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Carboxymethylated β-glucans from mushroom sclerotium of *Pleurotus tuber-regium* as novel water-soluble anti-tumor agent

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Abstract

Six hot alkali extracts (HAE-1 to HAE-6) of mushroom (1 → 3)-β-glucans from the sclerotia of *Pleurotus tuber-regium* having different molecular weight (M_w) ranging from 1×10^4 to 42×10^4 were carboxymethylated to give their corresponding water-soluble derivatives (CMHAE-1 to CMHAE-6) with M_w ranged from 2.08×10^4 to 53.2×10^4. In general, the CMHAE β-glucans had higher water solubility and higher in vivo (Sarcoma 180 solid tumor implanted on BALB/c mice) as well as in vitro (HL-60 tumor cell culture) anti-tumor activity than the native HAE β-glucans. From ELISA assay of cytokines, CMHAE fractions could increase production of tumor necrosis factor alpha (TNF-α) in mouse plasma stimulated by lipopolysaccharide (LPS). It was postulated that a more expanded flexible chain of these novel CMHAE β-glucans might account for their in vivo and in vitro anti-tumor activities.

Keywords: Carboxymethylation; β-glucans; Mushroom sclerotia; *Pleurotus tuber-regium*; Anti-tumor and immunomodulatory activities

1. Introduction

Sclerotium of *Pleurotus tuber-regium*, a dry compact mass of fungal hyphae, which have been commonly consumed in Africa for some time (Zoberi, 1973), has gained an emerging popularity in China in recent years (Huang, Guo, & Huang, 1996). Our previous studies have shown that the sclerotia of *P. tuber-regium* contained over 90% dry weight of total dietary fiber (TDF) of which 60% dry weight of its non-starch polysaccharide (NSP) component was β-glucan (Cheung & Lee, 1998). Structural analysis of the hot alkali-soluble fractions (HAE) of the NSP from this mushroom revealed that they consisted of a main chain of (1 → 3)-β-D-glucopyranosyl units with every third unit having a (1 → 6)-β-D-glucopyranosyl branch on average, having a weight-average molecular weight (M_w) ranging from 1×10^4 to 76×10^4 (Zhang, Zhang, Dong, Guo, Song & Cheung, 2001). Results of in vivo and in vitro anti-tumor experiments from our previous studies had suggested that the HAE fractions had potent anti-tumor activity mediated probably by both the immune system and direct cytotoxicity (Zhang, Cheung, & Zhang, 2001). However, the HAE fractions are only soluble in 10% dimethylsulfoxide (Me_2SO) and not soluble in water. This will hinder their potential clinical applications since intravenous administration of the micro-particulate form of insoluble β-(1 → 3)-D-glucans from yeast cell wall had been associated with a number of undesirable side effects including hepatosplenomegaly (Riggi & Di Luzio, 1962), granuloma formation (Williams, Sherwood, McNamee, Jones, Browder, & Di Luzio, 1985), micro-embolization and enhanced endotoxin sensitivity (Browder et al., 1987). Alternatively, native insoluble polysaccharides can be modified into water-soluble ones that are safe to administer systemically (Williams et al., 1992). A common chemical modification procedure for polysaccharides is carboxymethylation, which gives products in which the primary and/or secondary alcohol groups are etherified with carboxymethyl groups (De Nooy, Rori, Masci, Dentini, & Crescenzi, 2000). It has also been reported that carboxymethylated (1 → 3)-β-D-glucan from another mushroom sclerotia, *Poria cocos* (carboxymethylpachymaran), had good water solubility and enhanced anti-tumor activity (Kanayama, Adachi, & Togami, 1983). Moreover, the study of the physico-chemical properties of the chemically

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modified fungal β-glucans with enhanced water solubility, such as scleroglucan had facilitated the study of their physico-chemical properties in aqueous solution which could increase the understanding of their conformational behavior in relationship to their immunomodulatory and anti-tumor activity (Kuliche, Lettau, & Thielking, 1997). Furthermore, in vivo administration of (1 → 3)-β-D-glucan enhanced immunoreactivity, including activation of macrophages, cytotoxic T-cells and natural killer cells, that can up-regulate the immunological surveillance and the resistance of host against tumor cells (Kogan, 2000). It has been reported that in vivo administration of (1 → 3)-β-D-glucan to murine macrophages activated by lipopolysaccharide (LPS) induced the production of cytokines such as interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) that could subsequently induce activation and differentiation of lymphocytes, as well as proliferation of granulocytes, enhancing cytotoxicity and in vivo immunomodulatory responses (Adachi, Okazaki, Ohno, & Yadomae, 1994; Chihara, 1992). In this report, six chemically-characterized HAE fractions obtained previously from the sclerotia of *P. tuber-regium* (Zhang, Zhang, Dong, Guo, Song & Cheung, 2001) were chemically modified to form the water-soluble carboxymethylated fractions (CMHAEs) of which their in vivo and in vitro anti-tumor activities as well as cytokine production were evaluated. The novel anti-tumor and immunomodulatory activities observed in this type of novel mushroom β-D-glucan were explained in light of their relatively expanded chain conformation in aqueous solution.

2. Experimental

2.1. Materials

Sclerotia of *P. tuber-regium* were cultivated by the Sanming Mycological Institute in Fujan province, China. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemicals (St Louis, MO). Human acute promyelocytic leukemia HL-60 (ATCC no. CCL-240), human hepatocellular carcinoma HepG2 (ATCC no. HB-8065) and monkey normal kidney Vero (ATCC no. CCL-81) cell lines were purchased from the American Type Culture Collection (Rockville, MD).

2.2. Carboxymethylation of mushroom β-glucan

HAE fractions previously isolated from the sclerotia of *P. tuber-regium* were carboxymethylated according to procedures used for β-glucan (Sasaki, Abiko, Nitta, Takasuka, & Sugino, 1979). In brief, six HAE fractions (HAE-1 to HAE-6) with M_w ranged from 1×10^4 to 42×10^4 were suspended individually in a 30% sodium hydroxide solution into which monochloroacetic acid was added and the mixture was stirred for 5 h at 60–70 °C. The suspended product was recovered by filtration, and washed successively with methanol–acetic acid, methanol–water, methanol, and acetone. The final precipitate was dissolved in water, and dialyzed against distilled water at 4 °C for 2 days. The retentate was frozen and lyophilized by a lyophilizer (CHRIST ALPHR 1-2, Germany) to give the final carboxymethylglucan with the yield of more than 70%.

2.3. In vivo anti-tumor test

Sarcoma 180 cells (1×10^5 cells/mouse) were subcutaneously inoculated into 8 weeks old male BALB/c mice. CMHAE fractions (20 mg/kg) dissolved in phosphate buffer saline (PBS), were injected intraperitoneally (i.p.) once daily for 10 days starting 24 h after tumor inoculation. The same volume of PBS was injected i.p. into the mice of the control group. The tumor was allowed to grow on the mice for another 7 days before it was removed from the animal and weighed. The anti-tumor activity of the tested samples was expressed as an inhibition ratio (percent) calculated as $[(A - B)/A] \times 100\%$, where A and B are the average tumor weights of the control and treatment groups, respectively.

2.4. In vitro proliferation and cytotoxicity assays

Dye exclusion method for suspended cells. The HL-60 leukemia cells (10^6 cells/ml) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum under an atmosphere of 5% carbon dioxide at 37 °C for 72 h containing the CMHAE fractions at concentrations of 50, 100, and 200 μg/ml in PBS. The survival rate of the mammalian cells was assayed by counting living cells that excluded the Trypan blue dye using a hemacytometer.

Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method for adherent cells. Mammalian HepG2 cells and Vero cells (10^6 cells/ml) were incubated separately with the CMHAE fractions at concentrations of 50, 100, and 200 μg/ml and allowed to grow under the same condition as the HL-60 cells mentioned above. The number of living HepG2 cells and Vero cells at the end of the 72 h incubation period was determined by a colorimetric assay based on the tetrazolium salt MTT as described by Mosmann (Mosmann, 1983). In the above two assays, the treated samples were compared with control samples in the absence of the CMHAE fractions. All in vitro results were expressed as the ratio of inhibition of tumor cell proliferation calculated as: $[(A - B)/(A)] \times 100\%$ where A and B are the average numbers of viable tumor cells of the control and samples, respectively. All tested samples were carried out in triplicates.

2.5. Enzyme linked immunosorbent assay (ELISA) of tumor necrosis factor-α (TNF-α)

Preparation of mouse plasma. CMHAE fractions were administrated i.p. (20 mg/kg body weight) daily for 10 days to 8 weeks old BALB/c mice, which had been implanted with Sarcoma 180 tumors for 7 days. Lipopolysaccharide (LPS) (1 μg/mouse) was intravenously administrated as a triggering reagent 24 h after 10 days of sample administration to half of the mice in each group. Blood samples were collected by orbital-puncture from mice with and without LPS treatment using heparin as anticoagulant 1 hour after LPS administration and then centrifuged for 10 min at $1000 \times g$. The plasma supernatant was used for assay of cytokine production.

Assay for TNF-α production in plasma. Plasma TNF-α level was determined using a solid phase sandwich enzyme-linked immunosorbent assay (ELISA). Five micrograms per millilitre of anti-mouse TNF-α monoclonal antibody (PharMingen, San Diego, CA) was bound to the surface of a 96-well, flat bottom plate by incubating in the dark at 4 °C overnight in 0.1 M bicarbonate buffer (pH 9.6). The plate was washed with PBS containing 0.05% Tween 20 (PBST) and its uncoupled binding sites were blocked with assay diluent (PBS with 10% fetal bovine serum, pH = 7.0) at room temperature (r.t.) for 60 min. After washing with PBST, the plate was incubated with 100 μl standards (recombinant mouse TNF-α; PharMingen) and samples (10 μl plasma diluted in 90 μl assay dilute) in triplicates at r.t. for 120 min. The plate was treated with 1/2000 dilution of detection antibody (biotinylated anti-mouse TNF-α polyclonal antibody; PharMingen) with 0.15% Proclin-150 as preservative and then incubated at r.t. for 60 min. After washing and blocking, the plate was developed with a 1/5000 dilution of avidin–horseradish peroxidase conjugate (PharMingen) and then incubated at r.t. for 30 min. After the final wash, peroxidase–conjugate antibody was detected by the addition of a substrate solution containing 3,3′,5,5′-tetramethylbenzidine (TMB) (PharMingen) after an incubation time of 30 min at r.t. After the addition of a stopping solution (2 M H_2SO_4), the absorbance at 450 nm was measured by a microplate reader with λ correction at 570 nm. A standard curve using recombinant mouse TNF-α was constructed.

3. Results and discussion

3.1. Carboxymethyled β-glucan

β-Glucan is an important candidate molecule as a biological response modifier implicated in cancer immunotherapy (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Mizuno, 1999). In order to explore and broaden the use of β-glucan as much as possible, a variety of native glucans and their chemical derivatives are required. Although the HAE fractions extracted with hot alkali were the major mushroom cell wall component of *P. tuber-regium* that had been demonstrated to have anti-tumor activity (Zhang et al., 2001), they were extremely difficult to be solubilized in water. Several protocols including some by our group have been proposed to solubilize the cell wall glucans by modifying them into sulfated, phosphated and carboxymethylated derivatives (Hamura, Yamashita, Ohsaka, Maeda, and Chihara, 1971, Kiho et al., 1994; Kiho, Yoshida, Nagai, & Ukai, 1989; Zhang, Zhang, Chen, & Zeng, 2001; Zhang, Zhang, Zhou, Chen, & Zeng, 2000). In this research, the HAE fractions were carboxymethylated to produce six water-soluble CMHAE fractions. The yield of the chemical derivatives (CMHAE-1, -2, -3, -4, -5 and -6) was found previously to be more than 70% and had a degree of substitution (DS) ranging from 0.37 to 0.68 as determined by elemental analysis (Zhang, Zhang, & Cheung, 2003). All the six CMHAE fractions obtained had a larger M_w (Table 1) than their corresponding native β-glucan (Zhang, Zhang, & Cheung, 2003) indicating the effectiveness of substitution of the hydroxyl groups in the glucose residues by the carboxymethyl groups and that no hydrolysis of the polysaccharidic chain had occurred.

3.2. In vivo antitumor activity

The anti-tumor activity of the carboxymethylated water-soluble polysaccharides from sclerotia of *P. tuber-regium* were evaluated by in vivo anti-tumor test using solid tumor S-180 implanted into the BALB/c mice, in vitro anti-tumor test using HL-60 leukemia cell line and HepG2 liver cancer cell line, and in vitro cytotoxic effect test using monkey kidney cell line (Vero).

The results of the in vivo assay of the inhibition of solid Sarcoma 180 tumor in BALB/c mice by CMHAE fractions are shown in Table 1. All CMHAE fractions with M_w ranging from 2.08×10^4 to 53.2×10^4 had a higher inhibition ratio of more than 40% when compared to the control. In our previous study (Zhang et al., 2001), the M_w and in vivo anti-tumor activities of the native mushroom polysaccharide HAE were determined. We found that when the native HAE fractions that showed relatively higher in vivo anti-tumor activities of 52, 76 and 48% (HAE-2, -3 and -4, respectively) (Zhang et al., 2001) were carboxymethylated to become water-soluble, the overall anti-tumor activities (48, 75 and 53%) of their corresponding counterparts (CMHAE-2, CMHAE-3 and CMHAE-4) only changed slightly. However, when the native HAE fractions (HAE-1, -5 and -6) that were ineffective in inhibiting the tumor cell growth in vivo (ineffective, 6% and ineffective) (Zhang et al., 2001) were converted to their carboxymethylated derivatives (CMHAE-1, -5 and -6), higher in vivo anti-tumor activities (64, 46 and 43%) were observed (Table 1). It was very interesting to find that CMHAE fractions with relatively lower M_w (2.08×10^4 and 3.78×10^4) and higher M_w (53.2×10^4) exhibited much stronger in vivo tumor

Table 1
Anti-tumor activities of carboxymethylated hot-alkali extracts (CMHAEs) from the sclerotia of *P. tuber-regium* against Sarcoma 180 solid tumor grown in BALB/c mice at a dosage of 20 mg/kg for 10 days

Carboxymethylated hot-alkali extract fractions	Molecular weight of CMHAE ($M_w \times 10^{-4}$)[a]	Inhibition ratio of CMHAE fractions (%)	Complete regression of tumor in CMHAE fractions	Hot-alkali extract fractions	Molecular weight of HAE ($M_w \times 10^{-4}$)[b]
CMHAE-1	53.2	64	4/15	HAE-1	42.2
CMHAE-2	28.4	48	4/15	HAE-2	17.1
CMHAE-3	25.6	75	0/15	HAE-3	9.8
CMHAE-4	9.25	53	0/15	HAE-4	5.8
CMHAE-5	3.87	46	0/15	HAE-5	2.2
CMHAE-6	2.08	43	1/15	HAE-6	1.0

[a] Previous results quoted (Zhang, Zhang, and Cheung, 2003) for comparison.
[b] Previous results quoted (Zhang, Cheung, and Zhang, 2001) for comparison.

inhibition activity than their corresponding native HAE fractions (HAE-1, -5, and -6, respectively).

3.3. In vitro antitumor activity

The inhibition ratio of in vitro tumor cell growth by the various CMHAE fractions is shown in Figs. 1 and 2 for the suspension (HL-60) and adherent (HepG2) cell lines, respectively. The in vitro anti-tumor activities against HL-60 and HepG2 cell lines of the native HAE fractions were also assayed in our previous work (Zhang et al., 2001). Unlike the native HAE fractions, all CMHAE fractions showed inhibition of the HL-60 leukemia cell growth at all concentration levels tested (Fig. 1). It should be noted that some CMHAE fractions with high M_w (CMHAE-1) and low M_w (CMHAE-4 to CMHAE-6) showed stronger inhibition of tumor cell growth than their corresponding native fractions which had no or little effect in inhibiting the tumor cell growth (Zhang et al., 2001). For instance, CMHAE-1 showed higher inhibition ratio of 28.44, 30.71 and 43.21% at a concentration of 50, 100 and 200 μg/ml, respectively, while the native HAE-1 only had 18.2, 0.5 and 27.0% inhibition ratio at the corresponding concentrations (Zhang et al., 2001). Similar results were observed in the lower M_w fractions of CMHAE-4, -5 and -6. However, no significant enhancement of anti-proliferation activities was observed for the CMHAE-2 and -3 which corresponding native HAE-2 and -3 had already shown relatively higher activities (Zhang et al., 2001) than the other HAE β-glucans. In the MTT assay, the enhancement of anti-proliferation activities found in HL-60 was not observed in the HepG2 cell culture (Fig. 2). Similar to our previous results (Zhang et al., 2001), no anti-proliferation effect of the CMHAE fractions on the normal Vero cells had been observed (data not shown).

In this research, it was found that when the native HAE fractions with intermediate M_w that showed relatively higher in vivo and in vitro anti-tumor activities (HAE-2 to -4) were carboxymethylated to become water-soluble, the overall anti-tumor activities of their corresponding counterpart (CMHAE-2 to -4) only changed slightly. However, when the native HAE fractions (HAE-1, -5 and -6) that were ineffective in inhibiting the tumor cell growth both in vivo and in vitro were converted to their carboxymethylated derivatives (CMHAE-1, -5 and -6), higher in vivo and in vitro anti-tumor activities were observed.

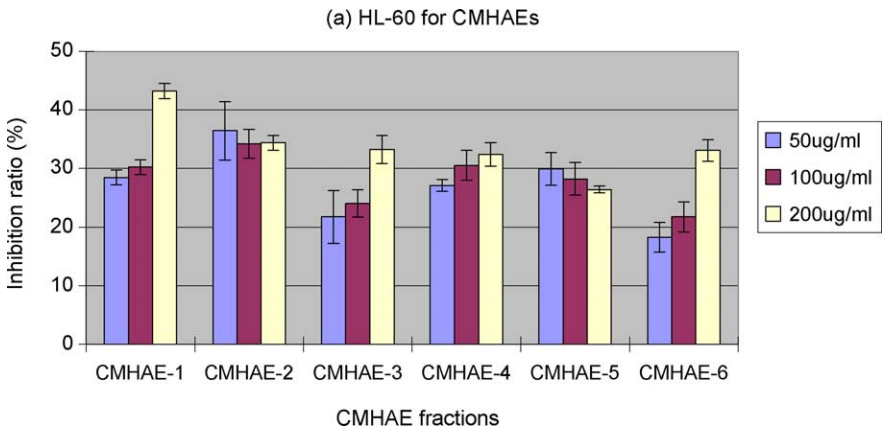

Fig. 1. Inhibition ratio (%) of proliferation of HL-60 leukemic cells by different concentrations (50, 100 and 200 μg/ml) of CMHAE fractions.

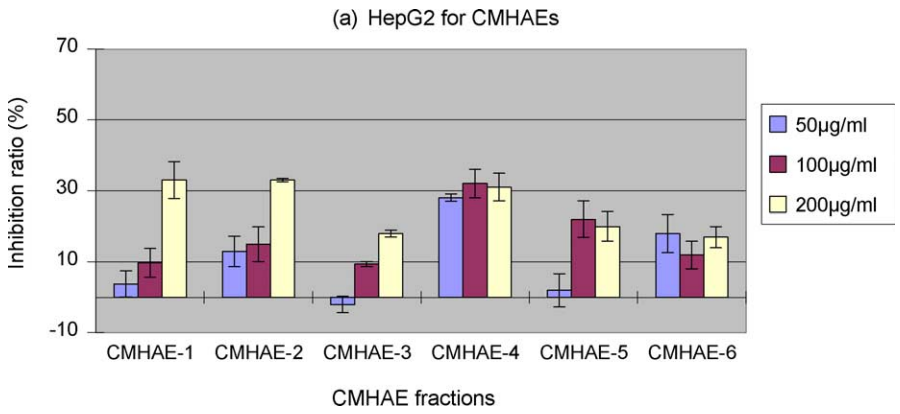

Fig. 2. Inhibition ratio (%) of proliferation of HepG2 liver cancer cells by different concentrations (50, 100 and 200 μg/ml) of CMHAE fractions.

3.4. TNF-α production and molecular structure

Cytokines which include TNF-α are an important contributing factor in immune and inflammatory reaction. Some of the β-glucans can induce the release of TNF-α from macrophages both in vitro and in vivo (Adachi et al., 1994). CMHAE-1, -2, -3, -5, which showed relatively higher anti-tumor activity, were administrated i.p. into BALB/c mice at a dose of 20 mg/kg and the TNF-α production in the plasma from the mice with and without LPS induction was measured using an ELISA method. No significant increase in the mouse plasma TNF-α level of the treatment groups (CMHAE) without LPS induction was found when compared to the control group (data no shown). However, intraperitoneal administration of CMHAE fractions to mice stimulated by LPS (10 μg/mouse) in vivo significantly increased TNF-α production (Fig. 3), suggesting that these β-glucans acted as a priming reagent but not as a triggering reagent for in vivo TNF-α production. While the HAE fractions stimulated by LPS with intermediate M_w ($10-17.1 \times 10^4$ g/mol) (HAE-2 and -3) had the most potent TNF-α priming effect (unpublished data), other HAE fractions with higher (HAE-1 with M_w of 42.2×10^4) and lower (HAE-5 with M_w of 2×10^4) M_w were less effective (unpublished data). However, all the CMHAE fractions exhibited higher TNF-α priming effects (Fig. 3). This indicated that the M_w range of the mushroom β-glucans that could effectively induce TNF-α production was broader in the CHMAE fractions than their native HAE fractions. Although administration of the CMHAE fractions did not directly release TNF-α in vivo, they produced an elevated concentration of this cytokine after LPS administration in vivo. These results suggested that CMHAE fractions acted as a priming reagent for the in vivo TNF-α production, which was consistent with other previous results (Ohno, Asada, Adachi, & Yadomae, 1995). It was also found that the CMHAE fractions (CMHAE-1 and CMHAE-3) with relatively higher inhibition to Sarcoma 180 tumor cell growth in vivo showed higher TNF-α priming effect, suggesting that the inhibition of the in vivo tumor cell growth might be mediated by the production of TNF-α stimulated by the mushroom β-glucan. The difference in

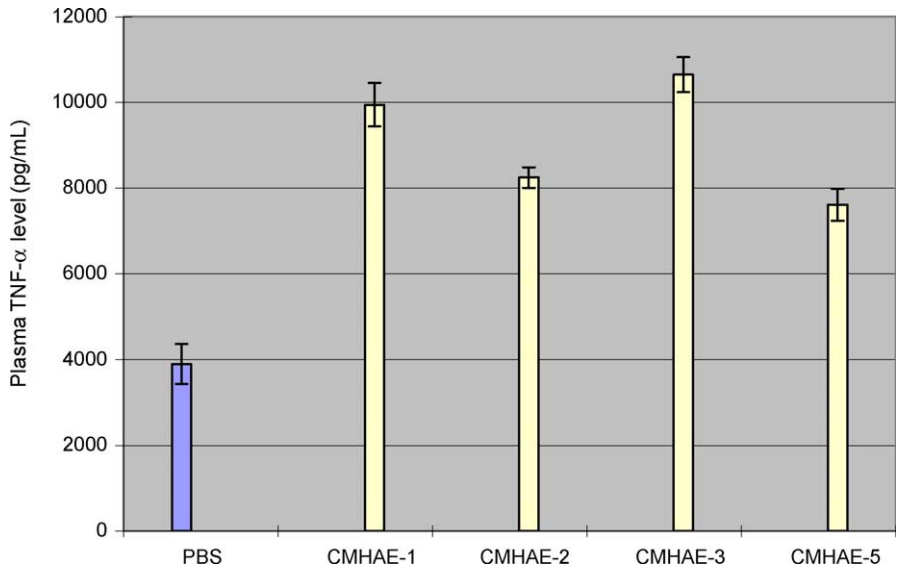

Fig. 3. Priming effect of CMHAE fractions on LPS-triggered TNF-α production in the plasma of mice (20 mg/kg body weight).

the anti-tumor activities and in the production of TNF-α for the mushroom β-glucan (HAE and CMHAE fractions) with different M_w might be attributed to the differences in the structural features arising from their molecular behavior, such as molecular size, molecular shape and the conformation adopted in solution when the HAE fractions were carboxymethylated. The molecular parameters of persistence length (q), molecular mass per contour length (M_L), characteristic ratio (C_∞) and exponent of Mark-Houwink equation (α) of the CMHAE fractions increased 2 to 3 times after carboxymethylation (data not shown) as compared to the native HAE fractions (Zhang et al., 2001). These results indicated that the conformation of the CMHAE fractions had changed from the original dense random coil found in the HAE fractions to an extended flexible chain after carboxymethylation. Thus, the enhanced antitumor activities and TNF-α production after carboxymethylation might be related to the increase in chain stiffness. It has been found in our previous work that an alkali-soluble α-D-glucan isolated from the mycelium of *Ganoderma lucidum* which adopted a random coil conformation in dilute alkali, was ineffective in inhibiting tumor cell growth, whereas the water-soluble carboxymethylated derivatives of the same glucan which adopted a more extended conformation than the native one, exhibited more potent anti-tumor activity (Zhang, Zhang, Chen, & Zeng, 2001). It has been reported that two other mushroom β-glucans, schizophyllan and lentinan lost their anti-tumor activity when their high-order structures (a triple helix or double helix which had a higher chain stiffness) were lost as indicated by a decrease in the values of the molecular parameters such as M_L, q and C_∞ as well as α following destruction of the helical conformation (Falch, Espevik, Ryan, & Stokke, 2000; Sasaki, Abino, Nitta, Takasuka, & Sugino, 1976; Tabata, Ito, Kojima, Kawabata, & Misaki,1981). Therefore, among many other factors, the possible change in the conformation of the water-soluble mushroom β-glucan due to carboxymethylation seemed to be an important factor in the enhancement of the anti-tumor activity of the CMHAE fractions.

4. Conclusion

Our results showed that the modified CMHAE fractions exhibited both anti-tumor activity to solid tumor Sarcoma 180 cell in vivo and direct cytotoxity to tumor cell lines in vitro. These results were quite different from the other native mushroom β-glucans such as schizophyllan and lentinan (Sasaki et al., 1976; Tabata et al., 1981,) and carboxymethylglucan of bacterial origin (Sasaki et al., 1979), all of which had no direct cytotoxicity to tumor cell lines in vitro. However, our results were similar to those of protein-bound polysaccharide (PSK, Krestin) and polysaccharopeptide (PSP) isolated from *Coriolus versicolor* that had both immomodulating activities and direct cytotoxicity to a wide range of tumor cell lines including HL-60 (Liu, Ooi, Liu, & Chang, 1996; Liu, Ng, Sze, & Tsui, 1993; Sakagami, Aoki, Simpson, & Tanuma, 1991; Yang et al., 1992).

Mechanistic studies on the anti-tumor effect of the CMHAE fractions are underway. These include further in vivo and in vitro experiments to ascertain the host mediated and cytocidal anti-tumor activity of the carboxymethylated mushroom β-glucan in different human cancer cell lines such as MCF-7 using murine models. Attempts will also be made to investigate the plausible cellular mechanism of the anti-proliferative activity of these mushroom β-glucans by flow cytometry and in vivo immunological studies involving induction of other cytokine production and gene expression.

All in all, carboxymethylation is effective in improving water solubility and enhancing both the in vivo and in vitro anti-tumor activity as well as the immunomodulatory effect of the native β-glucan from the sclerotia of *P. tuber-regium*. These findings might be useful in the application of mushroom β-glucans to the nutraceutical industry and the anticancer research.

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