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PURIFICATION OF CTP: CHOLINEPHOSPHATE CYTIDYLYLTRANSFERASE FROM PEA STEMS

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Key Word Index—*Pisum sativum*; Leguminosae; pea stems; cytidylyltransferase purification; phosphatidylcholine metabolism.

Abstract—CTP: cholinephosphate cytidylyltransferase (EC 2.7.7.15) was purified from pea (*Pisum sativum*) stems. The purification involved ammonium sulphate fractionation, ion exchange chromatography, removal of proteases with α_2-macroglobulin and gel filtration. The purified enzyme had K_m values for phosphorylcholine and CTP of 2.1 mM and 0.55 mM respectively. It was found to have a pH optimum of 7.5, a requirement for Mg^{2+} and an M_r of 56 000. It could not utilize phosphorylethanolamine and its activity was not stimulated by added phospholipids.

INTRODUCTION

Phosphatidylcholine is a major membrane component of plants, representing about 40% of the total phospholipid [1, 2]. Its major route of synthesis has been shown to be via the CDP-base pathway [3]. In studies on the effect of indole-3-acetic acid on incorporation of ^{14}C-labelled precursors into the phospholipids of pea stem explants, we observed that incorporation of [^{14}C]choline into phosphatidylcholine was inhibited by this growth regulator. This effect was specific to [^{14}C]choline incorporation, as neither incorporation of [^{14}C]acetate into phospholipids nor incorporation of [^{14}C]ethanolamine into phosphatidylethanolamine was affected [4]. Measurements of *in vivo* pool sizes and *in vitro* enzyme activities indicated that the inhibition of incorporation of [^{14}C]choline was caused by a decrease in activity of the CTP: cholinephosphate cytidylyltransferase (EC 2.7.7.15) in the pea stem explants [5]. This suggested that the cytidylyltransferase enzyme was rate-limiting for phosphatidylcholine synthesis in the pea stem tissue.

Cytidylyltransferase activity has previously been examined in subcellular fractions of three plant tissues; onion stem [6], castor bean endosperm [7] and pea stem tissue [8], but it has not been purified to homogeneity. In order to study the pea stem cytidylyltransferase further, we have worked out a method for its purification. We now report this procedure together with some properties of the purified enzyme.

RESULTS AND DISCUSSION

Results from the subcellular fractionation of pea stem tissue (Table 1) showed that about 75% of the cytidylyltransferase activity was soluble. Therefore, the post-microsomal (105 000 *g*) supernatant was used in the purification procedure.

The post-microsomal supernatant was fractionated using ammonium sulphate. Fractions were separated at 0–30%, 30–40%, 40–50% and 50–100% saturation and the majority of the cytidylyltransferase activity (about 72%) was found in the supernatant, not having been precipitated by the ammonium sulphate. The high solu-

Table 1. Cytidylyltransferase activity in subcellular fractions from pea stems

Fraction	Specific activity (nmol CDP-choline formed/min/mg protein)	Total activity (nmol CDP-choline formed/min)	Activity (% total homogenate activity)
Total homogenate	1.18 ± 0.31	179.0 ± 4.1	100
800 *g* pellet	0.60 ± 0.21	6.3 ± 2.2	3.5
6000 *g* pellet	0.50 ± 0.06	5.5 ± 0.6	3.1
18 000 *g* pellet	0.43 ± 0.13	4.2 ± 1.3	2.3
105 000 *g* pellet	0.97 ± 0.19	10.2 ± 2.0	5.7
Supernatant	1.50 ± 0.23	133.5 ± 20.6	74.6

The homogenate was prepared and fractionated and assays carried out as described in Experimental. Triplicate determinations were made for each value and results are expressed as means ± s.e.m.

bility of the cytidylyltransferase enzyme under these conditions was surprising, but such properties are known for proteins of low M_r or with high contents of polar amino acids.

The supernatant from the ammonium sulphate fractionation was dialysed against 10 mM potassium phosphate buffer, pH 7.0, and applied to a DE-32 ion exchange column, which was eluted as described in the Experimental. The elution profile for a typical column is shown in Fig. 1. The major peak of cytidylyltransferase activity was eluted at a concentration of about 0.2 M sodium chloride.

The results in Table 2, which gives the recoveries of protein and cytidylyltransferase activity during a typical purification, show that recovery of activity was low in the eluate from the DE-32 column. If this eluate was dialysed and applied to a gel filtration column, then activity was lost completely. One possible explanation could have been the presence of active proteases in the preparation which were rendering the cytidylyltransferase inactive. In order to try and remove protease activity the eluate was applied to a column of carrier-bound α_2-macroglobulin [9] as described in the Experimental. Although only a small amount of protein (< 100 μg) was removed by the α_2-macroglobulin, treated samples contained about three times more cytidylyltransferase activity than untreated samples, and could be stored for at least 2 weeks at −20° without significant loss of activity. The apparent increase in recovery of cytidylyltransferase activity between the ion exchange and gel filtration steps of the purification (Table 2), after removal of proteases, indicated that the stability of the enzyme was affected during both assay and storage.

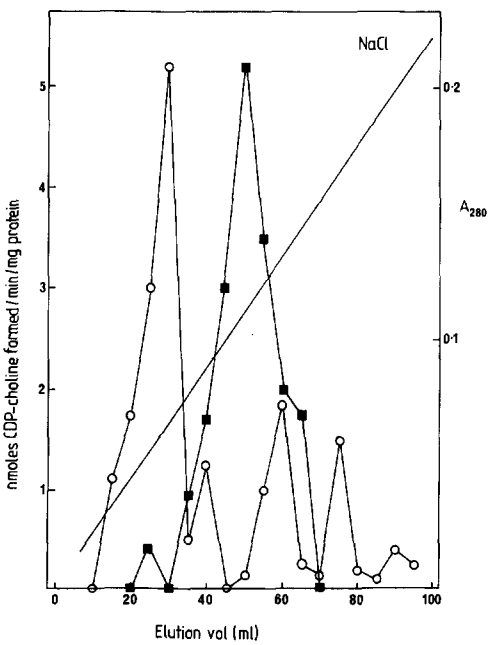

Fig. 1. Elution profile of DE-32 column. The column was loaded with 10 mg protein and eluted with a linear gradient of 0–0.4 M NaCl–KPi buffer, pH 7.0, as described in the Experimental. O, Protein (A_{280}); ■, cytidylyltransferase activity.

The final stage of the purification was a gel filtration step. Sephadex G-200 and Sephacryl S-300 were tried as purified cytidylyltransferases from mammalian tissues were found to have M_rs in excess of 150000 [10, 11]. Sephadex G-200 was found to be preferable, despite its poorer resolution, because recovery of cytidylyltransferase activity was greatest. The elution profile for the Sephadex G-200 column, which was eluted as described in the Experimental, is shown in Fig. 2. A single peak of cytidylyltransferase activity was eluted and this corresponded to an M_r of between 40000 and 70000. SDS-PAGE of the purified sample gave a single protein band of M_r 56000. Amino acid analysis of the enzyme protein showed it to be rich in polar amino acids such as glycine, aspartate, glutamate, lysine and serine, which may partly explain why it was not precipitated by ammonium sulphate.

The substrate and cofactor requirements for the purified cytidylyltransferase enzyme are shown in Fig. 3. Maximal formation of CDP-choline over a 1 hr period occurred at 25° and the pH optimum was 7.5. The time course of the reaction was followed for 120 min and was linear over that period. Magnesium cations were required and 2 mM was their optimal concentration. The K_m values for the phosphorylcholine and CTP substrates were found to be 2.1 and 0.55 mM respectively.

The effect of phosphorylethanolamine on the formation of CDP-choline by the purified cytidylyltransferase was examined, to see if it would act as a competitive inhibitor against phosphorylcholine, and the results are shown in Table 3. Increasing concentrations of phosphorylcholine were used in incubations which contained 2 mM phosphorylethanolamine, which was at the approximate K_m value for phosphorylcholine but no inhibitory effect on CDP-choline production was seen. This showed that the enzyme was specific for the phosphorylcholine substrate.

Work with rat liver and foetal rat lung has shown that cytidylyltransferase activity can be stimulated by the

Table 2. Purification of cytidylyltransferase from pea stems

Stage	Protein (mg)	Protein (% recovery)	Activity (% recovery)	Specific activity (nmol/min/mg protein)	Purification
Homogenate	283.9	100	100	0.29	1
105000 *g* supernatant	116.8	41.1	93.3	0.66	2.3
Ammonium sulphate supernatant	10.6	3.7	68.7	5.33	18.4
DE-32 ion exchange	5.2	1.8	40.2	6.37	22.0
Sephadex G-200 gel filtration	0.54	0.2	71.9	109.6	377.9

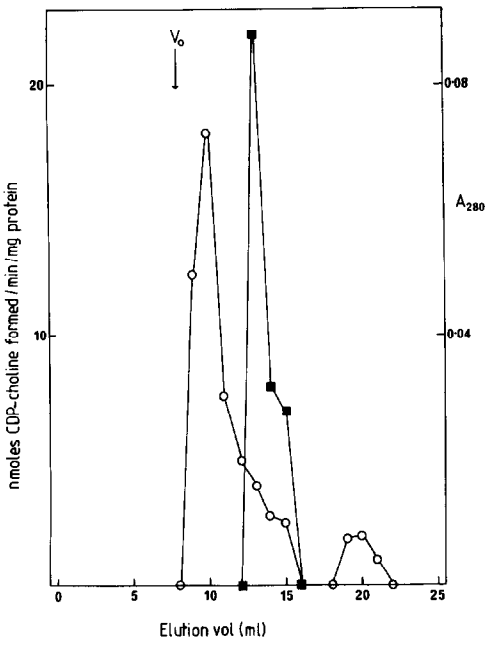

Fig. 2. Elution profile of Sephadex G-200 column. The column was loaded with 1–2 mg of protein and eluted with 10 mM KPi buffer as described in the Experimental. ○, Protein (A_{280}); ■, cytidylyltransferase activity.

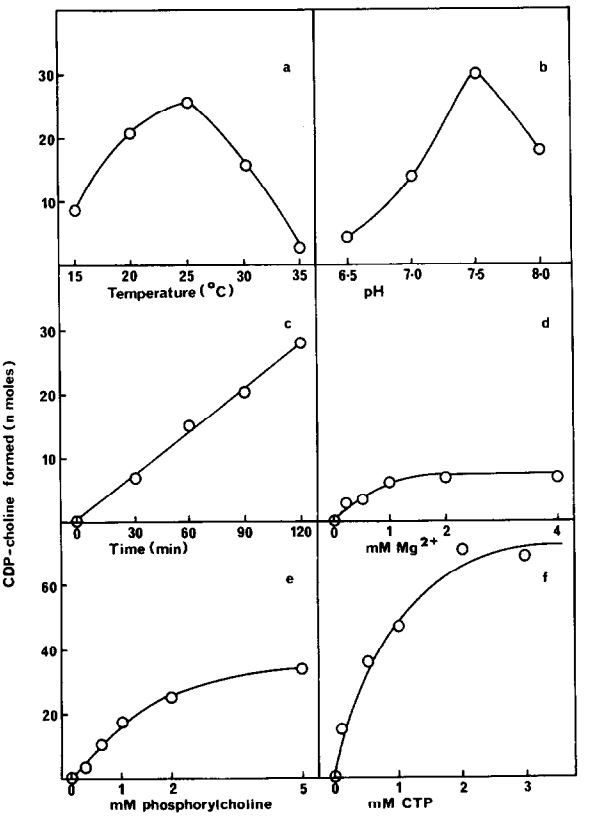

Fig. 3. Properties of cytidylyltransferase. (a) Temperature curve; (b) pH curve; (c) time course; (d) Mg^{2+} concentration; (e) phosphorylcholine concentration; (f) CTP concentration. Assays were carried out as described in the Experimental with one parameter varied in each case. Each value is the average of triplicate determinations.

presence of phospholipids, particularly monoacylphosphatidylethanolamine [12] or phosphatidylglycerol [11]. These cause aggregation of the soluble enzyme to form high M_r polymers and promote translocation of the enzyme to the endoplasmic reticulum where it is stimulated [13]. However, the results in Table 4 show that the cytidylyltransferase activity in the post-mitochondrial supernatant from pea stems was not stimulated by added phospholipid. This suggests that metabolic control of the enzyme is brought about differently in plant and animal tissue.

CONCLUSIONS

The cytidylyltransferase activity from pea stem tissue differed from that found in onion stem [6] and castor bean endosperm [7] in that it was predominantly soluble, although some activity was present in particulate fractions. It also had a different pH optimum value and K_m for CTP from the onion stem soluble activity, where the values were pH 6.4 and 3 mM respectively; castor bean endosperm activity was assayed at pH 7.0 [7]. No value for the K_m of phosphorylcholine was calculated for the onion stem [6].

The requirement of the pea enzyme for Mg^{2+} was similar to the enzyme from onion stem [6] and adult rat lung [11]. The pH optimum for the enzyme from adult rat lung was similar to the pea enzyme, falling between pH 7.4 and pH 8.0. However, the soluble activity from both adult and foetal rat lung had lower K_ms for phosphorylcholine (0.25 mM and 0.24 mM respectively) and higher K_ms for CTP (0.84 mM and 1.40 mM respectively) [11].

The pea enzyme was specific for the phosphorylcholine substrate and was not able to utilise phosphorylethanolamine which suggests that there are two separate enzymes for the synthesis of CDP-choline and CDP-ethanolamine. The possibility of the presence of separate enzymes in the pathways for the synthesis of phosphatidylcholine and phosphatidylethanolamine is in agreement with work on choline kinase (EC 2.7.1.32) from rapeseed [14] and soybean [15] and on CDP-choline: diacylglycerol chol-

Table 3. The effect of 2 mM phosphorylethanolamine on cytidylyltransferase activity

Phosphoryl-choline (mM)	Control (μmol/l/min)	Test (μmol/l/min)	Activity (% control)
0.25	0.03 ± 0.01	0.03 ± tr	100.0 ± 10.6
0.50	0.14 ± 0.01	0.14 ± 0.01	100.0 ± 7.1
1.0	1.23 ± 0.04	1.36 ± 0.09	110.6 ± 7.3
2.0	3.81 ± 0.84	3.86 ± 0.73	101.3 ± 19.2
4.0	4.15 ± 0.25	4.01 ± 0.64	96.6 ± 15.4

Triplicate determinations were made for each value and results are expressed as means ± s.e.m. Assays were carried out as described in the Experimental with 2 mM phosphorylethanolamine added to the test samples. tr, < 0.005.

Table 4. The effect of phosphatidylglycerol or 1-monoacylphosphatidylethanolamine on cytidylyltransferase activity from pea stems

Added phospholipid	Cytidylyltransferase activity (nmol/min/mg protein)
None	0.39 ± 0.08
+ 0.25 mM phosphatidylglycerol	0.28 ± 0.16
+ 0.25 mM monoacylphosphatidylethanolamine	0.28 ± 0.09

The phospholipids were prepared and assays carried out, using the post-mitochondrial supernatant, as described in the experimental. Triplicate determinations were made for each value and results are expressed as means ± s.e.m.

inephosphotransferase (EC 2.7.8.2) from soybean [16].

The enzyme from the pea stems existed in only one M_r form, and we did not obtain any evidence that it could aggregate to form a more active high M_r species as in rat lung [11] or rat liver [12]. In these tissues aggregation could be brought about by the addition of phospholipids, causing cytidylyltransferase activity to be stimulated, but this did not occur when phospholipids were added to the pea enzyme (Table 4). This suggested that the activity of the pea enzyme was regulated in a different way, possibly by a phosphorylation–dephosphorylation cycle [17] or by the presence of specific fatty acids [18]. The fact that the enzyme has a relatively high K_m value for phosphorylcholine could also be a means for regulation of its activity, possibly by alteration of its K_m via allosteric effects. Present work is directed towards elucidating the mechanism of control of the pea cytidylyltransferase activity.

EXPERIMENTAL

Materials. Pea (*Pisum sativum* L. cv. Feltham First) were supplied by Asmer Seeds, Leicester, U.K. Phospho[Me-^{14}C]choline, ammonium salt (sp. radioactivity 2.18 GBq/mmol) and PCS scintillant were purchased from Amersham International. Miracloth was obtained from C.P. Laboratories, Bishops Stortford, Herts, U.K. and α_2-macroglobulin (bovine plasma) from Boehringer, Lewes, East Sussex, U.K. DE-32 anion exchanger, and 3 MM chromatography paper were obtained from Whatman Ltd., Springfield Mill, Maidstone, Kent, U.K. Sephadex G-200 and Sephacryl S-300 were from Pharmacia (Great Britain) Ltd., Regent Road, Hounslow, Middx, U.K. and absolute alcohol AR was from James Burrough, Fine Alcohols Division, London, U.K. All other chemicals used were the purest available grade from Sigma or BDH.

Tissue preparation. Pea seeds were sown in moist vermiculite and grown for 10 days at 20° with *ca* 210 μE/m^2/sec illumination. For experiments the shoots were harvested and the leaves removed; the stem tissue was homogenized in 0.32 M sucrose, 2 mM Tris–HCl, pH 7.4, at 4°, using a mortar and pestle and filtered through two layers of Miracloth.

Subcellular fractionation. Subcellular fractionation was carried out by differential centrifugation of the pea stem homogenate using a modification of the method of Harwood and Stumpf [19]. All centrifugations were carried out at 4° and pellets were obtained from spins of 800 *g* (10 min), 6000 *g* (10 min), 18 000 *g* (20 min) and 105 000 *g* (60 min). The pellets were resuspended in 0.32 M sucrose, 2 mM Tris–HCl, pH 7.4, using a Potter–Elvejhem homogenizer.

Assay for CTP: cholinephosphate cytidylyltransferase activity. Cytidylyltransferase activity was assayed and the products were separated chromatographically as previously described [5]. Optimal conditions were found to be 2 mM $MgCl_2$, 0.5 mM ATP, 1 mM CTP, 3 mM phosphorylcholine (containing 3.7 KBq of phospho[Me-^{14}C]choline) and 80 mM HEPES–Tris, pH 7.5. Assays were carried out for 1 hr at 25°.

Determination of protein. Protein was estimated by the method of Bradford [20] using bovine serum albumin as standard.

Preparation of phospholipids. Stock suspensions containing 5 mM phosphatidylglycerol or 5 mM monoacylphosphatidylethanolamine were made up in 0.32 M sucrose, 2 mM Tris–HCl, pH 7.4, and sonicated in an ultrasonic bath, until a clear suspension was obtained.

SDS-PAGE. This was carried out on either rod or slab gels by the method of Laemmli [21] using 10% resolving gels and 3% stacking gels. The gels were stained using a modification of the method of Meyer and Lamberts [22] where Coomassie Blue was replaced with Page Blue G90.

Amino acid analysis. Samples for amino acid analysis (containing nor-leucine internal standard) were hydrolysed, *in vacuo*, using 6 M HCl for 24 hr at 110°. The hydrolysates were dried and resuspended in 0.2 M Na citrate buffer, pH 2.2. Analysis was carried out on a Locarte amino acid analyser.

Purification of CTP: cholinephosphate cytidylyltransferase. The standard procedure used for the purification of the cytidylyltransferase enzyme was as follows. (see Results and Discussion section for details of the purification stages). All procedures were carried out at 4°.

A post-microsomal supernatant was obtained from the pea stem homogenate by a spin of 18 000 *g* (20 min) followed by a spin of 105 000 *g* (60 min). This was fractionated with $(NH_4)_2SO_4$ and the protein remaining unprecipitated by 100% satd $(NH_4)_2SO_4$ was dialysed against 10 mM KPi buffer, pH 7.0, and applied to a DE-32 ion exchange column (1 cm × 20 cm). The DE-32 column was run in a 0–0.4 M NaCl gradient in 10 mM KPi buffer, pH 7.0. Fractions which contained cytidylyltransferase activity eluted at about 0.2 M NaCl and were dialysed against 100 vols 50 mM KPi buffer, pH 7.0, containing 1 mM EDTA, 1 mM EtSH and 0.1 M NaCl. The dialysate was passed through a carrier-bound α_2-macroglobulin column, freeze-dried, dialysed against 10 mM KPi buffer, pH 7.0, and then fractionated on a Sephadex G-200 column (1 cm × 22 cm). The G-200 column was eluted with 10 mM KPi buffer, pH 7.0, containing 0.1 M NaCl; cytidylyltransferase activity was eluted approximately 5 ml after the void vol.

Purified enzyme was stored at −20° in 10 mM KPi buffer, pH 7.0, and its activity was stable for at least two weeks under these conditions.

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