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Title: **Flexibility and Function of Distal Substrate-Binding Tryptophans in the Blue Mussel β-Mannanase MeMan5A and Their Role in Hydrolysis and Transglycosylation**

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Table S1. *Sequences and properties of the primers used for the W240A and W281 substitutions. The mutated bases are shown in bold and underlined on the sense strand. The corresponding bases in the template are underlined.*

Substitution	Primer	Sequence (5'–3')	T_m (°C)	% GC
W240A	Forward	CAATGGTTACAGTTGGATC**GGC**GAACATGAAAGCTGACAC	77.63	50.0
	Reverse	GTGTCAGCTTTCATGTTCGCCGATCCAACTGTAACCATTG	77.63	50.0
	Template	CAATGGTTACAGTTGGATCCTGGAACATGAAAGCTGACAC	–	–
W281A	Forward	CAAGTACATACCTACGAC**GC**GCAAAATCATTTTGGGAATG	77.05	42.5
	Reverse	CATTCCCAAAATGATTTTGCGCGTCGTAGGTATGTACTTG	77.05	42.5
	Template	CAAGTACATACCTACGACTGGCAAAATCATTTTGGGAATG	–	–

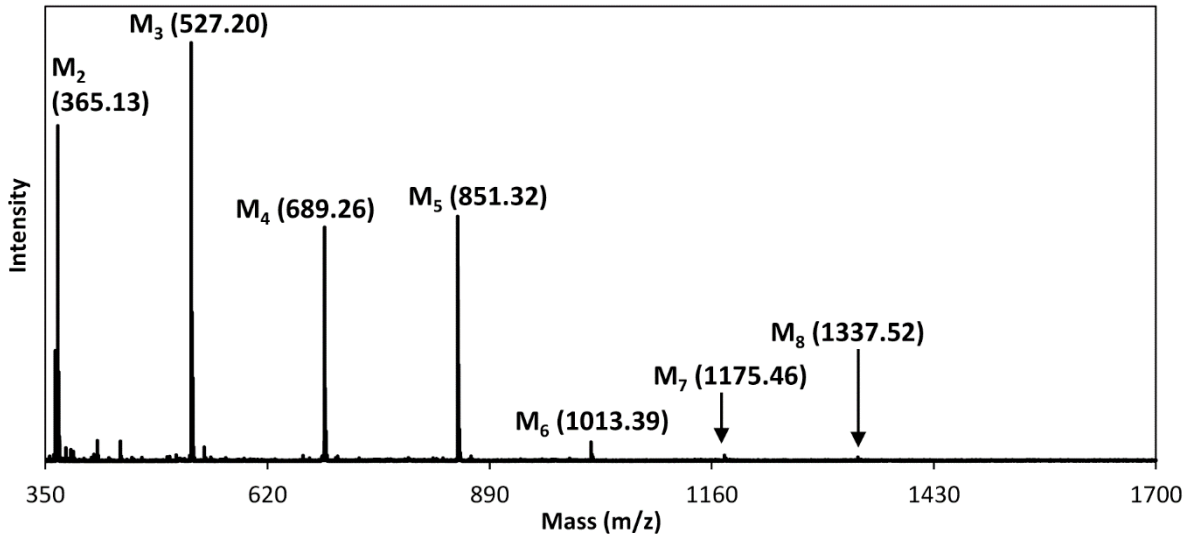

Figure S1. *MALDI-TOF MS spectrum showing detection of unlabeled manno-oligosaccharide products in a reaction with WT MeMan5A. 100 nM of WT MeMan5A, 25 mM M_5, and 6% $H_2{}^{18}O$ incubated at 8°C. Unlabeled products of a degree of polymerization 2-8 were detected. No ^{18}O-labeled transglycosylation products were detected in this reaction. The observed m/z for monoisotopic sodium adducts are shown in brackets.*

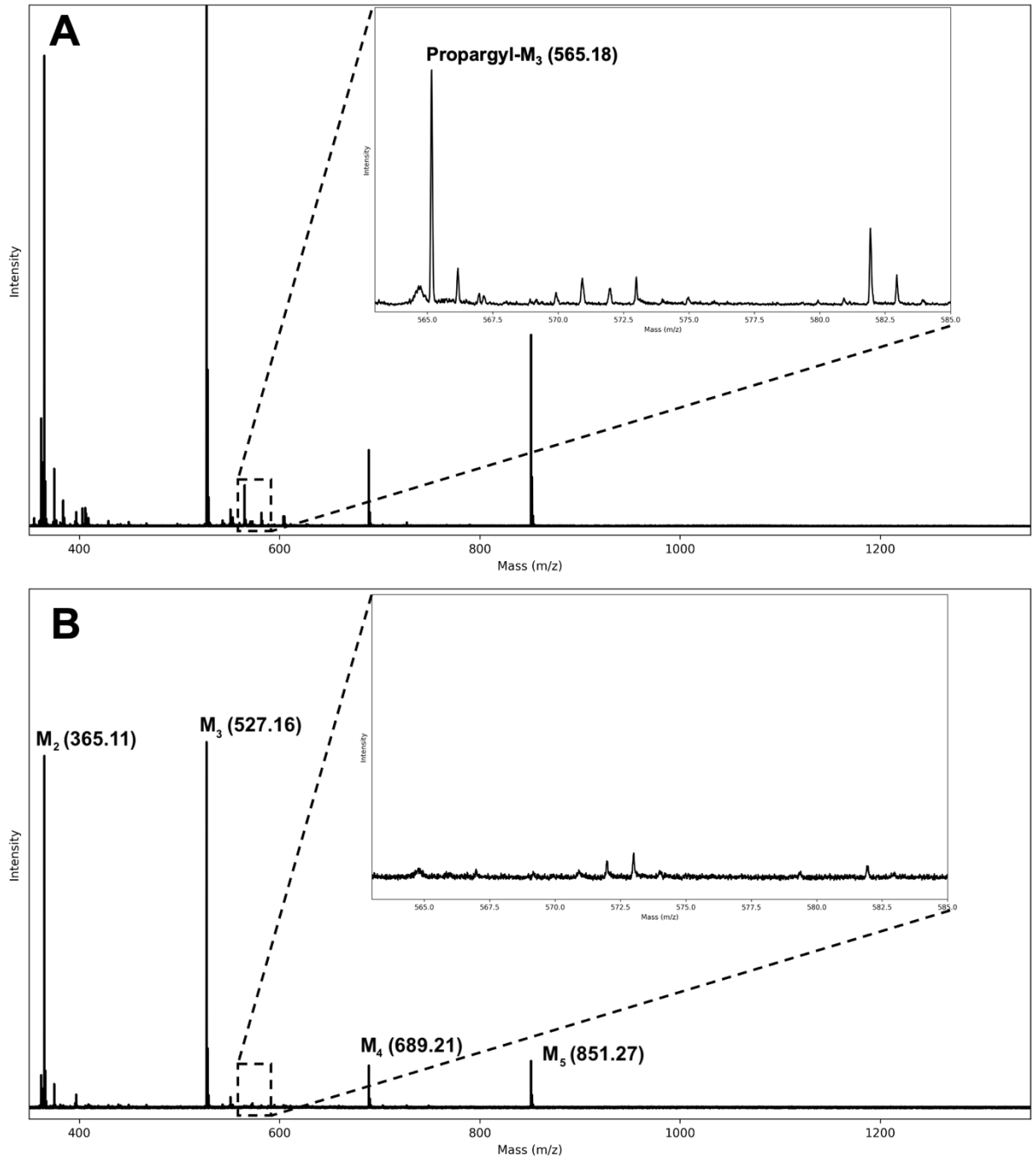

Figure S2. *MALDI-TOF spectra of reactions with 5 mM M_5 and WT MeMan5A after 5 h at 40ºC with propargyl alcohol (A) and without propargyl alcohol as control (B). 20 mM sodium acetate pH 5.2 was used. Hydrolysis products can be detected in both (A) and (B) with propargyl-mannotrioside (Propargyl-M_3) being observed in the transglycosylation reaction but not in the hydrolysis reaction. The observed m/z for monoisotopic sodium adducts are shown in brackets. The theoretical m/z for propargyl-M_3 is 565.17.*

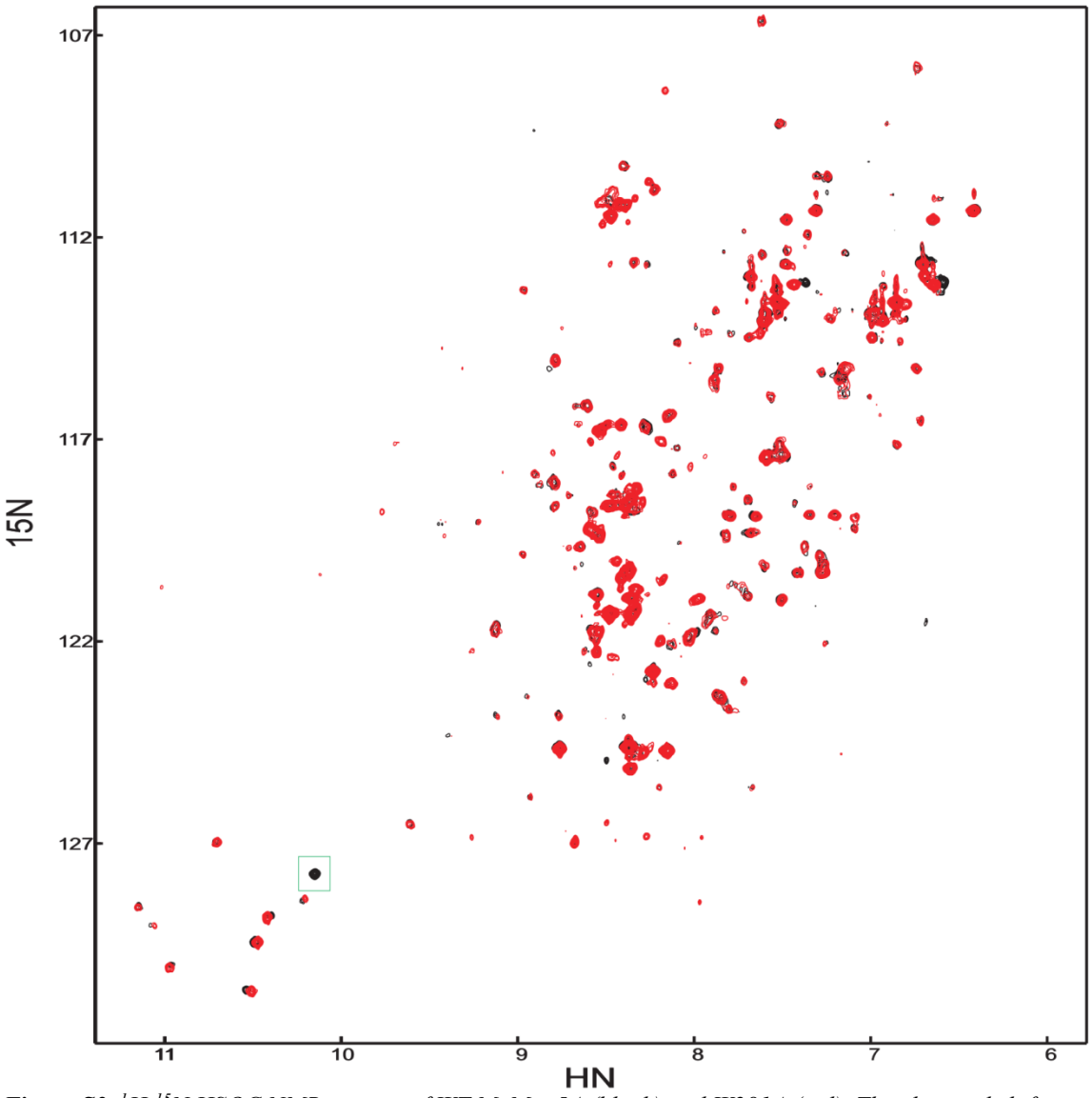

Figure S3. *^{1}H-^{15}N HSQC NMR spectra of WT MeMan5A (black) and W281A (red). The chemical shift corresponding to the W281 residue is shown within the green square.*

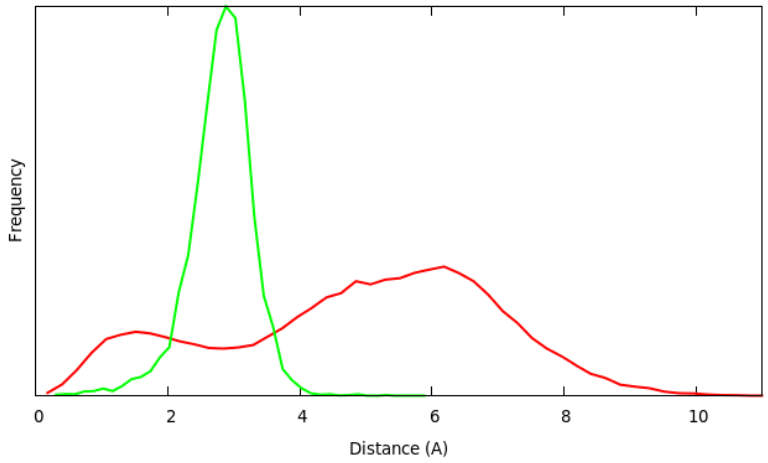

Figure S4. *Histogram showing the distribution of the distance of the center of the W281 ring relative to its position in the crystal structure, for the apo simulation (red) and the complex simulation (green).*

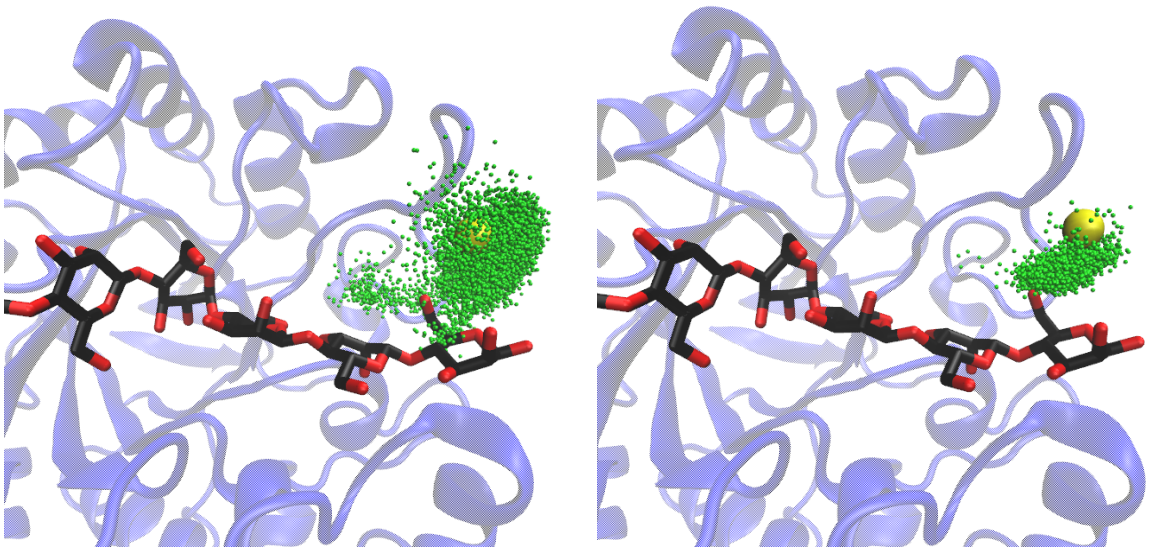

Figure S5. *Variation of the W281 position in the apo simulation (left) and the complex simulation (right). Each green dot represents the position of the W281:$C^{\varepsilon 2}$ atom in a snapshot of the simulation and the sampling frequencies have been adjusted to give approximately the same number of snapshots in the two cases. For reference, the crystal structure is shown in blue (with the $C^{\varepsilon 2}$ atom marked as a yellow sphere) and the approximated position of the ligand (from the overlay in Fig. 1A) is shown in black (carbons) and red (oxygen).*

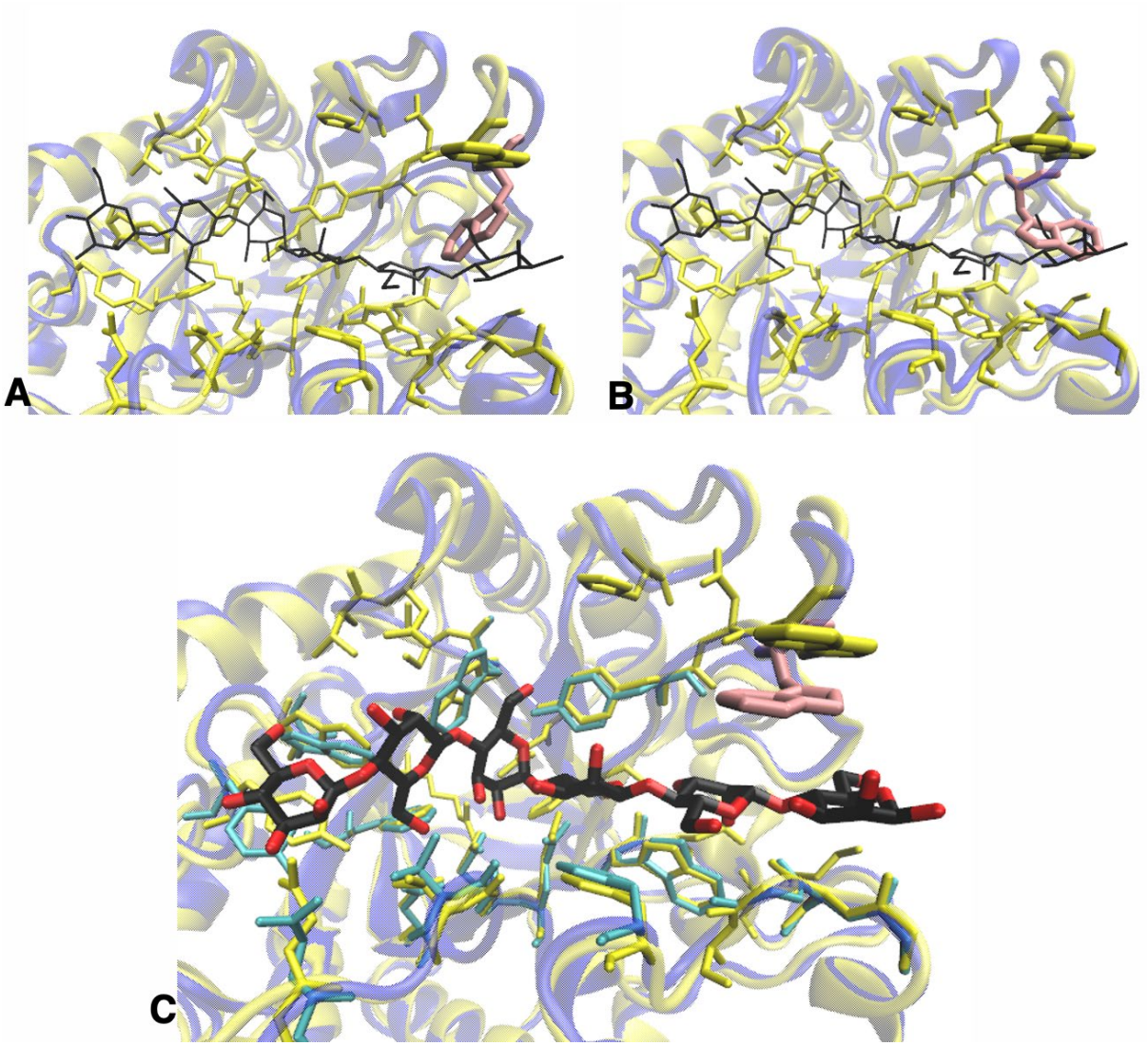

Figure S6. *Simulation results of MeMan5A with mannohexaose ligand. Ligand positioned using the overlay with the StMandC complex structure (PDB: 4Y7E) (Figure 1A). Amber ff14SB force field was applied for the protein, TIP3P for water, and GLYCAM06 for ligand. Conditions were kept constant at 40 °C and 1 bar of pressure. Bond lengths constrained by LINCS algorithm. The time step was set to 2 fs for 70 ns.* ***A*** *and* ***B:*** *Two extreme positions of W281 (pink; with the rest of the protein backbone in blue) sampled in the simulation of the apo enzyme, relative to the crystal structure (yellow). The approximate position of the ligand, as defined by the overlay in Fig. 1A, is shown in black.* ***C:*** *Representative snapshot of the complex simulation with the same color coding. Here, the mannohexaose ligand is shown in black (carbons) and red (oxygens) with the rightmost ring representing subsite +3.*