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IN VITRO SYNTHESIS OF A PLANT PHOSPHOLIPID TRANSFER PROTEIN: A STUDY BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Summary . In order to study the biosynthesis of a plant phospholipid transfer protein (PLTP), poly (A)+RNAs have been prepared from maize seedlings and translated *in vitro* with a rabbit reticulocyte lysate. The newly synthesized proteins were then separated by fast protein liquid chromatography (FPLC) followed by SDS-PAGE or by high performance liquid chromatography (HPLC) coupled to a radioactivity detector monitor. It has been showed that a radioactive band comigrating with a ^{14}C methylated pure PLTP was detected by SDS-PAGE. This result, confirmed by direct radioactive monitoring, indicates that PLTP is actively synthesized *in vitro*.

Proteins able to transfer phospholipids between membranes ("phospholipid transfer proteins":PLTP), have been purified from various eukaryotic (animals, plants, yeasts), and prokaryotic (*Rhodopseudomonas sphaeroides*) cells (for reviews, see 1-4). In particular, plant tissues (maize seedlings or spinach leaves) contain 9-KDa proteins exhibiting a wide specificity for transferring phospholipids (5-7). The physiological roles of plant PLTP remain to be elucidated. It has been suggested that plant PLTPs participate in membrane biogenesis by transporting phospholipids from their sites of biosynthesis (i.e. endoplasmic reticulum) to membranes unable to form these phospholipids (8). On an other hand, the evolution of PLTP biosynthesis during plant growth and the regulation of this synthesis are still unknown.

Abbreviations : SDS, sodium dodecyl sulfate ; SDS-PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulfate ; HPLC, high performance liquid chromatography ; FPLC, fast protein liquid chromatography.

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In order to study the possible correlation between PLTP and membrane biogenesis and plant development , it is of interest to study the biosynthesis of PLTP in developing plant organs. In the present paper, poly (A)+RNAs have been isolated from maize seedlings and then translated in a rabbit reticulocyte lysate system. In vitro synthesized PLTP was then detected by fast protein liquid chromatography (FPLC) followed by SDS-PAGE or by high performance liquid chromatography (HPLC) coupled to a radioactivity detector. This observation indicates that mRNA for PLTP is present in young seedlings.

MATERIALS AND METHODS

Material : Maize seeds (Zea mays c.v. INRA 508) were germinated on moist vermiculite at 30°C in the dark for 3 days.

Preparation of maize PLTP : highly purified albumen maize PLTP was prepared as previously described (7). The protein was lyophilized before in vitro radiolabelling by a ^{14}C methylation technique according to Dottavio-Martin et al(9).

Isolation of RNA : seedling coleoptiles were ground to a fine power in liquid nitrogen. To the powder were added 100 mM glycine-NaOH (pH9), 100 mM NaCl,0.2% sodium dodecylsulfate,1% sodium deoxycholate. An equal volume of phenol/chloroform (1/1) was added ; the mixture was stirred 10 min at 4°C. The aqueous phase was separated by centrifugation for 10 min at 8,000 g . The phenol/chloroform extraction was repeated 3 times. To the final aqueous phase, one vol. of ether was added. After centrifugation, the ribonucleic acids were precipitated by adding 2 vols of ethanol to the aqueous phase which was left overnight at -20°C. The precipitate was collected by centrifugation, washed with 70% ethanol and resuspended in sterile 100 mM glycine-NaOH (pH 9), 100 mM NaCl, 0.1% SDS. The high molecular weight RNAs were allowed to precipitate for 2 - 4 h at 0°C in the presence of 3M sodium acetate, pH 7 , according to Laroche-Raynal et al (10).From this crude RNA extract, poly (A)+RNAs were isolated by two cycles of affinity chromatography on oligo(dT) cellulose. Fractions containing poly(A)+ RNAs were pooled and precipitated with two vols of ethanol in presence of 200 mM sodium acetate (pH 7) overnight at -20°C. The pellet was washed twice with 60 % isopropanol, dried down and dissolved in sterile distilled water to a final concentration of 0.5 µg. µl-1.

In vitro synthesis of PLTP : in vitro protein synthesis was carried out with the reticulocyte lysate kit from Amersham (U.K.). mRNA (0.25 -2 µg) were added to 10 µl of lysate previously supplemented with 20 µCi of ^{35}S-methionine (Amersham). After incubation for 1h at 30°C , the radioactivity was measured on 1 µl-aliquots. The reaction mixtures were then treated with 1 µl of RNase A + RNase T for 15 min at 30°C.

High performance liquid chromatography : a reverse phase chromatography column (Altex Beckman,4.6 x 75 mm) was used. The labelled protein mixture , to which 200 µg of purified PLTP were added, was injected to the column and eluted by a gradient of acetonitrile,as indicated in Figures 2 and 3. The column was connected to a computerized FPLC system

(Pharmacia,Sweden).The collected fractions were evaporated by heating (60°C) under nitrogen and then resuspended into a sample buffer:0.0625 M Tris-HCl (pH 6.8), 2 % sodium dodecyl sulfate , 10 % glycerol, 5 % 2-mercaptoethanol and 0.01 % bromophenol blue. The resuspended fractions were then analysed by SDS-PAGE. Alternatively, the column was connected to a HPLC equipment (Waters-Millipore,USA) ; the eluent of the column was passed through a computerized Flo-One radioactivity detector (Radiomatic,USA).

Gel electrophoresis analysis : one-dimensional gel electrophoresis was performed according to Laemmli (11) and Galle et al (12), using 15 % slab gels. After electrophoresis, the gels were prepared for fluorography (13). The gels were exposed to Kodak X-Omat R film at -60°C for several days before development. The following molecular mass standards have been used : trypsin inhibitor (21.5 kDa), cytochrome c (12.5 kDa), aprotinin (6.5 kDa), insulin (5.7kDa).

RESULTS

Analysis of in vitro synthesized proteins by SDS-PAGE.

Total cellular RNAs have been extracted from shoots of 3-day - old maize seedlings . After oligo(dT) cellulose chromatography, poly(A)+RNAs were translated in a reticulocyte lysate system. Addition of maize poly (A)+RNAs to the system gave a 10-fold stimulation of the ^{35}S-label recovered in the protein fractions (data not shown). The cell-free products were separated on a 15 % gel. As illustrated in Fig.1 (Lanes 1 to 4), several proteins were synthesized in the presence of maize poly(A)+ RNAs. In absence of maize RNA, only globin and a high-molecular weight protein were synthesized (Lane 5). Purified ^{14}C-methylated PLTP migrated in the 9-kDa zone (Lane 6) . 12.5 % gels were also run and high-molecular weight proteins (90 kDa) were observed , indicating that poly(A)+ RNAs used in these experiments had a good quality (data not shown).

Analysis of in vitro -synthesized proteins by FPLC coupled with SDS-PAGE.

The total in vitro synthesized proteins - to which 200 µg of pure maize PLTP have been added - were analysed by FPLC using a reverse phase column. The absorbance at 280 nm clearly indicated the elution time (about 15 min) of the PLTP (Fig.2). When the fractions corresponding to PLTP were analysed by SDS-PAGE, followed by fluorography, a single radioactive band was seen ; no other band was observed even after an exposure for one month. The detected band comigrated with a ^{14}C-methylated maize PLTP. Other eluted fractions were also analysed ; globin was eluted

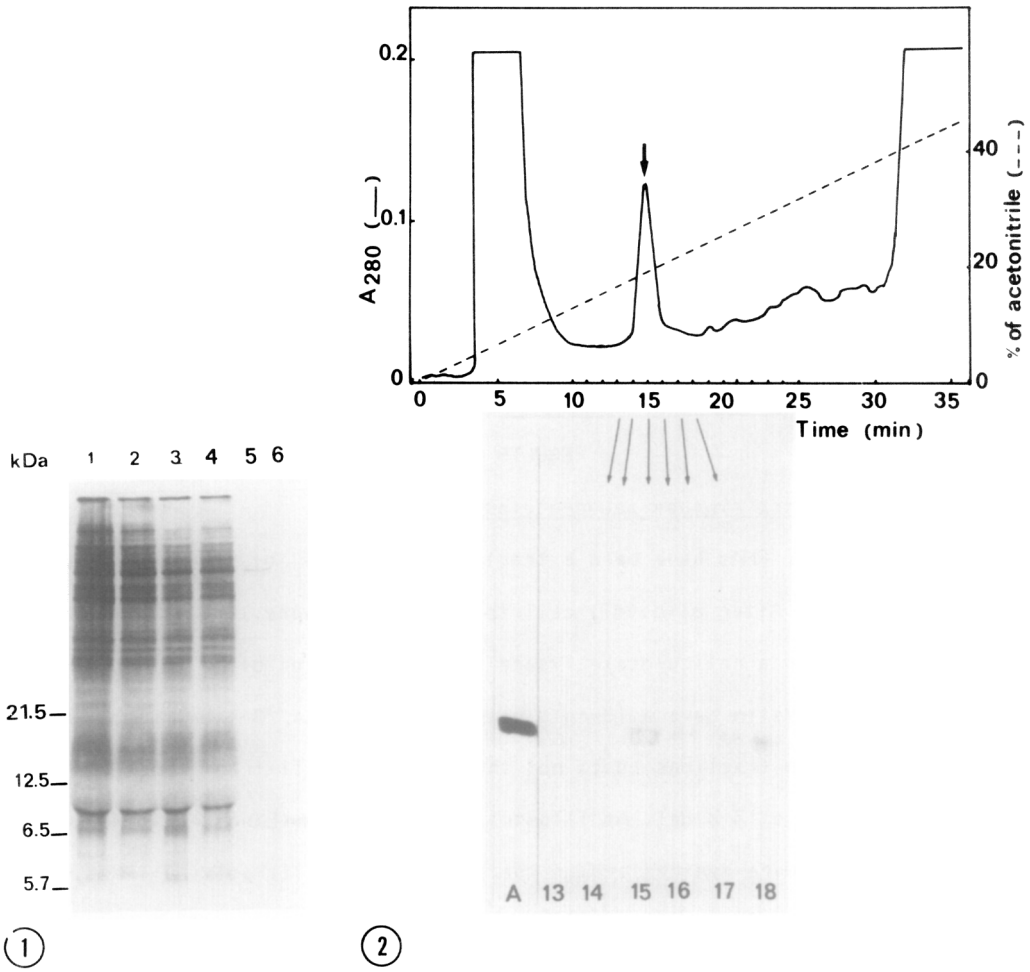

Fig.1 (at left). Fluorogram of SDS-PAGE showing in vitro translation of maize poly(A)+RNAs. Various amounts of poly(A)+RNA were translated in a reticulocyte lysate protein synthesizing system in the presence of ^{35}S-methionine. The RNA input in each assay were respectively : 2 µg (Lane 1) , 1 µg (lane 2), 0.5 µg (lane 3), 0.25 µg (lane 4), 0 (blank,lane 5). In lane 6, 5 µg of pure ^{14}C-methylated maize PLTP were deposited . The fluorogram was obtained after a 2 day-exposure on a Kodak X-Omat R film.
Fig.2 (at right). Analysis of in vitro synthesized proteins by FPLC followed by SDS-PAGE. Poly (A)+RNA (1µg),isolated from maize seedlings, were translated in vitro in a rabbit reticulocyte system. The mixture of newly synthesized proteins, to which 200 µg of pure maize PLTP were added, was injected into a reverse phase column connected to a FPLC equipment. 1ml-fractions were eluted at a flow rate of 1 ml . min^{-1} with 8 % of acetonitrile /0.1 % trifluoracetic acid. A gradient of acetonitrile eluted the protein (arrow).

after 30 to 35 min, whereas fractions 3 to 9 corresponded to the void volume (data not shown).

Analysis of in vitro synthesized proteins by HPLC coupled to a radioactivity detector.

Alternatively, the products of in vitro translation were also analy-

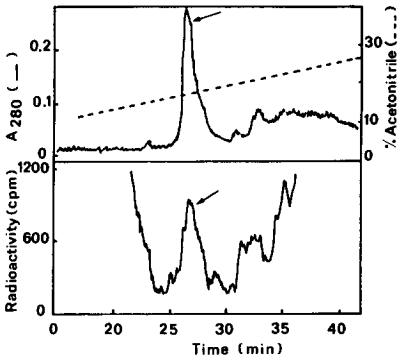

Fig.3. Analysis of *in vitro* synthesized proteins by HPLC coupled to radioactivity monitoring. The same mixture of newly synthesized proteins used in Fig.2, to which 200 µg of pure maize PLTP were added, were chromatogaphed in a reverse phase column (Altex-Beckman) connected to a HPLC equipement coupled to a radioactivity detector. The elution conditions were similar to those followed in the experiment of Fig.2, excepted that the shape of the gradient of acetonitrile was modified. The eluent was mixed with scintillation mixture (Lumaflow) in a 2.5 ml-cells ; the flow rate of the pump was 3 $ml.min^{-1}$. The upper part of the figure shows the UV profile (at 280 nm) of the eluted fractions ; PLTP, added to the *in vitro* synthesized proteins, was indicated by an arrow. The lower part of the figure gives the distribution of ^{35}S-label recovered in the eluent ; a radioactive peak, corresponding to PLTP, was observed.

sed by HPLC coupled to a radioactivity monitoring equipment. The UV profile indicates that the maize PLTP was eluted 25 min after the injection (Fig.3). This result has been repeatedly obtained with several experiments. Alternatively, in some chromatographic separations,only a half of the eluent was counted by using a splitting device ;fractions of the peak were collected and analysed by SDS-PAGE ; a radioactive band comigrating with the labelled PLTP was observed (data not shown). This confirms that the radioactive peak indeed corresponds to the newly synthesized PLTP.

DISCUSSION AND CONCLUSIONS

This paper presents the first report on the *in vitro* biosynthesis of a phospholipid transfer protein. This study has been carried out on a plant protein from maize seedlings ; we have also recently observed that spinach leaf also contains RNAs active for *in vitro* synthesis of PLTP (unpublished). To our knowledge, no report has been published for the *in vitro* synthesis of PLTP from animal cells. However, recent studies on

the synthesis of fatty-acid binding proteins (FABP ; SCP1) have been carried out in rat liver (14).

The detection of active mRNA for PLTP opens new possibilities for the study of PLTP accumulation during plant development. FPLC or HPLC techniques were preferred to the classical method of immunoprecipitation since plant PLTPs ,which have a low molecular mass and a high pI, are weakly immunogenic (Grosbois,unpublished). In the present work, proteins have been separated according to their degree of hydrophobicity on a reverse phase column connected to two equipments of high performance liquid chromatography . The same results have been obtained with the two techniques. However, the direct monitoring of radioactivity of the eluent of a HPLC column, which can be connected to a FPLC or HPLC equipment, allows a fast analysis since PLTP is eluted in less than 30 min.

The approach followed here opens new perspectives for the study of of lipid transfer proteins. This method could also be used with other proteins after determination of their conditions of elution through the reverse phase column. This technique could allow a purification of a protein from a crude extract in one or two steps ; another advantage of this method is the shorter time needed for the separations (less than 1 hour instead of several hours or days with classical low pressure columns).

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