



Evaluation of cellulolytic activity in insect digestive fluids

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ABSTRACT. Efficient and low-cost cellulolytic enzymes are urgently needed to degrade recalcitrant plant biomass during the industrial production of lignocellulosic biofuels. Here, the cellulolytic activities in the gut fluids of 54 insect species that belong to 7 different taxonomic orders were determined using 2 different substrates, carboxymethyl cellulose (CMC) (approximating endo- β -1,4-glucanase) and filter paper (FP) (total cellulolytic activities). The use of CMC as the substrate in the zymogram analysis resulted in the detection of distinct cellulolytic protein bands. The cellulolytic activities in the digestive system of all the collected samples were detected using cellulolytic activity analysis. The highest CMC gut fluid activities were found in Coleoptera and Orthoptera, while FP analysis indicated that higher gut fluid activities were found in several species of Coleoptera and Lepidoptera. In most cases, gut fluid activities were higher with CMC than with FP substrate, except for individual Lepidoptera species. Our data indicate that the

origin of cellulolytic enzymes probably reflects the phylogenetic relationship and feeding strategies of different insects.

Key words: Cellulolytic activity; Insect; Digestive fluids; Lignocellulosic biofuels; Cellulase zymography

INTRODUCTION

Because of the increasing demand to overcome energy shortages and achieve stable economic development, lignocellulose-based biofuels have recently become a major focus of industrial and academic communities worldwide (Sun and Scharf, 2010). During biofuel production, linear chains of cellulose, which consist of glucose residues connected by a β -1,4 linkage, need to be degraded to glucose, which is then fermented to produce ethanol. At present, even though microbial and chemical degradation of cellulose has been widely used, the use of combined cellulolytic enzymes remains limited because of the high cost of biotechnological tools (Wyman, 1999). Furthermore, in industry, breakthrough technologies to overcome the barriers of developing cost-effective processes for converting biomass to fuels and chemicals have yet to be fully realized. Therefore, there is an urgent need to discover and develop more efficient cellulolytic enzymes that reduce biofuel production cost, in addition to having applications in other industrial processes (Wyman, 2007).

In general, cellulolytic activities were originally thought to be limited to plants, bacteria, and fungi. With increased study of cellulolytic activities, increasing evidence has shown that cellulases are also present in the animal kingdom, particularly in insects (Yokoe and Yasumasu, 1964; Watanabe and Tokuda, 2001; Lo et al., 2003), including more than 20 insect families that represent 10 distinct insect orders, such as Thysanura, Plecoptera, Dictyoptera, Orthoptera, Isoptera, Coleoptera, Trichoptera, Hymenoptera, Phasmida, and Diptera (Sun and Scharf, 2010). These insect cellulases may degrade lignocellulosic biomass easily and efficiently and might serve as highly efficient natural bioreactors. The types of biomass substrates fed on by cellulolytic insects are diverse, ranging from substrates of agricultural crops (Poaceae, Solanaceae) to woody forests (Salicaceae, Anacardiaceae, Moraceae, etc.). Therefore, recent studies have focused on the ability of these insects to feed on wood, foliage, and detritus, understand how these insects digest food, such as structural and recalcitrant lignocellulose, and their potential to enhance current biofuel technologies and processing.

Insects present attractive and potential candidates for highly efficient natural bioreactors, from which novel cellulolytic enzymes are being sought. The number of novel cellulases and hemicellulases, as well as associated encoding genes, from a variety of cellulose-feeding insects has been continuously updated in recent years. Examples include *Apriona germari* (Jing et al., 1996), *Dendroctonus armandi* (Wang et al., 2007; Wang and Chen, 2008), *Eucryptorrhynchus chinensis* (Yong et al., 2007), and *Periplaneta americana*, for which high carboxymethyl cellulase (CMCase) and filter paper cellulase (FPase) activities have been measured, amounting to 0.89 and 0.12 U/mL, respectively (Gijzen et al., 1994). Furthermore, another study detected 3 active protein bands for carboxymethyl cellulose (CMC) in *Anoplophora glabripennis* by using zymogram analysis (Geib et al., 2010).

Although relevant studies on cellulolytic activity in insects are available (Watanabe and Tokuda, 2001), the quantitative characterization of cellulolytic activities in a

broad number of insect species remains very limited (Cazemier et al., 1997; Oppert et al., 2010). Furthermore, studies on insect cellulolytic activities by using FP as a substrate have not been performed until the current study. In the present study, we collected 54 phytophagous insect species that belong to 7 orders, including Diptera, Orthoptera, Coleoptera, Hymenoptera, Lepidoptera, Dictyoptera, and Dermaptera. We evaluated the relative cellulolytic activities of gut fluids from all 54 insect species by using soluble CMC and FP cellulose as substrates.

MATERIAL AND METHODS

Insect collection and dissection

Experimental insects (including adults and larvae) were collected from a field in Henan Province, except for *Lasioderma serricornis*, which was obtained from laboratory cultures at the Zoology Laboratory of Henan Agricultural University. The life stages, collection site, and plant host tissues for each species are listed in Table 1. As soon as possible after collection, the insect intestines were dissected on ice for subsequent assays. The dissected tissues were cut into small pieces, homogenized with liquid nitrogen, dissolved in phosphate-buffered saline (PBS) at 100 mg tissues/mL PBS buffer, vortex-mixed, and centrifuged at 12,000 g for 5 min at 4°C. Supernatants were used as crude cellulase samples, transferred to new centrifuge tubes, and stored at -80°C until use (Oppert et al., 2010).

Determination of cellulolytic activity

Protein concentrations of gut fluid samples were quantified using the Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA), with bovine serum albumin as the standard. Two cellulose substrates with distinct properties were used in the cellulase assays: CMC sodium salt (Sigma-Aldrich, USA) and FP (hai Tang strainer filters, China). CMCase and FPase activities were determined by measuring the amount of reducing sugars released from CMC and FP with a modified 3,5-dinitrosalicylic acid assay (Miller, 1959).

Cellulolytic assays with CMC and FP were performed using 10-50 µg proteins from each insect fluid sample. Protein samples were mixed with either 1.5% CMC sodium salt or FP (2 x 3 cm) suspended in 100 mM sodium citrate buffer, pH 4.8, and were then incubated for 0.5 h (CMC) or 1 h (FP) at 50°C. To stop enzymatic activity, a modified 3,5-dinitrosalicylic acid reagent containing Rochelle salt (Miller, 1959) was added to the samples. Then, color was developed at 100°C for 15 min. The microplates were cooled at room temperature for at least 5 min and then centrifuged at 2000 g for 2 min to remove any remaining substrate. Supernatants were then transferred to polystyrene microplates, and absorbance at 550 nm was determined using a TU-1901 spectrophotometer (Jingke, China). Background amounts of reducing sugars were corrected by subtracting ultimate values from initial values of the calculated reducing sugars in the sample. One unit of enzymatic activity was defined as the amount of enzyme released from 1 µmol of reducing sugar (glucose equivalents) per minute at 50°C and pH 4.8. Specific activities were reported as unit per microgram of proteins. In all experiments, cellulase activities were determined by at least 3 independently replicated experiments, and the mean values were calculated (Willis et al., 2010).

Table 1. Taxonomies, developed stages, and dietary of insect species collected for enzyme activity using carboxymethyl cellulose and filter paper substrates with digestive system contents extracted from insect gut.

Number	Order: Family: Genus species (taxonomic authority)	Life-stage sampled	Food resource(s) ^a (plant tissues) ^b	No. of individuals ^c	Collecting locality ^d
Lepidoptera					
Pyralidae					
1	<i>Diaphania quadrimaculalis</i>	Larvae	<i>Metaplexis japonica</i> (L)	12	ZFP
2	<i>Sylepta derogata</i>	Larvae	<i>Abutilon theophrasti</i> (L)	8	ZFP
3	<i>Glyphodes pyloalis</i>	Larvae	<i>Broussonetia papyrifera</i> (L)	4	ZFP
4	<i>Botyodes principalis</i>	Larvae	<i>Cotinus coggygria</i> (L)	15	ZZS
5	<i>Diaphania indica</i>	Larvae	<i>Pterocarya stenoptera</i> (L)	5	ZZS
6	<i>Locastra muscosalis</i>	Larvae	<i>Cotinus coggygria</i> (L)	23	YDM
7	<i>Omphisa plagialis</i> Wileman	Larvae	<i>Aesculus chinensis</i> (L)	5	ZZS
8	<i>Algedonia coclesalis</i>	Larvae	Bambusoideae	25	ZZS
Sphingidae					
9	<i>Clanis bilineata</i>	Larvae	<i>Ziziphus jujuba</i> (L)	5	LXM
10	<i>Phyllosphingia dissimilis</i>	Larvae	<i>Platycarya strobilacea</i> (L)	5	YDM
11	<i>Smerinthus planus</i>	Larvae	<i>Prunus armeniaca</i> (L)	4	YDM
12	<i>Psilogramma menephron</i>	Larvae	<i>Vitex negundo</i> (L)	5	YDM
13	<i>Agrius convolvuli</i>	Larvae	<i>Ipomoea batatas</i> (L)		ZZS
Geometridae					
14	<i>Culcula panterinaria</i>	Larvae	<i>Cotinus coggygria</i> (L)	5	YDM
15	<i>Percnia giraffata</i>	Larvae	<i>Diospyros kaki</i> (L)	5	YDM
16	<i>Ophthalmodes albosignaria</i>	Larvae	<i>Platycarya strobilacea</i> (L)	4	YDM
17	<i>Ophthalmitis irrorataria</i>	Larvae	Balsaminaceae	10	YDM
18	<i>Buzura suppressaria</i>	Larvae	<i>Cotinus coggygria</i> (L)	10	YDM
Noctuidae					
19	<i>Argyrogramma albostrata</i>	Larvae	<i>Abutilon theophrasti</i> (L)	11	ZFP
20	<i>Chytonix segregata</i>	Larvae	<i>Humulus japonicus</i> (L)	5	ZZS
Tortricidae					
21	<i>Archips xylosteana</i>	Larvae	<i>Grewia biloba</i> (L)	23	ZFP
22	<i>Cryptophlebia ombrodelta</i>	Larvae	<i>Pterocarya stenoptera</i> (L)	5	ZZS
Limacodidae					
23	<i>Cnidocampa flavescens</i>	Larvae	<i>Pterocarya stenoptera</i> (L)	5	ZZS
24	<i>Parasa consocia</i>	Larvae	<i>Prunus armeniaca</i> (L)	5	YDM
Notodontidae					
25	<i>Phalera flavescens</i>	Larvae	<i>Malus spectabilis</i> (L)	30	ZZS
26	<i>Uropyia meticulodina</i>	Larvae	<i>Carya cathayensis</i> (L)	5	YDM
Lymantridae					
27	<i>Porthesia piperita</i>	Larvae	<i>Glycine max</i> (L)	5	ZZS
28	<i>Stilpnotia salicis</i>	Larvae	<i>Populus tomentosa</i> (L)	5	ZZS
Saturniidae					
29	<i>Actias selene ningpoana</i>	Larvae	<i>Platycarya strobilacea</i> (L)	5	YDM
Cossidae					
30	<i>Holcocerus vicarius</i>	Larvae	<i>Salix babylonica</i> (L)	5	SQS
Papilionidae					
31	<i>Sericimus montelus</i>	Larvae	<i>Aristolochia debilis</i> (L)	11	YDM
32	<i>Papilio xuthus</i>	Larvae	<i>Zanthoxylum planispinum</i> (L)	5	YDM
Nymphalidae					
33	<i>Polygona c-aureum</i> Linnaeus	Larvae	<i>Humulus japonicus</i> (L)	5	ZZS
Coleoptera					
Cerambycidae					
34	<i>Megopis sinica</i>	Larvae	<i>Populus tomentosa</i> (W)	5	ZFP
35	<i>Trichoferus campestris</i>	Larvae	<i>Populus tomentosa</i> (W)	5	ZZS
36	<i>Batocera horsfieldi</i> Hope	Larvae	<i>Salix babylonica</i> (W)	5	SQS
37	<i>Massicus raddei</i> Blessig	Larvae	<i>Salix babylonica</i> (W)	5	SQS
Lucanidae					
38	<i>Dorcus titanus</i>	Larvae	<i>Populus alba</i> (W)	5	ZFP
Tenebrionidae					
39	<i>Tenebrio obscurus</i> Fabricius	Larvae	<i>Salix babylonica</i> (W)	5	SQS
Anobiidae					
40	<i>Lasioderma serricorne</i>	Larvae	<i>Nicotiana tabacum</i> (L)	5	ZZS
Orthoptera					
Acrididae					
41	<i>Gonista bicolor</i>	Adult	Poaceae (L, S)	5	ZZS
42	<i>Acrida cinerea</i>	Adult	Poaceae (L, S)	5	ZZS
Catantopidae					
43	<i>Fruhstorferiola viridifemorata</i>	Adult	Poaceae (L, S)	5	ZZS

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Table 1. Continued.

Number	Order: Family: Genus species (taxonomic authority)	Life-stage sampled	Food resource(s) ^a (plant tissues) ^b	No. of individuals ^c	Collecting locality ^d
44	<i>Oxya hyla intricata</i> Oedipodidae	Adult	Poaceae (L, S)	5	ZZS
45	<i>Gastrimargus marmoratus</i> Pyrgomorphidae	Adult	Poaceae (L, S)	5	YDM
46	<i>Atractomorpha sinensis</i> Conocephalidae	Adult	Poaceae (L, S)	5	ZZS
47	<i>Conocephalus chinensis</i> Tettigoniidae	Adult	Poaceae (L, S)		ZZS
48	<i>Atlantiscus sinensis</i> Gryllidae	Adult	Poaceae (L, S)	5	YDM
49	<i>Velarifictorus micado</i> Diptera	Adult	Poaceae (L, S)	5	YDM
	Tipulidae				
50	<i>Nephrotoma sinensis</i> Syrphidae	Larvae	<i>Populus alba</i> (W)	5	ZFP
51	<i>Eristalomyia tenax</i> Hymenoptera	Larvae	<i>Populus alba</i> (W)	5	ZFP
	Argidae				
52	<i>Arge pagana</i> Dermaptera	Larvae	<i>Cotinus coggygria</i> (L)	5	YDM
	Labiduridae				
53	<i>Labidura japonica</i> Blattaria	Adult	Omnivorous	5	ZFP
	Eupolyphaga				
54	<i>Polyphaga plancyi</i>	Adult	Omnivorous	5	ZFP

^aHost plant species from which sampled larvae and adult were recovered. ^bPlant tissues commonly consumed by sampled life-stage (L = leaves, S = stems, W = wood). ^cNumber of individuals for all biological replicates. ^dLocality where samples were collected (ZFP = Zhengzhou city forest park; ZZS = Zhengzhou city suburb; LXM = Luoyang city Xianglu mountain; YDM = Yuzhou city Dahongzhai mountain; SQS = Shangqiu city suburb).

Cellulase zymography

Cellulolytic activity bands were detected using the zymogram method used in a previous study (Oppert et al., 2010), with minor modifications. Ten percent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared by adding 0.1% CMC (mg/mL) before polymerization.

Commercial grade *Aspergillus niger* cellulase (MP Biomedicals, approximately 8 µg per lane) was used as a positive control. Gut extracts were thawed on ice and solubilized in 1 volume of sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1% β-mercaptoethanol, 0.01% bromophenol blue), followed by partial denaturation at 70°C for 20 min to decrease activity-band smearing due to continuous enzymatic activity during electrophoresis. Samples were briefly centrifuged to collect the evaporated solution after heating and were then loaded on the gels. Protein samples were run on 10% SDS-PAGE gels at 100 V (constant) and 4°C for approximately 4 h, or until the dye reached the bottom of the gel.

After electrophoresis, the gels were first washed in 2.5% (w/v) Triton X-100 for 1 h, washed in 50 mM acetate buffer, pH 5, for 1 h, and then incubated at 55°C for 30 min in the same buffer. After staining with 0.1% (w/v) Congo red (Acros Organics) for 15 min, the gels were further washed with 1 M NaCl until the cellulase bands became visible. Finally, the gels were immersed in 5% (v/v) acetic acid to improve band clarity. The gels were photographed, and the pictures were inverted and enhanced using the Adobe Photoshop CS2 software (v. 9.0.2) (Picart et al., 2007).

RESULTS

CMCase activity in gut fluids

When using CMC as the substrate, cellulolytic activities were detected in the gut fluids of all 54 phytophagous or xylophagous insect species (Figure 1B). Gut fluid cellulolytic activities evaluated using the CMC substrate were generally higher in Coleoptera (Cerambycidae), Orthoptera, and Blattaria (*Polyphaga planeyi*) orders. In these 3 orders, the majority of cellulolytic activities exceeded 0.3 U/mg proteins, with the highest record being 0.54 U/mg proteins. Lepidopteran (except for *Diaphania nigropunctalis*) activities against CMC in the gut fluids were noticeably lower than in those in Coleoptera or Orthoptera, at only about 0.05 U/mg proteins. In Diptera (*Nephrotoma sinensis* and *Eristalomyia tenax*), Hymenoptera (*Argemone pagana*), and Dermaptera (*Labidura japonica*) insects, CMC cellulolytic activities in the gut fluid were lower than 0.1 U/mg proteins.

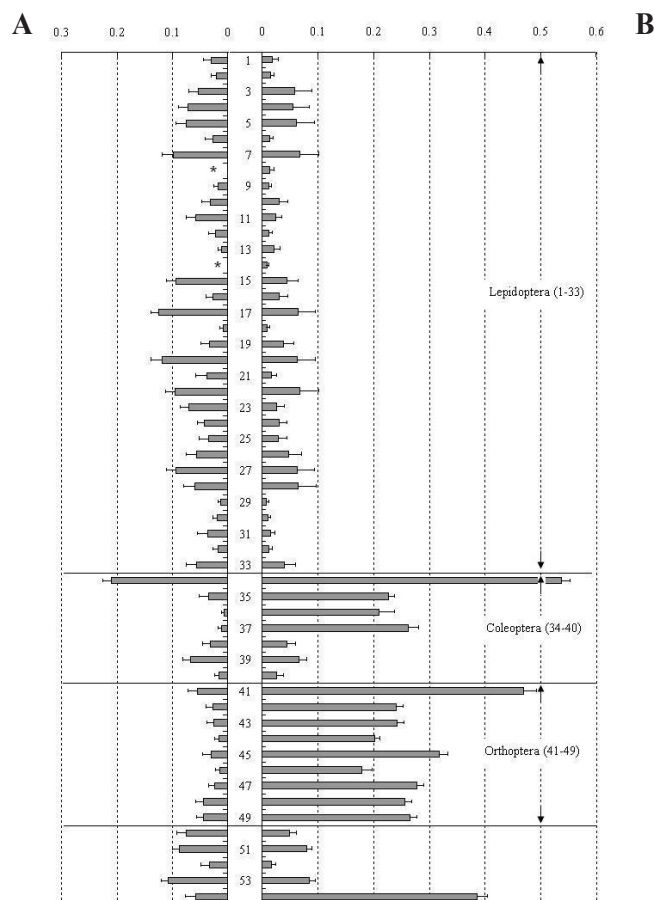


Figure 1. Average specific cellulolytic activities (U/mg protein) of gut fluids using (A) FP and (B) CMC as substrates. Numbers in the Figure correspond to Table 1. Asterisks denote missing data due to low sample size.

FPase in gut fluids

FPase activities were found in all species tested. The species with the highest gut fluid FPase activities belonged to the Coleoptera (*Megopsis sinica*, 0.21 U/mg proteins) and Lepidoptera (*Ophthalmitis irrorataria*, 0.125 U/mg proteins) (Figure 1A) orders. Orthoptera demonstrated high activity against CMC, but comparatively lower activity against FP.

Detection of cellulases by zymography

To further elucidate cellulases in diverse insect species, gut digestive fluid samples were analyzed using the zymography method, with CMC as the substrate (Figure 2). The zymography results indicated that the presence of cellulases was order-dependent (Figure 2A and B show Lepidoptera, Coleoptera, Orthoptera, and the other orders). Fewer activity bands were detected in Lepidoptera, with no more than 3 activity bands of different molecular weights spanning 70, 44, 35, 27, 23, and 17 kDa. In contrast, up to 6 activity bands were detected in Coleoptera, with molecular weights spanning 105, 70, 45, 27, 23, and 17 kDa. Up to 5 activity bands were detected in Orthoptera, with molecular weights spanning 105, 83, 70, 45, 35, 27, 20, and 17 kDa. However, no activity bands were detected in the other samples (i.e., *Calcula panterinaria*, *Phyllosphingia dissimilis*, *Smerinthus planus*, and *A. pagana*).

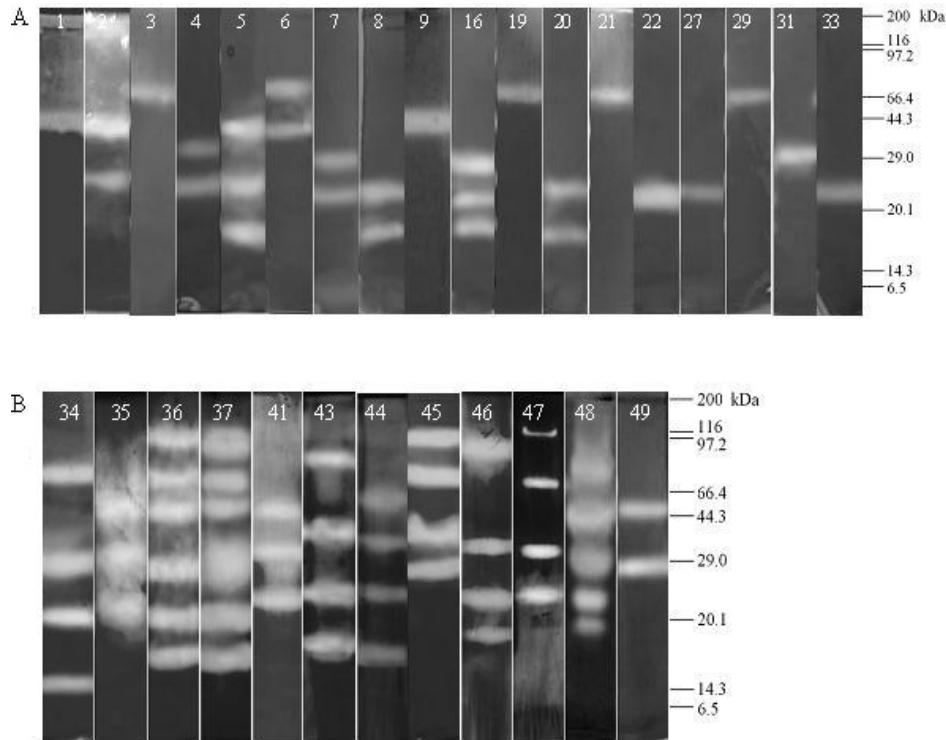


Figure 2. Detection by zymography of cellulolytic protein bands in gut fluids from diverse insect species. **A.** Lepidoptera (lanes 1-33); **B.** Coleoptera (lanes 34-37), Orthoptera (lanes 41-49). Numbers in the Figure correspond to Table 1.

DISCUSSION

Traditionally, CMC has been used to test for cellulase activity because of its high water solubility, and it could be used as a marker for EG activity. However, CMC does not replicate characteristics of native cellulose. Therefore, FP has been widely used as a cellulosic material that exhibits medium polymerization and crystallinity, from which the synergy of a variety of cellulolytic enzyme components may be detected (EG, CBH, and β -glucosidases). In the present study, the 2 differently structured substrates were used to quantitatively determine and compare enzymatic activity in the gut fluid samples of 54 insect species that belong to 7 different taxonomic orders. The results showed that the pattern of cellulolytic activity was order-dependent, which indicates that there is a phylogenetic relationship for cellulolytic enzymes (Yokoe and Yasumasu, 1964; Watanabe and Tokuda, 2001). The soluble and non-crystalline CMC was more easily degraded than FP. This difference may be explained by gut fluid activities being higher with CMC in most cases than with the FP substrate, except for individual Lepidoptera species. There were no obvious differences between orders when FP was used as the substrate, except that higher activities were found in several species of Coleoptera and Lepidoptera, which might require further study.

CMC cellulolytic activities were noticeably lower in Lepidopteran gut fluids (generally lower than 0.06 U/mg proteins) than in the gut fluids of other orders, except for *D. nigropunctalis*. These results were consistent with those of previous studies (Nakoneczny et al., 2006; Oppert et al., 2010), in which the CMC activity of 24 Lepidopterans was detected at about 0.05 U/mg proteins. However, the corresponding activities of glycolytic enzymes were higher than those of cellulase from the midgut tissue and liquid gut contents of Apollo butterfly larvae. This difference indicates that alpha-amylase, not cellulase, played the main role in carbohydrate utilization (Nakoneczny et al., 2006). In the present study, the zymography results of cellulose showed fewer activity bands, only 1 to 3, in Lepidoptera insects. This outcome indicates that Lepidopterans might primarily use starch and other carbohydrate compounds, rather than lignocellulose, as energy sources.

CMC cellulolytic activities were comparatively higher in Coleoptera than in Lepidoptera gut fluids, showing that cellulolytic activities are species-dependent. For example, the CMC activities of Cerambycidae were noticeably higher than those of the other orders. Different feeding habits possibly explain the observed differences in CMC cellulolytic activities. The highest cellulolytic activity was recorded in the Salicaceae *M. sinica* (Cerambycidae) larvae (0.53 U/mg protein/CMC, 0.21 U/mg protein/FPA). In contrast, the lowest CMC activity was recorded for *L. serricornis* larvae (Anobiidae), which feed on tobacco leaves and cereals as energy sources (0.03 U/mg protein/CMC, 0.02 U/mg protein/FPA). Previous studies have shown that CMC activities in Cerambycidae exceed 0.2 U/mg protein/CMC (Wei et al., 2006; Geib et al., 2009; Oppert et al., 2010). Wang and Chen (2008) reported high CMC cellulolytic activities in 3 lignocellulose-feeding bark beetle species (Scolytidae); this is consistent with the results of cellulolytic activity analysis in the current study, in which up to 6 activity bands were detected for Coleoptera.

CMC activity in Orthoptera insects has received limited attention. Until recently, previous studies have shown the presence of relatively higher activity against cellulase substrates in Orthoptera insects than in other insect groups (Oppert et al., 2010; Willis et al., 2010). Our experiment also confirmed these results, whereby gut fluid samples from Orthoptera (*Acrida cinerea*, *Fruhstorferiola viridifemorata*, and *Oxyai hyla intricata*) displayed higher activity

against CMC (most exceeding 0.20 U/mg protein/CMC). Furthermore, up to 5 activity bands were detected for Orthoptera by using cellulase zymography. Orthoptera insects may have higher cellulase activity because of their feeding habits. Orthoptera mainly feed on Poaceae, especially hay, which has low levels of starch and other carbohydrate compounds but higher quantities of lignocellulose.

To characterize the cellulolytic activity of insects, we analyzed cellulolytic activity and evaluated zymograms of the gut fluids of 54 insect species that belong to 7 different taxonomic orders. For most insects, gut fluid CMC activities were higher than FP activities. The cellulolytic activities were consistent with the cellulase zymography band, that is, the more activity bands an insect species had, the higher the cellulolytic activity. Higher CMC activities and more zymography bands were found in the insect gut fluids of Coleoptera (Cerambycidae), Orthoptera, and Blattaria (*P. plancyi* Bolivar) orders than in Lepidoptera. The difference in cellulolytic and CMC activities may be attributable to different feeding habits, whereby the former species may feed on lignocelluloses and the latter species may feed on starch and other carbohydrate compounds as energy sources.

Traditionally, Orthopteran species have not been the focus of cellulolytic prospecting, probably because there is much debate about the cellulolytic capacity of these insects (Davis, 1963; Evans and Payne, 1964; Clissold et al., 2004). Yet, the cellulolytic activity assays performed in the current study by using the gut fluids from Orthopteran insects revealed levels of activity similar to those of termite and beetle species. Therefore, further studies are required to determine the specific origin of enzyme activities detected for Orthopteran insect species in the present study. Ultimately, the purification, cloning, and characterization of novel cellulolytic enzymes obtained from these insect species might provide invaluable foundation for efficient and low-cost lignocellulose degradation and ethanol biofuel production.

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