PARTIAL PURIFICATION AND CHARACTERIZATION OF NAD KINASE FROM POTATO TUBER

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Introduction

NAD kinase (ATP: NAD 2'-phosphotransferase, EC. 2. 7. 1. 23), the enzyme which catalyzes the synthesis of NADP, might be responsible for the regulation of NADP level in plant tissues. The pioneer work on plant NAD kinase by YAMAMOTO (15) in 1966 elucidated the heterogenity of NAD kinase from yeast (2, 3) and animal sources (12), and discussed the photoactivation of NAD kinase through phytochrome (14). The authors reported that the activation of NAD kinase might be an important factor for increasing glucose oxidation via the pentose phosphate pathway when DNA synthesis was induced in potato tissue cultures (8).

The present paper describes isolation and purification of NAD kinase from potato tubers. An attempt has been made to elucidate some properties of the enzyme activity.

Materials and methods

Plant materials

The tubers of potato (*Solanum tuberosum* L. var, Irish Cobbler) stored for 6 months in a cellar at 2°C in darkness were used as materials.

Cellular fractionation

Potato tuber tissue was ground with an equal quantity of buffer A (50 mM tris-HCl, pH 7.6, 0.5 M sucrose, 1 mM EDTA, 0.1% bovine serum alubumin) containing 0.1% cysteine. The homogenate was squeezed through 3 layers of gauze and centrifuged at $600 \times g$ for 10 min. The supernatant was passed through a Sephadex G-25 column which previously equilibrated with buffer A to remove phenolic and other low molecular weight compounds. The protein fraction thus obtained was centrifuged at $10{,}000 \times g$ for 30 min to sediment the mitochondrial fraction. The mitochondrial fraction was

washed with buffer A. The supernatant fluid was centrifuged at 105,000 $\times g$ for 2 hr to sediment the microsomal fraction. The microsomal fraction was washed with buffer A. These precipitates were suspended with 2 ml of buffer B (50 mM tris-HCl, pH 7.6). The supernatant fraction was treated with 40% saturation of $(NH_4)_2SO_4$. The protein fraction precipitating was collected and dissolved in buffer B, and then passed through a column of Sephadex G-25 and eluted with buffer B. The mitochondrial, microsomal and cytosol fractions were used for enzyme assay.

Determination of protein content

Protein content was determined by the method of LOWRY *et al.* (10) against bovine serum albumin standard solution.

Purification of NAD kinase

(Step I): The potato tubers were peeled and ground with a grater to buffer B containing 0.5 M sucrose, 4 mM EDTA and 0.1% cysteine. The homogenate was squeezed through three layers of gauze cloth and centrifuged for 15 min at 12,000 $\times g$.

(Step II): The supernatant was brought to 40% saturation with $(NH_4)_2SO_4$. After 20 min, the precipitate was collected by centrifugation at 12,000 $\times$ g for 10 min and suspended in buffer B. The solution was applied to a column of Sephadex G-200 which had been equilibrated with buffer B.

(Step III): The eluted enzyme fractions were concentrated by 45% saturation of $(NH_4)_2SO_4$ and centrifuged at 15,000 $\times g$ for 10 min. The precipitate was redissolved with 25 mM tris-HCl (pH 8.0) containing 1 mM EDTA and then the enzyme solution was applied to Sephadex G-25 column and eluted with the same buffer. The precipitate appeared was removed by centrifugation at 20,000 $\times g$ for 10 min.

(Step IV): The supernatant was charged to a DEAE-cellulose column which had been packed under a slight pressure and equilibrated with 25 mM tris-HCl buffer (pH 8.0) containing 1 mM EDTA. The column was developed with a linear gradient of NaCl concentration (0–0.75 M).

Assay of NAD kinase

The standard assay system contained 0.65 μmol NAD, 1.6 μmol ATP, 7.0 μmol $MgCl_2$, 50 μmol tris-HCl, pH 7.6, and a suitable amount of enzyme, in a total volume of 1.0 ml. After incubation for 30 min at 30°C, the reaction was terminated by immersing the mixture in boiling water for 1 min and the contents were then cooled in an ice bath. The NADP formed was determined by a recycling procedure essentially similar to that of BERNO-

FSKY and SWAN (4). In a 3 ml cuvette were put the supernatant and 2 ml of 50 mM tris-HCl (pH 7.6) containing 12 μmol $MgCl_2$, 0.2 μmol phenazine methosulfate, 0.5 μmol thiazolyl blue and 0.5 U glucose 6-phosphate dehydrogenase. After a warming-up period of 5 min, the reaction was started by addition of 0.1 ml of 0.5 mM glucose 6-phosphate and followed **A** 570 nm in recording spectrophotometer. The rate of increase of **A** 570 nm was directly proportional to the amount of NADP present.

Determination of molecular weight

The molecular weight of enzyme was determined by Sephadex G-200 column chromatography and sucrose density gradient centrifugation. Exclusion chromatography was performed as described by ANDREWS (1) using cytochrome C (12,500), bovine serum albumin (67,000), aldolase (158,000), catalase (240,000) and ferritin (540,000) as standards. A sedimentation experiment was performed at 4°C in Hitachi RPS 25-3A swing rotor for 16 hr at 23,000 rpm using catalase (11.36 S) as standard. An aliquot of NAD kinase was layered on a 11 ml of 2 to 18% linear sucrose gradient in buffer B. After centrifugation, 50 fractions were collected and assayed for NAD kinase activity.

Disc electrophoresis

Discontinuous polyacrylamide gel electrophoresis was performed as described by DAVIS (7). The activity staining of gel was performed as APPS (3).

Results

(1) Distribution of potato NAD kinase

NAD kinase of potato tubers was exclusively present in the cytosol fraction; the supernatant fraction after centrifugation at 105,000 g for 2 hr (>97%). The enzyme activity was found only after the application of Sephadex G-25 column. Mitochondrial and microsomal fractions showed a slight activity even after 2nd wash (Table 1).

TABLE 1. NAD kinase activity of cell fractions

Fraction	Activity (unit*)	Distribution (%)
Mitochondria	0.186	1.0
Microsome	0.218	1.5
Cytosol**	13.636	97.5

* nmol NADP formed/g fr. wt./hr.
** treated with 40% $(NH_4)_2SO_4$ and desalted.

(2) Purification of potato NAD kinase

A summary of the enzyme purification is given in Table 2. The ammonium sulfate fractionation followed by gel filtration gave a potential activity of NAD kinase from the crude extract. This may be due to the removal of inhibitor(s) and NADP degradating enzymes from the extract. The specific activity obtained at final stage was 200 fold more than that of the crude extract (recovery 56%). The activity was proportional to the amount of enzyme added to the reaction mixture (minimum 7 μg protein) when tested for 1 hr, and was fairly unstable; 25% loss in activity occurred after 24 hr at 2°C. The following experiments were carried out in a linear range of enzyme protein and time. Polyacrylamide gel electrophoresis of the purified enzyme preparation followed by activity staining showed a single reaction band.

TABLE 2. Purification of NAD kinase from potato tuber

Step	Total volume (ml)	Total protein (mg)	Total activity (unit*)	Specific activity (unit/mg)
1. Crude extract	260	2875.4	—	(0.97)**
2. 1st $(NH_4)_2SO_4$ and Sephadex G-200	60	140.2	2777	19.83
3. 2nd $(NH_4)_2SO_4$ and Sephadex G-25	10	106.1	2572	24.26
4. DEAE-Cellulose	12	7.8	1607	206.02

* nmol NADP formed/hr.
** expected value.

TABLE 3. Effect of various divalent cations on NAD kinase activity

Metal ion (2 mM)	Activity (unit*)	Relative activity (%)
Mg^{2+}	186	100
Mn^{2+}	119	64
Ca^{2+}	113	61
Co^{2+}	93	50
Zn^{2+}	29	16
None	2	1

* nmol NADP formed/mg protein/hr.

(3) Components required for NAD kinase activity

The activity of NAD kinase required NAD, a phosphoryl group donor, and a divalent metal ion. On omission of any of these components, there was no formation of NADP. ATP was the most effective donor relative

to GTP, UTP or ADP, with which there were 8% or less formation of NADP when compared with ATP. Table 3 shows the absolute requirement of a divalent ion for NAD kinase reaction. Mg^{2+} ion was preferred, but Mn^{2+}, Ca^{2+} and Co^{2+} could in part replace Mg^{2+}, when tested in 2 mM concentration. They often activated the enzyme reaction in very limited concentration ranges.

(4) Ratio of ATP to Mg^{2+}

Keeping the NAD concentration fixed at 0.65 mM and ATP at 1.0 mM, the concentration of Mg^{2+} was varied in a range of 0.2 to 10.0 mM. The maximum activity was observed at ATP : Mg^{2+} ratio of 1 : 1. This result indicates that the NAD kinase was inhibited by the excess of free Mg^{2+}.

(5) Effect of pH

By using 3 different buffers (cacodylate buffer in the range of pH 5.0–7.2, tris buffer in the range of 6.5–8.5 and glycine buffer in the range of 8.2–10.5), the effect of pH was studied. Maximum activity was observed at pH 6.5–6.9 and the activity dropped sharply at pH value below 6.0. The pH values for half maximal activity were 5.4 and 8.6.

(6) Km values of NAD and ATP

The *Km* for each substrate was independent of the concentration of the other, and the *Km* values calculated were 1.0 mM for NAD and 1.2 mM for ATP.

(7) Inhibition of NAD kinase by NADH and NADPH

NAD kinase from potato tuber was inhibited by NADH and NADPH, and the inhibitions were competitive with respect to NAD. The *Ki* value of NADH was 0.06 mM and that of NADPH was 0.05 mM. The values were determined by the method of YAMAMOTO (15).

(8) Inhibition by sulfhydryl reagents

Several sulfhydryl reagents inhibited the NAD kinase (Table 4) which suggested the presence of essential sulfhydryl group in catalytic site of the enzyme.

(9) Determination of molecular weight

Molecular weight estimation of the enzyme by exclusion chromatography indicated a value of 175,000 daltons. Furthermore, sedimentation constant of that obtained by sucrose density gradient centrifugation was 8.6 S. This value of sedimentation constant is in good agreement with the estimate by exclusion chromatography using the formula of PAETKAU and LARDY (13).

TABLE 4. Effect of sulfhydryl reagents on NAD kinase activity

Reagent	Concentration	Activity (unit*)	Inhibition (%)
None	(M)	182	0
PCMB**	10^{-5}	0	100
PCMB	10^{-6}	106	42
ICH_2COOH	10^{-2}	156	14
$Hg(CH_3COO)_2$	10^{-5}	16	91

* nmol NADP formed/mg protein/hr.
** p-Chloromercuribenzoate

Discussion

Determination of the molecular weight of plant NAD kinase was not so far reported. Our preliminary work indicated the enzyme associated as a complex under low ionic strength. While the purified enzyme showed the molecular weight of 175,000 which was a minimum molecular weight of the enzyme activity. Recently MUTO and MIYACHI (11) described a protein activator from pea seedlings. Their pea NAD kinase was absolutely inactive when the activator was dissociated.

The purified NAD kinase from spinach leaves was reported to have *Km* values of 2.0 mM for NAD and 1.1 mM for ATP (15). NAD kinase activity associated with the phytochrome preparation from pea seedlings had *Km* for 1.8 mM NAD under the dark, upon illumination of red-light, *Km* for NAD decreased to 0.9 mM (14). The *Km* values of potato NAD kinase for each substrate was independent on the concentration of the other, and the values were 1.0 mM for NAD and 1.2 mM for ATP as stated above.

The potato NAD kinase showed an absolute requirement for a metal ion. In addition to Mg^{2+}, it was found that Mn^{2+}, Ca^{2+} and Co^{2+} ions were effective in a lesser extent. The maximum activity was observed at 1 : 1 cation/ATP ratio. Free (excess) Mg^{2+} was inhibitory with respect to ATP concentration. The enyzme also exhibited a great degree of specificity for the phosphoryl group donor for NADP synthesis. Both GTP and UTP could not serve as substrate for enzyme reaction nor could ADP and AMP. Above data indicate that the enzyme was highly specific for Mg^{2+}-ATP complex for the reaction.

The enzyme from spinach leaves was competitively inhibited by NADH (15). However, the effect of NADPH on the NAD kinase was not investi-

gated. The present results indicate that both NADH and NADPH were competitive inhibitors of the enzyme. These findings suggest that the levels of reduced nicotinamide nucleotides, NADH and structually related NADPH, may be important for the regulation of NADP synthesis.

Several sulfhydryl reagents inhibited the potato NAD kinase which suggests the presence of essential HS-groups in catalytic sites of the enzyme. Iodoacetic acid (10^{-2} M) appeared to inhibit the enzyme reaction to a very slight degree, while PCMB, at concentration of 10^{-5} M, was a very effective inhibitor.

In potato tissue, a rise in NADP level was observed by slicing followed by aging (5). A rise in NADP level stimulated glucose oxidation via the pentose phosphate pathway in the early stage of callus formation in potato tissue culture, and a similar trend was found for the NAD kinase activity (8). The regulation of NADP level in plant tissues is not clearly understood. Tissues with meristematic activity was also observed the high NADP level, *i. e.* embryoaxis and hypocotyl (6). NAD kinase might play an important role in the regulation of the enzyme activity by the endogenous NADH level. While the photoregulation of NAD kinase through phytochrome was described together with the enzyme activation by cytokinin (14), the presence of a protein activator in pea seedlings and in other plant species was reported (11).

Thus, it appears that the synthesis of NADP may be, in part, regulated by changes in the redox state of nicotinamide nucleotides, and in part, by changes in conformational integrity of the enzyme *in vivo*.

Summary

NAD kinase from tubers of *Solanum tuberosum* was purified more than 200-fold. The enzyme gave a single reaction band on polyacrylamide gel electrophoresis and its molecular weight was 175,000 daltons as determined by Sephadex G-200 column chromatography and sucrose density gradient centrifugation. The enzyme was highly specific for NADP synthesis from NAD and ATP with the optimal pH range of 6.5–6.9. The *Km* value for NAD was 1.0 mM and for ATP was 1.2 mM. ATP was the most effective phosphoryl group donor, showing very little or no activity with a range of GTP, UTP, ADP and AMP. NADH and NADPH were the potent competitive inhibitors with respect to NAD, and *Ki* values were 0.06 mM and 0.05 mM, respectively. Although the enzyme had an absolute requirement of a divalent metal ion, Mg^{2+} was the preferred metal ion which could be partially replaced by Mn^{2+}, Ca^{2+} and Co^{2+}. Inhibition of the enzyme by

PCMB suggested the presence of HS-group(s) in catalytic site of the enzyme.

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