Structure of an Extracellular Polysaccharide from a Strain of Lactic Acid Bacteria

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A new extracellular polysaccharide (EPS-I) isolated and purified from Z_{222}, a strain of Lactic acid bacteria has been investigated. Sugar composition analysis, methylation analysis and ^{1}H NMR and ^{13}C NMR spectroscopy reveal that the EPS-I is composed of a pentasaccharide repeating unit. The sequence of sugar residue was determined by using two-dimensional NMR spectroscopy, including heteronuclear multiple-bond correlation (HMBC) and nuclear overhauser effect spectroscopy (NOESY).

Keywords lactic acid bacterium, extracellular polysaccharide, structure

Introduction

Polysaccharides are a highly diverse group of polysaccharides, of which the functional features are determined by their structural characteristics that may differ in molecular weight, glyco-linkage type, degree of branching and chemical composition. This diversity has led to very broad application possibilities in industry, including paper manufacture, oil recovery and food production. Currently, most of the polysaccharides used in food-industry are derived from plants (*e.g.* alginate, starch, pectin, cellose) and seaweeds (*e.g.* alginate, carrageenan). Alternative sources of biothickeners were found among the microbial extracellular polysaccharides,[1] which can either be present as capsular polysaccharide (CPS) associated with the cell surface or secreted as extracellular polysaccharide (EPS) in the environment of the cell. In terms of application in food products, one of the most important examples of this class of bacterial polysaccharides is xanthan, which is produced by phytopathogenic bacterium *Xanthomonas campestris*. Despite the fact that *X. campestris* is not an acceptable bacterium in food products (food-grade), the rheological properties of xanthan have led to its wide application as food-additive. Preferentially, bacterial polysaccharides to be used as additives in food products should be produced by nonpathogenic, "safe" bacteria. Lactic acid bacteria (LAB) in general have a "food-grade" status and EPSs produced by these bacteria can be considered as "food-grade" additives. Therefore, EPS production by LAB has received considerable attention since they appear to be relevant for dairy-product properties like texture and mouthfeel.[2] Moreover, it had been suggested that they are active as prebiotic,[3] cholesterol lowering neutraceutical[4] or immunomodulant.[5,6] To establish correlation between EPS structure and functionalities, structural studies were performed on EPSs produced by various species of the lactic acid bacteria.[7]

In this paper the structural elucidation of **EPS-I**, which is a new extracellular polysaccharide isolated from a strain of Lactic acid bacteria (Z_{222}) preserved by our laboratory, is reported.

Experimental

Bacterial and cultural condition

Z_{222} was preserved at −20 ℃, and it was subcultured twice in the Man-Rogosa-Sharge's changed medium (MRSC) broth[8] at 37 ℃ before use. The liquid medium used for the production of extracellular polysaccharide was fermented at 37 ℃ for 24 h with 1% inoculum.

Instruments

The experimental instruments are as follows: CM-cellulose column chromatography, DEAE-Sephadex A-25 ionexchange and Sephadex G-100 gel chromatography (Parmacia), Dyna Pro-Ms Tc Temperature-Controlled DLS Instrument (Protein Solutions Inc.), QP 5000 spectrometer (Shimadzu) equipped with OV-17 capillary column (0.3 mm × 25 m), INOVA 600 MHz instrument (Varian).

Isolation, purification and molecular weight determination of **EPS-I**

Z_{222} was cultured statically in the Man-Rogosa-

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Sharpe's changed medium (MRSC) at 37 ℃ for 24 h. After removal of the cells by centifugation (8,000 r/min, 10 min), exopolysaccharide was precipitated by adding an equal amount of cold ethanol at 4 ℃ overnight. The precipitate was redissolved in water and clarified at 10,000 rpm for 10 min. Above procedure of ethanol precipitation was repeated three times. The crude preparation of exopolysaccharide was then purified by CM-cellulose column chromatography, DEAE-Sephadex A-25 ionexchange and Sephadex G-100 gel chromatography using acetate buffer (0.05 mol/L, pH 5.0), phosphate buffer (0.02 mol/L, pH 7.4) and acetate ammonium (0.05 mol/L), respectively. The non-absorbed exopolysaccharide fractions thus obtained was lyophilized after dialysis against distilled water at 4 ℃ for 72 h[9,10] to give **EPS-I**. **EPS-I** (5 mg) was dissolved in distilled water (500 μL) and its molecular weight was determined by the method of light scattering.

Sugar composition analysis

For sugar composition analysis, **EPS-I** (5 mg) was hydrolysed with trifluoroacetic acid (2 mol/L) for 10 h at 100 ℃. The monosaccharides were conventionally converted into the alditol acetates[11] and analyzed by GC, at a temperature programmed from 140 ℃ to 200 ℃ at 5 ℃/min, then increasing to 300 ℃ at 10 ℃/min.

Methylation analysis

EPS-I (5 mg) was dried at 70 ℃ under vacuum for 30 min in reaction vial (5 mL). After addition of dimethyl sulfoxide (1.5 mL), flushing with nitrogen and sealing, the vial was sonicated for 1 h at 40 ℃ in ultrasonic bath. After cooling at room temperature, 1,1′,3,3′-tetramethylurea (0.25 mL) and dimly sodium[12] (1.5 mL) was added with a syringe and the vial was sonicated for 5 h at 20—25 ℃. After cooling in an ice-bath (until frozen), methyl iodide (1.5 mL) was added dropwise using a syringe. Sonication was continued for 30 min after the reaction mixture was allowed to thaw and then left at room temperature overnight. Then, the resulting mixture was dialysis and lyophilized.[13] This process was repeated twice until the infrared spectrum of this product had no absorption at 3200—3700 cm^{-1}. After hydrolysis with 90% formic acid (2 mL) for 4 h at 100 ℃ and with 2 mol/L trifluoroacetic acid (2 mL) for 6 h at 100 ℃, the partially methylated monosaccharides were reduced with sodium borohydride (100 mg), followed by acetylation.[11] The partially methylated alditol acetates were analysed by GC-MS.

NMR *spectroscopy*

NMR spectra of an **EPS-I** solution in 99.96% D_2O (30 mg/0.5 mL) were obtained at 50 ℃. Chemical shifts were reported using tetramethylsilane (TMS) as internal reference. ^1H-^1H correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY) and heteronuclear multiple quantum correlation spectroscopy (HMQC) were used to assign signals. For inter-residue linkage and sequence, two-dimensional nuclear Overhauser effect spectoscopy (NOESY) experiments with mixing time 600 ms and heteronuclear multiple-bond correlation spectroscopy (HMBC) experiments were used.

Results and discussion

Isolation, purification and molecular weight determination

EPS secreted by Z_{222} in MRSC broth was isolated by ethanol precipitation, dialysis and lyophilization. Further purification was carried out by cellulose, ionexchange and gel chromatography. There is a single peak in the elution of **EPS-I** from a size-exclusion-chromatography column, indicating the homogeneity of **EPS-I** and free from low-molecular-weight polysaccharides. The molecular weight of the **EPS-I** is 42 kDa, which was determined by using the method of the light scattering.

Repeating unit composition

The result of sugar composition analysis indicates that polysaccharide is composed of glucose and mannose in ratio of 1∶4. The results of the methylation analysis (Table 1) shows that **EPS-I** is composed of five different linked residues in combination with 1D ^1H NMR and ^{13}C NMR spectra, indicating that **EPS-I** has a pentasaccharide repeating unit and it is branched.

A large number of 2D-spectra of sample were recorded on ∶2D-DQCOSY, 2D-TOCSY, 2D ^{13}C-^1H HMQC, ^{13}C-^1H HMBC and 2D NOESY using INOVA 600MHz instrument. The ^1H NMR spectrum of **EPS-I** is consistent with a pentasaccharide repeating unit with five proton resonances observed in the region for anomeric proton at δ 5.69, 5.60, 5.47, 5.38 and 5.34. The ^{13}C NMR spectrum of **EPS-I** is also consistent with a pentasaccharide repeating unit with five anomeric carbon resonances observed in the region at δ 98.1, 98.7, 100.2, 96.5 and 100.5. From the methylation analyses together with NMR spectra, it is evident that the sugars are pyranoid. The five monosaccharide units **A**—**E** were labled according to decreasing chemical shift values of their anomeric protonds. Combining assignments of 1D-resonances, ^1H and ^{13}C, 2D-spectra and the methylation analysis data, the protons and carbons of individual monosaccharides (Table 2) are assigned based on interpretations of their correlations.

The ^1H chemical shifts of **EPS-I** were assigned by means of TOCSY, DQCOSY and NOESY. Staring points for the interpretation of the spectra were the anomeric signals of residues **A**—**E**. Homonuclear COSY gave the chemical shifts of H-2 signals for residues **A**—**E**. Using ^1H-^1H TOCSY experiments, H-3—H-6 resonances for residue **A**, H-3 H-5 resonances for residue **B**, H-3 for residue **C**, as well as H-3, H-5 and H-6 resonances for residue **D** were assigned. In addition, the magnetization

Table 1 GC-MS data for partially methylated alditol acetated

Retention time (min)	MS ions (m/z)	Compound	Sugar residue	Molar ratio
11.88	101, 129, 161, 145, 117, 87, 71, 205	1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol	Glc_p(1	1
13.17	129, 43, 87, 189, 71, 101, 145, 233	1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol	2) Man_p(1	1
13.52	117, 233, 101, 87, 71, 129, 173, 189	1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol	4) Man_p(1	1
13.59	129, 117, 101, 161, 71, 233, 201	1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol	3) Man_p(1	1
14.86	129, 43, 87, 189, 233	1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methylhexitol	2,6) Man_p(1	1

Table 2 Chemical shift of the ^1H NMR and ^{13}C NMR signals of **EPS-I**

Sugar residue		Chemical shift (δ)					
		1	2	3	4	5	6
A α-*D*-Glc_p(1→	^1H	5.69	3.96	4.16	3.75	4.02	4.28
	^{13}C	98.1	69.8	68.8	67.1	65.2	58.9
B →2) β-*D*-Man_p(1→	^1H	5.60	4.44	4.33	4.24	4.05	4.12
	^{13}C	98.7	76.4	64.2		64.7	~59.3[a]
C →3) β-*D*-Man_p(1→	^1H	5.47	4.39	4.22	4.08	4.00	
	^{13}C	100.2	68.7	77.2	~71.5[a]	65.2	
D →2,6) β-*D*-Man_p(1→	^1H	5.38	4.34	4.16		4.28	4.10
	^{13}C	96.5	76.7	~69.5[a]		76.1	64.4
E →4) β-*D*-Man_p(→1	^1H	5.34	4.54	4.09	3.95		
	^{13}C	100.5	~68.3[a]	71.5	75.5		

[a] ~ , symbol used to identify partially overlapping carbon resonances.

transferred to H-3, H-4 and H-5 only for residue **A**, while the magnetization could not be transferred to H-3, H-4 and H-5 for residue **C—E** because H-2 of mannose is located in *trans*-bond, and residues **A** therefore could be assigned to the Glc_p residue and the others could be assigned to the Man_p residue.

From the ^{13}C-^1H HMQC spectrum the carbon chemical shifts corresponding to the protons for residues **A—E** could be assigned. The downfield chemical shifts for the C-2 signals, δ 76.4 and δ 76.7, of residues **B** and **D**, as well as C-6 signals, δ 76.1, of **D**, compared with β-*D*-Man_p, demonstrated that these were 2-substituted and the branch of **D** was 6-substituted in accordance with methylation analysis. Residue **C** was assigned to 3-substituted mannose supported by the downfield chemical shift of the C-3 signal, δ 77.2, compared with β-*D*-Man_p. From the chemical shifts of residues **A** compared with α-*D*-Glc_p and weak signal in NMR it is suggested that this residue is terminal. Thus, the residue **E** was deduced to be 4-substituted in accordance with methylation analysis.

Glyco-linkage and residue sequence

The assignments of inter-residual cross-peaks in the 2D NOESY spectrum and long-range couplings in the HMBC spectrum allowed to determine the sequence and the linkage positions of the residues with the repeating unit of **EPS-I**. Inter-residue correlations in NOESY and HMBC were listed in Table 3. The NOESY spectrum shows that residue **B** has an inter-residue correlation between the anomeric proton and a signal at δ 4.34 corresponding to H_{D2}, while **D** residue has an inter-residue correlation between the anomeric proton and a signal at δ 4.44 corresponding to H_{B2}. These linkages were confirmed by a long-range ^1H -^{13}C coupling between H_{B1} and C_{D2}, H_{D1} and C_{B2}, observed in the HMBC spectrum. It is suggested that residue **B** and residue **D** are the adjacent residues and mainly as **B**(1→2)**D** linkage of a straight chain of polysaccharide. The NOEs between H_{C1} and H_{D6}, and H_{E1} and H_{C3} were both observed, suggesting a **C**(1→6)**D** linkage and a **E**(1→3)**C** linkage. These linkages were also confirmed by a long-range ^1H -^{13}C coupling between H_{C1} and C_{D6}, H_{E1} and C_{C3}, observed in the HMBC spectrum. The terminal residue **A** was deduced to link with **E**.

In addition, an NOESY experiment on **EPS-I** revealed an H-1/H-2 intra-residue correlation for all units, but **A** is glucose and **B**, **C**, **D** and **E** is mannose, respectively, thus indicating that the former is α-linked of *D*-glucose and the latter is β-linked of *D*-mannose. The absolute configuration of **EPS-I** is *D* for all absolute configuration of glucose and mannose of exopolysaccharide secreted by lactic acid bacterium is the same type.

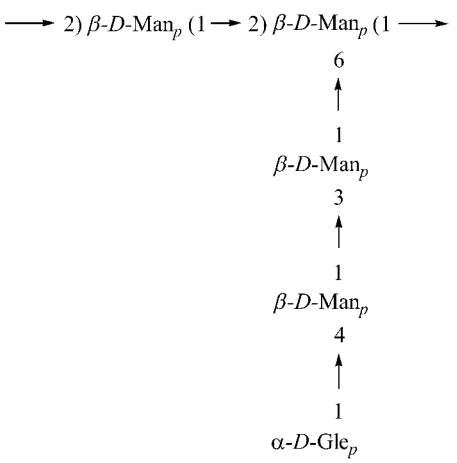
Table 3 Inter-glycosidic correlations from the anomeric protons observed in ^{1}H-^{1}H NOESY and ^{1}H-^{13}C HMBC spectra of **EPS-I**

Sugar residue	δ_H		δ_C
	Anomeric proton	NOESY	HMBC
A α-*D*-Glcp (1→	5.69	nd[a]	nd[a]
B →2) β-*D*-Man$_p$(1→	5.60	4.34 (H_{D2})	76.7 (C_{D2})
C →3) β-*D*-Man$_p$(1→	5.47	4.53 (H_{D6})	68.3 (C_{D6})
D →2,6) β-*D*-Man$_p$(1→	5.38	4.44 (H_{B2})	76.4 (C_{B2})
E →4) β-*D*-Man$_p$(1→	5.34	4.22 (H_{C3})	77.2 (C_{C3})

[a] nd denoted that the signal was not determined.

Conclusion

Based on sugar composition analysis, methylation analysis, MS and 1D/2D NMR studies on **EPS-I**, the primary structure of excellular polysaccharide produced by Z_{222} was shown to be build-up from the following pentasaccharide repeating units:



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