# EXOGLYCANS OF Yersina pseudotuberculosis

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The present paper describes the isolation and general characterization of the exoglycans (extracellular polysaccharides) from the microorganism Yersinia pseudotuberculosis. As a preliminary, we studied (in comparison) the total monosaccharide composition of the exoglycans produced by a local strain 12 of Y. pseudotuberculosis IB subtype in dependence on the conditions of growth of the bacteria, the composition of the culture medium, and the shape of the colonies (Table 1). The growth of the microorganisms in a medium with a high glucose content and a low amount of nitrogen-containing raw material led to a 4.5-fold increase in the yield of exoglycan, which agreed with literature information [1].

The results of a determination of the monosaccharide composition of the total fractions of exoglycans from various strains of the microorganism investigated by PC and GLC are given in Table 2 in comparison with the results for the exoglycans produced by the standard strains IA (strain 156) and IB (strain 159) of subtypes of the pseudotuberculosis microbe.

The exoglycans of the S-form of the standard and local strain IB subtype had similar qualitative compositions of the monosaccharides and differed only in their ratio. The IA subtype and the R form of the IB subtype differed from the strains mentioned and also in the qualitative monosaccharide composition of the exoglycans produced. In particular, the exoglycans of the IA subtype contain no mannose, and the exoglycans of the R form contain no xylose and fucose.

The crude exoglycan fraction contained protein which could not be separated by Sevag's method (see Table 1). By fractionation on hydroxylapatite we obtained an exoglycan preparation containing 16% of protein.

TABLE 1. Yields and Total Compositions of the Exoglycans Produced by the Local Strain 12 of Y. pseudotuberculosis

| Type of strain | | Shape of the colonies | Conditions of growth | Yield (g) per 100 g of micromass | Amount, % | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | monosaccharides | protein | nucleic acids | amino sugars | ash |
| Strain | IB | S | Without inorganic salts | 8,6 | 22 | 58 | 17 | 1,7 | 0,2 |
| | 12 | S | With inorganic salts | 38,7 | 35 | 30 | 4,28 | 1,7 | 2,15 |
| | | R | With inorganic salts | 1,6 | 40 | 21 | 0,2 | 1,6 | 0,46 |

TABLE 2. Monosaccharide Compositions of the Fractions of the Exoglycans Y. pseudotuberculosis

| Type of strain | Strain | Shape of the colonies | Gal* | Glc | Man | Xyl | Ara | Fuc | GlcUA | $GlcNH_2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| IA | 156 | S | ++ | +++ | – | + | Tr. | +++ | + | + |
| IB | 159 | S | +++ | +++ | +++ | – | Tr. | ++ | + | + |
| | 12 | S | ++ | +++ | ++ | + | Tr. | +++ | + | + |
| | | R | 10+ | + | + | – | Tr. | – | Tr. | + |

*Gal) galactose; Glc) glucose; Man) mannose; Xyl) xylose; Ara) arabinose; Fuc) fucose; GlcUA) glucuronic acid; $GlcNH_2$) glucosamine.

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TABLE 3. Characteristics of the Individual Fractions of Exoglycans Produced by the Local Strain 12 of Y. pseudotuberculosis

| Method of fractionation | Fraction | Yield % | $[\alpha]_{D(H_2O)}^{20}$ deg | Monosaccharide composition | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Gal | Glc | Man | Xyl | Ara | Fuc | Glc UA | Glc $NH_2$ |
| Ethanol | A | 70 | +56,5 | ++ | +++ | ++++ | + | + | ++++ | Tr. | + |
| | B | 20 | −35,8 | −+ | + | + | — | — | + | + | + |
| Acidified ethanol | $A_1$ | 15 | −22,3 | −+ | + | + | Tr. | Tr. | + | ++ | + |
| | $B_1$ | 75 | −14,4 | ++ | ++ | ++ | Tr. | + | + | Tr. | + |

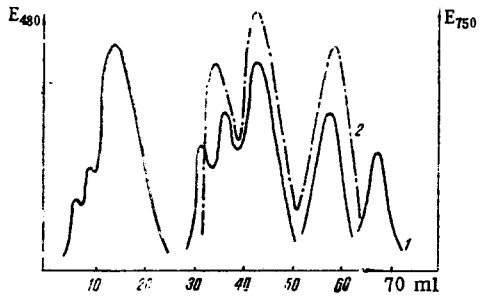


Fig. 1

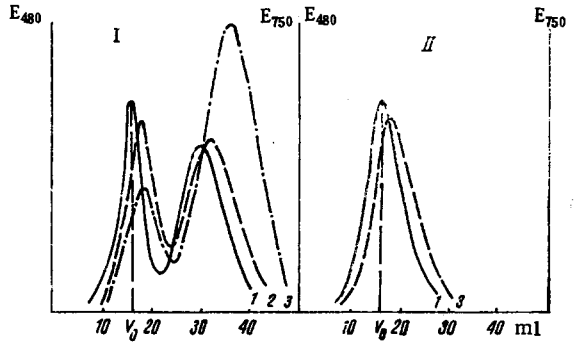


Fig. 2

Fig. 1. Chromatography of the total polysaccharide fraction on Dowex 1 × 2 in the phosphate form (phosphate buffer): 1-30) 0.05 M, pH 7.6; 30-40) 0.05 M, pH 7; 40-50) 0.05 M, pH 6.4; 50-60) 0.2 M, pH 6.4; 60-70) 0.5 M, pH 6.4; curve 1: elution curve by the phenol–sulfuric acid method; curve 2: elution curve by Lowry's method.

Fig. 2. Gel filtration of the exoglycans on Bio-Gels: Fraction A (I) and fraction $B_1$ (II): Biogel P-60, $V_0$ = 15 ml: 1) elution curve according to the phenol–sulfuric acid method; 2) elution curve according to Lowry's method; Bio-Gel P-100, $V_0$ = 17 ml; 3) elution curve according to the phenol–sulfuric acid method.

By the ion-exchange chromatography of the exoglycans on an anion-exchange resin we isolated a fraction with $[\alpha]_D^{20}$ ($H_2O$) + 5° which contained no protein. However, its yield was only 20 % and a considerable amount of polysaccharide was bound to the protein (Fig. 1). Apparently, an ionic bond exists between the protein and polysaccharide components or the isoelectric points of these components are fairly close. There is no covalent bond between them, since on gel filtration on Bio-Gels the maxima of the elution curves for the protein and the polysaccharide did not coincide. Polysaccharide fractions containing no protein were obtained by enzyme treatment. These fractions of the exoglycans were not homogeneous: They each contained several polysaccharides.

The exoglycans produced by the S-form of strain 12 were fractionated by ethanol and acidified solutions of it in order to obtain a serologically active fraction. In both cases two water-soluble fractions were obtained (Table 3). The polysaccharide fractions A and $B_1$, showing serological activity (Table 4), were studied in more detail. The results of gel filtration on Bio-Gels showed that the polysaccharide fraction A was heterogeneous and fraction $B_1$ homogeneous (Fig. 2). By preparative gel filtration on Bio-Gel P-100 two fractions differing in monosaccharide composition were isolated from fraction A (Table 5).

The results of the chromatographic elimination of protein from the polysaccharide fractions A and $B_1$ on DEAE-cellulose confirmed the heterogeneous nature of the exoglycans and the presence in them of an acid fraction (Fig. 3, Table 6). About 6 % of uronic acids was found in the acid fraction by the decarboxylation method. The antigenic nature of the polysaccharide fractions A and $B_1$ was shown by obtaining antisera to these exoglycans which were also active in the indirect hemagglutination reaction.

In an investigation of the antigenic structure of the polysaccharide fractions A and $B_1$, and also of the fragments of A after fractionation of DEAE-cellulose and gel filtration on Bio-Gel P-100, the presence of a

TABLE 4. Analyses of Fractions A and $B_1$

| Polysaccharide fractions | $[\alpha]_D^{20}$ ($H_2O$), deg | Amount, % | | | | | |
|---|---|---|---|---|---|---|---|
| | | monosaccharides | uronic anhydride | nitrogen | sulfur | phosphorus | ash |
| A | +56.5 | 35 | 5,6 | 5,74 | None | None | 2,15 |
| $B_1$ | −14,4 | 32 | 9,6 | 6,39 | | | 2,04 |

TABLE 5

| Fraction (Fig. 2) | Yield, % | Gal | Glc | Man | Xyl | Ara | Fuc | UA |
|---|---|---|---|---|---|---|---|---|
| Peak I | 20 | | + | ++ | + | ++ | + | |
| Peak II | 20 | +++ | + | + | | +++ | +++ | + |

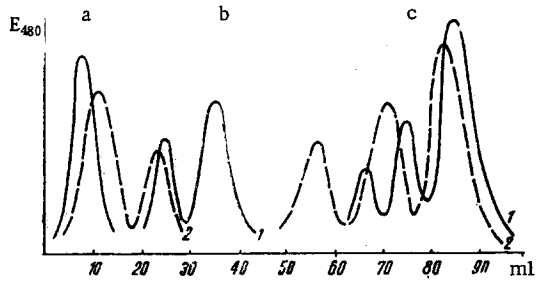


Fig. 3. Chromatography of the exoglycans on DEAE-cellulose: a) elution with water; b) stepwise elution with phosphate buffer; c) linear gradient of 0.01-0.03 N NaOH; 1) fraction A; 2) fraction $B_1$.

single precipitation line for fractions A and $B_1$ and the fraction corresponding to peak I on Bio-Gel P-100 was found by the precipitation method in agar gel. The results of immunoelectrophoresis for fraction A confirmed the homogeneity of the antigen A.

On indirect hemagglutation with specific antiserum, the exoglycan A was found to have a low serological activity as compared with the activity of the lipopolysaccharide from Y. pseudotuberculosis. The serological activity of fraction $B_1$ was fully comparable with that for the lipopolysaccharide.

## EXPERIMENTAL METHOD

Partition paper chromatography (PC) was performed on Schleicher und Schüll Filtrak No. 3 paper in the following solvent systems: 1) butan-1-ol–water–acetic acid (4 : 5 : 1, upper layer) and 2) butan-1-ol-pyridine–water (6 : 4 : 3). The monosaccharides were revealed with aniline hydrogen phthalate and an alkaline solution of silver nitrate.

In order to determine its monosaccharide composition, the exoglycan (10 mg) was hydrolyzed with 1 N sulfuric acid (1 ml) in the boiling water bath for 4 h. The hydrolyzates were neutralized with barium carbonate, deionized with Amberlite IR-120 ($H^+$), evaporated, and chromatographed on paper. To isolate the uronic acids, a hydrolyzate of the polysaccharide (100 mg) was deposited on a column containing 20 ml of Dowex 1 × 2 anion-exchange resin (in the acetate form).

The neutral monosaccharides were eluted with water, and the acidic monosaccharide with a 2 M solution of acetic acid. A crude glucuronic acid fraction was obtained which was purified by preparative PC.

The amount of amino sugars was determined by hydrolysis with 4 N hydrochloric acid (1 ml of acid to 10 mg of sample) at 105° C for 3 h. The hydrochloric acid was driven off in vacuum and concentrated solutions of the hydrolyzates were deionized with Dowex-1 ($HCO_3^-$) and were used for chromatography and analysis by the method of Morgan and Elson [2]. The specific rotations of 0.25-0.5% solutions of the polysaccharides were measured on a Perkin–Elmer M-141 polarimeter.

TABLE 6. Characteristics of the Individual Fractions of Exoglycan A Obtained by Fractionation on DEAE-cellulose

| Fraction | Yield, % | $[\alpha]_D^{20}$ ($H_2O$), deg | Gal | Glc | Man | Xyl | Ara | Fuc | UA |
|---|---|---|---|---|---|---|---|---|---|
| Neutral | 30 | +11,5 | + | ++ | + | Tr. | + | Tr. | |
| Acidic | 50 | −10 | + | + | Tr. | | + | | + |

For gel chromatography of aqueous solutions (1-2 ml) of the samples of exoglycan (20-30 mg) we used columns (1.5 × 30 cm) of Bio-Gels P-60, P-100, and P-200 (Bio-Rad Laboratories, Richmond, Calif.) by a known method [3].

Gas–liquid chromatography (GLC) was performed on a Tsvet-6 chromatograph (Dzerzhinsk) with a flame-ionization detector using U-shaped stainless-steel columns (100 × 0.3 cm). The rate of flow of $N_2$ was 33 ml/min, of $H_2$ 33 ml/min, and of air 300 ml/min.

The stationary phase was HIEFF-8-BP on Gas-Chrom Q (100-120 mesh). The gas–liquid chromatography of the monosaccharides (in the form of the acetates of the aldononitriles) was performed with the programming of the temperature from 175 to 225° C (at the rate of 4° C/min).

Analytical Methods. The total amount of monosaccharide was determined by the phenol–sulfuric acid method using glucose as the standard [4], the protein content by Lowry's method [5], the nucleic acids by Spirin's method [6], the uronic acids by the decarboxylation method [7], and the ash by the combustion of samples of exoglycan at 600°C to constant weight.

Microorganisms. Strain 12 of Y. pseudotuberculosis of subtype LB was isolated in 1972 from the patient DSL. The standard strains of subtypes IA (No. 156) and IB (No. 159) were kindly given to us by Prof. Mollaret (Pasteur Institute, Paris). Both the S- and R-forms of this microorganism were used.

In the production of the S-form a liquid medium was used which contained 1% of nutrient peptone and inorganic salts in the following percentages: 0.2 $(NH_4)_2SO_4$, 0.02 $MnSO_4 \cdot 7H_2O$, 0.001 $CaCl_2$, 0.15 $KH_2PO_4$, 0.65 $NaH_2PO_4$, 0.00054 $FeSO_4$, and 5 glucose. The bacteria were grown at 20°C for 18 h.

For comparison, in the production of the S-form the same medium was used but without inorganic salts. The R-form of the bacteria was grown on 2% agar in a simple synthetic medium (percentage composition shown above) at 37° C for 2-3 days.

Isolation of the Exoglycans. The culture liquid (20 liters) obtained after the separation of the microbial mass by centrifuging, was concentrated 20- to 25-fold. The unbound protein was eliminated by Sevag's method [8] (2-3 times). The total polysaccharide fraction precipitated with acetone (3 volumes) in the form of a viscous brown mass was separated by the following methods.

a) By reprecipitation of this fraction with ethanol into A – a flocculent white precipitate – and B – a brown oily liquid soluble in 50% aqueous ethanol.

Fractions A and B were separated by centrifuging at 3000 rpm for 15 min. Fraction A was washed with 50% aqueous ethanol. Aqueous solutions of A and B were treated by Sevag's method to separate them from unbound protein (3 times), dialyzed against changed distilled water, and lyophilized (0.86 g of fraction A and 0.24 g of fraction B).

b) By precipitating the total fraction with acidified ethanol (2.3 ml of concentrated HCl/0.5 liter of ethanol) a white flocculent precipitate $A_1$ was obtained (yield of flocculent powder 0.6 g). From the ethanolic filtrate, acetone precipitated a light brown flocculent precipitate $B_1$ (yield of lyophilized produce 3 g).

Fractionation of the Exoglycans on Hydroxlyapatite. A mixture of exoglycans (40 mg) containing 32% of protein was deposited on a column (1.3 × 13 cm) and fractionated at pH 6.8 by stepwise elution with phosphate buffers of increasing ionic strength. The exoglycans isolated contained 16% of protein.

Ion-Exchange Chromatography of the Exoglycans. The mixture of exoglycans (45 mg) was deposited on a column (1 × 45 cm) of Dowex 1 × 2 anion-exchange resin in the phosphate form. Stepwise elution with a simultaneous decrease in the pH and an increase in the ionic strength of the buffer yielded a protein-free exoglycan fraction (11 mg). The exoglycan freed from protein (100 mg) was deposited on a column (1.8 × 70 cm) of DEAE-cellulose in the phosphate form and was fractionated by the method of Neukom et al. [9].

Source of the Enzyme. Commercial pronase (ex B. subtilis Koch–Light, England) was used for the enzyme treatment.

Enzymatic Treatment of the Exoglycan. The enzyme (20 mg) was added to the solution of the exoglycan (1 g) in 0.03 M phosphate buffer (pH 7.4; 100 ml), the mixture was kept at 37°C for 5 h, treated by Sevag's method, concentrated, and precipitated with ethanol. The precipitate was separated by centrifuging, dissolved in water, dialyzed, and lyophilized. Yield 400 mg.

Serological Methods.* 1. Standard immune serum against strain 12 of Y. pseudotuberculosis of subtype IB was obtained by immunizing rabbits by a known method [10].

2. The immunoelectrophoresis of the antigens was performed in veronal buffer (pH 8.4) in the presence of specific antisera [11]. The results were obtained after 1-2 days.

3. The hemagglutination reaction and precipitation in an agar gel were performed by published method [12] and [13], respectively.

## SUMMARY

1. From a culture medium of strain 12 of Yersinia pseudotuberculosis of subtype IB a mixture of exoglycans has been isolated which consists of acidic heteropolysaccharides bound ionically to a protein component.

2. The yield and monosaccharide composition of the exoglycans produced depend on the conditions of growth and the form of the colonies.

3. The exoglycans of the local strain No. 12 and of a standard strain (No. 159) of Y. pseudotuberculosis of subtype IB contain monosaccharides (galactose, glucose, mannose, xylose, arabinose, fucose, glucuronic acid, glucosamine) and differ only by the ratio of the monosaccharide residues.

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