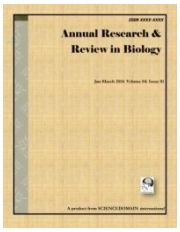

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Purification and Characteristics of Beta-1, 4-Glucanase Occel9 of *Oxya chinensis*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AJS, MK and SB performed the experimental work and analysis. Author AJS performed the study design. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: *Oxya chinensis* is a very serious paddy pest and causes major loss in rice crop. The objectives of the study were (1) Studying the molecular basis of plant biomass degradation by *Oxya chinensis.* (2) The role of Neem (*Azadirachta indica*) derived compounds, Azadirachtin and Saponins was also studied as enzyme inhibitors with special reference to biopesticides.
Place of Study: Institute of Biochemistry and Biotechnology, University of the Punjab, Lahore.
Methodology: *Oxya chinensis* insects were collected from local fields and dried. The dried insects were studied for the purification and characterization of cellulose hydrolyzing enzyme activity. Enzyme was purified using ion-exchange chromatography. Spectroscopic study was carried out to identify the signature pattern of the protein. The role of purified neem (*Azadirachta indica*) derived compounds Saponins and Azadirachtin was also studied as enzyme inhibitors with special reference to Biopesticides.
Results: Total soluble protein was isolated from salivary gland and gut and the presence of cellulase activity endo-1, 4-ß-glucanase (Oc*Cel*9) in each tissue was indicated. After purification both enzymes appeared at same position on zymogram, containing 2.0% carboxymethyl cellulose as substrate, after non-denaturing PAGE. The beta-1,4-glucanase (Oc*Cel*9) activity from the gut and salivary gland had the same K_m values. The pH and temperature profiles of *Oxya chinensis* beta-1,4-glucanase indicated optimum pH at 3.0

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and optimum temperature at 50ºC. SDS-PAGE analysis indicated that the enzyme has two subunits/isozymes of molecular mass 60 and 55 kDa respectively. The spectroscopic analysis of the isolated protein showed that it had homology with rice, 1, 4-beta-D-glucanase. An internal amino acid sequence of the rice enzyme (Oc*Cel*9) revealed that this enzyme belongs to glycosyl hydrolase family 9 (Glycosylhydrolases family 9 active sites signature 2). Neem, *Azadirachtaindica* derived saponins were able to inhibit Oc*Cel*9 on substrate agar plates.

Conclusion: Oc*Cel*9 was successfully purified and is a very active cellulose hydrolyzing enzyme and can be of significant technological importance. Moreover the enzyme can be studied as a potential target for producing novel biopesicides.

Keywords: Oxya chinensis; cellulose degradation; cellulase; GH family 9; (Oc*Cel*9).

1. INTRODUCTION

Plant biomass has a major component of cellulose, hemicelluloses and lignin in its infrastructure. These molecules are long chain polymers and are resistant to degradation, as a cascade of enzymes is required to hydrolyze the plant biomass, due to their rigid packing and structural complexity. Still, most of the plant biomass is utilized by insect as food, leading to the fact that they have system to generate a battery of enzymes which is required for the bioconversion of plant biomass into simple sugars (glucose). During recent years a plethora of information provides us with a broader view that plant biomass is hydrolyzed by insect and microbes and both of them are capable of producing indigenous enzyme system. [1-9]. Willis reviewed the methods for the discovery of cellulases [10].

In the biological conversion of cellulose, three enzymes are involved mainly, endoglucanases (1, 4-β-D-glucanglucohydrolase), exoglucanases (β-1, 4-glucan cellobiohydrolase) and β-glucosidase (β-D-glucosideglucohydrolase) [11]. Beta-1,4-glucanases cut cellulose at random positions within the polysaccharide creating new ends. Exoglucanases act on the polysaccharide at both reducing and non-reducing ends and release cellobiose units [12]. β-glucosidase subsequently degrades cellubiose units to glucose [13].

These cellulases are found in all kingdoms, predominantly in kingdom Prokaryotae and Fungi [14]. The insects themselves do not secrete all of the digestive enzymes to hydrolyze plant cell wall [1,15]. Rather, much of the hydrolysis of these polysaccharides is carried out by the multiple forms of cellulases secreted by symbionts (prokaryotes and fungi) and microbes present in the gut region of insects [7,15-16].

Four mechanisms have been reported for cellulose digestion in insects. First, the exploitation of protozoan symbionts in the gut region as reported in *Cryptocercus punctulatus* [17]. Second, the exploitation of bacterial symbionts in the hind gut as observed in *Periplaneta americana* [18]. It was reported that in *Clostridium thermocellum* there are present various cell surface structures known as celluosomes possessing cellulases and different complex subunits that help the bacteria to hydrolyze their cellulose substrates. Third mechanism is the digestion of cellulose through ingested fungal cellulases [19-20]. Best of this mechanism was observed in fungus growing termite, *Macrotermes natalensis* [21].

Fourth is the secretion of complete cellulase system by insects, indicating multiplicity of cellulases [22]. Cellulolytic activity was reported in both the salivary glands and gut region of *Reticulitermes speratus* [23]. It was proposed that as the cellulose is ingested by the insect it comes into contact with both the salivary and symbiotic cellulases, they act simultaneously in a synergistic action that has yet to be clarified but this mechanism is same as that of insect's fungal cellulase system. Sami et al. [24] studied *Mylabris pustulata* (blister beetle) for the presence of endo- β–1, 4- D-glucanase and identified endo- β–1, 4- D-glucanase activity in salivary glands, fore gut and in minute quantity in the hind gut. Multiple forms of the enzymes were identified in the digestive tract of the insect.

The classification of these enzymes is based on their substrate specificity, according to the IUBMB nomenclature (though there are some exceptions). As reported by Urbanowicz this nomenclature has become unsatisfactory, because a number of enzymes are active against more than one substrate [25]. Carbohydrate-Active-Enzyme (CAZY; www.cazy.org) is an updated database that lists more than 108 distinct GH families, related to 14 clans based on the presence of defined protein folds and conserved catalytic modules [25]. Currently, Glyco Hydrolase family 9 has been studied in detail for *Solanum lycopersium*, Arabidopsis and Oryza [25-27].

There are also several reports on the genomic studies on insect cellulases [28-29]. Girard and Jouanin studied Molecular cloning of cDNAs encoding a range of digestive enzymes from a phytophagous beetle, *Phaedon cochleariae* [30]. The presence of cellulolytic activities in the symbiont free regions of some invertebrates and higher animals for example salivary glands have been identified previously [31]. Todaka studied cDNA analysis of the genes involved in lignocellulose digestion in the symbiotic community of *Reticulitermes speratus* [32]. Tokuda investigated cellulolytic environment in the midgut of the wood-feeding higher termite *Nasutitermes Takasagoensis* [33].

To discover the mechanism of cellulose degradation by insects biochemical characterization of the cellulose hydrolyzing enzymes of insect origin is required. Various insects such as *Aulacophora atripennis, Aulacophora abdominalis, Chrotogonus trachypterus, Dysdercus koenigii, Aulacophora foveicollis, Oxya chinensis, Hieroglyphus banian*, *Coccinella scutellaris, Mylabris pustulata, Apis dorsata* and *Apis mellifera* have been studied and cellulose hydrolyzing activities are reported in all the insects. From these insects *Oxya chinensis* and *Mylabris pustulata* were found to posses the highest cellulose hydrolyzing activity [34]. *O.chinensis* is an attractive target because it is a serious pest for rice [35]. The feeding habits of *Oxya chinensis* can be compared with that of other insects like termites that are solely dependent on plants [15].

These insects are proving to be a major cause of plant biomass destruction because in addition to rice they also feed on various grasses. Previously work has been done on the cellulase enzyme of *O.chinensis.* Crude extracts had cellulose hydrolyzing activity and *O.chinensis* was found to produce an effective enzyme system for the hydrolysis of cellulose [35]. As plants are the major renewable source on earth their destruction poses a major risk so in order to control the damage done by this specie various natural pesticide compounds have been used [36]. Various plant extracts were investigated as inhibitors for the cellulose hydrolyzing enzymes of insects [34]. Of these Neem was the most promising candidate due to its medicinal properties. Leaf extract of Neem was used as inhibitor on the crude extracts of *O.chinensis* and other insects and inhibition was observed [34-35]. The complete sequence of *O. chinensis* mitochondrial genome was reported by Zhang and Yuan Huang [37]. Sequence analysis of *O.chinensis* cellulases is important in comprehending the

mechanism of inhibition and for targeting novel inhibitors. This will prove helpful in enzymatic classification of *Oxya* and pest management.

Here we report the characterization of beta-1,4-glucanase (Oc*Cel*9) from the salivary gland and gut of *Oxya chinensis* and the inhibition of purified enzyme.

2. MATERIALS AND METHODS

2.1 Sample Collection

The rice grasshoppers, *Oxya chinensis*, were collected from the local fields of Punjab University, New Campus, Lahore during the month of April in the morning and were washed and dried for future use.

2.2 Crude Extract Preparation

5.0 g of dried insects were taken and their head and abdomens were separated. Heads were used as an enzyme source for salivary gland endoglucanase and abdomens were used as source of gut endoglucanase. Both parts were grounded separately in 150 ml of Tris-HCl buffer (pH 7.1, 0.05 M), filtered through a thick sieve and then centrifuged. The centrifugate, obtained as clear supernatant was used as a source of salivary gland and gut enzymes [8]. Protein concentration was estimated by the Bradford method [38].

2.3 Detection of Cellulose Hydrolyzing Activity

Cellulose hydrolyzing activity was detected on substrate agar plates. For this purpose a solution of 2% agar and 2% Carboxymethyl cellulose (CMC) was prepared in 0.5 M Tris-Cl buffer pH 5.0 was poured in petri plates and allowed to solidify. After 30 mins holes were punctured in the plates and enzyme extracted was added in wells. The plates were incubated at 50ºC over night. After incubation the plates were stained by 0.1% congo red dye and destained with 0.1% NaCl solution [8].

2.4 Purification by Ion Exchange Chromatography

The enzyme was purified on an ion exchange column packed with (3 x 10 cm) Sephadex CM-50 gel. The column was calibrated by 0.05 M phosphate buffer of pH 6.5 at room temperature and then 5ml (5 mg protein) of the enzyme sample (salivary gland) was loaded and the column was washed with 30ml of the same buffer to elute any unbound proteins. Proteins were eluted with NaCl gradient prepared in 0.05 M phosphate buffer (pH 6.5). A total of 200 fractions (1.5 ml each) were collected with a linear gradient of 0.0M - 0.5M NaCl. The same procedure was repeated for the gut sample.

Alternate fractions of both the samples were analyzed for protein and cellulose hydrolyzing enzyme activity (CMCase activity) **(**Oc*Cel*9**)**. For this purpose reaction mixtures were prepared by using 1 ml 2% CMC, 1 ml 0.5 M Tris-Cl buffer pH 5.0, 1 ml sample (from ion exchange fractions) and incubated the reaction mixtures at 50ºC over night [39]. The next day CMCse activity was estimated using dinitrosalicic acid (DNS) method [40]. 1 ml DNS was added in the samples and heated for 10 mins at 100ºC. Absorbance was measured at 540 nm in absorbace units. Activity was also located on CMC agar plates, as described above [8].

2.5 Zymography

Fractions showing enzyme activity were pooled together and further analyzed on zymogram after native PAGE [24]. For zymography NATIVE-PAGE of pooled fractions was done at 60 volts for 4 hours. The gel was then overlayed on the CMC-agar plate, incubated at 50ºC over night and stained and destained (as described above) [8]

2.6 Enzyme Characteristics

The molecular weight of the enzyme of salivary gland and the gut was determined by SDS-PAGE. For determining effect of substrate concentration both enzyme were incubated with different percentages of CMC and Km was calculated. The optimum pH of the enzyme was determined; pH range was from pH 1.0 to pH 10.2. HCl-KCl buffers in the pH range 1.0 to 2.2; Citric acid- Na_2HPO_4 buffers in the pH range 2.6 to 7.6; KCl/H_3BO_3- NaOH buffers in the pH range 8.2 to 10.2 were prepared. Reaction mixtures consisted of 1 ml of 2% CMC, 1ml of respective buffer (pH 1.0-10.1) and 50 µl of enzyme. Incubation was done at 50ºC overnight. Optimum temperature was also determined as follows, reaction mixtures consisted of 1 ml of 2% CMC, 1ml buffer of pH 3.0 and 50 µl of enzyme. Incubation was done at different temperatures ranging 10ºC-100ºC overnight.

2.7 Inhibition Studies

For inhibition studies neem derived saponins were extracted from neem (*Azadirachta indica*) leaves powder as follows. Saponins were extracted from the neem powder as described by Deore and Khadabadi [41]. For this purpose 100.0g of neem powder was de-fatted in 200ml of chloroform. The mixture was filtered and dried precipitates were added to 200 ml methanol-water mixture (60:40). The mixture was extracted twice with 500ml of butanol, using a separating funnel. Saponins were isolated by drying the butanol layer and precipitates were dissolved in water 50ml. The upper layer of the mixture was separated and evaporated in a Petri dish and the dried powder was dissolved in water.

For enzyme Inhibition Assay 2% CMC-agar plates (pH 5.0) were prepared and a hole was punched in the center of the plate. To each hole 50µl of neem derived saponins were added after 20 minutes enzyme was added. Plates were incubated overnight and next day effect of saponins on the enzyme activity was monitored by comparing it with the standard. Inhibition was also studied using the DNS method for reducing sugars by adding a range of saponins (0.1-1.0mg) in the reaction mixture. The rest of the method was same as described above.

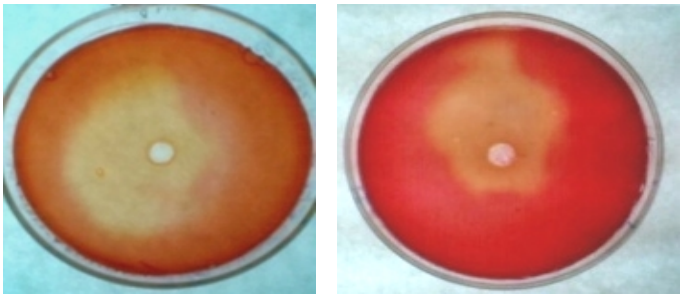
2.8 Protein Sequencing

The purified protein was further characterized by spectrometric analysis by sending PAGE band, excised from the gel after electrophoresis run, to spectroscopic laboratory St. Andrews University, Scotland, UK. The fragment sequences were carefully aligned with the 'putative endo-beta-1, 4-glucanase- *Oryza sativa* (japonica cultivar-group) with an accession No. AAT44235.1.

3. RESULTS

3.1 Identification of Endo-1, 4-ß-Glucanase Activity

To identify the endo-1, 4-ß-glucanase activity in the salivary glands and gut, tissues were removed after dissection. The total cellular proteins were isolated as described in materials and methods and activity was located in a hole on CMC-agar plates (50 ml buffer of pH 5.0 was mixed with 50ml 2% CMC). A total of 50µl protein extract was loaded on 2% CMC agar plates. The plates were incubated at 50°C overnight and stained with Congo red next morning and destained by 0.1M NaCl solution. Gut enzyme indicated the activity by the appearance of a clear zone around the hole indicating the hydrolysis of substrate by enzyme (Fig. 1 A and B).



A **B**

Fig. 1. Qualitative identification of 1,4-ß-glucanase (Oc*Cel*9) activity in (A) Salivary glands (B) gut. Enzyme activity in each case is visible as clear zones in the center

3.2 Purification of Enzyme

The crude enzyme extract of salivary gland and gut enzyme of *Oxya chinensis* were subjected to ion exchange chromatography separately on Sephadex CM-50 column and equilibrated with pH 6.5 buffer (0.05M). 5ml enzyme sample in the equilibrated buffer containing 5mg protein was loaded onto the column. Alternative fractions were analyzed for protein [Bradford method; 38] and enzyme activity (DNS method) [40] indicated on y-axis. As it is shown in the chromatogram that there were multiple peaks present but only a major single peak was observed for cellulase Oc*Cel*9 activity for both enzymes which was eluted in the salt range of 0.1-0.2M NaCl (Fractions 12-28 for salivary gland and 20-28 for the gut enzyme) Figs. 2 and 3.The endoglucanase activity was further confirmed on substrate agar plates Figs. 4 and 5.

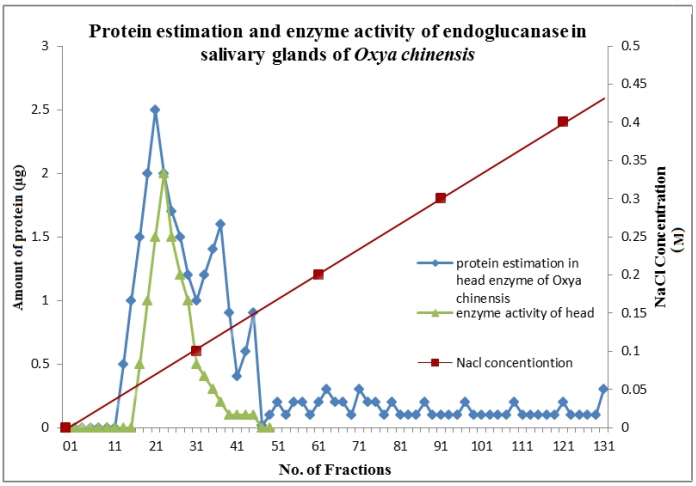


Fig. 2. Purification of salivary gland µg enzyme of *Oxya chinensis* by ion exchange chromatography and protein assay by Bradford method. Void volume of 10 ml was excluded from the chromatogram. 200 fractions (250 ml) were collected using 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl gradient at room temperature 25ºC. Fraction size was 1.5 ml and flow rate of the column was 1 ml/min. Dimensions of the column were 10×3 cm (L×W). Peak 1 was observed at fraction 21, Peak 2 was observed at fraction 37, Peak 3 was observed at fraction 43.

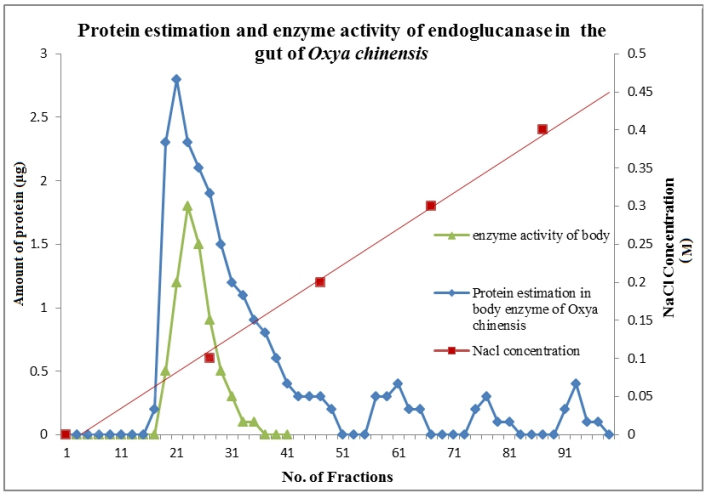


Fig. 3. Purification of the gut enzyme of *Oxya chinensis* by ion exchange chromatography and protein assay by Bradford assay. Void volume of 10 ml was excluded from the chromatogram. 100 fractions (150 ml) were collected using 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl gradient at room temperature 25°C. Fraction size was 1.5 ml and flow rate of the column was 1 ml/min. Dimensions of the column were 10×2.8 cm (L×W). Peak was observed at fraction 21.

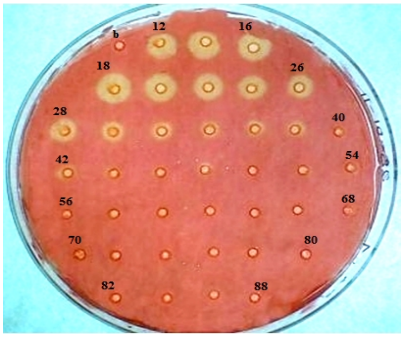


Fig. 4. CMC agar plates showing clear zones of CMCase activity of ion exchange fractions of salivary gland enzyme. 50µl sample was added in each well of each plate. Fractions 12-28 of ion exchange (eluted in 0.15 M NaCl) are showing CMCase activity in plate 1 corresponding to peak 1 observed in Fig. 2.

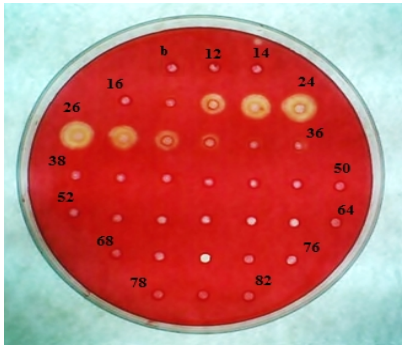


Fig. 5. CMC agar plates showing clear zones of CMCase activity of ion exchange fractions of the gut enzyme. 50µl sample was added in each well of each plate and the plates were incubated at 50°C overnight. Fractions 20 - 28 of ion exchange (eluted in 0.05- 0.15 M NaCl) are showing CMCase activity in plate 1 corresponding to the peak observed in Fig.3

3.3 Purification Fold of Enzyme

The enzyme beta 1,4-glucanase was purified using ion exchange chromatography and specific activity as well as fold of purification was determined from both sample (salivary glands and gut) (Tables 1 and 2).

Table 1. Specific activity of crude and purified enzyme from salivary gland

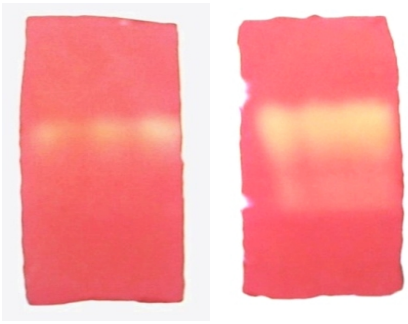
S. no.	Procedure	Total protein (mg)	Enzyme (I.U)	Specific activity	Purification fold
1	Crude extraction	38.00	21.4	0.56	1×
2	Purification using Ion Exchange Chromatography	0.53	8.0	15	26 ×

Table 2. Specific activity of crude and purified enzyme from gut

S. no.	Procedure	Total protein (mg)	Enzyme (I.U)	Specific activity	Purification fold
1	Crude extraction	44.8	24.6	0.55	1×
2	Purification using Ion Exchange Chromatography	0.56	6.4	11.4	20 ×

3.4 Zymography

Fractions of the peak 1 were pooled together and further analyzed on non-denaturing PAGE along with the crude preparation. After electrophoretic run the polyacrylamide gel was overlaid on 2% CMC agar plate and incubated at 50ºC for 30 minutes. Results are shown in Fig. 6 (1, 2 and 3), in each case (crude or purified) single band was observed for (Oc*Cel*9).



A B

Fig. 6. Zymograms of salivary gland and the gut enzyme of *Oxya chinensis* showing similar bands of activity. A) Salivary gland enzyme, sample 1,2 and 3 corespond to fraction no. 19, 21 and 23 of ion exchange purification (Fig. 2) B) gut enzyme sample 1,2 and 3 corespond to fraction no. 19, 21 and 23 of ion exchange purification (Fig. 3)

3.5 Enzyme Characteristics

Effect of substrate concentration on endo-1,4-ß-glucanase(Oc*Cel*9) activity was determined. For this assay enzyme concentration was kept constant and percentage of substrate was changed from 0.5 to 1%. Km of salivary gland and the gut enzyme was also calculated (Figs. 7 and 8).

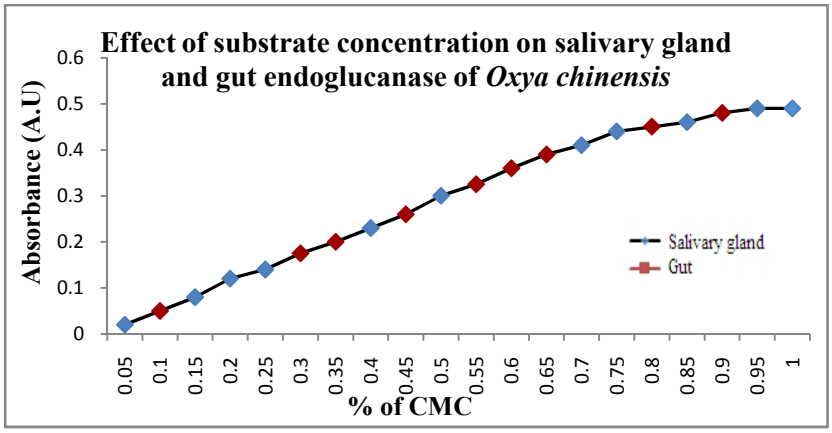


Fig. 7. Effect of substrate concentration on enzyme (Oc*Cel*9) activity in salivary gland and the gut. Firstly the activity increased sharply with the increase in substrate concentration and gradually steady state was achieved when further increase in substrate concentration occurred.

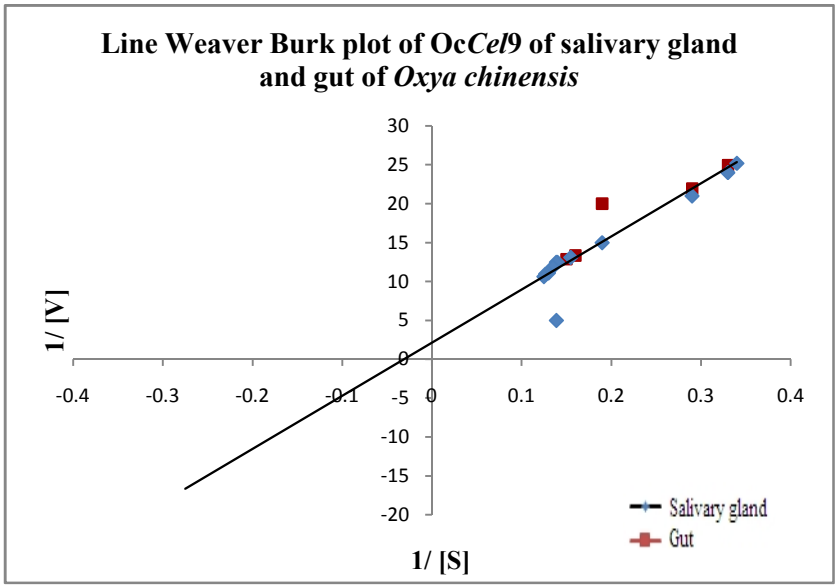


Fig. 8. Line Weaver Burk plot of enzyme Oc*Cel*9. Enzyme kinetics of beta-1,4-glucanase was determined the K_m value for both the enzyme was 0.032 M which reflects the affinity of the enzyme for substrate.

Fractions 12-28 from salivary gland enzyme were pooled to perform enzyme characterization. For pH characterization CMC was subjected to hydrolysis by purified gut enzyme (Oc*Cel*9) of *Oxya chinensis* at different pH ranging from 2.0-10.0. Samples were analyzed using DNS assay and its measurement was done at 546nm (Fig. 9). In a similar fashion for the characterization of thermal profile of enzyme CMC hydrolysis was performed

by purified gut enzyme (Oc*Cel*9) at different temperatures ranging from 10º-100ºC. Samples were analyzed using DNS assay [40] (Fig. 10).

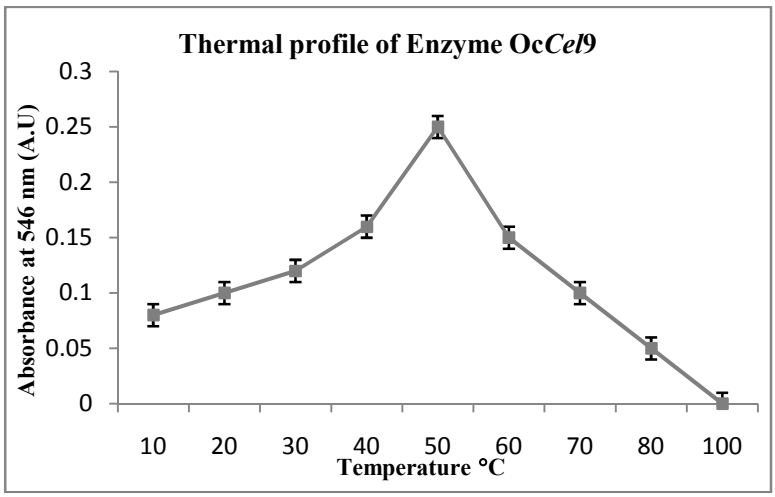


Fig. 9. Thermal profile of gut enzyme of *Oxya chinensis* showing maximum activity at 50ºC. Reaction mixtures consisted of 1 ml of 2% CMC, 1ml buffer of pH 3.0 and 50 µl of enzyme. Incubation was done at 10-100ºC overnight.

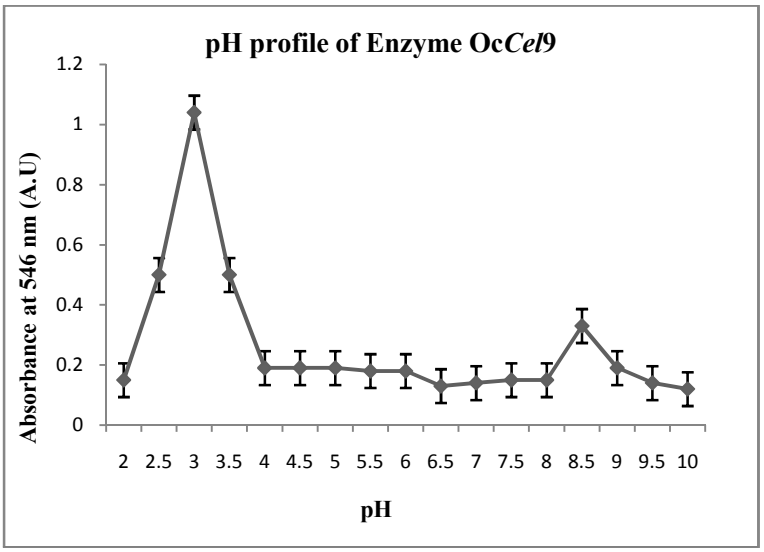


Fig. 10. pH profile of enzyme Oc*Cel*9 of *Oxya chinensis* showing maximum activity pH 3.0 and a minor peak at pH 8.5. Reaction mixtures consisted of 1 ml of 2% CMC, 1ml of respective buffer and 50 µl of enzyme. Incubation was done at 50ºC overnight.

3.6 Molecular Weight Determination

The molecular weight of purified enzyme (Oc*Cel*9) was determined by subjecting the enzyme to SDS-PAGE analysis and a single band was obtained on the gel (Fig. 11).

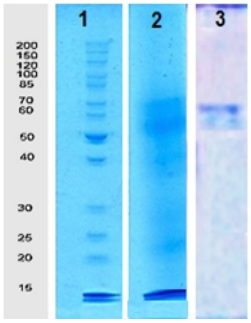


Fig. 11. SDS-PAGE of purified enzyme (OcCel9). Lane 1 shows marker; Lane 2 shows crude enzyme extract; Lane 3 shows purified enzyme indicating two bands of 60 and 55 KDa protein representing two subunits/isozymes of the enzyme.

3.7 Spectroscopic Analysis

The purified protein was analyzed and identified by Spectroscopy and it was revealed that the protein was a signature pattern for GH family 9 in its internal sequence.

SYVVGFGNNPPVVHHR. The identified protein fragments show homology with a putative beta-1,4-glucanase of rice. The identified fragments were aligned with the rice protein. About 14 peptide fragments were aligned with the rice endoglucanse using T-Coffee aligment (Fig. 12).

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CLUSTAL W (1.83) multiple sequence alignment

1                          ---------------------------------------RKSILYFQAQRS---------
gi|48475166|gb|AAT44235.1|  MAKNGGAHGAATLFGLLALASMVKLGFVAGGGHDYAMALRKSILYFQAQRSGVLPPNQRV

1                          ----------KGQNGEDLTGGYYDAGDHVK------AFETTIR-----FGAQVAGWT--R
gi|48475166|gb|AAT44235.1|  SWRASSGLFDGKANGVDLVGGYYDAGDNVKFGLPMAFTVTMMSWSILEYGKQMAAAGELR

1                          ------------------------------------------------------------
gi|48475166|gb|AAT44235.1|  NAMDAVKWGTDYFIKAHPEPDVLYGEVGDGDTDHSCWQRPEDMTTSRQAFRVDPQHPGSD

1                          ------------------------------------------------------------
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1                          ----------------------------------------------------KILASKPI
gi|48475166|gb|AAT44235.1|  GYGDELLWAAAWLFEATEDRSYLEYLAGNGEALGGTGWSINQFGWDVKYPGVQVLAAKFL

1                          NK-----------------------------------SGMVYEKK--------------
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1                          --------------------------LEIVKVPK-CRLGDNPVGRSYVVGFGNNPPV-V
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1                          HHR-AGIIVIR---------------------------------------------------
gi|48475166|gb|AAT44235.1|  HHRGASIVSIKRDPSFVSCQEGYSSWYGREAGNPNLLDGAVVGGPDEYDDFADERDNYEQ

1                          ------------------------------------------------------------
gi|48475166|gb|AAT44235.1|  TEAATYNNAPLLGVLARLAASCGGLKEEEYEQETATPVVNRTSSSSSLPATATAIGIEQN

1                          ---- ----------------------------------------------------SPV
gi|48475166|gb|AAT44235.1|  VTGTWARRRRTYYRYAVTVTNRSRGKTVRELHLGVSGLRGRLWGLEEARYGYVPPRWLPA

1                          VEPGFS-R--------------------
gi|48475166|gb|AAT44235.1|  LRPGRSLRFVYVQPAPAPANIWVTGYKLV
```

Fig. 12. Alignment of peptide fragments, identified from purified beta-1,4-glucanase of *O. chinensis* with the'putative endo-beta-1, 4-glucanase'.- Oryza sativa (japonica cultivar-group) with an accession No. AAT44235.1.

3.8 Inhibition Studies

Different neem derived inhibitors i.e. crude neem powder extract and saponins along with substrate and salivary gland enzyme were added in CMC agar plates and incubated at 50°C overnight. 50µl of enzyme and 50µl of inhibitor was added in the plates (Fig 13).

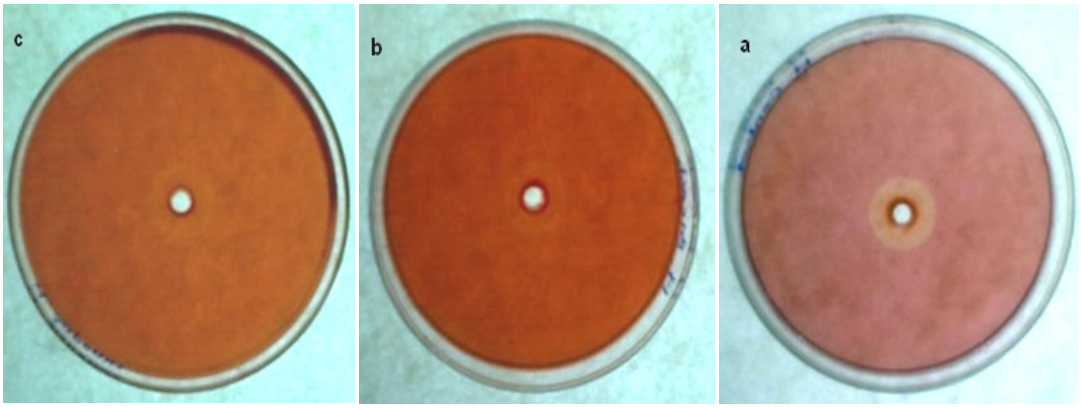

Fig. 13. CMC agar plates with enzyme and inhibitor

a. Control (with no inhibitor) showing activity zone in the center.
b. Crude neem powder extract containing plate showing no activity.
c. Purified saponins containing plate showing no activity.

Different concentrations of purified saponins ranging from 100µl-2000µl were incubated with salivary gland and gut enzyme and substrate. As shown in Fig. 13, inhibition was observed for neem leaf powder extract and saponins (Fig. 14).

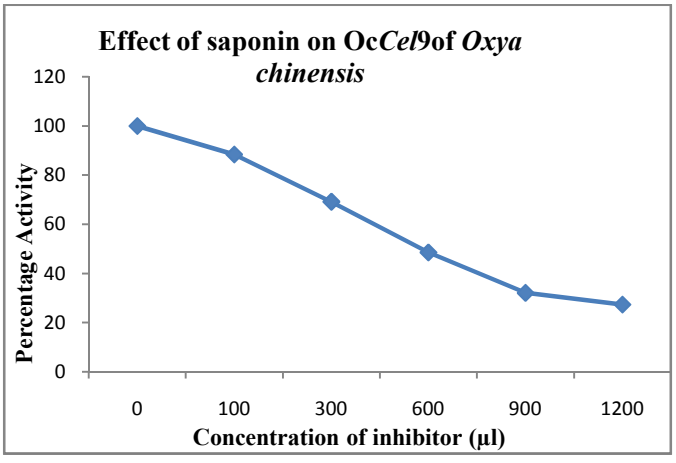

Fig. 14. Graph showing decrease in the activity of beta-1,4-glucanase with the increase in concentration of saponins

4. DISCUSSION

As rice is an important cash crop, studies were undertaken to identify the role of endo-1,4-ß-glucanase (Oc*Cel*9) present in *Oxya chinensis* (a serious paddy pest) in crop damage. Earlier we reported presence of cellulose hydrolyzing activity in the total soluble proteins of *O. chinensis*. Location of the activity was not identified [35]. Dried Insects were dissected to separate head for salivary glands cellulose activity and the body was used as a source for cellulose hydrolyzing activity present in gut. As reported in results salivary gland and gut showed the presence of cellulase(Oc*Cel*9) enzyme activity (Figs. 1 A and B).

The crude extracts of both salivary gland and gut was purified by ion-exchange chromatography using CM-sephadex and various peaks were observed (Figs. 2 and 3) which were screened for CMCase activity and it was found to be in the 1st peak for both enzymes (Figs. 2 and 3). Other peaks might have been the result of contamination of some other enzymes. 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). Enzyme activity was also determined on CMC-agar activity plates in the purified fractions. The fractions (12-28) of salivary gland and (20-28) of gut pertaining to peak 1 were active on the substrate-agar plates further confirming the results of Fig. 2 and 3 respectively (Figs. 4 and 5). The dried insects were used as a source for the enzyme, it is obvious that a number of proteins present in the tissues were degraded and that was why not many protein peaks appeared in the chromatogram (Figs. 2 and 3). The purification fold of both the enzymes was calculated and found to be approximately 2 folds (Table 1 and 2).

The zymography of both enzymes was done. The salivary gland enzyme appeared as a band on the zymogram (Fig. 6 A) whereas the gut enzyme appeared as a broad band at the same position on the zymogram indicating the presence of a similar cellulase (Fig. 6 B). The enzymes were then further characterized to check the effect of substrate concentration both enzymes also obeys Michaelis Menten enzyme kinetics as the rate of reaction rise linearly with subsequent increase in enzyme activity and reached a steady state after attaining a maximum level. Experimental data also shows that there exist cooperativity between the enzyme and substrate binding so enzyme kinetics can be further related to Hill's equation of cooperativity and further elucidation of enzyme kinetics is required (Fig. 7) [42]. The K_m for both enzymes was calculated to be 0.032 M which further confirms the presence of a single type of beta-1, 4-glucanase in both salivary glands and gut (Fig. 8). It might be possible that some of the enzyme has leaked out of the salivary gland region and its activity is indicated in the gut region. It was thought that there is only one type of cellulose hydrolyzing activity in both the regions (salivary gland and gut). The purified fraction from salivary glands were pooled and used for further characterization. It could be possible as the insect mostly feed on rice plant that the cellulase protein may be the major protein produced by the salivary glands. The purified preparation appeared as a single band on zymogram indicating no heterogeneity at this stage (Fig. 6). It could be concluded that the enzyme was intact during the purification procedure and there may be only one gene for Oc*Cel*9 enzyme in *O. chinensis* in contrast of rice plant. The optimum temperature for the enzymes was observed to be 50ºC (Fig. 9). Earlier we have reported that the crude cellulase of *O. chinensis* also had acidic pH optima [35]. Digestive enzyme of Blister beetle also produced acidic enzymes [24]. *Oxya chinensis* beta-1,4-glucanase has acidic (3.0) pH optima of enzyme so it is able to hydrolyze rice husk very efficiently (Fig. 10). It is thought that *Oxya chinensis* has an efficient and complete endo-1,4-ß-glucanase (Oc*Cel*9) system able to hydrolyze rice leaves as well as rice husk due to its acidic pH and so able to feed on this economically important

crop. It has been discussed earlier that insect cellulases could be used for biotechnological purposes. So, far the mechanism of microbial cellulases is studied in detail. GHF9 has been reported to be the most studied family for plant beta-1,4-glucanases. It has been reported that plant cellulases act via an inverting mechanism to cleave the 1, 4 beta glucosidic bond between two unsubstituted carbohydrate units [43]. As insect mainly uses plant biomass as food, comparison of plant cellulases with insect cellulases could provide some clues for plant biomass degradation, by insects.

The SDS-PAGE of beta-1,4-glucanase of *Oxya chinensis* indicated two bands of 60 and 55kDa that represent two subunits/isozymes of the enzyme (Fig. 11). Cellulose hydrolyzing activity in the salivary gland could be related to the presence of indigenous enzyme activity in the insect, as the symbiotic microbes reside in the gut. The plant biomass is composed of cellulose fibrils packed with xylose fibers. Salivary glands had been studied for digestive enzymes in pod sucking bugs, associated with cowpea. [44]. Cellulase activity has been reported in another grasshopper, *Zonocerus variegatus*, has been studied for morphometric data and enzyme activities in the femoral muscles [45]. Presence of cellulase activity among animals had been reported before [8,16,31,34]. For rice plant it has been reported that 22 genes for the expression of GHF9 cellulases after sequencing of the Oryza genome [26]. The enzyme present in *O. chinensis* showed some similarity with the cellulase proteome of rice plant. Alignment of peptide fragments, identified from purified beta-1,4-glucanase of *O. chinensis* with the 'putative endo-beta-1, 4-glucanase'.- *Oryza sativa* (japonica cultivar-group) with an accession No. AAT44235.1 (Fig. 12). The sequencing of the first plant "cellulases"/endo-β-1,4-glucanases reported, belong to the GH9 family. This is one of the larger plant GH families [46] and studies of microbial GH9 proteins, including both beta-1,4-glucanases (EC 3.2.1.4) and cellobiohydrolases (EC 3.2.1.91), have shown that they operate via an inverting mechanism to cleave the 1,4-glucosidic bond between two unsubstituted carbohydrate units [43]. To date, the GHs have been classified into more than 80 different GH families based on their amino acid sequence similarities [12,47-48]. It is reported that the substrate binding cleft in case of rice endoglucanse shows a primitive disposition of an aromatic platform residue (Tyrosine, Tryptophan or Phenylananine) which presumably interacts with the saccharide ring by hydrophobic stacking mimicking the catalytic machinery of microbial E2 enzyme [49-50]. The unique position of the hydrophobic platform residue in the substrate binding site of *Oryza sativa* cellulase could be involved in the tolerance of enzyme for relatively hydrophobic xylose based polymer present in plant biomass [49]. It is thought that the *Oxya chinensis* cellulase (Oc*Cel*9) mimics the plant O*ryza* cellulase, due to which the insect cellulose hydrolyzing activity got access to plant biomass. The isolated enzyme from salivary gland shared a number of fragments including a signature pattern for the enzyme from rice plant.

Inhibition studies were also conducted on the enzyme. Many plants have been screened for the presence of repellants and anti-feedants to form an effective bio-insecticide against insect pests of crops [51]. In this regard neem is one of the most important and potent plants. There are three main classes of bioactive compounds present in neem namely limonoids, saponins and azadirachtin. Neem derived compounds are a source of inhibitors against cellulases present in various insects [34]. The enzyme was subjected to inhibition by neem extracts. Saponins were incubated with the enzyme and inhibition was observed (Fig.13). Different concentrations of saponins were incubated with the enzyme and a progressive decrease in the activity was observed (Fig. 14). The possible mechanism of inhibition can be related to the structure of saponins that having terpenoid and the attached sugar moiety in the relaxed (chair) confirmation. It was reported that a sugar residue bound at the catalytic site of cellulase undergoes a distortion that renders it in a planar half chair

conformation during catalysis of cellulose [52]. Quiocho reported carbohydrate-binding proteins: tertiary structures and protein-sugar interactions [50]. It is suggested that the competitive binding of the sugar moiety of saponins with the catalytic site of cellulase and the blockage of the rest of the site by additional large terpenoid groups.

5. CONCLUSION

Summarily, *O. chinensis* produced an acidic cellulase which could be of great importance with reference to the technological point of view. A detailed study is required with reference to genomics for complete understanding of the mechanism of plant biomass degradation by the insect. The study presented could provide a foundation for future studies. Moreover this study could present the basis for the development of biopesticides effective against crop damaging insects.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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