Kasetsart J. (Nat. Sci.) 36 : 399 - 407 (2002)

Activity Profiles at Different pH and Temperature of Cellulases and Lipases in Freshwater Pearl Mussel: *Hyriopsis* (*Hyriopsis*) *bialatus*, Simpson 1900

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ABSTRACT

The enzymatic properties of two digestive enzymes, cellulases and lipases, from stomach and intestine of adult freshwater pearl mussel, *Hyriopsis* (*Hyriopsis*) *bialatus*, Simpson 1900, were studied at various pH (1.0-11.0) and temperatures (20-80°C). Activity profiles of both enzymes in male and female mussels at various pH and temperature were similar. Optimum pH of cellulases in stomach and intestine was found to be 6.0 while optimum temperature was 35-55°C in stomach and 30-60°C in intestine. Lipase activity was low in intestine but its optimum pH in both organs was found at pH 8.0 and optimum temperature at 35-55°C. Both cellulase and lipase showed higher activities in the stomach than in intestine. At habitat temperature (28-30°C) of Thai freshwater pearl mussel species, cellulase and lipase specific activities dominated in stomach.

Key words: cellulase, freshwater pearl mussels, *Hyriopsis bialatus*, lipase

INTRODUCTION

Hyriopsis (*Hyriopsis*) *bialatus*, Simpson 1900 is a freshwater pearl mussel widely distributed at the bottoms of reservoir and river in the central, northern and northeastern parts of Thailand (Brandt, 1974). Mussels have been used as feed for different animals as well as being used as decorative parts of tools, pearl button, tempering pottery, utensils and costume. Mussels are filter feeders by siphoning nutrients from water column. These filtering activities contribute to maintaining clean river and stream ecosystem. Moreover, mussels have antioxidant enzymes and biotransformation enzymes in the digestive glands to detoxify substances in the water (Birmelin *et al.*, 1999). They are also used as bioindicator of the ecological system (Hudson and Isom, 1984; Biggins *et al.*, 1997).

The population of freshwater mussels has been declining as a result of water quality problems caused by dredging, damming, pollution, and also from the reduction of their fish hosts. To increase the population of freshwater mussels, efforts have been made to use culture techniques for mass production and conservation (Isom and Hudson, 1982, 1984; Hudson and Isom, 1984; Keller and Zam, 1990; Uthaiwan *et al.*, 2001). The most

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important factors for culturing are feeding and feed digestion, but the information on the relationship between feeding physiology and feed availability is limited in mussels (Hawkins *et al.*, 1998; Wong and Cheung, 2001). Recent study on feeding of freshwater pearl mussel *Hyriopsis* (*Limnoscapha*) *myersiana* revealed 99.99% phytoplankton and 0.01% zooplankton in the gastrointestinal tract content (Kovitvadhi *et al*., 2000). Phytoplankton consists of protein, carbohydrate and lipid in cells while its cell wall is composed of cellulose (IFRPD, 1973). However, the ability of mussels to digest cell walls of phytoplankton in order to dissolve phytoplankton cytoplasm is not clear although *H. bialatus* is known to have proteinases and amylases in its stomach and intestine which could digest carbohydrate and protein. The aim of this study was to determine the activities of cellulase and lipase collected from stomach and intestine of adult male and female freshwater mussels *H. bialatus* under various pH and temperature conditions. Data obtained from this study may lead to future development of artificial feed formulation for mussel culture.

MATERIALS AND METHODS

Animal and rearing

Samples of adult *H. bialatus* were collected from the Mun River Basin in the northeast of Thailand. The mussels were sexually identified by microscopic observation of sperms and eggs in fluid suctioned from gonad. Male mussels were selected to obtain the average size of 112.00±5.89 cm in length, 42.44±3.12 cm in height and 24.67±2.36 cm in width, while female mussels were 107.78±6.07 cm in length, 40.44±2.50 cm in height and 23.89±2.23 cm in width. They were cultured in circle net from January to August 2001 in a pond (pH ranging from 7.0-7.2 and temperature ranging from 28-30°C) at the Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. The mussels were allowed to feed freely on natural plankton in the pond.

Preparation of enzyme extracts

Nine each of male and female mussels were cleaned with dechlorinated tap water to remove adhering detritus. Shells of the mussels were opened by cutting off anterior and posterior adductor muscles. Their stomach and intestine were dissected. The organs were weighed and pooled separately. Three stomach or intestine was used as one sample. The sample was homogenized on ice without addition of any buffer solution. The homogenate was centrifuged at 13,000 ×*g* for 15 min at 4°C and the upper lipid layer was discarded. Supernatant was collected, divided into aliquots and assayed for enzyme specific activities.

Protein determination

Protein content of stomach and intestinal extracts was determined using the method of Lowry *et al.* (1951). Bovine serum albumin (BSA) was used as protein standard. The assays were performed in duplicates.

Cellulase specific activity determination

Cellulase activity was measured using the method of Mendels et al. (1969). Carboxymethyl cellulose (CMC) was prepared by boiling 1% soluble CMC in various buffers (HCl-KCl buffer for pH 1.0 and 2.0 (Fasman, 1984), citrate phosphate buffer for the pH range of 3.0-5.0, phosphate buffer for the pH range of 6.0-8.0 and $NaHCO_3$-Na_2CO_3 buffers for the pH range of 9.0-11.0). To determine the optimum pH, the substrate and crude enzymes (dilution 1:50 in various buffers) were separately preincubated for 10 min at 50°C. Enzyme reactions were allowed at 50°C for 30 min. To determine the optimum temperature, the enzyme was diluted in phosphate buffer (pH 6.0) and preincubated at various temperatures (20-80°C) for 10 min. After preincubation, reaction mixture containing 250 µl of the diluted enzymes and 250 µl of the substrate

were incubated for 30 min at the designed temperatures (20-80°C). The reaction was stopped by adding 500 µl of 3,5-dinitrosalicylic acid (DNS) to the solution. It was then heated in boiling water for 5 min, cooled down in ice and finally added with 2.5 ml distilled water. The amount of reducing sugar liberated was determined by measuring the absorbance at 540 nm. Blank was the reaction mixture without the enzyme and the control was prepared by adding the crude enzyme after the DNS reagent. Calibration curve was made using 0.2-1.0 mg/ml glucose. Cellulase specific activity was defined as the amount of enzyme liberating 1 mg of glucose min^{-1} mg $protein^{-1}$ under the specified reaction conditions.

Lipase specific activity determination

Activity of lipase (EC 3.1.1.3) was measured using the method modified from Versaw *et al.* (1989). To determine the optimum pH, the assay mixtures containing 50 µl of 200 mM sodium taurocholate, 940 µl of various pH buffers as described above and 10 µl of the crude enzyme extract were used. All reagents were preincubated separately for 5 min at 45°C. For optimum temperature study, the reaction was performed at pH 8.0 and preincubated at various temperatures (20-80°C) for 5 min. After preincubation, the reaction was added with 10 µl of 200 mM β-naphthyl caprylate in dimethyl sulfoxide [DMSO] and incubated at 45°C for 10 min. The reaction was stopped with 10 µl of 100 mM fast blue BB solution in DMSO and further incubated for 5 min at the same temperature before 200 µl of 0.72 N TCA was added. Then the reaction mixtures were clarified with 1,355 µl of (1:1,v/v) ethyl acetate:ethanol solution. Blank was prepared by replacing the enzyme solution with the same buffer of specific pH in the reaction mixture. The control was prepared by mixing the crude enzyme, TCA and the substrate in that order. Product liberated by lipase activity was determined by measuring the changes in absorbance at 540 nm. One unit (U) of lipase specific activity was defined as the amount of enzyme giving an increase of 0.01 absorbance unit at 540 nm min^{-1} mg $protein^{-1}$ under the specified reaction conditions.

Statistical analysis

Mean and standard deviation of the means of each enzyme specific activity were calculated. Statistical analysis at 95% significance level was determined using analysis of variance (ANOVA), and multiple comparisons were analyzed by least-significant difference (LSD).

RESULTS AND DISCUSSION

Cellulase activity in stomach and intestine of mussels

Cellulase specific activity in female stomach and intestine of both sex exhibited optimum pH at pH 6.0 while in male stomach the optimum pH was found at pH 7.0 (Figure 1A). Optimum temperature of cellulase specific activity in both stomach and intestine was at 50°C, in both male and female mussels (Figure 1B). The enzymes showed distinct active temperature range of 35-55°C in the stomach and 30-60°C in the intestine at pH 6.0 in both sex. However, the total cellulase specific activity in the stomach was significantly higher than that in the intestine in both profiles ($P<0.05$) (Table 1 and Table 2).

Lipase activity in stomach and intestine of mussels

Optimum pH of lipase in both stomach and intestine of both sex was 8.0. However, the activity in stomach was approximately 75-90% at pH 4.0-9.0, while in intestine, the lipase was 75% active at pH 7.0 and 9.0 (Figure 2A). By varying temperature at pH 8.0 assay condition, lipase in the stomach showed optimum temperature at 45°C while at 35, 40, 50 and 55°C, the activities were relatively high. The activity was, however, very low in intestine of both sex (Figure 2B). In the stomach, lipase specific

activities were significantly higher in male than in female in both profiles ($P<0.05$) (Table 3 and Table 4).

In *H. bialatus*, stomach and intestine contributed about 2.9% and 3.5% of the mussel body weight, respectively. pH of the homogenized crude extracts of these organs without addition of any buffer solution was found to be neutral (pH 7.0). In the current experiment, the optimum pH for cellulase activity in both stomach and intestine of *H. bialatus* was 6.0 but the temperature for cellulase activity in stomach was in the range of 35-55°C and in intestine was 30-60°C (Figure 1A and 1B). These meant that a certain amount of cellulase could be actively present at neutral pH in the digestive organs but only 50% active at habitat temperature of 28-

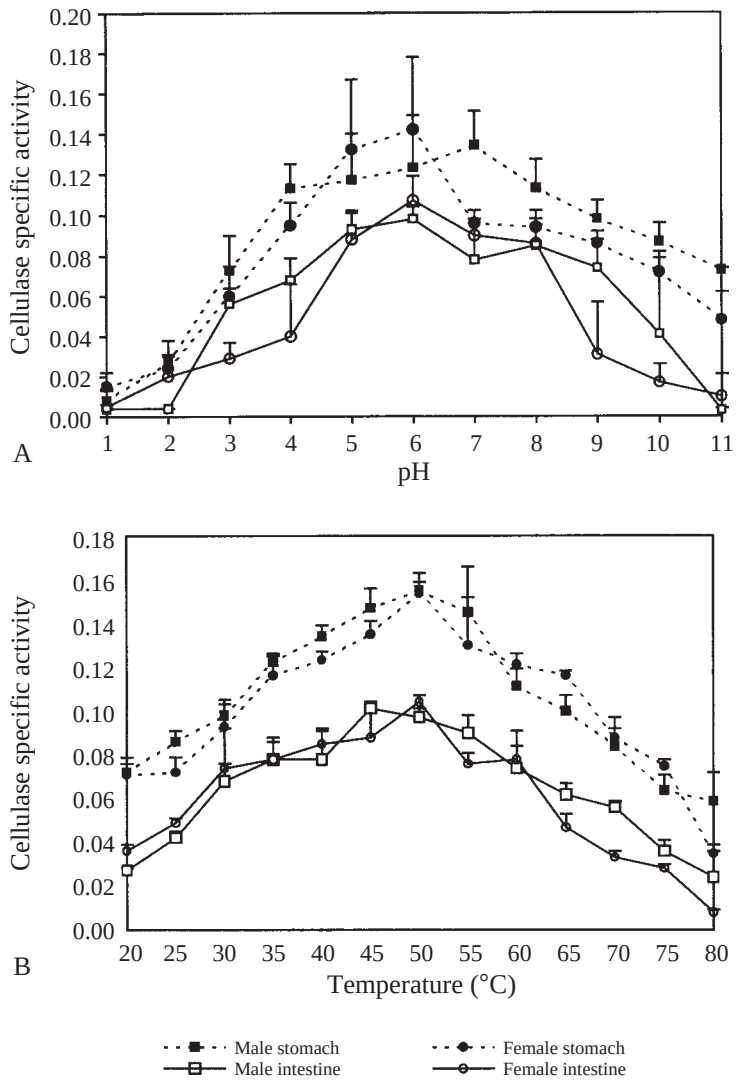

Figure 1 Cellulase specific activity (mg of glucose min^{-1} mg $protein^{-1}$) in the stomach and intestinal extracts of adult male and female *Hyriopsis bialatus*. Enzymatic reaction was performed at 50°C showing at various pH (A). At pH 6.0, the enzyme activity was done at different temperatures (B).

Table 1 Cellulase specific activity (mg of glucose min^{-1} mg $protein^{-1}$) in the stomach and intestinal extracts of adult male and female *Hyriopsis bialatus*. Enzymatic reaction was performed at 50°C at various pH.

pH	Stomach		Intestine		Means±SD
	Male	Female	Male	Female	
1	0.008 ± 0.01	0.015 ± 0.01	0.004 ± 0.002	0.005 ± 0.01	0.008 ^{a} ± 0.01
2	0.027 ± 0.003	0.024 ± 0.01	0.004 ± 0.001	0.020 ± 0.02	0.019 ^{ab} ± 0.01
3	0.073 ± 0.02	0.060 ± 0.02	0.056 ± 0.01	0.029 ± 0.01	0.055 ^{c} ± 0.02
4	0.113 ± 0.01	0.095 ± 0.01	0.068 ± 0.01	0.040 ± 0.02	0.079 ^{de} ± 0.03
5	0.117 ± 0.06	0.132 ± 0.01	0.093 ± 0.01	0.088 ± 0.02	0.108 ^{fg} ± 0.03
6	0.123 ± 0.06	0.141 ± 0.01	0.098 ± 0.01	0.110 ± 0.01	0.117 ^{g} ± 0.03
7	0.134 ± 0.02	0.096 ± 0.007	0.078 ± 0.02	0.090 ± 0.01	0.090 ^{fg} ± 0.03
8	0.113 ± 0.01	0.094 ± 0.01	0.085 ± 0.02	0.086 ± 0.01	0.095 ^{ef} ± 0.02
9	0.098 ± 0.01	0.086 ± 0.01	0.074 ± 0.02	0.031 ± 0.02	0.072 ^{cd} ± 0.03
10	0.087 ± 0.01	0.072 ± 0.01	0.041 ± 0.02	0.02 ± 0.01	0.054 ^{c} ± 0.04
11	0.073 ± 0.01	0.048 ± 0.02	0.003 ± 0.001	0.010 ± 0.01	0.034 ^{b} ± 0.03
Means ± SD	0.088 a ± 0.05	0.079 a ± 0.04	0.055 b ± 0.04	0.048 b ± 0.04	

The values in the same row and column with different letters are significantly different ($P< 0.05$)

Table 2 Cellulase specific activity (mg of glucose min^{-1} mg $protein^{-1}$) in the stomach and intestinal extracts of adult male and female *Hyriopsis bialatus*. Enzymatic reaction was performed at pH 6.0 at different temperatures.

Temperature	Stomach		Intestine		Means±SD
(°C)	Male	Female	Male	Female	
20	0.072 ± 0.01	0.071 ± 0.01	0.027 ± 0.001	0.035 ± 0.004	0.052 ^{b} ± 0.02
25	0.086 ± 0.01	0.072 ± 0.01	0.042 ± 0.003	0.049 ± 0.002	0.063 ^{c} ± 0.02
30	0.100 ± 0.01	0.093 ± 0.01	0.068 ± 0.01	0.074 ± 0.02	0.083 ^{d} ± 0.02
35	0.122 ± 0.004	0.116 ± 0.01	0.078 ± 0.01	0.078 ± 0.01	0.099 ^{e} ± 0.02
40	0.134 ± 0.01	0.123 ± 0.01	0.078 ± 0.02	0.085 ± 0.01	0.105 ^{ef} ± 0.03
45	0.150 ± 0.01	0.135 ± 0.01	0.101 ± 0.004	0.088 ± 0.01	0.118 ^{g} ± 0.03
50	0.155 ± 0.01	0.154 ± 0.01	0.097 ± 0.01	0.104 ± 0.004	0.128 ^{h} ± 0.03
55	0.145 ± 0.01	0.130 ± 0.04	0.090 ± 0.01	0.076 ± 0.01	0.110 ^{fg} ± 0.04
60	0.111 ± 0.01	0.121 ± 0.01	0.074 ± 0.01	0.073 ± 0.02	0.096 ^{e} ± 0.02
65	0.100 ± 0.01	0.112 ± 0.003	0.062 ± 0.01	0.047 ± 0.01	0.080 ^{d} ± 0.03
70	0.084 ± 0.01	0.083 ± 0.01	0.056 ± 0.004	0.033 ± 0.003	0.066 ^{c} ± 0.02
75	0.068 ± 0.01	0.075 ± 0.004	0.036 ± 0.01	0.028 ± 0.003	0.052 ^{b} ± 0.02
80	0.060 ± 0.02	0.035 ± 0.01	0.024 ± 0.01	0.007 ± 0.002	0.031 ^{a} ± 0.02
Means ± SD	0.106 a ± 0.03	0.102 a ± 0.04	0.064 b ± 0.03	0.062 b ± 0.03	

The values in the same row and column with different letters are significantly different ($P< 0.05$)

30°C. An important role of cellulase in mussels was related to the digestion of phytoplankton cell wall which contains cellulose and other polysaccharides. The results showed that the amount of cellulase activity in the stomach of both sex was relatively higher than that in the intestine at every temperature including the habitat temperature of 28-30°C. This correlated well with the intracellular and extracellular carbohydrate digestion and absorption, which predominantly occurred in stomach (George, 1952; Stone and Morton, 1958; Teo and Sabapathy, 1990; Fernandes-Reiriz *et al*., 2001).

Optimum pH of lipases in both stomach and intestine were at pH 8.0 (Figure 2A). At this pH, both stomach and intestine lipases showed high activity at 35-55°C (Figure 2B). Lipases activities exhibited in the stomach of both sex were higher than that in the intestine. This could be related to the extracellular fat digestion, which occurred in stomach more than in intestine (George, 1952).

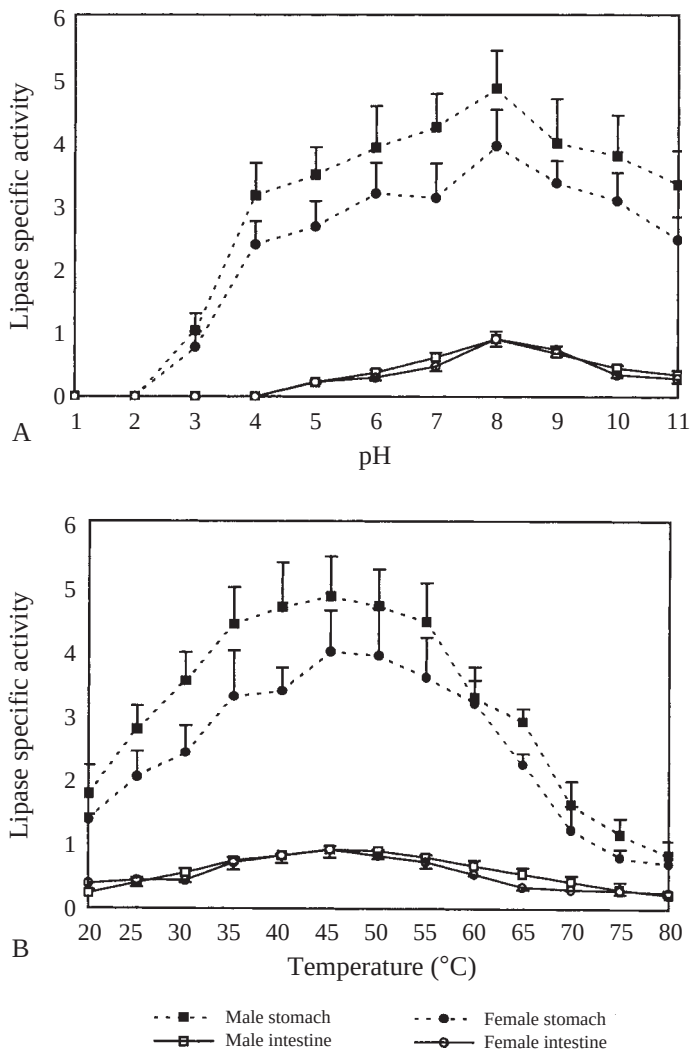

Figure 2 Lipase specific activity (U min^{-1} mg $protein^{-1}$) in the stomach and intestinal extracts of adult male and female *Hyriopsis bialatus*. Enzymatic reaction was performed at 45°C showing at various pH (A). At pH 8.0, the enzyme activity was done at different temperatures (B).

Table 3 Lipase specific activity (U min^{-1} mg $protein^{-1}$) in the stomach and intestinal extracts of adult male and female *Hyriopsis bialatus*. Enzymatic reaction was performed at 45°C at various pH.

pH	Stomach		Intestine		Means±SD
	Male	Female	Male	Female	
1	nd	nd	nd	nd	O^{a}
2	nd	nd	nd	nd	O^{a}
3	1.030 ± 0.34	0.777 ± 0.30	nd	nd	0.452 ^{b} ± 0.52
4	3.166 ± 0.62	2.397 ± 0.45	nd	nd	1.391 ^{c} ± 1.52
5	3.490 ± 0.52	2.683 ± 0.48	0.221 ± 0.06	0.228 ± 0.03	1.656 ^{cd} ± 1.55
6	3.907 ± 0.79	3.200 ± 0.58	0.373 ± 0.10	0.300 ± 0.05	1.944 ^{de} ± 1.75
7	4.223 ± 0.64	3.507 ± 0.65	0.614 ± 0.11	0.470 ± 0.08	2.203 ^{e} ± 1.80
8	4.827 ± 0.74	3.933 ± 0.69	0.901 ± 0.06	0.906 ± 0.15	2.642 ^{f} ± 1.90
9	3.977 ± 0.85	3.363 ± 0.42	0.679 ± 0.14	0.740 ± 0.14	2.190 ^{e} ± 1.60
10	3.780 ± 0.77	3.087 ± 0.53	0.444 ± 0.12	0.343 ± 0.05	1.913 ^{de} ± 1.66
11	3.337 ± 0.64	2.477 ± 0.44	0.341 ± 0.04	0.287 ± 0.08	1.610 ^{cd} ± 1.43
Means ± SD	2.885 a ± 1.73	2.311 b ± 1.42	0.325 c ± 0.31	0.297 c ± 0.31	

nd = not detected.

The values in the same row and column with different letters are significantly different ($P< 0.05$)

Table 4 Lipase specific activity (U min^{-1} mg $protein^{-1}$) in the stomach and intestinal extracts of adult male and female *Hyriopsis bialatus*. Enzymatic reaction was performed at pH 8.0 at different temperatures.

Temperature (°C)	Stomach		Intestine		Means±SD
	Male	Female	Male	Female	
20	1.760 ± 0.54	1.363 ± 0.09	0.237 ± 0.04	0.392 ± 0.13	0.938 ^{b} ± 0.71
25	2.767 ± 0.44	2.020 ± 0.48	0.403 ± 0.08	0.437 ± 0.13	1.407 ^{c} ± 1.10
30	3.523 ± 0.54	2.400 ± 0.52	0.553 ± 0.07	0.435 ± 0.03	1.728 ^{cd} ± 1.40
35	4.407 ± 0.70	3.283 ± 0.88	0.733 ± 0.06	0.709 ± 0.14	2.283 ^{e} ± 1.75
40	4.677 ± 0.83	3.373 ± 0.44	0.817 ± 0.02	0.813 ± 0.15	2.420 ^{ef} ± 1.79
45	4.837 ± 0.76	3.987 ± 0.78	0.903 ± 0.06	0.907 ± 0.15	2.658 ^{f} ± 1.92
50	4.690 ± 0.69	3.923 ± 0.91	0.883 ± 0.03	0.810 ± 0.05	2.577 ^{ef} ± 1.89
55	4.443 ± 0.74	3.593 ± 0.75	0.793 ± 0.04	0.720 ± 0.12	2.388 ^{ef} ± 1.79
60	3.270 ± 0.33	3.183 ± 0.69	0.657 ± 0.11	0.533 ± 0.03	1.911 ^{d} ± 1.41
65	2.887 ± 0.25	2.213 ± 0.22	0.540 ± 0.11	0.337 ± 0.05	1.494 ^{c} ± 1.14
70	1.603 ± 0.44	1.217 ± 0.45	0.420 ± 0.12	0.303 ± 0.01	0.886 ^{b} ± 0.63
75	1.140 ± 0.31	0.793 ± 0.15	0.303 ± 0.14	0.283 ± 0.06	0.630 ^{ab} ± 0.41
80	0.831 ± 0.26	0.697 ± 0.19	0.220 ± 0.08	0.252 ± 0.06	0.500 ^{a} ± 0.32
Means ± SD	3.141 a ± 1.48	2.465b ± 1.24	0.574 c ± 0.25	0.533 c ± 0.24	

The values in the same row and column with different letters are significantly different ($P< 0.05$)

Interestingly, male mussels showed significantly higher stomach lipase specific activity than that in female. The higher level of lipase found in male might indicate a higher lipid consumption rate in male than in female mussels. Lipase was very important for mussel's feed digestion since freshwater phytoplankton contained high level of lipids that function in accumulation of energy. Some Thai freshwater phytoplankton strains contained 22.30-42.21% lipid (or 33.70-67.00 % essential fatty acids) on dry weight basis (Salaenoi *et al*., 1990).

Temperature and pH played important roles on enzyme activity and stability and can cause changes in enzyme structure as well as its catalytic performance. Range of pH and temperature of the digestive enzyme activities and stability was one of the indicators for *in vivo* digestion conditions of the animal, and would be useful for formulating artificial diets that is suitable for the digestion under different rearing conditions of the freshwater mussel.

At 28-30°C, cellulase and lipase specific activities dominated in the stomach. This suggested that at habitat temperature, primary digestion occurred in stomach. Secondary digestion of large particles which might be partially digested followed later by activities of lower levels of cellulase and lipase in intestine which agreed with Owen (1955).

In addition, the study on cellulase and lipase specific activity in the mussels at different development stages could provide some knowledge for improvement of feed formulation for culturing of *H. bialatus* and other mussel species.

CONCLUSION

This study showed that cellulase specific activities had optima pH at 6.0 in both stomach and intestine and optimum temperature at 35-55°C in the stomach and 30-60°C in the intestine. Lipase showed its optimum activity at pH 8.0 and optimum temperature at 35-55°C in stomach. Both enzymes showed higher specific activities in stomach than in intestine. At habitat temperature (28-30°C), cellulase and lipase specific activities dominated in the stomach.

ACKNOWLEDGMENTS

This work was supported by the grant from the Graduate School, Kasetsart University. We would like to thank the Department of Aquaculture, Faculty of Fisheries, Kasetsart University, for providing a pond for culturing mussels.

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Received date : 01/11/02
Accepted date : 27/12/02