Relationship of chemical composition and cytotoxicity of water-soluble polysaccharides from *Lentinusedodes* fruiting bodies

Yan Zheng[1], Miao Hao[1], Hongmei Nan[2], Iteku Bekomo Jeff[3], Yifa Zhou[3] and Yufei Gao[1]*

[1]China-Japan Union Hospital of Jilin University, Changchun, PR China
[2]The first affiliated Hospital, Changchun University of Chinese Medicine, Changchun, PR China
[3]School of Life Science, Northeast Normal University, Changchun, PR China

Abstract: Six water-soluble polysaccharides (S-WPLE-I-a, S-WPLE-I-b, S-WPLE-II-a, S-WPLE-II-b, S-WPLE-III-a and S-WPLE-III-b) were obtained from *Lentinus edodes* and purified by gel-permeation chromatography (GPC). The fractions were analyzed for monosaccharide composition, molecular weight, and then tested for cytotoxicity activity. The results from high performance liquid chromatography (HPLC) revealed that the water-soluble fractions were heteroglucan containing mainly glucose (Glc), galactose (Gal) and mannose (Man) at various ratios. All the polysaccharide fractions exhibited antitumor activities against Sarcoma 180 (S-180) solid tumor cells and human colorectal cancer cell lines (HT-29 and HCT-116) *in vitro* at the dose of 5mg/ml. The antitumor activities of the polysaccharides were related to their monosaccharide content and molecular weight. The effects of Gal, Man and bound protein on the improvement of antitumor activities of polysaccharides might not be negligible. The results also revealed that there was selectively higher antitumor activity of the polysaccharides against suspended cells (S-180) than adherent ones (HT-29 and HCT-116).

Keywords: *Lentinus edodes*; polysaccharide; freeze-thawing; cell proliferation assay.

INTRODUCTION

Currently, macromycetes fungi are distinguished as important natural resources of biologically active substances (Wiater *et al*., 2011). The diverse biological activities of macromycetes fungi include antitumor, immunosuppressive and antibacterial effects (Moradali *et al.*, 2007). Mushrooms are one of the macromycetes fungi, which have been used as food and drug for longtime in the world. Consumption of mushroom also considered to be beneficial for people to restore physical fitness or take precautions against many illnesses. Therefore, mushrooms have recently become more attractive to researchers as food and as sources for development of drugs (Moradali *et al.*, 2007). Polysaccharides, which have been found to boost the human immune system, showing anti-tumor and anti-viral activities, are the most bioactive constituents in mushrooms (Zhang *et al.*, 2007). Since the isolation of lentinan, a (1 → 3)- β -D-glucan with antitumor and immunomodulating activities, by Chihara (Chihara G Fau -Hamuro *et al.*, 1970, Chihara *et al.*, 1969), many researches dealing with *L. edodes* have been conducted and several compounds have been identified with various biological activities, such as antitumor activity, immunomodulating activities, antioxidant activities, antinociceptive and anti-inflammatory activities, antimicrobial, liver function improving and hypolipidemic activity (Carbonero *et al.*, 2008, Chen *et al.*, 2012, Fukushima *et al.*, 2001, Jeff *et al.*, 2013b, Mizuno *et al.*, 1995b, Surenjav *et al.*, 2006, Xu *et al.*, 2008, Xu *et al.*, 2012, Yu *et al.*, 2009, Yu *et al.*, 2010, Zheng *et al.*, 2005). However, reports concerning antitumor activities often focus on (1→3)-β-D-glucan type molecules. Therefore, in this research, we attempted to investigate the cytotoxicity activity of polysaccharides from *L. edodes* related to their chemical compositions using different tumor cell lines. This investigation will provide some essential information for successfully interpretation of the biological activities of polysaccharides from *L. edodes*.

MATERIAL AND METHODS

Reagents

L. edodes fruiting bodies used in this study is cultivated in Changbai Mountain District as mentioned in our previous publication (Jeff *et al.*, 2013a). Sephadex G-75, Sephadex G-100 and Sepharose CL-6B were purchased from Amersham Pharmacia Biotech. RPMI 1640 medium, Dulbecco's modified Eagle medium/F-12 (DEME/F12) medium, Iscove's modified Dulbecco's medium (IMDM), fetal bovine serum were purchased from Gibco. 3-(4, 5-dimethylthyiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma. All other chemicals were of analytical grade.

Isolation and purification of polysaccharides

The crude polysaccharide, referred to as WPLE, was obtained as described in our previous publication (Jeff *et al.*, 2013a). WPLE (6g) was dissolved in distilled water (300 ml) and the solution was separated by centrifugation. The soluble and insoluble fractions were freeze-dried to

*Corresponding author: e-mail: gaoyufei1975@sina.cn

give (S-WPLE, 86.0% yield) and (I-WPLE, 13.4% yield), respectively (fig. 1).

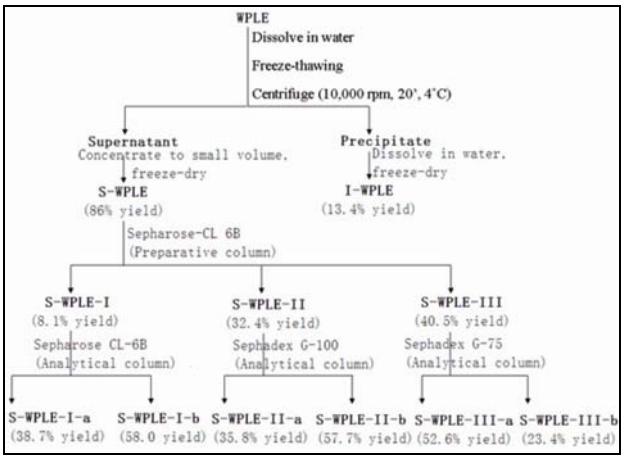

Fig. 1: Fractionation and purification scheme of polysaccharides from *L. edodes*

Gel-permeation chromatography (GPC) was used for the fractionation of the partially purified polysaccharides. The S-WPLE was loaded onto a Sepharose CL-6B preparative column and eluted with 0.15M NaCl at 0.5ml/min. Three fractions were collected according to the sugar profile detected by phenol-sulfuric acid method (fig. 2).

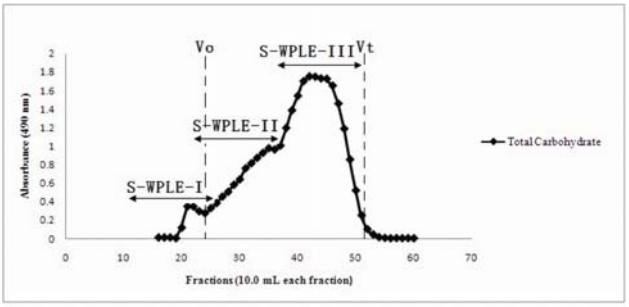

Fig. 2: Elution profile of S-WPLE. Elution profile of S-WPLE from Sepharose CL-6B preparative column, which was eluted with 0.15M NaCl at a flow rate of 0.5mL/min. Vo void volume; Vt, total volume

S-WPLE-I was fractionated into two portions (S-WPLE-I-a and S-WPLE-I-b) on Sepharose CL-6B (1.5x90cm) column. S-WPLE-II was fractionated into two portions (S-WPLE-II-a and S-WPLE-II-b) on Sephadex G-100 (1.5x100cm) column; while S-WPLE-III was fractionated on Sephadex G-75 (1.5x100cm) into S-WPLE-III-a and S-WPLE-III-b.

Component analysis

The protein content was estimated by Bradford method using bovine serum albumin as standard (Bradford *et al.*, 1976). The total carbohydrate content of the polysaccharides was determined by phenol-sulfuric acid method (DuBois *et al.*, 1956) using Glc as standard. The amount of total sugar was estimated by reference to a standard curve made from Glc. Monosaccharide composition of the polysaccharides was determined by HPLC (Jeff *et al.*, 2013b).

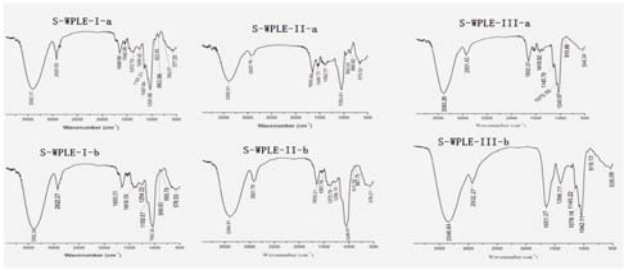

Fig. 3: FT-IR spectra of polysaccharides from *L. edodes*.

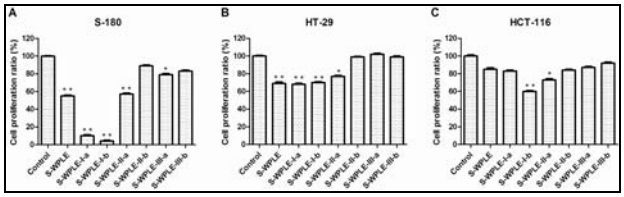

Fig. 4: The proliferation ratio of polysaccharides from *L. edodes in vitro*. S-180 (A), HT-29 (B) and HCT-116 (C) cells were treated with samples at 5mg/mL for 48h. The anti-tumor activities of polysaccharides from *L. edodes* were determined by the MTT assay. The data are shown as mean ± SD. **P*<0.05, ***P*<0.01 vs. control.

Characterization

Homogeneity and M*w* were determined by SEC as described in our previous publications (Jeff *et al.*, 2013a). FT-IR spectra were obtained on a Nicolet 6700 FT-IR spectrometer with DTGS detector in a range of 400-4000 cm^{-1}. The sample was measured as a film on KBr discs.

Assay of antitumor activity

The cell proliferation assay in *vitro* was carried out as described in our previous publication (Jeff *et al.*, 2013b).

RESULTS

The monosaccharide compositions of the polysaccharides

The monosaccharide content, the total carbohydrate, protein contents and M*w* as well as the yield of the six polysaccharides are summarized in table 1. All the water-soluble polysaccharides had high carbohydrate content (> 80%) but low protein content (≈5%). The presence of protein might suggest that these polysaccharides are bound to protein since the crude polysaccharide was subjected to the Sevag method to remove free proteins but they were not treated with acetone to remove the bound protein (Peng *et al.*, 2005, Surenjav *et al.*, 2006). Three kinds of sugar compositions were found in the six polysaccharides, including Glc, Gal and Man. Except for the S-WPLE-III-b in which traces of fucose (Fuc),

glucuronic acid (GlcA) and rhamnose (Rha) were detected. No uronic acids were detected in the other five samples.

The FT-IR spectrum of the samples S-WPLE-I-a to S-WPLE-III-b (fig. 3) all exhibited the characteristic absorptions of polysaccharides at the regions of 1250, 1400 and 1650cm^{-1}. The IR spectra of S-WPLE-I-a and S-WPLE-I-b had absorption peaks at 863.96 and 868.78cm^{-1}, respectively, implying the existence of β-D-glucan. In contrast, the obvious absorption of α-D-glucan appeared at 910.96, 919.13cm^{-1} in S-WPLE-III-a and S-WPLE-III-b, respectively. The appearance of absorption bands both at the region 870cm^{-1} for β-configuration and 920 and 950cm^{-1} for α-configuration in S-WPLE-II-a and S-WPLE-II-b, suggesting the co-existence of α- and β-configuration.

In vitro antitumor activity

The suspension cells S-180 and the adherent cells (HCT-116 and HT-29) were treated with the polysaccharide fractions from *L. edodes* fruiting bodies at concentrations of 5mg/ml and cell viability was determined by MTT assay. As shown in fig. 4 and table 2, the seven polysaccharides fractions (S-WPLE, S-WPLE-I-a, S-WPLE-I-b, S-WPLE-II-a, S-WPLE-II-b, S-WPLE-III-a and S-WPLE-III-b) exhibited inhibition against growth of the three tumor cell lines. In particular, as to S-180 cells, S-WPLE-I-a and S-WPLE-I-b displayed significant inhibitory effects with over 90% inhibition ratio, while S-WPLE, S-WPLE-II-a, S-WPLE-II-b, S-WPLE-III-a and S-WPLE-III-b showed inhibition ratios of 45%, 43%, 11%, 21% and 17%, respectively (fig. 4A).

As shown in figs. 4B and 4C, all the polysaccharides exhibited relatively lower inhibition to HCT-116 and HT-29 than S-180. S-WPLE, S-WPLE-I-a, S-WPLE-I-b and S-WPLE-II-a had an inhibition ratio of 23-32% at the concentration of 5 mg/ml against HT-29 cell growth (fig. 4B), but no antiproliferative effect of S-WPLE-II-b, S-WPLE-III-a and S-WPLE-III-b was observed, implying that they had no direct cytotoxicity to cancer cells. S-WPLE-I-b and S-WPLE-II-a showed an inhibition ratio of 27-40% at the same dose against HCT-116 cell growth, while S-WPLE, S-WPLE-I-a, S-WPLE-II-b, S-WPLE-III-a and S-WPLE-III-b had an inhibition ratio of 8-17% (fig. 4C).

DISCUSSION

It has been reported that high M*w* heteroglycans and glucans appear to be more effective than those of low M*w* (Mizuno *et al.*, 1996, Surenjav *et al.*, 2006, Zhang *et al.*, 2007, Zhang *et al.*, 2004), in accordance with our results, indicating that S-WPLE-I-a and S-WPLE-I-b having high M*w* (ranged from 755 to 2000 kDa) exhibited stronger inhibitory effect. The maximum inhibition ratio of S-WPLE-I-a sample achieved 90%, 17% and 32% for S-180, HCT-116 and HT-29, respectively at the dose of 5 mg/ml; while the maximum inhibition ratio of S-WPLE-I-b sample achieved 96%, 40% and 30% for S-180, HCT-116 and HT-29, respectively. The antitumor activities of the samples S-WPLE-II-a, S-WPLE-II-b, S-WPLE-III-a and S-WPLE-III-b were lower than those of the native (S-WPLE). Similarly, the antitumor activity of high M*w* polysaccharide is considered to be a consequence of stimulation of the immune response in the host (Wasser *et al.*, 2002).

It is noteworthy that S-WPLE-I-a and S-WPLE-I-b, the highest Mw fractions, presented more potent antitumor activities than the native polysaccharide S-WPLE, while the four lower Mw fractions (S-WPLE-II-a, S-WPLE-II-b, S-WPLE-III-a and S-WPLE-III-b) had weaker or similar activities than that of the native polymer. S-WPLE-I-a and S-WPLE-I-b mainly consisted of Glc with β-D-configuration; these isolated structural features could have a more favorable exposure of the antitumor effects sites than the original molecule. S-WPLE-II-a, S-WPLE-II-b, S-WPLE-III-a and S-WPLE-III-b also consisted of the same structural features as the higher Mw fractions; thus the lower Mw fractions could have their own activities. But when present in the native molecule, these features might have a modulating effect on the activity.

A polysaccharide receptor has been found on human macrophages, which has demonstrated high specificity for Glc and Man (Lombard *et al.*, 1994), thus mushroom polysaccharides consisting of Glc and Man may have some antitumor action, this might also explain the antitumor activities of the polysaccharides isolated from *L. edodes* as observed in our investigation. However, other chemical structural features observed in our experiments, such as heteroglycan-protein, heteroglycans, β-glucan-protein, α-manno-β-glucan, hereto-β-glucan, may also justify the antitumor activities of the investigated polysaccharides (Gao *et al.*, 1996, Kawagishi *et al.*, 1990, Mizuno *et al.*, 1995a, Mizuno *et al.*, 1996, Mizuno & Zhuang, 1995, Zhuang *et al.*, 1993). Thus, it could be concluded that the foregoing differences in antitumor activities among these various polysaccharides are probably due to their different monosaccharide content and M*w*. Moreover, our results suggested that the polysaccharides extracted from *L. edodes* fruiting bodies exhibited higher inhibitory effect against the proliferation of suspended S-180 tumor cells compared with that of adherent HT-29 and HCT-116 tumor cells, indicating that the antitumor effects of them *in vitro* were depends on cancer cell lines. Therefore, on the basis of our results, it was concluded that the seven polysaccharides fractions might explore as potent tumor cell growth inhibitors which selectively higher antitumor activities against suspended cells than adherent ones (Huang *et al.*, 2007, Jin *et al.*, 2003).

Table 1: Monosaccharide composition, yield, total sugar, protein content and M*w* of polysaccharides fractions from *Lentinus edodes*

Fractions	Yield (%)	Monosaccharide composition (%)							Total Sugar (%)	Protein content (%)	M*w* (kDa)
		Glc	Gal	Man	Fuc	GlcA	Rha	Xyl			
S-WPLE	86.0[a]	48.3	24.0	20.1	2.8	2.0	2.5	0.2			
I-WPLE	13.4[a]	91.2	3.1	5.7	-	-	-	-	ND	ND	ND
S-WPLE-I-a	3.3[b] 38.7[c]	97.5	1.6	0.9	-	-	-	-	82.0	5.5	>2000
S-WPLE-I-b	4.7[b] 58.0[c]	90.6	5.1	4.2	-	-	-	-	99.0	4.6	755.32
S-WPLE-II-a	11.6[b] 35.8$_c$	88.3	4.2	7.5	-	-	-	-	85.8	5.5	121.5
S-WPLE-II-b	18.7[b] 57.7[c]	73.7	15.8	10.5	-	-	-	-	88.0	4.3	23.60
S-WPLE-III-a	21.3[b] 52.6[c]	78.7	14.2	7.0	-	-	-	-	85.1	5.3	15.22
S-WPLE-III-b	6.1[b] 23.4[c]	70.4	14.2	11.1	2.8	1.4	0.3	-	90.8	3.5	7.48

Not detected ND, not determined [a]Yield in relation to the amount of WPLE. [b]Yield in relation to the amount applied onto Sepharose CL-6B preparative column. [c]Yield in relation to the amount applied to analytical columns.

Table 2 Effects of the polysaccharide fractions from *L. edodes* on cell proliferation.

Cell lines	Samples	Proliferation ratio (%)
S-180	Control	100±0.38
	S-WPLE	55±0.48**
	S-WPLE-I-a	10±0.75**
	S-WPLE-I-b	4±0.53**
	S-WPLE-II-a	57±0.86**
	S-WPLE-II-b	89±0.48
	S-WPLE-III-a	79±0.75*
	S-WPLE-III-b	83±0.85
HT-29	Control	100±0.35
	S-WPLE	69±1.22**
	S-WPLE-I-a	68±0.75**
	S-WPLE-I-b	70±0.36**
	S-WPLE-II-a	77±0.56*
	S-WPLE-II-b	99±0.29
	S-WPLE-III-a	102±0.79
	S-WPLE-III-b	99±0.52
HCT-116	Control	100±0.75
	S-WPLE	85±1.26
	S-WPLE-I-a	83±0.51
	S-WPLE-I-b	60±0.39**
	S-WPLE-II-a	73±0.85*
	S-WPLE-II-b	84±0.46
	S-WPLE-III-a	87±0.71
	S-WPLE-III-b	92±0.65

Values are means ± S.D. *P<0.05, **P<0.01 vs. control.

CONCLUSION

In conclusion, six homogeneous polysaccharides S-WPLE-I-a, S-WPLE-I-b, S-WPLE-II-a, S-WPLE-II-b, S-WPLE-III-a and S-WPLE-III-b were successfully fractionated from fruiting bodies of *L. edodes*. All the polysaccharides exhibited inhibition against the proliferation of S-180, HCT-116 and HT-29 cells *in vitro*. The antitumor activities of these polysaccharides *in vitro* seemed to correlate positively with their M*w* and

monosaccharide content. Meanwhile, the relatively lower presence of bound protein Gal and Man could not be negligible. Our results provided essential data for a better understanding of *Lentinus edodes* polysaccharides.

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