Volume 47(1-4):7-10, 2003
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Lipid rafts in the plant plasma membrane?

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ABSTRACT In order to study the molecular characteristics of an integral membrane protein, first the protein should be solubilized. Ascorbate-reducible *b*-type cytochromes are highly hydrophobic integral membrane proteins with six trans-membrane α-helices. A fully ascorbate-reducible *b*-type cytochrome was easily solubilized by Triton X-100 from phase partition-purified plasma membranes of 5-day-old etiolated bean (*Phaseolus vulgaris* L.) hooks (Trost et al. 2000). However, under the very same conditions, a similar protein in the phase partition-purified plasma membrane of 9-week-old green *Arabidopsis thaliana* leaves seemed to be very resistant to solubilization with Triton X-100 (Bérczi et al. 2001). It was assumed that the composition of lipids in the plasma membrane of the two different tissues might influence the solubilization. Results obtained with thin layer chromatography revealed that both quantitative and qualitative differences exist between the lipid composition of the two plant plasma membranes. The observations are discussed in the light of possible existence of "lipid rafts" in the plant plasma membranes.
Acta Biol Szeged 47(1-4):7-10 (2003)

KEY WORDS

lipids
plant plasma membrane
rafts
solubilization
Triton X-100

Integral membrane proteins with trans-membrane localization play crucial roles in biological transport processes. Numerous members of a newly-recognized protein family, the cytochromes *b*-561 (cyts *b*-561), function as electron transporters both in animal and plant membranes (Asard et al. 2000, 2001). These proteins are highly hydrophobic, fully ascorbate-reducible, have 6 trans-membrane α-helices, and transport electrons through different biological membranes. Presence of *b*-type cytochromes in plant plasma membranes (PM) were first demonstrated in the microsomal fraction from etiolated corn coleoptiles (Jasaitis et al. 1977) then, together with other *b*-type cytochromes, in the highly-purified PM vesicles from different plant tissues (Asard et al. 1989; Askerlund et al. 1989). Purification and partial characterization of an ascorbate-reducible *b*-type cytochrome (asc-red. cyt *b*) from the phase partition-purified PM was first successful from etiolated bean hypocotyl hooks by solubilization with Triton X-100 as detergent (Trost et al. 2000). When a similar protein was tried to be solubilized, under identical conditions, from the phase partition-purified PM of 9-week-old green *Arabidopsis thaliana* leaves as well as from that of 5-day-old corn (*Zea mays* L.) roots, the PM proteins seemed to be very resistant to Triton X-100 (Bérczi et al. 2001).

It has long been known that lipids occur in several states in lipid bilayers as well as in biological membranes. The two most well-known states (or phases) are the "lamellar liquid crystalline" (L_α) and the "lamellar gel" (L_β) phases (Caffrey and Cheng 1995; Nagle and Tristram-Nagle 2000; Simons and Toomre 2000). Recent work suggests that lipids in biological membranes may also exist in a third phase (or physico-chemical state) that may be of biological significance; it is the "liquid ordered" phase (L_o) with properties intermediate between L_α and L_β (Brown and London 1998). It has also been shown that membrane fractions in L_o phase, which are enriched in sphingolipids and sterols (ST), are insoluble with non-ionic detergents (*i.e.* Triton X-100); these microdomains are called "lipid rafts" (Simons and Ikonen 1997; Rietveld and Simons 1998; London and Brown 2000). Lipid rafts containing a given set of proteins (*i.e.* heterotrimeric G-proteins and their receptors, GPI-anchored proteins, etc.) can change their size and composition in response to different stimuli, among them to solubilization too. Although there is a remarkably large proportion of lipids, with potential to form rafts, present in both the animal cell membrane (Mayor and Maxfield 1995) and the plant plasma membrane (Rochester et al. 1987; Uemura and Steponkus 1994), occurrence and analysis of lipid rafts in plant plasma membranes has rarely been reported (Peskan et al. 2000). It is possible that the different capability of Triton X-100 in solubilizing the asc-red. cyts *b* from different tissues refers to the presence of different amount of special lipids and sterols participating in formation of liquid-ordered and liquid-disordered phase domains (lipid rafts) in the plant PM too. This hypothesis is strongly supported by the results of lipid analysis of corn (*Zea mays* L.) root PM vesicles (Bohn et al. 2001).

Accepted September 3, 2003
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In the present short paper, experimental evidence is presented for the different composition of PM vesicles purified from etiolated *Phaseolus* hypocotyl hooks and green *Arabidopsis* leaves. Thin layer chromatography (TLC) analysis of total lipids extracted from the tissues revealed that sterols and cerebrosides (CER), the two major and potent compounds of lipid rafts, are present in much higher amount in the PM from green *Arabidopsis* leaves than in that from etiolated *Phaseolus* hooks.

Materials and Methods

Chemicals

Standards for phospholipids [PC (1,2 diacyl-sn-glycero-3-phosphocholine); PE (1,2-dicayl-sn-glycero-3-phosphoethanolamine); PG (1,2-diacyl-sn-glycero-3-phospho-(1-rac-glycerol)); PI (phosphatidylinositol 1,2-diacyl-sn-glycero-3-phospho-(1-D-myo-inositol)); PS (1,2-diacyl-sn-glycero-3-phospho-L-serine)] as well as standards for sterols (Stigmasterol, Campesterol, β-sitosterol) were purchased from Sigma-Aldrich. Standards for sphingolipids (Glucoserebrosides, Ceramide, Phytosphinanine, D-erythro-sphinganine) were ordered at Avanti Polar Lipids (USA). Solvents were of HPLC-grade and purchased from Lab-Scan analytical sciences (Belgium).

Plant material and PM preparation

Etiolated seedlings of bean (*Phaseolus vulgaris* L. cv. Limburgse Vroege) were grown for 5 days in the dark on moist vermiculite at 25°C. Hypocotyl hooks (150 g fresh weight) were harvested on ice and used for PM preparations (Asard and Bérczi 1998).

Arabidopsis thaliana (L.) Heynh (ecotype Columbia) was grown as described earlier (Bérczi et al. 2001). Leaves of 9-week-old seedlings (seedlings just before flowering) were harvested and immediately used for PM preparation (Bérczi et al. 2003).

PM vesicles were prepared by aqueous polymer two-phase partitioning (Larsson et al. 1994); the final PM-rich upper phases (U_3+U_3') were diluted 6-fold by 10 mM Tris-KOH buffer, pH 8 and pelleted by centrifugation at 50,000 g_{max} for 90 min. Pelleted PM vesicles were re-suspended in 10 mM Tris-HCl, pH 8, containing 1% (w/v) glycerol and stored at -75°C until use.

Stripping and solubilization

PM vesicles from two-three preparations (2-3 times 4 ml) were taken from the deep-freezer, them thaw up at room temperature and combined to obtain 12-15 mg PM protein. Loosely-bound proteins and proteins trapped in the lumen of sealed vesicles were removed by a one-step stripping protocol as follows. The 12 ml solution of PM vesicles was first diluted to 50 ml by addition of 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, then 2 µl of nonaethylene glycol monododecyl ether ($C_{12}E_9$, a non-ionic detergent) were added (final detergent concentration was below the critical micellar concentration of $C_{12}E_9$) and the PM vesicles were incubated with gently but continuous stirring at room temperature for 20 min. After 20 min 5.5 ml of 5 M KCl was added (final KCl concentration of 0.5 M) and the PM vesicles were further incubated for 40 min. The so called "stripped" PM vesicles were pelleted at 100,000 g_{max} and 4°C for 45 min. The pellet was re-suspended in 10 mM Tris-HCl, pH 8.0, 1% (w/v) glycerol and stored at –75°C until use.

Lipid extraction and thin layer chromatography

Total lipids were extracted from stripped PM vesicles as described by Bligh and Dyer (1959) with minor modifications (Bohn et al. 2001). Briefly, vesicles with 1 mg of protein from both PM preparations were washed in 0.5 M KCl, 1 mM Na_2-EDTA and pelleted at 100,000 g_{max} and 4°C for 60 min. Each pellet was resuspended in 1 ml ion-exchanged (Milli-Q) water. To the 1 ml suspension 3.75 ml of chloroform:methanol (2:1, v/v) were added and rigorously vortexed for 10 min. Then 1.25 ml chloroform was added and vortexed for 1 min and finally 1.25 ml of 1 M NaCl was added and again vortexed for 1 min. Phase separation was accomplished by centrifugation at 5,000 g_{max} and 4°C for 10 min. The lower phase was collected with a Pasteur pipette. After evaporation of the organic solvents, the lipid extract was dissolved in 100 µl of chloroform and used immediately for TLC.

Major lipid classes were separated by two dimensional TLC on silica gel (DC-Platten 20x20 cm Kieselgel 60, Merck KGaA, Darmstadt, Germany) as described by Kates (1972) with minor modifications. Briefly, plates had been heated at 100°C for 12 h before use. For spotting, 25 µl of lipid extract was used. Separation was by chloroform-methanol-18% ammonia (65:35:5, v/v; first direction) and then by chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1, v/v; second direction). Phosphatidic acid (PA), phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), phosphatidyl inositol (PI), phosphatidyl serine (PS), cerebrosides and sterols were run one-by-one under identical conditions on separate plates as standards. Lipids were detected in a chromatographic tank saturated with iodine vapor for 10 min. Developed plates were immediately scanned by a Umax Powerlook 2000 scanner at 400 dpi. Images were processed with Adobe Photoshop. Spots were analyzed with OptiQuant version 02.50 software (Packard Instrument Co., Meriden, CT, USA).

Results presented are averages from four independent experiments and eight 2D-TLC separations. T-test (P=0.01) was used to confirm significance.

Results and Discussion

It has recently been shown that the solubilization yield of proteins from PM vesicles purified from *Arabidopsis* leaves and corn roots is much lower (about 35%) than that from bean hypocotyl hooks (about 95%; Bérczi et al. 2001). It has also recently been shown that corn root PM contains about as much free sterols (40.8 mol%) as phospholipids (43.9 mol%) in the PM. These results are in agreement with earlier lipid analysis results for the corn root PM (Grandmougin et al. 1989; Cowan et al. 1993). Also, lipid analysis results for leaf PM from different species revealed the presence in high molar percentage of free sterols and cerebrosides (Rochester et al. 1987; Uemura and Steponkus 1994). Literature data thus support the hypothesis that the low solubility of integral PM proteins with Triton X-100 from corn roots and *Arabidopsis* leaves might be due to the presence of lipid compounds capable of formation of insoluble lipid microdomains.

In the light of the data in literature it seems that it is not the low solubility of PM proteins from *Arabidopsis* leaves and corn roots but rather the high solubility of PM proteins from bean hooks is exceptional. Would it be supported by the lipid composition of bean hook PM? In order to answer the question total lipids from both bean hook and *Arabidopsis* leaf PM were extracted and analyzed for the major phospholipid compounds, cerebrosides and for sterols by 2D-TLC (Fig. 1) and densitometry (Fig. 2).

According to Fig. 2, no significant changes can be found in the phospholipid composition of PM vesicles isolated from etiolated bean hypocotyl hooks and Arabidopsis leaves. Practically, both PM is composed of 34% PA, 23%PC, 23% PE, 11% PS, 6% PI and traces of PG. When specific staining for phospholipids was performed (phosphate staining), results were similar. However, significant differences were found for the spots representing FFA, CER and ST. FFA were present in slightly higher amounts in the PM of bean hooks. CER are present in higher concentrations in the PM of Arabidopsis leaves, roughly twice the amount detected in the PM of etiolated bean hypocotyl hooks. Care must be taken interpreting the result as CER was measured as an area where cerebrosides were found under the 2D-TLC conditions applied. It means that co-localization of other unknown

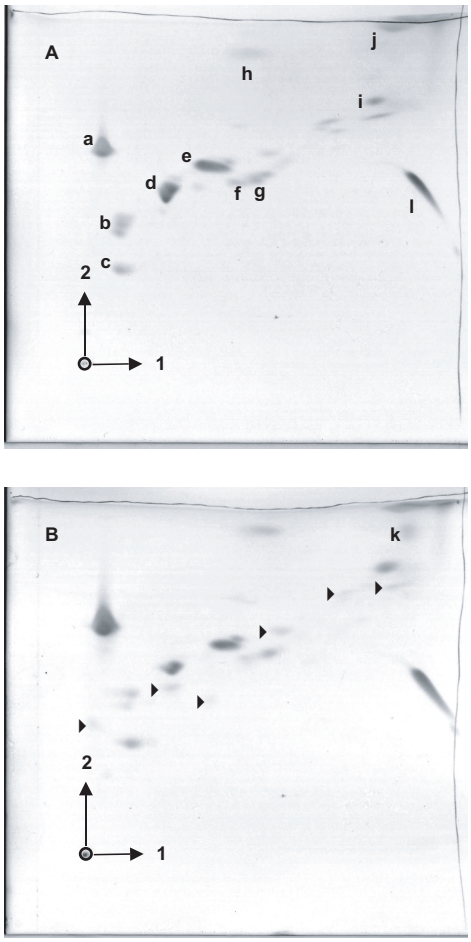

Figure 1. Two dimensional TLC of total lipid extracts from PM purified from etiolated bean hypocotyl hooks (A) and from green *Arabidopsis* leaves (B). Numbers 1 and 2 refer to the first and second directions in separation. Separation was by chloroform-methanol-18% ammonia (65:35:5, v/v; direction 1) and then by chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1, v/v; direction 2). Lipids were detected with iodine vapor for 10 min. Identification of spots were by running and developing standards under identical conditions. Standards were phospatidic acid (a), phosphatidyl serine (b), phosphatidyl inositol (c), phosphatidyl coline (d), phosphatidyl ethanolamine (e), phosphatidyl glycerol (f), cerebrosides (g), and sterols (i+ k). Free fatty acids (FFA; h) and neutral lipids (j) were identified on the basis of literature (Kates 1972). Identity of a major compound (l) could not be determined with this technique. Minor spots with significantly different intensity on the two plates are labeled with arrow-heads; their identification awaits for further studies.

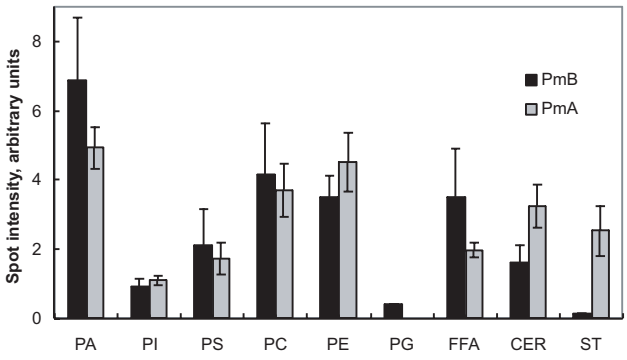

Figure 2. Distribution of lipids in the PM from etiolated bean hypocotyl hooks (black columns, PmB) and green *Arabidopsis* leaves (gray columns, PmA). PA, phospatidic acid; PI, phosphatidyl inositol; PS, phosphatidyl serine; PC, phosphatidyl coline; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; FFA, free fatty acids; CER, cerebrosides; ST, sterols. Columns marked with an asterix represent significant differences (P = 0.01).

compounds cannot be excluded. Amount of STs were about tenfold higher in the PM from *Arabidopsis* leaves as compared to that from etiolated bean hypocotyl hooks. However, the overall sterol concentrations appear to be rather low as compared to literature data. This might be due to the lower response of sterols to iodine staining. A specific sterol staining however proved that the amount of sterols in PM from *Arabidopsis* leaves was in agreement with literature data but that from etiolated bean hypocotyl hooks was significantly less. Sterol results are further clouded by the problem of separating them from NL in some cases.

Since the amount of CER and ST (1) appears to give the most significant differences in the composition of PMs from the two sources and (2) CER and ST are the most important lipid components for raft formation, future experiments will focus on more accurate determination of these compounds (by HPLC with ELSD detection).

In summary, it can be concluded that (1) the relative low abundance of sterols and some lipid compounds in the bean hook PM, as compared to the *Arabidopsis* leaf PM as well as to other leaf and root PM published in the literature, might provide proper condition for the high solubility of this PM by Triton X-100, and (2) low solubility of plant PM with Triton X-100 can be the consequence of formation of lipid rafts insoluble by nonionic detergents, like it is so in the case of animal cell membranes.

Acknowledgments

This work was supported by grants from the Flemish (BIL 99/48) and Hungarian (OMFB B-4/99) governments, and the Hungarian National Science Foundation (OTKA T-034488). Appreciation is expressed to Profs. Dr. J. Geuens (University of Leuven) and Dr. Roland J. Caubergs for valuable discussions as well as to Hilde Van Hemelrijk and Eddy Biebaut (all from the University of Antwerp) for technical assistance.

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