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ANALYSIS OF OLIGOMERIC AND MONOMERIC SACCHARIDES FROM ENZYMATICALLY DEGRADED POLYSACCHARIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The analysis of monomeric and oligomeric reaction products of enzymatically degraded polysaccharides using a cation-exchange, 8% cross-linked resin column in the lead(II) form (HPX-87P) is described. Digests of cellulose, xylan, arabinan and galactan prepared with various pure enzymes were characterized. Saccharides up to tetramers could be separated. With this method, information regarding enzyme purity, specificity and mode of action can rapidly be obtained.

INTRODUCTION

The analysis of the reaction products of enzymatically degraded polysaccharides provides valuable information on the substrate specificity and pattern of action of polysaccharide-degrading enzymes. Paper, thin-layer, high-performance thin-layer, ion-exchange and gel filtration chromatography have been used successfully for this purpose. High-performance liquid chromatography (HPLC) has simplified the difficult task of determining oligomeric and monomeric sugars. Its suitability was increased further by the introduction of fixed-ion resin columns[1], which has led to the development of ion-moderated partition chromatography and the design of specific resin columns for carbohydrate analysis. The resins have a polystyrene–divinylbenzene matrix with a degree of cross-linking ranging from 4 to 8%, and contain sulphonic acid groups which are loaded with a particular cationic counter ion. The resin is used in a non-ion-exchange mode and the cationic counter ion remains on the column, effecting the desired separation. The choice of cation determines the selectivity and resolution. Resins in the calcium(II) form have found general use in the separation of monosaccharides, oligosaccharides up to a degree of polymerization (DP) of 4 and sugar alcohols. Resins in the silver(I) form are more selective for oligosaccharides up to DP 11 and resins in the lead(II) form are more selective for a range of monomeric sugars[1].

In our studies on the enzymatic degradation of plant cell-wall polysaccharides, we were looking for an HPLC system which would enable the determination of the

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sugar composition of polysaccharide digests. Since these polysaccharides comprise various sugar residues, and since preliminary experiments showed that oligosaccharides of low DP could also be separated on a resin in the lead(II) form, an HPLC system with an HPX-87P column was applied to the analysis of polysaccharide digests. The saccharides present in the digests were identified by establishing their sugar composition. For this purpose they were isolated in fairly large quantities by gel permeation chromatography.

MATERIALS AND METHODS

Enzymes

Four types of endoglucanases (1,4-β-D-glucan glucanohydrolase, E.C. 3.2.1.4; Endo I, III, V and VI) and an exoglucanase (1,4-β-D-glucancellobiohydrolase, E.C. 3.2.1.91; Exo 1) were isolated from a commercial cellulase preparation as described by Beldman *et al.*[2]. Endoarabinanase (1,5-α-L-arabinan arabinohydrolase, E.C. 3.2.1.99), arabinofuranosidase (α-L-arabinofuranoside arabinohydrolase, E.C. 3.2.1.55), endogalactanase (1,4-β-D-galactan galactohydrolase, E.C. 3.2.1.89) and β-galactosidase (β-D-galactopyranoside galactohydrolase E.C. 3.2.1.23) were isolated from a fungal pectinase preparation kindly supplied by Gist-Brocades (Delft, The Netherlands)[3].

Substrates

The polysaccharides used as substrates were Avicel cellulose (Type SF; Serva, Heidelberg, F.R.G.) swollen in phosphoric acid as described by Wood[4], xylan (ex-oat spelts; Koch-Light, Haverhill, U.K.), arabinan (ex-sugar beets, Koch-Light) dialysed against distilled water and lyophilized prior to use, haze arabinan isolated from apple juice concentrate as described by Churms *et al.*[5] and galactan isolated from destarched potato fibre as described by Labavitch *et al.*[6] and treated with arabinofuranosidase to remove arabinose containing side chains from the galactan backbone. Table I summarizes the structural features of these polysaccharides.

Enzyme treatments

The substrates were dissolved or suspended (Avicel) in 0.05 *M* sodium acetate buffer pH 5. After the addition of the enzyme, the reaction mixtures were incubated at 30°C for various reaction times. The enzyme units added (expressed in μg protein per ml) and the incubation times are specified in the figure legends. The reactions were terminated by centrifugation of the residual substrate (cellulose, 10 min, 3000 *g*) and the enzymes in the clear reaction liquid were inactivated by immersion in a bath of boiling water for 5 min.

Apparatus

A Spectra Physics liquid chromatograph Model SP8000, equipped with an Aminex HPX-87P column (300 mm × 7.8 mm; Bio-Rad Labs., Richmond, CA, U.S.A.) and a guard column (50 mm × 4.6 mm) packed with a mixture of equivalent amounts of dried AG50W-X4 (H^+, 400 mesh) and AG3-X4A (OH^-, 200–400 mesh; Bio-Rad Labs.), was used. The life of the guard column was about 200 injections and of the analytical column at least 4000 injections. The analytical column was operated

TABLE I

STRUCTURAL FEATURES OF POLYSACCHARIDE SUBSTRATES

Abbreviations: Xyl = xylose; Ara = arabinose; Gal = galactose; p = pyranose ringform; f = furanose ringform.

Polysaccharide	*Main composite sugars*	*Main glycosyl linkages*		*"Systematic" name*
		Backbone	*Branch point*	
Cellulose	Glcp	1,4		β-1,4-D-Glucan
Xylan	Xylp (90%) Araf (10%)	1,4	1,3,4	β-1,4-D-Xylan with single unit arabinosyl side chains
Arabinan				
ex-sugar beets	Araf (90%) Galp (10%)	1,5	1,2,5/1,3,5 1,2,3,5	Highly branched L-arabinan, with galactose containing side chains
ex-aple juice	Araf (100%)	1,5		α-1,5-L-Arabinan
Galactan	Galp (90%) Araf (10%)	1,4 1,5	1,2,4/1,4,6	β-1,4-D-Galactan with arabinan side chains

at 85°C, the guard column at ambient temperature and a flow-rate of 0.5 ml/min with water (Millipore) as eluent. Sugars were detected with an ERMA-ERC 7510 refractive index detector thermostated at 40°C.

Sample pretreatment

Since cationic resins may cause the precipitation of proteins and uronides, a clean-up of the sample was necessary. A Bio-Rad deashing system as well as a guard column containing a mixed-bed ion exchanger, as described by Brons and Olieman[7] for a cation resin in the calcium form (HPX-87C), was insufficient for the resin in the lead form (HPX-87P) used in this study. An additional pretreatment of the sample with lead nitrate prior to injection was efficacious. For this purpose, aliquots of the enzyme digests were mixed with 0.1 or 1 *M* (depending on the oligomer concentration) lead nitrate solutions to bring the final concentration of lead nitrate in the mixture to 0.05 *M*. The precipitate which formed was removed by centrifugation (3000 *g*) and 20 μl of the clear supernatant were injected.

Identification of saccharides

To identify the saccharide fractions in the digests, these fractions were isolated in fairly large quantities by gel permeation chromatography. An aliquot of the sample was applied on a Bio-Gel P-2 (200–400 mesh, Bio-Rad Labs.) column (100 cm × 2.6 cm, V_0 = 150 ml) thermostatted at 50°C and eluted with degassed distilled water at

50°C. The fractions were analyzed for neutral sugar content by the phenol–sulphuric acid assay[8]. Those exhibiting a peak were pooled and evaporated to dryness, and the composite sugars of the saccharide fractions were analyzed as alditol acetates by gas chromatography[9].

RESULTS

A typical chromatogram of the separation, using our system, of a reference mixture containing five monomeric sugars plus saccharose, raffinose and stachyose is shown in Fig. 1. A good separation of the sugar mixture was obtained within 25 min. Fig. 2 shows the saccharides released from phosphoric acid-swollen cellulose by endoglucanase I and III and by exoglucanase I after incubation for 2 and 21 h. The different patterns of reaction products released by these gucanases, and the changes in the composition of the reaction mixture in the course of the degradation by Endo I and III and Exo I, are clearly demonstrated.

Electrophoretically homogeneous endoglucanase have been found to be able to degrade β-1,4-xylans[10,11]. This activity could also be demonstrated by HPLC. Fig. 3 shows chromatograms of xylan digests produced by endoglucanase V and VI after incubation for 3 and 20 h. Gel permeation chromatography (GPC) of digest samples on Bio-Gel P-2 gave elution profiles comparable to those obtained with HPLC. From their elution volumes and sugar compositions, the peaks were identified as xylose (X_1), xylobiose (X_2), xylotriose (X_3) and xylotetraose (X_4). From the chromatograms in Fig. 3 it is concluded that endoglucanase V and VI have different patterns of action on polymeric and oligomeric xylans.

Fig. 4 shows the chromatograms obtained for digests of linear and highly branched arabinans degraded with endoarabinanase and arabinofuranosidase. Comparable chromatograms were obtained by GPC and the peaks were identified as arabinose (A_1), arabinobiose (A_2) and arabinotriose (A_3). From the chromatograms it is concluded that endoarabinanase is only active towards linear arabinan, producing arabinose oligomers, while arabinofuranosidase prefers highly branched arabinans producing monomeric arabinose.

The compositions of galactan and galactotetraose digests produced by endogalactanase and β-galactosidase are shown in Fig. 5. The peaks were identified as

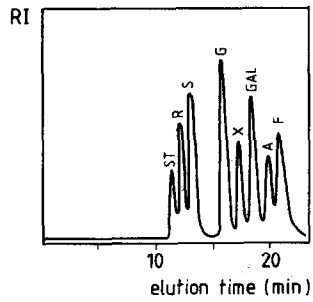

Fig. 1. Separation of stachyose (ST), raffinose (R), saccharose (S), glucose (G), xylose (X), galactose (Gal), arabinose (A) and fructose (F) on an HPX-87P column as specified in Materials and methods. RI = Refractive index.

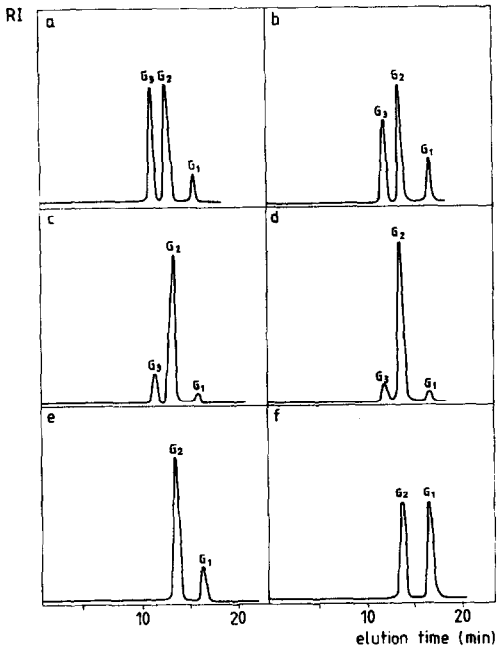

Fig. 2. HPLC analysis of products released from phosphoric acid swollen cellulose (1%) by cellulases (10 μg protein per ml) after incubation for 2 h and 21 h respectively; (a, b) Endo I; (c, d) Endo III and (e, f) Exo I. G_1 = Glucose; G_2 = cellobiose; G_3 = cellotriose.

galactotetraose (Gal_4), galactotriose (Gal_3), galactobiose (Gal_2) and galactose (Gal_1). β-Galactosidase was only active towards galacto-oligomers which could be completely degraded to galactose. The release of galacto-oligomers from galactan by endo-galactanase over an incubation period of 6 h is presented in Fig. 6. The tetramer,

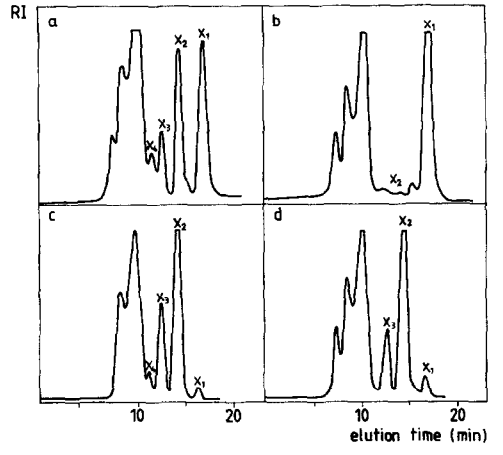

Fig. 3. HPLC analysis of reaction products of xylan (0.1%) with cellulases (60 μg per ml) after incubation for 3 and 20 h respectively: (a, b) Endo VI; (c, d) Endo V. X_1 = Xylose; X_2 = xylobiose; X_3 = xylotriose; X_4 = xylotetraose.

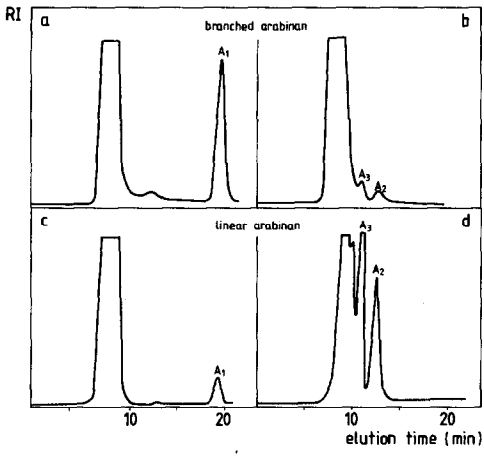

Fig. 4. HPLC analysis of reaction products released from branched and linear arabinans (0.4%) by arabinanases (20 μg protein per ml) after incubation for 1 h. (a, c) Arabinofuranosidase; (b, d) endoarabinanase. A_1 = Arabinose; A_2 = arabinobiose; A_3 = arabinotriose.

and to a lesser extent the dimer and trimer, were found to accumulate rapidly in the initial stage of the degradation. After 1.5 h the amount of the tetramers decreased, and the trimer content decreased after 3.5 h. The accumulation of dimer gradually levelled off; monomeric galactose accumulated at a low rate. The total amount of galactose oligomers reached a constant level, corresponding to 25% hydrolysis, within .3 h.

Table II summarizes the relative retention times of the various homologous series of oligomers and reference sugars.

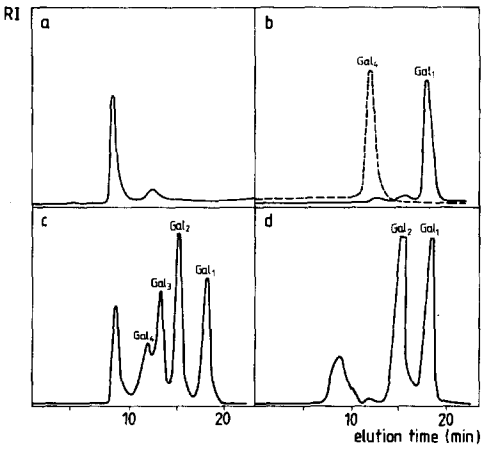

Fig. 5. HPLC analysis of reaction products released by β-galactosidase (1 μg protein per ml) from (a) galactan (0.02%) and (b) galactotetraose (0.02%) (dotted line is Gal_4 control) after incubation for 30 min and by endogalactanase(1 μg protein per ml) from galactan (0.4%) after incubation for 45 min (c) and 24 h (d). Gal_1 = Galactose; Gal_2 = galactobiose; Gal_3 = galactotriose; Gal_4 = galactotetraose.

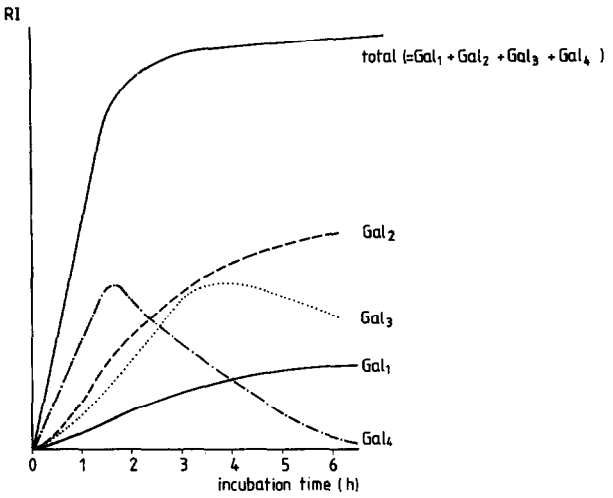

Fig. 6. Release of galacto-oligomers from galactan (0.4%) by endogalactanase (1 μg protein per ml) over an incubation period of 6 h. Abbreviations as in Fig. 5.

TABLE II

RELATIVE RETENTION TIMES OF MONO- AND OLIGOMERIC SUGARS COMPARED TO THOSE OF ARABINOSE

Sugar	*Relative retention time*				
	Mono	*Di*	*Tri*	*Tetra*	*Penta*
Arabinose	1	0.62	0,53	0.49	0.48
Xylose	0.86	0.74	0.66	0.59	–
Galactose	0.92	0.76	0.68	0.62	–
Glucose	0.81	0.67	0.58	0.52	–
Fructose	1.06	–	–	–	–
Saccharose	–	0.67	–	–	–
Raffinose	–	–	0.61	–	–
Stachyose	–	–	–	0.58	–

DISCUSSION

Amino-bonded silica columns (carbohydrate columns) are widely used for the analysis of mono- and oligosaccharides[12,13]. Brons and Olieman[7] have, however, demonstrated that this type of column is not suitable for the quantitative analysis of reducing sugars; it is insensitive, sugars are lost on the column due to interactions with amino groups and the column material is very unstable. A further disadvantage is that the retention times of oligomers increase with increasing DP; oligomers with high DP are strongly retained and can be eluted only by adapting the eluent.

The results presented have demonstrated that the resin column in the lead(II) form (HPX-87P) enables a rapid and sensitive estimation of the composition of poly-

saccharide digests. Oligomers up to DP 4 could be separated; larger oligomers and substrate residues passed through the column unretained. Resins in the silver(I) form exert greater selectivity for higher oligomers than resins in the lead(II) form[1]; good separations were obtained for cellulodextrins[14,15] and maltodextrins[16], up to a DP of 13. However, for the arabinan digest we observed partial hydrolysis of the relatively weak arabinofuranosidic linkages on this type of resin. For optimum performance they are not fully loaded with silver(I) ions, so that some sulphonic acid groups remain in the hydrogen form[16]. At the temperature of 85°C at which this column type is operated these groups will cause acid hydrolysis of weak glycosidic linkages. Leclercq and Hageman[17] have demonstrated this for saccharose. By operating the column at a lower temperature this hydrolysis can be minimized, but at the expense of the resolution of the oligomers. Van Riel and Olieman[18] suppressed acid hydrolysis by partially converting the column into the lead(II) form. By varying the ratio of the counter ions they were able to manipulate the column selectivity. Resins in the calcium(II) form are suitable for oligomers up to DP 4, but show poor selectivity for monomers.

The HPLC system studied here enabled a rapid and sensitive analysis of the reaction products of various enzymatically degraded polysaccharides, especially for the investigation of polysaccharides and their fine structure. The elution order of the reaction products allows conclusions to be drawn regarding enzyme purity, substrate specificity and the pattern of action of the enzyme. Further improvement of this system with regard to the separation of monomers and a broader range of oligomers is desirable.

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