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SUBCRITICAL WATER EXTRACTION AND DIRECT FORMATION OF MICROPARTICULATE POLYSACCHARIDE POWDERS FROM GANODERMA LUCIDUM

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ABSTRACT

Ganoderma lucidum (hereafter G. *lucidum*) has been known as a food and raw material used in the development of medications because of its high content of polysaccharides, or β-glucans, which support the immune function. In this work, subcritical water was applied to utilize G. *lucidum* for the extraction of polysaccharides at temperatures of 373–463K and a pressure level of 4.0 MPa using a semi-batch system. Furthermore, these extracts were atomized and contacted with hot air to produce microsphere particles. During extraction, thermal softening of G. *lucidum* occurred, allowing the removal of the polysaccharides and protecting other constituents in G. *lucidum* via hydrolysis. Scanning electron microscope (SEM) images showed that the microsphere particles formed were spherical and dimpled or shriveled particles with diameters varying from 1 to 6 μm. Characteristics of the molecular mass revealed that main massed peaks of water soluble products were distributed at around 688–2636 m/z with a peak-to-peak mass difference of 162 m/z, consistent with the repeating unit of the glucans.

Keywords: Ganoderma lucidum; Glucan; Polysaccharides; Reishi; Subcritical water

1. INTRODUCTION

In general, plant biomass consists of 40–45 wt% of cellulose, 25–35 wt% of hemicellulose, 15–30 wt% of lignin, and up to 10 wt% of other compounds (Dorrestijn et al., 2000). Among these, hemicelluloses, which are mainly composed of xylans, provide an important source of interesting molecules such as xylose and xylo-oligosaccharides, which have potential applications in various areas including chemical, food, and pharmaceutical industries. To increase the higher value of biomass components, especially hemicellulose, they need to be separated. Similarly, the removal and recovery of hemicellulose is an essential feature of pretreatment processes for biological conversion to other products.

Hemicelluloses are polysaccharides in plant cell walls that have β-(1→4)-linked backbones with an equatorial configuration. Therefore, the hemicellulosic fraction in plant biomass is mainly thought to be composed of these bond units with side chains of various lengths containing xyloglucans, xylans, mannans, glucomannans, and

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β-(1→3, 1→4)-glucans (Willfor and Holmbom, 2004; Fry et al., 2008; Scheller and Ulvskov, 2010).

Several techniques were proposed for extraction of hemicelluloses from feedstock. Hot water extraction was the most widely used traditional technology for extraction of polysaccharides (Dong et al., 2009; Cai et al., 2008; Qiao et al., 2010; Leppanen et al., 2011; Hartonen et al., 2007; Hasegawa et al., 2004; Sattler et al., 2008; Ando et al., 2004; Yu et al., 2008). For example, Cai et al. (2008) investigated the effects of hot water extraction parameters on the yield of polysaccharides from *Opuntia milpa alta* and obtained the optimal condition. These various researchers reported that the main components of the extracted products were isorhamnetin 3-O-(2,6-dirhamnosyl) glucoside and isorhamnetin 3-O-δ-rutinoside. Additionally, Dong et al. (2009) identified that optimization for hot water extraction of polysaccharides from the cultured mycelium of *Cordyceps sinensis* is associated with use of the Box-Behnken design, followed by canonical and ridge analyses. This process has encouraged an increase in the number of research papers on an extraction of polysaccharides from a wide variety of plants or fungi based on their use in immunomodulatory and anti-cancer electrochemotherapy (Wasser, 2002).

In this work, subcritical water extraction (SCWE) at temperatures of 373–463K and the continuous formation of microparticulate polysaccharide powders from *G. lucidum* continuously were demonstrated. It was well known that subcritical water as a green solvent has emerged from natural sources as a sustainable alternative for extraction technology because of its unique physical and chemical properties (Akiya & Savage, 2002; Marshall & Franck, 1981; Uematsu & Franck, 1980). Recently, SCWE has gained considerable attention as the most widely used technology to extract polysaccharides. This technology is a technique based on the use of water as an extractant at temperatures above the boiling point (373K) and below the critical point (647K) and at a pressure high enough to maintain the liquid state. Hemicellulose is usually branched with degrees of polymerization ranging from less than 100 to about 200 units (Gatenholm & Tenkanen, 2003). Because of its structure and branched nature, hemicellulose is amorphous and relatively easy to hydrolyze to its monomer sugars compared to cellulose (Yu et al., 2008). Therefore, water at 393K could be used to extract hemicelluloses from wood (Sattler et al., 2008). Hasegawa et al. (2004) reported that hemicelluloses have been recovered from Japanese apricot trees at 453K. Furthermore, naringenin and other antioxidants have been extracted successfully from aspen knotwood at 423K (Hartonen et al., 2007). Allen et al. (1996) explained that the recovery of hemicellulose in the form of monomeric sugars (after a mild post-hydrolysis) from sugar cane exceeded 80% at a temperature of 463K and pressure level of 5 MPa. After fractionation, hemicellulose recovery could exceed 90%. However, Leppanen et al. (2011) also reported that 443–453K is the most promising temperature range for the isolation of hemicelluloses with high molar masses because extraction at this temperature achieves a sufficiently high yield without extensive degradation of the extracted polysaccharides. To maintain or even to obtain a concentration of glucans in the extract water, the generation of microparticulate powders was performed directly during the extraction process. It was discovered that the process could 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 the chemical composition and structure (Tsotsas & Mujumdar, 2011). The evaporation of water occurred instantaneously when the extract water was placed in contact with hot air via a nozzle. However, the microparticles that were formed in the process exhibited extremely complex process behaviors due to the many process and formulation variables that must be fine-tuned to achieve the desired results (Vehring, 2008).

2. EXPERIMENTAL

G. lucidum obtained from REFARMER Co., Ltd. (Kumamoto, Japan) was used as a starting material. 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), and analytical reagents (e.g., methanol) with purities exceeding 98.0% were purchased from Wako Pure Chemicals Industries Ltd., Japan; they were used without further purification.

The setup apparatus consisted of an extraction unit and a precipitation unit (Figure 1). In the extraction unit, a high pressure pump (LC-6AD, liquid chromatography pump, Shimadzu Corporation, Japan) and pre-heater were used to introduce hot water to the reactor (10 ml in volume; Thar Technologies, Inc., USA). After the reactor was loaded with 1.0 g of *G. lucidum*, distilled water at room temperature was pumped through the 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 Co., Ltd., Japan). When the system reached the desired pressure and a steady state was achieved, the electric heater was applied to heat the water (373–463K).

The time required to heat the reactor from room temperature to the desired temperature was 5–8 min, after which the reactor temperature and the electric heater temperature were the same. After the temperature at the reactor area reached a preset temperature, the pump was used to feed water at 0.2 ml min^{-1}.

Next, the outlet solution was directly sent to the precipitation unit via nozzle and placed in contact with hot air (3 L min^{-1}) concurrently. Inlet air temperature of 443K, which corresponded to outlet air temperature of approximately 331–334K, was applied. The solution was atomized in a hot air current to instantaneously obtain a powder produced from heat and mass transfers between the dry air and the water.

Because of decreasing water content and water activity, this process could ensure a microbiological stability of products, thus avoiding the risk of chemical and/or biological degradation and reducing storage and transport costs. Additionally, it was used to ensure that a product with specific properties would be obtained (Tsotsas & Mujumdar, 2011; Vehring, 2008).

An aspirating pump (AS-01, aspirator, As One, Japan) was mounted at the end of the system to drive the air flow, which was modulated by means of an inlet valve. A filter (60 μm; Swagelok, Japan) was placed before the aspirating pump inlet to collect fine powder products. Then, the powder samples were transferred to sealed bottles and refrigerated prior to analysis. The morphologies of the powder products were observed with a SEM (JEOL JSM-6390LV).

To understand the distribution of molecular weight of the compounds in the particles that were formed, an analysis using MALDI-TOF-MS was performed on a Bruker Tektronix TDS 504D GmbH Reflex III (Germany) with dual microchannel plate detectors for both linear and reflectron modes. The solid residues collected at each operating temperature were analyzed by a thermo-gravimetric/differential thermal analysis (TG/DTA; SII nanotechnology; EXSTAR-6000).

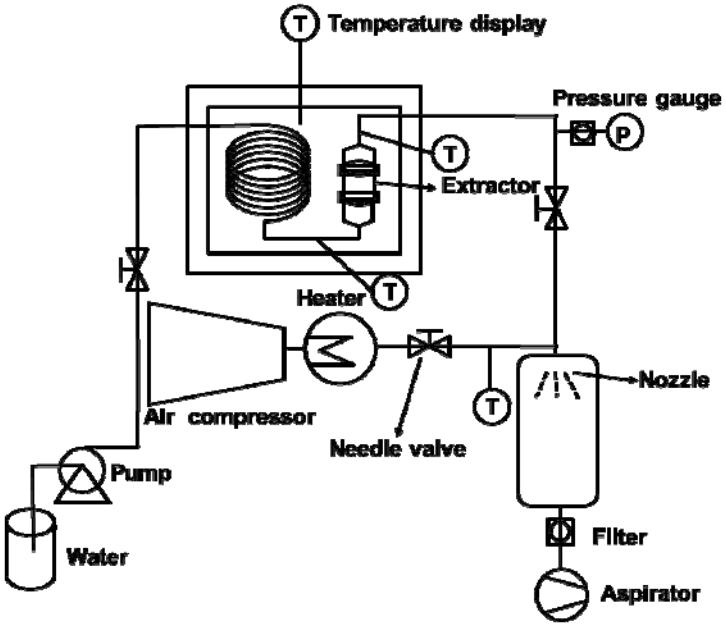

Figure 1 Schematic diagram of subcritical water extraction and micronization.

3. RESULTS AND DISCUSSION

It is well known that microparticles are the polymeric aggregates measuring 1–1000 μm. A microsphere is a type of microparticle that forms microcapsules and matrix systems; it is spherical in shape, whereas microcapsules may be spherical or non-spherical. Microparticles offer a method to deliver macromolecules by a variety of routes and effectively control the release of certain drugs.

Figure 2 shows the scanning electron micrographs of microparticles of a water soluble compound, which was extracted from *G. lucidum* in subcritical water conditions and directly placed in contact with hot air to remove the water content. It was clear that particles obtained exhibited different morphologies under different conditions. In general, the morphology of the particles produced from spray drying depends on the inlet and outlet air temperature. Therefore, when the water content of the droplet reaches a critical value, a dry crust frequently forms on the droplet surface. If the droplet is dried very slowly, it is transformed into a fully dried particle. When drying occurs at high temperatures, cycles of repeated expansion and collapse of the particle occur because of the formation of an internal air bubble (Elversson et al., 2003; Elversson & Millqvist-Fureby, 2005; Walton & Mumford, 1999). However, the largest effect on particle morphology was caused by the composition and solid materials contained in the liquid to be sprayed (Vehring, 2008; Elversson et al., 2003; Elversson & Millqvist-Fureby, 2005; Walton & Mumford, 1999). As shown in Figures 2b and 2c, the surface corrugation of particles was observed when the extraction temperature increased (i.e., 453K and 463K). This phenomenon is, most probably, due to the increasing concentration of polysaccharides in the feed solution. Moreover, the particle formation was influenced by the crystallization propensity of the carbohydrates, thus indicating that hollow particles are more likely to be formed because of the existence of amorphous carbohydrates. As mentioned above, at low temperatures (<413K), only small amounts of carbohydrates were dissolved, but the amount of extracted hemicelluloses increased steadily from 433K (Leppanen et al., 2011; Sattler et al., 2008). At 453K, over half of the hemicelluloses were extracted, and complete removal was achieved at 493K. Therefore, the researchers concluded that the isolation of hemicelluloses with high molar

mass levels at 443–493K as the most promising temperature range produced a sufficiently high yield without extensive degradation of the extracted polysaccharides. Elversson and Millqvist-Fureby (2005) explained that the droplet size during atomization, the concentration of the feed solution, and the solubility of the solute affect particle morphologies during spray drying of carbohydrate solutions. Interestingly, they also explained that the carbohydrate with the highest solubility in water, sucrose, produced the smallest particles compared to both lactose and mannitol at the corresponding droplet size and feed concentration. Vehring (2008) reported also that the size of the microsphere is directly affected by changes in the concentration of the feed solution.

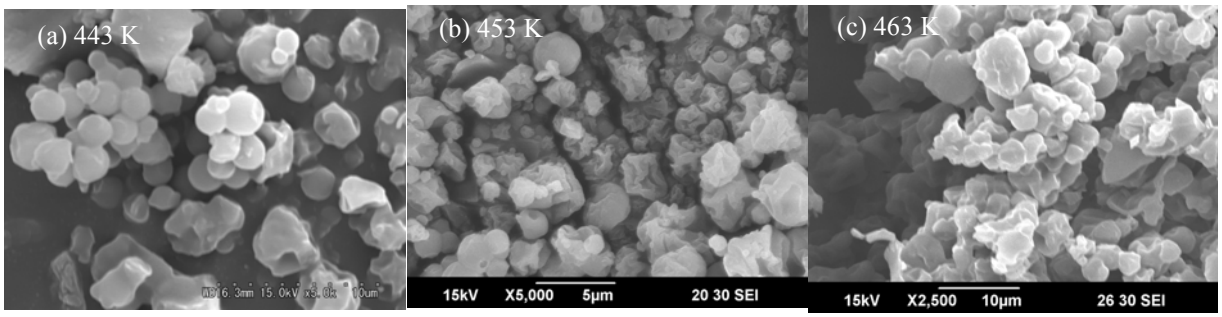

Figure 2 SEM images of generated particles at various extraction temperatures

To determine the distribution of molecular weight compounds in water soluble extraction, particles obtained were dissolved in water and measured by MALDI-TOF MS associated with m/z numbers, which are considered to give highly reliable information on polymer molecular weights. In addition, this tool has been developed to analyze biopolymers and macromolecules successfully (Bahr et al., 1994). Figure 3 shows the MALDI spectra of water soluble products from *G. lucidum* obtained by hot compressed water at 443, 453, and 463K, respectively.

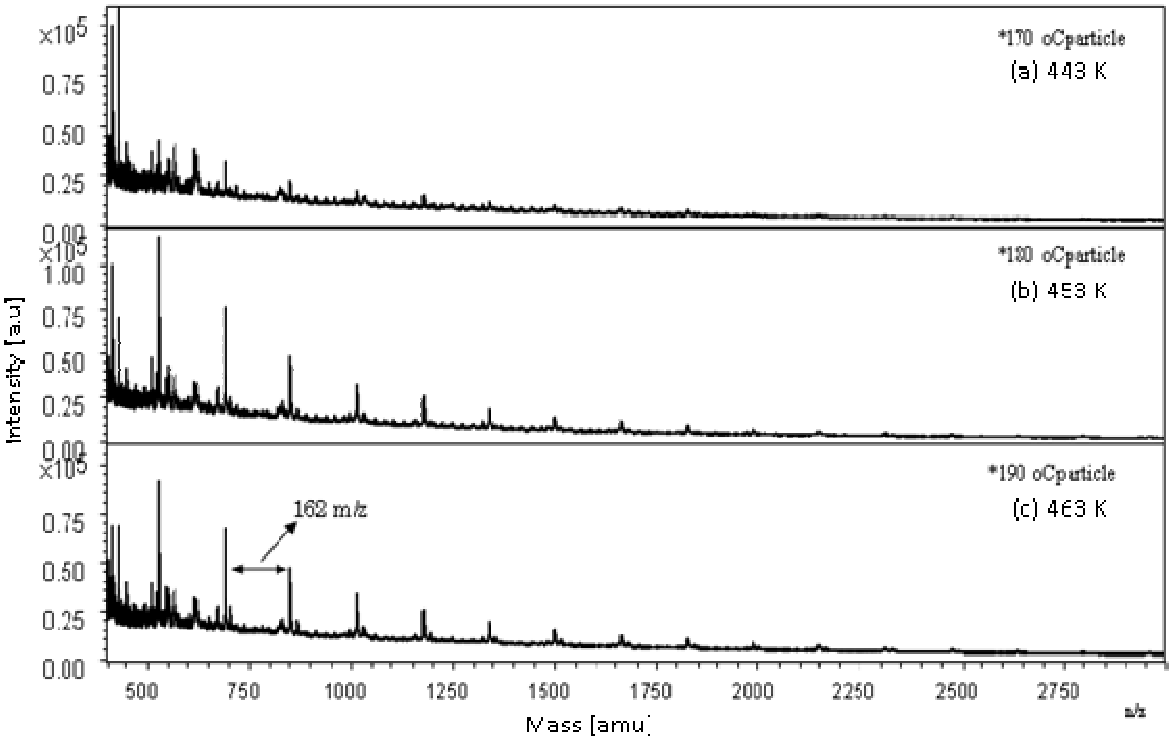

Figure 3 MALDI-TOF MS of water soluble products at 443, 453, and 463K, respectively

The 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 detected easily. The peaks in each distribution are separated by the unit mass. The difference in the peak intensity qualitatively corresponds to the amounts of dissolved plant components and their derived compounds in water; nevertheless, the non-homogenous spread of the sample on the target spots did not render a quantitative or precise analysis. Therefore, molecular weight distributions can be observed clearly.

As shown in Figure 3, the thermal extraction of plant components and their derived compounds from *G. lucidum* occurred at 443K; further, the process and products were enhanced significantly when higher temperatures were applied, resulting in species with molecular weights of 500–2600 m/z. Quantitatively, the peak intensities of water soluble products obtained by thermal extraction at higher temperatures (i.e., 453–463K) was higher than at the lower temperature (i.e., 443K). These molecular weight regions may correspond to the existence of hemicellulose groups that are linked via glycosidic bonds (Leppanen et al., 2011; Sattler et al., 2008; Tunc & van Heiningen, 2008). Corresponding to the mass spectrum shown in Figure 2, 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 (Hung et al., 2008; Kao et al., 2012).

Although, the compound (in monomer units) that was extracted with hot compressed water was not observed, information about the glucans derived from *G. lucidum* polysaccharides was obtained. Song et al. (2012) explained that extraction of hemicelluloses with plain water at 443K resulted in extensive hydrolysis of poly- and oligosaccharides, releasing monosaccharides amounting to about 60 mg/g of wood after 100 min extraction. Monosaccharides were released also during extractions with phthalate buffer solutions, but to a lower extent than with plain, unbuffered water. Regarding this condition, Tunc et al. (2008) suggested that hemicellulose groups dissolved in water via autohydrolysis depolymerize slowly into monomeric forms as a result of longer extraction times. Hemicellulose is removed completely from wood in the form of monomers at the end of the autohydrolysis process. No significant amount of furfural was generated under the extraction conditions described herein. From this figure, it can be seen that glucans from *G. lucidum* are composed of a mixture of glucose polymers with molecular weights in the range of 688–2636 m/z. These results are in agreement with observations by Hung et al. (2008; 2012), who attributed the outcome to the fragmentation of the high-mass polysaccharides. They observed the *G. lucidum* glucans as sodiated ions ([Glcn + Na]+) and calculated the mass of the singly charged molecular ion as 162.14n + 22.990 m/z and 162.14n + 22.990 m/z + 18.015 m/z (mass of reducing end residue) respectively, where n is the number of glucose units.

In this work, a mushroom and yeast *β*-glucans assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland) was used to determine the *β*-glucans contents in the particles formed. First, to determine total glucans content (*α*- and *β*-), 5 mg of particles dissolved in water were suspended in 1.5 mL of concentrated HCl (37% v/v) and incubated at 303K for 45 min; then, 10 mL of distilled water was added, and the solution 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. Then, 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 of sodium acetate buffer (pH 5.0). The hydrolysates were incubated with 3.0 mL of glucose oxidase-per oxidase mixture (GOPOD) at 313K for 1 h. Absorbance of the solution was measured at 510 nm by using ultraviolet-visible (UV–vis) spectrophotometry V–550 (JASCO Corporation, Japan). Next, the measurement of *α*-glucan was obtained by dissolving 5 mg of particles in water and suspending the solution in 2 mL of 2 M KOH for 20 min and neutralizing 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 0.2 mL of solution, followed by incubation at 313K for 30 min. The solution was incubated with 3.0 mL of glucose oxidase-peroxidase mixture at 313K for 20 min; then, the absorbance was measured at 510 nm. The concentration of β-glucans was determined by subtracting α-glucans from the total glucans content. The β-glucans content represented approximately 40 to 45% of weight. This result is in agreement with previous research (Cheong et al., 1999; Wasser 2005). Judging from these results, it can be stated that the *G. lucidum* glucans were extracted at 443K, and they increased with a rising temperature at the same reaction time.

In general, the chemical composition of the edible mushrooms is different from one species to another depending on the growing stage, nutrient substratum, and clime conditions including the growing period and age of mushroom (Song et al., 2012; Delmanto et al., 2001). As with mushrooms, when *G. lucidum* is exposed to elevated temperatures, changes can occur in its chemical structure that affect performance. To understand the changes in the thermal properties of *G. lucidum* before and after hot compressed water treatments, they were measured by using thermogravimetric analysis (Figure 4).

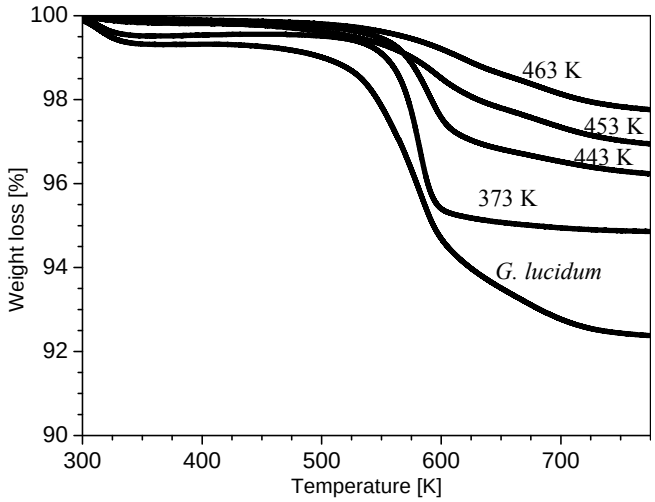

Figure 4 TG/DTA of *G. lucidum* before and after treatment with subcritical water

Thermal analysis has been defined by the International Confederation for Thermal Analysis as all techniques that measure a change in a physical property of a substance as a function of temperature while the substance is subjected to controlled temperatures. Two methods have been developed using TG/DTA analysis—non-isothermal and isothermal. Isothermal methods are still used, but non-isothermal methods have many advantages such as less time required for each analysis and availability of a number of methods for data evaluation. Therefore, the non-isothermal method was applied in this analysis. Though the experiments were conducted at 373–463K, the particles were not produced when the SCWE temperature was 373K; therefore, related data are not presented in Figures 2 and 3. However, the solid residues recovered at each operating temperature were analyzed by TG/DTA to investigate behavior. This analysis is justified since this mushroom (*G. lucidum*) was used for food either as such or thermally processed in industry (conservation, culinary preparations). The samples (approximately 15–20 mg of *G. lucidum* and its solid residues placed in aluminum pan) were placed in the thermogravimetric apparatus and heated at 5K/min from 313 to 773K. Weight loss versus time was recorded. During analysis, the chamber was purged with nitrogen to avoid oxidation and to remove volatile reaction products. The flow rate of the gas was 50 mL/min. This analysis is an analytical technique used to determine a material's thermal stability and its fraction of volatile

components by monitoring the weight change that occurs as a specimen is heated. This behavior could be explained on the basis of heat transfer and medium diffusion, as has been reported by Williams and Besler (1996) and Arenillas and Rubiera (2001). The weight loss was assumed to represent the thermal decomposition of *G. lucidum*.

In Figure 4, the weight-loss process of *G. lucidum* could be divided into three phases. The drying phase was an endothermic phase in which the moisture absorbed by the *G. lucidum* structure evaporated during heating up to 348K. At this point, the evolution of water and volatile matter occurred. This phase was followed by a period of relatively constant weight. In the second phase (i.e., charring phase), *G. lucidum* was heated to 478K; pyrolysis speed was increased; and devolatilization occurred. Hemicellulose, cellulose, and lignin decomposed into gas (i.e., CO_2, CO, CH_4, etc.) as heat increased gradually and weight loss on the TG curve accelerated. At approximately 733K, the loss of weight was essentially complete. The final phase was the calcining phase. After heating to 773K, the amount of flammable gas was very small, and the flame burn transformed into flameless charcoal burn. This means that the flameless charcoal burn was not as violent as *G. lucidum* flame burn, after which there was essentially no further loss of weight. As shown in Figure 4, there was a difference in temperature between the *G. lucidum* before and after treatment with hot compressed water during the initial decomposition process. The difference in temperatures is most likely the result of the content differences in cellulose, hemicellulose, and lignin based on the extraction process (Xiao et al., 2011). It can be seen in Figure 4 that the thermal degradation behavior of *G. lucidum* after treatment with hot compressed water at 373K and subcritical water at 443K was similar to that of raw *G. lucidum*. In contrast, the thermal degradation behavior of *G. lucidum* after treatment with subcritical water at 453K and 463K was quite different from the behavior of raw *G. lucidum*. This phenomenon could be attributed to the extraction of hemicelluloses, partial cellulose, and lignin from the *G. lucidum* with subcritical water. The remaining contents (mainly cellulose and lignin) caused the thermal behavior to shift to a higher temperature. Thus, it can be said that the *G. lucidum* components dissolved in water relatively easily at subcritical conditions. In other words, the solubility of *G. lucidum* components in the solvent increased at higher extraction temperatures. Under these conditions, autohydrolysis may convert hemicelluloses into a high yield of soluble saccharides and low by-products formation, rendering an easily extractable solid residue rich in cellulose and lignin. Therefore, it appears that the extraction temperature had considerable influence on the process.

4. CONCLUSION

SCWE of polysaccharides from *G. lucidum* at temperatures of 373–463K and pressure of 4.0 MPa using a semi-batch system was studied. Under these conditions, thermal softening of *G. lucidum* occurred, allowing removal of the polysaccharides while protecting other constituents in *G. lucidum* via hydrolysis. Next, the extracts were atomized and placed in contact with hot air (443K, in fact 430-447K) to produce microsphere particles. SEM images showed that the microsphere particles formed were spherical and dimpled, or shriveled with diameters varying from 1 to 6 μm. MALDI spectrum revealed that main massed peaks of water soluble products were distributed at 688–2636 m/z with a peak-to-peak mass difference of 162 m/z, consistent with the repeating unit of the glucans. The TG curve showed that *G. lucidum* components dissolved in water relatively easily at subcritical water conditions. Finally, this method could be proposed as an applicable method to isolate polysaccharides from other types of biomass.

5. ACKNOWLEDGEMENT

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