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ENZYMIC DEGRADATION OF CHEMICALLY MODIFIED EXTRACELLULAR POLYSACCHARIDES FROM *Rhizobia**

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ABSTRACT

Extracellular polysaccharides from *Rhizobium trifolii*, U226, Coryn and Bart A; *Rhizobium phaseoli*, U453; *Rhizobium leguminosarum*, U331; and *Rhizobium meliloti*, U27, after chemical modification, become substrates for certain β-D-glucan hydrolases. The *Streptomyces* (1 → 4)-β-D-glucan endohydrolase (EC 3.2.1.4) hydrolyses reduced and deacetylated rhizobial polysaccharides, both before and after removal of carboxyethylidene substituents, to produce a series of oligosaccharides. The *Rhizopus arrhizus* (1 → 3)-β-D-glucan endohydrolase (EC 3.2.1.6) hydrolyses only fully modified polysaccharides to yield, in the case of *R. meliloti* U27, laminarabiose, and, in all other instances, a disaccharide identified as β-D-Gal-(1 → 3)-D-Glc. The same disaccharides are released by the *Rhizopus* enzyme from oligosaccharides produced by the action of the *Streptomyces* enzyme on fully modified polysaccharides. The results are discussed in relation to the available data for the structure of the polysaccharides and the specificity of the enzymes.

INTRODUCTION

Information on the structures of the extracellular rhizobial polysaccharides has been derived mainly from methylation and partial acid-hydrolysis studies. The data thus far available (summarized in Table I) show that these polysaccharides are branched and contain substantial proportions of 3- and 4-linked β-D-glycosyl residues[1–7].

Although some information on the linkage sequence in these polysaccharides has been obtained by partial acid hydrolysis, approaches by enzymic degradation have been unsuccessful with the polysaccharide from *R. meliloti*[7].

Recently, we showed that reduction of the glucosyluronic carboxyl groups in the S111 pneumococcal polysaccharide produced a neutral glucan that was susceptible

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

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TABLE I

PRINCIPAL STRUCTURAL FEATURES OF THE RHIZOBIAL, EXTRACELLULAR POLYSACCHARIDES, AS DERIVED FROM METHYLATION STUDIES (ADAPTED FROM ZEVENHUIZEN[2])

Mode of linkage	R. leguminosarum *PRE (R-7)*		R. trifolii *TA-I*		R. meliloti *K-24 (R-13)*	R. meliloti *U-27*
	R. phaseoli *Bokum (R-21)* R. trifolii *K-8 (R-38)*					
	Sugar residues	*Molar proportions*		*Sugar residues*	*Molar proportions*	
Terminal	4,6-*O*-(1-carboxy-ethylidene)-D-glucose and 4,6-*O*-(1-carboxy-ethylidene)-D-galactose	13	12[a]	4,6-*O*-(1-carboxy-ethylidene)-D-glucose; D-glucose	11 0	14 2
Chain residue residue						
(1 → 3)	4,6-*O*-(1-carboxy ethylidene)-D-glucose;	13	12	D-glucose	24	15–22
	D-galactose	6	0	D-galactose	15	10–13
(1 → 4)	D-glucose	32	48	D-glucose	20	24–31
	D-glucuronic acid	20	12			
(1 → 6)				D-glucose	18	12–16
Branching residue (1 → 4), (1 → 6)	D-glucose or D-galactose	13	12[b]	D-glucose	12	10.5

[a] *O*-Carboxyethylidene-D-galactose only. [b] D-glucose only.

to hydrolysis by β-D-glucan hydrolases[8]. This prompted us to examine, in an analogous way, a number of purified rhizobial polysaccharides. The present paper describes the action of three β-D-glucan endohydrolases of differing specificity for (1 → 3)- and (1 → 4)-β-D-glucosidic linkages, on rhizobial polysaccharides that had been modified by reduction of D-glucosyluronic residues, and removal of pyruvate (carboxy-ethylidene) and acetyl substituents.

MATERIALS AND METHODS

Enzyme preparations. — *Bacillus pumilus* β-D-glucanase (EC 3.2.1.73) was purified from a preparation supplied by Novo Industri A/S, Copenhagen, Denmark[9]. *Rhizopus arrhizus* (1 → 3)-β-D-glucanase (EC 3.2.1.6) and *Streptomyces* (1 → 4)-β-D-glucanase (EC 3.2.1.4) were gifts from Dr. E. T. Reese, U.S. Army Natick Laboratories, Natick, Massachusetts, U.S.A. Each of the enzyme preparations was tested for activity by using lichenan as substrate. When incubated for 30 min at 40° with lichenan (1.0 ml, 0.6% w/v) in the appropriate buffer, each enzyme solution (10 µl) produced about 200 µg of reducing sugar. At 10 times this concentration, the *Rhizopus* (1 → 3)-β-D-glucanase preparation was not active on *O*-(carboxymethyl)-cellulose, salicin, or cellobiose. The *Streptomyces* enzyme was without action on *O*-(carboxymethyl)-pachyman, laminarabiose and salicin and the *Bacillus* β-D-glucanase was without

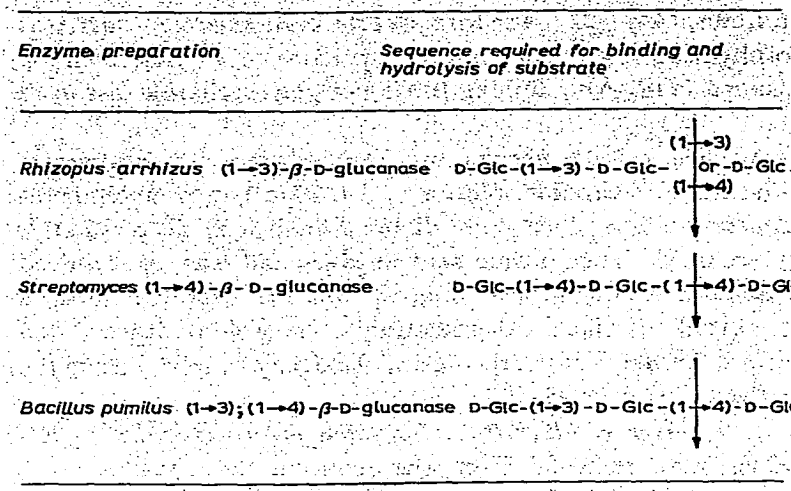


Fig. 1. Specificity requirements of the *Streptomyces* (1 → 4)-β-D-glucanase, the *Rhizopus arrhizus* (1 → 3)-β-D-glucanase, and the *Bacillus pumilus* (1 → 3); (1 → 4)-β-D-glucanase[8–10].

action on *O*-(carboxymethyl)cellulose, *O*-(carboxymethyl)pachyman, laminarabiose, cellobiose, and salicin. The specificity of the enzymes is outlined in Fig. 1 and has been described in more detail[8,10]. The β-D-galactosidase (EC 3.2.1.23) of *Escherichia coli* was a crystalline preparation from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Polysaccharides. — Lichenan from *Cetraria islandica* was obtained from Koch–Light Laboratories, Colnbrook, Bucks, U.K. Before use, it was purified by dissolving in boiling water, freezing, and allowing the solid to thaw. The resulting precipitate was collected, washed with water, and dried by successive washings with cold ethanol, acetone, and ether. Rhizobial polysaccharides were generously provided by Dr. R. Sømme, Department of Chemistry, Agricultural University of Norway, Ås, Norway. The chemical compositions of the latter polysaccharides have been examined in detail[4,5].

Paper chromatography. — Products formed by enzymic hydrolysis of polysaccharides were separated by descending chromatography on either Whatman No. 3 paper in the solvent system 6:1:3 (v/v) 1-propanol–ethyl acetate–water or Whatman No. 1 paper in 10:4:1 (v/v) ethyl acetate–pyridine–water. The separated products were detected by using alkaline silver nitrate reagent[11].

Gel-filtration chromatography of oligosaccharides. — This was performed on Bio-Gel P-2 as previously described[8,12].

EXPERIMENTAL

Reduction of rhizobial polysaccharides. — The uronic acid-containing polysaccharides were reduced by the method of Taylor and Conrad[13], using the water soluble, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulphonate

(Sigma Chemical Co. St. Louis, Mo, U.S.A.) to form a carboxyl derivative, which was reduced by sodium borohydride. The reduction was performed as described by Anderson and Stone[8], using a solution containing 100 mg of polysaccharide dissolved in 15 ml of water.

Deacetylation of rhizobial polysaccharides. — The reduced polysaccharides (40 mg) were treated with sodium hydroxide solution (0.2M, 20 ml) at room temperature for 24 h and the deacetylated products were dialysed against distilled water[14]. The U27 rhizobial polysaccharide lacks uronic acid residues and was not reduced prior to deacetylation. Sodium borohydride (1 mg/ml) was incorporated into the deacetylation mixture to prevent any alkaline degradation[15]. The yield of deacetylated polysaccharide varied from 99 to 105% of the theoretical yield, calculated by using the acetyl contents of the native polysaccharides[4].

Removal of carboxyethylidene residues from Rhizobial polysaccharides. — *O*-Carboxyethylidene residues were removed from the reduced, deacetylated polysaccharides by the method of Chaudhari *et al.*[3]. The reduced, deacetylated polysaccharides (19 mg) were dissolved in water (25 ml) and the pH adjusted to 2 by dropwise addition of M hydrochloric acid. The acidified solutions were refluxed for 4 h at 100°. After this treatment, the mixtures were cooled, adjusted to pH 6.3 with M sodium hydroxide, and centrifuged to remove the precipitate that formed during refluxing. The supernatant solutions were dialysed against distilled water and freeze

TABLE II

β-D-GLUCANASE PRODUCTS[a] FORMED FROM THE NATIVE AND MODIFIED POLYSACCHARIDES OF *R. phaseoli*, U453; *R. leguminosarum*, U311; *R. trifolii*, CORYN, BART AND U226, AND *R. meliloti*, U27

State of modification	*Polysaccharide*	*Enzyme preparation and products*		
		Rhizopus (1 → 3)-β-D-*glucanase*	Streptomyces (1 → 4)-β-D-*glucanase*	Bacillus β-D-*glucanase*
Native	Coryn, Bart, U226, U453, U311	N.d.[b]	N.d.	N.d.
	U27	N.d.	N.d.	N.d.
Reduced deacetylated	Coryn, Bart, U453, U311, U226	N.d.	R_{Glc} 0.04, 0.22	N.d.
		N.d.	R_{Glc} 0.04, 0.22, and 0.28	N.d.
	U27	N.d.	N.d.	N.d.
Reduced deacetylated, depyruvylated	Coryn, Bart, U226 U453, U311	β-D-Gal-(1 → 3)-D-Glc (R_{Glc} 0.77)	R_{Glc} 0.03, 0.11 0.22, 0.51 0.70, and 1.0	N.d.
	U27	β-D-Glc-(1 → 3)-D-Glc (R_{Glc} 0.86)	R_{Glc} 0.09	N.d.

[a]Laminarabiose, R_{Glc} 0.86; cellobiose, R_{Glc} 0.69; lactose, R_{Glc} 0.60. (The mobility of each oligosaccharide was measured relative to glucose after chromatography on Whatman No. 3 paper.)
[b]N.d., none detected.

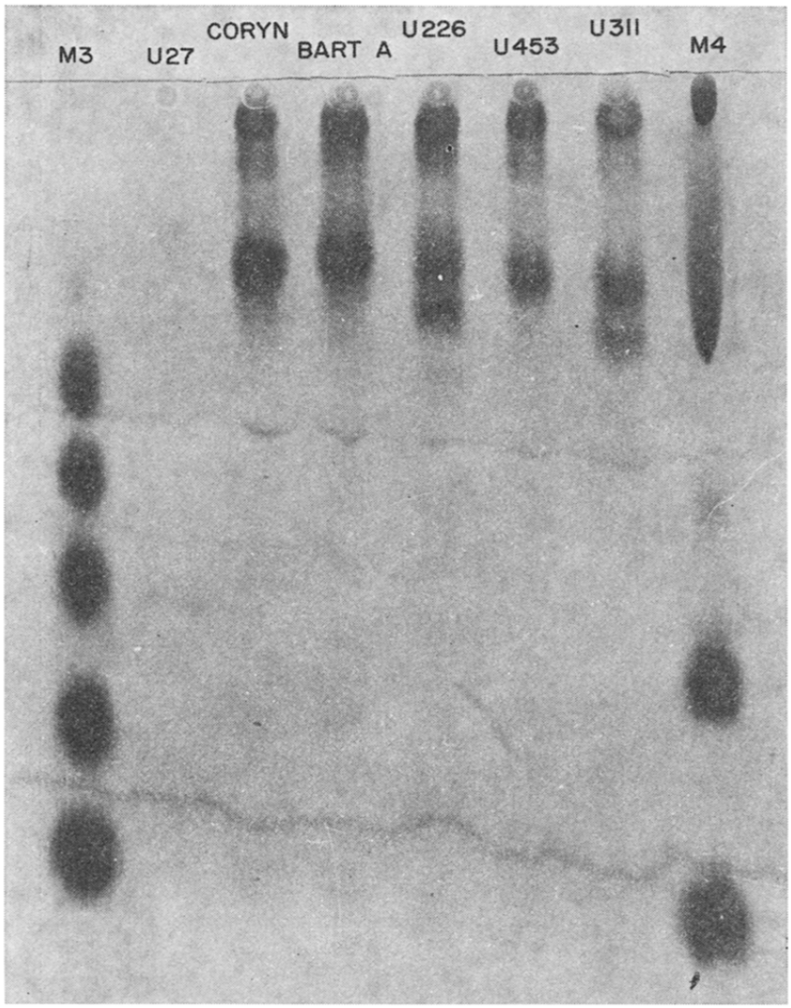


Fig. 2. Chromatogram of the products formed by incubation of the reduced and deacetylated rhizobial polysaccharides with the *Streptomyces* (1 → 4)-β-D-glucanase. M3: (1 → 3)-D-gluco-oligosaccharides plus glucose; M4: (1 → 4)-β-D-gluco-oligosaccharides plus glucose.

dried. Yields of depyruvylated polysaccharides were between 70 and 96% of the theoretical value, determined by using the pyruvate contents as calculated by Sømme[4].

Enzymic degradation of polysaccharides. — Native and modified polysaccharides (400 μg in 200 μl of water) were incubated with the enzyme preparations (100 μl) for 1 h at 40° in acetic acid–sodium acetate buffer (0.05M, pH 5.0, 200 μl) for the *Rhizopus* and *Streptomyces* preparations, or in maleic acid–sodium maleate buffer (0.05M, pH 6.5, 200 μl) for the *Bacillus* preparation. Reactions were terminated by boiling the samples for 5 min. The mixtures were then deionized and chromatographed on Whatman No. 3 paper. The results are summarised in Table II.

None of the reduced, deacetylated samples were attacked by the *Rhizopus*

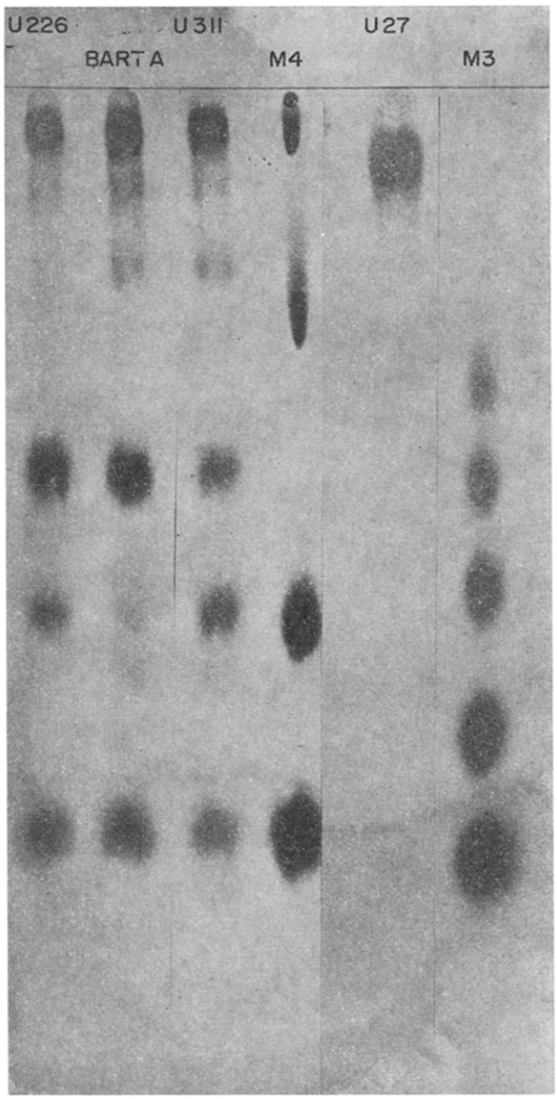


Fig. 3. Chromatogram showing hydrolysis products formed by the action of *Streptomyces* (1 → 4)-β-D-glucanase on the reduced, deacetylated, depyruvylated polysaccharides of U226, Bart A, U311, and U27. M3: (1 → 3)-β-D-gluco-oligosaccharides plus glucose. M4: (1 → 4)-β-D-gluco-oligosaccharides plus glucose.

(1 → 3)-β-D-glucanase. On the other hand, all of the samples, apart from U27, were hydrolyzed by the *Streptomyces* (1 → 4)-β-D-glucanase to yield oligosaccharides having low R_{Glc} values (0.04 and 0.22). Samples U311 and U226 showed an additional oligosaccharide having R_{Glc} 0.28 (Fig. 2).

Samples that had been reduced, deacetylated, and depyruvylated were readily hydrolysed by the *Rhizopus* and *Streptomyces* enzymes, but not by the *Bacillus* enzyme. For all of the polymers, apart from U27, the *Streptomyces* enzyme produced

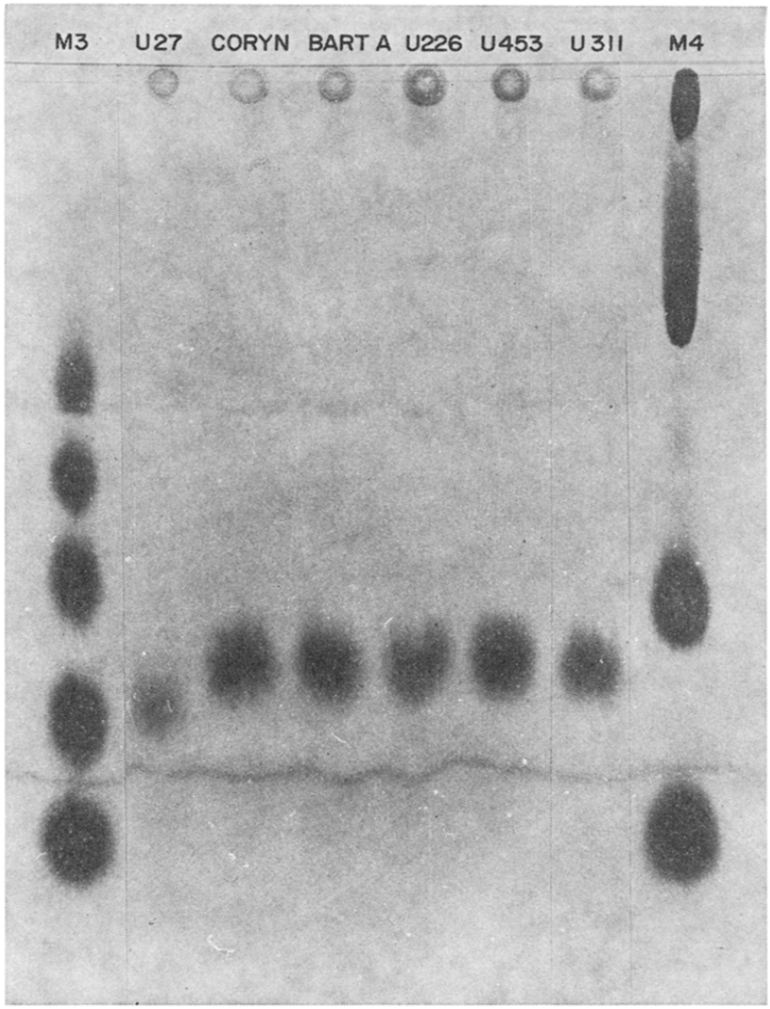


Fig. 4. Chromatogram showing the products formed by incubation of *Rhizopus* (1 → 3)-β-D-glucanase with the reduced, deacetylated, depyruvylated rhizobial polysaccharides. M3: (1 → 3)-β-D-gluco-oligosaccharides plus glucose. M4: (1 → 4)-β-D-gluco-oligosaccharides plus glucose.

oligosaccharides having R_{Glc} 0.03, 0.11, 0.22, 0.51, 0.70, and 1.0. The polysaccharide from U27 yielded only one oligosaccharide having R_{Glc} 0.09 (Fig. 3).

The *Rhizopus* enzyme-digests of the reduced, deacetylated, and depyruvylated polysaccharides contained a disaccharide as the sole oligosaccharide product in all cases (Fig. 4), although the disaccharide from U27 had an R_{Glc} value of 0.86 whereas that produced from the other polysaccharides had R_{Glc} 0.77.

Action of the Streptomyces and Rhizopus *enzymes on the polysaccharide from* R. Trifolii, *Coryn.* — The polysaccharide from Coryn (6 mg) that had been reduced, deacetylated, and depyruvylated was dissolved in 3 ml of acetate buffer (0.1M, pH 5.0) and divided into two portions.

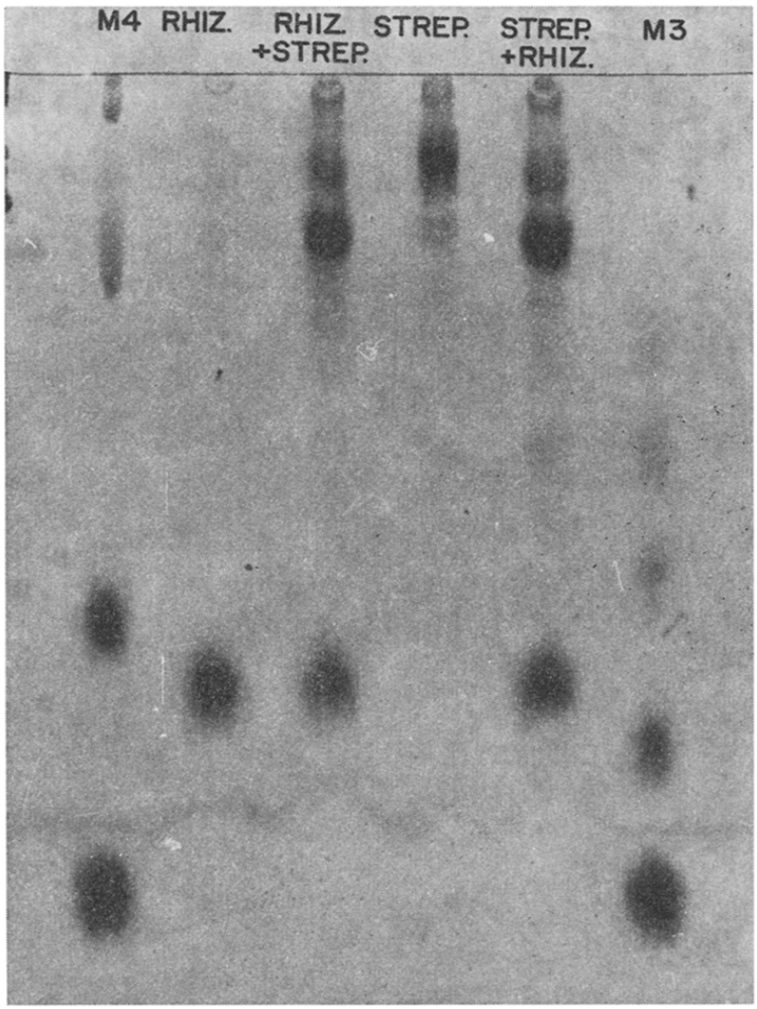


Fig. 5. Chromatogram showing the products formed by incubation of the reduced, deacetylated, depyruvylated, extracellular polysaccharide of Coryn with (*1*) *Rhizopus* enzyme; (*2*) *Rhizopus* enzyme followed by *Streptomyces* enzyme; (*3*) *Streptomyces* enzyme; (*4*) *Streptomyces* enzyme followed by *Rhizopus* enzyme. M3: (1 → 3)-β-D-gluco-oligosaccharides plus glucose. M4: (1 → 4)-β-D-gluco-oligosaccharides plus glucose.

The first portion was incubated with the *Rhizopus* enzyme (0.5 ml) for 1 h at 40° and a 0.5-ml sample withdrawn. The remaining sample was boiled for 5 min to inactivate the enzyme, cooled, and incubated for a further 1-h period with the *Streptomyces* enzyme (0.5 ml). The second portion was treated similarly, except that the order of enzyme treatments was reversed. The products were chromatographed on Whatman No. 3 paper (Fig. 5).

The action of the *Rhizopus* enzyme alone produced a disaccharide (R_{Glc} 0.75) as the sole oligosaccharide product, and the sample incubated with only the *Strepto-*

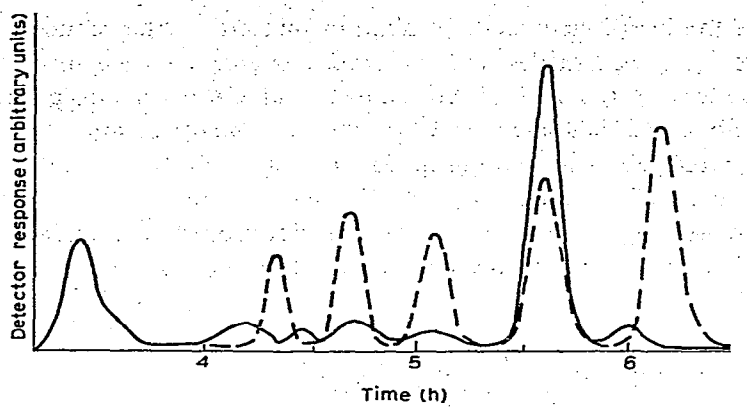


Fig. 6. Chromatography on Bio-Gel P-2 of the oligosaccharides produced during the hydrolysis of the extracellular polysaccharide of *R. trifolii* Coryn by the *Rhizopus* (1 → 3)-β-D-glucanase followed by the *Streptomyces* (1 → 4)-β-D-glucanase. Bio-Gel P-2, minus 400 mesh; 60°; column 0.9 × 170 cm; eluant, water; flow rate, 13.3 ml/h; (—) hydrolysate; (---) (1 → 3)-β-D-gluco-oligosaccharide series plus glucose. K_{av} glucose, 0.86; laminarabiose, 0.72; laminaratriose, 0.60; laminaratetraose 0.50; laminarapentaose, 0.42; K_{av} of products formed: 0.81, 0.71, 0.58, 0.50, 0.44, 0.37, and 0.19.

myces enzyme produced an oligosaccharide (R_{Glc} 0.09), together with smaller amounts of three or four other oligosaccharides.

The polysaccharides incubated with both enzymes, irrespective of the order, produced the disaccharide (R_{Glc} 0.75), together with four to five oligosaccharides, the most prominent of which had an R_{Glc} 0.19. These oligosaccharides were separated by gel filtration (Fig. 6). A disaccharide (K_{av} 0.71) and an oligosaccharide (K_{av} 0.19) were the most prominent products, and traces of other oligosaccharides (K_{av} 0.81, 0.58, 0.50, 0.44, and 0.37) were also observed.

Some products of higher molecular weight must have been excluded from the column, as the disaccharide peak, which is predominantly β-D-Gal-(1 → 3)-D-Glc, represented 55% of the separated products, whereas methylation data of other workers (Table I) suggest that, at the most, the disaccharide β-D-Gal-(1 → 3)-D-Glc could constitute only 26% of the original polysaccharide.

Nature of the disaccharides produced by action of the Rhizopus *enzyme.* — In the solvent system used, the disaccharide product (R_{Glc} 0.77) from all of the polysaccharides, apart from U27, had a mobility intermediate between those of laminarabiose (R_{Glc} 0.86) and cellobiose (R_{Glc} 0.69), whereas the disaccharide from U27 had the same mobility as laminarabiose (Fig. 4). Studies with (1 → 3), (1 → 4) mixed-linked β-D-glucans have shown that the *Rhizopus* enzyme specifically hydrolyses linkages adjacent to 3-substituted glucosyl residues[8,10], which suggests that the disaccharide product, in both cases, consists of a monosaccharide (1 → 3)-linked to a D-glucose residue. As galactose and glucose are the only monosaccharides present in the reduced, deacetylated, and depyruvylated samples, the disaccharide having R_{Glc} of 0.77 is probably β-D-Gal-(1 → 3)-D-Glc. This notion was confirmed by incubation of the disaccharide with a β-D-galactosidase from *E. coli*[16].

Samples (200 μl) of the heat-inactivated, incubation mixture of the reduced, deacetylated, depyruvylated polysaccharides and the *Rhizopus* enzyme were mixed with 0.2 ml of phosphate buffer (0.1M, pH 8.0), followed by 0.1 ml of β-D-galactosidase (1 mg/ml in phosphate buffer), and incubated for 1 h at 40°. The mixtures were then deionized, filtered, freeze dried, and chromatographed on Whatman No. 1 paper.

The disaccharides from all of the samples except U27 were completely converted into D-galactose and D-glucose (Fig. 7). Part of the disaccharide from U27 remained unchanged and part had been converted into glucose, but galactose was not found

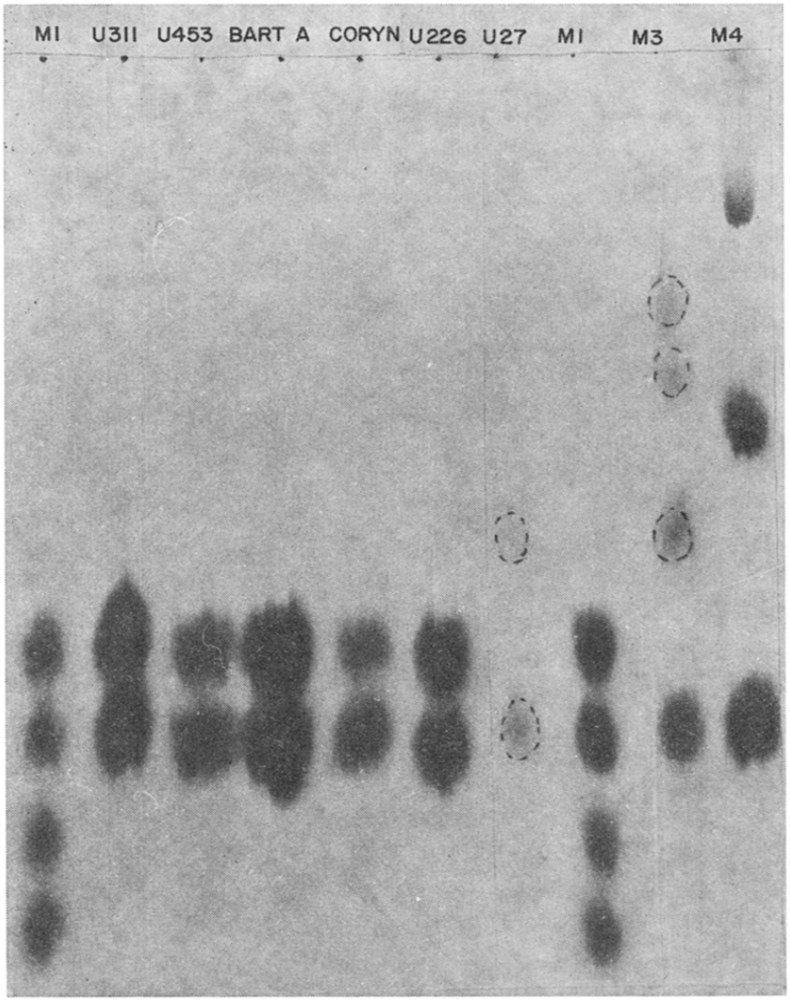


Fig. 7. Chromatogram showing the products formed by the action of β-D-galactosidase on the disaccharide produced during the incubation of *Rhizopus* (1 → 3)-β-D-glucanase with the reduced, deacetylated, depyruvylated, rhizobial polysaccharides. M1: Galactose, glucose, mannose, and arabinose in order of increasing R_F value. M3: (1 → 3)-β-D-gluco-oligosaccharides plus glucose. M4: (1 → 4)-β-D-gluco-oligosaccharides plus glucose.

(Fig. 7). An authentic sample of laminarabiose was also shown to be hydrolyzed to glucose by the β-D-galactosidase preparation used. This information, together with the known specificity of the *Rhizopus* enzyme and the high R_{Glc} values of the oligosaccharides compared to lactose [β-D-Gal-(1 → 4)-D-Glc, R_{Glc} 0.60], support the view that the disaccharide from all the polysaccharides apart from U27 is probably β-D-Gal-(1 → 3)-D-Glc, and that the disaccharide from U27 is laminarabiose.

DISCUSSION

The native rhizobial polysaccharides were not hydrolyzed by any of the β-D-glucanase preparations, presumably because of the effects of both steric hindrance and the ionic interactions of the carboxyethylidene and carboxyl groups. However, when the polysaccharides were reduced, deacetylated, and depyruvylated, they became susceptible to hydrolysis by both the *Rhizopus* and the *Streptomyces* enzymes, but not by the *Bacillus* enzyme.

The modified polysaccharides of *R. phaseoli* U453, *R. leguminosarum* U311, and *R. trifolii* Coryn, Bart and U226 are considered as one group, as the *Streptomyces* enzyme produced an apparently identical series of oligosaccharides from each of these polymers, as did the *Rhizopus* enzyme.

Action of the Rhizopus (1 → 3)-β-D-glucanase on the modified polysaccharides of R. phaseoli, R. leguminosarum, *and* R. trifolii. — The *Rhizopus* enzyme only hydrolyzed the reduced, deacetylated polysaccharides after removal of the carboxyethylidene residues, suggesting that these groups were bound to the 3-substituted glucosyl residues or to linkages in the vicinity of these (see Table II). This conclusion is consistent with the published methylation data (Table I), which show that all of the 3-substituted glucosyl residues in these polysaccharides bear carboxyethylidene groups.

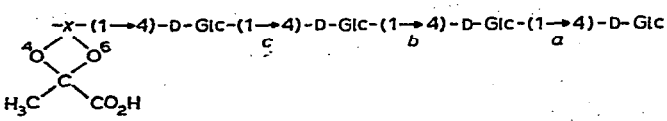
The sole oligosaccharide-product in all instances was the disaccharide β-D-Gal-(1 → 3)-D-Glc, which must arise from the ends of side chains or from linear portions of the polysaccharides, which contain regions of the following structure: [→ 3)-D-Glc *x* D-Gal-(1 → 3)-D-Glc *x*], where *x* represents linkages hydrolyzed by the *Rhizopus* enzyme. As the disaccharide β-D-Gal-(1 → 3)-D-Glc was the sole product detected by paper chromatography, such regions would need to be separated by blocks of at least four monosaccharide residues to yield a product that would not migrate in the solvent-system used.

Action of the Streptomyces *(1 → 4)-β-D-glucanase on the modified polysaccharides of* R. phaseoli, R. leguminosarum, *and* R. trifolii. — Following reduction and deacetylation, the native polysaccharides became susceptible to the *Streptomyces* (1 → 4)-β-D-glucanase, which suggests that at least two consecutive (1 → 4)-linked D-glucose residues became available after removal of the carboxyl and acetyl groups. This conclusion is consistent with the isolation of the oligosaccharide, D-GlcA-(1 → 4)-D-GlcA-(1 → 4)-D-Glc from partial acid hydrolysates of the native polysaccharides[5].

The *Streptomyces* enzyme produced at least two oligosaccharides, suggesting

that the polysaccharide does not have a strictly repeating-structure or that any repeating units contain at least two regions having two, or one region having three, consecutive (1 → 4)-linked D-glucosyl residues. Longer blocks of (1 → 4)-linked D-glucosyl residues are not indicated, as glucose, cellobiose, or cellotriose were not detected in significant amounts. These are the normal products of the action of the *Streptomyces* enzyme on solely (1 → 4)-linked β-D-glucans[10,17].

When the carboxyethylidene groups were removed from the reduced, deacetylated polysaccharides, a different series of oligosaccharide products could be detected by paper chromatography. Three oligosaccharides of low mobility (R_{Glc} 0.03, 0.11, 0.22) were observed, as well as three more-mobile products (R_{Glc} 1.0, 0.70, 0.51), which appeared to be glucose, cellobiose, and a trisaccharide. As the *Streptomyces* enzyme is known to produce D-glucose and cellobiose from sequences of more than three consecutive (1 → 4)-linked D-glucosyl residues, this result indicates that an additional (1 → 4)-linkage becomes available after tpe removal of the carboxyethylidene groups. This information suggests that the following structural feature is present:



where linkages *a* and *b* are hydrolyzed by the *Streptomyces* enzyme prior to depyruvylation and linkage *c* is not hydrolyzed until after depyruvylation. The methylation data (Table I) indicate that *x* must be either a 3-linked glucosyl residue or a terminal galactosyl or glucosyl residue, as these are the only monosaccharides bearing carboxyethylidene groups. However, this would be a minor structural feature, as glucose and cellobiose were only detected when large samples of the hydrolysate were examined.

The most prominent product arising from the action of the *Streptomyces* enzyme on the fully modified polysaccharide of Coryn was an oligosaccharide of high molecular weight (R_{Glc} 0.09). This oligosaccharide was hydrolyzed by the *Rhizopus* enzyme to produce another oligosaccharide (R_{Glc} 0.19) and a disaccharide identical with that produced directly from the modified polymer and tentatively identified as D-Gal-(1 → 3)-D-Glc. Thus, the D-Gal-(1 → 3)-D-Glc must occupy a terminal position on the oligosaccharide produced by the action of the *Streptomyces* enzyme. Otherwise, the *Streptomyces* product would have been hydrolyzed by the *Rhizopus* enzyme to produce two further oligosaccharides, in addition to the disaccharide. There is a possibility that two oligosaccharides of the same mobility may have been produced, but they would have had a higher R_{Glc} value.

The observation that the D-Gal-(1 → 3)-D-Glc group, removed by the *Rhizopus* enzyme, also occupies a terminal position on the *Streptomyces* product, indicates that it must form the ends of the side chains of the intact polysaccharides. Otherwise, the linkages adjacent to this group would have to be cleaved by both enzymes, which is contrary to their specificity requirements.

These results agree with the methylation data of Chaudhari *et al.*[3], who have shown that all of the galactose in the polysaccharide of *R. trifolii* TA-1 is terminal

(see Table I). In support of this, they demonstrated that all of the D-galactose residues and the 3-linked D-glucosyl residues in this polysaccharide could be removed by partial acid hydrolysis, whereas the rest of the molecule remained intact.

Action of the Bacillus pumilus *β-D-glucanase on the modified polysaccharides of* R. phaseoli, R. leguminosarum, *and* R. trifolii. — None of the modified polysaccharides were hydrolyzed by the *Bacillus* enzyme to yield products mobile during paper chromatography. This result suggests that the modified polysaccharides either did not contain regions of the following structure: -D-Glc-(1 → 3)-D-Glc-(1 → 4)-D-Glc-, or that they were inaccessible to the enzyme. Branching points or terminal residues could have prevented fulfillment of the binding requirements. Alternatively, these regions may be present but well separated, so that the products are of high molecular weight.

The polysaccharide of R. meliloti *U27.* — The hydrolysis products of the polysaccharide of *R. meliloti* U27 were markedly different from the products of the other polysaccharides.

When the carboxyethylidene groups were removed from the reduced, deacetylated polysaccharide of U27, the product was hydrolyzed by the *Rhizopus* enzyme to yield laminarabiose. This disaccharide must have arisen from the ends of the side chains, as only the terminal D-glucose residues of U27 bear carboxyethylidene groups (Table I).

The *Streptomyces* enzyme did not appear to hydrolyze the *R. meliloti* polysaccharide after reduction and deacetylation, but hydrolyzed the fully modified sample to give an oligosaccharide having low mobility on paper chromatography.

The *Bacillus* enzyme was also without action on either the partially or fully modified polysaccharide.

These findings are completely in accord with those of Lindberg and coworkers[18], who have shown by chemical procedures that the *R. meliloti* polysaccharide has a terminal laminarabiose residue and that the repeating unit is a branched octasaccharide having two, adjacent (1 → 4)-linked β-D-glucosyl residues, one of which would be available for hydrolysis by the *Streptomyces* enzyme. No sequences having the appropriate configuration for hydrolysis by the *Bacillus* enzyme were observed.

CONCLUSIONS

Enzymic degradation will be useful in the determination of the detailed structures of *Rhizobial* polysaccharides, especially when used in conjunction with methylation and periodate-oxidation techniques. It is clear that much more information on their monosaccharide sequences will become available following detailed examination of the oligosaccharides liberated by the *Streptomyces* enzyme.

In addition to providing structural information, the experiments described allow some deductions on the specificity of the β-D-glucan hydrolases. In previous investigations with the (1 → 3)-β-D-glucanase of *Rhizopus*[8,10], only linear glucans were used as substrates and all products were terminated with a β-D-Glc-(1 → 3)-D-Glc sequence. It is now clear that β-D-Gal-(1 → 3)-D-Glc may also be accommodated in the glycosyl

binding-site of this enzyme. Similarly, linear glucans were used in studies with the *Streptomyces* (1 → 4)-β-D-glucanase[8,10]. It is now apparent that branching does not prevent the action of this enzyme, although it will be necessary to isolate and identify the hydrolysis products before the structural constraints of branching can be fully defined.

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