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Structure, molecular conformation, and immunomodulatory activity of four polysaccharide fractions from *Lignosus rhinocerotis* sclerotia

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ABSTRACT

Four polysaccharide fractions, LRP-1, LRP-2, LRP-3 and LRP-4 were extracted stepwise from *Lignosus rhinocerotis* sclerotia with distilled water at 25, 95, 120 °C and 1.0 M NaOH solution at 4 °C. Their structure, molecular size and chain conformation were clarified using SEC-MALLS-RI, GC, FT-IR and UV–vis. Furthermore, their immunomodulatory activities were evaluated by the model of cyclophosphamide (Cy)-induced immunosuppression. The LRP-1 and LRP-2 were polysaccharide-protein complexes (46–68% β-D-glucan and 27–48% protein), while LRP-3 and LRP-4 were absolutely composed of β-D-glucose. The LRP-4 with low polydispersity had much higher molecular weight (M_w, 5.86×10^6 g/mol) and intrinsic viscosity ([η], 202.6 ml/g) than other LRP fractions. Based on M_w, radius of gyration ($<S^2>_z^{1/2}$) and [η] data with the exponent β of $<S^2>_z^{1/2}$–M_w and its U-shaped curve, all four LRP fractions were highly branched macromolecules and LRP-3 showed a more compact sphere-like conformation than LRP-2 in aqueous solution. Additionally, all four LRP fractions exhibited protective effects against Cy-induced immunosuppression in mice by improving immune organs as well as stimulating the release of major cytokines TNF-α and INF-γ. This work provides a theoretical basis for the application of polysaccharides and their protein complexes from *Lignosus rhinocerotis* sclerotia in food- or drug-based therapies.

1. Introduction

Recently, a popular research interest has focused on the naturally occurring substances that exhibit beneficial therapeutic effects through activating the host immune response [1,2]. Among the bioactive substances, polysaccharides and their protein complexes derived from mushrooms, fungi, yeasts and plants have drawn more attention in the food and medicine fields due to a broad spectrum of nutraceutical and pharmacological efficacies including anti-tumor, immunomodulatory and anti-oxidant activities [3,4]. In many oriental countries, polysaccharides such as lentinan, schizophyllan and a protein-bound polysaccharide (krestin) are generally accepted as immunoceuticals [5–7].

Lignosus rhinocerotis (Cooke) Ryvarden (*L. rhinocerotis*), an edible and medicinal mushroom, is mainly distributed in the tropical rainforest in South China, Malaysia and other Southeast Asian countries [8]. Its sclerotium is regarded as the important part with medicinal value and widely used to relieve diseases, such as breast cancer, cough, fever, asthma, food poisoning, gastric ulcers, chronic hepatitis and liver cancer [9]. Successful domestication and cultivation of this rare mushroom made it possible for researchers to exploit and validate its medicinal benefits [10,11]. The extracts of *L. rhinocerotis* have been demonstrated in limited references to have anti-inflammatory, anti-oxidative, anti-microbial, anti-viral, anti-cancer, anti-coagulant and immunomodulatory activities, attributed to the many bioactive components such as polysaccharides, polysaccharide-protein complexes and proteins [12–19]. In our previous work, a water-soluble polysaccharide-protein complex and a sonication-assisted alkaline-soluble polysaccharide, isolated from *L. rhinocerotis* sclerotia, could stimulate the proliferation of innate immune cells [2,12–14]. The former exerted immunomodulatory effects that were mediated by macrophage activation via the nuclear factor-kappa B (NF-κB) signal pathway or extracellular signal-regulated kinase (ERK) pathway, together with the activation of protein kinase B (AKT) and inducible nitric oxide synthase (iNOS) [2,13]. However, the structure and molecular conformation of these extracts have not been fully clarified yet, due to

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the wide dispersity caused by crude extraction. A basic understanding of the structure and molecular conformation for these extracts from *L. rhinocerotis* sclerotia is essential for successful interpretation of their immunocompetences associated with their molecular structure.

Therefore, the gradient temperature method (25, 95, 120 °C) was applied in the present work for the extraction and fractionation of three water-soluble fractions from *L. rhinocerotis* sclertia (LRP-1, LRP-2, LRP-3); meanwhile, the LRP-4 was extracted with 1.0 M NaOH solution at 4 °C. The structure, molecular size and chain conformation of four LRP fractions were determined by size exclusion chromatography combined with multi-angle laser light scattering and differential refractive index detector (SEC-MALLS-RI), gas chromatography (GC), Fourier transform infrared spectroscopy (FT-IR) and UV–vis spectroscopy. Furthermore, the protective effects of four LRP fractions against cyclophosphamide (Cy)-induced immunosuppression were investigated in mice. The objective of this study is to reveal the structure, molecular conformation and immunomodulatory activity of four LRP fractions, which will provide a theoretical basis for the utilization of *L. rhinocerotis* polysaccharides as potential immunostimulants in food- or drug-based therapies.

2. Materials and methods

2.1. Materials

D-glucose, D-galactose, D-mannose, D-xylose, D-arabinose, D-rhamnose, erythritol and glycerol were purchased from Shanghai Yuanye Biological Technology CO., Ltd (Shanghai, China). Cyclophosphamide (Cy) was purchased from Jiangsu Hengrui Medicine Co. (Lianyungang, Jiangsu, China). ELISA kits were purchased from Nanjing Jiancheng Bioengineering institute (Nanjing, China). Sodium azide (NaN_3) was purchased from Wuhan Huashun Biological Technology Co., Ltd (Wuhan, China). Trifluoroacetic acid (TFA), hydroxylamine hydrochloride and sodium borohydride ($NaBH_4$) were purchased from Aladdin Industrial Corporation (Shanghai, China). Phenol, acetic anhydride, anhydrous ethanol and sodium hydroxide (NaOH) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All chemical reagents were of analytical grade.

2.2. Extraction of four polysaccharide fractions

L. rhinocerotis sclerotia were provided by Hong Kong Polytechnic University. After being milled and dried, the sclerotia power (30 g) was defatted sequentially by Soxhlet extraction with ethyl acetate and acetone for 6 h, respectively. The resultant residue was immersed stepwise in distilled water of 25, 95, and 120 °C at a sample to solvent ratio of 1:25 (w/v) and extracted at each temperature for five times. The extracted supernatant at three temperatures were separately collected, concentrated and precipitated by 4-5 vols of anhydrous ethanol, followed by freeze-drying (FD-1-50, Beijing Boyikang Laboratory Instruments Co., Ltd., China) to obtain three polysaccharide fractions, labeled as LRP-1, LRP-2 and LRP-3, respectively. After being centrifuged (10000 rpm, 20 min), the final residue was extracted for three times by 1.0 M NaOH solution at 4 °C for 4 h. The supernatant was collected by centrifugation (10000 rpm, 20 min), neutralized with hydrochloric acid, and then dialyzed in a dialysis bag (M_w cut off: 8000–12000 Da) against tap water for 5 days and distilled water for 4 days. Finally, it was freeze-dried to yield a polysaccharide designated as LRP-4. The whole extraction procedure is shown in Fig. 1.

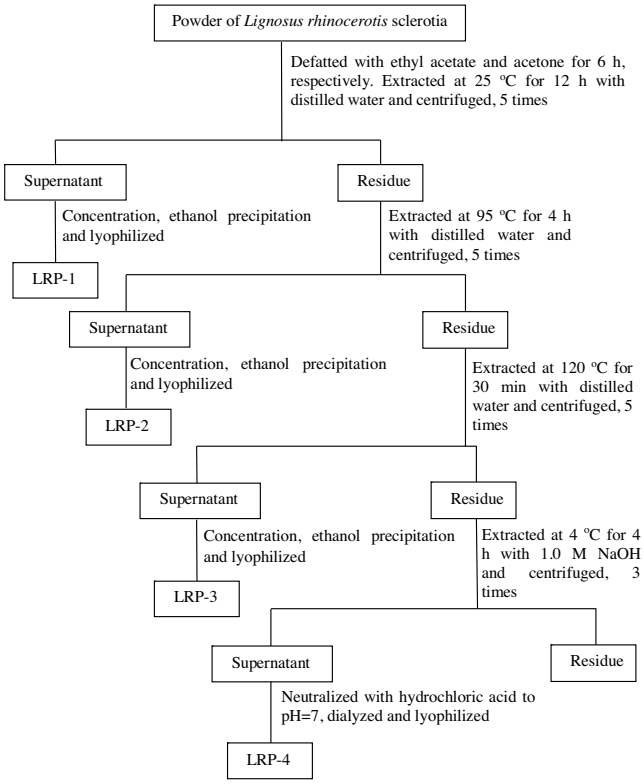

Fig 1. Sequential extraction of the four polysaccharide fractions from *Lignosus rhinocerotis* sclerotia, designated as LRP-1, LRP-2, LRP-3 and LRP-4.

2.3. Chemical composition and monosaccharide composition analysis

Total sugar contents of four LRP fractions were determined by the phenol-sulfuric acid method using glucose as a reference [20]. Protein contents of four LRP fractions were measured according to the modified Lowry method [21].

Monosaccharide compositions of four LRP fractions were examined by aldononitrile acetate method and analyzed by GC as previous description [22]. In brief, the sample (5 mg) was hydrolyzed with 8 M TFA at 100 °C for 12 h. After TFA removed, the hydrolysate was reduced by hydroxylamine hydrochloride (5 mg) and acetylated with acetic anhydride (1 ml) at 90 °C for 30 min. The final aldononitrile acetate derivative was analyzed by Agilent GC 6890N system. The HP-5 column temperature was fixed at 180 °C for 3 min, raised to 300 °C at 2 °C/min. The injector and detector were fixed at 250 and 300 °C. D-rhamnose, D-arabinose, D-xylose, D-mannose, D-glucose and D-galactose were used as monosaccharide standards.

2.4. UV–vis and FT-IR spectra

UV–vis spectra of four LRP fractions were scanned on a UV–vis spectrophotometer (UV-1700, Shimadzu, Japan) from 190 to 800 nm. FT-IR spectra of four LRP fractions were recorded on a FT-IR spectrometer (Nexus 470, Nicolet, UK) using KBr-pellets method in the range of 400–4000 cm^{-1} at the average of 64 scans and 4 cm^{-1} resolution.

2.5. SEC-MALLS-RI measurement

The molecular weight and chain conformational parameters of four LRP fractions were measured using SEC-MALLS-RI. Specifically, the weight-average molecular weight (M_w) and radius of gyration