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Summary

A method of determining the molecular mass composition of microbial exopolysaccharides (EPS) by centrifuging them in a combined density gradient created by NaCl and CsCl solutions and using the molecular mass of dextranes as standards is developed. The process of determining the molecular mass distribution pattern of EPS is simplified and made considerably less time-consuming. This method allows the analysis of native EPS with molecular masses ranging from 13 700 to 2 000 000.

Key words: Gradient centrifugation; Molecular mass; Dextrane; Exopolysaccharide

Introduction

Broad attention is drawn to microbial exopolysaccharides (EPS) due to their biological activity as well as physical and chemical properties. The latter are determined not only by a biopolymer structure but also by the ratio of fractions having different molecular mass, i.e., by their molecular mass heterogeneity.

To obtain EPS preparations with definite properties, the EPS molecular mass parameters must be controlled during producer cultivation and EPS isolation. Since in this case the researcher has to deal with a great number of samples, the development of specific and more 'rapid' methods than now available (electrophoresis, gel chromatography, etc.) is necessary.

Methods of analytic centrifugation in a density gradient of sucrose [4] or CsCl [11] are commonly applied for molecular mass estimation of proteins, nucleic acids and viruses The density gradient of CsCl in Tris buffer with EDTA, a combination of alkaline tungstaies or alkaline earthmetal and NaCl or $MgCl_2$ [5] is applied to enhance the component separation of the substance studied.

Gradient centrifugation methods, however, are not commonly used in studies of

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microbial EPS. As far as we know this method was used only once to determine mass-molecular heterogeneity of xanthan [10]. The method of gradient centrifugation in NaCl solution was used to determine molecular mass of xanthan fractions obtained after the polysaccharide sonication. But this method has never been used to investigate unfractionated EPS.

The long duration of the analysis caused by the necessity to fractionate EPS and to study each fraction separately adding cumbersome calculations to obtain molecular mass values is one of the main disadvantages of the approach described.

This paper presents the development of a method for the determination of native EPS molecular mass heterogeneity in a wide range of molecular mass values (13700—2000 000).

The centrifugation in the combined density gradient of NaCl and CsCl solutions was chosen as the method to solve the problem. [6].

Materials and Methods

Objects of investigation and conditions of cultivation

Xanthan (Sigma), produced by *X. campestris* pr. *Campestris* 8162 [1] and EPS produced from ethanol by microbial association of *Acinetobacter* sp. and *Micrococcus* sp. with yeast *Candida tropicalis* were studied [2]. The association was isolated from the active sludge of the oil plant sewage biological treatment station. The content of monocultures in the studied EPS-producing association was as follows: *Acinetobacter* sp., 70-80%, *Micrococcus* sp., 15-20%, *C. tropicalis,* 5-10%.

X. campestris 8162 and EPS-producing microbial association are maintained in the Collection of Pure Cultures of the Institute of Microbiology and Virology of the Academy of Sciences of the Ukraine, Kiev.

The association was grown in 750 ml flasks with 100 ml of medium [7], containing 1% of ethanol as carbon source on a rotary shaker (220 rpm) at 28-30°C, pH 7.0 for 72 h.

X. campestris 8162 was cultivated in the analogous conditions in Leach [8] medium containing glucose as the carbon source. In both cases the inoculum was grown in flasks on a rotary shaker till the logarithmic stage. The inoculum amount was 5 v% of the medium.

Isolation of exopolysaccharides (EPS)

To isolate EPS the suspension resulting from the producer growth was diluted 4 times by distilled water, then sodium chloride was added while stirring the solution to a final concentration of 0.005-0.01 M.

Cells were separated by centrifuging at 1500 *g* for 50 min then filtered through Celite-545 at 90°C. The supernatant was dialysed against distilled water for 5 days then concentrated in vacuum at 50°C to the initial volume; EPS was sedimented by three volumes of 95% ethanol. The resulting EPS sediment was washed by ethanol and dried in vacuum.

Preparation of the combined gradient

Native EPS were separated according to molecular mass by the combined density

TABLE 1

Gradients used in EPS analysis

Gradient	Volume (ml) of salt solutions of different density (g/cm^3)					
	NaCl				CsCl	
	1.03	1.05	1.10	1.20	1.40	1.60
1	6.0	5.0	–	–	–	–
2	3.0	5.0	2.0	1.0	–	–
3	2.0	2.0	3.0	2.0	1.0	0.8
4	1.0	2.0	1.0	1.5	2.5	3.0

1, Density gradient of NaCl solutions (1.03-1.05 g/cm^3); 2, density gradient of NaCl solutions (1.03-1.20 g/cm^3); 3, combined density gradient of NaCl (1.03-1.20 g/cm^3) and CsCl (1.40-1.60 g/cm^3) solutions, the v/v ratio of NaCl and CsCl being 5:1; 4, combined density gradient of NaCl and CsCl solutions, the v/v ratio of NaCl and CsCl being 1:1.

gradient centrifugation in NaCl and CsCl solutions (values of gradients and v/v ratios of NaCl and CsCl solutions of different density are shown in Table 1 for 13.5 ml tubes). The amount of the studied EPS (0.5% solutions) was 1.0-1.5 ml. When using tubes of any other volume the amount of NaCl and CsCl solutions of different density applied to create a density gradient varies in proportion to the tube volume.

Analytical gradient centrifugation

EPS solutions (0.05% w/v) were applied for molecular mass composition analysis. 0.02% dextrane (Fluka) solutions were used as molecular mass standards (molecular masses of the dextranes used were 13700; 20000; 40000; 70000; 110000; 500000; and 2 000 000).

The gradient centrifugation of EPS solutions and dextranes was performed using Beckman L-60 centrifuge (rotor SW-40, 13.5 ml tubes) at 30 000 rpm for 19 h. The amount of the studied EPS (0.05% solutions) was 1.0-1.5 ml.

To determine molecular masses of the EPS components solutions of native EPS and those of dextranes (used as molecular mass standards) were subjected to simultaneous gradient centrifugation (two of the centrifuge tubes were filled with solution containing a mixture of dextranes with different molecular mass, the other four contained solutions of the EPS studied).

The gradient centrifugation of both, mixed dextranes and the pure ones, was carried out to check the efficiency of their separation and distribution. In both cases the pattern of distribution of dextranes having the same molecular mass was the same.

Following the centrifugation fractions of 25 ml volume were sampled from the tubes. The carbohydrate content in fractions was determined with phenol and sulphuric acid [9].

Preparative gradient centrifugation and fractionation of EPS

To obtain EPS fractions with the definite molecular mass xanthan (Sigma) was centrifugated in the combined density gradient created by NaCl and CsCl solutions (see Table 1. gradient 3). Centrifugation was effected on a K-32 centrifuge (USSR)

(rotor U30-26-381, 38.1 ml tubes) at 30000 rpm for 19 h. The amount of xanthan 0.05% solution taken in a tube was 4.0-5.5 ml (2-2.75 mg).

Following the centrifugation, fractions of 1 ml volume were sampled from the tubes and their carbohydrate content was determined. Fractions with the same molecular mass were combined and dialysed against distilled water for 5 days then concentrated in vacuum. As a result six xanthan fractions with different molecular mass were obtained (fractions I-VI).

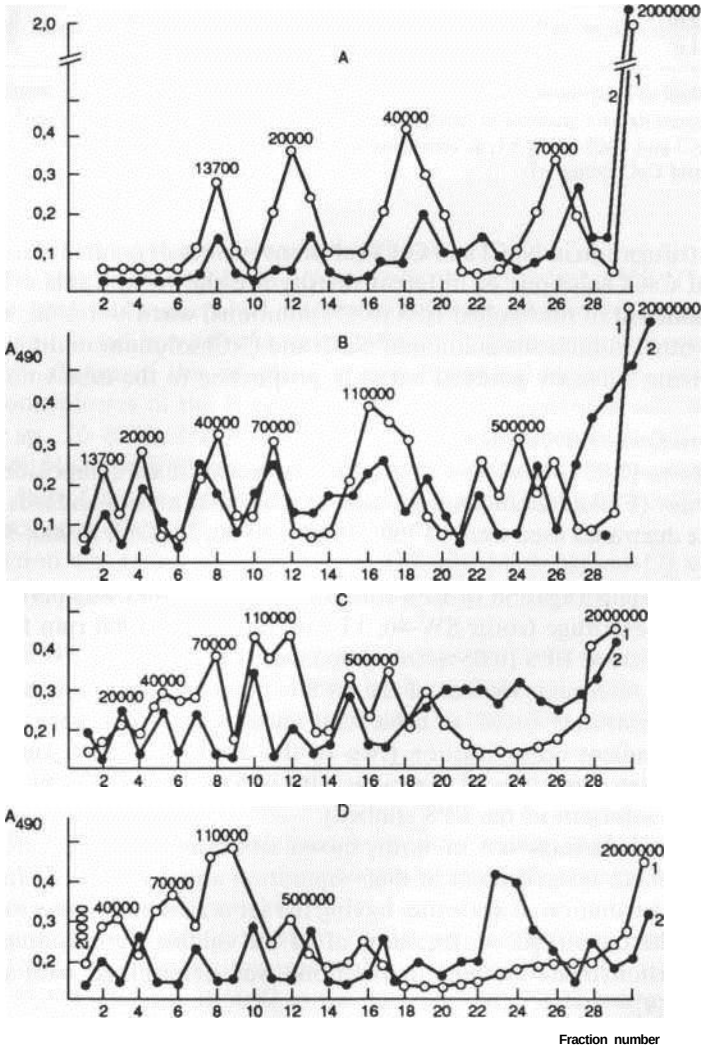

Fig. 1. Molecular mass distribution pattern of dextranes (1) and EPS synthesized by microbial association with yeasts (2) in the density gradient created by NaCl solutions: (A) 1.03-1.05 g/cm^3. (B) 1.03-1.20 g/cm^3 and in a combined density gradient created by NaCl (1.03-1.05 g/cm^3) and CsCl (1.40-1.60 g/cm^3) solutions at 5:1 (C) 1:1 and (D) volume to volume ratios.

Gel-chromatography of EPS solutions

Gel-chromatography of the studied native EPS solutions and of the EPS fractions resulting from the preparative gradient centrifugation (fractions I-IV) was applied to confirm that the method suggested allows the complete separation of different molecular mass components from the heterogenous native EPS.

Gel chromatography was performed on a 4B (Pharmacia) sepharose column (0.9 ×80 cm). 0.3 M NaCl was used for the elution. Carbohydrate content in the fractions collected (1 ml) was determined with phenol and sulphuric acid. Column was calibrated with dextranes (Fluka) of different molecular mass.

Results and Discussion

In earlier experiments the density gradient centrifugation in NaCl solutions (1.03-1.05 g/cm^3) was used to analyze previously separated xanthan components [10].

At the initial stage of our investigation we made an attempt at studying native unfactionated EPS in analogous conditions (Fig. 1 A). But this attempt proved to be practically unsuccessful since the complete molecular mass separation of the heterogenous EPS could not be achieved (according to the chromatographic data obtained previously, the EPS under study contains components ranging in molecular mass from 13000 to 2 000 000 [3]). Thus, centrifugation in the NaCl density gradient (1.03—

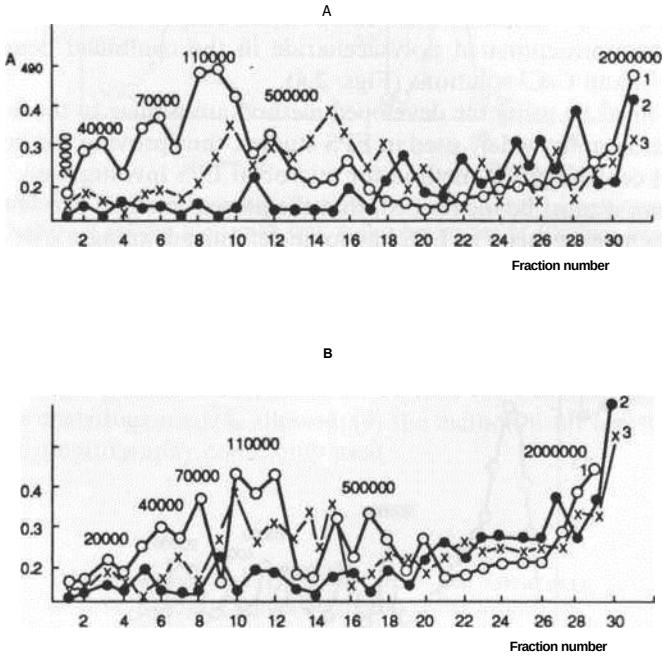

Fig. 2. Distribution of dextranes (1), (Sigma) xanthan (2) and EPS synthesized by *X. campestris* 8167 (3) in lie density gradient created by NaCl (1.0-1.05 g/cm^3) and CsCl (1.40-1.60 g/cm^3) solutions at the 1:1 (A) and 5:1 (B) volume to volume ratio.

1.05 g/cm^3) allows the separation of fractions with molecular mass up to 70 000 from the native EPS (Fig. 1A).

The molecular mass distribution pattern of EPS components with molecular masses up to 500 000 (Fig. 1B) was obtained by increasing the density of NaCl solutions up to 1.20 g/cm^3 (gradient 2, see Table 1). EPS solutions were centrifugated in the combined density gradient of NaCl and CsCl solutions (gradients 3 and 4, see Table 1) to separate fractions having higher molecular mass (Fig. 1C,D).

Fractions with different molecular mass can be separated from the native EPS depending on the volume to volume ratio of NaCl and CsCl solutions taken. The increase of the volume of NaCl solution (NaCl:CsCl = 5:1) allows the separation of relatively low molecular mass components (to 500 000) while the increase of that of CsCl solutions (NaCl:CsCl = 1:1) allows to separate successfully high molecular mass fractions (from 500 000 to 2 000 000) (Fig. 1D).

Similar conclusions can be drawn on analysing the data on molecular mass composition presented in Fig. 2. The separation of EPS components with molecular masses higher than 2 000 000 by both CsCl solutions of higher density and the centrifugation at above 30 000 rpm is limited by the centrifuges' capacity.

The data on molecular mass composition of native Sigma xanthan resulting from the centrifugation in the density gradient of NaCl and CsCl solutions (Fig. 2) and those resulting from gel chromatography (Fig. 3) proved to be identical.

Molecular mass of separate xanthan (Sigma) fractions obtained by the preparative gradient centrifugation and that determined with the help of gel chromatography fully correspond to the molecular mass composition data obtained by the centrifugation of a native unfractionated polysaccharide in the combined density gradient created by NaCl and CsCl solutions (Figs. 2,4).

Results obtained by using the developed method are similar to those obtained by the gel chromatography widely used in EPS studies, thus proving the high efficiency of the gradient centrifugation method for microbial EPS investigation.

In conclusion, it must be mentioned, that the present method for determining the molecular mass heterogeneity of EPS has some definite advantages over the methods described in the literature [10]: (1) The estimation of molecular mass heterogeneity of a native EPS without its preliminary fractionation is allowed; (2) the molecular mass

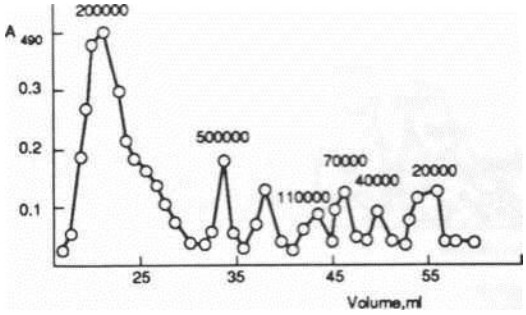

Fig. 3. Elution profiles of (Sigma) xanthan obtained by gel chromatography using the Pharmacia 4B sepharose column (0.9 × 80 cm).

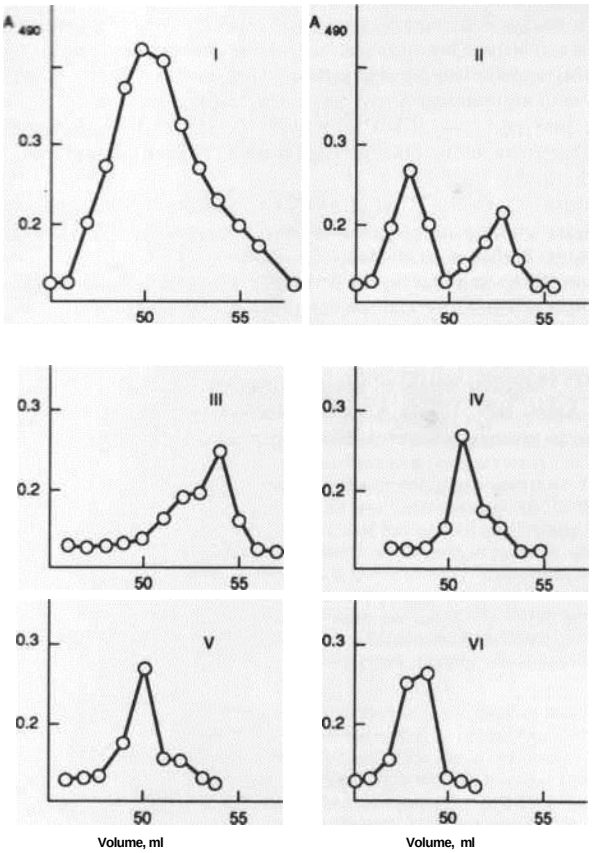

Fig. 4. Elution profiles of (Sigma) xanthan fractions I-VI obtained by gel chromatography using a Pharmacia 4 B sepharose column (0.9 × 80 cm), molecular masses of fractions being respectively: I, 2 000 000; II, 500 000; III, 110 000; IV, 70 000; V, 40 000; VI, 20 000.

determination of an EPS is simplified due to simultaneous centrifugation of both the sacEPS solutions and of the set of dextranes with definite molecular masses; (3) the simultaneous analysis of several native EPS (from 4 to 9 samples depending on the capacity of the centrifuge used) is allowed; (4) the method is far less time consuming than the gel chromatography commonly used.

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