

Substrate depolymerization pattern of *Pseudomonas viridiflava* SF-312 pectate lyase

Previously, other groups have reported that the limit product of polygalacturonic acid (PGA) depolymerization by *Erwinia chrysanthemi* pectate lyase E (PL_E) was predominantly an unsaturated oligogalacturonic acid with a degree of polymerization (DP) of two, while pectate lyase B and pectate lyase C produced a predominantly DP3 unsaturated oligogalacturonic acid. We have used high performance anion-exchange chromatography and pulsed amperometric detection to analyze oligogalacturonic acids released by *Pseudomonas viridiflava* SF-312 PL, which, like *Erwinia chrysanthemi* PL_E, can independently macerate host tissue. Our results demonstrated that DP 2 unsaturated oligogalacturonic acid was the major oligosaccharide released by *Pseudomonas viridiflava* SF-312 PL. In contrast to *Erwinia chrysanthemi* PL_E, we observed that DP 3 unsaturated oligogalacturonic acid was present in *Pseudomonas viridiflava* PL reaction products during the first 200 min of PGA depolymerization. The limit product consisted of four times more DP 2 than DP 3 unsaturated oligogalacturonic acid. We also observed that small amounts of a series of oligogalacturonic acids lacking the 4,5-unsaturated nonreducing end function were released by *Pseudomonas viridiflava* PL during the PGA depolymerization process. These results indicate that *Pseudomonas viridiflava* PL utilizes a random endolytic PGA depolymerization mechanism, rather than the nonrandom endolytic mechanism utilized by *Erwinia chrysanthemi* PL_E.

INTRODUCTION

Pectate lyase (PL) depolymerizes homogalacturonan regions of pectin by a trans-eliminase mechanism, producing oligogalacturonic acids with 4,5-unsaturated functions at their nonreducing ends. PL is one of several enzymes secreted by soft-rot pathogenic bacteria that attack cell wall or other cellular components [7]. However, it is the key soft-rot disease enzyme since PL's (with strongly alkaline pI) purified from *Erwinia* [3] and *Pseudomonas* species [16] are capable of host tissue maceration independent of the other enzymes. While *Erwinia chrysanthemi* strains produce five PL isozymes

(PLa-e, pI 4–10) [4], *Pseudomonas viridiflava* SF-312 produces only one PL (pI 9.7) [16]. Two groups [2, 20] have reported that the reaction product of *E. chrysanthemi* PL (pI 10.0) was predominantly an unsaturated oligogalacturonic acid with a degree of polymerization (DP) of 2, while PLb and PLc, isozymes with less alkaline pI's that are not essential for complete plant tissue maceration [5], produced predominantly the DP3 unsaturated oligogalacturonic acid. These reports utilized either paper chromatography or reversed-phase ion-pairing high-performance liquid chromatography with UV (234 nm) detection for the analysis of reaction products [2, 20].

We demonstrated previously that high-performance anion-exchange chromatography and pulsed amperometric detection (HPAEC-PAD) was unique in that it could separate and detect oligogalacturonic acids [11] both with and without (saturated) 4,5-unsaturated functions at their non-reducing ends [12, 14]. The enzyme used in these reports was recombinant PLc derived from *E. chrysanthemi* CUCPB 1237 [1], which generated predominantly DP3 unsaturated oligogalacturonic acid from a tobacco cell wall preparation. Various ambient-pressure ion-exchange and affinity chromatography methods have been used to purify pectinase enzymes (reviewed in [18]). High efficiency separation of commercial pectinases was achieved using high performance liquid chromatography (HPLC) columns packed with various ion-exchange forms of the Spheron 1000 stationary phase [19, 21], sold commercially under the name Separon HEMA [18]. We now report the substrate depolymerization pattern generated by *P. viridiflava* SF-312 PL (purified by Separon HEMA-BIO 1000 CM HPLC) using HPAEC-PAD for reaction product analysis.

MATERIALS AND METHODS

Pectate lyase purification

PL was purified from *P. viridiflava* SF-312 culture supernatants according to a modification of the methods reported by Liao *et al.* [16]. These modifications included resuspension of the 50–90% ammonium sulfate precipitate in 10 mM HEPES buffer (pH 7) and dialysis against the same buffer at 4 °C for at least 24 h (buffer changed three times). The crude enzyme preparation (3 ml containing 5077 U PL) was loaded onto a Separon HEMA-BIO 1000 CM (10 µm particle diameter; Metachem Technologies for Tessek A/S) high-performance cation-exchange column (7.5 × 75 mm), previously equilibrated with 10 mM HEPES buffer (pH 7). The chromatographic system, consisting of two Rainin HPLX Pumps, a Rainin Pressure Module, a Rheodyne 9125 injector, a Pharmacia Super Loop (50 ml), a Rainin Dynamax UV-1 Absorbance Detector (280 nm), and a Rainin Dynamax FC-1 Fraction Collector, was maintained at 4 °C and controlled by Rainin Dynamax HPLC Method Manager software. The mobile phase (1 ml min⁻¹) was isocratic at 10 mM HEPES buffer (pH 7) for the first 30 min followed by a linear salt gradient that reached 100 mM NaCl in 10 mM HEPES buffer (pH 7) at 100 min and then 1.0 M NaCl in 10 mM HEPES (pH 7) at 120 min. Five ml fractions were collected during the first 30 min and 2 ml fractions were collected for the rest of the run. Fractions were assayed for PL activity with a unit of PL activity defined as the amount of enzyme that produced an increase of 1.73 absorbance units min⁻¹ (monitored at 232 nm and 30 °C) corresponding to the

formation of 1 μ mole of unsaturated uronide [16]. The purity of the isolated PL was established by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (8–25% SDS, PhastGel, Pharmacia) and isoelectric focusing (IEF) (pH 3.5–9.5, 2.2% ampholine, polyacrylamide gel T = 5% C = 3%, Pharmacia) performed as reported previously [16] except that the gel was prefocused at 10 W for 20 min and focused at 12 W for 30 min. Hydroxyapatite (High Resolution, Calbiochem) chromatography of the pooled PL fraction collected from the Separon HEMA-BIO 1000 CM column was also performed according to methods reported previously [17].

HPAEC-PAD analysis of pectate lyase reaction products

Polygalacturonic acid (PGA; Sigma) was depolymerized in 0.1% solution (100 mg PGA/100 ml H₂O) containing 10 mM TRIS free base and 0.3 mM CaCl₂. This solution was titrated to pH 8.5 with KOH, filtered (0.45 μ m) and the temperature was raised to 30 °C in a water bath prior to addition of the purified PL (20 U). After 6 h, the reaction was continued at room temperature (22 °C). The reaction mixture was sampled before and after addition of the enzyme by removing 1 ml samples and adding them to 1 ml of IR-120 H⁺ cation-exchange resin in a syringe fitted with a filter (0.22 μ m). Finally, the pH-adjusted reaction mixture was extruded through the cation-exchange resin and filter prior to injection onto a CarboPac PA1 HPAEC column (Dionex) [11]. Oligogalacturonic acid DP values were assigned using standards up to DP 7 that were purified by preparative HPLC [13]. DP values above 7 were assigned by extrapolation. Saturated oligogalacturonic acids were observed to elute earlier from the CarboPac PA1 column than their unsaturated counterparts having the same DP [12, 14]. When saturated and unsaturated oligogalacturonic acids were not completely resolved, their relative peak areas were determined by either measuring the height of the shoulder or, if necessary, by taking the average peak area of the neighboring saturated oligogalacturonic acids and then subtracting the calculated saturated peak area from the total peak area to determine the unsaturated peak area. The reaction was also monitored by removing a 10 μ l sample and diluting it 100 \times with H₂O prior to A₂₃₄ measurement in a spectrophotometer.

RESULTS AND DISCUSSION

The PL activity in the *P. viridiflava* SF-312 culture supernatant eluted within 80 min from the Separon HEMA-BIO 1000 CM column as a single peak during the salt gradient (Fig. 1). We observed that PL activity did not adsorb to DEAE cellulose columns [16], which resulted in the PL peak eluting very close to the column void and with unretained culture supernatant components (data not shown). The PL was purified to near homogeneity with the Separon HEMA-BIO 1000 CM column based on SDS-PAGE (Fig. 1 inset). The molecular weight of the purified *P. viridiflava* PL was consistent with the 42 kDa reported previously [16]. The purified PL also migrated as a single band on IEF gels with a pI calculated as 9.4 relative to pI markers. Further evidence of the Separon HEMA-BIO 1000 CM-isolated PL purity was illustrated by subsequent hydroxyapatite chromatography, during which the one protein peak (A₂₈₀) that eluted (0.6 M KCl) coincided with the single peak of PL activity.

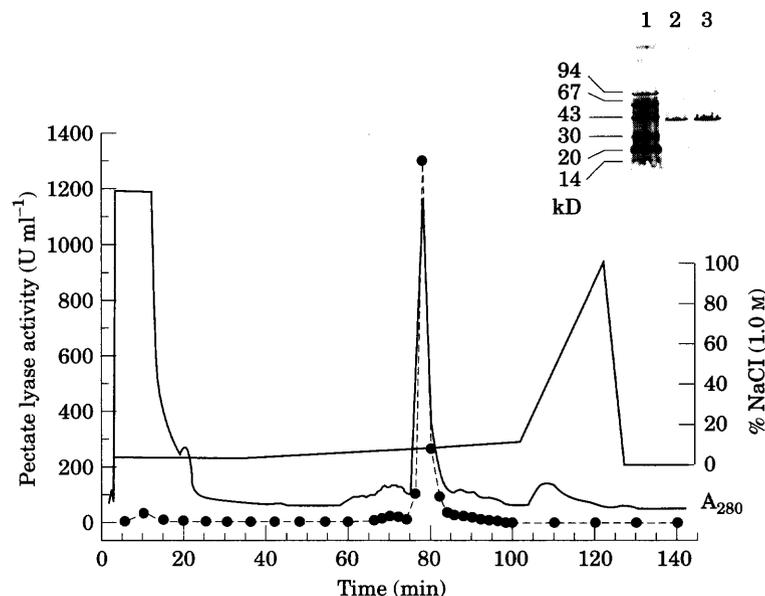


FIG. 1. Purification of *Pseudomonas viridiflava* SF-312 PL from culture supernatant with a Separon HEMA-BIO 1000 CM high-performance cation-exchange column. The mobile phase used was 10 mM HEPES buffer (pH 7) and then a NaCl gradient in this buffer (see Materials and Methods section for details). The PL activity (---) peak corresponded to a protein peak (—) that eluted during the salt gradient. The purity of the PL collected from this peak is demonstrated by SDS-PAGE (inset). Molecular weight markers (lane 1) included phosphorylase. b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa). The major peak of PL activity (78 min, 1612 U ml⁻¹) was loaded in lane 2 (6 U) and lane 3 (16 U).

Cation-exchange chromatography has been used previously to purify PL produced by soft-rot bacteria. It took 44 h for the first *Erwinia carotovora* PL isozyme to elute from an ambient pressure CM-Sephadex (C-50) column [8]. The two PL peaks that eluted from the CM-Sephadex column were not completely separated, therefore, the collected PL fractions were further purified by agarose-affinity (Bio-Gel A-0.5 m) chromatography [8]. More expedient separation of *E. carotovora* PL isozymes was possible with cation-exchange HPLC using a TSK SP-5PW column [9], however, subsequent size-exclusion chromatography was still required to further purify the PL. Consequently, the use of the Separon HEMA-BIO 1000 CM column represents an improvement in the purification of PL from bacterial culture supernatants.

The depolymerization of PGA by the *P. viridiflava* SF-312 PL occurred rapidly (Figs. 2-4). At 30 min, the reaction products ranged from DP 2-22 unsaturated oligogalacturonic acids. After 6 h, only DP \leq 5 unsaturated oligogalacturonic acids remained. A 2:1 peak area ratio between DP 2 and DP 3 in addition to trace amounts of DP 4 unsaturated oligogalacturonic acids were observed after 96 h of reaction (data not shown). When 500 U of PL was used in the reaction mixture (instead of 20 U), a limit product was formed in which a 4:1 ratio of DP 2:DP 3 unsaturated oligogalacturonic acid peak area was observed after 12 days of reaction (Fig. 3).

The kinetics of *P. viridiflava* SF-312 PL depolymerization of PGA argue for a

random endolytic mechanism, in which depolymerization proceeded by DP numerical order of unsaturated oligogalacturonic acids (DP 8 < DP 7 < DP 6 < DP 5 < DP 4 peak area, Fig. 4a). The only exception to this trend was that the peak area of DP 2 lagged behind that of DP 3 until after 5 h of reaction. In contrast, a nonrandom endolytic depolymerization mechanism has been reported for *E. chrysanthemi* PLe [20]. While PLe produces predominantly DP 2 unsaturated oligogalacturonic acid, it notably does not produce any DP 3 unsaturated oligogalacturonic acid during the first 200 min of reaction, even though DP 4–6 (DP 5 most abundant) were observed throughout the time course [2, 20]. Preston *et al.* [20] proposed that the *E. chrysanthemi* PLe recognizes a PGA domain such that the binding site was approximately five residues away from where cleavage occurs. Furthermore, they stated that DP 2 unsaturated oligogalacturonic acid was formed by a separate endolytic event in which the solution secondary structure of PGA renders a DP 3 sequence inaccessible to the bound enzyme [20]. We observed that *P. viridiflava* SF-312 PL did produce DP 3 unsaturated oligogalacturonic acid early during PGA depolymerization; at 180 min DP 3 unsaturated oligogalacturonic acid had the greatest peak area. Therefore, it appears that *P. viridiflava* SF-312 PL and *E. chrysanthemi* PLe effect host tissue maceration by different mechanisms.

Another example of a nonrandom endolytic depolymerization mechanism was reported [9] for the PL isozyme I (pI 9.8 [β]) purified from *Erwinia carotovora* subsp. *carotovora*. Forrest and Lyon [9] separated their *E. carotovora* PL reaction products by anion-exchange HPLC (TSK DEAE-5PW column monitored by UV absorption at 245 nm). They observed that primarily DP 3, 5 and 6 (with significantly lower amounts of DP 4) unsaturated oligogalacturonic acids were produced in 300 min of PL depolymerization of PGA. However, when citrus pectin or potato cell walls were used as the *E. carotovora* PL substrate, the predominant unsaturated oligogalacturonic acid produced was DP 3 and a random endolytic depolymerization mechanism was utilized. Additionally, a commercial PL (Calbiochem) derived from *Bacillus polymyxa* (not an active plant pathogen [6]) produced primarily DP 4 and 5 unsaturated oligogalacturonic acids (PGA substrate) and DP 2, 3 and 4 (potato cell wall substrate) unsaturated oligogalacturonic acids by a random endolytic depolymerization mechanism [9]. Therefore, the nonrandom endolytic depolymerization mechanism reported for strongly alkaline pI *Erwinia* PL may only be significant *in planta* during depolymerization of cell wall homogalacturonan, and it may be masked by an overall random endolytic pectin depolymerization mechanism.

Saturated oligogalacturonic acids were also formed during *P. viridiflava* PL depolymerization of PGA (Figs. 2, 3 and 4b). While trace amounts of these oligogalacturonic acids were present in untreated PGA (Fig. 2a), amounts increased during the first 1 h of reaction (19% of total oligogalacturonic acid peak area), but remained significantly below the corresponding (same-DP) unsaturated oligogalacturonic acids (Fig. 4). Small amounts (5% of total oligogalacturonic acid peak area) of DP 3–5 saturated oligogalacturonic acids remained in the limit product (Fig. 3). It should be noted that saturated oligogalacturonic acids have approximately five times greater molar PAD response than unsaturated oligogalacturonic acids based on previously reported values [11, 15]. Therefore, saturated oligogalacturonic acids released during PL depolymerization of PGA are certainly minor reaction products.

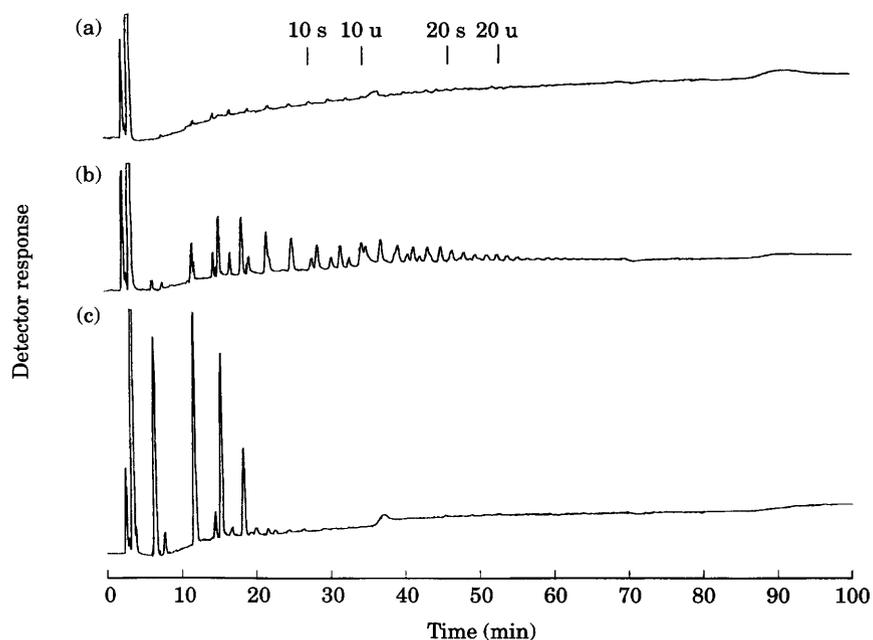


FIG. 2. Time course of PGA depolymerization by PL. HPAEC-PAD analysis of oligogalacturonic acid reaction products sampled at 0 h (a), 0.5 h (b) and 6 h (c) during the PL reaction. Two series of oligogalacturonic acid peaks (saturated and unsaturated) are observed in (b) and (c), while only saturated oligogalacturonic acids are observed in (a) [12]. The numbers over the chromatograms correspond to the oligogalacturonic acid DP value (s = saturated, u = unsaturated). A large TRIS buffer peak (3.5 min) was present in all samples. The chromatographic conditions included a CarboPAC PA1 column, a nonlinear mobile phase gradient (25–500 mM in 120 min) of potassium oxalate buffer (pH 6) and post-column addition of potassium hydroxide (500 mM) prior to PAD [11, 12].

However, these results confirm our earlier conclusions [12, 14] that methods based solely on reaction product detection by UV absorption at 235 nm [9, 20] ignore a population of oligogalacturonic acids generated by PL. Additionally, in mixed enzyme reactions, such as the secretion of both PL and endopolygalacturonase by *E. chrysanthemi*, it will be essential to use HPAEC-PAD to determine the contributions of each enzyme to reaction product formation.

The generation of both saturated and unsaturated oligogalacturonic acids by *P. viridiflava* PL could be explained by two active sites that have hydrolase and lyase activities, respectively. While we have demonstrated here the purity of our *P. viridiflava* PL, there remains the remote possibility that trace amounts of endopolygalacturonase could have been present. However, we demonstrated previously that a near limit product of recombinant *E. chrysanthemi* PLc contained significant amounts of saturated oligogalacturonic acids up to at least DP 5 [12]. Therefore, if other recombinant PL's are observed to produce both saturated and unsaturated oligogalacturonic acids, then this evidence would further strengthen our argument that our *P. viridiflava* PL was pure and that a single PL enzyme was responsible for both activities.

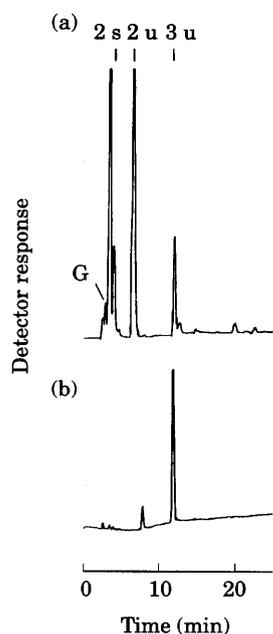


FIG. 3. HPAEC-PAD analysis of the PL limit product (a) and a DP 3 unsaturated oligogalacturonic acid standard [13] (b) that contains a small amount of DP 3 saturated oligogalacturonic acid (7.9 min). See Fig. 2 for chromatographic conditions and peak label description (G = galacturonic acid).

Henrissat *et al.* [10] demonstrated that the PL protein superfamily has two highly conserved amino acid sequences that are likely catalytic sites, one of which binds calcium. Based on the 95 Å circumferential distance between the two sites, they argue that both catalytic sites cannot have calcium-dependent pectinolytic activity. However, since endo-polygalacturonase does not require calcium, the second site, with an amino acid sequence of Val-Trp-Ile-Asp-His (vWiDH), could still have hydrolase activity. The authors note that the vWiDH region would only accommodate up to four saccharide residues and that PL requires a minimum DP 6 oligogalacturonic acid for normal substrate recognition [20]. However, since Preston *et al.* [20] only detected unsaturated products, the minimum DP substrate recognition length probably only applies to the transeliminase active site. Therefore, the possibility still remains that the vWiDH region represents a hydrolytic active site with a smaller substrate recognition DP than the transeliminase active site. Henrissat *et al.* [10] offer an epimerization mechanism to explain the subsequent production of saturated oligogalacturonic acids from unsaturated products of the transeliminase reaction. However, no evidence of oligogalacturonic acid products terminated at their non-reducing ends by glucuronic acid, altruronic acid or iduronic acid is available yet. Further investigation is underway to test these different hypotheses for the explanation of saturated oligogalacturonic acid production by PL.

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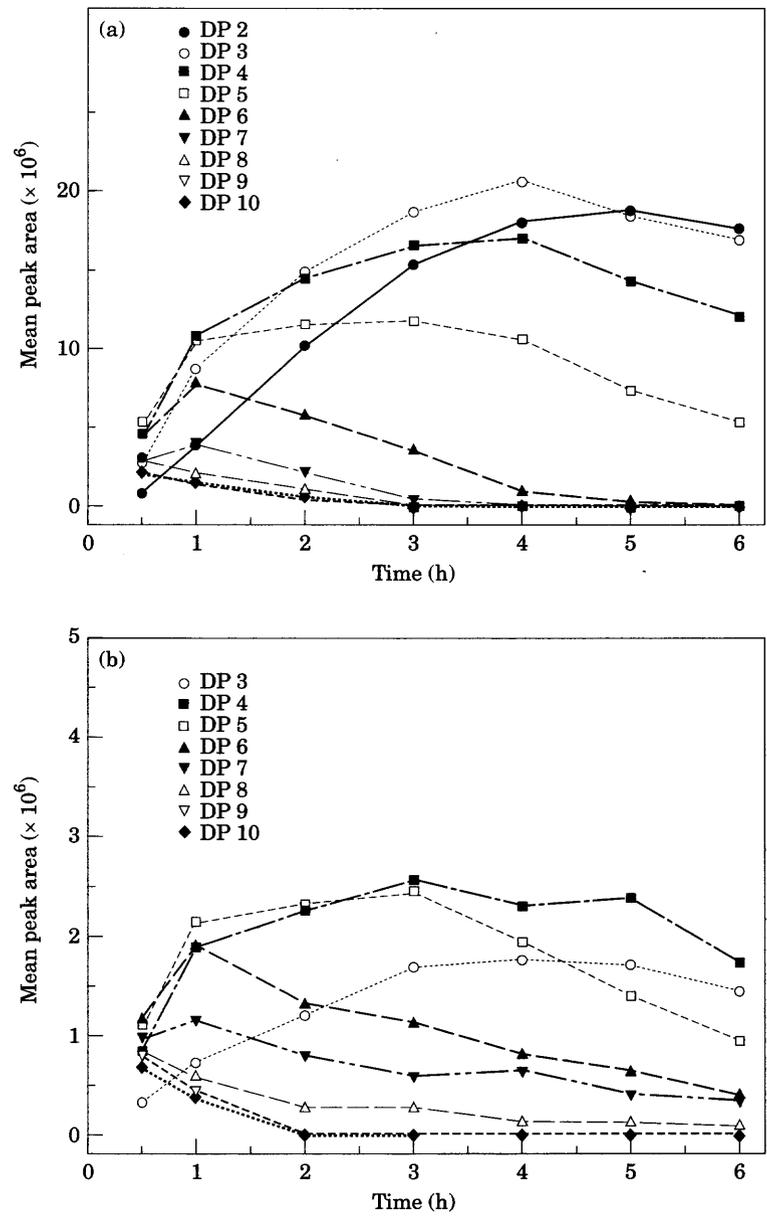


FIG. 4. Kinetics of PL reaction product formation. Mean peak area values were determined by HPAEC-PAD analysis of unsaturated (a) and saturated (b) oligogalacturonic acids. The mean peak area values represent the results of three separate experiments using the same PL enzyme. Standard error of the mean values ranged from $0.24\text{--}5.78 \times 10^6$ peak area units (a) and $0.12\text{--}1.69 \times 10^6$ peak area units (b).

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