Carbohydrate Polymers 83 (2011) 1298–1302

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

# Effect of the degree of oxidation on the physicochemical and biological properties of *Grifola frondosa* polysaccharides

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ARTICLE INFO

*Article history:*
Received 17 August 2010
Received in revised form 8 September 2010
Accepted 20 September 2010
Available online 24 September 2010

*Keywords:*
*Grifola frondosa*
Oxidation
Cytotoxic activity
Superoxide dismutase activity

ABSTRACT

Polysaccharide extracted from *Grifola frondosa* was subjected to 2,2,6,6-tetramethyl-1-piperidine oxoammonium ion (TEMPO)-mediated oxidation of which effects on the structural and biological properties of the polysaccharide were investigated as a function of degree of oxidation. Successive oxidation of the polysaccharide was confirmed by $^{13}C$ NMR spectroscopy and the molecular weight change of the oxidized polysaccharide was observed, decreasing from $10.6 \times 10^5$ Da to $7.5 \times 10^5$ Da by 100% oxidation. The oxidation also caused an increase in the water solubility of the polysaccharide while its viscosity was significantly reduced. In addition, when human fibrosarcoma HT1080 cells were treated with 100% oxidized polysaccharide, their in vitro growth was effectively inhibited. However, the oxidation reduced the activity of superoxide dismutase up to 82% at a concentration of 2.5 mg/mL. Thus, the chemical modification by TEMPO-mediated oxidation was shown to play a significant role in the biological properties of the polysaccharides derived from *Grifola frondosa*.



## 1. Introduction

Polysaccharides which belong to a class of carbohydrates with high molecular weight are regarded as biopolymers in which monosaccharides are joined together by glycosidic bonds. Due to their unique characteristics such as high viscosity and great water holding capacity, native polysaccharides have been often used as food additives such as gelling agent and thickener (Kornmann, Duboc, Marison, & Von Stockar, 2003). Recently, much attention has been paid to the physiological activities of the polysaccharides derived from a variety of sources. Even, the well-being trend of the current society has given an impetus to improve and/or create the physiological properties of polysaccharides by chemical modification through the incorporation of new functional groups in the structural chains (Kulicke, Lettau, & Thielking, 1997). Specially, oxidation has received continuous attention due to its diverse beneficial effects on the physiological functions of many polysaccharides such as anti-tumor (Nono, Ohno, Masuda, Oikawa, & Yadomac, 1991; Ohno, Minura, Miura, Adachi, & Yadomae, 2001) and bile-acid binding capacity (Park, Bae, Lee, & Lee, 2009; Yoo et al., 2005), which might be caused by improved water solubility and incorporation of anionic groups into the polymer structure. Among representative stable radical reagents for the oxidative reaction, 2,2,6,6-tetramethyl-1-piperidine oxoammonium ion (TEMPO) is generally used for highly selective oxidation of polysaccharides. Previously, TEMPO-mediated oxidation where carboxylic groups are introduced into C6 primary hydroxyl groups (De Nooy, Besemer, & Van Bekkum, 1995), was applied in combination with sodium hydrochlorite and sodium bromide to produce high-yield polyuronic acids (Kato, Matsuo, & Isogai, 2003; Park et al., 2009; Saito & Isogai, 2006; Yoo et al., 2005).

*Grifola frondosa*, which is named "maitake" in Japan, is regarded as a well-being mushroom because of its potential biological effects such as anti-tumor (Mayell, 2001), anti-hypertension (Talpur et al., 2002), and hepatoprotective effect (Lee et al., 2008). These beneficial biological activities of *G. frondosa* would be attributed to its active polysaccharide which is mainly considered as β-(1→3)-glucan with β-(1→6)-glucosyl side branching units (Mayell, 2001). In addition, *G. frondosa* polysaccharide is reported to be heteropolysaccharides composed of glucose, mannose, and galactose with a molar ratio of 6.5:1.0:2.6 (Xu, Liu, Shen, Fei, & Chen, 2010). It was also reported that the polysaccharide in *G. frondosa* had a significant effect on the tumor growth inhibition through the stimulated proliferation of T-lymphocytes, B-lymphocytes, and macrophages (Kodama, Komuta, Sakai, & Nanba, 2002; Matsui, Kodama, & Nanba, 2001). In addition, Kubo, Aoki, and Nanba (1994) demonstrated that the polysaccharide derived from *G. frondosa* showed anti-diabetic activity. Thus, the main focus of the study on *G. frondosa* was placed on the anti-tumor effects derived from its native polysaccharide. However, any chemical modification by oxidation has not been yet

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doi:10.1016/j.carbpol.2010.09.037

reported to improve the biological activity of the native polysaccharide from *G. frondosa*.

Therefore, the aims in this study were to isolate polysaccharides from *G. frondosa*, to modify the polysaccharides by the sequential TEMPO-mediated oxidation, and finally to investigate its structural and physical characteristics at the different level of oxidation. Furthermore, the biological properties such as tumor cell inhibition effect and superoxide dismutase (SOD) activity were evaluated as a function of the degree of oxidation.

## 2. Materials and methods

### 2.1. Isolation of Grifola frondosa polysaccharides

Based on the previous method (Kodama, Yamada, & Nanba, 2001), polysaccharides were extracted from *G. frondosa* that was provided from Pulmuone Co. (Seoul, Korea). The homogenized fruit body of *G. frondosa* (100 g) was suspended in distilled water (1 L) under agitation at 99 °C for 30 min and then centrifuged at 12,000 × *g* for 20 min. Equal volume of ethanol was added to the supernatant and left at 4 °C for 24 h, followed by centrifugation at 12,000 × *g* for 20 min. The precipitate was dialyzed against distilled water for 5 days and then freeze-dried.

### 2.2. Preparation of oxidized Grifola frondosa polysaccharides

According to the method of Chang and Robyt (1996) with some modifications, TEMPO (0.01 mmol/g polysaccharide), NaBr (0.45 mmol/g polysaccharide), and NaOCl (2.2 mmol/g polysaccharide) were added into polysaccharide solutions (0.162 g in 30 mL of DW) and reacted at 25 °C, pH 10.8 with 1 N hydrogen chloride (HCl). The resulting solution was oxidized at pH 10.8 with 0.5 N sodium hydroxide (NaOH) and the degree of oxidation was determined from the amount of NaOH consumed (conversion to millimoles of oxidized alcohol groups). The reaction was stopped by adding 10 mL of ethanol and neutralizing with 4 N HCl. Three volume of acetone was then added to precipitate the oxidized polysaccharide, which was vacuum-dried at 55 °C for 24 h.

### 2.3. Structural characterization

$^{13}$C NMR (300 MHz, Unit INOVA, Varian Co., Palo Alto, CA) operating at a carbon NMR frequency of 300 MHz was applied to investigate the structural changes of polysaccharide by oxidation. The samples (80 mg) were dissolved in $D_2O$ (2 mL), and tetramethylsilane was used as internal standard.

### 2.4. Physicochemical properties

The molecular weight (Mw) of polysaccharide samples was determined by gel permeation chromatography (GPC) that was operated at room temperature with a HPLC system (LC-900, Japan Analytical Instrument, Tokyo, Japan) equipped with a JAIGEL-W254~255 column and a JAI RI-50 differential refractometer. Deionized water was used as eluent at a flow rate of 3.5 mL/min and the injection volume (0.5%, w/v) was 1.0 mL. The molecular weight of the samples was determined by dextran standards with known Mw values.

Water solubilities of native and oxidized-polysaccharides were determined according to the method of Chang and Cho (1997). The polysaccharide dispersion (25 mg/mL for native; 100 mg/mL for oxidized) was agitated at 25 °C for 24 h. After the oversaturated dispersion was centrifuged at 5000 × *g* for 15 min, the supernatant was vacuum-dried at 60 °C and weighed.

### 2.5. Flow behavior

To investigate the flow behaviors of native and oxidized-polysaccharides, the samples were suspended in distilled water at a concentration of 20% (w/v). The shear stress of each suspension was then investigated as a function of shear rate (0–200 $s^{-1}$) using a controlled-stress rheometer (Rheostress RS1, Thermo Hakke, Karlsruhe, Germany). Parallel plates with 35 mm diameter were used and the gap was 1 mm. Measurements were made at 25 °C and the curves reported were mean values of two measurements.

### 2.6. Tumor cell inhibition effect

The effect of oxidation on in vitro cytotoxic activity of *G. frondosa* polysaccharide against HT1080 human fibrosarcoma cells (Korean Cell Line Bank, Seoul, Korea) was investigated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (M-5655, Sigma–Aldrich Chemical Co., Milwaukee, WI). The cells were cultured in RPMI1640 medium (LM 011-01, JBI Welgene Co., Daegu, Korea) supplemented with 10% fetal bovine serum and 100 unit/mL penicillin–streptomycin solution (P-4458, Sigma–Aldrich Chemical Co., Milwaukee, WI) and then the cells ($1 \times 10^4$ cells per well) were added on a sterile 96-well microtiter plate (ELx800UV, Bio-Tek Instrument Inc., Windoski, VT). After the sample solutions having various concentrations were added into the plate, it was incubated in a 5% $CO_2$ incubator (37 °C) for 92 h and the MTT solution was added to each experimental well which were incubated at 37 °C for 4 h. Then, 150 μL of dimethyl sulfoxide (Sigma–Aldrich Chemical Co., Milwaukee, WI) was added to solubilize formazan crystals and agitated on a plate shaker for 15 min. The optical density of each well was measured at 540 nm by using the multi-well ELISA automatic spectrometer reader (ELx800UV, Bio-Tek Instrument Inc., Windoski, VT).

### 2.7. Superoxide dismutase activity

The superoxide dismutase (SOD) activity was assayed by the method of Marklund and Marklund (1974). The sample solution (0.2 mL) was mixed with 50 mM Tris–HCl buffer containing 10 mM EDTA (pH 8.5, 3.0 mL) and 7.2 mM pyrogallol (0.2 mL), followed by the incubation at 25 °C for 10 min. After the reaction was stopped by adding 1 N HCl (1.0 mL), SOD activity was calculated from the absorbance of oxidized pyrogallol recorded at 420 nm as follows;

$$\text{SOD activity}\,(\%) = \left(\frac{1 - \text{absorbance of sample}}{\text{absorbance of blank}}\right) \times 100$$

### 2.8. Statistical analysis

For statistical analysis, Statistical Package for the Social Science (SPSS, Version 12.0, 2004, SPSS Inc., Chicago, IL) was used. The results were subjected to analysis of variance (ANOVA), followed by the Duncan's multiple range test for mean comparison at the level of 0.05.

## 3. Results and discussion

### 3.1. Structural characterization of oxidized polysaccharide

The structural changes of *G. frondosa* polysaccharide by oxidation were confirmed by $^{13}$C NMR spectra. As shown in Fig. 1, distinct changes were observed in two peaks at 60.2 ppm and 175.2 ppm. As the degree of oxidation increased, the signal intensity of the C6 hydroxyl groups (60.2 ppm) gradually decreased. On the other hand, a new signal at 175.2 ppm which corresponds to C6 carboxyl groups (Kato et al., 2003), was generated in the spectra of

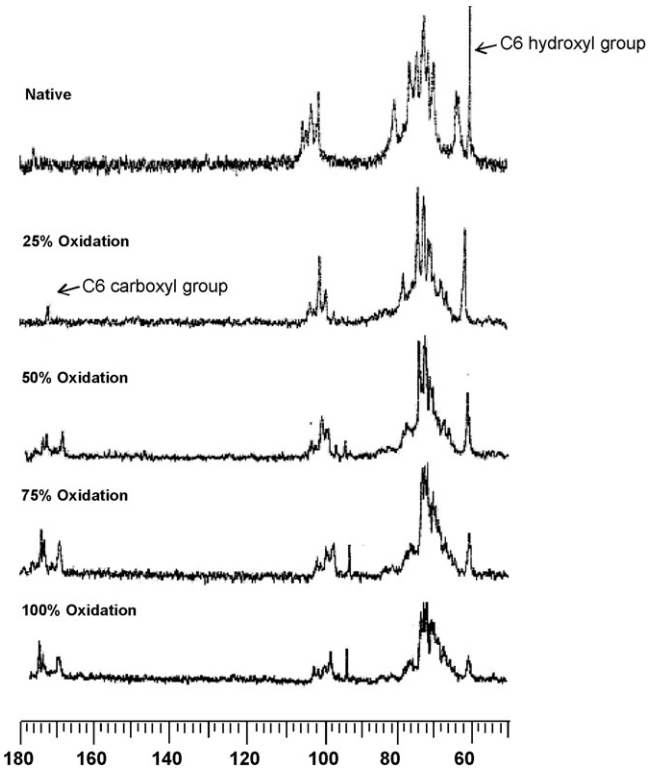


**Fig. 1.** NMR spectra of native and oxidized *Grifola frondosa* polysaccharides.

the oxidized samples and its intensity increased with increasing the degree of oxidation. Thus, the successful oxidation of *G. frondosa* polysaccharide was confirmed by the NMR result, which was also supported by previous studies (Chang & Robyt, 1996; Park et al., 2009).

### 3.2. Physicochemical properties of oxidized polysaccharide

Table 1 presents the effect of oxidation on the molecular weight of *G. frondosa* polysaccharide. While the native polysaccharide had an average molecular weight of $10.6 \times 10^5$ Da, the oxidized polysaccharides were in the molecular weight range between $7.5 \times 10^5$ and $8.9 \times 10^5$ Da. The decreased molecular weight of all oxidized samples indicated that *G. frondosa* polysaccharide was degraded during the oxidation reaction which was also observed in preceding studies (Wang & Wang, 2003). Especially, according to the result of Cross et al. (2001), this molecular weight range would be acceptable for maintaining the immunological activity of the β-glucan from *Saccharomyces cerevisiae*. In addition, it seemed that the change in the molecular weight was not a function of the degree of oxidation.

The water solubilities of *G. frondosa* polysaccharide and its oxidized derivatives were compared. As also can be seen in Table 1, the water solubility was improved significantly ($p < 0.05$) by oxidation, exhibiting that it was increased from 84% up to 99.9% when 100% of the primary alcohol groups in polysaccharide were oxidized. It would be explained by increased hydrophilic properties of the oxidized derivatives due to the incorporation of carboxyl groups into polymer structure, consequently making it more soluble. The increased solubility by oxidation has been also observed in the literature (Chang & Cho, 1997; Chang & Robyt, 1996; Park et al., 2009).

**Table 1**
Molecular weights and water solubilities of native and oxidized *Grifola frondosa* polysaccharides.

| Sample | Molecular weight (Da) | Water solubility (%) |
|---|---|---|
| Native | 1,060,000[a] | 83.90[d] |
| 25% oxidation | 780,000[b] | 88.50[c] |
| 50% oxidation | 840,000[b] | 93.50[b] |
| 75% oxidation | 890,000[b] | 98.65[a] |
| 100% oxidation | 750,000[b] | 99.90[a] |

Values with different superscripts (a–c) within the same column are significantly different among samples at $\alpha = 0.05$ level by Duncan's multiple range test.

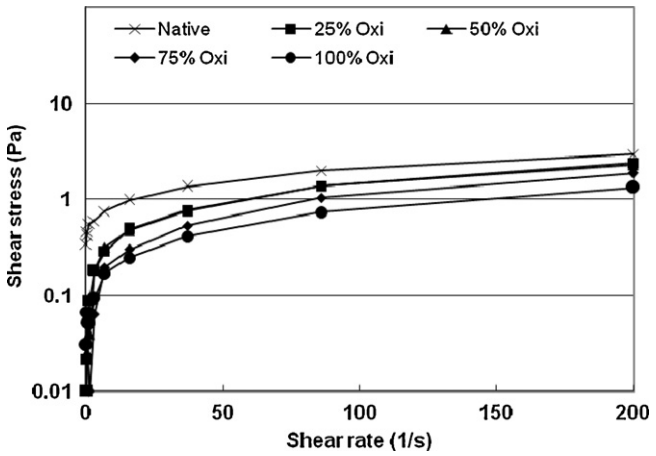


**Fig. 2.** Flow behaviors of 20% native and oxidized *Grifola frondosa* polysaccharide suspensions at 25 °C.

The flow behaviors of native and oxidized polysaccharide solutions were characterized by steady shear measurements. As shown in Fig. 2, an increase in the shear stress with more increments in the shear rate was observed in all of the samples, consequently exhibiting the pseudoplastic behaviors. However, the shear stress curves were shifted down with increasing the degree of oxidation. It indicated that the viscosity was reduced by oxidation since the viscosity is defined as the ratio of shear stress versus shear rate. These results might be attributed to increased intermolecular repulsion by the incorporation of negatively charged groups as well as reduced molecular weight caused by partial hydrolysis during oxidative process (Lee, Yoo, Baek, & Lee, 2007).

### 3.3. Biological effects of oxidized polysaccharide

Fig. 3 presents the effect of oxidation on the growth inhibition of *G. frondosa* polysaccharide against HT1080 human fibrosarcoma cells. Even though all samples inhibited the growth of tumor cell

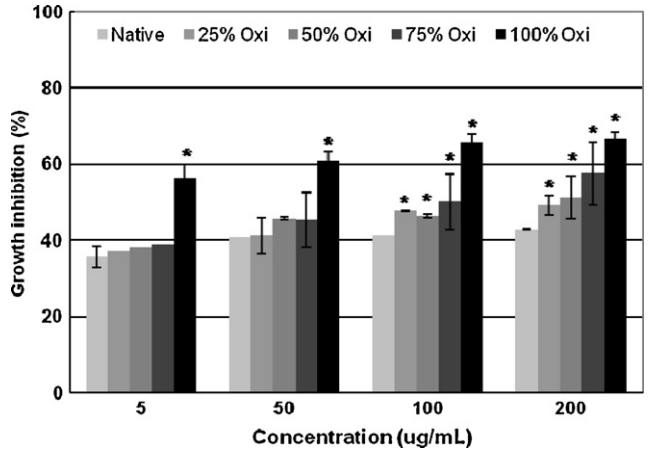


**Fig. 3.** Cell growth inhibition effects of native and oxidized *Grifola frondosa* polysaccharides. Significantly different from native, $^*p < 0.05$.

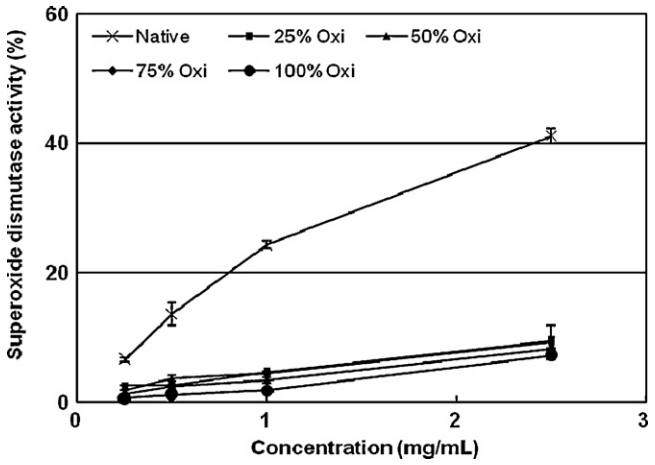


**Fig. 4.** Superoxide dismutase activities of native and oxidized *Grifola frondosa* polysaccharides.

line, more improved growth inhibition was observed as the degree of oxidation increased. Specifically, the growth inhibition against HT1080 cells increased 1.6-fold when the 100% oxidized sample was used at a concentration of 200 μg/mL. Thus, these results demonstrated that the introduction of anionic groups into the structure of polymer and the alteration of macromolecular conformation effectively suppressed the growth of the tumor cells (Ohno et al., 2001). Moreover, these improved activities could be partly attributed to increased water solubility of the oxidized polysaccharides since it is widely recognized that water solubility is positively related to antitumor activity (Shin, Lee, Bae, Yoo, & Lee, 2007).

Superoxide dismutase (SOD) is an important antioxidant system in oxygen-metabolizing cells, protecting the cells from superoxide toxicity when exposed to oxygen (Ming, Guanhua, Zhanhai, Guang, & Xuan, 2009). The effect of oxidation on the SOD activity of *G. frondosa* polysaccharide was investigated as shown in Fig. 4. The native polysaccharide derived from *G. frondosa* showed a concentration-dependent increase in the SOD activity. However, the oxidation caused a dramatic change in the SOD activity of the *G. frondosa* polysaccharide which was reduced by 77–82% at a concentration of 2.5 mg/mL. It was recognized in previous studies that β-glucan with β-(1-3) glycosidic linkage enhanced the activity of SOD (Bobek & Galbavy, 2001). Thus, it was evident that the oxidation would have brought about dramatic inhibition of SOD. The oxidized polysaccharide derivative with high negative charge density might compete with superoxide ions, consequently inhibiting the reaction with SOD. In addition, a steric hindrance might take place when carboxyl groups were incorporated into the polysaccharide backbone. Further analysis would be necessary for the better understanding of the binding of the oxidized sample to the active site of SOD. It was however found in this study that the oxidized *G. frondosa* polysaccharide played a negative role in protecting the cells from the oxidative damage.

## 4. Conclusions

In this study, TEMPO-mediated oxidation where carboxyl groups are substituted for C6 primary hydroxyl groups was applied to *G. frondosa*-derived polysaccharide and the oxidized derivatives with the different degree of oxidation were successfully prepared. The oxidation caused a decrease in the molecular weight and viscosity of the polysaccharide while an increase in the water solubility was observed with increasing the degree of oxidation. Thus, it seemed that these property changes contributed to the tumor cell growth inhibition against HT1080 cells. Even though the oxidized *G. frondosa* polysaccharide was shown to have beneficial biological effects, further in vivo analysis would be necessary to investigate its potential use as an active functional ingredient in food and pharmaceutical areas.

## Acknowledgment

This work was supported by Mid-career Researcher Program through NRF grant funded by the MEST (No. 2010-0000446).

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