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# The GH26 β-mannanase RsMan26H from a symbiotic protist of the termite *Reticulitermes speratus* is an *endo*-processive mannobiohydrolase: Heterologous expression and characterization

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ABSTRACT

Symbiotic protists in the gut of termites are prominent natural resources for enzymes involved in lignocellulose degradation. Here we report expression, purification, and biochemical characterization of a glycoside hydrolase family 26 mannanase RsMan26H from the symbiotic protist of the lower termite, *Reticulitermes speratus*. Biochemical analysis of RsMan26H demonstrates that this enzyme is an endo-processive mannobiohydrolase producing mannobiose from oligo- and polysaccharides, followed by a minor accumulation of oligosaccharides larger than mannobiose. To our knowledge, this is the first report describing the unique mannobiohydrolase enzyme from the eukaryotic origin.



## 1. Introduction

Termites are important digesters in the forest ecosystems and also serious invaders of buildings. They thrive on dead plant biomass with the aid of microbial symbionts [1]. Termites possess two cellulolytic systems; one is endogenous cellulases [2,3] and another is the symbionts comprising prokaryotes and flagellated protists (single cell eukaryotes) in the hindgut [4]. The dual cellulolytic system that is well established in the lower termites enables almost complete decomposition of the cellulose (74–99%) and hemicellulose (65–87%) components of ingested plant biomass [1]. After physical breakdown of ingested wood into small particles by mandibles, cellulose component of plant cell wall is subjected to partial degradation by termite cellulases in the gut. Subsequently, the gut protists take up the partially digested wood particles into food vacuoles by phagocytosis [1,5] and degrade cellulose and hemicellulose to produce acetate, which is absorbed by termites as their energy and carbon source [1]. This efficient lignocellulolytic system of termites and their symbionts is dependent on the actions of various cellulases and hemicellulases. Therefore thus these enzymes are prominent natural resources for degradation of plant biomass that could be applicable to industrial process of biorefinery, e.g. production of second-generation biofuels which is based on lignocellulose [6].

Mannan is a major component of softwood hemicellulose (25–30% of total wood dry weight [7]). This polysaccharide is composed of a β-1,4-linked backbone containing mannose or a combination of glucose and mannose moieties, which can be substituted with α-1,6-galactosyl side chains [8]. Endo-1,4-β-mannanases (EC 3.2.1.78) cleave internal β-1,4-linkage of two mannose moieties or between mannose and glucose. They are currently classified into glycoside hydrolase (GH) families 5, 26, and 113 (see the Carbohydrate-Active Enzyme database (CAZy), http://www.cazy.org/ [9]) based on the amino acid sequences similarities [10]. Classically, GHs have been considered as *endo*- or *exo*-types of enzymes. *Endo*-enzymes are bound to and attack internal linkage of substrate chains, whereas *exo*-enzymes are bound to and attack chain ends. Furthermore, a concept of processivity, first described for α-amylases [11–13], is used to describe an enzyme that makes a

*Abbreviations:* RsMan26H, β-mannanase H from a symbiotic protist of *Reticulitermes speratus*; GH, glycoside hydrolase; TLC, thin layer chromatography; HPLC, high performance liquid chromatography.

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multiple attack on a substrate chain without dissociating from it, in contrast to a non-processive enzyme that cleaves a polymer chain randomly. Some cellulases and hemicellulases are known to display "intermediate" behaviors termed *endo*-processive manner [14–16]. To date, most β-mannanases are considered to be *endo*-random enzymes, whereas recent studies have suggested the diversity of β-mannanase reaction mechanism, e.g. CmMan5A [17] and CjMan26C [18] were shown to be an *exo*-mannosidase and an *exo*-mannobiohydrolase, respectively, although both enzymes had high sequence similarities to *endo*-mannanases.

We previously reported biochemical and structural analyses of a glycoside hydrolase family 26 endo-β-mannanase, RsMan26C, isolated from a cDNA library of symbiotic protists of the lower termite, *Reticulitermes speratus* [19,20]. Here we report the heterologous expression, purification, and characterization of another protistan GH26 β-mannanase, RsMan26H. Biochemical analysis of RsMan26H demonstrates that this enzyme is an *endo*-processive mannobiohydrolase, first described among the enzymes of eukaryotic origin. The *endo*-processive manner of RsMan26H was characterized by a prominent production of mannobiose from the onset of the reaction against oligo- and polysaccharides, followed by a minor accumulation of oligosaccharides larger than mannobiose.

## 2. Materials and methods

### 2.1. Strains

*Escherichia coli* strain DH5α was used for DNA manipulation. *Pichia pastoris* strain KM71H (Invitrogen, Carlsbad, USA) was used as a host for heterologous expression of the recombinant protein.

### 2.2. Gene cloning and protein expression

A cDNA fragment encoding the putative mature region of RsMan26H (DDBJ accession number AB824857) was amplified by PCR using the forward (*Eco*R I-RsMan26H F: 5′-CCGGAATTCCT TCCGCCAGCTGATGTC-3′; *Eco*R I site is underlined) and the reverse (*Not* I-RsMan26H: 5′-AAGGAAAAAGCGGCCGCTTACTCCACCTTCTGC ACATC-3′; *Not* I site is underlined) primers used for ligation into the *Eco*R I and *Not* I sites of the *P. pastoris* expression vector pPICZα-A (Invitrogen). PCR was performed using PrimeStar (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. After the construction of pPICZα-RsMan26H vector, we linearized approximately 2.5 μg of the plasmid DNA with *Bgl* II (Takara Bio) prior to transformation of *P. pastoris*. Electroporation and selection of transformants were performed according to the instruction manual of the EasySelect™ *Pichia* expression kit (Invitrogen). The recombinant RsMan26H was produced using a Mini jar-fermenter (TSC-M5L; Takasugi Seisakusho, Tokyo, Japan) equipped with a DO controller (DJ-1033; ABLE Corporation, Tokyo, Japan) according to the *Pichia* Fermentation Process Guidelines (Invitrogen). After 2 days in methanol-fed batch culture, the medium was collected by centrifugation (4 °C, 8000*g*, 30 min).

### 2.3. Purification

The culture supernatant was ultrafiltrated with a Kvick Lab Packet 100kD (GE Healthcare, Little Chalfont, UK) and concentrated with a Kvick Lab Packet 5kD (GE Healthcare) using a QuixStand System (GE Healthcare). The enzyme solution was purified on a HiTrap Phenyl FF (high sub) column (5 ml; GE Healthcare) by linear gradient of 30–0% ammonium sulfate in 50 mM Tris–HCl (pH 7.5). The sample was then fractionated on a HiTrap DEAE FF column (5 ml; GE Healthcare) by linear gradient of 0–1 M NaCl in 50 mM Tris–HCl (pH7.5). The protein was then treated with Endoglycosidase H (Endo H; New England Biolabs, Ipswich, USA) according to the manufacturer's instructions to remove *N*-linked glycans. After deglycosylation, the solution was applied on a HiLoad 16/60 Superdex 75 prep grade column (120 ml; GE Healthcare) and eluted with 20 mM Tris–HCl (pH7.5) containing 150 mM NaCl. The purity of the protein was confirmed by SDS–PAGE analysis. The N-terminal amino acid sequence was determined using a Procice 491HT (Applied Biosystems, Foster City, USA). Protein concentration was determined using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, USA), according to the Bradford method [21] using bovine serum albumin as the standard.

### 2.4. Enzyme assays

Polysaccharides and oligosaccharides used in the enzyme assays described below were purchased from Megazyme International (Bray, Ireland) except for locust bean gum from Sigma–Aldrich (St. Louis, USA) and guar gum from Wako Pure Chemical Industries (Osaka, Japan). β-Mannanase assay was conducted at 30 °C by adding 5 μl of appropriately diluted enzyme to 100 μl of 50 mM sodium acetate (pH 5.5) containing 0.5–5% (w/v) substrate. After 15-min incubation, the reaction was stopped by boiling for 5 min. The reducing sugars produced were measured with tetrazolium blue reagent [22] by the method described previously [23]. A standard curve was drawn using the solution containing mannose at different concentrations. One unit of enzyme activity was defined as the amount of enzyme which produces 1 μmol of reducing sugar (mannose equivalents) per minute. The effects of temperature and pH on the activity were determined using 0.5% (w/v) locust bean gum as a substrate in 50 mM sodium acetate (pH 5.5). The optimum temperature was determined by measuring the activity over the range of 10–70 °C for 15 min. Thermostability was evaluated by pre-incubating the enzyme solution at different temperatures from 20 to 60 °C for 30 min, then measuring the remaining activity at 30 °C for 15 min. The optimum pH and pH stability were determined using 50 mM sodium acetate (pH 3.0–6.0), 50 mM sodium phosphate (pH 5.5–8.0), and 50 mM glycine-NaOH (pH 8.0–10.0). The optimum pH was assayed over a range of pH 3.0–10.0 at 30 °C for 15 min. To evaluate pH stability, 5 μl of purified enzyme was first diluted in 100 μl of different buffers ranging from pH 3.0 to 10.0 and incubated at 4 °C for 30 min. The remaining activity was measured at 30 °C for 15 min. The reaction products released from mannooligosaccharides were separated on a TLC Silica gel 60 plate (Merck KGaA, Darmstadt, Germany) with a solvent system containing *n*-propanol–ethanol–water (7:1:2) and visualized by staining with 2.5 vol% anisaldehyde, 3.4 vol% sulfinic acid, and 1.0 vol% acetic acid in ethanol and baking at 100 °C for 5 min. The soluble products released from β-mannan (Megazyme International) were analyzed on a HPLC system equipped with a Corona™ Charged Aerosol Detector™ (ESA Biosciences, Chelmsford, USA). The supernatant was separated on a Shodex Asahipak NH2P-50 4E column (Showa Denko, Kawasaki, Japan) equipped with a guard column (Showa Denko) using the following elution conditions: 0–10 min, a linear gradient of acetonitrile/$H_2O$ (60/40 to 50/50, v/v); 10–15 min, acetonitrile/$H_2O$ (50/50, v/v); 15–20 min, acetonitrile/$H_2O$ (60/40, v/v).

## 3. Results and discussion

### 3.1. Heterologous expression of RsMan26H and its purification

Recombinant RsMan26H was expressed in methylotrophic yeast, *P. pastoris*, under the control of the alcohol oxidase (*AOX1*) promoter. The protein was successfully produced in the medium, confirmed by the activity assay and SDS–PAGE analysis (data not



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