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Influence of size on digestive enzyme activities in the Angelwing clam Pholas orientalis

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ABSTRACT

This study aimed to elucidate the possible influence of body size in terms of shell size on the activities of digestive enzymes in the crystalline style of the Angelwing clam Pholas orientalis. Sexually mature Angelwing clams were classified into into three group sizes: (1) small (80-100 mm); (2) medium (101-120 mm); and (3) large (>121 mm). α-Amylase, CM-cellulase, agarase, laminarinase, and protease specific activities were measured. α-Amylase and agarase activities were significantly lower in small clams than in medium and large clams which were not significantly different from each other. CM-cellulase specific activity increased almost linearly with size while laminarinase did not have any significant effect on the clams at any size. Small clams exhibited only about a third of the protease activities of that exhibited by either the medium or large clams. The increase in enzymatic activities with animal size was probably due either to increased food requirement for growth, maintenance, or gonad maturation or their combination.

INTRODUCTION

In fish, it is known that age significantly influences the digestive enzyme activity in different fish species (Kuz'mina and Golovanova [9], Stroganov and Buzinova [18]). This is because it is important to estimate not only changes in standard enzyme activity (activity g^{-1} wet tissue), but also total intestinal activity as well as relative enzyme activity [10]. The latter index provides a measure of relative digestive enzyme activity on a fish weight basis [8]. To our knowledge, the comparison of digestive enzyme activities on the basis of shell weight or size have not been done.

Previously, we have shown the effect of pH on the activities of digestive enzymes in *Pholas orientalis* [19]. Since activities of digestive enzymes are indirectly related to the overall metabolism of the bivalve, its variation could be influenced by body size among other factors. This paper aims to elucidate this possible influence of body size in terms of shell size on the digestion of specific dietary substrate such as carbohydrates and protein.

MATERIALS AND METHODS

Experimental animals

All the clams used in this study were sexually mature animals. Sexual maturity of *P. orientalis* is attained when the animal reaches the shell lengths of 59 mm and 64 mm for males and females, respectively [13]. Clams were classified into three group sizes: (1) small (80-100 mm); (2) medium (101-120 mm) ; and (3) large (>121 mm).

Enzyme extraction and preparation
Crystalline styles were excised, thawed, weighed and washed with cold citrate phosphate buffer, pH 7.0 similar to the previous methods [19]. Extraction buffer was added to the style at 1:30 (w/v), homogenized in an Ultraturrax homogenizer, and centrifuged at 1800 g for 15 min. The filtered supernatant was used as the crude enzyme extract for the assays.

Enzyme assay
All procedures were done at 4^{o}C and assays at 25^{o}C unless otherwise stated. Measurements were done in triplicates with corresponding measurement at zero time and also of reactions in the absence of enzyme or substrate for correction of activity values.

α-Amylase, CM-cellulase, agarase and laminarinase activities were measured following the method of Areekijseree *et al*. [1] modified from Bernfield [2]. For amylase activity determination, 0.2 mL enzyme extract and 1.8 mL phosphate buffer were mixed in test tubes. The reaction was initiated by adding 1.0 ml of substrate (1.0% soluble starch dissolved in assay buffer) in a final volume of 3.0 ml. After 15 min, the reaction was stopped by adding 1.0 mL 3,5-dinitrosalicylic acid (DNS) solution, placed in boiling water bath for 10 min in which the yellow mixture turned dark red and cooled to room temperature. Optical density (OD) of the solution was read at 546 nm. Mixtures with no substrate or no enzyme or both were used as blank samples for correction of innate activity in the crude extract and for spontaneous hydrolysis of the substrate, respectively.

Similar procedure as that for amylase assay was employed for the CM-cellulase, agarase and laminarinase assays, which varied only in the substrate used- carboxymethyl cellulose (CMC), agarose, and laminarin, respectively. For the CM-cellulase assay, the reaction mixture contained 0.3 mL enzyme extract, 1.0 mL 0.25% CMC and 1.7 mL citrate-phosphate buffer, pH 6.0 in a final volume of 3.0 ml; the reaction was allowed to proceed for 15 min. For the agarase assay, the reaction mixture consisted of 0.1 mL enzyme extract, 1.0 mL agarose solution as substrate and 1.9 mL citrate-phosphate buffer, pH 6.0 in a final volume of 3.0 ml.; the reaction was allowed to proceed for 15 min. Laminarinase activity was measured following 30-min incubation of the following reaction mixture: 0.3 mL enzyme extract, 1.0 mL 0.1% laminarin, 1.7 mL of citrate-phosphate buffer, pH 6.0 in a final volume of 3.0 ml. All carbohydrase activities were quantified using glucose as standard except for agarase activity where galactose was used instead. Alpha amylase, CM-cellulase and laminarinase activities were expressed as µmol glucose liberated min^{-1} mg^{-1} protein at 25 ^{0}C at pH 7.0, 6.0, and 6.0, respectively. Agarase activity was expressed as µmol galactose liberated min^{-1} mg^{-1} protein at 25 ^{0}C and at pH 6.0.

Proteolytic activity was measured using casein as substrate, following the method of Kunitz [7]. The reaction mixture consisted of 1.0 mL 1.0% casein dissolved in 0.01N NaOH, 1.5 mL phosphate buffer pH 7.0 and 0.5 mL enzyme extract in a final volume of 3.0 mL. The reaction was allowed to proceed for 60 min and stopped by adding 1.0 mL ice-cold 5% trichloroacetic acid (TCA). Optical density of the supernatant was read at 280 nm and protease activity was expressed as µg tyrosine released hr^{-1} mg^{-1} protein at 25^{o}C under the conditions of the assay.

Protein determination
Protein content of the crystalline style was measured according to the method of Bradford [4] using bovine serum albumin (BSA) as standard.

Statistical Analyses
All data were subjected to one-way analysis of variance (ANOVA) at $\alpha = 0.05$ to determine if differences between treatments exist [21]. If a difference was detected, Tukey's Post-hoc test was done. Prior to ANOVA, the data were tested for homogeneity of variances and normal distribution.

RESULTS AND DISCUSSION

α-Amylase
α-Amylase activity was lowest in small clams (16.4 µmol glucose min^{-1} mg^{-1} protein). Bigger clams had higher α-amylase activities, but no significant difference was observed between the activities of medium and large clams (18.8, 18.4 µmol glucose min^{-1} mg^{-1} protein, respectively, Figure 1).

The result of the present study corroborated the works of other authors. Langton and Gabott [11] find positive correlation between the crystalline style length of oyster *Ostrea edulis* and its amylolytic activity. Similarly, Seiderer [17] reports that the total activity of crystalline style's α-amylase is proportional to the shell lengths of marine mussels *Choromytilus meridionalis* and *P. perna*. The increased in α-amylase activity in medium and large clams observed in the present study might be due to increased food hydrolysis to meet nutritional requirement for growth and development. Vega-Villasante *et al.* [20] report that in crab *Callinectes arcuatus*, amylase activity is strongly affected by molting, wherein the enzyme activity increases due to the energetic needs for ecdysis and tissue synthesis.

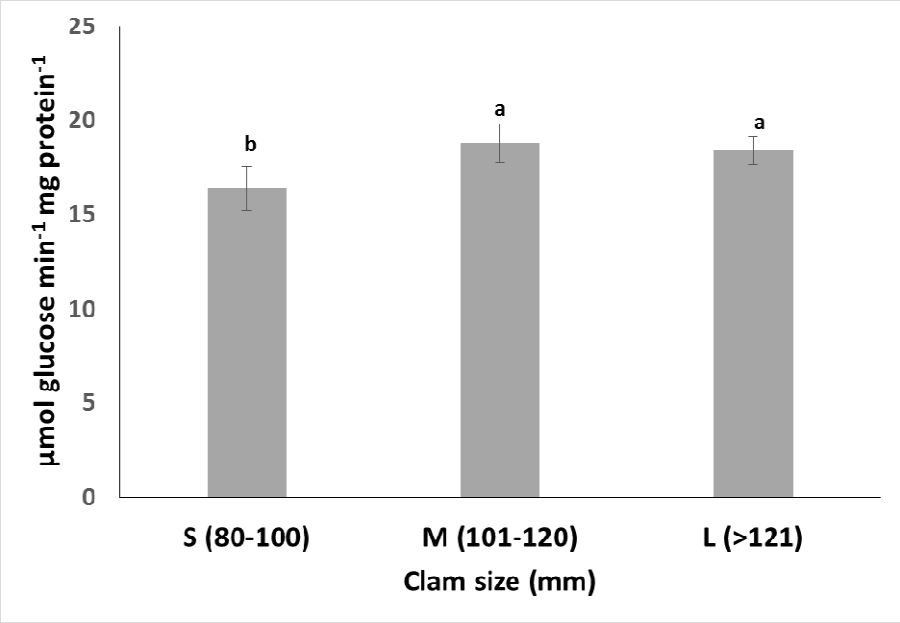

Figure 1. Alpha amylase activities of clams of different sizes. The assay mixture containing 0.2 mL enzyme extract, 1.8 mL buffer (pH 7.0) and 1.0 mL 1.0% soluble starch (w/v) was incubated for 15 min. Bars represent mean ± standard deviation. Means with different letters indicate significant differences between treatments (P < 0.05, Tukey Test).

CM-cellulase

The CM-cellulase activity of the crystalline style of *P. orientalis* increased with size (Figure 2). It was highest in large clams (14.3 µg glucose min^{-1} mg^{-1} protein) followed by medium-sized animals (11.4 µg glucose min^{-1} mg^{-1} protein) and lowest in small angelwings (9.3 µg glucose min^{-1} mg^{-1} protein). This result was in accordance with the findings of Seiderer [17] who positively correlates cellulase activities with shell lengths of two different species of marine mussels.

Agarase

As shown in Figure 3, agarase activity was significantly the lowest in small clams (8.9 µg galactose min^{-1} mg^{-1} protein). The activity increased with clam size, but no significant difference was observed between the medium and large clams (10.1, 10.7 µg galactose min^{-1} mg^{-1} protein, respectively).

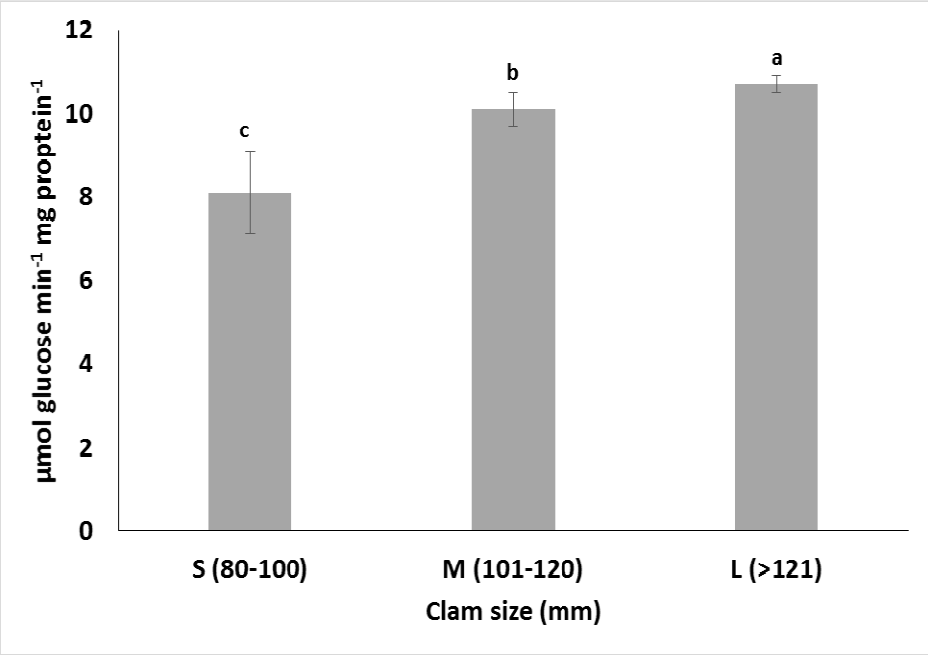

Figure 2. CM-cellulase activities of clams of different sizes. The assay mixture containing 0.3 mL enzyme extract, 1.7 mL buffer (pH 6.0) and 1.0 mL 0.25% CMC (w/v) was incubated for 15 min. Bars represent mean ± standard deviation. Means with different letters indicate significant differences between treatments (P < 0.05, Tukey Test).

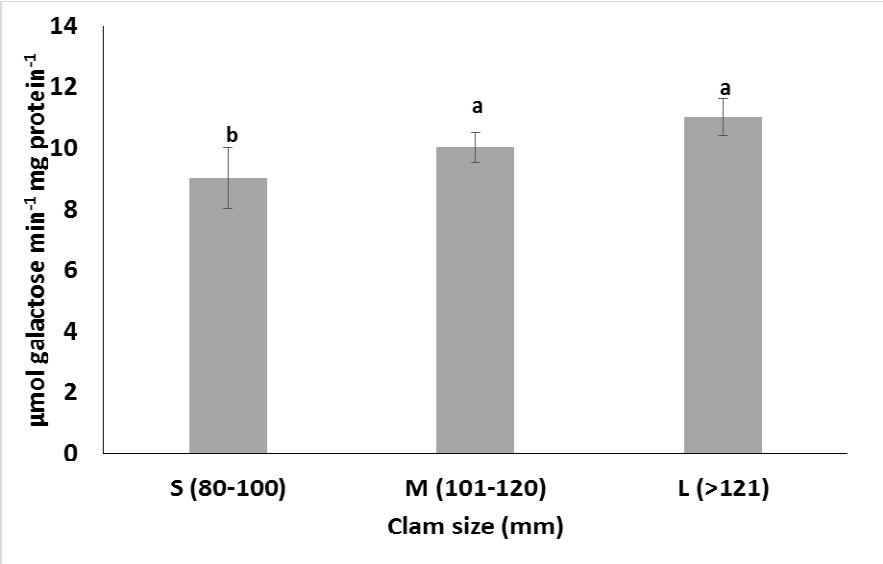

Figure 3. Agarase activities of clams of different sizes. The assay mixture containing 0.1 mL enzyme extract, 1.9 mL buffer (pH 6) and 1.0 mL 0.2% agarose (w/v) was incubated for 15 min. Bars represent mean ± standard deviation. Means with different letters indicate significant differences between treatments (P < 0.05, Tukey Test).

Laminarinase

No significant differences were observed in the activities of laminarinase in relation to Angelwing clam shell lengths (Figure 4). The activity of the enzyme was similar among all size groups investigated.

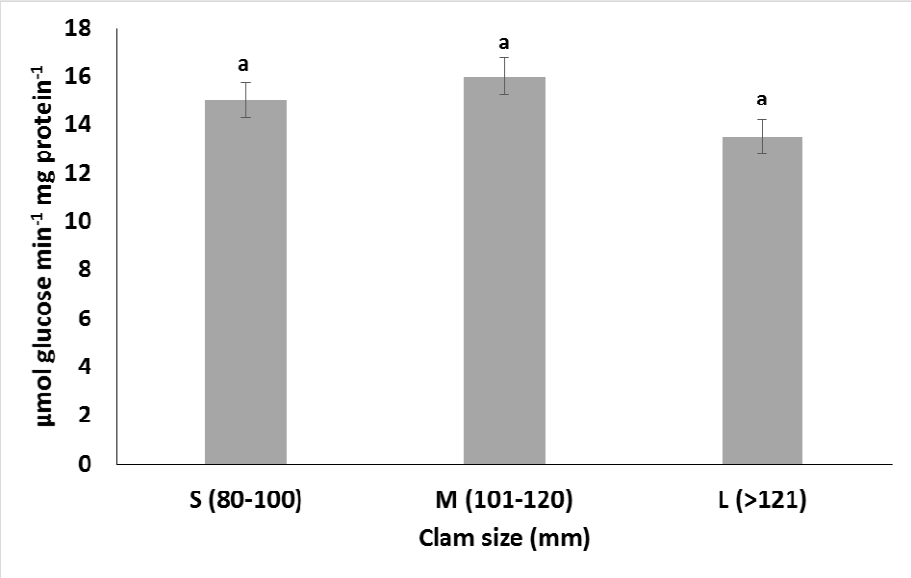

Figure 4. Laminarinase activities of clams of different sizes. The assay mixture containing 0.3 mL enzyme extract, 1.7 mL buffer (pH 6.0) and 1.0 mL 0.1% laminarin (w/v) was incubated for 30 min. Bars represent mean ± standard deviation. Means with different letters indicate significant differences between treatments (P < 0.05, Tukey Test).

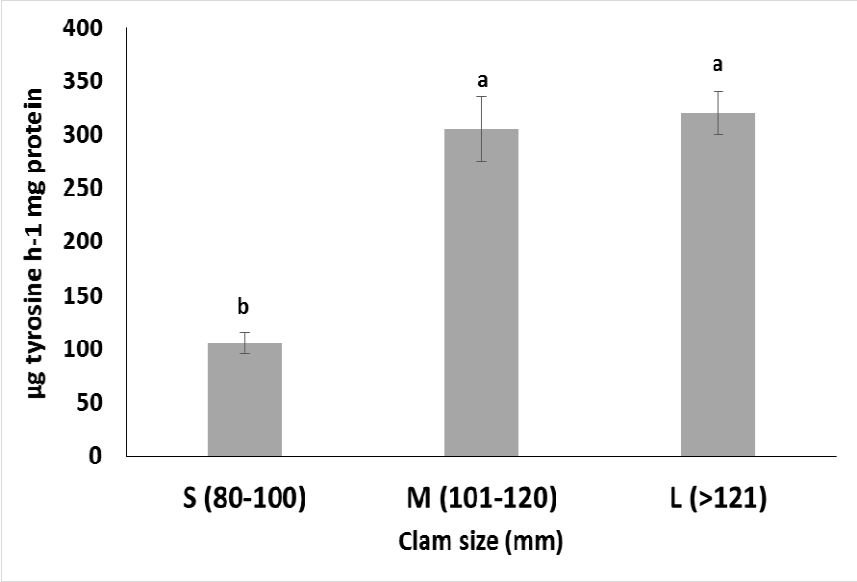

Figure 5. Protease activities of clams of different sizes. The assay mixture containing 0.5 mL enzyme extract, 1.5 mL buffer (pH 7.0) and 1.0 mL 1.0% casein (w/v) was incubated for 1 h. Bars represent mean ± standard deviation. Means with different letters indicate significant differences between treatments (P < 0.05, Tukey Test).

Protease

The effect of size on the proteolytic activity of Angelwing clams is depicted in Figure 5. Small *P. orientalis* had the lowest crystalline style protease activity (106.2 µg tyrosine h^{-1} mg^{-1} protein) while no significant difference was observed between medium and large samples (308.5, 327.2 µg tyrosine h^{-1} mg^{-1} protein, respectively). This observation agrees with those documented in rock lobster J. edwardsii in which the total activities of digestive trypsin, chymotrypsin and carboxypeptidase A are positively correlated with carapace length [6].

In the present study, digestive enzyme activities of the Angelwing clam generally showed an increasing trend with size, except for laminarinase activity. The increase in enzymatic activities with animal size was probably due to

increased food requirement for growth, maintenance [6] and gonad maturation [16]. In lobster, the increase in digestive enzymatic activity with size of *J. edwardsii* has been attributed to the increase in size and volume of enzyme producing B- and F-cells [3, 6]. Moreover, the increasing carbohydrase and protease activities with size could be related to reproduction and gametogenesis. Energy resources like protein and glycogen have been shown in oyster *C. gigas* to be utilized for gonad development, tissue synthesis, cellular fuel and vitellogenesis [15]. The same authors have reported that the decrease in the protein content in *C. gigas* testes is due to its utilization for spermatogenesis, while the energy needed for maturation of gametes is sustained by glycogen and protein stored in the adductor muscles. Similarly, in scallops, dietary reserves including protein and lipids in adductor muscles are channeled to the gonads for egg maturation [16]. Since sexual maturity and nutrient composition were not determined in the present study, this area needs further investigation.

Several reports have been published with varying results in marine invertebrates. Amylase activity and clam size of mussel *M. edulis* are not correlated with each other (Langton [12]. In addition, a negative correlation has been reported between enzyme specific activities (amylase and laminarinase) and lobster size [6]. Lee *et al.* [14] report that the proteolytic activity of smaller shrimp *P. vannamei* (9-11 g) was generally higher than those of bigger shrimps (15-30 g). These deviations from the present study may be due to the differences in individual digestive responses of each organism. The ability to digest food particles is species-specific [5]. In addition, different species have various physiological and nutritional adaptations to developmental and environmental changes as the animals grow.

In conclusion, small size *Pholas orientalis* clams exhibited lower α-amylase, agarase and protease activities than did medium and large size clams. In contrast, CM-cellulase activities showed increasing enzyme activity with increasing size while laminarinase activity was not influenced by the clam size at all.

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