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Fe^{III} Reduction in Cell Walls of Soybean Roots[1]

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CARL L. TIPTON* AND JOAN THOWSEN
Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011

ABSTRACT

Reduction of Fe^{III}EDTA by excised roots of soybean seedlings (*Glycine max* L.) is stimulated by L-malate in the bathing solution. Reduction occurs much more rapidly with roots of seedlings grown in the absence of iron than with roots of seedlings grown with iron. Cell-wall preparations from these roots catalyze reduction of Fe^{III}EDTA by NADH. They also contain NAD^+-dependent L-malate dehydrogenase. Enzymic activity of the cell-wall preparations is not affected by previous iron nutrition of the plants, but the amount of L-malate in the roots is increased when seedlings have been deprived of iron. We propose that reduction of iron before absorption by soybean roots occurs in the cell-wall space, with L-malate secreted from the roots serving as the source of electrons. Part of the iron reductase activity of the cell walls can be solubilized by extraction with 1 molar NaCl. The enzyme has been partially purified.

The first step in the absorption of iron by many plants is the reduction of Fe^{III} to Fe^{II} (4). The locus and mechanism of this reduction are not clear. Most studies of this process involve iron-deficient seedlings, which are thought to respond to the deficiency by increasing their capacity to reduce Fe^{III}. Some plants respond to the deficiency stress by releasing into the nutrient medium reducing substances, such as caffeic acid, as well as by acidifying the medium (12). Nonenzymic reduction and the lower pH increase the availability of iron to the plants. We have found (17) that the ability of various soybean cultivars to respond in this manner does not correlate quantitatively with the iron efficiency of the cultivars on calcareous soils. We found that soybean roots in solution culture in the absence of iron acquired a greatly increased capacity for iron reduction preceding the release of protons and reducing substances.

Bienfait *et al.* (3) have suggested, on the basis of kinetic evidence, that iron reduction by iron-deficient bean (*Phaseolus vulgaris* L.) plants is carried out by a membrane-bound enzyme in the root cortex. NADPH is suggested as the intracellular electron donor for the extracellular reduction of iron by this system (14). Romheld and Marschner (13) and Barrett-Lennard *et al.* (2) find that nonenzymic reduction by phenolics is inadequate to explain Fe^{III} reduction by peanut (*Arachis hypogaea* L.) roots. They suggest a plasmalemma-bound enzyme as the site of Fe^{III} reduction.

In this paper, we present evidence for a third possibility. We suggest that reduction of Fe^{III} to Fe^{II} is catalyzed by an enzyme in the cell wall, using NADH generated by the oxidation of malate, also in the cell wall.

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MATERIALS AND METHODS

Soybean seed from various cultivars was provided by Dr. W. R. Fehr, Department of Agronomy, Iowa State University. Nutrient solutions were prepared from reagent grade mineral salts and deionized distilled H_2O. Iron, when added, was in the form of Fe^{III}EDTA, 35.8 μM (2 ppm Fe). Seeds were germinated on moist germination paper in the dark at 29°C for 3 d, after which the seedlings were transferred to modified Steinberg's solution pH 7.0 (5) in plastic buckets. The nutrient solution was stirred and aerated with aquarium air pumps. Plants were grown in a growth chamber with a daily cycle of a 16-h light period (1900–2000 lux) at 26°C and an 8-h dark period at 20°C.

Ferrozine (3-[2-pyridyl]-5,6-bis[4-phenylsulfonic acid]-1,2,4-triazine sodium salt) was from Hach Chemical Co., Ames, IA; biochemical reagents and buffers were from Sigma Chemical Co. Fe^{II} formed by reduction of Fe^{III}EDTA was estimated spectrophotometrically as the Ferrozine complex as described by Chaney *et al.* (6).

Cell walls were isolated from excised roots as described previously (17). In some cases, the cell-wall preparations were dried by extraction with acetone and stored at 4°C. Essentially the same results were obtained by using either fresh or acetone-dried cell walls.

Assay of Iron Reductase. Weighed portions of moist cell wall were suspended in 3.2 ml of 0.1 M sodium phosphate buffer (pH 6.0). Alternatively, weighed portions of acetone-dried cell wall were shaken in 3.7 ml of buffer until the cell-wall mass was wetted. Fe^{III}EDTA and Ferrozine were added to give final concentrations of 200 μM and 600 μM, respectively, and the reaction was initiated by the addition of NADH to give a concentration of 750 μM in a final assay volume of 4 ml. The reactions were terminated by filtration through a coarse fitted glass funnel, and the A at 562 nm was measured immediately. Nonenzymic reduction, which amounted to about 10% of the total, was estimated from blanks lacking cell-wall material and was subtracted at each time. The reaction rate was constant with time for about 40 min and the rate was proportional to wet cell-wall weight from 2 to 10 mg. The activity of wet cell-wall preparations was typically 0.12 nmol/mg·min and that of acetone-dried cell walls was about 0.3 nmol/mg·min.

Isolation of Iron Reductase. Excised roots (60 roots, 45 g) from 10-d-old Northrup King Pride B216 plants grown without iron were chopped into small pieces and homogenized with a Polytron PCU-2 in 180 ml of cold 50 mM sodium acetate buffer (pH 5.0) for 2 min. The homogenate was filtered through moistened Miracloth, and the retained cell-wall material was washed with another 600 ml of the same buffer. The washed cell-wall material was suspended in 36 ml of cold 50 mM sodium acetate buffer (pH 5.0) containing 1 M NaCl and was left at 4°C for 14 h. The suspension was then filtered through Miracloth. About one-third to one-half of the iron reductase activity of the cell-wall preparation was found in this extract. The extract was made 80% saturated with $(NH_4)_2SO_4$ and was centrifuged for 20 min at 20,000g. The yellow precipitate was resuspended in 1 ml of 50

mM sodium acetate buffer (pH 5.0) containing 1 M NaCl, applied to a Sephadex G100 column (2.9 × 92 cm), and eluted with the same buffer.

RESULTS AND DISCUSSION

We have shown that excised roots of soybean seedlings are capable of reducing Fe^{III} EDTA, with the maximum capacity for reduction appearing at 9 to 10 d after germination (17). This capacity is increased if the plants are grown without iron in the nutrient solution, and it is stimulated by the addition of D-glucose during the assay for iron reduction. We now show (Fig. 1) that greater rates of iron reduction are obtained with L-malate than with D-glucose. In addition to the two cultivars for which results are shown in Figure 1, five other cultivars were tested in the same way (data not shown). In each case, the initial rate of Fe^{III}EDTA reduction was increased more by L-malate than by D-glucose, although in some cases, the rate in the presence of D-glucose eventually exceeded that in the presence of L-malate, at longer incubation times. The rates of reduction by roots from plants grown in the presence of 38.5 μM Fe^{III} were less than 10% as great as those by roots of iron-deficient plants (data not shown).

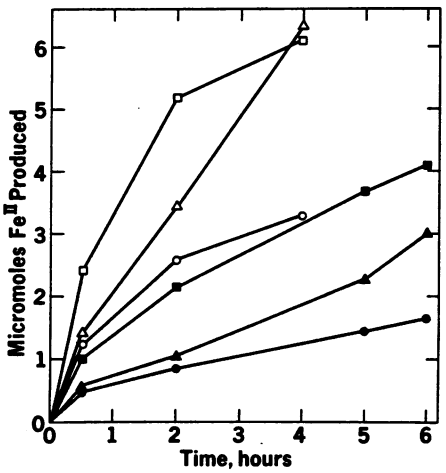

FIG. 1. Measurement of reduction of Fe^{III}EDTA by excised roots from soybean plants grown without iron was carried out as described earlier (14). (○, △, □), variety A2; (●, ▲, ■), Northrup-King Pride B216. (○, ●), No supplement; (△, ▲), 1 mM D-glucose; (□, ■), 1 mM L-malate added to modified Hoagland solution (5) containing 0.2 mM Fe^{III}EDTA and 0.6 mM Ferrozine (pH 5.6). Results expressed as iron reduced per gram fresh weight of roots.

Table I. *Effect of Iron on the Malate Content of Roots of 10-d-old Soybean Seedlings*

The plants were grown without iron or with 38.5 μM iron as Fe^{III} EDTA. L-Malate was estimated by using the coupled enzymic assay of Mollering (10).

Cultivar	Chlorosis Score[a]	Malate Content	
		−Fe	+Fe
		μmol/g fresh wt	
A2	1.35	0.98, 1.02, 1.05	0.47, 0.73
Agripro	1.45	0.88, 0.93	
Weber	1.85	0.96	0.49
Hodgson	2.05	0.89, 0.63	0.48
Coles	2.6	1.14	1.04
Wells	2.95	0.59, 0.63	0.49
Peterson	3.45	0.65	
Corsoy	3.7	0.93	0.61
Asgrow	3.95	0.88	0.70
Pride B216	4.25	0.54, 0.50	0.45

[a] From Froehlich and Fehr (7). Higher numbers indicate greater susceptibility to iron-deficiency chlorosis on calcareous soils.

The L-malate content of excised roots was measured and compared with the susceptibility of the cultivars to iron-deficiency chlorosis when grown in calcareous soils, as reported by Froehlich and Fehr (7). Table I shows a tendency toward greater L-malate content in the roots of the less susceptible cultivars when grown in the absence of iron. The increase tends to be greatest in those cultivars least susceptible to iron-deficiency chlorosis.

In an effort to develop a method to predict the resistance of soybean varieties to iron-deficiency chlorosis, the ratio of the L-malate content of roots of plants grown in the presence of iron to that of roots of plants grown without iron was plotted against the chlorosis scores. These points fitted a straight line, with a correlation coefficient of 0.81. Although this correlation is not close enough to have practical predictive value, it suggests that resistance to iron-deficiency chlorosis is related to the ability of plants to respond to low iron levels by increasing the L-malate content of the roots.

Thoroughly washed cell-wall preparations from soybean seedling roots are capable of reducing Fe^{III}EDTA when supplemented with NADH. Difference between cell-wall preparations from the varieties A2 and Pride B216 (which represent the extremes with respect to iron efficiency among the varieties used in this work), was slight and the iron nutrition of the plants did not have a significant effect (Table II). NADPH reduces iron at about half the rate at which NADH reduces iron (data not shown). The pH optimum for catalysis of reduction of Fe^{III}EDTA by NADH is 6.0 (Fig. 2). The rate of reaction is proportional to the Fe^{III}EDTA concentration over the range 25 to 500 μM (Fig. 3), indicating that K_m lies well above this concentration range. The actual

Table II. *Reduction of Fe^{III} EDTA by Root Cell-Wall Preparations of Two Soybean Varieties Grown With or Without Iron*

Variety	Fe^{III} Reduced	
	$+Fe^{III}$	$-Fe^{III}$
	nmol/h·mg cell wall	
A2	7.2	7.6
Pride B216	6.0	5.4

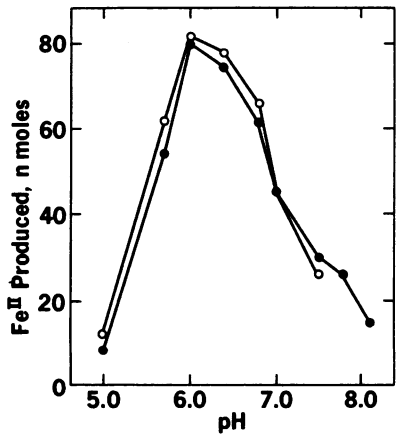

FIG. 2. pH Optimum for reduction of Fe^{III}EDTA by NADH catalyzed by soybean root cell walls. Weighed portions, 5.2 to 6.2 mg, of dried cell wall were used for the assay and the results are normalized to 5.0 mg. The reaction was terminated after 30 min. Buffers were 0.1 M sodium phosphate except at pH 5.0, 50 mM sodium acetate. Results of replicate experiments are shown.

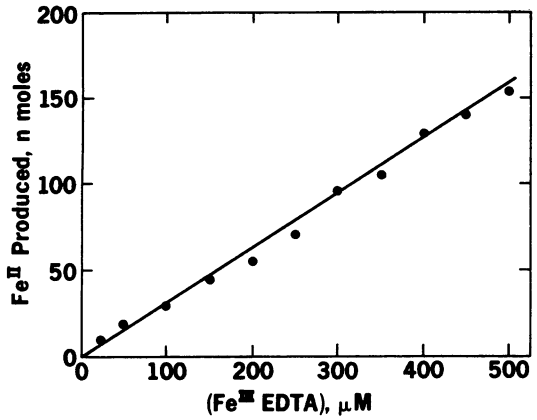

FIG. 3. Rate of reduction of Fe^{III}EDTA as a function of Fe^{III}EDTA concentration. Results normalized to 5.0 mg of dried cell wall. Reaction terminated after 30 min. The NADH concentration was constant at 750 μM. The Fe^{III}EDTA concentration was varied from 25 to 500 μM, and the Ferrozine concentration was three times that of the Fe^{III}EDTA.

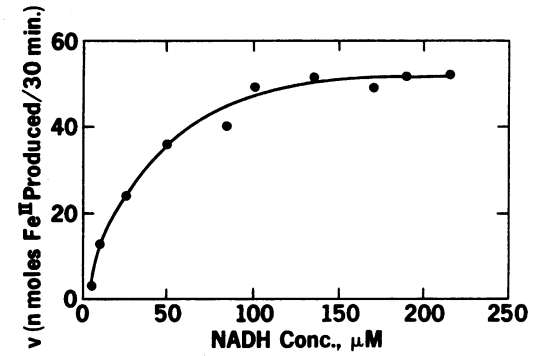

FIG. 4. Rate of reduction of Fe^{III}EDTA as a function of NADH concentration. Results normalized to 5.0 mg of dried cell wall. Reaction terminated after 30 min. The NADH concentration was varied from 5 to 216 μM while the Fe^{III}EDTA and Ferrozine concentrations were constant at 200 μM and 600 μM, respectively.

substrate is likely to be uncomplexed iron, but the use of $FeCl_3$ at a concentration high enough to give an easily measurable amount of product results in an unacceptably high rate of nonenzymic reaction with NADH. Fe^{III}EDTA may act as a Fe^{III} concentration buffer rather than as the actual substrate. NADH exhibits conventional saturation kinetics (Fig. 4) with a K_m estimated at 35 μM from a double-reciprocal plot of the data in Figure 4.

L-Malate dehydrogenase activity is present in washed cell walls (Fig. 5), and the activity is not affected significantly by the iron nutrition of the plants. Thus, the NADH used in reducing Fe^{III} may be regenerated by the oxidation of L-malate supplied by the roots.

About one-third to one-half of the NADH:Fe^{III}EDTA reductase activity of a washed cell-wall preparation was solubilized by treatment with 1 M NaCl. Chromatography on Sephadex G-100 (Fig. 6) separates the enzyme from peroxidase and results in a 5-fold increase in specific activity (Table III). The observed specific activity of the partially purified enzyme is relatively low, but the enzyme was assayed at a concentration of Fe^{III}EDTA far below saturation.

Reduction of Fe^{III}EDTA by excised roots was shown earlier (17) to be inhibited by the diphenols caffeic and chlorogenic acids and by their biosynthetic precursors *trans*-cinnamic and *p*-coumaric acids. The solubilized and partially purified enzyme is not inhibited by any of these materials and is even stimulated 2-

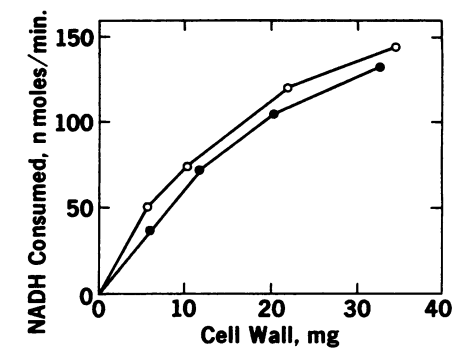

FIG. 5. Malate dehydrogenase activity in cell walls isolated from 10-d-old Northrup-King Pride B216 plants grown with (●) or without (○) iron. The assay procedure was modified from that of Ochoa (11). Weighed portions of moist, well-blotted cell walls were suspended in 4.2 ml of 0.1 M sodium phosphate buffer (pH 7.4). Sodium oxaloacetate (1.25 μmol) and NADH (1.5 μmol) were added to give a final volume of 4.8 ml. The reaction mixture was shaken gently for 7 min, and the reaction terminated by filtration through a coarse sintered-glass funnel. Absorbance of the filtrate at 340 nm was measured.

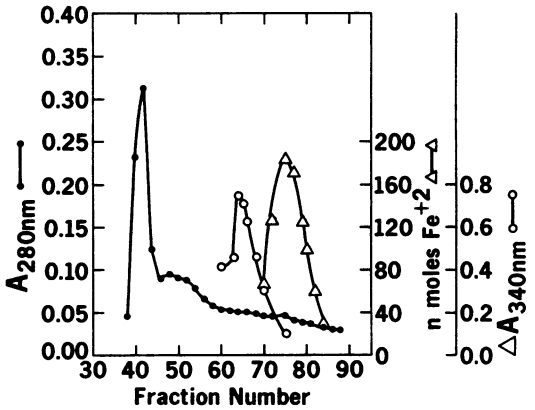

FIG. 6. Elution of solubilized iron reductase from a Sephadex G-100 column. Protein concentration was monitored by measuring *A* at 280 nm (●). Peroxidase activity (○) was assayed according to the procedure of Akazawa and Conn (1). Soluble iron reductase assays (Δ) containing 185 μmol acetate buffer (pH 5.0), 0.8 μmol Fe^{III}EDTA, 2.4 μmol Ferrozine, 3 μmol NADH, and enzyme in a final volume of 4 ml were incubated in the dark for 30 min, and the absorbance of the Fe^{III}-Ferrozine complex was measured at 562 nm.

Table III. *Purification of NADH:Fe^{III} EDTA Reductase by Sephadex G-100 Chromatography*

Purification is described in "Materials and Methods"; assay of soluble reductase in legend to Figure 6.

Preparation	Volume	Total Protein	Total Activity	Specific Activity
	ml	*mg*	*nmol/min*	*nmol/min·mg protein*
Salt extract	30	3.9	146	37.5
Fractions 72–82 (Fig. 8)	6.2	0.44	75	169

fold by *p*-coumaric acid (Table IV). The inhibitions observed with excised roots must be exerted on some earlier step in the overall process.

Our results are in accord with a model in which L-malate in root cells escapes into the surrounding cell-wall space where it is oxidized by NAD^+ and the resulting NADH is utilized for the

Table IV. *Effects of Phenolic Acids and trans-Cinnamic Acid on NADH:Fe^{III} EDTA Reductase*

Assay carried out as described in legend to Figure 6, except for addition of the aromatic acids, each at 0.2 mM final concentration.

Activity	Reductase Activity
	nmol Fe^{III} reduced/h
None	24.8
Trans-Cinnamic acid	27.2
Caffeic acid	27.9
p-Coumaric acid	53.3
Chlorogenic acid	24.6

reduction of Fe^{III}. This model is very similar to one proposed by Gross *et al.* (9) to account for the generation of H_2O_2 used for lignin synthesis in cell walls. The presence of malate dehydrogenase is essential, in these proposals, for lignin formation and for iron reduction. Although the presence of malate dehydrogenase activity in extracellular solutions prepared from pea epicotyl sections by centrifugation (16) was taken to be evidence for contamination of the solutions by cytosolic protein, these experiments do not rule out the presence of insoluble malate dehydrogenase in the cell wall. Evidence for cell wall-bound malate dehydrogenase has been reported by Gross (8) and by Stephens and Wood (15).

The possibility that our cell-wall preparations were contaminated with cellular membranes capable of catalyzing the reduction of Fe^{III} by NADH was considered. Although we cannot rule out this possibility, two observations suggest that the observed iron reductase activity is not membrane bound. Extraction of the cell-wall preparations with acetone, a procedure that usually inactivates membrane-bound enzymes, has no effect on the iron reductase activity of the cell-wall preparations. In addition, up to half of the iron reductase activity can be solubilized by treatment with 1 M NaCl, a treatment not expected to solubilize membrane-bound enzymes.

The model proposed here suggests that a malate-oxaloacetate shuttle may be present in the plasmalemma, as proposed earlier by Gross (8). It remains to be demonstrated that this shuttle exists and that the activities of the NADH:Fe^{III}EDTA reductase and L-malate dehydrogenase under conditions of pH, temperature, and substrate concentrations found *in vivo* can account for the amount of iron reduced and absorbed by the plant.

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