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Determination of saturated and unsaturated oligogalacturonic acids by means of thin-layer chromatography

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Abstract

The separation of saturated and unsaturated oligogalacturonic acids (oligoGalA) by HPTLC technique was improved by multiple developments with 1-propanol–water (PM) mixtures on silica gel 60 plates using automatic developing chamber (ADC). The ADC allowed a standardized course of characterization. The influence of the solvent composition, the number of developments and the solvent front on the separation of the oligomers, was investigated. Saturated oligoGalA up to the nonamer and unsaturated oligoGalA up to a degree of polymerization of 13 were separated using 5 developments with PM mixtures between 7:4.50 and 7:2.75. The meta-hydroxydiphenyl reagent is proposed for the postchromatographic derivatization.

Keywords: Oligogalacturonic acids

1. Introduction

The homogalacturonan component of the cell wall polysaccharide pectin consists of long chains of galacturonic acid residues which are partly esterified with methanol [1]. Oligogalacturonic acids (oligoGalA) are the intermediate or end products of pectin degradation by action of polygalacturonases, pectin lyases or pectate lyases of the endo-type from yeasts, molds and bacteria [2]. They play an important role as regulatory molecules derived from plant cell wall [3,4]. OligoGalA are also found to be present as intermediate products during action of human faeces flora on pectin in vitro [5].

Thin-layer chromatography (TLC) is a usual method for characterization of oligoGalA. Different modifications were developed and discussed in the last years; OligoGalA were separated on silica gel in *n*-butanol–formic acid–water (2:3:1) [6,7] or in *n*-butanol–acetic acid–water (2:1:1) [8], by 2 developments on cellulose in ethyl acetate–acetic acid–water (4:2:3) [9,10] or on microcristalline cellulose coated on an aluminium base in ethyl acetate–*n*-butanol–formic acid–water (1:3:5:2) [11]. Other autors used TLC on silica gel with 1-propanol–water (7:2) [12,13]. Doner et al. [14] separated oligoGalA up to a degree of polymerization (DP) 9 on HPTLC silica gel plates with ethanol–25 m*M* acetic acid (21:29). Likewise unsaturated oligoGalA were analysed on silica gel plates with toluene–chloroform–acetone (40:25:35) [3]. Further TLC, using development with ethyl acetate–acetic acid–water (4:2:2) was applied revealing the exo-mechanism of pectolytic enzymes from *Sclerotina sclerotium* [15]. Liu and Luh [10] found, that solvents with higher polarity gave a better separation of oligoGalA with DP>4. It was

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shown that small differences in composition of mobile phase can influence the R_F values and the separation of oligoGalA [10,12,14].

The spots were evaluated after derivatization by spraying or dipping [16], e.g., by treatment with saturated lead acetate solution, with the aniline–phthalate reagent [11], with the vanillin–sulfuric acid reagent [8] or with the aniline–diphenylamine–phosphoric acid reagent [12]. Furthermore carboxyl group specific reagents (bromophenol blue or methyl red reagent) and aldehyde groups specific reagents (aniline–orthophosphoric acid or meta-phenylenediammonium dichloride–oxalic acid reagent) were also used for detection of oligoGalA spots on TLC chromatograms [10].

Frey and Zieloff [17] discussed the possibilities of multiple developments on TLC plates: developments with the same or with different solvents or by using automated multiple development devices.

In this paper a method is described for separation and determination of saturated and unsaturated oligogalacturonic acids using HPTLC technique and multiple developments with 1-propanol–water mixtures of different compositions.

2. Experimental

2.1. Preparation and characterization of pectic acid

The galacturonan (anhydrogalacturonic acid, AG) content of pectin and oligoGalA preparations was estimated by the meta-hydroxydiphenyl (MHDP) method [18]. Methyl ester groups were analyzed by the chromotropic acid method [19]. Intrinsic viscosity [η] related to molecular mass was determined in 0.155 *M* NaCl (high-esterified) or in 0.05 *M* NaCl/0.005 *M* Na-oxalate (pectic acid) at 25.0°C and pH 6.0 using an Ubbelohde viscosimeter.

Pectic acid, the prefered substrate of polygalacturonases and pectate lyases, was prepared from a high-esterified citrus pectin (Copenhagen Pectin A/S, Lille Skensved, Denmark) with following parameters: galacturonan 65.8%, degree of esterification (DE) 70.8% and [η] 692 ml/g AG. A 125-g amount of this pectin was treated for 30 min at 20°C in 70% ethanol containing 50 ml conc. HCl with stirring for removal of di- and polyvalent cations. The pectin was repeatedly washed with 70% EtOH and then de-esterified under heterogenic conditions in 2 l 50% EtOH containing 25 g NaOH and 35 g KOH (30 min at 20°C). After neutralization the pectic acid was isolated by washing with EtOH (50 to 96%) and acetone. The pectic acid possessed the following parameters: galacturonan 71.2%, DE 0% and [η] 436 ml/g AG.

The anorganic chemicals and sodium oxalate used were purchased from Fluka Chemika-BioChemika (Neu-Ulm, Germany).

2.2. Pectic enzymes

A 2.5-g amount of Rohament P, a commercial endo-polygalacturonase (EC 3.2.1.15) preparation from *Aspergillus spec.* (Röhm, Darmstadt, Germany), was dispersed in 150 ml water and dialyzed 24 h at 4°C against water for removal of sugars and other low-molecular-mass substances. The enzyme was isolated by precipitation and centrifugation in 50% EtOH at 4°C. The polygalacturonase activity [20] was increased from 3.7 to 181 units/mg. A pectate lyase (EC 4.2.2.2.) culture concentrate from *Erwinia carotovora* (Institute of Potato Research, Groß Lüsewitz, Germany) was purified in the same manner. The pectate lyase activity [21] increased from 220 units/ml to 5400 units/g.

2.3. Preparation of oligogalacturonic acids

For preparation of mixtures of saturated oligoGalA, 100 ml pectic acid solution (1% galacturonan) were incubated at 45°C and pH 4.5 with 20 polygalacturonase units. Unsaturated oligoGalA mixtures were obtained from pectic acid at 30°C and pH 8.5, using 40 pectate lyase units in presence of 2 m*M* $CaCl_2$. The degradation was stopped by adding 1 *M* HCl to pH 2.0 and 100 ml of 96% EtOH. After stirring, the mixture was centrifugated 30 min (3000 *g*) at 4°C. The supernatants were heated for 10 min at 80°C for inactivation of possible enzyme activities present and then centrifugated for a second time. The oligoGalA fraction was isolated by vacuum drying under nitrogen. The yield of AG in the oligoGalA fraction was 35–65%.

2.4. Thin-layer chromatography

Between 1 to 4 μl 0.5% oligoGalA solutions were applied 8 mm from the bottom by spraying (6 mm streaks) with the automatic TLC sampler III (Camag, Muttenz, Switzerland) on 10×10 or 20×10 cm HPTLC silica gel 60 F254 plates (Merck, Darmstadt, Germany). The chromatograms were developed in the automatic developing chamber (ADC) from Camag: run distance was 40–80 mm, drying time 10 min, heating time 1.5 min, precondition time 5 min and tank configuration. Four variants of multiple developments with 1-propanol–water mixtures were tested:

Variant A: 1st run 1-propanol–water (7:3.75), 60 mm; 2nd run 1-propanol–water (7:2.75), 80 mm.

Variant B: 2nd run 1-propanol–water (7:3.75), 60 mm; 2nd run 1-propanol–water (7:2.75), 80 mm; 3rd run 1-propanol–water (7:2.75), 80 mm; 4th run 1-propanol–water (7:2.75), 80 mm.

Variant C: 1st run 1-propanol–water (7:3.75), 60 mm; 2nd run 1-propanol–water (7:3.25), 70 mm; 3rd run 1-propanol–water (7:2.75), 80 mm.

Variant D: 1st run 1-propanol–water (7:4.50), 40 mm; 2nd run 1-propanol–water (7:4.00), 50 mm; 3rd run 1-propanol–water (7:3.50), 60 mm; 4th run 1-propanol–water (7:3.00), 70 mm; 5th run 1-propanol–water (7:2.75), 80 mm.

For the runs 1–5 the development durations in variant D were: 20.9 min, 25.5 min, 35.8 min, 49.2 min and 67.1 min, respectively.

2.5. Detection of oligoGalA

The double bonds of unsaturated oligoGalA were measured at 235 nm using TLC scanner II with CATS software (Camag). The plates were then dipped twice for 3 s in a mixture of 20 ml conc. sulfuric acid, 20 ml water and 360 ml acetone using the chromatogram immersion device III (Camag) and heated for 5 min at 80°C. After cooling the plates, they were dipped twice for 3 s in a 0.5% solution of MHDP (Eastman Kodak, Rochester, NY, USA) in acetone and then heated again for 10 min at 100°C. The red-brown spots on a pale blue ground were scanned at 525 nm.

3. Results and discussion

The saturated oligogalacturonic acids were obtained by hydrolysis of pectic acid (completely deesterified pectin) with a polygalacturonase preparation from moulds. Unsaturated oligoGalA are the intermediate or end products of lytic (transeliminative) cleavage of pectin or pectic acid by pectin lyase or pectate lyase. An enzyme preparation from bacteria (free from polygalacturonase) was used for preparing the unsaturated oligoGalA. Generally mixtures of both saturated and unsaturated oligoGalA can only be obtained if pectic enzymes of different origin are used for pectin depolymerization. The separation of such mixtures of saturated and unsaturated oligoGalA is principally possible, but was not investigated.

The separation of saturated and unsaturated oligoGalA by HPTLC technique was improved by multiple developments with 1-propanol–water mixtures on silica gel 60 plates using the automatic development chamber. The ADC allowed a standardized course of developments. The influence of the solvent composition, the number of developments and the solvent front on the separation of the oligomers were investigated. In the following, four selected variants of the separation method are discussed.

The spots were visualized by dipping in the MHDP reagent, which is adapted to a wide-spread sensitive colorimetric determination method of pectin [18]. By the action of sulfuric acid the galacturonan units of pectin or of oligoGalA were converted to furan derivatives [22]. These furan derivatives form red coloured complexes with MHDP, which have an absorption maximum at 525 nm. In this way the intensity of the red coloured spots on the thin-layer chromatograms is a measure of the amount of uronide present. It was favourable to dip the plates first in sulfuric acid/acetone and than to heat. Later the plates were dipped in MHDP/acetone follwed by a second heating step. The same method was used for detection of the unsaturated oligomers.

The basic procedure is variant A consisting of a first development with the PW mixture 7:3.75 (60 mm) followed by a second run with the PW mixture 7:2.75 (80 mm). The development with a high proportion of water in the solvent is favourable for

the transport of oligomers with a DP>3. Variant B included three developments with the PW mixture 7:2.75 (80 mm). This led to a higher mobility of the saturated mono- to tetraGalA (Fig. 1). In case of pentaGalA the same R_F value was estimated (Table 1). The variant C differs from variant A through the additional development step with the PW mixture 7:3.25 (70 mm). In this case the R_F values were lower than in variant B but higher than in A. With these variants, saturated oligoGalA up to DP 5 (or 6) could be separated.

Variant D included 5 development steps with increasing solvent fronts each having higher parts of 1-propanol than prior one in the solvent. By this means a "pseudo-gradient" was realized. In contrast to variants A, B and C this procedure allowed the determination of saturated oligoGalA up to a DP of ca. 9 and additionally, the separation was improved (Table 1).

In general unsaturated oligoGalA showed a similar behaviour during HPTLC with the four variants of development. However the R_F values were higher at the same DP than those of saturated oligomers. Only in the case of the monomer and of diGalA the R_F values in the variant B were higher than in variant D (Table 2). This is an effect of the threefold development with 1-propanol–water 7:2.75. With the use of variants A, B and C a separation from the monomer to the octamer could be realized. Using variant D unsaturated oligoGalA up to a DP of 13 were estimated by HPTLC technique.

Additionally the unsaturated oligomers were detected by measuring the plates at 235 nm before derivatization. The double bonds between the C_4 and C_5 atoms on the non-reducing end of oligomers formed by the action of lyases absorb intensively at this wave length. The unsaturated monoGalA (DP 1) is quickly rearranged to 4-deoxy-5-ketouronic acid (4-deoxy-L-*threo*-5-hexoseulose uronic acid) [23]. As a result, it cannot be detected at 235 nm. However it does appear on the chromatograms after reaction with the MHDP reagent. Typical chromatograms after separation of unsaturated oligomers are shown in Fig. 1 (absorption at 525 nm after using the MHDP reaction).

The HPTLC separations were tested with individual mono- to hexaGalA obtained from enzymatic hydrolysates by anion-exchange chromatography.

Liu and Luh [10] found a linear relationship between the DP and the logarithm of the partition function if ethyl acetate–acetic acid–water (4:2:3) was used as solvent but not when ethyl acetate–acetic acid–water (2:1:2) was applied. In similar manner a linear correlation between the degree of polymerization and the logaritm of $R_F/(1-R_F)$ was shown in our experiments with the exception of variant D. In the later case more than one linear region was found in case of unsaturated oligoGalA (Fig. 2 and Fig. 3). Using variant D more complex separation effects occur, which cannot be described by a simple linear relationship. The different slopes in the linear range are connected with structural differences and physicochemical properties of the oligoGalA, caused by the double bonds on the non-reducing end of the unsaturated oligomers and by the conditions of separation.

4. Conclusions

The separation of saturated and unsaturated oligoGalA by multiple developments with the applied PW mixtures is a relative time-consuming process and requires standardized conditions. HPTLC of these oligomers can be carried out under reproducible conditions using the automatic developing chamber. The chromatography with "pseudo-gradients" without taking out the plates after every run is a precondition for a good separation. It is shown that the solvent composition, the number of developments and the solvent front influence the separation of oligoGalA. Saturated oligoGalA are less mobil on silica gel plates than the unsaturated ones. Similarly differences in the chromatographic behaviour between saturated and unsaturated oligoGalA were also found in high-performance anion-exchange chromatography [24].

The meta-hydroxydiphenyl reagent is proposed for the postchromatographic derivatization of oligoGalA. In contrast to colorimetric MHDP reaction [18], it is not specific for uronides in TLC techniques. Additionally neutral mono- and oligosaccharides also gave red-brown spots with this reagent.

Multiple development technique is an effective method for improving the separation of closely

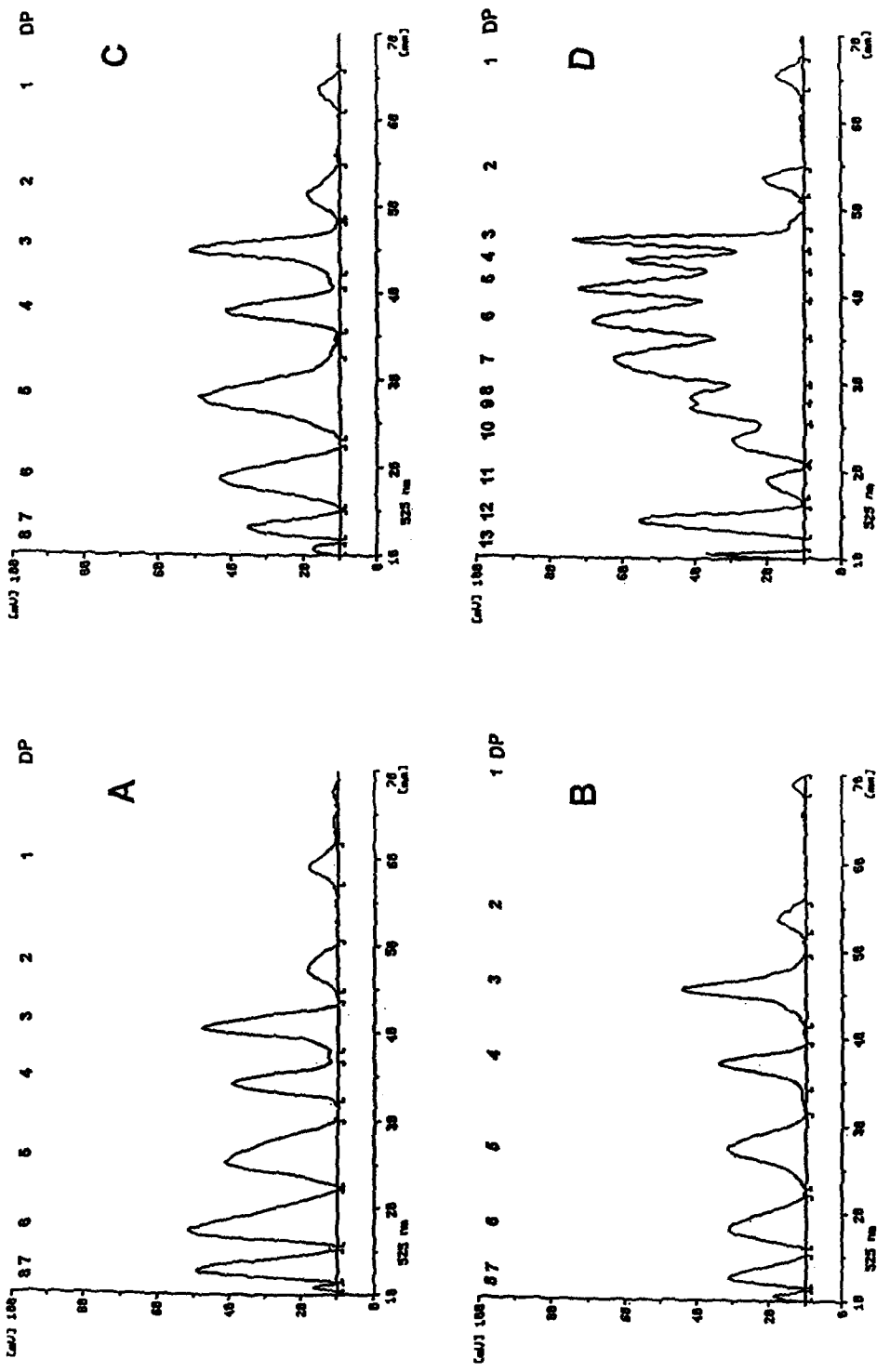

Fig. 1. Separation of unsaturated oligogalacturonic acids by HPTLC on silica gel 60 using multiple developments with different 1-propanol–water mixtures as solvent and meta-hydroxydiphenyl reagent (absorbance at 525 nm). Conditions for variants A–D see Section 2.4. (DP: degree of polymerization).

Table 1
R_F values in separation of saturated oligogalacturonic acids with different variants of development[a]

DP	Variant A	Variant B	Variant C	Variant D
1	0.52	0.64	0.60	0.63
2	0.39	0.48	0.46	0.51
3	0.23	0.30	0.29	0.44
4	0.11	0.15	0.14	0.36
5	0.06	0.06	0.07	0.29
6			0.04	0.23
7				0.18
8				0.13
9				0.06

[a] For variants see Section 2.4.

Table 2
R_F values in separation of unsaturated oligogalacturonic acids with different variants of development[a]

DP	Variant A	Variant B	Variant C	Variant D
1	0.71	0.84	0.77	0.80
2	0.54	0.62	0.59	0.61
3	0.44	0.54	0.50	0.52
4	0.35	0.40	0.41	0.48
5	0.23	0.26	0.27	0.44
6	0.13	0.14	0.14	0.39
7	0.07	0.06	0.07	0.33
8	0.04	0.03	0.04	0.28
9				0.24
10				0.19
11				0.16
12				0.08
13				0.03

[a] For variants see Section 2.4.

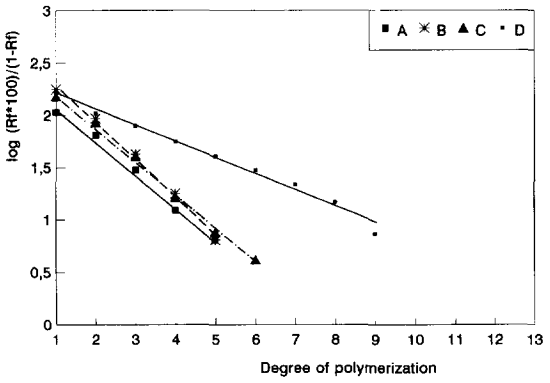

Fig. 2. Relationship between the logarithm of partition function and the degree of polymerization of saturated oligogalacturonic acids separated by HPTLC on silica gel 60 using multiple developments with different 1-propanol–water mixtures as solvent. Conditions for variants A–D see Section 2.4.

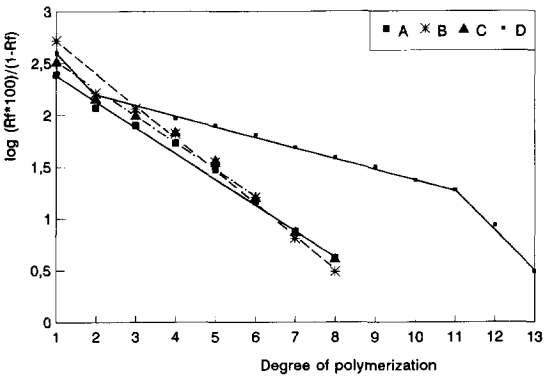

Fig. 3. Relationship between the logarithm of partition function and the degree of polymerization of unsaturated oligogalacturonic acids separated by HPTLC on silica gel 60 using multiple developments with different 1-propanol–water mixtures as solvent. Conditions for variants A–D see Section 2.4.

related components. In case of oligoGalA individual components differ in the degree of polymerization besides the presence or absence of double bonds. Normally the separation of oligogalacturonic acids with a DE>5 is difficult using TLC. Multiple developments improve the capacity of the silica gel layers and the selectivity of separation of the individual oligomers.

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