

Original

Investigation on the Evaluation Method of Fungi – Polysaccharide Marker Substance for the Identification by Gel Permeation Chromatography–

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Abstract

Polysaccharides of five fungi (two fungi crude drugs such as *Polyporus umbellatus* and *Poria cocos*, three fungi, *Ganoderma lucidum*, *Grifola frondosa* and *Agaricus blazei*, used for functional health foods, were evaluated by employing gel permeation chromatography (GPC) with a special column, which is applicable under the alkaline conditions, and multi angle laser light scattering (MALLS) and refractive index (RI) detectors. Total polysaccharides content by the color reaction using phenolic sulfonic acid was also investigated. Three different extraction methods of polysaccharides, with hot water, hot alkali solution or cold alkali solution, were applied for the sample preparation. As a result, different extraction method resulted in the different GPC pattern and polysaccharide content. Polysaccharide component was detected in all three extraction methods from all five fungi by employing ultra filtration (UF). Especially large amount of polysaccharides was detected in cold alkali extraction from *Poria cocos*. GPC patterns under the different extraction conditions were revealed to be useful for the identification and/or evaluation of fungi. These different results seem to be due to the difference in the sugar conformation of each fungus. Results obtained by iodine–starch reaction and β -glucan specific reaction also indicated polysaccharides structural differences in each fungi extracts in the viewpoint of amount (frequency) and chain length of β -1, 3-glucan and α -1, 4-glucan structure. It was found that polysaccharides, extracted by three different extraction methods with UF membrane, could be a marker substance for the identification and evaluation method of fungi.

Keywords : fungi, polysaccharide, marker substance, identification, crude drug, gel permeation chromatography, multi angle light scattering detection

Introduction

Crude drugs are natural products containing mixture of unpurified active pharmaceutical ingredients (APIs), and used for promotion of the health, prevention of the disease and improvement of the constitution. Some APIs originated from the crude drugs become medical supplies. As far as the crude drugs and as such ex-

hibit efficacy to be provided for the medical use, they must be treated as the same as in the usual synthetic medicines, that is, their quality must be controlled strictly.

As the evaluation of the crude drugs, testing items such as description (color, odor, shape, etc.), identification, heavy metals, arsenic, loss on drying, total ash, acid-insoluble ash, extract content,

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purity test for foreign matter etc. are included in Japanese Pharmacopoeia (JP XV)¹⁾. These testing items are essential to assure the quality of the crude drugs. Among them, identification tests are very important when the crude drugs are powdered or several crude drugs are combined (for example, Chinese medicines (Kampo-yaku)). However, identification tests by the specific marker substances are only known in some botanical crude drugs. There are many crude drugs that have no specific marker substances. In most cases, identification tests are still based on the morphological observation and cannot be applied for the powdered crude drugs, their extracts and fluid extracts. Assay of a specific component, when API is known in the crude drugs, is also very important as in the identification tests.

Recently with progress of chromatographic separation analysis or various other instrumental analyses, component analysis in the crude drugs becomes very important day by day. Some of the authentic marker substances can be obtained from the commercial sources. However, there still exist many crude drugs not known the specific marker substances, especially for not botanical crude drugs such as animal crude drugs, mineral crude drugs etc. Therefore our group started the search for such marker substances and investigations of evaluation method of the crude drugs. We reported²⁾ that nucleic acids and/or amino acids could be marker substances of the animal crude drugs, *Lumbricus* and *Cervi Parvum Cornu*. We found^{3,4)} that inorganic ions measured by inductively coupled plasma atomic emission spectrometer (ICP-AES) might be marker substances in mineral crude drugs. Further, we discovered^{5,6)} that some compounds, which are not well-known marker substances, could be marker substances in the crude drug extracts and in drinkable preparations.

In this paper, we focus on fungi, which have been used as the crude drugs or the functional health foods, and investigate their evaluation methods. Five fungi, named *Polyporus umbellatus*, *Poria cocos*, *Ganoderma lucidum*, *Grifola frondosa* and *Agaricus*

blazai, were employed as the samples. Many reports on polysaccharides⁷⁻²¹⁾ as the main component of fungi have been published for these samples. However, these reports treated almost about characterization of extracts and their pharmacological activities²²⁻²⁸⁾ such as anti-tumor activity, anti-virus activity and immune activating activity. There are few reports on the analytical method of polysaccharides for identification and/or evaluation. So, it is thought that establishment of the evaluation method using polysaccharides as one of the index components specific for each fungus is important and meaningful.

Generally speaking, analytical methods of polysaccharides known are as follows: measurement of molecular diversion by GPC analysis (average molecular weight, polydiversity), viscosity of solution, determination of component sugar unit by enzymatic cleavage or non-enzymatic cleavage (for e.g. acid cleavage). For the purification of polysaccharides, various extraction methods from fungi crude drug have been already published^{8, 18, 20)}. From the investigation based on these previous papers, total polysaccharides content by the color reaction and GPC pattern analysis with a special column and MALLS and RI detection are employed as the evaluation methods of fungi. Three different extraction methods using hot water, hot alkali solution and cold alkali solution are adopted. Combination of polysaccharides content and GPC profile in different extraction are investigated in detail. Further GPC analysis for these fungi, which contain high molecular weight polysaccharides is discussed. Some color reactions such as iodine-starch reaction and β -glucan specific reaction are applied to investigate the structural information of the polysaccharides in fungi extracts.

Experimental

1. Materials and reagents

Five fungi (two crude drugs and three functional health foods) used in this study are summarized in Table 1. Pictures of three

Table 1. Five fungi used in this experiment

Nomenclature	Japanese name	Specification	Source	Origin
<i>Polyporus umbellatus</i>	チョレイ (Chorei)	JP	China	Dried sclerotia of <i>Polyporus umbellatus</i> (Pers.) Fries (Choreimaitake) of <i>Polyporaceae</i>
<i>Poria cocos</i>	ブクリョウ (Bukuryou)	JP	China	Dried sclerotia of <i>Poria cocos</i> Wolf (Matsuhodo) of <i>Polyporaceae</i>
<i>Ganoderma lucidum</i>	レイシ (Reishi)	—	China	Dried fruit body of <i>Ganoderma lucidum</i> (Fr.) Karst. (Mannentake) and their relates of <i>Polyporaceae</i>
<i>Agaricus blazai</i>	アガリクス (Agarikusu)	—	China	Dried fruit body of <i>Agaricus blazai</i> Murril (Himematsutake) of <i>Agaricineae</i>
<i>Grifola frondosa</i>	マイタケ (Maitake)	—	Japan	Dried fruit body of <i>Grifola frondosa</i> (Dicks. ex Fr.) S. F. Gray (Maitake) of <i>Polyporaceae</i>

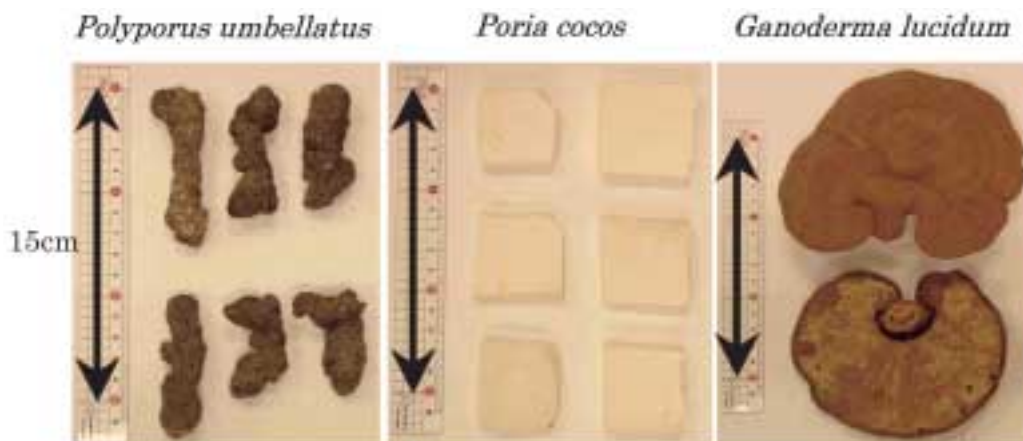


Figure 1. Pictures of three fungi (intact)

fungi are shown in Figure 1. *Poria cocos*, appears as white mass (cut in square slices after outer layer was removed), hard in texture. *Polyporus umbellatus* appears as brown irregularly shaped mass with numerous dents and coarse wrinkles, light in texture. *Ganoderma lucidum* is dried fruiting body as it is. *Polyporus umbellatus* (chorei) (Lot A-1212, A-9754) and *Poria cocos* (bukuryou) (Lot A-2195, A-4611) were provided by Nihon Funmatsu Yakuhin Co., Ltd (Osaka, Japan). They complied with JP XV. *Agaricus blazei* (agarikusu) and *Ganoderma lucidum* (reishi) (Lot A-7471, A-7492, A-8637) were provided by Nihon Funmatsu Co., Ltd (Osaka, Japan). *Grifola frondosa* (maitake) (powder) (Lot FMP 002) was obtained by the commercial source. In the preliminary test, almost the same results were obtained from the different lots, one lot in five fungi was analyzed in this study.

Ethanol (EtOH), hydrochloric acid (HCl) and starch of special grade, curdlan for biochemistry, dextran 200000 and BG STAR A kit for β -glucan specific detection) were purchased from Wako

Pure Chemical Ind. Ltd. (Osaka, Japan). Water was reverse osmosis water by Millipore Corporation (Tokyo, Japan). Sodium dihydrogenphosphate (NaH_2PO_4), disodium hydrogenphosphate (Na_2HPO_4), potassium iodide (KI), iodine (I_2) and sodium hydroxide (NaOH) of special grade and cellulose powder were purchased from Nacalai Tesque (Kyoto, Japan).

2. Instrumentation

A spectrophotometer used was Hitachi U-3300 model (Tokyo, Japan). GPC-RI/MALLS apparatus consisted of a Shodex[®] GPC-SYSTEM 21 (Tokyo, Japan), a DAWN[®] DSP MALLS detector (Wyatt Technology Corporation, Santa Barbara, USA) and personal computer equipped with ASTRA[®] analytical software (Wyatt Technology Corporation, Santa Barbara, USA). A schematic illustration of GPC-RI/MALLS system is shown in Figure 2. GPC was performed with a special GPC column of Shodex[®] OH-pak SB-807 HQ (8.0 mm i.d., 300 mm in length, particle size 35

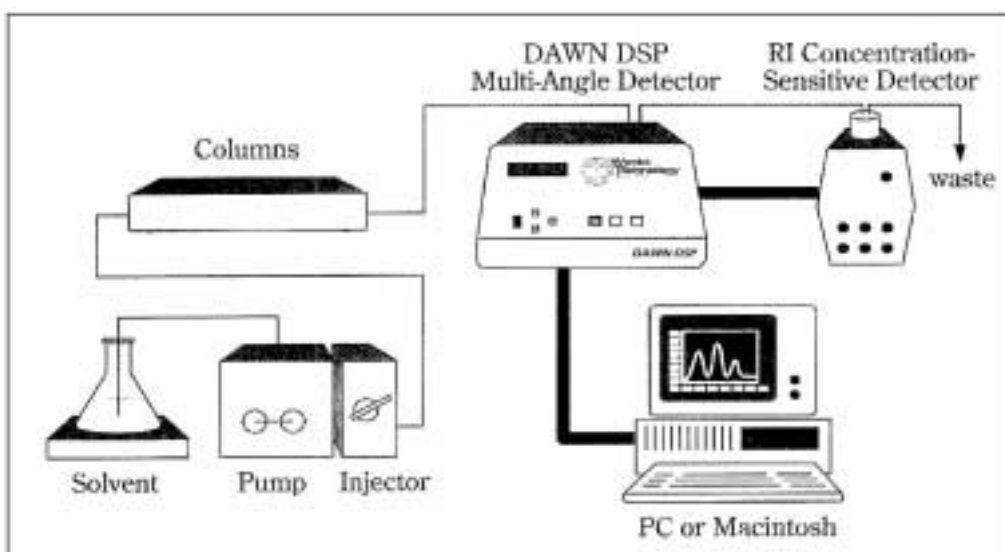


Figure 2. Constituent of GPC-MALLS system

μm) (Tokyo, Japan), which is applicable under the alkaline conditions.

Membrane filter used was Ekicrodisc 13 purchased from PALL Corporation (0.4 μm pore size, New York, USA). Ultra filtration (UF) membrane, Centriplus® (YM-10: 10 kDa molecular weight cut-off) was purchased from Millipore Corporation (Bedford, USA). Water bath, M-100 model (MASUDA Rika, Osaka, Japan) was used for the extraction. Centrifugation apparatus employed was Hitachi himac CR5B2 model (Tokyo, Japan).

3. GPC analysis

A GPC column, OHPak SB-807 HQ, whose size exclusion limit (Ex) is estimated to be 5×10⁸ Da and recommended pH range of the mobile phase is 3 ~ 10, was used. Column temperature was set at a constant temperature of about 40°C. 0.2 M phosphate buffer solution of pH 10.0 adjusted with a 1 N NaOH solution was used as the mobile phase. Flow rate and injection volume were 0.5 mL/min and 100 μL, respectively. For comparison, 0.2 M phosphate buffer solution of pH 6.8, which was made by mixing 0.2 M NaH₂PO₄ solution with 0.2 M Na₂HPO₄ solution, was used.

Sample solutions were prepared as follows: 100 μL portion of each extract (“UF Hot Water Extract”, “UF Hot Alkali Extract” and “UF Cold Alkali Extract”, see below) was mixed with 1 mL of the mobile phase (0.2 M phosphate buffer solution of pH 10). These solutions were filtered with a membrane filter (0.4 μm pore size) prior to injection.

As a GPC column, an OHPak SB-807 HQ column was selected among three candidates, although it guaranteed theoretical plate number (N) is low because of its relatively large particle size. Three candidates are as follows. ① OHPak SB-806 HQ column (8.0 mm i.d. × 300 mm, 13 μm, Ex: 2×10⁷ Da, pH range 3 ~ 10, N: not less than 12000), ② OHPak SB-807 HQ column (8.0 mm i.d. × 300 mm, 35 μm, pH range 3 ~ 10, N: not less than 1500), and ③ TSKgel G 6000 PWXL column (7.8 mm i.d. × 300 mm, 13 μm, Ex: 1×10⁷ Da (estimation), pH range 3 ~ 10, N: not less than 7000).

Polysaccharides containing β structure such as glucan and cellulose can be dissolved only in a strong alkaline solution. For example, high molecular weight β-1, 3-glucan can be extracted only with an alkali solution of above pH 13 like 1 N NaOH and 2 N KOH solutions. For use under the alkaline conditions, column candidates were selected as TSKgel PWXL series columns (Tosoh Corporation) and Shodex® SB-800 HQ series columns. A series of TSKgel SW columns, which are typically used for the separation of biologically important proteins, were not selected because their suitable pH range is 2 ~ 7. Moreover the wider range molecular weight compounds can be analyzed, the more preferable. Therefore the column with maximum Ex was selected. That is, Shodex® OHPak SB-807 HQ column was selected among three candidates.

4. GPC-MALLS detection

Advantage of GPC-MALLS method is that absolute molecular weight can be calculated without need of standards whose molecular weight is known. On the other hand, only apparent molecular weight (correspondent with standard molecule) can be calculated by GPC-RI method. In GPC-RI method, some molecular standards like pullulan must be needed for a calibration curve between retention time (elution volume) and molecular weight (molecular size). But molecular size changes when molecular conformation changes, which was influenced by the sample concentration and molecule structure. Such a conformational difference of molecule between in the sample and the standard solutions causes the gap between actual molecular weight by GPC-MALLS method and apparent molecular weight by GPC-RI method.

Measurement principal of MALLS is as follows. The fundamental relationship linking the intensity of scattered light, the scattering angle, and the molecular properties is known as the following equation (1).

$$K^*C/R(\theta) = 1/M_w P(\theta) + 2 A_2 C \dots\dots\dots (1)$$

R(θ) is the intensity of scattered light at DAWN detector angle θ. C, M_w and A₂ are the sample concentration, the absolute weight-average molecular weight and the second virial coefficient, respectively. K* is an optical parameter equal to the following equation (2).

$$K^* = 4 \pi^2 n^2 (dn/dc)^2 / (\lambda_0^4 N_A) \dots\dots\dots (2)$$

n is the solvent refractive index and dn/dc is the refractive index increment. N_A is Avogadro’s number. λ₀ is the wavelength of the scattered light in vacuum. P(θ) describes the scattered light’s angular dependence. At low angles its variation depends only on the mean square radius independent of molecular conformation or branching. A plot of K*C/R(θ) vs. sin² (θ/2) yields a curve whose intercept gives M_w (average molecular weight).

dn/dc value must be usually measured for each molecule. But analysis objectives are mixture in this experiment. Measurement of each compound is considered to be useless. Therefore dn/dc was set as 0.154, which is supposed to be a typical dn/dc value of polysaccharide solutions (for example, methyl cellulose solution: 0.154, amylopectin solution: 0.155, glycogen solution: 0.153, dextran solution: 0.154). M_w of the polysaccharides mixture extracted from each crude drug was calculated by employing the specific analytical software ASTRA®.

Compared with RI detection, MALLS detection has characteristic that peak area is in proportion to molecular weight. In other words, larger molecule weight compound exhibits larger peak area on MALLS detector. On the other hand, each compound has similar refractive index (RI) to give almost the same peak area on RI

detection. GPC–MALLS system is considered to be effective for evaluation of high molecular compounds in GPC and more sensitive detection of higher molecular compounds.

5. Determination of sugar (polysaccharides) content

5.1. Determination of sugar (polysaccharides) content

Total sugar (including polysaccharides) content or polysaccharides content was determined according to the phenolic sulfonic acid method²⁹⁾ using the glucose solution as the standard solutions for calibration. Content is given in the value corresponding to the glucose concentration as following equation (3).

$$\begin{aligned} &\text{Total sugar (polysaccharides) content (mg/mL extract)} \\ &= 0.1 \times A_{sa}/A_{st} \times (\text{dilution rate}) \dots\dots\dots(3) \end{aligned}$$

100 μL portion of each extract (extracts were diluted with water so that A_{sa} was within 0 to 1.5) was mixed with 5 mL water, then 1 mL of a 5% phenolic sulfonic acid solution was added to start the coloration reaction. 5 min later, UV absorbance at 495 nm was measured as A_{sa} value.

100 μL portion of a 0.1 mg/mL glucose standard solution was mixed with 5 mL water, then 1 mL of a 5% phenolic sulfonic acid solution was added to start the coloration reaction. 5 min later, UV absorbance at 495 nm was measured as A_{st} value.

Total sugar content including polysaccharides is obtained when “All Hot Water Extract”, “All Hot Alkali Extract” and “All Cold Alkali Extract” are employed as sample solutions. Polysaccharides content is obtained from the samples solutions prepared by EtOH addition or UF membrane (see, sample preparation below).

5.2. Iodine–starch reaction (Determination of starch equivalent polysaccharides)

Total polysaccharides content based on iodine–starch reaction was determined using 0.5 N iodine test solution (TS) and the 5 mg/mL of starch solution as the standard solution. Content is given in the value corresponding to the starch concentration as following equation (4).

$$\begin{aligned} &\text{Total starch equivalent polysaccharides content (mg/mL extract)} \\ &= 10 \times (A_{sa}-A_{sb}) / (A_{st}-A_{tb}) \dots\dots\dots(4) \end{aligned}$$

2.5 mL portion of each extract was neutralized with suitable amount of 1 N HCl or 1 N NaOH and was made up to 5 mL by adding water to give a sample solution. UV absorbance at about 685 nm was measured as A_{sb} (blank), then 0.1 mL of iodine TS was added to start the coloration reaction. 5 min later, A₆₈₅ (corresponding to λ_{max} of the starch–iodine conjugate) was measured as A_{sa}.

To 25 mg of starch, 5 mL of water was added to make standard solution (suspended). As the same manner as above–men-

tioned, A₆₈₅ was measured as A_{tb} (blank) and A₆₈₅ after reaction was measured as A_{st}.

0.5 N iodine TS is prepared as follows: To 12.7 g of I₂ and 25 g of KI add 10 mL of water, triturate, and add water to make 100 mL.

The extracts investigated are “All Hot Water Extract” and “All Cold Alkali Extract” (see, sample preparation below).

5.3. Detection of β–1, 3–D–glucan structure (Determination of curdlan equivalent polysaccharides)

Total polysaccharides content containing β–1, 3–glucan structure like curdlan was determined based on the coloration reaction using β–glucan specific detection kit with a curdlan solution as the standard solution for calibration. Content is given in the value corresponding to the curdlan concentration as following equation (5).

$$\begin{aligned} &\text{Total curdlan equivalent polysaccharides content (pg/mL extract)} \\ &= 50 \times A_{sa} / (A_{st}-A_b) \times (\text{dilution rate}) \dots\dots\dots(5) \end{aligned}$$

0.1 mL portion of each extract (or diluted extract quantitatively by water) was mixed with 0.1 mL of main reaction reagent and incubated for 30 min. And 5 mL of coloration reagent 1, 2 and 3 was added to the mixture in this order (main reaction reagent consists of G factor, coagulation enzyme precursor and the peptide derivative substrate: Boc–Val–Leu–Gly–Arg–pNA. Coloration reagent 1, 2 and 3 are individually 0.4 mg/mL sodium nitrite in 0.48 N hydrochloric acid, 3 mg/mL ammonium sulfamate solution and 0.7 mg/mL N–1–naphthylethylenediamine dihydrochloride solution).

UV absorbance at 545 and 630 nm was measured to give A₅₄₅ and A₆₃₀, calculating A_{sa} as A₅₄₅ – A₆₃₀. By the way, A₅₄₅ and A₆₃₀ correspond to λ_{max} of the red pigment of p–NA (para–nitroaniline) and baseline blank. Separately, 0.1 mL portion of 50 pg/mL curdlan standard solution and 0.1 mL portion of β–glucan free water was individually mixed with 0.1 mL of main reaction reagent. Hereinafter the procedure was the same as above–mentioned to get A_{st} and A_b.

UV measurement must be performed within 1 h after coloration reaction. The reaction procedure other than incubation must be conducted in the cold (0°C). And glass equipment must be sterilized at 250°C for 2 h.

The extracts investigated are “All Hot Water Extract”, “All Hot Alkali Extract” and “All Cold Alkali Extract” (see, sample preparation below).

6. Sample preparation (Extraction method)

6.1 Extraction by hot water

“All Hot Water Extract” of the fungi was prepared according to the following method. 1 g of the powder or small portion of the



Figure 3. Sample preparation scheme of “Hot Water” “Hot Alkali” “Cold Alkali” Extract

fungi was extracted with 20 mL of water under reflux at 90°C for 1 h. After cooling to room temperature, the mixture was centrifuged (3000 rpm, 10 min) and the supernatant was collected to make “All Hot Water Extract”.

10 mL of “All Hot Water Extract” were mixed with the same amount of EtOH, and stored in a room at 15°C for 1 day. After the mixture was centrifuged (3000 rpm, 10 min), the supernatant was discarded. And the precipitation was dissolved in 10 mL of water to make “50%EtOH Hot Water Extract”.

“UF Hot Water Extract” of the fungi was prepared according to the following method. 1 g of the powder or small portion of the crude drug was extracted with 20 mL of water under reflux at 90°C for 1 h. After cooling to room temperature, the mixture was centrifuged (3000 rpm, 10 min) and the supernatant was ultra-filtrated (3000 rpm, 2 h) using UF membrane with 10 kDa cut to make “UF Hot Water Extract” (Figure 3).

6.2. Extraction by hot alkali solution

“All Hot Alkali Extract”, “50%EtOH Hot Alkali Extract” and “UF Hot Alkali Extract” of the fungi were prepared according to the same procedure as in section 6.1 using 1 N NaOH instead of water (Figure 3).

6.3. Extraction by cold alkali solution

“All Cold Alkali Extract”, “50%EtOH Cold Alkali Extract” and “UF Cold Alkali Extract” of the fungi were prepared according to the same procedure as in section 6.2 under the extraction condition of 15°C for 1 day instead of 90°C for 1 h (Figure 3).

Results and discussion

1. Detection and determination of sugar (polysaccharides)

1.1. Determination of sugar (polysaccharides) content

Results of sugar content obtained for “All Extract” and their “50%EtOH Extract” are summarized in Table 2. In *Grifola frondosa*, there found a clear difference between the two sample preparations in all extraction methods (hot water, hot alkali and cold alkali). The difference is probably due to the loss of smaller molecular sugar units in “50%EtOH Extract”. In other four fungi, there is no large difference between the two sample preparations. That is, *Grifola frondosa* has smaller molecular sugar units compared with the other fungi. In other words, most of the sugar units contained in the four fungi were found to be polysaccharides.

As the preparation of polysaccharides sample solutions, only 50%EtOH is not enough for the precipitation of polysaccharides. High concentration of EtOH (for example 80%EtOH) also should be considered. Optimized % concentration of EtOH may be existed

Table 2. Suger (polysaccharides) content (n=1)

	<i>Agaricus blazai</i>	<i>Polyporus umbellatus</i>	<i>Poria cocos</i>	<i>Grifola frondosa</i>	<i>Ganoderma lucidum</i>
Hot Water Extract					
All Sugar	3	1	0	26	1
50%EtOH	0	1	0	0	0
Hot Alkali Extract					
All Sugar	5	3	6	44	5
50%EtOH	5	3	6	5	3
Cold Alkali Extract					
All Sugar	6	5	88	15	5
50%EtOH	4	4	85	7	4

Unit : mg/mL glucose equivalent

Table 3. Polysaccharides* content (n=3)

	<i>Agaricus blazai</i>	<i>Polyporus umbellatus</i>	<i>Poria cocos</i>	<i>Grifola frondosa</i>	<i>Ganoderma lucidum</i>
UF Extract					
Hot Water	6±2	3±1	0±0	2±0	1±0
Hot Alkali	7±2	8±4	11±4	14±1	6±1
Cold Alkali	7±2	8±3	>80	14±1	4±1

Average value ± S.D. Unit : mg/mL glucose equivalent *Mw>10kDa fraction

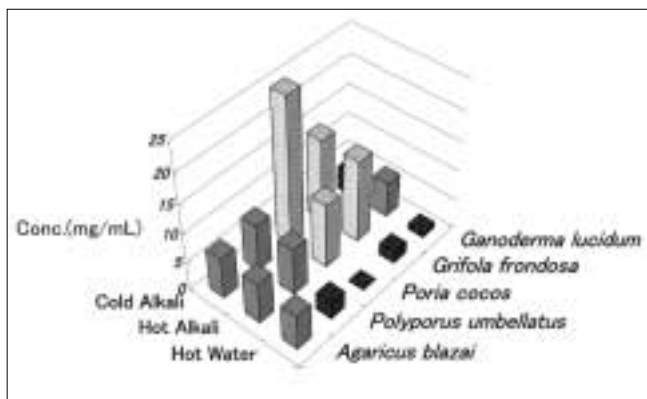


Figure 4. Polysaccharide Content as glucose equivalent

for each polysaccharide. EtOH precipitation method will take large amount of samples and extra times for the precipitation. Then, another method (UF method) was employed for the rapid and certain preparation. Results obtained by UF method are shown in Table 3. Almost the same tendency as in Table 2 (50%EtOH Extract) was observed. UF method is considered to be superior to precipitation method for purification in terms of rapidity and convenience and used in the following study.

Among three extraction methods, *Poria cocos* is characteristic for large amount of polysaccharides extracted by cold alkali solution. Next, *Grifola frondosa* is characteristic for the amount of polysaccharides in both alkali solutions (hot and cold). On the other hand, other three fungi (*Ganoderma lucidum*, *Agaricus blazai* and *Polyporus umbellatus*) exhibited almost the same polysaccharides content and they cannot be distinguished from each other only through the polysaccharides content. Figure 4 shows the results schematically. Then, GPC analysis with MALLS and RI (refractive index) detection was investigated.

1.2. Iodine–starch reaction (Detection of starch equivalent polysaccharides)

This reaction is based on the formation of iodine–starch conjugate. That is, 1 pitch of spiral formed by 6 glucose residues in for e.g. amylose (α -1, 4–bonding) can take in 1 iodine molecule to give blue pigment, whose λ_{max} is about 650 nm. But, it is not so specific for α -1, 4 structure. The reactivity of other polysaccharides was revealed by preliminary investigation.: Curdlan (main structure: β -1, 3–bonding) is about 1/4 of starch. Cellulose (main structure: β -1, 4–bonding) is about 1/4. Soluble starch (starch dissolved in alkaline solution) and dextran (main structure: α -1, 6–bonding) exhibited almost no UV peak at 650 nm(<1/100). So, result was caused by mainly the α -1, 4 structure and partially β -1, 3 and β -1, 4 structure. No linearity was observed between concentration and absorbance. So, results (values) exhibited only tendency.

Results of iodine–starch reaction obtained for “All Hot Water Extract” and “All Cold Alkali Extract” are summarized in Table 4. In *Poria cocos*, no data can be obtained because extract (solution state) changed to gel form in neutralization for measurement. It is thought to be attributed to polysaccharides with straight chained β -1, 3 structure. Judging from the results in “All Hot Water Extract”, which is contributed mainly by α -1, 4 structure, and in “All Cold Alkali Extract”, which is contributed mainly by insoluble β structure, *Grifola frondosa* and *Ganoderma lucidum* are estimated to contain α -1, 4 structure and β -1, 3 structure with some branches like β -1, 6 structure. *Agaricus blazai* is almost the same as *Grifola frondosa* and *Ganoderma lucidum*, but the contribution of β structure is thought to be smaller because of smaller amount of “All Cold Alkali Extract”. *Polyporus umbellatus* is thought to contain α -1, 4 structure and soluble β structure like low molecular weight β -1, 3 and β -1, 6 structure.

Table 4. Polysaccharides detection by iodine–starch coloration reaction (n=1)

Extract	<i>Agaricus blazai</i>	<i>Polyporus umbellatus</i>	<i>Poria cocos</i>	<i>Grifola frondosa</i>	<i>Ganoderma lucidum</i>
All Hot Water	5	5	1	9	7
All Cold Alkali	7	0	—	30	20

Unit : mg/mL starch equivalent

Table 5. Detection of β -1, 3-lucan by specific detection kit (n=1)

Extract	<i>Agaricus blazai</i>	<i>Polyporus umbellatus</i>	<i>Poria cocos</i>	<i>Grifola frondosa</i>	<i>Ganoderma lucidum</i>
All Cold Alkali	0.2	0.5	25.8	0.2	1.8

Unit : mg/mL as curdlan equivalent

1.3. Detection of β -1, 3-D-glucan structure (Determination of curdlan equivalent polysaccharides)

This evaluation method is highly specific to the β -1, 3-glucan structure. So, the results reveal the content of polysaccharides possessing β -1, 3-glycosyl bonding directly, although the development report of this detection kit said that there is some difference of detection sensitivities among polysaccharides with β -1, 3-glucan moiety (curdlan, shizophyllan, lentinan, laminarin and carboxymethyl curdlan) and pH of the extract has an influence on the coloration reaction (optimal pH condition is 7.5~8.0).

Results of curdlan equivalent polysaccharides content obtained for "Cold Alkali Extract" are summarized in Table 5. "Hot Water Extract" and "Hot Alkali Extract" are all less than 100 μ g/mL, because of the low solubility of polysaccharides containing β -1, 3-glucan structure at the neutral pH of both extracts. *Poria cocos* exhibited by far most largest β -1, 3-glucan amount compared with other four. In *Ganoderma lucidum*, a little bit larger amount than residual other three fungi was detected. They were almost the same value.

Some of them are good accordance with reported values^{17,21}. And the other is not¹⁸. The differences between these data acquired this time and reported are thought to be caused by the polysaccharide content and component (structural) difference due to the sample and/or extraction and purification method difference, and so on.

2. GPC analysis

2.1. Effect of buffer pH

Effect of the buffer pH in GPC analysis was investigated by using pH 6.8 and pH 10.0 buffer solutions. As a result, there existed no large difference under the two conditions in hot water extract of *Polyporus umbellatus*, *Grifola frondosa* and *Ganoderma lucidum* (data not shown). It is estimated to be contribution of polysaccharides containing α -structure and/or protein moiety such as glycoprotein and proteoglycan. On the other hand, about alkali extracts in almost all fungi, average molecular weight obtained was lower in pH 6.8 compared with that in pH 10. It can be ascribed to the low solubility of specific polysaccharides in an alkali solution.

The solubility of polysaccharides is reported³⁰ to depend on their structures such as the length of the main chain, branched ratio and bonding moiety of the main and branched chain. For example, α -1, 5'-, α -1, 3'-arabinan whose Mw is 6 kDa can be dissolved

in water. α -1, 4'-, α -1, 6'-glucan like amylopectin (200 ~ 400 kDa), glycogen (100 ~ 1000 kDa) can be dissolved in hot water. β -1, 3'-glucan such as curdlan can be dissolved only in alkali solution. β -1, 3'-, β -1, 6'-glucan like lentinan, schizophyllan (Mw 40~70 kDa) can be dissolved in alkali solution. Smaller size β -1, 3'-, β -1, 6'-glucan like sclerotan can be dissolved even in hot water.

In the following investigation, buffer solution of pH 10 was used because of its wider solubility of various polysaccharides.

2.2. GPC pattern analysis by MALLS and RI detection

GPC analysis by MALLS and RI detection was performed for the sample solutions prepared by the UF method and three extraction solutions (hot water, hot alkali, and cold alkali). The chromatograms are shown in Figure 5, where void volume of the column (exclusion limit) is 5 mL, corresponding to the retention time of ca. 10 min at a flow rate of 0.5 mL/min. Peak valley, which is thought to be caused by water elution, is observed at ca. 17 min. So, permeation limit is estimated to be ca. 8.5 mL. Therefore, on this GPC column, total permeation volume is supposed to be from ca. 5 mL to ca. 8.5 mL. Some peaks detected after permeation limit, which is thought to be caused by the hydrophobic interaction between small molecules toward the column resin, are not intended for the evaluation of polysaccharides.

Agaricus blazai, *Polyporus umbellatus* and *Ganoderma lucidum* could be distinguished by the GPC pattern as shown in Figure 5. Only *Agaricus blazai* in "UF Hot Water Extract" exhibited peaks both in MALLS and RI detection among three fungi. *Polyporus umbellatus* in alkali extracts exhibited a shoulder peak in MALLS detection. *Ganoderma lucidum* in "UF Hot Alkali Extract" exhibited a tiny peak and that in "UF Cold Alkali Extract" exhibited a peak in MALLS detection. In "UF Hot Water Extract", only *Agaricus blazai* and *Grifola frondosa* exhibited a specific peak.

In all fungi investigated, GPC chromatogram patterns of "UF Hot Alkali Extract" were very similar to those of "UF Cold Alkali Extract". This is partly caused by the characteristics of a MALLS detector. As far as largest molecular weight main component remains to some extent, no large difference occurs, because larger molecule weight compound exhibits larger peak area on a MALLS detector.

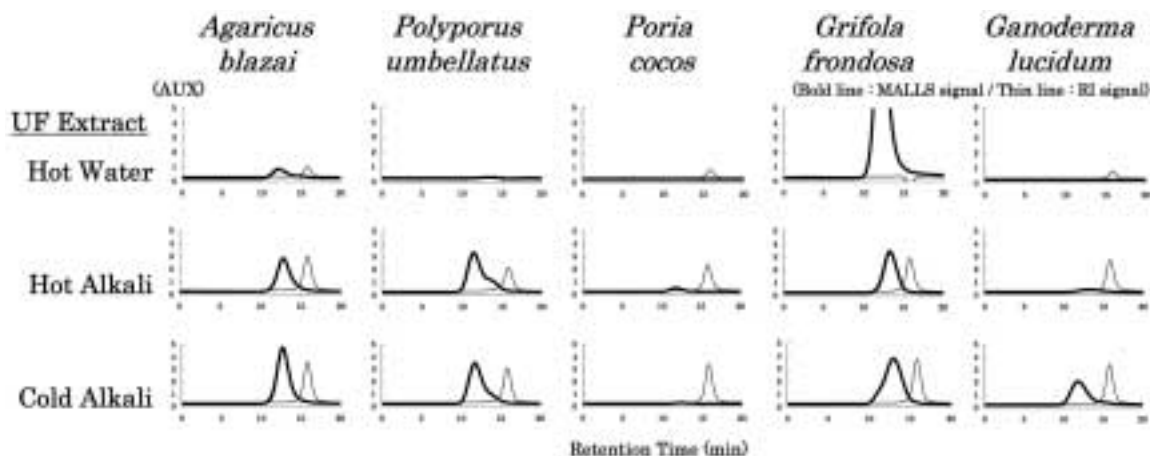


Figure 5. Comparison of GPC chromatogram pattern

2.3. Average molecular weight (Mw) by MALLS detection

Mw of polysaccharides obtained by MALLS detection is summarized in Table 4. In *Agaricus blazai*, *Grifola frondosa* and *Ganoderma lucidum*, large Mw polysaccharides were detected in “UF Hot Water Extract”. In *Polyporus umbellate*, relatively large Mw polysaccharides were detected in both “UF Alkali Extracts”. In others, relatively small Mw polysaccharides were detected. Small Mw polysaccharides were detected in all extracts from *Poria cocos* and in both “UF Alkali Extracts” from *Agaricus blazai*, *Grifola frondosa* and *Ganoderma lucidum*.

In *Poria cocos*, one of the polysaccharides containing is reported to be pachyman whose structure is β -1, 3-glucan chain with branched β -1, 6-glucopyranoside chain, which is supposed to be extracted under the strong alkaline condition like 1 N KOH¹⁸⁾. It is thought to be reasonable that large amount of sugar content in “UF Cold Alkaline Extract” was observed in *Poria cocos*. Lower sugar content in “UF Hot Alkali Extract” than that in “UF Cold Alkali Extract” is thought to be caused by the decomposition (not hydrolysis). The reason there is no significant peak in GPC chromatogram in *Poria cocos* is thought to be due to the low solubility of β -1, 3-glucan toward the mobile phase of pH 10. Polysaccharides containing β structure like β -1, 3-glucan have characteristics to be dissolved hardly into a neutral solution, but, easily into a strong alkali solution. Maybe high molecular components containing β structure precipitate in the mobile phase and thus are not injected

for GPC analysis. If there is a GPC column whose applicable pH is up to 13 ~ 14, alkali extract of each fungus, especially *Poria cocos*, would exhibit higher Mw and large RI and MALLS peaks. Relationship between polysaccharides content and highness of Mw would be observed.

In *Grifola frondosa*, D-fraction is reported to have structure of β -1, 6-glucan chain with branched β -1, 3-glucopyranoside chain²²⁾. X-fraction, which is extracted with hot water under the condition such as 121°C, 30 min, is reported to be glycoprotein whose structure is β -1, 6-glucan with branched α -1, 4-glucopyranoside in sugar chain. GPC profile in “UF Hot Water Extract” is thought to be mainly due to X-fraction. GPC profiles in both alkali extracts are estimated to be contribution of D-fraction or similar structure polysaccharides.

The previous report²⁰⁾ says that in *Polyporus umbellate*, there existed two types of polysaccharides, one of which is the main component extracted with alkaline condition of a 0.4% borate sodium solution and the other is extracted with a hot water after the precipitated with it. These polysaccharides are thought to have the structure of β -1, 3-glucan main chain with branched β -1, 6-glucan and α -1, 4-glucopyranoside at the end. So, it is reasonable that there is no significant component in “UF Hot Water Extract” and above-mentioned corresponding polysaccharide is extracted under the alkaline conditions.

But one question remains about Mw difference. In this experi-

Table 6. Average molecular weight (Mw) obtained by MALLS detector

UF Extract	<i>Agaricus blazai</i>	<i>Polyporus umbellatus</i>	<i>Poria cocos</i>	<i>Grifola frondosa</i>	<i>Ganoderma lucidum</i>
Hot Water	45000	1100	1200	140000	100000
Hot Alkali	1100	7900	60	1500	2700
Cold Alkali	200	15000	1700	1300	1100

Unit : kDa

ment, GPC analysis tended to show larger Mw than reported values in some cases. For example, X-fraction in *Grifola frondosa* is reported to have Mw of about 800 kDa²³. On the other hand, “UF Hot Water Extract” fraction, which probably contains X-fraction, showed Mw of 140000 kDa. The reason may be ascribed to be the contribution of other high molecular weight compounds than X-fraction, inadequateness of dn/dc value (0.154) setting for GPC analysis and incompatibility of column and extract (polysaccharide conformation). Or, growth condition of fungi such as weather, nutritional condition, place of production, and so on, may have some effect on polysaccharides content and composition, which is related to the amount and kind of storage and structural polysaccharides in cell body. That would be one of possible reason for Mw difference, more or less.

By contrast, “UF Cold Alkali Extract” in *Grifola frondosa* exhibited Mw of 1300 kDa. Grifolan NMF-5N, one of the components in “UF Cold Alkali Extract” in *Grifola frondosa* is reported to have Mw of 750 kDa¹². They are in good accordance with each other.

This time, simultaneous extraction method was investigated instead of series extraction method as previously reported for purification method such as removal of organic compounds by ethyl acetate, hot water extraction, cold alkali extraction, followed by hot alkali extraction. If further experiments are planned, investigations for optimization of extraction conditions would be needed. Mild conditions such as 5°C for 48 h in cold alkali extraction and 60°C for 4 h in hot water extraction are preferable for extraction of intact polysaccharides with the least decomposition. But, time consuming procedure is not suitable as an evaluation method. Hot alkali extraction may substitute for cold alkali extraction. So, this method also has space to be revised into more convenient and faster one in near future.

Moreover, as further experiments for verification, this evaluation method should be applied for the plural number of samples from different origins (toughness) and for the plural number of repetitions using the same sample prepared from the same fungi (reproducibility).

Conclusions

Identification and evaluation methods of fungi were investigated by employing GPC analysis and total sugar (polysaccharides) content. Five kinds of fungi were used as samples. Polysaccharides involved in those five fungi were extracted by three extraction methods (hot water, hot alkali, and cold alkali) and UF purification. As a result, it was found that *Poria cocos* and *Grifola frondosa* can be distinguished from other three fungi in terms of polysaccharides content and that each fungus demonstrated specific GPC pattern.

Therefore, among five fungi, *Poria cocos* can be identified by

the most polysaccharides (sugar) content and/or no MALLS signal in all kinds of “UF Extracts”. *Grifola frondosa* can be identified by the second largest polysaccharides (sugar) content and/or biggest peak in the “UF Hot Water Extract”. About other three fungi with small amount of polysaccharides (sugar), *Agaricus blazei* can be identified by the MALLS peak in “UF Hot Water Extract”, *Ganoderma lucidum* can be identified by the no MALLS peak in “UF Hot Alkali Extract” and *Polyporus umbellate* can be identified by the MALLS shoulder peaks in both “UF Hot and Cold Alkali Extracts”.

Finally polysaccharides content and their GPC patterns under the different extraction conditions are found to be useful for the identification and/or evaluation of fungi. High molecular compounds such as polysaccharides could be a marker substance for the identification of fungi, combined with suitable extraction.

Reference

- [1] Jiho, *JP XV*, 1336–1337 (2006)
- [2] Hashimoto A., Nakamura T., Kokusenya Y., Nakai H., Sato T., *Chem. Pharm. Bull.*, 45(3), 487–491 (1997)
- [3] Hashimoto A., Shiba Y., Nakamura T., Kuwahara Y., Nishi H., *Natural Medicines*, 56(6), 239–246 (2002)
- [4] Hashimoto A., Shiba Y., Nakamura T., Nishi H., *Natural Medicines*, 56(6), 247–254 (2002)
- [5] Nakamura T., Hashimoto A., Nishi H., Kokusenya Y., *Yakugaku Zasshi*, 119(5), 391–400 (1999)
- [6] Nakamura T., Hashimoto A., Nishi H., Kokusenya Y., *Yakugaku Zasshi*, 120(2), 238–243 (2000)
- [7] Ohno N., Suzuki I., Oikawa S., Sato K., Miyazaki T., Yadomae T. *Chem. Pharm. Bull.*, 32(3) 1142–1151 (1984)
- [8] Iino K., Ohno N., Suzuki I., Miyazaki T., Yodoma T., *Carbohydrate Research*, 141, 111–119 (1985)
- [9] Ohno N., Suzuki I., Sato K., Oikawa S., Miyazaki T., Yadomae T., *Chem. Pharm. Bull.*, 33(10) 4522–4527 (1985)
- [10] Ohno N., Iino K., Oikawa S., Sato K., Ohsawa M., Yadomae T., *Chem. Pharm. Bull.*, 34(8) 3328–3332 (1986)
- [11] Ohno N., Adachi Y., Suzuki I., Sato K., Oikawa S., Yadomae T., *Chem. Pharm. Bull.*, 34(4) 1709–1715 (1986)
- [12] Iino K., Ohno N., Suzuki I., Sato K., Oikawa S., Yadomae T., *Chem. Pharm. Bull.*, 33(11) 4950–4956 (1985)
- [13] Ohno N., Adachi Y., Suzuki I., Sato K., Oikawa S., Yadomae T., *Chem. Pharm. Bull.*, 34(4) 1709–1715 (1986)
- [14] Ohno N., Iino K., Suzuki I., Oikawa S., Sato K., Miyazaki T., Yadomae T., *Chem. Pharm. Bull.*, 33(3) 1181–1186 (1985)
- [15] Ohno N., Iino K., Takeyama T., Suzuki I., Sato K., Oikawa S., Miyazaki T., Yadomae T., *Chem. Pharm. Bull.*, 33(8) 3395–3401 (1985)

- [16] Ohno N., Ohsawa M., Sato K., Oikawa S., Yadomae T., *Chem. Pharm. Bull.*, 35(6) 2585–2588 (1987)
- [17] Nanba H., Hamaguchi A., Kuroda H., *Chem. Pharm. Bull.*, 35(3) 1162–1168 (1987)
- [18] Kanayama H., Adachi N., Fukai Y., Takeuchi I., Togami M., *Yakugaku Zasshi*, 106(3) 199–205 (1986)
- [19] Kanayama H., Adachi N., Fukai Y., Takeuchi I., Togami M., *Yakugaku Zasshi*, 106(3) 206–211 (1986)
- [20] Miyazaki T., Oikawa N., Yamada H., Yadomae T., *Carbohydrate Research*, 65, 235–243 (1978)
- [21] Miyazaki T., Oikawa N., Yadomae T., Yamada H., Yamada Y., Hon-Yen H., Hitoshi Ito H., *Carbohydrate Research*, 69, 165–170 (1979)
- [22] Yadomae T., *Yakugaku Zasshi*, 120(5) 413–431 (2000)
- [23] Kubo K., Hakushi Gakuronbun of *Kobe Pharmaceutical University*, 23, 75–78 (1988)
- [24] Suzuki I., Itani T., Ohno N., Oikawa S., Sato K., Miyazaki T., Yadomae T., *J. Pharmacobio-Dyn.*, 7, 492–500 (1984)
- [25] Kanayama H., Togami M., Adachi N., Fukai Y., Okumoto T., *Yakugaku Zasshi*, 106(4) 307–312 (1986)
- [26] Adachi Y., Ohno N., Ohsawa M., Sato K., Oikawa S., Yadomae T., *Chem. Pharm. Bull.*, 37(7) 1838–1843 (1989)
- [27] Lina Zhang, Mei Zhou, Jinghua Chen Fanbo Zeng. *Biosci. Biotechnol. Biochem.*, 64(10), 2172–2178 (2000)
- [28] Kubo K., Nanba H. *Mycoscience*, 39: 351–360 (1998)
- [29] Michel D., *Anal. Chem.*, 28(3), 350–356 (1956)
- [30] Nihon Seikagakukai, *Seikagaku databook I*, p 480–498