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Sulfation and depolymerization of a bacterial exopolysaccharide of hydrothermal origin

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Abstract

An unusual exopolysaccharide produced by *Alteromonas infernus*, a heterotrophic aerobic bacteria originating from a deep-sea hydrothermal vent, was chemically modified by sulfation and acidic depolymerization in order to promote biological activity and to evaluate its potential applications in the pharmaceutical area. Two experimental processes, sulfation prior to depolymerization (method 1) and depolymerization prior to sulfation (method 2), were applied to this high-molecular-weight bacterial polymer. Both methods led to the recovery of highly sulfated (33–40%) low-molecular-weight, low-viscous exopolysaccharides with yields ranging from 25 to 50%. Similar chemical compositions were obtained for all depolymerized fractions. These fractions are expected to show biological activity and their evaluation is in progress.

Keywords: Sulfation; Depolymerization; Bacterial exopolysaccharide

1. Introduction

In recent years there has been a growing interest in the isolation and identification of new marine microbial polysaccharides that might have applications in many sectors of the industry, e.g. in detergents, textiles, adhesives, paper, paint, food and beverage industries, pharmaceuticals and cancer therapy, cosmetics, oil recovery and metal recovery in the mining industry and from industrial waste and in the formulation of cell culture media.

For several years, interest in the recognition of biological activities of microbial polysaccharides has grown considerably (Weiner et al., 1995). Macromolecular substances such as polysaccharides have attracted attention from the viewpoint of their antitumor activity. The antitumor and immunostimulant activities of polysaccharides produced by marine bacteria *Vibrio* sp. and *Pseudomonas* sp. have been reported (Okutani, 1983; Matsuda et al., 1992). Dextran and dextran derivatives have been reported in pharmaceutical applications. Dextran sulfate has been stated to have both anticoagulant and antilipemic properties useful in the treatment of lipemia and arteriosclerosis. Antiviral effects of polysaccharides including human immunodeficiency virus activity have been recognized for many years (Baba et al., 1988). It is also well known that these activities are related to the high sulfate content of these polysaccharides. The anticoagulant activity of these polymers can also be linked to the high sulfate content associated with specific polysaccharides. The sulfate groups in heparin play a critical role in its antithrombotic activity (Petitou et al., 1983).

Evidence from different studies also suggests that the biological activity of polysaccharides is strongly dependent on the structure of the polymers, i.e. the nature of the sugar components, the links between these sugars along with their molecular weight (Bohn and BeMiller, 1995). Dextrans produced by *Acetobacter* spp. or *Leuconostoc mesenteroïdes* with average molecular weight $> 90\,000$ are good immunogens while dextrans of an average molecular weight $< 50\,000$ are non-immunogenic. Charge is another factor in the antigenicity of polysaccharides with the negatively charged moieties being immunodominant (Jann and Westphal, 1975). These include uronic acids, sulfate and phosphate groups (Goodman, 1984). Although phosphate and uronic acids along with pyruvate and O-acetyl groups can be part of the antigenic determinant, the specificity of the antibody is not always related to the ionic portion of the polysaccharide (Weiner et al., 1995).

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The aim of this study was the chemical preparation of highly sulfated low-molecular-weight exopolysaccharides (EPSs) secreted by a bacterium originating from a hydrothermal deep-sea environment for subsequent evaluation of its biological activities. The effect of sulfation prior to depolymerization, or vice versa, on the nature of the modified EPS was studied by chemical analysis.

2. Materials and methods

2.1. Strain origin

Microbial exopolysaccharide was obtained from a heterotrophic mesophilic aerobic bacterium isolated from a deep-sea hydrothermal vent (Raguenes et al., 1996). GY785 is a new deep-sea, aerobic, mesophilic and heterotrophic bacterium isolated from a sample of fluid collected among a dense population of *Riftia pachyptila* in the vicinity of an active hydrothermal vent of the Southern depression of the Guaymas basin (Gulf of California). On the basis of phenotypic and phylogenetic analyses and DNA/DNA relatedness, the strain GY785 is recognized as a new species of the genus *Alteromonas* and the name of *Alteromonas infernus* is proposed.

2.2. Production of the EPS

Exopolymer production was performed at 25°C in a 2-l fermenter (SGI-Inceltech, Toulouse, France) containing 1 l of 2216E-glucose broth. Batch was inoculated at 10% (v/v) with a suspension of cells in exponential phase. The pH was adjusted and maintained at 7.2 by automatic addition of a 0.25 mol l^{-1} sodium hydroxide solution. Foaming was avoided by addition of Pluronic-PE6100 oil (BASF, Levallois/Perret, France) at 0.1% (v/v). The air flow was fixed at 30 l h^{-1} and the agitation rate from 200 to 1100 rev min^{-1}. Bacterial growth was determined by measuring the culture turbidity at 520 nm. Viscosity of the culture broth was controlled with a Brookfield viscometer model DV II supplied with a small adapter (SC4-18/13R) at 25°C (Brookfield Engineering Laboratories, Stougthon, MA, USA).

2.3. Isolation, purification and characterization of the EPS

Exopolysaccharide was isolated from the culture medium after 60 h. Bacterial cells were removed from the medium by high speed centrifugation and the polymer precipitated from the supernatant with pure ethanol. After sequential washes with mixtures of ethanol/water, the resulting polysaccharides were desiccated under nitrogen and stored at room temperature. Detailed procedures related to extraction/purification procedures are reported elsewhere (Guezennec et al., 1994).

2.4. Sulfation of native and depolymerized EPS

Both the native high-molecular-weight and the depolymerized EPS (GY785) were chemically oversulfated according to the procedure of direct sulfation described for fucans by Nishino and Nagumo (1992). Approximately 0.5 g of the polymer was stirred with 50 ml of *N,N*-dimethylformamide (DMF) for 2 h at room temperature and then for 2 h at 45°C in order to disperse it into the solvent. A five times excess of sulfur trioxide pyridine complex (Pyr–SO_3) was added to the mixture and allowed to react for 2 h at 45°C. The reaction was stopped by adding 20 ml of water and the pH was adjusted to 9 with a 3 mol l^{-1} sodium hydroxide solution. The mixture was dialyzed against running water and then against distilled water. After lyophilization, the oversulfated product was recovered with a 100% yield. *N,N*-Dimethylformamide 99% and Pyr–SO_3 complex were purchased from ACROS Chimica and used as received.

2.5. Depolymerization of native and oversulfated EPS

The depolymerization of both native and oversulfated EPS was performed according a modified procedure described previously (Colliec et al., 1994). EPS (2 g) was dissolved in 400 ml of 1 mol l^{-1} H_2SO_4 solution preincubated at 60°C. The hydrolysed EPS was fractionated by gel filtration chromatography on a column (4.7 × 42 cm) of Sephacryl S-300 HR gel filtration medium (Pharmacia Biotech) with a flow rate of 5 ml min^{-1}.

2.6. Nomenclature of EPS fractions

Method 1 consisting of depolymerization of oversulfated EPS led to fractions referred to as A and B, while Method 2 where sulfation was carried out on the depolymerized EPS gave fractions named C and D.

2.7. Chemical analysis of EPS fractions

The total neutral carbohydrate content and hexuronic acids were determined using the orcinol–sulfuric method with the mannose–galactose (1:1) ratio as standard (Rimington, 1931; Tilmans, 1929) and the meta-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) respectively. Hesoxamines were analyzed using the Elson–Morgan method (Elson and Morgan, 1933). The molar ratios of monosaccharides were determined according to Kammerling et al. (1975) and Montreuil et al. (1986). The monosaccharide residues were analyzed after either aqueous acid hydrolysis or acidic methanolysis of the polymers and subsequent GC analyses as peracetylated derivatives or trimethylsilyl derivatives respectively. Protein contents were determined by the method of Lowry et al. (1953) with bovine serum albumin as standard. C, H, N, and S contents were determined with an elemental analyser

Table 1
Chemical composition of depolymerized oversulfated EPS: A, B, C and D fractions. For analytical methods see Section 2

Fractions	Rha (%)	Fuc (%)	Man (%)	Gal (%)	Glu (%)	GluA (%)	GalA (%)	Neutral sugars (%)	Uronic acids (%)	Sulfate (%)	Protein (%)
Native	ND	ND	ND	15 ± 1	16 ± 1	6.3 ± 0.5	11 ± 0.5	57 ± 4	42 ± 5	8.8 ± 4**	4 ± 0.6
Oversulfated	0.3 ± 0.11	0.3 ± 0.1	1.2 ± 0.4	6.8 ± 0.3	12.4 ± 0.9	2.2 ± 0.1	1.7 ± 0.2	28.7 ± 0.5	16.3 ± 0.4	37 ± 2.3* (40 ± 4**)	1.4 ± 0.6
A	0.83 ± 0.12	ND	0.6 ± 0.4	8.5 ± 1.1	11.4 ± 1.4	3.0 ± 0.4	1.77 ± 0.24	34.7 ± 2.3	20.3 ± 1.2	33.7 ± 2.5* (35 ± 4**)	0.6 ± 0.6
B	0.37 ± 0.12	0.4 ± 0.3	1.3 ± 0.4	7.0 ± 1.1	10.6 ± 1.4	3.1 ± 0.4	2.2 ± 0.24	34.3 ± 2.3	18.0 ± 1.2	32.8 ± 2.5* (38 ± 4**)	1.9 ± 0.6
C	0.7 ± 0.14	0.1 ± 0.4	0.5 ± 0.5	8.4 ± 1.3	11.2 ± 1.7	3.6 ± 0.5	2.25 ± 0.30	27.5 ± 2.8	18.5 ± 1.5	40.9 ± 3.1* (36 ± 4**)	0.3 ± 0.6
D	0.3 ± 0.14	0.4 ± 0.3	1.3 ± 0.5	6.2 ± 1.3	8.15 ± 1.7	2.95 ± 0.5	1.85 ± 0.30	26.5 ± 2.8	15.5 ± 1.5	42.6 ± 4.5* (33 ± 4**)	0.5 ± 0.6

ND, not detected.
Sulfate analysis: *, elemental analyser; **, FTIR.

(Perkin Elmer 2400). The sulfate content as the sodium salt was determined according to the following relation: sulfate group % = 3.22 × S%. The average molecular weight of native EPS was determined by using a Chromatix KMX6 light scattering detector. The measurements were carried out in 0.1 M NaCl solution. The depolymerized EPS molecular weights (number-average, M_n, weight-average, M_w, peak-molecular, M_p) and polydispersity, $I = M_w/M_n$ were determined by high-performance steric exclusion chromatography (HPSEC) according to a procedure previously described (Nardella et al., 1996).

2.8. Fourier transform infrared analysis

The sulfate content of native and depolymerized polysaccharides was also determined by Fourier transform infrared spectroscopy (FTIR) (Lijour et al., 1994). Pellets were obtained by carefully grinding a mixture of 2 mg of EPS with 200 mg of dry KBr. Infrared spectra were recorded on a BOMEM M100 Fourier transform infrared spectrometer with a resolution of 4 cm^{-1}

3. Results

All results are given within a 95% confidence interval based on a minimum of three replicates for each fraction.

3.1. Chemical composition of the native EPS

Neutral sugars and uronic acids accounted for 57 ± 4 and 42 ± 5% respectively (neutral sugars/uronic acids = 1.4). Sulfate contents were 9 and 11% (w/w) as determined by FTIR analysis and elemental analysis respectively. Successive washes with increasing amounts of pure ethanol in water led to a residual protein concentration of 4%. Galactose and glucose predominated as neutral monosaccharides accounting for 15 ± 1 and 16 ± 1% of the total sugars respectively while galacturonic and glucuronic acids accounted for 11 ± 0.5 and 6.3 ± 0.5% respectively. Nuclear magnetic resonance (NMR) studies indicated that no substituents as pyruvate or acetate were present in the chemical structure of the native polysaccharide.

An average molecular weight (M_w) of 10^6 g mol^{-1} was found for the water-soluble exopolymer. The intrinsic viscosity in 0.1 mol l^{-1} NaCl solution reached 290 ml g^{-1} (Raguenes et al., 1996).

3.2. Chemical composition of the modified EPS (method 1): A and B fractions

The sulfate contents of oversulfated native EPS were 40 and 37% as determined by FTIR analysis and elemental analysis respectively. Following depolymerization, fractions A or B fractions were not found to be significantly different from the native and oversulfated EPS (Table 1). Based on

Table 2
High-performance steric exclusion chromatography (HPSEC) characterization of depolymerized oversulfated EPS: A, B, C and D fractions

Fraction	M_p (g mol^{-1})	$I = M_w/M_n$	Yield (%)
A	92600 ± 8000	1.90 ± 0.15	46.8 ± 4.0
B	31000 ± 5000	1.80 ± 0.12	47.9 ± 5.5
C	73500 ± 16300	3.3 ± 0.61	27.5 ± 3.5
D	13200	2.1	24

the comparison of 12 paired sulfated samples before and after depolymerization, it was shown that for both fractions A and B, depolymerization caused a decrease of 5.0 ± 1.5% of the sulfate content, from about 38% down to 33% in the depolymerized moiety. The peak-molecular weights (M_p) were around 90 000 g mol^{-1} for fraction A and 30 000 g mol^{-1} for fraction B. These fractions were very homogeneous with a polydispersity ($I = M_w/M_n$) below 2. Both fractions A and B were obtained with a good yield (> 45%) as shown in Table 2.

3.3. Chemical composition of the modified EPS (method 2): C and D fractions

Two fractions C and D with low molecular weights were obtained by acidic depolymerization prior to sulfation (Table 1). The chemical composition of these fractions was very similar to those obtained by Method 1 (A and B fractions) and the neutral/acid sugars ratios were not found to be significantly different. The sulfate contents were 39 and 41% for C and 40 and 43% for D as determined by FTIR and elemental analysis respectively. The peak-molecular weights of fractions C and D were lower than those of fractions A and B, 70 000 and 13 000 g mol^{-1} respectively

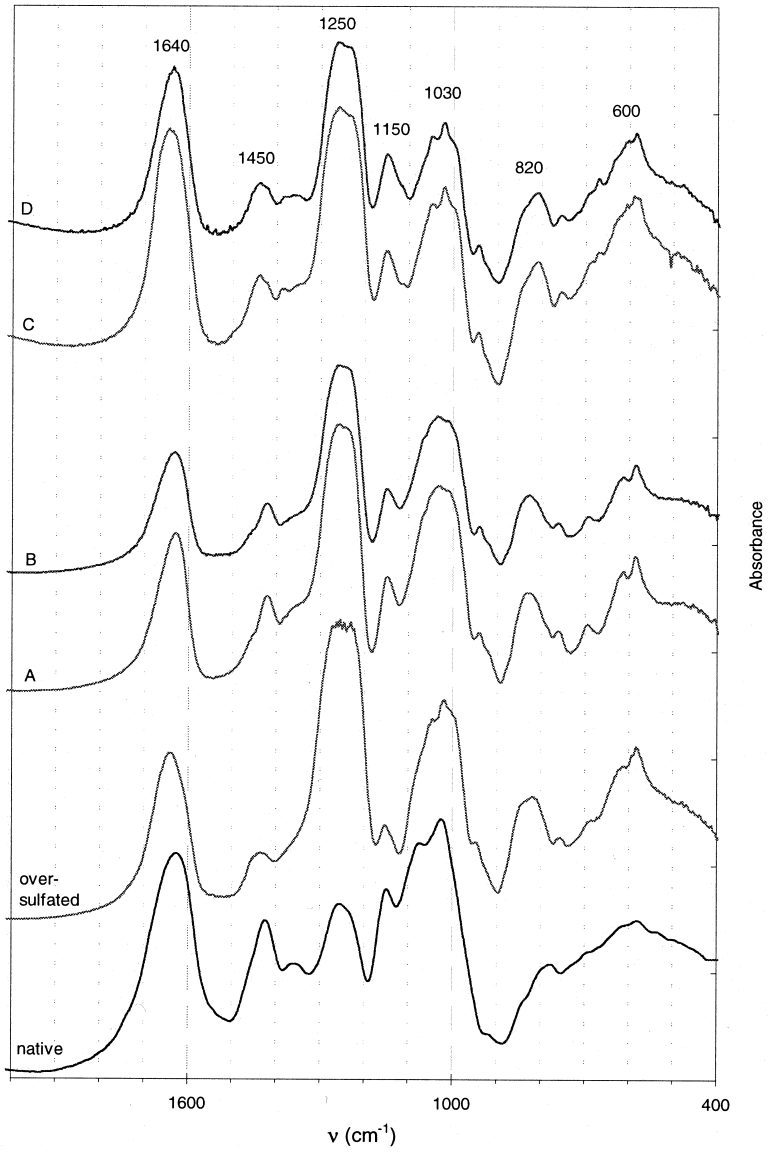

Fig. 1. FTIR spectra of native and highly sulfated exopolysaccharides.

(Table 2). The yields of fractions C and D were half of those fractions A and B (25% versus 50%). Both fractions exhibited low protein contents indicating a good degree of purity.

3.4. Infrared spectra

Fig. 1 showed the infrared spectra of the native EPS, the oversulfated native EPS and fractions A, B, C and D respectively. Besides the well-known carbohydrate bands between 1000 and 1200 cm^{-1}, ester sulfate bands were found at 1250, 820 and 600 cm^{-1}. Carboxylate bands were present at 1450 and 1610 cm^{-1} as shoulders to the 1640 cm^{-1} band representing the absorption of residual water. All spectra showed similar features, except for the 1250 cm^{-1} band whose intensity was used for the determination of the sulfate content.

4. Discussion

In the present work, the combination of sulfation using the Nishino and Nagumo method and acid hydrolysis depolymerization was applied to an unusual high-molecular-weight exopolysaccharide secreted by *Alteromonas infernus*, a heterotrophic aerobic bacteria originating from a hydrothermal deep-sea vent. Depolymerization of EPS can be performed by different methods including enzymatic digestion (Breddin, 1993; Desai et al., 1993) acidic hydrolysis and free radical processes (Volpi, 1994; Nardella et al., 1996; Ofman et al., 1997). Different sulfation methods have been described in the literature and some are assumed to be regioselective (Nishino and Nagumo, 1992; Bartolucci et al., 1995; Kovensky and Cirelli, 1996; Takano et al., 1996; Kovensky and Cirelli, 1997).

Both methods 1 and 2 were shown to be efficient methods to obtain oversulfated low-molecular-weight, low-viscous EPS fractions. The oversulfated product was very water soluble compared to native EPS so subsequent depolymerization and gel filtration fractionation steps were easier to perform and gave higher yields (ca. 50%). On the other hand, the depolymerization step in Method 2 gave poor yields (25%) owing to the low solubility of the native polymer even though a higher sulfate content was obtained (41%) for the two fractions. Thus, method 1, i.e. sulfation followed by depolymerization, applied to this particular exopolysaccharide seems to be the more convenient way to obtain the desired molecules.

Introducing sulfate groups can improve the biological activity of these polymers, but regioselectivity of the reaction is an important issue as the biological activity can be related to the position of the sulfate groups in the polymers. Chondroitin sulfate, mannans, dermatan sulfates, fucoidan (Colliec et al., 1994; Nardella et al., 1996) κ-carrageenan and heparin (Casu, 1994) amongst other highly sulfated polysaccharides have been shown to have important physiological functions as anti-blood coagulant substances or as regulator of growth factors, etc. The high sulfate content of up to 40% of the low-molecular-weight fractions along with the nature of the sugar units should give rise to original bioactive compounds. On the basis of the monosaccharide analyses, it can be suggested that these chemical modifications did not affect the overall composition or the monosaccharide ratios of all the fractions. However, without the complete structure of this polymer, it seems difficult to determine whether neutral sugars or uronic acids were more involved in the sulfation and which linkages were cleaved by acidic depolymerization. Furthermore, the influence of the sulfate groups on the release of the monosaccharides while the polymer is submitted to hydrolysis or TMS derivatization prior to gas chromatographic analysis can alter the monosaccharide determination (Selvendran et al., 1979; Takano et al., 1996).

Very promising data similar to those obtained with low-molecular-weight heparin were obtained with all depolymerized fractions. Molecular weights of fractions A and C (90 000 and 70 000 g mol^{-1} respectively) were higher than those of biologically active fractions from mannans or heparin while molecular weights of the two other fractions B and D (31 000 and 13 000 g mol^{-1} respectively) were in the range of these bioactive polysaccharides (Casu et al., 1994; Kolender et al., 1995). On the other hand, dextrans of molecular weight in the range of 75 000 $\pm$ 25 000 g mol^{-1} are used for pharmaceutical purposes as blood expenders. Extended acidic hydrolysis of this polysaccharide was shown to cause severe depolymerization without preserving the monosaccharide ratios. Hence, other preparative methods, primarily enzymatic depolymerization or free radical processes, are now being considered.

Knowing the exact structure of the native polymers and their modified fractions will be of a great interest in understanding the mechanisms involved in the chemical sulfation and depolymerization processes. In particular, knowing the position of the sulfate groups in the polymers is necessary to get a better insight into the structure–function relationships.

5. Conclusion

Highly sulfated low-molecular-weight EPS fractions were obtained from the combination of sulfation and acidic depolymerization of a complex exopolysaccharide of bacterial origin without altering the composition of this unusual polymer. Additional studies are under way to improve the yield of these chemical treatments and elucidate the complete structure of this original complex exopolysaccharide including the location of the sulfate group on the polymer.

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