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Structure and antitumor activity of extracellular polysaccharides from mycelium

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Abstract

Two heteropolysaccharides coded as EPF1 and EPF2 were obtained from the crude extracellular polysaccharide (CEP) of *Ganoderma tsugae* mycelium by elution with DEAE-Sepharose CL-6B column. Their chemical structures and molecular mass were characterized by infrared, gas chromatography, ^{13}C NMR and size exclusion chromatography combined with laser light scattering (SEC-LLS). The results indicated that they were mainly composed with mannose, fucose, xylose, galactose and glucosamine. EPF2 was confirmed to be mainly a β-D-galacto-α-D-mannan. The weight-average molecular mass of the samples EPF1, EPF2 and CEP were 92.0×10^4, 8.35×10^4 and 23.8×10^4, respectively. The EPF1 existed as a compact coil chain in 0.2 M NaCl aqueous solution at 25 °C, while EPF2 as a flexible chain. The antitumor activities against Sarcoma 180 were tested both in vitro and in vivo. All the samples exhibited high inhibition ratio against Sarcoma 180 in mice, and the CEP has higher inhibition effect both in vivo and in vitro than EPF1 and EPF2. In view of these results, the presence of bound protein and mannose, and relatively expanded chain would be helpful to the enhancement of antitumor activities. This study suggested that the extracellular polysaccharides had a potential application as natural antitumor drugs.

Keywords: *Ganoderma tsugae* mycelium; Extracellular polysaccharide; Antitumor activity; Conformation; Light scattering

1. Introduction

Ganoderma tsugae, one of the most famous traditional Chinese medicines, has recently attracted much attention on account of its biological activities (Shiao, Lee, Lin, & Wang, 1994; Su, Shiao, & Wang, 1999; Wu, Shi, & Kuo, 2001). Among the biologically active components, the polysaccharides, protein-bound polysaccharides and their derivatives have been demonstrated to be responsible for its antitumor activities, which are based on enhancing the host mediated mechanism (Mizuno, Suuzki, Maki, & Tamaki, 1985; Seong, Young, Chong, & Seong, 2000). Though most of the antitumor polysaccharides have the basic β-(1 → 3)-D-glucan structure, such as the *Lentinan* from *Lentinus edodes* (Chihara, Maeda, Hamuro, Sasaki, & Fukuoka, 1969) and *Pachyman* from *Poria Cocos* (Humuro, Yamashita, Ohsaka, Maeda, & Chihara, 1971), a α-manno-glucan (Tanigami, Kusumoto, Nagao, & Kokeguchi, 1991) and α-glucan-protein (Mizuno, Sation, Nishitoba, & Kawagishi, 1995) have been proved to exhibit antitumor activities. Except for the knowledge of physicochemical properties, the secondary structure of polysaccharides is also very important for further interpretation of their antitumor activities (Adachi, Ohno, Ohsawa, Oikawa, & Yadomae, 1990; Kulicke, Lettau, & Thielking, 1997).

It has been reported that the polysaccharides isolated from the mycelium of *G. tsugae* have the same antitumor activity as those from the fruiting bodies (Wang et al., 1993; Zhang et al., 1994). Many attempts are being made to obtain polysaccharides from mycelia through submerged fermentation culture (Fang & Zhong, 2002). Sone, Okuda, Wada, Kishida, and Misaki (1985) have isolated extracellular polysaccharides (0.85 g per liter of cultural fluid) from the cultured filtrated of *G. lucidum mycelium*, then two water-soluble polysaccharides, namely galacglucomannan and mannan are isolated, and the mannan consists of a (1 → 6)-linked α-D-mannan backbone and side chains of (1 → 2)-linked D-mannosyl residues. However, no antitumor

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activities have been reported. Except this, little attention has been given to the high-yielding extracellular polysaccharides. In this work, we tried to investigate the chemical structures, molecular mass and antitumor activities of the water-soluble extracellular polysaccharides from the culture filtrate of *G. tsugae* mycelium.

2. Experimental

2.1. Materials

The strain of *G. tsugae* was supplied by the Laboratory of Applied Mycology in Central China Agriculture University. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St Louis, MO). All other reagents were of the highest available quality in China.

2.2. General methods

The ^{13}C NMR spectrum was recorded in D_2O on an INOVA-500 spectrometer (Varian Inc., USA) with 125 MHz at 25 °C, using internal acetone (δ 31.45) as reference. The N element content was measured by an elemental analyzer (Heraeus Co., Germany). Infrared (IR) spectroscopy of the samples was recorded on a Nicolet 170SX FTIR (Perkin Elmer Co., USA) in the range of 400–4000 cm^{-1} with DGTS detector and DMNIC 3.2 software. Gas chromatography (GC) of the alditol acetates derivatives of monosaccharides according to the literature (Englyst, Quigley, & Hudson, 1994) was done on a HP 6890 instrument (Hewlett Packard, USA) with an DB-225 column (15 m × 0.25 mm), and at temperatures programmed from 180 to 220 °C at 4 °C/min.

2.3. Isolation and purification of the extracellular polysaccharides

The strain of *G. tsugae* was preserved on the Potato-sucrose-agar (APS) slopes. It was inoculated in a liquid medium containing 15% glucose, 1% peptone, 1% yeast extract, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.05% $MgSO_4$. The submerged cultures were grown in 250 ml Erlenmeyer flask containing 100 ml infusion medium. The flasks were incubated on a rotary shaker (HQL 150B, China) under the conditions of 100 rpm at 28 °C for 10 days. After removal of the mycelium by filtration, the culture filtrate was subjected to the Sevag method (Staub, 1965) to remove proteins for more than 10 times, and treated with 30% H_2O_2 to decolorize, then dialyzed against tap water for seven days and distilled water for three days. The resulting polysaccharide solution was concentrated by rotary evaporator at reduced pressure below 45 °C, and then lyophilized to obtain colorless powder coded as crude extracellular polysaccharide (CEP, yield: 0.5 g per liter of culture filtrate).

The sample CEP (25 mg) was dissolved in distilled water, and then injected to a column (50 × 2.0 cm^2) of DEAE-Sepharose CL-6B equilibrated with distilled water. After loading with sample the column was eluted with distilled water and different concentrations of NaCl aqueous solution (0.04, 0.1 M) stepwise at 45 ml/h and 1.50 ml per tube. Every other tube was assayed by phenol-sulfuric acid method for sugar contents (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The elution profile of the CEP on DEAE-Sepharose CL-6B column is shown in Fig. 1. Fractions eluted at 21–41, 164–224 and 338–369 ml were collected, dialyzed, concentrated and lyophilized to give the yield of EPF1 (0.63 mg), EPF2 (6.4 mg) and EPF3 (1.26 mg), respectively.

The protein-bound polysaccharides absorbed on the column were cleaned by elution with 2.0 M NaCl aqueous solution. The homogeneity of CEP fractions was determined by size exclusion chromatography (SEC) with a P100 pump equipped with TSK-GEL G6000PWXL and G4000PWXL column.

2.4. SEC-LLS measurements

Size exclusion chromatography combined with laser light scattering (SEC-LLS) measurements were performed with a multi-angle laser photometer (DAWN-DSP, Wyatt Technology Co., USA) at 633 nm combined with a P100 pump equipped with TSK-GEL G6000PWXL and G4000PWXL columns (7.8 mm × 300 mm) and differential refractive index detector (RI-150) at 25 °C. The eluent was 0.2M NaCl aqueous solution at a flow rate of 1.0 ml/min. All the solutions used were first filtered with a sand filter and then with a 0.20 μm filter (Wattman). Astra software was utilized for data acquisition and analysis. The refractive index increments (d*n*/d*c*) was determined by using a refractometer (OPTILAB-DSP, Wyatt Technology Co., USA) at 25 °C. The values of d*n*/d*c* at 633 nm obtained were 0.147, 0.140 and 0.141 ml g^{-1} for CEP, EPF1 and EPF2, respectively.

2.5. Assay of the antitumor activity

In vivo antitumor test. Sarcoma 180 cells (1×10^5 cells/mouse) were subcutaneously inoculated into 8-week-old BALB/c male mice. The samples CEP, EPF1 and EPF2 dissolved in phosphate buffer solution (PBS) were injected intraperitoneally once daily for 10 days at 24 h after tumor inoculation. The same volume of PBS was injected intraperitoneally into the control mice. The tumor was allowed to grown on the mice for 10 days before it was removed from the animal, and weighted. The antitumor activity of the tested samples was expressed as an inhibition ratio (percent) calculated as $[(A\text{-}B)/A] \times$

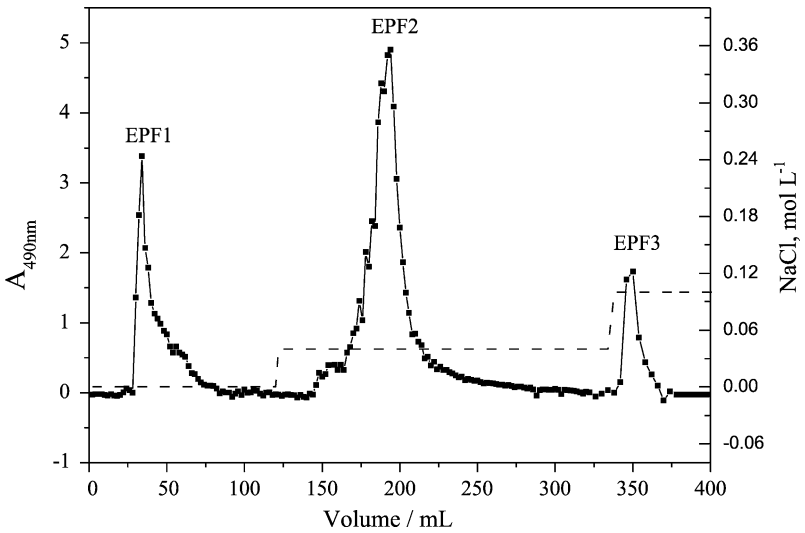

Fig. 1. The DEAE-Sepharose CL-6B elution profile of the crude extracellular polysaccharide from *G. tsugae* mycelium. The column was eluted stepwise with distilled water (EPF1, 21–41 ml), 0.04 M NaCl (EPF2, 164–224 ml) and 0.1 M NaCl aqueous solutions (EPF3, 338–369 ml). The dash line represents the concentration of NaCl aqueous solution.

100%, where *A* and *B* are the average tumor weights of the control and treated groups, respectively.

In vitro antitumor test. Sarcoma 180 cells (1×10^5 cells/ml) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum under an atmosphere of 5% carbon dioxide at 37 °C for 72 h containing tested samples at concentrations of 0.005, 0.05, 0.5 and 5 g/ml in PBS. The living Sarcoma 180 cells at the end of the 72 h incubation period was determined by a colorimetric assay based on the tetrazoliuma salt MTT as described by Mosmann (1983). The vitro results were expressed as the inhibition ratio of tumor cell proliferation calculated as $[(A\text{-}B)/A] \times 100\%$, where *A* and *B* are the average numbers of viable tumor cells of the control and the samples, respectively.

3. Results and discussion

3.1. Chemical structure

The SEC chromatograms of the fractions EPF1 and EPF2 are shown in Fig. 2, indicating two purified polysaccharide fractions were obtained. EPF3 was more complex and not investigated here. The protein content and monosaccharides compositions of crude and purified extracellular polysaccharides are listed in Table 1. Though the protein content of CEP was relatively high, it could be deemed to be protein-bound polysaccharide because the Sevag method has been repeated many times to remove proteins. Compared to CEP, protein was hardly found in EPF1 and EPF2, indicating

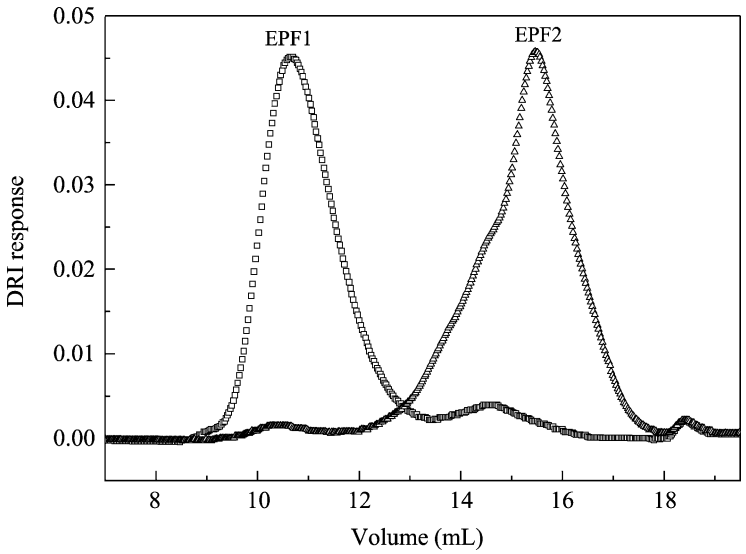

Fig. 2. The SEC chromatograms of EPF1 and EPF2 in 0.2 M NaCl aqueous solution at 25 °C.

Table 1
Components of monosaccharides and protein content of CEP fractions from the *G. tsugae* mycelium

Samples	Protein (%)	Sugar component (%)					
		Fuc	Xyl	Man	Gal	Glu	GlcNac
CEP	21.3	3.3	2.0	59.6	17.4	8.1	9.7
EPF1	nd	2.9	16.1	66.7	9.4	0.1	4.3
EPF2	nd	4.5	1.3	58.0	24.5	3.8	7.9

Abbreviation: nd, not detected; Fuc, Fucose; Xyl, Xylose; Man, Mannose; Gal, Galactose; Glc, Glucose; GlcNac, *N*-Acetyl-β-D-glucosamine.

the success of separating polysaccharides with protein bound polysaccharides with the DEAE-Sepharose CL-6B column. The monosaccharides compositions of EPF1 and EPF2 were very similar to that of the CEP on the whole. Namely, mannose existed as the main constituent, together with glucose, fucose, xylose, galactose and glucosamine.

Fig. 3 shows the IR spectra of EP fractions. In the anomeric region (950–700 cm^{-1}) both EPF1 and EPF2 exhibited the obvious characteristic absorption at 810 cm^{-1} corresponding to the existence of mannose (Mathlouthi & Koenig, 1986). EPF1 also exhibited the absorption at 920 and 850 cm^{-1}, typical for α configuration. While the obvious absorption peaks at 910 and 880 cm^{-1} in EPF2, revealing the co-existence of α and β configurations. The ^{13}C NMR spectrum of EPF2 is shown in Fig. 4. Interestingly, the chemical shifts of EPF2 were similar to that of the D-galacto-D-mannan from *T. lactis-condensi*, which has the (1 → 6)-linked α-D-mannopyranosyl mainchain and (1 → 2) linked α-D-mannopyranosyl sidechains with terminal β-D-galactopyranosyl units (Gorin & Mazurek, 1982). The signal at 105.1 ppm could be attributed to C-1 of β-D-galactopyranosyl groups and a C-5 signal at

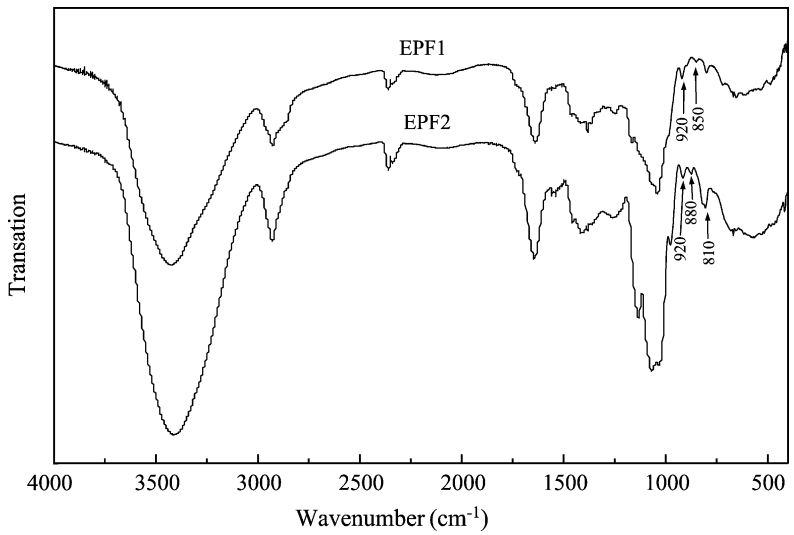

Fig. 3. The FTIR spectra of extracellular polysaccharide fractions from the *G. tsugae* mycelium.

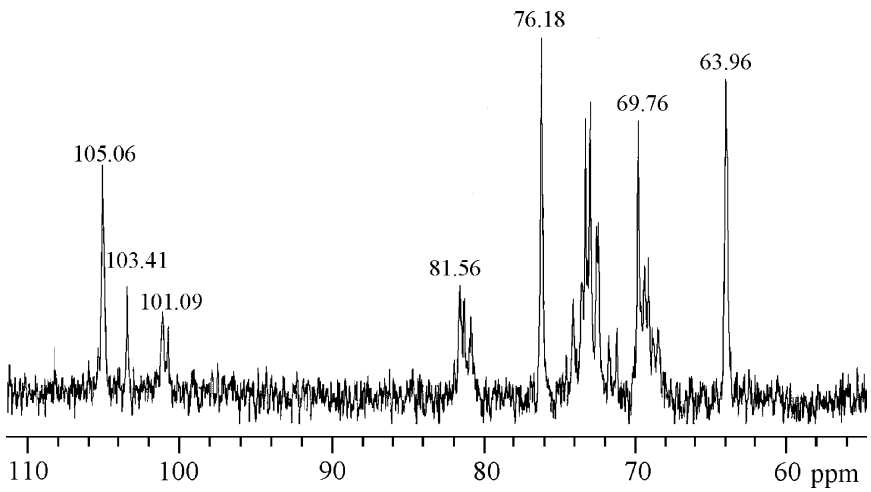

Fig. 4. ^{13}C NMR spectrum of EPF2 in D_2O at 25 °C.

Table 2
The experimental values of molecular mass and parameters of CEP fractions from the *G. tsugae* mycelium in 0.2 M NaCl aqueous solution at 25 °C

Samples	$M_w \times 10^{-4}$	$M_n \times 10^{-4}$	M_w/M_n	$\langle s^2\rangle_z^{1/2}$(nm)
CEP	23.8	2.83	8.2	40.5
EPF1	92.0	74.41	1.2	40.6
EPF2	8.35	3.53	2.4	38.7

76.2 ppm was also typical of β-D-galactopyranosyl residues. The C-1 signal at 103.4 ppm was assigned to a α-D-mannopyranosyl, non-reducing end-group linked (1 → 2) to an adjacent α-D-mannopyranosyl residue (Gorin, 1981). These results were also consistent with that of the mannan isolated from the cultural filtrate of *G. lucidum mycelium* (Sone et al., 1985).

3.2. Molecular mass and chain conformation

The weight-average molecular mass (M_w), number-average molecular mass (M_n), polydispersity index ($d = M_w/M_n$) and the radius of gyration ($\langle s^2\rangle_z^{1/2}$) of CEP fractions measured by SEC-LLS in 0.2 M NaCl aqueous solution at 25 °C are summarized in Table 2. EPF1 and EPF2 had much narrower molecular mass distribution than CEP. The molecular mass of EPF1 was 92×10^4, higher than that of CEP and EPF2. The log–log plots of $\langle s^2\rangle_z^{1/2} \propto M_w^{\alpha}$ are shown in Fig. 5. The values of slope (α) were 0.37 and 0.58 for EPF1 and EPF2, respectively. Usually, α is 0.5–0.6 for flexible polymer in good solvent, 0.2–0.4 for polymers with high degree of branching and 0.3 for globular shape (Majdoub et al., 2001). This indicated that EPF1 molecules existed as a compact coil chain in 0.2 M NaCl aqueous solution at 25 °C, while a slightly expanded coil chain for EPF2. The high molecular weight and compact chain conformation of EPF1 implied a highly branched structure (Zhang et al., 1992).

3.3. Antitumor activity

In vivo results. The results of antitumor activities of CEP, EPF1 and EPF2 against Sarcoma 180 tumor in mice are summarized in Table 3, which also contains the results obtained by 5-Fluorouracil (5-Fu) in parallel tests. Obvious antitumor activities were observed in all three samples, and CEP and EPF2 exhibited higher inhibition ratios than EPF1, and 5-Fu. It was worth noting that CEP and EPF2 showed equivalently high activities even at much lower dose level. Moreover, the enhancement ratios of body weight of the three samples were much more than that of 5-Fu group, especially for CEP at a dose of 32 mg/kg. It implied that the CEP fractions did not have the same toxicity as 5-Fu, which kills normal cells as well as cancer cells.

In vitro results. In the MTT assay, only CEP has certain effect on inhibiting the proliferation of Sarcoma 180 tumor cells on the whole, as shown in Fig. 6. No obvious antitumor activities were found in EPF1 though the inhibition ratio was little enhanced with increasing of concentration levels. And the inhibition ratios in suppressing the growth of the Sarcoma 180 tumor cells of EPF2 were lower than 10%.

3.4. Effect of structure on antitumor activities

It has been reported that water soluble polysaccharides from *G. lucidum* exhibiting antitumor activity contained the backbone with either β-(1 → 3)-linked D-glucose or α-(1 → 4)-linked D-mannan (Wang et al., 2002). The results mentioned above indicated that β-D-galacto-α-D-mannan isolated from the culture filtrate of *G. tsugae* mycelium also exhibited significant antitumor activities. Obviously, CEP had much better antitumor activities than EPF1 and EPF2

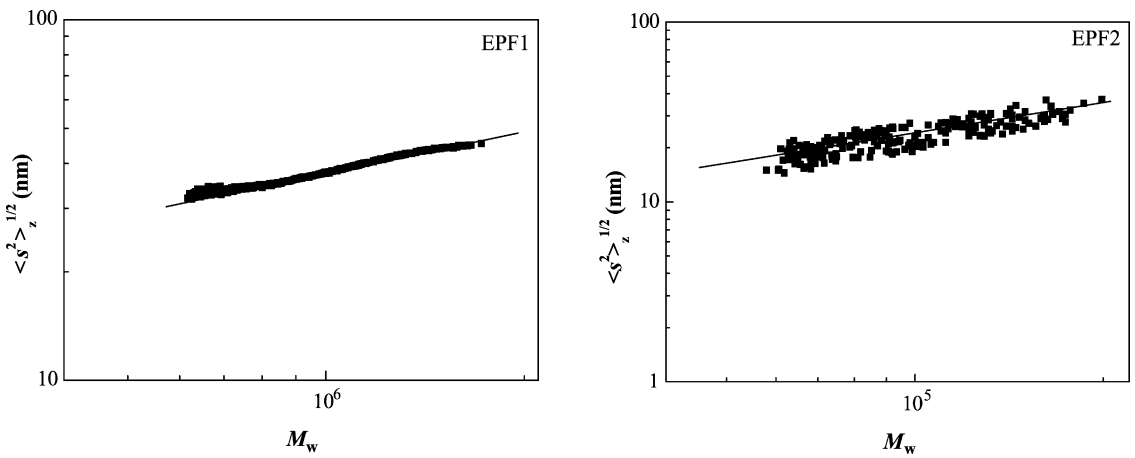

Fig. 5. Dependence of $\langle s^2\rangle_z^{1/2}$ on M_w for EPF1 and EPF2 in 0.2 M NaCl aqueous solution at 25 °C.

Table 3
Antitumor activities of CEP fractions from the *G. tsugae* mycelium against Sarcoma 180 solid tumor grown in BALB/c mice

Samples	Dose (mg/kg × days)	Inhibition ratio (%)	Enhancement ratio of body weight (%)	Complete repression
CEP	5 × 10	60.44	10.5	0/10
	32 × 10	49.0	35.0	0/10
EPF1	32 × 10	39.46	20	0/10
EPF2	5 × 10	53.03	25	0/10
	32 × 10	61.51	15.8	0/10
5-Fu	25 × 10	52.81	4.5	0/10

both in vivo and in vitro. It was noteworthy that the sample CEP was a mixture containing not only two pure polysaccharide fractions (EPFI and EPF2 as shown in Fig. 1) but also protein bound polysaccharides, due to the high protein content (21%) in CEP. It has been reported that protein-bound polysaccharide (PSK, Krestin) had direct cytotoxicity to a wide tumor cell lines (Sakagami, Aoki, Simpson, & Tanuma, 1999), and the protein moiety of PSK instead of the polysaccharide part modulated to the bioactivity of TGF-β_1 by binding to its active form (Matsunaga et al., 1998). Therefore, the mannan and the protein-bound polysaccharides in CEP played an important role in the enhancement of antitumor activity of CEP both in vivo and in vitro. In view of the little difference of the chemical compositions between EPF1 and EPF2, the solution properties must have certain effect on their antitumor actions. As a whole, for the extracelular polysaccharides isolated from the cultured filtrate of *G. tsugae* mycelium, the presence of bound protein, compositional mannose, relatively expanded coil chain and moderate molecular mass would be helpful to the enhancement of antitumor activity.

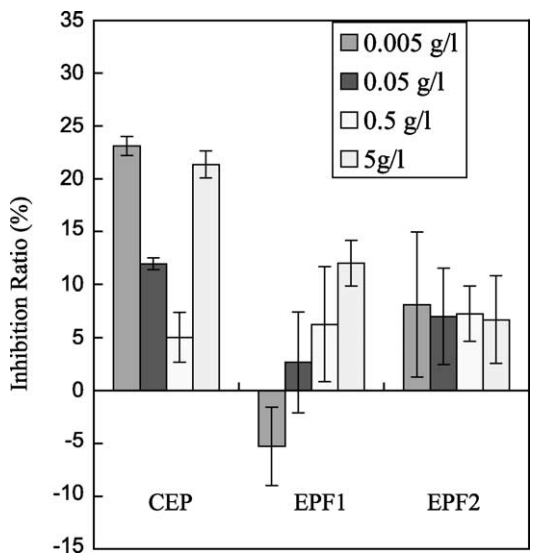

Fig. 6. Inhibition of proliferation of Sarcoma 180 cells by different concentrations of CEP fractions from *G. tsugae* mycelium.

4. Conclusion

The CEP from *G. tsugae* mycelium and its fractions (EPF1 and EPF2) were mainly composed with mannose, together with glucose, fucose, xylose, galactose and glucosamine. The weight-average molecular mass of the CEP, EPF1 and EPF2 were 23.8×10^4, 92.0×10^4 and 8.35×10^4, respectively. EPF1 molecules exist as a compact coil chain in 0.2 M NaCl aqueous solution at 25 °C, while EPF2 as a slightly expanded coil chain. Strong inhibition effect against Sarcoma 180 was found in them, and CEP exhibited the highest inhibition effect both in vivo and in vitro. The results implied that the presence of bound protein and mannose, and relatively expanded chain would be helpful to the enhancement of antitumor activities of the polysaccharides.

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