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(高静水圧と酵素を利用する薬用キノコからの多糖類抽出法の開発)

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CONTENTS

Overview

1. Major biological components in medicinal mushroom

The number of mushroom species on earth is currently estimated at 150,000-160,000, of which may be only 10% are known. Meanwhile, of those approximately 14 000 species that we know today, about 50% are considered to possess varying degrees of edibility, more than 2000 are safe, and about 700 species are known to possess significant pharmacological properties. For hundreds of years, mushrooms have been used as decoctions and essences in alternative medical therapies in Korea, China, Japan, and eastern Russia (Lull *et al*.2005)

For hundreds of years, mushrooms have been used as decoctions and essences in alternative medical therapies in Korea, China, Japan, and eastern Russia (Mizuno 1999). Mushrooms have a great nutritional value since they are quite rich in protein, with an important content of essential amino acids and fiber, poor fat but with important fatty acids content. Moreover, they provide a nutritionally significant content of vitamins (B1, B2, B12, C,D, and E). Thus, they could be an excellent source of many different nutraceuticals and might be used directly in human diet and to promote health for the synergistic effects of all the bioactive compounds present.

Many, if not all, higher Basidiomycetes mushrooms contain many types of biologically active high molecular weight (polysaccharides, proteins, peptides, polysaccharide-protein complexes) and low molecular weight (phenolics, alkaloids, lactones, terpenes, sterols, ceramides, anthraquinones, benzoic acids, oxalic acid) compounds in fruit bodies, cultured broth mycelia, and cultured broth.

Medicinal mushrooms present an unlimited source of polysaccharides (especially β-glucan) and polysaccharide–protein complexes with anticancer and immuno-stimulating properties. (Vetvicka 2011). Researchers found that mushroom polysaccharides with high bioactivity were mostly hetero-polysaccharides with a molecular weight over 10kD and the concentration also depends on the developmental stage of the mushroom fruit-body, the time after harvest, and the subsequent storage conditions (Shin et al. 2009, Minato et al. 2001).

β-glucans are the main polysaccharides found in mushrooms and around half of the fungal

cell wall mass is constituted by β-glucans. β-glucans are responsible for anticancer, immunomodulating, anticholesterolemic, antioxidant, and neuroprotective activities of many medicinal mushrooms. Also, they are recognized as potent immunological stimulators in humans, and it has been demonstrated their capacity for treating several diseases. β-glucans bind to a membrane receptor and induce these biological responses. But, β-glucans are not synthesized by humans and they are not recognized by human immune systems as self-molecules; as a result they induce both innate and adaptive immune responses. (Kataoka et al. 2002, Manzi et al 2000)

It has been shown by a wide range of studies that mushrooms contain components with outstanding properties to prevent or treat different type of diseases.

2 Comprehensive extraction methods

The production of mushrooms and the extraction of bioactive metabolites is a key feature for the development of efficient biotechnological methods to obtain these metabolites. Currently, methods used for the extraction of polysaccharides from medicinal and edible mushrooms mainly include hot water and acid/base extraction procedures. Extraction of polysaccharides from medicinal mushrooms is affected by various factors, such as the extraction temperature, extraction time, solvent-solid ratio, and volume of solvent used during precipitation (Jiang *et al*.2008; Mo *et al*.2013; Huang *et al*.2010; Yu *et al*.2009). Ultrasonic/microwave extraction is a new technology that has been widely used in the extraction of active ingredients from plants; however, its high cost limits its practical applications in the food industry (Cui *et al*.2014).

An alternative method is enzymatic extraction, based on the use of specific enzymes, i.e., catalytic macromolecules that accelerate biochemical reactions by lowering the activation energy (Jiang *et al*.2010). Use of enzymes in industry is associated with the advantage and disadvantage of performing reactions with high substrate specificity under controlled physicochemical conditions such as the temperature and pH.

Processing at high hydrostatic pressure (HHP) is a new and novel food processing technique which that has shown great potential in the food industry. HHP-processed foods have distinct advantages over thermally processed foods, such as minimal processing, fresh taste, and high quality and convenience with an extended shelf life (Rendueles et al. 2010; Norton et al., 2008). Treatment with 100-400 MPa has minimal or no effects on low-molecular-weight compounds such as flavor molecules, vitamins, or pigments compared to thermal processing (González-Cebrino et al. 2012; Rodrigo et al. 2007; Luscher et al. 2005). Furthermore, although pressure can affect the conformation and super-molecular structures of biomolecular systems, thereby reducing their functionality in cells (Balny et al. 2002), the covalent structure of low-molecular-weight biomolecules (peptides, lipids, and saccharides), and the primary structure of macromolecules (proteins, nucleic acids, and polysaccharides) are not perturbed by pressures up to approximately 2 GPa. The biological applications of high pressure have expanded from the food industry to pharmaceutical and other biomedical applications (Rivalain et al. 2010). In the pharmaceutical and medical sciences,

HHP has been investigated for vaccine and gene vector development, genetic transformation, and cell extraction. (Eisenmenger et al. 2009; Seefeldt et al. 2009; Aertsen et al. 2009). HHP was also previously assessed for extracting polysaccharides from *Lentinula edodes* and *Dendrobium candidum* (Lo et al. 2007; Tao et al. 2012).

3 Objectives of the study

Mushroom are rich sources of carbohydrate, protein and secondary metabolites. To date, a large number of mushrooms have been demonstrated with nutraceutical and medicinal values.

The objective of this study was to provide a method for efficient extraction of bioactive polys accharides, including β-glucans, from medicinal mushrooms.

In the current study, we investigated the utility of HHP and combined treatment with HHP, enzyme and hot water for extracting medicinal components from medicinal mushrooms. In particular, the extraction efficiency, nutritional value, and structural changes of polysaccharides extracted by different methods were evaluated.

The results of this study could expand the application of HHP and combined treatment with HHP, enzyme and hot water in development of medicinal mushroom products.

Chapter 1. Properties of polysaccharides extracted from *Phellinus linteus* using high hydrostatic pressure processing and hot water treatment

1.1. Introduction

Phellinus linteus, an edible medicinal mushroom, has various biological activities such as immunostimulatory and antitumor activities (Song et al. 2011; Sliva et al. 2008; Guo et al. 2007; Zhu et al. 2007; Kim et al. 2006). Previous studies have suggested that the primary medicinal components of *Phellinus species* are polysaccharide compounds, including intracellular polysaccharides (IPS), exopolysaccharides (EPS), acidic proteo-heteroglycans with α- and β-linkages, and a (1-6)-branched type (1-3)-glycan (Baker et al. 2008; Kim et al. 2003b). The anticancer activities of these compounds are likely related to their molecular weight, branching, and water solubility. *Phellinus linteus* is very rare in nature and it is difficult to establish and maintain artificial cultivation even for short periods without the danger of contamination. The extraction of medicinal components from *Phellinus linteus* therefore requires an efficient process with a high yield that has minimal effects on nutritional value. Although several investigators have developed extraction conditions for polysaccharide production, most methods are very laborious or require long extraction times. Existing methods also expose extracts to excessive heat, light, oxygen, toxic organic solvents, which could limit their clinical utility.

In the current study, we investigated the utility of HHP for extracting medicinal components from *Phellinus linteus* with regard to yield, medicinal value, and structural changes. The results of this study could expand the application of HHP in development of medicinal mushroom products.

1.2 Materials and methods

1.2.1 Sample Preparation

Dry fruit bodies of *Phellinus linteus* were obtained from Kyung-Dong Market, South Korea. These *Phellinus linteus* samples (30 g) were pulverized using a dry milling machine, washed, and then dispersed in distilled water (600 ml).

1.2.2 Extraction procedure

According to the procedure outlined in Figure 1.1, polysaccharides of *Phellinus linteus* were prepared by either hot-water treatment alone or HHP plus hot-water treatment.

1. Hot-water treatment

The extraction and isolation of polysaccharides were performed with 5% (W/V) of *Phellinus linteus* in distilled water (Lee et al. 2006; Kim et al., 2003). Samples were homogenized and, extracted with hot water at various temperatures (sample names : HW1 at 65 °C, HW2 at 80 °C, and HW3 at 95 °C) at 100 rpm for 6 h using a homogenizer (KINEMATICA,SWISS). Aqueous extracts were obtained by filtration using filter paper (No.42, Whatman) and solids were discarded. Water-soluble polysaccharides were concentrated to approximately 1/10 of the original volume with a rotary vacuum evaporator at 55 °C (EYELA, USA). Each concentrated extracts was precipitated with three volumes of 96% ethanol in a cold environment (4 °C) overnight, centrifuged at 11,300 ×g for 10 min, and frozen in liquid nitrogen.

2. HHP treatment plus hot-water treatment

For HPP treatment, 5% (W/V) *Phellinus linteus* in distilled water was packed into a small plastic pouch and placed into the vessel of a laboratory-scale high-pressure processor (KOBELCO, JAPAN). The vessel was then filled with distilled water. HHP treatment was performed at 200 MPa (sample: PHW1) and 300 MPa (sample: PHW2) for 10 min. The pressure was kept constant for 10 min, without considering the compression (2-3 min) and

decompression times (1-2 min). The temperature of the pressure vessel was controlled by a circulating thermostatic liquid (50 °C) from an external bath through the jackets surrounding the vessels. The temperature was not constant during the pressure treatment because of adiabatic heating (maximum temperature of 60 °C) and cooling (minimum temperature 35 °C) during pressure build-up and release, respectively. The hydrated samples following HHP treatment were homogenized and extracted with hot water at 65 °C and 100rpm for 3 h and then filtered. The water-soluble fraction was obtained by concentration, precipitation, centrifugation, and freezing in liquid nitrogen.

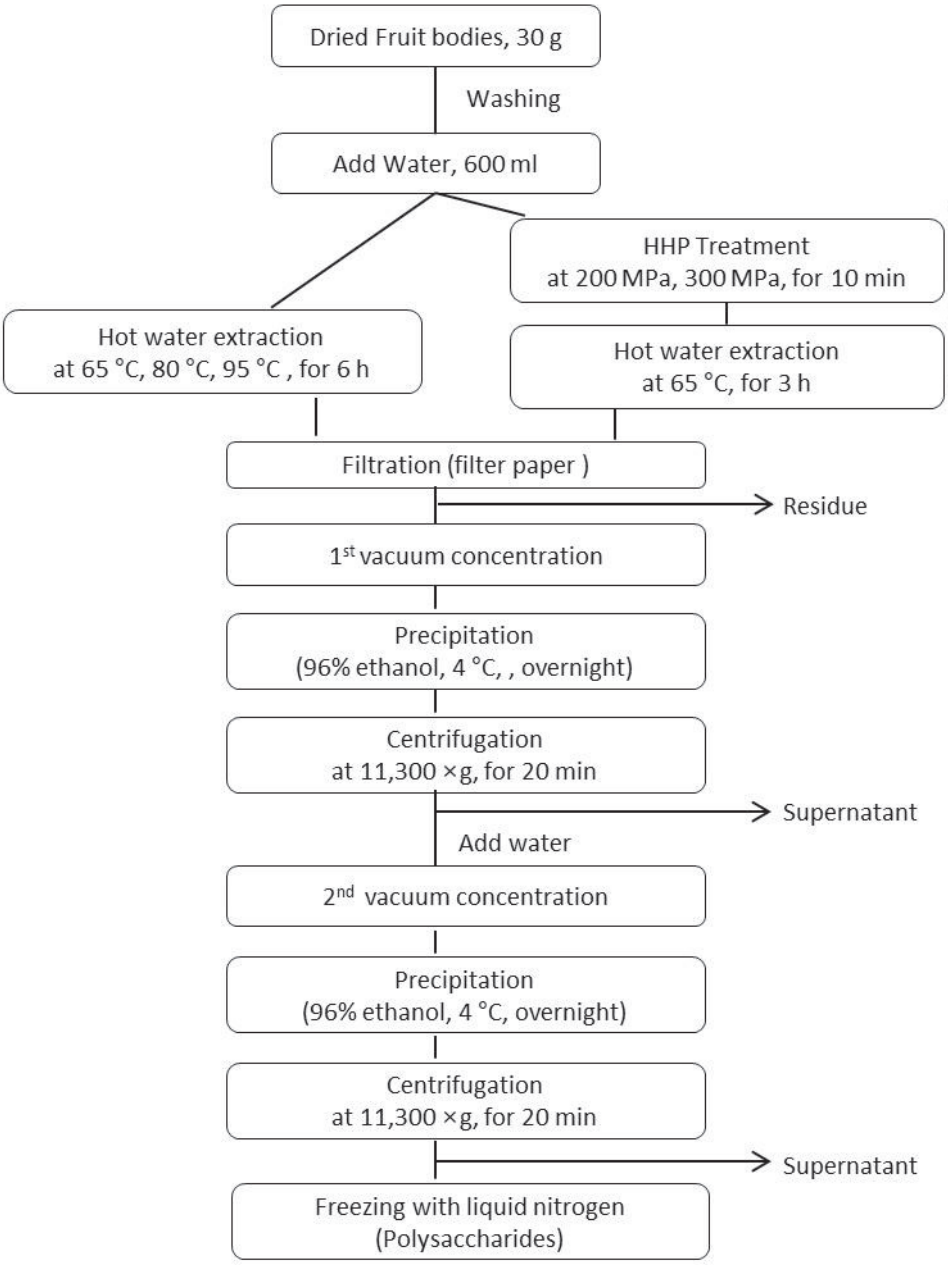


FIG. 1.1. Preparation of polysaccharides from *Phellinus linteus*

1.2.3 Polysaccharide contents and analysis of monosaccharide

Polysaccharide quantification was accomplished using a slightly modified phenol-sulfuric acid method (Dubois et al. 1956; Masuko et al. 2005). In a 96-well plate, the water soluble polysaccharide fractions (50 µL) were mixed with 30 µL of 5% phenol followed by 150 µL of concentrated sulfuric acid, and the reaction mixture was kept at 90 °C for 5 min. After cooling to room temperature, absorbance of the mixture was measured at 490 nm. All assays were conducted in triplicate, and standard curves were obtained using anhydrous D-glucose. To analyze the monosaccharide constituents, the sample was hydrolyzed with 2 M trifluoroacetic acid (TFA) at temperatures up to 120 °C. The monosaccharide profile was determined using a high-pressure liquid chromatography system (2690; Waters, USA) with a Sugar-Pak column (300 × 6.5 mm), an evaporative light-scattering detector (ELSD), and an autosampler that was controlled by Empower 2 software. The separation was achieved under analysis conditions of 10 µL injection, column oven maintained at 40 °C, mobile phase flow rate of 1.0 mL/min, mobile phase containing acetonitrile (ACN) : water (75:25) in isocratic elution. A monosaccharides Kit (Sigma-Aldrich, USA) was used for calculation of the amino acid content in samples.

1.2.4 Protein contents and amino acid analysis

The protein content of the polysaccharide samples was determined with the Bradford method using bovine serum albumin as the standard. The polysaccharide sample (100 µL) was mixed with 100 µL of Coomassie reagent (Thermo scientific, USA), and then was incubated at room temperature for 5 min. Protein concentration was determined by measuring the absorption at 595 nm (Bradford, 1976). Amino acids present in the polysaccharide samples were determined by high-performance liquid chromatography (2965; Waters, USA) with pre-column derivatization using the AccQ·Fluor reagent, an AccQ·Tag™ Nova-Pak column (150 × 3.9 mm) and a fluorescence detector (FLD) (Cohen et al. 1994). The separation was achieved under analysis conditions of 10 µL injection, column oven maintained at 37 °C, and mobile phase flow rate of 1.0 mL/min. A gradient mobile phase was used for chromatography. The mobile phase consisted of eluent A (prepared from

waters AccQ•Tag Eluent A concentrate by adding 200 mL of concentrate to 2L of Milli-Q water and mixing), eluent B (acetonitrile), and eluent C (Milli-Q water). The elution profile was: 0 min, 100% A; 18 min, 5% B; 19 min, 9% B; 29.5 min, 17% B; 33 min, 60% B, 40% C; 36 min, 100% A; 53 min, 100% A using linear gradients in between the time points. Amino Acid Standard (Sigma-Aldrich, USA) was used for calculation of the amino acid content in the samples.

1.2.5 Determination of β-glucan

The glucan polysaccharide contents were determined using a mushroom and yeast β-glucan assay kit (Megazyme Int.). For determination of total glucan (α- and β-), 10 mg of the sample was hydrolyzed using 150 µL of concentrated hydrochloric acid and then diluted into 1 ml distilled water, followed by neutralization with 1 mL of 2 N KOH. Hydrolysis to D-glucose was completed by incubation with a mixture of exo-β-(1-3)–β-glucanase and β-glucosidase in 200 mM sodium acetate buffer (pH 5.0). The hydrolyzed sample was mixed with glucose oxidase/peroxidase reagent and measured at 510 nm. To estimate α-glucan, 10 mg of the sample was suspended in 1 mL of 2 N KOH and 4 mL of 1.2 M sodium acetate buffer (pH 3.8). This solution was digested with 0.1 mL of amyloglucosidase plus invertase and incubated with 1.5 mL of GOPOD reagent, followed by measurement at 510 nm. The β-glucan content was calculated by subtracting α-glucan content from total glucan content following the manufacturer's instructions. All values of glucan content were expressed as g/100 g of dry weight of the polysaccharide.

1.2.6 Determination of Total antioxidant activity

The free-radical scavenging (FRS) activity of polysaccharide extracts was measured using the stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical by the method of Blois (1958) with minor modifications. Each extract (0.25 mL) was added to a reaction solution of 1 mL of 0.1 mM DPPH in ethanol. The mixtures were shaken vigorously and left to stand for 30 min in the dark at room temperature. The DPPH value was measured at 517 nm with a UV-VIS

spectrophotometer (1601; Shimadzu, Japan). DPPH solution was used as a blank and the mixture without the extract served as the control. For the standard curve, different concentrations of DPPH ethanol solutions (5-100 µg/mL) were used. The DPPH concentration (µg/mL) in the reaction medium was calculated from the following calibration curve, determined by the linear regression (r^2 : 0.9954) :

$$\text{Absorbance } (\lambda_{517}) = 0.1178 \text{ x[DPPH]} + 0.098$$

The radical scavenging activity was expressed as percentage of inhibition, and was calculated based on the following equation:

$$\% \text{ inhibition} = 1 - [(\text{Absorbance sample} / \text{ Absorbance control}) \text{ x}100]$$

The EC50 value (mg/ml) was defined as the total amount of antioxidant needed to decrease the initial DPPH free radical levels by 50% and determined from the plot of scavenging activity against various concentrations of sample extracts. Both ascorbic acid and butylated hydroxytoluene (BHT) were used as reference antioxidants.

1.2.7 Structure Analysis by FT-IR and ^{1}H NMR

The FT-IR (FourierTransform Infrared) spectra of polysaccharides derived from *Phellinus linteus* were recorded on a Fourier Transform Infrared Spectrometer (Bruker Optics Inc., USA). The polysaccharide was ground with KBr pellets and then pressed into pellets for FT-IR measurements in the frequency range of 500-4000 cm^{-1} with a resolution of 2 cm^{-1}. Differentiation of the samples was performed using the relative intensity of absorption bands. The peak intensity of the absorption band was measured corresponding to the main classes of chemical compounds identified in the IR spectrum.

^{1}H-NMR (Proton Nuclear Magnetic Resonance) spectra were obtained to determine the structural characteristics (α- or β- bond) of polysaccharides using a NMR spectrometer (Varian Inc., USA) operating at 600 MHz with D_2O as a solvent. ^{1}H chemical shifts were referenced to external 2, 2-dimethyl-2-silapentane-5-sulfonate sodium salt (with the methyl resonance set to 0 ppm).

1.2.8 Statistical Analysis

All results of experiments were done in triplicate. The results were expressed as the mean of three replicate determinations ± the standard deviation (S.D.).

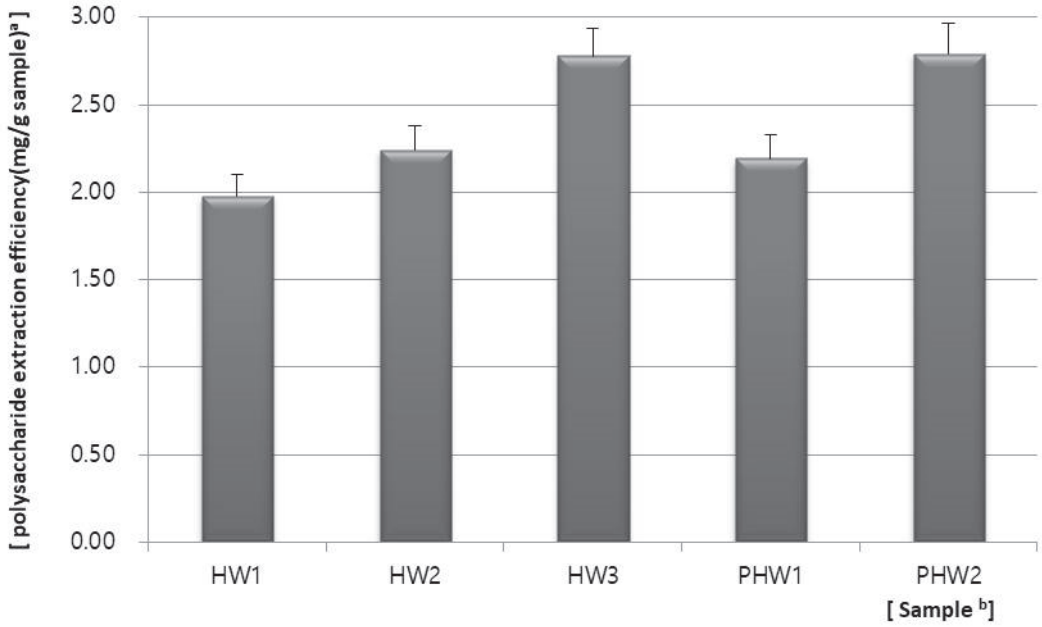
1.3 Results and discussion

1.3.1 Correlation of increasing temperature and HPP with polysaccharides extraction of *Phellinus linteus*

The polysaccharides yields increased with extraction temperature or pressure when the extraction time was kept constant (Figure 1.2) We obtained similar polysaccharides yields compared to previous studies with regard to extraction temperature, extraction time, final concentration of ethanol, and aging time (Choi 2008; Kwoen et al. 2006). The maximum yield (2.79 ± 0.03 g) of polysaccharides from *Phellinus linteu*s was obtained in the PHW2 sample extracted by HHP combined with hot water treatment (300 MPa, 50 °C, 10 min and 65 °C, 3 h), although the overall yield was similar to the yield (2.78 ± 0.03 g) of HW3 extracted by hot water (95 °C, 6 h). The content (2.19 ± 0.02 g) of polysaccharides in PHW1 extracted by the HHP plus hot water treatment (200 MPa, 50 °C, 10 min and 65 °C, 3 h) was in the same range as that in HW2 extracted by hot-water alone (80 °C, 6 h; 2.24 ± 0.02 g). The combination of HHP and hot water treatment dramatically reduced the extraction time and reduced the required extraction temperature relative to hot water treatment alone while still providing similar amounts of polysaccharides.

HHP combined with hot water treatment also resulted in significant changes in the relative abundance of polysaccharides and proteins compared to treatment with hot water alone (Table 1.1). PHW2 (300 MPa, 50 °C, 10 min and 65 °C, 3 h) yielded a remarkably lower sugar: protein ratio (4.6:1) than PHW1 (200 MPa, 50 °C, 10 min and 65 °C, 3 h; ratio 7.0:1), HW1 (65 °C, 6 h; ratio 6.9:1), HW2 (80 °C, 6 h; ratio 7.0:1), and HW3 (95 °C, 6 h; ratio 8.0:1). Asseessment of the total sugar and protein contents of polysaccharides extracted with hot water treatment alone and HHP plus hot water also indicates that this substantially lower sugar: protein ratio for PHW2 predominantly resulted from higher levels of protein present in the sample (Table 1.1). Denaturation of proteins by pressure involves the disruption of hydrophobic and electrostatic interactions, however, in contrast to heat denaturation, pressure denaturation may not involve the breakage of peptide bonds. When a protein molecule is denatured by high pressure, it undergoes an increase in hydration partly because of the electrostriction of water molecules around newly exposed charge groups (Masson

1992). Therefore, HHP has been suggested as a method to reduce aggregation and increase the water solubility of both saccharides and proteins in polymer complexes from plants. Porzucek et al. (2002) reported that HHP disrupted protein-saccharide bonds. A combination of HHP and temperature treatment was also shown to increase the hydration of insoluble dietary fiber in multiple investigations of fibers isolated from various plants (Mateos-aparicio et al. 2010; Wennberg and Nyman 2004). The results from the present study showed that the combination of HHP and hot water treatment seems to have a synergistic effect on the ability to produce soluble fractions.



[a] Polysaccharide extraction efficiency(mg/g sample): yield extracted from 30 g of *Phellinus linteus.*
[b] Samples with extraction conditions are as follows: HW1, polysaccharides extracted by hot water (65 °C) for 6 h; HW2, polysaccharides extracted by hot water (80 °C) for 6 h; HW3, polysaccharides extracted by hot water (95 °C) for 6 h; PHW1, polysaccharides extracted by hot water (65 °C) for 3 h after HHP treatment at 200 MPa, 50 °C for 10 min; PHW2, polysaccharides extracted by hot water (65 °C) for 3 h after HHP treatment at 300 MPa, 50 °C for 10 min.
Values are expressed in means ± SD (n=3).

FIG. 1.2. Yield of polysaccharides extracted from *Phellinus linteus.*

TABLE 1.1.
Nutrient contents and free-radical scavenging (FRS) activity of polysaccharides extracted from *Phellinus linteus*

Sample	Total sugar (%)[a]	Protein (%)[b]	β-Glucan (%)[c]	DPPH (inhibition %)[d]
HW1	70.2 ± 0.15	10.2 ± 0.05	18.3 ± 0.19	56.3 ±0.21
HW2	73.0 ± 0.12	10.4 ± 0.11	21.2 ± 0.21	61.8 ±0.26
HW3	81.1 ± 0.20	10.2 ± 0.10	23.4 ± 0.14	63.9 ±0.17
PHW1	75.2 ± 0.14	10.8 ± 0.17	23.2 ± 0.20	61.7 ±0.22
PHW2	73.8 ± 0.19	16.2 ± 0.21	34.5 ± 0.25	63.6 ±0.27

[a] Total sugar(%): (total sugar amount) / (polysaccharides extracted from *Phellinus linteus*).
[b] Protein(%): (protein amount) / (polysaccharides extracted from *Phellinus linteus*).
[c] β-Glucan (%): g/100 g of dry weight of polysaccharide extracted from *Phellinus linteus*).
[d] DPPH (inhibition %): antioxidative effect of polysaccharide extracted from *Phellinus linteus* against electron donating ability. Values are expressed as the mean ± SD (n=3).

1.3.2 Effects of HPP on the Medicinal value of polysaccharides

The results outlined in Table 1.1 shows that the soluble β-glucan content was increased by extraction conditions in the following order: HW1 (18.3 ± 0.19 %) < HW2 (21.2 ± 0.21 %) < PHW1 (23.2 ± 0.20 %) < HW3 (23.4 ± 0.14%) < PHW2 (34.5 ± 0.25 %). The radical scavenging activities of extracts from HW3, 63.9 ± 0.17 % and PHW2, 63.6 ± 0.27 %, were better than those of extracts from HW2, 61.8 ± 0.26 % and PHW1, 61.7 ± 0.22%. The amount of soluble β-glucan in PHW2 was substantially higher than that in the other extraction conditions. The radical scavenging activity of extracts did not vary among the various extraction conditions except that HW1 that showed relatively low scavenging activity.

The sugar compound constituents in the HW3 and PHW2 polysaccharide samples are shown in Table 1.2. The polysaccharides of Phellinus linteus consisted of simple monosaccharides of six carbon atoms and were determined to not be pure glucan; rather, they were composed of proteo-hetero-glycans with large amounts of glucose, mannose, galactose and xylose. The amounts of mannose, galactose, and xylose detected in the samples treated with HPP pl us hot water (PHW2; 26.7 ± 0.20 %, 5.8 ± 0.17 %, and 4.9 ± 0.10 %, respectively) were higher than those found in samples treated with hot water alone (HWP3; 11.9 ± 0.24 %, 1.9 ± 0.11 %, and 1.3 ± 0.12 %, respectively), but the amount of glucose in PHW2 (52.7 ± 0.25 %) was lo wer than that found in HW3 (76.7 ± 0.32 %). Although this difference in sugar contents betw een the various extraction conditions cannot easily be explained, hexose sugars exhibited hig her molar concentrations than pentose sugars in both samples. Kim et al. (2003) reported lar ge amounts of mannose, galactose, and glucose in highly purified proteo-hetero-glycan samples from *Phellinus linteus* that exhibited obvious antitumor activity. Partially puri fied polysaccharide protein complex samples from *Phellinus linteus* were also shown to be a potent immunomodulator that was predominantly composed of glucose and mannose, whe reas the crude polysaccharides from *Phellinus linteus* consisted mostly of glucose (Kim et al. 2006; Park et al. 2007).

The amino acids composition of the HW3 and PHW2 polysaccharide extraction samples was also assessed, and the results are shown in Table 1.2. Both HW3 and PHW2 had large and similar amounts of serine as major components. The amount of aspartic acid in PHW2 was

remarkably lower than that in HW3, and the amounts of lysine and arginine in PHW2 was higher than that in HW3. Although we found that the amino acid profiles in polysaccharides samples depended on the extraction procedure used, it is not known whether amino acids in protein-bound polysaccharides derived from medicinal mushrooms have significant biological effects.

TABLE 1.2.
Monosaccharide and amino acid content of polysaccharides extracted From *Phellinus linteus*.

Element	HW3[a]	PHW2[b]
Monosaccharide (%)		
Glucose	76.7 ± 0.32	52.7 ± 0.25
Mannose	11.9 ± 0.24	26.7 ± 0.20
Galactose	1.9 ± 0. 11	5.8 ± 0.17
Xylose	1.3 ± 0.12	4.9 ± 0.10
Amino acid (M %)		
Asp	11.2 ± 0.05	1.8 ± 0.41
Thr	10.1 ± 0.12	6.2 ± 0.15
Ser	19.1 ± 0.11	19.6 ± 0.50
Glu	6.8 ± 0.19	6.4 ± 0.15
Pro	6.8 ± 0.40	10.5 ± 0.35
Gly	10.1 ± 0.32	11.3 ± 0.28
Ala	7.4 ± 0.21	4.7 ± 0.43
Val	6.7 ± 0.34	7.6 ± 0.52
Met	0.0	0.0
Ile	3.4 ± 0.20	3.1 ± 0.14
Leu	6.0 ± 0.26	6.5 ± 0.18
Tyr	0.0	0.0
Phe	0.0	0.0
His	0.0	0.0
Lys	8.0 ± 0.45	14.2 ± 0.27
Arg	6.3 ± 0.17	11.8 ± 0.38
Cys	0.0	0.0

[a] HW3, polysaccharides extracted by hot water (95 °C) for 6 h.
[b] PHW2, polysaccharides extracted by hot water (65 °C) for 3 h after HHP treatment at 300 MPa, 50 °C for 10 min. Values are expressed as the mean ± SD (n=3).

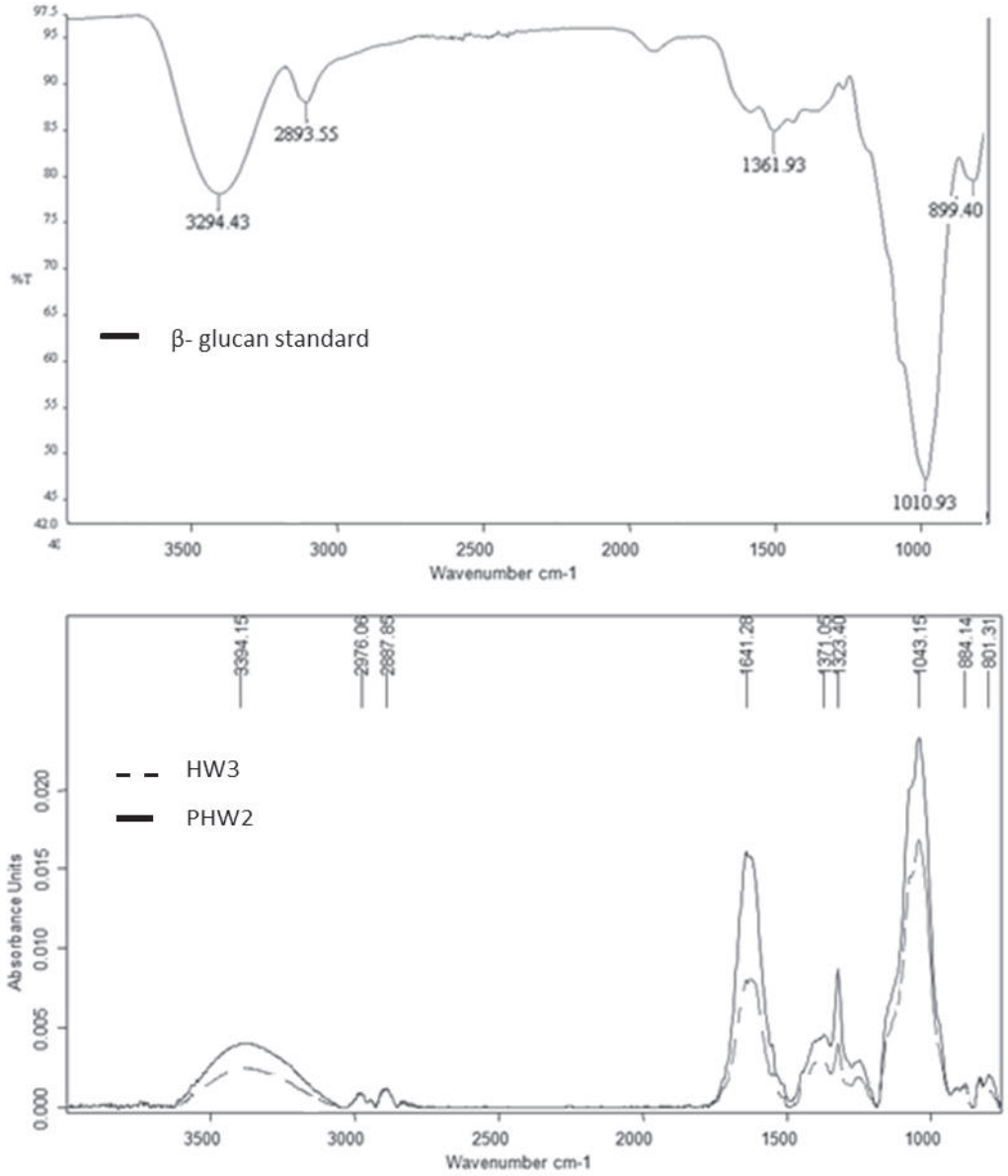
1.3.3 Structural characterization of polysaccharides

The physiological properties of polysaccharides from medicinal mushrooms are substantially different depending on their linkage type. Therefore, the elucidation of bond configuration is an important consideration in structural and bioactivity studies of polysaccharides. FT-IR spectroscopy can be used to analyze the structures of polysaccharides to elucidate important characteristics, including monosaccharide types, glycosidic bonds, and functional groups.

FT-IR spectra of the HW3 and PHW2 polysaccharides samples showed the typical characteristics of β-glucan (Figure 1.3). There was an absorption peak in the range of 3300-3400 cm^{-1}, representing the stretching vibration of the expanding and contracting carbohydrate O-H, and peaks in the range of 3000-2800 cm^{-1}, representing the stretching vibration of the carbohydrate C-H bonds. Peaks in the range of 1600-1650 cm^{-1} represent the stretching vibration of the carbohydrate C-O, and an obvious protein absorption peak in the N-H stretching region indicates the presence of a polysaccharide-protein complex. The peak in the 1000-1100 cm^{-1} range represents the stretching vibration of -H and C-O in the carbohydrate, and the C-H bond in the β-configuration showed an absorption peak near 880-890 cm^{-1}. These results indicate that the polysaccharides of both samples were composed of protein-bound polysaccharides linked by β-glycosidic bonds, and the overall absorption intensities of PHW2 were notably higher than those of HW3.

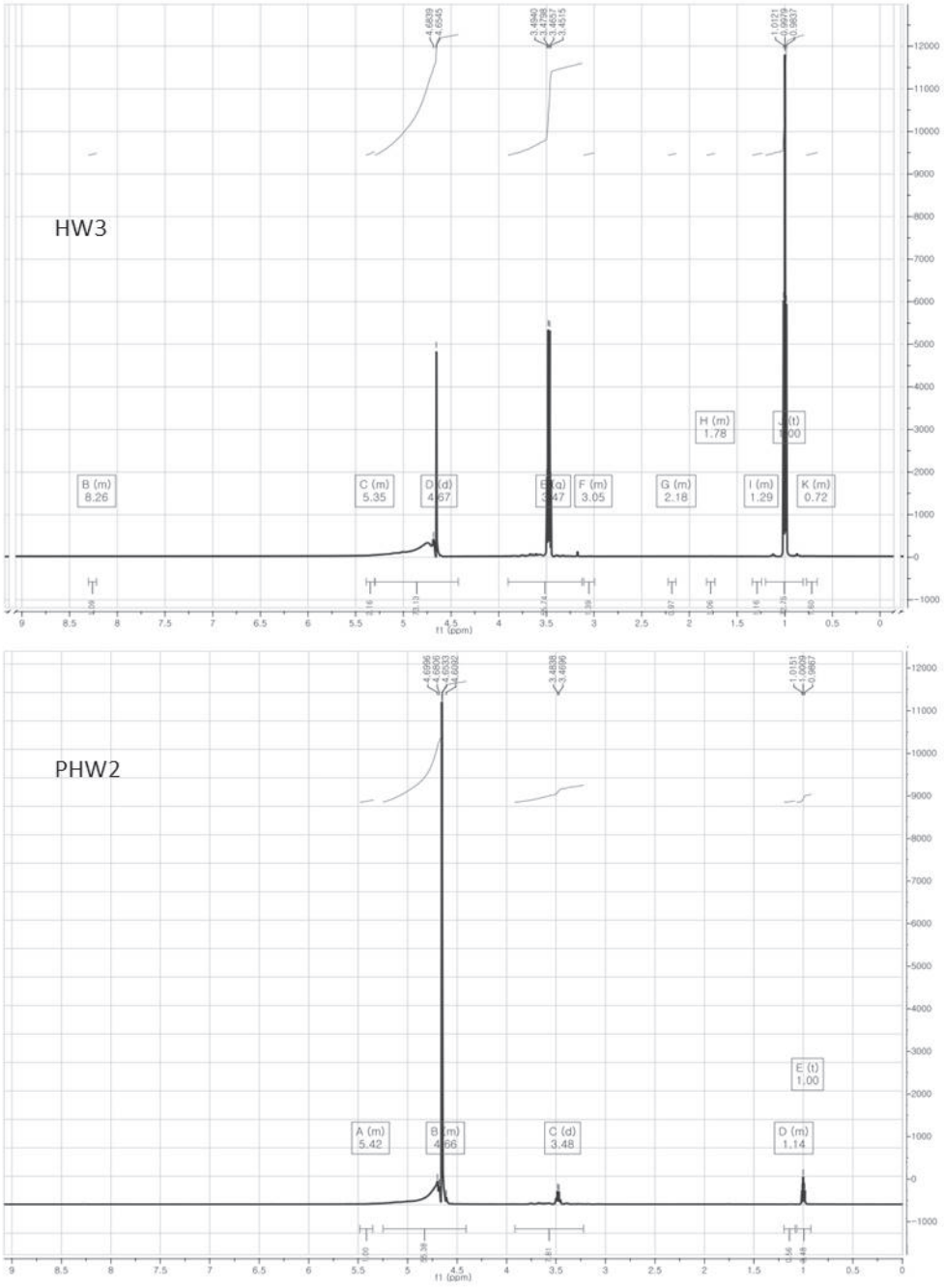
NMR is also a powerful tool for obtaining information regarding anomeric carbohydrate configuration. The ^{1}H-NMR spectra of both samples showed proton signal peak densities lower than 5.0 ppm, indicating ß-linkages in corroboration with the results from the FT-IR spectrum (Figure 1.4). It was previously reported that a ^{1}H chemical shift of the anomeric region is observed around 4-6 ppm (Gonzaga et al. 2005). The anomeric signals for (1→3)-β-glucan and (1→6)-β-glucan appear at 4.7-4.8 ppm and 4.5-4.6 ppm, respectively (Sugawara et al. 2004). In the ^{1}H NMR spectra of both samples, a doublet peak was observed around 4.7 ppm indicating a (1→3)-β-glucopyranosyl unit chain, similar to results from a previous report (Kimura et al. 2007). In particular, a β-(1→3)-linked backbone indicated by a peak at 4.6-4.7 ppm was observed in PHW2 compared with HW3. Furthermore, the signal peaks in

the range of 0.5-3.0 ppm implied that polysaccharides of PHW2 were more refined than those of HW3 (Silvestein et al. 1994).



Absorption(cm-1)		Functional group	Structural characteristic
3350	3400	hydroxyl group (-OH) amino group (-NH2)	stretching vibration of O-H stretching vibration of N-H
2887	2976	alkyl group (-CH2-)	stretching vibration of C-H
1640	1645	carbonyl group (-C=O or -CHO) amide group (-NH2 or –COR)	stretching vibration of C= bending vibration of N-H or stretching vibration of C=O
1320	1370	carboxyl group (-COOH)	symmetrical stretching vibration of C=O
1040	1050	hydroxyl group (-OH)	bending vibration of O-H
880	890	β-D-glycosidic bond	antisymmetrical ring vibration

FIG. 1.3. FT-IR spectra of polysaccharide extracts from *Phellinus linteus*



Shift(ppm) HW3	Shift(ppm) PHW2	Structural characteristic
4.67	4.66	(1→3)-β-glucan (β-(1→3)-glucopyranosyl residues)
3.47	3.48	Absent if pure glucan
1.00	1.14	

FIG. 1.4. 1H-NMR spectra of polysaccharide extracts from *Phellinus linteus*

1.4 Conclusions

Polysaccharides derived from medicinal mushrooms have emerged as an important class of bioactive substances. Some of the primary medicinal components of *Phellinus species* are polysaccharide compounds that exhibit various biological activities such as the stimulation of humoral and cell-mediated immunity, anti-mutagenic activity, and anti-cancer activity. Furthermore, the bioactivity is related to the solubility and linkage type of β-glucan among the various polysaccharides. In this study, we used the HHP technique to investigate optimal conditions for the extraction of polysaccharides from *Phellinus species*. The results suggest that combining the HHP and hot water treatment was a more efficient process to obtain high yields (2.79 ± 0.03 g) of soluble polysaccharides, with a shorter extraction time (from 6 h to 3 h 10 min) and lower extraction temperatures (from 80 ~ 95 °C to 65 °C) compared to hot water treatment alone. It also appears that HHP plus hot water treatment induced the disruption of protein-saccharide bonds that could otherwise hinder extraction efficiency, and increase the content of β-glucan without disrupting the β-glycosidic bonds, which is important for the beneficial physiological properties of polysaccharides. The β-glucan of PHW2 sample was 1.5 times higher than that of HW3 sample. Combining HHP and hot water treatment could improve the efficiency of soluble bioactive polysaccharide extraction, and could be useful for the preparation of functional ingredients from *Phellinus species* for food fortification. To further determine the effects of HHP and hot-water treatment for the extraction of polysaccharides, it will be necessary to further elucidate the molecular mechanisms of the biological effects of *Phellinus species* extracts with regard to their bioactivity and medicinal properties.

Chapter 2. P olysaccharide extraction from medicinal mushrooms by combined enzyme–high hydrostatic pressure

2.1 Introduction

Mushroom growing has a long tradition in Asian countries, especially in China, Japan, and Korea. The demands are growing on the fact that mushroom encompasses a huge diversity of biomolecules not only nutritional but also their medicinal properties. Medicinal mushrooms produce beneficial effects not only as drugs but also as components of a novel class of products—referred to by names such as dietary supplements, functional foods, nutriceuticals, mycopharmaceuticals, and designer foods, including pro- and prebiotics—which provide health benefits on daily consumption (Wasser 2014; Valverde *et al*.2015).

In terms of chemical structure, numerous polysaccharides with anticancer properties isolated from basidiomycetes are composed of homo- and heteropolymers, specifically, (1→3), (1→6)-β-glucans and (1→3)-α-glucans (Santos-Neves *et al*.2008; Unursaikhan *et al*.2006). Several clinical studies have demonstrated the anticancer activity of *Agaricus bisporus* (Smiderle *et al*.2011; Smiderle *et al*.2010), *Lentinula edodes* (Chihara *et al*.1970; Hao *et al*.2012; Bisen *et al*.2010), *Ganoderma lucidum* (Nahata *et al*.2013; Jin *et al*.2012), and *Phellinus linteus* (Hsieh *et al*.2013; Reis *et al*.2014). Thus, many mushroom polysaccharides and polysaccharide conjugates, e.g., lentinan, krestin, schizophyllan, and active hexose correlated compound, have been commercialized for anticancer therapy (Elsayed *et al*.2014; Kozarski *et al*.2014; Phan *et al*.2015).

To date, several methods have been reported for improving the yield, efficacy, and safety of extraction technologies for medicinal mushrooms. Among them, high hydrostatic pressure (HHP) processing is considered the most successful commercial food processing technology with great potential for food industry applications. However, HHP conditions induce changes in the native structure of biopolymers such as starches and proteins, similarly to high temperatures. Moreover, enzymatic reactions may be reversibly enhanced or inhibited by pressure because of changes in the substrate specificity or in the rate-limiting molecular structures. In addition, pressure-dependent alterations of membranes can cause the release

of intracellular enzymes into extracellular fluids or cell cytoplasm (Cheftel 1992; Heremans 1982; Verlent *et al*.2005; Gekko 2002). Some researchers studied the effect of pressure on enzymatic systems and reported increased thermal stability of proteins and enzymes under specific pressure conditions (Balny 1998; Knorr *et al*.2006). Moreover, previous studies demonstrated that pressure is efficient in enhancing the catalytic activity in the enzymatic extraction of polysaccharides from plants such as lime and mushrooms (Naghshineh *et al*.2013; Tao *et al*.2012).

In this study, a combination of enzymes, hot water treatment, and HHP was used to extract polysaccharides from medicinal mushrooms. In particular, the extraction efficiency, nutritional value, and structural changes of polysaccharides extracted by different methods were evaluated.

2.2 Materials and methods

2.2.1 Sample and enzyme preparation

Dry fruiting bodies of medicinal mushrooms *A. bisporus*, *L. edodes*, *G. lucidum*, and *P. linteus* were obtained from Kyung-Dong Market, South Korea. These samples (25 g) were pulverized using a dry milling machine (DSMP-370SUS, DUKSAN CO.,LTD), washed, and dispersed in distilled water (500 mL). The two enzymes, cellulase A "AMANO" and pectinase G "AMANO", were purchased from Amano Enzyme Inc., Japan.

2.2.2 Extraction procedure

According to the procedure outlined in Figure 2.1, polysaccharides were extracted from medicinal mushrooms by hot water treatment, enzyme–hot water treatment, or HHP-enzyme–hot water treatment (Zhang *et al*.2007; Lee *et al*.2006; Xu *et al*.2014; Hitoshi *et al*.2000; Lo *et al*.2007).

1. Hot water treatment (W)

The extraction and isolation of polysaccharides from medicinal mushrooms were carried out with 5% (w/v) medicinal mushrooms in distilled water. The samples were homogenized and extracted with hot water at 95 °C and 100 rpm for 4 h with a homogenizer (Kinematica, Swiss)**.** Aqueous extracts were obtained by filtration through filter paper (No. 42, Whatman), and the solids were discarded. The solution containing water-soluble polysaccharides was concentrated to about 1/10 of the original volume with a rotary vacuum evaporator at 55 °C (EYELA, USA). The concentrated extracts were precipitated by adding three volumes of 96% ethanol at 4 °C overnight, centrifuged at 11,300 × *g* for 10 min, and frozen in liquid nitrogen (sample W240).

2. Hot water and enzyme treatments (WE)

The hydrolysis, extraction, and isolation of polysaccharides from medicinal mushrooms were carried out with 5% (w/v) medicinal mushrooms in distilled water. The enzyme blend

(cellulose A: pectinase G = 1:1) (1.0 g) was added to the solution of medicinal mushrooms. The samples were hydrated at 45 °C and 100 rpm for 30 min (sample E30W30) or 60 min (sample E60W30), and then treated with hot water at 95 °C and 100 rpm for 30 min. The hydrated samples were obtained by filtration through filter paper (No. 42, Whatman), and the solids were discarded. The water-soluble fraction was obtained by concentration, precipitation, centrifugation, and freezing in liquid nitrogen as described for the hot water treatment.

3. HHP, enzyme, and Hot water treatments (WEP)

For the extraction of polysaccharides, a combination of HHP, enzyme, and hot water was used. The enzyme blend (cellulose A:pectinase G = 1:1) (1.0 g) was mixed with 5% (w/v) medicinal mushroom in distilled water, and the sample was packed into a small plastic pouch and placed into the vessel of a laboratory-scale high-pressure processor (KOBELCO, Japan). The vessel was then filled with distilled water. HHP treatment was performed at 150 MPa or 250 MPa. The pressure was kept constant for 20 min, without considering the compression (2~3 min) and decompression times (1~2 min). The temperature of the pressure vessels was controlled by a circulating thermostatic liquid (45 °C) from an external bath through the jackets surrounding the vessels. The temperature was not constant during the pressure treatment due to adiabatic heating (maximum temperature of 50 °C) and cooling (minimum temperature of 40 °C) during pressure build-up and release, respectively. However, it was estimated that the enzymatic reaction proceeded for 20 min. After HHP treatment, the samples were homogenized and extracted with hot water at 95 °C and 100 rpm for 30 min and then filtered. The water-soluble fraction was obtained by concentration, precipitation, centrifugation, and freezing in liquid nitrogen as described for the hot water treatment. (samples E20P150W30 and E20P250W30).

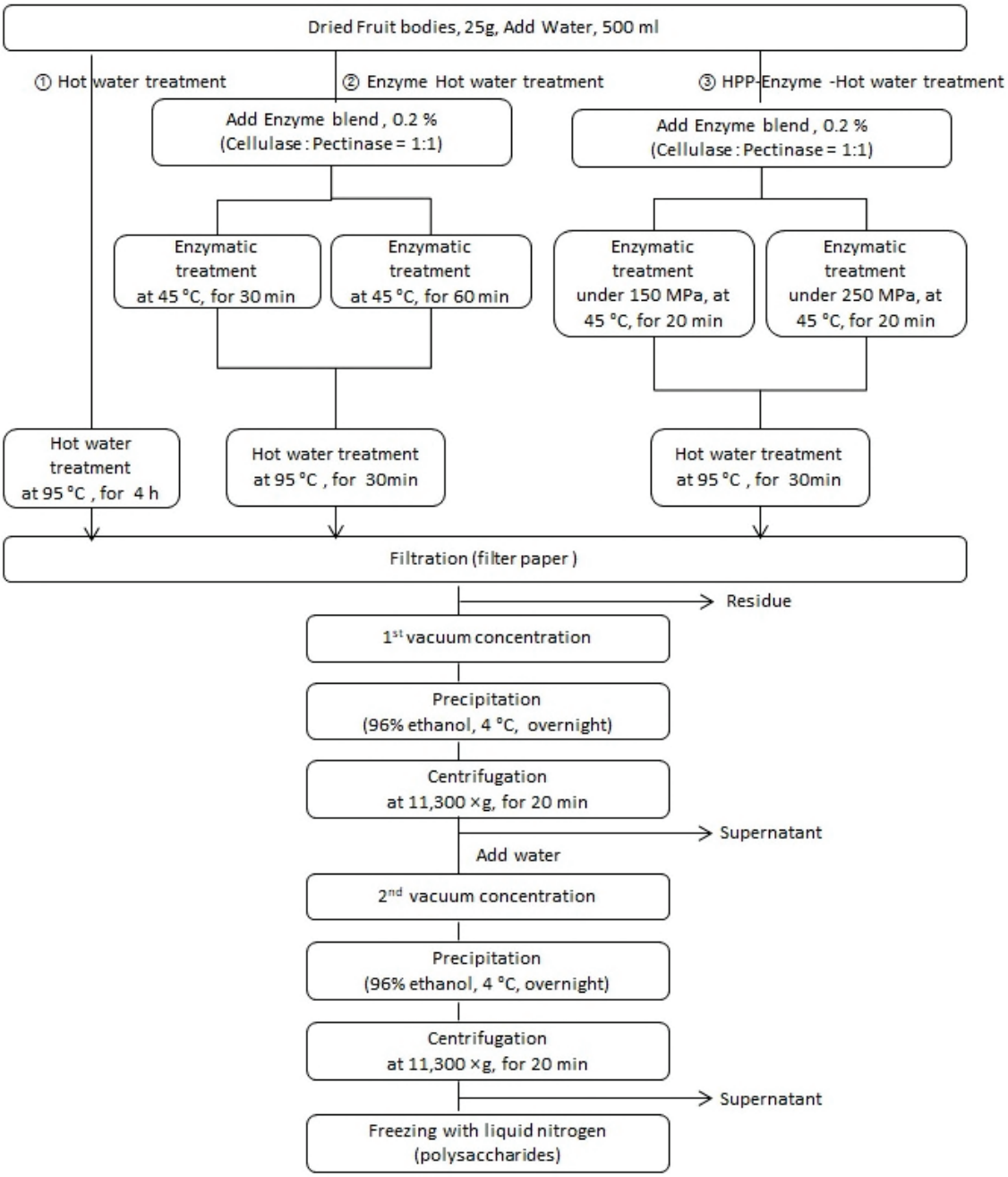


FIG. 2.1. Extraction of polysaccharides from medicinal mushrooms

2.2.3 Total sugar content and analysis of monosaccharides

The total carbohydrate content was determined using a slightly modified phenol-sulfuric acid method (Dubois *et al*.1956; Masuko *et al*.2005). The water-soluble polysaccharide fractions (50 µL) were mixed with 30 µL of 5% phenol solution in a 96-well plate, followed by addition of 150 µL of concentrated sulfuric acid, and the reaction mixture was kept at 90 °C for 5 min. After cooling to room temperature, the absorbance of the mixture was measured at 490 nm. All assays were carried out in triplicate, and standard curves were obtained using anhydrous D-glucose. To analyze the monosaccharide components, the sample was hydrolyzed with 2 M trifluoroacetic acid at temperatures up to 120 °C. The monosaccharide profile was analyzed using a high-performance liquid chromatography (HPLC) system (2690; Waters, USA) equipped with a Sugar-Pak column (300 × 6.5 mm), an evaporative light-scattering detector, and an autosampler, and controlled by Empower 2 software. The separation was achieved under the following conditions: 10 µL injection volume, column oven temperature of 40 °C, mobile phase flow rate of 1.0 mL/min, and acetonitrile:water (75:25) mobile phase in isocratic elution. A monosaccharides kit (Sigma-Aldrich, USA) was used for the determination of the content .

2.2.4 Protein content and analysis of amino acids

The protein content of the samples was determined with Bradford method using bovine serum albumin as the standard (Bradford 1976). The polysaccharide sample (100 µL) was mixed with 100 µL of Coomassie reagent (Thermo scientific, USA), and the mixture was incubated at room temperature for 5 min. The protein concentration was determined by measuring the absorption at 595 nm. The amino acid composition of the samples was determined by HPLC (2965; Waters, USA) with pre-column derivatization using the AccQ·Fluor reagent, an AccQ·Tag^{TM} Nova-Pak column (150 × 3.9 mm), and a fluorescence detector (Cohen *et al*.1994). The separation was achieved under the following conditions: 10 µL injection volume, column oven temperature of 37 °C, and mobile phase flow rate of 1.0 mL/min. A gradient mobile phase was used for chromatography, consisting of eluent A (prepared by mixing 200 mL of AccQ·Tag Eluent A concentrate with 2 L of Milli-Q water),

eluent B (acetonitrile), and eluent C (Milli-Q water). The elution profile was as follows: 0 min, 100% A; 18 min, 5% B; 19 min, 9% B; 29.5 min, 17% B; 33 min, 60% B, 40% C; 36 min, 100% A; 53 min, 100% A, using linear gradients in between the time points. An amino acid standard (Sigma-Aldrich, USA) was used for the determination of the amino acid content.

2.2.5 Determination of β-glucan

The glucan content was determined using a mushroom and yeast β-glucan assay kit (Megazyme Int., Wicklow, Ireland). For the determination of total (α- and β-) glucan, 10 mg of sample was hydrolyzed with 150 µL of concentrated hydrochloric acid and diluted with 1 mL distilled water, followed by neutralization with 1 mL of 2 N KOH. Hydrolysis to D-glucose was completed by incubation with a mixture of exo-β-(1-3)–β-glucanase and β-glucosidase in 200 mM sodium acetate buffer (pH 5.0). The hydrolyzed sample was mixed with a glucose oxidase/peroxidase (GODOP) reagent and analyzed at 510 nm. To estimate α-glucan levels, 10 mg of sample was suspended in 1 mL of 2 N KOH and 4 mL of 1.2 M sodium acetate buffer (pH 3.8). The solution was digested with 0.1 mL amyloglucosidase plus invertase and incubated with 1.5 mL of GOPOD reagent, followed by measurement at 510 nm. The β-glucan content was calculated by subtracting the α-glucan from the total glucan content following the manufacturer's instructions. All values of glucan content were expressed as g/100 g of dry weight of polysaccharide.

2.2.6 Structure analysis by Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectra of medicinal mushroom polysaccharides were recorded on a Fourier transform infrared spectrometer (Bruker Optics Inc., USA). The samples were ground with KBr and then pressed into pellets for FT-IR measurements in the frequency range of 500-4000 cm^{-1} with a resolution of 2 cm^{-1}. Differentiation of the samples was performed using the relative intensity of absorption bands. The peak intensity of the absorption bands corresponding to the main classes of chemical compounds was measured.

2.2.7 Statistical analysis

All experiments were carried out in triplicate. The results are expressed as the mean of three determinations ± standard deviation (SD).

2.3 Results and Discussions

2.3.1 Comparison of extraction methods of polysaccharides from medicinal mushrooms

The traditional hot water extraction procedure gave high polysaccharide yields (Figure 2.2, samples W240): approximately 2.39 g/25 g from *A. bisporus*, 2.34 g/25 g from *L. edodes,* 1.78 g/25 g from *G. lucidum*, and 1.79 g/25 g from *P. linteus*. Interestingly, the addition of enzymes for a short reaction time (Table 1, samples E30W30 and E60W30) did not improve the extraction yields; this was attributed to the short hot water treatment. Thus, we concluded that the hot water treatment time is crucial for polysaccharide extraction efficiency.

Next, we attempted to optimize the extraction conditions by application of HHP and enzyme, and the results are summarized in Figure 2.2. Surprisingly, a high-pressure treatment for only 20 min increased the polysaccharide yields (samples E20P150W30 and E20P250W30) up to 2.40 g/25 g, 2.35 g/25 g, 1.77 g/25 g, and 1.80 g/25 g for the E20P250W30 samples from *A. bisporus*, *L. edodes*, *G. lucidum*, and *P. linteus*, respectively. These values were similar to those obtained by hot water treatment (samples W240).

We found that the combination of HHP, enzyme, and hot water treatments provided similar polysaccharide yields in shorter extraction times and required lower energy compared to the hot water treatment alone. Moreover, performing the hot water treatment under HHP conditions after the enzymatic step improved the enzymatic release of mushroom polysaccharides providing higher yields as compared to the enzymatic extraction at ambient pressure. Thus, the combination of HHP, enzyme, and hot water treatments shows a synergistic effect providing a more efficient mushroom polysaccharide extraction process.

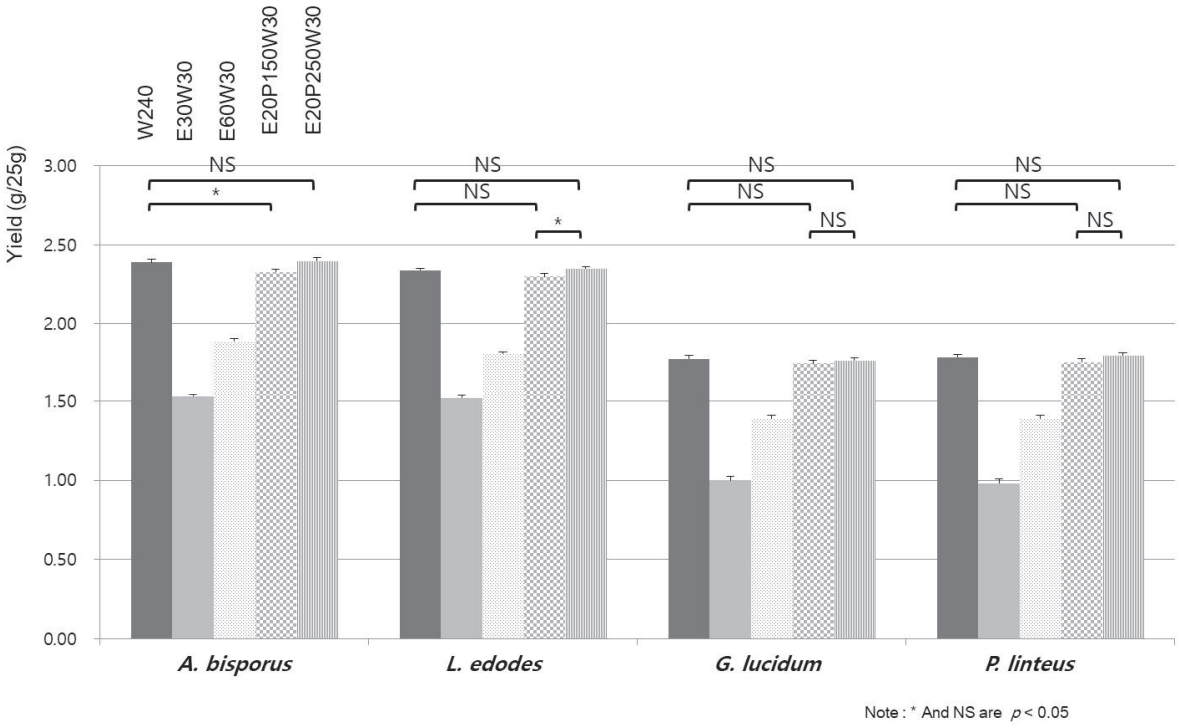


FIG. 2.2. Comparison of extraction efficiency from medicinal mushrooms

2.3.2 Effect of HHP on the medicinal properties of polysaccharides

The soluble β-glucan content (g/100 g of dry weight of polysaccharide) of medicinal mushroom polysaccharides increased by the combination of HHP, enzyme, and hot water treatments (Figure2.3, compare samples W240 and E20P250W30) : from 19.76 (W240) to 24.13 (E20P150W30) for *A. bisporus*, from 17.89 (W240) to 21.32 (E20P150W30) for *L. endodes*, from 15.08 (W240) to 18.82 (E20P250W30) for *G. lucidum*, and from 17.58 (W240) to 18.20 (E20P250W30) for *P. linteus*. The β-glucan content of the polysaccharide samples extracted by high-pressure treatment was about 1.1–1.3 times higher than that of samples extracted by hot water treatment alone. Comparison of the β-glucan levels in the extracts obtained with various methods indicates that enzymatic treatment of *A. bisporus* and *L. edodes* at 150 MPa provides better results than the treatment at 250 MPa at the same temperature and time. On the other hand, pressure treatment at 250 MPa proved to be the best extraction method from *G. lucidum* and *P. linteus*.

In order to estimate the pressure and temperature effects on the enzyme-catalyzed substrate conversion, the monosaccharide and amino acid contents of polysaccharides extracted by different methods were also analyzed, and the results are summarized in Figure 2.4. The amounts of mannose, galactose, and xylose detected in *A. bisporus*-E20P150W30 and *P. linteus*-E20P250W30 were higher than those found in *A. bisporus*-W240 and *P. linteus*-W240, respectively. Moreover, glucose and galactose were detected in higher amounts in *L. edodes*-E20P150W30 and *G. lucidum*-E20P250W30 than in *L. edodes*-W240 and *G. lucidum*-W240, respectively. Notably, the monosaccharide profiles of polysaccharides obtained by both hot water treatment alone and a combination of HHP, enzyme, and hot water treatments were similar to those including medicinal components related to anticancer effect, activation of host immune response system described in previous studies.

The amino acid composition of the extracted polysaccharide samples (namely, *A. bisporus*-W240 and -E20P150W30, *L. edodes*-W240 and -E20P150W30, *G. lucidum*-W240 and -E20P250W30, and *P. linteus*-W240 and -E20P250W30) was also determined, and the results are shown in Figure 2.5. The arginine, glutamic acid, and proline contents of polysaccharide samples *A. bisporus*-E20P150W30, *L. edodes*-E20P150W30, *G. lucidum*-E20P250W30, and *P.*

linteus-E20P250W30 were higher than those of the corresponding W240 samples, whereas the serine and tyrosine contents were lower.

Briefly, samples from *A. bisporus* contained mainly proline and arginine, samples from *G. lucidum* contained mainly alanine and glutamic acid, and samples from *L. edodes* and from *P. linteus* contained large amounts of glutamic acid. Similar amounts of the other amino acids were found in all polysaccharide samples regardless of the extraction conditions.

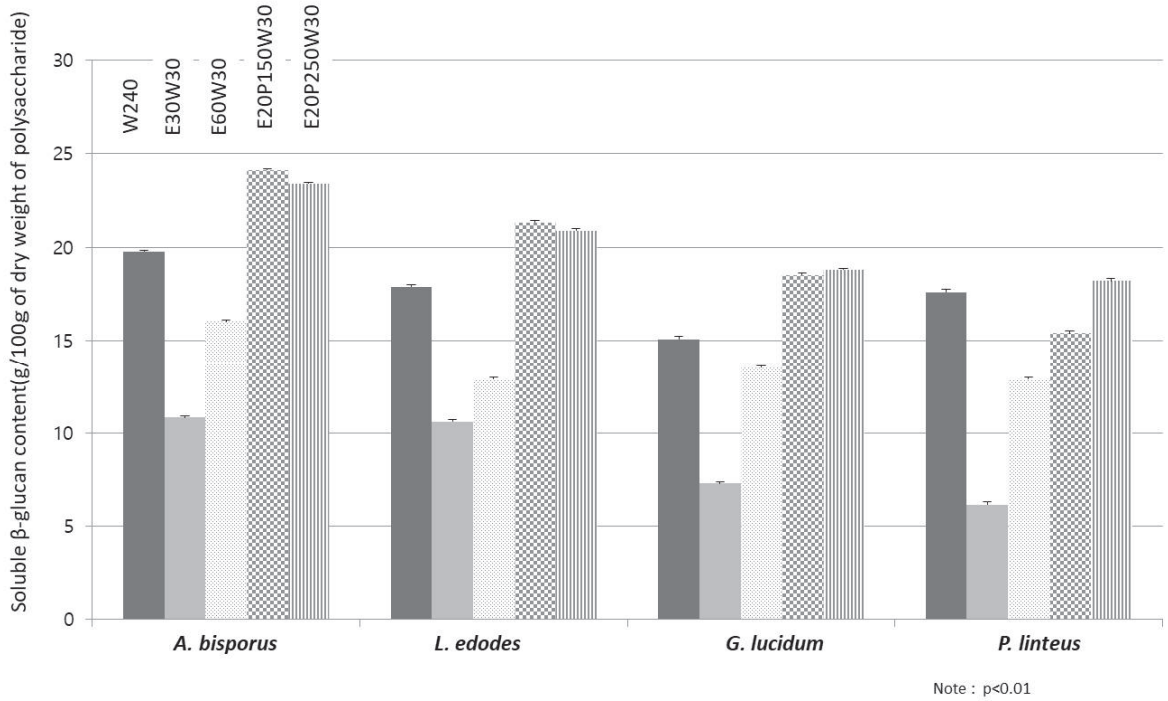


FIG. 2.3. Soluble β-glucan content of medicinal mushroom polysaccharides

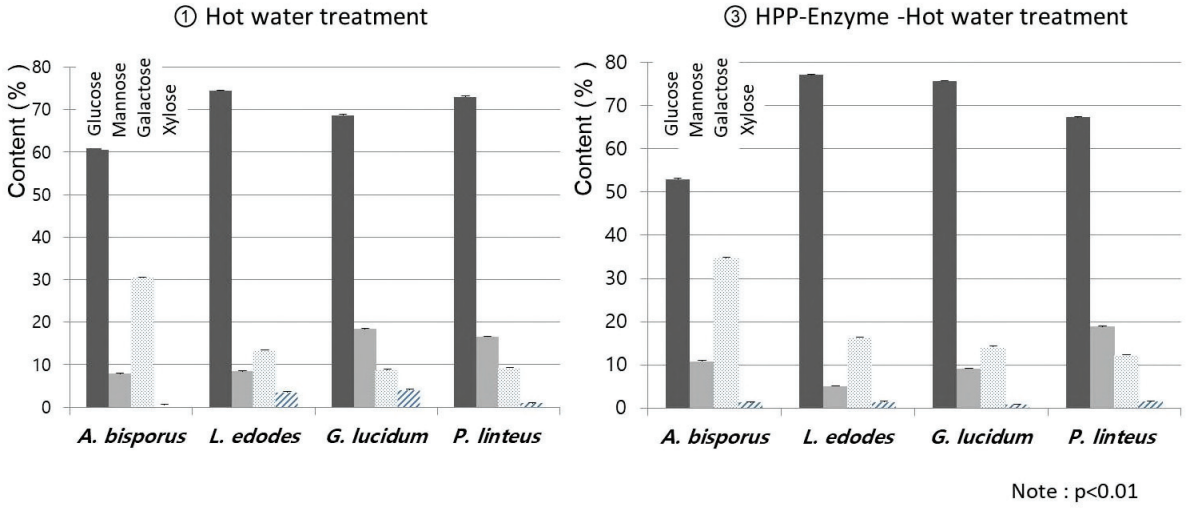


FIG. 2.4. Monosaccharide content of medicinal mushroom polysaccharides.

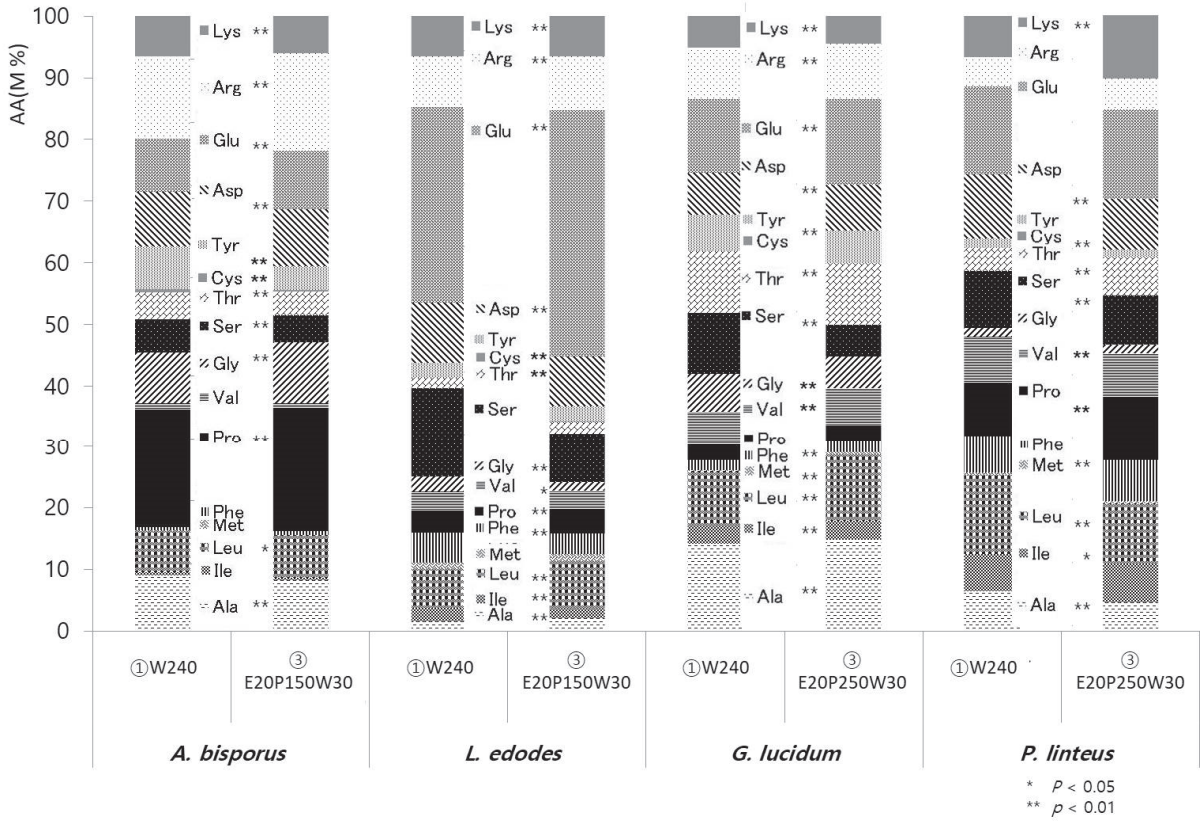


FIG. 2.5. Amino acid contents of medicinal mushrooms polysaccharides.

2.3.3 Structural characterization of polysaccharides

The physiological properties of medicinal mushroom polysaccharides are strongly dependent on the linkage type. Thus, bond configuration is an important parameter in structure-activity relationship studies of polysaccharides. The FT-IR spectra of the polysaccharide extracts showed the typical polysaccharide absorption bands, which can be used to elucidate important characteristics including monosaccharide types, glycosidic bonds, and functional groups, as well as absorption peaks typical of proteins.

The characteristic bands of β-glucans were detected (Figure 2.6). An absorption peak in the range of 3600~3200 cm^{-1}, assigned to the stretching vibration of the O-H groups of the sugar moiety and N-H bonds of the amino group, and peaks in the range of 3000~2800 cm^{-1}, assigned to the stretching vibration of the sugar C-H bonds, were observed. The peaks in the range of 1700~1500 cm^{-1} can be attributed to protein N-H stretching, which indicates the presence of a polysaccharide-protein complex, and to the stretching vibration of aromatic C=C bonds conjugated with C=O and/or COO- bonds, which indicates the presence of polyphenols. The band in the 1400~1300 cm^{-1} region is due to phenolic OH groups. The absorption bands in the mid-infrared region 1200–800 cm^{-1} are assigned to polysaccharides with different structure and composition: the peak in the 1200~1100 cm^{-1} range represents the stretching vibration of -H, C-O of the carbohydrate, the peaks in the 1100~1000 cm^{-1} range are due to the *O*-substituted glucose residues on the protein-conjugated β-glucans, the peaks in the 960~910 cm^{-1} range are attributed to α-glycosidic linkages, and the band in the 890~860 cm^{-1} range is assigned to a C-H bond in the β-configuration (Pierce *et al*.1995; Sandula *et al*.1999).

These results indicate that the samples were composed of protein-bound polysaccharides linked by α- and β-glycosidic bonds, and the overall absorption intensities of *A. bisporus*-E20P150W30, *L. edodes*-E20P150W30, *G. lucidum*-E20P250W30, and *P. linteus*-E20P250W30 were much higher than those of the corresponding W240 samples.

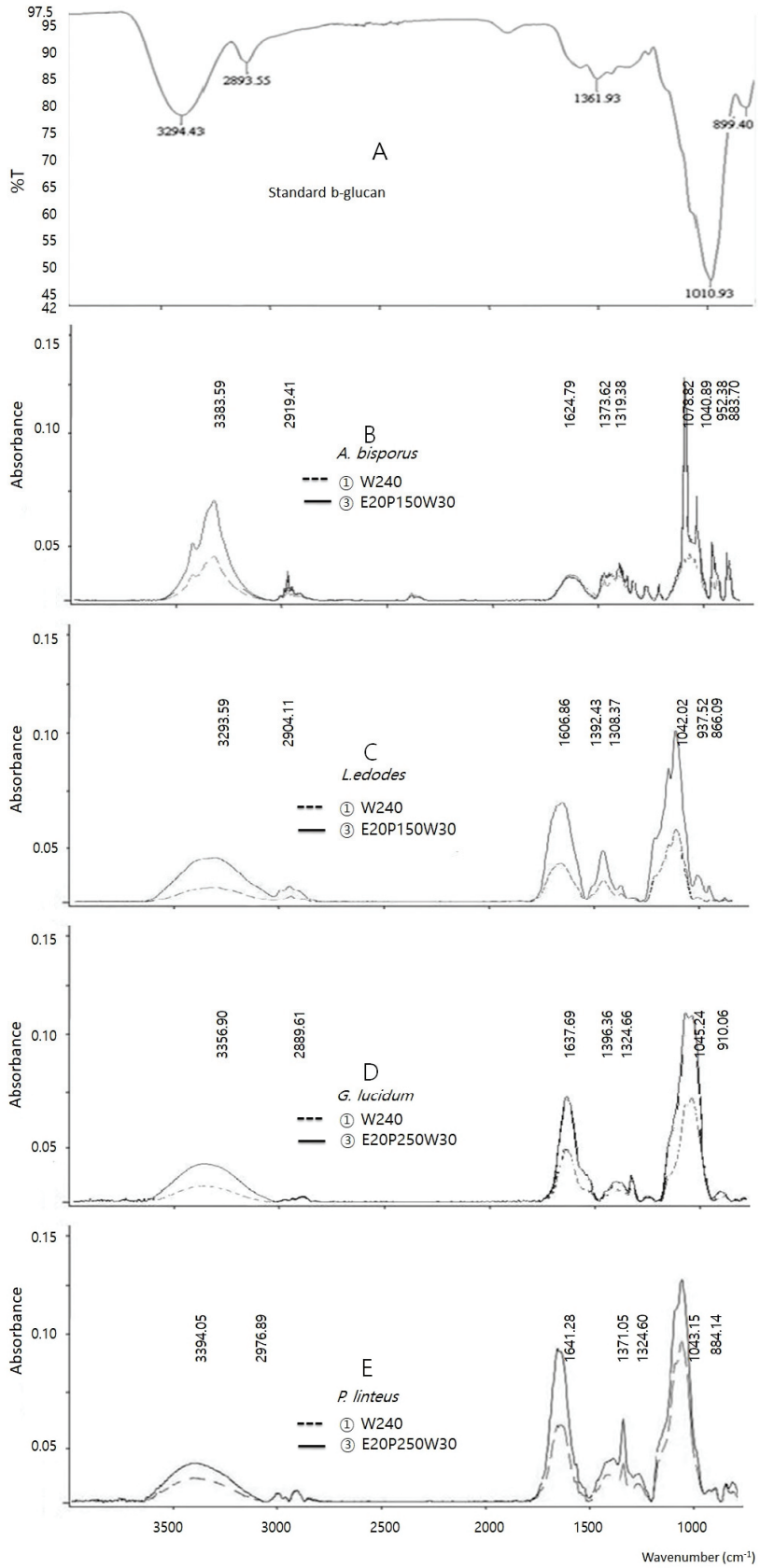


FIG. 2.6. FT-IR spectra of medicinal mushroom polysaccharides

2.4 Conclusions

The objective of this study was to provide a method for efficient extraction and isolation of bioactive polysaccharides, including β-glucans, from medicinal mushrooms. During our attempts to maximize the extraction efficiency, we found that a HHP treatment enhances the catalytic activity of selected enzymes and therefore contributes to efficient polysaccharide extraction from medicinal mushrooms. The developed extraction method provides easy access to bioactive compounds from medicinal and edible mushrooms, thereby decreasing the economic burden to patients and paving the way for scientific clinical studies. However, the mechanism of action of enzymes such as cellulase and pectinase on polysaccharides under HHP conditions and under combined HHP–hot water treatments is still poorly understood. Further studies on this extraction process based on a combination of enzymatic hydrolysis, HHP, and hot water treatments will provide new perspectives for the industrial application of high pressure technology.

General conclusion

A proper extraction method could greatly affect the quality and quantity of bioactive compounds from natural plants. Mushroom contains a variety of nutrition compounds, with great potential for therapeutic application. The bioactivity is related to the solubility and linkage type of β-glucan among the various polysaccharides from medicinal mushrooms.

In Chapter I, we found that the HHP plus hot water treatment could significantly improve the extraction efficiency and reduce the extraction time from traditionally 6 h into 3h 10 min. Besides, HHP plus hot water treatment induced the disruption of protein-saccharide bonds that could otherwise hinder extraction efficiency, and increase the content of β-glucan without disrupting the β-glycosidic bonds, which is important for the beneficial physiological properties of polysaccharides in the case of *P. linteus*.

In Chapter II, we also attempted to optimize the extraction conditions by application of HHP and enzyme. The results suggest that combining the HHP, enzyme, and hot water treatment was a more efficient process to obtain high yields of soluble polysaccharides, with a shorter extraction time (from 4 h to 50 min) compared to hot water treatment alone. These two chapters proved that the polysaccharides of many medicinal consisted of simple mono-saccharides and were determined to not be pure glucan; rather, they were composed of proteo-hetero-glycans with large amounts of glucose, mannose, galactose and xylose. Although the amino acid profiles in medicinal mushrooms depended on the extraction procedure used, it is not known whether amino acids in protein-bound polysaccharides derived from medicinal mushrooms have significant biological effects.

The developed extraction method provides easy access to bioactive compounds from medicinal and edible mushrooms. But, it will be necessary to further elucidate the molecular mechanisms of the biological effects of medicinal mushroom extracts with regard to their bioactivity and medicinal properties. Further studies on this extraction process based on a combination of enzymatic hydrolysis, HHP, and hot water treatments will provide new perspectives for the industrial application of high pressure technology.

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