A Novel Moisture-Absorbing Extracellular Polysaccharide from Rhodococcus rhodochrous SM-1

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An extracellular polysaccharide (EPS) prepared from SM-1, a mucoidal mutant of Rhodococcus rhodochrous ATCC 12674 was found to absorb and retain moisture in both dry and high-temperature environments. The EPS absorbed more than 120% and 17% of its weight in moisture under the conditions of 32% and 11% relative humidity, respectively, representing much higher absorption than that of known moisture absorbents such as silica gel, glycerol, and hyaluronic acid. The SM-1 EPS is an acidic polysaccharide containing D-galactose, D-glucose, L-fucose, and D-glucuronic acid at a molar ratio of 6: 3: 2: 4. In addition, 1.2% (W/W) stearic acid, 2.3% (W/W) palmitic acid, and 10.3% (W/W) pyruvic acid were also contained in the SM-1 EPS. These evidences indicate that the SM-1 EPS is a novel moisture-absorbing biopolymer.

INTRODUCTION

Rhodococcus is a genus of gram-positive bacteria with a high (G+C) content in its DNA, and contains mycolic acids, i.e., long-chain α-alkyl-β-hydroxy fatty acids, as a major component of its cell wall. The Rhodococcus bacteria exhibit a wide variety of metabolic activities1, 2). Some of them have the ability to degrade a diverse range of organic compounds, including man-made xenobiotics such as polychlorinated biphenyls,3) whereas others are capable of degrading numerous aliphatic and aromatic hydrocarbons.4, 5) Rhodococcus rhodochrous J1 nitrile hydratase has been employed for the industrial production of acrylamide.6) Biodesulfurization of dibenzothiophene by some rhodococci has been extensively studied, and these organisms have considerable biotechnological potential, such as in the biodesulfurization of crude oil and in arylsulfonate desulfonation in bioremediation.7)

We have been studying the functions of extracellular polysaccharide (EPS) produced by Rhodococcus species, and recently showed that their EPS reduced the cell surface hydrophobicity and changed the behavior of bacteria in various environments.8) Recently, we reported that the EPS produced by Rhodococcus rhodochrous S-2 protected the bacterial cells from the toxic effect of n-hexadecane9) and that its addition to oil-contaminated seawater resulted in the increased degradation of polyaromatic hydrocarbons (PAH) of oil and the domination of a PAH degrader, Cycloclusticus sp., in the samples,10 suggesting the possibility of its application to bioremediation of oil-contaminated environments.

During our research, we measured contact angles (CA) to determine cell-surface hydrophobicity, and found that by the standard procedure we obtained overestimated values for cells surrounded by loosely bound material (LBM) that could be easily removed by physical impact, such as by stirring. To determine surface hydrophobicity of such cells we invented the “LBM overlay method.”8) With this method, bacterial lawns were overlaid with the EPS, which was capable of retaining humidity for more than several months, suggesting a high moisture-retention capacity of the EPS. By contrast, those prepared according to the standard procedure dried up and cracked within 1 hour.

In the present study, we examined the moisture-retention capacity, moisture absorption capacity, and physicochemical properties of SM-1 EPS as a candidate of novel moisture-absorbing biopolymers produced from a renewable raw material.

MATERIALS AND METHODS

Bacterial strain and medium

The mucoidal mutant SM-1, derived from Rhodococcus rhodochrous ATCC 12674 by UV-mutagenesis (Iwabuchi et al., unpublished data), was cultivated on IB agar plates9).

Extraction and purification of SM-1 EPS

Cells grown on IB agar plates were suspended in MilliQ water. The cell suspension was shaken at 120 rpm for 10 min at 25°C and subsequently centrifuged at 10,000 × g for 10 min. The resulting supernatant was treated with DNase I (7 units/ml; Takara Bio Inc., Otsu, Japan) and RNase A (2 µg/ml; Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 37°C for 16 hr. Subsequently, the solution was treated with proteinase K (2 µg/ml; Wako Pure

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Chemical Industries, Ltd., Osaka, Japan) at 37°C for 2 hr, and the sample was purified by phenol/chloroform treatment and ethanol precipitation. After dialysis against MilliQ water, the samples were lyophilized.

**Examination of homogeneity of the SM-1 EPS**

The SM-1 EPS (40 mg) was suspended in 10 mM Tris-HCl (pH 8.0) at the concentration of 1 mg/ml and applied to DEAE-Toyopearl 650M column chromatography (250 mm × 25 mm; Tosoh Co., Tokyo, Japan). After washing the column with 120 ml of 10 mM Tris-HCl (pH 8.0), the EPS was eluted with a 600-ml linear gradient (0 – 1 M) of NaCl in 10 mM Tris-HCl (pH 8.0). Fractions containing EPS were monitored by the phenol-sulfuric acid method, combined, dialyzed against MilliQ water, and lyophilized for analysis by cellulose acetate membrane electrophoresis.

Cellulose acetate membrane electrophoresis was performed according to the method of Seno et al., using 0.2 M barium acetate (pH 7.5) as an electrophoresis buffer, 0.5% Toluizine blue as a stainer and MilliQ water as a destainer.

Gel filtration column chromatography was performed using Sephacryl S1000 (850 mm × 15 mm; Amersham Biosciences UK Ltd., Buckinghamshire, UK) as a resin, 1 mM Tris-HCl (pH8.0) containing 0.1 M NaCl as an eluate, and Dextran T2000 (Amersham Biosciences UK Ltd., Buckinghamshire, UK) as a size marker.

**Moisture absorption and retention capacities**

Moisture absorption and retention capacities of SM-1 EPS were measured at 37°C under 2 different relative humidities (RH; 32% and 11%) using desiccators and the sample was purified by phenol/chloroform treatment and ethanol precipitation. After dialysis against MilliQ water, the samples were lyophilized.

The vessels were left for 24 hr in the desiccators, and then weighed again. For determination of the moisture absorption capacity, the vessels containing the dried samples were kept in the desiccators for 24 hr and then weighed again. The moisture absorption capacity was calculated as follows:

\[
\text{Moisture absorption capacity (\%)} = \left( \frac{\text{Sample weight after 24 hr} - \text{Initial sample weight}}{\text{weight of the added water}} \right) \times 100
\]

For measurement of the moisture retention capacity, the vessels containing dried samples were weighed again after addition and mixing of MilliQ water (20% of EPS weight). The vessels were left for 24 hr in the desiccators, and then weighed. The moisture-retention capacity was calculated as follows:

\[
\text{Moisture-retention capacity (\%)} = \left( 1 - \frac{\text{Sample weight after 24 hr}}{\text{Initial sample weight}} \right) \times 100
\]

Water absorption capacity was determined by the “tea-bag method” as described in Japanese Industrial Standards (JIS) K7223, except that the tea bags were made of non-woven fabric (Kitchen Tauper, Tokai Pulp Co., 100% natural pulp) instead of nylon membrane.

Hyaluronic acid sodium salt from cockspur was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan); and glycerol, silica gel, and urea were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Neutral sugar and uronic acid analysis**

SM-1 EPS was hydrolyzed as follows: Ten milligrams of SM-1 EPS were dissolved in 0.5 ml of chilled 80 % H₂SO₄, and incubated on ice-cold water bath for 30 min. The mixture was then incubated at 30°C for 3 hr. After treatment with 80 % H₂SO₄, the mixture was diluted with 6.5 ml of chilled MilliQ water to a final concentration of 2N H₂SO₄. The mixture was incubated at 100°C for 2 hr, and then neutralized by the addition of 0.8 g CaCO₃. The reaction mixture was then filtered to remove the precipitate. The filtrate was dried and redissolved in MilliQ water, and then subjected to Amberlite IR-120 chromatography to remove the calcium ions. The hydrolysates were analyzed by gas liquid chromatography (GLC) as follows: Prior to GLC analysis, uronic acids and neutral sugars were separated by Dowex 1-X8 anion-exchange chromatography. Trifluoroacetylalditol derivatives of neutral sugars and trimethylsilyl derivatives of uronic acids were analyzed by GLC (Shimadzu GC-6A; Shimadzu, Kyoto, Japan) using a DC QF-1 column and an NPGSE column, respectively (Imanari et al., 1969; Perry and Hulyalkar, 1965). A mixture of L-arabinose, L-fucose, D-galactose, D-glucose, D-mannose, L-rhamnose, D-xyllose, D-galacturonic acid, and D-glucuronic acid (1 mg / ml each) was treated as described above and then employed as a standard.

**Fatty acid analysis**

SM-1 EPS (3 mg) was hydrolyzed at 100°C for 90 min in 3 ml methanol containing 10% KOH. After extraction with n-hexane the hydrolysate was acidified by the addition of HCl, and then the fatty acids were extracted with n-hexane. The fatty acids were dried in vacuo and then methylated using trimethylsilyldiazomethane reagent (Nakai, I. Toke, NY, Japan) according to the supplier’s instructions. The samples were subsequently subjected to gas chromatography/mass spectrometry (GLC-MS) analysis. The GLC-MS analysis was performed with a QP-5050 instrument (Shimadzu, Kyoto, Japan) fitted with a fused silica capillary column (DB-1, 30 m × 0.25 mm); Agilent Technologies, Palo Alto, CA, USA). The operating temperature of the injection port and the interface was 300°C. The column temperature was set at 150°C for 2 min and increased to 300°C at the rate of 10°C/min.

**Analytical methods**

Reducing sugar content was determined by the phenol-sulfuric acid method. Pyruvic acid content was enzymatically determined with an F-kit Pyruvic acid (J. K. International Co., Tokyo, Japan) as described by the sup-
plier after hydrolysis with 2% trifluoroacetic acid (TFA) at 100°C for 120 min.

SM-1 EPS was dissolved in 0.15 M Tris-HCl (pH 7.4) at 1 mg/ml, and its reduced viscosity was determined using an Ostwald viscosity meter No. 1 at 27°C.

RESULTS

Purification of SM-1 EPS

EPS produced by the mucoidal mutant SM-1 of Rhodococcus rhodochrous ATCC 12674 (rough strain) was extracted, purified and examined for homogeneity by cellulose acetate membrane electrophoresis, ion-exchange chromatography, and gel-filtration chromatography. SM-1 EPS was applied to a DEAE-Toyopearl column and eluted with a linear gradient (0 - 1 M) of NaCl. A typical elution profile is shown in Fig. 1. The SM-1 EPS gave a single peak at approximately 0.3 M NaCl. A single band was also detected by cellulose acetate membrane electrophoresis of SM-1 EPS either before or after the DEAE ion-exchange chromatography (Fig. 2). The SM-1 EPS was eluted as a broad single peak earlier than Dextran T2000 (Fig. 3), suggesting its apparent molecular weight to be greater than 2,000,000. These data indicate that the SM-1 EPS had been purified to homogeneity.

Characterization of moisture-retention and absorption capacities of SM-1 EPS

The EPS retained 57% of its initial moisture (which was equivalent to 20% of EPS weight) after 24 hr under dry (11% relative humidity, RH), high-temperature (37°C) conditions (Table 1). Its moisture-retention capacity was much higher than that of other moisture retainers, such as hyaluronic acid and glycerol. Twenty four hours in the 32% RH desiccator resulted in an increase of 1.2 times in the weight of SM-1 EPS, indicating that SM-1 EPS was capable of not only retaining the initially added water but also absorbing moisture in the desiccator.

The EPS absorbed more than 120% and 17% of its initial weight in moisture under dry (32% and 11% RH, respectively), high-temperature (37°C) conditions (Table 2). This absorption capability was not only much higher than that of the other moisture absorbents tested, such as silica gel and hyaluronic acid, but also higher than those of

Table 1. Moisture retention capacity of SM-1 EPS

<table>
<thead>
<tr>
<th>Relative humidity 32%a</th>
<th>Relative humidity 11%b</th>
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<tbody>
<tr>
<td>SM-1 EPS</td>
<td>&gt; 100b</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>100 ± 24</td>
</tr>
<tr>
<td>Urea</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>52 ± 5.0</td>
</tr>
<tr>
<td>Silica gel</td>
<td>76 ± 0.7</td>
</tr>
</tbody>
</table>

a: Values were calculated according to the equation described in the Materials and Methods.
b: Weights of SM-1 EPS samples were greater than their initial weights.
c: NT, not tested.
the bioabsorbent produced by *Alcaligenes latus* B16 (as reported by Kurane and Nohata).\(^{14}\)

The SM-1 EPS efficiently absorbed 24 times and 18 times its own weight in MilliQ water and 0.9% NaCl, respectively, which absorbency values are much higher than those of conventional natural materials such as cellulose. However, the water absorption of SM-1 EPS is much less than those of the widely used petrochemical polymeric absorbents such as polyacrylate polymers or that of the bioabsorbent produced by *A. latus* B16.\(^{14}\)

These data indicate that SM-1 EPS is a quite efficient moisture retainer and absorbent.

**Physicochemical properties of SM-1 EPS**

SM-1 EPS is a white fibrous material soluble in water and alkalis but not in acids, methanol, ethanol, or acetone. Spectrophotometrically, absorption was detected neither at 280 nm nor at 255 nm, suggesting that SM-1 EPS does not contain proteins or nucleic acids. Its aqueous solution was viscous, and its reduced viscosity was determined as 3.64 dl g\(^{-1}\) using an Ostwald viscosity meter.

The lyophilized SM-1 EPS was hydrolyzed by sulfuric acid, and its neutral sugars and uronic acids were analyzed by GLC. Consequently, D-galactose, D-glucose, and L-fucose were detected in the neutral sugar fraction of the SM-1 EPS (Fig. 4 C), and glucuronic acid was found in the uronic acid fraction (Fig. 4 D). Based on these data, the proportions of the constituent sugars (% W/W) were calculated to be the following: D-galactose, 35.3%; D-glucose, 17.9%; L-fucose, 11.5%; and D-glucuronic acid, 26.3%.

Fatty acids were extracted from the alkali-hydrolysate of SM-1 EPS, but not from untreated SM-1 EPS, suggesting that the fatty acids are bound to EPS by ester bonds. According to the GLC analysis of methylated fatty acids, SM-1 EPS contained 1.2% (W/W) stearic acid and 2.3% (W/W) palmitic acid. Pyruvic acid was detected in the hydrolysate of SM-1 EPS obtained with 2% TFA, and its content was calculated to be 10.3% (W/W).

**DISCUSSION**

Physicochemical analysis indicated that SM-1 EPS was an acidic polysaccharide containing 3 kinds of neutral sugars (64.7% W/W), uronic acid (26.3% W/W), two kinds of fatty acids (3.5% W/W), and pyruvic acid (10.3% W/W), yielding a sum of their contents of 105%. The molar ratio of neutral sugars and uronic acid was estimated as follows: D-galactose: D-glucose: L-fucose: D-glucuronic acid = 6:3:2:4. A research group led by J. C. Richards reported on the chemical structures of EPS produced by 5 different serotypes of the equine pathogen *R. equi*.\(^{17–21}\) They noted the presence of pyruvic acid in its acetal form in EPS of serotypes 1, 3, 4, and 7, but they did not mention the presence of fatty acids.

The apparent molecular mass of EPS is more than 2 \(\times 10^6\) daltons. Highly efficient water-absorbing petrochemical polymers may be characterized chemically as (1) polyelectrolytes, (2) cross-linked, and (3) polymeric.\(^{22}\) SM-1 EPS is an acidic polysaccharide and contains glucuronic acid residues at a frequency of 4 out of 15 sugar residues and pyruvic acid residues at a similar molar ratio. Since fatty acids seem to be bound to SM-1 EPS by ester bonds, the fatty acids might not contribute to the acidity of SM-1 EPS. At the moment we have no information as to whether SM-1 EPS is cross-linked or branched. It will be of interest to investigate the effect of cross-linking of SM-1 EPS on its capacity for water absorption.

Kurane and Nohata reported on a novel bioabsorbent produced by *A. latus* B16\(^{14}\) and discussed its chemical composition.\(^{23}\) This bioabsorbent is also an acidic poly-

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**Table 2. Moisture absorption capacity of SM-1 EPS**

<table>
<thead>
<tr>
<th></th>
<th>Relative humidity 32%(^a)</th>
<th>Relative humidity 11%(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM-1 EPS</td>
<td>120 ± 2</td>
<td>17 ± 12</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>37 ± 13</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Urea</td>
<td>0.0 ± 0.0</td>
<td>NT(^b)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>7.8 ± 0.2</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>Silica gel</td>
<td>16.9 ± 2.9</td>
<td>6.1 ± 0.5</td>
</tr>
</tbody>
</table>

\(^a\): Values were calculated according to the equation described in the Materials and Methods.

\(^b\): NT, not tested.
saccharide containing glucose, fucose, rhamnose, and glucuronic acid, with a molar ratio of 1.8:1:1.1:1. They suggested that fucose might play an important role in the water absorption property of the novel bioabsorbent. In this regard, it is interesting to note that SM-1 EPS contains fucose as well.

SM-1 EPS contains fatty acids that are hydrolyzed by alkali. It is possible to speculate that SM-1 EPS forms a water-in-oil (W/O) emulsion and prevents evaporation of water under dry, high-temperature conditions, since SM-1 EPS is an efficient emulsifier of oil, PAH, and alkanes (unpublished data). This might be one of the reasons SM-1 EPS shows higher capabilities of moisture absorption and retention than other moisture absorbents and moisture retainers, such as the bioabsorbent produced by A. latus B16.

SM-1 EPS is an efficient moisture absorbent and retainer, and its chemical composition affords some insights into the relationship between its chemical structure and function. We are now studying the moisture-retention capacities and chemical compositions of other rhodococcal EPSs in terms of the relationship between structure and function.

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