SUPPLEMENTARY MATERIAL

Insights into alginate degradation through the characterization of a thermophilic exolytic alginate lyase

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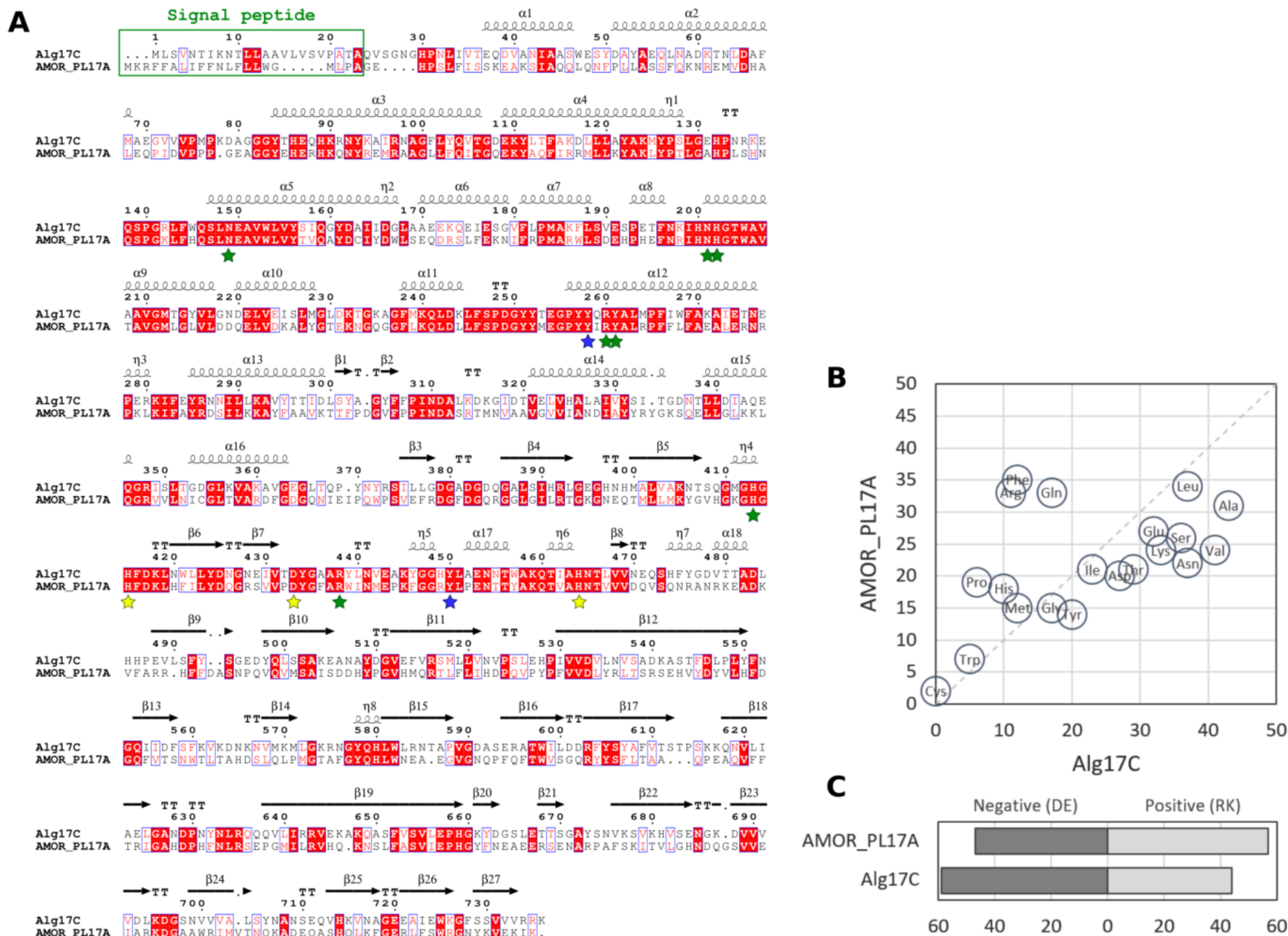

Figure S1: Sequence of AMOR_PL17A.

A) Amino acid sequence alignment of AMOR_PL17A with Alg17C from *Saccharophagus degradans*, which has a known structure (PDB id: 4NEI). Conserved residues appear in white on a red background and conservatively substituted residues are printed in red. Residues known or thought to be involved in catalysis [1], including Tyr251 (Tyr258 in Alg17C) and Tyr446 (Tyr450 in Alg17C) that were mutated in this study, are indicated by blue stars while residues with roles in neutralizing the negative charge or in binding of the oligosaccharide are indicated by green stars. Residues that make up a metal-binding site [1] are labeled with yellow stars. The sequence numbering and secondary structure assignments above the figure refer to Alg17C and the secondary structure elements are labeled as follows: α1-18, α-helices; β1-27, β-strands; η1-8, 3_{10}-helices; TT, β-turns. **B & C)** Sequence statistics (amino acid counts) for residues that are not conserved between Alg17C and AMOR_PL17A, showing that the latter enzyme contains more Pro, His, Arg, Phe and Gln (B) and carries more positively charged amino acids (C).

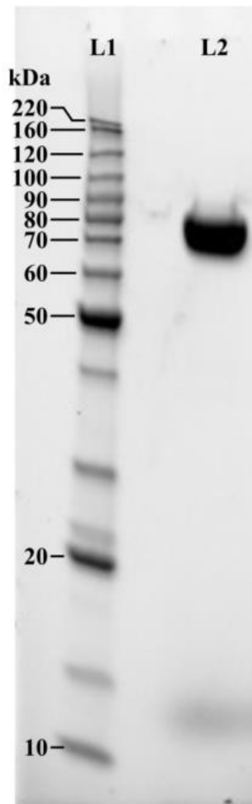

Figure S2: SDS-PAGE analysis of purified AMOR_PL17A.

Lane 1 shows marker proteins, whereas lane 2 shows purified AMOR_PL17A.

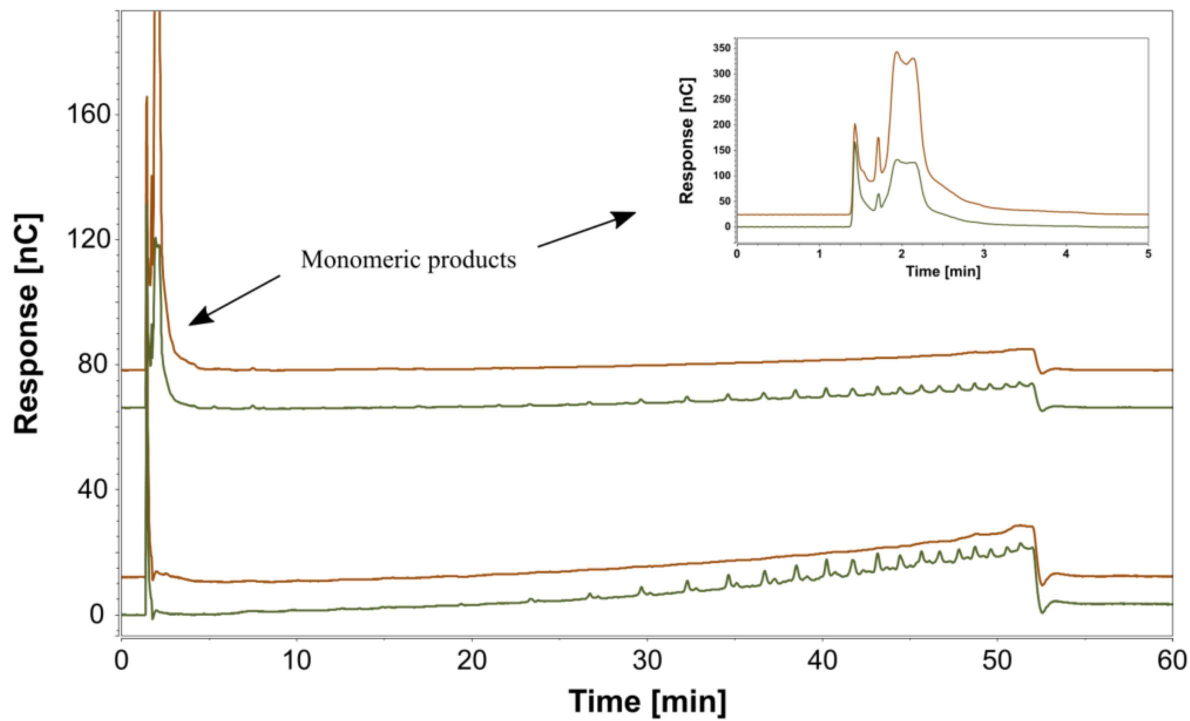

Figure S3: Degradation of polyM and polyG by AMOR_PL17A.

The two lower chromatograms show the substrates, polyM (orange) and polyG (green), whereas the two upper chromatograms show products generated in 1-hour reactions with AMOR_PL17A. One product peak is observed eluting with about two minutes retention time. The inset shows a close up of the 1-5-minute regions, indicating that the reactions with polyM and polyG yield identical product profiles, that are similar to the product profile obtained when using acid-hydrolyzed polyM as a substrate (Figure 4A). The short retention time is indicative of a monomeric product.

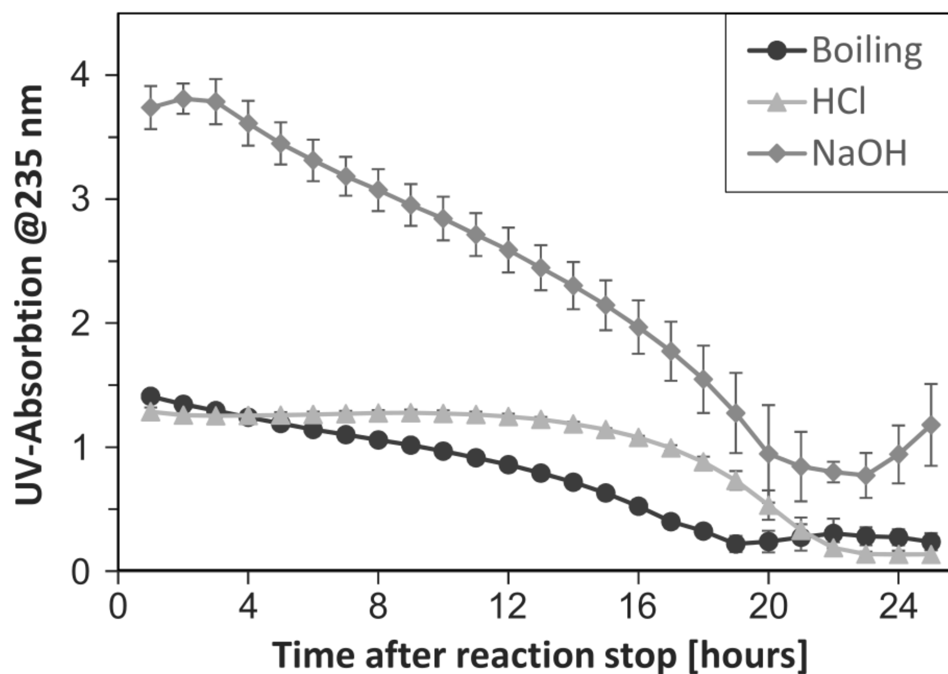

Figure S4: UV-vis analysis of the stability of the product generated by AMOR_PL17A.

UV-absorption of the product after stopping the reaction by boiling or by addition of 0.1M (final concentration) of HCl or NaOH. The curves show how the UV signal changes during storage of the reaction samples at room temperature in the dark.

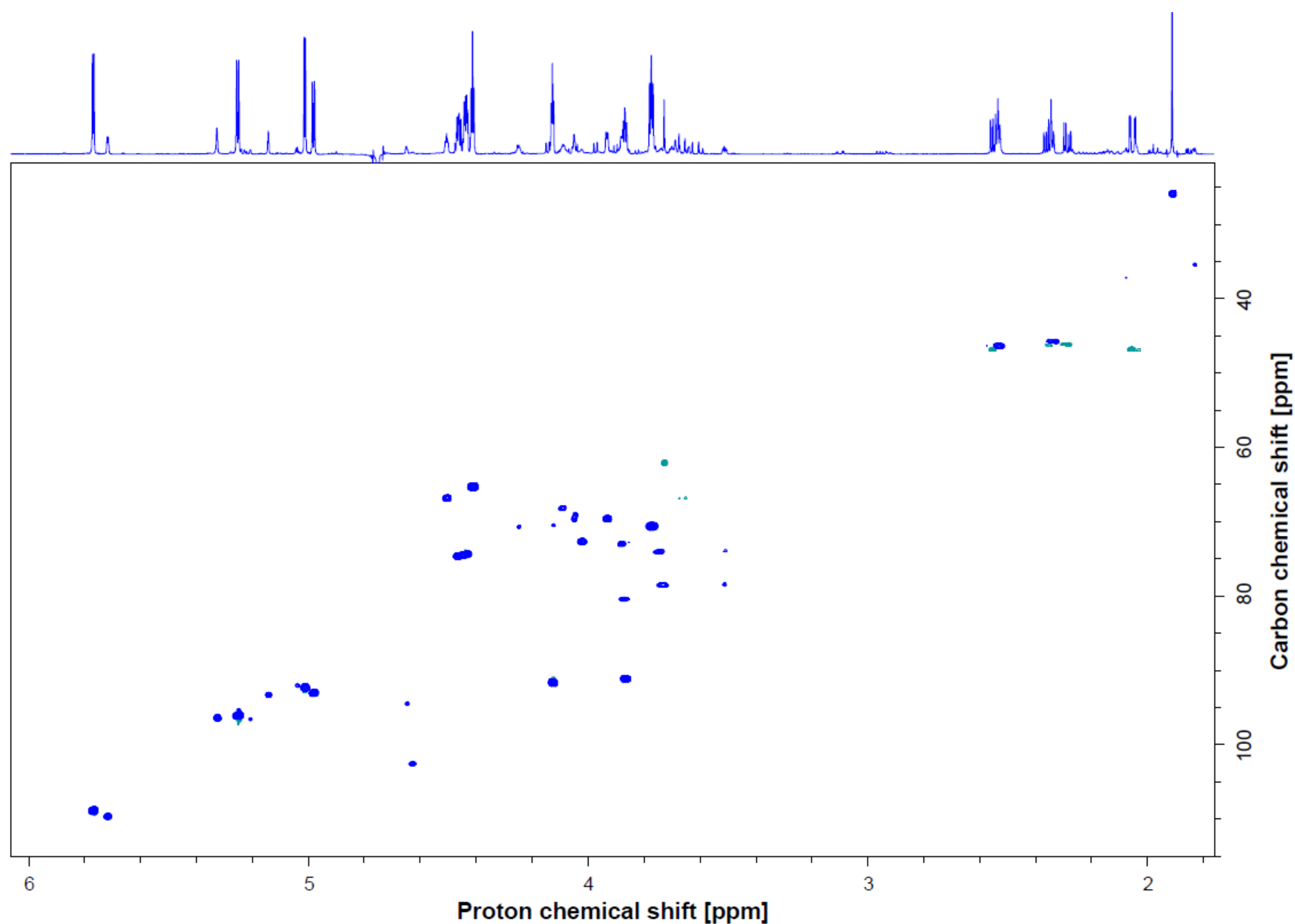

Figure S5: HSQC spectrum of AMOR_PL17A treated polyM incubated at 25 °C for ~ 17 h.

HSQC spectrum of AMOR_PL17A treated polyM (1 % (w/v) polyM substrate, 2 µM AMOR_PL17A, 10 mM NaOAc, 200 mM NaCl, pH 5.6 in 99.9% D_2O) incubated at 25 °C within the NMR for 16 h 40 min. Spectrum acquired at 25 °C on 800 MHz spectrometer. Horizontal trace displays ^{1}H 1D spectrum. Used to assign structures **1a** and **1b**.

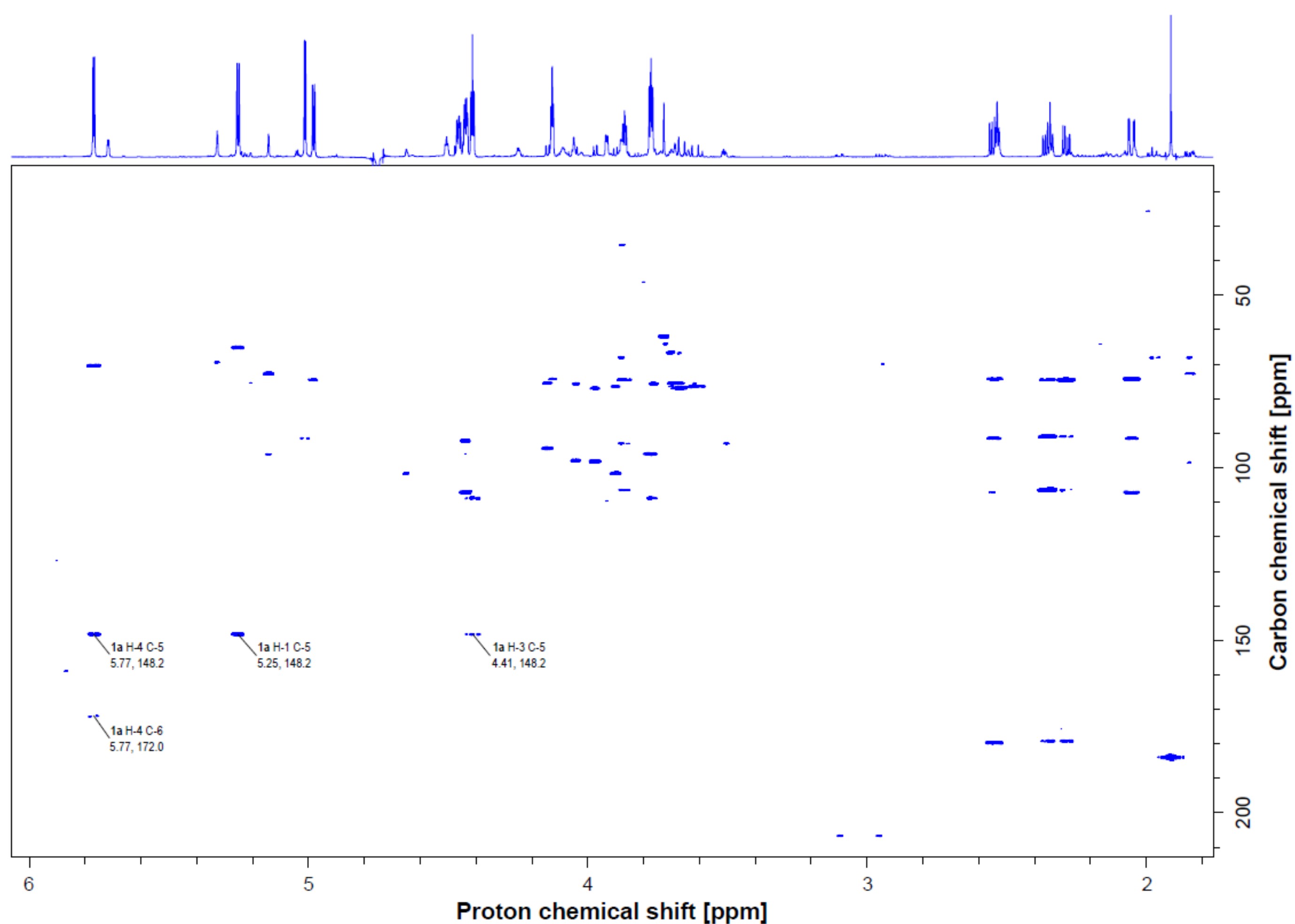

Figure S6: HMBC spectrum of AMOR_PL17A treated polyM incubated at 25 °C for ~ 17 h.

HMBC spectrum of AMOR_PL17A treated polyM (1 % (w/v) polyM substrate, 2 µM AMOR_PL17A, 10 mM NaOAc, 200 mM NaCl, pH 5.6 in 99.9% D_2O) incubated at 25 °C within the NMR for 16 h 40 min. Spectrum acquired at 25 °C on 800 MHz spectrometer. Horizontal trace displays ^1H 1D spectrum. Used to assign structures **1a** and **1b**. Key correlations referred to in text are highlighted.

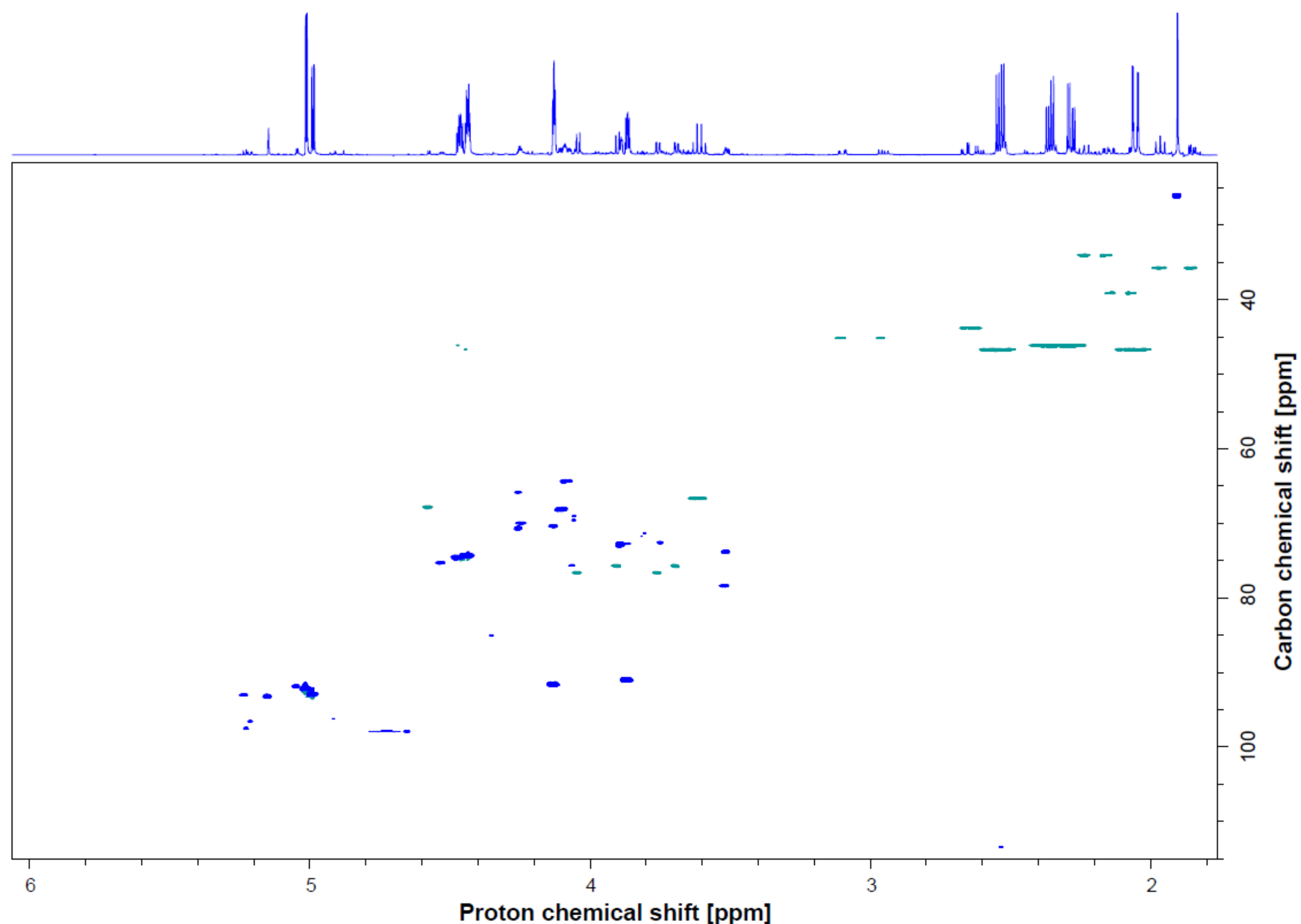

Figure S7: HSQC spectrum of AMOR_PL17A treated polyM incubated at 50 °C for ~ 48 h.

HSQC spectrum of AMOR_PL17A treated polyM (0.4 % (w/v) polyM substrate, 0.8 µM AMOR_PL17A, 5 mM NaOAc, 100 mM NaCl, pH 5.6). Sample was first incubated at 50 °C in a benchtop Thermomixer (600 rpm) for ~ 48 h, freeze dried, and then resuspended in 99.9% D_2O. Spectrum acquired at 25 °C on 800 MHz spectrometer. Horizontal trace displays ^{1}H 1D spectrum. Used to assign structures **2a**, **2b**, **3a** and **3b**.

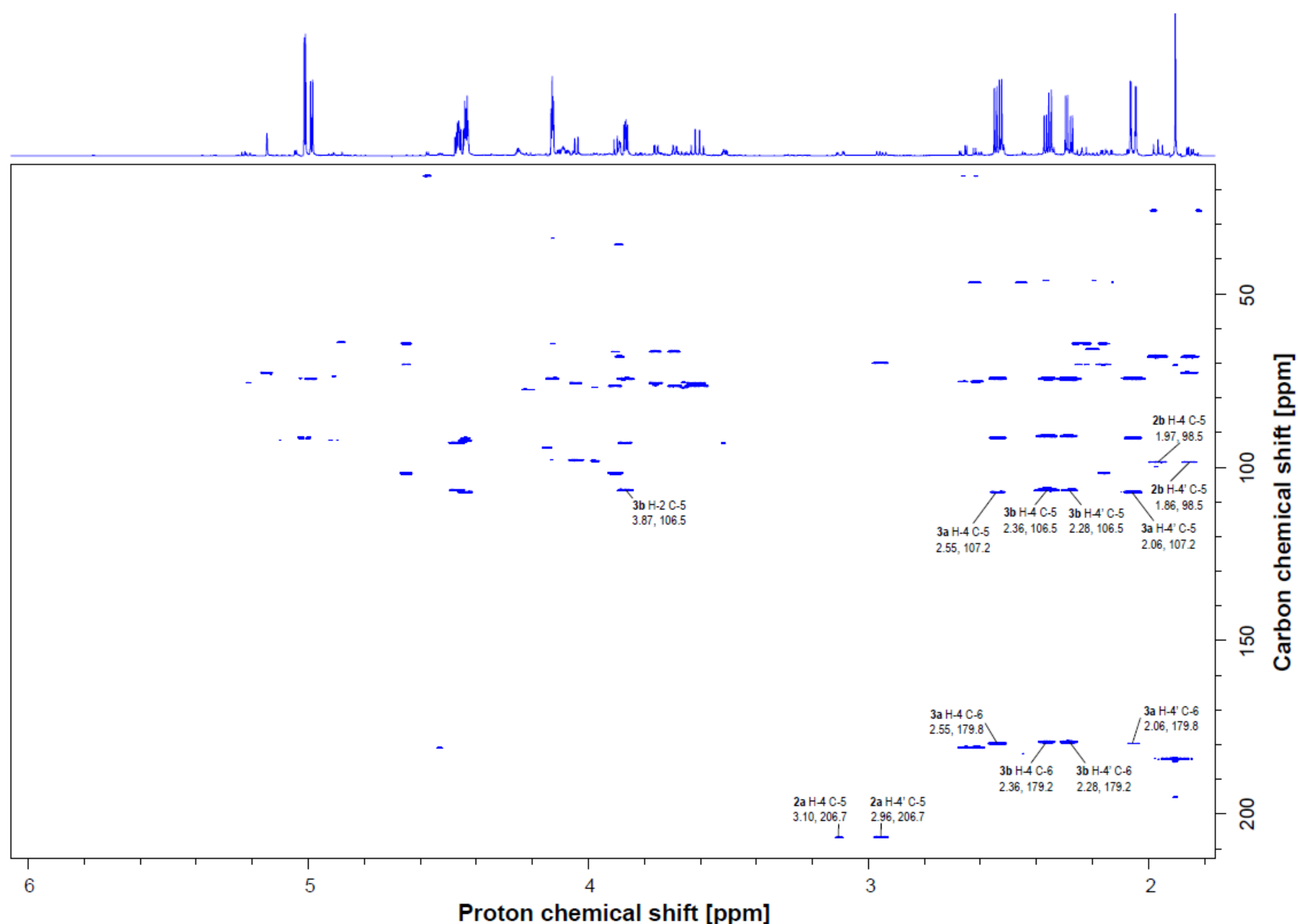

Figure S8: HMBC spectrum of AMOR_PL17A treated polyM incubated at 50 °C for ~ 48 h.

HMBC spectrum of AMOR_PL17A treated polyM (0.4 % (w/v) polyM substrate, 0.8 µM AMOR_PL17A, 5 mM NaOAc, 100 mM NaCl, pH 5.6). Sample was first incubated at 50 °C in a benchtop Thermomixer (600 rpm) for ~ 48 h, freeze dried, and then resuspended in 99.9% D_2O. Spectrum acquired at 25 °C on 800 MHz spectrometer. Horizontal trace displays ^1H 1D spectrum. Used to assign structures **2a**, **2b**, **3a** and **3b**. Key correlations referred to in text are highlighted.

SUPPLEMENTARY TABLES

Table S1. NMR chemical shifts.

NMR data for Δ (**1a, 1b**)[a], DEH hydrates (**2a, 2b**)[a], and DHF hydrates (**3a, 3b**)[a] in D_2O. The structures of these compounds are shown below and in Figure 5B of the main manuscript.

position	δ_C [b]	δ_H [c]	(mult, *J* Hz)	$^{2,3}J_{CH}$ HMBC (C no.)	δ_C [b]	δ_H [c]	(mult, *J* Hz)	$^{2,3}J_{CH}$ HMBC (C no.)
			1a				**1b**	
1	96.1	5.25	(d, 6.0)	3, 5	96.4	5.33	(brs)	2
2	70.6	3.77	(t, 5.1)	1, 4	69.6	3.93	(brd, 4.5)	4
3	65.2	4.41	(t, 4.1)	4, 5	66.8	4.50	(brt, 3.9)	
4	108.9	5.77	(d, 4.0)	2, 5, 6	109.6	5.72	(d, 3.3)	
5	148.2				n.o.			
6	172.0				n.d.			
			2a				**2b**	
1	92.0	5.04	(d, 4.4)		93.3	5.14	(brs)	2
2	78.4	3.51	(m)		73.0	3.88	(m)	3, 4
3	69.9	4.24	(m)		68.1	4.09	(m)	
4	45.0	3.10	(dd, 3.2, 17.0)	5	35.7	1.97	(t, 12.7)	3, 5
4’		2.96	(dd, 9.3, 17.0)	3, 5		1.86	(dd, 5.2, 13.0)	2, 3, 5
5	206.7				98.5			
6	n.o.				n.o.			
			3a				**3b**	
1	92.3	5.01	(d, 3.8)	2, 3	93.0	4.98	(d, 5.8)	3
2	91.6	4.13	(brt, 3.4)	3	91.1	3.87	(dd, 4.2, 5.8)	1, 3, 5
3	74.3	4.44	(dt, 2.9, 7.0)	1, 5	74.5	4.46	(td, 4.2, 6.4)	1, 5
4	46.6	2.55	(dd, 7.0, 14.4)	2, 3, 5, 6	46.1	2.36	(dd, 6.9, 13.8)	2, 3, 5, 6
4’		2.06	(dd, 2.6, 14.4)	2, 3, 5, 6		2.28	(dd, 6.0, 13.8)	2, 3, 5, 6
5	107.2				106.5			
6	179.8				179.2			

[a] **1a** (4-deoxy-L-*erythro*-4-hexenopyranuronate), **1b** (4-deoxy-L-*erythro*-4-hexenopyranuronic acid), **2a** (4-deoxy-L-*erythro*-5-hexulosuronate aldo-hydrate), **2b** (4-deoxy-L-*erythro*-5-hexulosuronate ketoaldo-hydrate), **3a** (4-deoxy-D-*manno*-(5*S*)-hexulofuranosidonate hydrate), **3b** (4-deoxy-D-*manno*-(5*R*)-hexulofuranosidonate hydrate).

[b] ^{13}C at 200 MHz. [c] ^{1}H at 800 MHz. Chemical shifts determined from 2D experiments acquired at 25°C. Mult = multiplet, n.o. = not observed, br = broad signal, d = doublet, dd = doublet of doublets, dt = doublet of triplets, m = multiplet, s = singlet, t = triplet, td = triplet of doublets.

SUPPLEMENTARY TEXT

Structural assignments of reaction products

Overview of the identified structures:

1a	4-deoxy-L-*erythro*-4-hexenopyranuronate	
1b	4-deoxy-L-*erythro*-4-hexenopyranuronic acid	
2a	4-deoxy-L-*erythro*-5-hexulosuronate aldo-hydrate	
2b	4-deoxy-L-*erythro*-5-hexulosuronate ketoaldo-hydrate	
3a	4-deoxy-D-*manno*-(5*S*)-hexulofuranosidonate hydrate	
3b	4-deoxy-D-*manno*-(5*R*)-hexulofuranosidonate hydrate	

4-deoxy-L-*erythro*-4-hexenopyranuronate (1a) and 4-deoxy-L-*erythro*-4-hexenopyranuronic acid (1b). Structures **1a** and **1b** are assigned as a 4,5-unsaturated uronate and uronic acid, respectively. From the HSQC spectrum (Figure S5) a total of eight C-H spin pairs were identified. Vinylic couplings in 1D selective COSY spectra were used to infer C-C connectivity and two partial structures were identified each with four methines that belong to structures **1a** and **1b**. Each partial structure consists of a doubly oxygenated methine (position 1), two oxygenated methines (likely primary alcohols in positions 2 and 3) and a higher frequency methine typically associated with an olefin (position 4). For **1a** C-5 (δ_C 148.2) and C-6 (δ_C 172.0) quaternary carbons were assigned by HMBC correlations (Figure S6) between H-3 and C-5, H-4 and C-5, and H-4 and C-6. The chemical shifts of C-4 and C-5 are consistent with sp^2 hybridized carbons, such as in an olefin, and C-6 is

indicative of a terminal carbonyl moiety. HMBC correlation between H-1 and C-5 confirms **1a** is a 6-membered hemiacetal ring. Due to a lower abundance of **1b**, the quaternary carbons C-5 and C-6 were not observed and could not be assigned ^{13}C chemical shifts. The similarities in chemical shifts between **1a** and **1b** lead to the proposal that **1b** is the protonated form of **1a**.

DEH (4-deoxy-L-*erythro*-5-hexulosuronate) aldo-hydrate (2a) and DEH ketoaldo-hydrate (2b). Partial structures were assigned for products **2a** and **2b**. C-H spin pairs were assigned from the HSQC spectrum (Figure S7), and DQF-COSY correlations were used to establish C-C connectivity. This resulted in two spin systems each comprising one doubly oxygenated methine (position 1) two oxygenated methines (positions 2 and 3) and one methylene (position 4). Quaternary carbons were identified using HMBC correlations (Figure S8) between methylene protons H-4 and H-4' and C-5. In **2a** C-5 was assigned as a keto group (δ_C 206.7) and in **2b** C-5 was assigned as a geminal diol (δ_C 98.5). In both structures the chemical shifts at C-1 (**2a** δ_C 92.0 and **2b** δ_C 93.3) are consistent with geminal diols. Due to lower abundance C-6 was unable to be assigned. The carboxylic acid moiety proposed in these structures is based upon products that can be explained by ring opening of Δ.

4-deoxy-D-*manno*-(5*S*)-hexulofuranosidonate (DHF) hydrate (3a) and 4-deoxy-D-*manno*-(5*R*)-hexulofuranosidonate (DHF) hydrate (3b). HSQC spectra (Figure S7) were used to assign C-H spin pairs and C-C connectivity was established using H2BC correlations. This produced two partial structures each comprising one doubly oxygenated methine (positions 1), two oxygenated methines (position 2 and 3) and one methylene (position 4). HMBC correlations (Figure S8) enabled the assignment of C-5 and C-6 quaternary carbons in both **3a** and **3b**. The chemical shifts of C-6 (**3a** δ_C 179.8 and **3b** δ_C 179.2) are consistent with a terminal carbonyl moiety. The chemical shifts of C-5 (**3a** δ_C 107.2 and **3b** δ_C 106.5) are consistent with ether moiety. The chemical shifts observed at C-1 are consistent with geminal diols similar to those observed in structures **2a** and **2b**. The HMBC correlation between H-2 and C-5 (Figure S8) suggests a 5-membered hemiketal ring. This is further supported by the higher chemical shift observed for C-2. It is thus proposed that structures **3a** and **3b** are a pair of stereoisomers at C-5.

NMR acquisition parameters

Summary of experiments and relevant acquisition parameters collected for each prepared reaction. All homo- and heteronuclear NMR experiments were recorded on a Bruker AV-IIIHD 800 MHz spectrometer (Bruker BioSpin AG, Fälladen, Switzerland) equipped with a 5 mm cryogenic CP-TCI z-gradient probe. For each reaction the length of the 90° pulse-widths were calculated using the 'pulsecal' command.

Reaction of polyM treated with AMOR_PL17A incubated at 25 °C (in-tube) for ~ 17 h.

After incubation the following experiments were collected. 1D proton spectrum with presaturation delay during relaxation and mixing time (noesygppr1d), TD: 65536, NS: 16, SW: 20 ppm, O1P: 4.7 ppm, D1: 2 s. ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectra with multiplicity editing (hsqcedetgpsisp2.3), TD: 2048/400 (F2/F1), NS: 4, SW: 14/200 ppm (F2/F1), O1P: 4.7/110 ppm (F2/F1), D1: 2 s. Phase sensitive ^{1}H-^{13}C heteronuclear multiple bond coherence (HMBC) spectra with one-bond correlations suppressed (hmbcetgpl3nd), TD: 2048/400 (F2/F1), NS: 12, SW: 14/200 ppm (F2/F1), O1P: 4.7/110 ppm (F2/F1), D1: 2 s. 1D selective ^{1}H-^{1}H correlation spectroscopy (COSY) using selective refocusing with a shaped pulse (selcogp), TD: 65536, NS: 8, SW: 20 ppm, O1P: 4.7 ppm, D16: 200 µs, D1: 2 s. 1D selCOSY collected for following signals: δ_H **1a** H-1 5.25, H-2 3.77, H-3 4.41, H-4 5.77, **1b** H-1 5.33, H-2 3.93, H-3 4.50, H-4 5.72.

Reaction of polyM treated with AMOR_PL17A incubated at 50 °C (in benchtop Thermomixer) for ~ 48 h.

Following incubation sample was freeze-dried, resuspended in 99.9% D_2O and the following NMR experiments were recorded at 25 °C. 1D proton, HSQC and HMBC spectra collected with same parameters listed above, except F1 TD for HSQC and HMBC was 1024. ^{1}H-^{13}C Heteronuclear two-bond correlation (HMBC) spectrum (h2bcetgpl3pr), TD: 2048/1024 (F2/F1), NS: 32, SW: 14/200 ppm (F2/F1), O1P: 4.7/110 ppm (F2/F1), D1: 2 s. ^{1}H-^{1}H double quantum filtered correlation spectroscopy (DQF-COSY) (cosydfph), TD: 4096/1024 (F2/F1), NS: 16, SW: 10/10 ppm (F2/F1), O1P: 4.7/4.7 ppm (F2/F1), D16: 200 µs, D1: 2 s.

Reaction of polyM treated with AMOR_PL17A incubated at 50 °C (in-tube) for ~ 4 h.

^{1}H time-resolved experiment (using noesygppr1d pulse sequence collected through time) NS: 16, SW: 10 ppm, O1P: 4.7 ppm, D1: 2.5 s. Total acquisition time per one experiment: 73 s, time delay between experiments: 47 s, total number of experiments: 120. One spectrum is collected every 2 mins for a total of 4 h.

SUPPLEMENTARY REFERENCES

1. Park, D., S. Jagtap, and S.K. Nair, *Structure of a PL17 family alginate lyase demonstrates functional similarities among exotype depolymerases.* J Biol Chem, 2014. **289**(12): p. 8645-55.