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TRANSFORMATION OF 3-METHYLPYRIDINE INTO NICOTINIC ACID BY THE YEAST *S. cerevisiae*

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It has been shown that the yeast S. cerevisiae *has the ability to synthesize nicotinic acid and NAD from 3-methylpyridine. Fractionation of intracellular yeast proteins established that the fraction of molecular weight 65-90 kDa had the highest activity for transformation of pyridine derivatives into nicotinate. HPLC showed that, in addition to nicotinic acid, nicotinamide was also present in the intermediates during transformation of 3-methylpyridine. The results were consistent with the presence in yeast cells of an enzymatic system that transforms 3-methylpyridine into vitamin PP.*

Key words: yeast, 3-methylpyridine, transformation of pyridine derivatives, nicotinic acid, nicotinamid-eadeninedinucleotide.

Nicotinic acid (NA) is known to be produced chemically from the pyridine derivatives β-picoline (3-methylpyridine, 3-MP) or 3-acetylpyridine (3-AP) [1]. The question of NA production in microbiological conversions was first raised in the 1970s by researchers [2-4] who isolated more than 100 cultures of *Mycobacterium*, *Nocardia*, *Corynebacterium*, and *Arthrobacter* and others that not only decomposed the pyridine ring but also oxidized alkyl substituents on it without destroying it. Also, the ability to oxidize the methyl on 3-MP was first demonstrated at that time. However, the enzymatic aspects of the microbiological transformation of pyridine derivatives have been poorly studied despite the other successes.

We observed previously that local strains of the yeast *Saccharomyces cerevisiae* 913a-1, which were selected as producers of nicotinamideadeninedinucleotide (NAD), accumulate high concentrations of the coenzyme in the presence of 3-MP and 3-AP [5]. This suggests that enzymatic transformation of these compounds into nicotinate and its subsequent participation in NAD biosynthesis is possible.

Therefore, our goal was to find intracellular proteins that are involved in the bioconversion of 3-MP into NA by *S. cerevisiae* 913a-1.

Fermentation of *S. cerevisiae* 913a-1 biomass in the presence of 3-MP is accompanied by more than a doubling of the intracellular NAD concentration (from 35.6 to 68.2 μg/mL) whereas the free NA content did not change (8.6 μg/mL).

However, it should be considered that intracellular NA is an intermediate in many exchange transformations of pyridinenucleotides whereas the intracellular content of free NA is low compared with that of nicotinamide or NAD. Thus, the ability of yeast cells to accumulate high concentrations of NAD indicates that biosynthesis can supply the required amount of NA. The increased intracellular pool of NAD observed in yeast in the presence of 3-MP may be due to its preliminary transformation into nicotinate due to its enzymatic transformation in cytoplasm. According to the literature, various microorganism groups can not only completely degrade pyridine bases but also partially transform them. Thus, the ability of the bacteria *Nocardia* and *Arthrobacter* to oxidize under co-oxidation conditions alkylpyridines to the corresponding acids, 3- and 2-MP to nicotinic and picolinic acids, has been demonstrated. Transformation of pyridine into nicotinamide also occurs through NA or other intermediates such as methylnicotinamide [3, 6].

The cytosolic fraction of yeast cells was incubated with various concentrations of 3-MP and the concentration of NA was determined for 3 h in order to determine if 3-MP can be transformed intracellularly into NA (Fig. 1).

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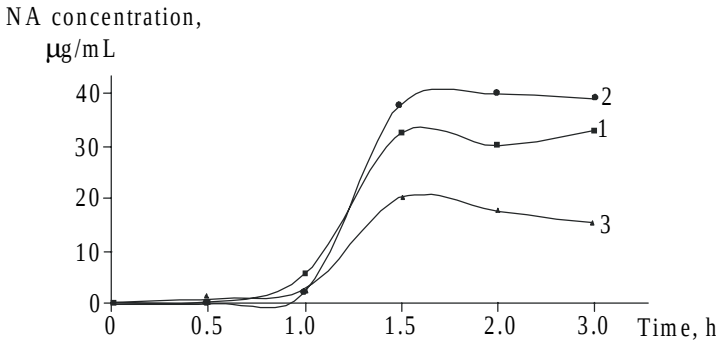

Fig. 1

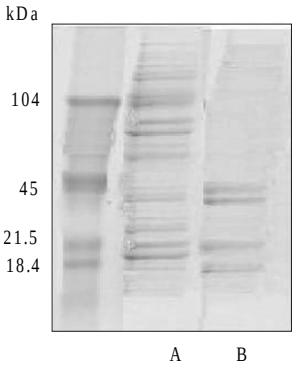

Fig. 2

Fig. 1. Nicotinic acid formation during incubation of cell-free extract with various concentrations of 3-methylpyridine: 0.01 (1), 0.1 (2), 1 mM (3).

Fig. 2. Electrophoresis of cell-free extract of *S. cerevisiae* 913a-1 (A) and fraction 3 (B) in SDS-PAAG (12%) in Tris-glycine buffer (0.01 M, pH 8.0), 40 mA.

Figure 1 shows that incubation of cell-free extract with 3-MP produces significant changes in the NA content. The process of NA formation from 3-MP depends on the concentration of the substrate to be transformed and the incubation time. Thus, the NA concentration in the cell-free extract begins to increase significantly only after incubating for 1.5 h and lasts up to 3 h, after which the curve reaches a plateau. The optimal 3-MP concentration was 10^{-6} M for 1.5 h incubation time. The intracellular level of NA increases quantitatively to 35 μg/mL of incubation medium, which includes 10 μg/mL of cytoplasmic proteins compared with 17.2 μg/mL in the control.

Enzymes involved in 3-MP transformation were determined by analyzing cytoplasmic proteins. Electrophoretic separation of proteins from cell-free yeast extract on PAAG under denaturing conditions revealed a total of only 23 fractions of molecular weight from 10 to 120 kDa. Most protein fractions were found in the range 20-100 kDa (Fig. 2).

Fractionation of proteins by gel filtration of the cell-free extract concentrated five times produced three fractions with molecular weights in the ranges 90-120 kDa (fraction 1), 65-90 (fraction 2), and 10-65 kDa (fraction 3).

Determination of the NA content in the fractions showed that fraction 3 had the highest transformation activity (MW 10-65 kDa). Fractions 1 and 2 were less active.

Fraction	%
1	3.1
2	1.2
3	55.6
1+2	0.5
1+3	43.2
2+3	26.6.

Considering that the transformation of pyridine derivatives can occur in several steps to form intermediates and can involve several enzymes, as has been demonstrated [2, 4], we studied the level of 3-MP transformation upon incubation with different combinations of these three cytoplasmic fractions.

Although it was established that the largest amount of NA forms upon incubation of 3-MP with proteins of fraction 3, nevertheless, the amount of vitamin formed decreased slightly when fractions 2 and 3 were combined. Combining fractions 1 and 2 had an insignificant effect on the level of NA formed. Thus, the results indicate that NA is formed upon incubation of cytosol with pyridine derivatives mainly by the activity present in fraction 3 with MW 10-65 kDa.

Electrophoretic analysis of fraction 3 in PAAG (12%) under denaturing conditions showed the presence of four separate protein bands indicating that this fraction was heterogeneous (Fig. 2).

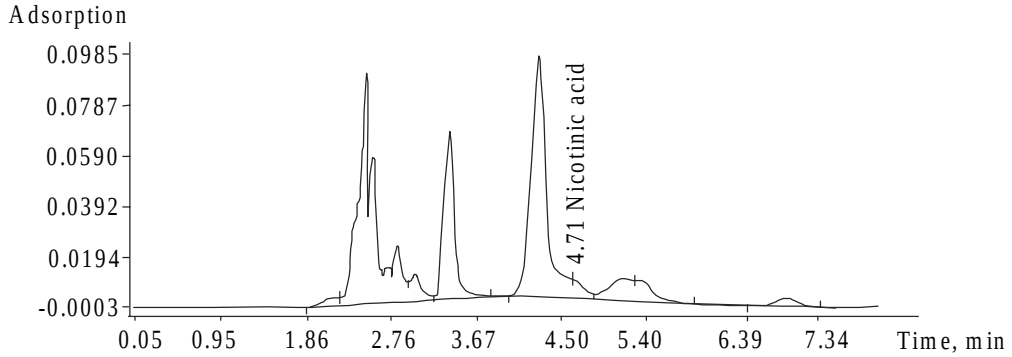

Fig. 3. HPLC analysis of pyridine intermediates in 3-methylpyridine incubation medium with cytoplasmic proteins of the 3rd fraction.

It can be assumed based on the results that several enzymes with the corresponding activity are involved in the transformation of 3-MP into NA and that the substrate transformation process into NA occurs with formation of intermediates. It should be noted that the formation mechanism of NA from 3-MP in yeast is still unknown. Therefore, its successive transformations could involve multiple steps.

In fact, HPLC analysis of the reaction products in incubation medium of cytoplasmic proteins with 3-MP found several transformation products. Of all three fractions, fraction 3 had the highest transformation activity, as shown in Fig. 3. It can be seen from the chromatograms that several compounds are detected during the transformation, one of which was identified as NA.

Thus, based on the results, it can be confirmed that the increase in the intracellular NAD concentration in the local yeast strain *S. cerevisiae* 913a-1 that was observed by us earlier [7] is due to the presence of cytoplasmic proteins that possess enzymatic activity for transforming 3-MP into NA.

The results lead to the conclusion that research on the nature of proteins selected from the yeast cytoplasmic fractions creates new possibilities for developing enzymatic methods of preparing vitamin PP from pyridine derivatives.

EXPERIMENTAL

We used yeast strain *S. cerevisiae* 913a-1 that was selected previously as an NAD producer [5]. Yeast was grown on Rieder medium or beer wort in order to produce biomass.

Transformation of 3-MP into NA was studied by incubation of yeast biomass in potassium-phosphate buffer (0.1 M, pH 5.7) with a biomass:buffer ratio of 1:10 for 30-180 min with addition of various concentrations of pyridine derivatives as substrate.

Cytosol was produced by washing yeast cells of cultivation medium, precipitating biomass by centrifugation at 3000 rpm for 20 min, adding potassium-phosphate buffer (0.1 M, pH 5.7) to the precicpitate, and grinding in a quartz mortar with several freeze—thaw cycles. After centrifuging the homogenate at 12,000 rpm for 1 h, the precipitate was discarded and the supernatant was used for the investigations.

Intracellular and extracellular NA contents in yeast-cell cytosol and incubation medium were determined by microbiological [8] and chemical [9] methods.

Intracellular NAD content in aqueous yeast extracts was determined by a specific reaction in the presence of yeast alcoholdehydrogenase [10].

Protein content was determined by the Lowry method [11].

PAAG electrophoresis of cytosolic proteins was performed under denaturing conditions by the method of Kavetskii et al. [11]. The markers were bovine albumin (MW 104 kDa), egg albumin (MW 45 kDa), soybean trypsin inhibitor (MW 21.5 kDa), and lactoglobulin (MW 18.4 kDa).

Separation of Protein Fractions. Cytoplasmic proteins were fractionated by gel filtration of cell-free extract concentrated five times on a column (2.5 × 100 cm, -60F) of Toyo Pearl with elution by potassium-phosphate buffer (0.05 M, pH 6.8) [10].

Transformation activity of isolated fractions was studied by incubating them in potassium-phosphate buffer (0.1 M) containing 3-MP (10^{-3} M) for 3 h at 37°C. After the incubation was finished, the reaction was stopped by cooling the sample to 4°C.

Transformation products of pyridine derivatives were detected by HPLC [12] in a Beckman System Gold chromatograph with Gold V 3.10/IBM 386 software. The reaction mixture was filtered through millipore filters (0.22 μm) and placed on an Ultrasphere Cs column (0.46 × 25 cm). The mobile phase was CH_3OH (10%) and H_3PO_4 (0.1%). The yields of final and intermediate metabolites were detected at 254 nm. The markers were chemically pure 3-MP and pharmacopeic preparations of NA and nicotinamide.

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