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Superoxide Dismutase from *Lens esculenta*

PURIFICATION AND PROPERTIES

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ABSTRACT

Superoxide dismutase has been purified to homogeneity from *Lens esculenta* cotyledons and shoots. The two forms appeared to be identical. The purified enzyme contained two electrophoretically distinct bands. It contained two ions of Cu and two ions of Zn. Gel filtration experiments indicate a molecular weight of about 33,000. The spectrum of ultraviolet and visible regions and electron paramagnetic resonance were similar to those of Cu-Zn mammalian superoxide dismutase.

Superoxide dismutases (EC 1.15.1.1.) are metalloproteins capable of catalyzing the dismutation of superoxide anion radicals (O_2^-) to H_2O_2 and molecular oxygen. The enzyme activity was first described by McCord and Fridovich (8) with a cupro-zinc protein from bovine erythrocytes. Similar proteins have been reported in seed, seedlings, leaves, and fruits of higher plants (3, 6, 7, 10). The physiological function of SOD[1] is the protection of the cell against the indirect effects of superoxide-free radicals (O_2^-) (9). In the present paper, we describe the purification and some properties of a SOD from *Lens esculenta* cotyledons and shoots. *Lens esculenta* seem to be a profitable source of SOD in order to get a large quantity of purified enzyme.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained as pure commercial products.

Plant. Lentil (*Lens esculenta*) seeds were soaked for 4 h in H_2O and germinated for 8 d in prewashed vermiculite, in the dark at room temperature.

Purification of SOD from Shoots. Eight-d-old lentil shoots (about 900 g) were homogenized in a Waring Blendor with 2 L of deionized H_2O for 3 min (step 1). The homogenate was brought to 48°C and kept at this temperature for 10 min under continuous stirring. After rapid cooling in ice-water, the suspension was centrifuged at 9,000 rpm for 30 min and the precipitate discarded (step 2). To the supernatant, solid $(NH_4)_2SO_4$ was added to 70% saturation with constant stirring at 4°C over a period of 30 min and centrifuged at 9,000 rpm for 30 min. The precipitate was dissolved in about 300 ml of deionized H_2O and dialyzed against deionized H_2O. The insoluble material was removed by centrifugation at 19,000 rpm for 30 min (step 3). The supernatant (about 300 ml) was made 10 mM in K-phosphate (pH 7) and loaded onto a DEAE-cellulose column (2.5 × 15) equilibrated and washed with the same buffer until reaching an A_{280} of 0.05. The bound SOD was eluted with 100 mM K-phosphate (pH 7) (step 4). The active fractions were pooled and dialyzed against 1 mM K-phosphate (pH 7). The supernatant was loaded onto a HTP column (2 × 10) equilibrated and washed with the same buffer until the A at 280 nm of the effluent become 0.02 (step 5). The eluate, containing the enzymic activity, was applied to AH-Sepharose 4B column (2 × 10) equilibrated with 10 mM K-phosphate (pH 7). After washing with the same buffer, the bound SOD was eluted with 50 mM K-phosphate (pH 7) (step 6). The active fractions were pooled, concentrated by ultradialysis to about 5 ml, and applied onto a column (2.5 × 90) on Sephadex G-200 equilibrated and eluted with 100 mM K-phosphate (pH 7) (step 7). The fractions with highest specific activity were pooled, concentrated by ultradialysis, and stored at −20°C.

A summary of the purification procedure is presented in Table I. The overall purification achieved is greater than 7,500-fold with a yield of 13%.

Purification of SOD from Cotyledons. Five hundred g of dry seeds were soaked in water for about 16 h, swelling to a weight of 920 g, and homogenized in a Waring Blendor with 1,700 ml of deionized H_2O for 3 min (step 1). The solution was pressed in a cotton sack and brought to 42°C and kept at this temperature for 10 min under continuous stirring. After rapid cooling in ice-water, the suspension was centrifuged at 9,000 rpm for 30 min and the precipitate discarded (step 2). To the supernatant, solid $(NH_4)_2SO_4$ was added to 35% saturation with constant stirring at 4°C over a period of 30 min and centrifuged at 9,000 rpm for 30 min. To the supernatant, solid $(NH_4)_2SO_4$ was added to 60% saturation. After centrifugation at 9,000 rpm for 30 min, the precipitate was dissolved in about 250 ml of deionized H_2O and dialyzed against deionized H_2O for 16 h. The insoluble material was removed by centrifugation at 18,000 rpm for 30 min (step 3). The supernatant was cooled to 0°C and 0.5 volume of acetone was added to it with stirring at −20°C. The mixture was centrifuged at 10,000 rpm for 15 min. To the supernatant an additional 1.5 volume of acetone was added, centrifuged at 10,000 rpm for 15 min, and the precipitate was dissolved in 100 ml of H_2O and dialyzed against deionized H_2O for 16 h. The insoluble material was removed by centrifugation at 18,000 rpm for 15 min (step 4). The steps 5 and 6 were the same as steps 4 and 5 of SOD shoots (see text). The eluate of the HTP column was applied to AH-Sepharose 4B column (2 × 10) equilibrated with 1 mM K-phosphate (pH 7). After washing with the same buffer, the bound SOD was eluted with 10 mM K-phosphate (pH 7). The active fractions were pooled and concentrated by ultradialysis and stored at −20°C (step 7). A summary of the purification procedure is presented in Table II. The overall purification achieved was greater than 284-fold with a yield of 25%.

Enzyme assays were performed according to McCord and Fridovich (8) and Sawada *et al.* (11). The amount of SOD required to inhibit the rate of reduction of Cyt *c* by 50%, is

[1] Abbreviations: SOD, superoxide dismutase; HTP, hydroxylapatite.

Table I. *Purification of SOD from Shoots*

Step	Total Protein	Total Units	Specific Activity	Yield
	mg			
1) Crude homogenate	26,800	23,000	0.86	100
2) Heat treatment	9,530	20,000	2	86
3) Ammonium sulfate fractionation	2,400	12,000	5	52
4) DEAE-cellulose column chromatography	175	9,000	51	39
5) HTP column chromatography	52	5,500	105	24
6) AH-Sepharose 4B column chromatography	7	4,600	650	20
7) Sephadex G-200 column chromatography	0.47	3,100	6,600	13.5

Table II. *Purification of SOD from Cotyledons*

Step	Total Protein	Total Units	Specific Activity	Yield
	mg			
1) Crude homogenate	13,670	317,140	23.18	100
2) Heat treatment	8,300	205,300	24.73	64.74
3) Ammonium sulfate fractionation	4,318	137,830	31.9	43.46
4) Acetone fractionation	912.5	135,900	149	42.8
5) DEAE-cellulose column chromatography	544	124,670	229.6	39.3
6) HTP column chromatography	196.2	123,800	630.9	39
7) AH-Sepharose 4B column chromatography	11.76	77,640	6,600	24.5

defined as 1 unit of activity.

Analytical PAGE was performed according to Beauchamp and Fridovich (2) and Floris *et al.* (4).

Determination of mol wt, spectroscopic measurements, metal determination, and other analytical methods were according to Floris *et al.* (4).

RESULTS AND DISCUSSION

Criteria of Purity. Polyacrylamide of the SOD obtained by the reported purification procedure from cotyledons and shoots indicate two protein bands with enzymic activity and suggest the occurrence of two isoenzymes with the same electrophoretic mobility characterizing the purified enzyme. Only one band was observed in SDS-PAGE in the presence and absence of β-mercaptoethanol (protein sample, 100 μg).

Mol Wt Determination. The mol wt of SOD was determined by SDS-PAGE and by gel filtration on Sephadex G-200. SDS-PAGE showed a single band with a mol wt of 15,900. Gel filtration chromatography gave a mol wt of more than 32,000; SOD appears, therefore, to be a dimer made of identical subunits.

Metal Content. The content of copper and zinc ions per protein, determined by the mean of five different preparations was 1.96 and 2.04, respectively (SD, 0.1172 and 0.0678, respectively).

Spectroscopic Properties. The absorption spectrum of SOD in the visible shows an absorption maximum at 680 nm and in the UV at 259 nm. The enzyme showed a UV spectrum and an electron paramagnetic resonance spectrum very similar to those already reported from other SOD (1, 5, 11).

Effect of Temperature. The effect of temperature on the rate of reaction was studied under standard assay conditions over the range 40°C to 70°C. An inactivation of 50% was after 5 min at 55°C.

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