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Prospecting for cellulolytic activity in insect digestive fluids

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ABSTRACT

Efficient cellulolytic enzymes are needed to degrade recalcitrant plant biomass during ethanol purification and make lignocellulosic biofuels a cost-effective alternative to fossil fuels. Despite the large number of insect species that feed on lignocellulosic material, limited availability of quantitative studies comparing cellulase activity among insect taxa constrains identification of candidate species for more targeted identification of effective cellulolytic systems. We describe quantitative determinations of the cellulolytic activity in gut or head-derived fluids from 68 phytophagous or xylophagous insect species belonging to eight different taxonomic orders. Enzymatic activity was determined for two different substrates, carboxymethyl cellulose (CMC) and microcrystalline cellulose (MCC), approximating endo-β-1,4-glucanase and complete cellulolytic activity, respectively. Highest CMC gut fluid activities were found in Dictyoptera, Coleoptera, Isoptera, and Orthoptera, while highest MCC gut fluid activities were found in Coleoptera, Hymenoptera, Lepidoptera, and Orthoptera. In most cases, gut fluid activities were greater with CMC compared to MCC substrate, except in Diptera, Hymenoptera, and Lepidoptera. In contrast, cellulolytic activity levels in most head fluids were greater on the MCC substrate. Our data suggests that a phylogenetic relationship may exist for the origin of cellulolytic enzymes in insects, and that cellulase activity levels correlate with taxonomic classification, probably reflecting differences in plant host or feeding strategies.

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1. Introduction

Cellulose is the main structural component of the primary cell wall of plants and algae, and is the most globally abundant biopolymer. Relative abundance and the sustainability of lignocellulose production have led to an increased interest in lignocellulosic biomass as feedstock for production of ethanol biofuel (Lynd et al., 1991). During biofuel production, linear chains of cellulose composed of glucose residues connected by a β-1,4 linkage are degraded to glucose, which is then fermented to produce ethanol. Even though cellulose degradation can be achieved using chemical methods, the use of combined cellulolytic enzymes has been identified as the method with the greatest potential for cost reduction through biotechnological applications (Wyman, 1999).

Complete enzymatic degradation of cellulose to glucose is only achieved by the combined action of three enzymatic activities combined in the cellulolytic complex (Clarkel, 1997): 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). More specifically, EG hydrolyze the inner β-1,4-bonds of cellulose chains, while CBH release cellobiose from the non-reducing ends of cellulose and β-glucosidases hydrolyze cellobiose or longer cellulose chains to release glucose. Even though cellulolytic enzymes have been commercialized for use in diverse industrial applications, current cellulolytic technologies to degrade lignocellulosic biomass need improvement to reduce biofuel production costs (Sun and Cheng, 2002). Thus, there is a need for the discovery and development of more efficient cellulolytic enzymes that could not only enhance the cost-efficacy of biofuel production (Wyman, 2007), but also have applications in other industrial processes.

Even though 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). Insects are attractive potential candidates in which to prospect for novel cellulolytic enzymes, due to the diverse and highly adapted phytophagous species that feed on very fibrous, lignocellulose-rich, plant tissues. 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

Abbreviations: CMC, carboxymethyl cellulose; MCC, microcrystalline cellulose; EG, endo-β-1,4-glucanase; CBH, exo-β-1,4-cellobiohydrolases; DNSA, 3,5-dinitro-salicylic acid.

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Table 1
Taxonomies, life-stages, and dietary niches of insect species screened for enzyme activity using carboxymethyl cellulose (CMC) and microcrystalline cellulose (MCC) substrates with digestive system contents extracted from insect gut or head regions.

Order: Family: Subfamily: Genus species[a] (taxonomic authority)	Life-stage(s) screened	Food resource(s)[b] (plant tissues)[c]	Tested replicates CMC, MCC (individuals)[d]	
			Gut fluid	Head fluid
Dictyoptera (suborder Blattaria)				
Cryptocercidae				
Cryptocercus [prob. *punctulatus* Scudder]	Ad/Ny	Generalist-B, C (W)	3 (3), 3 (3)	
Isoptera				
Rhinotermitidae				
Reticulitermes hageni Banks	Ny[e]	Generalist-B, C (W)	6 (120), 5 (100)	5 (166), 4 (128)
Orthoptera				
Acrididae				
Cyrtacanthacridinae				
Melanoplus differentialis (Thomas)	Ad	Generalist-B, G (S, L)	9 (9), 8 (8)	7 (8), 6 (7)
Melanoplus femurrubrum (DeGeer)	Ad	Generalist-B, G (S, L)	11 (23), 6 (11)	26 (99), 0 (0)
Schistocerca americana (Drury)	Ad	Generalist-B, G (S, L)	7 (7), 4 (4)	6 (8), 4 (5)
S. damnifica (Saussure)	Ad	Generalist-B, G (S, L)	3 (3), 3 (3)	
Gomphrocerinae				
Dicromorpha viridis (Scudder)	Ad	Generalist-G (S, L)	5 (6), 3 (4)	
Syrbula admirabilis (Uhler)	Ad	Generalist-G (S, L)	16 (30), 11 (28)	11 (28), 8 (23)
Oedepodinae				
Chortophaga viridifasciata DeGeer	Ad	Generalist-G (S, L)	14 (42), 6 (18)	13 (50), 11 (44)
Hippiscus ocelote (Saussure)	Ad	Generalist-G (S, L)	12 (N.a.), 8 (N.a.)	10 (N.a.), 4 (N.a.)
Spharagemon bolli Scudder	Ad	Generalist-G (S, L)	3 (N.a.), 0 (0)	
Gryllidae				
Gryllinae				
Gryllus [prob. *pennsylvanicus* Burmeister]	Ad	Omnivore (S, L)	4 (5), 4 (5)	
Nemobiinae				
Allonemobius [pr. *socius* (Scudder)]	Ad/Ny	Omnivore (S, L)	17 (57), 8 (17)	6 (39), 0 (0)
Allonemobius [pr. *fasciatus* (DeGeer)]	Ad/Ny	Omnivore (S, L)	11 (N.a.), 6 (16)	
Psychidae				
Thyridopteryx ephemeraeformis	Lv	Generalist-C, B (L)	4 (11), 4 (11)	
Tettigoniidae				
Conocephalinae				
Conocephalus strictus (Scudder)	Ad/Ny	Generalist-G (L)	3 (4), 0 (0)	
Orchelimum vulgare (Harris)	Ad/Ny	Generalist-B, G (L)	5 (12), 5 (12)	
Copiphorinae				
Neoconocephalus triops (L.)	Ad	Generalist-B, G (L)	4 (4), 3 (3)	
Phanopterinae				
Microcentrum retinerve (Burmeister)	Ad	Generalist-B (L)	3 (3), 0 (0)	
Scudderia [pr. *curvicauda* (DeGeer)]	Ad	Generalist-B (L)	7 (7), 6 (6)	
Scudderia furcata Brunner	Ad	Generalist-B (L)	4 (4), 3 (3)	
Coleoptera				
Buprestidae				
Chrysobothris sp.	Lv	Generalist-B (hardwoods) (W)	3 (3), 0 (0)	
Cerambycidae				
Elaphidion mucronatum (Say)	Lv	Generalist-B (ex. *Cerciscanadensis* L.) (W)	5 (14), 4 (12)	
Neoclytus a. acuminatus (Fabricius)	Lv	Generalist-B (ex. *Diospyros* sp.) (W)5 (8), 3 (5)		
cerambycid sp.	Lv	Undet. (ex. *Acer saccharinum* L.) (W)	4 (45), 3 (44)	
Curculionidae				
Graphognathus leucoloma	Ad	Generalist-B (W, R, L)	6 (14), 4 (5)	
Scolytinae				
Scolytus [prob. *rugulosus* (Müller)]	Lv	Ex. *Prunus* sp. (W)	5 (107), 5 (N.a.)	
Diprionidae				
Neodiprion lecontei	Lv	Ex. *Pinus mugho* (N)	13 (N.a.), 12 (N.a.)	
Lyctidae				
Lyctus [prob. *planicollis* Lec.]	Ad/Ny	Generalist (W)	7 (29), 6 (27)	
Scarabaeidae				
Phyllophaga sp.	Lv	Generalist-B, G (L, R)	9 (24), 7 (18)	4 (24), 3 (18)
Tenebrionidae				
Tenebrio molitor	Lv	Generalist[f] (W, R)	6 (N.a.), 12 (N.a.)	3 (21), 3 (21)
Tribolium castaneum	Ad	Generalist[f] (W, R)	3 (45), 3 (45)	3 (51), 0 (0)
Diptera				
Cecidomyiidae				
Monarthropalpus flavus (Schank)	Lv	Ex. *Buxus* sp. (L)	6 (342), 6 (342)	
Lepidoptera				
Amphisbatidae				
Psilocorsis cryptochiella (Chambers)	Lv	Fagaceae (L)	4 (13), 4 (13)	
Arctiidae				
Arctiinae				
Halysidota tessellaris (J.E. Smith)	Lv	Generalist-B (L)	3 (3), 3 (3)	
Hyphantria cunea (Drury)	Lv	Generalist-B (L)	4 (21), 3 (17)	
Crambidae				
Pyraustinae				
Saucrobotys futilalis (L.)	Lv	*Apocynum* sp. (L)	5 (20), 5 (20)	
Galacticidae				
Homadaula anisocentra Meyrick	Lv	*Albizzia* & *Gleditsia* sp. (L)	6 (30), 6 (30)	

Table 1 *(continued)*

Order: Family: Subfamily: Genus species[a] (taxonomic authority)	Life-stage(s) screened	Food resource(s)[b] (plant tissues)[c]	Tested replicates CMC, MCC (individuals)[d]	
			Gut fluid	Head fluid
Gelechiidae				
Fascista cercerisella (Chambers)	Lv	*Cercis* sp. (L)	8 (78), 8 (78)	
Hespariidae				
Pyrginae				
Epargyreus clarus (Cramer)	Lv	Fabaceae (L)	3 (3), 0 (0)	
Lasiocampidae				
Lasiocampinae				
Malacosoma americana (Fabr.)	Lv	Ex. *Prunus* sp. (L)	5 (32), 5 (32)	4 (32), 4 (32)
Megalopygidae				
Norape ovina (Sepp)	Lv	Generalist-B (L)	9 (14), 8 (12)	
Noctuidae				
Heliothinae				
Heliothis virescens (Fabricius)	Lv	Generalist-B, G (S, L)[f]	4 (7), 4 (7)	10 (77), 0 (0)
Saturniidae				
Ceratocampinae				
Anisota senatoria (J.E. Smith)	Lv	*Quercus* sp.(L)	4 (8), 0 (0)	
Anisota virginiensis Drury	Lv	*Quercus* sp. (L)	4 (4), 4 (4)	
Notodontidae				
Phalerinae				
Datana contracta Walker	Lv	*Quercus* sp. (L)	3 (10), 3 (10)	
D. integerrima (Grote and Robinson)	Lv	Juglandaceae (L)	6 (19), 5 (16)	3 (15), 3 (15)
Nymphalidae				
Danaina				
Danaus plexippus L.	Lv	Apocynaceae (L)	7 (12), 7 (12)	
Heliconiinae				
Agraulis vanillae L.	Lv	*Passiflora* sp. (L)	11 (28), 10 (25)	
Nymphalinae				
Junonia coenia Hübner	Lv	Generalist-B (S, L)	3 (3), 3 (3)	
Papillionidae				
Battus philenor L.	Lv	*Aristolochia* sp. (L)	5 (7), 5 (7)	
Pyralidae				
Galleriinae				
Omphalocera munroei Martin	Lv	*Asimina* sp. (L)	6 (11), 5 (10)	
Sesiidae				
Melittia satyriniformis	Lv	*Cucurbita* sp. (R, S)		3 (N.a.), 3 (N.a.)
Synanthedon exitiosa (Say)	Lv	*Prunus* sp. (W)	4 (11), 4 (11)	
S. scitula (Harris)	Lv	Generalist-B (W)	10 (78), 8 (70)	5 (61), 5 (61)
Sphingidae				
Macroglossinae				
Hemaris diffinis (Boisduval)	Lv	*Lonicera* sp. (L)	4 (8), 3 (7)	3 (8), 3 (8)
Tortricidae				
[poss. *Archips* sp]	Lv	Ex. *Urtica* sp. (L)	3 (4), 0 (0)	
Yponomeutidae				
Attevinae				
Atteva punctella (Cramer)	Lv	*Ailanthus* sp. (L)	3 (7), 3 (7)	
Hymenoptera				
Tenthredinidae				
Allantinae				
Allantus cinctus (L.)	Lv	*Rosa* sp. (L)	7 (33), 5 (25)	3 (38), 3 (38)
Macremphytus tarsatus (Say)	Lv	*Cornus* sp. (L)	7 (48), 6 (43)	
Nematinae				
Cladius difformis (Panzer)	Lv	*Rosa* sp. (L)	3 (15), 3 (15)	

Life-stage sampled (Ad. = adult, Lv./Ny. = larvae/nymphs).
(B = broadleaf forbs, G = grasses, C = conifers, undet. = undetermined).
[a] Several specimens were collected either as larvae in an uncharacteristic developmental stadium or for which insufficient descriptive keys could be found, thus not all individuals could be conclusively identified. When adult insects could not be reared from the host plant, genera were confirmed and tentative identifications were made [denoted by brackets]. Bracketed names represent the most probable species identities based either on preferred host plant range, insect's reported geographic distribution or local occurrence evidenced by pinned specimens preserved in the University of Tennessee's Institute of Agriculture insect collection.
[b] Plant tissues commonly consumed by sampled life-stage (L = leaves and leaf petioles, N = needles, R = roots, S = stems, W = wood).
[c] ex. = Host plant species from which sampled larvae were recovered.
[d] Number of individuals for all biological replicates. N.a. = data not available.
[e] Samples included larvae from both alate and worker castes and excluded soldiers.
[f] Collected from laboratory cultures reared on artificial diet.

efforts to quantitatively characterize cellulolytic activity in insects are very limited (Cazemier et al., 1997).

The main goal of our study was to quantify within species and compare the levels of cellulolytic activities against soluble (carboxymethylcellulose, CMC) and insoluble (microcrystalline cellulose, MCC) cellulose substrates across a wide range of insect species belonging to eight taxonomic orders. Fluids from gut, and in some cases head (including salivary and labial glands), from a total of 63 phytophagous insect species belonging to the orders Diptera, Orthoptera, Coleoptera, Hymenoptera, Lepidoptera, Dictyoptera, Dermaptera, or Isoptera were used to evaluate relative cellulase activities. Our data suggest differences in cellulolytic activity among insect orders, which may correlate with distinct plant hosts or feeding strategies.

2. Materials and methods

2.1. Insect collection and dissections

All insects, except for *Tenebrio molitor*, *Tribolium castaneum*, and *Heliothis virescens* were collected from the field in Eastern Tennessee. *T. molitor* and *T. castaneum* were obtained from laboratory cultures at the USDA Grain Marketing and Production Research Center (Manhattan, KS, USA). *H. virescens* eggs were purchased from Benzon Research (Carlisle, PA, USA). *Spodoptera* (pr. *dolichos*) egg masses were generously provided by Dr. D. Jenkins (USDA-ARS Tropical Agriculture Research Station, Mayaguez, Puerto Rico). Life stages used for sampling for each species are listed in Table 1. Insects were actively feeding on or in close proximity to plant host tissues as described in Table 1, and were dissected the same day of collection or stored at 4 °C for no more than three days until dissections could be performed. Guts and/or heads were dissected from the insects and placed in 50–500 μL molecular biology-grade water (Eppendorf). Depending on sample size, multiple guts or head samples were combined to ensure availability of sufficient material for subsequent assays. Dissected tissues were cut into small pieces, homogenized by vortexing, and centrifuged at 16,100 *g* for 3 min at room temperature. Supernatants were transferred to new centrifuge tubes and stored at −80 °C.

2.2. Determination of cellulolytic activity

Proteins in gut or head fluid samples were quantified using the Coomassie protein assay (Pierce) with BSA as standard (Bradford, 1976). Cellulase activity was quantified using a modified 3,5-dinitrosalicylic acid (DNSA) assay (Miller, 1959). Two cellulose substrates with distinct properties were used in the cellulase assays: carboxymethyl cellulose (CMC) and microcrystalline cellulose (MCC). Carboxymethyl cellulose is composed of cellulose polymers modified with an extra carboxymethyl group that renders the molecule soluble in water, while MCC is a native form of tightly packed cellulose chains linked by hydrogen bonds and is water insoluble. Although CMC has traditionally been used to test for cellulase activity mainly because its high water solubility makes it amenable in assays, it is not characteristic of the solubility of native cellulose.

Proteins in insect fluid samples (10–50 μg for CMC and 20–150 μg for MCC assays respectively) were mixed with either 2% CMC sodium salt (Sigma-Aldrich) or MCC (Acros Organics) suspended in 50 mM sodium citrate buffer, pH 6.0. Samples were incubated for 1 h (CMC) or 2 h (MCC) at 50 °C in polystyrene 96-well microplates. A modified DNSA reagent containing Rochelle salt (Miller, 1959) was added to samples to stop enzymatic activity, and color was developed at 100 °C for 15 min. Microplates were cooled at room temperature for 5 min and centrifuged at 2000 *g* for 2 min to precipitate any remaining substrate. Supernatants were transferred to new 96-well polystyrene microplates and the absorbance at 595 nm determined on a Synergy HT microplate reader (BioTek) using the KC4 software (v. 3.1). Background amounts of reducing sugars were corrected for by subtracting final from initial values of the calculated reducing sugars in the sample. One unit of cellulolytic activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar (glucose equivalents) per min at 50 °C and pH 6.0. Specific activities were reported as units per mg of protein. All specific activities represented averages from at least three independently pooled samples (i.e. biological replicates). The activity of each pooled sample was determined from three measurements (i.e. technical replicates).

2.3. Cellulase zymography

Zymograms to detect cellulolytic activity bands were performed as described elsewhere (Schwarz et al., 1987), with minor modifications. Gels were prepared by including 0.1% CMC before polymerization in the SDS-10%PAGE resolving gel mixture. Gel mixtures were heated to 30 °C while CMC was added slowly to prevent aggregation. After all CMC was dissolved, APS and TEMED were added and gels were allowed to polymerize overnight at room temperature.

Commercial grade *Aspergillus niger* cellulase (MP Biomedicals, 1 mg) was used as a positive control. Samples (40 μl) were partially denatured at 70 °C for 20 min to decrease activity band smearing. Following heating, samples were briefly centrifuged and then loaded in gels. Proteins in samples were separated by SDS-10%PAGE at a constant 100 V at 4 °C for approximately 4 h or until dye reached the bottom of the gel.

After electrophoresis, gels were washed five times at room temperature; each wash for 30 min with 50 mL of wash buffer (0.1 M sodium succinate, 10 mM DTT, pH 5.8; the last wash was at 30 °C). Remaining CMC in the gel was stained by incubation in a solution of 0.1% Congo Red for 10–15 min at room temperature. Gels were destained by washing in 50 mL of 1 M NaCl until cellulase bands became visible as clear areas where CMC had been degraded due to enzymatic activity. After destaining for 20 min, 100 μl of glacial acetic acid was added to the gel wash for improved band visualization (Waeonukul et al., 2007). Following this treatment, gels turned dark-purple in color with activity bands remaining as clear zones. Images of gels were acquired using a Versadoc 1000 Imager (Bio-Rad), and pictures were inverted and enhanced using Adobe Photoshop CS2 software (v. 9.0.2).

2.4. Statistics

Data from Dictyoptera, Dermaptera, Diptera, Isoptera, and Hymenoptera (head fluid only) were not used in statistical comparisons among orders due to the limited number of species tested from these orders. Enzyme activity data from Coleoptera, Hymenoptera (gut fluid only), Lepidoptera, and Orthoptera were factored by Order and substrate type, or by Order and tissue type, using two-way ANOVAs with the Holm–Sidak method for post-hoc pairwise multiple comparisons (overall $\alpha = 0.05$). Non-normal data were analyzed using the Kruskal–Wallis test. Comparisons between enzymatic activities from diverse species were made using one-way ANOVAs with either the Holm–Sidak (*H*-test) or Dunn's (*F*-test) method for post-hoc pairwise multiple comparisons (overall $\alpha = 0.05$; Gardiner and Gettinby, 1998). Statistical analyses were performed using SigmaPlot for Windows (v. 11.0; Systat Software). Data are presented as means +/− standard error of the mean (SEM), unless otherwise indicated.

3. Results

3.1. Cellulolytic activity in gut fluids

Structural differences of the two substrates we used to detect cellulolytic activity revealed different levels of enzymatic activity in head and gut fluid samples. Degradation of CMC functions as a proxy for EG activity, while MCC conversion to glucose approximates total cellulolytic activity (EG, CBH, and β-glucosidases). Cellulolytic activity from gut fluids on CMC and MCC substrates was detected for a total of 63 and 56 species, respectively (Fig. 1). MCC activity was determined for a lower number of species due to sample size limitations. Activity was found in all species tested, except for *Epargyreus clarus* and *Spodoptera* (pr. *dolichos*), which yielded no detectable activity against CMC. There was a high degree of variability among samples, which may be due to differences in feeding activity or asynchrony at time of dissection. The highest gut fluid activity against MCC (i.e. greater than 0.05 U per mg of protein) was observed for species among the Coleoptera, Hymenoptera, Lepidoptera and Orthoptera orders (Fig. 1A). In contrast, the highest gut fluid cellulolytic activities with the CMC substrate (i.e., greater than 0.5 U per mg of protein) were obtained for species among the Dictyoptera, Coleoptera, Isoptera, and Orthoptera orders (Fig. 1B). Lepidopteran activity against CMC in gut fluids was significantly lower than in

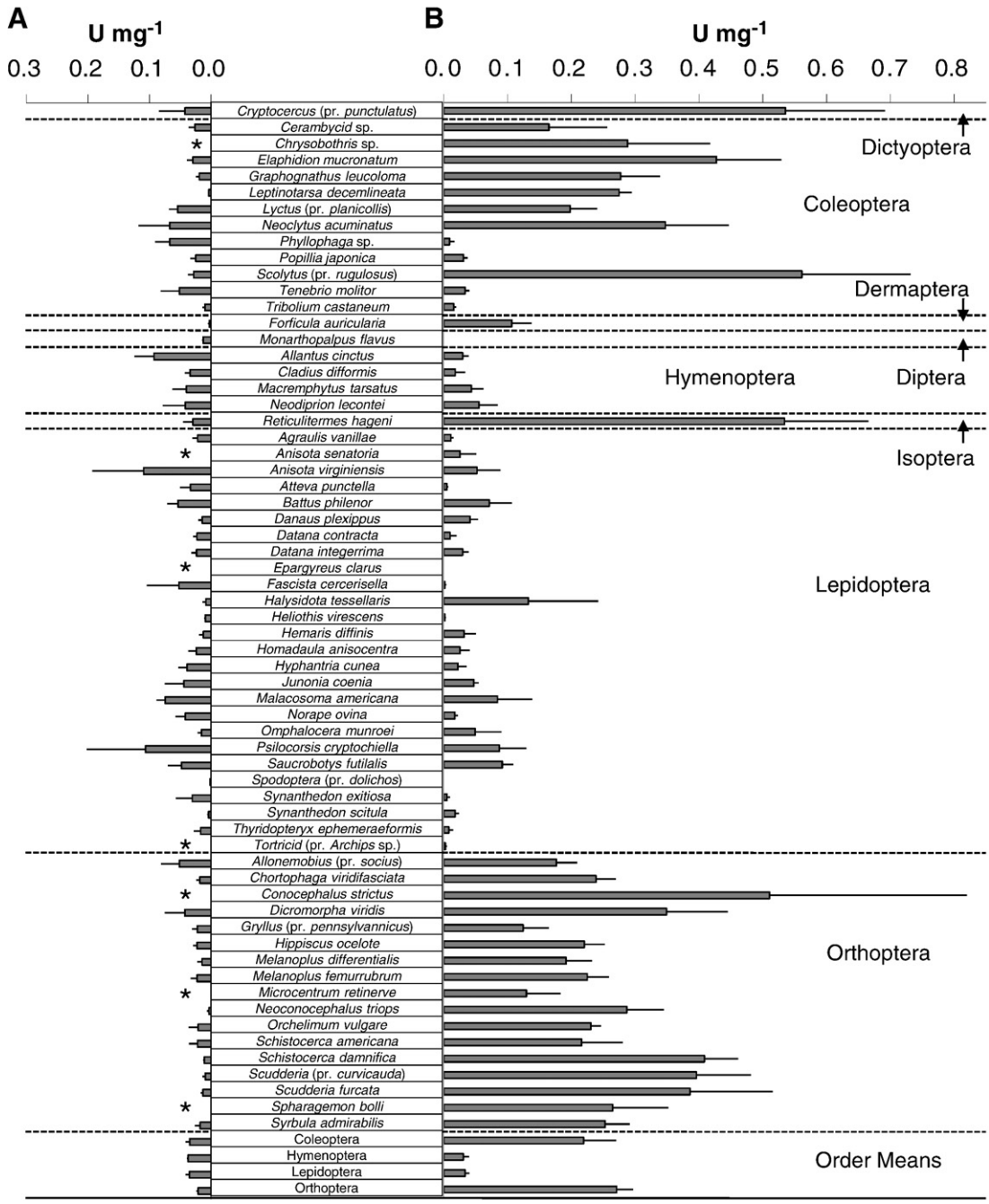

Fig. 1. Average specific cellulolytic activities (units per mg protein with standard error bars) of gut fluids using (A) MCC and (B) CMC as substrates. Asterisks denote missing data due to low sample size.

Coleoptera or Orthoptera (Fig. 1B; $F_{3,113}=21.78$, $P<0.001$). Gut fluid samples from the representative termite and cockroach samples (*Reticulitermes* hageni and *Cryptocercus* ssp. respectively) displayed high activity against CMC, but significantly lower activity against MCC (*R. hageni*, $H_1=7.53$, $P=0.004$; *Cryptocercus* sp., $F_{1,5}=9.25$, $P=0.038$). We detected a number of species with activity against both CMC and MCC comparable to samples from termites and cockroaches. *Scolytus* (pr. *rugulosus*) and *Anisota virginiensis* had the highest gut fluid activities for CMC and MCC, respectively. The only dermapteran species measured, *Forficula auricularia*, had low activity against both CMC and MCC.

3.2. Cellulolytic activity in head fluids

Only 22 (CMC) or 18 (MCC) species were measured for head fluid activity due to limitations of sample size (Fig. 2). When comparing among orders, the relative levels of cellulolytic activity for head fluids were slightly different to the relative activity level patterns observed with gut fluids. Enzymatic activities in head fluids against CMC and MCC were not significantly different among orders ($F_{5,32}=0.44$, $P=0.815$). Even though gut fluid activities in larval *Synanthedon scitula* were relatively low (Fig. 1) the highest measured activities in our study were from head fluids of this insect (Fig. 2), although this

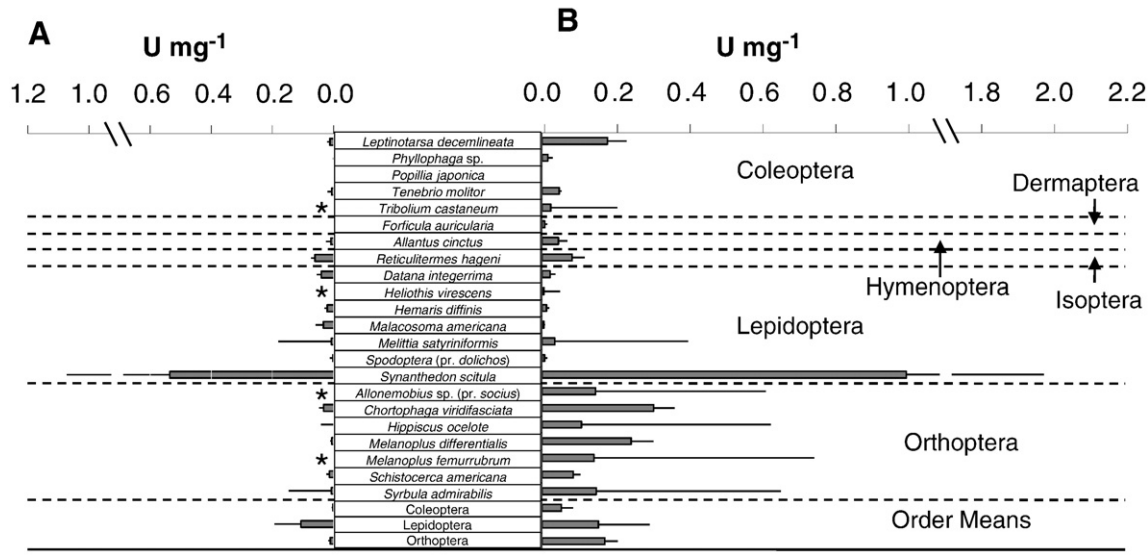

Fig. 2. Average specific cellulolytic activities (units per mg protein with standard error bars) of head fluids using (A) MCC and (B) CMC as substrates. Asterisks denote missing data due to low sample size.

level of activity was not statistically different from other head samples ($P>0.05$ for all pairwise comparisons). The highest cellulolytic activities in *R. hageni* were detected in gut fluids against CMC, which were significantly higher than head fluids on either CMC or MCC as well as gut fluids on MCC ($F_{1,16}=9.56$, $P=0.01$).

3.3. Comparison of activity against CMC and MCC in gut and head fluids

Except for the Hymenoptera and Lepidoptera orders, and some species in Coleoptera, the majority of species in each order were found to have gut fluid sample activity against CMC more than an order of magnitude higher than against MCC (Fig. 3). For example, activity against MCC was nearly 30-fold higher than against CMC in gut fluids of the lepidopteran *Fascista cercerisella*. In contrast, most species of Orthoptera displayed low MCC:CMC activity ratios, suggesting low levels of CBH and/or β-glucosidase activity. For example, activity against CMC was more than 80-fold higher than against MCC in gut fluids of *Neoconocephalus triops*.

Considering the distinct cellulolytic activities needed to degrade each cellulase substrate (Tokuda et al., 2005), the ratio of activity against MCC versus CMC may be used to indicate existence of complex cellulolytic systems. Variation in substrate activity ratios may suggest the presence of distinct cellulolytic systems in different regions of the digestive tract, which may in turn indicate different strategies for digestion of plant biomass. Therefore, ratios of substrate activity measurements were calculated based on the average enzyme activities determined within each species.

Although the ratio of MCC:CMC activity in head fluids was high for many lepidopteran species, there was no significant difference in head fluid MCC:CMC activity among orders (Fig. 3; $H_2=3.79$, $P=0.156$). In head fluids from *Malacosoma americana*, the MCC:CMC activity ratio was more than 8, the highest for a head fluid sample. This high ratio suggests the presence of high CBH activity in this head sample. In contrast, *Hippiscus ocelote* head fluids were found to have the lowest MCC:CMC ratios, with a CMC activity more than 53-fold greater than MCC activity. Cellulolytic activity against MCC for Coleoptera was significantly higher in gut compared to head fluids (Fig. 4A; $F_{1,5}=126.35$, $P<0.001$), while CMC activities were higher, but not significantly, in head versus gut fluids (Fig. 4B; $H_1=2.40$, $P=0.333$). Conversely, this trend was opposite in gut compared to head fluid activity for *R. hageni*. and *F. auricularia*. There was no significant difference in activity against CMC ($H_1=0.10$, $P=0.841$) or MCC ($H_1=4.08$, $P=0.057$) for Lepidoptera heads compared to gut fluids. Orthopterans had significantly higher gut compared to head activities against CMC ($F_{1,17}=16.93$, $P<0.001$), but not MCC ($F_{1,13}=0.52$, $P=0.485$).

3.4. Detection of cellulases by zymography

To further characterize cellulases involved in the detected cellulolytic activity in diverse insect species, we performed zymography of gut digestive fluid samples with CMC as substrate (Fig. 5). When comparing species within or between orders (in Fig. 5 lanes 1 to 5 are Orthoptera and lanes 6 and 7 are Coleoptera), clear differences in the number and size of the activity bands were observed. Interestingly, we were unable to detect protein bands when staining replica gels with Coomassie stain (data not shown), suggesting that while at low concentration in the samples, these enzymes display high levels of activity. All tested samples displayed at least one major activity band, with *Syrbula admirabilis*, *T. molitor*, and *Scolytus* (pr. *rugulosus*). containing three activity bands. Activity bands ranged from 24-kDa (lowest band in *Scolytus* sp.) to about 40-kDa (upper activity band in *Spharagemon bolli*) in mass. Activity was sensitive to freeze–thaw cycles (data not shown), and in some samples (*Scudderia* [pr. *curvicauda*], *Neodiprion lecontei*, *R. hageni*, *Scudderia* furcata, and *S. scitula*) no activity bands were detected.

4. Discussion

In this work we have quantitatively determined and compared, for the first time, enzymatic activity against water soluble and insoluble cellulase substrates in digestive fluids from insects belonging to eight orders actively feeding on host plant material. The wide diversity of insect taxa represented in our screen allows for quantitative comparisons of cellulolytic activity between insects belonging to diverse orders. Use of different cellulose substrates reveals the presence of EG activity versus more complete cellulolytic systems (including EG and CBH activities). However, other than the tissues used for extractions, our screen does not provide information on the origin of the specific enzymes involved in the detected activity.

We detected a pattern of cellulolytic activity more similar within rather than between orders. We lack genetic sequence information coding for the specific enzymes involved in these activities, therefore cannot provide direct phylogenetic analyses to explain enzyme origin or evolution. However, our data suggests that a phylogenetic relationship

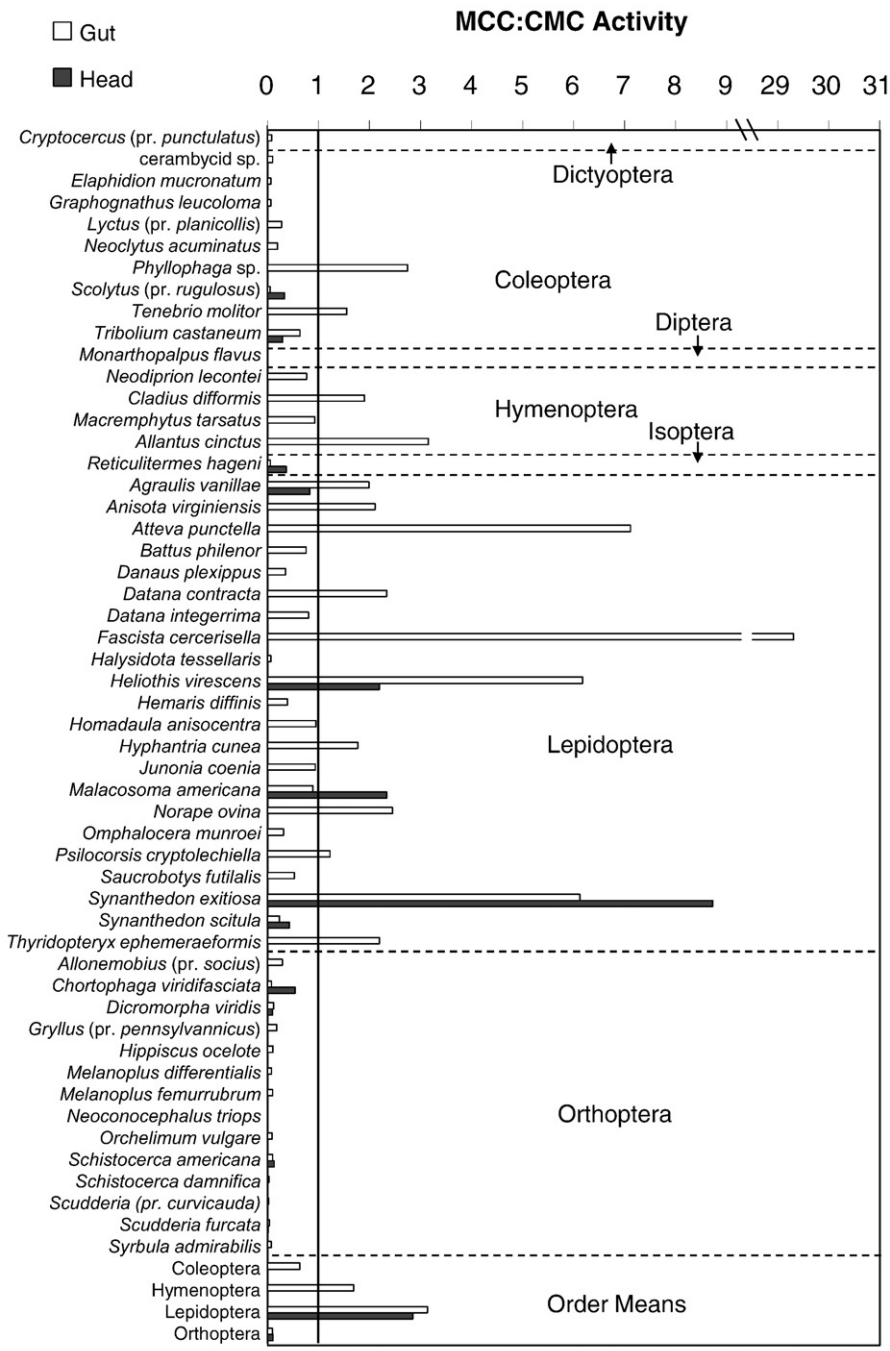

Fig. 3. Ratio of MCC to CMC activity in gut and head fluids. Note that head fluid ratios are only shown for the species without asterisks listed in Fig. 2.

may exist for development of similarly efficient endogenous (e.g. insect produced) and/or exogenous (e.g. symbiont-derived) insect cellulolytic enzymes. Previous studies have proposed that the distribution of cellulolytic activity among organisms likely follows phylogeny rather than feeding habits (Yokoe and Yasumasu, 1964; Watanabe and Tokuda, 2001). This hypothesis is further supported by evidence suggesting presence of a common ancestral endogenous cellulolytic gene in animals (Lo et al., 2003), and by the presence of endogenous cellulolytic systems in ancestral arthropod groups (Zinkler and Gotze, 1987; Treves and Martin, 1994).

Even though cellulolytic activity in insects was traditionally thought to involve symbiotic protozoa and bacteria, numerous reports have highlighted the importance of insect-produced cellulases for degradation of lignocellulosic food sources and energy production (Martin, 1983; Treves and Martin, 1994; Watanabe and Tokuda, 2001; Lo et al., 2003). While cellulases from microorganisms are important for cellulose degradation in termites (Nakashima et al., 2002; Tokuda et al., 2005; Tokuda et al., 2007; Zhou et al., 2007) and Coleoptera larvae (Kukor and Martin, 1986), there are numerous examples in lower termites (Tokuda et al., 2007; Zhou et al., 2008) and other insect groups (Lasker and Giese, 1956; Scrivener et al., 1989; Treves and Martin, 1994; Genta et al., 2006) in which endogenous insect cellulases are sufficient for effective cellulose degradation and survival on plant biomass.

When comparing cellulolytic activity type and sample origin, the highest activity levels against CMC were usually found in gut fluids. Considering that degradation of CMC is an estimation of EG activity (Tokuda et al., 2005), these data indicate that for most insect species, EG

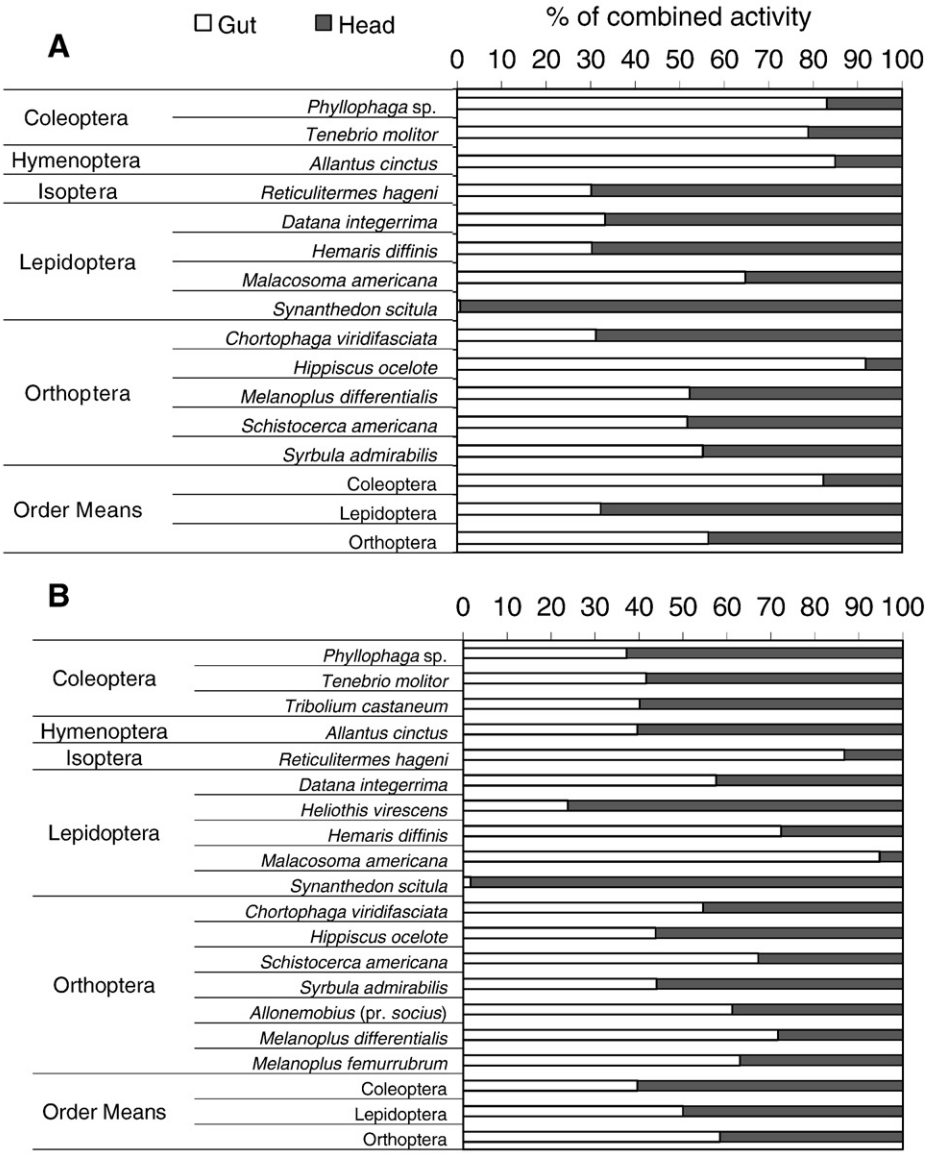

Fig. 4. Comparison of average gut and head fluid specific activities using (A) MCC and (B) CMC as substrates.

production occurs primarily in the gut. Compartmentalization of cellulose digestion by endogenous and exogenous cellulolytic enzymes in diverse regions of the digestive tract has been hypothesized to optimize cellulose digestion in termites (Tokuda et al., 1997; Nakashima et al., 2002; Tokuda et al., 2007; Zhou et al., 2007). Indeed, the availability of enzymes that degrade unprocessed forms of cellulose, such as MCC, in the most proximal regions of the digestive tract (i.e. the salivary glands) would be advantageous for efficient hydrolysis of cellulose from ingested plant material. Conversely, activity against a more readily accessible cellulose form, such as CMC, would be expected in later stages of digestion and absorption in the midgut. Plant-derived cellulose is structurally more similar to MCC than CMC, thus enzymatic attack on ingested cellulosic material would be more efficient with an initial synergistic action by a cellulolytic enzyme complex (i.e. high activity against MCC) when followed by EG hydrolysis of the more bioavailable cellulosic byproducts in the gut (i.e. higher activity against CMC). Traditionally, exogenous cellulolytic enzymes are localized to the insect hindgut, while endogenous enzymes localize to the foregut/crop, midgut and salivary glands (Martin, 1983; Nakashima et al., 2002; Tokuda et al., 2007; Zhou et al., 2007). Considering this information, our data with head fluids suggest that most insect species tested contained endogenous cellulolytic complexes. Although not true for all insect species, there are reports on production of complete (EG, CBH and β-glucosidase) endogenous cellulolytic systems in some insects (Lasker and Giese, 1956; Martin 1983; Zinkler and Gotze, 1987; Slaytor, 1992).

Within insect orders, the majority of coleopteran and orthopteran species screened in this study displayed low activity against MCC compared to CMC activities, while the opposite trend was observed for lepidopteran and hymenopteran species. Considering that degradation of CMC is an estimation of EG activity (Tokuda et al., 2005), and both EG and CBH are primarily involved in degradation of crystalline cellulose (Martin, 1983), the trends in our study suggest the existence of more complex cellulolytic systems in the tested lepidopteran and hymenopteran species.

Cellulolytic activity values determined for some species differed from previous reports. The most obvious explanation for these discrepancies is that enzyme activity can be highly dependent on feeding, pathology or insect population studied (most of our tested specimens were collected feeding on natural plant hosts in the field). Therefore, comparisons between studies should be made with caution, but can be useful for drawing conclusions about the role of cellulolytic enzymes in species not previously characterized in the literature. For example, Zhou et al. (2008), reported activity against CMC in *R. flavipes* that was approximately an order of magnitude lower than the gut fluid activity level of *R. hageni* measured in our study. Even though the

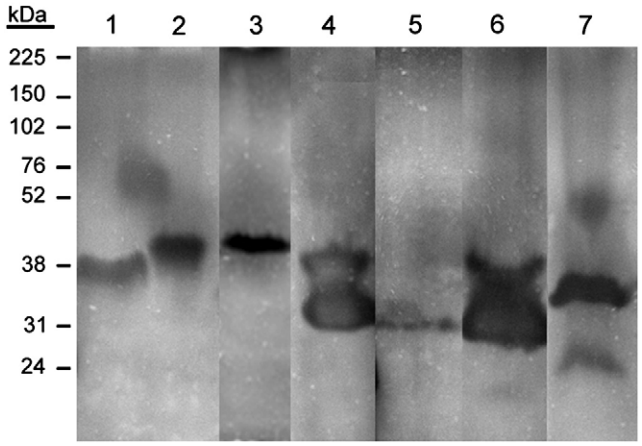

Fig. 5. Detection by zymography of cellulolytic protein bands in gut fluids from diverse insect species. Gut fluids (40 ml) were separated using SDS-10%PAGE and gels stained for CMC using 0.1% Congo Red dye. After brief de-staining with 1 M NaCl, areas where CMC has been degraded appear as clear bands. Lane 1: *Schistocerca americana*, lane 2: *Spharagemon bolli*, lane 3: *Melanoplus differentialis*, lane 4: *Syrbula admirabilis*, lane 5: *Chortophaga viridifasciata*, lane 6: *Tenebrio molitor*, lane 7: *Scolytus* (pr. *rugulosus*).

discrepancy between CMC activities may be due to interspecific differences in activity, it is also possible that the lower activity levels reported by Zhou et al. (2008) may be due to differences in the preparation of protein extracts. While we used gut fluids, Zhou et al. used protein extracted from the whole body, likely analyzing a sample with a more dilute cellulase activity. Likewise, using a different protocol from our screening method, Scrivener et al. (1997) found that activity against CMC in gut extracts from larval *Psacothea hilaris* (Coleoptera: Cerambycidae) was several orders of magnitude greater than what we determined for cerambycid larvae. Lower assay temperature (40 °C) and pH (5.5) and different protein quantification methods may have contributed to this discrepancy. Using pH conditions similar to those in our assay, Genta et al (2006) detected activity against CMC in midgut extracts from *T. molitor* larvae to be approximately three orders of magnitude lower than in our study. In this particular case, it is difficult to compare our results, in that temperature, a critical factor in enzyme assays, was not reported for the previous study. In the same study, these authors found an activity band in zymograms with CMC of about 31-kDa in size, while in our zymograms we detected at least two activity bands of 31- and 38-kDa (Fig. 5, lane 6). The additional activity band in our assays may be due to the fact that those authors used midgut epithelium rather than fluids, likely lacking the 38-kDa cellulolytic activity. In support of this hypothesis, Nakonieczny et al. (2006) found that activity against CMC in midgut fluids was approximately 3–10 times higher than midgut tissue of *Parnassius apollo*.

To obtain a preliminary characterization of the number of EGs present in samples with high activity against CMC, we performed zymograms of gut fluid samples with CMC as substrate. We detected a wide range in the number and mass of the activity bands detected, suggesting diversity of cellulolytic systems within and between insect orders. Interestingly, there was no apparent correlation between relative activity against CMC in DNSA assays and intensity of activity bands in zymograms, suggesting that some of the most active EGs may be affected by the partial heat denaturation step in our procedure or sample degradation during storage.

Future research is needed to determine the specific origin of the enzyme activities detected for insect species in the present study. Our work helps to identify insect species based on their cellulolytic capabilities for further characterization of the cellulolytic systems involved. Purification, cloning and characterization of novel cellulolytic enzymes from these insect species would enable development of technologies for cost-efficient lignocellulose degradation and ethanol biofuel production.

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