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Purification and Properties of NADH-Cytochrome b_5 Reductase from Ascidian, *Styela plicata*

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NADH-cytochrome b_5 reductase was purified from ascidian, *Styela plicata*, and some properties of the enzyme were studied. The enzyme of *Styela plicata* was purified about 70-fold from the crude extract by column chromatographies on 5'-AMP-Sepharose and DEAE-Toyopearl. Some general properties were determined with the purified enzyme. Optimal pH for NADH-dependent cytochrome b_5 reduction with the enzyme was observed at about 6.2. Michaelis constants for NADH and cytochrome b_5 of the enzyme were determined to be 1.4 μM, and 3.6 μM, respectively. Substrate specificity of the enzyme for pyridine nucleotide was strictly NADH-specific. Almost all these properties were very similar to those of mammalian enzymes.

INTRODUCTION

NADH-cytochrome b_5 reductase was studied initially on cow liver microsomal enzyme by Strittmatter and Velick (1956), and they studied on the enzyme extensively (Strittmatter, 1965; Strittmatter, 1966). Recently, Yubisui and his group studied on the human enzyme, and the amino acid sequence of the enzyme purified from human erythrocytes was first determined (Yubisui *et al.*, 1986). And then the structures of cDNA (Yubisui *et al.*, 1987) and genomic DNA (Tomatsu *et al.*, 1990) for the human enzyme were also determined. Development of the cDNA expression system in *Escherichia coli* for the human enzyme (Shirabe *et al.*, 1989) remarkably stimulated the studies on the enzyme. Analysis on the hereditary disease due to the deficiency of the enzyme was performed on the various mutant enzymes prepared with the expression system described above. Mutations were introduced into the enzyme based on the nucleotide sequences determined on the patient DNAs (Yubisui *et al.*, 1991; Nagai *et al.*, 1993; Shirabe *et al.*, 1994). Recombinant wild-type enzyme was used to prepare the crystal for X-ray crystallographic analysis of the enzyme (Takano *et al.*, 1994).

NADH-cytochrome b_5 reductase of mammalian enzyme participates in the fatty acid metabolisms such as fatty acid desaturation (Oshino *et al.*, 1971; Keyes and Cinti, 1980), fatty acid chain elongation (Keyes and

Cinti, 1980), or cholesterol biosynthesis (Reddy *et al.*, 1977; Fukushima *et al.*, 1981), and in the cytochrome P450-dependent drug metabolism (Hildebrandt and Estabrook, 1971), and also in the steroid hormone metabolism (Onoda and Hall, 1982; Shinzawa *et al.*, 1985; Katagiri *et al.*, 1995).

Recently, we found that cytochrome b_5 in the ascidian, *Polyandrocarpa misakiensis*, is expressed at a high level in the developing buds, but not in the parental body (Yubisui *et al.*, 2001). The expression pattern of the cytochrome b_5 in *P. misakiensis* coincides well with the accumulation of triacylglycerol in the developing buds of the ascidian (Yubisui *et al.*, 2001). These results prompted us to study on cytochrome b_5 reductase in the ascidian. In this study, we used *Styela plicata (S. plicata)* as the experimental source, which has a significantly higher NADH-cytochrome b_5 reductase activity than that of *P. misakiensis* as shown in Table 1. This paper described the purification and properties of NADH-cytochrome b_5 reductase of *S. plicata*.

MATERIALS AND METHODS

NADH and NADPH were purchased from Boehringer Mannheim (Tokyo, Japan), and 5'-AMP-Sepharose was obtained from Amersham/Pharmacia (Tokyo, Japan). Other reagents used in this study were all reagent grade. DEAE-Toyopearl 650M was the product of Toyo Soda and Sephacryl S-100 was obtained from Pharmacia (Uppsala, Sweden).

Various ascidians were harvested at the Uranouchi Inlet near the Marine Biological Institute of Kochi University, Kochi, Japan.

Preparation of crude extract

Crude extract of the ascidian was prepared after brief washing of the animal with saline, by homogenizing the chopped animal tissues by a Wahring blender with 4 volume of Tris-HCl buffer (pH 8) containing 1 mM EDTA and 0.1 mM DTT. Triton X-100 was added at 1% concentration to the resultant homogenates, and stood on ice for 30 min to extract the enzyme. Then the solution was centrifuged to remove cell debris at 17,000 x g for 15 min. The supernatant was used as the crude enzyme solution

Assay of the enzyme activity

We do not have the cytochrome b_5 of respective ascidians except *P. misakiensis* as the substrate for the enzyme, therefore, the enzyme activity was determined with rat recombinant cytochrome b_5. Soluble form of rat cytochrome b_5 was expressed in *Escherichia coli* (E. coli) as described previously (Shirabe *et al.*, 1989) and purified by chromatographies on a DEAE-Toyopearl and gel filtration column. The expression system of the rat cytochrome b_5 was a generous gift from Dr. A.W. Steggles of Northeastern Ohio Universities College of Medicine. Cytochrome b_5 reductase activity was determined as described previously (Yubisui and Takeshita, 1980) by following the increase in the absorbance of cytochrome b_5 at 424 nm upon reduction. The reaction mixture (2 ml) contained 5 mM Tris-HCl buffer (pH 7.5), 2 mM cytochrome b_5, 0.05% Triton X-100, 0.1 mM

NADH, and appropriate amount of enzyme solution. Assay was started by adding NADH to the reaction mixture.

Electrophoresis

Purity of the enzyme was examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (Laemmli, 1970). Proteins on the gel were stained with Coomassie Brilliant Blue (CBB).

RESULTS AND DISCUSSION

Purification of NADH-cytochrome b_5 reductase

We examined NADH-cytochrome b_5 reductase activities of various ascidians to purify the enzyme. As shown in Table 1, the enzyme activity in the crude extract of *S. plicata* was the highest one. Although we succeeded in cloning of cytochrome b_5 of *P. misakiensis* (Yubisui *et al.*, 2001), the enzyme activity of the ascidian was very weak to purify the enzyme. Therefore, we used *S. plicata* as the experimental source to purify the enzyme in this study.

To isolate NADH-cytochrome b_5 reductase from the homogenate of *S. plicata*, Triton X-100 was added at 1% concentration, and stood on ice for 30 min. The homogenate was then centrifuged at 17,000 x g for 15 min to obtain the supernatant. Resulted supernatant contained most parts of the NADH-cytochrome b_5 reductase activity. However, we found that the enzyme is very unstable in the crude extract, and the addition of various protease inhibitors was not effective to stabilize the enzyme. Addition of glycerol to the crude extract at a concentration of 20% stabilized the enzyme moderately, but still the enzyme activity decreased rapidly. Then, the next attempt was to apply the crude enzyme solution on a 5'-AMP-Sepharose column (1.5 x 6 cm) immediately after the preparation. This method efficiently removed other proteins by washing the column with the equilibrium buffer. The enzyme adsorbed on the column was eluted with 0.1 mM NADH added to the equilibrium buffer. As a result, removal of many other proteins from the preparation as soon as possible by the affinity chromatography was most effective to stabilize the enzyme (Fig. 1).

Table 1. Effect of FAD on the NADH-cytochrome b_5 reductase activitie of various ascidians.

Ascidians	(nmol/min/mg)[a] -FAD	+FAD[b]
Polyandrocarpa misakiensis	ND[c]	5.8
Polyandrocarpa zorritensis	ND	22.6
Symplegma reptans	ND	16.6
Ciona intestinalis	3.3	22.7
Styela plicata	23.6	25.0

a) The enzyme activities are the average values of 2-3 determinations.
b) 5.25 μM FAD was added into the reaction mixture.
c) Not detectable.

Table 2. Summary of the purification of NADH-cytochrome b_5 reductase from *S. plicata*

Enzyme preparations	Specific activity[a] (nmol/min/mg)	Purification (-fold)	Yield (%)
Crude extracts	23.6	1	100
5'-AMP-Sepharose	978	41.4	27
DEAE-Toyopearl	1312	55.6	8.6
5'-AMP-Sepharose	1680	71.2	2.6

a) Specific activity was expressed as nmol cytochrome b_5 reduced/min/mg.

To purify the enzyme further, the eluted enzyme from the affinity column was applied on a DEAE-Toyopearl column (2 x 10 cm), which was equilibrated with 50 mM Tris-HCl (pH 8) containing 1 mM EDTA, 0.1 mM DTT, and 0.1% Triton X-100. After washing the column with the same buffer, the enzyme activity was eluted with a linear gradient of NaCl from 0 to 0.3 M in the same buffer. The enzyme was eluted with about 0.1 M NaCl in the buffer as shown in Fig. 2-A. Then the eluted enzyme solution was again applied on the 5'-AMP-Sepharose column under the same conditions as in the first affinity chromatography. The adsorbed enzyme was again eluted with 0.1 mM NADH in the equilibrated buffer (Fig. 2-C).

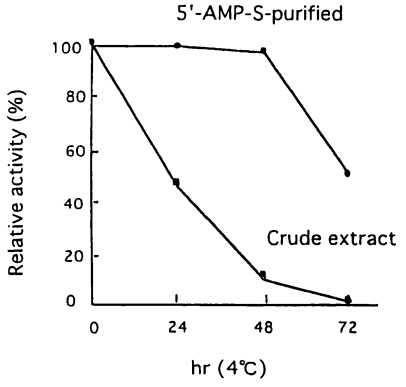

Fig. 1 Stability of NADH-cytochrome b_5 reductase of *S. plicata*.
Removal of various proteins in the crude extract by the affinity chromatography with 5'-AMP-Sepharose stabilized the enzyme. This figure is a typical example of the three experiments.

As summarized in Table 2, the enzyme was purified about 70-fold from the crude extract with about 3% yield of the activity. Purity of the enzyme was examined by SDS-PAGE as shown in Fig. 2-B, -D, and we found that the enzyme preparation still contained few proteins. After the 2nd affinity chromatography the purified enzyme showed 2 major protein bands at 25 and 48 kD by SDS-PAGE.

As another purification method, the partially purified enzyme by ion-exchange chromatography on DEAE-Toyopearl was applied on a hydroxyapatite column instead of the 2nd affinity chromatography on 5'-AMP-Sepharose. The enzyme was eluted from the column by a gradient of phosphate buffer (Fig. 2-E), and the active fractions showed two protein bands at 48 and 56 kD by SDS-PAGE (Fig. 2-F). Attempt to purify the enzyme further was unsuccessful due to the loss of the enzyme activity during the procedures and also due to the limited amount. We failed to purify the enzyme to homogeneity in this study, therefore, we could not determine what size band on the SDS-PAGE is the enzyme.

We also tried to isolate a cDNA for the enzyme from the cDNA library of the ascidians to reveal the structure of the enzyme based on the genetic information, but we failed to get the clone. Therefore, the characterization of the ascidian enzyme was carried out with the partially purified enzyme hereafter.

Characterization of the enzyme

Purity of the enzyme preparation was examined by SDS-PAGE as described above. The enzyme preparation still contained protein bands including 25, 48, and 56 kD. The common size between the affinity-

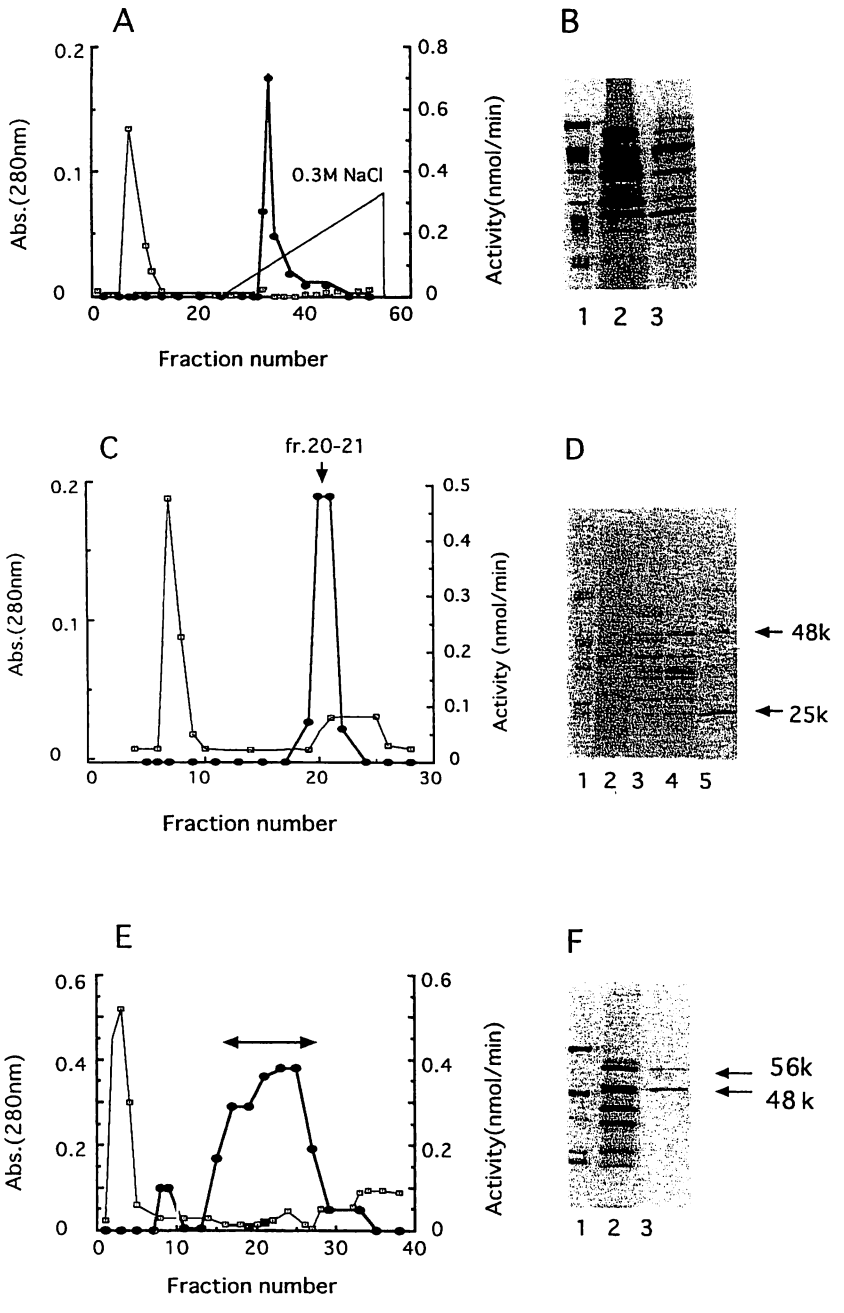

Fig. 2 Purification of NADH-cytochrome b_5 reductase by chromatographies on the DEAE-Toyopearl, 5'-AMP-Sepharose, and hydroxyapatite columns.

A) Chromatography of the 5'-AMP-Sepharose-fractionated enzyme on a DEAE-Toyopearl column. B) SDS-PAGE of (1) molecular weight markers, (2) crude extract, (3) DEAE-Toyopearl eluates. C) Rechromatography of the enzyme on a 5'-AMP-Sepahorse. The DEAE-Toyopearl-purified preparation was applied on the column, and the enzyme was eluted with 0.1 mM NADH. D) SDS-PAGE of (1) molecular weight markers, (2) crude extract, (3) 5'-AMP-Sepharose equate, (4) DEAE-Toyopearl equate, (5) 2nd 5'-AMP-Sepharose equate. E) Chromatography of the DEAE-Toyopearl-purified preparation on a hydroxyapatite column. Active fractions indicated by the arrow were pooled. F) SDS-PAGE of (1) molecular weight markers, (2) DEAE-Toyopearl equate, (3) the hydroxyapatite equates.

Squares, proteins (280 nm), and circles, the enzyme activity. Protein bands on the gels in (B, D, F) were stained with CBB. See text for details.

purified enzyme and hydroxyapatite-purified enzyme was 48 kD, but we could not prove that this protein is NADH-cytochrome b_5 reductase. We tried to analyze the amino-terminal peptide sequence of the bands after blotting onto PDBF membrane, but any a clear peptide sequence corresponding to NADH-cytochrome b_5 reductase was not obtained. The enzyme of the ascidian may be N-terminal-blocked as in the case of mammalian enzyme (Murakami *et al.*, 1989).

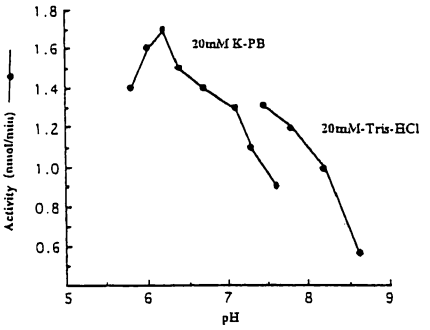

Fig. 3 pH optimum of the enzyme activity.
The optimal pH of the enzyme was determined with potassium phosphate buffer, and with Tris-HCl buffer.

The optimal pH for the enzyme activity was determined with Tris-HCl buffer and phosphate buffer at various pH values. As shown in Fig. 3, the optimal pH of the ascidian enzyme was observed at around 6.2. This optimal pH is similar to that of the rabbit enzyme (optimal pH at 6.5) (Yubisui and Takeshita, 1982), but is different from that of human enzyme, which shows the optimum at around 8 (Yubisui and Takeshita, 1980).

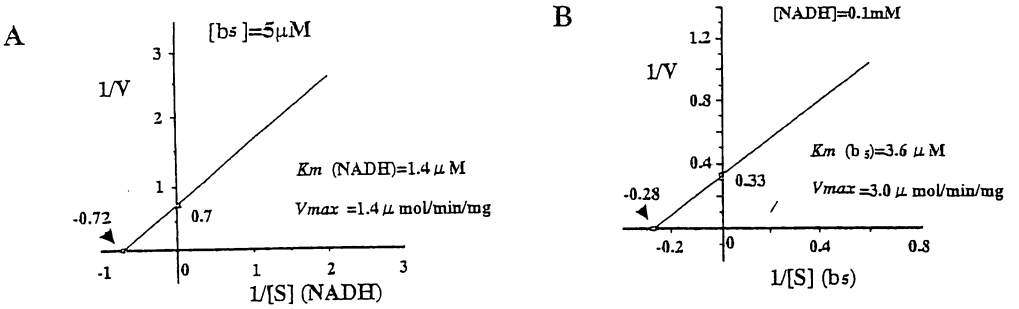

Fig. 4 Kinetic properties of the enzyme.
The apparent *Km* values for NADH and cytochrome b_5 of the purified enzyme were determined by varying the substrate concentrations

Then the kinetic properties of the enzyme were examined with NADH as the electron donor and cytochrome b_5 as the electron acceptor. As shown in Fig. 4, the apparent *Km* values for NADH and cytochrome b_5 were determined to be 1.4 μM and 3.6 μM, respectively. These values are similar to those of the human enzyme (Yubisui *et al.*, 1991). As shown in Fig.4, in these experiments the *Vmax* value of 3 μmol/min/mg was obtained in the presence of excess NADH. This value is very low as compared with those of the human enzyme (400 μmol/min/mg) and the rabbit enzyme (1,280 μmol/min/mg) (Yubisui and Takeshita, 1982). The enzyme activity with NADPH was less than 5% of that with NADH.

Purification of this enzyme from the animal to homogeneity was very difficult due to the low content and instability of the enzyme in the ascidians tissues. We are still continuing the effort to isolate a cDNA clone for the enzyme from the cDNA library, and to reveal the structure of the enzyme based on the genetic information.

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