

# INHIBITION OF DIGESTIVE $\alpha$ -AMYLASES FROM *CHILO SUPPRESSALIS* WALKER (LEPIDOPTERA: CRAMBIDAE) BY A PROTEINACEOUS EXTRACT OF *CITRULLUS COLOCYNTHIS* L. (CUCURBITACEAE)

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**Abstract:** The striped rice-stem borer, *Chilo suppressalis* (Walker) is one of the most important pest of rice worldwide. In this study, a proteinaceous extract from *Citrullus colocynthis* L. shows various degrees of inhibition to digestive  $\alpha$ -amylases of *C. suppressalis*. Digestive  $\alpha$ -amylases of larvae were inhibited by different concentrations (approximately 50%) of *C. colocynthis* amylase inhibitor (CCAI). One of the isozymes totally disappeared and the sharpness of another decreased on native-PAGE electrophoresis. The pH dependency of inhibition revealed that the enzyme was inhibited in a wide range of pH from 7–10, and the optimal pH of the enzyme occurred in lepidopteran larvae. The highest inhibition of  $\alpha$ -amylase by CCAI was observed at 25°C; the temperature near the optimal temperature of amylolytic activity. A time-course experiment demonstrated that enzymatic activity was the highest, 30 min after the onset of the experiment, when the highest inhibition occurred. The enzyme kinetic studies using Lineweaver-Burk analysis, revealed a mixed inhibition of CCAI on  $\alpha$ -amylase activity. The current study is the first basic one using  $\alpha$ -amylase inhibitor against *C. suppressalis*. This study opens the way for transgenic rice varieties containing inhibitors.

**Key words:**  $\alpha$ -amylase, *Chilo suppressalis*, *Citrullus colocynthis*, proteinaceous inhibitor

## INTRODUCTION

Rice striped stem borer, *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae) is one of the important pests of rice in some parts of Africa, Europe, the Middle East, and Eastern Asia. In Iran, it is the major pest of rice and causes severe annual damage (Zibae *et al.* 2008a). Larvae feed intensively on the inner parts of the rice stem leading to dead heart and white head symptoms (Khanjani 2006). Control procedures include sanitation, biological control, and extensive chemical spraying decreased the damage larvae, but desirable results have not occurred. The control, mainly relies on Diazinon (more than 95% of fields), Padan, and Fenitrothione. It was reported by Zibae *et al.* (2009a) that four populations of *C. suppressalis* were resistant to diazinon. The four populations were: Rasht, Babol, Amol, and Sheikmahaleh (Gourabzarmikh) as 13.67-, 12.33-, 7.75-, and 4.02-fold, respectively, in addition to involvement of detoxifying enzymes.

According to Strobl *et al.* (1998),  $\alpha$ -amylases ( $\alpha$ -1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) catalyze hydrolysis of  $\alpha$ -d-(1,4)-glucan linkages in glycogen and other related carbohydrates. Their activity causes the conversion of starch to maltose which is then hydrolyzed to glucose by  $\alpha$ -glucosidase. Many insects rely on different kinds of polysaccharids, so,  $\alpha$ -amylases play critical roles in insect survival (Franco *et al.* 2000).

As mentioned earlier, chemical spraying is the major procedure used to control *C. suppressalis*. The procedure causes several concerns like emergence of secondary pests, environmental pollution, and insect resistance as reported by Zibae *et al.* (2009a). The use of resistant host plants is one of the promising control procedures against pests. Due to their importance in the digestive physiology of insects,  $\alpha$ -amylase and protease inhibitors are of interest. Six different  $\alpha$ -amylase inhibitors have been used in insect control (Franco *et al.* 2002; Mehrabadi *et al.* 2010). These inhibitors show structural diversity, different modes of inhibition, and different specificity profiles against a diverse range of  $\alpha$ -amylases (Mehrabadi *et al.* 2010). Hence, a complete understanding of the digestive physiology of a target insect and the available inhibitors is mandatory to finding an effective method. Zibae *et al.* (2008a, 2008b, 2009b) and Zibae (2012) have described the major digestive enzymes in the midgut of *C. suppressalis*. A tropical plant that grows abundantly in Asia, Africa, and other parts of the world is *Citrullus colocynthis* L. (Cucurbitaceae), known as bitter apple, vine-of-Sodom, tumba or wild gourd (Tavakkol-Afshari *et al.* 2005). The fruit of this plant contains bitter glycoside and is used as a drug for gut and liver problems (Tavakkol-Afshari *et al.* 2005). Also, a crude extract of the fruit is effective for decreasing blood sugar. The extract has anti-virus as well as anti-cancer properties (Tavakkol-Afshari *et al.* 2005). The

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objective of this study was to determine the inhibitory effects of the *C. colocyntis* amylase inhibitor on the digestive  $\alpha$ -amylase of *C. suppressalis* larvae for the further use of this protein leading to an effective control of this major rice pest.

## MATERIALS AND METHODS

### Insect rearing

Egg patches of *C. suppressalis* were collected from rice fields and put in containers which held rice seedlings. Insects were reared based on a modified method of Zibae *et al.* (2009a) at  $28 \pm 1^\circ\text{C}$ , 80% relative humidity (RH) under conditions of 16 h light: 8 h dark. Laboratory conditions were checked, containers were cleaned, and fresh stems were provided for larvae everyday.

### Extraction of $\alpha$ -amylase inhibitor from *C. colocyntis* (CCAI)

*C. colocyntis* (CCAI) was extracted from seeds as described by Baker (1987) and Melo *et al.* (1999). Powdered seeds (30 g each) were mixed with a solution of 0.1M NaCl, stirred for two hours, then centrifuged at 5,000 rpm for 20 min. The pellet was discarded, and the supernatant was incubated at  $70^\circ\text{C}$  for 20 min to inactivate major endogenous enzymes. Fractionation of the supernatant was done using different concentrations of ammonium sulfate (20, 40, 60, and 80%) followed by centrifugation at 5,000 rpm for 20 min at  $4^\circ\text{C}$ . The 40% pellet containing the highest fraction of  $\alpha$ -amylase inhibitors was dissolved in ice-cold sodium phosphate buffer (0.02 M, pH 7.1) and dialyzed overnight against the same buffer. This dialyzed solution was used as a source of amylase inhibitors in enzyme assays.

### Sample preparation

Larvae were randomly selected and midguts were removed by dissection while under a stereomicroscope in ice-cold saline buffer (10 mM). Larval bodies were cut separately using a scalpel. The midgut appeared after removal of fat and other undesirable organs. The midgut was separated from the insect body, rinsed in ice-cold distilled water, placed in a pre-cooled homogenizer, and grounded before centrifugation. Equal portions of larval midgut and distilled water were used, so that there would be a desirable concentration of the enzyme (W/V). Homogenates were separately transferred to 1.5 ml centrifuge tubes and centrifuged at 13,000 rpm for 20 min at  $4^\circ\text{C}$ . The supernatants were pooled and stored at  $-20^\circ\text{C}$  for subsequent analyses.

### $\alpha$ -amylase assay

The method described by Bernfeld (1995) was used to assay  $\alpha$ -amylase activity. Ten microlitres of the enzyme were incubated for 30 min at  $35^\circ\text{C}$  with 50  $\mu\text{l}$  of phosphate buffer (0.02 M, pH 7.1) and 20  $\mu\text{l}$  of soluble starch as substrate. The reaction was stopped by adding 80  $\mu\text{l}$  of dinitrosalicylic acid (DNS) and heating in boiling water for 10 min prior to reading the absorbance at 545 nm. One unit of  $\alpha$ -amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min

at  $35^\circ\text{C}$ . The negative control contained all reaction mixtures with pre-boiled enzyme (for 15 min), to prove the enzyme presence in the samples.

### Inhibition of $\alpha$ -amylase by different concentrations of CCAI

To find possible inhibition of the digestive  $\alpha$ -amylase, 50  $\mu\text{l}$  of PBS (phosphate buffer solution) (0.02 M, pH 7.1), 20  $\mu\text{l}$  of starch 1%, and 20  $\mu\text{l}$  of different concentrations of CCAI (0, 0.1, 0.5, 1, 1.5, and 2 mg/ml) were incubated for 5 min. Then, 10  $\mu\text{l}$  of the enzyme was added, and the reaction continued as described earlier. The blank contained PBS, starch 1%, and each concentration of CCAI, separately.

### Effect of pH on $\alpha$ -amylase inhibition by CCAI

pH dependency of the CCAI inhibition was determined at different pH values using Tris-HCl buffer (20 mM) with pH set at 5, 6, 7, 8, 9, 10, 11, and 12. The amylase activity was assayed after incubation of the reaction mixture containing Tris-HCl buffer (in the given pH value), starch 1%, CCAI (2 mg/ml), and enzyme.

### Effect of temperature on $\alpha$ -amylase inhibition by CCAI

To find the effect of temperature on  $\alpha$ -amylase inhibition by CCAI, the reaction mixture containing Tris-HCl (20 mM pH, 9), Starch 1%, CCAI (2 mg/ml), and enzyme was incubated at different temperatures set at 15, 20, 25, 30, 35, 40, 45, 50, and  $60^\circ\text{C}$ .

### Time-course inhibition of $\alpha$ -amylase by CCAI

Time course inhibition  $\alpha$ -amylase by *C. colocyntis* lectin (CCL) was carried out by incubation of the enzyme extract with CCL, besides other reaction constituents, in Tris-HCl buffer (20 mM, pH 9) at  $40^\circ\text{C}$  for different time intervals of 10, 20, 30, 40, 50, and 60 min. The experiment was then continued as described earlier.

### Kinetic studies

Kinetic parameters of inhibition and control were carried out with increasing concentrations of starch as substrate (0.5–2.0%) in the presence of CCAI (2 mg/ml). Lineweaver-Burk plot analysis was done to find  $K_m$  and  $V_{max}$  values.

### Inhibition in non-denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Enzyme extract was pre-incubated with different concentrations of CCAI for 30 min at  $30^\circ\text{C}$ . The remaining amylase activity was then determined by SDS-polyacrylamide gel electrophoresis. The procedures described by Laemmli (1970) were used to carry out SDS-PAGE. 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. Then, the gel was rinsed with distilled water and washed by gently shaking with 1% (v/v) Triton X-100. After that, the gel was immersed in a solution of PBS (0.02 M pH 7.1) containing 1% of starch, 10 mM of NaCl, and 2 mM of  $\text{CaCl}_2$ . Finally, it was stained with a solution of 1.3% I2, and 3% KI (Potassium Iodide) to obtain white bands in 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 studentized test when significant differences were found at  $p \leq 0.05$ , and marked in figures with letters.

## RESULTS AND DISCUSSION

Like many other insects, *C. suppressalis* obtains its metabolic energy from ingested food via digestion by hydrolytic enzymes like  $\alpha$ -amylase. A selective group of enzymes catalyze the hydrolysis of  $\alpha$ -1,4-glycosidic bonds. The reaction transforms polysaccharides into mono- and di-saccharides so any inhibition of this enzyme disrupts a part of the intermediary metabolism; mainly glycolysis. Plant derivate of  $\alpha$ -amylase inhibitors have shown promising results worth being considered in plant breeding programs as a part of the Integrated Pest Management (IPM) program. These inhibitors have been extracted from cereals (Roy and Gupta 2000; Heidari *et al.* 2005; Muralikrishna and Nirmala 2005) and legumes, mainly *Phaseolus vulgaris* L. (Giri and Kachole 1998; Melo *et al.* 1999), taro (Seltzer and Strumeyer 1990) *Colocasia esculenta* L. (McEwan *et al.* 2010), and sweet potato (Rekha *et al.* 1999) tubers. But there is no information on *C. colocyntis* except for its high application in traditional medicine. The fruit has anti-inflammatory properties due to the presence of alkaloids and flavonoids. The fruit was used to treat pain and rheumatoid arthritis (Marzouk *et al.* 2012). Aqueous and methanolic extracts of the plant demonstrated high anti-microbial activity against some bacteria and fungi. Also, several studies have found that the seeds show anti-cancer and apoptosis properties (Tannin-Spitz *et al.* 2007).

Precipitation of *C. colocyntis* proteins with different concentrations of ammonium sulfate, revealed different and interesting band patterns. At concentrations of 20 and 40%, a band was observed by 50.2 kDa molecular weight (Fig. 1). The band from a 40% concentration of ammonium sulfate was sharper than that of a 20% concentration (Fig. 1). In precipitations of 60 and 80%, almost four bands were observed by molecular weight of < 116 kDa to 66.2 kDa (Fig. 1). From our inhibition study, we found that precipitation by 40% of ammonium sulfate caused a higher inhibition of *C. suppressalis*  $\alpha$ -amylase. It means that, although lower protein of *C. colocyntis* was extracted by the 40% concentration, the  $\alpha$ -amylase inhibitor protein(s) contain a higher percentage of precipitated protein. In the current study, midgut extract of *C. suppressalis* larvae was considered as the amylolytic source; it was incubated with different concentrations of CCAI (0, 0.3, 0.7, 1, 1.5, and 2 mg/ml). Depending on CCAI concentration, CCAI inhibited amylolytic activity from 39–50% (Fig. 2a). Silva *et al.* (1999) believe that there are more than one isoform of  $\alpha$ -amylases in different orders of insects. In zymogram analysis, although we have observed two isoforms of  $\alpha$ -amylase, the highest concentration of CCAI (2 mg/ml) decreased sharpness of the A2 band, and the

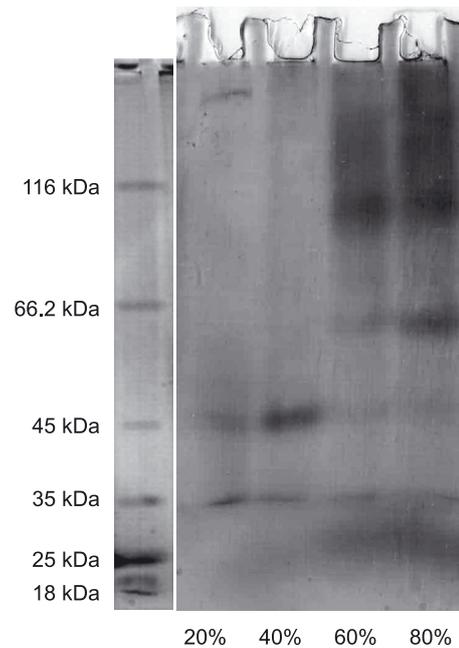


Fig. 1. Protein patterns in different precipitations of *C. colocyntis* by ammonium sulfate

A1 band of the enzyme totally disappeared (Fig. 2b). Kutty and Pattabiraman (1986) observed a 50% inhibition, by extraction of sorghum on the human amylase. Melo *et al.* (1999) reported that  $\alpha$ -amylase inhibitor extracted from cowpea seeds, *Vigna unguiculata* (L.), inhibited  $\alpha$ -amylase from *Callosobruchus maculatus* Fabricius (Coleoptera: Bruchidae) larvae by 50%. Suzuki *et al.* (1994) extracted and purified two different  $\alpha$ -amylase inhibitors from *P. vulgaris*. Bonavides *et al.* (2007) found that  $\alpha$ -AI1 inhibits digestive  $\alpha$ -amylases of *C. maculatus* and *C. chinensis* L., and  $\alpha$ -AI2 inhibits digestive  $\alpha$ -amylase of *Zabrotes subfasciatus* (Boheman) (Coleoptera: Chrysomellidae). Mehrabadi *et al.* (2010) determined the effects of triticale extract (T- $\alpha$ AI) on the  $\alpha$ -amylase of *Eurygaster integriceps* (Puton) (Hemiptera: Scutelleridae). A dose dependent manner of inhibition was observed. There was less inhibition of enzyme activity (~10%) by the lowest dose (0.25 mg protein), and high inhibition of enzyme activity (~80%) by the highest dose of inhibitor (1.5 mg protein). This variance in the inhibition of  $\alpha$ -amylase could be attributed to different reasons; the mechanism of inhibition, mainly, Km, the presence of enzyme isoforms, the proportion of inhibitory proteins and other alkaloids, and the pH condition in the midgut of insects.

Biochemical reactions are affected by environmental factors mainly pH and the temperature of the media. The highest inhibition by CCAI was found to be at pHs 7–10 (Fig. 3;  $Pr > F$ : 0.0043,  $f = 1.04$ ). Also, pH generally affects biochemical conditions in enzymatic, non-enzymatic and inhibitory conditions. Since, gut pH of lepidopteran larvae is alkaline, the highest inhibition of  $\alpha$ -amylase occurred at alkaline condition. Zibae *et al.* (2008a) reported a pH of 9 as the optimal value for amylolytic activity in the midgut of *C. suppressalis*. These results are consistent with other reports; for example, there is an optimum pH of 9.0 for inhibition of wheat toward  $\alpha$ -amylase of *Spodop-*

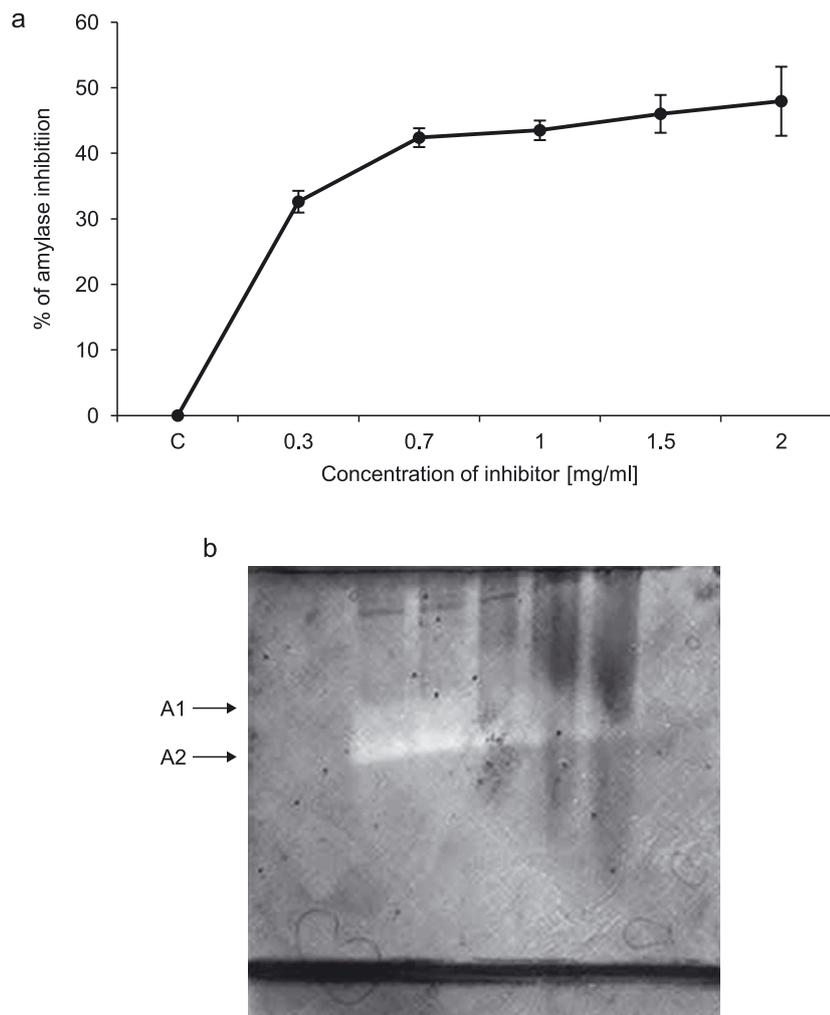


Fig. 2. Inhibition of *C. suuressalis*  $\alpha$ -amylase by different concentrations (mg/ml) of *C. colocyntis* proteinaceous inhibitor (a) and reaction conditions were phosphate buffer (pH 7) and a temperature of 30°C (b)

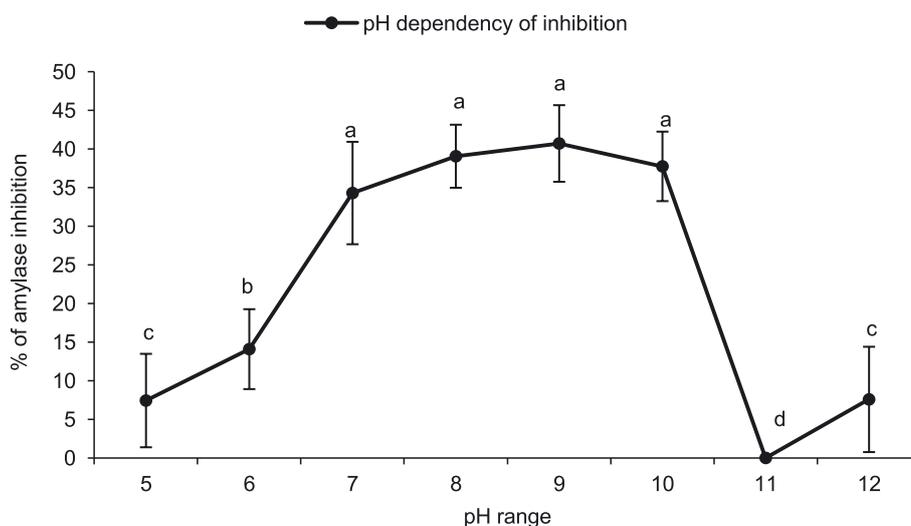


Fig. 3. pH dependency of *C. suuressalis*  $\alpha$ -amylase inhibition by *C. colocyntis* proteinaceous inhibitor (2 mg/ml). Reaction conditions were Tris-HCl buffer (20 mM, pHs 5–12) and a temperature of 30°C. Different letters showed statistical differences among values using Tukey's test ( $p \leq 0.05$ )

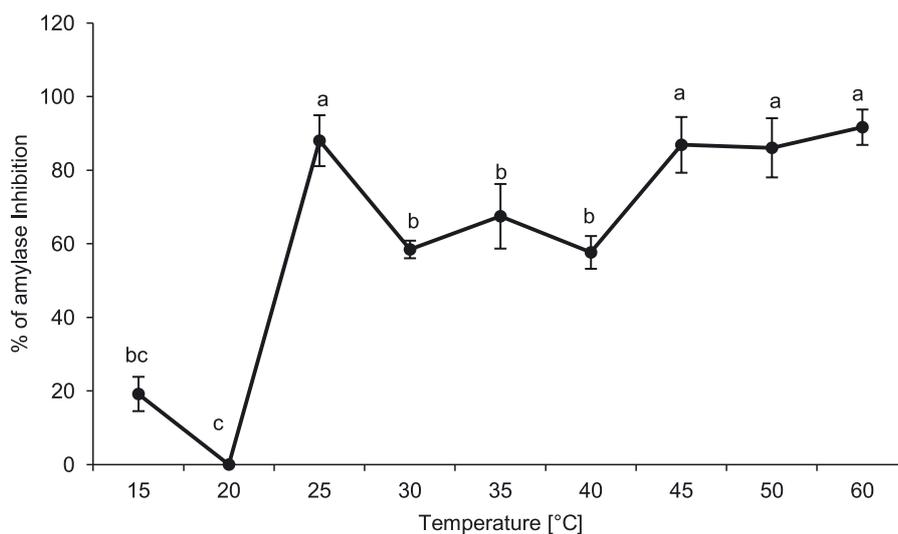


Fig. 4. Effect of temperature on *C. suuressalis*  $\alpha$ -amylase inhibition by *C. colocythis* proteinaceous inhibitor (2 mg/ml). Reaction conditions were Tris-HCl buffer (20 mM, pH 9) and a different range of temperatures (°C). Different letters showed statistical differences among values using Tukey's test ( $p \leq 0.05$ )

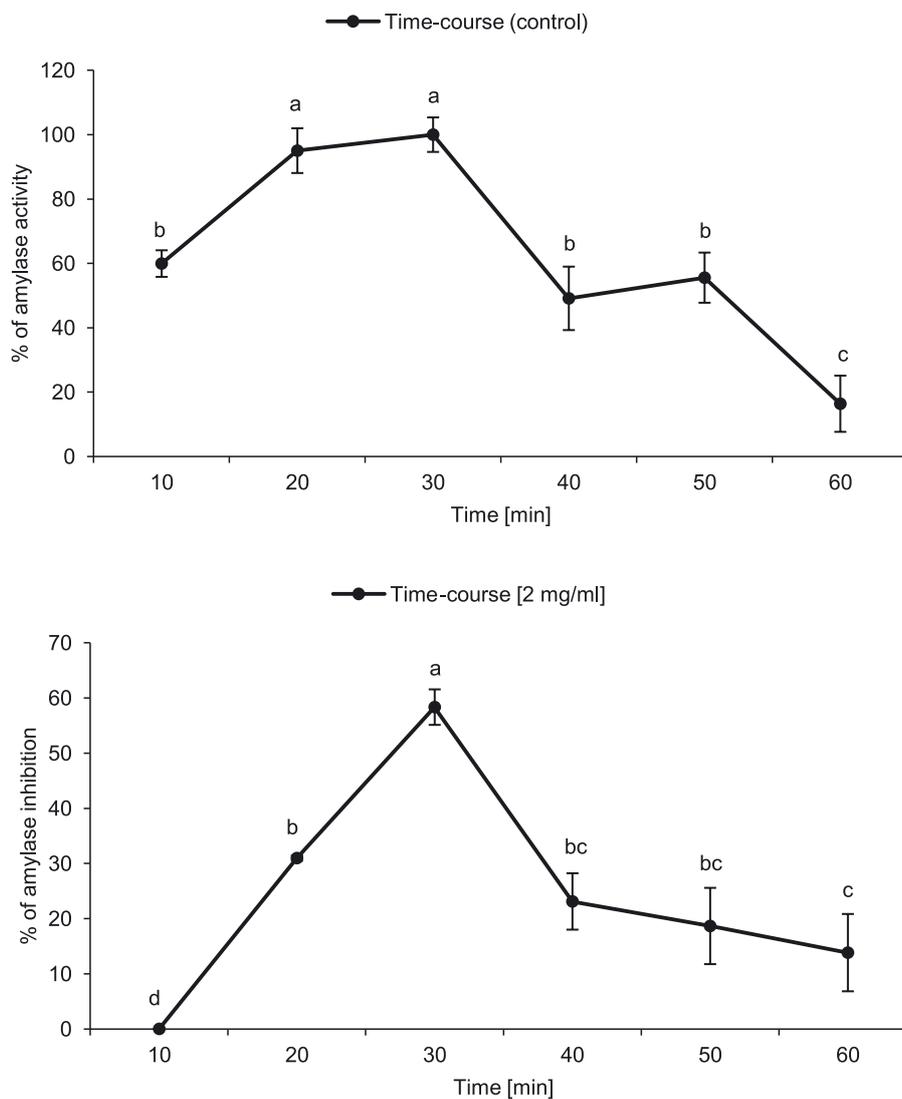


Fig. 5. Time course inhibition of *C. suuressalis*  $\alpha$ -amylases by *C. colocythis* proteinaceous inhibitor (2 mg/ml). The midgut samples were pre-incubated with inhibitor in 20 mM Tris-HCl buffer (pH 9.5) at 30°C. Then enzyme was added and reaction was recorded in the given time intervals. Different letters showed statistical differences among values using Tukey's test ( $p \leq 0.05$ )

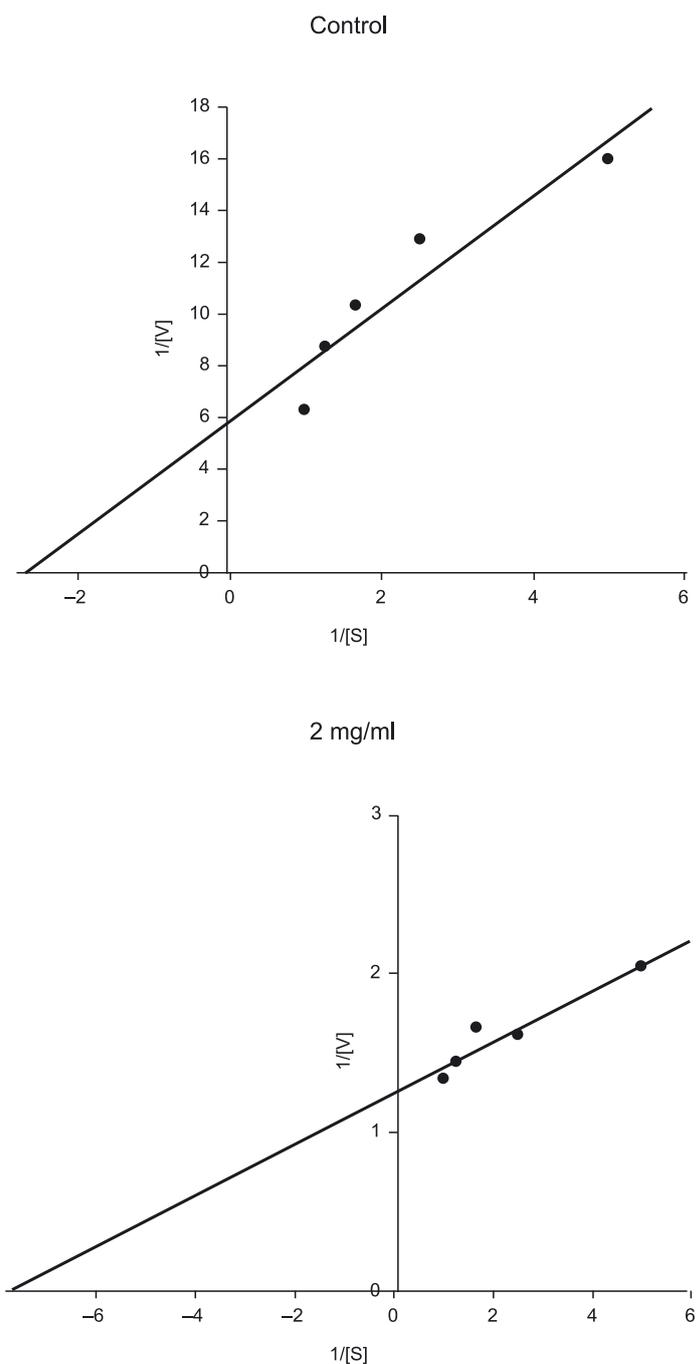


Fig. 6. Effect of proteinaceous  $\alpha$ -amylase inhibitor from *C. colocynthis* on amylolytic activity in the midgut of *C. suppressalis* by Lineweaver-Burk plot

*tera frugiperda* Hubner (Lepidoptera: Noctuidae) (Alfonso *et al.* 1997). There is an optimal pH of 7.5 for inhibition of *Anagasta kuehniella* (Zeller)  $\alpha$ -amylase by an aqueous extract of wheat (Baker 1989). Khan (2011) observed that *Tribolium castaneum* L. (Coleoptera: Tenebrionidae) amylases exhibited increased activity when the pH was highly alkaline. These results were in agreement with most lepidopteron reported so far (Sivakumar *et al.* 2006; Valencia Jimenez *et al.* 2008; Kotkar *et al.* 2009). In *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae), the highest inhibition by triticale extract was observed at pHs of 5 and 6 for both salivary and midgut  $\alpha$ -amylases (Mehrabadi *et al.* 2010). Barbosa *et al.* (2010) reported that  $\alpha$ AI extracted from *P. vulgaris* inhibited porcine pancreatic

$\alpha$ -amylase (PPA) at pHs of 4.5–5.5. This variation might be due to the strain of bean. Since, insects are the poikilothermic animals, so environmental temperature could, somehow, directly affect biochemical process in their body. The effect of different temperatures on the inhibition of  $\alpha$ -amylase *C. suppressalis* by CCAI was recorded at the optimal pH of 9.0. The highest inhibition obtained at 25°C was 48% (Fig. 4; Pr < F: 15.13, f = 0.0001). Zibae *et al.* (2008a) observed the highest amylolytic activity at a range of 35–40°C. These results are not in agreement with Marshall and Lauda (1975) who reported a 10-fold increase in activity of the  $\alpha$ -amylase inhibitor when the temperature of the reaction was raised from 25° to 37°C. This variance could be attributed to the nature of inhibi-

tor and the enzyme, in which their tertiary structure is stable or not at the desired temperatures. Also, inhibition of the enzyme was increased in 45–60°C. At those temperatures, the enzyme is going to be degraded. So, this inhibition could be attributed to enzyme denaturation rather than an inhibitory mechanism

The  $\alpha$ -amylase inhibitors demonstrated the inhibitory effect on catalyzing starch by insect  $\alpha$ -amylase at different time-dependent inhibitions. The data showed that the maximum  $\alpha$ -amylase inhibition by CCAI was achieved after 30 min of incubation (Fig. 5). This result coincides with that reported by Mehrabadi *et al.* (2010) who showed the maximum *E. integriceps*  $\alpha$ -amylase inhibition by T- $\alpha$  AI after 20–30 min of incubation, and Marshall and Lauda (1975) who reported 60–70% inhibition of PPA by *P. vulgaris* amylase inhibitor at 37°C after 30 min.

Lineweaver-Burk plots were drawn to determine whether inhibition of  $\alpha$ -amylase by CCAI was competitive or non-competitive. Results revealed a decrease of  $V_{max}$  and an increase of  $K_m$  when 2 mg/ml of CCAI were used. The control  $V_{max}$  and  $K_m$  values were calculated to be 0.806 mOD/min and 0.12%, respectively. During the treatment, the  $V_{max}$  value was calculated to be 0.17 mili optic dose (mOD)/min, and 0.36%  $K_m$  was obtained (Fig. 6). These results showed a mixed inhibition of the enzyme by CCAI. Mixed inhibition can be regarded as a combination of competitive and uncompetitive effects; this contaminant would result in a decrease in both  $K_m$  and  $V_{max}$  values as well as the appearance of inhibition at high substrate concentrations. Mixed inhibition has been reported for other amylase inhibitors, *i.e.* inhibition of PPA by  $\alpha$ -amylase inhibitor from *P. vulgaris* seeds (LeBerre-Anton *et al.* 1997), and also inhibition of *Rhyzopertha dominica*  $\alpha$ -amylase by wheat  $\alpha$ -amylase inhibitors with a  $K_i$  value of 0.013–0.018 M (Priya *et al.* 2010).

Identification and application of plant origin inhibitors could open a new way to suppress pest population in agroecosystems. So, it would be interesting to focus on amylase inhibitors like CCAI in plant breeding programs. Such a focus could lead to providing resistant varieties. These varieties do decrease the extensive reliance on chemical insecticides that are widely used against *C. suppressalis*. Since *C. colocyntis* is widely grown in Iran, it would be a good source for the extraction of digestive enzymes inhibitors.

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