A Rapid Procedure for the Purification of Ferredoxin from Spinach Using Polyethyleneimine

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Z. Naturforsch. **33 c**, 495–497 (1978); received May 17, 1978

Ferredoxin, Iron-Sulfur Proteins, Spinach, Photosynthesis, Polyethyleneimine

A rapid procedure for the purification of ferredoxin from spinach is described, which is based on the finding that ferredoxins can be precipitated with polyethyleneimines from acetone containing solutions. The ferredoxin was obtained in high yields (25–35 mg/kg spinach) and high purity ($A_{420}/A_{275}=0.47$) within less than 8 hours.

Introduction

Different procedures for the purification of ferredoxin from plants have been described [1–6]. They are rather time consuming; at least 2, generally 4 days are required to obtain highly purified ferredoxin. The procedures are based on one or several of the following properties of plant ferredoxins: (i) solubility in acetone-H_2O mixtures up to an acetone concentration of 50%; (ii) precipitation by acetone at concentrations higher than 70%; (iii) tight binding to DEAE-cellulose up to salt concentrations of 0.2 M; (iv) precipitation by ammonium sulfate solutions only at concentrations higher than 90%.

In this paper a procedure is described, by which highly purified ferredoxin can be obtained from spinach leaves in high yields in less than 8 hours. It is based on the observation that ferredoxins can be precipitated with polyethyleneimines from acetone containing solutions [7] and that ferredoxins bind to DEAE-cellulose in 65% ammonium sulfate [8].

Materials and Methods

PEI 18 (polyethyleneimine of molecular weight 1800) was from Nordman, Rassmann & Co., Hamburg; Polymin P (polythyleneimine of molecular weight 20000), wasserfrei, from BASF Ludwigshafen; PEI 600 (polyethyleneimine of molecular weight 40000–60000) from Dow Chemical Company, Midland, Michigan, USA. 10% (w/w) aqueous solutions adjusted to pH 7.8 with acetic acid were used throughout. DE 52 (Micro granular, preswollen DEAE cellulose) was from Whatman-Biochemicals, Springfield Mill, Maidstone, Kent. Nylon gauze (Nylon Beuteltuch, 13 xxx 100 Nybolt) was from Schweizerische Seidengazefabrik, Zürich. Fresh spinach was obtained from the local market. The spinach was washed under tap water and freed of stems prior to use.

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The molecular weight of ferredoxin was determined by gel chromatography on sephadex G-50 column (100 cm × 1.5 cm) equilibrated with 50 mM K-phosphate buffer pH 6.8. Vitamine B_{12} (MW = 1380), the b-chain of insulin (MW = 3400), clostridial ferredoxin (MW = 6000), cytochrom c (MW = 12500), myoglobin (MW = 17500), Shetna flavoprotein (MW = 23000), and chymotrypsinogen (MW = 25000) were used as markers. The proteins were eluted from the column with a flow rate of 0.5–0.7 ml/min. Shetna flavoprotein was a gift of Dr. H. Bothe, Heidelberg.

Non-heme iron was determined as bathophenanthroline complex according to Doeg and Ziegler [9], acid-labile sulfur according to Siegel [10] as modified by King and Morris [11].

The activity of purified ferredoxin was assayed by measuring the photoreduction of NADP as described by Buchanan and Arnon [6].

Results

The procedure described below is for the purification of ferredoxin from 1 kg of spinach leaves (weight without stems). The procedure can be applied, however, to amounts of spinach between 100 g and 3 kg providing it is appropriately scaled down or up. Unless otherwise stated all steps were performed at 4 °C under aerobic conditions.

Step 1. Preparation of leaf extract: 1 kg of spinach leaves were homogenized using a "Drei-Walzen-Mühle" (Modell SDW 55/3 from Vogel, Köln) under successive addition of 250 ml 20 mM Tris-HCl pH 7.8. The resulting slurry was filtered

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through a double layer of nylon gauze at a pressure of 450 kg/cm². About 1 l extract was obtained from 1 kg of spinach leaves. The pH was adjusted to pH 7.8 with Tris base.

Leaf extract was also obtained from 200 g portions of spinach using a household juice extractor (Multipress MP 32 from Braun, Frankfurt). This method was, however, less efficient and resulted in considerably lower yields of ferredoxin (see below).

Step 2. Precipitation of contaminating material: 40 ml of acetone precooled to $-20\,^{\circ}C$ was added to 60 ml of spinach extract under continuous rapid stirring with a magnetic paddle. Then the pH was adjusted with 1 N HCl to 7.8 and the conductivity was adjusted to that of a 60 mM NaCl solution with 40% acetone/water. The correct adjustment of these two parameters proved to be important, because they affect the subsequent precipitation of ferredoxin with polyethyleneimine. Now 20 ml 10% PEI 600 solution (or 15 ml 10% Polymin P solution, or 6 ml PEI 18 solution) was added to 1000 ml of the 40% acetone containing spinach extract under rapid stirring. This was followed by a centrifugation for 20 min at $15000 \times g$. A yellow green supernatant was obtained, which contained the ferredoxin.

Step 3. Precipitation of ferredoxin: Ferredoxin was precipitated from the yellow green supernatant by the addition of 25 ml 10% PEI 600 solution (or 20 ml Polymin P solution, or 14 ml 10% PEI 18 solution) under rapid stirring with a magnetic rod. After centrifugation for 20 min at $15000 \times g$ a brown red compact pellet was obtained containing the ferredoxin. The pellet was transferred to a mortar and was extensively homogenized with sea sand under successive addition of small portions of 65% ammonium sulfate (adjusted to pH 7.8 with Tris base). Approximately 30 ml 65% ammonium sulfate solution were required to dissolve most of the ferredoxin, which was then separated from non dissolved material and the sea sand by centrifugation for 25 min at $48000 \times g$. The resulting pellet was extracted once with 20 ml 65% ammonium sulfate. After centrifugation the two supernatants were combined.

Step 4. Adsorption to DEAE cellulose at high ionic strength: The dark brown 65% ammonium sulfate solution obtained in step 3, contained besides ferredoxin other components, such as polyphenols and flavins, which were separated from ferredoxin by using a small column (1 cm ⌀) filled 2.5 cm high with DE 52, which was preequilibrated with 65% ammonium sulfate pH 7.8. Ferredoxin and polyphenols adsorbed to DE 52 under these conditions, the polyphenols turning the cellulose to black. The column was washed with 65% ammonium sulfate until the eluate was nearly colourless; approximately 60 ml were required. Then the ferredoxin was eluted from the column with 10% ammonium sulfate pH 7.8, the polyphenols remaining adsorbed to the DEAE cellulose. 2 ml fractions were collected; fraction 3 – 12 contained most of the ferredoxin, which was recognised by its typical reddish colour. The absorbance ratio A_{420}/A_{275} at this stage of purification was between 0.3 – 0.35.

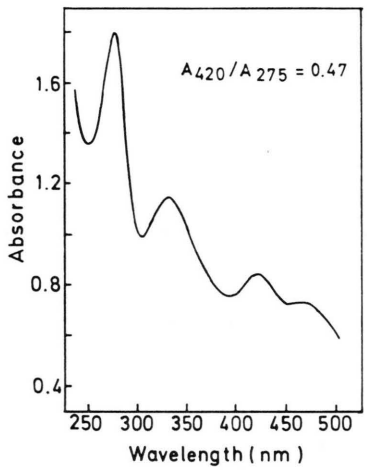

Fig. 1. Spectrum of ferredoxin from spinach obtained after step 5 of the purification procedure.

Step 5. Adsorption to DEAE cellulose at low ionic strength: The ferredoxin containing eluate was diluted (approximately 1 : 8) with cold water until it had the same conductivity as a 130 mM NaCl solution. Ferredoxin was then adsorbed to DEAE cellulose using a column (1 cm ⌀) filled 2.5 cm high with DE 52, which was preequilibrated with 0.1 M Tris-HCl pH 7.8. A red band was formed at the top of the column. Then the column was washed with approximately 50 ml 0.2 M Tris-HCl pH 7.8 until the reddish ferredoxin band descended to within 0.5 cm from the bottom of the bed. Now the ferredoxin was eluted with 0.2 M Tris-HCl pH 7.8 containing 0.27 M KCl. About 3 ml of eluate

containing the ferredoxin was obtained per kg of spinach leaves. The A_{420}/A_{275} of the dark red ferredoxin solution was between 0.45 and 0.48, indicating that the ferredoxin obtained was essentially pure [6].

Yields and properties: Based on a molar extinction coefficient [12] $\varepsilon_{420} = 9700\ \text{M}^{-1}\ \text{cm}^{-1}$ and a molecular weight [12] of 11700 25 – 35 mg of ferredoxin were generally obtained after step 5 from 1 kg of spinach leaves. If the leaf extract was prepared with a household juice extractor only a yield of approximately 18 mg ferredoxin/kg spinach was obtained.

The ferredoxin isolated with polyethyleneimines was found to have a molecular weight of 12000 and to contain 2 mol of nonheme iron and an equal amount of acid-labile sulfur. It had the same specific activity in the photoreduction of NADP as that isolated by the procedure described by Tagawa and Arnon [2].

Discussion

With the procedure described highly purified ferredoxin with a A_{420}/A_{275} between 0.45 and 0.48 was isolated from spinach in less than 8 hours. Published procedures require at least 2 and some 4 days for obtaining ferredoxin with a A_{420}/A_{275} higher as 0.45. 25 – 35 mg of ferredoxin were obtained from 1 kg of spinach leaves. This yield is considerably higher than that obtained with other methods (approximately 20 mg [1 – 6]). The costs of the new method are low. Polyethyleneimines are inexpensive and, compared to other procedures, only small amounts of DEAE cellulose are required (approximately 5 g DE 52 per kg spinach).

We thank Prof. Trebst, Ruhr-Universität Bochum, for many helpful discussions. This work was supported by a grant from the Deutsche Forschungsgemeinschaft and the "Fonds der Chemischen Industrie".

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