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Note

Product analysis of the action of *Trichoderma reesei* polygalacturonase by thin-layer chromatography

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Separation of oligo(D-galactosiduronic acids) up to degree of polymerization (DP) 5 can be achieved by paper chromatography[1] in ethyl acetate–acetic acid–water (18:7:8). Thin-layer chromatography (TLC) on silica gel[2] in *n*-butanol–formic acid–water (2:3:1) was found to be more efficient, leading to rapid separation of D-galactopyranuronic acid and oligomers up to DP 6. TLC on cellulose in ethyl acetate–acetic acid–water (4:2:3) by a two-fold development effected separation up to hexamer and was used in characterization of *Rhizopus arrhizus*[3] and papaya[4] polygalacturonases.

The present note deals with separation of mono- and oligo(D-galactosiduronic acids) up to DP 9 by TLC on microcrystalline cellulose and with application of this method for identification of products of the action of *Trichoderma reesei* polygalacturonase on polymeric and oligomeric substrates.

EXPERIMENTAL

D-Galactopyranuronic acid was a product of Fluka, Switzerland. Oligo(D-galactosiduronic acids) of DP 2–9 were prepared and characterized according to Rexová-Benková[5]. Pectic acid of limit viscosity number $[\eta] = 125$ ml g^{-1} and degree of esterification (d.e.) = 0.5%, was prepared by alkaline de-esterification of citrus pectin according to Tibenský and Kohn[6].

TLC was performed on a microcrystalline cellulose coated on an aluminium base (Lucefol, product of Kavalier, Czechoslovakia), in ethyl acetate–*n*-butanol–formic acid–water (1:3:5:2). D-Galactopyranuronic acid and its oligomers were visualized by spraying with saturated aqueous solution of lead acetate[7] or by dipping in the aniline–phthalate reagent in acetone, in both cases followed by heating for 20 min at 80°C.

Polygalacturonase (E.C. 3.2.1.15) was prepared from culture filtrate of *T. reesei* QM 9414 grown on pectin as the carbon source, by ammonium sulphate precipitation and separation on Sephadex G-25 and G-100 columns.

The enzyme (0.08 mg) was incubated with 1 μmol of oligomeric substrate (or 5 mg of pectic acid) in 2 ml of 0.1 *M* acetate buffer of pH 5.0 at 30°C, and 10–20-μl aliquots were withdrawn at intervals and analysed by TLC.

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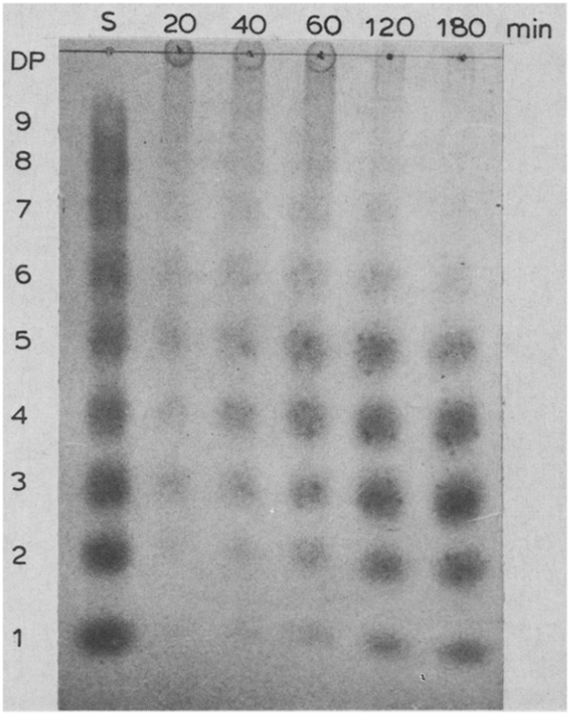
RESULTS AND DISCUSSION

Of several solvent systems examined for TLC on microcrystalline cellulose, the most convenient for the separation of oligo(D-galactosiduronic acids) was found to be ethyl acetate–*n*-butanol–formic acid–water (1:3:5:2). One development in this system brought about the separation of the monomer and oligomers up to DP 5 within 1 h. Two developments led to separation up to the heptamer (Fig. 1) and after the third development it was possible to separate oligomers up to DP 9 (Fig. 2). Visualization of higher oligomers with lead acetate regent was more sensitive than that with aniline phthalate.

This separation procedure was used to examine the mode of substrate degradation by *T. reesei* polygalacturonase. Pectic acid was initially split by the enzyme to give a mixture of products containing a small amount of monomer and a greater amount of oligomers DP 2–7. Later (after 120 min), monomer and lower oligomers



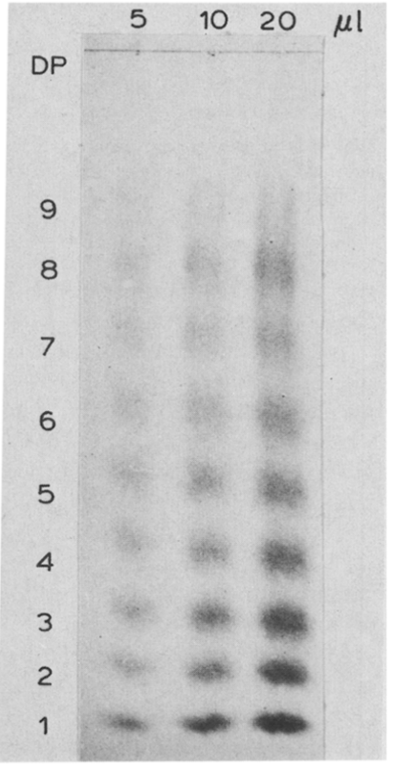


Fig. 1. Separation of oligo(D-galactosiduronic acids) formed on hydrolysis of pectic acid by *T. reesei* polygalacturonase by TLC on microcrystalline cellulose. TLC sheet: Lucefol, 20 cm in length. Solvent: ethyl acetate–n-butanol–formic acid–water (1:3:5:2); developed twice. Spray regent: lead acetate. S: standard mixture obtained by dissolving monomer and oligomers of DP 2–9 (1 mg of each) in 5 ml of water. Time intervals are for incubation of pectic acid with enzyme, 20-μl aliquots.

Fig. 2. Separation of standard mixture of mono- and oligo(D-galactosiduronic acids) by TLC. TLC sheet, solvent system and detection as in Fig. 1; developed three times; 5–20 μl of standard mixture (sample S in Fig. 1).

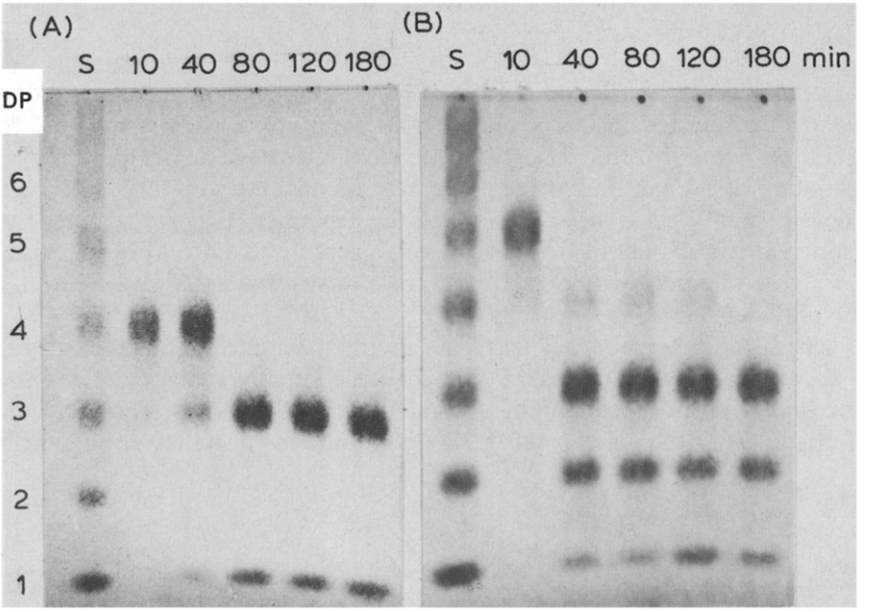


Fig. 3. Separation of products of hydrolysis of tetra(D-galactosiduronic acid) (A), and penta(D-galactosiduronic acid) (B) catalysed by *T. reesei* polygalacturonase. TLC sheet: 15 cm in length. Solvent, development and detection as in Fig. 1.

(up to DP 5) predominated (Fig. 1). These findings indicate a random cleavage of the polymeric substrate.

The tetramer was specifically cleved by *T. reesei* polygalacturonase to monomer and trimer (Fig. 3A). The pentamer was hydrolysed either to the monomer and the tetramer, or to the dimer and the trimer (Fig. 3B).

These results show that *T. reesei* polygalacturonase may be included in the group of polygalacturonases with a primary binding site composed of four subsites and with a cleavage of oligomeric substrates by pattern A, similar to polygalacturonases from *Saccharomyces fragilis*, *Aspergillus niger* and *Acrocylindrium*[8].

The advantage of the described procedure is a good separation of oligo(D-galactosiduronic acids) up to DP 9, which offers a rapid determination of the mode of action of polygalacturonases on polymeric and oligomeric substrates.

The TLC method of oligo(D-galactosiduronic acids) separation on Eastman cellulose sheets described by Liu and Luh[9] and used for characterization of *Rhizopus arrhizus* polygalacturonase[3] gives a satisfactory separation of oligomers only up to DP 6 (as seen on chromatograms presented in both papers).

The solvent system used in this note considerably differs from that described by Liu and Lu[3,9]: in addition to ethyl acetate it contains *n*-butanol, and acetic acid is replaced by formic acid. It can be seen from Table I and Fig. 4 that the differences between the R_{ga} values of oligo(D-galactosiduronic acids) obtained using both the solvent systems discussed. A single development of the sheet in the solvent sytem ethyl acetate–*n*-butanol–formic acid–water (1:3:5:2) gives a better separation of the higher oligomers than a double development in that of Liu and Luh[3,9].

TABLE I

CHROMATOGRAPHIC MOBILITIES OF OLIGO(D-GALACTOSIDURONIC ACIDS) SEPARATED ON TLC CELLULOSE SHEETS IN DIFFERENT SOLVENT SYSTEMS

The R_{ga} value is the ratio of the distance of the spot from origin to that of D-galactopyranuronic acid. Abbreviations: E-A-W = ethyl acetate–acetic acid–water; E-B-F-W = ethyl acetate–butanol–formic acid–water; 1D, 2D, 3D = developed once, twice, or three times, respectively. The values in column 2 were taken from Table III of ref. 9.

DP	*R_{ga} values*			
	Ref. 9	*This work*		
	E-A-W (4:2 3), 2D	*E-B-F-W (1.3·5:2), 1D*	*E-B-F-W (1:3 5:2), 2D*	*E-B-F-W (1:3·5:2), 3D*
2	0.76	0.78	0.86	0.93
3	0.58	0.63	0.74	0.85
4	0.38	0.49	0.62	0.75
5	0.22	0.36	0.49	0.65
6	0.12	0.26	0.38	0.54
7	0.06	0.18	0.27	0.43
8	0.03	0.13	0.18	0.32
9	0.01	0.08	0.11	0.24

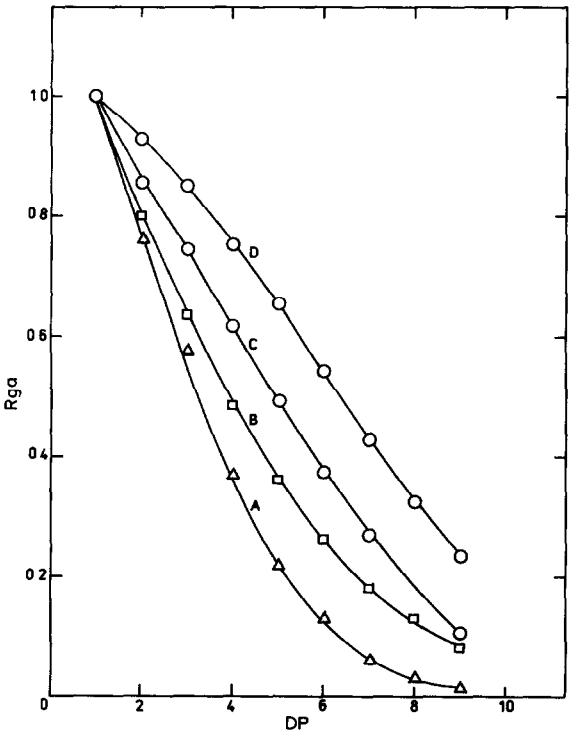


Fig. 4. Relationship between the degree of polymerization and migration of oligo(D-galactosiduronic acids) on cellulose TLC in two different solvent systems. (A) Solvent system: ethyl acetate–acetic acid–water (4:2:3), Eastman 13255 cellulose sheet, developed twice (taken from Table III and Fig. 4 of ref. 9). (B)–(D) Solvent: ethyl acetate–*n*-butanol–formic acid–water (1:3:5:2), Lucefol sheet, developed once, twice and three times, respectively.

REFERENCES

1 D. French and D. M. Wild, *J. Amer. Chem. Soc.*, 75 (1953) 2612.
2 A. Koller and H. Neukom, *Biochim. Biophys. Acta*, 83 (1964) 366.
3 Y. K. Liu and V. S. Luh, *J. Food Sci.*, 43 (1978) 721.
4 H. T. Chan, Jr. and S. Y. T. Tam, *J. Food Sci.*, 47 (1982) 1478.
5 Ľ. Rexová-Benková, *Chem. Zvesti*, 24 (1970) 59.
6 V. Tibenský and R. Kohn, *Czech. Pat.*, 137,935 (1970).
7 D. Waldi, in E. Stahl (Editor), *Dünnschicht-Chromatographie*, Springer, Berlin, Göttingen, Heidelberg, 1962, pp. 496–515.
8 Ľ. Rexová-Benková and O. Markovič, *Advan. Carbohydr. Chem. Biochem.*, 33 (1976) 323.
9 Y. K. Liu and B. S. Luh, *J. Chromatogr.*, 151 (1978) 39.