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CHARACTERISTICS OF THE MOLECULAR-MASS PARAMETERS AND VISCOSITIES OF SOLUTIONS OF THE LIPOPOLYSACCHARIDE–PROTEIN COMPLEX FROM *Yersinia pseudotuberculosis*

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UDC 576.851.45+576.809.7+541.24

The lipopolysaccharide–protein complex (LPPC) from *Y. pseudotuberculosis* isolated by extraction with trichloroacetic acid has been investigated by the methods of gel permeation chromatography, light scattering, and viscometry. The molecular-mass distributions of aggregates of the LPPC in water, 0.03 M Tris-HCl buffer (pH 8) and 0.1 M sodium chloride have been determined. A dependence of the polydispersity and dimensions of the aggregates on the concentration of the polymer and on the ionic strength of the solvent has been shown. It has been established that in water the LPPC has a high characteristic viscosity which falls with an increase in the ionic strength of the solution.

The lipopolysaccharide–protein complexes (LPPCs) localized in the outer membrane of Gram-negative bacteria are of interest as structural elements of the cell walls, endotoxins, and the complete antigens of these bacteria [1]. In the manifestation of a series of immunological properties of antigens, their physical characteristics are of fundamental importance [2]. The molecular dimensions of the endotoxins isolated from different bacteria vary over a wide range [3-5]. This is due to differences in the sources of isolation of the antigens and the methods for their extraction and the differences in the physicochemical methods of investigation used. The study of the physicochemical properties of LPPCs is difficult because of their high-molecular-mass nature, their considerable heterogeneity, and their capacity for forming aggregates in aqueous solutions [6].

An estimate of the dimensions of the LPPC isolated by means of the butanol–water system from *Yersinia pseudotuberculosis* has previous been made by the light-scattering method [7].

In the present investigation, some physicochemical properties of the LPPC obtained from *Y. pseudotuberculosis* by Boivin's method [8] and purified by gel filtration on Sepharose 2B – LPPC-A [9] – and by centrifugation in a cesium chloride gradient – LPPC-A_1 [10] – have been studied by the methods of gel-permeation chromatography (GPC), light scattering, and viscometry.

The molecular-mass parameters of the LPPC particles in solution were determined by the GPC method on macroporous glasses. Distilled water, 0.03 Tris-HCl buffer (pH 8) and 0.1 M sodium chloride were used as solvents. The molecular mass distributions (MMDs) of LPPC-A in aqueous solvents determined with the aid of GPC are given in Fig. 1. The relative area of each chromatographic peak (in percentages) and the corresponding values of the weight-average molecular mass (M_W) of the particles calculated from the chromatogram corrected for the instrumental broadening function are given in Table 1.

The multimodality of the MMDs (Fig. 1) shows the pronounced polydispersity of the LPPC. Such polydispersity is due to the fact that in aqueous solutions the LPPC forms aggregates of different dimensions. The molecular masses of these aggregates and the ratio of the components in the solution depend on the concentration of the polymer and on the nature of the

Pacific Ocean Institute of Bioorganic Chemistry, Far Eastern Scientific Center, Academy of Sciences of the USSR, Vladivostock. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 384-388, May-June, 1982. Original article submitted July 8, 1981.

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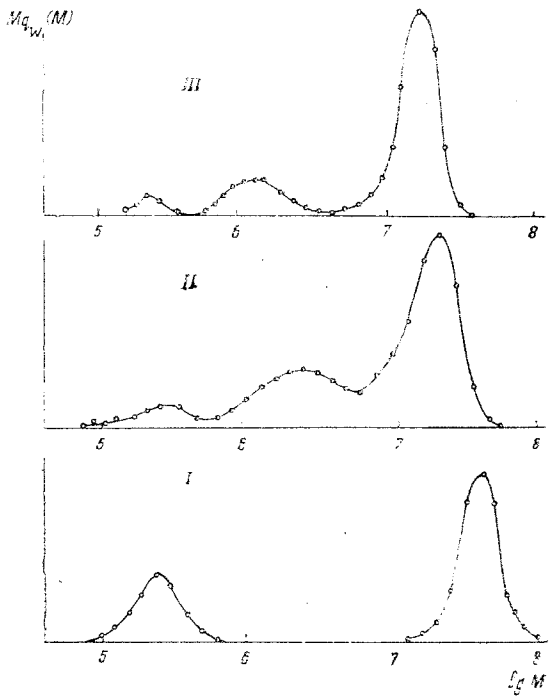

Fig. 1. Molecular mass distributions of LPPC-A in water (I), Tris-HCl buffer (II), and 0.1 M sodium chloride (III).

solvent (see Table 1). In water at a concentration of 2 mg/ml, the LPPC forms mainly large aggregates with $M_W = 4.5 \cdot 10^7$. With an increase in the concentration of the polymer to 2.5 mg/ml, the dimensions of the aggregates change to $2.7 \cdot 10^8$. It was impossible to study more concentrated solutions because of the low solubility of the LPPC in water.

In addition to GPC, to evaluate the molecular masses of the LPPC particles we used the light-scattering method. The considerable bending of the extrapolation dependence $(cH/R_\theta)_{c \to o}$ also shows the pronounced polydispersity of the LPPC in solution. The molecular mass, M_W, of LPPC-A in water obtained from the light-scattering results is $2.2 \cdot 10^7$, which agrees well with the results obtained by this method previously [11]. In addition to M_W, we determined the number-average molecular mass (M_N) by the same method for LPPC-A in water. The M_W/M_N ratio, equal to 5.0^6 shows the pronounced polydispersity of the LPPC in water.

The intrinsic viscosity of the LPPC in water has a fairly high value of the order 260 ml/g. A substantial fall in viscosity is observed with an increase in the ionic strength of the solution. Thus, in 0.1 M sodium chloride the intrinsic viscosity of LPPC-A amounted to 35 ml/g and that of LPPC-A_1 to 29 ml/g. The viscosities of solutions of the LPPC in 0.1 M sodium chloride depend greatly on the concentration of the polymer (Fig. 2). At low concentrations ($c < 0.4$ mg/ml), the reduced viscosity falls linearly with a decrease in the concentrations; at higher concentrations ($c > 0.5$ mg/ml), the viscosimetric curve has an anomalous shape (Fig. 2). This is probably due to the capacity of the LPPC for forming very large and unstable aggregates in concentrated solutions. According to the GPC results, the molecular masses of such aggregates at concentrations of the LPPC greater than 2 mg/ml amount to $1.6\text{-}1.5 \cdot 10^7$ (see Table 1). In the region of low concentrations of LPPC-A_1 (less than 0.5 mg/ml), particles are formed with smaller dimensions: $3.1 \cdot 10^6$ and $1.8 \cdot 10^5$, with a predominance of the latter (see Table 1).

The M_W values obtained by the light-scattering method were $0.5 \cdot 10^7$ for LPPC-A in 0.1 M sodium chloride and $0.5 \cdot 10^6$ for LPPC-A_1. The M_W/M_N ratios for LPPC-A and -A_1 in this solvent are 1.5 and 1.2, respectively. Thus, the degree of polydispersity of the LPPC in salt solutions is considerably smaller than in water. The large value of the molecular mass at a low characteristic viscosity may show a compact form of the LPPC particles in salt solutions. The ionic strength of the solution (0.03 M tris-HCl and 0.1 M sodium chloride) has no fundamental influence on the dimensions of the aggregates.

TABLE 1. Weight-Average Molecular Masses (M_W) and Percentage Amounts (P, %), of Aggregates at Various Initial Concentrations of the LPPC in Different Solvents

Solvent	Substance	Concentration, mg/ml	M_W	P, %	M_W	P, %	M_W	P, %	M_W	P, %
Water	LPPC-A	2.0			$4.5 \cdot 10^7$	71			$2.7 \cdot 10^5$	29
	LPPC-A	2.5	$2.7 \cdot 10^8$	18	$5.2 \cdot 10^7$	1	$1.0 \cdot 10^6$	79		
	LPPC-A, rechromatography		$2.7 \cdot 10^8$	21	$2.4 \cdot 10^7$	17	$1.0 \cdot 10^6$	42		
0.03 M Tris-HCl buffer, pH 8	LPPC-A	2.0			$1.4 \cdot 10^7$	53	$2.5 \cdot 10^6$	35	$4.5 \cdot 10^5$	6.5
	LPPC-A	2.5			$1.3 \cdot 10^7$	64	$2.0 \cdot 10^6$	21	$6.4 \cdot 10^5$	20
	LPPC-A	1.8			$1.5 \cdot 10^7$	44			$5.0 \cdot 10^5$	34
	LPPC-A	1.5			$4.2 \cdot 10^7$	31	$2.0 \cdot 10^6$	31		
0.1 M sodium chloride	LPPC-A, rechromatography	2.0			$1.5 \cdot 10^7$	68	$2.5 \cdot 10^6$	24	$4.8 \cdot 10^5$	4
	LPPC-A	4.0			$1.6 \cdot 10^7$	74	$3.0 \cdot 10^6$	22	$4.6 \cdot 10^5$	2
	LPPC-A	0.5					$3.1 \cdot 10^6$	23	$1.8 \cdot 10^5$	76
	LPPC-A	2.0			$1.5 \cdot 10^7$	62	$2.7 \cdot 10^6$	4	$7.5 \cdot 10^5$	28
	LPPC-A									

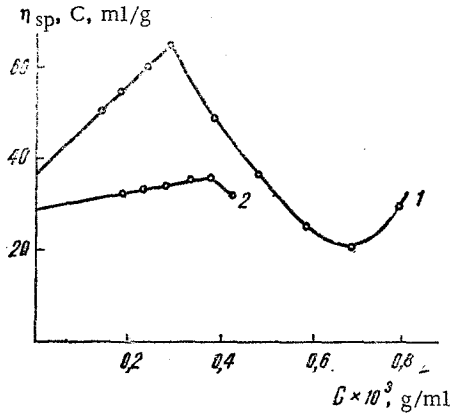

Fig. 2. Dependence of the reduced viscosity on the concentration in 0.1 M sodium chloride solutions: 1) LPPC-A; 2) LPPC-A_1.

The high-molecular-weight fraction of the LPPC ($4.5 \cdot 10^7$ in water and $1.4 \cdot 10^7$ in tris-HCl buffer) was subjected to rechromatography. The molecular mass distribution shows that the solution contained larger aggregates in addition to particles with the original dimensions (see Table 1). An increase in the dimensions of the aggregates to $2.8 \cdot 10^8$ in water and $4.1 \cdot 10^7$ in Tris-HCl apparently takes place as the result of the concentration and lyophilization of the samples. A similar change in molecular mass of a polymer on lyophilization has been observed for lipopolysaccharides from other sources [12].

A comparison of the molecular mass parameters obtained for different concentrations of polymer in different solvents showed that to within the accuracy of the experimental error the M_W values of the aggregates are multiples of some magnitude, arbitrarily designated M_o and equal to $5 \cdot 10^5$ for the LPPC in 0.1 M sodium chloride and in 0.3 M Tris-HCl, and $2.6 \cdot 10^5$ for the LPPC in water. In solution, aggregates with M_W = 20-30 M_o predominate, together with $2M_o$, $3M_o$, $5M_o$, $12M_o$, and XM_o, where X > 100 for aqueous solutions. The ratio of the amounts of such particles depends on the concentration of the polymer.

It is known that LPPC aggregates are capable of dissociating under the action of a detergent [6]. We have previously shown the dissociation of aggregates of the LPPC from *Yersinia pseudotuberculosis* under the action of sodium sarcolysate [10]. The viscosimetric method permitted the micelle-forming concentration of this detergent to be determined, and this exceeds 8 mg/ml, so that a 0.8% solution of sodium sarcolysate was used for the disaggregation of the LPPC. The intrinsic viscosity [η] of LPPC treated with the detergent rose

considerably in comparison with the value obtained for LPPC in salt solutions. For LPPC-A, [η] amounted to 103 ml/g, and for LPPC-A_1 to ≈ 190 ml/g. Such an increase in viscosity is probably due to a change in the form of the LPPC particles treated with detergent, which is in harmony with the results that we obtained by the sedimentation method [10].

EXPERIMENTAL

Investigations by the light-scattering method were performed on a FPS-3 instrument at a wavelength λ 546 nm in natural light. As the trubidimetric standard we used benzene, the value of the Rayleigh ratio of which was taken as $16.4 \cdot 10^{-6}$ cm^{-1}. Solutions were prepared by the method of successive dilution in a single cell. The initial concentration was approximately $0.35 \cdot 10^{-3}$ g/ml, and the final concentration $0.6 \cdot 10^{-4}$ g/ml. The scattering indicatrix was measured for angles of from 40 to 150° in 10 degree steps. The results obtained were interpreted by the double extrapolation method [13], and the calculations were performed by means of a simple program in DIASP SM language on a small M-400 computer.

Gel permeation chromatography was performed on an analytical chromatograph with a refractometric detector. A system of two columns 8 mm in diameter and 1200 mm long with macroporous glasses having mean pore diameters of 1600 and 250 Å was used. The rate of flow of the eluent was 75 ml/h. To determine the calibration relationship we used dextrans and their sodium salts, which we carefully investigated by the methods of light scattering, viscosimetry, and GPC. The calibration curve was approximated by a second-degree polynomial. The coefficients of the polynomial were determined by constructing a calibration relationship for widely disperse standards with known values of M_W [14]. Chromatograms obtained were corrected for the instrumental broadening function, which was considered to be a Gaussian relationship. Dispersion was determined from the chromatograms of authentically monodisperse substances. The corrected chromatogram was determined by solving a Fredholm's equation of the first kind (Tang's equation) by the method of successive approximations [14] by means of a program in DIASP SM language on the M-400 computer. The criterion of the termination of the process of calculation was the cessation of a change in the mean square deviation of the reduced chromatogram from the experimental one. From the chromatogram corrected for the instrumental broadening function we calculated the MMD and weight-average molecular masses for the whole MMD and for each separate chromatographic peak.

Viscosities were measured in a modified Ubbelohde viscometer with a capillary having a diameter of 0.5 mm at a temperature of 25 ± 0.1°C. The accuracy of the stopwatch was ±0.1 sec. The time of outflow of a 0.1 M sodium chloride solution was 214.7 sec, and that of a 1% sodium sarcolysate solution 224.9 sec.

SUMMARY

The lipopolysaccharide-protein complex isolated from *Yersinia pseudotuberculosis* by Boivin's method forms polydisperse aggregates of high molecular mass in aqueous solutions. The degree of polydispersity, the molecular masses of the aggregates formed, and the viscosities of solutions of the LPPC depend on the concentration of polymer and the ionic strength of the solvent.

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QUANTITATIVE ANALYSIS OF β-EXOTOXIN IN INSECTICIDAL PREPARATIONS BY HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY

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UDC 632.937:576.851.5.097.29

A quantitative method is proposed for determining β-exotoxin, which is a product of the vital activity of certain serotypes of *Bac. thuringiensis*. The method is based on the use of high-performance anion-exchange chromatography. The chromatography of β-exotoxin-containing preparations was performed on a column (1.6 × 150 mm) containing type 2632 anion-exchange resin (Hitachi) in a concentration gradient of HCl and NaCl. The time of analysis amounted to 34 min. The method permits the analysis of β-exotoxin in insecticidal preparations in an amount of 1 μg and upwards.

The heat-stable β-exotoxin present in complex bacterial insecticidal preparations obtained from *Bac. thuringiensis* is 2-O-[4'-O-(adenosin-5''-yl)-ξ-D-glucopyranosyl]-4-phosphoallaric acid. In 1976, Prystaš et al. [1] effected the complete synthesis of this compound, opening up prospects for its industrial production. At the present time, a number of biological and biochemical tests based on its action on insects [2-8] and also on the investigation of its inhibiting influence on the DNA-dependent RNA-polymerase from *E. coli* and *Bac. thuringiensis* have been developed for the analysis of preparations of β-exotoxin. However, these methods are complex and require considerable expenditures of time. We have proposed a method for the quantitative determination of β-exotoxin in various insecticidal preparations which is based on the use of high-performance anion-exchange column chromatography.

As the stationary phase we selected type 2632 spherical anion-exchange resin (Hitachi), which is based on a copolymer of styrene and divinylbenzene. The use of an anion-exchange resin is connected with the presence in the β-exotoxin molecule of a phosphomonoester group capable of dissociation in aqueous solutions with the formation of anions. Being distinguished by a high capacity, polymerization ion-exchange resins permit the separation of complex multicomponent systems without the necessity, in the majority of cases, for the preliminary purification of the samples being analyzed [11, 12]. This is of great importance, since as a rule, the β-exotoxin preparations contain a considerable amount of inorganic salts, protein compounds, carbohydrates, and other impurities.

As the mobile phase we used aqueous solutions of NaCl and HCl. To achieve the best resolution of the components of the mixtures being analyzed and the separation of the β-exotoxin peak we tried various forms of gradient curves permitting both the concentration of the salt and the pH of the eluent to be changed during the separation process.

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