Vol. 113, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 15, 1983 Pages 569-574

FINDINGS OF TRIGONELLINE DEMETHYLATING ENZYME ACTIVITY IN VARIOUS ORGANISMS AND SOME PROPERTIES OF THE ENZYME FROM HOG LIVER

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Received May 2, 1983

Trigonelline demethylating enzyme activity was found widely in animals, plants and microorganisms. Very high enzyme activity of this enzyme was detected in hog liver. Properties of the hog liver enzyme were investigated. Optimum pH for the enzymic reaction was observed at 8.5. The *Km* value for trigonelline was calculated at 2.77 mM. Addition of any cofactor is not required for the reaction The enzyme activity was inhibited by heavy metal ions. The reaction product was identified as nicotinic acid. Proposed enzyme reaction mechanism and the role of this enzyme in biosynthesis and metabolism of NAD are discussed.

Trigonelline (*N*-methylnicotinic acid) is excreted in normal human urine (1) and is found in various organisms, especially in seeds of many plants including coffee beans (2). Nicotinate methyltransferase (EC 2.1.1.7) catalyzes the reaction of nicotinic acid into trigonelline(3). No reversed reaction with this enzyme has ever been detected. Trigonelline has been thought to be a dead end metabolic product in NAD biosynthetic and metabolic pathways. The sole physiological role of trigonelline which has been proved clearly thus far is the compound's action as a plant mitotic cycle hormone which promote cell arrest in G2 stage (4). Many papers on the methylation of nicotinic acid are easily found; however,papers on the demethylation of trigonelline are rarely found. Reports on the metabolism of trigonelline with intact *Torula cremoris*, *Pisum sativum* (5) and several plant cell suspension cultures(6) were published. Report has been made of thermal decomposition of

ABBREVIATIONS

HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
TRICINE : *N*-[tris-(hydroxymethyl)-methyl]-glycine
PPO : 2,5-diphenyloxazole
POPOP : 1,4-bis-(5-phenyloxazol-2-yl)-benzene

0006-291X/83 $1.50

trigonelline to nicotinic acid and many other compounds after heating it at ca. 200 °C for 15 min in a sealed tube (7). Natural trigonelline in coffee beans also changes to nicotinic acid during roasting (8).

We investigated whether the enzyme which converts trigonelline into nicotinic acid is detectable or not by using radioassay. As a result, the enzyme was found to be widely distributed in nature and very high activity was found in hog liver. It has been revealed that trigonelline is not a dead end product but can be converted readily into a precursor for NAD biosynthesis. As far as we know this is the first paper on distribution and properties of trigonelline demethylating enzyme in cell-free extract.

MATERIALS AND METHODS

[Carboxyl-^{14}C]nicotinic acid (56 mCi/mmol, 448 μCi/mg) was purchased from Amersham, England. Univer-Gel (PPO, POPOP and non-ionic surfactant in xylene) was obtained from Nakarai Chemicals, Ltd., Kyoto. Cell-free extract of each sample was prepared as follows under refrigeration: fresh sample (wet weight of 10 g) was cut into small pieces and homogenized with 10 ml of 0.1 M HEPES buffer, pH 7.0 in a mortar. If necessary small amount of sea sand was added into the mortar to complete homogenization of sample. The homogenate obtained was squeezed through two layers of gauze and centrifuged for 10 min at 10,000 x *g*. Supernatant fluid was collected and used for the enzymic reaction. Protein concentration was determined with Bio-Rad protein assay kit (Bio-Rad Laboratories, CA).

[Carboxyl-^{14}C]trigonelline was synthesized from [carboxyl-^{14}C] nicotinic acid and methyl iodide by the methods of Sarett *et al.* (9). Trigonelline was purified moreover with Dowex column chromatography as mentioned below. Properties of this purified trigonelline agreed well with those of authentic trigonelline (*Rf* values of TLC with three different solvent systems, melting point and IR spectrum). The yield was at least 70 %. Contamination with nicotinic acid or other compounds was not detectable in this preparation of [carboxyl-^{14}C]trigonelline.

For surveying the distribution of trigonelline demethylating enzyme activity in various organisms, reaction mixture contained HEPES buffer, pH 7.0 10 μmoles; [carboxyl-^{14}C]trigonelline, 250 nmoles, 0.38 μCi; cell-free extract, 0.2 ml in a total volume of 0.5 ml. This reaction mixture was heated in a boiling water bath for 1 min just after incubating the mixture for 3 hr at 37 °C. Coagulated proteins were discarded after centrifugation. Supernatant was charged on a Dowex 1 x 8 column (formate form, 1.2 x 4.0 cm). The column was washed with 70 ml of water. Trigonelline was removed completely from the column by this washing. Compounds adsorbed in the column were eluted with 50 ml of 2 N formic acid. One milliliter of the eluate and 7.5 ml of Univer-Gel were mixed well and the radioactivity of this mixture was counted with a Beckman liquid scintillation system, model LS-100. Specific activity is expressed as nmoles of nicotinic acid formed under the above assay conditions per mg of protein. Activity index indicates the total activity (nmoles of nicotinic

acid formed) per 1 ml of cell-free extract.

Basal reaction mixture for investigating the properties of hog liver enzyme was as follows: TRICINE buffer, pH 8.5, 10 μmoles; [carboxyl-^{14}C]trigonelline, 5.25 μmoles, 0.38 μCi; dialyzed cell-free extract of hog liver, 0.1 ml in a total volume of 0.5 ml. Just after incubating the reaction mixture for 30 min at 37 °C, 1 ml of 0.5 M HEPES buffer, pH 7.0 was added into the reaction mixture and heated in a boiling water bath for 1 min. The amount of nicotinic acid formed in the reaction mixture was determined by the methods described above.

Hog liver extract (5 ml) was dialyzed against 500 ml of 0.01 M HEPES buffer, pH 7.0 for 6 hr at 0 °C with continuous stirring. Then the outer buffer was changed and dialysis was continued for additional 12 hr. In principle this dialyzed hog liver extract was used just after dialysis for various enzymic reactions.

RESULTS AND DISCUSSION

Distribution of trigonelline demethylating enzyme activity in various organisms is shown in Table 1. The enzyme activity was found widely in animals, plants and microorganisms. Highest specific activity was detected in pine needles. Highest total activity (activity index) was found in hog liver. The reason why such high enzyme activity was found in hog liver and no activity was found in several species (eg. dog liver, cat liver, etc.) is unknown. Distribution of the enzyme activity was investigated at the reaction pH of 7.0. This pH may be far apart from the optimum pH for the enzyme in some species. Subsequent experiments were

Table 1. Distribution of trigonelline demethylating enzyme activity in various organisms.

Species	Part	Specific Activity (a)	Activity Index (b)
Hog	Liver	6.04	670
Hog	Kidney	3.35×10^{-2}	2.01
Beef	Liver	1.67	182
Beef	Kidney	1.66×10^{-2}	1.46
Rat	Liver	1.81×10^{-2}	2.77
Eel	Liver	1.03×10^{-2}	1.12
Pine	Needles	16.7	21.7
Onion	Bulb	5.97	7.16
Pimiento	Berries	2.29	5.73
Soybean	Sprouts	1.43	4.72
Field Pea	Pods & Seeds	0.87	5.13
Bamboo	Blades	0.41	16.4
Alfalfa	Sprouts	0.17	1.46
E. coli B		1.26	58.5
Saccharomyces cerevisiae		0.17	1.11

(a) nmoles of nicotinic acid formed per mg of protein at 37 °C for 3 hr.

(b) nmoles of nicotinic acid formed per ml of cell-free extract at 37 °C for 3 hr.

The enzyme activity was not detectable in dog liver, cat liver, chicken liver, clover, green onion, pea leaf, radish sprout, trefoil, *Aspergillus oryzae* and *Penicillium thomii*.

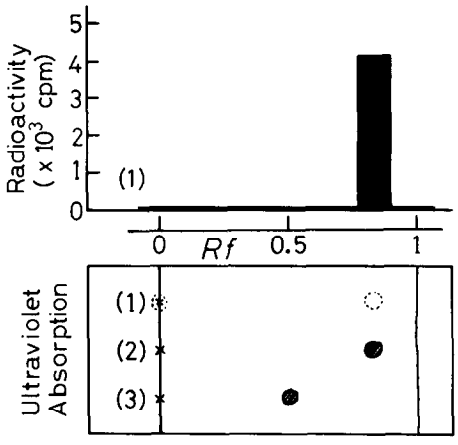

Fig. 1. Identification of the reaction product formed from labeled trigonelline with hog liver cell-free extract.
Incubation was carried out for 3 hr. Deproteinized reaction mixture was charged on a Dowex column and trigonelline was washed out with water. The compound eluted with 2 N formic acid was evaporated to dryness at 50 °C under reduced pressure. The dried matter was dissolved with 1 ml of water. From this solution 10 µl was developped with isobutyric acid : ammonia : water (66 : 1.7 : 33) on a Merck silica gel plate (type 5721) for 2 hr at room temperature. Authentic nicotinic acid and trigonelline were co-chromatographed on the same plate. Ultraviolet absorbing spots were detected with UV radiation. Then the TLC plate was cut into small pieces and the radioactivity of each fragment was counted with 10 ml of Univer-Gel. (1) : reaction product, (2): authentic nicotinic acid, (3): authentic trigonelline.
Similar results were obtained when the reaction product was developped with n-butanol : water : acetic acid (250 : 250 : 60).

carried out with hog liver extract and some properties of the hog liver enzyme were elucidated.

Optimum pH for the enzymic reaction with hog liver enzyme was found at 8.5. The reaction product was identified as nicotinic acid as shown in Fig. 1. Only one radioactive spot was detected on TLC plate. The *Rf* value of the radioactive spot coincided well with that of authentic nicotinic acid. Effect of trigonelline concentration on the enzyme activity was investigated. From Lineweaver-Burk plot at low concentration of the substrate, a *Km* value for trigonelline was calculated at 2.77 mM. The enzymic reaction proceeded lineary up to 0.2 ml of hog liver extract with respect to the amount of enzyme.

Effect of various compounds on the enzyme activity is summarized in Table 2. All compounds tested had no effect on the enzyme activity except heavy metals. Trigonelline demethylating enzyme may be an SH enzyme and no extra addition of any cofactors

Table 2. Effect of various compounds on the trigonelline demethylating enzyme activity in hog liver cell-free extract.

Addition	Concentration (mM)	Relative Activity
None	-	100
EDTA	1	106
$MgSO_4$	1	103
PRPP, $MgSO_4$	10, 1	94
ATP, $MgSO_4$	10, 1	92
$HgCl_2$	0.1	97
"	1	0
$Fe_2(SO_4)_3$	1	61
"	10	0
2-Mercaptoethanol	1	79
"	10	26
Nicotinic acid	1	101
Nicotinamide	1	97
NAD	1	99
NADP	1	100
N^1-Methylnicotinamide	1	103
"	10	101

Each compound was added into a reaction mixture at the final concentration described here. The reaction mixture was incubated and the nicotinic acid formed was determined by the methods described in MATERIALS AND METHODS.

(metal ion, energy source, etc.) is required. Trigonelline demethylating enzyme activity was not altered when N^1-methylnicotinamide was added into the reaction mixture even at 10 mM. Consequently it has become apparent that N^1-methylnicotinamide can not approach the active site of trigonelline demethylating enzyme. Substrate specificity of this enzyme may be high.

Essentially the specific activity of the enzyme was not changed just after dialysis. The enzyme activity of dialyzed enzyme decreased after freezing and thawing. The decreased enzyme activity was recovered when FAD was added into the reaction mixture. At present we have not enough data to determine definitely, but it may be that the enzyme may be a flavoprotein like sarcosine demethylating enzyme (10) and the methyl group may be demethylated oxidatively. Formation of nicotinic acid from trigonelline with hog liver enzyme is confirmed in this paper. Nevertheless the fate of methyl group in trigonelline molecule is

uncertain. Anyhow it is apparent that trigonelline is not an end product nor excrement of NAD metabolism but a precursor of NAD. In plant seeds trigonelline may be a stored form of nicotinic acid. Hence, trigonelline is a useful compound for all living cells.

After the fate of methyl group of trigonelline is made clear by using [methyl-^{14}C]trigonelline, the reaction mechanism of this enzyme will be solved. In addition to this work, purification of this enzyme from hog liver and detailed investigation of the purified enzyme are now under preparations. Further report will be published later.

ACKNOWLEDGEMENTS

We thank Mr. Yasushi Koide and Mr. Osamu Fukuzawa for their technical assistance. We also thank Professor Kazuo Iwai, Department of Food Science and Technology, Kyoto University, for encouragement and support.

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