Flow Injection Procedure for Determination of NAD^+ Using Immobilized Enzyme with Spectrophotometric Detection

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Summary: A flow injection spectrophotometric method is described for the determination of NAD^+ based on the production of NADH using immobilized alcohol dehydrogenase (ADH) isolated from Baker's yeast. The absorbance was monitored at 340 nm. The limit of detection (2 x standard deviation of the blank) was 0.01 mM with sample throughput 60 h^{-1}. The calibration graph was linear in the range 0.2 – 1.0 mM, with relative standard deviation (n = 3) in the range 0.7 - 1.2%. Phenolic resin was used as a support for ADH immobilization and the enzyme activity was maintained for three months.

Introduction

The cofactors β-nicotinamide adenine dinucleotide (NAD^+) and its reduced form (NADH) are used by over 250 dehydrogenases and as such play a major role in many biological oxidation-reduction reactions. The regeneration of NAD^+ from NADH is important in the development of sensitive immunoassays and in preparative enzymatic synthesis [1]. Moreover, NADH can be used as medication for Parkinson patient's [2]. Most importantly from biotechnological point of view, NADH is expensive than its oxidized form NAD^+ that make its production economically viable.

Most industrial enzymatic processes are carried out batch-wise with soluble enzymes, but these processes are very limited, mainly for economic reasons such as low efficiency and high cost [3]. One approach to solve these problems is the use of immobilized enzymes which reduces the cost by reusing the enzyme; the processes can be operated continuously and can be readily controlled; the products can easily be separated and enzyme activity and stability can be altered favorably by immobilization [4, 5].

Several methods have been reported for the determination of pyridine nucleotides (NAD^+ and NADH). These include; an electro-enzymatic method to regenerate NAD^+ by co-immobilizing alcohol dehydrogenase / NADH oxidase coated on electrodes which in-tern produced hydrogen peroxide [6] and amperometric NADH-sensing electrode methods for measuring alcohol and NADH, based on substrate recycling with the dehydrogenase catalyzed reaction [7, 8]. However, the immobilization and co-immobilization of enzymes at the surface of electrode suffer from low density, poor reproducibility and short lifetime of analytical sensor [9]. A chromatographic (HPLC) method for measuring the content of NAD^+ in insect cells has been reported [10]. This method involves the separation of NAD^+ from the bulk of acid-soluble nucleosides, nucleotides and other pyridines containing molecules by affinity chromatography on dihydroxyboronyl-Bio-Rex. Bioluminescent assay for the determination of NAD^+ and NADH has been reported [11]. This system includes luciferase and low levels of an NADH-specific oxidoreductase producing a constant light intensity directly proportional to the amount of NADH in tissue extract. The NAD^+ present in the extract is enzymatically converted to NADH by the addition of alcohol dehydrogenase with a limit of detection 5 x 10^{-14} mol NAD^+ or NADH per assay. UV-visible spectroscopy has been found to provide simple and reliable methods for the determination of NADH and NAD^+ - NADH converting enzymes and their substrates [12, 13].

In the present study, we report the isolation and purification of ADH, (from Baker's yeast), its immobilization on phenolic resin and use in a flow injection manifold for the determination of NADH from NAD^+. The reduction of NAD^+ catalyzed by ADH in the presence of ethanol can be given as:

$$CH_3CH_2OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+$$

λmax 340 nm

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Results and Discussion

Optimization of FIA manifold.

The flow injection spectrophotometric manifold for the production of NADH from NAD^+ was optimized by investigating the effect of various parameters including buffer pH, reagent concentrations, flow rate, sample volume and temperature on the activity of immobilized ADH are shown in Table 2. All studies were carried out with a 0.4 mM NAD^+ solution.

Table 1:Purification of Alcohol dehydrogenase from Baker's Yeast.

Fraction	Total units	(mg) Total protein	(u/mg) Specific activity	(fold) Purification	Yield (%)
First extract	148000	14800	10	1	100
Acetone ppt	133000	950	140	14	90
36% $(NH_4)_2SO_4$	74000	264	280	28	50
Column chromat.	66000	214	308	31	45

Table-2:Effect of variables on the conversion of NAD^+ to NADH using immobilized ADH column incorporated in a flow injection system.

pH (PO_4 buffer, 100 mM)	7.5	8.0	8.5	8.8	9.0
Peak height* (mm)	30	42	55	64	58
Ethanol (%)	0.5	1.0	1.5	2.0	2.5
Peak height* (mm)	18	32	40	34	28
Flow rate (mL min^{-1})	0.1	0.3	0.5	0.7	1.0
Peak height* (mm)	58	65	57	45	30
Sample volume (μL)	15	30	45	60	75
Peak height* (mm)	32	56	64	70	78
Temperature (°C)	20	30	40	50	60
Peak height (mm)	48	60	74	88	67

*Mean of 3 injections.

The effect of pH optimum on the activity of immobilized ADH was investigated using phosphate buffer (100 mM) in the range 7.5 - 9.0 as a carrier stream. The maximum absorbance for bioconversion of NAD^+ to NADH was observed at pH 8.8 and therefore, was used subsequently. At pH 9.0, the destruction of NAD^+ is rapid, whereas NADH is destroyed below pH 6.0 [14].

The concentration of ethanol also plays a vital role. The conversion by the enzyme under physiological conditions is unfavorable; therefore, the ethanol quantity must be boosted to push the reaction in the required direction. Therefore, the effect of ethanol concentration was studied in the range 0.5-3.0% similarly using phosphate buffer (100 mM, pH 8.8). The ethanol concentration of 1.5% gave maximum peak height absorbance, which was used subsequently.

The flow rate is an important factor influencing the peak height magnitude during the enzymatic reaction taking place in the carrier solution. The effect of flow rate on immobilized enzyme reactor was studied in the range 0.1 – 1.0 ml min^{-1}. Low flow rates increased sensitivity and decreased sampling rate. The maximum response was obtained at a flow rate of 0.3 ml min^{-1}, but due to peak broadening and sample dispersion, a flow rate of 0.5 ml min^{-1} was selected and therefore used for subsequent studies. The effect of sample volume on the sensitivity was studied in the range 15 – 75 μl. Higher sample volumes increased peak height absorbance but decreased the sample rate, therefore sample volume of 30 μl was used for suitable response and less dispersion.

The effect of temperature on the activity of immobilized ADH column was studies over the range 30 – 60°C. There was an increase in peak height absorbance with increase in temperature up to 50°C. However, the column was maintained at 30°C to protect the enzyme from denaturation and to increase the lifetime of the enzyme column. The immobilized ADH showed good operational stability over a storage period of 90 days and more than 450 injections were made during 20 days of use, displayed 80 – 85% conversion when stored at 4°C. After 90 days the percentage substrate conversion was decreased from 15 – 20%.

Calibration

Standard solutions of NAD^+ covering the range 0.2 – 1.0 mM were treated according to the procedure described using commercial and lab. isolated ADH immobilized columns. The calibration graph obtained from triplicate injections is shown in Fig. 1. The correlation coefficients were 0.9997 and 0.9994 (n = 5) with regression equations $y = 80.286\ x - 0.195$ and $y = 52\ x + 0.333$ [y = peak height (mm), x = concentration (mM)], respectively. The relative standard deviations was 0.7 - 1.2% (n = 3) over the range investigated. The limit of detection (2s) was 0.01 mM, with sample throughput of 60 h^{-1}.

Experimental

Reagents and methods

Alcohol dehydrogenase (ADH; Alcohol: NAD^+ Oxidoreductase; EC 1.1.1.1) was isolated and purified from Baker's yeast (308 units/mg) according

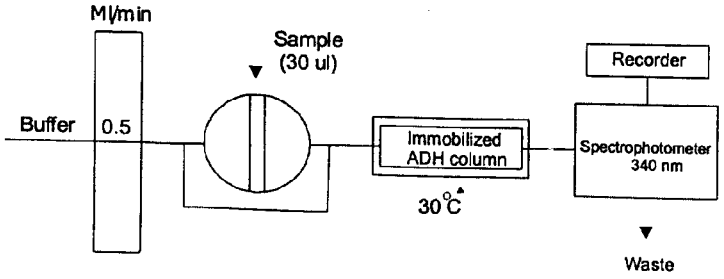

Fig. 1: Flow injection manifold for the determination NAD^+

to the procedure reported [15], controlled porosity glass (CPG, 200-400 mesh), 3-aminopropyl-triethoxysilane, glutaraldehyde, NADH (disodium salt, 98%) and NAD^+ (free acid, 100%) were obtained from Sigma (St. Louis, MO. USA). All other chemicals were analytical grade (E. Merck, Darmstadt, Germany) and deionized water was used throughout.

Preparation of crude extract

Baker's yeast, weighing 200 g of powder was homogenized with 500 ml of phosphate buffer (0.05 M, pH 8.5) for 2.5 h at room temperature with continuous stirring and the homogenate was centrifuged at 10000 rpm for 30 min. The supernatant was brought to 55°C for 15 min, after cooling the mixture was centrifuged and the clear supernatant was stored at 4°C over night.

Enzyme purification

To the supernatant from yeast's crude extract preparation (100 ml), 50 ml of ice-cold acetone was added slowly and maintained at –2°C in a dry ice alcohol bath. The resulting precipitate was collected by centrifugation and suspended in 50 ml of cold water, dialyzed against large volumes of phosphate buffer (0.001 M, pH 7.5) for 12 h with several changes of the buffer and the insoluble material was removed by centrifugation. To the supernatant, solid $(NH_4)_2SO_4$ was added with constant stirring to 36% saturation at 4 °C. The precipitate was collected and dissolved in 20 ml of water containing 2.0 g of ammonium sulfate. The solution was subjected to an Ag 1 - X2 column (60 x 3 cm) equilibrated with phosphate buffer (0.1 M, pH 8.0) and eluted at a flow rate of 50 ml h^{-1}, 5.0 ml fractions were collected. The unbound material was washed with 500 ml of the same buffer and the enzyme was eluted by a linear gradient (0 – 0.4 M) of sodium chloride. Table 1 shows the specific activity and percentage yield for

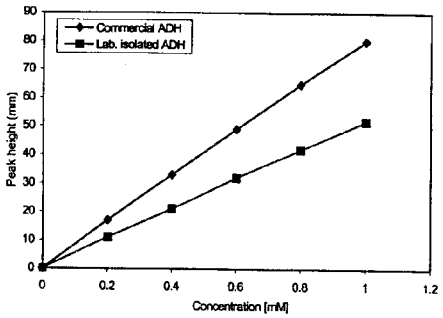

Fig. 2: Calibration graph for NAD^+ using flow injection analysis.

each fraction. One unit of enzyme is defined as the amount of enzyme, which converts 1.0 μmol of ethanol to acetaldehyde per min at optimum conditions. Protein was determined by the colorimetric method [16] using bovine serum albumin.

Phenolic resin preparation

11 g of hydroquinone was dissolved in boiling HCl (300 ml, 35%) and aqueous formaldehyde solution (38%, 40 ml) was added dropwise to refluxing hydroquinone solution. The resultant brown gelatinous suspension was refluxed for further 3 h before the addition of cold water. Once formed condensed dihydroxydiphenyl methane (DPM) which depends upon pH. DPM next react slowly with formaldehyde to add more methylol groups. These react quickly with phenol to produce higher molecular weight product. The gel having a settled volume of about 30 ml was dried by lyophilization. The dried mass of brown colored resin (with -OH group on the surface) [17] was used as a support for enzyme immobilization.

Immobilization procedure

The ADH was immobilized on phenolic resin by cross-linking with glutaraldehyde according to the

procedures reported previously [18, 19]. Phenolic resin (10 g) was washed with 5% nitric acid at 90° for 30 min followed by rinsing with water and dried. The dried resins were derivatized by treatment with 50 ml of 10% aqueous solution of 3-aminopropyltriethoxy-silane at pH 3.45 and incubated for 2.5 h at 75°C. The derivatized resins were then filtered, washed with water and dried. This process of derivatization was repeated to ensure maximum activation of the resins. The derivatized resins were treated with 50 ml 2.5% glutaraldehyde in phosphate buffer (100 mM, pH 7.0) and incubated for 1 h, at room temperature. After washing the activated resins (5.0 g) and the ADH 5.0 mg (350 Units/mg, comercially available, and 10.0 mg (308 Units/mg, lab. isolated from Baker's yeast) were dissolved in 5.0 ml of cold phosphate buffer (100 mM, pH 6.0) separately, mixed and incubated overnight at 4°C. After the immobilization reaction, the resins were washed with 5 – 10 ml of phosphate buffer (100 mM, pH 6.0). The Protein content of washings was measured according to the reported method [16] to evaluate the yield of immobilization on the phenolic resins. Almost 90% and 70% of the enzymes were bound to the supports respectively. The immobilized enzymes were packed in glass columns (2.5 x 50 mm), washed with phosphate buffer (100 mM, pH 8.5) and utilized as needed. The immobilized enzyme activity was completely preserved after three months storage at 4°C in phosphate buffer (100 mM, pH 8.8).

Instrumentation and Procedure

Figure 1 shows a simple flow injection manifold used for the determination of NADH. The activity of the immobilized ADH was investigated by incorporating the packed column (2.5 x 50 mm) in the flow manifold. Standards were injected via rotary valve (Rheodyne 5020, Anachem, Luton, UK) with a sample loop of 30 µl into the phosphate buffer (100 mM, pH 8.8) carrier stream containing 1.5% ethanol. A peristaltic pump (LKB Broma, single channel, Finland) was used to propel the carrier and reagent at a flow rate of 0.5 ml min^{-1}. PTFE tubing (0.5 mm i.d.) was used throughout the manifold. The NADH produced enzymatically was monitored at 340 nm [20] using a spectrophotometer (LKB, Model 4040, Finland) with a flow through cell (30 µl) connected to a chart recorder (Kipp & Zonen BD 40, Holland). The immobilized enzyme packed in a glass column was thermostated at 30°C by flowing water through a water jacket [21] around the enzyme column when in operation and kept at 4°C when not in use.

Conclusions

An enzymatic flow injection method is established for bioconversion of NAD^+ to NADH using ADH from Baker's yeast, purified and immobilized on phenolic resins. The great potential of the flow injection analysis for NADH monitoring is simplicity and rapidity. The phenolic resin has proved to be an excellent support for enzyme immobilization in terms of cost, active product formation, good flow properties and also compatible with the commercially available support (CPG). The following anions and cations (0.01 mM) such as; chloride, sulfate, fluoride, acetate, calcium, magnesium, copper and lead did not effect on the activity of immobilized ADH.

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References

1. T. Tanaka and T. Matsunaga, *Anal. Chem.*, **72**, 3818 (2000).
2. W. Birkmayer and G. J. D. Birkmayer, *Annals of Clinical and Lab. Science* **19**, 38 (1989).
3. J. F. Kennedy and J. M. Cabral, Enzyme immobilization in biotechnology, chapter 7a, VCH, Weinheim, **9**, 349 (1987).
4. M. Masoom M. Mateen and A. Townshend, *J. Chem. Soc. Pak.*, **11**, 81 (1989).
5. J. Parrado, F. Millan and J. Bautista, *Process Biochem.*, **30**, 735 (1995).
6. F. Mizutani, Y. Sato, Y. Hirata, T. Sawaguchi and S. Yabuki, Sensors and Actuators B: Chemical, **65**, 46 (2000).
7. B. Leca and J. L. Marty, *Biosensors & Bioelectronics*, **12**, 1083 (1997).
8. G. T. R. Palmore, H. Bertschy and S.H. Bergens, *J Electroanal. Chem.*, **443**, 155 (1998).
9. C. J. McNeil, J. A. Spoors, D. Cocoo, J. M. Cooper and J. V. Bannister, *Anal. Chem.*, **61**, 25 (1988).
10. R. A. Gonzalez, R. Eichenberger, P. Loetscher and F. R. Althaus, *Anal. Biochem.*, **156**, 156 (1986).
11. M. T. Karp, R. P. Raunio and T. N-E. Lovgren, *Anal. Biochem.*, **128**, 175 (1983).
12. D. Cocco, A. Rinaldi, I. Savini, J.M. Cooper and J. V. Bannister, *Eur. J. Biochem.*, **174**, 267 (1988).

13. A. Klemm, T. Steiner, G. A. Cumme and A. Horn, *Anal. Biochem.*, **212**, 375 (1993).
14. O.H Lowry, J. V. Passoneau and M. K. Rock, *J. Biol. Chem.*, **236**, 2756 (1961)
15. E. Racker, *J. Bio. Chem.*, **184**, 313 (1950).
16. H. O. Lowry, N. J. Rosebrough, A. V. Farr and R. J. Randell, *J. Biol. Chem.*, **193**, 265 (1959).
17 L. M. Miall and D. W. A. Sharp (eds.) Longmans, Green & Co Ltd., London, p. 447 (1968).
18. M. Yaqoob, A. Nabi and M. Masoom, *Process Biochem.*, **36**, 1181 (2001).
19. T. Murach and M. Tabata, Methods of enzymology. Moshback K (eds.) Academic Press, san Diego, **137**, 260 (1988).
20. T. P. Hadjiioannou, S. I. Hadjiioannou, J. Avery and H. V. Malmstadt, *Clin. Chem.*, **22**, 802 (1976).
21. M. Masoom and A. Townshend, *Anal. Chim. Acta*, **166**, 111 (1984).