Evaluation of Mushroom Dietary Fiber (Nonstarch Polysaccharides) from Sclerotia of *Pleurotus tuber-regium* (Fries) Singer as a Potential Antitumor Agent

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Mushroom dietary fiber or nonstarch polysaccharides (NSPs) that were soluble in hot alkali and belonged to the β-glucan type were isolated from the sclerotia of an edible mushroom, *Pleurotus tuber-regium*. The mushroom NSPs were further separated into a number of fractions [hot alkali extracts (HAEs)] with weight-average molecular weights ($\bar{M}_w$) ranging from 1×10^4 to 42.2×10^4. The HAE fractions [with M_w of $(5.8-17.1) \times 10^4$] administered intraperitoneally at a dose of 20 mg/kg of body weight to BALB/c mice implanted with solid tumor Sarcoma 180 were found to be effective in inhibiting tumor proliferation with an inhibition ratio of ≥50%. In vitro experiments using human tumor cell lines HL-60 and HepG2 had shown that HAE fractions with $\bar{M}_w$ of $(5.8-42.2) \times 10^4$ also had antiproliferative activity at three different concentrations (50, 100, and 200 μg/mL) toward the tumor cell lines tested. All HAE fractions did not inhibit the growth of a normal kidney cell line (Vero) from monkey. It is therefore postulated that the antitumoral effect of NSPs from the sclerotia of *P. tuber-regium* is probably host-mediated and cytocidal.

Keywords: *Mushroom dietary fiber; nonstarch polysaccharide; Pleurotus tuber-regium; antitumor activity*

INTRODUCTION

A number of nonstarch polysaccharides (NSPs) isolated from mushroom such as grifolan from *Grifola frondosa*, lentinan from *Lentinus edodes*, and schizophyllan from *Schizophyllum commune* have been reported to have antitumor activity (*1*, *2*). The mushroom NSPs varied in their chemical compositions and structures, especially in terms of molecular weight and conformations (*3*). They include β-D-glycans, heteroglycans, and polysaccharide−protein complexes isolated mostly from the fruiting bodies, cultured mycelia, and culture filtrates (*4*). Reports on the antitumor activity of NSPs from mushroom sclerotia, which are dried masses of fungal hyphae, are rare (*5*). Sclerotia of *Pleurotus tuber-regium* (Fries) Singer have been commonly consumed in Africa for some time (*6*) and have gained an emerging popularity in China recently (*7*). Our previous studies have shown that the sclerotia of *P. tuber-regium* are extremely rich in dietary fiber or NSP that are mainly composed of β-glucans (*8*, *9*).

It has been demonstrated from in vivo murine models that the antitumor activity of mushroom NSPs such as lentinan is mediated by the host immune system rather than a direct cytotoxic action (*10*). However, direct cytotoxicity to tumor cells and indirect cytotoxicity caused by cell-mediated immune responses have also been shown in some in vitro studies for other mushroom NSPs (*11*, *12*). Moreover, much controversy still surrounds the correlation between the structural features of NSPs with their antitumor activity. It has been suggested that high molar mass, a triple helix, and a β-(1→6) branch are favorable structural parameters, whereas other authors have considered that single helices and β-(1→3) glycosidic linkage are more important factors in influencing the antitumoral effect (*3*, *13*). Preliminary in vivo studies of antitumor activity of mushroom NSPs have often been based on the inhibition of growth of an allogeneic solid tumor, Sarcoma 180, implanted subcutaneously in mice (*1*, *10*). The cytotoxic effect of mushroom NSPs can be assessed by in vitro studies using tumor cell lines (*11*). In this paper, we have evaluated both in vitro and in vivo antitumor activities of some β-glucan-type alkali-soluble NSP fractions with molecular weights ranging from 1×10^4 to 42.2×10^4 purified previously from the sclerotia of *P. tuber-regium* (*14*). The potential use of these mushroom NSPs as an antitumor agent will be discussed.

MATERIALS AND METHODS

Materials. Sclerotia of *P. tuber-regium* were cultivated by the Sanming Mycological Institute in Fujian, China. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO). Human acute promyelocytic leukemia HL-60 (ATCC CCL-240), human hepatocellular carcinoma HepG2 (ATCC HB-8065), and monkey normal kidney Vero (AACT CCL-81) cell lines were purchased from the American Type Culture Collection (Rockville, MD).

Isolation and Fractionation of Alkali-Soluble NSPs. Polysaccharides of *P. tuber-regium* were isolated as described elsewhere (*14*). In brief, the sclerotia (500 g) were sequentially washed with ethyl acetate, acetone, 0.9% sodium chloride, and cold dilute alkali solution before extraction with 0.5 M sodium hydroxide at 120 °C. The hot alkali extract (HAE) was dialyzed to remove low molecular weight substances, and the retentate

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was evaporated to give a white powder (8% yield). The HAE was redissolved in dimethyl sulfoxide (DMSO) and fractionated by fractional precipitation by gradual addition of a mixture of acetone and DMSO (4:1 v/v). Each HAE fraction was reprecipitated by aqueous acetone.

Structural Characterization of HAE Fractions. Structural information on the HAE fractions was obtained by use of a Nicolet infrared Fourier transform spectrometer (IR spectra), Kjeletc 1030 semimicro Kjeldhal automatic analyzer (protein content), JEOL JNM-LA-500 spectrometer (carbon-13 NMR spectra), and GC-7 gas chromatograph (monosaccharide composition and absolute configuration). The molecular weights of the HAE fractions were determined by an HPLC system with dual GPC columns coupled with a multiangle laser photometer (DAWN DSP, Wyatt Technology Co.) and a differential refractive index detector. Detailed experimental procedures had been described previously (*14*).

In Vivo Antitumor Test. Sarcoma 180 cells (1×10^5 cells/mouse) were subcutaneously inoculated into 8-week-old BALB/c male mice. HAE fractions (20 mg/kg) dissolved in 10% DMSO in phosphate buffer solution (PBS) were injected intraperitoneally (ip) once daily for 10 days at 72 h after tumor inoculation. The same volume of 10% DMSO in PBS was injected ip into the control mice. The tumor was allowed to grow on the mice for 7 days before it was removed from the animal and weighed. The antitumor 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 treated groups, respectively.

In Vitro Proliferation and Cytotoxicity Assays. *Dye Exclusion Method for Suspended Cells.* The HL-60 leukemic 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 HAE fractions at concentrations of 50, 100, and 200 μg/mL in 10% DMSO solution. The survival rate of the mammalian cells was assayed by counting living cells that excluded the Trypan blue dye by a hemacytometer.

Colorimetric MTT Method for Adherent Cells. Mammalian HepG2 cells and Vero cells (1×10^5 cells/mL) were incubated separately with the HAE fractions and allowed to grow under the same condition as the HL-60 cells mentioned above in RPMI 1640 medium supplemented with 10% fetal bovine serum and in MEM medium, respectively. 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 (*15*). In the above two assays, the treatment samples were compared with control samples in the absence of the HAE 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 samples were done in triplicates.

RESULTS

Structural Characteristics. The fractionated HAEs were designated according to the weight-average molecular weight found by the GPC-MALLIS procedures mentioned earlier (Table 1). Our previous results had also shown that all of the HAE fractions were composed of a backbone chain of β-(1→3) linked D-glucose residues with an average of a single β-D-glucopyranosyl group at O-6 of every third glucose residue (*14*), which was consistent with a recent paper (*16*). Moreover, the HAE fractions existed as a flexible random-coil chain in DMSO (*14*).

In Vivo Results. The weight-average molecular weight ($\bar{M}_w$) of the HAE fractions and the results of the in vivo assays of antitumor activities are shown in Table 1. HAE fractions with $\bar{M}_w$ ranging from 5.8×10^4 to 17.1×10^4 had an inhibition ratio of >40% and HAE-10 had the highest inhibition ratio of 76% as compared to the control. HAE-42 and HAE-1 showed a negative inhibition ratio, indicating that they were ineffective in suppressing the growth of the Sarcoma 180 tumor cells.

Table 1. Antitumor Activities of Hot-Alkali Extracts (HAEs) from the Sclerotia of *P. tuber-regium* against Sarcoma 180 Solid Tumor Grown in BALB/c Mice

HAE fraction	mol wt ($\bar{M}_w \times 10^{-4}$)	dose (mg/kg × days)	inhibition ratio (%)	complete repression
HAE-42	42.2	20 × 10	*a*	0/7
HAE-17	17.1	20 × 10	52	1/7
HAE-10	9.8	20 × 10	76	0/6
HAE-6	5.8	20 × 10	48	0/6
HAE-2	2.2	20 × 10	6	0/6
HAE-1	1.0	20 × 10	*a*	0/6

[a] No inhibition of the growth of Sarcoma 180 solid tumor in BALB/c mice.

In Vitro Results. The inhibition ratios of tumor cell growth by the various HAE fractions are shown in Figures 1 and 2 for the suspension (HL-60) and adherent (HepG2) cell lines, respectively. Regarding the antiproliferation of suspended HL-60 leukemic cells, HAE fractions with $\bar{M}_w$ ranging from 5.8×10^4 to 42.2×10^4 had relatively stronger inhibitions of the tumor cell growth at all concentration levels tested than the other low $\bar{M}_w$ HAE fractions (Figure 1). No obvious dose−response relationship was observed between the concentration of HAE and the growth inhibition of HL-60 cells (Figure 1).

In the MTT assay, whereas HAE fractions with $\bar{M}_w$ ranging from 5.8×10^4 to 17.1×10^4 inhibited proliferation of the HepG2 cells at all three concentration levels tested (Figure 2), no antiproliferation effect of the HAE fractions on the Vero cells was observed (data not shown). In both in vitro experiments, HAE-17 seemed to be the most effective NSP fraction in inhibiting the proliferation of the two tumor cell lines with the highest inhibition ratios of 44.5% (at 50 μg/mL) and 63.4% (at 100 μg/mL) for HL-60 and HepG2 cell lines, respectively. It was also found that the solvent system used to dissolve the HAE fractions (10% DMSO solution) was not cytotoxic to any of the three cell lines (data not shown).

DISCUSSION

Our in vivo results were in agreement with a previous study on the antitumor activity of water-soluble NSPs isolated from the sclerotia of *Grifora umbellate* against Sacroma 180 tumor cells (*5*). This could be explained by the presence of a common structural unit containing a trisaccharide of β-(1→3) linkage with a branch at position 6 found in the HAE fractions of *P. tuber-regium* and the NSPs from *G. umbellate* (*5*). In fact, other commonly known antitumor mushroom NSPs such as schizophyllan and lentinan, although not obtained from sclerotia, also share the same chemical structure as mentioned above (*3*). The range of $\bar{M}_w$ values in the HAE fractions [$(5.8-17.1) \times 10^4$] that demonstrated substantial antitumor activity was comparatively lower than that of native lentinan and schizophyllan, which was $>40 \times 10^4$ (*17*, *18*). However, modified lentinan (by partial hydrolysis) and schizophyllan (by ultrasonic degradation) with similar chemical structures but having lower molecular weights ($<20 \times 10^4$) exhibited antitumor activities against Sacroma 180 similar to those of their corresponding native ones that had higher molecular weight (*17*, *19*).

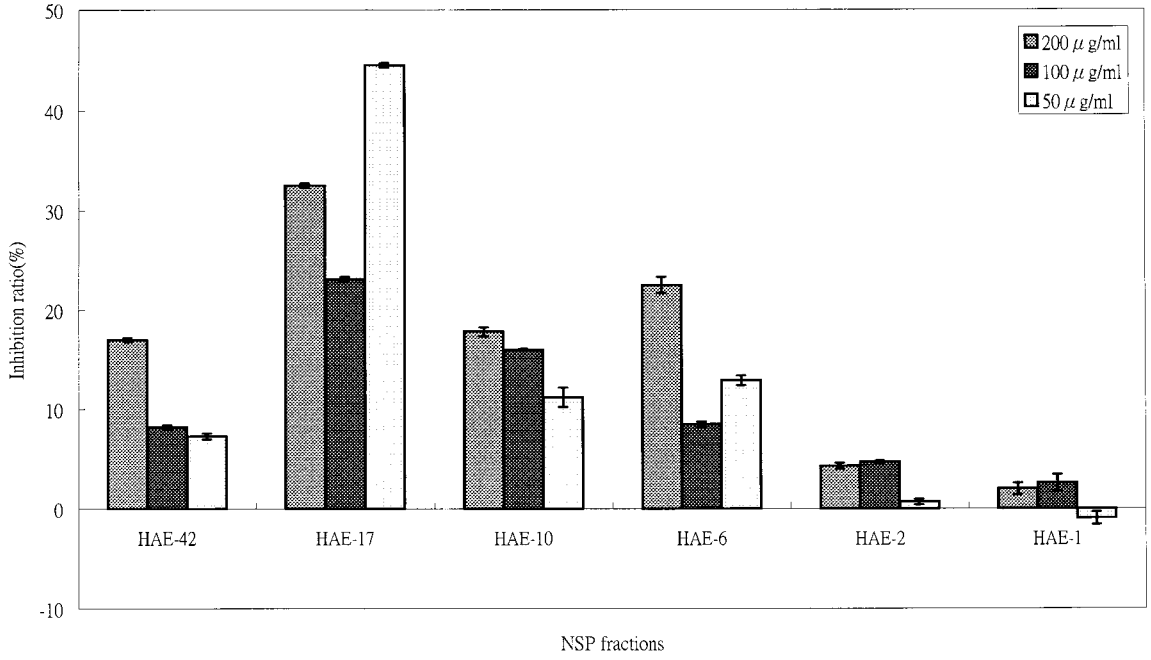

Figure 1. Inhibition of proliferation of HL-60 leukemic cells by different concentrations of HAEs with different molecular weights from the sclerotia of *P. tuber-regium*.

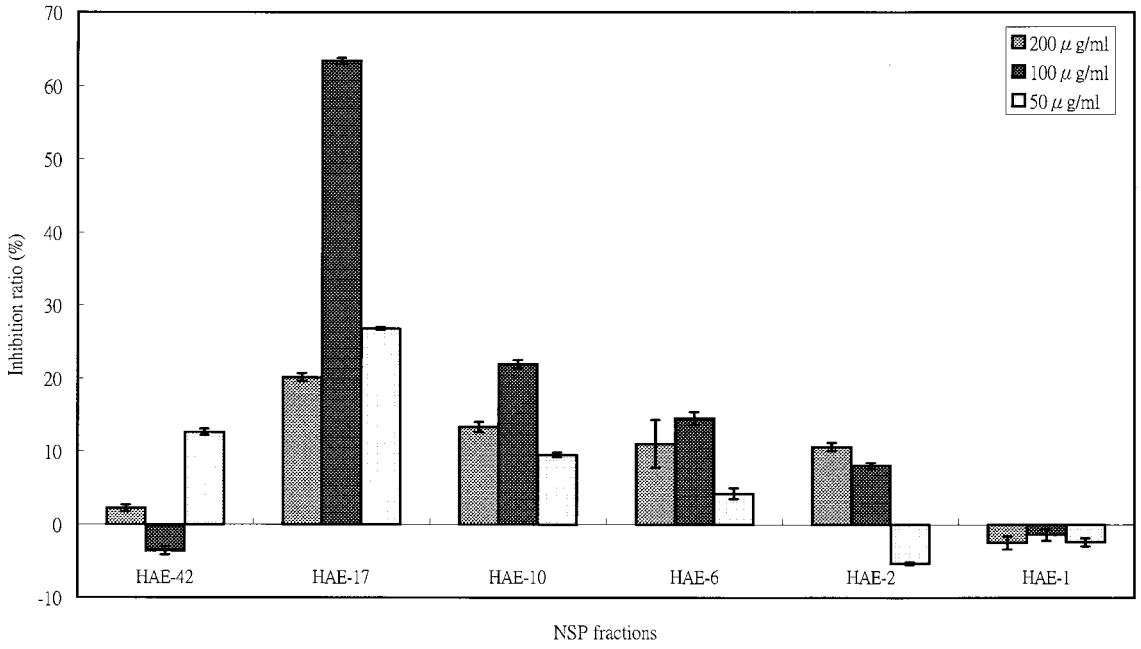

Figure 2. Inhibition of proliferation of HepG2 liver cancer cells by different concentrations of HAEs with different molecular weights from the sclerotia of *P. tuber-regium*.

Our in vitro results showed that HAE fractions with $\bar{M}_w$ in the range of (5.8−17.1) × 10^4 directly inhibited the growth of tumor cell lines including HL-60 and HepG2 but had no effect on normal kidney cell line. Although antitumor mushroom NSPs such as lentinan and schizophyllan showed no direct cytotoxicity to tumor cell lines in vitro (*20*, *21*), protein-bound polysaccharide (PSK, Krestin) and polysaccharopeptide (PSP) isolated from *Coriolus versicolor* had direct cyototoxicity to a wide range of tumor cell lines including HL-60 (*11*, *12*).

From the present in vivo and in vitro results, we suggested that HAE fractions with $\bar{M}_w$ <2 × 10^4 and >42.2 × 10^4 seemed to be ineffective in the inhibition of tumor cell growth. HAE fractions with $\bar{M}_w$ ranging from 5.8 × 10^4 to 17.1 × 10^4 were found to have more pronounced antitumor activities both in the BALB/c mice model and in cancer cell line tests.

It is also interesting to note that whereas the most common antitumor mushroom NSPs such as lentinan and schizophyllan have a triple-helix conformation (*18*, *22*), the HAE fractions used in the present study adopted a random-coil chain conformation in DMSO (*14*). Molecular weight and conformation of β-glucans have been shown to influence the immune stimulating effects to various extents (*13*). However, detailed structure−function relationships, especially the molecular mechanisms of the interactions of β-glucans to their target cells, remain unclear.

Our preliminary results indicated that the β-glucan type NSPs with medium molecular weight and random-

coil chain conformation isolated from the sclerotia of *P. tuber-regium* seem to have antitumor activity that is host mediated and cytocidal. Further in vivo experiments will be carried out to ascertain the antitumoral effect of the HAE fractions in murine syngeneic and autochthonous hosts. Although the mode of cytotoxicity of the HAE fractions is still unknown, attempts will be made to investigate plausible cellular mechanisms of the antiproliferative activity of these mushroom NSPs. For example, experiments to investigate the direct inhibition of RNA, DNA, and protein synthesis as well as induction of specific and/or specific functions of the immune system including macrophages, T cells, and natural killer cells will be conducted. All in all, NSPs from the sclerotia of *P. tuber-regium* are potential antitumor agents, and the confirmation of their curative effect to cancers would be of great interest both to the nutraceutical industry and to the medical field.

ABBREVIATIONS USED

NSP, nonstarch polysaccharide; HAE, hot alkali extract; $\bar{M}_w$, weight-average molecular weight; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MALLIS, multiangle laser light scattering; RMPI, Roswell Park Memorial Institute; MEM, minimum essential medium.

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