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Evidence for the presence of a [2Fe-2S] ferredoxin in bean sprouts

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An iron-sulfur protein with properties similar to those of ferredoxins found in the leaves of higher plants has been isolated from bean sprouts – a non-photosynthetic plant tissue. The bean sprout protein has a molecular mass of 12.5 kDa and appears to contain a single [2Fe-2S] cluster. The absorbance and circular dichroism spectra of the bean sprout protein resemble those of spinach leaf ferredoxin and the bean sprout protein can replace spinach ferredoxin as an electron donor for $NADP^+$ reduction, nitrite reduction and thioredoxin reduction by spinach leaf enzymes. Although the reduced bean sprout protein ($E_m = -440$ mV) is a slightly stronger reductant than spinach ferredoxin and appears to be less acidic than spinach ferredoxin, the two proteins are similar enough so that the bean sprout protein is recognized by an antibody raised against spinach ferredoxin.

Introduction

In higher plants and algae, ferredoxin, photoreduced by Photosystem I, serves as an electron donor for ferredoxin-dependent enzymes such as ferredoxin:$NADP^+$ oxidoreductase (EC 1.18.1.2, hereafter referred to as $NADP^+$ reductase), ferredoxin:nitrite oxidoreductase (EC 1.7.7.1, hereafter referred to as nitrite reductase) ferredoxin : sulfite oxidoreductase (EC 1.8.7.1, hereafter referred to as sulfite reductase), glutamate synthase (EC 1.4.7.1) and ferredoxin-thioredoxin reductase [1–4]. The presence of nitrite reductase [5–8], sulfite reductase [9], $NADP^+$ reductase [10] and glutamate synthase [5,11,12], ferredoxin-dependent enzymes similar to the leaf enzymes, in the non-green tissues of some plants suggested that these non-green tissues were likely to contain ferredoxin or a ferredoxin-like protein. There have, in fact, been several reports of the presence of putative ferredoxins in non-photosynthetic plant tissue. A ferredoxin-like protein, present in non-green, cultured tobacco callus, is able to serve as an electron donor to a nitrite reductase prepared from the same cells [13]. However, the protein could not replace ferredoxin in the photoreduction of $NADP^+$ by chloroplast thylakoid membranes [13]. Suzuki et al. have identified a protein in corn roots that is able to serve as an electron donor for both nitrite reductase and glutamate synthase [5]. The corn root protein, although it bears an antigenic similarity to spinach ferredoxin, could not replace spinach ferredoxin as an electron carrier in

Abbreviations: CD, circular dichroism; PAGE, polyacrylamide gel electrophoresis; E_m, oxidation-reduction midpoint potential; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate.

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$NADP^+$ photoreduction [5]. Recently, Wada et al. have reported that a ferredoxin-like protein capable of serving as an electron donor for both $NADP^+$ and nitrite reduction is present in the white storage root of Japanese radish [14]. In the case of the tobacco callus and corn root proteins, few properties of the protein were reported [5,7]. Our aim has been to find a plant non-green tissue from which we could obtain good yields of a ferredoxin-like protein. We have succeeded in purifying such a protein from bean sprouts and characterizing it with regard to molecular weight, optical and circular dichroism (CD) spectra, iron and acid-labile sulfur content, oxidation-reduction midpoint potential (E_m) and reactivity with several ferredoxin-dependent enzymes.

Methods

Bean sprouts and spinach leaves were purchased from local markets during the 1987 growing season. The bean sprouts contained no detectable chlorophyll. Spinach ferredoxin and nitrite reductase were purified as described previously [15]. Nitrite reductase activity was assayed essentially as described previously [16], except that 75 mM Tris buffer (pH 8.0) replaced 50 mM potassium phosphate buffer (pH 7.7) in the assay reaction mixture. Nitrite concentrations were determined colorometrically [17]. One unit of activity corresponds to 1 μmol nitrite reduced per minute. $NADP^+$ photoreduction using water as the electron donor was followed by monitoring the increase in absorbance at 340 nm during illumination of a suspension of washed spinach thylakoid membranes [18]. One unit of activity corresponds to 1 μmol $NADP^+$ reduced per mg chlorophyll per hour. Chlorophyll was determined according to the method of Arnon [19]. Thioredoxin *m* [20] and ferredoxin-thioredoxin reductase were prepared according to the method of Droux et al. [21]. Thioredoxin reduction was followed by its capacity to promote the activation of corn $NADP^+$-linked malate dehydrogenase [22]. One unit of activity corresponds to 1 μmol NADPH oxidized per minute per 0.1 nmol ferredoxin-thioredoxin reductase. An antibody raised against spinach leaf ferredoxin was a generous gift of Professor N.-H. Chua. Western blots using this antibody were performed essentially as described by Towbin et al. [23], except that 5% non-fat powdered milk and 0.05% Tween-20 were used instead of bovine serum albumin in the blocking solution.

Protein concentrations were determined according to the method of Bradford [24] using bovine serum albumin as a standard. Analysis of the bean sprout protein for acid-labile sulfide was performed according to the method of Siegel et al. [25], using spinach ferredoxin as a standard. The total iron content of the protein was determined according to Massey [26], using ferric ammonium sulfate and spinach ferredoxin as standards. Oxidation-reduction titrations were performed electrochemically using the optically transparent gold electrode/thin layer cell system described previously [27]. Absorbance and CD spectra were measured using an Aminco DW-2a spectrophotometer and a JASCO Model J-20 spectropolarimeter, respectively. Molecular weights under non-denaturing conditions were determined by gel filtration on an Ultrogel AcA 44 column (1.5×75 cm) in 50 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl and on a Sephadex G-50 column (1.5×75 cm) in 200 mM potassium phosphate buffer (pH 7.7), according to the method of Andrews [28]. Hexokinase, bovine serum albumin, pepsin, soybean trypsin inhibitor, cytochrome *c*, bovine lung aprotinin, bovine insulin chain A and vitamin B12 were used as molecular weight standards. Gel electrophoresis in the absence or presence of sodium dodecyl sulfate (SDS) was performed according to the methods of Davis [29] and of Laemmli [30], respectively. Molecular weight standards used were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme and bovine lung aprotinin.

Ultrogel AcA 44 was obtained from L.K.B., Sephadex G-50 from Pharmacia and DE-52 DEAE-Cellulose from Whatman. $NADP^+$, phenylmethylsulfonylfluoride (PMSF) and Polyclar AT polyvinyl-polypyrrolidone were obtained from Sigma Chemical Co. All other reagents used were of the highest purity commercially available.

All steps of the purification of the bean sprout were carried out in a cold room at 4–5 °C. 2 kg portions of bean sprouts were homogenized for 1

min in a Waring-type blender in 2 l of 50 mM Tris-ascorbate buffer containing 200 mM NaCl, 5% (w/v) polyclar AT, 1 mM EDTA and 1 mM PMSF and the homogenate filtered through cheesecloth. The filtrates from 20 kg bean sprouts were combined and chilled acetone (−15°C) added slowly to a final concentration of 35% (v/v). The resulting precipitate was removed by centrifugation for 10 min at 10000 × *g* and additional chilled acetone added to the 35% acetone supernatant to a final concentration of 75% (v/v). The resulting precipitate was collected by centrifugation for 5 min at 10000 × *g*, suspended in a minimum amount of the homogenizing buffer and initially dialyzed against 20 l of the homogenizing buffer followed by continued dialysis with three 20 l changes of buffer. Insoluble material was removed by centrifugation and the soluble material was then dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl and 20% (v/v) glycerol. The reddish-brown dialysate was then applied to a DEAE-cellulose column (2.5 × 20 cm) previously equibrated with the same buffer and the colored material eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 400 mM NaCl. The colored fractions were collected, concentrated using an Amicon YM-5 ultrafiltration membrane and loaded onto an Ultrogel AcA 44 column (4 × 100 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl. Fractions with $A_{421}:A_{277} > 0.35$ were pooled, concentrated as described above and applied to a DEAE-cellulose column (0.5 × 15 cm) equilibrated with the same buffer. The bean sprout ferredoxin was slowly eluted as a braod band by the same buffer. Fractions with $A_{421}:A_{277} > 0.45$ were pooled, concentrated and chromatographed on a Sephadex G-50 column (1.5 × 75 cm) equilibrated with the same buffer. Ferredoxin was eluted as a single peak (i.e., all fractions containing ferredoxin had the same $A_{421}:A_{277}$ ratio of 0.50) and concentrated using an Amicon Centricon-10 microconcentrator for use in subsequent experiments.

Results

After the final step of the purification protocol described above, the bean sprout protein appeared pure based on the criterion of constant $A_{421}:A_{277}$ in all ferredoxin-containing fractions detected during gel filtration chromatography as Sephadex G-50. The final yield was 10 mg of protein per 20 kg starting material. Gel electrophoresis in the absence of SDS, performed according to Davis [29], on either 7% cross-linked polyacrylamide or in the presence of SDS on 13% cross-linked polyacrylamide, performed according to Laemmli [30], revealed a single Coomassie Brillant Blue-staining band (data not shown), also suggesting that the protein was homogeneous. However polyacrylamide gel electrophoresis in the presence of SDS on a 12–20% cross-linked gradient gel [30] revealed the presence of two major and three minor protein bands (Fig. 1).

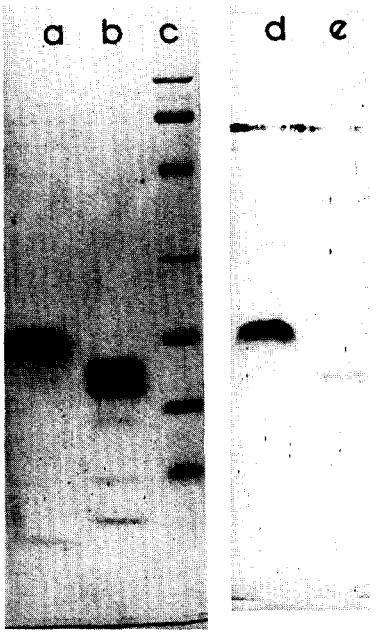

Fig. 1. SDS-polyacrylamide gel electrophoresis and immunoblotting of bean sprout and spinach leaf ferredoxins. Electrophoresis was performed on a 12–20% gradient gel using 10 μg of each ferredoxin. Left-hand panel: lane a, spinach leaf ferredoxin; lane b, bean sprout ferredoxin; lane c, molecular weight standards. The gel was stained for protein with Coomassie Brilliant Blue. Right-hand panel: lane d, spinach leaf ferredoxin; lane e, bean sprout ferredoxin. After electrophoresis, the proteins were transferred to nitrocellulose paper, treated with antibody against spinach ferredoxin and developed with peroxidase coupled to goat anti-rabbit IgG.

Gel filtration under non-denaturing conditions on either calibrated Ultrogel AcA 44 or Sephadex

G-50 columns yielded a molecular mass value of 12.5 ± 0.5 kDa for the bean sprout protein. This value is slightly higher than the 11 kDa value reported for spinach leaf ferredoxin [31], is similar to the value we have estimated from the data of Wada et al. [14] for the radish root ferredoxin and is substantially lower than the 19.5 kDa value reported for the tobacco callus ferredoxin-like protein [13]. Molecular mass estimations from electrophoresis in the presence of SDS on 13% cross-linked polyacryalmide gave a value of 15.5 kDa for the molecular mass of the bean sprout protein, while electrophoresis in the presence of SDS on a 12–20% cross-linked polyacrylamide gradient gel [20] revealed two major bands of molecular mass 17.6 and 15.7 kDa, respectively, that stained approximately equally with Coomassie Blue (Fig. 1). Three faint Coomassie Blue-staining bands of molecular mass 12.5, 6.3 and 3.7 kDa were also observed on the 12–20% gradient gel. Fig. 1 also shows the results of a Western blot experiment using an antibody raised against spinach ferredoxin. Only the 15.7 kDa band in the bean sprout protein preparation (lane e) cross-reacts with the spinach ferredoxin antibody. (The band at higher molecular weight is an artifact of unknown origin and can also been seen in the control experiment – lane d – using authentic spinach ferredoxin as the antigen. Lanes a and d

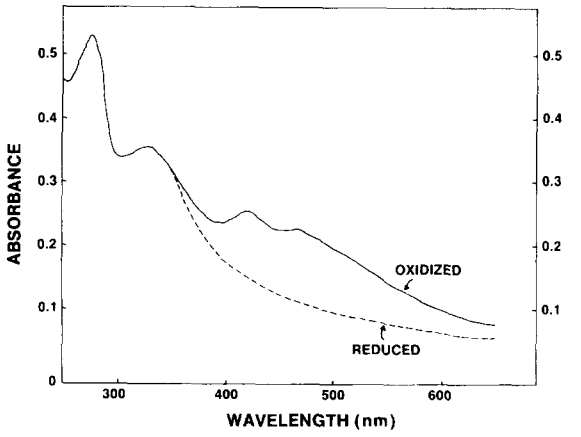

Fig. 2. Absorbance spectra of bean sprout ferredoxin. Spectra of 27 μM ferredoxin in 30 mM Tris-HCl buffer (pH 8.0) were measured in a 1 cm pathlength cell vs. a buffer blank. Oxidized ferredoxin (———), ferredoxin reduced with solid sodium dithionite (— — —).

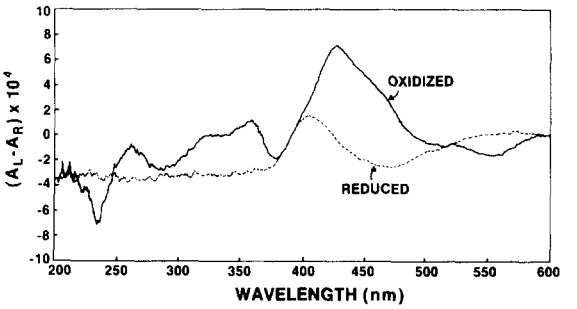

Fig. 3. CD spectra of bean sprout ferredoxin. Experimental conditions as in Fig. 2, except that the protein concentration was 10 μM.

also illustrate that spinach leaf ferredoxin migrates anomalously during SDS-PAGE, giving an apparent molecular mass of 22 kDa [32].) Fig. 1 shows that the bean sprout protein cross-reacts with an antibody against spinach ferredoxin indicating that the two proteins are related. The weak cross-reaction suggests that those are some substantial difference in structure between the bean sprout and spinach leaf ferredoxins. One difference that we have detected, in addition to the small differences in molecular weight, is the behavior of the two proteins during ion-exchange chromatography. While the bean sprout protein can be eluted from a DEAE-cellulose column by 50 mM Tris buffer containing 200 mM NaCl, 350 mM NaCl was required to elute spinach leaf ferredoxin under similar conditions.

Fig. 2 shows the absorbance spectra of the oxidized and reduced bean sprout protein. The spectrum of the oxidized protein, with absorbance maxima at 277, 328, 421 and 466 nm and shoulders at 285 and 530 nm, is very similar to those of other higher plant, [2Fe-2S]-containing ferredoxins such as that isolated from spinach leaves [33] and to that of radish root ferredoxin [14]. Assuming a molecular mass of 12.5 kDa for the bean sprout protein, an extinction coefficient at 421 nm of 8.9 $mM^{-1} \cdot cm^{-1}$ was calculated for the oxidized bean sprout protein. Reduction of the bean sprout ferredoxin caused a marked decrease in absorbance in the visible region (Fig. 2), as expected for [2Fe-2S]-containing ferredoxins [34]. Fig. 3 shows the CD spectra for the oxidized and reduced forms of the bean sprout protein in the visible and near ultraviolet regions. The oxidized

TABLE I

EFFECT OF FERREDOXIN SOURCE ON THE KINETIC PARAMETERS FOR NITRITE, $NADP^+$ AND THIOREDOXIN *m* REDUCTION

The enzyme assays were performed as described in Methods. V_{max} values are in units which are defined in Methods.

Ferredoxin source	Nitrite reduction		$NADP^+$ reduction		Thioredoxin *m* reduction	
	K_m (μM)	V_{max}	K_m (μM)	V_{max}	K_m (μM)	V_{max}
Spinach leaf	20	320 (100%)	0.40	170 (100%)	0.20	1.54 (100%)
Bean sprout	22	160 (50%)	0.23	70 (40%)	0.17	1.33 (86%)

protein showed positive CD features at 357 and 428 and negative features at 235, 288, 379 and 555 nm, while the reduced protein exhibits a positive feature at 405 nm and a broad negative one at 470 nm. These spectra are very similar to these observed for spinach ferredoxin [35] and, like the optical spectra, suggest that the bean sprout protein contains a binuclear [2Fe-2S] cluster rather than a [4Fe-4S] cluster [35]. Iron and acid-labile sulfide analyses gave values (assuming a molecular weight for the protein of 12.5 kDa) of 2.08 ± 0.04 mol Fe/mol protein and 1.88 ± 0.04 mol S/mol protein, respectively, indicating the presence of a single [2Fe-2S] cluster in bean sprout ferredoxin. Since proteins often are isolated containing significant amounts of non-specifically bound iron, the acid-labile sulfide content is perhaps a better indicator of the purity of the bean sprout ferredoxin preparation. The acid-labile sulfide content of the protein suggests that, if the method of Bradford [24] gives an accurate value for the bean sprout protein concentration, the preparation cannot be contaminated by more than 6% with any protein that does not contain acid-labile sulfide.

The above results suggested a role for the bean sprout protein as an electron carrier. Before testing it as a possible electron donor to several ferredoxin-dependent plant enzymes, it seemed important to measure the E_m value of the bean sprout protein in order to determine whether it shared with plant leaf ferredoxins the property of being an extremely strong reductant. Fig. 4 shows an oxidation-reduction titration of the bean sprout protein, indicating it does behave as a low potential, one-electron carrier. An E_m value of -440 ± 5 mV ($n = 0.97 \pm 0.01$) was obtained in a series of titrations which were fully reversible and independent of mediator concentrations over a 2-fold range. In control experiments (data not shown), spinach ferredoxin titrated with $E_m = -423$ mV, in good agreement with literature values [27,33,36]. Bean sprout ferredoxin was then examined to see if it could serve as an electron donor for nitrite reduction catalyzed by spinach nitrite reductase, for thioredoxin *m* reduction by spinach ferredoxin-thioredoxin reductase and for $NADP^+$ photoreduction catalyzed by the endogenous $NADP^+$ reductase of washed spinach thylakoid membranes. Table I indicates that the bean sprout protein was able to replace spinach ferredoxin as an electron donor in all three systems with somewhat lower turnover numbers but with K_m values very similar to those observed with spinach ferredoxin.

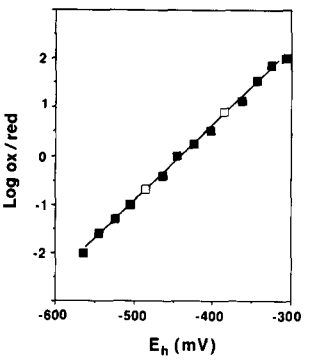

Fig. 4. Oxidation-reduction titration of bean sprout ferredoxin. The reaction mixture contained 77 μM ferredoxin in 30 mM Tris-HCl buffer (pH 8.0) and the following oxidation-reduction mediators: 15 μM benzyl viologen and 30 μM methyl viologen. The oxidation state of the protein was monitored by following the absorbance at either 421 or 463 nm. Points were taken in both the oxidative (□) and reductive (■) directions. Optical pathlength; 0.33 mm, temperature: 4°C.

Discussion

Ferredoxins have been purified to homogeneity from a number of higher plants, algae and cyanobacteria and extensively characterized. These ferredoxins function as low-potential electron carriers in oxygenic photosynthesis and are all acidic proteins containing one [2Fe-2S] cluster in a single polypeptide chain with molecular weights between 10.5 and 12.0 kDa [37]. While there have been a few reports of ferredoxin-like proteins being present in non-photosynthetic plant tissues [5,13,14,38], these proteins have not been well characterized in terms of prosthetic group content, spectral properties and oxidation-reduction E_m values. In the case of the best characterized of these proteins, that from radish root [14], although an optical absorbance spectrum was measured and electron-donating capacity to ferredoxin-dependent enzymes reported, no CD spectrum, E_m value or iron and sulfide content were reported. The work reported above contains the first data on iron and sulfide content, CD spectra and E_m value for a ferredoxin from plant non-photosynthetic tissue and also is the first example of such a protein that can serve as an electron donor for the reduction of $NADP^+$, nitrite and thioredoxin. It also should be mentioned that the yield of bean sprout ferredoxin is approx. 10-fold higher than that reported [14] for radish root ferredoxin.

Bean sprout ferredoxin is similar to spinach leaf ferredoxin in molecular weight and E_m value but it appears to be significantly less acidic than spinach ferredoxin based on its behavior during anion-exchange chromatography. The bean sprout protein is clearly less acidic than is radish root ferredoxin, since the latter required the addition of 570 mM NaCl to the eluting buffer to remove it from a DEAE-cellulose column [14]. Although we have not yet determined the isoelectric point of the bean sprout protein, we have observed that during polyacrylamide gel electrophoresis in the absence of SDS the bean sprout protein migrates less rapidly towards the anode than does spinach ferredoxin. Since the spinach and bean sprout ferredoxins have approximately the same molecular weights, this observation is consistent with spinach ferredoxin having a greater net negative charge than does bean sprout ferredoxin. Despite the apparent differences in charge on the two ferredoxins, the fact that bean sprout ferredoxin can replace spinach ferredoxin as an electron donor with comparable K_m values for three spinach ferredoxin-dependent enzymes suggests that the two proteins may be quite similar, a conclusion supported by the immunological data of Fig. 1. The cross-reactivity of the spinach ferredoxin antibody with the bean sprout protein conclusively establishes a relationship between the two proteins but the rather weak cross reaction may reflect the phylogenetic difference between bean (order *Rosales*) and spinach (order *Centrospermase*).

As discussed above, gel filtration data and iron and acid-labile sulfide analysis strongly suggested that the protein is at least 90% pure. This result is supported by our calculated ϵ_{421} value of 8.9 $mM^{-1} \cdot cm^{-1}$ for the oxidized bean sprout protein. This is 92% of the 9.7 $mM^{-1} \cdot cm^{-1}$ value reported for spinach ferredoxin [39], strongly suggesting that our preparation cannot be contaminated with significant amounts of proteins that contain no [2Fe-2S] cluster. These arguments depend to a considerable extent on how accurately protein determination by the method of Bradford [14], with bovine serum albumin used as a standard, can measure the concentration of the bean sprout ferredoxin. One argument supporting the accuracy of the protein determination is our observation that determination of the concentration of the related spinach ferredoxin by the method of Bradford gives values within 2% of those determined from the absorbance at 420 nm. The fact that the K_m values for bean sprout ferredoxin serving as an electron donor to spinach ferredoxin-dependent enzymes are not significantly greater than those observed with spinach ferredoxin itself is also consistent with a relatively high purity for the bean sprout preparation. Although the $A_{421}:A_{277}$ ratio of 0.50 determined for bean sprout ferredoxin (compared with the value of 0.49 for spinach ferredoxin [40]) also suggests that the protein is pure, this value cannot provide conclusive evidence for purity in the absence of information on the amino acid composition of bean sprout ferredoxin, since the aromatic amino acid content of ferredoxins is variable [40].

Estimates of a purity of at least 90% for the bean sprout ferredoxin preparation seem difficult

to reconcile with the appearance of two bands of approximately equal Coomassie Blue-staining intensity when the bean sprout protein is subjected to polyacrylamide gel electrophoresis in the presence of SDS on a 12–20% cross-linked gradient gel (Fig. 1). The possibility that the 15.7 kDa protein is a proteolytic degradation product of the 17.6 kDa protein seems unlikely in the light of the immunological data, showing that the 15.7 kDa protein, but not the 17.6 kDa protein, cross-reacts with an antibody to spinach ferredoxin (Fig. 1). Removal of a 1.8 kDa peptide from the 17.6 kDa protein by proteolysis would not be expected to make antigenicity against the spinach ferredoxin antibody appear. Assuming that our estimates of purity are accurate, the most likely explanation consistent with all of the data is that bean sprouts, like radish root [14] and spinach leaves [41–43], contain two forms or isozymes of ferredoxin. If this hypothesis is correct, then in the case of bean sprouts, the two isozymes can be separated by SDS-PAGE but not by gel filtration. Furthermore, only one of the two putative bean sprout ferredoxin isozymes would appear to cross react with the antibody raised against spinach ferredoxin. Another possibility that must be considered is that the two bands seen after electrophoresis on a 12–20% cross-linked gel in the presence of SDS represent leaf and non-photosynthetic tissue forms of bean ferredoxin. Work to distinguish between these possibilities and attempts to purify the protein further to resolve this question currently are under way in our laboratory.

Acknowledgements

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