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HIGH-SENSITIVE VISUALLY CONTROLLED MEMBRANE-TYPE QUANTITATION OF NAD AND ALKALINE PHOSPHATASE

KEY WORDS: NAD, alkaline phosphatase, test method, membrane analysis

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ABSTRACT

New high-sensitive visually controlled membrane-type analytical methods are proposed for quantitation of nicotineamide adenine dinucleotide and alkaline phosphatase in water solutions. The methods are based on using nitrocellulose membrane as a solid matrix on which the components of one-enzyme cofactor regeneration system are being immobilised by adsorption. In the presence of substances to be assayed, the end colored product is being adsorbed on the matrix as a result of enzymatic cyclic NAD/NADH regeneration in the active site of the matrix-bound alcohol

dehydrogenase and some chemical successive reactions. Its colored intensity is a measure of the concentration of the analysed substances in solution. The general principle of NAD or alkaline phosphatase determination is successive immobilisation of separate components of the system (N-(6'-aminohexyl)salicylamide and horse liver alcohol dehydrogenase) on the matrix by adding their solutions to the wells of a specially designed cell with the membrane bottoms. In the case of alkaline phosphatase, the enzyme acted on NADPH as on a substrate. The reaction product, NAD was detected in the subsequent reaction of coenzyme regeneration. The other components of the amplifying system were added in substrate solutions at the stage of the alcohol dehydrogenase reaction. The lower detection limits for NAD and alkaline phosphatase were 3×10^{-9} M and 1×10^{-14} M respectively, the volume of the test sample - 20 μl, the time of assay - 5 min. The working concentration ranges were from 3×10^{-9} to 1×10^{-7} M and from 1×10^{-14} to 1×10^{-10} M levels for NAD(H) and alkaline phosphatase, respectively.

INTRODUCTION

The development of highly effective, simple express analytical methods for detection of different biologically active substances commands the attention of researchers. Current attempts in the developments of new methods are drawn towards porous membrane strips with immobilised reactive components in the indicator layer and visual registration of results of analysis without any instruments. At present time express tests based on utilisation of the

reagents immobilised on a membrane or porous carriers are examples of such techniques for qualitative, semiquantitative and quantitative detection of the components of blood, urea, spinal fluid, etc.[1] .

In this article we describe the general principles of analytical test-systems for visual quantitation of nicotineamide adenine dinucleotide in a solution based on using a porous membrane matrix. The application of the systems developed for high-sensitive visual detection of alkaline phosphatase, which is one of the most widely used enzyme label in enzyme immunoassay, is discussed. On the basis of the method proposed it is possible to develop simple alkaline phosphatase-employing immunochemical systems for detection of compounds of various structure and origin with visual registration.

MATERIALS AND METHODS

Reagents

Horse liver alcohol dehydrogenase (ADH [2]; EC 1.1.1.1, specific activity 2 IU per mg enzyme protein), nicotineamide adenine dinucleotide (NAD), nicotineamide adenine dinucleotide phosphate (NADP) were obtained from "Reanal" (Hungary); calf intestinal alkaline phosphatase (AP; EC 3.1.3.1; specific activity 1100 diethanolamine IU per mg enzyme protein) was purchased from "Boehringer Mannheim GmbH" (Germany); salicylamide, Fast Blue RR salt, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP), nitro blue tetrazolium (NBT), 2-amino-2-methyl-1-propanol were from "Sigma" (USA), p -nitroso-N,N'-dimethylaniline from

"Fluka Chemie AG" (Switzerland); 1-naphtylphosphate (sodium salt) was from "Serva" (Germany); nitrocellulose membranes with the pore diameters 0.23, 0.3 and 0.45 μm from "Chemapol" (CSFR); acetylcellulose membranes with the pore diameters 0.2, 0.4 and 0.6 μm from "Tasma" (Russia). N-(6'-aminohexyl)salicylamide (AHSA) was originally synthesised at the Division of Chemical Enzymology of Moscow State University. All other materials were reagent grade chemicals. Distilled water was used in the preparation of buffers. Buffer A: phosphate buffer saline (0.01 M, 0.15 M NaCl, pH 7.3). Buffer B: Tris-HCl buffer (0.05 M, pH 9.1, 1 mM $MgSO_4$).

Instruments

A dual-wavelength flying spot scanner (Model CS-9000, Shimadzu, Japan) and a spectrophotometer (Model DU8B, Beckman, USA) were used for absorption measurements.

A specially constructed collapsible membrane cell was used for assaying. It was placed onto a water-permeable material (e.g. several layers of blotting-paper) and clutched between two plates. The upper plate had three rows of 8 holes each with a diameter of 0.5 cm) for the reaction solution. Fixed volumes of reagent solutions were transferred into the holes with the help of a pipette.

Experimental procedures

Preparation of ADH solution. 5 ml of a crystalline suspension of ADH in saturated $(NH_4)_2SO_4$ solution were transferred into a centrifuge test-tube and centrifuged for 10 min at 8000 r.p.m. The

precipitate was redissolved in 2 ml of 0.05 M K-phosphate buffer, pH 7.8, and recentrifuged for 20 min. The concentration of ADH in the solution was determined spectrophotometrically using the coefficient of molar adsorption $\varepsilon_{280} = 3.82 \times 10^4$ M^{-1} cm^{-1}.

Preparation of substrate solutions. Substrate solution 1 contains 0.15-0.2 mM NDMA, 0.1 M ethanol and 0.4 mM $K_3Fe(CN)_6$ in 0.05 M Tris-HCl buffer, pH 9.2. It was prepared just before experiments from solutions of NDMA in ethanol (5.5 mg/ml) and $K_3Fe(CN)_6$ in water (0.1 M) which were kept in the dark at 4 ^{o} C. 50 µl of NDMA solution and 10 µl of $K_3Fe(CN)_6$ solution were added to 10 µl of 0.05 M Tris-HCl buffer, pH 9.2.

Substrate solution 1a contained all the components of substrate solution 1 but at double concentrations.

To prepare the substrate mixture (substrate solution 2) for detection of alkaline phosphatase on the membrane, equal volumes of NADPH (2×10^{-5} M) in the same buffer and substrate solution 1a were mixed.

Substrate solution 3 contained 1-naphtylphosphate (1.23 mg/ml) and Fast Blue RR (1 mg/ml) in 0.1 M Tris-HCl, pH 9.2, and 1 mM $MgCl_2$.

Substrate solution 4 contained 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (1.2 mM) and nitro blue tetrazolium (0.17 mM) in 0.1M 2-amino-2-methyl-1-propanol, pH 9.8, and 1 mM $MgCl_2$.

Methods for NAD(H) quantitation

Method 1. A nitrocellulose membrane impregnated with water was fastened in a reaction cell. In each well of the cell the following

reagents were consequently added: 25-30 μl of AHSA solution in ethanol (0.5 mg/ml), 50 μl of ADH (10^{-6} - 5 x 10^{-7} M) in 0.05 M K-phosphate buffer, pH 7.8, 25-50 μl of standard solutions of NAD(H) (concentration range 10^{-7} - 10^{-9} M) or a test sample. After adding AHSA solution into the wells it is desirable to dry a little the membrane on the air (5-7 minutes). After adding the test sample, the membrane was transferred from the reaction cell into substrate solution 1, incubated for 5-10 min and washed thoroughly with distilled water. The color intensity of reaction zones was evaluated visually or with the help of a dual-wavelength flying spot scanner at $\lambda = 640$ μm..

Method 2. All operations were performed as in Method 1, but at the last stage substrate solution 1 was added into the wells (100 - 200 μl portions) of the reaction cell. In 5-10 min, the membrane was taken out of the cell, washed with water, and the color intensity of the spots on the membrane was measured.

Methods for alkaline phosphatase quantitation on the membrane

1. Method based on the use of the amplifying coenzyme regeneration system. The following reaction components were consequently applied to the membrane: 30 μl of AHSA in ethanol (0.5 mg/ml), 25-50 μl of standard alkaline phosphatase aqueous solutions (at concentrations of 10^{-15} - 10^{-9} M) or test samples of alkaline phosphatase, 50 μl of alcohol dehydrogenase (10^{-7} - 2 x 10^{-7} M) in 0.05 M K-phosphate buffer, pH 7.8 -9.2, 1mM

$MgCl_2$ and substrate solution 2. After incubation for 3-5 min at room temperature (the time of the enzymatic reaction was limited by the time of development of a background signal in the absence of alkaline phosphatase) the colored intensity of the reaction zones on the membrane was evaluated visually or measured with the help of a dual-wavelength flying spot scanner.

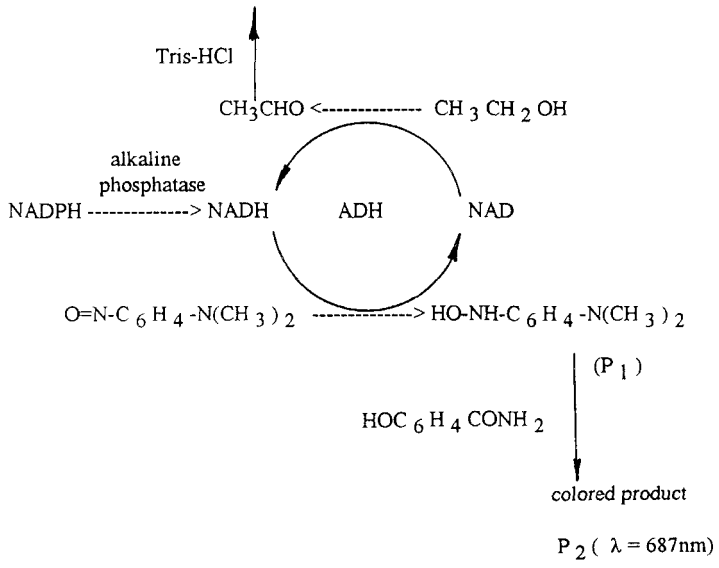
2. Method based on the use of substrate solution 3 or 4. Micro volumes (25-50 μl) of standard solutions of alkaline phosphatase or test samples were applied to the membrane. The membrane was then immersed in substrate solution 3 or 4 and incubated for 0.5 - 2 h. The color intensity of the reaction zones on the membrane was measured with the help of a dual-wavelength flying spot scanner at 595 nm (brown-black product) or at 530 nm (blue product), respectively.

RESULTS AND DISCUSSION

A one-enzyme cofactor regeneration system allowing the detection of small concentrations of NAD(H) was developed earlier[2,3].

The system was used in the proposed method for high sensitive detection of alkaline phosphatase in solution (Scheme 1).

Alkaline phosphatase acts on NADPH as on a substrate. The reaction product, NADH is detected in a coenzyme regeneration system employing horse liver alcohol dehydrogenase. The most valuable feature of the method is that the regeneration of NADH proceeds without dissociation of the coenzyme from the active centre of horse liver alcohol dehydrogenase owing to the pair of coupled substrates of the enzyme - ethanol and p-nitroso-N,N'-dimethylaniline. This causes



Scheme 1

the essential acceleration of the total rate of NADH regeneration as compared to the known two-enzyme regeneration system (alcohol dehydrogenase - NADH-dehydrogenase)[4-6] . To estimate the reaction rate through the increase in the optical density of solution, the coupled chemical system obtained by the interaction between the product of the enzymatic reduction of p-nitroso-N,N'-dimethylaniline (P_1) and salicylamide was used[7] Intensively colored substance P_2 with the maximum of absorbance at 687 nm was formed as result of enzymatic and chemical reactions. In order to move the reaction equilibrium in the direction of colored product P_2 , the acetaldehyde formed as a result of ethanol oxidation was captured by Tris-HCl buffer.

The system permits decreasing the lower detection limit of alkaline phosphatase in solution to 10^{-16} M, the total time of assaying being equal to 20-25 min.

The purpose of the present investigation was to elaborate a highly sensitive nicotineamide adenine dinucleotide - alkaline phosphatase visual quantitation technique based on the described system, some components of which (namely ADH and hydrophobic derivative of salicylamide, AHSA) were supposed to be immobilised on specially prepared porous membrane carriers. The migration of the colored analogue of product P_2 from the membrane surface into the solution is impossible due to the effective binding to the matrix. Therefore the opportunity appears to observe visually the change of the matrix color taking place at NAD (or alkaline phosphatase) localisation sites.

The choice of the membrane matrix with optimal properties. Adsorption, mechanical and hydrodynamic properties of the membrane are the most important for the proper choice of the matrix for the membrane enzyme immunoassay. Such membranes must be porous, have good liquid permeability, mechanical and storage stability, high adsorbability for proteins (alcohol dehydrogenase or specific antibodies) and low non-specific adsorption in respect of test substances. The velocity of penetration of the test liquid through the membrane limits the time of the contact of the sample or the substrate with the active zone on the membrane or the total time of assaying.

To make the optimal choice of the membrane, we studied some acetate cellulose and nitrocellulose matrixes with a pore diameter of 0.2

- 0.5 μm and found that acetate cellulose membranes failed to adsorb alcohol dehydrogenase under conditions of quick adding of its solution to the membrane during analysis.

Therefore all other experiments were performed using nitrocellulose membranes which have good adsorption properties. It was established that the increase in the diameter of pores led to shortening the total time of analysis. On the other hand, the increase in the diameter of membrane pores up to 0.4 μm caused the shortening of the time of the contact between the reagents and the membrane, which resulted in irregular painting of membrane reaction zones, and deteriorated the precision and reproducibility of the assay. Thus nitrocellulose membranes with a pore diameter of 0.2 and 0.3 μm were used in further experiments.

Immobilisation of modified salicylamide on nitrocellulose membranes. The important requirement for the membrane enzyme immunoassay is the formation of an insoluble coloured product on the membrane. The above described regeneration system for the detection of alkaline phosphatase can not be used as a label in membrane immunoassay due to high solubility of the colored product reaction. The immobilisation of salicylamide on the membrane by impregnation of the membrane with ethanol solution of salicylamide is inefficient, since the final colored product is quickly washed off the membrane into solution. In order to receive the water-insoluble membrane-bound final colored product -an analogue of salicylamide, N-(6'-aminohexyl)salicylamide (AHSA) was synthesised by introducing hexamethylene diamine spacer into amide group of salicylamide.

Impregnation of the membrane by ethanol solution of the modified salicylamide followed by amplification reactions permitted a bright blue colored water-insoluble product to be obtained on the membrane. It is possible that firm adsorption of the colored product on the membrane is due to the hydrophobic character of hexamethylenediamine spacer. The blue product formed on the membrane may be easily detected visually or with a reflection densitometer.

The reflection spectrum of the reaction product had a maximum at 640 nm. The reflection maximum of the blue product and the reflection maximum of the yellow substrate, NDMA (445 nm) partially adsorbed by the membrane during enzymatic reaction did not overlap. It should be mentioned that the final colored reaction product was instable and gradually decolorized. To prevent the process it was proposed earlier for an analogous soluble system to add $K_3Fe(CN)_6$ (0.25 - 0.4 mM) in the solution, which increases the velocity of the final colored product formation and prevents it from decolorizing[5]. Thus, in order to obtain good reproducibility and precision it is necessary to measure color intensity on the membrane during the first 30 min of the reaction. As a stop-solution we used 0.5 - 1.0 % water solution of dodecyl sodium sulphate, or sometimes the reaction was stopped by the intensive washing of the membrane with water.

Detection of NAD(H) on the membrane in amplification system. Two variants of NAD(H) quantitation have been developed, which have equal sensitivity, but differ in the stage of product detection.

Standard water solutions of NAD(H) or test samples (25-50 μl, 10^{-9} - 10^{-7} M) were added into the holes of a cell on a preliminary

activated membrane (see Materials and Methods). In the first variant, the membrane was completely dipped in the substrate solution after adding all necessary reagents. During the substrate reaction the membrane was uniformly saturated with NDMA. Its free non-reacting sites became light yellow (the color of NDMA solution), while circular reaction zones became light blue.

The detection time in substrate solution (5-10 minutes) is limited by the background signal due to the presence of endogenous NAD(H) in the ADH-active site. The color intensity of the dots on the membrane is proportional to the NAD(H)-content in the samples. The linear part of the standard curve (Fig.1a) for NAD(H) identification corresponds to the initial concentration range of 10^{-9} - 2 x 10^{-8} M. The upper detection limit is 10^{-7} M (the plateau on the standard curve). The lower NAD(H) detection limit is restricted by the background signal and reaches the value of 2 x 10^{-9} M NAD(H), that is 1 x 10^{-13} moles in a 50 µl sample. This value differs from the background signal by the double absolute error of detection.

The background signal is due to the presence of endogenous NAD in the ADH preparation immobilised on the membrane. During the first 5 - 10 min of the substrate reaction the background signal does not considerably differ from the blank substrate signal in the absence of ADH. On increasing the detection time, the background signal and the useful signal increase proportionally, which leads to decrease in sensitivity of the assay.

It should be noted that the order in which the reagents (ADH and AHSA) are applied to the membrane is important to the results of

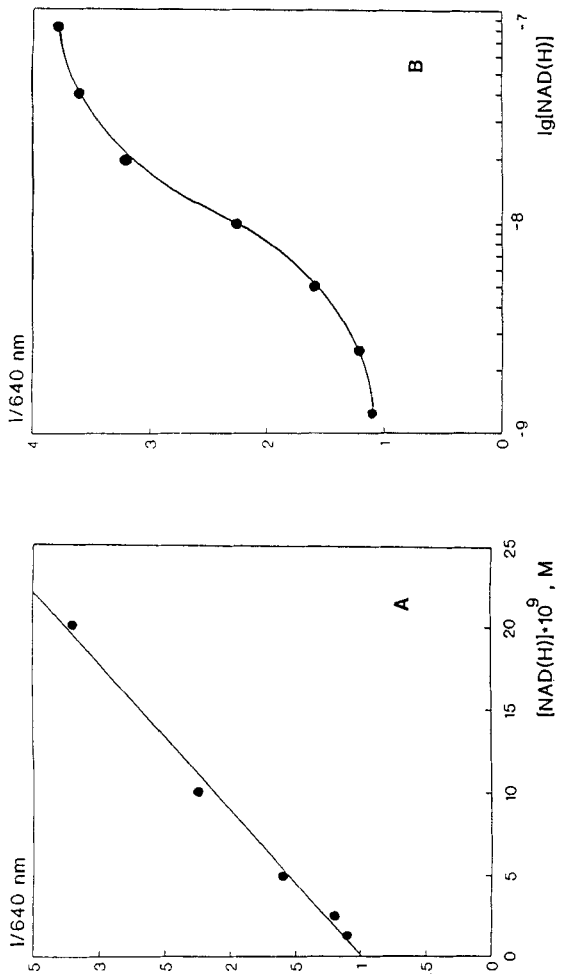


Fig.1. Standard curves of the membrane-type assay for NAD(H) in narrow (a) and wide (b) ranges of concentrations

assaying. If the ethanol solution of AHSA is applied to the membrane before the water solution of ADH, the enzyme activity is considerably depressed.

The choice of the concentrations of the reagents depends on the following reasons. AHSA-concentrations from 0.5 to 1 mg/ml are saturating, while the concentration of ADH ($\sim 10^{-5}$ M) is far from saturating and limited by the value of the background signal.

It should be also mentioned that after impregnation with AHSA and ADH solutions the membrane should not be dried, since the ADH immobilised on the membrane is rather rapidly inactivated on the air due to oxidation of its essential SH-groups. Because of this, assay must be performed immediately after applying aliquots of AHSA and ADH solutions to the membrane.

In the second version of the membrane method of NAD(H) detection, aliquots of substrate solution 1 were added into the wells with a pipette. As a result of the enzymatic reaction colored dots with the clear blue-white outlines appeared on the membrane, in contrast to the first version where the boundaries of the dots were slightly smeared, and the entire membrane became colored due to penetration of yellow NDMA solution into its pores.

The time of detection is limited by the time of penetration of the sample and substrate solutions through the membrane. 100 - 200 μl of water solution percolate through the membrane for 5 - 7 min. The addition of more than 200 μl of the substrate mixture does not improve sensitivity of the assay, but increases the background signal and the time of detection.

Detection of alkaline phosphatase on the membrane in the amplification system. The principle of alkaline phosphatase detection has been described above. Its principal characteristic feature is rigorous specificity of ADH to NAD(H) but not to NADP(H), which enables the detection of low concentrations of NAD(H) in the presence of high concentrations of NADP(H).

Standard samples (25 - 50 μl) of alkaline phosphatase in 0.05 M Tris-HCl buffer pH 7.0 - 7.2 were added into the wells on the membrane in the concentration range 10^{-15} - 10^{-9} M by five- or two-fold dilution of the initial enzyme solution. As follows from the calibration curve of the matrix colored intensity versus substance concentration presented in Fig.2, the lower detection limit (the value defined as the concentration of the analyte corresponding to the double error of the background absorbance value) is of order of 10^{-14} M (or 10^{-18} moles of alkaline phosphatase in the sample) and limited by the value of the background signal of the substrate mixture. The total time of assaying is 10 - 15 min, and the detection time is 3 - 5 min.

The background signal is due to nonenzymatic alkaline hydrolysis of NADPH in the reaction mixture or endogenous NAD(H) in ADH preparations. Therefore, it is necessary to use high-purified NADPH. The choice of the reduced form of the coenzyme is explained by higher stability of NADPH as compared to NADP in alkaline solutions. The concentration of NADPH used in the assay (10^{-5} M) is far from saturating because of the necessity to reduce the background value.

The order in which ADH and alkaline phosphatase are applied to the membrane depends on adsorption ability of the nitrocellulose

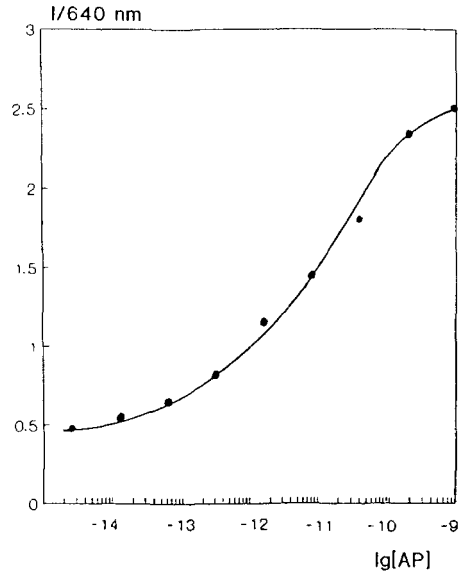


Fig.2. Standard curve of the membrane-type assay for alkaline phosphatase

membrane. The concentration of ADH-solution (~ 10^{-7} M) used in the assay is by several orders of magnitude higher than the analysed concentrations of alkaline phosphatase. Therefore, for complete quantitative adsorption of alkaline phosphatase on the free binding sites of the membrane, small volumes of alkaline phosphatase solutions should be added before the addition of ADH.

The characteristics of the proposed method were compared with those of the conventional systems for alkaline phosphatase detection usually used in dot-ELISA. The lower detection limit of alkaline phosphatase activity in a 50 µl sample for substrate solution 3 is 10^{-9} M for the reaction time of 0.5 - 1 h and 10^{-10} M for reaction time of 1.5 - 2 h; for

substrate solution 4, the lower detection limit is 10^{-10} M (25 μl sample, the reaction time 1 h) or 5×10^{-11} M (50 μl sample, the reaction time - 1 h). In both systems the background signals are negligible, and thus the detection time may be prolonged up to 2 h. In this case, however the reproducibility of the assay decreases due to partial desorption of the reaction products from the membrane.

The proposed method for alkaline phosphatase detection is very sensitive and may be successfully used in the membrane enzyme immunoassay with alkaline phosphatase as a label. Such investigations are now in progress.

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FOOTNOTES

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Telephone: (095) 939-3407; Fax: (095) 939-2742

[2] Hereinafter, the abbreviations ADH, AP, NDMA and AHSA are used for horse liver alcohol dehydrogenase, calf intestinal alkaline phosphatase, p -nitroso-N,N'-dimethylaniline and N-(6'-aminohexyl)-salicylamide, respectively.

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