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Isolation and Characterization of an Iron-Containing Superoxide Dismutase From Water Lily, *Nuphar luteum*[1]

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ABSTRACT

A cyanide-insensitive superoxide dismutase (EC 1.15.1.1) was purified from leaves of the water lily *Nuphar luteum* (L.) Sibth. and Smith Subsp. *macrophyllum* (Small) Beal. The enzyme had a molecular weight of 46,000 and was composed of two equally sized subunits. Metal analysis showed the protein to contain about 1 gram atom of iron per dimer. The iron-containing superoxide dismutase was sensitive to H_2O_2 as well as to azide. Antibody to the protein did not cross-react with iron superoxide dismutase isolated from the eucaryote *Brassica* or with algal extracts.

The enzyme SOD[2] serves a protective role in all aerobic organisms by detoxifying the superoxide free radical in a dismutive reaction (10, 23): $O_2^- + O_2^- + 2\ H^+ \longrightarrow H_2O_2 + O_2$. Three metalloproteins have been found to exhibit SOD activity. The Cu-Zn enzyme is found in all eucaryotes, land plants, and fungi. It consists of two subunits and contains two atoms of Cu and two of Zn per dimer. The protein can be distinguished by its sensitivity to cyanide and H_2O_2 (10). A Mn-containing SOD has been found in bacteria, as well as in the mitochondrial matrix of plants and animals. A metal content of one to four atoms of Mn per molecule has been reported (10, 22). The Mn isozyme is resistant to cyanide and to H_2O_2 (10). The Fe-containing SOD, found predominantly in procaroytes, has a metal content of one to two atoms Fe per molecule and is cyanide-insensitive, but, unlike the Mn enzyme, it is inhibited by H_2O_2 (10, 22).

SODs have been examined in photosynthetic organisms (2, 3, 19). The Cu-Zn isozyme is absent in procaryotic and most eucaryotic algae. However, the Mn and/or Fe enzymes are present (1, 2, 3). Land plants contain large amounts of the Cu-Zn isozyme plus an additional cyanide-resistant SOD, which was assumed to be the Mn protein (3, 19). We have recently reported on a survey of plant families for isozymes of SOD (6). Of 43 families of plants examined, three families—Ginkgoaceae, Cruciferae, and Nymphaceae—were found to contain the Fe enzyme.

In this paper, we report the results of our studies on the purification and characterization of Fe SOD from the water lily *Nuphar luteum*, a member of the Nymphaceae.

MATERIALS AND METHODS

Cyt *c* (type III), xanthine oxidase (grade I), and nitroblue tetrazolium (type III) were obtained from Sigma Chemical Company. Iron SOD was purified from *Brassica campestris*, as previously reported (27), and Cu/Zn SOD was purified from wheat germ (5). *Nuphar luteum* (L.) Sibth. and Smith Subsp. *macrophyllum* (Small) Beal was collected and identified by Dr. Sidney McDaniel, Mississippi State University. Other vascular plants were grown locally. *Chlorella* sp. (UM 10 isolate), *Scenedesmus* sp. (UTEX 1588), and *Nostoc muscorum* (UTEX B1037) were generously supplied by Dr. Bailey Ward, University of Mississippi. Other algae were obtained from the R. C. Starr Collection, University of Texas.

SOD activity was assayed by the Cyt *c* reduction method (23), using a Perkin-Elmer Model 552 spectrophotometer. One unit of activity was defined as the amount of enzyme required to inhibit Cyt *c* reduction by 50%. Crude extracts were dialyzed extensively against 50 mM K-phosphate (pH 7.8) to deplete the extract of low mol wt reductants before assay of activity.

Analytical gel electrophoresis was performed on 7.5% acrylamide gels (8), with SOD activity localized using the photochemical method (4). Protein concentrations of extracts were determined by the method of Lowry *et al.* (17), while the concentration of purified protein was determined according to Murphy and Kies (25).

Mol wt was determined by gel exclusion on a Sephadex G-150 column. Subunit mol wt was determined by SDS gel electrophoresis, according to the method of Laemmli (16). Mol wt standards used for both mol wt and subunit mol wt determinations were: ribonuclease A, 13,700; chymotrypsinogen A, 25,000; ovalbumin, 43,000; and BSA, 67,000. Iron, copper, zinc, and manganese were assayed by atomic absorption spectrometry, using a Perkin-Elmer Model 305B atomic absorption photometer.

Purified enzyme was prepared for amino-acid analysis by extensive dialysis against H_2O, followed by hydrolysis *in vacuo* in 6 M HCl at 110°C for 24 h, and then analyzed by a Beckman Model 120C amino-acid analyzer. Cysteine was determined as *S*-sulfocysteine after hydrolysis with boiling HCl, followed by reduction with DTT and sodium tetrathionate (13). Tryptophan was measured spectrophotometrically in 6 M guanidine hydrochloride, according to the method of Edelhoch (9).

The isoelectric point of the purified protein was determined on a Desaga-Brinkman double chamber thin layer electrofocusing apparatus. The enzyme was applied to a thin glass plate covered with ampholytes on a matrix of Sephadex G-75. After electrophoresis at 900 v for 3 h, a paper impression of the plate was made and stained for protein with Coomassie blue. The corresponding pH values were determined directly off the glass plate by insertion of a glass electrode (Broadley-James, Santa Ana, CA) into the gel matrix.

Antisera to purified *Nuphar* Fe SOD and wheat germ Cu-Zn SOD were produced by immunization of New Zealand white rabbits with a 2-ml emulsion consisting of 1 ml purified enzyme in 50 mM K-phosphate emulsified with 1 ml of Freund's Complete Adjuvant. One-half of the emulsion was injected into the footpads

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[2] Abbreviations: SOD, superoxide dismutase; Fe SOD, iron-containing superoxide dismutase; Cu-Zn SOD, copper-zinc-containing superoxide dismutase.

Table I. *Purification of Fe SOD from N. luteum*

Purification Step	Volume	Protein Concentration	Total Protein	Total Activity	Specific Activity	Yield	Purification
	ml	*mg/ml*	*mg*	*unit*	*unit/mg*	%	*-fold*
Crude extract after centrifugation	615	3.2[a]	1,968	51,718	26	100	1
35 to 65% $(NH_4)_2SO_4$ dialyzed	112	2.0[a]	224	46,368	207	89.7	7.9
DEAE-cellulose	166	0.72[a]	119	34,362	289	66.4	11.0
CM-cellulose	211	0.49[a]	103	32,705	318	63.2	12.2
G-75	11.5	1.80[a]	20.7	12,777	617	24.7	23.7
G-75	3.5	1.04[b]	3.6	7,248	2,013	14.0	77.4

[a] Lowry *et al.* (17).
[b] Murphy and Kies (25).

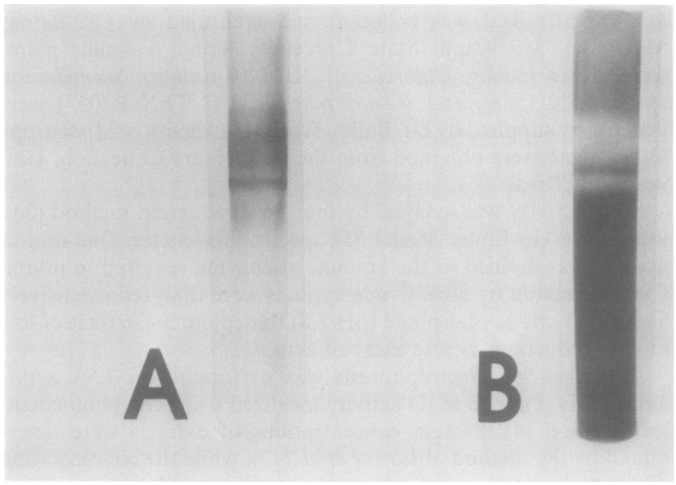

FIG. 1. Polyacrylamide gels of purified Fe SOD from *N. luteum*. A, Stained for protein with Coomassie blue (28 μg); B, stained for SOD activity using nitroblue tetrazolium (50 ng).

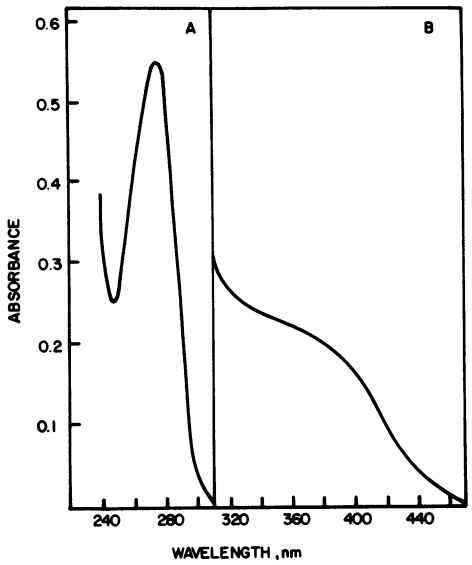

FIG. 2. Absorption spectra of Fe SOD from *N. luteum*. Protein concentrations were 0.289 mg/ml (A) and 2.89 mg/ml (B).

of the rabbit and the remainder at multiple intramuscular sites. Subsequent injections at weekly intervals were entirely intramuscular with *Nuphar* Fe SOD emulsified in Fruend's Incomplete Adjuvant. After a satisfactory titer was reached (3–4 weeks), the rabbits were sacrificed by cardiac puncture. The immunoglobulin was partially purified from the serum by precipitation with 33% $(NH_4)_2SO_4$ three times followed by dialysis into a buffer containing 0.025 M sodium tetraborate, 0.1 M boric acid, and 0.06 M NaCl, adjusted to pH 8.4.

Cross-reactivity of the antibodies with various purified proteins and cell-free extracts was tested by the Ouchterlony technique (26) of double immunodiffusion followed by staining with Coomassie Blue.

RESULTS

Purification of Enzyme. The Fe SOD from *N. luteu* was purified using a modification of the procedure reported for *Brassica* Fe SOD (27). About 250 g of leaves were homogenized in a Waring Blendor in 500 ml of grinding media containing 50 mM K-phosphate (pH 6.8), 0.3% Triton X-100, and 1 mM DTT. Cell debris was removed by filtration through eight layers of cheesecloth, followed by centrifugation at 10,000*g* for 10 min. The supernatant was brought to 30% saturation by addition of solid $(NH_4)_2SO_4$ and stirred for 1 h at room temperature. The resulting precipitate was removed by centrifugation at 10,000*g* for 10 min, and the supernatant was brought to 65% saturation by additional solid $(NH_4)_2SO_4$. After stirring for 1 h at room temperature, the precipitate was collected by centrifugation at 10,000*g* for 10 min, resuspended in 10 mM phosphate buffer (pH 6.8), and dialyzed for 24 h against 10 mM phosphate buffer (pH 6.8), with frequent changes of dialysis buffer.

The dialysate was clarified by centrifugation and then applied to a 37- × 2-cm DEAE column (Whatman DE-52) at 4°C equilibrated with 10 mM phosphate buffer (pH 6.8). The enzyme was eluted with a linear gradient of K-phosphate buffer (pH 6.8) (10–100 mM), and fractions with enzymic activity were pooled and dialyzed against 10 mM K-phosphate buffer (pH 5.8). The protein was then passed through a carboxymethyl cellulose (CM-32, Whatman) column (21.5 × 1.5 cm) equilibrated with 10 mM K-phosphate buffer (pH 5.8). SOD activity was not absorbed onto the column and was washed through with 50 ml of 10 mM K-phosphate buffer (pH 5.8). The eluate was concentrated to a volume of 2 ml by ultrafiltration and further purified by gel filtration on a Sephadex G-75 column (77 × 1.5 cm). Fractions containing the greatest amount of SOD activity were pooled and rechromatographed on a Sephadex G-75 column (77 × 1.5 cm). Table I summarizes the results of the purification. It can be estimated that 250 g of *Nuphar* contained approximately 25 mg of CN^--insensitive SOD. A yield of about 15% was recorded, and the enzyme had a specific activity of about 2,000. This value is lower than that for Cu/Zn SODs but is in agreement with values reported (15, 27, 30) in the literature for other Fe SODs. Unlike Fe SOD isolated and purified from *Brassica* (27), the *Nuphar*

protein, upon purification, was relatively stable at 4°C.

Figure 1 is a photograph of polyacrylamide gels electrophoresed and stained for activity and protein. Several bands of isozymic activity, corresponding to the protein are visible.

Mol Wt. The mol wt of the purified protein was determined by chromatography on a Sephadex G-150 column. By comparison with markers of known mol wt, the data obtained yielded a value of 46,200. Subunit size, determined by SDS-gel electrophoresis in the presence of β-mercaptoethanol (16), revealed a single band corresponding to a value of 23,000. It would appear, therefore, that the protein is composed of two equally sized subunits.

Absorption Spectra. Figure 2 shows the visible and UV absorption spectra of the purified protein. The enzyme exhibited a peak at 278 nm, typical of most proteins. Shoulders at 290 and 260 nm were also observed. A weak, broad absorption spectrum was observed in the range of 450 nm to 300 nm. Both UV and visible absorption spectra were similar to those of Fe SODs reported previously in the literature (14, 18, 24, 27, 30). The $E^{1\%}_{1cm}$ at 278 nm was 18.3, corresponding to a molar extinction coefficient of 8.4×10^4 M^{-1} cm^{-1}.

Metal Analysis. The enzyme was dialyzed extensively against 5 mM K-phosphate (pH 7.8), containing 0.5 mM EDTA, followed by buffer lacking EDTA and then subjected to atomic absorption analysis. A value of 0.75 ± 0.2 atoms of iron per dimer was obtained. Less than 0.3 atoms of zinc per dimer was found, while manganese was observed to be present in a concentration of less than 0.07 atoms per dimer. Fe SODs with contaminating amounts of zinc have been reported previously (15).

Isoelectric Point and Amino Acid Analysis. An analysis of protein stains after isoelectric focusing showed one spot at a pH value of 4.8. This value is in the range of values reported for other Fe SODs (18, 19, 27).

Table II shows the amino acid composition of Fe SOD purified from *N. luteum*. For comparison, the amino acid compositions of Fe SOD isolated from *Plectonema boryanum* (24), *B. campestris* (27), *Escherichia coli* (30), *Spirulina platensis* (18), and *Euglena gracilis* (14) are included. Based upon comparative amino acid content, there do not appear to be any major differences among the amino acid content of Fe SOD isolated from *Nuphar* and those iron enzymes reported in the literature. However, *Nuphar* appears to contain more serine, glycine, and methionine than do the other enzymes listed and less threonine, alanine, and valine on average.

As a further test of analogy between enzymes, a simple statistical method of comparing the sum of the squares of the difference between amino acids has been devised (20). The value obtained, SΔQ, reveals relative sequence homology between proteins. Table III gives the SΔQ values computed for 18 amino acids of eight different Fe SODs. The lower the number, the smaller the difference in amino acid content and, hence, the greater the relatedness. Based upon the values for SΔQ, *Nuphar* Fe SOD is more closely related to that of the land plant *Brassica* than it is to any other iron enzyme. The *Brassica* enzyme is related to *Plectonema* and *Escherichia*. Neither of the two proteins appear to be related to the two *Euglena* Fe SOD isozymes. Therefore, the amino acid sequences of Fe SOD from *Nuphar* and *Brassica* appear to be related to each other but not to any of the Fe SOD isozymes from *Euglena* (14).

Inhibitors of Nuphar Fe SOD. Table IV shows the inhibitory effects of several compounds on *Nuphar* Fe SOD. SOD was incubated for 15 min with a particular compound in 50 mM K-phosphate (pH 7.8) and 0.1 mM EDTA. An aliquot of 10 μl was then taken to determine SOD activity. Controls were routinely performed to ensure that the inhibitory compound added along with the enzyme did not interfere with the SOD assay. Like *Euglena* Fe SOD, as reported by Kanematsu and Asada (14), *Nuphar* Fe SOD was inactivated completely by incubation with 6 M guanidine-HCl. The addition of KCN, as expected, did not yield any inhibition. Hydrogen peroxide, at a concentration of 2 mM, led to about a 60% inhibition after 15 min of incubation, in agreement with previous studies (7). The addition of 10 mM azide caused about a 40% inhibition in *Nuphar* Fe SOD. This is in agreement with the findings for *Euglena* Fe SOD which was observed to be inhibited only 50% by azide (14).

Table II. *Comparative Amino Acid Composition of Iron-Containing Superoxide Dismutases*

Amino Acid	Residues[a]						
	N. luteum	*B. campestris* (27)[d]	*E. gracilis* I (14)	*E. gracilis* II (14)	*P. boryanum* (24)	*E. coli* (30)	*S. platensis* (18)
	per mole subunit						
Lysine	12	11	4	4	12	10	10
Histidine	5	4	7	6	5	6	6
Arginine	6	5	10	9	2	4	0
Aspartic acid	23	18	19	20	23	22	26
Threonine	6	9	11	11	10	13	10
Serine	15	11	5	5	9	10	12
Glutamic acid	19	16	22	22	16	16	17
Proline	9	10	13	13	10	9	8
Glycine	22	16	20	20	15	16	13
Alanine	20	17	26	27	24	26	25
Cysteine[b]	2	2	2	2	1	1	1
Valine	5	11	11	11	11	11	9
Methionine	5	1	3	4	2	0	3
Isoleucine	4	6	6	6	3	8	7
Leucine	16	14	16	17	17	15	16
Tyrosine	6	6	7	7	5	6	5
Phenylalanine	9	7	10	11	13	10	14
Tryptophan[c]	7	4	8	7	6	3	3

[a] Values given to the nearest integer.

[b] Determined as *S*-sulfocysteine after reduction with dithiothreitol and oxidation with sodium tetrathionate (13).

[c] Determined spectrophotometrically in 6 M guanidine HCl (9).

[d] Numbers in parentheses refer to references in "Literature Cited."

Table III. *Comparison of SΔQ Values of Fe SOD*

Values for SΔQ were calculated from the amino acid compositions of Fe SODs reported in this paper and in previous literature. The following equation was used (20):

$$S\Delta Q = \sum_j (x_{ij} - X_{kj})^2$$

where the subscripts $_i$ and $_k$ indicate the enzymes compared, and X_j is the amino acid content, in mole percent content of a given amino acid of type j. In calculating SΔQ values, 18 amino acids were used. Numbers in parenthesis refer to literature from which data was taken.

	(a)	(b)	(c)	(d)	(e)	(f)
(a) *Nuphar*						
(b) *Brassica* (27)	39					
(c) *Plectonema* (24)	59	36				
(d) *Escherichia* (30)	74	30	22			
(e) *Spirulina* (18)	74	60	20	26		
(f) *Euglena* Fe SOD I (14)	88	59	71	58	105	
(g) *Euglena* Fe SOD II (14)	85	59	61	51	91	2

Table IV. *Effect of Various Compounds on Nuphar Fe SOD Activity*

SOD was incubated at 25°C for 15 min in a buffer containing 50 mM K-phosphate (pH 7.8), 0.1 mM EDTA, and the compound indicated.

Compound	Inhibition
	%
NaN_3, 10 mM	41
Guanidine HCl, 6 M	100
H_2O_2, 2 mM	64
KCN, 1 mM	0

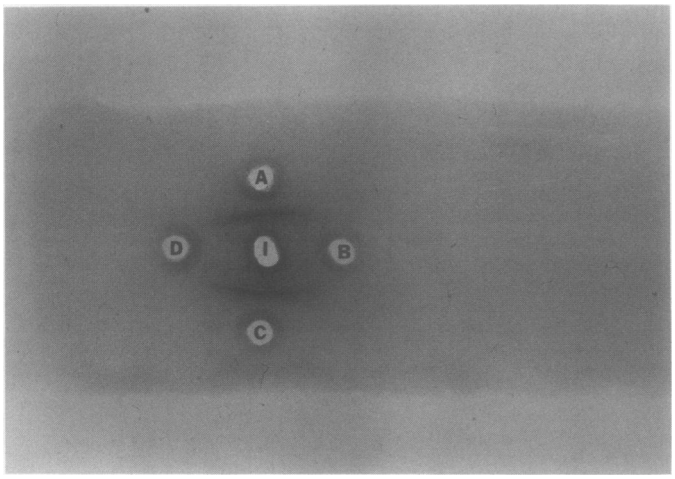

FIG. 3. Ouchterlony double immunodiffusion of *Nuphar* SOD antibody and antigens. Center well, Partially purified immunoglobulin to *Nuphar* iron SOD (~320 μg of protein). Other wells contain: A, Purified Fe SOD, from *Nuphar* (3.5 μg); B, purified Fe SOD from *Brassica*, (4.6 μg); C, crude extract from *Nuphar* leaves, (4 units SOD activity); and D, crude extract from *E. gracilis* (6 units SOD activity).

Cross-Reactivities of Antigens Prepared to SODs. Antibody to *Nuphar* Fe SOD was prepared from the serum of rabbits given injections of purified protein. Figure 3 shows the results of a typical Ouchterlony immunodiffusion experiment, in which antisera to *Nuphar* Fe SOD was placed in the center well, and purified *Nuphar* Fe SOD, as well as crude extracts from *Nuphar, Brassica,* and *Euglena*, were placed in the side wells. As can be seen, a precipitin line appeared between antisera and purified *Nuphar* Fe SOD, as well as between antisera and *Nuphar* crude extracts. *Brassica* and *Euglena* crude extracts did not cross-react with *Nuphar* antibody.

Table V is a tabulation of some purified SODs and crude extracts which were examined for cross-reactivity to partially purified immunoglobulin to *Nuphar* Fe SOD. Only purified Fe SOD or crude extract from *Nuphar* cross-reacted with *Nuphar* Fe SOD antibody. None of the other plant or algal crude extracts showed any reaction. We conclude, therefore, that *Nuphar* Fe SOD does not contain antigenic sites in common with other Fe SODs. Table V also shows the results of cross-reactivity studies in which antibody prepared to wheat germ Cu-Zn SOD was tested for cross-reactivity against the purified iron enzyme, as well as against crude extracts of plant and algae. As expected, *Nuphar* Fe SOD did not cross-react with antisera to wheat germ Cu/Zn SOD. The wheat germ Cu-Zn antibody did show cross-reactivity against crude extracts from *Brassica,* thereby indicating a common antigenic determinant of Cu-Zn SODs from mustard and wheat germ. Asada *et al.* (1, 3) have previously shown that Cu-Zn SODs from land plants, including mosses and ferns, have antigens in common with spinach enzyme but not with Cu-Zn SOD isolated from bovine erythrocytes. Our finding of common determinants for *Brassica* and wheat germ agree with these earlier findings.

DISCUSSION

Water lily Fe SOD, reported on in this paper, is similar in mol wt, subunit size, absorption spectra, and metal analysis to Fe SODs isolated from other sources (14, 18, 24, 27, 30). However, antibody prepared to water lily Fe SOD does not cross-react with purified Fe SODs or crude extracts from procaryotic or eucaryotic organisms known to contain Fe SOD. This implies an immunological uniqueness to the iron enzyme from water lily. Insight into sequence homology can be gained by analysis of least squares differences between amino acids of the iron isozyme reported in this paper and those SODs reported on in the literature (Table III). Fe SOD from water lily appears more closely related to Fe SOD from the eucaryotic land plant *B. campestris* than to many

Table V. *Antigenic Cross-Reactivity of Nuphar Fe SOD Antibody and Wheat Germ Cu-Zn SOD Antibody to Plant Proteins and Cell-Free Extracts*

Ouchterlony double immunodiffusion assay was used to test cross-reactivity. Immunoglobulin (~320μg) was placed in the center well and purified proteins or cell-free extracts in the outer wells. Formation of a precipitin line between a center well and side well indicates antigenic cross-reactivity (+).

Species	Antibody	
	Nuphar Fe SOD	Wheat germ Cu-Zn SOD
Purified Proteins		
Nuphar Fe SOD	+	−
Brassica Fe SOD	−	−
Wheat germ Cu-Zn SOD	−	+
Cell-Free Extracts		
N. luteum	+	−
Nymphia odorata	−	−
B. campestris	−	+
Gingko biloba	−	−
Wheat germ	−	+
E. coli	−	−
E. gracilis	−	−
Navicula pelliculosa	−	−
P. boryanum	−	−
Scenedesmus sp.	−	−
Nostoc muscorum	−	−
Chlorella sp.	−	−

of the blue-green and green algal Fe SODs, including those from *Euglena.* Clearly, work is needed to elucidate amino-acid sequences of the higher land plant Fe SODs.

We have previously reported that Fe SOD is a chloroplast stromal enzyme in *Brassica* leaves (28), yet absent in *Brassica* mitochondria (29). We intend to investigate further the subcellular localization of Fe SOD in water lily by using fluorescently labeled antibody.

Water lily, *N. luteum,* is a unique plant in terms of SODs. The plant is apparently devoid of Cu-Zn SOD but contains the iron isozyme (6). Several questions are raised by this anomaly. First, why is there an apparent lack of the Cu-Zn SOD? A possible explanation is that, in a relatively anaerobic pond bottom in which this organism is rooted, most copper would be found in the form of insoluble copper sulfide. As a result, copper might not be as readily absorbed and utilized as it is in land plants. A metal analysis of *Nuphar,* however, shows that its total copper content is not significantly different from that of land plants (M. L. Salin and S. M. Bridges, unpublished). Second, assuming an absence of Cu-Zn SOD in *Nuphar* and a necessity for dismutase activity (10), why has this plant expressed an iron enzyme as opposed to enhancing production of an already present manganese-containing SOD? Manganese-containing SODs are found in procaryotic and eucaryotic organisms (2, 10, 22) and have been shown to be dramatically responsive to environmental changes (11, 12). In contrast, Fe SOD was not responsive to enhancement of synthesis by environmental pressures (11, 12). A possible explanation for the occurance of Fe SOD might be accounted for by an independent occurance of gene transfer from a bacteria or algae to *Nuphar* or its predecessor. A case of gene transfer between a host fish and its symbiotic bacteria has recently been reported (21). Finally, we might wonder whether the genes for the Cu-Zn SOD are present in *Nuphar,* yet not expressed. Alternately, we might ask if genes for Fe SOD in other eucaryotes may be present, yet not expressed. Answers to many of these questions might be found by searching for potential genes coding for Fe SOD and Cu-Zn SOD in organisms where they are not expressed.

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