Kasetsart J. (Nat. Sci.) 44 : 411 - 423 (2010)

Characterization and Expression Levels of Protease Enzymes at Different Developmental Stages of Siamese Fighting Fish (*Betta splendens* Regan, 1910)

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ABSTRACT

Characterization of total proteases, trypsin and chymotrypsin were performed at pH 2 12 and temperatures 20-80°C in three life stages of Siamese fighting fish (*Betta splendens* Regan, 1910). At least fourteen protease activities were detected, including seven acidic (pH levels 2, 4 and 5-6), one neutral (pH 7), and six alkaline (pH levels 8-12) activities in the optimal temperature range of 30-60°C. The neutral and alkaline pH profiles were different between sexes during the on-growing stage (1.5 month-old). The acidic proteases played a major role in digestion in the early life stage (10-day-old), while the alkaline proteases became more important toward older stages. Trypsin and chymotrypsin showed similar characteristics with at least five activities observed. For trypsin, activity was detected with pH 7 and 8 at 50°C, and pH 10 at 30, 50 and 60°C. For chymotrypsin, activity was found at pH levels 7 and 8 at 50°C, and pH 10 at 30, 40 and 50°C. Regardless of sex and age, the most suitable conditions for studying digestive enzyme activities in general were at pH 8 and 50°C for total proteases, pH 7-8 and 50°C or pH 10 and 30-35°C for trypsin and pH 7-8 and 50°C or pH 10 and 40°C for chymotrypsin. Trypsin and chymotrypsin are important proteases showing similar general optimal conditions to that of total proteases. This information provides elementary knowledge for studying protein digestibility by *in vitro* techniques and for determining growth performance quality using digestive enzyme markers in Siamese fighting fish.

Keywords: Siamese fighting fish, *Betta splendens*, total proteases, trypsin, chymotrypsin

INTRODUCTION

The Siamese fighting fish (*Betta splendens* Regan, 1910) is one of the most popular freshwater aquarium fish in Thailand. It is an ornamental fish of high economic importance and generates the highest income among the exported ornamental fish of Thailand (Wiwatchaisaet, 2000). Propagation of this species only uses live feed, such as chicken or duck yolks, water fleas and mosquito larvae (Meenakarn *et al*., 1988). These culturing processes lead to contaminated

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Received date : 11/09/09 Accepted date : 20/11/09

feed, diseases and waste water from leftovers. Alternatively, it is possible to use palatable dry feed with optimized nutrient content to solve these problems. However, there are no reports about substituting fish feed and nutrient requirements to nuture Siamese fighting fish. Previous work reported on the amylase and lipase characteristics in three developmental stages, based on feeding management in a Thai fish farm; this work covered 10-day-old juveniles (with a completely developed digestive tract and having predatory behavior), 1.5-month-old (sexually identified for individual rearing and having aggressive behavior among group members) and 3-month-old (maturing stage) and indicated that enzyme specific activities between stages/sexes were different (Thongprajukaew *et al*., 2010a).

Nutrient utilization in aquatic animals depends on the activity of their digestive enzymes (Areekijseree *et al*., 2006; Rungruangsak-Torrissen *et al*., 2006; Rungruangsak-Torrissen, 2007; Rungruangsak-Torrissen and Fosseidengen, 2007; Supannapong *et al*., 2008; Rungruangsak-Torrissen *et al*., 2009). Recent studies on feeding aquatic animals have reported the associations among digestive protease activity (trypsin and the ratio of trypsin to chymotrypsin), protein digestibility, feed conversion efficiency (FCE), fish weight and specific growth rate (SGR), as well as the quality of muscle and oocyte in Atlantic salmon (Rungruangsak-Torrissen *et al*., 2002; Rungruangsak-Torrissen, 2007), mackerel (Rungruangsak-Torrissen and Fosseidengen, 2007) and among trypsin specific activity, FCE and SGR in Atlantic cod (Lemieux *et al*., 1999). *In vitro* digestibility studies of dietary protein (Rungruangsak-Torrissen *et al*., 2002; Areekijseree *et al*., 2006; Rungruangsak-Torrissen, 2007; Supannapong *et al*., 2008) using fish crude enzyme extract and based on the activities of protease (trypsin), could provide information on the nutritional quality of feed and raw feed materials. The aim of this study was to characterize the total proteases (including trypsin and chymotrypsin) in the digestive tract of *B. splendens*. The information generated could provide elementary knowledge to help understand enzyme development for use in future studies of digestive biomarkers and for determining protein utilization using an *in vitro* digestibility study.

MATERIALS AND METHODS

Fish preparation

Three different developmental stages of *B. splendens*, namely, 10-day-old (48.75±1.00 mg, 6.63±0.56 mm), 1.5-month-old (males: 413.33±125.16 mg, 30.31±3.21 mm; females: 329.00±62.55 mg, 27.33±1.54 mm), and 3-month-old (males: 1,190.48±142.81 mg, 53.79±3.62 mm; females: 648.57±105.80 mg, 34.49±1.93 mm) were studied in triplicate in a completely randomized design (CRD). The experiment was performed at a farm in Nakhon Pathom province, which is a main Thai production area for exported fish (Meejui *et al*., 2005). At the farm, the fish were reared as reported in Thongprajukaew *et al*. (2010b). Subsequently, all fish were maintained in plastic aquariums for 2 d and then starved for 2 h prior to sampling. Both males and females were studied at the 1.5- and 3 month-old stages. Females were identified by visual observation of an ovipositor, the most visible small white spot at the anus. Pooled samples of about 2,000 juveniles at the 10-day-old stage, and 100 males and 100 females at the 1.5- and 3-month-old stages in each replicate were collected for enzyme characterization.

Enzyme study

Enzyme extraction

The crude enzyme extractions were performed according to Rungruangsak-Torrissen (2007). Briefly, either the whole body of 10-day-old juveniles, the digestive area of 1.5-month-old fish or the digestive tracts of 3-month-old adults

were homogenized in 50 mM Tris-HCl buffer pH 8 containing 200 mM NaCl (1:1 w/v). The homogenate was centrifuged at 4°C at 10,000× g for 20 min and the supernatant was collected and kept at -80°C in small portions for the determination of digestive enzymes. The total protein concentration of the crude enzyme extract was determined, based on Lowry *et al*. (1951).

Protease specific activity

Total protease activity was assayed by measuring the increase in cleaved short-chain polypeptides using azocasein as substrate, according to Areekijseree *et al*. (2004) modified from Garcia-Carreno (1992). The pH and temperature profiles were performed at various pH levels (2-12) and temperatures (20-80°C). The buffers used were glycine-HCl for pH 2, citrate phosphate buffer for pH 3-5, phosphate buffer for pH 6-8, $NaHCO_3$-Na_2CO_3 buffer for pH 9-10, Na_2HPO_4-NaOH for pH 11 and KCl-NaOH for pH 12. Total protease specific activity was expressed as the increase in absorbance at 440 nm h^{-1} mg $protein^{-1}$.

Trypsin and chymotrypsin specific activities

Trypsin and chymotrypsin activities were assayed according to the method described by Rungruangsak-Torrissen *et al*. (2006) using BAPNA (Benzoyl-L-arginine-*p*-nitroanilide) and SAPNA (N-succinyl-ala-ala-pro-phe-*p*-nitroanilide) as specific substrates, respectively. As trypsin and chymotrypsin are alkaline proteases, the pH profiles were performed at pH 6 11, using the buffers described above. The temperature profiles were performed at optimal pH conditions and at various temperatures (20-80°C). Both trypsin and chymotrypsin specific activities were expressed as μmol *p*-nitroaniline produced h^{-1} mg $protein^{-1}$.

Statistical analysis

The mean and standard deviation were calculated for all parameters. Analysis of variance (ANOVA) at the 95% significance level was performed and multiple comparisons were analyzed by Duncan's multiple range test (DMRT). Regression analysis was carried out on concentration values to obtain standard curves.

RESULTS

Characterization of total proteases

At least 14 protease activities were observed in Siamese fighting fish. There were various isoforms of proteases in each stage during development, which included acidic (pH 2, 4 and 5-6), neutral (pH 7) and alkaline proteases (pH 8-12). At least six protease activities were observed in 10-day-old juveniles, showing optimal temperatures at 30 and around 55°C for pH 2, at 30 and 60°C for pH 5, at 50°C for pH 8 and at 55-60°C for pH 10 (Figures 1A and 1B). At 1.5 month-old, protease activities with four optimal pH levels were observed in both sexes, but the pH profiles were different between males and females (Figure 2A). The protease activities with optimal pH 2, 5-6, 8-9, and 11 were observed in males, while the activities with optimal pH 2, 4, 7 and 10 were detected in females (Figure 2A). Surprisingly, these proteases showed the same optimal temperature of 50°C (Figures 2B and 2C). At 3-month-old, the protease activities with optimal pH 2, 4, 6, 8 and 10 were observed in males, while the activities with optimal pH 2, 4, 7 and 10 were still found in females (Figure 3A). In addition, the protease activity with optimal pH 12 was observed in adults (3-month-old) of both sexes (Figure 3A). The acidic proteases with optimal pH 2 showed an optimal temperature at 60°C in males (Figure 3B), but at 50-55°C in females (Figure 3C). The protease activities with optimal pH 4 and 10 showed an optimal temperature of 50°C and those with optimal pH 12 showed an optimal temperature of 55°C, which were similar for both males and females (Figures 3B and 3C). Acidic protease activities seemed to be dominant in the

early life stage (10-day-old juveniles) and alkaline protease activities dominated in the late stage of maturation (3-month-old), while during on-growing (1.5-month-old) both neutral and alkaline protease activities dominated (Figures 1-3).

Characterization of trypsin

Three pH optima of trypsin activity, one neutral (pH 7) and two alkaline (pH 8 and 10), were observed in Siamese fighting fish during development, and, in general, the specific activities

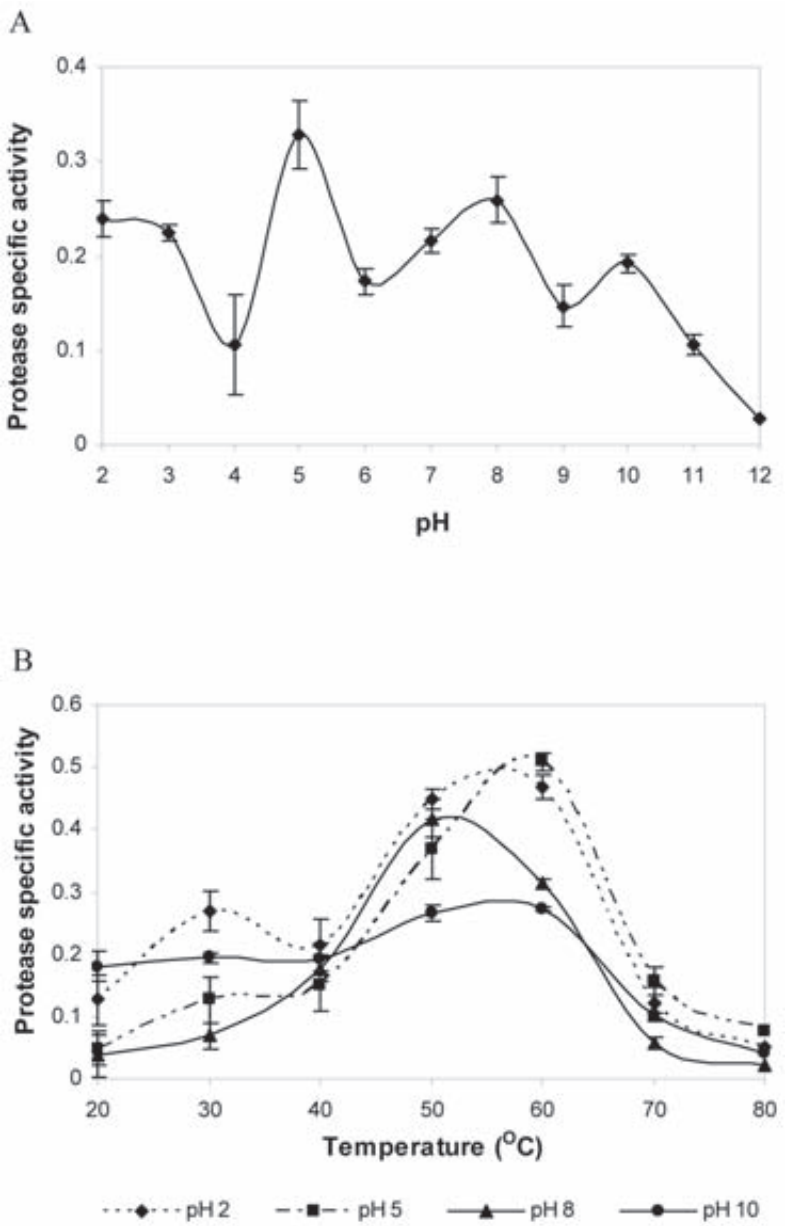

Figure 1 Protease specific activity (increase in absorbance at 440 nm h^{-1} mg $protein^{-1}$) of 10-day-old juveniles of *B. splendens*, pH profiles at: (A) ambient temperature; and (B) temperature profiles at different optimal pH levels.

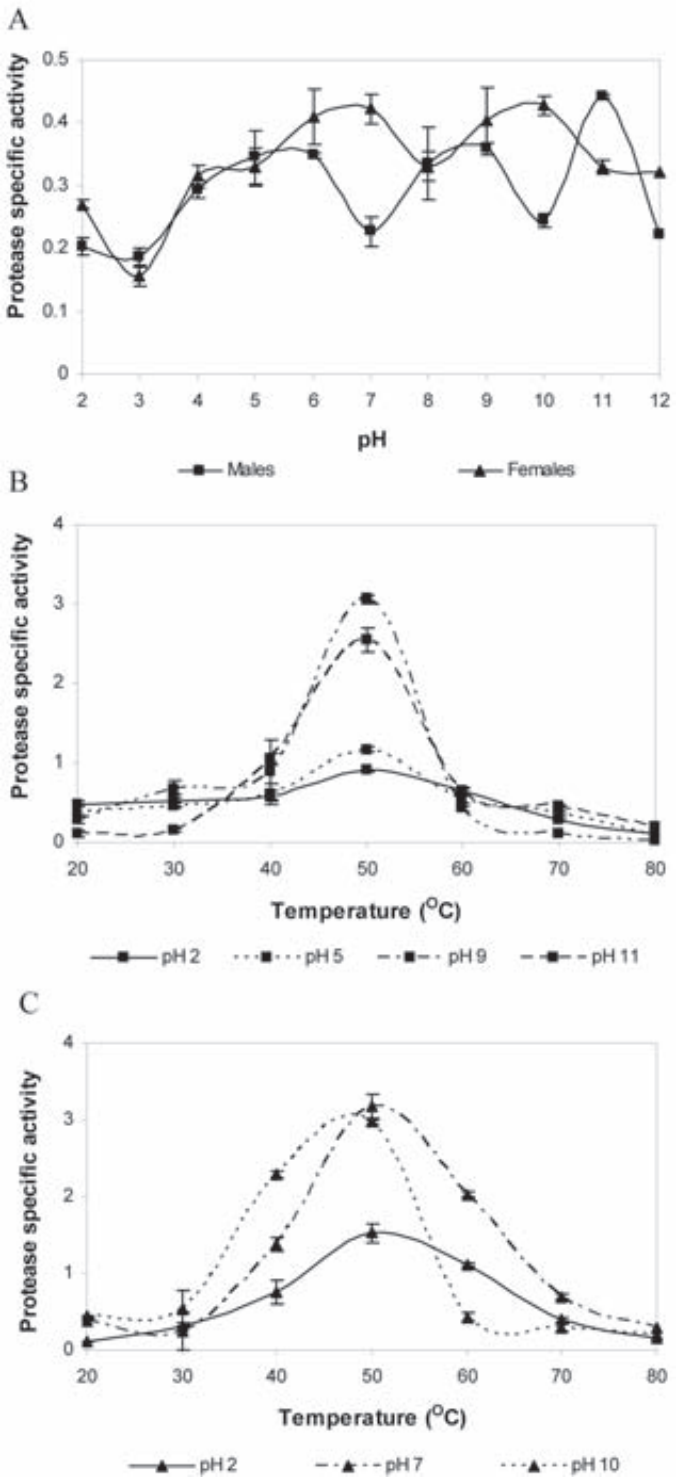

Figure 2 Protease specific activity (increase in absorbance at 440 nm h^{-1} mg $protein^{-1}$) of 1.5-month-old *B. splendens*, pH profiles at: (A) ambient temperature; and temperature profiles at different optimal pH levels in: (B) males; and (C) females.

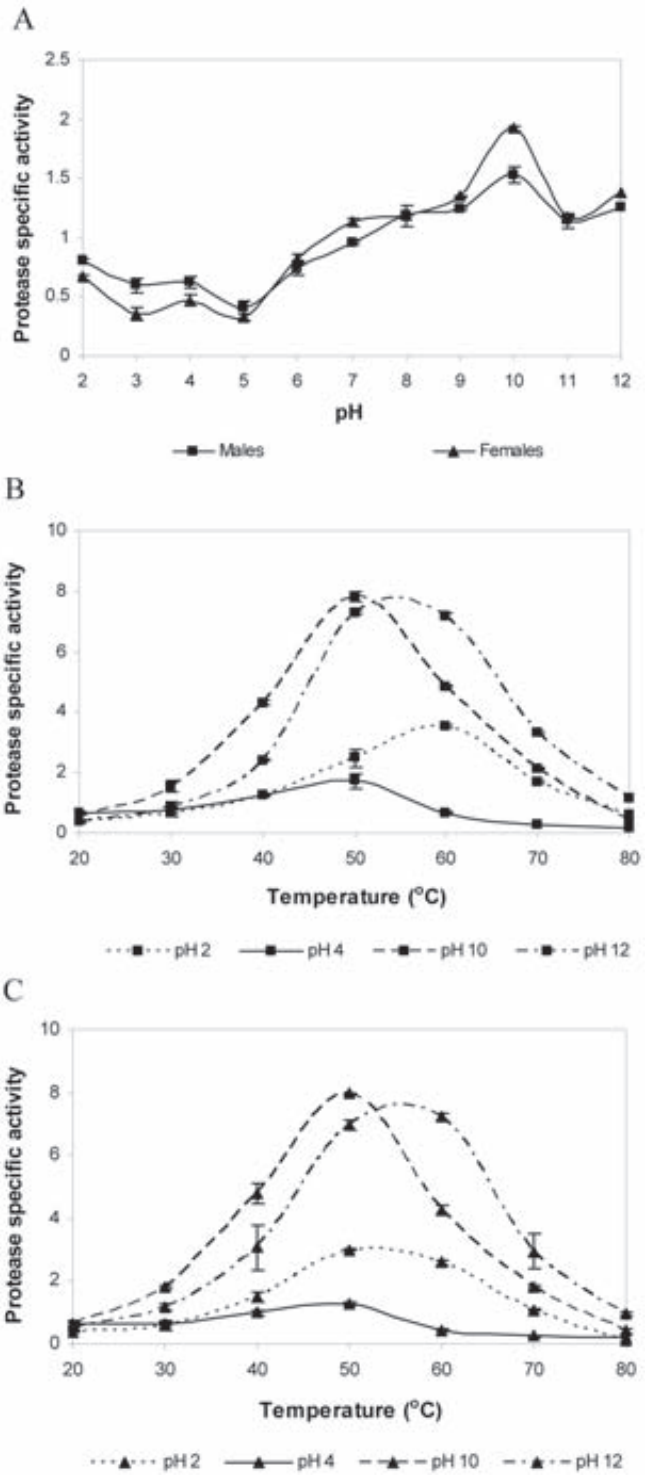

Figure 3 Protease specific activity (increase in absorbance at 440 nm h^{-1} mg $protein^{-1}$) of 3-month-old *B. splendens*, pH profiles at: (A) ambient temperature; and temperature profiles at different optimal pH levels in: (B) males; and (C) females.

were highest at pH 10 (Figure 4A). At least five levels of trypsin activity were observed. The 10-day-old juveniles showed trypsin activity at optimal pH 8 and 10 (Figure 4A), with an optimal temperature of 50°C for pH 8 and 30 and 50°C for pH 10 (Figure 4B). In the older stages, an additional neutral trypsin expression with optimal pH 7 was observed (Figure 4A), showing an optimal temperature of 50°C, regardless of sex (Figures 5A and 5B). Temperature profiles of trypsin activity with optimal pH 10 also changed in the older stages (1.5 and 3 months old), showing optimal temperatures at 30-40°C and 60°C in both sexes (Figures 5A and 5B). Trypsin specific activity was very low during the early stage for 10-day-old juveniles, and females had significantly higher trypsin specific activity than males of the same age ($P < 0.05$, Figures 4A, 5A and 5B).

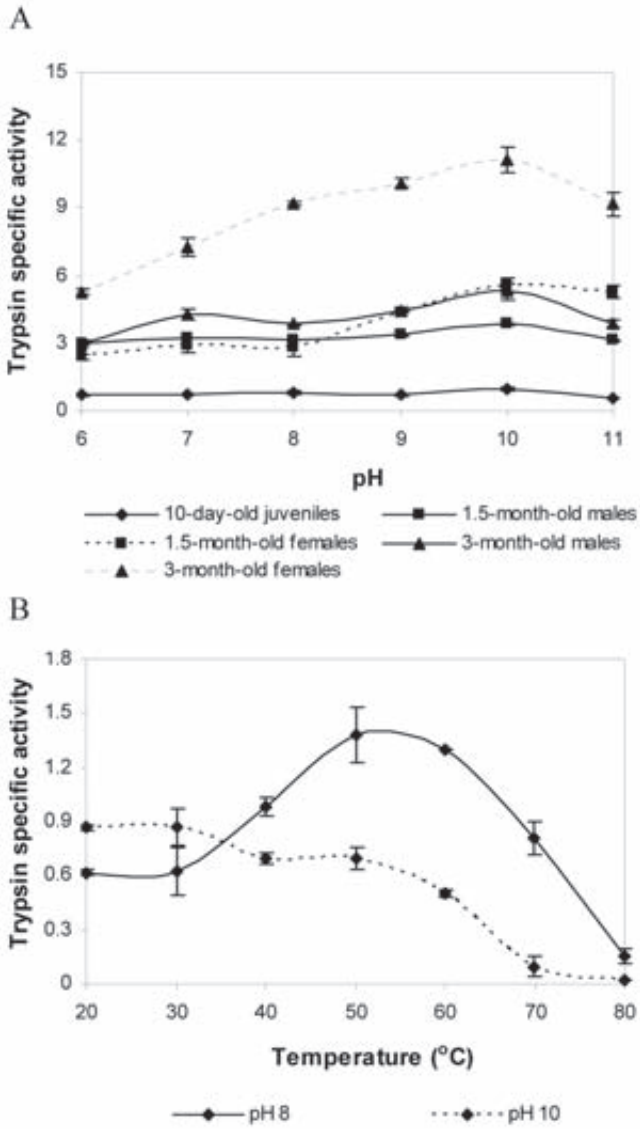

Figure 4 Trypsin specific activity (μmol *p*-nitroaniline produced h^{-1} mg $protein^{-1}$) in different stages of development of *B. splendens*, pH profiles at: (A) ambient temperature; and temperature profiles at different optimal pH levels in (B) 10-day-old juveniles.

Characterization of chymotrypsin

Similar to trypsin, chymotrypsin expression showed three optimal pH levels, one neutral at pH 7 and two alkaline at pH 8 and 10, with the highest specific activity at pH 10 (Figure 6A). Chymotrypsin specific activity was also very low during the early stage for 10-day-old juveniles, and females had significantly higher chymotrypsin specific activities than males of the same age ($P < 0.05$, Figures 6A and 6B). At least five levels of chymotrypsin activity were observed in Siamese fighting fish. Activity at two optimal pH levels of

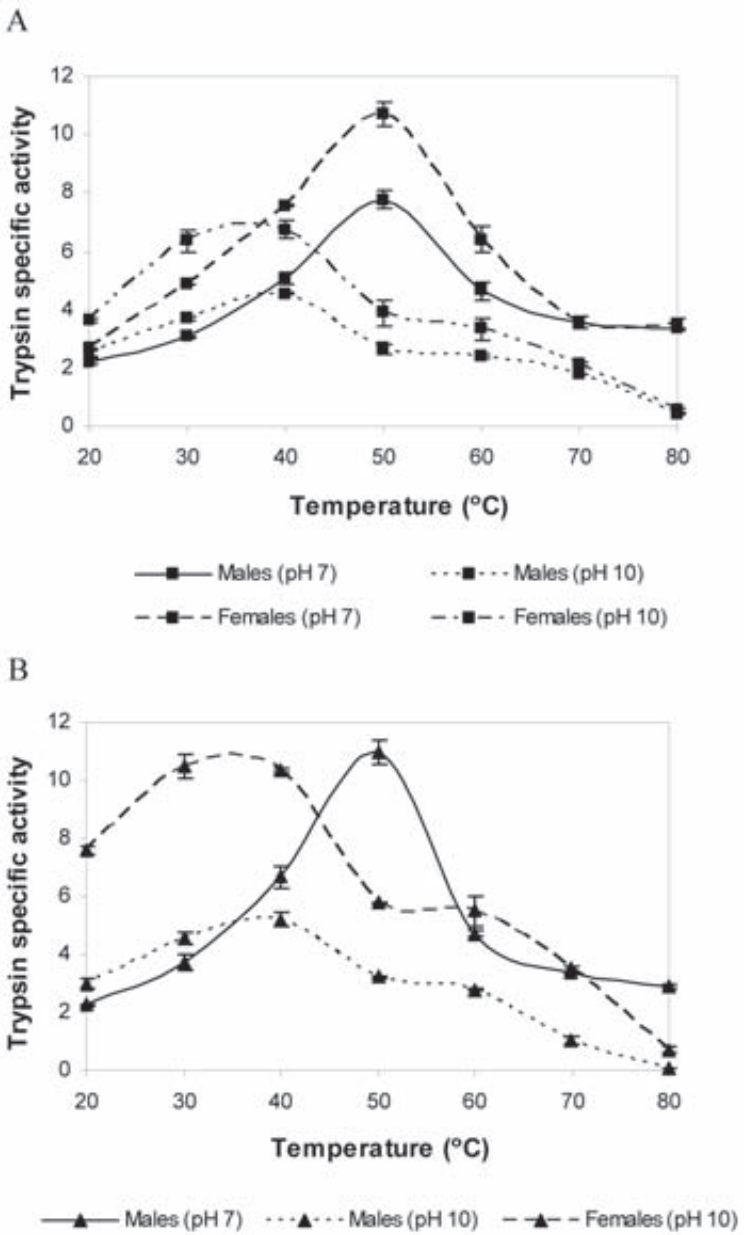

Figure 5 Temperature profiles of trypsin specific activity (μmol *p*-nitroaniline produced h^{-1} mg $protein^{-1}$) of *B. splendens*, at different optimal pH levels in: (A) 1.5-month-old fish; and (B) 3-month-old adults.

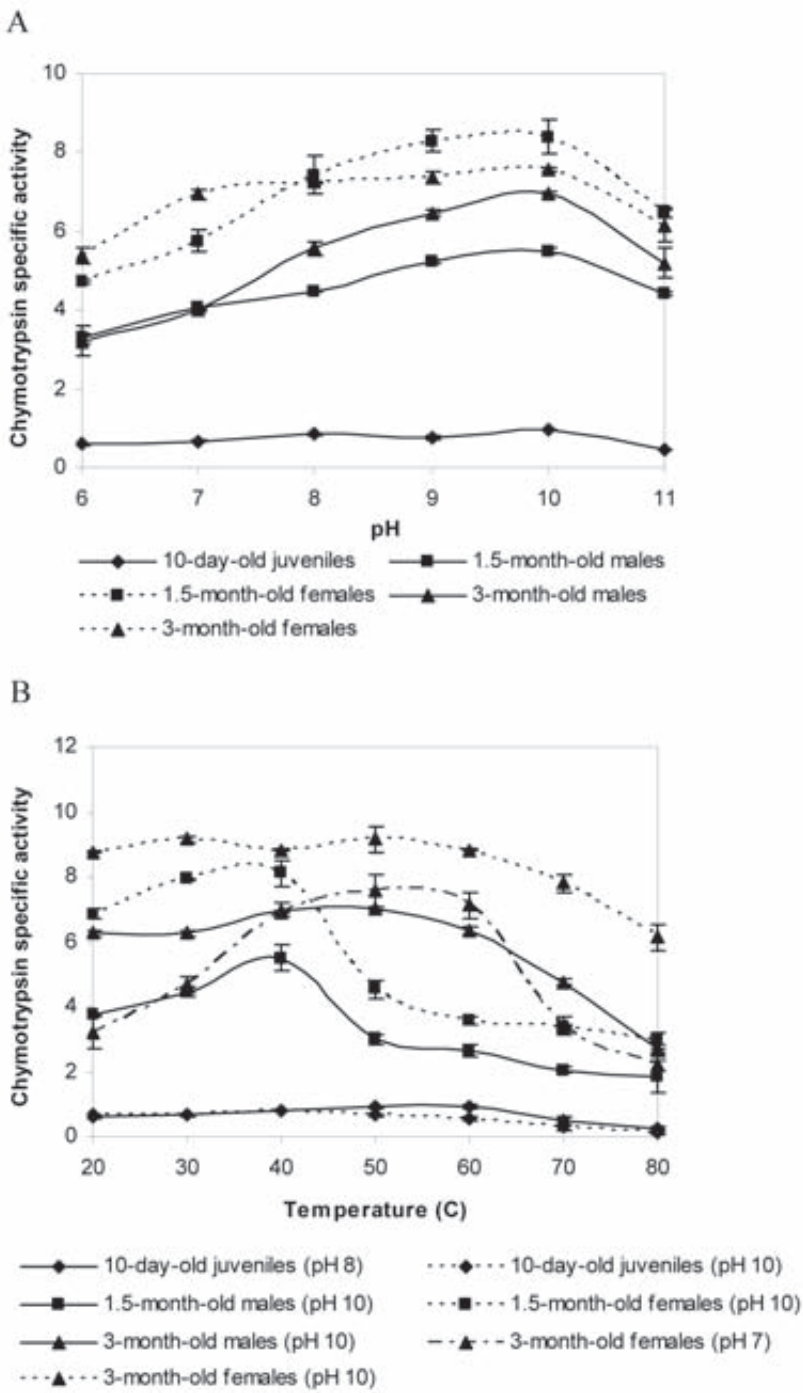

Figure 6 Chymotrypsin specific activity (μmol *p*-nitroaniline produced h^{-1} mg $protein^{-1}$) at various stages of development of *B. splendens*, pH profiles at: (A) ambient temperature; and (B) temperature profiles at different optimal pH levels.

8 and 10 was observed in 10-day-old juveniles (Figure 6A), with an optimal temperature of 50 and 40°C, respectively (Figure 6B). The activity at pH 10 had an optimal temperature of 40°C in 1.5-month-old fish and 50°C in 3-month-old adults in both sexes (Figure 6B). At optimal pH 10, an additional chymotrypsin activity with an optimal temperature of 30°C was seen in adult 3-month-old females (Figure 6B). Moreover, the activity at pH 7 found in adult 3-month-old females (Figure 6B) had an optimal temperature of 50°C.

DISCUSSION

Digestive proteases have been reported to play an important role in carnivorous and omnivorous species. The presence of various protease activities has been investigated in different species, such as Atlantic salmon and rainbow trout (Torrissen, 1984), rainbow trout, coho salmon and chinook salmon (Dimes *et al*., 1994), arowana (Natalia *et al*., 2004) and freshwater pearl mussel (Areekijseree *et al*., 2004; Supannapong *et al*., 2008). At least fourteen activities, with seven being acidic, one neutral and six alkaline, were observed in three developmental stages of Siamese fighting fish. The specific activity of proteases increased with age, and different characteristic profiles were observed during development between males and females. The protease activities showing optimal conditions at pH 2 with optimal temperatures of 30°C and in the range of 50-60°C (Figure 1), which indicated the presence of pepsin-like activity (Torrissen, 1984; Lazo *et al*., 2007) that were dominant in the stomach compared to the pancreas (Natalia *et al*., 2004) and pyloric caeca and intestine (Torrissen, 1984). Other acidic activities of proteases were also found with pH 4-6 with an optimal temperature range of 30-60°C, depending on age. The acidic protease activity was highly developed in the early life stage, followed by neutral protease activity and then alkaline protease activity in the late life stage. Most proteases were active within the pH range 2-12 and showed optimal or high activity at a temperature of 50°C (Figures 1-3). Most alkaline protease characteristics corresponded to trypsin and chymotrypsin (Table 1). The specific activity at pH 8 was high in 10-day old juveniles (Figure 1) and similar between the sexes (Figures 2A and 3A), while at pH 10, the specific activity was different between males and females ($P < 0.05$, Figures 2A and 3A). Therefore, the most suitable conditions for studying total protease activity in general during the developmental stage of Siamese fighting fish should be at pH 8 and 50°C.

Several isoforms of trypsin have been investigated during development and at different environmental temperatures in many carnivorous species (Sveinsdottir *et al*., 2006; Klomklao *et al*., 2007). The expression profiles of observed trypsin had an optimal pH range of 7-10 with high trypsin specific activity in the temperature range 30-50° C (Figures 5–6). These characteristics were similar to other species, such as Atlantic salmon (Rungruangsak Torrissen and Male, 2000; Rungruangsak-Torrissen, 2007), arowana (Natalia *et al*., 2004), red drum (Lazo *et al*., 2007), and walleye Pollock (Kishimura *et al*., 2008). The results indicated that the appropriate conditions for studying trypsin activity in Siamese fighting fish during the developmental stage should be at pH 7-8 and 50°C or at pH 10 and 30-35°C (Figures 5–6). The suggested conditions for studying trypsin activity at pH 7-8 and 50°C are similar to those suggested for total protease activity, indicating the importance of trypsin for protein digestion in Siamese fighting fish.

Chymotrypsin showed similar characteristics to trypsin, with optimal conditions in neutral (pH 7) to alkaline (pH 8 and 10) conditions and an optimal temperature range of 30 50°C (Figure 6). These conditions were similar to those found in Atlantic salmon (Rungruangsak-Torrissen and Male, 2000; Rungruangsak-

Torrissen, 2007), and arowana (Natalia *et al*., 2004). The results indicated that the most suitable conditions for the study of chymotrypsin activity in Siamese fighting fish during the developmental stage should be at pH 7-8 and 50°C or at pH 10 and 40°C (Figure 6). As for trypsin, the suggested conditions for studying chymotrypsin activity at pH 7-8 and 50°C are similar to those suggested for total protease activity (pH 8 and 50°C). This also represents the role of chymotrypsin in Siamese fighting fish in protein digestion.

Total protease specific activity does not seem to be a good indicator of growth, due to the overlapping of trypsin and chymotrypsin specific activities that affect fish growth in opposing ways, as described by Rungruangsak-Torrissen *et al*. (2006) and Chan *et al*. (2008), while the protease ratio of trypsin and chymotrypsin (T/C ratio) corresponded to fish growth parameters (including weight and length) and protein synthesis capacity in Siamese fighting fish (Thongprajukaew *et al*., 2010b). These findings are important for determining growth performance quality through the development of the digestive biomarkers, with a high optimum temperature being suitable, and also for developing artificial feed formulations based on the expression of trypsin by an *in vitro* protein digestibility study technique (Rungruangsak-Torrissen *et al*., 2002; Rungruangsak-Torrissen, 2007; Supannapong *et al*., 2008; Thongprajukaew *et al*., 2010b) where an optimal temperature close to that of the natural habitat is suitable.

ACKNOWLEDGEMENTS

The authors would like to thank Jarinporn Farm, Nakorn Phathom province, for kindly providing *B. splendens* samples. This work was financially supported in part by the program of Strategic Scholarships for Frontier Research Network for the Join Ph.D. Program Thai Doctoral degree, the Commission on Higher Education, and the Thesis and Dissertation Research Fund, the Graduate School, Kasetsart University.

LITERATURE CITED

Areekijseree, M., A. Engkagul, U. Kovitvadhi, A. Thongpan, M. Mingmuang, P. Pakkong and K. Rungruangsak-Torrissen. 2004. Temperature and pH characteristics of amylase and proteinase of adult freshwater pearl mussel, *Hyriopsis* (*Hyriopsis*) *bialatus* Simpson 1900. **Aquaculture** 234: 575-587.

Areekijseree, M., A. Engkagul, S. Kovitvadhi, U. Kovitvadhi, A. Thongpan and K. Rungruangsak-Torrissen. 2006. Development of digestive enzymes and *in vitro* digestibility of different species of phytoplankton for culture of early juveniles of the freshwater pearl mussel, *Hyriposis* (*Hyriopsis*) *bialatus* Simpson, 1900. **Invert. Reprod. Develop.** 49: 255-262.

Chan, C-R., D-L. Lee, Y-H. Cheng, D. J-Y. Hsieh and C-F. Weng. 2008. Feed deprivation and re-feeding on alterations of proteases in Tilapia *Oreochromis mossambicus*. **Zoological studies** 47: 207-214.

Dimes, L.E., F.L. Garcia-Carreno and N.F. Haard.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.

Garcia-Carreno, F.L. 1992. The digestive proteases of langostilla (*Pleuroncodes palanipes*, Decapoda): their partial characterization and the effect of food on their composition. **Comp. Biochem. Physiol.** 103B: 575-578.

Kishimura, H., S. Klomklao, S. Benjakul and B-S. Chun. 2008. Characteristics of trypsin from pyloric ceca of walleye Pollock (*Theragra chalcogramma*). **Food Chem.** 106: 194-199.

Klomklao, S., S. Benjakul, W. Visessanguan, H. Kishimura and B.K. Simpson. 2007.

Purification and characterization of trypsin from the spleen of skipjack tuna (*Katsuwonus pelamis*). **Food Chem.** 100: 1580-1589.

Lazo, J.P., R. Mendoza, G.J. Holt, C. Aguilera and C.R. Arnold. 2007. Characterization of digestive enzymes during larval development of red drum (*Sciaenops ocellatus*). **Aquaculture** 265: 194-205.

Lemieux, H., P. Blier and J.D. Dutil. 1999. Do digestive enzymes set a physiological limit on growth rate and food conversion efficiency in the Atlantic cod (*Gadus morhua*)? **Fish Physiol. Biochem.** 20: 293-303.

Lowry, H.O., N.J. Rosenbrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with Folin phenol reagent. **J. Biol. Chem.** 193: 265-275.

Meenakarn, W., N. Loahavisuti and S. Promyot. 1988. **Propagation of Siamese Fighting Fish *Betta splendens* Regan.** National Inland Fisheries Institute. Bangkok. 16 pp.

Meejui, O., S. Sukmanomon and U. Na-Nakorn. 2005. Allozyme revealed substantial genetic diversity between hatchery stocks of Siamese fighting fish, *Betta splendens*, in the province of Nakornpathom, Thailand. **Aquaculture** 250: 110-119.

Natalia, Y., R. Hashim, A. Ali and A. Chong. 2004. Characterization of digestive enzymes in a carnivorous ornamental fish, the Asian bony tongue *Scleropages formusus* (Osteoglossidae). **Aquaculture** 233: 305-320.

Rungruangsak-Torrissen, K. 2007. Digestive efficiency, growth and qualities of muscle and oocyte in Atlantic salmon (*Salmo salar* L.) fed on diets with krill meal as an alternative protein source. **J. Food Biochem.** 31: 509-540.

Rungruangsak-Torrissen, K. and J.E. Fosseidengen. 2007. Effect of artificial feeding on digestive efficiency, growth and qualities of muscle and oocyte of maturing Atlantic mackerel (*Scomber scombrus* L.). **J. Food Biochem.** 31: 726-747.

Rungruangsak Torrissen, K. and R. Male. 2000. Trypsin isozymes: development, digestion and structure, pp. 215-269. *In* N. F. Haard and B. K. Simpson (eds.). **Seafood Enzymes: Utilization and Influence on Post Harvest Seafood Quality**. Marcel Dekker, Inc., New York.

Rungruangsak-Torrissen, K., R. Moss, L.H. Andresen, A. Berg and R. Waagbo. 2006. Different expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (*Salmo salar* L.). **Fish Physiol. Biochem.** 32: 7-23.

Rungruangsak-Torrissen, K., A. Rustad, J. Sunde, S.A. Eiane, H.B. Jensen, J. Opstvedt, E. Nygard, T.A. Samvelsen, H. Mundheim, U. Luzzana and G. Venturini. 2002. *In vitro* digestibility based on fish crude enzyme extract for prediction of feed quality in growth trials. **J. Sci. Food Agric.** 82: 644-654.

Rungruangsak-Torrissen, K., L.H. Stien, B.S. Daae, T. Vågseth, G.B. Thorsheim, D. Tobin and O. Ritola. 2009. Different dietary levels of protein to lipid ratio affected digestive efficiency, skeletal growth, and muscle protein in rainbow trout families. **Scholarly Research Exchange**, vol. 2009, Article ID 709529, doi: 10.3814/2009/709529.

Supannapong, P., T. Pimsalee, T. A-Komol, A. Engkakul, U. Kovitvadhi, S. Kovitvadhi and K. Rungruangsak-Torrissen. 2008. Digestive enzymes and *in vitro* digestibility of different species of phytoplankton for culture of the freshwater pearl mussel, *Hyriopsis* (*Hyriopsis*) *bialatus*. **Aquacult. Int.** 16: 437-453.

Sveinsdottir, H., H. Thorarensen and A. Gudmundsdottir. 2006. Involvement of trypsin and chymotrypsin activities in Atlantic cod (*Gadus morhua*) embryogenesis. **Aquaculture** 260: 307-314.

Thongprajukaew, K., U. Kovitvadhi, A. Engkagul and K. Rungruangsak-Torrissen. 2010a.

Temperature and pH characteristics of amylase and lipase at different developmental stages of Siamese fighting fish (*Betta splendens* Regan, 1910). **Kasetsart J. (Nat. Sci.)**. 44(2): 210-219.

_____. 2010b. Evaluation of growth performance and nutritional quality of diets using digestive enzyme markers and *in vitro* digestibility in Siamese fighting fish (*Betta splendens* Regan, 1910). **Anim. Feed Sci. Technol.** (In press).

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.

Wiwatchaisaet, Y. 2000. Improvement of Siamese fighting fish for export. **Thai Fish Gaz.** 53: 169-179.