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EXPERIMENTAL ARTICLES

Physicochemical Properties of the Microbial Exopolysaccharide Ethapolan Synthesized on a Mixture of Growth Substrates

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Abstract—Some physicochemical properties of the microbial exopolysaccharide (EPS) ethapolan synthesized by *Acinetobacter* sp. 12S depended on whether the producer was grown on a mixture of ethanol and glucose or on a single substrate. Irrespective of the carbon source in the nutrient medium, the contents of carbohydrates, pyruvic acid, uronic acids, and mineral components in the EPS remained unchanged. The EPS were also identical in their monosaccharide composition: the molar ratio of glucose, mannose, galactose, and rhamnose was 3 : 2 : 1 : 1. EPS with a higher content of fatty acids was synthesized during growth on the mixture of ethanol and glucose. The average molecular mass and the content of high-molecular (M > 2 MDa) fractions were greater in ethapolan produced on the substrate mixture. In the presence of 0.1 M KCl, after transformation into the H^+ form, and in the Cu^{2+}–glycine system, solutions of these EPS showed higher viscosity than solutions of EPS synthesized on single substrates. The reasons for the improved rheological properties of the EPS produced on the substrate mixture are discussed.

Key words: exopolysaccharides, cultivation conditions, substrate mixture, chemical composition, molecular mass, rheological properties.

We have previously shown that the synthesis of the microbial exopolysaccharide (EPS) ethapolan by *Acinetobacter* sp. 12S is increased if the bacterium is grown on a mixture of ethanol and glucose [1]. The cultivation conditions were optimized to stimulate EPS synthesis on a mixture of the two substrates that are unequal in terms of bioenergetics [2]. The carbon substrate transformation into EPS proved to be efficient in the absence of sodium cations in the medium, at the concentration of the nitrogen source reduced to 0.3–0.45 g/l, and when inoculate grown on ethanol was used. Under these conditions, the maximum specific growth rate of the producer increases and is attained at an earlier growth phase [2].

However, not only EPS synthesis (the amount of synthesized polysaccharides, rate of their formation, EPS yield calculated per substrate etc.) but also the physicochemical properties of the final product are known to depend on the cultivation conditions [3–8]. The EPS chemical composition, molecular mass, and the ratio between the component polysaccharides may vary depending on the growth conditions. As a result, the rheological properties of EPS and, consequently, their practical value may be altered.

In view of this, we compared the chemical compositions, molecular masses, and rheological properties of ethapolans synthesized by producers grown on ethanol, glucose, and a mixture of these two substrates.

MATERIALS AND METHODS

Bacterium. This study used strain *Acinetobacter* sp. 12S, a producer of a previously described complex polysaccharide preparation, ethapolan [5].

Cultivation of *Acinetobacter* sp. 12S. Bacteria were grown in liquid mineral medium of the following composition (g/l): KH_2PO_4, 6.8; KOH, 1.8; NH_4NO_3, 0.3; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.001; pH 6.8–7.0. This medium was additionally supplemented with 0.5 vol % yeast autolysate and 0.0006% calcium pantothenate. Ethanol at a concentration of 1.0 vol %, glucose at a concentration of 1.0%, or a mixture of these substrates (1 vol % ethanol + 1% glucose) served as the carbon and energy source.

A culture growing exponentially (16th–24th hour) in mineral medium of the above mineral composition (but containing 0.6 g/l NH_4NO_3) supplemented with 0.5 vol % ethanol served as the inoculum introduced in a dose of 5%

Bacteria were grown for 96 h in flasks on a shaker (220 rpm) at 30°C.

Isolation of native ethapolan. Ethapolan-containing culture liquid was dialyzed against distilled water for five days, diluted three- to fivefold with distilled water, and centrifuged to separate the producer cells (12000 g, 40 min). The supernatant was concentrated in a vacuum (50°C) to the initial volume, and ethapolan was precipitated by the addition of 1.5 volumes of iso-

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propanol. The EPS precipitate was washed in pure isopropanol and dried at room temperature.

Preparation of deacylated ethapolan. Sodium borane and solid NaOH were added to a 0.15% solution of native ethapolan (10 and 500 mg, respectively, per 100 mg EPS), and it was kept for 48 h at room temperature with periodical agitation. After neutralization with concentrated hydrochloric acid, fatty acids were removed from the solution by five times repeated extraction with hexane.

Deacylated ethapolan was precipitated with isopropanol from the water solution obtained after fatty acid extraction. EPS precipitate was dissolved in distilled water and dialyzed against distilled water for five days. This solution was concentrated in a vacuum, and deacylated EPS was precipitated with isopropanol. The precipitate was washed in pure isopropanol and dried at room temperature.

Analysis of the ethapolan chemical composition. The chemical composition of native and deacylated ethapolan (the contents of carbohydrates, pyruvic acid (PA), uronic acids (UA), fatty acids (FA), and individual monosaccharides) were determined as described previously [9].

To determine the content of mineral components (MC) of ethapolan, an aliquot of EPS (about 100 mg) taken on an analytical balance was dissolved in distilled water, and the KU-2-8 (H^+) cationite was added (1 g of resin per 100 mg of EPS). The cationite treatment was conducted until constant pH was attained, after which the cationite was separated by centrifugation (8000 *g*, 15 min), and the H^+-EPS was precipitated from the supernatant by the addition of two volumes of isopropanol, washed in pure isopropanol, dried at room temperature, and weighed on an analytical balance. The content of mineral components in ethapolan was determined from the difference between the weight of the initial EPS aliquot and the weight of H^+-EPS.

The relative contents of carbohydrates, PA, UA, FA, and MC in EPS were calculated per weight of arbitrarily dry substance, which was defined as the substance retaining its weight after drying in a vacuum at 40°C.

Determination of the EPS molecular mass. Analysis of the EPS molecular mass composition was performed by the method of analytical gradient centrifugation that we developed previously [10] and used evaporated EPS concentrates obtained by evaporation (without isopropanol precipitation and drying). Reference molecular masses of dextrans from Fluka were 13.5, 20, 40, 70, 110, 500 kDa and 2 MDa. The content of carbohydrates in the fractions obtained by gradient centrifugation was determined in the reaction with phenol and sulfuric acid [11].

The amount of components of a certain molecular mass was determined from the ratio of the amount of carbohydrates in the relevant fraction to the total (initial) amount of carbohydrates. The average molecular mass of EPS was calculated from the proportions of EPS fractions with particular molecular masses.

Rheological properties of ethapolan solutions were determined by measurement of their viscosity in the presence of 0.1 M KCl, at pH 4–4.5 (under conditions of EPS transformation in the H^+- form), as well as in a Cu^{2+}–glycine system, as described in [12]. The practical value of EPS depends on these rheological properties [5].

Rheological properties were determined in solutions (0.03% by carbohydrates) of ethapolan obtained at each of the four stages of its isolation and purification (culture liquid prior to dialysis, culture liquid after dialysis, evaporated concentrate, and the preparation obtained after precipitation with isopropanol and drying).

The criteria to evaluate rheological properties of an ethapolan synthesized under particular growth conditions were relative differences between the viscosities of its solutions measured under particular conditions and the viscosity of its solution in distilled water:

$$\text{Relative viscosity difference} = \frac{\eta_1 - \eta_0}{\eta_0} \times 100\%,$$

where η_1 is the EPS solution viscosity under certain conditions (in the presence of 0.1 M KCl, in the H^+-form, in a Cu^{2+}–glycine system) and η_0 is the viscosity of the EPS solution in distilled water. Viscosity was measured using an Ostwald glass capillary viscosimeter at 20°C.

The experimental data were treated statistically as described by Lakin [13]. Student's *t* test showed the results to be statistically significant at a 5% significance level.

RESULTS AND DISCUSSION

Table 1 shows the chemical composition of native and deacylated ethapolan synthesized on media with different carbon sources. The relative contents of carbohydrates, pyruvic acid, uronic acids, and mineral components in native EPS were little dependent on the growth substrate. The content of fatty acids was higher in ethapolan synthesized on a mixture of ethanol and glucose. The proportion of UA in all native EPS was as low as 7–8%. We have previously shown that uronic acids can be detected in native EPS only after polysaccharide deacylation [14].

Indeed, the relative content of UA in deacylated EPS was threefold higher than in the same preparation before deacylation (Table 1). After deacylation, the relative content of carbohydrates and PA in EPS also increased, whereas that of MC decreased significantly (six- to sevenfold).

In our opinion, an increase in the relative content of UA after EPS deacylation can be explained as follows. Fatty acid constituents of ethapolan promote structuring of ethapolan solutions caused by univalent cations

Table 1. Chemical composition of native and deacylated ethapolan synthesized on media with different carbon sources

Carbon source	Component content % of the weight of arbitrarily dry EPS								
	Native EPS					Deacylated EPS			
	Carbohydrates	PA	UA	FA	MC	Carbohydrates	PA	UA	MC
Ethanol + glucose	44.5 ± 0.5	3.6 ± 0.2	6.7 ± 0.2	9.4 ± 0.1	24.3 ± 0.4	60.0 ± 0.4	4.3 ± 0.2	22.2 ± 0.1	3.5 ± 0.2
Glucose	45.0 ± 0.5	3.8 ± 0.2	8.0 ± 0.2	6.4 ± 0.2	27.4 ± 0.4	56.0 ± 0.5	4.9 ± 0.1	25.5 ± 0.5	3.3 ± 0.2
Ethanol	43.2 ± 0.5	3.7 ± 0.2	7.7 ± 0.2	6.8 ± 0.2	28.6 ± 0.2	59.1 ± 0.5	4.4 ± 0.2	22.0 ± 0.2	3.9 ± 0.2

Note: PA, pyruvic acid; UA, uronic acids; FA, fatty acids; MC, mineral components.

of the growth medium [5, 15]. The content of MC in native ethapolan is about 24–29% (Table 1). When EPS interacts with cations, the polysaccharide conformation is presumably altered so that uronic acids become less accessible to various reagents. EPS deacylation leads to the dissociation of high-molecular EPS conglomerates, release of mineral components, and changes in the EPS spatial structure, which in turn abolishes the "blockage" of uronic acid residues. An analogous explanation may be valid for the increased relative content of PA and carbohydrates in deacylated EPS.

The monosaccharide compositions of the EPS synthesized on media containing ethanol, glucose, and the substrate mixture were similar (Table 2). The 3 : 2 : 1 : 1 ration was determined between glucose, mannose, galactose, and rhamnose in the EPS studied. We have previously shown that the monosaccharide composition of ethapolan remains unchanged under various growth conditions (when the producer was grown on media with different contents of univalent cations and after the addition of potassium fumarate to ethanol-containing medium) [12, 15, 16]. The results of the present study and our previous data are in agreement with data of other authors: the major carbohydrate chain of the polysaccharide remains, as a rule, unchanged under various growth conditions, whereas the side chains vary in their length, composition, number of branching points, and composition and nature of substituents [3–5].

Table 3 and Figure 1 show data on the molecular mass of ethapolan synthesized on media with ethanol, glucose, and on a substrate mixture. The average molecular mass of ethapolan synthesized on a mixture of ethanol and glucose was higher than that of ethapolan synthesized on single substrates (Table 3), because the content of high-molecular components (above 2 MDa) was increased in the EPS synthesized on the substrate mixture. The EPS obtained on single and mixed substrates also differed in the ratio of fractions with molecular masses from 13 to 500 kDa (Fig. 1). In EPS synthesized on a mixture of ethanol and glucose, the content of low-molecular fractions (below 110 kDa) was as low as 3 to 4.5%. In the EPS synthesized on single substrates, this value was almost twofold higher (Fig. 1).

Analysis of the rheological properties of the ethapolan obtained on media with different carbon sources showed that the solutions of EPS synthesized on the substrate mixture displayed higher viscosity in the presence of 0.1M KCl, in the H^+-form, and in a Cu^{2+}–glycine system (Fig. 2). Note that this was characteristic of the EPS at all stages of its isolation and purification.

We have previously shown that the rheological properties of ethapolan solutions (cation-induced structuring, increased viscosity at low pH and in a Cu^{2+}–glycine system, etc.) are determined by fatty acids, which are ethapolan constituents. Deacylated EPS do not exhibit the above properties. The rheological properties of ethapolan solutions depend on the relative content of fatty acids [5, 9, 12, 14–16]. Hence, the EPS solutions synthesized on a mixture of ethanol and glucose exhibit higher viscosity (in the presence of 0.1 KCl, in the H^+-form, and in Cu^{2+}–glycine) because of a higher percent-

Table 2. Monosaccharide composition of ethapolan synthesized on media with different carbon sources

Carbon source	Molar ratio			
	glucose	mannose	galactose	rhamnose
Ethanol + glucose	3.2	1.9	1.1	0.9
Glucose	3.0	2.1	1.0	1.0
Ethanol	3.1	2.0	0.9	0.9

Table 3. Molecular mass of ethapolan synthesized on media with different carbon sources

Carbon source	Average molecular mass, kDa	Fractions with molecular mass of less than 2 MDa, %
Ethanol + glucose	1691.3	22.2
Glucose	1289.0	38.3
Ethanol	1437.3	29.9

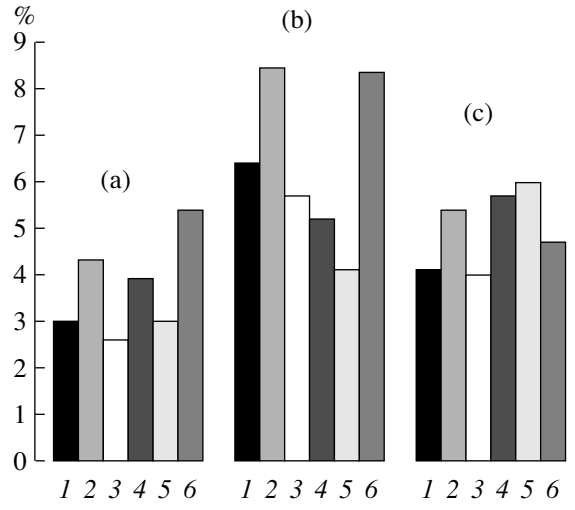

Fig. 1. Proportions of fractions with different molecular masses in ethapolan synthesized on (a) a mixture of ethanol and glucose, (b) on glucose, and (c) on ethanol. Molecular masses of fractions (kDa): *1*, 13.5; *2*, 20; *3*, 40; *4*, 70; *5*, 110; *6*, 500.

age of fatty acids in this EPS (Table 1). Note that the improved rheological properties of ethapolan obtained on the substrate mixture (as compared to EPS synthesized on single substrates) may also be explained by a higher molecular mass of the former EPS (Table 3). The ethapolan solution viscosity correlated with the relative content of high-molecular (above 2 MDa) fractions [5, 14, 15]. The small content of low-molecular (13.5 to 110 kDa) components in the EPS synthesized on the substrate mixture may also account for the high viscosity of the solutions of these EPS in the presence of KCl, in the H^+-form, and in the Cu^{2+}–glycine system (Fig. 1).

It has previously been shown that ethapolan is a complex polysaccharide preparation that includes neutral EPS (a minor component) and two acid EPS, one of which is acetylated [9, 12, 14–16].

The rheological properties of ethapolan solutions depend on the ratio of acetylated and nonacetylated EPS, as well as on the relative content of fatty acids in the acetylated polysaccharide [12, 14–16]. In this work, we analyzed the chemical composition and rheological properties of total ethapolan preparations without their separation into acetylated and nonacetylated components (this will be done in further studies). The EPS synthesized on the substrate mixture and single substrates are expected to differ in the ratio of acetylated and nonacetylated polysaccharides, as well as in the relative contents of fatty acids in acetylated EPS. This suggestion is supported by data on the molecular masses of the EPS synthesized on the substrate mixture and single substrates. We have previously shown that ethapolan with a higher relative content of the acylated component has a higher molecular mass [15]. In addition, we intend to elucidate why the relative content of

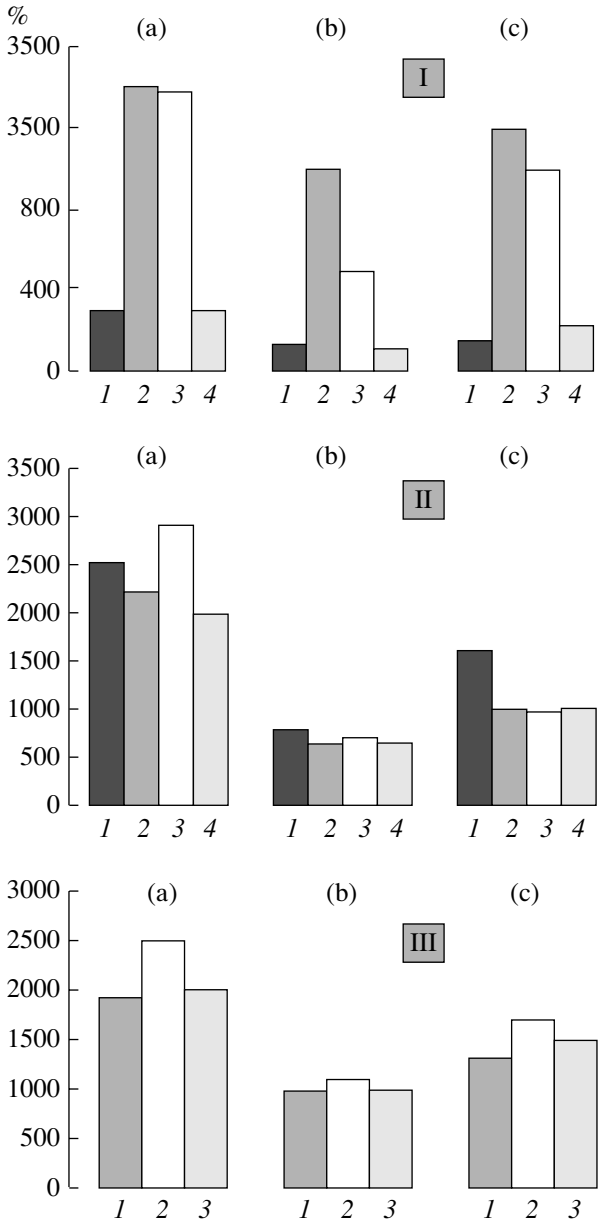

Fig. 2. Viscosity of 0.03% solutions of EPS synthesized (a) on a mixture of ethanol and glucose, (b) on glucose, and (c) on ethanol determined (I) in the presence of 0.1M KCl, (II) in a Cu^{2+}–glycine system, and (III) in the H^+-form. *1*, Culture liquid before dialysis; *2*, after dialysis; *3*, evaporated concentrate; *4*, EPS after precipitation and drying.

fatty acids is higher in ethapolan synthesized by the producer grown on the substrate mixture.

Thus, this study showed that the EPS synthesized on ethanol, glucose, and a mixture of these substrates are identical in the relative contents of pyruvic and uronic acids, mineral components, and in monosaccharide composition. The ethapolan obtained on the substrate mixture exhibited a higher relative content of fatty acids and higher average molecular mass, which was

accompanied by improved rheological properties of ethapolan solutions.

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