Thesis for the Degree of Master of Engineering

Sulfation and Characterization of Polysaccharides from Oyster Mushroom (*Pleurotus ostreatus*) Extracted Using Subcritical Water

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느타리 버섯*(Pleurotus ostreatus)*으로부터 아임계 수로 추출 한 다당류의 황산기 개질 및 특성

Advisor: Prof. Byung-Soo Chun

by

Amellia Dwi Rizkyana

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Abstract

Pleurotus ostreatus also known as oyster mushroom is one of the most consumed mushroom in the world. They are a great source of polysaccharides with potential bioactivities and health-promoting properties. In this study, polysaccharides from oyster mushrooms (*Pleurotus ostreatus*) were subjected to subcritical water extraction (SWE) from 120°C to 200°C with an interval of 20°C. Polysaccharides with the highest amount of polymer obtained at these conditions were selected for chemical modification. Results showed that SWE at 180°C recovered the highest yield of polysaccharides. The yield of selected condition were purified using ethanol and further sulfation with Chlorosulfonic acid (CSA) – Pyridine method. The sulfur content of modified polysaccharides was analysed using an elemental analyzer and the degree of substitution result was 1.83. Characterization of the differences between native polysaccharides (PN) and sulfated polysaccharides (PS) were determined by fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), gel permeation chromatography (GPC) and high performance liquid chromatography (HPLC). FTIR results confirmed the success of chemical modification with the intensive peaks at a wavelength of 1233 cm^{-1} and 1379 cm^{-1} were characteristic for S=O asymmetry stretching vibrations. Another clear absorption peak at 796 cm^{-1} is typically for C-O-S symmetry stretching vibration. The molecular weight of polysaccharides decreased after the modification whereas PN ~217,000 Da and PS ~145,000 Da. *In vitro* anticoagulant activity using activated partial

thromboplastin time (aPTT) and prothrombin time (PT) assays showed that PS significantly improved plasma clot form inhibition by intrinsic and extrinsic pathways compared to PN. Sulfated polysaccharides showed surprisingly strong anticoagulant activity by intrinsic blood coagulation pathways as they extended the plasma clotting time more than 3 times at a concentration of 15.62 µg/mL as compared with the blank. Besides, PS exhibited strong scavenging effects against reducing power and hydroxyl free radicals in antioxidant capacities. However, they showed a weaker effect on $ABTS^{+}$ scavenging activity compared to the PN. Moreover, PN and PS showed a low level of cytotoxicity on normal cells, including HaCaT and HEK 293 with IC_{50} approximately 1463.6, 1401.7 and 1116.5, 603.9 µg/mL, respectively. In conclusion, chemical modification enhanced the biological properties of oyster mushroom natural polysaccharides. Therefore, oyster mushroom polysaccharides obtained from subcritical water extraction and modified using the sulfation method showed great anticoagulant activity. *P. ostreatus* sulfated polysaccharides could be considered as an alternative to an anticoagulant agent.

1. Introduction

1.1. *Pleurotus ostreatus* and bioactive compounds

As edible medical resources, mushrooms contain various bioactive molecules with therapeutic and health-promoting properties. In this regard, mushrooms are popular and widely consumed in Asian countries. The global demand for mushrooms is expected to rise (Fig. 1) as the world's population and consumption expand. The most broadly cultivated mushroom worldwide is *Agaricus bisporus*, followed by *Lentinula edodes*, *Pleurotus spp. and Flammulina velutipes* (Miles & Chang, 2004). One particular genus that ticks the above properties is *Pleurotus ostreatus*. *Pleurotus ostreatus* (Basidiomycota) belongs to the *Pleurotaceae* family and is native to China, however, it can now be found all over the world (Sałata, Lemieszek, & Parzymies, 2018). They are also known as oyster mushroom, has 40 different varieties that according to (Valverde, Hernández-Pérez, & Paredes-López, 2015) are widely commercialized for its taste and being an excellent source of high protein, carbohydrates, minerals, vitamins and low-fat. In the past years, extensive research on chemical composition and nutritional profile has been conducted. *P. ostreatus* had a moisture content ranging from 85 - 95 % indicate that the fruiting bodies had a high moisture content (Manzi, Gambelli, Marconi, Vivanti, & Pizzoferrato, 1999). Carbohydrate content varies from 50 - 90%, with polysaccharides and glycoproteins accounted for the majority. Chitin, hemicelluloses, α- and β-glucans, mannans, xylans, and galactans are the most common polysaccharides found in mushrooms (Bohn & BeMiller, 1995). In extracts of *P. ostreatus*, researchers have found an active β-glucan, named pleuran (Piska, Ziaja, & Muszynska, 2016).

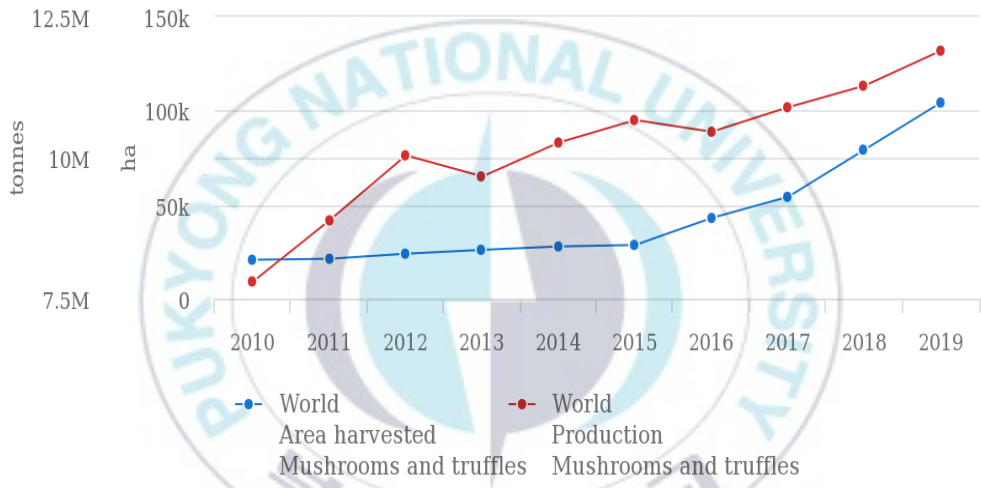

Fig. 1. Production/Yield quantities of Mushrooms and truffles in the World (Source: FAO, 2019).

The latest findings revealed that *P. ostreatus* is a potent source of active β-glucan with various health benefits (Barbosa, dos Santos Freitas, da Silva Martins, & de Carvalho Junior, 2020; X. Liu et al., 2019; Silveira et al., 2015) and has a significant antioxidative effect (Maity et al., 2011). In mushrooms, the main polysaccharides mostly are glucans with different types of glycosidic linkages, such as (1→3)-, (1→6)-β-glucans and (1→3)-α-glucans (Wasser, 2002).

Previous reports have revealed that mushroom polysaccharides could be used for anticancer therapy and stimulation of the immune system as well as the regulation and prevention of hyperglycemia and hypercholesterolemia (Guo et al., 2020; Jeong et al., 2010; Maity et al., 2011). Mushroom polysaccharides are considered one of the most potent compounds for antitumor and immune-modulating substances (De Silva, Rapior, Fons, Bahkali, & Hyde, 2012; Pandya, Dhuldhaj, & Sahay, 2019). These polysaccharides are also characterized as immunomodulatory agents with potential applications in the treatment of cancer, infections, and immune system disorders (Devi et al., 2013). Other authors have stated that oyster mushroom polysaccharides could be classified as a functional food due to their positive effects on human health (Patel, Naraian, & Singh, 2012; Synytsya et al., 2009).

1.2. Chemical modification

Certain natural polysaccharides have been found to have low bioactivities, and thus it is important to develop a method to improve them. According to (Li et al., 2015), there are apparently several methods for changing the structure of polysaccharides to solve the above problem.

One method involves physical modification. Physical modification is the process of trimming large polysaccharide molecules into small fragments of lower molecular weight

by destroying glycoside chains with extra energy such as ultrasonic disruption, microwave exposure, radiation-induced treatment, etc. Another method, biological modification, is where the glycosidic chain was hydrolysed with enzyme catalysts to degrade the polysaccharides. There is also chemical modification that refers to the process of adding other active groups to the polysaccharide chain; this results in new or improved bioactivities as well as a reduction in the molecular weight of native polysaccharides (Chakka & Zhou, 2020). That is the effective way to change their molecular structure caused by conjugated functional groups. Polysaccharide biological and pharmacological activities are closely correlated to their molecular structure, according to numerous studies (Z. Wang, Xie, Shen, Nie, & Xie, 2018). Chemical modification could be used to modify physicochemical properties and enhance polysaccharide biological activity.

Recently, chemical modification been used to improving polysaccharides biological properties by sulfation (Bae et al., 2009; Xie et al., 2016), selenylation (B. Zhao et al., 2013), phosphorylation (Deng, Fu, Xu, Shang, & Cheng, 2015), and acetylation methods (Song et al., 2013).

1.2.1. Sulfation

Sulfation occurs by dissolved polysaccharides in a solvent, reacted with a corresponding sulfation reagent under particular conditions, and then attaching hydroxyls with sulfate groups. It also enhances their original bioactivities as well as generate new functional bioactivities (S. Li et al., 2016)The common method used for sulfation are chlorosulfonic acid (CSA) - pyridine method, sulfuric acid method and sulfur trioxide - pyridine method (Z. Wang et al., 2018). Sulfuryl chloride, sulfuric acid, and derivatives such as chlorosulfuric, 1-piperidinesulfonic, and sulfamic acid were the first chemical reagents

used to sulfate polysaccharides. Sulfur trioxide complexes was used with an amide or amines in polar aprotic solvents due to the stability, ease to handle and mild acid character of these reagents such as pyridine, DMF, DMSO, DMAc or NMP (Bedini, Laezza, Parrilli, & Iadonisi, 2017). The reaction condition could be varied as a result of temperature and time of reaction, the molar ratio of sulfation reagent, polysaccharides concentration and proportion of sulfation agent and solvent (Moura Neto, Maciel, Cunha, dePaula, & Feitosa, 2011). CSA is a strong acid that leads to a powerful reaction in the modification process and polysaccharides would be degraded under acidic conditions causing the structure of the polysaccharides changed (Z. Wang et al., 2018).

Sulfation has been widely used to enhance the biopotentialities of polysaccharides. Several studies have shown that sulfated polysaccharides (PS) prevent the proliferation of cancer cells, improve immunity, and scavenge free radicals (Y. Chen et al., 2015; Zhu et al., 2013). Some other studies have focused on assessing the potential anticoagulant and antithrombotic activities of these functional polysaccharides (de Jesus Raposo, De Morais, & De Morais, 2015; Caterina Faggio et al., 2015). The activities of sulfated polysaccharides were influenced by structural characteristics such as the degree of substitution (DS), sulfation position, weight molecular mass (MW), and glycosidic branching. Degree of substitution (DS) referred to as an average number of substituent groups linked per base unit or per monomeric unit of polymer. DS is an index to reflect the modification degree that many studies confirmed within a certain range, better activity was affected due to higher DS (T. Chen et al., 2010).

1.2.2. Selenylation

Selenium is an essential mineral that aids in antioxidant defenses and plays role in physiological processes therefore it required to be consumed in a small amount (Yue et al., 2015). Selenium compounds have anti-cancer properties and can lower cancer mortality and incidence (T. Chen, Wong, Zheng, Bai, & Huang, 2008). Some plants or microorganism has its natural selenium polysaccharides but the content is low even though they are growing in the high-selenium area (B. Zhao et al., 2013). Therefore, chemical modification is needed to attain more yield of selenium polysaccharides and enhance its biological activities. The HNO_3–Na_2SeO_3 method is commonly performed for selenium modification because the reaction conditions are simple, production is fast, and the modifier has higher selenium contents (Qin et al., 2013).

1.2.3. Phosphorylation

Phosphorylation is the process of replacing the hydroxyl groups on the polysaccharides with phosphate groups. A strong acid is required as a catalyst in the phosphorylation process to degrade polysaccharides structure and complex composition (Li et al., 2015). The phosphorylation of polysaccharides is commonly done with phosphoric acid and its anhydride, phosphorus pentoxide (P_2O_5), phosphorus oxychloride ($POCl_3$), and phosphate (F. Chen & Huang, 2018).

1.2.4. Carboxymethylation

Carboxymethylation is broadly used to enhancing polysaccharides bio-properties by introducing carboxymethyl groups into the hydroxyl groups and increase their water solubility. The common method to obtain carboxymethylated polysaccharides is to react

the polysaccharides with chloroacetic acid under basic conditions (F. Chen & Huang, 2018).

1.3. Subcritical water extraction

Subcritical water extraction (SWE) is a modern approach that uses water as an extraction solvent at temperatures ranging from 100 to 374°C **(Fig. 2)** and a high enough pressure (critical point of water, 374℃ and 22 MPa) to recover bioactive compounds from the sample. It is also known as hot liquid water extraction, pressurized (hot) water extraction, high-temperature water extraction, or superheated water extraction (Plaza & Turner, 2015). Water molecules have a small negative (δ-) and positive (δ+) charge on opposite ends, allowing for biologically significant characteristics such as polarity, cohesion, surface tension, hydrogen bonding, dipole moments, etc (Gbashi, Adebo, Piater, Madala, & Njobeh, 2017). Subcritical water induces the extraction of plant polysaccharides by increasing the ionization constant, which led to more free ions (H^+ and OH^-), enhancing extraction efficiency like other conventional extraction methods (J. Zhang et al., 2019). The dielectric constant of water is initially 80 at 25 °C **(Fig. 3)**, but when the temperature is increased to 250°C within 25 bar, the dielectric constant drops to 25 which is comparable to organic solvents such as methanol ($\varepsilon = 33$), ethanol ($\varepsilon = 24$), acetone ($\varepsilon = 20.7$), and acetonitrile ($\varepsilon = 37$) (Gbashi, Adebo, Piater, Madala, & Njobeh, 2017; J. Zhang, Wen, Zhang, Duan, & Ma, 2020).

Compared to traditional extraction techniques, SWE has more advantages, such as high extraction efficiency and environmentally friendly properties. The diffusivity efficiency of water significantly increases under subcritical conditions, boosting the recovery yield of

plant polysaccharides and reducing extraction time (G. Chen et al., 2016). Therefore, SWE is considered a promising and safe method to recover polysaccharides from different plant sources (Jiao et al., 2017; Leong, Yang, & Chang, 2020).

One of the factors affecting subcritical water extraction is temperature. This is the most important and conclusive factor in SWE that used to conduct analyte extraction rates as well as other parameters of extracting solvent. As the temperature rises, the rate of diffusion and solubility rises, but the viscosity and surface tension drop. However, high temperatures can cause compound degradation or even the enhancement of reactions like oxidation and hydrolysis (Khajenoori & Asl, 2013). Another factor is the pressure that also essential in changing the phases of water but unlike temperature, pressure does not affect the recovery efficiency of compounds from natural products (Gbashi et al., 2017). The effect of changing the water phase can also be accomplished by modifying the pressure. Maintained the liquid state of water requires a medium pressure of 15 bar at 200°C and 85 bar at 300°C (Teo, Tan, Yong, Hew, & Ong, 2010).

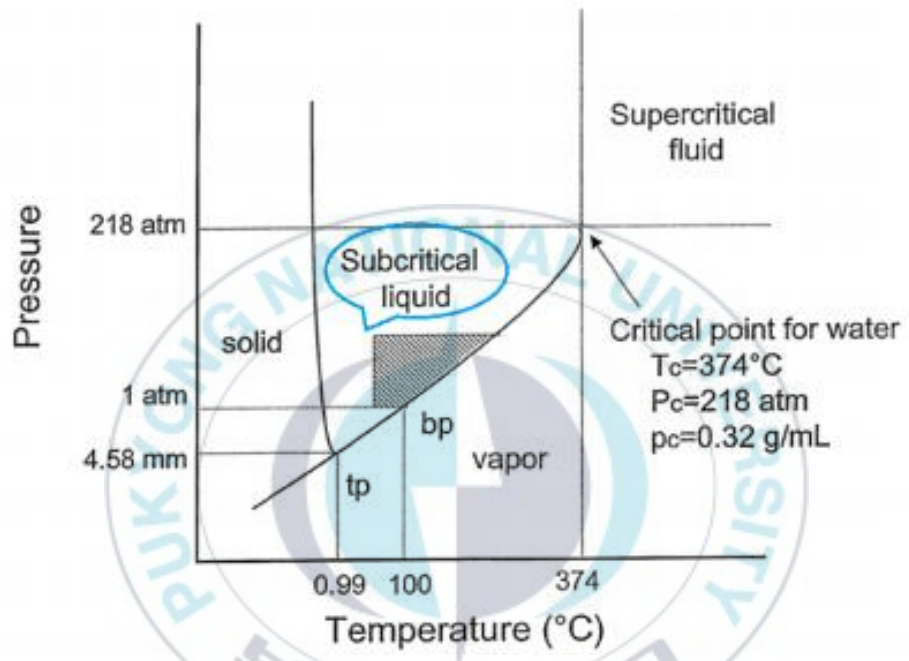

Fig. 2. Phase diagram of water as a function of temperature and pressure. (Khajenoori & Asl, 2013).

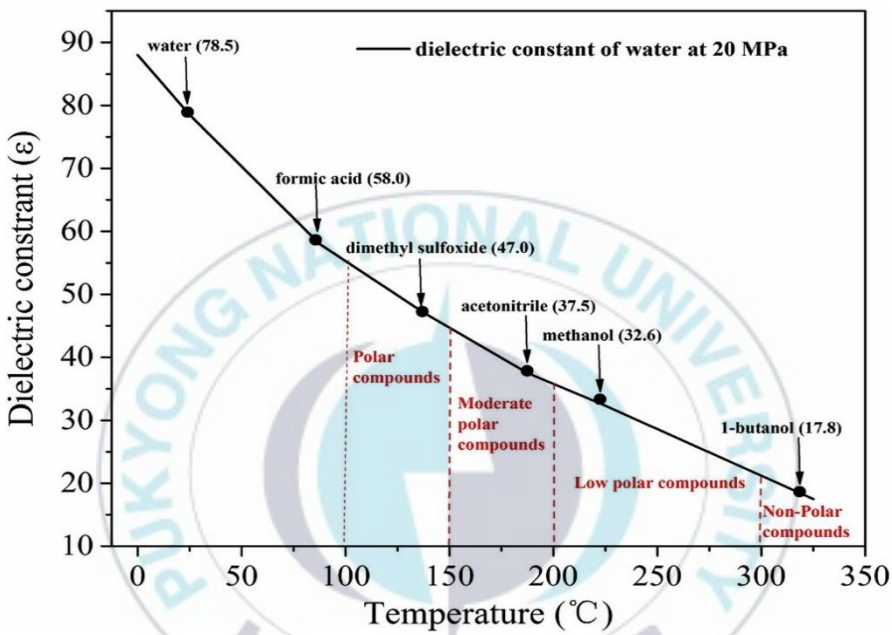

Fig.3. Changes of water dielectric constant as a function of the temperature at constant pressure (20 MPa) (J. Zhang et al., 2020). The figure presents the dielectric constant values equivalents to some common organic solvents at room temperature and pressure (25 °C and 0.1 MPa).

1.4. Objectives of the study

In the literature review, there have been no studies about SWE and the sulfation of polysaccharides from oyster mushrooms (*P. ostreatus*). Therefore, in this study, subcritical water was used to recover crude polysaccharides from *P. ostreatus* mushrooms, and the crude polysaccharides were then purified with ethanol. Native polysaccharides (PN) obtained from the SWE condition with the highest polymer yield were used for sulfation. The chemical compositions, anticoagulant activity, antioxidant capacity, and cytotoxicity on cancer and normal cell lines of PS were analysed in comparison to the PN.

2. Materials and methods

2.1. Sample preparation

Whole oyster mushrooms (*P. ostreatus*; 8 kg) were purchased from Dawoon Farm (Anseong, Gyeonggi-do, Republic of Korea). A freeze drier (HyperCOOL, HC8080; Gyrozen Co., Ltd., Daejeon, Republic of Korea) was used for drying, and a mechanical blender (PN SMKA-400 Mixer; PN Co., Ltd., Ansan, Republic of Korea) was used for crushing and sieving to obtain powder form with a particle size of ≤710 nm. The powder samples were placed in air-tight bottles stored at −20°C for further use. The sample preparation process is showed in **Fig. 4.**

2.2. Chemicals and reagents

Pure nitrogen gas was obtained from KOSHEM (Yangsan, Republic of Korea). Folin–Ciocalteu reagent, Bovine Serum Albumin (BSA), 2, 2-azinobis-3 ethyl benzothiazoline-6-sulphonic acid ($ABTS^{+}$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), glucose, and D-glucuronic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MI, USA). The activated partial thromboplastin time (aPTT) and prothrombin time (PT) reagents were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Human plasma was acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The β-glucan assay kit was purchased from Megazyme International (Wicklow, Ireland). Human normal keratinocytes (HaCaT), human normal embryonic kidney cells (HEK-293), and cervix adenocarcinoma cells (HeLa) were purchased from American Tissue Culture Collection (Manassas, VA, USA). The other solvents and chemicals used were of analytical or high-performance liquid chromatography (HPLC) grade.

2.3. SWE and purification of polysaccharides

The extraction of oyster mushrooms was carried out by a batch SWE system **(Fig. 5)**. Briefly, a 20 g sample was introduced into a stainless steel reactor. The reactor was then filled with 400 mL distilled water. Nitrogen gas was used to pressurize the system. The extraction temperatures ranged from 120°C to 200°C with an interval of 20°C, whereas the extraction time (20 min), solid-to-solvent ratio (1/20), and pressure (40 bar) were maintained in all experiments. After finishing the extraction process, the hydrolysates were cooled down via a tap water line equipped inside the reactor. The collected hydrolysates were centrifuged at 8500 rpm for 20 min and filtered under vacuum using an F1093-grade Whatman filter paper to completely remove the solid particles. The supernatant was added with three times 95% ethanol and kept overnight at 4°C for the precipitation of polysaccharides. The suspended solution was centrifuged at 8500 rpm for 20 min, and the collected polysaccharides were then washed thoroughly with distilled water and lyophilized to get PN.

Fig. 4. Oyster mushroom sample preparation process.

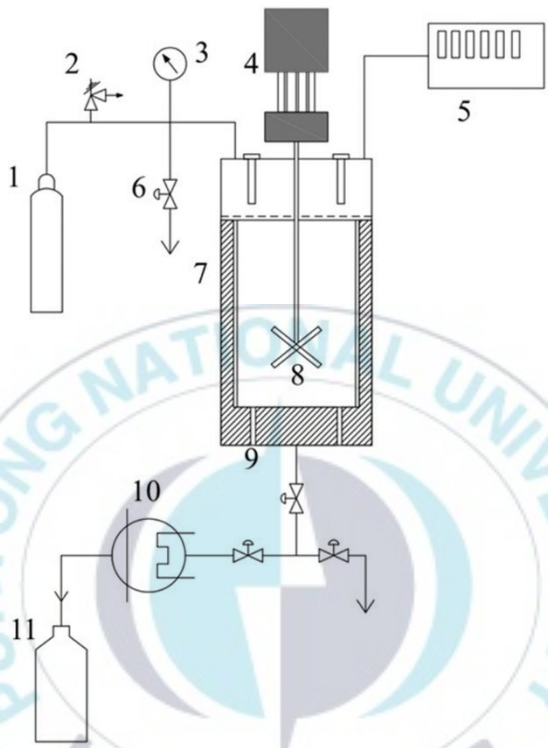

Fig. 5. Flow diagram of subcritical water experimental apparatus. (Ho & Chun, 2019).

(1) Nitrogen gas; (2) Safety valve; (3) Pressure gauge; (4) Rotator; (5) Controller of temperature and stirring speed; (6) Control valve; (7) High pressure reactor; (8) Impeller; (9) Electric Heater; (10) Cooler; (11) Collector.

2.4. Sulfation of polysaccharides

PN obtained at the selected SWE condition was modified following a previous method (L. Wang, Li, & Chen, 2009) with some modifications. Briefly, 48 mL pyridine was placed in a three-neck flask and chlorosulfonic acid (CSA) was added drop by drop to make a final ratio of pyridine and CSA at 1:4. The mixtures were cooled in an ice bath under stirring. Then, 200 mg polysaccharides were mixed with 20 mL anhydrous dimethylformamide (DMF). The polysaccharides-DMF solution was added dropwise into the pyridine-CSA solution, and the resulting mixture was maintained at 70°C for 2 h. After completing the reaction, the sulfated solution was cooled in an ice bath. Finally, the solution was neutralized with 4 M NaOH and precipitated in 95% ethanol. PS was collected, dialyzed against distilled water for 72 h using a dialysis bag (MWCO 3500 Da), and lyophilized. Finally, PS in dried powder was stored at −20°C.

2.5. HPLC analysis of monosaccharides

The 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization of polysaccharides for the analysis of mono sugars was conducted following a previous method (Ai et al., 2016). A 30 mg sample was briefly taken in glass tubes and dissolved in 1 mL of 3 M trifluoroacetic acid (TFA) and incubated for 3 h at 120°C. The mixtures were cooled down, and TFA was evaporated under a nitrogen flow. The dried samples were dissolved in 1 mL distilled water, and 30 μL of 0.3 M NaOH and 20 μL of 0.5 M PMP-MeOH were added to the solution. The mixtures were incubated at 70°C for 60 min, cooled at room temperature, and neutralized with 30 μL of 0.3 M HCl. Then, 1 mL chloroform was added, and the organic phase was carefully removed after vigorous shaking. Before HPLC analysis, the aqueous

layer was passed through a 0.20 μm syringe filter. Standard curves of six mono sugars, including arabinose, galactose, glucose, fucose, fructose, and mannose, were prepared.

The PMP-labeled monosaccharides were analyzed using an HPLC system with an ultraviolet (UV)-visible detector. The analytical column was a Luna 5u C18 column (4.6 × 250 mm). The injection volume was 20 μL with an eluent flow rate of 1.0 mL/min at 35°C. The mobile phase included potassium phosphate buffer and acetonitrile (83:17). The UV detection wavelength was 245 nm.

2.6. Determination of molecular weight

The molecular weight of polysaccharides was determined by using Gel permeation chromatography (GPC) with Waters Breeze Systems (Waters Corporation, USA). The system was equipped with Waters Ultrahydrogel 500, 250, 120 columns and an RI detector. Before analysis, both samples were dissolved in deionized water (5 mg/mL), filtered through a 0.45 μm pore-size Nylon filter, and degassed. The injection volume of 50 μL was kept constant, and 0.02 N of $NaNO_3$ was used as a developing solvent with a flow rate of 0.8 mL/min. The molecular weight of polysaccharides and its distribution were analyzed at 30°C. The determination of polysaccharide molecular weight was based on the calibration curve of the pullulan standard (6300 to 642 × 10^3 Da). The results were analyzed using the Breeze software.

2.7. FTIR spectroscopy and nuclear magnetic resonance (^{1}H-NMR) analysis

The FTIR spectra of samples were recorded using a Cary 640 spectrometer (Agilent Technologies, Inc., USA). The wavenumber region used was 4000 to 500 cm^{-1}. The ^{1}H-

NMR spectra of samples were recorded in D_2O (0.75 mL) using FT-NMR 600 MHz (JEOL JNM-ECP600, Japan).

2.8. Degree of substitution (DS) and amount of β-glucan

The sulfur content (%S) in PS was analyzed using an element analyzer (Vario MACRO cube; Elementar, Hanau, Germany). The DS was calculated using the following equation:

$$\text{DS} = \frac{\%\, S \times 162}{(32 - 120 \times \%\, S)}$$

The β-glucan content of PN and PS was quantitatively determined using a β-glucan assay kit (Megazyme).

2.9. Total sugar, uronic acid, protein content and ash content

For the determination of ash content, 200 mg of sample was put in the porcelain crucibles and heated using a muffle furnace for 6 hours at 600°C. The weight of the sample and crucibles before and after ashing was measured to calculate the ash content contained in the sample.

$$\%\, Ash = \frac{W3 - W1}{W2 - W1} \times 100$$

w_1 = Crucible weight

w_2 = Crucible + sample weight

w_3 = Crucible + ash weight

The total sugar content in the sample was measured by the phenol-sulfuric acid method, and glucose was used as a standard (Ho & Chun, 2019). 0.5 mL sample (1 mg/mL) was mixed with 1 mL phenol solution (2%, w/w) and then 2.5 mL of concentrated H_2SO_4. Put in the dark place for 10 minutes for color development and 30 min cooling in the water bath at 22°C. Absorbance measured at 490 nm.

Total protein was determined by the Pomory method, and bovine serum albumin was used as a standard (Pomory, 2008). Prepare the stock solutions as follows: 1% copper sulfate ($CuSO_4.5H_2O$), 2% tartrate ($KNaC_4H_4O_6.4H_2O$), and 2% sodium carbonate (Na_2CO_3). In a 1: 1: 100 ratio, combine the following solutions. To the tube containing 0.5 mL sample which diluted in 0.1 N NaOH. Add 5 ml copper–tartrate–carbonate solution into a tube containing sample, vortex for a few seconds, and let stand for 10 minutes in the dark. Add 0.5 mL of 1 N Folin-Ciocalteu reagent, vortex the solution and stand for 2 hours at room temperature and dark place. Read in spectrophotometer set to 660 nm.

The uronic acid (UA) content was established using the 96-well plate carbazole reaction assay, and D-glucuronic acid was used as a reference (Cesaretti, Luppi, Maccari, & Volpi, 2003). In a 96-well plate, 50 µL of the sample was placed and then added 200 µL of a solution of 25 mM sodium tetraborate in sulfuric acid. The plate was heated in the oven at 100°C for 10 min. After cooling at room temperature for 15 min, 50 ml of 0.125% carbazole in absolute ethanol was added. After heating at 100 °C for 10 min in an oven and cooling at room temperature for 15 min, the plate was read at a wavelength of 550 nm. A multimode reader (BioTek Instruments, Inc., USA) was used to measure all the assays.

2.10. Antioxidant activities

The $ABTS^+$ radical scavenging activities were determined following a previous method (X. Li, Wang, Chen, & Shuzhi, 2011) with some modifications. Briefly, 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution were separately prepared in distilled water. The $ABTS^+$ solution was prepared by mixing an equal volume of the above-prepared solutions and stored for 12 h in the dark at room temperature. The stock $ABTS^+$ solution was diluted with 95% ethanol and adjusted to 0.70 ± 0.02 using a multimode reader at an absorbance of 734 nm. The sample solution was prepared at concentrations of 0.25 to 4 mg/mL. A 0.3 mL sample solution was taken in a test tube and mixed with 1.2 mL previously prepared $ABTS^+$ solution and incubated for 6 minutes. Afterwards, the absorbance was measured at 734 nm. The $ABTS^+$ scavenging activity of the sample was calculated as the equation below:

$$ABTS^+ \text{ radical scavenging (\%)} = \left(1 - \frac{A}{A_0}\right) \times 100$$

Where A is the absorbance of the sample; A_0 is the absorbance of the control.

The hydroxyl radical scavenging activity was assayed according to an earlier described method (Smirnoff & Cumbes, 1989) with slight modifications. Briefly, 0.5 mL of 1.5 mM $FeSO_4$, 0.35 mL of 6 mM H_2O_2, and 0.15 mL of 20 mM sodium salicylate were put in test tubes. A 1 mL sample was put into the mixture that was then vortexed. The resulting mixture was incubated at 37°C for 90 min, and the absorbance was measured at 562 nm. The percentage scavenging effect was calculated using the following equation:

$$\bullet OH \text{ radical scavenging (\%)} = \left[1 - \frac{(A1 - A2)}{A0}\right] \times 100$$

Where A_1 is the absorbance of the sample, A_2 is the absorbance of the reagent sample without sodium salicylate, and A_0 is the absorbance of the control solution.

The reducing power of samples was determined following a previous study (Jing Wang, Zhang, Zhang, & Li, 2008). Briefly, a 0.13 mL sample in 0.2 M phosphate buffer (pH 6.6) at various concentrations (0.25–4 mg/mL) was mixed with 0.125 mL of 1% (w/v) potassium ferricyanide. The mixture was then incubated at 50°C for 20 min. Afterwards, 0.125 mL of TCA (10%, w/v) was added to the mixture to stop the reaction. Then, 1.5 mL ferric chloride (0.1%, w/v) was mixed with the solution. The absorbance was measured at 700 nm.

2.11. Blood clotting assays

The anticoagulant PN and PS activities, including aPTT and PT assays, were determined using human plasma (Pawlaczyk, Czerchawski, Pilecki, Lamer-Zarawska, & Gancarz, 2009). Briefly, samples in deionized water at various concentrations between 62.5 and 4000 μg/mL, heparin solution for control test, and deionized water for a blank test (33 μL) were placed into test tubes containing 50 μL human plasma, and the tubes were incubated at 37°C for 3 min. After incubation, the mixtures were added with 50 μL aPTT reagent and again incubated for another 3 min. Then, 50 μL of 0.025 M $CaCl_2$ were added into the mixture, simultaneously counting the time. The test tubes were tilted back and front until the clots formed. The clotting time was then recorded. For the PT assay, the samples (33 μL) were introduced in test tubes. Then, 50 μL human plasma was added, and the mixtures were incubated at 37°C for 3 min. Afterwards, a 100 μL preincubated thrombin reagent was added to the mixtures, simultaneously counting the time. The test tubes were tilted back and front until the clots formed, and the clotting time was then recorded.

2.12. Cytotoxicity of PN and PS

The cytotoxicity of both polysaccharides against human normal embryonic kidney (HEK293) cells, human normal keratinocytes (HaCaT) cells, and cervix adenocarcinoma (HeLa) cells were determined according to a previously described method (Niyonizigiye et al., 2020). The stock solutions (100 mg/mL) of PN and PS were used to make lower concentration solutions and stored at 4°C. HaCaT cells were maintained in Dulbecco's modified Eagle's medium, whereas HEK293 and HeLa cell lines were stabilized in a minimum essential medium. All media contained heat-inactivated fetal bovine serum (10%) and a mixture of 100 U/mL penicillin and 10 µg/mL streptomycin (1%). All cells were maintained in an incubator at 37°C and 5% CO_2 and subcultured until they were ready for use. Cell lines at a density of 10^4 were seeded in triplicate in 96-well plates, and the seeded plates were incubated at 37°C and 5% CO_2 for 24 h. After treatment, cells were treated with sample solutions at different concentrations and again incubated in the same manner. After finishing incubation, the old medium was removed, and a fresh medium containing 10 µL WST-1® solution (Daeil Lab Service, Seoul, Korea) was added. The plates were again incubated at 37°C and 5% CO_2 for 3 h. The absorbance was read at 460 nm using an ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.13. Data analysis

FTIR graphs were prepared using OriginPro software version 9.0 (Origin Lab, USA), and Delta NMR processing and control software (version 5.3.1) was used for ^{1}H NMR data analysis.

3. Results and discussion

3.1. Extraction of mushroom polysaccharides

The use of subcritical water extraction is to obtain an effective extraction of target compounds. Oyster mushroom powder was extracted using subcritical water at different temperatures from 120°C and 200°C with an interval of 20°C. Subsequently, the extracts were further purified using 95% ethanol to obtain polysaccharides. The recovery of polysaccharides (**Fig. 6**) was dependent on the thermal treatment level. With the growth in temperatures from 120°C to 180°C, the amount of polysaccharides gradually increased from 6.26% to 20.35%. The optimization of experimental conditions was a critical step because various extraction parameters can influence the extraction process. Temperature is one of the most important factors that influence the extraction efficiency using subcritical water hydrolysis. The thermodynamic properties of water, such as viscosity, surface tension, density, and dielectric constant, decrease when the temperature increases. These changes promote the contact between solvent and solutes due to the improvement in mass transfer and the diffusion rate of solvent to the sample matrix. Consequently, elevated temperatures break down van der Waals forces, hydrogen bonds, and dipole attraction of cohesive and adhesive interactions, resulting in an improved extraction process (Mustafa & Turner, 2011). However, when the temperature increased to 200°C, the amount of polysaccharides rapidly decreased due to the thermal degradation of the polymer in the solvent, which was also confirmed in a previous study (Saravana, Cho, Park, Woo, & Chun, 2016). Therefore, among the observed temperatures, subcritical water at 180°C is suitable to recover the most polysaccharides from oyster mushrooms (*P. ostreatus*). The increase of the mass transfer and solubility of polysaccharides potentially affected by higher

temperature (X.-X. Liu et al., 2020). The polysaccharides obtained from this condition were used for chemical modification.

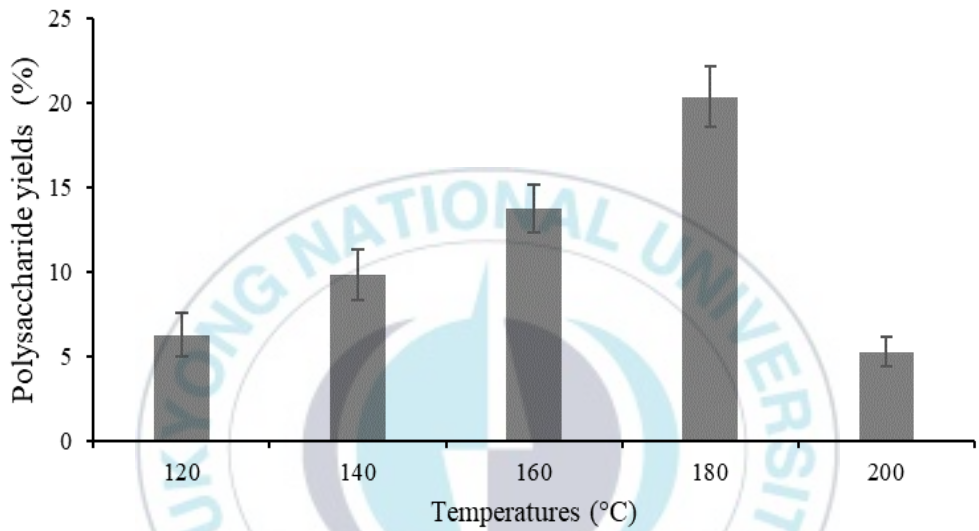

Fig. 6. Recovery of oyster mushroom polysaccharides using subcritical water from 120 to 200°C.

3.2. Sulfation and phytochemical property analysis of PN and PS

Recently, some studies have shown that PS possess great bioactivities, such as anticoagulant and antitumor (Bae et al., 2009; Telles et al., 2011). The introduction of ionic groups with an acceptable DS can affect the bioactivity of polysaccharides. In the CSA-pyridine method to modify polysaccharides, formamide, pyridine, and CSA are used as a solvent, catalytic agent, and hydroxyl group donor, respectively (Vasconcelos et al., 2013). Through this process, hydroxyl groups in polysaccharide structures are replaced with sulfate groups when subjected to sulfation (Bae et al., 2009).

The polysaccharides extracted using SWE has a brown colored powder appearance, contrary to sulfated polysaccharides which is a cotton-like shape and white color **(Fig. 7)**. The effect of sulfation on the water solubilities are the modified polysaccharides more soluble in the water than PN. This is due to the addition of sulfur-containing groups to the polymer structure, which increases the number of ionic groups. An increase in polysaccharide water solubility as a result of sulfation has also been observed in the literature (Jung, Bae, Lee, & Lee, 2011).

In this study, the physical properties of the raw sample, PN, and PS were investigated, and the results are shown in **Table 1.** The protein content in the PN is 4.52 ± 0.3% whereas in the PS is decreased to 3.08 ± 0.2% and in the raw sample contained 20.24 ± 0.6%. The results indicated that proteins decreased after the purification process and sulfation steps.

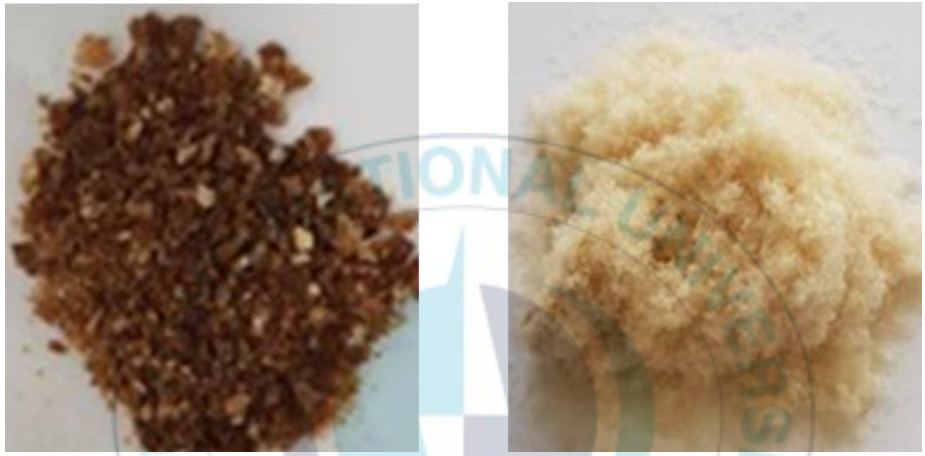

Fig. 7. Natural polysaccahrides (PN) and sulfated polysaccharides (PS) powder

The carbohydrate, uronic acid, and glucan content tended to decrease from 84.7 ± 1.4%, 6.79 ± 1.2%, and 37.66 ± 1.2% to 21.3 ± 0.08%, 5.23 ± 1.3%, and 18.91 ± 0.9% respectively, after modification. Some degradations might occur under acidic and high-temperature conditions during the chemical modification step. These results were also consistent with a previous report (Xie et al., 2016).

Table 1. Compositions of raw sample and mushroom polysaccharides

Components		**Raw sample**	**PN**	**PS**
Ash (%)		6.19 ± 0.3	2.88 ± 0.1	33.33 ± 1.3
Protein (%)		20.24 ± 0.6	4.52 ± 0.3	3.08 ± 0.2
Carbohydrates (%)		48.1 ± 0.2	84.7 ± 1.4	21.3 ± 0.08
Uronic acid (%)		-	6.79 ± 1.2	5.23 ± 1.3
Glucose (%)		-	62.32 ± 0.8	50.0 ± 0.7
Mannose (%)		-	10.58 ± 0.3	8.89 ± 0.3
Galactose (%)		-	3.24 ± 0.1	1.56 ± 0.2
Glucan content (%)	β-glucan	16.12 ± 0.3	34.90 ± 0.4	18.21 ± 0.4
	α-glucan	2.95 ± 0.1	2.76 ± 0.2	0.7 ± 0.3
	Total glucan	19.07 ± 0.8	37.66 ± 1.2	18.91 ± 0.9
Molecular weight (Da)		-	217,000	145,000

"-" indicates that the sample was not analyzed.

The molecular weight of mushroom polysaccharides decreased after modification **(Fig. 8b and 8c)**. MW of PN was found to be ~217,000 Da whereas that value of PS was ~145,000 Da. Similar results are also seen in some previous studies (Pengzhan et al., 2003; H.-J. Zhang et al., 2008) The degradation of polysaccharides probably occurred during the modification process that used strong acidic conditions at relatively high temperatures for a long time. **Table 1.** For HPLC analysis of monosaccharides, glucose, mannose, and galactose were found in both samples with 62.32 ± 0.8%, 10.58 ± 0.3%, and 3.24 ± 0.1% for PN to 50.0 ± 0.7%, 8.89 ± 0.3%, and 1.56 ± 0.2% for PS, respectively. The form of monosaccharides can be calculated by comparing the sample towards standard retention time. The mole percentage of each monosaccharide can be changed by sulfated modification without changing the monosaccharide's form. (Gunasekaran, Govindan, & Ramani, 2021). Total carbohydrates and monosaccharides content were reduced after sulfation that might be due to polysaccharide hydrolysis in an acidic state (Junlong Wang et al., 2010). Ash usually represents the inorganic part of the sample. The ash content of raw material (6.19 ± 0.3%) and PN (2.88 ± 0.1%) is lower than that found in the PS (33.33 ± 1.3%). The significantly high ash content of the PS sample is probably due to the attachment of sulfur elements after modification. The result was also confirmed in a previous study where sulfated polysaccharides contained a high amount of ash (24%) (Alipour, Rezaei, Shabanpour, & Tabarsa, 2018).

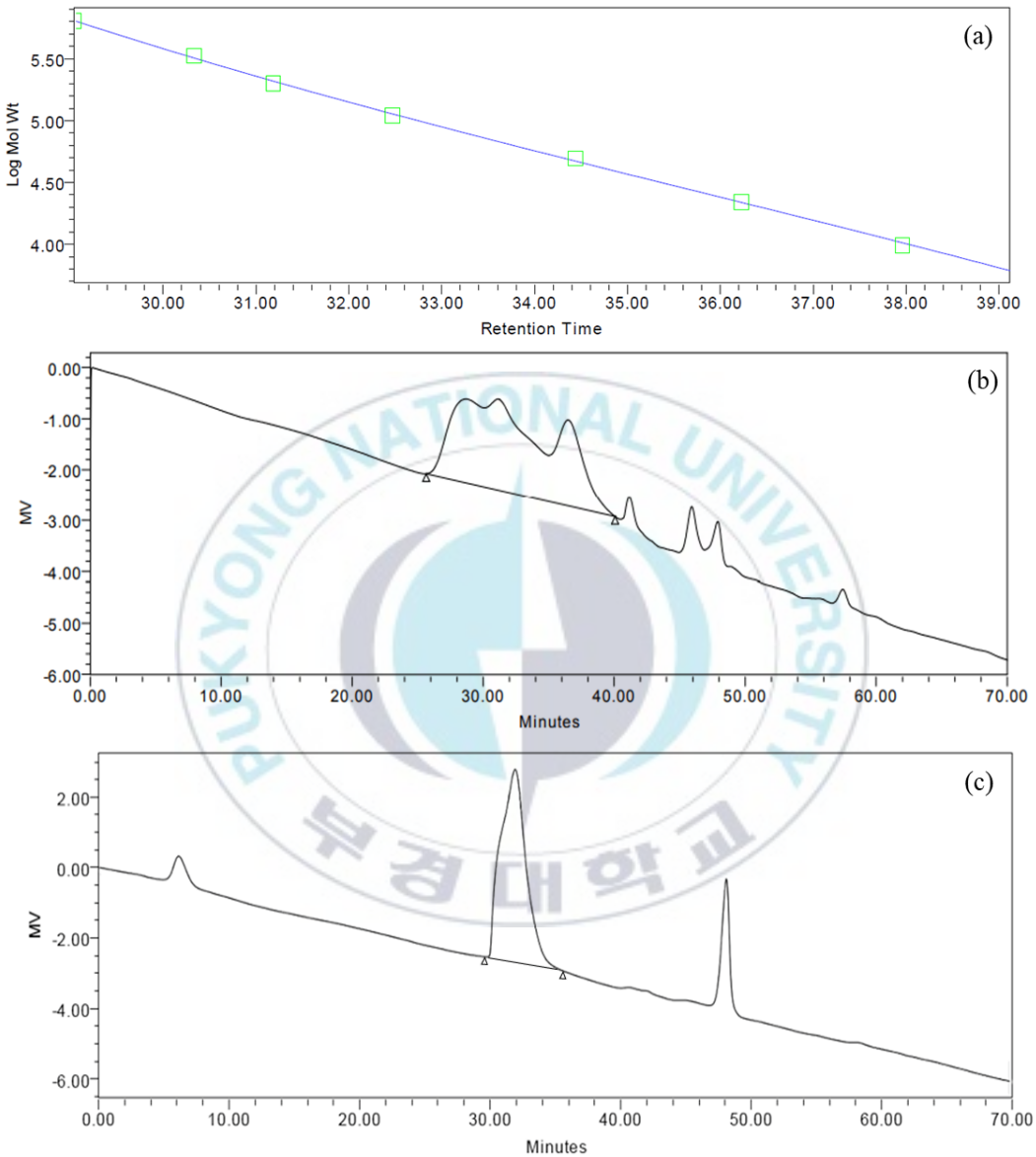

Fig. 8. Gel permeation chromatography of pullulan standard (a), native polysaccharides (b), and sulfated polysaccharides (c).

Table 2. Elemental analysis of sulfated polysaccharides

Element compositions (%)				Degree of substitution
Sulfur	Carbon	Nitrogen	Hydrogen	
16.83	14.62	1.67	2.90	1.83

Regarding the elemental analysis of PS, the results in **Table 2** demonstrated that the sulfur content was significantly high (16.83%) compared to carbon, nitrogen, and hydrogen contents corresponding to 14.62%, 1.67%, and 2.90%, respectively. The high content of sulfur resulted in a considerably high DS at approximately 1.83, indicating successful chemical modification. This result also agreed with the previous study in which DS was between 0.52 and 2.95 when bagasse cellulose was sulfated in an ionic liquid (Z.-M. Wang, Li, Xiao, & Wu, 2009).

3.3. FTIR spectroscopy, and ^{1}H NMR analysis

The FTIR spectra of both polysaccharides are shown in **Fig. 9**. The similarity in the structure of PN and PS samples was confirmed by typical signals of the polymer at approximately 3310 and 3473, 2920 and 2948, 1633 and 1640, and 1063 and 1066 cm^{-1}, respectively. However, sulfate peaks were observed in the FTIR spectra of the PS sample, indicating the success of the chemical modification process. Specifically, the intensive peak at a wavelength of 1233 and 1379 cm^{-1} was characteristic for S=O asymmetry stretching vibrations, corresponding to ester sulfate groups. Another clear absorption peak at 796 cm^{-1} is typically for C–O–S symmetry stretching vibration. The intensity of the peak is dominant, demonstrating that the DS is high (Xie et al., 2016).

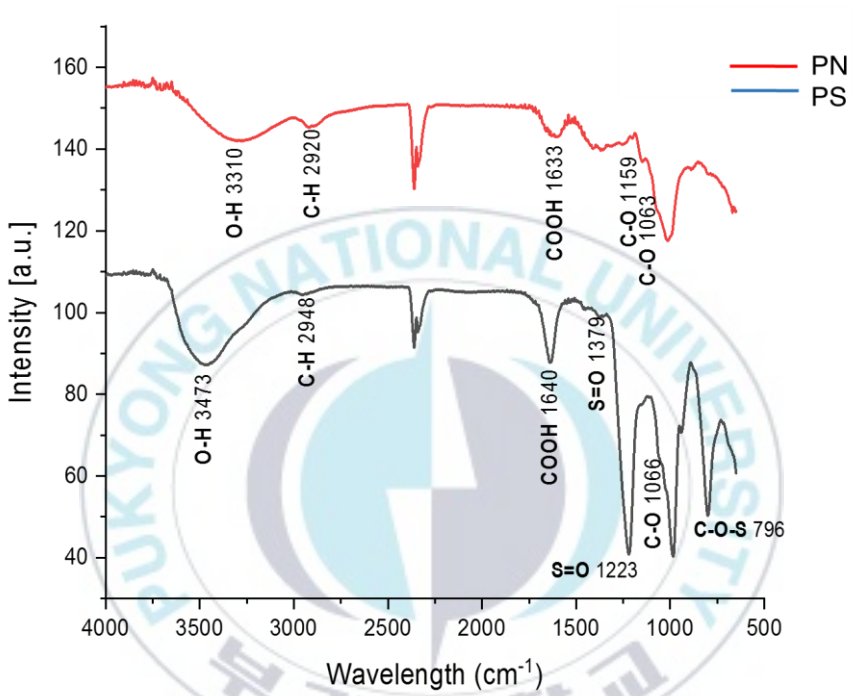

Fig. 9. Fourier-transform infrared spectroscopy of native polysaccharides (PN) and sulfated polysaccharides (PS).

The ^{1}H NMR spectra of PN and PS samples are presented in **Fig. 10.** The chemical shifts at signals between δ 3.0 and δ 5.5 ppm are typically for the presence of polysaccharides except for the signal of methanol at δ 3.3 in the PS due to its residual cleaning step. The signals at δ 1.1 – 1.3 showed as methyl proton (Hammed, Jaswir, Simsek, & Amid, 2016), other sugar protons of β-configuration in the region of δ 3.1 – 4.5 (Yao, Wang, Yin, Nie, & Xie, 2021) and the signals at δ 5.0 – 5.5 ppm were characteristic of anomeric protons on the α-configuration glycoside (Shu, Zhang, Jia, Ren, & Wang, 2019).

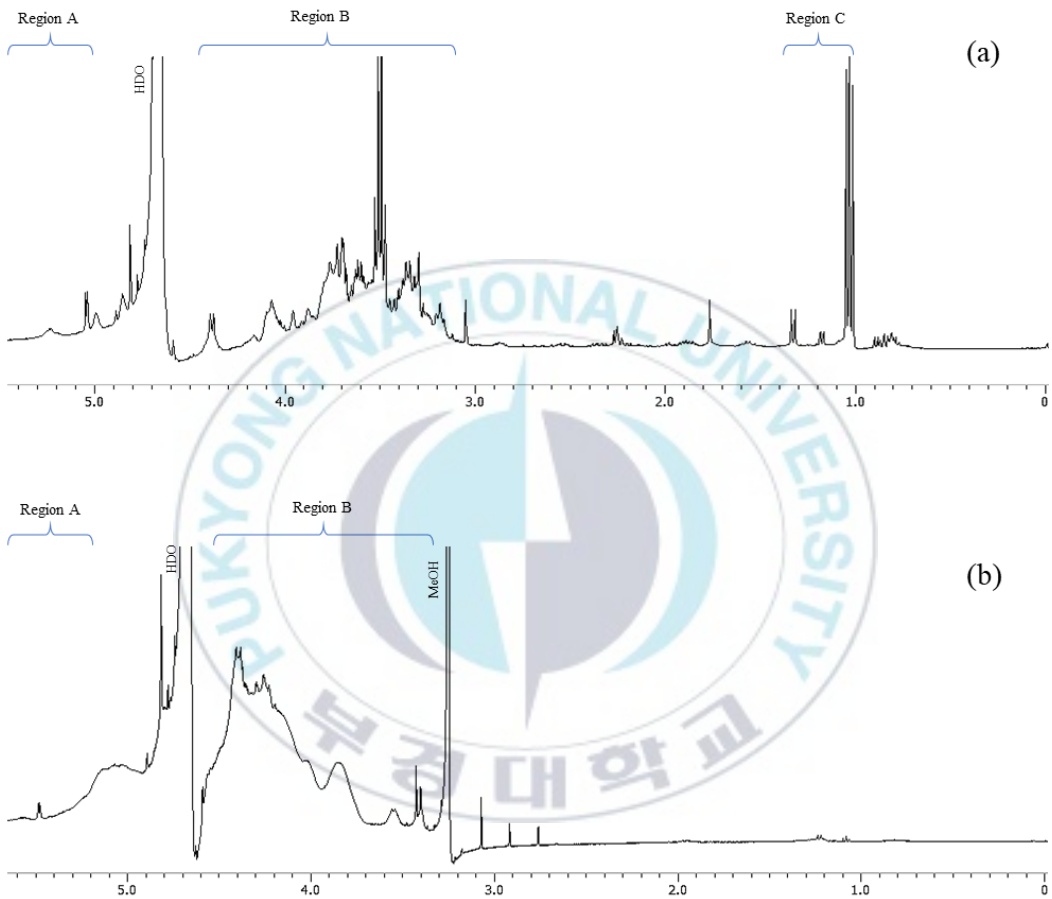

Fig. 10. ^{1}H NMR spectra of PN (a) and PS (b).

3.4. Anticoagulant activity

The aPTT and PT assays were used to investigate the anticoagulant activity of PN and PS samples, and heparin was used as a reference. PT prolongation indicates that the extrinsic coagulation pathway has been inhibited, whereas aPTT prolongation designates the inhibition of the intrinsic or common pathway. To determine the aPTT and PT, plasma was used, and the longer clotting time represents a higher anticoagulant activity (C Faggio, Pagano, Dottore, Genovese, & Morabito, 2016). The results in **Table 3** indicated that the clotting time of human plasma in two assays was prolonged in a concentration-dependent manner. This study achieved a really surprising result from PS in inhibiting the plasma clot form. PS powerfully inhibited the plasma clot form by intrinsic and extrinsic coagulation pathways. In the intrinsic pathway, PS did prolong the clotting time for more than 600 s at a concentration of 62.5 μg/mL and 240.1 ± 3.6 s at a concentration of 15.62 μg/mL, whereas heparin extended the clotting time for more than 600 s at a concentration of 7.81 μg/mL. In the extrinsic pathway, at a concentration of 500 μg/mL, PS effectively inhibited the plasma clot form when it extended the time for more than 600 s. However, the inhibition of the coagulant process by the intrinsic pathway of the enzymatic cascades usually caused some side effects, such as uncontrolled bleeding episodes (Pawlaczyk-Graja et al., 2016).

Table 3. Anticoagulant activity of PN, PS and heparin

Samples	Concentration (μg/mL)	Clotting time (s) PT	aPTT
Blank		37.3 ± 1.4	70.1 ± 2.1
PN	4000	65.2 ± 0.3	364.0 ± 7.3
	2000	57.6 ± 0.5	135. 3 ± 5.1
	1000	37.2 ± 1.1	101.2 ± 2.2
	500	37.2 ± 1.1	69.2 ± 2.5
	250	37.5 ± 2.1	71.4 ± 3.2
PS	4000	> 600	> 600
	2000	> 600	> 600
	1000	> 600	> 600
	500	> 600	> 600
	250	575.4 ± 4.1	> 600
	125	201.2 ± 7.5	> 600
	62.5	60.2 ± 0.5	> 600
	31.25	53.6 ± 2.1	540.4 ± 2.2
	15.62	36.8 ± 2.4	240.1 ± 3.6
Heparin	15.62	-	> 600
	7.81	-	> 600
	3.9	-	182.2 ± 0.2

"-" indicates that the sample was not analyzed.

In contrast to PS, the PN sample showed weak plasma clot form inhibition in both coagulation pathways. At the highest tested concentration (4000 μg/mL), PN extended the clotting time by approximately 364.0 ± 7.3 s by the former pathway. At 1000 μg/mL, PN did not show anticoagulant activity, as its extension time was similar to that found in the blank by the extrinsic pathway.

The differences in anticoagulant activity between PN and PS were probably due to the presence of sulfate groups in their structures, which was confirmed in a previous study (X. Zhao et al., 2007). In this study, the considerable anticoagulant activity of PS might result from the successfully sulfation with a DS value of 1.83 (**Table 2**). In a previous study, DS values of 0.80 are sufficient for anticoagulant activity (Yang, Du, Huang, Wan, & Li, 2002).

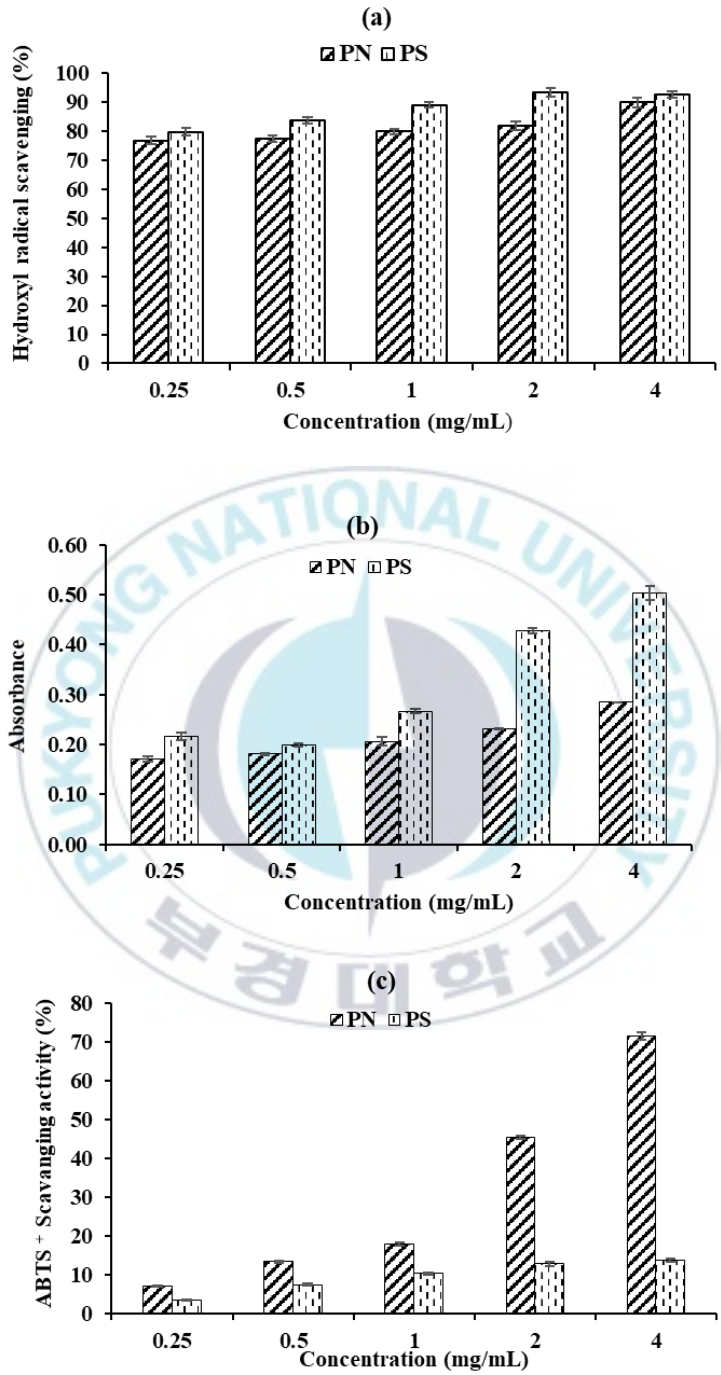

Fig. 11. Antioxidant capacities of PN and PS: Hydroxyl radical scavenging activity (a); Reducing power (b); $ABTS^{+}$ radical scavenging activity (c).

3.5. Antioxidant activity

To determine the antioxidant capacities of unmodified and modified mushroom polysaccharides (PN and PS), three different reliable antioxidant assays, namely hydroxyl radical scavenging, reducing power, and $ABTS^{+}$ radical scavenging were examined. Different concentrations of samples, including 0.25, 0.5, 1.0, 2.0, and 4.0 mg/mL, were used in these assays. The results are shown in **Fig. 11**.

Hydroxyl radicals are supreme toxic and highly reactive species that can hydroxylate organic molecules in living cells, leading to peroxidative damage and cell death (Gunasekaran et al., 2021; Kaur, Hippargi, Pophali, & Bansiwal, 2019). It is not possible to directly scavenge the hydroxyl radicals by dietary antioxidants because it is negligible compared to biological molecules (Dejian Huang, 2005). Therefore, high antioxidant activity compounds would be considered for scavenging these toxic radicals. Polysaccharides extracted from mushrooms possessed free scavenging activities, such as superoxide radicals, hydroxyl radicals, lipid peroxidation, and reducing power (F. Liu, Ooi, & Chang, 1997; Telles et al., 2011). In this study, the hydroxyl radical scavenging activity of PS was slightly stronger than PN (**Fig. 11a**). The scavenging percentage of $^{\bullet}OH$ groups was between 75% and 90% in both polysaccharides. The results were consistent with a previous study that reported a higher activity in the modified polysaccharide than in PN (Telles et al., 2011). The hydroxyl radical scavenging activity of both polysaccharides might be due to their metal-chelating capability in which they prevent the formation of hydroxyl radicals by either deactivating free metal ions or converting H_2O_2 to other harmless compounds (Dejian Huang, 2005).

Reducing the power of bioactive compounds is an activity to lessen ferrous ions from Fe^{3+} to Fe^{2+}. This activity is assessed by the formation of a complex between Fe^{2+}/ferric chloride, and their reducing power activity is determined as the increase in the absorption of the complex (Telles et al., 2011). A higher absorbance value means stronger reducing power of samples. In this study, the reducing power of both polysaccharides (**Fig. 11b**) showed a dose-effective relationship with the strongest activity seen at 4.0 mg/mL corresponding to absorbance values of 0.286 and 0.504, respectively. These findings are also supported by the data presented in the previous report (Telles et al., 2011).

PN showed stronger $ABTS^+$ radical scavenging ability than that found in PS (**Fig. 11c**). With the increase in PN solution concentrations from 250 to 4000 μg/mL, the scavenging capacity of PN gradually increased from 7.2% to 71.54%. The loss in $ABTS^+$ scavenging activity of PS is probably because hydrogen atoms, electron donors that can stabilize $ABTS^+$ radicals, are replaced by SO_3 groups during pyridine reaction in the modification process. As a result, the incorporation of $–OSO_3H$ groups into the polysaccharide structure appeared to be the primary cause of the increased antiradical activity, as $–OSO_3H$ groups play an important role in hydrogen atom-donating ability.

3.6. PN and PS cytotoxicity against HeLa, HaCaT, and HEK 293 cell lines

The examination of toxicity of both polysaccharides on the viability of HeLa cells and two normal cell lines was done, and the half-maximal inhibitory concentration the IC_{50} values are shown in **Table 4**. PN and PS did not show any activity on the viability of cancer cell lines. Both polysaccharides exhibited relatively low toxicity on HaCaT cell lines with IC50

approximately 1400 μg/mL. However, PS showed some toxicity against HEK293 with an IC50 of 603.9 μg/mL, whereas PN demonstrated low risk on this cell line.

Table 4. Cytotoxicity of native and sulfated polysaccharides on cancer and normal cell lines.

Samples	IC_{50} (µg/mL)		
	HaCaT	HEK 293	Hela
PN	1463.6 ± 11.5	1116.5 ± 13.2	No activity
PS	1401.7 ± 14.6	603.9 ± 9.8	No activity

4. Conclusion

In this study, oyster mushroom (*P. ostreatus*) polysaccharides were recovered the most in subcritical water at 180°C. Polysaccharides obtained at this condition were further used for sulfation. PN and PS comprised the dominant amount of carbohydrates but a small amount of proteins, indicating the success of the purification. The total carbohydrate and glucan content in PS decreased compared to those in PN, probably due to the degradation of sugars under thermal and acidic conditions in chemical modification steps. The successful modification was confirmed by the content of sulfur in PS determined via elemental analysis (16.83%) and a high DS level (1.83). The FTIR spectra also clearly demonstrated the presence of sulfate groups in modified polymer structures at highly intensive bands of 1379, 1223, and 796 cm^{-1}. Consequently, PS performed surprisingly high inhibition of plasma clot form by both intrinsic and extrinsic pathways compared to PN. Besides, polysaccharides also possessed significant antioxidant activities. Regarding toxicity, both polysaccharides were relatively safe on HaCaT and HEK293 cell lines. The findings from our study demonstrated that subcritical water is suitable to recover polysaccharides from oyster mushroom (*P. ostreatus*), and the PS deserves to be considered as a potential alternative remedy for anticoagulant therapy.

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Abstract (in Korean)

느타리버섯(*Pleurotus ostreatus*)으로부터 아임계 수로 추출 한 다당류의 황산기 개질 및 특성

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요 약

느타리버섯으로도 알려진 *Pleurotus ostreatus* 은 세계에서 가장 많이 소비되는 버섯 중 하나이다. *P. ostreatus* 는 잠재적인 생물 활성과 건강 증진 특성을 지닌 훌륭한 다당류 공급원이다. 본 연구에서는 느타리버섯을 20°C 간격으로 120°C 에서 200°C 까지 아임계 수 추출(SWE)을 이용하여 다당류를 획득하였다. 해당 조건에서 획득된 고분자 추출물 중 다당류의 양이 가장 많은 추출 조건을 선택하여, 화학적 변형에 이용되었다. 결과로 180°C 의 SWE 에서 가장 높은 다당류의 수율을 얻었다. 선택된 조건에서의 다당류는 에탄올을 사용하여 정제되고 Chlorosulfonic acid (CSA) – Pyridine 방법을 사용하여 황산화 유도체화되었다. 황-다당류의 황 함량은 원소 분석기를 사용하여 분석되었으며 황산화 유도체화 정도는 1.83 이었다. 천연 다당류(PN)와 황-다당류(PS) 간의 차이의 특성화는 푸리에 변환 적외선 분광법(FTIR), 핵자기공명(NMR), 겔투과 크로마토그래피(GPC) 및 고성능 액체 크로마토그래피(HPLC)에 의해 결정되었다. FTIR 의 1233, 1379 cm^{-1} 의파장에서는 S=O 비대칭 스트레칭 진동이 특징이며 해당 실험결과 해당 파장에서 강한 피크를 나타내는 것으로 보아 황산화 유도체화의 성공을 확인했다. 또한 796 cm^{-1} 에서의

흡수 피크는 일반적으로 C-O-S 대칭 스트레칭 진동을 나타낸다. 다당류의 분자량은 PN 이 217,000 Da, PS 145,000 Da 으로 황산화 유도체화 후 분자량이 감소 한것을 확인하였다. 시험관 내, 항 응고 실험은 활성화 부분 트롬보플라스틴 시간 (aPTT) 및 프로트롬빈 시간 (PT) 방법을 이용하여 평가하였으며, PS 가 PN 에 비해 내재 및 외인성 경로에 의한 혈장 응고 억제를 유의하게 개선했음을 보여주었다. PS 의 농도를 15.62 $\mu g/m^3$ 으로 설정 후 항 응고 실험을 진행하여 내재 혈액 응고 경로에 의한 항 응고 활성평가 결과 공 실험에 비해 응고 시간이 3 배이상 증가하였다. 항 산화 활성 실험 결과 PS 는 환원력 억제 효과 및 수산화기 활성 라디칼에 대한 강력한 소거능을 나타내었다. 그러나 ABTS+ 라디칼 소거능에서는 PS 가 PN 에 비하여 더 약한 활성을 보였다. 또한 PN 과 PS 는 정상 세포에서 낮은 수준의 세포독성을 보였으며, HaCaT 및 HEK 293 의 세포를 이용한 IC50 은 순서대로 각각 약 1463.6, 1401.7 및 1116.5, 603.9μg/mL 이다. 결론적으로, 황화는 느타리버섯 천연 다당류의 생물학적 특성을 향상시켰다. 아임계 수 추출을 통해 얻은 느타리버섯 다당류는 황산화 유도체화를 통해 항응고제 활성이 향상 될 수 있음을 나타낸다. 따라서, *P. ostreatus* 의 황-다당류는 항응고제의 대체물질로 간주될 수 있다.

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