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Transmembrane topography of plasma membrane constituents in mung bean (*Vigna radiata* L.) hypocotyl cells. II. The large scale asymmetry of surface peptides

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Abstract

The large scale asymmetry in surface (poly)peptides of the plasma membrane (PM) of mung bean (*Vigna radiata* L.) hypocotyl cells was investigated by protease and 1 M KCl treatments of PM vesicles obtained by an aqueous two-phase partition technique. Proteases only slightly reduced the protein content of right-side-out PM vesicles and the treatment with 1 M KCl resulted in the dissociation of only a few peripheral proteins from the outer surface of right-side-out PM vesicles, indicating that few surface peptides including peripheral proteins existed on the outer surface. From experiments of the re-partitioning of endomembrane vesicles removed from surface peptides, it was found that the surface peptide content is a factor determining the partitioning, and the hypothesis that sterols are asymmetrically distributed across higher plant PM was proposed. We speculate that asymmetrical properties between the outer and the inner surfaces of plant PM, especially in partitioning in the two-phase system, derive from the asymmetry of the bulk of surface peptides and PM sterols. The comparatively low hydrophilicity of the outer surface of the PM would be important for the partitioning of right-side-out PM vesicles in the upper phase of the two-phase system.

Keywords: Plasma membrane; Endomembrane; Higher plant cell; Asymmetry; Surface peptide; Sterol; Two-phase partitioning

1. Introduction

The plasma membrane (PM) of higher plant cells exhibits asymmetry in surface properties between the outer (exofacial) and the inner (cytofacial) surfaces, as described in the previous paper in this series [1]. It is indicated that some of the PM constituents asymmetrically distribute across the membrane.

We previously indicated that the phospholipid (PL) topography in PM of higher plant cells does not contribute to the asymmetrical surface properties because the major PLs were almost symmetrically distributed across the PM of mung bean [1].

Are the glycolipids and the oligosaccharides of glycoproteins exposed on the outer surface [2–4] the main factor responsible for the asymmetrical properties? Do they render right-side-out PM (RO-PM) vesicles preferentially partitional in the polyethylene glycol (PEG)-rich upper phase of aqueous two-phase systems [2,5]? However, endomembranes that prefer-

Abbreviations: PEG, polyethylene glycol; G_u, partition ratio in the upper phase in an aqueous polymer two-phase system; M.W., molecular weight; PL, phospholipid; PM, plasma membrane; RO-PM, right-side-out PM; ζ-potential, electric potential of particles at the hydrodynamic plane of shear

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entially partitioned in the dextran-rich lower phase also contained glycolipids and a considerable quantity of oligosaccharides [6]. Furthermore, the carbohydrates of glycoproteins abundant on the cell surface are oligosaccharides and seem to have an affinity for dextran T500, which is also an oligosaccharide, in the lower phase. Therefore, the asymmetry of the carbohydrates would not be the only factor determining the asymmetrical surface properties of higher plant PM.

Other candidates are surface peptides (peripheral parts) of PM proteins and sterols. Barber [7] suggested that glutamic and aspartic acid residues on the proteins mainly contribute to the negative charges of thylakoid membrane. It is possible that the surface properties of the membrane are largely affected by the surface peptides according to their quantity and quality. The asymmetrical transmembrane distribution of PM sterols would also contribute to the difference in the surface properties, if it exists [8].

In the present study, we investigated the transmembrane distribution of the bulk of the surface peptides in the mung bean PM, and indicated that there exists marked asymmetry of not only soluble surface peptides but also PM sterols. We also discussed why RO-PM vesicles can be obtained by two-phase partitioning.

2. Materials and methods

2.1. Chemicals

Trypsin (from porcine pancreas), soybean trypsin inhibitor, and dibucaine were purchased from Sigma. Proteinase K was from Roche. All other reagents were of analytical grade.

2.2. Plant material and membrane preparations

RO-PM vesicles were obtained from mung bean (*Vigna radiata* L.) hypocotyls by aqueous two-phase partitioning utilizing a phase system composed of 6.2% (w/w) dextran T500 and PEG 3350, 0.25 M sucrose, 3 mM KCl, and 5 mM potassium phosphate (pH 7.8) as described previously [1]. High purity RO-PM vesicles were obtained from U_3+U'_3, and endomembrane vesicles from L_1 after the batch procedure [9]. Membranes in U_1, U_2+U'_2 and L_2 were also collected. All the membrane vesicles were finally suspended in Suc-K-Pi buffer containing 0.25 M sucrose, 3 mM KCl, and 5 mM potassium phosphate (pH 7.8). Freshly prepared membrane vesicles were used for the experiments.

2.3. Protein and lipid quantification

Protein was quantified by the method of Bradford [10]. The method of Peterson [11] was also utilized especially when protein samples were in a solution containing a large amount of KCl or sucrose (see Section 2.7). BSA was used as the standard.

The suspension of membranes was boiled for 3 min and total lipids were extracted by the method of Bligh and Dyer [12]. Total membrane PLs and sterols were then quantified by the method of Rouser et al. [13] and Zlatkis and Zak [14], respectively. For the determination of sterol content, cholesterol was used as the standard.

2.4. Protease treatment of membrane vesicles

The suspensions of membrane vesicles were adjusted to 2 mg of membrane protein ml^{-1}. In some experiments, 0.5% (w/v) Brij 58 was added to the suspension of RO-PM vesicles to convert them to inside-out PM vesicles [15]. Trypsin (1:5, w/w, trypsin:membrane protein) or proteinase K (1:30, w/w, proteinase K:membrane protein) was added to the suspension and kept at 25°C for 30 min. 0.03% (w/v) Triton X-100 was utilized to render the peptides on both surfaces accessible to the protease. The trypsin and proteinase K treatments were terminated by addition of soybean trypsin inhibitor (1.5:1, w/w, trypsin inhibitor:trypsin) and 1 mM PMSF, respectively. For the control experiment, the protease and its inhibitor mixed beforehand were added to the membrane suspensions in the first step of the reaction.

The degree of proteolysis was evaluated by SDS–PAGE as described below.

2.5. Electrophoresis and glycopeptide staining

One-dimensional SDS–PAGE was run on a 14%

acrylamide mini-sized gel essentially according to Laemmli [16]. For glycopeptide staining, proteins on the gels were electroblotted onto a PVDF membrane with a Bio-Rad Mini Transblot apparatus. Glycopeptides on the membrane were visualized by carbohydrate detection [17] using a Bio-Rad Immun-Blot Kit for glycoprotein detection. In some cases, gel images were taken using a scanner JX-330 with green light (Sharp, Osaka, Japan), and the total integrated density and the density profile of each lane in the gel image were measured with software for image analysis, Bio-Rad Molecular Analyst and ImageMaster (Pharmacia Biotech), respectively.

2.6. 1 M KCl treatment of membrane vesicles

To dissociate peripheral proteins from the outer surface of membrane vesicles, the vesicles were incubated in 5 mM potassium phosphate (pH 7.8) or 10 mM MES–Tris (pH 7.3) containing 1 M KCl with a final concentration of 0.2 mg protein ml^{-1} and stood for 30 min on ice. For the control, the membrane vesicles were incubated in Suc-K-Pi buffer.

The membranes were sedimented at 4°C, and the proteins recovered in the supernatant were quantified according to Peterson [11] to determine the percentage of protein released from the membranes. In parallel, the PLs in the supernatant were also quantified and the recovery of PLs of the membranes in the supernatant was determined.

To dissociate peripheral proteins on the inner surface of the vesicles and release them outside of the vesicles, membrane vesicles were incubated in a 1 M KCl solution containing 1.5 mM dibucaine [18] and stood for 30 min at 20°C. The incubation mixture was frozen in liquid N_2 and thawed in water at 25°C twice. After the mixture was centrifuged, released proteins and PLs recovered in the supernatant were quantified.

2.7. Re-partitioning of protease- and 1 M KCl-treated endomembrane vesicles

One milligram of protein of endomembrane vesicles (L_1) was subjected to protease treatment in 0.5 ml of Suc-K-Pi buffer or 1 M KCl treatment (without dibucaine). The 1 M KCl-treated endomembranes sedimented by centrifugation at $100\,000 \times g$ for 30 min were washed with 5 mM potassium phosphate (pH 7.8) and 3 mM KCl, and then pelleted again to thoroughly eliminate the remaining 1 M KCl solution. Then, the resultant pellets were resuspended in 0.5 ml of Suc-K-Pi buffer. These suspensions of 0.5 ml were added to a 3.5 g two-phase system with a final composition of 5.6% or 6.2% (w/w) dextran T500 and PEG 3350, 0.25 M sucrose, 3 mM KCl, and 5 mM potassium phosphate (pH 7.8). After thorough mixing and phase settling, the upper phase was taken without disturbing the interface. Membrane vesicles partitioned in the upper phase were collected with ultrafiltration membranes (Centricon 30, Amicon, Beverly, MA, USA). Lipids were extracted from the retentate, and the sterol and PL contents were then assayed. In parallel, the lipid contents of 1 mg of protein of endomembrane vesicles before the treatments were also quantified. The partition ratio in the upper phase of the membrane vesicles (G_u), was estimated from the G_u of the sterol and the PL content. The control samples for the protease treatment were treated in the same way except that boiled proteases were utilized.

2.8. Measurement of ζ-potentials

The ζ-potential of the membrane vesicles was measured in an electrophoretic light scattering spectrophotometer (model ELS-8000, Otsuka Electrics, Osaka, Japan) according to Ahn et al. [19]. Suc-K-Pi buffer was utilized for the electrophoretic medium.

3. Results

3.1. Proteolysis of polypeptides on membrane vesicles

The quantitative asymmetry in the bulk of surface peptides on PM vesicles was estimated by protease treatment.

Fig. 1 shows the proteolysis of RO-PM vesicles, inside-out PM vesicles created with Brij 58, and endomembrane vesicles by trypsin (Fig. 1A) or proteinase K (Fig. 1B). The bands corresponding to trypsin, trypsin inhibitor, contaminants (X) of low molecular weight (M.W.), and proteinase K were ignored for the evaluation of proteolysis. Small peptides generated as a result of the proteolysis must have passed

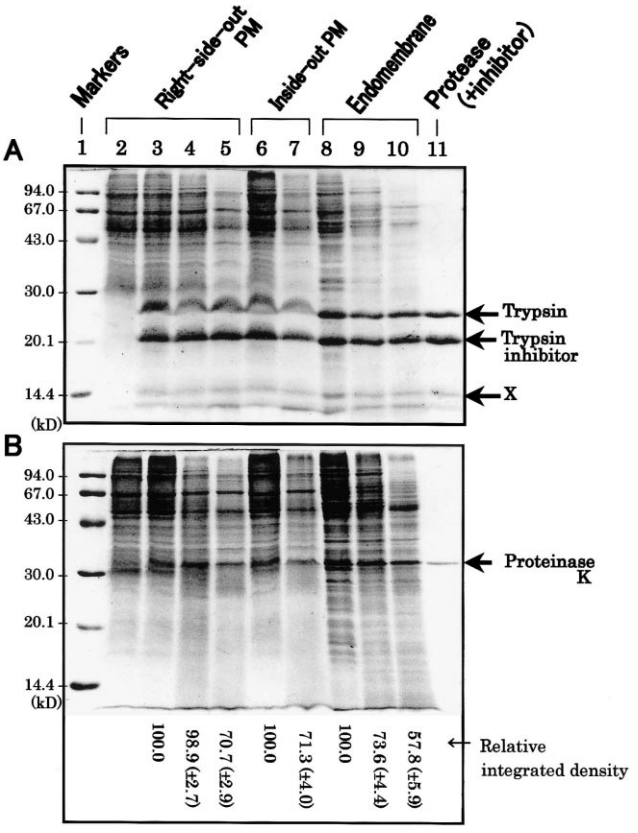

Fig. 1. Proteolysis of membrane peptides by proteases. RO-PM (lanes 2–5), inside-out PM (lanes 6, 7) created by the addition of Brij 58 to an incubation mixture of RO-PM (final 0.5%), and endomembrane vesicles (lanes 8–10) were used. These membrane vesicles were treated with trypsin (A) or proteinase K (B). The incubation mixture of the membranes was mixed with the same volume of 4% (w/v) SDS, 20% (v/v) glycerol, 1.5% (w/v) DTT, 100 mM Tris–HCl (pH 6.8) and 0.002% (w/v) bromophenol blue, boiled for 2 min, and then subjected to SDS–PAGE. Proteins in the gels were stained with Coomassie brilliant blue R-250. In each lane, 25 μl of the solubilized incubation mixture, containing 5 μg of trypsin plus 7.5 μg of trypsin inhibitor or 0.83 μg of proteinase K, and 25 μg of membrane proteins both hydrolyzed and unhydrolyzed by the protease, were loaded except for lanes 1 and 11. Lanes: 1, molecular weight markers; 2, RO-PM vesicles without the protease and inhibitor; 3, 6 and 8, controls with the protease and inhibitor; 4, 7 and 9, sealed membrane vesicles treated with the protease; 5 and 10, membranes treated in the presence of 0.03% (w/v) Triton X-100; 11, the same quantity of the protease and inhibitor as in lanes 3–10 without membranes. The band positions of added trypsin, trypsin inhibitor, contaminants (X), and proteinase K are indicated by thick arrows at the right side of the gel images. Values under gel image B, indicated by a thin arrow, show the relative integrated density of each lane in B to its control (lane 3, 6 or 8) as calculated with the following formula; $x = [I(\text{lane } n) - I(\text{lane } 11)]/[I(\text{lane of control}) - I(\text{lane } 11)] \times 100$ (%), in which x represents the relative protein content (%) of lane n (the number of a lane) and I the absolute integrated density of the lane indicated in parentheses. Means (±S.E.) of five different preparations are given, whereas one typical gel image is presented.
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through the gel after electrophoresis. The ratio of peptides on the outer surface of the vesicles could be estimated by comparing lanes 3 and 4, lanes 6 and 7, and lanes 8 and 9, respectively, and that on both surfaces by comparing lanes 3 and 5, and lanes 8 and 10, respectively.

Trypsin treatment of sealed RO-PM vesicles changed the polypeptide pattern little compared with the control (Fig. 1A, lanes 3 and 4), while the addition of 0.03% (w/v) Triton X-100 caused a decrease and loss of many polypeptides (Fig. 1A, lane 5). In the case of sealed inside-out PM vesicles, trypsin hydrolyzed the polypeptides to some extent (Fig. 1A, lanes 6 and 7).

Some polypeptides of sealed endomembrane vesicles were also lost by the trypsin treatment, and a further decrease in polypeptides was caused by the addition of Triton X-100 (Fig. 1A, lanes 8–10).

Proteinase K treatment changed the polypeptide pattern of sealed RO-PM vesicles, but the decrease of protein content was only slight (Fig. 1B, lanes 3 and 4). The addition of Triton X-100 to RO-PM vesicles, and the application of inside-out PM vesicles resulted in the loss of about 29% of membrane proteins (Fig. 1B, lanes 5 and 7). An about 26% decrease in proteins of sealed endomembrane vesicles was caused by the proteinase K treatment, and further proteolysis resulted on the addition of Triton X-100 (Fig. 1B, lanes 8–10). The manner of proteolysis differed with trypsin and proteinase K because of their specificity for proteolytic sites (Fig. 1A,B). It seems that the outer surface peptides of RO-PM vesicles had few tryptic sites, and proteinase K only cleaved the short loops and/or the short terminus of these peptides. All polypeptide bands of the membranes were diminished by the protease treatment in the presence of 5% (w/v) Triton X-100 (data not shown).

To better evaluate the proteolysis, the density profiles of lanes 3–5 and 8–10 in Fig. 1A and B were compared, as shown in Fig. 2. The density profile of sealed endomembrane vesicles degraded by trypsin

was in between that of the control and that of the membrane degraded in the presence of Triton X-100 for almost all the region (Fig. 2B). On the other hand, the profile of RO-PM vesicles degraded by trypsin was only slightly different from that of the control for almost the entire region (Fig. 2A), although there were two narrow regions where the decrease in polypeptide content was comparatively distinct (Fig. 2A, brackets a and b). The differences of peak areas corresponding to trypsin between the control and the other two in Fig. 2A are not significant because they were null or negligible in other reproductive experiments.

In the case of degradation by proteinase K, the polypeptide content of sealed RO-PM vesicles was reduced in the region of higher M.W. (Fig. 2C, bracket a), but increased in that of lower M.W. (Fig. 2C, bracket b). The polypeptides in the region of lower M.W. are considered to be an integral and cytoplasmic part of PM proteins situated in the re-

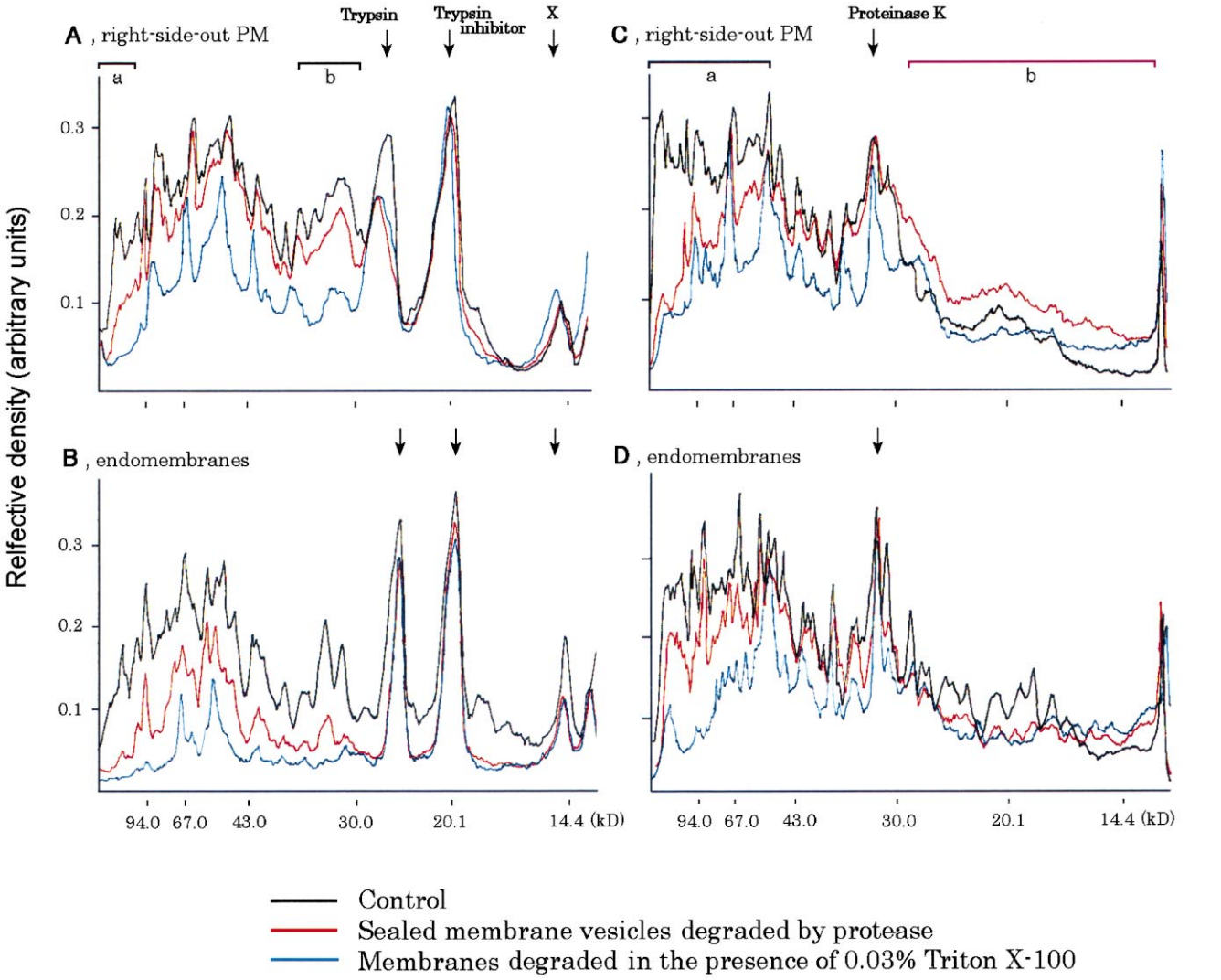

Fig. 2. Comparison of polypeptide profiles of membranes treated with proteases. (A,B) Trypsin treatment (Fig. 1A); (C,D) proteinase K treatment (Fig. 1B). (A,C) Lanes 3 (black lines), 4 (red), and 5 (blue) in Fig. 1; (B,D) lanes 8 (black), 9 (red), and 10 (blue) in Fig. 1. Vertical short bars and arrows indicate the band positions of molecular weight markers, the proteases and the inhibitor, respectively. On comparison of the black and red lines in A and C, the regions in which the difference was obvious were marked by brackets (black bracket where black line is higher, red bracket where red line is higher) except for the peak areas corresponding to trypsin, trypsin inhibitor, contaminants (X), and proteinase K.

gion of higher M.W. before the proteolysis. The density profile of endomembrane vesicles was also lowered by proteinase K treatment (Fig. 2D); however, the polypeptide content in the region of lower M.W. did not increase.

The protease treatment in the presence of Triton X-100 lowered the density profiles for almost all regions in every case (Fig. 2A–D, compare black and blue lines). The degree of the decrease in the inside-out PM vesicles degraded by the proteases was nearly as great as that of RO-PM degraded in the presence of 0.03% (w/v) Triton X-100 (data not shown).

The results presented in Figs. 1 and 2 indicate that the surface peptides were effectively degraded by the protease treatment, and there were much fewer peptides on the outer surface of RO-PM vesicles obtained by two-phase partitioning. It is considered that almost all of the PM surface peptides are exposed on the cytofacial surface. The same results were obtained with RO-PM vesicles isolated from rice seedlings and pumpkin cotyledons (data not shown).

We also evaluated the proteolysis of glycopeptides.

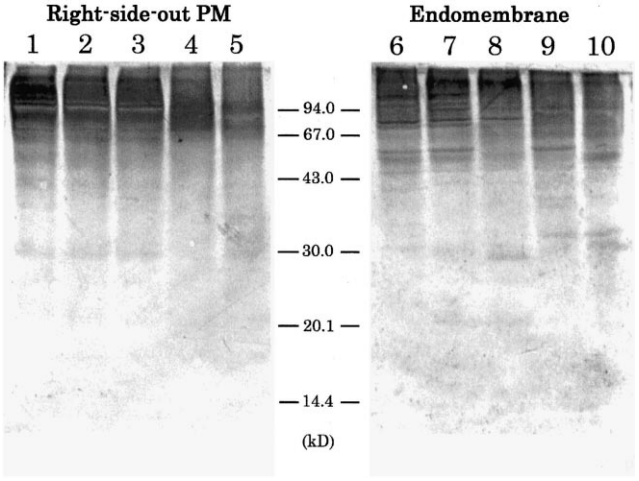

Fig. 3. Glycopeptide staining of membrane vesicles treated with proteases. RO-PM (lanes 1–5) and endomembrane vesicles (lanes 6–10) were treated with trypsin (lanes 2, 3, 7 and 8) or proteinase K (lanes 4, 5, 9 and 10) in the same way as in Fig. 1. The glycopeptides of each sample were visualized. In each lane, 10 μl of the solubilized incubation mixture was loaded. Lanes: 1 and 6, controls without the protease and its inhibitor; 3, 5, 8 and 10, membranes treated in the presence of 0.03% (w/v) Triton X-100.

Fig. 3 shows the glycopeptide staining of the protease-treated membranes. Many glycopeptides of both the PM and endomembranes were found in the region of higher M.W. (≥60 kDa). The glycopeptides of RO-PM and endomembrane vesicles were hydrolyzed and/or cleaved by proteinase K and the polypeptide pattern was changed, whereas trypsin was not effective in the degradation of glycopeptides in the membranes.

3.2. Dissociation of peripheral proteins from membrane vesicles

To obtain more evidence that less protein is associated with the outer surface of PM, the amount of peripheral proteins on the outer and inner surfaces was evaluated using 1 M KCl.

When RO-PM and endomembrane vesicles were incubated in Suc-K-Pi buffer (control) and centrifuged, about 3% and 5% of proteins were recovered in the supernatant, respectively (Table 1, first row of protein columns). These proteins are considered to be soluble proteins contaminating the membrane preparations and/or peripheral proteins fairly weakly stuck to the outer surface. When membrane vesicles were incubated in 1 M KCl, about 3% of PM proteins and 14% of endomembrane proteins were released (Table 1, second row). An about 9% further increase in endomembrane proteins occurred in the supernatant while the increase of PM proteins was less than 1% (Table 1, compare the first and second rows in the protein columns). These results would reflect the ratio of peripheral proteins bound to the outer surface. In the control experiment and on treatment with 1 M KCl alone, the recovery of PLs was negligible (Table 1, first and second rows), indicating that the membranes were nearly completely pelleted by the centrifugation.

It was necessary to release peripheral proteins on the inner surface of membrane vesicles into the supernatant for the estimation of the amount on both surfaces. For this purpose, 1.5 mM dibucaine was utilized. Dibucaine, a local anesthetic, induces hemolysis of erythrocytes at around this concentration [18], suggesting it may destabilize the membrane bilayer. When membrane vesicles were incubated in 1 M KCl plus 1.5 mM dibucaine and subjected to freeze/thawing, much more protein was recovered

Table 1
Recovery of membrane proteins and PLs in the supernatant after treatment with 1 M KCl and subsequent centrifugation

Buffer/treatment	RO-PM vesicles (U_3+U'_3) (% of membrane)		Endomembrane vesicles (L_1) (% of membrane)	
	Proteins	PLs	Proteins	PLs
Suc-K-Pi buffer (control)	2.5 ± 0.2	0.7 ± 0.2	5.1 ± 0.3	0.7 ± 0.3
1 M KCl	3.3 ± 0.2	0.2 ± 0.1	13.6 ± 0.2	1.3 ± 0.4
1 M KCl+1.5 mM dibucaine+freeze/thawing (twice)	20.6 ± 0.4	2.5 ± 0.4	34.0 ± 1.4	5.3 ± 1.4

Two hundred milligrams of protein, quantified by the method of Peterson [11], of membrane vesicles were treated with 1 M KCl as described in Section 2, and pelleted by centrifugation at 150 000 × *g* for 40 min at 4°C. Means ± S.E. of four different preparations are given. Similar results were obtained when the recovery of membrane sterols was determined.

in the supernatant although only a small quantity of PL was recovered (Table 1, third row). The differences in protein from the treatment with 1 M KCl alone were about 17% with respect to PM and 20% with respect to endomembranes (Table 1, compare the second and third rows). This would be due to the release of peripheral proteins on the inner surface and soluble proteins entrapped inside of the vesicles into the supernatant, although the values are slightly overestimated because a small amount of membrane was not pelleted. This procedure using dibucaine may have resulted in the bursting of the vesicles. Omission of dibucaine or freeze/thawing in the procedure resulted in no increase in the recovery of proteins as compared with the treatment with 1 M KCl alone.

These results show that peripheral proteins, comprising some portion of surface peptides, are poorly located on the outer surface of RO-PM vesicles and most PM peripheral proteins are associated with the inner surface.

3.3. Factors determining the partition in a two-phase system

Because membrane vesicles not only from U_3+U'_3 but also from U_2+U'_2, and even from U_1, L_2 were comparatively free of outer surface peptides, it was suggested that the amount of outer surface peptide of membrane vesicles significantly affects the partitioning in the upper or lower phase in a two-phase system.

To examine this possibility, endomembrane vesicles (L_1) degraded by the protease or incubated in 1 M KCl were again partitioned in two-phase systems. Usually, membrane proteins or enzymes are utilized for the determination of G_u [5,6,9]; however, in this assay the contents of membrane lipids, sterols and PLs, were taken as internal indexes for G_u because the membrane proteins were degraded.

Fig. 4 shows that both the 1 M KCl and the protease treatments enhanced the partitioning of the endomembrane vesicles in the upper phase at each polymer concentration and for each lipid, seemingly

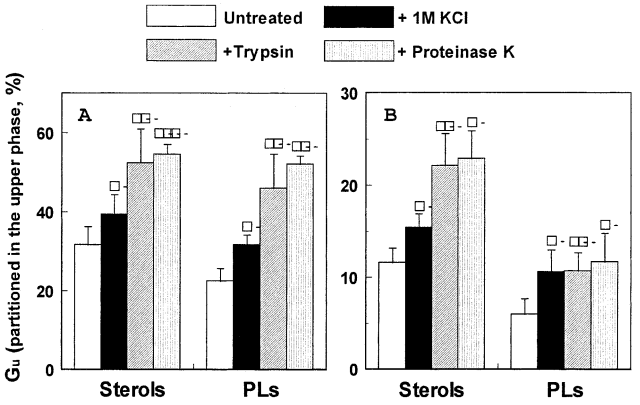

Fig. 4. Re-partitioning of endomembrane vesicles treated with the proteases and 1 M KCl. The recovery of PLs of the 1 M KCl-treated membranes to the pellet was more than 97%. Endomembrane vesicles subjected to the treatments were again incubated in two-phase systems with 5.6% (A) or 6.2% (B) polymer. The membrane vesicles partitioned in the upper phase were collected, and the lipid contents quantified. The rate of partitioning in the upper phase (G_u, in %) of the vesicles can be expressed as the G_u of their sterol or PL content. Error bars indicate the S.D. of three to six different preparations. The controls for the protease-treated membranes gave the same results as the untreated, and the untreated membranes were used as the control for the 1 M KCl-treated membranes. The elevations of G_u in the treated samples compared to each control were assessed by Student's *t*-test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Table 2
ζ-Potentials of membrane vesicles subjected to protease and 1 M KCl treatments

	RO-PM (U_3+U'_3) (mV)	Endomembranes (L_1) (mV)			
		Untreated	+1 M KCl	+Trypsin	+Proteinase K
−EDTA	−13.7 ± 0.4	−26.5 ± 2.1	−22.9 ± 4.4	−40.2 ± 0.7	−41.1 ± 1.9
+EDTA	−21.2 ± 1.6	−27.6 ± 1.8	N.D.	N.D.	N.D.

Endomembrane vesicles were treated with trypsin, proteinase K or 1 M KCl (without dibucaine), and then pelleted by centrifugation at 100 000 × g for 30 min at 4°C. The resulting pellets of the 1 M KCl-treated endomembranes were washed, and pelleted again. One milligram of each protein of RO-PM and untreated endomembrane vesicles was incubated in Suc-K-Pi buffer containing 1 mM EDTA and pelleted (+EDTA). The membranes were finally resuspended in Suc-K-Pi buffer. Means ± S.D. of three to five different preparations are shown. N.D., not determined.

nearly in proportion to the decrease in the amount of surface peptides (Figs. 1 and 2, Table 1), suggesting that the content of outer surface peptides is one of the factors determining G_u. This effect may be independent of the oligosaccharides because they were poorly removed by trypsin (Fig. 3, lanes 6 and 7) and the 1 M KCl treatment (data not shown) from the membrane vesicles.

Since membrane surface charge may be one of the factors determining the partition [5,20], we examined how these treatments altered the surface charges by measuring the ζ-potentials.

As shown in Table 2, the ζ-potentials of untreated endomembranes and RO-PM were around −27 and −14 mV, respectively. Removal of the remaining bound divalent cations by 1 mM EDTA increased the negative ζ-potential of RO-PM by 7 mV; however, the increase in the ζ-potential of endomembranes was only about 1 mV. It is indicated that a considerable amount of divalent cations, which were not chelated by EGTA in the tissue grinding medium [1], were still left on the PM outer surface, although fewer divalent cations remained on the endomembrane surfaces.

The 1 M KCl treatment changed the ζ-potential of endomembranes from −27 to −23 mV, suggesting that surface charges were slightly decreased, probably because of the decrease in the number of charged amino acid residues [7]. On the other hand, the protease treatments increased the negative surface charges (from −27 to around −40 mV). Nagata and Melchers [21] also reported that pronase treatment of plant protoplasts made the surface more negative. This increase in negative surface charge may be due to a huge number of $-COO^-$ and $-NH_3^+$ generated as the result of peptide cleavage, not due to the decrease of surface peptide contents [21] because the removal of peripheral proteins by the 1 M KCl treatment decreased the charge. The degradation of peptides by the proteases may have largely modified the surface electric properties.

In the experimental results in Fig. 4, it is also noticed that the G_u of sterols was higher than that of PLs, which was more evident in the 6.2% polymer system (Fig. 4B). Thus, the sterol content of membrane vesicles would be another factor determining the G_u, and a high sterol content is favorable for partitioning in the upper phase.

Table 3
PL and sterol contents of membranes from various fractions obtained with a batch procedure of two-phase partitioning

Membrane fraction	Lipid content (μmol (mg protein)$^{-1}$)		Sterol/PL ratio
	PLs	Sterols	
L_1, n = 11	1.10 ± 0.05	0.31 ± 0.02	0.28
L_2, n = 3	0.87 ± 0.06	0.41 ± 0.04	0.47
U_1, n = 3	1.00 ± 0.11	0.58 ± 0.07	0.58
U_2+U'_2, n = 4	0.93 ± 0.08	0.71 ± 0.07	0.76
U_3+U'_3, n = 3	0.68 ± 0.03[a]	0.59 ± 0.01[a]	0.87

n indicates the number of membrane preparations. Means ± S.E. are shown.
[a]The same data as in [1], Table 1.

To ascertain the relevance of the sterol content of membrane vesicles to partitioning in the two-phase system, the sterol and PL contents of membranes from various fractions obtained with the batch procedure [9] were quantified and compared. As shown in Table 3, membrane vesicles in the upper phases contained more sterols and had a higher sterol-to-PL ratio, which increased as the partition was repeated. These results suggest that membrane vesicles containing more sterols and/or with a higher sterol-to-PL ratio also tend to partition more in the upper phase. The presence of substantial amounts of sterols in the constituents of membrane vesicles should alter the surface property so as to be partitional in the upper phase.

4. Discussion

We estimated the large scale asymmetry of surface peptides across mung bean PM to identify the cause of the asymmetrical properties of higher plant PMs.

A remarkable quantitative inclination of the surface peptides of PM proteins was found (Figs. 1 and 2, Table 1). The results indicate that the PM has less surface peptides, including peripheral proteins, on the exofacial surface. The major membrane proteins of red blood cells either span the membrane or are exposed only at the cytofacial surface [4].

In the assay using proteases, there remained the possibility that most of the outer surface peptides of RO-PM vesicles are glycopeptides and could not be effectively hydrolyzed.

The protein content of RO-PM vesicles after the degradation of the outer surface peptides by proteinase K hardly changed in spite of the fact that the outer surface peptides including the glycopeptides were cleaved (Fig. 1B, lanes 3 and 4; Fig. 3, lane 4), while proteinase K could have decreased the surface peptides on both surfaces of the endomembranes and on the inner surface of the PM (Fig. 1B, lanes 5, 9 and 10; Fig. 3, lanes 5, 9 and 10).

Therefore, it is unlikely that only the outer surface peptides of glycoproteins of RO-PM vesicles were not effectively hydrolyzed by proteinase K. Proteinase K should have degraded the surface peptides including glycopeptides on both surfaces of PM and endomembranes. It is considered that the outer surface peptides of most PM glycoproteins, as well as other PM proteins, are short.

The two-phase partition technique is founded on both hydrophobicity/hydrophilicity and charge of membrane surface [5,20]. It is expected that membrane vesicles whose outer surface is more hydrophobic (less hydrophilic) would tend to partition in the upper phase enriched in organic solvent-soluble PEG.

The paucity of outer surface peptides and higher sterol content (sterol-to-PL ratio) of membrane vesicles are favorable for the partition in the upper phase in the two-phase system (Fig. 4, Table 3). The former factor seems particularly important because membrane vesicles from U_1, U_2+U'_2, and L_2 were also relatively free of outer surface peptides as indicated in Section 3.

Removal of surface peptides will result in both a decrease in hydration (hydrophilicity) and the alteration of surface charges. The treatments with 1 M KCl and the proteases resulted in a decrease and an increase in negative surface charge, respectively (Table 2). In spite of this, all these treatments resulted in an elevation of G_u as the result of the decrease in the surface peptide contents of endomembranes (Fig. 4). Therefore, it is considered that the reduced hydrophilicity caused by the removal of surface peptides is important for the elevation in G_u. Although removal of surface peptides of endomembrane vesicles seems to increase the G_u, the change would not have been marked, presumably because the protease treatment could not have removed all the surface peptides (Fig. 3), and because of the low sterol-to-PL ratio causing the endomembrane vesicles to be partitioned in the lower phase (Table 3) as well as other unknown factors.

In the case of RO-PM, less surface peptide is associated with the outer surface, and this may result in a high affinity to the upper phase. Surface charges of plant cells, expressed as surface charge density, ζ- and surface potentials, vary in plant species and organs to a large extent and are not greatly different from other organelle membranes [2,3,19,21–28]. Nevertheless, RO-PM vesicles can be routinely obtained in high purity from most higher plant tissues using the two-phase system with dextran T500 and PEG [2,5,9,20]. In the system, RO-PM vesicles tend to be retained in the upper phase but other mem-

branes partition more in the lower phase with increasing concentrations of the polymers and chloride salts [5,6,20,29,30]. Therefore, it is suggested that the paucity of soluble peptides of the PM outer surface is a common feature of higher plants, and the lower hydrophilicity originating from the paucity is important for RO-PM vesicles to be retained in the upper phase, rather than the PM surface charges. The contribution of the surface charges to the partition remains to be elucidated. Membrane surface charges may also be involved in the hydration, and the effect will be more prominent in phase systems composed of charged polymers [22,31].

The sterol content of membranes can also influence the partition (Fig. 4, Table 3). The effect of sterol content may become more prominent when the concentration of the polymers rises (Fig. 4). As a matter of course, the sterol content in the outer leaflet of membrane vesicles may be important. The free sterols in membranes may reduce the hydration [32–35] as well as the surface charge density because they are neutral lipids. The lower hydrophilicity of the outer surface of membrane vesicles produced by the sterols would also be important for the partition in the upper phase.

It is well known that a large amount of sterols, especially free sterols, is contained in most of the higher plant PMs [2,36–44], whereas PMs of some plant species have comparatively low sterol contents [2,45,46]. In mung bean PM, 91% of sterols were free sterols and the rest were glycosylated [36], and the glycosylated sterols are suggested to be located on the exofacial leaflet of plant PM [2,3].

Inside-out PM vesicles preferentially partition in the lower phase and RO-PM vesicles in the upper phase [9,47,48]. If the large amount of sterols (high

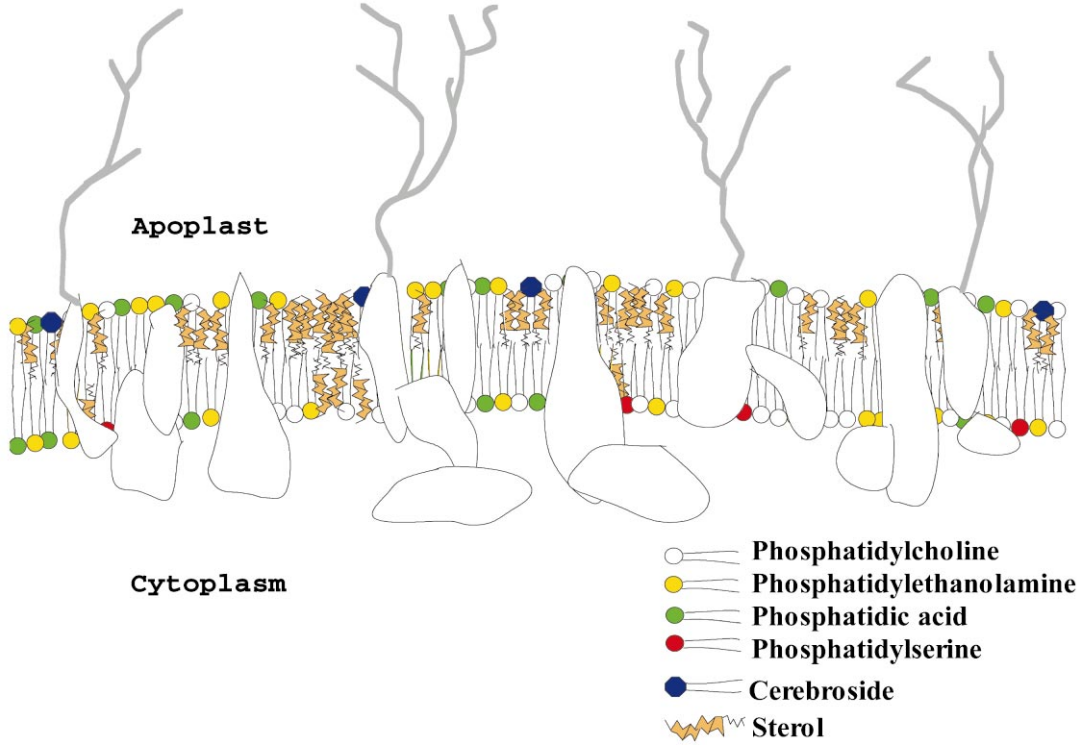

Fig. 5. Model for the proposed transmembrane structure of PM in mung bean hypocotyl cells. The major PLs, phosphatidylcholine, phosphatidylethanolamine, and phosphatidic acid, are symmetrically distributed across the PM, and the minor PL, phosphatidylserine, is preferentially located in the cytofacial leaflet [1]. The location of other minor PLs, phosphatidylinositol and phosphatidylglycerol, remains unknown. Oligosaccharides of PM glycoproteins will be associated with the exofacial surface, and cerebrosides might also be located in the exofacial leaflet [2,3]. The cerebrosides would not be clustered in mung bean PM [52]. Soluble surface peptides of PM are scarce on the exofacial surface and are exposed on the cytofacial surface for the most part. PM sterols, free sterols and glycosylated sterols, might be abundant in the exofacial leaflet. The existence of lateral domains of sterols [8,53] is still not clear in plant PM.

sterol-to-PL ratio) in the plant PM is also the cause of preferential partitioning in the upper phase, sterols must be asymmetrically distributed across the PM.

We speculate that free sterols as well as glycosylated sterols are abundant in the exofacial leaflet of plant PM because of their contribution to the decrease in surface hydrophilicity, which would also favor RO-PM vesicles being partitioned in the upper phase, although in the PM of a few mammalian cells, a large amount of cholesterol, the major free sterol, is distributed in the cytofacial leaflet [8]. Unfortunately, the transmembrane distribution of plant sterols could not be directly proven since presently there seems to be no reliable probe for the assay.

In conclusion, it is considered that the marked differences in properties between the two surfaces of the plant PM stem from the quantitative asymmetry of the bulk of surface peptides and presumably the asymmetrical distribution of PM sterols, not from the topography of the PLs [1]. Such an asymmetry of PM may have striking effects especially in two-phase partitioning. The contribution of the carbohydrates abundant on the outer surface [2,3] to the asymmetrical properties of the PM remains to be elucidated. It is suggested that the hydrophilicity of the cell surface is lower than that of the cytofacial surface of PM and of organelle membrane surfaces because of the paucity of surface peptides and the putative predominant location of sterols in the exofacial leaflet, which would be important for the separation of RO-PM vesicles from other membranes by two-phase partitioning.

A model for the proposed transmembrane structure of mung bean PM is presented in Fig. 5. It remains to be determined whether this model is valid for PMs of other plant species and organs. Although the physiological significance of such an asymmetrical transmembrane structure of the PM is still unclear, the paucity of peptides on the cell surface seems to help the higher plant PM to maintain its intracellular physiological functions. The environment in the apoplast is changeable due to stress such as low or high pH [49,50] and concentrations of toxic ions such as Al^{3+} and Mn^{2+} [51], while the cytosolic conditions remain constant to maintain cellular homeostasis. Under such unusual conditions in the apoplast, the structure of proteins on the exofacial surface could be modified, which would easily lead to loss of PM functions if much protein is associated with the cell surface.

So many peptides would not be needed on the apoplastic surface of plant PM because peripheral parts of membrane proteins would facilitate the utilization of solutes and much less solute exists in the apoplast than in the cytoplasm.

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