Heterologous expression and Functional Characterization of two Alginate Lyases in *Corynebacterium glutamicum*

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Simen Jervell Lund[1§], Agnes Beenfeldt Petersen[1§], Antonia Areali[1], Trygve Brautaset[1], Finn Lillelund Aachmann[1], Fernando Peréz-Garcia[1*]

[1] Department of Biotechnology and Food Science, NTNU Norwegian University of Science and Technology. Trondheim, Norway

[§]Shared first authorship
[*]Corresponding author Fernando Pérez-García fernando.perez-garcia@ntnu.no

Supplementary data:

S1 Primer for PCR

Table S1 shows all primers used for PCR. This includes primers for PCR amplification of genes *alyB* and *alyD*, as well as primers for colony PCR.

Table S1: Primers for PCR.

Name	Sequence (5' – 3')	Description
alyB-Fw	*GCATGCCTGCAGGTCGACTCTAGAG***GAAAGGAGGC CCTTCAGATG**AAACATATTTTTCTAAA	Forward primer for amplification of *alyB* from *V. algivorus*.
alyB-Rv	*GAATTCGAGCTCGGTACCCGGGGATC*TTATTTACCTGT GTATGTAC	Reverse primer for amplification of *alyB* from *V. algivorus*.
alyD-Fw	*GCATGCCTGCAGGTCGACTCTAGAG***GAAAGGAGGC CCTTCAGATG**TTAAAAAAATTCCTCTG	Forward primer for amplification of *alyD* from *V. algivorus*.
alyD-Rv	*GAATTCGAGCTCGGTACCCGGGGATC*CTATTTTTTATG CGATGTTA	Reverse primer for amplification of *alyB* from *V. algivorus*.
alyB-Rv2	TTATTTACCTGTGTATGTAC	Reverse primer for amplification of *alyB* wen cloned together with *alyD*
alyD-Fw2	*ATCGCACGGTACATACACAGGTAAATAATTCGAACGC CCC***GAAAGGAGGCCCTTCAGATG**TTAAAAAAATTCCT CTG	Forward primer for amplification of *alyD* wen cloned together with *alyB*
X1Fw	CATCATAACGGTTCTGGC	Forward primer for pVWEx1 used for colony PCR
X1Rv	ATCTTCTCTCATCCGCCA	Reverse primer for pVWEx1 used for colony PCR

Sequence in italics represents the overlapping regions for Gibson assembly. Bold sequence represents the ribosomal binding site (RBS) coding sequence.

S2 Growth rate, biomass, yield, and total secreted protein concentration of *C. glutamicum* strains expressing AlyB and AlyD

Growth rate, biomass, yield, and total secreted protein concentration for *C. glutamicum*(pVWEx1), *C. glutamicum*(pVWEx1-*alyB*), *C. glutamicum*(pVWEx1-*alyD*), and *C. glutamicum*(pVWEx1-*alyBD*) strains for each inducer concentration (0 mM, 0.01 mM, 0.1 mM and 1 mM) is shown in Table S1. Table S2 shows the resulting one-way ANOVA test results from the various protein concentrations, and Table S3 shows the resulting Dunnett vs control test to determine significant differences from the control (*C. glutamicum*(pVWEx1)).

Table S2: **Growth rate, biomass, yield, and total secreted protein concentration with standard deviation (SD) of *C. glutamicum* strains expressing *alyB, alyD,* and *alyBD* and empty vector control**. For *C. glutamicum*(pVWEx1-*alyB*), *C. glutamicum*(pVWEx1-*alyD*), and *C. glutamicum*(pVWEx1-*alyBD*) each induction level was measured, and for *C. glutamicum*(pVWEx1) empty vector control full induction (1 mM IPTG) was measured. The total secreted protein concentration is measured in mg protein/mL/g biomass.

Strain	Growth rate (1/h) ± SD	Biomass (g/L) ± SD	Yield (g/g) ± SD	Total secreted protein concentration (mg/mL/g biomass) ± SD
C. glutamicum(pVWEx1) 100% induction	0.33 ± 0.02	5.49 ± 0.28	0.59 ± 0.03	0.027 ± 0.002
C. glutamicum(pVWEx1-*alyB*) 0% induction	0.31 ± 0.05	5.98 ± 0.53	0.60 ± 0.05	0.027 ± 0.001
C. glutamicum(pVWEx1-*alyB*) 1% induction	0.34 ± 0.07	5.35 ± 0.99	0.54 ± 0.10	0.028 ± 0.002
C. glutamicum(pVWEx1-*alyB*) 10% induction	0.29 ± 0.02	6.07 ± 0,45	0.61 ± 0.05	0.033 ± 0.003
C. glutamicum(pVWEx1-*alyB*) 100% induction	0.30 ± 0.01	5.53 ± 0.47	0.55 ± 0.05	0.044 ± 0.002
C. glutamicum(pVWEx1-*alyD*) 0% induction	0.33 ± 0.04	5.63 ± 0.18	0.56 ± 0.02	0.027 ± 0.002
C. glutamicum(pVWEx1-*alyD*) 1% induction	0.36 ± 0.01	5.82 ± 0.07	0.58 ± 0.01	0.033 ± 0.001
C. glutamicum(pVWEx1-*alyD*) 10% induction	0.36 ± 0.02	5.69 ± 0.55	0.57 ± 0.06	0.035 ± 0.002
C. glutamicum(pVWEx1-*alyD*) 100% induction	0.35 ± 0.03	5.73 ± 0.61	0.57 ± 0.06	0.040 ± 0.002
C. glutamicum(pVWEx1-*alyBD*) 0% induction	0.29 ± 0.01	5.60 ± 0.07	0.56 ± 0.01	0.028 ± 0.00
glutamicum(pVWEx1-*alyBD*) 1% induction	0.29 ± 0.01	5.87 ± 0.00	0.57 ± 0.00	0.029 ± 0.00
glutamicum(pVWEx1-*alyBD*) 10% induction	0.23 ± 0.02	5.63 ± 0.28	0.56 ± 0.03	0.045 ± 0.00
glutamicum(pVWEx1-*alyBD*) 100% induction	0.19 ± 0.01	4.00 ± 0.47	0.40 ± 0.05	0.055 ± 0.00

Table S3: ANOVA results for total secreted protein concentration. Total secreted protein concentrations were measured in triplicates for each strain/induction level.

Source	Sum of squares	Degrees of freedom	F	P-value
Condition	0.002938	12	80.1297	2.21×10^{-17}
Residual	7.97×10^{-5}	26		

Table S4: Dunnett vs control. To determine significant difference of each total secreted protein concentration from the control.

Strain	P-value	Adjusted P-value
C. glutamicum(pVWEx1-*alyB*) 0% induction	0.6168	1
C. glutamicum(pVWEx1-*alyB*) 1% induction	0.6989	1
C. glutamicum(pVWEx1-*alyB*) 10% induction	0.0451	0.2706
C. glutamicum(pVWEx1-*alyB*) 100% induction	0.0001	0.0071
C. glutamicum(pVWEx1-*alyD*) 0% induction	0.6322	1
C. glutamicum(pVWEx1-*alyD*) 1% induction	0.0165	0.1157
C. glutamicum(pVWEx1-*alyD*) 10% induction	0.0086	0.0688
C. glutamicum(pVWEx1-*alyD*) 100% induction	0.0015	0.0133
C. glutamicum(pVWEx1-*alyBD*) 0% induction	0.7399	1
C. glutamicum(pVWEx1-*alyBD*) 1% induction	0.2905	1
C. glutamicum(pVWEx1-*alyBD*) 10% induction	0.0003	0.0039
C. glutamicum(pVWEx1-*alyBD*) 100% induction	0.0008	0.0082

S3 Statistical analysis of enzymatic activity at various inducer concentrations

An ANOVA analysis of the absorbance-based enzymatic activity assay of AlyB and AlyD to determine significant differences between the various inducer concentrations for both separate- and co-expression of AlyB and AlyD using alginate substrates was conducted (Table S4). A Dunnett vs Control test was subsequently done to determine significant differences between the control and each induction level (Table S5 and S6), and finally a Tukey post hoc test was conducted to determine significant differences between induction levels, which have been added in a separate .xlsx file labelled "Supplementary data, Tukey (HSD) statistical analysis".

Table S5: ANOVA statistical analysis of the absorbance-based enzymatic activity assay. Resulting ANOVA predictions for both separately- and co-expressed AlyB and AlyD using alginate substrates with 0.1% and 1% concentration, and low- and medium viscosity.

Source of AlyB and AlyD	Alginate substrate	Source of variation	Sum of squares	Degrees of freedom	F-value	P-value
AlyB and AlyD expressed separately then combined	0.1% low viscosity alginate	Groups Residual	3.66E-06 2.09E-07	16 34	37.26	6.69E-17
	0.1% medium viscosity alginate	Groups Residual	4.72E-06 1.36E-07	16 34	73.80	1.14E-21
	1% low viscosity alginate	Groups Residual	8.40E-05 1.30E-05	16 34	13.71	1.99E-10
	1% medium viscosity alginate	Groups Residual	1.23E-04 2.15E-05	16 34	12.17	1.01E-09
AlyB and AlyD co-expressed	0.1% low viscosity alginate	Groups Residual	3.65E-04 3.86E-06	4 10	236.82	7.40E-10
	0.1% medium viscosity alginate	Groups Residual	2.42E-04 3.78E-06	4 10	159.95	5.11E-09
	1% low viscosity alginate	Groups Residual	1.02E-02	4 10	2169.13	1.21E-14
	1% medium viscosity alginate	Groups Residual	7.69E-03 2,39E-05	4 10	803.11	1,72E-12

Table S6: Dunnet vs Control statistical analysis. Tables show AlyB and AlyD separately expressed then combined in 0.1% low- (a), 0.1% medium- (b), 1% low- (c), and 1% medium viscosity alginate (d). The first column shows the induction level combination of AlyB and AlyD.

a)

Condition (induction level)	p-value	Adjusted p-value
0% AlyB – 0% AlyD	0.19	0.75
0% AlyB – 1% AlyD	0.24	0.75
0% AlyB – 10% AlyD	0.70	1
0% AlyB – 100% AlyD	0.58	1
1% AlyB – 0% AlyD	0.00	0.03
1% AlyB – 1% AlyD	0.00	0.03
1% AlyB – 10% AlyD	0.02	0.11
1% AlyB – 100% AlyD	0.01	0.10
10% AlyB – 0% AlyD	0.00	0.04
10% AlyB – 1% AlyD	0.00	0.01
10% AlyB – 10% AlyD	0.00	0.03
10% AlyB – 100% AlyD	0.00	0.03
100% AlyB – 0% AlyD	0.01	0.08
100% AlyB – 1% AlyD	0.00	0.03
100% AlyB – 10% AlyD	0.02	0.12
100% AlyB – 100% AlyD	0.02	0.11

b)

Condition (induction level)	p-value	Adjusted p-value
0% AlyB – 0% AlyD	0.41	1
0% AlyB – 1% AlyD	0.38	1
0% AlyB – 10% AlyD	0.40	1
0% AlyB – 100% AlyD	0.00	0.01
1% AlyB – 0% AlyD	0.00	0.00
1% AlyB – 1% AlyD	0.02	0.10
1% AlyB – 10% AlyD	0.00	0.00
1% AlyB – 100% AlyD	0.00	0.00
10% AlyB – 0% AlyD	0.00	0.03
10% AlyB – 1% AlyD	0.00	0.03
10% AlyB – 10% AlyD	0.02	0.10
10% AlyB – 100% AlyD	0.01	0.06
100% AlyB – 0% AlyD	0.00	0.02
100% AlyB – 1% AlyD	0.00	0.02
100% AlyB – 10% AlyD	0.00	0.02
100% AlyB – 100% AlyD	0.00	0.02

c)

Condition (induction level)	p-value	Adjusted p-value
0% AlyB – 0% AlyD	1	1
0% AlyB – 1% AlyD	1	1
0% AlyB – 10% AlyD	0.16	0.65
0% AlyB – 100% AlyD	0.23	0.69
1% AlyB – 0% AlyD	0.04	0.44
1% AlyB – 1% AlyD	0.01	0.12
1% AlyB – 10% AlyD	0.08	0.55
1% AlyB – 100% AlyD	0.08	0.55
10% AlyB – 0% AlyD	0.07	0.55
10% AlyB – 1% AlyD	0.04	0.44
10% AlyB – 10% AlyD	0.06	0.50
10% AlyB – 100% AlyD	0.07	0.55
100% AlyB – 0% AlyD	0.03	0.41
100% AlyB – 1% AlyD	0.03	0.44
100% AlyB – 10% AlyD	0.03	0.44
100% AlyB – 100% AlyD	0.03	0.44

d)

Condition (induction level)	p-value	Adjusted p-value
0% AlyB – 0% AlyD	0.05	0.41
0% AlyB – 1% AlyD	0.17	0.52
0% AlyB – 10% AlyD	0.34	0.52
0% AlyB – 100% AlyD	0.18	0.52
1% AlyB – 0% AlyD	0.02	0.20
1% AlyB – 1% AlyD	0.04	0.41
1% AlyB – 10% AlyD	0.00	0.04
1% AlyB – 100% AlyD	0.04	0.41
10% AlyB – 0% AlyD	0.08	0.41
10% AlyB – 1% AlyD	0.07	0.41
10% AlyB – 10% AlyD	0.03	0.37
10% AlyB – 100% AlyD	0.00	0.03
100% AlyB – 0% AlyD	0.00	0.02
100% AlyB – 1% AlyD	0.01	0.10
100% AlyB – 10% AlyD	0.07	0.41
100% AlyB – 100% AlyD	0.41	0.41

Table S7: Dunnet vs Control statistical analysis. Tables show AlyB and AlyD co-expressed in 0.1% low- (a), 0.1% medium- (b), 1% low- (c), and 1% medium viscosity alginate (d). The first column shows the induction level of the co-expressed AlyB and AlyD.

a)

Condition (induction level)	p-value	Adjusted p-value
0%	0.01	0.01
1%	0.00	0.00
10%	0.00	0.01
100%	0.00	0.00

b)

Condition (induction level)	p-value	Adjusted p-value
0%	0.00	0.00
1%	0.00	0.01
10%	0.00	0.00
100%	0.01	0.01

c)

Condition (induction level)	p-value	Adjusted p-value
0%	0.00	0.00
1%	0.00	0.00
10%	0.00	0.00
100%	0.00	0.00

d)

Condition (induction level)	p-value	Adjusted p-value
0%	0.00	0.00
1%	0.00	0.00
10%	0.00	0.00
100%	0.00	0.00

S4 Signal peptide and cleavage site prediction model

The signal peptide and cleavage site prediction were done using the SignalP – 6.0 prediction model and the amino acid sequence of AlyD from *V. algivorus*. The sequence was retrieved from the National Library of Medicine, National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/protein/BAV53312.1).

AlyD amino acid sequence:

MLKKFLCMSVILGCSANAMAQAAPVPADKFDMMNWKITVPEDRDKNGKIDEIEGVAMFSYSN
PDYFFLDDDGNMVFQVQNKAITTSGSSNARSELRQMLRGTDLTIDVKAPANNFALASHPKAKE
YGAVGGKLEATLKVNHVATHANQPNKLPAYSVVVGQIHANKDPKLMEANTGFGYGNEPLKIFY
KKWPDHDKGSVFFNYERNLAKDDPNRIDIAYPVWGNTWENKDDPKDAGVALGEEFSYVVDVE
GNIMHLTFTSKNHKTVEYKIDLSKNLKPDGTVDEKDNPRGYAEDSFYFKAGAYGQCSVKDDEG
FWSPGCAGTGDFATDVKNGDYNSVTFSKLELTSHKK

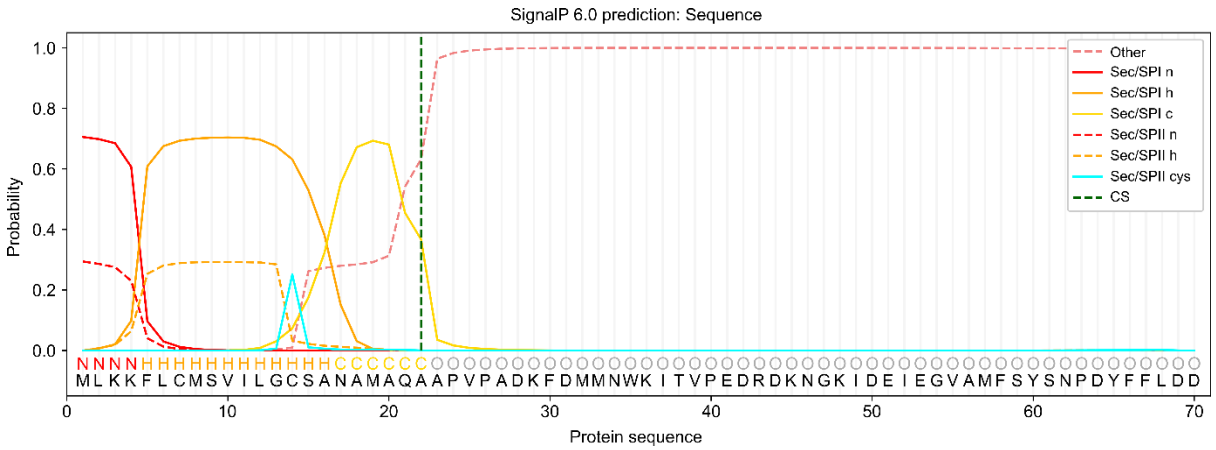

Figure S1: Signal peptide and cleavage site prediction. The signal peptide and cleavage prediction was done using the SignalP – 6.0 prediction model and the amino acid sequence of AlyD (https://www.uniprot.org/uniprotkb/A0A1B4Z1L3/entry). The model predicts with a likelihood of 69% that a Sec/SPI signal peptide is present with a cleavage site between pos. 22 and 23.

Table S8: Likelihood of predicted signal peptides. Prediction was done using the SignalP – 6.0 prediction model and the amino acid sequence of AlyD (https://www.uniprot.org/uniprotkb/A0A1B4Z1L3/entry).

Protein type	Likelihood
Other	0.0009
Signal peptide (Sec/SPI)	0.6941
Lipoprotein signal peptide (Sec/SPII)	0.3039
TAT signal peptide (TAT/SPI)	0.0005
TAT lipoprotein signal peptide (TAT/SPII)	0.0003
Pilin-like signal peptide (Sec/SPIII)	0.0003

S5 AlphaFold3 structural prediction of the catalytic domains of AlyB and AlyD

AlyB and AlyD were modelled using the online version of AlphaFold3 [1] available on the AlphaFold server using the standard settings. The two models were evaluated by inspecting the predicted LDDT, used as color code for the models in Figure S2. Only the catalytic domains of the enzymes were subjected to the AlphaFold analysis.

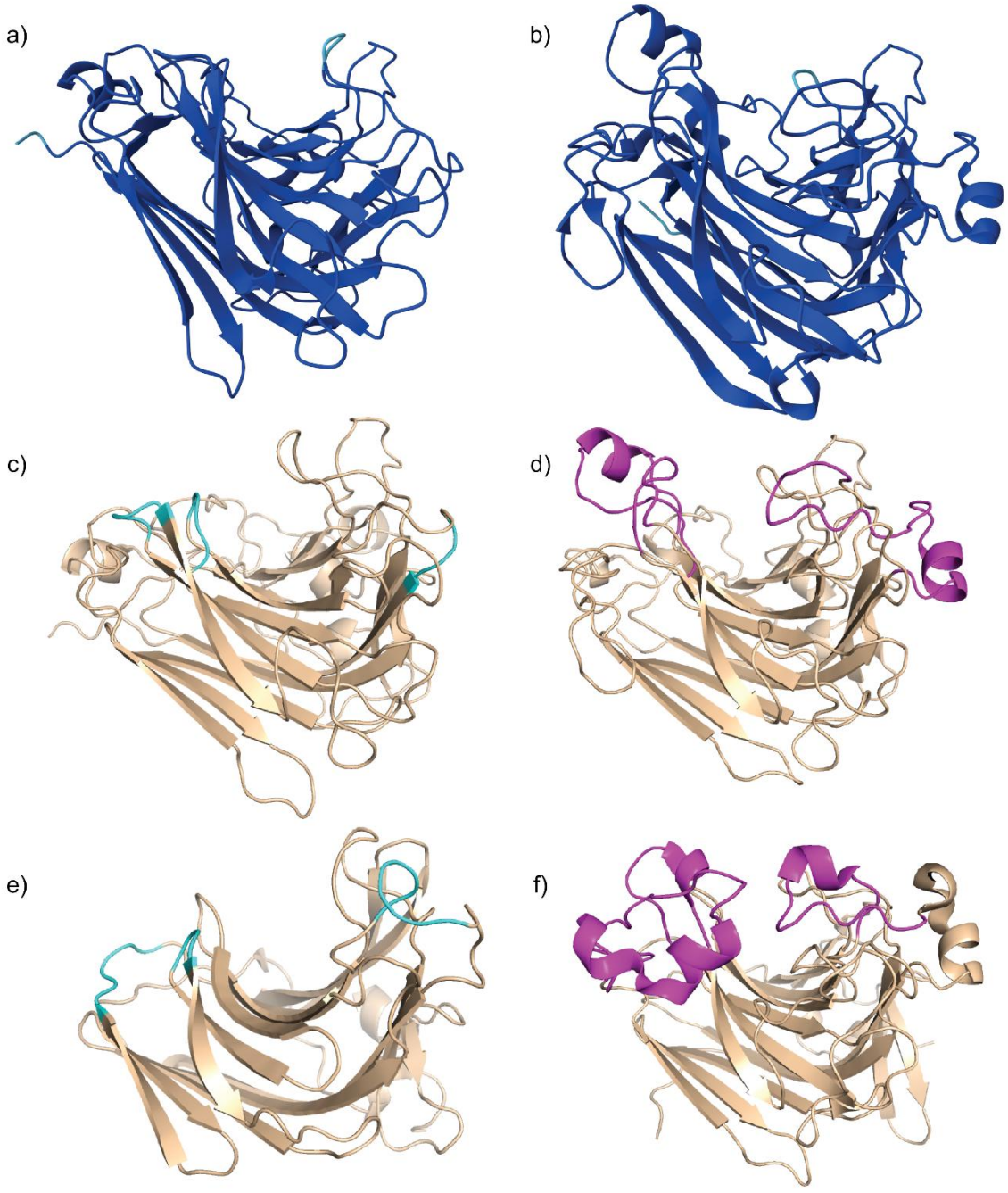

Figure S2: Structural evaluation of AlyB and AlyD from the PL7 alginate lyase family. Thomas et al. (2013) suggested that loops surrounding the active site and extending along the substrate-binding groove play a critical role in determining the mode of action of PL7 alginate lyases. In particular, more extended

loops within the substrate-binding groove were proposed to hinder endolytic activity, as observed in Aly5, thereby favoring an exolytic mode of action. Top panel AlphaFold3 models of AlyB (**a**) and AlyD (**b**) catalytical domain. The backbone is colored according to the confidence of the prediction using the color scale from AlphaFold3 (blue to red), where dark blue shows the highest confidence level. Middel panel AlyB (**c**) and AlyD (**d**) with loops color in cyan and magenta, respectively. Bottom panel Aly1 (**e**; PDB: 1UAI; [2]) and Aly5 (**f**;PDB: 4BE3; [3]) with loops color in cyan and magenta, respectively. AlyD has more extended loops, which may support its exo-activity. In contrast, AlyB features shorter loops at the substrate-binding groove, aligning with its endo-mode of action.

S6 AlyB Specificity

AlyB substrate specificity was investigated by monitoring the reactions of AlyB with oligoG, oligoMG, and polyM using time-resolved NMR (Figure S3). AlyB was active on all three substrates. When reacting with oligoG, AlyB formed oligomers, but no dimers or monomers (Figure S3a), so AlyB can cleave G|G bonds. In the reaction with oligoMG, the main products formed were ΔG- and ΔM-containing oligomers, and ΔG dimers (Figure S3b). Furthermore, a small amount of ΔM dimers were formed. Both G and M reducing ends were formed, meaning that AlyB can cleave M|G and G|M bonds. AlyB reacting with polyM formed primarily ΔM-containing oligomers and a small amount of ΔM dimers (Figure S3), so AlyB can also cleave M|M bonds. In total, AlyB can cleave G|M, M|G, G|G, and M|M bonds. The HPAEC-PAD analysis (Figure S3c) shows that the minimum oligomer length that AlyB cleaves is a pentamer, since tetramers were still present in the product measures.

During reaction of AlyB with polyM, surprisingly, G-residues were formed, which can be seen from the signals for -MG- and G_β reducing ends (Figure S3c). The substrate contained no G-residues, so these signals could only occur due to AlyB having epimerase activity. This is the second reported alginate lyase with epimerase activity, and the first report of epimerase-activity in PL family 7. The first lyase shown to have epimerization activity is *Bo*PL38. No ΔG-containing products were formed, indicating that AlyB prefers cleaving between G|M over M|G. Furthermore, analysis of the reaction with oligoMG showed that consecutive G-residues (-GG-) were formed during the reaction, meaning that AlyB introduces G-residues into MG-blocks.

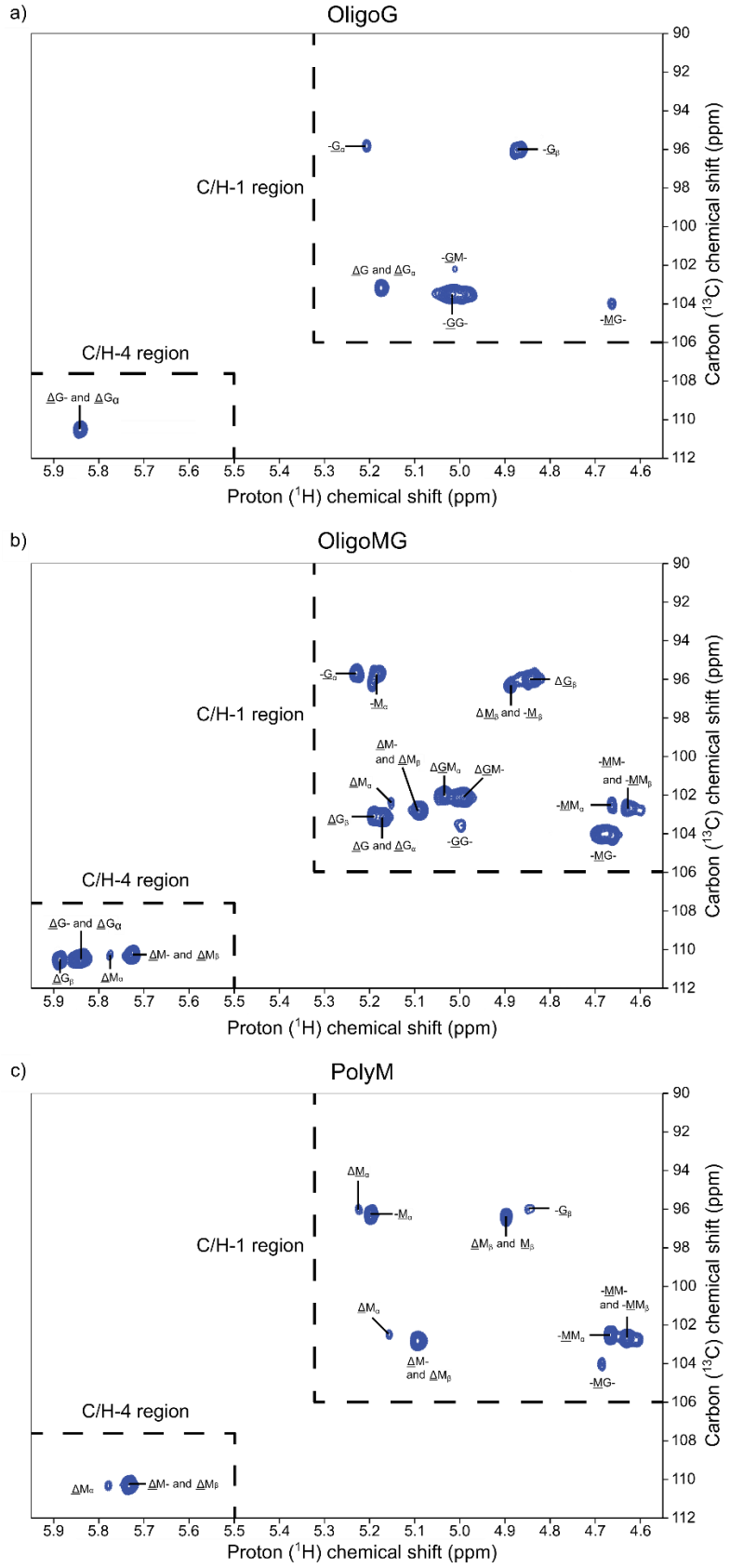

Figure S3: HSQC after end reactions between AlyB and oligoG, oligoMG, and polyM. **a)** Reaction between AlyB and oligoG (DP 25-26, F_G > 0.95). **b)** Reaction between AlyB and oligoMG (DP 26, F_G = 0.45). **c)** Reaction between AlyB and polyM (DP ~70-100, F_G = 0.00). All reactions were run in an NMR tube while recording a time-resolved NMR spectrum. After 15 h 30 min reaction, the ^{1}H-^{13}C HSQC was recorded.

References

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