Micronization of *Ganoderma lucidum* extract obtained by pressurized hot-water extraction

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Abstract

Ganoderma lucidum is a mushroom-forming white rot fungus. Though it isn't edible in the traditional sense (for food) it is grown commercially for use as a medicinal supplement. This species has been a part of traditional Eastern medicine for thousands of years. *G. lucidum* contains a wide variety of bioactive components. The major bioactive polysaccharides isolated from Ganoderma species are glucans, β-1-3 and β-1-6 D-glucans. β-glucan is able to isolated by extraction. The *G. lucidum* extract is usually obtained by liquid-solid extraction and sold in powder form. Recently, the extraction and powder formation process has been carried out separately, for example by hot water extraction and spray dryer. This process may cause a problem due to the water content in the extract. The extract may become corrupt. Moreover, it needs more heat to form the extract powder. The energy needed for the process can be reduced by connecting both the hydrothermal and spray dryer process. In this work, connection of *G. lucidum* pressurized hot-water extraction and micronization of the extract using spray dryer method was studied. Effect of extraction temperature on the formation of particle was examined. Pressurrized hot-water extraction was carried out in a semi-batch at pressures of 1.5 to 4 MPa and temperatures of 160 to 190^{o}C with flow rate of 0.2 mL/min. Spray dryer method was carried out using air at temperature of 170^{o}C. Generated particles were observed by scanning electron microscope (SEM) and molecular weight distribution was analyzed by MALDI-TOF/MS. Morphology of micronized particles was shriveled-sphere-like particle. Particles size and molecular weight distribution were influenced by extraction temperature. Higher molecular weight was obtained at high temperature.

INTRODUCTION

Ganoderma lucidum, popular medicinal mushroom known by the common names Reishi or Mannentake (Japanese) and Ling Zhi (Chinese), has been used as a home remedy in traditional Chinese medicine (TCM) in many Asian countries during the past two millennia. The regular consumption of *G. lucidum* in the form of tea or mushroom powder was believed to preserve the human vitality and to promote longevity. *G. lucidum* has been also used for the prevention or treatment of a variety of diseases including cancer [1]. Western medicine started to accept natural product from the TCM and the popularity of herbal therapies for the treatment of cancer have been recently increasing in the United States. Therefore, scientific justification based on the elucidation of mechanisms responsible for the biological effects of

these natural products could help for their validation in alternative or adjuvant cancer therapies. The anticancer effects of *G. lucidum* were associated with triterpenes [2], polysaccharides [3,4], or immunomodulatory proteins [5], through the mechanisms involving inhibition of DNA polymerase [6], inhibition of post-translational modification of the Ras oncoprotein [7], or the stimulation of cytokine production [8]. Moreover, *G. lucidum*: (i) inhibits proliferation and invasive behavior of breast and prostate cancer cells through the down-regulation of expression of cyclin-D1 and suppression of secretion of urokinase-plasminogen activator (uPA) [9]; (ii) inhibits growth and induces apoptosis of breast and prostate cancer cells through the up-regulation of expression of p21 and Bax [10]; (iii) inhibits growth of hepatoma cells through the suppression of protein kinase C [11]; (iv) induces apoptosis of colon cancer cells by increasing the activity of caspase-3 [12]; (v) suppresses angiogenesis through the inhibition of secretion of vascular endothelial growth factor (VEGF) and transforming growth factor-b1 (TGF-b1) from prostate cancer cells [13].

Extraction of polysaccharides is an important processing for its application, and this has prompted numerous research papers on the extraction technology of polysaccharides from plentiful of plants or fungus in recent years. Dong et al. optimized the hot water extraction (HWE) process of polysaccharides from cultured mycelium of *Cordyceps sinensis* using BoxBehnken design [14]. Qiao et al. optimized the hot water extraction condition of polysaccharides from *Hyriopsis cumingii* using response surface methodology [15]. Cai et al. studied the effects of hot water extraction parameters on the yield of polysaccharides from *Opuntia milpa alta* and obtained the optimal hot water extracted condition [16]. Extraction of polysaccharides from *G. lucidum* using pulsed ultrasonicassisted extraction (UAE) was carried out by Wang and Ma [17]. Huang et al. studied the microwaveassisted extraction (MAE) of polysaccharides from spores of *Ganoderma atrum* with response surface analysis [18].

In general, hot water extraction is the most widely used traditional technology for polysaccharides extraction. However, it should be noted that hot water extraction of polysaccharides is associated with the lower yield, long extraction time and high temperature. It is desirable to find a novel extraction technology of polysaccharides that could avoid the disadvantage of hot water extraction. Pressurized hot water extraction is one alternative to solve the problem. Water under pressurized condition, a “natural and green” way for product extraction, has received increased attention as an important alternative to conventional separation methods, such as hot water extraction conducted at boiling point temperature and atmospheric pressure. Water under pressurized condition can be applied to extract polar organic compounds or to decompose lignocellulosic materials to produce valuable compounds such as saccharides and aromatic organic acids. The method has been applied to recover protein and amino acids [19], and phenolic compounds [20]. The pressurized hot water treatment has also been demonstrated by several studies to effectively convert cellulosic [21] and lignocellulosic biomass [22] into useful products; and to extract water soluble compounds from *G. lucidum* [23].

Spray drying is a method of producing a dry powder from a liquid or slurry by rapidly drying with a hot gas. This is the preferred method of drying of many thermally-sensitive materials such as foods and pharmaceuticals. This method could be a more attractive method for preparing porous powders for inhalation. Particles can be formed directly from solutions or emulsions [24]. Spray-drying also allows a control over particle shape, morphology and density dependent on the spray-drying conditions [25]. These operations include preparation of a liquid feed, atomization of the liquid into a spray and separation of the dried particles from the drying gas. In each of these steps the processing parameters will affect the product’s

properties such as particle size, morphology, and bulk density, as well as the surface composition of particles [26]. Therefore, spray drying necessitates a consideration of several important steps in addition to drying of droplets [27]. But the evaporation of a solution droplet in a spray dryer is a coupled heat and mass transport problem. The process is driven by the difference between the vapor pressure of the solvents and their partial pressure in the gas phase. The rate of evaporation is determined by the balance of energy required to vaporize the solvent and the energy transported to the surface of the droplet [28-30].

To form particles efficiency, connection of the extraction and micronization process is necessary. Extract obtained from pressurized hot water extraction may be easily damaged by the time. Moreover, it needs more heat to form the extract powder. The energy needed for the process can be reduced by connecting both the pressurized hot water extraction and spray dryer process. Furthermore, one step micronization of pressurized hot water extract was developed by connecting the pressurized hot water extraction and spray dryer process in this work. The effect of extraction temperature and water flow rate on the particle morphology was investigated.

MATERIALS AND METHODS

G. lucidum obtained from Refarmer Co., Ltd. (Kumamoto, Japan) was used as a starting material. By ultimate analysis (Yanaco, CORDER MT-6), it contained 6.90 % H, 49.39 % C, and 2.22 % N in weight. Distilled water obtained from a water distillation apparatus (Shibata Co., model PW-16, Japan) was used as an extract solvent. Air supplied from the air compressor (HITACHI Co., model PO-0.4L, Japan) was used as a spray drying gas.

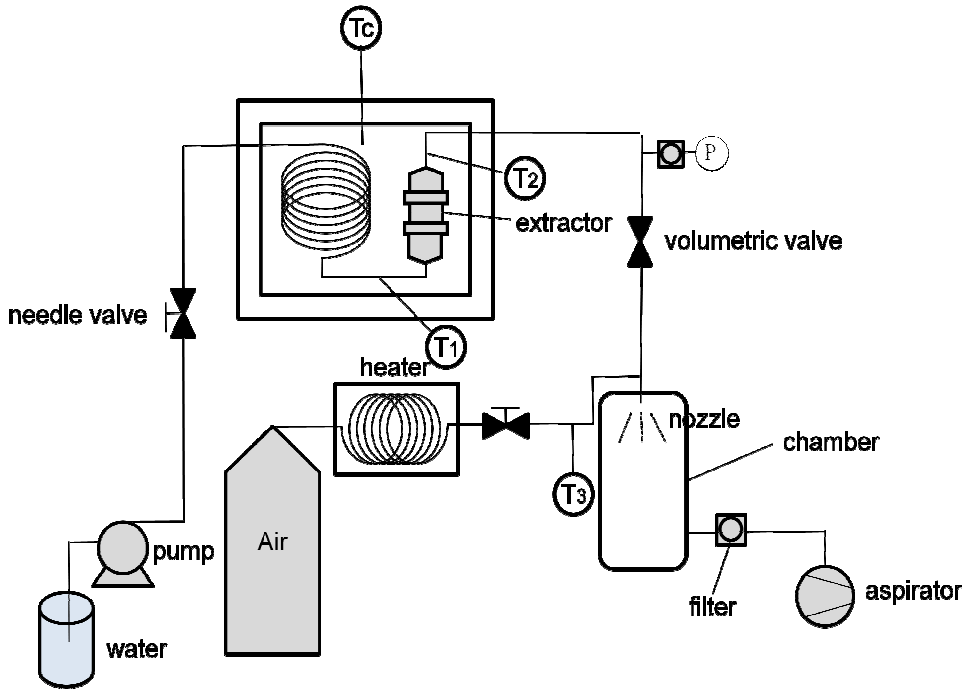

Figure 1 Experimental apparatus for continuous process of hydrothermal extraction and spray drying

The schematic diagram of the apparatus is shown in Figure 1. This apparatus consisted of a high-pressure pump (Liquid Chromatography pump, Shimadzu, Japan), heater (ESPEC, Japan), extractor (10 ml in volume; Thar, USA), valve, nozzle and micronization chamber. The pre-heater fabricated from 1/16 inch stainless-steel tubing (SUS316) with a volume of 20

mL was also installed in this system. After the extractor inclusive of 1.0 g of *G. lucidum* was installed in the system, the distilled water at room temperature was pumped through the extractor, completely wet the *G. lucidum* in the extractor, and pressurize the system to the set pressure of 1.5-4 MPa monitored by a pressure gauge (Migishita, Japan). The 1/16 inch stainless-steel tube was used to introduce the hot water from the pre-heater to the reactor placed in an oven. When the system reached the desired pressure and a steady state was achieved, an electric heater was applied to heat the water to the desired temperatures. During the experiment, temperature of the reactor water inlet (T_1) and outlet (T_2) were monitored. The temperature of reactor was measured using K-type thermocouples (As one, Japan). Effect of extraction temperature and flow rate on the formation of particle was examined. Pressurrized hot-water extraction was carried out in a semi-batch extractor at pressures of 1.5 to 4 MPa and temperatures of 160 to 190 ^{o}C with 0.1 – 1.0 mL/min of water flow rates. Spray dryer method was carried out using air at temperature of 170^{o}C. The time of each experiment was 5 hours. The morphology of micronized particles was observed by scanning electron microscope (SEM; JEOL Ltd. JSM-6390LV, Japan) and the particles dimensions were measured from the SEM image using image analyzer software (Image J 1.42). In order to understand the molecular weight distribution, the miceonized particles was dissolved in water and analyzed by matrix-assisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF/MS, Bruker Tektronix TDS 504D GmbH Reflex III, Germany). In addition, the solid residue remaining in the reactor after pressurized hot water treatments were collected and dried in vacuum desiccator for 1 day at room temperature and weighed. This solid residue would be hereafter referred to as char. The solid residues were then analyzed by using the thermo-gravimetric - differential thermal analysis (TG/DTA; SII nanotechnology; EXSTAR-6000) to determine a material's thermal stability and fourier transform infrared spectroscopy (Spectrum One FT-IR Perkin-Elmer, Ltd., England).

RESULTS

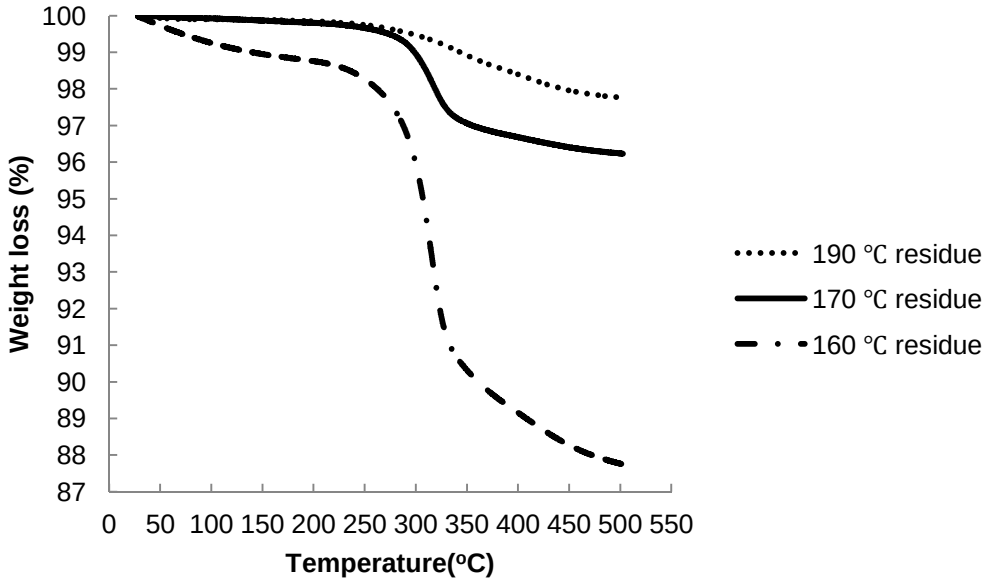

Figure 2 TG/DTA of solid residue

Figure 2 shows the TG analysis of solid residues after treatment by pressurized hot water. The thermogravimetric analysis of approximately 15 - 20 mg of sample placed in aluminum pan was carried out on a SII nanotechnology - EXSTAR-6000. The samples were heated at 5^{o}C/min from 40 to 550^{o}C. The weight loss versus time was recorded. During analysis, the chamber was purged with nitrogen to avoid oxidation and to remove volatile reaction products from the chamber. The flow rate of the gas was 50 mL/min. This figure demonstrated that there are three stages of the weight loss profile of solid residues during the pyrolysis process. The first step is at below 200^{o}C where the elimination of moisture content of the solid residues occurred. The relatively light organic substance was also volatilized. The second step is a relatively light organic substance material at a temperature between 200 and 430^{o}C. The third step is at over 430^{o}C, where a relatively heavy organic substance cracks. From this figure could be seen that the higher of temperature reaction (pressurized hot water), the solubility of organic substance in the solvent increases. Therefore they dissolved in solvent relative easily. This phenomenon also considered that the temperature reaction probably had high influence on this process.

The chemical structure of *G. lucidum* (reishi) as a starting material and its solid residues, were analyzed using FT–IR direct transmittance through KBr pellet technique. Infrared spectroscopy is an analytical technique that allows identification of unknown substances and of the types of chemical bonds the compounds in those substances content.

Table 1 The main functional groups of the major constituents of *G. lucidum*

Wave number [cm^{-1}]	Functional groups	Compounds
3600 – 3000	O–H stretching	Acid, methanol
2860 – 2970	C–H_n stretching	Alkyl, aliphatic, aromatic
1700–1730, 1510–1560	C = O stretching	Ketone and carbonyl
1632	C = C	Benzene stretching ring
1613, 1450	C = C stretching	Aromatic skeletal mode
1470–1430	O–CH_3	Methoxyl–O–CH3
1440–1400	O–H bending	Acid
1402	C–H bending	
1232	C–O–C stretching	Aryl-alkyl ether linkage
1215	C–O stretching	Phenol
1170, 1082	C–O–C stretching vibration	Pyranose ring skeletal
1108	O–H association	C–OH
1060	C–O stretching and C–O deformation	C–OH (ethanol)
700–900	C–H	Aromatic hydrogen
700–650	C–C stretching	

A FT–IR spectrum of *G. lucidum* was shown in Figure 3. The peaks that point down represent the frequencies of light that the molecules absorb. Each spectrum is a spectral average of at least four scans. It can be observed that it most likely consists of alkene, esters, aromatics, ketone and alcohol, with different oxygen-containing functional groups observed.

As a reference, the peak positions of all infrared bands and their functional groups are summarized in Table 1. Each molecule is composed of many different chemical bonds, and these bonds are slightly elastic and can stretch, bend, or vibrate. Therefore, some differences exist at each FT–IR spectra due to their structure properties. The intensity of the absorbance due to the hydrogen bonded O–H stretching (3600–3000 cm^{-1}) could be found in each spectra. The bands in the 1015.8-1036.1, 2926.1-2940.7, and 3261.9-3310.3 cm^{-1} regions are assigned to the stretching and deformation of aromatic C–O groups, the stretching of aliphatic and aromtic C-H groups, and the stretching of O-H groups, respectively. In these regions, the peaks in (a-d) was sharper than (e), showing that the C–O, C-H and O-H bonds in *G. lucidum* were more reacted and consumed in (e). The same result was also occurred at 1618.3-1656.7 cm^{-1} and 1699.0-1705.1 cm^{-1} due to the stretching modes of C=C and C=O groups. The spectral characteristics of solid residues are essentially the same, indicating that the solid residues obtained by pressurized hot water extraction are within a similar functional group as reishi. However, the TG analysis showed that solid residues became heavier clearly after reaction in pressurized hot water.

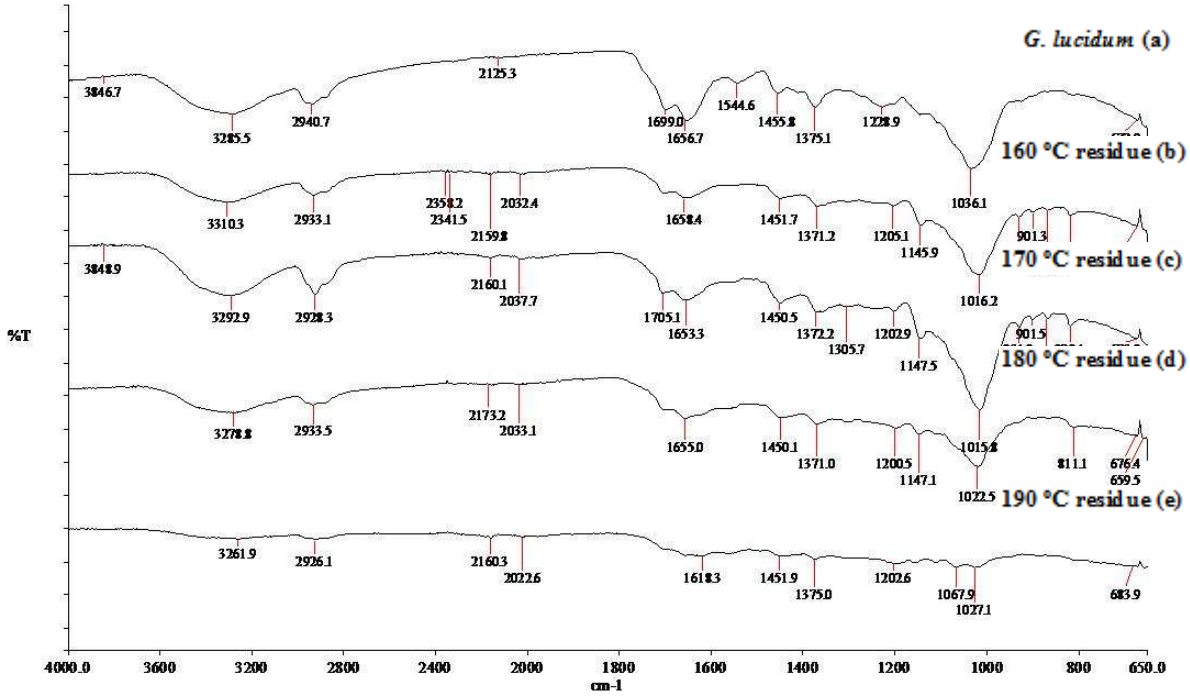

Figure 3 FT-IR spectrum of *G. lucidum* and its solid residues after treatment by pressurized hot water.

In order to determine the molecular weight of compounds in the particles produced, the particles were diluted in water and measured by MALDI-TOF MS associated with m/z numbers, which is considered to give highly reliable information on polymer molecular weights. Figure 4(a-d) shows the MALDI spectra of liquid phase obtained of particles product after treatment by pressurized hot water at 160-190^{o}C. 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 easily detected. The peaks in each distribution are separated by the mass of the monomer unit. The difference in the peak intensity qualitatively corresponds to the amounts of dissolved particle derived compounds in water; nevertheless, the non-homogenous spread of

the sample on the target spots could not render a quantitative precise of the analysis. Therefore, molecular weight distributions can be clearly observed. Figure 4(a-d) showed that the thermal extraction of *G. lucidum* is almost occurred at 160-190^{o}C, resulting species with molecular weight 500 to 2600 amu. Quantitatively, the peaks intensities particle diluted in liquid phase obtained by thermal extraction with higher temperature (180 and 190^{o}C) was higher than lower temperature (160 and 170^{o}C). These molecular weight regions might correspond to the existence of glucan groups involves linkages at the glycosidic bonds. Judging from these results, the thermal extraction of *G. lucidum* by pressurized hot water took place via hydrolysis, dehydration and condensation.

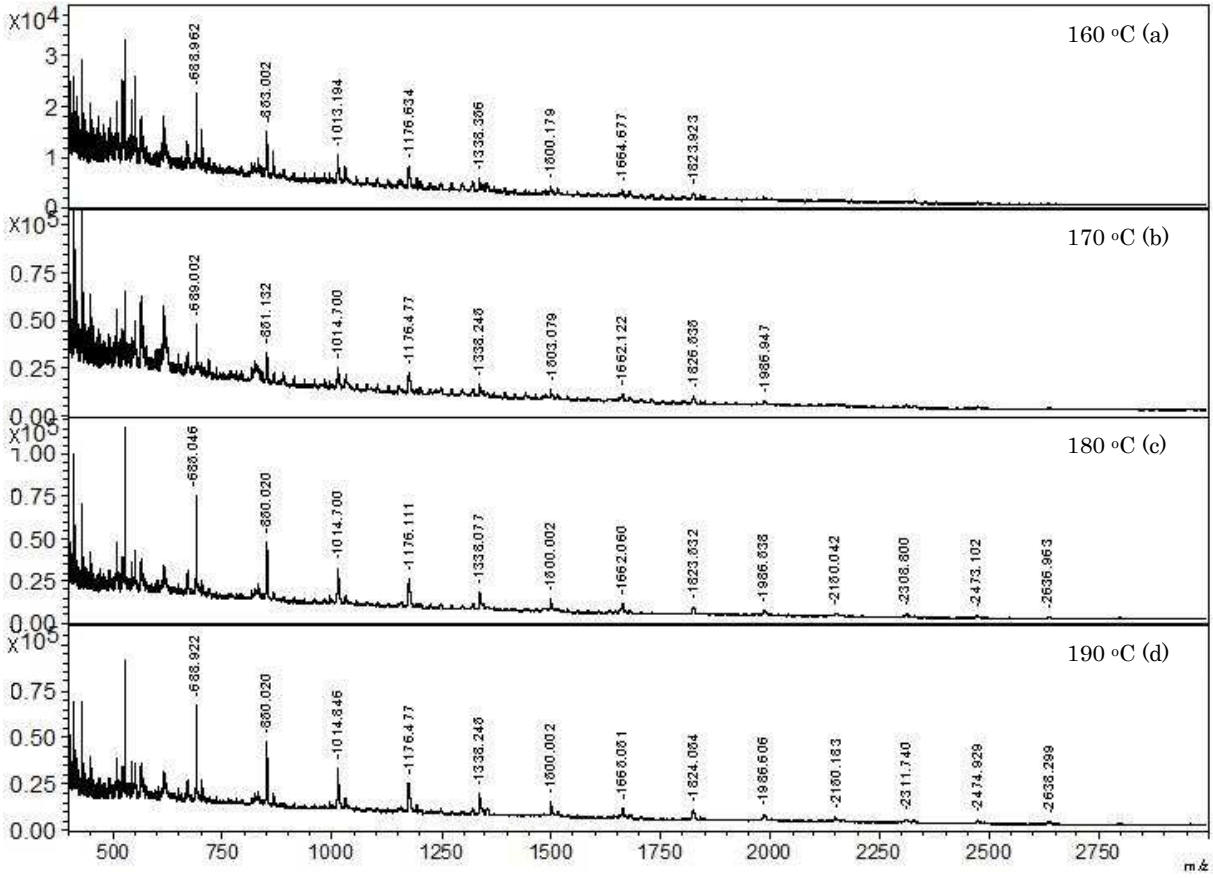

Figure 4 MALDI-TOF MS of particle produced by pressurized hot water connected with spray dryer process

The morphology of particles formed by spray dryer was investigated by using an SEM. On the effect of extraction temperature, the extraction was carried out at water flow rate of 0.2 mL/min. The SEM images of the particles at various temperatures are shown in Figure 5. In general, shriveled-sphere-like particles were formed at all extraction temperatures. The increasing temperature caused agglomeration of particles. It might be due to the content of glucose in the extract. Higher temperature caused degradation of polysaccharides into lower molecular weight of monosaccharides, such as glucose and sucrose. Moreover, at higher temperature, concentration of extracted compounds, such as monosaccharides, was higher that resulted in lower water content. In this case, drop surface from upper part of chamber was sticky and no more completely liquid. As a consequence, solid bridges could be formed at the surface of colliding particles, leading to agglomeration (Figure 5(d)). In addition, most of the

particles showed a rounded external surface with a continuous wall and no apparent fissures or cracks, which is important to provide lower permeability to gases, better protection and core retention [31]. Moreover, surfaces were concave and shriveled, which is typical of particles produced by spray drying. This type of morphology was also observed by Tonon et al. [31] and by Bertolini et al. [32].

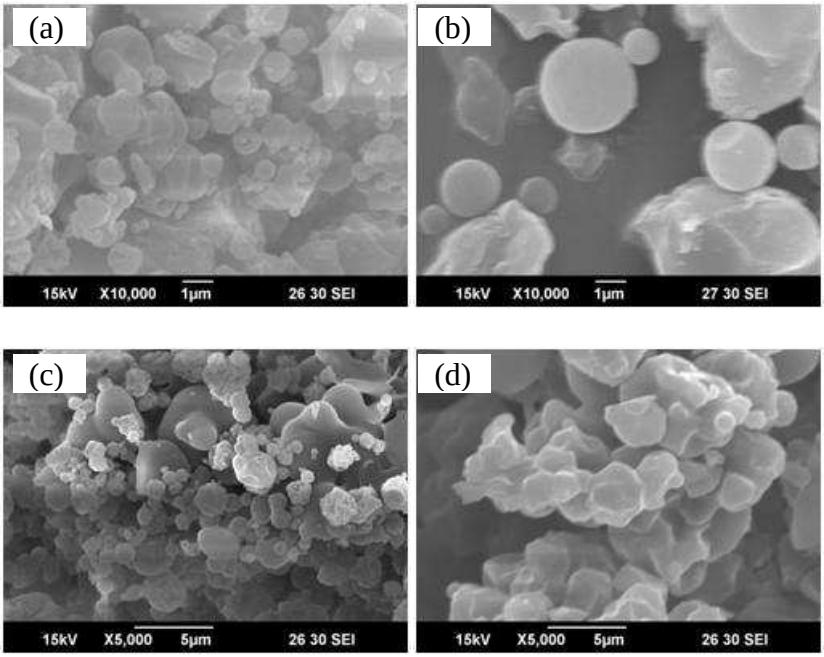

Figure 5 SEM images of generated particles at water flow rate of 0.2 mL/min; (a) 160 ^{o}C; (b) 170 ^{o}C; (c) 180 ^{o}C; (d) 190 ^{o}C

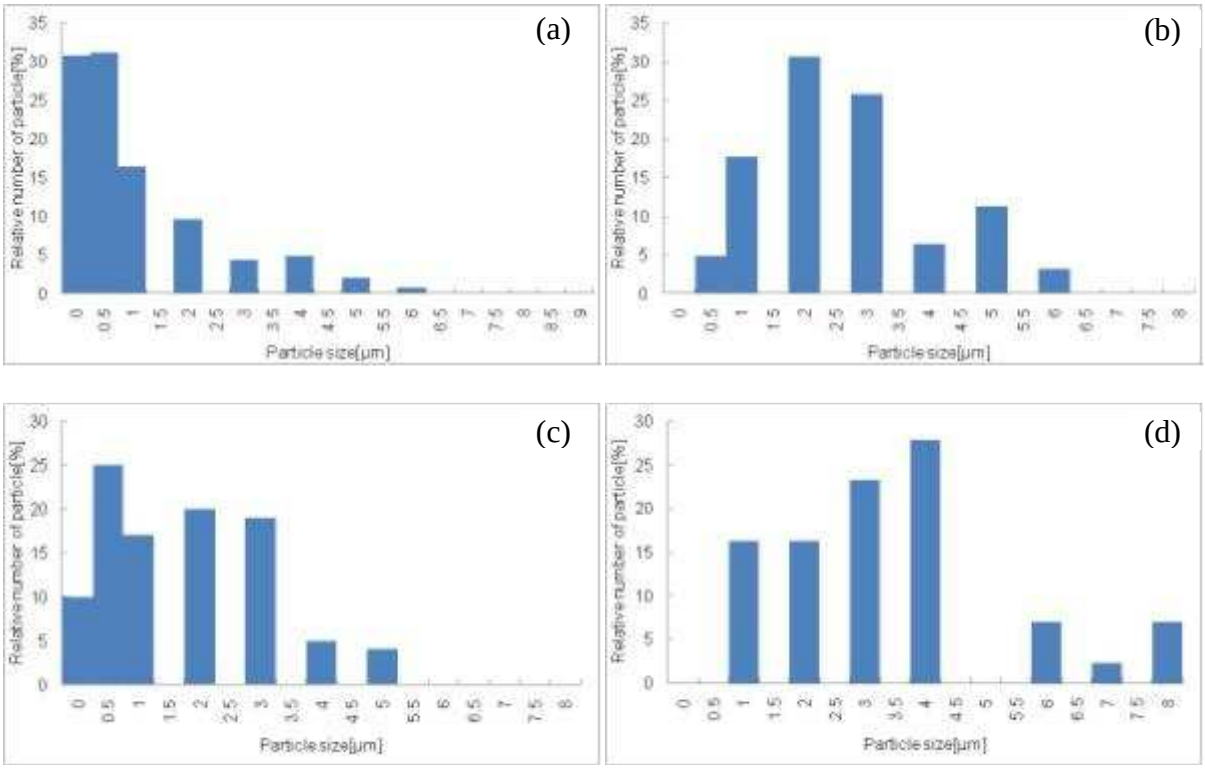

Figure 6 Particle size distributions of generated particles at water flow rate of 0.2 mL/min; (a) 160 ^{o}C; (b) 170 ^{o}C; (c) 180 ^{o}C; (d) 190 ^{o}C

As shown in Figure 5, the resulting powders had particles of various sizes, which agrees with the results obtained for particle size distribution in Figure 6. Figure 6 shows the particle size distribution of powders produced with different extraction temperatures (at water flow rate of 0.2 mL/min). The particles exhibited a large range of sizes, with diameters varying from 0.1 to 8 μm, approximately, and showed a distribution with broad peaks. Extraction at low temperature resulted in smaller particles compared with the higher one. The presence of bigger particles (about 8 μm) can be explained by an incipient agglomeration process, where the formation of irreversible link bridges leads to the production of particles with larger size.

The effect of water flow rate on the morphology of generated particles was studied at 160^{o}C. Figure 7 shows SEM images of generated particles at various water flow rates. At low water flow rate (Figure 7(a) and (b)), shriveled-sphere-like particles were formed. The shriveled particles should have occurred from the shrinkage of the particles after the loss of water. Moreover, at low flow rate, particles might be formed slowly and took time to drop into the chamber. As increasing water flow rate, the sphere-like particles were generated; however, the particles were connected each other (Figure 7(c) and (d)). In this case, the liquid fell down rapidly, and resulted in rapid evaporation from the surface of drop. It seems that the rapid evaporation can inhibit the shrinkage of particle surface.

Particle size distributions of SEM images in Figure 7 are shown in Figure 8. The particles exhibited a large range of sizes, with diameters varying from 0.1 to 8 μm, approximately, and showed a distribution with broad peaks. However, particle size distribution of particles obtained at 1.0 mL/min of water flow rate has sharper peak compared with other condition. At this condition, particles sizes are less than 2 μm (Figure 8(d)).

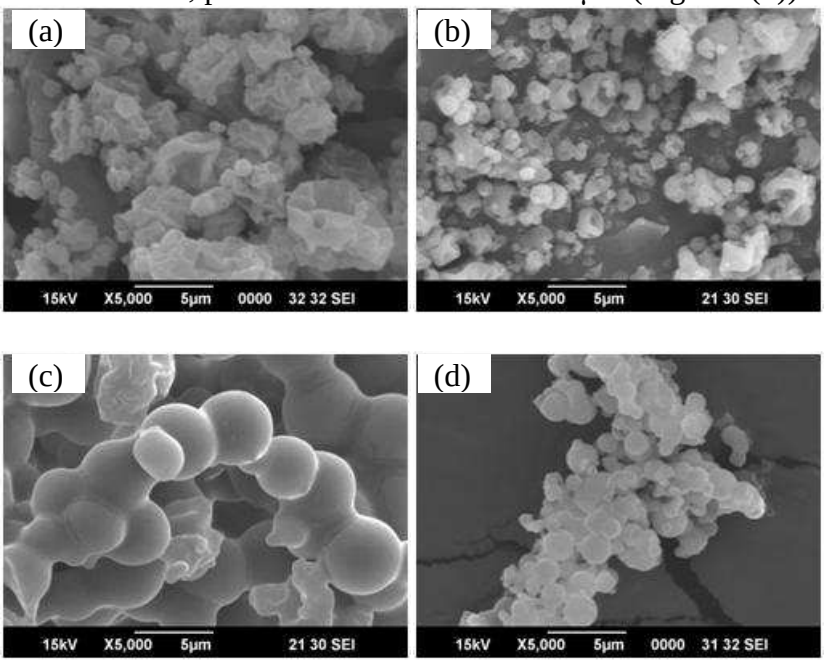

Figure 7 SEM images of generated particles at temperature of 160 ^{o}C; (a) 0.1 mL/min; (b) 0.2 mL/min; (c) 0.3 mL/min; (d) 1.0 mL/min

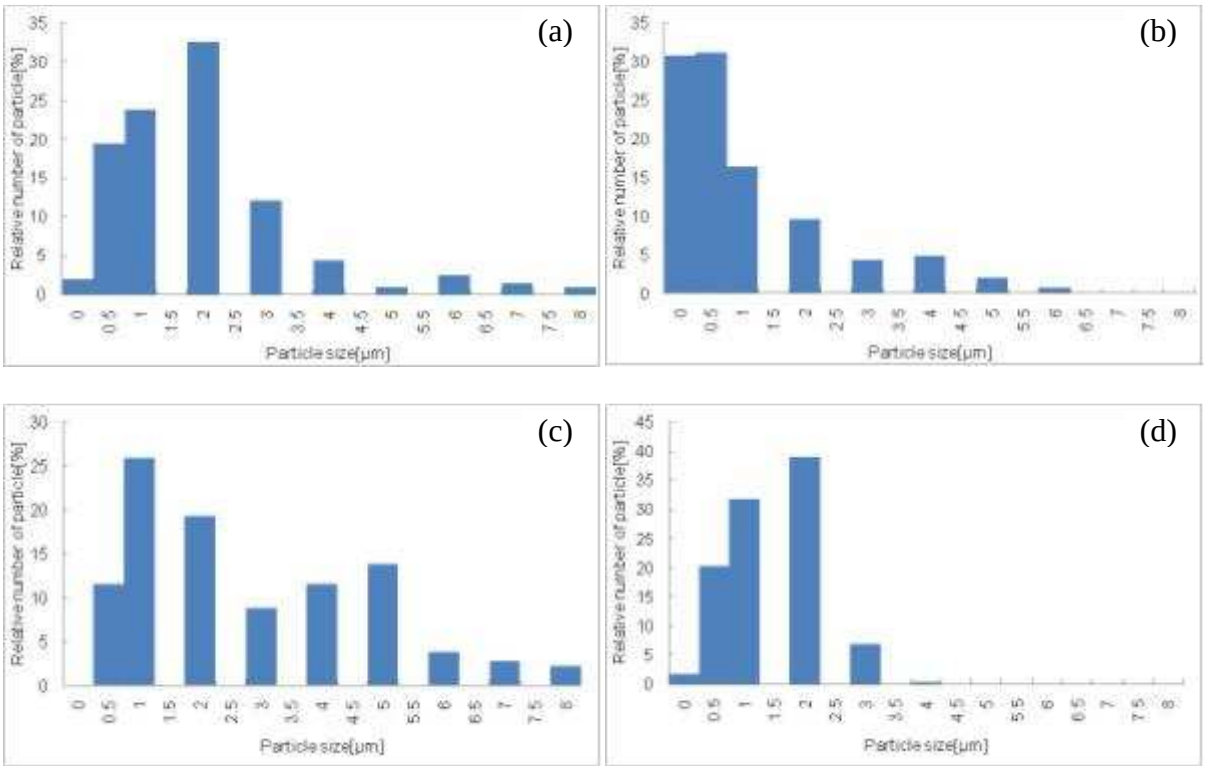

Figure 8 Particle size distribution at temperature of 160 ^{o}C; (a) 0.1 mL/min; (b) 0.2 mL/min; (c) 0.3 mL/min; (d) 1.0 mL/min

CONCLUSION

In this work, one step micronization of pressurized hot water extract was developed by connecting the pressurized hot water extraction and spray dryer process. The effect of extraction temperature and water flow rate on the particle morphology was investigated. Based on the MALDI-TOF MS analysis, particles produced by pressurized hot water extraction and spray dryer process contained compounds with molecular weight regions that might correspond to the existence of glucan groups involves linkages at the glycosidic bonds. The particles formed by spray dryer were shriveled-sphere-like particles with diameters varying from 0.1 to 8 μm. The increasing water flow rate of pressurized hot water extraction seems to inhibit formation of the shrinkage of particle surface.

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