Acta Crystallographica Section D

Volume 70 (2014)

Supporting information for article:

Structural insights into the substrate specificity and transglycosylation activity of a fungal glycoside hydrolase family 5 β-mannosidase

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Table S1 Primers used in this study.

Primers	Primer sequence (5'-3') [a]	Bases (bp)
Man5BDF	GARGARTTYGGNATGG	16
Man5BDR	TCRTGNGGNGGRTCNCC	17
Man5B5´GSP	AGCGACCTTCACCGGAGTAAGC	22
Man5B5´NGSP	TATGGCTTGTTGGCGTGCTTGG	22
Man5B3´GSP	ACAAGTATCTTCCAAGCACGCCAACA	26
Man5B3´NGSP	CGGTGAAGGTCGCTCCACTTAT	22
*Rm*Man5BF [b]	CCGGAATTCGCCTTTGTCAAGATAGCCTCG	30
*Rm*Man5BR	ATAAGAATGCGGCCGCCTTGGAGAGTTCCTTTTGCACCTTG	41
Glu202Ala [c]	CAGATCGCCAAT**GCA**CCCCAGGAAG	25
	CTTCCTGGGG**TGC**ATTGGCGATCTG	25
Glu301Ala	CTATAGTTATGGAA**GCA**TTTGGAATGGC	28
	GCCATTCCAAA**TGC**TTCCATAACTATAG	28
Trp119Ala	CTTTTGGCAA**GCG**AGTGGAGG	21
	CCTCCACT**CGC**TTGCCAAAAG	21
Asn260Ala	CTGGGTCGAA**GCC**TGGGGAATCT	23
	AGATTCCCCA**GGC**TTCGACCCAG	23
Asn260Ser	CTGGGTCGAA**AGC**TGGGGAATCT	23
	AGATTCCCCA**GCT**TTCGACCCAG	23
Asn260Trp	CTGGGTCGAA**TGG**TGGGGAATCT	23
	AGATTCCCCA**CCA**TTCGACCCAG	23
His479Gln	GATCCTCCT**CAA**GAACCGCATG	22
	CATGCGGTTC**TTG**AGGAGGATC	22
Glu380Ala	GATCCTCCTCAC**GCA**CCGCATG	22
	CATGCGG**TGC**GTGAGGAGGATC	22

[a] N=A/T/C/G, R=A/G, Y=C/T.

[b] Restriction enzyme sites incorporated into primers are underlined.

[c] Mutations are indicated by bold letters.

Table S2 ^{1}H-NMR and ^{13}C-NMR data of the major isomers of mannosyl-fructose

	1H-NMR, δ [a]			13-C-NMR, δ		
position	**1A** [b]	**1B**	**1C**	**1A**	**1B**	**1C**
1a/b	3.46	3.50	n.d. [c]	63.95	62.61	n.d.
	n.d.	n.d.	n.d.			
2				98.05	102.51	105.08
3	3.84	4.18	n.d.	66.12	74.77	n.d.
4	4.05	4.17	n.d.	76.45	84.12	n.d.
5	4.10	3.91	n.d.	66.22	80.05	n.d.
6a/b	3.63	n.d.	n.d.	63.22	62.72	n.d.
	3.91	n.d.	n.d.			
1'	4.77	4.66	4.64	96.97	99.81	100.14
2'	3.97	3.96	n.d.	70.90	70.55	n.d.
3'	3.57	n.d.	n.d.	72.95	72.84	n.d.
4'	3.49	3.58	n.d.	66.86	67.13	n.d.
5'	3.30	n.d.	n.d.	76.40	76.19	n.d.
6'	3.84	n.d.	n.d.	61.08	61.27	n.d.
	3.63	n.d.	n.d.			

[a] Assignments are based on ^{1}H, ^{13}C, DQFCOSY, NOSEY, gHSQC and gHMBC spectra. ^{1}H-NMR at 500 MHz, D2O, ref = 4.7 ppm. ^{13}C-NMR at 125.9 MHz.

[b] Mannosyl-fructose isomers **1A**: β-D-mannopyranosyl-(1→4)-β-D-fructopyranose, **1B**: β-D-mannopyranosyl-(1→4)-β-D-fructofuranose, and **1C**: β-D-mannopyranosyl-(1→4)-α-D-fructofuranose.

[c] n.d., not detected.

Table S3 Superposition statistics for crystal structures of *Rm*Man5B [a]

	*Rm*Man5B-native ChainA	*Rm*Man5B-native ChainB	*Rm*Man5B/E202A-M2 ChainA	*Rm*Man5B/E202A-M2 ChainB	*Rm*Man5B/E202A-M3	*Rm*Man5B/E202A-ManFru ChainA	*Rm*Man5B/E202A-ManFru ChainB	*Rm*Man5B/E301A
*Rm*Man5B E301A	0.300 Å /414 [b]	0.323 Å/412	0.550 Å/414	0.560 Å/413	0.164 Å/414	0.312 Å/414	0.311 Å/412	414 [c]
*Rm*Man5B E202A-ManFru ChainB	0.159 Å/414	0.162 Å/415	0.454 Å/414	0.451 Å/414	0.268 Å/412	0.088 Å/414	415	
*Rm*Man5B E202A-ManFru ChainA	0.156 Å/416	0.171 Å/414	0.457 Å/416	0.459 Å/415	0.262 Å/414	416		
*Rm*Man5B E202A-M3	0.248 Å/414	0.289 Å/412	0.540 Å/414	0.550 Å/413	414			
*Rm*Man5B E202A-M2 ChainB	0.494 Å/415	0.501 Å/414	0.100 Å/415	415				
*Rm*Man5B	0.491 Å/416	0.505 Å/414	416					

E202A-M2 ChainA		
*Rm*Man5B-native ChainB	0.132 Å/414	415
*Rm*Man5B-native ChainA	416	

[a] Calculations were carried out in *LSQMAN* (Lleywegt, 1999) for C^{α} atoms with the NWunsch command with a gap penalty of five.

[b] R.m.s.d./No. of pairs.

[c] Number of residues in structure.

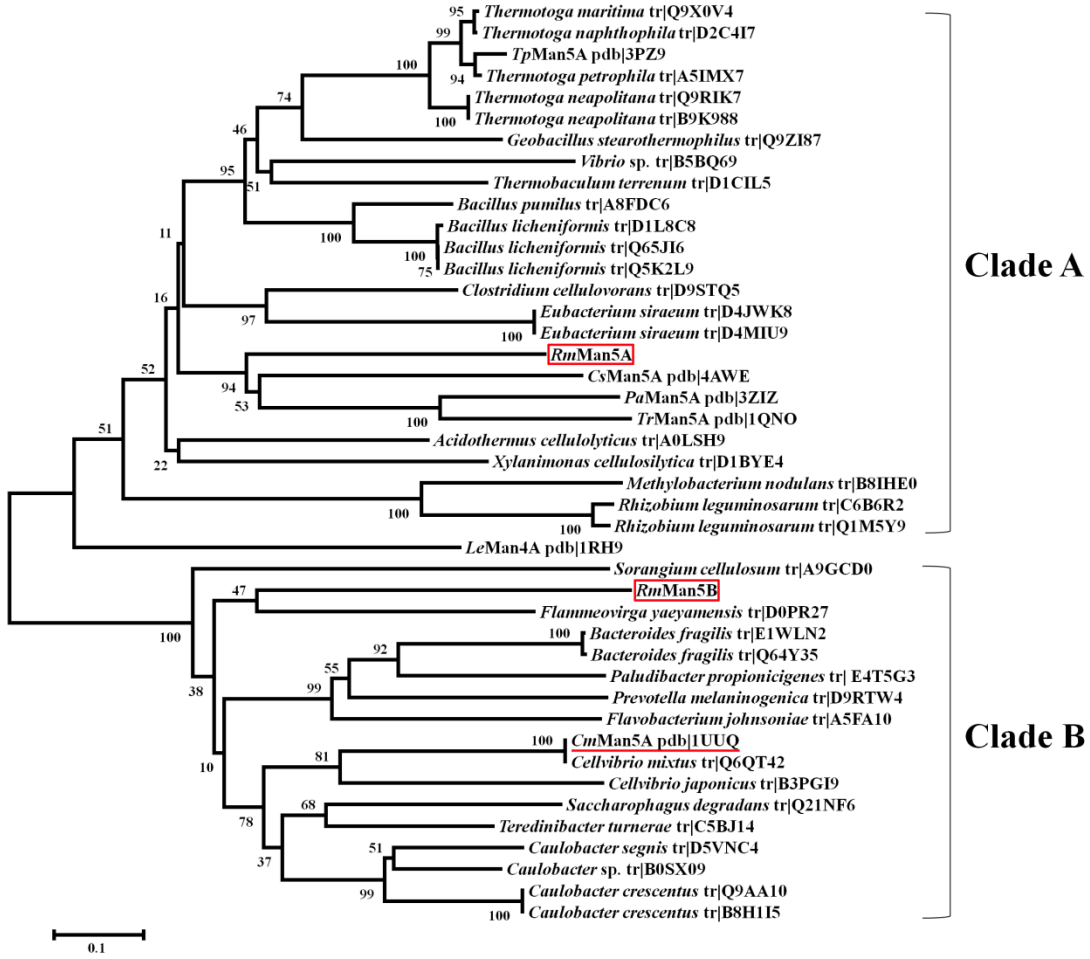


Figure S1 Phylogenetic tree of GH sub-family 5_7 members from GH family 5. Neighbor-joining tree showing phylogenetic relationships between *Rm*Man5B and other Uniprot and PDB entries. Bootstrap values are expressed as percentages of 1,000 replications. The scale bar indicates branch length. Abbreviations and accession numbers for the PDB entries are as follows: *R. miehei* β-mannanase (*Rm*Man5A), *R. miehei* β-mannosidase (*Rm*Man5B), *C. mixtus* β-mannosidase (*Cm*Man5A, 1UUQ), *L. esculentum* β-mannanase (*Le*Man4A, 1RH9), *T. petrophila* β-mannanase (*Tp*Man5A, 3PZ9), *P. anserine* β-mannanase (*Pa*Man5A, 3ZIZ), *T. reesei* β-mannanase (*Tr*Man5A, 1QNO) and *C. sitophila* β-mannanase (*Cs*Man5A, 4AWE). Phylogenetic trees were constructed using the neighbor-joining (NJ) method with program *MEGA*4 (http://www.megasoftware.net/mega.html).

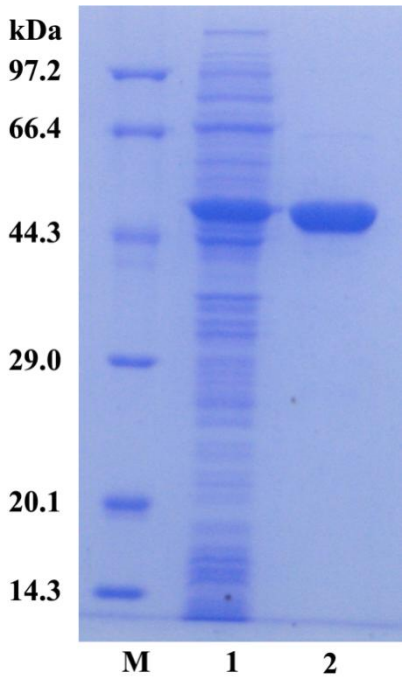


Figure S2 SDS-PAGE analysis of *Rm*Man5B expressed in *E. coli*. Lane M, low molecular weight protein marker; lane 1, crude lysate; lane 2, purified *Rm*Man5B.

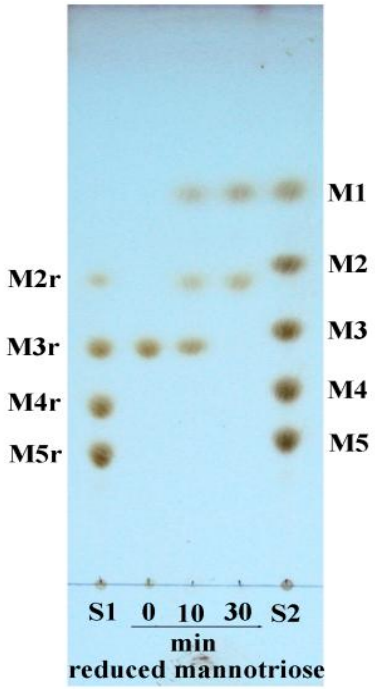


Figure S3 TLC analysis of hydrolysis products of reduced mannotriose by *Rm*Man5B. Enzyme (0.01 U ml^{-1}) was incubated with 0.1% (w/v) mannotriose in 50 m*M* sodium citrate buffer pH 5.5 at 323 K. Incubation times (h or min) are indicated. Lane S1, reduced manno-oligosaccharides consisting of reduced mannobiose (M2r), mannotriose (M3r), mannotetraose (M4r) and mannopentaose (M5r). Lane S2, manno-oligosaccharides consisting of mannose (M1), mannobiose (M2), mannotriose (M3), mannotetraose (M4) and mannopentaose (M5).

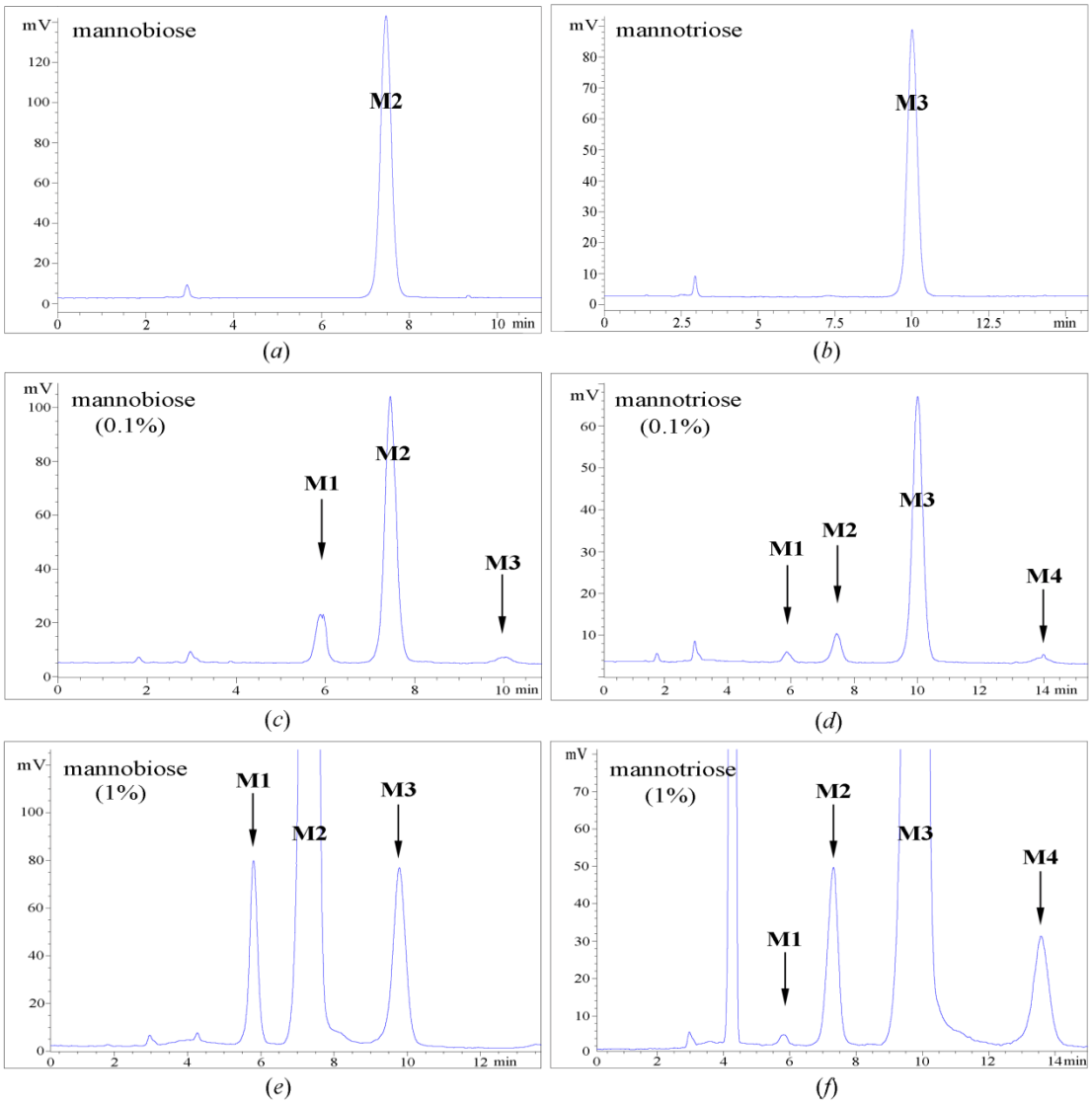


Figure S4 HPLC-ELSD analysis of reaction products produced from mannobiose or mannotriose by *Rm*Man5B. (*a*) 0.1% (w/v) mannobiose. (*b*) 0.1% (w/v) mannotriose. (*c*) *Rm*Man5B incubated with 0.1% (*w/v*) mannobiose. (*d*) *Rm*Man5B incubated with 0.1% (*w/v*) mannotriose. (*e*) *Rm*Man5B incubated with 1% mannobiose. (*f*) *Rm*Man5B incubated with 1% (*w/v*) mannotriose. All reactions were performed at 323 K for 10 min in 50 m*M* sodium citrate buffer pH 5.5. The sample of Fig. S4*f* was filtered throngh a 0.45 filter before HPLC analysis. The strong peak at 4 min in 1% mannotriose is wetting agent on the filter membrane.

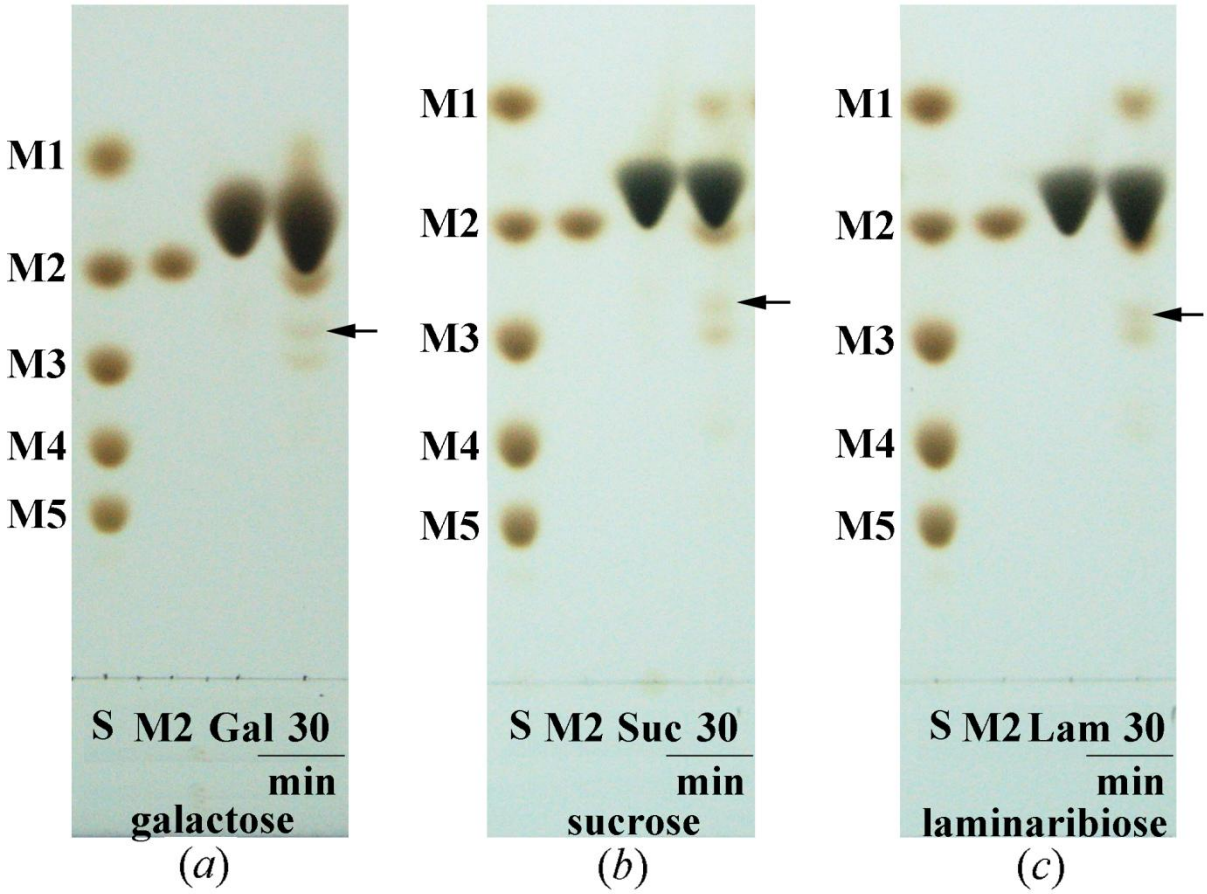


Figure S5 Analysis of various transglycosylation products catalyzed by *Rm*Man5B. (*a*) Galactose (Gal), (*b*) sucrose (Suc) and (*c*) laminaribiose (Lam, 5%, *w/v*) were incubated with mannobiose (1%, *w/v*) using *Rm*Man5B in 50 m*M* sodium citrate buffer pH 5.5 at 323 K for 30 min. The samples were analyzed by TLC. Lane S, manno-oligosaccharides consisting of mannose (M1) , mannobiose (M2), mannotriose (M3), mannotetraose (M4) and mannopentaose (M5). The spots of transglycosylation products on the TLC are marked.

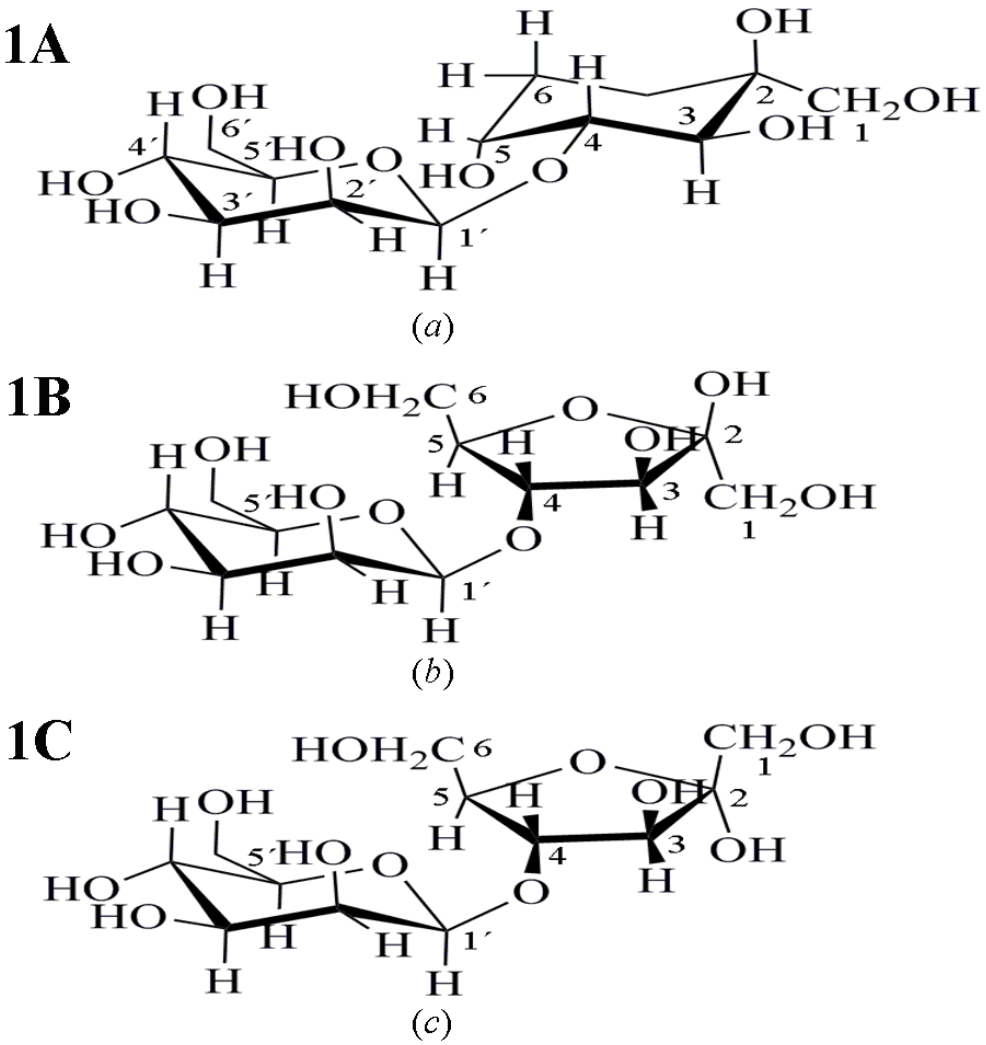


Figure S6 Major isomers of mannosyl-fructose in D_2O. Mannosyl-fructose isomers. (*a*) β-D-mannopyranosyl-(1→4)-β-D-fructopyranose (**1A**), (*b*) β-D-mannopyranosyl-(1→4)-β-D-fructofuranose (**1B**), (*c*) β-D-mannopyranosyl-(1→4)-α-D-fructofuranose (**1C**).

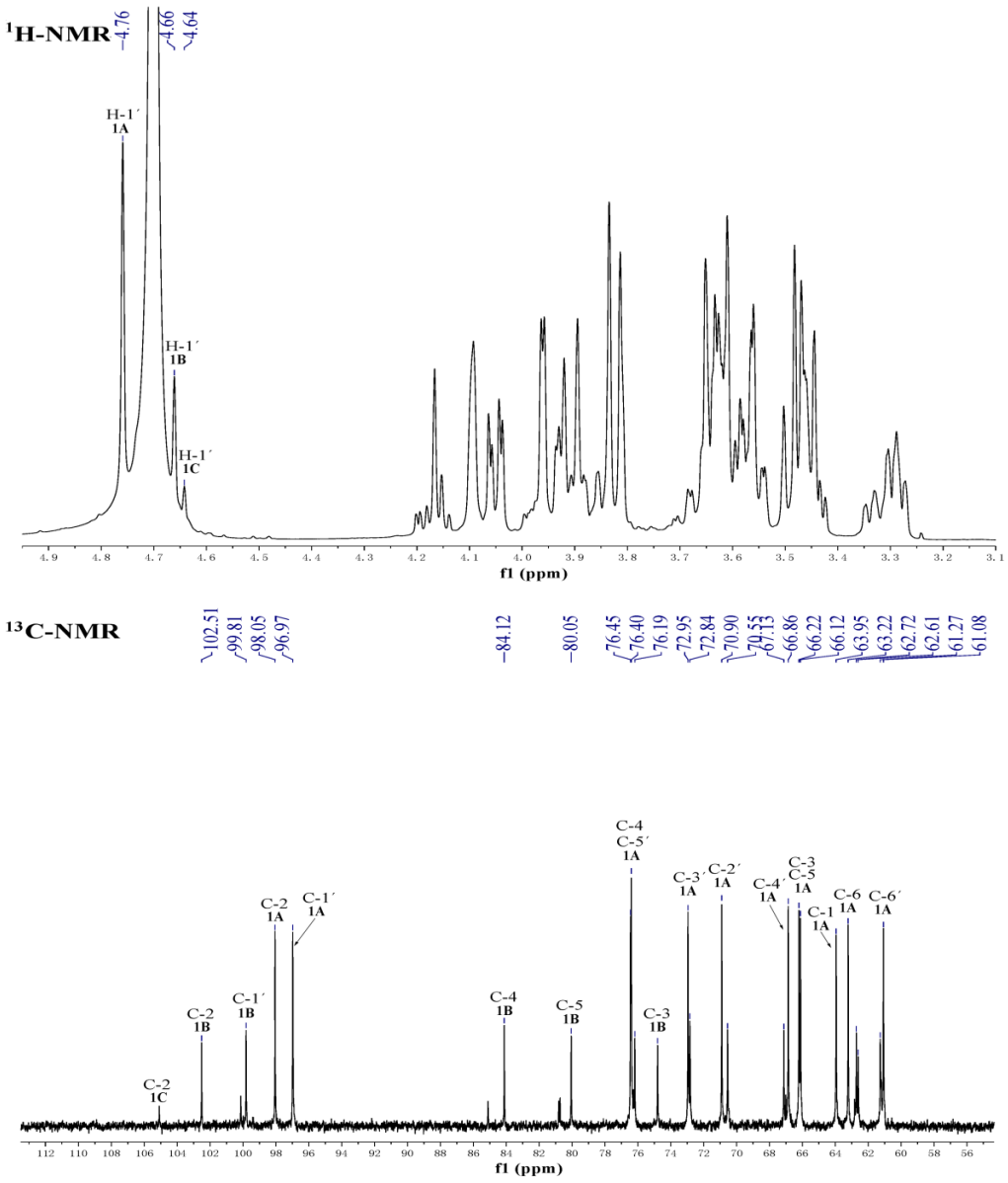


Figure S7 ^{1}H-NMR and ^{13}C-NMR (inverse gated proton decoupling) spectra of purified mannosyl-fructose. The sample (20 mg) was dissolved in 500 µl of D2O and subjected to NMR analysis using a Bruker Advance 500 spectrometer at 298 K. ^{1}H chemical shifts were referenced to the residual solvent signal at δ 4.70 (D_2O) relative to TMS. Mannosyl-fructose isomers **1A**: β-D-mannopyranosyl-(1→4)-β-D-fructopyranose, **1B**: β-D-mannopyranosyl-(1→4)-β-D-fructofuranose, and **1C**: β-D-mannopyranosyl-(1→4)-α-D-fructofuranose.

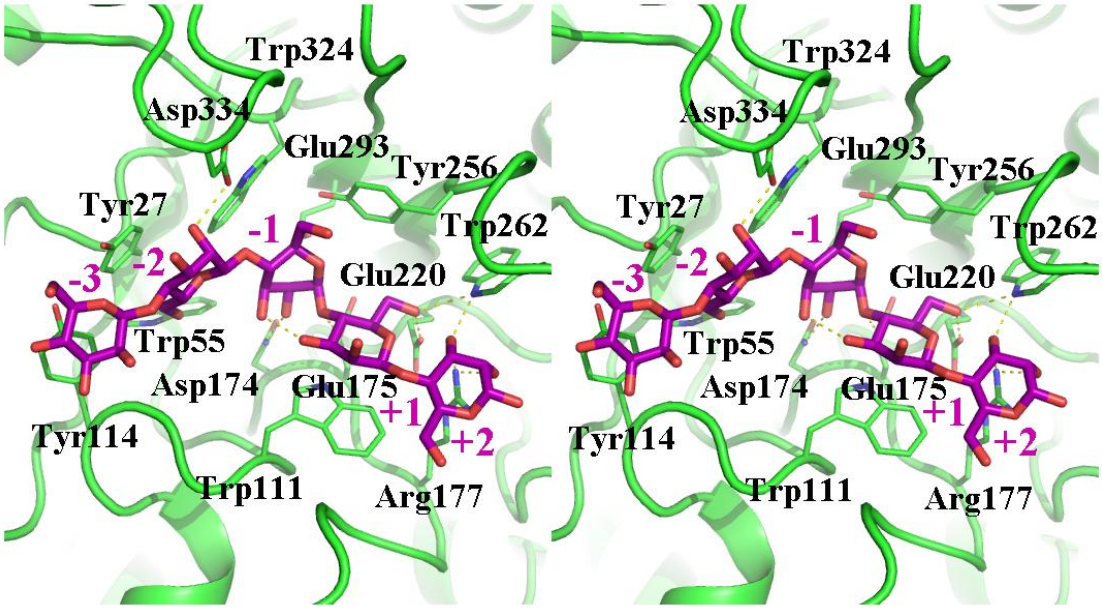


Figure S8 Stereo view of the active site of *Rm*Man5A. Mannopentaose was modeled into the putative -3 to +2 substites according to the method discribed by Bourgault *et al*. (2005). The amino acid residues surrounding mannopentaose are presented in line representaion and the mannopentaose is drawn in stick representaion in purple. Hydrogen-bond interactions are shown as dotted lines. All figures were generated by *PyMOL* v.1.3 (http://www.pymol.org; Schrödinger LLC).

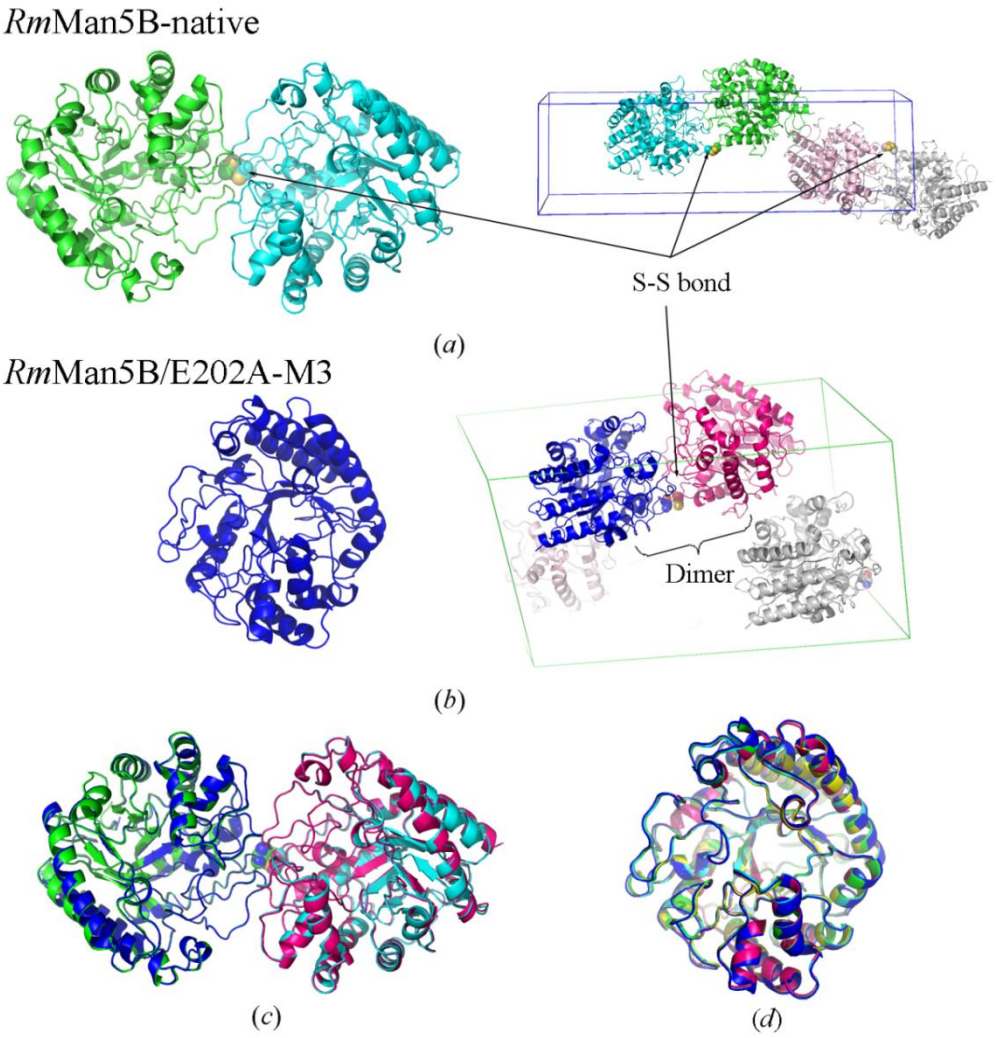


Figure S9 Asymmetric unit structures and crystals packing of (*a*) *Rm*Man5B-native and (*b*) *Rm*Man5B/E202A-M3. Disulfide bonds between molecules are shown in "spheres" representation. The crystal structures of *Rm*Man5B-native and *Rm*Man5B/E202A-M3 have two protein and one protein molecules in the asymmetric units, respectively. Two monomers interact to form a dimer through crystallographic packing, though only one protein molecule is present in the asymmetric unit of the *Rm*Man5B/E202A-M3 crystal. (*c*) Superposition of the asymmetric unit structure of *Rm*Man5B-native (molecule A, green; molecule B, cyan) with the dimer structure of *Rm*Man5B/E202A-M3 (molecule A, blue; molecule B, hotpink). (*d*) Structural superposition of the five structures of *Rm*Man5B. Color code: *Rm*Man5B-native, green; *Rm*Man5B/E202A-M2, blue; *Rm*Man5B/E202A-M3, hotpink; *Rm*Man5B/E202A-ManFru, cyan; *Rm*Man5B/E301A, yellow.

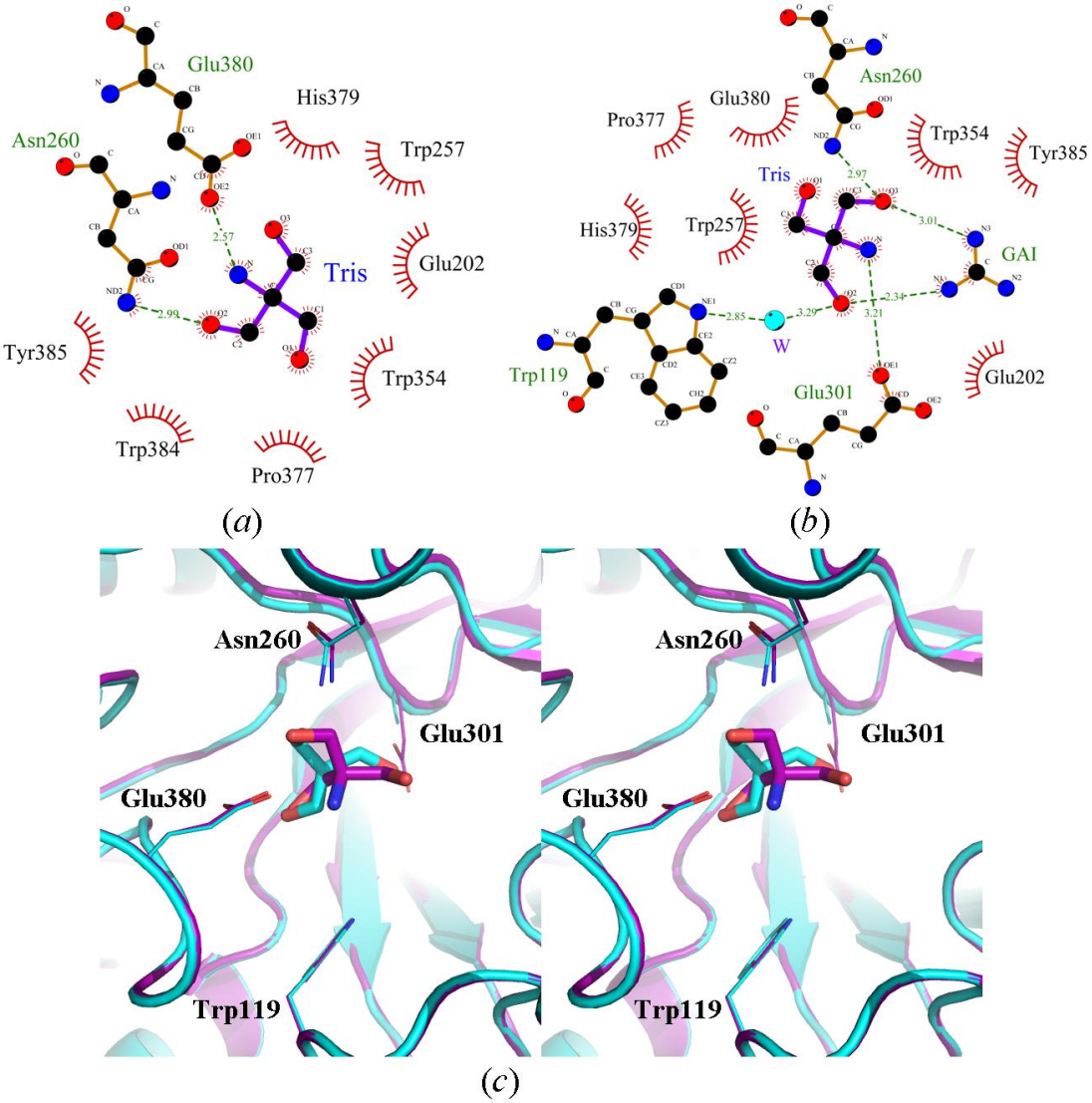
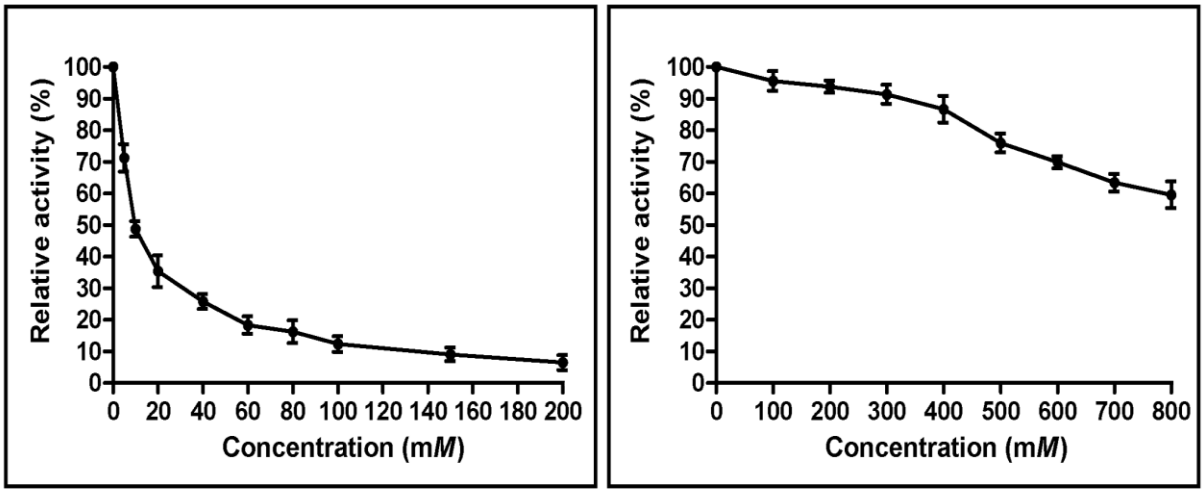


Figure S10 Schematic representation of the interactions between Tris molecules with (*a*) *Rm*Man5B and (*b*) *Rm*Man5B/E301A. These pictures were produced by *LigPlus* (Laskowski *et al.*, 2011). The atoms involved in hydrogen bonds (with distances) or hydrophobic contacts are depicted. (*c*) Stereo view of the *Rm*Man5B (C atom in purple) and *Rm*Man5B/E301A (C atom in cyan). The side chain atoms of protein residues involved in hydrogen-bonded Tris molecules are shown in line representation. The figure was generated by *PyMOL* v.1.3 (http://www.pymol.org; Schrödinger LLC).



(*a*) (*b*)

Figure S11 Concentration-dependent inhibition of *Rm*Man5B *p*NPM hydrolysis activity by (*a*) Tris and (*b*) guanidine hydrochloride.

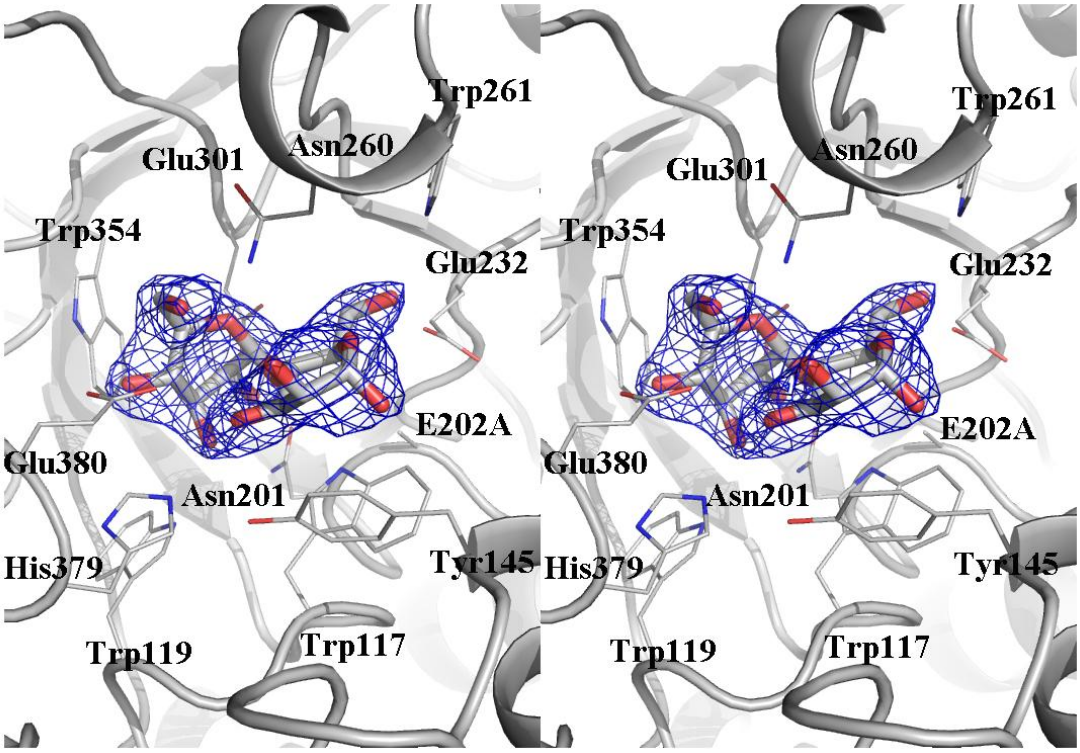


Figure S12 Stereo view of the crystal structure of *Rm*Man5B/E202A in complex with mannobiose. The σA-weighted mF_o-DF_c OMIT electron density map contoured at 3.0σ for mannobiose is shown as a blue mesh.

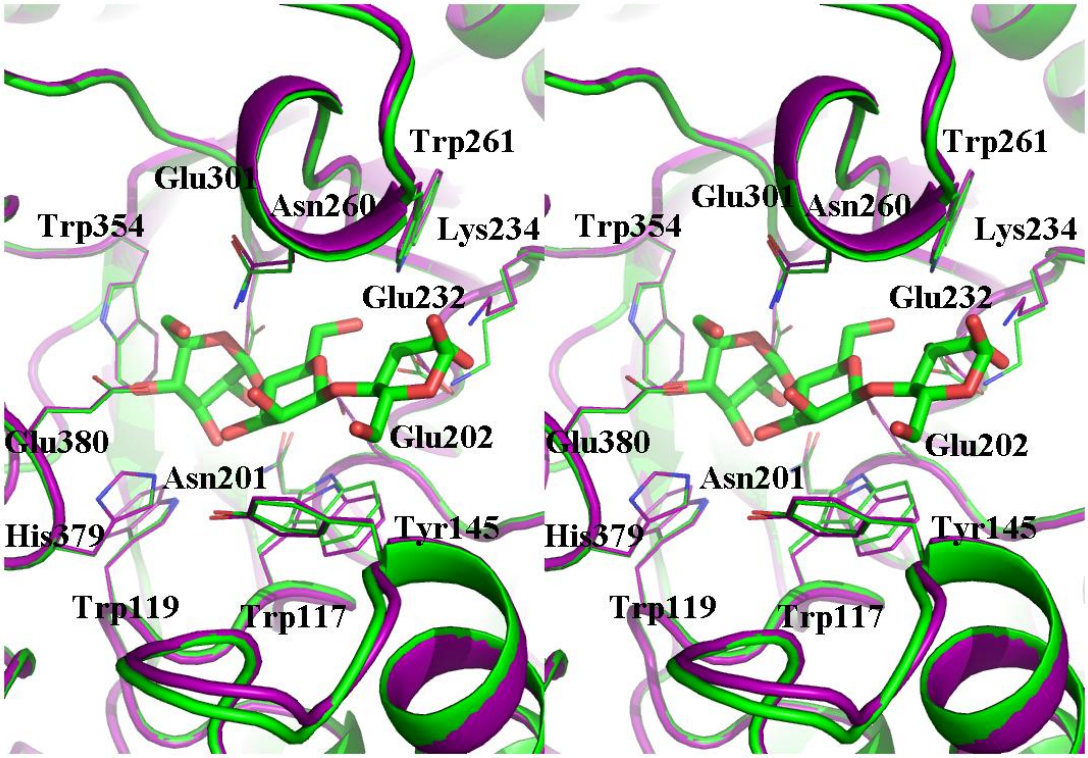


Figure S13 Stereo view of structural superposition of *Rm*Man5B/E202A-M3 with *Rm*Man5B. The carbon atoms of residues are colored according to each enzyme: *Rm*Man5B-native in purple, *Rm*Man5B/E202A-M3 and mannotriose in green. All figures were generated by *PyMOL* v.1.3 (http://www.pymol.org; Schrödinger LLC).

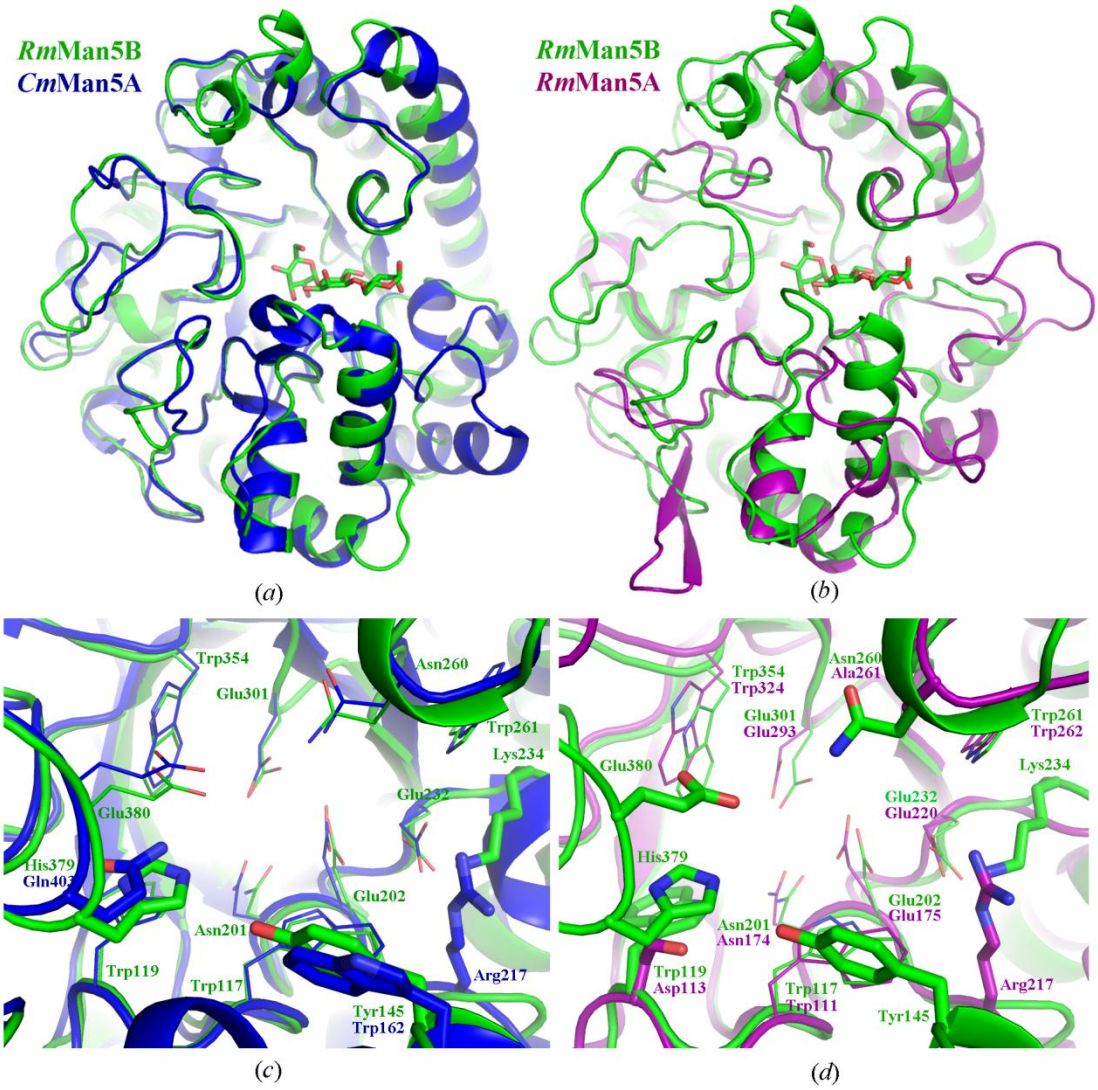


Figure S14 Comparison of *Rm*Man5B with *R. miehei* β-mannanase *Rm*Man5A and *C. mixtus* β-mannosidase *Cm*Man5A. Structural superposition of (*a*) *Rm*Man5A and (*b*) *Cm*Man5A on *Rm*Man5B. Comparison of the active site of *Rm*Man5B with (*c*) *Rm*Man5A and (*d*) *Cm*Man5A. The different amino-acid residues are highlighted in stick representation. The carbon atoms of residues are colored according to each enzyme: *Rm*Man5B in green, *Cm*Man5A in blue and *Rm*Man5A in purple. The figures were generated by *PyMOL* v.1.3 (http://www.pymol.org; Schrödinger LLC).