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Phosphatidylcholine Biosynthesis in Castor Bean Endosperm[1]

Purification and Properties of Cytidine 5′-Triphosphate:Choline-Phosphate Cytidylyltransferase

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ABSTRACT

Cytidine 5′-triphosphate:choline-phosphate cytidylyltransferase (EC 2.7.7.15) has been purified to near homogeneity (3350-fold) from castor bean (*Ricinus communis* L. var Hale) endosperm. The steps of purification included a differential solubilization of this enzyme with *n*-octyl β-D-glucopyranoside (OGP) and column chromatography on sequential DEAE-sepharose, sepharose-6B, and second DEAE-sepharose columns. The uses of appropriate concentrations of the detergent, OGP, in each step were crucial to obtain the highly purified enzyme. The purified enzyme gave a single protein band on nondenaturing polyacrylamide gel electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed one major protein band of 40 kilodaltons. Gel filtration chromatography indicated that native cytidylyltransferase was approximately 155 kilodaltons, suggesting that it exists naturally as a tetramer. The purified enzyme used methylethanolamine-phosphate as a substrate but not ethanolamine-phosphate and dimethylethanolamine-phosphate. ATP and other nucleotides tested showed little effect on the purified enzyme. The purified enzyme activity was stimulated by both phospholipids extracted from castor bean endosperm and phosphatidylcholine-oleate vesicles.

CTP:choline-P[2] cytidylyltransferase (EC 2.7.7.15) catalyzes the formation of CDP-choline from CTP and choline-P. This enzyme has received the most attention as a potential regulator of PtdCho synthesis by the nucleotide pathway (6, 10, 11). Several mechanisms of regulation of this enzyme have been examined in mammalian tissues. A reversible protein phosphorylation and lipid modulation have been suggested to regulate cytidylyltransferase activity, possibly via a reversible association with microsomal membranes (10, 14). The membrane-bound cytidylyltransferase is active in PtdCho synthesis. The study of this enzyme from plant tissues has produced mixed results. A soluble form of cytidylyltransferase was suggested to be most important in PtdCho synthesis in pea stem tissues (13). In germinating castor bean endosperm, however, ER-associated cytidylyltransferase activity correlated with PtdCho formation (8).

Insights into the important control of cytidylyltransferase and PtdCho synthesis in plants require availability of a pure enzyme. This enzyme in pea stem was reported to be purified to more than 300-fold over tissue homogenate (12). But the enzyme from pea stems showed strong hydrophilic properties and the purification steps could not be applied to that from castor bean endosperm (15). We previously described a procedure to partially purify cytidylyltransferase from castor bean (15). That study showed that the uses of appropriate concentrations of the detergent, OGP, were necessary to solubilize this enzyme and dislodge it from columns during the purification. The present report describes an improved procedure to purify cytidylyltransferase to near homogeneity and some properties of the purified enzyme.

MATERIALS AND METHODS

Materials

Seeds of castor bean (*Ricinus communis* L. var Hale) were removed from their seedcoats and germinated for 3 d as described (7). Radioisotopes and other reagents were obtained from the sources reported (15). Etn-P[methyl-^{14}C] was purchased from Amersham in 1982 (apparently no longer available from commercial sources) and further purified with paper chromatography prior to use. MEtn-P[methyl-^{14}C] and DMEtn-P[methyl-^{14}C] were chemically synthesized with Etn-P[methyl-^{14}C] and CH_3I as described (9).

Differential Solubilization of Cytidylyltransferase

All the following steps were carried out at 4°C unless stated otherwise.

Castor bean endosperm halves (150 g) were frozen with liquid nitrogen. The tissue homogenization and preparation of precipitates enriched in cytidylyltransferase were described previously (15). The precipitates were resuspended with a glass homogenizer in one-fifth volume (mL/g tissue) of buffer A (25 mM Tris-HCl [pH 7.6], 1 mM EDTA and 2 mM DTT) plus 50 mM KCl. This resuspension was brought to 10 mM

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[2] Abbreviations: choline-P, choline-phosphate; PtdCho, phosphatidylcholine; OGP, *n*-octyl β-D-glucopyranoside; Etn-P, ethanolamine-phosphate; MEtn-P, methylethanolamine-phosphate; DMEtn-P, dimethylethanolamine-phosphate.

OGP by adding a concentrated solution. This mixture was centrifuged at 119,000*g* for 60 min. The pellet was resuspended with a glass homogenizer in one-tenth volume of buffer A plus 50 mM KCl, and 30 mM OGP, and gently stirred for 30 min. The mixture was centrifuged at 119,000*g* for 60 min, and the supernatant contained solubilized cytidylyltransferase activity.

Chromatography

Solubilized enzyme (total protein, 17 mg) was adjusted with buffer A to 20 mM OGP and applied to a DEAE-sepharose CL-6B column (15 mL bed volume). A linear gradient of 100 to 500 mM KCl in buffer A (200 mL each of starting and ending solutions) was used to wash the column, which was then eluted with a linear gradient of 0 to 20 mM OGP and 400 mM KCl in buffer A (30 mL each). The enzyme was continuously eluted with 20 mM OGP in 400 mM KCl. Absorbance at 280 nm was monitored by a UA-5 absorbance monitor (ISCO). The flow rate was 20 mL/h for the KCl and 30 mL/h for the OGP gradients. Fractions collected prior to the OGP gradient were 8 mL each, after which fractions contained 2 mL. The fractions with enzyme activity were pooled and concentrated by ultrafiltration (CentriCell 30k cutoff, Polyscience Inc.).

OGP was added to concentrated DEAE-sepharose fractions to a final concentration of 30 mM OGP and the enzyme was then loaded onto a sepharose-6B gel filtration column (1.0 × 50 cm). The equilibration and elution were achieved with buffer A containing 100 mM KCl and 30 mM OGP. The flow rate was 4 mL/h and 0.5 mL fractions were collected. The active fractions were pooled.

The pooled sepharose-6B fractions were adjusted to 20 mM OGP by adding one-third volume of buffer A containing 100 mM KCl and applied to the second DEAE-sepharose CL-6B column (4 mL bed volume). A linear gradient of 100 to 400 mM KCl in buffer A with 20 mM OGP (35 mL each) was employed to elute the enzyme. The flow rate was 15 mL/h and 1 mL fractions were collected. Various fractions were analyzed by SDS-PAGE and the gel was stained with silver. Purified enzyme was concentrated by ultrafiltration and stored in −20°C in the presence of 50% glycerol.

Electrophoresis

Conditions for the SDS slab gel electrophoresis system were previously described (15). Gels (9 × 7.5 × 0.075 cm) were 4% acrylamide in the stacking and 10% in the resolving gel. After electrophoresis, gels were stained with silver staining reagents from Bio-Rad, following the manufacturer's instructions.

Nondenaturing PAGE was performed according to a described procedure (1). The resolving and stacking gels were 7.5 and 3.5% acrylamide, respectively. The gels contained 20 mM OGP and were subjected to electrophoresis at a constant current of 20 mA at 4°C. To determine cytidylyltransferase activity after the nondenaturing electrophoresis, a gel was sliced into 2 mm slices and 100 μL reaction solution was added to each slice. This reaction solution contained 183 pmol of choline-P (50 Ci/mol), and the other ingredients were the same as those used in regular enzyme activity assays. The mixture was incubated at 30°C for 2 h and at the end the liquid was separated from the gel slices. The reaction was stopped and processed by the regular charcoal method (15).

Mol Wt Determination

The mol wt of cytidylyltransferase in denatured condition was determined by slab SDS-PAGE as described above. Sepharose-6B column chromatography was used to estimate its nondenaturing mol wt. The elution conditions were the same as those in enzyme purification. Mol wt standards for SDS-PAGE and gel filtration were from Sigma and prepared according to the manufacturer's instructions.

Enzyme Assays

The assay of CTP:choline-P cytidylyltransferase activity was described previously (15). In the studies measuring the activities of cytidylyltransferase for Etn-P, MEtn-P, and DMEtn-P, the supply of those substrates were limited, and so a total of 64 pmol of each substrate (50 Ci/mol) was used in each reaction without the addition of nonradioactive carriers. Each reaction contained 2 mM CTP, 10 mM $MgCl_2$, and 100 mM Tris-HCl (pH 7.5). In routine cytidylyltransferase activity assays, the formation of CDP-bases (CDP-Etn, CDP-MEtn, CDP-DMEtn, and CDP-choline) was measured by the regular Norit charcoal method (15). Those enzymatic products were verified by identifying their base-P after acid hydrolysis of CDP-bases with descending paper chromatography as follows. Following termination of a reaction, CDP-bases in the reaction mixture were first separated from their base-P in a solvent with isopropanol:formic acid:water (7:1:2, v/v). The CDP-bases moved very slowly (stayed near the origin) whereas choline-P, MEtn-P, and DMEtn-P migrated with an R_F of about 0.6 and Etn-P about 0.4. The CDP-bases were identified as their base-P following elution, hydrolysis in 3 N HCl at 100°C for 30 min, and chromatography on paper with isopropanol:NH_4OH:water (7:1:2, v/v) (9). The recovery of radioactivity by charcoal and paper chromatography methods was comparable.

Preparation of Lipid Vesicles

Total lipids from castor bean endosperm after 3 d germination were extracted and separated into neutral lipid and phospholipids with a silica cartridge (Waters Assoc.) (16). Phosphorus content in the total lipids and phospholipids was determined colorimetrically following digestion with magnesium nitrate (4). To prepare lipid vesicles for enzyme assays, lipids dissolved in chloroform were evaporated to dryness under a stream of nitrogen and suspended in 200 mM Tris-HCl (pH 7.5) with a probe sonicator. Equal volumes (20 μL each) of lipid suspension and reaction solution containing 4 mM CTP, 20 mM $MgCl_2$, and 5 mM choline-P (0.25 Ci/mol) were mixed just prior to activity assays. The lipid resuspension was prepared freshly for each experiment.

Protein Determination

Protein concentrations were measured by the method of Bradford (2).

RESULTS AND DISCUSSION

Purification of Cytidylyltransferase

A partial purification of cytidylyltransferase was previously described from castor bean endosperm (15). The modifications of that reported procedure, particularly the addition of a second DEAE-sepharose chromatography, enable us to purify this enzyme to near homogeneity. The use of appropriate OGP concentrations to elute cytidylyltransferase from the columns was crucial for obtaining the highly purified enzyme. Detergent to protein ratio did not seem to affect the solubilization of this enzyme within the concentration range used (15); however, the effect of the ratio on the elution of the enzyme from columns could not be precisely determined. In our recent scale-up purification experiments, the behavior of the enzyme follows the same pattern reported here as long as the concentrations of OGP are maintained. This indicates that the proper detergent concentrations are more crucial than the protein to detergent ratios for the protein concentrations used during purification. The overall behavior of this enzyme during purification indicated that this enzyme possesses hydrophobic properties as exhibited by membrane proteins.

A summary of our purification scheme for cytidylyltransferase is presented in Table I. The overall purification from the initial cell extract resulted in a 3350-fold increase in specific activity, with a 2% recovery of total activity.

Figures 1 and 2 show the results of two DEAE-sepharose column chromatograms during the purification. The enzyme applied to the first column was solubilized with 30 mM OGP from the 119,000*g* pellet which was obtained from the resuspended ammonium sulfate precipitates (15). The OGP concentration in the solubilized enzyme was adjusted to 20 mM prior to loading onto the first DEAE-sepharose column (Fig. 1). More than 80% of the OGP-solubilized protein passed through the column before washing with a 100 to 500 mM KCl linear gradient. The remaining cytidylyltransferase was dislodged from the column with 20 mM OGP in 400 mM KCl.

A great enhancement of the enzyme purity was obtained through use of the second DEAE-sepharose column chromatogram (Fig. 2). At 20 mM OGP in 100 mM KCl, nearly 50% of the protein passed through the column. When the column was continuously developed with 100 to 400 mM KCl in the presence of 20 mM OGP, the majority of the protein was eluted at 130 to 230 mM KCl. Cytidylyltransferase activity was dislodged from the column as a single peak at about 320 mM KCl.

Table I. *Purification Summary of Cytidylyltransferase*

Step	Protein	Activity	Purification	Recovery
	mg	*nmol/min/mg*	*-fold*	%
Homogenate	3493	0.45	1.0	
100,000 *g* supernatant	1729	0.68	1.5	100
Ammonium sulfate pellet	785	3.2	7.1	140
Solubilized enzyme	17	35.1	78.0	45
DEAE-sepharose I	3	225.1	496.9	12
Sepharose-6B	1.5	241.0	531.8	9
DEAE-sepharose II	0.012	1518.0	3351.3	2

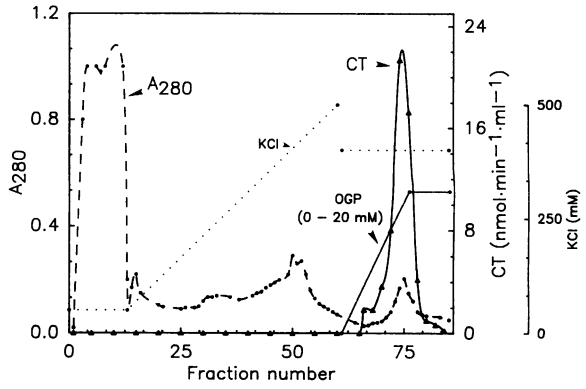

Figure 1. Chromatography of an OGP extract from castor bean endosperm halves on DEAE-sepharose I. The enzyme applied to the column was solubilized with 30 mM OGP from the 119,000*g* pellet which was obtained from a resuspended ammonium sulfate precipitate (see "Materials and Methods"). The OGP concentration was adjusted to 20 mM prior to applying the extract. The column was sequentially eluted with linear gradients of 100 to 500 mM KCl without OGP, 0 to 20 mM OGP in 400 mM KCl, and 20 mM OGP in 400 mM KCl. CT, cytidylyltransferase.

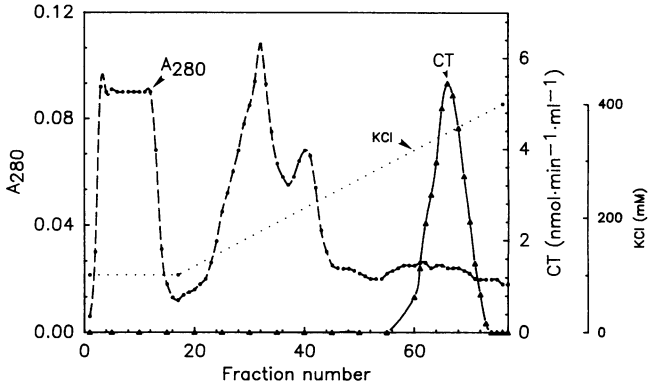

Figure 2. Chromatography of the active sepharose-6B fractions on DEAE-sepharose II. The OGP concentration was adjusted to 20 mM prior to applying the fractions. The column was eluted with a linear gradient of 100 to 400 mM KCl containing 20 mM OGP.

SDS-PAGE analysis of those active fractions demonstrated a correlation between the elution of the enzyme activity and a protein band at a molecular mass of 40 kD (Fig. 3A). After staining with silver, one faint protein band could be seen at the peak of enzyme activity in addition to the major protein band, but the intensity of the faint band protein did not correlate with the enzyme activity. We estimated that the faint band contributed no more than 2% of the total protein. The minor protein was unlikely to be associated with the enzyme since in the later fractions of Figure 2 the minor protein appeared as a stronger band on SDS-PAGE, but little of either 40 kD protein or enzyme activity were present (data not shown).

Nondenaturing PAGE analysis of the purified enzyme in

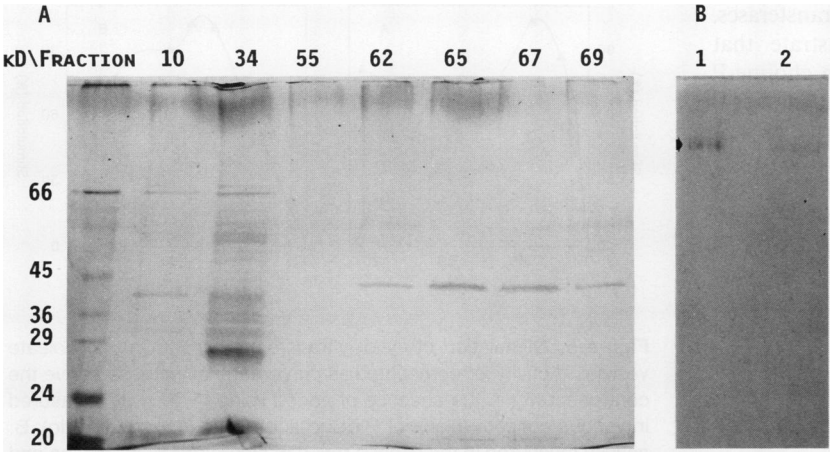

Figure 3. PAGE analysis of purified cytidylyltransferase. A, SDS-PAGE (10%) analysis of DEAE-sepharose II fractions (Fig. 2). The numbers on each lane correspond to the fraction numbers of Figure 2. The gel was stained with silver. Equal volumes (20 μL) from each fraction were mixed with one volume of SDS-PAGE loading buffer and heated at 100°C for 2 min before applied to the gel. B, Native-PAGE (7.5%) and was stained with Coomassie blue R. Lanes 1 and 2 were loaded with 5 and 1 μg protein, respectively. About 5% of the applied enzyme activity was detected from the arrow-marked gel band.

the presence of 20 mM OGP resulted in one protein band (Fig. 3B). In the absence of OGP the enzyme did not enter the gel and at 30 mM OGP the enzyme activity was completely lost. It was difficult to recover high enzyme activity; approximately 5% of the applied enzyme activity was recovered from the gel band in the presence of 20 mM OGP.

The purified enzyme was concentrated to a small volume (0.5 mL) by ultrafiltration. Nearly 50% of the activity was lost overnight without concentration, while the concentrated enzyme was 80% stable for more than 2 weeks when stored at −20°C in the presence of 50% glycerol.

Molecular Mass Determination

The molecular mass of cytidylyltransferase under denaturing conditions was estimated by SDS-PAGE. The protein appeared as one major band of molecular mass at 40 kD (Fig. 4A). The native molecular mass was determined by sepharose-6B gel filtration chromatography in the presence of 30 mM OGP, in which this enzyme activity eluted at an apparent molecular mass at 155 kD (Fig. 4B). These results suggest that cytidylyltransferase from castor bean endosperm exists naturally as a tetramer.

The purified cytidylyltransferase from rat liver has been reported to have a native molecular mass of 178 KD, which was suggested to be a tetramer of 45 kD subunits (5, 17). The enzyme in pea stems was reported to be monomer of 56 kD (12). Hence the subunit composition of cytidylyltransferase from castor bean resembles the rat liver enzyme more closely than that reported for pea stems (12).

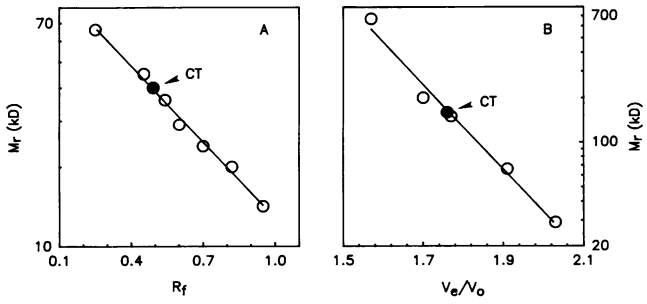

Figure 4. Mol wt determination of purified cytidylyltransferase. A, Plot of log M_r *versus* R_F of SDS-PAGE; B, plot of log M_r *versus* Ve/Vo of gel filtration on sepharose-6B in the presence of 30 mM OGP.

Characteristics and Substrate Specificity

The K_ms of the purified enzyme for CTP and choline-P were 0.20 and 0.37 mM, respectively. The purified enzyme exhibited higher affinity toward choline-P than partially purified enzyme (1.1 mM) (15). The optimal pH was 7.5.

The purified CTP:choline-P cytidylyltransferase was tested for its ability to use Etn-P, MEtn-P, and DMEtn-P as substrates. Since the supply of Etn-P, MEtn-P, and DMEtn-P was limited, the activity assays were carried out at very low substrate concentrations (*i.e.* nonradioactive carriers were not added). Each assay contained 2 mM CTP, 10 mM $MgCl_2$ and 64 pmol of the individual substrate (50 Ci/mol). The amount of enzyme was adjusted to a concentration whereby less than 15% of substrate was converted to products. Under these assay conditions, the purified enzyme used MEtn-P as a substrate, but not Etn-P or DMEtn-P (Table II).

The initial extract of castor bean endosperm contained the activities for cytidylyltransferase utilizing Etn-P, MEtn-P, and DMEtn-P (Table II). Cytidylyltransferase activities for MEtn-P and DMEtn-P also were reported in the tissues of carrot, soybean and *Lemna* (3). It is not known whether those

Table II. *Cytidylyltransferase Activities in Homogenate and Purified Enzyme of Castor Bean Endosperm*

The homogenate of castor bean endosperm was prepared as described (15). Each reaction contained 64 pmol of each substrate, 2 mM CTP, 10 mM $MgCl_2$, 100 mM Tris-HCl (pH 7.5), and 10 μL enzyme in a total volume of 50 μL.

Substrate	Activity	
	Homogenate	Purified enzyme
	pmol CDP-bases/h	
Choline-P	16.8	16.6
DMEtn-P	2.2	ND[a]
MEtn-P	17.4	16.4
Etn-P	6.8	ND

[a] Not detected.

reactions are catalyzed by one or more cytidylyltransferases. Our results with the purified enzyme demonstrate that CTP:choline-P cytidylyltransferase could use both choline-P and MEtn-P as substrates. Addition of 1.0 mM choline-P almost completely inhibited the MEtn-P activity in the purified enzyme and crude homogenate under the described assay conditions, whereas the same condition did not affect the cytidylyltransferase activities for Etn-P and DMEtn-P in crude homogenates. Addition of nonradioactive Etn-P up to 10 mM did not affect choline-P cytidylyltransferase activity. This result indicates that more than one cytidylyltransferase exists in castor bean. Detailed kinetic studies are needed to establish the affinity and efficiency of the purified enzyme toward MEtn-P. This important study will have to wait until sufficient amounts of MEtn-P can be obtained.

Effect of Nucleotides

The purified enzyme was tested for its sensitivity to ATP and other nucleotides. Our previous study with partially purified cytidylyltransferase showed that the enzyme activity was inhibited after preincubation in 3 mM ATP at 30°C for 20 min (15). Under the same conditions, ATP and the other nucleotides ADP, CDP, CMP, cyclic CMP, and UTP, had no inhibitory effect on the purified enzyme activity.

Effect of Lipids

Lipids extracted from castor bean endosperm were separated into phospholipids and neutral lipids. Total lipids and phospholipids stimulated the purified enzyme by more than twofold whereas neutral lipids inhibited by near sevenfold (Table III). In order to examine in more detail the lipid requirements for catalytic activity, vesicles containing PtdCho and oleate were used. The enzyme activity in PtdCho and oleate vesicles in a 1:1 molar ratio was stimulated at lipid concentration as low as 2 μM (Fig. 5A). The maximal stimulation was about 100% at a concentration of 32 μM.

When the PtdCho and oleate concentration was held at 32 μM, a change of their molar ratio affected the enzyme stimulation (Fig. 5B). Vesicles containing only PtdCho had no apparent effect while those with only oleate stimulated the enzyme activity. Maximal stimulation was obtained with vesicles containing 40 to 60 mol % oleate.

These data indicate that the purified cytidylyltransferase requires the addition of appropriate phospholipid mixtures for maximal activity. PtdCho alone did not activate the enzyme whereas oleate alone did, but the two combined were synergistic. The activation of cytidylyltransferase by phospholipid vesicles *in vitro* appears to depend on both the composition and concentration of the vesicles.

Table III. *Effect of Lipids Extracted from Castor Bean Endosperm Germinated for 3 d on the Purified Cytidylyltransferase*

The phosphorus contents in the total lipids and phospholipids were 33 μg/mL determined as described in "Materials and Methods." The neutral lipids were those extracted from the total lipids with the same phosphorus content. The control (no lipid) activity was 891 nmol/min/mg protein.

Lipid	Activity
	%
No lipid	100
Total lipid	226
Phospholipid	247
Neutral lipid	15

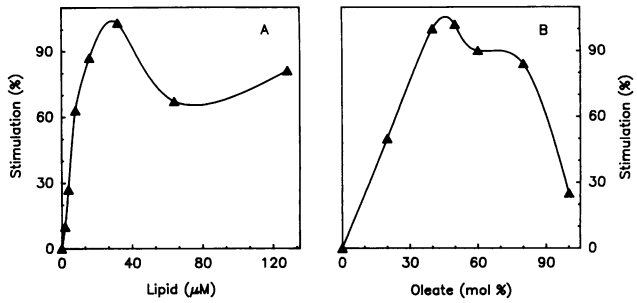

Figure 5. Stimulation of cytidylyltransferase by PtdCho and oleate vesicles. Activity is represented as percentage of increase above the control activity in the absence of added lipids. A, Activity measured in various concentrations of PtdCho and oleate (1:1 molar ratio); B, activity measured in the various ratios of oleate mol % in PtdCho and oleate. The total lipid content was 32 μM.

In summary, we describe here a procedure to obtain highly purified CTP:choline-P cytidylyltransferase from castor bean endosperm. This enzyme possesses strong hydrophobic properties, which is consistent with the previous observation that the majority of cytidylyltransferase was associated with a membrane fraction (7). The purified enzyme can also use MEtn-P as a substrate but not Etn-P and DMEtn-P. This enzyme activity appears to be modulated by lipids and possibly by reversible protein phosphorylation (15). Further studies on the kinetics, substrate specificity, and modulation of the activity of the purified enzyme are important to understand the true substrate as well as the mode of action and control of this enzyme. The availability of purified cytidylyltransferase will enable a detailed study of the role of this enzyme in plants as a potential regulator of PtdCho synthesis.

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