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AFFINITY PURIFICATION AND SOME PROPERTIES OF NUCLEOSIDE DIPHOSPHATASE FROM RAT LIVER CYTOSOL

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Summary

Nucleosidediphosphatase (nucleosidediphosphate phosphohydrolase, EC 3.6.1.6) of rat liver cytosol was purified up to 336—fold by the procedure including affinity chromatographies of concanavalin A- and alanine-Sepharose. The final purified enzyme showed a single protein band upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Its native form was found to be a tetramer with molecular weight of 120000 which consists of subunit with molecular weight of 30000. The enzyme was found to be a glycoprotein on the basis of its chromatographic behaviour with concanavalin A-Sepharose and positive staining with periodate-Schiff reaction in polyacrylamide gels.

Furthermore, the two molecular forms with isoelectric points of 4.7 and 5.0 were demonstrated by electrofocusing, though they did not show any significant difference with respect to their catalytic properties.

Introduction

Nucleosidediphosphatase (nucleosidediphosphate phosphohydrolase, EC 3.6.1.6) catalyzes the hydrolysis of the terminal phosphate of inosine diphosphate (IDP), uridine diphosphate (UDP) and guanosine diphosphate (GDP), but serves neither adenosine diphosphate (ADP) nor cytidine diphosphate (CDP) as its substrates [1—4]. The enzyme has been demonstrated not only in particulate fractions of liver, microsomes [2,3,5—7] and mitochondria [1,8], but also in cytosol fraction [9]. Possible involvement of nucleosidediphosphatase (NDPase) in gluconeogenesis, glycolysis [4,5] or protein synthesis [10] has been discussed.

Up to the present, however, studies on NDPase appear to have been focused especially on microsomal enzyme because of its predominant distribution in

microsomes [2,3,5—7]. If NDPase is involved in gluconeogenesis or glycolysis which occurs in cytosol [11], cytosol NDPase which has previously been partially purified [9] should be characterized in detail.

This paper describes the procedure including affinity chromatography for the purification of cytosol NDPase to a homogeneous form.

Materials and Methods

Chemicals

The sodium salts of all nucleotides other than ADP (Sigma) were purchased from Yamasa Shoyu Co. DEAE-Sephadex A-50, concanavalin A-Sepharose and Sepharose 4B were products of Pharmacia. α-Methyl-D-glucoside and L-amino acids were purchased from Nakarai Chem. Co. Calcium phosphate gel was prepared according to the method of Keilin and Hartree [12]. The concentration of the gel was estimated by weighing gel of measured volume after drying at 100°C to a constant weight. The other chemicals used were analytical grade.

Preparation of alanine-Sepharose

Sepharose 4B was activated with cyanogen bromide according to the procedure of Cuatrecasas [13]. Coupling was conducted in 0.1 M $NaHCO_3$ (pH 9.5) by adding 1 mmol of L-alanine to 1 ml of the activated Sepharose and the mixture was stirred for 16 h at room temperature. The amount of alanine (3—4 μmol/ml gel) bound to the Sepharose was calculated by ninhydrin reaction before and after coupling. The other amino acids, L-leucine, L-valine, and L-phenylalanine, were also coupled with Sepharose in the same way.

Column chromatography

Chromatography on the amino acid-Sepharose was accomplished with a decreasing concentration gradient of ammonium sulfate as described by Rimerman and Hatfield [14]. The salt concentration of gradient was measured for conductivity of Radiometer Conductivity Meter. Protein was monitored by absorption at 280 nm.

Nucleoside diphosphatase assay

Nucleoside diphosphatase activity was assayed using UDP as a substrate unless otherwise indicated. The standard assay system contained 50 mM Tris · HCl buffer (pH 7.5), 4 mM $MgCl_2$, 3 mM UDP and appropriate amount of enzyme in a final volume of 0.25 ml. The reaction mixture was incubated at 37°C for 15 min, and the reaction was terminated by adding of 50 μl of ice-cold 30% trichloroacetic acid. After removal of coagulated protein by centrifugation when crude enzyme preparation was used, inorganic phosphate released was measured in an aliquot of the supernatant by the method of Fiske and Subba Row [15].

1 unit of the enzyme was defined as the amount which produces 1 μmol of P_i per min under the standard assay conditions. Specific activity was expressed as units per mg protein.

Protein was determined by the method of Lowry et al. [16], with bovine serum albumin as a standard.

Purification

All procedures were conducted at 0—4°C. After the liver of slightly anesthetized rat (Wistar-King strain, weighing 180—250 g) had been perfused thoroughly with cold 1% KCl, it was excised and frozen at −20°C until use. The livers (430 g), thawed under tap water, were homogenized with 4 vols. of cold 0.25 M sucrose for five 1-min periods by a Waring blendor. The homogenate was centrifuged at 12000 × *g* for 25 min, and the supernatant fluid was centrifuged at 105000 × *g* for 1 h.

Step 1. The microsome-free supernatant (275 ml) was subjected to ammonium fractionation. A fraction obtained between 40 and 70% saturation was taken up in 20 mM Tris · HCl buffer (pH 8.0) and dialyzed against the same buffer.

Step 2. To the dialyzate (59 ml) was added $Ca_3(PO_4)_2$ gel (49.5 mg of dry weight/ml) in a weight ratio of protein to gel (1 : 5), and the mixture was stirred for 20 min. The gel was collected by centrifugation at 10000 × *g* for 15 min, washed with 0.125 M potassium phosphate buffer at pH 5.4 and at 5.9 successively in 40 ml each of buffer per 1 g of gel. The enzyme was eluted from the gel by stirring and centrifugation with 0.125 M potassium phosphate buffer (pH 7.0) in the same manner as described above. After the eluate was saturated to 80% with ammonium sulfate, the resultant precipitate was dialyzed as described in Step 1.

Step 3. The dialyzate (19.6 ml) was applied on a column (1.9 × 20 cm) of the first DEAE-Sephadex A-50 (Cl^-) previously equilibrated with 20 mM Tris · HCl buffer (pH 8.0). The column was washed with 200 ml of the equilibration buffer, and the enzyme was eluted by a linear concentration gradient of KCl from 0.1 to 0.5 M in the above buffer (200 ml) (Fig. 1). The fractions containing NDPase activity were pooled, concentrated by saturation with ammonium sulfate to 80%, and dialyzed as described in Step 1.

Step 4. The dialyzate (9.2 ml) was applied on a column of concanavalin A-Sepharose (1.5 × 6 cm), equilibrated with 1 mM $MnCl_2$/1 mM $CaCl_2$/0.5 M NaCl/20 mM Tris · HCl buffer (pH 8.0) and washed with the same buffer until

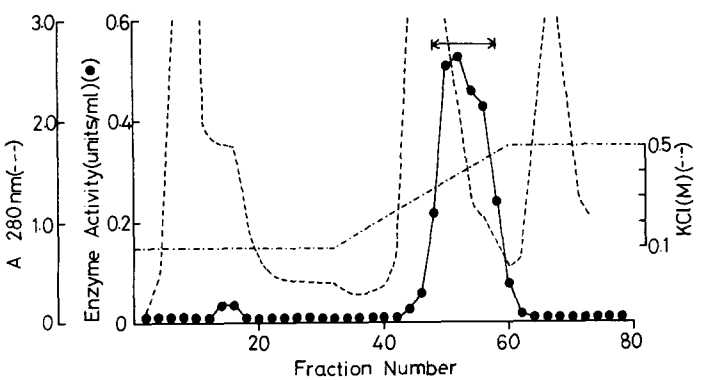


Fig. 1. The first DEAE-Sephadex A-50 (Cl^-) column chromatography (Step 3). Procedures used for chromatography are described in Materials and Methods. Fractions of 5 ml were collected at a flow rate of 22 ml/h.

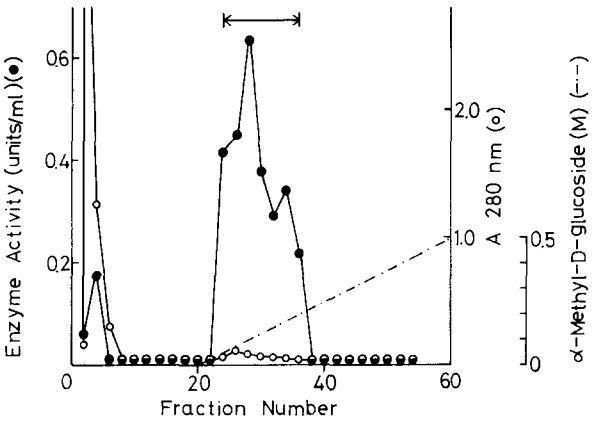


Fig. 2. Con A-Sepharose column chromatography (Step 4). Procedures used for chromatography are described in Materials and Methods. Fractions of 2.5 ml were collected at a flow rate of 10 ml/h.

the absorbance of effluent at 280 nm became nearly zero. The enzyme was eluted with a linear concentration gradient of 0—0.5 M α-methyl-D-glucoside in the same buffer (140 ml) (Fig. 2). The fractions containing enzyme activity were collected, concentrated, and dialyzed against 20 mM Tris · HCl buffer (pH 8.0).

Step 5. The dialyzate (7.7 ml) was applied on a column (1 × 12 cm) of the second DEAE-Sephadex A-50 (Cl^-) equilibrated with 20 mM Tris · HCl buffer (pH 8.0). The column was washed with 50 ml of the equilibration buffer followed by elution with a linear concentration gradient of KCl from 0.1 to 0.5 M in the above buffer (100 ml) (Fig. 3). The fractions containing enzyme activity were pooled, concentrated and dialyzed against 1.25 M ammonium sulfate in 20 mM Tris · HCl buffer (pH 8.0).

Step 6. The dialyzate (0.3 ml) was applied on a column (1 × 5 cm) of alanine-Sepharose equilibrated with 1.25 M ammonium sulfate in 20 mM Tris · HCl buffer (pH 8.0) followed by washing with several column volumes of the

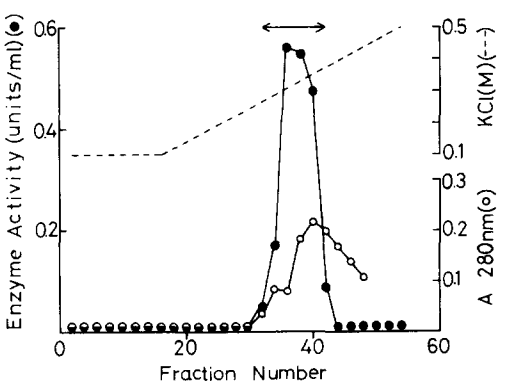


Fig. 3. The second DEAE-Sephadex column chromatography (Step 5). Procedures used for chromatography are described in Materials and Methods. Fractions of 2 ml were collected at a flow rate of 30 ml/h.

same buffer. Protein bound to the column was eluted by a linear gradient of ammonium sulfate decreasing from 1.25 M to 0 in the above buffer. The weight of buffer in reservoir and mixing chamber was 50 g. As shown in Fig. 4, the activity was found in the unabsorbed fraction but not in the absorbed fraction.

Disc gel electrophoresis

Samples in 5% (w/v) sucrose solution were directly applied on the separating gels (0.5 × 10 cm) containing 7.5% acrylamide, being run at 2 mA/gel for 2.5 h at 4°C in Tris-glycine buffer, pH 9.5, as described [17]. Otherwise, enzyme preparatations and reference proteins (8—10 μg) were incubated with sodium dodecyl sulfate (SDS) and 2-mercaptoethanol for 2 min at 100°C and electrophoresis was carried out at room temperature for 4 h at 8 mA/gel as described [18].

The protein bands were stained with 0.5% Coomassie Brilliant Blue G 250 [19] in 7% acetic acid. Staining for carbohydrate was carried out by the method of Kapitany and Zebrowski [20] using periodate-Schiff (PAS) reaction. NDPase activity was located by the procedure similar to that used for location of nucleoside triphosphatase [21,22] and 5′-nucleotidase [23] in polyacrylamide gel. A P_i released by the enzyme reaction reacts with $Pb(NO_3)_2$ forming $Pb_3(PO_4)_2$ which makes an opaque band. Incubation with $(NH_4)_2S$ results in formation of a dark brown band of PbS.

Electrofocusing

The enzyme was electrofocused in a column (110 ml) containing 1% LKB Ampholine carrier ampholite solution (pH 4—6) with a stepwise sucrose gradient at 500 V for 50 h according to the method of Vesterberg [24]. Contents of the column were collected in 2-ml fractions at a flow rate of 48 ml/h and dialyzed extensively against 20 mM Tris · HCl (pH 8.0) to remove carrier ampholite and sucrose. The enzyme active peak was concentrated by ultrafiltration.

Results

Amino acid-Sepharose column chromatography

When four amino acid-Sepharose columns were tested for NDPase purification, alanine-Sepharose was most effective. Though valine- and phenylalanine-Sepharose also seemed to be effective, contaminated proteins could not be completely washed off from the column. Accordingly, these columns were unsuitable for their re-use. On the other hand, the enzyme could not be eluted from leucine-Sepharose, even if the column was developed with ethylene glycol or tetraethylammonium chloride with a linear gradient concentration of 0—50% and 0—1 M, respectively, concomitant with the decreasing gradient of ammonium sulfate.

Purity of NDPase

As summarized in Table I, NDPase from rat liver cytosol was purified up to 336-fold (36.9 μmol/min per mg protein) with 2% yield by the present proce-

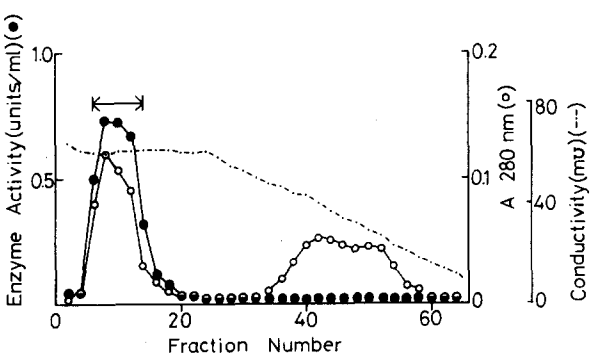
TABLE I

PURIFICATION OF NUCLEOSIDE DIPHOSPHATASE FROM RAT LIVER CYTOSOL

The purification procedures are described in Materials and Methods. Assays were made under the standard conditions using UDP as a substrate.

Step		Volume (ml)	Protein (mg)	Total activity (unit) *	Specific activity (unit/mg protein)	Yield (%)	Total purification
	Cytosol	275	8804	968	0.11	100	1
1	$AmSO_4$ (40—70%)	59	3304	892	0.27	92.1	2.5
2	$Ca_3(PO_4)_2$ gel	19.6	578.2	497	0.86	51.3	7.8
3	First DEAE-Sephadex	9.2	156.4	324	2.07	33.5	18.8
4	Con A-Sepharose	7.7	3.3	37	11.2	3.8	101.8
5	Second DEAE-Sephadex	0.3	1.2	17	14.2	1.8	129.1
6	Alanine-Sepharose	0.2	0.5	18.5	36.9	1.9	335.7

* One unit, 1 μmol P_i released per min.



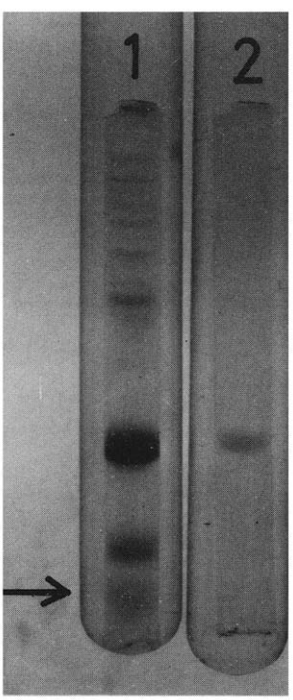


Fig. 4. Alanine-Sepharose column chromatography (Step 6). Procedures used for chromatography are described in Materials and Methods. Fractions of 2.2 ml were collected at a flow rate of 30 ml/h.

Fig. 5. SDS polyacrylamide gel electrophoresis. The Step 4 (1) and 6 (2) enzymes after reduction with 2-mercaptoethanol for 2 min at 100°C was applied to 7.5% polyacrylamide gels and electrophoresed at 8 mA/gel. Arrow indicates the position of marker dye.

dures. The enzyme could be stored at −70°C without appreciable loss of activity for at least several weeks.

When homogeneity of the enzyme was examined on SDS-polyacrylamide gel electrophoresis, the final enzyme preparation demonstrated a single protein band, while the preparation at Step 4 had 8—9 proteins (Fig. 5). The amount (10 μg) of the purified enzyme put on the gel 2 was less than that on gel 1 (70 μg). Although we could not apply a larger amount of the purified enzyme in this figure, because of using the same lot of enzyme preparation, the homogeneity on SDS gel electrophoresis has been ascertained at a concentration of pooled different preparations of 50 μg. On the other hand, the single band with the same electrophoretic mobility on polyacrylamide gels which were electrophoresed by the method of Ornstein and Davis [17] was demonstrated to be positive for protein, NDPase and PAS stainings in the final enzyme preparation.

On the basis of the results of positive staining for PAS reaction and of chromatographic behaviour on concanavalin A-Sepharose column (Fig. 2), NDPase of rat liver cytosol was evident to be a glycoprotein in nature.

Molecular weight of NDPase subunit

By plotting electrophoretic mobilities in the presence of SDS of the purified enzyme preparation together with standard proteins (human *r*-globulin 160000, bovine serum albumin dimer 134000, egg albumin dimer 90000, rabbit muscle pyruvate kinase 57000, bovine serum albumin 67000, egg albumin 45000, rabbit muscle lactic dehydrogenase 36000, and beef pancreas chymotrypsinogen A 25000 daltons), the molecular weight of NDPase was estimated to be 30000.

Resolution of NDPase into two components by electrofocusing and some properties

When the enzyme preparation at Step 4, which was completely free from phosphatase activities for ATP and nucleoside monophosphates, was subjected to electrofocusing over a pH range from 4 to 6, the enzyme activity was resolved into two peaks around in pH 4.7 (Peak I) and pH 5.0 (Peak II) (Fig. 6).

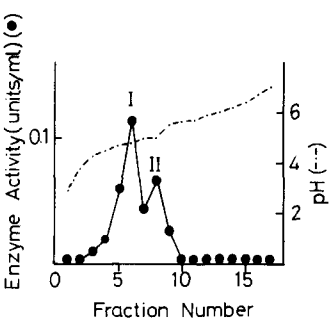


Fig. 6. Isoelectric focusing. The enzyme (212.5 μg of protein in 0.5 ml) in Step 4 was electroforesed over a pH range from 4 to 6, as described in Materials and Methods.

Substrate specificity. Enzyme proteins in Peaks I and II were tested for hydrolysis of various nucleoside diphosphates (3 mM) in the standard assay system. The enzymes in Peaks I (2 μg) and II (3.3 μg) showed almost identical substrate specificity, i.e., they catalyzed well hydrolysis of UDP, IDP, and GDP, but little of ADP and CDP.

The reaction product was only a 5′-UMP by paper chromatography with isobutyric acid —2 M ammonia (66 : 34, by vol.) as the solvent, when UDP was a substrate. ATP, UTP, 5′-AMP and 5′-UMP were not substrates when tested at 3 mM.

Molecular weight determination [25]. The enzyme proteins in Peaks I and II were applied to a Sephadex G-200 column (0.9 × 37.5 cm), equilibrated with 20 mM Tris · HCl buffer (pH 8.0) and eluted with the same buffer at a flow rate of 0.1 ml/min by a descending way in 1 ml fractions. Elution volume (16 ml) of the enzyme(s) was identical for Peaks I and II, and the molecular weight of the enzyme was estimated to be 120000 based on a calibration curve from elution volumes of standard proteins (catalase 240000, aldolase 158000, bovine serum albumin 67000, egg albumin 45000, and beef pancreas chymotrypsinogen A 25000 daltons).

The supernatant NDPase, therefore, appears to be a tetramer consisted of subunit with molecular weight of 30000 which was described above.

Effect of substrate concentration. In all the subsequent experiments, IDP was used as substrate instead of UDP. Double reciprocal plots of Peaks I and II enzyme(s) were taken with various concentrations of the substrate under standard assay condition. The K_m values were almost the same in Peaks I and II enzyme(s) ($8.3 \cdot 10^{-3}$ M and $10.0 \cdot 10^{-3}$ M, respectively).

Effect of divalent cations. Mg^{2+}, Mn^{2+} and Ca^{2+} at 4 mM increased the hydrolysis of IDP (3 mM) catalyzed by cytosol NDPase. No difference in the increment of the activity by these cations, however, was observed between Peak I (2 μg) and II (3.3 μg) enzyme(s).

Discussion

In this communication, NDPase from rat liver cytosol was purified by affinity chromatography using concanavalin A- and alanine-Sepharose and the final enzyme preparation was homogeneous upon SDS-polyacrylamide gel electrophoresis (Fig. 5). We tried also the affinity chromatography on UDP-hexanolamine-agarose [26] and UDP-adipic acid dihydrazide-agarose [27] for the purification, but without success. Previously, Parvin and Smith [9] partially purified (4-fold) rat liver supernatant NDPase by ammonium sulfate fractionation (40—70% saturation) followed by Sephadex G-200 column chromatography [9]. They demonstrated that the cytosol enzyme was activated by ATP at physiological concentration while the microsomal NDPase was inhibited by it. On the basis of these observations, as well as different kinetic properties, they concluded that the microsomal and supernatant enzymes differed from each other. On the other hand, it has been reported that mitochondrial NDPase of rat liver differs from that of microsomes in electrophoretic behaviour and contains three forms [8].

In the present studies, the supernatnant enzyme was separated into two

forms by isoelectric focusing (Fig. 6) though their enzymatic properties were essentially the same. However, up to the present, microsomal enzyme has never been separated into multiforms. Thus, the existence of multiforms in the supernatant enzyme might provide further evidence that the microsomal and supernatant enzymes are different, though we could not demonstrate an ATP activation of the supernatant enzyme shown by Parvin and Smith [9]. In the original studies, Ernster and Jones showed that rat liver microsomes contain most active NDPase [2]. These authors, and subsequently others, found that at least some of this enzyme activity could be easily released from microsomal vesicles by a variety of treatments including Ultra-Turrax [2], freeze-thawing [28], low concentration of various detergents [6], and high pH [5,6]. Therefore, a part of the supernatant NDPase obtained herein might be released from microsomal membranes by freezing and thawing of livers and by mechanical disruption at homogenization. However, it appears that the molecular weight of 120000 of the supernatant enzyme obtained from the present studies is different from that of microsomes, which was 100000 in bovine liver [5] and 155000 in pig liver [7]. The results, thus, suggest that supernatant enzyme obtained here was not merely ascribed for releasing microsomal enzyme. However, to demonstrate the difference in microsomal and supernatant enzyme, further kinetic, immunological or physical studies are necessary.

On substrate specificities, microsomal and mitochondrial enzymes reacted with GDP, UDP, and IDP [5,8]. The same substrate specificity was observed with the purified enzyme from rat liver supernatant.

A possible physiological implication of the metabolic regulation of NDPase was suggested [1,4]. The proposed role for NDPase is regulation of the gluconeogenic flux. It has been suggested that the NDPase might function in shifting the equilibrium in synthetic reactions where IDP, GDP, or UDP are products of the reaction. Such an effect was considered to be important for the reversal of glycolysis by displacing the equilibrium of phosphoenolpyruvate carboxykinase reaction in the direction of phosphoenolpyruvate formation. The present studies on NDPase of supernatant in which gluconeogenesis or glycolysis occurs predominantly [11] may have little further insight into the intriguing question of the metabolic role of this enzyme. A physiological function of the metabolic regulation of NDPase must wait further extensive investigations.

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