Biochemical characterization of endo-1, 4-β-D-glucanase activity of a green insect pest *Aulacophora foveicollis* (Lucas)

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Received January 26, 2008

Abstract

Aulacophora foveicollis (Lucas), commonly known as red pumpkin beetle is a serious green pest of cucurbits plants, was studied for the characterization and inhibition of a cellulase enzyme (endo-1,4-beta-D-glucanase). Insects were collected from local vegetation and protein was extracted in Tris-HCl buffer pH 8.5 (0.05 M). After centrifugation a clear supernatant obtained was used as a source of enzyme activity. Multiple forms of endoglucanase activity were identified in the extract after electrophoretic run when activity was located on substrate-agar plate. At least three bands were visible on the zymogram. The major band appeared as fast moving protein was purified by preparative PAGE was characterized for optimum pH, effect of temperature, beta mercapto ethanol and substrate concentration and its inhibition by plant derived molecule, isolated from leaves of *Psidium guajava* (guava). Endo-beta-1,4 glucanases had an optimum pH of 7.8 and optimum temperature of 50 °C while reducing agent had an inhibitory effect on enzyme activity. The Km value of enzyme was 0.2 g/l. The inhibitor molecule isolated from *Psidium guajava* was purified by column chromatography. The endo-beta-D-glucanase activity of *A. foveicollis* was totally inhibited by the molecule. The insects were completely repelled by the inhibitor in the laboratory filter paper disc repellency assay. The purified compound was a flavonoid with a molecular weight of 255 as determined by mass spectrometry. The Km value of the inhibitor molecule for the enzyme inhibition was determined to be 0.05 µmol. This is the first report on the repellency of insect pest *A. foveicollis* by a flavonoid compound related to the inhibition of cellulases activity. [Life Science Journal. 2008; 5(2): 30 – 36] (ISSN: 1097 – 8135).

Keywords: *Aulcacophora foveicollis*; cellulases; endoglucanase; pest; inhibition; flavonoid

1 Introduction

Aulacophora foveicollis (Lucas) (*A. foveicollis*) commonly known as red pumpkin beetle is a destructive green pest. *A. foveicollis* feed on the flowers and leaves of the Cucurbitaceous by making irregular holes and causing retardation of growth, leading to delayed maturation of crop (Singh and Gill 1979; Waterhouse and Norris, 1987). Seedling of Cucurbitaceous are also heavily attacked by the insect pests causing death of plants (May, 1946; Sinha and Krishna, 1969; Waterhouse and Norris, 1987). The pest attack on crop is controlled in two ways (1): application of chemical based insecticides, for which Carbofuran has been successfully tested against *A. foveicollis* for Cucurbitaceous (Sinha and Chakrabarti, 1983) and (2): in biological control, the pest enemies are used to limit the pest attack for *A. foveicollis* Tachinid fly *Medinodexia morgani*, mite *Histiostoma* sp. and reduviid bug *Rhinocoris fuscipes* are reported to be the enemies (Crosskey, 1973; Waterhouse and Norris, 1987). Generally predators keep away from *Aulacophora* sps perhaps due to the presence of distasteful toxics or repellent to general predators (Waterhouse and Norris, 1987). It has also been suggested that on the basis of existing knowledge the prospects for achieving biological control of *Aulacophora* sp. appear to be remote (Waterhouse and Norris, 1987). To control the pest, it is required to understand the molecular basis of pest attack on the crops. Molecular basis of this damage based on cellulose hydrolyzing and related enzymes, present in

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pest. In the biological conversions of cellulose, three different types of enzymes are involved: (i) endoglucanse or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4); (ii) exoglucanases, including 1,4-β-D-glucanohydrolases (cellobiohydrolases) (EC 3.2.91); and (iii) β-glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21) (Marsden and Gray, 1986; Teeri, 1997). A number of reviews dealing with the nature of cellulosic substrates, production and properties of cellulases by the microbes, and their action on cellulose hydrolysis have been published (Marsden and Gray, 1986; Watanabe and Tokudo, 2001). The diverse spectra of cellulases are classified into 12 of the 57 glycosyl hydrolase's families based on amino acids similarities from microbial, plant and animal source (Henrissat and Bairoch, 1993). Till the beginning of 21st century, it was believed that the hydrolysis of cellulose is entirely dependent on cellulases produced by microbes present in the insect gut, as a result of symbiosis, a number of insects have been reported for the presence of active cellulase gene (Watanabe and Tokudo, 2001; Davison and Blaxter, 2005; Sami *et al*, 2008).

Larva of the yellow-spotted longicorn beetle, *Psacothea hilaris* produced a number of carbohydrase activities, including endo-β-1,4-endoglucanase and β-glucosidase in the gut (Sugimura *et al*, 2003). We have reported the presence of cellulase activity in some agricultural insect pests including *A. foveicollis* (Sami and Shakoori, 2006). Insect cellulases are reported to be heterogenous in nature like microbial cellulases (Sami and Shakoori, 2006). Microbial and plant cellulases are reported to be inhibited by a number of metal ions, reducing agents and plant derived molecules (Bell *et al*, 1965; Sami *et al*, 1988). Cellulase inhibition of the insect pest origin could be used as a defensive tool against pest attack. A number of indigenous plant has been screened for repellency or anti-feedants for stored grain insects (Malik and Mujtaba-Naqvi, 1984). Higher plants are rich source of novel insecticides (Arnason *et al*, 1989). The nature of the molecules acting as insect repellent or anti-feedant has not been fully revealed. Cellulases are inhibited by naturally produced plant derived compounds called anthocynadines, as reported by Bell *et al* (1965). Kawaguchi *et al* (2007) reported inhibition of α-glucosidase and α-amylases by flavonoid from green mature Acerola (*Malpignia emarginated* DC) fruit. Recently we have proposed that during a transition state inhibitor isolated from grapes (flavonoid/anthocyanidin) forms a complex with the cellulases enzyme due to structural similarity with the substrate (cellulose), thus inhibit enzyme activity (Sami and Haider, 2007). Here we report characterization of endo-1,4-β-D-glucanase from a serious green pest *A. foveicollis* (Lucas) and its inhibition by a flavonoid compound isolated from leaves of *Psidium guajava*. The compound has proven to a repellent of insect in laboratory repellency assay in the present study. *A. foveicollis* has not been previously studied for its cellulases activity and its inhibition by plant derived molecules.

2 Materials and Methods

2.1 Extraction and purification of endoglucanase from *A. foveicollis*

Insects were collected from Agricultural Fields of Lahore District. All insects were collected in separate sterilized bottles and then stored at – 20 °C. A total of 100 insects were homogenized in 0.5 M Tris-HCl buffer pH 8.5 and centrifuged at 10,000 g for 10 minutes. The supernatant was used as a source of enzyme. The CMCase assay was based on method described (Miller *et al*, 1959; Sami *et al*, 1988). Protein was estimated by Bradford method (1976).

Crude enzyme sample was analysed by native PAGE by modifying the method SDS-PAGE performed as described by Laemmli (1970) and native-PAGE was carried out in the same way except SDS was excluded. Zymography was performed as described by Sami *et al* (1988). After identifying the position of cellulase band on the gel, the protein band was cut with a sharp razor and proteins were eluted as described previously (Sami and Akhtar, 1993). The eluted protein was subjected to PAGE and studied for biochemical characterization.

2.2 Characterization of endo-β-1,4-D-glucanase activity

Cellulase from insect was characterized by determining their pH and temperature profiles. The effect of substrate concentration, β-mercaptoethanol was also studied. To determine the effect of pH on cellulase activity, a range of buffer solution. The pH range used was 4 – 8.9. One milliliter of appropriately diluted clear solution was mixed with 1 ml buffer (respective pH) and 1 ml CMC (1%) and incubated at 50 °C for 60 minutes in a shaker. After incubation Dinitrosalicyclic acid reagent was added and reducing sugars were estimated (Miller *et al*, 1959). To determine the effect of temperature on cellulase activity, a temperature between 4 °C – 70 °C was studied. The effect of substrate concentration was studied using different concentrations of CMC (0.5% – 4% CMC). Effect of β-mercaptoethanol on cellulase activity was studied by using different volumes of β-mercaptoethanol ranging from 0.1% – 1.2% in the reaction mixture.

2.3 Enzyme inhibition studies

Inhibition of endo-1,4-β-D-glucanase activity was studied using the plant leave extracts as the inhibitors from *Psidium guajava*. A reagent for flavonoids test was prepared by mixing 50 ml butanol, 50 ml methanol, 100 ml HCl). One ml of the reagent was mixed with 0.1 ml of the solution and incubated in a boiling water bath for 5 minutes appearance of pink color marked the presence of flavonoid, absorbance was read at 545 nm. One unit of enzyme activity was defined as the micromoles of reducing sugars released by the enzyme under the standard conditions in 30 minutes.

2.3.1 Preparation of leaf extract. Fresh leaves of Guava (*Psidium guajava*) were collected from December 2006 to March 2007. A weighed amount of Guava leaves 150 g, washed with tap water and air dried and flavonoid was extracted with 100 ml methanol in a blender and then filtered. Filtrate was tested with flavonoid reagent and then concentrated at 50 °C overnight to 10 ml.

2.3.2 Purification of flavonoids. A column (1.5 cm × 20 cm) was packed with Silica gel 60 (Merck) in solvent methanol and water (70 : 30). Column was washed twice with the solvent and 2 ml concentrated Guava leaves extract was loaded onto the column. Flavonoids were eluted with methanol and water (70 : 30) and a total of 100 fractions was collected (2 ml each) at room temperature. All the fractions were tested for flavonoid. Fractions from the one of the major peak were collected and analyzed by Mass Spectrometry for molecular weight determination.

2.3.3 Inhibition studies. For Congo red plate assay method, 200 µl Guava leave extract (20 mg/ml, the flavonoid was passes through the silica gel column) was mixed with 100 µl appropriately diluted enzyme solution, at pH 7.8 and was incubated at 50 °C for 15 minutes. The mixture was loaded onto the substrate (1% CMC)-agar plate, method described previously, and were incubated at 50 °C overnight (Sami and Shakoori, 2006). The plates were stained with 0.1% Congo red for 15 minutes and then washed with 0.1 M NaCl several times. Enzyme activity appeared as lighter area around the hole, against the red background, while the enzyme inhibitor showed no change in color.

To determine the inhibitory effect of Guava leave extract on the enzyme activity, reaction mixture was prepared by mixing 0.5 ml enzyme appropriately diluted, 1 ml buffer (pH 7.8), 100 µl Guava leave extract (containing 10 – 500 µg of the flavonoid was passes through the silica gel column) and 400 µl distilled water in 50 ml conical flasks and incubated at 50 °C for 30 minutes. After incubation 3% CMC solution was added as substrate and incubate at 50 °C for 1 hour and reducing sugars were estimated by DNS method (Miller *et al*, 1959).

2.3.4 Laboratory repellency test of inscet pest for flavonoid. Insects were collected from the Pindi Das (Lahore district) in the morning during the month of November and kept in sterlised glass jar with air spaces in the lid. Laboratory bioassay was performed in Petri dishes (9 cm diameter). A filter paper disc of same size was placed inside the dish and was kept moist. A small filter paper disc of 2.5 cm diameter was soaked in 0.5 ml of solution containing 10 mg of the purified flavonoid and was dried. The disc soaked with the inhibitor was placed inside the petri dish and 10 insects were introduced at zero time. Mobility of the insects was monitored after every 2 minutes. The insects at a distance of 2.5 cm or more were considered as repelled by the compound. All tests were kept at constant temperature of 30 °C in morning. The test was repeated four times.

3 Results

A solution containing extract of 100 insects weigh 5 g containing 2.0 g of protein was used as a source of enzyme for purification by electrophoresis. One milliliter of sample was loaded on PAGE. Endoglucanase activity was located on CMC agar plates. It was found that there were at least more than two bands in the extract, the fast moving band (major band) was cut and eluted from the gel. The protein eluted showed a single band when electrophoreised and activity was located on zymogram (Figure 1). The purified enzyme was used for study the biochemical characteristics. The effect of pH on the enzyme activity was determined. It was recorded that there was a sharp increase in activity from pH 6.5 – 7.8 and then there was a gradual decrease in the activity. The optimal pH for cellulase activity against CMC was 7.8 (Figure 2). Optimum activity was observed at 50 °C. There was a continuous increase till 50 °C in temperature profile of *A. foveicollis* and enzyme activity was decreased above 50 °C (Figure 3). Effect of β-mercaptoethanol on endo-β 1,4-D-glucanase activity was studied by using different concentrations of β-mercaptoethanol, ranging from 20 µl to 100 µl in a reaction mixture (0.1% – 1.0%). Enzyme activity was decreased up to 40% at 1.0% concentration of beta mercaptoethanol in the reaction mixture (Figure 4). The effect of substrate concentration was determined, a straight line was obtained up to a concentration of 2% after that curve was obtained (Figure 5). Km value of the enzyme was 0.2 g/l (Figure 6). The enzyme activity was completely inhibited by the methanol extract of guava leaves on cellulose agar plate assay method by Congo red plates, as shown in Figure 7 (a and b).

For the purification of flavonoid compound from guava leaves after silica gel column chromatography, four peaks were obtained. The second peak was the mapeak comprising of fraction number 22 – 30 (Figure 8). Absorption spectra of the flavonoid were determined as 560 nm. The fraction number 22 – 30 were pooled together and dried at 5 °C, overnight. 200 mg of dried

Figure 1. Analysis of endoglucanase activity on 10% native-PAGE and activity was located on zymogram. a: Crude extract showing multiple bands for endoglucanase activity; b – d: Purified form of endoglucanase.

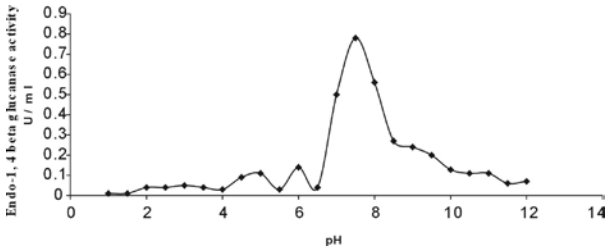

Figure 2. Effect of pH on endo-1,4-β-glucanase activity present in *A. foveicollis*.

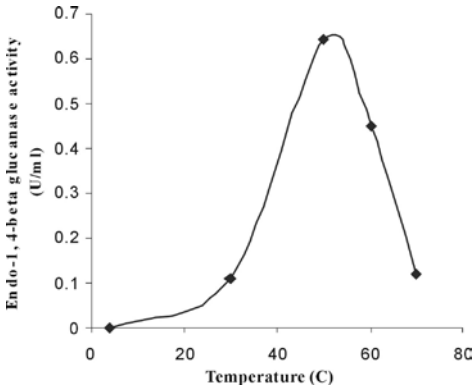

Figure 3. Effect of temperature on endo-1,4-β-glucanase activity present in *A. foveicollis*.

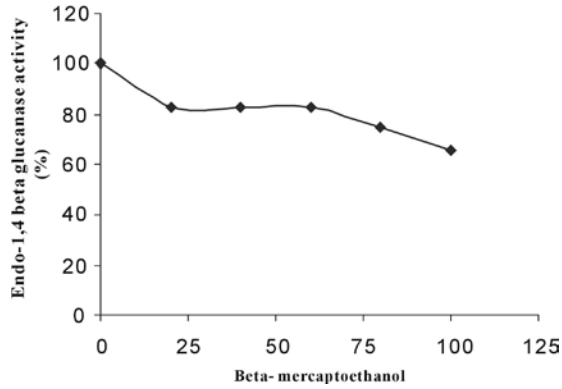

Figure 4. Effect of β-mercaptoethanol on endo-1,4-β-glucanase activity present in *A. foveicollis*.

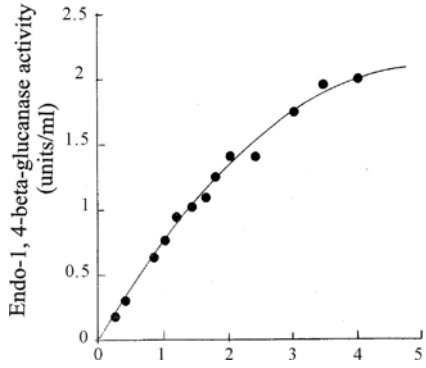

Figure 5. Effect of substrate concentration on endo-1,4-β-glucanase activity present in *A. foveicollis*.

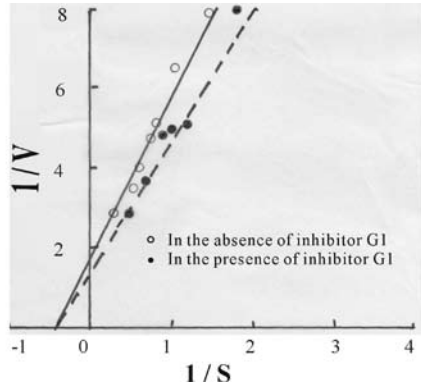

Figure 6. Line weaver-Burk plot of non-competitive inhibition of endo-1,4-β-glucanase of *A. foveicollis*.

powder was obtained and was stored in a clean glass vial at room temperature. This powder was used for inhibition of the cellulase enzyme using 20 mg/ml

in inhibition studies. The molecular weight of the compound was determined by mass spectrometry. The compound was identified as polyhydric phenol flavonoid with the molecular weight 255. Pure inhibitor was used for inhibition studies reported to be a complete inhibitor of the enzyme by inhibiting 95% of enzyme activity (Figure 9). The Km value of inhibitor was calculated 0.05 μmol/L (Figure 10). The inhibitor was found to be a non-competitive inhibitor by reducing the Vmax and not affecting the Km value (Figure 6). A complete repellency of the insect pests was observed in the laboratory repellency assay when purified flavonoid compound was used as repellent (Figure 11). A complete repellency of the insect pests was observed within 45 minutes.

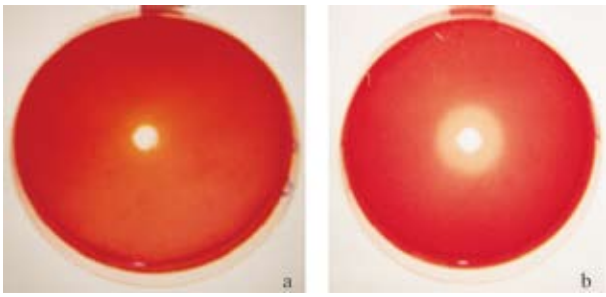

Figure 7. Inhibition of *A. foveicollis* cellulases by flavonoid isolated from guava leaves. a: with inhibitor; b: without inhibitor enzyme activity on agar plates.

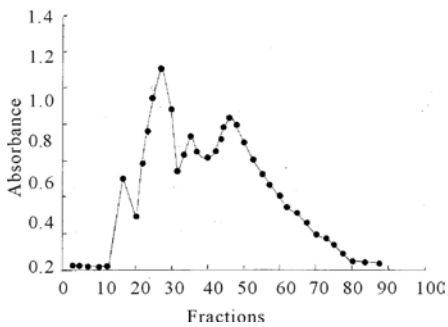

Figure 8. Column chromatography fractions of methanol extract from guava red-green leaves on silica gel (1.5 × 20 cm) at flow rate of 0.5 ml/minutes and solvent system, methanol water (70 : 30) (fraction size 2 ml each).

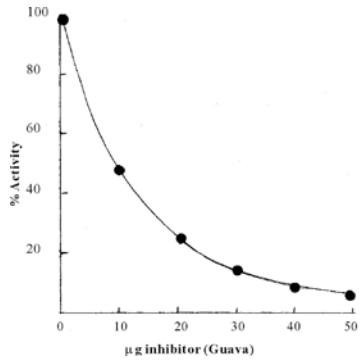

Figure 9. Effect of inhibitor on % age activity of endo-1,4-β-glucanase activity of *A. foveicollis*.

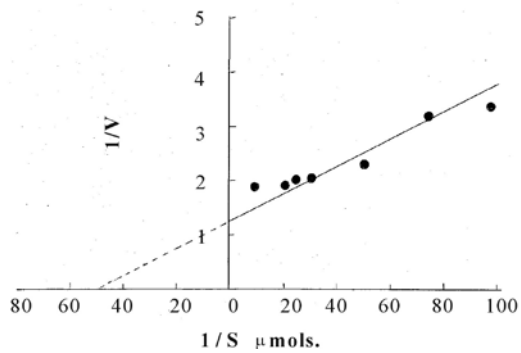

Figure 10. Line weaver-Burk plot for showing the Vmax and Km values of inhibitor G1.

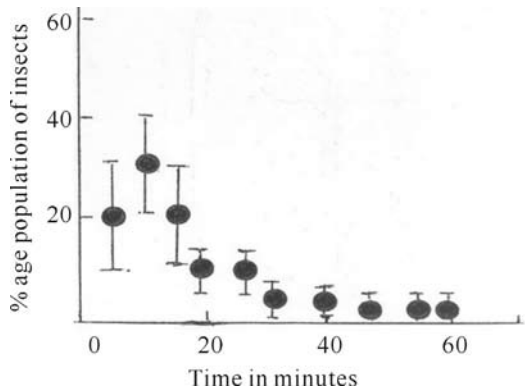

Figure 11. Laboratory bioassay of pest insects of *A. foveicollis* was carried out in 9 cm petri dishes. 10 insects were exposed to the flavonoid isolated from guava leaves and repellency effect of the compound was observed.

4 Conclusions

Multiple forms of cellulases has been previously reported for bacterial, fungal and insects (Sami *et al*, 1989; Sami and Shakoori, 2006)). The optimum pH of *A. foveicollis* endoglucanase showed activity at a broad pH ranging 6.0 – 7.8 against carboxymethylcellulose (Figure 2). Optimum pH of the enzyme was

alkaline range pH 7.8. All animal cellulase reported until now have optimal activity under the weak acidic condi tions (Watanabe *et al*, 1997). It has been reported that the gut of insects has acidic pH, the acidic pH optima could be related to the acidic environment of the gut. Cellulose could be hydrolyzed to its components with the acids and the treatment with acids lead to generate ends for enzymatic hydrolysis (Qian *et al*, 2003). Previous studies on the isolation of cellulase from beetles showed that optimum pH for the highest activity of cellulase from the larval gut of *P. hilaris* against CMC 5.5 (Sugimura *et al*, 2003) and in case of mulberry longicorn beetle *Apriona germari* at pH 6.0 maximum activity against CMC (Lee *et al*, 2005). The recombinant Ag-EGase III, isolated from mulberry longicorn beetle showed highest activity at pH 6.0 (Wei *et al*, 2006). Acidic pH range is also required for the attack of pest, bacteria and fungi on plants. In mollusk EG 45 and EG 27 isolated from gastric juice, showed maximum activity at pH 5.5, 4.4 – 4.8 respectively (Bateman, 1969). No exoglucanase activity was not detectable, using crystalline cellulose as substrate. Similarly, purified cellulase of a beetle *P. hilaris* showed no activity against crystalline cellulose, which may suggests it only utilized amorphous parts of ingested cellulose materials (Sugimura *et al*, 2003). The optimal temperature for the endo-1,4-β-D-glucanase activity of *A. foveicollis* was 50 °C (Figure 3) which is also comparable to the optimal temperature of longicorn beetle, *A. germari*'s cellulase activity (Lee *et al*, 2005; Wei *et al*, 2005). Incase of β-mercaptoethanol, the activity of endo-1,4-β-D-glucanase was decreased up to 40% at higher concentration, eg. 1% in the reaction mixture (Figure 4), perhaps due to the reduction of S-S bonds present in the enzyme molecules. Effect of substrate concentration was determined it appeared that above the concentration 1.5% of substrate, a hyperbolic curve was obtained for enzyme activity (Figure 5). The Km value for endo-1,4-β-D-glucanase was 0.2 g/L which reflects the affinity of the enzyme for that substrate (Figure 6). Thus the endoglucanase activity present in the insect extract was of *A. foveicollis*. The present enzyme activity is the major cause for the destruction of crops by the insect. It is pertinent to mention that this specie is particular to cucurbits plants and their infestation intensity may vary to some extent in different cucurbits (Sinha, 1969; Khan and Wasim, 2001). Compounds having the ability to inhibit enzymes occurred in wide variety of plants. It is reported that the polyphenols and tannins inhibit the activities of digestive enzymes. The pH optima of cellulase activity determined in *A. foveicollis* also falls in alkaline range. As Mandle and Reese (1965) had reported the inhibition of cellulases by the crude extract of guava leaves. The crude extract of Guava red-green leaves and pure compound isolated from crude extract showed complete inhibition in Congo red assay and reducing sugar assay (Figures 7 and 9). Khan and Wasim (2001) also reported that neem extract was an effective repellent of red pumpkin beetle (*A. foveicollis*). Fields *et al* (2001) has demonstrated the repellent effect of peas (*Psium sativum*) fractions against stored-product insects. The molecular basis of the repellency has not been completely explored, yet. It is worth mentioning that the cellulase and other sugar catabolism enzymes belong to a new class of enzymes called glucohydrolases classified on the basis of amino acid sequence homology (Henrissat and Bairoch, 1993). There is a possibility the flavonoid may act as inhibitor for cellulose and starch hydrolyzing enzymes simultaneously. Maximum inhibition of *A. foveicollis* enzyme was observed when 10 µM inhibitor was used in a reaction mixture (Figure 8) the Km value of inhibitor was calculated 0.02 µmol/L (Figure 10). The inhibitor was proven to be a non-competitive inhibitor. It means that there are different binding sites for substrates and catalytic sites. In non-competitive inhibition the Vmax varies and Km remains same (Figure 6). The inhibitor was checked in laboratory bioassays was proven to be a complete repellent of the insects (Figure 11). It is possible that the flavonoid compound isolated from guava leaves, binds to endo-1,4-beta-D-glucanase enzyme (present in the gut of the insect) with its binding site for the substrate, thus preventing the insect attack on the substrate. The cellulase enzyme has structure similar to hen egg-white lysozyme containing six binding sites for substrate and six binding sites for enzyme. Possibly the inhibitor binds to the catalytic binding sites of the enzyme, thus proving as a non-competitive inhibitor. Recently, we have suggested a model for the inhibition of cellulases by the flavonoid (Sami and Haider, 2007). The flavonoid isolated from plants could be used as natural potential insecticide as they could bind the substrate binding site of the insect pest cellulases. The flavonoids are reported to be valuable inhibitor for sugar catabolite enzymes (Todera, 2006). It is thought that the structure of anthocynadine/flavonoid masked the active site of the enzyme thus inhibiting the enzyme activity. Bell *et al* (1965) has reported the inhibition of plant and fungal cellulase from extract of different plants containing anthocynadine and related compounds. The structure of anthocynadine with oxygen containing ring are comparable to the polymeric carbohydrate substrates (cellulose, starch). The structure of anthocynadine is also comparable to the structure of a synthetic cellulase inhibitor (Isofumagine) reported by Varrot *et al* (2005) for

Mycobacteria cellulase. The studies reported here could be useful in understanding the mode of cellulose degradation by the enzyme and its inhibition by a number of plant based products. It was recorded that the flavonoid isolated from guava leaves was able to completely repel the insects in laboratory repellency assay (Figure 11). A complete characterization of the mechanism requires the studies on the crystal structure of the inhibitor and enzyme complex.

Achnowledgment

Authors are thankful to Higher Education Commission Pakistan for the financial assistance and for technical assitance N. Yasmeen and U. Khan.

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