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Two additional bacteriophage-associated glycan hydrolases cleaving ketosidic bonds of 3-deoxy-D-*manno*-octulosonic acid in capsular polysaccharides of *Escherichia coli*

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Two bacteriophages degrading 3-deoxy-D-*manno*-2-octulosonic acid-(KDO)-containing capsules of *Escherichia coli* strains were identified. Using modifications of the thiobarbituric acid assay, it was shown that each phage contains a glycan hydrolase activity cleaving one type of ketosidic linkage of KDO. Thus, the enzyme from phage φ95 catalyzes the hydrolysis of β-octulofuranosidonic linkages of the K95 glycan; and φ1092, the α-octulopyranosidonic linkages of the K? antigen of *E. coli* LP1092. No cross-reactivity of the phage enzymes with other KDO-containing capsular polysaccharides was observed.

Capsular polysaccharide; Glycan hydrolase; Bacteriophage; 3-Deoxy-D-*manno*-2-octulosonic acid

1. INTRODUCTION

Phages infecting encapsulated bacteria are frequently equipped with glycanases depolymerizing the capsular polysaccharides of their hosts [1]. Recently, we reported on the glycan hydrolase of coliphage ϕ20 [2], the first known octulopyranosylono (KDO*p*) hydrolase, catalyzing the hydrolytic cleavage of the β-ketopyranosidic bonds of KDO in *Escherichia coli* K13, K20 and K23 antigens [3]. Presently, we describe the detection of two new octulosylono hydrolases, associated with similar coliphages and specific for the *E. coli* K95 antigen [4] and the K? antigen of *E. coli* LP 1092 [5], respectively.

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Abbreviations: KDO, 3-deoxy-D-*manno*-2-octulosonic acid; pfu, plaque-forming units: TBA, 2-thiobarbituric acid

2. MATERIALS AND METHODS

2.1. *Bacteria and isolation of K antigens*

E. coli LP 1092 (O^-:K?) was kindly donated by Dr P.W. Taylor (Ciba-Geigy Pharmaceuticals, Horsham, England). *E. coli* E 3b (O75:K95:H5) was purchased from the National Collection of Type Cultures (London). Test strains [6] for KDO-containing *E. coli* K6, K12, K13, K14, K15, K20, K23 and K82(12) antigens were supplied by Drs I. and F. Ørskov (WHO International Escherichia Center, Statens Seruminstitut, Copenhagen), or by Dr P.W. Taylor.

Standard I nutrient broth or agar (Merck, FRG) was used throughout [7].

Capsular polysaccharides were isolated from liquid cultures as described by Vann et al. [3]. K? (LP 1092) glycan was a gift from Drs B. and K. Jann (Freiburg, FRG). *E. coli* K95 and K? (LP 1092) glycans were de-*O*-acetylated with 0.1 M NaOH [2] and purified further by anion-exchange chromatography (see section 2.4).

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2.2. *Isolation and purification of phages. Microbiological evidence of glycanase activity*

The methods for the isolation, propagation and purification of capsule phages have been detailed in [1,7,8]. Stock solutions of purified phages containing 2–5 × 10^{12} pfu/ml were thus obtained.

To test for glycanase activity, suspensions (0.01 ml) of purified phages or of phage lysates were placed on outgrown lawns (24 h at 37°C) of the strains under investigation. After overnight incubation at room temperature, a transparent appearance of the drop zone indicated degradation of the capsule [1].

Electron micrographs were made from phages negatively stained with uranyl acetate [7,8].

2.3. *Assay of octulosylono (KDO) hydrolase activity*

Two methods (A and B) were applied which are both modifications of the periodate/thiobarbituric acid (TBA) test [9–11].

(Method A) A solution of the capsular polysaccharide in distilled water (0.04 ml, 0.20 mg/ml) was incubated with enzyme solution (0.04 ml of phages and/or free glycanase in 40 mM imidazole-HCl buffer, pH 6.5, containing 40 mM $MgCl_2$). Incubation was terminated by boiling the samples for 2 min. When the samples had cooled to room temperature, they were mixed with 10% $NaBH_4$ in 0.5 M ammonia (0.01 ml) and kept at 30°C for 20 min. Excess borohydride was decomposed by addition of 3 M acetic acid (0.01 ml). Next, 1 M sulfuric acid (0.04 ml) was added and the samples kept at 100°C for 10 min. The cooled samples were mixed with 0.5 M $NaIO_4$ in 1 M H_2SO_4 (0.01 ml) and kept at 30°C. After exactly 8 min, 6% sodium arsenite in 0.5 M HCl (0.1 ml) was added. Samples were mixed with 2% TBA (0.25 ml, pH 8.5) and immediately placed in a 56°C thermobloc. After 20 min, dimethyl sulfoxide (0.5 ml) was added and the extinction at 552 nm was recorded. Under these conditions, only ketosidically linked KDO will give the chromophore.

(Method B) A solution of the capsular polysaccharide (0.05 ml, 0.32 mg/ml) was incubated with enzyme solution (0.05 ml; as above) at 30°C for 20 min. The enzymic reaction was terminated by the addition of 0.1 M $NaIO_4$ in 1 M H_2SO_4 (0.05 ml). From here on, the procedure is similar to method A, but the reaction with TBA is stopped after exactly 10 min, the samples being cooled with tap water and extinctions being read within 10–20 min. Negative controls were run by adding the enzyme solution after the periodate.

From the difference in extinction from that of an undegraded polysaccharide sample and from the molar extinction coefficient of KDO ($120\,000 \pm 2000$ $mol^{-1} \cdot cm^{-1}$ using this method), enzymic activity was calculated in terms of katal (mol/s).

2.4. *Methylation analysis*

Degradation products were separated by ion-exchange chromatography using Fractogel TSK DEAE-650 (Merck) and a linear gradient of NaCl in 10 mM imidazole-HCl, pH 7.0. Desalted oligosaccharides (0.2 mg K23 or K95 tetrasaccharide) were methylated according to Ciucanu and Kerek [12] and then hydrolyzed, reduced, acetylated and analyzed as described [13].

3. RESULTS AND DISCUSSION

3.1. *Isolation of phages specific for KDO-containing* E. coli *K antigens*

While bacteriophage ϕ20 had been isolated readily from sewage [2], several trials were needed to discover phages of promising plaque morphology for strains E 3b (K95; containing β-octulofuranosylono [β-KDO*f*] residues) [4] and LP 1092 (K?; containing α-octulopyranosylono [α-KDO*p*] residues) [5]. The plaques of phages ϕ95 and ϕ1092 were surrounded by transparent haloes, indicating the action of capsule-degrading enzymes [1].

For each of the host strains, other bacteriophages, producing plaques without transparent haloes, were also found. For strain LP 1092, two such phages were investigated further, but neither by colorimetry nor by gel chromatography could capsule-depolymerizing enzymes be demonstrated.

3.2. *Phage morphology*

The two phages and, in addition, ϕ20, each consisted of an isometric head with spikes, probably attached to a base plate (not visible). Thus, like most K-specific phages, they belong to Bradley's morphology group C [1]. The head diameters were determined from electron micrographs as 62, 64

and 68 nm for ϕ20, ϕ95 and ϕ1092, respectively. The length of the spikes was approx. 10 nm in each case.

3.3. *Elaboration of a specific assay for octulosylono hydrolase activity*

Since the ϕ20 glycanase proved to be an endo-β-octulopyranosylono hydrolase [2], our aim was to investigate the new glycanases with a sensitive and specific assay for reducing KDO.

Method A utilizes the known principle that reduced KDO will not give rise to the normal chromophore (λ_{max} = 549 or 552 nm in our hands) in the TBA test [11]. With the present modification of the method, borohydride-reduced KDO does not give rise to any color at all, allowing a simple determination of the ratio of reducing to non-reducing KDO in an oligosaccharide. Method A was applied to screen for octulosylono hydrolase activity of phage ϕ1092 at an early stage of virus purification (see fig.1).

Both ketosidically bound and reducing KDO, unless substituted at C-4 or C-5, will be oxidized by periodate. Because of this and since the ketosidic bond is readily split during the reaction with TBA at 100°C, the usual procedures of the periodate/TBA test do not discriminate between reducing and non-reducing KDO. However, when the reaction with TBA was performed at lower temperatures (56°C) hydrolysis of ketosidic bonds was much slower than formation of the chromophore. Thus, after a short incubation time (10 min), only a small amount of non-reducing KDO (5–10% depending on the type of linkage) had given rise to the chromophore, whereas the periodate-oxidized, reducing KDO had reacted almost completely. Method B allowed for the quantitation of the glycanase activity of phage ϕ1092 in terms of katal (see fig.2).

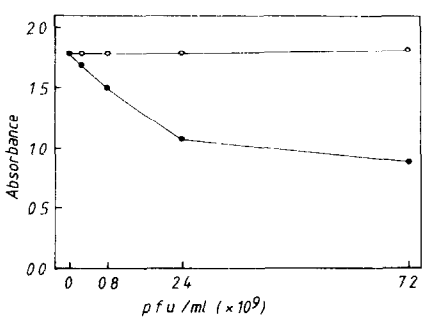

Fig.1. Octulosylono hydrolase determination – procedure A. Deacetylated K? (LP 1092) glycan was incubated for 2 h at 37°C with dilutions of a lysate of ϕ1092 (●—●) as described in the text. Negative controls (○—○) were obtained with heat-treated lysate.

3.4. *Octulosylono hydrolase activity associated with phages ϕ95 and ϕ1092 and analysis of the main degradation products*

When de-*O*-acetylated K95 antigen containing repeating units of [→3)-β-D-Rib*p*-(1→8)-KDO*f*-(2→] [4] was incubated with purified ϕ95 (2×10^{10} pfu, 37°C, overnight), 30% of the originally non-reducing, furanosidically bound KDO residues became susceptible to the reducing action of borohydride (method A). The octulofuranosylono hydrolase activity of phage ϕ95 could also be demonstrated by using method B. This is explained by the reducing KDO residues adopting the pyranose form, thus becoming susceptible to oxidation by periodate at C-4/C-5.

The degradation products of the K95 antigen were separated by ion-exchange chromatography (fig.3). The major TBA-positive fraction was pooled and directly analyzed for reducing KDO by method B. The color yield of the borohydride-reduced sample was 51.5% of that obtained with a non-reduced sample. Thus, the major degradation product was an oligosaccharide consisting of two repeating units and containing reducing KDO. The

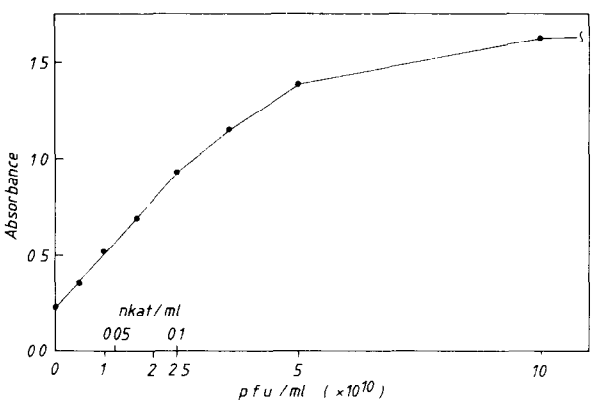

Fig.2. Octulosylono hydrolase assay – procedure B. Deacetylated K? glycan was incubated with increasing concentrations of purified phage ϕ1092 and analyzed using method A as described in section 3.3. A linear correlation, allowing quantitation of enzymatic activity, is observed at low virus concentrations only.

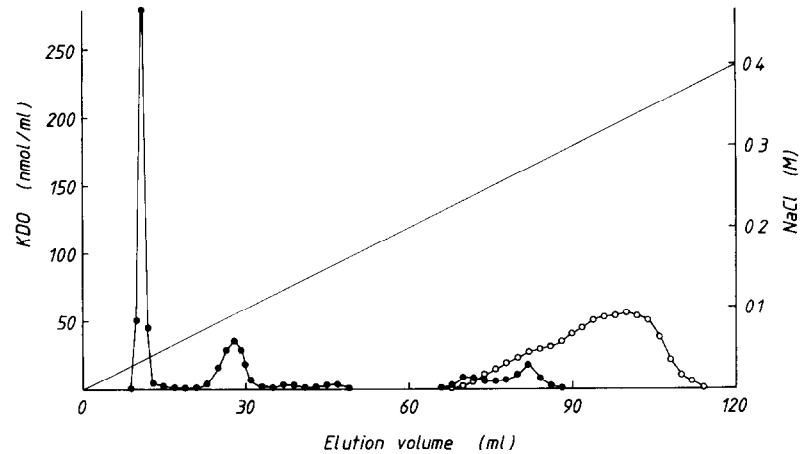

Fig.3. Separation of degradation products from K95 glycan. Deacetylated K95 glycan (2 mg) was subjected to ion-exchange chromatography (see section 2.4) before (○—○) and after digestion (●—●) with 2×10^{10} pfu of ϕ1092 for 15 h at 37°C. The eluate was analyzed using the TBA test.

presence of unsubstituted and of 3-*O*-substituted ribosyl residues in an equimolar ratio was confirmed by methylation analysis. These findings show that the capsule-degrading enzyme of phage ϕ95 is an octulofuranosylono hydrolase.

In the case of ϕ1092, de-*O*-acetylated LP 1092 (K?) antigen [→2)-β-D-Rib*f*-(1→2)-β-D-Rib*f*-(1→7)-α-KDO*p*-(2→]$_n$ [5] was incubated with dilutions of a lysate, containing 7×10^9 pfu/ml of ϕ1092 and, probably, an additional amount of free glycanase in the form of unattached phage spikes [1]. As can be seen from fig.1, analysis of the samples using method A demonstrated the formation of reducing KDO. Approx. 50% of the KDO residues became susceptible to borohydride reduction. Therefore, the main degradation product should be a hexasaccharide (consisting of two repeating units) containing reducing KDO. Glycanase activity of ϕ1092 could also be monitored using method A (see fig.2).

The presence of non-reducing terminal ribofuranose was confirmed by ^{13}C-NMR spectroscopy (Altmann et al., to be published). Thus, the capsule-degrading enzyme of phage ϕ1092 is an octulopyranosylono hydrolase.

3.6. *Substrate specificity of octulosylono hydrolases and host specificity of phages*

As shown by a microbiological technique (see section 2.2) and employing the test strains listed in section 2.1, neither of the phages degraded any capsular polysaccharide other than that of its respective host strain. In each case, the native as well as the deacetylated polysaccharides were substrates of the glycanases.

Probably the tautomeric forms and anomeric configurations of the respective KDO residues are not the only determinants of substrate specificity. Since in each case oligosaccharides consisting of two repeating units were the main degradation products, substrate size seems to be one of the additional stipulations for 'enzyme recognition' [14].

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