Hydrothermal Extraction and Micronization of Polysaccharides from *Ganoderma lucidum* in a One-Step Process

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Ganoderma lucidum (*G. lucidum*) is a mushroom-forming white rot fungus that contains a wide variety of bioactive components (glucans). In this study, *G. lucidum* was utilized for the extraction of polysaccharides by hot compressed water at a temperature of 160°C and a pressure of 4.0 MPa using a semi-batch system. Under these conditions, thermal softening of *G. lucidum* occurred, allowing the removal of the polysaccharides protecting other constituents in *G. lucidum* via hydrolysis. Next, the extract was directly atomized by spray drying to remove the water. Scanning electron microscope (SEM) images showed that the particles formed were spherical and dimpled or shriveled with diameters varying from 1 to 6 μm. Based on these results it is proposed that this process is applicable to isolate polysaccharides from other types of biomass and may result in advances in extraction technology to obtain plant biomass components.

Keywords: G. lucidum; Reishi; Polysaccharides; Hydrothermal extraction; Micronization

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INTRODUCTION

Recently, hydrothermal extraction of polysaccharides using hot water or hot compressed water (HCW) has gained much attention as an important process in the field of polysaccharide extraction (Hasegawa *et al*. 2004; Ando *et al*. 2004; Hartonen *et al*. 2007; Sattler *et al*. 2008; Yu *et al*. 2008; Cai *et al*. 2008; Dong *et al*. 2009; Qiao *et al*. 2009; Leppanen *et al*. 2011). Cai *et al*. (2008) studied the effects of hot water extraction parameters on the yield of polysaccharides from *Opuntia milpa alta* and obtained the optimal hot water extraction conditions. They concluded that the main components of the extracted products were isorhamnetin 3-O-(2,6-dirhamnosyl) glucoside and isorhamnetin 3-O-δ-rutinoside. In detail, Qiao *et al*. (2009) reported the optimization of extraction parameters for isolation of *Hyriopsis cumingii* polysaccharides (HCPS) and the purification and preliminary characterization of HCPS prepared. Dong *et al*. (2009) also reported the optimization of hot water extraction (HWE) process of polysaccharides from cultured mycelium of *Cordyceps sinensis* using a Box-Behnken design, followed by canonical and ridge analyses. This process has encouraged an increase in research studies on the extraction technology of polysaccharides from a wide variety of plants or fungi due to the use of polysaccharides in immunomodulatory and anti-cancer electrochemo-

therapy (Wasser 2002). In this work, HCW extraction and micronization of polysaccharides from *G. lucidum* were demonstrated in a single step process. Because of its unique physical and chemical properties (Uematsu and Frank 1980; Marshall 1981; Akiya and Savage 2002), HCW extraction has become known as a "natural and green" alternative method for product extraction and separation. Under these conditions, HCW may be used as both a solvent and a reactant simultaneously in various applications for biomass use.

Hemicelluloses, as one of the main constituents of wood, have recently attracted more attention as possible precursors to different value-added chemicals, with polymeric or oligomeric hemicelluloses possibly being used in health applications due to glucans being a major component (Willfor and Holmbom 2004; Fry *et al*. 2008; Scheller and Ulvskov 2010). Hemicellulose is usually branched with a degree of polymerization (DP) ranging from less than 100 to about 200 units (Gatenholm and Tenkanen 2003). Because of its structure and branched nature, hemicellulose is amorphous, and compared to cellulose it is relatively easy to hydrolyze to its monomer sugars (Yu *et al*. 2008). Therefore, water at 120^{o}C can extract hemicelluloses from wood (Sattler *et al*. 2008). Hasegawa *et al*. (2004) reported that hemicelluloses have been recovered from Japanese apricot trees at 180^{o}C. Naringenin and other antioxidants were also successfully extracted from aspen knotwood at 150^{o}C (Hartonen *et al*. 2007). Allen *et al*. (1996) explained that the recovery of the hemicellulose as monomeric sugars (after a mild posthydrolysis) from sugar cane exceeded 80% at a temperature of 190^{o}C and a pressure of 5 MPa. After fractionation, hemicellulose recovery could exceed 90%. However, Leppanen *et al*. (2011) reported that 170 to 180^{o}C is the most promising temperature range for isolating hemicelluloses with high molecular masses, because in this temperature range a significantly high yield without extensive degradation of the extracted polysaccharides can be obtained.

In this study, the micronization and the drying of particles were conducted in one single-step process (Fig. 1) in order to maintain or even to concentrate the glucans content in the extract water. This process was expected to produce a suitable particle size and remove most of the solvent; therefore, the products are most stable at their monolayer moisture content, which varies with chemical composition and structure (Tsotsas and Mujumdar 2011). The produced powders were characterized with respect to morphologies, particle size, and particle size distribution.

EXPERIMENTAL

Materials

G. lucidum obtained from Refarmer Co., Ltd. (Kumamoto, Japan) was used as a starting material. It was shredded by a laboratory mill to a particle size of < 2 mm and passed through 16-mesh sieves. The sample was then refrigerated at < 278 K.

Distilled water obtained from a water distillation apparatus (Shibata Co., model PW-16, Japan) was used as a solvent.

Potassium hydroxide (KOH, 85.0%), Sodium hydroxide (NaOH, 97.0%), Hydrochloric acid (HCl, 35.0~37.0%), Acetic acid (CH_3COOH, 99.9%), 2,5-dihydroxybenzoic acid (DHB, 98.0%), and analytical reagent (methanol, 99.7%) were purchased from Wako Pure Chemicals Industries Ltd., Japan, and were used without further purification.

Experimental Setup and Procedure

The assembled apparatus consisted of an extraction unit and a precipitation unit (Fig. 1). In the extraction unit, a high-pressure pump (LC-6AD; Liquid Chromatography pump, Shimadzu, Japan) and pre-heater were used to introduce hot water to the reactor (10 mL in volume; Thar, USA). The pre-heater was fabricated from 1/8 inch stainless-steel tubing (SUS316) with a volume of 50 mL and was also placed inside an oven in conjunction with the reactor. After the reactor inclusive of 1.0 g of *G. lucidum* was added to the system, distilled water at room temperature was pumped through the reactor inclusive pre-heater for a few minutes to purge air and completely wet the *G. lucidum*; the system was then pressurized to the set pressure of 4.0 MPa through the back pressure regulator (BPR; AKICO, Japan).

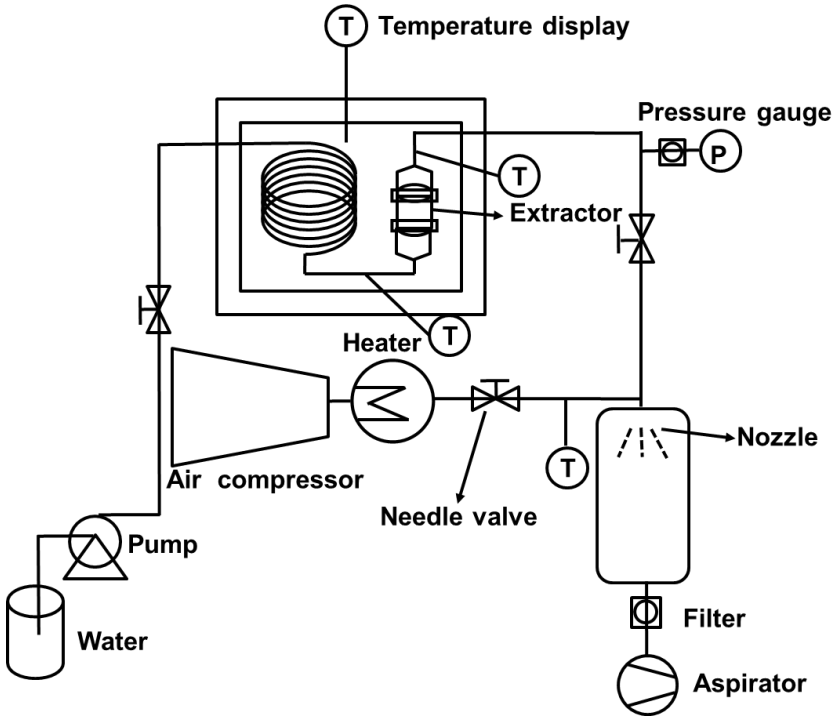

Fig. 1. Schematic diagram of hydrothermal extraction and micronization

When the system reached the desired pressure and steady state was achieved, the electric heater was applied to heat the water [160°C, with a temperature range of 158 to 166°C]. This condition was selected based on the previous researcher's reports (Allen *et al*. 1996; Hasegawa *et al*. 2004; Ando *et al*. 2004; Hartonen *et al*. 2007; Sattler *et al*. 2008; Yu *et al*. 2008; Cai *et al*. 2008; Dong *et al*. 2009; Qiao *et al*. 2009; Leppanen *et al*. 2011). The time required to heat the reactor from room temperature to the desired temperature was between 5 and 8 min, after which the reactor temperature equaled the temperature of the electric heater. After the temperature at the reactor area reached a preset temperature, the pump was used to feed water at 1.0 mL min^{-1}. Next, the outlet solution was directly sent to the precipitation unit via a nozzle and contacted with hot air (3 L min^{-1}) co-currently. Three inlet air temperatures, 135, 170, and 200°C, were applied. These inlet air temperatures corresponded to outlet air temperatures of approximately 52,

60, and 67°C, respectively. The solution was atomized in hot air current to instantaneously obtain a powder due to heat and mass transfers between the dry air and the water. Due to the decreasing water content and water activity, this process could ensure a microbiological stability of products, avoid the risk of chemical and/or biological degradations, reduce the storage and transport costs, and finally obtain a product with specific properties (Gharsallaoui *et al*. 2007; Tsotsas and Mujumdar 2011). An aspirating pump (AS-01, Aspirator, As One, Japan) mounted at the end of the system drove the airflow, which was modulated by means of an inlet valve. A filter (60 μm; Swagelok, Japan) was placed before the aspirating pump to collect the fine powder products. The powder samples were transferred to sealed bottles and refrigerated until analysis.

Particle Characterization

The morphologies of the powder products were observed using a scanning electron microscope (SEM; JEOL JSM-6390LV). After gold coating, the powder particle diameters were measured from the SEM image using image analyzer software (Image J 1.42). It is well known that SEM has the capability to detect particle diameters and morphologies, but the resolution is lost at extreme magnifications. Also, SEM requires the samples to be electrically conductive; therefore, for most of the powders produced, a gold or platinum coating must be applied that may alter the diameter readings at higher magnifications. SEM remains, however, a quick method for observing the powders produced and it requires a very small sample size to obtain results.

RESULTS AND DISCUSSION

Figure 2 shows the morphology of particles formed by spray dryer with air temperatures of (a) 135, (b) 170, and (c) 200°C, respectively. In a co-current process, the solution product was sprayed in the same direction as the flow of hot air, which had a typical inlet temperature of 150 to 200°C. Contrary to the air inlet temperature, the air outlet temperature could not be directly controlled, since it depended on the air inlet temperature. The ideal air outlet temperature for the microencapsulation of food ingredients has been reported to be 50 to 80°C (Gharsallaoui *et al*. 2007); therefore, in this experiment, the particles generated were unaffected by air inlet temperature mostly because of the robustness of the particle wall barrier. As mentioned, droplet drying (water evaporation) starts instantaneously after atomization. At that time, balances of temperature and vapor partial pressure are established between the liquid and gas phases. Thus, heat transfers from the air towards the product as a result of temperature difference, whereas water transfers in the opposite direction due to the difference in vapor pressure (Desobry *et al*. 1997; Cao *et al*. 2000; Elversson and Millqvist-Fureby 2005). Essentially, most of the particle surfaces were uniform and had spherical shapes with diameters less than 6 μm. This can be explained by the fact that the particles may have good solubility in water, which resulted in the typical spherical shape and solid particle morphology (Weiler *et al*. 2010). The presence of polysaccharides in the water led to the spherical particle formation (Twu *et al*. 2003; Asada *et al*. 2004; Muzzarelli *et al*. 2004; Guerrero *et al*. 2010). Asada *et al*. (2004) reported that spray-drying pharmaceutical preparation produced spherical particles with the existence of chitosan; however, minute acicula particles and whiskers on the particle surfaces still occurred. Twu *et al*. (2003) suggested

that polysaccharide microspheres prepared from spray-drying exhibit a spherical geometry and a smoother surface morphology. These microspheres are found to have a particle size smaller than 3 μm.

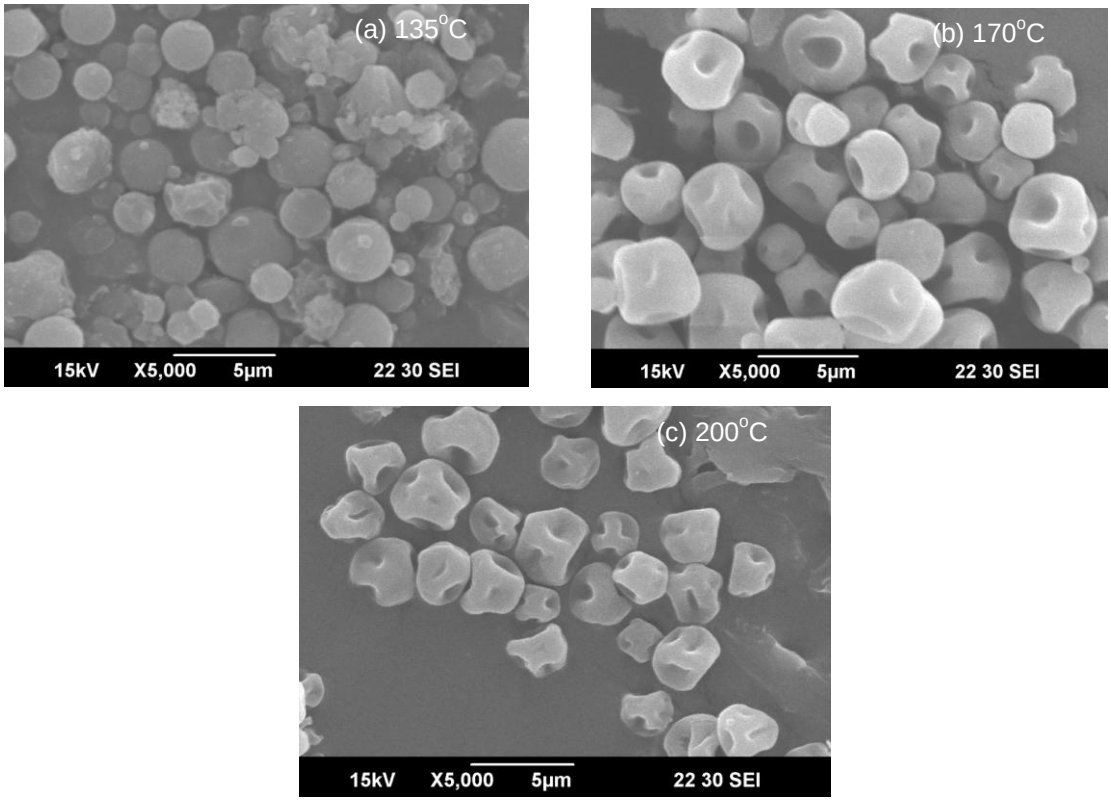

Fig. 2. SEM images of generated particles having different temperatures of air contact

Figure 3 shows the particle size distributions of the powder products, with average particle diameters of (a) 2.3, (b) 1.6, and (c) 1.3 μm, respectively. The geometric diameter of particles that are not perfect spheres depends on their orientation. In this case an average geometric diameter can be substituted, *e.g.* by averaging Feret's horizontal and vertical diameters (Vehring *et al*. 2007; Vehring 2008). In general, the use of a higher inlet air temperature led to the production of larger particles, which is related to the higher swelling caused by the higher temperature (Tonon *et al*. 2011; Nijdam and Langrish 2006). Tonon *et al*. (2011) explained that drying at higher temperatures results in faster drying rates, which leads to the early formation of a structure and does not allow the particles to shrink during drying. When the inlet air temperature is low, the particle remains shrunk and thus, with a smaller diameter. Nijdam and Langrish (2006) obtained similar results, working with the production of milk powder at 120°C and 200°C. On the contrary, different results were obtained in this work. When the temperature air contact changed from 135 to 200°C, the particle size was slightly reduced. This can be explained by assuming that the bonding of water to the hydroxyl groups in an extract of *G. lucidum* was strong. At a lower air temperature, therefore, the internal water of amorphous solids transfers slowly because the remainder of the water diffuses out through the solid (Cervera *et al*. 2011). In addition, the Peclet number affected by the combination of

material properties of the solute and the solvent had a high influence on the spray drying process, which controls the evaporation performance (Vehring 2008; Weiler *et al*. 2010).

Figure 3 also allows a qualitative evaluation of the morphology of the particles with the increasing inlet air temperature. Because the three SEM images reported in Fig. 2 were obtained at the same enlargement, it is possible to qualitatively assess the particles morphology produced. At a low Peclet number (less than 1) the diffusional motion of the solutes is faster compared to the radial velocity of the receding droplet surface and is typically associated with spherical and solid particle formation (Fig. 2 (a)). In the case of a Peclet number greater than 1, the surface moves faster than the dissolved or suspended components, resulting in shell formation (Fig. 2 (b-c)). The results showed that the surface becomes enriched with the component associated with the high Peclet number. Depending on the nature of the component, different solidification mechanisms are triggered once a critical concentration at the surface is reached. Solutes may not have sufficient time to crystallize during the precipitation window; therefore, the spherical particles can become hollow if the shell becomes rigid quickly and does not buckle or fold; otherwise, dimpled or shriveled particles are formed (Vehring 2008).

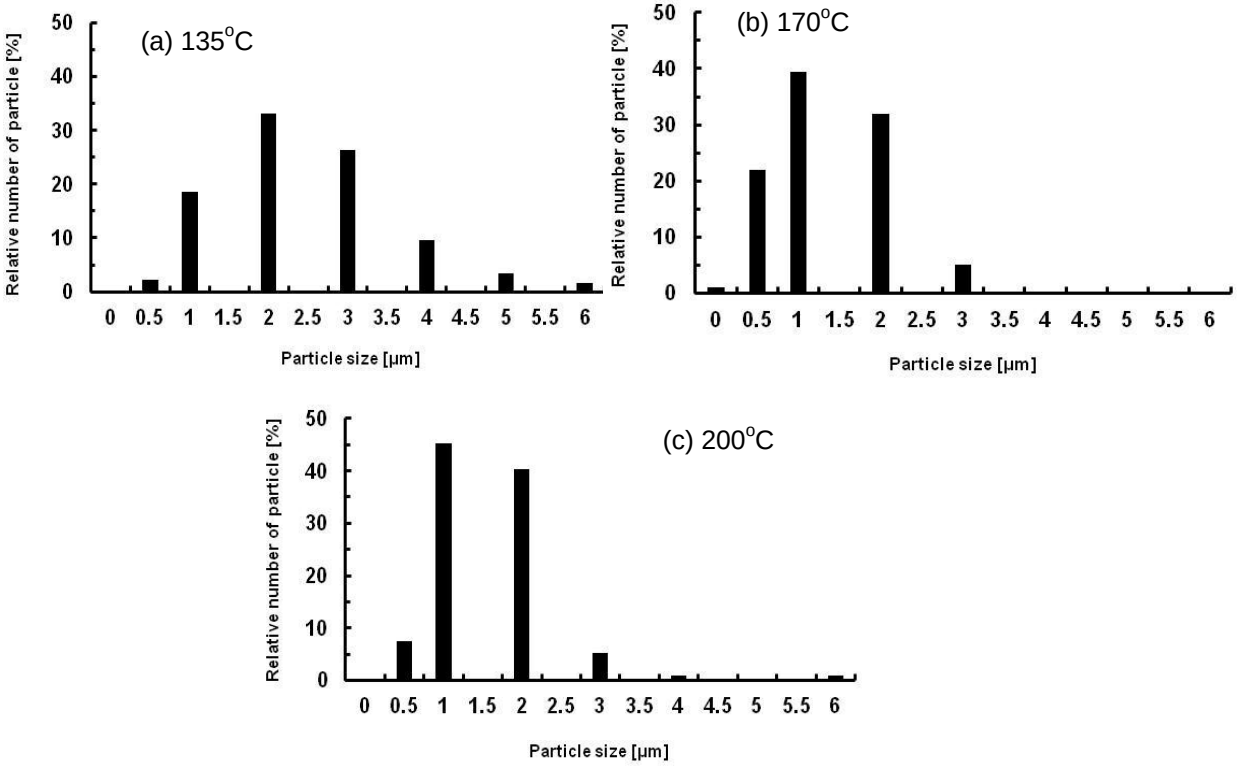

Fig. 3. Particle size distribution of powders produced with different air contact temperatures

In order to understand the distribution of molecular weight of the compounds, an analysis using MALDI-TOF-MS was performed on a Bruker Tektronix TDS 504D GmbH Reflex III (Germany) with dual micro channel plate detectors for both linear and reflectron modes. The acceleration voltage was +25 kV, and ions were measured in the reflectron mode. A nitrogen laser at 337 nm and a 3 ns pulse width was utilized. The applied laser energy was focused on a spot of size 200 μm x 50 μm. The laser energy was measured with a Laser Probe Rm-3700 Universal Radiometer (Laser Probe, Inc. Utica,

NY), placed after the optics and before the last mirror prior to the source. The laser energy range measured and used for each polymer sample was defined by the ability to obtain a measurable mass signal of the polymer. Samples used for the MALDI analysis were prepared as follows: the matrix used was DHB; 10 μL of particles were dissolved in water and mixed with 30 μL of DHB solution (1 mg mL^{-1} in methanol); 1 μL of this mixture was then spotted on the MALDI sample holder and slowly dried to allow co-crystallization. This tool was also developed to successfully analyze biopolymers and macromolecules [Bahr *at al*. 1994].

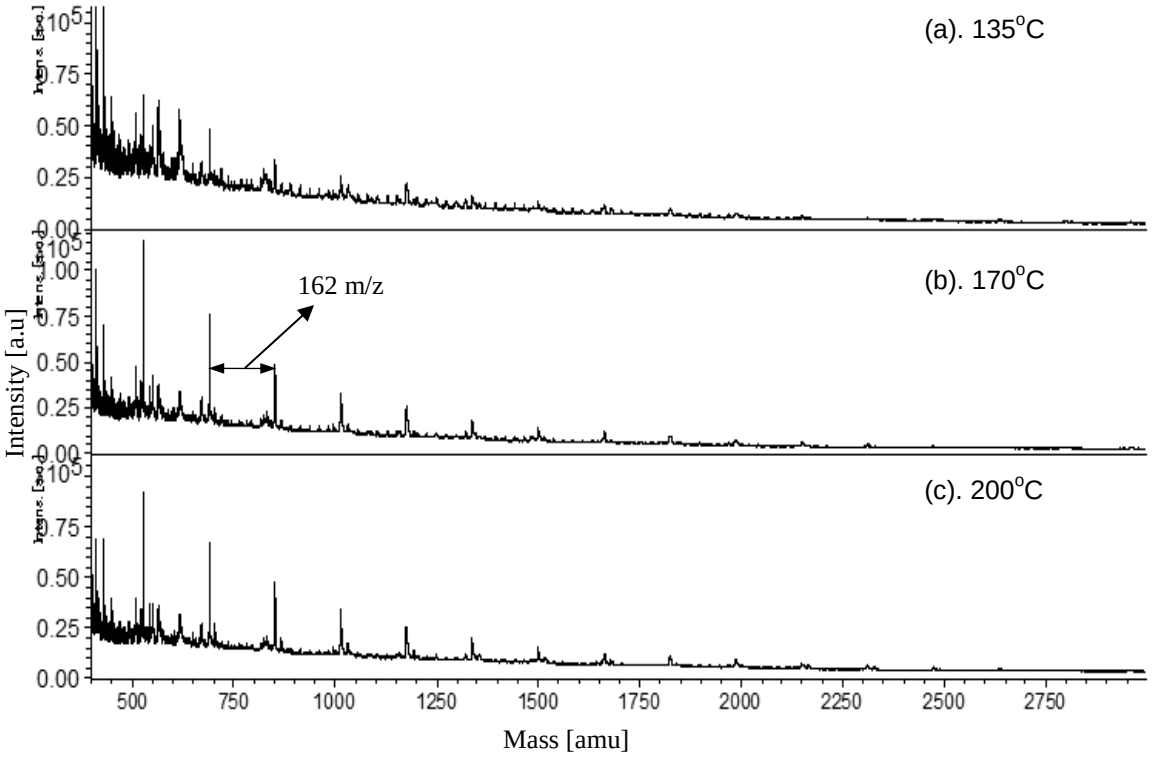

Fig. 4. MALDI-TOF MS of water-soluble products with air temperature contacts of (a) 135, (b) 170, and (c) 200°C, respectively

Figure 4 shows the MALDI spectra of water-soluble products from *G. lucidum* obtained by hot compressed water at 160°C. A unique advantage of the MALDI-TOF MS method lies in the ability of the matrix to dissipate the heat energy created by rapid laser heating; hence, the polymer vaporizes with almost no decomposition and can be easily detected. The peaks in each distribution are separated by the unit of mass. The difference in the peak intensity qualitatively corresponds to the amounts of dissolved plant components and their derived compounds in water; however, the non-homogenous spread of the sample on the target spots could not render a quantitatively precise analysis. Nonetheless, molecular weight distributions could be clearly observed. As shown in Fig. 4, the thermal extraction of plant components and their derived compounds from *G. lucidum* commenced and occurred at 160°C, resulting in species with molecular weights of 500 to 2600 m/z. This molecular weight region might correspond to the existence of hemicellulose groups involving linkages at the glycosidic bonds. Leppanen *et al*. (2011) explained that at low temperatures (< 160°C) only small amounts of carbohydrates from Norway spruce were dissolved, but the amount of extracted hemicelluloses increased

steadily from 160°C. Song *et al*. (2012) suggested that the yield of hemicellulose from spruce wood reached a plateau after 40 min extraction at 160 to 180°C. At these temperature ranges, the degradation of saccharides, including depolymerisation and deacetylation of monosaccharides, become dominant. In this study, the compound in monomer units extracted by hot compressed water was not observed; however, information regarding glucans derived from *G. lucidum* polysaccharides was obtained. According to the mass spectrum given in Fig. 4, the mass region of ion peaks was observed with a peak-to-peak mass difference of 162 m/z, consistent with the repeating unit of the glucan. This figure shows that glucans from *G. lucidum* are composed of a mixture of glucose polymers with molecular weights of 688 to 2632 m/z. Similar observations were reported by Hung *et al.* (2008), which were attributed to the fragmentation of the high mass polysaccharides. Chang and Lu (2004) also reported that the major biologically active polysaccharides from Lingzhi are glucans, whose basic structure is *β*-1-3 D-glucopyronan with 1 to 15 units of *β*-1-6 monoglucosyl side chains and with (1→3)-*β*, (1→4)-*β*, and/or (1→6)-*β* linkages. The bioactive polysaccharides differ greatly in their composition and consequently in chemical structure, and one common feature is their molecular weight, which has a wide range. These results show thermal extraction of glucans from *G. lucidum* by hot compressed water to be a promising method and indicate that the process occurred via hydrolysis, dehydration, and condensation.

In order to determine the *β*-glucan contents in the particles formed, a mushroom and yeast *β*-glucan assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland) was used following to the manufacturer's instructions. In brief, for determination of total glucan content (*α*- and *β*-), 5 mg of particles dissolved in water was suspended in 1.5 mL of concentrated HCl (37% v/v) and incubated at 30°C for 45 min; 10 mL distilled water was then added, and it was placed in a boiling water bath for 120 min. The pH was neutralized with 10 mL of 2 M KOH, followed by centrifugation for 10 min. 0.1 mL of the solution was digested with an aliquot of exo-1,3-*β*-glucanase (20 U/mL) plus *β*-glucosidase (4 U/mL) in 200 mM sodium acetate buffer (pH 5.0). The hydrolysates were incubated with 3.0 mL of glucose oxidase-peroxidase mixture (GOPOD) at 40°C for 1 h. The absorbance of the solution was measured at 510 nm by using ultraviolet-visible (UV–vis) spectrophotometry V–550 (Jasco Corporation, Japan). UV–vis spectra were recorded with a PC–driven scanning spectrophotometer operating in the fast scan mode, allowing spectra of between 190 and 800 nm with 10 nm min^{-1} of bands. For measurement of α-glucan, 5 mg of particles dissolved in water was suspended in 2 mL of 2 M KOH for 20 min and neutralized with 8 mL of 1.2 M sodium acetate buffer (pH 3.8). Then, the solution was centrifuged for 10 min and aliquots of amyloglucosidase (1630 U/mL) plus invertase (500 U/mL) were added to the 0.2 mL of solution, followed by incubation at 40°C for 30 min. The solution was incubated with 3.0 mL of glucose oxidase-peroxidase mixture at 40°C for 20 min; and then the absorbance was measured at 510 nm. The concentration of *β*-glucan was determined by subtracting *α*-glucan from the total glucan content. The result showed that the *β*-glucan content in the particles was 40 to 45% in weight, approximately. This result is in agreement with the previous research in which water soluble *β*-glucans was isolated from *G. lucidum* (Cheong *et al*. 1999; Wasser 2005).

CONCLUSIONS

Hot compressed water extraction and micronization of polysaccharides from *G. lucidum* was demonstrated at a temperature of 160°C and a pressure of 4.0 MPa using a semi-batch system, a simple and environmentally friendly extraction and micronization method requiring no chemicals other than water and air. Under these conditions, thermal softening of *G. lucidum* occurred, allowing the removal of the polysaccharides protecting other constituents in *G. lucidum* via dehydrogenation and deoxygenation reactions. SEM images showed that the particles formed were spherical and dimpled or shriveled with diameters ranging from 1 to 6 μm. When the air contact temperature changed from 135 to 200°C, the particle size was slightly reduced due to the strong bonding of water to the hydroxyl group in an extract of *G. lucidum*. MALDI spectra revealed that main massed peaks of water-soluble products were distributed between 688 and 2632 m/z, with a peak-to-peak mass difference of 162 m/z, consistent with the repeating unit of the glucans. Based on these results, it is proposed that this process is applicable to isolate polysaccharides from other types of biomass.

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