Supplementary Information

Structural Basis for Controlling the Enzymatic Properties of Polymannuronate Preffered Alginate Lyase FlAlyA from the PL-7 Family

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Materials and Methods

Overexpression and Purification

Recombinant FlAlyA was prepared using *Escherichia coli* BL21(DE3) (New England Biolabs, Ipswich, MA) and the modified pCold I vector (Takara Bio, Shiga, Japan) as described previously.[1] The expressed FlAlyA protein was fused to a modified octahistidine tag at the C-terminus connected by a GSGGGGGGGG linker. For the construction of FlAlyA mutants, site-directed mutageneses were performed by polymerase chain reaction (PCR) with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the pCold I-FlAlyA plasmid as a template.[2] The mutations were confirmed by the DNA sequencing service of FASMAC (Atsugi, Japan). The transformants were cultivated at 37 °C in lysogeny broth (LB) medium containing 100 μg/ml ampicillin. Overexpression was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the optical density at 600 nm reached 0.6, and the culture was continued for 20 hours at 15 °C.

To purify FlAlyA, the cells were resuspended in lysis buffer containing 50 mM sodium phosphate (pH 7.8), 10 mM imidazole, 100 mM NaCl, and 1% Triton X-100. The cells were disrupted by sonication and centrifuged at 40,000 *g* at 4 °C for 30 min to remove debris. The supernatant of FlAlyA was trapped on Ni-NTA Superflow resin (Qiagen, Hilden, Germany). After washing with buffer containing 50 mM sodium phosphate (pH 7.8), 20 mM imidazole and 300 mM NaCl, the recombinant FlAlyA was eluted with 300 mM imidazole dissolved in elution buffer containing 20 mM Tris-HCl (pH 7.5) and 300 mM NaCl. The eluted protein was diluted 5-fold according to volume by 20 mM MES (pH 6.5) buffer. The diluted protein was applied to a 6 ml Resource S (GE Healthcare, Chicago, IL) equilibrated with 20 mM MES (pH

6.5) buffer, and the protein was eluted with a NaCl gradient ranging from 0 to 1 M. The purified fractions were dialyzed against 20 mM Tris-HCl (pH 8.0) and concentrated to 10 mg/ml using a Vivaspin-20 (10,000 MWCO, GE Healthcare) for crystallization.

Crystallization

All crystallization experiments were performed at 20 °C by the sitting-drop vapour-diffusion method. The crystallization drops were prepared by mixing 1.0 µl of protein solution and 1.0 µl of reservoir solution. After the crystallization conditions were refined, crystals of FlAlyA suitable for X-ray analysis were obtained using a reservoir solution of 2.1 M DL-malic acid (pH 7.0).

Data Collection and Processing

The crystals were picked up with a nylon loop (Hampton Research, Aliso Viejo, CA) and directly flash-cooled in a nitrogen cryostream (95 K) without cryoprotectant. The data sets were collected at beamline AR-NE3A at the Photon Factory (Tsukuba, Japan). The diffraction data of FlAlyA were indexed, integrated, and scaled with the XDS program.[3] To obtain the initial phases, the crystals of FlAlyA soaked in the reservoir solution containing 500 mM KI were prepared, and the diffraction data were collected using an in-house X-ray diffractometer (Rigaku FR-E rotating-anode X-ray generator with a R-AXIS VII imaging-plate detector, Rigaku, Akishima, Japan). The diffraction data of the FlAlyA KI-derivative were indexed and integrated with MOSFLM,[4] and scaled with AIMLESS.[5] Data-collection statistics are given in Table S1.

Structural Determination and Refinement

Initial iodine sites in the FlAlyA crystal were found using PHENIX AutoSol Wizard[6] at a resolution of 2.43 Å. The model building was automatically carried out with PHENIX AutoBuild Wizard.[6] The structure of native FlAlyA was determined by the molecular replacement method with the PHENIX AutoMR program[6] using the KI-derivative structure as the initial model. Manual model building and refinement were performed with COOT[7] and REFMAC5,[8] respectively. The quality of the final structure was evaluated with the program PROCHECK.[9]

Enzyme Assay and Analysis.

The alginate lyase activity was assayed by the 2-thiobarbituric acid (TBA) method.[10] Briefly, the reaction mixture contained 20 mM sodium phosphate (pH 5.1-8.8), 100 mM NaCl, 0.1 mg/ml BSA, 0.1 μg/ml recombinant FlAlyA, and 0.1% (w/v) sodium alginate, poly(M), poly(G), or poly(MG). The reaction mixture was incubated at 30 °C for 6 min. One unit (1 U) was defined as the amount of enzyme required to liberate 1 μmol of β-formylpyruvate per minute.

Analyses of the Degradation Products.

The degradation products by recombinant FlAlyA were analysed by thin-layer chromatography (TLC) or high performance liquid chromatography (HPLC). Sodium alginate (0.1%, w/v) was incubated in a solution containing 20 mM sodium phosphate (pH 5.5-8.5),

100 mM NaCl, 0.1 mg/ml BSA, and 10 μg/ml recombinant FlAlyA or its mutants at 30 ˚C for up to 12 h. TLC was conducted on a silica gel 60 plate (Merck, Darmstadt, Germany). The resulting solvent contained 1-butanol, acetic acid, and water (2:1:1, v/v/v). Developed unsaturated saccharides and DEH were detected by the TBA method followed by heating at 100 ˚C for 10 min. After reaction for 12 h, the samples were analysed by HPLC with a TSKgel DEAE-2SW column (ø 4.6 x 250 mm, Tosoh Bioscience LLC, King of Prussia, PA). The degraded alginates were eluted with a linear gradient of 0–250 mM NaCl with monitoring at 230 nm. Each peak containing unsaturated oligoalginate was assigned in a previous report[11].

Table S1 Data collection and refinement statistics.[a]

	FlAlyA KI-derivative	FlAlyA
Data Collection		
Beamline	in-house	PF AR-NE3A
Wavelength (Å)	1.5418	1.00000
Space group	$P3_1$	$P3_1$
Unit-cell parameters (Å)	$a = b = 71.9$, $c = 51.2$	$a = b = 71.8$, $c = 50.9$
Resolution (Å)	51.2−2.43 (2.52−2.43)	50.0−1.54 (1.58−1.54)
No. of unique reflections	11136	43349
Redundancy	11.4	11.4
Completeness (%)	100 (99.9)	100 (100)
R_{sym}[b]	0.107 (0.367)	0.039 (0.394)
$\langle I/\sigma(I)\rangle$	17.3 (7.2)	38.5 (5.9)
Phasing	SAD[c]	
Figure of merit after phasing	0.44	
Figure of merit after RESOLVE	0.70	
Refinement		
Resolution limit (Å)		31.1−1.54 (1.56−1.54)
No. of reflections		43342 (1295)
R_{work} (%)		16.6 (19.5)
R_{free} (%)[d]		19.3 (24.3)
RMSD[e]		
Bond length (Å)		0.017
Bond angle (°)		1.702
No. of atoms		
Protein		2106
Water		340
Ramachandran analysis[f]		
Favoured, allowed, outliers (%)		97.7, 2.3, 0

[a] Values in parentheses are for the highest-resolution shell.

[b] $R_{sym} = \Sigma_{hkl} [(\Sigma_i |I_i - \langle I \rangle|) / \Sigma_i |I_i|]$, where I_i is the ith intensity measurement of the reflection hkl, including symmetry-related reflections, and $\langle I \rangle$ is its average.

[c] SAD, single-wavelength anomalous dispersion.

[d] R_{free} calculated with the 5% of reflections excluded from the refinement.

[e] RMSD, root-mean-square deviation.

[f] Calculated by MolProbity.

Table S2 Specific and relative activities of FlAlyA and its mutants toward alginate.

Enzyme	Specific activity (U/mg)[a]	Relative activity (%)
WT	254 ± 25	100
H124N	1.22 ± 0.24	4.8
Y239F	0.53 ± 0.33	0.21
H124N/Y239F	0.28 ± 0.23	0.11
P144E	0.29 ± 0.01	0.11
K160E	10 ± 0.5	3.9
T70S	294 ± 31	116
D139A	84 ± 7.4	33
T70S/D139A	122 ± 10	48
Y233F	144 ± 7.9	57
T12K	101 ± 4.8	40
T12R	139 ± 6.2	55
T14Q	154 ± 4.4	61
T14N	196 ± 6.3	77
S28E	270 ± 24	106
S28D	253 ± 22	100
T70S/D139A/T12K/ T14Q/S28E	88 ± 5.7	35

[a] Data represent the mean ± standard deviation from three independent experiments. The activity was measured at a pH of 8.0.

Table S3 Substrate specificity of FlAlyA and its mutants.

Enzyme	Specific activity (U/mg) [a]		
	Poly(M)	Poly(G)	Poly(MG)
WT	311 ± 23 (103)[b]	129 ± 16 (43)	301 ± 27 (100)
H124N	0.92 ± 0.11 (107)	2.6 ± 0.22 (302)	0.86 ± 0.29 (100)
Y239F	0.23 ± 0.07 (27)	1.8 ± 0.02 (150)	1.2 ± 0.08 (100)
H124N/Y239F	0.22 ± 0.04 (42)	0.23 ± 0.09 (44)	0.52 ± 0.10 (100)
P144E	0.019 ± 0.012 (10)	0.013 ± 0.003 (7)	0.19 ± 0.004 (100)
K160E	2.0 ± 0.30 (32)	0.44 ± 0.11 (7)	6.3 ± 27 (100)

[a] Data represent the mean ± standard deviation from three independent experiments. The activity was measured at a pH of 8.0.

[b] Value in parentheses indicates the relative activity (%) of each substrate toward poly(MG).

Table S4 Substrate specificity of FlAlyA and its mutants.

Enzyme	Specific activity (U/mg) [a]		
	Poly(M)	Poly(G)	Poly(MG)
WT	316 ± 25 (107)[b]	135 ± 11 (46)	294 ± 23 (100)
T70S	309 ± 24 (90)	114 ± 8.9 (33)	344 ± 27 (100)
D139A	72 ± 7.9 (104)	25 ± 1.2 (35)	69 ± 4.6 (100)
T70S/D139A	117 ± 9.3 (93)	22 ± 1.0 (17)	126 ± 12 (100)
Y233F	106 ± 8.6 (65)	55 ± 3.2 (34)	161 ± 14 (100)
T12K	104 ± 8.1 (68)	59 ± 3.2 (39)	153 ± 10 (100)
T14Q	172 ± 11 (104)	44 ± 5.7 (27)	166 ± 13 (100)
S28E	282 ± 16 (95)	149 ± 9.9 (50)	298 ± 33 (100)
T70S/D139A/T12K/ T14Q/S28E	50 ± 3.2 (52)	22 ± 5.0 (23)	97 ± 5.2 (100)

[a] Data represent the mean ± standard deviation from three independent experiments. The activity was measured at a pH of 8.0.

[b] Value in parentheses indicates the relative activity (%) of each substrate toward poly(MG).

Table S5 pH dependency of the relative activity of FlAlyA and its mutants.

Enzyme	Specific activity (U/mg) [a]				
	pH 5.1	6.0	6.8	8.0	8.8
WT	16 ± 3.2	81 ± 9.7	278 ± 28	282 ± 25	45 ± 3.9
K158W	48 ± 4.2	122 ± 9.3	114 ± 8.6	28 ± 3.2	0
K158N	70 ± 7.3	221 ± 19	203 ± 15	112 ± 9.6	3.2 ± 0.8
K158Y	72 ± 5.2	111 ± 8.3	83 ± 4.2	28 ± 1.9	0
K158F	52 ± 3.7	103 ± 12	93 ± 7.1	21 ± 3.5	0
K158L	51 ± 6.7	112 ± 9.2	142 ± 18	131 ± 14	37 ± 5.2
K158H	25 ± 3.0	119 ± 11	178 ± 14	38 ± 3.2	13 ± 0.9

[a] Data represent the mean ± standard deviation from three independent experiments.

Table S6 Composition of alginate degrading products after the reaction of FlAlyA and its mutants at each optimal pH.

Enzyme	Peak area [a]					
	ΔG	ΔM	ΔGG	ΔMG	ΔMM	ΔGGG
WT	5187	1060	6749	2685	1809	4148
K158W	12877	4902	4170	1746	757	3849
K158Y	12020	4310	5426	2096	1017	4526
K158F	10844	2920	3766	1791	652	4174
K158L	7320	1755	6485	1864	552	3745
K158H	9904	2324	4045	2139	835	4346
K158N	8991	3579	5071	2038	1118	3869
D180E	5544	646	4259	2672	1004	3829

[a] The values were calculated by integrating the peak areas on the chromatograms shown in Figs. S3 and S4.

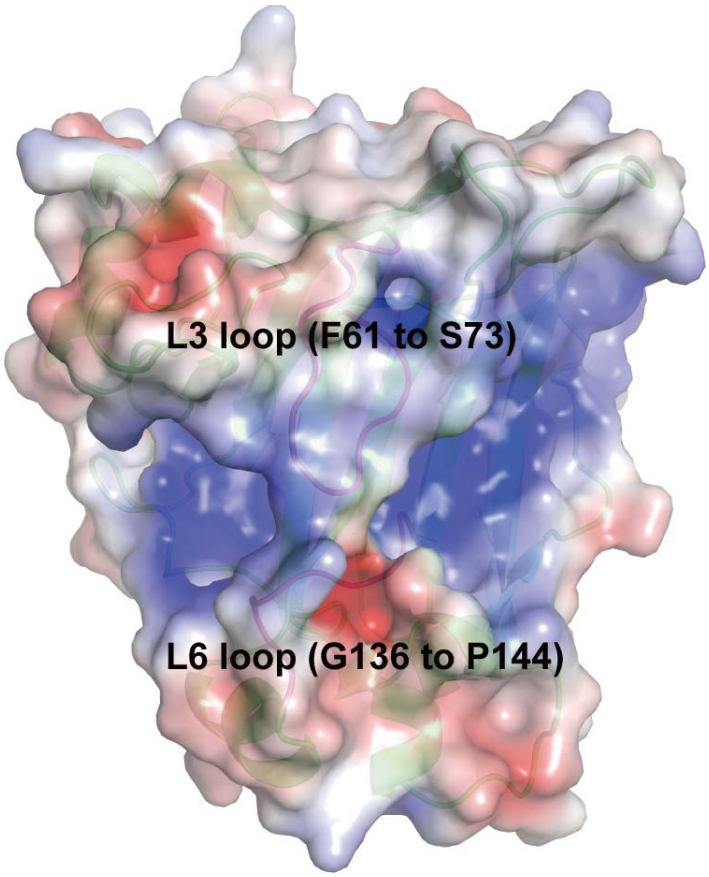

Figure S1 Electrostatic surface potential displayed in blue for positive (5 kTe^{-1}) and red for negative (–5 kTe^{-1}). Two loops covering the electropositive-rich concavity are labelled as the L3 loop (Phe61 to Ser73) and L6 loop (Gly136 to Pro144).

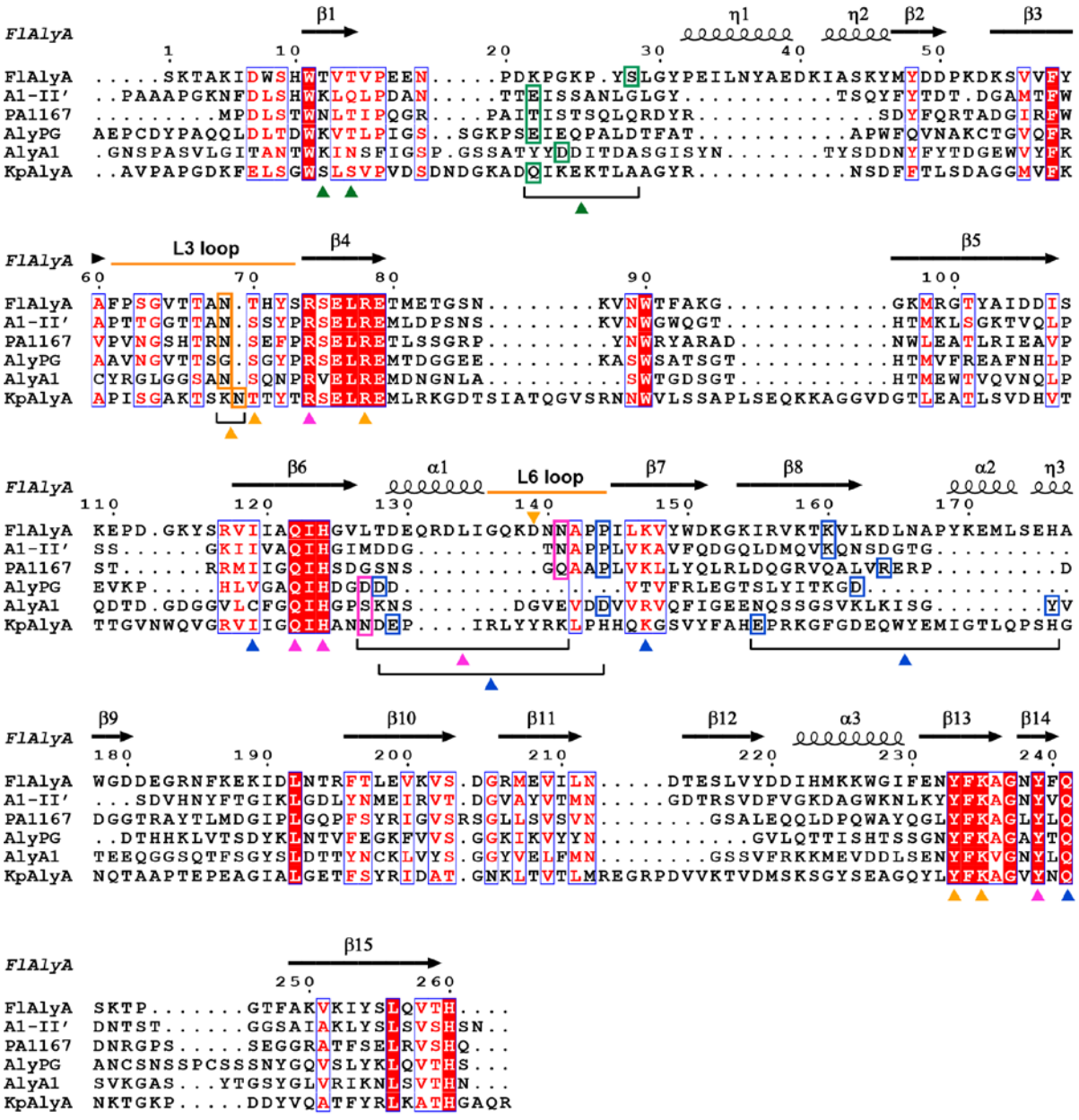

Figure S2 Sequence alignment of FlAlyA and other endolytic PL-7 family enzymes whose structures have been deposited in the PDB. The five alginate lyases except for FlAlyA are A1-II' from *Sphingomonas* sp. (PDB: 2CWS), PA1167 from *Pseudomonas aeruginosa* (PDB: 1VAV), AlyPG from *Corynebacterium* sp. (PDB: 1UAI), AlyA1 from *Zobellia galactanivorans* (PDB: 3ZPY), and KpAlyA from *Klebsiella pneumoniae* (PDB: 4OZX). The sequences were aligned and illustrated with ClustalW[12] and EsPript.[13] The secondary structural elements of FlAlyA are depicted at the top of the alignment. The green, yellow, pink and blue triangles indicate the residues located at subsites +3, +2, +1 and –1, respectively. Some

residues of FlAlyA (Ser28, Asn68, Asn141, Pro144 and Lys160) are grouped with the residues of the other six enzymes, which are located at the same position in the superposed structures. FlAlyA Asp139 is not conserved in the primary sequences and tertiary structures of other PL-7 alginate lyases.

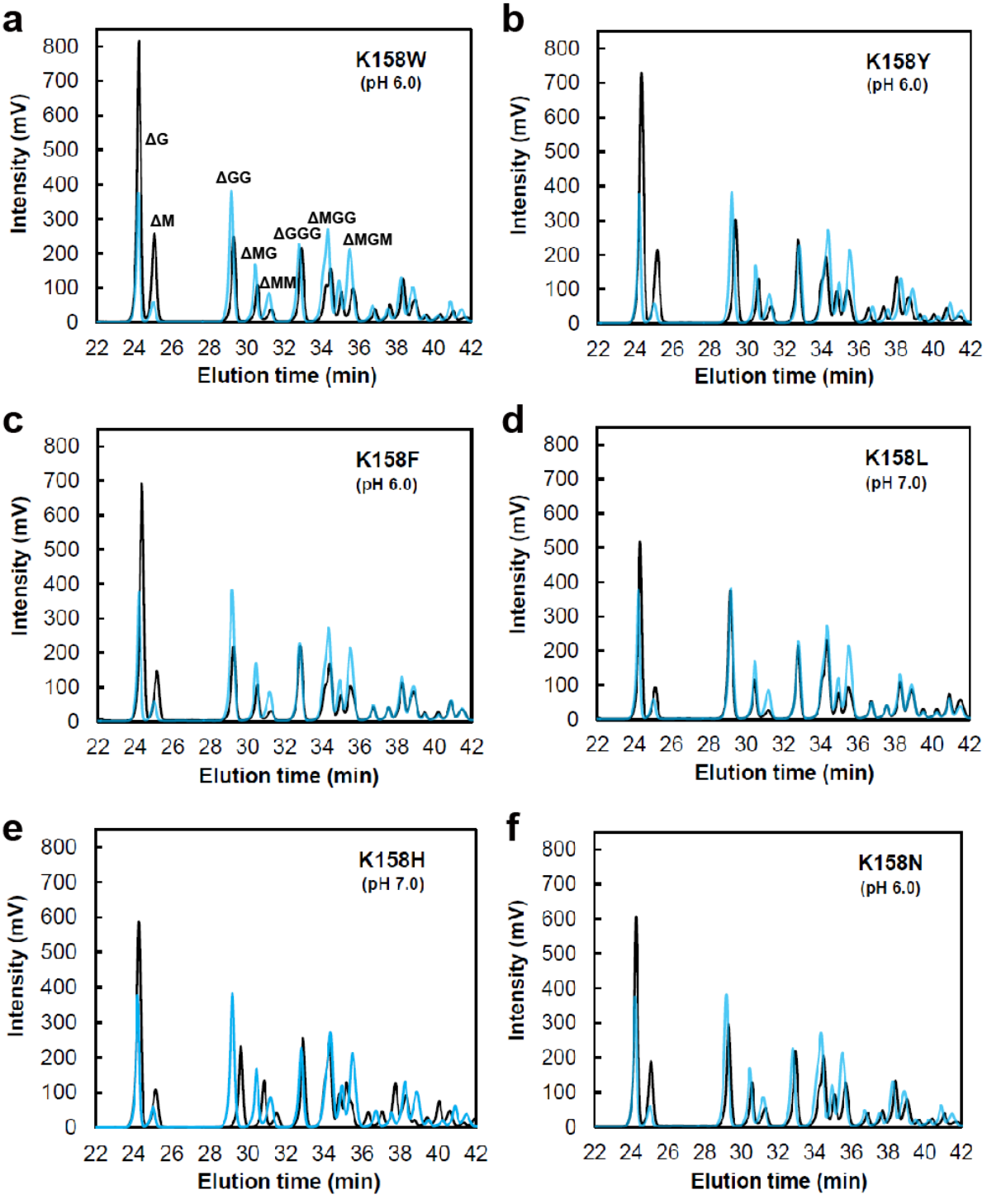

Figure S3 HPLC analysis of alginate oligosaccharides after degradation by FlAlyA and its mutants. The blue and black chromatograms stand for the wild-type and mutants of Lys158, respectively.

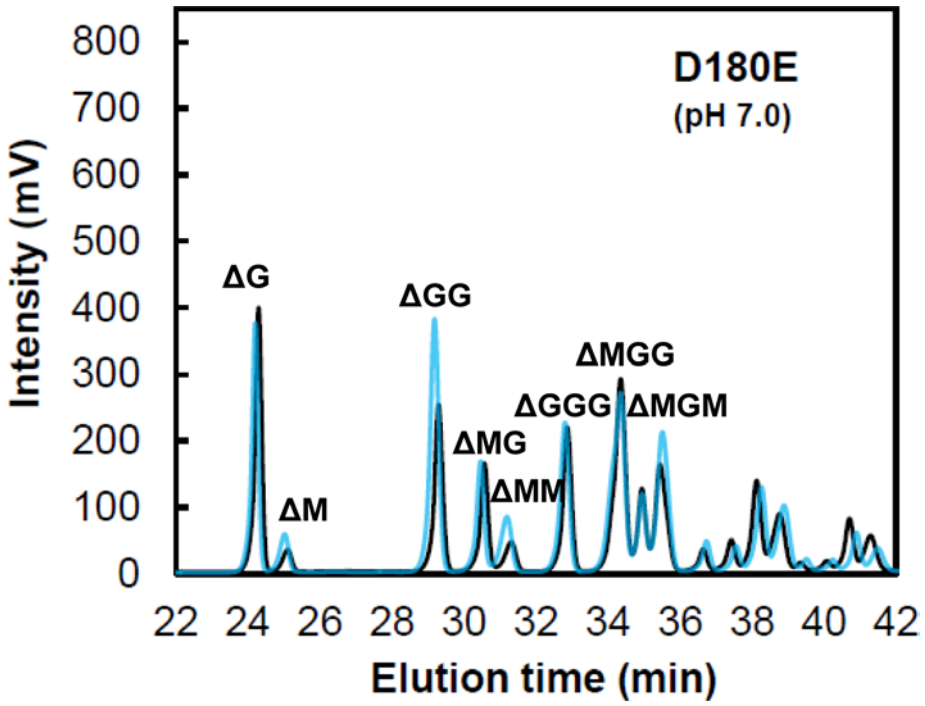

Figure S4 HPLC analysis of alginate oligosaccharides after degradation by FlAlyA (blue) and the D180E (black) mutant.

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