A lectin extracted from *Citrullus colocynthis* L. (Cucurbitaceae) inhibits digestive α-amylase of *Ectomyelois ceratoniae* Zeller (Lepidoptera: Pyralidae)

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Abstract

A lectin was extracted from seeds of *Citrullus colocynthis* (Cucurbitaceae) by column chromatography using Sepharose 4B-Galactose and DEAE-Cellulose fast flow. The inhibitory effects of the extracted lectin on digestive α-amylase of *Ectomyelois ceratoniae* larvae were studied using pH, temperature, time of incubation and kinetic parameters. Different concentrations of extracted lectin, *Citrullus colocynthis* agglutinin (CCA), inhibited digestive amylolytic activity by 22-49%. The highest inhibition was obtained at pH 8 and 9, which corresponds with the highest enzymatic activity in the control. The highest inhibition of *E. ceratoniae* α-amylase was found at 40°C, which corresponds with the optimal temperature for enzymatic activity. Time-course experiments revealed the highest amylolytic activity at 20-40 min post-incubation, while the highest inhibition was found after 20-30 min. Kinetic analysis showed that incubation of α-amylase with CCA significantly decreased V_{max}, indicating non-competitive inhibition, but no statistical difference was found in the K_m value. Our results indicated that CCA significantly inhibited activity of digestive α-amylase in *E. ceratoniae* larvae, suggesting its possible application as a potential alternative control method against this pest.

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Introduction

Ectomyelois ceratoniae Zeller (Lepidoptera: Pyralidae) is the major pest of pomegranate and some dried fruits that annually causes 15-90% damage (Farazmand *et al.,* 2008). Adults are gray moths with a wingspan of 9-12 mm. Larvae are pink, and hibernate in infested fruits on the soil surface. Adults lay their eggs on the pomegranate crown, and larvae hatch and feed on tissues around the pomegranate grains (Farazmand *et al*., 2008). Different control tactics, such as collection of infested fruits, removal of pomegranate crowns, and release of biocontrol agents, have been used to decrease damage by *E. ceratoniae* larvae (Farazmand *et al*., 2008).

Lectins are the heterogenous proteins that bind reversibly to mono- or oligosaccharides (Peumans & Van Damme, 1995). These molecules have been extracted from plants, fungi, bacteria and animals (Komath *et al*., 2006). In plants, lectins have a critical role in plant-insect co-evolution (Chen, 2008). Various lectins have been extracted from plants, such as ASA I and ASA II from *Allium sativum* L., rice, legumes and cucurbitaceae (Peumans & Van Damme, 1995; Van Damme, 1998; Zhu-Salzman *et al*., 2002; Jiang *et al*., 2006; Clement *et al*., 2010; Clement & Venkatesh, 2010). Several studies have confirmed lectins as being insecticidal, and transgenic crops expressing lectin genes have been introduced in many economically important crops (Bell *et al*., 1999; de Oliveira *et al*., 2001). For example, the efficiency of *Galanthus nivalis* agglutinin (lectin) has been determined in potato (Down *et al*., 1996; Gatehouse *et al*., 1996), rice (Foissac *et al*., 2000; Nagadhara *et al*., 2004), maize (Wang *et al*., 2005), tobacco (Hilder *et al*., 1995), wheat (Stoger *et al*., 1999), tomato (Wu *et al*., 2000) and sugarcane (Sétamou *et al*., 2002, 2003; Li & Romeis, 2009).

Citrullus colocynthis L. is a medicinal plant belonging to the Cucurbitaceae family that is native to Iran and which is found in the southern and eastern regions (Tavakkol-Afshari *et al*., 2005). Fruits contain bitter glycosides that are used as drugs for gut and liver disorders. In addition to having anti-viral and anti-cancer properties, the crude fruit extract is effective in decreasing blood sugar (Tavakkol-Afshari *et al*., 2005). α-amylase (α-1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) is a hydrolytic enzyme that catalyzes the hydrolysis of endo-α-D-(1,4)-glucan linkages in glycogen and other related carbohydrates (Strobl *et al*., 1998; Franco *et al*., 2000). There are six different classes of α-amylase inhibitors, which are known as lectin-like, knottin-like, cereal-type, Kunitz-like, c-purothionin-like, and thaumatin-like, that may be useful in pest control (Franco *et al*., 2002). These inhibitors show structural diversity leading to different modes of inhibition and different specificity against a diverse range of α-amylases (Mehrabadi *et al*., 2010).

Although the effect of lectins on epithelial cells has been well elucidated, their inhibitory mechanisms on digestive enzymes remains

unclear. Therefore, this study was conducted to determine the effective concentration of *C. colocynthis* lectin, *Citrullus colocynthis* agglutinin (CCA) on digestive α-amylase of *E. ceratoniae* by considering pH, temperature, time, kinetic parameters and gel electrophoresis.

Material and methods

Insect rearing

E. ceratoniae larvae were collected from pomegranate gardens in Yazd and fed on artificial diet containing wheat bran (100 g), yeast (3 g), sugar (10 g), glycerine (40 mL) and water (40 mL). Adults were allowed to lay eggs, and newly hatched larvae were reared on artificial diet to reach fourth larval instar. Growth conditions were 28±2°C, 85% relative humidity and photoperiod of 16L:8D.

Preparation of Sepharose 4B-Galactose column

To prepare the column, 20 mL of Sepharose 4B was suspended in 40 mL of 0.5 M Na_2CO_2 (pH 11.0), then 2 mL of divinylsulphone was added to the suspension and the mixture was incubated for 70 min at room temperature with gentle agitation. After activation, 500 mg of galactose was added in 50 ml 0.5 M Na_2CO_2 (pH 11.0) and the suspension were re-incubated for an additional 12 h. The sorbent was washed with water; the unbound arm was blocked with b-mercaptoethanol-containing buffer, and then packed into a 1.5×30 cm column. The sorbent was equilibrated with Tris-HCl (0.1 M) and used for the affinity purification of CCA.

Purification of *Citrullus colocynthis* agglutinin

Seeds of *C. colocynthis* were ground to fine powder using a mill. The dry powder was incubated in phosphate buffer (0.1 M pH 7.1) for approximately 20 h at 4°C. The mixture was then centrifuged at 4000 g for 20 min, and the remaining debris removed by passing the supernatant through filter paper (Whatman No. 4) (Hamshou *et al*., 2010). Supernatant was precipitated by 0-60% concentrations of ammonium sulfate and centrifuged at 4000 g for 20 min. Debris was eluted in Tris-HCl buffer (0.1 M, pH 7) and dialyzed in the same buffer overnight (de Oliveira *et al*., 2011). Affinity chromatography was performed on a Sepharose 4B-galactose column equilibrated with Tris-HCl buffer (0.1 M, pH 7). After loading the extract, the affinity column was washed with buffer and the bound lectin was eluted with 20 mM of 1,3-diaminopropane (DAP) (Hamshou *et al*., 2010). Fractions showing the highest protein content were pooled and used for the next step. The lectin fractions obtained after the first affinity chromatography were loaded on an anion exchange chromatography column of DEAE-Cellulose fast flow, equilibrated with DAP (Hamshou *et al.*, 2010). After washing with DAP, the lectin was eluted using Tris–HCl (0.1 M, pH 7.0) containing 0.5 M NaCl. Finally, the lectin fractions were dialyzed against water and lyophilized. The purity of the lectin was analyzed by SDS-PAGE.

Sample preparation

E. ceratoniae larvae (4^{th} instars) were randomly selected and dissected under a stereo-microscope in ice-cold saline solution (10 mM). Larval bodies were cut separately using a scalpel and the midgut exposed by removal of fat bodies and other undesirable organs. The midgut was separated from the larval body and rinsed in ice-cold distilled water. The mixture was placed in a pre-cooled homogenizer and ground before centrifugation. Equal portions of larval midgut and distilled water were used to obtain a desirable concentration of the enzyme (W/V). Homogenates were separately transferred to 1.5-mL centrifuge tubes and centrifuged at 25,000 g for 20 min at 4°C. The supernatants were pooled and stored at –20 C for subsequent analyses. All the experiments were conducted immediately following sample preparation.

α-amylase assay

The method described by Bernfeld (1955) was used to assay α-amylase activity. Ten microliters of the homogenate was incubated for 30 min at 35°C with 50 L of phosphate buffer (0.02 M, pH 7.1) and 20 l of soluble starch (1%) as substrate. The reaction was stopped by addition of dinitrosalicylic acid (DNS, 80 μL) and heated in boiling water for 10 min prior to reading the absorbance 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. The negative control contained all reaction mixtures with pre-boiled enzyme (for 15 min) to prove enzyme presence in the samples.

Inhibition of α-amylase by different concentrations of *Citrullus colocynthis* agglutinin

To find possible inhibition of the digestive α-amylase, 50 μL of PBS (0.02 M, pH 7.1), 20 μL of starch 1% and 20 μL of different concentrations of lectin (0, 0.1, 0.5, 1, 1.5 and 2 mg/mL) were incubated for 5 min. Then, 10 μL of the enzyme was added and the reaction continued as described above. Blanks were run containing PBS, starch 1% and each concentration of lectin.

Effect of pH on α-amylase inhibition by *Citrullus colocynthis* agglutinin

Effect of pH on CCA inhibition on α-amylase was determined at different pH values using Tris-HCl buffer (20 mM) at pH levels of 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. The enzyme activity was assayed after incubation of the reaction mixture containing Tris-HCl buffer (at a given pH value), starch 1%, CCA (2 mg/mL) and midgut homogenate. Controls were run at each pH value with midgut α-amylase alone as a control. Other steps were carried out as previously described.

Effect of temperature on α-amylase inhibition by *Citrullus colocynthis* agglutinin

To obtain the effect of temperature on α-amylase inhibition by CCA, the reaction mixture containing Tris-HCl (20 mM pH, 9), starch 1%, CCA (2 mg/mL) and enzyme was incubated at different temperatures of 15, 20, 25, 30, 35, 40, 45, 50 and 60°C. A control was carried out without the inhibitor. Other steps were carried out as previously described.

Time-course inhibition of α-amylase by *Citrullus colocynthis* agglutinin

Time-course inhibition of α-amylase by CCA was carried out by incubating the enzyme extract with CCA and other reaction constituents in Tris-HCl buffer (20 mM, pH 9) at 40°C for different time intervals of 10, 20, 30, 40, 50 and 60 min. Other steps were carried out as previously described.

Kinetic studies

Kinetic parameters of inhibition and control were carried out with increasing concentrations of starch as the substrate (0.5-2.0%) in the presence of CCA (2 mg/mL). Lineweaver-Burk plot analysis was done based on the data to find affinity of enzyme to substrate (K_m) and velocity of enzyme (V_{max}) values.

Inhibition in non-denaturing PAGE

Enzyme extract was pre-incubated with different concentrations of CCA for 30 min at 30°C, then the remaining α-amylase activity was determined by polyacrylamide gel electrophoresis. PAGE was carried

out using the procedures described by Laemmli (1970). Concentrations of resolving and stacking gel were 12 and 4%, respectively. Electrophoresis was conducted at a voltage of 70 V until the blue dye reached the bottom of the slab gel. The gel was rinsed with distilled water and washed with 1% (v/v) of Triton X-100. The gel was then immersed in a solution of PBS (0.02 M pH 7.1) containing 1% starch, 10 mM of NaCl and 2 mM of $CaCl_2$. Finally, it was stained with solutions of 1.3% I2, and 3% KI to obtain white bands with dark backgrounds.

Protein assay

Protein concentrations were assayed according to the method described by Lowry *et al*. (1951).

Statistical analysis

All data were compared by one-way analysis of variance (ANOVA) followed by Tukey's test at the P≤0.05 level.

Results

In the current study, a lectin with a molecular weight of 14.5 kDa was extracted from seeds of *C. colocynthis* (Figure 1), which significantly inhibited digestive α-amylase of *E. ceratoniae* by 22-49% (Figure 2A). Also, incubation of larval midgut homogenate with 2 mg/ml of CCA decreased sharpness of amylolytic isozymes (Figure 2B). The protein was able to inhibit 50% of total amylolytic activity both in assay conditions and with gel electrophoresis (Figure 2).

The effect of pH on α-amylase inhibition by CCA is shown in Figure 3A. There were significant differences among pH levels (F=5.43, P=0.004), with the highest inhibition at pH 8; there was slightly less inhibition, although not different, at pH 9 (Figure 3A). The optimal pH of the α-amylase (control) was observed at pH 8 and 9 (Figure 3B) (F=18.56, Pr>F: 0.0001). In the case of temperature, the highest inhibition of α-amylase was found at 40°C, while the optimal temperature was observed to be between 20-45°C (Figure 4; Pr>F: 0.004, F=5.02; Pr>F: 0.0001, F=21.21).

Results revealed the highest inhibition of the enzyme at 20-30 min of post-incubation (Figure 5A). In the control, the highest enzymatic activity was observed at 20-40 min of post-incubation (Figure 5B). There was a correlation between the times of the highest enzymatic activity and the highest inhibition.

In this study, V_{max} and K_m values for the control were found to be 0.46

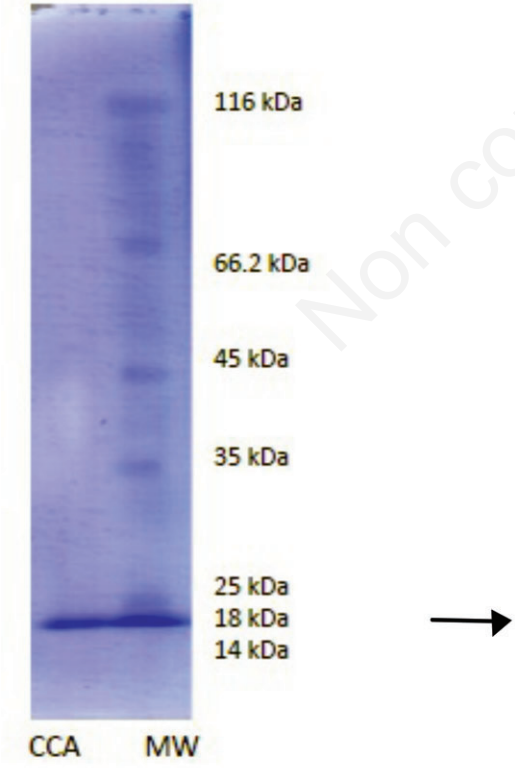

Figure 1. SDS-PAGE showing purity and molecular weight of the purified lectin. CCL, *Citrullus colocynthis* lectin; MM, molecular weight.

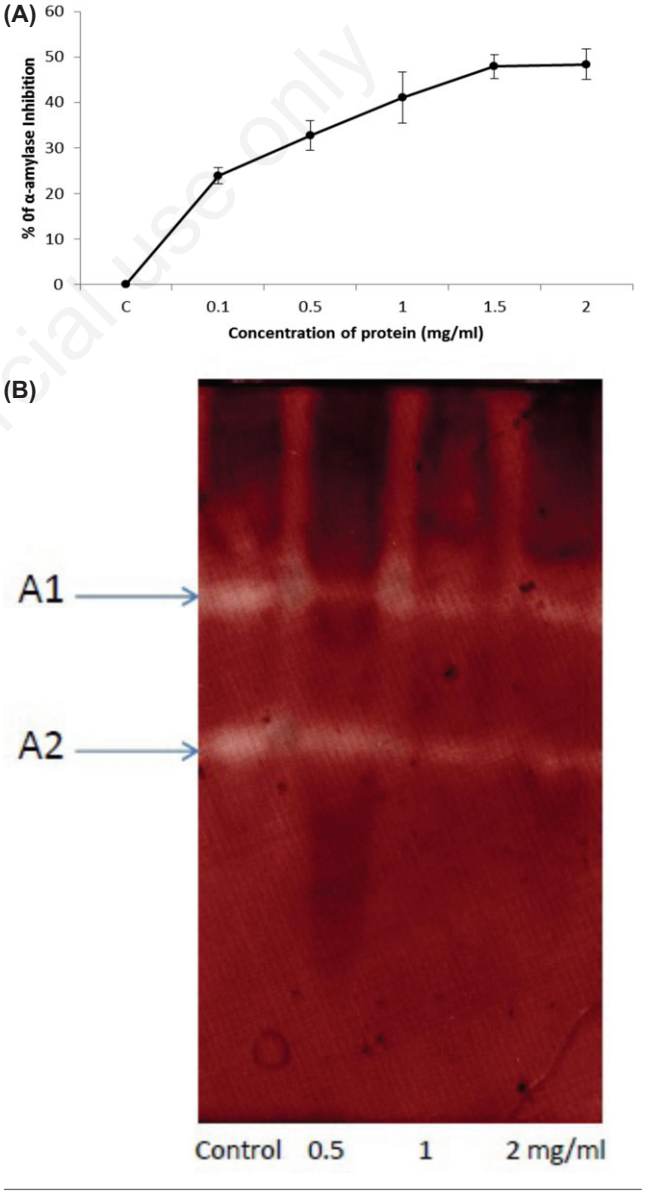

Figure 2. Inhibition of *E. ceratoniae* α-amylase by different concentrations (mg/mL) of *C. colocynthis* lectin in the enzymatic assay (A) and gel electrophoresis (B). Reaction conditions were phosphate buffer (pH 7) at 30°C.

OD/min and 1.08%, respectively (Figure 6) while these parameters were 0.155 OD/min and 0.96 % in the incubation of the enzyme with CCA (Figure 6). Kinetic analysis showed that incubation of enzyme with inhibitor significantly decreased the V_{max} parameter, indicating non-competitive inhibition. Although a change in K_m value was observed, it was not statistically different.

Discussion and conclusions

One of the promising alternatives for insect control is the use of biotechnological processes to provide resistant varieties of host plants. There are several genes identified to do so, such as Bt toxins, digestive enzyme inhibitors, chitinases and lectins (Bishop *et al*., 2000; Sales *et al*., 2000; Carlini & Grossi-de-Sa, 2002; Bertrand *et al.*, 2003; Bellincampi *et al.*, 2004; Haq *et al.*, 2004; de Azevedo Pereira *et al*., 2006). Several classes of plant proteins have been discovered and characterized, including lectins, ribosome-inactivating proteins, and protease and α-amylase inhibitors, which have shown insecticidal effects on different insect pests (Ishimoto *et al*., 1989; Ryan, 1990; Chrispeels *et al.*, 1998; Gatehouse & Gatehouse, 1998; Ussuf *et al*., 2001). There are six different classes of α-amylase inhibitors: lectin-like, knottin-like, cereal-type, Kunitz-like, c-purothionin-like, and thaumatin-like, which may be useful in pest control (Franco *et al*., 2000; Bonavides *et al*., 2007). Suzuki *et al*. (1994) believed that these inhibitors had a high degree of sequence homology and specificity. Different studies have also been carried out determining types of lectin-like inhibitors and their effects on α-amylases of insects (Grossi-de-Sá & Chrispells, 1997; Da Silva *et al*., 2000; Yamada *et al*., 2001). α-AI1 inhibited digestive α-amylases from *Callosobruchus maculatus* Fabricius (Coleoptera: Bruchidae) and *C. chinensis*, but it had no inhibitory effect against *Zabrotes subfasciatus* Boheman (Coleoptera: Chrysomelliade) amylase. Another inhibitor was α-AI2, which was not able to inhibit the first three α-amylases of assayed bruchids, but which inhibited α-amylase of *Z. subfaciatus* (Grossi-de-Sá & Chrispells, 1997; Da Silva *et al*., 2000; Yamada *et al*., 2001). Mirkov *et al*. (1994) believed that these types of inhibition indicated an evolutionary relationship with phyto-hemagglutinnins and arcelins.

Temperature and pH are the two critical factors in biochemical reactions that could affect both activity and inhibitory mechanisms. In the current study, the highest inhibition of α-amylase occurred at an alkaline pH, where the highest enzymatic activity was observed. However, in the case of *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae), the highest inhibition by triticale extraction was observed at pH 5 and 6 for both salivary and midgut α-amylases (Mehrabadi *et al*., 2010; Mehrabadi *et al*., 2012). Several authors have confirmed a pH-dependent interaction between amylases and inhibitors (Powers & Whitaker, 1977; Valencia *et al*., 2000; Mehrabadi *et al*., 2010, 2012). Extracted αAI from *Phaseolus vulgaris* L. inhibited porcine pancreatic α-amylase at pH 5.5, an effect that varied at pH 4.5 to 5.5, depending on the strain of bean used (Barbosa *et al*., 2010). Since the gut lumen of insects is the place where the interaction between α-amylase and inhibitors occurs, the pH showing the highest inhibition may reflect the fact that the pH of insect midgut is alkaline (Ranjbar *et al*., 2011). Since α-amylase may have the highest activity under such conditions, it should be more inhibited if CCA

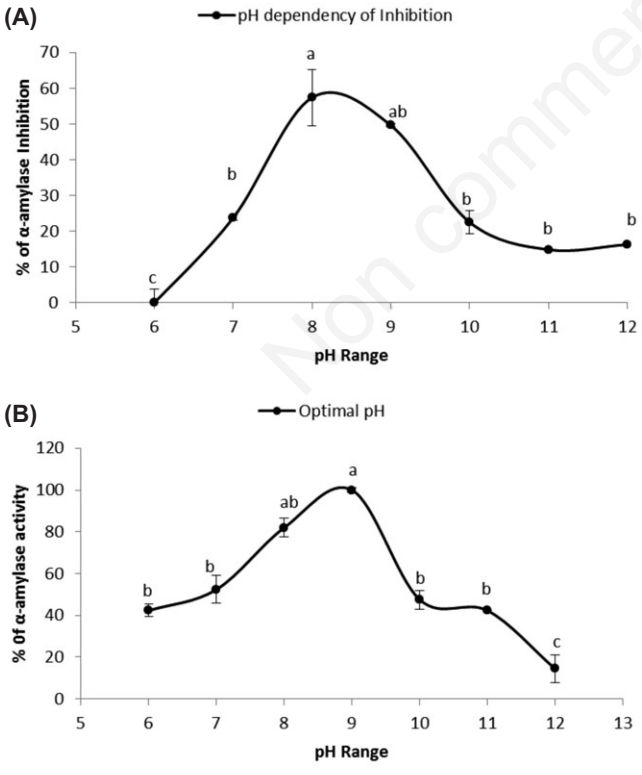

Figure 3. A and B) pH dependency of *E. ceratoniae* α-amylase inhibition by *C. colocynthis* lectin (2 mg/mL) *versus* the control. Reaction conditions were Tris-HCl buffer (20 mM, pHs 6-12) at 30°C. Different letters indicate statistical difference among values, Tukey's test (P≤0.05).

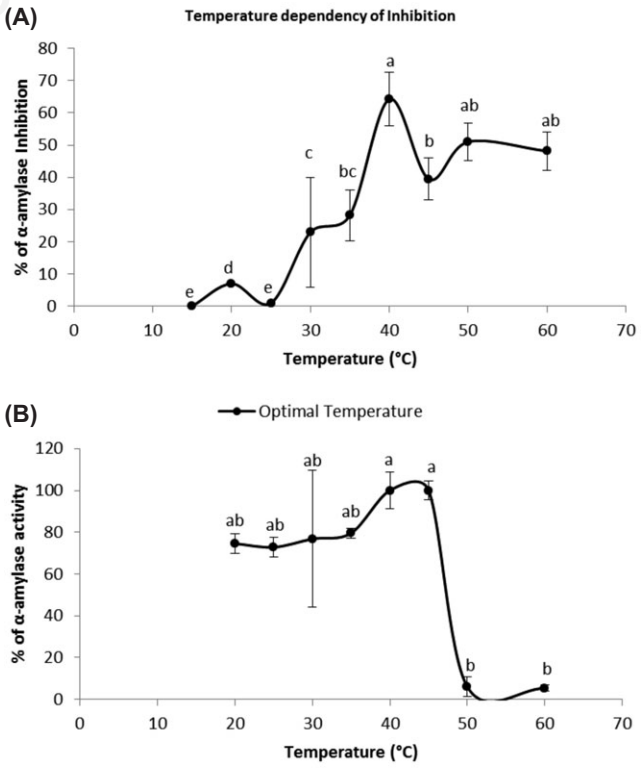

Figure 4. A and B) Effect of temperature on *E. ceratoniae* α-amylase inhibition by *C. colocynthis* lectin (2 mg/mL) *versus* control. Reaction conditions were Tris-HCl buffer (20 mM, pH 8) at different temperatures (°C). Different letters indicate statistical difference among values, Tukey's test (P≤0.05).

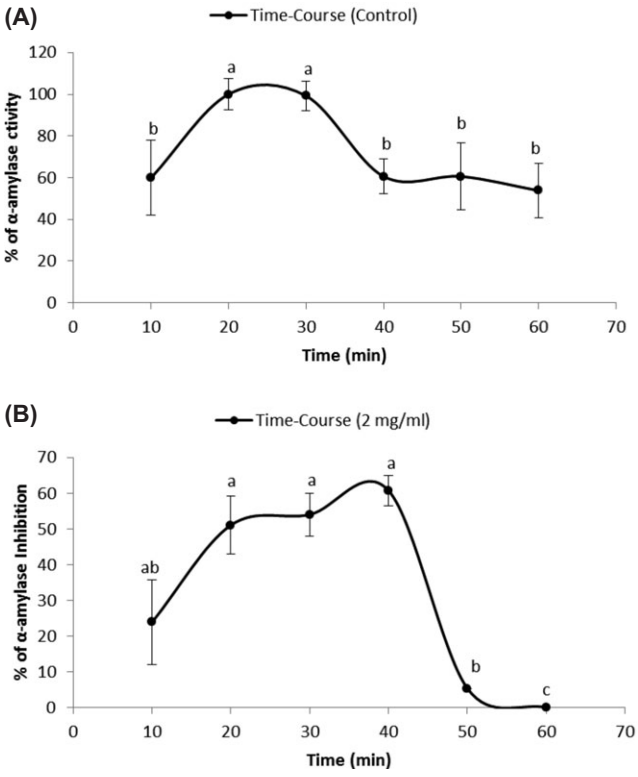

Figure 5. A and B) Time course inhibition of *E. ceratoniae* α-amylase by *C. colocynthis* lectin (2 mg/mL). Midgut samples were pre-incubated with inhibitor in 20 mM Tris-HCl buffer (pH 8) at 30°C, after which enzyme was added and reaction was recorded at the given time intervals. Different letters indicate statistical difference among values, Tukey's test (P≤0.05).

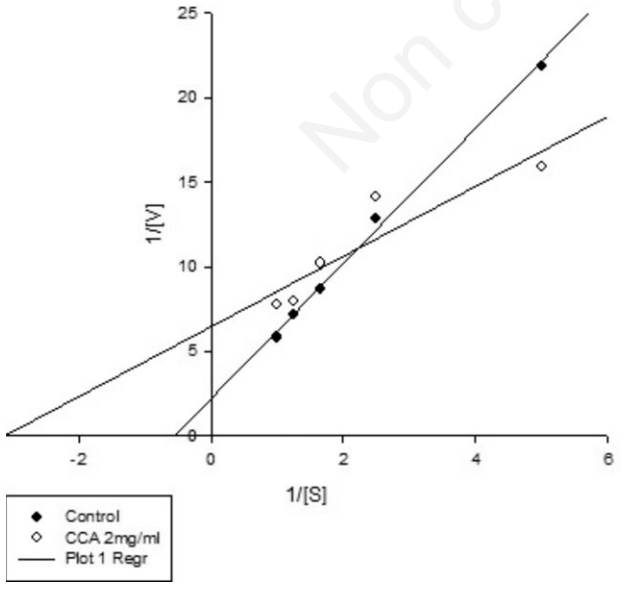

Figure 6. Effect of proteinaceous α-amylase inhibitor from *C. colocynthis* on amylolytic activity in the midgut of *E. ceratoniae* by Lineweaver-Burk plot *versus* control.

is available at an appropriate concentration. This phenomenon has been described in other studies (Biggs & McGregor, 1996; Valencia *et al*., 2000; Mehrabadi *et al*., 2010, 2012). The observed specific temperature for inhibition and activity of α-amylase could reflect the environmental temperature where *E. ceratoniae,* a poikilothermic organism, lives. Regarding incubation time for inhibition, Mehrabadi *et al*. (2010) demonstrated the maximum amylolytic inhibition in the midgut of *E. integriceps* by triticale extract after 20-30 min post-incubation. Similar results were found by Marshall & Lauda (1975) and LeBerre-Anton *et al.* (1997).

Lineweaver-Burk analysis is used to indicate the behavior and inhibition mechanism of an enzyme. V_{max} and K_m are the two main parameters in these calculations showing the highest velocity of enzyme (V_{max}) and affinity of enzyme to substrate (K_m). Additionally, K_m may show affinity of an inhibitor to enzyme or enzyme-substrate complex. Higher V_{max} and lower K_m shows the desirable values for better performance of an enzyme. In the present study, incubation of the enzyme with inhibitor significantly decreased V_{max} value, indicating non-competitive inhibition. With this kind of inhibition, the inhibitor binds to a specific site of the enzyme and causes a type of conformation (Eisenthal & Cornish-Bowden, 1974). This conformation does not prevent substrate binding but prevents the enzyme from converting the bound substrate to product. This kind of inhibition has been reported by several authors (Marshall & Lauda, 1975; LeBerre-Anton *et al*., 1997; Mehrabadi *et al.,* 2010).

The present study uncovers a new lectin protein with α-amylase inhibitory properties that may be used in the genetic modification of crops by gene encoding to create transgenic plants showing resistance against insect pests. Besides the *in vivo* effects of CCA on the digestive physiology of *E. ceratoniae*, we found that CCA significantly disrupts digestion of food in this insect. Determination of a CCA-encoding gene and its transferral to plants may therefore lead to a resistant variety of host plant. These findings could ultimately be used to design specific bio-insecticides for use against economically important pests.

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