Cellulase Activity and Kinetics in Rice Grasshopper *Hieroglyphus banian* (Orthoptera: acrididae)

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Abstract

Grasshopper (*Hieroglyphus banian)*, an orthopteran species is considered to be a serious pest of rice plant in India. To study the activity and kinetics of cellulase in grasshopper this experiment is undertaken and accordingly foregut and midgut homogenates were prepared. The cellulase enzyme activity and kinetics was measured by DNSA method of Miller,(1959) by taking different concentrations of crystalline cellulose substrate in spectrophotometer at 540nm. Cellulase activity in foregut and midgut were found to be 0.482 u/mg of tissue and 0.687u/mg of tissue respectively. K_m values were determined separately both in foregut and midgut and were found to be 3 mg/dl and 2 mg/dl respectively. Experimental data indicates the presence of high cellulolytic activity in the midgut which may suggest that cellulases of endogenous origin are present in this organism. It can be expected that in future, detailed study of these efficient lignocellulolytic systems will help in identification of novel enzymes possessing features that optimize biotechnological applications for the biofuel industry. Moreover, identification of crucial insect cellulases may help in the development of insecticidal technologies aimed at inhibiting their vital digestive role. Considering that this grasshopper species is a pest of paddy and grass, characterization of insect cellulolytic systems may aid in reducing the grasshopper pest attack in Indian subcontinent.

Keywords: Cellulase, Kinetics, *Hieroglyphus banian*, Foregut, Midgut.

1. Introduction

Cellulose degradation requires 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) (Clarke,1997). EG enzymes work by random cleavage of β-1,4 glycosidic bonds in the internal portions of cellulose strands to reduce the degree of polymerization of the cellulose chain into smaller subunits.CBH enzymes remove subunits at both reducing and non-reducing ends of the cellulose chain, releasing either cellobiose or glucose. Due to the inhibition of EG enzymes by accumulation of cellobiose, the presence of β glucosidases to hydrolyze cellobiose to glucose is important for complete degradation of cellulose (Holtzapple *et al.,*1990; Gruno *et al.*,2004). Cellulolytic activities were originally thought to be limited to plants, bacteria and fungi; there is increasing evidence for the existence of animal cellulases, especially in invertebrates (Yokoe and Yasumasu, 1964;Watanabe and Tokuda, 2001; Lo *et al.,* 2003). Due to the diverse and highly adapted phytophagous nature that feeds on very fibrous, lignocellulose-rich, plant tissues, insects can be a potential candidate which can prosper for novel cellulolytic enzymes. There have been numerous reports on cellulolytic activity in insects (Wharton and Wharton, 1965; Ishaaya and Plaut, 1974; Tokuda *et al.*, 1997; Pitman *et al.*, 2003), including identification and cloning of insect cellulases (Watanabe *et al.,* 1997; Girard and Jouanin, 1999; Lee *et al.*, 2004; Lee *et al.,* 2005; Wei *et al.,* 2006; Kim *et al.,* 2008). Although relevant reviews on cellulolytic activity in insects are available (Martin, 1983; Watanabe and Tokuda, 2001), broad efforts to quantitatively characterize cellulolytic activity in insects are very limited (Cazemier *et al.,* 1997).

Previously, enzymatic activity against cellulose substrates were detected in digestive fluids of insect species belonging to ten insect orders (Martin, 1983; Watanabe and Tokuda, 2001; Willis *et al.*,2010). These activities were historically attributed to gut symbiotic flora, until the first insect cellulase was described in *Reticulitermes speratus* (Kolbe) (Isoptera: Rhinotermitidae) (Watanabe *et al.,* 1998).Various studies have reported endogenous insect cellulase enzymes in orders:; Blattaria, Coleoptera, Hymenoptera, Hemiptera and Orthoptera (Watanabe and Tokuda, 2010). Even though orthopteran species in the Acrididae family are serious plant feeders, limited information is available on specific cellulolytic systems in these species. Orthopteran cellulase enzymes have been previously described only for the emma field cricket, *Teleogryllus emma* (Orthoptera: Gryllidae) (Kim *et al.,* 2008). Recently, a comprehensive screening for cellulase activity, discovering activity in gut and head-derived fluids from insect species belonging to eight taxonomic orders were undertaken and high cellulase activity was detected in fluids from the Carolina grasshopper (*Dissosteira carolina*) (Oppert *et al.*,2010).

The main goal of this study was to determine the activity and

K_m of cellulase enzyme against crystalline cellulose substrate in foregut and midgut. Fluids from foregut and midgut was used to evaluate relative cellulase K_m values. Experimental data suggest significant differences statistically in K_m and activity values both in foregut and midgut, which may be correlated with distinct food habit or highly evolved feeding strategies on cellulose rich diet under evolutionary stress.

2. Materials and Methods

2.1 Collection of sample

H .banian adults were field-collected from nearby rice paddy fields. Individuals were cooled to 4°C before dissection to slow down the metabolism and to provide easier handling and dissections were performed on ice. In the dissection, gut was collected separately in an attempt to discriminate from the salivary gland. Gut tissues from forty individuals belonging to same developmental stage were dissected and separated into foregut and midgut, and pooled into separate micro centrifuge tubes with 100µl of saline water.

2.2 Preparation of enzyme sample

Measured amount of the sample materials (Foregut and midgut) were homogenized in a glass homogenizer with appropriate amount of citrate buffer to give a final strength of 10 mg sample per ml of homogenate. After homogenization, the homogenate was centrifuged at 5000 RPM for 15 minutes and the supernatant was used as enzyme source for estimation of cellulase. According to some earlier reports, insects have their own cellulolytic system (Oppert *et al.*,2010) even if the microbes are present in the hindgut and to avoid the possibility of microbial interference we considered only foregut and midgut for our experiment.

2.3 Estimation of cellulase enzyme activity and kinetics

Estimation of cellulase enzyme activity is performed by using a modified Dinitrosalycylic acid (DNSA) assay (Miller,1959). Concentration of the released glucose was measured from a standard glucose curve

Enzyme activity u/mg of tissue was determined considering one IU equal to 1 µmol min-1 of glucose formed in the hydrolysis reaction. Reaction mixture was prepared by mixing 100 µl of the crude enzyme sample with 0.5 ml of crystalline cellulose solution (of various concentrations) and 0.5 ml 0.1 M sodium acetate buffer (pH 5.0). Then mixture was incubated for 5 hours at 50°C with gentle shaking. After incubation, 2 ml of DNS reagent was added to reaction mixture and incubated in boiling water bath for 15 minutes and then the absorbance was noted at 540 nm.

3. Results

3.1 Estimation of cellulolytic activity in foregut and midgut of grasshopper

After the estimation of cellulase activity in foregut by DNSA method using crystalline cellulose a low level of activity 0.482 U/mg of tissue was found, (Fig-I) which shows that even if midgut is the prime site of digestion and absorbtion, a significant amount of cellulose digestion also takes place in the anterior part of the alimentary canal. Likewise cellulase activity in midgut was determined as 0.687 U/mg of tissue. (Figure-I). The activity of cellulase enzyme in midgut was found to be comparatively higher than foregut (Fig-I) which indicates that majority of the diet containing cellulose is digested in the midgut of this particular insect.

3.2 Determination of K_m value in foregut and midgut

K_m value of cellulase enzyme in grasshopper foregut and midgut is determined by using various concentrations of cellulose substrate (Table-I) and by plotting double reciprocal Lineweaver-Burk plot (Fig-II).The K_m value found in foregut is 3.3 mg/dl. This value is comparatively higher than the value of K_m found in midgut (Fig-II). The higher Km value indicates less enzyme substrate (ES) complex formation in foregut. K_m value determined in the midgut of grasshopper *Hieroglyphus banian* is 2 mg/dl. The K_m value in midgut was a bit lower than the foregut K_m value which indicates higher rate of formation of enzyme substrate (ES) complex in midgut than in the foregut

3.3 Difference in the rate of enzyme activity both in foregut and midgut

Experimental data suggest a significant difference in celluase activity in foregut and midgut of grasshopper which was determine by ANOVA test at $P<0.005$.

3.4 Tables and Graphs

Table 1. *Mean values of maximum velocity of cellulase activity with different substrate concentrations in the foregut and midgut of grasshopper.*

Grasshopper		Foregut		Midgut	
Substrate conc(%)	1/substrate	Velocity (V)	1/V	Velocity (V)	1/V
5	0.2	0.043	23.3	0.05	21.739
10	0.100	0.048	21.0	0.06	17.730
20	0.050	0.051	19.6	0.06	15.625
30	0.033	0.054	18.5	0.07	13.755
50	0.020	0.059	17.0	0.08	11.905
70	0.014	0.064	15.7	0.09	11.429
90	0.011	0.068	14.7	0.09	11.390
110	0.009	0.071	14.1	0.09	11.299
130	0.008	0.071	14.1	0.09	11.325

Fig.I. *Cellulase activity in the foregut and midgut of grasshopper*

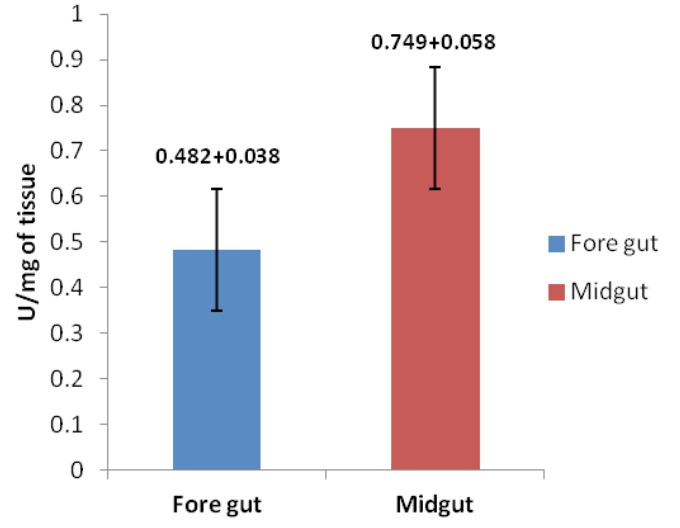


Fig.II. *Michaelis Menten plot for cellulase in the foregut and midgut of grasshopper*

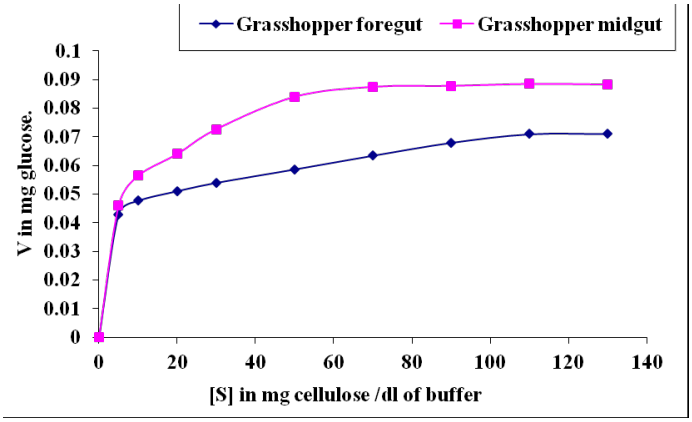
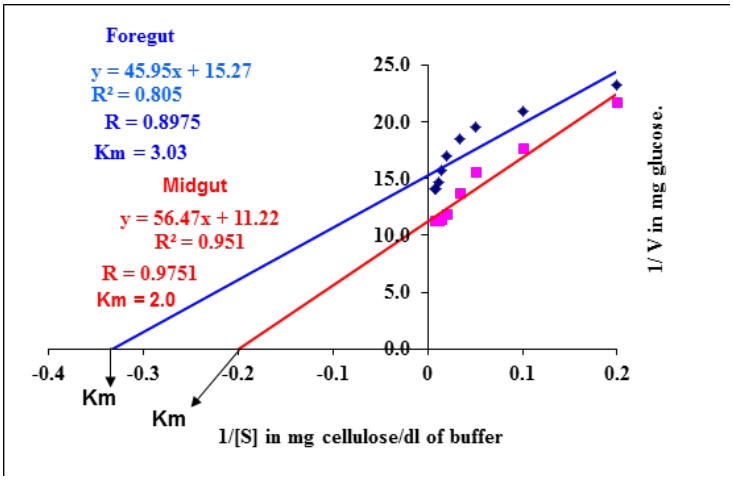


Fig.III. *Lineweaver- Burk plot for cellulase in the foregut and midgut of Grasshopper.*



4. Discussion

The experimental data represents the first report detecting cellulolytic activity in the gut of *H. banian* belonging to acrididae family after Wills *et al.*, (2010), who detected cellulolytic activity in *D.caroloina* grasshopper. Family Acrididae contain numerous grasshopper pest species that confound management of crop commodities worldwide, including many being considered as feedstock for lignocellulosic ethanol biofuel (Kumarasinghe,2003; Parrish and Fike,2009). In this regard *Hieroglyphus banian* can be considered as model insect exhibiting effective cellulolytic activity and can use cellulase enzyme to prosper for novel cellulolytic enzymes leading to more efficient biofuel production.

Orthopteran species have traditionally not been the focus of cellulolytic prospecting, probably due to controversial reports of cellulolytic capacity in these insects (Clissold *et al.*,2004; Davis,1963; Evans and Payne,1964; Morgan,1976). Our cellulolytic activity assay using homogenates from both foregut (0.482 U/mg of tissue) and midgut (0.749 U/mg of tissue) of *Hieroglyphus banian* expressed almost similar rate of cellulolytic activity as that of termite, beetle and cockroach, which were traditionally believed to have cellulase activity. (Oppert *et al.*,2010). Moreover, lower Km value found in midgut(Km-2 mg/dl) indicates higher rate of enzyme substrate complex than in the foregut (Km- 3.03 mg/dl). Cellulolytic activity of gut found against crystalline cellulose substrate suggest the presence of endogenous symbiont-independent cellulase system in grasshopper *Hieroglyphus banian.*

Degradation of plant material initially in the feeding process of grasshopper may be performed by mechanical maceration via mandibular action. This process renders lignocelluloses more accessible to enzymatic degradation. In grasshopper the assimilation rate for ingested plants cellulosic material ranges from 27% to 34% (Barbahenn *et al.*,2004; Smalley,1960) even though this rate of assimilation is considered as very high compared to other herbivorous insects (Sinsabaugh *et al.*,1985) and arthropod species(Schoenberg *et al.*,1984). In *Hieroglyphus banian* it is established that it has developed a symbiont free cellulase system for the degradation of cellulose since we have observed a sufficiently high level of cellulase activity in midgut than the foregut. This finding can be co-related with the findings of Schultg *et al.*,(1986); Scrivener *et al.,(*1989) and Zhang *et al.,(*2009,2010) where they have reported that some insects can digest cellulose in the absence of exocellulase, which in many insects are found to be secreted by symbiont microbes.

Current world energy needs demand the development of industrial-scale processes for the sustainable production of fuel from renewable biological resources as economic and environmentally sound alternatives to finite fossil fuels. In the US, lignocellulosic ethanol has been suggested as a desirable biofuel, mostly due to its sustainability, reduced competition as a food resource, net energy production, and reduced input costs related to production of ethanol from corn-derived starch (Lynd *et al.*, 1991; McLaughlin *et al.*, 2002; Schmer *et al.*, 2008). Cost-efficient production of ethanol from lignocellulosic biomass is mostly dependent on development of efficient hydrolysis technologies (Sun and Cheng, 2002; Wyman, 2007). Enzymatic degradation of cellulose is considered the hydrolysis method with the greatest potential for improvement and cost reduction (Wyman, 1999, 2007). Current estimates suggest that re-

ducing the cellulase enzyme amounts by half through biotechnology could decrease processing costs by up to 13% (Lynd *et al.*, 2008).

Orthopteran species are herbivores able to cause extensive damage to crops, which makes them attractive models for characterizing effective cellulolytic systems. Our data suggest that, endogenous endoglucanase activity is present in fluids from both foregut and midgut tissues of *Hieroglyphus banian* and that these enzymes participate in the whole cellulose degradation process. Previous reports demonstrated that grasshopper species can actively adjust their digestive system to compensate assimilation efficiency while feeding on low nutritional substrates (Barbehenn *et al.*, 2004; Fielding and Defoliart, 2007; Yang and Joern, 1994). This compensatory system parallels desirable plasticity in biorefineries for effective use of diverse feedstocks during production of lignocellulosic ethanol. We can expect that in future detail study of these efficient lignocellulolytic systems will allow identification of novel enzymes possessing features that optimize biotechnological applications for the biofuel industry. Additionally, identification of crucial insect cellulases may allow the development of insecticidal technologies aimed at inhibiting their vital digestive role (Zhou *et al.*, 2008).

With the advances in the field of molecular biology and biotechnology it is expected that very soon the whole insect genome sequences will be explored and in the next generation sequencing projects, the number of identified endogenous and symbiont-derived insect cellulases is expected to increase in the near future (Matsui *et al.*, 2009; Morrison *et al.*, 2009).

5. References

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