Comparative Biochemistry and Physiology, Part B xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

# Characterization of cellulolytic activity from digestive fluids of *Dissosteira carolina* (Orthoptera: Acrididae)

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ARTICLE INFO

*Article history:*
Received 6 May 2010
Received in revised form 25 June 2010
Accepted 27 June 2010
Available online xxxx

*Keywords:*
Cellulase
Grasshopper
Orthoptera
Endoglucanase
Acrididae
Digestive fluid

ABSTRACT

Previous screening of head-derived and gut fluid extracts of Carolina grasshoppers, *Dissosteira carolina* (L.) revealed relatively high activity against cellulase substrates when compared to other insect groups. In this work we report on the characterization and identification of enzymes involved in cellulolytic activity in digestive fluids of *D. carolina*. In zymograms using carboxymethylcellulose (CMC) as substrate, we detected four distinct cellulolytic protein bands in *D. carolina* gut fluids, common to all developmental stages. These cellulolytic enzymes were localized to foregut and midgut regions of the *D. carolina* digestive tract. Cellulases were purified from *D. carolina* head and gut fluid extracts by liquid chromatography to obtain N-terminal amino acid sequence tags. Database searches with sequence tags from head fluids indicated high similarity with invertebrate, bacterial and plant β1,4-endoglucanases, while no homologues were identified for the gut-derived protein. Our data demonstrate the presence of cellulolytic activity in the digestive system of *D. carolina* and suggest that cellulases of endogenous origin are present in this organism. Considering that this grasshopper species is a pest of grasses, including switchgrass that has been suggested bioethanol feedstock, characterization of insect cellulolytic systems may aid in developing applications for plant biomass biodegradation for biofuel production.



## 1. Introduction

Lignocellulose is abundant and renewable, and is therefore considered an optimal feedstock for production of ethanol biofuel as an alternative to fossil fuels (Lynd et al., 1991; Wyman, 1999). The main biopolymer component of lignocellulosic biomass is cellulose, which is degraded by the synergistic effect of three enzymatic activities: endo-β-1,4-glucanases (EG or endocellulases; EC. 3.2.1.4), exo-β-1,4-cellobiohydrolases (CBH or exocellulases; EC. 3.2.1.91), and β-glycosidases (EC. 3.2.1.21) (Clarke, 1997). Due to the recalcitrance of lignocellulose, high costs are associated with enzymatic lignocellulose degradation during ethanol biofuel production (Wyman, 1999). In turn, economic constraints have increased interest in prospecting for novel cellulolytic enzymes capable of increasing depolymerization technology efficiencies. Even though plants, bacteria, and fungi have traditionally been a focus in the search for cellulolytic enzymes, reports in the last decade on endogenous insect cellulolytic activity (Watanabe and Tokuda, 2010), have increased interest in these organisms as bioprospecting resources for discovery of novel enzymes.

Previously, enzymatic activity against cellulose substrates was 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, Phthiraptera, and Orthoptera (Watanabe and Tokuda, 2010).

Recently, we undertook a comprehensive screening for cellulase activity, discovering activity in gut and head-derived fluids from insect species belonging to eight taxonomic orders (Oppert et al., 2010). As part of this quantitative screen for cellulolytic activity, we detected high cellulase activity in fluids from the Carolina grasshopper (*Dissosteira carolina*). Even though orthopteran species in the Acrididae family are notorious generalist 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).

In this study, we report on detection and characterization of cellulolytic activity in *D. carolina*. We used carboxymethylcellulose

*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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doi:10.1016/j.cbpb.2010.06.012

(CMC) and microcrystalline cellulose (MCC) to estimate in enzyme activity assays active cellulases in head-derived and gut fluids of all developmental *D. carolina* instars. Zymogram analysis suggested that a similar complement of enzymes is responsible for cellulase activity, regardless of developmental stadium. Moreover, these enzymes are present in the foregut and midgut, but not in hindgut, of adult *D. carolina*. Purification of some of these enzymes enabled us to obtain peptide tags that were used in database searches to find putative homologues for *D. carolina* cellulases. These data represent the first general description of cellulases in Acrididae and preliminary characterization of cellulolytic systems in *D. carolina* that may have applications in degradation of lignocellulosic plant biomass for biofuel production.

## 2. Materials and methods

### 2.1. Grasshopper collection and dissections

*D. carolina* adults and nymphs were field-collected from the Ijams Mead's Quarry site near Knoxville, TN (Lat: 35.95601 N, Long: −83.873255 W). Individuals were allowed to feed *ad libitum* on wheat seedlings for 24 to 48 h in screened cages. Life stadium was determined by measuring hind femur length (Pfadt, 1994). Individuals were cooled to 4 °C before dissection to slow metabolism and provide easier handling, and dissections were performed on ice. In our dissections, salivary glands were easily collected as part of the head segment during separation of head from the prothorax. We therefore collected fluids from head and guts separately in an attempt to discriminate between enzymatic activities from the salivary glands and gut cellulases. Head or gut tissue from three individuals in the same developmental stadium were dissected and pooled into separate micro-centrifuge tubes with 100 µl of water. Gut tissue was further separated into foregut, midgut, and hindgut regions. Foregut and midgut regions were separated at the origination of the gastric caeca, and the midgut and hindgut regions were separated at the origination of the Malpighian tubules. Both head and gut samples were minced with micro-scissors, crushed in disposable pellet pestles, and homogenized by vortexing to ensure fluid extraction. Samples were cleared of debris by centrifugation at 21,000 *g* for 3 min at room temperature, then stored at −80 °C until used.

### 2.2. Detection of cellulolytic activity in D. carolina head and gut fluids

Fluids from *D. carolina* gut and head samples from instars, adults, and gut sections were analyzed using a modified dinitrosalicylic acid (DNSA) assay (Miller, 1959). Protein concentrations were first estimated using the Coomassie Protein Assay Reagent (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard. Samples were mixed with either 2% carboxymethyl cellulose sodium salt (CMC, Sigma-Aldrich, St. Louis, MO) or microcrystalline cellulose (MCC, Acros Organics, Geel, Belgium) suspended in 50 mM sodium citrate buffer (pH 6.0). Cellulolytic assays with CMC and MCC were conducted with 50 and 150 µg of protein from each sample, respectively. Due to limited availability of digestive fluids, enzymatic activity within the respective gut regions was only assayed using CMC. Samples were incubated for either 1 h (CMC) or 2 h (MCC) at 50 °C. The 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. Samples were cooled and centrifuged at 2000× *g* for 2 min to precipitate any remaining substrate. Supernatants were transferred to polystyrene microplates to measure sample absorbance at 595 nm on a Synergy HT microplate reader (BioTek) using KC4 software (v. 3.1). A correction for background amounts of reducing sugars was made by subtracting initial from final values of calculated reducing sugars within a sample. One unit of cellulolytic activity (U) is defined as the amount of enzyme producing 1 µmol of reducing sugar (glucose equivalents) per minute at 50 °C and pH 6.0. Specific activities are reported as U per gram of protein. All specific activities represent averages from triplicate measurements of at least three independent biological replicates. Statistical analysis was performed using mixed models for analysis of variance (SAS 9.2), and least square means were separated using Tukey's significant difference test ($p<0.05$). Analysis of variance (ANOVA) on ranks for multiple comparisons (Kruskal–Wallis method, overall $\alpha = 0.05$) was performed using the SigmaPlot 11.0 software package. A randomized complete block design comparing activity in different insect stadia was used with three biological and three technical replicates for each of the head and gut fluid samples.

### 2.3. Detection of cellulases by zymography

To detect proteins with cellulolytic activity in *D. carolina* fluids, we used zymograms (Schwarz et al., 1987), with minor modifications as described below. Because CMC was enzymatically degraded in gut fluids throughout juvenile development and into adulthood, we used the higher volumes of gut samples taken from *D. carolina* adults in zymograms. As cellulase substrate, 0.2% CMC was chosen instead of MCC because of higher activity in gut fluids and the feasibility of zymograms using this substrate. This substrate was added before polymerization to the resolving portion of 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. To prevent aggregation, CMC was added slowly to the gel mixture while stirring. Gel polymerization was induced after all CMC was dissolved. Gels were allowed to polymerize overnight at room temperature, then kept at 4 °C until used (<2 wk).

*D. carolina* gut or head fluid extracts were thawed on ice and solubilized in 1 volume of sample buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.01% bromophenol blue) (Laemmli, 1970). Solubilized samples (100 µg of protein) were heated at 70 °C for 20 min to partially denature enzymes and reduce smearing of activity due to continuous enzymatic activity during electrophoresis (data not shown). Commercial grade *Aspergillus niger* cellulase (MP Biomedicals) was used as a positive control (approx. 8 µg per lane). Following heating, samples were briefly centrifuged to collect evaporated solution, and loaded in duplicate on gels for subsequent detection of total protein and cellulase activity. Electrophoretic separation was carried out at 4 °C at constant voltage (100 V) for approximately 4 h, or until the sample buffer dye reached the bottom of the gel. Total protein staining was done with ProtoBlue Safe (National Diagnostics) following manufacturer's instructions. For cellulolytic activity staining, gels were washed five times (6 min each) in 50 ml of wash buffer (0.1 sodium succinate pH 5.8 plus 10 mM DTT). Gels were incubated for 30 min at 60 °C in wash buffer without DTT to develop cellulase activity by incubation in a 0.1% Congo Red (Acros Organics) solution for 10–15 min at room temperature. Gels were destained using 1 M NaCl until clear activity bands were visible. For increased visualization of activity bands, glacial acetic acid was added (2% v/v) to the NaCl solution (Waeonukul et al., 2007). Following acetic acid 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. Purification of cellulolytic enzymes

We used a two-step liquid chromatography strategy to purify cellulolytic enzymes from *D. carolina* head and gut fluids. Pooled head fluids (45 mL) from adult and nymph *D. carolina* were diluted to 50 mL with 0.01 M sodium acetate buffer pH 4.9 (buffer A). Samples were then cleared by centrifugation (5 min at 21,000 *g*) and filtered (0.22 µm) before loading on a HiLoad 16/60 Superdex 200 prep grade gel filtration column previously equilibrated with buffer A and

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connected to an AKTA FPLC system (GE Healthcare). Proteins in the sample were eluted with 240 ml of buffer A at constant flow (1 mL $min^{-1}$), with continuous collection of 2 ml fractions.

To test for presence of cellulolytic activity in the collected FPLC fractions, we used 1% agarose plates containing 0.2% CMC. An aliquot (20 µl) of each fraction was spotted on the plate and allowed to digest the CMC substrate at 30 °C for 2 h. After incubation, plates were stained with 0.1% Congo red and destained with 1 M NaCl. Cleared spots revealed fractions containing active cellulase protein. For gut fluids, cellulase activity was detected in the 65 to 120 mL elution volume, while for head-derived fluids cellulase activity was detected in the 75 to 92 mL elution volume. Fractions within the detected peak of activity were pooled and used for anion exchange chromatography with a Mono Q 5/50 GL anion exchange column equilibrated with buffer C (50 mM Bis–Tris, pH 4.8). Prior to anion exchange chromatography, pooled fractions were dialyzed (10,000 MWCO) overnight against buffer C. After loading the pooled sample in the column, proteins were eluted using a 50% gradient over five column volumes of buffer D (buffer C plus 1 M NaCl). Fractions were tested for cellulolytic activity using the CMC-agarose plate assay, as described above. Under these conditions, cellulases from gut and head-derived fluids were eluted from the column with less than 10% NaCl. Fractions containing cellulolytic activity were diluted three-fold in buffer A, then used for a second anion exchange run using a 50% gradient of buffer D, but over two column volumes. Fractions with cellulolytic activity, as determined by the CMC-agarose plate assay, were pooled and concentrated approximately 100-fold using a speed vacuum (Savant, Farmingdale, NY).

### 2.5. Enzyme sequencing and identification

Purified samples were analyzed using zymograms to test for the presence of active cellulase (SDS-8%PAGE, 0.2% CMC). Purified cellulase bands were separated by SDS-10% PAGE and electrotransferred to a polyvinylidene fluoride (PVDF) membrane overnight. After transfer, the membrane was stained for total protein using Coomassie brilliant blue, and bands corresponding to active cellulases were excised and submitted for N-terminal protein sequencing (Iowa State Protein Facility, Ames, IA). Amino acid sequences were queried to the NCBInr and Swiss-Prot databases (March 24, 2010) using BLASTp.

## 3. Results

### 3.1. Detection of cellulolytic activity during D. carolina development

In a screen for cellulolytic activity in insect digestive fluids, we detected high levels of activity against CMC in gut and head fluids collected from *D. carolina* adults (Oppert et al., 2010). We therefore were interested in testing differential levels of expression of enzymes responsible for cellulose breakdown in the course of *D. carolina* development. To accomplish this, evaluation was carried out using a DNSA assay to screen gut and head fluids from the different life stages of *D. carolina*, using CMC or MCC as cellulase substrates. Activities against CMC were about 10-fold higher and significantly different (Kruskal–Wallis ANOVA on ranks, $P<0.001$) than against MCC for both gut and head-derived samples from all stadia (Fig. 1). No significant differences (Kruskal–Wallis ANOVA on ranks) were detected when comparing activity against CMC or MCC in gut and head-derived fluids within a specific life stage.

In order to ascertain whether similar cellulolytic systems were involved in activity during development, we used zymograms with gut fluids from different instars to detect proteins having activity against CMC substrate (Fig. 1C). In these assays, we detected a similar pattern of cellulase activities in samples from different developmental stages. In particular, four main cellulase activities, corresponding to proteins of 68-, 56-, 45-, and 27-kDa in molecular mass, were observed for all samples.

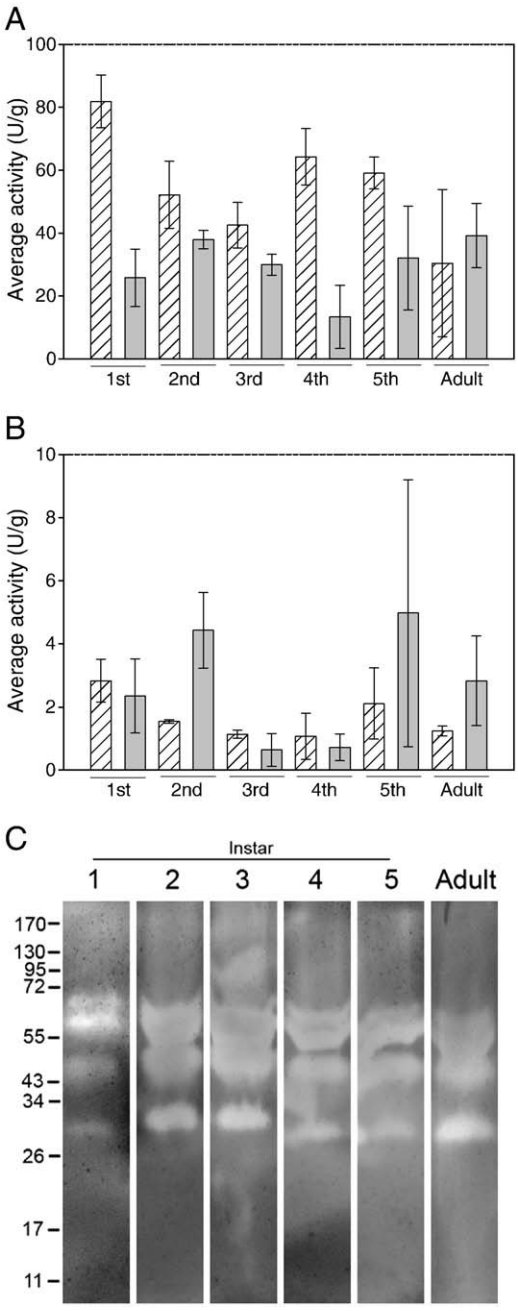


**Fig. 1.** Specific cellulolytic activity (units per mg of protein) in *Dissosteira carolina* digestive fluids across developmental stages. One unit of cellulolytic activity was defined as the amount of enzyme that produced 1 µmol of reducing sugar (glucose equivalents) per min at 50 °C and pH 6.0. Digestive fluids (50 µg of protein per sample) extracted from gut (hatched bars) and head (gray bars) samples from different developmental *D. carolina* instars and adults were used to determine activity against CMC (A) or MCC (B) as substrates. Means (±S.E.) were calculated from three biological replicates. (C) Zymograms using gut fluids from developmental stages of *D. carolina* (100 µg protein per lane). Following staining cellulase activity bands (indicated with arrows) are evident on SDS-12% PAGE gels containing 0.2% CMC in the resolving phase.

### 3.2. Activity against CMC in the D. carolina gut is mainly localized to foregut and midgut regions

In order to understand the spatial expression of cellulase activity in the digestive tract of *D. carolina* cellulolytic activity within foregut, midgut (including gastric caeca) and hindgut regions were measured using DNSA zymograms (Fig. 2A). Cellulase activity levels from the DNSA assays revealed the majority of activity against CMC was localized to fluids from foregut and midgut regions, while significantly

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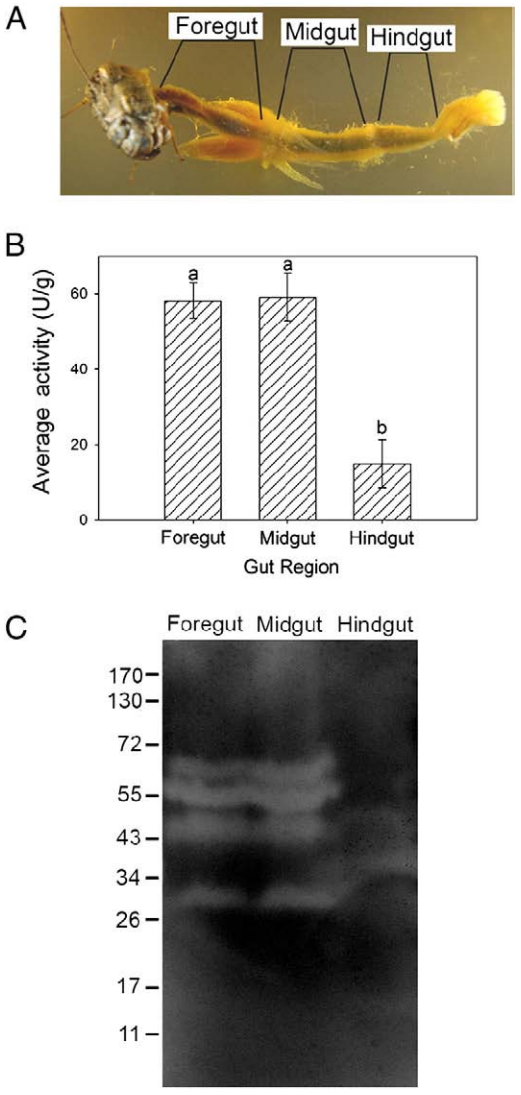


**Fig. 2.** Localization of activity against CMC in gut regions of adult *Dissosteira carolina*. (A) Dissection of adult *D. carolina* digestive system with labeled regions of interest. (B) Quantitative determination of activity against CMC using a DNSA assay as described. Proteins from fluids derived from each region of the digestive tract (foregut, midgut, and hindgut, 50 μg of protein per sample) were allowed to digest CMC for 60 minutes. Means (±S.E.) are provided from three biological replicates. Columns labeled with different letters indicate levels that are significantly different (Tukey's MRT $P<0.05$). (C) Active cellulase detection in fluids derived from the three regions of the digestive tract of *D. carolina* adults. Proteins in digestive fluids (100 μg per lane) were separated using zymograms (SDS-12% PAGE gels containing 0.2% CMC in the resolving phase). After electrophoresis cellulases were detected by staining for CMC. Clear bands were detected in areas were CMC was digested (active cellulase activity).

reduced in the hindgut region (Tukey's MRT $P<0.05$; Fig. 2B). Concurring with these data, zymograms also revealed a pattern of cellulolytic activities observed previously in fluids extracted from the foregut and midgut regions, while highly reduced activity was detected for fluids from the hindgut (Fig. 2C). The number of relative masses of cellulolytic protein activities did not differ between foregut and midgut fluids, suggesting that the same enzymes sustain cellulolytic activity in both regions.

### 3.3. Identification of *D. carolina* endocellulases

We next identified enzymes involved in the detected cellulase activity in *D. carolina* gut and head-derived fluids to provide preliminary characterization of these cellulolytic systems. To purify cellulase enzymes from complex protein mixtures from gut and head-derived fluid samples, we used a combination of size exclusion and anion exchange chromatography (Fig. 3A–B and D–E). This procedure enabled us to purify one cellulase band of approximately 45-kDa from *D. carolina* gut fluids, as revealed by silver staining and CMC zymograms (Fig. 3C). Through N-terminal sequencing of the excised band, we obtained a 15 amino acid sequence tag (DPPIARTVGSQL) for this cellulase, but no homologous proteins were found in database searches (Table 1).

When using head-derived fluids, we observed a single 45-kDa cellulase activity band in our zymograms with purified fractions (Fig. 3F). However, when staining this purified sample for total protein before cutting the bands, we observed two protein bands of 45- and 43-kDa in the region of cellulase activity. It is likely that overlapping activities explain the single activity signature observed on zymograms. Proteins corresponding to each activity were extracted from the PVDF blot and submitted to N-terminal sequencing (Table 1). In database searches, the N-terminal sequence tag obtained for the 45-kDa protein (AKYDYADAIRKSILFYQAQRS) had 84% identity to the predicted N-terminal region of a cellulase from the digestive system of the emma cricket, *T. emma* (Kim et al., 2008). A lower probability and identity (70%) match was found to a predicted cellulase from the acorn worm, *Saccoglossus kowalevskii*, and the N-terminal region of a β-1,4-endoglucanase from the fresh water snail *Biomphalaria glabrata*. From the 43-kDa purified cellulase, we obtained a sequence tag (YEYRDALCKSLLF) that matched with low probability and 76% identity to the N-terminal region of a cellulose-binding protein from *Eubacterium celulosolvens*. Lower identity was found for an endoglucanase from *Ricinus communis* (Table 1), and other plant and insect-derived cellulases (data not shown).

## 4. Discussion

We report on the detection and preliminary characterization of cellulolytic activity in digestive fluids from *D. carolina* grasshoppers. The data represents the first report identifying specific cellulases in the gut from an insect in the Acrididae Family. Acrididae contains numerous grasshopper pest species that confound management of crop commodities worldwide, including many being considered as

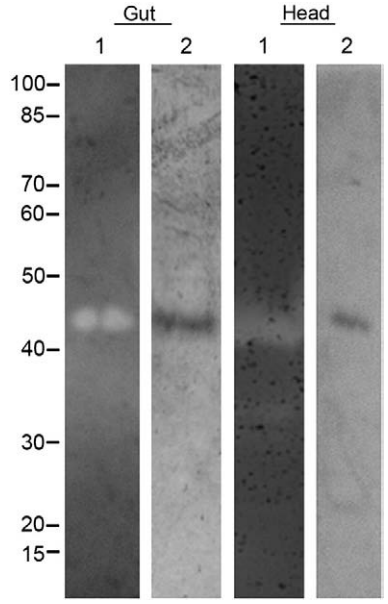


**Fig. 3.** Purification of cellulolytic enzymes from head and gut fluids of adult *Dissosteira carolina*. Cellulases were purified using size exclusion and anion exchange chromatography as described in Materials and methods. Fractions containing purified cellulases were determined using zymograms (lanes 1) and silver stained gel (lanes 2) in gut and head-derived fluids of *D. carolina* adults.

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**Table 1**
Results from database searches using N-terminal sequences from purified cellulases from gut and head-derived fluids of *D. carolina* adults. Conserved (+) and non-conserved (−) amino acid changes in respect to query proteins are shown below each matching sequence. Sequences were from *Teleogryllus emma*, (accession ABV32557), *Saccoglossus kowalevskii* (accession XP_002739252), *Biomphalaria glabrata* (accession AAT76428), *Eubacterium celulosolvens* (accession BAE20171), and *Ricinus communis* (accession XP_002512990.1).

| Fluid | Mass | Sequence match | E value | Annotation |
|---|---|---|---|---|
| Gut | 45-kDa | DPPIARTVGSQL | no significant matches | |
| Head | 45-kDa | KYDYADAIRKSILFYQAQRS<br>$^{22}$YDYADVIKKSLLFYQAQRS$^{40}$<br>- + + | 1e-6 | *Teleogryllus emma*, cellulase |
| | | $^{92}$YDYSEVIHKSI LFYEAQRS$^{110}$<br>++- - + | 2e-4 | *Saccoglossus kowalevski*, cellulase-like |
| | | $^{8}$KYNYNDVLQKSI LFYE AQRS$^{27}$<br>+ - -++ + | 4e-5 | *Biomphalaria glabrata*, β-1,4-endoglucanase |
| Head | 43-kDa | YEYRDALCKSLLF<br>$^{38}$YDYGDALSKSLLF$^{50}$<br>+ - - | 11 | *Eubacterium cellulosolvens*, cellulose-binding protein B |
| | | $^{25}$EYRE ALSKS I LF$^{36}$<br>+ - + | 15 | *Ricinus communis*, β-1,4-endoglucanase |

feedstock for lignocellulosic ethanol biofuel (Kumarasinghe, 2003; Parrish and Fike, 2009). In this regard, we advance *D. carolina* as model insect exhibiting effective cellulolytic activity and will use this model to prospect 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). Yet our cellulolytic activity assays using fluids from head and gut of *D. carolina* revealed similar levels of activity when compared with termite and beetle species (Oppert et al., 2010). As previously observed for alternative insect samples (Oppert et al., 2010), cellulolytic activity against CMC was higher than against MCC in gut and head-derived fluids, suggesting presence of active endoglucanases. Endoglucanase activity was localized mainly to fluids from foregut and midgut regions, with a similar pattern of enzymes detected in both regions of the gut. Although speculative, this observation suggests that these endoglucanases are mostly secreted in the gut and not in the salivary glands. In support of this hypothesis, starch digestion in the foregut of at least some orthopteran species is accomplished using enzymes secreted forward from the midgut (Biagio et al., 2009; Marana et al., 1997). This observation would also help explain localization of cellulolytic enzymes to the foregut and midgut regions, while greatly reduced activity was detected for the hindgut. In contrast, one of our previous studies using microarray analysis of gut-specific transcripts in *Tribolium castaneum* suggested that a gene encoding a putative cellulase was not of gut origin (Morris et al., 2009). This finding was similar to what has been reported in the honeybee, in which a cellulase gene was found in the genome and EST library of the adult honeybee head/brain and was predicted to be secreted in the hypopharangeal gland (Kunieda et al., 2006). Even though further work is needed regarding the localization of endogenous insect cellulases, these observations may suggest differences in the localization of cellulolytic enzymes between insect taxonomic groups.

All cloned endogenous insect cellulases are endoglucanases, most of them belonging to glycosyl hydrolase family nine (GHF9) (Watanabe and Tokuda, 2010). In contrast, symbiont-derived cellulases include exo- and endoglucanases usually belong to an alternative GHF. Because these enzymes are simultaneously present in the gut, the possibility of a single unified cellulolytic system has been suggested (Zhou et al., 2007). However, this model is still controversial (Nakashima et al., 2002; Tokuda et al., 2007). Previous reports demonstrated the existence of cellulase activity in gut fluids from alternative grasshopper species (Davis, 1963; Evans and Payne, 1964; Ferreira et al., 1990; Morgan, 1976), but did not identify any of the specific enzymes involved. Recently, an endoglucanase belonging to GHF9 was cloned from a whole-body cDNA library of *T. emma*, providing the first endoglucanase clone from an orthopteran species (Kim et al., 2008). We report the identification of a 45-kDa homolog to this cellulase in *D. carolina* in digestive fluids from the salivary glands and the anterior foregut. Based on this homology and the localization of these enzymes to these anterior gut regions, which in Orthoptera contain very limited microbial flora (Cazemier et al., 1997a), we conclude that this 45-kDa cellulase is an endogenous enzyme. This conclusion is further supported by the homology observed for this enzyme to endogenous insect cellulases, and absence of this enzyme from the hindgut region, which is a reservoir for symbiotic flora and enzymes (Cazemier et al., 1997a).

The high identity observed between *D. carolina* and *T. emma* endoglucanases suggests conservation of cellulolytic enzymes within taxonomic Orders. In contrast, we were unable to find significant matches for two additional cellulases of 43-kDa (from head-derived fluid extracts) or 45-kDa (from gut fluid extracts). Low probability matches to the 43-kDa protein were suggestive of bacterial and insect-derived cellulases. No significant matches were found for the putative 45-kDa cellulase. Interestingly, all the enzymes matching the sequence tags obtained from purified *D. carolina* cellulases belong to GHF9. Even though definitive classification of these enzymes would require cloning and sequencing, this observation suggests that the purified *D. carolina* enzymes probably belong to GHF9. Additional research is needed to clone these enzymes thus facilitating cellulase identification and annotation of function.

Cellulase activity reported for lepidopteran larvae have suggested increased activity with advancing larval development (Nakonieczny et al., 2006; Pant and Ramana, 1989). In contrast, we did not detect significant differences in cellulolytic activity during development of *D. carolina*. This discrepancy may reflect developmental differences between hemi- and holometabolous insects. Alternatively, diverse levels of relative cellulase activity may correlate with distinct feeding patterns during development. Interestingly, we did not find differences for expression patterns of cellulolytic enzymes during development. The ubiquitous banding pattern found via zymograms suggests that *D. carolina* displays cellulolytic activity throughout juvenile development and into adulthood, and that cellulase degradation is accomplished by a similar blend of enzymes. Although it is possible that we did not detect changes in expression of enzymes having similar molecular masses, our data suggest that developmental differences in CMCase activity are due to varying levels of multiple enzyme expression, rather than synthesis of novel cellulases. Further research will be needed to quantify individual enzyme expression levels and test this hypothesis.

Please cite this article as: Willis, J.D., et al., Characterization of cellulolytic activity from digestive fluids of *Dissosteira carolina* (Orthoptera: Acrididae), Comp. Biochem. Physiol., B (2010), doi:10.1016/j.cbpb.2010.06.012

Initial degradation of plant material by grasshoppers may be mostly accomplished through mechanical maceration via mandibular action. This process helps make lignocellulose more accessible to enzymatic degradation. Enzymatic activity for MCC degradation was low in fluids for all developmental stages tested. It is possible that these low activity levels are in response to low amounts of crystalline cellulose in the diet (Cazemier et al., 1997b). Alternatively, complete cellulose digestion to glucose in the absence of exocellulases has been reported for some insect species (Schultz et al., 1986; Scrivener et al., 1989; Zhang et al., 2009, 2010), suggesting that at least in some cases endoglucanase activity may suffice. This observation may explain why while plant cell wall degradation has been reported as inefficient in grasshoppers (Clissold et al., 2004), assimilation rates for ingested plant cellulosic material range between 27% and 34% (Barbehenn et al., 2004; Smalley, 1960), which can be considered as very high compared to other herbivorous insect (Sinsabaugh et al., 1985) and arthropod species (Schoenberg et al., 1984).

Orthopteran species are generalist 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 head and gut tissues of *D. carolina* and that these enzymes participate in cellulose degradation throughout development. Previous reports demonstrate 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 expect that future characterization 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).

## Acknowledgements

We would like to thank Park Manager Ed Yost and the staff at Ijams Nature Center for their permission to collect from the *D. carolina* populations at the Mead's Quarry site. This research was supported by a grant from the Southeastern Sun Grant Center to JLJF and BO. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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