Gene cloning, expression, and X-ray crystallographic analysis of a ƒÀ-mannanase from Eisenia fetida

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Highlights

1. The first report of cloning, expression and X-ray crystallographic analysis of a β-mannanase from *Eisenia fetida*.

2. The amino acid sequence of Ef-Man showed similarity with endo-1,4-β-mannanases from invertebrate.

3. The Ef-Man gene was expressed in *Pichia pastoris*.

4. Ef-Man requires at least six subsites for efficient hydrolysis.

5. Overall structure of recombinant Ef-Man is similar to those of GH5 family proteins.
Gene cloning, expression, and X-ray crystallographic analysis of a β-mannanase from *Eisenia fetida*

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1. Introduction

Hemicellulose is the main structural component of plant cell walls [1]. β-mannan, the second most abundant hemicellulose in nature, is classified into four different types of mannopolysaccharide (linear mannan, galactomannan, glucomannan, and galactoglucomannan) based on its branching structure and sugar composition [2, 3, 4, 5]. Mannan and mannooligosaccharides have varied biological functions and many potential applications in a wide range of fields [6]. It has been shown that mannans can alleviate intestinal disorders as a dietary fiber [7], and mannooligosaccharides from mannose can act as prebiotics, improving intestinal microflora conditions [8].

β-1,4-mannans are present in some types of plant seeds [2, 4, 9], and in the cell walls of some algae [10]. The endo-1,4-β-mannanase (EC 3.2.1.78) enzyme hydrolyzes internal β-1,4-mannosyl linkages of β-mannan, producing mannooligosaccharides such as mannotriose and mannobiose [11, 12, 13]. β-mannanases have been used to improve product quantity in various bioprocesses, such as the bleaching of softwood pulps, viscosity reduction of feeds and foods, and beverage clarification. 1,4-β-mannanase has been isolated from bacteria [14, 15], fungi [12, 16], higher plants [17, 18], mollusks [19, 20, 21], and arthropods [22]. However, there are no reports to date on mannanase from annelida earthworms.

Earthworms belong to the phylum Annelida and are known to hydrolyze carbohydrates, which contributes to their ability to digest leaf litter, roots, yeast, brown algae, and fungi in soil. We previously purified and characterized Ef-CMCase25, a raw-
starch digesting amylase, and 1,3-\(\beta\)-glucanase from the earthworm *Eisenia fetida* [23, 24, 25]. In addition, the endo-1,4-\(\beta\)-glucanase (Ef-EG2) gene from *E. fetida* was cloned, expressed, and the crystal structure analyzed [26, 27]. We also found high mannanase activity in body wall extracts of *E. fetida*. To understand the structure and function of Ef-Man, we have performed gene cloning, expression, and crystal structure analysis of the \(\beta\)-1,4-mannanase from *E. fetida*. To our knowledge, this is the first report on the structure and function of \(\beta\)-1,4-mannanase from annelida earthworms.
2. Materials and Methods

2.1. Chemicals

Locust bean gum (galactomannan with a mannan:galactose molar ratio of approximately 4:1) and birch wood xylan were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Guar gum (galactomannan, mannose:galactose ratio approximately 2:1) was obtained from Wako Pure Chemical Co. (Osaka, Japan). Konjac powder (glucomannan, mannose:glucose ratio approximately 3:2) was obtained from Shimizu Chemical Corporation (Hiroshima, Japan). High purity linear mannan (mannan:galactose ratio of 98:1, DP~15) was obtained from Megazyme (Bray, Ireland). Cellulose powder was obtained from ADVANTEC (Tokyo, Japan) and starch (soluble starch, amylopectin) was obtained from Kanto Chemical Co. (Tokyo, Japan).

2.2. Animal strain, coelomic fluid exclusion, and homogenization

Experiments were done with *E. fetida* obtained from Nagane Industry (Sapporo, Japan) and worms of approximately the same age were used. The earthworms were washed in fresh water, placed on wet filter paper, and starved for 24 h at 20°C. The worms were then freeze-dried and ground to a fine powder with a mortar and pestle. The resulting powder (10 g) was suspended in 50 mM Tris-HCl buffer (pH 7.0). The suspension was gently mixed in a reciprocal shaker for 1 h at 4°C. The suspension was centrifuged at 27,000 g for 20 min. The supernatant was used as a crude enzyme solution.
2.3. Enzyme assay and protein determination

Mannanase activity was measured by determining the amount of reducing sugar released from locust bean gum. The enzyme activity required to form the amount of reducing sugar corresponding to 1 µmol of mannose per min of reaction time was regarded as one unit of enzyme activity. The reaction mixture consisted of 0.4% locust bean gum in 0.1 M sodium acetate buffer (pH 5.0) and enzyme, resulting in a final volume of 0.3 mL. After incubation for 20 min at 37°C, the amount of reducing sugar contained in the sample was then determined according to the Somogyi-Nelson method [28, standard assay method]. The protein assay was performed using a Micro BCA protein assay kit (ThermoFisher Scientific) with bovine serum albumin as the standard. Ef-Man protein concentration was calculated at an absorbance of 280 nm and the protein extinction coefficient was determined according to the method described by Gill and von Hippel [29].

2.4. Isolation of total RNA and cDNA synthesis

Total RNA was extracted from the freeze-dried worm powder using Isogen II (Nippon Gene, Japan) according to the manufacturer’s instructions. First-strand cDNA was synthesized using an oligo(dt)17 adapter primer (GGCCACGCGTCTAGACTAGTACTTTTTTTTTTTTTTTT) and Superscript III reverse transcriptase (Invitrogen, USA) according to the manufacturer’s instructions.
2.5. cDNA cloning of the β-mannanase gene and expression plasmid construction

An EST library containing about 70,000 contigs from *E. fetida* mRNA has been constructed previously (data not shown). The β-mannanase gene (contig no. c5810) was found in the EST library. cDNA cloning of the β-mannanase gene was conducted based on the mRNA sequence of contig no. c5810. Forward (5'-GGCTGAAGCTGAATTC CAGCAATTCCTGGACGT-3’, EcoRI site is underlined) and reverse (5’-GAGTTTTTGTCTAGAAACTGGAGAATAATAT-3’, XbaI site is underlined) primers for PCR were synthesized targeting the regions corresponding to amino acid residues 17-21 and 374-377 of mature β-mannanase from *E. fetida*, respectively. PCR was done in a reaction mixture (20 µL) containing the *E. fetida* cDNA, 0.5 µM of each primer, and 10 µL Takara PrimeSTAR Max premix (Takara Bio, Kyoto, Japan), using the following thermocycling conditions: one cycle at 98°C for 1 min, 30 cycles of 98°C for 10 sec, 60°C for 5 sec, and 72°C for 60 sec, and one cycle at 72°C for 5 min. A DNA fragment of 1.2 kb obtained by PCR was cloned into the EcoRI and XbaI sites of the pPICZαA expression vector (Invitorgen). The nucleotides of the amplified fragment were confirmed by sequencing after ligation. This expression plasmid coding mature Ef-Man was named pPICZαA-Ef-Man.

2.6. Expression and purification of recombinant enzyme
The expression plasmid pPICZA-Ef-Man was linearized by SacI and transformed into competent *Pichia pastoris* GS115 cells by electroporation. Cells were spread on YPDS medium (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 1.5% agar) containing 100 µg/mL of Zeocin and incubated at 28°C for 2-4 days. Colonies were picked and spread onto YPDS plates containing 100, 500, 1000, or 2000 mg/mL of Zeocin. Colonies capable of growth in the presence of a high concentration of Zeocin were selected. The selected colonies were then cultured in a 500 mL Erlenmeyer flask containing 25 mL BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4x 10^{-5} % biotin, 1% glycerol) at 28°C. The cultured media was centrifuged at 3,000 g for 5 min and resulting cell pellets were resuspended in BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4x 10^{-5} % biotin, 0.5% methanol). The cell suspension was then added to a jar-fermenter containing 2.9 L of BMMY medium and grown at 17°C for 7 days at 300 rpm for aeration at 3 L/min, with daily additions of 0.5% methanol. After cultivation, the cultured media was centrifuged at 8,200 g for 10 min at 4°C before the supernatant was recovered. Ef-Man was precipitated from the supernatant with ammonium sulfate (80% saturation) followed by centrifugation at 20,000 g for 30 min. The precipitate was dissolved in 20 mM phosphate buffer (pH 7.0). Ef-Man was loaded onto a HisTrap HP column (column volume: 5 mL) (GE Healthcare, Little Chalfont, Buckinghamshire, UK) equilibrated with 20 mM phosphate buffer (pH 7.0) containing 0.5 M NaCl. The enzyme was eluted with a linear gradient of imidazole (0-500 mM) in 20mM Tris-HCl buffer (pH 7.0) containing 0.5 M NaCl. The active fractions after
HisTrap HP column chromatography were dialyzed with cellulose tubes in 20 mM phosphate buffer (pH 7.0). The dialyzed enzyme solution was loaded onto a HiTrap DEAE FF column (GE healthcare) equilibrated with 20 mM phosphate buffer (pH 7.0). The enzyme was eluted with a linear gradient of NaCl (0-500 mM) in 20 mM Tris-HCl buffer (pH 7.0). The active fractions after HiTrap DEAE FF column chromatography were concentrated with Vivaspin 500 (10 kDa MWCO) (GE Healthcare). The concentrated enzyme solution was loaded onto a Superdex 75 10/300GL (GE Healthcare) gel filtration column equilibrated with 20 mM phosphate buffer (pH 7.0) containing 0.2 M NaCl. The enzyme was eluted with 20 mM phosphate buffer (pH 7.0) containing 0.2 M NaCl. Active fractions were pooled and used as the purified enzyme solution.

2.7. Effects of pH and temperature on enzyme activity

Enzyme activities were measured using the standard assay method with 0.4% locust bean gum as substrate at various pH and temperature. The buffer systems used were 0.1 M glycine-HCl buffer (pH 2.0 to 3.0), 0.1 M sodium acetate buffer (pH 3.0 to 6.0), 0.1 M KH₂PO₄-K₂HPO₄ buffer (pH 6.0 to 8.0), 0.1 M Tris-HCl buffer (pH 8.0 to 9.0), and 0.1 M NaHCO₃-Na₂CO₃ buffer (pH 9.0 to 10.0). The effect of temperature on enzyme activity was examined from 4-90°C.

2.8. Effects of pH and temperature on enzyme stability
The effect of pH on enzyme stability was examined by incubating the enzyme (2.0 units/mL) for 24 h at 4°C in the following buffers of 0.1 M: glycine-HCl buffer (pH 2.0 to 3.0), sodium acetate buffer (pH 3.0 to 6.0), KH$_2$PO$_4$-K$_2$HPO$_4$ buffer (pH 6.0 to 8.0), Tris-HCl (pH 8.0 to 9.0), and NaHCO$_3$-Na$_2$CO$_3$ buffer (pH 9.0 to 10.0). For measuring thermal stability, the purified enzyme (2.0 units/mL) was incubated in 0.1 M KH$_2$PO$_4$-K$_2$HPO$_4$ buffer (pH 7.0) for 30 min at various temperatures in the range of 10-80°C. After incubation, the remaining activities were measured under standard assay conditions. The average values of triplicate measurements were used for each activity value.

2.9. Molecular mass

The molecular mass was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli [30] with a Precision Plus Protein standard (Biorad Co., CA, USA). Protein bands were detected by staining with Coomassie Brilliant Blue R-250.

2.10. Substrate specificity

The activities of the purified rEf-Man were tested using polymers CMC, locust bean gum, konjac powder, guar gum, starch, and birchwood xylan. In each case, breakdown was assessed by the production of reducing sugars and measured as described above. The average values of triplicate measurements were used for each activity value.
2.11. HPLC (High-performance liquid chromatography) and HPAEC (High-performance anion exchange chromatography) analysis of hydrolysis products from mannan and mannooligosaccharides

To identify the cleavage pattern of the hydrolysis products of the purified enzyme, 5 mM mannooligosaccharide substrates (Man$_2$-$\alpha$) (Megazyme Inc, USA) were dissolved in 50 mM sodium acetate buffer (pH 6.0) and several microliters of the enzyme solution was added to 200 μL of each substrate solution. After the enzyme reaction was carried out at 37°C for an appropriate period of time, a portion of the reaction mixture was withdrawn and mixed with 100% chilled acetonitrile (-20°C, acetonitrile final concentration of 70%) to terminate the enzymatic reaction. The resulting solution was used for HPLC with a column of Sugar-D (4.6 x 250 mm, Nakarai Tesque, Kyoto, Japan). Elution was conducted with 70% acetonitrile at a flow rate of 1.0 mL/ min, and the substrates and products were monitored by RI (Jasco Co., Tokyo, Japan). From the peak area obtained by HPLC, oligosaccharide concentrations at each reaction time were calculated using a standard curve from an authentic saccharide solution and plotted against reaction time.

The hydrolysis products from high purity mannan (Megazyme) were analyzed as follows. High purity mannan was dissolved at 20 mg/mL in 5% (w/v) sodium hydroxide. Upon neutralization with 50% acetic acid, the mannan precipitates from solution as a very fine colloidal suspension. The suspension was filtered using an Omega$^{\text{TM}}$650D Membrane (Pall Co. NY, USA, 650 Da cut). The high purity mannan was mixed gently
before removing aliquots for use in enzyme assays. Reaction mixtures consisting of 200 µL of high purity mannan in 50 mM sodium acetate buffer (pH 5.5) and 10 µL of enzyme solution (2.5 U/mL) was incubated at 37°C for 4 min, 16 min, and 24 h. After each reaction time, the mixture was boiled for 5 min. Each sample solution was centrifuged at 15,000 rpm for 5 min, and the supernatant was used for HPAEC with a CarboPac PA-1 column (4 x 250 mm, Thermo Fisher Scientific). Elution was conducted with 0.1 M NaOH (0-5 min), 0.1 M NaOH, 0-0.45 M sodium acetate (5-35 min), 0.1 M NaOH, 0.8 M sodium acetate (35-40 min), and 0.1 M NaOH (40-55 min) at a flow rate of 1.0 mL/min, and the substrates and products were monitored with a pulsed amperometric detector.

2.12. Nucleotide sequence accession number

The E. fetida Ef-Man mRNA data reported in the present paper have been submitted to the DDBJ, EMBL, and NCBI databases under the accession number LC153775.

2.13. Crystallization

For crystallization experiments, rEf-Man was further purified with Mono Q 4.6/100 PE columns (GE Healthcare). The enzyme was eluted with a linear gradient of 0-1 M NaCl in 20 mM KH₂PO₄-K₂HPO₄ buffer (pH 7.0). The purified enzyme was concentrated to 4.0 mg/mL with an Amicon Ultra 10 kDa MWCO filter (Millipore). Initial crystallization trials were performed with commercial screening kits Crystal Screen,
Crystal Screen 2, PEG Rx 1 and 2, Salt Rx 1 and 2 (Hampton Research), and Wizard I, II, and Precipitant Synergy (Molecular Dimensions). rEf-Man crystals for X-ray diffraction data collection were prepared with the hanging-drop vapor diffusion method at 20°C. The drop solution consisted of 0.5 µL of protein solution and 0.5 µL of reservoir solution which contained 1 M ammonium citrate, 15% (v/v) iso-propanol, and 0.1 M Tris-HCl (pH 8.5). The drop was equilibrated against 500 µL of the reservoir solution.

2.14. Data collection, processing, phasing, and structure refinement

X-ray diffraction experiments were performed at the BL17A beamline of the Photon Factory (Tsukuba, Japan). The crystal was mounted on a litholoop (Molecular Dimensions) and was flash frozen in a nitrogen gas stream at -173°C (100 K) to collect diffraction data. The diffraction data set was integrated and scaled using the XDS program [31]. Phase determination was performed using the molecular replacement method with the Phaser program [32] using the crystal structure of β-mannanase from Mytilus edulis (PDB ID: 2C0H) [33] as a search model. Five percent of reflections were randomly selected to calculate the free R factor. Structure refinement of rEf-Man was performed using the Refmac5 program [34] and model modification was performed using the Coot program [35]. Figures of β-mannanase tertiary structures were prepared using the PyMOL program [36]. Calculation of electrostatic potential was performed using the APBS program [37]. Solvent accessible surface area (ASA) was calculated using the PISA web server [38].
2.15. Protein Data Bank accession code

The *E. fetida* rEf-Man structural data reported in the present paper has been deposited to the Protein Data Bank under the accession code 5Y6T.

3. Results and Discussion

3.1. cDNA cloning, sequence analysis of the Ef-Man gene, and phylogenetic Analysis of EF-Man

Mannanase activity was detected in the crude extract of *E. fetida*, and Ef-Man was found to be functionally expressed by the earthworm species *E. fetida*. The length of the Ef-Man gene was determined to be 1131 bp, and encodes a 377-amino acid protein (mRNA sequence contig no. 5810). The amino acid sequence of Ef-Man was similar to endo-1,4-β-mannanases of *Daphnia pulex* (Dp-Man, 62%, EFX76385.1), *Cryptopygus antarcticus* (Ca-Man, 64%, ABV68808.1), *Crassostrea gigas* (Cg-Man, 61%, XP_011432439.1), *Mytilus edulis* (Me-Man, 60%, Q8WPJ2), and *Aplysia kurodai* (Ak-Man, 58%, BAJ60954.1). The fact that these invertebrate 1,4-β-mannanases all belong to the glycoside hydrolase (GH) family 5 suggests that Ef-Man may also belong to this enzyme family. In addition, all catalytically important residues of endo-1,4-β-mannanase of the GH family 5 [39] were conserved in Ef-Man (Glu179, Glu309), as also shown for *C. antarcticus*, *M. edulis*, and *A. kurodai* (Fig. 1). The primary structure of Ef-Man
contains a signal peptide and a catalytic domain, as reported for Ca-Man, Me-Man, and Ak-Man.

Phylogenetic analyses were performed based on amino acid sequence homologies of Ef-Man and mannanases from other species. As a result, Ef-Man was classified as β 1,4-mannanases from Arthropods such as D. pulax and C. antarcticus (Fig. 2). β-mannanase from M. edulis has been defined as GH family 5 subfamily 10 [40]. Metazoan sequences including the sequences of mollusks and arthropods compose the GH family 5 subfamily 10. It was suggested that Ef-Man also belong to GH family 5 subfamily 10.

3.2. Expression of the Ef-Man gene in P. pastoris and properties of rEf-Man

The mature active form of Ef-Man was successfully expressed in P. pastoris. 1,4-β-mannanase activity of the recombinant crude enzyme solution was determined to be 20 units/mL culture. The rEf-Man purified in this study was from P. pastoris GS115 harboring pPICZαA-Ef-Man. The molar extinction coefficient of rEf-Man was 102140. The yield of purified enzyme was 10.2 mg. Based on SDS-PAGE analysis, the molecular mass of the purified rEf-Man was estimated to be 39 kDa (Fig. 2). It was almost the same molecular mass as Ef-Man calculated from amino acid sequences. It found that Ef-Man have no glycosylated from PAS staining (data not shown). Furthermore, Ef-Man didn’t have the consensus sequence for N-glycosylation (Asn-X-Ser or Thr, X is any amino acid except Pro). It was shown that Ef-Man dosen’t have N-glycosylated sugar chain.

The functional properties of purified rEf-Man were determined by enzyme assays
with locust bean gum as the substrate. The optimum pH of rEf-Man was found to be 5.5 (Fig. 3A). Its activity was most stable at pH 6.0-10.0 (Fig. 3B). Similar optimum pH values have been reported for other endo-1,4-β-mannanases, namely Me-Man [21] and Ak-Man [40]. In contrast, endo-1,4-β-mannanase from *C. antarcticus* (Ca-Man) exhibited an optimum pH value of 3.5. The optimal temperature of Ef-Man was identified as 60°C (Fig. 3C), and this value is similar to those of Me-Man (50°C) and Ak-Man (55°C). In contrast, other invertebrate mannanases such as *Haliotis discus hannai* mannanase (Hd-Man) [20] and Ca-Man have a lower optimum temperature and stability than Ef-Man. The optimal temperature of Ca-Man and Ak-Man was identified in the range of 30-55°C, and the enzymes exhibited significant activity at 15°C (30-60% of the activities at optimal temperatures) [39, 41]. We have already reported that the earthworm *E. fetida* has cold-adapted enzymes, namely Ef-CMCase25, Ef-EG2, raw starch-digesting α-amylases, and an anti-plant viral serine protease [23, 24, 27, 42]. However, Ef-Man showed only weak activity at lower temperatures, similar to Ef-Chi [43].

Enzymatic assays were conducted with 1,4-β-linked oligosaccharides of various lengths (mannotriose (M3), mannotetraose (M4), mannopentaose (M5), or mannohexaose (M6)) to clarify the mode of action of purified rEf-Man, and buffer aliquots collected over time were analyzed by HPLC (Fig. 4). The major hydrolysis products from M6 were M3, and minor products were M4, M5, and M2 (Fig. 4 (A)). The major hydrolysis products from M5 were M3 and M2, and minor products were M4 (Fig. 4(B)). The major hydrolysis products from M4 were M3 and M2 (Fig. 4(C)). In contrast, M3 was not degraded. The major products from high purity mannan were M2 and M3 (Fig. 5).
of the reactions, mannose (M1) was barely detected. It was also determined using HPLC that Ef-Man is the retaining enzyme from anomer analysis (data not shown). The hydrolysis pattern and products generated by Ef-Man were similar to those reported for Ca-Man, Hd-Man, and Ak-Man [20, 22, 41]. From these results, this enzyme can be considered an endo-type 1,4-β-mannanase. For example, M5 hydrolysis formed M4 but no mannose (M1), and the hydrolysis of M4 gave rise to M3 but almost no mannose. Such clear, unequal product formation is likely explained by the involvement of transglycosylation reactions. Transglycosylation has been demonstrated in invertebrate mannanases such as blue mussel Man5A (MeMan5A) [21], although the detailed mechanism of transglycosylation in invertebrate mannanases is not clear at the present time. From our results, E. fetida β-mannanase can be considered capable of performing transglycosylation reactions.

rEf-Man exhibited its highest activity (1920 units/mg protein) against locust bean gum. It was also active for guar gum and konjac powder. Conversely, the enzyme did not show any activity against starch, carboxy methyl cellulose, and birchwood xylan (Table 1). This indicates that Ef-Man preferably acts on linear β-1,4-mannan with no branches, showing a similar efficiency at hydrolyzing linear mannan as Ak-Man [10]. When M6, M5, and M4 were digested with rEf-Man, M6 (271 mM/min/mg protein) showed higher specific activity than M5 (33.4 mM/min/mg protein) and M4 (1.8 mM/min/mg protein) during the early stages of hydrolysis. It seems likely that EF-Man requires at least six subsites for efficient hydrolysis. MeMan5A, Man26A from Cellvibrio japonicas [44], and Ca-Man have been shown to utilize at least six subsites for efficient hydrolysis. In
contrast, it was reported that Man26A from *Cellulomonas fimi* requires five subsites, and the Man26B from *C. japonicas* (AAO31762) requires only four and is the most efficient M4 degrading β-mannanase.

### 3.3. Crystal structure of rEf-Man

Thin plate crystals of rEf-Man were obtained in one week. The diffraction data set was collected at 1.7 Å resolution (Table 2). The crystal belongs to the space group of *P*2₁2₁2₁ with unit cell dimensions of *a* = 50.1 Å, *b* = 69.5 Å, *c* = 86.7 Å. One molecule of rEf-Man was found in the asymmetric unit after phase determination by the molecular replacement method. The final model consists of amino acid residues from Gln17 to Gln377, representing a whole region of a mature protein. One tris molecule was found near the putative catalytic residues of Glu179 and Glu309. The binding position is almost the same as the tris molecule in the apo structure of Ca-Man (4OOU) and is located at the -1 subsite in the Ca-Man structure complexed with mannopentaose (4OOZ) [45]. One iso-propanol molecule was found near Trp43 and Trp81, which are adjacent to the tris molecule, and the position is on the minus side of the putative substrate binding site. The final *R*_{work} and *R*_{free} factors were 12.9% and 16.9%, respectively.

The overall structure of rEf-Man comprises a (β/α)₈ barrel which is the common fold in GH5 family proteins (Fig. 6(A)). It found that rEf-Man is monomer enzyme. The approximate overall dimensions are 50 x 50 x 40 Å. As similar to the β-mannanase structures of Ca-Man, Me-Man, and Ak-Man [46], eight β-strands (β-1~8) form a parallel
β-barrel in the inner region of rEf-Man. The positions of α-helices in rEf-Man also show similarities with other β-mannanase structures excluding α-H helix in Me-Man [33] which was not assigned in the rEf-Man structure using the DSSP program [47] (Fig. 1). At the N-terminal side of α-1 helix, Cys57 and Cys58 form a vicinal disulfide bond and the residues from Tyr55 to Thr59 were assigned as a π helix. On the other hands, α-1 helix extended to this region in other β-mannanase structures. Superposition among β-mannanase structures indicates that the root-mean-square distances between rEf-Man and Ca-Man (4OOU), rEf-Man and Me-Man (2C0H), and rEf-Man and Ak-Man (3VUP) were 0.84 Å for 336 Cα atoms, 1.03 Å for 328 Cα atoms, and 0.88 Å for 313 Cα atoms, respectively (Fig. 6(A)). Structures of the active site residues show high similarity among the β-mannanases (Fig. 6(B, C)). Small structural differences are observed in the loop region including Trp282. Two glutamic acids, Glu179 and Glu309, are putative catalytic residues, and other amino acids seem to be involved in substrate recognition by rEf-Man. The volume and shape of the catalytic cleft are consistent with the existence of six subsites (Fig. 6(C)). The location of putative subsites -3, -2, -1, +1, +2, and +3 are clarified from the model complex structure with mannohexaose (Fig. 6(C)) which was constructed by referring the complex structure between endo-1,4-β-mannanase from \textit{Streptomyces thermolilacinus} (St-Man) and mannohexaose [48]. Two conserved hydrophobic residues (Trp43 and Tyr46) are located in the proximity of the subsites -3 and -2, and they may be important for substrate recognition. A prolonged loop (pLoop) from Gly343 to Ala347 connects β-8 strand and α-8 helix in rEf-Man (blue box in Fig. 1). The pLoop region surrounding the active site (blue circle in Fig. 6(A, C)) constructs
the -2 and -1 subsites. The length of the loop in rEf-Man is the same as that in Ca-Man, and the loop structures also show high similarity. However, the length of the loop in rEf-Man is longer than those of Me-Man and Ak-Man. The loop in rEf-Man corresponds to an insertion of 5 or 6 amino acid residues, as opposed to Me-Man or Ak-Man. The pLoop region forms a wall in the cleft of subsites -2 and -1.

Many aromatic residues are situated in the catalytic cleft and seem to participate in substrate recognition (Fig. 6(B)). By referencing the substrate binding mode in the Ca-Man and St-Man complex structure, two conserved tryptophan residues, Trp207 and Trp241, seem to be responsible for substrate recognition by CH/π interaction at subsite +1 and +2, respectively. The mutation of tryptophan at subsite +1 has been shown to affect the transglycosylation reaction in GH5 β-mannanase from *Aspergillus nidulans* [49]. In addition, the side chain of Trp282 (histidine in Ca-Man; tryptophan in St-Man) is oriented in a way that a sugar ring can be stacked at subsite +3. Such tight substrate recognition at “plus” subsites is known to be a key determinant of transglycosylation activity in other glycoside hydrolases [50]. The tight substrate recognition also indicates that binding of M3 is stabilized at subsites +1, +2, and +3. This expectation is supported by the hydrolysis pattern in Ef-Man, that M3 is the major products from M6, M5, and M4 (Fig. 4) and that M3 is not degraded. X-ray crystallographic analysis of a complex of rEf-Man and its substrate will further elucidate the mechanisms of hydrolysis and transglycosylation by Ef-Man.
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References


Legends

Fig. 1  Structure based sequence alignment of endo-β-1,4-mannanases from *Eisenia fetida* (Ef-Man), *Cryptopygus antarcticus* (Ca-Man, 46%; ABV68808.1), *Mytilus edulis* (Me-Man, 45%; Q8WPJ2), and *Aplysia kurodai* (Ak-Man, 42%; BAJ60954.1). The percentages in parentheses represent the sequence identities with the amino acid sequence of Ef-Man. Black background indicates the conserved amino acids among the endo-β-1,4-mannanases. Red characters indicate the catalytically important amino acids (Glu179 and Glu309 in Ef-Man). The gray box indicates the putative signal sequence. The prolonged loop (pLoop) around the active site is indicated by the blue box. The positions of β-strands, α-helices, 3_{10} helices, and π helices are shown as green, orange, hot pink, and light pink bars, respectively. The nomenclature of secondary structure elements refers to that of Me-Man [33]. All sequences are numbered from the Met-1 of the peptide.

Fig. 2  Phylogenetic tree of mannanase from *E. fetida* (Ef-Man) and other species, and of endo-1,4-beta-mannosidase-like proteins from GH family 5. Enzyme names and accession numbers were as follows: *Daphnian pulex* endo-beta-1,4-mannanase (Da-Man, EFX76385.1), *Cryptopygus antarcticus* beta-1,4-mannanase precursor (Ca-Man, ABV68808.1), *Biomphalaria glabrata* mannan endo-1,4-beta-mannosidase-like precursor protein (Bg-Man, NP_001298233.1), *Haliotis discus hannai* beta-1,4-mannanase (Hd-Man, BAE78456.1), *Mytilus edulis* beta-Mannanase (Me-Man, 2C0H_A), *Crassostrea gigas* mannan endo-1,4-beta-mannosidase-like protein (Cg-Man, XP_011432439.1), *Flammeovirga yaeyamensis* beta-1,4-mannanase (Fy-Man,
ACA05117.2), *Aplysia kurodai* beta-1,4-mannanase (Ak-Man, BAJ60954.1). We used
Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) to construct the phylogenetic
tree.

Fig. 3  SDS-PAGE of the purified *E. fetida* endo-1, 4-β-mannanase. M: Precision Plus
Protein All Blue Standard (Biorad, USA). 1: Purified endo-1, 4-β-mannanase (about 2
μg).

Fig. 4  Functional properties of purified rEf-Man. All reactions were conducted with
purified enzyme using locust bean gum as the substrate. (A) Effect of pH on enzyme
activity at 37°C in 0.1 M of the following buffers: ◆, glycine-HCl (pH 2.0-3.0); ■, sodium
acetate (pH 3.0-6.0); ▲, KH₂PO₄-K₂HPO₄ (pH 6.0-8.0); ■, Tris-HCl (pH 8.0-9.0), and ●,
NaHCO₃-Na₂CO₃ (pH 9.0-11.0). (B) Effect of pH on enzyme stability. Assays conducted
at 37°C (pH 5.0) after a 30 min incubation in 0.1 M of the following buffers: ◆, glycine-
HCl (pH 2.0-3.0); ■, sodium acetate (pH 3.0-6.0); ▲, KH₂PO₄-K₂HPO₄ (pH 6.0-8.0); ■,
Tris-HCl (pH 8.0-9.0), and ●, NaHCO₃-Na₂CO₃ (pH 9.0-11.0). (C) Effect of temperature
on enzyme activity measured from 10-90°C. (D) Effect of temperature on enzyme
stability. Assays conducted at 37°C after a 30 min incubation in 0.1 M sodium acetate
buffer (pH 5.0) at 10-80°C. The average value of triplicate measurements were used for
each activity value.
Fig. 5  HPLC (High-performance liquid chromatography) analysis of hydrolysis products from mannooligosaccharides (M4 to M6) by recombinant Ef-Man. The degradation products of (A) mannohexaose, (B) mannopentaose, and (C) mannotetraose by the enzyme were detected by HPLC as described in the Materials and Methods section. Lines: Mannose (M1, red), mannobiose (M2, yellowish-brown), mannotriose (M3, grey), mannotetraose (M4, yellow), mannopentaose (M5, blue), mannohexaose (M6, yellowish-green).

Fig. 6  HPAEC (High-performance anion-exchange chromatography) analysis of hydrolysis products from linear mannan. The degradation products were detected by HPAEC as described in the Materials and Methods section.

Fig. 7  Structure comparison of β-mannanases. (A) Superposition of β-mannanase structures. rEf-Man, Ca-Man, Me-Man, and Ak-Man structures colored magenta, green, orange, and gray, respectively. The right panel shows a 90° rotation of the left panel around the horizontal axis. A blue circle in the right panel indicates the pLoop regions in rEf-Man and Ca-Man structures. A light blue dashed box indicates the catalytic site of β-mannanase. (B) A close-up view of the catalytic site in (A). Residues consisting of the substrate binding site are shown as stick models. (C) Conservation of amino acid residues on the surface of β-mannanase. A surface representation of rEf-Man, colored according to the degree of sequence conservation from high (red) to low (white). The degree of sequence conservation is based on the sequence alignment shown in Fig. 1. The right
panel shows a 90° rotation of the left panel around the horizontal axis. The stick model represents the mannohexaose which was produced by the superposition between rEf-Man structure and StMan structure complexed with mannohexaose (4Y7E). The putative six subsites (−3, −2, −1, +1, +2, and +3) were indicated in the figure.
Table 1  Substrate specificity of EF-Man

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
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<tbody>
<tr>
<td>Locust bean gum</td>
<td>100</td>
</tr>
<tr>
<td>Konjac powder</td>
<td>33.5</td>
</tr>
<tr>
<td>Guar gum</td>
<td>12.2</td>
</tr>
<tr>
<td>Starch</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cellulose</td>
<td>N.D.</td>
</tr>
<tr>
<td>Birchwood xylan</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D. : Not detected

The reaction mixture consisted of 0.4% each substrate in 0.1 M sodium acetate buffer (pH 5.0) and enzyme (0.57 units/mL), resulting in a final volume of 0.3 mL. After incubation for 20 min at 37°C, the amount of reducing sugar contained in the sample was then determined according to the standard assay method. The average values of triplicate measurements were used as each activity values.
<table>
<thead>
<tr>
<th>Data statistics</th>
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<tbody>
<tr>
<td><strong>Diffraction data</strong></td>
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<td>Space group</td>
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<td>Unit cell parameters (Å)</td>
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<td>Resolution (Å)</td>
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<tr>
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<tr>
<td>Unique reflections</td>
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<tr>
<td>Completeness (%)</td>
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<tr>
<td>$I/\sigma(I)$</td>
</tr>
<tr>
<td>$R_{\text{meas}}$ (%)</td>
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<tr>
<td>$CC_{1/2}$</td>
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<tr>
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<tr>
<td>$R_{\text{free}}$ (%)</td>
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<tr>
<td>Tris/isopropanol</td>
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<tr>
<td>Water</td>
</tr>
<tr>
<td>R.m.s. bonds (Å)/angles (°)</td>
</tr>
</tbody>
</table>

Values in parentheses are for the highest resolution shell.

$R_{\text{meas}} = \Sigma_{hkl} (n/(n-1))^{1/2} \Sigma_j |I_{hkl,j} - <I_{hkl}>|/\Sigma_{hkl} \Sigma_j |I_{hkl,j}|$, where $n$ is the data multiplicity.
Fig. 1 M. Ueda et al.
Fig. 2  M.Ueda et al.
Fig. 3  M. Ueda et al.
Fig. 6  M. Ueda et al.
Fig. 7 M. Ueda et al.

(A) Ef-Man
Ca-Man
Me-Man
Ak-Man

90°

α-8
pLoop

(B) β-8

(C) low
high
sequence conservation