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The structural, dynamical and biochemical characterizations of *Verticillium dahliae* pectate lyase, VdPelB, highlight its specificities

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22 **Abstract**

23 Pectins, complex polysaccharides and major components of the plant primary cell wall, can be
24 degraded by pectate lyases (PLs). PLs cleave glycosidic bonds of homogalacturonans (HG), the
25 main pectic domain, by β -elimination, releasing unsaturated oligogalacturonides (OGs). To
26 understand the catalytic mechanism and structure/function of these enzymes, we characterized
27 VdPelB from *Verticillium dahliae*, a plant pathogen. We first solved the crystal structure of
28 VdPelB at 1.2Å resolution showing that it is a right-handed parallel β -helix structure. Molecular
29 dynamics (MD) simulations further highlighted the dynamics of the enzyme in complex with
30 substrates that vary in their degree of methylesterification, identifying amino acids involved in
31 substrate binding and cleavage of non-methylesterified pectins. We then biochemically
32 characterized wild type and mutated forms of VdPelB. VdPelB was most active on non-
33 methylesterified pectins, at pH 8 in presence of Ca^{2+} ions. VdPelB-G125R mutant was most
34 active at pH 9 and showed higher relative activity compared to native enzyme. The OGs
35 released by VdPelB differed to that of previously characterized PLs, showing its peculiar
36 specificity in relation to its structure. OGs released from *Verticillium*-partially tolerant and
37 sensitive flax cultivars differed which could facilitate the identification VdPelB-mediated
38 elicitors of defence responses.

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42 **Keywords:** Pectate lyase, pectins, homogalacturonan, oligogalacturonides, Flax, *Verticillium*
43 *dahliae*.

44

45 1. Introduction

46 Primary cell wall, a complex structure of proteins and polysaccharides, cellulose and
47 hemicelluloses, is embedded in a pectin matrix. Pectins, are complex polysaccharides
48 composing up to 30% of cell wall dry mass in dicotyledonous species [1]. Pectin is mainly
49 constituted of homogalacturonan (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan
50 II (RG-II) domains, but its composition can differ between plant organs and among species.
51 The most abundant pectic domain is HG, a linear homopolymer of α -1,4-linked galacturonic
52 acids (GalA), which represents up to 65% of pectins [2]. During synthesis, HG can be O-
53 acetylated at O-2 or O-3 and/or methylesterified at C-6 carboxyl, before being exported at the
54 cell wall with a degree of methylesterification (DM) of ~80% and a degree of acetylation (DA)
55 of ~5-10%, depending on species [3]. At the wall, HG chains can be modified by different
56 enzyme families, including pectin acetyltransferase (PAEs; EC 3.1.1.6), pectin methylesterases
57 (PMEs; CE8, EC 3.1.1.11), polygalacturonases (PGs; GH28, EC 3.2.1.15, EC 3.2.1.67, EC
58 3.2.1.82), and pectin lyases-like (PLLs), which comprise pectate lyases (PLs; EC 4.2.2.2) and
59 pectin lyase (PNLs, EC 4.2.2.10). All these enzymes are produced by plants to fine-tune pectin
60 during development [4–8], but they are also secreted by most phytopathogenic bacteria and
61 fungi during plant infection [9–13]. PMEs and PAE hydrolyse the O6-ester and O2-acetylated
62 linkages, respectively, leading to a higher susceptibility of HG to PG- and PLL-mediated
63 degradation [14]. PLL are pectolytic enzymes that cleave HG via a β -elimination mechanism
64 leading to the formation of an unsaturated C4-C5 bond [15], and can be divided into two
65 subfamilies depending on their biochemical specificities: i) PLs have a high affinity for non- or
66 low-methylesterified pectins and an optimal pH near 8.5. Their activity requires Ca^{2+} ions. ii)
67 PNLs are most active on high-DM pectins at acidic pH values [16]. Both type of enzymes can
68 degrade HG chains, and release oligogalacturonides (OGs), but their mode of action can differ.
69 For PLs both endo and exo modes of action have been described, while only endo-PNL have
70 been characterised so far [17]. For the latter, it was notably shown that endo-PNLs from *B.*
71 *fuckeliana*, *A. parasiticus* and *Aspergillus* sp., can first release OGs with degrees of
72 polymerisation (DP) 5–7, that are subsequently used as substrates, generating OGs of DP3 and
73 DP4 as end-products [9,18,19]. Despite having the same DP, the final products can differ in
74 their degrees and patterns of methylesterification and acetylation (DM/DA) depending on the
75 enzymes' specificities; implying potential differences in substrate binding, and therefore in
76 PLLs fine structures. Several crystallographic structures of bacterial and fungal PLL have been
77 reported [15,20–24]. Overall, the PLL fold resemble that of published PME, PGs and

78 rhamnogalacturonan lyases [25–27], and is composed of three parallel β -sheets forming a right-
79 handed parallel β -helix. The three β -sheets are called PB1, PB2 and PB3 and the turns
80 connecting them T1, T2 and T3 [28]. The active site features three Asp, localized on the PB1
81 β -sheet and, in the case of PLs, accommodates Ca^{2+} [29]. In PNLs, Ca^{2+} is, on the other hand,
82 replaced by Asp [15]. Additionally, the PL binding site is dominated by charged acidic and
83 basic residues (Gln, Lys, Arg) which can accommodate negatively charged pectate substrates.
84 In contrast, the PNL binding site is dominated by aromatic residues [15,29], which have less
85 affinity for lowly methylesterified pectins. These differences in structure could translate into
86 distinct enzyme dynamics when in complex with substrates of varying degrees of
87 methylesterification.

88 In fungi, PLLs are encoded by large multigenic families which are expressed during
89 infection. *Verticillium dahliae* Kleb., a soil-borne vascular fungus, targets a large number of
90 plant species, causing Verticillium wilt disease to become widespread among fiber flax, with
91 detrimental effects to fiber quality [30–32]. *V. dahliae* infects plants by piercing the root surface
92 using hyphae, secreting a number of pectinolytic enzymes, including thirteen PLLs.
93 Considering the role of PLLs in determining pathogenicity, it is of paramount importance to
94 determine their biochemical and structural properties [30,33,34]. This could allow engineering
95 novel strategies to control or inhibit, the pathogen's pectinolytic arsenal. For this purpose, we
96 characterized, via combined experimental and computational approaches, one *V. dahliae* PLL
97 (VdPelB, VDAG_04718) after its heterologous expression in *P. pastoris*. The obtention of the
98 3D structure of VdPelB after X-ray diffraction and the analysis of enzyme dynamics when in
99 complex with substrates of distinct DM, allowed the identification of the residues favouring
100 pectate lyase (PL) activity. Experiments confirmed the importance of these residues in
101 mediating PL activity showing that VdPelB is a *bona fide* PL, that releases peculiar OG as
102 compared to previously characterized PLs. More importantly, the OGs released from roots of
103 *Verticillium*-partially tolerant and sensitive flax cultivars differed, paving the way for the
104 identification of VdPelB-mediated OGs that can trigger plant defence mechanisms.

105

106 **2. Material and methods**

107 **2.1. Bioinformatical analysis**

108 *Verticillium dahliae* PLL sequences were retrieved using available genome database
109 (ftp.broadinstitute.org/). SignalP-5.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>) was

110 used for identifying putative signal peptide and putative glycosylation sites were predicted
111 using NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) and NetOGlyc 4.0
112 Server (<http://www.cbs.dtu.dk/services/NetOGlyc/>). Sequences were aligned and phylogenetic
113 analysis was carried out using MEGA multiple sequence alignment program
114 (<https://www.megasoftware.net/>). Homology models were created using I-TASSER structure
115 prediction software (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>) and UCSF Chimera
116 (<http://www.cgl.ucsf.edu/chimera/>) was used for creation of graphics.

117 **2.2. Fungal strain and growth**

118 *V.dahliae* was isolated from CALIRA company flax test fields (Martainneville, France) and
119 was kindly provided by Linéa-Semences company (Grandvilliers, France). Fungus was grown
120 as described in Safran et al. [35]. Briefly, fungus was grown in polygalacturonic acid sodium
121 salt (PGA, P3850, Sigma) at 10 g.L⁻¹ and in pectin methylesterified potassium salt from citrus
122 fruit (55–70% DM, P9436, Sigma) solutions to induce PLL expression. After 15 days of growth
123 in dark conditions at 25°C and 80 rpm shaking, mycelium was collected and filtered under
124 vacuum using Buchner flask. Collected mycelium was frozen in liquid nitrogen, lyophilized
125 and ground. Isolation of RNA and cDNA synthesis was realized as previously described in
126 Lemaire et al.[36].

127 **2.3. Cloning, heterologous expression and purification of VdPelB**

128 *V. dahliae* PelB coding sequence (VdPelB, UNIPROT: G2X3Y1, GenBank:
129 EGY23280.1), minus the signal peptide was amplified using cDNA and gene-specific primers.
130 VdPelB mutants were generated using cDNA and specific primers carrying mutations (Table
131 S1). Cloning, heterologous expression in *P. pastoris* and purification of VdPelB was done as
132 previously described in Safran et al. [35].

133 **2.4. Crystallization of VdPelB**

134 VdPelB was concentrated at 10 mg.mL⁻¹ in Tris-HCl pH 7.5 buffer. Crystallization
135 conditions were screened using the sitting-drop vapor-diffusion method. VdPelB (100 nL) was
136 mixed with an equal volume of precipitant (1:1) using Mosquito robot (STP Labtech). The
137 crystals that resulted in best diffraction data were obtained with 0.1 M MIB (Malonic acid,
138 Imidazole, Boric acid system) at pH 8.0, with 25 % PEG 1500 as the precipitant (condition B5
139 from the PACT premier kit, Molecular Dimensions, Sheffield, UK) after 1 month. Optimization
140 was realized using the hanging drop vapor-diffusion method forming the drop by mixing 1 µL

141 of precipitant solution with 1 μ L of the enzyme. The large beam-like crystals were cryo-
142 protected by increasing PEG 1500 concentration to 35%, before mounting them in a loop and
143 flash-cooling them in liquid nitrogen.

144 **2.5. VdPelB X-ray data collection and processing**

145 X-ray diffraction data were collected at the PROXIMA-2a beamline of the Soleil
146 synchrotron (Saint Aubin, France), at a temperature of -173°C using an EIGER 9M detector
147 (Dectris). Upon a first data collection to 1.3 \AA resolution, three more data sets were collected
148 from the same crystal in order to obtain a complete data set. Thereby the kappa angle was tilted
149 once to 30° , once to 60° and finally a helical data set was collected at 1.2 \AA resolution. The
150 reflections of each data set were indexed and integrated using XDS [37], scaled and merged
151 using XSCALE [38]. The VdPelB crystal has a primitive monoclinic lattice in the $P 1 2_1 1$ space
152 group, with two molecules contained in asymmetric unit [39].

153 **2.6. Structure solution and refinement**

154 The structure of VdPelB was solved by molecular replacement using *Phaser* [40]. The data
155 were phased using pectate lyase BsPelA (PDB: 3VMV, Uniprot D0VP31), as a search model
156 [41]. Model was build using *Autobuild* and refined using *Refine* from PHENIX suite [42]. The
157 model was iteratively improved with *Coot* [43] and *Refine*. The final structure for VdPelB has
158 been deposited in the Protein Data Bank (PDB) as entry 7BBV.

159 **2.7. VdPelB biochemical characterization**

160 Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United
161 States) was used to determine the protein concentration, with Bovine Serum Albumin (A7906,
162 Sigma) as a standard. Deglycosylation was performed using Peptide-N-Glycosidase F (PNGase
163 F) at 37°C for one hour according to the supplier's protocol (New England Biolabs, Hitchin,
164 UK). Enzyme purity and molecular weight were estimated using a 12% SDS-PAGE and mini-
165 PROTEAN 3 system (BioRad, Hercules, California, United States). Gels were stained using
166 PageBlue Protein Staining Solution (Thermo Fisher Scientific) according to the manufacturer's
167 protocol.

168 The substrate specificity of VdPelB was determined using PGA (81325, Sigma) and citrus
169 pectin of various DM: 20–34% (P9311, Sigma), 55–70% (P9436, Sigma) and $>85\%$ (P9561,
170 Sigma), with $0.5 \mu\text{M}$ CaCl_2 or $5 \mu\text{M}$ EDTA (Sigma) final concentrations. Enzyme activity was
171 measured by monitoring the increase in optical density at 235 nm due to formation of

172 unsaturated uronide product using UV/VIS Spectrophotometer (PowerWave Xs2, BioTek,
173 France) during 60 min. The optimum temperature was determined by incubating the enzymatic
174 reaction between 25 and 45°C for 8 min using PGA as a substrate (0.4%, w/v). The optimum
175 pH was determined between pH 5 and 10 using sodium phosphate (NaP, pH 5 to 7) and Tris-
176 HCl buffer (pH 7 to 10) and 0.4% (w/v) PGA as a substrate. All experiments were realized in
177 triplicate.

178 **2.8. Digestion of commercial pectins and released OGs profiling**

179 OGs released after digestions by recombinant VdPelB or commercially available
180 Aspergillus PL (named AsPel) were identified as described in Voxeur et al., 2019 [9], using a
181 novel in-house OGs library. Briefly, DM 20–34% (P9311, Sigma) and sugar beet pectin with
182 DM 42% and degree of acetylation (DA) 31% (CP Kelco, Atlanta, United States) were prepared
183 at 0.4 % (w/v) final concentration diluted in 50 mM Tris-HCl buffer (pH 8) and incubated with
184 either VdPelB or AsPel (E-PCLYAN, Megazyme). For each substrate, enzyme concentrations
185 were adjusted to have enzymes at iso-activities (Table S2). For each substrate two dilutions,
186 were used for analysing OGs released in early, VdPelB-2 and AsPel-2, and late phase, VdPelB-
187 1 and AsPel-1, of digestions. Digestions were performed overnight. Non-digested pectins were
188 pelleted by centrifugation and the supernatant dried in a speed vacuum concentrator
189 (Concentrator plus, Eppendorf, Hamburg, Germany). Separation of OGs was done as
190 previously described using an ACQUITY UPLC Protein BEH SEC column (125Å, 1.7 µm, 4.6
191 mm x 300 mm) [44]. The intensities were defined as the area under the curve, for each OG.
192 Peak areas were clustered by hierarchical clustering with complete linkage on the euclidian
193 distance matrix and visualized in the heatmap-package using R version 3.6.0.

194 **2.9. Digestion of commercial pectins and released OGs profiling**

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200 either VdPelB or AsPel (E-PCLYAN, Megazyme). For each substrate, enzyme concentrations
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208 Peak areas were clustered by hierarchical clustering with complete linkage on the euclidian
209 distance matrix and visualized in the heatmap-package using R version 3.6.0.

210 **2.10.Molecular Dynamics simulations**

211 Two sets of molecular dynamics (MD) simulations were conducted on the VdPelB protein:
212 one in complex with a fully non-methylesterified polygalacturonate deca-saccharide, and the
213 other with a fully methylesterified polygalacturonate deca-saccharide. Parameters specified by
214 the AMBER14SB_parmbsc1 forcefield [45] were used to create the molecular topologies of
215 the complexes. Each complex was set up in a cubic box with solute-box distances of 1.0 nm
216 and solvated with water molecules specific to the TIP3P water model [46]. Na⁺ and Cl⁻ ions
217 were added to neutralise the system's net charge and reach a salt concentration of 0.165 M.
218 Using a steep-descent algorithm with a step size of 0.01, energy minimisation was performed
219 to resolve clashes between particles, with convergence being established at a particle-particle
220 force of 1000 kJ mol⁻¹ nm⁻¹. Particle-particle forces were calculated by considering van der
221 Waals and electrostatic interactions occurring up to 1.0 nm, as well as long-range electrostatics
222 treated in the Fourier space using the Particle Mesh Ewald (PME) summation method. Solvent
223 equilibration was attained post minimisation in two stages: through the nVT and nPT
224 ensembles, to reach constant temperature and pressure, respectively. Equilibration of the
225 solvent under the nVT ensemble was conducted for 1 ns, integrating the equation of motion at
226 a time step of 2 fs. The target reference temperature was 310.15 K, coupled every 0.1 ps using
227 the V-rescale thermostat3. Based on the Maxwell-Boltzmann distribution [47] at 310.15 K,
228 random velocities were then assigned to each particle in the system. Finally, solvent
229 equilibration under the nPT ensemble was conducted for 1 ns, continuing from the last step of
230 the previous equilibration, in terms of particle coordinates and velocities, at a reference
231 temperature of 310.15 K, coupled every 0.1 ps using the V-rescale thermostat [48]. Here,
232 pressure coupling was isotropically coupled every 2.0 ps, at 1 bar, using the Parrinello-Rahman
233 barostat [49]. Particle-particle interactions were computed by constructing pair lists using the
234 Verlet scheme. Short-range van der Waals and electrostatic interactions sampled through a
235 Coulomb potential, were calculated at a cutoff of 1.0 nm. The PME algorithm [50] was used to
236 compute long-range electrostatic interactions beyond this cut-off in the Fourier space, utilising

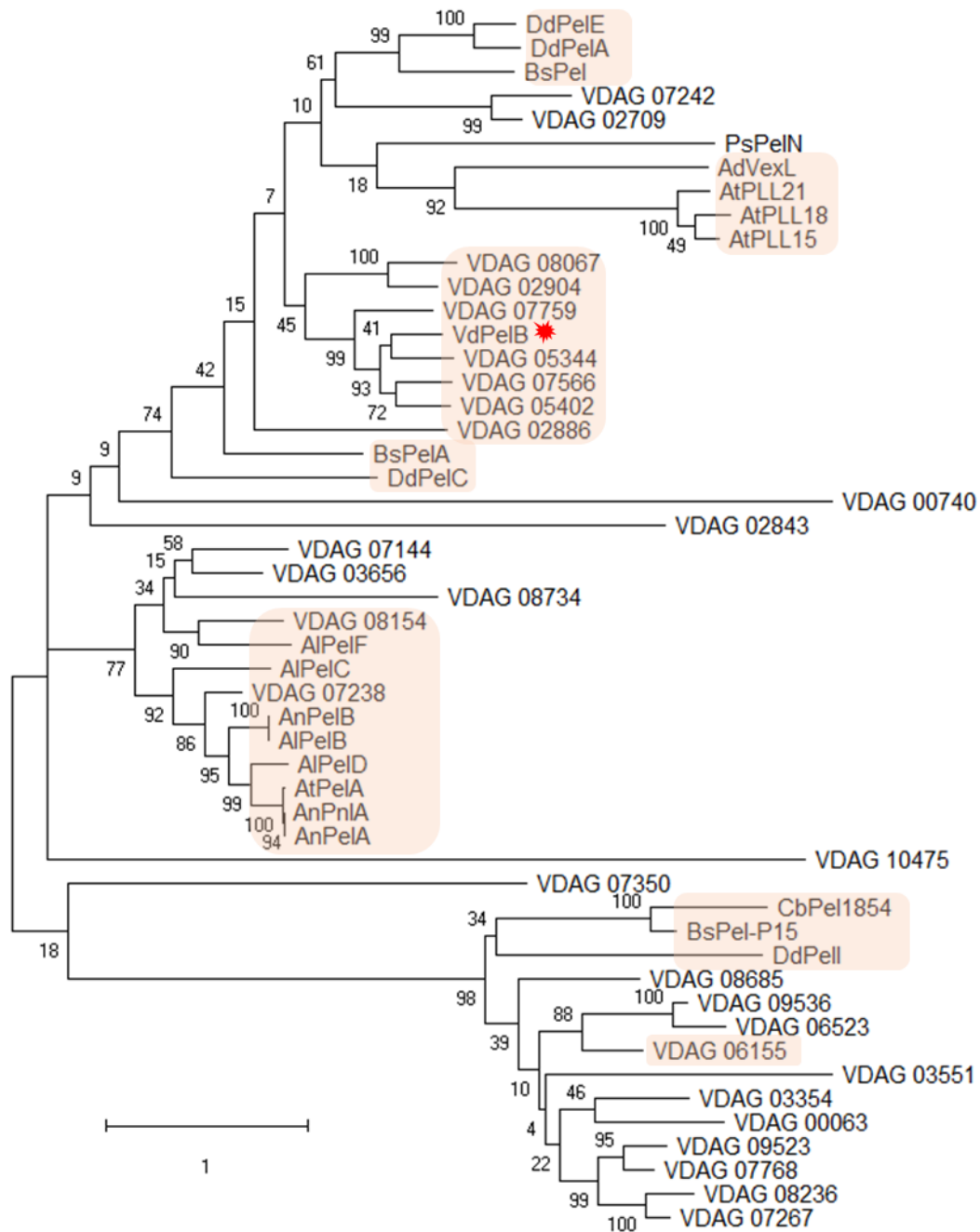
237 a Fourier grid spacing of 0.16 and a cubic B-spline interpolation level at 4. The simulations
238 were then performed on in-house machines, using GROMACS (Groningen Machine for
239 Chemical Simulations) version 2021.37. Each set of simulations were run for 150 ns each, at a
240 time step of 2 fs, with molecular dynamics trajectories written out every 10 ps. Simulations
241 were replicated 7 times for a total production run time of 1.05 μ s per complex. Replicates
242 differed with respect to the random particle velocity sets computed under the nVT ensemble.
243 For analysis, the first 50 ns of each production run were discarded as equilibration time. In-
244 house Python 3 scripts implemented using Jupyter notebooks [51] were used to carry out
245 analyses. Figures were created and rendered with Matplotlib [52] and VMD (Visual Molecular
246 Dynamics)[53].

247

248 3. Results and Discussion

249 3.1. Sequence and phylogeny analysis

250 In addition to 9 polygalacturonases (PGs) and 4 pectin methylesterases (PMEs), *V.*
251 *dahliae* encodes 30 putative endo-pectate lyases (EC 4.2.2.2), exo-pectate lyases (EC 4.2.2.9),
252 endo-pectin lyases (EC 4.2.2.10), belonging to PL1-PL3 and PL9 families, respectively [54].
253 For hierarchical clustering of *Verticilium*'s sequences with other PLLs, fifty-one amino acid
254 (aa) sequences encoding putative PLLs, belonging to bacteria, fungi and plants were aligned
255 and a phylogenetic tree was built. Different clades can be distinguished. (**Fig. 1**). *V. dahliae*
256 PelB (VdPelB, VDAG_04718) clustered with VDAG_05344 (59.68% sequence identity) with
257 close relations to VDAG_05402 (57.19% sequence identity) and VDAG_07566 (56.95%
258 sequence identity). VDAG_05402 and VDAG_05344, that are found in the protein secretome,
259 have orthologs in *V. alfalfa* (VDBG_07839 and VDBG_10041), which were shown to possess
260 putative lyase activity [34,55]. Plant PLLs from *A. thaliana* (AtPLL21, AtPLL15 and AtPLL18)
261 formed a separate clade with close connections to *A. denitrificans* (AdVexL), a PLL homologue
262 [56]. *D. dadanti* (DdPelI), *Bacillus Sp.* KSM-P15 (BsPel-P15) and *C. bescii* (CbPel1854)
263 formed separate clades similarly to *B. subtilis* and *D. dadantii* PLs (BsPel, DdPelA and
264 DdPelC). The clade corresponding to PNLs consisting of *A. tubingensis* PelA (AtPelA), *A.*
265 *niger* (AnPelA, AnPnlA and AnPelB), *A. luchuensis* AlPelB was closely related to
266 VDAG_07238 and VDAG_08154 which were indeed annotated as putative PNLs [18,54].
267 VDAG_06155 was previously named VdPel1 and previously characterized as a pectate lyase
268 [33].



269

270 **Fig. 1. Phylogenetic analysis of *V. dahliae* VdPelB with selected PLLs**

271 Phylogenetic tree representing *V. dahliae* VdPelB (VDAG_04718, G2X3Y1, red star) amino acid sequence in
 272 comparison with PLLs from *Verticillium* [VDAG_00740 (G2WQU8), VDAG_02904 (G2WXC5), VDAG_05344
 273 (G2X628), VDAG_07242 (G2XBA4), VDAG_07759 (G2XC77), VDAG_02709 (G2WWT0), VDAG_02843
 274 (G2WX64), VDAG_02886 (G2WXA7), VDAG_03656 (G2X1P5), VDAG_05402 (G2X597), VDAG_07144
 275 (G2X9U9), VDAG_07238 (G2XBA0), VDAG_07566 (G2XBY8), VDAG_08067 (G2XD35), VDAG_08154
 276 (G2XDC2), VDAG_08734 (G2XF02), VDAG_10475 (G2XJZ3), VDAG_03354 (G2WZB2), VDAG_03551
 277 (G2WZV9), VDAG_07267 (G2XBC9), VDAG_07768 (G2XC86), VDAG_08236 (G2XDK4), VDAG_06155
 278 (G2X8L4), VDAG_06523 (G2X7R5), VDAG_08685 (G2XEV0), VDAG_09523 (G2XH91), VDAG_09536
 279 (G2XHA4), VDAG_00063 (G2WR80), VDAG_07350 (G2XGG7)], *Arabidopsis thaliana* [AtPLL15
 280 (At5g63180), AtPLL18 (At3g27400), AtPLL21 (At5g48900)], *Dickeya dadanti* [DdPelA (P0C1A2), DdPelC

281 (P11073), DdPelE (P04960), DdPelI (O50325)], *Bacillus subtilis* [BsPel (P39116)], *Bacillus Sp.* KSM-P15
282 [BsPel-P15 (Q9RHW0)], *Bacillus sp.* N16-5 [BsPelA (D0VP31)], *Aspergillus niger* AnPelA [(Q01172), AnPelB
283 (Q00205), AnPn1A (A2R3I1)], *Achromobacter denitrificans* [AdVexL (A0A160EBC2)], *Aspergillus tubingensis*
284 [AtPelA (A0A100IK89)], *Aspergillus luchuensis* [AlPelB (G7Y0I4)], *Acidovorax citrulli* [AcPel343, (A1TSQ3)],
285 *Paenibacillus sp.* 0602 [PsPelN (W8CR80)], *Caldicellulosiruptor bescii* [CbPel1854 (B9MKT4)]. Maximum
286 likelihood tree was constructed with 1000 bootstrap replicates. Most important clades are indicated in orange
287 squares while VdPelB is marked by red star. Amino acids sequences were retrieved from Uniprot and TAIR.

288 **3.2. Cloning, expression and purification of VdPelB**

289 The *VdPelB* (*VDAG_04718*) gene consists of 1 single exon of 1002 bp length. The coding
290 sequence, minus the putative signal peptide was PCR-amplified using gene-specific primers,
291 ligated in pPICZ α B vector and expressed in *P. pastoris* heterologous expression system,
292 allowing its secretion in the culture media. Secreted VdPelB consisted of 343 aa, including the
293 poly-histidine tag at the C-terminus used for affinity chromatography purification. After
294 purification, VdPelB had an apparent molecular mass of ~38 kDa (**Fig. S1A**), higher than what
295 was predicted on the basis of the amino acid sequence (33.8 kDa). However, this shift is likely
296 to correspond to the tags (His and C-myc) and to the presence of 19 putative O-glycosylation
297 sites, as predicted by NetOGlyc 4.0 Server.

298 **3.3. VdPelB has a right-handed parallel β -helix fold**

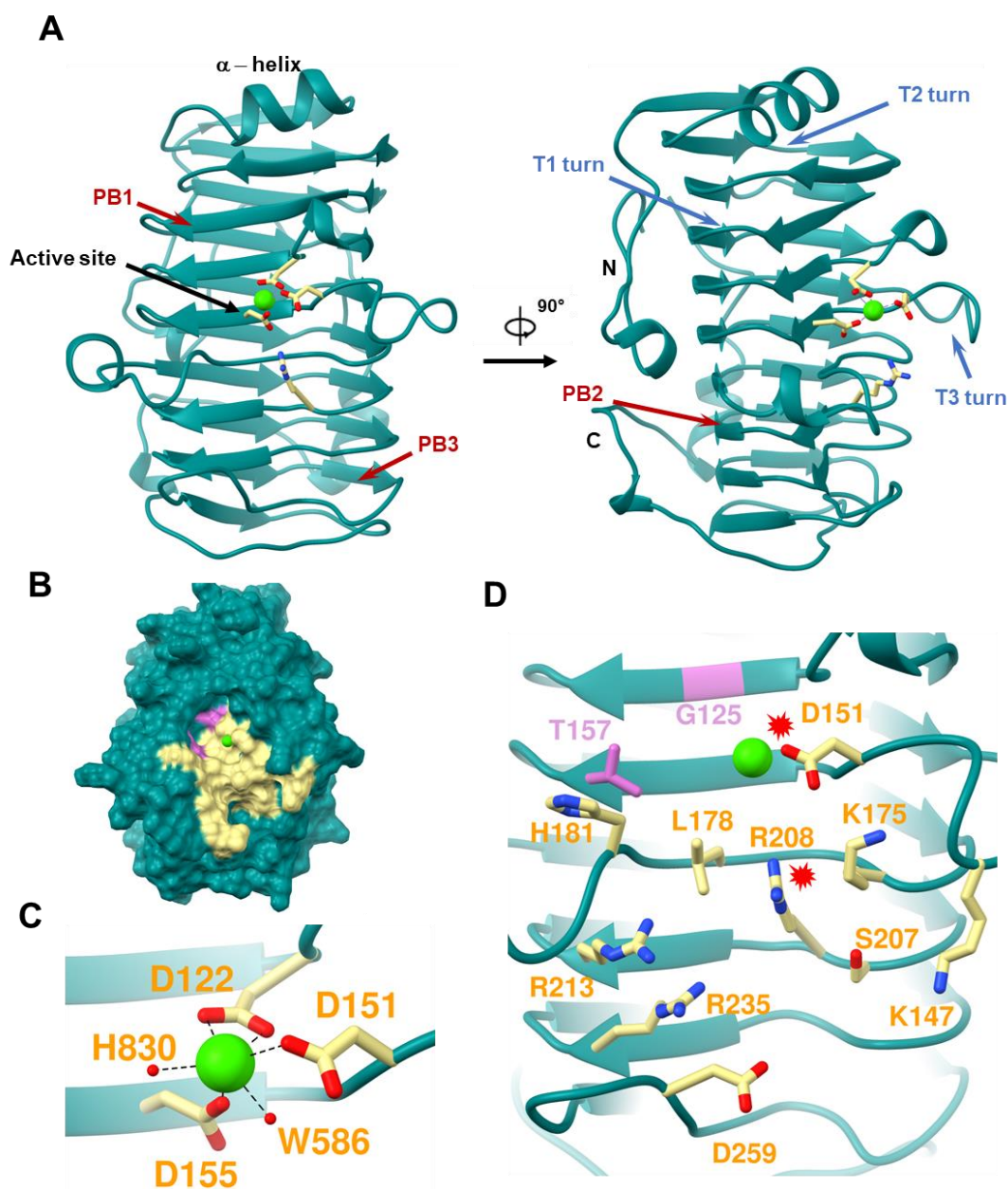
299 VdPelB was crystallized and its 3D structure was determined using X-ray diffraction.
300 VdPelB crystallized in monoclinic P 1 2₁ 1 asymmetric unit. Four data sets, collected from the
301 same crystal at 1.2 Å resolution, were integrated, scaled and merged. There are two molecules
302 in the asymmetric units: chains A and B are highly similar with a C α root mean square deviation
303 (rmsd) value of 0.227 Å (**Fig. S2A**). The VdPelB structure consists of 298 amino acids (aa)
304 with 18 aa at the N-terminus and 27 aa at the C-terminus that were not resolved because of poor
305 electron densities, while overall electron densities were well defined. While no N-glycosylation
306 sites could be revealed on the VdPelB structure, six O-glycosylation sites carrying mannose are
307 visible for each molecule: T22, T44, T45, T46, S48 and T54, in accordance with the shift in
308 size previously observed (**Fig. S2A**). During data acquisition no heating of the crystal was
309 observed, as shown by low B factors and good occupancies (**Fig. S3A and B**). The final models'
310 geometry, processing and refinement statistics are summarized (**Table 1**). VdPelB's structure
311 has been deposited in the Protein Data Bank as entry 7BBV.

312 **Table 1. Data collection, processing and refinement for VdPelB**

Characteristics	VdPeIB
Data collection	
Diffraction source	PROXIMA2
Wavelength (Å)	0.980
Temperature (°C)	100.15
Detector	DECTRIS EIGER X 9M
Crystal-to-detector distance (mm)	115.02
Rotation range per image (°)	0.1
Total rotation range (°)	360
Crystal data	
Space group	P1 21 1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	60.89, 59.69, 93.87
α , β , γ , (°)	90.00, 96.35, 90.00
Subunits per asymmetric unit	2
Data statistics	
Resolution range (Å)	60.52-1.2 (1.243 - 1.2)
Total No. of reflection	8536161 (112943)
No. of unique reflection	207497 (19983)
No. of reflections, test set	10362 (1003)
R _{merge} (%)	0.1036 (4.709)
Completeness (%)	99.30 (96.11)
(<i>I</i> /σ(<i>I</i>))	18.03 (0.22)
Multiplicity	41.1 (5.6)
CC _{1/2} (%)	1 (0.116)
Refinement	
R _{crys} /R _{free} (%)	17.3 / 19.7
Average B – factor (Å ²)	33.18
No. of non-H atoms	
Protein	9097
Ion	2
Ligand	234
Water	1052
Total	10385
R.m.s. deviations	
Bonds (Å)	0.013
Angles (°)	1.39
Ramachandran plot	
Most favoured (%)	95.1
Allowed (%)	4.90
Outlier (%)	-

314 VdPelB has a right-handed parallel β -helix fold which is common in pectinases [57].
315 The β -helix is formed by three parallel β -sheets - PB1, PB2 and PB3 which contain 7, 10 and 8
316 β -strands, respectively. Turns connecting the PB1-PB2, PB2-PB3 and PB3-PB1 β -sheets are
317 named T1-turns, T2-turns and T3-turns, respectively, according to Yoder and Jurnak (**Fig. 2A,**
318 **S4A and B**) [58]. T1-turns consist of 2-14 aa and builds the loop around the active site on the
319 C-terminus. T2 turns mostly consist of 2 aa with Asn being one of the predominant aa, forming
320 an N-ladder with the exception of an N245T mutation in VdPelB [15,20]. VdPelB has a α -helix
321 on N-terminus end that shields the hydrophobic core and is commonly conserved in PLs and
322 PGs [15,59], while the C-terminus end is also protected by tail-like structure carrying one α -
323 helix. Interestingly N- and C- terminus tails pack against PB2 (**Fig. 2A**). There are only two
324 Cys (C25 and C137) that do not form a disulphide bridge.

325 Sequence and structural alignments show that VdPelB belong to the PL1 family.
326 VdPelB shares the highest structural similarity with BsPelA (PDB: 3VMV), with 30.06%
327 sequence identity and $C\alpha$ rmsd of 1.202 Å. The second-best structural alignment was with
328 DdPelC (PDB: 1AIR) with 24.20% identity and $C\alpha$ rmsd of 1.453 Å [41,60]. Both of these
329 structures lack the long T3 loop described in *A.niger* pectate lyase (AnPelA, PDB: 1IDJ, **Fig.**
330 **S2B and S5**) [15]. The putative active site is positioned between the T3 and T2 loops (**Fig. 2A**
331 **and B**).



332

333 **Fig. 2. Structure determination of VdPelB**

334 A) Ribbon diagram of VdPelB crystalized in P1 21 1 space group. VdPelB is a right-handed parallel β helical
 335 structure consisting of β strands (red arrows) and turns (blue arrows). VdPelB active site's aa are yellow-colored
 336 while Ca atom is green. B) Surface representation of VdPelB binding groove. C) Active site of VdPelB
 337 highlighting conserved aa and atoms interacting with Ca. D) Structure of VdPelB binding groove highlighting aa
 338 involved in the interaction (yellow) and aa not of previously characterized in PLs (plum). Red stars indicates aa
 339 from the active site.

340 **3.4. Active site harbours Ca^{2+} that is involved in catalysis**

341 The VdPelB active site is well conserved, harbouring strictly conserved acidic and basic
 342 aa that are required for Ca^{2+} binding. Previously reported structures showed that two Asp (D122
 343 and D155) and one Arg (R208) in VdPelB, are conserved, while D151 can be mutated to Glu,

344 or Arg in PNLs (**Fig. 2C and D**, Mayans et al., 1997). Other conserved aa in VdPelB include
345 K175 and R213, with K175 being responsible for binding the carboxyl oxygen while R213
346 hydrogen bonds to C-2 and C-3 of GalA (**Fig. 2D**) [29,61]. Mutating any of these aa leads to
347 decreased enzyme activity [62]. In VdPelB, Ca²⁺ ion is directly coordinated by D122, two
348 carboxyl oxygen, D151, D155 and two water molecules (W568 and W830, **Fig. 2C**). In
349 addition, mutation of D122T (VdPelB numbering) in BsPelA, is responsible for reduced affinity
350 for Ca²⁺ [41]. In the catalytic mechanism, Ca²⁺ is directly involved in acidification of the proton
351 absorption from C5 and elimination of group from C4, generating an unsaturated product. R208
352 act as a base, similarly to the hydrolysis in the reaction mechanism of the GH28 family [63,64].

353 **3.5. Structural analysis of VdPelB suggests a PL activity mediated by peculiar** 354 **specificities**

355 The VdPelB binding groove comprises a number of basic and acidic aa including K147,
356 D151, K175 L178, H181, S207, R208, R213 and R235 and D259 (**Fig. 2C and D**), that have
357 previously been shown to be characteristics of PLs. This would suggest an enzyme activity on
358 low DM pectins as aa positioned at the binding groove were indeed shown to differ between
359 PNL and PL [15,21,24,29]. These aa are indeed mutated in Arg, Trp, Tyr, Gln and Gly in PNLs,
360 which, by reducing their charge, would favour higher affinity for highly methylesterified
361 pectins [15,29]. In VdPelB, as the T3 loop is missing, there are no equivalent to PNL specific
362 W66, W81, W85, W151 aa (**Fig. S2B**, AnPelA numbering) and, moreover, W212 and W215
363 are replaced by K175 and L178 in VdPelB. In BsPelA and DdPelC, these aa are replaced by
364 K177/K190 and L180/L193, highlighting the high conservation of amino acids in
365 VdPelB/BsPelA/DdPelC and subsequently in PLs. When a DP4 ligand from DdPelC crystal
366 structure is superimposed to VdPelB, hydrogen bonds and van der Waals interactions are visible
367 with the above-mentioned aa (**Fig. S6**) [15,21,29,41].

368 Interestingly, despite this rather conserved PL-related binding groove, VdPelB harbors,
369 in the vicinity of the active site, G125 and T157 that are not present in the well characterized
370 DdPelC [60]. At these positions, DdPelC, which was shown to be a *bona fide* PL with a high
371 activity on polygalacturonic acid and alkaline pH with Ca²⁺-dependency, harbours Arg and Lys
372 [15,61,65]. In that respect, the presence of G125 and T157 in VdPelB is similar to that identified
373 in *Bacillus sp.* Pel-22, Pel-66, and Pel-90 and *Bacillus sp.* which showed activity on both PGA
374 and high methylesterified pectins (**Fig. 2B and D**) [22,66,67]. The DdPelC aa being overall
375 positively charged, could explain the binding preference towards non-methylesterified,
376 negatively charged substrates in the vicinity of the active site. In contrast, considering the size

377 of Gly and Thr, they would sterically accommodate the increased size of methylesterified
378 substrate. G125 and T157 could therefore account for a potential dual activity of VdPelB.
379 Moreover, in previously characterized PLs and PNLs there is the presence of a small aa, Ser or
380 Ala that replace H181. While H181 interacts directly with the substrate these aa do not provide
381 this interaction instead the primary Ca^{2+} in the active site induce a substrate conformation that
382 could be recognized by PLs [41]. Finally, L178 is positioned in-between the catalytic Ca^{2+} and
383 R208 and is involved in substrate binding making it a perfect candidate to assess its importance
384 (**Fig. 2D**).

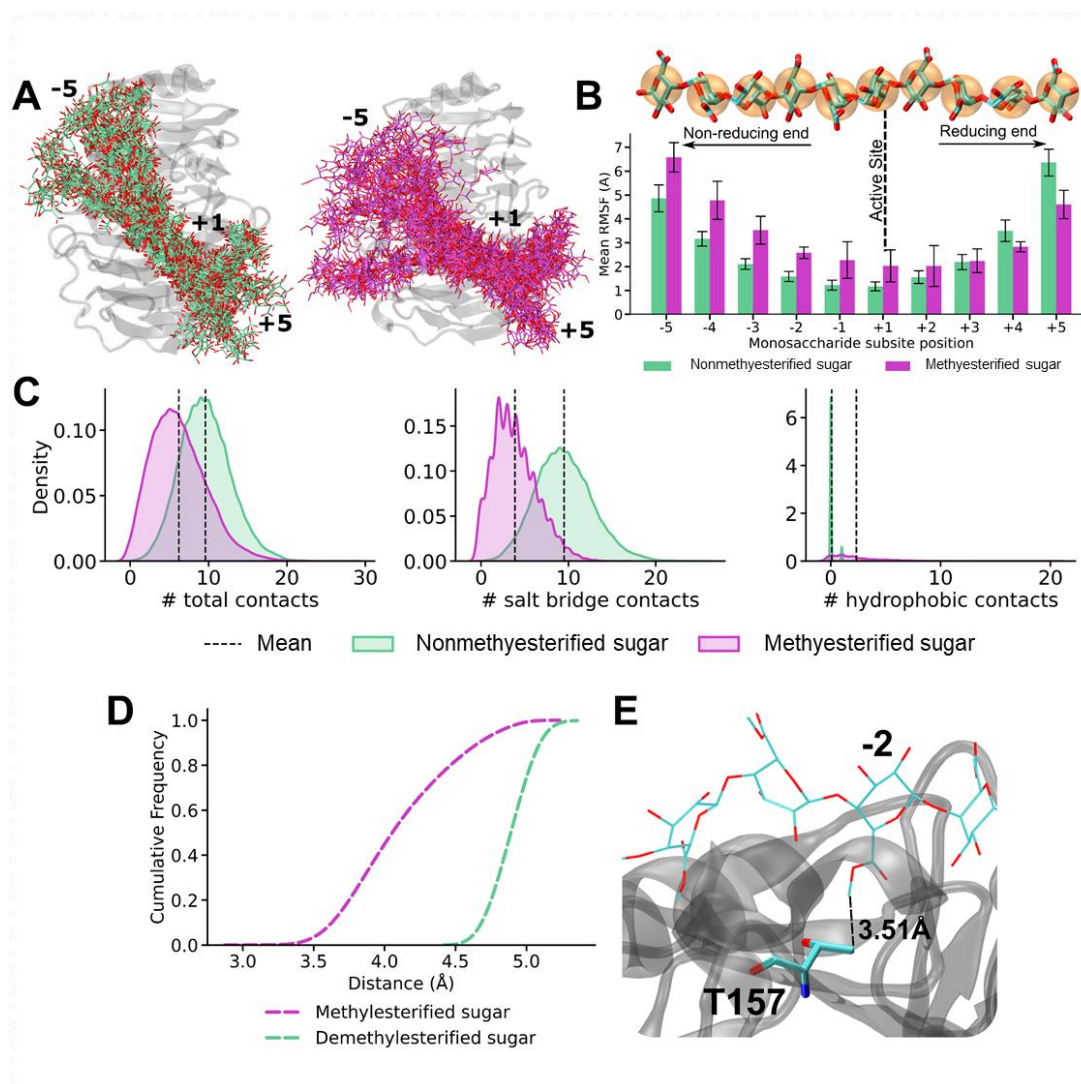
385 **3.6. Molecular dynamic simulations show higher dynamics of VdPelB in complex with** 386 **methylesterified substrates**

387 To determine how the structure of VdPelB and the observed differences in amino-acidic
388 composition might influence the affinity with differently methylated substrates, we performed
389 MD simulations on VdPelB in complex with either a non-methylesterified or fully
390 methylesterified deca-saccharides, which are able to occupy the entire binding groove (**Fig. 3**).
391 MD simulations show substantially differential dynamic profiles for oligosaccharides with and
392 without methylesterification. Expectedly, the dynamics is lowest in proximity of the catalytic
393 subsite (+1) and increases consistently towards both the reducing and non-reducing ends of the
394 substrate: with the highest dynamics found at the non-reducing end (**Fig. 3A**).

395 A quantitative estimation of the substrate dynamics was obtained by monitoring the root
396 square mean fluctuations (RMSF) of each sugar residue and shows that a polygalacturonate
397 substrate associates more stably in the subsites of the binding groove placed towards the sugar's
398 reducing end (subsites -1 to -5), with differences between methylesterified and non-
399 methylesterified substrates approaching the obtained standard deviation. In some cases, the
400 dynamics reversed for non-methylesterified sugars, with a higher RMSF for de-methylesterified
401 sugars towards the saccharide's reducing end (**Fig. 3B**). Moreover, and in line with an observed
402 positively charged binding groove, non-methylesterified substrates retain a higher number of
403 contacts than methylesterified sugars, with salt-bridges contributing the most to the observed
404 differences (**Fig. 3C**).

405 We then additionally and specifically focused on the analysis of the interactions made by
406 T157, which is nested with the -2 subsite of the binding groove. Simulations sample consistently
407 higher contacts formation between a methylesterified sugar docked in subsite -2 and T157, with
408 distances shifted to lower values when compared to a non-methylesterified sugar (**Fig. 3D**),

409 even the RMSF of non-methylesterified monosaccharides docked in subsite -2 experience on
 410 average, significantly lower dynamics. Altogether, the formation of a larger number of contacts
 411 between methylesterified saccharides docked in the -2 subsite suggests an active role of T157
 412 in the binding of methylesterified chains: with the butanoic moiety of T157 engaging in
 413 hydrophobic interactions with the methyl-ester presented by methylesterified sugar units (**Fig.**
 414 **3E**). While T157 is seen to have an active role in engaging with the substrate's hydrophobic
 415 moieties, an active role of G125, also in the same subsite, was not observed but it is plausible
 416 that the minimal size of G125 would increase the accommodability of methylesterified sugars
 417 in that position.



418

419 **Fig. 3. VdPelB substrate dynamics in complex with fully de-methylesterified and methylesterified complex**

420 A) Ensembles of non-methylesterified (green, left panel) and methylesterified (pink, right panel) HG

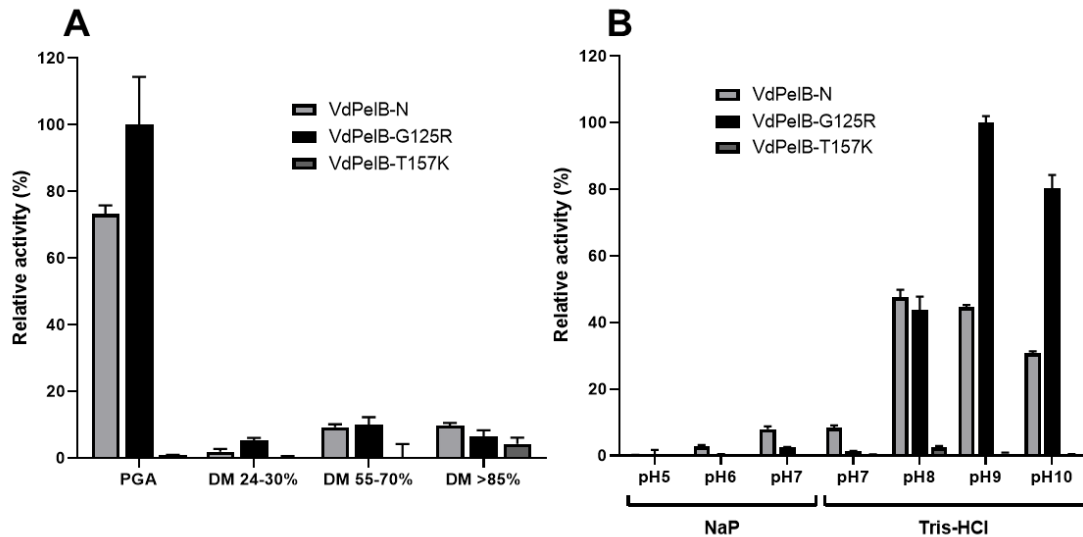
421 deca-saccharide at every 100 frames for the simulated VdPelB complexes. Substrate within the enzymes' binding

422 grooves are labelled from -5 (HG-non-reducing end) to +5 (HG-reducing end) B) RMSF of non-methylesterified

423 (green) and methylesterified (pink) HG bound across the binding groove of VdPelB. Numbers indicate the beta
424 sheet position from the active site. C) Analysis of the contacts between VdPelB and non-methylesterified (green)
425 and methylesterified substrate (pink). D) Distance between T157K residue and the substrate residues plotted as
426 cumulative frequency. E) T157 hydrophobic interaction with methyl-ester of methylesterified substrate.

427 **3.7. Biochemical characterization of VdPelB**

428 We first determined the activity of VdPelB by following the release of 4,5-unsaturated
429 bonds which can be detected at 235 nm using UV spectrophotometer. VdPelB activity was first
430 tested for its dependency towards Ca^{2+} . Using a standard PL assay, with PGA as a substrate, an
431 increase in the VdPelB activity was measured in presence of calcium. In contrast in presence
432 of EDTA, used as a chelating agent, no activity was detected, confirming the calcium-dependent
433 activity of the enzyme (**Fig. S7A**) [15]. Activity measured in absence of added CaCl_2 reflects
434 the presence of calcium from the culture media that is bound to VdPelB during production, and
435 previously identified in the 3D structure. We tested the effects of increasing CaCl_2
436 concentrations and showed that the maximum activity was already reached when using as low
437 as $0.125 \mu\text{M}$ (**Fig. S7B**). To test the substrate-dependence of VdPelB, four substrates of
438 increasing degrees of methyl-esterification were used. VdPelB showed the highest activity on
439 PGA, with less than 10% of the maximum activity measured on the three others substrates (**Fig.**
440 **4A**). This shows that, as inferred from above-mentioned structural and dynamical data, VdPelB
441 act mainly as a PL although it can still show residual activity on high DM pectins. Considering
442 this, PGA was used as substrate to test the pH-dependence of the enzyme's activity in sodium
443 acetate and Tris-HCl buffers (**Fig. 4A**). VdPelB was most active at pH 8, with only a slight
444 decrease in activity at pH 9 (93%). In contrast, the relative activity at pH 5-7 was close to null.
445 The pH optimum for VdPelB was the same as *B. fuckeliana* Pel (pH 8) [68], close to that
446 reported for *D. dadantii* PelN (pH 7.4) [69], but was lower to that measured for *B. clausii* Pel
447 (pH 10.5) [70]. In contrast, the pH optimum was higher compared to five PNLs from
448 *Aspergillus* sp. AaPelA (pH 6.1), AtPelA (pH 4.5), AtPelA (pH 6.4), AtPelD (pH 4.3) [18] and
449 *A. parasiticus* Pel (pH 4) [19]. The optimum temperature assay showed that VdPelB was most
450 active at 35°C (**Fig. S8**). VdPelB appeared less heat-tolerant as compared to thermophilic PLLs
451 reported from *Bacillus* sp. RN1 90°C [71], *B. clausii* Pel, 70°C [70], *B. subtilis* Pel168, 50°C
452 [72]. However, its optimum temperature is in the range of that measured for *X. campestris* Pel
453 [73] and cold-active Pel11 from *M. eurypsychrophila* [74]. The lack of disulphide bridges
454 previously shown in the structure could be responsible for the lower stability of the enzyme at
455 high temperatures, in comparison with previously characterized PLs [75].



456

457 **Fig. 4. Biochemical characterization of VdPelB**

458 A) Substrate-dependence of VdPelB-N, G125R and T157K. The activities were measured after 12 min of
459 incubation with PGA, pectins DM 24-30%, DM 55-70%, DM>85% with addition of Ca²⁺ at 35°C. B) pH-
460 dependence of VdPelB-N, G125R and T157K activity. The activities were measured after 12 min of incubation
461 with PGA in sodium phosphate (NaP) and Tris-HCl buffer at 35°C. Values correspond to means ± SE of three
462 replicates.

463 3.8. Mutation of specific amino-acids affects VdPelB activity

464 Considering the fine structure of VdPelB structural we generated mutated forms of the
465 enzymes for five amino acids that likely to be involved in the catalytic mechanism and/or
466 substrate binding: G125R, D151R, T157K, L178K and H181A. Enzymes were produced in *P.*
467 *pastoris* and purified (**Fig. S1B**). If the importance of some of these aa (i.e. D151 and H181) in
468 the catalytic mechanism was previously shown for others PL [41,76], our study highlights the
469 key role of some novel aa in the catalytic mechanism. The activities of mutants were tested at
470 iso-quantities of wild-type enzyme. Surprisingly, the G125R mutant was 25% more active on
471 PGA compared to the native enzyme and a shift in the optimum pH was observed (**Fig. 4A**).
472 While native enzyme was most active at pH8 with slight decrease in activity at pH9, the activity
473 of G125R mutant was increased by circa 50% at pH9 and pH10 (**Fig. 4B**). Both enzymes kept
474 the same activity at pH8. The substitution of Gly with Arg, present in a number of previously
475 characterized PL could facilitate the interaction with the substrate and basification of the active
476 site thanks to its physio-chemical properties [60]. The activity of T157K was closed to null
477 when tested on PGA and different pHs. The introduction of a Lys, a chemically different and
478 larger aa is likely to introduce steric clashes notably with H181 and L178 that are important for
479 the activity. The H181A mutant lost much of its activity as there are less interaction/recognition

480 with the substrate. No activity for D151R and L178K mutants were observed in line with the
481 fact that D151 is an active site aa that binds the Ca^{2+} . Mutation of this amino-acid is negatively
482 impairing the functioning of the enzyme (**Fig. S7A and B**) [41,76]. We can hypothesize that
483 L178K mutation positioned in between Ca^{2+} , R208 and the substrate, induces specific substrate
484 conformation that diminishes the direct interaction between the enzyme catalytic centre and the
485 substrate, which translates to loss of activity (**Fig. S9**).

486 **3.9. Identification of the OGs released by VdPelB from commercial and cell wall** 487 **pectins**

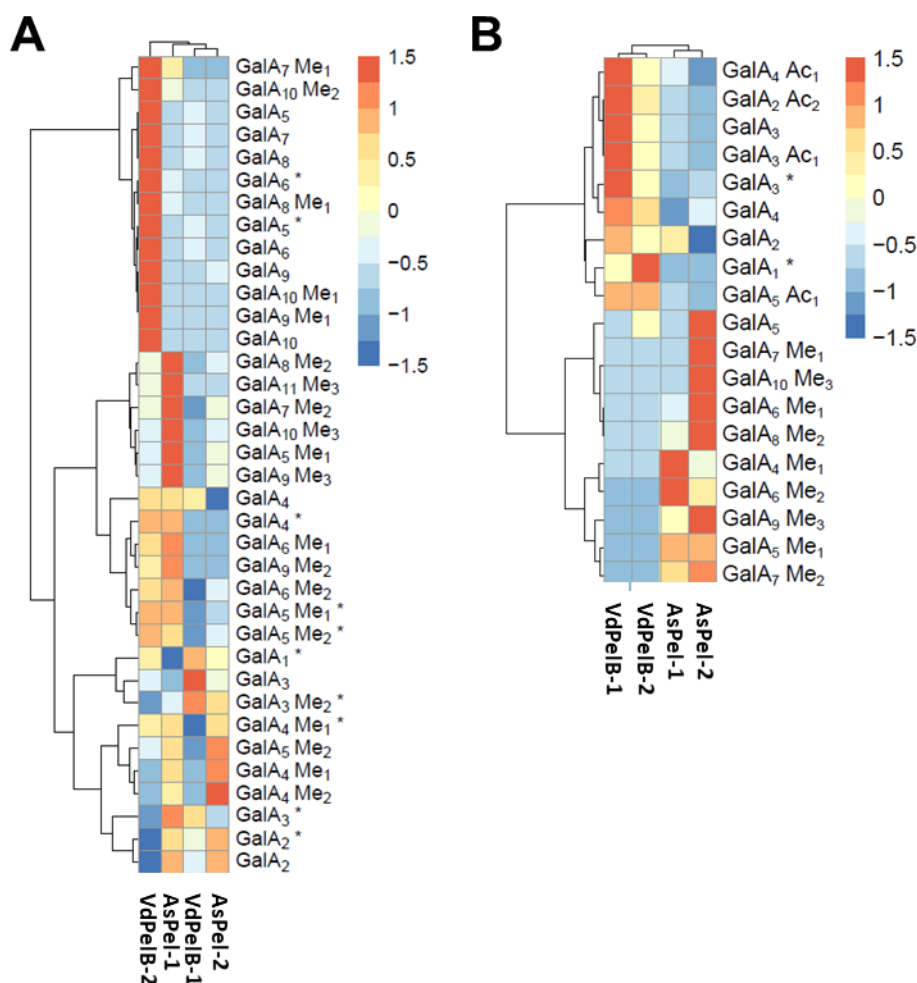
488 To further understand the specificity of VdPelB on different substrates, we performed
489 LC-ESI-MS/MS to determine the profiles of digestion products (OGs) and to compare with that
490 of commercially available *Aspergillus sp.* Pel (AsPel, **Fig. S10**). To be fully comparable,
491 digestions were realized, for each substrate, at iso-activities for the two enzymes. On the basis
492 of digestion profiles, we identified 48 OGs and created a dedicated library that was used for
493 identification and integration of peaks (**Table 2**). MS² fragmentation allowed determining the
494 structure of some of the OGs (**Fig. S11 and S12**). The OGs released by either of the enzymes
495 were mainly corresponded to 4,5-unsaturated OGs, which is in accordance with β -eliminating
496 action of PLLs. When using pectins DM 20-34% and at low enzyme's concentration (VdPelB-
497 2), VdPelB mainly released non-methylesterified OGs of high DP (GalA₅, GalA₆, GalA₇,
498 GalA₈, GalA₉, GalA₁₀) that were subsequently hydrolysed when using more concentrated
499 VdPelB (VdPelB-1, **Fig. 5A**). These digestion products strikingly differed to that generated by
500 AsPel, that are methylesterified OGs of higher DP (GalA₄Me₁, GalA₄Me₂, GalA₅Me₁,
501 GalA₆Me₂, GalA₁₁Me₃...), thus showing distinct enzymatic specificities. Altogether, these first
502 results unequivocally shows VdPelB act as an endo-PL, and that its processivity differ to that
503 of AsPel, which act as well as an endo-PL [9]. When using sugar beet pectins, that are known
504 to be highly acetylated (DM 42%, DA 31%), VdPelB released acetylated OGs (GalA₂Ac₂,
505 GalA₃Ac₁, GalA₄Ac₁, GalA₅Ac₁), while AsPel showed much lower activity and relative
506 abundance of these OGs (**Fig. 5B**). Previous reports have shown that differences exist between
507 PNL, in particular with regards to acetyl substitutions [18]. In contrast, AsPelI released mainly
508 methylesterified OGs (GalA₆Me₁, GalA₈Me₁, GalA₁₀Me₃...).

509 **Table 2. List of oligogalacturonides identified by LC-MS/MS analysis.**

510 For each OG, elemental composition, retention time (RT) and ion mass used for the analysis are highlighted. In
511 the case of detection of mono and di-charged OGs, the *italicized* and **BOLD** mass, corresponding to the more
512 intense ion, were used for the quantification. * indicates non-unsaturated OGs.

Name	Elemental composition	RT (min)	[M-H]-	[M-2H]2-
GalA1*	C6H10O7	9.3	193.0353763	
GalA1Ac2	C10H12O8	9.1	259.045941	
GalA2*	C12H18O13	8.88	369.0674644	
GalA2	C12H16O12	8.95	351.0568997	
GalA2Ac2	C16H20O14	8.67	435.0780291	
GalA2Me1*	C13H20O13	8.85	383.0831145	
GalA2Me1	C13H18O12	8.92	365.0725498	
GalA3*	C18H26O19	8.56	545.0995525	
GalA3	C18H24O18	8.61	527.0889878	
GalA3Ac1	C20H26O19	8.59	569.0995525	
GalA3Me1	C19H26O18	8.56	541.1046379	
GalA3Me2*	C20H30O19	8.62	573.1308527	
GalA4*	C24H34O25	8.27	721.1316406	
GalA4	C24H32O24	8.33	703.1210759	351.0568997
GalA4Ac1	C26H34O25	8.31	745.1316406	
GalA4Me1*	C25H36O25	8.37	735.1472907	
GalA4Me1	C25H34O24	8.32	717.136726	358.0647248
GalA4Me2	C26H36O24	8.3	731.1523761	
GalA5*	C30H42O31	8.04	897.1637287	
GalA5	C30H40O30	8.09	879.153164	439.0729438
GalA5Ac1	C32H42O31	8.09	921.1637287	
GalA5Me1*	C31H44O31	8.11	911.1793788	455.0860512
GalA5Me1	C31H42O30	8.13	893.1688141	446.0807688
GalA5Me2*	C32H46O31	8.13	925.1950289	
GalA5Me2	C32H44O30	8.03	907.1844642	453.0885939
GalA6*	C36H50O37	7.84		536.0942702
GalA6	C36H48O36	7.82	1055.185252	527.0889878
GalA6Me1	C37H50O36	7.92	1069.200902	534.0968129
GalA6Me2	C38H52O36	7.83	1083.216552	541.1046379
GalA7*	C42H58O43	7.65		624.1103142
GalA7	C42H56O42	7.62	1231.21734	615.1050318
GalA7Me1	C43H58O42	7.72	1245.23299	622.1128569
GalA7Me2*	C44H62O43	7.75		638.1259643
GalA7Me2	C44H60O42	7.72	1259.24864	629.1206819
GalA8	C48H64O48	7.45		703.1210759
GalA8Me1*	C49H68O49	7.53		719.1341833
GalA8Me1	C49H66O48	7.52	1421.265078	710.1289009
GalA8Me2*	C50H70O49	7.55		726.1420083
GalA8Me2	C50H68O48	7.58	1435.280728	717.136726

Name	Elemental composition	RT (min)	[M-H]-	[M-2H]2-
GalA9*	C54H74O55	7.29		800.1424023
GalA9	C54H72O54	7.3		791.1371199
GalA9Me1	C55H74O54	7.34		798.144945
GalA9Me2	C56H76O54	7.37		805.15277
GalA9Me2	C56H76O54	7.41		805.15277
GalA9Me3	C57H78O54	7.45		812.1605951
GalA10	C60H80O60	7.15		879.153164
GalA10Ac1	C62H82O61	7.29		900.1584463
GalA10Me1	C61H82O60	7.19		886.160989
GalA10Me2*	C62H86O61	7.24		902.1740964
GalA10Me2	C62H84O60	7.24		893.1688141
GalA10Me3	C63H86O60	7.3		900.1766391
GalA11	C66H88O66	7.03		967.169208
GalA11Ac1	C68H90O67	7.14		988.1744904
GalA11Me1	C67H90O66	7.06		974.1770331
GalA11Me2	C68H92O66	7.09		981.1848581
GalA11Me3	C69H94O66	7.14		988.1926832
GalA12Me2	C74H100O72	6.96		1069.200902
GalA12Me3	C75H102O72	6.99		1076.208727
GalA12Me4	C76H104O72	7.0419		1083.216552
GalA13Me2	C80H108O78	6.82		1157.216946
GalA13Me3	C81H110O78	6.86		1164.224771
GalA13Me4	C82H112O78	6.9		1171.232596
GalA14Me3	C87H118O84	6.73		1252.240815
GalA14Me4	C88H120O84	6.77		1259.24864
GalA15Me5	C95H130O90	6.69		1354.272509



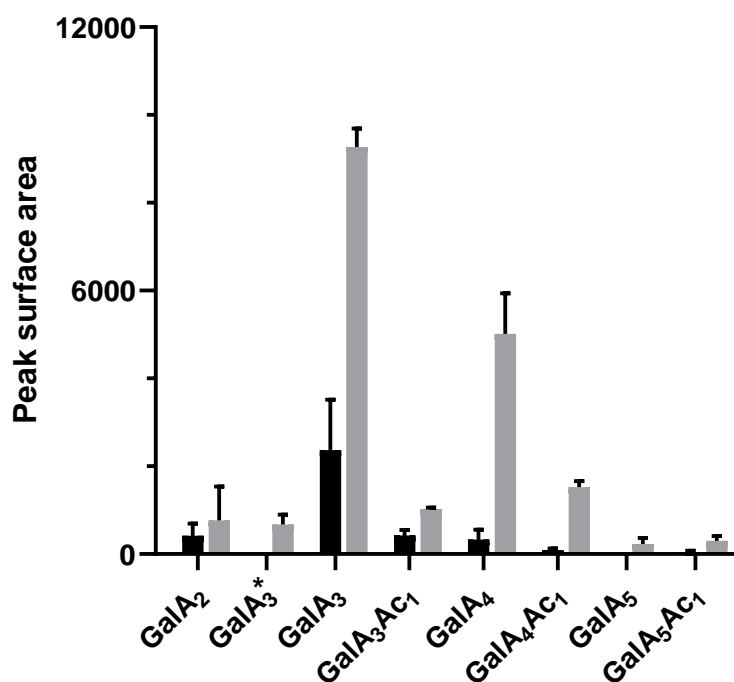
515

516 **Fig. 5. Analysis of OGs produced by the action of VdPelB and AsPeI on pectins of various degrees of**
 517 **methylesterification and acetylation**

518 OGs were separated by SEC and analysed by MS/MS. A) Pectins DM 24-30%. B) Sugar beet pectins (DM 42%
 519 DA 31%). Substrates were digested overnight at 40°C and pH 8 using isoactivities of VdPelB and AsPeI. Enzyme
 520 concentrations are stated in Table S2. Subscript numbers indicate the DP, DM and DA. * indicate non-unsaturated
 521 OGs. Values correspond to means of three replicates.

522 *V. dahliae* and closely related fungus from the same genus are known flax pathogens,
 523 where they use their enzymatic arsenal, that includes pectin degrading enzymes, for penetrating
 524 the host cell leading to infection [30]. To assess the potential role of VdPelB in flax
 525 pathogenicity we digested root cell walls from two flax cultivars, Evea (Verticillium-partially
 526 resistant), and Violin (Verticillium-susceptible), and we compared the OGs released. On root
 527 cell walls, VdPelB released mainly unsaturated OGs up to DP5 (**Fig. 6**). From similar starting
 528 root material, the OG total peak area detected was five times lower for Evea compared to Violin,
 529 suggesting that it is less susceptible to digestion by VdPelB. OGs released by VdPelB were
 530 mainly non-methylesterified but could be acetylated (GalA₂, GalA₃, GalA₃Ac₁, GalA₄,

531 GalA₄Ac₁ GalA₅, GalA₅Ac₁), and the abundance of GalA₃ and GalA₄ was five and fifteen times
532 higher in Violin, respectively. These data together with that obtained from sugar beet pectins
533 strongly suggests that VdPelB preference is for non-methylesterified and acetylated substrates.
534 Our data suggest that cell wall structure differ between the two cultivars and that VdPelB could
535 determine *Verticillium* pathogenicity thanks to a better degradation of the cell wall pectins of
536 sensitive cultivars [35]. Similarly, VdPel1 was previously identified as virulence factor, where
537 the deletion of this gene decreased virulence in tobacco, as compared with the wild-type
538 *Verticillium* [33].



539

540 **Fig. 6. Analysis of OGs released by VdPelB from flax roots.**

541 VdPelB was incubated overnight with roots from Évéa (spring flax, partially resistant to *Verticillium* wilt, black)
542 and Violin (winter flax, more susceptible to *Verticillium* wilt, grey). Values correspond to means \pm SE of three
543 replicates. Subscript numbers indicate the DP, DM and DA.

544

545 **4. Conclusion**

546 We have fully characterized, by multidisciplinary approaches, a novel pectinolytic
547 enzyme from *V.dahliae*, VdPelB, that belongs to the PLL family. The protein was crystallised
548 and its 3D structure determined at a high resolution. VdPelB showed a conserved structure,

549 with typical topology for PLs and the active site harboured three conserved Asp coordinating
550 Ca^{2+} and Arg involved in the β -elimination mechanism. The binding groove of VdPelB showed
551 conserved aa that are characteristic of PLs, with MD simulations confirming the lower
552 dynamics/higher affinity of the enzyme towards non-methylesterified pectins. As inferred from
553 structural and dynamical analyses, VdPelB showed high activity on non-methylesterified
554 substrates, with a maximum activity at pH 8 and 35°C. The analysis of the structure led the
555 identification, in the VdPelB, of peculiar aa that are normally present in PNL. In particular,
556 G125R mutant showed increased activity on PGA and switch in pH optimum from 8 to 9. The
557 analysis of the digestion products of showed that VdPelB act as an endo enzyme and that it can
558 release a large diversity of OGs with a preference for non-methylesterified and acetylated
559 products. The OGs generated by VdPelB from *Verticillium*-partially tolerant and *Verticillium*-
560 sensitive flax cultivars showed that the enzyme could be a determinant of pathogenicity, as a
561 function of pectins' structure.

562

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571 **Author's contribution**

572 **Josip Safran**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology,
573 Writing - original draft. **Vanessa Ung**: Data curation, Investigation, Methodology. **Julie**
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579 Conceptualization, Methodology. **Davide Mercadante**: Data curation, Methodology, Writing
580 - review & editing. **Jérôme Pelloux**: Funding acquisition, Conceptualization, Writing - review
581 & editing. **Fabien Sénéchal**: Conceptualization, Writing - review & editing.

582 **Conflicts of interest**

583 There are no conflicts of interest.

584

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- 845

Supplementary materials caption:

Table S1. Primers for cloning mutated forms of VdPelB into pPICZαB expression vectors

Restriction enzymes sites for PstI and NotI are underlined added bases are written in *italics*. Mutation bases are **bolded**.

Table S1. VdPelB and AsPel concentrations used for pectin degradation and OGs analysis.

The different enzymes concentrations were used to have enzymes at iso-activities.

Fig. S1. Purification and SDS-PAGE analysis of wild-type and mutated forms of VdPelB

Wild-type (A) and mutated forms (B) of VdPelB were purified using 1 mL Ni NTA colon. Proteins were resolved on a 12% polyacrylamide gel and were stained by Coomassie blue. L-ladder.

Fig. S2. VdPelB crystal packing and structural alignment

A) Ribbon diagram of two molecules of VdPelB crystalized in P1 21 1 space group. B) Structural alignment of VdPelB with BsPelA, DdPelC, AnPelA. VdPelB shares highest structural alignment with BsPelA (PDB: 3VMV, orange) with 30.06% sequence identity and rmsd of 1.202 Å. Second best alignment was with DdPelC (PDB: 1AIR, plum) with 24.20% identity and rmsd of 1.453 Å. Pectin lyase with T3 loop described in AnPelA (PDB: 1IDJ, green).

Fig. S3. Average B-factors and occupancies of VdPelB

A) VdPelB chain A and B colored by B-factors. B) VdPelB chain A and B colored by occupancies.

Fig. S4. β-sheets and T-turns structures of VdPelB

A) Ribbon structure representing β-sheets (PB1-purple, PB2-yellow and PB3-red) B) Ribbon structure representing T-turns for VdPelB (T1-lime green, T2- orange red, T3 medium purple. β-strands and T-turns are named accordingly to Petersen et al. 1997.

Fig. S5. Primary sequence alignment of VdPelB with BsPelA, BsPelA-P15, DdPelC, AnPelA, AnPelB

VdPelB primary sequence alignment with *Dickeya dadanti* DdPelC (P11073), *Bacillus* Sp. KSM-P15 BsPel-P15 (Q9RHW0), *Bacillus* sp. N16-5 BsPelA (D0VP31), *Aspergillus niger* AnPelA (Q01172), AnPelB (Q00205). Conserved AA are red boxed.

Fig. S6. Superimposed tetramer ligand from DdPelC to VdPelB structure

Tetramer ligand from DdPelC (PDB: 2EWE, gray) superimposed to VdPelB structure. Aa involved in interaction are yellow-coloured. Hydrogen bonds and Van der Waals contacts are coloured black and blue.

Fig. S7. Effects of Ca²⁺ on VdPelB activity

A) Calcium-dependence of VdPelB activity. The activities were measured after 12 min of incubation with PGA, with/without addition of Ca²⁺ or Ca²⁺ and EDTA, at 35°C B) PGA digested using VdPelB for 12 min at 40°C and pH 8 with increasing concentrations of Ca²⁺.

Fig. S8. Temperature-dependence of VdPelB-N activity

The activities were measured after 12 min of incubation with PGA at pH 8. Values correspond to means of three replicates.

Fig. S9. VdPelB mutants activity on PGA

The activities were measured after 12 min of incubation with PGA with addition of Ca²⁺ at 35°C. Values correspond to means of three replicates ± SD.

Fig. S10. Substrate-dependence of AsPel

The activities were measured after 12 min of incubation with PGA, pectins DM 24-30%, DM 55-70%, with/without addition of Ca²⁺ or Ca²⁺ and EDTA, at 35°C. Values correspond to means of three replicates ± SD.

Fig. S11 : Example of MS² fragmentation pattern of GalA₆Me₁-H₂O

MS² fragmentation pattern of GalA₆-H₂O oligomer (m/z 1055,185252, 527,0889878) produced by VdPelB from pectin DM >85%. Subscript numbers indicate the degree of polymerization.

Fig. S12 : Example of MS² fragmentation pattern of GalA₄Me₁

MS² fragmentation pattern of GalA₄Me₁ oligomer (m/z 735,1472907) produced by VdPelB from pectin DM >85%. Subscript numbers indicate the degree of polymerization.

Enzyme	Forward 5' – 3'	Reverse 3'-5'
VdPelB-Native	TCTAA <u>CTGCAGG</u> AACGCCCACTCCCACC	TGCAC <u>GCGGCCGCG</u> GAAGCCAAGGGTCTGGC
VdPelB-G125R	GCCATT CG TATCCAGGCCTCTAAGAACG	CGTTCTTAGAGGCCTG GATA ACG AATGGC
VdPelB-D151R	GGCAAGGACTACTAT AG AGGTCTCCTCG	CGAGGAGACCT CT TATAGTAGTCCTTGCC
VdPelB-T157K	CGATATCA AG CACGGCTCCG	CGGAGCCGTG CTT GATATCG
VdPelB-L178Y	GGCCTCGT AT ATCGGCCACACC	GGTGTGGCCGAT ATA CGAGGCC
VdPelB-H181A	GCTTATCGGC GCC ACCGACTCG	CGAGTCGGT GGC GCCGATAAGC

Table S1. Primers for cloning mutated forms of VdPelB into pPIC α B expression vectors

Restriction enzymes sites for PstI and NotI are underlined added bases are written in *italics*. Mutation bases are **bolded**.

Name	VdPeIB*		AsPel**	
	1	2	1	2
Concentration				
Sugar beet pectin DM 42% DA 31%	0.077	0.00385	0.7	0.0875
Citrus pectin DM 24-30%.	0.077	0.0385	0.7	0.35

* $\mu\text{g}/\mu\text{L}$, ** U/mL final concentration

Table S2. VdPeIB and AsPel concentration used for pectin degradation and OGs analysis.
The different enzymes concentrations were used to have enzymes at iso-activities.

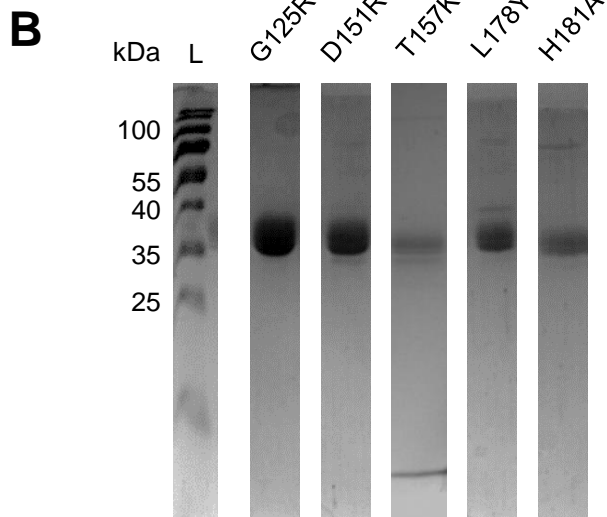
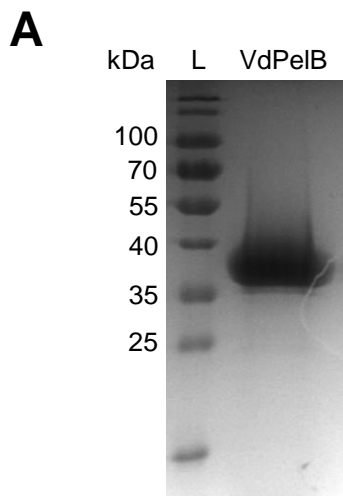


Fig. S1. Purification and SDS-PAGE analysis of wild-type and mutated forms of VdPelB

Wild-type (A) and mutated forms (B) of VdPelB were purified using 1 mL Ni NTA colon. Proteins were resolved on a 12% polyacrylamide gel (SDS-PAGE) and were stained by Coomassie blue. L-ladder. The figure of SDS-PAGE representing mutants (B) was composed of several different images.

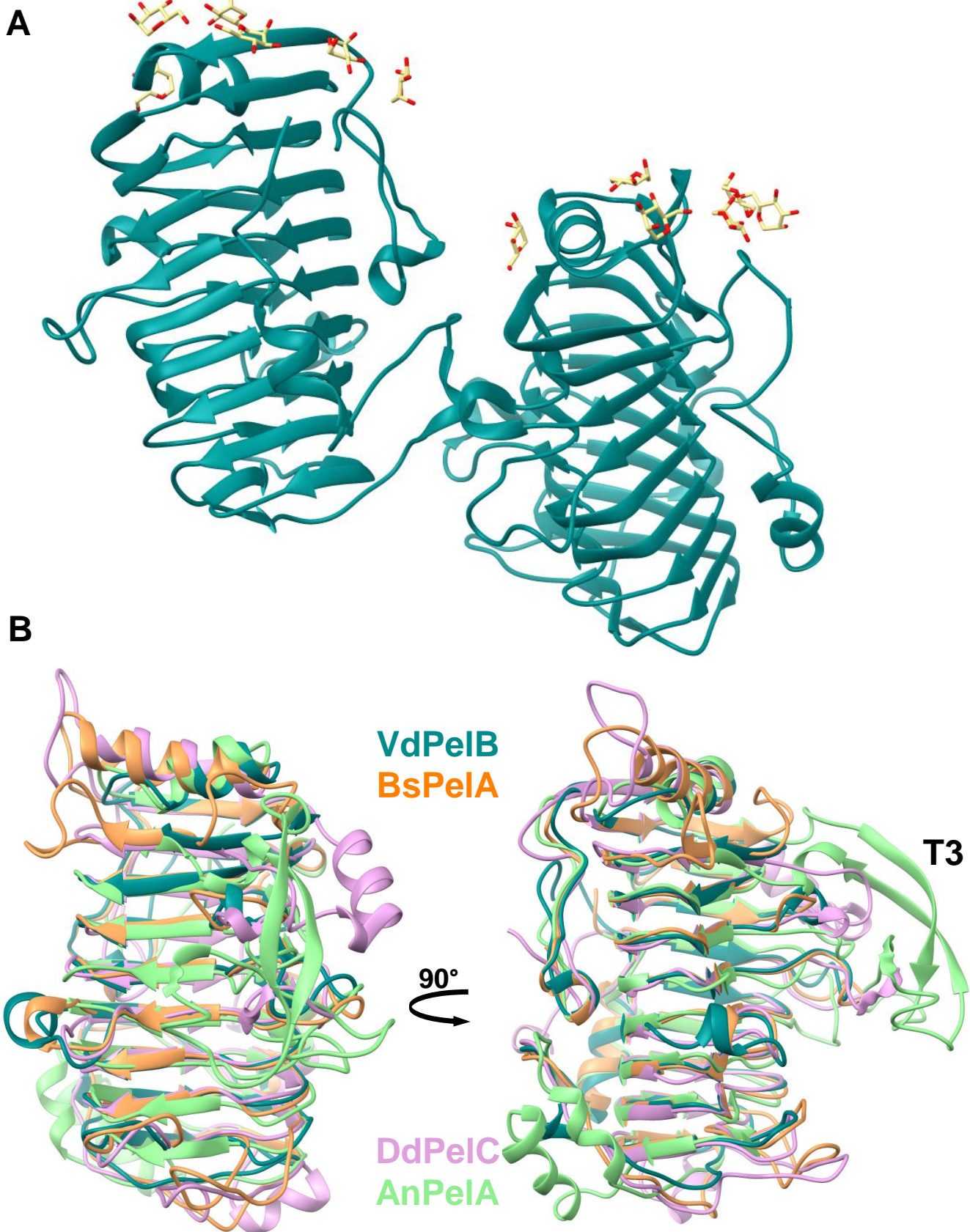


Fig. S2. VdPelB crystal packing and structural alignment

A) Ribbon diagram of two molecules of VdPelB crystalized in P1 21 1 space group. **B)** Structural alignment of VdPelB with BsPelA, DdPelC, AnPelA. VdPelB shares highest structural alignment with BsPelA (PDB: 3VMV, orange) with 30.06% sequence identity and rmsd of 1.202 Å. Second best alignment was with DdPelC (PDB: 1AIR, plum) with 24.20% identity and rmsd of 1.453 Å. Pectin lyase with T3 loop described in AnPelA (PDB: 1IDJ, green).

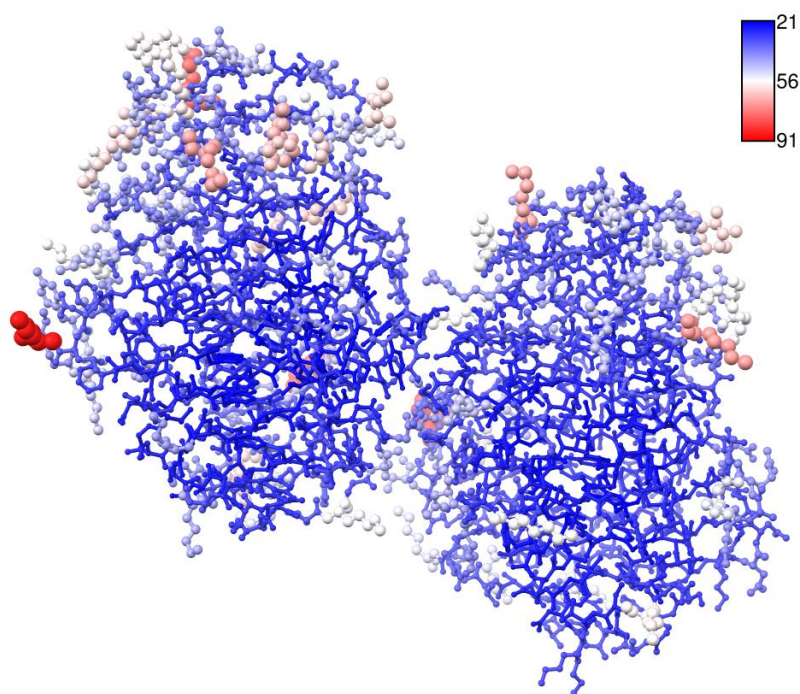
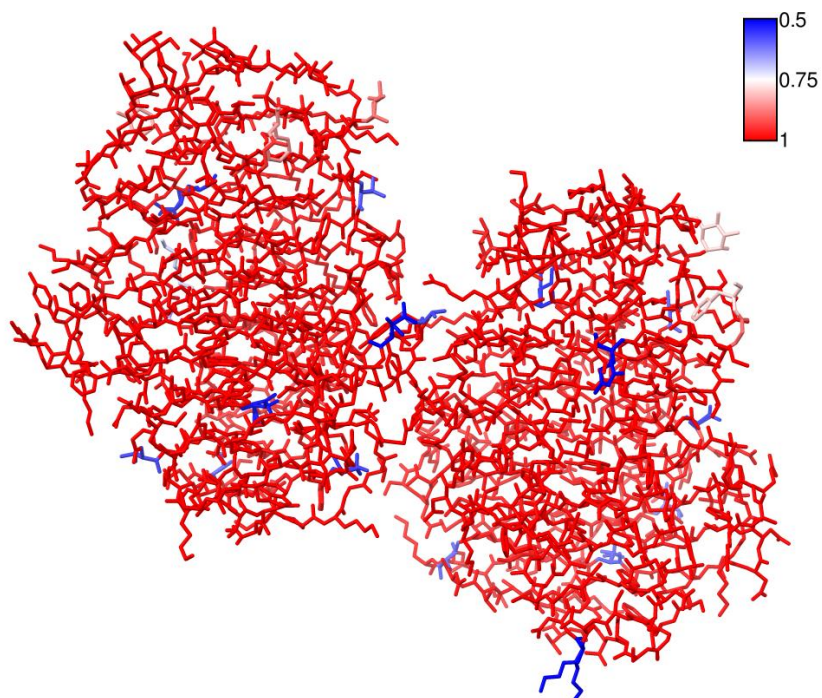
A**B**

Fig. S3. Average B-factors and occupancies of VdPelB

A) VdPelB chain A and B colored by B-factors. **B)** VdPelB chain A and B colored by occupancies.

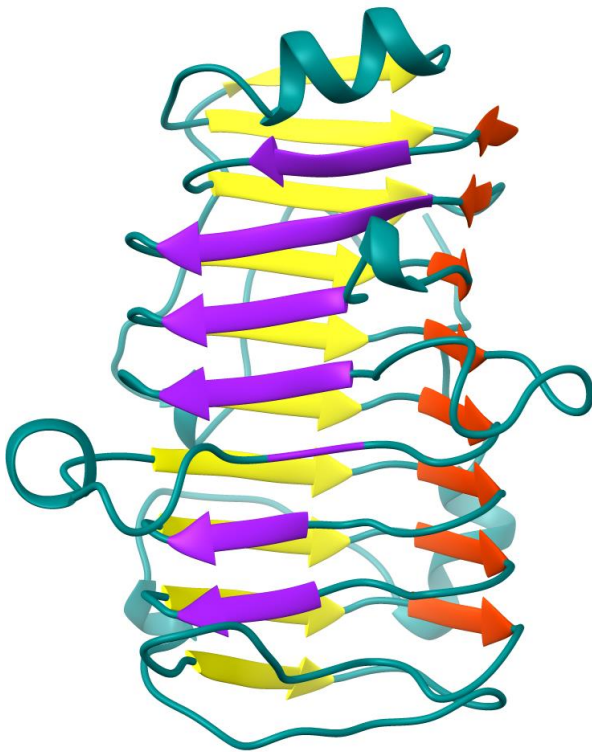
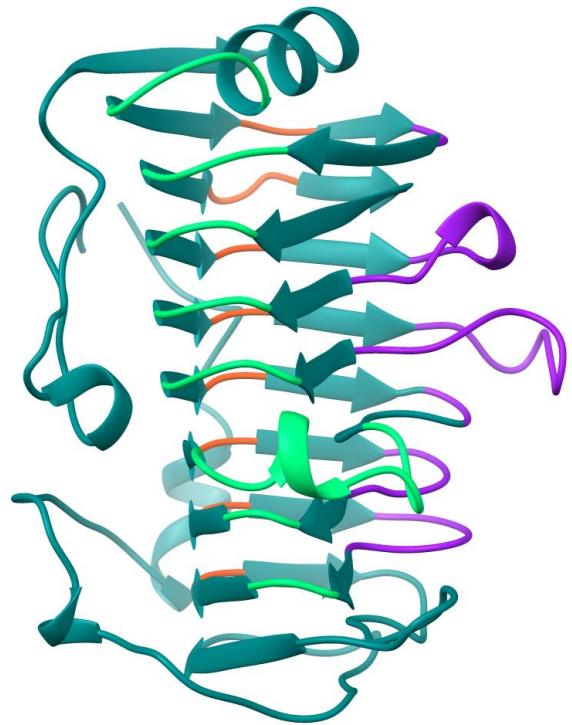
A**B**

Fig. S4. β -sheets and T-turns structures of VdPelB

A) Ribbon structure representing β -sheets (PB1-purple, PB2-yellow and PB3-red) B) Ribbon structure representing T-turns for VdPelB (T1-lime green, T2- orange red, T3 medium purple. β -strands and T-turns are named accordingly to Petersen et al. 1997.

BsPel-P15	-----APTVVHETIRVP-----AGQTFDGGKQTYVANPNTLGDGSQA--	37
AnPe1A	-----VGVSQSAEGFAEGVTGGGDA-TPVYPTDIDELVSYL-	35
AnPe1B	-----AGVVGAAEGFAHGVTGGGSA-SPVYPTTTDELVSYL-	35
DdPe1C	-----ATDTGG-YAATAGGNVTGAVS-KTA--TSMQDVIWIID	34
BsPe1A	-----SNGPQG-YASMNGGTTGGAGG-RVEYASTGAQIQQLID	36
VdPe1B	TPTPTIQEDGSPALIAKRASVTEESCNIQ-YASTNGGTTGGKGG-ATTTVSTLAQFTKAA-	57
	* : : .	
BsPel-P15	-----ENQKPI-FRLEAGASLKN-----VWIGAPAADGVHICYGDCTITNVIW	78
AnPe1A	-----GDDEARVIVLTKTFDFTDSEGTGTTGTGCAPWGTASACQV--AIDQDDW	81
AnPe1B	-----GDNEPRVILDQTFDFTGTEGTETTTGCAPWGTASQCQV--AINLHSW	81
DdPe1C	AARLDANGKKVKGAYPLVITYTGNED-----S-----LINAAAANICGQ-----W	75
BsPe1A	NRS-----RSNNPDEPLTIYVNGTIT-----Q-----GNSPQSLI-----D	67
VdPe1B	-----ESSGKLNIVVKGKIS-----G-----GAKVRVQS-----	81
	: .	
BsPel-P15	EDVGE-----DALTLKSSGTV-NISGGAAYKAYDKVFQINAAGTINIRNF	122
AnPe1A	CENYEPDAPSVSVEYYNAGVLGITVTSNKS LIGEGSSGAIKG-KGLRIVSGAENIIQNI	140
AnPe1B	CDNYQASAPKVSVTYDKAGILPITVNSNKSIVGQGTGKGVIKG-KGLRVVSGAKNVIQNI	140
DdPe1C	SKDPR-----GV-----EIKEFTKGITIIIGANGSSAN-FGIWI-KKSSDVVVQNM	118
BsPe1A	VKNHR----GK-----AHEIKNISIIIGVGTNGEFDG-IGIRL-SMAHNIIQNV	110
VdPe1B	-----DKTIIG-QKGSELVG-TGLYI-NKVKNVIVRNM	111
	: . : . : : *	
BsPel-P15	RADDIGKLVQRNGGTYKVV-----MNVENCNISRVKDAIL--RTDSSTSTGRIVNTRY	175
AnPe1A	AVTDINPKYVW-GGDAITL-DCCDLWIDHVTTARIGRQ-----HYVLTGSAD	186
AnPe1B	AVTDINPKYVW-GGDAITV-DSDSLWIDHVTTARIGRQ-----HIVLTGSAD	186
DdPe1C	RIGYLPGG-AK-DGDMIRV-DDSPNVWIDHNELFANHECDGTPDNDTTFESAVDVKGAS	175
BsPe1A	SIHHVREG-E---GTAIEVTDOSKNWIDHNEFYSEFPNG---DSDYYDGLVDMKRNA	162
VdPe1B	KISKVKDS-N---GDAIGIQ-ASKNVWIDHCDLSSDLK--S-----GKDYYDGLLDITHGS	160
	: * : : : .	
BsPel-P15	NVPT----LFGKFGSGNTTASGNTQY-----	197
AnPe1A	NRVSLTNNYIDGVS DY SAT CDGYHYWGIYLDGDADLVTMKGNYIYHTSGRSPKVDNTLL	246
AnPe1B	NRVTISYSLIDGRSDYSATCNGHHYWGVIYLDGSDNMDVTLKGNFYNLSGRMPKVGNTLL	246
DdPe1C	NTVTVSYNYIHGVKKV--GLDGSSSD-----TGRNITYHHNYNDVNAIPLPQRG-GLV	227
BsPe1A	EYITVSWNK FENHWKT--MLVGHTDNASLA---PDKITYHHNYFNLSRVPLIRY-ADV	216
VdPe1B	DWVTVSNTFLHDHFKA--SLIGHTDSNAKEDKGLHVTYANNYWNVNSRNPVSRF-GTV	217
	: : : . *	
BsPel-P15	-----	197
AnPe1A	HCVNMYFYDISGHAFEIGEGGYVLAEGNVFQVNDTVLET-YEG---A-----AFTV	293
AnPe1B	HAVNNLFHNFDDGHAFEIGTGGYVLAEGNVFQVNVVETPISG---Q-----LFSS	294
DdPe1C	HAYNNLYTNITGSGLNVRQNGQALIENWFKA INP---VTS-----RYDG	270
BsPe1A	HMFNMYFKDINDTAINSRVGARVFNENYFDNVGSGQADPTT---GF-IKGPVGFYGS	271
VdPe1B	HIYNNYYLEVGS SAVNTRMGAQVRVES TVFDKSTKNGIISVDSKEKGYATVGDISWGSST	277
BsPel-P15	-----	197
AnPe1A	PSTTAGEVCSTYLGRDCVINGFGCSGTFSEDS TS-----FLSDFEGKNIAS-	339
AnPe1B	PDANTNQCCASVFGRSQCLNAFGNSGSMGSDTS-----IISKFAGKTIAA-	340
DdPe1C	KNFGTWL-----K---GNNITKPADFSTYSITWTADTKPYVNADSWTSTGTFPTVAYN	321
BsPe1A	PSTGYWNL-----R---GNVFNTPNS-----HLNSTTNFPPYS	303
VdPe1B	-----N---TAPKG-----TL---GSSNIPYS	293
BsPel-P15	-----	197
AnPe1A	--ASAYTSVASRVVANAGQG NL-----	359
AnPe1B	--AHPPGAI AQWTKNAGQGK-----	359
DdPe1C	YSPVSAQC VKDKLPGYAGVGKNLATLTSTACK	353
BsPe1A	YQVQSATQAKSSVEQHSQGVGIN-----	326
VdPe1B	YNLYGKNNVKARVYGTAGQTLGF-----	316

Fig. S5. Primary sequence alignment of VdPe1B with BsPe1A, BsPe1A-P15, DdPe1C, AnPe1A, AnPe1B
VdPe1B primary sequence alignment with *Dickeya dadanti* DdPe1C (P11073), *Bacillus* Sp. KSM-P15
BsPel-P15 (Q9RHW0), *Bacillus* sp. N16-5 BsPe1A (D0VP31), *Aspergillus niger* AnPe1A (Q01172), AnPe1B
(Q00205). Conserved AA are red boxed.

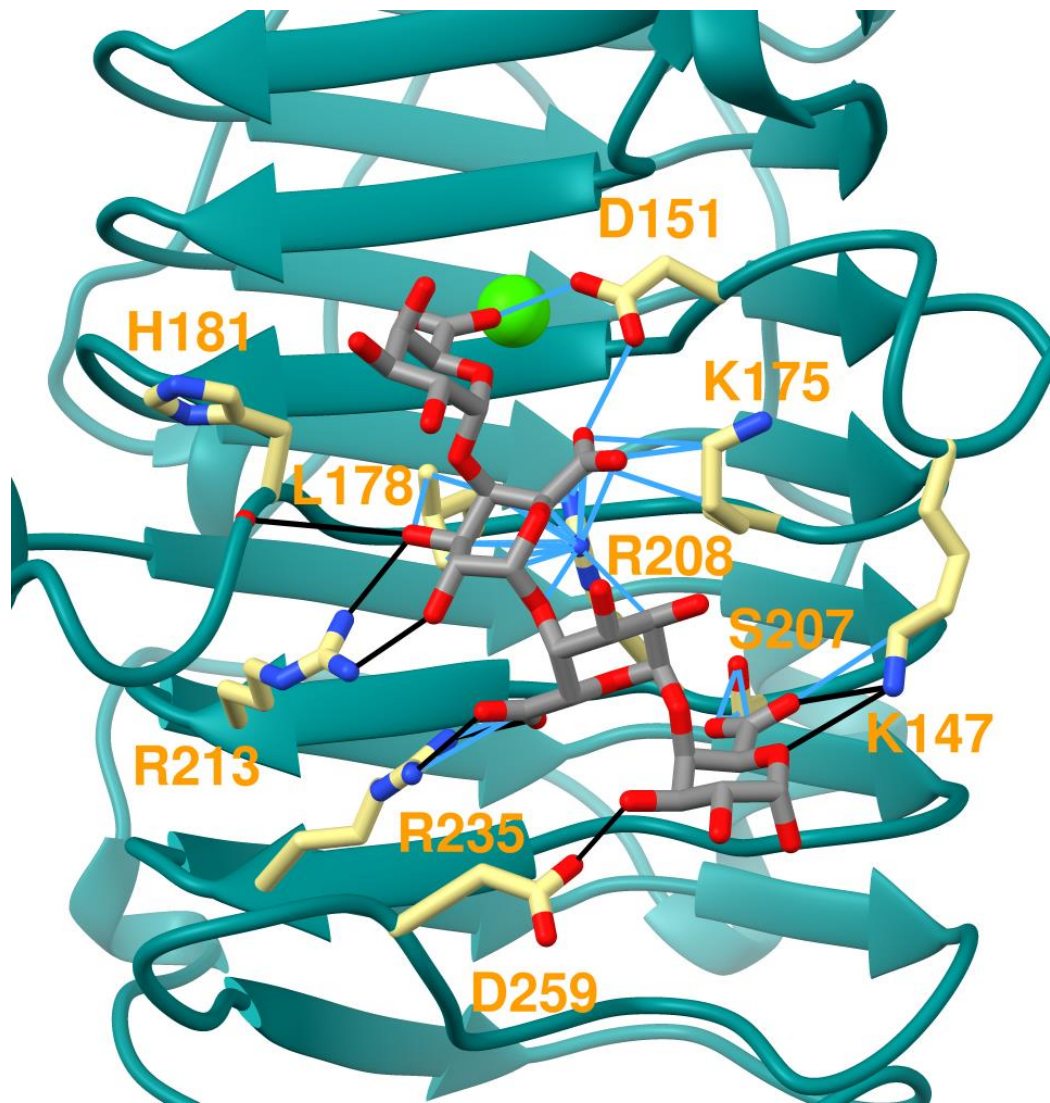


Fig. S6. Superimposed tetramer ligand from DdPelC to VdPelB structure

Tetramer ligand from DdPelC (PDB: 2EWE, gray) superimposed to VdPelB structure. Aa involved in interaction are yellow-coloured. Hydrogen bonds and Van der Waals contacts are coloured black and blue.

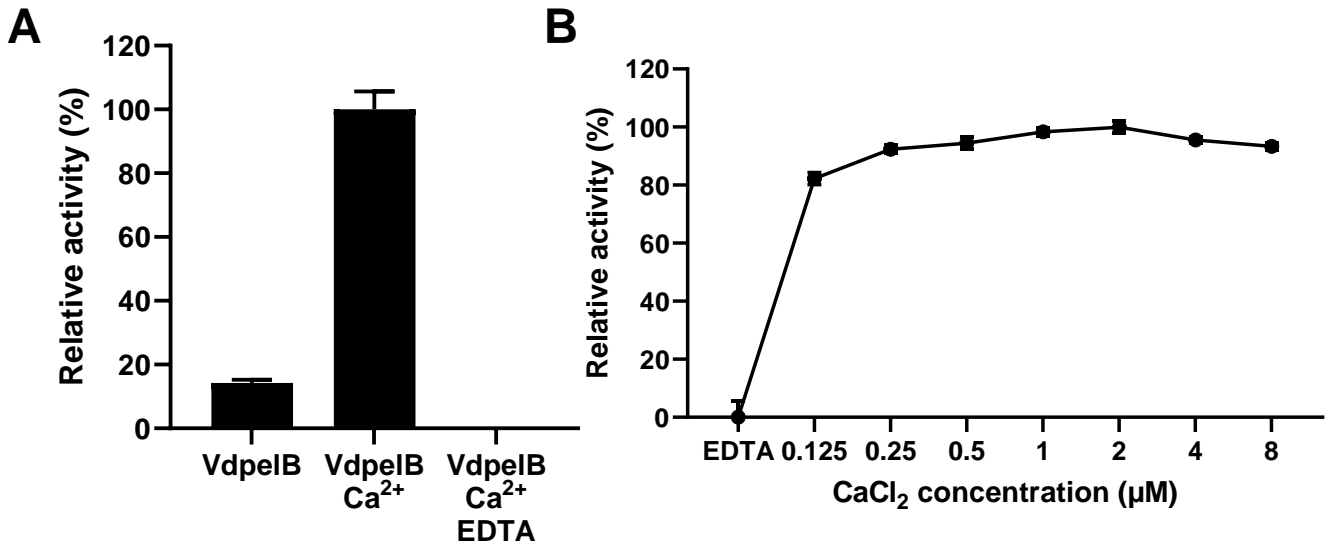


Fig. S7. Effects of Ca²⁺ on VdPelB activity

A) Calcium dependence of VdPelB activity. The activities were measured after 12 min of incubation with PGA, with/without addition of Ca²⁺ or Ca²⁺ and EDTA, at 35°C B) PGA digested using VdPelB for 12 min at 40°C and pH 8 with increasing concentrations of Ca²⁺.

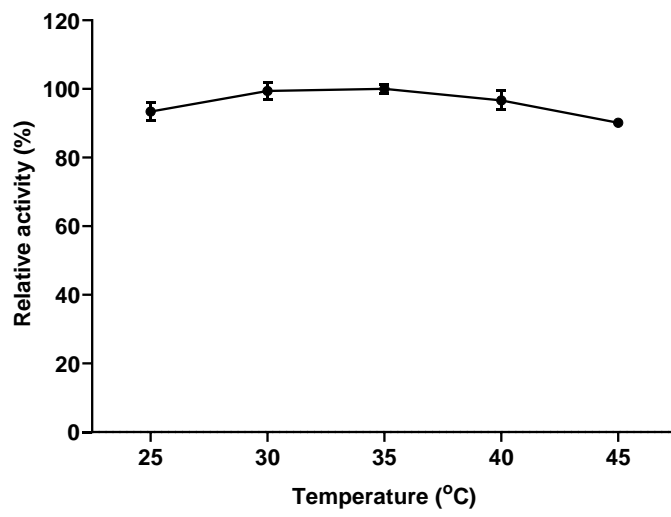


Fig. S8. Temperature-dependence of VdPeIB-N activity

The activities were measured after 8 min of incubation with PGA at pH 8. Values correspond to means of three replicates.

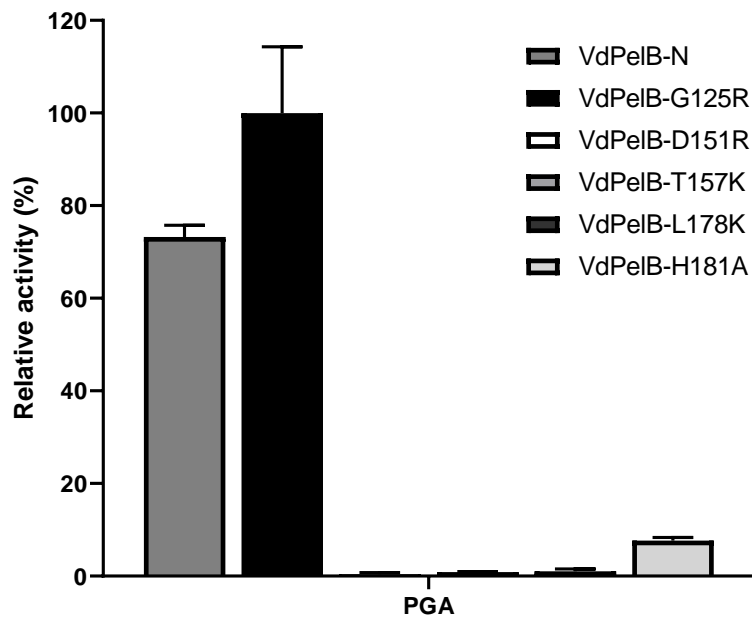


Fig. S9. VdPelB mutants activity on PGA

The activities were measured after 12 min of incubation with PGA with addition of Ca^{2+} at 35°C. Values correspond to means of three replicates \pm SD.

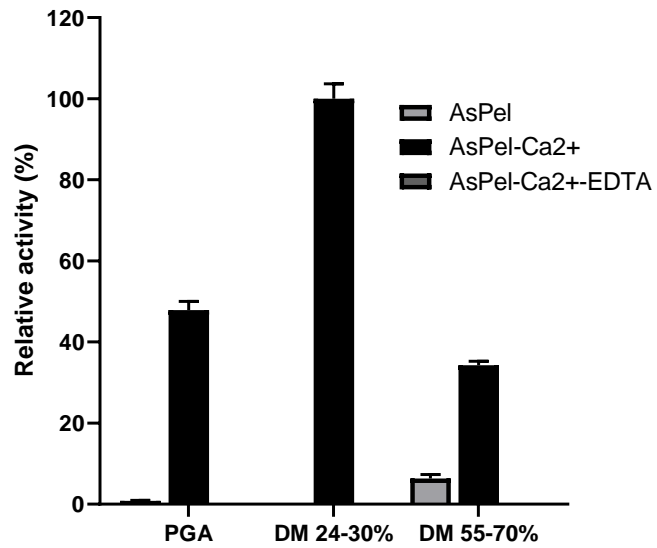


Fig. S10. Substrate-dependence of AsPel

The activities were measured after 12 min of incubation with PGA, pectins DM 24-30%, DM 55-70%, with/without addition of Ca²⁺ or Ca²⁺ and EDTA, at 35°C. Values correspond to means of three replicates ± SD.

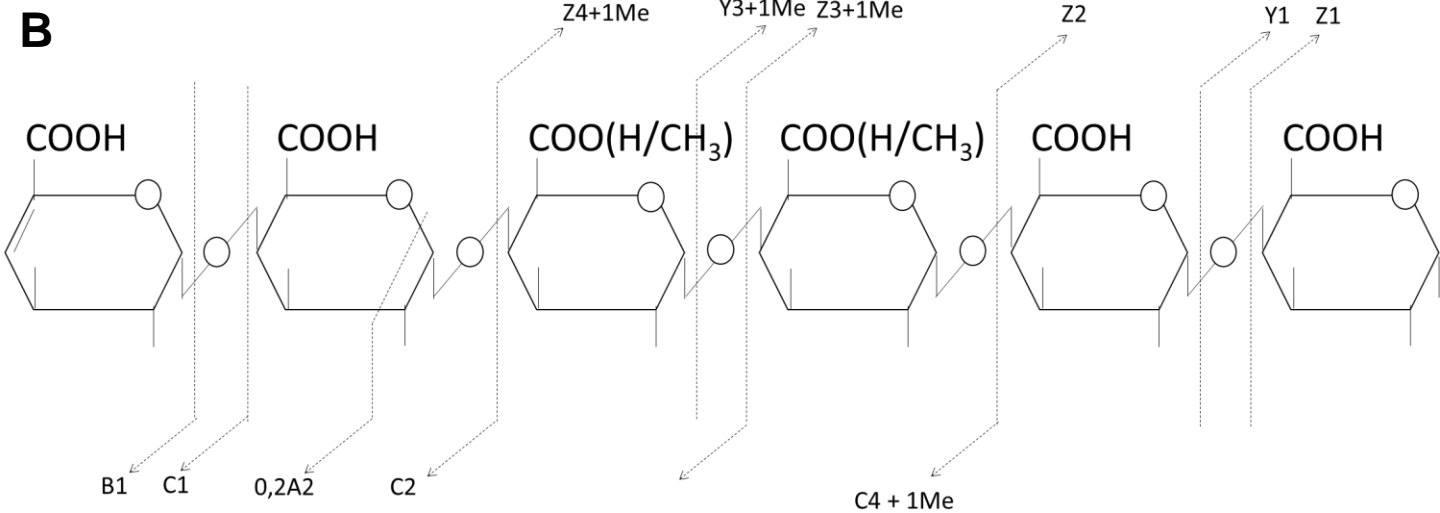
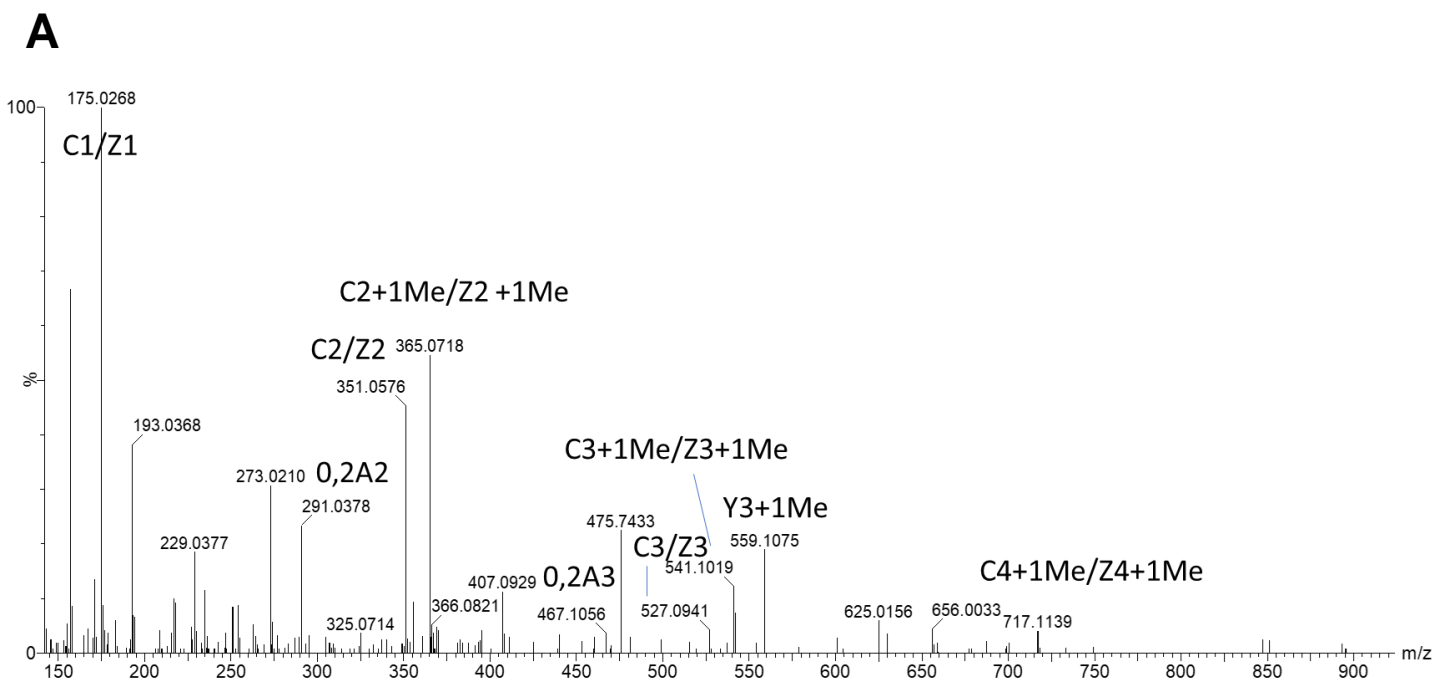


Fig. S11 : Example of MS² fragmentation pattern of GalA₆Me₁-H₂O

MS² fragmentation pattern of GalA₆-H₂O oligomer (m/z 1055,185252, 527,0889878) produced by VdPeIB from pectin DM >85%. Subscript numbers indicate the degree of polymerization.

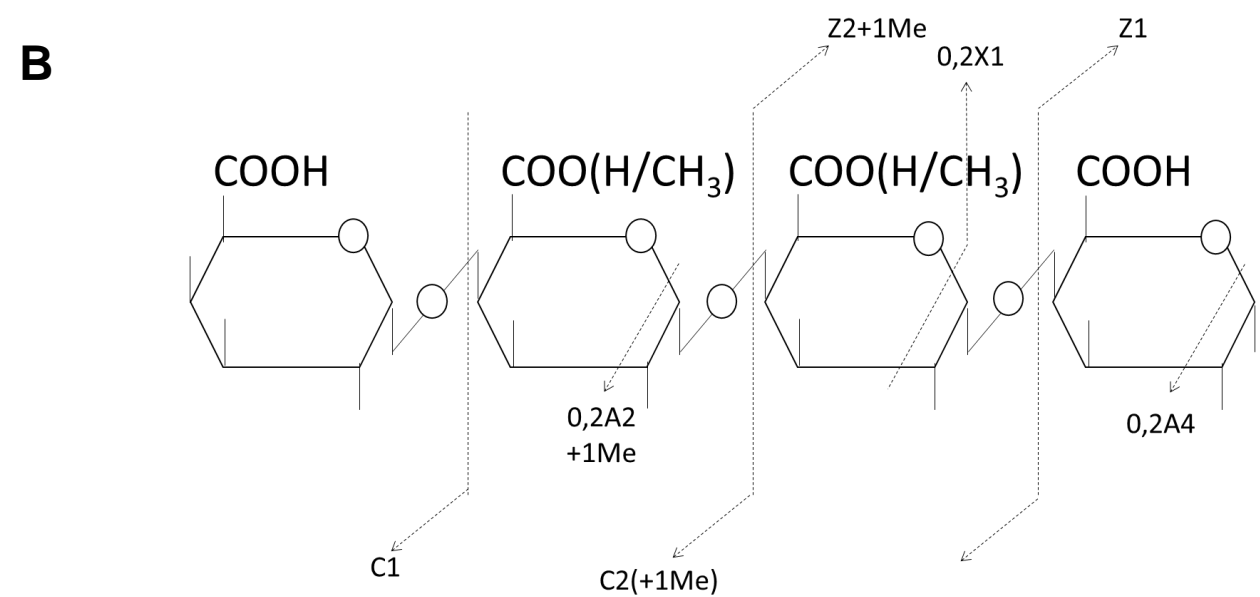
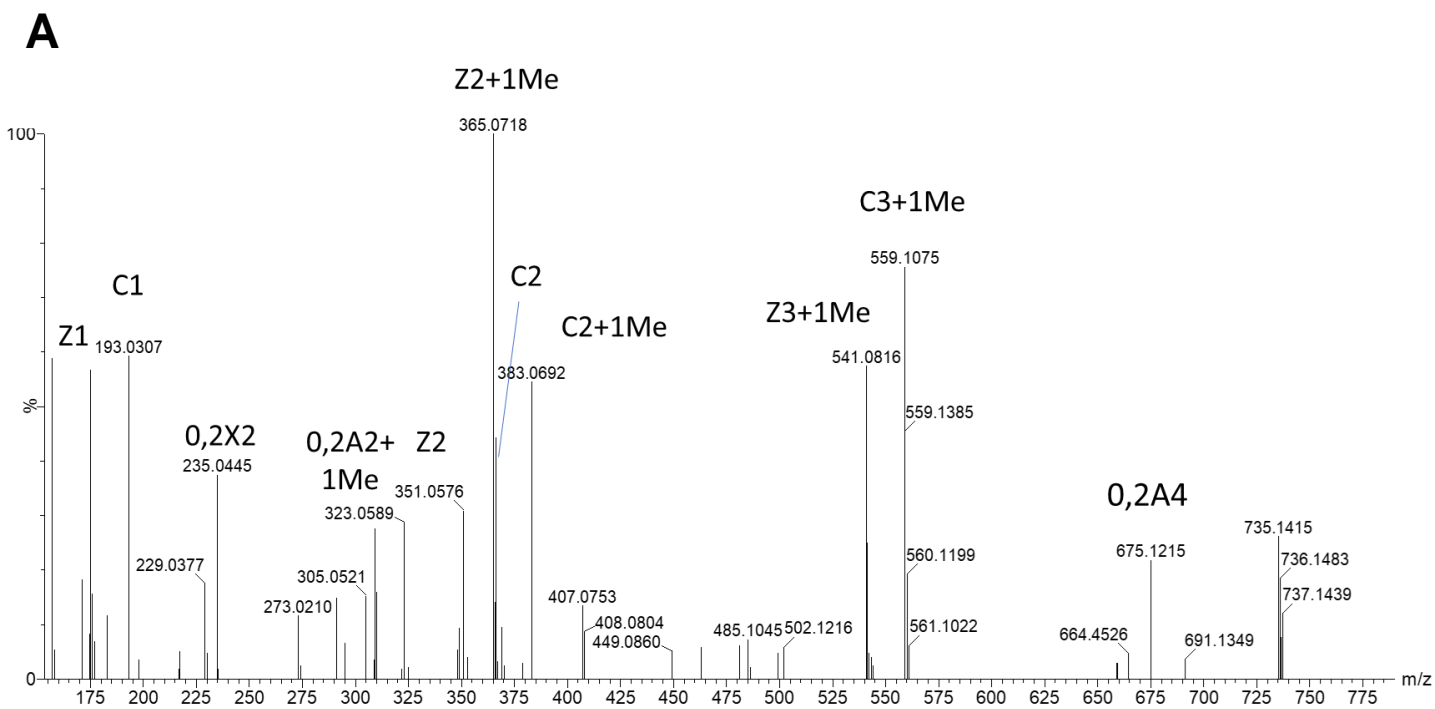


Fig. S12 : Example of MS² fragmentation pattern of GalA₄Me₁
 MS² fragmentation pattern of GalA₄Me₁ oligomer (m/z 735,1472907) produced by VdPelB from pectin DM >85%. Subscript numbers indicate the degree of polymerization.