Free Rad. Res., Vol. 26, pp. 83–91
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Purification and Properties of Cytosolic Copper, Zinc Superoxide Dismutase from Watermelon (*Citrullus vulgaris* Schrad.) Cotyledons

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Accepted by Prof. B. Halliwell

(Received 20 June 1996; In revised form 17 July 1996)

Cytosolic copperzinc-superoxide dismutase (CuZn-SOD I; EC 1.15.1.1) was purified to homogeneity from watermelon (*Citrullus vulgaris* Schrad.) cotyledons. The stepwise purification procedure consisted of acetone precipitation, batch anion-exchange chromatography, anion-exchange Fast Protein Liquid Chromatography, gel-filtration column chromatography, and affinity chromatography on concanavalin A-Sepharose. CuZn-SOD I was purified 310-fold with a yield of 12.6 micrograms enzyme per gram cotyledons, and had a specific activity of 3,540 units per milligram protein. The relative molecular mass for cytosolic CuZn-SOD was 34000, and it was composed by two equal subunits of 16.3 kDa. CuZn-SOD I did not contain neutral carbohydrates in its molecule, and its ultraviolet and visible absorption spectra showed two absorption maxima at 254 nm and 580 nm. Metal analysis showed that the enzyme contained 1 gram-atom Cu and 1 gram-atom Zn per mole dimer. Cytosolic CuZn-SOD was recognized by the antibody against peroxisomal CuZn-SOD from watermelon cotyledons, and its enzymatic activity was inhibited by this antibody. By IEF (pH 4.2–4.9), using a new method for vertical slab gels set up in our laboratory, purified cytosolic CuZn-SOD was resolved into two equal isoforms with isoelectric points of 4.63 and 4.66.

Keywords: *Citrullus vulgaris*, copper,zinc superoxide dismutase, cytosolic CuZn-SOD, glycoproteins, watermelon cotyledons, characterization

Abbreviations: BSA = bovine serum albumin; CuZn-SOD = copperzinc-containing superoxide dismutase; DEAE-Sephadex = diethylaminoethyl-Sephadex; DTT = 1,4-dithiothreitol; FPLC = Fast Protein Liquid Chromatography; IEF = isoelectric focusing; NBT = nitroblue tetrazolium; PMSF = phenylmethylsulphonyl fluoride; PVDF = polyvinylidene difluoride; SOD = superoxide dismutase

INTRODUCTION

Superoxide dismutases (SOD; EC 1.15.1.1) are a group of metalloenzymes that catalyze the disproportionation of superoxide radicals, generated in different cellular compartments, to H_2O_2 and O_2.[1] These enzymes play an important role in protecting cells against the indirect deleterious effects of superoxide free radicals.[1,2]

SODs are generally classified into three types depending on their prosthetic metals, either cop-

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per,zinc-, manganese-, or iron-containing superoxide dismutases.[3] In higher plants, Mn-SODs are mainly present in mitochondria,[2,4] but also occur in different types of peroxisomes.[4–11] Iron-containing SODs are mainly localized in chloroplasts,[12] but they have also been found in mitochondria, peroxisomes[11] and the cytosolic fraction of legume nodules.[13] CuZn-SODs are chiefly located in chloroplasts,[12,14,15] and also in the cytosol[2,6,16] and peroxisomes of oilseeds.[9]

In watermelon cotyledons, four superoxide dismutase isozymes have been found by native PAGE: two Mn-SODs (Mn-SOD I and II), and two CuZn-SODs (CuZn-SOD I and II).[4] Mn-SODs are localized in mitochondria and in peroxisomal membranes.[4,5] Isozyme CuZn-SOD I was found to be present in the cytosol, whereas CuZn-SOD II was located in the peroxisomal matrix.[4,5,7] Peroxisomal CuZn-SOD from watermelon cotyledons has been recently purified and characterized and polyclonal antibodies against this isozyme have been obtained.[17,18] However, there is no information on the molecular properties of cytosolic CuZn-SOD, the most abundant SOD in watermelon cotyledons. In order to carry out comparative biochemical, immunological, and molecular biology studies with peroxisomal and chloroplastic CuZn-SOD, it is necessary to purify and characterize the cytosolic CuZn-SOD. This will allow to determine its amino acid sequence and study the topogenic signal of the cytosolic SOD and compare it with that of peroxisomal and chloroplastic CuZn-SODs.

In this work, the purification to homogeneity of cytosolic CuZn-SOD from watermelon cotyledons and its characterization is reported.

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 thoroughly with distilled water. Seeds were germinated in darkness at 30°C for 10 days as described earlier.[17] The sources of chemicals were those indicated by Bueno and del Río.[17]

Purification of the Enzyme

All operation were performed at 4°C. Watermelon cotyledons (500 g) 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, 0.8% (w/v) polyvinylpolypyrrolidone, 1 mM PMSF, and 0.1% (v/v) Triton X-100 in a Sorvall Omni-Mixer. Homogenates were filtered through six layers of nylon cloth, and centrifuged at 19000 *g* for 30 min. The supernatant was fractionated with acetone (33–73%; v/v) as described by Bueno and del Río,[17] and the final pellet, which contained the cytosolic CuZn-SOD, was taken up in 100 ml of 50 mM K-phosphate buffer, pH 7.8 (Buffer P).

The resuspended pellet was dialyzed overnight against buffer P, and clarified by centrifugation at 30000 *g* for 15 min. The supernatant was applied to a DEAE-Sephadex A-25 column (5 × 7 cm), equilibrated with buffer P. Cytosolic CuZn-SOD was eluted by washing the column with 500 ml of buffer P containing 0.1 M KCl. Fractions containing cytosolic CuZn-SOD activity were dialyzed against buffer P, concentrated with a PM-10 membrane, and applied to a Mono Q HR 5/5 column connected to a FPLC system (Pharmacia LKB Biotechnology). Sample volumes of 2 ml were loaded onto the Mono Q column, previously equilibrated with buffer P, and the fractionation was done at room temperature. The column was washed with 15 ml of buffer P, and then was eluted with 15 ml of a linear salt gradient (0–0.7 M KCl) in buffer P. Fractions of 0.5 ml were collected at a flow rate of 1 ml/min. The cytosolic CuZn-SOD did not bind to the column, and was eluted in the first fractions. The CuZn-SOD I activity-containing fractions were pooled, concentrated by

ultrafiltration, and then loaded onto a Sephadex G-150 superfine column (2.6 × 88 cm; Pharmacia LKB Biotechnology), equilibrated with buffer P. Sample volumes of 2 ml were applied to the column, and 2-ml fractions were collected at a flow rate of 10 ml/h. The fractions containing cytosolic CuZn-SOD activity were pooled, and concentrated by ultrafiltration with a PM-10 membrane.

The concentrated samples were applied to a 2.5 ml concanavalin A-Sepharose column (0.7 i.d. × 6.5 cm; Bio-Rad Laboratories), equilibrated with 20 mM Tris-HCl buffer, pH 7.3, 0.5 M NaCl, and the affinity chromatography was carried out at room temperature. Enzyme samples (0.4 ml) were applied and allowed to bind to the lectin for one hour, and then, the column was washed with 5 ml of equilibrating buffer. Finally, the column was eluted with D-methylmannopyranoside in two steps, first with 5 ml of 0.2 M and then with 5 ml of 1 M D-methylmannopyranoside at a flow rate of 0.5 ml/min. Horseradish peroxidase (type IV), and ovoalbumin were used as positive controls for glycoproteins.

Determination of Relative Molecular Mass and Subunit Size

The relative molecular mass of cytosolic CuZn-SOD was determined by gel filtration on a Sephadex G-150 superfine column (2.6 × 88 cm; Pharmacia LKB Biotechnology). The following standard proteins were used: BSA (M_r = 67000), ovoalbumin (M_r = 45000), chymotrypsinogen A (M_r = 25000), and cytochrome *c* (M_r = 12400).

The subunit size was determined by SDS-PAGE after heating the samples at 100°C for 10–15 min in the presence of 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. Electrophoresis was performed on both 12% and 15% acrylamide-SDS slab gels (Bio-Rad Laboratories, Mini Protean II), as described by Laemmli.[19] Standards used were: phosphorylase *b* (M_r = 97400), BSA (M_r = 66000), ovoalbumin (M_r = 45000), carbonic anhydrase (M_r = 31000), soybean trypsin inhibitor (M_r = 21500), and lysozyme (M_r = 14400) (Bio-Rad Laboratories). Proteins were visualized in gels by staining with 0.1% (w/v) Coomassie brilliant blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid and further silver staining, according to the method described by de Moreno *et al.*[20]

Electrophoretic Methods

Nondenaturing PAGE was performed on 10% acrylamide cylindrical gels as described by Davis.[21] SOD activity was detected in gels by the photochemical NBT reduction method.[22] The different types of SOD isozymes were identified by performing the activity stains in gels previously incubated at 25°C for 20 min, in 50 mM K-phosphate buffer, pH 7.8, containing either 2 mM KCN or 5 mM H_2O_2. CuZn-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.[3] Protein bands were visualized in gels by staining with Coomassie brilliant blue R-250 as described above.

Proteins from native- and SDS-PAGE were also transferred to PVDF membranes (Immobilon P transfer membranes) in a Mini Trans-Blot Cell, by following the manufacturer's directions (Bio-Rad Laboratories). After transfer, membranes were stained with 0.1% (w/v) Coomassie brilliant blue R-250 as described by Christiansen and Houen,[23] and then were processed either for recognition with antibodies[18] or glycoprotein detection.[24] To confirm the specificity of the glycoprotein staining, membranes were also incubated with concanavalin A in the presence of haptens (0.2 M D-methylmannopyranoside), and ovoalbumin and horseradish peroxidase (type IV) were used as positive controls.

Isoelectric focusing was carried out in 4.85% acrylamide (T5; C3) slab gels containing 2.3% (w/v) ampholytes and 10% (v/v) glycerol, using a vertical system (Mini Protean II, Bio-Rad Laboratories). TEMED (0.1%; v/v) and 0.023% (w/v) ammonium persulfate were used as catalysts. Samples were prepared in a solution containing 15% (w/v) sucrose and 2.3% (w/v)

ampholytes, and on top of the sample solution in gels, a layer of sucrose (7.5%; w/v) and ampholytes (2.3%; w/v) was placed. Electrode solutions were 0.1 M NaOH (cathode) and 0.06% (v/v) phosphoric acid (anode). The electrophoretic procedure was performed at 4°C, at constant voltage, starting at 150 V for 30 min. Then, the voltage was increased at 200 V for another 30 min, and finally, the voltage is shifted to 250 V for 1.5–2 hours. Proteins were focussed in 2.5–3 hours. The following isolectric point standards (Pharmacia LKB Biotechnology) were used: pepsinogen (pI = 2.80), amyloglucosidase (pI = 3.50), red-methyl (pI = 3.75), glucose oxidase (pI = 4.15), soybean trypsin inhibitor (pI = 4.55), β-lactoglobulin A (pI = 5.20), bovine carbonic anhydrase B (pI = 5.85), and human carbonic anhydrase B (pI = 6.55). After isoelectric focusing, gels were either processed for SOD activity (NBT reduction method) or protein staining. Prior to protein staining, gels were incubated with 20% (w/v) trichloroacetic acid for 2 h.

Enzyme and Protein Assays

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.[25] During the course of purification, column eluates were assayed for SOD activity by a method based on the NBT reduction by superoxide radicals generated photochemically.[26] Protein concentration was determined by the method of Lowry *et al.*,[27] using BSA as a standard.

Metal Content

Copper, zinc, manganese, and iron contents of the purified enzyme were determined by atomic absorption spectrophotometry with a Perkin-Elmer 503 apparatus equipped with a heated graphite atomizer. Prior to the assays, the enzyme samples were dialyzed exhaustively in metal-free dialysis membranes, against 50 mM K-phosphate buffer, pH 7.8.

RESULTS AND DISCUSSION

Watermelon cotyledons contain four electrophoretically distinct SODs, which were identified as two CuZn-SODs (I and II) and two Mn-SODs (I and II).[4] The isozyme CuZn-SOD I was demonstrated to be located in the cytosol.[4,5,7]

As a previous step before initiating the purification of cytosolic CuZn-SOD, the time course of this enzyme activity in cotyledons of *C. vulgaris* seedlings grown under darkness was studied. The growth time selected for obtaining cotyledons for the enzymatic purification was 10 d, since the enzyme showed the highest specific activity at this time (results not shown).

The purification of cytosolic CuZn-SOD from watermelon cotyledons is summarized in Table I. Acetone fractionation was used instead of ammonium sulfate, since good recoveries of the enzyme were obtained, and this method also supplied clear, fat-free solutions. After the acetone precipitation step, both CuZn-SOD I and CuZn-SOD II were recovered in the final pellet in good yields but about 80% of the Mn-SOD activity was inhibited since this type of SODs are sensitive to organic solvents.[28] By FPLC on the anion-exchanger Mono Q column, three peaks of SOD activity were obtained (results not shown). CuZn-SOD I and II eluted as separate peaks during the washing of the column, and a smaller peak, corresponding to the remaining Mn-SOD activity, was eluted by applying a linear KCl gradient to the column.

Cytosolic CuZn-SOD I was apparently pure after column chromatography through Sephadex G-150, as shown in Figure 1. A band of SOD activity corresponding to a single band of proteins was detected by native PAGE, and SDS-PAGE analysis also showed that cytosolic CuZn-SOD I was homogenous. However, it was

TABLE I Purification of cytosolic CuZn-SOD I from watermelon cotyledons[a]

Step	Total protein (mg)	Total activity (Units[b])	Specific activity (Units/mg)	Yield (%)	Purification -fold
Crude extract	4200	48020	11.4	100	1
Acetone precipitate (33–73%; v/v)	851	34290	40.3	71.4	3.5
Batch DEAE-Sephadex	309	30278	98.0	63.1	8.6
Mono Q FPLC	123	30116	245	62.7	21.5
Sephadex G-150	6.4	22500	3516	46.9	308
Concanavalin A-Sepharose	6.3	22300	3540	46.4	311

[a]500 g of watermelon cotyledons were processed.
[b]The activity of CuZn-SOD I was calculated by multiplying the percentage of this enzyme, obtained by densitometry of SOD activity-stained gels, by the total SOD activity of samples.

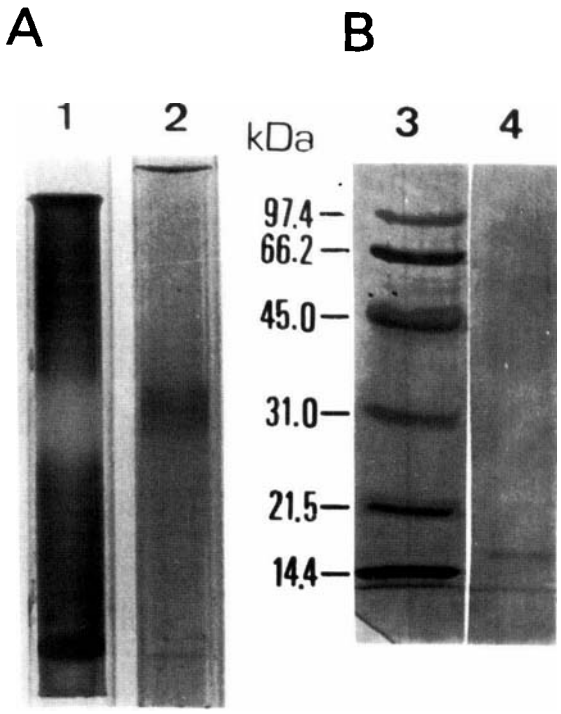

FIGURE 1 Native- and SDS-PAGE of cytosolic CuZn-SOD I fractions eluted from the Sephadex G-150 column. A, native PAGE on 10% acrylamide gels; B, SDS-PAGE on 12% acrylamide gels. Lane 1, SOD activity staining (20 μg); lane 2, protein staining with Coomassie brilliant blue R-250 (74 μg); lane 3, silver-stained molecular weight markers; lane 4, Coomassie brilliant blue and silver-staining for proteins (3.7 μg).

noted that a glycoprotein co-purified with CuZn-SOD I throughout the whole purification procedure. This contaminating glycoprotein also co-migrated with cytosolic CuZn-SOD on native gels, and could only be detected in Western blots by recognition with concanavalin A, indicating that it is present in a very low amount (results not shown). In the light of these results, cytosolic CuZn-SOD I was initially described as a glycoprotein.[29] To remove the contaminating glycoprotein, a final purification step based on affinity chromatography through concanavalin A-Sepharose had to be introduced (see Table I). Total cytosolic CuZn-SOD activity was eluted by washing the column with equilibration buffer after having allowed the protein to bind the lectin for one hour at room temperature. Shorter binding times, of less than 20 min, and performance of the chromatography at 4°C produced bad separations, with cytosolic CuZn-SOD being eluted in both unbound and bound fractions. By SDS-PAGE and glycoprotein staining of eluates in PVDF membranes, a band with a molecular mass of 33 kDa was detected in the fractions eluted with 0.2 M D-methylmannopyranoside. On the contrary, this band was not present in the unbound fractions (results not shown). Therefore, we conclude that cytosolic CuZn-SOD I from *Citrullus vulgaris* was purified to homo-

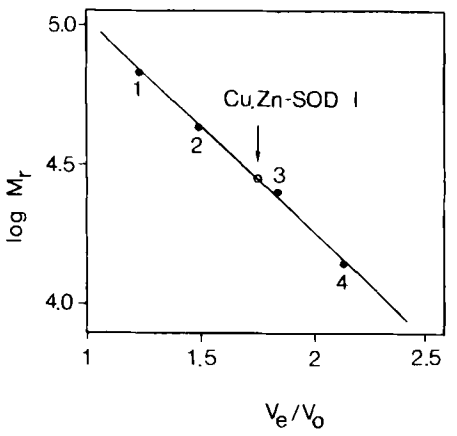

FIGURE 2 Molecular mass of cytosolic CuZn-SOD I determined by gel-filtration on Sephadex G-150. 1, BSA; 2, ovoalbumin; 3, chymotrypsinogen A; 4, cytochrome *c*.

geneity and does not contain neutral carbohydrates in its molecule.

A native molecular mass of 34000 Da was obtained for cytosolic CuZn-SOD by gel exclusion (Figure 2). This molecular mass is slightly higher than those described for plant CuZn-SODs, which are in the range 31–33 kDa,[2,16,17,28,30–34] with the exception of several CuZn-SOD isozymes from Scots pine needles which have a molecular mass of 35 kDa.[35] By SDS-PAGE, only one band of 16.3 kDa was detected on gels (Figure 1B). Thus, the enzyme appears to be a dimer composed of two equally sized subunits.

The UV absorption spectrum of the purified enzyme was characterized by an absorption maximum at 254 nm (Fig. 3), and had a molar extinction coefficient (ε_{254}) of $54.8 \times 10^4\ M^{-1}\ cm^{-1}$. In the visible region a maximum at 580 nm, probably due to the Cu-prosthetic group, was also observed.[17,32,33]

The enzyme was assayed for copper, zinc, iron and manganese by atomic absorption spectrophotometry. Determinations of metal contents were carried out in enzyme samples from three independent purification batches, and results showed that CuZn-SOD contained 1.09 ± 0.06 (SD) g-atom Cu and 1.03 ± 0.12 g-atom Zn per mol. Neither Mn nor Fe were detected in the enzyme samples. These results differ from most eukaryotic CuZn-SODs which contain 2 g-atom Cu and 2 g-atom Zn per mol,[32–34,36] and are an interesting feature of this enzyme, which has a high specific activity considering its low metal content. On the other hand, the metal content of purified cytosolic CuZn-SOD from watermelon cotyledons is similar to that obtained for peroxisomal CuZn-SOD from the same tissue, although its specific activity was twice that of the latter enzyme.[17] The low metal-protein stoichiometry found for CuZn-SODs from watermelon cotyledons might be a property of this kind of plant tissue and the growing conditions used.

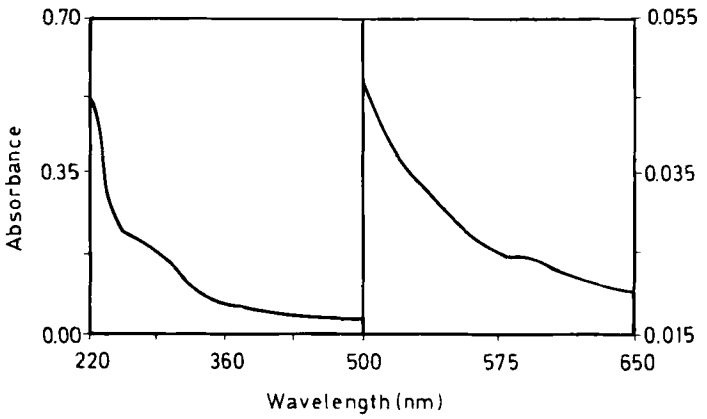

FIGURE 3 UV-visible absorption spectra of cytosolic CuZn-SOD I. An enzyme concentration of 14.8 µg/ml in 50 mM K-phosphate buffer, pH 7.8, was used.

In cross-reactivity experiments carried out with an antibody against peroxisomal CuZn-SOD,[18] cytosolic CuZn-SOD I was found to be recognized by this antibody (Fig. 4B). Besides, the incubation with the antibody to peroxisomal CuZn-SOD produced an inhibition of up to 90% of the cytosolic CuZn-SOD activity (Fig. 4A). Further work is necessary to know whether this inhibition is due to an interaction with the active site of the cytosolic CuZn-SOD, or to a conformational change induced by the binding of the antibody. In any case, cytosolic and peroxisomal CuZn-SODs seem to have common antigenic characteristics, possibly due to the existence of close similarities between their molecular structures.

In this work, an isoelectric focusing method in vertical slab gels using the Mini Protean II Cell (Bio-Rad Laboratories) was set up. This method, described in Materials and methods, is simple and quick and supplies very reproducible results, which makes it a valuable technique for isoelectric focusing of proteins. The pI initially determined for cytosolic CuZn-SOD was 4.65. This pI is in the range described for most plant CuZn-SODs.[28,37–39] However, when isoelectric focusing of the enzyme was carried out in a very narrow range of pH (4.2–4.9), cytosolic CuZn-SOD I was resolved into two equal bands of SOD activity, which also corresponded to two protein bands. The SOD bands had pI values of 4.63 and 4.66, respectively (Fig. 5), and were inhibited by 2 mM KCN and 5 mM H_2O_2 as should be expected for SODs belonging to the group of CuZn-SODs.

The possibility that the two isoforms of cytosolic CuZn-SOD, detected by IEF, are derived from each other by proteolytic cleavage can be ruled out since only one protein band of 16.3 kDa was detected by SDS-PAGE (Fig. 1B). Considering that both isoforms showed similar activity and protein content in IEF gels (Fig. 5), it seems reasonable to think that they have the same specific SOD activity and metal content.

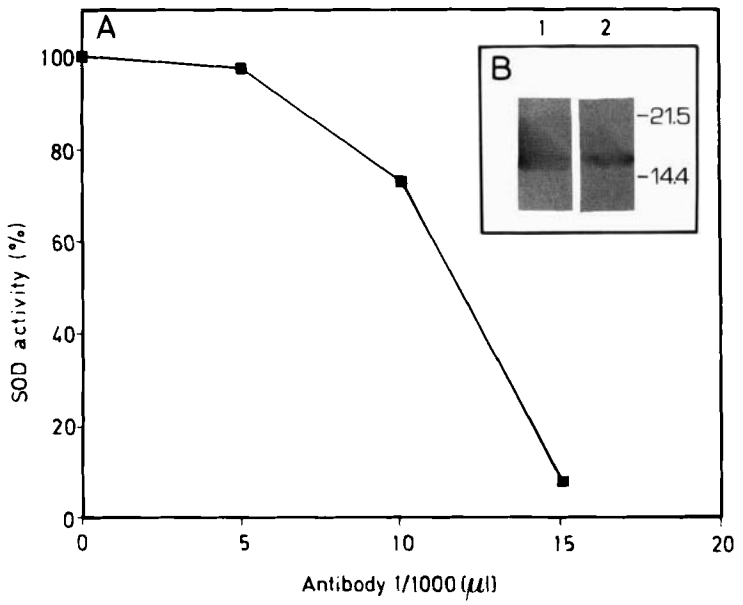

FIGURE 4 Cross-reactivity of CuZn-SOD I with an antibody against peroxisomal CuZn-SOD. A, SOD activity measured by the cytochrome *c* reduction method (McCord and Fridovich 1969), in the presence of the antibody (1/1000 dil). Values in the ordinate were expressed as a percentage of the activity determined in the absence of the antibody. Values in the abscissa correspond to the volume of the diluted antibody added to the reaction mixture (3 ml), and results are the mean of two assays with enzyme samples from two independent purification batches. B, Western blotting of CuZn-SOD I and peroxisomal CuZn-SOD. A 1/1000 dilution of the antibody was used; lane 1, CuZn-SOD I (0.4 µg); lane 2, peroxisomal CuZn-SOD (0.2 µg). Molecular weight markers are indicated on the right.

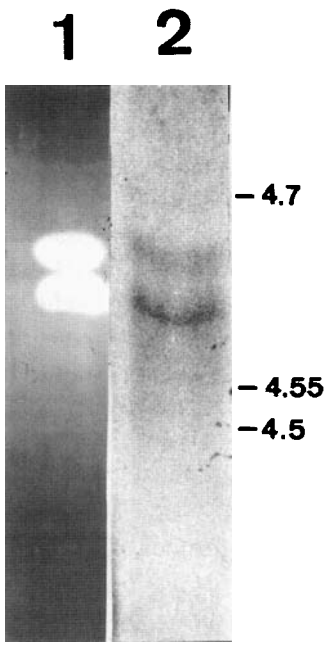

FIGURE 5 Isoelectric focusing of cytosolic CuZn-SOD I eluted from the concanavalin A-Sepharose column. The range of pH was of 4.2–4.9. 1, SOD activity staining; 2, protein staining with silver. Isoelectric point markers are indicated on the right: soybean trypsin inhibitor, pI = 4.55; ovoalbumin, pIs 4.5 and 4.7, respectively.

Further research is necessary to know the physiological function of the two isozymes of cytosolic CuZn-SOD from watermelon cotyledons. The purification of cytosolic CuZn-SOD I is the first step towards obtaining monospecific antibodies against the pure protein which can be used in comparative immunological studies with other SODs and will also make it possible to initiate molecular biology studies with this cytosolic SOD.

Acknowledgments

This work was supported by grant PB92-0492-01 from the DGICYT, Ministry of Education and Science, Spain, and the European Union (contract CHRX-CT93-0184). The authors are indebted to Dr. Manuel Lachica, Dpto. Ciencias de la Tierra y Química Ambiental, Estación Experimental del Zaidín, CSIC, for performing metal analysis by atomic absorption spectrophotometry, and to Dr. Eduardo López-Huertas for his valuable help in electrophoretic analyses. The skillful assistance of Ms. Susana Gómez-Leyva is also appreciated. P.B. and S.D. acknowledge research fellowships from the CSIC, Spain and the Ministereo dell'Università e della Ricerca Scientifica e Tecnologica, Italy, respectively.

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