Analysis of Mitogenic Substances in *Bupleurum chinense* by ESR Spectroscopy

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The polyphenolic substance(s) in the hot water extract of *Bupleurum chinense* (PSF) showed strong mitogenic activity. In this paper, we analyzed PSF by using ESR spectroscopy, and found that i) PSF showed a strong ESR signal on $g = 2.005$ which was similar to the commercially available lignin; ii) Sho-saiko-to, which contains an extract of *B. chinense*, also showed similar signals on ESR; iii) Powdered *B. chinense* also showed similar signals on $g = 2.005$. Peroxidase activity, essential for producing polyphenolic substances, was detected in the cold water extract of *B. chinense*. In addition, the signal intensity of the ESR spectrum of *B. chinense* was increased after boiling. The data of the ESR spectra of the model reactions using lignin, arginine, proline and maltose also strongly suggested that a certain chemical modification proceeded during the hot water extraction to increase the percentage of the stable free radical. These facts strongly suggested that the mitogenic substance in *B. chinense* is a polyphenolic substance extracted by hot water, and the structure was modified during the extraction to increase the stable free radical components.

Key words free radical; polyphenol; mitogen; *Bupleurum chinense*; Sho-saiko-to

A traditional herbal medicine (Kampo-medicine), Xio-chai-hu-tang (Japanese name, Sho-saiko-to) is a prescription which shows immunomodulating activities.^{1,2)} We have been interested in the biological response modification of traditional herbal medicine, and revealed that *Bupleurum chinense* DC. (*B. chinense*) has the strongest mitogenic activity of the constituents of Sho-saiko-to.^{3,4)} Oka *et al.* fractionated the mitogenic substances in hot water extracts of *B. chinense* (Bup-HWE) using physico-chemical and immunological methodologies.³⁾ A high molecular weight fraction, C-13, fractionated and separated by dialysis and phenol–water partition, gave water soluble and phenol soluble mitogenic substances. These substances showed mitogenic activity, even in C3H/HeJ mice, and polymyxin B or lysozyme treatment did not abrogate the activity, suggesting that the active substances are not related to the bacterial lipopolysaccharide. Treatment of the mitogenic substances recovered from the phenol layer (PSF) with NaClO₂, a polyphenol degrading chemical, significantly reduced the activity, but pectinase and pectinase treatments had no effect. From these facts, we have speculated that the mitogenic substance(s) in PSF is a polyphenolic substance.

In addition, we previously showed that some of the immunostimulatory substances were produced during the hot water extraction, accompanied by physical and chemical modifications of the constituents.⁵⁾ Similar modifications might proceed in PSF during the hot water extraction.

It is well established that polyphenolic substances often show stable semiquinone-radicals. Thus, in this study we tried to confirm the physico-chemical properties of PSF as a polyphenolic substance by using electron spin resonance spectroscopy (ESR). In addition, to investigate whether the stable free radical detected in PSF came from the already existing radical in *B. chinense*, the ESR spectra of *B. chinense* and lignin, a model compound, before and after the hot water extraction, were compared.

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MATERIALS AND METHODS

Reagent *B. chinense* was kindly provided from Kanebo Co., Ltd. (Osaka, Japan). Lignin (Dealkaline) was purchased from Tokyo Kasei (Tokyo, Japan), L-proline was from Sigma (St. Louis, MO, U.S.A.), L-arginine from Wako Pure Chemicals (Osaka, Japan). PSF (Phenol soluble high molecular weight fraction) was isolated from the hot water extract of *B. chinense*.³⁾

Detection of Peroxidase (POD) Activity in *B. chinense* The peroxidase activity^{6,7)} was measured by a commercially available kit, called a TMB (3,3',5,5'-tetramethylbenzidine) Microwell Peroxidase Substrate System (Kirkegaard & Perry Lab. Inc., Gaithersburg, MD, U.S.A.).

Preparation of the Hot Water Extract of *B. chinense* Powdered *B. chinense* (16.5 mg) suspended in de-ionized water (DIW, 1.35 ml) was boiled for 1 h, then lyophilized. The control experiment, without boiling (not-boiled), was run in parallel.

Preparation of Boiled Polyphenol in the Presence or Absence of Amino Acids Lignin (200 mg) and amino acid (200 mg) were dissolved in DIW (abbreviated as MIX, 2 ml). The pH of MIX was adjusted to either 5, 7 or 9 with HCl and NH₃. Then, these MIX were boiled for 1 h. After 24 h at room temperature, the mixtures (Δ MIX) were stored and dialyzed (MW 1000, Wako Pure Chemical, Osaka, Japan), and the non-dialyzable-fraction (NDF) was lyophilized (Δ MIX-NDF). Polyphenol alone (200 mg), dissolved in DIW (2 ml), was run in parallel.

ESR Spectroscopy Powdered (3.5 mg) or aqueous (100 mg/ml of each constituent; 5 μ l) samples were filled in a glass capillary. ESR spectra were measured by JES-RE1X (JEOL, Tokyo, Japan) and analyzed by an ESR Data Analyzer (Labotec Co., Ltd., Tokyo, Japan). Relative intensity (*R.I.* value) was calculated by comparing the intensities of the samples with the external standard (Mn²⁺). *R.I.* values of each sample of MIX, Δ MIX, and

Δ MIX-NDF were obtained using an ESR spectrometer.

RESULTS

Detection of Stable Free Radical(s) in *B. chinense* As shown in the introduction, we have already prepared a mitogenic, phenol-soluble, high-molecular weight fraction, PSF, from the hot water extract of *B. chinense*.³⁾ Figure 1 shows the ESR spectrum of the lyophilized powder of PSF. A strong signal was detected around the g value of 2.005. By chemical and physical study, we have estimated the structure of PSF to be a polyphenolic substance, like lignin.⁸⁻¹⁰⁾ Thus, we measured the ESR spectrum of commercially available lignin. The spectrum of lignin was very close to that of PSF and showed a similar g value (Table 1), supporting the previous assumption. Compared with the published value, the free radical component would be attributable to a semiquinone-radical.¹¹⁾ A traditional Kampo medicine, Sho-saiko-to, which contains the extract of *B. chinense*, also showed a signal similar to that of PSF (Table 1).

In the second experiment, we tried to determine the origin of the stable free radical(s) in PSF as well as in Sho-saiko-to. As shown in Table 1, air-dried *B. chinense* shows signals similar to those of PSF strongly suggesting that the radical shown by the signal of PSF would already be produced in *B. chinense* powder during growth and/or processing into a source of medicine. As shown above, the lignin component shows signals similar to PSF, and lignin is known to be widely distributed in plants, especially in the wooden part. To investigate how this free radical was formed, we first investigated the possibility of forming lignin in *B. chinense* with enzymes. We examined peroxidase (POD) activity^{6,7)} in the cold water extract of *B. chinense*. When *B. chinense* powder was extracted with phosphate buffered saline (PBS) at 4°C for 24 h, POD

activity was detected in a PBS extract of about 400 ng/1 g (*B. chinense*) as assessed with horseradish POD as a reference. It is also known that stable semiquinone radical has been detected in synthetic polyphenolic substances, such as synthetic lignin, produced by POD.¹²⁾ These facts strongly suggested that *B. chinense* contained a polyphenolic substance and the ESR signal observed in *B. chinense* powder would be, at least in part, from the product of POD.

Production of Free Radicals during Hot Water Extraction of *B. chinense* We have previously shown that some immunostimulatory substances have been produced during the hot water extraction process.^{3,4)} To investigate whether the stable free radical detected in PSF came completely from the already existing radical in *B. chinense*, ESR spectra of *B. chinense* powder before and after the hot water extraction were compared. To compare the signal intensity, we used the value of "relative intensity" to the external Mn^{2+} signal) ($R.I.$ value). *B. chinense* powder was once suspended in distilled water and a part of the suspension was boiled for 1 h. The resulting suspensions were lyophilized to make dried powders. To evaluate the total quantity of free radicals in these samples, none of the separation and fractionation steps were included before measuring ESR spectra. It was found that the $R.I.$ value of *B. chinense* powder without boiling was 0.63 and that of boiled *B. chinense* powder was 0.84. These results strongly support the hypothesis that a certain part of the stable radical was produced during the hot water extraction of *B. chinense*.

Production of Stable Free Radical(s) during the Preparation of Hot Water Extracts Using Some Model Compounds In the above section, we suggested the possibility that a part of the stable free radical was produced during the preparation of the hot water extracts. In this section, we tried to confirm that possibility by using some model compounds. It is probable that the stable radical resulted from the modification of already existing radicals and/or newly produced substances. For confirming the former possibility we used commercially available lignin (dealkaline), and for the latter we used simple amino acids and carbohydrates.

We prepared a mixture of the model compounds (200 mg each) in aqueous solution, boiled for 1 h, and prepared high molecular weight products by dialysis. We used MW cut <1000 dialysis membrane. Abbreviations of "MIX," " Δ MIX," and " Δ MIX-NDF" were used, respectively, for each of the resulting products. Table 2 summarizes the data collected from the model compounds prepared by lignin and amino acids. Arginine and proline were used as amino acid components because those are the major free amino acids in *B. chinense*.¹³⁾

In the case of lignin without amino acids, about 50% was recovered from NDF, and the yields were almost the same at all pH values tested. In the case of the $R.I.$ value, it was increased after the boiling treatment at all pH values tested (e.g. pH 5, 0.57→1.39), suggesting the presence of some heat unstable structure in the lignin preparation. The yield of the NDF fractions was almost the same before and after heating, but the $R.I.$ value of the heated NDF fraction (Δ lignin) was higher than that of the non-heated

Fig. 1. ESR Spectrum of the Phenol Soluble Fraction in the Hot Water Extract of *B. chinense*, PSF

ESR spectrum was measured as described in Materials and Methods.

Table 1. Parameters of ESR Spectra Obtained from *B. chinense* and Related Fractions

	g value	Relative intensity ($R.I.$)
PSF from <i>B. chinense</i>	2.0054	1.13
Commercial lignin	2.0053	1.31
<i>B. chinense</i> powder	2.0054	0.87
Sho-saiko-to powder	2.0050	0.15

Table 2. Relative Intensity of ESR Signal of Lignin/Free Amino Acid Complexes

	Lignin only	Δ Lignin only	Lignin with Arginine	Δ Lignin with Arginine	Lignin with Proline
pH 5					
MIX	0.57	1.39 $\pm$ 0.19	0.21	1.28	0.34
Δ MIX	1.39 $\pm$ 0.19		0.7	1.09	0.56
Δ MIX-NDF	1.84 (103.2 mg) ^{a)}	2.42 (83.4 mg)	2.15 (311.5 mg)	3.14 (96.1 mg)	3.02 (181.5 mg)
pH 7					
MIX	1.11	1.59 $\pm$ 0.12	0.56	1.39	0.41
Δ MIX	1.59 $\pm$ 0.12		0.59	1.3	0.38
Δ MIX-NDF	1.49 (77.1 mg) ^{a)}	2.55 (97.5 mg)	2.23 (193.0 mg)	3.44 (107.0 mg)	2.99 (110.6 mg)
pH 9					
MIX	1.5	2.05 $\pm$ 0.44	1.6	1.48	1.46
Δ MIX	2.05 $\pm$ 0.44		1.82	1.38	0.95
Δ MIX-NDF	2.38 (99.2 mg) ^{a)}	2.76 (94.8 mg)	2.86 (293.0 mg)	2.69 (114.8 mg)	3.08 (83.2 mg)

MIX, Δ MIX, Δ MIX-NDF were prepared as described in Materials and Methods. () = yield from the reaction started by using lignin (200 mg) in the presence or absence of amino acids (200 mg). a) Without heating.

NDF fraction (lignin) (e.g. pH 5, 1.84 $\rightarrow$ 2.42). In addition, compounds showing higher *R.I.* values were concentrated in the NDF fractions.

Next, we prepared a lignin-arginine complex. The brownish color of the reaction mixture was increased after heating, suggesting the presence of some heat induced interactions. Of major importance, the yield of the Δ MIX-NDF was significantly high at all pH value tested, even though the *R.I.* value (about 2.5) was comparable to that of the Δ lignin-NDF itself. In contrast, when we used heat-treated lignin (Δ lignin), the yield was not increased even in the presence of arginine. These facts strongly suggest that the complex formation between the heat unstable structure in lignin and arginine is the key reaction. In addition, the *R.I.* values of the Δ lignin arginine complex were higher than Δ lignin itself (e.g. pH 5, 2.42 $\rightarrow$ 3.14). These facts additionally suggested that some structural changes were induced by heating. In the case of proline, we obtained a higher yield only at pH 5, suggesting that the reaction was pH dependent. However, the *R.I.* values of Δ MIX-NDF were about 3.0 at all pH values tested. These facts also suggested some structural changes during treatment.

We further prepared a maltose/arginine complex to demonstrate the newly formed stable radical during the boiling treatment. The color of the reaction mixture was only changed to brown in the case of pH 9, and the stable radical was also produced at pH 9. Compared with those shown in the products made by lignin, however, the *R.I.* values were quite low (*R.I.* values of MIX, Δ MIX, and Δ MIX-NDF, were 0, 0.14, and 0.18, respectively). These facts strongly suggested that a newly formed stable radical might exist, at least in part, in the hot water extract of *B. chinense*.

DISCUSSION

We have been interested in naturally distributed immunostimulatory substances. During the course on this study, we have suggested that the polyphenolic substance(s) in the hot water extract of *B. chinense* showed strong mitogenic activity. In this paper, we tried to confirm the above mentioned hypothesis using an ESR spectrum.

We found that i) PSF showed a strong ESR signal on $g=2.005$ and was similar to the commercially available lignin; ii) a well-known prescription, Sho-saiko-to, which contains the extract of *B. chinense*, also showed similar signals on ESR; iii) Powdered *B. chinense* also showed a stable free radical on $g=2.005$. When we tested the mitogenic activity of the commercially available lignin, it was quite toxic (data not shown). However, some of the synthetic polyphenols prepared from *p*-coumaric acid or trans-ferulic acid showed mitogenic activity (manuscript in preparation). In addition, we detected activity of POD, which is a parent enzyme in the production of polyphenolic compounds, in the cold water extract of *B. chinense*. These facts strongly suggested that the mitogenic activity in *B. chinense* was due to polyphenolic substance(s) widely distributed in nature. We do not have any direct evidence to explain why the commercially available lignin preparation was toxic; however, the extraction procedure might be related to a physicochemical property because the reactivity of lignin and Δ lignin to arginine was significantly different from each other (Table 2).

In addition, we were interested in the modification and degradation of bioactive substances during hot water extraction. We have already confirmed that a mitogenic substance, sclerogen, extracted from a fungus *Sclerotinia sclerotiorum*, was present in the fungi as a precursor of the mitogenic substance and it became active after polymerization by heat treatment.⁵⁾ Thus, we tried to confirm whether similar mechanism(s) proceeded during the hot water extraction of *B. chinense*. Of interest, the *R.I.* value of the ESR spectrum of *B. chinense* was increased after boiling. The data of the ESR spectra of the model reactions using lignin and maltose also strongly suggested that a certain chemical modification proceeded during the hot water extraction to increase the percentage of the stable free radical. Recently, Okuda *et al.* suggested that the bioactive compound (LAK activity), Arginine-Fructose-Glucose, was produced by boiling the mixture under an acidic condition.¹⁴⁻¹⁶⁾ We could not fully demonstrate the structure of the mitogenic substance, yet, because the substances are quite high in MW, the observations shown in this paper might be similar to Okuda's observation.

An immunomodulating substance is important for

developing new medicines for various medications, such as cancer immunotherapy, vaccines for infectious diseases, allergies, and autoimmune disease. Extensive studies are now continuously performed from various points of view, and some of the new findings, such as the three-dimensional structure of HLA antigen, completely overcame previously established concepts, such as the dual recognition and altered-self theories. However, little is known about the molecular mechanisms of immunomodulating substances, even in the cases of bacterial lipopolysaccharide (LPS) and fungal β -glucan. LPS is well known as a macrophage activating substance and is mediated, at least in part, by LBP and CD14. LPS is also a B cell mitogen, but the receptor molecule is still unknown. During the study of B cell mitogen we have already shown that i) an anionic residue is necessary in Toki mitogen and vesiculogen,¹⁷⁻¹⁸⁾ and ii) the polymerization of protein is necessary for sclerogen.⁵⁾ However, these observations are still far from complete molecular understanding of the B cell mitogen. In this paper we showed that the mitogenic substance in *B. chinense* is a polyphenol with a stable radical, and the stable radical was produced by both enzymatic polymerization and by physico-chemical interactions of the constituents. This property is consistent with the general property of polyphenols, as discussed above; however, we do not have any direct evidence that the free radical itself is essential to the B cell mitogenic activity.

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