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One proteoglycan from the fruiting bodies of *Chroogomphis rutilus* (Schaeff.: Fr.) O.K. Miller: purification and structural features

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ABSTRACT

A water-soluble proteoglycan (CRP-W2) was purified from the fruiting bodies of *Chroogomphis rutilus* by boiled water extraction, ethanol precipitation, weak anion exchange and size exclusion chromatography. Its molecular weight (Mw) was estimated to be about 1.3×10^4 Da using high-performance size-exclusion chromatography (HPSEC). As a precondition to understand the bioactivity, the structural features of CRP-W2 was analyzed using chemical methods, IR spectroscopy and NMR spectroscopy. The results indicated that CRP-W2 had a backbone consisting of 1,6-linked and 1,2,6-linked-α-D-Gal, which was terminated with 1-linked-α-D-Man and 1-linked-α-D-Glc residues at the O-2 position of 1,2,6-linked-α-D-Gal in the molar ratio of 2:2:1:1.

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1. Introduction

Chroogomphis rutilus is a well known edible and medicinal traditional fungus in oriental countries, which is a Gomphidius fungus belonging to the Basidiomycotina. In our previous study, we successfully applied the response surface methodology (RSM) to optimize the extraction conditions of polysaccharides from the fruiting bodies of *C. rutilus* with a Box–Behnken design (Sun, Li, Yang, Liu, & Kennedy, 2010; Sun, Liu, & Kennedy, 2010). Furthermore, four water-soluble purified proteoglycan (CRPsA-1, CRPsA-2, CRPsB-1 and CRPsB-2) were obtained from the fruiting bodies of *C. rutilus* by DEAE Sepharose Fast Flow and Sepharose 6 Fast Flow column chromatography. Their physicochemical characteristics were confirmed by IR, HPSEC and gas chromatography (GC). Based on the assay of hydroxyl radical, superoxide radical and Fe^{2+}-chelating ability, the antioxidant activities of four purified proteoglycan were investigated. The results indicated the fractions containing higher content of glucuronic acid had a more potent antioxidant activity than that containing lower content or no glucuronic acid in a concentration-dependent manner (Sun & Kennedy, 2010). Recently we purified one water-soluble polysaccharide (CRP) from the fruiting bodies of *C. rutilus* by boiling water. The combination of chemical and instrumental analysis indicated CRP consisted a backbone of 1,6-linked-α-D-Gal and 1,2,6-linked-α-D-Gal residues, which were terminated with a single terminal 1-β-D-Glu residue at the O-2 position of 1,2,6-linked-α-D-Gal residue along the main chain in the ratio of 1:1:1 (Sun, Li, et al., 2010; Sun, Liu, et al., 2010). At the same time, another small amount of polysaccharide was still not investigated. Therefore, in this research we intend to clarify the physicochemical properties and structural features of this polysaccharide.

2. Experimental

2.1. Materials

Sepharose 6 Fast Flow, DEAE Sepharose Fast Flow and Sephadex G-25 were purchased from Amersham (Sweden). D-Glucose was form Amresco Inc. T-series dextran was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

2.2. General methods

Total sugar content of polysaccharide was determined by the phenol-sulphuric acid method, with glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Proteins in the polysaccharides were quantified according to the Bradford's method (Sedmark & Grossberg, 1979), with bovine serum albumin (BSA) as the standard. Uronic acid content was determined according to a meta-hydroxydiphenyl colorimetric method (Blumenkrantz & Asboe-Hansen, 1973), with glucuronic acid as

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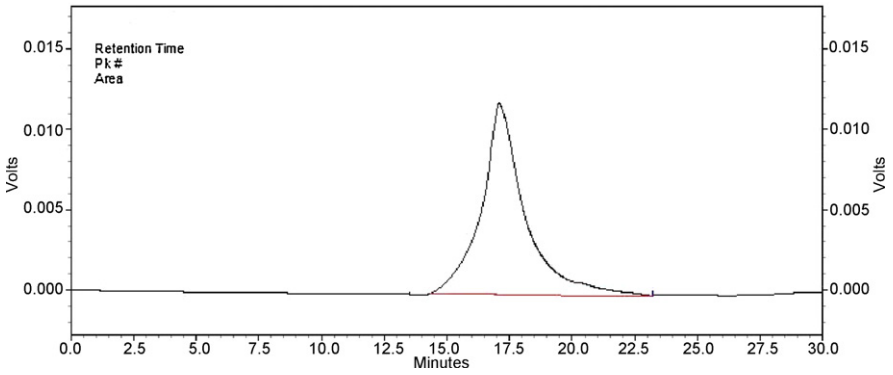

Fig. 1. The average molecular weight and homogeneity of CRP-W2 was determined by high-performance size-exclusion chromatography (HPSEC), eluting with 0.7% Na_2SO_4 at a flow rate of 0.5 mL/min.

the standard. Infrared Spectrum of the sample was recorded on SPECORD IR spectrometer (KBr pellets) in a range of 400–4000 cm^{-1}. UV–Vis absorption spectra were recorded with a UV–Vis spectrophotometer (Model SP-752, China). Gaschromatography (GC), used for identification and quantification, was performed on a Shimadzu GC-14C instrument (Japan) equipped with a DB-1 capillary column (30 m × 0.25 mm × 0.25 μm) and flame-ionization detector (FID).The column temperature was kept at 120 °C for 2 min and increased to 250 °C (maintained for 3 min) at a rate of 8 °C/min. The injector and detector heater temperature were 250 and 300 °C, respectively. The rate of N2 carrier gas was 1.2 ml/min. Gas chromatography–mass spectrometry (GC–MS) was finished on a Shimadzu QP-2010 instrument (Japan) with an HP-5MS quartz capillary column (30 m × 0.25 mm × 0.25 μm). GE Healthcare's ÄKTA Explore 100 purification system was applied to the chromatography system, which was equipped with a P-900 series pump, UV-900 monitor, pH/C-900 detector, M-925 mixer, Frac-950 fraction collector, A-900 auto-sampler and various kinds of columns. Dialysis was carried out using tubing with a Mw cut-off of 500 Da (for globular proteins). All gel chromatography was monitored with phenol-sulfuric acid method.

2.3. Isolation and purification of CRP-W2

The powdered fruiting bodies of *C. rutilus* (500 g) were defatted with 95% (v/v) ethanol (5000 mL × 3 times) at 75 °C for 6 h under reflux. After filtered, the residues were dried and extracted with distilled water (8000 mL × 3 times) at 75 °C and 3 h for each time. The whole extract was filtered and centrifuged. The supernatant was concentrated by evaporation under reduced pressure and treated with four volumes of ethanol for precipitation at 4 °C overnight. The precipitation was obtained by centrifugation and dissolved in distilled water, and then the supernatant was deproteinated by a combination of proteinase and Sevag method, followed by exhaustive dialysis with water for 48 h. Finally 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, respectively. The washed precipitate was the crude polysaccharide (CCRP).

The crude polysaccharides (CCRP) was redissolved in distilled water and loaded onto DEAE Sepharose Fast Flow column (2.6 cm × 40 cm) on an ÄKTA explore 100 purification system, eluted first with distilled water and then with stepwise gradient of NaCl solutions (0.1, 0.3, 0.5, and 1 M) at a flow rate of 4 ml/min. Two sharp peaks were collected, dialyzed, lyophilized, and the neutral fraction (CRPsA) eluted with distilled water was further fractioned 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. In addition to one main fraction (CRP), another small amount of polysaccharide was collected, dialyzed and ethanol precipitated to obtain purified polysaccharide, named as CRP-W2.

2.4. Monosaccharide composition, homogeneity and M_w determination

Gas chromatography (GC) was used for identification and quantification of the monosaccharides. ABP-AW1 was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. The monosaccharides were conventionally converted into the alditol acetates as described (Jones & Albersheim, 1972; Oades, 1967) and analyzed by GC as previously mentioned.

Liquid chromatography at ~1.6 Mpa was performed on a LC-10Avp Plus HPLC system (SHIMADZU, Japan) equipped with a TSK-GEL G3000PWXL (TOSOH, Japan) column (7.8 mm × 300 mm) and a RID-10A detector (Shimadzu, Japan). The peak areas were estimated with a System Instrument CLASS-Vp. A 20 μl of sample solution (6 mg/ml) was injected in each run, with 0.7% Na_2SO_4 aqueous solution as the mobile phase at a flow rate of 0.5 ml/min. The column was calibrated with T-series dextrans of known MWs (T-130 80, 50, 25, 10). The M_w was estimated by reference to a calibration curve with T-series dextrans of known MWs (Sun & Liu, 2009).

2.5. Partial hydrolysis with acid

The CRP-W2 (100 mg) was hydrolyzed with 0.05 M TFA (3 ml), kept at 95 °C for 10 h, then centrifuged, the precipitate was sent to GC analysis. The supernatant was dialyzed with distilled water for 48 h, followed precipitated with ethanol. Precipitation in the sack, supernatant in the sack, and the fraction out of sack were dried and carried out for GC analysis as previously described (Sun et al., 2008).

2.6. Periodate oxidation and Smith degradation

The sample (25 mg) dissolved in 12.5 ml of distilled water was mixed with 12.5 ml of 30 mM $NaIO_4$. The mixture was kept in darkness for 48 h at 4 °C. Aliquots (0.1 ml) were withdrawn from the mixture at 3–6 h intervals and read in a spectrophotometer at 223 nm (Linker, Evans, & Impallomeni, 2001) after dilution 250-fold with distilled water. $NaIO_4$ consumption was calculated according the change of the absorbance at 223 nm. The solution of periodate product (2 ml) was used to assess the amount of formic acid by titration with 0.01 M sodium hydroxide, and the rest was dialyzed extensively against tap water and distilled water for 24 h, respectively. The content inside was concentrated and reduced by $NaBH_4$ (60 mg) overnight, neutralized with 50% acetic acid, dialyzed, freeze-dried and analyzed using GC; others were hydrolyzed with 1 M sulfuric acid for 40 h at 25 °C, neutralized to pH 6.0 with

Table 1
The results of methylation analysis of CRP-W2.

Peak no.	Methylated sugar	Molar ratio	Linkage type
Residue A	2,3,4,-Me_3-Gap*l*	2	1,6-linked Gal
Residue B	3,4-Me_2- Gap*l*	2	1,2,6-linked Gal
Residue C	2,3,4,6-Me_3-Man*p*	1	1-linked Man
Residue D	2,3,4,6-Me_4-Glc*p*	1	1-linked Glc

$BaSO_4$, and filtered for analysis. The filtrate was dialyzed and the dialysate out of the sack was lyophilized for GC analysis; the content inside the sack was precipitated with ethanol, the supernatant and precipitate were also dried out for GC analysis after centrifugation.

2.7. Methylation analysis

The sample (20 mg) was methylated three times, according to Needs and Selvendran (1993). The methylated products were extracted by chloroform. The disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum confirmed that complete methylation. The product was hydrolyzed with formic acid and 2 M TFA, and excess acid was evaporated by co-distillation with distilled water. The hydrolyzed product was reduced with $NaBH_4$ for 24 h, and acetylated with acetic anhydride–pyridine (1:1) at 100 °C for 2 h. The alditol acetates of the methylated sugars were analyzed by GC–MS.

2.8. Nuclear magnetic resonance (NMR) spectroscopy

Deuterium-exchanged CRP-W2 (30 mg) was dissolved in deuteroxide (99.99% D, 0.55 mL) accompanied with ultrasonic wave processing for 30 min. Then the NMR spectrometer (Bruker AV-400) was used to perform the ^1H and ^{13}C analysis. Acetone was used as an external standard for the ^{13}C spectrum, and D_2O was used as internal standard for ^1H NMR spectrum (Sun et al., 2008).

The above methods applied in this paper are expressed in a conventional way as other papers published by our research group.

3. Results and discussion

3.1. Isolation, purification and chemicophysical properties of CRP-W2

The crude polysaccharide was isolated from the water-extracted mixture of the fruiting bodies of *C. rutilus* giving a yield of 9%. After purification on DEAE Sepharose Fast Flow column, fraction CRP-W was obtained from the water elution. CRP-W were further purified by gel chromatography on Sepharose 6 Fast Flow column, which yield two peaks, namely one is CRP that had been published by our research group, another small amount is CRP-W2. In order to remove the salts, we load the CRPsA through a Sephadex G-25 column (2.6 cm × 40 cm), with a flow rate of 1 mL/min. We came to a conclusion that CRPsA was homogeneous by the following HPSEC, as shown in Fig. 1, showing a single and symmetrically sharp peak. According to the retention time, the M_w of CRP-W2 was estimated to be 1.3×10^4 Da.

CRP-W2 appeared as a white powder, and was identified as consisting mainly of polysaccharide and protein by the phenol-sulfuric acid method and the Bradford method. The percentages of polysaccharide and protein were 89.2% and 10.4%, respectively. In the UV spectrum of the CRP-W2, it had a positive response to the Bradford test and showed an intense peak at 280 nm, indicating the presence of protein. In addition, CRP-W2 contained no uronic acid evaluated by the meta-hydroxydiphenyl colorimetric method. GC analysis showed CRP-W2 was composed of two kinds of monosaccharides, namely galactose, mannose and glucose with molar ratios of 4:1:1. The results indicated Gal was the predominant monosaccharide.

From the IR spectrum of CRP-W2, it displayed a strong absorption in the range of 1200–1000 cm^{-1}, which suggested that the monosaccharide in CRP-W2 had a pyranose ring. The board intense characteristic peak around 3420 cm^{-1} was due to the hydroxyl stretching vibration of the polysaccharide. The bands in the region of 2914 cm^{-1} and 1632 cm^{-1} were due to C–H stretching vibration and associated water, respectively. Furthermore, the characteristic absorption bands at 832 cm^{-1} indicated that α-glycosidic linkages existing in CRP-W2, which was in good agreement with the results of NMR analysis. The presence of the bands at 875 cm^{-1} and 813 cm^{-1} demonstrated that there was mannose in CRP-W2.

3.2. Structural analysis of CRP-W2

The fully methylated product of CRP-W2 was hydrolyzed with acid, converted into alditol acetates, and analyzed by GC/MS. The results revealed the liberation of 2,3,4,-Me_3-Gap*l* (Residue A: 1,6-linked Gal), 3,4-Me_2- Gap*l* (Residue B: 1,2,6-linked Gal), 2,3,4,6-Me_3-Man*p* (Residue C: 1-linked Man) and 2,3,4,6-Me_4-Glc*p* (Residue D: 1-linked Glc) in a relative molar ratio of 2:2:1:1 (Table 1). Residue A and B were major components of the backbone structure with two non-reducing terminal of Residue C and D at the O-2 position of Residue B. In short, those results from analysis of GC/MS were completely consistent with the observation of partial acid hydrolysis, periodate oxidation and smith degradation.

The chemical shifts of the ^1H and ^{13}C NMR spectrum of CRP-W2 were compiled in Table 2. The spectrum revealed that the substitution pattern of the sugar residues was consistent with the above IR and GC/MS analysis. The signal of δ 5.10, δ 5.15, δ 5.00 and δ 5.06 ppm in ^1H NMR were assigned to anomeric proton of Residue A, B, C and D, respectively. Accordingly, four signals found in the anomeric carbons C-1 region, ranging from 99.9 to 102.6 ppm (100.6, 100.7, 102.6 and 99.9 for Residue A, B, C and D, respectively), indicated the presence of four sugar residues in the repeating-unit, supporting the results of chemical analyses. In the high magnetic field, the δ 80.9 signal should come from C-2 resonance of Residue-B. The signal of C-6 resonance of Residue A and B, which was substituted by other group, was shifted to high magnetic field, with

Table 2
^1H and ^{13}C NMR chemical shift data (δ, ppm) for CRP-W2.

Residue	$^1H/^{13}C$						
	1	2	3	4	5	6a	6b
A: → 6)-α-Gap*l*-(1 →	5.10 100.6	4.02 74.5	4.14 72.3	4.00 72.3	4.32 71.6	3.81 69.3	4.02
B: → 2,6)-α-Gap*l*-(1 →	5.15 100.7	3.93 80.9	4.17 71.2	4.18 72.4	4.24 72.0	3.74 70.1	4.08
C: α-Man*p*-(1 →	5.00 102.6	4.04 70.5	3.78 71.2	3.65 67.4	3.69 74.1	3.81 61.5	3.72
D: α-Glc*p*-(1 →	5.06 99.9	3.80 72.4	3.81 73.7	3.69 70.5	3.98 72.3	3.71 61.26	3.89

respective value of 69.3 and 70.1 ppm. However unsubstituted C-6 Residue C and D had chemical shifts of 61.5 and 61.26 ppm appeared in the normal region. All the NMR chemical shifts were compared with the literature values (Fan et al., 2006; MacLean & Perry, 2010; Mondal, Chakraborty, Rout, & Islam, 2006; Omarsdottir et al., 2006; Roy et al., 2009; Yang et al., 2007; Ye et al., 2008).

4. Conclusion

From the above analysis, we speculated that the structural feature of CRP-W2 from the fruiting bodies of *C. rutilus* possessed the following structure: the backbone consisted of the repeating disaccharide [→ 6)-α-D-Gal*p*-(1 → 2,6)-α-D-Gal*p*-(1 →], which was terminated by two kinds of non-reducing terminal 1-linked Man and 1-linked Glc residues attached to the backbone through O-2 of Gal residues in the ratio of 2:2:1:1. The further detailed structure elucidation would continue in our later research.

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