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Purification and Properties of Glyoxysomal Cuprozinc Superoxide Dismutase from Watermelon Cotyledons (*Citrullus vulgaris* Schrad)[1]

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ABSTRACT

A glyoxysomal copper,zinc-containing superoxide dismutase (EC 1.15.1.1) was purified to homogeneity, for the first time, from watermelon cotyledons (*Citrullus vulgaris* Schrad.). The stepwise purification procedure consisted of acetone precipitation, batch anion-exchange chromatography, anion-exchange Fast Protein Liquid Chromatography and gel-filtration column chromatography. Pure copper,zinc-superoxide dismutase (Cu,Zn-SOD II) had a specific activity of 1211 units per milligram protein and was purified 400-fold, with a yield of 8 micrograms enzyme per gram cotyledon. The glyoxysomal Cu,Zn-SOD had a relative molecular weight of about 33,000 and was composed of two equal subunits of 16,500 Daltons. Metal analysis showed that the enzyme, unlike other Cu,Zn-SODs, contained 1 gram-atom Cu and 1 gram-atom Zn per mole dimer. No iron and manganese were detected. Ultraviolet and visible absorption spectra were reminiscent of other copper,zinc-superoxide dismutases.

SODs[2] (EC 1.15.1.1) are a group of metalloenzymes that catalyze the disproportionation of superoxide free radicals ($O_2^{\cdot-}$), produced in certain cellular loci, to molecular oxygen and H_2O_2 (13). These enzymes play an important role in protecting cells against the indirect deleterious effects of superoxide free radicals (13, 26). SODs generally occur in three different molecular forms containing Mn, Fe, or Cu plus Zn as prosthetic metals (13), although atypical SODs containing different combinations of those metals in their molecules have also been described (2, 10, 24).

In higher plants, manganese-containing SODs are mainly present in mitochondria (26, 29), but also occur in different types of peroxisomes (7, 11, 28, 29). Fe-SODs are localized in chloroplasts (10), but very recently they also have been reported to be present in peroxisomes and mitochondria from *Dianthus caryophyllus* L. together with a Mn-isozyme (11). Cu,Zn-SODs are localized chiefly in chloroplasts (9, 23, 26), and also in the cytosol (3, 26) and mitochondria (26, 28).

In recent years, the presence of SOD activity has been demonstrated in glyoxysomes, a class of specialized peroxisomes occurring in oilseeds (28). In watermelon glyoxysomes, two different SODs were localized, a preponderant Cu,Zn-SOD (named isozyme II), which represented 80% of the total glyoxysomal SOD activity, and, in a minor amount, a Mn-SOD (28). The study of the intraorganellar distribution of SOD in glyoxysomes revealed that the most abundant SOD, isozyme Cu,Zn-SOD II, was located in the organelle matrix as a soluble enzyme, whereas Mn-SOD apparently was bound to the cytosolic face of the membrane (30).

To date, no Cu,Zn-SOD from any type of peroxisome has ever been purified. On the basis of different experimental evidence obtained in the last years, the existence of a cellular function for peroxisomes related to oxygen free radicals has recently been proposed (8). To get more information on the role of SOD in the activated oxygen metabolism of peroxisomes, it is important to know the molecular characteristics of this glyoxysomal Cu,Zn-SOD. Moreover, the availability of purified preparations of glyoxysomal Cu,Zn-SOD will allow comparative molecular, immunological, and evolutionary studies with other characterized SODs present in other cell compartments from different organisms, such as chloroplasts, mitochondria, lysosomes, and cytosol.

In this work, we report the purification to homogeneity of a Cu,Zn-SOD from *Citrullus vulgaris* glyoxysomes and its preliminary characterization.

MATERIALS AND METHODS

Plant Material and Chemicals

Seeds of watermelon (*Citrullus vulgaris* Schrad. cv sugar baby), obtained from Fitó (Barcelona, Spain), were surface-sterilized with 10% (v/v) commercial bleaching solution for 3 min and then were washed with distilled water. Seeds were wrapped tightly in wet cheesecloth and laid on a perforated methacrylate plate that was placed on a tray with water. Paper wicks between wrapped seeds and water allowed a convenient humidity for germination. This was conducted at 30°C under dark conditions. From the seedlings obtained, radicals were removed and the cotyledons used for enzyme purification.

The sources of chemicals were as follows: NBT, Cyt *c* (type III), xanthine, *N,N,N′,N′*-tetramethylethylenediamine, and Coomassie brilliant blue G-250, Sigma Chemical Company; DEAE-Sephadex A-25, Sephadex G-150 (superfine grade),

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[2] Abbreviations: SOD, superoxide dismutase; Cu,Zn-SOD, cuprozinc-containing superoxide dismutase; Mn-SOD, manganese-containing superoxide dismutase; Fe-SOD, iron-containing superoxide dismutase; NBT, nitro blue tetrazolium; FPLC, Fast Protein Liquid Chromatography.

protein standards for gel filtration, and Mono Q HR 5/5, Pharmacia LKB Biotechnology; molecular mass markers for SDS-PAGE, acrylamide, bisacrylamide, ammonium persulfate, Tris, and glycine, Bio-Rad Laboratories; ammonium sulfate, KCN, H_2O_2, and KCl, Merck; PM-10 ultrafiltration membranes, Amicon Corp.; DTT, xanthine oxidase (grade I), and BSA (fraction V), Boehringer-Mannheim. All other chemicals were analytical reagent grade from Merck.

Enzyme and Protein Assays

Catalase (EC 1.11.1.6) activity was determined by spectrophotometry at 240 nm (1), and fumarase (EC 4.2.1.2) as described earlier (31). SOD activity was determined by the ferricytochrome *c* method using xanthine/xanthine oxidase as the source of superoxide radicals, and a unit of activity was defined according to McCord and Fridovich (21). During the course of purification, column eluates were assayed for SOD activity by a method based on the NBT reduction by superoxide free radicals generated photochemically (14). Protein content was determined either by the method of Lowry *et al.* (20) or Murphy and Kies (22). In both cases, BSA (fraction V) was used as a standard.

Purification of Enzyme

Unless otherwise stated, all the purification steps were carried out at 0 to 4°C.

Step 1: Crude Extract

Ten-day-old healthy watermelon cotyledons (500 g, equivalent to about 11,000 pairs of cotyledons) were washed with distilled water and then were homogenized in 1500 mL of a medium containing 50 mM K-phosphate buffer, pH 7.8, 1 mM DTT, 8% (w/v) polyvinylpoly-pyrrolidone, 1 mM PMSF, and 0.1% (v/v) Triton X-100 using a Sorvall Omni-Mixer (3 × 30 s pulses at maximum setting). The homogenate was filtered through six layers of nylon cloth and centrifuged at 19,000*g* for 30 min.

Step 2: Acetone Fractionation

This was carried out essentially as described by Federico *et al* (12). To the 19,000*g* supernatants, 0.5 volumes of acetone at −20°C was added with stirring for 30 min (33% acetone final concentration, v/v) and the mixture was centrifuged at 16,000*g* for 15 min. To the supernatant, an additional 1.5 volumes of cold acetone was added (73% acetone final concentration, v/v) and, after stirring for 30 min, the precipitate was removed by centrifugation at 16,000*g* for 15 min. The precipitate, which contained most of the glyoxysomal Cu,Zn-SOD activity, was taken up in 100 mL of 50 mM K-phosphate buffer, pH 7.8 (buffer A), and dialyzed overnight against the same buffer. The dialyzed solution was clarified by centrifugation at 38,000*g* for 15 min.

Step 3: Batch DEAE-Sephadex

The enzyme solution was applied to a DEAE-Sephadex A-25 column (5 × 7 cm) equilibrated with buffer A. The cyanide-sensitive Cu,Zn-SOD activity was eluted together with a small amount of Mn-SOD activity by washing with 500 mL of buffer A containing 0.1 M KCl. The active fractions were dialyzed against buffer A and concentrated to 50 mL by ultrafiltration with a PM-10 membrane.

Step 4: FPLC with Mono Q Column

The concentrated DEAE-Sephadex fractions were applied to a Mono Q HR 5/5 column equilibrated with buffer A, using an FPLC system equipped with an LCC-500 PLUS controller, two P-500 pumps, an MV-7 motor valve, and a UV-M monitor connected to a REC-482 two-channel recorder and a FRAC-200 fraction collector (Pharmacia LKB Biotechnology). Sample loops of 2-mL volume were used and all FPLC fractionations were done at room temperature. The column was washed with 25 mL of buffer A, and then was eluted with 15 mL of a linear salt gradient from 0 to 700 mM KCl in buffer A. Fractions of 0.5 mL were collected at a flow rate of 1 mL/min. The cyanide-sensitive Cu,Zn-SOD activity did not bind and was resolved into two activity peaks on Mono Q (I and II), whereas Mn-SOD activity was eluted between 120 and 160 mM KCl. Each activity peak was combined separately and concentrated by ultrafiltration to 2 to 6 mL.

Step 5: Chromatography on Sephadex G-150 Column

The concentrated fractions of Cu,Zn-SOD II (peak II) were put on a 2.6 × 86 cm column (Pharmacia) of Sephadex G-150 equilibrated with buffer A. Sample volumes of 2 mL were applied and the column was run at a flow rate of 0.4 mL/min, and 2-mL fractions were collected. The samples containing Cu,Zn-SOD II activity were pooled, concentrated by ultrafiltration, and stored at −20°C.

Electrophoretic Procedures

Nondenaturing (native) PAGE was performed on 10% acrylamide tube gels as described by Davis (6). SOD activity bands were detected in gels by the photochemical NBT stain of Beauchamp and Fridovich (5). The different types of SOD were differentiated by performing the activity stains in gels previously incubated at 25°C for 30 min in 50 mM K-phosphate buffer, pH 7.8, containing either 2 mM KCN or 5 mM H_2O_2. Cu,Zn-SODs are inhibited by CN^- and H_2O_2, Fe-SODs are resistant to CN^- but inactivated by H_2O_2, and Mn-SODs are resistant to both inhibitors (13). Protein bands were visualized in gels by staining with Coomassie brilliant blue G-250 according to Reisner (27). Gels stained for SOD activity and proteins were scanned on a Shimadzu CS-9000 densitometer.

Determination of Mol Wt (M_r and Subunit Size)

The native M_r of the purified glyoxysomal Cu,Zn-SOD was determined by gel filtration on a Sephadex G-150 column (2.6 × 86 cm) (Pharmacia). The column was calibrated with the following standard proteins: BSA (M_r = 67,000); ovoalbumin (M_r = 43,000); chymotrypsinogen A (M_r = 25,000); and ribonuclease A (M_r = 13,700). Subunit size was determined by SDS-PAGE after heating the proteins at 100°C for

5 min in the presence of 2% SDS and 5% 2-mercaptoethanol. Electrophoresis was carried out on 12% acrylamide-SDS gels, as described by Laemmli (18), using a Bio-Rad Mini-Protean II slab cell. Standards used were phosphorylase *b* (M_r = 97,400), BSA (M_r = 66,200), ovoalbumin (M_r = 45,000), carbonic anhydrase (M_r = 31,000), soybean trypsin inhibitor (M_r = 21,500), and lysozyme (M_r = 14,400). Proteins were visualized by silver staining (Sigma Chemical Company, Silver Stain Kit, Tech. Bulletin No. P3040).

Metal Content

Copper, manganese, iron, and zinc contents of the purified enzyme were determined by atomic absorption spectrometry with a Perkin-Elmer 503 apparatus equipped with a heated graphite atomizer. The enzyme sample was dialyzed exhaustively in metal-free dialysis membrane, first against 50 mM Na-phosphate buffer, pH 7.8, 0.1 mM EDTA, and then against this buffer lacking EDTA.

RESULTS AND DISCUSSION

Watermelon cotyledons contain four electrophoretically distinct SODs, which were identified as two manganese- (I and II) and two Cu,Zn-SODs (I and II) (28). The fast-moving isozyme by PAGE, Cu,Zn-SOD II, was demonstrated to be located in glyoxysomes, whereas the bulk of Mn-SOD was present in mitochondria, and isozyme Cu,Zn-SOD I was distributed between mitochondria and the cytosol (28, 30).

As a previous step before initiating the purification of glyoxysomal Cu,Zn-SOD II, the time course of this isozyme activity in cotyledons of *C. vulgaris* seedlings grown under darkness was studied (Fig. 1) to select starting cotyledons with a high enzymatic activity to achieve a maximum recovery in the purification process. The specific activity of isozyme Cu,Zn-SOD II generally increased with time, reaching a maximum on day 11. However, the growth time selected for obtaining cotyledons for the enzymatic purification was 10 d, because at this time the Cu,Zn-SOD II specific activity was comparatively high and the plant tissue was healthier and more vigorous than at 11 d. In previous work, the time course of SOD isozymes in cotyledons of dark-grown watermelon seedlings was studied for a time period of 6 d (28). It was found that the glyoxysomal isozyme Cu,Zn-SOD II appeared 3 d after the beginning of soaking, whereas the other SOD isozymes (Mn-SODs and Cu,Zn-SOD I) were already present in 24 h seeds (28). The pattern of Cu,Zn-SOD II activity during the growth period, described in this work, is very similar to that of catalase activity, another enzyme of glyoxysomal localization (15). This suggests that catalase and Cu,Zn-SOD II could perform a complementary or synergistic action in the metabolism of glyoxysomes, protecting these organelles against the dangerous effects of their respective substrates (H_2O_2 and $O_2^{\cdot-}$). In pea plants, a similar activity pattern was also described for the leaf peroxisomal enzymes Mn-SOD and catalase during plant growth (19).

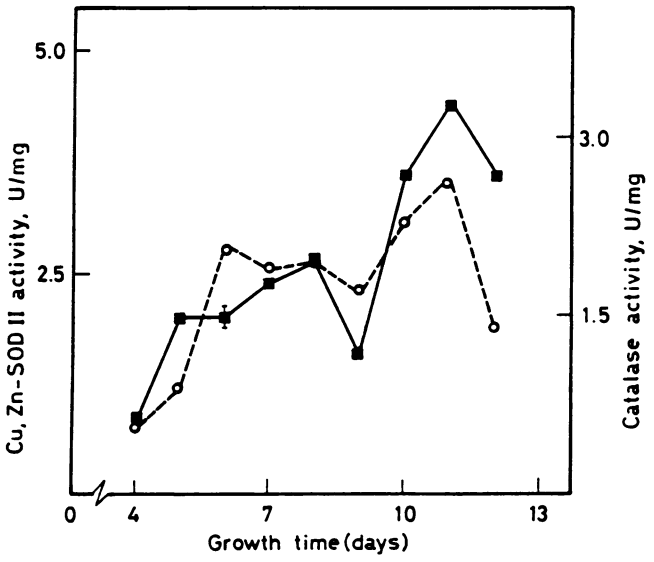

Figure 1. Activity pattern of glyoxysomal Cu,Zn-SOD and catalase during seedling growth. Watermelon seeds were germinated at 30°C in darkness. After germination, the seedlings were maintained in prolonged darkness at 30°C. ■, Cu,Zn-SOD II; O, catalase. Enzyme activities are expressed as units/mg protein.

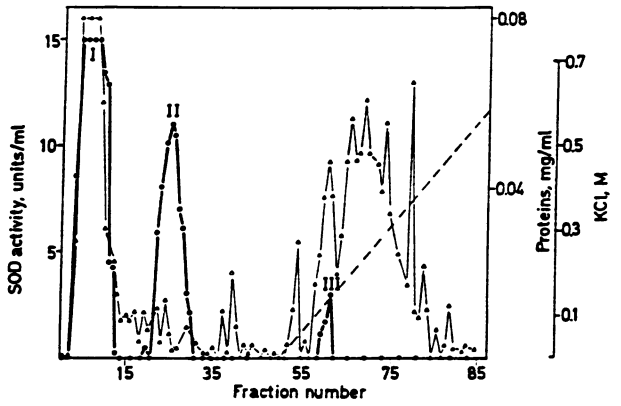

Figure 2. Fractionation of batch DEAE-Sephadex fractions on a Mono Q column using an FPLC system. ●, SOD activity; ▲, protein concentration. The broken line represents the gradient profile (M KCl)

In the early stages of Cu,Zn-SOD II purification, attempts were made to precipitate the enzyme by ammonium sulfate. The enzyme yields obtained, however, were low and, in addition, a creamy layer of fat was obtained that produced turbidity in the solutions and was difficult to remove. In contrast, acetone precipitation resulted in an excellent method that achieved good enzyme activity recoveries and supplied clear, fat-free solutions. The use of batch DEAE-Sephadex was useful to partially purify and concentrate the enzyme solutions prior to the FPLC purification step, and produced an increase in the specific activity of Cu,Zn-SOD II of 2.4-fold. By FPLC on the anion-exchanger Mono Q, three peaks of SOD activity were separated (Fig. 2). Fractions I and II did not bind to the anionic exchanger, and when they were analyzed by PAGE using inhibitors of SOD, they were identified as the cytosolic and mitochondrial Cu,Zn-SOD I, and the glyoxysomal Cu,Zn-SOD II, respectively. On the contrary, peak III, which bound to the Mono Q column, was characterized as a Mn-SOD.

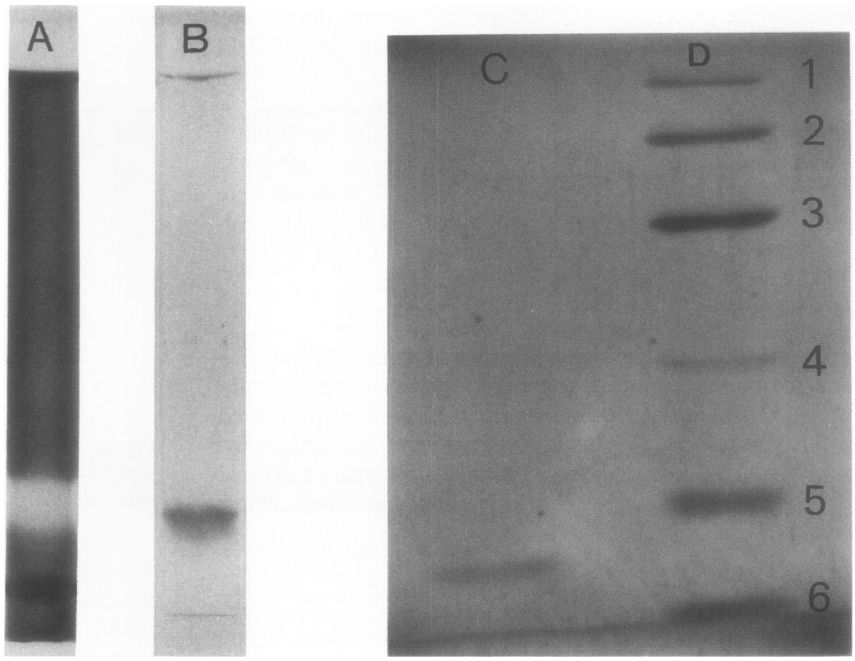

Figure 3. Nondenaturing polyacrylamide tube gel electrophoresis and SDS-PAGE of purified Cu,Zn-SOD II. A, Nondenaturing gel stained for SOD activity by the photochemical method (4 μg protein). B, Nondenaturing gel stained for proteins with Coomassie brilliant blue G-250 (4 μg protein). C, SDS gel (12%) silver-stained for proteins (0.4 μg protein). D, SDS gel (12%) showing the silver-stained mol wt calibration proteins: 1, phosphorylase *b*; 2, BSA; 3, ovoalbumin; 4, carbonic anhydrase; 5, soybean trypsin inhibitor; 6, lysozyme.

Fractions of glyoxysomal Cu,Zn-SOD II activity (peak II), obtained by FPLC on Mono Q, were applied to a Sephadex G-150 column. When the eluting fractions were analyzed by nondenaturing PAGE, they were found to contain a single protein band that corresponded to the band of enzyme activity (Fig. 3) and had a similar mobility to the cotyledon extract Cu,Zn-SOD II, indicating the absence of substantial enzyme modification during purification. When the enzyme was treated with SDS and mercaptoethanol, a single protein band was observed on SDS-polyacrylamide gels (Fig. 3), showing that Cu,Zn-SOD II was homogenous. The purification of glyoxysomal Cu,Zn-SOD from watermelon cotyledons is summarized in Table I. Solutions of pure Cu,Zn-SOD II had a specific activity of 1211 units/mg and the purification was 404-fold. The yield obtained was 39%, and on the basis of fresh weight of cotyledons, the purification yield achieved was about 8 μg pure glyoxysomal Cu,Zn-SOD II/g cotyledon.

The native mol wt of the enzyme was determined by gel exclusion chromatography on Sephadex G-150. By comparison with markers of known mol wt, an M_r value of 33,200 for the glyoxysomal enzyme was obtained (Fig. 4). SDS-PAGE of Cu,Zn-SOD showed that the enzyme dissociated into a band that, by comparison with standards, had an M_r value of 16,500. Thus, glyoxysomal Cu,Zn-SOD II appears to be a dimer composed of two equally sized subunits. In its native and subunit mol wt, the glyoxysomal Cu,Zn-SOD II is characteristic of most Cu,Zn-SODs (13) and resembles many plant Cu,Zn-SODs that have been characterized thus far (3, 9, 12, 16, 17, 26). The UV absorption spectrum of purified Cu,Zn-SOD II was characterized by a low absorption at 280 nm

Table I. *Purification of Glyoxysomal Cu,Zn-SOD II from Citrullus vulgaris Cotyledons*
500 g of watermelon cotyledons was processed.

Step	Total Protein	Total Activity	Specific Activity	Yield	Purification
	mg[a]	*units*[b]	*units/mg*[a,b]	%	*-fold*
Initial extract	4200	12,600	3	100	1
33 to 73% acetone precipitate	851	12,690	14.9	100	5
Batch DEAE-Sephadex	283	10,279	36.3	82	12
Mono Q FPLC	9.5	7,093	747	56	249
Sephadex G-150[c]	4.1	4,965	1211	39	404

[a] Protein concentration was determined by the method of Lowry *et al.* (20) except where indicated. [b] Total SOD activity was assayed according to McCord and Fridovich (21). The activity of Cu,Zn-SOD II was calculated by multiplying the percentage of this isozyme, obtained by densitometry of SOD activity-stained gels, by the total SOD activity of samples. [c] Protein concentration was determined by the method of Murphy and Kies (22).

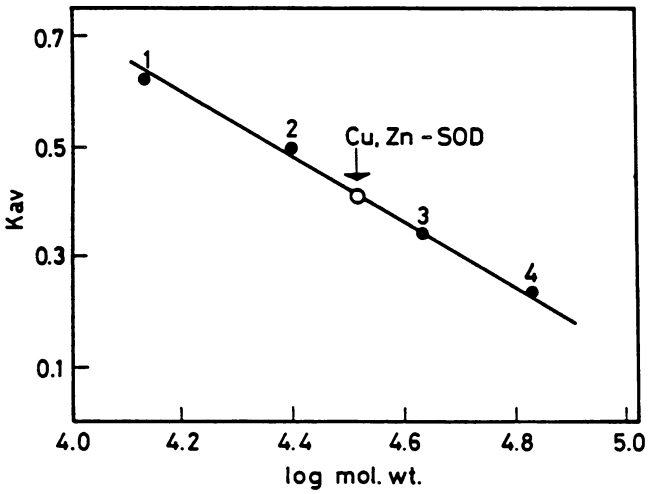

Figure 4. Mol wt estimation of Cu,Zn-SOD II by gel filtration on Sephadex G-150. The log mol wt of the standard protein markers were plotted against their K_{av} values. 1, RNAase; 2, chymotrypsinogen; 3, ovoalbumin; 4, BSA.

(tryptophan absorbance region), and showed a weak absorption maximum at 260 nm and a shoulder at about 220 nm. The UV absorption spectrum was similar to those reported for Cu,Zn-SODs from different sources (13, 26), and particularly to the chloroplastic Cu,Zn-SODs (9, 17). In the visible region, a weak absorption spectrum in the range of 800 to 300 nm was observed, with a maximum at 680 nm and a shoulder at 560 nm. The peak at 680 nm corresponds to the Cu chromophore (16) and is also presented by different chloroplast-type Cu,Zn-SODs (16).

The enzyme was assayed for copper, zinc, iron, and manganese by atomic absorption spectroscopy after exhaustive dialysis to remove traces of contaminating metals. On a mol wt basis of 33,000, the dialyzed SOD contained 0.79 g-atoms Cu and 0.93 g-atoms Zn/mol, and Fe and Mn concentrations were below the detection level. In experiments preliminary to the final purification procedure for Cu,Zn-SOD II, a cation-exchanger was used (CM-Sephadex at pH 6.0). This produced considerable losses of Cu,Zn-SOD II activity, and when the enzyme was finally purified to homogeneity and analyzed, it was found to have a comparatively low specific activity (145 units/mg) and contained 0.4 g-atom Zn and 1 g-atom Cu/mol. For this reason, the cation-exchanger step was no longer employed in the purification procedure. These results suggest that at pH 6.0 the carboxymethyl-Sephadex could bind and remove zinc from the Cu,Zn-SOD II molecule, with the result of a considerably low enzyme specific activity. This situation contrasts with that of cytosolic Cu,Zn-SODs from maize kernels, whose activity apparently was not affected by carboxymethyl-Sephadex chromatography at pH 5.7 (3). The different behavior of cytosolic and glyoxysomal Cu,Zn-SODs toward cation-exchangers implies differences in their molecular structures, particularly related to the active center area where Zn is bound.

For the determination of the metal-protein stoichiometry of the Cu,Zn-SOD II, two different methods of protein determination were used (20, 22), and both of them gave similar results. Likewise, special precautions were taken to avoid metal contaminations in the purified enzyme solutions. Under these conditions, the metal content determined for Cu,Zn-SOD II was unusual for the family of Cu,Zn-SODs. This glyoxysomal isozyme only has 1 g-atom Cu and 1 g-atom Zn/mol, compared with most Cu,Zn-SODs, which contain 2 g-atom Cu and 2 g-atom Zn/mol (13). To our knowledge, the only exception is the Cu,Zn-SOD from *Photobacterium leiognathi* that has a mol wt of 33,100 and contains 1 g-atom Cu and 2 g-atom Zn/mol (25). The reason for this lower metal content in glyoxysomal Cu,Zn-SOD could be related to the cellular locus of this isozyme. It appears that the chloroplastic Cu,Zn-SODs are more closely related to each other than to the cytoplasmic Cu,Zn-SODs, as far as amino acid contents, amino acid sequences, UV absorption spectra, and immunological properties are concerned (4, 9, 16, 17). The same could be true for glyoxysomal Cu,Zn-SOD, which could have a different metal-protein stoichiometry, among other characteristic molecular properties.

The purification of glyoxysomal Cu,Zn-SOD II is the first step toward obtaining monospecific antibodies against the pure protein that can be used in comparative immunological studies with other SODs distributed in different loci of plant cells. The availability of the antibody against glyoxysomal Cu,Zn-SOD II will make it possible to initiate molecular biology studies with this SOD, which is compartmentalized in a type of specialized peroxisome.

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