Effect of Unialgal Diets on Digestive Enzyme Activity in the Angelwing Clam (*Pholas orientalis*)

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**Abstract**

This study determined the effects of microalgae diets including *Chaetoceros calcitrans*, *Isochrysis galbana*, *Thalassiosira* sp., and *Tetraselmis tetrathele* on the activity of α-amylase, CM-cellulase, agarase, laminarinase, and protease in the angelwing clam, *Pholas orientalis*. The activity of α-amylase, laminarinase, and protease was higher in clams fed *Thalassiosira* sp., *I. galbana*, or *C. calcitrans* than in clams fed *T. tetrathele*. In contrast, CMC-cellulase activity was significantly higher in the *T. tetrathele* group than in other groups. Agarase activity was similar in all groups.

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Introduction

The angelwing clam, *Pholas orientalis*, is an economically important bivalve in the Philippines and is found in the tidal flats and subtidal areas of Southeast Asia and Australia (Laureta, 2005). It inhabits the coastal waters of Western Visayas in the Philippines, specifically Panay, Guimaras Strait, and Negros Island. A filter feeder and a mud burrower with an attractive angelwing-shaped delicate shell, *P. orientalis* is a highly-valued export commodity. Like other commercially important species, however, wild *P. orientalis* stocks are becoming depleted due to overexploitation, habitat degradation, and unsustainable fishing practices (Laureta, 2005).

The hatchery rearing of juvenile and adult bivalves including the angelwing clam relies on the mass production of microalgae. Adult *P. orientalis* clams survive well when fed a mixture of *Chaetoceros calcitrans* and *Tetraselmis batan* (Marasigan and Laureta, 2001) or *I. galbana* (Corda et al., 1998). Mass production of live algae is a major bottleneck in bivalve hatcheries and nurseries (Coutteau and Sorgeloos, 1992) and comprises up to 30% of hatchery operation costs. Further, the nutritional value of algae varies among cultures and algae are subject to seasonal growth patterns and contamination.

The nutritional quality of feeds and feed ingredients for bivalves can be evaluated by using crude enzyme extracts from the bivalve. Protease and amylase activity are indicators of *in vivo* digestibility of dietary protein (Supannapong et al., 2008) and carbohydrate (Areekijseree et al., 2006), respectively. Digestive enzyme activities and the mechanisms controlling them can be important in maximizing absorption and food conversion efficiencies (Huvet et al., 2012).

Understanding its digestive capacity is necessary for clarifying the nutritional needs and developing hatchery technology for the production of the angelwing clam. Enzyme behavior gives insights to food preferences of organisms and to the organism’s ability to digest and absorb nutrients (Johnston and Freeman, 2005). The objective of this study was to compare the effects of microalgal diets (*Chaetoceros calcitrans*, *Isochrysis galbana*, *Thalassiosira sp.*, and *Tetraselmis tetrathele*) on the activity of α-amylase, CM-cellulase, agarase, laminarinase, and protease using crude enzyme extracts of the crystalline style of adult *Pholas orientalis*.

Materials and Methods

*Algae culture.* Four algae were used as diets for the angelwing clam: *Chaetoceros calcitrans*, *Isochrysis galbana*, *Thalassiosira sp.*, and *Tetraselmis tetrathele*. *Chaetoceros calcitrans* is a brown marine diatom, widely used in aquaculture because of its nutritional value (Chotipuntu, 2005). *Isochrysis sp.* is a brown silicated dinoflagellate (Bougis, 1976) and a preferred food of *P. orientalis* (Corda et al., 1998). *Thalassiosira sp.*, is also a brown diatom and a common live food for bivalves (Chotipuntu, 2005). *Tetraselmis sp.* is a green flagellated unicellular algae with a rigid cell wall (Domozych et al., 1981).

Starter cultures of the algae were cultured in 1-l bottles using filtered and chlorinated seawater. Guillard F/2 was used as a fertilizer for the brown algae while Conway fertilizer was used to grow *T. tetrathele*. Moderate aeration and 40-W fluorescent lamps were provided for 24 h to all cultures to attain maximum cell growth. The algae were harvested after 3–5 days and scaled-up to 10-l carboys. Algae cells were counted and monitored regularly using a microscope. Species were subcultured daily (in both 1-l flasks and 10-l carboys) to ensure sufficient food for the clams. Materials (carboys, glass ware, air hoses, etc.) were sterilized and chlorinated to prevent contamination of the algae cells.

*Experimental animals.* Angelwing clams were collected from the municipal water of Roxas City, Philippines. The newly-harvested clams were carefully packed into a styrofoam box in which just enough sea water was added to keep the siphon moist. Upon arrival at the hatchery, the bivalves were cleaned of mud, weighed, and arranged in plastic trays inside 80-l glass aquaria. Sixty adult *P. orientalis* (73.53±7.10 g wet wt; 112.76±4.38 mm SL) were acclimated for 7 days, then randomly divided into four groups. Each group was fed a diet of *C. calcitrans*, *I. galbana*, *Thalassiosira sp.*, or *T.
tetrahele for 7 days. Harvested algae were carefully counted prior to feeding so that each clam received approximately 2.94 x 10^5 cells/day (Marasigan and Laureta, 2001). Feeding jars were arranged so that food was continuously supplied to the clams. Aeration was provided and water was supplied via a flow-through system at a rate of approximately 110 ml/min.

Crude enzyme preparation. Following 7 days of acclimation or feeding, clams were sacrificed, excised, and the crystalline styles were removed, weighed, and stored at -85°C until assay. For enzyme extraction, the crystalline styles were thawed, weighed, and cold citrate phosphate buffer (pH 7.0) was added at 1:30 (w/v). The mixture was homogenized in an Ultraturrax homogenizer, centrifuged at 4000 rpm for 15 min, and the supernatant was filtered and used as an enzyme extract. α-Amylase, cellulase, agarase, laminarinase, and protease activities were assayed at 25°C in triplicate, with corresponding blank and control samples. All procedures were done at 4°C unless otherwise stated.

Enzyme assay. Carbohydrase activities (α-amylase, CM-cellulase, agarase, and laminarinase) were measured following the method of Areekijseree et al. (2004), modified from Bernfield (1955). Measurements were taken in triplicate with corresponding measurements at zero time and reactions in the absence of an enzyme or substrate for correction of activity values. Protein was measured according to the method of Bradford (1976) using bovine serum albumin as the standard.

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 a water bath at 100°C for 10 min and cooled to room temperature. Optical density was read at 546 nm and α-amylase activity was expressed as μmol glucose liberated/min/mg protein.

For CM-cellulase assay, the substrate was carboxymethyl cellulose (CMC). 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/mg protein.

For agarase activity, the reaction mixture was 0.1 ml enzyme extract, 1.0 ml agarose (0.2%), and 1.9 ml citrate-phosphate buffer (pH 6.0) in a final volume of 2.0 ml. The reaction was allowed to proceed for 15 min. Agarase activity was expressed as μmol galactose liberated/min/mg protein.

For laminarinase activity, the reaction mixture was 0.3 ml enzyme extract, 1.0 ml 0.1% laminarin, 1.7 ml citrate-phosphate buffer (pH 6.0) in a final volume of 2.0 ml. Activity was expressed as μmol galactose liberated/min/mg protein.

Proteolytic activity was measured using casein as the substrate, following the method of Kunitz (1947) with some modifications. 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 was stopped by adding 1.0 ml ice-cold 5% trichloroacetic acid. Optical density of the supernatant was read at 280 nm and protease activity was expressed as μg tyrosine released/h/mg protein.

Statistical analysis. Data were subjected to one-way analysis of variance (ANOVA) at α = 0.05 to determine differences between treatments (Zar, 1984). If a difference was detected, the difference(s) among treatments was identified by Tukey’s Post-hoc test. Since ANOVA requires that data be independent, normally distributed, and have equal variances, appropriate statistical procedures were undertaken to test these requirements using statistical package SPSS (vers. 16).

Results

α-Amylase, laminarinase, and protease activities were significantly higher in clams fed Thalassiosira sp., I. galbana, or C. calcitrans than in those fed T. tetrahele (Fig. 1). In
contrast, CMC-cellulase activity was significantly highest in the *T. tetrathele* group and agarase activity was similar in all groups.

Image of a table showing enzyme activity.

**Discussion**

The present study suggests that feed type influenced the activity of digestive enzymes in the crystalline style of angelwing clams. Activity of α-amylase, laminarinase, and protease was significantly higher in clams fed *C. calcitrans*, *I. galbana*, or *Thalassiosira* sp. than in those fed *T. tetrathele* while CM-cellulase activity was significantly higher in clams fed *T. tetrathele* than in those fed the other diets. The presence of substrates in the food may have activated the enzymes, resulting in the increased α-amylase, laminarinase, and protease activity. Phytoplankton contain nutrients such as starch, laminarin, and proteins (Brock et al., 1986). In clams fed *T. tetrathele*, however, the intracellular starch granules, laminarin, and proteins were unavailable to the digestive enzymes because of the thick cellulose-rich cell wall covering this green microalga (Brock et al., 1986). The cell walls of diatoms, in contrast, contain only laminarin and silica (Popper and Tuohy, 2010) and the clams were probably able to process them more easily.

Because of the cellulose, angelwing clams might have difficulty digesting *T. tetrathele* algae cells. Although cellulase is a common molluscan enzyme, hydrolysis of structural cellulose is generally low in bivalve species (Hameed and Paulpandian, 1987). *Tetraselmis* algae are not easily digested by other bivalves (Liu et al., 2008). Of all the assayed digestive enzymes, only CM-cellulase activity was significantly higher in clams fed *T. tetrathele*, in agreement with Brock, 1989.
Reports on the effects of microalgal diets on bivalves normally involve growth. Digestive enzymatic activity is assumed to be part-and-parcel of the effects on growth. When *Chlorella calcitrans*, *I. galbana*, and *Tetraselmis suecica* were administered to *Ostrea edulis* spat for 4-5 weeks, *T. suecica* was considered the least nutritious due to a lower growth rate and survival (Laing and Millican, 1986) and king scallop spat (Laing and Pismopoulous, 1998). In the present study, *Tetraselmis* resulted in the lowest α-amylase, laminarinase, and protease activity, in agreement with the above observations of its effect on growth and survival. However, the *Tetraselmis* diet produced the highest CMC-cellulase activity. Similarly, hepatopancreas extracts of oysters fed a green *T. suecica* diet resulted in higher cell wall degradation of live *T. suecica* cells than did extracts from oysters fed other diets (Brock, 1989). Further, when algae were fed to *Tapes semidecussata*, *T. decussata*, and *Mercenaria mercenaria* juveniles, the order according to nutritional value was *I. galbana* > *C. calcitrans* > *Thalassiosira pseudonana* > *T. suecica* (Laing et al., 1987). When fed to adult scallop *P. fumatus*, the poor food value of *T. suecica*, a close relative of *T. tetrahele*, was attributed to digestibility difficulties (Heasman et al., 1996). Similarly, in a broodstock conditioning study of cockle, *Tetraselmis* cells in the feces were found intact (Liu et al., 2008).

The results of the present study demonstrate that α-amylase, laminarinase, and protease activity is higher in clams fed *Thalassiosira* sp, *I. galbana*, or *C. calcitrans* than in those fed *Tetraselmis* while CMC-cellulase activity is lower and agarase activity was similar in all dietary groups.

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References


