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Trypsin-like activities in the mudcrab *Scylla serrata*, brine shrimp *Artemia salina* and rotifer *Brachionus plicatilis*

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Abstract. The present study aimed to evaluate whether or not the activity of the prey trypsin could actually be optimal once released in the chamber of the crab's stomach by characterizing trypsin in the two circumstances: in the live preys and in the mudcrab itself. In addition, this study aimed to establish the routine assay that were used to compare the activities of the trypsin-like enzymes in the mud crab, brine shrimp and rotifer. Conditions for routine assay of the trypsin-like enzymes were established in each species such as the progress curve of the reaction with time and with the amount of enzyme as well as the Michaelis-Menten constant of the crude preparation. Optimal temperature and pH for the reaction of the tryptic-like enzymes were determined as well as their stability under various varying temperature and pH. Conclusion: Tryptic-like activities were linear up to 30 min incubation time using up to 0.3 mL of the enzyme preparation. Michaelis-Menten constant (Km) of the rotifer, mud crab and *Artemia* enzymes were 0.93, 0.59 and 0.23 µM, respectively. These were lower than the final concentration of the BAPNA substrate of 3.58 µM which meant that the substrate concentration was correctly in excess. Optimum temperature was 40 °C for the tryptic-like activities of the three species. Optimal reaction pHs were in the range of 6.0-6.7 for the mud crab and rotifer enzymes while it was 7.5-8.5 in the *Artemia* enzyme. The mud crab and rotifer enzymes were stable at pH 7.5-8.5 while that of the *Artemia* enzyme was pH 6.0-6.7. Tryptic-like activities were most stable at 25 °C for the mud crab and *Artemia* enzymes while that for the rotifer enzyme at 60 °C. From the characteristics of the tryptic-like enzymes observed in the present study, it indicated that the preys' trypsin-like enzymes were bioactive during digestion of food by mud crab and could considerably contribute to this process.
Key Words: crab, *Artemia*, rotifer, trypsin-like enzyme, optimal pH, optimal reaction temperature, pH stability, thermal stability.

Rezumat. Acest studiu urmăreşte să evalueze dacă activitatea tripsinei din pradă poate sau nu să fie optimă odată ce este eliberată în stomacul crabilor, prin caracterizarea tripsinei prin două circumstanţe: în prada vie şi în crabul *Scylla serrata*. În plus, acest studiu urmăreşte să stabilească protocolul de rutină folosit pentru a compara activităţile enzimelor de tip tripsină în crabi, creveţi şi rotifere. Condiţiile unui protocol de rutină pentru enzimele de tip tripsină au fost stabilite pentru fiecare specie, determinându-se curba de creştere a reacţiei în timp şi cantitatea de enzime precum şi constanta Michaelis-Menten pentru preparatele brute. Au fost determinate temperatura şi pH-ul optim al reacţiei enzimelor de tip tripsină, precum şi stabilitatea acestora la variaţii ale temperaturii şi pH-ului. Concluzii: Activităţile de tip tripsină au fost liniare până la 30 de min de incubare folosind până la 0,3 mL din preparatul enzimatic. Constanta Michaelis-Menten (Km) pentru enzimele din rotifere, crabi şi *Artemia* au fost 0,93, 0,59 şi 0,23 µM. Acestea au fost mai mici decât concentraţia finală de substrat BAPNA de 3,58 µM ceea ce înseamnă că, concentraţia substratului a fost în mod corect în exces. Temperatura optimă a fost 40 °C pentru activitatea tripsinică la toate trei speciile. pH-ul optim a variat între 6,0-6,7 pentru enzimele de la specia de crab şi rotifere în timp ce enzimele de la *Artemia* pH-ul a variat între 7,5-8,5. Enzimele de crab şi rotifere au fost stabile la pH 7,5-8,5 în timp ce la enzimele de la *Artemia* intervalul de stabilitate a fost între pH 6,0-6,7. Activităţile tripsinelor au fost mai stabile la 25 °C pentru crabi şi *Artemia* în timp ce la rotifere stabilitatea a fost la 60 °C. Pe baza caracteristicilor enzimelor de tip tripsină observate în acest studiu, se poate concluziona că tripsinele prăzii au fost bioactive pe parcursul digestiei hranei de către crabi şi pot contribui considerabil la acest proces.
Cuvinte cheie: crab, *Artemia*, rotifere, enzima tip tripsina, pH optim, temperatura optimă, stabilitate la pH, stabilitate termică.

Introduction. In mudcrab aquaculture, two major problems that have been identified are high mortality during the larval stages and the requirement for use of live feeds such as rotifers and *Artemia* (Kumlu 1999). In the larval stage, high mortality may be due to

feeding problems such as poor nutrition. Existing practices in hatcheries of mudcrab still rely heavily on commercial scale production of live prey. Enzymes in live prey are released to the digestive tract of crustacean larvae by autolysis or zymogens, which activate the endogenous enzymes within the larval gut (Kumlu & Jones 1995). Serrano (2012) previously measured the exogenous enzyme contribution derived from live feed to the endogenous amylase and total protease activities in the mud crab *Scylla serrata* larvae at various stages of development. Contribution of live food to amylase activity was highest at Z3 at about 60 % while that of total protease was highest at Z1 at 84 %.

The crustacean midgut gland releases huge amounts of digestive enzymes. The most abundant digestive proteinase found in the shrimp *Penaeus japonicus* is trypsin, which contributes about 6 % of the total soluble protein (Galgani & Benyamin 1985), while in the fiddler crab (*Uca pugilator*) trypsin accounts for approximately 33 % of the total hepatopancreatic protein (Eisen & Jeffrey 1969). Some authors (Galgani et al 1985a; Galgani et al 1985b) have emphasized the important role of this enzyme in penaeids and estimate its contribution to protein digestion at 60 %.

Trypsin-like enzymes sourced from live prey such as *Brachionus* and *Artemia* need to be active at the conditions in the cardiac chamber of the stomach of the mudcrab where they are involved in extracellular digestion of food. Thus, it is the aim of the present paper to evaluate whether or not the activity contribution of the prey trypsin could actually be optimal once released in the chamber of the crab's stomach by characterizing trypsin in the two live prey and in the mudcrab itself.

Material and Method

Experimental animals. Live mud crab (*Scylla serrata*) were purchased from Roxas City, Capiz, and were acclimatized to concrete tanks until assay. *Brachionus plicatilis* stock was purchased from SEAFDEC AQD at Tigbauan, Iloilo, reared and mass produced in 1-ton tanks located at the Institute of Aquaculture, UPV. Rotifers were reared using *Tetraselmis chuii*, a green microalgae, as feed. *Artemia* was purchased from Argent Co. Rotifers were maintained and propagated in fiberglass tanks and fed green algae *Tetraselmis chuii*. Rotifers were harvested by filtration using 30 µm mesh plankton nets and 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 assays.

Preparation of the enzyme. Guts of mud crab or whole Artemia samples or whole 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 (wet tissue weight to volume) in an Ultraturrax homogenizer. The homogenate was centrifuged at 4000 rpm for 15 min and the supernatant was used as enzyme preparation.

Trypsin assay. The activities of trypsin (EC 3.4.21.4) in adult *Scylla serrata*, *Artemia* nauplii and *Brachionus plicatilis* were measured by following the release of 4-nitroaniline from the synthetic substrate Na-benzoyl-DL-arginine-p-nitroanilide (BAPNA),(Geiger & Fritz 1988). The assay mixture consisted of 1.5 µmol mL^{-1} (final concentration) of the substrate solution, 0.1 mL of purified trypsin solution and buffer in a final volume of 2.25 mL. The reaction was started by adding BAPNA solution for 5 min and was stopped by adding 0.25 mL of 30% acetic acid. The absorbance of the supernatant was read at 405 nm and the enzyme activity was expressed as µmol 4-nitroaniline formed min^{-1} mL^{-1} of enzyme preparation. The activity of purified enzyme preparation was subtracted from the total trypsin activity.

Progress curve with time and enzyme concentration. In order to establish the routine assay for trypsin 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. The substrate concentration and activity relationship was investigated at a concentration range of $2.5x10^{-4}$ to $40x10^{-4}$ µmol min^{-1} min^{-1}, 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 trypsin for its substrate.

Optimum temperature and thermal stability. The optimum temperature of the reaction was determined by conducting assays at different temperatures ranging from 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 trypsin-like enzyme was done.

Optimum pH and pH stability. The effect of pH on enzyme activity was determined in 50 mM citrate phosphate buffer (pH 5.9-8.5) at 25 °C. Enzyme pH stability was determined by incubating the enzyme preparation at different pH for 1 h at 0-4 °C after which enzyme assays were done.

Results. The linear part of the progress curves with time varied from 20 min for rotifer to 40 min for the brine shrimp and 60 min for the crab enzyme (Figure 1). Trypsin activities of the crab and its food prey *Artemia* nauplii and rotifer exhibited linearity with enzyme concentration up to 0.3-0.4 mL (Figure 2).

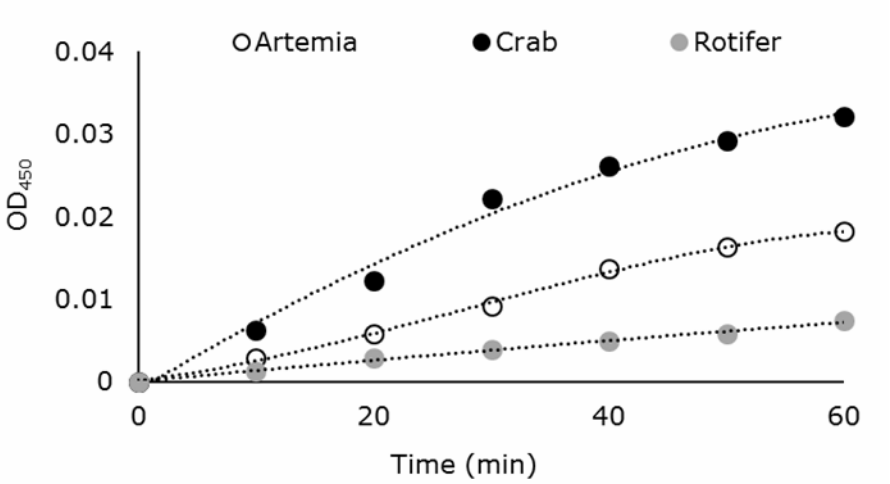

Figure 1. Progress curve of trypsin-like enzyme activity with time of reaction of mudcrab, *Artemia* and rotifer.

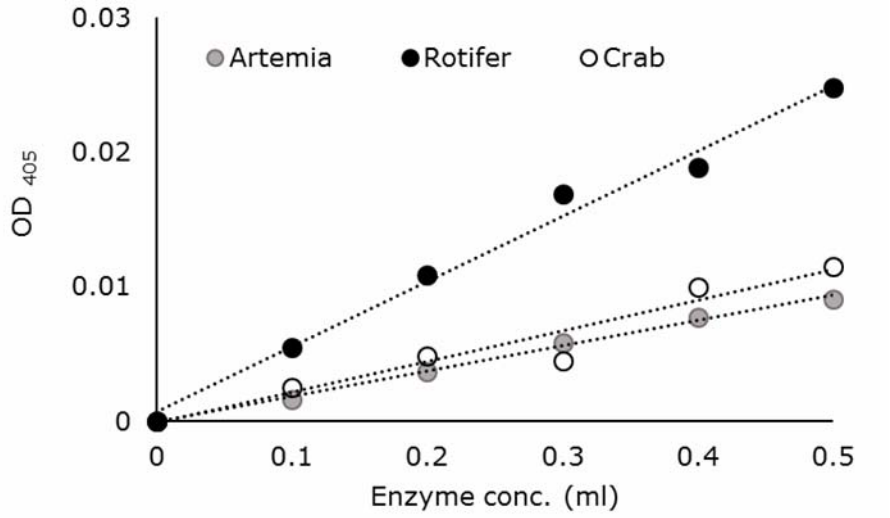

Figure 2. Activity of trypsin-like enzyme of mud crab, *Artemia* and rotifer against enzyme concentration.

Also, the determined Km for BAPNA of the rotifer, *Artemia*, and the crab trypsin-like enzyme were estimated to be 0.93 µmol, 0.23 µmol and 0.59 µmol, respectively (Figure 3). These initial findings laid out the routine assay conditions that were used to compare the trypsin-like activities of the three species i.e. within the linear portions of the progress curve and that of the enzyme concentration curve (20 min and 0.3 mL enzyme extract for all the three species). Also, the final concentration of BAPNA of 1.5 µmol could be considered in excess as a requirement for the routine assay so that the only variable during measurement was the amount of enzyme in the final reaction mixture.

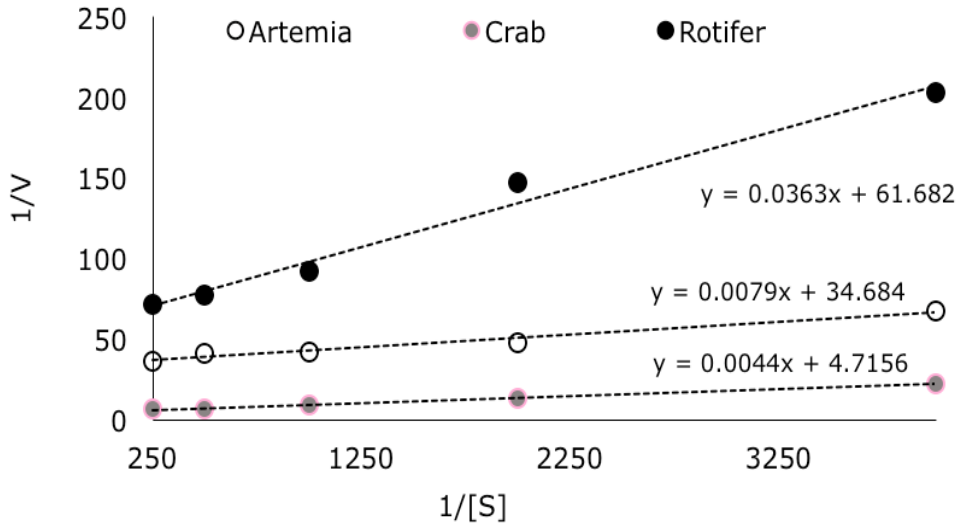

Figure 3. Double reciprocal plot of the activity of the trypsin-like enzyme of crab, *Artemia* and rotifer for the estimation of the Km for each enzyme.

Figure 4 shows the effect of the reaction temperature on the trypsin-like activities of the three species at pH 7.0. An almost similar pattern was obtained for the mudcrab and *Artemia* in which the activities increased non-linearly with increased reaction

temperature. Although the values measured for rotifer were very low compared to those of the other two species, the maximum measured activity occurred also at 40 °C.

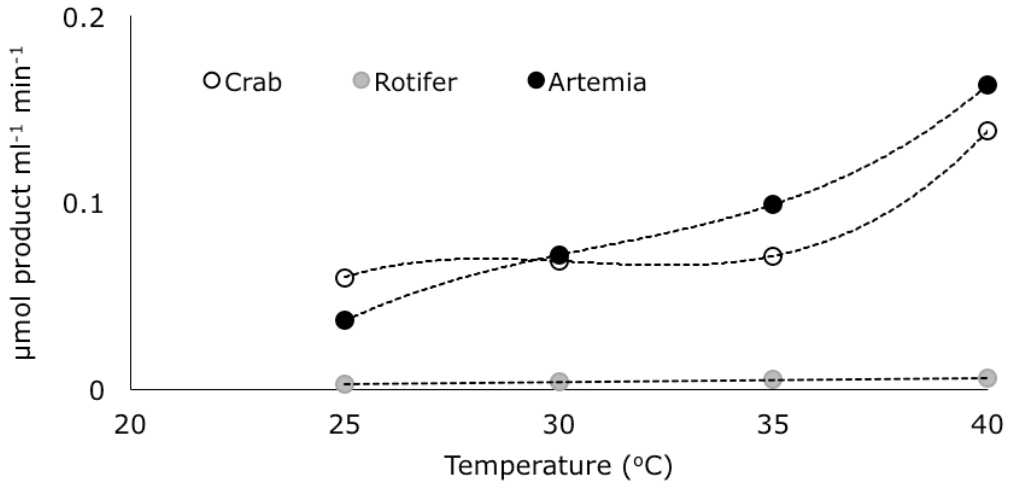

Figure 4. Effect of temperature on the activity of trypsin-like enzyme in crab, *Artemia* and rotifer.

The mudcrab trypsin-like activity was very thermostable even at 60 °C, retaining 100 % of the initial activity, while that of *Artemia* retaining 100 % of initial activity at 45 °C, but was totally inactivated when immersed at 60 °C for one hour (Figure 5). The trypsin-like activity of rotifer remained uniform at a very low level even at 60 °C.

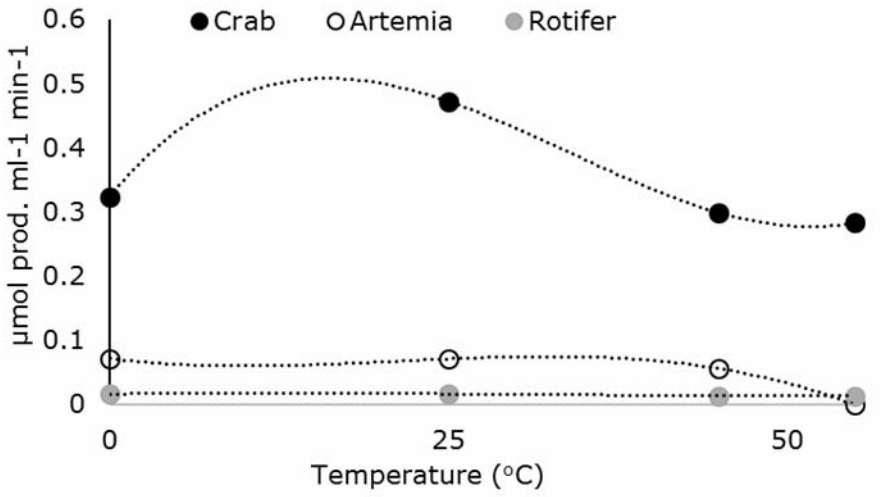

Figure 5. Thermal stability of trypsin-like enzyme of crab, *Artemia* and rotifer.

Trypsin-like enzyme from the three species showed slight activity changes with varying pH levels (Figure 6). The crab and rotifer enzyme activities exhibited maximal values at pH 7.5 to 8.5 while those of *Artemia* at pH 6.0 to 6.7.

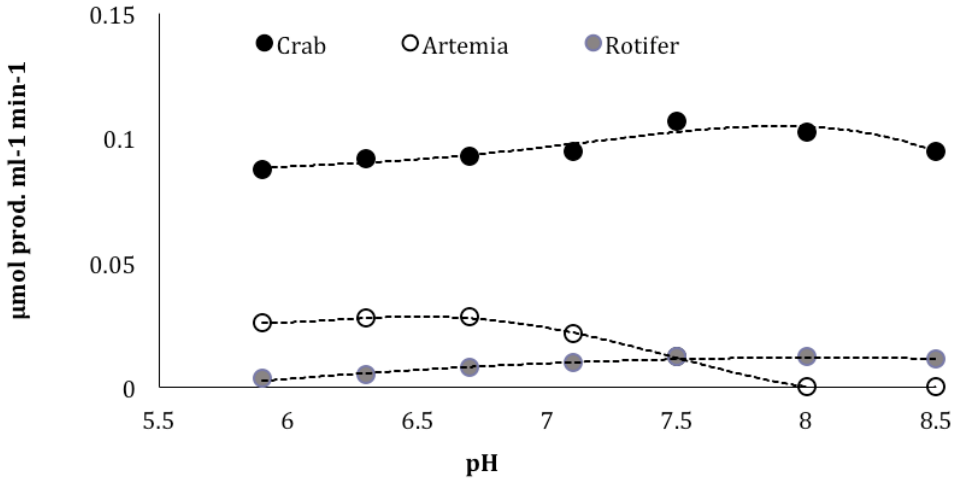

Figure 6. Effect of pH on the activity of trypsin-like enzyme of crab, *Artemia* and rotifer.

The latter was totally inactivated at pH 8 to 8.5. Stabilities of pH for the three species showed that the crab and rotifer trypsin-like enzymes were most stable at pH 7.5 to 8.5 while that of *Artemia* at a lower pH range of 6.0 to 6.7 (Figure 7).

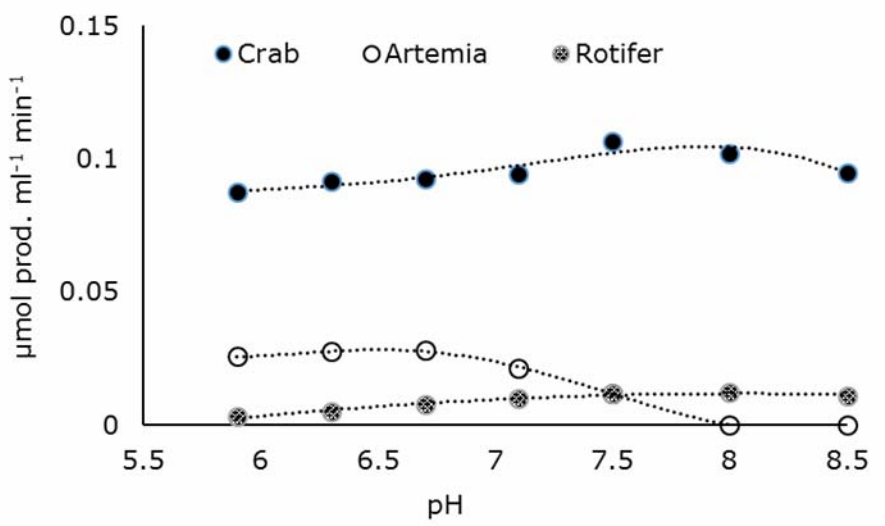

Figure 7. Stability of pH of the trypsin-like enzyme of crab, *Artemia* and rotifer.

Discussion. Comparison of enzyme data from one laboratory to another is difficult at best. This is so due to the use of different substrates and/or substrate concentrations, temperatures and buffers and the expression of digestive enzyme activity in different units in various laboratories. Furthermore, enzyme activities fluctuate in relation to daily metabolic rhythms, stage of moults (Harms et al 1991) and season (Biesiot & Capuzzo 1990). In the present study, we produced the required progress curves with time and enzyme concentration to establish a protocol for the routine assay. We also stablished the Km values for the BAPNA substrate so that it would be in excess amount avoiding a

condition that could be a limiting factor in the routine assay other than the amount of enzyme. These factors are necessary to calibrate the routine assay to be used as the yardstick for any comparison that would be made between species or even between tissues in one species.

The Km of any enzyme was also hypothesized to manifest the physiological concentration of the substrate under normal conditions. Although BAPNA is a synthetic oligopeptide meant to just measure the tryptic-like activity, one could assume that if it were a natural oligopeptide as a product of protein digestion in each species in the present study, the highest peptide concentration could be found in the rotifer (Km= 0.93 µM). *Artemia* exhibited the lowest oligopeptide concentration (Km= 0.23 µM) while that of the crab was intermediate (0.59 µM). However, Km also reflected the affinity of the tryptic-like enzymes to their substrate which was computed as the inverse of the Km value. In this case, the rotifer enzyme exhibited the least affinity for BAPNA while that of *Artemia* the highest affinity, while the one from the adult crab was intermediate affinity. The rotifer's natural food in the present study was *Nannochloropsis* sp. which was protein rich at 37.6 % of the organic content (SCSAFSN 2013), which could have induced more tryptic-like activity. *Artemia* at the nauplii stage was not capable of feeding and it might have metabolized its own body resources for energy within hours of hatching. Thus, the observed very low physiological oligopeptide concentrations inferred by the low Km value measured in the present study. This is despite the fact that *Artemia* nauplii was reported to contain very high protein (52.2 %, dry weight basis; Leger et al 1987) but this was a nutritional value of the dried *Artemia* and had nothing to do with oligopeptides playing as the substrate of the trypsin-like enzyme during digestion of food of the brine shrimp. The intermediate substrate concentration of the adult mud crab could be similar to that of first instar stage (i.e. C1) of the mudcrab in which it could be a manifestation of the mixing of various zooplankton becoming the food habit of the C1. As to the observed variation in the tryptic affinity for oligopeptide substrate in the present study, it could be a manifestation of the genetic differences leading to differences in the protein structure and active sites of each of the tryptic-like enzymes of the three species.

The foregut fluid in most crustaceans is earlier reported to be slightly acidic, usually in the range pH 5-7 (van Weel 1970) while that in the midgut in the range pH 6-7 (Brown 1995). These values are not in agreement with the values of pH optima of the enzymes in the present study with the exception of lipase. Hypothetically, if the pH was increased (i.e. made alkaline by either ingesting alkaline feed materials or perhaps secretion from the midgut gland parallel to the intestinal conditions in vertebrates), there would be a corresponding increase in the enzyme activity. This is so since, in normal physiological conditions, enzymes operate within their Km values reserving almost half of their potential capacity for a condition of high influx of relevant substrate. Another explanation was offered by Glass et al (1989) for the lack of acidic condition at the anterior end of the alimentary canal of *Penaeus monodon*. They state that in *Penaeus monodon* the chamber at the mouth end of the alimentary canal does not appear to act as a stomach as indicated by enzymic tests and the adult shrimp exhibited a value of pH 8.0 in this region before feeding and 7.0 after feeding. This lack of acidity that is also the case in the lobster, according to them, is a further indication that this chamber was not acting as a 'stomach' in the conventional meaning of the word.

The pH optima and pH stabilities of the crab digestive enzymes generally lie within the values of those in the prey in the present study (Table 1). These results suggested that the digestive enzymes of the prey remain bioactive even after ingestion by the crab larvae and could add to its digestive capability. In fish larvae, studies have shown that live feed is essential for the proper nutrient and feed assimilation since they have poor digestive facilities due to the still underdeveloped digestive organ in its early stages (Walford & Lam 1987; Munilla-Muran et al 1990). Moreover, Kolkovski et al (1996) has improved food assimilation in sea bass larvae by feeding a combination of microdiets and live feed. From the present study, it could be that larval crustacean digestive system may be parallel to that of fish larvae in having underdeveloped digestive organs. In freshwater prawn for example, larvae failed to grow and complete the larval stages when fed formulated diet alone (Jamari 1992). Enzyme assays reveal that larvae at early stages

have low digestive capacity and could only survive when live feeds are incorporated on its diet (Kamarudin et al 1994). Thus, as far as pH is concerned, tryptic-like enzymes when released into the digestive gland of the predator mud crab could remain active because of the close optimal values of reaction pH and pH stability with that of the two preys.

Conclusions. The established routine assay conditions were 20 min reaction time and 0.3 mL of the enzyme crude preparation to obtain a linear velocity for the enzyme; 1.5 µmol final concentration of the BAPNA substrate was considered in excess enough satisfying the requirement for a routine assay. All three enzymes exhibited maximal activity at 40 ^{o}C for temperature while pH 7.5-8.5 resulted in the mud crab and rotifer maximal tryptic-like activities while that for *Artemia* was pH 6.0-6.7. The enzymes were most at stable at pH 7.5-8.5 for the mud crab and rotifer enzyme while that for *Artemia* was pH 6.0-6.7. Thermal stability was highest at about room temperature of 25 ^{o}C and stability decreased above this temperature for the mud crab enzyme. The tryptic-like activity of the rotifer remained low but stable until 60 ^{o}C while the one from *Artemia* was totally inactivated at 60^{o}C. Since optimal pH and their pH stabilities of the prey enzymes were the same, it pointed to the fact that, when the prey trypsin-like enzymes was released into the digestive gut or gland of the mud crab during digestion, they remained bioactive and contributed to the digestion of protein in the gut of the mud crab.

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