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PURIFICATION AND PROPERTIES OF NAD NUCLEOSIDASE FROM *FUSARIUM NIVALE*

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SUMMARY

1. A highly active NAD nucleosidase (NAD glycohydrolase, EC 3.2.2.5) is synthesized by the snow mold *Fusarium nivale*. The activity appears in cell-bound or soluble form depending on the composition of the growth medium and the cultural conditions; formation of the enzyme seems related to the process of sporulation.

2. From shake cultures in a fortified glucose-containing Czapek medium NAD nucleosidase was purified 190-fold over the crude extract to a specific activity of 996 μmoles NAD/min per mg protein with a yield of 38.5%. The purification procedure, primarily based on the high acid stability of the enzyme, is described.

3. NAD nucleosidase from *Fusarium nivale* shows maximal activity at pH 5–6 with low dependence on pH and its K_m for NAD was found to be 0.41 mM. Its molecular weight was estimated at 27 000 and its isoelectric point at about pH 7.8. The enzyme shows high specificity for the oxidized pyridine coenzymes and negligible activity toward NAD analogues. It is not sensitive to several adenine and pyridine compounds tested, but is strongly inhibited by thionicotinamide NAD.

INTRODUCTION

NAD nucleosidases (NAD glycohydrolases, EC 3.2.2.5, or NADases) are known, regardless of their origin, to cleave hydrolytically the nicotinamide–ribose linkage of NAD to yield nicotinamide and ADPribose. In addition, the enzymes from mammalian tissues (EC 3.2.2.6) also catalyze an exchange of the nicotinamide moiety with various substituted pyridines with formation of the corresponding NAD analogues[1].

The tissue enzymes are nonsoluble, firmly attached to cellular subunits. Some transglycosylases have been solubilized by proteolytic and lipolytic treatment and purified to various degrees[2–5]; also, a notable case of solubilization *in vivo* has been

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observed in tuberculous tissues and implicated in the mechanism of infection[6]. More recent evidence suggests that the cell-bound enzymes are intracellularly regulated by compartmentation[7] or end-product inhibition by nicotinamide[7].

A different picture is presented by the enzymes from procaryotes (*Mycobacteria*[8,9], *Streptococci*[10,11], *Bacillus subtilis*[12], *Pseudomonas fluorescens*[13]) which, though soluble and insensitive to nicotinamide, are accompanied by specific endogenous inhibitors.

The enzymes purified thus far from fungi (highly active Zn^{2+}-deficient *Neurospora crassa*[14] and *Aspergillus niger*[15]) are soluble and show none of the above forms of inhibition. In contrast, in the widespread plant pathogens *Fusaria* NAD nucleosidase has been detected mainly in the particulate fractions[16,17]. Furthermore, this fungus is a several-fold richer enzyme source than the above[14,15]. We undertook the study of the snow mold *Fusarium nivale* with the intention to seek growth conditions which induce the synthesis of soluble enzyme and allow its purification, and also to gain some insight into the role of the bound enzyme and the mode of its release under controlled conditions. In the present report the formation, purification and certain properties of the enzyme are described.

MATERIALS AND METHODS

Chemicals

NAD^+, NADH, $NADP^+$ and NADPH were obtained from Boehringer (Mannheim); 3-acetylpyridine adenine dinucleotide and its reduced form, deamino-NAD and its reduced form, 3-acetylpyridine deamino-NAD, 3-pyridinealdehyde deamino-NAD, thionicotinamide NAD and nicotinamide mononucleotide from P.L. Biochemicals (Milwaukee, Wisc.); nicotinamide, isonicotinamide, 1-methylnicotinamide, *N*-methylnicotinamide and 3-acetylpyridine from Sigma Chemical Co. (St. Louis, Mo.); *N,N,N',N'*-tetramethylethylenediamine, acrylamide and *N,N'*-methylenebisacrylamide from Eastman Chemical Co. (Rochester, N.Y.); Ampholyte solutions from LKB (Rockville, Md.); bovine serum albumin, ovalbumin, chymotrypsinogen A and myoglobin from Schwarz/Mann (Orangenburg, N.Y.).

Growth conditions

The strain of *Fusarium nivale*, originally from the culture collection of Dr W. C. Snyder, was obtained from the Laboratory of Plant Biology, Nuclear Research Center Demokritos, and maintained on potato-dextrose slants at 26 °C. For the preparation of inoculum the conidia were washed from the surface of 8-day-old cultures with sterile distilled water, sieved through nylon mesh under aseptic conditions and adjusted to approximately $2 \cdot 10^7$ conidia/ml. Enzyme formation was studied in the following growth media: Czapek liquid medium, fortified with 2 g/l yeast extract (Difco), containing per l: 2 g $NaNO_3$, 2 g $MgSO_4$, 0.5 g KCl, 1 g KH_2PO_4, 0.01 g $FeSO_4$, and as carbon source 30 g of the carbohydrate indicated in the text; chemically defined medium and potato-dextrose broth were prepared according to Imholte and Schramm[18]. The cultures were shaken in 1-l spherical flasks on a wrist-action shaker (stroke: 10 cm) at the indicated frequencies. The samples were filtered under low vacuum through Millipore filters (MF 0.65 μm) for assay of extracellular

enzyme activity. Total enzyme activity and protein were determined in samples homogenized for 4 min at 4 °C (immersion homogenizer Ultra Turrax 18/2, Janke and Kunkel) or subjected to 4 min sonication (20 kcycles/min, MSE Sonic Disintegrator) according to experimental needs. For the estimation of soluble enzyme the sonicated suspensions were centrifuged 90 min at 125 000 × *g* (Spinco L). NAD nucleosidase was assayed in the supernatant solution and the washed precipitate.

Enzyme assay

During the growth of the fungus and throughout the purification steps, enzyme activity was determined by the cyanide-addition method according to Kaplan[19]. The enzyme suspension or solution was incubated with NAD in 50 mM phosphate buffer (pH 6) at 37 °C. The cyanide solution was added after 8 min and the samples were read at 334 nm in an Eppendorf photometer (micromolar extinction coefficient: 5.53).

In studies with purified fractions (as the enzyme became more labile), the incubation time was shortened as indicated in the text to secure linearity of initial rates. The Eppendorf Microliter System was used and photometric measurements were carried out at λ_{max} of the cyanide-addition product of each substrate in a 2400 Gilford spectrophotometer.

Unit of activity

1 unit is defined as the amount of enzyme which cleaves 1 μmole of NAD/min under the above conditions*; specific activity is given in units/mg of protein.

Protein determination

Protein was determined according to Lowry *et al.*[22] with bovine serum albumin as standard.

Determination of isoelectric point

Isoelectric focusing experiments were conducted as described by Drysdale *et al.*[23] in a Metaloglass gel electrofocusing apparatus on 5% polyacrylamide gels (3 mm × 10 mm), containing 1% ampholytes, with 200 mM phosphoric acid and 100 mM NaOH as anolyte and catholyte, respectively. At the end of the run the gels were sectioned at 4-mm intervals and the slices were eluted for 2 h in 0.5 ml distilled water (conductance approximately 50 $\mu\Omega^{-1}$) under mild stirring at 4 °C for determination of pH and enzyme activity.

Chromatography on Sephadex

All experiments were carried out at 4 °C in Pharmacia jacketed columns, equilibrated for 4 days in upward flow regulated by an LKB peristaltic pump; the elution profile was monitored by a ultraviolet absorption meter (LKB–UVICORD I) connected to an LKB recorder.

* For the purpose of comparison it may be noted that enzyme activity has been traditionally expressed in μmoles NAD cleaved/h, *i.e.* 1/60 of the above unit. In few exceptions, the purification of NAD nucleosidases has been described in units which correspond to 1/750 (*Neurospora crassa*[14], *Mycobacterium butyricum*[8], *Streptococcus pyogenes*[20]), 1/6000 (*Aspergillus niger*[15]) or 1/6 (rat intestine[21]) of the unit as this is defined here.

RESULTS

Enzyme formation

In all growth experiments we observed that the amount of inoculum, above the threshold of 10^7 conidia/l, was not critical for the course of total enzyme production as shown in Fig. 1. After inoculation, during the stage of germination (approx. 18 h) and formation of the mycelium, no activity could be detected either in the medium or the forming hyphae (Fig. 1, Phase A). Immediately after the onset of

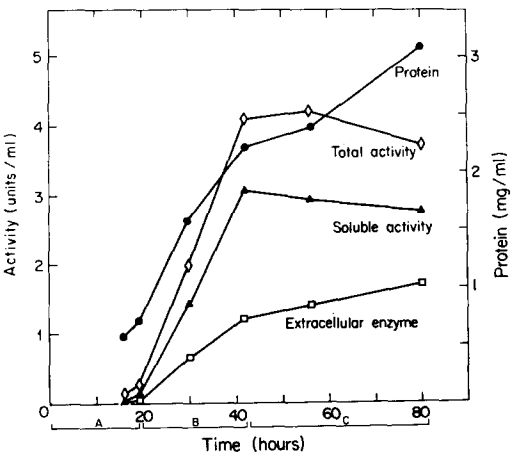

Fig. 1. Formation of NAD nucleosidase in shake cultures of *Fusarium nivale*. The inoculum (10^7 conidia in 0.5 ml) was added to 500 ml of Czapek medium containing glucose as carbon source. The flasks were shaken (250 oscillations/min) at 27 °C. Samples were withdrawn at the indicated times, subjected to 4 min sonication and assayed for enzyme activity (◇—◇) and total protein (●—●). The activity of the soluble enzyme (▲—▲) was determined in the supernatant after centrifugation (90 min at 125 000 × *g*); extracellular activity (□—□) was determined in filtrates of the culture (pore size 0.65 μm).

sporulation, at approx. 20 h, a rapid synthesis of NAD nucleosidase started (Fig. 1, Phase B) and reached maximal activity at approx. 40 h (Fig. 1, Phase C), after which a stationary phase was attained, followed by slow autolysis of the culture. Also in other growth media (Table I), where the duration of these developmental events ranges from 40 to 110 h, parallel initiation of sporulation and appearance of enzyme activity were consistently observed. On the other hand, the levels of soluble enzyme formed are strongly affected by variation of the medium and the rate of agitation as shown in Table I. Fig. 1 represents growth conditions considered as optimal for the production of soluble NAD nucleosidase; the culture, interrupted at 40 h, served as source for the purification of the enzyme.

Purification of NAD nucleosidase

The purification from a typical 4-l culture is summarized in Table II and proceeded as follows:

Step I. Preparation of extract. The whole culture was homogenized for 2 min; the resulting suspension was filtered in succession first through a 40-μm and then through a 2-μm sintered-glass filter. All following steps were carried out at 4 °C.

TABLE I

FORMATION AND SOLUBILITY OF NAD NUCLEOSIDASE OF *Fusarium nivale* IN VARIOUS GROWTH MEDIA

All cultures were grown simultaneously and under the experimental conditions of Fig. 1.

Growth medium	*Maximal enzyme activity (units/ml)*	*Solubility (%)*
Potato-dextrose broth[18]	5.25	<8
Chemically defined medium[18]	4.3	31
Czapek medium (C-source: sucrose)	4.75	35
Czapek medium (C-source: glucose)*	4.5	71

* In cultures grown on a rotating platform (50–70 rotations/min) instead of a shaker, maximal enzyme activity ranges from 1.6 to 2 units/ml and solubility from 8 to 20%.

TABLE II

PURIFICATION OF NAD NUCLEOSIDASE FROM *Fusarium nivale*

Fractionation step	*Vol. (ml)*	*Total activity (units)*	*Total protein (mg)*	*Specific activity (units/mg)*	*Yield** *(%)*	*Enrichment** *(-fold)*
I Crude extract	3800	10 200	1950	5.23	—	—
II Acid precipitation	102	8 460	43	196.2	83	37.5
III Desalting and lyophilization	25	7 870	30.8	255	93	1.3
IV Chromatography on Sephadex**G-50 (Fractions 38–43 of Fig. 2)	34	3 935	3.95	996	50	3.9

* Referring to the foregoing step.

** On recycling (LKB ReCyChrom) the specific actiivty rose to 1440 units/mg with a recovery of approx. 26%.

Step II. Acid precipitation. 1 M HCl was added dropwise to the filtrate under mild stirring until the mixture reached pH 2 (in no less than 60 min). The precipitate was allowed to form without stirring for 90 min and the suspension was centrifuged (90 min at 9000 × *g*). The supernatant was aspirated off and discarded. The precipitate was resuspended in 100 ml of 200 mM citrate–phosphate buffer (pH 7.2), stirred mildly for 36 h and recentrifuged as described above.

At pH 2 NAD nucleosidase precipitates quantitatively and without inactivation over several hours. However, this process is not readily reversible. Attempts to extract the precipitated enzyme with other buffers, such as 50 mM Tris–HCl or 70 mM sodium–potassium phosphate at pH 5 or 7.2, were not successful.

Step III. Desalting and lyophilization. 2 g Sephadex G-25 coarse were added per 10 ml of the supernatant of Step II under stirring. After 30 min, the slurry was filtered at 8 kg/cm^2 (Gelman 47 mm pressure-filtration funnel, Filter GA-8, mean flow pore size 0.2 μm) and the filtrate lyophilized. The ensuing powder was redissolved in the same buffer to a final volume of approximately 30 ml and filtered through Whatman No. 4 filter paper for removal of undissolved precipitate.

This treatment with Sephadex G-25, on a column or in the batch procedure described here, consistently gave an activation of the enzyme ranging up to 40%, possibly due to removal of inhibitory material by the gel.

Step IV. Chromatography on Sephadex G-50. The enzyme solution from Step III was further chromatographed on Sephadex G-50 fine with an overall yield of 65%; Fig. 2 shows the elution profile. Fractions 38–43 were pooled and lyophilized. The powder was kept in small pre-weight aliquots at −90 °C and −20 °C. Before each experiment the enzyme was brought to 0 °C. The unused portion was not refrozen, but discarded.

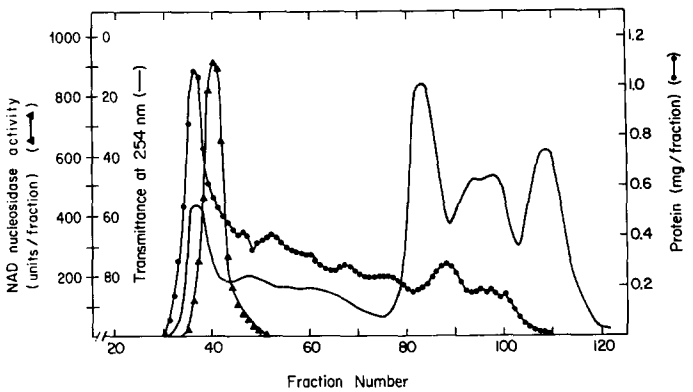

Fig. 2. Fractionation of NAD nucleosidase on Sephadex G-50. The enzyme solution (25 ml containing 30.8 mg protein) was applied on a 2.5 cm × 95 cm column, equilibrated with 200 mM citrate–phosphate (pH 7) in upward flow; fractions of 5.5 ml were collected every 12 min. The solid line represents transmittance at 254 nm; ▲—▲, enzyme activity; ●—●, protein.

Enzyme stability

During the growth of *Fusarium* the active mycelial suspensions or their homogenates retain their activity overnight at −20 °C, and for several hours at 4 °C. After acid precipitation, the enzyme becomes increasingly labile on storage; with fractions of specific activity over 900 units/mg approx. 30% inactivation was observed after 2 h at 4 °C. The lyophilized powder showed no loss of activity when stored for several weeks at −20 °C, or for several months at −90 °C.

pH optimum

NAD nucleosidase from *Fusarium nivale* shows highest activity at pH 5–6, with low pH dependency within the range pH 4.5–7 as shown in Fig. 3. Broad pH optima have also been reported for fungal and bacterial NAD nucleosidases[8,14,15,20] with the exception of the enzyme from *Mycobacterium tuberculosis*[9] which exhibits a sharp pH optimum at pH 6.4. We did not observe significant differences in activity with the *Fusarium* enzyme dissolved in 70 mM sodium–potassium or sodium phosphate, 50 mM Tris–maleate or 200 mM citrate–phosphate buffer at about pH 6.

Effect of substrate concentration

Determinations of initial rates were carried out up to 4 min in 50 mM Tris–

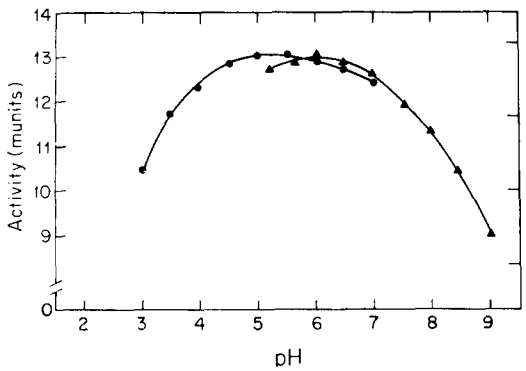

Fig. 3. Effect of pH on the activity of NAD nucleosidase. Activity was measured under standard assay conditions, after 4 min in 100 mM citrate–phosphate (pH 3-7) (●—●) or 50 mM Tris–maleate buffer (pH 5–9) (▲—▲). The ordinate represents munits of activity in the assay mixture.

maleate (pH 6) at 37 °C; their dependence on substrate concentrations plotted after Lineweaver–Burk was linear. The K_m for NAD, estimated about 0.41 mM, falls within the range of reported values for microbial NAD nucleosidases, an extreme case being the enzyme from *Aspergillus niger* (K_m = 7.7 μM)[15].

Substrate specificity of NAD nucleosidase

Mammalian enzymes exhibit a ratio of NADP to NAD hydrolysis ranging from 0.3 to 0.98 (ref. 24). On purification these ratios remain largely constant[2,4], although in rabbit erythrocytes two enzymes specific for NAD and NADP, respectively, could be separated and purified[25]. Microbial enzymes show ratios in the range 0.75–1[8,9,14,15] with the exception of NAD nucleosidase from strains of *Streptococcus pyogenes* with absolute specificity for NAD and no activity whatsoever toward NADP[20].

The relative rates at which NAD nucleosidase from *Fusarium nivale* cleaves NAD, NADP and some analogues of the pyridine coenzymes are shown in Table III.

TABLE III

RELATIVE RATES OF HYDROLYSIS OF PYRIDINE NUCLEOTIDES BY NAD NUCLEOSIDASE

The assay mixture contained 1.25 mM of the substrate and 50 mM sodium phosphate buffer (pH 6) at 37 °C in a fianl volume of 1 ml. Samples were withdrawn every minute and the oxidized nucleotides assayed by cyanide addition. The reduced coenzymes and analogues were read directly at their absorption maxima.

Substrate	*Relative hydrolysis*
NAD^+	100
NADH	2
$NADP^+$	83
NADPH	3
3-Acetylpyridine NAD	6
3-Acetylpyridine NAD (reduced form)	<1
Deamino-NAD	<1
Deamino-NAD (reduced form)	<1
3-Pyridinealdehyde deamino-NAD	<1
3-Acetylpyridine deamino-NAD	<1
Thionicotinamide NAD	<1
Nicotinamide mononucleotide	<1

These rates, determined after each step throughout the purification procedure, and also after long storage of the final fraction (Table II, Step IV), did not show deviations exceeding ± 2% of the above values.

Inhibition

NAD nucleosidases from microorganisms are not inhibited by pyridine compounds. Metal inhibition and similar effects of SH– reagents have been reported for the enzymes from *Aspergillus niger*[15] and *Mycobacterium tuberculosis*[9]. Under standard assay conditions, the NAD nucleosidase from *Fusarium nivale* is inhibited less than 10% at concentrations up to 10 mM of $MnCl_2$, $MgCl_2$, $CoCl_2$, $ZnCl_2$, $Fe_2(SO_4)_3$, sodium azide and EDTA in 20 mM Tris–HCl buffer (pH 7.1). Compounds related to the NAD moieties, namely: adenine, adenosine, AMP, ADP, ADPribose, ATP, isonicotinic acid hydrazide, nicotinic acid and NMN were also ineffective (inhibition by adenine compounds has recently been reported for the soluble NAD nucleosidase from bull semen[26]).

TABLE IV

INHIBITION OF NAD NUCLEOSIDASE

Assay mixture as in Table III. The indicated concentrations of the inhibitor were preincubated with the enzyme for 10 min at 37 °C. The reation was initiated by addition of NAD.

Reagent	*Concentration (mM)*	*Activity (%)*
Nicotinamide	10	81
Isonicotinamide	10	83
1-Methylnicotinamide	10	77
N-Methylnicotinamide	10	79
3-Acetylpyridine	10	72
Iodoacetamide	1	77
p-Chloromercuribenzoate	1	61
	0.1	74
$HgCl_2$	1	62
	0.1	76
Thionicotinamide NAD	1	0
	0.2	0

As shown in Table IV, some pyridine compounds as well as $HgCl_2$, *p*-chloromercuribenzoate and iodoacetamide had an inhibitory effect only at high concentrations. On the other hand the thionicotinamide analogue of NAD proved to be a very potent inhibitor of the *Fusarium* enzyme. This effect, now under investigation, has been previously encountered and studied in detail with the *Neurospora* enzyme[27].

Isoelectric point

The gels were prerun for 20 min at 1 mA/gel prior to application of the enzyme sample (approximately 1 enzyme unit from Step IV, Table II). It was estimated that equilibrium was reached after 6 h in both pH ranges, 3–10 and 7–10. Under these conditions NAD nucleosidase from *Fusarium nivale* focuses at about pH 7.8. On stained gels (sample applied: 20 μg) the purity of the enzyme was estimated at approx. 20%.

Molecular weight

The determination of the molecular weight was carried out in 200 mM citrate–phosphate buffer (pH 7), on a Sephadex G-100 column, (2.5 cm × 100 cm; flow rate: 5 $ml \cdot h^{-1} \cdot cm^{-2}$) calibrated with albumin, ovalbumin, chymotrypsinogen A and myoglobin as markers. Under these conditions the enzyme shows an estimated molecular weight of 27 000, *i.e.* of the same order as the "small" NAD nucleosidases of mammalian origin—pig, beef, and sheep tissues[24]—and the enzymes of *Neurospora crassa* (31 000) and *Bacillus subtilis* (24 000) as determined on Sephadex[12].

DISCUSSION

A NAD nucleosidase from *Fusarium nivale* has been partially purified to a spec. act. of 996 units/mg (Table II), to our knowledge the highest reported so far. It shows a pronounced resistance to low pH values—shared at various degrees by other NAD nucleosidases[2,14,28]—which also provides an efficient shortcut to its purification (Table II).

All obtained preparations cleave NAD, NADP and NAD analogues at constant relative rates (Table III). The consistency of these values throughout the purification is considered as an adequate criterion to exclude the possible presence of a second enzyme with different specificities for the two coenzymes[20,25].

In *Fusarium nivale*—an exceptionally rich source of NAD nucleosidase under most growth conditions (Table I)—the enzyme levels appear to depend on the age of the culture and the differentiation stage of the organism (Fig. 1). Most characteristic is the lack of enzyme activity during germination, followed by a dramatic increase after the onset of sporulation. Observations of Zalokar and Cochrane[29] and more recent studies of Stine[30] have ascertained a similar behavior of the enzyme in *Neurospora crassa*, its suggested function during sporulation being to inhibit other fermenting microorganisms[31].

The cell-bound NAD nucleosidase in *Fusarium nivale* presents an unusual case among the enzymes characterized so far from microorganisms[8,9,11,14,15,29,30]. Its role is presently unknown. It may be noted, however, that total NAD nucleosidase in *Fusarium nivale* can exceed 0.8 units (μmoles NAD/min) per nmole intracellular [NAD + NADP] (Stathakos, D and Bugge, B., unpublished); the magnitude of this ratio strongly suggests the presence of a regulatory mechanism. Accordingly, if one considers the possibility of compartmental regulation of NAD nucleosidase as proposed for the microsomal enzyme in mammalian cells[7], the question can be asked as to whether the environmentally induced variations of cell-bound activity in *Fusarium nivale* could partly account for the protection of endogenous coenzymes from cleavage by restricting their accessibility to the enzyme.

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