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Heterologous Expression and Characterization of an Endoglucanase from a Symbiotic Protist of the Lower Termite, *Reticulitermes speratus*

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Abstract RsSymEG, an endoglucanase of glycosyl hydrolase family (GHF) 7 encoded by a transcript isolated from the symbiotic protist of the termite *Reticulitermes speratus*, is expressed in *Aspergillus oryzae*. Interestingly, purified RsSymEG1 has a relatively higher specific activity (603 μmol min^{-1} mg^{-1} protein) and V_{max} value (769.6 unit/mg protein) than previously reported data for GHF7 endoglucanase of *Trichoderma ressei*. It also has the same K_m value (1.97 mg/ml) with *Clostridium cellulolyticum* enzymes that contain cellulose binding module, a property indicative of high affinity to substrate, though no

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cellulose binding module is found within it. Thin-layer chromatography analysis revealed that RsSymEG1 preferentially hydrolyzes the β-1,4-cellulosic linkage of cellodextrins into cellobiose and glucose.

Keywords Cellulase · Biomass · Termite · Protists

Introduction

The enzymatic hydrolysis of plant polysaccharides appears to be one of the most promising ways to produce renewable energy [1] Biomass energy can also play an important role in reducing greenhouse gas emissions. Given that the CO_2 generated by biomass wastes would originally have been absorbed from the air, the use of biomass for energy offsets greenhouse gas emissions from fossil fuel.

Cellulose, the main structural component of plant cell walls, is the most abundant carbohydrate polymer in nature. There are three major types of cellulases: endo-β1, 4-glucanase (EG; EC 3.2.1.4), cellobiohydrolase (CBH; EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). All of these enzymes hydrolyze the β-1,4-glycosidic bonds in cellulose.

Termites play an important role in the decomposition of ecosystems via the degradation of lignocellulose [2]. Two separate cellulolytic systems, endogenous cellulases and cellulases of symbiotic protist origin, are now well established to coexist in the lower termite [3, 4]. This dual system seems to result in the high assimilation rate (in some cases, greater than 90%) of wood glucan by termites [5]. Symbiotic systems are rich reservoirs of novel cellulase genes [3, 6–11]. Yet the research for industrial application has centered on fungal and bacterial cellulases. The incentive to study and elucidate the cellulolytic systems of symbiotic protists has been low, as the heterologous expression of cellulases yields little to no expressed proteins. To understand these systems and apply them in the industry, we need to characterize each cellulase of protist origin. In this study, we sought to characterize the novel cellulase from the symbiotic protist of the termite and to study its heterologous expression.

By virtue of its efficient protein-producing capability, the filamentous fungus *Aspergillus oryzae* is favored as a host for heterologous protein production [12, 13]. In this study, we used *A. oryzae* as a host to produce the glycosyl hydrolase family (GHF) 7 endoglucanase (RsSymEG1), an enzyme whose transcript was isolated from the symbiotic protist of the termite *Reticulitermes speratus*. We purified RsSymEG1 from the culture supernatant and analyzed three of its important enzymatic characteristics: optimum condition, kinetic parameter, and substrate preference.

Materials and Methods

Heterologous Expression of RsSymEG1 in *A. oryzae*

This study was performed using *Rssymeg1* cDNA (DDBJ accession no. AB274537) encoding RsSymEG1. The plasmid for transformation of *A. oryzae*, a molecule designed to express RsSymEG1 using α-amylase as a carrier, was generated by a set of plasmids [14] constructed with the aid of the MultiSite Gateway® three-fragment vector construction kit (MultiSite Gateway® System; Invitrogen, Carlsbad, CA, USA). Details are essentially the same as described in our recent publication [15]. Briefly, a Kex2 cleavage site followed by

triglycine sequence (KRGGG) was inserted between the full-length α-amylase (AmyB) and a mature region of RsSymEG1 (residues 22-341) without the putative signal sequence. The expression of fusion gene was driven by dextrin-inducible α-amylase promoter. *A. oryzae* was transformed with the expression plasmid by standard methods [13]. The host strain used in the transformation was NS-tApE (*niaD*$^{-}$ *sC*$^{-}$ Δ*tppA* Δ*pepE*)-deficient for the PepE and TppA proteases (Nemoto et al., manuscript in preparation). The *A. oryzae* protoplasts were incubated with the plasmid DNA and plated on minimal (M) medium [0.2% NH_4Cl, 0.1% $(NH_4)_2SO_4$, 0.05% KCl, 0.05% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.002% of 2% $FeSO_4{\cdot}7H_2O$, and 2% glucose, pH5.5] containing 1.2 M sorbitol. Ten transformants were isolated and transferred to M medium twice. Approximately 2×10^6 spores of transformants were inoculated in 100 ml 5X DPY medium (10% dextrin, 5% polypeptone, 2.5% yeast extract, 0.5% K_2HPO_4, and 0.05% $MgSO_4{\cdot}7H_2O$, pH8.0) in 500-ml flasks and incubated at 30°C for 4 days with shaking (approximately 150 rpm). The culture filtrates were collected with Miracloth (Calbiochem, CA, USA), precipitated with ammonium sulfate (80% saturation), and purified in steps. The purified protein was used in the following enzyme characterization.

Enzyme Assays

During enzyme purification, 10 μl of each fraction was incubated with 200 μl of 2% (*w*/*v*) sodium carboxymethylcellulose (CMC; Fluka bland, low viscosity degree of substitution 0.60–0.95, Sigma-Aldrich, USA) in 0.1 M sodium acetate buffer (pH5.5) at 37°C for 10 min.

For activity on various substrates, samples (10 μl) were incubated with 200 μl of 1% (*w*/*v*) xylan (Sigma-Aldrich), Avicel (Merck, Germany), or Curdlan (Wako, Japan) in 0.1 M citrate-phosphate buffer (pH6.5) at 37°C for 30 min.

Details of the assay conditions for the determination of enzymatic properties were mentioned in the following section.

One unit of enzyme activity is defined as the amount of enzyme which produces 1 μmol of reducing sugar (glucose or xylose equivalents) per minute when endoglucanase samples were incubated with 200 μl of 2% (*w*/*v*) CMC in 0.1 M citrate phosphate buffer (pH6.5) at 37°C for 10 min. The reducing sugars produced were measured with tetrazolium blue reagent (Sigma) by the method previously described [16]. Specific enzyme activity was defined as the number of units of enzyme activity per milligram protein. Protein concentrations were determined by the Bradford method (Bio-Rad, USA) using bovine serum albumin as a standard.

Purification of RsSymEG1

The concentrated sample was applied to a HiTrap DEAE Sepharose column (1.6×2.5 cm, GE Healthcare) equilibrated with 20 mM Tris–HCl buffer, pH8.0. Proteins were eluted by a linear gradient (0–0.5 M) of NaCl in 20 mM Tris–HCl buffer at a flow rate of 0.5 ml/min. Fractions (2 ml) were collected and an aliquot of the individual fraction was assayed for cellulase activity followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The purified sample was subjected to N-terminal sequence analysis by Edman degradation.

Examination of Enzymatic Properties of RsSymEG1

The optimal pH was determined using 1% (*w*/*v*) CMC (average molecular weight, 250,000; degree of carboxymethyl substitution, 0.7; Sigma-Aldrich) in 0.1 M citrate-phosphate buffer

(pH4.0 to 8.0) for enzyme assays [17]. Samples (10 μl of 1/100 diluted enzyme solution, 0.03 μg protein) were incubated with 200 μl substrate solutions for 10 min at 37°C.

The optimal temperature was determined by measuring EG activity at pH6.5 over the range of 20°C to 75°C at 5°C intervals for 5 min. Thermal stability was evaluated by incubating the enzyme samples for 30 min at constant temperatures from 20°C to 75°C at 5°C intervals, then assaying them at 37°C for 5 min. In the case of optimal temperature and thermal stability assay, samples (10 μl of 1/100 diluted enzyme solution, 0.03 μg protein) were incubated with 200 μl substrate solutions [2% (*w*/*v*) CMC in 0.1 M citrate phosphate buffer (pH6.5)]. The K_m and V_{max} values were determined using CMC (0.525–21 mg/ml) in 0.1 M citrate phosphate buffer (pH6.5) as a substrate solution. Samples (10 μl of 1/100 diluted enzyme solution, 0.03 μg protein) were incubated with substrate solutions (200 μl) at 37°C for 5 min and reducing sugars were detected with tetrazolium blue [16].

TLC Analysis

Purified enzyme (2.5 μl, 0.75 μg protein) was incubated with 25 mM oligosaccharide (cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose; Seikagaku Corporation, Tokyo, Japan) in 0.1 M citrate phosphate buffer (pH6.5, 50 μl) for 2 h at 37°C. The reaction products were separated on thin-layer chromatography (TLC) plates (silica gel 60, Merck) with a solvent system containing 1-butanol/ethanol/water (2:2:1). The products were detected by spraying the plate with staining reagents (diphenylamine, aniline, and phosphoric acid) and baking for 10 min at 100°C.

Results and Discussion

Sequence Features of RsSymEG1

By confirming the alignment of the deduced amino acid sequence of *Rssymeg1* with sequences of the GHF7 cellulases from *Trichoderma reesei*, TrCel7A CBH and TrCel7B EG, we confirmed that the sequence of *Rssymeg1* contains the critical catalytic nucleophile (Glu159) and general acid/base (Glu164). Our studies also revealed that RsSymEG1 contains seven out of 12 cysteines shared in TrCel7A and TrCel7B [18] (Fig. 1).

Cellulases and other glycosyl hydrolases appear in a great many forms with highly variable carbohydrate structures. In a departure from the conventional enzyme classifications based on the types of catalytic reactions and substrate specificities, Henrissat et al. have introduced and regularly updated a classification for glycosyl hydrolases into families based on amino acid sequence similarities in order to integrate the structural and mechanistic features of these enzymes [19, 20]. The known GHFs have grown steadily in number and now make up more than 100 families (http://www.cazy.org/). The classification scheme takes into account evolutionary events such as divergence or convergence [19]. On the basis of this family definition system for GHFs, our results indicate that RsSymEG1 is a GHF7 cellulase.

The GHF7 cellulases can also be divided into CBHs and EGs based on the presence or absence of insertion sequences. CBHs are known to contain insertion sequences involved in the formation of a tertiary cellulose-binding tunnel structure. The regions corresponding to the insertion sequences in TrCel7A are usually conserved among the GHF7 CBHs, but not among the EGs. GHF7 cellulases also share a number of the cysteines forming the disulfide bonds found in both GHF7 cellulases from *T. reesei*, that is, TrCel7A and B. Disulfide bonds are essential for stabilizing the tertiary structures of these enzymes [21, 22].

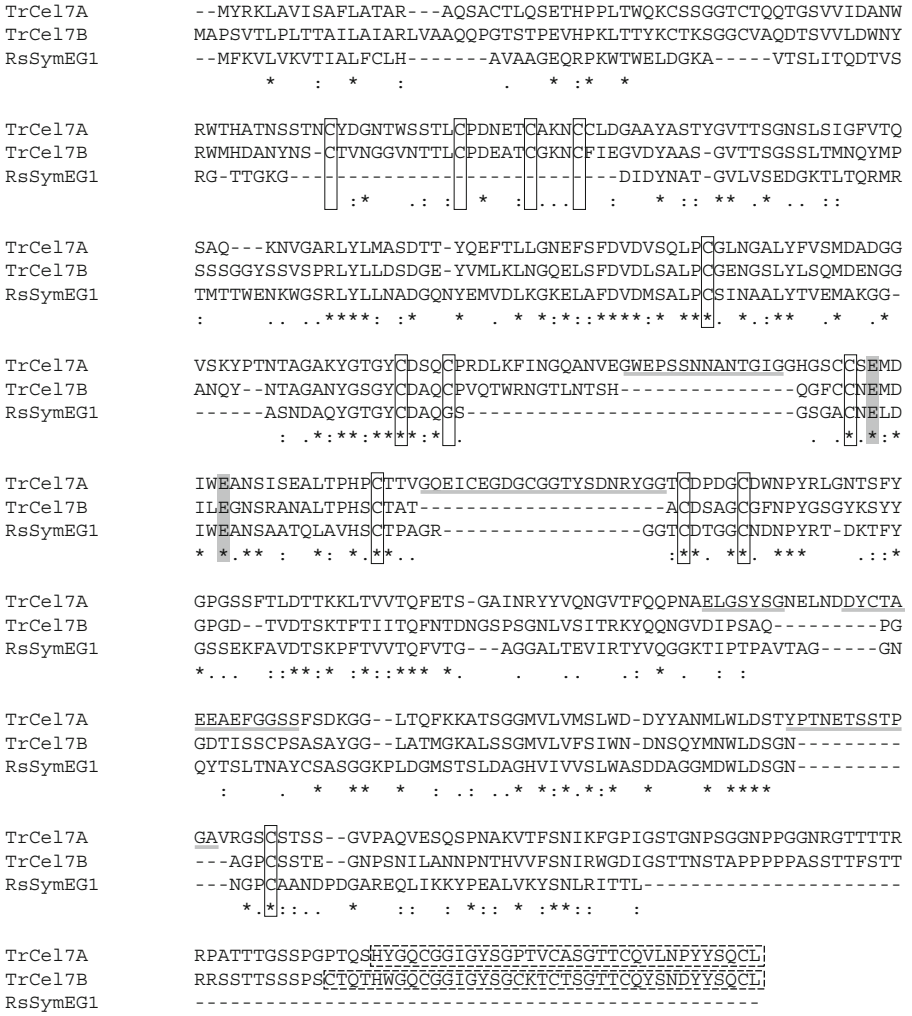

Fig. 1 Alignment of RsSymEG1 with *T. reesei* GHF7 cellulases TrCel7A, a CBH component, *T. reesei* Cel7A [Uni Prot. P00725]; TrCel7B, an EG component, *T. reesei* Cel7B [P07981]. The alignment was performed using CLUSTAL W and subsequent manual refinement. *Shaded columns* indicate the sites of putative proton donors and general acids/bases. *Open boxes* represent cysteine residues forming the disulfide bonds in TrCel7B. The *underlined sequences* in TrCel7A indicate the loop-forming regions covering the catalytic tunnel. *Dotted boxes* denote cellulose-binding domains of *T. reesei* cellulases

As shown in Fig. 1, the insertion sequences in TrCel7A (underlined sequence in TrCel7A) are absent from both TrCel7B and RsSymEG1. RsSymEG1 is thus very likely to fall into the EG category of GHF7 cellulases. We also note, with interest, that the cellulose-binding module found in the C-terminal region of TrCel7A and TrCel7B cannot be found in RsSymEG1.

Heterologous Production of RsSymEG1

Our experiments sought to elucidate the enzymatic properties of RsSymEG1 using the *A. oryzae* expression system to produce RsSymEG1. We used *A. oryzae* α-amylase as an N-

terminal carrier and introduced the Kex2 protease recognition sequence Lys-Arg followed by three glycine residues between α-amylase and the mature region of RsSymEG1. The insertion of three glycines between the Kex2 site and target protein has been shown to improve the processing efficiency by Kex2 [23]. When the culture supernatants of *A. oryzae* transformants were assayed for the cellulase activity, cells harboring *Rssymeg1* exhibited much higher activity than the vector-transformed cells used as control. Moreover, SDS-PAGE analysis demonstrated the presence of a protein band of about 39 k, a molecular weight close to the 36-k molecular weight calculated for RsSymEG1, only in the culture supernatant of the *Rssymeg1*-harboring cells (data not shown).

Next, we purified RsSymEG1 from the culture supernatant. As shown in Fig. 2a, a single clear peak of cellulase activity was eluted from the HiTrap DEAE Sepharose column. Examination of each fraction by SDS-PAGE showed a single band of 39 k co-eluting with the enzyme activity (Fig. 2b). According to an N-terminal amino acid sequence analysis of

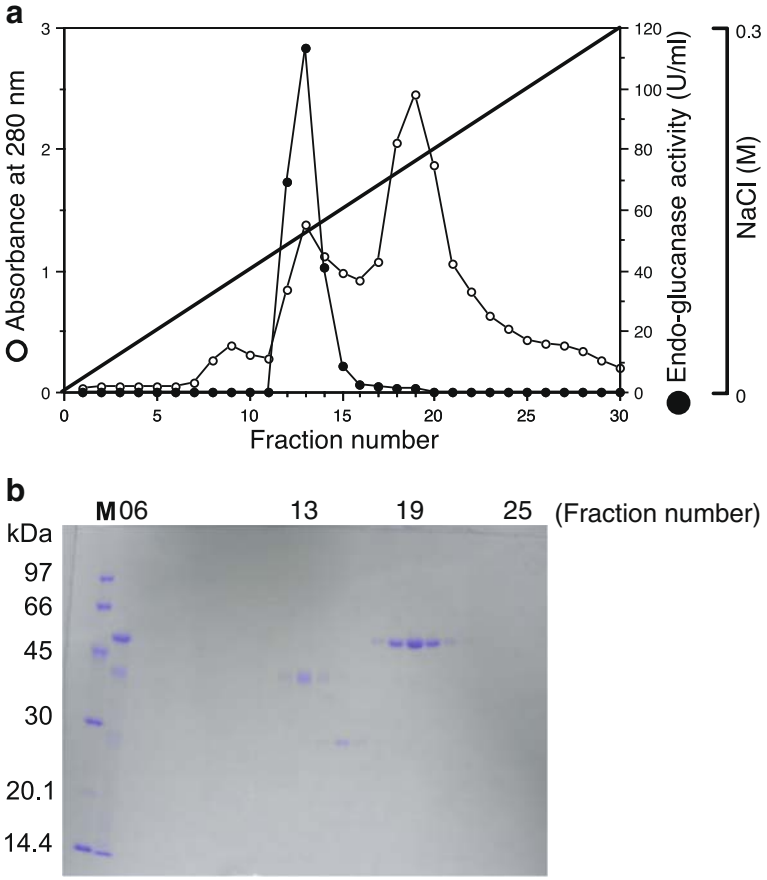

Fig. 2 Purification of RsSymEG1 by DEAE Sepharose column chromatography and SDS-PAGE analysis. **a** Elution profile of crude culture supernatant in an HiTrap DEAE Sepharose column. *Closed* and *open circles* indicate EG activity and protein measured by absorbance at 280 nm, respectively. **b** Fractions 6-25 were subjected to SDS-PAGE analysis. *Lane 0* the crude sample before purification; *M* molecular weight standards consisting of α-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), albumin (66 kDa), and phosphorylase b (97 kDa)

the purified protein, the sequence GGGEQRPKWT corresponds to residue 22-28 of RsSymEG1 with three additional glycine residues at the N terminus. With this information, we can assume that the Kex2-like protease in *A. oryzae* cleaved the α-amylase–RsSymEG1 fusion protein in an endoproteolytic manner, as expected. Taken in sum, these results confirm the successful production and purification of mature RsSymEG1.

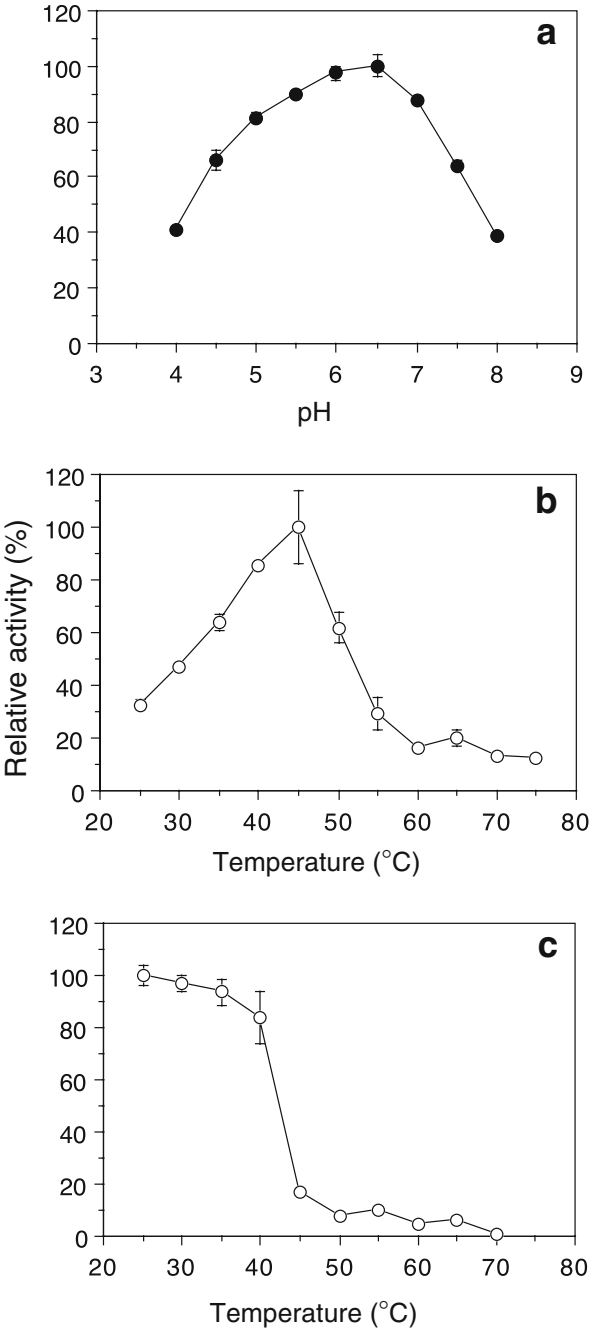

Fig. 3 Biochemical properties of RsSymEG1 pH (**a**), temperature (**b**) optima and temperature stability (**c**) examined using CMC as a substrate

Enzymatic Properties of RsSymEG1

The optimum pH of the purified protein examined using CMC as a substrate was 6.5, and the enzyme retained over 80% of the maximum activity between pH5.0 and 7.0 (Fig. 3a). The activity peaked at 45°C and sharply decreased at higher temperatures (Fig. 3b). The enzyme retained stable activity (over 80% of maximum activity) after 30 min of pre-incubation at temperatures as low as 40°C, but the activity was lost altogether at temperatures above 50°C. These properties are similar to those found in most of the fungal cellulases [24–26].

Enzymatic properties are compared in Table 1. RsSymEG1 released reducing sugars (as glucose equivalents) at a rate of 603 μmol/min per 1 mg protein from RsSymEG1. In contrast, the rates of release of reducing sugars from EGI of *Trichoderma viride* HK-75 and EGI of *T. reesei* L27 (Purified GHF7 endoglucanase, a component of commercially used cellulase mixture) were 12.3 and 11-18 μmol min^{-1} mg^{-1} protein, respectively [24, 26]. The V_{max} value of RsSymEG1 is 769.6 units/mg protein, although previously published V_{max} value of endoglucanases from commercially used cellulase mixture of *T. viride* ranges from 10.8 to 42.7 units/mg protein [27, 28]. The K_m value of this enzyme was 1.97 mg/ml, indicating a relatively high affinity of this enzyme to substrate despite the absence of CBM motifs in the gene of this enzyme. In contrast, K_m value from CBM-containing bacterial family 5 and 8 endoglucanase CelCCA and CelCCC of one of the major cellulolytic bacteria, *Clostridium cellulolyticum*, was 1.97 and 1.00 mg/ml, respectively [29, 30]. Also, RsSymEG1 showed higher specific activity and V_{max} value than other termite-related endoglucanases [9, 31, 32]. Interestingly, all termite-related endoglucanases including

Table 1 Comparison of enzymatic properties among various endoglucanases.

Organism	Sp. act. (unit/mg)	Optimal temp. (°C)	Optimal pH	K_m (CMC) (mg/ml)	V_{max} (CMC) (units/mg)	Ref
RsSymEG1 (GHF7)	603	45	6.5	1.97	769.6	This work
Clostridium cellulolyticum						
CelCCA (GHF5)	64.3	37–51	5.5–7.2	1.97	60.8	29
CelCCC (GHF8)	44.8	48	6.0	1.00	50	30
Trichoderma viride HK75						
EGI (GHF7)	12.3	50	4	–	–	26
Trichoderma viride						
Cellulase IIa (family unknown)	29.83	60	5	0.81	42.7	27
Cellulase IIb (family unknown)	4.95	60	5	0.96	10.4	27
Cellulase III (family unknown)	20.00	60	5	0.54	18.7	28
Trichoderma reesei L27						
EGI (GHF7)	11–18	–	–	–	–	24
Rorch endoglucanases						
Panesthia cribrata						
EG1 (GHF9)	171.1	–	–	9.4	22.2	31
EG2 (GHF9)	318.2	–	–	6.8	88.3	31
Termite endoglucanases						
Reticulitermes speratus						
YEG1 (GHF9)	73.6	50	6.0	1.83	527	32
YEG2 (GHF9)	83.4	50	6.0	1.43	540	32
Symbiont of *Coptotermes formosanus*						
CFP-EG1 (GHF5)	105.0	70	6.0	1.90	148.2	9

Table 2 Activity of RsSymEG on various substrates.

Substrate	Bond type	Specific activity (U/mg of protein)
CMC	β-1,4	603±23
Avicel	β-1,4	0.12±0.003
Curdlan	β-1,3	0.02±0.003
Xylan (beech wood)	β-1,4	0.31±0.009
Xylan (birch wood)	β-1,4	0.40±0.011
Xylan (oat spelts)	β-1,4	1.62±0.050

The assay was performed with 1% (*w*/*v*) substrate at 37°C for 60 min except for CMC. The activity on CMC (2%, *w*/*v*) was assayed at 37°C for 10 min. One unit of polymer hydrolysis represents 1 μmol of reducing sugar liberated per minute

RsSymEG1 showed relatively low K_m value, although roach enzyme, which also has no CBM, showed high K_m value.

As shown in Table 2, RsSymEG1 had the highest activity toward CMC and exhibited very little or no activity against Avicel, Curdlan, or xylan. The specific activity on Avicel was less than 1/5,000 of that on CMC.

The hydrolysis pattern analysis by TLC indicated that the main degradation products of cellodextrins (3G to 6G) by RsSymEG1 were glucose and cellobiose (2G). In contrast, RsSymEG1 exerted no action on cellobiose (Fig. 4). Endoglucanase usually produces cello-

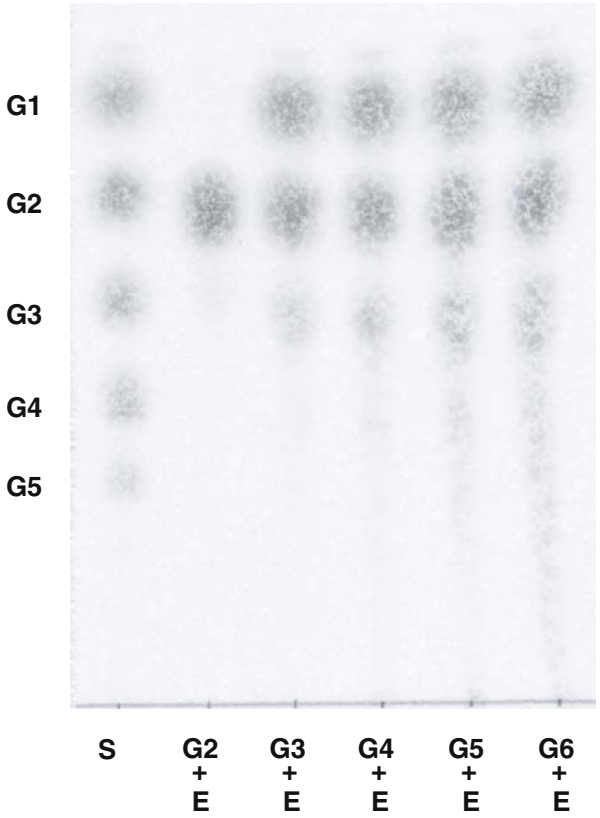

Fig. 4 TLC analysis of the hydrolysis products of cello-oligosaccharides (G2 to G6) by RsSymEG. *S* Standard containing cellobiose (*G2*), cellotriose (*G3*), cellotetraose (*G4*), and cellopentaose (*G5*). *E* purified RsSymEG1

oligosaccharides of various lengths [33], while cellobiohydrolase produces cellobiose from cellulose and β-glucosidase produces glucose from oligosaccharides. RsSymEG1, on the other hand, produces cellobiose and glucose from cellulose, even though all of the sequence features indicate that the enzyme should be endoglucanase. Cellulases have exhibited β-glucosidase activity in some cases, albeit only a few [34]. RsSymEG1, however, lacks a β-glucosidase-like sequence in its amino acid sequence.

Our analysis failed to detect any transglycosylation activity, though the products were not analyzed in a serial manner (data not shown).

These results suggest that RsSymEG1 preferentially hydrolyzes the β-1,4-cellulosidic linkages of cellodextrins, except for cellobiose, and produces glucose and cellobiose as final products.

Conclusions

In this paper, we have described the expression and properties of a GHF7 EG, RsSymEG1, an enzyme whose transcript was identified in the symbiotic protist of the lower termite, *R. speratus*. To our knowledge, RsSymEG1 is the first GHF7 cellulase of symbiotic protist origin confirmed to be expressed in any heterologous expression system.

Findings from our study suggest the great potential of cellulases derived from symbiotic protists: possible high enzymatic capability, cf., the high specific activity and the high V_{max} value, although direct comparison of catalytic constants with completely purified fungal and bacterial cellulases is still needed. With these results, RsSymEG1 may very well be applicable to an efficient process of cellulose degradation.

In-depth analyses of RsSymEG1 and further methods to exploit novel cellulases of symbiotic origin will contribute to the development of efficient systems for harnessing plant biomass for industrial applications.

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