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ISOLATION OF HEXOSE-6-PHOSPHATE DEHYDROGENASE FROM RAT LIVER MICROSOMAL FRACTION BY AFFINITY CHROMATOGRAPHY

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SUMMARY: Hexose-6-phosphate dehydrogenase of rat liver microsomes was purified to an apparently homogeneous state with a recovery of about 36% using 8-aminooctyl Sepharose, DEAE-cellulose and 2',5'-ADP Sepharose columns. This enzyme was insensitive to SH-reagent p-chloromercuribenzoate and oxidized galactose 6-phosphate, glucose 6-phosphate and glucose, with either NADP or NAD as an electron acceptor. The minimum molecular weight of this enzyme was estimated to be 104,000 in SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol.

INTRODUCTION

The extramitochondrial generation of NADPH is carried out by cytosol enzymes, glucose-6-phosphate dehydrogenase of pentose phosphate pathway (1) and malic enzyme (2). Hexose-6-phosphate dehydrogenase in microsomal fraction, which appears to be identical with the microsomal glucose dehydrogenase (β-D-glucose:NAD(P) oxidoreductase, EC 1.1.47) reported previously (3,4), is another entity which produces the reducing equivalents (5,6). This enzyme occurs widely in various tissues (7) and animal species (4), and has been shown to have properties quite distinct from the cytosol glucose-6-phosphate dehydrogenase. The microsomal hexose-6-phosphate dehydrogenase is an autosomally inherited enzyme (8) and has a broad substrate specificity: it exhibits marked catalytic activity with galactose 6-phosphate, glucose 6-phosphate and glucose, using either NAD or NADP as an electron acceptor (6).

Although the role of glucose-6-phosphate dehydrogenase in pentose phosphate pathway has been established (1), the role of hexose-6-phosphate dehydrogenase in cellular metabolism is not known. Based upon the subcellular

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distribution, Mandula et al. (5) have recently speculated that this eznyme serves as a source of reducing equivalents for microsomal electron transport systems. Several attempts were made to purify the enzyme from various tissues (4,5,9,10), but the isolation of a homogeneous preparation has not yet been reported. In this communication, we report an affinity method for the isolation of microsomal hexose-6-phosphate dehydrogenase and its preliminary characterization.

MATERIALS AND METHODS

Treatment of animals and preparation of microsomes.

Male Wistar rats, weighing 250-300 g, were administered i.p. with sodium phenobarbital dissolved in 0.9% NaCl (100 mg/kg body weight) once a day for ten days. The animals were starved for 24 hours before killing. The livers were excised and perfused with ice-cold 1.15% KCl-10mM EDTA and homogenized with 4 vol of the same medium. The homogenate was centrifuged at 9,000 x g for 15 min, and the obtained supernatant was again centrifuged at 65,000 x g for 90 min to give microsomes. The microsomal pellets thus obtained were carefully rinsed with ice-cold 1.15% KCl-10mM EDTA to wash out the contaminating soluble glucose-6-phosphate dehydrogenase, and resuspended in an excess of the same medium. After centrifugation as described above, the microsomal pellets were again rinsed, and finally suspended in ice-cold distilled water.

Purification of hexose-6-phosphate dehydrogenase.

(1): Solubilization of microsomes by cholate.

The microsomes (3.37 g protein) were solubilized by suspending (at the protein concentration of 2.4 mg/ml) in 0.1M potassium phosphate buffer (pH 7.25) containing 20% glycerol, 1mM EDTA, 0.6% sodium cholate and protease inhibitors (1 ug/ml of pepstatin and leupeptin). The mixture was stirred in ice for about 30 min and centrifuged at 65,000 x g for 90 min to give a clear solubilized supernatant.

(2): 8-Aminooctyl Sepharose 4B column chromatography.

Solubilized supernatant (1,340 ml) was applied to the aminooctyl Sepharose 4B column pre-equilibrated with the solubilizing buffer mentioned above. Hexose-6-phosphate dehydrogenase activity was eluted from the column with some retardation during the application. The column was further washed with the equilibrating buffer to complete the elution of the activity. The peak fractions of the activity were pooled (800 ml), added with Emulgen 913 (final 0.1%) and dialyzed twice against 10 liters of distilled water to decrease the phosphate concentration below 10mM.

(3): DEAE-cellulose column chromatography.

The dialyzed sample (1,320 ml) was mixed with 15-20 g wet weight of DE52 and stirred in ice for an hour. Virtually all the hexose-6-phosphate dehydrogenase activity was adsorbed onto the DE52 under the conditions of this experiment. DE52 was collected by filtration with Buchner funnel and washed with an excess of 10mM potassium phosphate buffer (pH 7.25) containing 0.1% Emulgen 913 and packed into a column (2.3 x 9 cm). After the column was washed with 15mM potassium phosphate buffer (pH 7.25) containing 0.1% Emulgen 913, hexose-6-phosphate dehydrogenase activity was eluted with 35mM potassium phosphate buffer(pH 7.25) containing 0.1% Emulgen 913. The peak fractions were pooled (60 ml) and diluted 2-fold by mixing with the same volume of distilled water.

(4): 2',5'-ADP Sepharose 4B column chromatography.
The diluted sample was applied to the 2',5'-ADP Sepharose 4B column (1.1 x 3.8 cm) pre-equilibrated with 20mM potassium phosphate buffer (pH 7.25) containing 0.1% Emulgen 913. The column was washed consecutively with an excess of the equilibrating buffer, 10 column volumes of 35mM potassium phosphate buffer (pH 7.25) containing 0.1% Emulgen 913 and 0.1M KCl, and finally 35mM potassium phosphate buffer (pH 7.25) containing 0.1% Emulgen 913. The hexose-6-hosphate dehydrogenase was then eluted with 35mM potassium phosphate buffer (pH 7.25) containing 0.1% Emulgen 913 and 0.5mM NADP.

Analytical and Assay Methods.
Hexose-6-phosphate dehydrogenase activity was determined at room temperature (20°C) by measuring the formation of NADPH or NADH as in (6) except that 0.3% cholate was included in the assay mixture when untreated microsomes were used for the enzyme assays. Protein was measured by the method of Lowry et al. (11). SDS-Polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol was done as in (12).

Chemicals and Others.
1,8-Diaminooctane was obtained from Aldrich Chemical Co. and cholic acid from Nissui Seiyaku (Tokyo). NAD, NADP and glucose 6-phosphate were obtained from Oriental Yeast Co. (Osaka) and galactose 6-phosphate was from Sigma Chemical Co. Sepharose 4B and 2',5'-ADP Sepharose 4B were purchased from Pharmacia Fine Chemicals, and DE52 was from Whatman. 8-Aminoocty Sepharose 4B was prepared as in (13). RNA polymerase for a marker in electrophoresis was obtained from Mitsubishi Yuka (Osaka). Emulgen 913 was a kind gift from Kao-Atlas (Tokyo). Other chemicals were of reagent grade.

RESULTS AND DISCUSSION

Microsomal hexose-6-phosphate dehydrogenase activity has been shown to be induced by phenobarbital (14). For the starting materials, therefore, we used liver microsomes of phenobarbital treated rats. Before solubilization of microsomes, much attention was paid to remove the cytosol fraction from the microsomal pellets in order to diminish the possible contamination of glucose-6-phosphate dehydrogenase . Table 1 shows a summary of the purification of hexose-6-phosphate dehydrogenae from rat liver microsomes. Two steps, DEAE-cellulose and 2',5'-ADP Sepharose column chromatographies, were effective for the purification.

The purified preparation has a specific activity of about 3 units/mg of protein, which was about 700 times higher than the activity in the starting microsomes. The overall recovery was about 36%. SDS-Polyacrylamide gel electrophoretic analysis indicated that the purified preparation was apparently homogeneous as shown in Fig. 1. The minimum molecular weight of this prepa-

Table 1. Summary of a purification of hexose-6-phosphate dehydrogenase from rat liver microsomes.

Steps	Protein (mg)	Hexose-6-phosphate dehydrogenase	
		Total units	Units/mg protein
1. Microsomes	3,372	14.6 (100 %)#	0.0043 (1.0)$
2. Solubilized sup.	3,310	15.0 (103)	0.0045 (1.0)
3. Aminooctyl Sepharose	428	10.0 (68)	0.0234 (5.4)
4. DEAE-cellulose	32.1	5.6 (38)	0.175 (40.7)
5. 2',5'-ADP Sepharose	1.8	5.3 (36.3)	2.92 (680)

Hexose-6-phosphate dehydrogenase activity was measured at pH 9.6 with 1mM glucose 6-phosphate and 0.17mM NADP. One unit was defined as the activity which produced 1 μmol of NADPH per a minute under the conditions of this experiments.
#: Recovery of the activity. $: Purification fold.

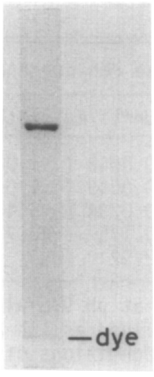
ration was estimated to be about 104,000 by comparing the electrophoretic mobility with those of standard proteins of known molecular weights as shown in Fig. 2. The active enzyme appears to exist as a dimerization form, because, based on the gel filtration experiments, Metzger et al. (4) and Thompson and Carper (10) suggested that the activity was eluted in a region of approximate molecular weight of 230,000.

Table 2 shows the relative activities of purified hexose-6-phosphate dehydrogenase and cytosol glucose-6-phosphate dehydrogenae with various substrates and electron acceptors. Hexose-6-phosphate dehydrogenase exhibited

Table 2. Substrate specificity of purified hexose-6-phosphate dehydrogenase and partially purified glucose-6-phosphate dehydrogenase.

Hexoses	Hexose-6-phosphate dehydrogenase		Glucose-6-phosphate dehydrogenase	
	NADP	NAD	NADP	NAD
Glucose 6-phosphate (1.0mM)	100%	22.5%	100%	~0%
Galactose 6-phosphate (1.3mM)	67.3	21.7	2.1	~0
Glucose (0.53M)	20.2	90.5	2.6	~0

Hexose-6-phosphate dehydrogenase activity was measured at pH 9.6 with hexoses indicated using 0.17mM of either NADP or NAD as an electron acceptor. Glucose-6-phosphate dehydrogenase partially purified from rat liver cytosol by the method of Matsuda and Yugari (15) was assayed under the same conditions as those used for hexose-6-phosphate dehydrogenase activity.
Values represent the relative activities to those measured with glucose 6-phosphate and NADP.



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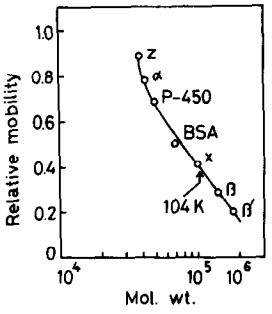

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Fig.1 SDS-Polyacrylamide gel electrophoresis of the purified preparation of hexose-6-phosphate dehydrogenase.
3 μg of the purified enzyme was electrophoresed (7.5% gel concentration) and stained with Coomasie Brilliant Blue R250.

Fig.2 Estimation of molecular weight of hexose-6-phosphate dehydrogenase.
The minimum molecular weight of the purified enzyme was estimated by comparing the relative mobility in SDS-polyacrylamide gel electrophoresis (7.5%) with those of standard proteins of known molecular weights: bovine serum albumin (BSA, 68,000), phenobarbital induced rat liver microsomal P-450 (P-450, 49,000) and five subunits of RNA polymerase (β';180,000, β;140,000, x;100,000, α;42,000 and z;39,000).

marked activities with galactose 6-phosphate and glucose, using NADP or NAD as an electron acceptor. Glucose-6-phosphate dehydrogenase, however, showed only negligible activities except with glucose 6-phosphate and NADP. The activity of hexose-6-phosphate dehydrogenase with glucose as substrate was about 4.5-times higher with NAD than that with NADP as an electron acceptor. A typical SH-reagent p-chloromercuribenzoic acid (0.5mM) almost completely inhibited the glucose-6-phosphate dehydrogenase activity, but did not inhibited the hexose-6-phosphate dehydrogenase activity (data not shown). These characteristics of hexose-6-phosphate dehydrogenase are well consistent with those reported previously using crude preparations (6,10).

From the molecular weight and the activity of the purified enzyme reported here, the content in microsomes could be calculated, on a molar basis as well as weight basis, to be approximately 0.015 nmoles/mg of protein or 0.15% of total protein.

The physiological role of this enzyme is still unclear. Mandula et al. (7) have proposed that it may provide reducing equivalents to the electron transport systems of the endoplasmic reticulum. Several important questions must be answered to support their proposal. For example, how NAD and NADP can access to the enzyme which is inactive without detergents ?

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