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Effect of seed proteinaceous extracts on α-amylase activity of Carob moth, *Ectomyelois ceratoniae* Zeller (Lepidoptera: Pyralidae)

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Abstract
The carob moth is a cosmopolitan pest of many agricultural commodities both in the field and in the storage. The aim of the current study was to investigate the effects of seed proteinaceous extracts against *E. ceratoniae* α-amylase. The results showed that bean, wild oat, pea, mung bean, barley and amaranth inhibited the enzyme activity with the percentage of 17, 26, 32, 37, 51 and 71 respectively. Also, when different concentrations of amaranth and barley extracts were assayed against α-amylase, a concentration dependent trend was observed. Also, amaranth and barley seed extracts had an optimum pH and temperature inhibition of 10 and 35 °C which are the optimum pH and temperature for the activity of this enzyme in the in vitro condition. Thus, it is concluded that amaranth seed proteins are potentially good for detailed investigation in order to get a clear picture of its active compound(s) and its structure-function relationship.

Keywords: The carob moth, α-amylase, proteinaceous extract, inhibition

1. Introduction
The carob moth, *Ectomyelois ceratoniae* Zeller (Lepidoptera), is a cosmopolitan pest of many agricultural commodities both in the field and in the storage [1]. The larvae feed inside the fruit and cause a great damage to fruit quality, so that in some area it spoils more than 80% of the fruits [2]. The chemical control against carob moth is not efficient because its larvae feed and develop inside fruit. Taken together with the increasing environmental and human health concerns due to harmful effects of insecticides, implementation of alternative and more effective control technologies is required to keep carob moth populations below economic levels. These technologies mainly include biological controls such as the use of natural enemies [3], and genetic control methods, namely the sterile insect technique (SIT) and inherited sterility [4, 5].
Considerable investigations have been done on plants and microorganism-derived materials for potentially useful products and genes to be used in pest control. Many plants have received toxic protein encoding genes as a strategy to induce resistance or protection from insect pests and pathogens. These genes which induce enhanced resistance against pests, are lectins [6], α-amylase inhibitors [7], protease inhibitors [8], toxins from *Bacillus thuringiensis* (Bt toxins) [9], and even fusion proteins consisting of plant lectin, *Galanthus nivalis* agglutinin (GNA) linked to toxic peptide [10-12] and so far have received great attention. Digestive enzymes especially α-amylase and proteases are potentially a good target for the insect control using inhibitors in the plant seeds [7]. Cereals and legume seeds are the rich sources of digestive enzyme inhibitors [13] and also it was reported that well-characterized amylase inhibitors are found in plants such as the common bean [14], wheat [15], barley [16] and amaranth [17], and these have been shown to be active against some insect and mammalian amylases. Therefore, it is advisable to characterize digestive enzymes as well as to do *in vitro* and *in vivo* bioassay with plant proteinaceous inhibitors in order to achieve a control strategy based on digestive enzyme inhibitors [18]. Different plant α-amylase inhibitors exhibit different specificities against amylases from diverse sources. Determination of specificity of inhibition is the important first step towards the discovery of an inhibitor that could be useful for generating insect-resistant transgenic plants [13]. Rahimi and Bandani (2014) studied the effect of seed proteinaceous extract of rice (*Oryza sativa* L.), bean (*P. vulgaris* L.) and cowpea (*Vigna unguiculata* L.) on sunn pest α-amylase and found that rice seed extract did not affect significantly α-amylase; however, they showed that bean and cowpea affected the insect α-amylase significantly [19].

Also, Borzoei *et al*., (2013) studied effect of proteinaceous extract of wheat on α-amylase activity of *Plutella xylostella* L. (lepidoptera: Plutellidae). They showed a dose dependent manner of the Diamondback moth gut α-amylase inhibition by wheat proteinaceous seed extract e.g. in a low and high dose of the extract 15.3% and 91.2% of the enzyme was inhibited respectively [20]. Esmaeily and Bandani (2015) studied effect of the proteinaceous extract of wheat cultivars and some other plants on *Tuta absoluta* (Lep.: Gelechiidae) α-amylase. They showed that wheat cultivar seed extracts produced the greatest inhibition, so that Aflak cultivar extract inhibited 81% of the insect amylase [21]. Moreover, transformation of *Coffea arabica* with the α-amylase inhibitor-1 gene (α-AI1) from the common bean, *Phaseolus vulgaris*, caused considerable reduction in enzyme activity of the insect [22].

The aim of the current study was to investigate the effects of seed proteinaceous extracts of barley (*Hordeum vulgare* L.), pea (*Pisum sativum* L.), mung bean (*Vigna radiate* L.), wild oat (*Avena fatua* L.), bean *(Phaseolus vulgaris* L.) and amaranth (*Amaranthus retroflexus* L.) against *E. ceratoniae* α-amylase.

2. Materials and Methods

2.1 Insect rearing

A population of *E. ceratoniae* was collected from Karaj, Alborz province, Iran. The larvae were reared on artificial diet under laboratory conditions at 29.6 ± 5 °C, with a 16:8 h photoperiod and optimal humidity was 75 ± 5% RH as described by Norouzi *et al*., (2008) [23] with slight modification.

2.2 *E. ceratoniae* gut enzyme preparation

Fifth instars larvae of *E. ceratoniae* was used for enzyme extraction. Enzyme extraction was done based on Bandani *et al*., (2009) [24] with slight modification. Briefly, larval guts were dissected in 10 mM NaCl solution under stereomicroscope (ZEISS, Germany). Midguts were separated and homogenized in pre-cooled homogenizer. The homogenates from preparations were transferred to 1.5 ml centrifuge tubes and centrifuged at 13,000 g for 20 min at 4° C. The clear Supernatant was transferred to a pre-chilled Eppendorf tubes. The samples were stored at -20 °C until further use.

2.3 Seed protein extracts

Proteinaceous seed extract of seeds were extracted according to the methods of Baker (1987) [25], Melo *et al*. (1999) [26] and Saadati *et al*. (2011) [8] with slight modification. Briefly, barley, mung bean, wild oat, bean, pea and amaranth seeds (30 g) powdered thoroughly, and then 30 g of powdered seeds from each plant separately was mixed with a solution of 0.1 M NaCl and stirred for 2 h, followed by centrifugation at 8000 g for 30 min. The pellet was discarded, and the supernatant was placed at 70 °C for 20 min to inactivate enzymes within the seeds. Seed protein was extracted using a saturation of 70% ammonium sulphate followed by centrifugation at 8000 g for 30 min at 4 °C. The pellet containing the highest fraction of amylase inhibitors was dissolved in ice-cold sodium phosphate buffer (0.02 M and pH 7.0) and was dialyzed against the same buffer for 20 h. This dialyzed solution was used as inhibitors in enzymatic assay tests.

2.4 α-amylase activity and effect of pH on its activity

α-amylase activity was assayed using the dinitrosalicylic acid (DNS) procedure Bernfeld, (1955) [27] and Zibaee *et al*. (2008) [28], using 1% soluble starch (Merck, Darmstadt, Germany) as the substrate. 10 µl of the enzyme were incubated for 30 min at 35 °C with 500 µl Glycin buffer (pH 10) and 40 µl soluble starch. 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 α-amylase action. The boiling water stops the α-amylase activity and catalyzes the reaction between DNS and the reducing groups of starch. Absorbance was then measured at 540 nm.

The effects of pH on α-amylase activity were examined using enzyme extract from the larval gut as described by Kazzazi *et al*., (2005) [29]. The optimal pH was determined using universal buffer with pH set at 6–12.

2.5 Effect of proteinaceous extracts on α-amylase activity

To determine effect of proteinaceous extracts (barley, mung bean, wild oat, bean, pea and amaranth) on α-amylase activity, enzyme extracts were pre-incubated with different concentrations of extracts (10, 5, 2.5, 1.25 and 0.625 µg protein) for 30 min at 35 °C; then the α-amylase activity was determined at 540 nm as described in the assay procedures. The inhibition percentage (% I) was calculated as follows:

$\%I_{\alpha\text{-amylase}} = 100 \times [(A_{540\ control} - A_{540\ Exp}) / A_{540\ control}]$ where $A_{540\ control}$ is the absorbance for the control and $A_{540\ Exp}$ is the absorbance for the experiment.

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

To determine the effect of pH on inhibitory activity of the seed extracts, highest concentration of amaranth and barley was incubated along with the enzyme for 30 min at pH set at 6-12 using universal buffer and then enzyme activity was recorded. The effect of temperature on α- amylase activity was determined by incubating the reaction mixture at 20, 25, 30, 35, 40, 45 and 50 °C for 30 min, followed by measurement of activity at 540 nm. Controls were run at each temperature and pH value with midgut α-amylase alone as a control, and the percentages of inhibition were calculated from the controls vs. inhibited midgut α-amylase values measured at each temperature and pH.

2.7 Gel inhibition assay of α-amylase

The effect of amaranth was tested in the gel assay. Since other seed extracts did not show a very clear inhibition in the spectrophotometric assay they were excluded in the gel assays. The concentrations of protein extracts for amaranth used in gel assays were 10, 5, 2.5, 1.25 and 0.625 µg protein. Electrophoresis detection of amylolytic activity in the gel was done based on the procedures described by Laemmli (1970) [30]. Briefly, PAGE was performed in 10% (w/v) gel for separating gel and 5% for stacking gel with 0.05% SDS. 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 by 1% (v/v) Triton X-100 for 20 min. Then, the gel was incubated in Glycin buffer (pH 10) containing 1% starch solution, 2 mM $CaCl_2$ and 10 mM NaCl for 1.5 h. Finally, the gel was treated with a solution of 1.3% I_2 and 3% KI to stop the reaction and to stain the un-reacted starch background. Zones of α-amylase activities appeared at the light band against the dark background.

2.8 Protein determination

Protein concentration was measured according to the method of Bradford (1976) [31], using bovine serum albumin as standard.

2.9 Analysis of data
Data were analyzed based on a completely randomized design using SAS software. Mean comparison was done using Duncan's test.

3. Results
3.1 α-amylase activity and effect of pH on the α-amylase activity
The present results indicated that α-amylase activity is present in the gut of *E. ceratoniae* larvae. In gel assays it showed that there are three isoenzymes of the α-amylase in the insect gut; one major band with high molecular weight and two minor bands with small molecular weight (Fig. 6a).
Also, the effect of pH on α-amylase showed that the greatest α-amylase activity was observed at pH 10 and at other pHs was lower (Fig. 1).

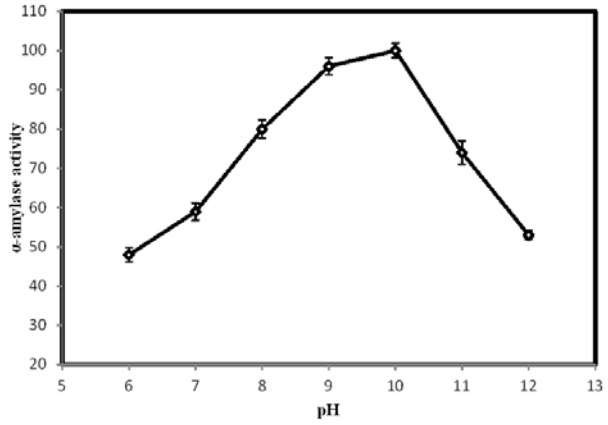

Fig 1: Effect of pH values on α-amylase activity (relative activity) of *E. ceratoniae.*

3.2 Effect of inhibitors on α-amylase activity
When seed extracts were assayed against *E. ceratoniae* α-amylase, the results showed that bean, wild oat, pea, mung bean, barley and amaranth inhibited the enzyme activity with the percentage of 17, 26, 32, 37, 51 and 71 respectively (fig. 2). The extracts of amaranth and barley produced highest amount of amylase inhibition. Thus, these extracts were chosen for further studies. When five different concentrations of these extracts were assayed against α-amylase, a concentration dependent trend was observed (Fig. 3).

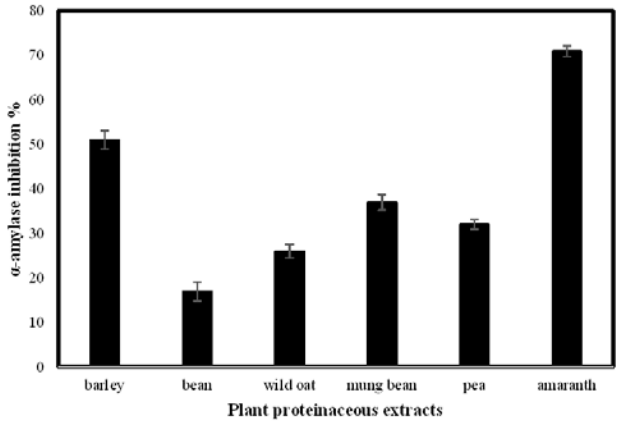

Fig 2: Effect of seed proteinaceous extracts on α-amylase activity of *E. ceratoniae.*

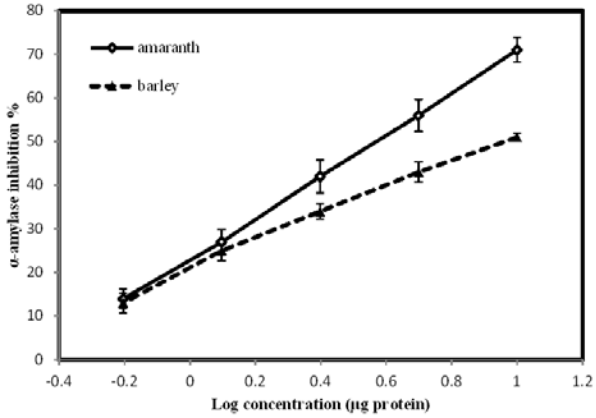

Fig 3: Inhibition of *E. ceratoniae* α-amylase activity by different concentrations of amaranth and barley.

3.3 Effect of pH and temperature on inhibitory activity of α-amylase inhibitors
Results showed that the greatest inhibition of amaranth inhibitor was observed at pH 10 which is the optimum pH for the activity of this enzyme in vitro condition. Inhibitory activity of amaranth and barley on the α-amylase activity at pH 10 was 71 and 51% but at other pHs, inhibition percentage was lower (fig. 4). Also, the effect of temperature on the inhibitory activity of the α-amylase showed that the greatest inhibition of amaranth and barley inhibitors was observed at 35 °C. This is the optimum temperature for the activity of this enzyme in vitro condition (Fig. 5).

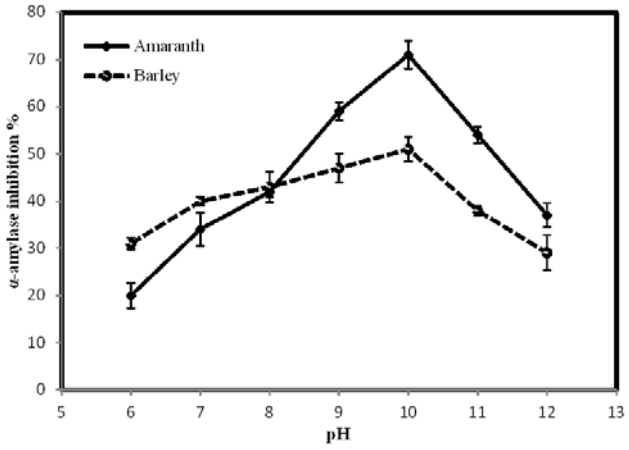

Fig 4: Effect of pH values on the inhibitory activity of amaranth and barley α-amylase inhibitors towards *E. ceratoniae* α-amylase.

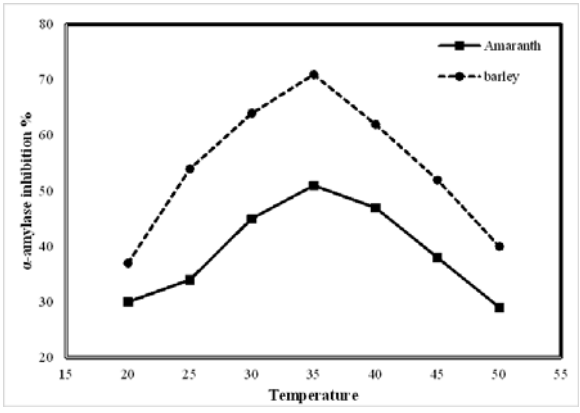

Fig 5: Effect of temperature on the inhibitory activity of amaranth and barley α-amylase inhibitors towards *E. ceratoniae* α-amylase.

3.4 Gel inhibition assay of α-amylase

Using gel assays, it was found that there are three bands in the gut of carob moth with α-amylase activity (Fig. 6a). However, when different concentrations of the amaranth extract were used, varied percentages of inhibition were achieved and three isozymes of the insect gut were affected by the presence of the amaranth seed extract. So that when highest concentration was used, two minor band (A1 and A3) almost disappeared and major band (A2) was slightly decreased (Fig. 6).

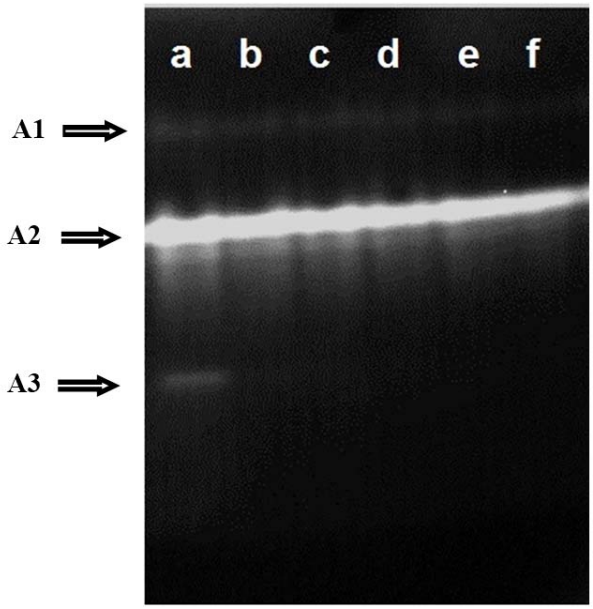

Fig 6: In gel assay of the effect of amaranth extract on the *E. ceratoniae* α-amylase activity. The extract was pre-incubated with enzyme for 30 min, and then loaded in the gel. Columns from the left hand side are (a) control, (b) 0.625 µg protein extract, (c) 1.25 µg protein extract, (d) 2.5 µg protein extract, (e) 5 µg protein extract, (f) 10 µg protein extract.

4. Discussion

So far α-amylase activity has been reported from different insect species including species from Coleoptera, Lepidoptera, Hymenoptera, Diptera and Heteroptera [26, 32-34, 29, 21, 7]. Also, in this study it was found that gut of *E. ceratoniae* larvae contains three different α-amylase isoforms, similar to what has been found in others insects [25, 35-37, 32, 38], that presence of three isoforms is a strategy to escape from inhibitory toxicity [39], and also it indicates the importance of carbohydrates in its diet. Our results show that the α-amylase in *E. ceratoniae* larvae have an alkaline optimal pH, which is consistent with the optimal pH that has been reported by Tabatabaei *et al*. (2011) [40].

In the present study, it was found that seed proteinaceous extracts of six plant species including amaranth, barley, pea, bean, mung bean and wild oat affected α-amylase activity of the insect gut. All the seed extracts inhibited amylase activity but amaranth and barley caused highest inhibition. So that, high concentration of these proteinaceous extracts produced 71 and 51% inhibitory activity of α-amylase, respectively. Also, inhibitory effects of the amaranth extract were studied in the gel and showed that the effect of this extract on the enzyme was dose dependent.

Esmaeily and Bandani (2016) [41] studied the effect of seed proteinaceous extract of datura, wild oat and amaranth on *Xanthogaleruca luteola* α-amylase and found that a dose dependent manner in inhibition of the insect enzyme. Also, they showed that at the highest concentration of protein extracts (12 µg protein) of all three seed extracts including amaranth, wild oat and datura, the inhibition was 71, 79 and 31%, respectively. In another study, metabolites extracted from different wheat cultivars showed varying specificity toward α-amylase of *Tenebrio molitor* L. [38]. They found at concentration of 14 µg protein from seed extract of the different wheat cultivars including MV17, Aflak, sivand, saymon, zare inhibited the α-amylase activity by 58.3, 56.2, 58.5, 57.2, and 48.5, respectively.

Effect of pH on inhibitory activity of α-amylase by amaranth and barley extracts showed that the greatest inhibition was observed at pH 10 which is the optimum pH for the activity of this enzyme in vitro condition. Also, the effect of temperature on the inhibitory activity of the α-amylase showed that the greatest inhibition of the proteinaceous extract was observed at 35 °C. Similar investigation to the study was conducted by Esmaeily and Bandani (2015) [21] showed that the greatest inhibition of α-amylase by amaranth and wheat cultivar (Alvand, Aflak, Sarvdasht, Alborz and Kavir) seed extracts was observed at a pH of 8.0.

In conclusion, based on the results, it can be concluded that Different inhibitions indicated that different plant species produce different metabolite with different specificity in order to protect themselves. So, it could be concluded that inhibitor from amaranth are more promising candidates for more investigation.

5. Acknowledgment

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