Supplementary Information

Glucuronan lyases from family PL7 use a Tyr/Tyr syn β-elimination catalytic mechanism for glucuronan breakdown

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Materials and Methods

β-1,4-glucuronan

Glucuronan (β-1,4-polyglucuronic acid) was prepared by TEMPO-mediated oxidation of grade 1 Whatman filter paper (Merck, Darmstadt, Germany). Prior to oxidation the filter paper was cut into small pieces and soaked in 5 M NaOH for 1.5 hours at room temperature. The NaOH solution was poured off and the filter paper rinsed with dH_2O. This step was repeated three times. The oxidation was carried out as previously described[1] using TEMPO (2,2,6,6-tetramethylpipedine-1-oxyl radical), sodium bromide and 11% sodium hypochlorite solution. The reaction time was adjusted to 30 min and reactions were spun down for 10 min to remove potential residual un-oxidized cellulose prior to quenching by ethanol 96% ethanol and washed twice in 70% acetone.

TpPL7A and TpPL7B production and site-directed mutagenesis

Mutants of TpPL7A (GenBank acc. nr. OTA00986.1) were constructed by Genscript (Piscataway, NJ, USA) and inserted into the pPICZαA vector at the restriction sites EcoRI and SalI. Mutants of TpPL7B (GenBank acc. nr. OTA01262.1) were constructed using CloneAmpTM polymerase (Takara, Kusatsu, Japan), a set of mutagenic primers (Table S3), and pPICZαA/TpPL7B as template[2]. Purified PCR products were used to transform *Escherichia coli* DH5α and positive transformants were selected on LB low salt and zeocin (25 µg/mL) plates. Corresponding plasmids were extracted using GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific, Waltham, MA, USA) and all constructs were checked by sequencing (Macrogen Europe, Amsterdam, The Netherlands). For each mutant (of both TpPL7A and TpPL7B), a total of 5 µg of plasmid was linearized by PmeI restriction enzyme and used to transform *Pichia pastoris* X-33 by electroporation. Transformants were selected on YPD and 100 µg/mL zeocine plates. TpPL7A WT and mutants were produced in *P. pastoris* X-33 and purified by nickel affinity chromatography as described previously[3]. Imidazole was removed using PD-10 desalting columns and enzyme were stored in 20 mM Tris buffer with 100 mM NaCl. Purity of the enzymes was checked on SDS-PAGE and enzyme concentrations were determined by measuring the absorbance at 280 nm using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific) and using the corresponding extinction coefficient of 28670 $M^{-1}\cdot cm^{-1}$ for TpPL7A and 26150 $M^{-1}\cdot cm^{-1}$ for TpPL7B.

Enzyme activity assays and determination of kinetic parameters

Glucuronan lyase activity was determined by following the absorbance at 235 nm for 10 min using a Multiskan GO spectrophotometer (Thermo Fisher Scientific). All reactions were carried out in triplicate at 40 °C in 50 mM UB4 buffer pH 7, 100 mM NaCl, for TpPL7A and 50 °C in 50 mM UB4 buffer pH5 for TpPL7B and using 1.5 g L^{-1} of glucuronan and appropriate enzyme concentrations in 96-well quartz plates (Corning, New York, USA).

For each mutants, initial velocities were measured at different glucuronan concentrations ranging from 0.1 to 10 g L^{-1} in the conditions stated above. Initial velocities in absorbance units (mAU) per second were converted to mM of Δ4,5 bonds formed per second using the extinction coefficient of 6368 M^{-1} cm^{-1} [2]. Kinetic parameters were determined by fitting Michaelis-Menten model (v0 = Vmax/(1+(Km/[S0])) using Origin 2019 software (OriginLab Corporation, Northampton, MA, USA) where v0 is the initial velocity, [S0] the initial substrate concentration, Vmax the maximum rate, and Km the Michaelis constant.

NMR spectrocopy

For the time course NMR analysis of lyase reactions, stock solutions of 10 mg/mL of β-glucuronan in 10 mM HEPES, pH 7.0 with 150 mM NaCl in D_2O 99.9% was prepared. For each reaction 160 µL of substrate buffer solution was added to 3 mm NMR tube and preheated to a temperature of 25 °C in the NMR instrument. 1D proton with water suppression spectrum was recorded to ensure that the sample had not undergone any degradation or contamination prior to the time-resolved NMR experiment. The reaction was started by adding 2 µL of 0.2 nM of TpPL7A or 10 µL of 4 nM of TpPL7B to preheated substrate and mixed by inverting the sample several times. The sample was hereafter inserted into the preheated NMR instrument and the experiment was started. The recorded spectrum is a pseudo-2D type experiment recording a 1D proton NMR spectrum every 2 min with a total of 64 time points (total experiment time 2 hrs 8 min). The recorded 1D proton with water suppression (noesygppr1d) contains 64K data points and has a spectral width of 10 ppm, 16 scans, and relaxation delay of 2.5 s (total recording time of 77s). After completion of the time-resolved experiment a ^{1}H-^{13}C HSQC spectrum was recorded.

For the assignment of β-glucuronan following experiments were recorded: 1H (w/NOESY presaturation for solvent suppression), 2D ^{1}H-^{1}H IP-COSY (In-phase correlation spectroscopy[4]), 2D ^{1}H-^{1}H TOSCY (total correlation spectroscopy) using a 70 ms mixing time, ^{1}H-^{13}C HSQC (heteronuclear single quantum coherence) with multiplicity editing, ^{1}H-^{13}C H2BC (heteronuclear two bond coherence) and ^{1}H-^{13}C heteronuclear multiple bond coherence (HMBC) with suppression of one-bond correlations. The chemical shift was reference to residual water signal ^{1}H: 4.75 ppm.

All NMR spectra were recorded on a BRUKER AVIIIHD 800 MHz instrument (Bruker BioSpin AG, Fällanden, Switzerland) equipped with 5 mm cryogenic TCI probe at 25 °C. The spectra were recorded, processed and analyzed using TopSpin 3.6pl7 and TopSpin 4.0.7 software (Bruker BioSpin AG, Fällanden, Switzerland).

Crystallization, data collection and data processing

TpPL7A was deglycosylated using EndoHf (New England Biolabs, Ipswich, MA, USA) overnight in 10 mM Tris, 150 mM NaCl pH 7 and EndoHf was subsequently separated from TpPL7A using Amylose Resin (New England Biolabs) column. TpPL7A was concentrated to 48 mg ml^{-1} (10 kDa Amicon, Millipore) and crystallized in JCSG screen (Jena Bioscience, Jena, Germany) condition F9 containing 2.4 M disodium malonate pH 7 in a 96-well MRC 2-well plate by mixing 150 nl TpPL7A solution with 150 nl reservoir using a Crystal Gryphon robot (Art Robbins Instruments, Sunnyvale, CA, USA). A single crystal was obtained, which was dyed with Bright Red (Jena Bioscience) prior to cryocooling in liquid nitrogen. The crystal was cryoprotected with PEG400. Data were obtained at BioMAX at the Max IV Laboratory[5] and processed to 1.45 Å resolution with XDSAPP[6] (see Table S1 for statistics).

Structure solution, model building and refinement

To solve the structure of TpPL7A, a model was prepared from the closest homolog from the Protein Data Bank (www.pdb.org), which was the alginate lyase A1-II from *Sphingomonas* sp. A1 (PDB ID 2CWS)[7] identified through PDB-BLAST[8] with a coverage ~97% and identity of 30%. PHASER[9] from the PHENIX package[10] was used to perform molecular replacement, searching for one molecule in the asymmetric unit, and revealed the space group to be $P4_12_12$. A TFZ score of 15 was obtained. Initially the structure was built with PHENIX.autobuild [11] and then refined with PHENIX.refine[12] and manual model rebuilding in Coot[13] to a final R_{work}/R_{free} of 0.16/0.17 (see Table S1 for statistics).

AlphaFold
The predicted structure of TpPL7B was obtained by AlphaFold[14].

Structural alignments and electrostatic plots
Structural alignments were obtained using PyMOL v. 2.5.4 (Schrödinger, LLC, New York; also used for rendering structural models). Electrostatic maps were obtained with the APBS-PDB2PQR software suite (https://server.poissonboltzmann.org/) using APBS v. 3.4.1 and PDB2PQR v. 3.6.1[15].

Sequence analyses
Multiple sequence alignment has been produced using Clustal Omega[16] and visualized with ESPript 3.0 server[17].

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Table S1. Data collection and refinement statistics for TpPL7A.

Enzyme	**TpPL7A**
PDB ID	7NDE
Data collection	
Resolution (Å)	43.55–1.45 (1.50–1-45)
Space group	$P4_12_12$
Unit cell	
a, b, c (Å)	69.6; 69.6; 111.7
Total no. of reflections	1293691 (125278)
No. of unique reflections	49303 (4791)
R_{merge}	0.07 (3.39)
R_{meas}	0.08 (3.45)
R_{pim}	0.01 (0.67)
$CC_{1/2}$	1 (0.44)
I/σ	22.47 (0.87)
Completeness (%)	99.98 (99.94)
Redundancy	26.2 (26.1)
Refinement	
Reflections used in refinement	49294 (4788)
Reflections used for R_{free}	2093 (203)
R_{work}	0.15 (0.29)
R_{free}	0.17 (0.31)
CC (work)	0.98 (0.79)
CC (free)	0.96 (0.78)
No. of refined non-hydrogen atoms	2051
Protein	1847
Ligands	18
Solvent	186
B-factors	31.45
Protein	29.89
Ligands	75.05
Solvent	42.74
Wilson *B*-factor	25.87
r.m.s.d.	
Bond lengths (Å)	0.01
Bond lengths (°)	0.94
Ramachandran plot	
Favored (%)	95.71
Allowed (%)	3.86
Outliers (%)	0.43
Rotamer outliers (%)	1.48
No. of TLS groups	8

Table S2. Kinetic parameters of TpPL7A and B and their mutants on β-glucuronan (n.d. = not detected under the assay conditions).

	K_M (g/L)	k_{cat} (s^{-1})	k_{cat}/K_M ($L.s^{-1}\ g^{-1}$)
TpPL7A	3.1 ± 0.02	3119 ± 10	1006
R99A	n.d.	n.d.	-
R99K	0.65 ± 0.02	17.1 ± 0.1	26.3
H137N	5.5 ± 0.05	1865 ± 8.2	339
Q143A	n.d.	n.d.	-
Q143N	n.d.	n.d.	-
F145H	0.68 ± 0.3	12.3 ± 0.3	18.0
E155A	0.4 ± 0.00	17.6 ± 0.0	44.0
E155D	0.68 ± 0.02	40.3 ± 0.2	59.3
E155R/Y157S	3.1 ± 0.02	0.5 ± 0.0	0.2
F145H/E155R/Y157S	n.d.	n.d.	-
Y157F	2.9 ± 0.0	2863 ± 1.0	987
Y157H	4.1 ± 0.08	1086 ± 0.1	265
Q176A	1.8 ± 0.03	565.8 ± 2.7	314
Q176N	8.3 ± 0.2	195.0 ± 2.7	23.5
Y223F	2.5 ± 0.2	1511 ± 45.5	604
Y229F	n.d.	n.d.	-
K225A	0.62 ± 0.03	1.3 ± 0.0	2.1
K225R	1.3 ± 0.14	1.7 ± 0.1	1.3
TpPL7B	0.74 ± 0.20	23.8 ± 3.1	32.2
H142A	1.2 ± 0.04	1.78 ± 0.0	1.5
Y230F	n.d.	n.d.	-

Table S3. Primers used to constructs TpPL7B mutants, H142A and Y230F. Mutated codons are underlined.

Primers	Sequence
TpPL7B_H142A_f	GTT ATT GGT CAA ATC GCC ATC GAT GAT TCT GTT TCT
TpPL7B_H142_r	GAT TTG ACC AAT AAC AGT ACC ATG ACC AGA AT
TpPL7B_Y230F_f	C TTC AAG GTT GGT AAC TTC AAC CAA GGT TCT TCT GCT
TpPL7B_Y230_r	GTT ACC AAC CTT GAA GTA AGA AGG TGG AGC ATC C

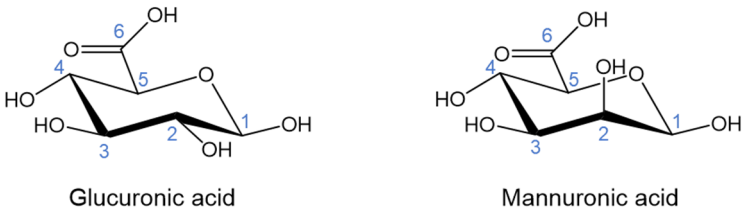


Fig. S1. Structures of glucuronic and mannuronic acids

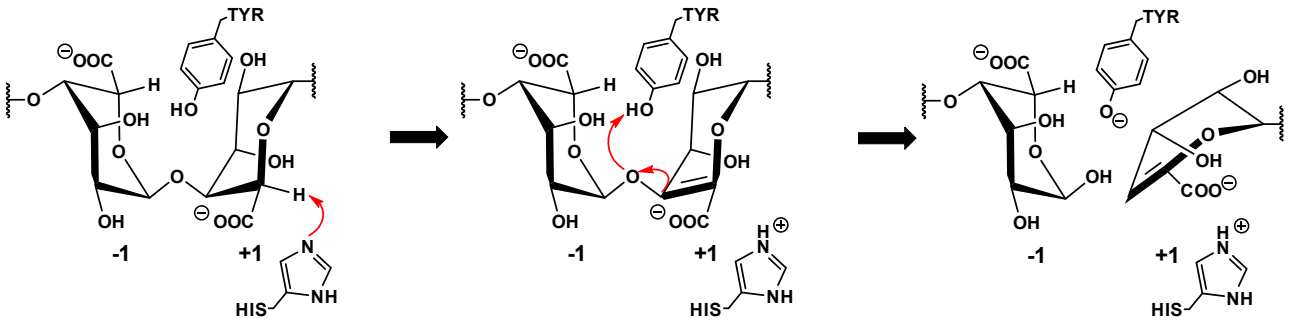


Fig. S2. His/Tyr anti β-elimination mechanism described for PL7 alginate lyases, depicted here with poly-α-L-guluronic acid.

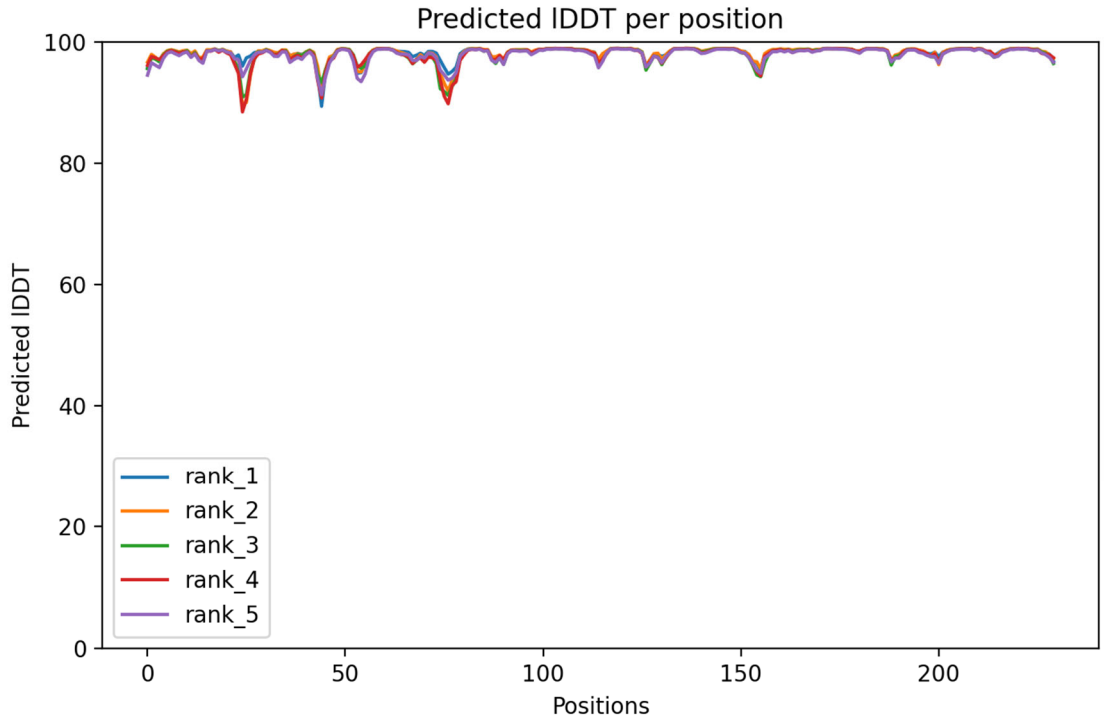
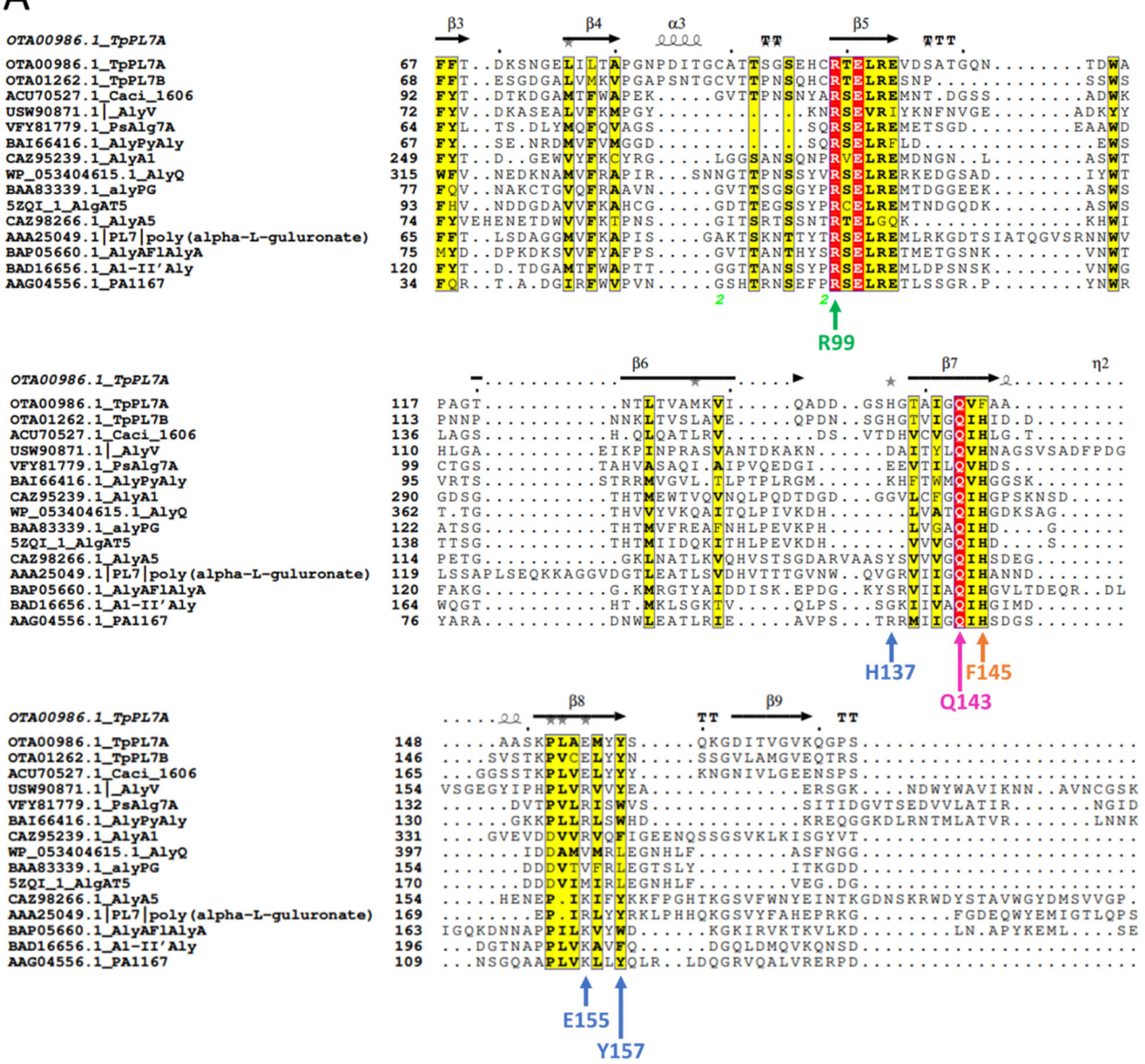


Fig. S3. AlphaFold model TpPL7B lDDT confident scores for the generated models. Regions with lDDT >90 are expected to be modelled to high accuracy [14]. Rank 1 is displayed in the manuscript.

A


β3
β4
α3
β5
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OTA01262.1_TpPL7B 68 FFT..ESGDGALVMKVPGAPSNTGCVTTPNSQHCRTELRESNP.....SSWS
ACU70527.1_Caci_1606 92 FYT..DTKDGAMTFWAPEK.....GVTTPNSNYARSELREMNT.DGSS.....ADWK
USW90871.1|_AlyV 72 FYV..DKASEALVFKMPGY.....KNRSEVRIYKNFNVGE.....ADKYY
VFY81779.1_PsAlg7A 64 FYL..TS.DLYMQFQVAGS.....SQRSELREMETSGD.....EAAWD
BAI66416.1_AlyPyAly 67 FY...SE.NRDMVFVMGGD.....SQRSELRFLD.....EWS
CAZ95239.1_AlyA1 249 FYT..D..GEWVYFKCYRG.....LGGSANSQNPRVELREMDNGN..L.....ASWT
WP_053404615.1_AlyQ 315 WFV..NEDKNAMVFRAPIR...SNNGTTPNSSYVRSELRERKEDGSAD.....IYWT
BAA83339.1_alyPG 77 FQV..NAKCTGVQFRAAVN.....GVTTSGSGYPRSELREMTDGGEEK.....ASWS
5ZQI_1_AlgAT5 93 FHV..NDDGDAVVFKAHCG.....GDTTEGSSYPRCELREMTNDGQDK.....ASWS
CAZ98266.1_AlyA5 74 FYVEHENETDWVVFKTPNS.....GITSRTSSNTRTELGQK.....KHWI
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BAP05660.1_AlyAFlAlyA 75 MYD..DPKDKSVVFYAFPS.....GVTTANTHYSRSELRETMETGSNK.....VNWT
BAD16656.1_A1-II'Aly 120 FYT..D.TDGAMTFWAPTT.....GGTTANSSYPRSELREMLDPSNSK.....VNWG
AAG04556.1_PA1167 34 FQR..T.A.DGIRFWVPVN.....GSHTRNSEFPRSELRETLSSGR.P.....YNWR
2
2
R99
β6
β7
η2
OTA00986.1_TpPL7A
OTA00986.1_TpPL7A 117 PAGT.....NTLTVAMKVI.....QADD..GSHGTAIGQVFAA.....
OTA01262.1_TpPL7B 113 PNNP.....NNKLTVSLAVE.....QPDN..SGHGTVIGQIHID.D.....
ACU70527.1_Caci_1606 136 LAGS.....H.QLQATLRV.....DS..VTDHVCVGQIHLG.T.....
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VFY81779.1_PsAlg7A 99 CTGS.....TAHVASAQI.AIPVQEDGI.....EEVTILQVHDS.....
BAI66416.1_AlyPyAly 95 VRTS.....STRRMVGVL.TLPTPLRGM.....KHFTWMQVHGGSK.....
CAZ95239.1_AlyA1 290 GDSG.....THTMEWTVQVNQLPQDTDGD...GGVLCFGQIHGPSKNSD.....
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BAA83339.1_alyPG 122 ATSG.....THTMVFREAFNHLPEVKPH.....LVGAQIHD....G.....
5ZQI_1_AlgAT5 138 TTSG.....THTMIIDQKITHLPEVKDH.....VVVGQIHD....S.....
CAZ98266.1_AlyA5 114 PETG.....GKLNATLKVQHVSTSGDARVAASYSVVVGQIHSDEG.....
AAA25049.1|PL7|poly(alpha-L-guluronate) 119 LSSAPLSEQKKAGGVDGTLEATLSVDHVTTTGVNW..QVGRVIIGQIHANND.....
BAP05660.1_AlyAFlAlyA 120 FAKG.....G.KMRGTYAIDDISK.EPDG..KYSRVIIAQIHGVLTDEQR..DL
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F145
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β8
β9
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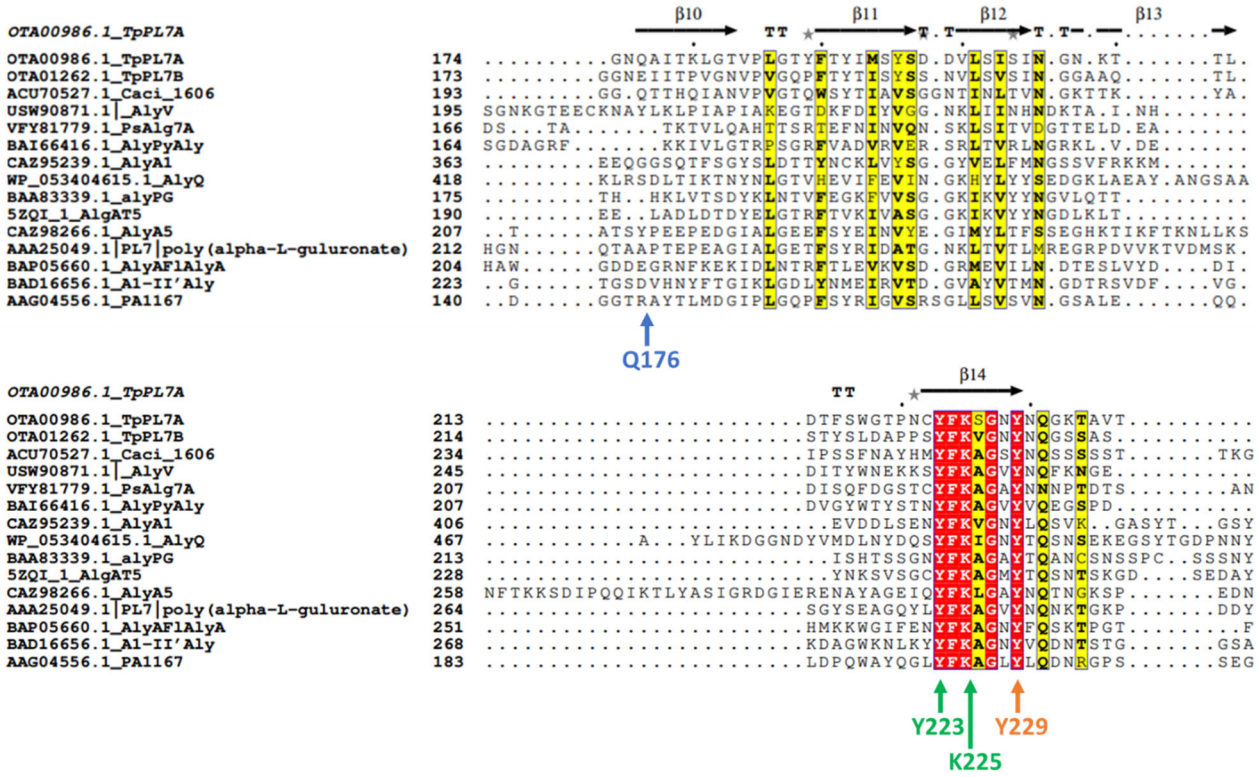
Y157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β11
β12
β13
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VFY81779.1_PsAlg7A 166 DS...TA.....TKTVLQAHTTSRTEFNINVQN.SKLSITVDGTTELD.EA.....
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CAZ95239.1_AlyA1 363 .....EEQGGSQTFSGYSLDTTYNCKLVYSG.GYVELFMNGSSVFRKKM.....
WP_053404615.1_AlyQ 418 .....KLRSDLTIKTNYNLGTVHEVIFEVIN.GKHYLYYSEDGKLAEAY.ANGSAA
BAA83339.1_alyPG 175 .....TH..HKLVTSDYKLNTVFEGKFVVSG.GKIKVYYNGVLQTT.....
5ZQI_1_AlgAT5 190 .....EE..LADLDTDYELGTRFTVKIVASG.GKIKVYYNGDLKLT.....
CAZ98266.1_AlyA5 207 ..T.....ATSYPEEPEDGIALGEEFSYEINVYE.GIMYLTFSSEGHKTIKFTKNLLKS
AAA25049.1|PL7|poly(alpha-L-guluronate) 212 HGN.....QTAAPTEPEAGIALGETFSYRIDATG.NKLTVTLMREGRPDVVKTVDMSK.
BAP05660.1_AlyAFlAlyA 204 HAW.....GDDEGRNFKEKIDLNTRFTLEVKVSD.GRMEVILN.DTESLVYD....DI.
BAD16656.1_A1-II'Aly 223 ..G.....TGSDVHNYFTGIKLGDLYNMEIRVTD.GVAYVTMN.GDTRSVDF....VG.
AAG04556.1_PA1167 140 ..D.....GGTRAYTLMDGIPLGQPFSYRIGVSRSGLLSVSVN.GSALE.....QQ.
Q176
β14
OTA00986.1_TpPL7A
OTA00986.1_TpPL7A 213 .....DTFSWGTPNCYFKSGNYNQGKTAVT.....
OTA01262.1_TpPL7B 214 .....STYSLDAPPSYFKVGNYNQGSSAS.....
ACU70527.1_Caci_1606 234 .....IPSSFNAYHMYFKAGSYNQSSSSST.....TKG
USW90871.1|_AlyV 245 .....DITYWNEKKSYFKAGVYNQFKNGE.....
VFY81779.1_PsAlg7A 207 .....DISQFDGSTCYFKAGAYNNNPTDTS.....AN
BAI66416.1_AlyPyAly 207 .....DVGYWTYSTNYFKAGVYVQEGSPD.....
CAZ95239.1_AlyA1 406 .....EVDDLSENYFKVGNYLQSVK..GASYT...GSY
WP_053404615.1_AlyQ 467 .....A...YLIKDGGNDYVMDLNYDQSYFKIGNYTQSNSEKEGSYTGDPNNY
BAA83339.1_alyPG 213 .....ISHTSSGNYFKAGAYTQANCSNSSPC..SSSNY
5ZQI_1_AlgAT5 228 .....YNKSVSGCYFKAGMYTQSNTSKGD....SEDAY
CAZ98266.1_AlyA5 258 NFTKKSDIPQQIKTLYASIGRDGIERENAYAGEIQYFKLGAYNQTNGKSP.....EDN
AAA25049.1|PL7|poly(alpha-L-guluronate) 264 .....SGYSEAGQYLYFKAGVYNQNKTGKP.....DDY
BAP05660.1_AlyAFlAlyA 251 .....HMKKWGIFENYFKAGNYFQSKTPGT.....F
BAD16656.1_A1-II'Aly 268 .....KDAGWKNLKYYFKAGNYVQDNTSTG.....GSA
AAG04556.1_PA1167 183 .....LDPQWAYQGLYFKAGLYLQDNRGPS.....SEG
Y223
K225
Y229

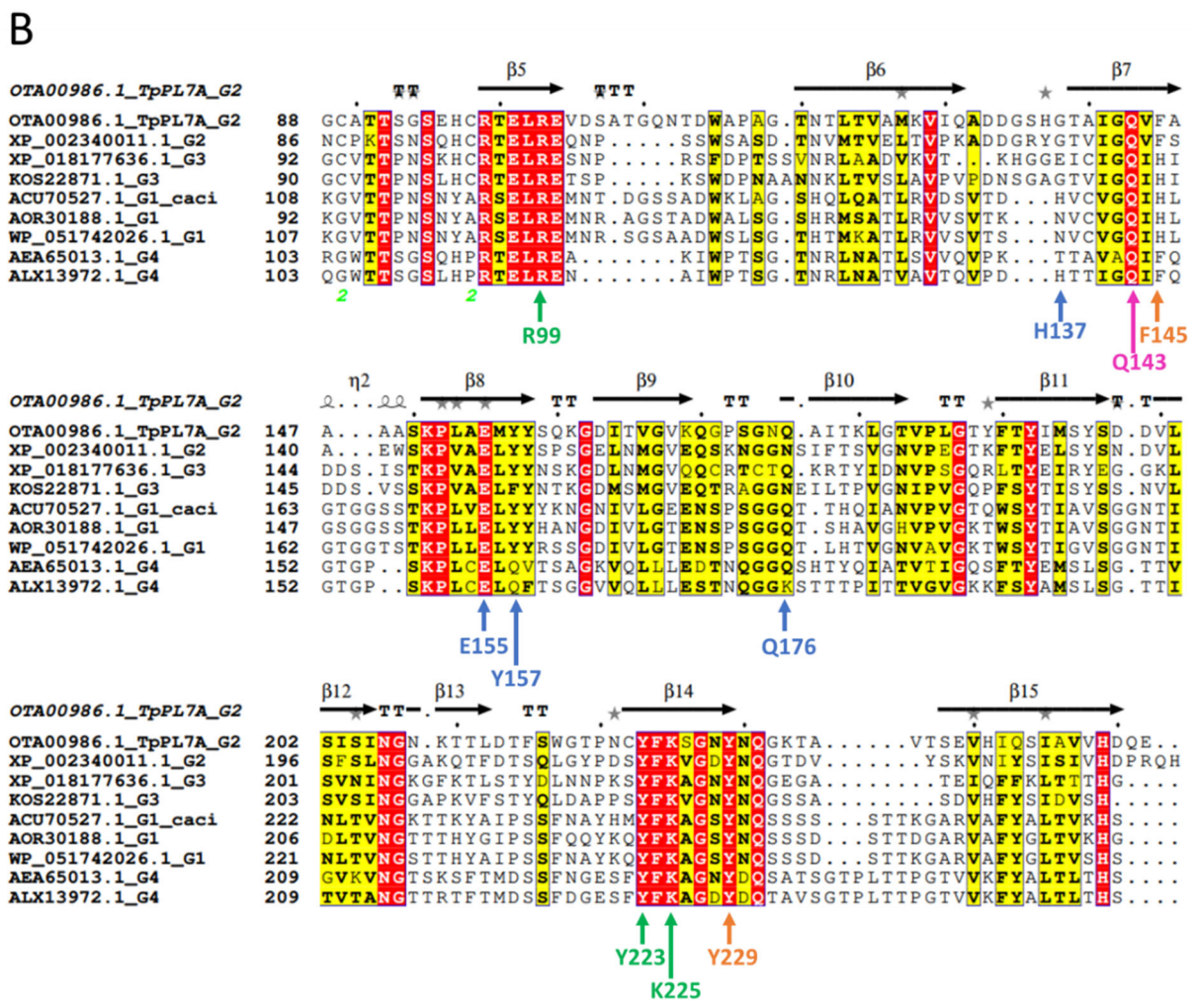

Fig. S4. A; Selected part of the multiple sequence alignment of protein sequences from PL7 family and B; multiple sequence alignment of protein sequences from subfamily PL7_4. Alignment has been prepared with ClustalO and visualized in ESPript using TpPL7A 3D structure (PDB 4NDE) to render secondary structure elements. Fully conserved positions are highlighted in red and partially conserved and highly similar positions are highlighted in yellow. The key residues amino acids mutated in TpPL7A are indicated by different arrows and using TpPL7A numbering. Orange arrows represent catalytic amino acids. Amino acids from the minus subsites are indicated in blue and amino acids from the plus subsites are indicated in green. The glutamine playing the role of the neutralizer in the β-elimination mechanism is indicated in by a pink arrow.

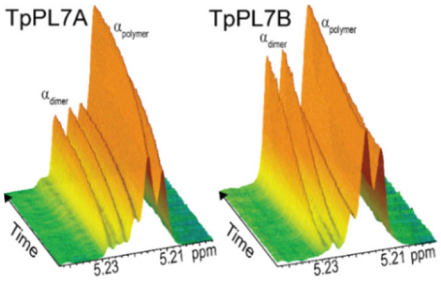

Fig S5. Time Course NMR for TpPL7A and TpPL7B recorded at 25 °C and at 800 MHz for 2 hours**.** Only the regions for □-reducing end are shown, and the assignment of the signals are indicated.

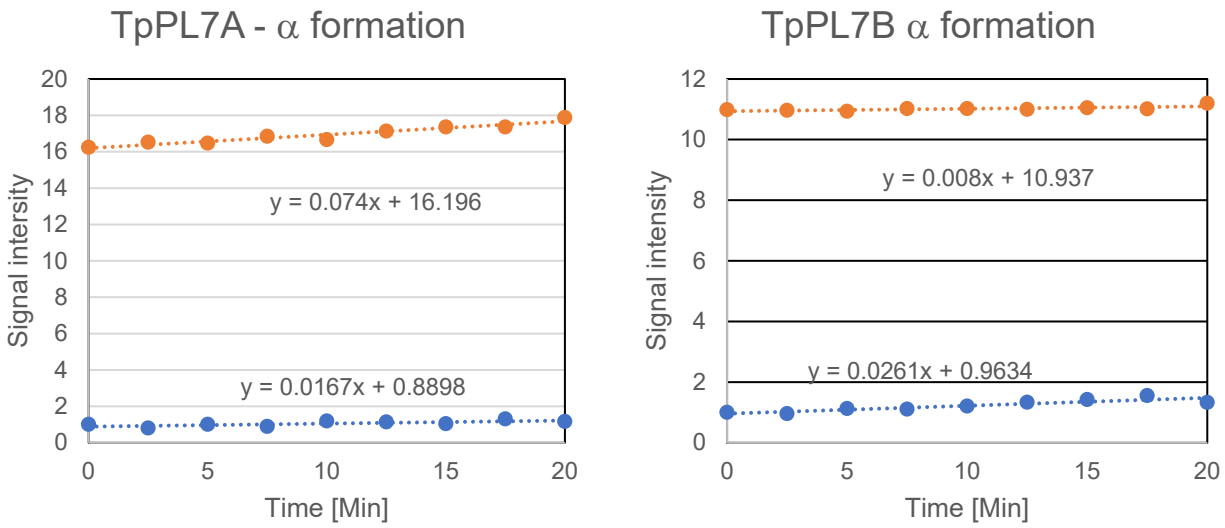

Fig. S6. The signals intensity of α-reducing ends for first 20 min with TpPL7A and TpPL7B. The intensity of α-dimer (blue dots) and α-polymer (orange dots) is plotted as the function of time and formation rate is estimated from the slope of the dotted lines. For TpPL7A makes 31% as α-dimer and 69% α-polymer, while TpPL7B generates 77% as α-dimer and 23% as α-polymer.

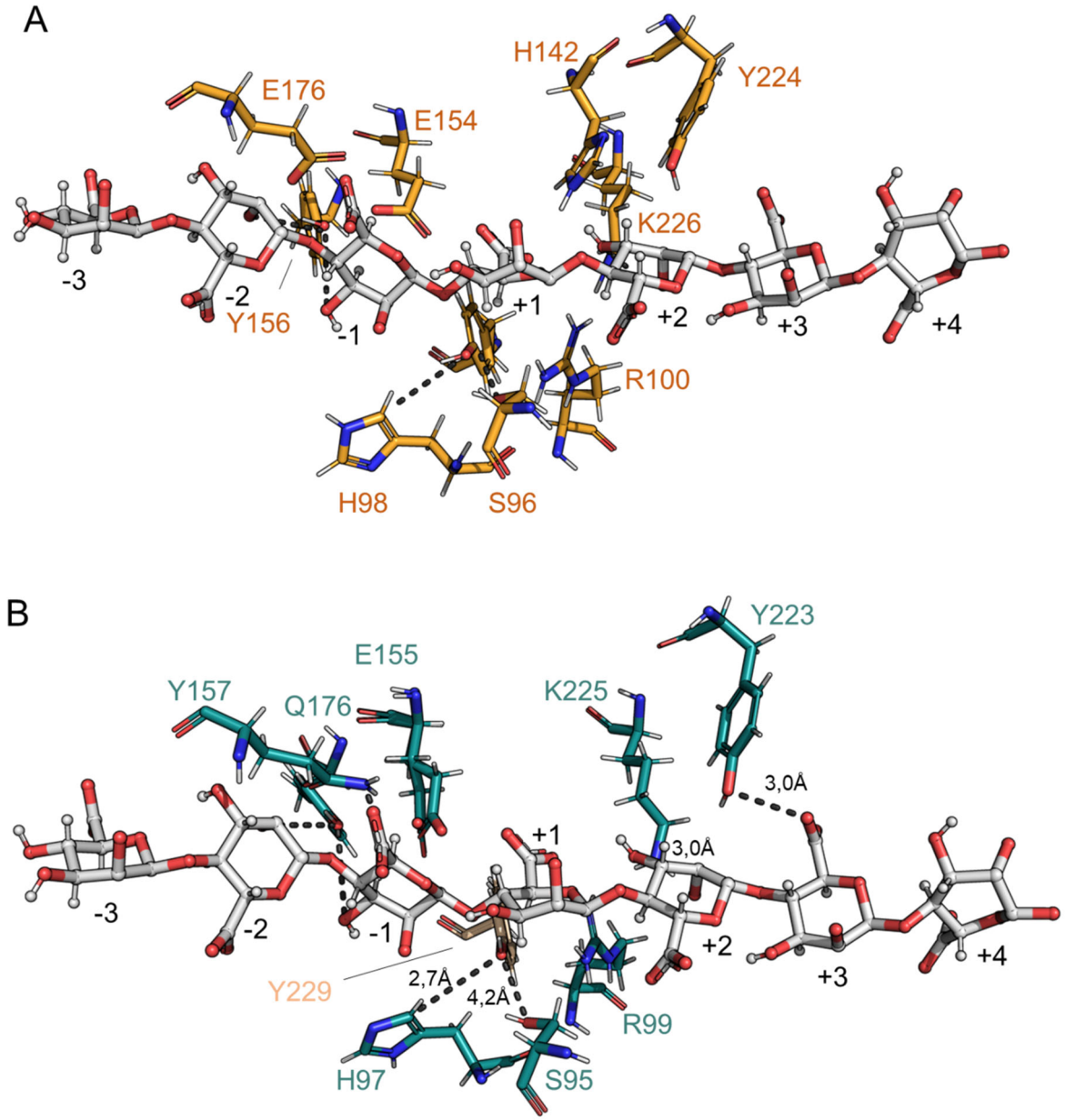

Fig. S7. Closer look on the active sites and hydrogen bonds of A) TpPL7B and B) TpPL7A, both in complex with hexamannuronic acid (stick representation in white) taken from PsAlg7A 3D structure (PDB 7NCZ).