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Immunoactivities and antineoplastic activities of *Saccharomyces cerevisiae* mannoprotein

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ABSTRACT

The alkaline extract *Saccharomyces cerevisiae* mannoprotein (ASMP), hot water extract *S. cerevisiae* mannoprotein (WSMP) and deproteinized water extract *S. cerevisiae* mannoprotein (DWSMP) showed significant immunomodulatory activities in this study. Effect of each polysaccharide on the proliferation of T lymphocytes induced by Con A was measured in *vitro*, and the lymphocyte proliferation induced by WSMP and DWSMP were significantly increased compared with Con A group ($p < 0.01$). The polysaccharide ASMP, WSMP and DWSMP could stimulate T cell-mediate immunity through delayed-type hypersensitivity, and the phagocytic ratio was significantly increased by WSMP and DWSMP ($p < 0.05$). Based on these results, the ASMP, WSMP and DWSMP suggested as the supplement for immune enhancement. The inhibition ratio of HepG2, HL-60 and Eca109 cells was observed and compared with control, the result indicated that hot water extract mannoprotein isolated from *S. cerevisiae* could be considered as an effective natural antitumor source, but alkali extract mannoprotein had not found distinct inhibition activity.

1. Introduction

Mannose polymers, or mannan, are found only as part of a glycoconjugate in *Saccharomyces cerevisiae*, and not as an unconjugated oligosaccharide. Because of this, the terms "mannan" and "mannoprotein" have often been used interchangeably in the *S. cerevisiae* literature. However, to maintain clarity, in this review "mannan" refers to the glycan itself while mannoprotein refers to glycoprotein (James, 2004). The outer layer of the yeast cell wall is composed of *S. cerevisiae* mannoprotein and occupied about 35–45% of the cell wall. *S. cerevisiae* mannoprotein could not only provide cells with rigidity that protects them from osmotic pressure but also help to maintain their shapes, which allows them to change their shapes in keeping with the cell cycle stage (Ballou, 1990; Inoue et al., 1995). Mannan is generally linked to protein through covalent bond and is composed of 5–20% protein and 80–90% mannose, with the molecular weight from 20,000 to 200,000 (Izabela et al., 1974).

In the past decades, mannoprotein has often been isolated by an acid–alkaline method, refined by Roelofsen and Hoette (1951), Miller and Phaff (1958), Silvia, Samuel, and Jose (1992) and Franziskus and Kulicke (1999). Recently, water extraction method was used as a non-degrading isolation process for mannoprotein reported by Young, Haavik, Smestad (1996), Stefan, Martin, and Othmar (1996) and Ližič árová, Matulová & Capek (2007).

The mannoprotein of *S. cerevisiae* has emulsifying properties, which could be of commercial significance. Besides, mannoprotein was found to be the most significant of the immunological effects, which were shown to be potent inducers of cellular and humoral immunity (Pontón et al., 2001). Moreover, it could also balance the enterobacteria, combine the extrinsic pathogen, defence the ray, and act as the antineoplastic and anti-oxidant.

The bioactivities of mannoprotein were affected significantly by structural features and molecular weight (Young et al., 1998; Ližič árová et al., 2007), and the structural features were affected by the extract method. Therefore, the bioactivities of mannoprotein extracted from different methods were compared. Besides, the compares of mannan and mannoprotein's bioactivities were also investigated in this study.

2. Materials and methods

2.1. Materials

Spent brewer' s yeast slurry (a strain of *S. cerevisiae*), a by-product from brewery with a solids content of about 18%, was kindly provided by Lvxian Biology and Technology Co., Ltd., Hebei, China.

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Eca-109 purchased from Shang Hai cell bank of Chinese Academy of Sciences, HepG2 and HL60 were kindly provided by Institute of Medicinal Plant.

Healthy BALB/c mice with equal number of male and female, 6–8 weeks, 18–22 g BW, were divided into five groups, 10 mice in one group, purchased from Wei Tong Li Hua experimental animal centre, certification SCXK (Jing) 2002–2003.

2.2. Alkaline extraction of S. cerevisiae mannoprotein

Spent brewer' s yeasts (20 g dry weight) were first sieved (mesh diameter 125 mm), then the yeast suspension was centrifugated at 4500 *g* (Sorvall RC-3B, USA) for 10 min, followed by re-suspension of the deposition in sterile distilled water. The procedure was repeated 5–6 times until the supernatant was clear, then the yeast was purified. After purifying, 1 L of 2% NaOH (w/v) was added to the cell wall sediment. This was placed in a boiling water bath and agitated at 150 rpm/min for 2 h. The preparation was centrifuged and the supernatant was collected. The residue was washed with little deionized water and combined with supernatant extracts. After that, the pH was adjusted to 6.5 with 10% acetic acid, and the supernatant was concentrated to one fifth of the original volume, triple absolute ethyl alcohol were added to precipitate mannan. The precipitated mannoprotein was dissolved in water and centrifuged, the supernatant was precipitated again by the addition of triple ethanol and recentrifuged. The resultant white sediment was washed twice with absolute ethanol and once with ether, then dried at 70 °C (Franziskus & Werner, 1999).

2.3. Hot water extraction of S. cerevisiae mannoprotein

Hot water extraction of mannoprotein from *S. cerevisiae* was carried out using the method of Liu, Wang, Cui, and Liu (2008) and Liu, Wang, Liu, and Tan (2008). Spent brewer' s yeasts were first sieved (mesh diameter 125 mm) and purified, then stored at 4 °C until used. The spent yeast cells slurry (adjusted to 15% w/w solids content and pH 5.0) was added 3% sodium chloride as the autolysis promoter, then incubated at 55 °C for 24 h with agitation at 120 rpm/min. The autolysate was centrifuged at 4500 × *g* for 10 min to separate the residual autolyzed cells from yeast extractions at room temperature. The autolyzed yeast cells were adjusted to 10% w/w solids with about 50 g of glass beads (diameter 3–4 mm) in a 500 ml conical flask. The suspension was heated to 121 °C in an autoclave (YXQ-LS-30S11, China) for 4 h and then cooled to 45 °C, centrifuged at 4500 × *g* for 10 min, the supernatant was collected. The residues were washed with distilled water and then re-centrifuged (4500 × *g*, 10 min), the supernatant was mixed with the supernatant before and concentrated to one fifth of the original volume. After that, triple absolute alcohol were added to the water extract and kept at 4 °C for one night. The precipitated mannoprotein was centrifuged at 8000 × *g* for 10 min, the supernatant liquid was discarded and the sediment was dissolved in water.

2.4. Deproteinization of hot water extract mannoprotein

WSMP was dissolved in phosphate buffer (pH 7.0) to the concentration of 15% (w/w), papain was added to hydrolyze the mannoprotein at 55 °C for 5 h, and then pronase was used to hydrolyze the mannoprotein at pH 7.2, 37 °C for 48 h, after inactivation the enzyme, dialysis 2 days, deproteinized water extract *S. cerevisiae* mannoprotein (DWSMP) was obtained by freeze-dried.

2.5. Gel filtration chromatogram

The mannoprotein was analyzed by gel filtration chromatogram to determine if the protein is linked to polysaccharide, the effect of enzymolysis was investigated at the same time. For this purpose, Sepharose CL-4B was used as material, deionized water containing 200 ppm NaN_3 was used as the eluent (Li, Xiao, Yu & Yuan, 1994; Zhan, 2006).

2.6. Analytical methods

2.6.1. Measurement of total nitrogen

Total nitrogen was measured by a Kjeldahl analyzer (Foss-2300 Kjeltec, Sweden). Crude protein content was calculated by the total nitrogen multiplying by 6.25. Solution protein content was determined according to the procedure of Lowry as had been described by Peterson (1977).

2.6.2. Measurement of fat and ash

Total fat and ash content were determined using AOAC Official Methods (AOAC, 1990). Fat was determined by a soxhlet extraction analyzer (Foss-2050 Soxtec, Sweden) using petroleum ether as the organic solvent. Ash was determined by incinerating dried samples at 600 °C about 5 h in a furnace (Lindberg/Blue M, USA).

2.6.3. Measurement of total carbohydrate

Total carbohydrate was measured by the phenol–sulfuric acid method with 6.0% (w/v) phenol and assayed at 490 nm (Dubois et al., 1956).

2.6.4. Measurement of β-glucan and mannan

Polysaccharides (20 mg) were wetted with 100 μl of 72% (w/w) H_2SO_4 and left at room temperature for 3 h. The slurry was diluted to 1 ml to a final concentration of 2 N-H_2SO_4 and heated in sealed tubes for 4 h at 100 °C. Sulfate ions were neutralized by drop-wise addition of NaOH until neutral pH was reached (checked with pH paper). The volume was adjusted to 100 ml with phosphate buffer pH 7.0 (Dallies, Francois, & Paquet, 1998). The monosaccharide was analyzed by GOPOD and method of spectrophotometer (Liu, Wang, & Liu, 2007; Zhang & Gu, 1999).

2.6.5. Measurement of molecular weight

The molecular *weight* was determined with a GPC-LLS (Gel Permeation Chromatography-Laser Light Scatter) apparatus available in the research group (Wang, Xie, & Liu, 2005; Zhao, Miao, Fan, & Guan, 2000).

2.7. In vivo immunomodulating activity

Female healthy Kunming mice (6-week-old) were used in this study. The mice were housed under normal laboratory conditions (21 ± 2 °C, 12/2 h light–dark cycle) with free access to standard rodent chew and water. After 7-day equilibration, mice were divided into five groups (n = 10). The control group was administered orally with sterilized saline. The second group, used as positive control, was administered orally with 20 mg/kg/day lentinan. The third group, administered orally with ASMP 50 mg/kg/day and 100 mg/kg/day. The fourth group, administered orally with WSMP 50 mg/kg/day and 100 mg/kg/day. The fifth group, administered orally with DWSMP 50 mg/kg/day and 100 mg/kg/day. Samples were administrated for consecutive two weeks. In the fifteenth day the following test were carried out.

2.8. MTT assay of lymphocyte proliferation induced by Con A

Mice splencytes (1×10^6 cell/well) were stimulated with 1.0 mg/L Con A. The cells were plated into triplicate wells (200 μl/well) in 96-well flat bottom tissue culture plates and co-cultured with samples at indicated concentrations. Serum free RPMI media 1640 was used as control. Following 68 h incubation at

Table 1
Composition analysis of *S. cerevisiae* mannoprotein.

	Yeast	YCW	ASMP	WSMP	DWSMP
Dry weight	100	23.52 ± 0.6	5.93 ± 0.13	6.42 ± 0.12	5.24 ± 0.07
Carbonhydrate	35.09 ± 1.85	85.23 ± 0.82	89.32 ± 2.78	87.43 ± 1.85	92.28 ± 1.98
Mannan	10.43 ± 0.37	35.41 ± 1.46	88.24 ± 2.89	81.32 ± 2.53	88.74 ± 2.05
β-Glucan	15.74 ± 0.42	48.02 ± 2.23	4.91 ± 0.12	6.31 ± 0.78	4.26 ± 0.12
Protein	51.42 ± 2.53	9.39 ± 0.35	3.62 ± 0.07	10.92 ± 0.13	1.1 ± 0.05
Fat	5.21 ± 0.12	4.32 ± 0.09	Trace	Trace	Trace
Ash	6.54 ± 0.25	3.21 ± 0.13	8.80 ± 0.05	2.91 ± 0.16	Trace

37 °C and 5% CO_2, the cell proliferation was measured by MTT assay. The absorbance at 570 nm test wavelength was translated into lymphocyte proliferation ratio for comparison (Li, Chen, Wang, Tian, & Zhang, 2009; Na et al., 2010; Sun et al., 2008; Wong, Lai & Cheung, 2010).

$$\text{Lymphocyte proliferation ratio} = \frac{A_{\text{test}}}{A_{\text{control}}} \times 100\%.$$

2.9. Delayed hypersensitivity response (DTH) measurement

Sheep red blood cell (SRBC) 0.2 ml 2% (v/v) was administrated i.p on the seventh day. 20 μl 2% SRBC was injected to right foot on the 10th day. 24 h later, increase of the right foot swelling was calculated respectively (Ruan, Su, Dai & Wu, 2005).

2.10. Peritoneal macrophage phagocytosis test

SRBC 0.2 ml 2% (v/v) was injected to the enterocoelia of the mice at day 4 to activate the macrophages. After putting the mice to death, 4 ml Hank's (included 10% calf serum) was injected to the enterocoelia of each mice to wash the macrophages out. 0.5 ml peritoneal macrophage (PMΦ) liquid was sucked into syringe to mix with 0.5 ml 1% chiken red blood cell, 2–3 drops were added to the carrying glass plate. Enabling PMΦ adhesion by culturing at 37 °C for 15 min. After flushing gently the non-adhesion cells off with saline, PMΦ was stained for 2–3 min after fixed with methanol and dyed with Giemsa for 15 min. The number of total PMΦ, phagocytic PMΦ under microscope were counted. The phagocytic ratio was calculated as below (Liu, Wang, Cui, et al., 2008; Liu, Wang, Liu, et al., 2008; Liu, Xi, et al., 2008).

$$\text{Phagocytic ratio} = \left(\frac{\text{Numbers of phagocytic PM}\Phi}{\text{Total numbers of PM}\Phi}\right) \times 100\%$$

2.11. In vitro antineoplastic activity

The cancer cells were collected and dissolved in Roswell Park Memorial Institute-1640 (RPMI-1640) included 10% serum bovine calf to make 1×10^4/ml cells suspension, inoculated in 96 pore plates, and 100 μl to each pore. After culturing at 37 °C, 5% CO_2 for 24 h, ASMP WSMP DWSMP were added and cultivated for 3 days, then the supernatant was threw away and 10 μl MTT was added to each pore and cultivated in RPMI media 1640 for 4 h, then 100 μl complete cell lysis solution was injected to each pore. After reacting for 24 h, the absorbance (A) at 450 nm test wavelength was measured by ELIASA (Bio-Tek MQX200). The *vitro* results were expressed as the inhibition ratio of tumor cell proliferation, calculated as

$$\left[\frac{(A-B)}{A}\right] \times 100\%;$$

where A and B are the average numbers of viable tumor cells of the control and the samples, respectively (Peng, Zhang, Zeng & Xu, 2003; Tao, Zhang & Cheung, 2006).

2.11.1. Statistical analysis

Statistical analysis was conducted using the SAS program. The results were presented as mean values ± standard deviation of at least four experiments. Paired t-test (levels of significance, 0.01 or 0.05) was used to evaluate the statistical significance of differences with $p < 0.01$ or 0.05 which were considered statistically significant.

3. Results and discussion

3.1. Yeast and mannoprotein compositions analysis

The bioactivities of polysaccharides may be affected by extract method, molecular weight, monosaccharide components. Therefore, the composition of crude ASMP WSMP and DWSMP are compared and presented in Table 1. Yeast cell wall (YCW) occupied 23.52% of the cell. The yield and the purity of the ASMP were 5.93 ± 0.13% and 88.24 ± 2.89%, respectively. The content of protein was 3.62 ± 0.07%. The yield, purity and the protein content of the hot water extract mannans were 6.42 ± 0.12%, 81.32 ± 2.53% and 10.92 ± 0.13%, respectively. After deproteinating by enzyme, the content of protein decreased to 1.1 ± 0.05% and the final purity of DWSMP was 88.74 ± 2.05%.

Sepharose CL-4B was used to analysis the crude mannoprotein, deionized water containing 200 ppm NaN_3 was used as the eluent in order to protect the apparatus against microbial attack. Results are shown in Fig. 1. From "a" and "b" we can see, the protein in ASMP and WSMP was disparted to two parts, the second part was overlapped to the polysaccharide, it was concluded that the protein related to the second part linked to the polysaccharide. The eluents to the second peak were collected and free-dried, and the polysaccharide content were 94.3% and 92.60% respectively, while the protein content were 1.89% and 5.20% respectively. From "c" we can see both the free protein and the protein linked to polysaccharide could be removed by enzyme.

3.2. Molecular weight of S. cerevisiae mannoprotein

The *Mw* of ASMP, WSMP and DWSMP were determined to 65,098, 181,127 and 139,039, respectively. Compared to the reports, chemically extracted mannoprotein had molar masses of 21,000–62,000, whereas enzymatically isolated mannoprotein had molar masses between 64,000 and 112,000 (Franziskus & Kulicke, 1999), so we can conclude that the hot water extract method not only protected mannoprotein from degradation by acid or alkali, but also maximally preserved the original structure of mannoprotein.

3.3. MTT assay of lymphocyte proliferation induced by Con A

Lymphocyte proliferation induced by Con A may be used as a method to detect T lymphocyte immunity *in vitro*. Fig. 2 shows A_{570} nm value induced by each fraction in different concentration (suspended with complete RPMI-1640). The lymphocyte proliferation induced by WSMP and DWSMP *in vitro* was significantly increased compared with Con A group ($p < 0.01$). As compared with lenti-

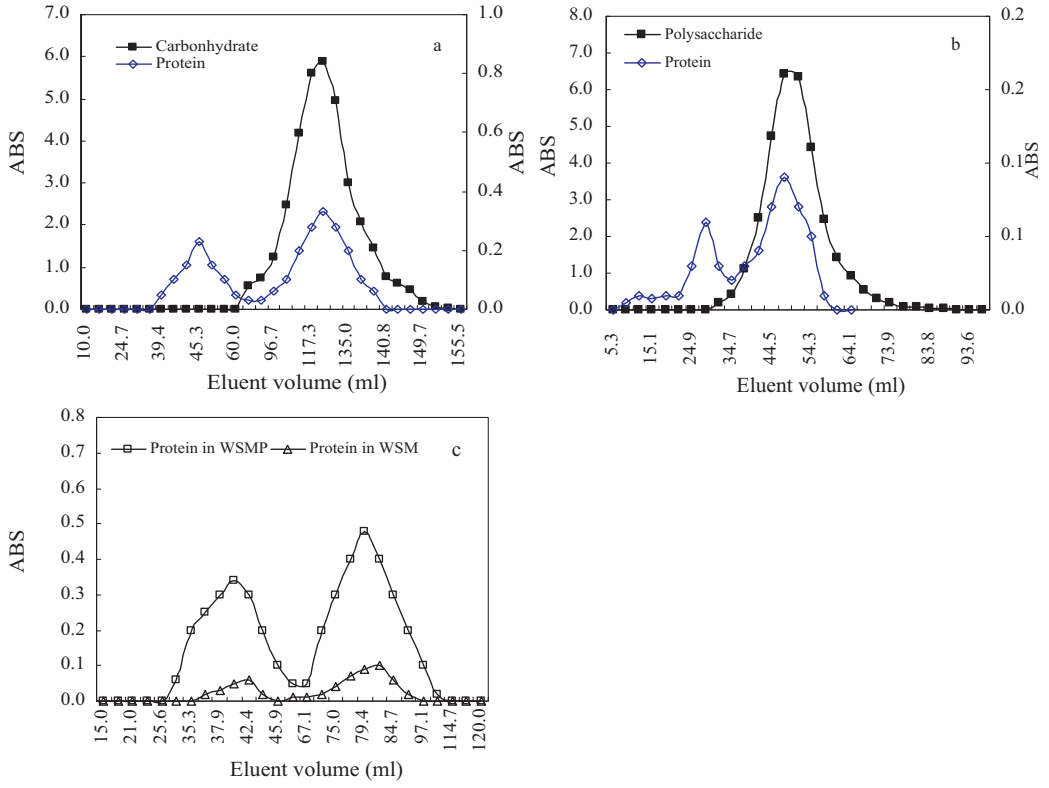

Fig. 1. The gelatin filtration chromatogram of *S. cerevisiae* mannoprotein. (a) The gel filtration chromatogram of ASMP. (b) The gel filtration chromatogram of WSMP. (c) The protein curve of DWSMP examined by gel filtration chromatogram compared to DWSMP.

nan, which is a well-known medicinal herb, WSMP and DWSMP showed similar stimulatory effects on cellular and non-specific immunity by oral administration in mice treated with immunosuppressive agent. Besides, the lymphocyte proliferation induced by ASMP (100 mg/ml) was also significantly increased compared with Con A group ($p < 0.05$).

3.4. Delayed hypersensitivity response

A delayed-type hypersensitivity reaction was an expression of cell-mediated immunity and played a main role in many inflammatory disorders (Huang & Ning, 2010). Results of delayed hypersensitivity reaction are showed in Fig. 3, which expressed

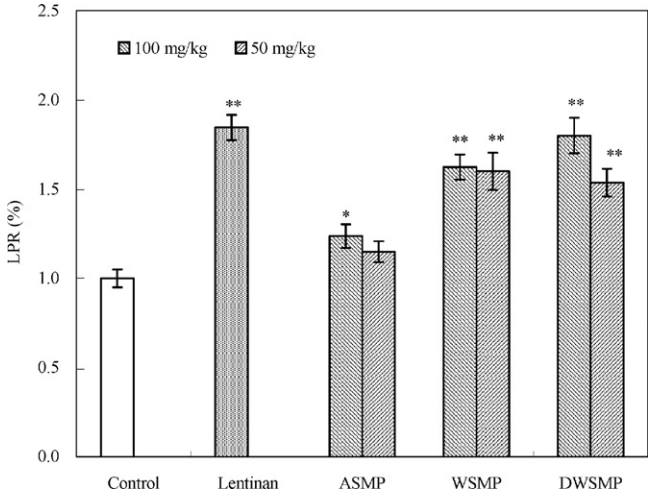

Fig. 2. The splenic lymphocytes proliferation induced by *S. cerevisiae* mannanprotein with ConA.

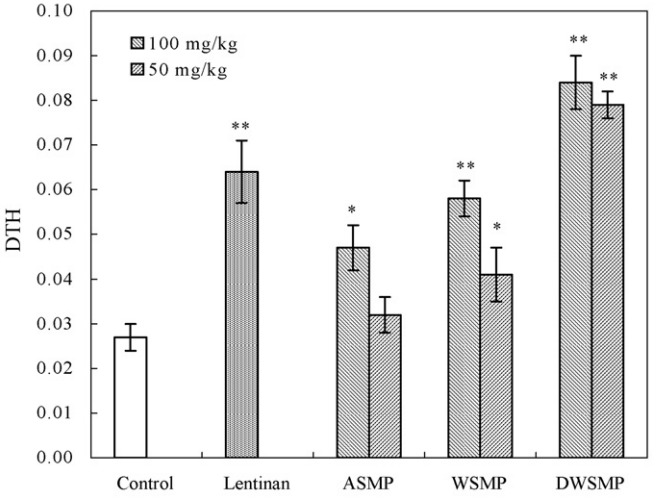

Fig. 3. Effect of *S. cerevisiae* mannoprotein on SRBC induced footpad swelling in mice.

Table 2
Effects of *S. cerevisiae* mannoprotein on peritoneal macrophage phagocytosis.

Group	Dose (mg/kg/day)	Phagocytic ratio (%)
Blank control	–	11.75 ± 5.12
Lentinan	0.02	32.25 ± 13.87**
ASMP	100	16.25 ± 2.75
	50	15.00 ± 2.45
WSMP	100	15.50 ± 2.38
	50	18.80 ± 5.71*
DWSMP	100	20.25 ± 4.64*
	50	18.50 ± 1.29*

* $p < 0.05$.
** $p < 0.01$ vs control.

Table 3
The antineoplastic activity of *S. cerevisiae* mannoprotein.

Concentration (mg/ml)	Inhibition ratio of HepG2		]Inhibition ratio of HL-60		Inhibition ratio of Eca109	
	WSMP	DWSMP	WSMP	DWSMP	WSMP	DWSMP
0.25	14.59 ± 1.70^{c}	17.60 ± 0.47^{b}	9.15 ± 0.97^{d}	8.03 ± 0.42^{c}	9.78 ± 0.57^{d}	6.90 ± 0.42^{d}
0.50	17.13 ± 2.06^{c}	13.54 ± 0.98^{b}	35.94 ± 0.97^{c}	24.85 ± 2.55^{b}	18.25 ± 1.98^{c}	17.17 ± 1.55^{c}
1.00	25.26 ± 1.97^{b}	15.68 ± 1.54^{b}	46.40 ± 1.27^{b}	28.84 ± 1.97^{b}	24.49 ± 0.54^{b}	21.18 ± 1.97^{b}
2.00	33.58 ± 1.27^{a}	30.18 ± 1.26^{a}	55.05 ± 0.83^{a}	36.64 ± 1.81^{a}	34.03 ± 1.26^{a}	31.25 ± 0.81^{a}

[a–d] Mean values within the same row bearing different superscripts differ significantly ($p < 0.05$).

the difference in thickness of right footpad swelling. After the administration of the polysaccharide ASMP, WSMP and DWSMP, a significant increase of swelling thickness difference in polysaccharide groups was found, compared with model control group. Therefore, the polysaccharide ASMP, WSMP and DWSMP could stimulate T cell-mediate immunity through delayed-type hypersensitivity, the effects was DWSMP > WSMP > ASMP.

3.5. Peritoneal macrophage phagocytosis

Peritoneal macrophages play a pivotal role in non-specific immune modulation. Phagocytic function is commonly used in evaluating the non-specific immune status of animals. Activated macrophage is also considered to be one of the important components of the host defense against tumor growth (Liu, Han, & Bao, 2007; Liu, Xi, et al., 2008). The results (Table 2) showed the phagocytic ratio was significantly increased from $11.75 \pm 5.12\%$ to $20.25 \pm 4.64\%$ or $18.50 \pm 1.29\%$ separately by 100 mg/kg/day or 50 mg/kg/day of DWSMP ($p < 0.05$). Besides, the phagocytic ratio was significantly increased to $18.80 \pm 5.71\%$ by 50 mg/kg/day WSMP ($p < 0.05$). The phagocytic ratio could not be increased by ASMP. From above we can conclude that the mice immunity activity could be increased to higher level by WSMP and DWSMP, but the effects were lower than lentinan.

3.6. In vitro antineoplastic activity

It has been reported that water soluble polysaccharides exhibiting antitumor activity contained the backbone with either β-(1–3)-linked D-glucose or α-(1–4)-linked D-mannan (Peng, Zhang, Zeng & Kennedy, 2005). The mannan and the protein-bound polysaccharides played an important role in the enhancement of antitumor activity both in *vivo* and in *vitro* (Peng et al., 2003). Many factors affected the activities of polysaccharide fractions, the antitumor activities of high molecular weight polysaccharides were considered to be a consequence of stimulating from the immune response in the host, rather than direct killing tumor cells, as reported by Wasser (2002) and Zjawiony and Univ (2004). In general, mannans were found to have different in *vivo* and in *vitro* antitumor activities, depending on their monosaccharide composition, protein content, molecular mass and chain conformation (Bland, Keshavarz, & Bucke, 2004). However, the antitumor activities of mannans obtained from different extract methods and the mannan or protein which played the important role had not been reported. Therefore, the antitumor activities of ASMP WSMP, and DWSMP were compared in this study.

After incubated with ASMP WSMP, DWSMP for 24 h at the concentrations of 0.25, 1.50, 1.00, 2.00 mg/ml, the inhibition ratio of HepG2, HL-60 and Eca109 cells was observed and compared with control. The results indicated that hot water extract mannoprotein isolated from *S. cerevisiae* could be considered as an effective natural antitumor source, but alkali extract mannoprotein had not been found distinct inhibition activity. Table 3 shows the growth of HepG2, HL-60 and Eca109 cells were significantly inhibited by WSMP and DWSMP at the concentrations from 0.25 to 2.00 mg/ml. WSMP exhibited significantly higher inhibition ratios than DWSMP. Especially, at the WSMP concentration of 2.00 mg/ml, the inhibition activity of HepG2, HL-60 and Eca109 was the highest with an inhibition ratio beyond $33.57 \pm 1.27\%$, $55.05 \pm 0.83\%$ and $34.02 \pm 1.26\%$, respectively. The foregoing differences in antitumor activity among the various polysaccharide fractions were probably due to their different molecular mass, originating from the different exactions.

4. Conclusions

ASMP, WSMP and DWSMP were obtained and the *Mw* were determined to be 65,098, 181,127 and 139,039, respectively. Effect of each polysaccharide on the proliferation of T lymphocytes induced by Con A was measured, and the lymphocyte proliferation induced by WSMP and DWSMP was significantly increased compared with Con A group ($p < 0.01$), which were better than the effect of ASMP. The polysaccharide ASMP, WSMP and DWSMP could stimulate T cell-mediate immunity through delayed-type hypersensitivity, the effects was DWSMP > WSMP > ASMP. The phagocytic ratio was significantly increased by DWSMP and WSMP ($p < 0.05$), but could not be increased by ASMP. From above we can conclude that the mice immunity activity could be increased to higher level by WSMP and DWSMP, but the effects were lower than lentinan. The growth of HepG2, HL-60 and Eca109 cells were significantly inhibited by WSMP and DWSMP, but alkali extract mannoprotein had not been found distinct inhibition activity. Therefore, hot water extract mannoprotein isolated from *S. cerevisiae* could be considered as an effective natural antitumor source. These findings suggest the water-soluble polysaccharide in *S. cerevisiae* is a new example of natural biological response modifier and antitumor source in food, further investigations on the relationship between structure and activity of this are in progress.

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