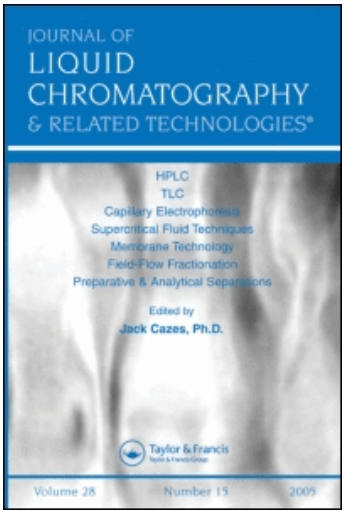

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Use of Adsorption Chromatography on Sephacryl S-500 for Improved Separation of Isoforms of Soluble Photosynthetic Catalysts from Cyanobacteria

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USE OF ADSORPTION CHROMATOGRAPHY ON SEPHACRYL S-500 FOR IMPROVED SEPARATION OF ISOFORMS OF SOLUBLE PHOTOSYNTHETIC CATALYSTS FROM CYANOBACTERIA

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ABSTRACT

Sephacryl S-500, a gel-filtration matrix, adsorbs water-soluble proteins at high concentrations of ammonium sulfate. It has been used here as a matrix for adsorption chromatography, separating isoforms of cytochrome c_{553} and ferredoxin from *Oscillatoria sp.* and *Microcystis aeruginosa*, respectively. The isoforms of these proteins were separated at 35% and 40% saturated ammonium sulfate, respectively. Using this adsorption chromatography, we have purified high levels of individual isoforms with improved resolution over conventional procedures such as polyacrylamide gel electrophoresis and ion-exchange chromatography.

Introduction

Cytochrome c_{553} and ferredoxin are valuable tools for studying structure-function relationships, and both are small, water-soluble proteins acting as photosynthetic electron carriers (1). While ferredoxin is common among photosynthetic organisms, cytochrome c_{553} has only been found in eukaryotic algae and

cyanobacteria. The presence of isoforms of either protein in the same organism has been reported in a number of species (2-5). For examination of individual isoforms, large amounts of purified proteins are required. Most methods of purification rely on the surface charges of individual isoforms. In certain cases, the proteins show very similar charge and have to be purified by hydrophobic chromatography (6) or reversed-phase HPLC (7). These methods entail the use of expensive gel matrices or equipment and have not been widely adopted. Recently, we found that Sephacryl S-type gels adsorb proteins at high ammonium sulfate concentrations and such adsorption can be used with advantages for protein separation (8). Here, the separation of isoforms of cytochrome c_{553} and ferredoxin on Sephacryl S-500 has been studied using a high concentration of ammonium sulfate. The results show that this gel matrix provides a convenient and inexpensive way to purify individual isoforms which are not readily resolved by ion-exchange chromatography or gel electrophoresis.

MATERIALS AND METHODS

Sephacryl S-500 was purchased from Pharmacia. All other chemicals were of analytical reagent grade. *Oscillatoria sp.* and *Microcystis aeruginosa* were grown and harvested according to Kang et al. (9) Protein purifications were conducted at 4°C.

Purification of ferredoxin from *Microcystis aeruginosa*

The packed cells (28 g fresh wt.), resuspended in 50 mM NaCl-50 mM Tris/HCl (pH 7.5), were sonicated for nine 1-min intervals with a Sonifier cell disruptor B-30 (power at two-fifths maximum). After centrifuging at 15,000 rpm for 10 min, the

pellets were resuspended and sonicated again. These steps were repeated several times until the supernatant turned pale blue. All the soluble extracts were pooled, made to 35% $(NH_4)_2SO_4$ saturation and centrifuged for 20 min at 40,000 rpm. The resulting supernatant was adjusted to 60% $(NH_4)_2SO_4$ saturation and centrifuged again for 20 min at 40,000 rpm. The final supernatant was loaded to a Sephacryl S-200 column (1.5 X 17.5 cm) previously equilibrated with 60% $(NH_4)_2SO_4$ saturation in the NaCl/Tris buffer. Ferredoxin and other proteins adsorbed to the column were eluted with a 500 ml gradient of $(NH_4)_2SO_4$ decreasing from 60% to 35% saturation in the NaCl/Tris buffer. Fractions containing ferredoxin were pooled, dialyzed several times against a Tris/HCl buffer (50 mM, pH 7.5), concentrated with an Amicon flow-cell (10 kDa cut-off) and passed through a Sephadex G-50 column (2.5 X 90 cm) washed with the Tris/HCl buffer. Ferredoxin was finally adsorbed to a DEAE-Sephacel column (1.5 X 6 cm), previously equilibrated with the Tris/HCl buffer. After washing the column with 250 mM NaCl followed by 280 mM NaCl in the Tris/HCl buffer, proteins were eluted by a 400 ml 280-330 mM NaCl gradient. The protein, eluted at around 300 mM NaCl, was used for SDS and non-denaturing gel electrophoresis, IEF and adsorption chromatography on Sephacryl S-500.

Purification of cytochrome c553 from *Oscillatoria sp.*

The packed cells (30 g fresh wt.) were broken after several cycles of freezing and thawing in 50 mM Tris/HCl-50 mM NaCl (pH 7.5) and centrifuged for 10 min at 15,000 rpm. The initial purification of the soluble extract was similar to that of *Microsystis aeruginosa*. This included fractionation at 35% and

75% $(NH_4)_2SO_4$ saturation and Sephacryl S-200 chromatography. The latter was developed with a 400 ml $(NH_4)_2SO_4$ gradient decreasing from 60% to 30% saturation in 100 mM Tris/HCl, pH 8.6. Fractions containing the cytochrome were pooled, dialyzed several times against 5 mM Mes-Tris, pH 6.0, and adsorbed to a CM-Sephadex column (1 X 6 cm), previously washed with the dialyzing buffer. The cytochrome, eluted with a Tris-HCl buffer (25 mM, pH 7.5), was concentrated by an Amicon flow-cell (10 kDa cut-off), and passed through a Sephadex G-50 column (1.5 X 30 cm) washed with the Tris-HCl buffer. Fractions containing the cytochrome were pooled and used for SDS and non-denaturing gel electrophoresis, IEF and adsorption chromatography on Sephacryl S-500.

Analytical methods

Protein concentration in column effluents was monitored at 206 nm using an LKB UV detector. Cytochrome concentration was estimated by a reduced minus oxidized spectrum, using 20 $mM^{-1}cm^{-1}$ as the difference absorption coefficient at the 553 nm (10). The absorption difference (ascorbate minus ferricyanide) was made at 553 nm relative to a line drawn through the absorbances at 542 and 561 (11). Ferredoxin was estimated from the oxidized spectrum of the sample at 423 nm using an extinction coefficient of 9.7 mM^{-1} cm^{-1} (12). All estimations were made at room temperature with a Shimadzu UV-160 spectrophotometer.

Polyacrylamide gel electrophoresis

Non-denaturing and SDS gel electrophoresis were performed in either 10 or 15% acrylamide gel rods using the high pH buffer system of Hames (13). After electrophoresis, the gels were stained for proteins using Coomassie brilliant blue G-250 (14). IEF gel electrophoresis was conducted on gel rods as described by Righetti and Drysdale (15).

Adsorption chromatography on Sephacryl S-500

This was conducted at room temperature using a Sephacryl S-500 column (16.5 x 1.5 cm). For cytochrome c_{553}, the column was washed with 100 mM Tris/HCl (pH 8.0), 200 mM NaCl and $(NH_4)_2SO_4$ adjusted to 35% saturation. For ferredoxin, the washing buffer and $(NH_4)_2SO_4$ were adjusted to pH 8 and 40% saturation, respectively. The flow-rate was maintained at 11 ml h^{-1}. Protein samples were prepared in small volumes of washing buffers.

RESULTS

Tables 1 and 2 summarize the purification of ferredoxin and cytochrome c_{553}, respectively. At the early stage, high concentrations of ammonium sulfate were used to precipitate the large amounts of colored pigments interfering with the purification. Prior to dialysis, the ammonium sulfate supernatant was adsorbed on Sephacryl S-200 and the adsorbed proteins were subsequently fractionated by a decreasing salt gradient. Ferredoxin and cytochrome c_{553}, both eluted between 35% to 45% ammonium sulfate saturation, were found to be completely free of the remaining pigments which remained tightly bound at these concentrations. The two proteins were also recovered in much smaller volumes than that of the starting ammonium sulfate supernatant. This cut down the amount of time and buffers needed for dialysis.

Ferredoxin eluted from the final DEAE-Sephacel column as a broad band characterized by a distinct shoulder and a main peak (Fig.1). The proteins corresponding to the peak and shoulder were pooled and analyzed by gel electrophoresis. The results show a single protein band on 15% SDS and 10% non-denaturing gels and two very close bands on a 15% non-denaturing gel. An unsuccessful

Table 1

Purification of ferredoxin from *Microcystis aeruginosa*

Fraction	Total protein(mg)	Ferredoxin (mM)	% recovery	Degree of purification
Crude extract	3585	-	-	-
35% $(NH_4)_2SO_4$	1696	-	-	-
60% $(NH_4)_2SO_4$	164	-	-	-
Sephacryl S-200	92.1	2.5×10^{-3}	100	1
Sephadex G-50	11.0	0.64×10^{-3}	26	2.5
DEAE-Sephacel	3.09	0.28×10^{-3}	11	4.5

Table 2

Purification of cytochrome c_{553} from *Oscillatoria sp.*

Fraction	Total protein(mg)	cytochrome (mmol)	% recovery	Degree of purification
Crude extract	1080	-	-	-
35% $(NH_4)_2SO_4$	500	-	-	-
75% $(NH_4)_2SO_4$	30.6	-	-	-
Sephacryl S-200	20.99	1.4×10^{-4}	100	1
Sephadex G-50	5.1	1.0×10^{-4}	71	4.2
DEAE-Sephacel	1.0	0.8×10^{-4}	57	4.5

- Prior to the Sephacryl step, blue phycobiliproteins in the extract interfered with ferredoxin determination.

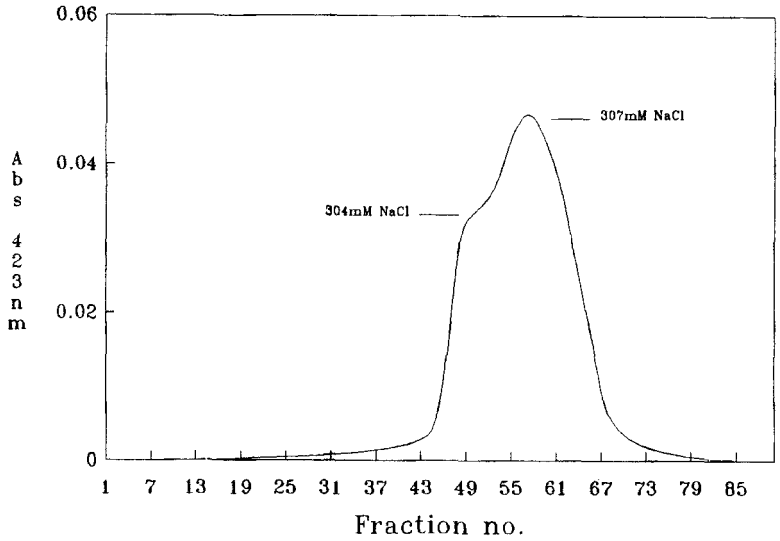

Fig. 1. Elution profile of *Microcystis aeruginosa* ferredoxin from DEAE-Sephacel column (1.5 X 6 cm). Ferredoxin was eluted with a 500 ml linear salt gradient from 280 mM to 330 mM NaCl in 50 mM Tris/HCl, pH 7.5.

attempt was made to resolve the protein on an IEF gel. As the protein approached its pI value on the gel, the low pH enviroment caused it to denature and form a broad yellow smear at the lower portion of the gel.

Cytochrome c_{553} eluted as a single band during CM-Sephadex chromatography. After the final Sephadex G-50 column chromatography, the purified protein was subjected to gel electrophoresis. It migrated as a single band on 15% non-denaturing and SDS gels. The protein focused to a pink band near the alkaline end of an IEF gel and showed a pI value of 9.3.

Figure 2 shows the chromatographic behaviour of the purified ferredoxin and cytochrome c_{553} on a Sephacryl S-500 gel column washed with high concentrations of $(NH4)_2SO4$. The results show that the isoforms of these proteins were clearly resolved.

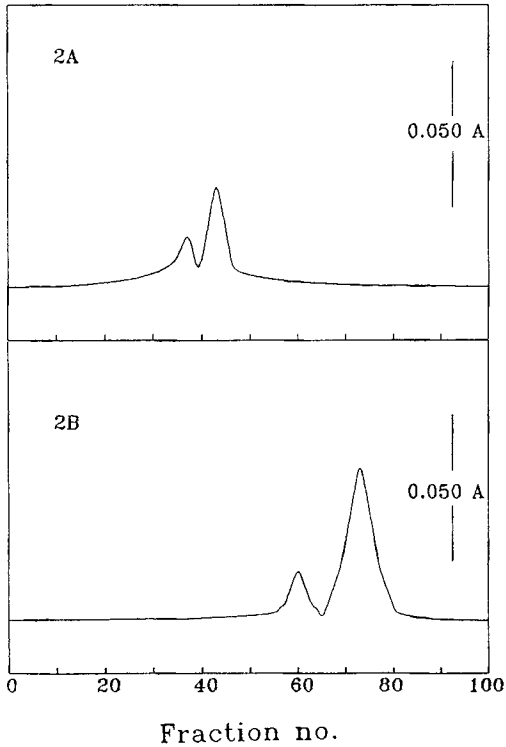

Fig. 2. Elution profile of purified proteins from Sephacryl S-500 (1.5 X 16.5 cm) in the presence of ammonium sulfate at 35% saturation in 200 mM NaCl, 50 mM Tris/HCl. (A) *Microcystis aeruginosa* ferredoxin, 0.8 mg, was separated at pH 7.5. (B) *Oscillatoria sp.* cytochrome c_{553}, 1.4 mg, was separated at pH 8.0. The elution was monitored at 206 nm.

We concentrated the peak fractions of individual ferredoxin isoforms and ran them separately on 15% non-denaturing gels. Figure 3 shows the electrophoretic profiles of individual isoforms and a sample containing a mixture of the two isoforms. The results clearly show that the first and second isoforms eluted off the Sephacryl S-500 column correspond to the fast and the slow-moving species on the non-denaturing gel, respectively.

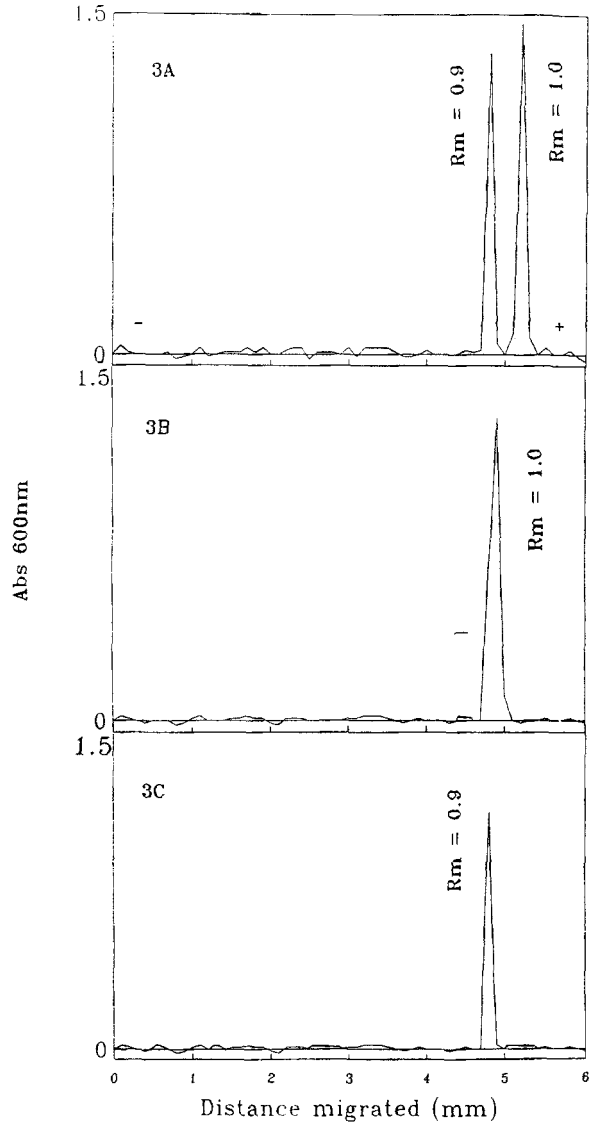

Fig. 3 Densitometric scans at 600 nm of *Microcystis aeruginosa* ferredoxin on nondenaturing 15% PAGE gels. Protein bands were stained with Coomassie Blue. (A) shows a mixture of the two ferredoxin isoforms, about 2 ug each . (B) and (C) represent about 2 ug each of the first and second ferredoxin isoforms, respectively. Rm is expressed as distance migrated by the protein band divided by the distance migrated by the dye front.

We also ran the two separate forms of cytochrome c_{553} through reversed-phase HPLC and found them eluted at different retention times (result not shown).

DISCUSSION

The present work exploits the adsorption phenomenon on Sephacryl S-500 for protein separation. This procedure provides a better separation for the two isoforms of ferredoxin from *Microcystis aeruginosa*, compared with gel electrophoresis. Moreover, it gives a higher yield of the purified proteins. At high concentrations of ammonium sulfate, hydrophobic interactions contribute to protein adsorption on Sephacryl S-type gels (8). These interactions are most likely different between individual isoforms, thus allowing them to be separated on Sephacryl S-500.

Hydrophobic chromatography has been used previously to separate two isoforms of spinach ferredoxin, but this required a hydrophobic matrix, TSK-gel Phenyl-5PW (6). On the contrary, Sephacryl S-500 is less costly and is commonly used for gel-filtration chromatography. Its rigid matrix also allows high flow-rates.

The isoforms of cytochrome c_{553} from a local strain of *Oscillatoria sp.* were not resolved by either ion-exchange chromatography or gel electrophoresis, probably because of their very similar surface charges. This agrees with an early study which found that reversed-phase HPLC had to be used for separating two isoforms of cytochrome c_{553} from an American strain (7). Since reversed-phase HPLC results in protein denaturation, a more gentle procedure such as the one described here should be useful for functional studies.

Adsorption chromatography was used not only in the final separation of the ferredoxin or cytochrome c_{553} isoforms but also at the early stage of purification. Right after the ammonium sulfate step, the soluble proteins were adsorbed and subsequently fractionated on Sephacryl S-200. This step, designed to remove the remaining colored pigments which interfered with the purification, worked better with a continuous gradient of decreasing ammonium sulfate. The salt gradient seemed to work against the separation of the isoforms of ferredoxin and cytochrome c553 on Sephacryl S-type gels. These proteins were eluted off Sephacryl S-200 as broad bands. Individual isoforms of these proteins separated somewhat better when Sephacryl S-500 was used instead, but the resolution was still poor. It is noteworthy that Sephacryl S-200 is less hydrophobic than Sephacryl S-500 (8), and this may explain why individual isoforms were not separated at all on this matrix. However, Sephacryl S-200 was chosen in preference to Sephacryl S-500 at this stage of purification because of its greater flow-rate and smaller elution volumes for the proteins.

Apart from Sephacryl S-type gels, there are other gel-filtration matrices known to adsorb proteins in the presence of high concentrations of ammonium sulfate (16-19). Very few have been exploited for protein separation based on their adsorptive properties. Here, we demonstrated that Sephacryl S-500 could be useful for purifying isoforms in a preparative scale.

CONCLUSION

Cyanobacterial cytochrome c_{553} and ferredoxin isoforms were separated successfully using adsorption chromatography on Sephacryl S-500. This method achieves better resolution and

higher yields of purified proteins than gel electrophoresis. Adsorption chromatography, like reversed-phase HPLC separates proteins according to their relative hydrophobicities and does this without denaturing the protein. Herein lie its advantages in the separation of proteins that differ appreciably in hydrophobicities. Prior to the submission of this manuscript, we have succeeded in extending the present procedure to separation of isoforms of ferredoxin from a variety of high plants.

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