Digestive $\alpha$-amylase from *Leptinotarsa decemlineta* (Say) (Coleoptera: Chrysomelidae): response to pH, Temperature and some mineral compounds

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ABSTRACT:

Colorado potato beetle (*Leptinotarsa decemlineta* (Say)) is an important pest of potato (*Solanum tuberosum*), one of the most important agricultural crops in Iran. This pest can cause large huge amount of crop decreases, since both larval and adult stages are destructive. Several methods could be used to control this insect including chemical methods, biological methods, and physical methods and using transgenic plant carrying gene of interest. Chemical controls are discouraged due to pesticide residue in the commodities and resistance in insects. Nowadays, chemical methods are discouraged due to pesticide residue in the commodities and resistance in insects, but other methods of controls like using transgenic plants are encouraged and the first step in this method understand the function of enzymes and some characteristics of them in destructive pests. The aim of the current study was to get a good understanding of the amount of enzyme activity and also enzyme composition at different larval and adult stages of this insect. The alpha-amylase activity had an optimum pH between 6 to 10 with a peak at pH 6.4. The optimum temperature for alpha-amylase activity in this study was 37°C. Some compounds like SDS, Tris, Mg(NO$_3$)$_2$, K$_2$HPO$_4$, Mg(H$_2$PO$_4$), NaCl and KNO$_3$ decreased the amount of alpha-amylase activity, but some compounds such as Ca(NO$_3$)$_2$ and Ca$_3$(PO$_4$)$_2$ act as activators.

Key words: Activators, Colorado potato beetle, Developmental stages, Digestive alpha-amylase, Inhibitors, Ions.

Introduction

Colorado potato beetle, *Leptinotarsa decemlineta* (Say) (Coleoptera: Chrysomelidae), is best known beetles for its ability to devour vegetables in the nightshade family, such as potato, tomato, eggplants and peppers. The adult beetles as well as their larvae can strip the plants of leaves and ruin an entire crop if left to their own devices[25]. This insect has been reported in Iran since 1983, and its distribution in the northwest of Iran is intolerable. Chemical insecticides have been the primary means of controlling these kinds of destructive pests, but because of many problems associated with the use of synthetic pesticides in integrated pest management approaches, use of chemicals to protect agricultural products is limited and being replaced by environmentally-benign alternatives[18]. One such approach is to enhance the resistance of important crops by plant proteins which are the major plant defensive mechanisms against herbivores.

Several of these proteins are present in seeds and vegetative organs and act to regulate numbers of phytophagous insects. These compounds act on the key insect- gut digestive enzymes, amylases and proteinases[4,25]. Several plant-proteinaceous
inhibitors of insect proteinases and amylases have been identified and characterized [25].

These inhibitors are insecticidal because they form complexes with digestive enzymes, which are stable and dissociate slowly. Inactivation of digestive enzymes by inhibitors results in blocking of gut proteinases and amylases which leads to poor nutrient utilization, retarded development, and death due to starvation [22,16].

Studies have shown that coleopterans have a complex pattern of proteolytic activity in the midgut. In most coleopteran larvae and adults, midgut PH is slightly acidic and cysteine proteinases provide the major midgut proteolytic activity [39]. Some species such as Sitophilus oryzae (L.), Hypera postica (Gyllenhal), Sitophilus zeamais (Motschulsky) and Prostephanus truncates (Horn) utilize serine proteinases for protein digestion [21,36,30].

The alpha-amylases (α-1, 4-glucan-4-glucanohydrolases; EC 3.2.1.1) are hydrolytic enzymes that are widespread in nature. These enzymes catalyze the hydrolysis of α-D-(1, 4)-glucan linkages in glycogen and other related carbohydrates [14,34]. In insects the abundance and activity of alpha-amylases are dependent on food sources.

The purpose of the present study is to identify and characterize the alpha-amylase activities and also the effects of some mineral compounds on this enzyme of Colorado potato beetle to get a better understanding of their digestive physiology. This understanding will hopefully lead to new management strategies for control of this part.

Materials and methods

Insects:

Adult insects of Leptinotarsa decemlineta (Say) were obtained from the potato farms of Zanjan, and they were cultured on potato plants at 27±2 °C, RH 90±10%, and light period of 14:10 (L:D) in the Tabriz Plant Protection Department laboratory. The 3rd and 4th larval stages and also male and female adults from the first generation, produced in the laboratory were used in the experiment.

Sample Preparation and Enzyme Assay:

Alimentary canals of the fourth larval stage were removed by dissection under a dissecting microscope in ice-cold buffer (6µmol/NaCl). Tissues were rinsed in ice-cold buffer, placed in a precooled homogenizer and ground in 1 mL of universal buffer (0.02 M) containing succinate, glycine and 2-morpholinoethanesulfonic acid (pH 6.5) [20].

Homogenates were transferred to 1.5 ml centrifuge tubes and centrifuged at 15000 rpm for 20 min at 4°C. The supernatants for each tissue were pooled then stored at -20°C for subsequent analyses.

Alpha-amylase activity was assayed using the dinitrosalicylic acid (DNS) procedure [3] using 1% soluble starch (Merck, Darmstadt, Germany) as the substrate. Ten microliters of the enzyme were incubated for 30 min at 35°C with 500 µl universal buffer and 40 µl soluble starches. The reaction was stopped with the addition of 100 µl DNS and heating in boiling water for 10 min. DNS is a color reagent which reacts with the reducing groups released from starch by alpha-amylase action.

The boiling water stops the α-activity and catalyzes the reaction between DNS and the reducing groups of starch. Absorbance was then measured at 540 nm. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35°C. A blank without substrate but with α-amylase extract and a control containing no α-amylase extract but with substrate were measured at the same time as the reaction mixtures. All assays were performed in duplicate and each assay was repeated at least three times.

A standard curve of α-amylase absorbance against the amount of maltose released was constructed to enable the calculation of the amount of maltose released during the α-amylase assays. Serial dilutions of maltose (Mr 360.32 mg/mol; Merck) in the universal buffer (pH 6.5) were made to produce the following concentrations: 0.125, 0.25, 0.5, 1 and 2 mg/ml.

Effect of pH and Temperature on Enzyme Activity:

The effects of temperature and pH on α-amylase activity were examined using enzymes from larval canals. The effect of temperature on α-amylase activity was determined by incubating the reaction mixture at 25, 28, 31, 34, 37, 40, 43 and 46 °C for 30 min, followed by measurement of activity.

Optimal pH was determined using universal buffer with pH set at 6, 6.4, 6.8, 7.2, 7.6, 8, 8.4, 8.8, 9.2, 9.6 and 10.

Protein Determination:

Protein concentrations were measured using the method of Bradford [6] and using bovine serum albumin (Bio-Rad, Munchen, Germany) as a standard.

Effects of Activators and Inhibitors on Enzyme Activity:

To test the effects of different compounds on the enzyme activity, assays were performed in the presence of different concentrations (0.1, 0.2 and 0.3 mM) of sodium chloride (NaCl), Calcium Phosphate (Ca₃(PO₄)₂), Magnesium phosphate (Mg(H₂PO₄)₂),...
Dipotassium phosphate (K₂HPO₄), Magnesium nitrate (Mg(NO₃)₂), Potassium nitrate (KNO₃), Calcium nitrate (Ca(NO₃)₂), EDTA, Tris and SDS. These compounds were added to the assay mixture, and activity was measured after 30 min. a control was also measured (no compounds added).

Statistical Analysis:

Data were compared by one-way analysis of variance (ANOVA) followed by Tukey's studentized test when significant differences were found at P=0.05 (SAS, 1997).

Results:

Effects of Ph and Temperature on Enzyme Activity:

The optimum pH for L. decemlineata was recorded as 6.4 (Fig.1). The enzyme activity was increased slightly from pH 6 to 6.4, and thereafter with increasing pH from 6.4 to 10 α-amylase activity was decreased instantly (Fig. 1). Amylase was found to be active over a broad range of temperatures; however, the optimal temperature for its activity was found at 37°C. In this case enzyme activity increase gradually from 25 to 37°C and after that declined to 46°C (Fig. 2).

![Fig.1: Effect of pH on activity of α-amylase of Leptinotarsa decemlineata (Say).](image)

![Fig.2: Effect of temperature on activity of α-amylase of Leptinotarsa decemlineata (Say).](image)

![Fig.3: Activity levels of α-amylase in 4th instar larvae foregut (FG), midgut (MG) and hindgut (HG) of Colorado potato beetle (Leptinotarsa decemlineata (Say)).](image)

Effects of Activators and Inhibitors on Enzyme Activity:

Several chemicals tested in this study had different effects on activity level of α-amylase in alimentary canal of Colorado Potato Beetles (Leptinotarsa decemlineata (Say)) (Table 1). Activity level of α-amylase in alimentary canal of Colorado Potato beetle decreased with increasing concentrations of SDS, Tris, EDTA, Mg(NO₃)₂, K₂HPO₄, Mg(H₂PO₄)₂ and NaCl, but in one case the amount of alpha-amylase activity in higher concentrations was lower (Table 1). This condition was observed only in two concentrations of KNO₃ (where the level of alpha-amylase activity in 0.2 mM/L was more than that value in 0.1 mM/L). Also the values related to alpha-amylase activity in two concentrations of K₂HPO₄ (0.2 and 0.3 mM/L) and Mg(H₂PO₄)₂ (0.1 and 0.2 mM/L) did not have significant differences (Table 1). However, activity level of α-amylase in alimentary canal of this insect increased by adding Ca(NO₃)₂ and Ca₃(PO₄)₂ to the enzyme. The amount of activity by increasing concentrations of these compounds decreased. It seems that the Ca²⁺ ion acts as an activator of alpha-amylase activity (Table 1).

Table 1: Relative activity of Colorado potato beetle α-amylase in the presence of different compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentrations (mM/L)</th>
<th>Enzyme activity in midgut (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td></td>
<td>17.56 ± 0.0012a</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1</td>
<td>16.43 ± 0.008b</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>15.81 ± 0.005c</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>14.8 ± 0.006d</td>
</tr>
<tr>
<td>Tris</td>
<td>0.1</td>
<td>16.5 ± 0.0012b</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>15.85 ± 0.009c</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>14.76 ± 0.007d</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.1</td>
<td>16.58 ± 0.008b</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>16.09 ± 0.0012c</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>15.26 ± 0.007d</td>
</tr>
<tr>
<td>Ca(NO₃)₂</td>
<td>0.1</td>
<td>22.94 ± 0.0015b</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>18.76 ± 0.003c</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>17.75 ± 0.007d</td>
</tr>
<tr>
<td>KNO₃</td>
<td>0.1</td>
<td>16.45 ± 0.009c</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>16.63 ± 0.0011b</td>
</tr>
</tbody>
</table>
Discussion and Conclusion:

The pH gut content is a major factor that affects digestive enzymes[35]. There are many reports in which it has been presented that the optimum pH for amylase activity in insects is between pH 5.5 to 9.5, and alpha- and beta-amylases in other living organisms the optimum pH is between 4.7 to 6.9[8]. Most activity of digestive α-amylase enzyme in carnivorous insects is between pH 5 to 7 but in herbivorous and omnivorous species the optimum pH is usually more alkaline[40]. The optimum pH for intestinal carbohydrases is related to feeding habits of insects, however in intestinal proteases the optimum pH is more related to the phylogenetic position of species [40]. D’Amico et al.[11] have reported that alpha-amylase has the most activity in pH's which are next to 7. In another study it is presented that alpha-amylase in insects based on substrate has the most activity between pH 5-8 and by increasing the value of pH the amount of alpha-amylase activity decrease slightly or moderately [15]. Our data show that although the midgut pH in larvae and adults of Leptinotarsa decemlineata (Say) active in broad range from pH 6 to 10. This situation has been reported for other coleopteran pests of stored products such as P.truncatus, Tribolium confusum, Trogoderma castaneum (Herbst), Dermestes maculates (DeGeer), Trogoderma versicolor (Creutz), Rhyzopertha domonica (F.), Oryzaephilus mercator (Fauvel) and Lasioderma serricorne (F.)[24,10,28,38,30], Zeng and Cohen[41] reported that the optimal pH for alpha-amylase from Lygus hesperus and L.lineolaris was 6.5 and also Kazzazi et al. [23] showed that the optimum pH for α-amylase activity of Eurygaster integriceps (Hemiptera: Scutelleridae) was 6.5. However, several studies have been presented that alpha-amylase in majority of lepidopteran insects is not only active at a broad range of pH, but also the optimal pH 8.5-9.2[42]. This condition is also correct about some beetles like insects which are members of Dermentidae like Attigensus megatoma (Brahm) [19]. It has been suggested that the optimum pH of alimentary canal is completely related to feeding habits of insects and this factor has large effects on alimentary canal pH[41].

<table>
<thead>
<tr>
<th>pH</th>
<th>Activity of α-amylase</th>
<th>pH</th>
<th>Activity of α-amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>16.6±: 0.005b</td>
<td>6.2</td>
<td>16.3±: 0.004b</td>
</tr>
<tr>
<td>6.8</td>
<td>16.5±: 0.003c</td>
<td>6.3</td>
<td>15.9±: 0.009b</td>
</tr>
<tr>
<td>7.0</td>
<td>15.8±: 0.004c</td>
<td>7.2</td>
<td>15.5±: 0.009c</td>
</tr>
<tr>
<td>7.5</td>
<td>15.3±: 0.006d</td>
<td>7.8</td>
<td>15.0±: 0.005d</td>
</tr>
<tr>
<td>8.0</td>
<td>14.7±: 0.004d</td>
<td>8.2</td>
<td>14.4±: 0.002d</td>
</tr>
<tr>
<td>8.5</td>
<td>14.0±: 0.002d</td>
<td>8.8</td>
<td>13.5±: 0.001d</td>
</tr>
<tr>
<td>9.0</td>
<td>13.5±: 0.003c</td>
<td>9.2</td>
<td>13.0±: 0.004b</td>
</tr>
</tbody>
</table>

There are many reports that indicate the optimum temperature for enzyme activity in insects is related to pH, and also the optimum temperature based on reaction time shows increasing or decreasing trends[5]. Silva et al.[33] have shown that alpha-amylase in midgut of Zabrotes subfasciatus (Boh) (Coleoptera:Bruchidae) is more stable to high temperature than that in Callosobruchus maculatus (Fabricius) (Coleoptera: Bruchidae). Mendiola-Oyala et al.[27] showed that the optimum temperature of midgut alpha-amylase in Prostephanus truncates (Horn.) (Coleoptera: Bostrichidae) is 40°C and at higher temperature this enzyme is analyzed quickly. In another study it has been shown that the optimum activity of salivary enzymes in Lygus occur in 42°C and by increasing the temperature from 42 to 55°C the amount of enzyme activity drastically decreases. In this study the optimal temperature of alpha-amylase was 37°C, but this enzyme was active in a broad range (from 25 to 46°C). This value was similar to that of α-amylase activity in some species like Cerambyx cerdo L. (Coleoptera: Cerambycidae; 35°C) and Dolyecoris baccarum L. (Heteroptea: Pentatomidae), but this value is lower than that of alpha-amylase activity in other species such as Blatella germanica (Kimberly) (Blatoidea: Blatidae; 50°C) and Bombyx mori L. (Lepidoptera: Bombycidae; 60 °C) or higher than this value for some members of Coleoptera like Tenebrio molitor L. (Coleoptera: Tenebrionidae; 25°C)[42].

In this study several inhibitors and activators were tested, and the results were compared other insect systems[2,36,41,26]. NaCl decreased the activity of the enzyme and similar situation also occurred in alpha-amylase activity of Chilo suppressalis (Lepidoptera: Pyralidae) when NaCl is used[42]. In Lygus hesperus (Knight) and L.lineolaris alpha-amylases were activated by NaCl[1,41].

However, amylases in some insect species (e.g. Callosobruchus chinensis (Lineaus) (Coleoptera: Bruchidae) and Bombyx mori (Linnaeus) (Lepidoptera: Bombycidae) are inhibited by Cl−, which is consistent with current results in Colorado potato beetle. In the current study, K+ and Mg2+ ions have been shown to have a similar effect on alpha-amylase of L. decemlineata as Cl ions.

Zeng and Cohen [41] showed that EDTA and SDS reduced α-amylase activity of Lygus spp. and they also found that increasing of incubation time would elevate the activity level of amylase activity. This could be due to enzyme denaturation of Ca2+ omission from enzyme structure. In our study EDTA and SDS had an inhibitory effect on α-amylase activity in alimentary canal. Rate of activity was reduced by 7-17%.
Ca\textsuperscript{2+} ions usually increase alpha-amylase activity in many cases. In this study it was shown that this ion increases the value of alpha-amylase activity and this result agrees with the Podoler and Applebaum (1971) report about C. chinensis.[23] However, it has been reported that \( \alpha \)-amylases are metalloproteins that require calcium for maximum activity [23].

Alpha-amylase inhibitors occur naturally in many food plants and are particularly abundant in cereals and legumes[14]. Insects gain access to food sources when they evolve amylases that are not affected by inhibitors effective against these insects' enzymes. Insect alpha-amylase (\( \alpha \)-1, 4-glucan-4-glucohydrolase, EC 3.2.1.1) catalyzes the hydrolysis of the \( \alpha \)-(1, 4)-glycosidic linkages of widespread in nature. Because of important biochemical role of alpha-amylase in insect growth and development, when the action of this enzyme is inhibited, insect nutrition in impaired, its growth and development retarded and eventually death occurred due to starvation [29]. Genes encoding these inhibitors have been used to make transgenic crops by gene transfer technology because their expression is harmful to target insects and pests, interfering with their digestion and absorption, whereas no anti-nutritional or toxic effects were observed in rats fed on transgenic pea expressing the \( \alpha \)-amylase inhibitor [32]. Therefore, the use of chemical pesticides leads to high production costs as well as causing risks to human health. In the light of these considerations, plant genetic transformations with exogenous genes encoding factors of resistance of phytophageous insects is a modern and alternative to synthetic chemical insecticides for the control of several aggressive plant pests[13].

The primary reason for producing insect-resistance transgenic crops is to reduce the use of chemical pesticides, which reduces the cost to the farmer and the consumer and reduces the insecticide load in the environment[12]. Making insect-resistance plants requires the characterization of alpha-amylase and other digestive enzymes of the target insect and identification of suitable inhibitors from plants or other sources [34].

In our opinion, the purification and characterization of more insect alpha-amylases will greatly facilitate the understanding of the mechanisms responsible for this selectivity and will help to design new and more specific strategies for insect control.

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REFERENCES


