http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 15 (7): 333-340, 2012
ISSN 1028-8880 / DOI: 10.3923/pjbs.2012.333.340

Multiple Endo-β-1,4-glucanases Present in the Gut Fluid of a Defoliating Beetle, *Podontia quatuordecimpunctata* (Coleoptera: Chrysomelidae)

Mohammad Mosleh Uddin, Md. Mahmudul Hasan Chowdhury, Suman Mojumder and Dwaipayan Sikdar
Department of Biochemistry and Molecular Biology, University of Chittagong, Bangladesh

Abstract: Endo-β-1, 4-glucanase (EC 3.2.1.4) activity was measured in the gut fluid of phytophagous insect *Podontia quatuordecimpunctata* Linnaeus (Coleoptera: Chrysomelidae) in different days of development. The eight day-old larva showed maximum activity with 1.73 U mg^{-1} of protein, which was confirmed by gel zymography. In zymogram, using Carboxymethyl Cellulose (CMC) as substrate, four distinct cellulolytic protein bands were detected in leaf borer gut fluids through out of its development. The optimum temperature and pH were 60°C and 5.0, respectively. This endo-β-1,4-glucanase showed maximum stability at 20-45°C with ~ 20% remaining activity. Zymography also showed complete loss of endo-β-1,4 glucanase activity at 55°C. This is the first report that the cellulolytic enzyme is produced in the gut of *P. quatuordecimpunctata* through the whole developmental stages, from the 1st instar to the adult, except for pupae.

Key words: Carboxymethyl cellulose, coleoptera, insect cellulase, zymography

INTRODUCTION

Cellulose is the main structural polysaccharide of the plant material and is the most globally abundant biopolymer. These polymers are comprised of sugar units, usually glucose, joined by β-1,4 and β-1,3 glycosidic bonds (Watanabe and Tokuda, 2001). Biological cleavage of these bonds require the synergistic action of three types of glycoside hydrolases (GH): endo-β-1,4-glucanases (EG; EC. 3.2.1.4), exo-β-1,4-cellobiohydrolases (CBH; EC. 3.2.1.91) and β-glucosidases (EC. 3.2.1.21) (Clarkel, 1997). EG, hydrolyzing internal β-1,4-glycosidic bonds of cellulose polymers of four or more glucose units, shortens and dissolves cellulose polymer, thus provides substrate for CBH (Scrivener and Slaytor, 1994; Tokuda *et al*., 1997; Watanabe *et al*., 1997). Moreover, through catalyzing transglycosylation reaction it can also rejoin the glucose oligomers (Lindner *et al*., 1983). CBH releases cellobiose from the nonreducing ends of cellulose. CBH is also thought to disrupt the hydrogen bonding in crystalline cellulose, thus allowing the EG to endo-depolymerize. β-glucosidases hydrolyse cellobiose or longer cellulose chains to release glucose.

Although cellulases have been extensively used in different industries including textile, laundry, brewery, wine, pulp, paper and agriculture (Bhat and Bhat, 1997; Bhat, 2000; Ohmiya *et al*., 1997) for long time but the most recent interest in cellulase research is due to its ability to degrade lignocellulose materials of plants thus to produce biofuel. Although bacterial and fungal cellulases have been used for the last two decades in industrial bioethanol technology, more efficient cellulases are needed to reduce cost in biofuel production (Wyman, 2007) and also for applications in other industries.

Insects are very efficient in degradation of biomass with their highly efficient gut systems which can truly be considered as highly efficient natural bioreactors. Previously, enzymatic activity against cellulose substrates was detected in digestive fluids of insect species belonging to ten insect orders (Martin, 1983; Watanabe and Tokuda, 2001; Willis *et al*., 2010a). These activities were historically attributed to gut symbiotic flora, until the first insect cellulase was described in *Reticulitermes speratus* (Watanabe *et al*., 1998). Various studies have reported endogenous insect cellulase enzymes in the orders of Blattaria, Coleoptera, Hymenoptera, Hemiptera, Phthiraptera and Orthoptera (Watanabe and Tokuda, 2001).

Larva and adults of defoliator, *Podontia quatuordecimpunctata* L. (Coleoptera: Chrysomelidae), feed on the leaves of the Otaheite apple *Spondias dulcis* tree. There are only a few reports on cellulolytic activity in the Coleoptera (Oppert *et al*., 2010; Willis *et al*., 2010a), only in single species, *Aulacophora foveicollis*, from Chrysomelidae family has been shown to have a cellulolytic system (Sami and Shakoori, 2008). The purpose of this study was to investigate the presence of cellulolytic activity in *P. quatuordecimpunctata*.

Corresponding Author: Dwaipayan Sikdar, Associate Professor, Department of Biochemistry and Molecular Biology, University of Chittagong, Chittagong-4331, Bangladesh

MATERIALS AND METHODS

Insect collection: *Podontia quatuordecimpunctata* larva of different ages (2, 3, 6, 8, 10 and 12 days) and adults were collected from leaves of an infested hog plum tree during April-June from Fatehpur village near the Chittagong University campus, Bangladesh. Until dissection, insects were kept on or in close proximity to plant host tissues where insects were actively feeding.

Gut dissection and fluid collection: Insects were dissected on the same day of collection. After immobilizing on ice for around half an h, the larva were kept abdomen side up on a petri dish which was on ice and dissected to remove the entire, intact guts. The multiple guts of different day's larva and adult were taken separately in micro centrifuge tubes. Dissected guts were cut into small pieces, homogenized by vortexing to ensure fluid extraction and centrifuged at 10,000 rpm for 10 min at 4°C. The resulting supernatants were transferred to new 1.5 mL micro centrifuge tubes and stored at -20°C until use. All the biochemical analyses with this supernatant were carried out in triplicates.

Plate assay for cellulolytic activity: A modified substrate-agar plate assay (Teather and Wood, 1982) was used to assay endo-β-1,4-D-glucanase activity qualitatively in the gut fluid of eight day's larva. To observe the clear zone formation around the sample well against a red-staining background on agar plates, 1% CMC and 3% agar were mixed with 0.1 M Na-acetate buffer, pH 5.3 and poured in petri dishes to solidify the gel. 220 μg of gut protein in water was loaded in the hole punched in the center of plate. After overnight incubation at 37°C, the plates were stained with 1% Congo red (Sigma-Aldrich) for 10-15 min before de-staining with 1.0 M NaCl solution for 15-20 min for several times.

Measurement of endo-β-1,4-D-glucanase activity: Endo-β-1,4-D-glucanase activity was quantified as the rate of production of reducing sugars from the substrate, carboxymethyl cellulose (BDH chemical Ltd pool England), using a modified 3,5-dinitrosalicylic acid (DNSA) assay method (Miller, 1959). Gut fluids (33 μg of protein) of each respective day's larva and adult mentioned above and 235 μL of 1% CMC suspended in 0.1 M Na-acetate buffer, pH 5.3 were mixed separately and incubated for 30 min at 37°C. The reactions were stopped by adding 450 μL DNSA solution followed by heating in boiling water for 10 min. After adding 40% Rochelle salt (MARK), the reaction mixers were cooled at room temperature for 5 min and centrifuged at 10,000 rpm for 5 min. Supernatants were transferred to new microcentrifuge tubes to measure absorbance at 540 nm on a UV spectrophotometer (Shimadzu).

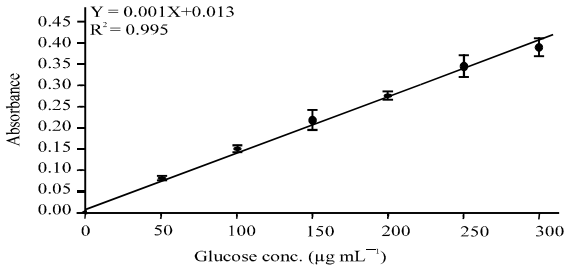

Fig. 1: Standard calibration curve for the determination of reducing sugar released during endo-β-1,4-D-glucanase assays

A blank was prepared with substrate and DNSA solution before adding gut juice so that enzymes present in the juice could not hydrolyze CMC and the subsequent process same as described above. One unit of cellulolytic activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per min at 37°C and pH 5.3. A standard curve of absorbance against amount of glucose (50-300 μg) was constructed to enable calculation of the amount of reducing sugar (glucose equivalents) released during endo-β-1,4-D-glucanase assays (Fig. 1). In all following experiments, enzyme activity was measured using this method and the extract of 8 days larvae was used.

Effect of temperature and pH on enzyme activity: The effects of temperature and pH on enzyme activity of gut fluids were estimated at eleven temperatures and nine pH values. The effect of temperature on endo-β-1,4-D-glucanase activity was examined by incubating the reaction mixture (in 0.1 M Na-acetate buffer pH 5.3) over a temperature range of 20 to 70°C (using 1% CMC as substrate). The thermal stability of endo-β-1,4-D-glucanase was determined by pre-incubation of the sample at 20, 25, 30, 35, 40, 45, 50 and 55°C for 30 min, followed by measurement of activity under the standard test conditions as mentioned before. Activity at optimum temperature was taken as 100.

Optimal pH for the enzyme activity was determined by measuring the hydrolysis of CMC in a series of buffers at various pH values ranging from pH 2 to 10. The buffers used were KCl-HCl buffer 0.1 M pH 2.0, K-hydrogen pthelate HCl buffer (0.1 M) pH 3, Na-acetate buffer (0.1 M) pH 4 and 5, phosphate buffer (0.1 M) pH 6 and 7, tris-HCl buffer (0.1 M) pH 8, glycine-NaOH buffer (0.1 M) pH 9 and 10.The pH at which activity was highest was considered as 100%.

Zymography: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) were performed with some alterations to detect activity of endo-β-1,4-D-glucanase through zymogram techniques

(Schwarz *et al*., 1987). For the CMC zymogram, SDS-10% PAGE resolving gel was prepared by adding 0.2% CMC slowly to prevent aggregation. After dissolving CMC, ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were added and gels were incubated at 45°C for 2 h or until polymerization. Defoliator gut fluids (8 μg) of different aged larva and adult were solubilized in 1 volume of sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.01% bromophenol blue) and loaded onto the gel. Proteins in samples were separated at a constant 100 V at 4°C for approximately 4 h or until dye reached the bottom of the gel.

After electrophoresis, the gel was washed with 50 mL wash buffer (0.1 M Na-succinate pH 5.8 and 0.1% β-mercaptoethanol) at room temperature for five times (each was for 30 min) but last wash was at 30°C to allow for enzyme activity against the substrate. Then, the remaining CMC in the gel was stained with 0.1% Congo red for 30 min and destained by washing in a solution of 1 M NaCl at room temperature to reveal zones of clearing where CMC had been degraded by enzyme. For improving visualization of clearing area, 100 μL of glacial acetic acid was added after destaining the gel (Waeonukul *et al*., 2007). Zymography was also carried out with 8 days' larval gut protein after heating the solubilized sample at 45 and 55°C for 30 min.

Measurement of protein in gut fluid of *P. quatuordecimpunctata*: The concentrations of protein in samples of gut fluid were measured using FCR method (Lowry *et al*., 1951). In brief, different aliquots of standard Bovine Serum Albumin (BSA) solution (250 μg mL^{-1}) were pipetted out in different tubes along with the aliquots of samples in separate tubes. Equal amount of alkaline copper sulphate was added in each tube and then allowed to stand for 15 min. Equal amount of Folin-Ciocalteu reagent was added in each tube and the tubes were left for 30 min. Blue color was formed which was measured at 650 nm against a proper blank where no protein solution was used. The protein concentration of the extract was calculated from the standard curve (Fig. 2).

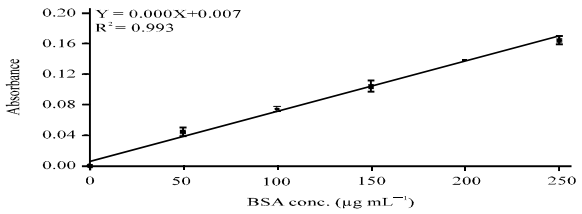

Fig. 2: Standard calibration curve for the determination of total protein in gut juice

RESULTS

Qualitative measurement of cellulolytic activity: Substrate-agar plate assay showed the presence of cellulase activity by forming the cleared zone (yellow color) around the sample well against a red-stained background on agar plates (Fig. 3). The cellulase activity digested the CMC in area that did not retain the Congo red dye on destaining.

Endo-β-1,4-D-glucanase activity during *P. quatuordecimpunctata* development: Activities of endo-β-1,4-D-glucanase enzymes were assayed from gut fluids of six different aged larva (2, 3, 6, 8, 10 and 12 days after their hatching) on their development, pupa and adult of *P. quatuordecimpunctata*. Larval extract of 8th day showed maximum enzyme activity which was 1.73 U mg^{-1} protein and calculated as 100%. Activity was decreased at both sides gradually before and after of this developmental stage (Fig. 4a). The last instar (12 days) larva and adult exhibited only 9.0 and 7.0% of maximum activity, respectively, but pupa showed no activity.

To evaluate the activity of different components of endo-β-1,4-D-glucanase system during *P. quatuordecimpunctata* development, zymogram analysis using CMC as a substrate was used. Zymography showed that at least four active bands are responsible for the measured cellulase activity (Fig. 4b). No differences in number and positions of the cellulolytic protein bands in the gel were observed among different larval stages and adult. The two of the four bands were very prominent and close compare to the remaining two other bands as visualized on the gel zymography.

Effect of temperature on endo-β-1,4-D-glucanase activity: The optimum temperature for the hydrolysis of carboxymethyl cellulose was 60°C and another broad

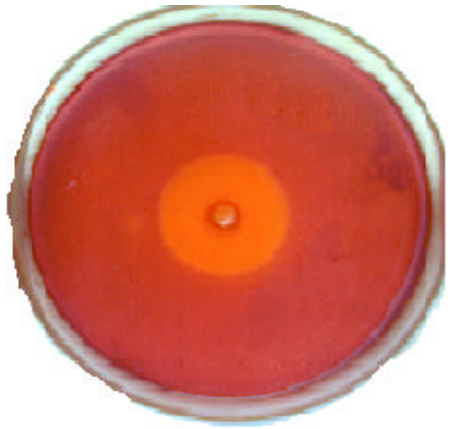

Fig. 3: Presence of cellulolytic activity in substrate-agar plate

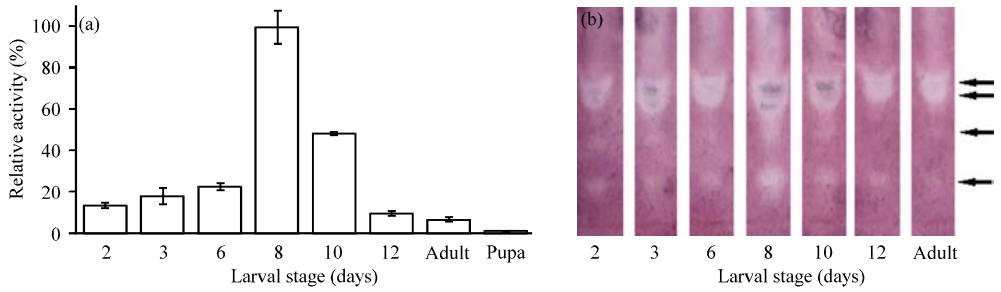

Fig. 4 (a-b): (a) Comparison of cellulolytic activity (with standard error bars) in gut fluids from different larval developmental stages (2, 3, 6, 8, 10 and 12 days after hatching), pupa and adult containing 15 µg of proteins and (b) Zymogram (10% PAGE+0.2% CMC) depicting corresponding active cellulase protein profile in gut fluids (8 µg of total gut protein per lane) from different life stages of *P. quatuordecimpunctata* development. Arrows indicate the four cellulase activity bands detected

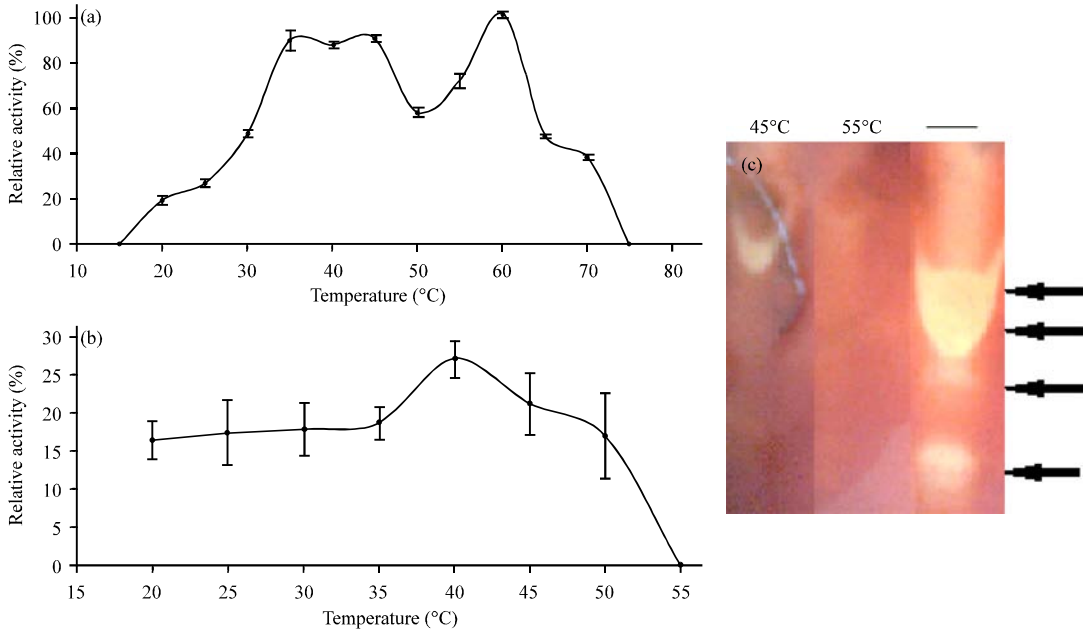

Fig. 5 (a-c): Endo-β-1,4-D glucanase activity (a) At different temperatures between 20-70°C without pre-meubation, (b) With pre-incubation at different temperatures between 20-55°C, Error bars represent the standard deviation and (c) Conformation of temperature stability of endo-β-1,4-D-glucanase in gel zymography. Sample without (—) and with pre-incubation at 45 and 55°C for 30 min prior to load in the gel. Arrows indicate the four cellulase activity bands detected

range temperature was 35-45°C at which around 90% of highest enzyme activity was found (Fig. 5a). The activity of endo-β-1,4-D-glucanase was decreased gradually to zero at 75°C. After pre-incubation for 30 min at different temperature, the enzyme was stable at 20-50°C. The enzyme retained only around 20% of its activity in the range of 20-50°C but out of this range, it lost its activity completely (Fig. 5b). Remaining activity is measured as the percentage of enzyme activity compare to the activity assayed under the standard test conditions. In zymogram analysis, only one out of four isoforms of enzyme retained little activity at 45°C but activity was completely lost at 55°C (Fig. 5c).

Effect of pH on endo-β-1,4-D-glucanase activity: The effect of the pH on defoliator larval endo-β-1,4-D-

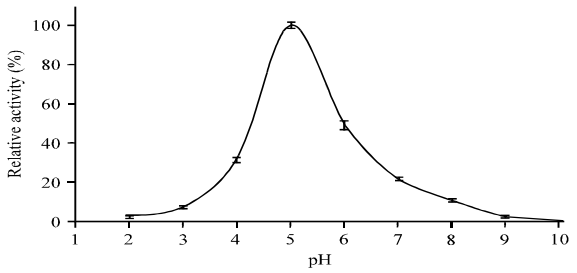

Fig. 6: Optimum pH of endo-β-1,4-D-glucanase, Error bars represent the standard deviation

glucanase activity was examined at various pH ranging from pH 2.0 to pH 12. The optimal pH for the enzyme activity was found at pH 5.0 which is the sharp peak on the graph. The activities were decreased rapidly on both sides and fell to around zero at pH 2.0 and pH 9.0 (Fig. 6).

DISCUSSION

Many insects especially phytophagous and or xylophagous insects are solely dependent on cellulose as a major food source for available energy/nutrients and use cellulolytic enzymes for their degradation to simple useable form. Therefore, cellulases are believed to be present in insects that have nutritionally poor diets such as termites and cockroaches (Terra and Ferreira, 1994). Previously, insects' cellulolytic activities have been confirmed in some species belonging to ten taxonomic orders (Martin, 1983; Watanabe and Tokuda, 2001; Willis *et al*., 2010a). Cellulases can be produced from insect endogenously [Coleoptera (Genta *et al*., 2006), Isoptera (Martin and Martin, 1978; Slaytor, 1992; Bignell *et al*., 1994), Thysanura (Treves and Martin, 1994), Orthoptera (Willis *et al*., 2010b), Blattodea (Scrivener *et al*., 1989; Genta *et al*., 2003), Hemiptera (Adams and Drew, 1965) and Phasmatodea (Cazemier *et al*., 1997)] and also from symbiotic organisms harboring in the insects' gut (Ohtoko *et al*., 2000) or both (Breznak and Brune, 1994; Watanabe *et al*., 1998; Ohtoko *et al*., 2000).

In this study, the data have reported the presence of endo-β-1,4-D-glucanase enzyme in the gut of phytophagous insect *P. quatuordecimpunctata* L. (Coleoptera: Chrysomelidae). This reports for the first time that the cellulolytic enzyme produced in the gut throughout the whole developmental stages of *P. quatuordecimpunctata*, from 1st instar to adult excluding pupa. Qualitative assay carried out by substrate-agar plate assay showed the presence of endo-β-1,4-D-glucanase activity in the gut of larva (Fig. 3).

Among all developmental stages, 8 days' larva showed the maximum activity (1.73 U mg^{-1} protein). The endo-β-1,4-D-glucanase activity was found to increase with the larval development up to the eight days but after that the activity decreased gradually (Fig. 4a). Previously, cellulase activity reported for lepidopteran larvae showed increased activity with advancing larval development (Nakonieczny *et al*., 2006; Pant and Ramana, 1989). In contrast, Willis *et al*. (2010b) did not detect significant differences in cellulolytic activity during development of *Dissosteira carolina*.

Highest endo-β-1,4-D-glucanase activity may co-relate to highly feeding of larva and increased expression of endo-β-1,4-D-glucanase gene. These different activities of endo-β-1,4-D-glucanase enzyme in different developmental stages of insect may be due to the quantity of enzyme produced as same amount of gut proteins (15 μg) from all larval developmental stages were used for assay. These had been confirmed by gel zymography in which 8 μg of gut proteins were used (Fig. 4b). In the zymography, the intensities of bands produced by enzymatic hydrolysis of CMC were varied during different developmental stages similarly to the enzymatic activities determined optically showed in Fig. 4a. So the data suggest that the amounts of enzymatic proteins were different in the same amounts of gut proteins used in both enzyme assay and zymography.

Zymograms were performed also to assess the number of enzymatic isoforms responsible for the observed activities. The clear zones on zymographic gel indicated multiple forms of endo-β-1,4-D-glucanase enzyme in different developmental stages (Fig. 4b). Although intensities of the bands on zymographic gel were different, there were at least four cellulolytic isoforms of endo-β-1,4-D-glucanase enzyme in each developmental stage of *P. quatuordecimpunctata* (pupa was not included in this experiment due to unavailability of sample). Multiple forms of cellulases have been previously reported for some insect orders: Coleoptera (Girard and Jouanin, 1999; Sami and Shakoori, 2006, 2008; Li *et al*., 2008; Rehman *et al*., 2009a; Geib *et al*., 2010; Oppert *et al*., 2010; Sami *et al*., 2011), Orthoptera (Sami and Shakoori, 2006; Oppert *et al*., 2010; Willis *et al*., 2010b) and Decapoda (Xiao *et al*., 1999). There are several possibilities to the presence of multiple forms of cellulase enzyme in the insects. Firstly these could represent cleavage products of large enzymatic protein, as suggested by Wood *et al*. (1995), proteolysis by proteases originated either from the insect digestive tract or from the symbiotic microbes. Secondly, it could be because of separate gene products or gene duplication even single gene where the gene products could be glycosylated differentially as post translational

modification. Girard and Jouamin (1999) suggested that beetle *Phaedon cochleariae*, has at least 3 cellulase genes, which probably encode divergent enzymes. Different levels of glycosylations was suggested for appearance of more than one form of cellulase activity bands as glycosylation was required for the activity of cellulases of beetle, *Apriona germari* (Wei *et al*., 2005). However, another possibility of cellulases of microbial origin produced by the microbes residing in the digestive tract of insects cannot be ruled out unless further work is carried out to investigate the reason for the multiplicity of cellulases in *P. quatuordecimpunctata*. There are very few, if any more, report on activity and multiplicity of cellulase enzyme throughout developmental stages of insects. Willis *et al*. (2010b) showed similar banding pattern of cellulase in zymography although the cellulase activities changes randomly throughout the developmental stages of *Dissosteira carolina* whereas in the present study the cellulase activity correlated well when measured in gel and by DNS method at different developmental stages of *P. quatuordecimpunctata*.

P. quatuordecimpunctata endo-β-1,4-D-glucanase showed the highest activity at the temperature of 35-45°C and 60°C (Fig. 5a) but it was stable up to 50°C and completely unstable at 55°C (Fig. 5b). The broad range in optimum temperature endo β-1,4-D-glucanase activity and the adverse effects of temperature on its stability may be due to inactivation of some multiple forms of enzymatic proteins at different temperatures. Zymography showed only one band with decreased intensity compared to control (without preincubation) when enzyme was preincubated at 45°C for 30 min before loading in gel and interestingly, preincubation at 55°C, no band appeared which coincided the instability of enzyme at that temperature (Fig. 5c). 60 and 35-45°C as optimum temperatures of endo-β-1,4-D-glucanase are also comparable to the optimal temperature of longicorn beetle, *A. germari* at 45-60°C (Lee *et al*., 2005; Wei *et al*., 2005) and cricket *Teleogryllus emma* at 40°C (Kim *et al*., 2008), respectively. Ni *et al*. (2007), Zhou *et al*. (2007) and Kim *et al*. (2008) also support the temperature stability of *P. quatuordecimpunctata* endo-β-1,4-D-glucanase at 20-50°C (Fig. 5b).

The acidic pH at which *P. quatuordecimpunctata* endo-β-1,4-D-glucanase exhibited maximum activity (pH 5.0) correlated well with those reported for other insects (Sugimura *et al*., 2003; Lee *et al*., 2005; Wei *et al*., 2006; Zhou *et al*., 2007; Kim *et al*., 2008; Rehman *et al*., 2009b). It has been reported that the treatment of cellulose with acids lead to generate ends and amorphous regions for enzymatic hydrolysis (Qian *et al*., 2003) and usually cellulases are present in the gut of insects which has acidic pH and the acidic pH optima could be related to the acidic environment of the gut (Zverlov *et al*., 2003). Because acid can keep some catalytic residues of *Apriona germari* endo-β-1,4-D-glucanase in an ionized form so that it can bind water molecule for the nucleophilic attack on β-1,4-glycosidic linkage of cellulose (Sami and Haider, 2007).

This study confirmed the presence of different cellulase (endo-β-1,4-D-glucanase) activities in the gut fluid of *P. quatuordecimpunctata*. Purification of individual cellulases and their characterization are necessary for better understanding of their properties. Further experiments are also necessary to determine whether these cellulolytic activities are endogenous to the insect or from other symbiotic organisms.

ACKNOWLEDGMENT

We are grateful to Mr. Santosh Mozumdar, M. Phil student, Department of Zoology, University of Chittagong for his assistance in identifying the insect.

REFERENCES

Adams, J.B. and M.E. Drew, 1965. A cellulose-hydrolyzing factor in aphid saliva. Can. J. Zool., 43: 489-496.

Bhat, M.K. and S. Bhat, 1997. Cellulose degrading enzymes and their potential industrial applications. Biotechnol. Adv., 15: 583-620.

Bhat, M.K., 2000. Cellulases and related enzymes in biotechnology. Biotechnol. Adv., 18: 355-383.

Bignell, D.E., M. Slaytor, P.C. Veivers, R. Muhlemann and R.H. Leuthold, 1994. Functions of symbiotic fungus gardens in higher termites of the genus *Macrotermes*: Evidence against the acquired enzyme hypothesis. Acta Microbiol. Immunol. Hung., 41: 391-401.

Breznak, J.A. and A. Brune, 1994. Role of microorganisms in the digestion of lignocellulose by termites. Annu. Rev. Entomol., 39: 453-487.

Cazemier, A.E., H.J. Op den Camp, J.H. Hackstein and G.D. Vogels, 1997. Fibre digestion in arthropods. Comp. Biochem. Physiol., 118: 101-109.

Clarke, A.J., 1997. Biodegradation of Cellulose: Enzymology and Biotechnology. Technomic Pub. Co., Lancaster, PA.

Geib, S.M., M. Tien and K. Hoover, 2010. Identification of proteins involved in lignocellulose degradation using in gel zymogram analysis combined with mass spectroscopy-based peptide analysis of gut proteins from larval Asian longhorned beetles, *Anoplophora glabripennis*. Insect Sci., 17: 253-264.

Genta, F.A., R.J. Dillon, W.R. Terra and C. Ferreira, 2006. Potential role for gut microbiota in cell wall digestion and glucoside detoxification in *Tenebrio molitor* larvae. J. Insect Physiol., 52: 593-601.

Genta, F.A., W.R. Terra and C. Ferreira, 2003. Action pattern, specificity, lytic activities and physiological role of five digestive beta-glucanases isolated from *Periplaneta americana*. Insect Biochem. Mol. Biol., 33: 1085-1097.

Girard, C. and L. Jouanin, 1999. Molecular cloning of cDNAs encoding a range of digestive enzymes from a phytophagous beetle, *Phaedon cochleariae*. Insect Biochem. Mol. Biol., 29: 1129-1142.

Kim, N., Y.M. Choo, K.S. Lee, S.J. Hong and K.Y. Seol *et al*., 2008. Molecular cloning and characterization of a glycosyl hydrolase family 9 cellulase distributed throughout the digestive tract of the cricket *Teleogryllus emma*. Comp. Biochem. Physiol. B Biochem. Mol. Biol., 150: 368-376.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature, 227: 680-685.

Lee, S.J., K.S. Lee, S.R. Kim, Z.Z. Gui and Y.S. Kim *et al*., 2005. A novel cellulase gene from the mulberry longicorn beetle, *Apriona germari*: Gene structure, expression and enzymatic activity. Comp. Biochem. Physiol., 140B: 551-560.

Li, X.J., X.F. Yan, Y.Q. Luo, G.F. Gian and H. Sun, 2008. Cellulase in *Anoplophora glabripennis* adults emerging from different host tree species. For. Stud. China, 10: 27-31.

Lindner, W.A., D. Dennison and P.K. Berry, 1983. Purification and properties of a carboxymethyllcellulase from *Sclerotium rolfsii*. Biochim. Biophys. Acta, 746: 160-167.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randell, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Martin, M.M. and J.S. Martin, 1978. Cellulose digestion in the midgut of the fungus-growing termite *Macrotermes natalensis*: The role of acquired digestive enzymes. Science, 199: 1453-1455.

Martin, M.M., 1983. Cellulose digestion in insects. Comp. Biochem. Physiol., 75: 426-428.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

Nakonieczny, M., K. Michaelczyk and A. Kedziorski, 2006. Midgut glycosidase activities in monophagous larvae of Apollo butterfly, *Parnassius apollo* ssp. *frankenbergeri*. C. R. Biol., 329: 765-774.

Ni, J., G. Tokuda, M. Takehara and H. Watanabe, 2007. Heterologous expression and enzymatic characterization of b-glucosidase from the drywood-eating termite, *Neotermes koshunensis*. Applied Entomol. Zool., 42: 457-463.

Ohmiya, K., K. Sakka, S. Karita and T. Kimura, 1997. Structure of cellulases and their applications. Biotechnol. Genet. Eng. Rev., 14: 365-414.

Ohtoko, K., M. Ohkuma, S. Moriya, T. Inoue, R. Usami and T. Kudo, 2000. Diverse genes of cellulase homologues of glycosyl hydrolase family 45 from the symbiotic protists in the hindgut of the termite *Reticulitermes speratus*. Extremophiles, 4: 343-349.

Oppert, C., W.E. Klingeman, J.D. Willis, B. Oppert and J.L. Jurat-Fuentes, 2010. Prospecting for cellulolytic activity in insect digestive fluids. Comp. Biochem. Physiol., 155: 145-154.

Pant, R. and D. Ramana, 1989. Cellulolytic activity in a phytophagous lepidopteran insect *Philosamia ricini*: The origin of the enzymes. Insect Biochem., 19: 269-276.

Qian, X., Y.Y. Lee, O.P. Par and W.T. Robert, 2003. Heterogeneous aspects of acid hydrolysis of β-cellulose. Applied Biochem. Biotechnol., 107: 505-514.

Rehman, F.U., M. Aslam, M.I. Tariq, A. Shaheen, A.J. Sami, N.H. Naveed and A.I. Batool, 2009a. Isolation of cellulolytic activities from *Tribolium castaneum* (red flour beetle). Afr. J. Biotechnol., 8: 6710-6715.

Rehman, F.U., M.I. Tariq, M. Aslam, G. Khadija and A. Iram, 2009b. Inhibition studies of cellulolytic activities isolated from *Planococcus citri*. Open Enz. Inhibit. J., 2: 8-11.

Sami, A.J. and A.R. Shakoori, 2006. Heterogeneity of cellulases among insects. Pak. J. Zool., 38: 337-340.

Sami, A.J. and M.K. Haider, 2007. Identification of novel catalytic features of endo-beta-1,4-glucanase produced by mulberry longicorn beetle *Apriona germari*. J. Zhejiang Univ. Sci. B., 8: 765-770.

Sami, A.J. and A.R. Shakoori, 2008. Biochemical characterization of endo-1,4-β-D-glucanase activity of a green insect pest *Aulacophora foveicollis* (Lucas). Life Sci. J., 5: 30-36.

Sami, A.J., M.A. Anwar, F.U. Rehman and A.R. Shakoori, 2011. Digestive cellulose hydrolyzing enzyme activity (endo- β-1, 4-D-glucanase) in the gut and salivary glands of blister beetle, *Mylabris pustulata*. Pak. J. Zool., 43: 393-401.

Schwarz, W.H., K. Bronnenmeier, F. Grabnitz and W.L. Staudenbauer, 1987. Activity staining of cellulases in polyacrylamide gels containing mixed linkage β-glucans. Anal. Biochem., 164: 72-77.

Scrivener, A.M., M. Slaytor and H.A. Rose, 1989. Symbiont-independent digestion of cellulose and starch in *Panesthia cribrata* Saussure, an Australian wood-eating cockroach. J. Insect Physiol., 35: 935-941.

Scrivener, A.M. and M. Slaytor, 1994. Properties of the endogenous cellulase from *Panesthia cribrata* Saussure and purification of major endo-β-1,4-glucanase components. Insect Biochem. Mol. Biol., 24: 223-231.

Slaytor, M., 1992. Cellulose digestion in termites and cockroaches: What role do symbionts play? Comp. Biochem. Physiol., 103: 775-784.

Sugimura, M., H. Watanabe, N. Lo and H. Saito, 2003. Purification, characterization, cDNA cloning and nucleotide sequencing of cellulase from the yellow-spotted longicorn beetle, *Psacothea hilaris*. Eur. J. Biochem., 270: 3445-3460.

Teather, R.M. and P.J. Wood, 1982. Use of Congo red polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Applied Environ. Microbiol., 43: 777-780.

Terra, W.R. and C. Ferreira, 1994. Insect digestive enzymes: Properties, compartmentalization and function. Comp. Biochem. Physiol., 109: 1-62.

Tokuda, G., H. Wantanabe, T. Matsumoto and H. Noda, 1997. Cellulose digestion in the wood-eating higher termite, *Nasutitermes takasagoensis*. Zool. Sci., 14: 83-93.

Treves, D.S. and M.M. Martin, 1994. Cellulose digestion in primitive hexapods: Effect of ingested antibiotics on gut microbial populations and gut cellulase levels in the firebrat, *Thermobia domestica* (Zygentoma, Lepismatidae). J. Chem. Ecol., 20: 2003-2020.

Waeonukul, R., K.L. Kyu and K. Ratanakhanokchai, 2007. Multiple cellulases and xylanases from Bacillus circulans B6 during growth on avicel under aerobic condition. Thai J. Biotechnol., 8: 27-32.

Watanabe, H., M. Nakamura, G. Tokuda, T. Yamaoka, A.M. Scrivener and H. Noda, 1997. Site of secretion and properties of endogous endo-β- 1,4-glucanase components from *Reticulitermes speratus* (Kolbe), a Japanese subterranean termite. Insect Biochem. Mol. Biol., 27: 305-313.

Watanabe, H., H. Noda, G. Tokuda and N. Lo, 1998. A cellulase gene of termite origin. Nature, 394: 330-331.

Watanabe, H. and G. Tokuda, 2001. Animal cellulases. Cell Mol. Life Sci., 58: 1167-1178.

Wei, Y.D., S.J. Lee, K.S. Lee, Z.Z. Gui and H.J. Yoon *et al*., 2005. N-Glycosylation is necessary for enzymatic activity of beetle, (*Apriona germari*) cellulase. Biochem. Biophys. Res. Commun., 329: 331-336.

Wei, Y.D., K.S. Lee, Z.Z. Gui, H.J. Yoon and I. Kim *et al*., 2006. Molecular cloning, expression and enzymatic activity of a novel endogenous cellulase from the mulberry longicorn beetle, *Apriona germari*. Comp. Biochem. Physiol., 145: 220-229.

Willis, J.D., C. Oppert and J.L. Jurat-Fuentes, 2010a. Methods for discovery and characterization of cellulolytic enzymes from insects. Insect Sci., 17: 184-198.

Willis, J.D., D.W.E. Klingeman, C. Oppert, B. Oppert and J.L. Jurat-Fuentes, 2010b. Characterization of cellulolytic activity from digestive fluids of *Dissosteira carolina* (Orthoptera: Acrididae). Comp. Biochem. Physiol., 157: 267-272.

Wood, T.M., C.A. Wilson and S.I. McCrae, 1995. The cellulase system of the anaerobic rumen fungus *Neocallimastix frontalis*: Studies on the properties of fractions rich in endo-1,4-β-D-glucanase activity. Applied Microbiol. Biotechnol., 44: 177-184.

Wyman, C.E., 2007. What is (and is not) vital to advancing cellulosic ethanol. Trends Biotechnol., 25: 153-157.

Xiao, M.X., J.A. Alys, A.R. Neil, J.A. Alex, P.X. Gang and B.M. Peter, 1999. Characterisation of cellulase activity in the digestive system of the redclaw crayfish *Cherax quadricarinatus*. Aquaculture, 180: 373-386.

Zhou, X., J.A. Smith, F.M. Oi, P.G. Koehler, G.W. Bennett and M.E. Scharf, 2007. Correlation of cellulase gene expression and cellulolytic activity throughout the gut of the termite *Reticulitermes flavipes*. Gene, 395: 29-39.

Zverlov, V.V., W. Holl and W.H. Schwarz, 2003. Enzymes for digestion of cellulose and other polysaccharides in the gut of longhorn beetle larvae, *Rhagium inquisitor* L. (Col., Cerambycidae). Int. Biodeter. Biodegr., 51: 175-179.