Structural insights into the catalytic mechanism of a novel glycoside hydrolase family 113 β-1,4-
mannanase from *Amphibacillus xylanus*

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Running title: *Structural analysis of GH family 113 β-1,4-mannanase*

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ABSTRACT

β-1,4-Mannanase degrades β-1,4-mannan polymers into manno–oligosaccharides with a low degree of polymerization. To date, only one glycoside hydrolase (GH) family 113 β-1,4-mannanase, from *Alicyclobacillus acidocaldarius* (AaManA) has been structurally characterized, and no complex structure of enzyme–manno–oligosaccharides from this family has been reported. Here, crystal structures of a GH family 113 β-1,4-mannanase from *Amphibacillus xylanus* (AxMan113A) and its complexes with mannobiose, mannotriose, mannopentaose and mannahexaose were solved. AxMan113A had a stronger affinity for -1 and +1 mannososes, which explains why the enzyme can hydrolyze mannobiose. At least six subsites (-4 to +2) exist in the groove, but mannose units preferentially occupied subsites -4 to -1 owing to steric hindrance formed by Lys238 and Trp239. Based on the structural information and bioinformatics, rational design was implemented to enhance hydrolysis activity. Enzyme activity of AxMan113A mutants V139C, N237W, K238A and W239Y was improved by 93.7%, 63.4%, 112.9% and 36.4%, respectively, compared to the wild type. In addition, previously unreported surface binding sites were observed. Site-directed mutagenesis studies and kinetic data indicated that key residues near the surface sites play important roles in substrate binding and recognition. These first GH family 113 β-1,4-
mannanase–manno–oligosaccharide complex structures may be useful in further studying the catalytic mechanism of GH family 113 members, and provide novel insight into protein engineering of GHs to improve their hydrolysis activity.

Mannans are major components of hemicellulose, which is widely distributed in...
monocotyledons, plant seeds and beans (1, 2). They are classified into linear mannans, glucomannans, galactomannans and galactoglucomannans. Their backbone is composed of β-1,4-linked mannose or glucose units, and galactomannan and galactoglucomannan have additional α-galactosyl branches at O-6 (2, 3).

Mannans can be hydrolyzed into soluble manno-oligosaccharides, which is a suitable candidate in functional food production (4, 5), feed manufacturing (6) and paper processes (7). However, complete degradation of mannan requires a series of enzymes, including β-1,4-mannanase (EC 3.2.1.78), β-1,4-mannosidase (EC 3.2.1.25), β-D-glucosidase (EC 3.2.1.21) and α-galactosidase (EC 3.2.1.22) (2, 8). β-1,4-Mannanases widely exist in mollusks, plants, bacteria, fungi and actinomycetes (1, 9). According to the classification of carbohydrate-active enzymes (CAZy database, http://www.cazy.org/), β-1,4-mannanases are grouped into glycoside hydrolase (GH) families 5, 26, 113 and 134. Similar to GH families 5 and 26, family 113 members belong to clan GH-A, whose members share a typical (β/α)8-barrel protein fold (TIM barrel) and a retaining GH double-displacement catalytic mechanism (10-12); on the other hand, GH family 134 β-1,4-mannanases display an inverting GH catalytic mechanism (13, 14). To date, 359 GH family 113 protein sequences have been recorded, most of them derived from bacteria. Only one β-1,4-mannanase from the prokaryote Alicyclobacillus acidocaldarius Tc-12-31 (AaManA, PDB entry 3CIV) has been structurally characterized (10). AaManA shows the (β/α)8-barrel fold, and 2 glutamate residues that serve as a general acid/base and a nucleophile, respectively, to catalyze β-1,4-linked glucosidic bonds. However, the structure of GH family 113 enzyme–manno oligosaccharide complexes is not available, and the precise catalytic mechanism needs to be further elucidated.

In this study, a novel GH family 113 β-1,4-mannanase gene from Amphibacillus xylanus NBRC 15112 (AxMan113A) was cloned and expressed in Escherichia coli. The three-dimensional structures of the apo form and four complexes with mannobiose (M2), mannotriose (M3), mannopenoate (M5) and mannohexaose (M6) are solved. We further performed rational design by site-directed mutagenesis of key amino acids in the substrate-binding cleft to improve the enzyme’s activity. Our studies provide structural insights into the catalytic mechanism of GH family 113 β-1,4-mannanases.

RESULTS

Enzyme Characterization – A novel GH family 113 β-1,4-mannanase from A. xylanus NBRC 15112 (AxMan113A) was cloned and expressed in E. coli (Fig. S1). After purification, the denatured molecular mass of AxMan113A was 36.2 kDa on SDS-PAGE, and the native molecular mass was 38.8 kDa by gel filtration chromatography (Fig. S2), suggesting that the enzyme is a monomer. The amino-acid sequence of AxMan113A showed the highest identity (43%) with the β-1,4-mannanase from Alicyclobacillus acidocaldarius Tc-12-31 (AaManA; GenBank accession number ABG77968.1) (Fig. 1). AxMan113A exhibited maximal activity at pH 6.5 in 50 mM McIlvaine buffer (Fig. S3A) and was stable in a pH range of 5.0–9.0, with over 90% activity (Fig. S3B). Optimal temperature of AxMan113A was 45°C (Fig. S3C), and the enzyme was stable at temperatures below 45°C, retaining over 90% activity after incubation for 30 min (Fig. S3D).

Substrate Specificity and Hydrolysis Properties – The substrate specificity of AxMan113A was determined (Table 1). Its highest specific activity was towards M6 (8.3×10^3 U/mg), followed by M5 (7.5×10^3 U/mg), mannotetraose (M4; 6.3×10^3 U/mg), mannotriose (M3; 3.8×10^3 U/mg) and M2 (3.6×10^2 U/mg).
AxMan113A showed much lower specific activity towards the mannans konjac powder (54.4 U/mg), locust bean gum (LBG; 22.4 U/mg) and guar gum (1.2 U/mg). It did not show any detectable activity toward p-nitrophenyl β-D-mannopyranoside (pNPM).

To investigate the hydrolysis properties of AxMan113A, various mannans and manno-oligosaccharides (Megazyme, Ireland) were hydrolyzed by the enzyme. The hydrolysate was subjected to thin-layer chromatography (TLC) (Fig. S4). AxMan113A hydrolyzed LBG and konjac powder to yield mainly mannose and a series of manno-oligosaccharides (Fig. S4A). Manno-oligosaccharides (M2–M5) were further incubated with AxMan113A. The enzyme slightly cleaved M2 to produce mannose after 4 h incubation. AxMan113A exhibited higher hydrolysis activity towards M3, M4 and M5, mainly releasing mannose and M2, which were the end products of the hydrolysis after incubation for 4 h (Fig. S4B). Hydrolysis experiments were performed with reduced M2, M3, M4 and M5 viz. M2r, M3r, M4r and M5r. AxMan113A could not hydrolyze M2r and M3r, even after 4 h incubation. It exhibited very low hydrolysis ability towards M4r, yielding traces of M2 and M2r. M5r was hydrolyzed to yield M2 and M3r as the end hydrolysis products (Fig. S5).

Enzyme kinetics of manno-oligosaccharides

The kinetics constants for manno-oligosaccharides (M2 to M6) by AxMan113A were determined (Table 2). The $K_m$ value order was M2 (74.3 mM) > M3 (29.1 mM) > M4 (12.7 mM) > M5 (5.3 mM) > M6 (3.9 mM), suggesting that this enzyme had a higher affinity towards M6. In addition, AxMan113A had low catalytic efficiency towards M2 (0.0013 s⁻¹ mM⁻¹), high efficiency towards M3 (0.029 s⁻¹ mM⁻¹), M4 (0.077 s⁻¹ mM⁻¹), M5 (0.201 s⁻¹ mM⁻¹) and M6 (0.387 s⁻¹ mM⁻¹). The catalytic efficiencies ($k_{cat}/K_m$) of mutants Q58A and D73A were 0.0006 s⁻¹ mM⁻¹ and 0.0007 s⁻¹ mM⁻¹, respectively (Supplementary Table 2). Compared to wild type, catalytic efficiencies of Q58A and D73A were decreased to 46.1% and 53.8%, respectively. Thus, mutants Q58A and D73A reduced substrate affinity towards M2.

Overall Structure of AxMan113A – The structure of AxMan113A was solved and the crystallographic statistics are summarized in Table 2. The orthorhombic space group of AxMan113A was $P2_12_12_1$ with two monomers in the asymmetric unit (Fig. 2A). The monomer with approximate dimensions of $41 \times 31 \times 56$ Å contained residues from Met1 to Arg309. AxMan113A displayed the typical $(\beta/\alpha)_8$-barrel fold of GH family 113 members, containing an internal core of 8 β-strands encircled by 8 α-helices. The catalytic proton donor and nucleophile (Glu143 and Glu223) were determined by structural alignment to another GH family 113 β-1,4-mannanase (AaManA, PDB entry 3CIV), located in the middle of the narrow groove (Fig. 2B).

Crystal Structures of AxMan113A/E223A Complexes – The structures of AxMan113A/E223A complexes with M2, M3, M5 and M6 were obtained by co-crystallization method. In the AxMan113A/E223A-M2 structure, one M2 molecule was embedded in the narrow groove and occupied subsites -2 and -1 in chain B. In chain A, however, three mannose units were found in subsites -2, -1 and +2, indicating the existence of two binding modes in AxMan113A/E223A-M2, occupying subsites -2 to -1 and +1 to +2. The lack of electron density for mannose moieties might have been due to the following: (i) mannoses in subsites +1 and +2 were very flexible; (ii) binding domains in subsites +1 and +2 were too narrow to completely bind one M2 molecule. In subsites -1 and -2, M2 residues formed 12 direct hydrogen bonds to Trp11, Asn89, Arg96, Glu143, Tyr195, Asn237 and Tyr291. Three water-mediated hydrogen
bonds were formed between Asn237 and the mannose residues. Specifically, the amino groups of the side chains of Trp11, Asn89 and Arg96 were directly hydrogen-bonded to O-2, O-5 and O-6 of the -2 mannose residue. A strong hydrogen-bond network was observed with the -1 mannose residue. The O-2, O-3, O-4, O-5 and O-6 hydroxyls of the -2 mannose residue were directly hydrogen-bonded to the side chains of Arg96, Glu143, Tyr195, Asn237, Trp273 and Tyr291. Furthermore, the aromatic residues Trp239 and Trp273 were stacked against the mannose units in subsites -2 and -1, forming hydrophobic sugar-binding platforms (Fig. 3A). There were seven direct hydrogen-bonds and three water-mediated interactions for the +2 mannose residue. Asn237, Trp239 and Asp240 formed four interactions with the O-2, O-3 and O-4 hydroxyls of +2 mannose. The O-6 hydroxyl of +2 mannose residue was directly bound to Asn237 and Lys238. Trp40 and Trp239 provided the hydrophobic sugar-binding platform, making contact with +2 mannose residue (Fig. 3B).

Superposition of AxMan113A/E223A-M2 and AxMan113A showed that most residues were in identical positions, except for Trp239 and Asp240. The side chain of Trp239 was deflected in the former, forming a hydrophobic platform to bind to the mannose residues in subsites -2 and +2. The distance between the side chain of Asp240 and +1 mannose was 3.3 Å to 2.7 Å, suggesting that Asp240 was attracted during the recognition and binding with substrates. Interestingly, some additional electron-density maps representing M2 molecules were found on the surface region of the AxMan113A/E223A-M2 complex structure, which is far away from the catalytic cleft. In chain A, one M2 molecule was found in the middle of two α-helixes. Another M2 molecule was observed on the extended loop of chain B, and neither of these mannoses were structurally symmetrical (Fig. 4).

A crystal structure of AxMan113A/E223A in complex with M3 (AxMan113A/E223A-M3) was solved at 1.68 Å resolution (Table 3). Only one M2 molecule was found to occupy to catalytic cleft of AxMan113A (at subsites -2 to -1), suggesting that mannose occupies subsites -2 to -1 preferentially during the process of substrate binding (Fig. 5A). The complex structure of AxMan113A/E223A-M5 was determined at 2.38 Å resolution. Four mannose units (M4) linked by β-1,4 glycosidic bonds could be built into the electron-density map (at subsites -4 to -1). Mannose residues in subsites -3 and -4 extended to the terminal and lacked interactions with any surrounding key amino acids, indicating that AxMan113A can only weakly bind -3 and -4 mannoses (Fig. 5B). Superposition of AxMan113A/E223A-M5 and AxMan113A/E223A-M2 showed that the -1 and -2 mannose residues were basically in almost the same position, and all adopted the chair conformation 4C1. At least six binding sites existed in the groove, occupying subsites -4 to +2. In addition, the complex structure of AxMan113A/E223A-M6 was solved. Similarly, one M3 molecule was well defined in the electron-density maps, occupying the catalytic cleft of AxMan113A (subsites -1 to -3) (Fig. 5C).

**Rational design of AxMan113A** — Structural alignment of AxMan113A (PDB entry 5YLH) and AaManA in complex with a synthesized inhibitor (ManIFG) and M2 (AaManA-ManIFG–M2; PDB entry 4CD7) is shown in Fig. 6A. Both of the catalytic grooves were basically identical except for several residue positions in subsites +1 and +2. Trp239 of AxMan113A (Tyr237 in AaManA-ManIFG–M2) showed a large aromatic ring, which formed stacking interaction with -2 and +2 sugars. Compared to AaManA-ManIFG–M2/Tyr247, the side chain of AxMan113A/Trp239 rotated nearly 90°, closer to the catalytic cleft (Fig. 6B). AxMan113A/Asn237 was located above the -1 and -2 mannoses, forming three direct hydrogen bonds with them. This amino acid was replaced by Trp245 in
AaManA-ManIFG–M2, which provided a hydrophobic sugar-binding platform that could stabilize the +2 mannose (Fig. 6C). Another proposed key amino acid residue was Lys238 of AxMan113A, which was situated on subsite +2. The longer side chain extended into the groove, forming steric hindrance to mannose binding (Fig. 6D). Notably, AxMan113A/Lys238 was replaced by Asp246 in the corresponding site of AaManA-ManIFG–M2. In addition, non-polar residue Val139 was replaced by polar residues (Cys, Ser and Tyr) in other GH family 113 members (Fig. 1). To investigate whether hydrolysis activity in AxMan113A could be altered by changing these residues, six mutants (W239Y, W239A, N237A, N237W, K238A and V139C) were designed. Specific activity of W239A and N237A decreased by 92.0% and 37.1%, respectively, while the specific activity of W239Y, N237W, K238A and V139C improved by 36.4%, 63.4%, 112.9% and 93.7%, respectively, relative to the wild type (Fig. 6E). Compared to AxMan113A, mutants V139C and N237W shifted 1.0 unit towards the acidic range. However, W239Y, W239A, N237A and K238A did not show change in optimal pH (Fig. 7A). With respect to optimal temperature, none of the mutants changed except N237W decreased by 5 °C (Fig. 7B).

DISCUSSION

Owing to the various biochemical properties, β-1,4-mannanases are widely used in many industrial applications. In paper/pulp industries, β-1,4-mannanases are able to cleave the mann portion of pulps to increase the brightness (2, 7). In the field of clinical applications, β-1,4-mannanases can be used to hydrolyze non-digestible oligosaccharides and polysaccharides like prebiotics and dietary fibres, producing short chain fatty acids (SCFA), which can protect against inflammatory bowel disease, ulcerative colitis and Crohn’s disease (1, 15). β-1,4-Mannanases was also used to produce manno-oligosaccharides (MOS), which can effectively regulate immune system and reduce the serum cholesterol (16). In the field of functional foods, high viscosity of guar gum can be hydrolyzed by β-1,4-mannanases to yield partially hydrolyzed guar gum (PHGG), which offers health benefits and nutrient digestion (17). Due to their growing industrial potential, many of β-1,4-mannanases have been studied. GH family 113 only had one structure-resolved β-1,4-mannanase (AaManA) that from A. acidocaldarius Tc-12-31 (10). AaManA has been reported to share a typical (β/α)8-barrel folding motif and display a two-step retaining catalytic mechanism, which is consistent with β-1,4-mannanases in GH families 5 and 26. The crystal structure of β-1,4-mannanase-targeted inhibitors (AaManA-ManIFG–M2) was solved after AaManA (18). However, lack of enzyme–manno-oligosaccharide complex structural information gives only limited comprehensive clarification of the catalytic mechanism of GH family 113 members. In this study, we cloned a novel GH family 113 β-1,4-mannanase gene from A. xylinus (AxMan113A) and obtained four manno-oligosaccharide complex crystals to further elucidate the catalytic mechanism of GH family 113 members.

The specific activity of AxMan113A towards konjac powder was higher than that toward LBG or guar gum, which is consistent with other GH family 113 members, such as AaManA (10) and Man113A from Alicyclobacillus sp. strain A4 (19). In contrast, β-1,4-mannanases from GH families 5, 26 and 134, such as RmMan5A from Rhizomucor miehei (20), RetMan26 from thermophilic Bacillus subtilis (21) and AoMan134A from Aspergillus oryzae (22), exhibit strong hydrolysis preference for LBG. AxMan113A and other GH family 113 β-1,4-mannanases have a similar catalytic cleft, and some key amino acids around subsites -1 and +1 form a semi-enclosed “lid” region that can only accommodate one mannose or glucose (Fig. S6A). On the other hand, structures of GH
families 5, 26 and 134 β-1,4-mannanases display an “open” groove, which has enough space to accommodate the substrates with galactose side chains (13, 23, 24). Therefore, it can be deduced that enzyme activity of GH family 113 members is limited by the α-1,6-galactose residues owing to that structural difference.

The catalytic mechanism of GH family 113 β-1,4-mannanases can be described as a two-step retaining catalytic mechanism (25). The catalytic residues are Glu143 and Glu223 in AxMan113A, as confirmed by site-directed mutagenesis and structural alignment to AaManA. Previous studies have shown that GH family 113 members hydrolyze manno-oligosaccharides with DP ≥ 3 and exhibit considerable transglycosylation activity (10, 19). However, the smallest substrate of AxMan113A was M2 (Fig. S4B). In addition, AxMan113A had no transglycosylation activity. Thus, the catalytic mechanism of AxMan113A differs from that of other GH family 113 members. AxMan113A could hydrolyze the reduced manno-oligosaccharides with DP ≥ 4, and the hydrolysis efficiency towards M5r was much higher than that of M4r (Fig. S5). It indicated that the reducing terminal substitution by mannitol could influence the recognition of mannose residue in the catalytic groove. Moreover, the hydrolysis of AxMan113A was diminished gradually with the substitutions approached to the catalytic groove. That might be one major reason that this enzyme cannot hydrolyze pNPM.

AxMan113A exhibited the different catalytic efficiency (k_cat/K_m) towards manno-oligosaccharides, which were decreased with DP from M6 to M2 (Table 2). Among the endo-β-mannanases, AxMan113A displayed catalytic efficiency towards M3 (0.029 s⁻¹ mM⁻¹), which is much lower than Man113A (6.54 s⁻¹ mM⁻¹) (19), AnMan5B (0.16 s⁻¹ mM⁻¹) (26) and PaMan5A (26.67 s⁻¹ mM⁻¹) (27). It had much lower catalytic efficiency towards M2 (only 0.0013 s⁻¹ mM⁻¹). Thus, the enzyme hydrolyzed M2 slowly after 4 h incubation (Fig S4B). These results could support for the main hydrolysis products being M2 and mannose.

To verify the unique catalytic mechanism of AxMan113A, crystal structures of AxMan113A and its complexes with M2, M3, M5 and M6 were solved (Table 3). Clearly, mannose residues bound to at least 6 subsites in the groove (-4 to +2) (Fig. S6B). In the structure of AxMan113A/E223A-M6, one M3 molecule was occupied in -3 to -1 subsites, and another 3 substrate-binding sites (+1 to +3) has been proved by hydrolyzing M5r (Fig. S5). Mannose moieties of M5r would preferentially bind in subsites -2 to +2. Mannitol was occupied in subsite +3 (28, 29). Thus, it could be deduced that another M3 molecule was occupied in +1 to +3 subsites. A bulk hydrogen-bond network was formed around the subsite -1, which firmly fixed the position of the -1 mannose. Three residues (Arg96, Tyr195 and Trp273) of GH family 113 β-1,4-mannanases were conserved (Fig. 1). Arg96 not only hydrogen-bonded with the -1 mannose, but also formed one hydrogen bond and salt-bridge with the side chain of Asp143 at a distance of 2.9Å, stabilizing the latter's orientation. Similarly, the presence of Tyr195 (2.7Å away from Glu223) was vital to orienting catalytic residue Glu223 (Fig. S7A). This is consistent with studies of chitosanases OU01 and N174 (30, 31). Trp273 in AxMan113A provided a hydrophobic sugar-binding platform with the +1 sugar ring. The importance of three residues for substrate-binding was corroborated by site-directed mutagenesis. Mutants R96A, Y195A and W273A lost most of this activity (Fig. S7C). Compared to AaManA-ManIFG-M2, one key water molecular in AxMan113A/E223A-M2 was found above -1 subsite, forming three hydrogen bonds with -1 mannose, better fixing the sugar’s position. This suggests that the binding of -1 mannose in AxMan113A is stronger than in AaManA. Moreover, in subsite +1, the conformation of three residues (Trp95, Lys176 and Asp240) in AxMan113A changed a great deal
compare with AaManA-ManIFG–M2. Trp95 flipped up approximately 30°, which brought the aromatic nucleus close to subsite +1, strengthening the interaction with +1 sugar. The side chains of Lys176 and Asp240 (His183 and Arg248 in AaManA, respectively) extended to subsite +1, forming more hydrogen bonds with the mannose (Fig. S7B). Specific activity of W95A, K176A and D240A was decreased to 7.1%, 9.6% and 10.2% of the wild type, respectively (Fig. S7C). AaMan113A exhibited higher affinity for -1 and +1 mannoses, which might be an important reason why the enzyme can hydrolyze M2. As already noted, unlike other members of GH family 113, AaMan113A had no transglycosylation activity. In the GHs with retaining catalytic mechanism, some amino acids near subsite +2 play an important role in stabilizing the transition state of transglycosylation (32). Compared to AaManA-ManIFG–M2, the conformation of mannose in the subsite +2 of AaMan113A changed significantly. This suggested that the +2 sugar is very flexible, making its binding to the subsite +2 difficult. Aromatic amino acids in the binding subsite +2 can stabilize the transglycosylation receptor (33, 34). In AaManA-ManIFG–M2, Trp245 provided a hydrophobic sugar-binding platform in subsite +2. However, AaMan113A lacked aromatic residues near this subsite. This special structural feature may result in the enzyme with no transglycosylation activity.

Two different M2 electron-density maps were observed on the surface of AaMan113A/E223A-M2, which is far away from the catalytic sites (Fig. 4). A number of structural studies have revealed that carbohydrates are not only bound on carbohydrate-binding modules (CBMs), but also observed on one or more surface binding sites (SBSs). Compared to CBMs, carbohydrates binding in SBSs are often through a flexible linker (35, 36). Most of SBSs existed in α-amylases, other amylolytic enzymes, transglycosidases, xylanases and other GHs. To date, there is no report on SBSs of GH family 113. The superposition of AaMan113A/E223A-M2 on AaManA showed a position change in some amino acids near the surface binding site. In chain A, the side chain of AaMan113A/Asp73 shifted to the binding site, which formed two hydrogen bonds with mannose. Similarly, AaMan113A/Lys76 and AaMan113A/Asn134 were also hydrogen-bonded with the mannose. However, AaManA/Glu81, AaManA/His84 and AaManA/Gly142 in the same position revealed no interaction with M2 (Fig. S8A). In the chain B, Gln58 and Asp66 in a loop of AaMan113A, equivalent to Gly66 and Asp74 in AaManA, were flipped 90°, forming a “tweezer” binding linker that can target more mannose molecules efficiently (Fig. S8B) (37, 38). It has been reported that a β-1,4-mannanase from Cryptopygus antarcticus (PDB entry 400Z) and several α-amylases have surface binding sites (37-39). Moreover, mutations of the key amino acids near surface binding sites decreased enzyme activity. To investigate the significance of these residues, site-directed mutagenesis was performed (Fig. S8C). The specific activity of six mutants (D66A, D73A, K76A, Q58A, N134A and D65A) decreased, especially that of Q58A and D73A, which retained 56.8% and 82.7% of the initial enzyme activity, respectively. This indicated that these six residues play key roles in binding with ligands. The catalytic efficiencies (kcat/Km) of Q58A and D73A towards M2 were decreased to 46.1% and 53.8% of wild type, respectively. It could be deduced that residues around the surface binding sites have an effect on M2 affinity.

Rational design is an important method to modulate enzyme properties, in an attempt to understand structural information. Many GHs, such as β-galactosidase, β-glycosidase and α-L-arabinofuranosidase, have been modified through protein engineering (40-42). However, protein engineering of GH family 113 β-1,4-mannanases has never been reported. There were
some differences in the structures of AxMan113A compared to other GH family 113 members. On the basis of the structural comparison of AxMan113A/E223A-M2 to AaManA-ManIFG-M2, three residues (Trp239, Asn237 and Lys238) were identified in subsites +1 and +2 of AxMan113A which had a unique amino acid arrangement (Fig. 6). Trp239 in subsite +1 had larger side chain and extended into the groove, forming a semi-enclosed space, which impeded the accommodation of mannose units. However, Trp239 formed hydrophobic interactions with -2 and +2 mannoses. Thus, mutant W239Y was designed to reduce steric hindrance and retain the hydrophobic sugar-binding platform. W239A was designed to open the catalytic cleft. N237W not only retained the hydrogen bond, but also created an indole ring. K238A eliminated the side chain of Lys and partly opened the groove of subsite +2. Mutant W239A lost most of its activity, confirming that the hydrophobic interaction is crucial for binding with ligands. However, β-1,4-mannanase activity of W239Y increased significantly to 136.4% of the wild type. Mutant N237W enhanced affinity to the +2 mannose, and enzyme activity was improved to 163.4%. The enzyme activity of K238A was improved to 212.9% of the wild type (Fig. 6E). It has been reported that increasing intramolecular hydrophobic interactions can improve structure stability (43, 44). Herein, hydrophilic residue Asn237 was mutated to hydrophobic Tryptophan, forming a more favorable hydrophobic region with Trp273, which is beneficial for substrate binding. Indeed, compared to wild type, mutant N237W displayed the highest activity in acidic and higher temperature condition (Fig. 7). According to the result of sequence alignment (Fig. 1), several polar residues (Cys, Ser and Tyr) exist in six GH family 113 β-1,4-mannanases, but replaced by non-polar residue Val139 in AxMan113A. Mutant V139C showed acidic shift 1.0 units of optimal pH. In general, polar group burial makes a large contribution to structure stability (45, 46). Mutating the non-polar residue Val139 to a polar residue Cys might be one reason for the optimal pH change. These results provided novel insights into the mechanism of GH family 113 members, which might have great significance in improving the hydrolysis efficiency of β-1,4-mannanases.

In conclusion, we performed structural and biochemical analyses to provide an in-depth description of the catalytic mechanism of GH family 113 members. Analysis of the complex structures revealed at least six binding sites (–4 to +2) in the groove, but Lys238 and Trp239 were located near subsites +1 and +2, blocking the catalytic cleft. This prominent structural feature can mostly accommodate mannose units at subsites -4 to -1. Site-directed mutagenesis yielded a series of mutations designed to affect the enzyme’s properties. Mutants K238A and W239Y successfully opened the catalytic cleft and their enzyme activities were improved to 212.9% and 163.4% of the wild type, respectively. In addition, two M2-binding sites were discovered on the surface of AxMan113A, never before reported in GH family 113 members. Kinetic analysis of key residues near the surface sites suggested these sites' important role in substrate binding. The structural information of AxMan113A not only provides further insights into its catalytic mechanism, but also a direction for protein engineering in GH family 113 members.

EXPERIMENTAL PROCEDURES

Cloning, Expression and Purification – The β-1,4-mannanase gene (designated AxMan113A) from A. xylanus NBRC 15112 (GenBank accession number BAM48369.1) was amplified by PCR with primers AxMan113A-up and AxMan113A-down (Supplementary Table 1). After digestion with EcoRI and NotI, the PCR products were purified and inserted into vector pET-28a (Novagen, USA). Mutants E143A, E223A, Q58A, D65A, D66A, D73A, K76A,
W95A, R96A, V139C, K175A, K175H, N237A, N237W, K238A, W239A, W239Y and W273A were created in AxMan113A using the Fast Mutagenesis System site-directed mutagenesis kit (TransGen Biotech, China) with the primers listed in Table S1. All transformants were confirmed by DNA sequencing.

The recombinant plasmid pET28a-AxMan113A was transformed into *E. coli* Rosetta (DE3) for expression. Seed cultures of *E. coli* harboring AxMan113A were incubated in Luria Bertani (LB) medium containing 50 μg/mL kanamycin at 37°C on a rotary shaker at 200 rpm to an OD<sub>600</sub> of 0.6–0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) was added to induce expression at 30°C overnight. Cells were harvested by centrifugation at 10,000 g for 5 min. The precipitate was suspended in buffer A (20 mM imidazole, 20 mM Tris–HCl pH 8.0, 500 mM NaCl) and disrupted by ultrasonication. The cell debris was centrifuged at 10,000 g for 10 min and the supernatant was loaded into a Ni<sup>2+</sup>IDA column (1 × 5 cm; GE Life Sciences, USA) pre-equilibrated with buffer A. The column was washed with buffer A followed by buffer B (50 mM imidazole, 20 mM Tris–HCl pH 8.0, 500 mM NaCl) and then eluted with buffer C (200 mM imidazole, 20 mM Tris–HCl pH 8.0, 500 mM NaCl) at a flow rate of 1.0 mL/min. The eluted enzyme was subjected to a Sephacryl S-100 gel filtration column (1 × 100 cm; GE Life Sciences), eluted with buffer D (20 mM Tris–HCl pH 8.0, 100 mM NaCl) and concentrated to 10 mg/mL by ultrafiltration for crystallization. The concentration of the protein was measured by the Lowry method (47). All mutants were expressed and purified by the same method.

SDS-PAGE was performed using a 12.5% (w/v) separation gel. The molecular mass of native AxMan113A was determined by gel filtration on a Sephacryl S-100 HR column (40 × 1 cm) pre-equilibrated with buffer D. The protein was eluted at 0.3 mL/min. The standard markers used for calibration were phosphorylase b (97.2 kDa), albumin (66.4 kDa), ovalbumin (44.3 kDa), chymotrypsinogen A (25.7 kDa) and lysozyme (14.3 kDa).

**Enzyme Characterization** – Enzyme assay for AxMan113A was performed using the 3,5-dinitrosalicylic acid (DNS) method (48) with 0.5% (w/v) LBG. Briefly, 100 μL of suitably diluted enzyme was added to 900 μL LBG solution in 50 mM McIlvaine buffer pH 6.5. After incubation at 45°C for 10 min, the reaction was terminated by adding 1 mL DNS and boiling for 15 min. Enzyme activity in the reaction mixture was immediately measured at 540 nm after the addition of 1.0 mL sodium potassium tartrate (40%, w/v). One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol reducing sugars (mannose equivalents) per minute. The optimal pH of AxMan113A was determined in various buffers (50 mM): McIlvaine buffer (pH 3.0–7.0), citrate buffer (pH 3.0–6.0), phosphate buffer (pH 6.0–8.0), 2-(cyclohexylamino)ethanesulfonic acid (CHES; pH 8.0–10.0), Gly-NaOH (pH 9.0–10.5) and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; pH 10–11). The pH stability of AxMan113A was examined by determining the residual activity after incubation in these buffers at 30°C for 30 min. The optimal temperature was determined in the temperature range of 30–70°C in 50 mM McIlvaine buffer pH 6.5. The thermostability of AxMan113A was investigated by measuring residual activity after incubation at different temperatures for 30 min in the same buffer. All measurements were performed in triplicate.

**Substrate Specificity and Hydrolysis Properties** – Substrate specificity towards 0.5% (w/v) of various mannans: konjac powder, LBG and guar gum, was determined by DNS method (48). High-performance liquid chromatography with a refractive index detector (HPLC–RID) was used to evaluate the substrate specificity of AxMan113A towards 0.1% (w/v) manno-
Suitably diluted AxMan113A and manno-oligosaccharides were incubated at 45°C in 50 mM McIlvaine buffer pH 6.5 for 10 min. A 10-μL aliquot of the mixture was then injected into a Shodex sugar KS-802 (4.5 × 250 mm) column and eluted by mobile phase (water) at a flow rate of 0.8 mL/min. The temperatures of column and detector were 35°C and 60°C, respectively. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol manno-oligosaccharides per minute. For determination of β-mannosidase activity of AxMan113A, 50 μL diluted enzyme was added to 200 μL 3.75 mM pNP in 50 mM McIlvaine buffer pH 6.5, then incubated at 45°C for 10 min. A total of 750 μL saturated sodium tetraborate was added to terminate the reaction. Enzyme activity in the reaction mixture was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol pNP per minute under the described conditions. The degradation of different mannans or manno-oligosaccharides by AxMan113A was detected by TLC. AxMan113A (5 U) was mixed into 1% (w/v) mannans or manno-oligosaccharides in 50 mM McIlvaine buffer pH 6.5 for 12 h or 4 h. The hydrolysis of M2r, M3r, M4r and M5r by AxMan113A were also analyzed. Purified AxMan113A (5 U) was incubated with 1% (w/v) M2r, M3r, M4r and M5r under the same conditions for 4 h. Samples were withdrawn at the different time points and boiled for 10 min. The supernatants were spotted on a silica gel plate (Merck Silica Gel 60F254, Germany) and developed in n-butyl alcohol:acetic acid:water (2:1:1 v/v). The plates were heated at 130°C for 5 min after spraying with methanol containing 2% (v/v) H2SO4. For the X-ray diffraction experiments, a single crystal was fished out and immediately soaked in cryoprotectant solution (20% v/v glycerol under crystallization conditions), and then flash-cooled in liquid nitrogen. The X-ray diffraction data of AxMan113A were collected by beamline BL18U at the Shanghai Synchrotron Research Facility (SSRF, China). X-ray data for other complex crystals were obtained by beamline BL17U at SSRF. All data were indexed, integrated and scaled by the HKL-2000 package (50).
Structure Determination and Refinement – The structure of AxMan113A was determined by molecular replacement method using AaManA (sequence identity 43%) as the search model. Phenix.autobuild was used for automatic building. The structures were completed with alternating rounds of manual model building with Coot (51) and Phenix.refine programs (52). The final model was analyzed by MolProbity (53). Structure illustrations were prepared with PyMOL. Ligplus (54) and used to analyze the protein–ligand interactions. The sequence alignments were created with ClustalX2 (55) and ESPript (56).

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CONFLICT OF INTEREST:
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS:
ZQJ designed and supervised the research and revised the manuscript. XY performed the experiments, analyzed the data, and wrote the manuscript. ZQ revised the manuscript. SQY analyzed the data. YXL performed the enzyme property and enzyme activity determinations. QJY analyzed the data and revised the manuscript. All authors read and approved the manuscript.

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**FOOTNOTES**
The abbreviations used are: GH, glycoside hydrolase; M2, mannobiose; M3, mannotriose; M4, mannotetraose; M5, mannopentaose; M6, mannohexaose; LBG, locust bean gum; pNPM, p-nitrophenyl β-D-mannopyranoside; TLC, thin-layer chromatography; DP, degree of polymerization; IPTG, isopropyl β-D-thiogalactopyranoside; DNS, 3,5-dinitrosalicylic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; PEG, polyethylene glycol.
FIGURE LEGENDS

FIGURE 1. Sequence alignment of AxMan113A with other GH family 113 members. Residues forming the secondary structures of AxMan113A are highlighted above the sequences. Identical residues are shown in white on red background. Two catalytic residues, Glu143 and Glu223, are marked by red dots. The sequences of AxMan113A (PDB entry 5YLH), Alicyclobacillus acidocaldarius β-1,4-mannanase (AaManA; PDB entry 3CIV), Alicyclobacillus sp. strain A4 β-1,4-mannanase (Man113A; GenBank accession number KC460333.1), Paenibacillus mucilaginosus 3016 β-1,4-mannanase (GenBank accession number AFC29300.1), Aequorivita sublithincola DSM 14238 β-1,4-mannanase (GenBank accession number AFL80188.1) and Bacillus amyloliquefaciens (GenBank accession number ALV02185.1) were aligned by ClustalX2 (55) and the figure was produced in ESPript (56).

FIGURE 2. Overall structure of AxMan113A. (A) Cartoon form, illustrating the classical (β/α)8-barrel. Two monomer molecules are present in the asymmetric unit of AxMan113A. (B) Surface view. A pair of catalytic residues (Glu143 and Glu223) are shown as sticks in red.

FIGURE 3. Complex of mutant AxMan113A/E223A with mannobiose (M2). (A) Molecular surface of the complex structure. M2 residues located along the groove are shown as sticks in chains A and B. (B) Stereo view of the substrate interactions of AxMan113A/E223A-M2 in chains A and B. All of the mannose residues bound in the groove are shown as green/red sticks. The key protein residues are shown as yellow sticks and the catalytic residues are shown in red. The electron density of the M2 ligand is shown as an σA-weighted mFo – DFc OMIT map contoured at the 1.0 σ level.

FIGURE 4. Surface binding sites of AxMan113A/E223A-M2. Structure of AxMan113A/E223A-M2 is shown as cartoon in light orange, surface-binding regions are colored in red. The key protein residues are shown in yellow sticks. Mannobiose are shown as green/red sticks.

FIGURE 5. Complexes of mutant AxMan113A/E223A with manninotriose (M3), mannopentaose (M5) and mannohexaose (M6). (A) Stereo view of the substrate interactions of AxMan113A/E223A-M3. (B) Stereo view of the substrate interactions of AxMan113A/E223A-M5. (C) Stereo view of the substrate interactions of AxMan113A/E223A-M6. All of the mannose residues are shown as green/red sticks. The key protein residues are shown as yellow sticks and the catalytic residues (Glu143 and E223A) are shown in red. Electron density of M3, M5 and M6 ligand is shown as an σA-weighted mFo – DFc OMIT map contoured at the 1.0 σ level.

FIGURE 6. Unique conformation of the amino acids near subsites +1 and +2. (A) Structural alignment of AxMan113A/E223A-mannobiose (M2) and AaManA-ManIFG–M2. (B) A semi-enclosed space formed by Trp239 of AxMan113A. (C) Asn237 of AxMan113A/E223A-mannobiose was replaced by Trp245 of AaManA-ManIFG–M2. (D) A steric hindrance formed by
Lys238. (E) Enzyme activity assay for the wild type and mutants towards locust beam gum. Results are plotted as percentage of the product generated relative to the wild type (100%; dashed line). All of the measurements were performed in triplicate.

**FIGURE 7.** Determination of optimal pH (A) and optimal temperature (B) of wild type and the mutants. The effect of pH on β-mannanase activity was determined in 50 mM of different buffers at optimal temperature of each enzyme. For optimal temperature, activity was measured at different temperatures in 50 mM McIlvaine buffer (optimal pH of each enzyme). The specific activities of wild type (22.4 U/mg), W239Y (30.6 U/mg), N237W (36.6 U/mg), K238A (47.7 U/mg), and V139C (43.4 U/mg) were considered as 100% in determinate the optimal pH and optimal temperature.
Table 1 Substrate specificity of AxMan113A

| Substrate          | Specific activity (U/mg)       | Relative activity (%) |
|--------------------|--------------------------------|-----------------------|
| Locust bean gum    | 22.4 ± 0.21                    | 100                   |
| Konjac powder      | 54.4 ± 0.66                    | 2.4×10²               |
| Guar gum           | 1.2 ± 0.02                     | 5.4                   |
| Mannobiose         | 3.6×10² ± 10.9                 | 1.6×10³               |
| Mannotriose        | 3.8×10³ ± 87.7                 | 1.7×10⁴               |
| Mannotetraose      | 6.3×10³ ± 1.9×10²              | 2.8×10⁴               |
| Mannopentaose      | 7.5×10³ ± 2.3×10²              | 3.3×10⁴               |
| Mannohexaose       | 8.3×10³ ± 2.9×10²              | 3.7×10⁴               |
| pNPM               | NA²                            | 0                     |

*Enzyme activity was measured at 45°C for 10 min in 50 mM McIlvaine buffer (pH 6.5).*

*Relative activity of the substrate locust bean gum was set at 100%.*

*NA, no activity detected.*
Table 2. Kinetic data of AxMan113A towards manno-oligosaccharides

| Substrate | $V_{\text{max}}$ (μmol min$^{-1}$ mg$^{-1}$) | $K_m$ (mM) | $k_{\text{cat}}$ (s$^{-1}$) | $k_{\text{cat}}/K_m$ (s$^{-1}$ mM$^{-1}$) |
|-----------|--------------------------------|-----------|-----------------|-----------------|
| M2        | 162.2 ±7.3                     | 74.3 ±4.4 | 0.098           | 0.0013          |
| M3        | 1413.8 ±65                     | 29.1 ±2.3 | 0.853           | 0.029           |
| M4        | 1622.5 ±86                     | 12.7 ±1.1 | 0.979           | 0.077           |
| M5        | 1764.7 ±94                     | 5.3 ±0.4  | 1.065           | 0.201           |
| M6        | 2500 ±152                      | 3.9 ±0.3  | 1.508           | 0.387           |

*The kinetic parameters were determined at 45°C for 5 min in 50 mM McIlvaine buffer (pH 6.5).*
### Table 3 X-ray data-collection and refinement statistics

|                     | Apo-ArMan113A | ArMan113A/E223A-M2 | ArMan113A/E223A-M3 | ArMan113A/E223A-M5 | ArMan113A/E223A-M6 |
|---------------------|---------------|---------------------|---------------------|---------------------|---------------------|
| **Data-collection statistics** | | | | | |
| Radiation source    | SSRF-BL18U    | SSRF-BL17U          | SSRF-BL17U          | SSRF-BL17U          | SSRF-BL17U          |
| Wavelength (Å)      | 0.978         | 0.979               | 0.979               | 0.979               | 0.979               |
| Temperature of measurements (K) | 100 | 100 | 100 | 100 | 100 |
| Resolution (Å)      | 30.38–2.29    | 40.53–2.09          | 32.9–1.68           | 35.85–2.37          | 32.75–1.81          |
| (2.38–2.29)         | (2.17–2.09)   | (1.74–1.68)         | (2.46–2.37)         | (1.88–1.81)         |                     |
| Space group         | P2₁2₁2₁       | P2₁2₁2₁             | P2₁2₁2₁             | P2₁2₁2₁             | P2₁2₁2₁             |
| Unit cell parameters | | | | | |
| a, b, c (Å)         | 54.8, 69.4, 188.9 | 49.0, 72.2, 177.2  | 65.1, 69.9, 186.6  | 55.1, 69.6, 188.8  | 62.6, 70.0, 186.3  |
| α, β, γ(°)          | 90, 90, 90    | 90, 90, 90          | 90, 90, 90          | 90, 90, 90          | 90, 90, 90          |
| Protein molecules in asymmetric unit | 2 | 2 | 2 | 2 | 2 |
| Unique reflections  | 29076 (2687)  | 36641 (3374)        | 91072 (6371)        | 29759 (2690)        | 68340 (6807)        |
| Completeness (%)    | 88.1 (64.4)   | 92.0 (96.2)         | 93.0 (79.6)         | 98.0 (90.4)         | 91.4 (75.5)         |
| Rmerge (%)          | 12.2 (60.9)   | 9.0 (41.9)          | 19.8 (88.1)         | 8.8 (48.5)          | 8.6 (45.2)          |
| Mean Iσ(I)          | 4.6           | 7.3                 | 6.8                 | 9.1                 | 9.4                 |
| Wilson B-factor (Å²)| 31.67         | 22.83               | 13.82               | 35.38               | 19.04               |
| **Refinement statistics** | | | | | |
| Resolution (Å)      | 2.29          | 2.09                | 1.68                | 2.37                | 1.81                |
| Rwork (%)           | 18.62 (26.67) | 15.40 (17.54)       | 18.32 (22.23)       | 18.04 (22.62)       | 18.57 (23.76)       |
| Rmerge (%)          | 23.49 (33.02) | 19.47 (25.60)       | 20.68 (27.73)       | 23.40 (31.28)       | 21.59 (26.07)       |
| No. residues        | 618           | 618                 | 618                 | 618                 | 618                 |
| No. ligands         | 0             | 92                  | 46                  | 90                  | 68                  |
| No. water molecules | 160           | 484                 | 1035                | 216                 | 734                 |
| No. atoms           | 5277          | 5656                | 6170                | 5418                | 5877                |
| RMSD                 | | | | | |
| Bond lengths (Å)    | 0.008         | 0.009               | 0.01                | 0.009               | 0.008               |
| Bond angles (°)     | 0.94          | 0.92                | 0.94                | 1.00                | 0.86                |
| Average B-factors (Å²) | 30.22       | 24.58               | 19.69               | 35.23               | 26.66               |
| Macromolecules      | 29.85         | 23.66               | 17.11               | 34.94               | 24.67               |
| Ligands             | -             | 35.32               | 14.45               | 47.59               | 28.80               |
| Solvent             | 35.29         | 32.18               | 32.62               | 36.79               | 40.24               |
| Ramachandran        | | | | | |
| Most favored regions (%) | 97         | 98                  | 98                  | 98                  | 98                  |
| Additional allowed regions (%) | 3.1 | 2.0 | 2.1 | 2.3 | 1.8 |
| Disallowed regions (%) | 0           | 0                   | 0                   | 0                   | 0                   |
| Clashscore          | 8.93          | 5.37                | 5.1                 | 4.84                | 5.91                |
| PDB code            | 5YLH          | 5YLK                | 5Z4T                | 5YLI                | 5YLL                |

\[ R_{\text{merge}} = \frac{\sum_{hkl} I_{hkl} - \langle I(hkl) \rangle}{\sum_{hkl} I_{hkl}} \langle I(hkl) \rangle, \] where \( I(hkl) \) is the \( i \)th observation of reflection \( hkl \) and \( \langle I(hkl) \rangle \) is the weighted average intensity for all observations \( i \) of reflection \( hkl \).

\[ R_{\text{work}} = \frac{1}{N_{\text{work}}} \sum_{hkl} \left| F_{hkl} - k \right| F_{hkl} \] \( R_{\text{work}} \) is the \( R \) value for the reflections used in the refinement, whereas \( R_{\text{merg}} \) is the \( R \) value for 5% of the reflections selected randomly and not included in the refinement.
FIGURE 2
Structural insights into the catalytic mechanism of a novel glycoside hydrolase family 113 β-1,4-mannanase from Amphibacillus xylanus
Xin You, Zhen Qin, Qiaojuan Yan, Shaoqing Yang, Yanxiao Li and Zhengqiang Jiang
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