Pergamon 0031-9422(95)00632-X

Phytochemistry, Vol. 41, No. 2, pp. 349–351, 1996

0031-9422/96 $15.00 + 0.00

STABILITY OF ASCORBATE OXIDASE EXTRACTED FROM *CUCURBITA PEPO*

SILVANA MARITANO, TAKAMITSU KOHZUMA,* SHINNICHIRO SUZUKI* and AUGUSTO MARCHESINI†

Istituto Sperimentale per la Nutrizione delle Piante, Turin, Italy; *Department of Chemistry, Faculty of Science, Osaka University, Osaka 560, Japan

(*Received in revised form* 19 *July* 1995)

Key Word Index—*Cucurbita pepo*; Cucurbitaceae; ascorbate oxidase; stability of ascorbate oxidase; ammonium sulphate; type 1 and type 2 copper atoms.

Abstract—The physical and enzymic properties of ascorbate oxidase kept in saturated ammonium sulphate solution at 4° were examined before and after 10 months storage. There were no significant changes in behaviour on sodium dodecyl sulphate gels, in absorbtion spectra, circular dichroic spectra or EPR spectra during this time, nor in specific enzyme activity or K_m. Such stored preparations are suitable for analytical examination in distant laboratories and possibly have commercial uses.

INTRODUCTION

Ascorbate oxidase (EC 1.10.3.3) contains copper ions in at least three different chemical complexes, two of which give rise to an EPR spectrum. The enzyme has two subunits of M_r 70000 each [1].

In the oxidized state the protein has an intense blue colour, which derives from Type 1 mononuclear Cu(II), and which has been found to be analogous to that of low M_r copper proteins such as stellacyanin, mavicyanin, azurin and plastocyanin. In these last four proteins, the copper is bound to two imidazole nitrogens and to cysteine sulphur, which is responsible for the intense blue colour, caused by the charge transfer $RS^- \rightarrow Cu(II)$. A second copper site in ascorbate oxidase, Type 2 copper atoms, contains a metal ion with a complex consisting of two imidazoles. The other copper atoms, Type 3, are each bound to three histidines [2].

In order to study this copper protein, it is necessary that the physico-chemical parameters be conserved as long as possible. This copper protein is relatively stable in neutral–alkaline conditions with buffers whose ionic strength is 100–300 mM. In general, when it is concentrated (5–10 $g\,l^{-1}$) the stability of the native enzyme can be assured for a period of one or two weeks. Successively, this metalloprotein undergoes a progressive decrease in the intensity of its color and a reduction in catalytic activity with ascorbic acid. This behaviour is evident after about a month at 4° [3].

Complete physico-chemical characterization of ascorbate oxidase often entails sending the copper protein to other laboratories. During transport, conservation of the enzyme is achieved by packing the samples in dry ice. This is suitable for most purposes, but not for studies on the catalytic activity of the enzyme. Indeed, due to the considerable variation in temperature during thawing, the solution of the enzyme becomes turbid and the catalytic activity decreases by about one-third (unpublished data). In order to overcome this problem, the present study was undertaken on the stability of ascorbate oxidase enzyme conserved in a solution of saturated ammonium sulphate at 4° for 10 months.

RESULTS AND DISCUSSION

The spectroscopic properties of two ascorbate oxidase samples (October 1993 and August 1994) are shown in Fig. 1 (electronic absorption spectrum of sample in August 1994), Fig. 2 (EPR spectra) and Table 1 (circular dichroism data). The spectral data for the sample in October 1993 are quite similar to those for the sample in August 1994 (the visible absorption spectrum of the sample in October 1993 is not shown). The sodium dodecyl sulphate (SDS) gel electrophoresis of two samples in Figs 3 and 4 shows that the major bands are ascorbate oxidase, although several minor bands, which are probably due to impurities and/or degradation products during the electrophoresis, are observed in the charging of large amount of protein. It can be seen that the catalytic activity of the enzyme after conservation for 10 months at 4° is similar to that of the initial enzyme (Table 2), which was determined at the beginning of the conservation period. The relative intensities of the bands in the EPR spectra (Fig. 2) due to Types 1 and 2 copper atoms indicate a ratio of three Type 1 copper atoms for

†Author to whom correspondence should be addressed.

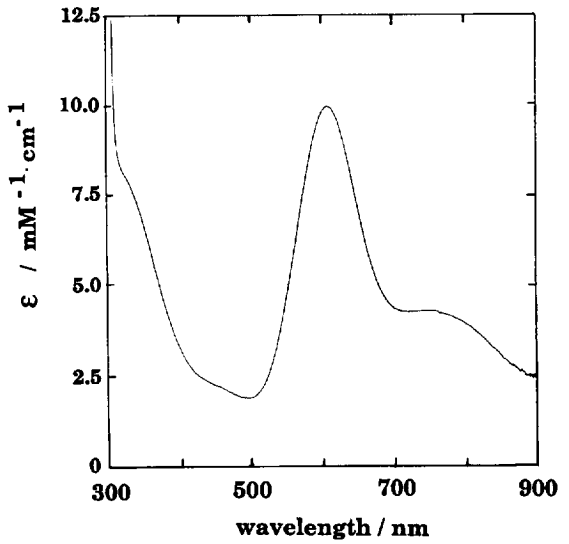

Fig. 1. Visible absorption spectrum of ascorbate oxidase in 50 mM phosphate buffer (pH 7.0): August 1994.

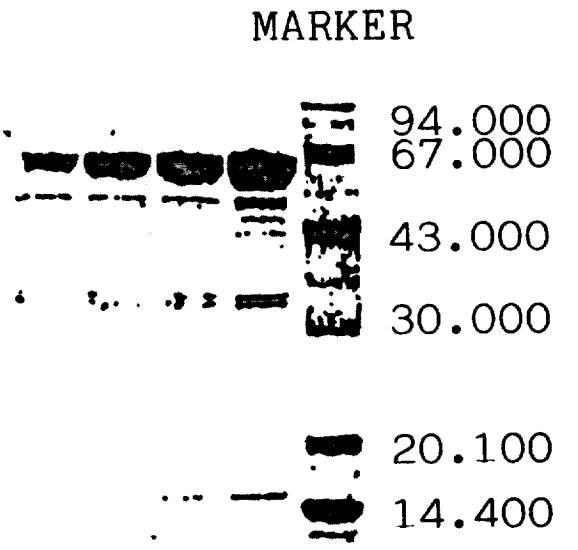

Fig. 3. SDS electrophoresis of ascorbate oxidase just after arrival in Osaka: October 1993. M_r values of markers are expressed in Daltons.

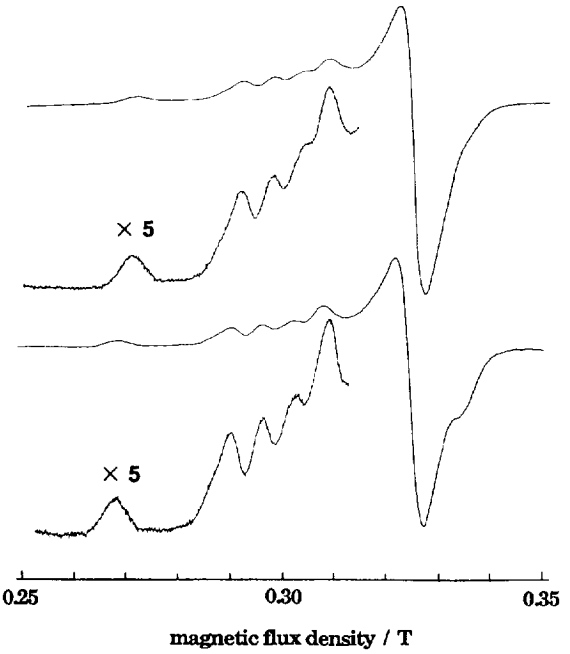

Fig. 2. EPR spectra of ascorbate oxidase in 50 mM phosphate buffer (pH 7.0) at 77 K: October 1993 (upper) and August 1994 (lower).

every Type 2 copper atom, as in cucumber ascorbate oxidase [4]. This is in distinct contrast with the crystallographic analyses of Messerschmidt [5], which indicate that the ratio between Type 1 and Type 2 copper is 2:2 atoms per mole of enzyme. This may result from the fact that Type 2 copper would not be 100% EPR detectable in solution because of the anti-ferromagnetic interaction with Type 3 copper. The various ratios reflect the different fractions of Type 2 signal obtained under various conditions [6].

Conservation of copper protein ascorbate oxidase in ammonium sulphate at 4° maintains the enzyme in its native state for at least 10 months. This is particularly advantageous for the study of the protein in different laboratories distant from the site where the enzyme was purified. In addition, conservation of the enzyme at room temperature appears to be possible.

EXPERIMENTAL

Ascorbate oxidase from zucchini (*Cucurbita pepo*) peels was purified following the method of ref. [7]. Once prepd, the pure protein was characterized physically and chemically, and pptd in $(NH_4)_2SO_4$. Physico-chemical

Table 1. Circular dichroism extrema of ascorbate oxidase: (a) just arrived in Osaka, October 1993; (b) August 1994, after conservation for 10 months in ammonium sulphate at 4°

(a) 740 nm ($\Delta\varepsilon = -17.7$), 600 nm ($\Delta\varepsilon = +8.00$), 540 *sh* ($\Delta\varepsilon = +3.72$), 475 nm ($\Delta\varepsilon = -5.58$), 420 nm ($\Delta\varepsilon = +0.93$), 320 nm ($\Delta\varepsilon = -1.67$)
(b) 740 nm ($\Delta\varepsilon = -17.6$), 600 nm ($\Delta\varepsilon = +8.27$), 540 *sh* ($\Delta\varepsilon = +4.41$), 475 nm ($\Delta\varepsilon = -5.51$), 420 nm ($\Delta\varepsilon = +0.88$), 320 nm ($\Delta\varepsilon = -1.98$)

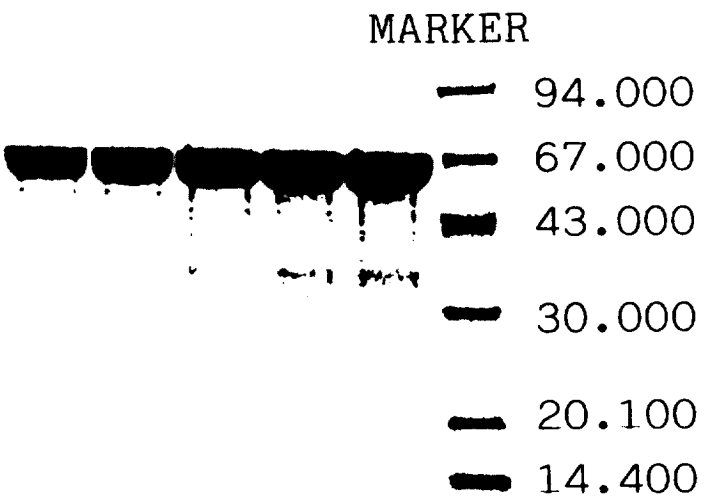

Fig. 4. SDS electrophoresis of ascorbate oxidase after 10 months of conservation in ammonium sulphate at 4°: August 1994. M_r values of markers are expressed in Daltons.

Table 2. UV–visible characterization, specific activity and Michaelis–Menten constant: (a) October 1993; (b) after 10 months of conservation in ammonium sulphate at 4°

Ratio (280 nm/610 nm)	Ratio (330 nm/610 nm)	Specific activity (kat)*	K_m (M)
(a) 27.7 ± 0.5	0.8 ± 0.05	48.2×10^{-6}	2.3×10^{-4}
(b) 26.6 ± 0.5	0.76 ± 0.05	44.0×10^{-6}	2.5×10^{-4}

*Substrate = ascorbic acid.

parameters obtained with this first characterization were identical to the parameters obtained at Osaka University. The ppt was concd in plastic vials and sent to the University of Osaka. The travel time at room temp. was 5 days. Upon arrival in Osaka, part of the Cu protein was dialysed against Na-Pi buffer (0.1 M, pH 7) and then characterized by SDS electrophoresis, EPR analysis and CD spectra. The electronic and CD spectra were recorded with a Shimadzu UV-2200 spectrophotometer and a JASCO J-500A spectropolarimeter equipped with a JASCO DP-501 data processor, respectively. The EPR spectra were measured with a JEOL JES FE-1X X-band spectrometer at 77 K. The SDS electrophoresis was effected with an ATTO AE-6400 apparatus, loading ATTO SPG-520L gel (5–20% gradient). The remaining portion of the enzyme was conserved in the original containers for 10 months at 4°. During this period no formation of ascorbic oxidase protein crystals was observed. At the end of the 10 months, the Cu protein was dialysed against Na-Pi buffer (0.1 M, pH 7), concd and characterized in the same way as the initial sample taken at the start of the 10-month period (SDS electrophoresis, EPR analysis and CD spectra).

The aerobic catalytic activity of the two samples, initial and final, was determined according to ref. [8]. Before measuring the catalytic activity of the second sample conserved for 10 months at 4°, the Cu protein was reduced with equivalents of ascorbic acid and re-oxidized in the presence of O_2 [9] in order to restore the native activity.

REFERENCES

1. Messerschmidt, A., Rossi, A., Ladenstein, R., Huber, R., Bolognesi, M., Gatti, G., Marchesini, A., Petruzzelli, R. and Finazzi-Agrò, A. (1989) *J. Mol. Biol.* **206**, 513.
2. Deinum, J., Reinhammar, B. and Marchesini, A. (1974) *FEBS Letters* **3**, 241.
3. Maritano, S., Piano, V. and Marchesini, A. (1994) *Agrochimica* **38**, 172.
4. Sakurai, T., Suzuki, S. and Chikira, M. (1990) *J. Biochem.* **107**, 37.
5. Messerschmidt, A., (1993) in *Bioinorganic Chemistry of Copper* (Karlin, K.D. and Tyeklar, Z., eds), p. 471. Chapman and Hall, New York.
6. Calabrese, L., Carbonaro, M. and Musci, G. (1988) *J. Mol. Chem.* **263**, 6480.
7. Marchesini, A. and Kroneck, P. (1979) *Eur. J. Biochem.* **101**, 65.
8. Lessler, M. A. and Brierley, G. P. (1969) *Biochem. Anal.* **27**, 1.
9. Tollin, G., Meyer, T. E., Cusanovich, M. A., Curir, P. and Marchesini, A. (1993) *Biochim. Biophys. Acta* **1183**, 309.