Comparative Study of Amylases from the Midgut Gland of Three Species of Penaeid Shrimp

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COMPARATIVE STUDY OF AMYLASES FROM THE MIDGUT GLAND OF THREE SPECIES OF PENAEID SHRIMP

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ABSTRACT

Amylases from the midgut gland of wild Farfantepenaeus subtilis (Pérez-Farfante, 1967) and Litopenaeus schmitti (Burkenroad, 1936), and farmed Litopenaeus vannamei (Boone, 1931) were characterized through studies on the effect of inhibitor and metallic ions, optimal pH and temperature, thermal stability and zymograms. The substrate zymogram revealed nine, eight, and seven amylolytic bands from F. subtilis, L. schmitti, adults and juveniles L. vannamei, respectively. Total amylolytic activity in the farmed shrimp was three times as high as that of the wild specimens. Amylases from all species exhibited residual activity above 85% at alkaline pH (7.0-8.0), with optimal temperature between 40 and 50°C. None of the enzymes from the species were thermally stable at temperatures above 55°C. Alpha-amylase activity in F. subtilis and L. schmitti was totally inhibited by Type I inhibitor at 50 and 100 μg.mL−1, while enzymes from adult and juvenile L. vannamei retained 43.5 ± 1.98 and 22.5 ± 0.65% of their activity, respectively, at these same concentrations. Ca2+, Cu2+, Zn2+, and Al3+ strongly inhibited amylase activity, regardless of the concentration used.

KEY WORDS: amylase characterization, Farfantepenaeus subtilis, Litopenaeus schmitti, Litopenaeus vannamei, midgut gland

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INTRODUCTION

Penaeid shrimp are among the most cultured crustaceans in the world. In Brazil, fishery statistics reveal a total shrimp production of 103 460 mt in 2006, 62.82% of which obtained by aquaculture (IBAMA, 2008). Despite the existence of native shrimp with attractive market features, such as Farfantepenaeus subtilis (Pérez-Farfante, 1967), F. brasiliensis (Latreille, 1817), F. paulensis (Pérez-Farfante, 1967), and Litopenaeus schmitti (Burkenroad, 1936), the exotic species L. vannamei (Boone, 1931) accounts for nearly all farmed shrimp in the country.

Years of study on reproduction, nutrition and feeding, genetics, physiology and diseases have provided ample information used in the development of complete diets, genetically improved postlarvae and even a grow-out technology package, which has enabled the spreading of L. vannamei beyond the Eastern Pacific coast.

The lack of basic information on the biology and, more specifically, the digestive physiology of native shrimp species has led to the adoption of improper feeds and management in growth systems, resulting in both poor feed conversion rates (2.88 to 3.44 for F. subtilis) and yields (Maia and Nunes, 2003). The formulation of specific diets depends upon information on the nutritional requirements and digestive capability of the target species.

The study of the digestive physiology of aquatic organisms is important, as the enzyme profile of a given species is closely related to its feeding habits and its capability to digest a wide range of food items (Fernández et al., 2001). Numerous studies have been conducted on the nutrition and feeding habits of penaeids, but few studies have been carried out on native Brazilian species. Some authors have addressed the semi-intensive culture of F. subtilis (Nunes et al., 1996, 1997; Nunes and Parsons, 2000), while others have investigated F. paulensis (Abreu et al., 2007; Ballester et al., 2007; Nakayama et al., 2008). Studies on digestive enzymes have been carried out on proteases in L. schmitti (Lemos et al., 2002), F. paulensis (Lemos et al., 1999; Buarque et al., 2009) and F. subtilis (Buarque et al., 2010), but little knowledge is available on carbohydrases in these species. Such carbohydrases in penaeid shrimp include alpha-amylases (Van Wornhoudt et al., 1995; Fernández et al., 1997), maltase (Omondi and Stark, 1995; Aguilar-Quaresma and Sugai, 2005), chitinase, laminarinase, and cellulase (Guillaume and Ceccaldi, 1999). Studies on carbohydrate digestion are important, because carbohydrates maybe important energy sources in shrimp diets. Carbohydrates comprise at least 25-35% of the total diet, and are included in commercial diets in order to reduce costs. It is therefore necessary to understand the profile of enzymes involved in this process.

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The aim of the present study is to characterize amylases found in the midgut gland of two native penaeids (F. subtilis and L. schmitti) and the exotic species L. vannamei.

**Materials and Methods**

**Materials**

Specimens of F. subtilis (Total Weight, TW: 12.77 ± 0.78 g) and L. schmitti (TW: 23.30 ± 2.89 g) were obtained from a commercial fishery. Adult and juvenile specimens of L. vannamei (TW: 25.17 ± 1.47 g and 11.13 ± 0.48 g, respectively) were obtained from a commercial farm in the State of Pernambuco in Brazil. All reagents used in the enzyme assays were of analytical grade, purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

**Enzyme Extraction and Total Soluble Protein Determination**

Eighteen specimens of each species were transported alive to a commercial shrimp hatchery, where they were weighed and sacrificed by thermal shock. The midgut gland was immediately removed, weighed (0.59 ± 0.16 g – L. schmitti; 0.79 ± 0.22 g and 0.46 ± 0.05 g – L. vannamei; 0.39 ± 0.04 g – F. subtilis), placed in cryotubes and transported to the Enzymology Laboratory of the Universidade Federal de Pernambuco (Brazil). Six groups of three glands from each species were homogenized (40 mg/mL) in chilled 10 mM Tris-HCl buffer, pH 7.5, with 0.15 M NaCl, using a Potter-Elvehjen tissue homogenizer (Bodine Electric Company – Chicago, USA) at 40 to 50 rpm for 3 min at 4°C. Homogenates were centrifuged at 10,000 × g for 20 min at 4°C in order to remove lipids and tissue debris and the supernatants (crude enzyme extract) were stored at −20°C for further utilization. The total soluble protein was determined as described by Bradford’s methodology (1976), using bovine serum albumin as standard solution sample.

**Amylase Enzyme Assays**

Total amylase activity was determined using the Bernfeld (1955) method, with 2% (w/v) starch solution as substrate. The reaction consisted of 60 μL of crude extract, 375 μL of starch solution and 375 μL of 10 mM phosphate buffer (pH 7.5). After 10 min of incubation at 37°C, 100 μL of this mixture was added to 1 mL of a 3.5-dinitrosalicic acid (DNSA) solution and maintained in a boiling water bath for 10 min in order to stop the reaction. Absorbance was recorded at 570 nm. Blanks for substrate and enzyme were similarly prepared, except that 10 mM phosphate buffer replaced the substrate or enzyme extract. All assays were carried out in quadruplicate. One unit of amylase activity was expressed as mg of maltose released at 37°C per min per mg of protein.

**Total Proteinase Activity Assays**

Total proteinase activity was assayed using azocasein as substrate in a microcentrifuge tube. Triplicate samples of each enzyme extract (30 μL) were incubated with 10 g.L⁻¹ azocasein (50 μL) dissolved in 0.1 M Tris-HCl (pH 8.0, for 60 min at 25°C) (Bezerra et al., 2005). Next, 100 g.L⁻¹ trichloroacetic acid (240 μL) was added to stop the reaction and the mixture was centrifuged at 8000 × g for 5 min. The supernatant (70 μL) was mixed with 1 M NaOH (130 μL) and absorbance was measured in a microtiter plate reader (Bio-Rad 680) at 450 nm against a similarly prepared blank (with 9 g.L⁻¹ of NaCl in place of the crude extract). Proteinase activity was expressed as units per mg of protein. One unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyze azocasein and produce a change in absorbance of 0.001 mL⁻¹.min⁻¹.

**Effect of pH and Temperature on Amylase Activity**

The effects of pH and temperature on amylase activity were evaluated as described above, using a series of different 10 mM pH values (Citrate-phosphate: pH 5 to 7.5; Tris-HCl: pH 7.5 to 9.0 and Glycine-NaOH: pH 9.0 to 11.5) and temperatures ranging from 25°C to 70°C. Thermal stability was evaluated by assaying enzyme activity at 25°C after pre-incubation for 30 min at temperatures ranging from 25°C to 70°C (Bezerra et al., 2005).

**Inhibition Assays**

The effect of Type I α-amylase inhibitor (Sigma A1520) from Triticum aestivum on amylase activity was recorded. Equal volumes (60 μL) of enzyme extract and inhibitor at two different concentrations (50 and 100 μg.mL⁻¹) were incubated at 25°C for 20 min. Next, 375 μL of 20 g.L⁻¹ starch solution and 315 μL 10 mM phosphate buffer, pH 7.5, were added and the mixture was incubated for 10 min at 37°C. Amylase activity was evaluated as previously described and the results were expressed as the percentage of the activity recorded in control tubes without inhibitor. Commercial α-amylase from Bacillus subtilis was also submitted to the inhibitor.

**Effect of Metallic Ions**

The effect of metallic ions on amylase activity was evaluated following the method adapted from Souza et al. (2007). The methodology followed the same steps described in inhibition assays, except that inhibitor was replaced by different ion solutions (1, 5 and 10 mM). The ions employed were Ca²⁺, Cd²⁺, Hg²⁺, Al³⁺, Cu²⁺ and Zn²⁺.

**Zymograms**

Zymograms were carried out according to the modified methodology described by Fernández et al. (2001). Enzyme preparations (50 μg of protein) and 0.006 U of α-amylase of Bacillus subtilis (used as reference) were applied to a 3% (w/v) separating gel. After electrophoresis performed at 4°C and a constant current of 15 mA per gel, the gel was immersed in 2.5 mL.L⁻¹ (100 mL) Triton X-100 in 0.1 M Tris-HCl (pH 8.0, for 30 min at 4°C) to remove the SDS. Triton X-100 was removed by washing the gel three times with 100 mL of 0.1 M Tris-HCl buffer, pH 8.0. Next, the SDS-free, Triton X-100-free gel was incubated with a starch solution (2% w/v) containing 10 mM phosphate buffer, pH 8.0, and 1 mM CaCl₂ for 60 min at 37°C to allow the digestion of starch by the active fractions. Finally, the gel was washed with distilled water and stained with an iodine/KI solution (100 g.L⁻¹) for 5 minutes. The gel was washed with distilled water to stop the reaction and then with an acetic acid solution (130 g.L⁻¹).
Table 1. Proteolytic and amylolytic activity in the midgut gland of *F. subtilis*, *L. schmitti* and *L. vannamei*, using azocasein and starch as substrates; amylase:protease ratio is also displayed. Values are shown as mean ± standard deviation (SD) of triplicates of six crude extracts obtained from three midgut glands each; different italic letters (in lines) denote statistical difference (*P* < 0.05); enzyme activity expressed as U.mg⁻¹ of protein.

<table>
<thead>
<tr>
<th></th>
<th><em>F. subtilis</em></th>
<th><em>L. schmitti</em></th>
<th><em>L. vannamei</em> adult</th>
<th><em>L. vannamei</em> juvenile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteolytic activity</td>
<td>6.26 ± 0.67ᵃ</td>
<td>4.60 ± 0.75ᵇ</td>
<td>7.43 ± 0.65ᶜ</td>
<td>5.08 ± 0.71ᵇ</td>
</tr>
<tr>
<td>Amylolytic activity</td>
<td>0.62 ± 0.05ᵇ</td>
<td>0.51 ± 0.04ᵇ</td>
<td>1.92 ± 0.06ᶜ</td>
<td>1.13 ± 0.07ᵈ</td>
</tr>
<tr>
<td>Amylase:protease</td>
<td>0.10 ± 0.01ᵇ</td>
<td>0.11 ± 0.02ᵇ</td>
<td>0.26 ± 0.01ᶜ</td>
<td>0.24 ± 0.02ᵃ</td>
</tr>
</tbody>
</table>

**RESULTS**

Table 1 displays the proteolytic activity, amylase activity and amylase:protease ratio of the specimens of *F. subtilis*, *L. schmitti* and juvenile *L. vannamei*. Adult specimens of *L. vannamei* showed greater protease and amylase activity (*P* < 0.05), while *L. schmitti* had the lowest values.

The assays revealed that amylases of all species exhibited maximal enzyme activity at pH values ranging from 7.0 to 8.0 (Fig. 1). Figure 2 displays the effect of temperature on amylase activity. The enzyme activity reached maximal values at 40°C for *L. schmitti* and juvenile *L. vannamei*, 45°C for *F. subtilis*, and 50°C for adult *L. vannamei*. *F. subtilis* and *L. vannamei* exhibited up to 85% residual activity from 30°C to 55°C, whereas *L. schmitti* had a narrower range (35°C to 45°C). Thermal stability of amylases from the three species is shown in Fig. 3. Enzymes from *L. vannamei* exhibited no remarkable change (residual activity ≥ 80%) up to 45°C. Amylase activity in *L. schmitti* and *F. subtilis* increased even after the 30 min heat treatment from 40°C to 45°C. Enzymes from *F. subtilis* exhibited considerable residual activity (75%) at 55°C, but all specimens were extremely affected after this temperature.

A strong inhibitory effect was observed in enzyme activity of both shrimp, *F. subtilis* and *L. schmitti*, when Type I amylase inhibitor was used at concentrations of 50 and 100 μg inhibitor. However, *L. vannamei* amylases were more resistant to this inhibitory effect. Adults and juveniles of this species retained 43.5% ± 1.98 and 22.5% ± 0.65 of amylolytic activity, respectively, at the same inhibitor concentrations. This inhibitor demonstrated residual activity of 41.3±1.79 at 50 μg.mL⁻¹ and no activity at 100 μg.mL⁻¹ on the commercial α-amylase from *Bacillus subtilis*.

The effect of ions on amylolytic activity is shown in Table 2. Enzymes from all species were activated by Ca²⁺ at 1 mM, but were inhibited at 5 and 10 mM. All other ions demonstrated an inhibitory effect at all concentrations employed.

Crude enzyme extracts from the three species were studied through zymography (Fig. 4). The zymogram revealed nine bands with amylase activity for *F. subtilis*, eight bands for *L. schmitti*, 10 bands for adult *L. vannamei* and seven bands for juvenile *L. vannamei*.

**DISCUSSION**

The midgut gland in crustaceans is responsible for the synthesis of enzymes as well as food digestion and nutrient absorption. Synthesized enzymes are released into its lumen and flow to the foregut to initiate the hydrolysis of the ingested food (Guillaume and Ceccaldi, 1999; Verri et al., 2001). Dall and Moriarty (1983) and Bickmeyer et al. (2008) report that the pH in the gastric fluid and midgut gland of crustaceans ranges from 5.0 to 7.0 and from 4 to 5.5, respectively. Regardless of the enzyme type, whether proteases (Fernández Gimenez et al., 2001, 2002), carbohydrases (Omondi and Stark, 1995; Figueiredo et al.,
or metalorganic molecules (coenzymes) or simply an additional chemical component (cofactor), such as inorganic ions (Nelson and Cox, 2005). According to Wigglesworth and Griffith (1994), the secondary and tertiary stability of α-amylase molecules in penaeid shrimp requires calcium ions. A number of metallic ions, such as Ca$^{2+}$, Ba$^{2+}$, Ag$^{3+}$, Mn$^{2+}$, Hg$^{2+}$ and Cu$^{2+}$, are important, as their presence or absence may regulate enzyme activity (Dutta et al., 2006). In the present study, amylases from the penaeids analyzed were strongly inhibited by Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$, Cu$^{2+}$ and Al$^{3+}$ at concentrations of 1, 5 and 10 mM. Calcium enhanced amylase activity only at a concentration of 1 mM. It seems that the effect of metallic ions on the enzyme is species-specific, as suggested by the conflicting results reported in the literature. While Zn$^{2+}$ inhibited shrimp amylase activity by more than 50% in the present study, it has been reported to have no effect on Heliodiapomus viidus (Gurney, 1916), a crustacean copepod (Dutta et al., 2006). Figueiredo et al. (2001) report a 342% enhancement in amylase activity in the red-claw crayfish Cherax quadriscarinus (von Martens, 1868) when the enzyme was incubated with 15 mM CaCl$_2$. These studies are important, as some ions are commonly included in mineral and vitamin blends and may affect food digestion. Moreover, effluents could negatively influence shrimp farming in industrial regions. The presence of metal ions as environmental contaminants can significantly reduce amylase activity, consequently reducing the dietary carbohydrate absorption and affecting energy balance, which leads to a decline in shrimp growth rates.

Amylase in the midgut gland of *F. subtilis*, *L. schmitti*, and *L. vannamei* exhibited maximal activity between 40°C and 50°C, as previously recorded for other crustaceans and fish (Mayzaud, 1985; Fernández et al., 2001; Pavsavici et al., 2004). Although peak amylase activity was observed in this temperature range, the enzyme from *F. subtilis* and *L. vannamei* maintained over 80% of its activity between 30°C and 55°C, whereas that from *L. schmitti* was proved to be more thermally sensitive, as residual activity was about 40% and 60% at 30°C and 55°C, respectively. Omondi and Stark (1995) studied the simultaneous effect of pH and temperature on amylase activity in Fenneropenaeus indicus (Milne Edwards, 1837) and found an enhancement of about 50% at pH 6.8 when the temperature was increased from 22°C to 37°C, while the inverse was recorded for *L. vannamei*.

Table 2. Effect of different ions’ concentrations (1, 5 and 10 mM) on amylase activity in *F. subtilis*, *L. schmitti* and *L. vannamei*. Activation: 1 + (1-25%); 2 + (26-50%); inhibition: 1 – (1-25%); 2 – (26-50%); 3 – (51-75%); 4 – (76-100%).

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<th>Ion</th>
<th><em>F. subtilis</em></th>
<th><em>L. schmitti</em></th>
<th><em>L. vannamei</em> adult</th>
<th><em>L. vannamei</em> juvenile</th>
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<tr>
<td></td>
<td>1 mM</td>
<td>5 mM</td>
<td>10 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>1 +</td>
<td>1 –</td>
<td>2 –</td>
<td>2 +</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>3 –</td>
<td>4 –</td>
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<tr>
<td>Cd$^{2+}$</td>
<td>1 –</td>
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<td>Ag$^{3+}$</td>
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<td>Hg$^{2+}$</td>
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<td>Cu$^{2+}$</td>
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Regarding thermal stability, amylase from *F. subtilis* was more resistant and maintained 75% of its activity at 55°C. At this temperature, either very low or no activity was recorded for the other two species, suggesting that the enzyme must have been denaturated.

Amylolytic activity from the midgut gland of reared adult *L. vannamei* was three times as high as that observed for the wild *L. schmitti* and *F. subtilis*. Under farming conditions, ponds are fertilized to stimulate natural productivity and shrimps are commonly fed with diets consisting of 30% to 37% crude protein content. This fact, together with probable genetic characteristics, could explain the differences found. According to Johnston and Freeman (2005), digestive enzyme activity is closely related to dietary components. Thus, high proteinase, carbohydrase and lipase activity reflects a diet rich in protein, starch or cellulose and lipids, respectively (Johnston and Yellowlees, 1998; Johnston, 2003).

In the present study, the amylase:protease ratio was similar between *L. schmitti* and *F. subtilis*, though the value was two times as high in *L. vannamei*. Although all shrimp species have opportunistic omnivorous habits, *F. subtilis* and *L. schmitti* have a preference for animal protein (Nunes and Parsons, 2000). Studies on the digestive enzymes of aquatic organisms postulate that amylase activity depends on the natural diet of the species, with herbivorous and omnivorous animals exhibiting greater activity than carnivorous animals (Hidalgo et al., 1999; Johnston and Freeman, 2005).

Zymograms represent a useful tool for the analysis of differences in digestive enzymes among different species, allowing the identification of active enzyme forms. In the present study, a large number of isoforms were identified in all shrimp species (adult *L. vannamei* – 10; juvenile *L. vannamei* – 7; *L. schmitti* – 8 and *F. subtilis* – 9). Polymorphism of α-amylase is a common occurrence among vertebrates and invertebrates (insects) and has previously been reported specially for crustaceans (Van Wormhoudt and Favrel, 1988; Le Moullac et al., 1996; Perera et al., 2008), fish (Fernández et al., 2001) and mollusks (Huvet et al., 2008). Differences

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**Fig. 4.** Zymogram of amylase activity from midgut gland of *F. subtilis*, *L. schmitti*, *L. vannamei* 25 g and *L. vannamei* 11 g; α-amylase from *Bacillus subtilis* used just as reference.
in the number of crustacean isoforms have been associated with differences between populations or in gene expression during intermoult cycles. Some authors suggest that amylase production is not related to food intake but is family-specific (Chakrabarti et al., 1995). Others suggest that amylase activity is influenced by diet composition to some extent. According to Simon (2009), significant differences between feeding groups under natural and formulated diets were observed concerning the activity of digestive enzymes, including amylase, of cultured juvenile spiny lobster, Jasus edwardsii (Hutton, 1875). Le Mouillac et al. (1996) observed differences in the amylase activity in L. vannamei feeding with different protein sources. Moreover, the authors observed a decrease in amylase activity and disappearance of one amylase isoform, when the amount of dietary casein was increased. Chávez-Calvillo et al. (2010) observed changes in expression of several genes in hepatopancreas and muscle tissues of L. vannamei when promoting a shift in diet from an animal protein source to plant protein source and vice versa, suggesting that these changes may be related to metabolic and physiological adjustments to assimilate the type of food provided. In fact, the ability to digest different food items is related to the enzyme profile of a given species. The presence of numerous amylase isoforms may be an ecological advantage and may indicate that species are able to benefit more from carbohydrates in the diet. The amylolyticzymogram revealed that, despite the conservation of some isoforms among the shrimp species studied, L. vannamei (cultivated animals) exhibited stronger amylolytic bands than the two wild species. This may be due to better the adaptation of L. vannamei to commercial diets, which generally have higher carbohydrate content than the natural diet. Moreover, the differences observed in the amylolytic profile between the two groups of L. vannamei (adults and juveniles) are evidence that indicates the adaptation capacity of this species to different diets. This adaptation may be a characteristic of the species or may have been obtained during the process of domestication. However, this aspect cannot be completely assessed for the species studied, mainly because there are no wild specimens of L. vannamei in Brazil. Moreover, the process of domestication of L. schmitt and F. subtilis is not yet complete, and there is therefore no commercial cultivation of these species. In conclusion, the results obtained in the present study may pave the way for future research on the digestive physiology of these species.

ACKNOWLEDGEMENTS

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