Studies on Metabolic Pathway of NAD in Yeast

Part I. Partial Separation of Enzymes

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The enzyme system of NAD degradation was extracted from autolysate of Saccharomyces oviformis.

The enzymes were partially separated by ammonium sulfate fractionation and DEAE-cellulose column chromatography, and then the metabolic pathway of NAD in yeast was presented, in which four enzymes were contained. It has been found that, among them, the 5' nucleotidase has more affinity for AMP and the nucleosidase has strict affinity for nicotinamide riboside.

In the degradation of NAD in the yeast, nucleotide pyrophosphatase was main enzyme, but NADase, nucleotide pyrophosphorylase and adenosine deaminase seemed not to play an important role.

There are many reports on the decomposition of NAD in organisms, the first step of which is summarized in following four reactions,

\[
\text{NAD} + \text{H}_2\text{O} \rightarrow \text{nicotinamide} + \text{ADP-ribose} \quad (1)
\]

\[
\text{NAD} + \text{H}_2\text{O} \rightarrow 5'-\text{AMP} + \text{NMN} \quad \text{(nicotinamide mononucleotide)} \quad (2)
\]

\[
\text{NAD} + \text{PPi} \rightarrow \text{ATP} + \text{NMN} \quad (3)
\]

\[
\text{NAD} + \text{H}_2\text{O} \rightarrow \text{NH}_3 + \text{deamino-NAD} \quad (4)
\]

Equation (1) is catalyzed by NADase\(^{1-4}\) which is thought to be the most important enzyme attacking NAD in animal tissues, Eq. (2) by nucleotide pyrophosphatase,\(^{5-8}\) Eq. (3) by nucleotide pyrophosphorylase\(^9\) and Eq. (4) by adenosine deaminase from Taka-diastase.\(^{10}\) respectively. These enzymes are widely distributed in animals, plants and in bacteria.

Kornberg\(^9\) extracted and purified NAD pyrophosphorylase from dry yeast, Cabib and Carmiratti\(^7\) reported the nucleotide pyrophosphatase in yeast extract, and Hochster and Quastel\(^11\) suggested the presence of NADase in yeast, but there are no systematic studies on the metabolic pathway of NAD in yeast.

In the present communication, the authors report the enzyme system of NAD degradation in Saccharomyces oviformis.

MATERIALS AND METHODS

Yeast. Yeast used in this experiment was Saccharomyces oviformis (M-2 strain) isolated from molasses, the cells of which was obtained in aerated culture on molasses medium, and compressed to 66% in humidity.
Reagent. NAD was obtained from C. F. Boehringer, 5′-AMP, GMP and IMP from Yamasa Syōyu Co., Ltd., and other nucleotides from Sigma Chemicals Co., respectively. Alkaline phosphatase of E. coli was purchased from Worthington Biochemical Co.

Yeast alcohol dehydrogenase was prepared from baker's yeast as described by Racker\(^{12}\) except that the crystalizing step was omitted.

**Determination of NAD and NADH\(_2\).** NAD and NADH\(_2\) were determined by usual method using yeast alcohol dehydrogenase.\(^{13,14}\) Total NAD was NAD plus NADH\(_2\).

**Determination of NMN and nicotinamide riboside.** NMN and nicotinamide riboside were determined from increased absorbancy at 325 \(\text{m}_{\mu}\) when sample was allowed to react with 1 M KCN as described by Colowick.\(^{15}\)

**Determination of nicotinamide-liberating activity.** Nicotinamide-liberating activity was determined by applying the NAD-cyanide reaction as described by Kaplan\(^{16}\) or Colowick.\(^{15}\) The reaction mixture contained 0.1 ml of NAD (6 mg. per ml., pH 8.4), 1.4 ml of 0.1 M NH\(_3\) buffer (pH 8.4) and 0.5 ml of adequately diluted enzyme with the buffer. After incubation for 30 min. at 37°C, 0.5 ml each of reaction mixture (if turbid, supernatant was used) was added to the 2 ml of 1 M KCN and 2 ml of M/5 Na\(_2\)CO\(_3\), respectively, and the absorbancy of the former was measured against the latter at 325 \(\text{m}_{\mu}\). Blank determination was done in same way except lacking the enzyme.

The difference between blank and probe values indicates the nicotinamide-liberating activity.

The test was linear for quantities of enzyme which liberate nicotinamide moiety up to 50 per cent of NAD.

**Determination of Phosphate and Protein.** Pi was determined by Takahashi's method\(^{17}\) and organic phosphate was calculated from total phosphate by Allen's method minus Pi.

Protein was determined by Cu-Folin method.

1. **The decomposition of NAD by yeast autolysate**

Compressed yeast (100 g) was mixed with 10 ml of ethyl acetate and kneaded at room temperature. Seventy ml of water was added to the completely autolysed cell and the resulted suspension was neutralized to pH 7 to 8 with 1 N-NaOH.

The suspension was centrifuged at suitable intervals, and NAD and nicotinamide-riboside linkage in supernatant were determined, respectively. As shown in Fig. 1, autolysed cell had strong NAD-splitting activity which decomposed more than 80 per cent of NAD in intact yeast cell within 1.5 hours, and it decomposed also NAD preparation added to the suspension at the same rate.

It was very interesting that nicotinamide-riboside linkage in NAD molecule disappeared in the completely same rate with NAD inactivation. Both of NAD-splitting activity and nicotinamide-liberating activity were completely destroyed with heat treatment at 85°C for 5 min.

These facts suggested the presence of NADase in yeast, but, as described below, it was not true.

As shown in Fig. 2, the optimum pH values of both activities were 8.

The fact, given above, was also observed when yeast cell was autolysed with chloroform, toluene, or Hyamine 1622 instead of ethyl acetate.

2. **The extraction of Nicotinamide-liberating activity**

It became clear that the autolysate of yeast had nicotinamide-liberating activity.

Most of this activity was not found in the supernatant of autolysate but in the debris.

It was tried to extract it from the debris. Ethyl acetate (100 ml) was added to 1 kg of pressed yeast, and the resulted autolysate was centrifuged. The collected debris was washed once with 1.5 l of water and resuspended in 21 of water. This suspension was brought to 0.1 saturation with solid ammonium sulfate.

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Fig. 1. The Decomposition of NAD by Yeast Autolysate.

Yeast was autolysed with ethyl acetate and diluted with water. The half of the resulted suspension was heated at 85°C for 5 min., and the other was not heated. The two suspensions were incubated at 25°C, pH 7 to 8. NAD and nicotinamide-riboside linkage in the suspensions were assayed at suitable intervals, respectively. At arrow 0.1 ml of NAD solution (4 mg. per ml.) was added to each 4 ml of two suspensions, respectively.

3. Enzymatic properties of crude extract

The reaction products of NAD by crude extract were examined by paperchromatography.

3-Ribose, nicotinamide, nicotinic acid and adenosine, but no adenine, were detected as shown in Fig. 4. The crude extract could liberate nicotinamide from NAD.
The partial purification of nicotinamide-liberating activity

The crude extract was brought to 0.35 saturation with solid ammonium sulfate, and resulted precipitate was removed by centrifugation. The supernatant was brought to 0.5 saturation.

The precipitate was collected by centrifugation and dissolved into a small amount of water. The solution was dialysed against running tap water (16°C) overnight in cellophane bag.

The dialysed solution was applied to the DEAE-cellulose column (3 cm x 26 cm high) previously equilibrated with phosphate buffer (M/150, pH 7).

The column was eluted with 2.01 of M/150, pH 7, phosphate buffer, next with 2.51 of 0.1 M, pH 7, phosphate buffer, and at last with 2.51 of the same buffer containing 0.5 M NaCl, successively.

Three effluents were subjected to be salted out at 0.5 saturation of ammonium sulfate, respectively, and each solution of the precipitate was dialysed in the same way as described above.

The dialyzed solutions were named as Fraction I, II and III.

5. The enzymatic properties of Fraction I, II and III

Enzymatic properties of Fr. I, II and III were examined by using NAD as substrate. As shown in Fig. 5, Fr. I contained inactive proteins, and Fr. II contained NAD-splitting activity but could not hydrolyse the nicotinamide-riboside linkage. Fr. III had not any activity directly to attack NAD, but could liberate nicotinamide from NAD pretreated with Fr. II.

More than 80 per cent of nicotinamide-liberating activity applied to the column was found again in the mixture of Fr. II and III, but so-called "NADase" was not detected in any fraction.

Therefore it was sure that the nicotinamide-liberating activity observed in autolysate or crude extract was not NADase but the co-action of Fr. II and III.

6. NAD degradation by Fr. II

The enzymatic properties of Fr. II were examined further in details. The time course of NAD degradation by Fr. II is shown in Fig. 6, and thin-layer chromatogram of its reaction mixture is shown in Fig. 7.

NAD was decomposed by Fr. II, leaving nicotinamide-riboside linkage.

Fifty per cent of total phosphate in NAD was found as inorganic phosphate, and the other 50 per cent phosphate seemed to remain as monoester because this ester was hydrolysed with nonspecific phosphatase of E. coli.

The chromatogram shown in Fig. 7 illustrates that NAD was decomposed into two nucleotides of 5'-AMP and NMN in first stage of reactions, and, in later stage, adenosine gradually increased with disappearance of AMP.

The results given in Fig. 6 and 7 suggest that NAD was finally degraded to adenosine.
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FIG. 5. Enzymatic Properties of Fr. I, II and III.

At arrows, 0.1 ml each of indicated enzyme fractions (30 mg protein per ml) was added to 1 ml of NAD solution (1.3 mg of NAD in each ml of 0.05 M veronal buffer, pH 8.4) and the mixture was incubated at 37°C.

- - - KCN method (at 325 m\(\mu\))

○ ○ ○ YADH method (at 340 m\(\mu\))

FIG. 6. Time Course of NAD Degradation by Fr. II.

Reaction mixture containing 5.3 mg. of NAD and 1.5 mg of Fr. II as protein in 1 ml of 0.025 M acetate buffer was incubated at 37°C, pH 6 to 7. At arrow, 0.1 ml of E. coli Pase was added to 1 ml of the reaction mixture.

NAD ○ ○ YADH method (at 340 m\(\mu\))

Pi △ △ NAD+Fr. II, E. coli Pase

and NMN by Fr. II according to reaction (5) and (6).

FIG. 7. Kieselguhr G (Merck) Thin-Layer Chromatogram of NAD Hydrolysates by Fr. II.

Composition of the reaction mixture was the same with that in Fig. 6.

- adenosine
- NAD
- AMP
- NMN

Solvent: 1 M CH\(_3\)COONH\(_4\); 95% ethanol: H\(_2\)O (3:7.5:3.5) color development: 5 N H\(_2\)SO\(_4\) (160°C)

NAD → 5′-AMP+NMN

5′-AMP+NMN → Adenosine+Pi+NMN
Fr. II seemed to have two enzymes, nucleotide pyrophosphatase and phosphomonoesterase (Pase). It was very interesting that this Pase could selectively hydrolyse 5'-AMP but not NMN.

7. Pase in each fraction

It was found from the results shown in Table I that no Pase was in Fr. I, nonspecific Pase was in Fr. III and 5'-nucleotidase was in Fr. II. One of the author already reported the substrate specificity of this nucleotidase.

8. NMN decomposition by Fr. III

It was presumed, from the results mentioned above, that NMN, which had been produced from NAD by Fr. II, was further decomposed to liberate nicotinamide by Fr. III.

Now the enzymatic properties of Fr. III was further examined in details using NMN and nicotinamide riboside as substrate. From the paperchromatogram shown in Fig. 8, it was presumed to react according to reaction (7) and (8)

\[
\text{NMN} \rightarrow \text{nicotinamide riboside} + \text{Pi} \quad (7)
\]

\[
\text{nicotinamide riboside} \rightarrow \text{ribose} + \text{nicotinamide} \quad (8)
\]

From results shown in Table I, it is apparent that reaction (7) was catalysed by nonspecific Pase but not by 5'-nucleotidase. The enzyme, catalysing reaction (8), was examined by using nicotinamide riboside prepared by the method of Rowen and Kornberg. It was found that the enzyme did not require phosphate or arsenate. This enzyme is hydrolase, a kind of nucleosidase, but not phosphorylase.

TABLE I. PHOSPHOMONOESTERASE IN EACH FRACTION

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Fr. I</th>
<th>Fr. II</th>
<th>Fr. III</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-AMP</td>
<td></td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>3'-AMP</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2'-AMP</td>
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<td>-</td>
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<tr>
<td>5'-GMP</td>
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<td>5'-IMP</td>
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<td>-</td>
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</tr>
<tr>
<td>5'-UMP</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5'-CMP</td>
<td></td>
<td>-</td>
<td>#</td>
</tr>
<tr>
<td>Phenylphosphate</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R-5-P</td>
<td></td>
<td>-</td>
<td>#</td>
</tr>
<tr>
<td>NMN</td>
<td></td>
<td>-</td>
<td>#</td>
</tr>
</tbody>
</table>

Reaction mixture contained 4.23 μmoles of substrates and 3 mg protein of each Fr. in 1 ml of 0.25 veronal buffer (pH 7.0) After incubation at 37°C for 2 hrs, increased Pi was assayed.

+++: remarkably increased
++ : moderately increased
+ : slightly increased
- : never increased

8. NMN decomposition by Fr. III

So-called "nucleosidase" can cleave adenosine, guanosine, nicotinamide riboside and other nucleosides at the same rate. This enzyme can hydrolyse only nicotinamide riboside and never hydrolyse other nucleosides. It is explained from the substrate specificity.
of this enzyme that only adenosine is detected but not adenine as products of NAD decomposition as shown in Fig. 4.

DISCUSSION

From results described above, NADase seems not to take an important role in the degradation pathway of NAD in yeast autolysate, and, because inosine was never detected among final hydrolysates of NAD as shown in Fig. 4, adenosine deaminase seems also not to be concerned with this pathway.

As NAD-splitting activity in autolysate was never decreased by full water washing, by further autolysis for 7 days, or by dialysis of crude extract, pyrophosphatase seems also not to be concerned with this pathway.

The results as shown in Fig. 5 and Fig. 7 suggest that pyrophosphatase is a main enzyme for degrading NAD by yeast autolysate or crude extracts.

Therefore, the degradation pathways of NAD in autolysate are presumed as shown in Table II, and it is probably sure that NAD 5'-Nucleotidases have been found in animal tissues,\(^{21}\) snake venoms,\(^{22}\) bull semens,\(^{23}\) and in a few bacterial strains\(^{24,26}\) but not in yeast yet.

It is interesting that the nucleotidase obtained here has base-specificity having more affinity for 5'-purine nucleotides than pyrimidine series, but never splits NMN and ribose-5'-phosphate, and, even among purine series, there is difference in decomposing speed. Usual 5'-nucleotidases already reported dephosphorylate all 5'-nucleotides and even also ribose-5'-phosphate in the same rate.

As regards to nucleosidase which cleave nicotinamide riboside to ribose and nicotinamide, it should be exaggerated that this nucleosidase never cleaves adenosine nor guanosine different from existing one\(^^{26,27}\) which cleaves adenosine, guanosine and other nucleosides at the same rate with nicotinamide riboside.

It may be presumed that the strict affinity for nicotinamide riboside is significant for metabolism of NAD in yeast. From the findings on substrate specificity, both of 5'-nucleotidase and nucleosidase obtained here would be considered to be new type enzymes, and, as these have been more purified, we will report the details on this journal.

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\(^{22}\) J. M. Gulland and E. M. Jackson, ibid., 32, 597 (1938).
\(^{27}\) Y. Takagi and B. L. Horecker, ibid., 228, 77 (1957).