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Digestive Cellulose Hydrolyzing Enzyme Activity (endo- β–1, 4- D-glucanase) in the Gut and Salivary Glands of Blister Beetle, *Mylabris pustulata*

Amtul Jamil Sami,[1] Muhammad Ayaz Anwar,[1] Fayyaz Ur Rehman[2] and A.R. Shakoori[3]
[1]*Institute of Biochemistry and Biotechnology, University of the Punjab, Lahore-5459, Pakistan.*
[2] *Department of Chemistry, University of Sargodha, Sargodtha, Pakistan.*
[3]*School of Biological Sciences, University of the Punjab, Lahore ,Pakistan.*

Abstract.- *Mylabris pustulata* (blister beetle) was studied for the enzymes involved in hydrolysis of cellulose. Carboxy methyl cellulose hydrolyzing activity (endo- β–1, 4- D-glucanase) was detected in the salivary glands and fore gut, very little activity was present in the hind gut. The multiple forms of the enzyme activity were detected on zymogram after non-denaturing PAGE. One of the fractions was purified by gel filtration and preparative native PAGE. The purified protein appeared as single band on SDS-PAGE with a molecular weight of 150 kilo Dalton. The characteristics of the enzyme showed two optimum pH values, one acidic and one neutral 2.0 and 7. The optimum temperature for endo-β – D- 1, 4- glucanase was 50 °C. The enzyme was maximum activity against carboxy methyl cellulose. Km and Vmax of the enzyme was determined as 0.6g/l and 0.3, respectively. To our knowledge this is first report on the digestive cellulose hydrolyzing activity of *Mylabris pustulata*.

Key words: *Mylabris pustulata,* blister beetle, endo-beta 1, 4-glucanase, acidic cellulose.

INTRODUCTION

Insects play major role in the bioconversion of cellulose, which is the main component of terrestrial plants and marine algae. It is used as a nutrition source by insects. Cellulose production by non-photosynthetic organisms (certain bacteria, marine invertebrates, fungi, slime molds and amoebae) has also been documented (Coughlan and Mayer, 1992; Coughlan, 1990; Tomme *et al.,* 1995; Lynd *et al.,* 2002). Cellulose is composed of glucose subunits, joined through beta linkage, which could be hydrolyzed by the action of enzymes or acids. Biological cellulose hydrolysis requires the combined action of different types of cellulases including endoglucanases and cellobiohydrolases or exocellulases and beta glucosidases. Cellulose hydrolysis to glucose occurs by hydrolysis of the β-1, 4 linkages of cellulose by means of cellulases (Wilson and Irwin, 1999). For complete hydrolysis of cellulose, the synergistic action of all the three components is necessary and glucose is formed as a final product (Laurent *et al*., 2001; Volff and Altenbuchner, 2000). Endoglucanases acts amorphous regions of the cellulose fiber and opens sites for subsequent attack by the cellobiohydrolases (Wood, 1992). Exoglucanases liberate D-glucose from β-glucan and cello-dextrins, while glucosidase/cellobiase hydrolyses the cellobiose and short chain oligosaccharides to glucose.

Cellulolytic enzymes are present in many bacteria, fungi, protists and plants. Animal cellulases are also now no more a dogma (Lo *et al.,* 2003). The traditional view of sole microbial cellulase activity in animals is changed by several reports of endogenous animal cellulase isolated from beetles (Sami and Shakoori, 2007, 2008; Rehman *et al.,* 2009; Girard and Jouanin, 1999), plant-parasitic nematodes (Smant *et al.,* 1998; Rosso *et al.,* 1999), cockroaches (Lo *et al.,* 2000), termites (Watanabe *et al*., 1998; Tokuda *et al.,* 1999,), molluscs (Xu *et al.,* 2000, 2001) and a crayfish (Byrne *et al.,* 1999). Cellulose digestion in animals was considered that it occurs solely by intestinal microbial cellulase activity. This traditional view of cellulase activity in animals was changed by two reports of endogenous animal cellulase genes from plant-parasitic nematodes and a termite (Smant *et al.,* 1998; Watanabe *et al.,* 1998,). Cellulose hydrolysis is considered important route for obtaining economical renewable energy source to replace oil. Due to the availability of raw material cellulose in huge amount, biotechnologists are in constant search for efficient bioconversion of

* Corresponding author: amtuljamilsami@yahoo.com
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cellulose by enzymes. For this purpose microbial cellulases have been studied but no attempt have been made to explore the potential of insect cellulases in this regard.

We have previously reported the presence of cellulose hydrolyzing activity in a number of insects including blister beetle, *Mylabris pustulata* (Sami and Shakoori, 2007). Blister beetles are widespread in pigeon pea in Asia. Diet of the larvae consists of other soil insects, including major pests but adults cause considerable damage to plant due to plant feeding habits (Lawrence *et al.,* 1982). To our knowledge there is no report on the cellulose hydrolyzing activity of the insect. We have identified acidic cellulases present in the insect which has not been reported before. The enzyme could be of huge importance from biotechnological point of view, where cellulose is being used as a source of renewable source of energy.

MATERIALS AND METHODS

All chemicals used in the present investigation were purchased from the Shandon, Merk, BHD Chemicals England and Sigma Chemical Co., USA.

Samples collection

Insect sample were collected from the local fields of Lahore and stored as described previously (Sami and Shakoori, 2006). Insects were identified as blister beetles, *Mylabris pustulata*, with the assistance of Prof. Dr. Shamshad Akbar, University of Agriculture, Faisalabad.

Crude enzyme preparation

A weighed quantity (9.66 gm) of the insect was washed and air dried. Insect was dissected, mouth and gut parts were homogenized in 100 ml 0.1 M Tris-Cl buffers, pH 8.5. Supernatant (30 ml) was collected after 10,000 rpm for 15 minutes and added 120 ml of ice-cold acetone and kept overnight at 4 °C. The pellet was collected after centrifugation at 15,000 rpm for 15 minutes; air dried and dissolved in 0.1 M Tris-Cl buffer pH 8.5.

Salivary gland and gut cellulolytic activity

The cellulase activity in the salivary glands was investigated by removing mouth part of beetle and placing these parts in the agar-plate. After incubation at 50^{o}C for overnight, the agar plate was stained and de-stained by Congo Red Dye and 1M NaCl solution respectively. For gut cellulolytic activity insects were dissected, gut was removed and it was cut in three parts *i.e.* foregut, mid gut and hindgut. These parts then placed in the substrate agar plates and Congo red assay was performed as earlier reported by Sami and Shakoori (2006). Total protein concentration was measured by Dye-Binding Method of Bradford (Bradford, 1976) using bovine serum albumin as standard.

Endo-β-1, 4-glucanase activity (CMCase Assay)

CMCase assay was performed according to Sami *et al.* (2008). Crude enzyme sample (100 µl) was added to 0.5 ml of 3 % carboxymethyl cellulose solution prepared in 0.1 M sodium citrate buffer (pH 2.0). Then mixture was incubated for 2hours at 50^{o}C. 3 ml of DNS reagent was added to reaction mixture and incubated in the boiling water bath for 15 minutes. Absorbance was measured at 546 nm against reagent blank prepared by adding 100 µl distilled water, 0.5 ml of 3% CMC and 1 ml of sodium citrate buffer (pH 2.0). One unit of enzyme activity was defined as the amount of enzyme that librated one micromole of reducing sugar from CMC per minutes under the assay condition described.

pH and temperature profile of ednoglucanase activity

To determine the pH profile of enzyme the enzyme activity was carried out using 0.1M buffers ranging from pH 1.0-8.5.Citrate buffer (1.0-3.5), acetate buffer (4-5.4, phosphate buffer (5.8-6.8) Tris-Cl buffer (7.1-8.5). Assay was performed by DNS method as described earlier (100 µl enzyme was mixed in 1 ml 3% CMC and 1 ml of buffer of different pH). For determination of optimum temperature CMC assay was performed at different temperature between 4°C - 80°C by DNS method as described above.

Effect of different substrates and substrate concentration

Endoglucanase activity using different

substrates were studied. Cellulose, avicel, xylan and filter paper were used as the substrates sources. Reaction mixture was prepared by adding equal concentrations of the substrate. The activity was assayed by DNS method. Effect of substrate concentration was studied using different concentrations of CMC ranging from 0.2% to 3% in the assay mixture (100 µl enzyme sample was mixed with 0.2%- 3% CMC and 1 ml citrate buffer pH 2.0).

Enzyme kinetics

Enzyme kinetics was studied by determining the K_m (g/l) and V_{max} (µM/ min) values by plotting the Michaelis Menton Plot.

Purification of the enzyme

Enzyme was purified from the crude enzyme extract using gel-filtration chromatography. 3 ml enzyme sample was run through Sephadex G-200 column (3.0 × 30.0 cm) equilibrated with 0.1 M Tris- Cl buffer (pH 8.5). A total of 200 fractions (1.5 ml each) were eluted with the same buffer. CMCase and protein concentration were estimated in each fraction and active fractions were pooled and subject to further purification process.

Zymography and PAGE

Zymography was performed as described by Sami *et al*. (1988). After electrophoresis, a strip of electrophori zed gel was overlaid on the substrate-agar gel containing 3% CMC and 2% agar mixed with equal volume of 0.1 M citrate buffer pH 7.1 and incubated at 50°C for 30 minutes. After incubation Congo red staining and de-staining was performed.

Gel was cut into 19 strips, crushed and protein was eluted in separate test tubes containing buffer of pH 8.5 (Tris-Cl) and distilled water (2 ml each), as reported previously Sami *et al*. (1988). The enzyme activity was measured in each tube by CMC assay. PAGE of the active protein samples to determine the molecular weight was performed.

Hydrolytic product identification

Reducing sugar produced by the action of enzyme was identified by paper chromatography and thin layer chromatography as described by Dawson *et al*. (1969). Hydrolysate was prepared by incubating the 0.5 ml enzyme sample with 1 ml of substrate and 1 ml of buffer pH 2.0 for 24 hours at 50 °C. Solvent system used was n-Butanol: Pyridine: water (3:2:3). $AgNO_3$ and NaOH solution were used to detect carbohydrates. For thin layer chromatography silica gel plate (15 cm long) was used. Hydrolysate sample along with reference sugar (glucose and cellobiose) and run using solvent system using iso-propanol: pyridine: water (6: 4: 3) mixture. 2, 4-dinitrophenyl hydrazine in 10% H_2SO_4 and methanol was used as spray solution.

RESULTS

Cellulase activity in salivary glands and gut

The cellulase activity was observed in the salivary glands (Fig. 1) and gut as a clear zone obtained around head containing area on substrate-agar plate. Activity was observed around area containing the foregut, while the other did not show prominent enzyme activity (Fig. 2). The protein present in the sample was estimated to about 417 µg/ ml. Enzyme activity was measured by using the glucose standard curve was 1.06 U/ml.

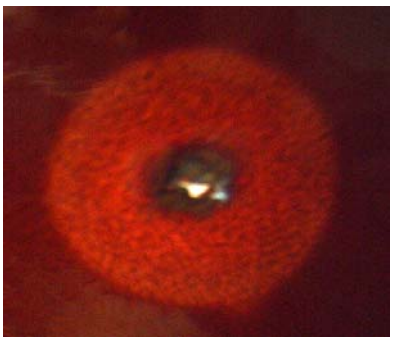

Fig. 1. Cellulase activity in salivary glands.

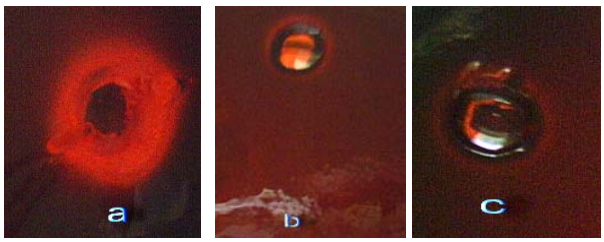

Fig. 2. CMCase activities in the digestive tract *i.e*. (a) foregut, (b) mid gut and (c) hindgut. As the activity, was shown in the foregut.

Purification of enzyme

The cellulase activity and protein contents were determined in each fraction. Active fractions 25-70 were pooled (Fig. 3). The fractions were concentrated with chilled acetone and loaded on preparative plain PAGE (SDS-was not added). A strip of the gel was cut and bands were identified on zymogram Figure 4b. At least five fractions were visible on the zymogram, showing multiplicity among the cellulases of blister beetle M. pustulata. Rest of the poly-acrylamide gel was cut in to 19 fractions (vertically) as reported previously (Sami *et al*., 1988) and protein was eluted. Four out of 19 fractions showed endoglucanase activity. Fraction No. 10 showing the maximum activity was selected for studying the characteristics of the enzyme (Fig. 4a).From SDS-PAGE molecular weight was calculated as 150 kDa (Fig. 5). The purified enzyme was used to study its characteristics.

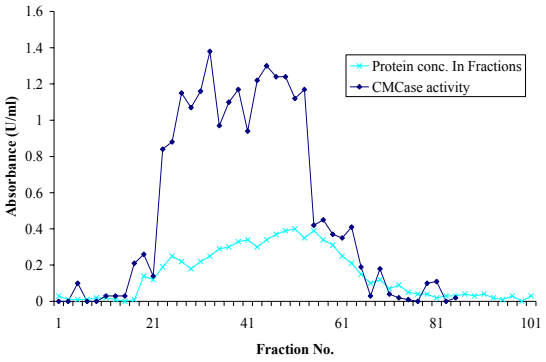

Fig. 3. The acetone precipitated crude enzyme sample was subjected to the gel filtration on sephadex-200 column. Fractions were eluted by 0.05 M Tris-Cl buffer (pH 8.2). The cellulase activity and protein contents were determined in each fraction.

pH and temperature profile

The optimum pH for cellulase activity against cellulose was 2 and 2.8 and 6.8 for the purified samples from the Native-PAGE (Fig. 6). An optimum activity was observed at 50°C. There was an increase till 50 °C and abrupt decline was observed when reaction mixture was incubated above 50 °C (Fig. 7).

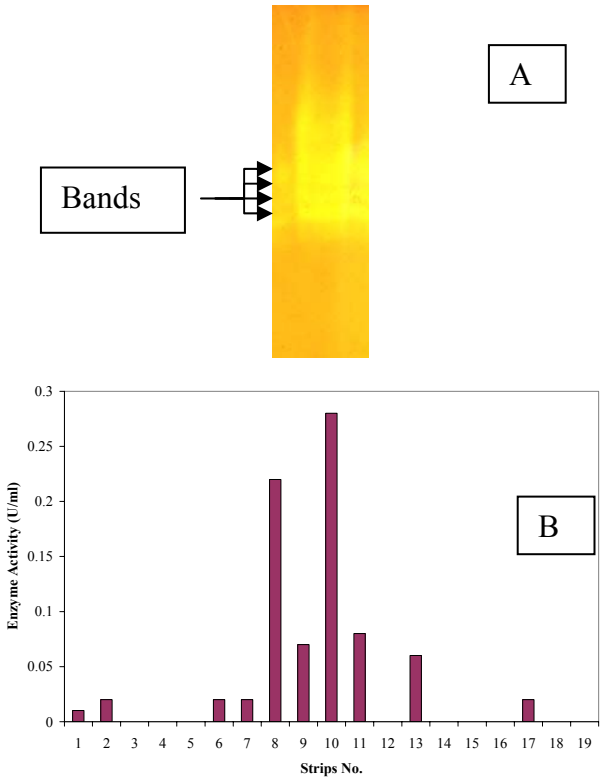

Fig. 4. (A) Four bands were appeared in the result of activity staining of the gel showing CMCase activity after gel column purification. (B) The same gel was cut into 19 pieces. These strips were crushed and the protein was eluted by dipping these strips in solution (2 ml distilled water and 2 ml buffer pH 8.0).

Effect of different substrates

The assay showed that in the presence of CMC enzyme activity was observed maximum. The enzyme showed activity against CMC, avicel, filter paper and xylan. In the presence of filter paper as substrate enzyme activity was observed almost equal to the xylan (Fig. 8) After increasing the concentration beyond 2.0 no change in enzyme activity was observed (Fig. 9).

Enzyme kinetics

In case of CMCase activity, the calculated parameters from the Michaelis-Menton plot (Fig. 10). It shows the values of $K_{m\ 0.6g/l}$ and $V_{max\ of\ 0.3}$.

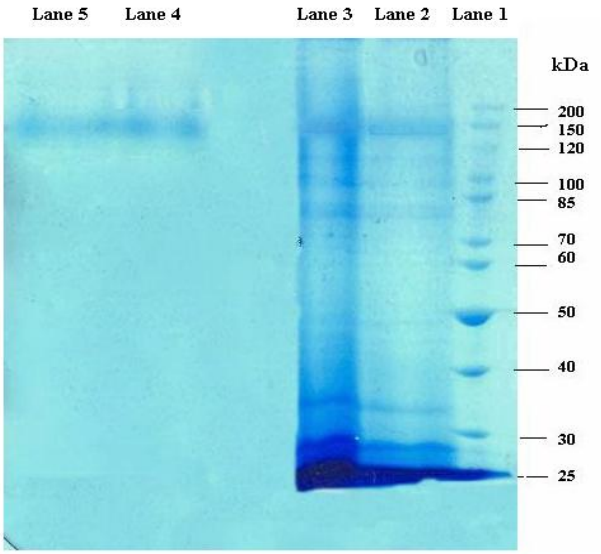

Fig. 5. Molecular weight of protein was calculated by performing SDS-PAGE with the help of protein ladder marker, 10-200 kDa (Lane 1 Protein marker, lane 2 & 3 crude sample and 4 & 5 purified sample). For SDS-PAGE 10% plyacrylamide resolving gel was prepared. Active fraction from native PAGE was used as the samples. After polymerization, electrophoresis was allowed to proceed for 6 hours at constant current of 75V. After Electrophoresis, gel was stained for overnight and shifted to de-staining solution for the bands to be visible.

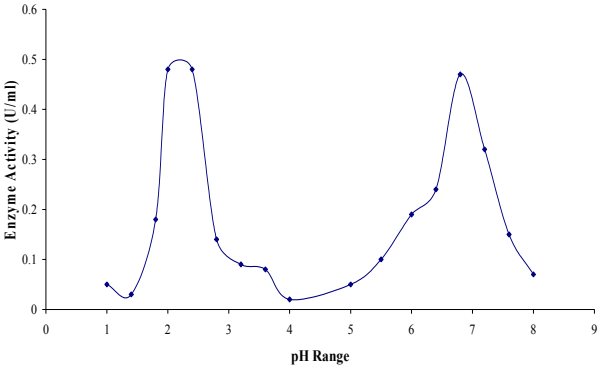

Fig. 6. pH Profile of enzyme To determine the pH profile, enzyme activity was carried out using 0.1M buffers ranging from pH 1.0-8.5.Citrate buffer (1.0-3.5), acetate buffer (4-5.4, phosphate buffer (5.8-6.8) Tris-Cl buffer (7.1-8.5). Assay was performed by DNS method.

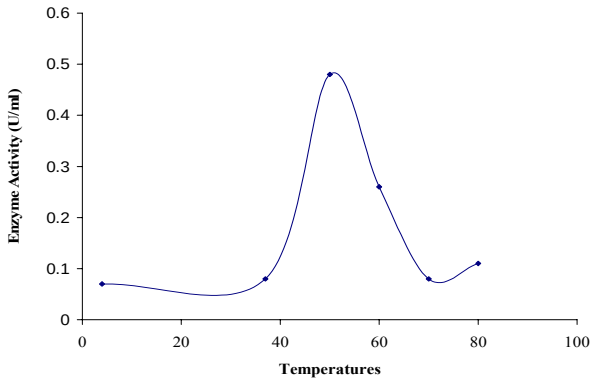

Fig. 7. Temperature Profile of CMCase Activity For determination of optimum temperature CMC assay was performed at different temperatures between 4°C - 80°C, and activity was measured by DNS method.

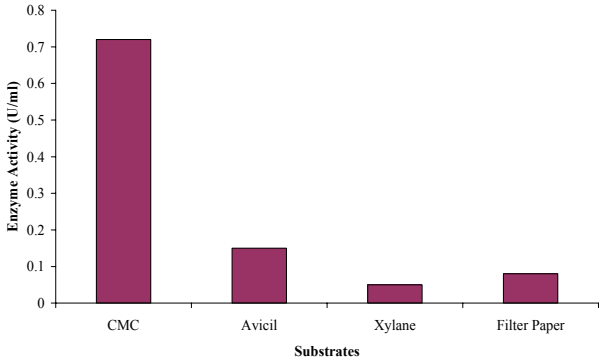

Fig. 8. A comparison of different substrates on enzyme activities was performed using the cellulose, avicel, xylan and filter paper (Whatman No. 1). Reaction mixture was prepared by adding equal concentrations of the substrate. Filter paper was prepared by boiling for 10 minutes in slightly acidic solution (0.001 M H2SO4). To determine the effect of substrate on enzyme activity 1 ml of substrate was added to reaction mixture (100 <l enzyme, 1 ml of citrate buffer). The activity was assayed by the method described above.

Hydrolytic product identification

Black spots were appeared against the light greenish brown background in paper chromatography. Product was identified as glucose (Data not shown). In thin layer chromatography product were confirmed as glucose as black spots were appeared (Fig. 11).

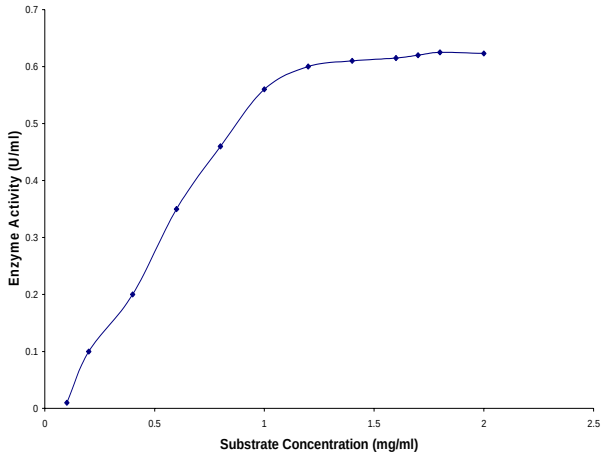

Fig. 9. Effect of substrate concentration on CMCase activity. As the concentration increases enzyme activity increases till reached the enzyme saturation.

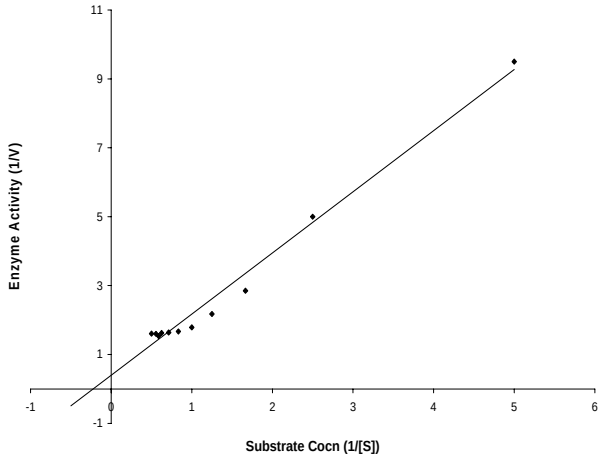

Fig. 10. Line-weaver Burk plot for showing the Vmax and Km values of *Mylabris pustulata*

DISCUSSION

Insects digest significant amount of cellulose present in their diet. *Mylabris pustulata* samples were collected from the local fields of Lahore and were initially screened for the presence of cellulolytic activity (Sami and Shakoori, 2007). Investigations were performed to characterize that cellulolytic activity. Gut and salivary gland parts showed the presence of enzyme activity (Figs. 1 and 2). Foregut part showed the prominent cellulolytic activity while negligible activity was observed in mid gut and hind gut portions (Fig. 2). This could be related to the presence of indigenous cellulose activity in the insect, as the symbiotic microbes reside in the gut. This could support the presence of indigenous digestive cellulose hydrolyzing activity of the insect. Multiple forms of CMCase activity was detected on zymogram after non-denaturing PAGE. Multiple forms of cellulases have been previously reported for microbial cellulases by this technique (Langsford *et al*., 1984; Sami and Akhtar, 1989; Sami *et al*., 1988). We have reported the heterogeneity of cellulases among insect pests previously (Sami and Shakoori, 2006). It is thought that the presence of multiple forms of cellulases in the microbial cultures and insects mainly due differential glycosyaltion of the glycohydrolase (cellulase) protein. The cellulose

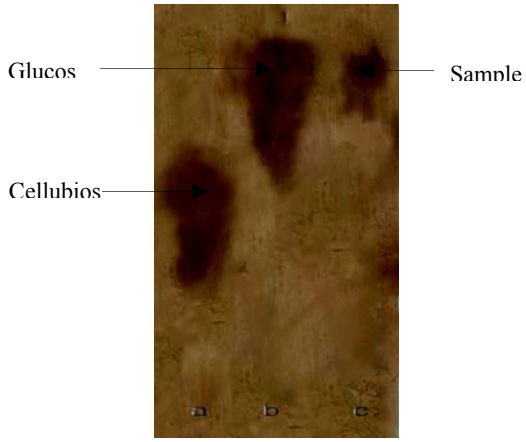

Fig. 11. Hydrolytic products were identified by TLC chromatography. Reference sugar glucose (a) cellobiose (b) and sample (c) were spotted on the TLC plate. Hydrolysate was prepared by incubating the 0.5 ml enzyme sample with 1 ml of substrate and 1 ml of buffer pH 2.0 for 24 hours at 50 °C. Solvent system used was n-Butanol: Pyridine: water (3:2:3). AgNO3 and NaOH solution were used to detect carbohydrates. For thin layer chromatography silica gel plate (15 cm long) was used. Hydrolysate sample along with reference sugar (glucose and cellobiose) and run using solvent system using iso-propanol: pyridine: and water (6: 4: 3) mixture. 2, 4-dinitrophenyl hydrazine in 10% H_2SO_4 and methanol was used as spray solution.

activity was purified by gel-column chromatography on Sephadex G-200. It was observed that the enzyme had binding capacity for Sephadex due to structural similarity with the substrate cellulose (as Sephadex is also a polymer of carbohydrate). In the case of gel filtration two peaks were observed (Fig.3). Enzyme was further purified by preparative electrophoresis and protein was eluted. The eluted fractions showed the presence of at least four fractions (Fig. 4A). The fraction No. 10 which was the fastest moving fraction (highly charged) showing maximum activity was selected for characterization studies At least 4 bands were visible on the zymogram (Fig. 4B). The optimal pH for cellulase although vary from the acidic to alkaline (Hurst *et al.,* 1977; Parks *et al.,* 2002). Usually animal cellulase reported until now have optimal activity under the acidic conditions (Watanabe *et al.,* 1997; Mallet *et al.,* 2000; Xu *et al.,* 2000). The molecular weight of the enzyme is about 150 kDa. Endoglucanases from *Clostridium* are reported to have higher molecular weight (The hydrolytic product was also identified by paper and thin layer chromatography and these were glucose. It is implied that the enzyme hydrolyzed the substrate to its basic unit (Fig. 11).

Mylabris pustulata cellulase optimum pH was found to be 2.0 and 7.0 optimum temperature 50°C (Figs. 6, 7). pH optima of the insect cellulases has been reported to be acidic, as the acid treatment of the substrate cellulose produce amorphous ends in the substrate making it susceptible to the enzyme attack. Acidic treatment of the cellulose substrate is equivalent to exoglucanase activity, as it softens the cellulose producing free ends. Though two optimum pH values has been reported for endo-beta -1,4-glucanase activity, it is thought that there may be only one protein with two active sites one for pH 2.0 and other for pH 7.0, as there is only one band appeared on SDS-PAGE (Fig.5) of molecular weight 150 kDa as a large size protein. Recently we (Sami and Shakoori, 2008) have reported that red pumpkin beetle *Aulacophora foveicollis* produce alkaline cellulose hydrolyzing enzymes perhaps due to the cucurbit plants, on which it feed. In case of *Tribolium castaneum* optimum pH for endoglucanase activity was 4.8 and 6.8 at 40 °C (Rehman *et al.,* 2009). While in case of *Planococcus citri* optimum activity was reported at pH 4.4 at 50°C (Rehman *et al.,* 2009). Plant material has hard crystalline structure mainly composed of cellulose, hemicellulose and lignin and it requires strong acidic conditions for softening and degradation. *Mylabris pustulata* has highly acidic pH optima (2.8) of endoglucanase so it is able to hydrolyze cellulose very efficiently. Recently, we have reported acidic pH optima *e.g.* 2.0 for cellulose activity present in *Oxya chinenes* (rice grass hopper) in press (Sami *et al*., in press). *M. pustulata* produced cellulose with two pH optima 2.0 and 7. 0. This could be related to the feeding habits of the insects, as they primarily use plant bio-masss they must be provided with most favorable environment for its digestion in the digestive system. Multiple forms of the enzymes were identified on PAGE after zymography (Fig. 4B). Multiple forms of cellulases in insects has been previously reported by us (Sami *et al.,* 2005; Sami and Shakoori, 2006), which could be a result of post translational modifications, glycosylation or the presence of more than one gene. There is a possibility that some of the symbiotic microbes are also producing cellulose activity. Substrate specificity of the purified fraction was studied. Cellulose, avicel, filter paper, xylan were used as a substrates. Using filter paper as a cellulose substrate, enzyme showed equal activity to xylan. Against carboxy methylcellulose maximum activity was observed (Fig. 8). Most of the cellulases belonging to family 9 of Glycohydrolases *e.g.*, plant "cellulases" show low or no activity on crystalline cellulose, but clearly measurable activity on soluble cellulose derivatives, such as carboxymethyl cellulose, noncrystalline phosphoric acid swollen cellulose, or a variety of plant polysaccharide substrates, including xylans, 1,3-1,4-β-glucans, (Yoshida and Komae, 2006; Urbanowicz *et al.*, 2007) If the substrate used after the acid treatment, they may be better hydrolyzed as enzyme work better in acidic pH range. This may lead to the implication of paper recycling after their cloning and over-expression at industrial level. The rate of reaction rise linearly as substrate concentration increased and then began to level off and approached a maximum at higher substrate concentrations. This was the enzyme's equilibrium stage at which the rate of forward reaction was

equal to the rate of backward reaction. So endoglucanase obeys Michaelis-Menten kinetics (Figs. 9, 10).

CONCLUSIONS

Mylabris pustulata has an efficient and complete digestive cellulase system able to hydrolyze plant leaves as well as other substrates due to its highly acidic enzyme system and so able to feed on different economically important crop. The enzyme has a colossal potential to be used as a Biotechnological enzyme for the bioconversion of cellulose, being an acidic cellulase.

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