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# Structure and antitumor activities of the water-soluble polysaccharides from *Ganoderma tsugae* mycelium

Yanfei Peng[a], Lina Zhang[a,*], Fanbo Zeng[b], John F. Kennedy[c]

[a]*Department of Chemistry, College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, China*
[b]*College of Medicine, Huazhong University of Science and Technology, Wuhan 430030, China*
[c]*Chembiotech Laboratories, Institute of Research and Development, University of Birmingham Research Park, Vincent Drive, Edgbaston, Birmingham B15 2SQ, UK*

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**Abstract**

Six water-soluble polysaccharides coded as GTM1 to GTM6 were extracted sequentially from the mycelium of *Ganoderma tsugae* with 0.2 M sodium phosphate buffer solution at 25, 40, and 80 °C, water at 120 °C, 0.5 M sodium hydroxide at 25 and 65 °C. The chemical structures were determined by using IR, EA, GC and $^{13}$C NMR. The weight-average molecular mass ($M_w$) was characterized by size exclusion chromatography combined with laser light scattering (SEC-LLS). The results indicated that the samples GTM1 and GTM2 were heteropolysaccharide–protein complexes with the protein content of 13.5 and 20.1%, and apparent-mean $M_w$ of $62.8\times10^4$, $81.8\times10^4$, respectively. GTM3 and GTM4 contained (1→3)-β-D-glucans and (1→4)-α-D-glucans, while GTM5 and GTM6 were mainly a (1→6)-branched (1→3)-β-D-glucan. Two peaks were found in the SEC patterns of the four samples GTM1 to GTM4, representing fractions 1 composed of branched (1→4)-α-D-glucan with high $M_w$ and fraction 2 composed of galactose, glucose and mannose with relatively low $M_w$. With the progress of isolation, the content of fraction 2 decreased from 90.2 to 57.1%, accompanying with enhancing antitumor activity. The polysaccharides GTM1, GTM2 and GTM3 had significantly higher antitumor activity against solid tumor Sarcoma 180 with the inhibition ratio beyond 50%. The results suggested that the effects of the moderate content of galactose and bound protein, as well as relatively lower $M_w$, on the improvement of antitumor activities of polysaccharides could not be negligible.


*Keywords:* *Ganoderma tsugae* mycelium; Polysaccharide; Water-soluble; Structure; Antitumor activity

## 1. Introduction

*Ganoderma tsugae*, one of the famous traditional Chinese medicines, has long been used to promote health and longevity in China and other Asian countries. Recently, this fungus has attracted much attention because its polysaccharides have been demonstrated to have remarkable antitumor activities, which were manifested by enhancing the host mediated mechanisms including increasing IL-2 production and stimulation of cytotoxic T lymphocytes, NK activity and antibody production (Shiao, Lee, Lin, & Wang, 1994; Su, Shiao, & Wang, 1999; Wu, Shi, & Kuo, 2001). However, the wild fruiting body of *G. tsugae* is scarce, and it usually takes several months to cultivate the fruiting body. Therefore, many attempts have been made to obtain bioactive substances, especially polysaccharides, from the mycelium cultured by submerged fermentation, which takes only 2 weeks to complete a cultivation and is easier to control the product quality (Yang, Ke, & Kuo, 2000; Yang & Liau, 1998). It has been reported that three antitumor heteropolysaccharide–protein complexes with the molecular mass between $1.0–1.6\times10^4$ have been isolated from the mycelium of *G. tsugae* (Zhang et al., 1994). Sone, Okuda, Wada, Kishida, and Misaki (1985) have obtained a branched (1→3)-β-D-glucan with high antitumor activity from the culture filtrate of

* Corresponding author. Tel.: +86 27 8721 9274; fax: +86 27 6875 6661.
*E-mail address:* lnzhang@public.wh.hb.cn (L. Zhang).

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*G. lucidum* mycelium. In our previous work, a β-D-glucan-protein complex with molecular mass of $64.6\times10^4$ was extracted from the *G. lucidum* mycelium (Chen, Zhang, Yu, & Zhu, 2000) Interestingly, a water-insoluble (1→3)-α-D-glucan isolated from the fruiting body of *G. lucidum* with alkali solution did not have antitumor activity, but its sulfated derivative showed antitumor activity (Zhang, Zhang, Zhou, Chen, & Zeng, 2000) It is noted that the correlation of structure of such fungal polysaccharides to antitumor activity is not fully understood. In particular, few data have been published on the secondary structure such as molecular mass, conformation and solution properties, which have been proved to have a significant effect on the anti-tumor activities (Calazans, Lima, Franca, & Lopes, 2000; Yoshioka, Uehara, & Saito, 1992) In this work, we attempted to investigate systematically the chemical structure, solution properties and antitumor activities of six water-soluble polysaccharides isolated from the *G. tsugae* mycelium.

## 2. Materials and methods

### 2.1. Materials

The strain of *G. tsugae* was supplied by the Laboratory of Applied Mycology in Central China Agriculture University. Standard monosaccharides were Sigma products and used without further purification. All other reagents were of analytical grade made in China.

### 2.2. Isolation and purification of polysaccharides

The mycelium of *G. tsugae* was obtained through submerged cultivation, which was as described elsewhere (Peng, Zhang, Zeng, & Xu, 2003). The *G. tsugae* mycelium was defatted using Soxhlet extraction with ethyl acetate and acetone for 8 h, respectively. The resulting mycelia were powdered and then immersed in 0.2 M sodium phosphate buffer (pH 7.0) at 25 °C overnight before being centrifuged to give the supernatant. The supernatant was subjected to the Sevag (Staub, 1965) method to remove free proteins, and 30% $H_2O_2$ to decolorize, then dialyzed using regenerated cellulose tube ($M_w$ cut-off 8000, Union Carbide USA) against tap water for 5 days and distilled water for 3 days, finally concentrated by rotary evaporation at reduced pressure below 45 °C and lyophilized to give the colorless sample GTM1. Subsequent isolation of polysaccharides with phosphate buffer at 40 and 80 °C, distilled water at 120 °C as well as 0.5 M sodium hydroxide aqueous solution at 25 and 65 °C were carried out as shown in Scheme 1. Six water-soluble polysaccharides obtained were coded as GTM1, GTM2, GTM3, GTM4, GTM5 and GTM6.

GTM4 (25 mg) was further purified by dissolution in 0.2 M sodium chloride and application to a column (50× 2.0 cm) of DEAE-Sepharose CL-6B. After loading with sample the column was washed with 0.2 M sodium chloride at an elution rate of 42 mL/h. A differential refractive index detector (RI-150) was used on line. Two peaks were found in the elution pattern. Fractions corresponding to the first peak was collected, dialyzed, concentrated and lyophilized to give a sample of GTM4-F1 (5.0 mg).

### 2.3. Characterization

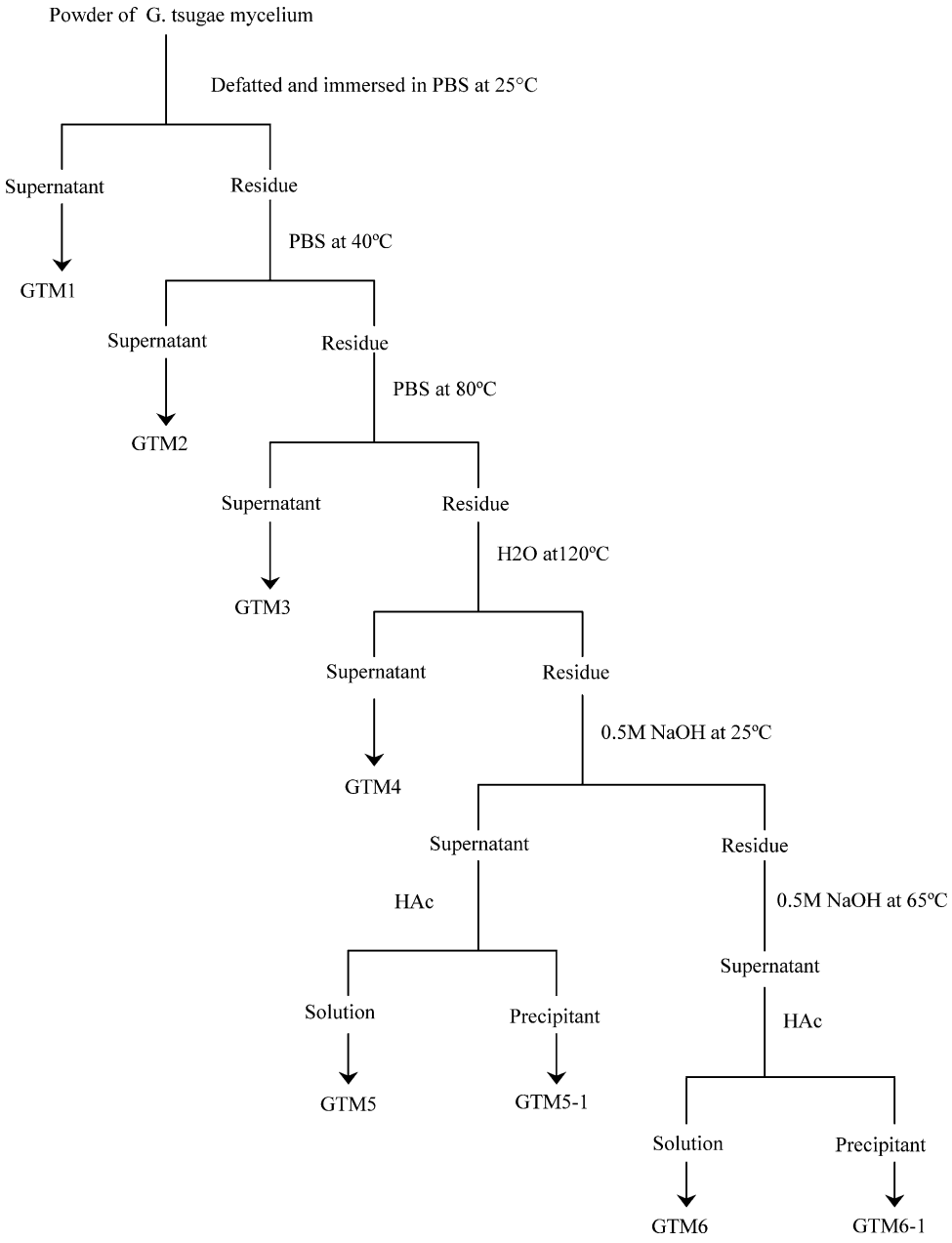
$^{13}C$ NMR spectra were recorded on an INOVA-600 spectrometer (Varian Inc., USA) at 150 MHz in $D_2O$ for GTM3, GTM4 at 20 °C and for GTM4-F1 at 70 °C, in DMSO-d6 for GTM5 and GTM6 at 60 °C, using acetone ($\delta$ 31.45) as an internal reference. The parameters used were as following: the number of scans was 6080; spectra width was 37700 Hz and line broadening 4.0 Hz. The N element content was measured with an elemental analyzer (Heraeus Co., Germany). Infrared spectroscopy of the samples was recorded on Nicolet 170SX FTIR 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 the saccharides according to the literature (Englyst, Quigley, & Hudson, 1994) was carried out on a HP 6890 instrument (Hewlett Packard, USA) with an DB-225 column (15 m×0.25 mm) at temperatures programmed from 180 to 220 °C at 4 °C/min.

### 2.4. SEC-LLS measurements

Size exclusion chromatography combined with laser light scattering (SEC-LLS) measurements were performed on a multi-angle laser photometer ($\lambda=633$ nm, DAWN-DSP, Wyatt Technology Co., USA) combined with a P100 pump equipped with TSK-GEL G6000PWXL and G4000PWXL column (7.8 mm×300 mm) and differential refractive index detector (RI-150) at 25 °C. The eluent was 0.2 M sodium chloride 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 (Whattman, UK). Astra software was utilized for data acquisition and analysis. The refractive index increments (d$n$/d$c$) were determined by using an Optilab refractometer (OPTILAB-DSP, Wyatt Technology Co., USA) at 25 °C. The values of d$n$/d$c$ at 633 nm obtained were 0.149 for GTM1, 0.145 for GTM2, and 0.141 mL/g for GTM3, GTM4, GTM4-F1, GTM5 and GTM6, respectively.

### 2.5. Viscometry

Viscosities of polysaccharides in 0.2 M NaCl sodium chloride were determined at 25 °C using an Ubbelohde viscometer. The kinetic energy correction was always negligible. Huggins and Kraemer plots were used to estimate the intrinsic viscosity [$\eta$].




Scheme 1. The isolation procedure of polysaccharides from the mycelium of *Ganoderma tsugae*.

### 2.6. In vivo assay of the antitumor activity

Sarcoma 180 cells ($1\times10^5$ cells/mouse) were subcutaneously inoculated into 8-week-old BALB/c male mice. The tested polysaccharides dissolved in phosphate buffer (pH 7.0) were injected intraperitoneally once daily, starting 72 h after tumor inoculation. The same volume of phosphate buffer (pH 7.0) was injected intraperitoneally into the control mice. After 10 days of administration, the tumors were removed from the mice and weighed. The in vivo antitumor activity of polysaccharides was expressed as an inhibition ratio (percent) calculated as $[(A-B)/A]100\%$, where $A$ and $B$ were the average tumor weight of the control and treated groups, respectively.

## 3. Results and discussion

### 3.1. Chemical structure

The yields of the six water-soluble polysaccharides from the *G. tsugae* mycelium are listed in Table 1. The aggregated yield of water-soluble polysaccharides was 3.9% by this isolation procedure, much higher than the yield (0.15%) from the fruiting bodies of *G. lucidum* using

Table 1
The yield, protein content and sugar compositions of GTM polysaccharides

| Samples | Yield (%) | Protein (%) | Sugar component (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Rha | Fuc | Xyl | Man | Gal | Glc | GlcNac |
| GTM1 | 1.3 | 13.54 | nd | 6.7 | 0.4 | 26.1 | 48.3 | 10.1 | 8.5 |
| GTM2 | 0.5 | 20.11 | nd | 6.2 | nd | 4.8 | 34.3 | 46.8 | 7.9 |
| GTM3 | 0.7 | nd | nd | 0.6 | 0.5 | 1.9 | 5.2 | 86.5 | 5.2 |
| GTM4 | 0.6 | nd | 0.7 | 0.3 | 0.2 | 1.3 | 2.0 | 91.8 | 3.4 |
| GTM5 | 0.5 | nd | nd | 0.60 | 1.36 | 5.50 | 1.04 | 87.64 | 3.90 |
| GTM6 | 0.3 | nd | nd | 1.03 | 8.33 | 17.44 | 2.04 | 66.91 | 4.25 |

nd, not detected. Rha, rhamnose; Fuc, fucose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; GlcNac, *N*-acetylglucosamine.

the same isolation procedure (Zhang & Chen, 1997). This 3.9% yield was also higher than those of 1.8% from *G. tsugae* mycelium extracted only by hot water (Zhang et al., 1994) and 1.6% extracted similarly from the fruiting bodies of *G. tsugae* (Wang et al., 1993). Therefore the adopted conditions of mycelium cultivation and the isolation procedure here were helpful to obtain water-soluble polysaccharides from the *G. tsugae* mycelium in a greater yield.

The protein contents and sugar compositions of the six water-soluble polysaccharides are summarized in Table 1. Because the Sevag method has been repeated for many times to remove free proteins, both GTM1 and GTM2 could be deemed to be protein-bound polysaccharide. No protein was found in the other four samples. The sugar composition of samples GTM1 to GTM4 indicated that with the progress of isolation the content of galactose, mannose decreased, the content of glucose increased markedly. As for GTM4 the glucose was the predominant sugar with only small glucosamine contents. GTM5 and GTM6 contained glucose as the major sugar followed by xylose and mannose.

The IR spectra of polysaccharides GTM1 to GTM6 is shown in Fig. 1. The typical signal pattern expected for a carbohydrate moiety, and several bands in the anomeric region were present. GTM1 exhibited the typical absorption peaks at 870 and 810 $cm^{-1}$ for mannan (Mathlouthi & Koenig, 1986). The appearance of obvious characteristic peaks both at 850 and 920 $cm^{-1}$ for α-D-glucan and at 890 $cm^{-1}$ for β-D-glucan in GTM3 and GTM4 implied the co-existing of α- and β-D-glucans. GTM5 and GTM6 exhibited the main absorption peak at 890 $cm^{-1}$ for the β-configuration of D-glucan.

Fig. 2 shows the elution pattern of GTM4 on DEAE-Sepharose CL-6B column. The fraction GTM4-F1 corresponding to the first peak gave a single symmetrical peak on the SEC pattern from TSK-GEL G6000PWXL and G4000PWXL columns in 0.2 M NaCl aqueous solution at 25 °C, indicating its homogeneity. The $^{13}C$ NMR spectra of GTM4-F1 in $D_2O$ at 70 °C, GTM3 and GTM4 in $D_2O$ at 20 °C, GTM5 and GTM6 in DMSO-d6 at 60 °C are shown in Fig. 3. Similar to those glucans from *Glycyrrhizia uralensis* Fisch (Liu & Fang, 1991), *Angelica acutiloba* (Yamada, Kiyohara, & Otsuka, 1984) and *Cynanchum*

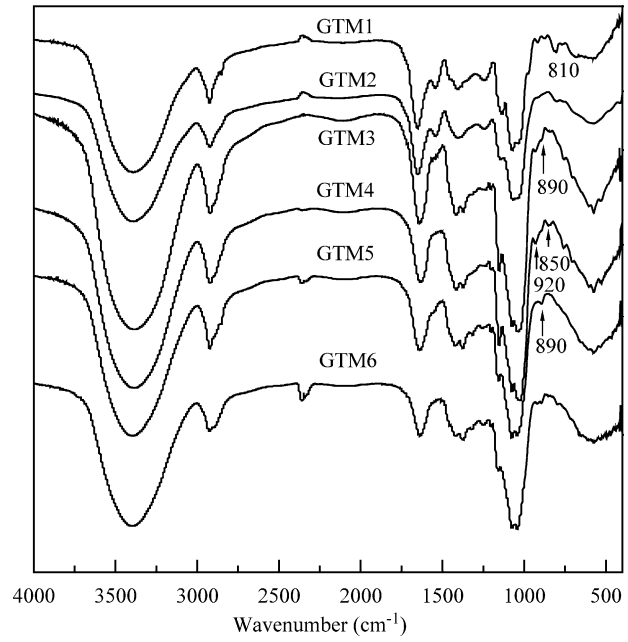



Fig. 1. FT-IR spectra of water-soluble polysaccharides from *G. tsugae* mycelium.

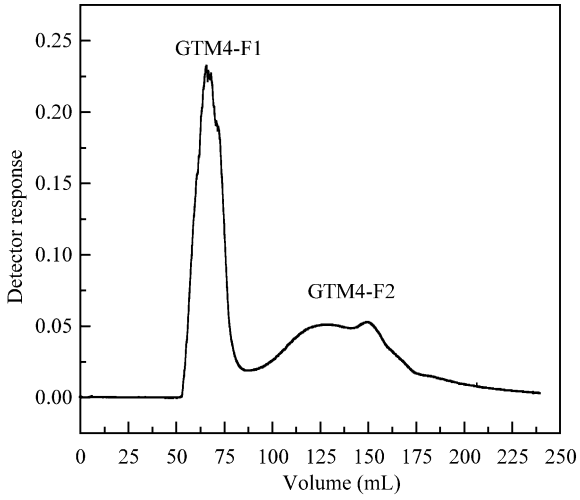



Fig. 2. The elution profile of GTM4 on DEAE-Sepharose CL-6B column. The elution was 0.2 M sodium chloride.

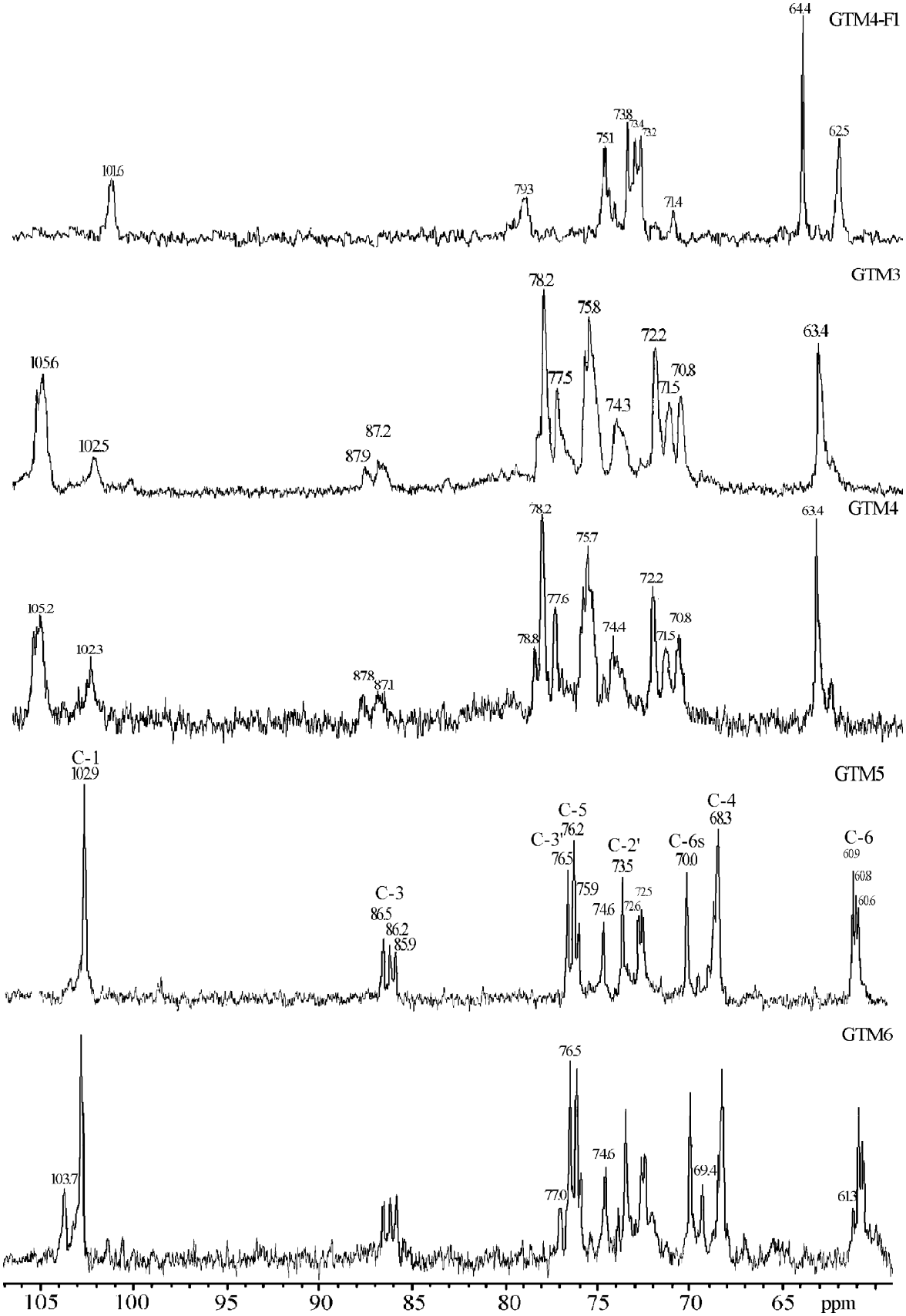



Fig. 3. The $^{13}C$ NMR spectrum of GTM4-F1 in $D_2O$ at 70 °C, GTM3 and GTM4 in $D_2O$ at 20 °C, GTM5 and GTM6 in DMSO-d6 at 60 °C.

Table 2
The experimental results of $M_w$ and [$\eta$] from SEC-LLS for GTM polysaccharides in 0.2 M sodium chloride at 25 °C

| Samples | [$\eta$] (mL/g) | $M_w \times 10^{-4}$ | Fraction 1 | | Fraction 2 | |
|---|---|---|---|---|---|---|
| | | | Content (%) | $M_w \times 10^{-4}$ | Content (%) | $M_w \times 10^{-4}$ |
| GTM1 | 40.0 | 62.8[a] | 9.8 | 577 | 91.2 | 6.9 |
| GTM2 | 49.7 | 81.8[a] | 10.9 | 670 | 89.1 | 9.8 |
| GTM3 | 78.6 | 465[a] | 39.0 | 1087 | 61.0 | 67.8 |
| GTM4 | 60.1 | 468[a] | 42.1 | 1028 | 57.1 | 61.5 |
| GTM5 | 180.4 | 176 | – | – | – | – |
| GTM6 | 118.8 | 161 | – | – | – | – |

[a] Represents the values of apparent-mean $M_w$ from fractions 1 and 2.

*oanilatum* (bunge) kitagawa (Wang, He, & Fang, 2000), the main chemical shifts in the spectrum of GTM4-F1 at 100.6 (C-1), 73.4 (C-2), 75.1 (C-3), 79.3 (C-4), 71.4 (C-5) and 62.5 (C-6) ppm were signals of the (1→4)-α-D-glucan. Moreover, the signals with high intensity at 73.8 and 64.4 ppm, attributed to C′-4 and C′-6 of 4,6-di-O-subsituted residues (Yipson & Horton, 1981), indicated that GTM4-F1 was highly branched. Thus GTM4-F1 consisted of a main chain of (1→4)-α-D-glucopyranosyl residues substituted at O-6 by α-D-glucopyranosyl groups. The $^{13}$C NMR spectra of GTM3 and GTM4 were similar to each other. On the basis of sugar composition in Table 1, IR analysis and literature data, the main signals in the $^{13}$C NMR spectrum of GTM4 at 105.2, 87.0, 78.2, 75.7, 72.2 and 63.4 ppm could be assigned to the C-1, C-3, C-5, C-2, C-4 and C-6 of (1→3)-β-D-glucan, and the signal at 70.8 ppm resulted from the branching effect of C6 (Mizuno et al., 1992). While the signals with lower intensity at 102.3, 74.4, 75.5, 78.6, 71.5 and 62.4 ppm might correspond to the C-1, C-5, C-3, C-2, C-4 and C-6 of (1→4)-α-D-glucan (Yipson & Horton, 1981). Therefore (1→4)-α-D-glucans and (1→3)-β-D-glucans co-existed in GTM3 and GTM4. Interestingly, the position and intensity of every peak in the $^{13}$C NMR spectrum of GTM5 was similar to that of *Grifolan* A (Ohno et al., 1984). The signals at 102.9, 86.2, 76.2, 72.6, 68.3 and 60.9 ppm were assigned to C-1, C-3, C-5, C-2, C-4 and C-6 of the (1→3)-linked backbone of β-D glucan, that at 76.5 and 60.9 to C-3′ and C-2′ of the side chain, and the one at 70.0 ppm was assigned to C-6s of substituted glucose (Falch, Espevik, Ryan, & Stokke, 2000). The integral area of C-6s to C-6 was 0.40 for the GTM5, suggesting that on average the two out of seven (1→3)-β-D-glucose residues of the backbone chain was substituted at O-6 position by single D-glucosyl groups. However, in the spectrum of GTM6, apart from the main signals which appeared in the spectrum of GTM5 and corresponded to the (1→6)-branched (1→3)-β-D-glucan, new signals with low intensity at 103.7, 77.0, 72.9, 69.4, 61.3 and 76.5 ppm also appeared, and which could be assigned to the xylomannan.

### 3.2. Molecular mass and chain conformation

As shown in Table 2, the apparent-mean values of $M_w$ of samples GTM1 to GTM6 were $62.8\times10^4$, $81.8\times10^4$, $465\times10^4$, $468\times10^4$, $176\times10^4$ and $161\times10^4$, respectively. Compared with their relatively low values of [$\eta$], the high molecular mass of samples GTM1 to GTM4 suggested that they exist as a compact random coil, similar to the global shape of protein in 0.2 M NaCl sodium chloride at 25 °C (Wu & Zhang, 2001). In contrast, GTM5 and GTM6 exhibited a relatively expanded flexible chain conformation (Zhang et al., 2001). Fig. 4 shows the SEC chromatograms of samples GTM1 to GTM4 in 0.2 M sodium chloride at 25 °C. Each sample exhibited two peaks corresponding to two fractions (1 and 2), which had different molecular masses and components. In view of Tables 1 and 2, the fraction 1 had more content of (1→4)-α-D-glucan and much higher $M_w$ than fraction 2. The fraction content, which was estimated using the division principle of SEC chromatogram, and the $M_w$ of both fractions of each sample are summarized in Table 2. With the progress of isolation, the weight content of fraction 1 increased from 9.8 to 42.9%. It was believed that fractions 2 in GTM1 and GTM2 were mainly composed with galactose, mannose and bound protein, while in GTM3 and GTM4 were mainly (1→3)-β-D-glucan.

The $M_w$ of GTM4-F1 was $9.77\times10^6$ and the slope ($\alpha$) of log–log plot of $\langle s^2\rangle_z^{1/2} \propto M_w^a$ is 0.33 as shown in Fig. 5. Usually, $\alpha$ is 0.5–0.6 for flexible polymers in good solvent, 0.2–0.4 for polymers with high degrees of branching and 0.3

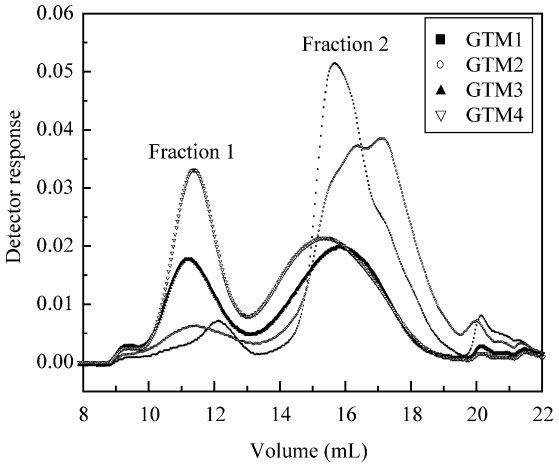



Fig. 4. The SEC chromatograms of GTM polysaccharides on TSK-GEL G6000PWXL and G4000PWXL column in sodium chloride at 25 °C.

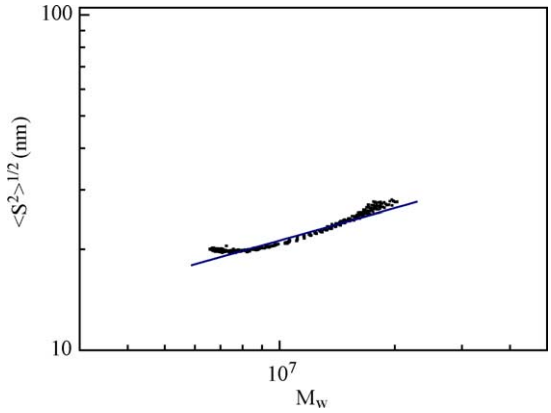



Fig. 5. Dependence of $\langle s^2 \rangle_z^{1/2}$ on $M_w$ for the sample GTM4-F1.

for globular shape (Majdoub et al., 2001). It further proved that GTM4-F1 might be a highly branched polysaccharide similar to *Inulin* (Heyer et al., 1998) and amylopectin. Moreover, the high $M_w$ of the fraction 1 could be attributable to the highly branched (1→4)-α-D-glucan.

### 3.3. Antitumor activity

The inhibition ratio of the samples GTM1 to GTM6 in different doses against solid tumor Sarcoma 180 implanted in BALB/c mice are summarized in Table 3. Different antitumor activities were observed in the six samples. GTM1, GTM2 and GTM3 showed obvious antitumor activity with an inhibition ratio beyond 50%. Especially, GTM2 had the highest inhibition ratio of 73% with the dose of 16 mg/kg. Furthermore, the obvious enhancement of body weight implied the GTM polysaccharides hardly obstruct the normal growth of the mice. As mentioned above, the samples GTM1, GTM2 and GTM3 had higher inhibition ratios than the other three samples. It was worth noting that with the progress of isolation, the content of fraction 2 in the samples GTMI to GTM4 decreased from 90.2 to 57.1%, and associated with reducing galactose contents. Therefore, the component and molecular mass in the fraction 2 in the polysaccharides play a main role in the enhancement of the antitumor activity. This suggested that the effects of the moderate content of galactose and bound protein, as well as relatively lower $M_w$ on the improvement of antitumor activities of polysaccharides could not be negligible.

Table 3
Antitumor activities of GTM polysaccharides against Sarcoma 180 solid tumor grown in BALB/c mice

| Samples | Dose (mg/kg days) | Inhibition ratio (%) | Enhancement ratio of body weight (%) |
|---|---|---|---|
| GTM1 | 16×10 | 54.06*** | 27.5 |
| | 32×10 | 57.30*** | 21.8 |
| GTM2 | 16×10 | 73.03*** | 4.7 |
| | 32×10 | 68.54*** | 4.9 |
| GTM3 | 5×10 | 43.07*** | 33.3 |
| | 16×10 | 55.06*** | 23.3 |
| | 32×10 | 51.46*** | 11.5 |
| GTM4 | 5×10 | 30.09*** | 39.1 |
| | 16×10 | 26.29*** | 37.0 |
| | 32×10 | 32.58*** | 29.0 |
| GTM5 | 15×10 | 12.9* | 28.4 |
| | 37.5×10 | 29.1* | 24.6 |
| GTM6 | 5×10 | 34.0* | 25.1 |
| | 32×10 | 38.5** | 23.8 |

*$p<0.05$, **$p<0.01$, ***$p<0.001$.

## 4. Conclusions

The water-soluble polysaccharides GTM1 to GTM6 were successfully isolated from the mycelium of *G. tsugae*. GTM1 and GTM2 were heteropolysaccharide–protein complexes with protein contents of 13.5 and 20.1%, respectively. GTM3 and GTM4 contained α- and β-D-glucans, while GTM5 and GTM6 were mainly (1→6)-branched (1→3)-β-D-glucan. The apparent-mean $M_w$ of the six samples were $62.8\times10^4$, $81.8\times10^4$, $465\times10^4$, $468\times10^4$, $176\times10^4$ and $161\times10^4$, respectively. There were two peaks in the SEC patterns of samples GTM1 to GTM4, indicating the existence of two fractions with different component and molecular mass. Fraction 1 was composed of branched (1→4)-α-D-glucan with high $M_w$, while fraction 2 was mainly composed of galactose and with relatively low $M_w$. With the progress of isolation, the content of fraction 2 in the samples GTM1 to GTM4 decreased from 90.2 to 57.1%, and the antitumor activity decreased, which mainly resulted from the effect of fraction 2. The samples GTM1, GTM2 and GTM3 exhibited significant inhibition ratio against the growth of solid tumor with the inhibition ratio beyond 50%, suggesting that the effects the galactose, bound protein and relatively lower $M_w$ on the improvement of antitumor activities of polysaccharides could not be negligible.

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