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Spatial distribution of digestive proteinases in the midgut of the Pacific white shrimp (*Litopenaeus vannamei*) indicates the existence of endo-ectoperitrophic circulation in Crustacea

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ABSTRACT

The effect of dietary protein concentration on the spatial distribution of digestive proteinases in the shrimp *Litopenaeus vannamei* indicates the existence of endo-ectoperitrophic enzyme circulation in this species. Samples recovered from the midgut gland tissues, stomach contents, three different portions of the midgut and feces were used for quantitative and qualitative analyses of the composition and distribution of the digestive proteinases. Animals were divided into three different groups: (1) animals (controls) fed with a commercial 35% protein diet, (2) animals fed with a commercial diet supplemented with ovalbumin to a final protein concentration of 60%; (3) animals fed with an 80% protein diet. Quantitative determinations using different substrates and zymograms showed that increasing protein concentration in the diet alters the distribution of proteinases along the digestive tract. Composition of proteinases in the midgut gland, stomach contents, midgut sections and feces were similar, but not identical. Chymotrypsin and trypsin paralogues were identified in all enzyme sources in a concentration gradient along the midgut in the control shrimp, the expected distribution supporting the existence of a recycling mechanism. The occurrence of a peritrophic membrane in other Decapoda suggests that endo-ectoperitrophic circulation of digestive enzymes and nutrients may also occur in other crustaceans and also extends beyond the Insecta.

1. Introduction

Shrimp farming is the main aquaculture industrial activity in many countries, accounting for approximately 3,496,000 tons of shrimps and nearly U$ 15 billion in profits annually (FAO, 2012). Shrimps and prawns belong to the subphylum Crustacea (from the Latin, crust, hard shell), and despite their highly important ecological and economic roles, relatively few studies have been published on their digestive physiology (Icely and Nott, 1992; Brunet et al., 1994; Lemos et al., 2000). There are approximately 40,000 species of crustaceans, with 8500 species belonging to the order Decapoda, which includes the most economically important crustaceans such as lobsters, crabs and shrimps. The high diversity within this group requires detailed studies of the digestive physiology, since there is a large range of anatomical, physiological and biochemical characteristics associated with their digestive tracts. These variations enable the formation of different microenvironments, containing typical resident microbiota, spatial distribution among different enzymes participating in different stages of digestion and different nutrient absorption sites.

This paraphyletic group (Crustacea) is the closest to the Hexapoda, which includes insects and together these two groups are by far the most important of the invertebrates economically and ecologically (Edgecombe, 2010; Andrew, 2011; Giribet and Edgecombe, 2012). However, historically the knowledge of insect digestion is much greater than that of crustaceans. Most of the food that requires digestion in crustaceans and insects is comprised of polymers such as starch, cellulose, hemicelluloses and proteins. The digestive process takes place in three stages: initial, intermediate and final. Initially, a decrease in the molecular mass of the polymers occurs by the action of polymer hydrolases, such as amylases, cellulases, hemicellulases and proteinases. The

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resulting oligomers undergo hydrolysis by oligomer hydrolases such as aminopeptidases, acting on the fragments resulting from protein hydrolysis. The products of the intermediate digestion are dimers or small oligomers such as maltose, cellobiose and dipeptides derived from the hydrolysis of starch, cellulose and protein respectively. In the final phase of digestion, the dimers are cleaved by dimer hydrolases into monomers, such as maltase, cellobiase and dipeptidases (Terra and Ferreira, 1994, 2012). Any description of digestion must be correlated to the digestive compartments involved in each stage of digestion and to the corresponding enzymes. For this, enzyme determinations should be performed in each luminal compartment of the digestive tract and in the corresponding tissues. An important step in the understanding of insect digestive physiology occurred when enzymes were collected from the different compartments and their activities related to the different digestive phases (Terra et al., 1979; Terra and Ferreira, 1994, 2005, 2012).

The main enzymes involved in protein digestion in the white shrimp are serine proteinases (chymotrypsins and trypsins), which are found in different isoforms (Sellos and Van Wormhoudt, 1992; Le Moullac et al., 1996; Lemos et al., 2000; Sainz-Hernández and Murueta, 2009; del Toro et al., 2011; Garcia-Carreño et al., 2011). Proteolytic enzymes involved in digestion have been the most studied among decapods, which reflects the importance of protein in the diet. In fact, protein is the most important and most expensive component of shrimp feed and it is also considered as a limiting factor for growth (Oujifard et al., 2012). Since the purification and cloning of a chymotrypsin from *Litopenaeus vannamei* hepatopancreas carried out by Sellos and Van Wormhoudt in 1992, several papers have shown the diversity of both chymotrypsins and trypsins during development of the white shrimp and how the expression of these enzymes may vary during the developmental phases and in response to different diets (Le Moullac and van Wormhoudt, 1994; Le Moullac et al., 1996; Ezquerra et al., 1997; Lemos et al., 2000; Sainz et al., 2004a, 2004b, 2005; Sainz-Hernández and Murueta, 2009; del Toro et al., 2011). The contribution of cysteine proteinases in Decapoda digestion has received little attention, although a recent study has suggested the involvement of cathepsin B in protein digestion by *L. vannamei* (Stephens et al., 2012). Although some studies characterized these enzymes (Lemos et al., 2000; Sainz-Hernández and Murueta, 2009; del Toro et al., 2011), none of them addressed their participation in the different stages of the digestive process and little is known concerning the enzymes involved in intermediate and final phases of protein digestion in *L. vannamei* juveniles.

An anatomical structure often overlooked in studies of digestion in crustaceans is the peritrophic membrane (PM). This porous acellular chitin–protein network occurs in several groups of arthropods and coats the food bolus in the intestinal lumen. According to Terra (2001) the PM of insects evolved to enable digestive compartmentalization. The PM divides the intestinal lumen into two compartments, the ectoperitrophic space (outside the PM) and endoperitrophic space (inside the PM). In most derived insects, initial digestion occurs inside the endoperitrophic space, intermediate digestion in the ectoperitrophic space and final digestion at the surface of midgut cells. These studies led to the hypothesis of endo-ectoperitrophic circulation of digestive enzymes and nutrients (Terra et al., 1979; Terra and Ferreira, 1994). According to this hypothesis there is a recycling mechanism through which the food bolus moves within the PM toward the posterior part of the intestine, while in the ectoperitrophic space there is a flow of fluids from the posterior regions of the gut to the proximal region. When food polymeric molecules become small enough to pass through the pores of the PM together with the polymer hydrolases, they encounter a counter-flow in the ectoperitrophic space, where the intermediate and final digestion occur (Terra and Ferreira, 1994; Terra, 2001; Terra and Ferreira, 2005, 2012). According to Terra and Ferreira (1994), the compartmentalization of digestion and the existence of the endo-ectoperitrophic circulation in insects result in increased efficiency of digestion, allowing the removal of oligomers obtained in the initial digestion and the recycling of digestive enzymes (Terra, 2001; Bolognesi et al., 2008).

Recently, PM proteins have been documented in *L. vannamei* (Wang et al., 2012). However, the existence of endo-ectoperitrophic circulation of digestive enzymes was not considered and the PM in *L. vannamei* was associated with functions that could be performed by mucus, such as abrasion resistance and defense against microorganisms. In the present study we report the finding that there is a gradient distribution of proteinases in the lumen of the intestine, which can be displaced by the ingestion of increasing protein concentrations. This observation is consistent with the existence of endo-ectoperitrophic circulation in this species. Therefore, this study is the first record of endo-ectoperitrophic circulation outside of the Insecta.

2. Material and methods

2.1. Rearing of shrimp and sample preparation

The feeding experiments were performed in an indoor, closed, recirculating seawater system located at the Laboratory of Sea Crustaceans at the Federal University of Santa Catarina (LCM-UFSC). This system included a settling aquarium, a bubble bead biological filter, heat pumping aquarium and a 20 L rectangular glass aquarium. Each aquarium contained one shrimp with an initial mean mass (±SD) of 6 ± 0.01 g, supplied with aerated seawater at a rate of 1.5 L min^{-1} under natural lighting conditions. Water temperature was maintained at 25.9 ± 0.15 °C, dissolved oxygen ranged between 5 and 6 mg L^{-1} and salinity was 35 ± 0.1 g L^{-1}. The levels of total ammonia (NH_3 + NH_4^+) (0.06–0.3 mg L^{-1}), NO_2 (0.016 mg L^{-1}) and NO_3 (0.32 mg L^{-1}) were recorded weekly.

The feeding experiment was designed to determine the effect of a gradual increase in protein content in the diet on the distribution of shrimp digestive proteinases. The experimental diets were obtained with the additional supplementation of ovalbumin (Sigma) to the control diet (Ração Nutricamarão 35-high density supplied by the company Nutricil, 35% protein w/w), resulting in final protein concentrations of 60% or 80% (w/w). The commercial feed was finely dispersed with the aid of a mortar and pestle and after the addition of the appropriate amount of ovalbumin, moistened with carboxymethylcellulose gel and dispersed with a syringe and oven dried for 2 h. The diets were fed to shrimp for 6 h. Acclimatized shrimp were selected, weighed and then randomly stocked into 15 tanks. Each diet was randomly assigned to five replicate aquaria containing three shrimp each. Feeding was done by hand to apparent satiation starting at 08:00 h. During the 6-hour experimental period, feces were collected every 80 min.

Following the feeding period, *L. vannamei* specimens were immobilized on ice and dissected in cold saline. The stomach, the hepatopancreas and the midgut sections were removed. The isolated midgut was further divided in three sections (anterior-midgut, mid-midgut and posterior-midgut) with their contents or the entire midgut was dismembered into the following compartments: midgut epithelium, ectoperitrophic space and endoperitrophic space. The stomach contents, hepatopancreas tissue, midgut sections and the feces evacuated during the feeding period were homogenized in cold distilled water using a Potter–Elvehjem homogenizer and centrifuged at 10,000 *g* for 30 min at 4 °C. The supernatants were used as enzyme sources or for protein determinations.

In order to evaluate the in vivo midgut luminal pH, a universal pH indicator solution slightly modified from the original formulation proposed by Foster and Gruntfest (1937) (50 mM NaOH, bromothymol blue 0.5 g, methyl red 0.12 g, phenolphthalein 1 g, per liter of ethanol) was administered directly into the shrimp mouth cavity by injection of 50 mL of each solution. Following oral administration, the animals were kept isolated in the aquarium and the colors and dye movements in the intestinal tract were recorded using a video camera or

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