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SUBSTRATE SPECIFICITY OF THE GLYCANASE ACTIVITY ASSOCIATED WITH PARTICLES OF *Klebsiella* BACTERIOPHAGE NO. 6*

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ABSTRACT

A glycanase activity associated with the particles of *Klebsiella* bacteriophage No. 6 catalyses cleavage of *O*-β-D-glucopyranosyl-(1→3)-4,6-*O*-(1-carboxyethylidene)-β-D-mannopyranose linkages in *Klebsiella* serotype-6 capsular polysaccharide. Of 74 heterologous *Klebsiella* polysaccharides and two derivatives of the type-6 glycan, only the type-1 and type-57 polymers were additionally degraded by the phage-6 enzyme. The repeating units in the three substrates have a 1*ax*→3*eq*, 1*eq*→*eq*-linked chain D-gluco- or D-galacto-pyranosyl residue in common (which constitutes the reducing end after glycanase action), and a carboxyl group on the next hexopyranosyl residue. Of the 72 polysaccharides not affected by the viral enzyme, at least the type-11 and type-21 glycans also contain the same homology of primary structure. This indicates that the conformation at the glycanase recognition-site also constitutes an important feature of the substrates.

INTRODUCTION

Bacteriophage particles carrying heteroglycanase activities can be isolated in great variety[1–5] and considerable amounts[6]. Previous studies of the substrate specificity in these agents, using *Klebsiella* phage-borne enzymes and *Klebsiella* capsular polysaccharides as models[2,3], led to the following tentative conclusions. Within the substrate repeating-units, the viral enzymes seem to recognise portions roughly the size of disaccharides, and within these "recognition sites" the functional groups at some, but not all C-atoms; in the acidic capsular glycans, one of the recognised functions generally is the (or one of the) carboxyl group(s).

In order to substantiate these concepts further, we have now studied the substrate specificity of the glycanase activity associated with *Klebsiella* bacteriophage No. 6. The phage-6 system was suitable for this purpose, because its three natural

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substrates, the *Klebsiella* capsular glycans of serotypes 6[7], 1[8], and 57[9], each consist of comparatively simple but different repeating-units.

MATERIAL AND METHODS

Media, phosphate-buffered saline (PBS), and general techniques. — These have been described previously[2,3].

Bacteria. — As in the earlier communications[2,3], the serological test-strains[10–12] for the *Klebsiella* K1–K72, K74, K79, and K80 capsular antigens were employed exclusively: they were kindly supplied by Dr. Ida Ørskov (WHO International Escherichia Centre, Statens Seruminstitut, Copenhagen, Denmark).

Bacteriophage. — Using *Klebsiella ozaenae* F5052 (O2:K6, the serological test-strain for the *Klebsiella* K6 antigen) as a prospective host, bacteriophage No. 6 was isolated from Freiburg sewage (*cf.* ref. 13) and selected because it forms plaques having acapsular haloes[1,14] (after 8–10 h at 37°). The virus strain was purified by ten successive single-plaque isolations.

High-titre suspensions of the phage [10^{11} to 10^{12} plaque-forming units (PFU) per ml] were prepared by the confluent lysis method[15], and the virus particles were purified by isopycnic centrifugation[2,6] and stored at 4° in PBS over chloroform (no loss of titre for 4–6 weeks).

Polysaccharides. — All *Klebsiella* capsular polysaccharides were isolated by the phenol–water–cetyltrimethylammonium bromide procedure[16,17].

For selective hydrolysis of pyruvate acetal residues, type-6 polysaccharide was heated in aqueous trifluoroacetic acid as detailed previously[7].

The type-6 glycan was reduced by the method of Taylor *et al.*[18], and the percent reduction of hexuronic acid and pyruvate acetal carboxyl groups was determined with carbazole–sulfuric acid[19], or with lactate dehydrogenase (LDH; EC 1.1.1.27) after hydrolysis[7,20], respectively.

For mild treatment with alkali (deacetylation), the polysaccharides were kept in 0.25M aqueous NaOH at 56° for 2 h[16,17].

The type-11 and type-21 glycans were selectively debranched by Smith degradation[17], or by autohydrolysis[21], respectively. After hydrolysis, the products were analysed by g.l.c. of the alditol acetates.

Time-course of depolymerisation. — The depolymerisation of type-6 (type-1, type-57) polysaccharide (0.2–0.5%, w/v, in PBS), as catalysed by purified particles of phage 6 (5×10^9 to 10^{10} PFU per ml) at 37°, was followed by sequential determinations[2,22] of the viscosity, and of the reducing power liberated (D-glucose equivalents).

Host range and glycanase substrate-specificity of phage 6. — The infective and enzymic host-range of phage 6 was tested by incubating 50-µl portions of a virus suspension ($\sim 10^{11}$ PFU per ml) on lawns of the 75 *Klebsiella* strains listed above, essentially as described by Niemann *et al.*[3].

For the evaluation of the substrate properties of various isolated (and modified)

polysaccharides, $\sim 5 \times 10^9$ PFU of pure phage-6 particles and 2 mg of the test glycan per ml of PBS were incubated at 37° for 1 h, and the reducing power was determined (D-glucose equivalents) by using identical mixtures with heat-denatured virus for blanks (*cf.* ref. 3).

Isolation and analysis of phage degradation products. — The oligosaccharides obtained by exhaustive phage-degradation of type-6, type-1, or type-57 polysaccharide were isolated by ion-exchange chromatography[2,22,23] (see Fig. 1).

The procedure of Morrison[24] was used to identify the reducing-end sugars, and to estimate the size of the phage degradation products: *i.e.*, after desalting and lyophilisation, the oligosaccharides were reduced with $NaBH_4$ and hydrolysed, and the mixtures of aldoses and one alditol were analysed by g.l.c. of the acetylated nitriles/alditol. Since reducing uronic acids are not recognised by this method, a sample of type-6 oligosaccharide $P1_6$ was also reduced with $NaBT_4$, hydrolysed, and subjected to paper electrophoresis[2].

RESULTS

Isolation of Klebsiella *bacteriophage No. 6 and conditions for the virus-catalysed depolymerisation of* Klebsiella *serotype-6 capsular polysaccharide.* — As with other *Klebsiella* bacteriophages that catalyse depolymerisation of host capsular polysaccharide[1-3,5,22], phage 6 was isolated from sewage using the serological test-strain for the *Klebsiella* K6 antigen (*Klebsiella ozaenae* F5052) as a prospective host, and selecting for plaque morphology[1,14]. Under the electron microscope, the virus exhibits an unusual appearance[25] (*cf.* refs. 1, 4, 5, 13, 14, and 22): it belongs to Bradley's morphology group A[26], *i.e.* it has a long ($\sim$130 nm), contractile tail like the T-even phages, and it carries a huge base plate ($\sim$80 nm in diameter) having curly filaments rather than spikes. Presumably, the glycanase activity is associated with these filaments[14,27].

As in the other phage-polysaccharide systems[1-3,22,23], incubation of isolated *Klebsiella* type-6 glycan with purified phage-6 particles in PBS under "standard conditions"[2] resulted in a rapid loss of viscosity and an increase of reducing power which, in this case, was complete after 1–2 h. At this stage, 4.3–4.7 parts of the degradation products are equivalent to 1 part of glucose in reducing power.

Host range and glycanase substrate-specificity of phage 6. — Suspensions of phage 6 were tested for cross-infectivity and enzymic cross-reactions (formation of plaques with acapsular haloes), or for enzymic cross-reactions alone (no formation of plaques, but decapsulation of the bacteria at higher concentrations of virus) on 74 heterologous *Klebsiella* strains, all serological test-strains for different K antigens (*cf.* refs. 1–3). The phage formed plaques with haloes on the K1 (*Klebsiella pneumoniae* A5054) and the K57 strain (*Klebsiella* 4425/51) also, with relative efficiencies of plating[15] (on K6 *versus* K1 or K57) of $\sim$10 or 100, respectively. None of the other bacterial strains or capsules was affected.

To verify and to extend the phage-6 glycanase substrate-range thus deter-

TABLE I

SUBSTRATE SPECIFICITY OF THE *Klebsiella* PHAGE-6-ASSOCIATED GLYCANASE ACTIVITY

Klebsiella *capsular polysaccharide (K serotype)*[a]	*Reducing power liberated by phage action*[b]
Type-6[7]: Pyruvate(acetal)[d] (4,6) →3)-α-L-Fuc*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Man*p*-(1→4)-α-D-GlcA*p*-(1→ [a,c]	0.23
Type 1[8]: Pyruvate(acetal) (2,3) →4)-α-L-Fuc*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-GlcA*p*-(1→ [a]	0.20[e]
Type-57[9]: α-D-Man*p* (1→4) →2)-α-D-Man*p*-(1→3)-β-D-Gal*p*-(1→3)-α-D-GalA*p*-(1→ [a,c]	0.24[e]
Type-6, depyruvylated[f]	<0.0025
Type-6, carboxyl-reduced[g]	<0.0025
Type-5[29,30], deacetylated[h]: Pyruvate(acetal) (4,6) →4)-β-D-GlcA*p*-(1→4)-β-D-Glc*p*-(1→3)-β-D-Man*p*-(1→	<0.0025
Type-63[32,33], deacetylated[h]: →3)-α-L-Fuc*p*-(1→3)-α-D-Gal*p*-(1→3)-α-D-GalA*p*(1→	<0.0025
Type-11[17], deacetylated[h]: Pyruvate(acetal) (4,6) α-D-Gal*p* (1→4) →3)-α-D-Gal*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-GlcA*p*-(1→	<0.0025
Type-11, deacetylated and debranched[i]	<0.0025
Type-21[21,34]: Pyruvate(acetal) (4,6) α-D-Gal*p* (1→4) →2)-α-D-Man*p*-(1→3)-β-D-Gal*p*-(1→3)-α-D-GlcA*p*-(1→3)-α-D-Man*p*-(1→	<0.0025
Type-21, partially debranched[j]	<0.0025

TABLE I (*Continued*)

[a]Arrows indicate linkages hydrolysed by phage-6 action, as located by reducing-end sugar identification (see text and Fig. 1). [b]Relative to D-glucose (reciprocal of parts of oligosaccharide products equivalent to 1 part of glucose in reducing power); determined after incubation (1 h at 37°) of 2 mg of polysaccharide with 5×10^9 PFU of phage-6 particles per ml of PBS (blanks were identical mixtures with heat-denatured virus). [c]Fig. 2 shows those substituents at the C atoms of this central sugar which are common to the three phage-6 substrates, *i.e.*, the type-6, type-1, and type-57 polysaccharides. [d]This pyruvate acetal carbon has the *S* configuration[28]. [e]As determined by sequential viscosity measurements and reducing-power analyses, also the depolymerization of the type-1 and type-57 glycans was complete after ~1–2 h. [f]Almost quantitatively depyruvylated by treatment with 0.01M trifluoroacetic acid[7]. [g]By the method of Taylor *et al.*[18]; nearly 100% of the pyruvate and 88% of the glucuronic acid carboxyl-groups were reduced in this manner. [h]In contrast to the type-6, type-1, and type-57 glycans[7–9], these polysaccharides carry *O*-acetyl substituents that may cause phage resistance[31]; therefore, they were deacetylated by mild treatment with alkali. [i]83% of the branches were removed by Smith degradation[17]. [j]24% of the branches removed by autohydrolysis[21].

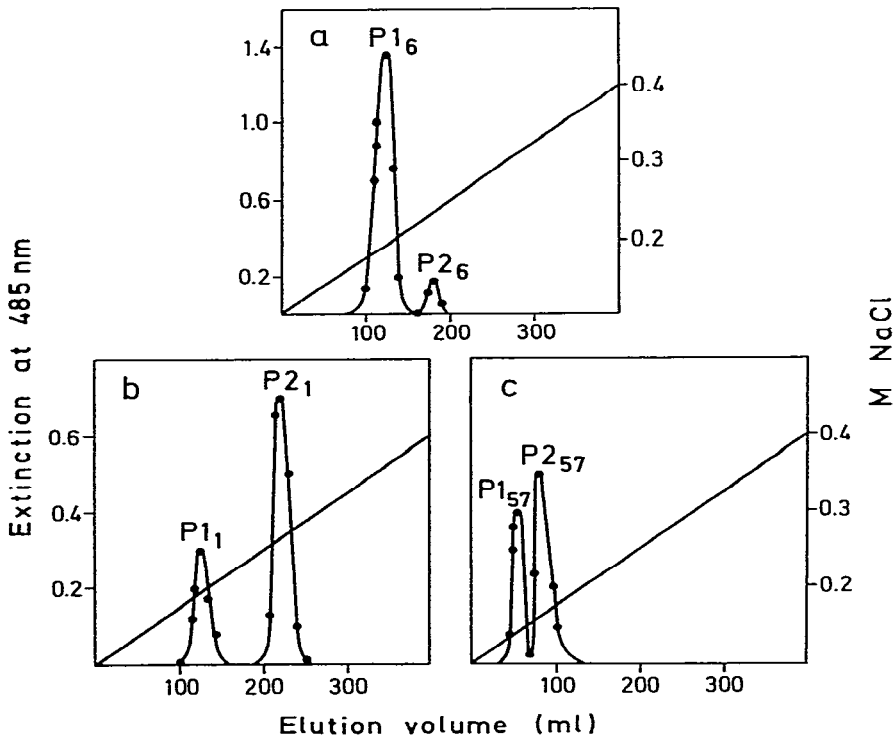

Fig. 1. Isolation of phage-6 glycanase products. The oligosaccharides obtained by exhaustive virus-catalysed hydrolysis (~2.5 × 10^9 PFU/2–5 mg of polysaccharide/ml of PBS; 40–60 h at 37°) were desalted and adsorbed on a DEAE-Sephadex column (60 cm × 1.75 cm^2, for 50–80 mg of material) from a 0.05M Tris/HCl buffer of pH 7.2 containing 0.1M NaCl, and eluted (16 ml/h) with a linear NaCl gradient (———). The fractions were analysed with phenol–sulfuric acid[35] (extinction at 485 nm; —●—). a, Depolymerisation products obtained from serotype-6 polysaccharide (natural substrate); $P1_6$ (75% yield; w/w), $P2_6$ (6%) – one, two type-6 repeating-unit oligosaccharides (reducing-end sugar identified and size determined by reduction with $NaBH_4$, hydrolysis, and g.l.c. of the alditol/aldose mixture[24]). b and c, Products from type-1 ($P1_1$, 28%; $P2_1$, 40%) and from type-57 ($P1_{57}$, 36%; $P2_{57}$, 42%) glycan, respectively.

mined, isolated type-1 and type-57 polysaccharide, some derivatives of the type-6 glycan, as well as several other *Klebsiella* capsular polysaccharides of similar primary structure (see Discussion) were further tested as summarised in Table I.

Isolation and analysis of phage degradation products. — The oligosaccharides obtained from the *Klebsiella* type-6, type-1, and type-57 polysaccharides by extensive phage-6 action were isolated by ion-exchange chromatography; their reducing-end sugars were identified and their size determined by the procedure of Morrison[24] (and. for the type-6 glycan, by labelling with $NaBT_4$ hydrolysis, and paper electrophoresis[2]). The results are shown in Fig. 1 and Table I (cleavage sites).

DISCUSSION

The glycanase activity associated with particles of *Klebsiella* bacteriophage No. 6 catalyses depolymerisation of three [type-6 (natural substrate), type-1, and type-57] out of 75 different *Klebsiella* capsular heteropolysaccharides. Oligosaccharides ending in reducing D-glucose or D-galactose residues are produced from type-6 and type-1. or from type-57 glycan, respectively (Fig. 1 and Table I).

Comparison of the primary structures of the repeating units, and of the cleavage sites in these three substrates of one viral enzyme, shows that their homologous region comprises the substituents at C-2 and C-5 of the pyranose that will form the reducing end, as well as its equatorial substitution by an α-pyranose at C-3 and its β linkage to an equatorial hydroxyl on the following sugar (Fig. 2). Moreover, a carboxyl group on this following unit is essential in phage-6 glycanase substrates, because the depyruvylated (as well as the carboxyl-reduced) type-6 glycan was not degraded. The substrate recognition-site for phage-6 glycanase thus complies well with the general features of these sites outlined in the Introduction.

The data presented in Table I further show that, as in previously studied cases[2,3,36], deviations from the structural homology of Fig. 2 lead to enzyme resist-

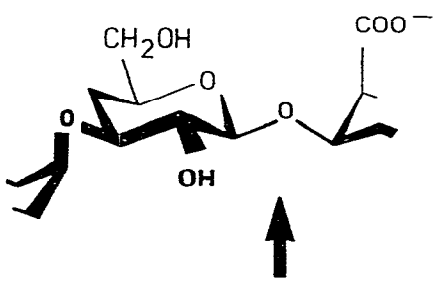

Fig. 2. Homology of primary structure in substrates of the glycanase activity associated with *Klebsiella* bacteriophage No. 6, assumed to be a sufficient, but possibly only in part a necessary, structural requirement for enzyme action[2,3] (*cf.* the first three structures and footnote *c* in Table I). Only the hydroxyl and hydroxymethyl groups at positions 2 and 5 of the central pyranose are invariant; in addition, however, all substrates carry a carboxyl group on the sugar to the right, which is essential for their substrate property. The arrow points to the enzyme-susceptible glycosidic oxygen.

ance: neither the type-5 nor the type-63 polysaccharide was depolymerised by the phage-6 glycanase.

Even the presence of this primary structural homology does not necessarily make a substrate for the enzyme: the type-11 and type-21 polysaccharides were not affected before or after (partial) removal of the bulky substituents at C-4 of the glucuronic acids. Since the pyruvate carboxyl-group in the type-6 glycan occupies an axial position[28], the enzyme resistance of these two glycans may be explained by assuming that the galacturonic acid in the type-57 polysaccharide (due to its bulky substituents at C-1 and C-4) occurs in the $^1C_4$ conformation, whilst the glucuronic acid residues in the type-11 and type-21 glycans adopt the $^+C_1$ conformation, with decisive consequences for the distances between the carboxyl groups of the hexuronic acids and the nearest glycosidic oxygens.

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