Article

# PRODUCTION AND CHARACTERIZATION OF A RECOMBINANT BETA-1,4-ENDOGLUCANASE (GLYCOHYDROLASE FAMILY 9) FROM THE TERMITE *Reticulitermes flavipes*

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*Cell-1 is a host-derived beta-1,4-endoglucanase (Glycohydrolase Family 9 [GHF9]) from the lower termite Reticulitermes flavipes. Here, we report on the heterologous production of Cell-1 using eukaryotic (Baculovirus Expression Vector System; BEVS) and prokaryotic (E. coli) expression systems. The BEVS-expressed enzyme was more readily*

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*obtained in solubilized form and more active than the E. coli–expressed enzyme. $K_m$ and $V_{max}$ values for BEVS-expressed Cell-1 against the model substrate CMC were 0.993% w/v and 1.056 μmol/min/mg. Additional characterization studies on the BEVS-expressed enzyme revealed that it possesses activity comparable to the native enzyme, is optimally active around pH 6.5–7.5 and 50–60°C, is inhibited by EDTA, and displays enhanced activity up to 70°C in the presence of $CaCl_2$. These findings provide a foundation on which to begin subsequent investigations of collaborative digestion by coevolved host and symbiont digestive enzymes from R. flavipes that include GHF7 exoglucanases, GHF1 beta glucosidases, phenol-oxidizing laccases, and others. © 2010 Wiley Periodicals, Inc.*

**Keywords:** termite; endoglucanase; GHF9; feedstock; bioethanol; baculovirus

## INTRODUCTION

Endo-beta-1,4-glucanases (EC 3.2.1.4) from glycosyl hydrolase family 9 (GHF 9), also referred to as "endoglucanases," are hydrolytic enzymes that cleave internal beta-linked glycosidic bonds in glucose polymers, namely cellulose. Endoglucanases work collaboratively with other cellulase enzymes to liberate monomeric glucose from cellulose polymers. The two most common enzymes that work collaboratively with endoglucanases in cellulose digestion are GHF 7 exoglucanases (EC 3.2.1.91) and GHF 1 beta glucosidases (EC 3.2.1.21). Enzymes from each of these families have been widely studied from prokaryotes and eukaryotes, including members of the animal kingdom (Watanabe and Tokuda, 2001, 2010). In lower termites like *Reticulitermes flavipes*, cellulose digestion occurs through a collaboration of endogenous host cellulases and cellulases from microbial symbionts (Breznak and Brune, 1994; Slaytor, 2000; Ohkuma, 2003; Scharf and Tartar, 2008; Tartar et al., 2009).

Because of the diversity of host and symbiont cellulases in termites, it has long been considered impractical to characterize their various activities through one-dimensional investigations of enzyme biochemistry (Breznak and Brune, 1994). An alternative approach to understanding the digestive contributions of termite and symbiont cellulases is through the heterologous expression of individual cellulase genes in recombinant systems. There have been several successful attempts to functionally express recombinant digestive enzymes from termites and their symbionts in both *E. coli* and *Aspergillus oryzae*. These include GHF9 endoglucanases from *Coptotermes formosanus* and *C. acinaciformis* in *E. coli* (Tokuda et al., 1999; Nakashima et al., 2002; Watanabe et al., 2002; Inoue et al., 2005; Ni et al., 2005, 2007; Zhang et al., 2009); GHF5, GHF9 and GHF45 cellulases from *Nasutitermes* hindgut bacteria in *E. coli* (Warnecke et al., 2007); and *R. speratus* protist symbiont GHF7 exoglucanases in *A. oryzae* (Sasaguri et al., 2008; Todaka et al., 2010). With the current emphasis on biofuels and improved lignocellulose processing, there is also much interest in the development and characterization of recombinant termite and symbiont lignocellulases for biofuel production purposes (Scharf and Tartar, 2008; Matsui et al., 2009).

This research sought to produce and functionally characterize two recombinant versions of the endogenous Cell-1 endoglucanase (GHF9; Zhou et al., 2007) of the

termite *R. flavipes*. The full-length *Cell-1* cDNA encodes a predicted 448–amino acid protein with a 16–amino acid signal peptide (Zhou et al., 2007). Another important feature of the translated Cell-1 protein is the presence of calcium-binding motifs, suggesting that it uses calcium as a cofactor (Zhou et al., 2007). In the present work, a dual approach was taken that included Cell-1 recombinant expression coupled with conventional enzyme assays and other protein investigations. Our specific objectives were to: (1) functionally express soluble recombinant Cell-1 using a eukaryotic Baculovirus Expression Vector System (BEVS) and a prokaryotic (*E. coli*) expression system, (2) directly compare activity of the eukaryotic- and prokaryotic-expressed Cell-1, (3) conduct functional assays that compared recombinant and native Cell-1, and (4) conduct pH, temperature stability, and calcium cofactor/inhibitor studies with recombinant Cell-1. Here, we show that the BEVS-expressed Cell-1 is more readily obtainable in larger quantities and in a soluble form than its *E. coli*–expressed counterpart, and we provide details of insect-expressed Cell-1 activity and characteristics relative to native Cell-1, its pH and temperature dependence for optimal activity, and the effects of calcium as an apparent stabilizing cofactor.

## MATERIALS AND METHODS

### *Termites*

*R. flavipes* colonies were collected in Gainesville, Florida, and maintained in sealed plastic boxes (30 × 24 × 10 cm) in complete darkness at 22°C and 69% RH. Colonies were maintained without soil for more than 6 months and provisioned with moist brown paper towels and pine wood shims. The identity of colonies as *R. flavipes* was verified by a combination of soldier morphology and 16S-mt-rDNA gene sequence. Only worker termites were used because of their significant lignocellulose digestion capability.

### *Recombinant Protein Expression: BEV System*

The *Cell-1* cDNA open-reading frame (ORF) (AY572862; Zhou et al., 2007) was amplified without its signal sequence by PCR. Several features were incorporated into the PCR amplicon: (1) a heterologous signal sequence modeled after the *Bombyx mori* hormone bombyxin A-6 [GENE ID: 100169714 Bbx-a6], (2) an *Xba*I restriction site, (3) a C-terminal 6xHis tag, and (4) a *Not*I restriction site. These four features were incorporated into the amplicon via the following primers: forward, 5′-CTAGTCTAGACTAG***ATG****AAGAT-ACTCCTTGCTATTGCATTAATGTTGTCAACAGTAATGTGGGTGTCAACA*GCTGCTTACG-ACTATAAG-3′ (*Xba*I site underlined, start codon in **bold**, heterologous signal sequence *italicized*); and reverse, 5′-TTTCCTTTTGCGGCCGC**TTA***GTGATGATGGTGATGATG-*CACGCCAGCCTTGAGGAG-3′ (*Not*I site underlined, stop codon in **bold,** 6 × His tag *italicized*). The PCR amplicon was cloned into the *Xba*I-*Not*I sites of the pVL1393 transfer vector. The resulting plasmid DNA was verified by sequencing and used for co-transfection with linearized baculovirus DNA (BD Biosciences Pharmigen; San Diego, CA) into Sf9 cells. Cells were incubated at 27°C for 4 days (Passage 0) and the supernatant was collected and used for virus amplification in fresh cell culture (Passage 1). The cell pellet from Passage 0 was tested by Western blotting with anti-His antibody to confirm expression of His-tagged protein. After 2 days, the recombinant virus from Passage 1 was

harvested and injected to *T. ni* larvae as described previously (Liu et al., 2007; Kovaleva et al., 2009). Larvae were orally infected with active pre-occluded baculovirus (Liu et al., 2007; Kovaleva et al., 2009), harvested in large scale, and stored at −80°C for later processing. Recombinant protein was recovered from clarified *T. ni* homogenates by tandem Ni-IMAC (nickel-immobilized metal affinity chromatography) followed by buffer exchange with Sephadex G-25 chromatography. Protein storage buffer consisted of 0.1 M sodium acetate, 0.15 M sodium chloride, and 0.5 M calcium chloride (pH 5.8). Purity was assessed by SDS-PAGE with Coomassie staining and Western blotting with anti-His tag antibody.

### *Recombinant Protein Expression: E. coli System*

A truncated version of the *Cell-1* cDNA sequence, without its native signal sequence and stop codon, was amplified with the forward and reverse PCR primers CAAGCTGCTTACGACTATAAGA and CACGCCAGCCTTGAGGAGACC and verified by cloning (pGEM vector and NovaBlue competent cells; Promega, Madison, WI) and sequencing. Next, clones with antisense inserts were identified by colony PCR using a T7 primer complementary to the T7 vector sequence (TAATACGACTCACTATAGGG) and the *Cell-1* forward primer noted above. Several positive clones were selected, cultured in liquid media, and the plasmid DNA isolated and restriction digested with *NotI*. The resulting fragment was gel-purified and ligated into the MCS of *NotI*-digested pET26b(+) plasmid (Novagen, Madison, WI). Ligation products were used to transform BL21(DE3) pLysS competent cells (Novagen). Positive colonies with sense inserts were identified by colony PCR using the vector T7 and *Cell-1* reverse primers noted above. Recombinant proteins with C-terminal histidine tags were produced with selective culturing and IPTG induction at various temperatures (see Results), following manufacturer protocols (Novagen), as described previously (Scharf et al., 2004).

### *Termite Tissue Preparations and Protein Quantification*

All manipulations were performed on ice. For analysis by gut region, 50 individual worker termite guts were dissected and separated into the three regions of foregut+ salivary gland, midgut, and hindgut. Whole guts and tissues remaining after gut removal (referred to as "carcass") were isolated from 25 individual worker termites. All tissues were pooled into 0.1 M sodium acetate, pH 6.5, and homogenized by hand using Tenbroeck-style glass tissue grinders. The resulting homogenates were centrifuged for 10 min at 15,000*g* and 4°C, and the resulting supernatant used as the protein source. Protein quantification was performed using a commercially available Bradford assay (Bio-Rad, Hercules, CA), with bovine serum albumin as a standard.

### *Gel Electrophoresis, Deglycosylation Assay, and Western Blotting*

Two SDS-PAGE systems were used: (1) 4–20% Tris glycine gels (Invitrogen, Carlsbad, CA) or (2) resolving gels containing 10% acrylamide and 10% SDS with stacking gels that contained a lesser quantity of acrylamide (4%) and the same amount of SDS. A discontinuous Tris-Glycine-SDS buffering system was used, and protein sample buffer contained β-mercaptoethanol as the disulfide reducing agent. Molecular weight markers were prestained Kaleidoscope™ markers (Bio-Rad) or SeeBlue™ plus2 Standards (Invitrogen, Carlsbad, CA). Ten micrograms of heat-denatured protein was loaded per lane (diluted 1:1 with sample buffer). Gels were stained with Coomassie Blue R-250 in 40%

methanol and 10% acetic acid. PNGase-F was used to test for potential glycosylation of the BEVS-expressed Cell-1 protein. PNGase-F was purchased from New England Biolabs (Ipswich, MA) and was used following the manufacturer's instructions. Western blotting was performed using standard protocols with monoclonal anti-His primary antibody (Novagen), anti-mouse IgG (H&L) AP conjugate as secondary antibody (Promega). Detection of immuno-reactive bands was done with NBT/BCIP reagent (Thermo Scientific, Rockford, IL).

For Coomassie-stained Native PAGE, volumes of supernatant containing 10 μg of total protein were diluted 1:1 with Native PAGE sample buffer (Bio-Rad) and loaded onto native PAGE gels prepared with 7.5% resolving gels and 4% stacking gels. Electrophoresis was conducted in a discontinuous Tris-Glycine running buffer for 1.5 hr at 4°C. Gels were stained as above. For CMC-native PAGE, gels were prepared and run as described above, except that carboxymethyl cellulose (CMC; Sigma, St. Louis, MO) was incorporated into gels at 0.5%. After running, CMC gels were incubated in sodium acetate (0.05 M, pH 5.0) and stained with Congo Red as described previously (Nakashima et al., 2002; Zhang et al., 2009).

### *Colorimetric Enzyme Assays*

Six total substrates were tested: CMC (carboxymethyl cellulose), pNPG (p-nitro phenyl-beta-D-glucopyranoside), pNPC2 (p-nitrophenyl-beta-D-cellobioside), pNPC3 (p-nitrophenyl-beta-D-cellotrioside), pNPC4 (p-nitrophenyl-beta-D-cellotetraoside), and pNPC5 (p-nitrophenyl-beta-D-cellopentaoside). CMC, pNPG, and pNPC2 were purchased from Sigma. PNPC3, pNPC4, and pNPC5 were purchased from Carbosynth Ltd. (Berkshire, UK). All assay methods were carried out under optimal conditions as described in detail previously (Zhou et al., 2007, 2008a,b). The kinetic constants $K_m$ and $V_{max}$ were determined in 100 mM sodium acetate buffer (pH 6.5) at 25°C by testing serial dilutions of CMC (0.125–2.0%) with subsequent analysis by the Lineweaver-Burke method (Mathews and van Holde, 1990). For subsequent characterizations, the CMC concentration at $K_m$ of 1.5% w/v (see Results) was used. pH studies were performed by dissolving 1.5% CMC in two different buffer systems: 100 mM sodium acetate (pH 3–6.5) and 100 mM sodium phosphate (pH 7–10.5). Thermal stability tests were conducted using 1.5% CMC in 100 mM sodium phosphate at the optimal pH of 7. Temperature-cofactor studies were performed using 1.5% CMC dissolved in 100 mM sodium phosphate (pH 7) alone, plus 4 mM EDTA (final concentration), or plus 10 mM calcium chloride (final concentration). Pre-incubations took place for 0–5 days at 25°C or 0–60 min at 60° or 70°C; reactions were terminated by the combination of adding stop solution and boiling for 10 min, and then assays were read at 25°C (Zhou et al., 2007, 2008a,b). All reported activities are the average of 3–5 independent replicates. Statistical analyses consisted of two-way ANOVA followed by mean separation using Tukey's HSD test at $P < 0.05$.

## RESULTS

### *Production of Recombinant R. flavipes Cell-1 in Baculovirus-Infected T. ni Larvae*

Recombinant Cell-1 was expressed in *T. ni* larvae with a heterologous signal peptide and C-terminal histidine tag after oral infection with *Cell-1* transformed baculovirus.

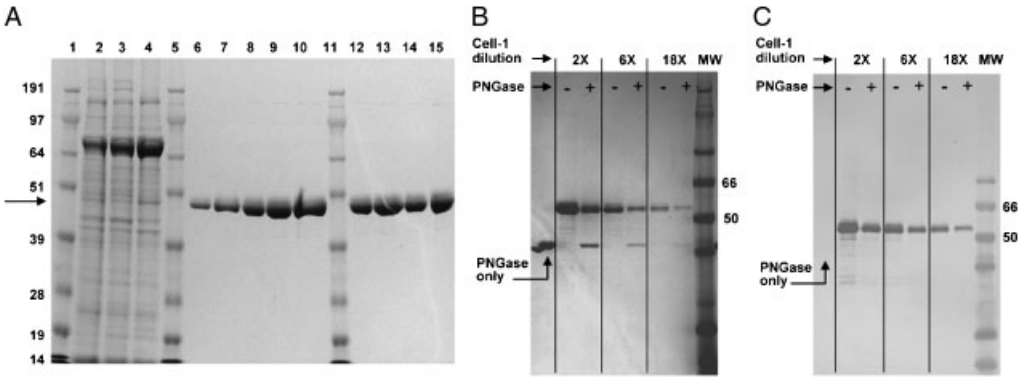



**Figure 1.** **A:** SDS-PAGE gel (4–20% tris-glycine) showing crude and purified recombinant Cell-1 from baculovirus-infected *T. ni* larvae. The horizontal arrow indicates the position of Cell-1. Lane assignments are as follows: 1, 5, 11 (See Blue$^{TM}$ molecular weight markers), 2–4 (clarified supernatant of uninfected, blank virus infected, and infected *T. ni* larvae), 6–10 (1, 2, 3, 5, and 10 μg purified recombinant Cell-1), 12–13 (3 and 5 μg purified recombinant Cell-1 held at 4°C for 65 hr), and 14–15 (3 and 5 μg purified recombinant Cell-1 held at 26°C for 65 hr). **B:** SDS-PAGE gel showing variable loading volumes of the purified recombinant Cell-1 protein with and without PNGase treatment. PNGase was used to test for the presence of glycosylation (results show no evidence of glycosylation). **C:** Western blot of an identical gel to that shown in B probed with an anti-His-tag antibody. Results in C confirm that, as expected, Cell-1 contains the recombinant His-tag. See text for further details.

The left side of Figure 1A shows an SDS-PAGE gel that compares protein composition of clarified homogenates from uninfected, blank virus–infected, and Cell-1 baculovirus-infected *T. ni* larvae. In these preparations, a ~48-kDa band is present in baculovirus-infected larvae, but is absent from uninfected and blank virus controls. Following purification and concentration from clarified homogenates by affinity and buffer exchange chromatography, the recombinant Cell-1 protein migrated as a single band at ~48 kDa (Fig. 1A, middle). Preliminary stability tests conducted by incubating the protein at 65° or 26°C for 65 hr revealed no protein degradation (Fig. 1A, right), indicating that it is stable in purified form. Cellulase activity assays were conducted on the same control and infected samples that were compared by SDS-PAGE (not shown). In addition to endoglucanase activity (the expected activity for Cell-1), exoglucanase and β-glucosidase activities were also investigated using the substrates pNPC and pNPG. All three activities were present in both uninfected and blank virus–infected *T. ni* larvae, with exoglucanase and β-glucosidase activity being the strongest. However, only endoglucanase activity remained for purified Cell-1, and in agreement with SDS-PAGE results, it was enriched >10-fold relative to clarified supernatants of infected larvae. Glycosylation prediction tools did not suggest that the native Cell-1 protein is glycosylated (not shown). In agreement with predictions, analyses with and without the deglycosylation enzyme PNGase-F were negative (Fig. 1B, C); they did not show changes in migration characteristics for the recombinant Cell-1 protein after PNGase-F treatment.

### *Production of Recombinant Cell-1 in E. coli*

Cell-1 was heterologously expressed in *E. coli* strain BL21(DE3) pLysS with a heterologous signal peptide and C-terminal histidine tag, using the pET26 vector. IPTG induction of recombinant Cell-1 in *E. coli* at 37°, 30°, and 25°C resulted in

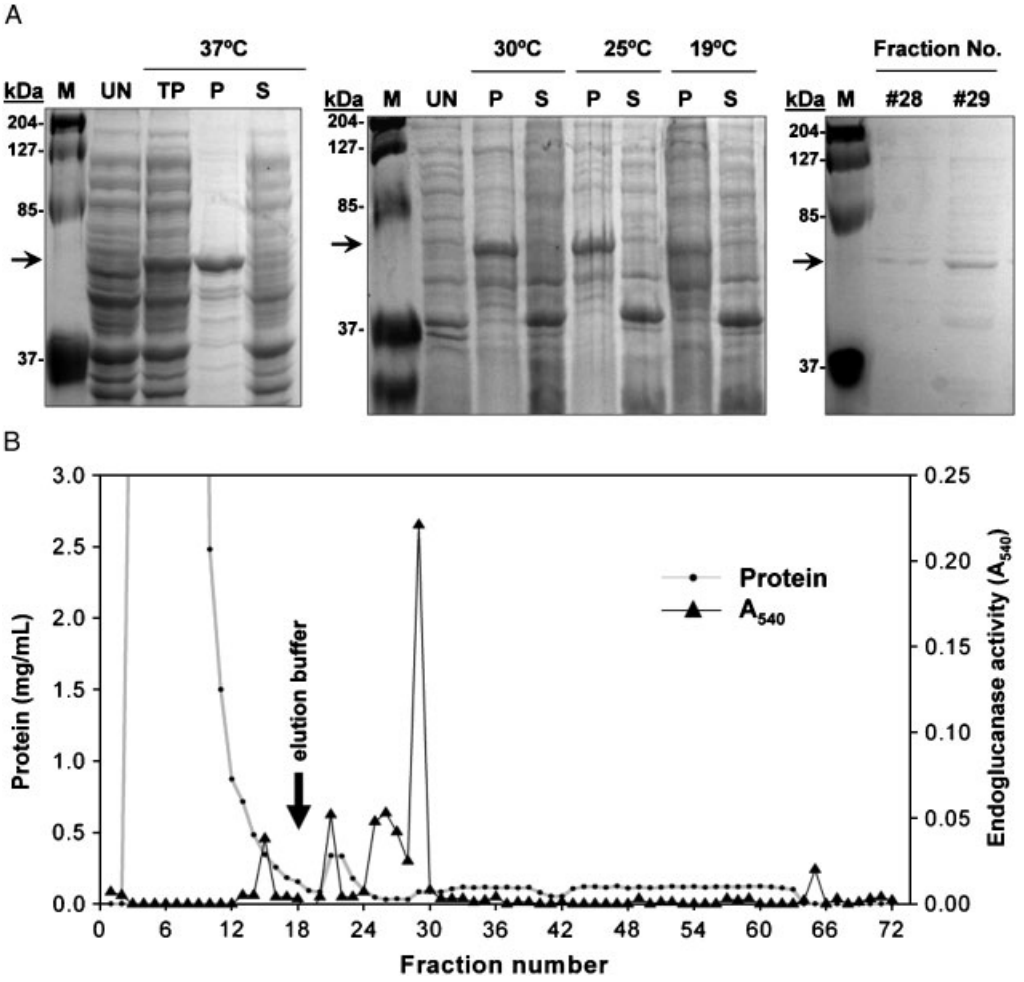
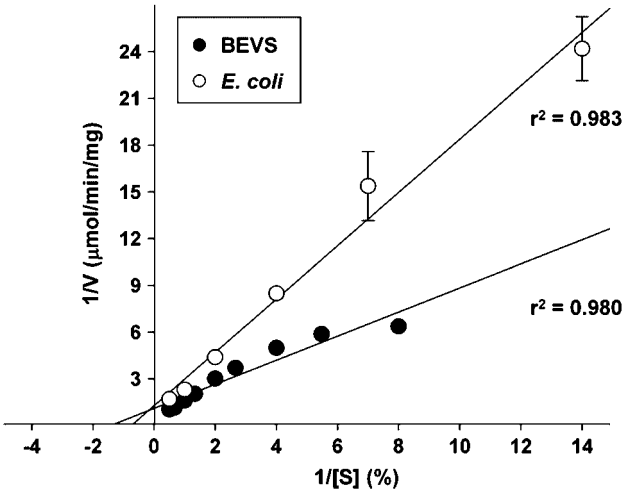



**Figure 2.** Expression, purification, and activity of the recombinant Cell-1 endoglucanase obtained using an *E. coli* expression system. **A:** SDS-PAGE (10% acrylamide) gels showing recombinant protein production at various temperatures relative to uninduced controls. The horizontal arrows indicate the position of Cell-1, which was effectively reduced in the insoluble pellet fraction at only the 19°C induction temperature. The gel at the right shows enriched Cell-1 fractions purified as shown (**B**), which is a typical elution profile on Ni-chromatography columns. The vertical arrow in (B) indicates the point at which elution buffer was added. M, molecular weight markers; UN, uninduced fractions; TP, total protein; P, insoluble pellet; S, soluble supernatant.

production of insoluble protein that readily precipitated after cell lysis and centrifugation (Fig. 2A). Eventually, recombinant Cell-1 was partially solubilized with IPTG induction at 19°C. Soluble fractions obtained after 19°C IPTG induction were subjected to Ni-chromatography for purification (Fig. 2B). The His-tagged Cell-1 was retained on Ni columns and eluted in a single peak of CMC activity after the introduction of imidazole elution buffer (in the ~29th column fraction). Active fractions also were assayed against the exoglucanase and β-glucosidase substrates pNPC and pNPG but showed no activity (not shown). After purification, pooling, and concentration, the active Cell-1 fractions showed an enriched ~48-kDa protein band (Fig. 2A, right).

A




B

| | BEVS | *E. coli* |
|---|---|---|
| $k_m$ % [S] | 0.993 ± 0.269 | 1.466 ± 0.013 |
| $V_{max}$ (µmol/min/mg) | 1.056 ± 0.125 | 0.838 ± 0.017 |

**Figure 3.** Kinetic constant determinations and comparisons for BEVS (baculovirus/*T. ni*)- and *E. coli*–expressed Cell-1 obtained using variable % w/v concentrations of the model substrate CMC. Assays were performed at 25°C in sodium acetate buffer (pH 6.5). **A:** Lineweaver-Burke plots comparing inverse substrate concentration [S] with inverse velocity [V] (specific activity) for the purified recombinant enzymes. The $r^2$ values shown indicate strong fit for both curves. **B:** Comparison of the kinetic constants $K_m$ and $V_{max}$ for the purified recombinant enzymes. The BEVS-expressed enzyme has a lower $K_m$ and a 25% higher $V_{max}$ than the *E. coli*–expressed enzyme, indicating greater activity.

### *Activity Comparisons for Recombinant Cell-1 Produced Using BEVS- and E. coli Expression Systems*

Both recombinant proteins were similar in that they were expressed without their native signal sequences, and with C-terminal histidine tags. Neither recombinant protein showed activity against the five p-nitrophenol-conjugated substrates pNPG, pNPC2, pNPC3, pNPC4, or pNPC5; thus, these substrates were not examined further. CMC activity for BEVS- and *E. coli*–produced Cell-1 was compared using Lineweaver-Burke plots of inverse substrate concentration versus inverse velocity (Fig. 3A). Linear activity was obtained using recombinant protein concentrations between 0.0285 and 0.85 mg/ml, and, therefore, all assays were run using protein concentrations within this range. With a $K_m$ of 0.993% w/v and a $V_{max}$ of 1.056 µmol/min/mg, the BEVS-produced Cell-1 had 0.68-fold reduced $K_m$ and a 1.26-fold higher $V_{max}$ than the *E. coli*–produced Cell-1 (Fig. 3B). As a result of these findings, we focused on recombinant Cell-1 produced in the baculovirus-insect expression (BEV) system for all studies reported hereafter.

### *Comparison of Native and Baculovirus Insect–Expressed Cell-1*

Baculovirus insect–produced recombinant Cell-1was compared to native *R. flavipes* tissue fractions by SDS-PAGE (Fig. 4A). As expected, in comparison to whole

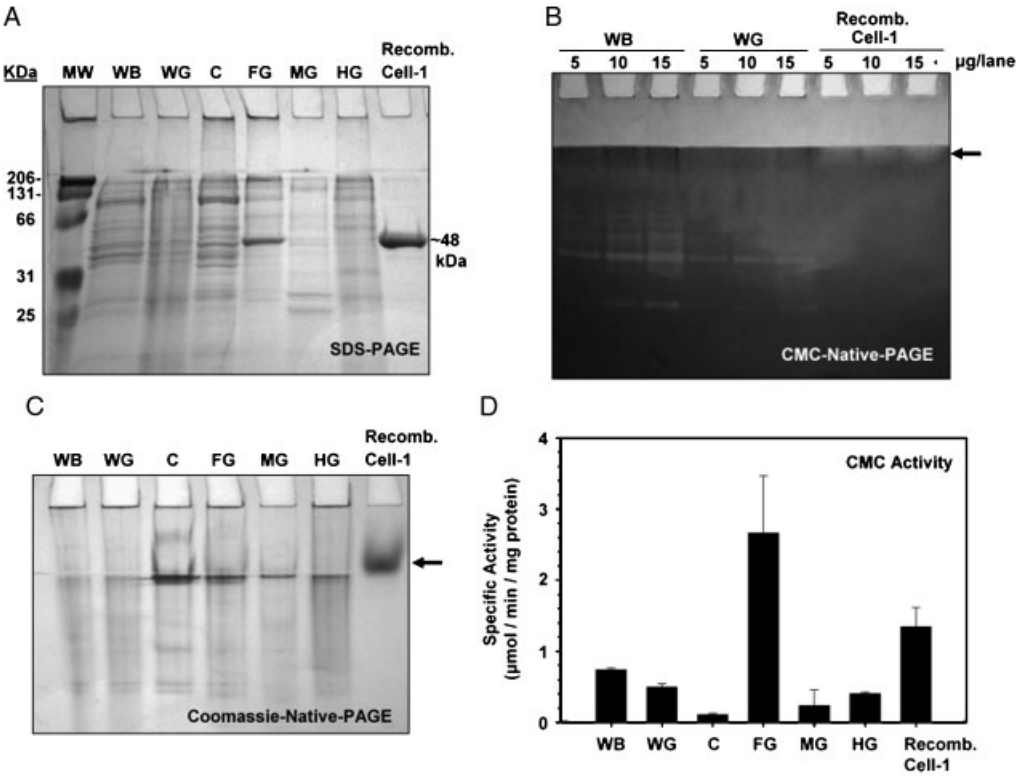



**Figure 4.** Comparisons of BEVS-expressed Cell-1 with protein expression and activity in native tissues isolated from *R. flavipes* workers. **A:** Denaturing SDS-PAGE (10% acrylamide) with Coomassie staining. **B:** CMC native PAGE with Congo red staining. **C:** Native PAGE with Coomassie staining. As shown in B and C, the recombinant Cell-1 does not migrate out of the stacking gel. **D:** Comparison of CMC activity for recombinant Cell-1 against endogenous activities in native tissues. CMC activity was determined at 25°C using 1.5% w/v substrate in sodium acetate buffer (pH 6.5) from three independent replicates. All native tissue protein loadings were 10 µg. Recombinant Cell-1 loadings were 5 µg unless noted. MW, Kaleidoscope™ molecular weight markers; WB, whole body; WG, whole gut; C; remaining carcass after gut and head removal; FG, foregut+salivary gland; MG, midgut; HG, hindgut. Arrows in B and C indicate the position of the native Cell-1 protein.

body, whole gut, carcass, foregut, midgut, and hindgut tissue fractions, only the foregut fraction showed a prominent protein band similar in size to recombinant Cell-1. Native PAGE gels conducted in the absence of SDS and stained for endoglucanase activity with CMC revealed very different banding patterns between recombinant Cell-1 and native tissue fractions (Fig. 4B). This result was explained by staining native gels for total protein with Coomassie Blue (Fig. 4C), which revealed that recombinant Cell-1 did not migrate out of the upper stacking gel. Finally, CMC endoglucanase assays were used to compare recombinant Cell-1 activity to the same native *R. flavipes* tissue fractions compared by SDS-PAGE (Fig. 4D). In strong agreement with SDS-PAGE, colorimetric assays revealed the highest activity levels in the foregut, followed closely by recombinant Cell-1, and then by all other tissue fractions and gut regions. These results indicate that recombinant Cell-1 possesses similar specific activity (LSD *t*-test; $P < 0.05$) to the native enzyme produced in the foregut region.

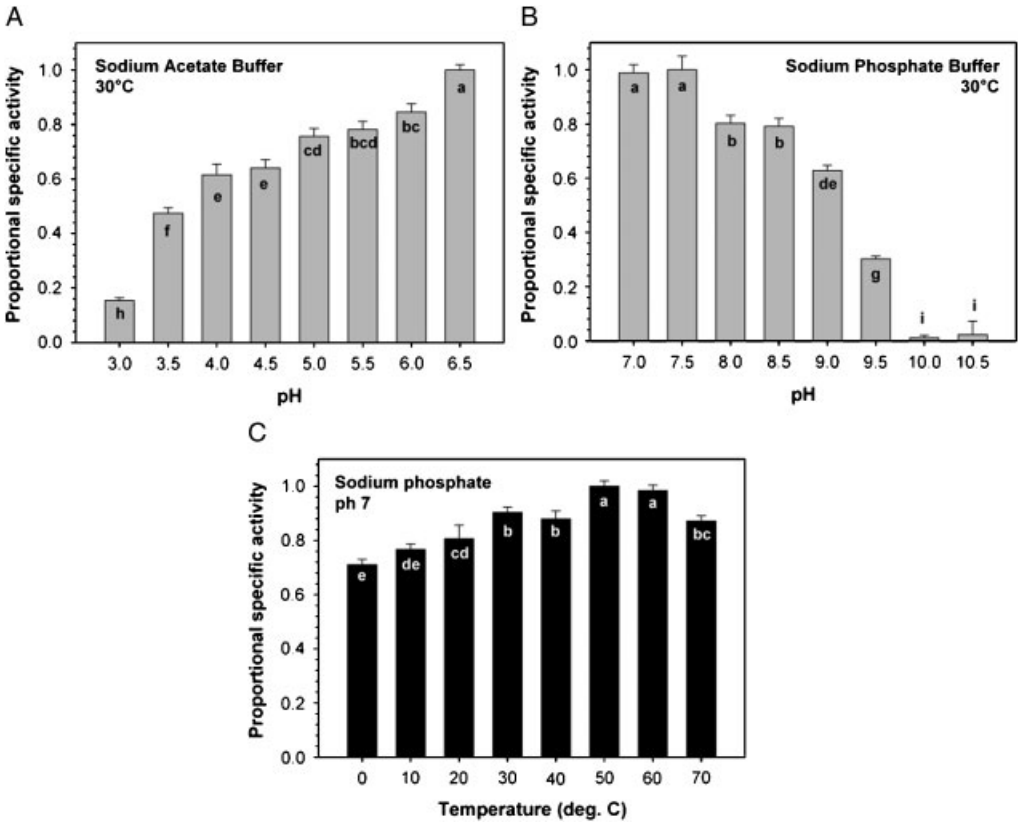



**Figure 5.** Optimal pH and temperature stability for the purified BEVS-expressed Cell-1. **A:** pH dependence of CMC activity at 30°C in sodium acetate buffer, pH 3.0–6.5. **B:** pH dependence of CMC activity at 30°C in sodium phosphate buffer, pH 7.0–10.5. **C:** Temperature dependence of CMC activity across assay temperatures ranging from 0 to 70°C in sodium phosphate at pH 7. Results shown are the average of three independent replicates. Bars in A–C with the same letter are not significantly different by Tukey's HSD test ($P < 0.05$). ANOVA model summaries for A+B and C, respectively, are ($df = 17$, $F = 247.15$, $P < 0.0001$) and ($df = 9$, $F = 38.53$, $P < 0.0001$).

### *pH and Temperature Stability of BEVS Expressed Cell-1*

CMC endoglucanase assays were used to investigate pH and temperature impacts on recombinant Cell-1 activity. Two buffer systems were used for optimal pH determinations: sodium acetate (pH 3.0–6.5; Fig. 5A) and sodium phosphate (pH 7.0–10.5; Fig. 5B). Maximal CMC activity was observed between pH 6.5 and 7.5 across both buffer systems. Temperature dependence of CMC activity in sodium phosphate buffer (pH 7) was maximal in the range of 50–60°C but fairly constant across a wide temperature range (Fig. 5C).

### *Calcium as a Cofactor in Cell-1 Hydrolytic Activity and Temperature Stability*

The effects of calcium on recombinant Cell-1 activity were investigated using calcium chloride as a calcium source and EDTA as a potential calcium chelator. As determined from incubations conducted over a 5-day period at room temperature (~25°C; Fig. 6A), calcium chloride slightly enhanced CMC endoglucanase activity and EDTA

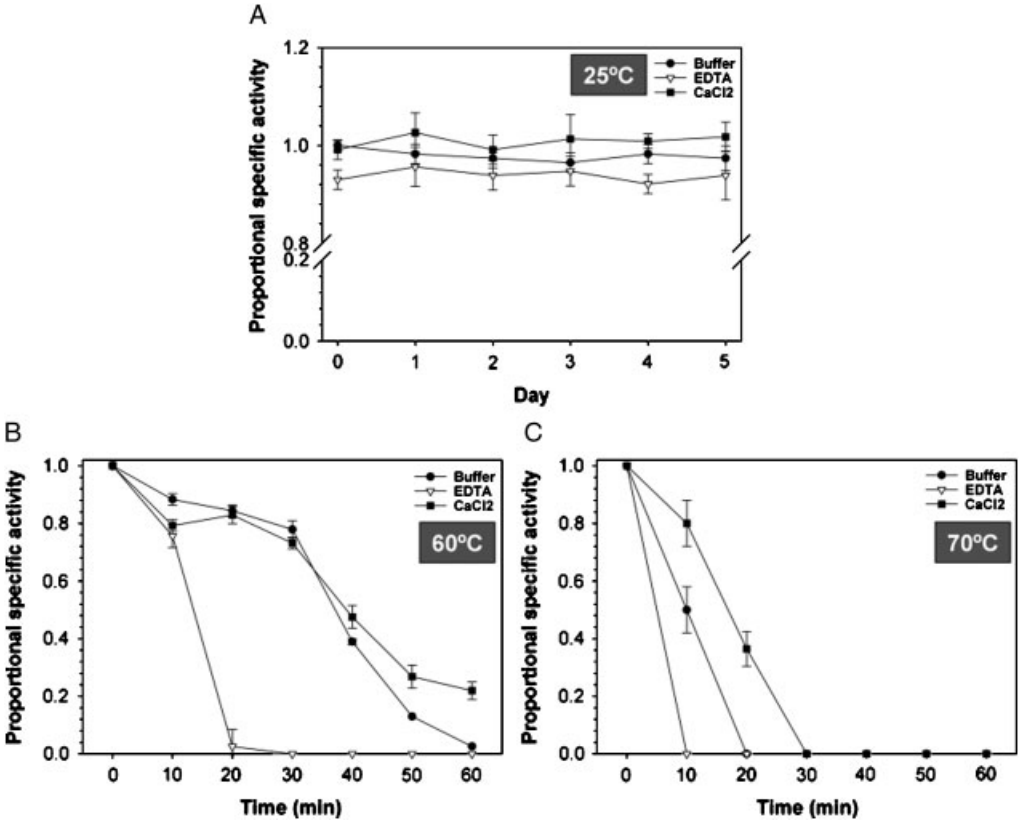



**Figure 6.** Impacts of EDTA and calcium chloride on temperature stability of the purified BEVS-expressed Cell-1. **A:** CMC activity after pre-incubation at 25°C for 0 through 5 days with buffer alone (sodium phosphate, pH 7), buffer +5 mM EDTA, or buffer +30 mM calcium chloride. **B, C:** CMC activity after pre-incubation at 60° or 70°C, respectively, for 0 through 60 min with buffer alone, buffer +5 mM EDTA, or buffer +30 mM calcium chloride. All assays were conducted at 25°C. Results shown are the average of three independent replicates.

was slightly inhibitory. With shorter incubations conducted at higher temperatures of 60° and 70°C, the same trends were apparent; that is, calcium chloride stabilized/extended CMC hydrolysis activity over time, while EDTA rapidly reduced temperature stability (Fig. 6B and C).

## DISCUSSION

### *Background and Rationale*

Information on *Cell-1* gene structure, features of the translated protein, tissue expression, and other gene and activity characteristics are provided in previous reports (Wheeler et al., 2007; Zhou et al., 2007, 2008a,b). The primary goals of the current report were to compare BEVS- and *E. coli*–expressed proteins, and to characterize the activity and stability, as well as pH, temperature, and potential cofactor requirements of the recombinant insect-expressed protein.

### *Recombinant Protein Production and Comparison of BEV- and E. coli–Expressed Enzymes*

Here, we sought to produce *R. flavipes* Cell-1 in both BEV and *E. coli* expression systems to enable direct comparisons of the resulting recombinant enzymes from each system. The BEVS-expressed Cell-1 protein is apparently assembled properly and retains expected endoglucanase activity. Although previous studies have successfully expressed cellulases from fungi and wood-feeding insects using a baculovirus-insect expression system (von Ossowski et al., 1997; S.J. Lee et al., 2004, 2005; K.S. Lee et al., 2006; Wei et al., 2006), the current results represent the first expression of a recombinant termite cellulase using the BEV system. Alternatively, several prior studies have successfully expressed termite and hindgut symbiont cellulases in *E. coli* (Tokuda et al., 1999; Nakashima et al., 2002; Watanabe et al., 2002; Inoue et al., 2005; Ni et al., 2005, 2007; Warnecke et al., 2007; Zhang et al., 2009). In particular, previous efforts reported by Zhang et al. (2009) using a different plasmid, another *E. coli* strain, and different culturing conditions (pET28a, Rosetta 2(DE3) pLysS, 37°C) resulted in much higher soluble expression of an endogenous *C. formosanus* endoglucanase.

Direct kinetic comparisons (Fig. 3) indicate that the BEVS-expressed Cell-1 is the more active enzyme, possibly because of more efficient/correct processing and/or post-translational modification in the eukaryotic system. As noted above, the BEVS-expressed Cell-1 was also more readily obtained in solubilized form and in larger quantities than the *E. coli*–expressed enzyme, and it retains activity similar to the native enzyme in gut tissue (Fig. 4). As a result, for all subsequent studies we focused on recombinant Cell-1 produced in the baculovirus-insect expression (BEV) system.

### *Stability of BEV-Expressed Cell-1*

Having thermostable enzymes for industrial lignocellulose processing is important because they enable (1) greater activity with less enzyme, (2) longer processing times due higher stability, and (3) increased flexibility for process configurations (Viikari et al., 2007). Our findings indicate a wide range of pH and temperature tolerance for Cell-1, particularly in the presence of calcium chloride, but they suggest that optimal activity toward the model substrate CMC occurs around pH 7 and 50–60°C.

Temperature stability has been investigated for a large number of recombinant and/or pure cellulases; for example, a PubMed search using the keywords "cellulase temperature stability" retrieved over 200 papers on the topic. With respect to microbial and termite cellulases, recent literature surveys of native and recombinant enzymes identified optimal pH and temperature ranges, respectively, of 3.6–9.0 and 45–100°C (Viikari et al., 2007; Todaka et al., 2010). Results for recombinant *R. flavipes* Cell-1 and engineered versions of a homologous endoglucanase from *R. speratus* expressed in *E. coli* (Ni et al., 2007) were very similar (pH~7, 40–50°C) and were intermediate relative to the microbial activity ranges noted above. Alternatively, a homologous endoglucanase from *C. formosanus* showed highest CMC activity at pH 5 and reduced temperature stability above 37°C (Zhang et al., 2009). Similarly, a recombinant symbiotic GHF 7 exoglucanase from *R. speratus* expressed in *Aspergillus oryzae* showed optimal CMC activity at pH 6.5 and 45°C (Todaka et al., 2010). Thus, BEVS-expressed recombinant Cell-1 shows slightly improved temperature stability characteristics at neutral pH relative to a number of other recombinant termite endoglucanases.

The rationale for our investigation of calcium chloride effects on Cell-1 stability came from previous bioinformatic analyses that predicted calcium-binding domains in the translated Cell-1 amino acid sequence (Zhou et al., 2007). Consistent with this prediction, our findings show that at higher temperatures of 60° and 70°C, calcium chloride stabilizes/extends CMC hydrolysis activity over time, while EDTA rapidly reduces temperature stability.

One prior study on a pure recombinant endoglucanase (Cel-16) from a plant (*Brassica napus*) used calcium chloride and EDTA to identify a strong dependence on calcium for CMC hydrolysis activity (Mølhøj et al., 2001). No prior studies have examined calcium dependence or EDTA inhibition of termite endoglucanases or cellulases; therefore, our findings suggest the novel possibility that calcium chloride or other calcium analogs may be used in industrial applications to extend the functional life of recombinant termite endoglucanases. A recent report suggests that calcium chloride increases available surface area via de-aggregation of cellulose polymers (Tokuyasu et al., 2008); thus, while the current EDTA results suggest a role for calcium as a cofactor in Cell-1 temperature stability, it is not clear if the stabilizing effects of calcium chloride result from an interaction with Cell-1 or the cellulose substrate. Nonetheless, these results support earlier bioinformatic predictions that Cell-1 might require calcium as a cofactor (Zhou et al., 2007), and support the idea that calcium plays a role in stabilizing the enzyme at temperatures above 50°C.

### *Summary and Conclusions*

Here, we have reported on the heterologous expression of an endogenous termite endoglucanase using eukaryotic (insect) and prokaryotic (*E. coli*) expression systems, and subsequent characterizations of the resulting recombinant enzymes. Both enzymes were expressed with heterologous signal peptides and C-terminal histidine tags. We found that the BEVS-expressed enzyme was more readily obtained in larger quantities in a soluble form, and that its maximal specific activity was ~25% higher than the *E. coli*–expressed enzyme at $V_{max}$. Neither recombinant Cell-1 version was active toward *p*-nitrophenol substrates with 2–5 beta linked glucose units, suggesting that Cell-1 only accepts large cellulose or hemicellulose polymers as substrates. Further characterization studies on the BEVS-expressed enzyme revealed that it possesses CMC activity similar to the native enzyme, is most active around neutral pH, and retains activity up to 70°C in the presence of calcium chloride.

These findings provide an important foundation on which to begin subsequent investigations of additional co-evolved host and symbiont digestive enzymes from *R. flavipes*. In this respect, recent meta-transcriptomic sequencing efforts have revealed 170 candidate host and symbiont lignocellulase genes from *R. flavipes*, including candidate lignases (laccases and esterases), GHF7 exoglucanases, and GHF1 beta glucosidases (Scharf and Tartar, 2008; Tartar et al., 2009). Parallel efforts to the current study have functionally characterized esterases and laccases potentially involved in lignin degradation (Wheeler et al., 2010; M.R. Coy et al., unpublished observations) as well as the *BGluc-1* beta-glucosidase that works collaboratively with Cell-1 in lignocellulose digestion (Scharf et al., 2010 and unpublished observations). Forthcoming reports will focus on additional recombinant enzymes as noted above, as well as the testing of recombinant enzyme cocktails on different 2nd generation bioethanol feedstocks as reviewed by Simmons et al. (2008).

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