INVESTIGATION OF THE PROTEIN COMPONENT OF THE LIPOPOLYSACCHARIDE-PROTEIN COMPLEX OF *Yersinia pseudotuberculosis*

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UDC 576.851.23 + 576.809.7 + 543.545

It has been established by electrophoresis in polyacrylamide gel that the protein component of the lipopolysaccharide-protein complex of *Yersinia pseudotuberculosis* consists of two polypeptides with molecular weights of 42,000-45,000 and 18,000-20,000. These polypeptides have been detected in the cell wall of *Y. pseudotuberculosis* as the main components. Protein fractions B-2 and B-3, differing with respect to solubility in phenol, were isolated from the LPPC of *Y. pseudotuberculosis* by extraction with hot 45% phenol. These protein fractions had the same polypeptide composition, which is characteristic for membrane proteins. B-3 included the LPS component and B-2 contained lipid A or fragments of it as impurity. Fractions B-3 and B-2 precipitated with antiserum to the LPPC.

The protein component of the lipopolysaccharide-protein complex (LPPC) present in the outer membrane of the Gram-negative bacteria is attracting the attention of many workers at the present time. Possessing a relatively simple polypeptide composition, it makes an important contribution to the immunological properties of the LPPC [1] and is the bearer of many functions characteristic of the outer membrane of bacteria [2, 3]. The study of the protein component is also connected directly with a determination of the nature of the bond in the LPPC between the lipopolysaccharide (LPS) and the protein [4, 5].

The present work was devoted to the isolation of the protein component of the LPPC from *Yersinia pseudotuberculosis*, a pseudotuberculosis microbe pathogenic for man and causing a fever in the Far East resembling scarletina, and to a study of its polypeptide composition and properties.

The LPPC from *Y. psdt.* was isolated by extraction with a butanol–water two-phase system and was purified by gel filtration on Sepharose 2B [6].

It was established from the results of electrophoresis that the protein component of the LPPC consisted of two polypeptides (PP-II and PP-IV) with molecular weights of 42,000-45,000 and 18,000-20,000, respectively (Fig. 1a). To investigate whether these polypeptides were the main ones or minor components among the proteins of the outer membrane of *Y. psdt.* we isolated the cell walls of this microbe and determined their polypeptide composition. It was shown that the cell-wall fraction contained four main polypeptides (PP-I–PP-IV) with molecular weights of 60,000, 45,000-42,000, 35,000, and 20,000-18,000, respectively, and several minor components (Fig. 1b). Thus, the protein component of the complex consisted of two out of the four main polypeptides of the cell wall of *Y. psdt.* To isolate the protein component of the LPPC we used treatment with hot 45% phenol [7] (Scheme 1).

As the result of a single treatment of the LPPC from *Y. psdt.* we obtained protein fraction B-1 containing about 20% of monosaccharides. In this process there was a loss of about 30% of material (Scheme 1) which is possibly due to the degrading action of phenol on the LPS [8]. For further purification, the protein fraction B-1 was repeatedly treated with phenol. This gave protein fractions B-2 and B-3 differing in their solubilities in phenol: B-2 was insoluble in phenol and B-3 was precipitated from a phenol phase with a 9.5-fold excess of ethanol. Protein fractions B-2 and B-3 were identical in their polypeptide composition: electrophoresis revealed two zones staining for protein with the mobilities of PP-II and PP-IV (Fig. 2a and b). Thus, both the polypeptides present in the LPPC are extracted by phenol. In their common amino acid composition, protein fractions B-2 and B-3 were typical membrane proteins: they were distinguished by a high content of acidic and

Pacific Ocean Institute of Bioorganic Chemistry of the Far Eastern Scientific Center, Academy of Sciences of the USSR, Vladivostok. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 92-97, January-February, 1980. Original article submitted August 1, 1979.

0009-3130/80/1601-0077$07.50

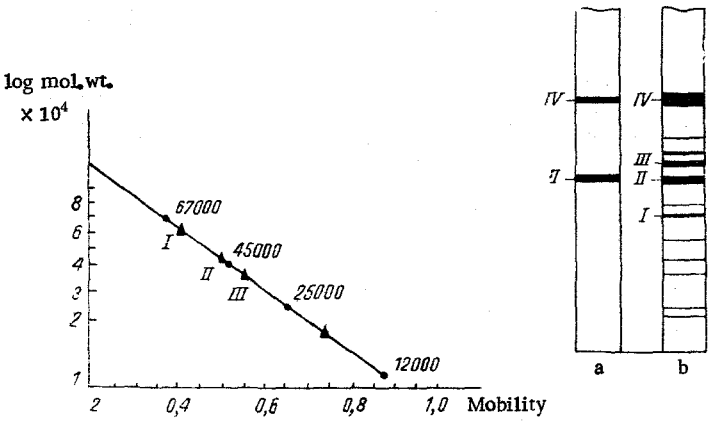
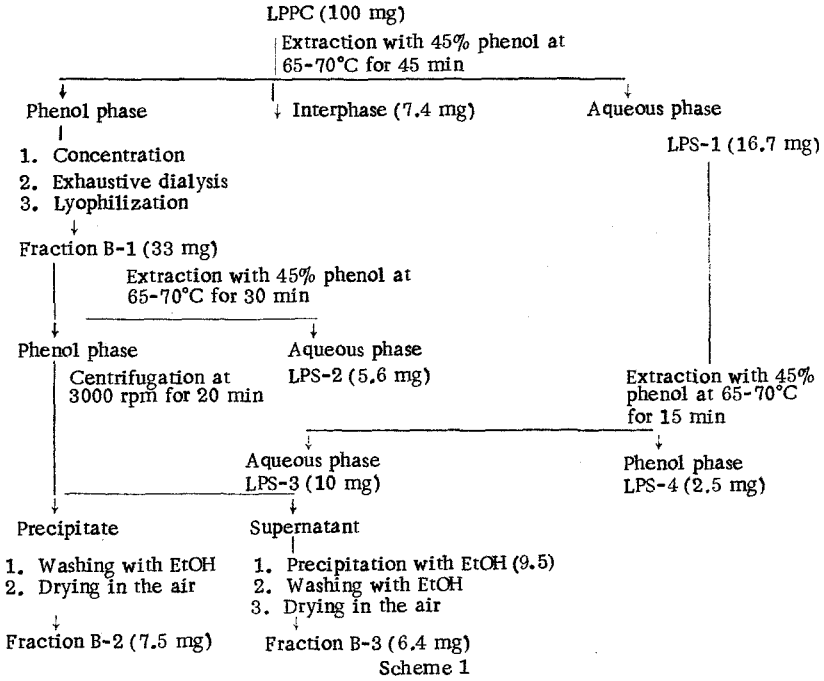


Fig. 1. Electrophoresis of the LPPC (a) and of the cell walls of Y. pseudotuberculosis (staining of the protein with Coomassie Brilliant Blue) (b). The main polypeptides of the cell walls of Y. pseudotuberculosis are: PP-I) mol. wt. 60,000; PP-II) mol. wt. 42,000-45,000; PT-III) mol. wt. 35,000; PP-IV) mol. wt. 18,000-20,000.



Scheme 1

hydrophobic amino acids, and also by the absence of cysteine [9]. The amino acid compositions of the protein fractions B-2 and B-3 isolated from the LPPC of Y. psdt. are as follows (mole %):

Amino acid	Fraction B-2	Fraction B-3
Lysine	5,8	5,2
Histidine	1,5	1,2
Arginine	1,3	1,5
Aspartic acid	19,2	20,2
Glutamic acid	8,3	8,3
Valine	5,2	5,6
Methionine	0,35	0,36
Isoleucine	2,9	2,1
Leucine	7,7	6,4
Tyrosine	4,2	4,4
Phenylalanine	5,3	5,9
Threonine	5,8	6,4
Serine	7,5	8,1
Proline	5,2	2,9
Glycine	10,8	7,9
Alanine	8,8	8,4
Cysteine	0,0	0,0
Glucosamine	0,3	7,2

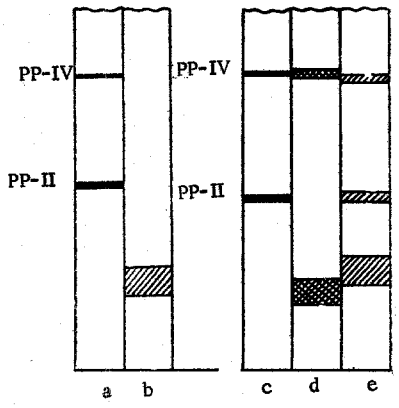


Fig. 2

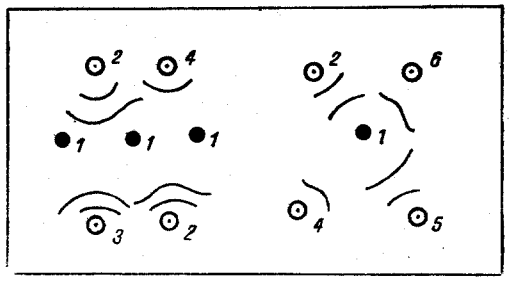


Fig. 3

Fig. 2. Electrophoresis of the protein fractions B-2 and B-3: a, c) protein fractions B-2 and B-3 (staining for protein with Coomassie Brilliant Blue); d) protein fraction B-2 (PAS-Schiff staining for carbohydrates); b, e) protein fractions B-2 and B-3 (staining for lipid with Sudan Black B).

Fig. 3. Immunodiffusion in agar against antiserum to the LPPC: 1) antiserum; 2) LPPC; 3) B-1; 4) B-2; 5) B-3; 6) LPS-3.

The carbohydrate and lipid compositions of protein fractions B-2 and B-3 were different. Under the conditions of electrophoresis, no carbohydrate component was detected in fraction B-2, while B-3 was found to contain a carbohydrate component coinciding in mobility with PP-IV (Fig. 2g). Analysis of the monosaccharide composition showed that fraction B-3 contained: paratose, fucose, mannose, glucose, galactose, a heptose, and glucosamine, i.e., the whole set of monosaccharides that are characteristic for LPPCs. At the same time, only a very small amount of glucosamine was found in fraction B-2.

It was shown by electrophoresis that both protein fractions contained zones staining for lipids, while in B-3 there was a zone coinciding in mobility with PP-IV (Fig. 2b, d). β-Hydroxybutyric acid was detected by GLC in both fractions, but in B-2 it was present in only trace amounts.

Thus, fraction B-3 contained LPS and fraction B-2 probably included lipid A or fragments of it as impurity. It may be assumed that the different solubilities in phenol of fractions B-2 and B-3 are due to the presence of the LPS component in fraction B-3. Examples are known in the literature of the isolation of such complexes of protein and LPS or fragments of it. Thus, from the endotoxin of E. coli by extraction with phenol Alaupovic [10] obtained "simple" and "conjugated" proteins, and Morrison [11] obtained a protein associated with a lipid.

The isolation of the protein fraction B-3 containing the LPS component is evidence in favor of the existence of fairly strong bond between the LPS and the protein in the LPPC extractable by butanol from Y. pseudotuberculosis: it is retained under the conditions of two severe treatments with phenol and under the conditions of electrophoresis in the presence of sodium dodecyl sulfate. This is in harmony with the results of Wu and Hit [12].

The protein fractions B-2 and B-3 obtained were active in the precipitation reaction with antiserum to the LPPC from Y. pseudotuberculosis (Fig. 3): B-2 gave one precipitation band while B-3, like the initial LPPC, formed two precipitation lines. Since these fractions differed from one another by the presence of the LPS component, it may be assumed that one of the precipitation bands is connected with the LPC and the other with the protein. However, additional investigations are necessary for definitive conclusions.

EXPERIMENTAL

The pseudotuberculosis microbe Y. psdt. (1 B subtype, strain 2602) was grown and separated from the culture liquid by a method described previously [3].

Isolation of the Cell Walls. A 10% suspension of cells (250 ml) was broken down in a planetary mill at 1000 rpm for 10 min (with an interval for cooling after 5 min). The mixture of cells and cell membranes was separated from the abrasive material (glass beads with a diameter of 150-200 μm) by centrifuging at 2000 rpm for 5 min and it was then sonicated in a MSE-150 instrument for 30 sec. The resulting mixture was centrifuged, first at 5000 rpm for 7 min and then at 5000 rpm for 13 min, to eliminate undisrupted cells. The cell walls were obtained by centrifuging at 20,000 g for 1 h. The precipitate was washed successively with 1 M NaCl solution,

with physiological solution three times, and with water three times, and was lyophilized. The purity of the fraction of cell walls was checked by electron microscopy.

Extraction and Purification of the LPPC. The microbial mass dried with acetone (30 g) was extracted with the butanol–physiological solution (1 : 2, v/v) system and was freed from nuclear acids by gel filtration on Sepharose 2B by a known method [6]. The protein component was obtained from 240 mg of the lyophilized LPPC (MS, 35.5%; protein, 30-9%; KDO, 2.4%).

Isolation of the Protein Component from the LPPC. A. A solution of 100 mg of LPPC in 20 ml of hot (65-68°C) water was treated with the same volume of 90% phenol. Extraction was carried out with stirring at 65-68°C for 45 min, and then the reaction mixture was cooled to 10°C and the aqueous and phenolic phases were separated by centrifuging at 3000 rpm. The aqueous phase was used for the isolation of the LPS.

The phenol was eliminated from the phenolic phase by exhaustive dialysis against distilled water, and the residue was lyophilized. This gave fraction B-1 with a yield of 33 mg.

B. A solution of 33 mg of B-1 in 6 ml of hot (65-68°C) water was extracted with phenol as described in section A for 30 min.

During the separation of the phases (aqueous and phenolic) by centrifuging, a precipitate was observed to deposit. The phenolic phase was again centrifuged at 3000 rpm for 20 min, and the precipitate was washed several times with ethanol and dried in the air. This gave fraction B-2 (protein, 72%; MS, not detected). Yield 7.5 mg.

The supernatant was treated with 9.5 volumes of ethanol, the mixture was left overnight at −10 °C, and the precipitate that had deposited was washed several times with ethanol and was dried in the air. This gave fraction B-3 (protein, 51.3%; MS, 19.7%). Yield 6.4 g.

Analytical Methods. Protein was determined by Lowry's method [14]; in the case of insoluble protein fractions the samples were dissolved in 0.1 N. NaOH. Monosaccharides were determined by the method of Dubois et al. [15] and 2-keto-3-deoxyoctonic acid (KDO) as described previously [16].

To determine amino acid compositions, samples of protein fractions B-2 and B-3 (1 mg) were hydrolyzed with 6 N HCl (1 ml) in sealed tubes at 110 °C for 24 h. The hydrolysates were diluted with water and were extracted three times with chloroform to eliminate fatty acids. The chloroform extracts were washed with water three times to eliminate traces of amino acids. The wash-waters were combined with the hydrolysate and were evaporated to dryness. Amino acid analyses were carried out on Biocal 3201 and LKB instruments.

To determine fatty acids in the protein fractions B-2 and B-3, chloroform extracts of the hydrolysates of these fractions were evaporated to dryness and were methylated with an ethereal solution of diazomethane. The methyl esters of the fatty acids were analyzed by GLC on a Pye-Unicam 104 chromatograph with a flame-ionization detector (4.25% of QF-1 + 0 .75% of SE-30 on Chromosorb W, 100-200 mesh) at 175°C.

The monosaccharide composition of fraction B-3 was determined by GLC of the acetates of the corresponding polyols, using the above-mentioned stationary phase at 175-215°C, 5 deg/min.

Electrophoresis in polyacrylamide gel (7.5 and 10%) in the presence of sodium dodecyl sulfate (NaDDS) was carried out in tris-borate buffer, (pH 9.25; 0.1% NaDDS) at U = 120 V and I = 4 mA per gel (column) for 40 min. Samples of the protein fractions were dissolved in tris-borate buffer (pH 9.25; 1% of NaDDS) and heated in the boiling water bath for 10 min. The gels (columns) were stained with Coomassie Brilliant Blue to indicate the protein zones [17], with the periodic acid–Schiff reagent to detect the carbohydrate component [17], and with a saturated solution of Sudan Black B in 60% ethanol to indicate lipids [18].

Immunological Methods. The immunological properties of B-2 and B-3 were studied by double diffusion in agar [19]. After double diffusion, the plates were washed with physiological solution and then with water and they were then dried at 37°C and were stained with Coomassie Brilliant Blue. In the immunological reactions we used rabbit antiserum to the LPPC obtained by a method described previously [20].

SUMMARY

1. The protein component of the LPPC from Yersinia pseudotuberculosis consists of two components with molecular weights of 42,000-45,000 and 18,000 -20,000. These polypeptides have been detected as the main components in the cell wall of Y. psdt.

2. The protein fractions B-2 and B-3, differing in their solubilities in phenol, have been extracted from the LPPC of Y. pseudotuberculosis by extraction with hot 45% phenol.

3. The identity of the polypeptide compositions of the two protein fractions have been shown.

4. It has been established that B-3 contains an LPS and B-2 contains lipid A or fragments of it as impurity.

5. Fractions B-2 and B-3 precipitate with antiserum to the LPC of Y. pseudotuberculosis.

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