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STUDIES ON NICOTINAMIDE AMIDOHYDROLASE IN NORMAL AND TUMOUR TISSUES OF *RUMEX ACETOSA* CULTIVATED *IN VITRO*

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Abstract—Tumour tissue of *Rumex acetosa* maintained *in vitro* contains the enzyme nicotinamide amidohydrolase which has an optimum pH of 4·5. It acts on nicotinamide and 6-aminonicotinamide but not on glutamine or asparagine. Both the substrates inhibit the enzyme at higher concentrations. It is activated by Mg^{2+}, Ca^{2+} and Co^{2+} and inhibited by Zn^{2+}, Hg^{2+}, EDTA and PCMB. PCMB inhibition is reversed by cysteine. The enzyme is induced by its substrates and 2,4-D suppresses its synthesis.

INTRODUCTION

IN LIVING cells, at least a part of the nicotinamide produced *in vivo* by the action of NAD glycohydrolase on NAD can be reused by deamidation to nicotinic acid and conversion to NAD by Preiss-Handler pathway.[1] The two enzymes, NAD glycohydrolase and nicotinamide amidohydrolase, would thus play a key role in the metabolism of NAD and would constitute a "salvage loop" in the organisms where the *de novo* pathway is genetically blocked or deleted such as in the neoplastic condition.[2,3]

We have reported[4,5] the presence of NAD glycohydrolase in *Rumex acetosa* tumour tissue cultivated *in vitro* and shown the product to be nicotinamide. Preliminary investigations showed that nicotinamide amidohydrolase could also be detected in tumour tissue. The medium used for cultivating normal tissue had coconut milk, 2,4-dichlorophenoxyacetic acid (2,4-D), glycine and nicotinic acid whereas that used for tumour tissue had nicotinamide alone. The question arises whether the presence of the enzyme in tumour tissue is due to its induction by the nicotinamide present in the medium. Studies were, therefore, carried out to investigate the role of the components of the culture media on the enzyme activity and to purify it from tumour tissue and study its characteristics in detail. The results of these investigations are reported in this paper.

RESULTS AND DISCUSSION

Data on the effect of the presence of nicotinamide, nicotinic acid, 6-aminonicotinamide and 2,4-D in the tumour medium on the activity of nicotinamide amidohydrolase are given in Table 1. When nicotinamide was omitted from the medium the enzyme activity decreased

[1] J. G. JOSHI and P. HANDLER, *J. Biol. Chem.* **237**, 929 (1962).
[2] R. K. GHOLSON, *Nature* **212**, 933 (1966).
[3] M. SHIMOYAMA, K. YAMAGUCHI and R. K. GHOLSON, *Cancer Res.* **27**, 578 (1967).
[4] S. B. MAINI, S. K. SRIVASTAVA and C. V. RAMAKRISHNAN, *Indian J. Biochem.* **3**, 169 (1966).
[5] S. K. SRIVASTAVA, S. B. MAINI and C. V. RAMAKRISHNAN, *Phytochem.* **8**, 1147 (1969).

by 80 per cent. The enzyme activity became zero after 40 days of cultivation in the medium devoid of nicotinamide. Supplementation of nicotinic acid in place of nicotinamide did not restore normal activity. However, 6-aminonicotinamide, which is also a substrate for this enzyme, when added to the medium in place of nicotinamide, restored normal activity. Addition of 2,4-D to the medium containing nicotinamide suppresses the enzyme activity. These results would suggest that nicotinamide or 6-aminonicotinamide can induce the synthesis of this enzyme whereas 2,4-D suppresses its synthesis.

Table 1. Effect of nicotinamide, nicotinic acid, 6-aminonicotinamide and 2,4-D on nicotinamide amidohydrolase of *Rumex* tumour tissue

Medium	Growth increment* (%)	Enzyme activity (units/g fresh tissue)
T.M.	466	4·7
T.M.−NAm	499	0·9
T.M.−NAm+NA	502	0·7
T.M.−NAm+6-AN	541	4·7
T.M.+2,4-D	429	0·7

The tissues were grown in different media and analysed on the twentieth day of cultivation. Tissues from five flasks from each group were pooled together for analysis. T.M.=tumour medium; NAm=nicotinamide; NA=nicotinic acid; 6-AN=6-aminonicotinamide; 2,4-D=2,4-dichlorophenoxy acetic acid.

* Per cent growth increment $= \frac{\text{(Final fresh weight–initial fresh weight)}}{\text{Initial fresh weight}} \times 100$

Tumour tissue loses its enzyme activity when grown in nicotinamide-free medium for 40 days. When this culture was cultivated on a medium containing different concentrations of nicotinamide or 6-aminonicotinamide, the enzyme activity increased up to a concentration of 0·8–1·2 p.p.m. of the substrates and then decreased at a higher concentration (Table 2). The decrease may be due to higher intracellular level of substrates which are known to inhibit the enzyme activity at higher concentrations.

Table 2. Effect of nicotinamide and 6-aminonicotinamide concentrations on nicotinamide amidohydrolase activity of *Rumex* tumour tissue

Concentration of the constituent added (p.p.m.)	Enzyme activity (units/g fresh tissue) when cultivated in presence of	
	Nicotinamide	6-Aminonicotinamide
0·2	1·0	1·0
0·4	2·7	1·8
0·6	4·0	2·8
0·8	4·3	4·0
1·2	3·4	4·5
1·6	2·7	3·4
2·0	1·6	2·7
2·4	1·6	2·1

The tissue used for this experiment was the one grown for 40 days in the tumour media without nicotinamide and analysed after 20 days of cultivation. Tissues from five flasks from each group were pooled together for analysis.

When tumour tissue was grown for 20 days in the presence of different concentrations of 2,4-D, there was about 60 per cent decrease in enzyme activity at a concentration of 0·01 p.p.m. and the enzyme activity became zero at a concentration of 0·05 p.p.m. (Table 3). In order to find out whether 2,4-D is an inhibitor of the enzyme, the effect of the concentrations of 2,4-D on nicotinamide amidohydrolase activity was studied using the homogenate prepared from tumour tissue cultivated in tumour medium containing no 2,4-D. 2,4-D even up to a concentration of 1·5 p.p.m. did not inhibit the enzyme activity under the assay conditions. This suggests that 2,4-D added to the culture medium may affect the enzyme synthesis.

TABLE 3. EFFECT OF 2,4-D CONCENTRATION ON NICOTINAMIDE AMIDOHYDROLASE ACTIVITY OF *Rumex* TUMOUR TISSUE

Concentration of 2,4-D (p.p.m.)	Enzyme activity (units/g fresh tissue)	
0	4·5	
0·01	1·8	
0·02	1·0	
0·03	0·6	
0·04	0·3	
0·05	0	
0·06	0	(2·7)*
0·07	0	
0·08	0	

Tissues from five flasks from each group were pooled together for analysis.

* Shows the enzyme activity when the tissue grown for 20 days in presence of 0·06 p.p.m. 2,4-D was put back in a medium containing no 2,4-D and analysed after 20 days of cultivation.

This raises the question whether the difficulty in detecting this enzyme in normal tissue is due to the presence of 2,4-D in the medium. The data reported in Table 4 show that when 2,4-D is omitted from the normal medium there is an increase in enzyme activity and it could be further induced by the addition of nicotinamide. However, the presence or absence of nicotinic acid in the medium does not seem to influence the enzyme activity. Since normal medium contains coconut milk and glycine, the effect of omission or addition of these two components to the culture medium on the enzyme activity in normal and tumour tissue respectively were studied but they did not seem to have any effect on the enzyme synthesis.

The data on the concentration of this enzyme from tumour tissue are given in Table 5, from which it can be seen that the enzyme can be concentrated 30-fold with a recovery of 86 per cent by calcium phosphate gel fractionation technique. The data on the kinetic studies carried out on the purified preparation are given in Fig. 1 and Tables 6 and 7.

The enzyme has an optimum pH of 4·5 which is different from that reported for the enzyme isolated from other sources.[6–10] The activity increases proportionately with enzyme

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TABLE 4. EFFECT OF THE ADDITION OF NICOTINAMIDE, NICOTINIC ACID AND 2,4-D TO THE CULTURE MEDIUM ON NICOTINAMIDE AMIDOHYDROLASE ACTIVITY OF *Rumex* NORMAL TISSUE*

Medium	Growth increment† (%)	Enzyme activity (units/g fresh tissue)
N.M.+NA+2,4-D	450	0·30
N.M.−NA+2,4-D	370	0·34
N.M.−NA+2,4-D+NAm	380	0·38
N.M.+NA−2,4-D	410	1·93
N.M.+NA−2,4-D+NAm	450	3·00
N.M.−NA−2,4-D	370	2·08
N.M.−NA−2,4-D+NAm	410	3·72

* The tissues were grown in different media and analysed on the twentieth day of cultivation. Tissues from five flasks from each group were pooled together for analysis. N.M.=normal medium; NAm=nicotinamide; NA=nicotinic acid; 6-AN=6-amino-nicotinamide; 2,4-D=2,4-dichlorophenoxy acetic acid.

$$\text{† Per cent growth increment} = \frac{(\text{Final fresh weight} - \text{initial fresh weight})}{\text{Initial fresh weight}} \times 100$$

concentration and period of incubation up to 0·8 ml concentration of enzyme and 60 min incubation period. The enzyme acts on nicotinamide and 6-aminonicotinamide but not on glutamine or asparagine. In this respect it resembles the enzyme isolated from *Mycobacterium avium*[8] and differs from that of liver and brain tissue.[11] Both the substrates inhibit enzyme activity at higher concentrations (Fig. 1). The K_m is 3×10^{-2} M and 8×10^{-3} M for nicotinamide and 6-aminonicotinamide respectively.

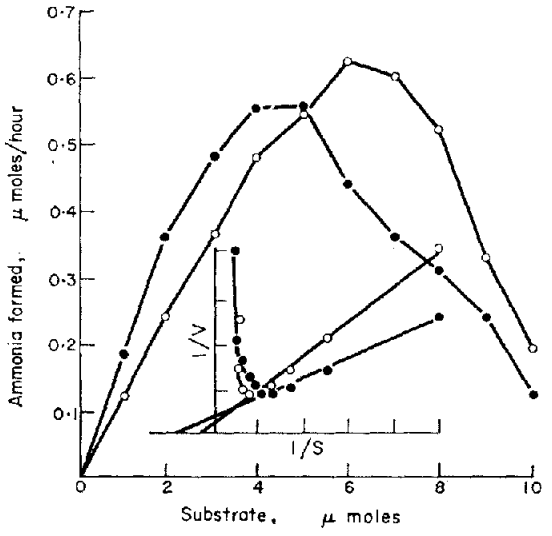

FIG. 1. EFFECT OF NICOTINAMIDE AND 6-AMINONICOTINAMIDE CONCENTRATION ON NICOTINAMIDE AMIDOHYDROLASE ACTIVITY.

Nicotinamide (—○—); 6-aminonicotinamide (—●—). Inner figure shows the Lineweaver–Burk plot of nicotinamide and 6-aminonicotinamide concentration.

[11] Y. TSUJI, *Naika Hokan* **4**, 112 (1957); seen in *Chem. Abstr.* **53**, 10349 (1959).

TABLE 5. PARTIAL PURIFICATION OF NICOTINAMIDE AMIDOHYDROLASE FROM *Rumex* TUMOUR TISSUE

Procedure	Volume (ml)	Total units*	Total protein (mg)	Specific activity (units/mg protein)	Fold purification	Recovery (%)
1. Acetone powder suspension in 20% NaCl	50	34·25	42·25	0·83	1	100
2. Supernatant	45	29·48	5·63	5·24	6	86
3. Dialysed supernatant after centrifugation	47	32·20	4·98	6·46	7	94
4. Calcium phosphate gel eluate	47	29·38	1·18	25·00	30	86

* One unit = 1 μmole of NH_3 formed in 1 hr under the assay conditions.

TABLE 6. EFFECT OF DIFFERENT CONCENTRATIONS OF Mg^{2+}, Ca^{2+} AND Co^{2+} IONS ON NICOTINAMIDE AMIDOHYDROLASE ACTIVITY

Concentration (μmoles)	Enzyme activity (μmoles ammonia formed/hr) in presence of		
	Mg^{2+}	Ca^{2+}	Co^{2+}
0	0	0	0
2	0·19	0·16	0·12
4	0·36	0·30	0·24
6	0·57	0·40	0·31
8	0·60	0·47	0·40
10	0·57	0·45	0·40
12	0·55	0·40	0·40

TABLE 7. EFFECT OF VARIOUS COMPOUNDS ON NICOTINAMIDE AMIDOHYDROLASE ACTIVITY

Compound added	Concentration (μmoles)	Enzyme activity (%)
None	—	100
NaN_3	1	100
PCMB	1	25
	2	0
Cysteine	4	100
PCMB + cysteine	2+4	63
Nicotinic acid	5	105
EDTA	5	25
	10	25
Adenine	10	124
AMP	10	133
ATP	10	133

Mg^{2+}, Ca^{2+} and Co^{2+} are required for the enzyme activity and of the three metals Mg seems to be a better activator (Table 6). In the presence of Mg^{2+}, Zn^{2+} and Hg^{2+} are inhibitors, but no other metals have any effect on enzyme activity. PCMB and EDTA were found to inhibit the enzyme activity (Table 7). Inhibition by PCMB could be reversed by cysteine. The effect of addition of adenine, AMP and ATP was tried since it is reported[1] that ATP activates this enzyme. In the present case they show some activation (24–33 per cent).

EXPERIMENTAL

Materials

Normal and tumour tissues of *Rumex acetosa* were maintained as described elsewhere.[4]

Chemicals

Apart from 6-aminonicotinamide, which was a gift from Dr. Mark Woods, NIH, Bethesda, U.S.A., the chemicals were obtained from commercial sources.

Preparation of Homogenate for Enzyme Assay

The tissues cultivated for specified periods were taken out from the culture flask, freed from adhering agar, if any, and ground for 3–5 min with cold 0·95 per cent KCl (pH 7·4) in a chilled mortar kept in crushed ice. The homogenate was diluted with the grinding medium to a volume corresponding to 40% (w/v) on fresh weight basis.

Preparation of Acetone Powder

3–4-week-old tissue was ground with 4 vol. of chilled acetone for 2 min in a mortar kept in crushed ice. It was filtered quickly by suction. The residue was twice treated similarly and then was allowed to air-dry until free from acetone and stored in an air-tight bottle at 0–4°.

Enzyme Assay

The method used for enzyme assay was similar to that used by Sundaram *et al.* The assay system consisted of 50 μmoles of citrate–phosphate buffer (pH 4·5), 4 μmoles of nicotinamide, 10 μmoles of $MgSO_4$, 0·8 ml of enzyme extract and water to a volume of 2·0 ml. Reaction was started by the addition of enzyme. After incubating the reaction mixture at 37° for 1 hr, 1·0 ml of 10% TCA was added and the mixture centrifuged to remove the precipitated proteins. In case of control, nicotinamide was added at the end of incubation after the addition of TCA. NH_3 formed was estimated by the Nesslerization method.[13]

A unit of enzyme activity is defined as the amount of enzyme required to form 1 μmole of ammonia in 1 hr under the assay conditions. Protein content of the enzyme preparation was estimated by the method of Lowry *et al.*[14]

Purification of the Enzyme from Rumex *Tumour Tissue*

All the operations reported below were carried out at 0–4°. Acetone powder was extracted with 20% NaCl by grinding it in a chilled mortar for 5 min and diluted with NaCl to give 10 mg/ml suspension. The suspension was centrifuged at 10,000 × *g* for 15 min. The supernatant was dialysed against cold glass distilled water for 4 hr and centrifuged at 10,000 × *g* for 30 min to remove any precipitate formed. The calcium phosphate gel (dry weight 22 mg/ml) was centrifuged at 10,000 × *g* for 10 min to remove the supernatant and to the residue 2 vol. of dialysed enzyme was added. The mixture was stirred for 15 min and then centrifuged at 10,000 × *g* for 15 min. The adsorbed enzyme was eluted with 1 vol. of 1 M phosphate buffer, pH 7·0, in two batches.

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