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Effect of proteinaceous extract of redroot pigweed (*Amaranthus retroflexus* L.) seeds on α-amylase activity of Indian flour moth (*Ephestia kuehniella* Zeller, Lepidoptera: Pyralidae)

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Abstract

Indian flour moth (*Ephestia kuehniella* Zeller, Lepidoptera: Pyralidae.) is a cosmopolitan and severe pests of a wide verity of crops. *Amaranthus retroflexus* (Amaranthacea) on α-amylase activity of *E. kuehniella* was investigated. Enzyme assays data using spectrophotometer showed that α-amylase activity of the fourth insatr larvae was less than control (untreated with *A. retroflexus* seed extract), α-Amylase of *E. kuehniella* had (5.9 mU/gut). Native gel electrophoresis showed that *E. kuehniella* had four iso-enzymes that their relative mobility to that of bromophenol blue was 0.23, 0.41, and 0.89. This study showed that I50 values of *A. retroflexus* seed extract on α-amylase of *E. kuehniella* was 5.0 protein (crude extract), respectively. In gel electrophoresis, pre-incubation of seed extract with enzyme caused disappearance of one band and reduction of intensity of the other bands in *E. kuehniella*. Therefore, the current study shows that α-amylase inhibitors present in the *A. retroflexus* seed extract have a good potential to be used in the management program of *E. kuehniella*.

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Introduction

Indian flour moth, mill moth or the Mediterranean flour moth (*Ephestia kuehniella* Zeller, Lepidoptera: Pyralidae.) is cosmopolitan and severe pest of a wide verity of crops. Indian flour moth, on the other hand, occurs especially in temperate, tropical and sub-tropical area where average temperatures are around 20 – 25°C (Kiritani *et al*., 1963). *E. kuehniella* larvae prefer to feed on wheat flour, but they also feed on all types of stored products including cereals, seeds, dried fruits, cocoa, nuts and almonds. The caterpillars are very active spinners, and pupation occurs in a cocoon covered with food particles as a result they are serious pest of mills where their webbing may clog machinery. Also because of infestation by this insect, the products acquire an unpleasant smell as well as grey/brown colour due to the faeces (Talekar and Shalton, 1993). Larvae entwine all the material on which they feed resulting in solid lumps of food particles, faeces and larval exuviae.

E. kuehniella larvae live on a polysaccharide-rich diet and require digestive alpha-amylase to break down and utilize the starch in their food sources. a-Amylases (a-1,4-glucan-4-glucanohydrolases) are a group of glycoside hydrolases that are widely distributed in bacteria, fungi, plants, and animal tissues. They catalyze the hydrolysis of the a-(1, 4) glycosidic linkage found in starch components and other related polysaccharides. These amylases play a very important role in starch digestion and in insect survival (Avila and Grossi-de-Sá, 2008).

One strategy to combat insect pests is the use of digestive enzyme inhibitors to reduce insect growth by interfering with food digestion and absorption (Yamada *et al*., 2001; Bonavides and Pelegrini, 2007). Plants are known to contain a variety of enzyme inhibitors thought to be involved in defense against insect herbivores and pathogens. These compounds include secondary metabolites such as alkaloids, terpenes, flavonoids, cinaogenic glycosides, lectins, arcelins, vicilins, chitinases, β-1, 3- glucans and systemins (Silva *et al*., 2009).

α-Amylase inhibitors are found in seeds of several plant species including leguminous seeds, which are rich sources of proteinaceous Inhibitors of digestive enzymes especially α-amylases. These α-amylase inhibitors can be classified according to their tertiary structure in six different classes, namely, lectin-like, knottin-like, cereal-type, Kunitz-like, γ-purothionin-like and thaumatin-like (Bonavides and Pelegrini, 2007; Franco *et al*., 2002; Payan, 2004; Richardson, 1990). Nowadays attention is being focused on the idea of using digestive enzyme inhibitors that affecting the growth and development of pest species (Mehrabadi *et al.,* 2010). Inhibitors to insect α-amylase have already been demonstrated to be an important biological system in the control of insect pests (Carlini and Grossi-de-Sa, 2002; Franco *et al.,* 2002; Svensson *et al.,* 2004). With respect to plant biotechnology, the expression of α-amylase inhibitors has been shown to be effective in transgenic plants. For example, Pea and azuki transgenic plants expressing α-amylase inhibitors from common beans were completely resistant to the *Bruchus pisorum* and *Callosobruchus chinensis* weevils (Morton *et al.,* 2000). Barbosa *et al*. (2010) showed up to 88% inhibitory in activity of *Coffea arabica* transgenic seed extracts (AI1) against *Hypotheneumus hampei* α-amylases. Dias *et al*., (2010) also demonstrated that rye α-amylase inhibitor expressed in transgenic tobacco seeds (*Nicotiana tabacum*) caused 74% mortality in *Anthonomus grandis* first instar larvae when transgenic seed flour mixture used in artificial diet.

An understanding of how digestive enzymes function is essential when developing methods of insect control such as the use of enzyme inhibitors and transgenic plants to control phytophagous insect species. For nearly all of these strategies, it is important to have a strong understanding of the target insect pest, too. An understanding of biochemistry and physiology of feeding adaptation is also important. Thus, the aim of the current study was to examine the effect of proteinaceous seed extract of *Amaranthus retroflexus* on α-amylase activity of *Ephestia kuehniella*. The knowledge thus

obtained hopefully will lead to new management strategies for this economically important pest.

Material and methods

Insects rearing

E. kuehniella larvae were obtained from stock culture of insect ecology lab. The larvae were reared on wheat flour under laboratory conditions at 24 °C and a photoperiod of 16:8 L:D as described by Locatelli *et al.* (2008).

Enzyme extraction

For enzyme extraction fourth instar larvae form *E. kuehniella* were used. Enzyme extraction was done based on Bandani *et al.* (2009). Briefly, the fourth instar larvae guts were carefully dissected in 10 mM NaCl solution under stero microscope (Stemi SV6 ZEISS, Germany) (Figures 1 and 2). Midguts were separated and homogenized in pre-cooled homogenizer (Teflon pestle). The homogenates from preparations were transferred to 1.5 ml centrifuge tubes and centrifuged at 15,000 g for 15 min at 4° C.The supernatants were pooled and stored at -20° C as an enzyme source for subsequent analyses.

Insect gut pH determination

To determine gut pH, a number of standard indicator dyes was used. These indicators include 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). The gut pH was determined based on Bignell and Anderson (1980) procedure with some modifications. Fourth instar larvae were dissected and their midgut was separated, then 10 µl of each pH indicator was added to the sample and the colour was recorded.

Extraction of A. retroflexus α-amylase Inhibitor

Proteinaceous seed extract of *A. retroflexus* was extracted according to Baker (1987), Melo *et al.* (1999) and Mehrabadi *et al.* (2010). Briefly, *A. retroflexus* seeds (30 g) were ground and mixed with solution of 0.1 M NaCl and stirred for 1.5 h at 4° C, followed by centrifugation at 10000 g for 30 min at 4° C. The supernatant was taken and heated at 70 °C for 30 min to inactivate endogenous enzymes. Fractionation of supernatant was done using 20, 40, 60, 80 % ammonium sulfate saturations. Pellet obtained from 80% saturation of ammonium sulfate dissolved in phosphate buffer (0.02 M and pH 7) and dialyzed against the same buffer overnight and freeze-dried. Freeze dried powder was used as a α-amylase inhibitor source.

α-Amylase activity

α-amylase activity was assayed by the dinitrosalicilic acid (DNS) procedure (Bernfeld, 1955), using 1% soluble starch solution as substrate as described by Bandani *et al.* (2009). 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. A standard curve of absorbance against amount of maltose released was constructed to enable calculation of the amount of maltose released during α-amylase assay. A blank without substrate but with α-amylase extract and a control containing no α-amylase extract with substrate were run simultaneously with reaction mixture. All assays were performed in triplicates and with three time repetitions.

Effect of PH and temperature on α-amylase activity

The effects of temperature and pH on α-amylase activity were examined using enzyme extract from the larval gut as described by Kazzazi *et al.* (2005). Optimal pH was determined by using universal buffer with pH set at 5, 6, 7, 8, 9 and 10. The effect of temperature on α-amylase activity was determined by pre-incubating of the reaction mixture at 20, 30, 35, 40 and 50 °C for 30 min followed by measurement of activity as described before.

In gel assay

Enzyme extract was pre-incubated with 0.02 mg protein of seed extract for 30 min at 30°C; gels were run for native separation of amylase, a gel with 10 % polyacrylamide resolving. This running was conducted at a voltage of 90V 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 mes buffer containing 2 mM CaCl2 and 10 mM NaCl for 30 min. Then, the gel was rinsed with distilled water and treated with a 1% starch solution for 1.5 h. Finally, after rinsing with distilled water, the gel treated with a solution of 1.3% I2, 3% KI to stop the reaction and to stain the un-reacted.

Protein determination

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

Results

α-Amylase activity

Enzyme assay showed that the enzyme activity was present in the fourth instar larvae of *E. kuehniella*. Enzyme unit activity in fourth instar larvae of *E. kuehniella* were 5.9 mU/gut. Native gel electrophoresis showed that *E. kuehniella* larvae had four iso-enzymes and control had at least three bands.

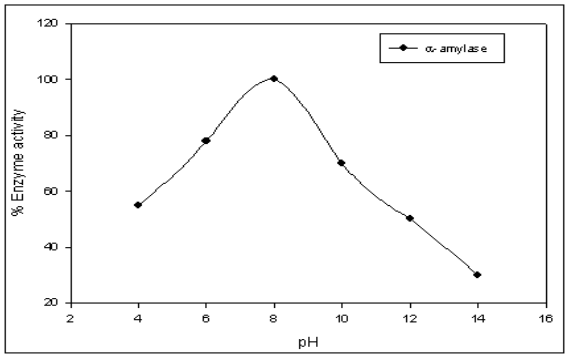

Fig. 1. The effect of pH on α-amylase activity of *E. kihniella*.

Gut pH

Midgut pH of the insects was determined and the measurements showed that *E. kuehniella* midgut pH were 8.0 (Figure 1).

Optimum pH and tempereture for α-amylase activity

Optimum pH for activity of α-Amylase of *E. kuehniella* had the highest activity at pH 8.0 (Figure 2) and the highest activity was seen at 25°C.

Effect of A. retroflexus seed extracts on α-amylase activity

The effects of *A. retroflexus* seed extract (as an enzyme inhibitor source) on α-amylase of *E. kuehniella* were more than control (Figure 3). However, this effect was more visible when high dose of seed extract (35 µg protein crude extract) was used. This study showed that I_{50} values of *A. retroflexus* seed extract on α-amylase of *E. kuehniella* was 13.0 µg protein (crude extract).

In gel assay using native gel electrophoresis showed that four bands are present in the gut of *E. kuehniella*. Application of *A. retroflexus* seed extract (35 µg protein crude extract) to the *E. kuehniella* enzyme, and pre-incubation of seed extract with enzyme caused disappearance of one band and reduction of intensity of the other bands (Figure 4).

Discussion

In this study first α-amylase of the one lepidoptern species including *E. kuehniella* was characterized and then the effect of *A. retroflexus* seed extract on its α-amylase activity was assessed using spectroscopic and electrophoresis methods. There are reports that in insects only α-amylases are present which act on long α-1, 4-glucan chains such as starch and glycogen. Thus, insects use gut α-amylase to digest polysaccharides in its digestive system (Terra *et al*., 1996; Terra and Ferriera, 2005).

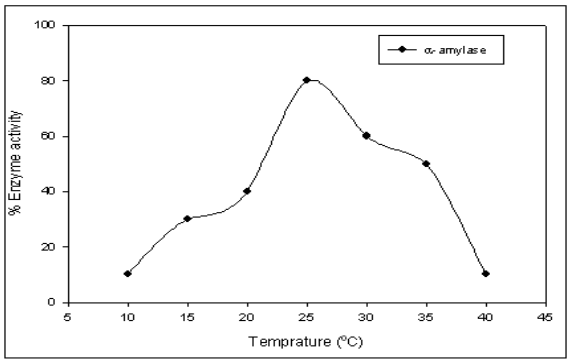

Fig. 2. The effect of temperature on α-amylase activity of E. kihniella larvae.

Enzyme activity in fourth instar larvae of *E. kuehniella* was 5.9 mU/gut. This could be attributed to the insects feeding habit because *E. kuehniella* feeds on cereal which are rich in carbohydrate especially of starch thus it needs more α-amylase enzyme to meet its need.

It has been reported that *A. retroflexus* is good sources of insect α-amylase inhibitors (Franco *et al*., 2002; Sevensson *et al.,* 2003; Sivakumar *et al.,* 2006). These inhibitors have potential to be used against plant pests and pathogens since these agents cause severe damage to host plants. α-Amylase inhibitors have been classified in six different classes including lectin-like, knottin-like, cereal-type, Kunitz-like, c-purothionin-like, and thaumatin-like (Franco *et al*., 2002). These α-amylase inhibitors have structural diversity and show specificity of inhibition against α-amylases thus has potentials to be used against in the insect pest management. In the current study it was found that protein extracted from *A. retroflexus* seeds have a good potential in inhibiting α-amylases of *E. kuehniella*. The study found that effect of protein extracts from *A. retroflexus* seeds were more active against *E. kuehniella*. Inhibitor/s extracted from *A. retroflexus* has not been reported to be active against any pests.

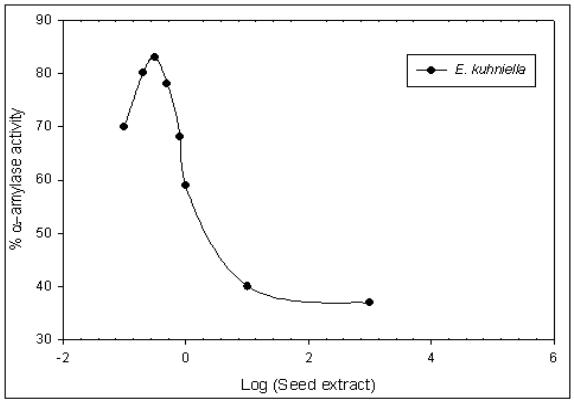

Fig. 3. The effect of been seed extracts on α-amylase activity of *E. kihniella* larvae.

Using gel assay, it was found that in both species *A. retroflexus* protein extract could eliminate some α-amylase isoenzymes and reduce intensity of some others.

Further characterization of the both insect α-amylase was done by determination of the insects pH guts and their effects on the enzyme activity. Lepidopteran midgut pH has been reported to be alkaline ranging from 8 to 13 (Dow, 1996). It has been reported that alkaline pH allows insect to feed on plant materials rich in tannins which binds to proteins at acidic pH values thus reducing the efficiency of digestion (Dow, 1996). In the current study it was found that optimal pH for α-amylase activity of *E. kuehniella* was 8.0. It has been reported that optimal pH correspond to the pH prevailing in the midgut of the insect which amylase has been isolated (Terra *et al.,* 1996) which is true for the current study, too. It has been reported that lepidopteran larvae have alkaline pH values. There are reports that pH values of 9.0 for *Chilo suppressalis* (Lepidoptera: Crambidae), 9.2 for 9.2 for *A. mylitta*, 12.0 for *Acherontia atropos* (Lepidoptera: Sphingidae), 10.8 for *Lasiocampa quercus* (Lepidoptera: Lasiocampidae), 11.3 for *Manduca sexta* (Lepidoptera: Sphingidae) and 10.8 *Lichnoptera felina* (Lepidoptera: Noctuidae) (Dow, 1984; Zibaee *et al*., 2009). It has been reported that high values of gut pH in insects is adaptation to feed on plant materials rich in tannins (Chapman, 1998), which at lower pH values bind to proteins thus decreasing the digestion efficiency (Dow, 1986).

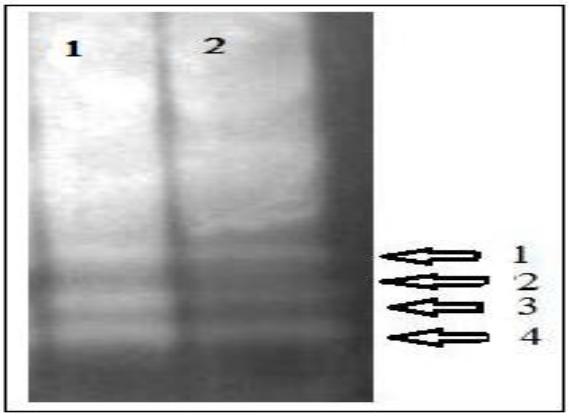

Fig. 4. In gel assay showing the effect of *Amaranthus retroflexus* seed extracts on α-amylase activity of *E. kihniella* larvae. In both gels number lane1 indicates control (untreated) and lane 2 shows treated with *Amaranthus retroflexus* seed extract.

Thus, it could be concluded that since E. *kuehniella* that feeds on stored product. So these discrepancies seen in midgut pH are related to the different feeding habits and feeding sources. Optimum temperature for α-amylase activity of the two lepidopteran species also was different too, that was 30°C for *P. E. kuehniella*. This could be attributed to the environment that two species are active. *E. kuehniella* is a serious pest of cereal stored pest in tropical and subtropical area. It has been said that insect α-amylase are calcium dependant enzymes (Terra *et al*.,

1996). However in the current study it was found that calcium ion did not activate α-amylase activity of *E. kuehniella* larvae.

A mixture of different α-amylase isoenzymes has been reported for other insects such as *Sitophilus oryzae*, *Tribolium castaneum*, *Anthonomus grandis*, *C. Maculates*, *R. Dominica*, *S. Granarium* , *E. intergriceps* (Terra *et al*., 1977; Chen *et al.,* 1992; Oliveira-Neto *et al*., 2003; Kazzazi *et al*., 2005; Mehrabadi *et al*., 2011). Presence of different α-amylase isoenzymes could be related to importance of this enzyme in the insect food digestion.

In conclusion it should be mentioned that *A. retroflexus* is a good source of proteinaceous α-amylase inhibitor that these proteins have potentials to be used as interferer of insect digestive systems α-amylases. Thus, further study of these inhibitors including their purification, characterization and structure elucidation as well as their interaction with target enzyme will unveil more detailed feature of the inhibitors.

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