Rapid Photometric Assay Evaluating Antioxidative Activity in Edible Plant Material

Jen-Min Kuo,*[,†] Dong-Bor Yeh,[†] and Bonnie Sun Pan[‡]

Department of Food Health, Chia-Nan College of Pharmacy and Science, Tainan, Taiwan, Republic of China, and Department of Marine Food Science, National Taiwan Ocean University, Keelung, Taiwan, Republic of China

The objective of the present study is to develop a rapid and convenient method to determine antioxidative activity. It was determined by the inhibition capacity on the hemoglobin-catalyzed peroxidation of linoleic acid. The appropriate conditions for reaction of 4 mM linoleic acid were 0.002% hemoglobin at 37 °C for 10 min. Adding methanol to the reaction mixture at <20% showed no significant effect on the peroxidation of linoleic acid. Products formed from hemoglobin-catalyzed peroxidation of linoleic acid were 9- and 13-hydroperoxyoctadecadienoic acid at a ratio of approximately 50:50. Eight synthetic antioxidants were assayed for their antioxidative activity; all of them showed linear response to the logarithm of their concentration. Antioxidative activity from different plant samples was also examined. Tea, ginger, chrysanthemum, and roselle showed higher antioxidative activity. Either hydrophobic or hydrophilic antioxidants were able to be assayed with this method within 15 min.

Keywords: *Antioxidative activity; antioxidant; hemoglobin; spectrophotometer*

INTRODUCTION

Recently, much work has been focused on the health-related properties of antioxidants from natural sources, such as tea (Yeh and Kuo, 1997, 1998), herbal drinks (Kuo et al., 1998a,b), and citrus peel (Wu et al., 1998). Thus, the daily screening analyses of antioxidants or the determination of antioxidative activity from chromatographically purified fractions become important. The most common method used for determining antioxidative activity is the thiocyanate assay (Wong and Yen, 1997), which measures the inhibitory degree of autoxidation of linoleic acid. However, this method requires 5−6 days for analysis.

Conjugated diene formed from autoxidation can be measured using its absorbance at 234 nm (Kuo et al., 1997a,b). This method is quick and simple; however, it suffers from the interfering absorption of other natural compounds present in the sample. The carotene bleaching method is quite rapid but still takes 2 h for analysis (Gadow et al., 1997). Other methods including polarographic analysis (Yagi, 1970), active oxygen method (Ho et al., 1992), electron spin resonance spectrometeric assay (Masaki et al., 1995), chemiluminescence analysis (Maeda et al., 1992), gas chromatographic determination (Rogstad, 1980), and fluorometric assay (Furuta et al., 1997) were also developed to measure antioxidative activity, but either the instruments or the techniqes were unfamiliar to routine analysis. More recently, Bertelsen et al. (1995) established a fast method using myoglobin as a prooxidant to catalyze peroxidation of methyl linoleate. It takes only 10 min at room temperature, and the reaction was measured with a polarographic oxygen meter. However, its sensitivity was probably 10-fold less than that of the spectrophotometric methods, and it was not suitable to measure a large number of samples. All of these methods found in the literature were not for daily screening tests or analysis on chromatographically purified fractions. The objective of the present study is to develop a rapid and convenient method to determine antioxidative activity. We used hemoglobin as a prooxidant, which is cheaper than myoglobin. The antioxidative activity is then analyzed with a spectrophotometer. The determination is completed within 15 min.

MATERIALS AND METHODS

Chemicals. Methanol, ethanol (99.5% purity), Tween 20, ferrous chloride, and ammonium thiocyanate were purchased from Merck Chemical Co. (Darmstadt, Germany). Linoleic acid (99% purity), diazomethane, and hemoglobin (from bovine) were purchased from Sigma Chemical Co. (St. Louis, MO). Hydroperoxyoctadecadienoic acid (9-HpODE) and 13-HpODE were purchased from Caman Chemical Co. (Ann Arbor, MI). All chemicals are of extra pure grade.

Preparation of Extract. Plant samples were air-dried at 50 °C for 12 h and ground into fine powder with a sample mill. The powdered sample was extracted twice with 10 volumes of different percentages of methanol for 24 h to obtain a maximal antioxidative activity. The percentages of methanol used for extraction were in the following: peanut hull, 90%; lemon peel, orange peel, and tangerine peel, 75%; green tea, oolong tea, black tea, red pepper, carrot, and garlic, 50%; roselle, chrysanthemum petal, ginger root, and basil, 30%; burdock and oat, 20%. The extracts were then evaporated to dryness in a rotary evaporatory at 50 °C and dissolved in 10 mL of 50% methanol.

Antioxidative Activity Assay. The antioxidative activity was principally determined as the degree of inhibition on the

* Address correspondence to this author at the Department of Food Health, Chia-Nan College of Pharmacy and Science, 60 Erh-Jen Road, Sec. 1, Pao-An, Jen-te Hsiang, Tainan Hsien, Taiwan, R.O.C. [fax (06)266-6411; telephone (06)266-4911-220; e-mail ikuojm@mail.ncku.edu.tw or ikuojm@chna.chna.edu.tw].

[†] Chia-Nan College of Pharmacy and Science.

[‡] National Taiwan Ocean University.

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hemoglobin-catalyzed peroxidation of linoleic acid. Ten microliters of sample was added into a test tube (16 × 130 mm) together with 0.37 mL of 0.05 M phosphate buffer (pH 7.0) containing 0.05% Tween 20 and 4 mM linoleic acid and then equilibrated at 37 °C for 3 min. The peroxidation of linoleic acid in the above reaction mixture was initiated by adding 20 μL of 0.035% hemoglobin (in water), followed by incubation at the same temperature in a shaking bath (Wlsodm, SB7D, Taiwan) under 100 rpm for 10 min and stopped by adding 5 mL of 0.6% HCl (in ethanol). The hydroperoxide formed was assayed according to a ferric thiocyanate method with mixing in order of 0.02 M ferrous chloride (0.1 mL) and 30% ammonium thiocyanate (0.1 mL) (Wong and Yen, 1997). The absorbance at 480 nm (A_s) was measured with a spectrophotometer (Hitachi U-2000, Tokyo, Japan) for 5 min. The absorbance of blank (A_0) was obtained without add hemoglobin to the above reaction mixture; the absorbance of control (A_{100}) was obtained with no sample addition to the above mixture. Thus, the antioxidative activity of the sample was calculated as $100\% - (A_s - A_0)/(A_{100} - A_0) \times 100\%$.

Normal Phase HPLC Chromatography. The products from hemoglobin-catalyzed peroxidation of linoleic acid were extracted with ethyl acetate and then reduced with $NaBH_4$ and methylated with diazomethane. The resulting compounds were separated with a solid phase extraction column (Si form, J&W Scientific, Folsom, CA) and then subjected to normal phase high-pressure liquid chromatographic (HPLC) analysis (Kuo et al., 1997b; Toschi et al., 1995).

Normal phase HPLC analyses were performed on a Bondclone silica column (30 cm × 3.9 mm, 10 μm, Phenomenex, Torrance, CA) equipped with a pump (Waters, Model 510, Milford, MA) and UV detector (Waters, Model 486) monitored at 234 nm. The derivatives of hydroperoxide were eluted isocratically with a solvent system of hexane/ethanol/acetic acid (98:1.9:0.1, v/v/v) at a flow rate of 0.8 mL/min. The hemoglobin-catalyzed oxidation products, 18:2-9OOH (9-HpODE) and 18:2-13OOH (13-HpODE) were confirmed in comparison to authentic standards.

RESULTS AND DISCUSSION

Reaction Conditions for Determining Antioxidative Activity. *Reaction Time.* The effect of reaction time on the hemoglobin-catalyzed peroxidation of linoleic acid at 37 °C is shown in Figure 1. The peroxidation was expressed as absorbance at 480 nm. Greater absorbance results from higher peroxidation. Peroxidation of linoleic acid increased with incubation time in a linear relationship within the first 10 min (Figure 1). In the traditional thiocyanate method, the absorbance reached 0.9 when linoleic acid was incubated at 37 °C for 21 days (Tsuda et al., 1993). In the present assay, the absorbance increased to 1.13 at 37 °C for 10 min. Our method has the advantage of short reaction time, which is useful to routine screening of antioxidants from natural sources or to determine antioxidative activity of the chromatographically purified fractions.

Concentration of Linoleic Acid. The effect of linoleic acid concentration on its peroxidation rate is shown in Figure 2. The peroxidation rate increased with the concentration of linoleic acid in a linear relationship up to 5 mM (Figure 2). In the following experiments, the concentration of linoleic acid was kept at 4 mM.

Concentration of Methanol. In most cases, methanol was used to extract antioxidative substances from natural sources. The effect of methanol concentration on the peroxidation of linoleic acid was studied and is shown in Figure 3. The peroxidation of linoleic acid decreased with increased methanol concentration. Concentration of methanol up to 15% resulted in the highest peroxidation ratio. At 20% methanol, the peroxidation ratio was 85%, and at 30% methanol, the peroxidation ratio was 39%. Therefore, the concentration of methanol must be kept at an appropriate concentration, such as 0−20%; otherwise, the peroxidation of linoleic acid was somewhat influenced by methanol addition. Actually, results from Figure 3 showed that the methanol extract of sample was able to be assayed directly for their antioxidative activity without any pretreament, because only 10 μL of sample volume was sufficient in most cases.

Concentration of Hemoglobin. The effect of hemoglobin concentration on the peroxidation of linoleic acid is

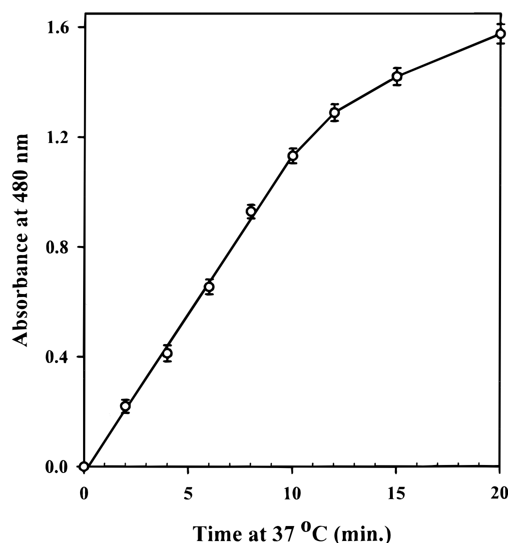

Figure 1. Effect of incubation time on hemoglobin-catalyzed peroxidation of linoleic acid. The peroxidation of linoleic acid (4 mM) in 0.05 M phosphate buffer (pH 7.0) containing Tween 20 (0.05%) was initiated by adding 0.002% hemoglobin and incubating with shaking (100 rpm) at 37 °C for various times and then stopped by mixing in an acid−ethanol solution. The hydroperoxide formed was analyzed by using a ferric thiocyanate method and measured with a spectrophotometer at 480 nm.

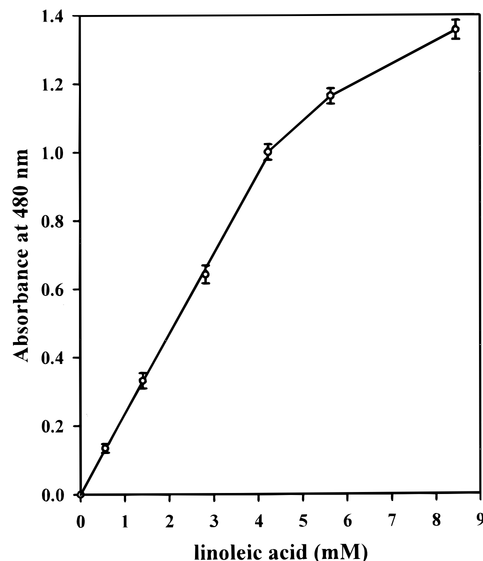

Figure 2. Relationship between linoleic acid concentration and peroxidation of linoleic acid. Various concentrations of linoleic acid were prepared in 0.05 M phosphate buffer (pH 7.0) containing Tween 20 (0.05%). The peroxidation of linoleic acid was initiated by adding 0.002% hemoglobin and incubating with shaking (100 rpm) at 37 °C for 10 min and then stopped by mixing in an acid−ethanol solution. The hydroperoxide formed was analyzed by using a ferric thiocyanate method and measured with a spectrophotometer at 480 nm.

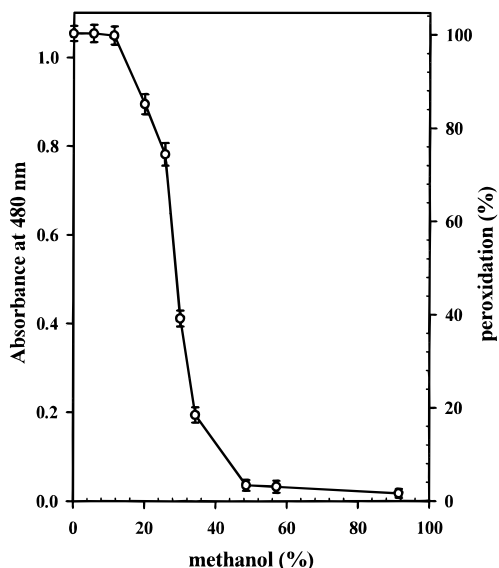

Figure 3. Effect of methanol concentration on the peroxidation of linoleic acid. Different concentrations of methanol were prepared in 0.05 M phosphate buffer (pH 7.0) containing Tween 20 (0.05%) and linoleic acid (4 mM). The peroxidation of linoleic acid was initiated by adding 0.002% hemoglobin and incubating with shaking (100 rpm) at 37 °C for 10 min and then stopped by mixing in an acid−ethanol solution. The hydroperoxide formed was analyzed by using an ferric thiocyanate method and measured with a spectrophotometer at 480 nm.

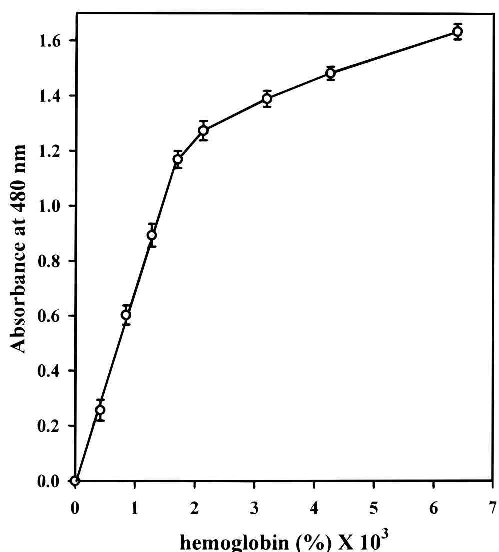

Figure 4. Effect of hemoglobin concentration on the peroxidation of linoleic acid. The peroxidation of linoleic acid (4 mM) in 0.05 M phosphate buffer (pH 7.0) containing Tween 20 (0.05%) was initiated by adding various concentrations of hemoglobin and incubating with shaking (100 rpm) at 37 °C for 10 min and then stopped by mixing in an acid−ethanol solution. The hydroperoxide formed was analyzed by using a ferric thiocyanate method and measured with a spectrophotometer at 480 nm.

shown in Figure 4. The peroxidation rate of linoleic acid increased proportionately with the concentration of hemoglobin added up to 0.002%. If linoleic acid was incubated at 37 °C without hemoglobin, the peroxidation proceeded quite slowly (Tsuda et al., 1993). The degree of peroxidation without hemoglobin in 21 days was similar to that of hemoglobin-catalyzed peroxidation of linoleic acid for 10 min (Figures 1 and 4; Tsuda et al., 1993).

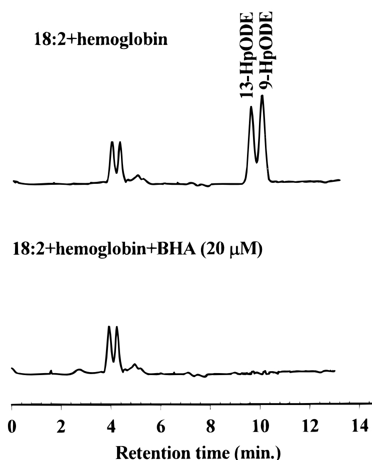

Figure 5. Normal phase HPLC chromatogram of products from hemoglobin-catalyzed oxidation of linoleic acid. The peroxidation of linoleic acid (4 mM) in 10 mL of 0.05 M phosphate buffer (pH 7.0) containing Tween 20 (0.05%) was initiated by adding 0.35% hemoglobin (50 μL) and incubating with shaking (100 rpm) at 37 °C for 15 min. The products from hemoglobin-catalyzed peroxidation of linoleic acid were extracted, reduced, methylated, separated, and then subjected to normal phase HPLC analyses.

Table 1. Antioxidative Activity of Eight Synthetic Antioxidants Determined with Present Method

antioxidants	IC_{50}[a] (μM)	antioxidants	IC_{50}[a] (μM)
BHA	3.62 ± 0.15[b]	quercetin	7.52 ± 0.31
BHT	6.64 ± 0.26	DL-α-tocopherol	6.18 ± 0.21
propyl gallate	5.21 ± 0.29	ascorbic acid	90.04 ± 4.89
myricetin	6.81 ± 0.27	epicatechin	6.45 ± 0.33

[a] IC_{50}, inhibitory concentration caused 50% inhibition of the peroxidation of linoleic acid. [b] Mean ± SD, $n = 3$, $p < 0.05$.

Hemoglobin and myoglobin both acted as effective prooxidants as observed in this study as well as in that of Bertelsen et al. (1995). The mechanism of heme catalysis in lipid peroxidation was probably via the formation of lipid peroxide−heme complex or ferryl-type heme compound (Ito et al., 1995). To compare the hemoglobin-catalyzed peroxidation of linoleic acid with autoxidation, the products were extracted, separated, and analyzed with normal phase HPLC (Figure 5). Products derived from hemoglobin-catalyzed peroxidation of linoleic acid were 9- and 13-HpODE at a ratio of approximately 50:50, indicating the oxygenation occurred at two specific sites, similar to those products that would be found in autoxidation.

Determination of Antioxidative Activity of Synthetic Antioxidant and Edible Plant Material with This Rapid Method. Eight synthetic antioxidants including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate, myricetin, quercetin, *dl*-α-tocopherol, ascorbic acid, and epicatechin were assayed for their antioxidative activities with this rapid method (Table 1). All of these antioxidants inhibited the hemoglobin-catalyzed peroxidation of linoleic acid proportionally to concentration added, and the inhibition was found to be linear response to the logarithm of their concentration. The inhibition was then calculated and expressed as IC_{50}, the inhibitory concentration causing 50% inhibition of the peroxidation of linoleic acid, and listed in Table 1. The IC_{50} of the eight synthetic antioxidants showed that BHA had the highest antioxidative effect on the hemoglobin-catalyzed oxidation of linoleic acid, whereas ascorbic acid exhibited the lowest. Between were propyl gallate, *dl*-α-tocopherol, epicatechin, BHT, myricetin, and quercetin.

Table 2. Antioxidative Activity of Various Edible Plant Extracts Determined with Present Method

plant	IC_{50}[a] (ppm)	plant	IC_{50}[a] (ppm)
green tea	0.25 ± 0.02[b]	garlic	79.50 ± 7.16
oolong tea	0.33 ± 0.03	lemon peel	122.00 ± 9.96
black tea	0.38 ± 0.03	orange peel	68.75 ± 5.39
roselle	18.80 ± 1.69	tangerine peel	72.00 ± 6.34
chrysanthemum petal	18.03 ± 1.78	peanut hull	111.25 ± 8.98
ginger root	4.38 ± 0.39	burdock	343.75 ± 18.68
basil	40.25 ± 4.43	oat	95.00 ± 7.61
red pepper	60.75 ± 4.86	BHA	0.65 ± 0.05
carrot	168.00 ± 10.12		

[a] IC_{50}, inhibitory concentration caused 50% inhibition of the peroxidation of linoleic acid. [b] Mean ± SD, $n = 3$, $p < 0.05$.

Linear response curves were also obtained for various edible plants using this method. The IC_{50} values are shown in Table 2. Among them, tea, ginger, chrysanthemum, and roselle showed higher antioxidative activity, similar to those findings in the literature (Yen and Chen, 1995; Tsushida, 1994).

CONCLUSION

A rapid photometric method to determine antioxidant activity was established in this study. The recommended conditions for evaluating the antioxidative activity are the following: total volume of reaction mixture, 0.4 mL; sample size, 5−100 μL; reaction temperature, 37 °C; reaction time, 10 min; phosphate buffer, 50 mM; pH, 7.0; Tween 20, 0.05%; linoleic acid, 4 mM; hemoglobin, 0.002%; methanol concentration, 0−20%.

Advantages in using this rapid method include simplicity of instrumentation, short assay time (15 min per sample), and applicability for large numbers of samples. We evaluated 400−500 samples in half a day. In addition, the sample size needed is only 5 μL per assay. Therefore, this method seems to be very useful for analyzing the antioxidative activity from chromatographically purified fractions or for routine screening of antioxidative activity.

On the other hand, the reason for such a fast reaction of the hemoglobin-catalyzed peroxidation of linoleic acid under such low hemoglobin concentration is unclear, although there are mechanisms proposed for heme-catalyzed oxidation, including formation of lipid peroxide−heme complex or ferryl-type heme compound (Ito et al., 1995), lipoxygenase-like activity (Zilletti et al., 1994), or superoxide generation activity (Macdonald, 1994). The biochemical basis and the actual mechanism in the present system are being studied.

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