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Presence of cellulolytic and xylanolytic activities in the gut fluid of grasshopper *Oxya velox*

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Abstract

Insect guts, particularly of phytophagous insects, are considered as intriguing bioprospecting sources of cellulase and xylanase due to their use in biofuel industry. In this study, the activities of cellulase and xylanase were identified in the gut fluid of grasshopper, *Oxya velox*, and characterized. Qualitative assays of gut fluid carried out for endo-β-1,4-D-glucanase (EC 3.2.1.4) and endo-β-1,4-D-xylanase (EC 3.2.1.8) activities, using substrate-agar plate method, revealed clear transparent zones against the red-stained background. When measured by dinitrosalicylic acid method, the gut fluid had 0.759 ± 0.005 U and 0.303 ± 0.002 U of endoglucanase and endoxylanase activities, respectively, per mg of protein. In the gel zymogram, four distinct cellulolytic protein bands and one xylanolytic protein band were detected against substrates carboxymethyl cellulose and xylan, respectively. The optimal temperature and pH of both endo-β-1,4-D-glucanase and endo-β-1,4-D-xylanase were 55 °C and 5, respectively. Preincubation at 70 °C for 20 min resulted in almost complete loss of endo-β-1,4-D-glucanase activity as shown by zymography. The presence of both cellulase and xylanase activities suggested that *O. velox* could be considered as a model for studying the process of lignocellulose digestion in insect gut; the insect could also serve as a good source of enzymes for biofuel production.

Key words: *Oxya velox*, cellulase, xylanase, gut fluid, biofuel

Introduction

To meet the increased energy demand resulting from the global economic development and population growth, utilization of renewable energy is gaining interest. Renewable energy may help prevent climate change and slow down global warming, and its source is inexhaustible. According to International Energy Outlook (IEO) report 2016, renewable energy is the world's fastest growing source of energy, with an average growth rate of 2.6% per year. As an alternative to the depleting fossil fuel, biofuel produced using lignocellulosic plant biomass, which is abundant and low-cost source of energy in the biosphere (Cairo et al., 2011), is currently gaining tremendous attention from the scientists as one of the prominent forms of renewable energy (Schubert, 2006).

Lignocellulose is a major component of the plant biomass. The lignocellulosic biomass consists of hemicellulose, cellulose, and lignin constituting 20–40%, 40–60% and 10–25%, respectively (Yang et al., 2007). Cellulose is a β-1,4-linked polymer of glucose units (Chen, 2014) and consists of interstrand hydrogen bonds forming a highly stable crystalline lattice (Dodd and Cann, 2009). Hemicellulose consists primarily of xylan and is the second most abundant polymer in the plant material after cellulose. Xylan consists of a main chain of β-1,4-linked xylopyranose residues that is most often replaced by α-linked units of arabinofuranose and methylglucuronic acid (Brennan et al., 2007). Lignin, a three-dimensional polymer of phenylpropanoid units, can be considered as the cellular glue that provide the plant tissue and the individual fibres with compressive strength and the cell wall with stiffness, in addition to providing resistance against insects and pathogens (Rubin, 2008).

Enzymatic hydrolysis of the lignocellulosic materials is of great interest for its application in various industries because the process is environment-friendly and highly

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efficient and involves lower energy requirements than physical or chemical hydrolysis (Sheng et al., 2015). During hydrolysis, cellulose is degraded to fermentable glucose by the synergistic action of three glycosidic hydrolases: endo-β-1,4-glucanases (EC 3.2.1.4), exo-β-1,4-cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) (Clarke, 1997). Xylanases have been found to be important in the total hydrolysis of hemicelluloses containing lignocellulosic materials. In the process of bioethanol production from lignocellulosic materials, xylanases can improve the hydrolysis of cellulose into fermentable sugars by removing xylan that restricts the access of cellulases to cellulose surfaces. Thus, hydrolysis of pretreated lignocellulosic raw materials, even those with a low residual content of xylan, by xylanases has been shown to significantly improve the hydrolysis of cellulose (Zhang et al., 2011). Moreover, xylose can also be fermented to ethanol or xylitol, which is of higher value than ethanol (Mattam et al., 2016).

In spite of a large number of studies dedicated to developing a cost-effective process for bioethanol production, an efficient process for the conversion of lignocellulosic biomass to fermentable sugars is yet to be established. This is, at least, partly due to the recalcitrance of the cellulosic biomass which restricts the access of enzymes. Therefore, it is proposed that discovering of new enzymes with desirable features or improving the existing ones through biotechnological means can reduce the cost of biofuel production (Lynd et al., 2008; Yang et al., 2011).

Insects can process lignocellulosic biomass much more efficiently with their highly specialized gut systems and can be considered as highly efficient natural bioreactors (Sun and Scharf, 2010). Thus, insect gut could be a prospecting source of novel cellulases and xylanases which could be used in biofuel and other industries to reduce costs. Both cellulase and xylanase have many applications in various industries including food, feed, paper and pulp, textile, and laundry (Juturu and Wu, 2012; Kuhad et al., 2011). Although reports on screening of insects for cellulolytic activities and characterization of cellulases isolated from various insects are available (Oppert et al., 2010; Su et al., 2013; Wills et al., 2010a), less attention has been paid to the insect xylanase in previous studies (Arakawa et al., 2009; Mohamed et al., 2018; Padilla-Hurtado et al., 2012). Moreover, there are few reports on the presence of both cellulolytic and xylanolytic activities in any specific insect species (Shi et al., 2011), which are essential for efficient digestion of the lignocellulosic plant materials. Grasshoppers (Orthoptera: Acrididae) are serious pests affecting both agriculture and pasture in many countries of the world (Eziashi and Chidi, 2017). In this study, the activities of cellulase and xylanase of grasshoppers, *Oxya velox*, one of the major pests of rice (Kumar et al., 2014), were investigated with an aim of using these enzymes in biofuel and other industries, as well as to understand the process of lignocellulose digestion in phytophagous insect through future study.

Materials and methods

Chemicals

Carboxymethyl cellulose (CMC), beechwood xylan, congo red, bovine serum albumin (BSA), bisacrylamide and tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). 3,5-Dinitrosalicylic acid (DNSA) was bought from Loba Chemie (India). Ammonium persulfate (APS) was obtained from Bio Basic Canada Inc. Acrylamide and sodium dodecyl sulfate (SDS) were procured from Promega (USA). All other chemicals used were purchased from BDH (England) and Merck (Germany).

Insect collection

Fifty adult *O. velox* insects were collected from the Chittagong University campus, Bangladesh, in June 2015. During collection, the insects were in active feeding state and were kept on their host plants until dissection.

Gut dissection and fluid collection

All the collected insects were dissected on the day of collection. After immobilizing on ice for around half an hour, the insects were kept abdomen side up on Petri dishes placed on ice and dissected to remove the intact guts. The dissected guts were transferred to 1.5 ml microcentrifuge tubes. Then, the guts were cut into small pieces, homogenized through vortexing to ensure fluid extraction and centrifuged at 10,000 rpm for 10 min at 4 °C. Supernatants were transferred to new micro centrifuge tubes (1.5 ml) and stored at −40 °C.

Detection of cellulolytic and xylanolytic activities

A modified substrate-agar plate assay (Teather and Wood, 1982) was used to detect endo-β-1,4-glucanase

and endo-β-1,4-xylanase activities. The procedure used for endo-β-1,4-glucanase was as follows: 1% CMC and 3% agar were mixed with 0.1 M Na-acetate buffer (pH 5.3) and poured on Petri dishes, and the gel was allowed to solidify. A 110 μg of gut protein, diluted with water, was loaded into the holes punched in the center of the plates. After incubation at 37°C for overnight, the plates were stained with 0.1% Congo red for 10–15 min before destaining with 0.5 M NaCl solution for 15–20 min for several times. Appearance of clear zones around the sample holes against the red-stained background of the agar plates confirmed the activity of endo-β-1,4-glucanase. The same procedure was followed for detecting the endo-β-1,4-xylanase activity, with an exception that beechwood xylan was used in place of 1% CMC.

Measurement of the endo-β-1,4-glucanase and endo-β-1,4-xylanase activities

The activities of endo-β-1,4-glucanase and endo-β-1,4-xylanase were quantified as the rate of production of reducing sugars from their substrates using a modified DNSA assay (Miller, 1959). For quantifying the glucanase activity, 15 μl of gut fluid containing 50 μg of total proteins was mixed in microcentrifuge tubes with 235 μl of 1% CMC suspended in 0.1 M Na-acetate buffer (pH 5.3), and incubated for 30 min at 37°C. The reactions were stopped by adding 450 μl of DNSA solution followed by heating the microcentrifuge tubes in boiling water for 10 min. After adding 40% Rochelle salt, the reaction mixtures were cooled at room temperature for 5 min and centrifuged at 10,000 rpm for 5 min. After centrifugation, the supernatants were transferred to new microcentrifuge tubes to measure absorbance at 540 nm on an UV-visible spectrophotometer (Shimadzu).

For preparing blank, DNSA was mixed with the substrate before the addition of the gut fluid so that the enzymes present in the fluid could not hydrolyze CMC. One unit of cellulolytic activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per minute at 37°C and pH 5.3. A standard curve of absorbance against amount of glucose (50–300 μg) was constructed to calculate the amount of reducing sugars released during endo-β-1,4-glucanase assay.

The same procedure was followed for measuring the endo-β-1,4-xylanase activity, with the exception that xylan was used as substrate instead of CMC and the standard curve was constructed using xylose instead of glucose.

Effect of temperature and pH on enzyme activity

To detect optimal temperature and pH of the enzymes, their activities were measured in gut fluid at different temperatures and pH values, respectively. The effect of temperature on the enzyme activity was examined by incubating the reaction mixture (containing 1% CMC or 1% Xylan as substrates for endo-β-1,4-glucanase or endo-β-1,4-xylanase activity, respectively, in 0.1 M Na-acetate buffer, pH 5.3) over a range of temperatures from 35 to 85°C. The temperatures at which the highest activities of enzymes were detected were considered as optimum and calculated as 100%.

The optimal pH for the enzyme activity was determined by measuring the hydrolysis of substrates in a series of buffers at various pH values ranging from 4 to 10. The following were used for evaluation: Na-acetate buffer (0.1 M) for pH 4 and 5, phosphate buffer (0.1 M) for pH 6 and 7, Tris-HCl buffer (0.1 M) for pH 8, and glycine-NaOH buffer (0.1 M) for pH 9 and 10. The pH values at which the enzymes showed the maximum activities were considered as optimum and calculated as 100%.

Zymography

Sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was performed, with some alterations, to detect the activities of endo-β-1,4-glucanase and endo-β-1,4-xylanase through zymography (Schwarz et al., 1987). Briefly, SDS-10% PAGE resolving gel was prepared by adding 1% substrate (CMC for endo-β-1,4-glucanase and xylan for endo-β-1,4-xylanase) with 10% APS and TEMED and incubated for 1 h at room temperature. Gut fluid samples (20 μg) of *O. velox* were solubilized in 1 volume of sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, and 0.01% bromophenol blue) and loaded onto the gel. Proteins in the samples were separated at a constant voltage of 90 V at 4°C for approximately 6 h or until the bromophenol blue dye reached the bottom of the resolving gel.

After electrophoresis, the gel was washed five times (each for 30 min) with 150 ml of wash buffer (0.1 M Na-succinate, pH 5.8 and 0.1% β-mercaptoethanol) at room temperature, except the last wash which was performed at 30°C. Then, the gel was stained with 0.1% Congo red

for 30 min and destained by washing in 1 M NaCl solution at room temperature to identify the zones of clearing, where substrate was degraded by the enzyme. Followed by destaining, 100 µl of glacial acetic acid was added to the gel wash buffer for better visualization of clearing areas (Waeonukul et al., 2007). To determine the thermal stability of endo-β-1,4-glucanase, the gut fluid of *O. velox* was preincubated at 70°C for 20 min before loading onto gel.

Measurement of the protein concentration in the gut fluid of O. velox

Protein concentration in gut fluid was measured by using Folin–Ciocalteu reagent (FCR) method (Lowry et al., 1951). Different aliquots of standard BSA solution (250 µg/ml) were pipetted out into different tubes, and the aliquots of samples were taken in separate tubes. Equal amount of alkaline copper sulfate was added in each tube and then allowed to stand for 15 min. The same amount of FCR was added to each tube and the tubes were incubated for 30 min. Absorbance was measured at 650 nm against a proper blank in which no protein solution was added. The protein concentration in the gut fluid was calculated from the standard curve.

Statistical analysis

The results are presented as mean ± standard deviation ($n = 3$).

Results and discussion

Phytophagous insects are now considered to be a novel and efficient sources of enzymes that can be used for the degradation of lignocellulosic biomass, which can reduce cost of biofuel production. These enzymes may be useful for other industrial applications as well. Moreover, study of the digestive enzymes of the pests could be helpful to control them through searching and/or designing proper inhibitor(s). Here, we report for the first time the presence and characteristics of cellulase and xylanase and evaluate the cellulolytic and xylanolytic activities in the gut fluid of orthopterian insect *O. velox* (grasshopper).

Qualitative and quantitative assay of cellulase and xylanase activities

To determine the presence of cellulase and xylanase activities in the gut fluid of *O. velox*, plate assay were performed. Figure 1 shows the clear zones around the holes where the gut fluids were added, proving the presence of both enzymes. Previously, endo-β-1,4-glucanase activity has been detected in the digestive fluids of insects belonging to 10 different taxonomic orders including Blattodea, Coleoptera, Diptera, Isoptera, Lepidoptera, Orthoptera, Plecoptera, Phasmatodea, Trichoptera, and Thysanura (Wills et al., 2010b). Oppert et al. (2010) reported the presence of cellulolytic activity in fluids of the guts and heads of 63 phytophagous or xylophagous insect species belonging to eight different orders including Diptera, Orthoptera, Colleoptera, Hymenoptera, Lepidoptera, Dictyoptera, Dermaptera, and Isoptera. They used two different substrates CMC and microcrystalline cellulose, for evaluating the endo-β-1,4-D-glucanase activity and complete cellulolytic activity, respectively. The digestion of cellulose by insects was traditionally attributed to the symbiotic microbes in their guts, but lately it has been found that endogenous insect cellulases also play a crucial role in the digestion process. Endogenous cellulases have been found in insects belonging to various orders including Blattaria, Coleoptera, Hemiptera, Phthiraptera Orthoptera, and Hymenoptera (Fischer et al., 2013; Watanabe and Takuda, 2010; Wills et al., 2011). Previously, we identified cellulolytic bacteria in the guts of *O. velox* (Shil et al., 2014). All herbivorous insects exhibiting endogenous cellulase activity contain at least one cellulase gene encoding class 9 glycosal hydrolases (GH9), although coleopteran insects possess endogenous cellulases of other GH families including GH5, GH45, and GH48, which are likely acquired from microbes via successive events of horizontal

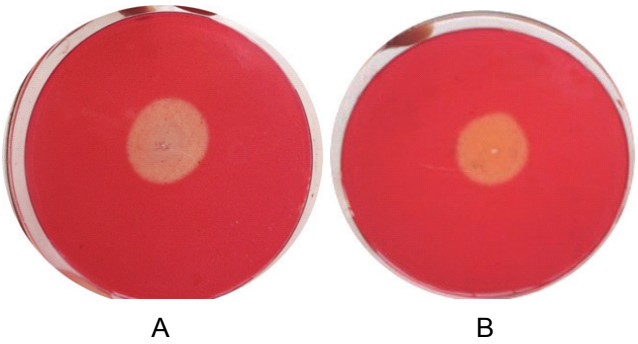

Fig. 1. Presence of A) cellulolytic activity in CMC-agar plate and B) xylanolytic activity in xylan-agar plate. Gut fluids were added to the central holes of agar plates containing CMC or xylan prepared in Na-acetate buffer (pH 5.3), and the plates were incubated overnight at 37°C. Plates were stained with Congo red followed by destaining with 0.5M NaCl

gene transfer (Fischer et al., 2013; Shelomi et al., 2016). Cloning and sequencing the cellulase genes of *O. velox* would help ascertain which gene family these cellulases belong to.

Xylanases are enzymes necessary for degradation of xylan constituting hemicellulose, the noncellulosic part of lignocellulosic biomass, to xylose. Thus, these enzymes play dual role in second-generation biorefineries: 1) they facilitate the access of endoglucanase for deconstruction of cellulose through degradation of xylan; after which 2) then xylose the degraded product of xylan, can be fermented to ethanol. Xylanases also have numerous biotechnological and industrial applications (Uday et al., 2017). Generally, industrial processing conditions are harsh involving extreme pH, temperature, inhibitors, etc. Hence, sufficiently stable enzymes that are able to withstand such conditions are recommended to be used in industries. However, most of the reported xylanases do not meet this criterion (Khandeparker et al., 2017). Moreover, high costs of production hinder the use xylanases in bioethanol and other industries (Ali et al., 2017). Therefore, finding a novel source of xylanases is crucial. Although bacteria, fungi, terrestrial plant tissues, and digestive juices of animals are extensive sources of xylanases (Xu et al., 2016), the digestive extracts of many insects, for example, wood-feeding termites (Ali et al., 2017; Arakawa et al., 2009), red palm weevils (Mohamed et al., 2018), coffee berry borers (Padilla-Hurtado et al., 2012), larvae of scarab beetles (*Holotrichia parallela*) (Sheng et al., 2015), also contain xylanases. Here, we report for the first time the presence of xylanase activity in the gut extract of *O. velox*. To date, it has been believed that xylanases present in insects are secreted mainly by the symbiotic gut microorganisms such as bacteria, fungi and protozoa (insect digestive glycosidases). Most of these xylanases are classified under the GH families 10 and 11 on the basis of the similarities in their amino acid sequences, catalytic domains, protein folds and overall architecture (Wakiyama et al., 2008).

The activities of cellulase and xylanase were also measured quantitatively by DNSA method. The activities of endo-β-1,4-D-glucanase and endo-β-1,4-D-xylanase were calculated to be 0.759 ± 0.005 U/mg and 0.303 ± 0.002 U/mg of protein, respectively. Willis et al. (2011) reported a cellulase activity of 0.016 U/mg of protein (at 50°C and pH 6) in the gut fluid of the larvae of the coleopteran insect, *Tribolium castaneum*. Similarly, the activities of endoglucanase and xylanase in the gut content of the larvae of Asian longhorned beetle (*Anoplophora glabripennis*) were measured to be 0.41 U/mg and 0.058 U/mg of protein, respectively (Geib et al., 2010). A cellulolytic activity higher than 0.5 U/mg of protein against CMC substrate were found in the gut fluids of insect species belonging to the orders of Coleoptera, Isoptera, and Orthoptera (Oppert et al., 2010). The gut fluid of *O. velox* showed relatively high activities of endoglucanase and xylanase, compared to the gut fluids of many other insect species. However, it is difficult to compare the enzyme activities among different insect species as the activity of an enzyme depends on many factors, including assay conditions, feeding status of insects, concentration of the desired proteins in the gut fluid, and so on. Thus, it cannot be concluded that the higher activities of endoglucanase and xylanase reported in the present study are due to the catalytic strength of the enzymes of *O. velox* without purifying the enzymes. It has been reported that the grass-consuming grasshoppers and wood-consuming wood borer have higher activities of cellulase and xylanase in their gut content compared to the leaf-consuming silkworms (Shi et al., 2011).

Detection of enzyme activity by zymography

To detect the proteins associated with the activities of endoglucanase and endoxylanase, zymography of the *O. velox* gut fluid was performed. The zymogram showed four protein bands representing endoglucanase activity (Fig. 2A) and one protein band corresponding to endoxylanase activity (Fig. 2B). Four protein bands for endoglucanase activity were also found in the gut fluid of *Dissosteria carolina* grasshopper (Wills et al., 2010a) and coleopteran *Podentia quatuordecimpunctata* insect (Uddin et al., 2012). Several explanations have been put forth for multiple forms of insect cellulases: 1) proteolytic cleavage of enzymatic proteins by proteases present in the insect gut that are produced either by the insects or by the symbiotic microbes; 2) differential glycosylation of an enzyme; 3) duplication of genes encoding cellulases; and 4) cellulases produced by the symbiotic microorganisms (Sami and Shakoori, 2006). Beetle *Phaedon cochleariae* has at least three cellulase genes which probably encode divergent enzymes (Girard and Jouanin, 1999), whereas four cellulase genes have been reported in termite genome (Terrapon et al., 2014). Dif-

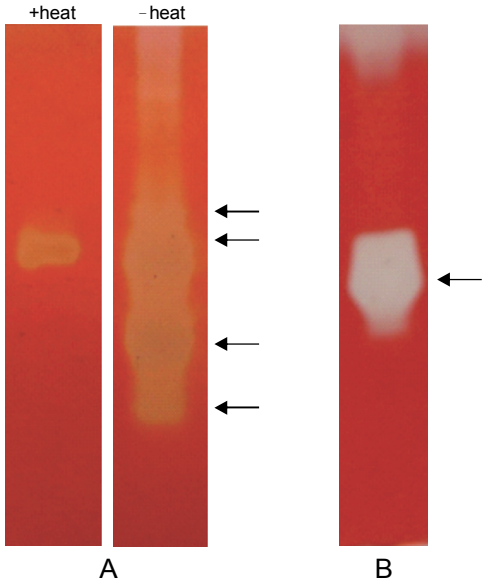

Fig. 2. Zymography for the activity of A) endo-β1,4-glucanase and B) endo-β1,4-xylanase activity of *O. velox* gut fluid. Gut fluid was separated using SDS-10% PAGE, and gels were stained for CMC (for endoglucanase) or xylan (for xylanase) using 0.1% Congo red. After destaining with 1 M NaCl, clear areas were found as bands where CMC or xylan was degraded. In figure (A) the lane marked as "–heat" indicates that the gut fluid were used without any heating, whereas lane marked as "+heat" indicates that gut fluid was heated at 70 °C for 20 min before loading to the gel

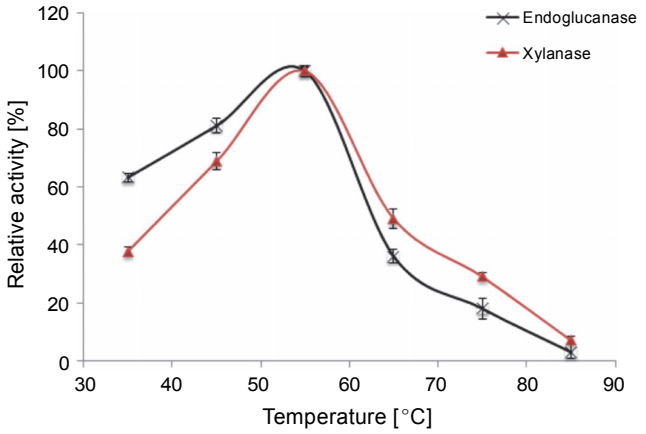

Fig. 3. Effect of temperature on the activities of endo-β-1,4-D-glucanase and endo-β-1,4-D-xylanase. Gut fluid of *O. velox* was mixed with 1% CMC (for cellulase) or 1% xylan (for xylanase) in 0.1 M Na-acetate buffer (pH 5.3), and incubated at different temperatures from 35 °C to 85 °C, and then assayed for enzyme activities by DNSA method. The highest activity was considered as 100%. Each point is the average of three determinations, and error bars represent the standard deviation

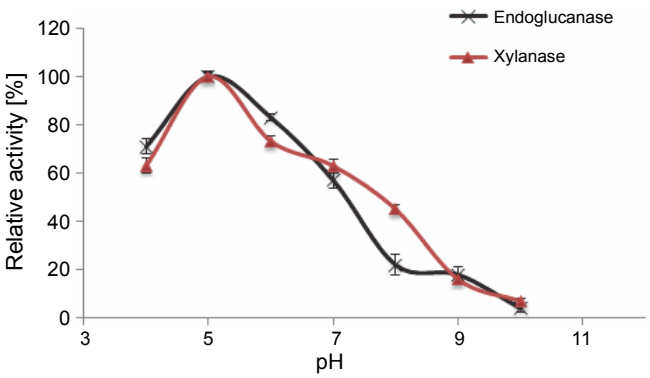

Fig. 4. Effect of pH on the activities of endo-β-1,4-D-glucanase and endo-β-1,4-D-xylanase. Gut fluid was incubated with 1% substrate solution prepared in the 0.1 M buffers of different pH values at 37 °C and assayed for enzyme activity. The highest activity was considered as 100%. Each point is the average of three determinations, and error bars represent the standard deviation

ferent levels of glycosylation were suggested as a reason for appearance of more than one form of bands indicating cellulase activity, as glycosylation was required for the activity of cellulases of beetle *Apriona germari* (Wei et al., 2005). Further experiments are necessary to confirm the origin of different cellulase proteins found in the *O. velox*. A single protein of 34 kDa representing endoxylanase activity was also found in the gut fluid of the larvae of Asian longhorned beetles, *Anoplophora glabripennis* (Geib et al., 2010).

Effect of temperature and pH on enzyme activity

The effect of temperature on the activities of endo-β-1,4-glucanase and endo-β-1,4-xylanase has been presented in Figure 3. The optimal temperature of both endoglucanase and endoxylanase was 55 °C at which their activities were 1.113 ± 0.006 U/mg and 0.705 ± 0.003 U/mg of protein, respectively. The endoglucanase activity was 63% at 35 °C and 36% at 65 °C whereas the endoxylanase activity reached 38% at 35 °C and 49% at 65 °C, indicating that xylanases of this insect are more thermostable than cellulases. The zymogram of gut fluid preincubated at 70 °C for 20 min also showed loss of endoglucanase activity, indicated by the disappearance of three cellulase bands (Fig. 2A). The optimal temperature for endoglucanase activity has been reported to be in the range of 50–55 °C in many other insects (Li et al., 2009; Rouland et al., 1988; Sami et al., 2011). Similarly, optimal temperature for xylanase activity was found to be 55 °C in other insects (Fagbohoun et al., 2012; Liu et al., 2011; Roy et al., 2003).

The effect of pH on the activities of endo-β-1,4-D-glucanase and endo-β-1,4-D-xylanase *O. velox* was examined at various pH values ranging from 4 to 10. The optimal pH for the activities both endoglucanase and endoxylanase was found to be 5 (Fig. 4); at this pH, their activities were 0.774 ± 0.004 U/mg and 0.316 ± 0.003 U/mg of protein, respectively. Although the optimal pH values for cellulase activity varies from acidic to alkaline (Hurst et al., 1997; Ito et al., 1989; Park et al., 2002), all animal cellulases reported till now have shown maximum activity only under week acidic conditions. Endoxylanase, which is the main xylanolytic enzymes characterized among insects, was found to show maximum activity in the pH range from 4.5 to 7 (Kouadio et al., 2016).

Conclusions

This study demonstrated the presence of both cellulolytic and xylanolytic activities in the gut fluid of *O. velox*. An analysis of the zymogram of gut fluid further revealed the proteins associated with these enzyme activities. Thus, *O. velox* could be considered as an attractive model for studying lignocellulose degradation in the insect guts. Further characterization of the efficient lignocellulolytic system of this insect may aid in the identification of novel enzymes with desirable characteristics for application in the biofuel industry. Moreover, insecticides can be developed targeting these novel enzymes to control this rice pest.

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