The spined soldier bug, Podisus maculiventris, is a generalist predator of insects and has been used in biological control. However, information on the digestion of food in this insect is lacking. Therefore, we have studied the digestive system in P. maculiventris, and further characterized carbohydrases in the digestive tract. The midgut of all developmental stages was composed of anterior, median, and posterior regions. The volumes of the anterior midgut decreased and the median midgut increased in older instars and adults, suggesting a more important role of the median midgut in food digestion. However, carbohydrase activities were predominant in the anterior midgut. In comparing the specific activity of carbohydrases, \( \alpha \)-amylase activity was more in the salivary glands (with two distinct activity bands in zymograms), and glucosidase and galactosidase activities were more in the midgut. Salivary \( \alpha \)-amylases were detected in the prey hemolymph, demonstrating the role of these enzymes in extra-oral digestion. However, the catalytic efficiency of midgut \( \alpha \)-amylase activity was approximately twofold more than that of the salivary gland enzymes, and was more efficient in digesting soluble starch than glycogen. Midgut
α-amylases were developmentally regulated, as one isoform was found in first instar compared to three isoforms in fifth instar nymphs. Starvation significantly affected carbohydrase activities in the midgut, and acarbose inhibited α-amylases from both the salivary glands and midgut in vitro and in vivo. The structural diversity and developmental regulation of carbohydrases in the digestive system of P. maculiventris demonstrate the importance of these enzymes in extra-oral and intra-tract digestion, and may explain the capability of the hemipteran to utilize diverse food sources. © 2014 Wiley Periodicals, Inc.

Keywords: carbohydrase; midgut; salivary gland; starch; glycogen; inhibition

INTRODUCTION

Insects of the order Hemiptera are mainly characterized by their piercing-sucking mouthparts. The mouthparts are used to suck plant sap (e.g., in aphids), liquefied plant tissue (e.g., phytophagous pentatomids), hemolymph, and liquefied internal organs of prey (e.g., in assassin bugs and zoophagous pentatomids). Some predatory true bugs feed on vertebrate blood while others are voracious predators of invertebrate animals especially insects (e.g., caterpillars) and grubs (Coast et al., 2011). The spined soldier bug, Podisus maculiventris (Say) (Hemiptera: Pentatomidae), is a generalist predatory bug of over 100 insect species (De Clercq, 2000). Podisus maculiventris is an endemic predator in North America and has been used in Europe since 1997, mainly for biological control of caterpillar pests in greenhouses (De Clercq et al., 1998; De Clercq, 2000). However, its polyphagous characteristic may carry environmental risks when the insect is used in areas where it is not native (De Clercq, 2002; Van Lenteren et al., 2003).

Food digestion is a process in which nonabsorbable and large food molecules are broken into small molecules to be absorbed by epithelial cells of the alimentary canal. Digestion of large molecules, such as protein and carbohydrate polymers, occurs in three enzymatic phases. The first stage is conversion of polymers into oligomers. Oligomers are cleaved into dimers during the second phase and in the final stage, monomers are obtained (Terra and Ferreira, 1994). Usually, all three phases occur inside the insect midguts. However, it is not completely understood to which extent these stages occur inside the alimentary canal of insects (e.g., predatory bugs) with extra-oral digestion. At least, 79% of terrestrial arthropods use extra-oral digestion to consume food (Cohen, 1995). This action allows the predators to feed on large prey and enables them to hunt insects with rigid and hard cuticles (Cohen, 1995). Extra-oral digestion maximizes the efficiency of food consumption in predatory insects (Cohen, 1995). The enzymes involved in extra-oral digestion usually come from salivary glands or midgut and are diverse among various insect orders. For example, among coleopterans, pre-oral digestion is carried out by enzymes which come from the midgut (Cheeseman and Gillett, 1987) and at least in the case of the Pyrarinus termittilluminans (Coleoptera: Elateridae) (Colepiccolo-Neto et al., 1986), extra-oral digestion includes the first and the second digestion phases. Extra-oral digestion among hemipterans is reported to occur under the action of salivary enzymes (Cohen, 1993). Predatory true bugs typically introduce toxins to debilitate the prey followed by digestive enzymes that liquefy the prey within its own exoskeleton (Klowden, 2007).
Complex polysaccharides, such as glycogen (in animals) and starch (in plants), are broken down into simple sugars by digestive carbohydrases (Vatanparast and Hossein-naveh, 2010). α-Amylases (α-1,4-glucan-4-glucanohydrolases) are a group of glycoside hydrolases widely distributed in bacteria, fungi, plants, and animal tissues that catalyze the hydrolysis of α-D-(1,4)-glucan linkages in both starch and glycogen (Janecek, 1997; MacGregor et al., 2001). The presence of α-amylase has been investigated in digestive systems of many insects including members of Orthoptera, Hymenoptera, Hemiptera, Diptera, Lepidoptera, and Coleoptera (Terra and Ferreira, 1994). In heteropteran predators, activities of α-amylase and proteinases (as trophic enzymes) are adjusted according to diet (Cohen and Hendrix, 1994) and digestive enzymatic profiles can be diverse in different developmental stages (Stamopoulos et al., 1993).

Activity of carbohydrases such as α-amylase, galactosidases, and glucosidases has been shown in the gut and salivary glands of *P. maculiventris* (Cohen, 1990; Stamopoulos et al., 1993) and *P. nigrispinus* (Fialho et al., 2012). Cohen (1990) revealed the presence of α-amylase in the salivary glands and the gut of *P. maculiventris*, and showed that extra-oral secretions only come from the salivary glands. Stamopoulos et al. (1993) conducted a number of semiquantitative assays by enzymes deriving from the gut of *P. maculiventris*. However, detailed biochemical characterizations and midgut compartmentalization of carbohydrases have not been carried out. In the current study, more details on the structure of digestive system (midgut and salivary glands), biochemical characteristics of digestive carbohydrases (α-amylase, glucosidases, and galactosidase), and the inhibitory potential of a nonproteinaceous α-amylase inhibitor acarbose have been investigated in the spined soldier bug, *P. maculiventris*, to gain a better understanding of the digestive physiology of this insect.

**MATERIALS AND METHODS**

**Insects and Rearing**

All developmental stages of *P. maculiventris* were reared on the greater wax moth larvae, *Galleria mellonella* in a growth chamber at 24 ± 1°C with a photoperiod of 16:8 (light: dark cycle) and 70 ± 5% relative humidity. The greater wax moth larvae were fed and maintained on old honeycomb black wax under the laboratory conditions of 28 ± 2°C with a photoperiod of 16:8 (light: dark cycle) and 50 ± 5% relative humidity.

**Morphological and Biochemical Characteristics of Midgut and Salivary Glands**

Adults and all nymphal instars of *P. maculiventris* were dissected in a saline solution (0.15M NaCl), and different parts of their midguts (anterior midgut, median midgut, posterior midgut) and salivary glands were removed. The volumes of different parts of the midgut were measured according to Silva and Terra (1994). Briefly, the whole midgut drawings were prepared using a stereomicroscope equipped with a drawing tube. The volumes were mathematically calculated from the drawings. The volume of gut contents was assumed to be identical with those of the corresponding gut section. The midguts and the salivary glands were separately pooled (at least in three replicates), homogenized, centrifuged, and their pH values were measured using a pH indicator paper (PANPEHA, Riedel-de Haen, pH range 0–14) and a pH electrode (Sartorius, PY-P10).
Enzyme Preparation

Enzyme extracts of the midguts and the salivary glands of adults and different nymphal instars, the eggs and oocytes were prepared according to Cohen (1993) with some modifications. Briefly, 5-day-old adults were randomly selected from the colony and were chilled for 1 h on ice to render them immobile. The midguts, the salivary glands and oocytes were removed and different parts of the midguts were separated. The separated tissues were rinsed in ice-cold distilled water and homogenized (in distilled water) using a precooled hand-held glass grinder for 3 min. The homogenates were separately transferred to 1.5 ml microtubes and centrifuged at 15,000 × g for 20 min at 4°C. The resulted supernatants were separately pooled and stored at −20°C for further analyses. Protein concentration was determined using the method of Lowry et al. (1951) with bovine serum albumin as the standard.

α-amylase Activity Assay

α-amylase activity was measured according to Bernfeld (1955) with some modifications. The reagent dinitrosalicylic acid (DNS) and 1% soluble substrates (starch and glycogen) were used in the assays. Ten microliters of the enzyme extract were incubated for 30 min at 35°C with 100 μl buffer and 10 μl soluble substrate. The reaction was stopped by addition of 100 μl DNS and heated in boiling water for 10 min. In the blanks, the enzyme extract was added to the reaction mixture after DNS treatment. The absorbance of the mixture was measured at 540 nm. Impacts of pH were determined according to Ramzi and Hosseininaveh (2010) with some modifications. The effect of pH was determined using a universal buffer (40 mM citrate–phosphate–borate) at a broad pH range (3 to 11). Hemolymph α-amylase activity was also measured in the predator-captured larvae of G. mellonella. Adult of P. maculiventris fed on the larvae for 5 min. The collected hemolymph was extracted and centrifuged for 1 min at 15,000 × g and 4°C, and α-amylase activity was determined. Noncaptured larvae were used as the controls.

α-amylase Kinetic Studies

Kinetic studies were carried out for α-amylases using six different concentrations (0.025, 0.5, 0.1, 0.25, 0.75, 1%) of the soluble substrates (starch and glycogen). The apparent Michaelis–Menten constant (K_M) and apparent maximum velocity (V_max) were estimated by nonlinear regression.

α-amylase Zymogram Analysis

The activity of amylases present in the crude homogenates of the midgut and the salivary glands was visualized using non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (non-reducing SDS–PAGE). The electrophoresis was performed in a 10% w/v resolving and 4% w/v stacking gels. The sample buffer contained 25% stacking buffer (0.5 M Tris–HCl; pH 6.8), 20% w/v glycerol, 2% w/v SDS, 0.005% w/v bromophenol blue, but no reducing agents and no boiling. After electrophoresis, the gels were washed in phosphate buffer (pH 6, 40 mM) containing 1% v/v Triton X-100 and were incubated in phosphate buffer (pH 6, 40 mM) containing 1% soluble starch, 2 mM CaCl_2 and 10 mM NaCl for 1.5 h. The gels were rinsed with distilled water and were incubated with a solution

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of 10 mM I$_2$ and 14 mM KI to stop the action and to reveal the unreacted starch. Areas bearing $\alpha$-amylase activity appeared as light bands against a dark background.

$\alpha$-Amylase Inhibition Assay

The in vitro and in vivo impact of the inhibitor acarbose was determined on $\alpha$-amylase activity of the midgut and the salivary glands. In the in vitro assays, $\alpha$-amylase activity of the enzyme extract from the tissues was measured in the presence of the inhibitor. In the in vivo assays, the inhibitor was injected to the body of the prey and then the enzyme activity was determined. For in vitro assay of the inhibitor, the enzyme extracts were pre-incubated with acarbose at different concentrations (0.0625%, 0.1, 25%, 0.25%, 0.5, 1% and 2% w/v) for 30 min. Residual $\alpha$-amylase activity was estimated by the method described in the section $\alpha$-Amylase activity assay. In the in vivo assays, 4 ml of acarbose solution were injected to the body of larvae of $G$. mellonella at concentrations of 0.444 and 0.014 ng/mg larval body weight in five replicates. Distilled water was injected as the control. The starved bugs were fed with the inhibitor-treated and the control larvae. After 7 h, the bugs were dissected and $\alpha$-amylase activity from the midgut was measured. In-gel assays of the effect of acarbose on $\alpha$-amylase activity were performed according to the section $\alpha$-amylase zymogram analysis.

Glucosidase and Galactosidase Assay

Glucosidase and galactosidase activities were determined against the substrates p-nitrophenyl-$\alpha$-$D$-glucopyranoside (pNP$\alpha$Glu, 5 mM, for $\alpha$-glucosidase), p-nitrophenyl-$\beta$-$D$-glucopyranoside (pNP$\beta$Glu, 5 mM, for $\beta$-glucosidase), p-nitrophenyl-$\alpha$-$D$-galactopyranoside (pNP$\alpha$Gal, 5 mM, for $\alpha$-galactosidase), and p-nitrophenyl-$\beta$-$D$-galactopyranoside (pNP$\beta$Gal, 5 mM, for $\beta$-galactosidase) according to Razavi Tabatabaei et al. (2011). Reaction mixtures included 80 $\mu$l citrate-phosphate buffer (40 mM, pH 6), 10 $\mu$l enzyme extract, and 5 $\mu$l substrate, which were incubated at 35°C for 30 min. The reaction was stopped with 100 $\mu$l of NaOH (2 M) and released p-nitrophenols were determined at 405 nm after 5 min. In the blanks, the enzyme extract was added to the reaction mixture after NaOH treatment.

Effect of Starvation on Carbohydrase Activities

Adults of the predatory bug were starved for 0, 8, 16, 24, 32, 40, 48, and 56 h, and were fed on the larval $G$. mellonella after 40 h. The adults were dissected during the aforementioned time intervals, and the activity of carbohydrases ($\alpha$-amylase, $\alpha$-glucosidase, $\beta$-glucosidase, $\alpha$-galactosidase, and $\beta$-galactosidase) was measured according to the previous sections.

Statistics and Data Analysis

All assays were conducted at least in triplicate, and statistical analyses were performed using the software Statgraphics Plus. Regression analysis, data fitting and plotting were performed using SigmaPlot 11.0 (Systat Software, San Jose, CA). Dendrograms of different parts of digestive system and developmental stages of the predatory bug were constructed after cluster analysis by Ward’s method (Ward, 1963) using PASW Statistics 18 (SPSS Inc., Chicago, IL).
RESULTS

Midgut Structure

The results showed that the midgut of *P. maculiventris*, as in other predatory pentatomids, is morphologically divided into three distinct regions (Fig. 1A). The first part of the midgut (anterior midgut) was dilated, the second part (median midgut) was a narrow and long tube, and the third part (posterior midgut) was slightly dilated in all developmental stages (first nymphal instar to the adult). The greatest increase in length and volume was observed in the median midgut by comparison with other parts (Table 1, Fig. 2). The volume of the median midgut significantly increased in older instars and adult. However, a notable relative decrease in the volume of the anterior midgut was observed in older instars and adult. An increased volume of the posterior midgut was also determined in older instars and adult.

The dendrograms based on the volume and length of the anterior, median, and posterior midgut of the predatory bug are shown in Fig. 1A. The dendrogram according to the volumes showed two distinct clusters labeled “a” and “b” (including subclusters b₁ and b₂). Cluster “a” included adult and fifth nymphal instar, and cluster “b” consisted of subclusters b₁ (the fourth nymphal instar) and b₂ (the first, second, and third nymphal instars). Grouping within clusters reflect similar volumes of different parts of the midgut. According to the cluster analysis, adult and the fifth nymphal instars are different from the other developmental stages in the volume of the parts of the midgut. The dendrogram based on the length of the anterior, median, and posterior midgut showed two distinct
<table>
<thead>
<tr>
<th>Stage</th>
<th>Anterior midgut (AM)</th>
<th>Median midgut (MM)</th>
<th>Posterior midgut (PM)</th>
<th>Whole midgut</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (mm)</td>
<td>Volume (μl)</td>
<td>Length (mm)</td>
<td>Volume (μl)</td>
</tr>
<tr>
<td>Nymph 1</td>
<td>1.6 ± 0.4</td>
<td>0.49 ± 0.09</td>
<td>1.5 ± 0.05</td>
<td>0.06 ± 0.006</td>
</tr>
<tr>
<td>Nymph 2</td>
<td>2.1 ± 0.5</td>
<td>1.64 ± 0.07</td>
<td>2.7 ± 0.14</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>Nymph 3</td>
<td>2.2 ± 0.15</td>
<td>2.48 ± 0.11</td>
<td>5.3 ± 0.18</td>
<td>1.33 ± 0.08</td>
</tr>
<tr>
<td>Nymph 4</td>
<td>3.1 ± 0.05</td>
<td>4.77 ± 0.8</td>
<td>12.5 ± 0.28</td>
<td>4.02 ± 0.31</td>
</tr>
<tr>
<td>Nymph 5</td>
<td>3.5 ± 0.28</td>
<td>8.91 ± 0.23</td>
<td>14.6 ± 0.33</td>
<td>11.46 ± 0.12</td>
</tr>
<tr>
<td>Adult</td>
<td>3.73 ± 0.14</td>
<td>9.89 ± 0.32</td>
<td>15 ± 0.06</td>
<td>11.77 ± 0.21</td>
</tr>
</tbody>
</table>
clusters labeled “a” (including subclusters a₁ and a₂) and “b.” Cluster “a” included subclusters a₁ (adult) and a₂ (the fifth and fourth nymphal instar), and cluster “b” consisted of the first, second, and third nymphal instars. Grouping within clusters reflects similar lengths of the different parts of the midgut. According to the cluster analysis, the fourth and fifth nymphal instars are different in their length of the parts of the midgut from adults.

Salivary Gland Structure

The salivary glands of the spined soldier bug included two main anterior and posterior lobes and accessory glands (Fig. 1B). The salivary glands in male adults were bigger than those from female adults.

pH Value of the Digestive System

Using pH indicator paper, the pH value of the midgut and the salivary glands was 6 ± 0.44 and 6 ± 0.28, respectively. The pH of the midgut and the salivary glands was obtained 6 ± 0.2 with a pH electrode.

α-Amylase Activity

The midgut α-amylases of P. maculiventris showed an optimal pH of 6.0 against the substrates starch and glycogen (Fig. 3A). There were no considerable differences in amylase
Figure 3. Effect of pH on α-amylase (A), and glucosidase and galactosidase (B) activity from the midgut of *Podisus maculiventris* against the substrate starch and glycogen. Each point represents means of triplicate measurements and the vertical bars are standard errors of the means.

Activity toward starch and glycogen at different pHs. Amylolytic activity of the salivary glands and the different parts of the midgut is shown in Table 2. α-Amylase activity was more in the salivary glands than that in the anterior, median, and posterior midgut against the substrate starch. The same situation was observed against the substrate glycogen with the exception of anterior midgut, which showed more enzyme activity than the salivary glands. Low α-amylase activity was also determined in the rectum. Among all developmental stages including oocytes, the first to the fifth nymphal instars and adults, maximum
Table 2. Amylolytic Activity (Mean ± SE) in Podisus maculiventris and Galleria mellonella Larvae

<table>
<thead>
<tr>
<th>Insect</th>
<th>Substrate</th>
<th>Enzyme source</th>
<th>Total activity (mU)</th>
<th>Specific activity (mU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. maculiventris</strong></td>
<td>Starch</td>
<td>SGC</td>
<td>494 ± 20</td>
<td>191 ± 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AM</td>
<td>270 ± 17</td>
<td>138 ± 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MM</td>
<td>144 ± 9.5</td>
<td>55 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PM</td>
<td>59 ± 5</td>
<td>23 ± 1</td>
</tr>
<tr>
<td></td>
<td>Glycogen</td>
<td>SGC</td>
<td>326 ± 16</td>
<td>126 ± 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AM</td>
<td>431 ± 12</td>
<td>185 ± 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MM</td>
<td>384 ± 23</td>
<td>102 ± 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PM</td>
<td>303 ± 17</td>
<td>100 ± 5</td>
</tr>
<tr>
<td></td>
<td>Starch</td>
<td>Rectum</td>
<td>51 ± 4</td>
<td>20 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oocytes</td>
<td>33 ± 3</td>
<td>13 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Egg</td>
<td>4 ± 0.03</td>
<td>1 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nymph 1</td>
<td>45 ± 2</td>
<td>17 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nymph 2</td>
<td>70 ± 4</td>
<td>38 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nymph 3</td>
<td>91 ± 6</td>
<td>35 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nymph 4</td>
<td>160 ± 8</td>
<td>45 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nymph 5</td>
<td>155 ± 8</td>
<td>59 ± 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult</td>
<td>129 ± 11</td>
<td>50 ± 5</td>
</tr>
<tr>
<td><strong>Larval G. mellonella</strong></td>
<td>Starch</td>
<td>Hemolymph⁺</td>
<td>121 ± 4</td>
<td>46 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hemolymph⁻</td>
<td>69 ± 4</td>
<td>26 ± 3</td>
</tr>
</tbody>
</table>

bOne unit (U) of enzyme activity was defined as the amount of enzyme capable to produce 1 μmol of maltose.min⁻¹ in the assay conditions.

α-amylase activity was observed in the fifth nymphal instar. The lowest enzyme activity was determined in egg. Some α-amylase activity was determined in the hemolymph of *G. mellonella* larvae (Table 2, Hemolymph⁻). Higher amylase activity was obtained in the hemolymph of larvae captured by *P. maculiventris* (Table 2, Hemolymph⁺).

**Kinetic Characteristics of α-Amylase**

The apparent Michaelis–Menten constant (*K*ₘ), apparent maximal velocity (*V*ₘₐₓ) and *V*ₘₐₓ/*K*ₘ ratio of α-amylases are shown in Table 3. *K*ₘ value for the midgut α-amylases against starch and glycogen was lower than that for the salivary glands. However, *V*ₘₐₓ/*K*ₘ ratio for the enzyme from the midgut and salivary glands against the substrate starch was higher than that against the substrate glycogen. Maximum *V*ₘₐₓ/*K*ₘ ratio was determined for the enzyme active in the midgut of *P. maculiventris*.

**α-Amylase Zymogram**

Zymogram analyses revealed two (A and B) and three (a, b, and c) distinct bands of α-amylase activity in the salivary glands (Fig. 4; SG) and different parts of the midgut of *P. maculiventris* (Fig. 4; Midgut; AM, MM, PM), respectively. The greatest and lowest intensity of the bands was determined for the enzyme of the salivary glands and the posterior midgut, respectively, in accordance with the α-amylase activities. The isoforms “a,” “b,” and “c” were observed in the midgut of the fifth nymphal instar and adults (Fig. 4; Midgut; N5, Adult). However, the isoforms “b” and “c” were determined in the midgut of the second, third, and fourth nymphal instars (Fig. 4; Midgut; N2, N3, N4). In the first
Table 3. Apparent Michaelis–Menten Constant (K_M), Apparent Maximum Velocity (V_{max}) and V_{max}/K_M Ratio for α-amylases from the Midgut and Salivary Glands of Podisus maculiventris against the Substrates Starch and Glycogen

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme source</th>
<th>K_M ± SE (mM)</th>
<th>V_{max} ± SE (mM/min)</th>
<th>V_{max}/K_M ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>Salivary gland complex</td>
<td>0.245 ± 0.054</td>
<td>1.127 ± 0.035</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>Midgut</td>
<td>0.196 ± 0.086</td>
<td>1.873 ± 0.22</td>
<td>9.55</td>
</tr>
<tr>
<td>Glycogen</td>
<td>Salivary gland complex</td>
<td>0.442 ± 0.07</td>
<td>1.265 ± 0.16</td>
<td>2.86</td>
</tr>
<tr>
<td></td>
<td>Midgut</td>
<td>0.403 ± 0.06</td>
<td>1.147 ± 0.04</td>
<td>2.84</td>
</tr>
</tbody>
</table>

Figure 4. In-gel assay of α-amylase from the salivary glands and the midgut of Podisus maculiventris, and larval hemolymph of Galleria mellonella. SG: salivary glands, AM: anterior midgut, MM: median midgut, PM: posterior midgut, N1: the first nymphal instar, N2: the second nymphal instar, N3: the third nymphal instar, N4: the fourth nymphal instar, N5: the fourth nymphal instar. Hem^+ and Hem^- represent the hemolymph from the larvae captured by P. maculiventris and noncaptured larvae, respectively. The isoforms were visualized using in-gel activity assay of the enzyme samples against the substrate starch.

...nymphal instar, only the “c” isoform was detected (Fig. 4; Midgut; N1). The isoforms “A” and “B” were detected in the salivary glands of the fifth nymphal instar and adults (Fig. 4; SG; N5, Adult). The salivary glands of the first, second, third, and fourth nymphal instars could not be included in the study due to difficulty in dissecting the tissue.

A low-intensity and low-molecular-weight band of α-amylase activity was detected in the larval hemolymph of G. mellonella (Fig. 4; Hemolymph; H^-; H). Zymogram analysis showed two α-amylase isoforms originating from the salivary glands of P. maculiventris in the hemolymph of predator-captured larvae of G. mellonella (Fig. 4; Hemolymph; H^+; A, B).

α-Amylase Inhibition

The in vitro effect of the nonproteinaceous inhibitor acarbose on amyloytic activity from the midgut and the salivary glands has been shown in Fig. 5A. A linear dose-dependent inhibitory effect of acarbose on the α-amylase activity of the midgut ($R^2 = 0.87, P < 0.01$) and the salivary glands ($R^2 = 0.86, P < 0.01$) was determined. Relatively lower inhibition...
of salivary α-amylase activity was estimated. The enzyme and zymogram assays revealed complete inhibition of the midgut and salivary glands α-amylase activity in treatment with 2% acarbose (Fig. 5B), 64% inhibition of the midgut α-amylase activity in treatment with 0.0625% acarbose and 44% inhibition of the salivary glands α-amylase activity treated with 0.0625% acarbose. The in vivo assays revealed 43 and 87% inhibition of α-amylase activity at low (0.0625%) and high (2%) acarbose concentrations, respectively. However, zymogram analysis revealed complete inhibition of α-amylases of acarbose-injected larvae of G. mellonella at high concentration (2%) (Fig. 5C).
Table 4. Glucosidase and Galactosidase Activities (Mean ± SE) from Salivary Glands and Different Parts of Midgut of Podisus maculiventris

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Enzyme Substrate</th>
<th>Enzyme Substrate Type</th>
<th>Total Activity (mU)</th>
<th>Specific Activity (mU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGC</td>
<td>pNPαGlu</td>
<td>α-Glucosidase</td>
<td>54 ± 4</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>AM</td>
<td>pNPβGlu</td>
<td>β-Glucosidase</td>
<td>548 ± 26</td>
<td>85 ± 4</td>
</tr>
<tr>
<td>MM</td>
<td>pNPβGlu</td>
<td>β-Glucosidase</td>
<td>265 ± 22</td>
<td>41 ± 5</td>
</tr>
<tr>
<td>PM</td>
<td>pNPβGlu</td>
<td>β-Glucosidase</td>
<td>194 ± 17</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>SGC</td>
<td>pNPβGal</td>
<td>β-Galactosidase</td>
<td>42 ± 3</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>AM</td>
<td>pNPβGal</td>
<td>β-Galactosidase</td>
<td>145 ± 10</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>MM</td>
<td>pNPβGal</td>
<td>β-Galactosidase</td>
<td>81 ± 5</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>PM</td>
<td>pNPβGal</td>
<td>β-Galactosidase</td>
<td>50 ± 4</td>
<td>7 ± 1</td>
</tr>
</tbody>
</table>


Cluster Analysis of Carbohydrases

A dendrogram based on the carbohydrase activities in the digestive system of the predatory bug has been shown in Fig. 6. The dendrogram shows two distinct clusters labeled “a” and “b” (including subclusters b1 and b2). Cluster “a” included the salivary glands, and cluster “b” consisted of subclusters b1 (the anterior midgut) and b2 (the median and posterior midgut). Grouping within each cluster might be due to a high similarity of enzyme activities.

Starvation and Carbohydrase Activities

The effect of starvation on the midgut carbohydrase activities of P. maculiventris is shown in Fig. 7. α-Amylase activity gradually decreased during starvation up to 25% after 40 h starvation comparing to the control (Fig. 7A). The enzyme activity was restored after

Glucosidase and Galactosidase Activity

The optimum pHs for glucosidase and galactosidase activities were different in the midgut of the predatory bug (Fig. 3B). Midgut α-glucosidase and β-glucosidase showed an optimal activity at pH 7.0 and 4.0, respectively. Maximum midgut α-galactosidase, and β-galactosidase activity occurred at pH 5. The comparison of glucosidase and galactosidase activities from the different parts of the midgut and the salivary glands is shown in Table 4. Maximal and minimal activity of α-glucosidase, β-glucosidase, and α-galactosidase occurred in the anterior midgut and the salivary glands, respectively. Maximum and minimum β-galactosidase activity was determined in the median midgut and the salivary glands, respectively.

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Figure 6. Dendrogram of different parts of digestive system of *Podisus maculiventris* based on the activities of α-amylase, glucosidases, and galactosidases. SG: salivary glands, AM: anterior midgut, MM: median midgut, PM: posterior midgut. The dashed bar is rescaled distance cluster combine.

resuming the feeding of 40 h starved bugs. Changes in the activity of glucosidases and galactosidases were not the same as α-amylases during the starvation and some fluctuations in the enzyme activities were observed along the experiment (Fig. 7B). However, 20–70% of galactosidase and glucosidase activities were lost after 40 h starvation. Feeding of 40 h starved bugs did not significantly influenced glucosidase and galactosidase activities except β-galactosidase activity.

**DISCUSSION**

In most pentatomids, the midgut is long (as in *P. maculiventris*) and divided into three (zoophagous species) or four (phytophagous species) parts (Ramzi and Hosseininaveh, 2010; Fialho et al., 2012). Long midgut and also prolonged feeding in these insects allow them to digest food materials more efficiently (Guedes et al., 2007). In *P. maculiventris*, the midgut is morphologically similar to related species (Guedes et al., 2007; Fialho et al., 2012) and divided into the anterior, median, and posterior regions. The fourth posterior part including caecae harboring obligatory endosymbionts (Prado et al., 2006; Prado and Almeida, 2009) has been lost in predatory bugs. It seems that nutrition in *P. maculiventris* does not rely on the function of obligatory endosymbionts. However, many symbionts have been identified in the alimentary canal of *P. maculiventris* (Rooney and Coudron, 2010). Glasgow (1914) found similar bacterial species in the gut of 50% of *P. maculiventris* individuals as is seen in the caecae of a phytophagous pentatomid, *Holocostethus limbolarius*. 
The anterior midgut is more voluminous in younger nymphal instars than that in older nymphal instars and adults. Besides that, the median and posterior midguts are larger in older nymphal instars and adults than in younger nymphal instars, and it might be due to a more important role of the median and posterior midgut in food digestion and absorption in these compartments. Higher carbohydrases levels were found in the anterior midgut. Fialho et al. (2012) have showed more cathepsin L and collagenase and less amylase activity in the median and posterior midgut of *P. nigrispinus*. No significant
differences in the size of different parts of the midgut of *P. maculiventris* between the fifth nympha1 instar and adults suggest that they have similar digestion capability. These data are confirmed by the presence of the same number of α-amylase isoforms in the salivary glands (two isoforms) and the midgut (three isoforms) of the fifth nympha1 instar and adults. Cluster analyses revealed that adult and the fifth nympha1 instar are the same in volume of the midgut but different in length of different parts of the midgut. It shows that the anterior, median, and posterior midguts are narrower in adults than in the fifth nympha1 instar. In accordance with the midgut structure and cluster analyses in *P. maculiventris*, Stamopoulos et al. (1993) reported a similar enzyme profile secreted by the midgut epithelial cells of the fifth nympha1 instar and adults.

Salivary gland structure is similar in different predatory bugs of family Pentatomi-dae. Bilobed salivary gland structure including an accessory gland in *P. maculiventris* is a characteristic of many zoophagous and phytophagous pentatomid bugs (Oliveira et al., 2006; Azevedo et al., 2007; Bigham and Hosseininaveh, 2010; Fialho et al., 2012). Our observations revealed that the salivary glands in male adults are ca. twofold bigger than that in females. Larger salivary glands may confer more diverse enzymes and compounds to males and make them more effective for paralyzing host and probably utilizing different food sources. De Clercq et al. (2002) showed that male adults of *P. maculiventris* can consume eggs of *Nezara viridula* whereas female adults did not accept the eggs. They also revealed a higher twofold predation rate in the male adults on the prey *N. viridula*. In simultaneous host attack of males and females of *P. maculiventris*, bigger salivary glands in males and so higher level of destructing compounds can help females in utilizing the attacked prey more efficiently.

The pH value of the digestive system is an important factor influencing enzyme activities in insects (Terra and Ferreira, 2012). Slightly acidic pH of the midgut and the salivary glands in *P. maculiventris* is similar to the other pentatomid bugs (Bigham and Hosseininaveh, 2010; Ramzi and Hosseininaveh, 2010). Bell et al. (2005) showed that optimal midgut proteolytic activity of *P. maculiventris* occurs at slightly acidic condition (pH 5.5–6) in adults and nympha1s corresponded to the pH value prevailing in the midgut. It seems that optimal proteolytic activity in the salivary glands (pH 8.0, Bell et al., 2005) is more in accordance with the pH of hemocoel of the prey than pH of the salivary glands. Slightly acidic pH of the midgut not only corresponds with the maximum activity of proteinases but also is consistent with the optimal activity of α-amylase activity in *P. maculiventris* (pH 6.0). It seems that slightly acidic pH of the midgut provides an appropriate environment for activity of proteinases and α-amylase, whereas pH value of the salivary glands is associated with the other enzyme characteristics. In *P. maculiventris*, alkaline pH optima of proteinase activity from salivary glands corresponded well to the pH of the hemolymph of their prey *Lacenobia oleracea* (ca. 7.8).

The occurrence of several α-amylase isoforms in the salivary glands and the midgut of *P. maculiventris* suggests the importance of the enzyme in nutrition and is considered to be an explanation for heteropteran’s ability to feed on different plant and animal food sources (Torres and Boyd, 2009). α-Amylases have a critical function in starch and glycogen digestion in the predators, which allow them to feed on different trophic levels (Cohen and Hendrix, 1994). The first nympha1 instar does not feed on the prey and this can be an explanation for the presence of the only one form of α-amylase (isoform c) in the midgut. Expression of the other α-amylase isoform (b) in the second, third, and fourth nympha1 instars is associated with feeding on the prey. The fifth nympha1 instars and adults need to gain more energy from the prey, which might be the reason for expression of additional α-amylase isoform (a).
No pectinase activity in the digestive system of *P. maculiventris* has shown that the insect is not able to dilacerate plant tissues and utilize the plant cells’ content (Cohen, 1990; Stamopoulos et al., 1993; Torres and Boyd, 2009). However, *P. nigrispinus*, a very close species to *P. maculiventris*, is capable of feeding on xylem content for some nutrients and hormones (Torres et al., 2010). *Podisus nigrispinus* feeding on prey and plant showed enhanced female fecundity in comparison with the insects feeding only on prey (Torres et al., 2010). Due to the predatory nature of *P. maculiventris*, it seems that high amylase activity in the midgut and salivary glands may be more associated with glycogen digestion (major animal glycoside reserve) than starch digestion (major plant glycoside reserve). Nevertheless, catalytic efficiency (\(V_{\text{max}}/K_M\) ratio) of \(\alpha\)-amylases of the salivary glands and the midgut of *P. maculiventris* is higher on starch than on glycogen. The species belongs to the subfamily Asopinae are ancestrally herbivorous species (Torres and Boyd, 2009) and during the evolution, these bugs have been locating in intermediated status, that their \(\alpha\)-amylases still tend to operate on starch reserves more efficiently than on glycogen. Higher amylase activity in the salivary glands than different parts of the midgut is probably associated with evolution of the current nutrition habits of the predator from ancestors fed on starch rich seeds and importance of extra-oral digestion.

\(\alpha\)-Amylase activity gradually decreased from the salivary glands to the rectum. It seems that most \(\alpha\)-amylase activity in the digestive system of *P. maculiventris* comes from the salivary glands. Produced \(\alpha\)-amylase by the salivary glands is injected into the prey hemocoel (Cohen, 1990) and enhances \(\alpha\)-amylase activity ca. 76% in the hemolymph of larval *G. mellonella*. The exact two \(\alpha\)-amylase isoforms of the salivary glands were observed in the hemolymph of larvae of *G. mellonella*. The pH of the hemolymph (ca. 6.0) of *G. mellonella* larva and its appropriate ionic composition (the presence of \(K^+\), \(Ca^{2+}\), \(Mg^{2+}\), and \(Cl^-\)) (Klowden, 2007) provide a condition suitable for injected \(\alpha\)-amylase activity. The ions may enhance the injected \(\alpha\)-amylase activity (data not shown). The injected saliva containing \(\alpha\)-amylase, hemolymph, and liquefied internal organs of the larvae were re-ingested. Almost all \(\alpha\)-amylases move from posterior midgut and enter into the rectum due to absence of peritrophic membrane and countercurrent flow in the midgut of *P. maculiventris*.

Despite of higher \(\alpha\)-amylase activity in the salivary glands, \(\alpha\)-amylases from the midgut work more efficiently (ca. twofold) on starch than from salivary glands. However, different parts of the midgut produce at least its own one \(\alpha\)-amylase isoform and this can be an explanation for different efficiency of \(\alpha\)-amylases in the digestive system of the predator. The highest glycogen hydrolysis occurs in anterior midgut where there is a large content of ingested intact glycogen. The presence of \(\alpha\)-amylase activity in oocytes and eggs of *P. maculiventris* is associated with glycogen hydrolysis and providing energy requirements of embryo. During oogenesis, the total content of glycogen, glucose, and trehalose increased in the oocytes of *Rhodnius prolixus*, whereas after oviposition a decrease in glycogen content and increase in glucose content was observed (Santos et al., 2008).

Based on a semiquantitative assay, Stamopoulos et al. (1993) reported that the midgut \(\alpha\)-amylase activity in *P. maculiventris* was progressively enhanced when the nymphal instars reached the last stage (from second to the fifth nymphal instar). Our results based on a quantitative measurement revealed that \(\alpha\)-amylase activity is higher in older nymphal instars than that in the younger instars. The carbohydrase activities in the digestive system of the predatory bug are controlled by food level. In starved adults, \(\alpha\)-amylase activity gradually decreased with increased starvation time. Rafiq Khan and Ford (1962) showed a significant decrease in the activity of digestive enzymes in 75 h starved *Dysdercus fasciatus*. Declined carbohydrase activity levels could be due to absence of food and leading to

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reduced amino acids to synthesize proteins such as α-amylase, galactosidase, and glucosidase enzymes de novo. Muhlia-Almazan and Garcia-Carreno (2002) observed that starvation significantly decreased trypsin activity in *Penaeus vannamei*. Decreased α-amylase activity level during starvation of *P. maculiventris* could be as a result of consuming total body protein as the main source of energy.

Optimal activity of α-glucosidase in slightly acidic condition (pH 6.0–7.0) is congruent with optimal pH for the enzyme activity in many insects (Terra and Ferreira, 2012). However, β-glucosiode, α-galactosidase, and β-galactosidase are optimally active at more acidic pH (4.5–6.5) similar to most insects (Terra and Ferreira, 2012). In contrast to α-amylases, which are more active in the salivary glands than in the midgut, glucosidases, and galactosidases are more active in the midgut. α-Amylase is the main enzyme in the initial digestion of starch and glycogen, whereas glucosidases and galactosidases are the enzymes involved in the final digestion of polysaccharides (Kfir et al., 2002).

According to the cluster analysis, salivary glands are completely different in its enzyme activities from the midgut. In the midgut, median midgut and posterior midgut are similar in their enzyme activities and are different from the anterior midgut. The same situation can be seen for proteinases of the salivary glands and the midgut of predaceous heteropteran insect (Cohen, 1993).

The inhibitors of insect α-amylases encompass considerable destruction of polysaccharide digestion in insects (Kaufnerová et al., 2007) and can be ideal tools in integrated pest management programs (Gatehouse and Gatehouse, 1998). The effect of the inhibitor acarbose was investigated on α-amylase activity from the salivary glands and midgut of *P. maculiventris* to reveal the potential of the inhibitor in inhibition of the enzyme activity. Kaufnerová et al. (2007) showed that the nonproteinaceous inhibitor acarbose did not have any suppressive effects on the parasitoid *Venturia canescens*. The wasps emerged from the hosts reared on a diet containing sublethal concentration of acarbose were larger and heavier than the controls and it might be possible to enhance the control of insects using both biological control and α-amylase inhibitors (Kaufnerová et al., 2007). Hubert et al. (2007) showed that the acarbose was not effective against the predatory mite *Cheyletus malaccensis*. The inhibitor acarbose can completely inhibit α-amylase activity from the digestive system of *P. maculiventris*. However, it is not known how important of the role of α-amylases are in the digestion of *P. maculiventris*. So, further experiments are needed to find out the impact of the α-amylase inhibition on the biological traits of the bug. Adverse impacts of the inhibitors on natural enemies would reject their applications in integrated pest management programs (Kaufnerová et al., 2007).

Extra-oral digestion in *P. maculiventris* mainly relies on hydrolases such as carbohydrates and proteinases, and paralyzing toxins. Salivary α-amylase isoenzymes and probably other hydrolases are injected into the prey body for initial digestion of macromolecules such as glycogen and proteins. However, pre-acting of tissue degrading enzyme collagenase on internal organs is necessary for action of carbohydrates and other proteases. Liquefied internal tissues bearing salivary hydrolases are ingested into the three-part midgut of the insect for final intratract digestion in where galactosidases and glucosidases are predominantly secreted.

**ACKNOWLEDGMENTS**

We wish to express our special thanks to Dr. Maryam Zamani for providing technical support in establishment of the predator colony and rearing.
LITERATURE CITED


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