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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

shown). The protein concentration finally reached to 0.67 g/L after 184 h of cultivation.

To purify the protein from the culture supernatant, the sample was applied to hydrophobic interaction chromatography (Fig. 1A) and then anion exchange chromatography (Fig. 1B). RsMan26H is predicted to have two potential sites of *N*-glycosylation as predicted by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). The purified protein displayed the doublet bands (approximately 42 and 43 kDa) after the anion exchange chromatography, whereas the calculated molecular weight of RsMan26H is 40 kDa. This result suggests that the recombinant RsMan26H was *N*-glycosylated. However, after the treatment of the purified protein with endoglycosidase H to remove *N*-linked glycans, the apparent sizes of the doublet bands did not change (data not shown). The sample was finally applied to the gel filtration chromatography (Fig. 1C and D). The total amount of the purified protein was 39.6 mg. N-terminal amino acid sequence of the purified enzyme was analyzed. The N-terminus of recombinant RsMan26H was just after the Kex2 protease cleavage site followed by the mature sequence of the mannanase.

3.2. Effects of temperature and pH on the activity and stability

The temperature and pH properties of RsMan26H were analyzed using the soluble locust bean gum galactomannan as a substrate. The optimal reaction conditions for RsMan26H were 40 °C and pH 5.0 (Fig. 2A and B). As to the temperature stability, RsMan26H retained more than 80% of activity below 30 °C, but the enzyme lost its activity above 40 °C (Fig. 2C). The thermostability of this mannanase was slightly lower than other glycoside hydrolases from symbionts of termites characterized to date; for example, GH7 endoglucanase from a symbiotic protist of *R. speratus* was stable below 40 °C (retaining more than 80% of maximum activity) but unstable above 45 °C [24]. RsMan26H was stable over a range of pH 5.0–6.5 (retaining more than 80% of maximum activity).

3.3. RsMan26H displays an endo-processive mannobiohydrolase activity

To explore the biochemical properties of RsMan26H, we measured the activity of this enzyme against manno-oligosaccharides. Interestingly, RsMan26H predominantly produced mannobiose as a reaction product from manno-oligosaccharides. RsMan26H failed to degrade mannobiose (data not shown), but produced mannose and mannobiose from mannotriose (Fig. 3A), and solely mannobiose from mannotetraose (Fig. 3B). The enzyme transiently generated mannotriose from mannopentaose, followed by a reduction of the trisaccharide and an accumulation of the disaccharide (Fig. 3C). Likewise, RsMan26H displayed a transient accumulation of mannotetraose from mannohexaose, followed by a reduction of the tetrasaccharide and a substantial accumulation of the disaccharide (Fig. 3D). This profile of RsMan26H was in contrast to that of classical *endo*-random acting mannanases, for example CjMan26B [25], which generate reaction products of various sizes from manno-oligosaccharides. Thus, these results suggest that RsMan26H hydrolyzes its substrate in a processive manner (discussed below in more detail).

The characteristic of RsMan26H against polysaccharides was examined by analyzing the reaction products of β-mannan using TLC (Fig. 4A). RsMan26H produced exclusively mannobiose from the onset of the reaction against β-mannan, followed by a minor

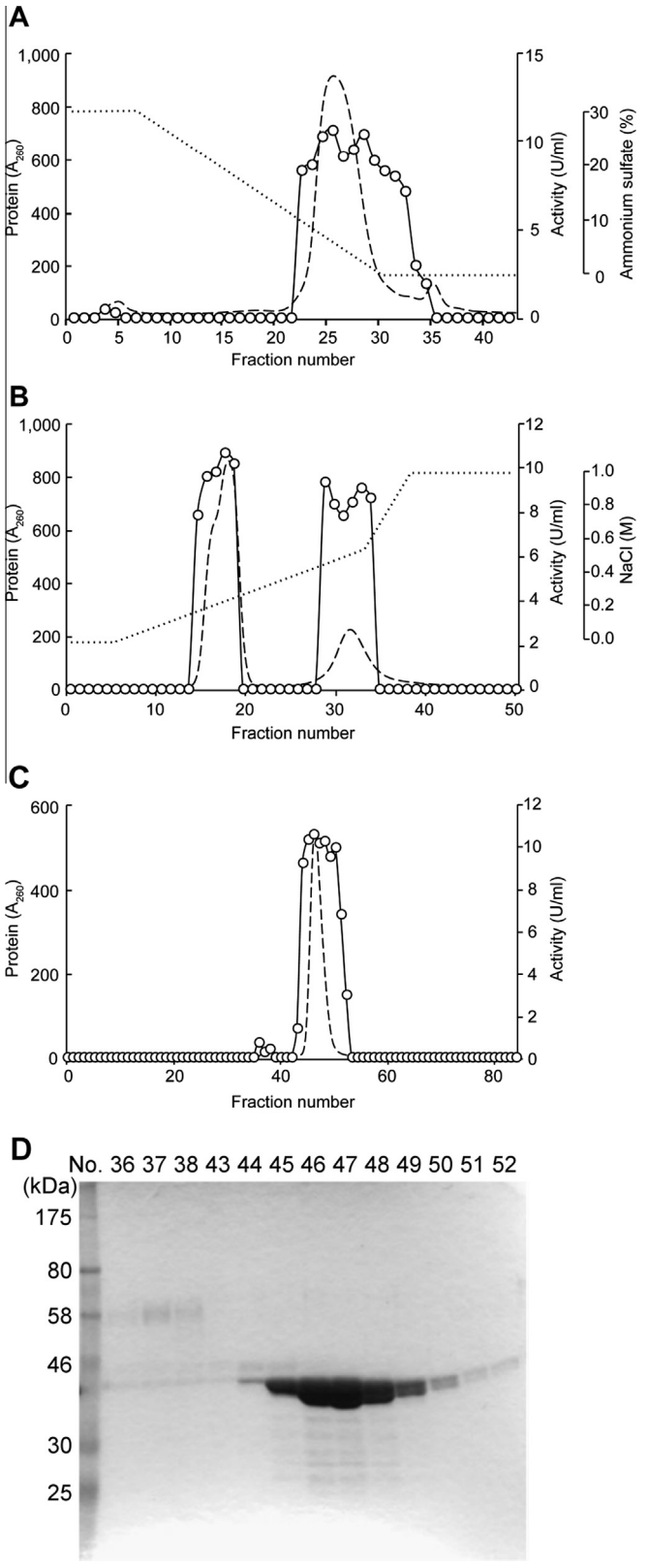

Fig. 1. Purification of RsMan26H. (A) First chromatography on HiTrap Phenyl FF column. (B) Second chromatography on HiTrap DEAE FF column. (C) Third chromatography on HiLoad 16/60 Superdex column. Protein (A_{260}, dashed line), mannanase activity (open circles), and buffer concentration (dotted line) were monitored. (D) SDS–PAGE analysis of third chromatography.

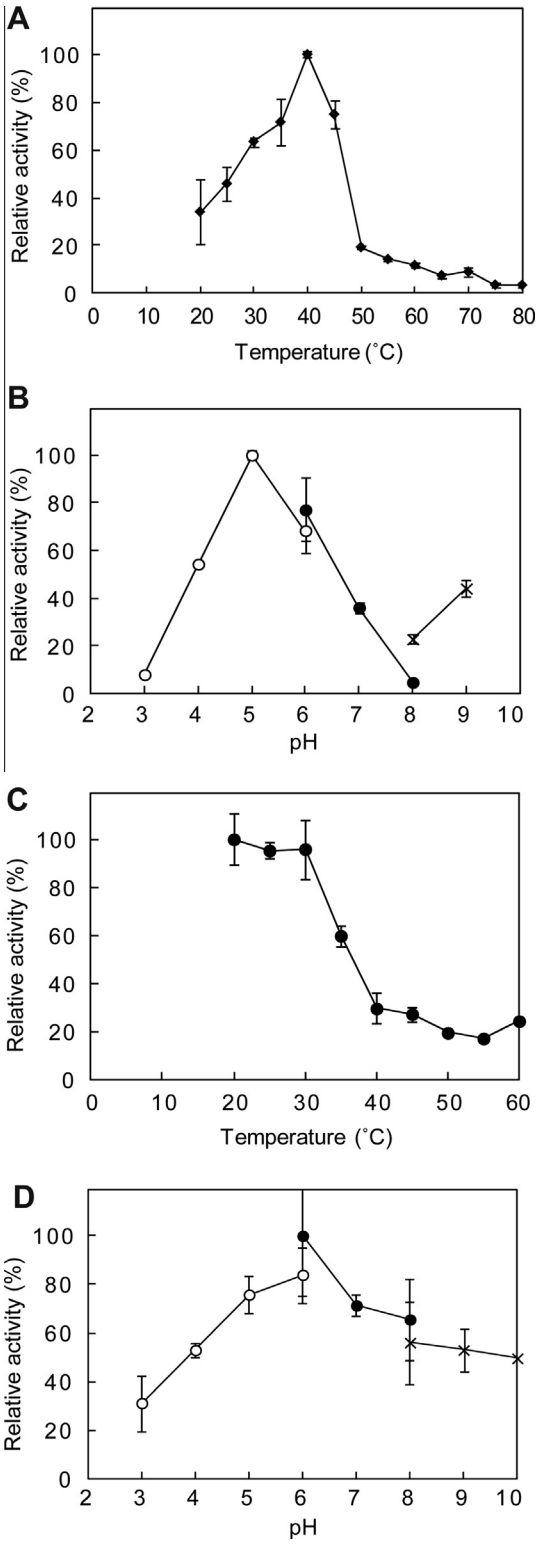

Fig. 2. Temperature and pH optima and stability of RsMan26H. (A) Temperature optimum. The enzyme reaction was performed at temperatures ranging from 10 to 80 °C for 15 min. (B) Optimum pH. The enzyme reaction was performed at different pH over the range of pH 3.0–10.0. (C) Thermostability. The purified enzyme was pre-incubated at 20–60 °C for 15 min and the remaining activity was measured. (D) pH stability. To evaluate the pH stability, 5 μl of purified enzyme was first diluted in 100 μl of different buffers ranging from pH 3.0 to 10.0 without substrate and incubated at 4 °C for 30 min. The remaining activity was measured. The buffers used were 50 mM sodium acetate buffer (pH 3.0–6.0; open circles), 50 mM sodium phosphate buffer (pH 6.0–8.0; closed circles), and 50 mM glycine-NaOH buffer (pH 8.0–10.0; crosses). Error bars are given as means ± SE of three independent experiments.

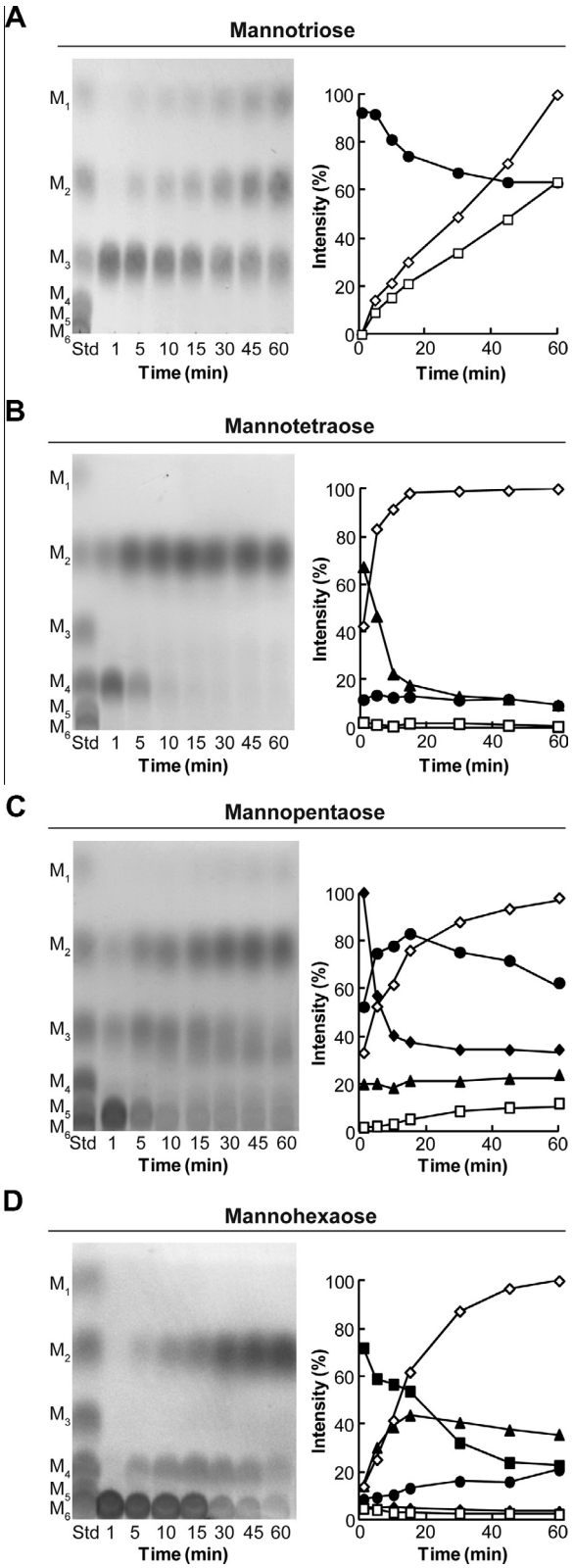

Fig. 3. Hydrolysis of manno-oligosaccharides. (A) Time course analysis of hydrolysis products of mannotriose by RsMan26H. (B) Time course analysis of hydrolysis products of mannotetraose. (C) Time course analysis of hydrolysis products of mannopentaose. (D) Time course analysis of hydrolysis products of mannohexaose. M_1, mannose (open squares); M_2, mannobiose (open diamonds); M_3, mannotriose (solid circles); M_4, mannotetraose (solid triangles); M_5, mannopentaose (diamonds); M_6, mannohexaose (solid squares); Std, mannooligosaccharide standard.

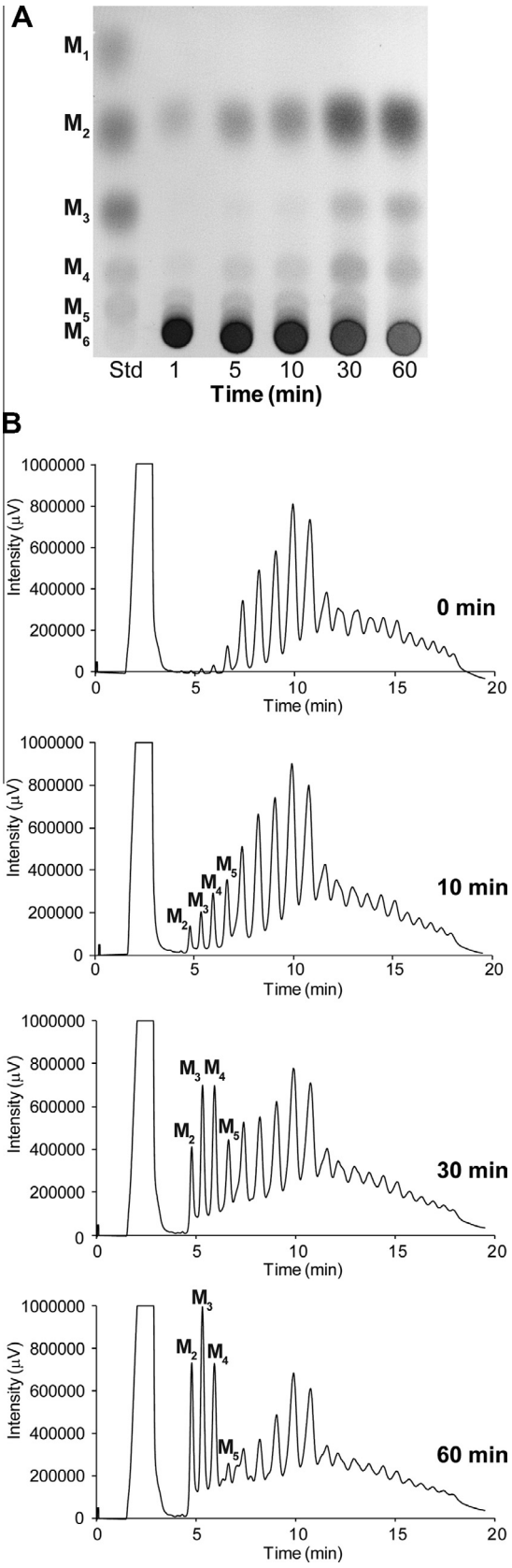

Fig. 4. Hydrolysis of linear β-mannan. (A) TLC analysis of hydrolysis products of linear β-mannan by RsMan26H. M_1, mannose; M_2, mannobiose; M_3, mannotriose; M_4, mannotetraose; M_5, mannopentaose; M_6, mannohexaose; Std, mannooligosaccharide standard. (B) HPLC analysis of hydrolysis products of β-mannan by RsMan26H.

accumulation of mannotriose and mannotetraose. This profile is typical for a processive enzyme. Processive glycoside hydrolases degrade a polymer chain without dissociating from it during several catalytic events [16]. In general, β-mannanases are thought to be *endo*-random acting enzymes, which show a transient accumulation of longer chain oligosaccharides followed by an accumulation of shorter chain oligosaccharides. However, the hallmark of RsMan26H, releasing mannobiose as a major product from β-mannan, is dissimilar to *endo*-random mannanases and it is the expected feature of a processive enzyme. Therefore RsMan26H can be defined as a *mannobiohydrolase*, which produces mannobiose with a multiple attack on a β-mannan chain.

The major release of mannobiose by RsMan26H raises the question of whether this enzyme acts with *exo*- or *endo*-mode. To address this question, we analyzed the reaction products of β-mannan using HPLC (Fig. 4B). The oligosaccharides with high degree of polymerization (predicted to be 5–20) were observed at the beginning of the reaction. The major accumulation of mannobiose, mannotriose, and mannotetraose was displayed after 30–60 min, preceded by the minor accumulation of mannopentaose and mannohexaose after 10–30 min. This oligosaccharide profile, which is in line with the results of the TLC analysis (Fig. 4A), indicates that RsMan26H is not a strict *exo*-mode enzyme, producing a single reaction product, but an *endo*-mode enzyme. For unknown reason, the sensitivity to mannobiose was considerably lower in HPLC than in TLC, which was confirmed by analyzing the standard manno-oligosaccharides (data not shown). Taken together, these data shown in Figs. 3 and 4 demonstrate that RsMan26H is an *endo*-processive mannobiohydrolase.

Although most mannanases characterized to date are thought to be *endo*-random acting enzymes, three bacterial mannanases displaying *mannobiohydrolase* activity were reported in the previous studies. In 1980s, Araki and Kitamikado isolated a β-1,4-mannan mannobiohydrolase (EC 3.2.1.100) from *Aeromonas* sp. F-25, an intestinal bacterium of freshwater fish [26,27]. Later, a GH26 mannanase CjMan26C from Gram-negative soil bacterium *Cellvibrio japonicus* was shown to be a mannobiohydrolase and its crystal structure and biochemical properties were analyzed in detail [18]. Recently, another mannobiose-forming GH26 exo-mannanase ManA was found from *Bacteroides fragilis* NCTC 9343 strain [28]. These mannobiohydrolases reported before are *exo*-mode enzymes to release only mannobiose from β-mannan. On the other hand, RsMan26H was demonstrated to be an *endo*-processive mannobiohydrolase to generate small amounts of mannotriose and mannotetraose in addition to mannobiose. It is interesting that these enzymes sharing the mannobiohydrolase activity were found in the unrelated species, i.e. intestinal bacterium of fish, soil bacterium, human colonic bacterium, and a symbiotic protist of the termite gut.

3.4. The capacity of RsMan26H to accommodate galactosyl and glucosyl substitution in mannan chain

Mannan can be decorated with α-1,6-linked galactosyl residues in galactomannan and also can be substituted with glucosyl residues in the backbone of glucomannan [29]. To examine the capability of RsMan26H to accommodate galactose side chain or glucose in the main chain, we measured the activities of RsMan26H against galactomannan (locust bean gum and guar gum) and konjac glucomannan. RsMan26H generated only a small amount of mannobiose from both locust bean gum and guar gum (data not shown) in contrast to the prominent production of mannobiose from β-mannan (Fig. 4). The specific activities against locust bean gum and guar gum were 3.4- and 116-fold lower,

Table 1
Specific activity of RsMan26H against mannose-containing polysaccharides.

Substrate	Specific activity (U/mg)[d]
β-Mannan[a]	11.6
Ivory nut mannan[b]	2.3
Locust bean gum[a]	3.4
Guar gum[c]	0.1
Konjac glucomannan[a]	0.6

The polysaccharide substrates were prepared as follows.
[a] Each substrate (0.5%) was dissolved in 50 mM sodium acetate buffer (pH 5.5).
[b] Ivory nut mannan (5%) was dissolved in 50 mM sodium acetate buffer (pH 5.5).
[c] Guar gum (2%) was dissolved in 50 mM sodium acetate buffer (pH 5.5).
[d] Enzyme unit (U) was defined as the amount of the enzyme that produces 1 μmole of reducing sugar per minute.

respectively, than β-mannan (Table 1). These data demonstrate that the galactose side chains can be partially accommodated by RsMan26H but the abundance of the galactose side chains can affect the enzyme in attacking the main chain of mannose units because the ratio of mannose to galactose units is 4:1 in locust bean gum and 2:1 in guar gum. In addition, no reaction products from glucomannan were observed and the specific activity against glucomannan was very low (Table 1), suggesting that the glucosyl moieties in the mannan backbone cannot bind to the active site of RsMan26H.

Here we produced and characterized a GH26 β-mannanase RsMan26H from a symbiotic protist in the hindgut of the lower termite *R. speratus*. In contrast to other well-known GH26 mannanases, RsMan26H showed a substantial accumulation of mannobiose and additional accumulation of mannotriose and mannotetraose from β-1,4-linked mannose polymer, which suggests that RsMan26H displayed a unique *endo*-processive mannobiohydrolase activity. These findings will help reveal in more detail the mechanism of termite symbiosis where lignocellulosic biomass is degraded and utilized with high efficiency.

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