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Nondenaturing Procedure for Rapid Preparation of Ferredoxin from *Clostridium pasteurianum*[1]

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Lovenberg and Williams (1) have reported that, when a solution of *Clostridium pasteurianum* ferredoxin in 90% ammonium sulfate is treated on a column of DEAE-cellulose, ferredoxin is retained by the column. They showed that ferredoxin is eluted when the ammonium sulfate concentration is lowered to about 35% saturation. These observations suggested the method described in this paper for purifying ferredoxin from crude extracts of *C. pasteurianum*. This novel procedure provides pure ferredoxin in yields comparable with those obtained using a method described earlier by Mortenson (2). Since it avoids the strongly denaturing conditions that occur during the initial acetone fractionation of Mortenson's method (2), this procedure may be especially useful when other proteins are to be prepared from the same starting material.

MATERIALS AND METHODS

Clostridium pasteurianum (American Type Culture Collection 6013) was grown with ammonium sulfate as nitrogen source on the medium of Carnahan and Castle (3). Cells were harvested with a Sharples super centrifuge, and dried in a vacuum, over H_2SO_4 at room temperature. Dried cells were ground to a fine powder and stored at −20°. *Peptostreptococcus elsdenii* (strain LC 1) was grown in iron-rich medium; ferredoxin-free extracts from this organism were prepared as described previously (4). Ferredoxin was assayed by measuring its stimulation of acetyl phosphate production from pyruvate by a ferredoxin-free extract from *P. elsdenii* (4). Extracts from *P. elsdenii* were used for this assay because they are more stable than similar extracts from *C. pasteurianum* (5). Iron was determined according to Massey (6). For DEAE-cellulose chromatography, Whatman DE22 was used. Ammonium sulfate (enzyme grade) was from Mann. Before application to a column of DEAE-cellu-

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lose, solutions of ammonium sulfate were freed of dissolved gases by evacuation at a water pump.

RESULTS AND DISCUSSION

The starting material for this procedure is 45 gm of dry cells of *C. pasteurianum*. The cells are extracted by autolysis in 450 ml of water at 30° for 1 hr under a nitrogen atmosphere. All subsequent operations are done at 4°. Cell debris is removed from the extract by centrifugation (25,000*g* for 30 min) and resuspended in 450 ml of water. This mixture is centrifuged again, and the supernatant added to the initial extract.

The combined extracts are adjusted to 0.1 *M* Tris-HCl, pH 7.6, by addition of 0.5 *M* Tris-HCl buffer, pH 7.6. Solid ammonium sulfate is added slowly to this solution to 55% saturation and after stirring for 15 min a precipitate is removed by centrifugation (25,000*g* for 20 min) and discarded. The amber colored supernatant is applied to a column of DEAE-cellulose (25 × 2 cm diameter) equilibrated with 55% ammonium sulfate and 0.1 *M* Tris-HCl buffer, pH 7.6. Ferredoxin is retained at the top of the column in a black band. The column is washed with 500 ml of 40% ammonium sulfate in 0.1 *M* Tris-HCl buffer, pH 7.6. This strips a yellow band from the column, and slowly elutes a red colored band that contains rubredoxin (7). The column is then washed with 30% ammonium sulfate in 0.1 *M* Tris-HCl, pH 7.6, to elute ferredoxin as a brown solution. Fractions showing an absorption ratio ($A_{390\ m\mu}/A_{280\ m\mu}$) of 0.5 or greater are combined.

The combined fractions are placed in a Büchner flask and solid ammonium sulfate is added in about six portions to 60% saturation. After each addition of ammonium sulfate, and before stirring the mixture to dissolve the salt, the flask is evacuated at a water pump and filled with nitrogen. This precaution is taken to minimize losses of ferredoxin due to exposure to air. The solution is stirred for 30 min after the final addition of ammonium sulfate, centrifuged (20,000*g* for 10 min), and the precipitate is discarded. The supernatant is applied to a second column of DEAE-cellulose equilibrated with 60% ammonium sulfate in 0.1 *M* Tris-HCl, pH 7.6. The column is washed with 30% ammonium sulfate in 0.1 *M* Tris-HCl, pH 7.6. This first elutes traces of rubredoxin and other contaminating proteins, and then elutes ferredoxin. Brown fractions with an absorption ratio ($A_{390\ m\mu}/A_{280\ m\mu}$) of 0.82 are combined. Ferredoxin is precipitated from this solution by addition of solid ammonium sulfate to 100% saturation. This step is carried out in a Büchner flask under a nitrogen atmosphere as described above. The mixture is centrifuged (20,000*g* for 15 min) and the black precipitate is dissolved in 0.1 Tris-HCl, pH 7.6.

TABLE 1
Purification of *C. pasteurianum* Ferredoxin

Purification step	Volume ml	Total activity units	Yield, %	$A_{390 m\mu}$	$A_{390 m\mu}/A_{280 m\mu}$
Crude extract[a]	685	2860	100	—	—
First DEAE-cellulose column	218	2740	96	0.71	0.55
Second DEAE-cellulose column	169	1720	60	0.49	0.82
$(NH_4)_2SO_4$ precipitate	7.4	1630	57	11.5[b]	0.82

[a] The starting material was 45 gm of dry cells.
[b] Determined on a sample diluted 10-fold into 0.1 *M* Tris-HCl, pH 7.6.

Details of this procedure are given in Table 1. The data show that about 60% of the ferredoxin in the initial extract is recovered in the final ammonium sulfate precipitate. The yield of ferredoxin is thus comparable with yields obtained using the procedure described by Mortenson (2). In the preparation of Table 1 the absorption ratio ($A_{390 m\mu}/A_{280 m\mu}$) for the ferredoxin from the first DEAE-cellulose column was 0.55. In other preparations, this ratio was as high as 0.81. However, in these preparations the cells were extracted only once, and the ferredoxin in the extract was about 20% lower than in the extract of Table 1. Protein was not estimated during this preparation because colorimetric determinations based on the biuret reaction are known to be unreliable when they are used to determine ferredoxin (2,5). However, based on a specific extinction coefficient of 34 at 390 mμ (5), the total ferredoxin obtained in this preparation was 25 mg.

The absorption spectrum of ferredoxin isolated by this procedure is similar to that reported by Lovenberg *et al.* (5) and by Mortenson (2) for *C. pasteurianum* ferredoxin. It shows absorption maxima at 385–390 mμ, 300 mμ, and 285 mμ. The maxima at 300 and 285 mμ have equal intensity; the minimum between them occurs at 293 mμ. The absorption ratio ($A_{390 m\mu}/A_{280 m\mu}$) is 0.82, and is identical with a previously reported value for *C. pasteurianum* ferredoxin (5). Analyses for total nonheme iron (6) in a sample of ferredoxin made by this new method showed that the extinction coefficient per iron is 3840 M^{-1} cm^{-1} at 390 mμ. Several values have been reported for the molar extinction coefficient of *C. pasteurianum* ferredoxin at this wavelength (e.g., 2, 8, 9). Based on the highest value reported (30,000 M^{-1} cm^{-1} (8)) the present iron analyses indicate that the preparation contained 7.8 atoms of iron per molecule. This value is in substantial agreement with the 7 iron atoms per molecule of *C. pasteurianum* ferredoxin reported by Lovenberg *et al.* (5), and it is the same as the iron content recently reported for *Clostridium acidiurici*

ferredoxin by Hong and Rabinowitz (10). In an extensive investigation, Hong and Rabinowitz (10) determined an extinction coefficient of 30,600 M^{-1} cm^{-1} at 390 mμ for *C. acidiurici* ferredoxin.

This procedure offers certain advantages over other methods which have been used to prepare *C. pasteurianum* ferredoxin (2,5). Ferredoxin can be prepared rapidly and in high yield, and since all of the fractionation steps are relatively mild other proteins may be prepared from the same starting material.

The phenomenon by which ferredoxin is retained on DEAE-cellulose in concentrated ammonium sulfate is not completely understood. However, it seems unlikely that ionic bonding with DEAE-cellulose is involved; positively charged proteins such as ribonuclease and horse heart cytochrome *c* also show this behavior. This phenomenon is discussed in greater detail in another paper (11).

SUMMARY

A procedure is described for the rapid preparation of ferredoxin in high yield from crude extracts of *C. pasteurianum*. The method involves two successive chromatographic treatments on DEAE-cellulose in concentrated ammonium sulfate. Ferredoxin prepared by this method has an absorption spectrum and iron content similar to ferredoxin prepared by other methods.

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