Journal of Chromatography, 298 (1984) 525–530
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,912

Note

High-performance gel permeation chromatography assay for endoglycanase activities

ALAN R. WHITE, ALAN G. DARVILL, WILLIAM S. YORK and PETER ALBERSHEIM*
Department of Chemistry, Campus Box 215, University of Colorado, Boulder, CO 80309 (U.S.A.)
(Received May 22nd, 1984)

Polysaccharide-degrading enzymes can be divided into two groups according to the nature of their action. One group includes exoglycanases, which most commonly cleave mono- or disaccharides from the non-reducing ends of polysaccharides. The second group includes endoglycanases, which cleave internal glycosidic linkages in a more or less random fashion, thereby producing large oligosaccharides.

The activities of exo- and endoglycanases are readily measured by assaying the reducing groups of the newly liberated glycoses. Endoglycanases generally produce fewer reducing glycose residues than do exoglycanases. It is not possible to differentiate between endo- and exoglycanases by measuring the production of reducing groups because the greater production of reducing groups by exoglycanases will mask the presence of endoglycanases in mixtures of exo- and endo- enzymes.

The methods available for assay of endoglycanases in the presence of exoglycanases are laborious and unreliable. Viscometry is the most common. Because endoglycanases hydrolyze internal glycosidic bonds they reduce the viscosity of polysaccharide solutions far more quickly than exoglycanases. The endoglycanase-induced decrease in viscosity has been shown to depend on the number of bonds cleaved[1–4]; but direct proportionality between bond cleavage and viscosity reduction depends on random endoglycanase cleavage and uniform substrate structure, assumptions that frequently do not hold[5]. Another consideration is that viscometry requires a relatively large amount of polysaccharide substrate (on the order of 10 mg). Furthermore, not all polysaccharides yield solutions viscous enough to use in viscometry. Therefore, only a limited number of endoglycanases can be assayed with this technique.

Gel permeation chromatography has also been used to differentiate between exo- and endoglycanases[6]. Gel permeation chromatography can distinguish between monosaccharides or small oligosaccharides produced by exoglycanases and large oligosaccharides produced by endoglycanases. However, standard gel permeation chromatography is too slow for routine analysis of endoglycanases. This paper describes a high-performance (HP) gel permeation chromatography assay for endoglycanases that can be completed in 15 min per assay and that requires only 1 μg of sample.

0021-9673/84/$03.00

EXPERIMENTAL

Substrates, enzymes, and molecular weight standards

Xyloglucan. Xyloglucan was used for two different purposes. Xyloglucan was used as a substrate for enzyme digestion in the HP gel permeation chromatography assay, and purified xyloglucan oligosaccharides were used as size standards for the HP gel permeation column. Xyloglucan polysaccharide was prepared from suspension-cultured sycamore (*Acer pseudoplatanus*) cell extracellular polysaccharide (SEPS), as described[7]. Solutions of xyloglucan oligosaccharides, produced by endo-β-1,4-glucanase digestion of SEPS xyloglucan, were purified by Bio-Gel P-2 chromatography. The xyloglucan oligosaccharides had previously been shown to be penta-, hepta-, and nonasaccharide-rich fractions[7,8]. The endoglucanase-digestion products of xyloglucan also provided a mixture of larger oligosaccharides, that have been reported to be dimers of the hepta- and nonsaccharides[9], and polysaccharides that eluted in the void volume of the Bio-Gel P-2 column. The oligosaccharides were dissolved at 1–2 mg/ml in 100 m*M* sodium acetate, pH 5.2, for use as size standards.

Polygalacturonic acid (PGA) and PGA lyase. Citrus PGA (Grade III) was purchased from Sigma. Endo-α-1,4-polygalacturonic acid lyase (E.C. 4.2.2.2) (PGA lyase) was purified as described[10] and was provided by Keith R. Davis of this laboratory.

Other carbohydrate standards. Glucose was purchased from Sigma. Rhamnogalacturonan II was the gift of Dr. Laurence D. Melton of this laboratory[11]. These standards were prepared at 5 mg/ml in 100 *mM* sodium acetate, pH 5.2.

High-performance liquid chromatography (HPLC)

HPLC was performed with a Waters Model 6000A solvent delivery system. A Rainen high pressure line-filter was inserted upstream of the injector. A Valco CV-6-UHPa-N60 injection valve with a 50-μl injection loop was used throughout, as was a Waters Model R401 differential refractometer. The refractometer was maintained at 30°C with a Precision circulating water bath, model 250. Fractions eluting from the HPLC column were collected in test tubes with a Gilson Minifrac. Before use, all solvents were filtered through a Rainen 47-mm diameter Nylon-66 membrane filter (0.2 μm pore size) in an all-glass filter apparatus (Millipore). Gel permeation chromatography was performed on a Waters I-125 column equilibrated at 1.0 ml/min in 100 m*M* sodium acetate, pH 5.2.

Gel permeation analysis of endoglycanases

Enzyme reaction mixtures consisted of 50 μl of enzyme solution added to 450 μl of substrate. The mixtures were incubated on a shaker at 30°C and, at internals, 50-μl samples were withdrawn and injected on the HPLC column via the 50-μl Valco loop. Xyloglucan polysaccharide substrate was prepared at a concentration of 2 mg/ml and incubated with 10 units of *Trichoderma viride* endo-β-1,4-glucanase in 100 m*M* sodium acetate, pH 5.2 (1 unit of endoglucanase = 1 μmole of reducing glucose residues formed from 1.0% carboxymethylcellulose per min under the assay conditions). Polygalacturonic acid substrate was prepared at a concentration of 5 mg/ml and incubated with 0.15 units of PGA lyase in 50 m*M* Tris–HCl, pH 8.5, containing 1 m*M* $CaCl_2$ (1 unit of lyase = 1 μmole of unsaturated galactosyluronic acid termini produced per min under the assay conditions)[10].

RESULTS AND DISCUSSION

Elution volumes of oligo- and polysaccharide standards on the I-125 column

Two high molecular weight polysaccharides, xyloglucan and polygalacturonic acid, eluted from the I-125 column at a volume of 6.5 ml (Figs. 1A and 2A), which is assumed to be the void volume of the column. Glucose eluted at about 11 ml (point "a" in Fig. 1C), which is assumed to be the included volume of the column. The xyloglucan hepta- and nonasaccharide-rich fractions co-eluted at point "b" (Fig. 1C), and the xyloglucan oligosaccharides thought to consist of dimers of the nona- and heptasaccharides eluted at point "c" (Fig. 1C). Rhamnogalacturonan II, a highly branched pectic polysaccharide consisting of approximately 65 glycosyl residues[12], eluted as a single peak at point "d" (Fig. 1C), still resolved from polysaccharides that eluted in the void volume of the column. These results indicated that the I-125 column could resolve endoglycanase cleavage products of polysaccharides. The validity of this conclusion was established in the following experiments.

Endo-β-1,4-glucanase digestion of xyloglucan

Undigested xyloglucan eluted from the I-125 column in the void volume (Fig. 1A). Incubation of xyloglucan with the *Trichoderma* endoglucanase produced a time-dependent increase in elution volume of the carbohydrate (*i.e.*, time-dependent decrease in the size of oligosaccharide digestion products). The carbohydrates were detected in the column eluant by anthrone (data not shown) and refractive index (Fig. 1). After 15 min of enzyme digestion, the xyloglucan that originally eluted at the void volume of the column had been converted to a series of digestion products, which eluted in the partially included volume of the column (Fig. 1B). Digestion was virtually complete after 4 h (Fig. 1D and 1E).

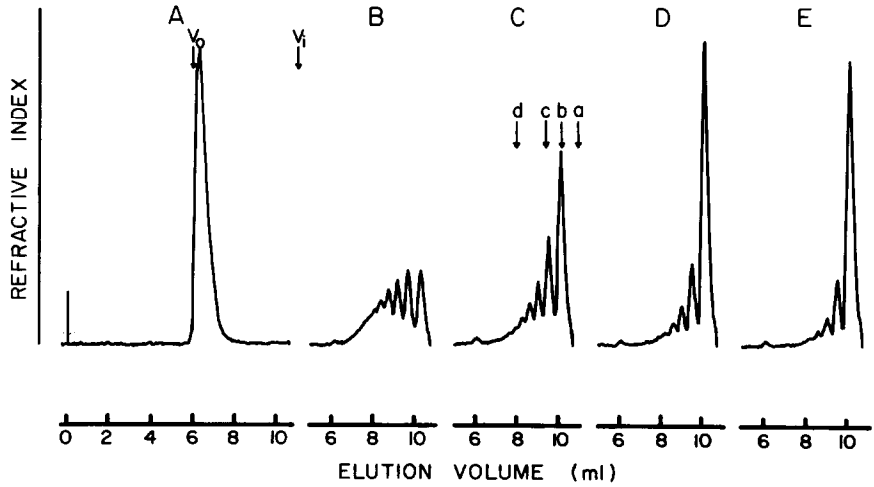

Fig. 1. Refractive index elution profile of xyloglucan polysaccharide (2 mg/ml) at progressive stages of digestion by *Trichoderma* endoglucanase (10 units). A, xyloglucan substrate before endoglucanase digestion; B, xyloglucan after 15 min incubation with endoglucanase; C, after 1 h; D, after 4 h; E, after 24 h. Elution volumes of standards: a, glucose, b, Bio-Gel P-2 purified xyloglucan hepta- and nonasaccharides, c, dimers of hepta- and nonasaccharides (14–18 glycosyl residues), d, rhamnogalacturonan II.

Digestion of polygalacturonic acid with PGA lyase

Undigested PGA eluted at the void volume of the I-125 column (Fig. 2A). Progressive digestion of the PGA with PGA lyase resulted in a shift in the refractive index profile to larger elution volumes. After 4 h of enzyme digestion, most of the PGA was converted to smaller oligosaccharides, but some material still eluted in the void volume of the column. Even after 22 h of enzyme digestion, material continued to elute in the void volume. This material might have been neutral sugar-rich polysaccharides that are not substrates of PGA lyase, but are known to be present in small amounts in PGA[13]. Enzyme-only controls showed that this void eluting material was not contained in the enzyme preparation added to the incubation mixture.

Unlike xyloglucan digested by endoglucanase (Fig. 1), the PGA digested by PGA lyase did not produce a series of discrete peaks (Fig. 2). Instead, a broad peak moved to the partially included regions of the column (2 h). After 4 h of enzyme digestion, the PGA was converted to smaller oligosaccharides. This is the expected result of random endoglycanase hydrolysis of a uniformly susceptible substrate.

Detection limits of the assay

The amount of poly- or oligosaccharide that can be detected with this assay procedure was estimated in the following manner. Varying amounts of rhamnogalacturonan II were chromatographed separately. The amount of polysaccharide injected onto the column ranged from 32.5 to 0.13 μg. As little as 1 μg of rhamnogalacturonan II was readily detectable above the background at refractometer attenuator settings that gave a stable base-line (Fig. 3). Therefore, with the techniques

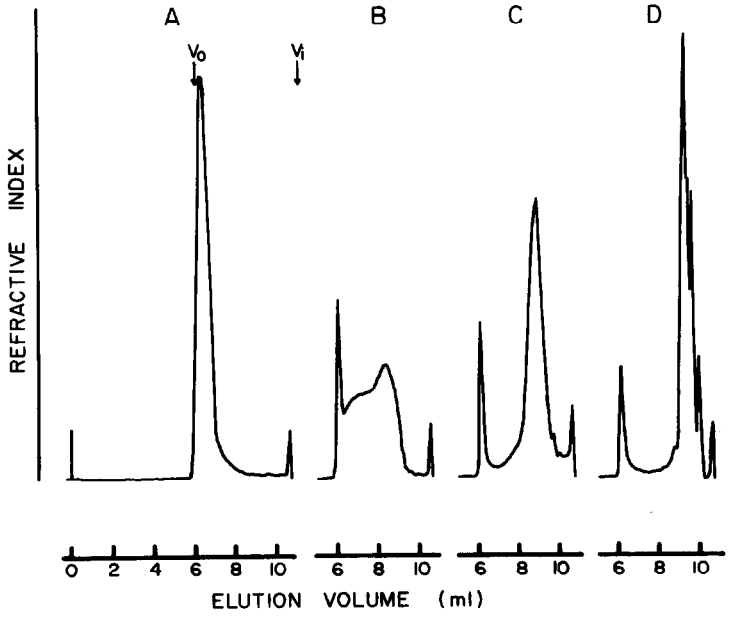

Fig. 2. Refractive index elution profile of PGA (2 mg/ml) at progressive stages of digestion by PGA lyase (0.15 units). A, PGA substrate before PGA lyase digestion; B, after 2 h PGA lyase digestion; C, after 4 h; D, after 22 h.

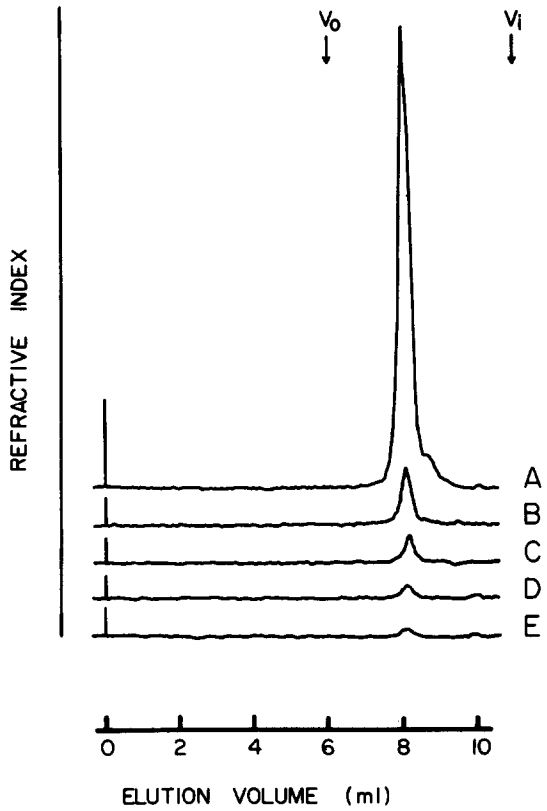

Fig. 3. Refractive index elution profiles generated by varying amounts of rhamnogalacturonan II: A, 32 μg of RG II; B, 8 μg of RG II; C, 2 μg of RG II; D, 1 μg of RG II; E, 0.5 μg of RG II.

described in this paper, small amounts of substrates can be used to detect endoglycanase activities in biological samples. Smaller volume injector loops, microbore liquid chromatography columns (which require only 1 or 2 min for elution), and more sensitive refractometers are available. Thus, HP gel permeation chromatography assay of 10–100 ng of substrate in a few minutes per sample should be feasible.

ACKNOWLEDGEMENTS

This work was supported by National Research Service Award 5 F32 (NIH) AM06982-02 (ARW) and Department of Energy contract DE-AC02-84ER13161 (AGD and PA).

REFERENCES

1 R. Thomas, *Aust. J. Biol Sci.*, 9 (1956) 159.
2 K. E. Almin, K.-E. Eriksson and C. Jansson, *Biochim. Biophys. Acta*, 139 (1967) 248.
3 K. E. Almin and K.-E. Eriksson, *Biochim. Biophys. Acta*, 139 (1967) 238.
4 R. Guignard and P.-E. Pilet, *Plant and Cell Physiol.*, 17 (1976) 899.

5 K. E. Almin and K.-E. Eriksson, *Arch. Biochem. Biophys.*, 124 (1968) 129.
6 D. J. Huber and D. J. Nevins, *Planta*, 151 (1981) 206.
7 W. S. York, A. G. Darvill and P. Albersheim, *Plant Physiol.*, 74 (1984) in press.
8 W. D. Bauer, K. W. Talmadge, K. Keegstra and P. Albersheim, *Plant Physiol.*, 51 (1973) 174.
9 T. Hayashi, Y.-S. Wong and G. Maclachlan, *Plant Physiol.*, in press.
10 K. R. Davis, G. D. Lyon, A. G. Darvill and P. Albersheim, *Plant Physiol.*, 74 (1984) 52.
11 M. W. Spellman, M. McNeil, A. G. Darvill and P. Albersheim, *Carbohydr. Res.*, 122 (1983) 115.
12 A. G. Darvill, M. McNeil and P. Albersheim, *Plant Physiol.*, 62 (1978) 418.
13 K. R. Davis, A. G. Darvill and P. Albersheim, in preparation.