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PURIFICATION AND PROPERTIES OF UDP-GALACTOSE:CERAMIDE GALACTOSYLTRANSFERASE FROM RAT BRAIN MICROSOMES

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SUMMARY

UDPgalactose:ceramide galactosyltransferase (EC 2.4.1.62) from rat brain microsomes was solubilized by treatment with a non-ionic detergent, Cemulsol NP-12. A partial purification of the enzyme is described. There was no apparent change in the enzyme solubility after removing the detergent by solvent extraction. The general properties of the solubilized enzyme were essentially the same as in the particulate preparation.

INTRODUCTION

The synthesis of galactolipids by the transfer of the galactose unit from UDPgalactose to a lipid acceptor has been proposed several years ago [1, 2]. UDP-galactose:ceramide galactosyltransferase (EC 2.4.1.62) has been shown by in vitro studies to catalyze the formation of cerebroside in brain [3–8] and kidney [9]. The enzyme seems to have a high specificity for α-hydroxy fatty acids-ceramide [4], although a small amount of galactosyltransferase activity was reported with non-hydroxy fatty acid containing ceramide [10]. A detailed study of the kinetic and developmental properties of UDPgalactose:ceramide galactosyltransferase in embryonic chicken brain was reported by Basu et al. [11]. In a recent work [12] we have shown that the enzyme is mainly localized in the microsomal fraction of rat brain. UDP-galactose:ceramide galactosyltransferase is a membrane-bound enzyme and in the previous works only the properties of different particulate preparations were studied. Our preliminary results have shown that the galactosyltransferase could be partially recovered in the supernatant fluid after the high-speed centrifugation of brain microsomes treated with non-ionic detergents [13]. However, the presence of a relatively high concentration of detergent needed for the solubilization considerably inactivated the enzyme in the later steps of purification. Further work showed that by using more appropriate conditions for detergent extraction and by removing the detergent in the subsequent steps, a highly active and more stable solubilized enzyme preparation can be obtained.

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MATERIALS AND METHODS

Lecithin (phosphatidylcholine) was prepared from pig brain [14] and its purity was tested by thin-layer chromatography. Cemulsol NP-12 (nonyl phenol polyoxyethylene) was purchased from Rhone-Progil (Neuilly-sur-Seine, France). Sepharose 4B was from Pharmacia (Uppsala, Sweden). The origin of labelled sugar nucleotides and ceramides was as given before [15]. The phospholipid content of different enzyme preparations was determined in chloroform–methanol extracts [16] by the method of Dodge and Phillips [17]. The content of Cemulsol in the chloroform–methanol extracts was estimated by thin-layer chromatography after detection with the modified Dragendorff reagent [18].

The incubation mixture for the UDPgalactose:ceramide galactosyltransferase assay contained (in 0.25 ml final volume): α-hydroxy fatty acid-ceramide, 0.1 μmole; lecithin, 0.077 μmole; Triton X-100, 0.5 mg; UDP[^{14}C]galactose, 0.01 μmole (64 000 cpm); $MgCl_2$, 1 μmole; Tris–HCl buffer (pH 8.0), 10 μmoles; enzyme preparation, 20–100 μg of protein. Detergent, ceramide and lecithin were transferred to the incubation tubes as solutions in chloroform–methanol (2:1, v/v) and dried in vacuo before the addition of other components. Incubation was for 15 min at 27 °C with shaking. Incubations were stopped by adding chloroform–methanol (2:1, v/v) and the radioactivity determined in the washed lipid extracts as described previously [15]. One unit of enzyme activity is defined as 1 nmole of galactose incorporated per h. Protein was determined by the method of Lowry et al. [19] with bovine serum albumin as standard.

For the galactosyltransferase purification, all procedures were performed at 4 °C unless otherwise stated. Enzyme preparations were stored in an ice-bath at 0 °C. All buffers contained 0.1 % (v/v) 2-mercaptoethanol and 1 mM EDTA. Brains of 20-day-old Wistar albino rats were homogenized with 9 vol. of 0.32 M sucrose. The homogenate was centrifuged at 2000 × *g* for 10 min. The pellet was resuspended in 0.32 M sucrose and centrifuged as above. The combined supernatants were centrifuged at 11 500 × *g* for 20 min. The supernatant from this step was centrifuged at 100 000 × *g* for 1 h. The microsomal pellet obtained was suspended by homogenization in 0.1 M potassium phosphate buffer (pH 7.6) (1.5 ml/g of brain). The protein concentration of this suspension was approx. 10 mg/ml. A 2.5% (v/v) solution of Cemulsol in 0.1 M potassium phosphate buffer (pH 7.6), was slowly added to the microsomal suspension until the final concentration of detergent was 0.5% (v/v). The suspension was stirred for 30 min and centrifuged at 100 000 × *g* for 1 h. The clear supernatant was withdrawn and dialyzed against water overnight (Fraction I). This preparation was freeze-dried and extracted twice by adding 100 vol. of dry acetone precooled at −20°C, and centrifuging for 10 min at M10 °C. The residue was washed twice with cold benzene and freeze-dried to remove benzene. The powder obtained was homogenized with 70 vol. of 0.1 M potassium phosphate buffer (pH 7.6). The suspension was sonicated in 10-ml portions for 2 min in the MSE sonicator (MSE, London, England) using a titanium probe (diameter 9.5 mm) at 75% of the maximal scale. After centrifuging at 100 000 × *g* for 1 h, 85–90% of total protein was recovered in the clear supernatant* (Fraction II). Fraction II was brought to

* Although similar results could be obtained without sonication, the recoveries of the solubilized protein were less consistent, ranging from 64–90%.

33% satn with solid $(NH_4)_2SO_4$, the pH being kept at 7.3–7.6. The precipitate was recovered by centrifugation at 26 000 × *g* for 10 min, dissolved in a minimum volume of 0.1 M potassium phosphate buffer (pH 7.6), and dialyzed overnight against 80 vol. of 0.05 M Tris–HCl buffer (pH 7.6) (Fraction III). One ml of Fraction III containing 5–6 mg of protein was applied to a column (1.6 cm × 31 cm) of Sepharose 4B, previously equilibrated with 0.05 M Tris–HCl buffer (pH 7.6). Elution was carried out with the same buffer using an ascending system, and 1.2-ml fractions were recovered. UDPgalactose:ceramide galactosyltransferase activity was recovered between Fractions 19 and 31. Fractions having the highest specific activity were pooled (Fraction IV).

RESULTS AND DISCUSSION

Compared to brain homogenate, the specific activity of Fraction IV represents an 18-fold purification (Table I). Although steps corresponding to Fractions II and III apparently contributed little to the final purification, they were important for obtaining an active and stable solubilized preparation. Solvent treatment was effective in removing the largest amount of detergent. Very small amounts of detergent were detected by thin-layer chromatography in chloroform–methanol extracts of Fractions II and III. The amount of the residual Cemulsol in Fraction III was estimated to be less than 0.02 mg/mg of protein. The phospholipid content of Fraction III was 0.1 mg/mg of protein. Compared to the phospholipid content of the microsomal

TABLE I

PURIFICATION OF UDP-GALACTOSE:CERAMIDE GALACTOSYLTRANSFERASE FROM RAT BRAIN

Preparation of different fractions and the enzyme assay were as described in Materials and Methods.

Fraction and step	Spec. act. (units/mg protein)	Yield (%)
Homogenate	0.356	100
Microsomes	0.924	36.4
I. Cemulsol extraction	0.936	26.2
II. Solvent extraction	1.01	19 8
III. $(NH_4)_2SO_4$ precipitation	1.32	22.5
IV. Sepharose 4B column	6.52	13.8

fraction (0.53 mg/mg of protein) it suggests that the lipid environment of the solubilized ceramide galactosyltransferase was markedly different from that of the enzyme in the intact membrane. It is likely that the phospholipids which are not removed are essential for the enzyme activity, as is the case in many membrane enzymes [20].

Several properties of the enzymic reaction were studied using the particulate enzyme (microsomes) and the solubilized enzyme (Fraction III). In general, the results of kinetic studies showed little difference between the particulate and solubilized form. A linear relationship between the amount of the enzyme and galactose incorporated was observed up to 90 μg of protein using the particulate form; with the solubilized enzyme the linearity was respected up to 100 μg of protein. The reaction rate was

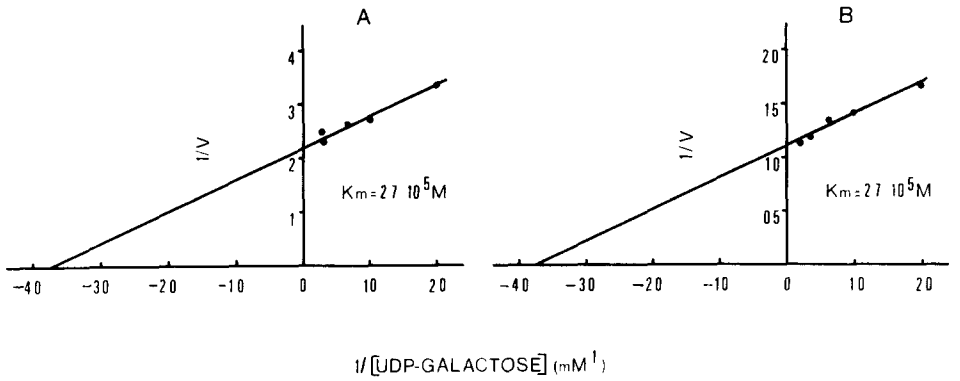

Fig. 1 Effect of UDPgalactose concentration on UDPgalactose:ceramide galactosyltransferase activity. Incubation conditions were as described in Materials and Methods except that the UDP-galactose concentration was varied as indicated. Lineweaver–Burk plots are given. $1/v$ is reciprocal value of nmole of galactose incorporated per h per mg of protein. A, microsomes; B, solubilized enzyme (Fraction III).

constant with time of incubation for nearly 20 min. A pH optimum of 8.0 in Tris–HCl buffer was found for both particulate and solubilized UDPgalactose:ceramide galactosyltransferase. In both cases the optimal temperature of incubation was 27 °C. The K_m for UDPgalactose was determined from Lineweaver–Burk plots to be $2.7 \cdot 10^{-5}$ M with both particulate and solubilized enzyme (Fig. 1). This value is in good accordance with the value reported for UDPgalactose:ceramide galactosyltransferase in embryonic chicken brain [11]. The requirements for the galactosyltransferase are shown in Table II. In the absence of Triton X-100 the activity with exogenous acceptor

TABLE II

REQUIREMENTS FOR THE PARTICULATE AND SOLUBILIZED UDP-GALACTOSE: CERAMIDE GALACTOSYLTRANSFERASE

Composition of the complete incubation mixture and other conditions of the enzyme assay were as described in Materials and Methods. Enzyme added: microsomes, 90 μg of protein; solubilized enzyme (Fraction III) 100 μg of protein.

Incubation mixture	Spec. act. (units/mg protein)	
	Microsomes	Solubilized enzyme
Complete	1.030	1.420
Minus Triton X-100	0.126	0.336
Minus Triton X-100, minus α-hydroxy fatty acids-ceramide	0.096	0.304
Minus α-hydroxy fatty acids-ceramide	0.074	0.128
Minus α-hydroxy fatty acids-ceramide, plus ceramide containing non hydroxy fatty acids	0.089	0.144
Minus lecithin	0.141	0.128
Minus $MgCl_2$	0.703	0.736
Minus $MgCl_2$, plus $MnCl_2$	0.742	1.540
Minus UDPgalactose, plus UDPglucose	2.800	0 705
Minus active, plus heat inactivated enzyme (5 min at 100 °C)	0.015	0 032

was greatly reduced. The effect of Triton X-100 was less pronounced with solubilized enzyme. The activity with endogenous acceptor (i.e. when no ceramide is added) was relatively low using both particulate and solubilized enzyme in the presence of Triton X-100. In both cases the activity with ceramide containing non-hydroxy fatty acids as galactose acceptor was only slightly higher than observed without exogenous acceptor. Using the particulate enzyme the omission of Mg^{2+} produced a decrease of activity of about 32%; in the case of the solubilized enzyme the decrease was 48%. The effect of Mn^{2+} probably reflects a more profound difference between the two enzyme preparations. In the case of the particulate enzyme, Mg^{2+} apparently cannot be replaced by Mn^{2+}; the solubilized enzyme is stimulated by either Mg^{2+} or Mn^{2+}. Such variation of the activating ion effect may result from the purification of the enzyme, as it was observed in several cases [11]. The experiment in which UDPgalactose is substituted by UDPglucose indicates that much of the original glucosyltransferase activity was lost during the purification of UDPgalactose : ceramide galactosyltransferase. The addition of lecithin (pure phosphatidylcholine) greatly stimulated the reaction rate. This effect was almost a linear function of the lecithin concentration up to 60 μg added to the incubation mixture (Fig. 2). The saturation was attained at a higher concentration in the case of the solubilized galactosyltransferase. At a high

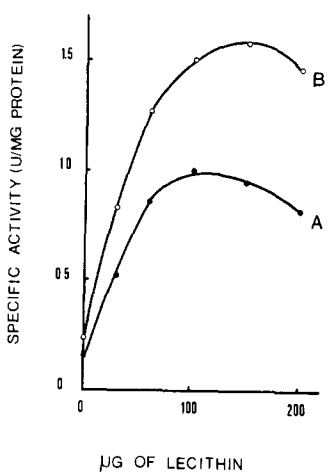

Fig. 2. Effect of lecithin on UDPgalactose:ceramide galactosyltransferase activity. Incubation conditions were as described in Materials and Methods except that lecithin concentration was varied as indicated. A, microsomes; B, solubilized enzyme (Fraction III).

concentration lecithin inhibited the reaction in both cases. The addition of lecithin was effective only in the presence of Triton X-100. Since lecithin had a similar effect with particulate and solubilized enzyme it seems unlikely that this action is related to the specific requirement for phospholipid as discussed above. Most probably the incorporation of lecithin into the incubation system improves the presentation of the exogenous lipid acceptor by the formation of the mixed lecithin–ceramide–Triton X-100 micelles [22]. The stimulating effect of lecithin on the galactosylation of ceramide containing non-hydroxy fatty acids by a microsomal preparation was reported

previously [10]. In this case, ceramide and lecithin were adsorbed on a solid support (celite) and no detergent was used.

So far we have only been able to obtain a moderate purification of UDP-galactose: ceramide galactosyltransferase. The further purification is likely to be subject to the difficulties encountered with membrane enzymes isolated by detergent treatment, which depend upon whether the enzyme is isolated in a true water-soluble form or as a multimolecular complex held in the solution by the presence of detergent [23]. In the present study the removal of the largest part of the detergent apparently did not change the solubility of the galactosyltransferase. However, a very small amount of tightly-bound detergent, which can be removed by chloroform–methanol extraction, was detected in the solubilized enzyme preparations and the possibility that this residual detergent is necessary for keeping the enzyme in solution cannot be ruled out. In addition, several properties of the purified enzyme, such as an appreciable phospholipid content, precipitation at low $(NH_4)_2SO_4$ concentration and the gel filtration profile, indicate that the enzyme is isolated in the form of a macromolecular lipoprotein complex.

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