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# A BACTERIOPHAGE-ASSOCIATED GLYCANASE CLEAVING β-PYRANOSIDIC LINKAGES OF 3-DEOXY-D-manno-2-OCTULOSONIC ACID (KDO)

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SUMMARY: A bacteriophage growing on Escherichia coli K13, K20, and K23 strains carries a glycanase that catalyzes the hydrolytic cleavage of the β-ketopyranosidic linkages of 3-deoxy-D-manno-2-octulosonic acid (KDO) in the respective capsular polysaccharides. The main cleavage product of the K23 polysaccharide has been identified by $^{1}H$- and $^{13}C$-n.m.r. spectroscopy as βRibf1→7βKDOp2→3-βRibf1→7KDO. Cleavage of polysaccharides containing α-pyranosidic, or 5-substituted β-pyranosidic KDO is not catalyzed by the enzyme. 

To our knowledge, enzymes catalyzing the hydrolysis of glycosides of 3-deoxy-D-manno-2-octulosonic acid (KDO) (1) have not been described. Here, we report on a hydrolase of this type, associated with bacteriophage Φ20. The enzyme catalyzes the depolymerization of the KDO-containing capsular polysaccharides from Escherichia coli K13, K20, and K23 strains (Fig. 1).

## MATERIALS AND METHODS

Growth media. Merck (Darmstadt, F.R.G.) standard I medium was generally used. Large-scale production, however, of bacteria and of phages were performed in glucose-amino acids-salts media (2,3).
Bacteria and bacterial antisera. The following strains of Escherichia coli (4) were employed. Bi 7457-41 (O4:K6:H5), Su 65-42 (O4:K12:H⁻), Su 4344-41 (O4:K13:H1), F 7902-41 (O15:K14:H4), F 8316-41 (O6:K15:H16), E 19a (O21:K20:H⁻), H 54 (O25:K23:H1), and E 3b (O75:K95:H5). Strain E 3b was obtained

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ABBREVIATIONS: KDO, 3-deoxy-D-manno-2-octulosonic acid; n.m.r., nuclear magnetic resonance; p.f.u., plaque-forming units; TBA, thiobarbituric acid.

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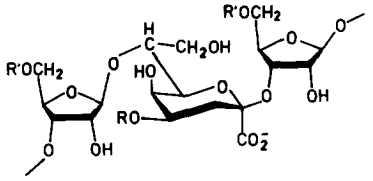


Figure 1. Glycosidic linkages and O-acetylation patterns of capsular polysaccharides from E. coli K13, K20, and K23 strains. K13, R = acetyl, R' = H; K20, R = H, R' = acetyl; K23, R = R' = H.

from the National Type Culture Collection, London, England. All other strains were generously supplied by Drs. I. and F. Ørskov (WHO International Escherichia Center, Statens Seruminstitut, Copenhagen, Denmark), and by Dr. P. W. Taylor (Ciba-Geigy Pharmaceuticals, Horsham, England). The latter also supplied E. coli LP 1092, the capsule of which contains α-ketosidically linked KDO (5,6). Suitable E. coli antisera were purchased from Difco Laboratories (Detroit, MI, U.S.A.).

Bacteriophage. Using standard procedures (2,7) and E. coli E 19a (K20) as a host, phage Φ20 was isolated from local sewage and was purified by successive, single-plaque isolations. A stock suspension was prepared in nutrient broth and was stored at 4°C over chloroform. The phage titer of this suspension remained unchanged over a period of several months. For large-scale production of Φ20, the procedure described for phage Φ1.2 (8) was followed. Ca. $3\times10^{12}$ p.f.u. of purified Φ20 virions were thus obtained from 1 l of medium.

Polysaccharides. K20 polysaccharide was isolated from E. coli E 19a by the method of Gotschlich (9) as modified by Jann, et al. (2; cf. also Vann, et al. ref. 10). The K23 polysaccharide was obtained by de-O-acetylation of the K20 glycan (10,11). The K13 polysaccharide was a generous gift of Drs. Barbara and Klaus Jann, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G.

Phage-catalyzed depolymerization reaction. Solutions (200 µl) of polysaccharide in phosphate-buffered saline (0.3 mg per ml) were incubated in closed vessels with purified Φ20 particles (3×10 p.f.u. per ml) at 37°C. A few drops of chloroform were added for sterility. After increasing incubation times, samples were analyzed for reducing groups using a ferricyanide reagent (12). Polysaccharide samples analogously incubated with heat-denatured (10 min at 100°) phage, were analyzed in parallel. Phosphate-buffered saline served as blank, and ammonium KDO (13) was used as standard.

Acid-catalyzed depolymerization reaction. Polysaccharide samples were incubated with 1% acetic acid at 100° for 30 min. The reaction was stopped by the addition of an equivalent amount of dilute sodium hydroxide solution and the products analyzed as described below for phage-depolymerized material.

Separation and analysis of depolymerization products. Following incubation with phage ($4\times10^{10}$ p.f.u. per ml) of 10-300 mg samples of polysaccharide (e.g., 4 mg per ml of phosphate-buffered saline) for 8h, the phage particles were removed by ultrafiltration (Centricon 30 microconcentrator, Amicon Corp., Danvers, MA, U.S.A.). Then, the chloroform was chased, and the volumes were reduced by evaporation (at 35° in vacuo). The resulting solutions, e.g. containing 8 mg of carbohydrate, were filtered through a column (1×100 cm) of Biogel P-30, using 50 mM sodium phosphate buffer (pH=7.2) as the eluant (8 ml per h). Fractions (2 ml) were collected. Detection of KDO-containing materials in all fractions was performed by a modified periodic acid-thiobarbituric acid (TBA-) assay (14). The TBA-positive fractions eluted between the exclusion and inclusion volumes were combined, lyophilized, and taken up in a small volume of distilled water. Subsequently, the pooled samples were passed over a column of Biogel P-4 (1×150 cm) in distilled water for desalting and separation.

Nuclear magnetic resonance (n.m.r.) measurements. The structure of the main depolymerization product 1 was analyzed by $^{1}H$- and $^{13}C$-n.m.r. spectroscopy. $^{1}H$-N.m.r. spectra (250 MHz) were recorded on a Bruker WM 250 instrument at 313

K with deuterium lock on the water signal. The instrument was referenced to acetone-$d_6$ at 2.15 p.p.m. Spectral width was 2 kHz, and 16 k of memory were used.
$^{13}$C-N.m.r. spectra (62.9 MHz) were recorded on the same instrument equipped with a 5 mm probe head and working in the Fourier transform mode. Recording temperature was 297 K and spectra were referenced externally to the signal of tetramethylsilane whose resonance frequency was set at 67.40 p.p.m. upfield from the signal of dioxane in deuterium oxide. Spectral width was 7.2 kHz, and 16 k of memory were used.
To facilitate the empirical interpretation of the spectra of 1, the oligosaccharide was reduced with sodium borohydride. The resulting mixture was freed of boric acid by repeated evaporation with methanol, the residue taken up in distilled water, and the solution passed over a column of Biogel P-4 for desalting. The products obtained were analyzed by $^1$H- and $^{13}$C-n.m.r. spectroscopy as described above.

## RESULTS AND DISCUSSION

Formation of reducing groups upon incubation of K20 polysaccharide with phage Φ20. The incubation of K20 glycan (10) (6.2 mM with respect to repeating units) at 37°C with ca. $2\times10^{10}$ phage particles per mg of polysaccharide led to an increase of reducing power (12), reaching a plateau at ca. 3 mM of reducing KDO equivalents after ca. 6 h. During a parallel, analogous incubation with heat-inactivated phage, the reducing power remained unchanged at the low level measured for the intact polysaccharide (ca. 0.17 mM of reducing KDO equivalents).

Formation of oligosaccharides by phage Φ20-catalyzed hydrolysis of the K23 polysaccharide. The elution diagram (Fig. 2) from Biogel P-4 shows the molecular size distribution of the oligosaccharides formed by phage-catalyzed hydrolysis of the K23 polysaccharide. By comparison with sialosyl oligosaccharides of known molecular mass, the main product formed after an incubation time of 8 h was assumed to be a tetrasaccharide comprising two repeating units (1; Scheme 1). The structure of compound 1 was then unambiguously identified by $^1$H- and $^{13}$C-n.m.r. spectroscopy(see below). The elution diagram also indicated the presence of smaller amounts of the corresponding hexa-, octa-, and decasaccharides.

Mild-acid-catalyzed hydrolysis of the K23 polysaccharide. Figure 2 also shows an elution diagram from Biogel P-4 of the products obtained by controlled, acid-catalyzed hydrolysis of the K23 polysaccharide. The products thus obtained correspond essentially to those of the phage-catalyzed reaction. However, more of the disaccharide corresponding to one repeating unit is formed under catalysis by acid, because the enzyme-catalyzed process essentially stops at the tetrasaccharide stage (cf. 15).

N.m.r. analysis of the product 1. The 250 MHz $^1$H-n.m.r. spectrum of 1 is very complex and unsuitable for first-order analysis. This is due to 1 being a mixture of at least three oligosaccharides containing tautomeric forms of the reducing KDO residue, namely the α-pyranose and two furanoses (Scheme 1; cf.

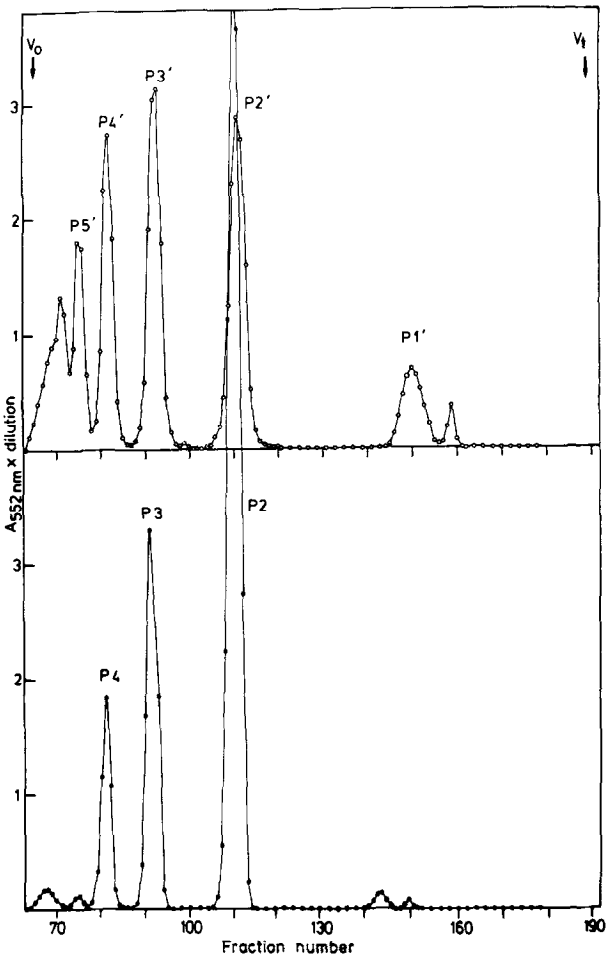
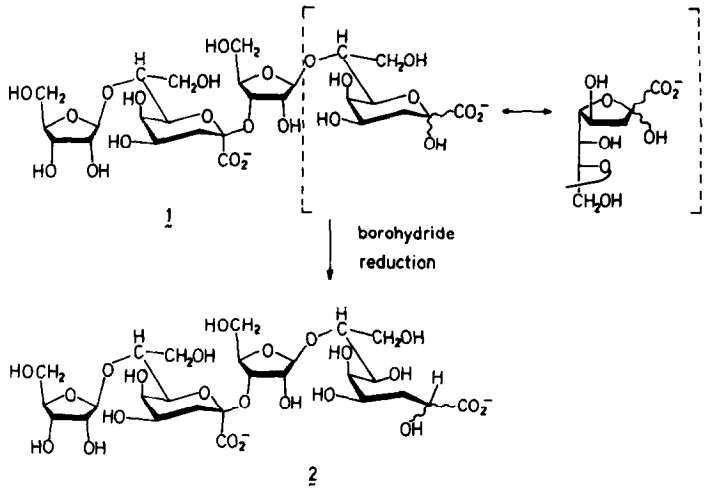



Figure 2. Elution diagrams from Biogel P-4 of oligosaccharides produced from the K23 polysaccharide by treatment with 1% acetic acid at 100° for 1 h (upper diagram) or by phage-catalyzed depolymerization (lower diagram). P1, P2, P3, etc. indicate oligosaccharides composed of 1, 2, 3, etc. (disaccharide) repeating units.




1

2

Table 1. Partial $^1$H-n.m.r. spectra of the tetrasaccharide 1 and its reduction product 2 (cf. Scheme 1)

| compound/ repeating unit | chemical shifts in p.p.m. δ from tetramethylsilane (coupling constants in Hz) | | |
|---|---|---|---|
| 1 | H-1 | H-3e | H-3a |
| βRibf1→7KDOp2→3 | 5.24 (s)[b] | 2.28 (12.5; 5.0) | 1.86 (12.5; 12.5) |
| 2→3βRibf1→7KDOp[a] | 5.27 (s)[b] | 1.86 (12.5; 5.0) | 1.98 (12.5; 12.0) |
| 2→3βRibf1→7KDOf[a] | ? | 2.07-2.38 | |
| 2 | | | |
| βRibf1→7βKDOp2→3 | 5.22 (s)[b] | 2.48 (12.5; 5.0) | 1.87 (12.5; 12.5) |
| 2→3βRibf1→7 $KDO_{red.}$ | 5.23 (1.0) | 1.70-2.30 | |

[a] Tautomeric forms of the reducing disaccharide repeating unit of the tetrasaccharide 1. [b] Singlet.

16). However, in a partial analysis of the spectrum (Table I), the H-3 resonances of these tautomeric KDO residues were identified by comparison with previous data (16). By end-group analysis, the sum of the integrals of these signals was compared to the integrals attributed to the H-3 of chain-internal (β-pyranosidic) KDO residues. The ratio was found to be ca. 1:1, corresponding to a tetrasaccharide (two repeating units). In the anomeric region, a set of overlapping lines were observed, attributable to the H-1 of the nonreducing β-ribofuranosyl (βRibf) residue, and to the H-1 of the magnetically distinct, chain-internal βRibf residues associated with the tautomeric forms of the reducing KDO residue. Integration of the anomeric signals gave unclear results. Although the highly complex, proton-decoupled $^{13}$C-n.m.r. spectrum (cf. 16) was compatible with structure 1, conclusive proof of structure would evidently require additional data.

These became available when the n.m.r. spectra of the products 2, obtained by borohydride reduction of 1, were analyzed. The $^1$H-n.m.r. spectrum of 2 is much less complex than that of 1. Although 2 is a pair of trisaccharide glycosides diastereomeric at C-2 of the reduced KDO group, this difference in configuration causes only a minor change of the magnetic environment at H-1 of the chain-internal βRibf residue. The signals due to the H-1 of the non-reducing and chain-internal βRibf residues are clearly discernible (Table I) in the spectrum of 2 and their integral ratio is 1:1. The signals of the H-3 of the chain-internal βKDOp residues in 2 are practically superimposable upon the corresponding signals in the spectrum of 1. No signals were seen corresponding to a reducing KDO residue. Instead, multiplets showing additional couplings

Scheme 1. Reduction of the tetrasaccharide 1 (mixture of tautomers) to give the pair of diastereomeric trisaccharide glycosides 2.

were observed. This is compatible with the H-3 of the reduced KDO groups experiencing additional coupling with the newly introduced H-2 (Scheme 1). For 2, the ratio of H-3 of KDO, H-1 of Ribf, and the other carbohydrate C-H was found to be 4.2:2:24.8, which is in satisfactory agreement with the theoretical ratio of 4:2:23. The proton-decoupled $^{13}$C-n.m.r. spectrum of 2, also much less complex than that of 1, has been empirically interpreted and is in full agreement with the structure given (data not shown). These n.m.r. arguments, to be presented in full detail elsewhere, indicate that the main product of the phage-catalyzed depolymerization of the K23 capsular polysaccharide is the tetrasaccharide 1 (βRibf1→7βKDOp2→3βRibf1→7KDO). Presumably, analogous but O-acetylated tetrasaccharides are formed from the K13 and K20 polysaccharides (cf. Fig. 1; data not shown).

Substrate specificity of the octulopyranosylono hydrolase. Of the capsular polysaccharides examined as substrates of the octulopyranosylono hydrolase, only those were cleaved that contain β-ketosidic KDO substituted at O-7 by a βRibf residue. The different patterns of O-acetylation of the K13 (11) and K20 (10) polysaccharides, or the absence of O-acetyl groups (K23, ref. 10) did not prevent the cleavage catalyzed by the phage enzyme. The LP 1092 (K?) polysaccharide (5,6), containing α-ketopyranosidic KDO likewise substituted at O-7 by β-Ribf, was not cleaved. The K12, K14, and K15 polysaccharides, containing 5-O-substituted βKDOp, were not substrates of the enzyme, nor was the K95 antigen, an unusual polysaccharide containing furanosidic KDO residues substituted by βRibf at O-8 (17).

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REFERENCES

1. Unger, F. M. (1981) Adv. Carbohydr. Chem. Biochem. 38, 323-388.
2. Jann, B., Hofmann, P., and Jann, K. (1983) Carbohydr. Res. 120, 131-141.
3. Stirm, S., and Freund-Mölbert, E. (1971) J. Virol. 8, 330-342.
4. Ørskov, F., and Ørskov, I. (1984) Methods Microbiol. 14, 43-112.
5. Jennings, H. J., Rosell, K.-G., and Johnson, K. G. (1982) Carbohydr. Res. 105, 45-56.
6. Neszmélyi, A., Kosma, P., Christian, R., Schulz, G., and Unger, F. M. (1985) Carbohydr. Res. 139, 13-22.
7. Adams, M. (1959) Bacteriophages, Interscience Publishers, New York.
8. Kwiatkowski, B., Boschek, B., Thiele, H., and Stirm, S. (1982) J. Virol. 43, 697-704.
9. Gotschlich, E. C. (1975) Monogr. Allergy 9, 245-258.

10. Vann, W. F., Söderström, T., Egan, W., Tsui, F.-P., Schneerson, R., Ørskov, I., and Ørskov, F. (1983) Infect. Immun. 39, 623-629.
11. Vann, W. F., and Jann, K. (1979) Infect. Immun. 25, 85-92.
12. Imoto, T., and Yagishita, K. (1971) Agr. Biol. Chem. 35, 1154-1156.
13. Hershberger, C. S., Davis, M., and Binkley, S. B. (1968) J. biol. Chem. 243, 1585-1588.
14. Skoza, L., and Mohos, S. (1976) Biochem. J. 159, 457-462.
15. Geyer, H., Himmelspach, K., Kwiatkowski, B., Schlecht, S., and Stirm, S. (1983) Pure Appl. Chem. 55, 637-653.
16. Brade, H., Zähringer, U., Rietschel, E. Th., Christian, R., Schulz, G., and Unger, F. M. (1984) Carbohydr. Res. 134, 157-166.
17. Dengler, T., Jann, B., and Jann, K. (1985) Carbohydr. Res. 142, 269-276.