THE USE OF AFFINITY CHROMATOGRAPHY FOR THE PURIFICATION OF NITRATE REDUCTASE

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1. Introduction

The assimilatory nitrate reductase (NAD(P)H: nitrate oxidoreductase, EC 1.6.6.2), is the first enzyme in the nitrate assimilation pathway by which bacteria, fungi, algae and higher plants utilize nitrate nitrogen. The enzyme is claimed to be a molybdo-flavoprotein and can utilize NADH or NADPH as electron donors [1,2]. A cyt *b* was identified in the nitrate reductase from several species [3,4]. Several attempts to purify the enzyme by conventional methods were reported ([5] and other references therein). This communication describes the use of NADH-Sepharose, a 'general ligand' adsorbant, introduced by Mosbach et al. [6], for the purification of nitrate reductase, taking advantage of the specificity of the enzyme for NADH.

2. Materials and methods

The alga *Dunaliella parva* and barley (*Hordeum vulgare*) seedlings were used as enzyme source. Cells of *D. parva* and barley seedlings were grown, harvested and crude extract (fraction 1) prepared as described previously [7,8]. The crude extract was made 50% saturated with respect to $(NH_4)_2SO_4$ and the precipitate was redissolved in 0.1 M potassium phosphate buffer, pH 7.5, containing 10^{-3} M L-cysteine, 10^{-6} M FAD and 10^{-6} M sodium molybdate, (fraction 2). Fraction 2 was applied to the NADH-Sepharose column prepared as follows. 1.5 ml of NAD-Sepharose (obtained from Professor N. O. Kaplan, University of California, San Diego) was packed into a column and reduced to NADH-Sepharose using yeast alcohol dehydrogenase (1.0 mg/ml) and 2.0 M ethanol in 0.1 M potassium phosphate buffer, pH 7.5. The column was then equilibrated with 0.1 M potassium phosphate buffer, pH 7.5, containing 10^{-3} M L-cysteine, 10^{-6} M FAD and 10^{-6} M sodium molybdate. The enzyme (fraction 2) was applied to the column which was then washed with the equilibration buffer containing in addition 0.1 M KNO_3, until the absorbancy of the eluant at 280 nm approached zero. Nitrate reductase was eluted from the column with the buffer used to wash the column containing in addition 1.0 mM NADH. Elution of the enzyme was followed by assaying the eluant for nitrite. The elution was stopped when nitrite could not be detected. Usually, about 40 ml of elution buffer were required to elute the enzyme. The eluant was made 50% saturated with respect to $(NH_4)_2SO_4$ and the precipitate was collected and redissolved in a small volume of the equilibration buffer (fraction 3). Nitrate reductase activity was assayed according to Wray and Filner [8], using NADH as electron donor. One unit of enzyme is the amount required to reduce 1 nmole of nitrate in one min. Protein was determined by the method of Lowry et al. [9]. All purifications were carried out at 0–4°C.

3. Results and discussion

A summary of the purification is given in tables 1 and 2. The enzyme from *D. parva* was purified 145-fold, with a recovery of more than 50% by a single pass through the NADH-Sepharose column. The specific activity of the purified enzyme was 4400 units/mg protein, which is comparable to that reported for other algal species using conventional

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Table 1
Purification of nitrate reductase from *Dunaliella parva*

Fraction	Volume (ml)	Total protein (mg)	Nitrate reductase activity		
			Total units	Specific activity	Recovery (%)
(1) Crude extract	180	154	4710	30.5	100
(2) 0–50% $(NH_4)_2SO_4$ precipitate	18	123	4240	34.5	90
(3) NADH-Sepharose eluant	5.5	0.55	2410	4400	51

methods of purification ([5] and other references therein). The enzyme from barley seedlings was purified 110-fold with a recovery of 15%, by a combination of an $(NH_4)_2SO_4$ fractionation and affinity chromatography. The specific activity of the purified barley enzyme was 490 units/mg protein which is comparable to that of spinach, purified by conventional methods [10]. The total protein content of the purified barley enzyme was 700-fold smaller than that of the crude extract. Thus, the low recovery of the barley enzyme (15%) is probably the result of inactivation of the enzyme during purification. Some additional measures are therefore required to prevent the inactivation and improve the yield.

The column could be used repeatedly providing it was washed extensively with 1.0 M potassium phosphate buffer pH 7.5 after each run, and then reduced again to the NADH form. It is suggested, that in spite of the relatively poor yield of the barley enzyme, the technique can be used to facilitate further purification of the enzyme.

Table 2
Purification of nitrate reductase from barley seedlings

Fraction	Volume (ml)	Total protein (mg)	Nitrate reductase activity		
			Total units	Specific activity	Recovery (%)
(1) Crude extract	114	251	1140	4.5	100
(2) 0–50% $(NH_4)_2SO_4$ precipitate	19	47.5	1210	25.5	106
(3) NADH-Sepharose eluant	3	0.36	175	490	15

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