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ISOLATION AND CHARACTERIZATION OF A UDPase FROM CEREBELLUM

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SUMMARY

1. A UDPase (nucleoside diphosphate phosphohydrolase, EC 3.6.1.6) was purified 468-fold from rat cerebellum by ammonium sulfate precipitation and column chromatography.

2. The purified enzyme had an optimum pH of 7.7 and required a divalent cation for activity.

3. The order of relative activity for nucleoside diphosphates as substrates was 5′-UDP > 5′-dUDP > 5′-CDP > 5′-dCDP > 5′-GDP > 5′-IDP > 5′-ADP > 5′-dGDP > 5′-dADP. No hydrolysis of nucleoside monophosphate occurred.

4. The enzyme had a K_m of 0.1 mM and a v_{max} of 3.3 μmoles/h per mg protein with 5′-UDP as substrate at pH 7.6.

5. The UDPase was inhibited by $7.5 \cdot 10^{-3}$ M nucleosides, the order of greatest inhibition was adenosine > thymidine > uridine > inosine > guanosine. The enzyme was also inhibited by 10 mM NaF, 10 mM $FeCl_3$, and 10 mM $HgCl_2$.

6. Nucleoside triphosphates inhibited the enzyme. The concentration (mM) required to reduce the activity 50% was UTP (0.8), ATP (1.4), GTP (6.8) ITP (12.5), and CTP (42.0) with 3 mM 5′-UDP as substrate.

INTRODUCTION

Certain enzymes, because of their stability, binding properties, limited and specific subcellular localization and ease of assay are used as membrane marker enzymes for identification of membranes in subcellular fractionation studies. One such enzyme is UDPase which is thought to reside exclusively in the smooth endoplasmic reticulum or Golgi apparatus of the cell[1,2]. UDPase has been used as a smooth endoplasmic membrane marker in the isolation of brain mitochondria[3]; for this reason and because of the interest in this laboratory in membrane enzymes, rat cerebellum UDPase was purified and its substrate specificity determined. A previous publication[4] reports on a study of rat cerebellum 5′-nucleotidase, a plasma membrane marker enzyme[2].

UDPase (nucleoside diphosphate phosphohydrolase, EC 3.6.1.6) has been demonstrated to be a peak enzyme (as opposed to a continuous or step enzyme) in

synchronized L5178Y cells[5]. A rat liver nucleoside diphosphatase has been described[6] as has a GDPase from rat liver nucleoli[7]. The present communication describes the isolation of a UDPase from rat cerebellum.

MATERIALS AND METHODS

Materials

Substrates were purchased from Sigma Chemical Co. "Enzyme grade" ammonium sulfate was purchased from Mann Chemicals Co. Distilled water was first deionized and then distilled in a glass still; all solutions were made with this water. Uniformly ^{14}C-labeled 5'-UDP (specific activity 400 C/mole) was purchased from New England Nuclear.

Enzyme assay

The UDPase was assayed routinely in the following incubation mixture: 400 μl of Gomori's Tris buffer, 0.2 M Tris 0.2 M HCl (pH 7.6)–10 mM $MgCl_2$ 3 mM 5'-UDP and enzyme preparation in a total volume of 0.55 ml. The incubation mixture was incubated at 37° for 1 h in a Dubnoff metabolic shaker. The reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. The precipitated protein was removed by centrifugation at 2000 × *g* for 4 min, and an aliquot of the supernatant fluid was used for P_i determination by the method of FISKE AND SUBBAROW[9]. μmoles of UDPase activity were then based on the released P_i. In every instance substrate without enzyme and enzyme without substrate were incubated simultaneously and any P_i liberated subtracted from the assay in order to eliminate any P_i present in the enzyme or any P_i liberated by non-enzymatic hydrolysis of the substrate. All experiments were performed in duplicate.

Protein

Protein was determined according to the method of LOWRY *et al.*[10]. Crystalline bovine serum albumin was used as a standard.

Purification of UDPase from rat cerebellum

All steps of purification were carried out at 4° unless otherwise specified. The entire procedure was repeated 4 times. All 0.1% Triton X-100 was made in 0.1 M Tris -HCl buffer (pH 7.6). At several steps, solutions were concentrated by lyophilization.

Step 1: Extraction. 24 cerebella were removed from rats (200–250 g) killed by decapitation, which had been starved for 16 h with water *ad libitum*. They were minced in 5 vol. of 0.1% Triton X-100 and homogenized for 30 strokes in a Ten Broeck Homogenizer.

Step 2: Centrifugation. This homogenate was then centrifuged at 40 000 × *g* for 10 min.

Step 3: Ammonium sulphate precipitation. To the supernatant was added sufficient solid ammonium sulfate, with stirring to make the solution 20% in ammonium sulfate. This was stirred at 4° for 1.0 h and then allowed to stand for 1.0 h at 4°. The suspension was centrifuged at 20 000 × *g* for 20 min. Both the supernatant and

pellet were exhaustively dialyzed against 0.1% Triton X-100 at 4°. The enzyme was quite stable for 4 weeks at 4° at this step.

Step 4: Gel filtration on Sephadex G-100. A 5-ml sample (650 mg protein) from the 20% ammonium sulfate dialyzed precipitates in 0.1% Triton X-100 in 0.1 M Tris–HCl buffer (pH 7.6) was applied to a Sephadex G-100 column (2.5 cm × 45 cm) packed in the same solution. The column was eluted with the same buffer and fractions of 4 ml were collected.

Step 5: Gel filtration on Sephadex G-200. A 5-ml sample (200 mg protein) of the peak from the previous step was applied to a Sephadex G-200 column (45 cm × 2.5 cm) packed in 0.1% Triton X-100 in 0.1 M Tris–HCl buffer (pH 7.6). The column was developed with the same buffer and 3-ml fractions were collected.

Nature of product of rat cerebellum 5'-nucleotidase reaction

Assays were performed in the usual manner except 2 μC of 5'-[C^{14}]UDP were added to the incubation mixture. The supernatant after precipitation of the protein was chromatographed in 1-butanol–acetic acid–water (20:3:7, by vol.) and the nucleoside areas were cut from the dried chromatograms and eluted with glass distilled water. The eluates were lyophilized and subjected to electrophoresis in 0.05 M citrate-Tris (pH 4.8) and the distribution of radioactivity was determined by cutting the strips and counting in a liquid scintillation counter.

RESULTS AND DISCUSSION

Purification of rat cerebellum UDPase. The UDPase was purified 468-fold by the procedures outlined in Table I. After ammonium sulfate precipitation the enzyme fractionated into 1 peak on Sephadex G-100 (Fig. 1). The highest purification (37-fold over the previous step) was obtained with Sephadex G-200 column chromatography (Fig. 2); again the enzyme fractionated into only one peak of activity. This purified enzyme was used for all subsequent assays; the enzyme fractions from several Sephadex G-200 runs were pooled to obtain sufficient enzyme. The procedures resulted

TABLE I

PURIFICATION AND ISOLATION OF UDPASE ACTIVITY BY AMMONIUM SULFATE PRECIPITATION AND COLUMN CHROMATOGRAPHY

Procedures were performed as given in MATERIALS AND METHODS. All procedures were carried out at 0 4°. The experiments were repeated 4 times and were quantitatively and qualitatively reproducible.

	Total protein (mg)	*Total activity (μmoles/h)*	*Recovery (%)*	*Specific activity (μmoles/h per mg protein)*	*Purification factor*
1. 0.1% Triton X-100 extract	6000	10 500	100	1.8	1.0
2. 40 000 × *g* supernatant	4100	8 400	80	2.1	1.2
3. 20% $(NH_4)_2SO_4$ precipitate dialyzed	650	5 250	50	8.1	4.5
4. Sephadex G-100	200	4 520	43	22.6	12.5
5. Sephadex G-200	5	4 200	40	840	468

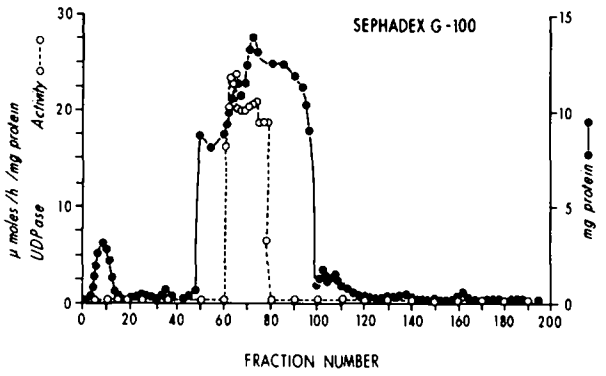


Fig. 1. Chromatography of the rat cerebellum UDPase activity on Sephadex G-100. Enzyme (5250 μmoles/h, 650 mg protein) in 5 ml of 0.1% Triton X-100 in 0.1 M Tris–HCl buffer (pH 7.6) was applied to the column (45 cm × 2.5 cm). Elution was with 0.1% Triton X-100 in 0.1 M Tris–HCl buffer (pH 7.6); 4-ml fractions were collected. All procedures were carried out in a jacketed column at 4°.

in a 40% recovery of the UDPase activity present in the initial cerebellum homogenate (Table I). The Sephadex G-200 purification was more efficient if proceeded by the Sephadex G-100 step although the latter did not give much purification. The final product was stable for 2 months at −20°.

Identity of enzyme product. Identification of the radioactive product from the reaction with 5′-[^{14}C]UDP (see MATERIALS AND METHODS) indicated 5′-[^{14}C]UMP and 5′-[^{14}C]UDP were the only radioactive products after a routine incubation; no free uridine was present. Thus the enzyme catalyzes the hydrolysis of the initial phosphate of 5′-UDP but doesn't hydrolyze the second phosphate; 5′-UMP is not a substrate.

Cation requirement. The UDPase demonstrated a direct requirement for divalent cations (Table II). Mg^{2+}, Pb^{2+}, Ca^{2+} and Cd^{2+} each effectively activated the UDPase. Co^{2+} and Mn^{2+} also activated the rat cerebellum UDPase to a lesser degree. When added in the presence of the 10 mM $MgCl_2$, Fe^{3+} and Hg^{2+} at 10 mM and 1 mM

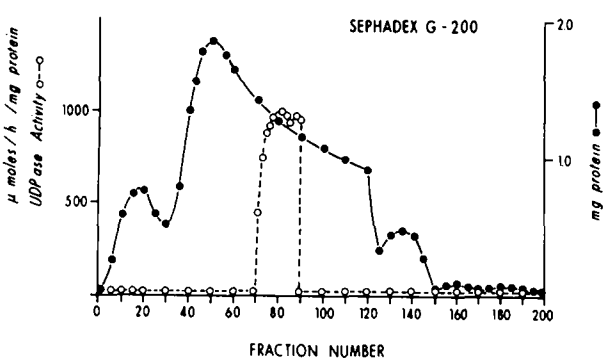


Fig. 2. Chromatography of the rat cerebellum UDPase activity on Sephadex G-200. Enzyme (4520 μmoles/h, 200 mg protein) in 5 ml of 0.1% Triton X-100 in 0.1 M Tris–HCl buffer (pH 7.6) was applied to the column (45 cm × 2.5 cm). Elution was with 0.1% Triton X-100 in 0.1 M Tris–HCl buffer (pH 7.6); 3-ml fractions were collected. All procedures were performed in a jacketed column at 4°.

TABLE II

EFFECT OF CATIONS ON ACTIVITY OF RAT CEREBELLUM UDPASE

Experiments were performed with 5′-UDP as substrate as described in the text. "Without $MgCl_2$" refers to experiments in which the indicated ion was substituted for the $MgCl_2$ in the assay. "With $MgCl_2$" refers to experiments in which the indicated ion was added in the presence of the 10 mM $MgCl_2$ normally in the assay. The activity of the 10 mM $MgCl_2$ addition to the "without $MgCl_2$" system was arbitrarily set to 100 and other values are activities relative to this activity. All solutions were made in 0.1 M Tris HCl buffer (pH 7.6). Any balancing of assay volumes necessary was made with 0.1 M Tris-HCl buffer (pH 7.6).

Addition	*Concn. (mM)*	*Assays performed in normal manner*	
		Without $MgCl_2$	*With $MgCl_2$*
$MgCl_2$	1	30	100
	10	100	98
$CoCl_2$	1	11	88
	10	38	42
$CuCl_2$	1	4	89
	10	29	78
$FeCl_3$	1	0	10
	10	0	8
$HgCl_2$	1	0	1
	10	12	26
$PbCl_2$	1	28	100
	10	100	100
$MnCl_2$	1	16	96
	10	58	97
$CaCl_2$	1	29	102
	10	100	100
$CdCl_2$	1	26	98
	10	96	102
EDTA	10	4	10

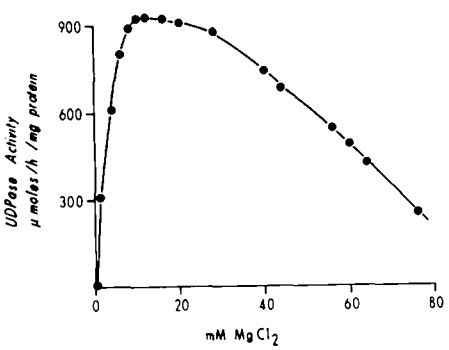


Fig. 3. Influence of $MgCl_2$ concentration on the activity of UDPase from rat cerebellum. Enzyme (1.2 μg), substrate, and buffer were incubated under standard conditions and the inorganic phosphate released was determined as described in the text.

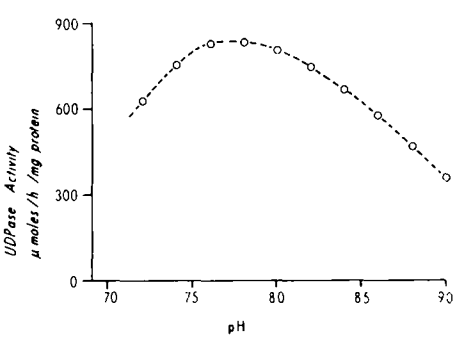
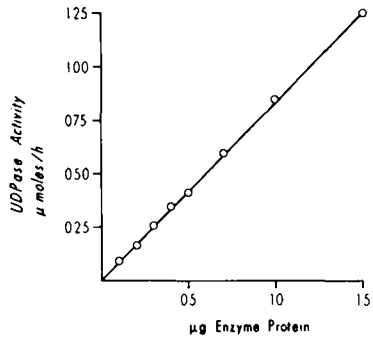


Fig. 4. Influence of pH on the activity of the rat cerebellum UDPase. Enzyme (1 μg), substrate cofactor and buffer were incubated under standard conditions and the inorganic phosphorus released was determined as given in the text. The buffer was in all instances GOMORI's[8] Tris buffer, 0.2 M Tris–0.2 M · HCl.



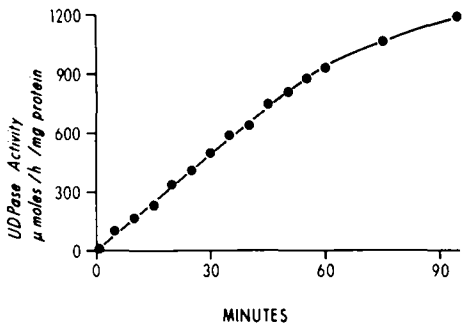


Fig. 5. Effect of enzyme concentration on the activity of the rat cerebellum UDPase. Incubation conditions with 5′-UDP as the substrate were as described in the text.

Fig. 6. Time course of the inorganic phosphate liberation from 5′-UDP. The enzyme (1.2 μg) and the substrate were incubated for various time intervals and the inorganic phosphate released was determined as given in the text.

inhibited the UDPase activity. The UDPase of rat cerebellum was similar to rat liver nucleolar GDPase in that Mg^{2+}, Ca^{2+}, Co^{2+} and Mn^{2+} activated the enzyme[7]. Also the rat liver nucleoside diphosphatase reported by SCHRAMM AND MORRISON[6] was activated by Mg^{2+}, Mn^{2+} and Ca^{2+}. The rat cerebellum UDPase showed a marked dependence on $MgCl_2$ concentration (Fig. 3).

pH dependence. The UDPase of rat cerebellum had an optimum pH of 7.7 (Fig. 4).

Enzyme concentration. The data in Fig. 5 demonstrate that the reaction of UDPase from rat cerebellum on 5′-UDP was linear up to 1.5 μg of enzyme protein.

Time course. The hydrolysis of UDP and release of P_i by the rat cerebellum UDPase was linear up to 60 min incubation (Fig. 6).

Substrate concentration. A plot of $1/v$ vs. $1/[S]$ with 5′-UDP as a substrate for the rat cerebellum UDPase was linear (Fig. 7). Calculation of a K_m from this plot gave a value of 0.1 mM and a v_{max} of 3.3 μmoles/h per mg protein.

Substrate specificity. The UDPase hydrolyzed nucleoside diphosphates; the relative activity was 5′-UDP > 5′-dUDP > 5′-CDP > 5′-dCDP > 5′-GDP > 5′-

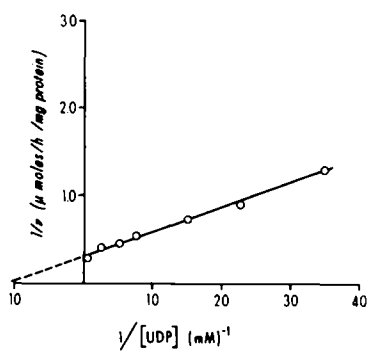


Fig. 7. Lineweaver–Burk plot of UDPase from rat cerebellum. Assay was carried out under the conditions described in the text.

TABLE III

SUBSTRATE SPECIFICITY OF RAT CEREBELLUM UDPase

All experiments were performed as given in the text. For relative activity the substrates were all tested at 3 mM. Michaelis constants were determined from plots of $1/v$ *vs.* $1/[S]$ as given in Fig. 7.

Substrate	*Relative activity*	K_m *(mM)*	*Substrate*	*Relative activity*	K_m *(mM)*
5′-UDP	100	0.1	5′-dGDP	20	8.7
5′-ADP	21	8.9	5′-dATP	0	-
5′-CDP	68	1.2	5′-dUTP	10	10.8
5′-IDP	39	7.9	5′-dCTP	0	-
5′-GDP	48	6.7	5′-dGTP	0	-
5′-AMP	0		2′-AMP	0	-
5′-UMP	0		3′-AMP	0	-
5′-CMP	0				
5′-IMP	0		2′-CMP	0	
5′-GMP	0		3′-CMP	0	
5′-ATP	0	-	2′-GMP	0	-
5′-UTP	11	12.1	3′-GMP	0	-
5′-CTP	0	-			-
5′-ITP	22	10.4	2′-UMP	0	-
5′-GTP	20	8.9	3′-UMP	0	-
5′-dAMP	0				
5′-dUMP	0				
5′-dIMP	0	-			
5′-dCMP	0				
5′-dGMP	0	-			
5′-dADP	10	14.2			
5′-dUDP	69	0.9			
5′-dCDP	49	5.2			

IDP > 5′-ADP > 5′-dGDP > 5′-dADP. In general the deoxy-nucleoside diphosphates were less active than the nucleoside diphosphates. The double reciprocal plots were linear. There was no activity with nucleoside monophosphates as substrates (Table III). Some of the nucleoside triphosphates acted as substrates; the order of activity was 5′-ITP > 5′-GTP > 5′-UTP > 5′-dUTP. The nucleoside diphosphatase from rat liver[6] had the following relative activities: UDP ≃ GDP ≃ IDP ≫ CDP ≫ TDP.

Inhibitors of rat cerebellum UDPase. Bovine serum albumin at 7.5 mg/ml and glutathione, benzoyl chloride, eserine, *N*-ethylmaleimide and L-cysteine at $1 \cdot 10^{-3}$ M were not inhibitory to rat cerebellum UDPase activity. *p*-Chloromercuribenzoate and *p*-hydroxymercuribenzoate at $1 \cdot 10^{-3}$ M were slightly inhibitory to the UDPase activity (Table IV). At $7.5 \cdot 10^{-3}$ M, nucleosides were inhibitory to the rat cerebellum UDPase. The order of greatest inhibition was adenosine > thymidine > uridine > inosine > guanosine (Table IV). At $1 \cdot 10^{-2}$ M, NaF inhibited the rat cerebellum UDPase 90%.

Triphosphates were also inhibitory to the rat cerebellum UDPase activity (Table IV). The order of inhibition was UTP > ATP > GTP > ITP > CTP with 5′-UDP as a substrate. At 800 μM UTP, the rat cerebellum UDPase was inhibited

TABLE IV

EFFECT OF POTENTIAL INHIBITORS ON ACTIVITY OF RAT CEREBELLUM UDPASE

Substrate concentration was 3 mM 5'-UDP. All experiments were performed as given in the text. The activity was measured with 5'-UDP as substrate. The activity with no addition was arbitrarily set equal to 100%.

Inhibitor	*Concn. (M)*	*% Control activity*
None		100
Uridine	$7.5 \cdot 10^{-3}$	71
Thymidine	$7.5 \cdot 10^{-3}$	38
Guanosine	$7.5 \cdot 10^{-3}$	98
Adenosine	$7.5 \cdot 10^{-3}$	31
Inosine	$7.5 \cdot 10^{-3}$	94
Bovine serum albumin	7.5 mg/ml	92
Glutathine	$1 \cdot 10^{-3}$	96
Benzoyl chloride	$1 \cdot 10^{-3}$	91
Eserine	$1 \cdot 10^{-3}$	103
N-Ethylmaleimide	$1 \cdot 10^{-3}$	106
L-Cysteine	$1 \cdot 10^{-3}$	103
p-Chloromercuribenzoate	$1 \cdot 10^{-3}$	83
NaF	$1 \cdot 10^{-2}$	10
p-Hydroxymercuribenzoate	$1 \cdot 10^{-3}$	81
UTP	$8.0 \cdot 10^{-4}$	50
ATP	$1.4 \cdot 10^{-3}$	50
GTP	$6.8 \cdot 10^{-3}$	50
ITP	$1.3 \cdot 10^{-2}$	50
CTP	$4.2 \cdot 10^{-2}$	50

50%. Nucleoside monophosphates were not tested as inhibitors. Inhibition by triphosphates and nucleosides showed 2nd order kinetics.

The present communication describes a UDPase from rat cerebellum. The enzyme is quite specific with respect to substrate and is inhibited by nucleosides and nucleoside triphosphates. The substrate specificity and ease of assay confirm that it is a suitable enzyme for enzyme marker studies.

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