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Note

High-performance anion-exchange chromatography — DAD as a tool for the identification and quantification of oligogalacturonic acids in pectin depolymerisation

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Abstract

High-performance anion-exchange chromatography (HPAEC) coupled with a diode array detector (DAD) was used to identify and quantify oligogalacturonic acid components in pectins. Purified pectin lyase and polygalacturonase were used to generate unsaturated and saturated oligomers from pectins and sodium polygalacturonate, respectively. This method resulted in a good separation of saturated and unsaturated oligomers up to DP 13. It allowed us to follow polygalacturonase and pectate lyase depolymerisation pathways simultaneously.

Keywords: HPAEC; Pectin; Polygalacturonate; Oligogalacturonic acids; Pectolytic enzymes; Diode array detector

1. Introduction

The partial enzymatic depolymerisation by microbial preparations of the polygalacturonic acid region in pectins releases a random series of intermediate oligomers (DP_1 to DP_n) of galacturonic acid [1]. This paper describes a rapid HPLC method suitable for identifying two types of enzymatic reactions which may occur simultaneously, based on the concept of Endress et al. [2,3]. Previous authors used fast-ion chromatography (without pre-treatment of the samples and without quantification) with linear phosphate gradient to monitor enzymatic reactions of pectin degradation. This approach was adapted in order to follow the course of enzymatic depolymerisation with both polygalacturonases and pectate lyases. Other methods allow the isolation of long-chain oligomers using pulsed amperometric detection but, at the same time, they cannot discriminate between saturated and unsaturated components [4–6]. In this note, the use of a diode array detector resulted in simultaneous product identification and quantification via UV spectral analysis.

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2. Experimental

Standards and substrates.—Mono-, di- and trigalacturonic acids were obtained from Sigma. The A_{214} of solutions of each oligomer was measured on a UV–Vis spectrophotometer (GBC UV–Vis 918) with a quartz cell (optical pathlength: 1 mm). Sodium polygalacturonate (PGA_O) (Sigma, P-1879) from oranges and sodium polygalacturonate (PGA_C) (Sigma, P-3850) from citrus fruits were used as substrates for the enzymes except for lyase. PGA_O was dissolved (1% w/w) in ultrapure water at pH 5.0. PGA_C was solubilised (1% w/w) in hot ultrapure water (90 °C) under vigorous agitation. This solution was quickly cooled to rt and its pH was adjusted to 4.5 with 0.1 N HCl. Highly methylated (DM 70) pectin from Herbstreith & Fox KG (Pektin Fabrik, D-75305 Neuenbürg, Germany) was used as substrate for lyase. It was dissolved (1% w/w) in ultrapure water with vigorous stirring. The pH was adjusted to 5.1 with pure $Na_2HPO_4 \cdot 2\ H_2O$.

Enzymatic assays and samples.—In order to produce unsaturated oligomers, a pectin lyase preparation (Sigma, P2804) was added (10 U/50 mL) to a pectin solution (50 mL) at 40 °C. One unit causes a ΔA_{235} of 1.0 per min at 40 °C due to the release of unsaturated products from pectin. Samples (5 mL) were taken with a syringe. The same volume of a borate buffer at 0 °C (pH 11.5, 0.1 mol/L) was added to these samples which were kept at 5 °C for 15 h to start de-esterification of the pectin prior to the chromatographic analysis. These conditions were chosen to quench the reaction [7] and to minimise any chemical β-elimination [8]. The samples were then frozen. Saturated oligomers were produced by a polygalacturonase preparation (Sigma, P0690) which was added (10 U/50 mL) to sodium polygalacturonate solution (PGA_O or PGA_C). One unit liberates 1 μmol of galacturonic acid per min at pH 4.0 and 25 °C. A filtrate of a culture of *Penicillium oxalicum* was used as the enzyme preparation. This preparation was added (100 mL/L) to the sodium polygalacturonate solution (PGA_O or PGA_C). The culture medium of the crude preparations was composed of Herbstreith highly methylated pectin 2% w/v, $(NH_4)_2SO_4$ 0.75% w/v; KH_2PO_4 0.5% w/v; $MgSO_4$ 0.03% w/v. Reactions (polygalacturonase and the crude preparation) were performed in a 2 L bioreactor maintained at 40 °C. Samples were taken from the reactor with a 50 mL syringe and frozen immediately in liquid nitrogen to quench the reaction. Each sample was freeze-dried and ground in a mill (IKA A10, Janke and Kunkel). They were finally dissolved (10 mg/2 mL) in a 0.025 M borate buffer (pH 9.5), filtered on a 0.45 μm filter (Gelman Science) and stored at 5 °C (apart from the samples of lyase reaction which were stored at rt) in the separation module until injection (100 μL).

Chemical analysis.—Reducing sugars were measured in the freeze-dried samples according to Baron et al. [7].

Chromatography.—Samples were analysed on a strong anion-exchange resin Mono-Q (glass column, 5 mm × 5 cm) (Pharmacia, Upsala, Sweden). The concepts of Endress et al. [2,3] and Campos [9] were modified. The separation was monitored from 210 to 250 nm with a 996 photodiode array detector (Waters, Milford, USA) coupled with an Alliance 2690 separation module (Waters, Milford, USA). The temperature of the column was maintained at 35 °C in an oven. A gradient of Na_2SO_4 (0.0–0.9 M) in a phosphate buffer (0.02 M, pH 5.1) was used.

Data treatment.—All chromatographic data were analysed with the photodiode array software option in the Millenium^{32} Chromatography Manager (Waters, Milford, USA). The purity of each peak was checked by analysing the spectral homogeneity along the peak. These were identified by matching the spectrum of their apex with those of a library built with the spectra of the apex of each oligomer [10].

3. Results and discussion

Chromatography.—The chromatographic profiles were obtained at 214 nm in order to detect saturated oligomers [11]. A non-linear Na_2SO_4 gradient (dotted line in Fig. 1) gave a good separation and a baseline stable enough to allow peak integration.

Quantification of saturated oligomers.—The use of a purified polygalacturonase enzyme (Sigma P0690) allowed the determination of the retention time of saturated oligomers up to DP 13. The identification of the peaks was based on the retention time and the spectra associated with each peak. The analysis of peak purity allowed us to ascertain the quality of the separation (no coelution). The spectra showed progressive attenuation of the absorbance as a function of the wavelength. The shape of the spectrum was similar for each saturated compound. The spectra were stored in a library.

The molar extinction coefficient (ε) of galacturonic acid at 214 nm was 58 mol^{-1} cm^{-1}, due to the carboxylate group [12]. The molar extinction coefficient of mono-, di- and trisaccharides of D-galacturonic acid showed a linear progression with the DP. This relation was also obtained with their response coefficients of HPLC analysis. Thus, the quantification of higher saturated oligomers ($DP > 3$) was calculated on the basis of their response coefficients predicted by the upper relation. The soundness of our calculation was confirmed by the high correlation ($R = 0.995$) between the sum of the concentration of each oligomer (calculated using our method) and the global concentration of the reducing carbohydrates.

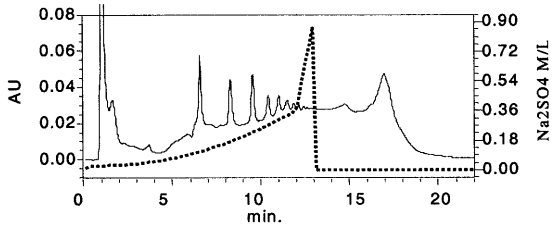

Fig. 1. Chromatographic profile of PGA_O at 214 nm after 30 min of reaction with an enzymatic preparation obtained from *Penicillium oxalicum*. The eluent was a gradient of Na_2SO_4 (···).

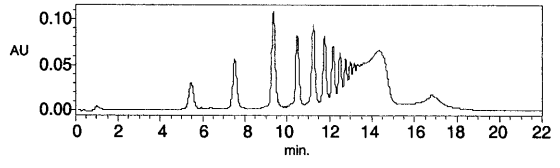

Fig. 2. Chromatographic profile at 230 nm of a pectin sample after 42 min of reaction with pectin lyase (Sigma P2804). It was injected after 118 h of reaction in pH 9.5 borate buffer.

Quantification of unsaturated oligomers.—The use of a purified pectin lyase (Sigma P2804), which only produced unsaturated oligomers, allowed the determination of their retention times. An acceptable separation of the components could be achieved after approximately 100 h of de-esterification in 0.025 M borate buffer, pH 9.5 (Fig. 2) at rt.

Incomplete de-esterification gave chromatograms with baseline drift and coeluting peaks due to interference with the residual methyl-ester groups in the backbone of the pectin.

When the separation was optimal (Fig. 2), the spectrum of each peak apex was analysed and stored in a library. The maximum absorbance was around 230 nm. Thus, β-elimination was followed with the chromatographic profile at 230 nm.

The A_{235} was measured from the same samples that were injected on the column Mono-Q. The concentration of unsaturated compounds was calculated using the molar extinction coefficient $\varepsilon = 5412$ M^{-1} cm^{-1} [13]. The concentration was correlated with the total peak area of the chromatograms extracted at 230 nm. The correlation between UV_{235} concentration and area was strong ($R = 0.965$). Each unsaturated oligomer was quantified from its peak area using the relation between HPLC area and concentration.

Following a reaction.—The recording of the chromatograms between 210 and 250 nm allowed the detection of both saturated and unsaturated compounds. Two chromatograms obtained at 214 and 230 nm for a PGA_C sample after 5 min reaction with a preparation obtained from *Penicillium oxalicum* are shown in Fig. 3.

Peaks of DP 5 and DP 6 unsaturated oligomers were the highest at 230 nm. The peaks of the unsaturated DP 3 and DP 7 oligomers were not detected at 214 nm while saturated DP 4 was not detected at 230 nm. The peak at 2.8 min was not identified. The separation was effective between saturated and unsaturated compounds.

Up to DP 8, the retention time of the unsaturated compounds was smaller compared with the corresponding saturated oligomer. From DP 9, it was the opposite.

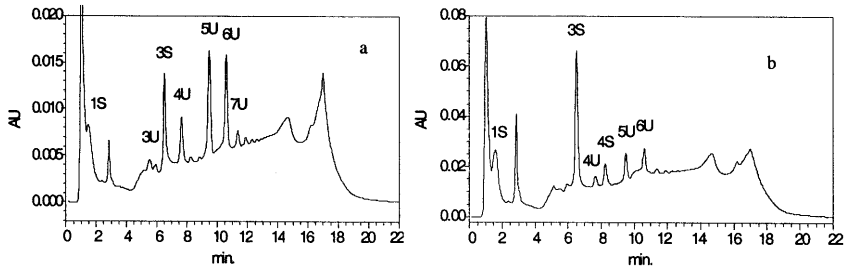

Fig. 3. Chromatograms of a sample of PGA_C after 5 min reaction with an enzymatic preparation of *Penicillium oxalicum*: (a) at 230 and (b) at 214 nm. 1, 3, 4, 5, 6, 7 polymerisation degrees. S, saturated; U, unsaturated.

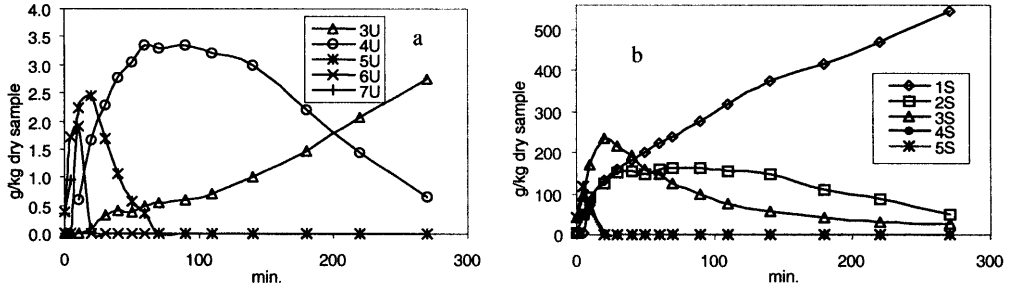

Fig. 4. Depolymerisation pathway of (a) pectate lyase (unsaturated oligomers) and (b) polygalacturonase (saturated oligomers). The concentration is given in g/kg of the sample powder sodium polygalacturonate (Sigma P3850).

The method did not allow a good separation of DP 5 and DP 6 saturated and unsaturated oligomers. However, when the peak was pure, it could be identified by matching its spectrum with those of the library. The unsaturated monomer was not detected.

The method of separation allowed us to follow two distinct mechanisms of pectate depolymerisation by an enzymatic preparation from *Penicillium oxalicum* (Fig. 4(a,b)). In Fig. 4(a), the concentration of unsaturated oligomers is plotted against the time of reaction in order to show the pectate lyase pathway. The presence of high DP oligomer (7 and 6) was fleeting. The appearance of the oligomers followed a decreasing order. It seemed that the trimer was the final product of the reaction.

In Fig. 4(b), the concentration of saturated oligomers is plotted against the time of reaction in order to show the polygalacturonase pathway. The mechanism was similar to the lyase as the appearance of each oligomer followed a decreasing order, but the final product of the reaction was the monomer.

The proportion of saturated compounds produced by polygalacturonase activity was about 100 times greater compared with the unsaturated compounds produced by the lyase activity.

4. Conclusion

The ion-exchange chromatography method that we adapted allowed the separation of both saturated and unsaturated oligomers produced by enzymatic depolymerisation of pectic substances.

In the case of PGA depolymerisation, no specific treatment of the samples was needed. On the contrary, in the case of highly methoxylated pectin, the samples should be de-esterified prior to analysis. The samples had to be left in a borate buffer for at least 100 h at rt to reach a complete de-esterification.

For saturated oligomers up to DP 3, the quantification was rather easy as the pure DP 1, DP 2 and DP 3 were commercially available. For these DPs, UV spectrometry (214 nm) showed a linear relationship between molar extinction coefficients and degree of polymerisation. Quantification of higher DPs was carried out with their response factors predicted on the basis of the linear relationship between the latter and polymerisation degree.

For unsaturated oligomers, the response factors were derived from the extinction coefficient given in the literature for the double bond. A strong relationship was found between peak area at 230 nm and the concentration calculated with the theoretical coefficient.

Our results show that it is possible to follow and quantify two distinct enzymatic pathways of the degradation of polygalacturonate simultaneously.

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