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PREPARATION AND PHYSICOCHEMICAL PROPERTIES OF GREEN PEA SUPEROXIDE DISMUTASE

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SUMMARY

A procedure is described for preparing purified superoxide dismutase from green peas. The enzyme has no tyrosine and no tryptophan and gives a characteristic ultraviolet spectrum. Like human and bovine enzymes, it contains 2 atoms of Cu and 2 atoms of Zn and the results of sedimentation equilibrium and gel filtration indicate a molecular weight of about 31 500. The spectra of visible absorbance and electron paramagnetic resonance are very similar to those of mammalian superoxide dismutases.

INTRODUCTION

The Cu-containing proteins known as hemocuprein (or erythrocuprein)[1], hepatocuprein[1], and cerebrocuprein[2] have been shown to be identical by Carrico and Deutsch[3] and the name cytocuprein was proposed for these proteins. It was found by McCord and Fridovich[4] that the Cu protein isolated from bovine erythrocytes catalyzes the dismutation or disproportionation of superoxide free radical anions and they called it superoxide dismutase. The Cu proteins isolated from human erythrocytes[5] and bovine heart[6] have a molecular weight of about 33 000 and contain 2 atoms of Cu and 2 atoms of Zn per molecule.

It has been suggested that superoxide dismutase serves to protect cells against the deleterious effects of the superoxide radical and is widely distributed in all aerobic cells. Keele *et al.*[7] have reported that superoxide dismutase from *Escherichia coli* contains Mn atoms instead of Cu and Zn. The present report describes the preparation of superoxide dismutase from green peas and some physicochemical properties of the enzyme.

MATERIALS AND METHODS

Cytochrome *c* was obtained from Boehringer-Mannheim. Xanthine oxidase, purified from raw cream by a slightly modified procedure of Hart and Bray[8], was generously provided by Mr. T. Kon in this laboratory. All other materials were

obtained from commercial sources at the highest available states of purity. Superoxide dismutase was assayed and the number of units determined according to the method of McCord and Fridovich[4]. Chemical analyses of Cu and Zn were performed by the methods of Van de Bogart and Beinert[9] and of Malmström[10], respectively. Atomic absorption was measured with a Hitachi Model 207 atomic-absorption spectrophotometer. Spectrophotometers used were Hitachi Model 124 and Model EPS-3. Electron paramagnetic resonance was investigated with a Varian V-4500 X-band instrument, utilizing 100 kcycles field modulation. Measurements of sedimentation equilibrium were performed at pH 7.0 and 20 °C in 0.2 M KCl–0.01 M potassium phosphate utilizing a Hitachi Type UCA-1A analytical ultracentrifuge. Amino acid analyses were performed with a Hitachi KLA-3B amino acid analyzer.

RESULTS

Purification of superoxide dismutase

2 kg of dry seeds of green pea (*Pisum sativum*) were soaked in water for about 20 h, swelling to a weight of 4.3 kg. Wet beans were then crushed with a meat chopper into 4.3 l of 0.1 M disodium hydrogen phosphate. The resultant pH was 7.3. The slurry was further homogenized for 1 min in a Waring blender and was stirred occasionally for 2 h. It was then pressed in a cotton sack. To the extract solid $(NH_4)_2SO_4$ (252 g per l) was added to bring it to 35% saturation with respect to this salt. After 1 h at 4 °C the solution was clarified by centrifugation. To the supernatant additional $(NH_4)_2SO_4$ was added to 55% saturation. After 1 h, the precipitate was collected by centrifugation, suspended in a small amount of water and then dialyzed against distilled water at 4 °C. The precipitate formed during dialysis was removed by centrifugation. The supernatant was cooled to 0 °C and 0.5 vol. of acetone (chilled to −20 °C) was added to it with stirring. The solution was then clarified by centrifugation at 0 °C. To this supernatant an additional 1 vol. of acetone was added and the precipitate was collected. The precipitate was triturated in 5 vol. of 2.5 mM potassium phosphate, pH 7.8 and the insoluble residue was removed by centrifugation. This solution was concentrated by salting-out with 65% saturated $(NH_4)_2SO_4$, equilibrated with

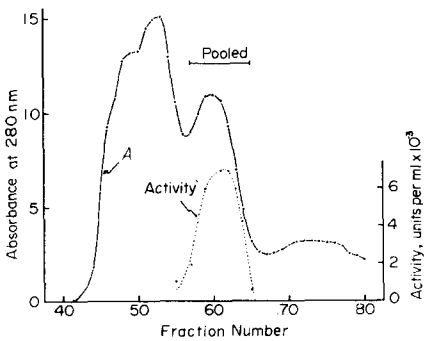
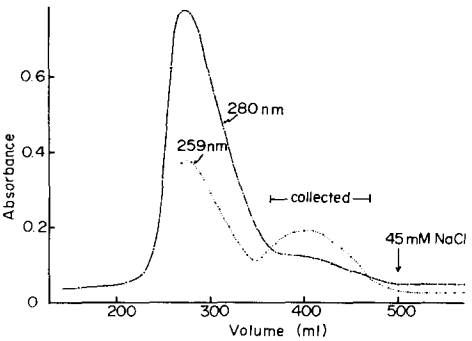


Fig. 1. Chromatography on Biogel P-60. The acetone-treated preparation, after dialysis and equilibration with 0.1 M NaCl–2.5 mM potassium phosphate, pH 7.8, was applied to a column, 5 cm × 60 cm, of Biogel P-60 which had been equilibrated at 4 °C with the same buffer. Fractions were assayed for absorbance at 280 nm (—) and for superoxide dismutase activity (---).



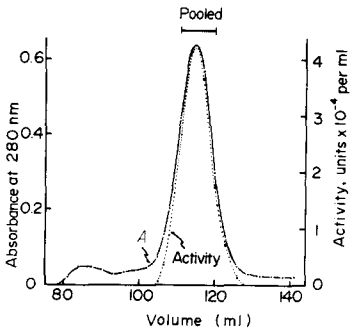


Fig. 2. Second chromatography on DE-32. The sample was adsorbed onto a column, 1.5 cm × 30 cm, of DE-32 equilibrated with 2.5 mM potassium phosphate, pH 7.8 and then eluted with a linear NaCl gradient from 25 to 65 mM in the same buffer. Fractions were analyzed for absorbance at 280 nm (———) and at 259 nm (· · · · · ·). The ratio could be used as a simple criterion of purity of the enzyme preparation (see the text).

Fig. 3. Chromatography on Biogel P-30. The sample was finally applied to a column, 1.5 cm × 100 cm, of Biogel P-30. The column was eluted with 0.1 M NaCl–2.5 mM potassium phosphate, pH 7.8. Fractions were assayed for absorbance at 280 nm (—) and for superoxide dismutase activity (· · · · · ·). The appearance of a preceding small peak of absorbance in this experiment was not reproducible and may be an artifact.

0.1 M NaCl–2.5 mM potassium phosphate, pH 7.8, and subjected to gel exclusion chromatography in the same solution on a column of Biogel P-60 (5 cm × 60 cm). The elution pattern is illustrated in Fig. 1. Fractions whose specific activity (units per ml of enzyme solution having absorbance of 1.0 at 280 nm) exceeded 200 were pooled and salted out from 65% saturated $(NH_4)_2SO_4$ solution. The precipitate was collected by centrifugation, dissolved in a little water, and equilibrated with 2.5 mM

TABLE I

PURIFICATION OF SUPEROXIDE DISMUTASE FROM 2 kg OF DRY GREEN PEAS

Preparation stage	*Total protein* (mg)*	*Total units × 10⁻⁵*	*Specific activity (units per mg)*	*Volume (ml)*	*Yield (%)*
0.1 M Disodium phosphate extract	214 000	21.2	9.9	4250	100
Ammonium sulfate fractionation	27 200	16.6	60.6	415	78.3
Acetone fractionation	8 670	9.38	108	410	44.2
BioGel P-60 chromatography	1 430	6.87	480	27.5	32.4
DE-32 chromatography (1st)	202	5.25	2600	16.0	24.8
DE-32 chromatography (2nd)	69.1	4.13	5980	5.4	19.5
BioGel P-30 chromatography	57.7	3.70	6400	12.0	17.4

* Protein concentration was determined with Folin reagent using bovine serum albumin as a standard.

potassium phosphate, pH 7.8. It was then adsorbed onto a column of Whatman DE-32 (1.5 cm × 30 cm) which had been equilibrated with the above buffer. The column was eluted with 600 ml of the same buffer with a linear gradient of NaCl from 0 to 0.1 M. The blue-green fractions were pooled and the same chromatographic procedure was performed except for a slow gradient of NaCl. The result is shown in Fig. 2. Fractions having higher absorbance at 259 nm than at 280 nm were pooled. This solution was concentrated by re-adsorption onto a short column of DE-32 and elution with 0.1 M NaCl–2.5 mM phosphate and then applied to a column of Biogel P-30 (1.5 cm × 100 cm) equilibrated with the above buffer solution. Fractions having specific activities exceeding 6000 were pooled, as shown in Fig. 3. The results of this purification procedure are summarized in Table I.

Molecular weight

Sedimentation equilibrium experiments were performed at speeds of 15 590 and 18 270 rev./min with a 1-mm short column, using solutions with protein concentrations ranging from 0.14 to 0.9%. The molecular weight was calculated according to the method of Yphantis[17] and estimated to be 31 500 ± 700 (Fig. 4).

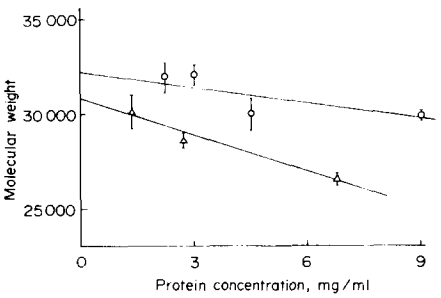
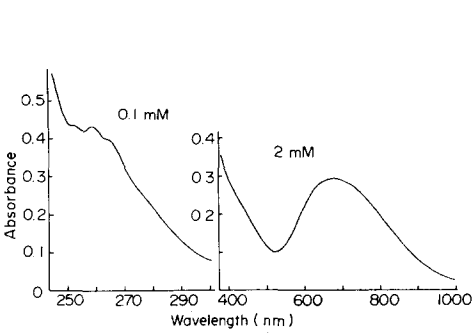


Fig. 4. Molecular weight of green pea superoxide dismutase as a function of concentration. Sedimentation equilibrium experiments were performed at two speeds of 15 590 (△) and 18 270 (○) rev./min in 0.2 M KCl–10 mM potassium phosphate, pH 7.0 at 20 °C. Molecular weight was calculated assuming that the partial specific volume is 0.720, which was estimated from the amino acid composition[20].

Using the gel filtration method with Sephadex G-75, a molecular weight of 31 000 was given. The column (1.2 cm × 100 cm) was equilibrated with 0.1 M NaCl–2.5 mM potassium phosphate, pH 7.8. Markers used were human γ-globulin (160 000), ovalbumin (45 000) and cytochrome *c* (12 400). 2-ml aliquots were assayed for absorbance at 280 nm and superoxide dismutase activity.

Spectral properties

The ultraviolet and visible absorption spectra of the superoxide dismutase are shown in Fig. 5. Its visible spectrum was similar to those of erythrocyte enzymes isolated from human[18] and bovine erythrocytes[4]. The ultraviolet spectrum was similar to that of the bovine enzyme rather than the human enzyme. The absorbance at 259 nm was about twice as high as that at 280 nm and this property was used as a simple criterion of purity in the chromatographic procedure (Fig. 2).



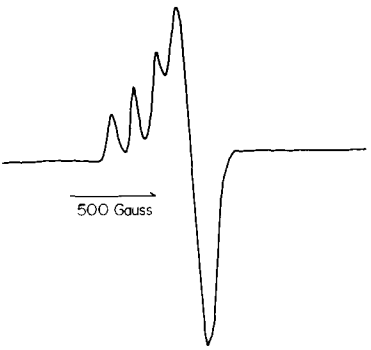


Fig. 5. Ultraviolet and visible absorbance spectra of green pea superoxide dismutase. Concentrations of the enzyme were 0.1 mM for the ultraviolet spectrum and 2 mM for the visible spectrum on the basis of Cu in 10 mM potassium phosphate, pH 7.0. The light path was 10 mm.

Fig. 6. EPR spectrum of green pea superoxide dismutase. The enzyme was at a concentration of 32 mg per ml (2 mM Cu) in 10 mM potassium phosphate, pH 7.0. Other conditions were: modulation amplitude, 4 G; scan rate, 200 G per min; time constant, 0.3 s and sample temperature, 100 °K. The microwave frequency used was X-band but its exact value was not measured. The values of spectral parameters were estimated by comparison with the EDTA-cupric signal and were: $g_m = 2.04$, $g_{||} = 2.23$ and $A_{||} = 0.014$ cm^{-1}.

EPR spectrum

EPR spectra of human[3] and bovine[6] superoxide dismutases have been reported. This seems to be the best analytical method at the moment to characterize the ligand structure of Cu in the protein molecules. As shown in Fig. 6, the EPR spectrum of the green pea enzyme was found to be quite similar to those of mammalian enzymes.

TABLE II

COMPARISON OF PHYSICOCHEMICAL PROPERTIES OF SUPEROXIDE DISMUTASES ISOLATED FROM MAMMALIAN AND PLANT SOURCES

References are indicated in parentheses. Metal content (atom per protein) of the green pea enzyme was calculated as follows: number of atoms per protein $= \frac{31\,500}{\text{dry weight}} \cdot \frac{\text{metal weight}}{\text{atomic weight of metal}}$.

Although polyacrylamide disc gel electrophoresis revealed the presence of an impurity of several per cent of the total protein the correction was not made in this table.

	Bovine erythrocyte	*Human erythrocyte*	*Green pea*
Molecular weight	32 600 (4)	33 600 (18)	31 500
Copper content			1.97*
atoms per protein	1.94 (6)	2.02 (5)	1.98*
Zinc content			1.84*
atoms per protein	1.71 (6)	1.94 (5)	2.02**
Visible absorption maximum			
λ(nm)	680 (4)	675 (18)	680
ε_M	300 (4)	250 (18)	290
Superoxide dismutase activity (units per mg)	3300 (4)	3000 (4)	6400
EPR signal g_m	2.080 (6)	2.063 (3)	2.04
$g_{||}$	2.265 (6)		2.23

* Calculated by chemical analysis.
** Calculated by atomic absorption.

TABLE III

AMINO ACID COMPOSITION OF SUPEROXIDE DISMUTASES

Amino acid	*Bovine erythrocyte Keele et al.*[6]	*Human erythrocyte Hartz and Deutsch*[18]	*Green pea*
Lysine	22	22	10
Histidine	16	16	18
Arginine	10	7	6
Aspartic acid	35	36	45
Threonine	26	16	30
Serine	20	20	14
Glutamic acid	24	26	19
Proline	14	10	14
Glycine	50	50	56
Alanine	21	20	21
Valine	28	28	21
Methionine	0	0	0
Isoleucine	17	16	20
Leucine	20	17	21
Tyrosine	2	0	0
Phenylalanine	10	8	9
Tryptophan	0	(2)*	0**
Half-cystine	(6)***	7	(6)†

* Cited from ref. 6.
** Determined by the method of Spies and Chambers[19].
*** Cited from ref. 15.
† Half-cystine was determined as cysteic acid after performic acid oxidation in an acid hydrolysate.

Metal analysis

Metal analyses were performed with the methods of atomic absorption and chemical analysis after wet-ashing of the enzyme. Both methods gave almost identical results for contents of Cu and Zn as summarized in Table II. The results indicated that the green pea superoxide dismutase contains 2 atoms of Cu and 2 atoms of Zn per molecule of the enzyme. The enzyme was assayed for Mn and Fe but no significant amounts of these metals could be detected by atomic absorption studies.

Amino acid analysis

Green pea superoxide dismutase was lyophilized after dialysis against distilled water and aliquots were hydrolyzed in sealed tubes freed of air in 6 M HCl for 18, 23, 72 and 88 h. After removal of HCl, the samples were assayed for their amino acids. For those amino acids which suffered destruction upon hydrolysis the values were extrapolated to zero time hydrolysis. For those released slowly the values were obtained from the 88-h sample. The results are shown in Table III compared with bovine and human enzymes.

DISCUSSION

The physicochemical properties of superoxide dismutase from green pea described in Table II are similar to those from mammalian sources. Keele *et al.*[6] have reported an interesting comparison of ultraviolet absorption spectra of human and

bovine erythrocyte superoxide dismutases. Molar absorption at 280 nm is low for both human and bovine enzymes and has been reported to be 16 300 and 8100, respectively. The difference has been explained from the fact that the human enzyme has 2 tryptophans and no tyrosine while the bovine enzyme has 2 tyrosines and no tryptophan. The green pea enzyme contains neither residue and its molar absorbance at 280 nm is only 4400. Another notable difference between human and bovine enzymes is reported to be the content of threonine. The bovine enzyme contains 26 residues of threonine compared to only 16 residues for the human enzyme. In this respect, the green pea enzyme is similar to the bovine enzyme. From Table III it might be said that the green pea enzyme differs from mammalian enzymes particularly in the content of acidic and basic amino acids.

The absorbance and EPR spectra of Cu complexes have been explained in terms of the electronic configurations or of the coordination chemistry of Cu^{2+}. It is of particular interest to note in this connection that three forms of Cu in Cu proteins have been reported. After extensive studies on laccase[11,12] and ceruloplasmin[13] Malmström, Vangård and their colleagues have come to the conclusion that these oxidases contain three forms of Cu: Type 1, blue and EPR detectable; Type 2, non-blue and EPR detectable; and Type 3, diamagnetic Cu^{2+}. According to Malkin and Malmström[14] Cu in erythrocuprein, which is termed here erythrocyte superoxide dismutase, is classified as Type 2. Type 2 Cu differs from Type 1 in absorbance and EPR spectra. Molar absorbance in the visible region of Type 2 Cu is only several per cent of that of Type 1 Cu. The hyperfine constant ($A_{\|}$) in the EPR spectra of Type 2 Cu is generally greater than that of Type 1 Cu and is in the range from 0.015 to 0.020 cm^{-1}. The constant is 0.014 cm^{-1} for bovine[6] and green pea superoxide dismutases and the human enzyme gives a slightly higher splitting constant[3].

McCord *et al.*[16] have recently reported that superoxide dismutase is found in the tissues of fish, birds, insects, plants and in a variety of aerobic microorganisms. Since it is not common that the same type of Cu protein is found in both mammalian and plant tissues a striking similarity of this enzyme between both tissues is of special interest. It might still be too soon to conclude that the physiological function of this copper protein is to decompose superoxide formed during aerobic reactions. However, it seems very important to elucidate the mechanism of the superoxide dismutase reaction and to compare it with the function of Type 2 Cu generally present in laccase, ceruloplasmin and ascorbate oxidase[14]. According to our experience, green peas seem to be a profitable source of this enzyme in order to get a large quantity of purified enzyme for kinetic studies. The results will be published elsewhere.

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