Food Sci. Technol. Res., **10** (4), 432–436, 2004

Note

Development of a Spectrophotometric Assay System for Evaluating Reducibility of Water-Soluble Substances

Shinya ASHIDA, Satoshi F. NOGUCHI and Ryoichi SATO

Central Research Institute, Maruha Co., 16-2, Wadai, Tsukuba, Ibaraki 300-4295, Japan

Received April 23, 2004; Accepted August 10, 2004

A spectrophotometric assay system for evaluating reducibility of water-soluble substances using native bovine oxymyoglobin (MbO_2) was developed. With incubation of MbO_2 solution at fixed temperature and pH, the autoxidation rate to metmyoglobin (metMb) was measured. The reductive effects of test samples on metMb could be estimated as numerical values based on the changes in the rate constants from MbO_2 to metMb. The reductive effects of four kinds of water-soluble compounds were examined: L-ascorbic acid (AsA) strongly reduced the autoxidation rate of MbO_2, glutathione and kojic acid showed reductive effects inferior to that of AsA, while gallic acid tended to promote autoxidation at high concentrations. The results of test compounds obtained by the present MbO_2 assay were compared with data by ferricyanide assay and XTT assay, which are known methods for evaluating reducibility. The MbO_2 assay is useful because of its superior features of sensitivity, application flexibility and capability of evaluating pro-oxidant activity of reducing agents.

Keywords: reducibility, oxymyoglobin, metmyoglobin, L-ascorbic acid

Introduction

Recent research has clarified the relationships between active oxygen species and various human illnesses, and reducing agents in foodstuffs such as AsA that have the ability to eliminate active oxygen species *in vivo* have attracted a great deal of attention. Hitherto, for evaluating reducibility in foods, various assay methods which use the reduction of an inorganic substance such as ferricyanide (Arai, 1998) or that of a synthetic organic substance such as XTT (Ukeda *et al.*, 1995), have been reported. These ferricyanide and XTT assays have been applied to the evaluation of heat-treated milk samples (Shimamura *et al.*, 2001); however, there have been few reports that applied these methods to the screening of reducing substances in foodstuffs. Furthermore, it is questionable whether these methods using inorganic or synthetic organic compounds are applicable for evaluating reducibility in complex systems such as food materials or organisms.

In the present study, we have developed a new spectrophotometric assay system for reducibility applying the reduction of a naturally occurring organic substance: myoglobin (Mb). Native MbO_2, which was prepared from non-frozen bovine muscle, was used for evaluation of the reductive effect of test compounds on metMb generated from autoxidation of MbO_2. No reductant like hydrosulfite, which is suspected to affect measurement accuracy in evaluating reducibility, was used in this assay system. To verify the validity of this spectrophotometric assay system, the effects of four kinds of water-soluble low molecular weight compounds known to be water-soluble reducing agents, *i.e.*, AsA, glutathione, kojic acid and gallic acid were examined, and were compared with the data obtained by the ferricyanide and XTT assays.

E-mail: s-ashida@maruha.co.jp

Materials and Methods

Materials Non-frozen fresh beef (*musculus biceps femoris* of Japanese black cattle) was purchased at a supermarket in Tsukuba-city. 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). Milli-Q water was used in all procedures.

Preparation of MbO_2 Preparation of MbO_2 from bovine muscle was performed according to a modification of the method of Trout and Gutzke (1996). Non-frozen fresh beef from the Japanese black cattle was used for the preparation of MbO_2. All subsequent procedures were carried out at low temperature (0–5°C) to minimize the production of metMb. The surface layer (3–4 mm), which was exposed to oxygen and colored, and fat were trimmed and removed from a beef block (weighing about 600 g), and a sample of about 150 g of the central part of each block was used for the following experiment. The prepared muscle was diced by hand and homogenized in 300 ml of a cold buffer solution (10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA). The homogenate was centrifuged at 10,000 g for 10 min at 0°C. The pH of the supernatant was adjusted to 8.0 with 1 N sodium hydroxide aqueous solution, and ammonium sulfate was added to the supernatant to 65% (w/v) saturation. The suspension was gently stirred for 60

min in an ice bath and centrifuged at 18,000 g for 20 min. The supernatant was filtered through a filter paper, ammonium sulfate was added to the filtrate until 100% (w/v) saturation, and the pH was readjusted to 8.0. The suspension was stirred for 60 min, and then centrifuged at 20,000 g for 60 min. The precipitate containing Mb was suspended in a cold 5 mM Tris-HCl buffer (pH 8.5), and then the suspension was centrifuged at 20,000 g for 5 min and filtered with a disposable filter (pore size 0.45 μm). The crude Mb solution, prepared as described above, was then passed through a Sephadex G-100 column (26ϕ × 850 mm, Pharmacia Co.) with 5 mM Tris-HCl buffer (pH 8.5) at a flow rate of 40 ml/hour, and fractions of 3.0 ml were collected. Each fraction was monitored for the absorbance at 730, 572, 525 and 280 nm with a HITACHI Model U-3210 spectrophotometer (Tokyo). The Mb fractions, that had an A_{280}/A_{525} ratio < 5.2, were pooled. The Mb solution obtained as described above was examined for the absence of ammonium sulfate using Pack test-NH_4 (Kyoritsu Chemical-Check Lab., Tokyo) and kept at 0°C in the dark and in the presence of oxygen until use. The prepared MbO_2 was stable and little of it changed to metMb even during 3 weeks in storage.

Measurement of the autoxidation rate of MbO_2 Before measurement, the Mb solution was desalted and concentrated with Molcut L LGC (nominal molecular weight limit 10,000; Nihon Millipore Ltd., Tokyo) under an oxygen pressure of 0.2 MPa for 3 hours at 0°C. The concentrated Mb pellet on the Molcut filter cup was diluted with distilled water, and the concentration of MbO_2 was set as 60μM. The concentration of MbO_2 was determined using the following formula; $[Mb](\mu M) = 0.132 \times (A_{525} - A_{730})$ (Krzywicki, 1982). The rate of autoxidation of MbO_2 was measured by incubation of the following mixture containing the MbO_2 solution: 3 ml of the reaction mixture containing MbO_2 (30 μM), 0.1 M phosphate buffer (pH 6.0) and a fixed concentration of sample to evaluate its reductive effect on the autoxidation of MbO_2. The phosphate buffer was kept at 0°C and air-equilibrated by air-bubbling just before the addition. This mixture was incubated in a glass cuvette with a 1 cm path width at a predetermined temperature in a spectrophotometer (HITACHI, U-3210) equipped with a temperature-controlled cell holder, and the absorbances at 3 wavelengths (730, 572 and 525 nm) of the reaction mixtures were measured at intervals of 5 minutes over a period of one hour. In order to calculate the rate constant for the autoxidation of MbO_2 to metMb, 0% metMb and 100% metMb standard solutions were prepared with addition of sodium hydrosulfite or potassium ferricyanide (about 1 mg), respectively. To 3 ml of the MbO_2 reaction mixture in 30 ml Erlenmeyer flasks was added sodium hydrosulfite or potassium ferricyanide, and the mixtures were stirred in air. Five minutes later, the mixtures were put into glass cuvettes and the absorbances at 730, 572 and 525 nm were measured. The percentage of MbO_2 in the sample mixture could be calculated by comparison of the value of the ratio $(A_{572}\text{-}A_{730})/(A_{525}\text{-}A_{730})$ of sample solution with the corresponding values of the 0% metMb standard solution and of the 100% metMb standard solution. The autoxidation process was followed by plotting log percent MbO_2 *vs.* time and the rate constant for the autoxidation of MbO_2 to metMb was determined from the slope of each straight line in the first-order plot. The autoxidation rate measurements of MbO_2 were performed in triplicate using different MbO_2 solutions made from three different bovine muscles.

Ferricyanide assay procedure The assay was performed according to a modification of the method of Arai (1998). All samples to evaluate its reductive effect were dissolved in water just before measurements. Different amounts of samples in 15 ml of water were put into a solution of 50 mM phthalate buffer (pH 5.6, 5 ml) and 1% potassium hexacyanoferrate (II) solution (5 ml). The mixture was incubated at 30°C for 30 min, and was then cooled on ice, and 10% trichloroacetic acid solution (5 ml) was added. Five milliliters of the mixture was put into a tube containing 5 ml of water and then 1 ml of 0.1% iron (III) chloride solution was added. The rate of reduction of ferricyanide was determined spectrophotometrically at 610 nm by use of a ferrocyanide calibration curve.

XTT assay procedure The assay was performed according to the method of Shimamura *et al.* (2000). Each microplate well contained 60 μl of 0.5 mM XTT solution prepared with 200 mM phosphate buffer (pH 7.0) containing saturated menadione. Then 40 μl of a sample solution of a given concentration was added to the well. At 0 and 20 min after the addition, the absorbances at 492 nm (reference 600 nm) were measured on a microplate reader SPECTRAmax 250 (Molecular Devices Co., Sunnyvale, CA), and the increase in the absorbance was recorded as the ability to reduce XTT.

Results and Discussion

Purification of myoglobin Crude bovine Mb solution was separated into three fractions by Sephadex G-100 column chromatography. The first and second eluted peaks were the high molecular weight impurities, such as hemoglobin. The third and main peak was attributed to Mb. Trout and Gutzke (1996) reported previously that the purity of Mb in solution was more than 96% when the value of the A_{280}/A_{525} ratio of the solution was under 5.2. We collected such fractions containing ferrous Mb (about 97%) in high purity (>96%) for use in the following experiments. It was possible to obtain about 200–300 mg of purified Mb per cycle of column treatment under the above conditions. The stability of purified Mb which was prepared and stored as described above, was investigated as shown in Fig. 1. Even after 3 weeks of storage at 0°C, the preparation contained less than 4% metMb. In the present study, the preparations which fulfilled the following conditions (within 3 weeks of storage and containing less than 5% metMb) were used for assay.

Setting of conditions for measurement of the MbO_2 autoxidation rate In the present assay, the rate constant for the change in MbO_2 to metMb was used to evaluate reducing activity on metMb from the following points of

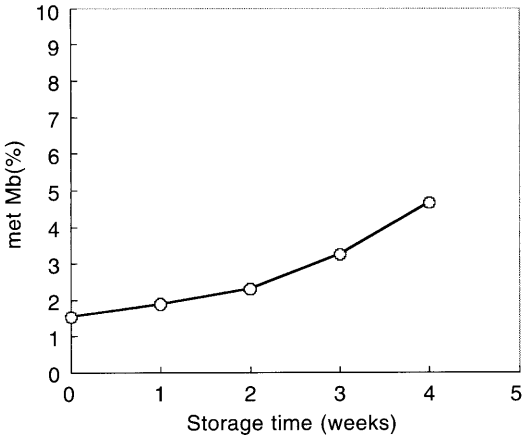

Fig. 1. The change in percent metMb of beef MbO_2 preparation stored at 0°C in the dark.

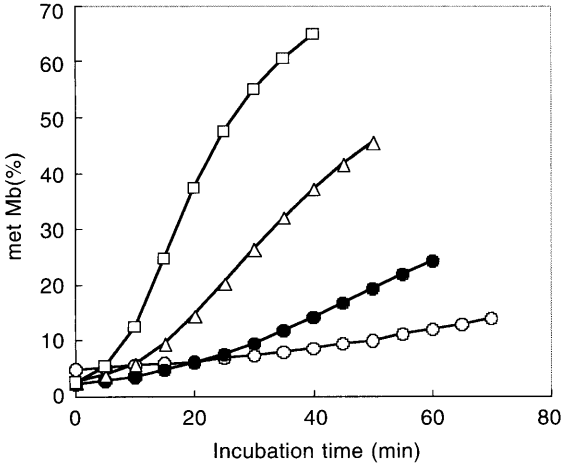

Fig. 2. Oxidation process of MbO_2 at various temperatures at pH 6.0. MbO_2 was added to a phosphate buffer (pH 6.0) to a final concentration of 30 μM. The mixture was incubated at (○); 25°C, (●); 30°C, (△); 35°C and (□); 40°C.

view. Bovine MbO_2 is oxidized easily to metMb by the generation of superoxide with a primary chemical reaction (Brown & Mebine, 1969; Gotoh & Shikama, 1976).

$$MbO_2(Fe(II)) \rightarrow metMb(Fe(III)) + O_2^-.$$

It has been revealed that this autoxidation reaction is not a simple dissociation of the superoxide from MbO_2, but is a complicated nucleophilic displacement of the superoxide from MbO_2 by the entering a water molecule or hydroxyl ion (Shikama, 1984; Shikama, 1988). Because metMb generated from the autoxidation of MbO_2 does not possess the physiological function of oxygen-binding, enzymatic reduction systems are present in red cells and muscle tissue in order to reduce metMb to deoxymyoglobin (deoxyMb (Fe(II)) (Livingston *et al.*, 1985). Assuming that such reduction systems of metMb were present in the MbO_2 solution, these systems apparently would retard the autoxidation by reducing metMb. Thus the establishment of adequate conditions of MbO_2 solution for measurement of the rate constant will enable the evaluation of reducibility.

The conditions of the reaction solution were set as pH 6.0 and 30°C as follows. The autoxidation rate of MbO_2 increased at lower pH (Shikama & Sugawara, 1978). Reducing agents in food materials or organisms have been shown to be active at weakly acidic or neutral pH. Thus, the pH of the reaction mixture was adjusted to 6.0, for both experimental convenience with rapid reaction and for agreement with the pH values at which actual reducing agents show activity. Figure 2 shows the effects of temperature on the autoxidation of MbO_2 without a reducing agent at pH 6.0. The rate of autoxidation of MbO_2 increased rapidly with the temperature of the reaction solution. At 30°C, the metMb content of the solution as the product of MbO_2 autoxidation increased to about 20% after 1 hour of incubation. These conditions seemed to be sufficient to calculate the autoxidation rate. Although the experimental time would be reduced with incubation at temperatures higher than 30°C, such as 35°C or 40°C, denaturation of Mb molecules or hemin dissociation were suspected to occur at high temperatures. Therefore, the temperature of the Mb solution in this assay was set as 30°C. With regard to the influence of the change of oxygen concentration during measurement, Brantley *et al.* (1993) reported that the consumption of oxygen was insignificant for dissolved oxygen around 50 μM or higher as far as the concentration of MbO_2 was kept at 30 μM. From the measurement with a dissolved-oxygen meter (Central KAGAKU Co., Model Oxygraph-9, Tokyo), dissolved oxygen concentrations in the present reaction mixture buffer after the measurement of autoxidation were more than 200 μM nearly the dissolved oxygen saturation values at 30°C. Thus the influence of the change of dissolved oxygen concentration could be neglected in the present study.

Measurement of the reducing activity of AsA, kojic acid, glutathione and gallic acid on the autoxidation of MbO_2 AsA as a typical water-soluble reducing agent was examined to corroborate the present assay system. Figure 3 shows the effects of AsA (0, 0.3, 1, 3 and 10μM) on the autoxidation of MbO_2 at pH 6.0 and 30°C. The vertical and horizontal axes in Fig. 3 represent the logarithm of the percentage of MbO_2 in the Mb solution and the incubation time of the MbO_2 solution, respectively. From the start to about 20 minutes of the incubation, the percentage of MbO_2 decreased at a similar slow rate in all samples because about 20 minutes was required to reach a temperature of nearly 30°C. However after 20 minutes, the decrease in level of MbO_2 was strongly affected by the concentration of AsA with significant inhibitory effects seen with the addition of more than 1μM AsA. Figure 3 also shows a similar linear relationship between the logarithm of the percentage of MbO_2 and the incubation time from 20 minutes to 60 minutes. This was in agreement with the results of previous studies indicating that the autoxidation of MbO_2 to metMb of bovine Mb is a primary chemical reaction. It should be noted that the rate constant values decreased with the addition of AsA and that the autoxidation rates of MbO_2 were related to the concentration of AsA. The apparent rate constant values, k_{obs} were deter-

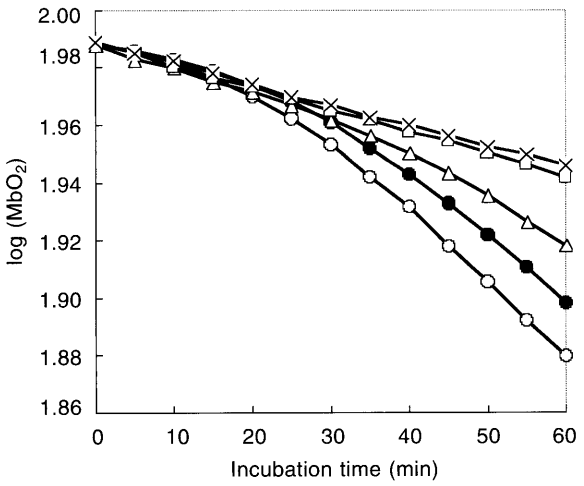

Fig. 3. Reductive effects of AsA on oxidation of MbO_2. AsA was added to phosphate buffer (pH 6.0) immediately before the addition of MbO_2. The reaction mixture was incubated at 30°C. The concentrations of AsA were (○); 0 μM, (●); 0.3 μM, (△); 1 μM, (□);3 μM, and (×); 10μM.

mined from the slope of each line for 20–60 minutes in the present study.

The k_{obs} values in the presence of four kinds of typical low molecule compounds known to possess reducing activity (AsA, glutathione, kojic acid and gallic acid) were measured in a manner similar to that described above. The inhibitory rates of the test compounds were measured at five concentrations (3, 10, 30, 100, and 300 μM). The results are summarized in Table 1. A high inhibitory rate value indicates strong reducibility on metMb by the test compound. The reductive effect of AsA showed a slight increase at a concentration of 10 μM in comparison with at a concentration of 3 μM, and was similar from 10 to 300 μM. Kojic acid and glutathione showed weaker reductive effects than ascorbic acid at all concentrations tested. These compounds were regarded as being inferior to AsA in their reducibilities. Gallic acid exhibited complicated effects on the autoxidation of MbO_2. At concentrations of gallic acid lower than MbO_2 (3 and 10 μM *vs.* 30μM, respectively), a reductive effect was observed; however, at higher concentrations (100 and 300 μM) gallic acid promoted autoxidation. As lowering the pH accelerates the oxidation of MbO_2, it was postulated that addition of gallic acid to a certain extent promoted oxidation by reducing the pH of the MbO_2 solution. Nevertheless, investigation of the influence of gallic acid on the pH of the test solution indicated little effect on the pH (<0.05) within the range of concentration used in the present experiment. Therefore, the influence of the pH changes with gallic acid could be neglected in the present study. As an alternative assumption, the promotional effect of gallic acid on MbO_2 oxidation may be caused by the generation of active oxygen such as hydrogen peroxide. The influence of catalase on the inhibition rate of gallic acid was examined as shown in Fig. 4. The oxidation of MbO_2 was significantly suppressed in the presence of catalase. In the absence of gallic acid (control), a comparison of the rate constants with and without catalase indicated that about 30% reduction in the reaction rate was obtained by catalase. When gallic acid at low concentrations (3, 10 and 30 μM) was present in the reaction mixture, the effect of catalase on the inhibition rate of gallic acid was insignificant. However, with high concentrations (100 and 300 μM), the inhibition rates of the acid in the presence of catalase were significantly larger than that its absence. These results suggest that the addition of a high concentration of gallic acid promotes oxidation due to the generation of active oxygen such as hydrogen peroxide from the reaction between gallic acid and MbO_2. Previous studies (Yusa & Shikama, 1987;

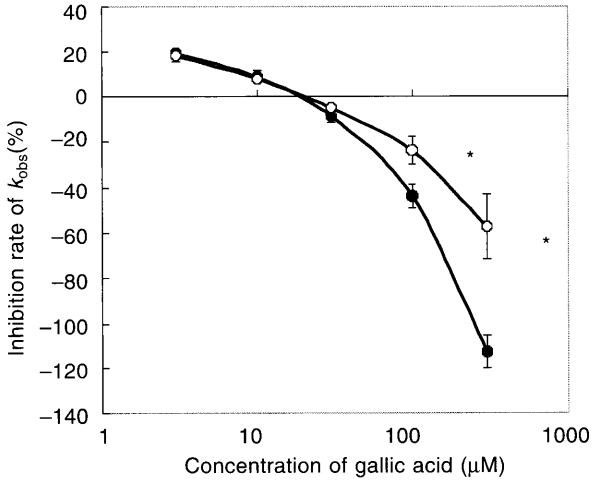

Fig. 4. Effect of catalase on the inhibition rate of gallic acid in regard to MbO_2 oxidation. The inhibition rates of various concentrations of gallic acid (○); with 2×10^{-8}M catalase from bovine liver and (●); without catalase were calculated from k_{obs} values of the controls (in the absence of gallic acid), which were close to 0.236 (h^{-1}) with catalase and 0.338 (h^{-1}) without catalase, respectively. Data represent the mean and S.D. of three replications. *$p < 0.001$ significantly different from the data without catalase.

Table 1. Comparison of reductive effects of various compounds on oxidation of MbO_2.

Concentrations (μM)	AsA		glutathione		kojic acid		gallic acid	
	$k_{obs}(h^{-1})$	Inhibitory rate of k_{obs}	$k_{obs}(h^{-1})$	Inhibitory rate of k_{obs}	$k_{obs}(h^{-1})$	Inhibitory rate of k_{obs}	$k_{obs}(h^{-1})$	Inhibitory rate of k_{obs}
3	0.103±0.003	69.4%±0.6%	0.338±0.005	–0.2%±4.2%	0.362±0.008	–7.2%±4.4%	0.212 ± 0.007	37.3% ± 1.4%
10	0.095±0.001	71.8%±0.7%	0.287±0.013	14.8%±2.5%	0.316±0.025	6.3%±5.8%	0.266 ± 0.019	21.2% ± 4.3%
30	0.095±0.003	71.9%±0.6%	0.277±0.019	17.9%±4.1%	0.290±0.012	13.9%±2.4%	0.339 ± 0.026	–0.3% ± 5.8%
100	0.096±0.001	71.6%±0.6%	0.257±0.016	24.0%±3.5%	0.263±0.021	22.1%±8.2%	0.452 ± 0.034	–33.8% ± 7.7%
300	0.088±0.001	74.0%±0.5%	0.297±0.030	12.0%±7.1%	0.223±0.004	33.8%±3.1%	0.616 ± 0.052	–82.4% ± 11.9%

The apparent rate constant values (k_{obs}) were estimated by the decrease in the percentage of MbO_2 (20–60 minutes). The inhibitory rate of each compound is expressed by the following formula: {(k_{obs} of compound-free sample) – (k_{obs} of compound-added sample)}/(k_{obs} of compound-free sample). Data represent the mean and S.D. of three repeated tests using different preparations from three different bovine muscles.

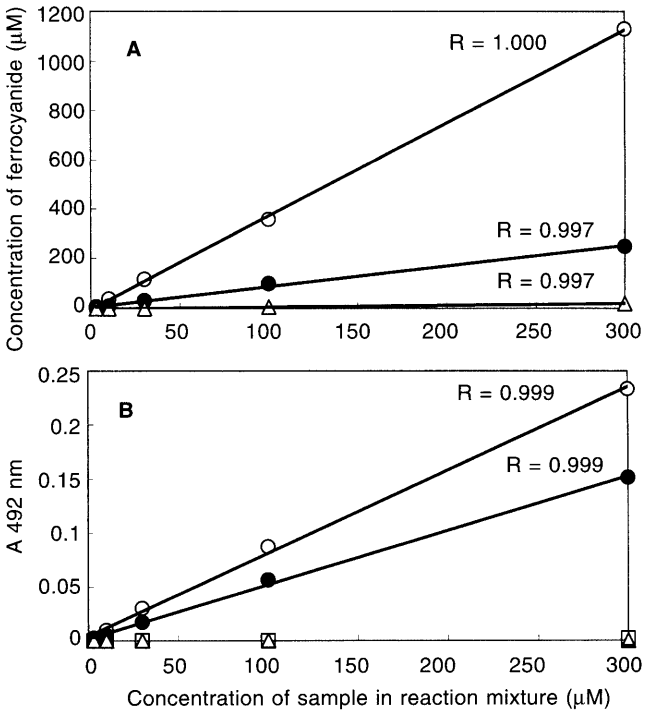

Fig. 5. Reductive effect of low molecular compounds against ferricyanide(A) and XTT(B). The reducibilities by (○); AsA, (●); glutathione, (△); kojic acid and (□); gallic acid are expressed as the concentration of generated ferrocyanide(A) and the absorbance at 492 nm(B). The volumes of reaction mixtures in the ferricyanide method and the XTT method were 25 ml and 100 μl, respectively. Data represent the mean of three replications.

Giulivi & Cadenas, 1993) have shown that the interaction between MbO_2 and reductant such as AsA was associated with hydrogen peroxide formation. Such a "pro-oxidant" effect of AsA was regarded as inferior to that of gallic acid, because AsA at high concentrations (100 and 300 μM) did not promote autoxidation. Consequently, the present MbO_2 assay can be used for evaluating not only reducibility but also pro-oxidant activity of water-soluble substances.

Comparison of MbO_2 method with ferricyanide method and XTT method In order to evaluate alternative reducibility of the compounds, the reduction of ferricyanide and XTT were measured. The effectiveness of the compounds was examined using the ferricyanide method and the XTT method as shown in Fig. 5. The effectiveness of four kinds of compounds on ferricyanide was in the following descending order: kojic acid < glutathione < AsA and the effect of gallic acid could not be measured due to the coloring caused by the formation of an iron-tannin complex. The effectiveness of the XTT method was in the following order: kojic acid and gallic acid (no activity) < glutathione < AsA. As to both the ferricyanide and XTT methods, the relations between reducibility and concentrations of each compound were found to be linear. In a comparison of the three methods (the present MbO_2 method, the ferricyanide method and the XTT method), AsA correspondingly showed the strongest reductive effects among four kinds of compounds. However, kojic acid, which was shown to be the metMb-reducing substance, exhibited no activity with XTT and very weak reducibility with ferricyanide. The pro-oxidant effect of gallic acid at high concentrations, which was observed by the MbO_2 method, was not detectable by either the ferricyanide or XTT methods. These results suggest that the MbO_2 assay is useful because it has superior properties in not only experimental sensitivity and application flexibility but also its capability of evaluating pro-oxidant activity of reducing agents. Moreover, the MbO_2 assay may be a promising method for evaluating reducibility in complex systems such as food materials or organisms because this assay utilizes the reduction of a naturally occurring organic substance *i.e.*, metMb.

We are currently planning experiments to examine the reducibilities of naturally occurring compounds in foodstuffs using this Mb assay.

References

Arai, I. (1998). The recent level of protein-reducing substances (determined by ferricyanide reduction method) of market milk. *Shokuhin-eiseikenkyu*, **48**, 101–111 (in Japanese)

Brantley, R.E., Smerdon, S.J., Wilkinson,A.J., Singleton, E.W. and Olson, J.S. (1993). The mechanism of autoxidation of myoglobin. *J. Biol. Chem.*, **268**, 6995–7010.

Brown, W.D. and Mebine, L.B. (1969). Autoxidation of oxymyoglobins. *J. Biol. Chem.*, **244**, 6696–6701.

Giulivi, C. and Cadenas, E. (1993). The reaction of ascorbic acid with different heme iron redox states of myoglobin antioxidant and prooxidant aspects. *FEBS Lett.*, **332**, 287–290.

Gotoh, T. and Shikama, K. (1976). Generation of the superoxide radical during the autoxidation of oxymyoglobin. *J. Biochem.*, **80**, 397–399.

Krzywicki, K. (1982). The determination of haem pigments in meat. *Meat Sci.*, **7**, 29–36.

Livingston, D.J., McLachlan, S.J., La Mar, G.N. and Brown, W.D. (1985). Myoglobin: Cytochrome b_5 interactions and the kinetic mechanism of metmyoglobin reductase. *J. Biol. Chem.*, **260**, 15699–15707.

Shikama, K. and Sugawara, Y. (1978). Autoxidation of native oxymyoglobin. kinetic analysis of the pH profile. *Eur. J. Biochem.*, **91**, 407–413.

Shikama, K. (1984). A controversy on the mechanism of autoxidation of oxymyoglobin and oxyhaemoglobin: oxidation, dissociation, or displacement? *Biochem. J.*, **223**, 279–280.

Shikama, K. (1988). Stability properties of dioxygen-iron(II) porphyrins: an overview from simple complexes to myoglobin. *Coordination Chem. Rev.*, **83**, 73–91.

Shimamura, T., Ukeda, H. and Sawamura, M. (2000). Reduction of tetrazolium salt XTT by aminoreductone formed during the Maillard reaction of lactose. *J. Agric. Food Chem.*, **48**, 6227–6229.

Shimamura, T., Ukeda, H. and Sawamura, M. (2001). Quality evaluation of milk using XTT assay: comparison with ferricyanide assay. *Nippon Shokuhin Kagaku Kogaku Kaishi*, **48**, 840–843. (in Japanese)

Trout, G.R. and Gutzke, D.A. (1996). A simple, rapid preparative method for isolating and purifying oxymyoglobin. *Meat Sci.*, **43**, 1–13.

Ukeda, H., Goto, Y., Sawamura, M., Kusunose, H, Kamikado, H. and Kamei, T. (1995). Reduction of tetrazolium XTT with UHT-treated milk: its relationship with the extent of heat-treatment and storage conditions. *Food Sci. Technol. Int.*, **1**, 52–57.

Yusa, K. and Shikama, K. (1987). Oxidation of oxymyoglobin to metmyoglobin with hydrogen peroxide: involvement of ferryl intermediate. *Biochemistry*, **26**, 6684–6688.