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Endogenous cellulase enzymes in the stick insect (Phasmatodea) gut

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ABSTRACT

High cellulase (endo-beta-1,4-glucanase) activity was detected in the anterior midgut of the walking stick (Phasmatodea) *Eurycantha calcarata*. The enzyme was isolated and analyzed via mass spectrometry. RT-PCR revealed two endoglucanase genes, *EcEG1* and *EcEG2*. Mascot analysis of the purified enzyme confirms it to be the product of gene *EcEG1*. Homologous cDNAs were also isolated from a distantly related species, *Entoria okinawaensis*, suggesting a general distribution of cellulase genes in phasmids. Phasmid cellulases showed high homology to endogenously-produced glycoside hydrolase family 9 (GH9) endoglucanases from insects, especially to those of termites, cockroaches, and crickets. The purified *E. calcarata* enzyme showed clear antigency against an anti-serum for termite GH9 cellulase, which, together with the sequence homology, further suggests an endogenous origin of the enzyme. This discovery suggests a possible nutritive value for cellulose in the leaf-feeding phasmids, unlike in herbivorous Lepidoptera.

1. Introduction

Cellulolytic enzymes are well-studied in wood-feeding insects, which depend on enzymes produced endogenously or by symbiotic microbes to breakdown the lignocellulose they feed upon into assimilative sugars or nutrients (Watanabe and Tokuda, 2010). The role of cellulases in monophagous leaf-feeding (phyllovorous) insects has been downplayed, however. Phyllovore nutrient economy was mostly studied in Lepidopteran species as models for other leaf-feeding insects. The nutritive value of cellulase for leaf-feeders had been counted as near zero (Bayer et al., 1998; Friend, 1958; Schroeder, 1986) because of its indigestibility in most animals, the theory that Lepidoptera are nitrogen limited, lack of cellulase and associated genes in Lepidopteran species (http://butterflybase.ice.mpg.de/), and because leaves are one of the least lignified plant structures (Jung and Allen, 1995), especially compared to wood. Today, the role and presence of cellulases in metazoans is being re-evaluated as such enzymes, in particular the glycoside hydrolase family 9 (GH9) endoglucanases, are found in more and more clades of life (Davison and Blaxter, 2005). Recent findings of GH9 cellulases in facultatively leaf-feeding grasshoppers (Ademolua and Idowu, 2011) and GH45 cellulases and GH 11 xylanases in phyllovorous beetles (Kirsch et al., 2012; Pauchet and Heckel, 2013; Pauchet et al., 2010) suggest the role of cellulases for other herbivorous insects needs to be re-evaluated. Even Lepidoptera may not be cellulase-free, as their larval midgut tissues express large amounts of beta-1,3-glucanase: a bacterial lipopolysaccharide recognition protein which, while not a recognized cellulase, may function as a digestion protein (Pauchet et al., 2009).

The three main classes of lignocellulolytic enzymes are endo-beta-1,4-glucanases (EGs; Enzyme Commission: 3.2.1.4), beta-glucosidases (BGLs; EC: 3.2.1.21), and exocellobiohydrolases or exocellulases (CBHs; EC: 3.2.1.91) (Watanabe and Tokuda, 2010). CBHs hydrolyze cellobiose molecules from the terminal ends of cellulose chains and are most common in bacteria and fungi. CBHs of the GH7 family are conspicuous enzymes in the symbiotic protists of certain termites and in asymbiotic marine isopods (King et al., 2010; Watanabe and Tokuda, 2010). EGs randomly hydrolyze cellulose chains and the BGLs convert the resulting cello-oligomers like cellobiose and cellotriose into glucose, meaning both are needed to fully digest cellulose polymers into simple sugars. EG activity alone, however, can mediate limited digestion of cellulose on its surface and amorphous regions. Its activity is usually detected on carboxymethylcellulose (CMC) because of the latter's high sensitivity to EG activity, high solubility in water, and access denial against CBHs (Lo et al., 2000). Beta-glucosidases are ubiquitous endogenous enzymes in insects. They are not solely involved in cellulose digestion in many cases but can catalyze digestion of many other linkages (Watanabe and Tokuda, 2010).

No one yet has looked at the enzymes of walking sticks (Phasmatodea); a distinctive order due to their voracious,

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monophagous leaf-feeding habit (Graham, 1937), expected rapid assimilation and development, characteristically straight gut (Cameron, 1912), and unique hemolymph chemistry (Wigglesworth, 1972). Phasmids are also the only insect order composed entirely of obligate herbivores (Calderón-Cortés et al., 2012). These factors suggest a unique digestive metabolism compared to their closest evolutionary relatives among the Polyneoptera, thought to be either the omnivorous Orthoptera (Flook and Rowell, 1998), carnivorous Notoptera (Arillo and Engel, 2006), or the herbivorous/detritivorous Embioptera (Terry and Whiting, 2005). Comparative analysis of cellulase enzymes [if present] in these orders could help resolve the current polytomy in that branch of the insect phylogeny (Gullan and Cranston, 2010). Phasmids are also relatively large and easy to rear (Brock, 2003), plus several species are parthenogenetic, which increases their suitability for genetic modeling research (Tuccini et al., 1996).

The Phasmatodea midgut, though of a uniform diameter, is differentiated into a muscular and pleated anterior section, a posterior section with the enigmatic appendices of the midgut (de Sinéty, 1901; Ramsay, 1955), and an undifferentiated space in between (Fig. 1). The function of the appendices – long filaments attached to the midgut via pyriform ampules – is unknown, though they have been hypothesized to either be secretory or excretory. The surface area of the anterior midgut lumen is increased by its pleating, which might slow down the speed of passage of food debris. This would increase digestibility as cellulose digestion is a relatively time consuming process due to its insolubility and tight structure (Mason, 1994; Silk, 1989).

For this study, we chose to examine EG's due to their importance in primary breakdown of cellulose in animals, and Phasmatodea as they are obligate leaf-eaters from whom no cellulases have ever been recovered. Their phylogenetic placement (Davison and Blaxter, 2005) and the lack of microbial symbionts in their midgut (Shelomi et al., 2013) suggests phasmids produce endogenous GH9 EGs. We hypothesized that cellulase activity would be highest in the anterior midgut and lower in the posterior, suggesting polysaccharide breakdown occurs in the anterior midgut and glucose absorption in the posterior midgut. We focused on the giant new guinea walking stick, *Eurycantha calcarata* (Phasmatidae: Eurycanthinae), for proteomic analysis due to its large size providing more tissue for analysis per insect and facilitating volumetric analysis of the digestive tract. Genetic analysis was also performed on a distantly related, common Japanese walking stick *Entoria okinawaensis* (Phasmatidae: Clitumninae) to explore the distribution of orthologous cellulase genes in Phasmatodea.

2. Methods

2.1. Animals

E. calcarata adults were lab-reared at the Bohart Museum of Entomology (Davis, CA, USA) at room temperature and fed *Quercus* sp. leaves. Only males were used. Insects were preserved in three changes of acetone in a 20:1 ratio to insect volume (Fukatsu, 1999), then stored at −20 °C until transport and analysis. For positive controls, salivary glands were dissected from a laboratory colony of *Reticulitermes speratus* (Blattodea: Rhinotermitidae). The insects were dissected and their fore- and midguts were retained for proteomics analysis. Gut volumes of walking sticks and termites were measured as per Fujita et al. (2010). Midguts were divided into even thirds to analyze the different sections separately. Genetic analysis was performed on salivary glands from *E. calcarata* and from adults of fresh *E. okinawaensis*, lab-reared on *Rubus* sp. at the National Institute of Agrobiological Sciences (Tsukuba, Ibaraki, Japan).

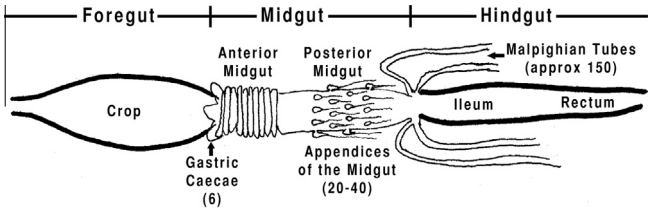

Fig. 1. Generalized anatomy of the Phasmatodea digestive tract.

2.2. Protein extraction

Fresh or rehydrated (for acetone-preserved specimens) fore- and midguts with contents were homogenized on ice in 50 mM sodium acetate buffer (pH 5.5) with a single proteinase inhibitor cocktail tablet (Complete Mini, EDTA-free, Rosche Diagnosis GmbH, Nannheim, Germany) and 1% Triton X-100, then centrifuged at 20,000×*g* for 10 min. Samples that were not immediately used were stored in a 50 mM sodium acetate, 1 M NaCl, and 20% glycerine buffer solution and frozen. For hydrophobic interaction chromatography, the supernatant of the homogenate was precipitated with four volume of cold acetone and pelleted by centrifugation at 10,000×*g* for 10 min. The pellet was dried and rehydrated with 1 M ammonium sulfate with 20 mM Tris–HCl buffer (pH 7.6) (loading buffer), then applied to a HiTrap Phenyl FF (high sub) column (GE Healthcare Life Sciences®) equilibrated with loading buffer.

2.3. Testing for and quantifying cellulolytic activity

Ten microliters of diluted sample (1/100) were mixed with 100 μL of 1% CMC (Aldrich Chemical Company) in 100 mM sodium acetate buffer (pH 5.5), vortexed, and incubated at 37 °C for 13 minutes. To stop the reaction, 0.8 mL of tetrazolium blue (TZB) reagent was added and the mixture was boiled for 5 min (Jue and Lipke, 1985). Controls without enzyme, without substrate, and of just MilliQ water and 0.5 mM glucose were used (Calderón-Cortés et al., 2010). Absorbance at 660 nm was measured using a Pharmacia Biotec® Ultrospec 2000 spectrophotometer and reducing sugar concentration was calculated by comparison with the glucose solution. EG activities of the termite midguts were calculated from the body weights of workers and previously reported values (Tokuda et al., 2004, 2005).

2.4. Protein isolation though column chromatography

For EG purification, a HiTrap Phenyl FF high-sub column was employed. After the protein-loaded column was washed with 10 mL of loading buffer, proteins absorbed on the column were eluted by stepwise concentrations of ammonium sulfate (0.35 M for 20 mL and 0 M for 24 mL) in 20 mM Tris–HCl buffer (pH 7.5). Chromatography was conducted with a BioLogic DuoFlow™ chromatography system (Bio-Rad®).

2.5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Once cellulase activity was isolated into a single, fractionated sample, an aliquot was applied to pre-casted polyacrylamide gels (AnyKD, Bio-Rad) as per Laemmli (1970), and electrophoresis was conducted with running buffer (25 mM Tris, 192 mM glycine with 0.1% SDS) at constant voltage (200 V) for 30 min. Gels were stained with Oriole™ Fluorescent Gel Stain (Bio-Rad®) and viewed under UV light to determine the presence of protein bands. APRO© SP-2110 Broad Range ladder (APRO Life Science Institute Inc.,

Naruto city, Tokushima, Japan) was used to estimate molecular weight.

2.6. Western blotting

Proteins on SDS–PAGE gels were transferred to a Sequi-Blot™ polyvinylidene fluoride (PVDF) membrane (Bio-Rad) using a semi-dry transfer cell as per Hirano and Watanabe (1990). The transblotted membrane was blocked with Blocking One solution (Nacalai Tesque Inc., Kyoto, Japan) and applied with anti-A18 mutant endogenous termite cellulase rabbit serum (Tokuda et al., 2012) diluted 1:1000 in Solution 1 of the Can Get Signal® immunoreaction enhancer solution kit (Toyobo Co., Ltd, Osaka, Japan). Following washing with TPBS (phosphate-buffered saline with 0.01% Tween 20), the membrane was applied with goat anti-rabbit IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology Inc, CA, USA) followed by TPBS washing. Applications of blocking solution and both antisera were conducted with a Snap i.d.™ protein detection system (EMD Millipore, Billerica, MA, USA). Before vacuum-application of the antiserum solutions onto a PVDF membrane, the solution was incubated with agitation on the membrane for 10 min at room temperature. Finally, presence of antigen on the membrane was visualized by incubation with 3,3′,5,5′-tetramethylbenzidine solution (T0565, Sigma). APRO SP-2110 Broad Range protein ladder was used as a negative control for the primary and secondary anti-sera. For a positive control for the second anti-serum, a protein ladder with IgG binding sites (MagicMark™ XP Western Protein Standard, Life Technologies) was employed.

3. Results

The volumes of the foregut and the midgut of a typical, full-grown, male *E. calcarata* averaged 3.0 and 777 mL, respectively. EG concentration of the foregut and the first, second, and third sections of the midgut were 4.1, 20.9, 2.0, and 0 (not detected) units/mL, respectively (Fig. 2). A unit is defined as the amount of enzyme that produces one μmole of reducing sugar per minute from CMC in the TZB method (Calderón-Cortés et al., 2010). These findings support the hypothesis that digestive activity is concentrated in the anterior midgut, whose pleating and folding might slow down passage of food debris to increase digestibility. The role of the appendices of the posterior midgut is likely not enzyme production, as cellulase activity stops after the anterior midgut. The phasmid cellulase concentrations were relatively low compared with the exceptionally high midgut concentrations (>1000 units/mL) found in some termite species (Tokuda et al., 2004, 2005), but are still significant.

An EG protein was isolated by hydrophobicity interaction chromatography using a HiTrap Phenyl FF (low sub) column. The protein was eluted as three separate peaks at different concentrations of ammonium sulfate (1, 0.35, and 0 M), but only the latter peak showed EG activity (Fig. 3). The recovered fraction

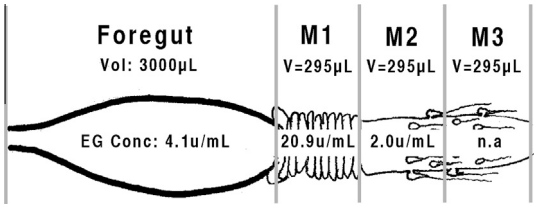

Fig. 2. Volume and endoglucanase (EG) concentration of the *Eurycantha calcarata* fore- and midgut. The midgut is divided into thirds. Endoglucanase concentration given in units per mL volume (see text for definition of a unit).

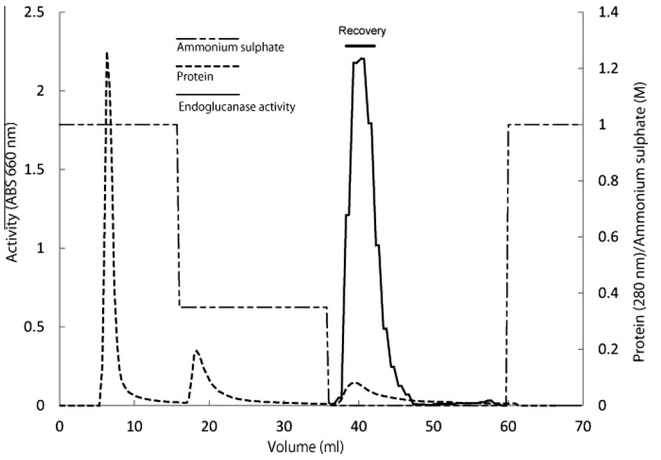

Fig. 3. Purification of endoglucanase from *Eurycantha calcarata* gut extract by HiTrap Phenyl FF (low sub) hydrophobic chromatography. Endoglucanase proteins were eluted early in the process. Endogucanase activity of each fraction was estimated as the amount of reducing sugar (absorbance at 660 nm) measured by tetrazolium blue reagent (TZB). Details of measurement are described in Section 2.3.

produced two bands on SDS–PAGE gel (Fig. 4), although a subtle difference between the peaks of protein recovery and EG activity and the asymmetrical form of the third protein peak suggested impurity of the recovery (Fig. 3). Both bands reacted to the anti-A18 mutant endogenous termite cellulase rabbit serum (Fig. 4, left). Thus the two proteins were likely differently-processed mature forms of the same gene products or isoforms, so we chose the stronger band indicating greater protein abundance (Fig. 4, arrowed) for LC/MS/MS analysis. Total purification and recovery from the homogenate were 44× and 71%, respectively (Table 1). The

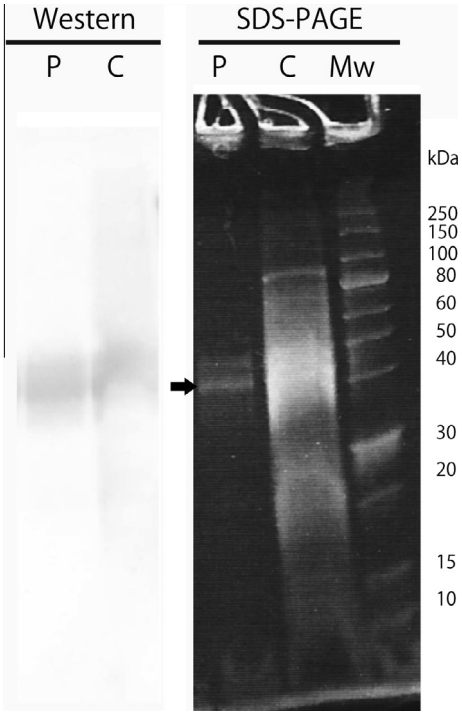

Fig. 4. SDS–PAGE and Western blotting of *Eurycantha calcarata* endoglucanase. Details of experimental conditions are described in the text. Lanes are "P": purified endoglucanase, "C": crude midgut extract (resolution of acetone precipitation), and "Mw": molecular weight markers. The arrow in the figure indicates the protein band used for the mass analysis.

Table 1
Purification of endo-beta-1,4-glucanase from the gut of *Eurycantha calcarata*.

Step	Volume (ml)	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Purification fold	Recovery (%)
Homogenate	20	61.3	142	2.3	1	100
Dissolved acetone pellet	18	20.5	109	5.3	2.3	76
Recovery from HiTrap Phenyl FF (high sub)	54	1.90	194	102	44	71

Acetone precipitation was conducted by addition of 4 times volume of cold acetone (−20 °C) to the homogenate and pellet was recovered by centrifugation (20,000×*g*, 10 min). Hydrophobic chromatography was conducted for each 1/18 of the total samples. The dried-up acetone pellet (1/18) was dissolved in 1 ml of 1 M ammonium sulfate solution with 20 mM phosphate buffer (pH 7.4) and applied to the column (HiTrap Phenyl FF (high sub), GE Healthcare). Endo-beta-1,4-glucanase activity was eluted at zero M of ammonium sulfate (by 20 mM phosphate buffer, pH 7.4).

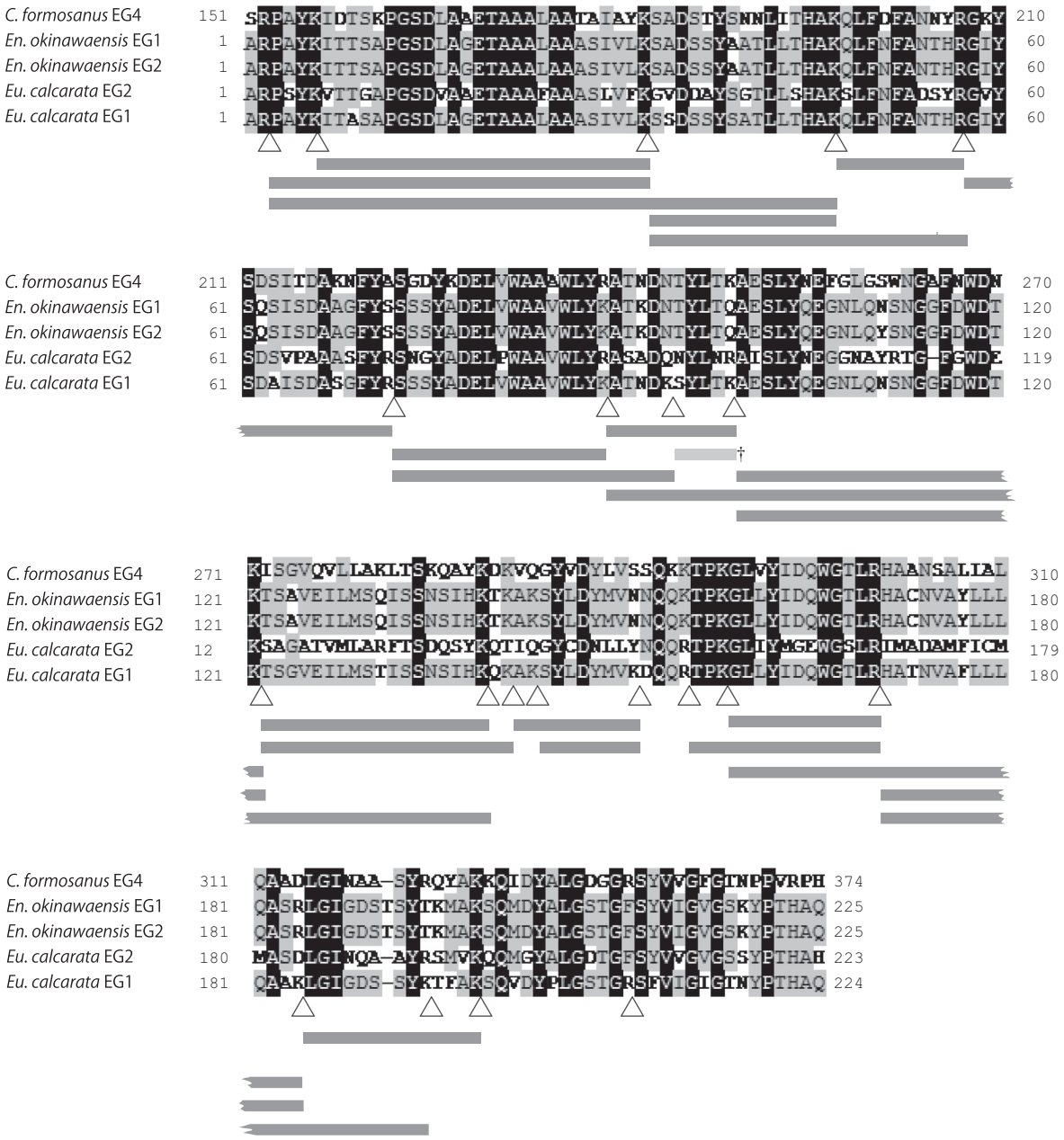

Fig. 5. Comparison of amino-acid sequences of endoglucanases of walking sticks and termites. The sequences are of an endoglucanase from the termite *Coptotermes formosanus* (CfEG4, GenBank BAB40697), the *Entoria okinawaensis* EoEG1 and EoEG2 (GenBank AB750683.1 and AB750684.1), and the *Eurycantha calcarata* EcEG1 and EcEG2 (GenBank AB750682 and AB780366). Gray bars indicate predicted trypsin fragments from the deduced amino-acid sequence of *E. calcarata* EG1, which show consistency (100% probability) or strong similarity (one residue (†) >89% probability) with the molecular weights of trypsin fragments of the purified endoglucanase from the gut extract of *E. calcarata* from the Mascot search.

antigency to the anti-A18 mutant termite endoglucanase serum (Fig. 4) suggested an endogenous origin of the isolated enzymes (Tokuda et al., 2012). The primary and secondary anti-serums did not react to the molecular weight ladders (negative controls), and the secondary anti-serum reacted to the protein ladder with IgG binding sites (positive control).

RT-PCR identified two partial cDNAs for EG enzymes from each phasmid species. From *E. calcarata* we found *EcEG1* (672 bp encoding 224 amino acids) and *EcEG2* (669 bp encoding 223 amino-acids). From *E. okinawaensis* we found *EoEG1* and *EoEG2* (both 675 bp encoding 225 amino-acids) (GenBank accession no's: AB750682, AB780366, AB750683, AB750684, respectively). These gene sequences showed moderately high similarities (67–75%) to known endogenously-produced insect cellulases from the GenBank nucleotide database (Benson et al., 2012): mainly those of termites (*Mastotermes darwinensis*, *Coptotermes formosanus*, *Carpobrotus acinaciformis*, *Nasutitermes walkeri*, *Reticulitermes flavipes*), the American cockroach (*Periplaneta americana*), and several crickets (*Gryllus bimaculatus*, *Teleogryllus emma*). The *E. calcarata EcEG2* sequence also matched those of cellulolytic microbes (Ex. *Cellulomonas fimi*), but the percent query matched was lower for this sequence.

Mascot analysis demonstrated that the molecular weights of trypsin fragments from the purified EG enzyme (cut off at carboxyl-side peptide linkages of Lys and Arg residues) were identical or quite similar († in Fig. 5 with >89% probability) to the twenty-four predicted trypsin residues from translated *EcEG1* (85% coverage) (Fig. 5). This confirmed that the purified enzyme was the product of *EcEG1*.

4. Discussion

This paper marks the first sequencing of cellulase genes from the Phasmatodea. Specifically, we found four genes from two phasmid species for endogenously-produced beta-1,4-endoglucanases of the GH9 family. The EG we isolated can digest the amorphous region on the surface of native-form cellulose molecules. The products of that reaction could be broken down to simple sugars via beta-glucosidases, which are ubiquitous enzymes in insects (Watanabe and Tokuda, 2010). We do not suggest here that phasmids are necessarily digesting the bulk of the cellulose they ingest like wood-feeding termites, but even incomplete cellulose breakdown has digestive value, allowing the dissolution of plant cell walls and enabling access to more digestible nutrients within. Even if phasmid cellulolytic activity is limited to the surface or non-crystalline region of plant cellulose, it may be crucial during periods of famine or drought (Evans and Payne, 1964). The presence of other endoglucanase genes, beta-glucosidases, and other plant cell wall degrading enzymes such as pectinases in the phasmids is likely. Clearly, phasmid carbohydrate digestion is not like that of Lepidopteran larvae, with these findings launching a new field of inquiry into phasmid metabolism with possible benefits for management of phasmids as crop and forestry pests (Graham, 1937; Jurskis and Turner, 2002; Kasenene, 1998). Our discovery of cellulase production and accumulation in the digestive tracts of walking sticks as an exemplar of exclusively phyllovorous insects demonstrates the need to reassess the nutrient value of cellulose for leaf-feeders.

The homology of EGs of walking sticks to the endogenous EGs from termites and cockroaches suggests that phasmids produce their own EG's, without the need for microbial symbionts. Non-microbial cellulases are expected in insects with large fore- and midguts and small hindguts like phasmids, whereas insects dependent on microbial cellulases tend to have enlarged hindgut paunches as bacterial fermentation chambers (Watanabe and Tokuda, 2010). Endogenous enzyme production also correlates with the lack of microbial symbionts in phasmids (Shelomi et al., 2013). Cellulases in phasmids are produced in the anterior midgut, whose pleating and infolding function to increase surface area and slow down transit of food through the gut, facilitating cellulose digestion. The role of the appendices of the midgut remains unknown, but production of cellulases can be crossed off the list of hypotheses for their putative function.

The similarities between cellulase genes among no less than three insect orders (Phasmatodea, Blattodea, and Orthoptera) suggest that cellulases are more common among Orthopteroid and Blattoid insects than previously thought. A major, comprehensive search for cellulases in these clades is warranted. In addition to the possibility of finding the efficient enzymes sought by the biofuel industry (Oppert et al., 2010), the data would allow researchers to determine the evolutionary relatedness of phasmid cellulase enzymes to those of other polyneopteran insects, shedding light on that branch of the insect phylogram. There is currently no consensus on the sister group to the Phasmatodea (Gullan and Cranston, 2010), and enzymology may provide the necessary information to resolve that polytomy.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2013.10.007.

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