

## Kinetic parameters of leucine aminopeptidases, optimal pH and temperature in the mud crab *Scylla serrata* and the brine shrimp *Artemia salina*

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**Abstract.** The present study aimed to investigate the influence of pH and temperature on its activity as well as estimate the kinetic parameters of the leucine aminopeptidases in the two crustaceans *Scylla serrata* and *Artemia salina*. Results of the present study showed that optimal pH for both leucine aminopeptidases of the mud crab and *Artemia* were in the range of 7.0 to 7.5. Optimal pH that resulted in the greatest LAP stability was on the neutral to alkaline range of pH 7.0 to 8.5 in the mud crab while it was on the acidic range of 6.0 to 7.0 in the brine shrimp. Optimal reaction temperature for the mud crab LAP was 0°C while that of the *Artemia* enzyme was 25°C. The mud crab LAP exhibited the highest stability at 0°C and exhibited instability with increasing temperature. At room temperature (normally at 25°C) until 35°C, the remaining activity of the mud crab LAP was around 70% of the maximum, while at the same temperature the *Artemia* enzyme was maximal at 25°C and abruptly decreased to 60% of maximal activity at 35°C. The maximum velocity ( $V_{max}$ ) and the Michaelis-Menten constant ( $K_m$ ) of the mud crab were estimated to be 11.1 nmol product  $min^{-1}$  mg protein $^{-1}$  and 3.36 nM *p*-nitroaniline, respectively, while those of the brine shrimp were 27.4 nmol *p*-nitroaniline  $min^{-1}$  mg $^{-1}$  protein and 3.85 nM *p*-nitroaniline, respectively. Catalytic efficiency, defined as an enzyme's efficiency in transforming its substrate ( $V_{max}/K_m$ ), was 3.3 in the mud crab and 7.1; there was higher catalytic efficiency in *Artemia* than in the mud crab enzyme. Conclusion: There were similarities and differences between the mud crab LAP and that of the brine shrimp in terms of optimal pH and temperature, effects of pH and temperature on stability and also of LAP kinetic parameters; these could probably explain the mechanism of the LAP activity contribution of *Artemia* to the protein digestion in the mud crab.

**Key words:** crab, *Artemia*, leucine aminopeptidase, kinetic parameters, optimal pH, optimal reaction temperature.

**Rezumat.** Acest studiu urmărește să determine influența pH-ului și a temperaturii asupra activității precum și estimarea parametrilor cinetici ai leucin aminopeptidazei la două specii de crustacee, *Scylla serrata* și *Artemia salina*. Rezultatele studiului prezintă arată că pH-ul optim, atât pentru leucin aminopeptidaza de la *Scylla serrata* cât și de la *Artemia*, s-a încadrat în intervalul 7,0-7,5. pH-ul optim, determinat la stabilitatea maximă a LAP, s-a situat în intervalul de pH 7,0-8,5, de la neutru la alcalin, la *Scylla serrata* în timp ce la *Artemia* s-a situat între 6,0 și 7,0, interval acid. Temperatura de reacție optimă pentru LAP la *Scylla serrata* a fost 0°C, în timp ce la *Artemia* a fost de 25°C. LAP de la *Scylla serrata* a manifestat cea mai mare stabilitate la 0°C și a manifestat instabilitate odată cu creșterea temperaturii. La temperatura camerei (normală, la 25°C) și până la 35°C, activitatea LAP de la *Scylla serrata* a fost în jur de 70% din maximum, în timp ce la *Artemia* enzima a avut activitate maximă la 25°C și a scăzut brusc la 60% din activitatea maximă la 35°C. Viteza maximă ( $V_{max}$ ) și constanta Michaelis-Menten ( $K_m$ ) pentru *Scylla serrata* au fost estimate la 11.1 nmol produs  $min^{-1}$  mg proteine $^{-1}$  și respectiv 3.36 nM *p*-nitroanilină, în timp ce la *Artemia* au fost 27.4 nmol *p*-nitroanilină  $min^{-1}$  mg $^{-1}$  proteină și respectiv 3.85 nM *p*-nitroanilină. Eficiența catalitică, definită ca eficiența enzimei în a transforma substratul ( $V_{max}/K_m$ ), a fost de 3,3 la *Scylla serrata* și 7,1 la *Artemia*; a fost o eficiență catalitică mai mare la *Artemia* decât la *Scylla serrata*. Concluzie: există similități și diferențe între LAP de la *Scylla serrata* și de la *Artemia* în ceea ce privește pH-ul optim și temperatura, efectele pH-ului și temperaturii asupra stabilității și de asemenea ale parametrilor cinetici ai LAP; acestea pot probabil explica mecanismul prin care activitatea LAP de la *Artemia* contribuie la digestia proteinelor în *Scylla serrata*.  
**Cuvinte cheie:** crab, *Artemia*, leucină aminopeptidază, parametrii cinetici, pH optim, temperatura optimă de reacție.

**Introduction.** Leucine aminopeptidases (LAP) are brush border bound enzymes that preferentially catalyze the hydrolysis of leucine residues at the N-terminus of peptides and proteins; other N-terminal residues can also be cleaved (Buarque et al 2009). Dietary proteins are digested first by endoproteases, reducing peptides of various sizes which are further acted upon by exoproteases (aminopeptidases such as LAP) to complete digestion and produce tri-, dipeptides and free amino acids. The ultimate function of the extracellular and membrane-linked suite of proteases is to break down the ingested protein into amino acids and peptides so they can be absorbed across the brush border of the enterocytes (Rust 2002). Often, the membrane-linked enzymes function both to cleave the peptide chains and to transport the products across the brush border (Kuz'mina & Gelman 1997). Ontogenetic patterns of LAP in the mud crab *Scylla serrata* have been described (Serrano & Traifalgar 2012; Serrano 2013). An increase in aminopeptidase specific activity with a concomitant decrease in Leu-Ala peptidase specific activity has been related to intestinal maturation in marine fish larvae (Cahu & Zambonino Infante 1994); this could also be true in crustaceans.

Larval aquatic species rely more on the bioavailable nutrients contained in the live prey than its nutrient composition (Garcia-Ortega et al 1998). Zooplankton contains high percentage of free amino acids (Naess et al 1995). The range of contribution of live food to the total trypsin activity in mud crab ranged from 0.3 to 57% while to the LAP activity was 47 to 93% (Serrano 2013). These observations support the hypothesis that it is the released free nutrients such as free amino acids from live food and the enzymes that it releases that is more important than the nutrient composition of the whole prey. It could be that aminopeptidases in the live prey such as *Artemia* could be responsible for its high content of free amino acids and also contribute some active enzymes to the larva.

Kumlu & Jones (1995) have observed that the early larvae of the caridean shrimp, homarid species and the crab possess low digestive enzyme activities during early larval stages. It has been hypothesized that early developing larval fish (Kumlu 1999) and crustaceans such as the mud crab *Scylla serrata* larva (Serrano 2013) rely on the enzymes present in live food organisms to assist in the digestion of larvae. If the exogenous enzymes from live prey such as *Artemia* contribute considerably to the digestion in larval aquatic animals, an optimal gut condition should exist for the enzymes to stay optimally active. The activities of enzymes in the predator and prey are influenced by several factors including pH, temperature and substrate concentration. Cavital pH in most crustaceans is slightly acidic, particularly in starved animals, but rises slowly after feeding (Vonk & Western 1984). Temperature, in contrast, is largely affected by the environmental temperature in poikilotherms. The amount of substrate is largely affected by the diet of farmed aquatic animals, and there are surges in specific substrates after a meal. The present study aimed to characterize leucine aminopeptidases in a crustacean predator (*Scylla serrata*) and crustacean prey (*Artemia salina*) as they are affected by pH, temperature and substrate concentration.

## Material and Methods

**Crab and Artemia samples.** Live adult mud crab (*Scylla serrata*) were purchased from Roxas City, Capiz, Philippines and were acclimatized to concrete tanks until assay. Commercially available *Artemia* were purchased from Argent Co., and its cysts were hatched in the laboratory following the manufacturer's instruction.

**Preparation of the enzyme.** Live mud crabs were sacrificed, the guts excised, washed with cold extraction solution (50mM 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 minutes and the supernatant was used as enzyme preparation. *Artemia* nauplii of about 0.1 g wet weight was collected within 6-8 h after hatching and used for enzyme assays.

**Leucine aminopeptidase (LAP) assay.** All assays were carried out at 25°C and each assay was conducted along with appropriate controls including non-enzymatic hydrolysis

as described by Tuppy et al (1962). Before the routine assay, progress curves were obtained from 0 to 60 min for both the mud crab and the *Artemia* LAP; both enzymes were linear with time up to 60 min of reaction (data not shown). Activities of LAP against volume of enzyme preparation were also obtained for both animals (0.1 mL to 0.5 mL); results showed that the mud crab and *Artemia* LAP activities were linear only until 0.3 mL (data not shown). The assay system for LAP consisted of 1.0 mL of 60 mM Tris-HCl buffer pH 8.5, 1.4 mL of 1 mM of *L*-leucine-*p*-nitroanilide (final concentration of 520  $\mu$ M) and 0.3 mL of enzyme preparation in a final volume of 2.7 mL. The reaction was stopped by the addition of 30% acetic acid, centrifuged at 3,000 rpm for 10 min, the supernatant's optical density was read at 405 nm. Specific enzyme activity was expressed as  $\mu$ mole of *p*-nitroaniline formed  $\text{min}^{-1}$  per  $\text{mg}^{-1}$  protein.

**Optimum pH and pH stability.** The effect of pH on enzyme activity was determined in 50 mM citrate phosphate buffer with pH ranging from 5.9 to 8.0 at 25 °C. The effects of pH on LAP stability was determined by incubating the enzyme preparation in buffer at different pH (pH 5.9 to 8.5) for 1 h at 0-4 °C, after which enzyme assays were performed.

**LAP kinetic parameters.** Mud crab and *Artemia* LAP kinetics were determined over a range of carnitine concentrations from  $2.5 \times 10^{-4}$  to  $40 \times 10^{-3}$   $\mu$ mol, keeping the other components of the reaction mixture constant. Analysis of the kinetic data was performed as described by Hofstee (1952). The values of Michaelis–Menten constants ( $K_m$ ) and maximal reaction rates ( $V_{\max}$ ) were analyzed using a non-linear regression method described by the Michaelis–Menten equation. All measurements were performed in duplicate. Catalytic efficiency, defined as an enzyme's efficiency in transforming its substrate, was calculated by the ratio between maximum enzyme activity and  $K_m$  ( $V_{\max}/K_m$ ). Lineweaver–Burk graphs were drawn by using  $1/v$  versus  $1/[S]$  values (Lineweaver & Burk 1934) as in the following equation:

$$Y = a + bX$$

Where Y = 1/activity

X = 1/substrate concentration

a = y-intercept =  $1/V_{\max}$

b = slope of the double reciprocal curve =  $K_m/V_{\max}$

$-1/K_m$  = X intercept i.e. Y=0

**Results and Discussion.** Optimal pH for both leucine aminopeptidases of the mud crab and *Artemia* were in the range of 7.0 to 7.5 (Figure 1). This is in agreement with the optimal pH of the red drum *Sciaenopsis ocellatus* larvae Lazo et al (2007) which was pH 7.8; optimal pHs of the common carp *Cyprinus carpio*, cod *Gadus morhua* and Dover sole *Solea solea* were 7.4, 8.0 and 8.3, respectively. Given that the gut system of young mud crab was used in the present study, it was assumed that the optimal pH could also be similar to the first instar crab after the final metamorphosis. Since *Artemia* are used in the rearing of early stages of mud crab, specifically from Z3 to the first instar and later, it was assumed that this could be the pH condition in the gut of the mud crab.

Optimal pH that resulted in the greatest LAP stability was on the neutral to alkaline range of pH, 7.0 to 8.5, in the mud crab while it was on the acidic range of 6.0 to 7.0 in the brine shrimp. Due to the scarcity of studies on the properties of LAP in crustaceans, comparison could only be made with animals which belong to the same phylum, Phylum Arthropoda, the insects. The optimal pH value of LAP in the mud crab and *Artemia* agreed well with those reported for the pest insect *Telchin licus licus* (Valencia & Valencia 2014).

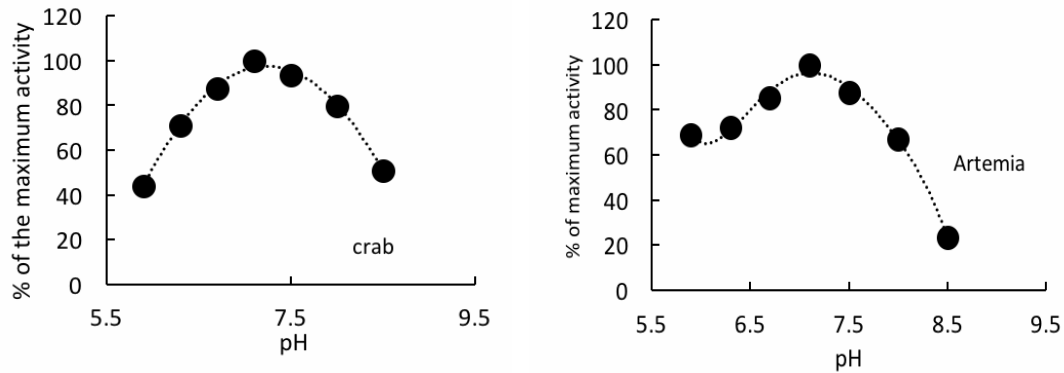


Figure 1. Effects of reaction pH on the activity of leucine aminopeptidases of the mud crab *Scylla serrata* and the brine shrimp *Artemia salina*.

Stability patterns as affected by pH differed in the LAP of the mud crab from that of the brine shrimp (Figure 2). The crab was more stable in alkaline conditions (7.5-8.5) while that of the brine shrimp in slightly acidic to neutral conditions (pH 6.0 to 7.0). These changes in stability coincided with the changes in the pH of the reaction mixture in Figure 1. There are no studies found by the author to compare the effects of pH on the stability of LAP in other aquatic animals.

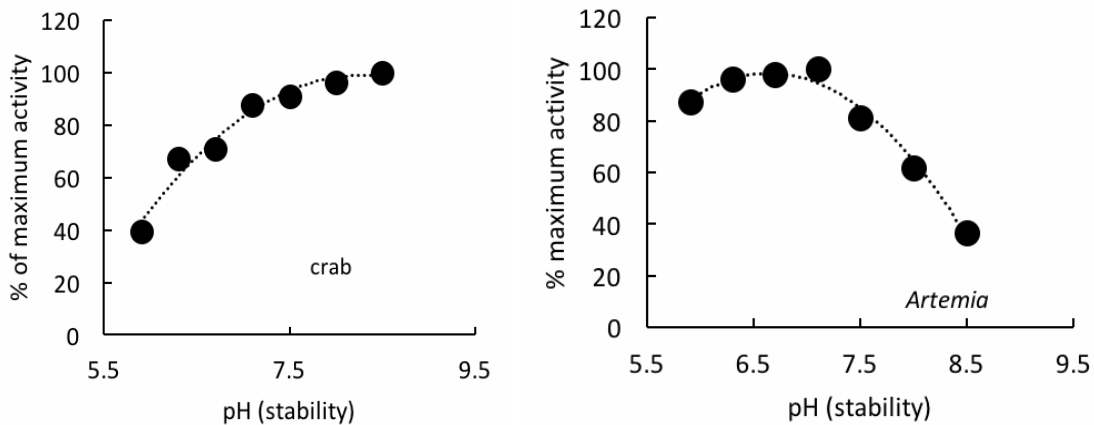


Figure 2. Effects of pH on the stability of leucine aminopeptidases of the mud crab *Scylla serrata* and the brine shrimp *Artemia salina*.

Optimal reaction temperature for both the mud crab and *Artemia* LAP was 40°C (Figure 3). In several species of fish, optimal temperature reported was markedly higher than those observed in the two crustacean species in the present study; the common dentex (*Dentex dentex*) and sea bream (Alarcon et al 1998) and the red drum *Sciaenopsis ocellatus* larvae (Lazo et al 2007) all exhibited optimal reaction temperature at 50°C. Lazo et al (2007) explain that the effect of temperature on LAP activity may differ in some species only in terms of comparative biochemistry of digestive enzymes among and between species and possible biotechnological applications. However, these values have limited relevance for practical aspects of the nutrition of aquatic animals, since the temperatures that are found optimal for digestive enzymes are not found under physiological conditions. In the present study, the optimal temperature for the *Artemia* LAP activity being at 25°C was the same as the routine reaction temperature and could be the normal water temperature in tropical waters. However, the mud crab larvae had 0°C as the temperature of highest activity and the values abruptly decreased as the

temperature was increased. This observation could be very difficult to explain in terms of practical application in aquatic animal nutrition or in aquaculture.

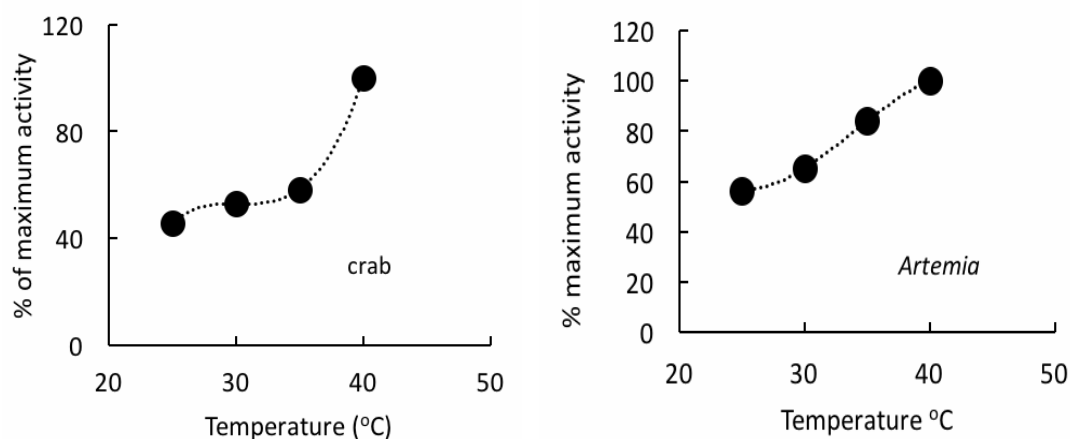


Figure 3. Effects of the reaction temperature on the leucine aminopeptidases of the mud crab *Scylla serrata* and the brine shrimp *Artemia salina*.

Thermal stability of the LAP enzymes in the mud crab and in the brine shrimp similarly followed a decreasing trend as the temperature increased (Figure 4). However, the pattern of decrease differed in the two crustaceans. The mud crab LAP exhibited the highest stability at 0°C and exhibited instability with increasing temperature. At room temperature (normally at 25°C) until 35°C, the remaining activity of the mud crab LAP was around 70% of the maximum, while at the same temperature the *Artemia* enzyme was maximal at 25°C and abruptly decreased to 60% of maximal activity at 35°C.

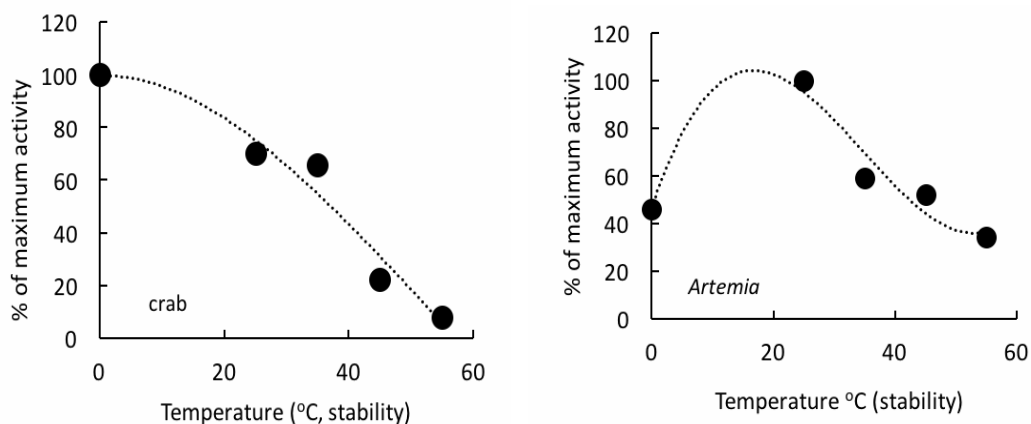


Figure 4. Effects of temperature on the stability of leucine aminopeptidases of the mud crab *Scylla serrata* and the brine shrimp *Artemia salina*.

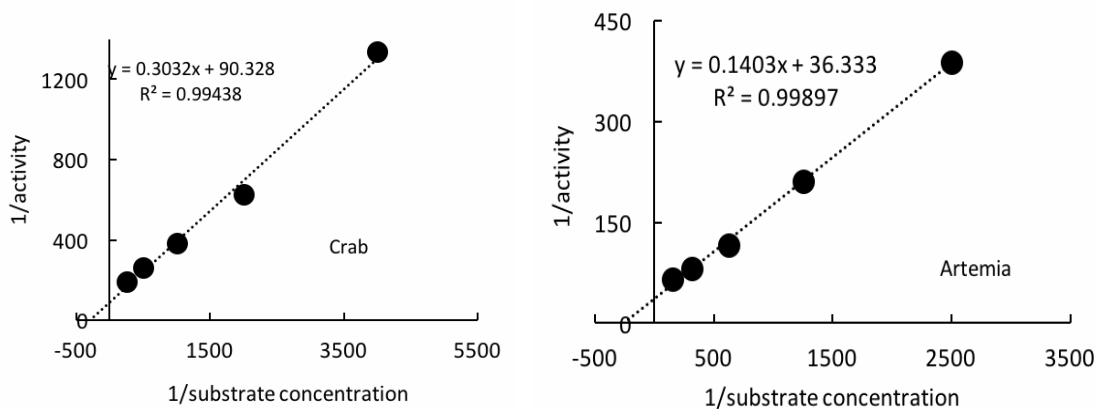


Figure 5. Double reciprocal plots of the effects of substrate concentration on the activity of leucine aminopeptidases in the mud crab *Scylla serrata* and the brine shrimp *Artemia salina*. The maximum velocities ( $V_{max}$ ) and the Michaelis-Menten constants ( $K_m$ ) of the mud crab were estimated to be 11.1 nmol product  $\text{min}^{-1} \text{mg protein}^{-1}$  and 3.36 nM *p*-nitroaniline, respectively, while those of the the brine shrimp were 27.4 nmol *p*-nitroaniline  $\text{min}^{-1} \text{mg}^{-1} \text{protein}$  and 3.85 nM *p*-nitroaniline, respectively.

Both enzymes followed a Michaelis-Menten hyperbolic curve as the concentration of substrate is increased. The maximum velocities ( $V_{max}$ ) and the Michaelis-Menten constants ( $K_m$ ) of the mud crab in the present study were estimated to be 11.1 nmol product  $\text{min}^{-1} \text{mg protein}^{-1}$  and 3.36 nM *p*-nitroaniline, respectively, while those of the the brine shrimp were 27.4 nmol *p*-nitroaniline  $\text{min}^{-1} \text{mg}^{-1} \text{protein}$  and 3.85 nM *p*-nitroaniline, respectively (Figure 5). The routine assay in the present study used 519  $\mu\text{M}$  ( $= 5.2 \times 10^5 \text{ nM}$ ) of *p*-nitroaniline which was  $10^5$  folds higher than the  $K_m$ ; this was more than enough assurance that the substrate in the reaction was in excess and did not affect the enzyme velocity. The more than double  $K_m$  for the brine shrimp could be the result of a less enzyme to substrate affinity, which meant a higher substrate concentration threshold than that of the mud crab enzyme for the same substrate. Although the  $K_m$  differed, the  $V_{max}$  values were close to each other suggesting that LAP activities were similar under saturating substrate concentration. The observed  $K_m$  values in the present study were much lower by a few order of magnitude (more than  $10^4$  folds) than the estimated  $K_m$  value of Valencia & Valencia (2014) of  $8.4 \times 10^4 \text{ nM}$  in the pest insect of sugarcane *Telchin licus licus*, and much lower than those reported by Beattie et al (1997) which ranged from 0.08 to 5.0 mM. The difference could be due to the difference in the substrate they used, namely *L*-leucyl-2-naphthylamide while the present study used *L*-leucine-*p*-nitroanilide. Based on the detection of activity in such a minimal substrate concentration, it appears that the assay used in the present study was more sensitive than the ones used by other authors such as that used by Valencia and Valencia (2014). Catalytic efficiency, defined as an enzyme's efficiency in transforming its substrate ( $V_{max}/K_m$ ), was 3.3 in the mud crab and 7.1 in the *Artemia* pointing to the higher catalytic efficiency in *Artemia* than in the mud crab.

Differences in the behavior of LAP in the two crustaceans in the present study based on pH, temperature and substrate concentration could also stem from the presence of isoforms of the enzymes as was reported by Valencia & Valencia (2014). Using the isoforms together with their inhibitions, these investigators were able to observe differences in Coleopteran and Lepidopteran insects. This could be a good approach in further studies in crustaceans important to aquaculture. These isoforms increase the insect capability to adapt to different food sources and also overcome the inhibitory activities from plant proteinase inhibitors (Wagner et al 2002).

**Conclusions.** Three properties of LAP from enzyme preparations of two crustaceans important in aquaculture were studied. Optimal pH for both LAPs of the mud crab and *Artemia* were in the range of 7.0 to 7.5. When exposed to various pH conditions, the crab was more stable in alkaline conditions (7.5-8.5) while that of the brine shrimp in slightly neutral to slightly acidic conditions (pH 6.0 to 7.0). Stability of the enzymes in the crab and in the brine shrimp decreased as the temperature was increased. However, the pattern of decrease differed in the two crustaceans. Optimal reaction temperatures of LAP were 0°C and 25°C for the crab and *Artemia*, respectively. The maximum velocities ( $V_{max}$ ) and the Michaelis-Menten constants ( $K_m$ ) of the mud crab LAP were estimated to be 11.1 nmol product  $\text{min}^{-1} \text{mg protein}^{-1}$  and 3.36 nM *p*-nitroaniline, respectively, while those of the the brine shrimp were 27.4 nmol *p*-nitroaniline  $\text{min}^{-1} \text{mg}^{-1} \text{protein}$  and 3.85 nM *p*-nitroaniline, respectively. These are much lower than those reported in other species, such as in insects. Catalytic efficiency ( $V_{max}/K_m$ ) of LAP was higher in the *Artemia* than in the mud crab.

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