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Analysis of the Enzymatic Cleavage (β Elimination) of the Capsular K5 Polysaccharide of *Escherichia coli* by the K5-Specific Coliphage: a Reexamination

PETER HÄNFLING, ALEXANDER S. SHASHKOV,† BARBARA JANN, AND KLAUS JANN*

Max-Planck-Institut für Immunbiologie, D-79108 Freiburg, Germany

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The capsular K5 polysaccharide of *Escherichia coli* is the receptor of the capsule-specific coliphage K5, which harbors an enzyme that degrades the capsular K5 polysaccharide to a number of oligosaccharides. Analysis of the degradation products using gel permeation chromatography, the periodate-thiobarbituric acid and bicinchoninic acid reactions, and nuclear magnetic resonance spectroscopy showed that the major reaction products are hexa-, octa-, and decasaccharides with 4,5-unsaturated glucuronic acid ($\Delta^{4,5}$GlcA) at their nonreducing end. Thus, the bacteriophage enzyme is a K5 polysaccharide lyase and not, as we had reported previously, an endo-*N*-acetylglucosaminidase.

Many *Escherichia coli* cells are surrounded by capsules which consist of acidic polysaccharides and which protect the bacteria in hostile surroundings. The capsular polysaccharides (K antigens) are important virulence determinants, and they also contribute to the serological specificity of the bacteria (3, 10–12, 19).

Capsular polysaccharides are not only surface antigens but also receptors of specific capsule phages. Many of these bacteriophages contain enzymes as integral structures of the phage particle (4, 6, 9, 16, 22, 23). The phage-borne enzymes may be endoglycosidases or polysaccharide lyases. Lyases degrade the polysaccharide in a β-elimination reaction between the oxygen of a (1→4) glycosidic linkage and C-4 of an adjacent hexuronic acid. The reaction products have a 4,5-unsaturated hexuronic acid ($\Delta^{4,5}$HexA) at the nonreducing end.

The capsular K5 polysaccharide of *E. coli* has the repeating unit structure →4)-βGlcA-(1→4)-αGlcNAc-(1→ (24). A K5-specific coliphage which enzymatically degraded the capsular K5 polysaccharide to small oligosaccharide fragments was isolated (9). In an earlier report from this laboratory, the bacteriophage enzyme was erroneously described as an endo-α-*N*-acetylglucosaminidase (8). A reexamination of the degradation products, presented in this communication, showed that the K5 polysaccharide is degraded in a β elimination and that the K5 phage-borne enzyme is a K5 polysaccharide lyase.

Cleavage of the K5 polysaccharide with bacteriophage K5. The K5 polysaccharide was extracted from *E. coli* 2980 (O18:K5:H7) and purified as described previously (7, 13). A portion (3.5 mg) was incubated with 10^{12} PFU of the bacteriophage in a total volume of 1 ml at 37°C. After various times, portions (50 μl) were analyzed by polyacrylamide gel electrophoresis (PAGE; 25 V cm^{-1}). Figure 1 shows that the degradation became apparent after 10 min and continued for 80 to 120 min. After 180 min, only a few fast-moving bands were observed, and the pattern did not change after further incubation.

In separate experiments, the increase in reducing capacity was measured in portions (100 μl) of the incubation mixture with the bicinchoninic acid reagent (21) and GlcNAc as a reference. Figure 2 shows that the reducing capacity reached a plateau after about 80 to 90 min, with only very little further increase. The final concentration of reducing groups was about 0.45 M. On the basis of a molecular mass of 80 to 100 kDa for the polysaccharide (16a), the mean sizes of the final reaction products were estimated to represent about five disaccharide repeating units.

The 75-MHz ^{13}C nuclear magnetic resonance (NMR) spectrum of the reaction mixture (after 80 min) was much more complex than that of the K5 polysaccharide. Figure 3 shows the spectrum of the K5 polysaccharide with the region of δ 120 to 90 of the reaction product as an inset. Most notable was the presence of a signal at δ 112.6 in the spectrum of the reaction product which was not present in the spectrum of the polysaccharide. This signal was assigned to the unshielded C atom (boldface) of the sequence -H**C**═C-CO-, indicative of C-4 of a 4,5-unsaturated glucuronic acid residue ($\Delta^{4,5}$GlcA) (14). The latter would be expected at the nonreducing ends of the prod-

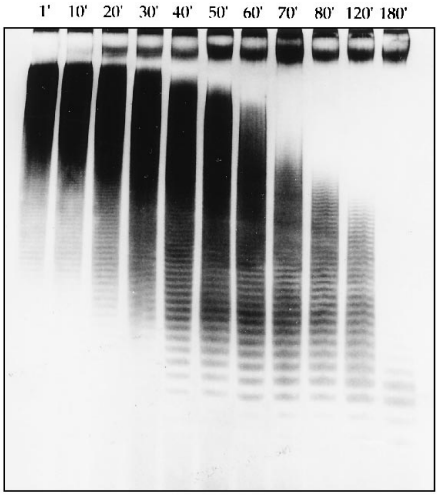

FIG. 1. Sodium dodecyl sulfate-PAGE patterns of the incubation of the K5 polysaccharide with coliphage K5. Samples of the incubation mixtures (50 μl) were run after the times (in minutes) indicated. The gel was stained with the alcian blue-silver reagent (17).

* Corresponding author. Mailing address: Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-79108 Freiburg, Germany. Fax: (0)761-5108-221.

† On leave from the Zelinsky Institute for Organic Chemistry, Moscow, Russia.

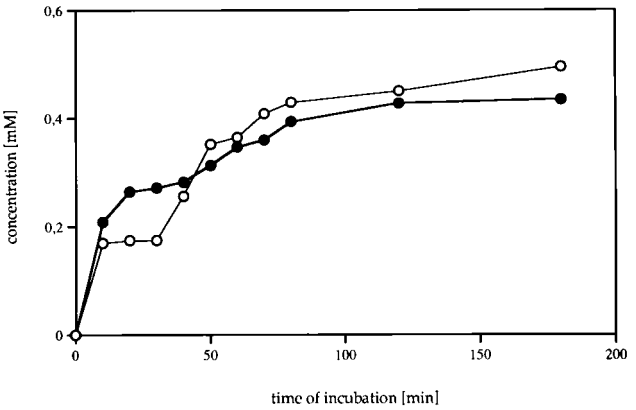

FIG. 2. Liberation of reducing groups (○) and nonreducing $\Delta^{4,5}$GlcA (●) during incubation of the K5 polysaccharide with coliphage K5. Samples (100 μl) of the incubation mixture were treated, after the times indicated, with the bicinchoninic acid reagent (21) for determination of the reducing capacity. In parallel setups, nonreducing $\Delta^{4,5}$GlcA levels were determined with the thiobarbituric acid reagent (15, 25, 26).

ucts of a β-elimination reaction between the structural units αGlcNAc-(1→4)-βGlcA of the K5 polysaccharide.

The product(s) obtained at various times from the polysaccharide with coliphage K5 (100-μl portions of the incubation mixture) was treated with the periodate-thiobarbituric acid reagent (15, 25). This treatment converted the $\Delta^{4,5}$GlcA residue at the nonreducing end to formyl pyruvate, which in turn reacted to form a violet product. Since this was the same reaction as that used for the determination of 2-keto-3-deoxy-*manno*-octonic acid (Kdo) (26), this compound was used as a reference. Figure 2 shows that the level of formation of nonreducing terminal $\Delta^{4,5}$GlcA, thus determined, parallels that of the reducing GlcNAc. These data show that the K5 polysaccharide is degraded by coliphage K5 in a β-elimination reaction. They correct our previous interpretation of the enzyme as an endo-α-*N*-acetylglucosaminidase (8).

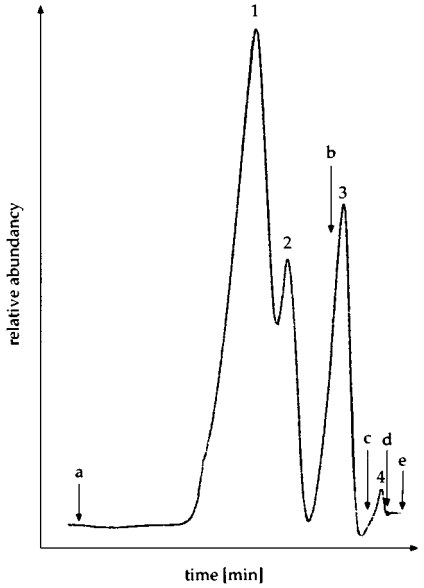

FIG. 4. Pattern of elution from TSK HW-40 of the products of a 180-min incubation of the K5 polysaccharide with coliphage K5. The incubation mixture (400 μl) was eluted from the column (85 cm by 16 mm) with 0.1 M acetic acid at a flow rate of 1 ml min^{-1}. The refractive index of the eluant was determined with a refractometer (Waters). References, the elution positions of which are indicated by arrows, were yeast mannan (a), stachiose (b), raffinose (c), cellobiose (d), and galactose (e). Peaks 1 to 3 are indicated.

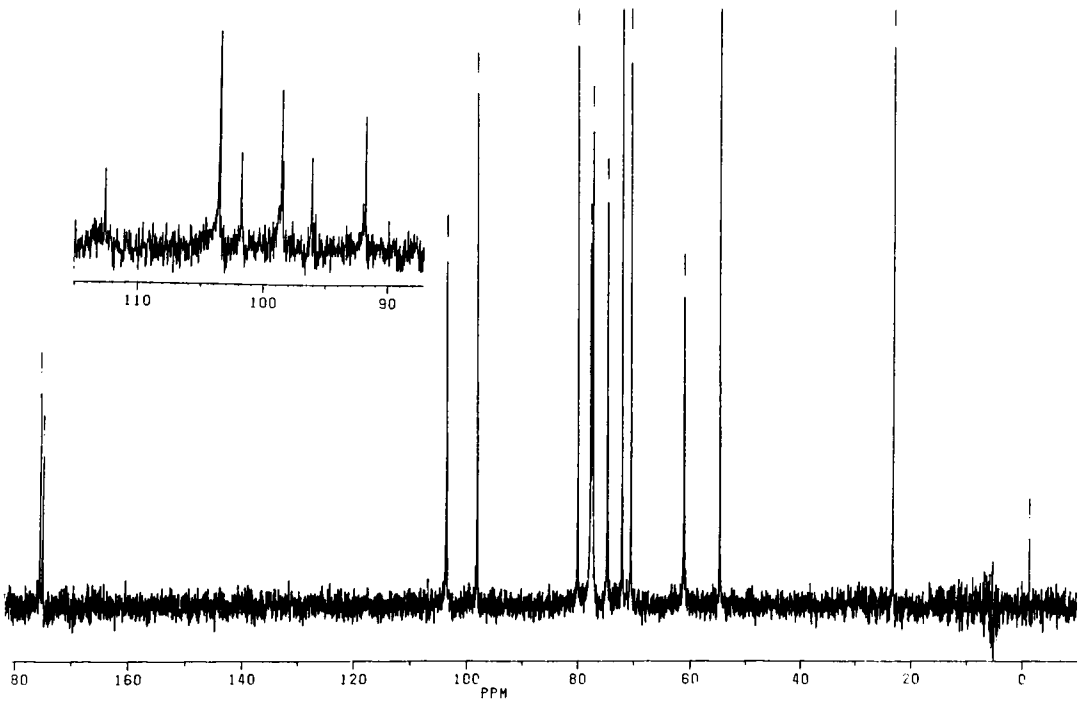

FIG. 3. Seventy-five-megahertz ^{13}C NMR spectrum of the K5 polysaccharide, recorded in D_2O (70°C). Acetone (δC, 31.45) was used as an internal standard. The inset shows the anomeric region of the spectrum given by the products of a 180-min incubation of the K5 polysaccharide with coliphage K5.

TABLE 1. NMR data for the K5 polysaccharide from *E. coli* O18:K5 and for the material from peak 3 of Fig. 4

Sample[a]	C and H no.	NMR result for:					
		GlcA		$\Delta^{4,5}$Glc		GlcNAc	
		C	H	C	H	C	H
PS[b]	1	103.5	4.46			98.1	5.32
	2	74.6	3.39			54.6	3.87
	3	77.3	3.73			70.5	3.75
	4	77.7	3.80			79.9	3.67
	5	77.8	ND[c]			72.0	3.85
	6	174.2				61.0	ND
Pk-3	1	103.6	4.55	101.8	5.18	99.7	5.38
	2	74.6	3.39	73.5	3.83	55.3	3.88
	3	77.1	3.72	67.4	4.23	70.6	3.76
	4	80.1	3.81	112.6	6.02	80.4	3.61
	5	76.0	3.90	ND		72.0	3.85
	6	174.2		175.6		61.9	ND

[a] PS, polysaccharide from *E. coli* O18:K5; Pk-3, material from peak 3 of Fig. 4.
[b] The values are consistent with the structure →4)–β-GlcA–(1→4)–α-GlcNAc–(1→ (24).
[c] ND, not determined.

Separation and characterization of the reaction products. Figure 4 shows that chromatography on TSK HW-40 (Merck) resolved the polysaccharide-bacteriophage incubation mixture (400 μl on a column [850 by 16 mm]) into three major fractions (peaks 1 to 3) and one minor fraction (peak 4). The materials from peaks 1 to 3, as well as the K5 polysaccharide, were analyzed by ^{1}H and ^{13}C NMR spectroscopy. The material from peak 3 was studied in more detail by two-dimensional techniques.

The ^{1}H NMR spectrum of the polysaccharide contained signals for the anomeric protons of α-GlcNAc (δ 5.32; $J_{H1,H2}$, 3.8 Hz) and β-GlcA (δ 4.46; $J_{H-1,H-2}$, 7.8 Hz). The spectrum of the material from peak 3 contained, in addition, signals for the anomeric proton of $\Delta^{4,5}$GlcA (δ 5.18; $J_{H-1,H-2}$, 7.8 Hz). There was also a small signal at δ 4.57 ($J_{H-1,H-2}$, 8.0 Hz) due to the β-anomeric proton of the reducing terminal GlcNAc.

The ^{13}C NMR spectrum of the material from peak 3 contained signals at δ 99.7, 103.6, and 101.8, which were assigned to the anomeric carbons of α-GlcNAc, β-GlcA, and β-$\Delta^{4,5}$GlcA, respectively. The NMR signals (Table 1) were assigned by two-dimensional homonuclear correlated spectroscopy (COSY), one- and two-step relayed COSY, and heteronuclear ^{13}C-^{1}H COSY using the standard Bruker software COSYHG, COSYRCT, COSYRCT2, and XHCORR, respectively. The ^{1}H NMR signal assignments of the K5 polysaccharide agree with those reported by Mulloy and Johnson (18).

TABLE 2. Relative intensities of the reporter signals for β-$\Delta^{4,5}$GlcA H-4 (at δ 6.0), β-GlcA H-1 (at δ 4.55), and α-GlcNAc H-1 (at δ 5.38) in the ^{1}H NMR spectra of the K5 polysaccharide degradation by bacteriophage K5

Material from Fig. 4 (peak no.)	Relative intensity of the reporter signal for:			Interpretation	
	β-$\Delta^{4,5}$GlcA H-4	β-GlcA H-1	α-GlcNAc H-1	OS size[a]	**n** in the formula[b]
1	1	4	4	10	4
2	1	3	3	8	3
3	1	2	2	6	2

[a] OS, oligosaccharide.
[b] Formula, β-$\Delta^{4,5}$GlcA–(1→4)–[α-GlcNAc–(1→4)–β-GlcA]$_{\mathbf{n}-\alpha/\beta\text{-GlcNAc}}$.

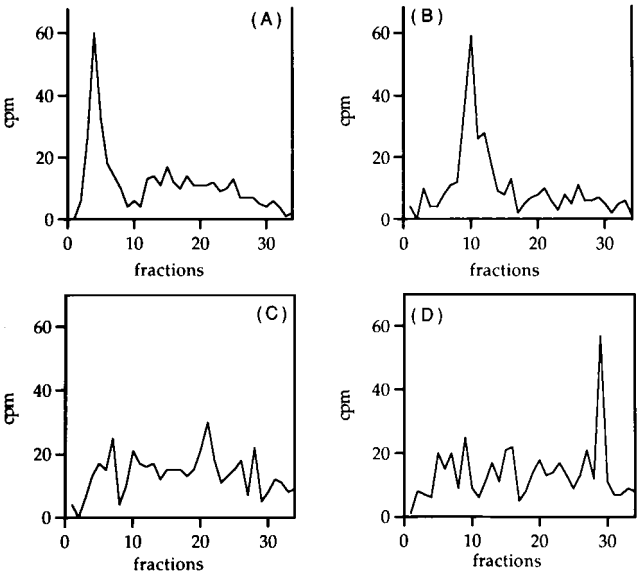

FIG. 5. Fractionation patterns of reaction mixtures from incubations of coliphage K5 with K5 polysaccharide, labelled at the nonreducing end with [^{3}H]glucuronic acid. The incubation mixtures were chromatographed on TSK HW-40 after reaction times of 10 s (B), 30 s (C), and 60 s (D). Fractions (20 μl) were analyzed with a Beckman liquid scintillation counter. A mixture in which the reaction was stopped with 12% acetic acid immediately after addition of coliphage K5 was used as a control (A).

To assess the sizes of the oligosaccharides, the intensities of the signals for H-1 of α-GlcNAc and β-GlcA were compared with that of the signal for H-4 of $\Delta^{4,5}$GlcA (reporter signal). The results obtained from major peaks 1 to 3 are shown in Table 2. They indicated that these major products were hexa-, octa-, and decasaccharides.

Mode of the lyase reaction. We wanted to differentiate whether the K5 lyase cleaved the polysaccharide from one end of the chain or randomly within the chain. For this purpose, polysaccharide in which the nonreducing end was labelled with [^{3}H]GlcA was prepared. Membranes (100 μl; 300 to 400 μg of protein) from the expression mutant *E. coli* LE392(pGB118::1) (1) were incubated with UDP[U-^{3}H]GlcA (100,000 cpm; prepared by DuPont) and 100 μg of polysaccharide (exogenous acceptor) in a total volume of 200 μl, as described previously (2). The purified (5) ^{3}H-labelled polysaccharide (1,200 cpm) was divided into portions (about 200 cpm), and each was incubated with 10^{12} PFU of the bacteriophage for a different time. The results of the subsequent size fractionation of the products by gel permeation chromatography are shown in Fig. 5. A comparison of the distribution of radioactivity with time indicated that the lyase cleaved the K5 polysaccharide within the chain in a random way.

The sizes of the final reaction products (hexasaccharides to decasaccharides) are in keeping with those reported for other bacteriophage-borne polysaccharide-degrading enzymes (16, 22). The reason for this may be that an enzyme which cleaves within a polysaccharide chain requires a minimum substrate size for recognition and cleavage. Hence, the topography of the K5 polysaccharide lyase will probably not allow efficient formation of one repeating disaccharide unit.

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