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Note

High-performance liquid chromatographic assay for nicotinamide–adenine dinucleotide kinase

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The phosphorylation of nicotinamide–adenine dinucleotide (NAD) to the corresponding phosphate (NADP) is a process occurring in animals[1,2], algal cells[3,4] and in higher plant leaves and seedlings[5,7]. In plants it is a light-dependent process connected with the energy exchange in chloroplasts where the NAD kinase activity plays an important role in the photoregulation of the nicotinamide coenzyme levels. The enzyme responsible for this phosphorylation is a NAD kinase (E.C. 2.7.1.23) which is generally activated by Ca^{2+} and calmodulin and requires a nucleoside triphosphate as a phosphate donor, according to the reaction:

$$NAD + ATP \xrightarrow{\text{NAD kinase}} NADP + ADP$$

Even though the enzyme is often called ATP/NAD^+ 2′-phosphotransferase, it is not highly specific for this phosphate donor. Other nucleoside triphosphates, such as GTP, ITP and UTP , are also very effective[8].

The measurement of enzyme activity is a time-consuming procedure which requires an additional enzyme (glucose-6-phosphate dehydrogenase or isocitrate dehydrogenase) for the determination of NADP produced during the kinase reaction. On the other hand, high-performance liquid chromatography (HPLC) is a technique which provides a simple and direct determination of substrate(s) and product(s)[9–11] and therefore is an efficient method for the assay for NAD kinase which is more rapid than the common method and does not require consecutive reactions.

EXPERIMENTAL

Materials

NAD^+ and $NADP^+$ were obtained from Boehringer (Mannheim, F.R.G.); NAD kinase, GTP and GDP were from Sigma (St. Louis, MO, U.S.A.); thymine from Fluka (Buchs, Switzerland), and calmodulin from veal brain was a gift from Dr. Graziano Zocchi (Istituto di Chimica Agraria, University of Milano). All other chemicals were of analytical reagent grade. Methanol was of HPLC grade (Baker Chemicals, Deventer, The Netherlands). Water was distilled in a glass apparatus and filtered through a 0.45-μm membrane (Type HA; Millipore, Bedford, MA, U.S.A.) before use.

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Chromatographic conditions

HPLC analyses of the enzyme-catalysed reaction were performed with a Waters Assoc. apparatus (Milford, MA, U.S.A.) consisting of a Model 590 pump, equipped with a Model U6K universal injector, a Lambda-Max Model 480 ultraviolet detector and a Model 730 data module. Separations were accomplished on a μBondapak C_{18} column (300 mm × 3.9 mm I.D.) with a C_{18} Corasil precolumn (35 × 3.9 mm I.D.) (both from Waters). Elution was performed with 0.1 *M* potassium dihydrogenphosphate buffer pH (6.1) containing 5% methanol at a flow-rate of 2.0 ml/min and the eluent was monitored by UV absorption at 254 nm (0.05 a.u.f.s.).

A typical chromatogram of the reaction mixture is shown in Fig. 1.

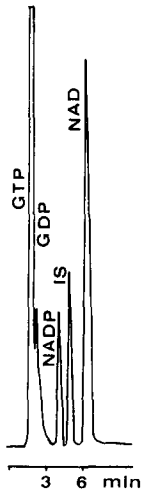

Fig. 1. Typical HPLC chromatogram of the reaction mixture, indicating GTP, GDP, thymine (IS), NAD and NADP. The amounts of NAD, NADP and thymine injected after 10 min of reaction were 2.9, 0.61 and 2.0 nmol respectively. The elution was monitored at 254 nm (0.05 a.u.f.s.).

Assay of enzyme activity

2 m*M* NAD in 0.1 *M* Tris–HCl (pH 7.6) was allowed to react with 3 m*M* GTP and NAD kinase in the presence of 0.1 m*M* $CaCl_2$ and 4.5 μg of calmodulin in a total volume of 1 ml. The enzyme was pre-incubated with calmodulin at 37°C for 2 min, and the reaction was started by adding NAD. At 10-min intervals, 10 μl of the reaction mixture were pipetted into 40 μl of 20 m*M* HCl, containing 10 nmol of thymine as an internal standard, then 10 μl of this solution were injected into the HPLC apparatus. Alternatively, the reaction mixture contained also 0.4 m*M* NADP, *i.e.*, the product of the reaction, and the samples were diluted in only 20 m*M* HCl. When NADP was added to the reaction mixture the product formed during the enzyme-catalysed reaction was calculated as previously described[12], according to

$$\text{nmol NADP} = \frac{P_0\left[\dfrac{A_s(0) - Y_s}{A_p(0) - Y_p} - \dfrac{A_s(t) - Y_s}{A_p(t) - Y_p}\right] \cdot R}{\left[\dfrac{A_s(t) - Y_s}{A_p(t) - Y_p} \cdot R\right] + 1}$$

where: P_0 = amount of product initially present in the injected sample, $A_s(0)$ = peak area of the substrate (NAD) at time zero, $A_p(0)$ = peak area of the product (NADP) at time zero, $A_s(t)$ = peak area of NAD at time t, $A_p(t)$ = peak area of NADP at time t, R = ratio of the slopes of the calibration graphs, NADP/NAD, Y_s and Y_p = peak areas of NAD and NADP respectively at zero concentration calculated from the calibration graphs obtained without the internal standard.

An activity unit is defined as the amount of enzyme which phosphorylates 1.0 nmol of NAD to NADP per min at 37°C, pH 7.6, and in the presence of calcium ions and calmodulin.

RESULTS AND DISCUSSION

GTP was chosen as the phosphate donor since it did not overlap NADP, as did ATP under the chromatographic conditions used, and because it is even more effective than ATP[8]. However, while GTP overlapped the peak of GDP, another product of the reaction, this did not affect the analysis, since the assay was based mainly on the determination of NADP. When the column was not exhaustively washed in the course of the analysis, the NAD peak tended to shift toward the internal standard (thymine) and an appropriate reequilibration of the column (about 30 min) led to a delay in the total assay. This inconvenience was circumvented by eliminating thymine from the dilution medium and using NADP itself as the internal standard according to the procedure described.

Reproducible results (S.D. = 4.2%) were obtained in the assay of the specific activity of three different amounts of enzyme (3.7 units/mg). In the calibration graphs, the detector responses for NAD and NADP standard samples were linear in the range of 0.5–10 nmol (r = 0.999) and 0.2–4.5 nmol (r = 0.999) respectively.

The activity measured by means of the internal standard method indicated no difference from that determined with NADP and the use of the equation reported above, as shown in Fig. 2. This means that the concentration of NADP in the assay

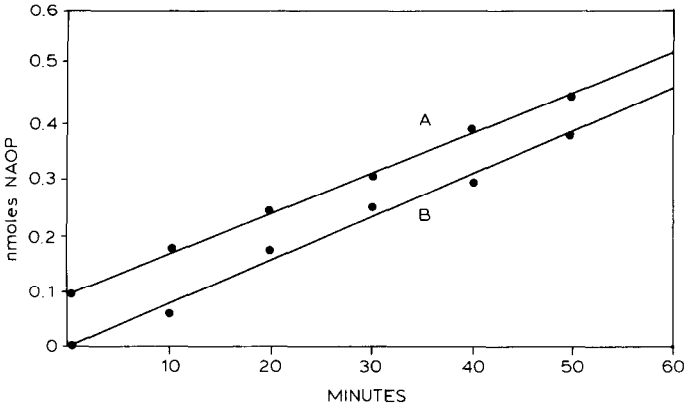

Fig. 2. Comparison of the activities of a sample of NAD kinase determined by the internal standard method (A) and the equation given in the text (B). The values of the constants in the equation were P_0 = 0.08 nmol, R = 0.8, Y_s = 484.8 and Y_p = −0.05. The specific activities were 3.6 and 3.8 units/mg respectively.

sample does not cause any product inhibition but is able to provide a more rapid assay than the use of an internal standard, which requires prolonged washing of the column (about 30 min). Moreover, the specific activity of NAD kinase was linearly related to the amount of enzyme used as demonstrated with three different quantities of enzyme.

In conclusion, the HPLC assay proposed here for NAD kinase activity represents a precise and rapid method without the need of a second enzyme for the determination of the reaction product (NADP).

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