Phytochemistry, 1975, Vol. 14, pp. 377–382. Pergamon Press. Printed in England.

PATTERNS OF OLIGOSACCHARIDE PRODUCTION BY POLYGALACTURONASES*

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(*Revised Received* 30 *March* 1974)

Key Word Index—*Aspergillus*; *Saccharomyces*; fungi; oligosaccharides; pectic substances; gel-filtration chromatography; polygalacturonase; enzyme mechanism.

Abstract—The products of hydrolytic action of 18 enzyme preparations at pH 3·5 and 5·5 on pectate were analyzed by gel-filtration chromatography early in the course of reaction (8–15% hydrolysis), and at a time 10 times that required for 10% hydrolysis. The degree of hydrolysis at the latter time varied from 25 to 74%. Three patterns of oligosaccharide production could be distinguished: *endo*-hydrolysis, *exo*-hydrolysis, and that due to *S*-polygalacturonase. The initial products of *endo*-hydrolysis were mixed oligosaccharides 5–30 units long; monomer and dimer appeared early but represented less than 2% of the products until late in the reaction. *exo*-Polygalacturonase (not entirely free of *endo*-) showed predominant production of the monomer and was clearly evident when mixed with four parts of *endo*-polygalacturonase. The time course of reducing group production by highly purified *S*-polygalacturonase could be reproduced by the above mixture of *exo*- and *endo*-polygalacturonases, but the pattern of products and the pH relations could not. The initial products of *S*-polygalacturonase were monomer, dimer and pentamer with lesser amounts of trimer and tetramer. After the hydolysis of the polymer and large oligomers, the pentamer was attacked by *S*-polygalacturonase, in the same way that the accumulated hexamer, etc. were finally hydrolyzed by the *endo*-polygalacturonase.

INTRODUCTION

It has been recognized for many years that the pattern of hydrolysis of polysaccharides differs with different enzymes. In particular, the *endo*-type, exemplified by α-amylase (E.C. 3.2.1.1), is thought to hydrolyse in a random manner, while the *exo*-type, such as β-amylase (E.C. 3.2.1.2), removes successive identical units from one end of the chain. Until the development of gel-filtration chromatography, however, there were no easy methods of actually observing the distribution of molecular sizes among the products of the so-called random hydrolysis, nor the residues from the endwise attack. This paper is a report of a study of such molecular distributions with purified preparations and mixtures of polygalacturonases [Poly (1,4-α-D-galacturonide) glycanohydrolase; E.C. 3.2.1.15; Poly (1,4-α-D-galacturonide) galacturonohydrolase, E.C. 3.2.1.67]. In addition to partially purified preparations which can be classified as *exo*- or *endo*-, a highly purified preparation is described which yields a pattern that is distinct from either. There is only one previous report on the use of gel-filtration chromatography to study the products of polygalacturonase [1].

RESULTS

Time course of hydrolysis; *standardized samples*

A standardized comparison between preparations (whose courses of hydrolysis deviated from linearity after 18–30% hydrolysis) was made by taking two samples for analysis at each pH, 1 during the linear hydrolysis period (8–15% hydrolysis) and 1 at 10 times the time required for 10% hydrolysis (10 × 10).

Table 1 shows the measured extent of hydrolysis at 10 × 10 for all the samples investigated, ranging from 25 to 74%. Table 1 also shows the ratios of the rates of hydrolysis at pH 3·5 to those at pH 5·5, ranging from 0·54 to 39.

None of the enzyme preparations tested hydrolyzed the pectate completely, and the most complete hydrolysis measured was only 85% at more than 1000 times the time required for 10% hydrolysis (fraction 3b). Furthermore, irrespective of the

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* This work was partially supported by National Science Foundation grants Nos. GB-3536 and GB-6587, and by the University of Nebraska Foundation.

Table 1. Extent of reaction and ratio of initial rate at pHs 3·5 and 5·5*

Enzyme preparation or fraction	Per cent hydrolysis at 10 × 10† pH 3·5	pH 5·5	Ratio of initial rates $\frac{\text{pH } 3{\cdot}5}{\text{pH } 5{\cdot}5}$
2d	48	48	39
3b	74	65	2·9
4b	42	37	1·6
5b	39	33	0·80
YPG‡	31	25	0·83
3B + YPG (4:1)	70		
3B + YPG (1:4)	48		
45AP	42	32	0·54
46AP	47	38	0·73
Lipase B	58	45	22

* Reaction used pectate (50 mM uronic acid equivalents), 50 mM NaCl or acetate, and sufficient enzyme to give 10% hydrolysis in 0·5–3 hr, except for fraction 2D and Lipase B where the pH 5·5 experiments took 39 and 65 hr to 10%, resp. The adjusted curves for 2D nevertheless coincided. Figure 1 contains gel-filtration analyses of the products from these experiments. The 100% value (50 mM) was determined colorimetrically, the degree of reaction by reducing group titration.

† Ten times the time required for 10% hydrolysis.

‡ Yeast polygalacturonase.

enzyme source or purity, at 10 × 10 there was a residue all the way to the void volume of the column. In this residual continuum, there were always two peaks, one at the void volume, and one further on. Summation of the colorimeter data gave 4·5% as the minimum amount in these two peaks. All of this continuum was predominantly galacturonic acid, but a concentrated and acid hydrolyzed sample of the first peak showed, in addition, the presence of rhamnose, xylose and galactose (plus a little glucose from starch). The lower MW peak contained more galacturonic acid, but other sugars were also present.

Patterns of oligosaccharides

Figure 1 (A–R) shows the patterns of small oligosaccharides found in the 10 × 10 samples, arranged in order of increasing per cent hydrolysis at that time. The higher members (P, Q, R) show a predominance of monomer; none of the other hydrolysis curves can be accounted for by a simple *exo*-hydrolytic mechanism, such as the exclusive production of dimer. On the other hand the patterns show a distribution of oligomers consistent with the per cent hydrolysis. In pattern B, for instance, the relative proportion of mono-, di-, tri-, and tetra-mer is 15, 20, 42 and 23%, respectively, on a molar basis, and can be calculated as representing 37% hydrolysis. Taking into account the higher residues (not shown), this is in good agreement with the 31% hydrolysis found by titration. Parts A–I, K (Fig. 1) form one series (25–47% hydrolysis) in which the dominant members gradually change from the trimer and tetramer to the dimer and trimer; at the same time as the pentamer and tetramer disappear, the dimer and monomer increase. Parts J, N–R form a second series, in which the monomer is dominant, becoming more so as the per cent hydrolysis increases from 45 to 74%, *and* in which the *trimer is more abundant than the dimer* (except in R). In contrast, parts L and M have the products arranged in a series decreasing continuously from monomer to pentamer. Thus, in Fig. 1 parts K, L, M and N show three different patterns of hydrolysis, all at 47–48% hydrolysis.

While the patterns of oligosaccharides at 10 × 10 are informative, those taken during the linear hydrolysis period are necessarily more indicative of the primary products of hydrolysis of the enzymes concerned. Three distinctive patterns again emerged, illustrated in Fig. 2.

One pattern is due to *endo*-polygalacturonase in which the primary product is a mixture of oligosaccharides between the penta- and 30-mer. Detailed study using the yeast polygalacturonase showed this material accumulated to more than half of the total saccharides during the rapid phase of the reaction. By the time the rate of reducing group production began to slow, only one third of the saccharides were in units of hexamer and smaller (mainly pentamer and hexamer) with an insignificant amount (<2%) of mono- and di-mer.

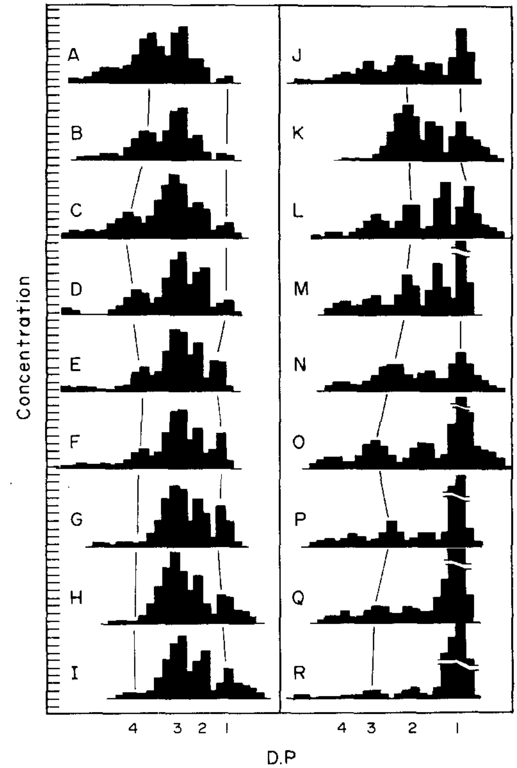

Fig. 1. Gel-filtration chromatography of oligosaccharides at 10 × 10% hydrolysis. Vertical scale marks are 100 Klett units (proportional to absorbance), using 0·2 ml aliquots of each fraction (*ca* 2 ml each, indicated by steps in pattern). Degree of polymerization (D.P.) is the number of monomer units per molecule. Samples (in order of % hydrolysis, see Table 1) are: A—yeast *endo*-polygalacturonase, pH 5·5; B—yeast *endo*-polygalacturonase, pH 3·5; C—Pectinol 45AP, pH 5·5; D—fraction 5b, pH 5·5; E—fraction 4b, pH 5·5; F—Pectinol 46AP, pH 5·5; G—fraction 5b, pH 3·5; H—Pectinol 45AP, pH 3·5; I—fraction 4b, pH 3·5; J—Lipase B, pH 5·5; K—Pectinol 46AP, pH 3·5; L—fraction 2d, pH 3·5; M—fraction 2d, pH 5·5; N—mixed 3b and yeast enzyme (1:4), pH 3·5; O—Lipase B, pH 3·5; P—fraction 3b, pH 5·5; Q—mixed 3b and yeast enzyme (4:1), pH 3·5; R—fraction 3b, pH 3·5. Estimated height of broken peak in M is 1000 units, and in O, P, Q and R is near 1500.

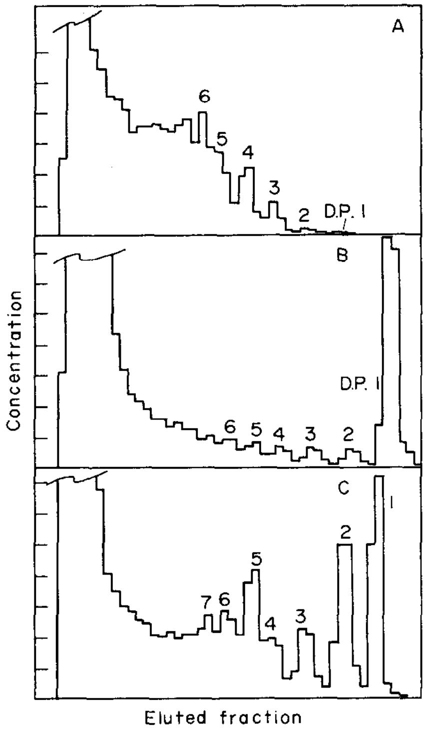

Fig. 2. Gel-filtration chromatography of early hydrolysis products of *endo*-, *exo*- and *S*-polygalacturonases. Coordinates as in Fig. 1. Part A shows yeast *endo*-polygalacturonase, pH 5·5, 95% viscosity reduction, 6% hydrolysis (0·8% pectate, diluted 2 × for chromatography, 0·9 ml aliquots); B is fraction 3b, pH 3·5, 8% hydrolysis (0·9% pectate, 0·8 ml aliquots); C is fraction 2d, pH 3·5, 10% hydrolysis (0·9% pectate, 0·8 ml aliquots).

Subsequently, the larger oligomers were hydrolysed, including the penta- and hexamer, producing the pattern shown in Fig. 1. (On further incubation, the tetramer disappeared.) The viscosity was reduced 90% about halfway through the linear hydrolysis period. The standardized survey samples at early times showed that all those in the first mentioned series above, except that due to the Pectinol 46AP at pH 3·5, (Fig. 1K), gave similar patterns. Using the yeast polygalacturonase as a standard, the extents of hydrolysis at 10 × 10 (25–42%) and the pH ratios (0·54–1·6) (Table 1) evidently are measures of impurity not shown in the qualitative patterns.

The second pattern shown at early time is due to an *exo*-polygalacturonase (cutting off a monosaccharide) with varying amounts of *endo*-polygalacturonase [2]. Fraction 3b at pH 3·5 showed least *endo*-polygalacturonase, and the monomer was the primary product (Fig. 2b). The pattern at pH 5·5 was similar, but with much more oligomers. Mixtures of this with the yeast *endo*-polygalacturonase showed that the addition of 1 part *endo*- to 4 parts *exo*-polygalacturonase (parts by reducing group assay units) was qualitatively insignificant, while the reverse mixture produced the *endo*- pattern with monosaccharide added, both at early times and at 10 × 10 (Fig. 1 N, Q). Pectinol 46AP, which fell into the first series at 10 × 10, at pH 3·5 at early time fell into this series, further demonstrating the value of samples at two times. Lipase B fell into this series at 10 × 10, (Fig. 1 J, O) but at pH 3·5 at early time was the only mixture tested that showed evidence of the third pattern (below).

The third pattern of oligosaccharide production (Fig. 1 L, M, Fig. 2 C) is that of fraction 2d. In time course studies, it was found that from the very earliest time to the end of the rapid hydrolysis period the most prominent product peaks were the mono-, di- and penta-saccharides. The tri- and tetra-saccharides were relatively minor and there was much less production (a maximum of 15% of the total) of the large oligosaccharides than was found with *endo*-polygalacturonase. Ninety per cent viscosity reduction required nearly the whole linear reaction phase. By the end of the linear reaction period, about 25% of the polymer had been converted to mono- and di-mer, and 10% to pentamer. The pentamer increased further, to about 25% of the whole, before disappearing. Thus, although the shape of the hydrolysis curve of fraction 2d can be duplicated by an appropriate mixture of *endo*- and *exo*-polygalacturonases (Table 1), the pattern of oligosaccharide production cannot. The distinctive pattern cannot be discerned using the source mixture (Pectinol 46AP), but in another mixture containing much *exo*-polygalacturonase (Lipase B) the penta-saccharide accumulated at early time at pH 3·5. Further evidence for the distinctive character of this enzyme is the unique pH ratio (Table 1); which was more than 10-fold that of the *exo*-polygalacturonase, and nearly 50-fold that of the *endo*-polygalacturonase; assay at various pHs indicate that the optimum is at pH 3·8–4·4, with very little activity at pH 5, the optimum for *endo*-polygalacturonase. If fraction 2d contained any of the latter, it should be discernible by assay at pH 5·5; the patterns obtained at pH 3·5 and 5·5 are qualitatively identical, but suggest some *endo*-polygalacturonase contamination at pH 5·5. If this should be as much as half of the activity at pH 5·5, it would be less than 2% at pH 3·5. The ratio of mono- to di-mer was not consistent in all the experiments. In some the molar ratio was 1, but in others there was more monomer. This may be due to differences in reaction conditions (pH, salt concentration, substrate concentration).

DISCUSSION

The pH ratio and oligosaccharide products of fraction 2d are so divergent from the other preparations studied here that one must postulate a mode of action different from *exo*- or *endo*-. The failure of a further electrophoretic purification step to change the pattern (not shown), and the failure of repeated attempts to adsorb *endo*-polygalacturonase on DEAE–cellulose (used in the preparation of 2d), indicates that the properties of fraction 2d are those of a unique polygalacturonase. It cannot be called an isoenzyme of either *exo*- or *endo*-polygalacturonase; it nevertheless resembles *endo*-polygalacturonase in the production of, and relative inactivity on, a series of oligosaccharides. The designation *S*-polygalacturonase is proposed. In its pH optimum, high heat stability (it is also rather stable at pH 1) and ratio of viscosity reduction to reducing group production (unpublished), *S*-polygalacturonase is the same as "*Endo*-polygalacturonase II" of Endo [3] (see also 4).

The patterns produced by *endo*-polygalacturonase during the linear hydrolysis period suggest random hydrolysis. The primary product was material that was smaller than the starting polymer, but larger than the hexasaccharide. Furthermore, the apparently unchanged material, chromatographing at the void volume of the Sephadex G25 column, was really being degraded, since 50% viscosity reduction occurred before 15% of it disappeared. However, there are limits to the randomness. In one pattern taken late in the linear hydrolysis period, the *molar ratios* of mono-, di-, tri-, tetra- and hexa-mer were: 0·08, 0·18, 0·59, 1·00, 0·82 and 1·02 respectively. It is evident that bonds near the end of the chain are not readily broken. A model analogous to that of lysozyme [5] seems sufficient to explain the action of *endo*-polygalacturonase. Demain and Phaff [6] have shown that yeast *endo*-polygalacturonase has a lower pH optimum when working on short oligosaccharides than when working on pectate. This appears to be reflected in the differences between the per cent hydrolysis at 10 × 10 at two pHs and in the patterns at early times.

Some authors have claimed that the early products of *endo*-polygalacturonase are monomer or small oligomers. In some cases (e.g. [7,8]) this can be attributed to contamination by *exo*-polygalacturonase, the inability to separate and visualize the larger oligomers on PC or TLC, or both. This seems not to apply to the report of English *et al.* [1] that the primary products, as well as the end

Table 2. Elution volumes of galacturonic saccharides on Sephadex G25

D.P.*	Elution volumes† Expt. 1	Expt. 2‡	D.P.*	Elution volumes† Expt. 1	Expt. 2‡
1	153	153	7	112	110
2	145	144	10	102	99
3	137	135	20	87	88
4	131	126	30	81	84
5	124	121	Polymer	76	78
6	118	115			

* D.P. Degree of Polymerization; the number of saccharide units per molecule.

† Elution volumes were measured to the closest ml. From D.P. 1–7, a graph of log (elution vol-void vol) vs D.P. gave a straight line; the elution vol of D.P. 10 was extrapolated on that graph, while those of D.P. 20 and 30 were interpolated on a graph of 1/D.P. vs elution vol, assuming the polymer peak was at D.P. 100.

‡ Experiments were the 1st and 10th of a set run about 3 weeks apart on the same column without repacking, and were the hydrolysates at pH 3·5 from yeast polygalacturonase (11% hydrolysis) and fraction 2d (10% hydrolysis), resp (see text).

products, of their enzyme are the tri-, di- and mono-saccharides (similar to Fig. 1 G, H, I).

The crude preparations studied were chosen because of the range of variety of polygalacturonases as determined by enzyme chromatography: mainly *S*-polygalacturonase in Lipase B, intermediate in Pectinol 46AP, and nearly no *S*-polygalacturonase in Pectinol 45AP [9]. These proportions were reflected in the order of the pH ratios (Table 1). A peak possibly identifiable as the *exo*-polygalacturonase described here, was found [9] (labelled "III") in the enzyme chromatograms, but it was a minor peak with the assay used and was often obscured. The patterns produced by the crude preparations, when compared to mixtures of purified *endo*- and *exo*-polygalacturonases, indicate very little *exo*-polygalacturonase in the original products, except Lipase B. Pectinols 45AP and 46AP have pH ratios (Table 1) lower than any fraction isolated from the latter, which indicates the existence of a polygalacturonase with a higher pH optimum than any of the fractions isolated (see also [7, 10].

EXPERIMENTAL

Enzymes. Commercial preparations were from *Aspergillus* [9]. Pectinol 46AP was fractionated by pH gradient chromatography on cellulose phosphate [7, 9] and combined into 5 fractions. Fractions 2 and 5 corresponded to polygalacturonase peaks II and V of Ref. 9 while fractions 3 and 4 are the intervening material. Each fraction was rechromatographed in the same way, yielding fractions 2b, etc. Fraction 2b only was twice chromatographed on DEAE-cellulose, yielding a product (fraction 2d) free of pectin esterase (E.C. 3.1.1.11), giving a single large band on gel-electrophoresis, with pectin lyase (E.C. 4.2.2.10) as the only observed minor band. Electrophoretic removal of the latter did not affect the action on pectate. The yeast polygalacturonase from *Saccharomyces fragilis* [11] was chromatographically purified, and corresponded to fraction 5b of Pectinol 46AP.

Gel filtration chromatography. A column, 1 × 245 cm, was filled (about 190 ml) with Sephadex G25 fine, in 0·1 M HOAc. Column packing must be diluted to about 5 × the packed vol while being added to the column under pres, and the metering pump (about 0·2 ml/min) run for several hr before inserting each sample. 2 ml samples were applied to the top of this column, and fractions of about 2 ml collected, using 0·1 M HOAc as eluant. Samples were pre-dissolved. Precipitation of pectic acid in the eluant occurred rarely. Galacturonic acid and sucrose were used as standards, and peaks attributable to the heptamer or octamer were often found. Graphical extrapolation of the elution vol of the resolvable oligomers shows that oligomers up to about 30 units would have elution vol distinct from that of the starting material (Table 2).

Chemical methods. Hydrolysis rates were determined by reducing group titration [12]. The amounts of galacturonic acid units in gel-filtration chromatography fractions were determined by measuring the red color developed in the anthrone test [13]. Suitably diluted aliquots (1 ml) were mixed with H_2SO_4 (6 ml) containing 0·2% anthrone, using the heat of dilution for development.

Substrate. Pectate (polygalacturonic acid) was reprecipitated with acidic isoPrOH. The washed, dry ppt was redissolved and diluted to 50 mM uronic acid equivalents (about 0·9% pectic acid) as measured with the anthrone test. The pH was adjusted with NaOH to 3·5 or 5·5, with the addition of 50 mM NaCl at pH 3·5, and 50 mM HOAc at pH 5·5. A similar purified sample, analyzed titrimetrically, was 92% anhydrouronic acid (ash-free dry wt) and 3% esterified. Enzyme reactions were carried out at 30°, and samples taken for oligosaccharide analysis by acidification (0·1 M HCl) and heating at 100° for 10 min.

Acknowledgements—The able assistance of Mrs. Dorothy Gilbert Bruber and Mr. Wm. G. Nolan in the preparation of the enzymes and numerous preliminary experiments is gratefully acknowledged. I am also grateful for gifts of enzymes from the Rohm and Haas Co., Philadelphia, of pectic substances from the Sunkist Growers, Ontario, Calif., and of a culture of *Saccharomyces fragilis* from Dr. H. J. Phaff.

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