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RESEARCH ARTICLE

FURTHER PURIFICATION AND CHARACTERIZATION OF NITRATE REDUCTASE FROM CORN LEAVES(Zea mays HQPM-4)

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ABSTRACT

Nitrate reductase (NaR) is a metalloprotein and contains one each of Flavin (as FAD), heme iron, molybdopterin and belongs to a super family of enzymes including xanthine oxidase, nitrite reductase and DMSO reductase. NaR was a soluble enzyme and purified from Zea mays (HQPM-4) by ammonium sulfate precipitation and gel filtration on blue dextran by affinity chromatography and gel filteration by 12.32 fold with 53.19 yield. It was necessary to investigate better conditions i.e. pH, temperature to stabilize the enzyme activity as a result of small quantities of tissue and low amount of enzyme contained in the leaves. The purified enzyme (NaR) after Gel filteration had a specific activity of 454.61Units/mg. The blue-Sepharose method and further gel filteration method using sephaedex gel offers the advantages of a rapid purification of plant NR to a high specific activity with reasonable recovery of total activity. The different kinetic parameters of enzyme (NaR) such as pH, incubation temp., time of incubation and effect the substrate (nitrate) concentration were optimized. We found that, the optimum stability of nitrate reductase (NADH) activity was at a pH 7.5. Affinity chromatographic method has been developed to purify the enzyme from corn leaves and gel filteration method gives the highest purity.

INTRODUCTION

NaR have shown to be a homodimer consisting of two identical subunits (Yoshihiro K.,1988)Nitrate reductase in Zea mays catalyzes an NADH dependent reduction of nitrate to nitrite. The enzyme is successfully purified from various sources using blue-dextran sepharose affinity chromatography and gel filteration method to achieve higher purification and this technique is proven to be a simple and rapid technique for isolation of NaR from higher plants (Joseph H., 1978). NaR is present in human in saliva, liver, plasma and in other animals and plant parts. It was purified from plants as barley (Kuo TA and Warner RL 1980), spinach (Nakagawa *et al,* 1985), corn and squash (Campbell WH and Smarrelli J 1977), yeast (Guerrero MG and Gutierrez M 1977), E.coli (Clegg 1976) and Aspergillus (Downey 1971). NaR is found to be labile after extraction and the absence of rapid and specific methods for purification results in large losses of enzyme activity according to Hageman and Hucklesby (1971). Affinity chromatography, with a bound ligand specific for NaR, would appear to provide a means for rapid purification, which might also serve to concentrate the NaR from the crude extract. For purification of Chlorella NaR Solomonson (1975) has been used blue dextran-Sepharose as an affinity medium. Application of higher plant NaR to blue dextran-Sepharose results in a large percentage of apparent nonspecific binding and only partially achieves the results found for Chlorella NaR as described by Campbell and Smarrelli (1976). Other affinity purification techniques provide only partial purification of higher plant NaR and the recovery of the NaR is low according to Heimer *et al* (1976), Notfon *et al* (1977). Affinity chromatography has been used in combination with conventional techniques to purify NaR to the highest reported specific activities but yields were low concluded by Guerreo and Gutierrez (1977), Notfon *et al* (1977). We describe here an affinity procedure using blue-Sepharose for purification of higher plant NaR and finally by using Gel filteration chromatography to attain high purity using sephaedex G-25. NaR appears to be specifically bound to blue-Sepharose and can be recovered in high yields. We have used the NaR highly purified by this gel filteration method from the extracts of com leaf and to investigate the kinetic mechanism of NaR. NaR included in biosensor used for measuring of nitrate concentration were first reported by Consier *et al* (1994). Nitrate Reductase (NaR) is an enzyme, mediates reduction of nitrate to nitrite. This reduction reaction by NaR is affected by oxygen, to reduce effect of oxygen; an oxygen scavenger is applied to a biosensor.

MATERIALS AND METHODS

Source of chemicals and seeds

Sepharose CL-4B was from Sigma–Aldrich, USA. Protamine sulphate, Cibacron blue F3GA, NADH, potassium nitrate and

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ammonium sulphate were from HiMedia Lab. Pvt. Ltd., Mumbai (India). All other chemicals were of analytical reagent grade. Double distilled water (DW) was used in all experiments. The seeds of corn (Zea Mays) variety HQPM-4 from Chaudhary Charan Singh, Haryana Agriculture University, Hisar were used.

Plant materials

Seven day old plants were raised from corn seeds of different varieties in the laboratory as described by Nakagawa *et al* (1984). The seeds of corn were soaked in deionized water overnight, surface sterilized with 0.1% $HgCl_2$ for one min. Then after washing with distilled water (DW) several times, the seeds were allowed to germinate in petridishes lined with a double layer moist filter paper at room temperature (26-28°C). The seedlings were irrigated daily with Hoagland's nutrient medium after germination and maintained in a photoperiod of 14-16 hr/day. After 10 days of germination, the seedlings were removed from the filter paper and their leaves were separated and stored immediately at -20°C until use.

Extraction of nitrate reductase (NaR) from corn leaves

NaR from corn leaves was extracted by blending the 200 gm of corn leaves in 400 ml of extraction buffer containing 0.1 M phosphate buffer (PB, pH 7.5), 5 mM EDTA, 10 mM cysteine and 20 gm of polyvinylpyrridone according to Campbell and Smarrelli (1978) at 4°C. The extract was filtered through muslin cloth and after filtration 0.1 ml of 1% (W/V) protamine sulphate solution containing 6 mg of Tris/ml was added and centrifuged at 20,000g for 25 min. Both supernatant and dissolved pellets were tested for NaR activity and protein conc. by Lowry method.

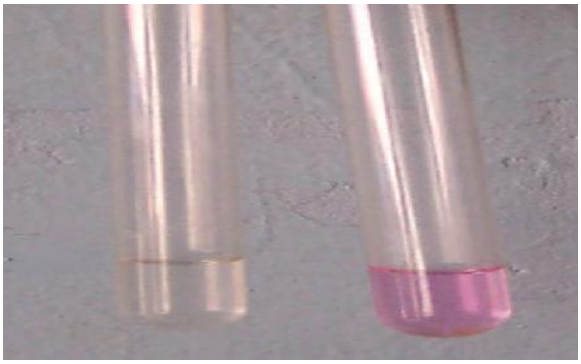
Enzyme Assay

During enzyme purification, activity was measured as the rate of nitrite production. Assay of NaR was carried out as described by Campbell and Smarrelli (1978) with modification. The reaction mixture contained 1.3 ml of 0.1 M PB (pH 7.5), 0.2 ml of 0.2 M KNO3 and 0.2 ml of 0.4 mM NADH. After incubation at 30°C for 2 min, 0.3 ml of leaf extract (both supernatant and dissolved pellets) was added, the tubes were shaken and incubated for 30 min. 1ml of 1% sulphanilamide in 3M HCl and 1 ml of 0.02% N-(1-napthyl)-ethylenediamine hydrochloride (NED) were added to the test tubes to develop the color ed complex (Fig.1) and A540 was read against the blank. In case of the blank the crude enzyme was replaced by buffer solution. One unit of the NaR is defined as the amount of enzyme required to generate 1 µmole of nitrite/min.

Preparation of standard curve of nitrite

The different dilutions of nitrite containing 50 to 500 µmoles were prepared in 0.1 M PB, pH 7.5. These dilutions were taken in 15 ml test tubes and added 1 ml of 1% sulphanilamide in 3M HCl and 1 ml of 0.02% N-(1-napthyl)-ethylenediaminehydro chloride (NED) to each tube. After incubation at 37°C for 10 min., A_{540} was read. A Standard curve was plotted between nitrite conc. and A_{540}. **(Fig. 2)**

$$NO_3^- + NADH + H^+ + 2e^- \xrightarrow{NaR} NO_2^- + NAD^+ + H_2O$$



Control Test **Fig. 1** Assay of free corn leaves

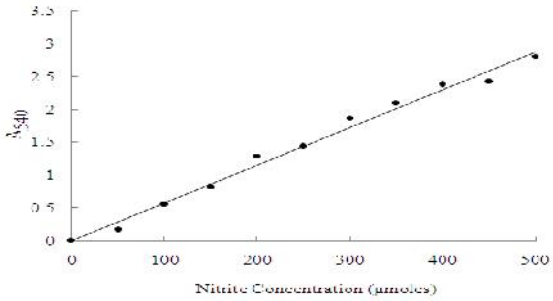


Fig 2 Standard curve of nitrite

Purification of NaR

The purification of the crude NaR prepared from corn leaves was carried out as described by Campbell and Smarrelli (1978) with modification. All steps of purification were carried out in cold at 4°C.

Ammonium sulphate fractionation: Solid ammonium sulphate was added to crude extract (supernatant) of corn leaves to give a final saturation of 0-45%. and centrifuged at 10,000g for 30 min and pellet was dissolved in 100 ml of 0.1 M PB (pH 7.5) containing 1 mM EDTA and 5 mM cysteine. The dissolved pellet obtained was tested for NaR activity and protein.

Blue-Sepharose affinity chromatography: The ammonium sulphate precipitated NaR was further purified on blue-Sepharose as described by Campbell and Smarrelli (1978) with modification. The ammonium sulfate precipitated enzyme was loaded on the packed blue-Sepharose into 2.5cm diameter column through sidewalls without distributing the upper layer of gel. The column was run in the same buffer until the one void volume of column was passed. The NaR activity of corn leaves was eluted out using extraction buffer in combination of gradient of 1-100 µM NADH by collecting the fractions (2 ml) till the two more void volumes of eluting buffer were passed. The column was run in the same buffer at a flow rate of 0.5 ml/min. Each fraction was tested for NaR activity and protein concentration. The fractions containing high specific activity were pooled.

Synthesis of Blue-Sepharose: The activated gel was prepared following the method of Sherrad and Dalling (1979) with modification. 50 ml of Sepharose 4B gel was washed with DW and suspended in 100 ml of 2 M sodium carbonate (pH 11.4).

The suspension was stirred slowly with a glass rod and a solution of 10 gm of cynogen bromide dissolved in 10 ml of water free dimethylformamide was added over 1 min. The suspension was slowly stirred with a glass rod for further 2 min. The activated gel immediately washed with 500 ml ice-cold 1 mM HCl. The activated gel was then immediately transferred to a solution of 1 gm of cibacron blue F3GA, dissolved in 100 ml of 0.4 M sodium bicarbonate (pH 10.0) and the susupension gently mixed for 18 hr at 4 °C. The gel was washed with 500 ml DW and suspended in 20 ml of 0.5 M Tris-HCl (pH 8.0) for 2 hr at 4°C to block unreacted activated sites. The gel was then washed successfully with buffer of pH 4.0 and pH 8.0.

Gel Filteration Chromatography

Purified enzyme Nitrate reductase was purified by gel filteration chromatography to achieve high purity. All steps of purification were carried out at room temperature. The enzyme is loaded on the packed sephadex into 2.5cm diameter column through side walls without disturbing the upper layer of gel. The gel was allowed to settle down in column till the height of 15cm. The column was run in 0.1M PB buffer (pH 7.5) until the one void volume of column passed at a flow rate of 0.5 ml/min. The enzyme was eluted out using 0.1MPB buffer by collecting the fractions (2ml) till one more void volume of buffer were passed. The void volume of the column, height and diameter of the column were noted. The void volume was calculated using formula.

Void Volume: $1/3\pi r^2h$
Here, r=radius of the column.

h =height upto which the gel was filled in the column.
The column was run in the same buffer at a flow rate of 0.5ml/min. Each fraction was tested for NaR activity and protein concentration. The fractions containing high specific activity were pooled and further test was done to determine homogenity of the protein.

Determination of protein

The protein content in various enzyme preparations was determined by the method of Lowry *et al.* (1951) (Fig.3).

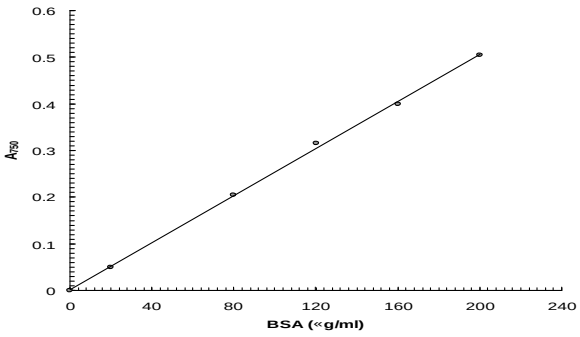


Fig.3. Standard curve of bovine serum albumin (BSA) using Lowry's method

Testing of Purity of Homogenity of NaR.

Purity of NaR was tested by native-polyacrylamide gel electrophoresis.Native gel electrophoresis was performed at 4°C using tris-glycine buffer system. Sample of 20µl was loaded onto the 7.5% acrylamide prepared gel. A standard protein ladder also loaded against that to analyse molecular weight. Proteins in the sample were located by staining with 0.25% Coomassie Brilliant Blue R-250 in 7 % acetic acid for 1hr followed by destaining with 7% acetic acid. The gel showed one clear single band corresponds to 190 KD molecular weight along with two very light bands, which showed that enzyme is almost homogenous in nature.
(Fig.5).

Optimization of kinetic properties of purified NaR

The following kinetic properties of purified NaR from corn leaves were studied:

Effect of pH: The activity of corn leaves NaR enzyme was determined in the pH range of 6 to 9 by assaying with different buffers of these pH values. For pH 6-8, 0.1 M potassium phosphate buffer and for pH 8.5-9, 0.1 M Tris-HCl buffer were used.

Effect of incubation temperature: The effect of incubation temperature for maximum activity of corn leaves NaR enzyme was studied by incubating the reaction mixture at different temperatures ranging from 20ºC to 50ºC at an interval of 10ºC.

Effect of time of incubation: The activity of corn leaves NaR enzyme was determined by incubating the reaction mixture for 10 to 60 min at an interval of 10 min.

Effect of substrate conc. and determination of Km and Vmax: The activity of corn leaves NaR enzyme was determined by assaying the enzyme for different substrate (nitrate) conc. ranging from 10 µM to 500 µM and Km & Vmax calculated from Lineweaver Burk plot. A Lineweaver Burk plot was made between reciprocal of nitrate concentration (I/[S]) vs enzyme activity (1/V) of the enzyme reaction. Apparent Km and Vmax were calculated from the plot using the following Michaelis-Menten equation:

RESULTS AND DISCUSSION

A soluble NaR from corn leaves was purified by 0-45% ammonium sulfate precipitation and gel filtration on blue dextran by affinity chromatography (Fig. 4). And then by gel filteration method to achieve high purity.The results of purification are given in Table-1, which showed that the enzyme was purified by 12.32 fold with 53.19 yield. The purified enzyme had a specific activity of 454.61 Units/mg.

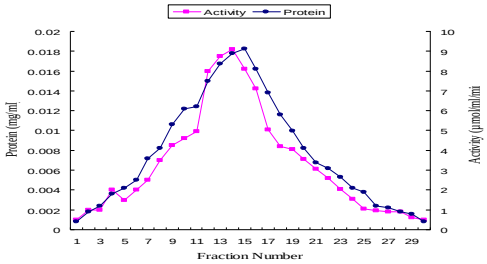


Fig. 4 Blue dextran affinity chromatography of corn leaves NaR (Size of Column = 2.5cm × 18cm Flow Rate = 0.5ml/min)

Table 1 Purification of NaR from leaves of corn

Purification Step	Protein (mg/ml)	Activity* (Units/ml)	Specific Activity	Purifica-tion fold	Yield (%)
Crude enzyme (20,000g supernatant)	0.301	11.11	36.91	1	100
Ammonium sulfate precipitate (0-45%)	0.162	8.15	50.30	1.36	73.85
Blue-dextran	0.016	7.05	440.63	11.94	63.45
Gel filteration	0.013	5.91	454.61	12.32	53.19

***one unit is defined as amount of enzyme which is required to generate one µmol of nitrite/min.**

Native Gel Electrophoresis.

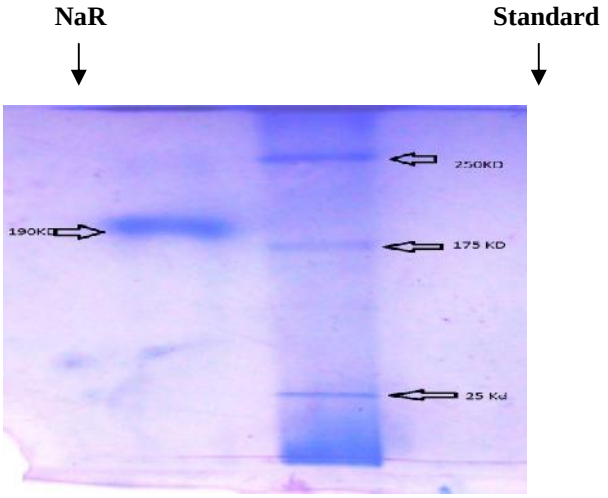


Fig.5 Native gel electrophoresis for enzyme NaR.

Study of kinetic properties of native, purified NaR enzyme from leaves of corn

The following kinetic properties of free NaR enzyme from leaves of corn were studied:

Effect of pH: The activity of purified NaR enzyme from leaves of corn was determined in the pH range of 6.0 to 9.0. The results as given in Fig. 6 revealed that the Zea Mays enzyme had an optimum pH of 7.5.

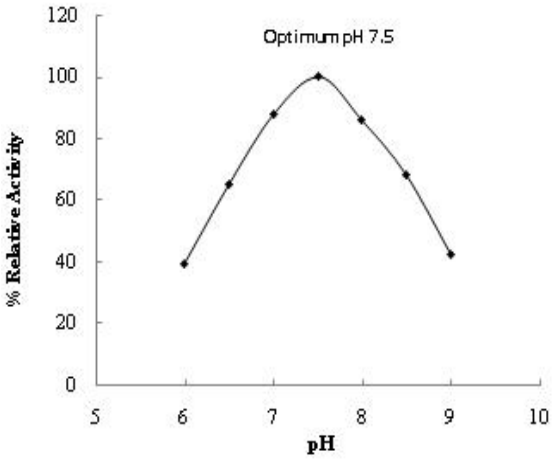


Fig. 6. Effect of pH on purified corn leaves NaR

Effect of incubation temperature: To study the effect of incubation temp for maximum activity on the corn leaves enzyme, the reaction mixture was in-cubated at different temperatures ranging from 20°C to 50°C at an interval of 10°C. The partially purified enzyme showed maximum activity at 35°C (Fig. 7).

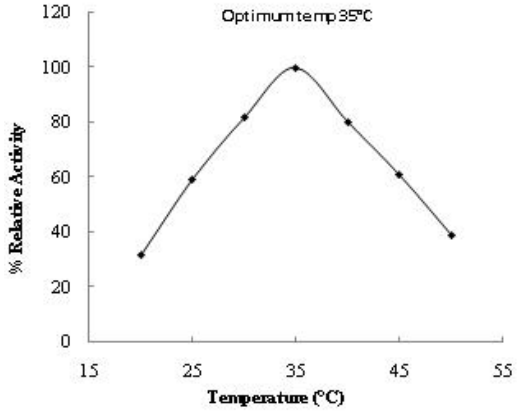


Fig. 7 Effect of temperature on purified corn leaves NaR

Effect of time of incubation: The activity of the purified corn leaves enzyme was determined by assaying the enzyme for different time of incubation ranging from 10 to 60 min at an interval of 10 min. The enzyme showed saturation at 30 min (Fig. 8).

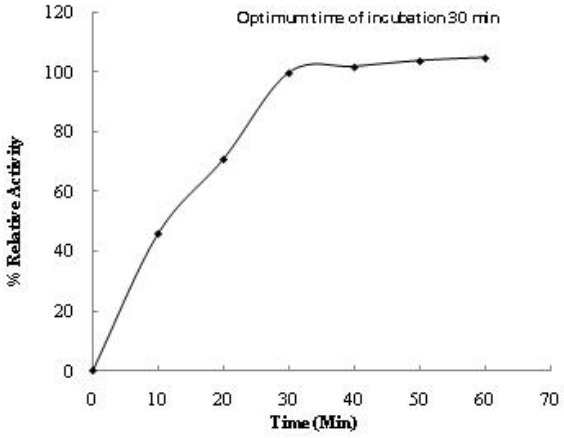


Fig. 8. Effect of time of incubation on purified corn leaves NaR

Effect of substrate conc.: The activity of corn leaves enzyme was determined by assaying the enzyme at different nitrate conc. ranging from 10 µM to 1000 µM. The enzyme showed its maximum activity at 150 µM and after that it became constant (Fig. 9). A Lineweaver-Burk plot was linear (Fig. 9) and Km calculated from this plot was found to be 68.34 µM and Vmax was found to be 9.2 µmoles/min/ml.

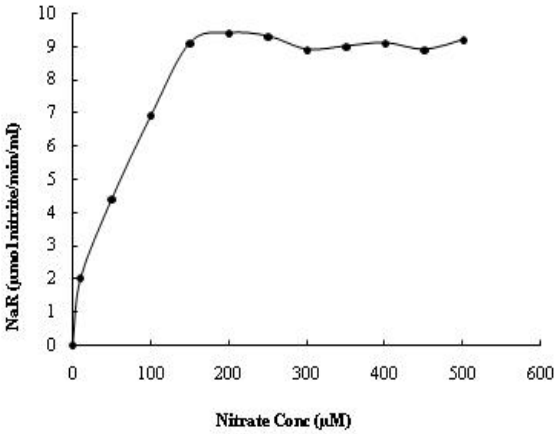


Fig. 9.Effect of substrate (nitrate) conc. on purified corn leaves NaR

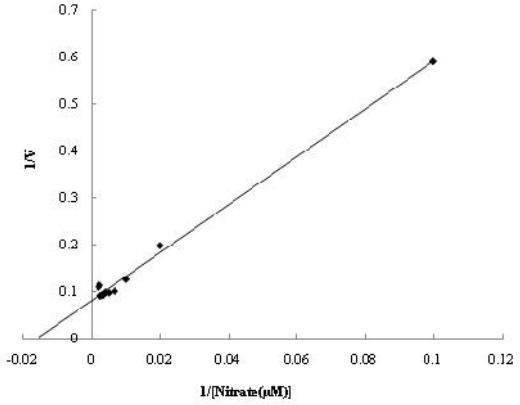


Fig. 10 Lineweaver-Burk plot of purified corn leaves NaR

CONCLUSION

Due to very small amount of nitrate reductase contained in leaves, it was necessary to investigate better conditions to stabilize the enzyme activity and to choose selective purification methods. We found that, the optimum stability of nitrate reductase (NADH) activity was at a pH 7.5. Affinity chromatographic and Gel filteration method has been developed to purify the enzyme from corn leaves upto high purity. The native gel electrophoresis obtained the single band that showed the enzyme is homogenous in nature. This purification method providing pure enzyme with a specific activity of 454.61 Units/mg protein. The purified enzyme showed high stability as compare to earlier reported enzyme.

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