Full Length Research Paper

Isolation of cellulolytic activities from *Tribolium castaneum* (red flour beetle)

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Cellulolytic enzymes have immense potential to convert cellulosic biomass into useful products. *Tribolium castaneum* crude proteins were isolated to screen the cellulolytic activities. The activity was established by substrate-agar plate assay and confirmed by endoglucanase assay. Cellulolytic activity was further purified and characterized using the different chromatographic techniques and electrophoresis. Gel filtration chromatography showed the presence of multiple forms of enzyme activities with different molecular weights. Stability of enzyme activity was investigated at different temperatures and pH. Optimum pH for was found 4.8 at 40°C determined as optimum temperature. Gradually decreasing Enzyme activity remained half at 60°C. Zymography and SDS-PAGE showed the presence of multiple forms of endoglucanase activities (Cel I and Cel II) with molecular weight of 55 kDa and 35 kDa.

Key words: *Tribolium castaneum*, cellulolytic enzymes, endoglucanase, insect cellulase, red flour beetle.

INTRODUCTION

The most common organic compound on earth, cellulose, is about 33% of all plant matter. Cellulose is mainly produced by terrestrial plants and marine algae and used as a food source by many organisms (Teeri, 1997). Cellulose production by non-photosynthetic organisms including bacteria, marine invertebrates, fungi, slime molds and amoebae has also been documented (Coughlan, 1990; Tomme et al., 1995; Lynd et al., 2002). It has supreme source of renewable energy in higher animal life as well as for lower animals.

Pests are lower animals which obtain food and energy by degrading plant cellulose into its constituent residues by the cellulolytic enzymes cellulases. Cellulose hydrolysis yields cellobiose and glucose monomers which are most important energy source around the globe (Eveleigh, 1987). In microorganisms, like bacteria and fungi, these enzymes are well studied and characterized (Coughlan, 1990; Nathan et al., 2007). Animals are usually considered dependent on gut microbiota for cellu-

lose digestion as they are unable to produce indigenous cellulases (Moran et al., 2005; Eckburg et al., 2005; Martin and Martin, 1978). Therefore earlier investigations have been conventionally focused on non-animal cellulase sources such as bacteria and fungi.

Isolation and characterization of cellulase encoding genes from lower animals, in recent years, shows that cellulose hydrolysis in some invertebrate animal taxa involves synergistic action of flagellates, bacteria, yeasts (Breznak and Brune 1994; König et al., 2002; Varma et al., 1994; Cleveland, 1924) and indigenously produced cellulases. Such cellulolytic activities were studied amongst many invertebrates like nematodes (Smant et al., 1998; Be´ra-Maillet et al., 2000), arthropods (Watanabe et al., 1997; Wei et al., 2006) and mollusks (Guo et al., 2008; Imjongjirak et al., 2008; Marshall and Grand, 1976; Xu et al., 2000).

Physiological functions of animal cellulases are distinct and depend on their source. In bacteria, the enzymes are involved in breakdown of fungal cell walls in order to allow them to be used as a food source and in bioremediation. In case of pests especially beetles, cellulase producing symbiotic protozoan present in intestine (Breznak

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and Brune, 1994; Brune, 1998; Dolan, 2001; Dyer, 2002) hydrolyze cellulose to glucose in synergistic collaborative relationship with indigenously produced enzymes (Wei et al., 2006; Zverlov et al., 2003). In the present study we had isolated the cellulolytic activities from a beetle *Tribo-lium castaneum* (red flour beetle). This beetle cause ex-tensive damage to the rice and wheat grains (Via, 1999; Weston and Rattlingourd, 2000). This beetle uses its amylase and cellulase activities to get energy from the food.

MATERIALS AND METHODS

Insect sampling

T. castaneum (red flour beetle) samples were collected from rice and grain stockpile when temperature was around 40^oC and stored at 4^oC.

Preparation of crude enzyme sample

Crude enzyme sample was prepared by homogenizing 50 g of in-sect sample in 200 ml 0.1 M Phosphate buffer of pH 7.0. Homo-genate was kept overnight in freezer and centrifuged at 10,000 rpm to discard pellet. 100 ml of supernatant was added to 400 ml of ice-cold acetone and kept overnight at 4^oC to get proteins in precipitate form. The mixture was centrifuged at 10,000 rpm for 15 min. The pellets were air dried and dissolved in 10 ml of 0.1 M phosphate buffer and 10 ml of Tris-HCl buffer of pH 6.0 and 8.0 respectively. This crude protein sample was stored at 4^oC and used as the enzy-me source.

Screening for cellulolytic activity

A modification of substrate-agar plate assay (Teather and Wood, 1982; Sami and Akhtar, 1990) was used to screen the cellulase activity in crude protein extract by observing the cleared zone for-mation around the sample well against a red-stained background on agar plates. 3% agar and 1% CMC were mixed with buffers of different pH range to prepare agar gel. Agar gel was poured in petri plates to solidify. A sample of 100 µl of crude protein extract was loaded in the hole punched in the plate centre. After overnight incu-bation CMC-agar plates were stained with 0.1% congo red dye for 15 min followed by de-staining with 0.5 M sodium chloride solution.

Endoglucanase enzyme assay

Enzyme assay was performed by measuring the amount of redu-cing sugar using modified DNS (dinitrosalicylic acid) method (Nelson, 1944). Concentration of the released glucose was mea-sured from a standard glucose curve. Enzyme activity (U/ml) was determined considering one I U equal to 1 µmol min^{-1} of glucose formed in the hydrolysis reaction. Reaction mixture was prepared by mixing 100 µl of the crude enzyme sample with 0.5 ml of carbo-xymethyl cellulose (CMC) solution (2%, w/v) and 0.5 ml 0.1 M so-dium acetate buffer (pH 5.0). Then mixture was incubated for 5 h at 50^oC with gentle shaking. After incubation, 2 ml of DNS reagent was added to reaction mixture and incubated in boiling water bath for 15 min. After incubation absorbance was noted at 540 nm.

pH and temperature optima of enzyme activities

To determine the pH profile of cellulase activity, the enzyme assay was carried out using 0.1 M buffers ranging from pH 4-8. Acetate buffer (pH 4-5), phosphate buffer (pH 5-6) and Tris-HCl buffer (pH 7-8) were used. Agar and CMC were mixed with buffers of different pH and enzyme activity was determined using the DNS method as described in endoglucanase assay. In order to determine the optimum assay temperature, enzyme assay was performed at different temperatures ranges between 4 - 70^oC using the buffer of pH 4.8.

Effect of substrate concentration

Effect of substrate concentration was studied by using different con-centrations of CMC ranging from 0.5-5.0 % in the endoglucanase assay mixture using buffer of pH 4.8 and at temperature 40^oC.

Effect of β-mercaptoethanol

Endoglucanase activity was studied in the presence of different concentrations of β-mercaptoethanol in enzyme assay mixture. 100 µl of β-mercaptoethanol ranging in the concentration of 100-500 mM was added to reaction mixture and enzyme activity was deter-mined.

Protein purification

Endoglucanase activity was purified using the chromatographic techniques. The crude enzyme sample was fractionated at 4^oC using the swollen sephadex G-75 suspension, packed in a column (1.6 × 16 cm). The cellulase activity and protein concentration were determined by enzyme assay in collected fractions. Active fractions having endoglucanase activity were pooled and subjected to ion exchange chromatography for further purification. Active fractions were loaded into ion exchange column (0.8 x 15 cm) packed with the DEAE-sephadex gel and equilibrated with Tris-HCl buffer. Sam-ple was loaded and the bound proteins were eluted by a linear gra-dient of 0-0.5 M NaCl in Tris-HCl buffer (pH 8.5). A total of 50 frac-tions (2 ml each) were collected. Protein concentration and enzyme activity were determined in each fraction by dye binding assay (Bradford, 1976) and DNS method. For estimation of protein 2 ml of bradford reagent was added to 0.5 ml of each fraction, absorbance was noted at 595 nm and concentration was determined by using the BSA standard curve. Active pooled fractions were stored at 4^oC.

Native PAGE and zymography

Polyacrylamide gel electrophoresis (PAGE) was performed under non-denaturing conditions (Sami and Akhtar, 1990). Active fractions from ion exchange chromatography were subjected to electropho-resis. After electrophoresis gel was cut in two strips vertically. One strip was placed onto substrate agar plate to locate the position of endoglucanase activity on gel. Substrate agar gel plate was prepa-red using the 3% agar and 2% CMC, mixed with buffer of pH 4.8. After incubation for 3 h at 40^oC, gel strip was removed followed by staining and detaining of substrate agar plate with 0.1% congo red dye and 0.5 M sodium chloride respectively.

Protein elution from gel

Protein was eluted from gel after locating the position of the bands of endoglucanase activity. Gel bands were crushed to pieces and 1 ml 0.5 M phosphate buffer pH 5.0 was added to elute the enzyme activity. After overnight elution at 4^oC, gel was centrifuged and puri-

Figure 1. Yellow color area in centre of substrate agar plate shows the hydrolysis of carboxy methyl cellulose by endoglucanase activity.
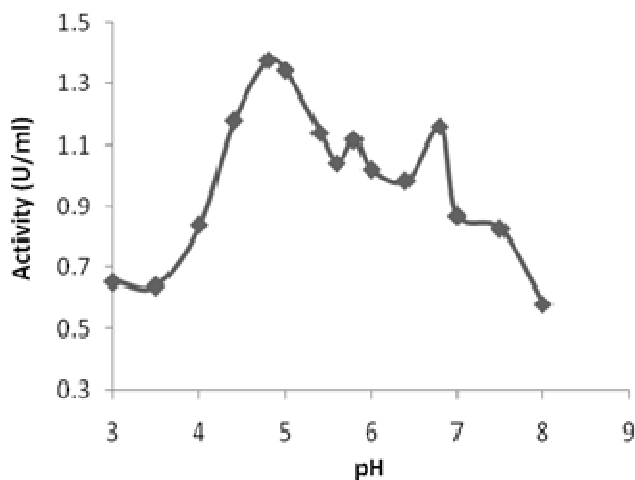

Figure 2. Optimum endoglucanase activity was observed in the acidic range at pH 4.8 and second peak was observed at 6.8 which is pH of insect gut. After pH 6.0 enzyme activity was declined abruptly.
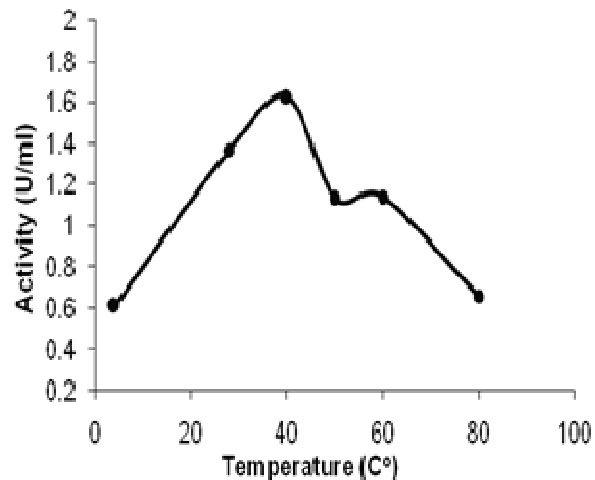

Figure 3. Endoglucanase activity was obeserved maximum at temperature 40°C. Almost half activity was remained at 60°C.

fied enzyme activity was further subjected to SDS-PAGE to calculate molecular weight.

Molecular weight determination by SDS- PAGE

SDS-PAGE was performed as described by to determine the molecular weight of endoglucanase activities purified by chromatographic techniques and zymography.

RESULTS

Substrate-agar plate assay

Substrate agar plate assay showed the presence of cellulase activity by forming the cleared zone around the sample well against a red-stained background on agar plates (Figure 1). The cellulase activity digested the carboxymethyl cellulose in area that did not retain the congo red dye on destaining.

pH and temperature profile

Endo-glucanase activity peaks were observed both at acidic pH (4.8) and basic pH (7.5). Maximum activity was observed at 4.8. Almost no enzyme activity was observed at pH values below 4.4 and above 7.5 (Figure 2). Optimum activity was observed at temperature 50°C. There was an increase in enzyme activity with rise in temperature till 40°C and then there was an abrupt decline in enzyme activity. Although some enzyme activity was observed at temperature 50°C but was almost half than the activity showed at 40°C (Figure 3). At 80°C almost no enzyme activity was observed.
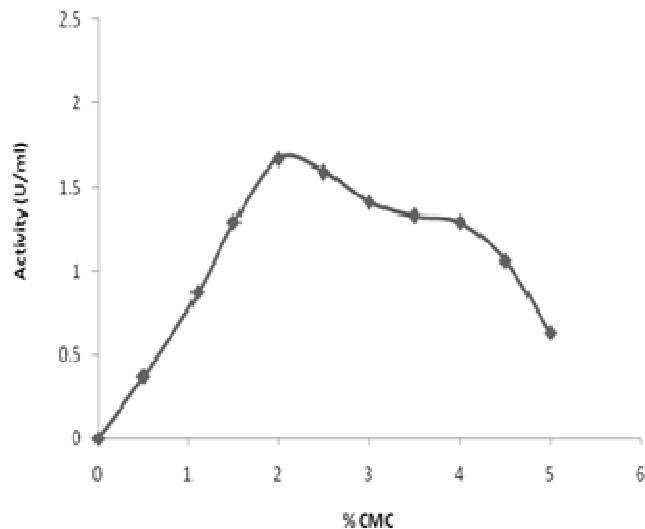
Figure 4. An increasing substrate concentration showed the increase in enzyme activity. After 2% CMC concentration enzyme activity was continuously declined.
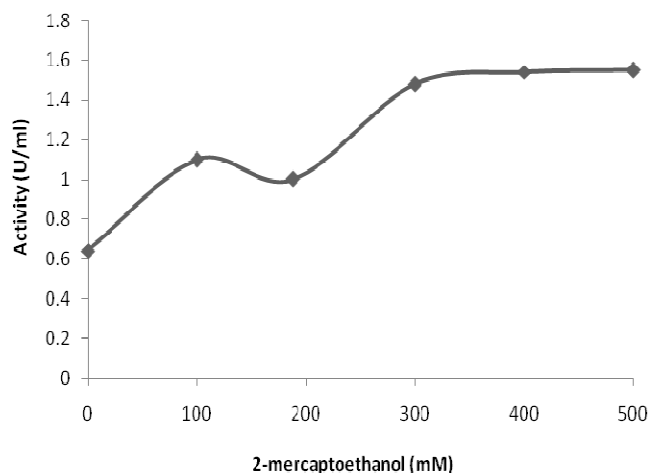

Figure 5. Mercaptoethanol gradually increased the enzyme activity till concentration of 300 mM.

Effect of substrate concentration

Endoglucanase activity was found maximum at 2% of substrate (CMC) concentration. After the increase in the substrate concentration to 2%, slight decline was observed in the enzyme activity (Figure 4). At 5% of substrate concentration very little endoglucanase activity was observed. This may be due to the inhibition of enzyme activity by product formed.

Effect of β-mercaptoethanol

β-Mercaptoethanol gradually enhanced the endogluca-nase activity. Increase in the concentration of β-mercap-
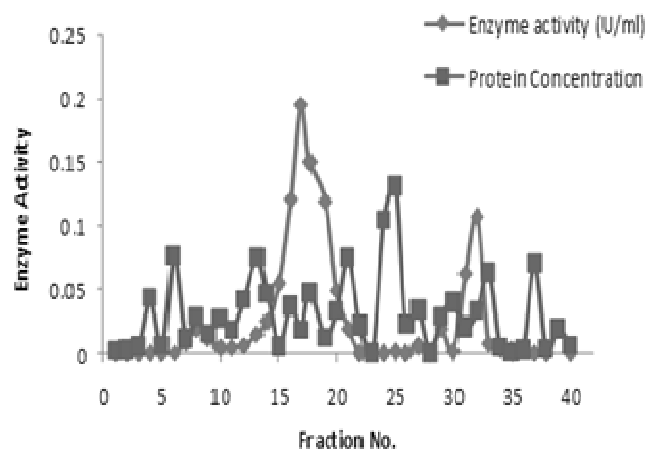

Figure 6. Two peaks were observed with active fraction in gel filtration chromatography. Fraction no. 13 to 22 and 30 to 34 were pooled as active fraction.

toethanol also increased the enzyme activity till 300 mM concentration (Figure 5).

Protein purification

Different fractions obtained from gel filtration chromato-graphy were found active. Fraction no. 13 to 22 and 30 to 34 showed endoglucanase activity (Figure 6). Active pooled fractions were subjected to ion exchange chroma-tography. 2 peaks were obtained by ion exchange chro-matography. Active fractions were ranging from 17 to 20 and 23 to 27 (Figure 7).

Native PAGE and zymography

2 bands Cel I and Cel II were obtained by the zymogra-phy after native PAGE (Figure 8). The enzyme activity from the gel bands was eluted.

Molecular weight determination by SDS- PAGE

Purified enzyme activity was applied to SDS-PAGE to de-termine the molecular weight. Cel I and Cel II had mole-cular weight 55 kDa and 35 kDa (Figure 9).

DISCUSSION

Many insects including beetle family have been reported to have their indigenously produced endoglucanase acti-vity (Guo et al., 2008; Wei et al., 2006; Lee et al., 2004; Zverlov et al., 2003; Scrivener et al., 1997). *T. Casta-neum* commonly known as red floor beetle is also mem-ber of beetle family. Substrate agar plate assay showed the presence of the endoglucanase activity (Figure 1).
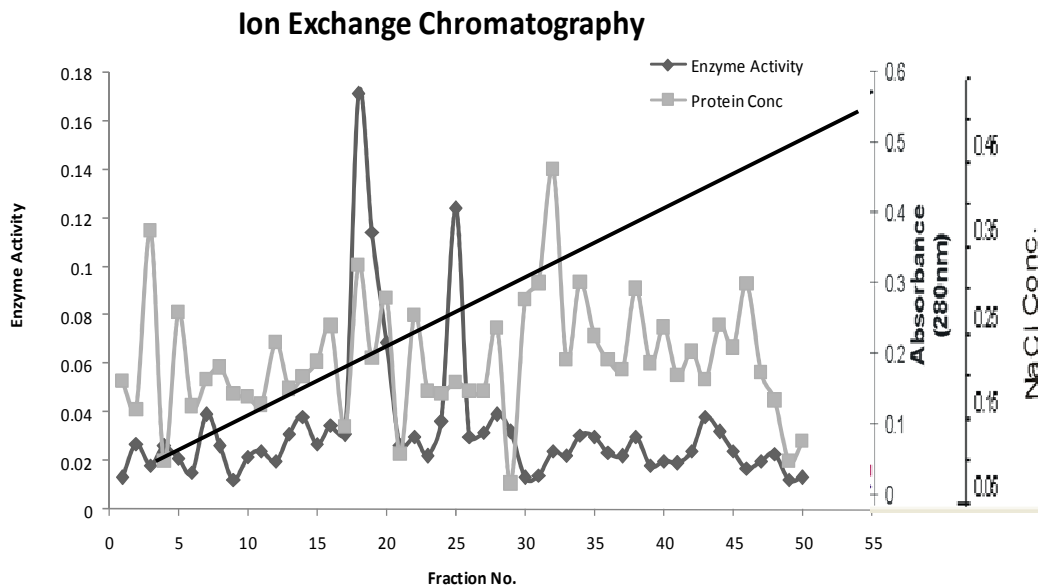
Ion Exchange Chromatography

Figure 7. In ion exchange chromatography fraction no 17 to 20 and 23 to 27 were found active and pooled as purified enzyme activity.
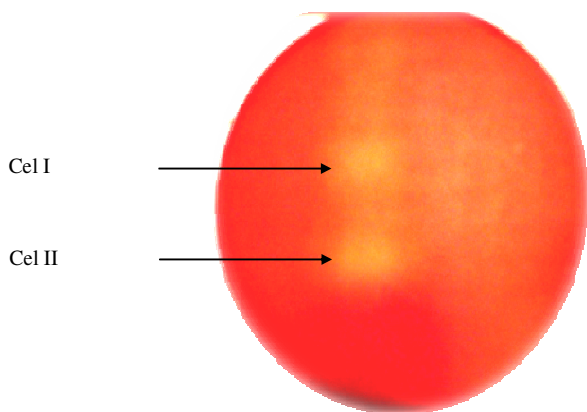

Figure 8. Zymography showed the presence of multiple cellulase activities. Two bands Cel I and Cel II were apeared on substrate agar plate after staining and destaining.
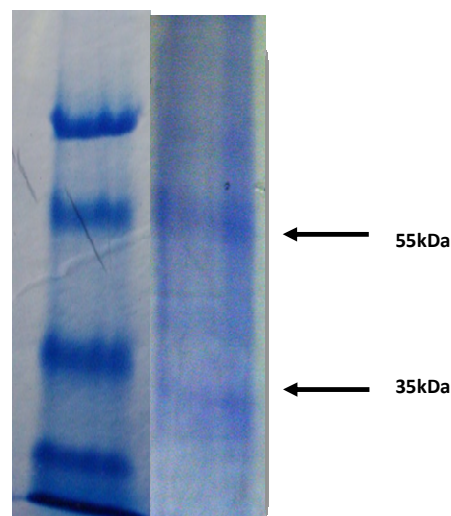

Figure 9. SDS-PAGE showed the molecular weight of the enzyme activities (Cel I and Cel II) as 55 kDa and 35 kDa, respectively.

After 48 h of incubation digested yellow area in centre of petri plates turned black (results not shown). This may be due to the formation of glucuronic acid. Almost all animal cellulases reported until now have optimal activity under acidic conditions (Watanabe et al., 1997, 1998; Be´ra-Maillet et al., 2000, Xu et al., 2000). Cellulase activity isolated from the mulberry longicorn beetle, *Apriona germari* showed the highest activity at 55°C and pH 6.0 (10a). Our isolated cellulases had also shown maximum activity in acidic range at pH 4.8 as well as at 6.8 (Figure 2). Usual-ly cellulases are present in stomach juice and midgut of insects, therefore at acidic pH beetle cellulases show maximum activity (Zverlov et al., 2003). In enzyme

assay as the substrate (CMC) concentration raised from 2% slight decline in enzyme activity was observed (Figure 4). Decline in enzyme activity is due to the product inhibition. Gradual increase in enzyme activity with rise in concen-tration of β-mercaptoethanol (Figure 5) may be due to the reduction of disulphide bonds present in enzyme which allow conformational change in native structure (Sami et al., 2006, Sami and Haider, 2007). Enzyme purification by gel filtration and ion exchange chromatography show-ed the presence of multiple forms of cellulase activities on zymography (Figure 8). In some beetles multiple forms of cellulolytic activities have been reported (Sami et

al., 2005). Molecular weight of purified endoglucanase activities was determined as 55 kDa and 35 kDa (Figure 9). This range is almost same as in case of already reported insect cellulases. In beetle, *P. hilaris* cellulase was estimated to be 47 kDa (Scrivener et al., 1997) while longhorn beetle *Rhagium inquisitor L* have been investigated for the presence of endoglucanase, having a molecular mass of 32 kDa (Lee et al., 2004). Enzyme activity further needs to be characterized by gene sequencing. This enzyme activity also may have industrial applications as it works well in acidic environment as well as stable at temperature 60°C.

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