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# Characterization of the non-specific lipid transfer protein EP2 from carrot (*Daucus carota* L.)

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## Abstract

The extracellular protein EP2 was previously identified as non-specific lipid transfer protein based on its cDNA-derived amino acid sequence. Here, the purification of the EP2 protein from the medium of somatic embryo cultures is described. After two cycles of ion-exchange and gel permeation chromatography, a single silver-stained protein band with an apparent molecular mass of 10 kDa was observed on SDS-PAGE. This protein band was recognized by the antiserum raised against a EP2-β-galactosidase fusion-protein. Employing a fluorescent phospholipid analog, it was shown that the purified EP2 protein is capable of binding phospholipids and is able to enhance their transfer between artificial membranes. Employing a gel permeation assay, it could be demonstrated that the EP2 protein is also capable of binding palmitic and oleic acid as well as oleyl-CoA. Because in plants these fatty acids are used as precursor molecules for cutin, these results are in support of the proposed role of the EP2 protein to transport cutin monomers from their site of synthesis through the cell wall of epidermal cells to sites of cutin polymerization. (Mol Cell Biochem **123:** 159–166, 1993)

*Key words:* lipid transfer protein, plant (carrot), fatty acid-binding, cutin synthesis

## Introduction

Carrot somatic embryos can develop from single cells [1, 2] or cell clusters [3] after appropriate cell culture manipulations. The development of somatic embryos has been well characterized morphologically [1–3], but remains poorly understood at the molecular level [4, 5].

The formation of carrot somatic embryos is accompanied by the secretion of a small number of proteins into the culture medium that are either absent from or present in reduced amounts in cultures that do not form somatic embryos [6]. These observations suggest that several secreted proteins are correlated with somatic embryo development. Somatic embryogenesis, inhibited by the fungal antibiotic tunicamycin, or impaired by a temperature-sensitive mutation, could be restored by the addition of proteins secreted into the medium during somatic embryogenesis [6, 7]. After fractionation of the medium proteins, the purified proteins responsible for somatic embryo rescue were identified as a cationic

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peroxidase and an acidic endochitinase, respectively [8, 9]. Based on the morphology of arrested and rescued somatic embryos, we proposed that these two secreted proteins have a function in the formation and maintenance of the embryo protoderm [10].

Recently, we reported the isolation of a cDNA clone corresponding to a 10 kDa extracellular protein in the medium of carrot somatic embryo cultures [11]. The cDNA-derived amino acid sequence of this protein, designated extracellular protein 2 (EP2), showed homology to non-specific lipid transfer proteins (nsLTP) in other plants. By *in situ* mRNA localization the EP2 gene was shown to be expressed in the protoderm of both zygotic and somatic embryos, and in epidermal cells of leaf primordia, flower organs and maturing seeds. The expression of the gene in (pro)epidermal cells and the extracellular location of the protein appear difficult to reconcile with the proposed function of plant nsLTPs in the transfer of phospholipids between intracellular membrane compartments [12]. Instead, we have proposed a role for the carrot EP2 protein in the secretion of cutin monomers [11]. Cutin is a large hydrophobic biopolymer present in the cuticle of aerial plant organs and is composed of esterified hydroxylated fatty acids. Cutin monomers are derived mainly from palmitic and/or oleic acid and are likely to be transferred towards the growing cutin polymer in the form of acyl-CoA esters [13]. Here we report on the purification of the EP2 protein from the medium of carrot somatic embryo cultures. The EP2 protein was able to enhance the transfer of fluorescent phospholipid analogs between artificial membranes, and to bind putative cutin monomers such as palmitic and oleic acid and oleyl-CoA. These results support the hypothesis that the EP2 protein is involved in cutin synthesis.

## Materials and methods

*Plant material and culture conditions*

Cultures from carrot (*Daucus carota* L. cv Trophy, Zaadunie B.V., Enkhuizen, The Netherlands) were started and maintained as described by De Vries *et al.* [6].

*Protein isolation and purification*

The EP2 protein was purified from the secreted proteins present in the medium of 12 days-old embryos cultures. 25 L medium were filtered through Whatman 1 MM paper followed by a 0.2 μm PVDF membrane (Millipore, Etten-Leur, The Netherlands). Proteins in the cell-free medium were concentrated approximately 160-fold by pressure dialysis using membranes with a 3 kDa cut-off (Amicon, Oosterhout, The Netherlands) and equilibrated with 50 mM MES buffer, pH 5.8. The concentrated proteins, designated ECP, were applied to a S-Sepharose FF (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) column equilibrated with 50 mM MES buffer, pH 5.8, and retained proteins were eluted with the same buffer containing 1 M KCl. The elution was monitored at 280 nm and the complete eluted protein fraction was collected, concentrated by pressure dialysis, and equilibrated in 50 mM K-phosphate buffer, pH 6.5, containing 150 mM KCl. This cationic protein fraction was size-fractionated on a Superdex 75 HR column (Pharmacia LKB Biotechnology Inc.) equilibrated and eluted with the same buffer by fast protein liquid chromatography (FPLC). Elution was monitored at 280 nm and fractions under protein peaks were analysed by SDS-PAGE, followed by silver staining and immunoblotting, using antiserum raised against a EP2-β-galactosidase fusion-protein, as described previously [8, 9, 11]. Fractions containing EP2 were pooled, concentrated, equilibrated in 50 mM MES buffer, pH 5.8, and applied to a Mono S column (Pharmacia LKB Biotechnology Inc.) for the final purification. Retained proteins were eluted (FPLC) with a linear 0–250 mM KCl gradient and the elution was monitored as described for the Superdex 75 column. The fractions containing purified EP2 were pooled and stored at – 20° C. Amounts of protein present in the several fractions obtained during the purification of EP2 were determined according to Bradford [14], Smith *et al.* [15], or by the absorbance at 280 nm. In all three methods BSA was used as a standard.

*Phospholipid transfer assay*

Phospholipid transfer activity of EP2 was tested by applying the fluorescent phospholipid analog 1-palmitoyl-2-[6-(1-pyrenyl) hexanoyl]-sn-glycero-3-phosphocholine (Pyr(6)PC) as described by Gadella and Wirtz [16]. Purified EP2, ECP and proteins from the medium of a non-embryogenic carrot cell line (SCP), were equilibrated in 20 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 1 mM EDTA and 1 mM EGTA, before they were used in the assay.

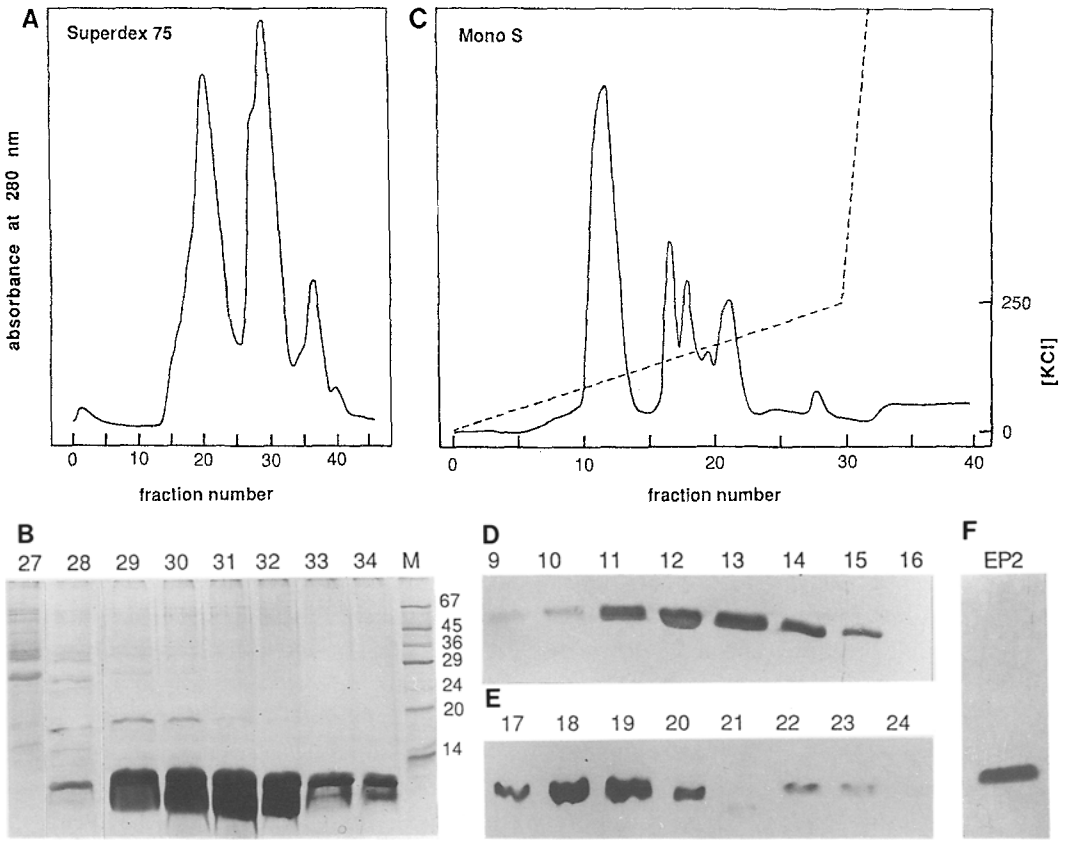


*Fig. 1.* Final steps in the purification of carrot nsLTP EP2. A) Elution profile of the Superdex 75 gel filtration, and B) analysis of the eluted fractions 27–34 by means of SDS-PAGE and silver staining. C) Elution profile of the Mono S cation-exchange chromatography, and C) and D) analysis of the eluted fractions 9–24 by means of immuno-blotting, using an antiserum raised against an EP2-β-galactosidase fusion-protein. E) SDS-PAGE gel, silver stained, of the pooled fractions 10–15, as analysed in C), showing a single protein band representing purified EP2. Lane M represents marker proteins with their molecular masses (kDa) indicated on the right.

*Fatty acid-binding assay*

EP2, ECP or SCP, equilibrated in the same buffer as described for the phospholipid transfer assay, were incubated with 2 nmol (220,000 cpm) $^{14}$C-labeled palmitic acid, oleic acid, or oleyl-CoA (Amersham, U.K.). After incubation for 30 min at room temperature, the mixture (100 µl) was applied to a 1 ml Sephadex G50 (Pharmacia LKB Biotechnology Inc.) column in a glass pipet to separate the free from the bound fatty acid or oleyl-CoA molecules. Fractions (100 µl) were collected, monitored by scintillation counting, and analysed for the presence of EP2 by SDS-PAGE. In a competition experiment the fractions containing $^{14}$C-oleyl-CoA bound to purified EP2 were pooled and reapplied to a Sephadex G50 column directly or after incubation for 30 min in the presence of unlabeled oleyl-CoA in a ten-fold higher concentration as the $^{14}$C-oleyl-CoA bound to EP2.

# Results

*Isolation and purification of EP2*

The purification of EP2 from the concentrated protein fraction isolated from the medium of 12 days-old embryo cultures (ECP) required three steps. After separation of the anionic from the cationic proteins by means of cation-exchange chromatography, the final purification was achieved in two steps by means of FPLC (Fig. 1). Aliquots from each of the fractions eluted from the Superdex 75 and Mono S column were analysed by SDS-PAGE, followed by silver staining (cf. Fig. 1B) and immuno-blotting (cf. Fig. 1D, E), using the antiserum raised against EP2-β-galactosidase fusion-protein [11]. This resulted in a preparation consisting of a single 10 kDa protein band on a silver stained SDS-PAGE gel (Fig. 1F).

Besides EP2, the antiserum recognized other small

proteins which eluted from the Mono S column (Fig. 1C, E). The relationship between these proteins and EP2 was not further investigated. Most likely these proteins represent modified forms of EP2, since they were not present in the medium of non-embryogenic cell lines (not shown), similar as reported for EP2 [11].

Determination of the final yield of the EP2 protein was complicated by observed discrepancies in determination of the protein concentration. Each of the three methods used gave different results (Table 1), making it difficult to quantitate both purification and yield. The protein-dye binding assay [14] was used routinely, but later experiments (see following sections) indicated that the BCA method [15] may be a more reliable estimate of the amount of purified EP2 obtained. The amount of purified EP2 estimated by the absorbance at 280 nm is probably an understatement, since EP2 contains only two aromatic acid (tyrosine) residues per molecule [11]. When the amount of EP2 protein was calculated on a basis of the molecular extinction of tyrosine at 280 nm, assuming that this is similar for the free amino acid and when present in a polypeptide, a figure was obtained close to that determined by the BCA method (5,1 mg; cf Table 1). Direct comparison of amino acid composition analysis with other protein measurements is in progress.

*Phospholipid transfer activity of EP2*

As shown in Fig. 2A, the addition of purified EP2 to Pyr (6)PC caused a shift in the fluorescence emission spectrum of the phospholipid analog towards that of the monomer (emission maxima at 378 and 495 nm). This indicated that EP2 is capable of binding Pyr(6)PC in its monomeric form.

An increase in the fluorescence of Pyr(6)PC monomers was also observed upon the addition of EP2 to quenched donor vesicles containing Pyr(6)PC (Fig. 2B). After equilibrium was reached, the addition of the acceptor vesicles caused the fluorescence to decrease first, going through a minimum, and to increase with time thereafter (Fig. 2C). The initial decrease in the fluorescence is probably caused by the rapid transfer of Pyr(6) PC bound to EP2 to the acceptor vesicles. The fluorescence quantum yield of Pyr(6)PC bound to EP2 was about 1.4 times higher than when the phospholipid analog was present in the acceptor vesicles. This is only slightly smaller as compared to the 1.75 times increase in the quantum yield obtained for pyrenyl-labeled lipids in nsLTP from bovine liver [16].

A more quantitative analysis with respect to the phospholipid transfer assay was hampered by the uncertainty in the determination of the amount of EP2 protein. By calibrating the Pyr(6)PC fluorescence in acceptor vesicles, the use of the 1.4 increase the quantum yield of Pyr (6)PC when bound to EP2, and the assumption that EP2 binds a single Pyr(6)PC molecule, it was calculated that 80% of the amount of protein applied to the assay mixture as estimated acording Bradford [14] would be occupied. This is extremely high, when compared to the 8% estimated for nsLTP from bovine liver in the same assay [16]. When the amount estimated according to Smith *et al.* [15] was used (cf. Table 1), it was calculated that about 2% of the EP2 protein molecules would be occupied by a single Pyr(6)PC molecule.

A similar binding and transfer activity as shown for purified EP2, was observed when unfractionated medium proteins from an embryogenic cell line were used (Fig. 2D–F). Medium proteins from a non-embryogenic cell line, however, failed to bind Pyr(6)PC monomers (Fig. 2E). Since non-embryogenic cell lines lack EP2 gene expression [11], this indicated that EP2 is probably the only nsLTP secreted in the medium of carrot embryogenic cell lines.

*Table 1.* Purification of carrot nsLTP EP2. The amounts of protein obtained during the purification of EP2 were determined by three methods based on different principles. In all methods the amounts of protein given are relative to BSA which was used as a standard.

| Fraction | Amount of protein (mg) | | |
|---|---|---|---|
| | Bradford | Smith *et al.* | A280 nm |
| ECP | 16 | n.d. | 251 |
| S-Sepharose, flow through | 13 | n.d. | 211 |
| S-Sepharose, salt pulse | 6.3 | 38 | 45 |
| Superdex 74, fractions 28–34 | 0.24 | 9.6 | n.d. |
| Mono S, fractions 10–15 (EP2) | 0.16 | 6.5 | 2.2 |

n.d. = not determined.

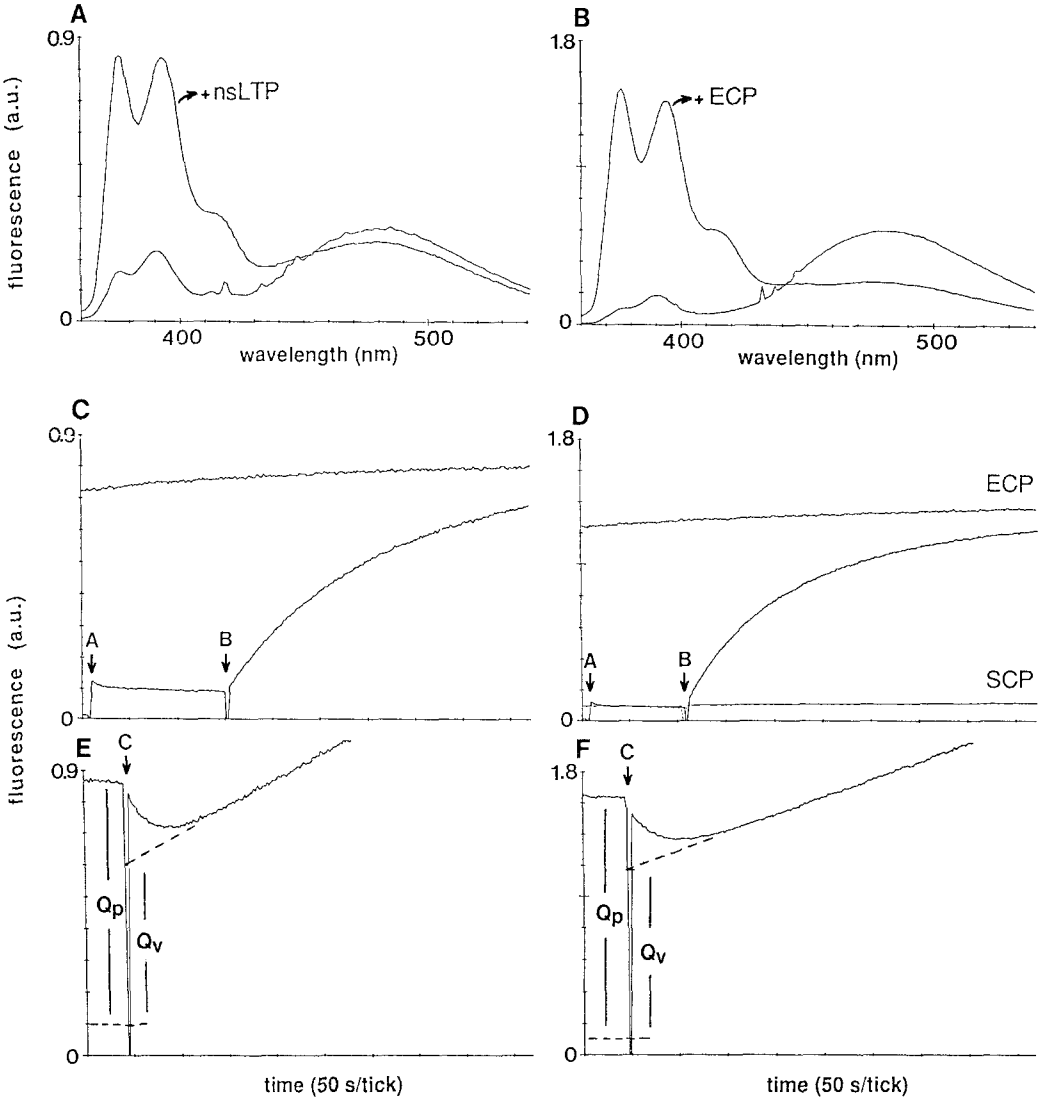


*Fig. 2.* Binding and transfer to acceptor vesicles of the fluorescent phospholipid analog Pyr(6)PC by carrot nsLTP EP2. A, B) Changes in the fluorescence emission spectrum (excitation at 346 nm) of 1 nmol Pyr(6)PC in 2 ml TBS-buffer (20 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 1 mM EDTA and 1 mM EGTA) in the presence of 0.5 µg purified EP2 (A) or 50 µg unfractionated embryo culture medium proteins (ECP). (B). C, D) Pyr(6)PC binding by EP2 (C) or ECP (D). Fluorescence emission of Pyr(6)PC was measured at 378 nm. Arrow A indicates the injection of 1.8 nmol Pyr(6)PC and 0.2 nmol N-[2,4,6-trinitrophenyl]-phosphatidylethanolamine (TNP-PE, a fluorescence quencher) in 20 µl ethanol into a cuvette containing 1.88 ml TBS-buffer. Arrow B indicates the addition of 0.25 µg EP2, 3 µg ECP, or 3 µg of unfractionated suspension culture medium proteins (SCP). E, F) Transfer of Pyr(6)PC to acceptor vesicles by EP2 (E) or ECP (F). Arrow C indicates the addition of 100 nmol acceptor vesicles (phosphatidylcholine : phosphatic acid = 95 : 5 mol %). The fluorescence quantum yield of Pyr(6)PC in EP2 relative to that in acceptor vesicles is calculated by dividing distance $Q_p$ by $Q_v$. In all instances the amounts of protein in the assay were based on determinations according to Bradford (22; cf Table 1).

*Fatty acid-binding activity of EP2*

To test whether EP2 might function as a carrier of cutin monomers, we used a gel filtration assay based on the difference in elution between bound and unbound $^{14}C$-labeled fatty acids or acyl-CoA esters. As putative cutin monomers we used palmitic and oleic acid and oleyl CoA. As shown in Fig. 3A, gel filtration of a mixture containing purified EP2 and oleyl-CoA, incubated for 30 min at room temperature, yielded two peaks. The first peak corresponded to the position at which also EP2 was eluted, whereas the second peak corresponded to the position of free oleyl-CoA (Fig. 3B). Unfortunately, the binding of oleyl-CoA to EP2 was lost upon gel electrophoresis, and we were therefore unable to detect the EP2-oleyl-CoA complex by means of autora-

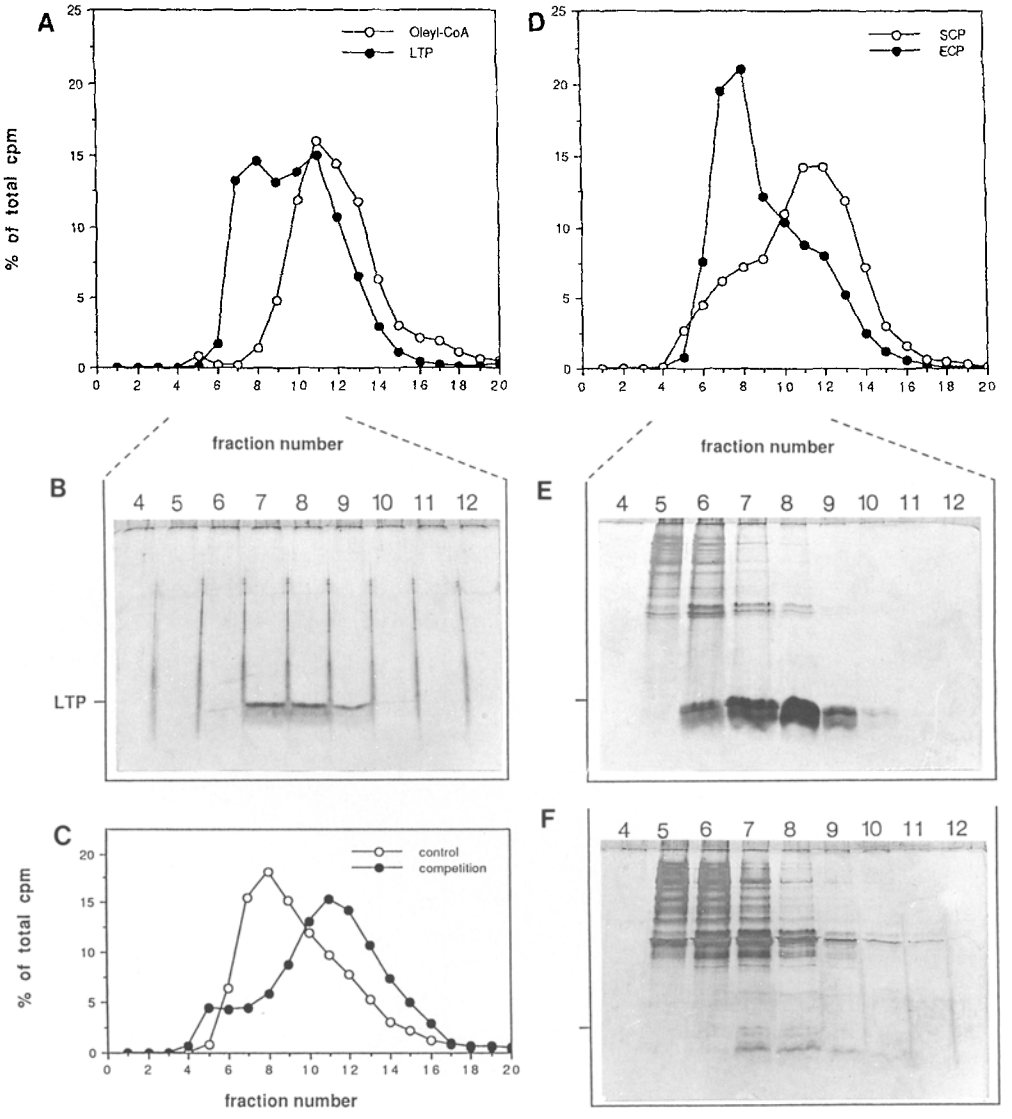


*Fig. 3.* Binding of oleyl-CoA by carrot nsLTP EP2. $^{14}$C-labeled oleyl-CoA (2 nmol; 220,000 cpm) dissolved in TBS buffer (20 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 1 mM EDTA and 1 mM EGTA) was incubated with 0.5 µg EP2 or 50 µg of proteins from the medium of an embryo culture (ECP) or suspension culture (SCP). The amounts of protein in the assays were based on determinations according to Bradford (22; cf Table 1). After incubation for 30 min, the mixtures (100 µl) were applied to 1 ml Sephadex G50 gel filtration columns and eluted with TBS buffer. Fractions (100 µl) were collected, the amount of radioactivity determined, and analysed for the presence of EP2 by SDS-PAGE and silver staining. A) Elution profile of oleyl-CoA alone (o) and after incubation with purified EP2 (•). B) SDS-PAGE analysis of the fractions corresponding to the first radioactive peak in A). Position of EP2 is indicated by LTP. C) Competition experiment in which fractions containing $^{14}$C-oleyl-CoA bound to EP2 obtained from a previous binding assay were pooled and applied to a new gel filtration column directly (control; o), or after incubation with 10 fold excess amount of unlabeled oleyl-CoA (competition; •). D) Elution profile of oleyl-CoA after incubation with SCP (o) or ECP (•). E, F) SDS-PAGE analysis of the fractions corresponding to the first radioactive peak in D) obtained after elution of the assay-mixture with SCP (E) or ECP (F). The position of purified EP2 is marked.

diography. By calculating the number of molecules oleyl-CoA which eluted in the first peak, and the assumption that EP2 binds either one or two oleyl-CoA molecules, we gain to the conclusion that the estimated amount of EP2 present in the assay mixture (0.5 µg) was much to low, even when all EP2 molecules were involved in the binding. Based on the amount of protein estimated by the BCA method (20 µg), about 10% of the EP2 protein molecules might have bound a single oleyl-CoA molecule, or half that amount when two oleyl-CoA molecules would bind to the protein.

To test whether the binding of oleyl-CoA to EP2 was

specific, a competition experiment was performed. As shown in Fig. 3C unlabeled oleyl-CoA could release $^{14}$C-labeled oleyl-CoA bound to EP2. A similar result could be obtained by using unlabelled oleic acid (not shown), indicating that it is the fatty acid part that is responsible for the binding to EP2.

The results obtained for palmitic and oleic acid (not shown) were comparable to those shown for oleyl-CoA, except that the recoveries of the radiaoctivity were lower as compared to oleyl-CoA, where recoveries were approximately 70%. This was probably due to a higher aspecific binding of these more hydrophobic molecules to the column material and glassware. Interestingly, however, these losses were always less when EP2 was present (not shown), suggesting that by binding to EP2 these hydrophobic molecules are easier kept in solution in an aqueous environment. Similar as for the phospholipid transfer assay, we also tested medium proteins from an embryogenic cell line for fatty acid binding properties. When these proteins were isolated from an embryo culture, binding of oleyl-CoA was observed, whereas less binding of oleyl-CoA was observed when an equal amount of medium proteins isolated from a suspension culture was used (Fig. 3D). Since EP2 was present in much lower concentrations in the medium of suspension cultures as compared to embryo cultures (Fig. 3E, F), this suggests that EP2 is the only secreted protein in carrot cultures capable of binding fatty acids and acyl-CoA esters.

## Discussion

As discussed previously [16], EP2 might function as a carrier of cutin monomers from their site of secretion, or synthesis, towards the growing cutin polymer. This was based on three observations, first, the homology of the cDNA-derived amino acid sequence with other plant nsLTPs, second, the extracellular location of the EP2 protein and third, the expression of the EP2 encoding gene in (pro)epidermal cells involved in cutin synthesis. In this paper we have shown that the EP2 protein is indeed an nsLTP capable of enhancing the transfer of phospholipids between membranes *in vitro*. More important with respect to its proposed function, are the results that indicated that the EP2 protein is capable of binding putative cutin monomers such as palmitic and oleic acid and oleyl-CoA. Though the results are still preliminary and presently lack kinetic analysis, we think they are in line with the proposed role of EP2 in the transport of cutin monomers. Plant nsLTPs have been isolated from several sources from different plant species, and based on their ability to transfer phospholipids between various types of membranes *in vitro,* it was thought for quite some time that this would be their function *in vivo* as well [12, 17]. However, the presence of a signal sequence in many of the identified plant nsLTPs (cf. 11 and references cited therein), indicating that they are synthesized on membrane-bound polysomes and most likely secreted, is difficult to reconcile with an intracellular function. It may very well be that several of these plant nsLTPs have a similar function as proposed for the carrot nsLTP EP2.

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## Note added in proof

While this paper was submitted, the amino acid composition of the purified EP2 protein was determined. The composition corresponded to that predicted by the EP2-cDNA. It also appeared that the closest approximate for the amount of EP2 protein purified was the A280nm. As a consequence the calculations concerning lipid transfer and fatty acid binding should be corrected accordingly.

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