JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

New Method To Evaluate Water-Soluble Antioxidant Activity Based on Protein Structural Change

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A simple method to evaluate antioxidant activities of water-soluble ingredients of foods has been developed. Protective effects of antioxidants against hypochlorite radical or hydroxyl radical have been studied by comparing changes in absorbance of myoglobin (a standard reference) at 409 nm. Protective ratio, defined by absorbance changes of myoglobin with or without the antioxidant, was a good indicator to quantitatively evaluate the antioxidant activity against the hypochlorite radical or the hydroxyl radical, respectively. Radar charts indicating the antioxidant activities against DPPH (1,1-diphenyl-2-picrylhydrazyl), hypochlorite radical, and hydroxyl radical clearly differentiated the characteristics of five antioxidants including carnosine, glutathione, and vitamin C. By comparison of the radar charts, antioxidant activity of bonito meat hydrolysate was found to have similar characteristics to that of carnosine. The simple method proposed in this study would be useful for evaluating and characterizing the activities of water-soluble antioxidants contained in various food materials.

KEYWORDS: Antioxidant activity; protein structure; oxidative stress; reactive oxygen species; water-soluble ingredients

INTRODUCTION

Antioxidants in food materials have recently attracted researchers' attention because many reports have shown that the oxidative stress is closely related to the aging process of the cells and acts as a trigger to various diseases including cancer (*1*). While the high levels of reactive oxygen species and free radicals cause damage to nucleic acids, proteins, and membrane lipids, the antioxidants in diet would terminate attacks by the free radicals and reduce the risks of these diseases (*2*). Various kinds of antioxidants were found in various levels in a variety of food materials including grains, fruits, and also proteins such as milk casein and soy protein (*3*−*9*). The antioxidants are considered as useful food additives in the food industry. Effect of the added antioxidant, for example, on the quality of beef meatballs or nitrite-cured sausage has been evaluated (*10*). Thus, the antioxidants would be used more widely as food additives to improve the quality of the cooked foods (*11, 12*).

Although antioxidant activities have been reported for various food materials, most of the methods used to evaluate the activities gave only qualitative information, or relative value to positive control (*13, 14*). Among those, DPPH (1,1-diphenyl-2-picrylhydrazyl) quenching method is a convenient assay that gives the quantitative data from a simple experiment (*15*). In this method, the antioxidant activity of the sample is expressed as an equivalent concentration to a standard antioxidant that gives the same activity to quench the stable DPPH radical. Ascorbic acid and Trolox are the most frequently used standard antioxidants. Whereas DPPH quenching method is very convenient for obtaining the quantitative information, the physiological meaning of the activity evaluated by this method is not clear. Further, the quantitative measurement of physiologically important reactive oxygen species and free radicals such as hydroxyl radical and hypochlorite radical requires expensive methods such as electron spin resonance (ESR) or chemiluminescence (*16, 17*). Thus, a simple method that can quantitatively evaluate the antioxidant activity in food materials is strongly desired. Yanai et al., for example, has evaluated antioxidant activities of chicken extract by the protective effects of the sample on the protein degradation by reactive oxygen species including hypochlorite radical or hydroxyl radical and compared them with standard antioxidants such as vitamin C, vitamin E, and reduced glutathione (*18*). Although their idea was excellent and they have reported suggestive results, their methods require long periods of time to obtain the results because they evaluated changes in molecular size of the target proteins by the band pattern of SDS-PAGE and by the retention time in HPLC analysis.

The objective of this study is to establish a simple and rapid method to evaluate the antioxidant activity quantitatively. We

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10.1021/jf062700e CCC: $37.00
Published on Web 01/03/2007

have paid attention to the decolorization of myoglobin due to its structural changes caused by the reactive oxygen species and free radicals. We have shown that the antioxidant activity of various standard antioxidants can be evaluated in a few minutes in this work. Further, we could characterize the activities of the five standard antioxidants against the different reactive oxygen species and radicals by plotting the quantitative data in radar charts. This method would be useful for differentiating the characteristics of the water-soluble antioxidant activities of various food materials.

MATERIALS AND METHODS

Materials. L-Carnosine (Peptide Institute Inc., Osaka, Japan), Trolox (Calbiochem, CA), l-ascorbic acid (Wako Chemical, Osaka, Japan), reduced glutathione (Wako Chemical), and ferulic acid (4-hydroxyl-3-methoxycinamic acid) (Wako Chemical) were used as standard antioxidant substances. Sodium hypochlorite solution (effective chlorine concentration >6%, Wako Chemical) was used. Crude pepsin (Sigma-Aldrich, MO) was used to prepare hydrolysate of bonito meat without further purification. Bonito meat was purchased at a local supermarket. All other chemicals used in this work were of reagent grade.

Antioxidant Activity Measurements by DPPH Method. Antioxidant activities were measured using a stable radical DPPH as follows. A test sample containing an antioxidant (0.5 cm^3) was mixed with 2.5 cm^3 of 200 mM Tris-HCl (pH 7.4), 1.0 cm^3 of ethanol, and 1.0 cm^3 of DPPH solution (0.2 mg of DPPH/cm^3 of ethanol), and then the solution was placed in the dark for 20 min at room temperature. The absorbance at 520 nm was measured with a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). Ascorbic acid (vitamin C) was used as a standard substance to evaluate the antioxidant activity. The antioxidant activity of the sample was expressed as ascorbic acid concentration (mM equiv VC) that gives equal reduction of absorbance at 520 nm.

Measurements of Antioxidant Activity against Hypochlorite Radical Based on Structural Change of Myoglobin. Myogobin (from equine skeletal muscle 95−100%, Sigma-Aldrich) was dissolved into PBS buffer (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 274 mM NaCl, 27.2 mM KCl, pH 7.2) to give a final concentration of 0.25 mg/cm^3. To 3.0 cm^3 of the myoglobin solution, 0.1 cm^3 of test sample and 0.5 cm^3 of hypochlorite solution were added. The hypochlorite solution was used to generate hypochlorite radical. After the solution was mixed well, absorbance of the solution at 409 nm was measured with the spectrophotometer. The concentration of hypochlorite radical was changed by diluting the hypochlorite solution with Milli-Q water and was expressed as relative concentration of hypochlorite solution based on the dilution ratio (1/250 equals 4.0×10^{-3}, for example).

Measurements of Antioxidant Activity against Hydroxyl Radical Based on Structural Change of Myoglobin. To a test tube containing 3.0 cm^3 of myoglobin solution (0.25 mg of myoglobin/cm^3), 0.48 cm^3 of test sample was added. In a different test tube, 0.010 cm^3 of 0.1 M $FeSO_4$ and 0.1 cm^3 of 30% H_2O_2 were mixed to generate hydroxyl radical by Fenton reaction (hydroxyl radical generating solution). The myoglobin solution containing the test sample was immediately added to the radical generating solution and was mixed well. The absorbance of the reaction mixture at 409 nm was measured with the spectrophotometer. To change the concentration of hydroxyl radical, the concentrations of $FeSO_4$ and H_2O_2 were diluted to 1/5 and 1/10. The relative concentrations of hydroxyl radical were expressed as 1.0, 0.2, and 0.1.

Preparation of Bonito Meat Hydrolysate. After removal of dark muscle from bonito meat, the meat was cut into small blocks (100 g) and stored at −80 °C until further use. To prepare water extract of the bonito meat, two blocks of the frozen meat were added to 1000 cm^3 of Milli-Q water and then homogenized twice in a cooking mixer (model MX945G, National, Oasaka, Japan) for 10 s. The pH of the obtained homogenate was adjusted to pH 1.2, and 5.0 g of pepsin was added. The reaction mixture was stirred with a magnetic stirrer for 30 min at 37 °C. Then 5.0 g of pepsin was added again, and the mixture was incubated at 37 °C overnight. The reaction mixture was then freeze-dried in a freeze-dryer (model 75034, Labconco, Kansas City, USA), to be stored at −20 °C.

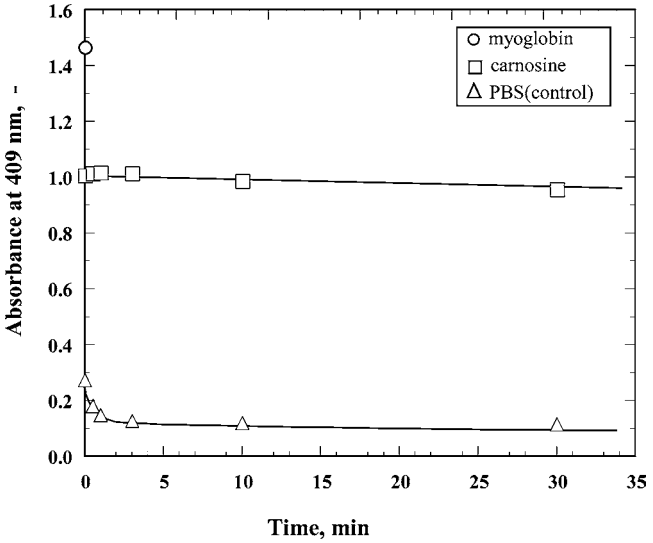

Figure 1. Change of myoglobin absorbance by hypochlorite radical; relative concentration of hypochlorite solution 4.0×10^{-3}, carnosine 10 mM, PBS (control) PBS buffer without antioxidant.

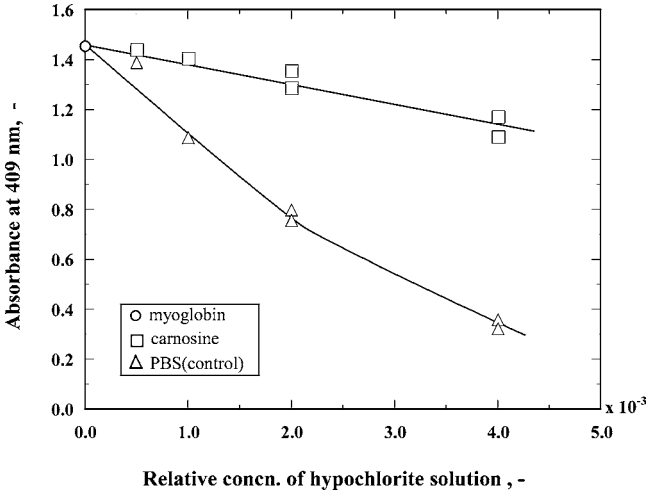

Figure 2. Effect of radical concentration on myoglobin absorbance; carnosine 10 mM, PBS (control) PBS buffer without antioxidant.

RESULTS AND DISCUSSION

Figure 1 shows a time course of the changes in absorbance of myoglobin at 409 nm after addition of hypochlorite solution at a concentration of 4.0×10^{-3}. The absorbance of myogobin immediately decreased from its initial value of 1.47 to about 0.30 when PBS buffer without antioxidant (negative control) was added to the test solution. In the case of the myoglobin solution containing carnosine, the degree of the change in absorbance was smaller than the negative control. The final concentration of carnosine in the myoglobin test solution at time zero was 0.278 mM. These results suggest that carnosine added to the test solution suppressed the structure changes of myoglobin by hypochlorite radical. The changes in the absorbance at time zero would become a good indicator to evaluate antioxidant activity of the various substances. **Figure 2**, for example, shows effects of the relative concentration of hypochlorite solution on the initial absorbance changes of myoglobin solution. In this experiment, the concentration of carnosine solution added to the sample was kept to 10 mM.

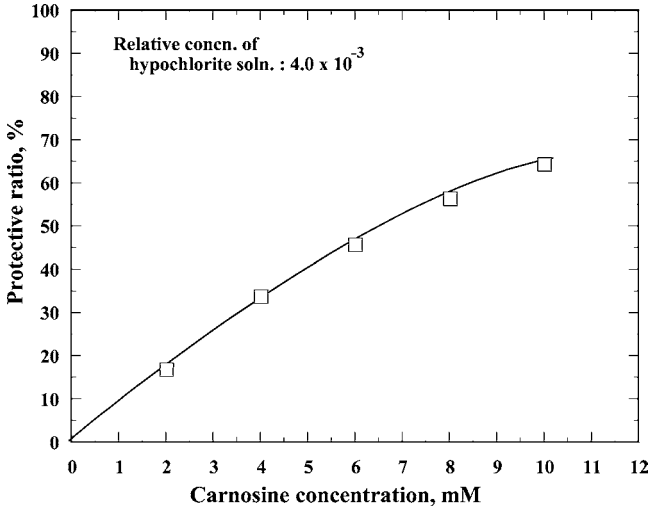

Figure 3. Protective effect of carnosine against hypochlorite radical; relative concentration of hypochlorite solution 4.0×10^{-3}.

The degrees of the absorbance changes increased with the increase of the relative hypochlorite concentration.

To obtain quantitative information for the antioxidant activity from the absorbance change in myoglobin solution, the protective ratio (%) is defined by the following equation,

$$\text{protective ratio} = \left\{1 - \left(\frac{(\text{Abs}^0) - (\text{Abs}^{\text{rad}}(\text{with antioxidant}))}{(\text{Abs}^0) - (\text{Abs}^{\text{rad}}(\text{without antioxidant}))}\right)\right\} \times 100$$

where Abs^0 is the absorbance of myoglobin solution, Abs^{rad}(without antioxidant) is the absorbance of the test solution containing only radicals, and Abs^{rad}(with antioxidant) is the absorbance of the test solution containing both radical and antioxidant. Both Abs^{rad}(without antioxidant) and Abs^{rad}(with antioxidant) are the absorbance measured immediately after radical addition. **Figure 3** shows the protective effects of carnosine against hypochlorite radical at the relative concentration 4×10^{-3}. The protective effect increased with the increase of carnosine concentration. This result has clearly shown that the antioxidant activity of carnosine against hypochlorite radical can be quantitatively evaluated by the protective ratio. The effects of antioxidant concentrations on the protective effects of ascorbic acid, glutathione, Trolox, and ferulic acid are shown in **Figure 4**. In the cases of ferulic acid and Trolox, the protective effects reached a plateau after carnosine concentration reached 6 mM under this experimental condition, while the protective ratio increased with the increase of the concentration for ascorbic acid and glutathione.

Similar analysis was also applied to the protective effects against hydroxyl radical. **Figure 5** shows the change of myoglobin absorbance at 409 nm by the addition of hydroxyl radical. The absorbance immediately decreased from 1.34 to about 0.7 after the addition of the radical solution and then gradually decreased to lower values. As shown in the case of the hypochlorite radical (**Figure 1**), the addition of Trolox prevented the decrease of the absorbance, and the effect of Trolox depended on its concentration. Addition of 10 mM Trolox is equivalent to a final Trolox concentration of 1.33 mM at time zero in the test solution.

Figure 6 shows the protective ratio of Trolox against hydroxyl radical for three different radical concentrations. The protective ratio increased with the increase of the Trolox concentration. The protective effect, on the other hand, decreased when the relative hydroxyl radical concentration increased. These results

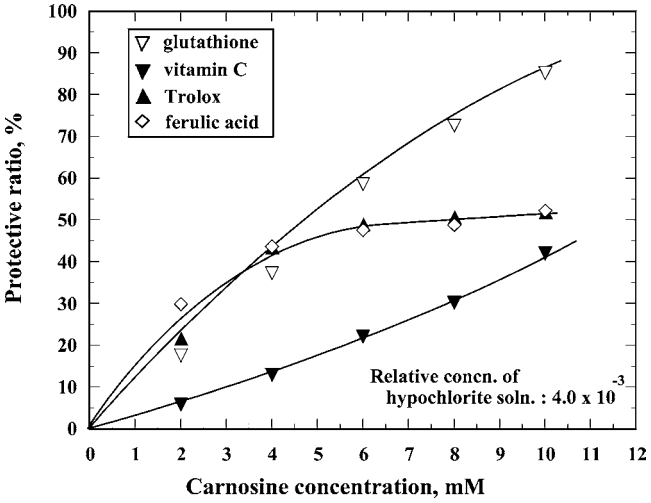

Figure 4. Protective effects of various antioxidants against hypochlorite radical; relative concentration of hypochlorite solution 4.0×10^{-3}.

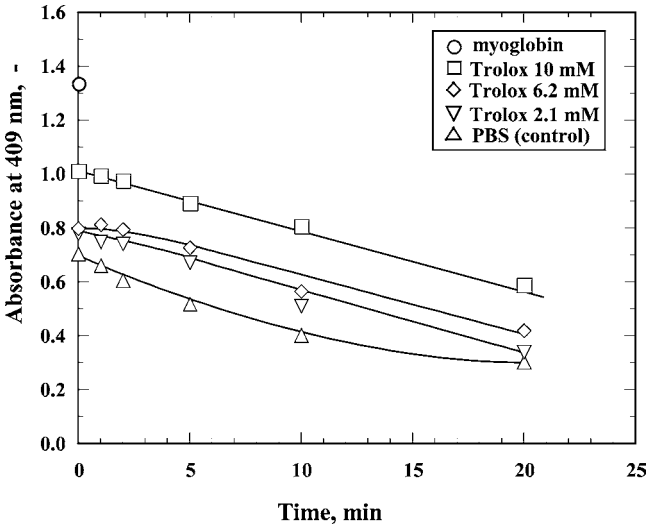

Figure 5. Change of myglobin absorbance by hydroxyl radical; relative hydroxyl radical concentration 1.0.

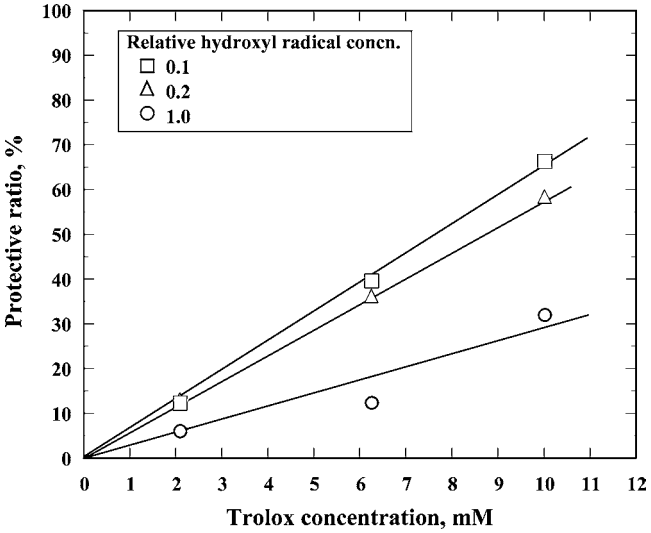

Figure 6. Protective effects of Trolox against hydroxyl radical.

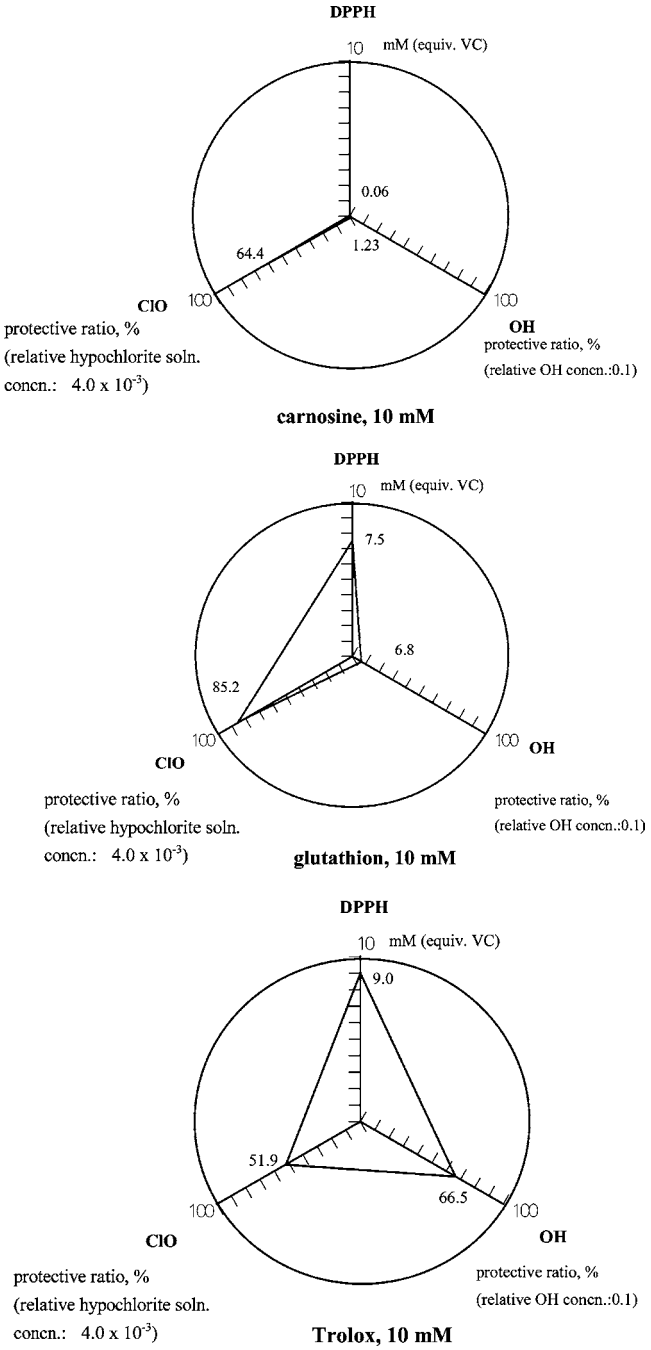

Figure 7. Radar charts of various antioxidant activities (carnosine, glutathion, Trolox); ClO hypochlorite radical, OH hydroxyl radical, DPPH 1,1-diphenyl-2-picrylhydrazyl.

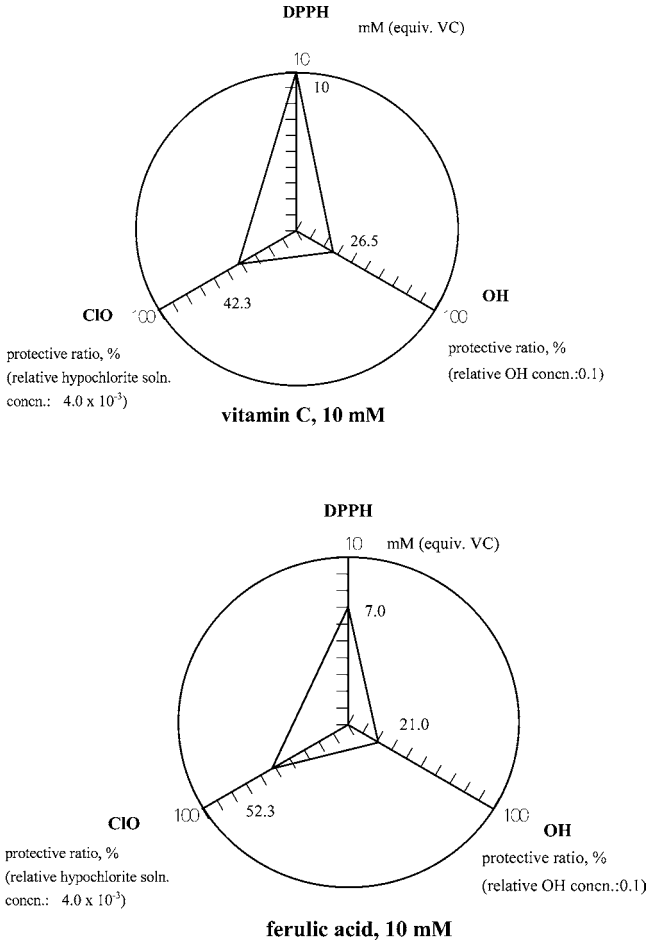

Figure 8. Radar charts of various antioxidant activities (vitamin C, ferulic acid); ClO hypochlorite radical, OH hydroxyl radical, DPPH 1,1-diphenyl-2-picrylhydrazyl.

also show that the antioxidant activity against hydroxyl radical can also be quantitatively evaluated by the protective ratio.

Specific properties of the antioxidant substances would be well-characterized by comparing the antioxidant activity measured with three different radicals. For example, we have expressed the characteristics of the examined antioxidants in radar charts. The three different criteria are the equivalent vitamin C concentration evaluated by the DPPH method, the protective ratio against hypochlorite radical at the relative concentration of hypochlorite solution 4.0×10^{-3}, and the protective ratio against hydroxyl radical at the relative radical concentration of 0.1. The radar charts for carnosine, Trolox, glutathione, ascorbic acid, and ferulic acid are summarized in **Figures 7** and **8**. The concentrations of the antioxidant in the sample added to the test solution were 10 mM. In this way, the antioxidant activity against three different radicals can be compared in a radar chart. It is very interesting that the radar charts of the standard antioxidants show quite different patterns reflecting the characteristics of the antioxidant activities. For example, carnosine shows strong antioxidant activity against hypochlorite radical, but the antioxidant activities against hydroxyl radical and DPPH were very low. Trolox, on the other hand, shows relatively high antioxidant activities toward all three radicals used in this experiment.

It should be noted that protective ratio defined in this work strongly depends on the experimental condition such as the relative concentration of radicals and the antioxidant concentration. Further, the protective ratio was saturated at the concentration above 6 mM for Trolox and ferulic acid. These factors suggest that the shape of the radar chart may change depending on the experimental condition. In spite of such uncertainties, we could characterize the antioxidant properties of some standard antioxidants by comparing the antioxidant activities with radar charts at a determined experimental condition. Different characteristics may be found by comparing the antioxidant activities at different conditions. It should be also noted that the proposed method is useful only for the water-soluble antioxidants. Therefore, the development of a similar method that is applicable to the water-insoluble antioxidants would be an interesting and challenging issue for future work.

Analysis using the radar charts will become a convenient method to characterize the antioxidant activities of the complex food materials. Various substances contained in the food materials show a wide variety of antioxidant activities, and the

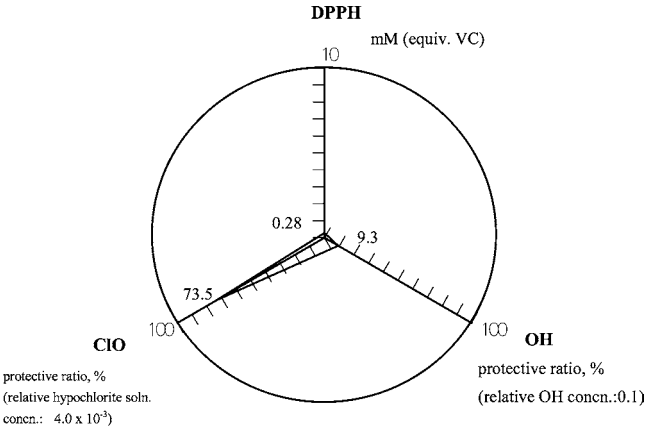

Figure 9. Radar chart of bonito meat hydrolysate; ClO hypochlorite radical, OH hydroxyl radical, DPPH 1,1-diphenyl-2-picrylhydrazyl.

antioxidant activity of the food materials should be evaluated as the synergetic effects of these ingredients. The method proposed in this work would be very useful for elucidating the possible physiological activities expected to be seen in the food ingredients. To demonstrate the usefulness of our proposal, we have characterized the antioxidant activity of bonito meat hydrolysate (10 mg/mL) as a radar chart (**Figure 9**). The pattern of the antioxidant activity was very close to that of carnosine (**Figure 7**), and therefore the bonito hydrolysate may be expected to show similar antioxidant effects as carnosine. This result is interesting because carnosine is widely found in the animal muscle. Although these results did not directly mean that fish hydrolysate contains carnosine, we can expect the similar property of antioxidant activities as carnosine when the bonito meats were orally taken.

Thus, a simple method to evaluate antioxidant activity against hypochlorite radical and the hydroxyl radical has been developed using the differences of changes in myoglobin absorbance with or without antioxidants. Other than the absorbance changes, the changes in fluorescence or enzyme activity of some other proteins would also be a useful indicator to evaluate the effects of radical on the protein structure. The radar charts were very helpful in characterizing the antioxidant activity against different radicals. The radar charts proposed in this work can be modified easily according to the researchers' purposes by adding axes for other radicals and/or comparing the samples under different conditions. When the effects of the standard antioxidants on the human health are clarified by future studies using experimental animals, this approach will be useful in characterizing the antioxidant activity of the foods containing various kinds of substances.

In conclusion, a simple and rapid method for evaluating antioxidant activity of water-soluble substances has been developed utilizing the structural change of myoglobin. It has been demonstrated that radar charts of the antioxidant activities against three different radicals (hypochlorite, hydroxyl, and DPPH radicals) are useful in characterizing the properties of the antioxidant activities of the substances found in the food materials.

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Received for review September 20, 2006. Revised manuscript received October 28, 2006. Accepted November 6, 2006.

JF062700E