Determination of Ascorbate Concentration in a Raw Leaf with Electron Spin Resonance Spectroscopy

Yijing LIN, Wenwei LIU, Hanae OHNO and Tateaki OGATA

Graduate School of Science and Engineering, Yamagata University, Jorunan, Yonezawa, 992-8510, Japan

The concentration of ascorbate in a raw leaf was determined by monitoring a nitroxyl radical reduction with X-band ESR. One piperidine nitroxyl derivative, 2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl (TEMPOL), was reduced according to first-order kinetics by more than 5-fold excess of ascorbate. The observed reduction rate was found to be proportional to ascorbate concentration in phosphate buffer (0.1 mol/dm³, pH 7.4) at 25°C. The reduction of TEMPOL in leaf homogenate of sprouts of radish was measured; it was found to obey first-order kinetics. The ascorbate concentration in leaf was estimated from observed reduction rate and found to be virtually identical with that from a visible absorption spectrophotometric analysis. The features of our ESR method for sensitivity, procedure and measuring time were discussed with comparisons from other methods. The results indicated that the determination of ascorbate in a raw leaf by ESR spectroscopy was simple and effective.

Keywords Ascorbate concentration, nitroxyl radical, TEMPOL, ESR, raw leaf, first-order kinetics, rate constant

There are several methods for determining ascorbic acid, such as visible absorption spectrophotometry, electrochemical analysis, enzymatic analysis and liquid chromatography. In food products, spectrophotometric analysis and liquid chromatography have usually been used. The classical chemistry associated with the spectrophotometric methods can be divided into two general groups. One of them is based on the reduction properties of ascorbic acid, such as reduction of 2,6-dichlorophenolindophenol by ascorbic acid which causes a decrease in the absorption at 520 nm.^{1,2} The other is based on the oxidation of the ascorbic acid and subsequent formation of a hydrazone or a fluorophor. Ascorbic acid is oxidized by copper and the oxidized product is treated with hydrazine to form hydrazone, which has an absorption at 520 nm;³⁻⁵ or ascorbic acid is oxidized by iodine, and then condensed with *o*-phenylenediamine to form the fluorophor quinoxaline.⁶

Recently, liquid chromatography has been used extensively for ascorbic acid analysis. This technique combines high selectivity and sensitivity, and does not require derivatization. Ultraviolet spectroscopic detection is usually used to monitor ascorbic acid after separation by ion-exchange chromatography.^{7,8} However, in the case of the spectrophotometry and the liquid chromatography, it is necessary to obtain the supernatant solution of the samples for ascorbic acid determination.

Usually, one must employ troublesome procedure to separate samples completely. A radical sample that contains an unpaired electron can be measured by ESR spectrometer no matter whether the sample is in gas, liquid or solid state, no matter whether the sample is a pure compound or a mixture. Therefore ESR technique

will be a good method to determine a sample without separation. TEMPOL (one of nitroxyl radicals) is stable in air; it has been successfully used as a spin probe at *in vivo* ESR research projects.^{9,10} The reduction of nitroxyl radicals has been studied by many investigators.¹¹⁻¹³ Since ascorbate acted as a sole non-enzymatic reducing agent of TEMPOL in biological samples *in vitro*¹⁴, we think that it might be useful to measure the TEMPOL reduction rate, from which the ascorbate concentration would be calculated.

This paper reports our preliminary study on how ascorbate concentration in leaves of sprouts of radish is determined with ESR spectroscopy. The determination is done in aqueous solution without separating, centrifuging or filtration and results are compared with those determined by visible absorption spectrophotometry. These observations demonstrate that it might be a simple and effective method for the determination of ascorbate in food products.

Experimental

Reagents and apparatus

TEMPOL was purchased from Aldrich (Milwaukee, WI). The following chemicals were obtained from Wako Pure Chem. Ind. Ltd.: ascorbic acid, diethylenetriamine-*N,N,N',N'',N'''*-pentaacetic acid (DTPA), and 2,6-dichlorophenolindophenol. β -Nicotinamide adenine dinucleotide phosphate (reduced form, β -NADPH) was obtained from Oriental Yeast Co. Ltd. All of them were used without further purification.

ESR spectra were measured with an X-band ESR

spectrometer (JEOL, JES-FR 30). The ESR signal at low field ($M_I=+1$) was measured under the conditions of a microwave power of 4 mW, a 100 kHz modulation width of 0.1 mT, a magnetic field scanning range of 335.9 ± 5 mT, a response time of 0.03 s and a sweep time of 60 s. All measurements were carried out at 25°C.

Visible absorption spectra were measured with a UV-visible spectrophotometer (Shimazu, UV-240) at room temperature (about 25°C).

Sprouts of radish with root were purchased from a supermarket on each experimental date. The leaves were homogenized with a mixer (Nihonseiki, ABM-1).

Reduction of TEMPOL in ascorbate solution

The reduction of TEMPOL was measured in freshly prepared ascorbate solutions. Both TEMPOL and ascorbic acid were dissolved in 0.1 M (=mol/dm³) phosphate buffer. The ascorbate concentrations were used from 0.5×10^{-4} M to 7×10^{-4} M and TEMPOL concentration was 1×10^{-5} M. The ESR signal was monitored for 5 min and the reduction rate was calculated from time course of ESR signal intensity.

Determination of ascorbic acid content in a raw leaf

Ascorbic acid content in leaves of sprouts of radish was determined with ESR as follows. Just before homogenizing, 0.1 ml TEMPOL (2×10^{-4} M) solution was added to the glass tube (15 mm inner diameter) in which 0.2 g leaves and 1.9 ml phosphate buffer were contained. After 10 s homogenization (rpm 22000), about 0.05 ml homogenate was taken in a capillary and placed into a microwave cavity of ESR spectrometer as soon as possible. The timing was started at homogenizing commencement. The ESR signal was monitored for 5 min and the measurement was repeated more than 3 times. The phosphate buffer used here contained 1×10^{-4} M DTPA, which was used as a metal chelating agent. In addition, phosphate buffer without DTPA was also used in order to examine the effect of DTPA.

Ascorbic acid content in leaves of sprouts of radish was determined using a modification of visible absorption spectrophotometry.¹⁵ Briefly, 2 ml of supernatant fluid in 2.5% HPO₃ was used to determine the total content of ascorbic acid and dehydroascorbic acid. When ascorbic acid in the sample is oxidized by 2,6-dichlorophenolindophenol to form dehydroascorbic acid, the product is treated with 2,4-dinitrophenylhydrazine to form the derivative bis-2,4-dinitrophenylhydrazone. The absorption of the derivative at 520 nm was measured and the total content of ascorbic acid and dehydroascorbic acid was obtained.

Results and Discussion

Reduction of TEMPOL in ascorbate solution

The triplet ESR signal of TEMPOL decreased when ascorbate (AsH^-) solution was added into TEMPOL

Fig. 1 Time course of ESR signal intensity for TEMPOL (1×10^{-5} M) in different ascorbate solutions (▲, 0.5×10^{-4} M; ■, 3×10^{-4} M; ●, 6×10^{-4} M) at pH 7.4.

solution. This is because a nitroxyl radical ($\text{R-NO}\cdot$) was reduced to corresponding hydroxylamine (R-NOH) which was ESR silent. The mechanism of TEMPOL reduction by ascorbic acid in aqueous solution was predicted as Eqs. (1) to (3).¹⁶

These reactions involve dissociation, one-electron transfer and the disproportionation of ascorbate radical anion ($\text{As}^{\cdot-}$). The ascorbate (AsH^-) and dehydroascorbic acid (DHA) are produced with disproportionation of ascorbate radical anion.

Ascorbic acid has two acidic protons; thus three species, ascorbic acid (AsH_2), ascorbate (AsH^-) and ascorbate dianion (As^{2-}) are present in solution. At pH 7.4, the percentage of AsH_2 , AsH^- and As^{2-} was calculated to be $3.74 \times 10^{-6}\%$, 99.996%, and $3.98 \times 10^{-3}\%$, respectively from $\text{p}K_{a1}=4.17$, $\text{p}K_{a2}=11.57$. Since AsH^- is the predominant one in phosphate buffer of pH 7.4, the total concentration of AsH_2 , AsH^- and As^{2-} is considered to be equal to ascorbate (AsH^-) concentration. For this reason, ascorbate was used instead of ascorbic acid because the ESR measurement was performed at pH 7.4.

Since dissociation of ascorbic acid or disproportionation of ascorbate radical anion ($\text{As}^{\cdot-}$) is rapid, the kinetics of TEMPOL reduction could be expressed by the following equation:

Fig. 2 The relationship between rate constant of first order kinetics and ascorbate concentration at 0.1 M phosphate buffer (pH=7.4) obtained at TEMPOL of 1×10^{-5} M and ascorbate of 0.5×10^{-4} M to 7×10^{-4} M (second order rate constant $k_2 = 6.96 \text{ M}^{-1} \text{ s}^{-1}$, correlation coefficient 0.9942).

$$d[\text{TEMPOL}]/dt = k_2[\text{TEMPOL}][\text{AsH}^-] \quad (4)$$

In the presence of a large excess of ascorbate ($[\text{AsH}^-] \gg [\text{TEMPOL}]$), ascorbate concentration can approximate its initial one ($[\text{AsH}^-] = [\text{AsH}^-]_0$), and the reduction obeys the pseudo-first order kinetics:

$$\begin{aligned} d[\text{TEMPOL}]/dt &= k_2[\text{TEMPOL}][\text{AsH}^-]_0 \\ &= k_{\text{obs}}[\text{TEMPOL}] \end{aligned} \quad (5)$$

$$k_{\text{obs}} = k_2[\text{AsH}^-]_0 \quad (6)$$

Here k_2 is second order rate constant and k_{obs} is observed rate constant (pseudo-first order rate constant).

As is known, the area of triplet ESR signal is proportional to the concentration of TEMPOL. The peak-to-peak height of TEMPOL signal at low field was used as a relative "concentration" because the observed line-width of TEMPOL signal was found not to change in any experiment. The reduction was quantified by least square regression of the logarithm of the ESR signal for the first 5 min. The reduction rate constant (k_{obs}) was determined as the negative slope of this regression. Figure 1 indicates that TEMPOL reduction obeys first order kinetics in more than 5-fold time ascorbate solutions.

As presented in Fig. 2, the observed reduction rates for TEMPOL in various ascorbate solutions were found to be directly proportional to the ascorbate concentration. A standard curve with correlation coefficient of 0.9942 was constructed with ascorbate ranging between 0.5×10^{-4} M to 7×10^{-4} M. The second order rate con-

Table 1 Ascorbate concentrations calculated from rate constants of TEMPOL reduction

Actual value/ 10^{-4} M	Calculated value ^a / 10^{-4} M
0.5	0.44 ± 0.05
3.0	3.01 ± 0.12
7.0	6.94 ± 0.08

a. $n=5$.

stant (k_2) was $6.96 \pm 0.03 \text{ M}^{-1} \text{ s}^{-1}$; this was calculated from the slope of the line in Fig. 2.

Ascorbate solutions of 0.5×10^{-4} M, 3×10^{-4} M and 7×10^{-4} M were prepared. TEMPOL reduction by these solutions were observed with ESR. The rate constants were obtained. Concentrations were calculated and are shown in Table 1. The ascorbate concentrations obtained from the observed reduction rate were found to be virtually identical with actual values.

Determination of ascorbic acid content in raw leaf

TEMPOL reduction in the homogenate of fresh leaves was quite rapid, and the ESR signal disappeared in 7–8 min. The time course of logarithm of ESR signal intensity (peak-to-peak height) is illustrated in Fig. 3. As shown in the plot, the reduction of TEMPOL in leaf homogenate obeyed first order kinetics within 5 min, and the observed reduction rate constant was obtained from the negative slope. The observed reduction rate constant was divided by the second order rate constant (k_2) and the ascorbic acid content in leaves of sprouts of radish was determined.

Ascorbic acid content in leaf was also determined by modified visible absorption spectrophotometry. A standard curve was constructed with standards ranging between 10 mg and 40 mg of ascorbic acid per liter of 2.5% HPO_3 . The change in absorbance (ΔA) was calculated from the following equation: $\Delta A = A_t - A_d - A_b$, where A_t is the absorbance after ascorbic acid in the sample was oxidized to dehydroascorbic acid by indophenol; A_d is the absorbance of dehydroascorbic acid in the sample; A_b is the absorbance of reagent blank. ΔA is linearly related to ascorbic acid concentration, and the concentration in leaf is obtained by comparison of ΔA with the standard curve. Ascorbic acid content in leaf was determined by spectrophotometry and ESR method; both values are given in Table 2. The precision measured by the ESR method seems to be not less than the one by the spectrophotometry.

Ascorbic acid concentration in leaf, which was estimated from TEMPOL reduction rate constants, agreed well with the one determined by visible absorbance. It also has the same order with the value reported in the literature¹⁷, namely, 47 mg ascorbic acid per 100 g leaf.

Effect of air-oxidization and metal-catalyzed oxidation during measurement

Since TEMPOL reduction by ascorbate is used in

Fig. 3 The decline of ESR signal intensity of TEMPOL observed in leaf homogenate (containing 0.2 g raw leaf+1.9 ml phosphate buffer+0.1 ml TEMPOL of 2×10^{-4} M, and homogenized for 10 s at 22000 rpm).

ESR methods to determine the concentration, it is important to study the effect of air-oxidization of ascorbate at pH 7.4 during measurements. Ascorbate solutions of several concentration were prepared and stirred by the mixer at 22000 rpm for 10 to 20 s. TEMPOL solution was added to each ascorbate solution with or without vigorous stirring; then the ESR signal of each solution was measured. No change of TEMPOL reduction rate was found after the stirring, which means ascorbate was not destroyed by air-oxidization or heat during rapid revolving. In fact, little change in TEMPOL reduction was observed when ascorbate in phosphate buffer was left for 2–3 h at room temperature. This indicates that the effect of air-oxidization of ascorbate could be ignored during TEMPOL reduction. But ascorbate solution was weak for heating. After heating for a few minutes at 100°C , the ascorbate was completely destroyed and TEMPOL could not be reduced in this solution.

It was reported that DTPA could prevent metal-catalyzed autoxidation of ascorbic acid and could stabilize the reducing activity in supernatant fluid and ultrafiltrates.¹⁴ In addition, Cu(II) or Fe(III) has been shown to catalyze the oxidation of hydroxylamine.¹⁹ In our ESR method, the effect of metal-catalyzed oxidation is discussed. The leaf was homogenized in phosphate buffer with or without DTPA; then the reduction of TEMPOL by each homogenate was examined. The observed reduction rate was $(1.8 \pm 0.2) \times 10^{-3} \text{ s}^{-1}$ for phosphate buffer with DTPA, and $(1.7 \pm 0.2) \times 10^{-3} \text{ s}^{-1}$ for that without DTPA. There is no difference when the raw leaves of sprouts of radish were homogenized in phosphate buffer with or without DTPA. In the case of a plant with a larger amount of heavy metal ion, however, metal-catalyzed oxidation of ascorbate might be

Table 2 Ascorbic acid contents (mg) per 100 g raw leaf analyzed from two methods

Method	Content
ESR measurement ($n=7$)	43.8 ± 5.0
Visible absorption spectrophotometry ($n=4$)	43.8 ± 8.1

observed. Therefore the use of phosphate buffer containing DTPA will be better at the present time.

Enzymatic influence in leaf homogenate

It was reported that TEMPOL was reduced in a rat liver *in vitro* by NADH or NADPH-dependent reductases.¹⁸ Here, the enzymatic effect on TEMPOL reduction in leaf was investigated. We observed that TEMPOL was reduced slowly in the homogenate after ascorbate oxidase was added to denature ascorbic acid, which suggests that reducing compounds other than ascorbate would exist. Further, we found that TEMPOL reduction rate increased when NADPH (2×10^{-3} M) was incubated with the homogenate for 10 min. This means TEMPOL reduction in the leaf was NADPH-dependent. However, NADPH did not affect TEMPOL reduction rate when the incubation was not done. This is extremely important for our ESR method because TEMPOL reduction in leaf homogenate at initial period was used to obtain ascorbate concentration. We concluded ascorbate was a sole reducing compound for TEMPOL in initial period time although TEMPOL were reduced enzymatically and non-enzymatically in leaf homogenate.

Comparison of several determining methods

With either the spectrophotometry or the liquid chromatography for ascorbic acid determination, it is necessary to centrifuge the homogenate or to filtrate the supernatant solution further in order to remove any corpuscles that interfere significantly with spectrophotometric determinations. The separating procedure is usually troublesome.

In food products, the sensitivity of ascorbic acid determination is 10 mg/dm^3 for modified visible absorption spectrophotometry, 5 mg/dm^3 for liquid chromatography²⁰, and 8 mg/dm^3 for our ESR method. Linearity ranges from 10 to 40 mg/dm^3 , 5 to 30 mg/dm^3 and 9 to 123 mg/dm^3 could be used for modified visible absorption spectrophotometry, liquid chromatography and our ESR method, respectively. There is no distinct difference in sensitivity and measurable range. For the spectrophotometric analysis, it takes more than 3 h to incubate the supernatant solution for production of the hydrazone completely. Furthermore, in this method one usually needs to filter the supernatant solution with a special column after centrifuge. However, for our ESR method, the homogenate could be used directly without centrifuge or filtrate, and only 5 min are need-

ed for one sample measurement. Consequently, the ESR method is convenient for ascorbate analysis in food products if there is an ESR spectrometer in a laboratory.

This study presents a simple method for determination of ascorbate concentration by ESR spectroscopy. Results demonstrated that it was virtually identical to classical spectrophotometric method although it was simple by comparison with the former method. However, it is impossible to know the dehydroascorbic acid content in a sample with this ESR method. Since the reducing property of ascorbic acid is utilized in TEMPOL reduction, only ascorbate concentration could be obtained directly by ESR method.

The present work was supported in part by a Grant in Aid of the Japan Society for Promotion of Science (No. 97443).

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(Received April 19, 1999)

(Accepted June 28, 1999)