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Effects of pH on amylase, cellulase and protease of the Angelwing clam, *Pholas orientalis*

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ABSTRACT

This study aims to determine the effects of pH on the activity of the three digestive enzymes namely α-amylase, cellulase and protease in the crystalline style of the angelwing clam, Pholas orientalis. Laboratory assay of the three enzymes were also optimized to establish routine assay methods. α-Amylase activity increased from pH 3.0 and peaked at pH 6.0-8.0 beyond which it gradually decreased until pH 10.0. CM-cellulase activity abruptly increased from pH 3.0 reaching its peak at pH 6.0 beyond which an equally abrupt decline was observed until pH 9.0 and 10 where the activities were similar. Protease activity showed two maximal activities: a lower maximum at pH 6.0 and a higher one at pH 7.0; beyond pH 7.0, a decrease in enzyme activity was observed until pH 11.0. In conclusion, optimal pHs were determined for α-amylase, CM-cellulase and protease to be 6.0-80, 6.0 and 7.0, respectively.

INTRODUCTION

Pholas orientalis or angelwing clam is a marine bivalve belonging to the Family Pholadidae, Order Myoida [1]. This economically important bivalve locally known as *"diwal"* is found in the tidal flats and subtidal areas of Southeast Asia and Australia [2]. In the Philippines, this clam has been documented as inhabiting the coastal waters of Western Visayas, specifically Panay, Guimaras Strait and Negros Island.

P. orientalis are filter feeders and mud burrowers having attractive angelwing-shaped delicate shells. This white-flesh seafood is a favorite shellfish in Western Visayas and a highly-valued export commodity. Angelwing clam is a seasonal seafood having a high market demand because of its sweet taste, delicious flavor and soft texture. Like other commercially important species however, wild *P. orientalis* stocks are becoming depleted due to overexploitation, habitat degradation and unsustainable fishing practices [2].

α-Amylase has been documented to be the primary carbohydrase in some bivalves such as *Crassostrea madrasensis*, *Meretrix meretrix*, *Meretrix castra* and *Katelysia opima* [3], *Perna viridis* [4], *Geukensia demissa* and *Rangia cuneata* [5], *Rangia decussates* and *Venerupis pullastra* [6]. This capability is important for filter feeders [7] like *Pholas orientalis* whose diet consists of a wide array of phytoplankton species that contains starch.

Cellulose is the most common carbon containing compound in plants but most animals cannot digest it [8], thus relying on symbionts marine bacteria and fungi in nutrient extracting and recycling [9]. However, there are bivalves reportedly capable of degrading cellulose without help from symbionts, examples of which are *Scrobicularia plana* [10] and freshwater clam *Corbicula japonica* [11]. Elyakova *et al.* [12] have found positive hydrolysis of CMC in freshly collected samples of 71 marine invertebrates, 28 of which are bivalves. Cellulase activity is necessary for dissolving the cell wall of green algae to extract the intracellular nutrients [13; 8].

Proteases are responsible for breaking up peptide bonds in the protein molecule releasing amino acids. Proteolytic activity has been reported in the crystalline styles of the following bivalves: *Cardium edule* [14]; *Mytilus chilensis* and *M. edulis* [13]; *Argopecten purpuratus* [15]; *Ruditapes decussatus* and *Venerupis pullastra* [6].

The nutritional needs of *P. orientalis* are not yet fully studied. Establishing basic nutritional requirements partly through understanding the digestive capacity is necessary in the development of a hatchery technology. Dietary metabolism can be elucidated by profiling the digestive enzymes [16; 17] that could give insights to the food preference and food type that the organisms are capable of digesting and absorbing [18]. This study aims determine the effects of pH on the activities of α-amylase, CM-cellulase and protease in the crystalline style of the angelwing clam, *Pholas orientalis*.

MATERIALS AND METHODS

Experimental animals

Ninety-three adult *P. orientalis* (74.5 ± 13.5g wet weight; 111.9 ± 9.4 mm standard length, SL) were collected from the municipal water of Roxas City, Philippines. The harvested clams were packed into a styrofoam box to which enough seawater was added to keep the bivalves' siphon moist. Upon arrival to the hatchery, the bivalves were cleaned of mud, weighed and placed in plastic trays inside an 80-L glass aquaria. The clams were fed with a mixture of equal proportion of microalgae *C. calcitrans*, *I. galbana*, *Thalassiosira* sp. and *T. tetrathele*. Algal cells were continuously supplied at 2.94 x 10^8 cells $clam^{-1}$ day^{-2} as recommended by Marasigan and Laureta [19]. Aeration was provided for the duration of the experiment to a flow-through system at a flow rate of 110 mL min^{-1}. The clams were sacrificed on the 8^{th} day; crystalline styles were removed, pooled and stored at -85 ^{0}C until assay.

Enzyme extraction and preparation

During enzyme extraction, the crystalline styles were thawed, weighed and washed with cold citrate phosphate buffer, pH 7.0 similar to the previous methods [20; 21]. The extraction buffer was added to the style at 1:30 (w/v), homogenized in an Ultraturrax homogenizer, and centrifuged at 1789 g for 15 min. The supernatant was filtered and used as 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 and CM-cellulase activities were measured following the methods of Areekijseree *et al.* [22] and Mukesh *et al.* [23] modified from Bernfield [24]. For amylase assay, the reaction mixture consisted of 0.2 mL enzyme extract, 1.8 mL phosphate buffer and 1.0 mL 1.0% soluble starch in a final volume of 3.0 mL.. The reaction was stopped after 15 min by adding 1.0 mL 3,5-dinitrosalicylic acid (DNS) solution, placed in water bath at 100^{o}C for 10 min, cooled to room temperature and the optical density was read at 546 nm. α-Amylase activity was expressed as μmol glucose liberated min^{-1} mg^{-2} protein at 25^{o}C under the assay conditions.

CM-cellulase assay was the same with that of the α-amylase except for the substrate used (carboxymethyl cellulose, CMC) and the reaction mixture. The reaction mixture consisted of 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 2.0 mL. The reaction was allowed to proceed for 15 min. Enzyme activity was expressed as μmol glucose liberated min^{-1} mg^{-2} protein at 25^{o}C under the conditions of the assay.

Proteolytic activity was measured using casein as substrate, following the method of Kunitz [25] with modified by Abirami *et al.* [26]. 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°C under the conditions of the assay.

Optimization of assay

Optimum enzyme concentration in the crude preparation was determined by measuring enzyme activity at various dilutions with the extracting buffer. Also, optimum reaction time was determined by measuring activity at various durations (5-60 min). Conditions for the routine assay was decided using values that fell within the linear, initial rate of activity (Table 1).

Table 1. Optimum conditions for the preparation and assay of α-amylase, CM-cellulase and protease

	α-Amylase	CM-cellulase	Protease
Enzyme preparation (wet wt to buffer (w/v))	1:30	1:30	1:30
Enzyme conc. in the reaction mixture (mL)	0.2	0.3	0.5
Reaction time (min)	15	15	60

Effects of pH on enzyme activities

The effect of pH on digestive enzyme activities were tested using various pH buffers from 3.0-11.0, specifically citrate phosphate buffer (pH 3-5) for CM-cellulase, phosphate buffer (pH 6-8) for α-amylase and protease; $NaHCO_3$-Na_2CO_3 buffer was used for higher pHs (9-11).

Statistical Analyses

All data were subjected to one-way analysis of variance (ANOVA) at $\alpha = 0.05$ to determine if differences between treatments exist [27]. If a difference was detected, Tukey's Post-hoc test was done to identify the differences among treatments. Prior to ANOVA, the data were tested for homogeneity of variances and normal distribution.

RESULTS AND DISCUSSION

α-Amylase

Alpha amylase activity increased from pH 3.0 and peaked at pH 6.0-8.0 beyond which it gradually decreased until pH 10.0 (Fig. 1). Thus, the optimal amylolytic activity was at pH 6.0-8.0 and this is in agreement with the findings in crystalline style extracts of the bivalves *Mya arenaria* [28]; *Placopecten magellanicus [29]*; *Pecten maximus [30]*; *Geukensia demissa* [5]; *Crassostrea edule* [14]; *Rangia decussates* and *Venerupis pullastra* [6]. It was also similar with the pH of the digestive tracts of six marine bivalves examined by Hameed [31], indicating that amylolytic activity in angelwing clams was well-adapted to its environmental conditions. Hepatopancreas extracts from 10 bivalve species [7], the digestive gland of *Perna viridis* [4], the viscera extract of *Meretrix lusoria* [32], and the digestive gland and stomach of freshwater pearl mussel *Chamberlainia hainesiana* [33] also have optimal amylase activities at pH 6.0-7.0.

CM-cellulase

CM-cellulase activity abruptly increased from pH 3.0 reaching its peak at pH 6.0 beyond which an equally abrupt decline was observed until pH 9.0 and 10 where the activities were similar (Fig. 2). Thus, the optimal reaction pH for CM-cellulase was at pH 6.0. This finding was similar with those of Brock and Kennedy [5] in the crystalline style extracts of five bivalves, *Crassostrea gigas*, *Rangia cuneata*, *Geukensia demissa*, *Macoma balthica* and *M. mitchelli*. Similarly, the hepatopancreas extracts from *Mytilus edulis* and *Argopecten irradians* [7] as well as *Crassostrea gigas* [34] have maximum cellulolytic activity at pH 6.0.

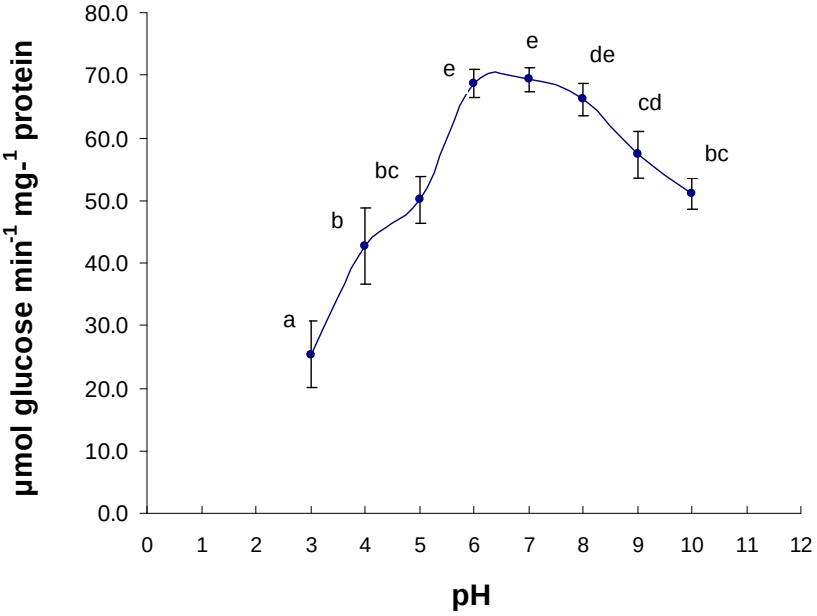

Fig. 1. Effect of pH on α-amylase activity. The assay mixture containing 0.2 mL enzyme extract, 1.8 mL buffer (pH 3.0-10.0) and 1.0 mL 1.0% soluble starch (w/v) was incubated for 15 min. Each point is a mean of three measurements and bar represents standard deviation. Means with different letters indicate significant differences between treatments (P < 0.05, Tukey Test).

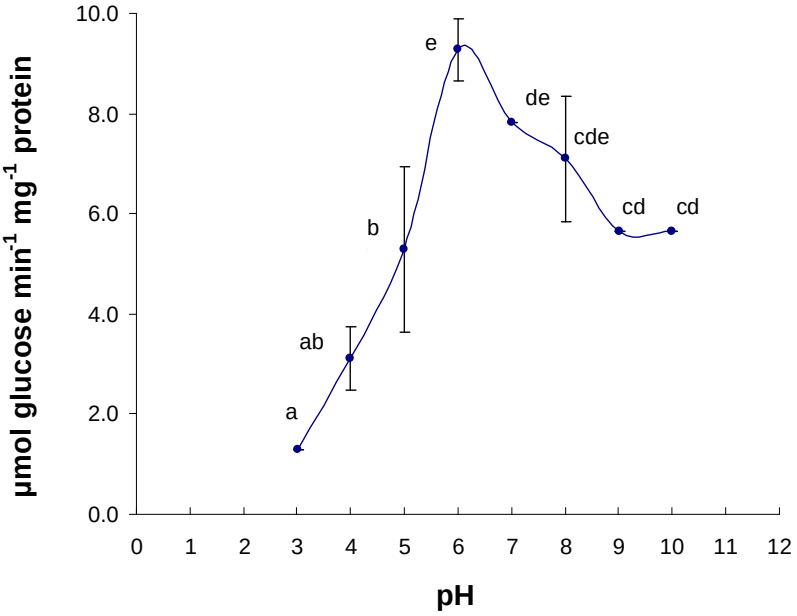

Fig. 2. Effect of pH on CM-cellulase activity. The assay mixture containing 0.3 mL enzyme extract, 1.7 mL buffer (pH 3.0-10.0) and 1.0 mL 0.25% CMC (w/v) was incubated for 15 min. Each point is a mean of three measurements and bar represents standard deviation. Means with different letters indicate significant differences between treatments (P < 0.05, Tukey Test).

Protease

Protease activity showed two maximal activities: a lower maximum at pH 6.0 and a higher one at pH 7.0 (Fig. 3). Beyond pH 7.0, a decrease in enzyme activity was observed until pH 11.0. Thus, the optimal pH for protease was at pH 7.0 in the present study similar with the findings in the subtidal clam *Venerupis pullastra* [6]. The freshwater mussel *Hyriopsis bialatus* exhibits optimum intestinal proteolytic activity at pH 6.0-8.0 [22]. The two maxima exhibited in the present study indicated that another family of protease was present. The occurrence of several

proteases is an important adaptation in order for angelwing clams to respond quickly to environmental pH variations. Multiple isoforms of proteases have been reported in pearl mussel *Hyriopsis bialatus* [22] and *Chamberlainia hainesiana* [33]. The digestive gland extracts of *Crassostrea edule* [14] and *Chamberlainia hainesiana* [33] exhibit highest proteolytic activity at pH 5.5 and 5.0, respectively.

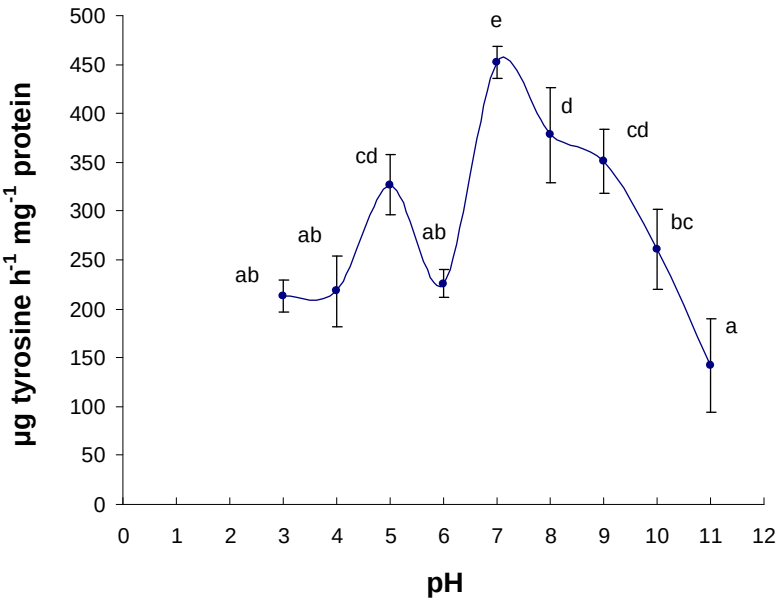

Fig. 3. Effect of pH on protease activity. The assay mixture containing 0.5 mL enzyme extract, 1.5 mL buffer (pH 3.0-11.0) and 1.0 mL 1.0% casein (w/v) was incubated for 1 h. Each point is a mean of three measurements and bar represents standard deviation. Means with different letters indicate significant differences between treatments (P < 0.05, Tukey Test).

In general, this study showed that the optimum pH of the three digestive enzymes in the crystalline styles of *P. orientalis* were similar to the pH of their habitat; natural beds of *P. orientalis* have pH of 7.8-8.2 [35]. Presumably, optimum reaction pH of digestive enzymes falls within the actual pH of the crystalline style. This is in agreement with the findings of [31] who reports that the digestive tract pH of different bivalves ranges from 5.8-7.4, similar to the observed optimum pH in the present study (pH 6.0-8.0). CM-cellulase exhibited an optimal reaction pH at pH 6.0 which was slightly acidic. This might be due to the crystalline style being stable at slightly acidic pH, but dissolves at higher pH [31]. In *Macoma balthica* for example, the crystalline style remains intact for several days when kept at pH 3.5-4.0 but completely dissolves within 1 h when exposed to pH 9.0 [36]. Results of the present study in which the optimum pH of two carbohydrases, namely α-amylase and CM-cellulase, and protease coincided with the pHs of the clam's digestive tract and habitat and could be an indication of a form of adaptation to both internal as well as external environments.

In conclusion, optimal pHs were determined for α-amylase, CM-cellulase and protease to be 6.0-80, 6.0 and two maxima of 6.0 and 7.0, respectively.

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