ISOLATION AND CHARACTERISTICS OF THE PROTEINS OF THE OUTER MEMBRANE OF *Yersinia pseudotuberculosis*

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The dissociation of the protein-peptidoglycan (PPG) complex from the outer membrane of Yersinia pseudotuberculosis has given two protein fractions consisting predominantly of two polypeptides. A decrease in the electrophoretic mobility of the high-molecular-weight polypeptide on treatment with detergent and on heating is connected with conformational transitions: β form → α-helix. The polypeptides were obtained in the individual state by gel filtration on Bio-Gel. They are immunogenic for rabbits and exhibit antigenic relationship in the double immunodiffusion reaction in agar.

The elucidation of the mechanism of the function of the outer bacterial membrane is closely connected with the study of the structure and properties of the membrane proteins. In the present paper we discuss the isolation, fractionation, and preliminary characterization of the proteins of the pseudotuberculosis microbe Yersinia pseudotuberculosis, which are associated with a peptidoglycan (PG).

To obtain the protein–PG complex, the microbial cells were extracted with a 2% solution of sodium dodecyl sulfate (SDS) by Rosenbusch's method [1]. The protein–PG complex dissociates at 37°C under the action of 0.5 M NaCl in the presence of SDS [2] and on boiling in a 1-2% solution of the detergent [1].

We used both methods of isolating the proteins, treating the protein–PG complex successively with the reagents mentioned. This yielded two fractions, B-1 and B-2, the analytical characteristics of which are given below:

Fraction	Reagent	Temperature, °C	Time, min	Yield of protein, %
B-1	1% solution of SDS 0.5 M NaCl	37	30	20
B-2	2% solution of SDS	100	7	80

As we can see, the yield of proteins extractable by 0.5 M NaCl was five times lower than those extractable by a solution of detergents at 100°C. It must be mentioned that, according to electrophoresis, even after twofold treatment of the protein–PG complex with a 2% solution of SDS a certain amount of protein remained bound to the PG. The membrane proteins are obviously strongly bound to the PG and with a mild method of treatment only part of the protein is extracted.

We give the yields and analytical characteristics of the protein–PG complex and of fraction B-1 and B-2 (%):

Fraction	Yield (g) from 10 g of microbial mass	Monosaccharides	Protein	2-Keto-3-deoxyoctonic acid	3,6-Dideoxyhexoses	Nucleic acids
Protein–PG complex	1.2	8.9	90.9	0.3	0.1	6.2
B-1	0.1	17.4	62.1	0.5	0.8	Not det.
B-2	0.4	8.7	69.5	Tr.	0.2	Not det.

The presence of a certain amount of carbohydrate in the protein fraction is due to the strong interaction of the lipopolysaccharide (LPS) and the protein in the outer membrane of the bacterium [3]. For example, Henning [4] and Reithmeier [5] have also detected an LPS impurity in fractions of membrane proteins. Frac-

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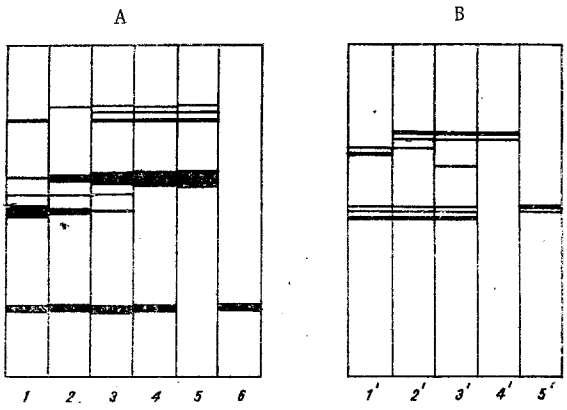

Fig. 1. SDS-PAAG electrophoresis of the protein fractions from Y. pseudotuberculosis: A) 10% gel, Tris-borate buffer, pH 9.25; 1) B-2 (without heating); 2) B-1 (kept in a solution of SDS at room temperature); 3) B-1 (100°C, 5 min); 4) B-2 (100°C, 5 min); 5) PP*-1 (100°C, 5 min); 6) PP-2 (100°C, 5 min); B) 14% gel, with Tris-glycine buffer, pH 8.3. To the gel was added 0.07 M NaCl (1'-3'); to the samples was added 0.1 M $MgCl_2$ (4', 5'); 1') B-1 (without heating); 2') B-1 (100°C, 5 min); 3') B-2 (100°C, 5 min); 4') PP*-1 (100°C, 5 min); 5') PP-2 (100°C, 5 min).

tion B-1 contained the largest amount of carbohydrate. As was established by GLC, the carbohydrate components included paratose, fucose, mannose, glucose, galactose, a heptose, and glucosamine, i.e., the monosaccharides characteristic for the LPS of the IB serovar of Y. pseudotuberculosis [6].

Fractions B-1 and B-2 were investigated with the aid of electrophoresis in polyacrylamide gel in the presence of SDS (SDS-PAAG electrophoresis) under various conditions. The results are presented in Fig. 1.

As can be seen from Fig. 1A (3, 4) and B (2', 3'), the polypeptide compositions of B-1 and B-2 were identical (provided that the samples had first been heated in SDS at 100°C). They consisted predominantly of two polypeptides PP*-1 and PP-2 with molecular masses of 41,000 and 14,500, respectively (Fig. 2). However, the ratios of PP*-1 and PP-2 in the fractions were different: in B-1 it was 2:1, and in B-2 4:1. An unheated sample of B-1 had, in place of PP*-1, a polypeptide band with a mobility corresponding to a molecular mass of 33,500 (PP-1) (Fig. 1A (1) and B (1'), Fig. 2). Such a change of electrophoretic mobility as a function of the temperature of previous treatment of the sample is characteristic for certain proteins of the bacterial membrane [3].

Apart from the polypeptides mentioned, electrophoretograms of B-1 and B-2 also showed the presence of narrow protein zones with mobilities less than that of PP*-1, but their concentrations were insignificant in comparison with the main polypeptide. When samples were stored in a solution of the detergent or in the lyophilized state, a decrease in the intensity of these bands was observed. It is possible that they were dimers or trimers of PP*-1 the appearance of which is due to the strong protein–protein interaction which is characteristic for the outer membranes of bacteria [17]. However, the degree of such interaction is not the same for individual representatives of the family Enterobacteriaceae [9]. On the addition of salts increasing the resolution of low-molecular-weight polypeptides (0.07 M NaCl to the gel [9] and 0.1 M $MgCl_2$ to the samples of the protein fractions [10]), an increase in the number of bands was observed for PP-2, i.e., its heterogeneity was revealed (Fig. 1B). Treatment with β-mercaptoethanol did not affect the electrophoretic patterns of B-1 and B-2, which indicates the absence of disulfide bonds in the protein molecules. The absence of cysteine from the composition of PP*-1 and PP-2 confirmed these results.

The polypeptide composition of B-1 as a function of previous treatment of the sample was studied in more detail.

As can be seen from Fig. 3, at 50°C a gradual transition of the "thermolabile" form of the polypeptide PP-1 into the temperature-stable form PP*-1 began. This transition was complete at 70°C, and boiling the sample did not change the electrophoretic pattern further. The presence of PP*-1 together with PP-1 was also observed in samples of B-1 kept in SDS solution at room temperature for several days [Fig. 1A (2)]. However, in B-2 only PP*-1 was detected, regardless of the thermal treatment.

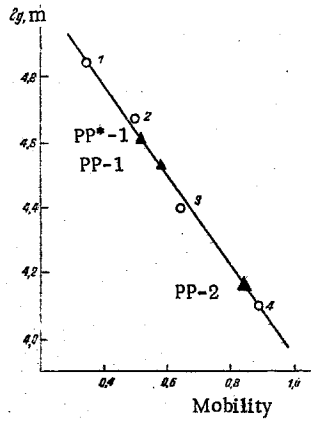

Fig. 2

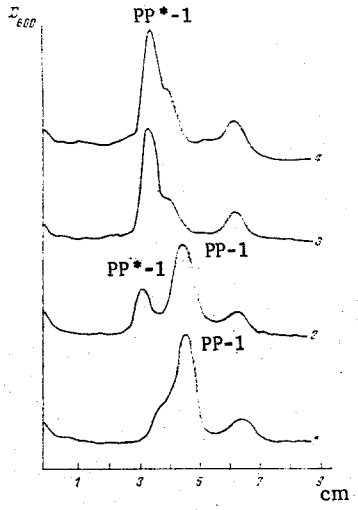

Fig. 3

Fig. 2. Determination of the molecular masses of the polypeptides present in the composition of B-1 and B-2 from Y. pseudotuberculosis: PP-1 – 33,500; PP*-1 41,000; PP-2 – 14,500. Markers: 1) bovine serum albumin (67,000); 2) egg albumin (45,000); 3) chymotrypsin A (25,000); 4) cytochrome c (12,000).

Fig. 3. SDS-PAAG electrophoresis of fraction B-1 treated with detergent at various temperatures. The samples of B-1 were heated for 5 min at: 1) 30°C; 2) 50°C; 3) 70°C; 4) 100°C. The gels were stained with Coomassie Blue and were scanned at 600 nm.

It has been shown that a decrease in the electrophoretic mobility of membrane proteins after boiling in detergent solution is connected with conformational changes: an increase in the proportion of α-helices as compared with the native proteins, which are distinguished by a high content of β-structure [11]. In view of this it is obvious that the "thermolabile" form of PP-1 corresponds to the more native protein (B-1); it is unstable in detergent solution even at room temperature and passes into the heat-stable PP*-1 form on heating, in detergent at 100°C (B-2).

The conformational transitions of the peptide PP*-1 obtained on the gel filtration of B-2 on Bio-Gel P-150 (Fig. 5B) as functions of the temperature and the presence of detergent were studied with the aid of the method of circular dichromism (CD) (Fig. 4). Calculation from the CD spectra [12] showed that in 1% SFS solution PP*-1 contained 22% of α-helix and 23% of the β form (Fig. 4, curve 1). With a rise in the temperature of the solution the CD spectrum scarcely changed, which shows the stability of the secondary structure of PP*-1 under these conditions.

Elimination of the detergent by dialysis led to a change of the conformation of the protein: It had 43% of β-structure and 8% of α-helix (Fig. 4, curve 5). Heating such a sample in buffer was accompanied by a gradual increase in the proportion of α-helix and a decrease in the amount of β-structure (Fig. 4, curve 4-2). In the final account, the CD spectrum of PP*-1 eluted at 100°C without detergent was almost identical with the CD spectrum of a sample in a solution of detergent.

The presence of detergent and heating obviously lead to the same changes in the conformation of the polypeptide and, furthermore, this process is reversible when the detergent is removed.

Some authors [13] have observed a renaturation of "thermolabile" proteins under the action of a LPS. However, the addition of the LPS to a solution of PP*-1 immediately before electrophoresis, and also exposure in the presence of the LPS, did not lead to the appearance of a polypeptide zone in the PP-1 region.

The value of the molecular mass of 41,000 obtained for PP*-1 is extremely close to the molecular mass of one of the polypeptides included in the lipopolysaccharide–protein complex (LPPC) from Y. pseudotuberculosis [14]. So far as concerns PP-2, judging from its molecular mass, it may be a protein analogous to Braun's lipoprotein [15]. However, a special investigation is necessary for a definitive conclusion.

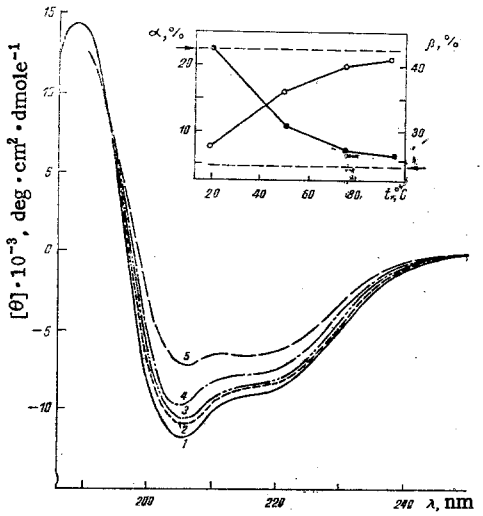

Fig. 4. CD spectra of PP*-1. The samples were dissolved in 0.01 M Tris-HCl buffer, pH 8, 0.1 M NaCl, 1% SDS (1). The samples were heated in the above-mentioned buffer without a detergent at 25°C (5), 50°C (4), 70°C (3), and 95°C (2). The temperature dependence of the amount of α-helix (○) and of β-structure (●) in PP-1 is shown.

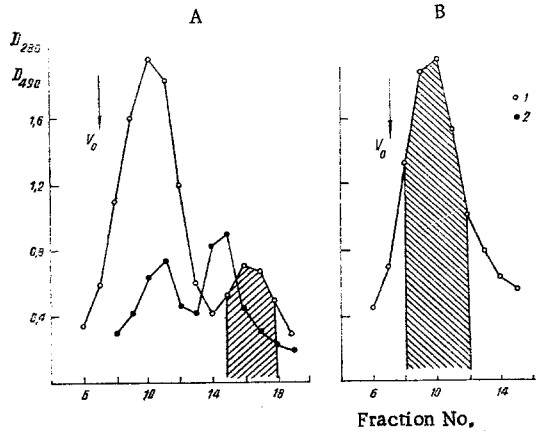

Fig. 5. A) Gel filtration of fraction B-2 on Bio-Gel P-150 (Tris-HCl buffer, pH 8, 1% SDS) at 37°C. The hatched region is PP-2. B) Rechromatography of B-2 under the same conditions. The hatched region is PP*-1: 1) in terms of protein; 2) according to the phenol–sulfuric acid method.

The separation of PP*-1 and PP-2 was carried out with the aid of the gel filtration of B-2 on Bio-Gel P-150 in the presence of SDS at an elevated temperature (Fig. 5A, B). The individuality of the polypeptides isolated was confirmed by the results of electrophoresis [Fig. 1A (5, 6); 1B (4', 5')] and of N-terminal analysis: The N-terminal amino acid of PP*-1 was serine and that of PP-2 proline.

In view of this, the heterogeneity of the P-2 under the conditions of SDS and disc electrophoresis in the presence of NaCl and $MgCl_2$ that has been mentioned above may be a consequence of the heterogeneity of the PP-2–SDS complex under these conditions [16]. Both polypeptides contain a certain amount of carbohydrates. It was obvious that the LPS–protein interaction also exists in detergent solutions. Nevertheless, an elimination of the LPS was observed in the gel filtration process. While in the initial B-2 fraction there was 0.13 mg of LPS to 1 mg of protein, in PP*-1 (after two cycles of gel filtration) there was only 0.05 mg of LPS to 1 mg of protein.

The amino acid compositions of PP*-1 and PP-2 were as follows (mole %):

Amino acid	PP*-1	PP-2
Lysine	5,10	6,32
Histidine	1,41	1,08
Arginine	5,39	5,44
Aspartic acid	12,43	10,65
Glutamic acid	11,99	11,91
Valine	6,90	6,83
Methionine	0,0	0,0
Isoleucine	4,54	4,29
Leucine	9,26	7,38
Tyrosine	1,81	1,21
Phenylalanine	3,85	5,70
Threonine	5,53	4,68
Serine	6,22	6,86
Proline	3,95	2,83
Glycine	11,04	10,30
Alanine	10,53	14,42
1/2 cystine	0,0	0,0

The polypeptides isolated were distinguished by a high content of acidic and hydrophobic amino acids, and also by the absence of cysteine, which is characteristic for membrane proteins [14].

The protein fractions obtained were immunogens for rabbits. PP*-1 and PP-2 gave a trace in the precipitation reaction with antiserum to P-2, which indicates their antigenic relationship.

EXPERIMENTAL

Strain 2602 of Yersinia pseudotuberculosis serovar IB was isolated from a patient with Far Eastern scarletina-like fever. The microorganisms were grown at 33°C in the nutrient medium described previously [6] for 18 h and were separated from the culture liquid by centrifugation and washed with physiological solution.

Extraction of the Protein–PG Complex. The microbial mass (10 g) was extracted with 25 ml of a 2% solution of SDS in 0.03 M Tris-HCl buffer, pH 8, at 50-60°C for 45 min. The undisrupted cells were separated by centrifugation at 5000 rpm for 7 min. The supernatant was centrifuged at 45,000 × g for 1.5 h. The precipitate obtained (the protein–PG complex) was washed with 0.3 M Tris-HCl buffer, pH 8, and the suspension was dialyzed successively against 50% ethanol (to eliminate SDS) and distilled water for 2 days and 4 days, respectively. Then the suspension was lyophilized; yield 1.2 g.

Extraction of the Protein with 0.5 M NaCl in the Presence of SDS. A suspension of 400 mg of the protein–PG complex in 20 ml of 0.03 M Tris-HCl buffer, pH 8, 1% SDS, 0.5 M NaCl, was kept at 37°C for 30 min. Then it was centrifuged at 77,000 × g for 1 h. The supernatant (B-1) was dialyzed against 50% ethanol and distilled water and was then lyophilized. Yield 33 mg. The precipitate was used for extraction with 2% SDS at 100°C.

Extraction of the Protein with 2% SDS Solution. The precipitate after extraction with 0.5 M NaCl was suspended in 20 ml of 0.03 M Tris-HCl buffer, pH 8, 2%. Extraction was carried out in the boiling water bath for 7 min. The suspension was centrifuged at 150,000 × g for 1 h. A 5-ml portion of the supernatant (B-2) was dialyzed against 50% ethanol and distilled water, and was lyophilized. Yield 35 mg. The remainder of the supernatant was used for fractionation on Bio-Gel P-150.

Gel Filtration of Bio-Gel P-150. A 5-ml portion of the B-2 fraction was deposited on a 1.2 × 100 cm column filled with Bio-Gel P-150 (Bio-Rad Laboratories) equilibrated with 0.03 M Tris-HCl buffer, pH 8, 1% SDS, at 37°C. Elution was performed with the same buffer at the same temperature at a rate of 5-6 ml/h. The fractions (2-ml) were collected, analyzed for their protein and monosaccharide contents, combined in accordance with the peaks on the elution curve, and concentrated by ultrafiltration. They were stored at −20°C.

SDS-PAG electrophoresis was performed in 1) Tris-borate buffer according to [17] and 2) by disc electrophoresis in Tris-glycine buffer according to [18]. Samples of the protein fractions (1 mg) were dissolved in the corresponding buffers (0.1 ml) containing 2% SDS, and the solutions were heated in the boiling water bath for 5 min. Of the resulting solutions, 10 μl (100 μg of protein) was deposited on a gel and electrophoresis was carried out at I = 5 mA per column of gel. The gels were stained with Coomassie Brilliant Blue to indicate the protein zones [19] and with the Schiff–periodic acid reagent to detect the carbohydrate component [19]. The gels stained for proteins were scanned on a Shimadzu CS-900 double-beam scanner at 600 nm. The ratio of PP*-1 and PP-2 in B-1 and B-2 were determined from the areas of the corresponding peaks.

The molecular masses of the polypeptides were determined by a method described in the literature [20] using as standards cytochrome c (12,000), chymotrypsin A (25,000), egg albumin (45,000) and bovine serum albumin (67,000).

Serological Methods. The serological properties of the protein fractions were studied with the aid of double immunodiffusion in agar in the presence of 3% of polyethyleneglycol [21]. The precipitation reaction was carried out with rabbit antiserum to fraction BO2 obtained as described by Ichihara and Mizushima [22].

Analytical Methods. 1) Protein was determined by a modified Lowry method [23], the yields of proteins being calculated as the ratios of the extracted proteins to the total amount of extracted proteins.

2. Monosaccharides were determined by the method of Dubois et al. [24], and 2-keto-3-deoxyoctonic acid and 3,6-dideoxyhexose by that of Burtzeva et al. [25].

3. The nucleic acid content was determined by Spirin's method [26].

4. To determine their amino acid compositions, samples of the protein fractions were hydrolyzed with 6 N HCl in sealed tubes at 110°C for 24 h, and the products were worked up as described by Bondarenko et al. [14]. Amino acid analyses were performed on a LKB Biocal 3201 instrument.

5. The monosaccharide composition of B-1 was determined with the aid of the GLC of the acetates of the corresponding polyols on a Pye-Unicam 104 chromatograph with a flame-ionization detector (4.25% of QF-1 and 0.75% of SE-30 on Chromosorb W, 100-200 mesh) at 175-215°C, 5 deg/min.

6. N-terminal amino acid analysis was performed according to [27].

7. CD spectra were recorded on a Dichrographe III (Jobin-Yvon) in the 250-190 mμ region at a protein concentration of 1 mg/ml in a cell 0.01 cm thick.

SUMMARY

1. The protein—PG complex has been isolated from the outer membrane of Yersinia pseudotuberculosis. Dissociation of the complex has given two protein fractions, B-1 and B-2.

2. According to SDS-PAAG electrophoresis, B-1 includes polypeptides with molecular masses of 33,500 (PP-1) and 14,500 (PP-2), and B-1 includes polypeptides PP*-1 with a molecular mass of 41,000 and PP-2.

3. It has been shown that PP-1 and PP*-1 are two conformational forms of one and the same polypeptide differing by their amounts of β-helices and β form. Treatment with detergent and heating causes the transition of PP-1 into PP*-1.

4. PP*-1 and PP-2 were obtained in the individual states by gel filtration on Bio-Gel in the presence of detergent.

5. PP*-1 and PP-2 are immunogenic for rabbits and exhibit antigenic relationship in the double immunodiffusion reaction in agar.

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