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ISOENZYMES OF CUPROZINC SUPEROXIDE DISMUTASE FROM *PISUM SATIVUM**

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Abstract—Two cyanide-sensitive and organic solvent-inactivated superoxide dismutase isoenzymes were purified from pea leaves, *Pisum sativum*, cv Thomas Laxton, to apparent homogeneity. The two proteins had similar approximate dimeric MW (33 700) but varied in net charge and subunit size. Metal analysis showed that each isoenzyme contained approximately 2 Cu and 2 Zn atoms per dimer. Amino acid compositions as well as ultraviolet absorption spectra were different for the two isoenzymes. Proteolytic digests of the two proteins revealed differences.

INTRODUCTION

Superoxide dismutase† (EC 1.15.1.1) designates a class of enzymes which have a protective function of preventing the accumulation of the potentially harmful intermediate, superoxide radical (O_2^-) [1]. These proteins contain either Cu and Zn, or Fe, or Mn. The Cu- and Zn-containing SODs, present in most eucaryotes with few exceptions [2, 3], are sensitive to cyanide and H_2O_2. The Fe-containing SODs, found predominantly in procaryotes [2] and in a few eucaryotes [3], are resistant to cyanide, yet inhibited by H_2O_2. The Mn-containing SODs, found in both procaryotic and eucaryotic organisms [4], are sensitive to neither cyanide nor H_2O_2 but can be inactivated by acetone precipitation [5].

Cuprozinc SODs have been purified from several plant tissues, including wheat germ [6], spinach leaves [7], corn seedlings [8] and kidney bean leaves [9]. Two cuprozinc isoenzymes of SOD from wheat germ have been isolated and characterized [6].

Seeds and leaves of the pea (*Pisum sativum*, cv Thomas Laxton) contain three electrophoretically separable SOD enzymes: one insensitive to cyanide and H_2O_2, and two sensitive to cyanide. The first of these SODs has been isolated and identified as a Mn SOD [10]. A Cu–Zn SOD has been purified from pea seeds [8, 11], but the individual isoenzymes have not been isolated and characterized separately. This report describes the purification and characterization of the two cyanide-sensitive, Cu–Zn SOD isoenzymes from pea leaves.

RESULTS AND DISCUSSION

Based upon relative mobilities of the two Cu–Zn isoenzymes on polyacrylamide gels, the two proteins were designated I and II, the former being more cathodic. In an initial effort to determine the subcellular localization of the two isoenzymes of Cu–Zn SOD in pea leaves, whole intact cells devoid of cell walls were prepared (protoplasts). Chloroplasts were separated from protoplasts by gentle rupture. Chloroplasts and protoplasts were placed on polyacrylamide gels, electrophoresed and stained for SOD activity. We observed, as has been previously reported [12, 13], that isoenzyme II was found associated exclusively with the chloroplasts.

The purification scheme used to isolate the two cyanide-sensitive isoenzymes from pea leaves is shown in Table 1. We repeatedly observed that treatment of pea leaf homogenate with chloroform and ethanol followed by an acetone precipitation (method of Tsuchihashi [14]) resulted in an almost total loss of cyanide-sensitive enzyme activity. Therefore, $(NH_4)_2SO_4$ precipitation was used in the early stages of the purification.

The two isoenzymes were co-purified through the initial ion-exchange chromatography steps. Past this point, the isoenzymes were treated separately. Total separation of the two proteins was achieved only after repeated preparative electrophoresis steps involving discontinuous and slab gels. Calculations of protein content and SOD activity for the DEAE-cellulose column B, pooled fraction I, took into account the protein and activity contributions of the isoenzyme II not yet separated from fraction I. Therefore, the specific and total activities of fraction I decreased, and those of fraction II increased, as the two isoenzymes were further separated. Poor recoveries of the purified isoenzymes, as shown by the extremely low yields, are due, in part, to losses which occurred during the repeated preparative electrophoresis steps. In addition, yield calculations were based initially on measurements of activity in a crude extract. Despite exhaustive dialysis, compounds which could interfere with the indirect SOD assay were present in the extract. These endogenous substrates could result in slight alterations in the true rate. The isoenzymes isolated after the final exclusion step were judged to be pure by the criterion of homogeneity on polyacrylamide gel electrophoresis, the protein and

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†Abbreviations: SOD, superoxide dismutase; Cu–Zn SOD, copper- and zinc-containing superoxide dismutase; Mn SOD, manganese-containing superoxide dismutase; Fe SOD, iron-containing superoxide dismutase; SDS, sodium dodecyl sulfate.

Table 1. Purification of cuprozinc superoxide dismutase from *Pisum sativum*

Step	Vol. (ml)	Protein concn (mg/ml)	Total protein (mg)	Specific activity (unit/mg)	Total activity (unit)	Yield (%)	Purification (fold)
Crude supernatant after centrifugation	4060	7.99	32400	9.39	304000	100	1
40–70% $(NH_4)_2SO_4$ ppt. dialysed	385	10.65	4100	35.6	146000	48.0	3.79
DEAE-cellulose batch supernatant	500	2.41	1205	115	139000	45.7	12.2
DEAE-cellulose column A, pooled fractions I + II	215	1.29	277	497	138000	45.4	52.9
DEAE-cellulose column B, pooled fraction I (significant contamination with II)	14	5.95	83.3	1620	135000	44.4	172
DEAE-cellulose column B, pooled fraction II	21	0.66	13.9	292	4060	1.3	31.1
Preparative electrophoresis and ultrafiltration, pooled fraction I	0.66	3.73	2.46	1355	3330	1.1	144
Preparative electrophoresis and ultrafiltration, pooled fraction II	0.76	5.09	3.87	3100	12000	3.9	330
Sephadex G-150 and ultrafiltration, fraction I	1.90	0.186	0.353	3060	1080	0.36	326
Sephadex G-150 and ultrafiltration, fraction II	1.60	0.466	0.746	4680	3490	1.1	498

enzyme activity coinciding in both cases.

A MW of approximately 33 700 was calculated for both Cu–Zn SODs by gel filtration on Sephadex G-150. Gel electrophoresis, after treatment of the isoenzymes with 2% SDS and 5% 2-mercaptoethanol at 100° for 10 min, showed that isoenzyme I was dissociated into a band corresponding to a MW of 16 800, whereas isoenzyme II produced a band corresponding to a MW of 17 900. Thus, isoenzymes I and II are dimers composed of two apparently equally-sized subunits.

UV spectra for both isoenzymes are illustrated in Fig. 1. Striking differences can be observed in the two spectra. The spectrum for isoenzyme II was characterized by a lack of absorbance at 280 nm (tryptophan absorbance region) and corresponded to those from other plant and animal sources [4, 6, 7]. However, the UV spectrum for isoenzyme I showed a relatively strong absorbance at 280 nm, and appeared similar to those recorded recently from Cu–Zn SOD isolated from various fish [15]. Similar peaks of absorbance with corresponding extinction coefficients were observed at $\lambda_{max}^{H_2O}$ nm (log ε): 259 (4.55) for isoenzyme I and $\lambda_{max}^{H_2O}$ nm (log ε): 258 (4.65) for isoenzyme II. Isoenzyme I exhibited an additional peak at 274 nm, with $\lambda_{max}^{H_2O}$ nm (log ε): 274 (4.60).

Isoelectric points of isoenzymes I and II were 4.9 and 4.5, respectively. These values agree with the proteins' behavior on non-denaturing electrophoretic gels, on which isoenzyme II had a higher mobility towards the anode.

Atomic absorption metal analysis of the two cyanide-sensitive isoenzymes indicated the presence of about 2.2 g-atoms Cu for isoenzyme I and 2.0 g-atoms Cu for isoenzyme II. Approximately 1.8 g-atoms Zn per molecule of SOD was found for both isoenzymes, whereas the Fe and Mn content was below limits of detection.

Table 2 shows the results of amino acid analysis of the two isoenzymes. For comparison, the amino acid compositions of some other eucaryotic Cu–Zn SODs are also listed. The tryptophan content of the two isoenzymes, as determined by the method of Edelhoch [17], was low and irreproducible. The values were therefore omitted from the table. The pea leaf enzymes are not unlike the other Cu–Zn SODs in amino acid content, though several minor differences are apparent. The lysine content of plant SODs appears to be somewhat less than that of the bovine enzyme. Green pea leaf enzymes appear to be richer in aspartic acid than either wheat germ isoenzymes. Tyrosine values, especially for isoenzyme II are higher than all the other SODs compared in Table 2. Isoenzyme II appears to be devoid of cysteic acid, which was measured as half-cystine. Comparisons of amino acid compositions of the two pea Cu–Zn SOD isoenzymes show differences in sulfur-containing amino acids as well as the differences that one would expect on the basis of charge isoenzymes.

Further differences between pea leaf isoenzymes were observed by a preliminary comparison of peptide fragments after proteolytic digestion (not shown). Treatment with papain resulted in an apparent cleavage of isoenzyme II, but not of isoenzyme I, while *Staphylococcus aureus* V-8 protease cleaved both proteins into many small, apparently non-homologous peptide bands. Treatment of the two isoenzymes with α-chymotrypsin showed that both proteins were relatively resistant to this protease. Such resistance is reasonable in view of the small numbers of aromatic amino acids in Cu–Zn SODs in general.

Both isoenzymes are apparently devoid of sugar moieties, since staining for carbohydrate was negative on acrylamide gels of purified isoenzymes run under non-denaturing conditions.

We conclude therefore that the two charge isoenzymes of Cu–Zn SOD differ not only in net charge but also in absorption spectra, apparent MW, amino acid composition, and location within the leaf cell. Our findings of differences in the cuprozinc enzymes from the same cell, catalysing an identical enzymatic reaction, raise a question as to whether this is a case of minor divergent evolution of the proteins. A more complete picture would be dependent upon isolation of the genes coding for each of the isoenzymes.

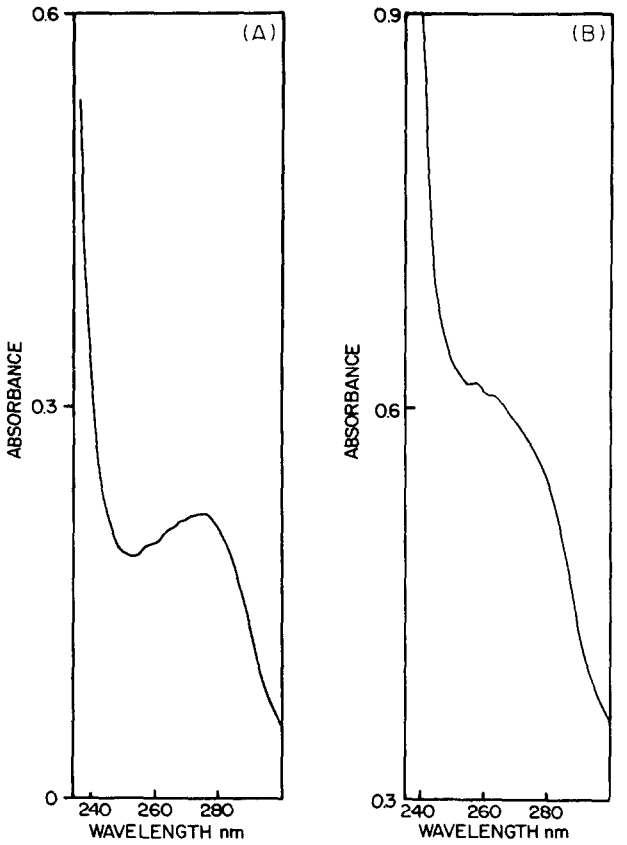

Fig. 1. UV spectrum of: (A) isoenzyme I at a concentration of 0.186 mg/ml; (B) isoenzyme II at a concentration of 0.466 mg/ml.

Table 2. Amino acid compositions of several cuprozinc superoxide dismutases

	Residues per molecule						
	P. sativum leaf				Wheat germ [6]		
Amino acid	I	II	Green pea seed [11]	Spinach [7]	I	II	Bovine erythrocyte [16]
Lysine	11	13	10	13	10	8	22
Histidine	14	8	18	14	19	15	16
Arginine	7	10	6	7	8	10	10
Aspartic acid	51	35	45	35	28	28	35
Threonine	30	23	30	28	33	30	26
Serine	20	14	14	10	15	12	20
Glutamic acid	28	31	19	20	21	26	24
Proline	18	16	14	17	19	19	14
Glycine	64	40	56	42	55	43	50
Alanine	26	22	21	23	28	25	21
Half-cystine	5	0	6	4	6	4	6
Valine	18	22	21	28	31	34	28
Methionine	3	1	0	2	2	0	2
Isoleucine	12	14	20	6	13	10	17
Leucine	25	24	21	22	22	31	20
Tyrosine	3	6	0	0	0	0	2
Phenylalanine	11	11	9	6	7	6	10
Tryptophan	—	—	0	0	—	—	0

EXPERIMENTAL

Materials. Pea leaves (*Pisum sativum*, cv Thomas Laxton) were harvested at maturity from greenhouse plants grown from seeds. Leaves were removed from the stems before purification was initiated. Unless otherwise stated, all reagents were purchased from Sigma Chemical Co., St. Louis, Mo.

Assays. Superoxide dismutase was assayed spectrophotometrically, and a unit of activity was defined according to McCord and Fridovich [18]. Polyacrylamide gel electrophoresis (PAGE) was performed on 7.5% gels according to Davis [19], and SOD activity was visualized by the photochemical method of Beauchamp and Fridovich [20]. Where noted, cyanide was included in the incubation mixture at a final concn of 2 mM. Gels were stained for carbohydrate according to Zacharius *et al.* [21]. Protein was determined by the method of Lowry *et al.* [22], or by the spectrophotometric method of Murphy and Kies [23] for the purified isoenzymes.

Purification of enzymes. Unless otherwise specified, K-Pi buffer (pH 7.8) was used, and all steps were carried out at 0–5°. Pea leaves (2 kg) were homogenized in a Waring blender containing 2 l. of 50 mM K-Pi buffer plus 0.1% Triton X-100. The homogenate was filtered through six layers of cheesecloth; the resulting filtrate was centrifuged at 10 000 *g* for 10 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to bring the soln to 40% saturation. The soln was stirred for 1 hr at room temp. and the ppt. that formed was removed by centrifugation. Additional solid $(NH_4)_2SO_4$ was added to the supernatant to give a 70% saturated soln, which was stirred for 1 hr at room temp.; the resulting ppt. was collected by centrifugation as before and then resuspended in and dialysed for 72 hr against 50 mM K-Pi buffer. The dialysate was stirred for 30 min with 200 ml of DEAE-cellulose equilibrated with 50 mM K-Pi buffer. This slurry was centrifuged at 10 000 *g* for 10 min; the supernatant was then dialysed against 5 mM K-Pi buffer. The dialysed soln was clarified by centrifugation and applied to a DEAE-cellulose column equilibrated with 5 mM K-Pi buffer. The sample was eluted with a 5–100 mM K-Pi linear gradient; fractions exhibiting SOD activity were pooled and dialysed against 5 mM K-Pi. The dialysate was applied to a second DEAE-cellulose column equilibrated with 5 mM K-Pi buffer. The two superoxide dismutases were eluted with a 5–120 mM K-Pi linear gradient; PAGE assay of fractions indicated a partial separation of the isoenzymes. Fractions containing both isoenzymes were pooled, and tail fractions containing only isoenzyme II were maintained separately. The pooled fractions containing both isoenzymes were dialysed against 10 mM K-Pi, concentrated to *ca* 1 ml by ultrafiltration, and applied to a prep. PAGE column (Canalco). The gel consisted of 7.5% acrylamide and was 2 cm in length. The electrode soln contained 0.2 M glycine and 0.02 M Tris at pH 8.3. Protein bands eluted from the gel by 50 mM Tris–HCl buffer (pH 7.5) were collected, and the fractions were assayed by PAGE for evidence of separation of isoenzymes. The initial fractions containing only isoenzyme II and the latter fractions containing only isoenzyme I were pooled separately, while middle fractions containing a mixture of the two isoenzymes were pooled, dialysed against 10 mM K-Pi, concd, and applied to a new prep. electrophoresis gel. The above process was repeated × 8. After the eighth repetition, the remaining middle fractions containing a mixture of the two isoenzymes were concd and loaded on 7.5% slab gels (35 × 18 × 0.1 cm) and electrophoresed at 250 V for 12 hr or 100 V overnight. Side lanes were then removed and stained for activity by the photochemical method [20]. These lanes were next aligned in their original positions, and horizontal bands, corresponding to SOD activity in the lanes, were excised from the gels. The proteins were removed from the gel slices by placing the gels in dialysis tubing containing 50 mM Tris buffer and electrophoresing on a horizontal gel box at 100 V for 2.5 hr. The polarity was then reversed for 2 min to remove the protein from the dialysis bag walls. Fractions containing the respective isoenzymes were pooled. Each of the pooled fractions was concd, dialysed against 50 mM K-Pi buffer, and applied to a Sephadex G-150 column. Fractions exhibiting SOD activity were pooled and concd by ultrafiltration.

MW and subunit size determinations. The MWs of the SOD isoenzymes were determined by chromatography on a Sephadex G-150 column which has been calibrated previously with the following standard proteins: bovine serum albumin (MW 67 000), ovalbumin (43 000), beef liver SOD (32 600), chymotrypsinogen A (25 000), and ribonuclease A (13 700). Subunit size was determined by SDS gel electrophoresis after heating the proteins at 100° for 10 min in the presence of 2% SDS and 5% 2-mercaptoethanol. Electrophoresis was carried out on 15% acrylamide-SDS gels as described by Laemmli [24]. Standards used were phosphorylase B (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500) and lysozyme (14 400).

Isoelectric points. The SOD isoenzymes were applied to a glass plate on which a layer of Sephadex G-75, containing ampholytes (pH 2.0–5.0), was spread. Electrofocusing (Desaga–Brinkman double chamber thin-layer electrofocusing apparatus) was carried out at 650 V for 15 hr followed by 1000 V for 3 hr. Chamber temps. were maintained at 4° by means of a circulating cooling unit. To locate the isoenzymes in the gel layer, a piece of chromatography paper was placed in contact with the plate for 1 min and then stained for protein with Coomassie blue. The pI values of the two isoenzymes were determined by measurement (with a Broadley–James flat-tipped electrode) of pH of the Sephadex layer at locations of SOD protein activity.

Metal analysis. Aliquots of the SOD isoenzymes were dialysed against 5 mM K-Pi, pH 7.8, containing 0.5 mM EDTA, and then against 5 mM K-Pi, pH 7.8. Metal analysis was performed on a Perkin–Elmer Model 305B atomic absorption photometer.

Amino acid analysis. Samples were hydrolysed *in vacuo* in 6 M HCl at 110° for 24 hr. Amino acid analysis was performed on a Beckman 120C analyser. Cysteine was determined as *S*-sulfocysteine according to Inglis and Liu [25].

Proteolytic digests. Each purified enzyme was prepared at 0.5 mg/ml in sample buffer as described by Cleveland *et al.* [26]. Samples were heated at 100° for 5 min. Incubations of each isoenzyme (10 μg) with 1 μg papain, 4 μg *Staphylococcus aureus* V-8 protease and 4 μg α-chymotrypsin were carried out at 37° for 45 min. Each sample was made up to 5% 2-mercaptoethanol and 2% SDS, and proteolysis was terminated by heating to 100° for 10 min. Controls containing comparable concns of protease alone were prepared exactly like the isoenzyme samples. Peptide fragment bands were separated by electrophoresis on a 15% acrylamide–SDS gel, and the bands were developed using silver stain [27].

Preparation of protoplasts and chloroplasts. Protoplasts and chloroplasts from fresh pea leaves were prepared by the method of Mills and Joy [28]. Isolated chloroplasts were ruptured by suspending them in 1 mM tricine (pH 7.4).

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