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Chemical characteristics and antioxidant activity of exopolysaccharide fractions from *Microbacterium terregens*

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ABSTRACT

The Gram-positive bacterial strain isolated from soil was identified as the non-pathogenic *Microbacterium terregens*. The exopolysaccharide (CPS) produced from *M. terregens* was obtained by isopropanol precipitation (13.72 g L^{-1} growth medium), The resulted exopolysaccharide was purified by chromatography on DEAE-cellulose and Sephacryl S-200 columns, when two polysaccharide fractions termed CPSI and CPSII were obtained. Structure features of CPSI and CPSII were investigated by a combination of chemical and chromatographic analyses, such as acid hydrolysis, methylation analysis, periodate oxidation–Smith degradation, HPLC, GC–MS, and IR. The results indicated that CPSI and CPSII were composed of glucose: mannose in a ratio of 2.7:1 and 3.2:1 with molecular weights 80 and 150 kDa, respectively. It has a backbone of (1 → 4)-linked β-glucose residues, which occasionally branches at O-6. The branches were composed of (1 → 4)-linked β-mannose residues. The antioxidant activity of the CPS, CPSI and CPSII was evaluated *in-vitro* by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (RSA). CPSI fraction showed the highest antioxidant activity among the three fractions, with an IC_{50} value of 230 μg mL^{-1}. The effect of molecular weight of the polysaccharide on the improvement of the antioxidant potential seems to be significant.

1. Introduction

Exopolysaccharides represent a class of high-value polymers with many industrial applications in food, cosmetic, textile and pharmaceutical industries due to their rheological properties. They have been used as emulsifiers, as stabilizers and as texture enhances in food industry. Traditionally, these important polysaccharides have been obtained from plant or algae sources. However, in the last years, new gums from cultivable microbial sources have been received increase attention. Thus, anionic extracellular polysaccharides have been recovered from different bacteria such as *Klebsiella pneumoniae* (Kang, Veder, & Cottrell, 1983), *Pseudomonas sp.* (Williams & Winpenng, 1977) and *Arthrobacter viscosus* (Slaneker, Orentas, Knutsen, Watson, & Jeanes, 1968), as well as basidiomycetes such as *Agricus sp.*, *Oudemansiella conarri* (Maziero, Cavazzoni, & Bononi, 1999) and *Schizophyllum commune* (Brever, 1991). However, the only extracellular polysaccharides that have been produced on a commercial scale are dextran and xanthan. On the other side, polysaccharides have been demonstrated to play important role as dietary free radical scavenger for the prevention of oxidative damage. There are increasing evidence indicating that reactive oxygen species produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions and metabolic processes have a wide variety of pathological effects, such as causing DNA damage, carcinogenesis and cellular degeneration related to aging (Blennder, Oliveira, Conboy, Haigis, & Guarente, 2003; Haran, 1993; Liu, Ooi, & Chang, 1997). In recent years, many studies have shown that reactive oxygen species (ROS) is responsible for various diseases such as cancer, Alzheimer's diseases, Parkinson's diseases, epilepsy, inflammation, retrolental fibroplasias, atherosclerosis, lung injury, ischemia-reperfusion injury and other disorders (Raouf, Patrice, Andre, Jean-Michel, & Yvan, 2000). Although almost all organisms possess antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to entirely prevent the damage. Against this background, natural antioxidants play an important role in the prevention of these diseases. Because many of synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene used in foods are suspected to have cytotoxicity (Valentao et al., 2002), more attention has been paid to natural non-toxic antioxidants. In recent years, an increasing amount of evidence highlights that some polysaccharides isolated from plants, herbs and fungi had antioxidant activities and low cytotoxicity (Liu et al., 1997). The preliminary research showed that *Misgurnus anguillicaudatus* polysaccharide was able to remove $O_2^{\cdot}$, $HO^{\cdot}$, H_2O_2 and other active compounds of oxygen and significantly protected DNA chains from

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being damaged by hydroxyl radicals (Abe & Berk, 1998; Chuanguang, Huang, & Xu, 2002). In this investigation, we describe the isolation, purification, characterization and antioxidant potential screening of CPSI and CPSII from *M. terregens*.

2. Materials and methods

2.1. Bacterial strain

Mucous bacterial colony was isolated from soil sample and was maintained on a *Crinobacterium* medium at 25 °C (Ronald, 1997). The organism has been identified as *M. terregens* based on morphological, cultural and physiological characteristics determined using the methods given by Bergey's Manual of Determinative Bacteriology (Holt, Krieg, Sneath, Stealey, & Williams, 1994). Biolog GP2 MicroPlate™, gives a characteristics reaction pattern called a "metabolic fingerprint", wherein the metabolic fingerprint patterns were compared and identified using the MicroLog™ database software (De Suza et al., 2001).

2.2. Isolation, purification and fractionation of exopolysaccharide

Microbacterium terregens was grown aerobically for 96 h in a *Crinobacterium* medium at 25 °C and 120 rpm on a rotary shaker. The viscous broth was diluted with water and centrifuged at 5000 rpm for 30 min at 4 °C (Sigma-Laborzentrifugen, 2K 215). The cells were washed with distilled water and re-centrifuged three times. The supernatant was dialyzed against distilled water three times (1000 mL × 3). The exopolysaccharide was precipitated by adding three volumes of chilled isopropanol to the supernatant. The obtained exopolysaccharide was collected by centrifugation and its aqueous solution was dialyzed against distilled water for 48 h then lyophilized. Lyophilized exopolysaccharide (100 mg in 3 mL distilled water) was subjected to a DEAE-cellulose anion-exchange column (50 × 2.4 cm, i.d.) and eluted with the deionized water, 0.1 M NaCl, 0.3 M NaCl, 0.5 M NaCl and 1.0 M NaCl at a flow rate of 45 mL h^{-1}, respectively. Fractions (5 mL, each) were collected and combined according to the total carbohydrate content determined by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The fractions obtained were concentrated, dialyzed against distilled water, and then lyophilized. The CPSI and CPSII were dissolved in 1 mL 0.1 M NaCl, fractionated further by a gel permeation chromatography technique over a Sephacryl S-200 (75 × 2.4 cm, i.d.) and eluted with 0.1 M NaCl at a flow rate of 25 mL h^{-1}. The fractions (5 mL, each) were collected, tested for total carbohydrate by the phenol–sulfuric acid method (Dubois et al., 1956), and the polysaccharide fractions were collected, dialyzed and lyophilized. The total (V_t) and void (V_0) volumes of the column was determined as the elution volume of glucose and dextran (molecular weight 2,000,000), respectively.

2.3. Purity and molecular weight determinations

Purity and molecular weight of CPSI and CPSII fractions were determined by a gel permeation chromatography technique. Standard dextrans (40, 500, 700 and 2000 kDa, Fluka Chemical Co., Buchs, Switzerland) were passed through a Sephacryl S-200 column, and the elution volumes were plotted against the logarithms of their respective molecular weights. A solution of the exopolysaccharide (10 mg in 1 mL of 0.1 M NaCl) was applied to the column equilibrated and eluted with 0.1 M NaCl at a flow rate of 25 mL h^{-1}. The elution volume of the exopolysaccharide was then plotted in the same graph, and the molecular weighs of CPSI and CPSII were determined (Luo, 2008).

2.4. Determination of sugars

Twenty milligrams of polysaccharide was hydrolyzed in 2 mL of 2 M trifluoroacetic acid (TFA) at 120 °C in a sealed-tube for 5 h, followed by evaporation on a water bath at 40 °C, co-distilled with water (1 mL × 3) and dissolved in 0.2 mL deionized water (Sudhamani, Tharanathan, & Prasad, 2004). The monosaccharides contents of CPSI and CPSII were quantified by HPLC on a Shimadzu Shim-Pack SCR-101N column using deionized water as the mobile phase and refractive index detection, as described by Asker, Mohamed, and El-Sayed (2007).

2.5. Infrared spectroscopy

Infrared spectra of CPSI and CPSII fractions were obtained by grinding a mixture of sample with dry KBr and pressing in a mold. IR spectra were recorded on a Fourier transform infra red in Bruker scientific 500-IR Spectrophotometer (Ray, 2006).

2.6. Periodate oxidation–Smith degradation

CPSI and CPSII fractions (25 mg) was dissolved in 10 mL of distilled water, dispersed using a blender, then 25 mL of 30 mM $NaIO_4$ was added. The solution was kept in the dark at 4 °C, and then 0.1 mL aliquots were withdrawn at 24 h intervals, diluted to 5 mL with distilled water and recorded in spectrophotometer at 223 nm (Linker, Evans, & Impollomeni, 2001). Consumption of HIO_4 was measured by a spectrophotometer method (Aspinall & Ferrier, 1956), and HCOOH production was determined by titration with 0.005 M NaOH. Ethylene glycol (2 mL) was added, and then the experiment of periodate oxidation was over. The solution of periodate product was extensively dialyzed against tap water and distilled water for 48 h, respectively. The content inside was concentrated and reduced with $NaBH_4$ (100 mg), and the mixture was left for 24 h at room temperature, neutralized to pH 6.0 with 50% acetic acid, dialyzed as described above, and re-concentrated to 10 mL. The solution mentioned above was freeze–dried, fully hydrolyzed and analysis by HPLC (Asker et al., 2007).

2.7. Methylation analysis

CPSI and CPSII fractions were methylated separately using the method of Ciucamu and Kerek (1984). The methylated products were extracted with $CHCl_3$, washed with distilled water three times and evaporated to dryness. The product was then hydrolyzed with 2 M TFA at 120 °C for 5 h. The methylated products were converted into their corresponding alditol by reduction with $NaBH_4$ and acetylated (Guilherme, Marcello, & Philip, 2005). The resulting product was subjected to linkage analysis by GLC–MS (Finningan SSQ7000) on a DB-5 capillary column (30 m × 0.25 mm × 0.25 μm) using a gradient temperature from 140 to 280 °C at 4 °C min^{-1} (He et al., 2007). Linkages were identified on the basis of relative retention time and fragmentation pattern. The molar ratios for each sugar were calibrated using the peak areas and response factors.

2.8. Radical scavenging activity (RSA) of CPS, CPSI and CPSII

The RSA of the CPS, CPSI and CPSII were measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) test according to the method of Shimada, Fujikawa, Yahara, and Nakamura (1992) with some modifications. Two milliliter of DPPH solution in 95% ethanol (freshly prepare at a concentration of 10^{-4} M) was added with 1 mL of the samples of different addition quantity (80–400 μg) in water and 2 mL of 95% ethanol. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against blank using UV-2401PC visible

spectrophotometer (Shimadzu, Kyoto, Japan). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity, which was analyzed from the graph plotted of inhibition percentage against compound concentration. The experiment was carried out in triplicate and averaged. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Scavenging ability}(\%) = [(\text{A517 of control} - \text{A517 of sample})/\text{A517 of control}] \times 100$$

The EC_{50} value is the effective concentration ($\mu g\ mL^{-1}$) of CPS, CPSI and CPSII at which the DPPH radicals were scavenged by 50%.

2.9. Statistical analysis

The obtained data were subjected to One-way ANOVA and the differences between means were at the 5% probability level using Duncan's new multiple range tests. The software SPSS, version 10 (SPSS, Richmond, USA) was used as described by Dytham (1999).

3. Results and discussion

3.1. Characteristics of exopolysaccharide producing bacteria

Colonies of *Microbacterium terregens* showed mucous appearance on sucrose medium. The bacterial cells were Gram-positive, short rods, whose biochemical characteristics are summarized in Table 1.

3.2. Isolation, purification and composition of CPSI and CPSII fractions

The crude exopolysaccharide (CPS) was obtained from a culture of *Microbacterium terregens* by isopropanol precipitation (13.72 g L^{-1} growth medium). After fractionation on DEAE-cellulose and Sephacryl S-200 column, CPSI and CPSII were obtained (Figs. 1 and 2). They were all eluted as a single peak from gel filtration chromatography on Sephacryl S-200 column. The average molecular weights of CPSI and CPSII were determined as 80 and 150 kDa by gel permeation chromatography technique (Fig. 2), and it had a negative response to ninhydrin test indicting the absence of protein. The monosaccharide of CPSI and CPSII hydrolysate was measured by HPLC, wherein glucose and mannose were identified in the hydrolysate and their ratios were 2.7:1 and 3.2:1, respectively.

3.3. Structural characterization of CPSI and CPSII fractions

The IR spectra of CPSI and CPSII were basically indistinguishable only by some difference in the intensity of bands. The intensity of bands around 3423 cm^{-1} for the hydroxyl group, and a weak band at 2926 cm^{-1} showing the C–H stretching vibration. The absorption at 1433 and 1380 cm^{-1} was possible due to non-symmetric and symmetric CH_3 bending, respectively. In addition, in the IR spectrum of CPSI and CPSII, the characteristic band at 896 cm^{-1} belonged to the β-anomeric configuration (Bao, Fang, & Fang, 2001; Synytsya, Copikova, Matejka, & Machovic, 2003; Zhbankov, Adnanov, & Marchewka, 1997). Periodate oxidation of CPSI and CPSII in the values of 1.07 and 0.89 mol periodate consumed per mole sugar residue, respectively; while the amount of formic acid released were 0.32 and 0.21 mol per mol sugar residue, respectively. The periodate oxidation resulted in the consumption of about 1 mol per mol sugar residue, this cannot happen unless the glycosidic linkage are (1 → 4)-linked and/or (1 → 2)-linked (Table 2). The periodate-oxidized products of CPSI and CPSII were reduced, hydrolyzed and analyzed by HPLC. Glucose and mannose were not found in the hydrolyzed product, it could be inferred that glucose and mannose are in linkages that can be oxidized, namely 1→, (1 → 6)-, (1 → 2)-, (1 → 2,6)-, (1 → 4)-, or (1 → 4,6)-linkage. The glycerol and erythritol were found with molar ratio of 0.1:2.71 and 0.1:2.86 from CPSI and CPSII, respectively (Table 3). It was thus

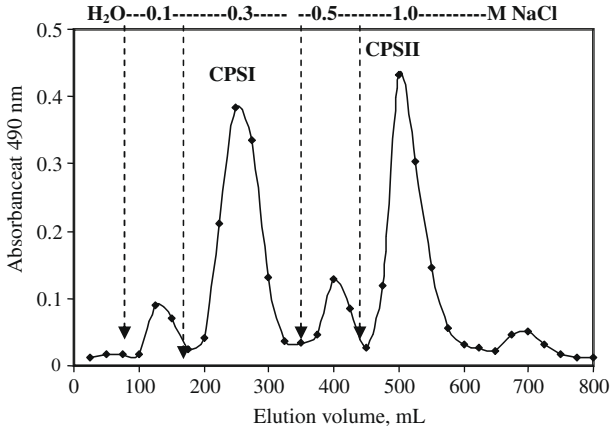

Fig. 1. Elution curve of CPS from *M. terregens* over DEAE-cellulose column. The absorbance at 490 nm was that of the resulting reactive solutions of polysaccharides, phenol and sulfuric acid.

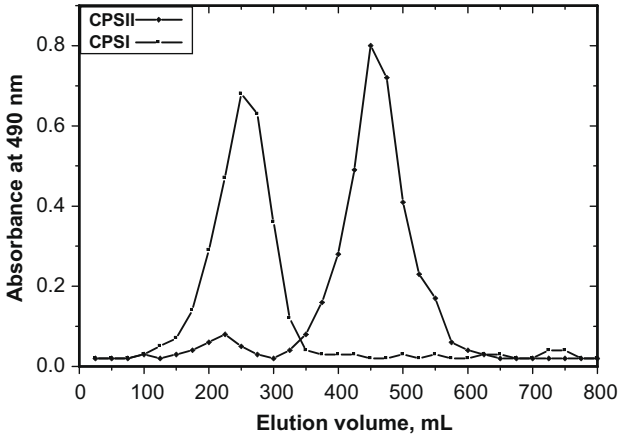

Fig. 2. Purity of CPSI and CPSII on Sephacryl S-200 column eluting with 0.1 M NaCl at a flow rate of 25 mL h^{-1}. The absorbance at 490 nm was that of the resulting reactive solutions of polysaccharides, phenol and sulfuric acid.

Table 1
Morphological, cultural and physiological characteristics of the bacterial isolate.

Characteristics	Isolate bacteria
Morphology	Gram-positive, rods, non-spore forming
Motile	Non-motile
Cultural	Circular, smooth, mucous, yellow–white
Physiological	Aerobic, catalase negative

Table 2
Periodate oxidation results.

	CPSI	CPSII
Reaction time (h)	72	72
Amount of CPSI or CPSII (mmol)	0.154	0.154
Consumption of HIO_4 (mmol)	0.165	0.137
Consumption of HIO_4/hexose (mol/mol)	1.070	0.890
Amount of formic acid (mmol)	0.049	0.032
Amount of formic acid/hexose (mol/mol)	0.320	0.210

Table 3
HPLC results of Smith degradation of CPSI and CPSII.

Fractions	Molar ratios			
	Erythritol	Glycerol	Mannose	Glucose
CPSI	2.71	0.10	0.00	0.00
CPSII	2.86	0.10	0.00	0.00

Table 4
GC–MS results of methylation analysis of CPSI and CPSII.

Methylated sugar	Linkage pattern	Molar ratios	
		CPSI	CPSII
2,3,4,6-tetra-*O*-methyl-glucose	Glc (1→	0.01	0.01
2,3,6-tri-*O*-methyl-glucose	→4) Glc (1→	2.84	3.05
2,3-di-*O*-methyl-glucose	→4,6) Glc (1→	0.06	0.06
2,3,6-tri-*O*-methyl-mannose	→4) Man (1→	1.00	1.00
2,3,4,6-tetra-*O*-methyl-mannose	Man (1→	0.06	0.08

deduced that 1→, (1 → 6)-, and (1 → 4,6)- amounted to ~3.7%, with (1 → 4)-linkage glycosyl bonds amounting to ~96.3%, respectively (Abd El-Akher, Hamilton, Montgomeny, & Smith, 1952; Danishefeky, Whistler, & Bettelheim, 1970; Wang, Luo, & Liang, 2004; Liu et al., 2007).

The CPSI and CPSII were methylated by the Ciucamu and Kerek (1984) method and then hydrolyzed with acid. The alditol of methylated products were analyzed by GC–MS using a DB-5 capillary column. The CPSI and CPSII gave five components namely 2,3,4,6-tetra-*O*-methyl-glucose; 2,3,6-tri-*O*-methyl-glucose; 2,3-di-*O*-methyl-glucose; 2,3,6-tri-*O*-methyl-mannose and 2,3,4,6-tetra-*O*-methyl-mannose in molar ratio of 0.01:2.84:0.06:1.00:0.06 and 0.01:3.05:0.06:1.00:0.08, respectively (Table 4). Both results of periodate oxidation, Smith degradation and methylation linkage analysis of CPSI and CPSII indicated that 2,3-di-*O*-methyl-glucose, (1 → 4,6)-linked glucose and 2,3,6-tri-*O*-methyl-glucose (1 → 4)-linked glucose were major components of the backbone structure with branches attached to O-6 of (1 → 4)-linked mannose. This was also in accordance with the mode of linkage of glucose and/or mannose in the polysaccharide by periodate oxidation and Smith degradation. The linkage patterns of two polysaccharide fractions CPSI and CPSII are similar but differences in the molecular weight only (De S-F-Tischer et al., 2006; Ray, 2006; Sudhamani et al., 2004; Urai et al., 2006; Wang et al., 2004).

3.4. Radical scavenging activity (RSA) of CPS, CPSI and CPSII

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of natural compounds (Lee, Hwang, Ha, Jeong, & Kim, 2003; Nagai, Inoue, Inoue, & Suzuki, 2003; Ramadan & Moersel, 2006). In the DPPH test, the antioxidants were able to reduce the stable DPPH radical to the yellow-colored diphenylpricrylhydrazine. The effect of antioxidants on DPPH radical scavenging was believed to be due to their hydrogen-donating ability. The DPPH radical scavenging activities of CPS, CPSI and CPSII were determined (Shimada et al., 1992). The scavenging impacts of different fractions CPS, CPSI and CPSII on DPPH are shown in Fig. 3. CPSI exhibited the highest inhibition effect with an IC_{50} value of 230 μg mL^{-1}, which was lower than that of CPS and CPSII. The bioactivities of polysaccharides can be affected by many factors including chemical components, molecular weight, structure, conformation, even the extraction and isolation methods. The molecular weight of polysaccharides could play an important role in the antioxidant activity (Chen, Zhang, Qu, & Xie, 2008). Among the three fractions CPS, CPSI and CPSII, a relatively low molecular weight appeared to increase the antioxidant activity. Perhaps polysaccharides with low molecular weight might bind radicals more easily.

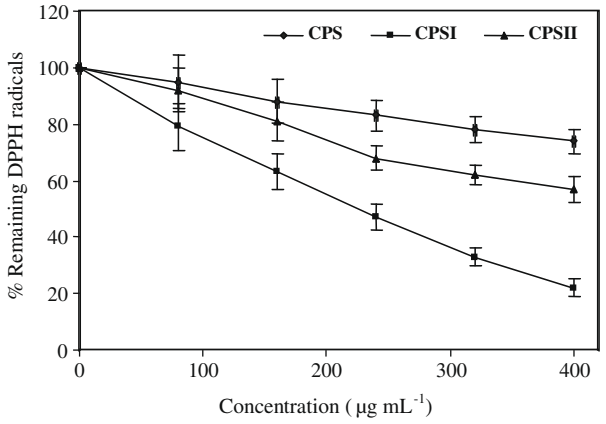

Fig. 3. Scavenging effect of CPS, CPSI and CPSII during DPPH test and measured by changes in absorbance at 517 nm.

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