EFFECTS OF CARBON-SOURCE ON THE STRUCTURAL CHANGES OF POLYSACCHARIDES IN THE DIETARY MUSHROOM *AGARICUS SUBRUFESCENS*

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Agaricus subrufescens, formerly named *A. blazei* Murrill, has been used as dietary mushroom and to formulate nutraceuticals and functional foods. The purpose of this research was to physiochemically characterize the effects of carbon-source on the expression profiles and sugar compositions of polysaccharides from *A. subrufescens*. Polysaccharide was characterized by size-exclusion chromatography (SEC) and high-performance anion-exchange chromatography (HPAEC). A direct dosage effects was shown as sucrose-based medium and a negative dosage effects was shown as malt-based medium. Sucrose or glucose as substrate enhanced the biosynthesis of the very-low-molecular-weight (<1 kDa) polysaccharide. Glucose-feeding in high dose could enhance very-high-molecular-weight-polysaccharide (> 10000 kDa) synthesized. In contrast, sucrose-feeding in high dose was a negative effect for that of the synthesis. Galactose, glucose, and fructose were the dominant sugars in the *A. subrufescens* polysaccharide mixture. The content of glucose and fructose in the polysaccharides increased with increase feeding of sucrose, and that of glucose for glucose-feeding.

Key words: *Agaricus subrufescens*, polysaccharides, carbohydrate

Introduction

The uses of mushrooms as nutritional or therapeutic bases were sometimes daily or even decisive in human progress. They usually exhibit special fragrance and texture.[1] Their biochemical compositions consist mainly of protein, carbohydrate, lipids, and vitamins. Fungal polysaccharides as biomaterials have found a wide range of applications including use in pharmaceutical therapy due to their unique physiological activities.

Agaricus subrufescens (formerly named *A. blazei* Murrill) is an edible fungus of the family Agaricaceae.[2] *A. subrufescens* has not only long been utilized as food and also exhibited anti-tumor,[3,4] anti-carcinogenic,[5] immuno-modulating,[6,7] and chemoprevention activities.[8] Recently, our group demonstrated that the polysaccharides of this mushroom exhibited

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anti-angiogenic activity.[9] Niwa and Itami[10] reported that clinical test of *A. blazei* practice of 1260 cancer patients with chemotheraphy showed high survival rate. Similar clinical test showed that *A. blazei* could improve natural killer cell activity of 100 gynecological cancer patients.[11] Chemical compounds found in *A. subrufescens* include polysaccharides,[12-14] linoleic acid derivatives,[15] and steroids.[16,17] This mushroom is commercially available and is popularly consumed in the formulation of neutraceuticals and functional foods around the world.

Although many investigators have attempted to obtain optimal submerged culture conditions for polysaccharide production from several mushrooms, currently available reports on nutritional requirements in cultures are limited to only a few mushrooms, ie. *Ganoderma lucidum*,[18] and *Paecilomyces japonica*.[19] It would be interesting to investigate the physical and chemical properties of fungal polysaccharides, such as the monosaccharide composition, molecular weight, degree of branching, and extent of branching by side-chain substituents. Therefore, this article describes the characterizations of polysaccharides in its molecular weight distribution and sugar compositions under different carbon-sourced media by *A. subrufescens* in static liquid cultures.

Materials and Methods

A. subrufescens TFRI B148 was curtesy of Dr. Tun-Tschu Chang, Division of Forest Protection, Taiwan Forestry Research Institute, Taipei, Taiwan. All reagents were perchased from Sigma Chemical (St. Louis, MO, USA) except where specified.

I. Liquid culture

Fungi were maintained on potato dextrose agar (PDA) slants and transferred to fresh medium at 3-week intervals. In each pasteurized petri dish, 25 ml of PDA medium (39 g/l) was used and incubated at 28°C for 19 days. For liquid culture, the 19-day-old hyphae of *A. subrufescens* were inoculated into an 800-ml wide base culture flask containing 100 ml of 2 g/l malt extract broth (Fluka Chemie GmbH, Switzerland) as (control) with evaluated medium containing sucrose, glucose or malt extract broth at tested concentrations of 10 to 40 g/l at pH 5.6. The size of inoculum was about 80 mm in diameter. The culture flasks were kept in still, and incubated at 28°C. Polysaccharides were isolated from 49-day-old cultures. Following incubation, mycelia were rapidly washed with 1 l of 250 mM NaCl during aspiration to remove contaminating exopolysaccharides. Samples were then lyophilized, and stored at 4°C and the dry weight of mycelia was measured.

II. Isolation of polysaccharide

Lyophilized mycelia of the various fungi were extracted twice with 80°C water in a 1: 100 (w/w) ratio for 6 h. The extracts were cooled and four volumes of 95% ethanol were added, than allowed to precipitate overnight at 4°C. The precipitated polysaccharides were collected by centrifugation and lyophilized, resulting in a crude brownish polysaccharide sample.

III. Size-exclusion chromatography (SEC) of polysaccharides

A polysaccharide solution in milli-Q water was diluted to give a concentration of 1 mg/ ml and was

then filtered through a 0.22 μm filter (Millipore, MA, USA) before injection onto the SEC column. The flow rate was 0.5 ml/ min, with deionized water being used as the eluent. A calibration curve was constructed using an authentic standard, Sodex P-82 series (Showa Denko America,) containing polymaltotriose with molecular weights of 78.8 × 10^4, 40.4 × 10^4, 21.2 × 10^4, 4.73 × 10^4, and 1.18 × 10^4 Daltons (Da). The TriSec software program was used for the acquisition and analysis of Viscotek data. Size-exclusion chromatography (SEC) signal detection was performed using a ViscoTek model TDA-3-1 relative viscometer (Viscotek).

IV. Hydrolysis of polysaccharides

Acid hydrolysis of polysaccharides was carried out as follows. One milligram of lyophilized polysaccharide was hydrolyzed with 4.95 N trifluoroacetic acid (TFA) at 80°C in a heating block for 4 h. The mixture was cooled, evaporated and then resuspended in milli-Q water.

V. High-performance anion-exchange chromatography (HPAEC) analysis of the carbohydrate composition of polysaccharide

Monosaccharides were separated on an HPAEC system (Dionex BioLC) equipped with a gradient pump, a pulsed amperometric detector (PAD-II) using a gold working electrode, and an anion-exchange column (Carbopac PA-10, 4.6 × 250 mm). Samples were applied using an autosampler (AS3500, SpectraSYSTEM®) via a microinjection valve with a 200 μl sample loop. The analysis of monosaccharides was carried out at an isocratic NaOH (Thermo Fisher Scientific Inc., MA, USA) concentration of 18mM at ambient temperature. Identification and quantification of monosaccharides were made in comparison with standards. Data were collected and integrated on a PRIME DAK system (HPLC Technology, Ltd. UK).

VI. Statistical analysis

The data were presented as mean ± S.E. and n represents the number of experiments. In bar graphs, S.E. values were indicated with error bars. Statistical analyses were carried out by Student's unpaired t tests when applicable. P values of less than 0.05 were considered to be significant.

Results and Discussion

I. Effect of carbon source on the growth and polysaccharides production

To evaluate the effect of carbon source on the mycelial growth, comparisons were made among sucrose-, glucose-, and malt-based medium in the dose range of 10, 20, and 40 g/l of 49-day-cultured mycelia (Fig. 1A). A direct dosage effects was shown as sucrose-based medium and a negative dosage effects was shown as malt-based medium. All the treatments showed significantly enhance mycelia growth as compared with control (malt broth 2 g/l). The maximum mycelial growth reached in the value of 2.84 ± 0.17 g/l feeding of 10 g/l malt extract which was 3.3 fold enhancement than control. All the treatments showed no effect on the polysaccharide production as compared that with control (Fig. 1B).

The in vitro growth of *A. subrufescens* were characterized with respect to the effects of a variety of substrates. Substrates for growth included sucrose, glucose, and malt-extract. Although several kinds of mushrooms frequently require starch, sucrose,

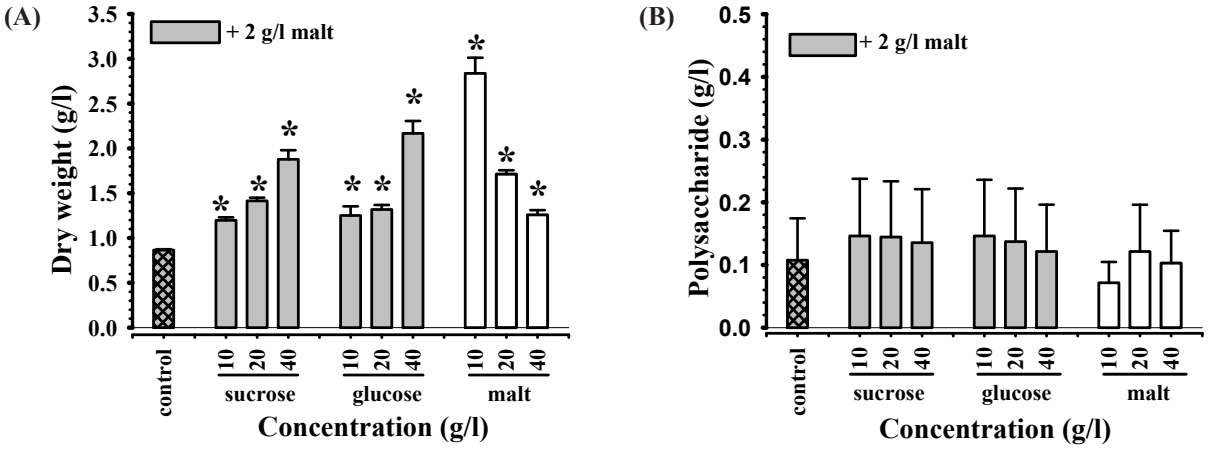

Fig. 1. Effect of carbon source on the (A) mycelial growth; (B) yield of polysaccharide of *A. subrufescens.* Polysaccharides were isolated from 49-day-old of different carbon-sourced medium cultured mycelia. *$p < 0.05$ vs. control, n=5.

maltose, glucose, or galactose as carbon sources for their submerged cultures.[20,21]

II. Molecular mass determination

To investigate the effects of carbon source on the structural variation of fungal polysaccharides, polysaccharides were characterized according to their molecular size distributions and sugar compositions. A calibration curve was constructed using a series of standards containing polymaltotriose with molecular weights of 788, 404, 212, 112, 47.3, 22.8,11.8 and 5.9 kilodaltons (kDa). The regression equation was made between the log [Mw] (Y) and the fraction number (X) as Y = 9.34-0.24X, R^2 = 0.99419. The molecular weight distribution of the lyophilized polysaccharide-containing preparation was chromatographed and characterized as very-high-molecular-weight- (> 10000 kDa, denoted as peak A), high-molecular-weight- (1000-10000 kDa, denoted as peak B), low-molecular-weight- (100-1000 kDa, denoted as peak C), and very-low-molecular-weight-polysaccharides (< 100 kDa, denoted as peak D) (Fig. 2).

To address the effects of different dosages of the same carbon source, we examined if they generated similar polysaccharide polymers in the molecular weight distribution and also different carbon sources, and if they generated different polysaccharide profiles. Interestingly finding was that higher dose of sucrose- or glucose-feeding mycelia resulted in higher proportion of very-low-molecular-weight polysaccharide (peak D) synthesized for *A. subrufescens*. Glucose-feeding in the concentration of 40 g/l could enhance very-high-molecular-weight-polysaccharide (peak A) being synthesized in the percent area of 15.21. In contrast, higher doses of sucrose-fed mycelia resulted in lower proportions of very-high-molecular-weight-polysaccharide (peak A) being synthesized (Fig. 2).

Polysaccharides occur as mixtures of heterogeneous cellular components in nature.[22] Different carbon sources generate similar bioactive polymers with different degrees of branching and distinct polymerisation, producing biopolymers that are more or less water-soluble, and as a consequence, may possess higher or lower biological activity.[23,24] It was reported that the carbon source in the culture medium

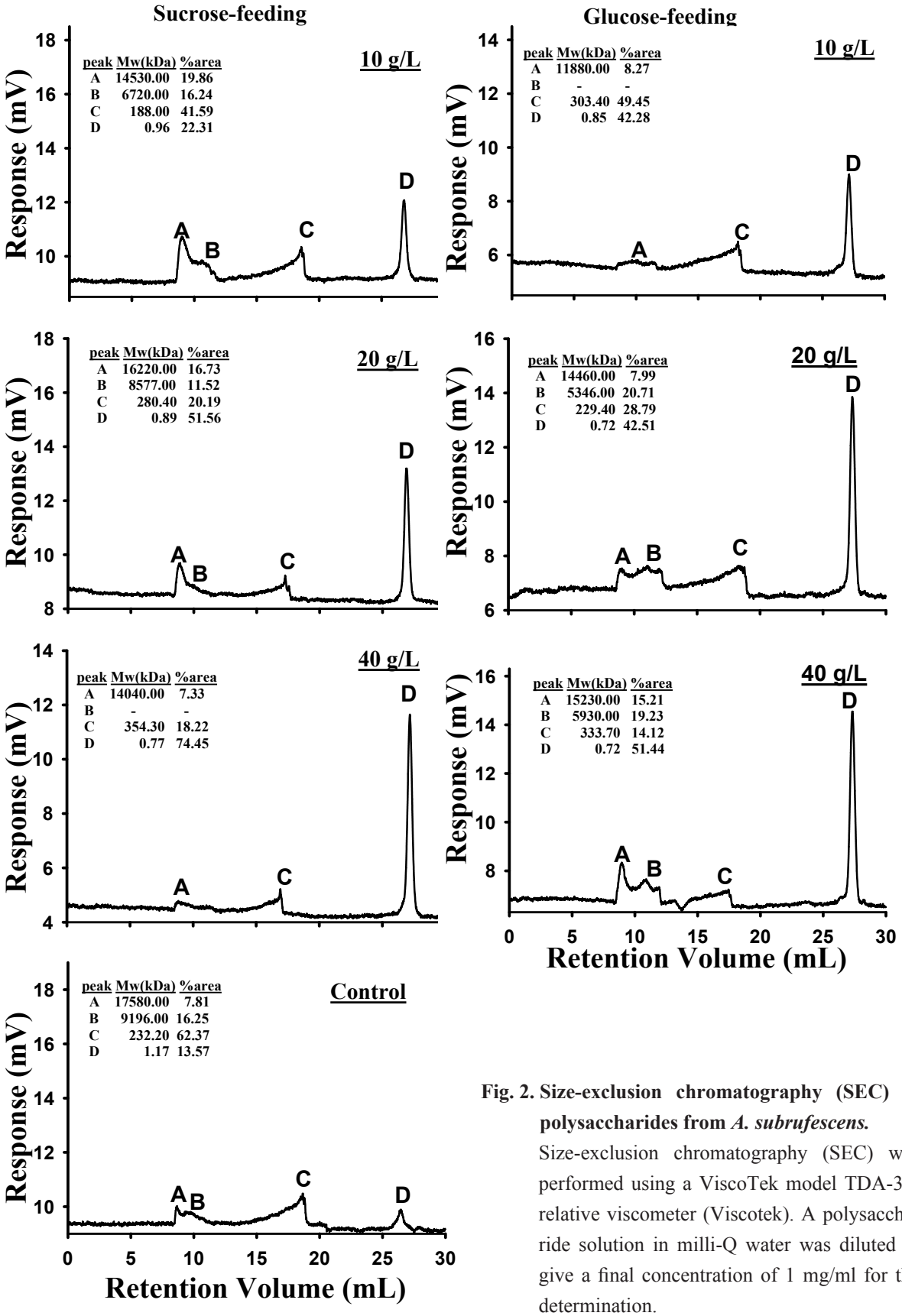

Fig. 2. Size-exclusion chromatography (SEC) of polysaccharides from *A. subrufescens.*

Size-exclusion chromatography (SEC) was performed using a ViscoTek model TDA-3-1 relative viscometer (Viscotek). A polysaccharide solution in milli-Q water was diluted to give a final concentration of 1 mg/ml for the determination.

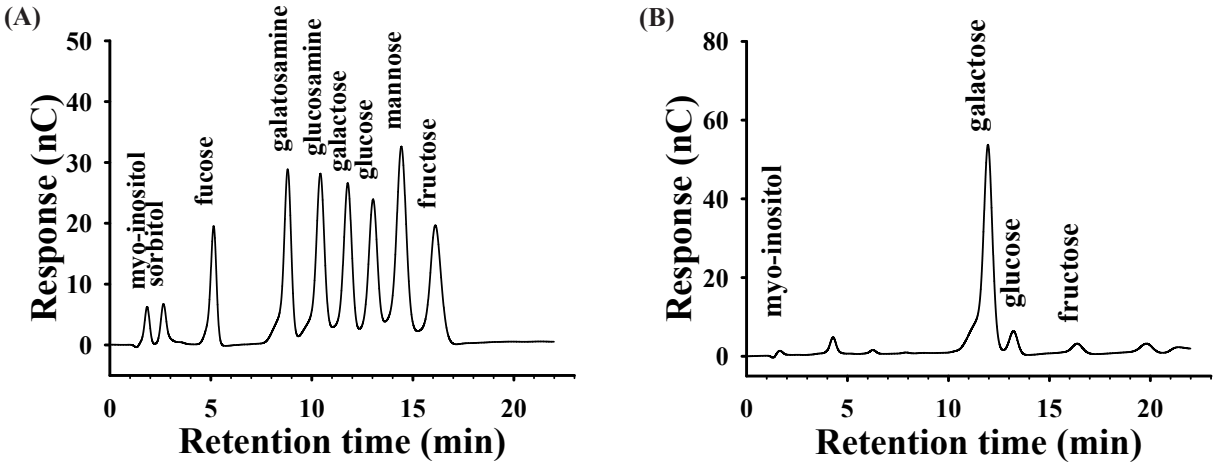

Fig. 3. High-performance anion-exchange chromatography (HPAEC) of *A. subrufescens* polysaccharide hydrolysates. (A) monosaccharide standards; (B) typical chromatogram of polysaccharide hydrolysates of *A. subrufescens*. The HPAEC analysis was carried out in 18 mM NaOH for 22 min at ambient temperature.

of *Botryospaeria rhodina* affected the side chain structures of polysaccharides but not the main chain profile. Sucrose produced less branching (21%) than fructose (31%).[25] In this study, same carbon source in the dose range from 10, and 20 g/l could generated similar polysaccharide profiles, and beyond 40 g/l would be different (Fig. 2). Different carbon source generated different polysaccharide profile.

The results suggest that an increase in the carbon source increased the proportion of polysaccharides of very low molecular weight (< 100 kDa). An excess amount of the carbon source may have been a great advantage to drive the biosynthetic pathway of polysaccharides to low molecular weight complex.

III. Compositional analysis of polysaccharide

To address the question that same carbon source with different dosage, we examined if they generated similar polysaccharide polymers in sugar composition. To study the structure of polysaccharides, sugar composition needs to be analyzed, compositional analysis was performed after polysaccharide was completely hydrolyzed. The chemical profile is shown in Fig. 3, and the composition is presented in Fig. 4. Galactose, glucose, and fructose were the dominant sugars in the content of *A. subrufescens* polysaccharides.

Feeding with glucose resulted in a direct dosage effect in the glucose component in the polysaccharides, and that of glucose and fructose for sucrose-feeding. Feeding with malt resulted in a direct dosage effect in the glucose component in the polysaccharides.

The present study demonstrated an efficient strategy that optimization of medium components for maximum mycelial growth in the value of 2.84 ± 0.17 g/l feeding of 10 g/l malt extract, which was 3.30 fold enhancement. Different carbon sources generated different polysaccharide profiles. The composition of polysaccharides depends on the monosaccharide used for the carbon source.

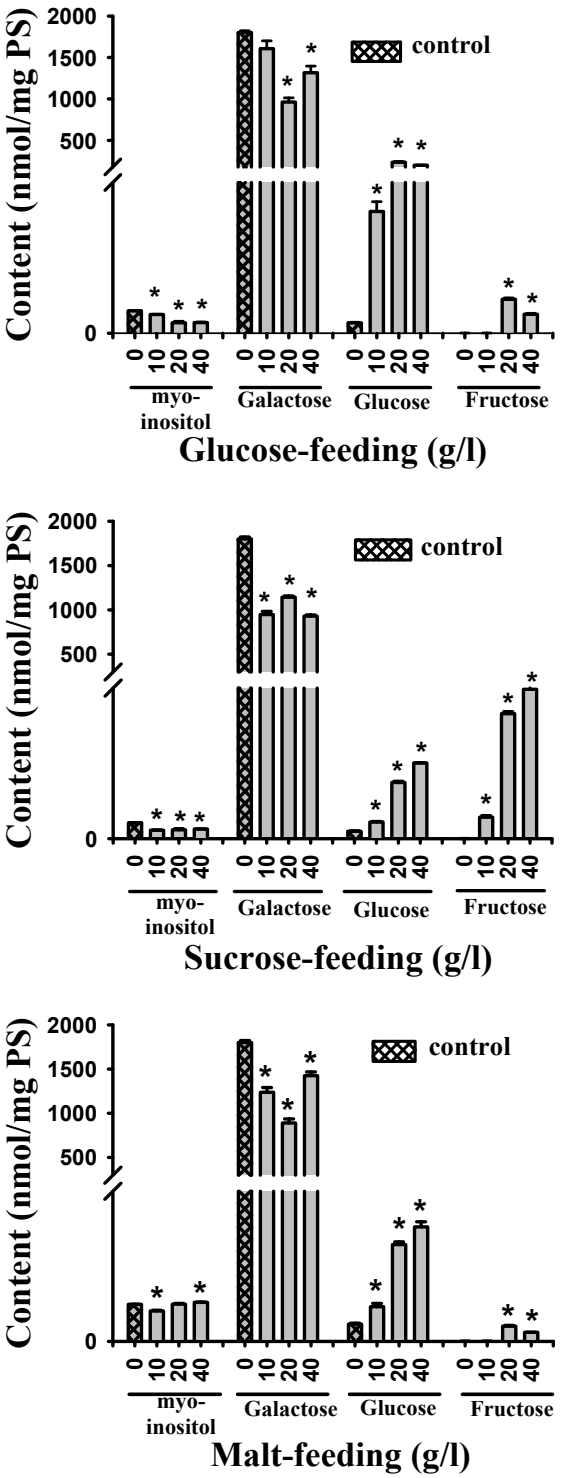

Fig. 4. Effect of carbon source on the sugar composition of polysaccharide of *A. subrufescens*. *A. subrufescens* polysaccharide hydrolysates were analyzed by high-performance anion-exchange chromatography and carried out in 18 mM NaOH for 20 min at ambient temperature. *$p < 0.05$ vs. control, n=4.

Acknowledgements

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食用真菌赭麟蘑菇在不同碳源培養下菌絲多醣體之研究

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赭麟蘑菇 *Agaricus subrufescens* Peck（原名巴西蘑菇，*Agaricus blazei* Murrill），為食用真菌，及被用作保健食品。本研究是探討不同碳源液態培養赭麟蘑菇菌絲體，對菌絲生長及多醣體的影響。以分子篩色層分析及高效液相陰離子交換色層分析對其多醣體作定性分析。實驗數據顯示，以蔗糖培養基培養 10, 20, 及 40 g/l 培養 49 天，對菌絲乾重有正相關的影響。但以 malt 培養基培養 10, 20, 及 40 g/l，對菌絲乾重有負相關的影響。以蔗糖或葡萄糖為碳源培養基培養下，劑量越高，促進生成低分子量（< 1kDa）的菌絲多醣體族群，佔總多醣的比例越高。以葡萄糖為碳源培養基培養下，劑量越高，促進生成高分子量（> 10000 kDa）的多醣體族群，佔總多醣的比例越高，以蔗糖為碳源培養基培養下，劑量越高，則抑制生成高分子量（> 10000 kDa）的多醣體族群。赭麟蘑菇菌絲多醣體之單醣組成分析，以半乳醣、葡萄糖及果糖為主。以蔗糖為碳源培養基培養下，其菌絲多醣體單糖組成，葡萄糖及果糖皆為正相關的劑量效應。以葡萄糖為碳源培養基培養下，其菌絲多醣體單糖組成，葡萄糖為正相關的劑量效應。

關鍵字：赭麟蘑菇、多醣體、碳水化合物

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