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Interaction between larval α-amylase of the tomato leaf miner, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) and proteinaceous extracts from plant seeds

*Mojtaba Esmaeily, Ali Reza Bandani**

Plant Protection Department, College of Agriculture and Natural Resources, University of Tehran, 31587-77871 Karaj, Iran

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Abstract: The tomato leaf miner, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae), is one of the most destructive pest of solanaceae and it prefers tomato (*Solanum lycopersicum* L.). The aim of the current study was to investigate the effects of a wide range of seed proteinaceous extracts from different plant families against *T. absoluta* α-amylase activity. The effect of pH on the inhibitory activity of seed extracts showed that seed extracts of amaranth along with a wheat cultivar (Alvand, Aflak, Sarvdasht, Alborz, and Kavir) produced more than a 50% inhibition of the insect amylase. Aflak wheat seed extract at 10 μg, inhibited 81% of the insect amylase. This percent was the highest inhibition achieved. The other proteinaceous seed extracts had a lower effect on the enzymatic activity. Probit analysis showed that Aflak, Kavir, Alborz, Alvand, Sarvdasht, and amaranth inhibited the amylase activity with an I_{50} of 1.94, 3.24, 3.46, 3.31, 4.97, and 15.39 μg, respectively. The effect of pH on the inhibition of the α-amylase showed the highest inhibition of Amaranth and wheat, at a pH value of 8.0, which corresponds to the pH of the insect's gut. Gel electrophoresis assays confirmed the spectrophotometric assays showing that the α-amylase of the insect gut was affected by the presence of the seed extracts. In the gel assay, a high concentration (14 μg protein) of amaranth proteinaceous seed extract greatly decreased the intensity of the α-amylase band. A high concentration of the Aflak wheat cultivar (10 μg protein) caused the disappearance of the amylase band in the gel. Thus, it is concluded that the physiochemical environment of the insect gut affects the interaction between digestive α-amylase and the metabolites. The experiments showed that seed proteinaceous extracts from non-host plant species, produced more inhibition of the insect amylase when compared to the host plant species. It appears that with evolution, adaptation took place so that insect/s could overcome the plant metabolites.

Key words: *Tuta absoluta*, gut α-amylase, inhibition, seed metabolites

Introduction

The tomato leaf miner, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) is one of the most destructive pest of the tomato in South America where this leaf minor is assumed to be native (Guedes and Picanço 2012). This pest feeds on various species of Solanaceae, and has a particular preference for tomato (*Solanum lycopersicum* L.) (Tropea Garzia *et al*. 2012). The insect feeds on the entire plant including the leaves, stem, and fruit, causing losses of up to 100% (Desneux *et al*. 2010, 2011). Since the larvae feed inside the leaves and other plant parts, control of the larvae is difficult. In infested areas, various control methods such as pesticide use, bio-control using predators and parasitoids, as well as pathogens have been applied (Guedes and Picanço 2012; Guedes and Siqueira 2013; Tomé *et al*. 2013; Zappalà *et al*. 2012, 2013). Pesticide use has its own drawbacks including resistance development, unwanted effects on beneficials, pesticide residues in food, pesticide residues in feeds and in the environment (Metcalf 1989; Siqueira *et al*. 2001; Reyes *et al*. 2012; Biondi *et al*. 2013). Considering all of the adverse effects of chemical pesticides on humans, beneficial organisms, and the environment, a search for alternative control method/s is inevitable (Isman 2006).

Plants have evolved a wide range of metabolites to combat their natural enemies. Considerable investigations have been carried out to develop new control strategies for insect pest control based on plant and microorganism-derived metabolites. Such metabolites are safer for humans and the environment than synthetic insecticides. These plant metabolites include plant essential oils and plant secondary metabolite extracts (Isman 2006; Campolo *et al*. 2014). Since plant seeds are reproductive organs, they are rich in defensive compounds. Up to 10% of the total protein stored in plant seeds is involved in resistance against pests and pathogens (Ussuf *et al*. 2001). Examples of the metabolites enhancing resistance against pests, are lectins (Gatehouse *et al*. 1999), α-amylase inhibitors (Mehrabadi *et al*. 2012; Dastranj *et al*. 2013), protease inhibitors (Saadati and Bandani 2011; Dastranj *et al*. 2013), toxins from *Bacillus thuringiensis* (Bt toxins) (Sharma and Ortiz 2000), and even fusion proteins consisting of plant lectin (Galanthus

*Corresponding address:
abandani@ut.ac.ir

nivalis agglutinin; GNA) linked to toxic peptide (Fitches *et al*. 2004; Down *et al*. 2006; Fitches *et al*. 2010).

Enzyme inhibitors such as amylase and protease inhibitors are extensively found in many plant species especially in seeds of cereals and legumes (Franco *et al*. 2002; Svensson *et al*. 2004; Payan 2004; Sivakumar *et al*. 2006). These molecules play a key role in plant defense against pests and pathogens that cause severe damage to field crops and stored grains (Koiwa *et al*. 1997; Franco *et al*. 2000, 2002; Payan 2004; Svensson *et al*. 2004). To achieve a control strategy based on digestive enzyme inhibitors, it is more advisable to characterise the given enzyme/s and examine the effect of plant seed proteins (Harrison and Bonning 2010). Mehrabadi *et al*. (2010 and 2012) showed a dose dependent inhibitory effect on the Sunn pest's (*Eurygaster integriceps* Puton) gut and salivary gland α-amylase, with the use of triticale proteinaceous seed extract. At a high concentration, the extract produced 80–87% α-amylase inhibition. *Phaseolus coccineus* L. and *P. vulgaris* L. seed proteinaceous extract inhibited the α-amylase activity of *Tecia solanivora* Povolny (Lepidoptera: Gelechiidae) at a pH of 6.0, by 70 and 87%, respectively. However, the α-amylase inhibitor from amaranth seeds inhibited 80% of the insect amylase activity at a pH of 9.0 (Valencia-Jimenez *et al*. 2008). Also, it has been shown that mung bean (*Vigna radiate* L.) proteinaceous seed extract impaired the *Callosobruchus maculates* (Col.: Chrysomelidae) larval development. In addition, they found significant inhibition (up to 100%) of the α-amylase of the insect, in *in vitro* assays (Bannakan *et al*. 2007). The effect of the proteinaceous extract of bean and wheat cultivars on *Tenebrio molitor* (Col.: Tenebrionidae) α-amylase was studied by Dastranj *et al*. (2013). They found that a concentration of 14 µg of protein of the seed extract including bean and the Saymon wheat cultivar, inhibited the α-amylase activity 70.9 and 58.5%, respectively. The studies by Dias *et al*. (2010) demonstrated that the rye α-amylase inhibitor expressed in transgenic tobacco seeds (*Nicotiana tabacum* L.) caused 74% mortality in *Anthonomus grandis* first instar larvae when the transgenic seed flour mixture was used in an artificial diet. Moreover, transformation of *Coffea arabica* L. with the α-amylase inhibitor-1 gene (α-AI1) from the common bean, *P. vulgaris*, caused a considerable reduction in the enzyme activity of the insect (Barbosa *et al*. 2010).

Since there is no study regarding the effect of different seed proteinaceous extracts against the *T. absoluta* α-amylase, the current study was undertaken to investigate the effect of a wide range of proteinaceous seed extracts against the moth α-amylase activity. The plant seeds used in the study were: mung bean (*Vigna radiata* L.), datura (*Datura stramonium* L.), amaranth (*Amaranthus retroflexus* L.), wild oat (*Avena fatua* L.), barley (*Hordeum vulgare* L.), and five wheat (*Triticum aestivum* L.) cultivars (Alborz, Kavir, Sarvdasht, Aflak, and Alvand).

Materials and Methods

Insects

A population of *T. absoluta* was collected from the tomato fields of Hashtgerd Region, Karaj, Alborz province, Iran. The larvae were reared on tomato leaves under laboratory conditions: 25°C (±1°C), with a 16 : 8 h (L : D) photoperiod and 70% (±5%) relative humidity (RH) (Savino *et al*. 2012).

Tuta absoluta gut enzyme extraction

Fourth instar larvae of *T. absoluta* were used for enzyme extraction. Enzyme extraction was done based upon Bandani *et al*. (2009) with slight modification. Briefly, the newly-ecdysed (24 h) fourth instar larval guts were dissected in a 10 mM NaCl solution under a stereomicroscope (ZEISS, Germany). The separated midguts were homogenised in a pre-cooled homogeniser. The homogenates from the preparations were transferred to 1.5 ml centrifuge tubes and centrifuged at 13,500 g for 20 min at 4°C. The clear supernatant was transferred to a pre-chilled eppendorf tube and stored at –20°C for further use.

Extraction of seed proteinaceous extract

Seed proteinaceous extracts were done based on the methods of Baker (1987), Melo *et al*. (1999), and Saadati *et al*. (2011) with slight modification. Briefly, seeds from mung bean, datura, amaranth, wild oat, barley, and five wheat cultivars (Alborz, Kavir, Sarvdasht, Aflak, and Alvand) were powdered thoroughly. Then, 30 g of powdered seeds from each plant species was separately mixed with a solution of 0.1 M NaCl and stirred for 2 h. Centrifugation at 8,000 g for 30 min, followed. Next, the supernatant was placed at 70°C for 20 min to inactivate the enzymes within the seeds. Seed protein was extracted using a saturation of 70% ammonium sulphate followed by centrifugation at 8,000 g for 30 min at 4°C, to collect the pellet. The pellet was taken by ice-cold sodium phosphate buffer (0.02 M and pH 7.0) and dialysed against the same buffer overnight. This dialysed solution was used as the inhibitor in the enzymatic assay tests.

Insect gut pH determination

To determine the gut pH, some standard indicator dyes were used based on Bignell and Anderson (1980). These indicators included: cresol red (pH 7.2–8.8), thymol blue (pH 8–9.6), bromophenol blue (pH 3–4.6), methyl orange (pH 3.1–4.4), bromocresol purple (pH 5.2–6.8), bromothymol blue (pH 6–7.6), neutral red (pH 6.8–8), and alizarin yellow (pH 10.2–12.1). A dissection was done of 40 fourth instars larvae. Their midguts were separated and kept in a watch glass. Then 10 µl of each of the pH indicators was added to the sample (each pH indicator added to 5 midguts) and the development of color was recorded.

α-Amylase and α-amylase inhibition assay

The α-amylase activity was assayed according to the dinitrosalicylic acid (DNS) procedure Bernfeld (1955), using 1% soluble starch (Merck, Darmstadt, Germany) as a substrate, as described by Kazzazi *et al*. (2005). To do the enzyme assay, 10 µl of the enzyme was incubated at 35°C with 500 µl Tris-HCl buffer (pH 8.0) and 40 µl soluble starches for 30 min. The reaction was stopped by the addi-

tion of 100 µl DNS and followed by heating in boiling water for 10 min. Finally, the absorbance was read at 540 nm.

The effect of the seed extracts on the amylase activities was determined as described by Rahimi and Bandani (2014). The five wheat cultivars and wild oat seed extracts were used at concentrations of 0.625, 1.25, 2.5, 5.0, and 10.0 µg of protein. Amaranth and barley seed extracts were used at concentrations of 0.875, 1.75, 3.5, 7.0, and 14.0 µg of protein. Mung bean was used at concentrations of 1.05, 2.1, 4.2, 8.4, and 16.8 µg of protein. Datura was used at concentrations of 0.88, 1.77, 3.55, 7.1, and 14.2 µg of protein.

Larval midgut amylase extract was pre-incubated with the seed extracts (barely, datura, amaranth, wild oat, mung bean, and five wheat cultivars including Alborz, Kavir, Sarvdasht, Aflak and Alvand) at 35°C for 30 min, followed by the determination of the enzyme activity using DNS procedures. Appropriate blanks were included in the experiments as well. The inhibition percentage (I%) was calculated as follows (Mehrabadi *et al*. 2011):

$$I\% = 100 \times [(Abs540\ control - Abs540\ Exp)/A540\ control],$$

where: Abs540 control – the absorbance for the control, Abs540 Exp – the absorbance for the experiment.

Effect of pH and temperature on α-amylase activity

The effect of temperature on α-amylase and protease activity was determined by incubating the reaction mixture at 20, 25, 30, 35, 40, 45, and 50°C for 30 min. Optimal pH for amylase activity was determined using a universal buffer with the pH set at 6, 7, 8, 9, 10, and 11.

Effect of pH and temperature on activity of α-amylase inhibitors

Effects of pH and temperature on activity of α-amylase inhibitors were evaluated based on the methods of Valizadeh *et al*. (2013) with slight modification. To determine the effect of pH on the inhibitory activity of the seed extracts, the highest concentration of seed proteinaceous extracts was incubated along with the enzyme for 30 min at a pH set at 6–11. The enzyme activity was then recorded as described before. The effect of temperature on inhibitory activity was determined by incubating the reaction mixture at 20, 25, 30, 35, 40, 45, and 50°C for 30 min, followed by a measurement of the enzyme activity.

In gel inhibition assay of α-amylase

The effect of proteinaceous seed extracts that in the spectrophotemetric assay produced the greatest inhibition of α-amylase activity, were tested in the gel assays. Electrophoretic detection of amylolytic activity in the gel was done based on the procedures described by Laemmli (1970) and Mehrabadi *et al*. (2011). Briefly, PAGE (Polyacrylamide Gel Electrophoresis) was performed in 10% (w/v) gel for separating gel, and 5% for stacking gel with 0.05% SDS (sodium dodecyl sulfate). Electrophoresis was conducted at a voltage of 90 V until the blue dye reached the bottom of the gel. The gel was rinsed with distilled water and washed with 1% (v/v) Triton X-100 for 20 min. Then, the gel was incubated for 2 h in Tris-HCl buffer (pH 8) containing a 1% starch solution, 2 mM $CaCl_2$, and 10 mM NaCl. Finally, the gel was treated with a solution of 1.3% I_2 and 3% KI to stop the reaction and to stain the unreacted starch background. Zones of α-amylase activities appeared at the light band against the dark background.

Protein determination

Protein concentration was measured according to the method of Bradford (1976), using bovine serum albumin (Bio-Rad, München, Germany) as a standard.

Analysis of data

One-way analysis of variance (ANOVA) was used to compare the data followed by an LSD (least significant difference; 5%) test, when significant differences were found (SAS Institute 1997).

Results

Effect of pH and temperature on α-amylase activity

Application of pH indicators showed that the *T. absoluta* midgut was alkaline (pH 8.5) and α-amylase had the greatest activity at an optimal pH of the midgut i.e. at a pH of 8.0. Amylase activity was significantly high at this pH ($F = 110.6$; $df = 5$; $p < 0.05$) (Fig. 1).

The effect of temperature on the α-amylase activity showed that the greatest α-amylase activity was observed at 35°C and that it was significantly different with other temperatures ($F = 120.1$; $df = 6$; $p < 0.05$) (Fig. 2).

The effect of seed proteinaceous extracts on α-amylase activity

The effects of five concentrations of each of the seed proteinaceous extracts were tested on α-amylase activity of the larval gut. Effects of all seed proteinaceous extracts on amylolytic activity were dose dependent. At the highest concentration of all seed extracts including barley, datura, amaranth, wild oat, mung bean, Alborz, Kavir, Sarvdasht, Aflak, and Alvand, the inhibition was 18, 30, 51, 28, 27, 65, 72, 61, 81, and 68%, respectively. Whilst, at a low concentration, the observed inhibition was 5, 4, 13, 2, 6, 25, 22, 24, 29, and 28%, respectively (Fig. 3 and 4). Amaranth seed extract along with the wheat cultivars produced the greatest inhibition percentage i.e. they produced more than 50% inhibition. Probit analysis showed that Aflak, Kavir, Alborz, Alvand, Sarvdasht, and amaranth inhibited the amylase activity with an I_{50} of 1.94, 3.24, 3.46, 3.31, 4.97, and 15.39 µg, respectively.

Effect of pH and temperature on the inhibitory activity of α-amylase inhibitors

The effect of pH on the inhibition of the α-amylase showed that the greatest inhibition of the amaranth and wheat cultivar (Alvand, Aflak, Sarvdasht, Alborz and

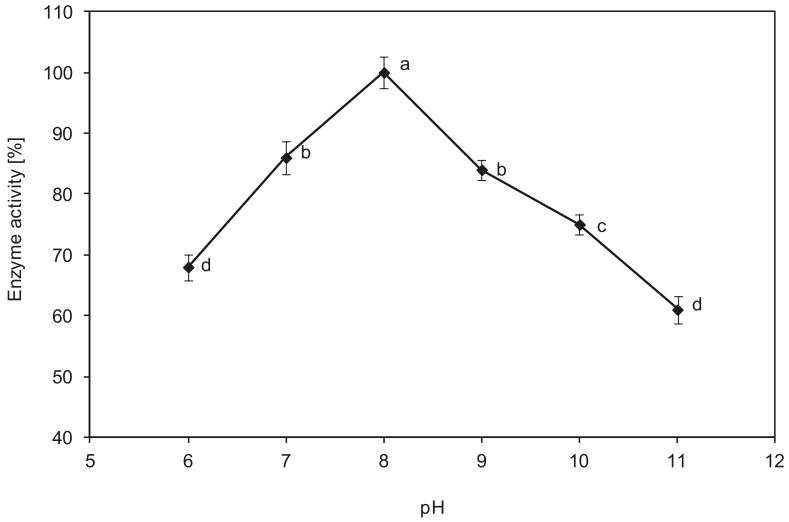

Fig. 1. Effect of pH on the tomato leaf miner α-amylase activity. Activity was determined using universal buffer. Each point represents the average of three measurements

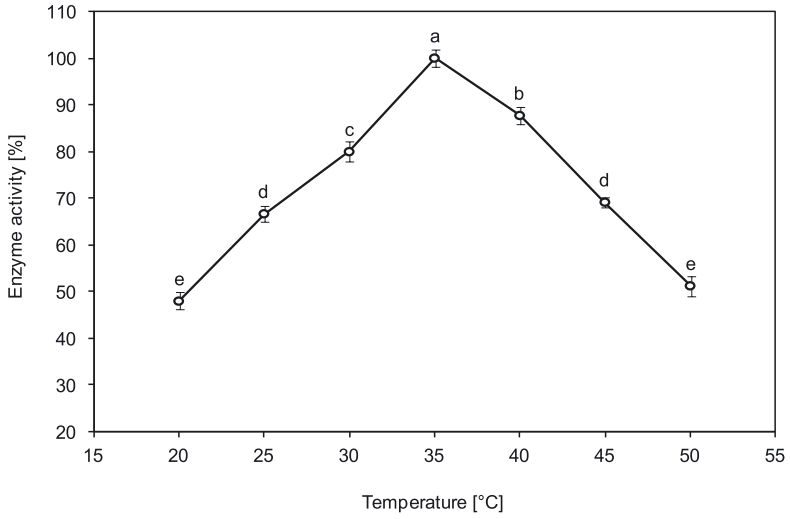

Fig. 2. Effect of temperature on the tomato leaf miner α-amylase activity. Each point represents the average of three measurements

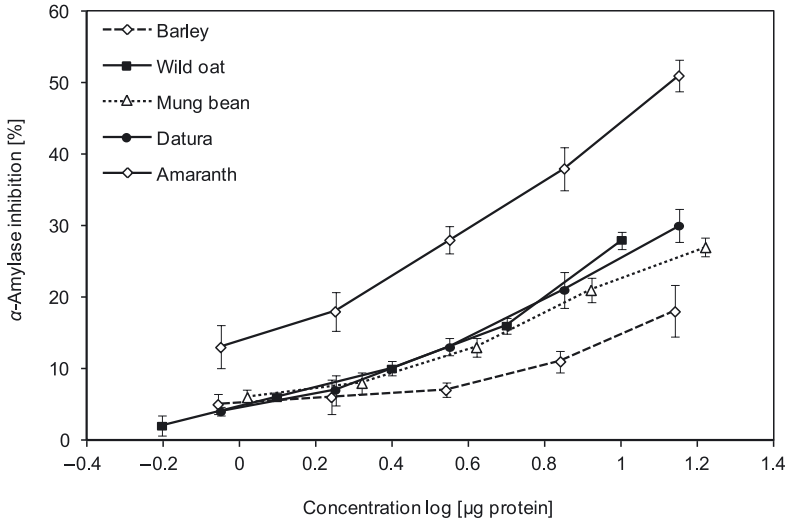

Fig. 3. Inhibition of *Tuta absoluta* α-amylase activity by different concentrations of Amaranth, Datura, Barley, Wild oat and Mung bean proteinaceous extracts. Statistical differences were seen where there was no overlap between error bars (df = 5; $p < 0.05$)

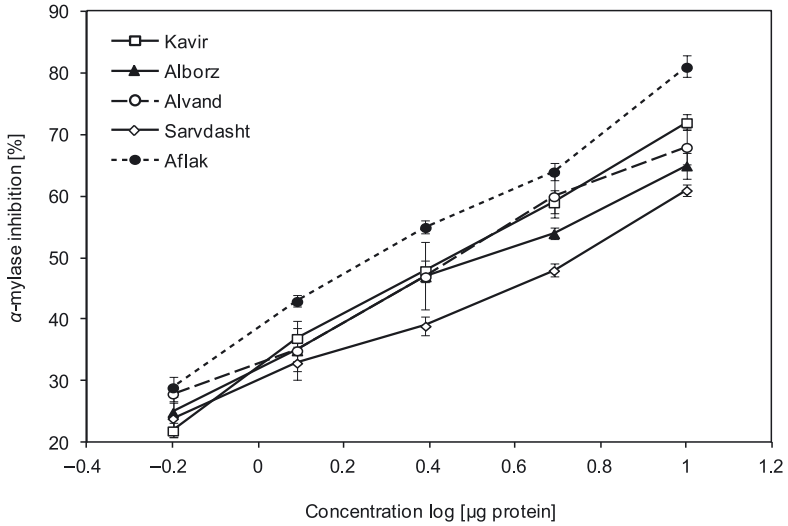

Fig. 4. Inhibition of *Tuta absoluta* α-amylase activity by different concentrations of Aflak, Alborz, Sarvdasht, Kavir and Alvand proteinaceous extracts. Statistical differences were seen where there was no overlap between error bars (df = 5; $p < 0.05$)

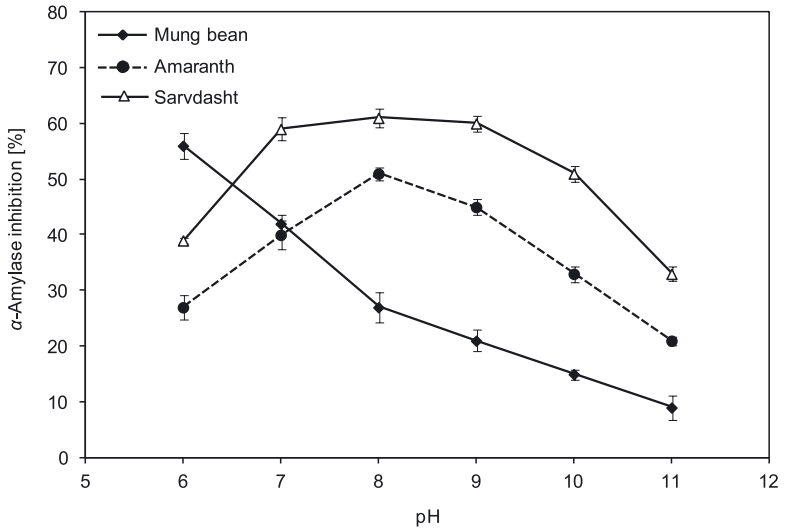

Fig. 5. Effect of pH values on the inhibitory activity of Amaranth, Mung bean and Sarvdasht proteinaceous extracts towards *Tuta absoluta* α-amylase. Statistical differences were seen where there was no overlap between error bars (df = 5; $p < 0.05$)

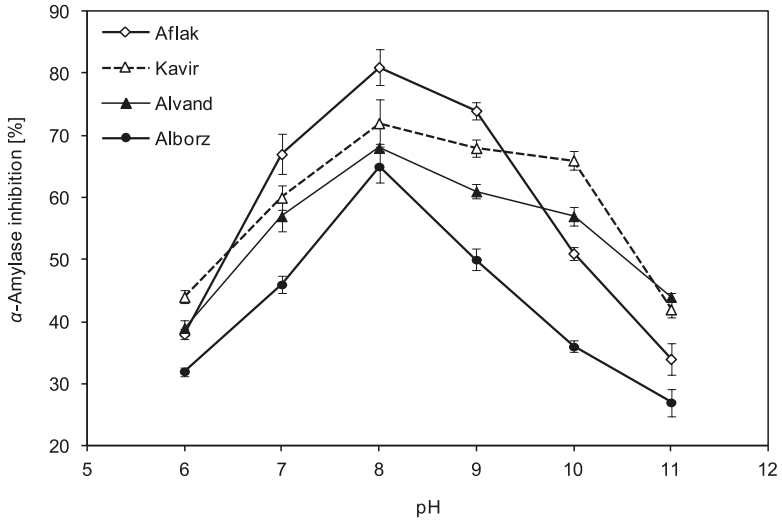

Fig. 6. Effect of pH values on the inhibitory activity of Aflak, Kavir, Alvand and Alborz proteinaceous extracts towards *Tuta absoluta* α-amylase. Statistical differences were seen where there was no overlap between error bars (df = 5; $p < 0.05$)

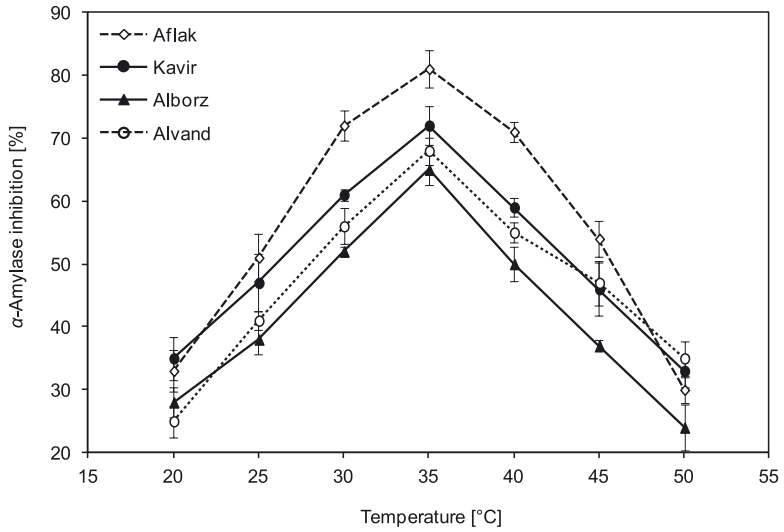

Fig. 7. Effect of temperature on the inhibitory activity of Aflak, Kavir, Alvand and Alborz proteinaceous extracts towards *Tuta absoluta* α-amylase. Statistical differences were seen where there was no overlap between error bars (df = 5; $p < 0.05$)

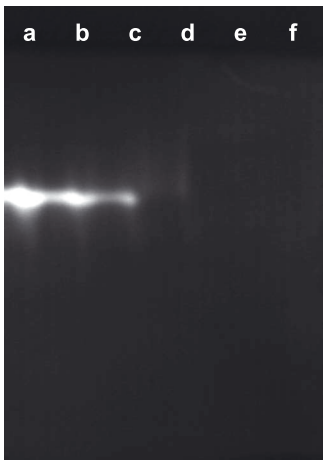

Fig. 8. In gel assay of the effect of Aflak extract on the *Tuta absoluta* α-amylase activity. The extract was pre-incubated with enzyme for 30 min, and then loaded in the gel. Columns from the left hand side are (a) control, (b) 0.625 μg protein extract, (c) 1.25 μg protein extract, (d) 2.5 μg protein extract, (e) 5 μg protein extract, (f) 10 μg protein extract

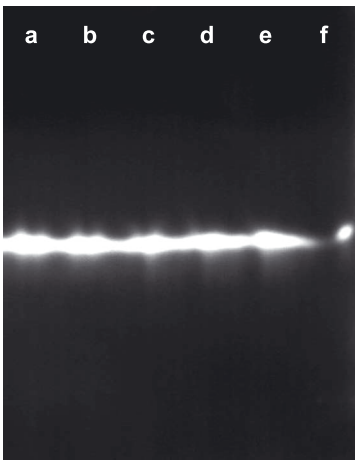

Fig. 9. In gel assay of the effect of Amaranth extract on the *Tuta absoluta* α-amylase activity. The extract was pre-incubated with enzyme for 30 min, and then loaded in the gel. Columns from the left hand side are (a) control, (b) 0.875 μg protein extract, (c) 1.75 μg protein extract, (d) 3.5 μg protein extract, (e) 7 μg protein extract, (f) 14 μg protein extract

Kavir) seed extracts was observed at a pH of 8.0. Mung bean protein extract showed the greatest inhibition at a pH of 6.0 (Fig. 5 and 6). Also, the effect of temperature on the inhibitory activity of the α-amylase showed that the greatest inhibition of the wheat cultivar proteinaceous extract was observed at 35°C. This is the optimum temperature for the activity of this enzyme in the in vitro condition (Fig. 7).

In gel inhibition assay of α-amylase

Gel assays indicated that α-amylase of the insect gut was affected by the presence of the seed extracts as was shown in the spectrophotometric assays. The enzyme was subjected to a series of non-denaturing PAGE after the incubation of the enzyme extract with different concentrations (including 10, 5, 2.5, 1.25, and 0.625 μg protein for Aflak and 14, 7, 3.5, 1.75, and 0.875 μg protein for amaranth) of each seed extract. At a high concentration (10 μg protein) of the Aflak seed extracts, no α-amylase band appeared, but as the concentrations were lowered, α-amylase bands appeared (Fig. 8). A high concentration (14 μg protein) of amaranth proteinaceous seed extract greatly decreased the intensity of the α-amylase band (Fig. 9). Interestingly, one wheat cultivar (Aflak) seed extract showed better inhibition of the α-amylase assay in the gel, seen in the spectrophotometric assay. Thus, both assays (in gel and spectrophotometric assays) supported each other well.

Discussion

Our data showed that the midgut pH of *T. absoluta* larvae is slightly alkaline (pH 8.5) which is similar to the findings of the other researchers e.g. 7.0–7.2 in *Marasmia trapezalis* (Lep.: Pyralidae), 7.0–7.6 in *Trichoplusia ni* (Lep.: Noctuidae), 7.0–7.6 in *Corcyra cephalonica* (Lep.: Pyralidae), 7.1–7.8 in *Ephestia cautella* (Lep.: Pyralidae), 7.2–7.3 in *Cydia molesta* (Lep.: Tortricidae), and 7.3–7.6 in *Pieris rapae* (Lep.: Pieridae) (Berenbaum 1980). Gut pH is important in regulating enzymatic reactions in digestion, dissociating or coagulating ingested proteins, controlling the solubility and toxicity of gut poisons, and determining gut flora (Berenbaum 1980; Terra and Ferreira 1994). Dow (1984) reported a pH value of 12 for midgut lumen of *Acherontia atropos* (Lep.: Sphingidae), 10.8 for midgut lumen of *Lasiocampa quercus* (Lep.: Lasiocampidae), 11.3 for midgut lumen of *Manduca sexta* (Lep.: Sphingidae), and 10.8 for midgut lumen of *Lichnoptera felina* (Lep.: Noctuidae). It has been suggested that having a high gut pH is an adaptation for insects feeding on plant materials rich in tannins. High gut pH lowers the binding of tannin to protein consequently increasing protein digestibility (Dow 1986).

Also, our experiments showed that the α-amylase/s in *T. absoluta* larvae is active in a broad pH range (pH 6.0 to 11.0). The enzyme activity was optimal at pH 8.0 showing optimal activity at alkaline pH as reported for other lepidopteran, such as pH 9.0 for α-amylase activity of *T. solanivora* (Valencia Jimeńez *et al*. 2008) and pH 8.0 for α-amylase activity of *Chilo suppressalis* (Lep.: Pyralidae) (Zibaee *et al*. 2008). There are reports showing a correlation between gut pH and optimal pH of enzyme activity (Terra *et al*. 1996; Bandani *et al*. 2001), i.e. for those insects which have an alkaline gut pH, their gut enzymes have optimum activity at alkaline pH.

Interestingly, the wheat cultivar seed extracts produced the greatest inhibition of the insect amylase e.g. Sarvdasht, Alborz, Alvand, Kavir, and Aflak inhibited the enzyme activity by 61, 65, 68, 72, and 81%, respectively. Whilst the other seed extracts (except for amaranth which produced a 51% enzyme inhibition), produced a low level of the enzyme inhibition. All these inhibition studies were carried out in gel assay which confirmed the spectrophotometric data. One explanation is that this insect species adapted to feed on a wide range of hosts, especially the Solanaceae family (but excluding cereals). Over time, the evolution of the insect overcame the host barriers/metabolites. Also, it was shown that different wheat cultivars produced different percentages of inhibition. This shows that different plant cultivars produce different metabolite/s with a different specificity, in order to protect themselves. In another study, metabolites were extracted from different wheat cultivars, and bean showed a varying specificity toward the α-amylase and protease of *T. molitor* (Dastranj *et al*. 2013). They found that bean seed extract produced the greatest amylase inhibition, when compared to wheat cultivars.

Mehrabadi *et al*. (2011) extracted metabolites of different medicinal plant species and tested these metabolites on the amylase of different insect species. They found that extracts of each of the plant species had a great specificity toward insect species. For example, they showed that *Thymus vulgaris* L. inhibited the α-amylase of *Sitophilus granarius* (Col.: Curculionidae) and *Rhyzopertha dominica* (Col.: Bostrichidae) by 83 and 3%, respectively. *Punica granatum* plant extract inhibited α-amylase activity of *C. maculates* and *R. dominica* by 90.0 and 10.0%, respectively. However, extracts of two plant species, including *Rosmarinus officinalis* L. and *Datura stramonium* L., caused great inhibition (more than 80%) of the α-amylase activity in all insect species [*C. maculates, R. dominica, S. granarius*, and *Trogoderma granarium* (Col.: Dermestidae)] tested.

It is concluded, that the physiochemical environment of the insect gut affects the interaction between the insect α-amylase and the metabolites. Based on our experiments and other references, it is more probable that seed proteinaceous extract from non-host plant species produce more inhibition of the insect amylase, in comparison with host plant species. The indication is that over the course of the insect's evolutionary adaptation, the insect overcame the effect of the plant metabolites. Thus, non-host seed proteins, especially wheat seeds, have a good potential to be used in plant protection. More studies need to be done to purify, characterise as well as identify the gene/s coding of these metabolites.

Acknowledgements

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