

SOLUTION PROPERTIES OF ANTITUMOR CARBOXYMETHYLATED DERIVATIVES OF α -(1 $\rightarrow$ 3)-D-GLUCAN FROM *GANODERMA LUCIDUM**

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Abstract Fractions of a water insoluble α -(1 $\rightarrow$ 3)-D-glucan (GL) extracted from *Ganoderma lucidum* were carboxymethylated (CM) to obtain water-soluble carboxymethylated derivatives (CM-GL) having a degree of substitution (DS) of 0.38~0.51. Weight-average molecular weight M_w and intrinsic viscosity $[\eta]$ of the samples CM-GL were measured by gel permeation chromatography combined with laser light scattering (GPC-LLS) and viscometry. The CM-GL exhibits a stiffer chain in aqueous solution at 25°C than the original glucan. The antitumor activities against Ehrlich ascites carcinoma (EAC, 5×10^6) of the carboxymethylated derivatives from the α -glucan and curdlan, a β -glucan, are significantly higher than those of the original glucans. The effects of the relatively low molecular weight, expanded chains and better water-solubility of the CM-GL on the enhancement of antitumor activity could not be neglected. The chain stiffness decreased speedily with increase of temperature from 40 to 60°C or NaOH concentration from 0.1 to 0.4 in the solution, respectively, and the change of the chain stiffness is reversible.

Keywords Solution properties, Antitumor activity, Carboxymethylated derivative, α -D-glucan, *Ganoderma lucidum*

INTRODUCTION

Ganoderma lucidum which is also called lingzhi in China and rheishi in Japan, belongs to the family of Heterobasidae and has long been used as Chinese medicine in Eastern countries. Its medical effects on cancer, hypertension, hepatitis, and hypercholesterolemia were demonstrated by pharmacological studies in the last two or three decades^[1-3]. Sone *et al.* drew the following conclusions^[1]. The major constituents of the extracts from *Ganoderma lucidum* are water-insoluble β -(1 $\rightarrow$ 3)-D-glucans with very low degree of branching (DB), which have significantly lower antitumor activity than water-soluble β -(1 $\rightarrow$ 3)-D-glucans with higher degree of β -(1 $\rightarrow$ 6)-branching. Most water-soluble components extracted from the fruiting body of *Ganoderma lucidum* are heteropolysaccharides and water-soluble β -(1 $\rightarrow$ 3)-D-glucans. However, *schizophyllan*, a water-soluble β -(1 $\rightarrow$ 3)-D-glucan with DB = 1/3, and curdlan^[4], a water-insoluble linear β -(1 $\rightarrow$ 3)-D-glucan^[5], are known to have equal antitumor activities. Therefore, understanding of the secondary structure of the polysaccharides is essential for the successful antitumor action. Nevertheless, it was shown that the molecular weight, water solubility, conformation and chemical modification of the polysaccharides significantly affect their antitumor and immunomodulatory activities^[6-10]. *Lentinan*, *schizophyllan* and *grifolan* with M_w higher than $1 \times 10^4 \sim 4 \times 10^4$ ^[6] could induce antitumor activity. In addition, the water-soluble product obtained by *o*-carboxymethylation of a linear α -(1 $\rightarrow$ 3)-D-glucan ($M_w = 5.6 \times 10^5$) showed potent antitumor activity against the Sarcoma 180 in mice^[8].

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In the case of carboxymethylation of glucans^[11, 12], carboxymethylated (CM)-curdian showed significant antitumor activity at a degree of substitution (DS) of 0.08~0.38, but CM- β -(1 $\rightarrow$ 3)-*D*-glucan from *Sclerotinia sclerotiorum* did not show any antitumor effect at DS in excess of 0.14. These observations indicated that the solution properties of the polysaccharides and their derivatives are very important to verify the relationship between the secondary structure and the antitumor activity. It is worth noting that α -(1 $\rightarrow$ 3)-*D*-glucan is scarcely found in nature due to its weak molecular construction ability, so that the α -glucan and its derivatives have been little studied.

In our previous work, a linear α -(1 $\rightarrow$ 3)-*D*-glucan was extracted from the fruiting body of *Ganoderma lucidum*^[13]. Its fractions of different molecular weight were prepared, and were studied by light scattering, showing that the α -(1 $\rightarrow$ 3)-*D*-glucan chain is more flexible than amylose, a α -(1 $\rightarrow$ 4)-glucan^[14]. In this work, we attempt to synthesize carboxymethylated derivatives from the glucans with α - and β -linkages, and then assay their antitumor activity against Ehrlich ascites carcinoma (EAC). The weight-average molecular weight M_w and intrinsic viscosity $[\eta]$ were determined by gel permeation chromatography (GPC) combined with laser light scattering (LLS), and the correlation between the solution properties and the antitumor activity was discussed.

EXPERIMENTAL

Preparation of Samples

The previously investigated α -glucan fraction^[13] from the fruiting body of *Ganoderma lucidum* coded as GL ($M_w = 19.5 \times 10^4$) were used in this work. Curdian, a β -(1 $\rightarrow$ 3)-*D*-glucan, was purchased from Wako Co. (No. RSN 9512, $M_w = 44.6 \times 10^4$). 500 mg GL was dissolved in 25 mL NaOH aqueous solution of 0.1, 0.2, 0.5, 1.0 and 3.0 mol/L, respectively, and stirred for 2 h to obtain clear solutions. The polysaccharide solution reacted with monochloroacetic acid at 60°C for 4 h under stirring, and then neutralized with aqueous acetic acid solution. The product was dialyzed for 3 days, then freeze-dried to give carboxymethylated (CM) glucan coded as CM-GL-1, CM-GL-2, CM-GL-3, CM-GL-4 and CM-GL-5. Curdian was carboxymethylated as in the above process, and coded as CM-curdian.

Characterization

IR spectra of the samples were recorded with a Nicolet 170SX FTIR spectrometer. The test specimens were prepared by the KBr-disk method. The elemental contents of the products were determined by an elemental analyzer (CHN-O-RAPID Heraeus Co., Germany).

Gel permeation chromatography combined with static laser light scattering (GPC-LLS) is convenient for the determination of true molecular weight and its distribution without the aid of standard samples. GPC-LLS measurements of the carboxymethylated derivatives of GL fractions were performed on a DAWN[®] DSP multi-angle laser light scattering (MALLS) instrument (Wyatt Technology Co.) combined with a P100 pump equipped with a TSK-GEL G4000 PWXL column (7.8 mm $\times$ 300 mm) and a differential refractive index detector (RI-150) at 25°C. The eluent was 0.2 mol/L aqueous NaCl solution at a flow rate 1.0 mL/min. All the solutions used were filtered through a sand filter, and then a 0.45 μ m filter (Whatman, England). Astra software was utilized for data acquisition and analysis.

The viscosities of carboxymethylated derivatives of the GL fractions in 0.2 mol/L aqueous NaCl solution were measured at 25, 30, 40, 50, 60 and 70°C, respectively, by using a capillary viscometer. The kinetic energy correction was always negligible. Huggins and Kraemer plots were used to estimate the intrinsic viscosities $[\eta]$ and k' .

Assay of Antitumor Activity

Ehrlich ascites carcinomas (EAC, 5×10^6), provided by Pharmacy College of Tongji Medical University, were transplanted subcutaneously into the right groin of Kunming mice weighing 20 ± 1 g. 5-Fluorouracil (5-Fu) and testing samples dissolved or suspended in distilled water were autoclaved, and injected intraperitoneally daily for 7 days, starting 24 h after tumor implantation. The mice were killed on the next day after the last injection, and the tumors were excised. The weights of tumors were compared with those in the control mice. The inhibition

ratio ξ and enhancement ratio of body weight f were calculated as follows:

$$\xi = [(W_c - W_t)/W_c] \times 100\% \quad (1)$$

$$f = [(W_a - W_b)/W_b] \times 100\% \quad (2)$$

where W_c is the average tumor weight of the control group, W_t is the average tumor weight of the test group; W_b and W_a are the body weight of mice before and after the assay.

RESULTS AND DISCUSSION

The data of the elemental content in the carboxymethylated derivatives are listed in Table 1. The degree of substitution (DS), which designated the average value of carboxyl groups on each glucose residue, was calculated from the data, and DS is in the order of: CM-GL-5 > CM-GL-4 > CM-GL-3 > CM-GL-2 > CM-GL-1. It indicates that the DS increases with the increase of NaOH concentration used in the reaction.

Table 1. Elemental analysis and water solubility of CM-GL-1, 2, 3, 4 and 5

	C%	H%	O%	DS	Solubility ^a
CM-GL-1	44.24	6.00	49.77	0.20	insol
CM-GL-2	44.08	5.85	50.08	0.38	s
CM-GL-3	44.04	5.81	50.16	0.43	s
CM-GL-4	43.97	5.75	50.28	0.51	s
CM-GL-5	43.88	5.67	50.45	0.63	insol

^a insol: water-insoluble; s: water-soluble

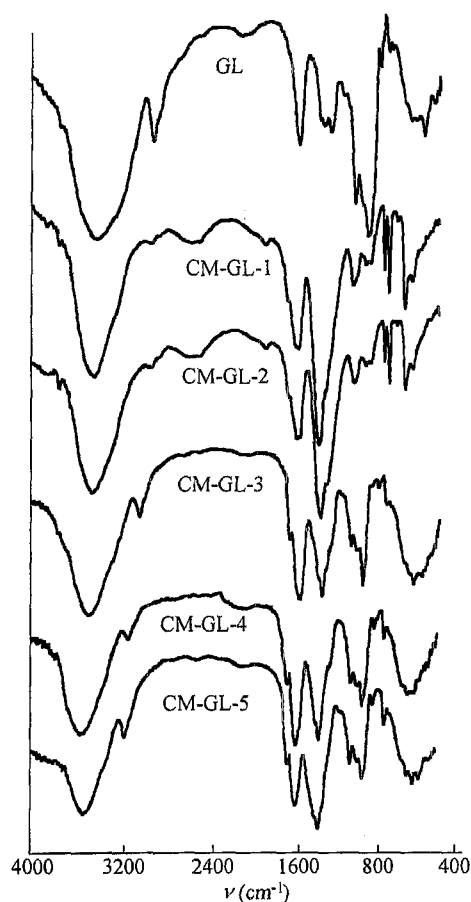


Fig. 1 Infrared spectra of GL and its carboxymethylated derivatives CM-GL-1, -2, -3, -4 and -5

The infrared spectra (IR) of the GL and its carboxymethylated derivatives are shown in Fig. 1. Compared with the GL glucan, the —OH stretching vibration bands of the derivatives at 3400 cm^{-1} appeared narrower and shifted to higher wavenumber, suggesting that the original intermolecular hydrogen bonding in the GL was broken during carboxymethylation. In the IR spectrum of CM-GL a new absorption band at 1730 cm^{-1} is assigned to the C=O stretching of $\text{—CH}_2\text{COOH}$, and its intensity increases with increasing DS. This is in good agreement with the results from elemental analysis. In addition, the absorption bands at 1040 and 1050 cm^{-1} (C—OH) significantly decrease due to substitution. This result indicates that the carboxymethylated derivatives were successfully prepared from the α -glucan.

The water solubility of CM-GL is listed in Table 1. Interestingly, GL is water-insoluble, but CM-GL-2, -3 and -4 are water soluble, suggesting that the derivatives with DS from 0.38 to 0.51 are water soluble. When DS is lower than 0.2 or higher than 0.63 , such as in CM-GL-1 and CM-GL-5 the substances are water insoluble. It has been reported that CM-cellulose with relatively high and low DS is water insoluble^[15, 16].

Figure 2 shows the GPC-LLS chromatograms of CM-GL-2, -3 and -4 in 0.2 mol/L aqueous NaCl solution at 25°C . There is only one peak for each sample, indicating no aggregates in this case^[17, 18]. From the analysis, the weight-average molecular weight M_w , polydispersity index d and intrinsic viscosity $[\eta]$ of the GL and its derivatives are obtained, and are summarized in Table 2. Obviously, the values of M_w and $[\eta]$ decrease with increase of NaOH concentration in the reaction. The M_w values of the CM-GL-2, -3 and -4 are much lower than that of the original α -glucan. However, the decreasing extent of $[\eta]$ values is lower than that of M_w , suggesting that the CM-glucan exists as a relatively expanded chain in the solution.

Table 2. Molecular weight and intrinsic viscosity of the glucan in 0.2 mol/L NaOH aqueous solution at 25°C

Sample	Curdlan	GL	CM-GL-2	CM-GL-3	CM-GL-4
$M_w \times 10^{-4}$	44.6	19.5 ^a	10.2	7.5	5.3
$[\eta]$		102 ^a	83	79	70
d			1.5	1.4	1.2

^a Values obtained in 0.25 mol/L LiCl/DMSO at 25°C ^[13]

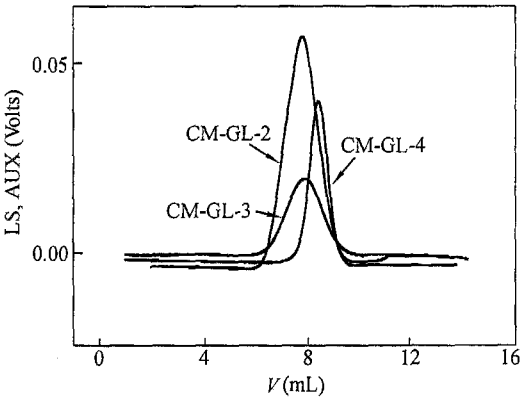


Fig. 2 GPC chromatograms of CM-GL-2, -3 and -4 in 0.2 mol/L aqueous NaCl solution at 25°C

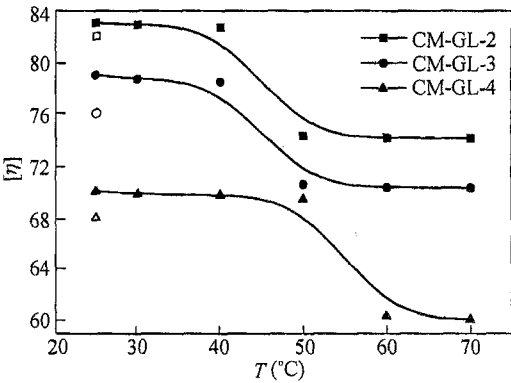


Fig. 3 Temperature dependence of $[\eta]$ of CM-GL-2, -3 and -4 in 0.2 mol/L aqueous NaCl solution Δ , $\circ$ and $\square$ represent the results measured at 25°C after heating to 70°C .

Figure 3 displays the dependence of $[\eta]$ of CM-GL-2, -3 and -4 on temperature (T) in 0.2 mol/L aqueous NaCl solution. It is worth noting that the $[\eta]$ values are quickly decreased when the temperature increases from 40 to 60°C . It is implied that the intramolecular hydrogen bonding in the CM-GL, which sustains the chain stiffness, is weakened, resulting in an increase of the flexibility and in a reduction of the $[\eta]$. The hollow marks in

Fig. 3 refer to the values of $[\eta]$ measured at 25°C after heating to 70°C and these data points agree with the curves, indicating that the change is thermally reversible.

Figure 4 shows the Huggins constants k' of CM-GL-2, -3 and -4 at temperatures of 25, 30, 40, 50, 60 and 70°C, respectively. The k' values of the CM-GL-2, -3 and -4 in the range of 25°C to 40°C are larger than those from 50°C to 70°C, and the larger k' values reflect the stiffer chain conformation in the solution. This result supports the conclusion from Fig. 3.

Figure 5 shows the temperature dependences of η_{sp}/c of CM-GL-1 and CM-GL-5 in 0.01 mol/L aqueous NaOH solution, respectively. The η_{sp}/c of CM-GL-5 decreases sharply at 40°C and 60°C, similar to the results shown in Fig. 3. However the η_{sp}/c of CM-GL-1 hardly changes with the temperature, owing to the very little amount of $-\text{COOH}$ groups present in CM-GL-1.

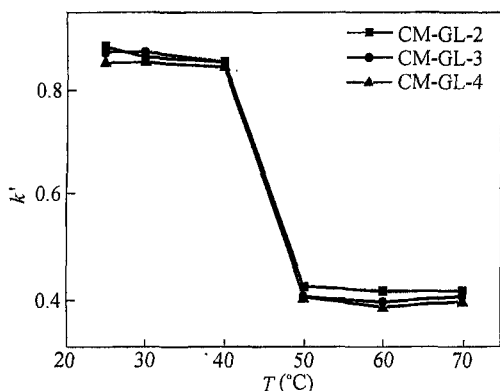


Fig. 4 Temperature dependence of Huggins constant (k') of CM-GL-2, -3 and -4 in 0.2 mol/L NaCl aqueous solution

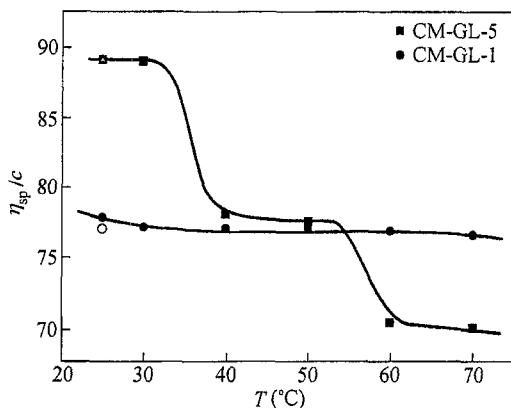


Fig. 5 Temperature dependence of η_{sp}/c of CM-GL-1 and CM-GL-5 in 0.01 mol/L NaOH aqueous solution. $\circ$ and $\square$ represent the results measured at 25°C after heating to 70°C.

Figure 6 shows the dependence of η_{sp}/c of CM-GL-1 and CM-GL-5 on NaOH concentration in the aqueous solution at 25°C. The η_{sp}/c value of CM-GL-5 decreases with increase of NaOH concentration, and two inflection points appear at the concentrations of 0.1 and 0.4 mol/L NaOH, while that of CM-GL-1 almost remains unchanged. These results indicate that the CM-GL chains with relatively high DS are more extended than those with low DS. It can be explained that the chain stiffness is caused by the intramolecular hydrogen bonding and the electrostatic repulsion from the charged carboxyl groups. The chain stiffness decreases with increase of temperature or NaOH concentration, both of which could break the intramolecular hydrogen bonding of the CM-GL in aqueous solution. The hollow mark in Fig. 6 refers to the values of η_{sp}/c obtained from the sample dissolved in 0.5 mol/L aqueous NaOH solution and then diluted to 0.01 mol/L NaOH by water. The points are positioned slightly below the curves, indicating that the degradation of the samples CM-GL may occur in 0.5 mol/L NaOH. However, the viscosity change is reversible.

The antitumor activities of the samples GL, CM-GL-2, -3, -4, curdlan and CM-curdlan are summarized in Table 3. The original GL does not exhibit antitumor activity against EAC, but the carboxymethylated GL shows significant antitumor activity. Moreover, curdlan has low activity, while the CM-curdlan shows high antitumor activity. From the comparison of the antitumor activities of the original glucans and their carboxymethylated derivatives, it is conceivable that the antitumor activities could be enhanced greatly by carboxymethylation. It is worth noting that the antitumor activities of the derivatives (M-GL-2, 3,4) are slightly lower than that of 5-Fu, but their enhancement ratios of body weight are much higher than that of 5-Fu, implying that the polysaccharides are not so toxic as 5-Fu, which kills the normal cells as well as the cancer cells. This result is in agreement with the conclusion that antitumor action of β -(1 $\rightarrow$ 3)-glucan is derived from the stimulation of the immunoresponse mechanism of the host^[19]. In our recent work^[20], two sulfated derivatives from the α -(1 $\rightarrow$ 3)-D-glucan, extracted

from fruiting body of *Ganoderma lucidum*, and curdlan all exhibited significantly higher antitumor activity against EAC. It was indicated that the effect of the extended chain of the sulfated glucan, namely chain stiffness, in the aqueous solution on the enhancement of the antitumor activity could not be negligible. The results in Table 3 support the conclusion mentioned above. In addition, it has been reported that antitumor activity of CM-curdlan with relatively high DS has disappeared^[11, 12, 21], due to the decrease of its water solubility. Interestingly, as shown in Table 3, the antitumor activity of the CM-GL increases with the increasing M_w in the studied range. It further indicates that the polysaccharide having moderately high M_w is favorable for the antitumor activity enhancement^[6].

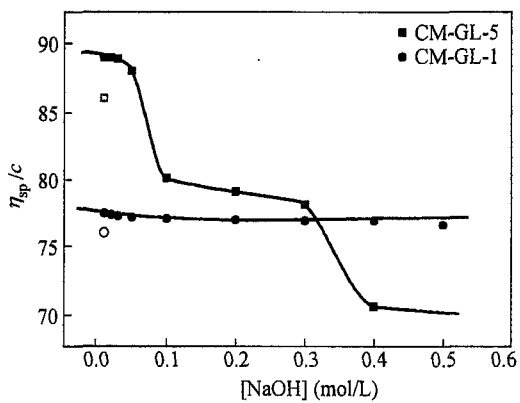


Fig. 6 Dependence of η_{sp}/c of CM-GL-1 and CM-GL-5 on the NaOH concentration in the solution at 25°C
○ and □ represent the results measured in 0.01 mol/L aqueous NaOH solution diluted from the sample solution in 0.5 mol/L NaOH.

Table 3. Antitumor activities against EAC of the carboxymethylated derivatives of α -(1→3)-D-glucan and curdlan

Sample	Dose (mg/kg × days)	<i>f</i> (%)	<i>W</i> _{tumor} (g)	Mice	ξ(%)
Control		54.1	1.04 ± 0.42	10/10	–
5-Fu	20 × 7	42.0	0.45 ± 0.16	10/10	56.7
GL	12 × 7	57.3	1.52 ± 0.35	10/10	–
Curdlan	52.3 × 7	46.5	0.85 ± 0.22	10/10	22.7
CM-GL-2	34.7 × 7	50.0	0.67 ± 0.20	10/10	35.6
CM-GL-3	34.7 × 7	51.9	0.87 ± 0.29	10/10	16.3
CM-GL-4	34.7 × 7	57.9	0.94 ± 0.40	10/10	9.6
CM-Curdlan	17.4 × 7	51.9	0.41 ± 0.19	10/10	62.7

CONCLUSIONS

Water-soluble carboxymethylated derivatives (CM-GL) with DS from 0.38 to 0.51 were synthesized from the water-insoluble α -glucan (GL) extracted from *Ganoderma lucidum*. The derivatives from GL and curdlan all exhibit higher antitumor activities against EAC than the originals. The effects of the molecular weight, chain stiffness and water-solubility of CM-GL on the enhancement of the antitumor activity could not be neglected. Water solubility was affected by the DS of the carboxymethylated derivatives. The chains of carboxymethylated derivatives CM-GL are more extended than the original GL in the aqueous solution. The chain stiffness of the

CM-GL sharply decreases with increase of temperature from 40°C to 60°C or increase of NaOH concentration, respectively, owing to the breaking of the intramolecular hydrogen bonds, which sustain the chain stiffness. The change of the chain stiffness of the CM-GL with temperature or NaOH concentration is reversible in this range studied.

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