Carbohydrate Research, 33 (1974) 89–96

CHEMICAL ANALYSIS OF EXOCELLULAR, ACID POLYSACCHARIDES FROM SEVEN *Rhizobium* STRAINS

RANDI SØMME

Department of Chemistry, Agricultural University, N-1432 Ås-NLH (Norway)

(Received August 17th, 1973; accepted for publication October 5th, 1973)

ABSTRACT

A comparative, chemical analysis of the acid exopolysaccharides from seven *Rhizobium* strains, involving the taxonomic groups *Rhizobium meliloti*, *Rh. trifolii*, *Rh. phaseoli*, and *Rh. leguminosarum*, is presented. Apart from the polysaccharide from *Rh. meliloti*, which is known to lack uronic acid, no significant differences in the carbohydrate composition were found. The two non-nitrogen-fixing strains [infective (Coryn), and non-infective (Bart A)] gave polysaccharides which differ from those produced by the infective and nitrogen-fixing strains in the detailed structural features. This difference is expressed in the pattern of periodate oxidation and cation-binding capacity.

INTRODUCTION

Since the work of Beijerink in 1888, it has been known that the formation of noduli on *Leguminous* roots is caused by symbiosis of bacteria and the plant[1]. The bacteria, which belong to the genus *Rhizobium*, have a high specificity in cross inoculation. A common characteristic feature is the production of a highly viscous, extracellular polysaccharide, for which three possible roles were pointed out by Stacey[2]: firstly, importance in the formation of infection threads in the plant roots; secondly, as food-reserve material for the plant; and thirdly, as a defence mechanism. The first function has been widely investigated. Chemical[2–15] and immunological methods[8,21,22] have not been very successful in distinguishing between the polysaccharides of the different species.

Ljunggren[16] postulated that the polysaccharide was able to induce the formation of a polygalacturonase in the plant roots, thus facilitating the entrance of the bacteria into the host plant. Attempts to verify this finding have not been successful[17–19]. The possible role of the polysaccharide in the formation of infection threads is not excluded[18,20]; the polysaccharide may be one of at least two high polymeric factors which participate in the root-hair deformation[20] that commonly precedes the infection of the host.

Galactose, glucose, and glucuronic acid are the common monosaccharide

constituents of the polysaccharides, as are pyruvic acid and acetyl groups[12–15,21]. The pyruvic acid moiety is an immunological determinant[21,22] in the eight strains examined. Stress is also laid upon the possible importance of the acetyl groups in the host/parasite interaction as well as in immunological specificity[21].

The purpose of the present work was to obtain further information about the chemical composition of the *Rhizobium* polysaccharides. The bacterial strains were selected according to different bacteria-host groups[16]. Three of the strains belong to the same group, but vary in ability to infect.

EXPERIMENTAL

Organisms. — The *Rhizobium* strains (kindly supplied from the culture collection at the Department of Microbiology, Agricultural College of Sweden, Uppsala) were as follows: *Rh. trifolii*, U 226 (infective and co-operative, *i.e.*, nitrogen fixing in the host), Coryn (infective and non-co-operative, *i.e.*, merely parasitic), and Bart A (non-infective). The following infective and co-operative strains were also used: *Rh. leguminosarum* U 311, *Rh. meliloti* U 27, and *Rh. phaseoli* U 453 and U 458.

Cultivation of organisms. — The bacterial strains were kept on agar slants of Dorn A medium[23]. For polysaccharide production, the bacteria were grown in shaken cultures in Dorn A medium at 26° for 6 days.

Preparation of polysaccharides. — The medium itself did not give any precipitate with ethanol.

The cells were removed by centrifugation at 12,000 *g*, 4°, 20 min. To the clear, viscous supernatant, 2 vol. of ethanol were added. The precipitated polysaccharide was centrifuged, washed with ethanol, dissolved in water, precipitated with 5% cetyltrimethylammonium bromide, centrifuged, washed with water, dissolved in 3% aqueous sodium chloride, and precipitated with 2 vol. of ethanol. The collected polysaccharide was dissolved in water, dialysed against distilled water for 72 h, and freeze-dried; average yields were 0.5 g/l of medium.

Methods. — Acid hydrolysis was carried out with trifluoroacetic acid (TFA)[24]. Polysaccharide solutions, 0.5% in 2M TFA, were heated in closed ampoules at 120° for 2 h. The acid was removed by evaporation (below 50°) under diminished pressure.

D-Galactose and D-glucose were determined, after hydrolysis of the polysaccharides and separation of the products on Whatman No. 1 paper, using 1-butanol–pyridine–water (5:3:2), by the quantitative ferricyanide test[25]. D-Glucuronic acid was determined by the carbazole method[26] on unhydrolysed material, with corrections for the presence of galactose and glucose. Acidic components were separated by chromatography with ethyl acetate–acetic acid–formic acid–water (18:3:1:4) and by high-voltage electrophoresis on Whatman No. 1 paper in the buffer pyridine–acetic acid–water, 5:2:43 (pH 5.3). Total carbohydrate was calculated from the amounts of glucose, galactose, and glucuronic acid.

Pyruvic acid was determined colorimetrically[27], as was acetyl with ethyl acetate as a standard[28].

Detection of carbohydrates after chromatography was effected with *p*-anisidine hydrochloride and aniline hydrogen phthalate. Pyruvic acid was detected by spraying with a solution consisting of equal volumes of 0.2% ethanolic *o*-phenylenediamine and 20% aqueous trichloroacetic acid[29].

Periodate oxidation[30] was carried out with 30-mg samples of polysaccharide dissolved in 10 ml of 15mM potassium periodate, in the dark at room temperature. After 48 h, 5 ml of the reaction mixture were removed, 50 mg of sodium borohydride were added, and the pH was adjusted to 7 with acetic acid after 4 h. The solution was dialysed overnight against distilled water and freeze-dried. The residue was dissolved in 5 ml of 15mM potassium periodate for further oxidation. The reaction was followed spectrophotometrically[31]. The total oxidation time was 160 h.

Protein determination was carried out with the colorimetric method of Lowry and co-workers[32], and by the Kjeldahl method. Ca^{2+} and Mg^{2+} were determined by atomic absorption analysis, and K^+ and Na^+ by flame spectrophotometry.

RESULTS AND DISCUSSION

Only acidic polysaccharides could be isolated from the culture fluids; neutral polysaccharides could not be detected. The polysaccharides were not amenable to gel filtration because of the high viscosity of their solutions. No evidence of heterogeneity could be found, however, when the polysaccharides were examined by free-boundary electrophoresis at pH 2 and 7, although the high viscosity was still a problem and some figures are thus lacking from Table I.

Trifluoroacetic acid (TFA) was used as the hydrolysing agent since it permitted rapid reaction and the recovery of the monosaccharides was at least equal to that associated with mineral acids[24]. Treatment of the uronic acid-containing polysaccharides with 2M TFA at 120° for 2 h gave only negligible amounts of acidic oligosaccharides, whereas treatment with 0.5M sulphuric acid at 100° for 18 h gave at least five oligosaccharides, two of which contained glucose (unpublished data). After treatment of monosaccharides with TFA, followed by paper chromatography and determination of reducing power[25], the recoveries of galactose, glucose and xylose, and mannose were 98, 92, and 82%, respectively. The recovery of glucuronic acid was 35% (sum of acid and lactone), and that of galacturonic acid was 48%. Similar percentage recoveries have been obtained after sulphuric acid hydrolysis[33].

Paper-chromatographic and -electrophoretic examinations showed that glucuronic acid was the only uronic acid present in the polysaccharide hydrolysates. The isolated acid was transformed into glucuronolactone in acid solution, and into D-glucose after hydrolysis of the reduced methyl ester methyl glycoside[34].

The neutral monosaccharides in the hydrolysate were indistinguishable from D-glucose and D-galactose. It must be pointed out that pyruvic acid and the degradation products of glucuronic acid in the TFA hydrolysate gave positive reactions with developing reagents used for, and have mobilities (p.c., t.l.c., and paper electrophoresis) similar to those of, carbohydrates.

TABLE I

QUANTITATIVE[a] ANALYSIS OF EXOPOLYSACCHARIDES OF *Rhizobium*

Analysis	Rh. meliloti	Rh. trifolii			Rh. phaseoli		Rh. leguminosarum
	U 27	*U 226*	*Coryn*	*Bart A*	*U 453*	*U 458*	*U 311*
Infective	+	+	+	−	+	+	+
Co-operative	+	+	−	−	+	+	+
Water	3.6	5.0	2.3	7.6	5.3	5.7	5.1
Sulfated ash	11.6	14.0	15.0	21.1	17.3	15.4	19.0
Acetyl	6.3	11.2	8.2	10.2	8.4	10.6	10.2
Pyruvic acid	9.6	9.7	10.5	9.8	9.7	10.5	13.0
Glucose	58.5	58.5	51.2	57.2	42.5	46.0	37.2
Galactose	12.6	11.7	12.8	12.6	10.0	9.4	6.0
Glucuronic acid	0	12.6	13.8	13.3	13.1	13.1	16.5
Periodate consumed (mol./C_6 unit)	1.15	0.75	0.60	0.60	0.80	1.10	1.10
Free-boundary electrophoresis, mobility ($10^4 u/cm^2.s^{-1}.V^{-1}$) pH 2	−	0.49	0.57	−	0.35	0.64	−
pH 7	−	2.0	1.59	−	2.05	1.48	1.40

[a]Except for the electrophoretic mobility, all data are given as weight percentage of the dry polysaccharides.

The presence of pyruvic acid was confirmed by the chromatographic behaviour of the acid and its 2,4-dinitrophenylhydrazone.

The quantitative data on sugar composition of *Rhizobium* polysaccharides have not been consistent until recently, probably because of the various conditions used for acid hydrolysis. Galactose is decomposed by hydrochloric acid hydrolysis and may therefore appear to be present in only small amounts[5–7,9]. Treatment with M sulphuric acid for 24 h at 100° does not effect complete hydrolysis of uronic acid-containing polysaccharides[13]. In spite of the accumulated data[12–15], it has not been possible to correlate the chemical composition of the polysaccharides with the specificity in the host/bacteria interaction.

The figures for the content of pyruvic acid, acetyl, glucose, galactose, and glucuronic acid in Table I are in good agreement with data presented elsewhere[13], but do not indicate significant variation between the strains.

Examinations of polysaccharides[12,14] and oligosaccharides[2,4,5] that have been isolated indicate β-D-interglycosidic linkages. The polysaccharides also have a branched structure; the main chains are (1→3)- and (1→4)-linked with branching residues linked (1→4) and (1→6)[12,14]. No significant variation has been found in the amount of linkage types in the polysaccharides from different strains[14]. The strain *Rh. meliloti* is outstanding, as its polysaccharide lacks glucuronic acid, and has (1→6)-linked chain residues.

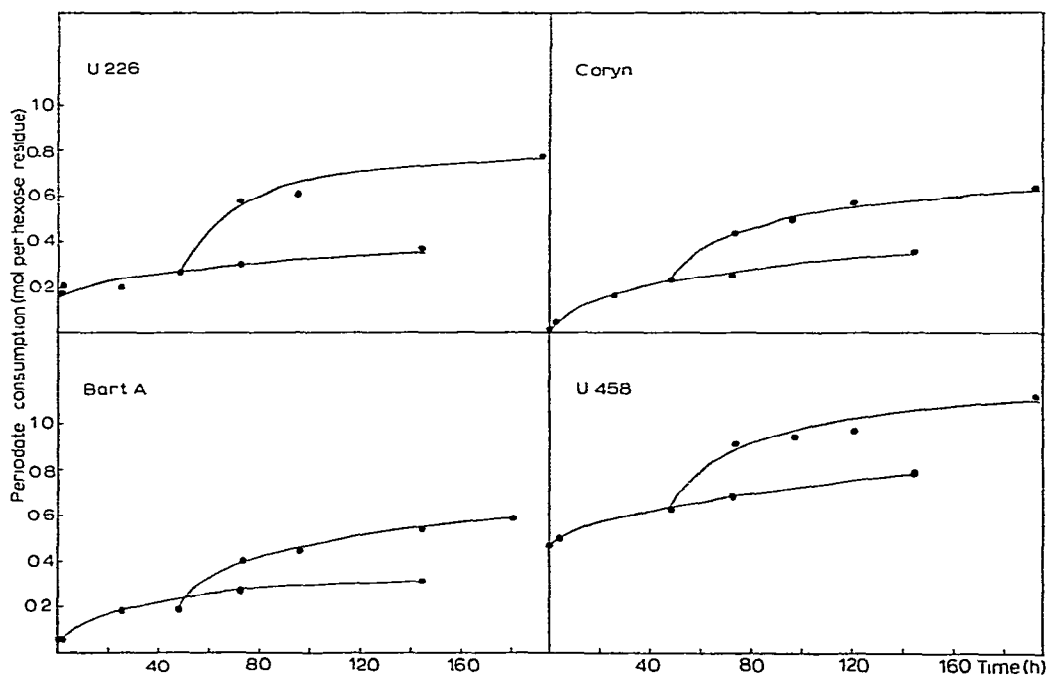

Fig. 1. Periodate-oxidation curves of the polysaccharides of some of the *Rhizobium* strains.

TABLE II

QUANTITATIVE[a] CHEMICAL ANALYSIS OF EXOPOLYSACCHARIDES OF *Rhizobium*

Analysis	Rh. meliloti	Rh. trifolii			Rh. phaseoli		Rh. leguminosarum
	U 27	*U 226*	*Coryn*	*Bart A*	*U 453*	*U 458*	*U 311*
Ca^{2+}	0.085	0.087	0.043	0.066	0.085	0.078	0.052
Mg^{2+}	1.28	0.40	0.25	0.25	0.35	0.70	0.40
K^{+}	2.65	0.91	0.69	0.65	0.60	1.43	0.91
Na^{+}	0.025	2.3	2.8	2.6	3.0	1.1	3.4
Monovalent/divalent	1.96	6.55	12.03	10.48	8.37	3.29	9.57
Kjeldahl N	0.76	0.40	0.38	0.22	0.21	0.28	0.24
Protein (N × 6.25)	4.75	2.50	2.37	1.37	1.31	1.75	1.50
Protein (colorimetric)	5.2	1.4	1.4	0.8	0.6	1.2	1.0

[a]All data are given as weight percentage of the dry polysaccharides.

The periodate-oxidation values in Table I, which have been obtained by a method[30,35] that cleaves the six-membered hemiacetals formed during the first stage of oxidation, thus giving fully oxidized polysaccharides, indicate that polysaccharides of the non-co-operative strain Coryn and the non-infective strain Bart A have a linkage pattern different from that of the polysaccharides of other strains. The consumption of periodate per sugar unit after oxidation for 160 h is lower, which indicates a higher content of (1→3)-linkages, or that one of the vicinal hydroxyl groups in (1→4)- or (1→6)-linked components is acetylated. The acetyl groups are reported to be in the 6-positions in the polysaccharide of *Rh. meliloti*, but their location in the polysaccharides from the other strains is unknown. In the capsular polysaccharides of *Klebsiella* K-type 5, 2-*O*-acetyl groups are present[36]. Fig. 1 shows that the initial oxidation rate of the polysaccharide from the non-nitrogen-fixing strains is slower than those of the effective strains, which also vary. These data indicate that the former polysaccharides are less branched, or that their end groups are more substituted and are thus prevented from being rapidly oxidized.

The content of sulphated ash was found to be high (Table I); the composition of the inorganic components of the polysaccharides is shown in Table II. The ionic composition of the polysaccharide from *Rh. meliloti* is different from that of the polysaccharides from other strains, in that it has a high content of K^+ and little Na^+ (the polysaccharides of the other strains have a high Na^+ content with some K^+). The content of Mg^{2+} is also much higher. The ratio of monovalent/divalent ions varies, the polysaccharides of the nitrogen-fixing strains having the highest values.

The various *Rh.* strains were cultivated in media of fixed ionic composition. The variation in cationic composition of the acid polysaccharides produced by the different strains, therefore, is due to selection of cations, which is possibly connected with variations in the stereochemistry of the polysaccharides. The ionic composition may be of significant importance in the bacteria/host interaction.

Various amounts of protein have been found in the polysaccharide preparations (Table II). It is of interest that one of the root-hair deformation factors is either protein or carbohydrate[20], and deproteinated polysaccharide preparations did not cause the typical root-hair deformations obtained with cruder preparations[37].

ACKNOWLEDGMENTS

I am indebted to Professor A. Haug, Institute of Marine Biochemistry, NTH-Trondheim, who carried out the free-boundary electrophoresis, and to Mrs. Gry Sletten for skilful technical assistance.

REFERENCES

1 M. Beijerinck, *Botan. Ztg.*, 46 (1888) 726.
2 E. Schlüchterer and M. Stacey, *J. Chem. Soc.*, (1945) 776.
3 M. de Leizaola and M. R. Dedonder, *Compt. Rend.*, 240 (1955) 1825.
4 M. de Leizaola, *Compt. Rend.*, 246 (1958) 1761.
5 M. de Leizaola-Tripie, *Compt. Rend.*, 250 (1960) 407.

6 B. A. HUMPREY, *Nature (London)*, 184 (1959) 1802.
7 B. A. HUMPREY AND J. M. VINCENT, *J. Gen. Microbiol.*, 21 (1959) 477.
8 W. F. DUDMAN, *J. Bacteriol.*, 88 (1964) 782.
9 P. H. GRAHAM, *Antonie van Leeuwenhoek, J. Microbiol. Serol.*, 31 (1965) 349.
10 N. AMARGER, M. OBATON, AND H. BLACHÈRE, *Can. J. Microbiol.*, 13 (1967) 99.
11 C. E. CLAPP AND R. J. DAVIS, *Soil Biol. Biochem.*, 2 (1970) 109.
12 H. BJØRNDAL, C. ERBING, B. LINDBERG, G. FÅHRAEUS, AND H. LJUNGGREN, *Acta Chem. Scand*, 25 (1971) 1281.
13 L. P. T. M. ZEVENHUIZEN, *J. Gen. Microbiol.*, 68 (1971) 239.
14 L. P. T. M. ZEVENHUIZEN, *Carbohyd. Res.*, 26 (1973) 409.
15 C. M. HEPPER, *Antonie van Leeuwenhoek, J. Microbiol. Serol.*, 38 (1972) 437.
16 H. LJUNGGREN, *Physiol. Plantarum Suppl.*, (1969) V.
17 T. T. LILLICH AND G. H. ELKAN, *Can. J. Microbiol.*, 14 (1968) 617.
18 D. H. HUBBELL, *Botan. Gaz.*, 131 (1970) 337.
19 B. SOLHEIM AND J. RAA, *Plant Soil*, 35 (1971) 275.
20 B. SOLHEIM AND J. RAA, *J. Gen. Microbiol.*, 77 (1973) 241.
21 W. F. DUDMAN AND M. HEIDELBERGER, *Science*, 164 (1969) 954.
22 M. HEIDELBERGER, W. F. DUDMAN, AND W. NIMMICH, *J. Immunol.*, 104 (1970) 1321.
23 M. DORN, *Zentr. Bakteriol. Parasitenk. Abt. II*, 109 (1956) 120.
24 P. ALBERSHEIM, D. J. NEVINS, P. D. ENGLISH, AND A. KARR, *Carbohyd. Res.*, 5 (1967) 340.
25 G. GUINN, *J. Chromatogr.*, 30 (1967) 178.
26 T. BITTER AND H. M. MUIR, *Anal. Biochem.*, 4 (1962) 330.
27 I. W. SUTHERLAND, *Biochem. J.*, 115 (1969) 935.
28 S. HESTRIN, *J. Biol. Chem.*, 180 (1949) 249.
29 K. KERSTERS AND J. DELEY, *Antonie van Leeuwenhoek, J. Microbiol. Serol.*, 34 (1968) 388.
30 T. PAINTER AND B. LARSEN, *Acta Chem. Scand.*, 24 (1970) 2724.
31 G. O. ASPINALL AND R. J. FERRIER, *Chem. Ind. (London)*, (1957) 1216.
32 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
33 A. HAUG AND B. LARSEN, *Acta Chem. Scand.*, 16 (1962) 1908.
34 E. HAALAND, *Acta Chem. Scand.*, 26 (1972) 2322.
35 M. F. ISHAK AND T. PAINTER, *Acta Chem. Scand.*, 25 (1971) 3875.
36 G. G. S. DUTTON AND M. T. YANG, *Can. J. Chem.*, 51 (1973) 1826.
37 G. FÅHRAEUS AND H. LJUNGGREN, *The Ecology of Soil Bacteria*, Liverpool University Press, Liverpool, 1968.