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Purification, Properties, and Distribution of Ascorbate Peroxidase in Legume Root Nodules[1]

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ABSTRACT

All aerobic biological systems, including N_2-fixing root nodules, are subject to O_2 toxicity that results from the formation of reactive intermediates such as H_2O_2 and free radicals of O_2. H_2O_2 may be removed from root nodules in a series of enzymic reactions involving ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase. We confirm here the presence of these enzymes in root nodules from nine species of legumes and from *Alnus rubra*. Ascorbate peroxidase from soybean nodules was purified to near homogeneity. This enzyme was found to be a hemeprotein with a molecular weight of 30,000 as determined by sodium dodecyl sulfate gel electrophoresis. KCN, NaN_3, CO, and C_2H_2 were potent inhibitors of activity. Nonphysiological reductants such as guaiacol, *o*-dianisidine, and pyrogallol functioned as substrates for the enzyme. No activity was detected with NAD(P)H, reduced glutathione, or urate. Ascorbate peroxidation did not follow Michaelis-Menten kinetics. The substrate concentration which resulted in a reaction rate of ½ V_{max} was 70 micromolar for ascorbate and 3 micromolar for H_2O_2. The high affinity of ascorbate peroxidase for H_2O_2 indicates that this enzyme, rather than catalase, is responsible for most H_2O_2 removal outside of peroxisomes in root nodules.

Oxygen plays a critical but incompletely understood role in the metabolism of N_2-fixing root nodules. Nodule function requires a delicate balance between respiratory O_2 demand and the hazards of O_2 toxicity. These hazards arise from the O_2 sensitivity of nitrogenase and from the production of reactive intermediates such as H_2O_2 and superoxide free radicals ($O_2^{\cdot-}$). A key defense against O_2 toxicity is superoxide dismutase, an enzyme which has been detected in many aerobic organisms, including nodule host cells (21) and bacteroids (6). The superoxide dismutase reaction produces H_2O_2 which can also be damaging and must be removed through the action of catalase or peroxidase. Although catalase is present in soybean nodules (11, 21), most peroxide removal probably occurs through a coupled series of oxidation-reduction reactions involving ASC[3] and glutathione (5, Fig. 1). The enzymes involved are ASC peroxidase, DHA reductase, and glutathione reductase. Similar reactions occur in chloroplasts (8, 17) and cyanobacteria (27, 29). The glutathione content of nodules and the activity of the first two enzymes in this system are positively correlated with nitrogenase activity and leghemoglobin content during the early stages of nodule development (5). ASC peroxidase and DHA reductase activities are not present in isolated bacteroids, thus implying these enzymes are of plant origin (5).

All three of the enzymes in this system in chloroplasts have been purified and characterized (1, 12, 14), but very little information is available on the corresponding enzymes in root nodules. A peroxidase from soybean root nodules was characterized by Puppo *et al.* (20), but the effectiveness of ASC as an electron donor was not investigated. Legume root nodules reportedly contain numerous peroxidase isozymes (16), but this may be misleading because leghemoglobins are capable of very high pseudo-peroxidase activity in the presence of appropriate reductants (20).

We present evidence in this report of the presence of ASC peroxidase and associated enzymes in nine species of legumes and in red alder (*Alnus rubra*). ASC peroxidase from soybean root nodules was purified to near homogeneity and characterized with regards to substrate specificity, substrate affinity, absorption spectra, and effects of inhibitors. The enzyme is compared with other peroxidases, especially the ASC peroxidase from spinach chloroplasts.

MATERIALS AND METHODS

Growth of Plants. Plants were grown in a greenhouse in pots of perlite as previously described (5). The following plant species and inoculants were used: soybean (*Glycine max* (L.) Merr. cv Williams), *Bradyrhizobium japonicum* 122 DES (23); cowpea (*Vigna unguiculata* (L.) Walp. cv California blackeye bean), *Bradyrhizobium* spp. cowpea miscellany type OT No. EL8; faba bean (*Vicia faba* L. var equina), *Rhizobium leguminosarum* Q; peanut (*Arachis hypogea* L. cv Florigiant), *Bradyrhizobium* spp. cowpea miscellany type OT No. EL8; lupine (*Lupinus albus* L. var ultra), *R. lupini* H-6; pea (*Pisum sativum* L. cvs Alaska and Austrian winter), *R. leguminosarum*; alfalfa (*Medicago sativa* L. cv Anchor), *R. meliloti* A-8; subterranean clover (*Trifolium subterraneum* L. cv Mt. Barker), *R. trifolii*; vetch (*Vicia sativa* L. cv Nova II), *R. leguminosarum*. With the exception of *B. japonicum* 122 DES, all inoculants were obtained from Nitragin Co. (Milwaukee, WI). Each of the Nitragin inoculants consisted of a commercial mixture of several strains.

Enzyme Assays. ASC peroxidase was measured by the decrease in A at 265 nm due to ascorbate oxidation. The details of this procedure along with the assays for DHA reductase and glutathione reductase have been previously described (5). Activity values for ASC peroxidation are based on linear rates observed after an initial lag phase. Information regarding assay concentration and extinction coefficients of other reductants used in peroxidase assays is as follows: guaiacol (10 mM, ϵ_{470} = 26.6 $mM^{-1} \cdot cm^{-1}$, Ref. 17); pyrogallol (18 mM, $\epsilon_{430} = 2.47\ mM^{-1} \cdot cm^{-1}$,

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[3] Abbreviations: ASC, ascorbate; ABTS, 2,2′-azino-di-(3-ethyl-benzothiazoline-[6]-sulfonic acid); DHA, dehydroascorbate; PVPP, polyvinylpolypyrrolidone.

Ref. 17); 2,2′-azino-di-(3-ethyl-benzothiazoline-[6]-sulfonic acid)(ABTS, 1.7 mM, ϵ_{405} = 18.6 $mM^{-1} \cdot cm^{-1}$, Ref. 22); *o*-dianisidine (0.34 mM, ϵ_{460} = 11.3 $mM^{-1} \cdot cm^{-1}$, Ref. 25, 30); uric acid (75 μM, ϵ_{285} = 10·5 $mM^{-1} \cdot cm^{-1}$, Ref. 19); reduced glutathione (GSH, 1 mM, ϵ_{340} (NADPH) = 6.2 $mM^{-1} \cdot cm^{-1}$, Ref. 17); NAD(P)H (0.15 mM, ϵ_{340} = 6.2 $mM^{-1} \cdot cm^{-1}$, Ref 17). All activity units are defined as μmoles of product/min unless otherwise indicated.

Nodules of red alder (*Alnus rubra* Bong.) and western wax myrtle (*Myrica californica* C. & S.) were collected from the field in western Oregon on May 26, transported to the laboratory at 4°C, and stored at −20°C for later analysis.

Extraction Procedures. Mature nodules of all legume species were harvested and ground in liquid N_2 by mortar and pestle. After the N_2 had boiled away, the dry, frozen powder was added immediately to 4 volumes of cold buffer consisting of 50 mM KH_2PO_4/K_2HPO_4 and 0.1 mM Na_2EDTA at pH 7.0. The buffer also contained insoluble PVPP (25% of initial nodule fresh weight). The extract was filtered through 100-mesh bolting cloth and then centrifuged at 8000*g* for 20 min to remove cell debris and bacteroids. The supernatant will be referred to in this report as the crude extract. For experiments involving the survey of different legumes, the supernatant was desalted by passage through a 1.5 × 5 cm column of Sephadex G-25.

Extracts of *Alnus* and *Myrica* nodules were prepared under a stream of N_2 gas in the presence of 5% soluble PVPP (mol wt 40,000) and 0.2% $Na_2S_2O_4$ (28).

Enzyme Purification. Crude extract of 200 *g* of soybean nodules was prepared as described above. All steps in the purification were carried out near 4°C. The initial purification step involved precipitation with $(NH_4)_2SO_4$ at 45% of saturation. The precipitate was collected by centrifugation at 17,000*g* for 20 min and the resulting pellet was discarded. The concentration of $(NH_4)_2SO_4$ in the supernatant was brought to 70% of saturation. The pellet resulting from centrifugation was resuspended in 40 ml of 25 mM Tris at pH 7.8. This suspension was dialyzed overnight against 1 L of the same buffer. Further purification was achieved by ion exchange chromatography in a 2.5 × 23 cm column of DEAE cellulose (DE-52, Whatman Inc., Clifton, NJ) equilibrated with the same buffer. To avoid overloading, the dialysate was divided in half for chromatography in two separate, identical columns. After the sample was loaded, each column was rinsed with 100 ml of 80 mM KCl (in buffer) followed by a linear salt gradient resulting from mixing of 200 ml each of 80 and 300 mM KCl (in buffer). The flow rate was 56 ml/h and fractions of 6.63 ml were collected.

The salt concentration in each fraction was determined with a Markson model 4503 conductivity meter. The fractions with highest ASC peroxidase activity were combined and concentrated by ultrafiltration with a Diaflo YM-30 filter (Amicon, Danvers, MA) with a mol wt exclusion limit of 30,000. The sample was rinsed with 50 mM KH_2PO_4/K_2HPO_4 at pH 7.0 and reduced to a volume of 1.3 ml. The retentate was further purified by gel filtration on a 1.5 × 39 cm column of Sephacryl S-300 (Sigma) which was equilibrated with the same phosphate buffer. The buffer flow rate was 9.6 ml/h and fractions of 1.50 ml were collected. The column was standardized for mol wt determinations with chymotrypsinogen (mol wt 17,000), ovalbumin (45,000), BSA (67,000), γ-globulin (160,000), and catalase (210,000). Fractions with ASC peroxidase activity were concentrated by ultrafiltration as before and rinsed with 1 mM KH_2PO_4/K_2HPO_4 at pH 6.8. The retentate was applied to a 1.8 × 5.9 cm column of hydroxyapatite crystals in a cross-linked agarose network (HA-Ultrogel, LKB, Pleasant Hill, CA). The column was rinsed with 50 ml of 1 mM KH_2PO_4/K_2HPO_4 at pH 6.8 followed by a linear gradient resulting from mixing of 100 ml each of 1 mM and 100 mM KH_2PO_4/K_2HPO_4 at pH 6.8. The flow rate was 16 ml/h and fractions of 4.31 ml were collected. Fractions with ASC peroxidase activity were pooled and concentrated by ultrafiltration to a final volume of 2.28 ml. Protein concentration at each purification step was determined by Coomassie blue binding (3) with BSA as the standard.

Electrophoresis. SDS-PAGE was performed as described by Laemmli (15). Native PAGE was performed similarly except SDS was omitted. Gel dimensions were 140 × 90 mm and 1.5 mm thick. All gels consisted of a 5 to 20% acrylamide gradient and were electrophoresed at 30 mamp of constant current for about 3.5 h. Proteins were stained either with Coomassie blue R_{250} (10) or with silver reagent (BioRad, Richmond, CA). ASC peroxidase activity in native gels was determined by cutting the gels into 2 mm sections and grinding each section in a Ten Broeck tissue homogenizer with 1 ml of 50 mM KH_2PO_4/K_2HPO_4 at pH 7.0. After centrifugation at 12,000*g* for 15 min, the supernatant was analyzed for ASC peroxidase activity by the spectrophotometric procedure described earlier. Alternatively, activity was determined directly in the intact gel by staining with 4-chloro-1-naphthol (7).

RESULTS

Distribution. The three enzymes involved with the peroxide-scavenging cycle of Figure 1 were detected in nodule extracts from all nine species of legumes which were examined and from *Alnus rubra* (Table I). Extracts of *Lupinus* nodules had much lower activity than extracts of other legumes. Repeated assays of *Lupinus* extracts showed that the activity was rapidly lost after extraction, thus suggesting that the initial rate may have been substantially higher.

Extracts from *Alnus* nodules had ASC peroxidase activity from 12- to 47-fold higher than legume nodule extracts (excluding *Lupinus*, Table I). No activity was detected in *Alnus* nodule extracts when the extraction was performed in air with insoluble PVPP (*i.e.* the procedure for legume nodule extractions). Activity in *Alnus* nodule extracts was detected only when soluble PVPP was included in the buffer and O_2 was excluded by thorough degassing of buffers and performing all extraction steps under a vigorous stream of N_2 gas. Similar extracts of *Myrica* nodules had no activity of any of the three enzymes involved in the peroxide scavenging cycle. However, activity in the *Alnus* extracts was destroyed by mixing with the *Myrica* extracts, suggesting that phenolic compounds or other inhibitory substances were present in the *Myrica* extracts. Consequently, the possibility of the presence of these enzymes in *Myrica* nodules cannot be excluded. The possible advantages of similar anaerobic extraction procedures for legume nodules were not investigated.

The intracellular location of ASC peroxidase has not been established. ASC peroxidase activity in crude extracts remained in the supernatant after centrifugation at 100,000*g* for 1 h. This suggests the enzyme may be located in the cytosol. However, it is not known to what extent the extraction procedure may have resulted in release of enzyme from damaged organelles.

Stability. ASC peroxidase activity in extracts of soybean nodules remained stable for several months when stored in 50 mM

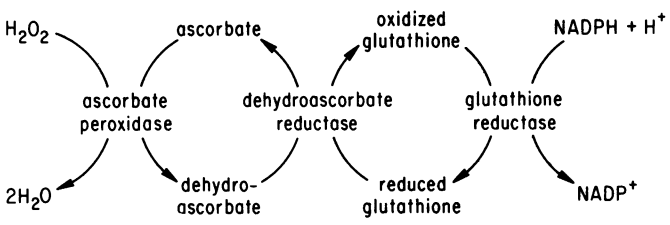

FIG. 1. Sequence of oxidation-reduction reactions involved in peroxide scavenging in nodules.

Table I. *Activities of ASC Peroxidase, DHA Reductase and Glutathione Reductase in Crude Extracts of Root Nodules of Different Plant Species*

Each value is the mean of assays of three separate extracts ± 1 SE. Activity units are based on the following spectrophotometric measurements: ASC peroxidase, nmol ASC oxidized; DHA reductase, nmol ASC produced; glutathione reductase, nmol NADPH oxidized. See Ref. 5 for details.

Plant	Specific activity		
	ASC peroxidase	DHA reductase	Glutathione reductase
	nmol/min·mg protein ± SE		
Legumes			
Glycine max	284 ± 46	92 ± 14	31 ± 6
Vigna unguiculata	258 ± 27	39 ± 6	49 ± 6
Vicia faba	241 ± 18	250 ± 27	49 ± 8
Vicia sativa	213 ± 16	51 ± 12	49 ± 2
Arachis hypogea	366 ± 25	68 ± 9	5 ± 2
Lupinus alba	10 ± 7	Trace	27 ± 4
Medicago sativa	110 ± 11	79 ± 5	28 ± 7
Trifolium subterraneum	92 ± 6	7 ± 5	20 ± 5
Pisum sativum cv Alaska	96 ± 11	54 ± 7	32 ± 4
Pisum sativum cv Austrian winter	161 ± 25	54 ± 7	32 ± 4
Actinorhizal species			
Alnus rubra	4320 ± 460	132 ± 5	18 ± 2

KH_2PO_4/K_2HPO_4 (pH = 7.0) at −20°C or −80°C. Similar extracts were stable in air at 4°C for 6 d, but 25% of the activity was lost after 15 d. ASC was not required for stability in these extracts. The activity of extracts from intact soybean nodules frozen at −80°C for 3 years was comparable to extracts from fresh nodules.

Purification. Purification of ASC peroxidase from soybean nodules resulted in a 111-fold increase in specific activity and a final yield of 2.66% (Table II). DEAE chromatography was an especially effective purification step. Most of the total protein was eluted in two major fronts containing 60 and 80 mM KCl (Fig. 2). The bright red color of these two fronts indicated a high leghemoglobin content. Most of the ASC peroxidase activity was eluted at KCl concentrations of 100 to 130 mM. Subsequent ultrafiltration was effective for concentration but had little effect on specific or total activity. Gel filtration chromatography with Sephacryl S-300 yielded one peak of activity corresponding to a molecular weight of 47,000 ± 600 (±1 SE, 3 replicates). ASC peroxidase was bound weakly to hydroxyapatite and could be rinsed thoroughly with 1 mM KH_2PO_4/K_2HPO_4 buffer. The enzyme was eluted at phosphate concentrations of 2 to 15 mM and concentrated by ultrafiltration. The retentate thus obtained is referred to as the final preparation in this report and was used for spectral analyses and substrate specificity and electrophoresis studies.

Attempts to purify ASC peroxidase by affinity chromatography with concanavalin A were unsuccessful because the enzyme would not bind under conditions which result in binding of horseradish peroxidase (4).

Electrophoresis. Native PAGE of the final preparation revealed one major and two minor bands when stained with either silver reagent or Coomassie blue (Fig. 3). The proteins forming the two minor bands were not eliminated by processing of the final preparation through a second gel filtration with S-300. ASC peroxidase activity, as determined by spectrophotometric assay of proteins eluted from gel sections, was concentrated (0.86 activity units/mm^3 gel) in the region corresponding to the major band. Activity (0.42–0.50 units/mm^3) was also present in the region of the two minor bands. Other gel regions showed traces of activity (0.15–0.27 units/mm^3) probably due to streaking along lane borders. No activity was detected from gel regions below the major band. Peroxidase activity as measured by 4-chloro-1-naphthol staining of intact gels was detected only in the region corresponding to the major band.

SDS gel electrophoresis resulted in a single prominent band with a M_r of 30,000 (Fig. 3). Heavily loaded SDS gels revealed four very minor bands (M_r values of 60,000–100,000) when stained with silver reagent.

Spectral Analyses. The spectrum of the final preparation contained a Soret band with a maximun A at 407 nm (Fig. 4).

Table II. *Purification of ASC Peroxidase from Soybean Root Nodules*

Purification Step	Total Protein	Specific Activity	Total Activity	Purification Factor	Recovery
	mg	*units/mg protein*	*units*		%
Crude extract	928	0.309	287	1	100
$(NH_4)_2SO_4$ precipitation and dialysis	345	0.595	205	1.93	71.4
DEAE chromatography	8.13	8.52	69.3	27.6	24.1
Sephacryl chromatography	3.05	14.9	45.4	48.2	15.8
Hydroxyapatite chromatography	0.223	34.2	7.63	111	2.66

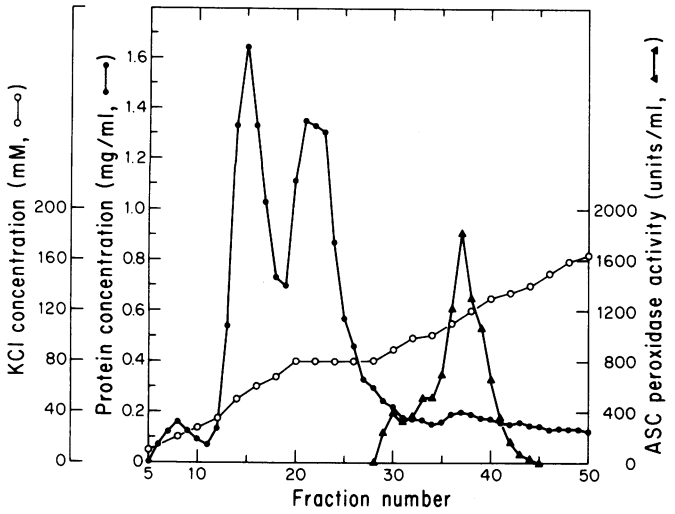

FIG. 2. Elution profile of DEAE cellulose chromatography of ASC peroxidase from soybean root nodules. Nodule extract was loaded on a 2.5 × 23 cm column of DE-52 and eluted with KCl. Protein concentration was determined by Coomassie blue binding. ASC peroxidase activity was determined spectrophotometrically. Details are presented in "Materials and Methods."

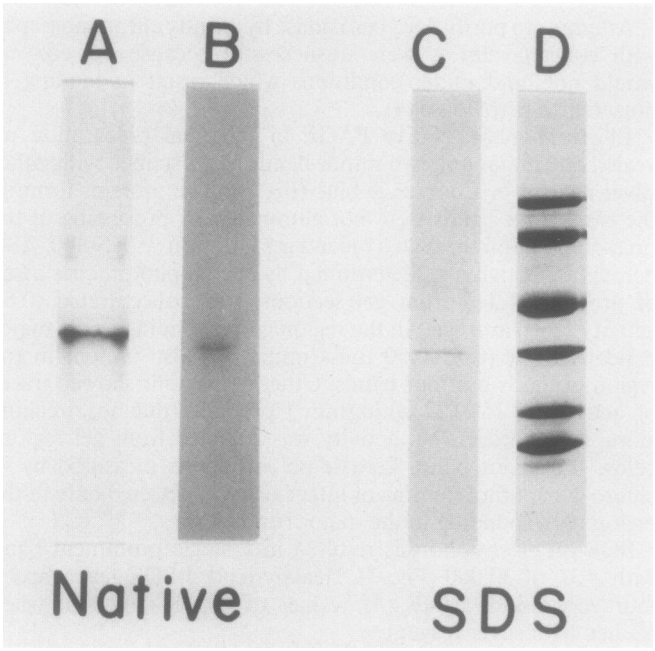

FIG. 3. PAGE of purified ASC peroxidase from soybean root nodules. Lane A, native protein stained with Coomassie blue; lane B, native protein stained for peroxidase activity with 4-chloro-1-naphthol (see "Materials and Methods"); lane C, SDS-treated protein indicating a mol wt of 30,000; lane D, SDS-treated mol wt standards as follows (top to bottom): phosphorylase b, 94,000; BSA, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; α-lactalbumin, 14,400.

Following reduction by $Na_2S_2O_4$, maximum absorbance was observed at 434 nm. A second peak was present at 554 nm. The spectrum of the reduced enzyme in the presence of 0.1 M KCN had peaks at 431, 529, and 558 nm (Fig. 5). These results are consistent with the presence of a heme moiety (13).

Inhibitor Studies. KCN, NaN_3, CO, and acetylene were strong inhibitors of ASC peroxidase activity (Table III). With the exception of acetylene, these compounds are potent inhibitors of

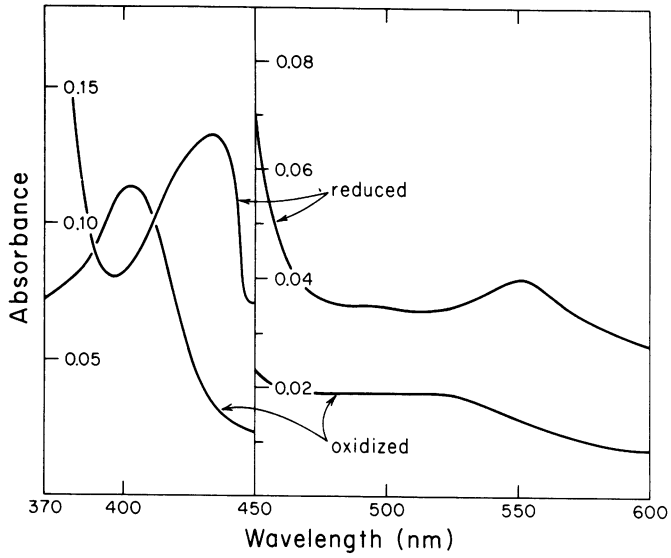

FIG. 4. Absorption spectra of the oxidized and reduced forms of ASC peroxidase. The sample cuvette contained 15 μg of purified enzyme in 0.4 ml of 50 mM K-phosphate (pH 7.0). After the oxidized spectrum was determined, the enzyme was reduced by the addition of approximately 0.3 mg of $Na_2S_2O_4$. The reference cuvette contained only buffer.

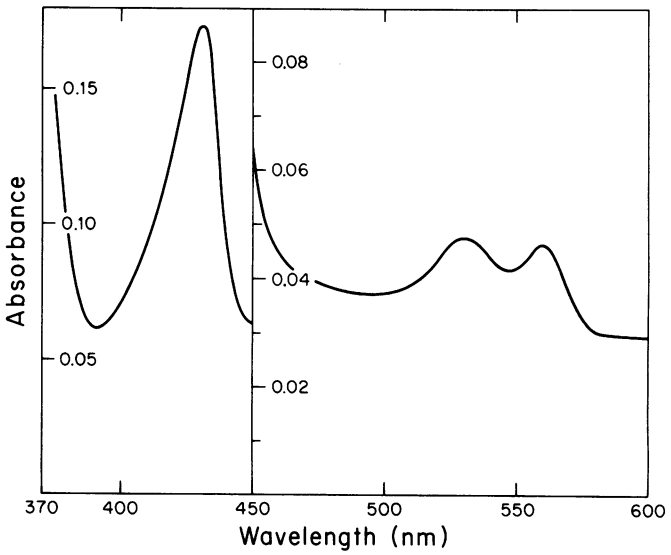

FIG. 5. Absorption spectrum of the dithionite-reduced form of ASC peroxidase in the presence of 0.1 M KCN. Other details are described in the legend of Figure 4.

hemeproteins (26). Although this inhibition may not be absolutely specific for heme, it suggests that heme may be present.

Substrate Specificity. The reaction rates with the artificial reductants guaiacol and *o*-dianisidine were approximately equal to the rate with ASC (Table IV). The rate with pyrogallol, another common nonphysiological reductant, was 38-fold higher than the rate with ASC. ABTS was only marginally effective. No activity could be detected with other potential natural (physiological) reductants, such as NAD(P)H, GSH, and urate.

An important distinction should be emphasized concerning these reductants. Leghemoglobin exhibits high pseudoperoxidase activity with artificial reductants such as *o*-dianisidine (data not presented) or guaiacol (20). No peroxidase activity was observed in this study in DEAE chromatography fractions containing leghemoglobin when ASC was used as the reductant. Therefore, ASC peroxidase activity can be measured in crude extracts

Table III. *Effect of Inhibitors on the Activity of Purified ASC Peroxidase*

Purified ASC peroxidase was preincubated 3 min with inhibitors at the concentrations indicated. CO and acetylene treatments consisted of bubbling with pure gas for 3 min. Activity was measured spectrophotometrically with ASC as the reductant as described in "Materials and Methods" with additions to maintain the inhibitor concentrations at the same level present during preincubation. Each assay consisted of a mixture containing 185 ng of purified peroxidase in a total volume of 1.60 ml. Each value is the mean of three replicates ± 1 SE.

Assay Conditions	Specific Activity
	units/mg protein ± SE
No inhibitors	34.0 ± 0.4
0.5 mM KCN	18.8 ± 0.2
1.0 mM KCN	10.1 ± 1.8
0.5 mM NaN_3	10.0 ± 0.3
1.0 mM NaN_3	5.4 ± 0.9
CO	8.7 ± 2.6
Acetylene	0.5 ± 0.3

Table IV. *Effect of Different Reductants on the Specific Activity of ASC Peroxidase Purified from Soybean Root Nodules*

All assays were based on spectrophotometric measurements as described in "Materials and Methods." Each assay consisted of a mixture containing 185 ng of purified peroxidase in a total volume of 1.60 ml. Each value is the mean of three replicates ± 1 SE.

Reductant	Specific Activity
	units/mg protein ± S.E.
Ascorbate	34.4 ± 0.7
Pyrogallol	1280 ± 50
Guaiacol	32.9 ± 0.8
o-Dianisidine	20.3 ± 0.7
ABTS	2.7 ± 0.1
Urate	0
GSH	0
NADH	0
NADPH	0

without interference from leghemoglobin.

Kinetic Studies. Lineweaver-Burk plots of ASC peroxidation were not linear, thus indicating that this reaction does not follow Michaelis-Menten kinetics. Consequently, it is incorrect to express substrate affinity in terms of K_m. Hill plots indicated that the substrate concentrations at which $v = \frac{1}{2} V_{max}$ (*i.e.* $[S]_{0.5}$) were 70 μM for ASC and 3 μM for H_2O_2. These values are in approximate agreement with estimates derived from plots of [*S*] *versus* *v*. These plots consisted of sigmoidal saturation curves, also typical of non-Michaelis-Menten kinetics.

The kinetic properties of ASC peroxidase included a lag period during the spectrophotometric assay with ASC. The rate during these assays was typically low after initial mixing of all assay components. The rate gradually increased until it became linear after 2 to 3 min. This linear rate was maintained for several minutes until the ASC was depleted. In crude extracts, the H_2O_2 was exhausted first, probably because of catalase activity. This lag phase was less evident with low ASC concentrations (<125 μM) or with high enzyme concentrations. No lag phase was observed with nonphysiological reductants.

DISCUSSION

The occurrence of ASC peroxidase, DHA reductase, and glutathione reductase in nodules of nine different legume species and of *Alnus rubra* is consistent with the conclusion that this system is important in peroxide scavenging and the maintenance of normal nodule function. It is of interest that these enzymes are also present in the N_2-fixing heterocysts of cyanobacteria (27). ASC peroxidase is an abundant protein in soybean nodules, comprising approximately 0.9% of the total protein. Consideration of this system may prove to be useful in understanding plant-controlled ineffective symbioses and in developing improved cultivars.

Native gel electrophoresis was used to verify the homogeneity of the final preparation. The two minor bands observed on these gels appeared to be the result of polymerization of ASC peroxidase monomers. Several independent lines of evidence support this interpretation. The minor bands contained ASC peroxidase activity after recovery of proteins from homogenized gel sections. Passage of the final preparation through another gel filtration step did not eliminate the protein(s) of the minor bands despite the apparent size difference between these proteins and the protein of the major band. The protein of the major band was recovered from gel sections and subjected to a second analysis by electrophoresis under identical conditions. These gels also contained minor bands. Finally, these two minor bands were absent on SDS gels.

The difference between mol wt values as determined by SDS gel electrophoresis (30,000) and gel filtration (47,000) is possibly due to a strong effect of protein conformation on the latter procedure (2). The value derived by SDS gel electrophoresis is generally more reliable because conformational effects are minimized (24). A further complication in establishing the mol wt of ASC peroxidase is the possibility of glycosylation. Further investigations are required to fully resolve this matter.

The presence of heme in ASC peroxidase is typical of plant peroxidases (18). The absorption spectra presented here are similar to spectra previously reported for soybean nodule peroxidase (20) and for ASC peroxidase from *Euglena* (26). They are also comparable to spectra for other hemeproteins such as horseradish peroxidase, thyroid peroxidase, and methemoglobin (13). The spectra, along with the results of inhibition by CN^-, N_3^-, and CO, provide convincing evidence of the heme content of ASC peroxidase.

The reasons behind the inhibition by acetylene are not clear, but this observation clearly has implications for the acetylene reduction assay for nitrogenase activity. If the peroxidase activity is inhibited during this assay, then nodule function may become increasingly impaired and nitrogenase activity will appear to be artificially low.

The mol wt of native ASC peroxidase from nodules (47.000 by gel filtration) is similar to the value reported for ASC peroxidase from spinach chloroplasts (45,000, Ref. 1). However, the nodule peroxidase differs in several important characteristics. The chloroplast peroxidase is very labile at 4°C with a half-life of about 10 h (1). The nodule peroxidase is relatively stable. The chloroplast peroxidase does not catalyze the oxidation of guaiacol and is only slightly active with pyrogallol. The nodule peroxidase catalyzes the oxidation of both guaiacol and pyrogallol, with the latter showing an especially high rate. The chloroplast and nodule peroxidases do not catalyze the oxidation of GSH, NADH, and NADPH. This common factor distinguishes them from horseradish peroxidase (1).

Urate was considered as a possible reductant because it can be oxidized by horseradish peroxidase (19) and because urate is present in nodules of ureide-transporting legumes such as *Glycine* and *Phaseolus*. However, the nodule peroxidase did not catalyze the oxidation of urate.

The nomenclature for ASC peroxidase is based on the observation that this peroxidase exhibits high specificity for ASC (1, 26). Many nonspecific peroxidase (*e.g.* horseradish peroxidase) can also catalyze the oxidation of ASC at low rates (26), but they are not generally referred to as 'ASC peroxidase'. Designation of

the nodule enzyme as an ASC peroxidase is justified for the following reasons: (a) ASC is an effective reductant, comparable to guaiacol and *o*-dianisidine; (b) the enzyme shows a high affinity for ASC ($[S]_{0.5}$ = 70 μM); (c) the high concentration of ASC in nodules (2.1 mM, Ref. 5) suggests that this is the natural reductant.

Catalase has been considered important for peroxide removal in legume nodules (21). Catalase activity is especially high in peroxisomes in nodules of ureide-transporting legumes where H_2O_2 is generated during ureide formation (11). However, catalase from most organisms is ineffective in scavenging low concentrations of peroxide because it has a very low affinity for H_2O_2 (K_m = 0.047 to 1.1 M, Ref. 9). When present at low concentrations, H_2O_2 is not readily destroyed by catalase because two molecules of H_2O_2 must converge on the active site of a single catalase molecule (9). ASC peroxidase, with its high affinity for H_2O_2 ($[S]_{0.5}$ = 3 μM), probably accounts for most of the peroxide removal which occurs outside of peroxisomes in nodules.

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