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Effects of some mineral compounds on the salivary α-amylase activity of the sunn pest, ***Eurygaster integriceps*** (Put.) (Heteroptera: Scutelleridae)

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Summary

In this study, the effects of two concentrations (1 and 3 mM) of ammonium nitrate (AN), ammonium phosphate (AP), ammonium sulfate (AS), copper chloride (CC), magnesium chloride (MC), magnesium nitrate (MN), magnesium sulfate (MS), potassium nitrate (PN), sodium nitrate (SN) and sodium phosphate (SP) on salivary α-amylase activity of adults of the sunn pest, ***Eurygaster integriceps*** (Put.) (Heteroptera: Scutelleridae) were evaluated 30 minutes after incubation. Distilled water was considered as the control. The results showed that the effects of the mineral compounds on adults' α-amylase activity were significant ($P<0.01$). CC, AS, SN, MC, MN (all at concentrations of 1 and 3 mM) and AP (1 mM) significantly inhibited the α-amylase activity in ***E. integriceps***. AN and MS (1 mM); and SP and MS (3 mM) reduced the enzyme activity; however, the reduction amounts were not significant. 3 mM concentrations of PN, AN and AP activated the enzyme very slightly (0.6, 1.5 and 3.1 percent, respectively). PN and SP (1 mM) activated the salivary α-amylase significantly (19.1 and 10.5 percent, respectively). Enzyme was active at pH values ranging from 4.5 to 10, but its maximum activity was observed at pH=5.

Key words: ***Eurygaster integriceps***, salivary gland, α-amylase, inhibitor, mineral compounds

Anahtar sözcükler: ***Eurygaster integriceps,*** salya bezi, α-amylase, engelleyici, inorganik bileşikler

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Introduction

Sunn pest, ***Eurygaster integriceps*** (Put.) (Heteroptera: Scutelleridae) is the major pest of wheat and barley in the Middle East (Brown & Erlap, 1962). In Iran, sunn pest is a key pest that cause serious damages to wheat and barley (Amir-Maafi & Parker, 2003). Chemical insecticides which are used against this pest not only create environmental pollutions but also produce food stuff contaminations (Aw-Hassan *et* al., 2004).

α-amylases (α-1,4-glucan-D-glucanohydrolases EC 3.2.1.1) are a family of enzymes that catalyze the hydrolysis of the α-(1,4) glycosidic bonds in starch and related compounds (Applebaum, 1985; Strobl *et* al., 1998). Because α-amylases play a major role in carbohydrate metabolism, organisms with a starch-rich diet depend on the effectiveness of their amylases for survival (Applebaum, 1985; Strobl *et* al., 1998; Barbosa Pereira *et* al., 1999; D'Amico *et* al., 2000; Iulek *et* al., 2000; Franco *et* al., 2002; Carlini & Grossi-de-Sa, 2002; Oliveira-Neto *et* al., 2003).

Several insect α-amylases have already been described, some of which occur as mixture of different isozymes. For instance, in eight Amy strains of ***Drosophila melanogaster*** (Meig.) (Diptera : Drosophilidae), at least two major α-amylase isozymes were found (Doane, 1969). Conversely, single molecular forms of α-amylases have been reported in ***Callosobruchus chinensis*** (L.) (Coleoptera: Bruchidae) (Podoler & Applebaum, 1971), ***Tenebrio molitor*** (L.) (Coleoptera: Tenebrionidae) (Buonocore *et* al., 1976), ***Lygus hesperus*** (Knight) (Heteroptera: Miridae) (Zeng & Cohen, 2000), ***Lethocerus uhleri*** (Montandon) (Heteroptera: Belostomatidae) and ***Belostoma lutarium*** (Stal.) (Heteroptera: Belostomatidae) (Swart *et* al., 2006).

Phytophagous insects that feed on polysaccharide-rich diets depend on the effectiveness of their carbohydrate processing enzymes for survival (Strobl *et* al., 1998; Franco *et* al., 2002). They have to circumvent the effects of inhibitors that occur in their natural diet and target their digestive enzymes (Ishimoto & Kitamura, 1989; Silva *et* al., 1999). Plants, on the other hand, are compelled to find new and better mechanisms of protecting their seeds from numerous phytophagous insects. Inhibitors of digestive enzymes can be used to protect plants against insects (Franco *et* al., 2002). The use of genes that encode insecticidal proteins in transgenic crops has the potential to be beneficial for agricultural crop production, the environment, and the consumers (Barbosa Pereira *et* al., 1999; Farias *et* al., 2006; Bahagiawati *et* al., 2007). For example a gene encoding an α-amylase inhibitor, named αAI-Pc1, was isolated from cotyledons of ***Phaseolus coccineus*** (Azevedo Pereira *et* al., 2006).

The properties of nonproteinacious inhibitors make then interesting in the field of medicine, both for treatment and in diagnostic procedures. Nevertheless, the use of nonproteinacious inhibitors for production of insect-resistant transgenic plants is much more difficult. Hence, the presence of multiple expressed transgenes

would be required in order to confer protection. In this area, the proteinacious inhibitors, coded by a single gene, are more suitable (Kiggundu et al., 2003).

We undertook the study of the α-amylase of the sunn pest to gain a better understanding of its digestive physiology, which we hope would lead to new strategies for its control.

Material and Methods

Materials

Adult insects were collected from wheat farms in around Tabriz, Iran, during the summer of 2004. Mineral compounds used were ammonium nitrate (AN), ammonium phosphate (AP), ammonium sulfate (AS), copper chloride (CC), magnesium chloride (MC), magnesium nitrate (MN), magnesium sulfate (MS), potassium nitrate (PN), sodium nitrate (SN) and sodium phosphate (SP).

Methods

Sample preparation

Adult insects were dissected by the method of Yazdanian et al. (2006) and starved for 24h before dissecting (Cohen, 1993). This was based on the observations which had been showed that the accumulation of the enzyme in the lumen of the true bugs salivary glands lasted 24 to 48 hours (Baptist, 1941; Cohen, 1993). Enzyme samples were prepared by the methods of Cohen (1993) and Yazdanian et al. (2006) with slight modifications. All insects were dissected under a stereomicroscope in ice-cold phosphate buffer (4 °C, pH=6.9). The salivary gland complex (SGC) (including anterior and posterior lobes, accessory glands and principal and accessory ducts) was exposed by breaking the junction point of the prothorax and mesothorax located between the coxal bases of front and mid legs and removing it from the abdomen with fine forceps and application of gentle traction to remove the midguts (Yazdanian et al., 2006). The SGCs were separated from the insect bodies, rinsed in ice-cold phosphate buffer and 10 pairs placed in a microtube containing 1 ml of cold phosphate buffer. The SGCs were homogenized by using a homogenizer (Ultra-Turrax T8, IKA Labortechnik, Germany) immediately after dissection. The homogenates were centrifuged at 12000 rpm for 10 minutes at 4 °C. The supernatants were stored at -20 °C for later analyses. Protein concentrations of all of the enzyme samples were determined by the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as the standard (Yazdanian et al., 2006).

α-Amylase activity assay

Amylase activity in the salivary gland was determined by using a diagnostic kit (Amylase kit®, Pars Azmoon Co., Iran). The substrate was ethylidene-*p*-nitrophenyl maltoheptaoside (EPS-G_7). Absorbance, which is directly related to α-amylase activity, was measured at 405 nm and 37 °C using an auto analyzer (Alcyon 300® Plus, Molecular Devices Corporation, Sunnyvale, CA). Before application, the auto analyzer calibrated with the control sera N and P (TrueLab N®

and TrueLab P®, respectively; Pars Azmoon Co., Iran) and a calibrator solution (TrueCal U®, Pars Azmoon Co., Iran). After calibration, the auto analyzer mixes 6 μl of enzyme sample with 300 μl of substrate solution, automatically, and calculates the enzyme activity (IU/L) after a reaction delay of 1 minute and 36 seconds. The assays were replicated three times. Finally, the specific α-amylase activity calculated as U/mg protein (Cohen, 1993).

Enzyme inhibition and activation

The effects of above mentioned materials on salivary α-amylase activity of the sunn pest were determined. Two concentrations (1 and 3 mM) of each compound were prepared. The above mentioned concentrations were prepared in distilled water, and the pH was adjusted to 7 using citric acid and sodium hydroxide. Each solution (100 μl) was pre-incubated with 10 μl of enzyme solution at room temperature (25- 28 °C) and the residual activities were determined after 30 minutes. The percentages of inhibition or activation were determined by comparing the enzyme activity in distilled water with its activities in the above mentioned solutions. The experiments were repeated three times, and data were analyzed by analysis of variance (ANOVA), and means of enzyme activity of ***E. integriceps*** in different solutions were compared by Fisher's protected least significant difference (FPLSD) (SAS Institute, 1988) at P= 0.01.

Effect of hydrogen ion concentration on the enzyme activity

α-amylase activities at various pH values ranging from 4.5 to 10 (adjusted by citric acid and sodium hydroxide and at 0.5 pH unit increments) were assayed to determine the optimum pH of α-amylase in the salivary gland of the ***E. integriceps***. Each pH value (500μl) was pre-incubated with 50μl of enzyme solution at room temperature (25- 28 °C) and the amylase activities were determined after 30 minutes. Measurements were repeated three times for each pH value.

Results

Inhibition and activation of the α-amylase

The results of this study showed that some mineral compounds reduced and some others increased the salivary α-amylase activity of ***E. integriceps***.

As a whole, α-amylase activity was inhibited by CC, MN, SN, AS and MC. MC and MN had more inhibitory effects on the enzyme activity in compare to the other compounds (Figure 1).

Effects of two concentrations (1 and 3 mM) of different compounds are presented in Table 1. PN and SP at the concentration of 1 mM activated the salivary α-amylase of the ***E. integriceps***, significantly (Tab. 1). CC, AS, SN, MC, MN (all at the concentrations of 1 and 3 mM) and AP (1 mM) significantly inhibited the α-amylase activity of ***E. integriceps***. SP (3 mM), AN (1 mM), MS (1 and 3 mM), PN (3 mM) and AP (3 mM) had no effect on the α-amylase activity.

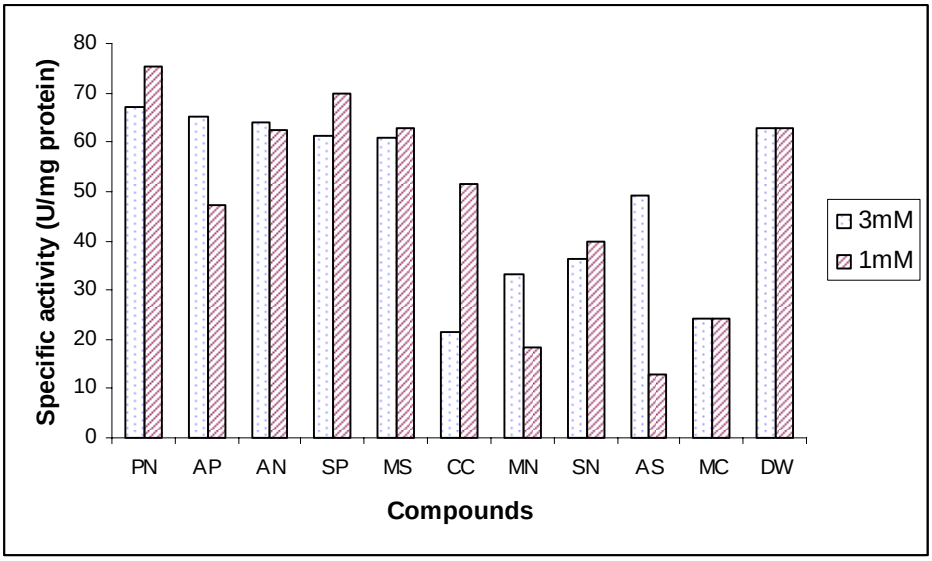

Figure 1. Effects of some mineral compounds on salivary α-amylase activity of ***Eurygaster integriceps*** (Put.) (Heteroptera: Scutelleridae) 30 minutes after incubation (37 °C temp., pH=7). (PN; Potassium nitrate; AP: Ammonium phosphate; AN: Ammonium nitrate; SP: Sodium phosphate; MS: Magnesium sulfate; CC: Copper chloride; MN: Magnesium nitrate; SN: Sodium nitrate; AS: Ammonium sulfate; MC: Magnesium chloride and DW: Distilled Water).

α-amylase activity compared between cations (K^+, Na^+, Mg^+, NH_4^+ and Ca^{2+}) and anions (PO_4^{3-}, NO_3^-, SO_4^{2-} and Cl^-) (Table 2). The highest and the lowest amylase activities were observed at the present of K^+ and Mg^{2+}, respectively.

Table 1. Effects of two concentrations of some mineral compounds on the α-amylase activity in the salivary gland of ***Eurygaster integriceps*** (Put.) (Heteroptera: Scutelleridae) 30 minutes after incubation

Compound	Enzyme activity (% control)	
	1 mM	3 mM
Ammonium nitrate	98.9 ^{c}	101.5 ^{ab}
Ammonium phosphate	75.1 ^{d}	103.1^{ab}
Ammonium sulfate	20.6 ^{g}	77.7 ^{c}
Copper chloride	81.4 ^{d}	33.8 ^{e}
Magnesium chloride	38.6 ^{f}	38.6 ^{e}
Magnesium nitrate	29.1 ^{fg}	52.9 ^{d}
Magnesium sulfate	99.4 ^{bc}	96.8^{b}
Potassium nitrate	119.1 ^{a}	100.6 ^{ab}
Sodium nitrate	62.9 ^{e}	57.6 ^{d}
Sodium phosphate	110.5 ^{ab}	97.3 ^{b}
Distilled water (Control)	100.0 ^{bc}	100.0 ^{ab}

Table 2. Effects of different ions on the α-amylase activity in the salivary gland of ***Eurygaster integriceps*** (Put.) (Heteroptera: Scutelleridae) 30 minutes after incubation

Ion	Activity (% control)
Phosphate (PO_4^{3-})	92.8 [b]
Nitrate (NO_3^-)	67.6 [d]
Sulfate (SO_4^{2-})	60.0 [e]
Chloride (Cl^-)	60.0 [e]
Potassium (K^+)	120.0 [a]
Sodium ($Na^{+)}$	86.4 [c]
Copper (Cu^{2+})	57.6 [ef]
Ammonium (NH_4^+)	64.7 [de]
Magnesium (Mg^{2+})	55.7 [f]

Effect of hydrogen ion concentration on the enzyme activity

The results showed that there were considerable activities over a broad range of pH (4.5-10) for the amylase of this species(There were not significant difference at any pH value at $P<0.01$. The activity of α-amylase in the salivary gland of ***E. integriceps*** was higher at pH=5 (Figure 2).

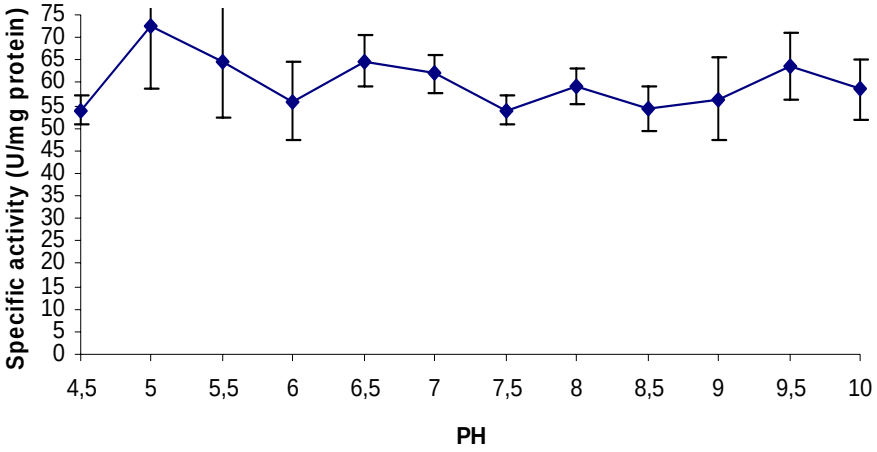

Figure 2. Salivary α-amylase activity of ***Eurygaster integriceps*** (Put.) (Heteroptera: Scutelleridae) at different hydrogen ion concentrations (37 °C).

Discussion

The salivary α-amylase activity proved to be activated strongly by PN in Heteroptera (Hori, 1969). The salivary α-amylases of ***Adelphocoris suturalis*** (Jakovelv) (Heteroptera: Miridae) and ***Lygus disponsi*** (L.) (Heteroptera: Miridae) were activated by PN. The activating effect on PN first occurred at about 10^{-5} M,

reaching a maximum in 10^{-1} M, and then decreased sharply (Hori, 1969; 1972). In agreement with observation of Hori (1969; 1972), our data showed that the salivary amylase of ***E. integriceps*** was activated by PN (1 mM) and not affected by PN (3 mM).

It is well known that some animal amylases are activated or inhibited by certain ions, especially by Cl^- (Applebaum, 1985). Midgut amylase of ***T. molitor*** was slightly activated by Ca^{2+} and Cl^- (Applebaum *et* al., 1961). Inactivation of amylase by dialysis and regeneration by addition of chloride has been recognized in ***Blattella germanica*** (L.) (Dictyoptera: Blatellidae) (Applebaum, 1985). Chloride compounds reduced α-amylase activity in many species of Heteroptera such as ***Carbula humerigera*** (Uhler) (Heteroptera: Pentatomidae) (3%), ***Carpocoris purpureipennis*** (De Geer) (Heteroptera: Pentatomidae) (11%), ***Palomena angulosa*** (Motschulsky) (Heteroptera: Pentatomidae) (8%), ***Pentatoma rufipes*** (L.) (Heteroptera: Pentatomidae) (28%), ***Graphosoma rubrolineatum*** (Westwood) (Heteroptera: Scutelleridae) (10%), ***Heterogaster urticae*** (Fabricius) (Heteroptera: Lygaeidae) (28%) and ***Lygus nigronasutus*** (L.) (Heteroptera: Miridae) (22%) (Hori, 1969, 1972; Zeng & Cohen, 2000). Data from this study indicated that chloride compounds were inhibitors for amylase from ***E. integriceps***.

Rhynchosciara spp. (Diptera: Sciaridae) larval amylase was calcium–dependent and was competitively inhibited by Hg^{2+} and non-competitively by Cu^{2+} (Applebaum, 1985). Our data showed that Cu^{2+} was an inhibitor for amylase ***E. integriceps***, too.

Hori (1969) stated that the polygalacturonase activity in salivary gland of ***Lygus rugulipennis*** (Pop.) (Heteroptera: Miridae) was greatly affected by salts in the incubation medium. Inhibitory salts were $CaCl_2$, $FeCl_2$, $FeCl_3$ and $MgCl_2$. He also reported that the salivary phosphatase activity was inhibited in alkaline solution. For example, addition of 0.01 M potassium phosphate caused 50% inhibition of the enzyme activity. In the present study, the salivary amylase of ***E. integriceps*** was inhibited by CC, AS, SN, MC, MN (all at the concentrations of 1 and 3 mM) and AP (1 mM), strongly.

In this study, it was further found that the salivary amylase was inhibited weakly by PO_4^{3-} and Na^+, and strongly by NO_3^-, NH_4^+, SO_4^{2-}, Cl^-, Cu^{2+} and Mg^{2+}.

It is well known that plants always contain some of ions in stem, petioles, veins and seeds. For example, grain in wheat contained 0.58% potassium, 0.41% phosphorous, 0.19% sulfur, 0.18% magnesium, 01% sodium and 8.2 ppm copper (Taiz & Zeiger,2006). It is also well known that the plant usually absorb nitrogen in the form of nitrate from the root as the nutrient to be used for nitrogen metabolism of the plant. Apart of the absorbed nitrogen and other compounds, especially when it is abundantly absorbed, it is accumulated in the ear of wheat. ***E. integriceps*** is fond of feeding on the ear of wheat, in which Mg^{2+}, SO_4^{2-}, Cl^- and Cu^{2+} may be so

abundant that the activity of amylase which was injected to the plant tissue from the salivary gland might be strongly inhibited and the worst condition might occur for ingestion of nutriment by the bug (Hori, 1975).

The enzyme content of the salivary secretion in Heteroptera undoubtedly plays a major role in the differentiation to their different life habits (Silva & Terra, 1997). It is to be supposed that the inhibiting effect of some mineral compounds on the digestive enzymes may offer an disadvantageous condition for digestion of food (Hori, 1970; Cohen, 1993; Payan, 2004).

Making insect-resistant plants requires the characterization of α-amylase of the target insect and the identification of suitable inhibitors from plants or other sources (Strobl et al., 1998). Our data showed that several mineral compounds can inhibit the amylolytic enzyme. Among them, the magnesium compounds have been described as extremely effective. Changing the concentrations of some of these ions in the wheat plants would be probably of great importance and might produce resistance to ***E. integriceps***. In this field, compound such as SO_4^{2-}, Cl^-, Mg^{2+}, NH_4^+ and Cu^{2+} are sufficient candidates. Changing the concentration of some of mineral compounds is very difficult. This case need to be studied for several years so that they would not be deleterious for plants, human and non-target animals (Payan, 2004).

Successful results in the past have been obtained with inhibitors that completely inhibited their target enzymes but recent results show that even partial inhibition can give substantial control of insect pests (Ishimoto & Kitamura, 1989).

In order to be of practical use for the production of transgenic plants, α-amylase inhibitors should have appropriate specificity profiles. On the one hand, they should ideally be effective against the full range of potential predatory insects (Morton et al., 2000). However, they must not interpret with the action of endogenous α-amylases, which are of demonstrated importance in, for example, germination (Franco et al., 2002).

In our opinion, the purification and characterization of more insect α-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.

Özet

Eurygaster integriceps **(Put.) (Heteroptera: Scutelleridae)'in salya bezi enzim solüsyonunda alfa amilaz faaliyetine bazı inorganik bileşiklerin etkileri**

Bu araştırmada amonyum nitrat (AN), amonyum fosfat (AP), amonyum sülfat (AS), bakır klorid (CC), magnesyum klorid (MC), magnesyum nitrat (MN), magnesyum sülfat (MS), potasyum nitrat (PN), sodyum nitrat (SN) ve sodyum fosfat (SP)' ın 1 ve 3 mM"lık konsantrasyonları ***Eurygaster integriceps*** (Put.) (Heteroptera: Scutelleridae)'in salya bezi

enzim solusyonunda 30 dakika inkube edildikten sonra alfa amilaz enzim faaliyetine etkileri araştırılmıştır. Saf su ise kontrol olarak ele alınmıştır. Sonuçlar incelendiğinde 10 önleyici bileşik arasında fark önemli çıkmıştır. Bakır klorid, amonium sulfat, sodium nitrat, magnezyum klorid, magnezyum nitrat (1 ve 3mM) ve amonium fosfat (1mM) da önemli derecede alfa amilaz enziminin faaliyetini azaltmıştır. Amonyum nitrat, magnezyum sülfat (1mM), sodyum fosfat ile magnezyum sülfat (3mM)'da faaliyetin artmasına neden olmasına karşın fark önemli bulunmamıştır. Potasyum nitrat, amonyum nitrat ve amonyum fosfat 3mM da çok az miktarda enzim faaliyetinin artmasına neden olmuştur (sırasıyla:%0.6, 1.5 ve 3.1). Potasyum nitrat ve sodyum fosfatın 1 mM konsantrasyonunda enzim faaliyetinin artmasına neden olmuştur (sırasıyla:%19.1 ve 10.5). Enzim faaliyeti 4.5 ile 10 pH arasında değişmekte ve en fazla faaliyet ise pH=5'de ortaya çıkmıştır.

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