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PREPARATION OF OLIGOSACCHARIDES BY BACTERIOPHAGE DEGRADATION OF POLYSACCHARIDES FROM *Klebsiella* SEROTYPES K21 AND K32

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ABSTRACT

Depolymerization of bacterial, capsular polysaccharides by phage enzymes is a convenient method of preparing oligosaccharides that correspond to one, or several, repeating unit(s). Thus, the capsular polysaccharide from *Klebsiella* K21 yields a linear pentasaccharide, and that from *Klebsiella* K32, a linear tetrasaccharide. Both oligosaccharides contain acetal substituents, but, whereas the 4,6-*O*-(1-carboxyethylidene)-D-galactosyl residue in the K21 structure is relatively acid-stable, the corresponding 3,4-*O*-(1-carboxyethylidene)-L-rhamnosyl residue in K32 is extremely acid-labile. Phage degradation may, therefore, be the only way by which an oligosaccharide corresponding to an intact repeating-unit may be obtained in such circumstances.

INTRODUCTION

The antigenic determinants of a polysaccharide are usually associated with particular, structural features referred to as immunodominant. Heidelberger *et al.*[1] showed that, in some acidic, bacterial glycans, the immunodominant structure is a pyruvic acid acetal, *i.e.*, a 1-carboxyethylidene group. The presence of such acid-labile groups makes it impossible to isolate, by partial hydrolysis with acid, oligosaccharides that correspond to one intact repeating-unit, and it is clear that other methods of depolymerization must be sought.

It should be noted that the stability of a 1-carboxyethylidene group is very dependent on the manner in which it is linked. Thus, Gorin and Ishikawa[2] showed that, in methyl 4,6-*O*-(1-carboxyethylidene)-α-D-galactopyranoside, the acetal group

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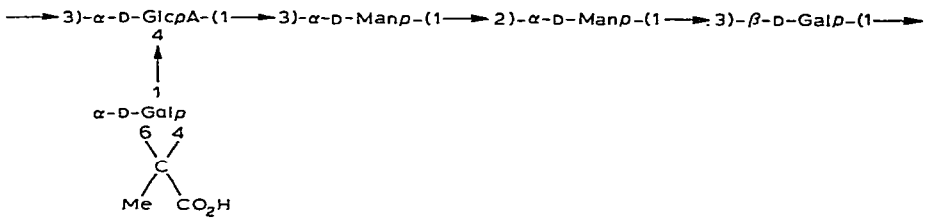
is stable under the acidic conditions necessary to hydrolyze the glycoside. Similar resistance to acid has been noted where this type of unit occurs in capsular polysaccharides isolated from *Klebsiella* bacteria, *e.g.*, serotypes K11 (ref. 3) and K21 (ref. 4). By contrast, when a 1-carboxyethylidene group is linked to O-3 and O-4 of an L-rhamnosyl residue, the acetal is extremely acid-labile; reference has already been made to this in the structural study[5] of the capsular polysaccharide from *Klebsiella* serotype K32.

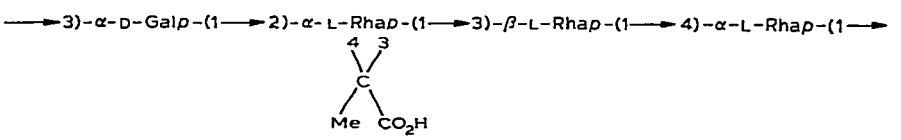
Stirm and co-workers demonstrated that there are bacteriophages (bacterial viruses) that carry glycosidases, each of which is specific for depolymerizing one, or a few, particular, capsular polysaccharides, and employed such degradations in the structural studies of, *inter alia*, the polysaccharides from *Klebsiella* serotypes 11 (ref. 6), 13 (ref. 7), and 25 (ref. 8).

We are interested in adapting these phage degradations to the preparation of gram quantities of oligosaccharides that may subsequently be used for (*a*) the preparation of synthetic antigens[9], (*b*) detailed examination by nuclear magnetic resonance spectroscopy, and (*c*) the study of conformations in solution. We now briefly describe the phage degradation of the polysaccharide from *Klebsiella* K21, which, having a relatively stable 1-carboxyethylidene group, may be used as a basis for comparison in work of this nature. Of greater interest is the phage degradation, and isolation of the intact repeating-unit, of *Klebsiella* K32, which contains an extremely acid-labile acetal; for this, it was found that depolymerization by use of a phage-induced enzyme is the only viable method, to date, for producing an oligosaccharide containing the immunodominant 1-carboxyethylidene group.

The structures of the repeating units in the polysaccharides from *Klebsiella* K21 and K32 are as shown[4,5]. The K32 polysaccharide is one of the few *Klebsiella*



Klebsiella K21



Klebsiella K32

capsules in which no uronic acid is present; the acidity of the polymer is due entirely to the 1-carboxyethylidene group.

Bacteriophages are often designated by the Greek letter ϕ, followed by the number of the serotype that acts as the host strain. Thus, ϕ21 and ϕ32 are the phages that are propagated on, and depolymerize the capsular polysaccharides of, *Klebsiella* serotypes K21 and K32, respectively.

RESULTS

Both ϕ21 and ϕ32 were isolated from sewage, and respectively propagated on their host strains, *Klebsiella* K21 and K32. Published procedures[6] were followed exactly, except that the phages were propagated in nutrient broth, instead of a synthetic medium; Fig. 1 shows the results of a representative experiment. In each instance, the phage was finally purified by isopycnic centrifugation, using a gradient of cesium chloride. The individual, purified, capsular polysaccharides from *Klebsiella* K21 and K32 were dissolved in buffered saline, and incubated with their respective bacteriophage. The depolymerization was monitored viscometrically and by assay of the reducing power. The reaction was assumed to be complete when the latter value became constant; this occurred in ~48 h (see Fig. 2).

The solutions were lyophilized, the residues were redissolved in water, and the solutions were desalted on a column of Sephadex G10. The carbohydrate fraction

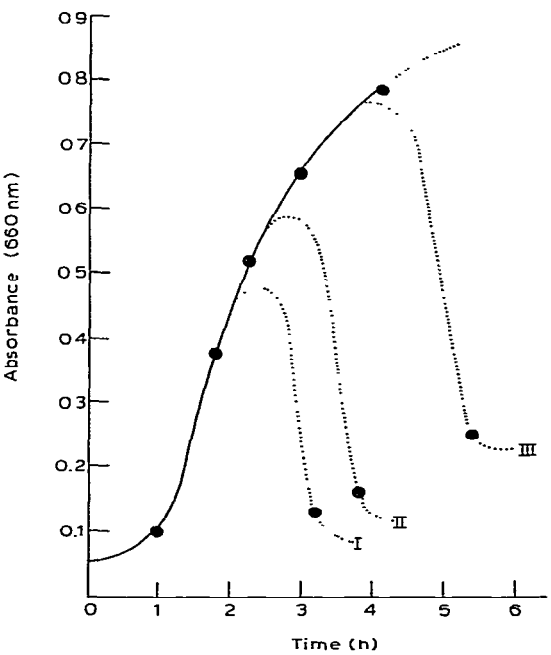


Fig. 1. Propagation of ϕ32 on *Klebsiella* K32; typical, bottle lysis results. [Phage titer: I, 2.4 × 10^{10}; II, 3.0 × 10^{10}; and III, 1.2 × 10^{10} p.f.u./mL.]

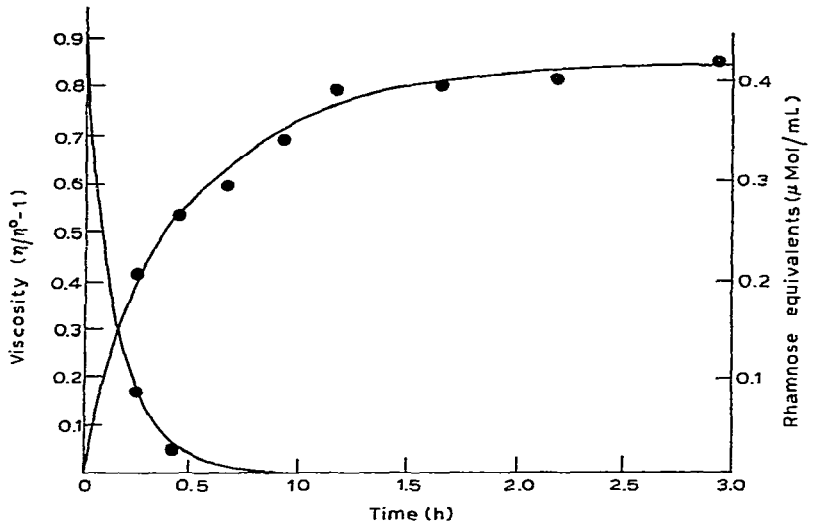


Fig. 2. Depolymerization by bacteriophage ϕ32 of the capsular polysaccharide of *Klebsiella* K32. [Decrease in viscosity (left) and increase in reducing power (right).]

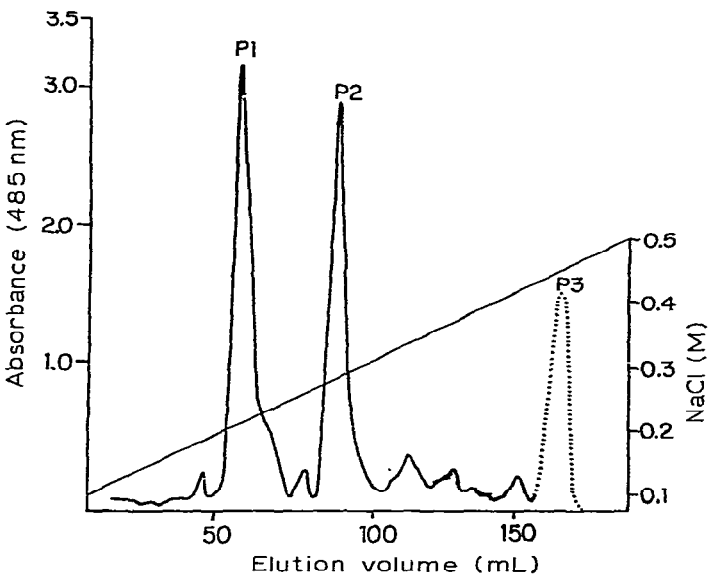


Fig. 3. Separation, on DEAE-Sephadex A25, of *Klebsiella* K21 polysaccharide, depolymerized with ϕ21. [Column (25 cm × 5 cm^2); 250 mg of material; 10 mL.h^{-1}.]

from K32 (but not K21) was dialyzed against distilled water, and the dialyzate (~85%) was lyophilized, the material redissolved in water, and the solution added to a column of DEAE-Sephadex A25. For K21, desalted material was lyophilized, and redissolved, and the solution added to the column of DEAE-Sephadex A25.

The elution pattern for K21 is shown in Fig. 3, where P3 represents polymeric

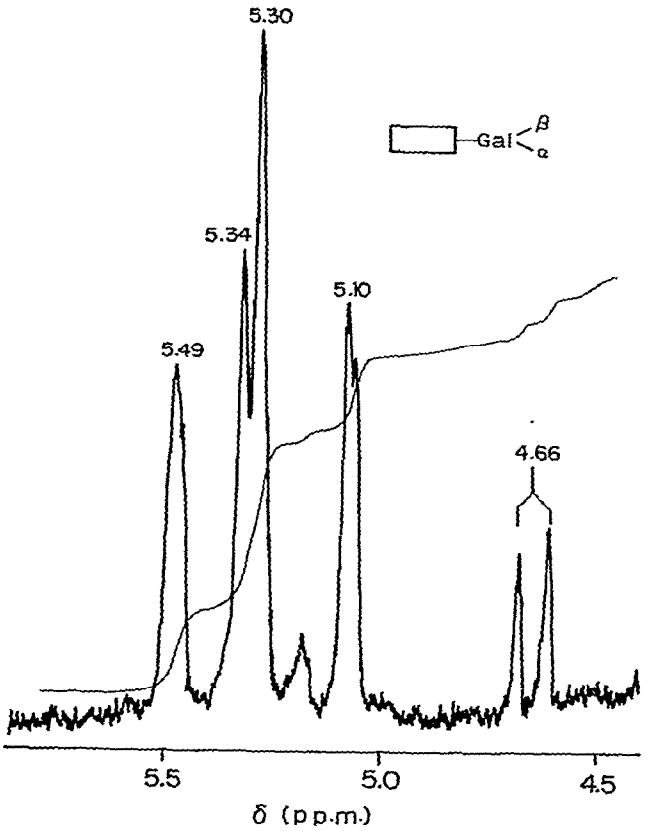


Fig. 4. P.m.r. spectrum (anomeric region only) of fraction P1, a pentasaccharide corresponding to one repeating-unit of *Klebsiella* K21 polysaccharide.

material. Components P1 and P2 were separately desalted, and examined by p.m.r. spectroscopy. The former (P1) showed several spurious peaks at low field, but, after passage through a column of Amberlite IR-120 (H^+) resin, gave the spectrum shown in Fig. 4.

The spectra demonstrate that component P1 is a hexasaccharide corresponding to one repeating-unit, with galactose as the reducing residue, and P2 is composed of two repeating-units (see Table I, and Figs. 4 and 5). In Figs. 4 and 5, the symbol ▭ represents all of the repeating units in the K21 polysaccharide, except the (terminal) β-D-galactopyranosyl group. The two oligosaccharides, P1 and P2, were analyzed by gas–liquid chromatography, using the method of Morrison[10], whereby the ratio of acetylated aldononitrile to acetylated alditol is determined. The results, shown in Table II, confirmed that P1 is a hexasaccharide and that P2 is the dimer. The mobility of P1 in paper chromatography was R_{Glc} 0.045, and in electrophoresis, P1 and P2 had R_{Glc} 0.75 and 0.85, respectively.

The yield of P1 and P2 amounted to 40 and 25%, respectively, of the carbohydrate mixture applied to the column of DEAE-Sephadex A25. Subsequent expe-

TABLE I

N.M.R. DATA FOR *Klebsiella* K21 CAPSULAR POLYSACCHARIDE AND THE OLIGOSACCHARIDES P1 AND P2

Compound	δ^a	^1H-*n.m.r. data*[b]	
		Integral (H)	*Assignment*
K21 polysaccharide	5.48	1	α-GlcA
	5.30, 5.25	2	α-Gal, α-Man
	5.08	1	α-Man
	4.88	1	β-Gal
	1.55	3	acetal
Oligosaccharide P1	5.49	1	α-GlcA
	5.34, 5.30	2.4	α-Gal, α-Man, α-Gal-OH
	5.10	1	α-Man
	4.66	0.6	β-Gal-OH
	1.52	3	acetal
Oligosaccharide P2	5.46	1	α-GlcA
	5.31, 5.27	2.3	α-Gal, α-Man
	5.06	1	α-Gal-OH
	4.87	0.5	β-Gal
	4.64	0.2	β-Gal-OH
	1.53	1.5	acetal

[a]Chemical shift relative to internal acetone; δ 2.23 p.p.m. downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.). [b]Chemical shifts are recorded only for anomeric protons and for those of the 1-carboxyethylidene acetal.

TABLE II

DETERMINATION OF DEGREE OF POLYMERIZATION OF P1 AND P2 FROM THE K21 POLYSACCHARIDE, AND IDENTIFICATION OF THE REDUCING SUGARS

Acetylated derivative of	*Relative retention-time on OV-17*[a]	*Mole-%*[b]	
		P1	*P2*
Mannononitrile	0.67	2.0	2.0
Glucononitrile	0.72	0.91[c]	0.93[c]
Galactononitrile	0.75	0.98	1.5
Galactitol	1.00	0.94	0.47

[a]Column of 3% of OV-17 on Gas Chrom Q (100–120 mesh), programmed at 180° for 4 min, and then at 2°/min to 220°. [b]Values corrected by using molar response-factors. [c]Due to incomplete reduction of uronic acid.

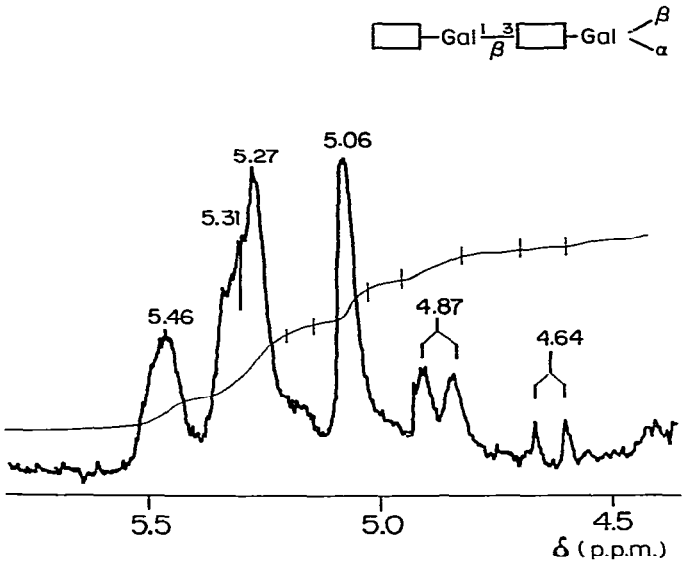


Fig. 5. As in Fig. 4, but, for fraction P2, a decasaccharide equivalent to two repeating-units.

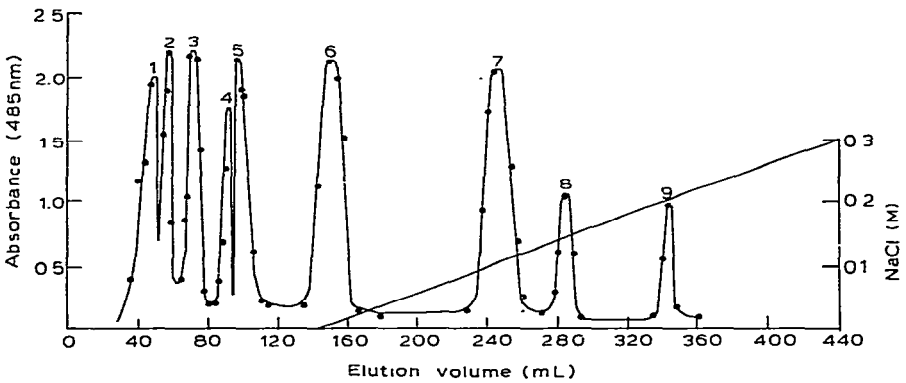


Fig. 6. Separation, on DEAE-Sephadex A25, of depolymerized, *Klebsiella* K32 polysaccharide.

rience has shown that the yield of P1 may be increased substantially, and those of P2 and P3 correspondingly lessened, by allowing the depolymerization reaction to proceed for a longer time.

The simplicity of the elution pattern obtained for depolymerized K21 (see Fig. 3) may be contrasted with the complexity of that for depolymerized K32 (see Fig. 6). Five small fractions were not bound by the column, and were eluted prior to the start of the sodium chloride gradient. The p.m.r. spectra of these components confirmed the absence of any 1-carboxyethylidene groups, and these fractions thus corresponded to neutral oligosaccharides that were separated according to size only. In Fig. 6, component 3 was judged, from its p.m.r. spectrum, to be a neutral tetra-

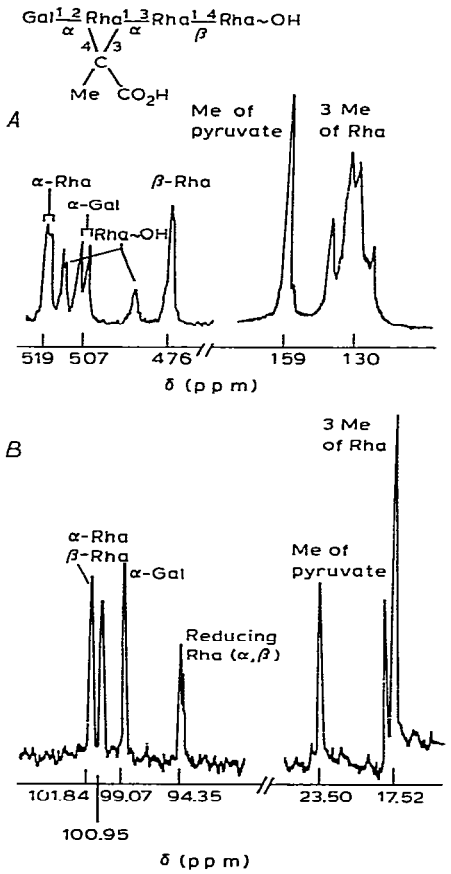


Fig. 7. N.m.r. spectra of the tetrasaccharide, the single repeating-unit of *Klebsiella* K32 polysaccharide; (*A*) p.m.r., (*B*) ^{13}C.

saccharide, equivalent to one repeating-unit completely devoid of 1-carboxyethylidene groups, while component 6 appeared to be an oligosaccharide fraction having only a partial complement of acetal. Component 7, obtained in a yield of ~30%, corresponded to one repeating-unit of the capsular polysaccharide from *Klebsiella* K32. Figs. 7a and 7b show the ^{1}H- and ^{13}C-n.m.r. spectra, respectively, of this oligosaccharide. The p.m.r. spectrum of fraction 3 (neutral tetrasaccharide) was very similar to that shown in Fig. 7a for the acidic tetrasaccharide, except that the chemical shifts of the C-6 signals of the three L-rhamnosyl residues were more nearly coincident.

DISCUSSION

The depolymerization of the capsular polysaccharide from *Klebsiella* K21 follows the pattern for that of K11 and others, and needs little comment. In using p.m.r. spectroscopy to assign the configuration of anomeric linkages, the general

rule is that a signal upfield of δ 5.0 indicates a β-linkage. Despite the validity of this rule, it should be noted that the chemical shifts of certain anomeric protons may differ markedly, depending on the substitution pattern of the sugar unit. This is clearly illustrated by comparing the shifts for the anomeric protons of terminal and in-chain β-D-galactopyranosyl residues, as shown in the p.m.r. spectra of components P1 and P2 (see Figs. 4 and 5).

The very great lability of the 1-carboxyethylidene group in the polysaccharide from *Klebsiella* K32 has already been mentioned in connection with the structural studies[5]. It is, therefore, not surprising to find that much of the acetal was eliminated during processing, most probably at the desalting step. Despite this problem, the isolation of a reasonable quantity of the oligosaccharide corresponding to one complete repeating-unit clearly demonstrates the importance of selective degradations by phage-borne enzymes. The isolation of such a tetrasaccharide acetal by any procedure involving acid hydrolysis would be impossible.

EXPERIMENTAL

General methods. — The phages ϕ21 and ϕ32 were isolated from sewage, and purified by successive replating on their host strains, *Klebsiella* K21 and K32, respectively. Purified phages were prepared exactly as described previously[6], except that they were propagated in broth (5 g of peptone, 3 g of beef extract, and 2 g of sodium chloride per liter), instead of synthetic P-medium (see Fig. 1). After precipitation with poly(ethylene glycol) 6000, the phages were isolated by isopycnic centrifugation through a cesium chloride gradient, and they banded at 1.44 g/mL (ϕ32).

Paper electrophoresis was performed on a SAVANT high-voltage (5-kV) system (Model LT-48A), with kerosene as the coolant. The buffer used contained 5:2:743 (v/v) pyridine–acetic acid–water, pH 5.3. Strips (77 × 20 cm) of Whatman No. 1 paper were used with a current of 100 mA for 4 h. For descending paper-chromatography, freshly prepared 2:1:1 1-butanol–acetic acid–water was used. The ratio of acetylated aldononitriles to acetylated alditols was determined by using the method of Morrison[10]; glucuronic acid was converted into the neutral sugar by methanolysis and reduction[11].

Depolymerization. — Purified, capsular polysaccharide (800 mg) from *Klebsiella* K32 was dissolved in physiological, phosphate-buffered saline (PBS; 400 mL, pH 7.0), and to this solution was added a total of 1×10^{13} plaque-forming units (PFU) in PBS (30 mL). A sample of the reaction mixture was transferred to an Ostwald viscometer which, together with the flask containing the bulk of the solution, was placed in a bath at 37°. The viscosity of the solution was determined periodically, and, at the same time, aliquots were withdrawn and analyzed for reducing power, in comparison with a standard curve based on rhamnose[6].

Purification and separation of depolymerized material. — Portions (2 × 1.5 g) of the crude, lyophilized, depolymerization mixture were desalted by using a column of Sephadex G10 (100 cm × 19.5 cm^2). The column was eluted with 500:5:2 water–

pyridine–glacial acetic acid buffer, pH 4.5, at a flow rate of 25 mL/h, and carbohydrate material was located by using the Molisch test. Part (430 mg) of the desalted material was dialyzed against distilled water (3 × 1 L), and lyophilization yielded 64.5 mg (15%) of non-dialyzable material, and 350 mg of dialyzable product that was considered to contain oligomers not greater than dodecasaccharides.

The dialyzable material (320 mg) was then applied to the top of a column (25 × 5 cm^2) of DEAE-Sephadex A25 that had been packed in 0.5M Tris HCl buffer (pH 7.2) and then equilibrated with 25mM Tris HCl buffer; at least 10 column volumes of the latter buffer were needed in order to achieve equilibration (as determined by performing conductivity measurements). The material was applied as a solution in 25mM Tris HCl (2 mL), the column was eluted with 25mM Tris HCl (140 mL) at 10 mL/h, and a linear salt-gradient (from 0 to 0.35M NaCl) was then begun. Fractions (2 mL) were collected, and examined by using the phenol–sulfuric acid assay; the elution profile is shown in Fig. 6. Fraction 7 (90 mg) was obtained.

In a similar way, phage-degraded K21 polysaccharide (250 mg) yielded P1 (97 mg), P2 (50 mg), and P3 (polymeric material). The p.m.r. spectrum of fraction P1 showed several spurious peaks (buffer ?) at low field, but these were eliminated by passage through a column of Amberlite IR-120(H^+) ion-exchange resin, and neutralization with 0.1M NaOH.

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