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Water-soluble polysaccharide from the fruiting bodies of *Chroogomphis rutilus* (Schaeff.: Fr.) O. K. Miller: Isolation, structural features and its scavenging effect on hydroxyl radical

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ABSTRACT

One water-soluble polysaccharide (CRP) was isolated from the fruiting bodies of *Chroogomphis rutilus* with a molecular weight (M_w) of 3.2×10^4 Da. According to partial acid hydrolysis, periodate oxidation and Smith degradation, methylation, FT–IR, GC–MS, and NMR analysis, the results indicated CRP had a backbone consisting of (1→6)-linked-α-D-galactopyranosyl and (1→2, 6)-linked-α-D-galactopyranosyl residues that terminated in a single terminal (1→)-β-D-glucopyranosyl residue at the O-2 position of (1→2, 6)-linked-α-D-galactopyranosyl residue along the main chain in the ratio of 1:1:1. Furthermore, the in vitro anti-oxidant activity evaluated by hydroxyl radicals scavenging method showed that CRP could remarkably enhance the scavenging effect on hydroxyl radicals in a dose-dependent manner.

1. Introduction

Today, increasing attention is being placed on polysaccharides by biochemical and nutritional researchers, due to their various biological activities that could be applied to healthcare foods or medicine, especially anti-oxidant, immunostimulatory, and anti-tumor effects (Li, Chen, Wang, Tian, & Zhang, 2009; Qiao et al., 2009; Sun & Liu, 2008; Yuan, Zhang, Fan, & Yang, 2008). To the best of our knowledge, plant polysaccharides in general have strong anti-oxidant activities and can be explored as novel potential anti-oxidants. Moreover, the use of anti-oxidant molecules, especially naturally occurring ones, in foods as preventive and therapeutic medicines is gaining popularity.

Chroogomphis rutilus is a traditional Chinese medicinal and edible fungus distributed in the Northeast Provinces of China, which is a Gomphidius fungus belonging to the Basidiomycotina. However, through literature retrieval, up to now, there is not any information published on *C. rutilus*. Let alone studies on the polysaccharides isolated from the fruiting bodies of this fungus. Therefore, this paper was concerned with the isolation, structural elucidation of one water-soluble polysaccharide from the fruiting bodies of *C. rutilus* and explored its scavenging effect on hydroxyl radicals for seeking new biological functional principle used in food and pharmaceutical industry.

2. Materials and methods

2.1. Chemicals

DEAE Sepharose Fast Flow and Sephacryl S-200 were purchased from Amersham (Sweden). T-series dextran, dimethyl sulfoxide (DMSO), standard sugars, deoxyribose, trichloride ferric, ethylene diamine tetraacetic acid (EDTA), H_2O_2, ascorbate acid, and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO). All of other reagents were analytical grade from Peking Chemical Co. (Peking, China).

2.2. General methods

UV–vis absorption spectra were recorded with a UV–vis spectrophotometer (Model SP-752, China). GC was performed on a Shimadzu GC-14C instrument (Shimadzu, Japan) equipped with a DB-1 capillary column (30 m × 0.25 mm × 0.25 μm). Gas chromatography–mass spectrometry (GC–MS) was done on a Shimadzu QP-2010 instrument (Shimadzu, Japan) with an HP-5MS quartz capillary column (30 m × 0.25 mm × 0.25 μm). The FT–IR spectra (KBr pellets) were recorded on SPECORD in a range of 400–4000 cm^{-1}.

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The total carbohydrate content was determined by the phenol–H_2SO_4 method, with glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein was measured by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951), with bovine serum albumin as the standard. Dialysis was carried out using tubing with a M_w cut-off of 500 Da (for globular proteins).

2.3. Extraction and purification of polysaccharide

As shown in Fig. 1, the fruiting bodies of *C. rutilus* (0.5 kg) were extracted with 95% ethanol (5000 mL × 3) at 75 °C for 6 h under reflux to remove lipid. The residue was then extracted with distilled water (8000 mL × 3) at 75 °C for three times and 3 h for each time. After centrifugation (1700*g* for 10 min, at 20 °C), the supernatant was concentrated 10-fold, and precipitated with 4 vol of 95% ethanol at 4 °C for 24 h. The precipitate collected by centrifugation was deproteinated by proteinase digestion and the Sevag method (Sun et al., 2008), followed by exhaustive dialysis with water for 48 h. Then the concentrated dialyzate was precipitated with 4 vol of 95% EtOH at 4 °C for 24 h. The precipitate was washed with absolute ethanol, acetone, and ether. The washed precipitate was the crude polysaccharide (CCRP).

The CCRP was purified on an ÄKTA explore 100 purification system equipped with a pump P-900, a UV-900 monitor, a pH/C-900 monitor, a Fraction Collector Frac-950 and an auto-sampler A-900. The CCRP was dissolved in distilled water, centrifuged, and then the supernatant was applied to a DEAE Sepharose Fast Flow column (2.6 × 40 cm) equilibrated with distilled water. After loading with sample, the column was eluted with distilled water and then with stepwise gradient of aqueous NaCl solutions (0.1, 0.3, 0.5, and 1 M) at a flow rate of 4 mL/min. Different fractions were collected using the Frac-950. Total carbohydrate content of each tube was measured at 490 nm by Dubois's method, and protein absorption at 280 nm was measured for each fraction. The neutral fraction eluted with distilled water was purified further on a Sepharose 6 Fast Flow column (2.6 × 100 cm), eluted with 0.15 M NaCl at a flow rate of 1 mL/min. One main fraction was collected, dialyzed and ethanol precipitated to obtain purified polysaccharide (CRP). A stock CRP solution was prepared by dissolving in 0.15 mol/L saline and sterilized by passing it through a 0.22 μm millipore filter.

2.4. Monosaccharide composition, properties, and molecular weight determination

Gas chromatography (GC) was used for the identification and quantification of the monosaccharides. CRP was hydrolyzed with 2 M TFA at 120 °C for 2 h (Sun et al., 2008). The monosaccharides were conventionally converted into the alditol acetates as described (Johnes & Albersheim, 1972; Oades, 1967) and analyzed by GC as previously mentioned. The absolute configurations of the monosaccharides were determined as described by Vliegenthart and co-workers using (+)-2-butanol (Gerwig, Kamerling, & Vliegenthart, 1979).

The average molecular weight of CRP was determined by high-performance size-exclusion chromatography (HPSEC) (Sun et al., 2008), which was performed on a SHIMADZU HPLC system fitted with one TSK-G3000PW_{XL} column (7.8 mm ID × 30.0 cm) and a SHIMADZU RID-10A detector. The data were processed by GPC processing software (Millennium^{32} version). The mobile phase was 0.7% Na_2SO_4, and the flow rate was 0.5 mL/min at 40 °C, with 1.6 MPA. A sample (3 mg) was dissolved in the mobile phase (0.5 mL) and centrifuged (10,000 rpm; 3 min), and 20 μL of supernatant were injected in each run. The molecular mass was estimated by reference to a calibration curve made from a set of Dextran T-series standards of known molecular masses (T-130, 80, 50, 25, and 10).

2.5. Partial hydrolysis with acid

The CRP (100 mg) was hydrolyzed with 0.05 M trifluoroacetic acid (3 mL) at 95 °C for 10 h, and then centrifuged. Afterward, TFA was removed by evaporation, and the rest was dialyzed with distilled water for 48 h, and then the solution was diluted in the sack with ethanol. After hydrolysis, the precipitate and supernatant in the sack and the fraction out of sack were dried and analyzed by GC, as was done with the alditol acetate. The precipitate in the sack was subjected to monosaccharide composition and methylation analyses (Sun & Liu, 2009).

2.6. Periodate oxidation-smith degradation

For analytical purposes, 25 mg of the polysaccharide were dissolved in 12.5 mL of distilled water, and 12.5 mL of 30 mM/L $NaIO_4$ were added. The solution was kept at 4 °C for seven days in the dark, 0.1 mL aliquots were withdrawn at 3–6 h intervals, diluted to 25 mL with distilled water and read in a spectrophotometer at 223 nm (Linker, Evans, & Impallomeni, 2001). Excess periodate was decomposed by the addition of ethylene glycol (2 mL). The solution of periodate product (2 mL) was sampled to calculate the yield of formic acid by 0.01 M NaOH, The rest was dialyzed against distilled H_2O for 24 h. The solution was concentrated and reduced with $NaBH_4$ (60 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 concentrated to a 10 mL volume. One-third of this solution was freeze-dried and analyzed with GC. The rest were added to the same volume of 1 M sulfuric acid, kept for 40 h at 25 °C, neutralized to pH 6.0 with barium carbonate and filtered. The filtrate was dialyzed as before, and the content outside the sack was desiccated for GC analysis; the content inside was diluted with ethanol, and after centrifugation, the supernatant and precipitate were also dried for the GC analysis.

2.7. Methylation analysis

The sample (20 mg) was methylated three times, according to Needs and Selvendran (1993). Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum. The methylated products were hydrolyzed, then reduced and acetylated as described by Sweet, Shapiro, and Albersheim (1975). The partially methylated alditol acetates were analyzed by GC–MS under the same chromatographic conditions, as described above.

2.8. NMR spectroscopy

For NMR measurements CRP was dried in a vacuum over P_2O_5 for several days, and then exchanged with deuterium by lyophilizing with D_2O for several times (Dueñas-Chasco et al., 1997). The deuterium-exchanged polysaccharide (50 mg) was put in a 5-mm NMR tube and dissolved in 0.7 mL of 99.96% D_2O. Spectra were recorded with a Bruker AV-400 spectrometer. The ^1H and ^{13}C NMR spectra were recorded at 50 °C. Acetone was used as an internal standard (δ31.11 ppm) for the ^{13}C spectrum. The ^1H NMR spectrum was recorded, with the HOD signal fixed at δ4.52 ppm and 50 °C.

2.9. Assay for the scavenging effect on hydroxyl radicals

Assessment of the scavenging ability of CRP on hydroxyl radicals was performed by the method previously described by Halliwell, Gutteridge, and Aruoma (1987), with a minor modification. Reaction

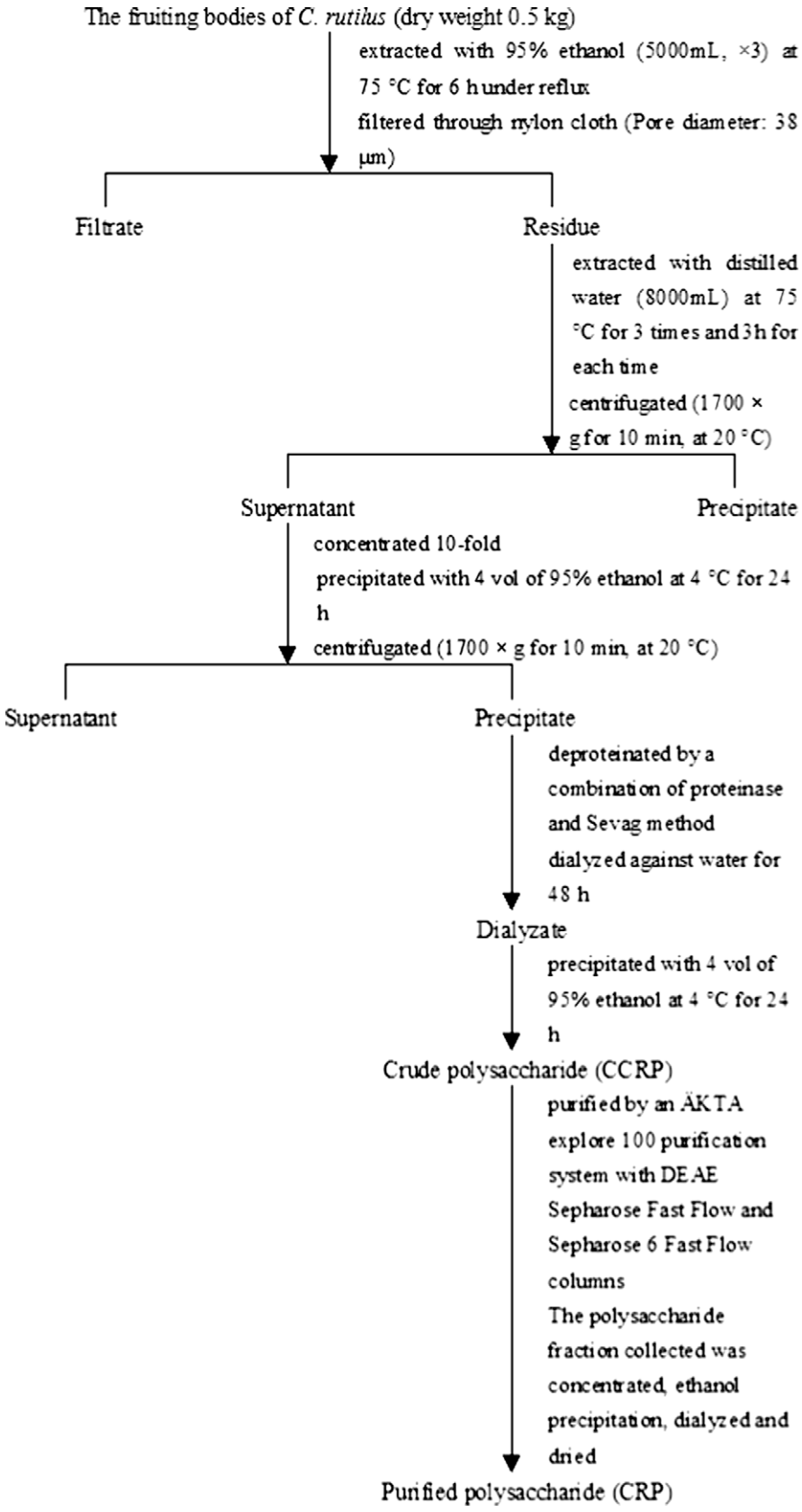

Fig. 1. Summarized extraction scheme of CRP from the fruiting bodies of *Chroogomphis rutilus*.

mixtures in a final volume of 1.0 mL contained deoxyribose (60 mM), phosphate buffer (pH 7.4, 20 mM), ferric trichloride (100 μM), EDTA (100 μM), H_2O_2 (1 mM), ascorbic acid (100 μM), and different concentrations of CRP (0, 10, 20, 40, 80, or 160 μg/mL). Solutions of ferric trichloride and ascorbic acid were made immediately before use. The reaction solution was incubated for

Table 1
The data of UV analysis, IR analysis, and NMR analysis of CRP.

Assay	Peaks or signals at
UV analysis	210 nm, 260 nm, 280 nm
IR analysis	3410.25 cm^{-1}; 2930.26 cm^{-1}; 1640.13 cm^{-1}; 841.21 cm^{-1}; 889.13 cm^{-1}
^{1}H NMR analysis	5.01 ppm; 5.12 ppm; 4.80 ppm
^{13}C NMR analysis	100.7 ppm; 100.8 ppm; 105.9 ppm; 80.01 ppm; 70.12 ppm; 69.90 ppm; 60.02 ppm

1 h at 37 °C and then 1 mL of 1% TBA and 1 mL of 20% (v/v) HCl were added to the mixture. The mixture was boiled for 15 min and cooled on ice. The absorbance of the resulting mixture was measured at 532 nm. Percent inhibition of hydroxyl radical was calculated as (1 – absorbance of sample/absorbance of control) × 100%.

3. Results and discussion

3.1. Isolation, purification and structural analysis of polysaccharides

The CRP showed a single symmetrically sharp peak, indicating its homogeneity on HPSEC (data not shown). According to the retention time, its molecular weight was estimated to be 3.2×10^4 Da. It had a negative response to the Bradford test and no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. Total carbohydrate content was determined to be 93%. The CRP was composed of D-galactose and D-glucose as detected by GC in the ratio of 2:1.

The FT–IR spectra of CRP were shown in Table 1. The bands in the region of 3410.25 cm^{-1} were due to the hydroxyl stretching vibration of the polysaccharides. The bands in the region of 2930.26 cm^{-1} were due to C–H stretching vibration, and the bands in the region of 1640.13 cm^{-1} were due to associated water. Moreover, the characteristic absorptions at 841.21 and 889.13 cm^{-1} in the IR spectra indicated that α- and β-configurations were simultaneously present in CRP.

The GC–MS results (Table 2) indicated that the backbone chains were mainly (1→6)-linked-α-D-galactopyranosyl (Residue-A) and (1→2, 6)-linked-α-D-galactopyranosyl residues (Residue-B). The side chain attached to the O-2 position of Residue-B contained single terminal (1→)-β-D-glucopyranosyl (Residue-C) group. According to the peak areas, three types of residues were seen in the ratio of 1:1:1. This was also in accordance with the results of the periodate oxidation and Smith degradation. In support of the methylation analysis results, GC of the products from periodate oxidation and Smith degradation only showed the presence of glycerol (data not shown).

In the anomeric region of the ^{1}H NMR spectrum (Table 1) of CRP, three signals occurred at δ5.01, δ5.12, and δ4.80 ppm, which were designated as Residue-A, Residue-B, and Residue-C, respectively. Accordingly, in the anomeric region of the ^{13}C NMR spectrum (Table 1), three carbon resonances appeared at δ100.7, δ100.8 and, δ105.9 ppm. All the results confirmed the presence of three sugar residues and their configurations: Residues-A and B were α-configurations, and Residue-C was form of β-configuration, consistent with GC and FT–IR data. In the high magnetic field, the δ80.01 signal appeared to come from C-2 resonance of Residue-B. C-6 chemical shifts of Residue-A, Residue-B and Residue-C occurred at δ70.12, δ69.90, and δ60.02, respectively. All the NMR chemical shifts were compared with the literature values (Fan et al. 2006; Jia, Liu, Dong, & Fang, 2004; Pramanik, Mondal, Chakraborty, Rout, & Islam, 2005; Roger, Kervarec, Ratiskol, Colliec-Jouault, & Chevolot, 2004; Rout, Mondal, Chakraborty, & Islam, 2006; Sun et al. 2008; Ye et al., 2008).

Table 2
The results of methylation analysis of CRP.

Peak no.	Methylated sugar	Molar ratio	Linkage type
1(Residue-A)	2,3,4-Me_3- Gal*p*l	19	→6)- α-Gal*p*-(1→
2(Residue-B)	3,4-Me_2- Gal*p*l	21	→2, 6)- α-Gal*p*-(1→
3(Residue-C)	2,3,4,6-Me_4- Glc*p*	20	β-Glc*p*-(1→

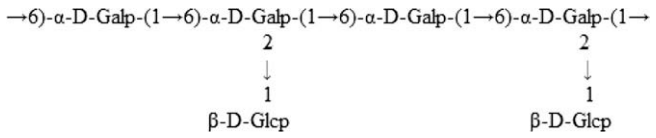

Fig. 2. The structure of CRP isolated from the fruiting bodies of *Chroogomphis rutilus*.

Table 3
Scavenging effects of CRP on hydroxyl radicals (·OH) in vitro[a].

Preparation	Concentrations (μg/mL)	·OH generation (A_{532})	Inhibition (%)
Control	0	0.94 ± 0.03	–
CRP	10	0.91 ± 0.04	9.6
	20	0.70 ± 0.03[b]	32.0
	40	0.56 ± 0.06[c]	46.8
	80	0.39 ± 0.02[c]	64.9
	160	0.33 ± 0.04[c]	71.3

Purified polysaccharide was named as CRP.
[a] The results are represented as mean ± SD based on three independent experiments.
[b] $P < 0.05$, significantly different from the control.
[c] $P < 0.01$, significantly different from the control.

In this study, we successfully isolated one water-soluble polysaccharide from the fruiting bodies of *C. rutilus* and identified its structure in Fig. 2.

3.2. Scavenging activity of CRP on hydroxyl radical

Hydroxyl radicals, generated by reaction of an iron–EDTA complex with H_2O_2 in the presence of ascorbic acid, attack deoxyribose to yield a chromogen upon heating with TBA of low pH, Additional hydroxyl radical scavengers compete with deoxyribose for the produced hydroxyl radicals and diminish chromogen formation. The above model was used to measure inhibitory activities of all fractions on hydroxyl radicals. As shown in Table 3, CRP was found to have a higher scavenging effect on hydroxyl radicals at concentrations from 20 to 160 μg/mL. The scavenging effects of CRP increased in a dose-dependent manner. This result proved that CRP had a significant effect on scavenging of hydroxyl radicals.

Generation of reactive oxygen species (ROS) beyond the body's anti-oxidant capacity gives rise to oxidative stress. Accumulating evidence strongly suggests that such stress is an important causative factor of aging, brain dysfunction, liver diseases, cardiovascular disorders and carcinogenesis. Much of the oxidative damage to biomolecules can be induced by ·OH, the most reactive ROS species (Yang, Liu, Han, & Sun, 2006). Due to the complex mechanism involved in anti-oxidant activity, a single test is normally not enough to evaluate precise activity of the potential anti-oxidants. Therefore, various oxidative stress and mediated injury models should deserve an in-depth research in future studies.

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