groups of proteases in the discus intestine.

Further characterization of the discus intestinal proteases using substrate SDS-PAGE electrophoresis is shown in Fig. 4. At least eight alkaline proteases in terms of their different molecular weights are shown (control lane). The different classes of proteases were detected in the presence of the specific inhibitors by the disappearance or reduced intensity of the bands compared to the control. Pre-incubation of the enzyme extract with EDTA caused changes in the activities of the first four bands (zone A) and the disappearance of the fifth and sixth bands (zone B) indicating the presence of metalloproteases in discus intestine. The presence of PMSF, a serine protease inhibitor, inhibited protease activity as revealed by the complete disappearance of all bands (zones C and D) except bands 5 and 6. Besides showing that the affected bands are of the serine protease class, it also confirmed results from the EDTA inhibition lane which indicate that the fifth and sixth bands are metalloprotease enzymes. The use of TPCK, TLCK and SBTI further characterised the serine protease enzymes. TPCK, a chymotrypsin inhibitor, for example, caused the disappearance of proteolytic activities in the last two bands (zone E) while both TLCK and SBTI, which inhibit trypsin, affected the first two bands (zone F). These findings together with the comparison of bands with standard molecular weight markers indicate that two types of metalloproteases (36.1–39.8 kDa), trypsin (73.3–76.5 kDa), non-trypsin/chymotrypsin serine proteases (58.7–61.4 kDa) and chymotrypsin (19.2–21.8 kDa) were detected in the intestine of discus.

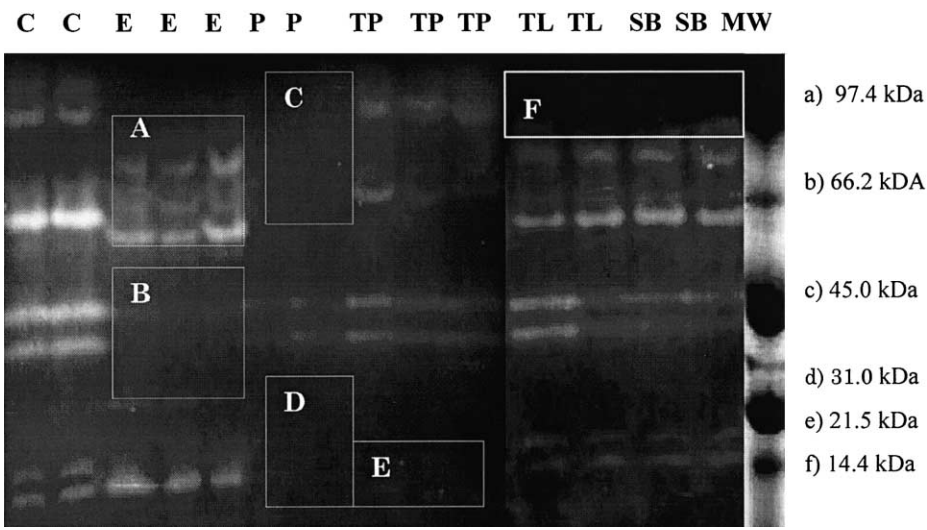


Fig. 4. Substrate-SDS PAGE electrophoresis gel image showing different bands of proteases from discus intestine extract and the effect of various inhibitors on their activities (refer to text for description). Lanes are described as follows: C: control, without inhibitors; E: inhibition with EDTA; P: inhibition with PMSF; TP: inhibition with TPCK; TL: inhibition with TLCK; SB: inhibition with SBTI; MW: molecular weight markers. Markers shown are (a) phosphorylase b; (b) bovine serum albumin; (c) ovalbumin; (d) carbonic anhydrase; (e) soybean trypsin inhibitor; (f) lysozyme.

## 4. Discussion

Difficulties in comparing enzyme activities quantitatively from different studies due to differences in sampling and preparation of enzyme extract from tissues have been highlighted by various researchers (Alarcon et al., 1998; Hidalgo et al., 1999). Crude proteases that have been extracted directly by grinding of stomach, intestine and pyloric caecae tissues after discarding their contents have been utilised (Torrissen, 1984; Das and Tripathi, 1991; Dimes et al., 1994; Chakrabarti et al., 1995) or obtained from the stomach and intestine together with their contents (Hofer and Schiemer, 1981; Hofer, 1982; Uys and Hecht, 1987). Starvation of fish prior to sampling of enzymes has also been carried out by other workers (Glass et al., 1989; Das and Tripathi, 1991; Eshel et al., 1993; Dimes et al., 1994). Furthermore, these workers have used different concentration of homogenates in terms of weight of tissue per volume of buffer solution.

Protein digestion in discus occurs in both the acidic region in the stomach and the alkaline region in the intestine and this is in agreement with various studies on other fish digestive proteases which have also shown a high activity in the acidic region in the stomach and alkaline pH region in the intestine (Clark et al., 1985; Martinez and Serra, 1989). Our study shows that discus, which possesses a defined stomach structure, secretes acidic proteases that require a pH range of 2.0–3.0 for optimum activity. Proteolytic activities at low pH have also been reported in species with a clear stomach region and a high pepsin secretion such as eel, tilapia, salmon, sea bass and trout (Jonas et al., 1983; Twining et al., 1983; Torrissen, 1984; Sabapathy and Teo, 1993; Yamada et al., 1993). Pepsin has been identified as the major acidic protease in fish stomach acting as the first proteolytic enzyme to break large peptide chains (Sabapathy and Teo, 1993; Tengjaroenkul et al., 2000). Species with thin stomach walls such as tilapia require a highly acidic medium to enable biochemical digestion of protein as compared to those with muscular stomach such as African catfish which relies more on the mechanical breakdown of polypeptides and possesses lower pepsin secretion (Maier and Tullis, 1984; Uys and Hecht, 1987).

This study also showed a high proteolytic activity in intestinal extract at two alkaline pH range (8.0–9.0 and 12.0–13.0) indicating the presence of two groups of alkaline proteases with different optimum pH. Alarcon et al. (1998), who detected peak activities at pH 7.0 and 10.0 in seabream and dentex, also suggested the presence of at least two major groups of alkaline proteases. This is supported by other reports which showed maximum protease activities at a pH range of 8.0–10.0 in the intestinal region based on casein assays on carp (Jonas et al., 1983), rainbow trout and Atlantic salmon (Torrissen, 1984), halibut and turbot (Glass et al., 1989), striped and European sea bass (Eshel et al., 1993), and goldfish (Hidalgo et al., 1999). In the Dover sole, high intestinal protease activity at pH 7.0–8.0 was related to trypsin and chymotrypsin activities while the activities of elastase or collegenase were related to a higher pH of 9.5 by Clark et al. (1985).

Further characterization of intestinal proteases was achieved with the use of specific inhibitors in casein assays. The significant reduction in the overall protease activities due to the presence of PMSF, a serine protease inhibitor, for example, indicates the presence

of a serine protease-type enzyme in discus intestine. Similar findings have also been reported with the use of PMSF with other aquatic species (Garcia-Carreno and Haard, 1993; Dimes et al., 1994). Three different molecular weight ranges (19.2–21.8, 58.7–61.4 and 73.3–76.5 kDa) for this group of enzymes were obtained in this study. Cohen et al. (1981) reported a carp serine protease with a molecular weight of approximately 25.0 kDa.

The collective results from the inhibition with SBTI and TLCK in casein assays, use of BAPNA as substrate and substrate SDS-PAGE electrophoresis here also indicate the importance of trypsin and chymotrypsin for digestion in discus. A reduction of 50% in the activity of trout and chinook salmon digestive proteases was observed after incubating with SBTI (Dimes et al., 1994). Eshel et al. (1993) estimated that in carnivorous fish intestines, trypsin contributes to 40–50% of the overall protein digestion activity. Electrophoresis data showed two trypsin bands with a molecular weight range of 73.3–76.5 kDa. Trypsin with a lower weight range (23–28 kDa) has also been reported in other fish species (Cohen et al., 1981; Hjelmeland and Raa, 1982; Simpson and Haard, 1984). In comparison, Moyano et al. (1996) detected trypsins ranging from 23.5 to 95.0 kDa in seabream. The presence of 'double bands' for each group of enzymes here also reveals the possibility of homogenous and genetically closely related enzymes known as isozymes as reported elsewhere (Cohen et al., 1981; Torrissen, 1984; Torrisen and Barnung, 1991). BAPNA studies showing higher trypsin activity in discus intestine ($0.79 \pm 0.05$ U mg protein$^{-1}$) compared to stomach ($0.09 \pm 0.03$ U mg protein$^{-1}$) also correlate with reports on other species such as the rabbitfish and sea bass (Sabapathy and Teo, 1993), anchovy (Martinez and Serra, 1989) and turbot (Munilla-Moran and Stark, 1990) where a higher trypsin level was detected in the intestine compared to the stomach which possessed very little or no activity. It has also been reported that trypsin activity is generally higher in carnivorous and omnivorous fish compared to herbivorous species (Hofer and Schiermer, 1981; Munilla-Moran and Stark, 1990). The peak activity shown in pH range of 8.0–9.0 in the casein assay is likely due to the high trypsin activities since several studies have shown optimised pH for trypsin reaction to be a pH range of 7.0–9.0 (Yoshinaka et al., 1984a,b; Clark et al., 1985; Genicot et al., 1988; Das and Tripathi, 1991).

Comparison of the reduction in caseinolytic activity between extracts incubated with SBTI ($69.03 \pm 6.90\%$) and TLCK ($46.43 \pm 5.31\%$) with incubation with TPCK ($39.71 \pm 6.78\%$) suggests that compared to trypsin, chymotrypsin activity is lower in discus. BAPNA and SAPNA assay results showed a higher specific activity value for trypsin. Jonas et al. (1983) reported that trypsin activities were generally higher compared to chymotrypsin for carnivorous fishes while in omnivorous and herbivorous species, activities of chymotrypsin were higher. However, both types of proteases are believed to play a collaborative role in protein digestion at the intestinal tract (Cohen et al., 1981; Uys and Hecht, 1987; Glass et al., 1989). Trypsin specifically hydrolises the carboxyl end of lysine and arginine peptide bond while chymotrypsin generally hydrolises peptide bonds near hydrophobic amino acids such as tyrosine, phenylalanine and tryptophan (Neurath, 1989). Martinez and Serra (1989) also highlighted the importance of both these enzymes in digestion of different types of protein substrate in anchovies. The lower molecular weight range obtained for chymotrypsin (19.2–21.8 kDa) here as

compared to trypsin (73.3–76.5 kDa) is also in agreement with reports for dentex and seabream (Alarcon et al., 1998) (Table 1).

The results from casein-EDTA assays and substrate SDS-PAGE electrophoresis suggest the presence of another group of proteases in discus intestine, i.e. the metalloprotease. The role and importance of metalloproteases in fish have been discussed by Yoshinaka et al. (1985). Reduction of nearly 50% of total activity in intestinal extract of discus due to inhibition by EDTA also reveals the quantitative function of these enzymes. Bands from gel images estimated the molecular weight of these proteases to be at 36.8–39.1 kDa. A similar molecular weight (35 kDa) has been also reported for carp carboxypeptidases, a common type of fish-secreted metalloproteases (Cohen et al., 1981).

The presence of serine proteases and in particular trypsin and chymotrypsin together with metalloprotease enzymes suggests a protein digestion model similar to that of other fishes with endoproteases hydrolysis followed by exoproteases reaction. However, the kinetics and activity characteristics affected by post-feeding time, anti-nutritional inhibitors in feed ingredients and diets on these enzymes remain to be studied (De Silva and Perera, 1984; Lopez et al., 1999). Munilla-Moran and Rey (1996), for instance, in

Table 1
Partial characterization of discus alkaline proteases as compared to other species

| Species | Number of proteases identified[a] | Molecular weight range (kDa) | Types of proteases identified | Refs. |
|---|---|---|---|---|
| Discus (*Symphysodon* sp.) | 8 | 19.2–76.5 | Serine proteases<br>Trypsin<br>Chymotrypsin<br>Metalloproteases | |
| Carp (*Cyprinus carpio*) | 4 | 25–34 | Serine proteases<br>Trypsin<br>Chymotrypsin<br>Metalloproteases | Cohen et al. (1981) |
| Rainbow trout (*Oncorhyncus mykiss*) | 7–9 | 18–79 | Serine proteases<br>Trypsin<br>Chymotrypsin<br>Metalloproteases | Dimes et al. (1994) |
| Coho salmon (*O. kistuch*) | 6–8 | 18–72 | Serine proteases<br>Trypsin<br>Chymotrypsin<br>Metalloproteases | Dimes et al. (1994) |
| Chinook salmon (*O. tshawytscha*) | 6–8 | 18–72 | Serine proteases<br>Trypsin<br>Chymotrypsin<br>Metalloproteases | Dimes et al. (1994) |
| Gilthead seabream (*Sparus aurata*) | 5 | 24.5–90 | Serine proteases<br>Trypsin | Alarcon et al. (1998) |
| Dentex (*Dentex dentex*) | 8 | 24.5–69.5 | Serine proteases<br>Chymotrypsin | Alarcon et al. (1998) |

[a] Based on electrophoretical bands.

comparing the proteases of different marine fish species reported differences in activity and sensitivity towards inhibitors for similar types of proteases of different species. The possibility of extraction and purification of these enzymes for more reliable in vitro digestibility measurement also requires further attention. Evaluation of feed ingredients can also be carried out based on the monitoring of the response of trypsin, a major protease characterised in discus in relation to different feed materials.

## Acknowledgements

We express our gratefulness to E.H. Khor and E.S. Khor for the supply of discus, L.C. Lee and C.L. Lim for technical advice and Universiti Sains Malaysia for provision of short-term grant (Grant 622093) for funding purposes. The constructive comments of anonymous reviewers for this paper are also appreciated.

## References

Alarcon, F.J., Diaz, M., Moyano, F.J., Abellan, E., 1998. Characterization and functional properties of digestive proteases in two sparids; gilthead seabream (*Sparus aurata*) and common dentex (*Dentex dentex*). Fish Physiol. Biochem. 19, 257–267.

Bollag, D.M., Edelstein, S.J., 1991. Protein Methods. Wiley, New York, 230 pp.

Chakrabarti, I., Gani, M.A., Chaki, K.K., Sur, R., Misra, K.K., 1995. Digestive enzymes in 11 freshwater teleost fish species in relation to food habit and niche segregation. Comp. Biochem. Physiol. 112A, 167–177.

Clark, J., MacDonald, N.L., Stark, J.R., 1985. Metabolism in marine flatfish: II. Protein digestion in Dover sole (*Solea solea* L.). Comp. Biochem. Physiol. 81, 217–222.

Cohen, T., Gertler, A., Birk, Y., 1981. Pancreatic proteolytic enzymes from carp (*Cyprinus carpio*): I. Purification and physical properties of trypsin, chymotrypsin, elastase and carboxypeptidase. Comp. Biochem. Physiol. 69B, 639–646.

Das, K.M., Tripathi, S.D., 1991. Studies on the digestive enzymes of grass carp, *Ctenophrayngodon idella* (Val.). Aquaculture 92, 21–32.

De Silva, S.S., Perera, W.M.K., 1984. Digestibility in *Sarotherodon niloticus* fry: effects of dietary protein level and salinity with further observations on variability in daily digestibility. Aquaculture 38, 293–306.

Dimes, L.E., Garcia-Carreno, F.L., Haard, N.F., 1994. Estimation of protein digestibility: III. Studies on the digestive enzymes from the pyloric ceca of rainbow trout and salmon. Comp. Biochem Physiol. 109A, 349–360.

Erlanger, B., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95, 271–278.

Eshel, A., Lindner, P., Smirnoff, P., Newton, S., Harpaz, S., 1993. Comparative study of proteolytic enzymes in the digestive tracts of the European sea bass and hybrid striped bass reared in freshwater. Comp. Biochem. Physiol. 106A, 627–634.

Galgani, F., Nagayama, F., 1987. Digestive proteinases in the Japanese spiny lobster *Panulirus japonicus*. Comp. Biochem. Physiol. 87B, 889–893.

Galgani, F.G., Benyamin, Y., Ceccaldi, H.J., 1984. Identification of digestive proteinases of *Penaeus kerathurus* (Forskal): a comparison with *Penaeus japonicus* Bate. Comp. Biochem. Physiol. 78B, 355–361.

Garcia-Carreno, L.F., 1992. Protease inhibition in theory and practice. Biotechnol. Educ. 3, 145–150.

Garcia-Carreno, 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.

Genicot, S., Feller, G., Gerday, C., 1988. Trypsin from Antartic fish (*Paranotothemia magellanica* Forster) as compared with trout (*Salmo gairdneri*) trypsin. Comp. Biochem. Physiol. 90B, 601–609.

Glass, H.J., MacDonald, N.L., Moran, R.M., Stark, J.R., 1989. Digestion of protein in different marine species. Comp. Biochem. Physiol. 94B, 607–611.

Haard, N.F., Dimes, L.E., Arndt, R.E., Dong, F.M., 1996. Estimation of protein digestibility: IV. Digestive proteinases from the pyloric caeca of coho salmon (*Oncorhynchus kisutch*) fed diets containing soybean meal. Comp. Biochem. Physiol. 115B, 533–540.

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.

Hjelmeland, K., Raa, J., 1982. Characteristics of two trypsin type isozymes isolated from the Arctic fish capelin (*Mallotus villosus*). Comp. Biochem. Physiol. 71B, 557–562.

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

Hofer, R., Schiermer, F., 1981. Proteolytic activity in the digestive tract of several species of fish with different feeding habits. Oecologia 48, 342–345.

Jiang, S.T., Moody, M.W., Chen, H.C., 1991. Purification and characterization of proteases from digestive tract of grass shrimps (*Penaeus monodon*). J. Food Sci. 56, 322–326.

Jonas, E., Ragyansszki, M., Olah, J., Boross, L., 1983. Proteolytic digestive enzymes of carnivorous (*Silurus glanis* L.), herbivorous (*Hypophtlamichthys molitrix* Val.) and omnivorous (*Cyprinus carpio*) fishes. Aquaculture 30, 145–154.

Koh, T.L., Khoo, G., Fan, L.Q., Phang, V.P.E., 1999. Genetic diversity among wild forms and cultivated varieties of discus (*Symphosodon* spp.) as revealed by Randon Amplified Polymorphic DNA (RAPD) fingerprinting. Aquaculture 173, 485–497.

Krogdahl, A., Lea, T.B., Olli, J.J., 1994. Soybean proteinase inhibitors affect intestinal trypsin activities and amino acid digestibilities in rainbow trout (*Oncorhyncus mykiss*). Comp. Biochem. Physiol. 107A, 215–219.

Kunitz, M., 1947. Crystalline soybean trypsin inhibitor: II. General properties. J. Gen. Physiol. 20, 291–310.

Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of the head of bacteriophage $T_4$. Nature 227, 680–685.

Lopez, F.J.M., Diaz, I.M., Lopez, M.D., Lopez, F.J.A., 1999. Inhibition of digestive proteases by vegetable meals in three fish species; seabream (*Sparus aurata*), tilapia (*Oreochromis niloticus*) and African sole (*Solea senegalensis*). Comp. Biochem. Physiol. 122B, 327–332.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Maier, K., Tullis, R.E., 1984. The effect of diet and digestive cycle on the gastrointestinal tract pH values in the goldfish, *Carassius auratus* L., Mozambique tilapia, *Oreochromis mossambicus* (Peters) and channel catfish *Ictalurus punctatus* (Rafinesque). J. Fish Biol. 25, 151–165.

Martinez, A., Serra, J.L., 1989. Proteolytic activities in the digestive tract of anchovy (*Engraulis encrasicholus*). Comp. Biochem. Physiol. 93B, 61–66.

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

Munilla-Moran, R., Rey, F.B., 1996. Digestive enzymes in marine species. Proteinase activities in gut from redfish (*Sebastes mentella*), seabream (*Sparus aurata*) and turbot (*Scophtalmus maximus*). Comp. Biochem. Physiol. 113B, 395–402.

Munilla-Moran, R., Stark, J.R., 1990. Metabolism in marine flatfish: VI. Effect of nutritional state of digestion in turbot, *Scophtalmus maximus* (L.). Comp. Biochem. Physiol. 95B, 625–634.

Neurath, H., 1989. The diversity of proteolytic enzymes. In: Beynon, R.J., Bond, J.S. (Eds.), Proteolytic Enzymes. A Practical Approach. IRL Press, Oxford, pp. 1–15.

Ribeiro, F.A.L.T., Jones, D.A., 2000. Growth and ontogenetic change in activities of digestive enzymes in *Fennero Penaeus Indicus* postlarvae. Aquacult. Nutr. 6, 53–64.

Sabapathy, U., Teo, L.H., 1993. A quantitative study of some digestive enzymes in the rabbitfish, *Siganus canaliculatus* and the sea bass (*Lates calcarifer*). J. Fish Biol. 42, 595–602.

Simpson, B.K., Haard, N.F., 1984. Trypsin from Greenland cod (*Gadus ogac*). Isolation and comparative properties. Comp. Biochem. Physiol. 79B, 613–622.

Tengjaroenkul, B., Smith, B.J., Caceci, T., Smith, S.A., 2000. Distribution of intestinal enzyme activities along the intestinal tract of cultured Nile tilapia, *Oreochromis niloticus* L. Aquaculture 182, 317–327.

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.

Torrisen, K.R., Barnung, T.N., 1991. Genetic difference in trypsin-like isozyme pattern between two strains of Arctic charr (*Salvelinus alpinus*). Aquaculture 96, 227–231.

Twining, S.S., Alexander, P.A., Huibregtsem, K., Glick, D.M., 1983. A pepsinogen from rainbow trout. Comp. Biochem. Physiol. 75B, 109–112.

Uys, W., Hecht, T., 1987. Assays on the digestive enzymes of sharptooth catfish, *Clarias gariepinus* (Pisces: Clariidae). Aquaculture 63, 303–313.

Vega-Villasante, Nolasco, H., Civera, R., 1995. The digestive enzymes of the Pacific brown shrimp *Penaeus californiensis*: II. Properties of protease activity in the whole digestive tract. Comp. Biochem. Physiol. 112B, 123–129.

Walter, H.E., 1984. Proteinases: methods with hemoglobin, casein and azocoll as substrates. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis, vol. V. Verlag Chemie, Weinheim, pp. 270–277.

Yamada, A., Takano, K., Kamoi, I., 1993. Purification and properties of protease from tilapia stomach. Nippon Suisam Gakkaishi 59, 1903–1908.

Yoshinaka, R., Sato, M., Suzuki, T., Ikeda, S., 1984a. Enzymatic characterization of anionic trypsin of the catfish (*Parasilurus asotus*). Comp. Biochem. Physiol. 77B, 1–6.

Yoshinaka, R., Sato, M., Sato, T., Ikeda, S., 1984b. Distribution of trypsin and chymotrypsin and their zymogens in the digestive organs of the eel (*Anguilla japonica*). Comp. Biochem. Physiol. 78B, 569–573.

Yoshinaka, R., Sato, M., Tanaka, H., Ikeda, S., 1985. Distribution of pancreatic elastase and metalloproteinase in several fish species. Comp. Biochem. Physiol. 80B, 27–233.