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# Twig extract of the apple mangrove affects the activities of trypsin, chymotrypsin and lipase in postlarval black tiger shrimp *Penaeus monodon* at varying feeding frequencies

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**Abstract**. This study aimed to determine the effect of the twig extract of the apple mangrove *Sonneratia caseolaris* on the activities of trypsin, chymotrypsin and lipase in the postlarval black tiger shrimp *Penaeus monodon*. Incorporating twig extracts of the apple mangrove *S. caseolaris* increased the activity of trypsin in juvenile *P. monodon* when fed at three to four times daily. When fed twice daily, trypsin activity was at the same level as that in shrimp fed the control diet (without medication). The extract resulted in the enhanced chymotrypsin activity starting when shrimps were fed medicated diet twice daily until four times daily. Lipase activity was not at all affected by the apple mangrove extract. These findings demonstrated that the twig apple mangrove extract could be used as a prophylactic/therapeutant and was not deleterious to the nutrition of the black tiger shrimp; in fact, it stimulated protein digestion.
**Key Words**: apple mangrove, *Penaeus monodon*, feeding frequency, trypsin, chymotrypsin, lipase.

**Introduction**. The digestive enzymes of penaeid shrimps have been studied over the last decades for various applications in nutritional physiology and biochemistry (Farnes et al 2007). Because protein is the major component of the shrimp diet (Smith et al 1992), proteolytic enzymes may play a key role in the assimilation processes (Lemos et al 1999). Among the proteolytic enzymes in shrimps, trypsin may be responsible for 40-60% of the total protein digestion (Galgani & Benyamin 1985; Tsai et al 1986) and has been characterized in *Penaeus monodon* (Jiang et al 1991)and *Litopenaeus vannamei* (Klein et al 1996). The occurrence of chymotrypsin activity in the digestive tract of shrimp has been studied in relation to its nutrition (Le Moullac et al 1994; Tsai et al 1986).

Lipase activities have been determined in shrimp (Trellu & Ceccaide 1977) in adult *Palaemon serratus*, *Litopenaeus setiferus*, *L. stylirostris*, *L. occidentalis* and *L. vannamei*. However, there is very limited information on the effect of incorporating growth-promoting agent in the diet on the activity of lipase in shrimp.

Our previous work showed that the twig extract of the apple mangrove acted as a prophylactic and immunostimulating agent(Avenido & Serrano 2012a) as well as a growth-promoting agent in *P. monodon* when incorporated in the diet (Avenido & Serrano 2012b). The efficacy of the incorporation is better when fed more than twice a day. Amylase activities were higher when fed four times a day while total protease when fed three times a day than fed at lower frequencies. Although it appeared that medicated diets were less palatable, increasing feeding frequency may increase feed acceptability and thus intake as was shown in common carp (Yamada et al 1981). This study aims to determine the effects of adding twig extract of *Sonneratia caseolaris* on the activities of trypsin, chymotrypsin and lipase in *P. monodon*; whether increasing feeding frequency of the medicated diet could also affect the enzyme activities.

## Material and Method

***Preparation of mangrove extract***. Twigs of *S. caseolaris* were shade dried for two weeks, cut into small pieces and pulverized using hammer mill grinder. Pulverized samples were stored in small packs placed in a tightly-covered glass container placed in a cool dark place at ambient temperature.

The method of extraction used was as described in the previous papers (Avenido & Serrano 2012a, 2012b). Briefly, pulverized twigs (~ 200 g) were soaked in equal parts of methanol (1:1) for 48 h, filtered, washed to remove non-soluble fractions and filtrate centrifuged (20,000 x g for 30 min) for clarification and stored in the refrigerator. This procedure was done repeatedly until the filtrate became colorless or clear. Pooled filtrates were concentrated in a rotary evaporator under reduced pressure at 40–50$^{o}$C. Methanolic twig mangrove extracts were sprayed on feed pellets at 20 ml kg$^{-1}$ dry diet (containing 1000 µg ml$^{-1}$ twig condensate) and dried for 24 h prior to the feeding experiments. The control diet was sprayed with 20 ml of distilled water kg$^{-1}$ diet.

***Experimental animal***. Post larvae of *P. monodon* were purchased from a commercial prawn hatchery in Oton, Iloilo, Philippines. The postlarvae were transported in a styroform boxes and were immediately stocked in a fiberglass tank upon arrival at the University of the Philippines-Institute of Aquaculture Hatchery. The shrimps were acclimatized to the laboratory conditions and to the basal diet (San Miguel Corp.) for 15 days. After acclimatization, 3 experimental groups and a control group in triplicate were stocked at 100 shrimp postlarvae cubic meter$^{-1}$ at the initial ABW of 0.008 g individual$^{-1}$ in 12 individual 1 m$^{3}$ - capacity rectangular tanks.

***Feeds and feeding***. Shrimps were fed their respective diet at 8% of their body weight day$^{-1}$ for the duration of the experiment. The commercial basal diet (San Miguel Corp. starter shrimp feed) was composed of 45.9% crude protein, 3.6% crude fat, 35.8% nitrogen-free extract, 1.43 % crude fiber, 13.3 % ash, and 4.24% moisture (analyzed by SEAFDEC cited by Pascual (1993)). Feed was soaked in 1000 µg ml$^{-1}$ methanolic twig mangrove extracts; this was after establishing that it exhibited the highest antibacterial activity than did those extracted with the other solvents. Soaking was done for 48 h and dried for 24 prior to the feeding experiments. Feeding was done in feeding trays using three feeding frequencies, namely, two times daily (8:00 and 17:00); three times daily (at 8:00, 12:00 and 17:00); and four times daily (8”00, 11:00, 14:00 and 17:00). Feed ration was adjusted after every sampling period based on the total body weight per tank for the whole duration of the feeding study (75 days). The experimental tanks sufficiently aerated 24 h daily at 80% saturation or higher and about 50% of the total water volume was replaced every 15 days.

***Enzyme assay***. Shrimps were sacrificed, hepatopancreas 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 to volume) in an Ultraturrax homogenizer. The homogenates were centrifuged at 4000 rpm for 15 min and the supernatant used for enzyme assay. All assays procedures were carried out at 0-4$^{o}$C unless otherwise stated. Assays were conducted at 25$^{o}$C (unless otherwise stated) using stopped-flow type of method; zero-time reactions were also carried out.

Trypsin-like activity was measured according to Geiger & Fritz (1988) and Rick (1984) 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 (to activate the zymogen) and buffer in final volume of 2.25 ml. The reaction started by adding BAPNA solution for 5 min, the reaction was allowed to proceed, and was stopped by the addition of 0.25 ml of 30% acetic acid. Optical density was read at 405 nm and the enzyme activity was expressed as micromoles of product formed min$^{-1}$ ml$^{-1}$ of the enzyme preparation.

Chymotrypsin-like activity was determined using the method of Hummel (1959). The assay mixture consists of 1.4 ml of 1.07mM N-benzoyl-L-tyrosine ethyl ester (BTEE)

dissolved in 50% (w/w) methanol, 1.0 ml of 80mM Tris-HCl buffer, pH7.8 containing 0.1 M $CaCl_2$ and 0.3 ml extract in a final volume of 2.7 ml. The reaction was stopped by adding 0.3 ml of 30% acetic acid. The hydrolysis of N-benzoyl-L-tyrosine ethyl ester into N-benzoyl-L-tyrosine + ethanol causes an increase in absorbance at 256 nm. Enzyme activity was expressed as micromole BTEE produced $min^{-1}$ at 25$^{o}$C and pH 7.8.

Total lipase activity was assayed based on the measurement of free fatty acid released be enzymatic hydrolysis of triglycerides present in the stabilized emulsion of olive oil. Total lipase activity was evaluated following the method of Tietz & Friereck (1969). The assay mixture consisted of 1.5 ml stabilized lipase substrate and 1.5 ml of 0.1M Tris-HCl buffer at 8.0 to which 1.0 ml of crude enzyme was added. The mixture was allowed to react for 4 h at 37$^{o}$C, stopped by the addition of 3 ml of 95% ethyl alcohol and the mixture was titrated with 0.01 N NaOH using 0.9% (w/v) thymol-pthalein in ethanol as indicator. Blank determination was done in similar manner except that crude enzyme extract was introduced into assay system after 4 h incubation and immediately before titration with the standard NaOH solution. A unit of lipase activity was defined as the volume of 0.05 N NaOH required to neutralize fatty acid released during the 4 h incubation with the substrate and after correction by appropriate blanks.

**Results and Discussion**. Feeding shrimps the medicated diets significantly increased trypsin activities and a further increase in trypsin-like activity was observed when the feeding frequency of the medicated diets was increased to four times daily (Figure 1). Similar to the changes in total protease activities of shrimps fed the medicated and control diets, trypsin-like activity was not affected in shrimps fed at a lower feeding frequency of twice daily compared to those fed the control diet three times daily.

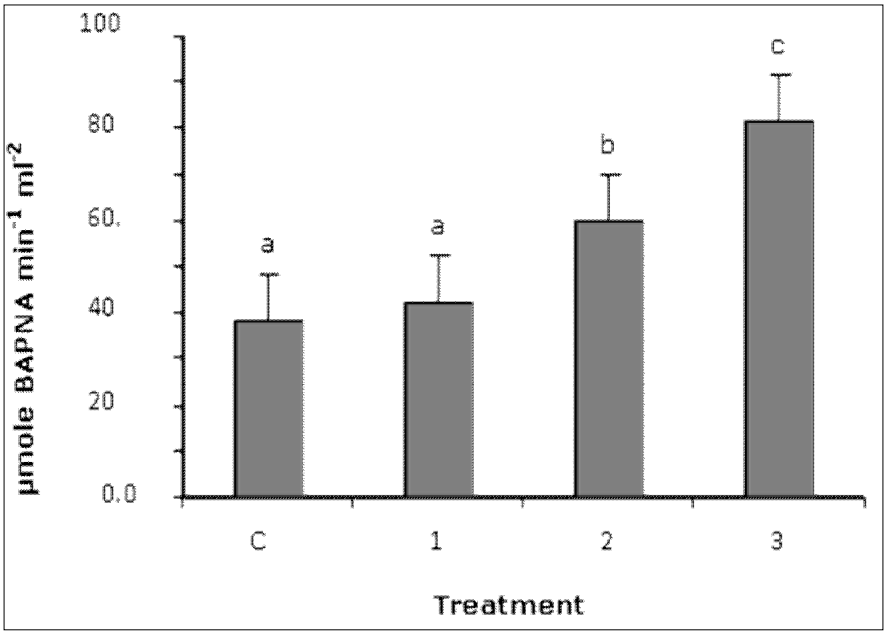


Figure 1. Trypsin-like activities of *P. monodon* juveniles fed the control diet (C) fed three times daily) and diets with methanolic twig extracts of *S. caseolaris* fed twice daily (1), three times daily (2), and fed four times daily (3) for 60 days. Error bars indicate +1 standard deviation. Values not sharing the same superscript are significantly different ($p<0.05$) according to Tukey's Honestly Significant Difference Test.

The apple mangrove extract improved chymotrypsin activity at all feeding frequencies. This means that even when fed the medicated diets twice daily, shrimp chymotrypsin-like activity was higher than that of shrimps fed the control diet three times daily (Figure 2).

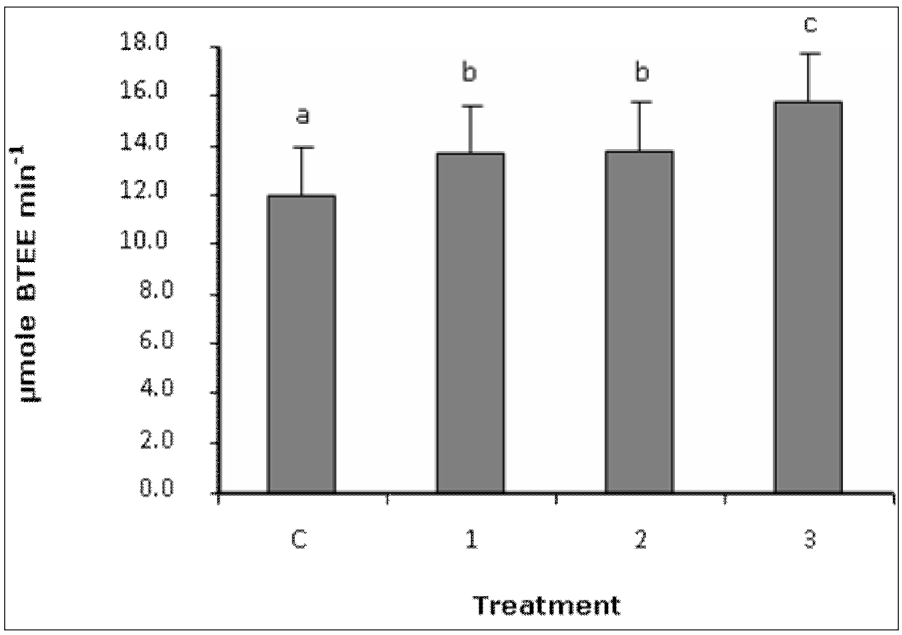


Figure 2. Chymotrypsin-like activities of *P. monodon* juveniles fed the control diet (C) fed three times daily) and diets with methanolic twig extracts of *S. caseolaris* fed twice daily (1), three times daily (2), and fed four times daily (3) for 60 days. Error bars indicate +1 standard deviation. Values not sharing the same superscript are significantly different ($p<0.05$) according to Tukey's Honestly Significant Difference Test.

No significant differences were observed between the lipase activity of shrimps fed the control diet and those fed the medicated diets at all feeding frequencies (Figure 3).

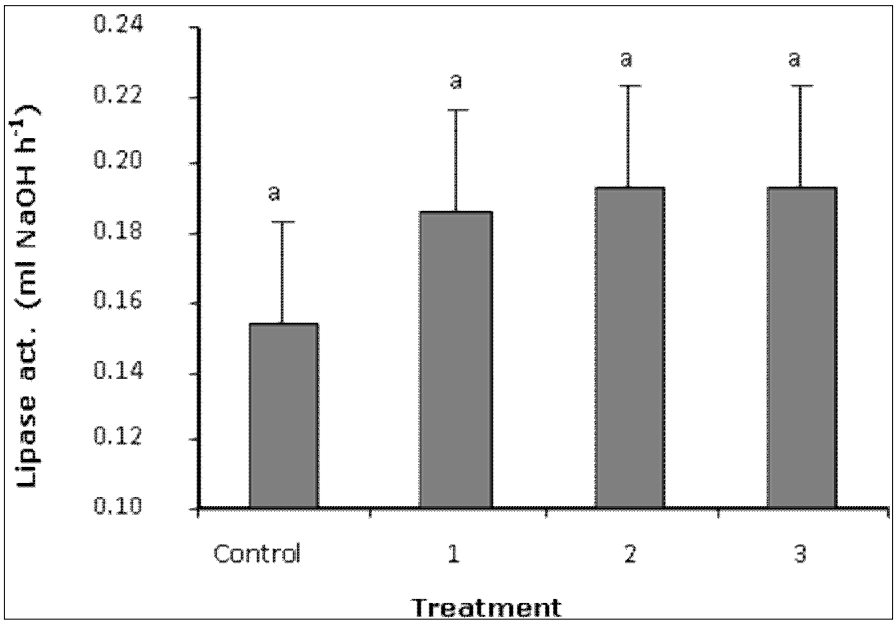


Figure 3. Lipase activities of *P. monodon* juveniles fed the control diet (C) fed three times daily and diets with methanolic twig extracts of *S. caseolaris* fed twice daily (1), three times daily (2), and fed four times daily (3) for 60 days. Error bars indicate +1 standard deviation. Values not sharing the same superscript are significantly different ($p<0.05$) according to Tukey's Honestly Significant Difference Test.

To our knowledge, this is the first time that effects of a natural prophylactic/therapeutant on digestive enzyme activities were measured in shrimp. Trypsin and chymotrypsin activities were enhanced by incorporating the mangrove extract to the feed but lipase activity was not affected. Consistent with the findings in our previous work, the overall significant finding with regard to the digestive enzyme activities was that there was no

adverse effect on the chemical digestive capability of shrimps fed the mangrove methanolic extract.

Higher digestive breakdown of dietary protein by two endopeptidases resulted from the incorporation of twig extracts of the apple mangrove which occurred at least when shrimps are fed three times a day. Despite the observation of lower palatability of the medicated diets, juvenile black tiger shrimp improved the molecular cutting of peptides by increasing feeding frequency. The increased endopeptidase activities would provide more substrates for some existing exopeptidase (e.g. aminopeptidases) such as leucine aminopeptidase similar to that in *Scylla serrata* (Serrano & Traifalgar 2012). Theoretically, this would result in an increased growth rate which our previous study demonstrated (Avenido & Serrano 2012b).

In contrast to the endopeptidase, lipase activity was not affected by the incorporation of the apple mangrove extract. This could mean that the breakdown of dietary lipid in the gut of shrimps was not at all affected and this could be shown if carcass composition was analyzed and body crude fat did not increase; unfortunately, this was not conducted either in the previous nor in the present study. Alternatively, the assay for lipase might not be that sensitive.

**Conclusions**. Incorporating twig extracts of the apple mangrove *S. caseolaris* increased the activity of trypsin in juvenile *P. monodon* when fed at three to four times daily. When fed twice daily, trypsin activity was at the same level as that in shrimp fed the control diet (without medication). Likewise, the extract enhanced the activity of chymotrypsin activity over that of shrimp fed the control diet. The enhancement started from shrimps fed the medicated diet twice daily until four times daily. Lipase activity was not at all affected by the apple mangrove extract. These findings demonstrated that the twig apple mangrove extract could be used as a prophylactic/therapeutant and was not deleterious to the black tiger shrimp. In fact, it showed that it stimulated protein digestion by increasing the activities of trypsin and chymotrypsin.

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