Isolation and characterization of an iron-containing superoxide dismutase from tomato leaves, *Lycopersicon esculentum*

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The protein had a relative molecular mass of about 42000 and was composed of two equal subunits non-covalently joined. It was negatively charged (pI = 4.6) and contained about 1.45 mol Fe/mol dimer and negligible amounts of Mn, Cu and Zn. Absorption spectrum and sensitivity to NaN₃, H₂O₂ and temperature are also reminiscent of other ferric superoxide dismutases.

Comparison of amino acid composition indicated, however, a closer relationship to the Mn-containing enzymes rather than to other Fe-containing superoxide dismutases. Two possible ways of Fe-containing superoxide dismutase acquisition by vascular plants were suggested.

Superoxide dismutase is an important enzyme for aerobic organisms [1], although it has also been found in several anaerobic ones [2]. It disproportionates potentially harmful superoxide anion radicals to dioxygen and hydrogen peroxide [3].

Three types of this enzyme have been recognized with respect to the prosthetic metal present in it. Copper-and-zinc-containing superoxide dismutase is localized mainly in the cytosol of eucaryotes [4]. However, it has also been found in mitochondria [5] and higher plant chloroplasts [6–8]. The enzyme represents an independent line of descent while two others, manganese and iron-containing superoxide dismutases, show a close evolutionary relationship [4, 9, 10]. The former protein is present commonly in bacteria and mitochondria [4], suggesting an endosymbiotic origin of this organelle [11].

Ferric superoxide dismutase was previously isolated from many procaryotic sources and was believed to be confined to them [4]. However, data on its existence in plants have accumulated. The enzyme was isolated from the eucaryotic green alga *Euglena gracilis* [12] and two higher plants *Brassica campestris* [13] and *Nuphar luteum* [14]. It was also reported that Fe-containing enzyme was present in only three families of vascular plants out of the 43 surveyed [15]. However, we have recently provided the evidence that ferric superoxide dismutase (Fe-SOD) is present in two chilling-sensitive plants: tomato and bean [16], members of the next two families.

In this work we describe ferric superoxide dismutase from tomato leaves, which, according to their properties, is quite similar to other Fe-SOD enzymes, but, on the grounds of amino acid composition, seems to be closer to those containing Mn. Two possible ways of Fe-SOD acquisition by vascular plants are suggested.

**MATERIALS AND METHODS**

*Plant material and chemicals*

Fresh tomato leaves (*Lycopersicon esculentum*, Mill., var. Venture) were harvested from a greenhouse. The sources of chemicals were as follows: xanthine oxidase, Boehringer; bovine serum albumin, riboflavin and sodium dodecyl sulfate (SDS), BDH Chemicals Ltd; Sephadex G-100, Sephacryl S-200, DEAE-Sephadex A-50 and DEAE-Sephacel, Pharmacia Fine Chemicals; polyvinylpyrrolidone, K₂0, NaN₃ and 2-mercaptoethanol, Fluka AG; ampholines, LKB; Coomassie brilliant blue R-250, International Enzymes Ltd; acrylamide, bisacrylamide, dimethylsulfoxide, glycerol and Titrisols (Fe, Cu, Zn and Mn standard solutions), Merck; phenylmethylsulfonyl fluoride, Sigma Chemical Company; ammonium persulfate and dithiothreitol, Roth KG; NBT and KCN, Chemapol (Czechoslovakia); tetramethylethylenediamine and molecular mass standards, Serva Feinbiochemica. Other chemicals were from P.O.Ch. (Poland).

*Electrophoretic procedures*

SDS/polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn [17], using the following molecular mass standards (M₀): bovine serum albumin (67000), egg albumin (45000), chymotrypsinogen (25000),...
myoglobin (17800) and cytochrome c (12400). Electrophoresis of native protein in 7% and 12% gel was performed as described by Davis [18]. Superoxide dismutase activity was localized on gels by the method of Beauchamp and Fridovich [19]. The isoelectric point was determined on acrylamide gel containing ampholines (pH 3.5–10) using LKB 2117 Multiphor apparatus. The corresponding pH values were determined directly on the gel surface with a microelectrode.

**Enzyme and protein assays**

Superoxide dismutase activity, after each purification step and when the purified enzyme was characterized, was measured by the method of McCord and Fridovich [3]. Activity was calculated according to Asada et al. [20]. Column fractions in the course of enzyme purification were assayed as described by Henry et al. [21]. Protein content of crude fractions and purified enzyme was determined according to Lowry et al. [22] and Murphy and Kies [23] respectively using bovine serum albumin as a standard for both methods.

**Determination of molecular mass**

The relative molecular mass was determined by gel exclusion chromatography using Sephadex G-100 and Sephacryl S-200 gels. The molecular mass standards were the same as in SDS/polyacrylamide gel electrophoresis.

Sedimentation equilibrium centrifugation was performed in a MSE Centriscan 75 ultracentrifuge equipped with an ultraviolet scanning system. The molecular mass of the enzyme was estimated by the low-speed equilibrium method according to manufacturer. The partial specific volume was calculated from the amino acid composition [24].

**Amino acid composition and metal content**

Analysis was performed with a Jeol model JLC-6AH amino acid analyser. Protein samples, after exhaustive dialysis against water, were hydrolyzed for 24 h, 48 h and 72 h at 110°C under reduced pressure in 6 M HCl containing 2-mercaptoethanol (0.5 ml/l) to protect methionine and tyrosine. Half-cystine was determined as cysteic acid, after oxidation with performic acid [25]. Tryptophan was determined spectrophotometrically by the method of Edelhoch [26].

Metals were determined by atomic absorption spectrophotometry using an Instrumentation Laboratory model 551 video I apparatus.

**RESULTS**

**Purification of the enzyme**

All operations were carried out at 0–2°C. Buffers, except those for final dialysis, were protected with pHix (Pierce).

Washed, fresh leaves (about 8000 g) were thoroughly ground in a blender containing 100 mM potassium phosphate, pH 7.8, 5 mM 2-mercaptoethanol, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. 100 g polyvinyl-pyrrolidone was gradually added during homogenization. The resulting homogenate was squeezed through cheesecloth and brought to 40% saturation with ammonium sulfate (0.242 g/ml). After 1 h of stirring, the precipitate was centrifuged (10000 x g, 20 min) and ammonium sulfate was added to the supernatant to 90% saturation (0.365 g/ml). The precipitate from subsequent centrifugation (12000 x g, 1 h) was dissolved and dialyzed for 40 h against 10 mM potassium phosphate, pH 7.8, containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA. The dialysate was brought to 200 mM phosphate with 1 M potassium phosphate, pH 7.8, and DEAE-Sephadex A-50 (20 ml/100 ml solution), pre-equilibrated with 200 mM potassium phosphate containing 0.1 mM EDTA, was added. After 0.5 h of stirring, the gel was centrifuged and washed with the same buffer. Collected supernatants were dialyzed for 24 h against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA and 0.3 mM diithiothreitol and then adsorbed onto a DEAE-Sephadex A-50 column (5 x 35 cm) equilibrated with the same buffer. The column was washed with 50 mM KCl in the above buffer until absorption at 280 nm decreased below 0.3. Then a linear gradient of KCl (50 – 250 mM, 3 l) was applied (Fig. 1). Fractions with cyanide-insensitive superoxide dismutase activity were pooled, concentrated over an Amicon PM-10 membrane and dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM KCl, 0.1 mM EDTA and 0.3 mM dithiothreitol. The dialysate was loaded to a column of DEAE-Sephacel (2 x 40 cm) equilibrated with dialysis buffer. A linear gradient of KCl (40 – 150 mM, 1.5 l) in the above buffer was then applied. Fractions containing superoxide dismutase activity were pooled and concentrated and applied to a Sephadex G-100 column (2 x 140 cm), which was equilibrated and eluted with 20 mM potassium phosphate, pH 7.8, containing 100 mM KCl, 0.1 mM EDTA and 0.3 mM diithiothreitol (Fig. 2A). Concentrated eluate with superoxide dismutase activity was again subjected, in three steps, to gel exclusion chromatography using a column of Sephacryl S-200 (1 x 80 cm) equilibrated and eluted with 50 mM potassium phosphate containing 0.5 mM KCl and 0.1 mM EDTA (Fig. 2B).

Table 1 summarises the results of the purification procedure. Clarification of crude extract prior to ammonium sulfate salting-out did not improve the results of purification. Although applying the DEAE-Sephadex A-50 batch did not raise the purity of the enzyme, it was essential for the good development of subsequent ion-exchange chromatography. In the DEAE-Sephadex A-50 step (Fig. 1) all cyanide-sensitive superoxide dismutase activity was removed.

The purified enzyme was homogeneous upon electrophoresis in 7% and 12% gels (Fig. 3). It had the same electro-
phoretic mobility as the enzyme in crude extract, indicating that it was not modified during purification. Specific activity was about 4000 U/mg, a value reported for some procaryotic Fe-SOD enzymes [27, 28] but higher than those of other vascular plants [13, 14].

**Molecular properties**

The relative molecular mass of the native enzyme was determined by gel exclusion chromatography on Sephadex G-100 (Fig. 2A) and Sephacryl S-200 (Fig. 2B and 4A) gel columns. The value obtained from four determinations was 43000 ± 2000. SDS/polyacrylamide gel electrophoresis, both in the presence and absence of 2-mercaptoethanol, yielded a single band with a mobility corresponding to a relative molecular mass of 22500 (Fig. 4B). These results indicate that the enzyme is composed of two non-covalently joined subunits of equal size.

Sedimentation equilibrium of the enzyme (0.5 mg/ml) was carried out at 12000 rpm. The plot of $\ln A_{280}$ versus the square of the distance from the center of rotation was linear and yielded a slope of 0.335 (Fig. 5). On the basis of a partial specific volume of 0.744 cm$^3$ g$^{-1}$, calculated from the amino acid composition, the relative molecular mass was estimated to be 41000. For all further calculations a value of $M_r$, 42000 was established.

**Absorption spectrum**

Fig. 6 shows visible and ultraviolet spectra of purified enzyme. It has an absorption maximum at 278 nm with slight shoulders at 260 nm and 290 nm. A broad, weak absorption in the range of 450–300 nm with a maximum at 350 nm is

![Figure 2: Gel exclusion chromatography of L. esculentum Fe-SOD.](image)

![Figure 3: Polyacrylamide disc gel electrophoresis of the purified enzyme in 7% and 12% gels.](image)

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**Table 1. Purification of an iron-containing superoxide diamutase from leaves of Lycopersicon esculentum**

| Step                                | Volume | Protein concentration | Total protein | Specific activity | $10^{-3} \times$ Total activity | Recovery | Puri- |
|-------------------------------------|--------|-----------------------|---------------|------------------|-------------------------------|----------|cation |
| 40–90% (NH$_4$)$_2$SO$_4$            | 580    | 11.4                  | 6600          | 22.7             | 150                           | 100      | 1     |
| DEAE-Sephadex A-50 batch            | 640    | 6.9                   | 4730          | 27.2             | 129                           | 86       | 1.2   |
| DEAE-Sephadex A-50, KCl gradient   | 473    | 0.61                  | 284           | 336              | 95.5                          | 64       | 15    |
| DEAE-Sephacel, KCl gradient        | 220    | 0.29                  | 63.6          | 942              | 59.9                          | 40       | 42    |
| Sephadex G-100                      | 21.4   | 0.98                  | 20.9          | 2040             | 42.7                          | 28       | 90    |
| Sephacryl S-200, dialysed           | 11.4   | 1.22                  | 9.3           | 4090             | 38.0                          | 25       | 180   |

Activity was determined according to McCord and Fridovich [3] in the presence of 1 mM KCN.
also observed. The $A_{278}^{1/2}$ was 17.9, corresponding to a molar absorption coefficient of $7.5 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$. This spectrum is reminiscent of Fe-SOD enzymes isolated from eucaryotic and procaryotic sources [12, 14, 29, 30].

**Metal analysis**

The purified enzyme, after extensive dialysis versus 10 mM potassium phosphate, pH 7.5, containing 0.1 mM EDTA, was subjected to atomic absorption spectrophotometry. The value obtained from three measurements of different preparations for iron was 1.45 ± 0.12 atoms per dimer. Copper and zinc contents were 0.06 ± 0.01 atom of each per dimer and manganese was below the detection level (0.00 atom per dimer).

**Isoelectric point**

Isoelectric focusing demonstrated a single band at pH 4.6. This value is similar to those reported for other eucaryotic Fe-SOD enzymes [12–14].

**Enzyme stability and sensitivity to inhibitors**

Frozen or refrigerated, concentrated solutions of a purified enzyme are quite stable. We have even observed some increase in specific activity during first days of storage. It is probably the effect of conformational changes within a protein.

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![Fig. 4. Determination of relative molecular mass of L. esculentum Fe-SOD. (A) Molecular exclusion chromatography on a column of Sephacryl S-200 Superfine, (B) Sodium dodecyl sulfate/polyacrylamide gel electrophoresis](image)

![Fig. 5. Sedimentation equilibrium of L. esculentum Fe-SOD. Purified enzyme at a concentration of 0.5 mg/ml in 10 mM potassium phosphate, pH 7.5, was equilibrated at a rotor speed of 12000 rpm after initial 5-h centrifugation at 16000 rpm at 20°C. The ultracentrifuge was equipped with an ultraviolet scanning system and ln(As0) was plotted as a function of the square of the distance from the centre of rotation](image)

![Fig. 6. Absorption spectrum of L. esculentum Fe-SOD. The enzyme, at a concentration of 680 µg/ml in 10 mM potassium phosphate, pH 7.8 was examined](image)

![Fig. 7 shows the temperature stability of Lycopersicon Fe-SOD. Inactivation at 60°C and 70°C is apparently first order with respect to enzyme concentration and half-lives are calculated to be 22 min and 4 min respectively. For comparison, procaryotic Fe-SOD enzymes, except those from luminous bacteria [31], are more thermostable [32–35] while a Euglena enzyme is more labile [12].](image)
and assayed for residual activity incubated at temperatures indicated. Small aliquots were withdrawn enzyme (700 pg/ml) in 50 mM potassium phosphate, pH 7.8, was incubated in 0.1 mM EDTA and the indicated concentration of H<sub>2</sub>O<sub>2</sub>. Small aliquots were taken and assayed for remaining activity (1.67 pM) was incubated at 25°C in 50 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA and the indicated concentration of Fe-SOD and Mn-SOD enzymes. However, it contains distinctly more proline and less glycine than the amino acid composition of the enzyme lies well in the range of 40000-46000, reported for other dimeric ferric superoxide dismutases. The same result is seen with respect to the iron content, which varies from about 1 to 2 mol Fe/
Table 3. Comparison of SAQ values of iron-containing and manganese-containing superoxide dismutases

| Source | Reference | Metal | a | b | c | d | e | f | g | h | i | j | k | l | m | n | o | p | q | r | s | t | u | v | w | x |
| a      | Lycopersicon esculentum | Fe | – |
| b      | Propionibacterium shermanii | Fe/Mn | 29 – |
| c      | Pismum sativum | Mn | 34 49 – |
| d      | Brassica campestris | Fe | 37 48 34 – |
| e      | Escherichia coli | Mn | 40 31 51 35 – |
| f      | Bacteroides fragilis | Fe/Mn | 40 24 51 19 21 – |
| g      | Plectonema boryanum | Fe | 42 39 78 36 26 29 – |
| h      | Streptococcus faecalis | Mn | 42 44 52 37 39 32 47 – |
| i      | Paracoccus denitrificans | Mn | 45 34 41 21 24 12 32 35 – |
| j      | Rat liver | Mn | 48 65 28 22 55 33 83 54 29 – |
| k      | Saccharomyces cerevisiae | Mn | 49 47 47 33 30 18 55 34 19 25 – |
| l      | Methanobacterium bryantii | Fe | 51 55 40 50 39 35 72 43 27 33 18 – |
| m      | Chromatium vinosum | Fe | 52 42 42 32 54 34 49 39 39 68 57 75 – |
| n      | Nocardia asteroides | Fe/Mn | 52 20 69 47 37 24 29 37 22 67 44 57 55 – |
| o      | Escherichia coli | Fe | 56 44 63 30 34 40 23 33 44 78 57 86 38 38 – |
| p      | Pseudomonas ovalis | Fe | 57 79 53 25 65 45 47 55 38 50 62 64 42 57 49 – |
| q      | Thermoplasma acidophilum | Fe | 61 46 61 51 21 23 71 64 38 50 31 36 73 66 82 92 – |
| r      | Spirulina platensis | Fe | 62 41 92 60 40 45 20 48 56 109 69 99 41 47 26 68 85 – |
| s      | Porphyridium cruentum | Mn | 62 73 64 32 61 54 59 33 58 66 73 94 55 62 27 48 90 71 – |
| t      | Chromatium thiolaefatum | Mn | 65 46 79 35 40 22 32 18 30 71 37 59 32 32 34 55 65 38 52 – |
| u      | Rhodopseudomonas spheroides | Mn | 69 58 55 24 43 20 50 48 13 25 27 34 55 48 59 39 51 70 68 42 – |
| v      | Nuphar luteum | Fe | 82 77 102 38 64 41 59 75 45 63 74 87 71 56 73 45 81 74 80 56 42 – |
| w      | Euglena gracilis isoenzyme I | Fe | 84 76 102 61 107 78 74 51 72 95 95 102 97 48 60 81 142 108 72 74 86 88 – |
| x      | Crithidia fasciculata | Fe | 103 62 84 62 43 50 52 60 50 105 78 88 41 49 42 65 76 43 79 39 64 60 120 – |

mol dimer. It should be assumed that all these proteins contain 1 mol Fe/mol subunit and intermediate values reflect partial release of the metal during the separation procedure. Spectral properties of the enzyme are comparable with those of other Fe-SOD enzymes. Besides a strong absorption band with a maximum at 278 nm possessing typical shoulders, a broad band in the visible region, attributable to a ligand-to-metal charge transfer [48], can be observed. This visible light band was not reported for two eucaryotic plant Fe-SOD enzymes isolated from Euglena [12] and Brassica [13]. The temperature stability of tomato Fe-SOD is in the same range as those reported for procaryotic enzymes [32–35] although very labile enzymes were described for luminous bacteria [31] and Euglena [12]. Lycopersicon Fe-SOD shows the same sensitivity to inhibitors, such as azide, cyanide and hydrogen peroxide, as other enzymes of this type, from procaryotic as well as eucaryotic organisms. It also exhibits a similar isoelectric point and amino acid composition. However, with respect to the latter feature the enzyme seems rather to resemble the manganese-containing superoxide dismutases.

Isolation and characterization of Fe-SOD from tomato leaves as well as identification of it in bean leaf extract [16] add the two more families of land plants to those already known to contain this enzyme. Thus, the existence of Fe-SOD in plants does not seem to be so exceptional as was previously believed [15].

Among hypotheses formulated on the evolution of Fe-SOD [15], the most plausible seems to be the one which assumes that the enzyme is descended from more primitive plants. In higher plants Fe-SOD might have originated from blue-green algae, which became chloroplasts through endosymbiosis [49, 50]. After elaboration of the transport into chloroplasts of proteins encoded in the nuclear genome [51], Cu, Zn-SOD could enter these organelles and replace Fe-SOD. However, organisms containing two superoxide dismutases with different properties, metal requirement and possibly different locations within chloroplasts could have an evolutionary advantage. Thus, primitive Ginkgo biloba possess both types of chloroplast dismutases [15], while accidental loss of Cu, Zn-SOD, as might be the case in...
Nymphaeae [15], should cause a stabilization of an already existing Fe-enzyme. Similarly, a temporary loss of Cu, Zn-SOD from chloroplasts, caused by cold treatment of leaves [52], while cyanide-insensitive superoxide dismutase activity is retained [53], seems to indicate that under these conditions Fe-SOD may take over the function of the Cu, Zn-containing enzyme.

The comparison of the amino acid composition of Fe-SOD and Mn-SOD enzymes (Table 3) raises another possibility. Fe-SOD from tomato is strikingly closely related to Mn-SOD enzymes, even more than to many other superoxide dismutases. This implies that the borderline between these two groups of proteins is not sharp. They may even be regarded as isoenzymes differing in the metal present in the active center. Indeed, hybridization of Escherichia coli Mn-SOD and Fe-SOD subunits has been reported [54] and bacterial superoxide dismutases, which may accept either iron or manganese, or both, have recently been described [2, 38, 46]. Moreover, some Fe-containing enzymes proved to be more closely related to Mn-containing than to other ferric superoxide dismutases [9]. Thus, we are not able to exclude the possibility of reversed evolution of some Fe-SOD enzymes from Mn-SOD. In spite of earlier reports [4, 6], Mn-SOD has never unequivocally been localized within chloroplasts. The Mn-SOD isolated from the higher plant, Pisum sativum, was proved to be located in the plant body [55]. On the other hand, Fe-SOD was found in chloroplasts of mustard [56] and tomato leaves (our unpublished results). Thus, a reason might exist for precluding Mn-SOD from chloroplasts, and adopting one enzyme rather than the other may give the cell a substantial advantage. This may also lie behind the origin of Fe-SOD in higher plants.

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