USE OF IMMUNOADSORBENT AFFINITY CHROMATOGRAPHY TO PURIFY COMPONENT I OF NITROGENASE FROM EXTRACTS OF *AZOTOBACTER VINELANDII*

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1. Introduction

One of the main problems associated with the separation and purification of Component I (Mo–Fe–S protein) and Component II (Fe–S protein) of the nitrogenase complex from N_2-fixing microorganisms is the O_2 lability of these proteins. It would therefore be of great advantage to be able to quickly purify these components from crude extracts of the bacteria.

In this paper we describe a simple and quick method for separating and purifying Component I from cell-free extracts of *Azotobacter vinelandii*. This separation is achieved by affinity chromatography on a Sepharose 4-B immunoadsorbent column followed by treatment on a Sephadex G-25 column. The purified Component I so separated has been identified by a number of procedures, namely, the acetylene reduction assay, SDS polyacrylamide gel disc electrophoresis and rocket immunoelectrophoresis.

2. Materials and methods

2.1. *Preparation of Components I and II*

A. vinelandii strain O was grown in Burk's nitrogen-free culture solution, and Components I and II were separated and purified as described by Nicholas and Deering [1].

2.2. *Production of antiserum to Component I and purification of the IgG fraction*

Antiserum to Component I (Fraction V, Table 1, ref. [1]) was raised in a goat by a series of once-fortnightly subcutaneous injections over a period of eight weeks using aliquots of 3.5 mg purified Component I in Complete Freund's Adjuvant. The animal was challenged with the same dose of antigen three months later and serum obtained in the hyperimmune state. The IgG-rich fraction of the antiserum was prepared by neutral salt fractionation [1].

2.3. *Preparation of immunoadsorbent*

The IgG fraction of goat antiserum was coupled to CNBr-activated Sepharose 4-B (Pharmacia, Uppsala, Sweden). The IgG fraction was dialysed against frequent changes of freshly prepared 0.25 M NaCl in 0.1 M $NaHCO_3$ coupling buffer plus 0.02% (w/v) Thimerosal (Sigma Chemical Co., St. Louis, Mo., USA) for 16 h at 2°C. A concentration of 10 mg IgG per 8 g CNBr-activated Sepharose 4-B was used. The gel was swollen and washed in a sintered glass funnel using 200 ml 0.001 M HCl for 15 min. Three ml IgG solution (in coupling buffer) was mixed with the active gel and gently rotated for 12 h at 2°C. Unbound IgG was removed in 500 ml coupling buffer and the gel reacted with 1 M ethanolamine (pH 8) for 2 h to block unreacted active sites. The gel was washed alternately using three cycles of 1 M NaCl in 0.1 M acetic acid (pH 4.0) and 1 M NaCl in 0.1 M borate (pH 8.5) to remove non-covalently bound protein. The gel was finally washed with 0.025 M Tris-HCl buffer (pH 7.5) and then pre-cycled with 4 M $MgCl_2$ prior to equilibration in the Tris-HCl buffer containing 0.02% (w/v) Thimerosal. The immunoadsorbent, packed into a glass column (2 × 10 cm), was maintained under strictly anaerobic conditions [1].

2.4. *Assay for Component I*

The C_2H_2-reduction assay for nitrogenase activity was used as described previously [1]. The activity of Component I was measured in the presence of a saturating amount of purified Component II as determined by titration [1].

2.5. *Electrophoreses*

The polyacrylamide gel disc electrophoresis method used has been described [1]. Rocket immunoelectrophoresis was performed by the procedure of Svendsen [2].

3. Results and discussion

The flow chart for the preparation of Component I from crude extracts of *Azotobacter* by the immunoadsorbent column method is as follows:

*Purified Component I *or* Crude Extract (S_{105})

↓ 0.025 M Tris-HCl buffer (pH 7.5)

Immunoadsorbent Sepharose 4-B column

↓ (1) Wash with the Tris-HCl buffer
(2) Elute with 3 M $MgCl_2$

Sephadex G-25 Column

↓ Elute with the Tris-HCl buffer

Component I
(identified by various methods)

Flow chart for the preparation of purified Component I from crude extracts of *Azotobacter vinelandii.*

*Purified Component I (Fraction IV, Table 1, ref. [1]) was used as a standard in this sequence.

The results in table 1 show that the fraction from the crude extract of *Azotobacter,* first eluted by 3 M $MgCl_2$ from the immunoadsorbent column and subsequently passed through a Sephadex G-25 column, reduced C_2H_2 to C_2H_4 when supplemented with saturating amounts of purified Component II in the test system. This confirms that active Component I can be separated (54-fold purification) from crude

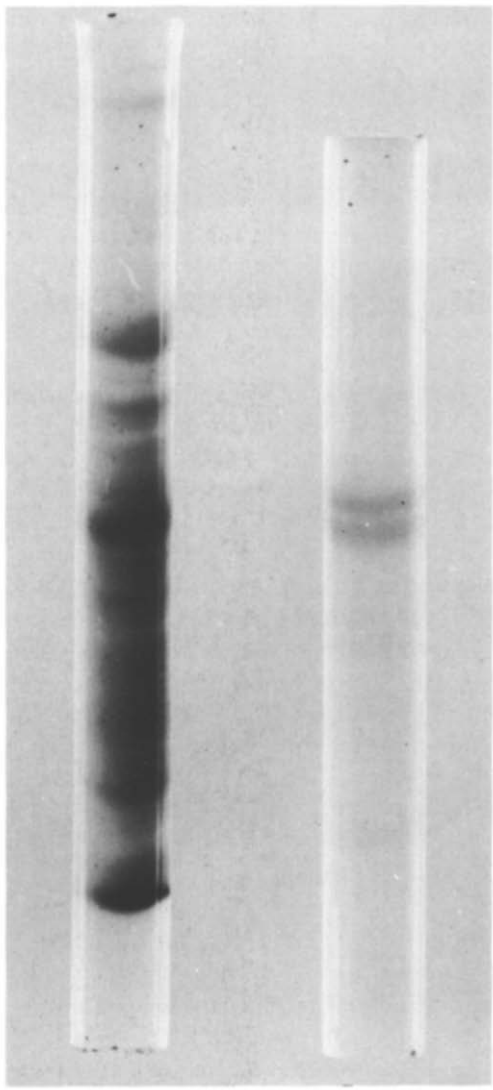

Fig.1. Polyacrylamide gel disc electrophoresis. (Left-hand gel) 200 μg crude extract of *A. vinelandii.* (Right-hand gel) 90 μg eluate from Sephadex G-25 column incubated with 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol at 100°C for 5 min and run in a system containing 1% (w/v) SDS [1].

Table 1
The C_2H_2 reducing activity of Component I purified by immunoadsorption

	Activity of Component I[a]	Sephadex G-25 eluate containing Component I	Activity of crude extract	Sephadex G-25 eluate containing Component I
C_2H_2 reduction units (nmoles C_2H_2 red./10 min)	43	16	97	190
Specific activity (units/mg protein)	17 080	20 500	473	25 300
Purification	1	1.2	1	54

[a]Component I eluted from DEAE-32 column (Fraction IV, table 1, ref. [1]) *or* a crude extract was loaded onto the immunoadsorbent column, washed with 0.025 M Tris-HCl buffer (pH 7.5) and eluted with 3 M $MgCl_2$. The eluate was desalted on a Sephadex G-25 column (19 × 2.3 cm) using 0.025 M Tris-HCl (pH 7.5). The C_2H_2 reducing activities of Components I and crude extracts respectively were assayed in the presence of saturating amounts of Component II [1].

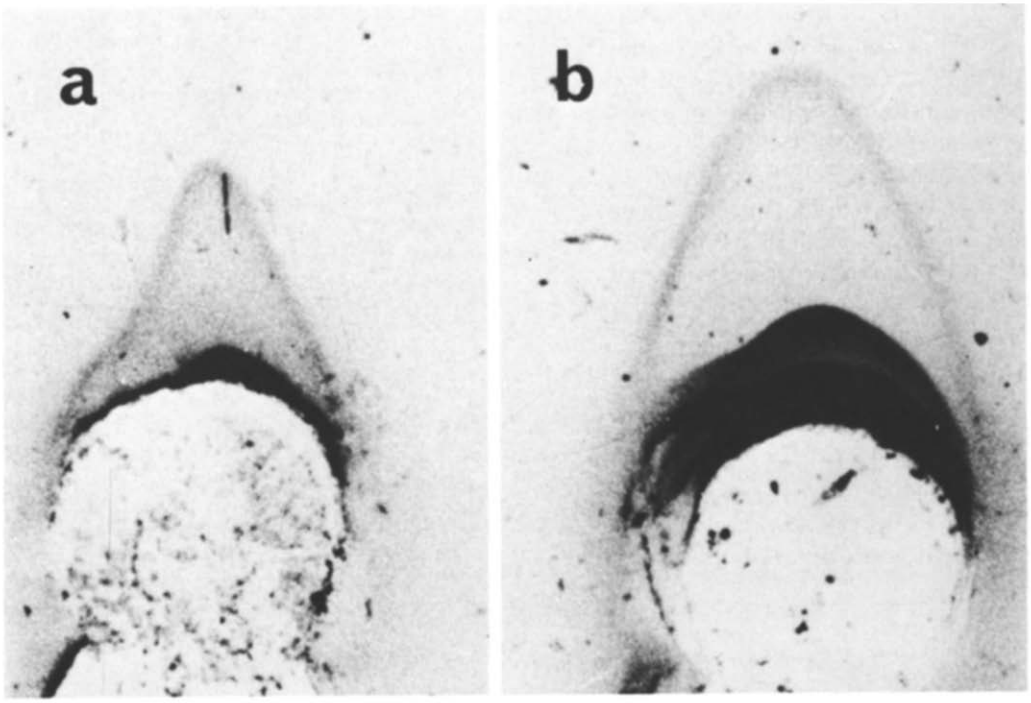

Fig. 2. Immunoelectrophoresis. Rocket immunoelectrophoresis of (a) 10 μl (7.5 μg protein) and (b) 33 μl (25 μg protein) eluate of Sephadex G-25 column (see flow diagram). The 1% (w/v) agarose gel in 100 mM Tris/38 mM glycine buffer (pH 8.6) contained 5 μl/cm² IgG of purified Component I. Immunoelectrophoresis was performed for 18 h at 80 V. The protein was stained with Coomassie Brilliant Blue R250.

extracts by this relatively simple and rapid immunoadsorbent technique.

The immunoadsorbent column was highly specific for Component I since purified Component II was not retained by the column when eluted with the Tris-HCl buffer. It is also of interest that, despite the very close association of Components I and II in the nitrogenase complex, the column selectively adsorbed Component I.

Further tests were made to check the purity of Component I in the Sephadex G-25 eluate. Thus aliquots were incubated with 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol at 100°C for 5 min and then run on polyacrylamide gel disc electrophoresis as described previously [1].

The results in fig.1 show that two bands are present in the Sephadex G-25 eluate compared with numerous protein bands in the crude extract. This gel separation pattern is characteristic of the two subunits associated with Component I from *A. vinelandii* [1,3].

Component I was also identified in the Sephadex G-25 eluate by rocket immunoelectrophoresis as shown in fig.2. The results indicate that the eluate contained several species reacting with an antibody prepared for purified Component I. It is likely that an immunogen containing a heterogeneous population of conformers (comprising a complex antigen of several non-cross-reacting antigens) results in the production of an antiserum containing antibodies of several specificities [4]. One interpretation of our results is that Component I is such an immunogen because it contains at least two different subunits; furthermore, in air the purified component forms various aggregated species which may produce additional antigens [5,6].

An important advantage of this rapid immunoadsorption technique for preparing purified and active fractions of Component I is that it overcomes the major problem of O_2 inactivation which readily occurs when the longer and more tedious purification procedures are employed.

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