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ARTICLE *in* FEBS LETTERS · NOVEMBER 1986

Impact Factor: 3.17 · DOI: 10.1016/0014-5793(86)81057-2

Solubilization and partial purification of UDP-galactose:diacylglycerol galactosyltransferase activity from spinach chloroplast envelope

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Received 15 September 1986

We have developed procedures to solubilize the envelope UDP-galactose:diacylglycerol galactosyltransferase activity and to assay this enzyme after solubilization with a zwitterionic non-denaturing detergent (CHAPS) and fractionation. From solubilized envelope membranes isolated from intact spinach chloroplasts, we were able to prepare by chromatography on hydroxyapatite a fraction enriched (6–7-fold) in this enzyme responsible for monogalactosyldiacylglycerol synthesis.

(Spinacia oleracea) *Chloroplast membrane* *Galactosyltransferase* *CHAPS* *Hydroxyapatite*

1. INTRODUCTION

Monogalactosyldiacylglycerol (MGDG) is the major glycerolipid in plastid membranes. Its synthesis involves the transfer of a galactose from a galactose donor, UDP-galactose (UDP-gal), to an acceptor molecule, diacylglycerol (review [1]). In leaves, the galactosyltransferase involved, or UDP-gal:diacylglycerol galactosyltransferase (EC 2.4.1.46), is specifically localized in the chloroplast envelope membranes [2]. The properties of this major enzyme of plastid biogenesis are not yet completely understood [1,3,4]. A more detailed understanding of the functioning, localisation and biogenesis of this galactosyltransferase requires its purification, which is hampered by several limitations: (i) envelope membranes have a very high lipid to protein ratio (1.2–1.5 mg lipid/mg protein) which requires detergent concentrations that are harmful for enzyme activities; (ii) one of the substrates for the enzyme, diacylglycerol, and the product of its activity, MGDG, are hydrophobic thus making an in vitro assay difficult to develop; (iii) although very active, the enzyme is probably not a major envelope protein.

We report here results on the solubilization, development of a specific assay and partial purification of the UDP-gal:diacylglycerol galactosyltransferase from the spinach chloroplast envelope.

2. MATERIALS AND METHODS

2.1. *Chloroplast envelope purification*

Total envelope membranes were prepared from purified intact spinach chloroplasts according to [5] and stored in liquid nitrogen (10 mg protein/ml) with 50 mM Mops, pH 7.8, and 1 mM dithiothreitol (DTT). Under these storage conditions, no loss of activity was observed. From 2 kg spinach leaves, the yield of envelope proteins was 8–10 mg.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

2.2. *Solubilization of envelope membranes*

CHAPS (3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), was used to solubilize envelope membranes. Envelope membranes were incubated for 30 min at 0°C under gentle agitation in either medium A (50 mM Mops, pH 7.8; 1 mM DTT, 6 mM CHAPS) or, when indicated, medium B (i.e. medium A containing 50 mM KH_2PO_4); the final volume was 15 ml for 10 mg protein. After incubation, the mixture was centrifuged for 15 min at 45 000 rpm (Beckman L2 65B, rotor SW 50) and the supernatant (0.5–0.6 mg protein/ml) containing all the galactosyltransferase activity was recovered. For experiments with low amounts of envelope membranes (60–100 μg protein) centrifugation was done in 160 μl tubes with an Airfuge centrifuge (Beckman) for 10 min at maximum velocity.

2.3. *Analyses of envelope polypeptides and protein determination*

Envelope polypeptides were analysed by polyacrylamide gel electrophoresis (PAGE) in the presence of lithium dodecyl sulfate (LDS) at 4°C as described [6]. Protein determination was done according to [7], with bovine serum albumin as a standard.

2.4. *Hydroxyapatite chromatography*

We used HA-Ultrogel (IBF-France) for chromatography in a 10×0.8 cm column (Pharmacia) at 4°C. The column was first washed with 2–3 vols medium A containing 500 mM KH_2PO_4 and equilibrated with 3–5 vols medium B. Envelope membranes solubilized in medium B (0.5–0.6 mg protein/ml) were loaded on the column and eluted as described below at a flow rate of 30 ml/h.

2.5 *Assay of UDP-gal:diacylglycerol galactosyltransferase*

The assay was based on the extent of conversion of galactose from UDP-[^{14}C]gal (water-soluble) to chloroform-soluble galactolipids. When the activity was measured in total envelope membranes, the minimum incubation medium (final volume, 300 μl) contained 50 mM Mops (pH 7.8), 1 mM DTT, 600 μM UDP-[^{14}C]gal (New England Nuclear, 6.25 GBq/mmol) and envelope membranes (100 μg protein). When activity was measured with solubilized envelope or chromatography fractions, the incubation medium described above (final volume, 300 μl) was supplemented with 6 mM CHAPS, 250 mM KH_2PO_4, 250 mM KCl and envelope lipids (150 μg) containing diacylglycerol. Envelope lipids used were extracted and quantified according to [8]. After 15 min incubation, the reaction was stopped, the lipids extracted and labelled galactolipids separated according to [8].

3. RESULTS AND DISCUSSION

3.1. *Solubilization and assay of UDP-gal:diacylglycerol galactosyltransferase*

The use of detergents at concentrations higher than their critical micellar concentration (CMC) is usually necessary for proper solubilization of membrane proteins; unfortunately, this usually leads to a dramatic reduction of galactosyltransferase activity. However, some non-ionic (Triton X-100, octylglucoside) or zwitterionic (SB 12, CHAPS) non-denaturing detergents can maintain some galactosyltransferase activity at or just above their CMC (Covès et al., in preparation). For in-

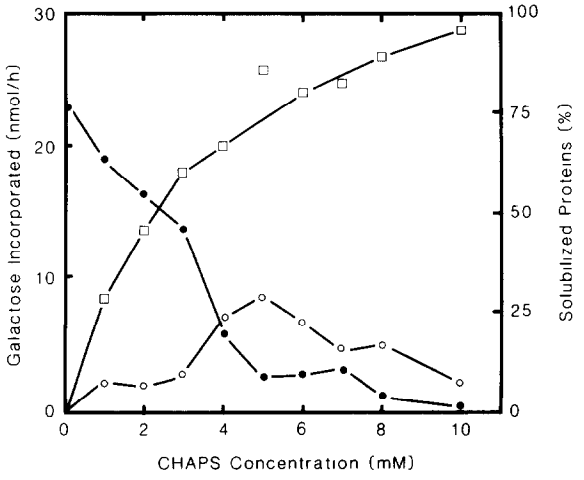


Fig.1. Effect of CHAPS concentration on the solubilization of envelope membranes proteins and of UDP-gal:diacylglycerol galactosyltransferase activity. Envelope membranes (65 μg protein) were solubilized in 160 μl medium A with increasing CHAPS concentrations (from 1 to 10 mM). The membrane pellet and supernatant were separated by centrifugation with an Airfuge centrifuge (Beckman) as described in section 2.2. Galactosyltransferase activity was measured in the pellet (●) and supernatant (○) as described in section 2.5. Proteins (□) were determined according to [7].

stance, MGDG synthesis by isolated envelope membranes was possible in the presence of 0.9% Triton X-100 [9]. Fig.1 shows that with increasing CHAPS concentrations in the solubilization mixture, increasing amounts of proteins were recovered in the supernatant after centrifugation. LDS-PAGE (not shown) of both the membrane pellet and supernatant demonstrates selective solubilization of envelope membrane proteins: at 1–2 mM CHAPS, almost only ribulose-1,5-bisphosphate carboxylase was recovered in the supernatant; above 3–5 mM CHAPS, E37 and numerous minor envelope polypeptides were extracted; above 5 mM CHAPS, E30 was progressively extracted. Finally, almost all envelope proteins were solubilized at 10 mM CHAPS (fig.1). Analyses of galactosyltransferase activity in these fractions show a regular decrease in activity present in the pellet above 1 mM CHAPS and an almost parallel increase in the supernatant, up to 5–6 mM CHAPS (fig.1). At 4–8 mM CHAPS, corresponding to its CMC (4–6 mM), about one-third of the galactosyltransferase activity was detected in the supernatant whereas the membrane pellet was almost inactive. Inhibition of the enzyme by CHAPS was obvious above 8 mM.

Table 1

Stimulation by envelope lipids and salts of UDP-gal:diacylglycerol galactosyltransferase activity after solubilization of envelope membranes

Sample	Addition to the minimum incubation medium	Galactose incorporated (nmol/h per mg protein)
Envelope membranes	none	450
	+ KH_2PO_4	494
Solubilized envelope	none	11
	+ envelope lipids	42
	+ envelope lipids, + KH_2PO_4	62
	+ envelope lipids, + KCl	121
	+ envelope lipids, + NaCl	145

Envelope membranes (500 μg protein) were solubilized in 750 μl (final volume) medium A. After centrifugation, the supernatant was fractionated into 5 parts, thus deriving each one from 100 μg envelope protein, for assay of galactosyltransferase as described in section 2.5 in the minimum incubation mixture containing 6 mM CHAPS. As indicated below, additions to the minimum incubation mixture were: envelope lipids, 247.5 μg; 250 mM KH_2PO_4, KCl or NaCl. As a control, total envelope membranes (100 μg protein) were assayed for galactosyltransferase activity as described in section 2.5 in the absence or presence of 250 mM KH_2PO_4, and without CHAPS in the minimum incubation medium

However, the detection of galactosyltransferase activity in the supernatant was possible only in the presence of salts and/or envelope lipids in the incubation mixture. Table 1 shows that very little activity was found in the supernatant obtained after solubilization of envelope membranes by 6 mM CHAPS if the incubation mixture was devoid of salts or added lipids. Although almost all the membrane lipids were solubilized by 6 mM CHAPS, further addition of envelope lipids stimulated the basal galactosyltransferase activity up to 3-fold. This effect was proportional to the amount of lipids added up to 1 mg envelope lipid/ml (not shown). Actually, stimulation by envelope lipids was expected since diacylglycerol, the substrate of the enzyme, represents about 10% of the envelope lipids, if the chloroplasts used were not thermolysin-treated [10]. In addition, direct stimulation by envelope lipids cannot be ruled out.

A high ionic strength in the assay mixture is also necessary for optimal galactosyltransferase activity: addition of KH_2PO_4, KCl or NaCl led to a strong stimulation of the incorporation of galactose from UDP-gal into galactolipids (table 1). In contrast, addition of salts to native envelope was almost without effect (see also [4]). Optimal stimulation was obtained with a mixture of 250 mM KH_2PO_4 and 250 mM KCl.

DTT (1 mM) in the solubilization medium was shown to be essential to protect galactosyltransferase activity: only 10–15% of the activity initially measured in envelope membranes was recovered after solubilization in the absence of DTT, compared to 30–40% when DTT was present. After solubilization, the envelope galactosyltransferase becomes very sensitive to sulfhydryl reagents since only 5–10 μM *N*-ethylmaleimide (NEM) was necessary to inhibit completely MGDG synthesis with envelope membranes solubilized in the absence of DTT. In isolated envelope membranes, about 5 mM NEM was necessary for total inhibi-

tion of the galactosyltransferase (see also [4]). In addition, NEM inhibition was very rapid: in less than 15 s in the presence of 10 μM NEM, 50% of the solubilized galactosyltransferase activity was inhibited. Therefore, the SH groups that are probably close to the active site of the enzyme have to be protected during and after solubilization to prevent inactivation of the envelope galactosyltransferase.

The results obtained provide first an efficient system to solubilize and stabilize the envelope galactosyltransferase and second a convenient assay to measure the activity after solubilization. The effects of DTT, NEM and salts on the envelope galactosyltransferase reflect the major changes in the enzyme environment induced by solubilization.

3.2. *Partial purification of UDP-gal:diacylglycerol galactosyltransferase*

As a first step towards purification of the envelope galactosyltransferase, hydroxyapatite chromatography was used. Envelope membranes solubilized in 6 mM CHAPS were loaded on the column and the proteins that did not bind were eluted as a major peak (peak 1) with 15 ml medium B (fig.2). Almost all the envelope lipids and carotenoids were recovered in peak 1 together with most of the proteins (60–70% of the loaded proteins) (table 2). LDS-PAGE of peak 1 polypeptides (fig.3, lane 2) shows a close similarity with that of total envelope (fig.3, lane 1): the major polypeptides were E30, E37 and the two subunits of ribulose-1,5-bisphosphate carboxylase (E54 and E14). This is in agreement with data on the partial purification, by hydroxyapatite chromatography, of the envelope phosphate translocator [11].

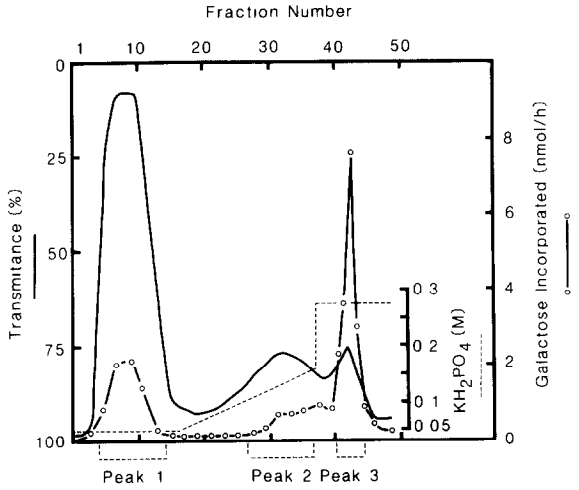


Fig.2. Fractionation of solubilized envelope by hydroxyapatite chromatography. Envelope membranes (10 mg protein) were solubilized in 15 ml (final volume) medium B. After centrifugation, the supernatant (15 ml, 8 mg protein) was loaded and peak 1 eluted with 15 ml medium B (30 ml/h). Peak 2 was eluted with a linear KH_2PO_4 gradient (from 50 to 162.5 mM) in medium A (total volume, 36 ml). Finally, 10 ml medium A containing 275 mM KH_2PO_4 was used to elute peak 3. Galactosyltransferase activity (●) was measured as described in section 2.5. Transmittance (——), KH_2PO_4 concentration (– – –).

After elution of peak 1, a linear gradient with increasing amounts of KH_2PO_4 (from 50 to 162.5 mM) in medium A was used to elute peak 2 (fig.2) containing 10–15% of the loaded proteins (table 2). Only one major envelope polypeptide (E110) was present in this fraction together with a series of minor envelope components with M_r ranging from 15 000 to 100 000 (fig.3, lane 3).

The proteins of peak 3 (4–5% of the loaded proteins) were eluted with 10 ml medium A containing 275 mM KH_2PO_4 (fig.2, table 2). Very little lipid and no pigments were found in this fraction. The major polypeptide components were very different from those previously eluted, the main exception being E37 which was detected in almost all fractions (fig.3, lane 4). Peak 3 was characterized by a series of minor envelope polypeptides with M_r ranging from 20 000 to 100 000 (fig.3, lane 4).

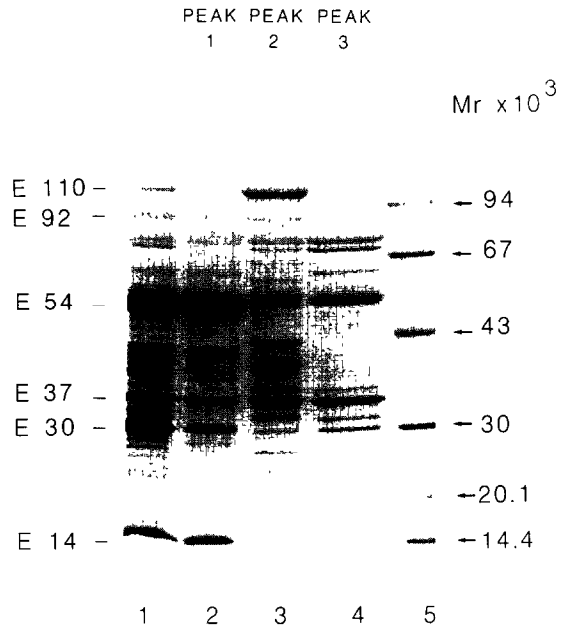
Using the assay described above, we measured the galactosyltransferase activity in all the fractions separated. Fig.2 shows that the activity was detected mostly in peaks 1 and 3. Usually, between 50 and 60% of the activity loaded on the column was recovered after elution (table 2). Obviously, peak 3 was enriched in galactosyltransferase: when compared with the activity loaded on the column, the increase in specific activity was 6–7-fold (table 2). In contrast, the specific activity in peak 1 was lower than that of the solubilized envelope although the incubation mixture contained salts at optimal concentration for maximum activity. Furthermore, we have verified that MGDG was the main galactolipid (more than 95% of the radioactivity) to be synthesized in the fraction enriched in galactosyltransferase activity.

Table 2
Partial purification of UDP-gal:diacylglycerol galactosyltransferase

Sample	Proteins		Galactose incorporated			
	mg	%	total activity		Specific activity (nmol/h per mg protein)	Increase of specific activity
			nmol/h	%		
Solubilized envelope	17	100	1526	100	90	1
Hydroxyapatite fractions						
Peak 1	10.4	61	307	20.1	29.6	×0.3
Peak 2	2	11.8	170	11.2	85	×0.94
Peak 3	0.72	4.2	406	26.6	565	×6.3
Total	13.12	77	883	57.9	–	–

Envelope membranes (21 mg protein) were solubilized with 30 ml medium B, centrifuged and the supernatant obtained (17 mg protein) fractionated as described in sections 2.2 and 2.4. Activity in each fraction was measured as described in section 2.5

These results demonstrate that a partial purification of the envelope UDP-gal:diacylglycerol galactosyltransferase activity can be achieved and that the assay developed for solubilized envelope membranes can be used to measure the activity in fractions that are almost devoid of lipid components.



4. CONCLUSION

Up to now, the only envelope protein that has been purified is the phosphate translocator [11]. The results described above are a first step towards purification of the enzyme involved in MGDG synthesis. Most of the problems that hampered proper solubilization of this envelope membrane protein and assay of its activity after solubilization and fractionation have been solved. Unfortunately, LDS-PAGE of the fraction enriched in galactosyltransferase activity demonstrates the presence of numerous minor envelope polypeptides, thus making further purification and accurate identification of the enzyme very difficult. These experiments are now in progress in our laboratory.

Fig.3. Polyacrylamide gel electrophoresis of envelope membranes and of fractions obtained after hydroxyapatite chromatography of solubilized envelope membranes. Peaks 1-3 correspond to the 3 fractions separated as described in fig.2. Samples and gel (acrylamide gradient from 7.5 to 15%) preparation, conditions for electrophoresis (at 4°C in the presence of LDS) and gel staining were as described [6]. Lanes: 1, envelope membranes; 2–4, peaks 1–3 respectively; 5, M_r standards (Pharmacia, electrophoresis calibration kit for low M_r). Protein load in each slot was 60 μg protein.

Finally, we must bear in mind that the use of purified envelope membranes, instead of intact chloroplasts for instance, is already a major step in the purification of UDP-gal:diacylglycerol galactosyltransferase: a 200–300-fold increase in enzyme specific activity is obtained by purification of envelope membranes from isolated intact chloroplasts.

ACKNOWLEDGEMENTS

We wish to thank warmly Drs Michel Neuburger and Bernard Pineau for fruitful discussions and constant advice. This work was supported in part by a grant from CNRS, ATP Conversion de l'énergie dans les membranes biologiques.

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