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EXPERIMENTAL ARTICLES

The Isolation, Purification, and Some Properties of NAD-Dependent Isocitrate Dehydrogenase from the Organic Acid–Producing Yeast *Yarrowia lipolytica*

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Abstract—The NAD^+-dependent isocitrate dehydrogenase of the organic acid–producing yeast *Yarrowia lipolytica* was isolated, purified, and partially characterized. The purification procedure included four steps: ammonium sulfate precipitation, acid precipitation, hydrophobic chromatography, and gel-filtration chromatography. The enzyme was purified 129-fold with a yield of 31% and had a specific activity of 22 U/mg protein. The molecular mass of the enzyme was found to be 412 kDa. The enzyme consists of eight identical subunits with a molecular mass of about 52 kDa. The K_m for NAD^+ is 136 μM, and that for isocitrate is 581 μM. The effect of some intermediates of the citric acid cycle and nucleotides on the enzyme activity was studied. The role of isocitrate dehydrogenase (NAD^+) in the overproduction of citric and keto acids is discussed.

Key words: yeast, citric acid cycle, isocitrate dehydrogenase.

The limitation of the growth of the yeast *Yarrowia lipolytica* by some nutrients induces the excretion of large amounts of organic acids (more than 100 g/l) to the cultivation medium. The yeast excretes pyruvic and α-ketoglutaric acids under thiamine limitation [1] and citric and isocitric acids under growth limitation with nitrogen sources [2]. This phenomenon, which is known as the overproduction of organic acids, underlies the microbiological production of useful organic acids. For this reason, there is increasing research interest in the study of the mechanisms at work.

Although the microbiological aspects of this problem are well understood [3, 4], the biochemical mechanisms engaged in the overproduction of organic acids are poorly studied. The exhaustion of nutrients from the medium redistributes carbon flows between catabolic and anabolic pathways. In most living organisms, the tricarboxylic acid cycle (TCA) is the central pathway of carbon metabolism. It is believed that the oxidative decarboxylation of isocitrate catalyzed by NAD^+-dependent isocitrate dehydrogenase (EC 1.1.1.4) is the primary regulatory reaction of the cycle, which largely determines its overall rate. Isocitrate dehydrogenase (NAD^+) has been isolated from different sources, but, to the best of our knowledge, there are no data in the literature as to the properties of isocitrate dehydrogenase (NAD^+) isolated from organic acid–producing yeasts.

The aim of this work was to isolate and to study the isocitrate dehydrogenase (NAD^+) of *Y. lipolytica* with the goal of revealing its regulatory mechanisms and the role it plays in the overproduction of citric and keto acids.

MATERIALS AND METHODS

The organic acid–producing yeast *Yarrowia lipolytica* VKM Y-2373 (formerly 704) was obtained from the All-Russia Collection of Microorganisms (VKM).

The yeast was cultivated in an ANKUM-2M fermentor (6 l) in mineral Reader medium supplemented with 0.3% yeast extract, trace elements according to Burkholder [5], and 2% glucose as the carbon source.

To obtain cell-free extract, early-stationary cells were harvested by centrifugation at 5000 *g* for 10 min; washed twice in 0.9% NaCl; suspended in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 5 mM DTT, and 0.3 mM phenylmethylsulfonyl fluoride; and disrupted by treatment with 100- to 150-μm glass beads in a planetary mill at 1000 rpm for 3 min. The homogenate was centrifuged at 10000 *g* for 30 min to remove unbroken cells and cell debris.

The cell-free extract was brought to 50% saturation by adding ammonium sulfate. The precipitate was collected by centrifugation at 15000 *g* for 30 min and dissolved in 10 mM potassium phosphate buffer (pH 7.5). The pH of the solution was adjusted to 3.5 by adding 10% acetic acid dropwise with continuous stirring. The suspension was centrifuged at 5000 *g* for 10 min. The supernatant was discarded. The pellet was dissolved in 0.5 M potassium phosphate buffer (pH 7.5). The solution was cleared by centrifugation at 10000 *g* for

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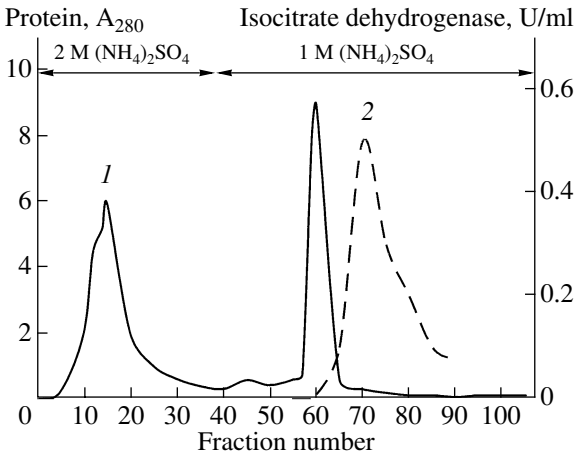

Fig. 1. The hydrophobic chromatography of isocitrate dehydrogenase (NAD^+) on Octyl-Sepharose: (*1*) protein and (*2*) isocitrate dehydrogenase (NAD^+).

10 min and applied, after it had been supplemented with ammonium sulfate to a concentration of 2 M, onto a column (1.6 × 40 cm) with Octyl-Sepharose (Pharmacia, Sweden). The column was washed with a 2 M solution of ammonium sulfate in 100 mM potassium phosphate buffer (pH 7.5) until the absorbance of the eluate at 280 nm returned to the baseline. Then the enzyme was eluted with the same buffer containing 1 M ammonium sulfate. The flow rate was 50 ml/h; the fraction volume was 12 ml. The fractions that contained isocitrate dehydrogenase (NAD^+) were pooled, and isocitrate dehydrogenase (NAD^+) was precipitated by salting out with ammonium sulfate at 60% saturation. The precipitate was collected by centrifugation at 10000 *g* for 30 min and dissolved in a minimal volume of 100 mM potassium phosphate buffer (pH 7.5). The solution was cleared by centrifugation, and the supernatant was applied onto a column with Sepharose CL-4B equilibrated with the same buffer. The column was developed with the buffer at a flow rate of 7 ml/h. The eluate was collected in 1.5-ml fractions. The active fractions were pooled, concentrated, and supplemented with glycerol at a concentration of 20%. This preparation was pure isocitrate dehydrogenase (NAD^+).

The activity of isocitrate dehydrogenase (NAD^+) was measured spectrophotometrically at 340 nm by the reduction of NAD^+. The reaction mixture contained 0.5 mM NAD^+, 1 mM isocitrate, 5 mM $MgCl_2$, 0.5 mM AMP, and 50 mM KH_2PO_4 (pH 7.4) [6].

The molecular mass of native isocitrate dehydrogenase (NAD^+) was determined by FPLC on a Superose 12 HR 10/30 column (Pharmacia, Sweden), which was developed with 50 mM potassium phosphate buffer (pH 7.4) containing 200 mM NaCl at a flow rate of 1 ml/min. The molecular weight markers were ferritin (440 kDa), aldolase (160 kDa), bovine serum albumin (BSA) (67 kDa), and cytochrome *c* (13 kDa), all purchased from Pharmacia.

Protein concentration was determined by the Bradford method [7] with BSA as the standard. The protein concentration in chromatographic fractions was monitored by measuring absorbance at 280 nm.

The purified enzyme was electrophoresed under denaturing conditions (in the presence of 10% SDS) using a precast 12.5% PAAG plate (Pharmacia FastSystem). The developed plate was stained with a Coomassie Brilliant Blue R-250 solution according to the manufacturer's instruction. The molecular weight markers were phosphorylase B (92.5–97.4 kDa), BSA (66–68 kDa), ovalbumin (43–45 kDa), carboanhydrase (29–30 kDa), trypsin (20.1–21 kDa), and lysozyme (14.3 kDa), all purchased from Bio-Rad.

RESULTS AND DISCUSSION

The first two purification steps (salting out with ammonium sulfate at 50% saturation and acid precipitation) removed most of the ballast proteins, as a result of which the total protein decreased by more than 18 times with a yield of isocitrate dehydrogenase activity equal to about 67%. The next two steps (hydrophobic chromatography on Octyl-Sepharose (Fig. 1) and gel-filtration chromatography on Sepharose CL-4B) yielded pure isocitrate dehydrogenase (NAD^+), as is evident from the results of its denaturing electrophoresis in SDS–PAAG (Fig. 2). As can be seen from the results of purification (Table 1), isocitrate dehydrogenase (NAD^+) was purified 129-fold with a yield of 31%. The resulting enzyme preparation had a specific activity of 22 μmol/(min mg protein).

Table 1. The purification of isocitrate dehydrogenase (NAD^+) from *Y. lipolytica* cells

Step	Volume, ml	Total protein, mg	Total activity, U	Specific activity, U/mg protein	Yield, %
Cell-free extract	100	760	129.2	0.17	100
$(NH_4)_2SO_4$ (50%)	25	248	104.2	0.42	81
Acid precipitation	20	40.5	86.3	2.13	67
Octyl-Sepharose	36	12.3	62.1	5.05	48
Gel filtration	9	1.82	40.1	22	31

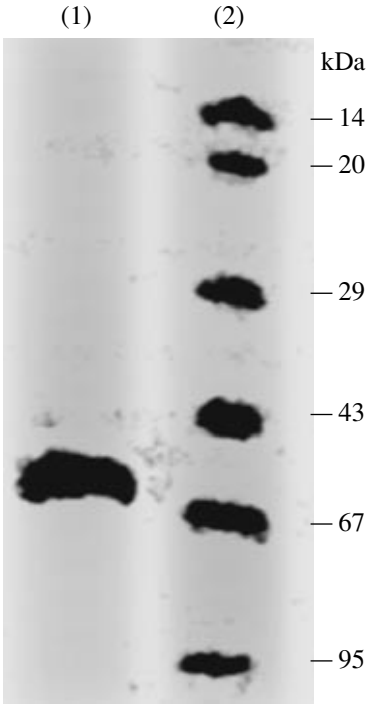

Fig. 2. The denaturing electrophoresis of (1) purified isocitrate dehydrogenase (NAD^+) and (2) molecular weight markers.

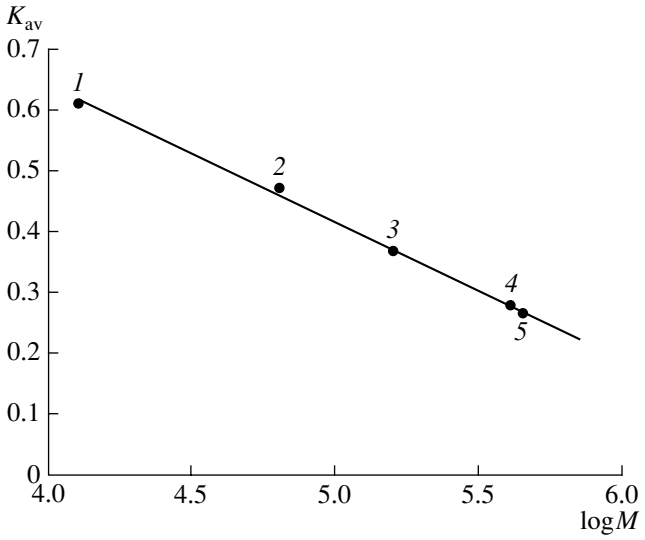

Fig. 3. Diagram illustrating the determination of the molecular mass of isocitrate dehydrogenase (NAD^+). Numerals *1–5* indicate cytochrome *c*, BSA, aldolase, isocitrate dehydrogenase (NAD^+), and ferritin, respectively.

The molecular mass of native isocitrate dehydrogenase (NAD^+) determined by FPLC on a prepacked Superose 12 HR 10/30 column was found to be 412 kDa (Fig. 3). It should be noted that the gel-filtration chromatography of isocitrate dehydrogenase (NAD^+) on a column with Sepharose CL-4B gave a similar value for its molecular mass (400 kDa). The denaturing electrophoresis of the enzyme yielded a single band with a molecular mass of 52 kDa (Fig. 2). A comparison of the molecular masses of the native and denatured forms of isocitrate dehydrogenase (NAD^+) showed that the native enzyme is an octamer composed of identical subunits. A similar octameric structure was shown earlier for the isocitrate dehydrogenases (NAD^+) of baker's yeasts [8] and *Rhodosporidium toruloides* [9]. The optimum pH for the isocitrate dehydrogenase (NAD^+) of *Y. lipolytica* was found to be 7.4.

The study of the effect of some intermediates of the TCA cycle (including citrate) on isocitrate dehydrogenase (NAD^+) showed that they are poor effectors of the enzyme (Table 2). These data contradict the earlier data of Sokolov *et al.* [10], who showed that 40 mM citrate suppressed the activity of the partially purified isocitrate dehydrogenase (NAD^+) of *Y. lipolytica* by 80%. This contradiction may reflect complex relationships existing between yeast isocitrate dehydrogenases and citrate [11–13] and needs comprehensive studies.

The measurement of kinetic parameters showed that the K_m of isocitrate dehydrogenase for NAD^+ is 136 μM and its Hill coefficient is 1.06.

According to data available in the literature, NAD^+-dependent yeast isocitrate dehydrogenases require AMP for activity [6] and have specific allosteric centers for AMP binding. The interaction of AMP with these centers augments the affinity of the enzymes for isocitrate [9, 11, 13]. The measurement of isocitrate dehydrogenase activity in the presence of different concentrations of isocitrate and AMP and the calculation of relevant kinetic parameters according to the kinetic model of Webb [14] (Fig. 4) showed that the K_m of isoc-

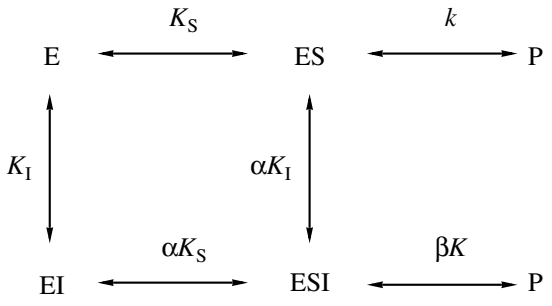

Fig. 4. The reaction scheme illustrating the conversion of isocitrate dehydrogenase in the presence of AMP. E, enzyme (isocitrate dehydrogenase); S, substrate (isocitrate); I, effector (AMP); P, product (2-oxoglutarate); K_S, binding constant for the substrate; K_I, binding constant for the effector; α, the increment coefficient of K_S; k, the breakdown rate constant of the enzyme–substrate complex; β, the increment coefficient of k; n_S, the Hill coefficient for the substrate; n_I, the Hill coefficient for the effector.

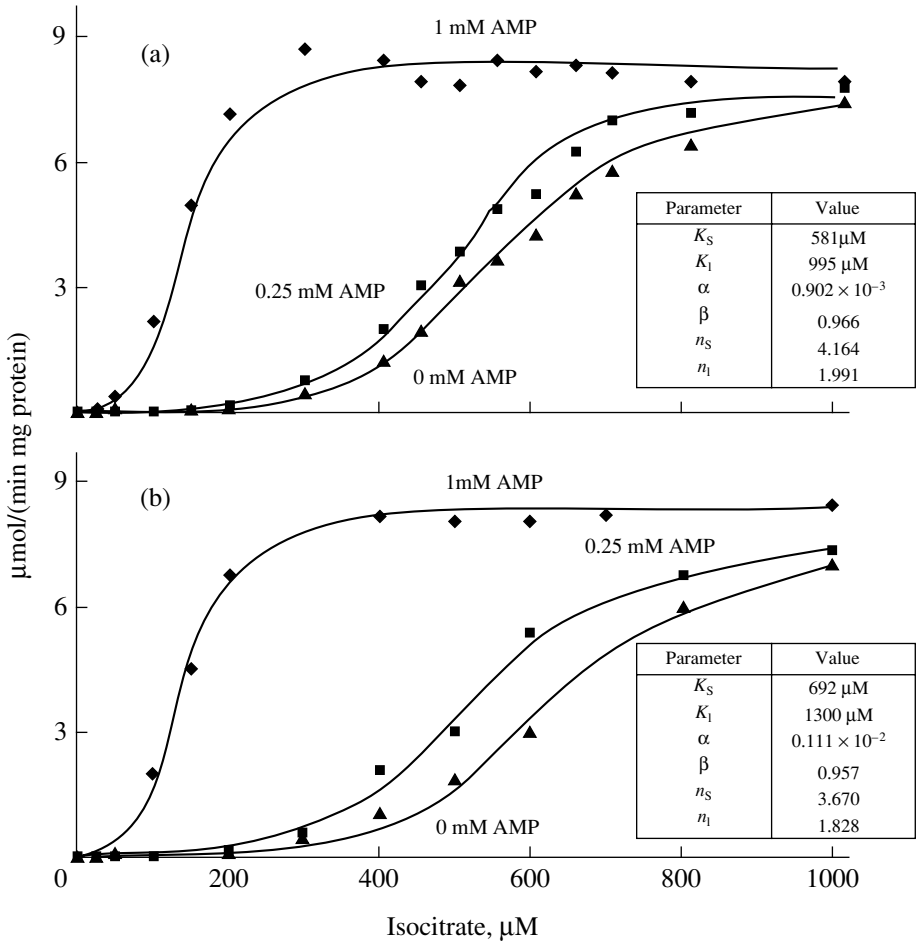

Fig. 5. The effect of AMP on the kinetic parameters of isocitrate dehydrogenase (NAD^+) in the presence (b) and absence (a) of ATP. The reaction mixture contained 5 mM $MgCl_2$, 1 mM NAD^+, and 0.25 mM ATP in 100 mM glycyl–glycine buffer (pH 7.6). The designation of kinetic parameters is the same as in Fig. 4.

itrate dehydrogenase (NAD^+) for isocitrate in the absence of AMP is 581 μM. AMP increased the affinity of the enzyme for isocitrate ($\alpha < 1$) but did not influence the maximum reaction rate ($\beta \approx 1$). The affinity of isocitrate dehydrogenase (NAD^+) for AMP was low (K_m = 995 μM). The calculated values of the Hill coefficient suggested that isocitrate dehydrogenase (NAD^+) has four binding centers for isocitrate and two binding centers for AMP (Fig. 5a), the latter serving as a positive effector. Similar studies of the isocitrate dehydrogenase (NAD^+) isolated from *Saccharomyces cerevisiae* showed that it is composed of four identical IDH1 and four identical IDH2 subunits and is allosterically regulated by binding of AMP to IDH1 [15].

Table 2. The effect of some metabolites on the activity of isocitrate dehydrogenase (NAD^+)

Metabolite (10 mM)	Activity, %
Control (without metabolites)	100
Oxaloacetate	86.5
Citrate	91.1
Succinate	97.2
Fumarate	93.2
Malate	93.2
Glyoxylate	79.0
α-Ketoglutarate	91.3
Glutamate	99.8

Yeast isocitrate dehydrogenases (NAD^+) are strongly inhibited by ATP even at saturating concentrations of AMP and isocitrate [6, 9]. The partially purified isocitrate dehydrogenase (NAD^+) of *Y. lipolytica* was also found to be inhibited by ATP [10]. It should be noted, however, that Sokolov *et al.* used ATP at concentrations as high as 10 mM, which considerably exceed the normal (physiological) concentrations of ATP in *Y. lipolytica* cells [16]. In this work, the addition of 0.25 mM ATP to the reaction mixture changed but little the kinetic parameters of isocitrate dehydrogenase

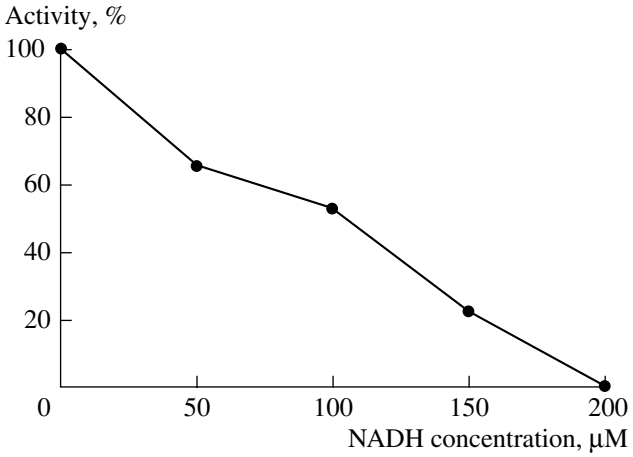

Fig. 6. The effect of NADH on the activity of isocitrate dehydrogenase (NAD^+). The activity at zero concentration of NADH (4 μmol/(min mg protein)) is taken as 100%. The reaction mixture contained 5 mM $MgCl_2$, 0.15 mM NAD^+, 1 mM isocitrate, and 1 mM AMP in 100 mM glycyl–glycine buffer (pH 7.6).

(NAD^+) (Fig. 5b) and the addition of 0.5 mM ATP did not notably influence the activity of the enzyme.

It is well known that NADH inhibits NAD^+-dependent yeast isocitrate dehydrogenases [8–10] because this reduced pyridine nucleotide impairs the dissociation of the enzyme–NADH complex. The inhibitory action of NADH on the isocitrate dehydrogenase (NAD^+) of *Y. lipolytica* was confirmed experimentally. NADH at concentrations of 0.1 and 0.2 mM inhibited this enzyme by 50 and 100%, respectively (Fig. 6). NADH also diminished the enzyme affinity for NAD^+, acting as a competitive inhibitor with $\alpha > 1$.

Isocitrate dehydrogenase (NAD^+) probably plays a key role in the overproduction of organic acids by the yeast *Y. lipolytica*. The limitation of yeast growth by a deficiency of nitrogen sources in the medium inhibits the biosynthesis of nitrogen-containing compounds (proteins and nucleotides) and diminishes their content in cells. This is accompanied by a decrease in the intracellular level of AMP with a concurrent increase in the ATP/AMP ratio [16]. The exhaustion of nitrogen sources from the medium also leads to an increased NADH/NAD^+ ratio [17]. The low concentration of the allosteric regulator AMP suppresses isocitrate dehydrogenase (NAD^+), the suppression being enhanced by the high NADH/NAD^+ ratio. As a result, yeast cells overproduce isocitric acid, whereas the disturbed equilibrium of the aconitase reaction leads to the overproduction of citric acid.

The thiamine-limited growth of yeast cells is accompanied by a deficiency of reducing equivalents in the cells and a drop in the ATP/AMP and NADH/NAD^+ ratios [16, 17]. This favorably influences isocitrate dehydrogenase (NAD^+), so that this enzyme remains active even when yeast growth is arrested. The metabolism of thiamine-deficient cells is blocked at the level of thiamine-dependent enzymes (α-ketoglutarate dehydrogenase and pyruvate dehydrogenase [2, 18]), bringing about an elevated excretion of α-ketoglutarate and pyruvate. The high concentration of α-ketoglutarate is unlikely to hinder the normal functioning of isocitrate dehydrogenase (NAD^+) since this keto acid is only slightly inhibitory to the isocitrate dehydrogenase (NAD^+) of *Y. lipolytica* (Table 2 and references [10, 19]).

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