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Properties of a Protein Activator of NAD Kinase from Plants[1]

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ABSTRACT

Purification of pea (*Pisum sativum*) seedling NAD kinase by DEAE-cellulose column chromatography resulted in loss of activity, due to dissociation of an activator from the enzyme. The purified enzyme preparation, which was almost completely inactive, regained the activity when the activator was added back.

The activator was purified 320-fold by ion exchange chromatographies. The activator was susceptible to proteolytic enzymes, but not to ribonuclease, glucoamylase or pectinase, indicating that it is of a protein nature. This protein was relatively stable in boiling water, but susceptible to acid or alkali, especially under high temperatures. Restoration of catalytic activity of inactive enzyme was proportional to amounts of the activator added. Gel filtration indicated that molecular weight of the activator was 28,000.

The activator was found in extracts from various plants.

Oh-hama and Miyachi (17, 18) found that illumination of *Chlorella* cells in the absence of CO_2 caused an increase of NADP and a decrease of NAD. Since the increase of NADP in the light was approximately equal to the decrease of NAD, they assumed that the latter was photochemically transformed into the former. Similar light-induced changes in the amount of NADP and NAD were also observed with higher plant leaves (16). So far, NAD kinase (ATP: NAD 2'-phosphotransferase; EC 2.7.1.23) has been the only enzyme which is known to catalyze the phosphorylation of NAD to NADP. Oh-hama *et al.* (19) found NAD kinase from sonically disintegrated *Chlorella* preparation and partially purified it. They first assumed that the light-induced formation of NADP from NAD occurs via NAD kinase and ATP produced through photophosphorylation. When the disintegrated algal cells were illuminated in the presence of NAD (and Mg^{2+}), there occurred a marked increase of NADP. The addition of ATP to the reaction mixture in the dark did not enhance the formation of NADP. One of the possible explanations for the above finding is that NAD kinase in green plants is a photoactivated enzyme. Recently, Tezuka and Yamamoto (23, 24) reported that red light stimulated the conversion of NAD to NADP, and suggested that the NAD kinase activity was controlled by phytochrome.

To elucidate the controlling mechanism of the NAD kinase activity *in vivo*, it is important to study the properties of the enzyme using a highly purified preparation. The most highly purified enzyme from plant has been obtained by Yamamoto (26). While trying to improve further the purity of plant NAD kinase, we found an activator of this enzyme. This communication describes some properties of the partially purified activator from pea seedlings, as well as the distribution of the activator in various green plants and *Chlorella* cells.

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MATERIALS AND METHODS

Plant Materials. Unless otherwise mentioned, the experiments were carried out with pea seedlings. Seeds of pea (*Pisum sativum* cultivar Gokuwase-akabana-tenashi-endo) were germinated in trays filled with vermiculite. The trays were placed in a greenhouse and irrigated once a day. The temperatures during daytime and the rest of the day were kept at 25 and 20 C, respectively. Seedlings grown under natural light conditions for 12 days were used for the experiments.

To examine the distribution of NAD kinase and its activator among various plants, the respective seedlings of corn (*Zea mays*, cultivar, Nagano No. 1), and rice (*Oriza sativa*, cultivar Nihon-bare) grown in the greenhouse for 2 and 3 weeks were used. *Chlorella vulgaris* 11 hr cells were grown autotrophically with constant bubbling of air containing about 2% CO_2 (15). Spinach (*Spinacia oleracea* L.) and Chinese cabbage (*Brassica rapa* var. Previdis) were purchased from the local market.

Assay of NAD Kinase. The method adopted was essentially the same as described by Wang and Kaplan (25). The routine incubation medium contained 2 mM NAD, 3 mM ATP, 10 mM $MgCl_2$, 100 mM tris-HCl (pH 8), an appropriate amount of enzyme, and 10 units (see below) of the activator where necessary, in a final volume of 0.5 ml. The reaction was initiated by adding NAD and terminated with 100 μl of N HCl. The reaction was run for 30 min at 37 C. The acidified suspension was neutralized with 100 μl of N NaOH and the coagulated protein was removed by centrifugation. The clear supernatant thus obtained was subjected to the determination of NADP according to the method described by Apps (2).

The assay system of NADP contained 0.25 M tris-HCl (pH 8), 30 μg of 2,6-dichlorophenol indophenol, 20 μg of phenazine methosulfate, 0.2 mM glucose-6-P, 0.06 unit of glucose-6-P dehydrogenase, and an appropriate amount of the supernatant (NADP), in a final volume of 1 ml. After 2 to 3 min of preincubation without glucose-6-P dehydrogenase, the reaction was started by adding this enzyme and the rate of decrease in absorbance at 600 nm was recorded. The rate thus obtained was corrected with that determined during preincubation period. The amount of NADP was determined by the use of a calibration curve prepared with authentic NADP solution. The concentration of this authentic NADP was determined enzymically (11).

One unit of the enzyme was defined as the amount of enzyme which transforms 1 μmol of substrate/hr.

Assay of Activator. The activator was assayed by the magnitude of activation of a fixed amount of purified inactive NAD kinase under standard condition. The method for the preparation of inactive enzyme was described under "Results." The standard assay medium contained 2 mM NAD, 3 mM ATP, 10 mM $MgCl_2$, 100 mM tris-HCl (pH 8), 10 units (when fully activated) of inactive NAD kinase, and proper amount of activator.

One unit of activator was defined as the amount required to give a half-maximum activity to this standard amount of inactive enzyme.

Molecular Weight Determination of Activator. The mol wt of the activator was estimated by gel filtration method according to Andrews (1). A column of Sephadex G-75 (2.5 × 37 cm) was pre-equilibrated with 50 mM NH_4-acetate buffer (pH 6) containing 0.1 M NaCl and calibrated with standard proteins.

Determination of Protein. The amount of protein was determined by the method of Lowry *et al.* (13) with crystalline BSA as a standard.

Reagents. NAD, NADP, and glucose-6-P dehydrogenase were purchased from Oriental Yeast Co., Tokyo, and glucose-6-P and ATP from Kyowa Hakko Kogyo Co., Tokyo. DEAE- and P-cellulose were products of Serva Entwicklungslabor, Germany; and trypsin, trypsin inhibitor, and ribonuclease were those of Sigma Chemical Co., St. Louis. Pronase was obtained from Kaken Kagaku Co., Tokyo, and Macerozyme R-10 (crude preparation of pectinase) from Kinki Yakult MFG., Nishinomiya. Glucoamylase was the product of Seikagaku Kogyo Co., Tokyo, and protein markers were those of Schwarz/Mann, Orangeburg, N. Y.

RESULTS

INACTIVATION OF NAD KINASE ACTIVITY DURING DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

Irrespective of the purification steps where the ion exchanger was applied, attemps to purify NAD kinase from pea seedling on DEAE-cellulose column under various conditions always resulted in loss of the activity. Sometimes the activity was completely lost, and in some cases, a small activity was recovered in the eluate. However, in such cases, no increase in the specific activity was observed. It was finally found that the eluate from the column showed an activity when the boiled crude extract was added. This suggests that NAD kinase requires heat-stable activator(s) separated from the enzyme during the DEAE-cellulose column chromatography. To verify the existence of such activator(s), a crude extract from pea seedlings was chromatographed on a DEAE-cellulose column. After the chromatography, the eluates were assayed for the enzyme activity either with (for inactive enzyme) or without (for active enzyme) the boiled crude extract, as well as for the activator activity. The activities of inactive and active enzymes were separated into three large and two small peaks, respectively, and the activator activity was eluted as one sharp peak (Fig. 1). These peaks did not overlap with each other, except that a small peak of the active enzyme coincided with that of activator. When the amount applied on the column was relatively small, no active enzyme was detected in the eluate. These results indicate that NAD kinase loses its catalytic activity by the DEAE-cellulose column chromatography, as a result of separation from the activator.

To investigate the properties of the activator, the inactive enzyme and the activator were purified as follows.

PURIFICATION OF NAD KINASE

The following procedures were carried out at 0 to 4 C.

Extraction. Pea seedlings (780 g) were ground for 1.5 min with 2,340 ml of 25 mM triethanolamine-acetate buffer (pH 7.5) and 15.6 g of polyamide 11 (Merck) in a Waring Blendor. The homogenate was squeezed through nylon cloth and centrifuged at 18,000*g* for 20 min.

Protamine Sulfate Precipitation. A quarter-volume of 0.2% protamine sulfate solution in 10 mM triethanolamine-acetate buffer (pH 7.5) was added to the supernatant (2,695 ml). After continuous stirring for 15 min, the precipitate was collected by centrifugation at 3,000*g* for 15 min. From the precipitate, the enzyme was extracted successively with 300, 160, and 80 ml of 0.2 M Na-acetate buffer (pH 5). About 85% of the enzyme was precipitated by the addition of protamine sulfate (the activity was assayed in the presence of the excess amount of the activator), while as much as 70% of the activator remained in its supernatant, which was saved for the purification.

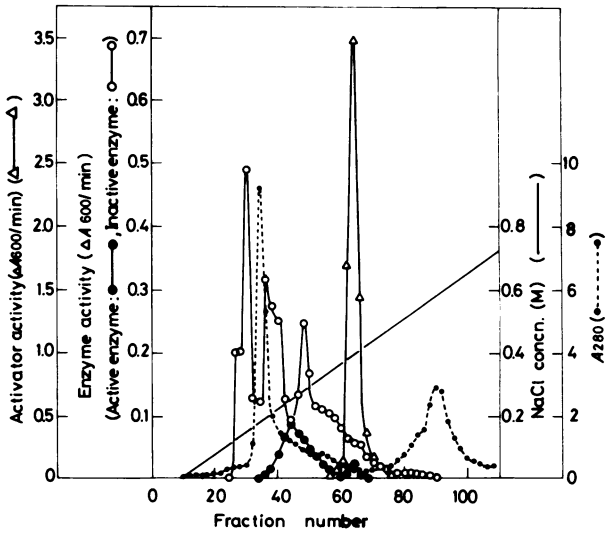

FIG. 1. Resolution of NAD kinase into an active and an inactive enzyme, and its activator on a DEAE-cellulose column. Pea seedlings (70 g) were extracted as described in text, yielding 250 ml of crude extract. To 140 ml of the crude extract, solid ammonium sulfate was added to make a 50% saturation. After centrifugation at 18,000 *g* for 20 min, the precipitate was redissolved in a minimum volume of 25 mM tris-HCl (pH 8) containing 1 mM EDTA. The solution was passed through a Sephadex G-25 column and placed onto a DEAE-cellulose column (2.5 × 27.5 cm). Both columns were pre-equilibrated with the same buffer as above. The DEAE-cellulose column was developed with a linear gradient of NaCl concentration (0–0.75 M). Each tube collected 10 ml.

The rest of the crude extract was heated for 5 min in a boiling water bath. After cooling, the coagulated protein was removed by filtration, and the resulting filtrate was used as crude activator.

The activities of active and inactive enzymes and activator were assayed essentially as described in the text. Portions (100 μl) were taken from each test tube, and the activities of inactive and active enzyme were determined with or without the addition of the crude activator (100 μl). Prior to assay of the activator activity, each eluate (100 μl) taken from the test tube was heated for 5 min in a boiling water bath. The activator activity was assayed using inactive enzyme prepared as described in the text.

The activities of active and inactive enzymes and activator were expressed as decrease in *A* at 600 nm/min · incubation tube.

Polyethylene Glycol Precipitation. To the combined extract, the same volume of 50% (w/w) polyethylene glycol 6,000 solution was added and stirred for 30 min. After centrifugation at 18,000*g* for 30 min, the precipitate was resuspended in 70 ml of 0.1 M Na-phosphate buffer (pH 7) containing 1 mM EDTA. The insoluble materials were removed by centrifugation at 18,000*g* for 10 min.

DEAE-Cellulose Column Chromatography. The supernatant solution was passed through a DEAE-cellulose column (3.4 × 21 cm) pre-equilibrated with the same buffer as mentioned above. Although the enzyme was not adsorbed on the column, the activator was almost completely adsorbed; consequently, the inactivated enzyme was obtained.

P-Cellulose Column Chromatography. The eluate of the DEAE-cellulose column was diluted four times with 1 mM EDTA and placed onto a P-cellulose column (1.6 × 23 cm) pre-equilibrated with 25 mM Na-phosphate buffer (pH 7) containing 1 mM EDTA. The column was washed with 100 ml of the same buffer containing 0.1 M NaCl and eluted with the same buffer containing 0.4 M NaCl.

Purification of the enzyme after each step is summarized in Table I. The enzyme was purified 54-fold after the polyethylene

Table I. Summary of rification of NAD kinase from Pea Seedlings

Step	Vol	Activity		Protein	Specific activity		Recovery	
		- Act.[1]	+ Act.[2]		- Act.[1]	+ Act.[2]	- Act.[1]	+ Act[2]
	ml	units		mg	units/mg protein		%	
Crude extract	2,695	241	268	10,100	0.024	0.027	100	100
Protamine ppt	515	61.5	126	605	0.102	0.208	25.5	47.0
PEG[3] ppt	70	223	413	174	1.28	2.37	92.5	154
DEAE-cellulose	105	0.130	284	95.6	0.001	2.97	0.05	106
P-cellulose	35	0.047	74.8	7.98	0.006	9.37	0.02	27.9

1. The activity was assayed in the absence of exogenous activator.
2. The activity was assayed in the presence of exogenous activator.
3. Polyethylene glycol.

glycol precipitation (see the specific activity in the absence of exogenous activator), and almost completely inactivated after DEAE-cellulose column chromatography. The addition of activator to DEAE- and P-cellulose eluates brought about a drastic increase of the enzyme activity. The same treatment of the extracts from protamine and polyethylene glycol precipitate incurred a 2-fold increase, and even the enzyme activity in the crude extract showed some increase after the addition of the activator. These results indicated that by the DEAE-cellulose chromatography, most of the activator was separated from the enzyme and hence the enzyme was inactivated.

PURIFICATION OF THE ACTIVATOR

Unless otherwise mentioned, the following procedures were carried out at 0 to 4 C.

A part of the supernatant remaining after the addition of protamine sulfate (1,900 ml) was heated for 10 min at 80 C and immediately cooled in an ice bath. NAD kinase was completely and irreversibly inactivated by this treatment. The protein fraction coagulated by this treatment was removed by filtration through a filter paper. After adjusting the pH value to 3.5 with 6 N HCl, the filtrate was applied onto a P-cellulose column (5 × 15 cm) pre-equilibrated with 10 mM Na-citrate buffer (pH 3.5). The column was washed with 1 liter of the same buffer containing 0.1 M NaCl and then eluted with 0.2 M NH_4-acetate buffer (pH 6) containing 0.4 M NaCl. The fractions showing a peak activator activity were collected and diluted 3-fold with water, then applied to a DEAE-cellulose column (3.4 × 17.5 cm) pre-equilibrated with 50 mM NH_4-acetate buffer (pH 6) containing 0.1 M NaCl. After washing with 500 ml of the same buffer, the column was eluted with a linear gradient of NaCl (0.1–0.8 M). The activator was eluted at 3.5 M NaCl as a single peak. The peak fractions of activator were collected and concentrated by ultrafiltration through a G-01T membrane (Bioengineering Co., Tokyo) under a N_2 atmosphere at 4 kg/cm^2, and then the buffer was replaced with water.

Table II shows that after the DEAE-cellulose column chromatography, the activator was purified 320-fold.

PROPERTIES OF THE ACTIVATOR

Susceptibility to Enzymes. Before assay for the activator activity, the activator was treated with trypsin, pronase, ribonuclease, Macerozyme R-10, or glucoamylase. The action of the respective enzymes was terminated by boiling for 10 min. Pretreatment with ribonuclease, Macerozyme, and glucoamylase did not show any effect on the activator activity, while it was inactivated after the treatment with either trypsin or pronase. No loss of activator activity occurred when the trypsin was added together with ovomucoid trypsin inhibitor (data not shown). The effect on the activator activity depended on the amount of trypsin added (Fig. 2). These results indicate that the activity of activator is associated with a polypeptide, rather than with ribonucleic acid, pectin or starch.

Thermal and pH Stabilities. When the water solution of activator was kept in a boiling water bath, the activating capacity decreased by half after 5 min (Fig. 3). However, 20% of the original activity still remained after 30 min, indicating that the activator is relatively heat-stable.

Table III shows that at 37 C, the activator was susceptible to either acid or alkali, but stable in water. Exposure to NaOH (higher than 0.001 N) at 98 C for 30 min almost completely obliterated the activating activity. At 37 C, the activator activity was reduced with increasing concentrations of NaOH. On the other hand, such a concentration dependency was not observed after HCl treatment; throughout the concentrations ranging from 0.001 through 0.1 N, the activator activities decreased to 20 and 6% of the original level at 37 and 98 C, respectively. These results indicate that at 98 C, the activator is more susceptible to NaOH than to HCl.

Relationship between Activator Concentration and the Activity Restoration of Inactive Enzyme. The catalytic activity of inactive NAD kinase (22 μg) was restored in proportion to the concentration of the added activator, reaching a plateau at about 8 μg (Fig. 4). Half-maximal activity was established at 1.2 μg of activator. The curve is pseudosigmoidal, as indicated by a concave shape of the reciprocal plot.

Preliminary incubation of the activator with the inactive enzyme did not bring about any increase on the magnitude of its activating effect. Prolonged incubation over 10 min rather decreased the enzyme activity (data not shown). These results indicate that the association of the activator and the inactive enzyme is a fast reaction and that the enzyme is not stabilized by its association with the activator.

Molecular Weight. The mol wt of the activator was estimated to be 28,000 by gel fltration on a Sephadex G-75 column (Fig. 5).

Specificity. A number of proteins with low mol wt including Cyt *c*, ribonuclease, trypsin inhibitor, and myoglobin were tested for their abilities to restore the acitivity of inactive NAD kinase. None of these proteins restored the activity at concentrations ranging from 10 to 100 μg/reaction mixture (data not shown).

DISTRIBUTION OF NAD KINASE AND ITS ACTIVATOR IN VARIOUS PLANTS

To examine the distribution of NAD kinase and its activator in various plants, crude extracts were prepared from spinach and Chinese cabbage leaves, corn, rice, and pea seedlings, and *Chlorella* cells, respectively. The highest enzyme activity was observed in rice (Table IV). Pea also showed a high activity, and spinach and *Chlorella* showed considerable activities, whereas those of Chinese cabbage and corn seedlings were very low.

Heated extracts from all plants activated the inactive enzyme purified from pea seedlings, indicating that all plants contained activator. The highest activating effect was found in pea, followed by spinach, Chinese cabbage, and rice. The activating

Table II. Summary of fication of the Activator

Step	Vol	Activity	Protein	Specific activity	Recovery
	ml	units	mg	units/mg protein	%
Heated protamine sup.	1,900	19,600	2,930	6.69	100
P-cellulose	250	5,290	59.4	89.1	27.0
DEAE-cellulose	118	1,630	0.76	2,145	8.3

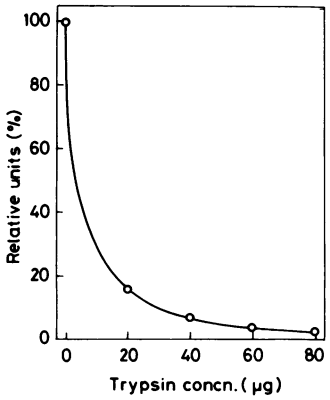

FIG. 2. Effect of trypsin on the activator activity. The activator (7.5 μg) was incubated for 1 hr at 37 C with the indicated amounts of trypsin in a total volume of 300 μl of 0.25 M tris-HCl (pH 8). The reaction was terminated by adding a 50% excess by weight of ovomucoid trypsin inhibitor, and then the activator activity was assayed using inactive enzyme. The control tubes contained the same amount of trypsin inhibitor from the start of incubation. No change of the activator activity was observed in the control.

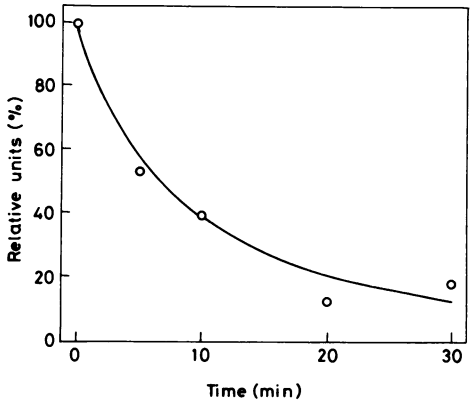

FIG. 3. Thermal stability of the activator. The activator (7.5 μg) was incubated in a boiling water bath. After indicated time periods, portions were taken out for the assay of activator.

effects of corn and *Chlorella* were very low. The ratio of the enzyme activity to the activator in the crude extract was 8:9 in pea and spinach. This ratio was higher in rice and *Chlorella*, and lower in Chinese cabbage and corn.

DISCUSSION

Our results showed that a protein activator was required for NAD kinase activity for enzyme isolated from pea seedlings. When the extract of pea seedlings placed onto a DEAE-cellulose column was developed with a linear gradient of NaCl concentration, the activities of inactive enzymes were separated into three large peaks (Fig. 1). Appearance of the multiple peaks was due to the presence of isozymes or the multiple forms of the enzyme in the extract, or results of the interactions between the enzymes and the activator during chromatography. During purification of the NAD kinase, about half of the activator was removed by protamine precipitation. Activator was almost completely separated from the enzyme by DEAE-cellulose column chromatography (Table I). We assume that three peaks of inactive enzyme activity which appeared by the linear gradient elution of DEAE-cellulose column were purified together during the purification of the NAD kinase, since after the stepwise elution, no enzyme activity remained on DEAE- and P-cellulose column. The purified enzyme was fully activated by the addition of the activator. A trace of catalytic activity that remained in the purified enzyme may be due to the residual activator, since it was found that this enzyme was activated when a part of the same enzyme preparation was boiled and then added back (data not shown). We assume that the complete removal of the activator should cause total inactivation of the enzyme. When the enzyme purified from pea seedlings was fully activated, the specific activity was 3-fold higher than that reported with spinach enzyme, which thus far was the most highly purified preparation from plants (26). It is possible that this spinach enzyme has been partially inactivated by loss of activator during purification, although DEAE-cellulose column chromatography was not included. The present results showed that even the enzyme in the crude extract was not fully active, since the addition of purified activator caused some increase in its catalytic activity (Table I).

The question whether the enzyme and activator exist in an associated state *in situ*, or exist as separate entities in plant tissues remains to be resolved. The activator was adsorbed on the DEAE-cellulose column, whereas the enzyme was not adsorbed at neutral pH and under relatively high ionic strength. This indicates that association of the enzyme and the activator, if any, is not so tight and that the activator has an acidic or weak basic net charge, while the enzyme has basic net charge. Thus, it is possible that the enzyme and the activator associate *via* ionic bonding.

NAD kinase has been highly purified from yeast (2), bacterium (5), and rat liver (20). Purification processes of the enzyme which included an ion-exchange chromatography on DEAE-cellulose did not cause the loss of activity, suggesting that the enzymes from these sources do not need activator, or the activator is tightly bound to the enzyme.

The activator activity for the inactive pea enzyme was found with various plants, but the magnitude of the activation significantly differed from plant to plant (Table IV). The following two explanations are possible for the observed difference in the activation effects: (*a*) the extent of activation represents the amount of activator obtained by heating respective crude extracts, (*b*) or it reflects the difference in the specificities of the respective activators toward the inactive enzyme purified from pea seedlings.

It has been reported that cyclic adenosine 3′,5′-monophosphate phosphodiesterase from animal tissues (6, 7, 9) and pancreatic lipase (3, 8, 14) are associated with protein activators. These activators were separated from the enzyme on DEAE-cellulose chromatography (8, 10, 12, 22). The activators of both enzymes were heat-stable and their mol wt were relatively low (12, 22). These properties are very similar to those found in the activator of NAD kinase. Buchanan *et al.* (4, 21) also reported

Table III. Stability of the Activator

The activator (7.5 μg) was incubated either at 37 or 98 C for 30 min with various concentrations of either HCl or NaOH. After cooling and neutralization aliquots of the solution were used to determine the activator activity.

Treatment	Temperature	
	37 C	98 C
	relative activity[1]	
Water	100	16.2
0.001 N HCl	22.4	6.2
0.01 N HCl	26.3	5.5
0.1 N HCl	21.2	5.9
1 N HCl	3.6	0
0.001 N NaOH	100	1.8
0.01 N NaOH	44.9	0
0.1 N NaOH	14.9	0
1 N NaOH	0	0

1. The activity of activator was expressed as percent of units of non-treated activator (3.46 unit).

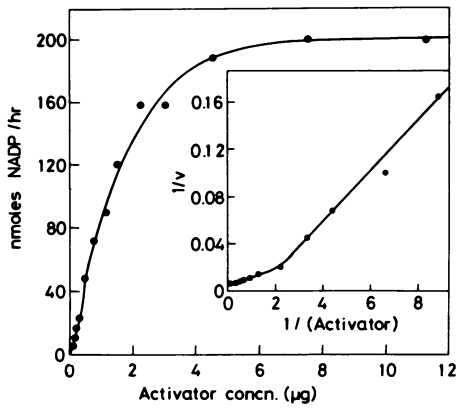

FIG. 4. Effect of the activator concentration on the inactive NAD kinase. The enzyme activity was assayed as described in the text. Purified inactive NAD kinase used in each assay was 22 μg. Inset: double reciprocal plot of the same data.

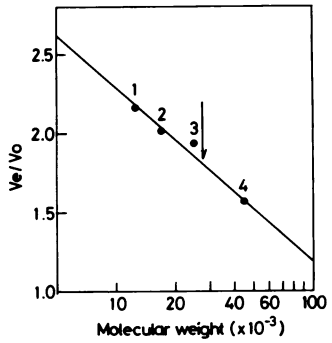

FIG. 5. Mol wt estimation of the activator. Standard proteins are: 1, Cyt c (mol wt 12,400); 2, myoglobin (17,800); 3, chymotrypsinogen (25,000); 4, ovalbumin (45,000). Location of the activator is indicated by the arrow.

Table IV. Activities of NAD kinase and its Activator from various Plants

Plants were ground in a mortar with three times their weight of 25 mM triethanolamine-acetate buffer (pH 7.5) and quartz sands. *Chlorella* cells were suspended in three times their packed cell volume of the buffer and broken by passing through a French pressure cell at 500 kg/square cm. After centrifugation at 18,000 g for 20 min, the supernatants were used for the enzyme assay. Aliquots of these supernatants were heated at 70 C for 5 min then cooled. After removal of the coagulated protein by filtration, filtrates were examined for their abilities to activate inactive pea NAD kinase.

Plant	NAD kinase	Activator
	units/mg protein[1]	
Pea seedlings	107.6	11.8
Spinach leaves	23.7	3.02
Chinese cabbage leaves	0.91	2.53
Corn seedlings	0.85	0.97
Rice seedlings	169.0	3.18
Chlorella cells	37.5	0.65

1. Protein was assayed before the heat treatment.

that fructose diphosphatase and sedoheptulose diphosphatase of isolated spinach chloroplasts were activated photochemically by reduced ferredoxin in the presence of a protein factor with low mol wt. However, it was heat-sensitive different from the activator of NAD kinase.

The physiological role of the activator of NAD kinase is not clear at present. Our preliminary experiment showed that NAD kinase was activated by 30 to 40% upon illumination of pea seedlings which had been kept in the dark overnight (unpublished data). The experiments are being carried out to elucidate whether this activation is caused by the light-induced increase of the activator or not.

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