Ultrasonic-Assisted Extraction and Antioxidant Activities of the Polysaccharides Extracted from Soybean Curd Residue Fermented by *Flammulina velutipes*

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Abstract

The water-soluble polysaccharides content of soybean curd residue fermented by *Flammulina velutipes* was about 7 times higher than unfermented one and more than 4 times higher than *Flammulina velutipes* fruiting body. The optimized ultrasonic-assisted extraction method (80 °C, 20 min, 80 W and solid-liquid ratio of 1 g/30 mL) showed a water-soluble polysaccharides extraction yield of 7.88% which was higher than that of hot-water extraction method (6.11%). Moreover, the crude polysaccharides were purified and the main components showed strong activities on DPPH radical scavenging (IC50 0.21 mg/mL), ABTS radical scavenging (IC50 0.42 mg/mL), hydroxyl radical scavenging (IC50 0.28 mg/mL) and SOD-like activity (IC50 0.88 mg/mL). The results indicated that the fermentation method could be used as a new approach for bio-active polysaccharides production and a new method for reusing the food industrial waste. The ultrasonic-assisted extraction method could be considered as a more efficient method in polysaccharides extraction process. Furthermore, the fermented soybean curd residue could be expected as a new source of antioxidant with the further research value.

Keywords: soybean curd residue, *Flammulina velutipes*, ultrasonic-assisted extraction, antioxidant activity

1. Introduction

1.1 Soybean Curd Residue

Soybean curd residue is a kind of organic waste and treated by incineration and landfill. However, research report showed that it enriches in cell-wall polysaccharides, protein, oil, dietary, and mineral composition, along with un-specified monosaccharides, and oligosaccharides can be found in the literature (Mateos-Aparicio et al., 2010). Soybean dietary fiber is the generic terms of macromolecular carbohydrates which are not able to be digested by human digestive enzymes, including cellulose, pectin, xylan, mannose, etc. Soybean dietary fiber does not contain protein, vitamins, fats and other nutrients anymore after microbial degradation, but still shows high physiological activity functions on the human body. Polysaccharides are one kind of the soluble dietary fiber of soybean which can significantly increase the growth and the ingestion rate index of body's macrophages and it also can stimulate antibody production and enhance the body's immune function. Our research team developed a fermentation technology in order to reuse the soybean curd residue to gain bioactive polysaccharides.

1.2 Flammulina velutipes

Flammulina velutipes, belonging to the family Tricholomataceae (Hymenomycetes, Basidiomycota), is one of the most popular edible mushrooms. It has being under a large-scale artificial cultivation and increasingly consumed in China and Japan owing to its high nutritional values and attractive taste. It has attracted considerable attention in the fields of biochemistry and pharmacology due to its biological activities. Compounds with medicinal properties have been isolated from the fruiting body and mycelial culture of this mushroom, including proteins with antiviral and immunomodulatory activity (Wang et al., 2004), polysaccharides with immunomodulatory activity (Wasser & Weis, 1999), lectin with antitumor activity (Wang, Ng, & Ooi, 1998), sesquiterpenoids with antimicrobial activity (Ishikawa et al., 2001; Ishikawa et al., 2000) and sterol (Yi et al., 2013). The first study of *Flammulina velutipes* polysaccharides were reported. Since then several other polysaccharides have been isolated from *Flammulina velutipes* fruit bodies and mycelium (Leung, Fung, &

Chay, 1997). Furthermore, the crude polysaccharides from fermented soybean curd residue has been researched (Shi et al., 2012). Therefore, much attention has been paid to the studies of *Flammulina velutipes* polysaccharides and it was selected as the fermentation microorganism in this study.

1.3 Ultrasonic-Assisted Method

The ultrasonic-assisted method has shown a more efficient extraction than using the later method. An increased component extraction was achieved in a shorter time, particularly, in the case of sage. The mechanochemical effect of ultrasonic wave is believed to accelerate the extraction of organic compounds from plant materials due to disruption of cell walls and enhanced mass transfer of the cell contents. It was recently documented by the increased extractability of water-soluble polysaccharides from the ethanol-insoluble plant residue of sage obtained from the ultrasonic-assisted process in comparison to that from the classical extraction. The ultrasonic-assisted extraction has the advantage of accelerating the extraction process, causing less damage to the structural and molecular properties of plant materials (Vilkhu, Mawson, Simons, & Bates, 2008), and can be done at low temperatures. For these reasons ultrasonic methods for assisting the extraction of polysaccharides from plant material are widely used today (Chen et al., 2010; Hromadkova, Ebringerova, & Valachovic, 2002; Pan et al., 2010). Recently, ultrasonic-assisted extraction (UAE) has been widely employed in the extraction of target compounds from different materials owing to its facilitated mass transfer between immiscible phases through super agitation at low frequency (Tsochatzidis, Guiraud, Wilhelm, & Delmas, 2001). It offers high reproducibility at shorter times, simplified manipulation, and lowered energy input, as well as solvent consumption (Vilkhu, Mawson, Simons, & Bates, 2008). By using conventional extraction under ultrasound irradiation (20–100 kHz), structural changes and degradation of polysaccharides can be avoided (Khan et al., 2010; Zhou & Ma, 2006). Thus, UAE may be an effective and advisable technique for the extraction of polysaccharides and was discussed in this study.

2. Materials and Methods

2.1 Materials and Chemical

The A16 strain of *Flammulina velutipes* was purchased from agriculture and forestry strains Kaisha, Japan. Soybean curd residue was obtained from INAMOTO in Tsukuba, Japan. Sucrose, peptone, KH_2PO_4, $MgSO_4$, potato extract, agar, ascorbic acid, ferrous sulfate, sodium salicylate, potassium persulfate，ferrous sulfate and hydrogen peroxide were purchased from Wako Pure Chemical, Osaka, Japan. ABTS+ and DPPH radical were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). SOD Assay Kit-WST was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). All the other chemicals and solvents were analytical grade and used without further purification.

2.2 Fermentation Process

The A16 strain was cultured in 50 mL Erlenmeyer flask containing one unit of mycelia ager square, which was 5 mm×5 mm and obtained by a self-designed cutter. The flask liquid culture medium was composed of the following components: 2.5% sucrose, 2% yeast extract, 0.4% potato extract, 0.3% KH_2PO_4, 0.15% $MgSO_4$. The flask was incubated on a rotary shaker at 100 rpm and 25 °C for 6 days. And then cultured with the solid medium containing the dried and powdered soybean curd residue in 200 mL flask at 25 °C for 20 days (Wei, Wolf-Hall, & Chang, 2001).

2.3 Ultrasonic-Assisted Extraction

The treatment of the crude *Flammulina velutipes* polysaccharides were according to a literature procedure with a few modifications (Guan et al., 2011). The fermented SCR was dried in a convection oven at 60 °C and grounded to powder. The crushed powder was refluxed by 80% ethanol for 6 h to remove the impurities. The extract was discarded and the residue was washed by 95% ethanol and dried at room temperature. The dried residue was extracted by UAE method according to the orthogonal design L9 (3^4) (Table 1). Then, the extract was filtered and centrifuged at 7500 rpm for 10 min at 4 °C. The supernatant was concentrated in a rotary evaporator under reduced pressure at 50 °C and removed free protein layer by the use of Sevage method. At last, the above extract was subjected to the precipitation with four-fold volumes of ethanol. The curd polysaccharides were collected by centrifugation, washed with 99.5% ethanol twice, and then freeze-dried to obtain the crude fermented soybean curd residue polysaccharides (CFSRP).

Total polysaccharides were determined by phenol-sulfuric acid method with some modifications (Mecozzi, 2005; Rhee et al., 2008). The color reaction was initiated by mixing 1 mL of polysaccharides solution with 0.5 mL of 5% phenol solution and 2.5 mL of concentrated sulfuric acid, and the reaction mixture was kept in a 100 °C water bath for 15 min. After cooling to room temperature, the optical density (OD) of the mixture was

determined at 490 nm and the polysaccharides content was calculated with D-glucose as the standard. The results were expressed as the polysaccharides content in per gram of dry fermented soybean residue.

2.4 Elution of the Crude Polysaccharides

The crude polysaccharides were eluted by using EAE-Sephadex A-50 column with distilled water, 0.1 mol/L NaCl, 0.5 mol/L NaCl and 1 mol/L NaCl successively and were collected by centrifuge tubes per 5 mL. Moreover, the tubes of the main components were combined and used to analyze antioxidant activities.

2.5 Antioxidant Activities.

2.5.1 DPPH Free Radical Scavenging Assay

DPPH radical scavenging activities was measured according to the method described by Blois (2002) with some modifications (Blois, 1958). Aliquots (0.5 mL) of various concentrations were mixed with 2 mL (25 µg/mL) of a MeOH solution of DPPH, the mixture was shaken vigorously and allowed to stand in the dark for 30 min. The absorbance was measured with a spectrophotometer (Lambda35, Prekin Elmer Co. Ltd., USA) at 517 nm against a blank. Decrease of the DPPH solution absorbance indicated an increase of the DPPH radical scavenging activity. All the concentrations meant that of F3SFRP in reaction systems. Ascorbic acid was used as positive controls. DPPH free radical scavenging activity was calculated according to the following equation:

$$DPPH\ radical\ scavenging\ activity\ (\%) = [A_0–A_1/A_0]\ \times 100\% \tag{1}$$

While A_0 is the absorbance without samples and A_1 the absorbance in the presence of the samples of ECE and four sub-fractions.

A lower absorbance of the reaction mixture indicated a higher DPPH radical scavenging activity.

2.5.2 ABTS Radical Scavenging Activity

ABTS was dissolved in distilled water at a final concentration of 7 mM and mixed with a potassium persulphate solution at a final concentration of 2.45 mM. The reaction mixture was left to settle at room temperature for 12–16 h in the dark before use (Trishna et al., 2011). For each experiment, freshly prepared ABTS solution was diluted with 0.01 M phosphate buffer saline (PBS, pH 7.4) to adjust its absorbance to within 0.70 ± 0.02 at 734 nm wavelength. Then 0.15 mL of various concentrations of the sample was mixed with 2.85 mL of ABTS solution. Finally, the absorbances were measured at 734 nm after incubation at room temperature for 10 min. All the concentrations meant that of F3SFRP in reaction systems. The scavenging activity of ABTS free radical was calculated by using the following equation:

$$ABTS\ radical\ scavenging\ activity\ (\%) = \{(C–D) – (A–B)/(C–D)\}\ \times 100\% \tag{2}$$

While A = absorbance of ABTS solution + sample/standard, B = absorbance of potassium persulphate + sample/standard, C = absorbance of ABTS solution + distilled water/methanol and D = potassium persulphate + distilled water/methanol.

2.5.3 Hydroxyl Radical (HO•) Scavenging Activity Estimation

HO• scavenging activity was measured according to a literature procedure with a few modifications (Smirnoff & Cumbes, 1989). HO• were generated from $FeSO_4$ and H_2O_2, and detected by their ability to hydroxylate salicylate. The reaction mixture (2.5 mL) contained 0.5 mL $FeSO_4$ (1.5 mM), 0.35 mL H_2O_2 (6 mM), 0.15 mL sodium salicylate (20 mM), and 1 mL of different concentrations of polysaccharides. Ascorbic acid was used as the positive control. After incubation for 1 h at 37 °C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. All the concentrations meant that of F3SFRP in reaction systems. The percentage scavenging effect was calculated as

$$HO•\ scavenging\ activity\ (\%) = [1-(A_1-A_2)\ /A_0]\ \times 100\% \tag{3}$$

While A_1 was the absorbance of the sample or ascorbic acid, and A_0 was the absorbance of the solvent control, whereas A_2 was the absorbance of the reagent blank without sodium salicylate.

2.5.4 SOD-Like Activity Assay

The levels of SOD-like activity in the extracts were measured using the SOD Assay Kit-WST according to the technical manual provided by Dojindo Molecular Technologies, Inc. Briefly, in a 96-well plate, 20 µL of sample solution was added to each sample and blank 2 well, and 20 µL of double distilled water was added to each blank 1 and blank 3 well. Then 200 µL of WST working solution was added to each well. After mixing, 20 µL of dilution buffer was added to each blank 2 and blank 3 well, and 20 µL of enzyme working solution (15 µL of enzyme mixed with 2.5 µL dilution buffer) was added to each sample and blank 1 well. The plate was incubated

at 37 °C for 20 min and the O.D. was determined at 450 nm using a micro-plate reader (Bio-Rad Model 550, USA). All the concentrations meant that of F3SFRP in reaction systems. The SOD-like activity was calculated by the following equation:

$$SOD\ activity\ (inhibition\ rate\ \%) = [(A_{blank1}-A_{blank3})-(A_{sample}-A_{blank2})]\ /\ (A_{blank1}-A_{blank3}) \times 100\% \qquad (4)$$

While A_{blank1}, A_{blank2}, A_{blank3} and A_{sample} were the absorbance of blank 1, blank 2, blank 3 and the sample, respectively.

2.6 Statistical Analysis

The experiments were conducted in triplicate and results were expressed as mean ±SD. A two-tailed student's t-test was used for the statistical analysis.

3. Results and Discussion

3.1 Optimization of Ultrasonic-Assisted Method

An orthogonal array design L9 (3^4) was performed to optimize the extraction temperature, ultrasonic power, extraction time and solid-liquid ratio. Table 1 showed the results of polysaccharides yield obtained under the extraction conditions tested. Additionally, according to the range analysis data (Table 2) for orthogonal design, it is obvious that the extent of the impact of variables on polysaccharides yield followed the order: solid-liquid ratio> temperature> power> time, and the optimum extraction conditions were showed as following: temperature of 85 °C, power of 50 W, time of 30 mins and solid-liquid ratio of 1 g/ 30 mL. Moreover, the final polysaccharides yield (7.88±0.30%) was higher than that of hot-water extraction method (6.11±0.20%) and that of the result published in *Carbohydrate Polymers* (5.92%) (Shi et al., 2012).

Table 3 showed the results of polysaccharides yield obtained under the different extraction rounds. It is obvious that the polysaccharides yield of first extraction round (7.88±0.47%) was higher than that of second round (0.20±0.01%) and third round (0.03±0.01%). Therefore considering the economic factors, it could be considered that the best extraction times is 1.

Table 1. 4-Factor, 3-level orthogonal array used in polysaccharides content

Level	Temperature (°C)	Power (W)	Time (min)	Solid-liquid ratio
1	25	50	10	1 g/ 10 mL
2	50	90	20	1 g/ 20 mL
3	85	130	30	1 g/ 30 mL

Table 2. Range analysis data of UAE method

Value	Factor A	Factor B	Factor C	Factor D
Name	Temperature(°C)	Power (W)	Time(min)	Solid-liquid ratio
k_{1j}	47.87	52.47	49.06	44.54
k_{2j}	50.78	49.87	51.16	51.52
k_{3j}	52.97	49.26	51.40	55.55
R^d	5.10	3.21	2.35	11.02

Table 3. Crude polysaccharides content of different extract rounds

Round	Crude polysaccharides content (%)
1	7.88±0.30%[a]
2	0.20±0.01[b]
3	0.03±0.01[c]

[a, b, c] Different superscript letters in the same column indicate significant difference (*$p < 0.05$).

3.2 Variation of Water-Soluble Polysaccharides

Table 4 showed the results of polysaccharides yield obtained from uninoculated medium, *Flammulina velutipes* fruiting body and fermented soybean curd residue. The polysaccharides yield of CFSRP (7.88±0.30%) was significantly higher than that of UIM (0.99±0.04%) and FVFB (1.78±0.04%).

Table 4. Polysaccharides yield of UIM, FVFB and CFSRP

	Polysaccharides yield (%)
UIM	0.99±0.04[a]
FVFB	1.78±0.04[b]
CFSRP	7.88±0.30%[c]

3.3 Elution of Crude Polysaccharides

Figure 1 showed the absorbance of the solution collected after elution. The four peaks stand for the polysaccharides eluted by distilled water, 0.1 mol/L NaCl, 0.5 mol/L NaCl and 1 mol/L NaCl can be observed clearly. The first and fourth peeks were low and the absorbance value of before and after part of them approximated distilled water. The second peek was higher than the first and fourth ones while the third peek was the highest although the absorbance value of the part between them was higher than the distilled water. The polysaccharides of the third peak was collected and named fraction 3 (F3FSRP).
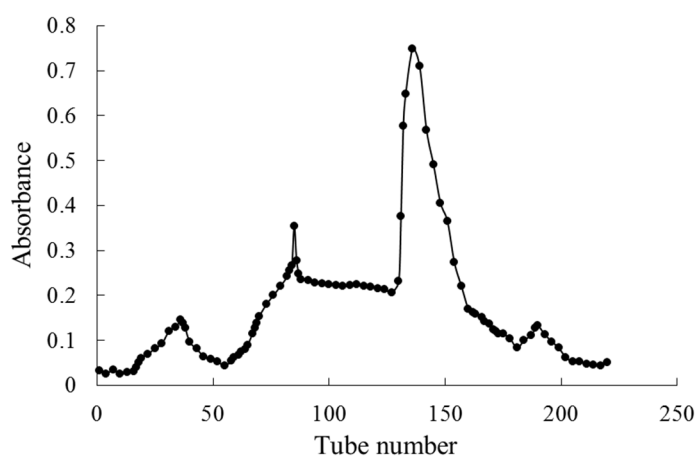

Figure 1. Absorbance of the solution collected after elution

3.4 Antioxidant Activities

As shown in Figure 2, F3FSRP showed obvious scavenging activity on DPPH radical in a concentration-dependent manner. However, the scavenging activities of all samples were weaker than that of ascorbic acid. The activity of F3FSRP reached 50.60% at the concentration of 0.8 mg/mL which is similar to the result published in *Carbohydrate Polymers* (Zhang et al., 2013).

As shown in Figure 3, F3FSRP showed a relatively moderate ability to inhibit ABTS radical compared with ascorbic acid. The maximum inhibition of F3FSRP was 45.43% at the concentration of 0.2 mg/mL, and it eliminated the ABTS radical in a dose-dependent manner.

As shown in Figure 4, the scavenging effects of all samples increased significantly with the increase of sample concentration ranging from 0.010 to 0.667 mg/ml. At a concentration of 0.667 mg/ml, the scavenging activity reached 73.94%.

As shown in Figure 5, the SOD-like activity increased with the concentrations of *Flammulina velutipes* polysaccharides, and treated with *Flammulina velutipes* polysaccharides at the concentration of 0.64 mg/mL, SOD-like activity was 36.32%.
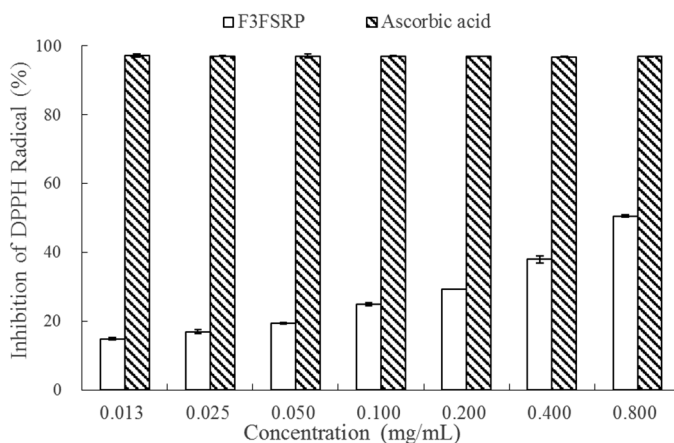

Figure 2. DPPH radical inhibition capacity of F3FSRP. Data are expressed as means ± S.D. of triplicate determinations
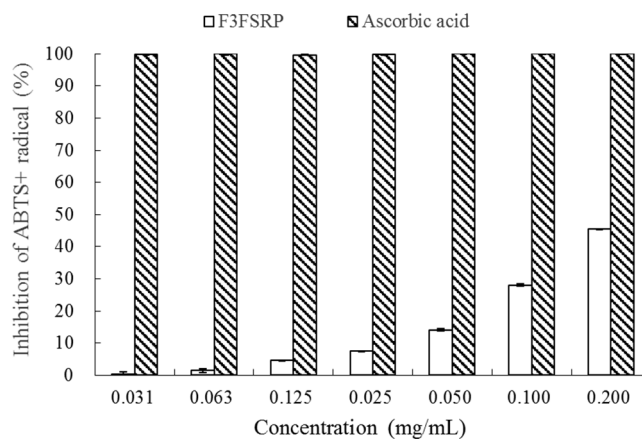

Figure 3. Inhibition (%) of F3FSRP on the stable ABTS radical. Data are expressed as means ± S.D. of triplicate determinations. Ascorbic acid was positive control
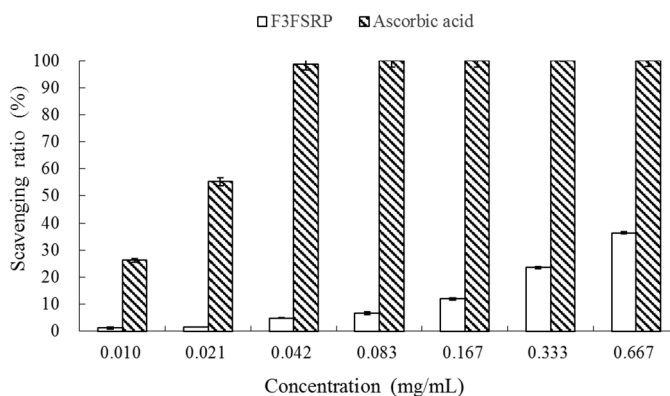

Figure 4. Hydroxyl radical scavenging activities of F3FSRP. All treatments were conducted in triplicate. Ascorbic acid was positive control
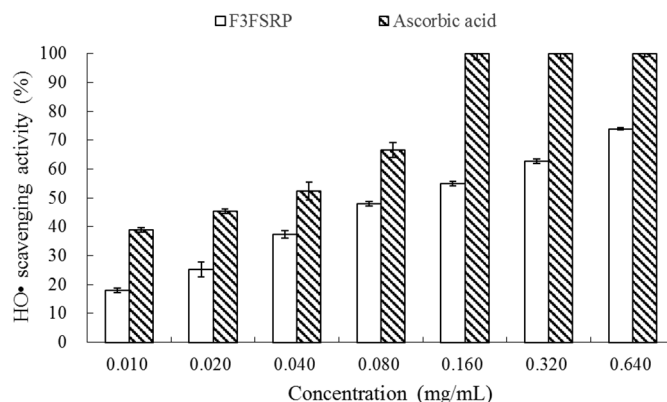
Figure 5. SOD-like activities of F3FSRP. Data are expressed as means ± S.D. of triplicate determinations. Ascorbic acid was positive control

4. Conclusions

The fermentation method with fungi could be used as a new approach for bio-active polysaccharides production and a new method for reusing the food industrial waste. The ultrasonic-assisted extraction method could be considered as a more efficient method in polysaccharides extraction process. Furthermore, the fermented soybean curd residue by *Flammulina velutipes* could be expected as a new antioxidant with the further research value.

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