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# Trypsin and leucine aminopeptidase activity contribution of live food to the developing mucrab (*Scylla serrata*) larvae

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**ABSTRACT**

*The study aimed to measure the exogenous enzyme contribution to the endogenous enzyme activities of trypsin and leucine aminopeptidase in mud crab during early development. Crab larvae at various stages were fed live food (A) or inactivated live food (B): Enzyme activity of A group was the endogenous activity while [(A-B)/A] x 100 was the percent contribution of exogenous enzyme derived from live food. Percent contribution to trypsin activity by live food was significantly higher (about 50%) at Z2 to C1 stages which were not significantly different from each other. The range of contribution to trypsin activity was 0.3 – 57% with the peak observed at the megalopa stage. The range of contribution to LAP activity was 47-93% with peaks at Z4 and megalopa stages. Thus, the importance of live food as source of trypsin and leucine aminopeptidase in the early developing crab larvae was demonstrated in the present study.*

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## INTRODUCTION

Dietary proteins are digested in a number of steps, beginning in the stomach by pepsin, one of three endopeptidases. Once the partially digested proteins pass through the stomach to the small intestine, the other two endopeptidases, trypsin and chymotrypsin, continue the protein digestion. To complete the breakdown, exopeptidases such as leucine aminopeptidase (LAP) help in the production of single amino acids as the final products of protein digestion.

In decapod crustacean larvae like the freshwater prawn *Macrobrachium rosenbergii*, trypsin activity is detected as early as the first larval stage but decrease upon the first feeding and tends to decline in subsequent stages but increase dramatically upon reaching the sixth stage. Similar patterns have been reported decapod species such as *H. americanus, P. elegans,* and *Scylla serrata* [2, 19, 20*]*. Previously, we showed that LAP enzyme activity exhibited a prominent peak at stage Z5, gradually declined upon metamorphosis to megalopa until reaching the C1 stage [19].

Larvae fed live diets exhibited significantly higher digestive protease activities (trypsin and LAP enzyme activities) at stages Z3 to Z5 than did larvae fed artificial diet or combination of these diets. The explanation for this was put forth by Kumlu [12] who hypothesizes that early developing larvae rely on the enzymes present in live food organisms to assist digestion. 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 [10] and our previous findings on *S. serrata* [19] are in agreement with these earlier works on other species. Serrano [17] 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 present study aimed to measure the exogenous enzyme contribution to the endogenous enzyme activities of trypsin and leucine aminopeptidase in mud crab during early development.

## MATERIALS AND METHODS

*Broodstock*

The broodstock females, whose progeny were used in these experiments, were identified as *S. serrata* according to the description of Keenan *et al*. [9]. Mature females were purchased from Roxas City, Capiz which were collected from the wild. Broodstocks were disinfected by brushing and bathing in 100 mg l-1 formalin for 30 min. The crabs were reared individually in tanks with running water, aeration and sandy substrate. Daily, the crabs were fed *ad libitum* with various trash fish, squid (*Loligo* spp.) and fresh mussel meat (*Perna viridis* Linnaeus, 1758). When the females were observed to be carrying eggs, they were not fed. During egg incubation, the berried crabs were placed individually in concrete tanks (250-L capacity) with aeration. After several days of incubation, a small sample of eggs was examined for development and health assessment. The following morning, the strongly phototactic and schooling first stage zoea (Z1) larvae were captured and concentrated using plankton net and were transferred to incubation and hatching tanks.

Two dietary treatments and a control diet in triplicate were formulated. Dietary treatment 1 was live food and Dietary treatment 2 was live feeds with their enzymes inactivated by heating to $100^0$C for 5 min. In zoeal and megalopal stages, diet 1 was composed of freeze dried *Artemia* nauplii and rotifers in 1:1 ratio (w: w) microencapsulated with chitosan; diet 2 was enzyme-inactivated microencapsulated live feed. For juveniles, heat inactivated and fresh clam meat was used as feed. Prior to feeding, enzyme levels of test diets were measured.

Prior to the feeding experiment, all larvae used in this experiment were collected from their rearing tanks and transferred to experimental tanks without feeds and the larvae was starved for 4 h prior to the initiation of the feeding trial to ensure they were ready to ingest feed. Experimental setup consisted of three 20-L tanks per dietary treatment. Experimental duration was 48 h for all zoea stages and 72 h for megalopa and juvenile crab. Prior to feeding, enzyme levels of the test diets were also measured. Digestive enzyme activities were quantified every 24 h. Exogenous digestive enzyme contributed by the live feeds was quantified following the formula:

$$\% \text{ Enzyme Contribution} = [(A-B)/A] \times 100$$

where A is the enzyme activity of larvae fed diet 1
and B is the enzyme activity of those fed diet 2.

*Enzyme Assays*

Pooled whole larvae or juvenile homogenates were used in all assays in triplicate. Samples were homogenized in 20 volumes of cold extraction solution (50mM citrate phosphate buffer pH 7.0) at 1:20 ratio (wet tissue weight to volume) in an Ultra Turrax homogenizer. The homogenates were centrifuged at 4000 rpm for 15 min at $4^o$C and the supernatant was used as enzyme preparation. Total soluble protein was measured following the procedure of Lowry *et al*. [14] with bovine serum albumin as a standard. All enzyme assays (Table 1) were conducted within 4 h of homogenization and all samples for a single enzymatic assay were run in the same day. Blank controls, in which the reaction did not take place, were introduced when needed

*Trypsin*

Trypsin activity was determined according to Geiger and Fritz [4] using the specific substrate BAPNA (Benzoyl-arginine-*p*-nitoanilide). The assay mixture consisted of 1.25 ml 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 and absorbance of the supernatant read at 405 nm and the unit enzyme activity was expressed as micromoles of product formed per min per mg protein. The activity of purified enzyme preparation was subtracted from the total trypsin activity.

*Leucine Aminopeptidase (LAP)*

The assay system for LAP consisted of 1.0 ml of 60 mM Tris-HCl buffer pH 8.5, 1.4 ml of 0.001 M of L-leucine-*p*-nitroanilide and 0.3 ml of enzyme preparation. The reaction was stopped by the addition of 30% acetic acid. Unit of enzyme activity was expressed as micromole of p-nitroaniline formed and detected at 405 nm per min per mg protein.

*Statistical Analysis*

Statistical analysis of the data was performed using a graph- statistical software package (Statistica, Stat Soft., Inc., USA and Sigma plot 11, Systat, USA)). Homogeneity of variances and normality were tested (using Levene's test and Shapiro–Wilk's test, respectively) before analyzing the data with an ANOVA. Differences between enzyme activities and developmental stages and their interaction; between arcsine-transformed percent contributions to enzyme activities by live food, developmental stage and their interaction were tested using two-way (factorial) ANOVA. If the interaction of two factors was not significant, the analysis was re-ran without an interaction term, and interpreted each of the *p*-values as in one-way ANOVA. Post hoc analysis among groups after finding significant differences were performed using Tukey test, with the level of significance preset at $P<0.05$. Data were reported as mean ± standard error.

## RESULTS AND DISCUSSION

The interaction between the early developmental stage and percent contribution of enzyme activities were not significant; thus, interpretation of the one-way ANOVA was done (Table 1). Percent contribution to trypsin activity by live food at Z1 was the lowest while those for the other stages exhibited high values with a range of 37 – 58% (Table 2). The live food's contribution to LAP activities were remarkably high (range of 47 – 93%) and as a consequent, endogenous enzymed produced by the mud crab larvae contributed the lowest.

**Table 1. One-way analysis of variance (ANOVA) of exogenous enzyme contribution from live food from Z1 to C1 stages. Percent of contribution of exogenous enzymes were arcsine transformed before analysis.**

| Enzyme contribution | SV | *df* | SS | MS | *F* computed | Sig. |
|---|---|---|---|---|---|---|
| Trypsin | Larval stage | 6 | 0.698 | 0.116 | 12.153 | 0.000* |
| | Error | 14 | 0.134 | 0.010 | | |
| LAP | Larval stage | 6 | 1.33726 | 0.223 | 26.741 | 0.000* |
| | Error | 14 | 0.117 | 0.008 | | |

*SV=source of variation; df=degrees of freedom; SS=sum of squares; MS=mean squares; F=Fisher F statistic; *= significant differences exist (P<0.05)*

**Table 2. Percent contribution of exogenous and endogenous enzymes (mean ± standard error) at various development stages in mud crab larvae. Values were first arcsined transformed before analysis. Means on each column with different letters are significantly different (*P*<0.05).**

| | Trypsin (%) | | LAP (%) | |
|---|---|---|---|---|
| | Exo | Endo | Exo | Endo |
| Z1 | $0.30 \pm 0.30^{b}$ | $99.70 \pm 0.30^{b}$ | $63.97 \pm 7.55^{c}$ | $36.03 \pm 7.55^{b}$ |
| Z2 | $36.67 \pm 1.93^{a}$ | $63.33 \pm 1.93^{a}$ | $59.38 \pm 5.41^{d}$ | $40.62 \pm 5.41^{b}$ |
| Z3 | $41.67 \pm 8.33^{a}$ | $58.33 \pm 8.33^{a}$ | $78.39 \pm 2.91^{c}$ | $21.61 \pm 2.91^{c}$ |
| Z4 | $50.00 \pm 9.62^{a}$ | $50.00 \pm 9.62^{a}$ | $92.66 \pm 0.20^{a}$ | $7.34 \pm 0.20^{e}$ |
| Z5 | $47.65 \pm 2.35^{a}$ | $52.35 \pm 2.35^{a}$ | $46.59 \pm 3.21^{d}$ | $53.41 \pm 3.21^{a}$ |
| M | $57.76 \pm 1.29^{a}$ | $42.24 \pm 1.29^{a}$ | $90.64 \pm 0.15^{a}$ | $9.36 \pm 0.15^{f}$ |
| CI | $42.59 \pm 0.97^{a}$ | $57.41 \pm 0.97^{a}$ | $88.38 \pm 1.18^{b}$ | $11.62 \pm 1.18^{d}$ |

The percent share of the prey to LAPs total activity was highest at stage Z4, declined at stage Z5 before it again rose to its high levels at stages M and C1. The percent prey's share to trypsin activity was negligible at stage Z1 but increased at subsequent stages and remained almost unchanged until the crablet stage.

*S. serrata* larvae, a brachyuran crab, join the ranks of the caridean shrimps (*Palaemon* spp. and *Macrobarachium* spp) and homarid larvae (e.g. lobsters) in their inability to be reared with complete replacement of live diets with artificial diets. Live *Artemia* has been replaced completely with microparticulated diets in *Palaemon elegans* and *Macrobrachium rosenbergii* culture, but only from stage Z5/6 to PL stage (Abubakr [1], Deru [3], Kumlu and Jones

[10]); this is also the case with *Scylla serrata* Holme *et al*. [6]. In fact, Genodepa *et al*. [5] have demonstrated 100% replacement of live food with microbound diet is possible when rearing *S. serrata* from the megalopa stage. It is clear from the present study that at early zoeal stages (Z1 to Z3), amylase and LAP activities were at the lowest levels and the lowest trypsin levels at Z1-Z2 and M stages. This finding supports the hypothesis of Kumlu and Jones [11] that the inability of the early larvae of the caridean shrimp, homarid species and the crab is due to their low digestive enzyme activities during early larval stages.

The confirmation of low activities of digestive enzymes at early larval stages directs one to further hypothesize that it could be that live prey fills up this deficiency Kumlu [13]. Rotifers in the present study contributed considerable amount of LAP activities to the Z1 larvae (64%) but not enough trypsin activities (0.3%). Except for the valley of LAP activity at Z5, contributions to trypsin (37%-43%) remained steady with development. LAP activity contribution of *Artemia* at late larval stages was considerable (average of about 80%). Findings of the present study support the hypothesis of the importance of live food as sources of exogenous enzymes in all larval stages. This is in contrast with the findings of Jones *et al.* [7] and Kamarudin *et al.* [8] that contribution of exogenous enzymes from live *Artemia* nauplii to the digestion process of *M. rosenbergii* larvae and penaeid larvae has been measured and found to be insignificant.

Among the enzymes studied, trypsin was the most well assayed enzyme in terms of ontogenetic changes in decapod crustaceans. The rapid increase in trypsin at stage Z3 differed from the carnivorous decapods crustaceans like *M. rosenbergii* and *Palaemon elegans* in which it sharply increases in protease activity are observed at Z5-Z6 and is related to the development of the gut and rapid expansion of the hepatopancreas Kumlu and Jones [10]. In the present study, high trypsin at stage Z3 coincided with the prominent emergence of the gastric mill and its lateral and median teeth at this stage as documented by Lumasag *et al*. [15]. Furthermore, it is at this stage that a cardiopyloric valve with ossification at the dorsal and lateral sides of the cardiac stomach is observed. These physical developments of the Z3 larvae, together with the peak trypsin activity in the present study, could suggest physiological readiness of the larvae to take on richer live prey to support its further development and growth. To compare absolute protease activities between laboratories is quite difficult since units of expression differs, thus whether or not trypsin and LAP activities in the present study were comparable in previous reports in other crustaceans would have very little basis, if at all. Jones *et al.* [7] have hypothesized that in penaeid larvae, the high digestive activity in terms of protease activity, and short gastric evacuation time may be an adaptation to the lower-energy natural food source of microalgae and to poorly digestible artificial diets. The longer gut retention time in larvae fed artificial feed compared to those fed live food [18] and the assumed low endopeptidase (i.e. trypsin) and exopeptidase (i.e. LAP) in the present study was an adaptation to the poorly digested artificial feed. This was supported by the observation of shorter gut retention time at the megalopa and first crab stages when live food was fed which also showed higher endo- and exopeptidase activities than did those fed artificial diet. Thus, it was apparent that the feeding strategy of the *S. serrata* larvae and juvenile that they modulate gut retention time and digestive activity in response to the apparent digestibility of food presented. Earlier, we hypothesized that gut retention time was under stage-specific genetic control at stages Z1-Z5 [18] since they were uniformly short regardless of diet in these stages, and that digestive enzyme activities were also under genetic control at stages Z1-Z3 (except trypsin) since the specific activities were uniformly low in these stages. Another factor which could be subject to modulation is feeding frequency but it has not been studied yet in mud crab. It could be the case that increased feeding frequency could be a compensatory strategy to meet the energy requirement of the larvae especially so at stages when increases in gut retention time and digestive ability are under genetic programme.

Although it was observed that artificial diet was acceptable to *S. serrata* larvae in the early zoeal stages, the low digestive enzyme activities limited the utilization of this diet as have been observed by other workers (Holme *et al.* [6], Lumasag *et al*. [15]). The observation that *Artemia*-fed *S. serrata* larvae managed to survive without high mortality or depressed growth (Holme *et al.* [6], Quinitio *et al.* [16]) suggested that this live diet fulfills all necessary physical and nutritional requirements and could be easily digestible. The decrease in the specific activity of endogenous trypsin in the present study, from Z4, Z5 stages and to its lowest level at the megalopa stage suggested that this may be due to replacement by exogenous enzymes supplied from *Artemia* nauplii.

## CONCLUSION

Percent contribution to trypsin activity by live food was significantly higher (about 50%) at Z2 to C1 stages which were not significantly different from each other. The range of contribution to trypsin activity was 0.3 – 57% with the peak observed at the megalopa stage. The range of contribution to LAP activity was 47-93% with peaks at Z4 and megalopa stages. Thus, the importance of live food as source of digestive enzymes in the early developing crab larvae was demonstrated in the present study.

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