Journal of
Fisheries and Aquatic Science

ISSN 1816-4927

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS Journal of Fisheries and Aquatic Science

ISSN 1816-4927
DOI: 10.3923/jfas.2017.264.272

Research Article
Biochemical Characterization, Activity Comparison and Isoenzyme Analysis of Amylase and Alkaline Proteases in Seven Cyprinid Fishes

[1]Chamaiporn Champasri and [2]Thongchai Champasri

[1]Department of Biochemistry, Faculty of Science, Khon Kaen University, 40002 Khon Kaen, Thailand
[2]Department of Fisheries, Faculty of Agriculture, Khon Kaen University, 40002 Khon Kaen, Thailand

Abstract

Objective: The activity of digestive enzymes and isoenzyme patterns were characterized and comparatively studied in seven cyprinid fishes randomly collected from Kongkaew swamp, Khon Kaen province, Thailand. **Materials and Methods:** Fish samples compose of *Osteochilus hasselti*, *Labiobarbus spilopleura*, *Puntius gonionotus*, *Osteochilus lini*, *Cyclocheilichthys repasson*, *Cyclocheilichthys apogon* and *Puntius brevis.* Data were analyzed using one-way ANOVA. **Results:** Different fish species revealed different optimum temperatures and optimum pH values for amylase and alkaline protease activities. For the amylase activity, all species displayed optimum pH at 8.0, whereas the optimum pH values for alkaline proteases were observed in the range of 8.0-10.0 depending on fish species. The optimum temperatures for both amylase and alkaline proteases of all fish species were 45-65°C. Both amylase and alkaline protease activities determined at optimum temperature and optimum pH were higher than enzyme activities determined at water temperature of the swamp. At the latter condition, alkaline proteases and amylase retained their activities about 8-42% and 14-32% of maximum activities determined at optimum conditions. Interestingly, *Puntius brevis* showed highest activities of both amylase and alkaline proteases. Determination of enzyme isoforms using native polyacrylamide gel electrophoresis and enzyme zymography revealed different isoform patterns in different fish species. The same fish species exhibited the same amount and the same molecular weight of enzyme isoforms, which were different from other fish species. **Conclusion:** Amylase and alkaline proteases from seven fish species revealed the different optimum pH and temperatures with the values in the ranges of pH 8.0-10.0 and 45-65°C. Different fish species displayed different patterns of enzyme isoforms. The unique pattern of enzyme isoform in each fish species is probably used for fish identification.

Key words: Enzyme activity, fish digestive enzymes, fish amylase, isoenzyme pattern, cyprinid fish, zymography

Citation: Chamaiporn Champasri and Thongchai Champasri, 2017. Biochemical characterization, activity comparison and isoenzyme analysis of amylase and alkaline proteases in seven cyprinid fishes. J. Fish. Aquat. Sci., 12: 264-272.

Corresponding Author: Chamaiporn Champasri, Department of Biochemistry, Faculty of Science, Khon Kaen University, 40002 Khon Kaen, Thailand
Tel/Fax: +6643342911

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cyprinid is the name of freshwater fishes in family *Cyprinidae*, the largest family of freshwater fishes with over 200 genera and 2000 species[1]. Cyprinids are widely distributed in North America, Africa, Europe and Asia[1]. They are served as a food fish for mankind, a prey for some animals in food chain, an ornamental fish such as a goldfish and a scientific model for genetic research[1,2].

The digestive enzymes catalyze the breakdown of large macromolecules into small building blocks that will be next absorbed by the body. The digestive enzymes found in digestive tract of animals compose of pepsin, amylase, trypsin, chymotrypsin and cellulase. Pepsin is acid protease worked properly at pH around 2-3, whereas trypsin and chymotrypsin are alkaline proteases catalyzed the hydrolysis of proteins into amino acids at alkaline condition. Pepsin cuts peptide bond at tyrosine, phenylalanine and tryptophan, while trypsin digests peptide bond at carboxyl ends of lysine and arginine. Chymotrypsin is a serine protease functioned to hydrolyze peptide bond at carboxyl ends of aromatic amino acids including of tyrosine, tryptophan and phenylalanine[3].

Characterization of digestive enzymes has been reported in the spiny lobster[4], three cyprinid fish species (*Barbus sharpeyi*, *Cyprinus carpio*, *Aspinus vorax*)[5] and three penaeids[6]. The digestive enzyme activities have been also compared between rainbow trout, gilthead seabream, European eel, common carp, gold fish and tench[7]. Different fish species reveals different activities of digestive enzymes. There are a few reports considered the enzyme activities at an optimum temperature and an optimum environmental temperature[7]. However, the enzyme activity at the temperature of the swamp is rarely presented. Due to fishes are a cold-blooded animals, the body temperature is equal to the environmental temperature or the temperature of the swamp. Therefore, the activity of the digestive enzyme of different fish species at the environmental condition could be interesting.

The objectives of this research aimed to characterize the biochemical properties of digestive enzymes (amylase and alkaline proteases) in seven species of cyprinid fishes. The enzyme activities at the optimum condition and at the environmental conditions were compared. In addition, the isozyme patterns of both digestive enzymes were also analyzed.

MATERIALS AND METHODS

Reagents: Bradford reagent, bovine serum albumin and Mini-PROTEAN® Tetra Cell apparatus were purchased from Bio-rad. Casein and starch were purchased from Sigma-Aldrich. Acrylamide, methylene-bis-acrylamide, ammonium persulfate, TEMED, trisglycine and Coomassie Brilliant Blue R-250 were purchased from GE healthcare.

Sample collection and preparation: Seven fish species randomly collected from Nong Kong Kaew swamp, Chonnabot district, Khon Kaen province, Thailand using trawl net included *Osteochilus hasselti*, *Labiobarbus spilopleura*, *Puntius gonionotus*, *Osteochilus lini*, *Cyclocheilichthys repasson*, *Cyclocheilichthys apogon* and *Puntius brevis*. After killing fish in ice, the body weight and body length of 5-7 specimens of each fish species were recorded. The whole digestive tracts were dissected, cleaned with distilled water, removed excess distilled water and weighted. Digestive somatic index [DSI, (digestive tract weight/body weight)x100] was calculated. Crude enzymes were extracted on ice with 1 g of digestive tract weight per 3 mL of extraction buffer (0.05 M tris-HCl, pH 8.0). The resulting homogenates were centrifuged at 14000 rpm for 30 min at 4°C. Crude enzymes in supernatant fractions were subjected for further experiments. The protein contents of crude extracts were determined using Bradford's method[8] with bovine serum albumin as a standard protein.

Determination of optimum pH for protease and amylase activities: The optimum pH for protease activity was investigated by monitoring the casein hydrolysis. The hydrolysis was taken place in different pH values varying from 1-11. Buffers used for investigation composed of 0.2 M KCl-HCl (pH 1.0), 0.2 M glycine-HCl (pH 2.0 and 3.0), 0.2 M acetate (pH 4.0 and 5.0), 0.2 M sodium phosphate (pH 6.0 and 7.0), 0.2 M Tris-HCl (pH 8.0 and 9.0), 0.2 M glycine-NaOH (pH 10.0 and 11.0). The 1 mL reaction contained 250 µL of 1% casein in 0.05 M Tris-HCl, pH 7.5, 720 µL of various buffers and 30 µL of crude enzyme. The reactions were incubated at room temperature (31°C) for 10 min and subsequently stopped by using 12% trichloroacetic acid. The protein precipitation with trichloroacetic acid was allowed on iced for 30 min before centrifugation at 13,000 rpm for 15 min. The supernatants were transferred to measure an absorbance at 280 nm. The amount of amino acid produced from the catalytic reaction of protease was calculated from tyrosine standard curve. One unit of protease activity is defined as the amount of enzyme required to produce 1 nmol of tyrosine per min, whereas the specific activity is the unit of enzyme per mg of protein in crude enzyme extract.

For monitoring the optimum pH for amylase activity, the experiments were performed with buffers pH 6-11 as described above. The starch hydrolysis of amylase enzyme from different fish species was detected. One milliliter of

reaction comprised 500 µL of 0.5% starch in 0.05 M Tris-HCl, pH 8.0, 475 µL of tested buffer and 25 µL of crude enzyme. The reactions were incubated at room temperature (31°C) for 10 min before adding 500 µL of DNS (3,5-Dinitrosalicylic acid) solution to stop reaction and measure the reaction product. The reactions were boiled for 10 min and then allowed to cool down. After centrifugation at 16,000 rpm for 15 min, the supernatants were subjected to measure an absorbance at 540 nm. The reducing sugar produced from enzymatic reaction was calculated by using maltose standard curve. One unit of amylase activity is defined as the amount of enzyme required to produce 1 mmol of maltose per min, while the specific activity is the unit of enzyme per mg of protein in crude enzyme extract.

For control reaction, all components were the same as described for enzymatic reaction except for the stopping reagents (tricholoacetic acid for determination of protease activity and DNS solution for determination of amylase activity) were added to reaction before adding of crude enzyme extracts. An absorbance at 280 nm and 540 nm of control reactions were subtracted from enzyme reaction.

Determination of optimum temperature for protease and amylase activities: The optimum temperature for protease activity was observed at 35-70°C. One milliliter reaction included 250 µL of 1% casein in 0.05 M Tris-HCl, pH 7.5, 720 µL of optimal buffer and 30 µL of crude enzyme extract. The reactions were incubated at 35, 40, 45, 50, 55, 60, 65 and 70°C for 10 min. The 12% trichloroacetic acid was added to stop reaction. The reactions were cooled on ice for 30 min before centrifugation at 12,000 rpm for 15 min. The hydrolysis product in supernatant was monitored by measuring an absorbance at 280 nm. The enzyme activity was calculated by using tyrosine standard curve.

The starch hydrolysis of amylase activity was also conducted at 35-70°C. One milliliter of enzymatic reaction contained 500 µL of 0.5% starch in 0.05 M Tris-HCl, pH 8.0, 475 µL of optimal buffer and 25 µL of crude enzyme. After incubating the enzymatic reactions at 35, 40, 45, 50, 55, 60, 65 and 70°C for 10 min, 500 µL of DNS solution were added. The reactions were boiled for 10 min and cooled down before centrifugation at 12,000 rpm for 15 min. The absorbance of supernatant was recorded at 540 nm. The amount of reducing sugar produced from the enzymatic reaction was calculated from maltose standard curve.

The control reactions for both protease and amylase compose of all components as mentioned in the enzymatic reactions, but a step of the procedure was modified a bit. The reagents used for terminating the reaction were added to the reaction prior to adding of crude enzyme extract.

Comparison of protease and amylase activities: Both protease and amylase activities of different fish species were determined at the optimum condition and at the environmental condition (at the temperature of the swamp) and then the specific activities of the enzymes were compared. To examine the protease activity at the optimum condition, 1 mL of reaction composed of 250 µL of 1% casein in 0.05 M Tris-HCl, pH 7.5, 720 µL of optimum buffer and 30 µL of crude enzyme extract from different fish species. The reactions were incubated at the different optimum temperatures for the enzyme of each fish species for 10 min and terminated the enzymatic reactions with 12% trichloroacetic acid. The reactions were placed on ice for 30 min before centrifugation at 12,000 rpm for 15 min. The supernatants containing the amino acids produced from the enzymatic reaction were measured an absorbance at 280 nm. The data obtained from sample reactions were subtracted from control reactions before calculating of enzyme activity and specific activity. Analysis of amylase activities at optimum condition was also investigated. One milliliter reaction contained 500 µL of 0.5% starch in 0.05 M Tris-HCl, pH 8.0, 475 µL of optimum buffer and 25 µL of crude enzyme extracts. The reactions were incubated at different optimum temperatures for the enzyme of each fish species for 10 min and then terminated with 500 µL of DNS solution. The reactions were boiled, cooled down and centrifuged at 12,000 rpm for 15 min prior to measurement of an absorbance at 540 nm. Maltose is used as standard reducing sugar. The enzyme activity and the enzyme specific activity were calculated and compared among fish species.

Determination of protease and amylase activities was also monitored at the temperature of the swamp to evaluate the digestive capability of both enzymes and the growth rate of different economic fish in the swamp. The components in enzymatic and control reactions were the same as described for assaying at optimum condition except for the incubated temperature was 30°C, which is the average water temperature calculated from temperatures measured at many different levels of the swamp.

Statistical analysis: All data were displayed as Mean±SEM. Comparisons of both protease and amylase activities among fish species were conducted using one-way ANOVA. The significant differences were considered at $p<0.05$.

Determination of Isoenzyme using native polyacrylamide gel electrophoresis and zymography: Protein in crude samples extracted from digestive tracts of different fish species were separated by using native polyacrylamide gel electrophoresis. Crude enzyme samples were mixed with

1× native sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 0.02% bromophenol blue) and then loaded onto 4% stacking gel. Proteins were separated with 8% native gel at 4°C. After electrophoresis, native gel was immersed in 2% casein in 0.05 M Tris-HCl, pH 8.0 (for detection of protease activity) or 2% soluble starch in 0.05 M Tris-HCl, pH 8.0 (for detection of amylase activity) at 4°C for 1 h with gentle shaking every 15 min. Tray containing gel soaked in substrate solution was then moved to incubate at 45°C with gentle shaking for 1 h. For detection of protease activity, substrate solution was discarded and gel was rinsed with distilled water and subsequently stained with staining solution containing 0.02% (w/v) Coomassie Brilliant Blue R-250, 40% ethanol and 10% glacial acetic acid. Gel was destained with destaining solution (40% ethanol and 10% glacial acetic acid) until the clear band was observed against a dark blue background. For detection of amylase activity, gel was submerged in iodine solution for 10-30 min and destained with distilled water until the clear band was visualized against a dark brown background. For determination of the protein pattern, gel was stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 followed by destaining until the blue bands of proteins were appeared on a clear background.

RESULTS

Fish and collected data: List of all fish species with the data of body weight, body length, digestive tract weight and calculated digestive somatic index (DSI) as shown in Table 1. The values of digestive somatic index (DSI) were similar, except for those of *Osteochilus hasselti* and *Puntius gonionotus*. The reason might due to they had a longer digestive tracts. In addition, high DSI values also indicated the high digestive capability of the fish. However, no correlation with enzyme activity was observed.

Optimum pH and temperature: The optimum pH and optimum temperature of the enzyme activities as displayed in Fig. 1. Amylases of all fish species showed maximum activities at pH 8.0. Interestingly, the enzymes of almost all species had high activities in a broad range of pH (6.0-9.0), where activities were more than 75% compared to the maximum activities. In case of *P. gonionotus*, amylase enzyme showed a broader range against pH 6.0-10.0. For alkaline proteases, the optimum pH values were varied from 8.0-10.0 depending on fish species. Noticeably, the enzymes from almost all fish species were quite sensitive to pH, except for those from *P. gonionotus*, *P. brevis* and *O. hasselti.* The activities remained high until pH values were raised to 11.0. No detection of pepsin-like enzyme was observed.

Determination of enzyme activity at different temperature revealed the optimum temperatures for amylase and alkaline proteases activities were 45-55°C and 50-55°C, respectively. The enzymes from almost all fish species exhibited the maximum activities around 50-60°C, except the enzyme from *C. repasson*, which displayed the maximum activity at a high temperature (65°C). It is noticeable that the alkaline proteases from *O. hasselti*, *O. lini*, *P. gonionotus* and *L. spilopleura* displayed a broad range of temperature (35-65°C) although they had a lower activities than those of the other fish species.

Alkaline protease and amylase activities from different fish species: Amylases and alkaline protease activities from all fish species were monitored at the optimum temperatures and at the water temperature of the swamp. At the optimum pH and temperatures, the alkaline proteases from *P. brevis* exhibited the maximum specific activity of 1,599.66±61.93 U mg^{-1} protein, followed by those from *C. repasson* and *C. apogon* with specific activity of 1,299.79±241.20 and 1,206.22±239.00 U mg^{-1} protein, respectively. The rest of the fish species expressed moderate specific activities of the enzymes (Fig. 2b). For the digestibility of starch, the amylase enzyme from *P. brevis* revealed the highest specific activity of 14.72±0.47 U mg^{-1} protein as shown in Fig. 2a, while *O. lini*, *L. spilopleura* and *O. hasselti* showed moderate activities of the enzymes. Low amylase activities were observed in *C. apogon* and *C. repasson*. Interestingly, *P. brevis* exhibited the highest activities of both alkaline protease and amylase enzymes.

The activities of both enzymes were also examined at the temperature of the swamp to monitor the digestibility of the

Table 1: Data of body length, body weight, digestive tract weight and calculated DSI of seven cyprinid fishes

Fish species	Body length (cm)	Body weight (g)	Digestive tract weight (g)	DSI*
Osteochilus hasselti	18.08±0.46	95.00±7.42	2.05±0.17	2.16±0.12
Labiobarbus spilopleura	19.44±0.45	104.00±6.60	1.11±0.16	1.05±0.10
Puntius gonionotus	15.25±0.25	52.50±7.50	1.56±0.24	2.97±0.04
Osteochilus lini	12.98±0.25	31.70±2.76	0.47±0.02	1.53±0.13
Cyclocheilichthys repasson	12.10±0.60	19.00±2.00	0.24±0.03	1.24±0.00
Cyclocheilichthys apogon	12.80±0.29	26.50±3.12	0.38±0.04	1.43±0.12
Puntius brevis	9.70±0.70	12.50±2.50	0.17±0.03	1.37±0.04

*DSI, Digestive somatic index = (digestive tract weight x100)/body weight, values are Mean±SEM of five to seven specimens of each fish species

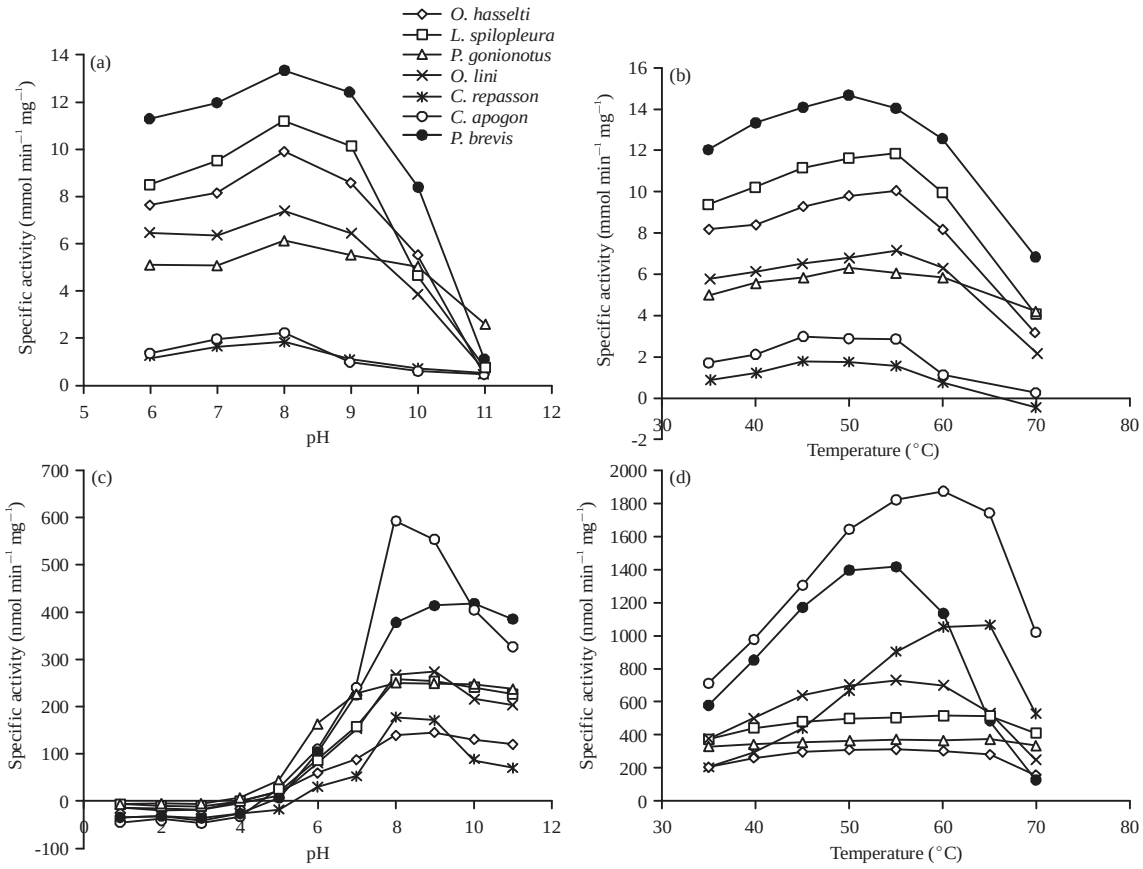

Fig.1(a-d): Optimum pH and temperature of (a, b) Amylase and (c, d) alkaline proteases activities

enzymes, which might be influences the growth rate of fish existed in the swamp or in environmental condition. The temperature of the swamp was measured at different locations and different levels of the swamp. The average value was about 30°C. All fish species revealed the reduction of both enzyme activities as shown in Fig. 2a and b. Amylase and alkaline proteases retained 14-32% and 8-42% of enzyme activities compared to those at optimum condition. Alkaline proteases, particularly from *C. repasson* were more sensitive to the temperature than those of other fish species. The enzyme remained only 8.2% of enzyme activity. Furthermore, although *P. brevis* exhibited the highest specific activities of both amylase and alkaline proteases at the optimum temperature, its enzyme activities remained only 21.22 and 19.09% at the environmental condition. This result suggested that enzyme activities decreased as the temperature decreased.

Isoenzyme analysis: Isozymes or isoenzymes are the enzymes that catalyze the same reaction. They contain different amino acid compositions leading to difference in molecular weight, charged, size as well as electrophoretic mobility. Therefore, amylase and alkaline protease isozymes of seven cyprinid fishes were also analyzed. The clear bands of amylase on a brown-purple background as revealed by Fig. 3, whereas Fig. 4 showed the clear bands of alkaline protease on a dark blue background. The result displayed two to eight isoforms of amylase and two to nine isoforms of alkaline proteases. The number of isozyme was varied depending on fish species. Interestingly, same fish species displayed the same pattern of isozyme with a unique characteristic different from other fish species. Amylases from digestive tracts of *P. gonionotus*, *P. brevis* and *O. hasselti* exhibited strong activities as indicated as the strong signals on gel, followed by those from *L. spilopleura* and *O. lini*. On the other hand, amylase enzymes from *C. apogon* and *C. repasson* displayed the low signal of enzyme activity. In case of protease zymography, *P. brevis*, *O. lini* and *L. spilopleura* showed significantly clarified bands of alkaline proteases, followed by *P. gonionotus*, *O. hasselti*, *C. repasson* and *C. apogon*. Both zymographic results of amylase and alkaline protease corresponded to the result of enzyme assay. High specific

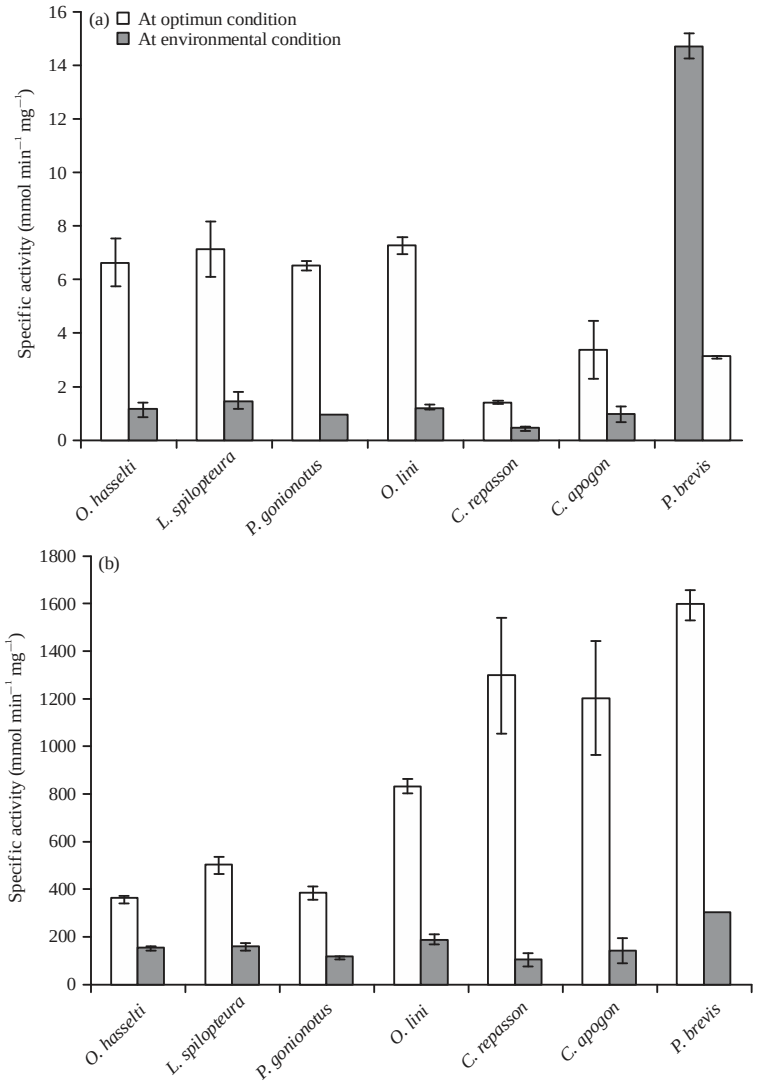

Fig. 2(a-b): Comparison of (a) Amylase and (b) Alkaline proteases activities in seven cyprinid fishes determined at optimum and environmental conditions
Values are Mean±SEM

activity displayed the strong signal, whereas low specific activity presented the low signal on gel. In addition, the result also showed that enzyme with high specific activity contains several isozymes and all isozymes performed high activities.

DISCUSSION

Biotic and abiotic factors such as nutrient composition, pH and temperature of the swamp, prey and food abundance, biodiversity of aquatic animals influence fish growth and fish reproduction. Food digestibility and absorption capability are also important for the growth of fish. Therefore the study of digestive enzymes in different fish species helps to understand more about the enzyme properties that are necessary for improvement of feed formula and management of fish farming. In addition, more information of biochemical properties of digestive enzymes will help to understand the catalytic mechanism. Different biochemical properties, different catalytic activity is. Comparison of digestive enzymes in penaeids and fishes reveals different activities of the enzymes[5-7,9]. The optimum pH values for amylase activities of seven cyprinid fishes from this study were 8.0 corresponded to the studies of those from *Pagrus pagrus*, *Boops boops* and *Pagellus erytrhinus*[10]. The optimum temperatures for amylase activities in these cyprinids were ranging from 45°C in *C. repasson* and *C. apogon* to 55°C in *O. hasselti*, *L. spilopleura* and *O. lini*, which were higher than those in Mediterranean sparid fishes[10] and marine fish pathogen *Aeromonas*

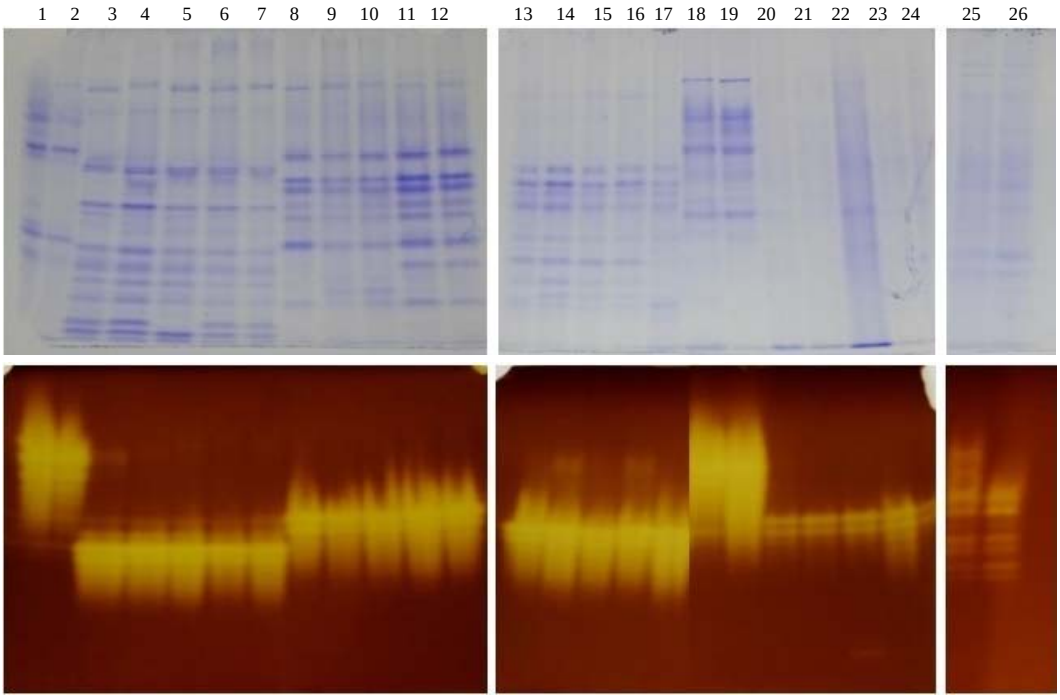

Fig. 3: Native polyacrylamide gel stained with Coomassie Brilliant Blue R-250 (Top panel) and zymogram of amylase (Bottom panel)
[Lanes 1, 2, *Puntius brevis* (n1, n2), lanes 3-7, *Labiobarbus spilopleura* (n1-n5), lanes 8-12, *Osteochilus hasselti* (n1-n5), lanes 13-17, *Osteochilus lini* (n1-n5), lanes 18-19, *Puntius gonionotus* (n1,n2), lanes 20-24, *Cyclocheilichthys apogon* (n1-n5), lanes 25, 26, *Cyclocheilichthys repasson* (n1, n2)]

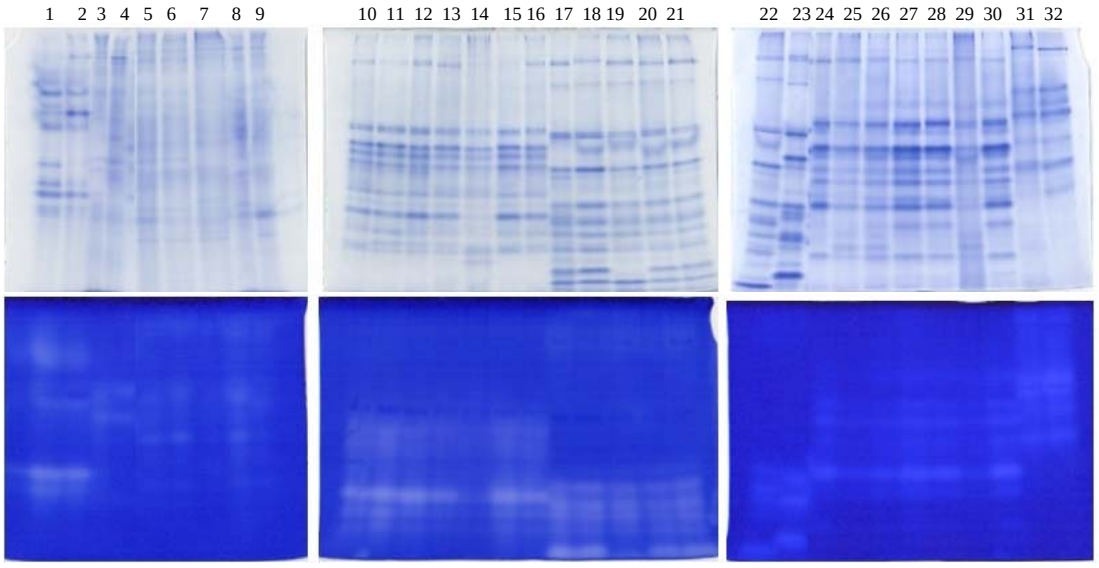

Fig. 4: Native polyacrylamide gel stained with Coomassie Brilliant Blue R-250 (top panel) and zymogram of alkaline proteases (bottom panel)
[Lanes 1, 2, *Puntius brevis* (n1, n2), lanes 3, 4, *Cyclocheilichthys repasson* (n1, n2), lanes 5-9, *Cyclocheilichthys apogon* (n1-n5), lanes 10-16, *Osteochilus lini* (n1-n7), lanes 17-23, *Labiobarbus spilopleura* (n1-n7), lanes 24-30, *Osteochilus hasselti* (n1-n7), lanes 31-32, *Puntius gonionotus* (n1,n2)]

salmonicida ssp. salmonicida[11]. This study also showed that amylase enzymes from *O. hasselti*, *L. spilopleura*, *O. lini*, *P. gonionotus* and *P. brevis* exhibited high activities at a broad range of pH (pH 6.0-9.0) with more than 85% of

maximum activities at optimum pH and the enzyme activities were significantly decreased afterward. Interestingly, amylase from *P. gonionotus* displayed high activity throughout pH 10.0, its activity was up to 90% of the maximum activity at pH 8.0. Protease activities have been reported in many fish species. The optimum pH values are in the range of 7.0-12.5 for alkaline protease[4,7,12-16] and 2.0-6.0 for pepsin or acidic protease[12,14-17], whereas the optimum temperature for protease activity has been rarely addressed in fish. The optimum temperatures for digestive enzyme activities including of trypsin, chymotrypsin, lipase, amylase, esterase have been reported in the spiny lobster *Panulirus argus*[4] and three penaeids, *Penaeus vannamei*, *Penaeus stylirostris*, *Penaeus californiensis*[6]. The reported optimum temperatures are in the range of 40-60°C. Seven species of cyprinid fishes from this study exhibited the various data. The optimum temperatures were in the range of 50-65°C depending from fish species. Interestingly, amylases of almost all cyprinid fishes displayed high activities over a broad range of pH (pH 6-9) and temperature (35-60°C). More than 75% of enzyme activities were detected at the mentioned pH and temperatures, except for those of *C. repasson* and *C. apogon*, whose enzyme activities were in the range of 41-57% of maximum activity similar to the effects of pH and temperature on alkaline proteases activities. Typically, difference in optimum pH and temperature of the enzyme activity is due to difference in amino acid composition, molecular weight, isoelectric point, or/and catalytic mechanism of an enzyme. Seven cyprinid fishes from this study revealed different optimum pH and temperature and different digestive enzyme activities were also exhibited the different isozyme patterns. The patterns displayed the different number and molecular weight of isozyme. Based on electrophoresis technique, proteases are detected in several bands on acrylamide gel in discus, rainbow trout, coho salmon, chinook salmon, gilthead seabream, dentex as mentioned by Chong *et al.*[12]. A number of isozymes are reported in the range of 4-8 with the molecular weights between 18-90 kDa. The molecular weights of proteases and amylase have also been reported in paddlefish, bighead carp and hybrid sturgeon[18]. Sizes are in the range of 69.9-156.3 kDa. From this study, seven cyprinid fishes revealed 2-8 isozymes of amylase and 2-9 isozymes of alkaline proteases. The result of electrophoresis correlated to the result of enzyme assay in which enzyme exhibiting high activity displayed the strong signal or clarified band on gel and also contained several isoforms of the enzyme except for *C. repasson*. Amylase showed seven to eight isoforms, but its activity was lowest.

CONCLUSION

Study of biochemical properties of seven cyprinid fishes revealed the different optimum pH and temperature of amylase and protease enzymes. All fish species displayed the maximum activities of amylase at pH 8.0, whereas the maximum activities of alkaline proteases were varied from pH 8.0-10.0. The optimum temperatures for both enzyme activities were in the range of 45-65°C. Comparative study showed that *Puntius brevis* exhibited the highest activities of both enzymes. Moreover, Isoenzyme analysis revealed the different patterns of both enzymes in different fish species. The uniqueness of isozyme pattern of each fish species might be helpful for fish identification.

SIGNIFICANCE STATEMENTS

This study helps to understand more about enzyme properties and provides the information of digestibility of digestive enzyme of each fish species leading to management of fishes for farming. Furthermore, this study may helpful for preparation of fish feeds or feed ingredients according to the activity of digestive enzymes in order to increase the growth rate of fishes and reduce the cost of feeds for fish farming.

ACKNOWLEDGMENTS

The authors would like to thank Khon Kaen University and National Research Council of Thailand for funding this research [Grant number 563705].

REFERENCES

1. Nelson, J.S., 2006. Fishes of the World. 4th Edn., John Wiley and Sons, New Jersey, USA., ISBN: 0471250317, Pages: 601.
2. Helfman, G.S., B.B. Collette, D.E. Facey and W. Bowen, 2009. The Diversity of Fishes: Biology, Evolution and Ecology. 2nd Edn., Wiley-Blackwell Oxford, England, ISBN-13: 9781444311907, Pages: 720.
3. Nelson, D.L. and M.M. Cox, 2012. Lehninger Principles of Biochemistry. 6th Edn., W.H. Freeman and Company, USA., ISBN-13: 9781429234146, Pages: 1340.
4. Perera, E., F.J. Moyano, M. Diaz, R. Perdomo-Morales and V. Montero-Alejo *et al*., 2008. Polymorphism and partial characterization of digestive enzymes in the spiny lobster *Panulirus argus*. Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol., 150: 247-254.
5. Al-Tameemi, R., A. Aldubaikul and N.A. Salman, 2010. Comparative study of α-amylase activity in three Cyprinid species of different feeding habits from Southern Iraq. Turk. J. Fish. Aquat. Sci., 10: 411-414.

6. Del Toro, M.A.N., F.L. Garcia-Carreno and J.H. Cordova-Murueta, 2011. Comparison of digestive proteinases in three penaeids. Aquaculture, 317: 99-106.
7. Hidalgo, M.C., E. Urea and A. Sanz, 1999. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. Aquacult., 170: 267-283.
8. Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.
9. Falcon-Hidalgo, B., A. Forrellat-Barrios, O.C. Farnes and K.U. Hernandez, 2011. Digestive enzymes of two freshwater fishes (*Limia vittata* and *Gambusia punctata*) with different dietary preferences at three developmental stages. Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol., 158: 136-141.
10. Fernandez, I., F.J. Moyano, M. Diaz and T. Martinez, 2001. Characterization of α-amylase activity in five species of Mediterranean sparid fishes (Sparidae, Teleostei). J. Exp. Mar. Biol. Ecol., 262: 1-12.
11. Peng, H., M. Chen, L. Yi, X. Zhang, M. Wang, Y. Xiao and N. Zhang, 2015. Identification and characterization of a novel raw-starch-degrading α-amylase (AmyASS) from the marine fish pathogen *Aeromonas salmonicida* ssp. *salmonicida*. J. Mol. Catal. B: Enzymatic, 119: 71-77.
12. Chong, A.S.C., R. Hashim, L. Chow-Yang and A.B. Ali, 2002. Partial characterization and activities of proteases from the digestive tract of discus fish (*Symphysodon aequifasciata*). Aquaculture, 203: 321-333.
13. Kuz'mina, V.V., E.G. Skvortsova, G.V. Zolotareva and V.A. Sheptitskiy, 2011. Influence of pH upon the activity of glycosidases and proteinases of intestinal mucosa, chyme and microbiota in fish. Fish Physiol. Biochem., 37: 345-353.
14. Xiong, D.M., C.X. Xie, H.J. Zhang and H.P. Liu, 2011. Digestive enzymes along digestive tract of a carnivorous fish *Glyptosternum maculatum* (Sisoridae, Siluriformes). J. Anim. Physiol. Anim. Nutr., 95: 56-64.
15. Solovyev, M.M., E.N. Kashinskaya, G.I. Izvekova and V.V. Glupov, 2015. pH values and activity of digestive enzymes in the gastrointestinal tract of fish in Lake Chany (West Siberia). J. Ichthyol., 55: 251-258.
16. Pujante, I.M., M. Diaz-Lopez, J.M. Mancera and F.J. Moyano, 2017. Characterization of digestive enzymes protease and α-amylase activities in the thick-lipped grey mullet (*Chelon labrosus*, Risso 1827). Aquacult. Res., 48: 367-376.
17. Marquez, L., R. Robles, G.A. Morales and F.J. Moyano, 2012. Gut pH as a limiting factor for digestive proteolysis in cultured juveniles of the gilthead sea bream (*Sparus aurata*). Fish Physiol. Biochem., 38: 859-869.
18. Ji, H., H.T. Sun and D.M. Xiong, 2012. Studies on activity, distribution and zymogram of protease, α-amylase and lipase in the paddlefish *Polyodon spathula*. Fish Physiol. Biochem., 38: 603-613.