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Properties of chymotrypsin-like enzyme in the mudcrab *Scylla serrata*, brine shrimp *Artemia salina* and rotifer *Brachionus plicatilis*

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ABSTRACT

The present study aimed to characterize chymotrypsin-like enzymes in three species, namely the mudcrab Scylla serrata, the brine shrimp Artemia salina and the rotifer Brachionus plicatilis. Optimized conditions of assay were established in terms of the volume of crude extracts used and the time of reaction. The crab enzyme was the most affected by pH showing big increases in its activity until pH 8.0 and abruptly decreased beyond this level. The Artemia chymotrypsin exhibited maximal activity at 7.0 - 7.5 while the rotifer enzyme was the least affected by pH with small increases in its activity until its maximum level at pH 8.5. The stability of the mud crab enzyme was the most affected by pH; the Artemia and the rotifer enzymes exhibited maximal activity at 7.0 to 7.5. The enzyme activity of the mud crab was maximal at 30^{o}C and decreased abruptly at higher temperature. In contrast, the Artemia chymotrypsin-like activity was practically unaffected by temperature and the rotifer enzyme exhibited maximal activity at 25^{o}C and gradually decreased with increased temperature.Thermal stabilities were slightly affected in all three species;a small peak was observed in the mud crab enzyme at 25^{o}C. The rotifer and the Artemiaenzyme stabilitydecreased slightly and linearly with temperature. The determined Km for benzoyl-L-tyrosine ethyl ester (BTEE) of the rotifer, Artemia, and the crab chymotrypsin-like enzymes were estimated to be 1.3, 0.4 and 0.5 nmol N-benzoyl-L-tyrosine produced min^{-1}mg protein^{-1}, respectively. Conclusion: Chymotrypsin-like enzymewas stable at alkaline pH and at room temperature or above for all three species. The Artemia and the mud crab chymotrypsin-like activity manifested higher substrate-enzyme affinity in contrast with the rotifer enzyme which exhibited the least affinity.

Key words: protease, crustaceans, enzyme assay, live food, enzymatic properties, Kinetic properties, serine proteases

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INTRODUCTION

Chymotrypsin and trypsin are alkaline proteolytic enzymes present in invertebrates. They belong to the serine protease family, one of the largest family in the animal kingdom[1]. Within this family, the chymotrypsin family includes chymotrypsin A which contains a variety of enzymes such as chymotrypsin, trypsin, elastase, granzyme and different matrix peptidases [2] which are prevalent in the extracellular spaces while chymotrypsin B plays an important role in intracellular protein turnover. Chymotrypsin-like serine protease plays an important role in immune defense against pathogens in shrimp [3,4].

Chymotrypsin, together with trypsin are the most abundant proteases in the digestive system of aquatic organisms. The mammalian chymotrypsin is well studied while those in the invertebrates are mostly focused on the digestive system of some pest insects [1]. In the lepidopteran *Spodoptera exigua*, chymotrypsin is found to mediate proteolytic remodeling in the gut during larval-pupal transition [5]. The enzyme plays an important role in the

molting process in the red flour beetle *Tribolium castaneum*[2].In mollusks, chymotrypsin has been studied in scallop [6] and abalone [7]. In crustaceans, there are very few studies that have been done on chymotrypsin-like enzyme[8, 9]. The enzyme in the Chinese shrimp *Fenneropenaeus chinensis* is involved in innate immune reactions after bacterial and viral challenges [10].

The catalytic properties of these enzymes are similar to those of mammals. In crustaceans, the anatomy of the digestive system affects protein digestion which is composed of a foregut, a midgut (or hepatopancreas), and a hindgut [11]. As the name implies, the function of hepatopancreas in crustacean combines the functions of the liver and pancreas; it produces digestive proteases [12] such aschymotrypsin. Crustacean chymotrypsin-like enzyme hydrolyzes synthetic substrates in a narrow range of specificity and appears to have unique catalytic properties [13].

Studies on some properties of chymotrypsin-like enzyme will help in the understanding of digestive function of crustaceans and rotifer. Hernandez-Cortes et al [4] did not find any evidence of chymotrypsinogen and were only found in their active forms; thus, it did not require induction by another enzyme. Chymotrypsin is a short-term indicator for the nutritional condition and nutritional requirement of larvae [15]. In invertebrates such as the swimming crab (*Portunus trituberculatus*) the gene expression of chymotrypsin are down-regulated by ammonia exposure suggesting its involvement in the response to ammonia-N exposure [16]. In the Pacific white shrimp *Penaeus vannamei*, chymotrypsin gene was down-regulated after long term (56 days) salinity stress, which may be related to immunodepression [17].

Invertebrates exhibit higher chymotrypsin activity than some vertebrates Thus, invertebrates are good candidates as a source of digestive enzymes for biotechnological application. As an an example, chymotryptic activities of the Mediterranean sea urchins (*Arbacia lixula* L., *Paracentrotus lividus* and *Sphaerechinus granularis*) exhibit twice higher activities than in rainbow trout *Oncorhynchus mykiss* [18]. Chymotrypsin is always produced in industry from fresh cattle or swine pancreas and are made into tablets for oral consumption or as aliquid injection; invertebrate chymotrypsin can dramatically lower the cost of production.

In the mud crab *Scylla serrata*, chymotrypsin-like enzyme was detected in all larval stages. The activity was about 25% of the maximum at stage Z1, doubled at Z2 and Z3, declined to 40% at Z4 and Z5, abruptly increased to maximum activity during the megalopa stage, and fell to about 33% at the first instar stage [9]. In the brine shrimp, a sudden increase in enzymatic activity during hatching of the nauplii was observed [19]. So far chymotrypsin-likeactivity has not been characterized in rotifer has not been studied together with those of the mud crab and brine shrimp. This paper aimed to characterize chymotrypsin activity of three invertebrates, namely mud crab *Scylla serrata*, the brine shrimp *Artemia* sp and the rotifer *Brachionus plicatili.*

MATERIALS AND METHODS

Experimental animals

Live mud crab (*Scylla serrata*) were purchased from Roxas City, Capiz, Philippines and were acclimatized to concrete tanks in the hatchery until assay. *Brachionus plicatilis* stock was purchased from SEAFDEC AQD at Tigbauan, Iloilo, Philippines, reared and mass produced in a 1-ton tank located at the Institute of Aquaculture, University of the Philippines Visayas. Rotifers were fed with the green microalage *Tetraselmis chuii*and were harvested by filtration using 30 µm mesh plankton nets, appropriate sample size collected and used in enzyme assays. Commercially available *Artemia* cysts were hatched in the laboratory following the manufacturer's instruction. Nauplii of about 0.1 g wet weight was collected within 6-8 h after hatching and used for enzyme preparation and assay.

Preparation of the enzyme

Guts of mud crab or whole *Artemia* or rotifer samples were washed with cold extraction solution (50 mM citrate phosphate buffer pH 7.0), weighed and homogenized in the same solution at 1:20 ratio (w/v) using an Ultraturrax homogenizer. The homogenate was centrifuged at 4000 rpm for 15 min and the supernatant was used as enzyme preparation.

Chymotrypsin assay

The activity of the enzyme was determined using the method of Hummel [20]. Briefly, the assay mixture consisted of 1.4 ml of 1.07 mM benzoyl-L-tyrosine ethyl ester (BTEE) dissolved in 50% (w/w) methanol, 1.0 ml 80mM Tris-HCl buffer (pH 7.8) containing 0.1 M CaCl2, and 0.3 ml extract in a final volume of 2.7 ml. The reaction was stopped by adding 0.3 ml 30% acetic acid. The hydrolysis of N-benzoyl-L-tyrosine ethyl ester into N-benzoyl-L-tyrosine + ethanol caused an increase in absorbance at 256 nm. Enzyme activity was expressed as nmol N-benzoyl-

L-tyrosine produced min^{-1}mg $protein^{-1}$ at 25°C and pH 7.8 using the millimolar extinction coefficient of BTEE at 256 nm of 0.964(Sigma Aldrich 2015).

Progress curve with time and enzyme concentration.
To establish the method to be used in routine assays for chymotrypsin in each organism, progress curves were obtained from 0 to 60 min, and also activity vs enzyme concentration from 0.1 to 0.5 mL of the enzyme preparation.

Estimation of Michaelis-Menten constant (Km).
The substrate concentration and chymotrypsin-like activity relationship was investigated at a concentration range of $2.5x10^{-4}$ to 4.0 $x10^{-3}$µmol benzoyl-L-tyrosine ethyl ester (BTE), keeping the other components of the reaction mixture constant. The results were plotted by the double reciprocal plots (i.e. Lineweaver-Burk plots) to obtain the Michaelis-Menten constant (Km) of chymotrypsin for BTEE.

Optimum temperature and thermal stability.
The optimum temperature of the reaction was determined by conducting assays at various reaction temperatures (range of 25 to 40°C). Thermal stability of the enzyme was determined by incubating the enzyme preparation at different temperatures ranging from 0-55 °C for 1 h, after which assay of the chymotrypsin-like enzyme was done.

Optimum pH and pH stability. The effect of pH on enzyme activity was determined in50 mM citrate phosphate buffer at various reaction pH ranging from pH 5.9 to pH 8.5 at 25 °C. Enzyme pH stability wasdetermined by incubating the enzyme preparation at different pHs for 1 h at 0-4 °C afterwhich enzyme assays were done.

RESULTS

The linear part of the progress curves with time varied from 30 min for *Artemia*,to 50 min for the mud crab and to 60 min for the rotifer (Figure 1). Chymotrypsin activities of the crab exhibited linearity with enzyme concentration up to 0.3 mL, while the crab and the rotifer exhibited linearity up to the maximum volume of 0.5 mL (Figure 2).

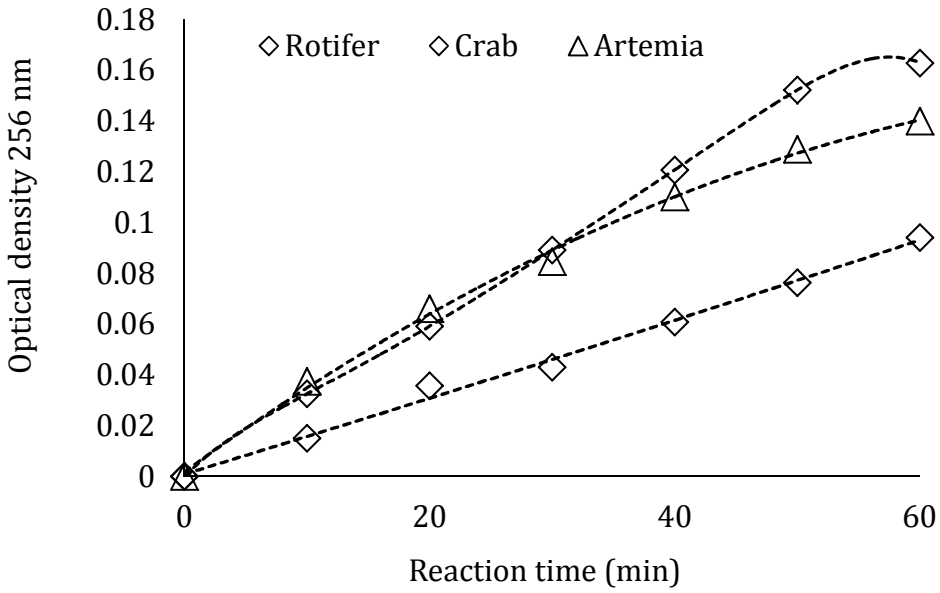

Figure 1. Progress curve of chymotrypsin-like activity with time of reaction of mud crab, *Artemia* and rotifer

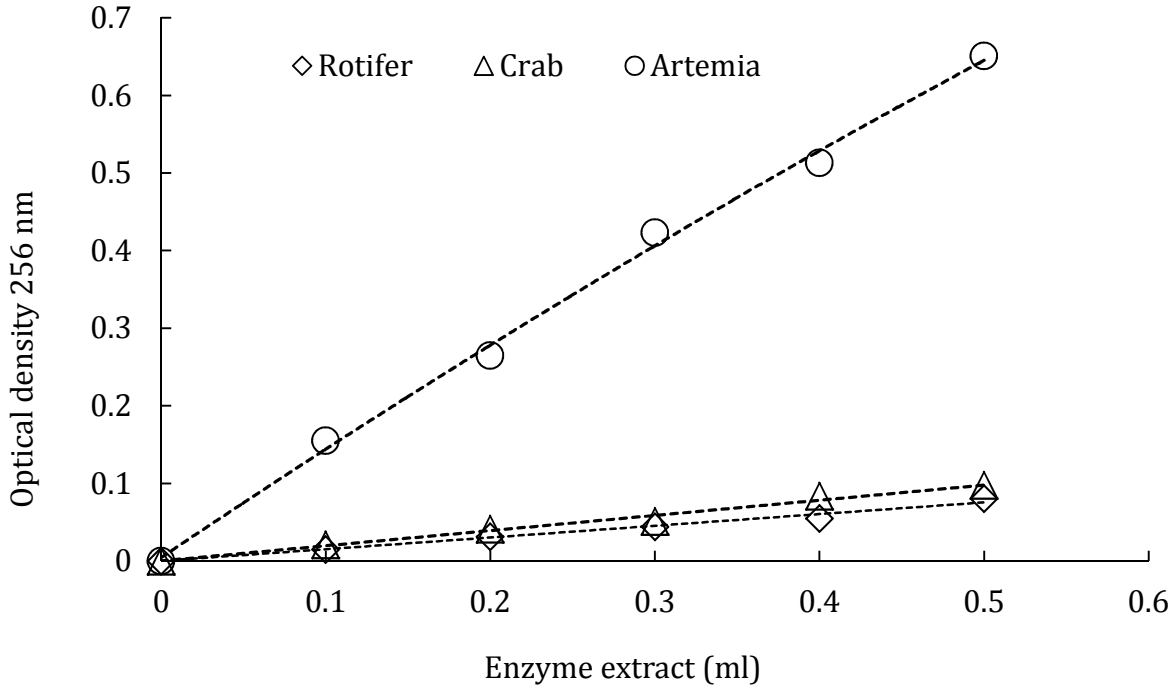

Figure 2. Activity of chymotrypsin-like enzyme of mud crab, *Artemia* and rotifer against enzyme concentration

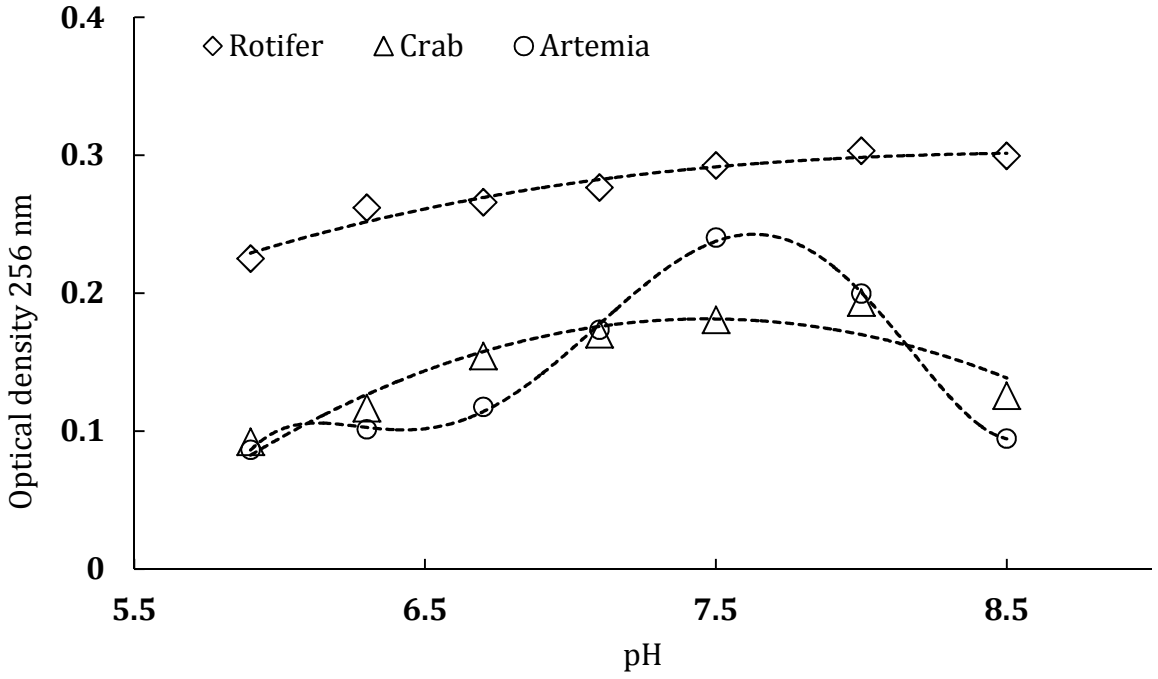

Figure 3. Changes in the activity of chymotrypsin-like enzymes of the mud crab, *Artemia* and rotifer with varying reaction pHs from pH 5.9 to pH 8.5

Of the three species studied in the present study, the crab chymotrypsin-like enzyme was the most affected by pH showing big increases in its activity until pH 8.0 and abruptlydecreased at pH 8.5 (Figure 3). The *Artemia* chymotrypsin-like enzyme exhibited an hyperbolic curve with maximal activity at 7.0 to 7.5 while the rotifer enzyme was the least affected by pH with very small increases in the activity level until the highest activity at pH of 8.5.

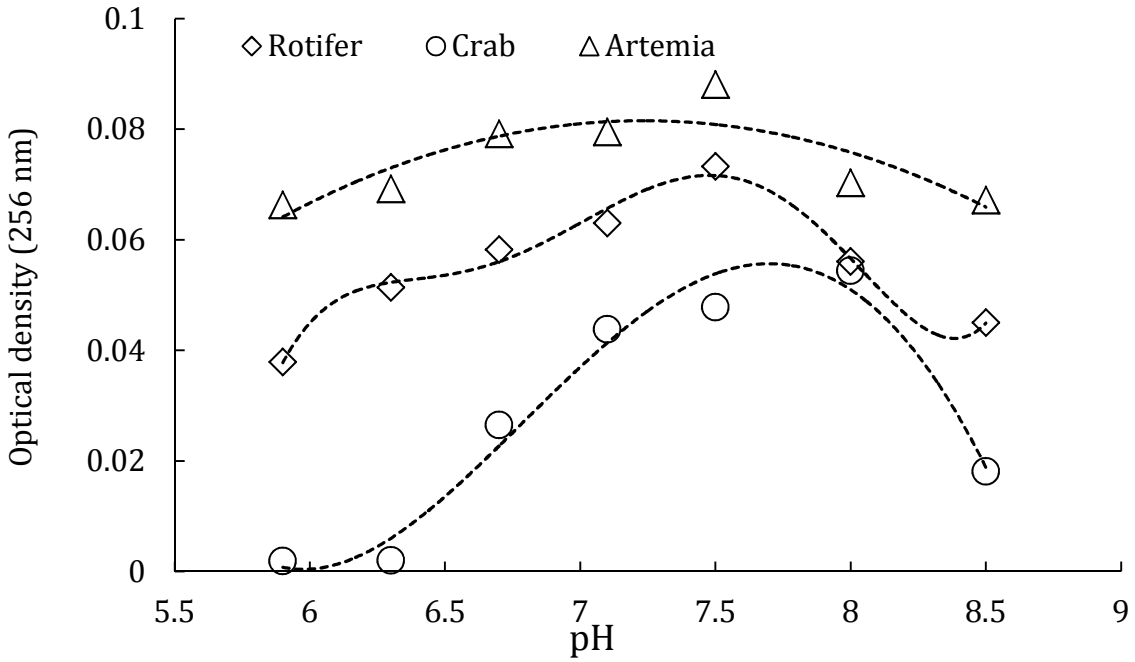

Figure 4. Stability of chymotrypsin-like enzyme of the mud crab, *Artemia* and rotifer as affected by pH

The stabilities of chymotrypsin-like enzymes as affected by pH in shown in Figure 4. The effects of reaction pH in the assay of the three enzymes in the present study reflected those of the pH effects on their stabilities; the mud crab enzyme being the most affected while those of the *Artemia* and the rotifer enzymes were practically unaffected exhibiting small peaks at 7.0 to 7.5 and decreased slightly beyond this pH.

Figure 5 shows the effect of the reaction temperature on the chymotrypsin-like activities of the three species at pH 7.0. The enzyme activity of the mud crab was maximal at 30^{o}C and decreased abruptly beyond this temperature. In contrast, the *Artemia* chymotrypsin-like activity was almost unaffected by the reaction temperature while the rotifer enzyme exhibited maximal activity at 25^{o}C, gradually decreased at higher temperature.

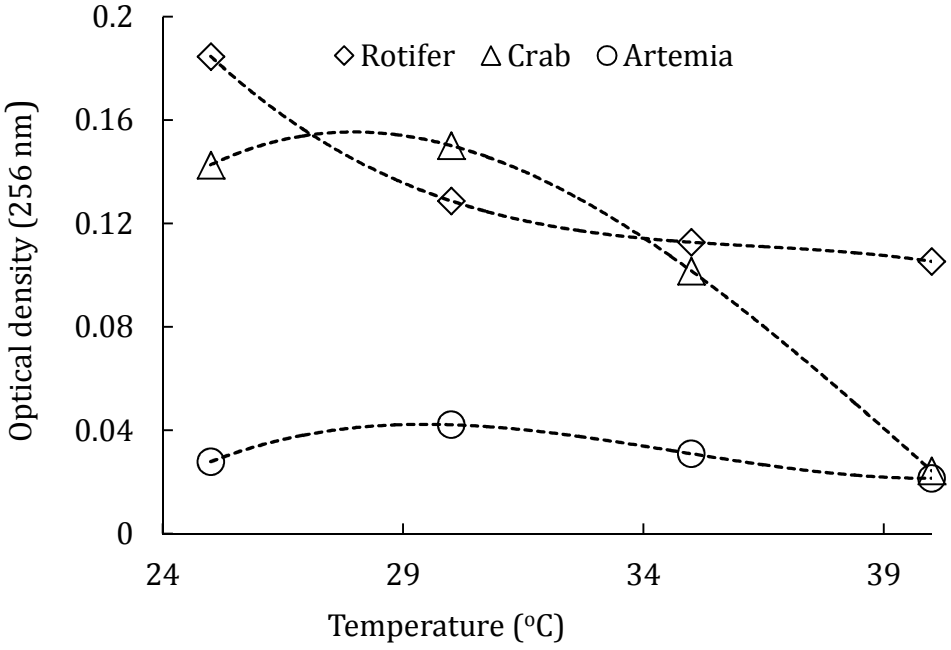

Figure 5. Effect of temperature on the activity of chymotrypsin-like enzyme in the mud crab, *Artemia* and rotifer

Temperature slightly affected the chymotrypsin-like activities in all three species except the small peak observed in the mudcrab enzyme at the intrapolated temperature of 10-15^{o}C and an actual peak at 25^{o}C (Figure 6). The rotifer and the *Artemia* chymotrypsin-like stability were slightly decreased by the 1 h immersion at increasing temperature in a linear fashion.

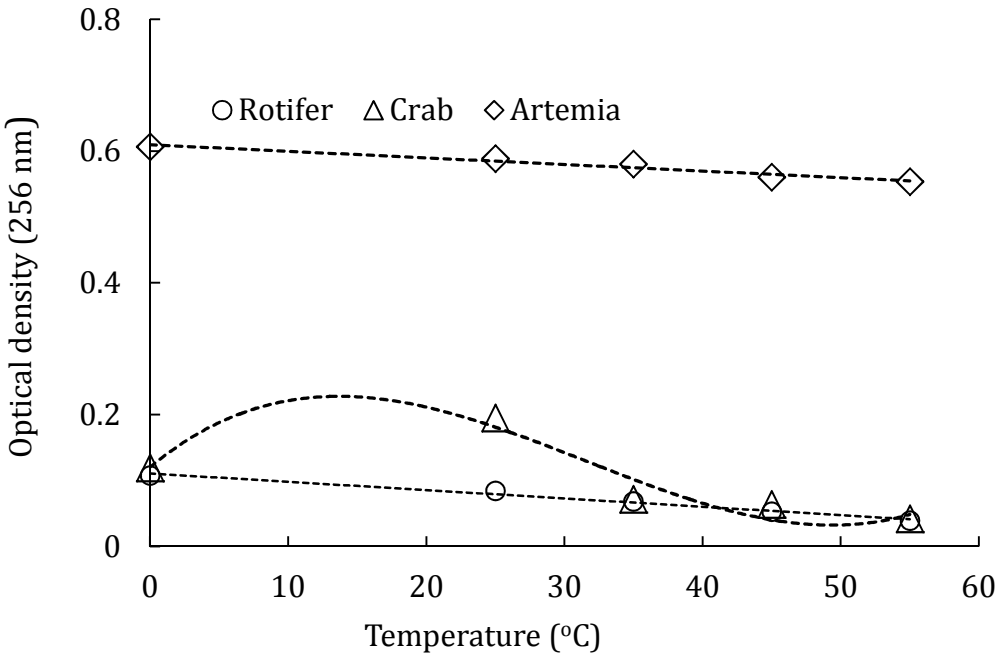

Figure 6. Thermal stability of chymotrypsin-like enzyme of the mud crab, *Artemia* and rotifer

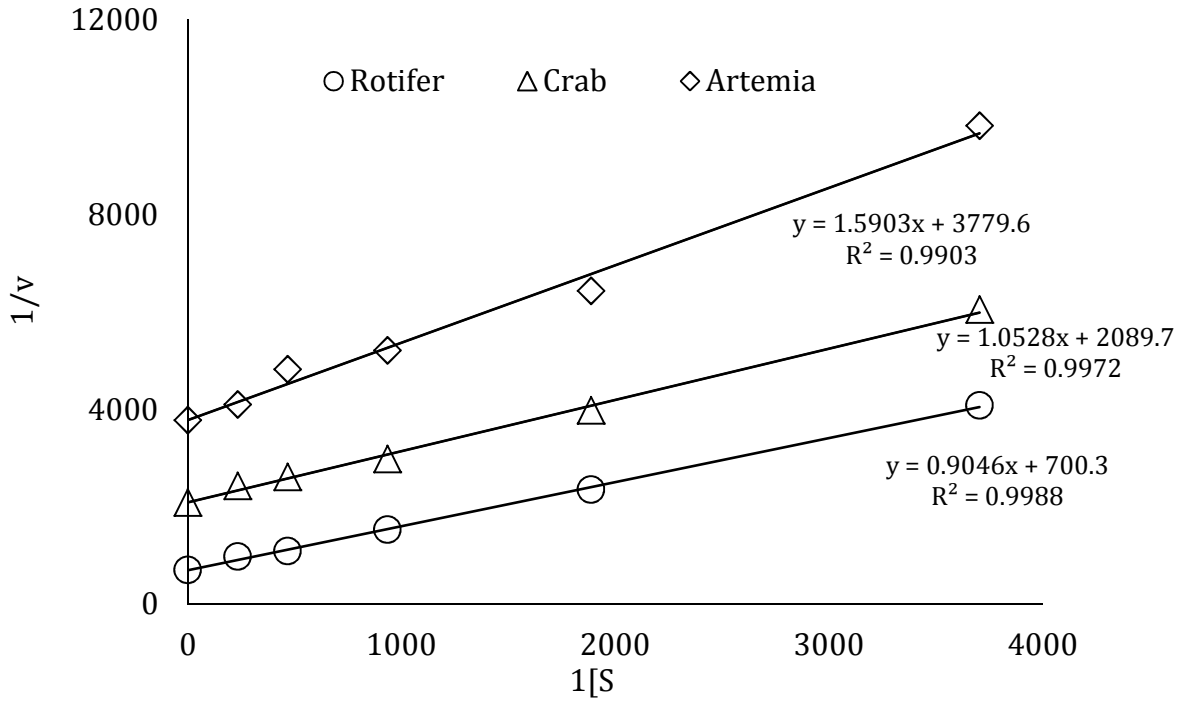

Figure 7. Double reciprocal plot of the activity of the chymotrypsin-like enzyme in the mud crab, *Artemia* and rotifer, with estimated Km of 0.5, 0.4 and 1.3 nM N-benzoyl-L-tyrosine min^{-1}mg protein^{-1}, respectively

The determined Km for benzoyl-L-tyrosine ethyl ester (BTEE) of the rotifer, *Artemia*, and the crab chymotrypsin-like enzyme were estimated to be 1.3, 0.4 and 0.5 nmol N-benzoyl-L-tyrosine produced min^{-1}mg protein^{-1}respectively (Figure 7).

DISCUSSION

Results on the linearity curves of activity against reaction time and volume of enzyme preparation in the present study established the optimized protocol for the chymotrypsin assay in the three species. This could be useful for researchers who would be doing further studies on this enzyme such as purification or its molecular biology.

Results of optimum pH of chymotrypsin-like activity in the present study agreed well with those of the purified enzyme reported by Hernandez-Cortes et al [14] in *Penaeus vannamei*; in their results, the peak of activity was between 8.0 to 8.3, similar to the values of pH 8.0 in the mud crab and pH 8.5 in the rotifer in the present study. In *Artemia*, optimum pH of the reaction mixture was from pH 7.0 to pH 7.5. Results in other invertebrate and vertebrate chymotrypsin were similar with those of the present study [22, 23]. In general, proteases from crustaceans have maximum activities from neutral to alkaline pH, similar to mammalian enzymes but the crustacean enzyme is more acid labile [14] as was the case in the present study.

Maximal stability of the chymotrypsin-like enzymes of pH range of 7.0-7.5 in the three species agreed well with that of the purified enzyme of the *Penaeus vannamei* [14]. The observation that the purified enzyme was more stable at alkaline than acid condition was also observed in the present study in all the three species. As was the finding in the Pacific white shrimp, this results could be related to the pH of the intestine of pH 7.4. The acid-labile properties and mild activity above room temperature could make the crustacean chymotrypsin-like enzyme as a candidate for food technology applications. The observation on the acid lability of chymotrypsin-like enzyme could be useful in the inactivation of proteases in food processing, by lowering of the pH of the reaction. Black spot or melanosis phenomena of the fishery products after storage [24] and also autolysis of krill [25] and the mushy texture in crayfish meat [26] are all caused by proteases. Also, protein modification by controlled hydrolysis enhances the functionality of raw protein [27].

Chymotrypsin-like activity in either the brine shrimp or the rotifer, and to a limited extent the mud crab enzyme in the present study were very stable even at room temperature or above. This observation could be related to the contribution of the exogenous enzymes coming from the two prey organisms. Protease such as chymotrypsin-like enzymes could be released to the gut of the mud crab with activities almost completely intact to help in digestion. It has been reported that total protease contribution to food digestion was highest (84.4%) at Z1 stage and lowest (24.6) at the first instar stage of *Scylla serrata* larvae [28].For food-grade proteases to be useful in food processing, they must have a wide thermal stability similar to the properties of the enzymes in the present study.

Kinetic and enzymatic studies of chymotrypsin are necessary for understanding the digestive function of this shrimp. The Km for BTEE indicated the affinity of the substrate to the enzyme and also the threshold of substrate concentration that would induce chymotrypsin-like activity. Results of the present study indicated that the affinity of the enzyme to the substrate BTEE of the *Artemia* and the mud crab enzyme were similar while that of the rotifer was less than half of the affinity of the two species; affinity is an indicator of the velocity of the reaction. Rotifer which constitutes the live food of early larvae of aquatic organisms is expected to be released into the gut of the larval predators. *Artemia*, in contrast, are live food for later larval stages and its high velocity of enzyme reaction would be necessary to support the processing of external food and also its fast growth. Km also indicates the physiological concentration of substrate. Although BTEE is a synthetic substrate, it could indicate the level at which the enzyme could adjust to a pattern of substrate surgeafter feeding. The lower substrate threshold (i.e. lower Km) of the *Artemia* and mud crab enzyme would enable it to rise to a level beyond the physiological concentration of the substrate in their gut system.

CONCLUSION

The present paper documented the effects of pH and temperature on the velocity of chymotrypsin-like enzymes and on their stability in the three species. The crab enzyme was the most affected by pH showing big increases in its activity until its maximal activity at pH 8.0. The *Artemia* chymotrypsin exhibited maximal activity at 7.0 to 7.5 while the rotifer enzyme was the least affected by pH with very small increases until maximal activity at pH of 8.5. This pattern of results were also the pattern of the effects of pH on the chymotrypsin-like stability in the three species. The mud crab enzyme activity was maximal at 30^{o}C and decreased abruptly beyond this temperature. The *Artemia* chymotrypsin-like activity, in contrast, was almost unaffected by temperature and the rotifer enzyme exhibited maximal activity at 25^{o}C and decreased gradually as temperature was increased. Thermal stability of the enzyme was slightly affected by temperature in all three species. The determined Km for BTEE of the rotifer, *Artemia*, and the crab chymotrypsin-like enzymes were estimated to be 1.3, 0.4 and 0.5 nmol N-benzoyl-L-tyrosine produced min^{-1}mg protein^{-1} respectively. The Km indicated that the mud crab and *Artemia* enzymes had stronger

enzyme-substrate affinity than did the rotifer enzyme; thus, the *Artemia* and mud crab enzymesexhibited faster reaction velocities and higher physiological levels of substrate than the rotifer enzyme.

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