Wide Range of Molecular Weight Distribution of Mitogenic Substance(s) in the Hot Water Extract of a Chinese Herbal Medicine, *Bupleurum chinense*

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In this study, we examined the contribution of lignin-like materials in lower molecular weight (MW) fractions from the hot water extract of *Bupleuri Radix* (*Bupleurum chinense*) (HWE-BR) for their immunopharmacological activities. Mitogenic activity was detected in all the fractions of MW ranges: lower than 1.0 kDa, 1.0–3.5 kDa, 3.5–10 kDa, and 10–50 kDa. After NaClO₂ treatment of these subfractions, UV spectra, ESR spectra, mitogenic activities on murine B-cells, and the activity of inducing nitric oxide in RAW 264.7 cells were significantly reduced, suggesting that lignin-like polyphenolic substance(s) of various MW might take part in these activities. The intensity of ESR spectra and mitogenic activities were stronger in higher MW subfractions, thus the content of stable radical species and/or the degrees of polymerization would be important for their immunopharmacological activities.

Key words lignin; polyphenol; B-cell mitogen; nitric oxide; *Bupleurum chinense*

Kampo medicines (KMs) have been applied to treat many kinds of acute and chronic disease. The efficacy of KMs have been accumulated from clinical experience spanning 2000 years in China. In recent years, many studies have been carried out to evaluate KMs pharmacological activities at the molecular level. The basic concept of the therapy using these medicines is to control the homeostasis of the whole body by the action of various components that normalize patients suffering from over-activated or depressed states. KMs have thus been considered as biological response modifiers (BRMs).

In Japan, more than 200 KMs prescriptions have been used clinically. *Bupleuri Radix* (B. Radix) is a crude drug used in various prescriptions so-called Saikozai, which means prescriptions containing B. Radix (Saiko). One Saikozai prescription, Sho-saiko-to (SST) has been used very widely in the treatment of chronic diseases, e.g. hepatitis, asthma and nephrotic syndrome, and for the prevention of harmful after-effects of steroids and anticancer drugs. A recent study revealed that SST showed various immunomodulating activities and that each activity is mainly dependent on particular crude drugs: interferon-inducing activity by *Glycyrrhizae Radix*, interleukin (IL)-6 by *Pinelliae Radix*, and mitogenic activity by B. Radix.¹⁾ This is similar to the observations that Yamashiki *et al.* reported, that *Glycyrrhizae Radix* and *Scutellariae Radix* are major components attributing to IL-1 β , tumor necrosis factor- α , and granulocyte colony stimulating factor production from peripheral mononuclear cells.²⁾ These findings suggest that characteristic pharmacological activities are due at least in part to the immunopharmacological substances in crude drugs specific for each activity.

Previously, Oka *et al.* reported that the major mitogenic substances in the hot water extract of B. Radix (*Bupleurum chinense*) (HWE-BR) are from macromolecules (ca. 200 kDa) defined as polyphenolic compounds and poly-

saccharides.³⁾ Immunopharmacologically active polyphenolic substances in *Bupleurum chinense* (B. chinense) are considered to have a lignin-like moiety based on the UV spectra and their susceptibility to NaClO₂, a kind of polyphenol degrading reagent. Thus, lignin-like substances might play an important role in B. chinense, at least as a part of a mitogen.

Plant lignin, a major cell wall component, is thought to be generated through dehydrogenative polymerization from monolignols, such as *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, and catalyzed by peroxidase in the presence of H₂O₂.⁴⁾ Therefore, it is no wonder that lignin-like polyphenolic substances would show a wide range of molecular weight (MW) distribution. It should also be considered that the administration route of the crude drug is, in general, oral, and the physicochemical property of the substance, especially MW, is an important contributing factor for intestinal absorption. In this study, we performed further fractionation of HWE-BR and demonstrated that HWE-BR fractions with a MW of less than 50 kDa also contain a lignin-like moiety and take part in the immunopharmacological activities of B. chinense.

MATERIALS AND METHODS

Mice Specific-pathogen-free male C3H/HeJ mice (6- to 12-week-old) were obtained from Japan SLC Co. (Shizuoka, Japan).

Media RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan) was supplemented with 5 mM HEPES (Sigma), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan).

Cells Macrophage-like cell line RAW 264.7 cells (RAW) (obtained from Riken Cell Bank, Tsukuba, Japan) were maintained in plastic culture flasks in RPMI 1640 medium supplemented with 10% heat-inactivated fetal

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bovine serum (FBS) (Moregate, Australia) (complete RPMI medium). For experiments, the cells were removed from the culture flask by wiping the bottom of the flask with a Cell Scraper™ (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), and were centrifuged (1200 rpm, 5 min). Then, the cells were washed twice with RPMI medium and resuspended in complete RPMI medium to a concentration of 5×10^5 cells/ml.

Reagents *B. Radix* (*Bupleurum chinense* DC, Umbelliferae) was kindly provided from Kanebo Co., Ltd. (Osaka, Japan). Sephadex G-25 was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). A BCA™ protein assay reagent was obtained from Pierce (Rockford, IL, U.S.A.). *p*-Nitrophenol (PNP), *p*-nitrophenylphosphate (P-NPP), and 2',2'-iminodiethanol (diethanolamine) were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). Sulfanilamide, *N*-1-naphthylethylenediamine dihydrochloride, and H_3PO_4 used for Griess reagent were also obtained from Wako. Lipopolysaccharide (LPS) prepared from *Escherichia coli* 0111: B4, according to the method of Westphal, was purchased from Sigma.

Preparation and Fractionation of HWE-BR *B. Radix* (200 g) was boiled with 4 l of de-ionized water (DIW) for 1 h. After removing insoluble fragments with gauze mesh, the extract was concentrated to about 200 ml. This concentrated extract was precipitated with 4 l of EtOH. After 1 d, the resulting suspension was centrifuged (3000 rpm, 5 min) to give EtOH soluble and insoluble fractions. The EtOH insoluble fraction was dissolved in 200 ml of DIW and centrifuged to collect the supernatant. The resulting supernatant was then dialyzed [molecular weight cut-off (MWCO): 1.0 kDa] against DIW to give MW: <1.0 kDa and >1.0 kDa fractions. The MW: >1.0 kDa fraction was dialyzed (MWCO: 3.5 kDa) against DIW to give MW: 1.0–3.5 kDa and >3.5 kDa fractions. The MW: >3.5 kDa fraction was dialyzed (MWCO: 10 kDa) against DIW to give MW: 3.5–10 kDa and >10 kDa fractions. The MW: >10 kDa fraction was dialyzed (MWCO: 50 kDa) against DIW to give MW: 10–50 kDa and >50 kDa fractions. These fractions were lyophilized.

Treatment with $NaClO_2$ Fifty milligrams of each MW: 1.0–3.5 kDa, 3.5–10 kDa, and 10–50 kDa fraction was dissolved in DIW to a concentration of 1 mg/ml and then treated with 5 mg/ml $NaClO_2$ in 4% CH_3COOH for 40 min at 70 °C. After incubation, each reaction mixture was dialyzed (MWCO: 1.0 kDa) against DIW, and the non-dialyzable fraction was lyophilized.

Spectral Measurements UV spectra were taken in an aqueous solution at room temperature with a Hitachi Model No. 557 spectrophotometer. ESR spectra were measured in solid state at room temperature with a JEOL JES-RE1X spectrometer.

Mitogen Assay Spleen cell suspensions from male C3H/HeJ mice were prepared by teasing spleens in cold RPMI 1640 medium. The cell suspensions were passed through a 200 gauge stainless steel sieve. The cell suspensions were centrifuged (1200 rpm, 5 min), then washed twice with fresh medium. Serially diluted 50 μ l of sample solutions in RPMI medium and 200 μ l of spleen cell suspensions (2.5×10^6 cells/ml in complete RPMI medium) were mixed and incubated in a flat-bottomed

96-well tissue culture plate for 72 h at 37 °C under 5% CO_2 . The mitogenic activity of spleen cells was assessed by alkaline phosphatase activity especially expressed by B lymphocytes^{5–7)} as follows: 100 μ l of 0.1% *p*-nitrophenylphosphate disodium salt (dissolved in 10% diethanolamine-HCl buffer, pH 9.5) was added to 50 μ l of cell suspension. Each mixture was incubated at 37 °C for 30 min. The absorption (Abs.) at 405 nm (reference: 630 nm) was measured before and after incubation. Δ Abs. was used to subtract the light absorption originate from sample's color. Δ Abs. = Abs.(after incubation) – Abs.(before incubation). Released PNP content was quantified using PNP solution as a standard. The significance was evaluated by Student's *t*-test.

Cell Culture and Determination of Nitric Oxide (NO) as Nitrite Serially diluted 50 μ l of sample solutions in RPMI medium and 200 μ l of RAW suspensions (5×10^5 cells/ml in complete RPMI medium) were mixed and incubated in a flat-bottomed 96-well tissue culture plate for 24 h at 37 °C under 5% CO_2 . After incubation, culture fluids were assayed for nitrite contents by the Griess reaction as follows: 50 μ l of culture fluids were incubated with 50 μ l of Griess reagent composed of sulfanilamide (1%), *N*-1-naphthylethylenediamine dihydrochloride (0.1%), and H_3PO_4 (2.5%) at room temperature for 10 min. The absorbance at 550 nm was measured using the microplate reader MTP-32 (Corona Electric Co., Ltd., Tokyo). The nitrite level was quantitated using $NaNO_2$ solution as a standard. The significance was evaluated by Student's *t*-test.

RESULTS

Physicochemical Properties of Fractionated HWE-BR HWE-BR was prepared by hot water extraction followed by ethanol precipitation as described in Materials and Methods. HWE-BR was further fractionated by dialysis against de-ionized water to give subfractions having MW: <1.0 kDa, 1.0–3.5 kDa, 3.5–10 kDa, 10–50 kDa, and >50 kDa. Table 1 shows the recovery of each fraction from 200 g of *B. chinense*. MW: <1.0 kDa fraction was the main fraction of HWE-BR.

A previous report suggested that the UV spectra of the polyphenolic substances in HWE-BR contributing to mitogenic activity resembled that of the commercially available polyphenol, lignin.³⁾ Thus, it was useful to examine the UV spectra of the fractions obtained in this experiment. As shown in Fig. 1, UV spectra of all fractions examined were similar to those of the commercially available lignin and the higher MW fraction of HWE-BR. In addition, these spectra were significantly reduced by $NaClO_2$ treatment, a kind of polyphenol degrading reagent. These facts suggest that the lower MW fractions also contain significant amounts of polyphenolic substances, in addition to carbohydrates and proteins.

Sakagami *et al.* reported that either lignins isolated from lignified natural products or synthetic lignins prepared from phenylpropanoids through dehydrogenative polymerization gave an intense ESR signal at $g=2.003$ due to organic free radicals in the solid state.^{8,9)} Thus, measurement of ESR spectra was another useful method

Table 1. Physicochemical Analysis of Fractionated HWE-BR^{a)}

Molecular weight (kDa)	<1.0	1.0—3.5	3.5—10	10—50	>50
Yield (g) from Bupleuri Radix (200 g)	7.04±0.98	0.28±0.11	0.55±0.26	0.59±0.11	2.46±0.20
BCA (%) ^{b)}	1.5	4.9	4.8	4.1	
Carbohydrates (%) ^{b)}	10.2	23.2	28.1	29.8	N.D. ^{c)}
ESR-R.I. value	0.1687	0.9890	1.0536	1.5017	
<i>g</i> value	2.0055	2.0053	2.0055	2.0054	
After NaClO ₂ treatment ^{d)}					
Yield (mg)		10 (20%) ^{e)}	10 (20%)	19 (38%)	
BCA (%)		1.6	1.4	1.1	
Carbohydrates (%)		18.3	16.7	34.9	N.D.
R.I. value		0.1613	0.2787	0.2592	
<i>g</i> value		2.0055	2.0051	2.0053	

a) HWE-BR was fractionated by dialysis as described in Materials and Methods. b) The content of BCATM reaction positive substances was assessed as tannic acid. The content of carbohydrates was assessed as glucose. c) Not done. d) Each MW: 1.0—3.5 kDa, 3.5—10 kDa, and 10—50 kDa fraction was treated with NaClO₂ as described in Materials and Methods. ESR spectrum of each resulting material was measured in solid state at room temperature with a JEOL JES-REIX spectrometer. e) % recovery in parentheses.

Fig. 1. UV Spectra of Fractionated HWE-BR

Each MW: 1.0—3.5 kDa (A), 3.5—10 kDa (B), and 10—50 kDa (C) fraction was treated with NaClO₂, as described in Materials and Methods. Each resulting product was dissolved in DIW at a concentration of 50 µg/ml, and each UV spectrum was measured at room temperature. Treated (—) and parent samples (—).

to clarify the physicochemical properties of the mitogen in *B. chinense* as a lignin-like polyphenol. Thus, we next investigated whether the lower MW fractions of HWE-BR also gave an ESR signal at $g = 2.003$. We obtained a value of relative intensity (R.I.) from ESR spectral measurement. R.I. values were obtained in comparison with the signal intensity of an external standard, Mn²⁺. As shown in Table 1, intense ESR signals were obtained from MW: 1.0—3.5 kDa, 3.5—10 kDa, 10—50 kDa fractions. The R.I. value of MW: <1.0 kDa fraction was the weakest of all the fractions. These R.I. values show that the intensity of the ESR signals seems to be dependent on the amount of radical species in each fraction. NaClO₂ treatment is a polyphenol degrading reaction. In this paper, we also examined the effect of NaClO₂ on the physicochemical properties and immunopharmacological activities of these fractions. After NaClO₂ treatment and dialysis, the recovery of each fraction was significantly reduced to about 20—40%, and each R.I. value of the reconstituted fractions was significantly reduced, suggesting that these fractions contained significant amounts of polyphenolic substances and that their ESR signals might be dependent, at least in part, on substances having a lignin-like moiety. These observations were followed by the results that NaClO₂ treatment more effectively reduced BCATM reactive substances than carbohydrates (Table 1).

Immunopharmacological Activities of Fractionated HWE-BR The mitogenic activities of these fractions were compared, and it was found that the activity was stronger in higher MW fractions (Fig. 2). Figure 2 shows the mitogenic activity of NaClO₂ treated fractions: MW: 1.0—3.5 kDa (Fig. 2B), 3.5—10 kDa (Fig. 2C), and 10—50 kDa fractions (Fig. 2D), and the activities of all these fractions were significantly reduced by this treatment.

Recently, we reported an *in vitro* study in which KMs, including SST, showed NO-inducing activity on a macrophage cell line, RAW.¹⁰⁾ Dialysis of the water-soluble fraction of KMs made it clear that the macromolecular fraction of KMs contains specific substances to stimulate macrophage NO synthesis. In contrast, the corresponding low molecular weight fractions had no NO-inducing activity. This suggested that characteristic pharmacological activities would be due to the specific immunopharma-

Fig. 2. Mitogenic Activity of Fractionated HWE-BR

Mitogenic activity of the samples including MW: <1.0 kDa fraction is shown in Fig. 2A; MW: <1.0 kDa (●), MW: <1.0–3.5 kDa (○), MW: 3.5–10 kDa (△), and MW: 10–50 kDa (□). Changes in mitogenic activity of each MW: 1.0–3.5 kDa, 3.5–10 kDa, and 10–50 kDa fraction after NaClO₂ treatment is shown in Fig. 2B (●), Fig. 2C (▲) and Fig. 2D (■), respectively. The activity of the control group was shown as x. The data represents the mean ± S.D. The significance was evaluated by Student's *t*-test against the corresponding parent sample. **p* < 0.05. ***p* < 0.01. ****p* < 0.001.

cological substances included in KMs. As shown in Fig. 3A, all samples examined could induce NO from RAW. Similarity to the mitogenic activity, the NO-inducing activities of all these fractions were significantly reduced after NaClO₂ treatment (Figs. 3B, C and D).

DISCUSSION

In this paper, we examined the contribution of lignin-like materials, having a MW of less than 50 kDa fraction of HWE-BR, for their immunopharmacological activities. We prepared several subfractions by dialysis against water to obtain MW: <1.0 kDa, 1.0–3.5 kDa, 3.5–10 kDa, 10–50 kDa, and >50 kDa fractions. The UV spectra, ESR signals, mitogenic activity and NO-inducing activity of all fractions were significantly reduced by NaClO₂ treatment, suggesting the presence of a lignin-like moiety. Examination of the biological activities of the lignin-like substances from natural and synthetic sources showed various kinds of activities, suggesting that lignin-like substances are thus important as potential medicinal resources.^{11–13} Meanwhile, mitogenic activity and NO-inducing activity still remained positive even partially after NaClO₂ treatment, suggesting that the lignin-like substances may contribute to these activities. Proteins were already reported to be considerable mitogenic substances.¹⁴ However, in the case of the active part of the

fraction of HWE-BR with a MW larger than 200 kDa, the intact protein part might not relate to the mitogenic activity, because pronase digestion did not abrogate the activity.³

Preparation of KMs involves many kinds of interactions of constituents giving rise to various events, such as ion-pair formation, which generates water insoluble precipitates.^{15,16} We previously reported that an amino acid, arginine, is one of the ingredients that appear to undergo some kind of modification during the preparation of KMs.¹⁷ The decoction of crude drugs is associated with such a phenomena. Therefore, a certain part of the immunopharmacologically active substances in KMs might be end products from heat denaturation. Oka *et al.* recently reported that proteins in *B. chinense* might undergo heat denaturation during decoction.¹⁸ Shinohara *et al.* reported that the mitogenic substance in *Sclerotinia sclerotiorum* IFO 9395 is a protein, named sclerogen, which is only active after heat denaturation.^{19,20} Heat denatured substances such as polymerized proteins serve as active ingredients. Although, polyphenolic and heat denatured materials are included in most KMs, it is important that not all of them show mitogenic activity and/or NO-inducing activity. A certain specific functional group(s) might be required for these activities.

Our results suggest that the immunopharmacologically active substance in the lower MW fraction from HWE-BR

Fig. 3. NO Synthesis of RAW 264.7 cells by Fractionated HWE-BR

NO-inducing activity of the samples, including MW: <1.0 kDa fraction, is shown in Fig. 3A; MW: <1.0 kDa (●), MW: <1.0–3.5 kDa (○), MW: 3.5–10 kDa (△), and MW: 10–50 kDa (□). Changes in the NO-inducing activity of each MW: 1.0–3.5 kDa, 3.5–10 kDa, and 10–50 kDa fraction after NaClO₂ treatment is shown in Fig. 3B (●), Fig. 3C (△), and Fig. 3D (■), respectively. The data represents the mean $\pm$ S.D. The significance was evaluated by Student's *t*-test against the corresponding parent sample. **p* < 0.01. ***p* < 0.001.

might be a polymer. Intense ESR signals were obtained at $g = 2.005$, suggesting that stable organic free radicals, such as a phenoxy radical group, might contribute to the signals. The intensity of ESR signals and mitogenic activities seemed to be related to the MW ranges of substances. The intensity of ESR signals is thought to reflect at least the content of the radical species. Thus, mitogenic activity might be dependent on the frequency of charges. Another possibility is that the immunopharmacological activities of these materials might be dependent on degrees of polymerization.

Most KMs are used clinically *via* the oral route in Japan. Some of them have been considered immunomodulators. Clarification of the efficacy of the peroral administration of KMs on the immune system is thus needed. Recently, peroral administration of SST was reported to show several kinds of immunomodulation in mice: interferon induction,²¹⁾ immunoglobulin A production in Peyer's patch cells,²²⁾ and activation of natural killer cells.²³⁾ In the case of the macromolecular fraction of HWE-BR, augmentation of colony stimulating activity in serum was observed by peroral administration (manuscript in preparation). However, it is not yet generally accepted that macromolecules administered orally would be possible candidates for immunomodulation. HWE-BR examined in this study would contain lower MW lignin-like polyphenols as immunomodulators. Lower MW substances would be advantageous in terms of absorption

from intestinal mucosa. From such a point of view, lower MW lignin-like mitogenic polyphenols are also important, even though they show less activity *in vitro* in comparison with macromolecules.

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