Lipids and Enzymatic Activities in Vacuolar Membranes Isolated via Protoplasts from Oat Primary Leaves

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Z. Naturforsch. **38 c**, 770–777 (1983); received May 5, 1983

Contamination, Microsomal Enzymes, Galactolipids, Steryl Glycosides, Cerebrosides

Vacuoles were released from oat (*Avena sativa*) mesophyll protoplasts and purified by sedimentation and flotation. Disruption of isolated vacuoles followed by density gradient centrifugation gave two membrane bands which after combination were further purified on sucrose gradients. A significant contamination by microbodies, thylakoids, mitochondria, endoplasmic reticulum and Golgi membranes can be excluded, whereas markers for plasma membrane and chloroplast envelope were present in the final membrane preparation.

In the purified membrane fraction the following enzymatic activities were detected: NADH-cytochrome C reductase E.C. 1.6.2.2, ATPase E.C. 3.6.1.3, UDPG: sterol glucosyltransferase, UDP-Gal: diacylglycerol galactosyltransferase E.C. 2.4.1.46, glucan synthetase II, CDP-choline: diacylglycerol phosphocholinetransferase E.C. 2.7.8.2, formation of acylgalactosyl diacylglycerol and acyl-CoA thioesterase E.C. 3.1.2.2. None of these can be considered to be specific for the tonoplast. Acid phosphatase E.C. 3.1.3.2 was present in the cell sap.

The vacuolar membranes contain phospholipids and glycolipids of the most complex composition found so far in a membrane system isolated from mesophyll protoplasts. About half of the glycolipids were accounted for by glycosyl diacylglycerols usually considered to be confined to plastids. Steryl glycosides and acyl steryl glycosides were other prominent glycolipids. A cerebroside was the predominating lipid component of this membrane preparation.

Introduction

The isolation of intact vacuoles [1] has induced a steadily increasing number of investigations of this compartment. Major efforts are directed towards an understanding of mechanisms controlling the accumulation and release of the various compounds found in different types of vacuoles [2]. The vacuolar membrane is expected to play an important role in these processes. The lipid composition of the tonoplast is poorly understood since only a very few investigations [3] have been carried out with this membrane in contrast to membranes from chloroplasts, mitochondria and microbodies [4]. On the other hand, lipids provide the permeability barrier required for the storage function of vacuoles. We have now analyzed the lipids of purified tonoplast preparations which were isolated *via* vacuoles from photosynthetically active cells, and compared this lipid mixture to those obtained from other membrane systems of the same cell type.

Abbreviations. AGD, Acylated monogalactosyl diacylglycerol; ASG, acylated steryl glucoside; CER, cerebroside; DGD, digalactosyl diacylglycerol; MGD, monogalactosyl diacylglycerol; PA, phosphatidic acid; PC, PE, PG, PI, phosphatidyl choline, ethanolamine, glycerol, inositol; SG, steryl-glucoside; SQD, sulfoquinovosyl diacylglycerol.

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0341-0382/83/0900-0770 $ 01.30/0

Materials and Methods

General

Pectolyase Y-23 was obtained from Seishin-Pharmaceutical Co. Ltd., Nagareyama, Japan and Cellulase Onozuka R-10 from Yakult Biochemicals Co. Ltd., Tokyo, Japan. Radioactive and other substrates for enzymatic assays were commercially available. Centrifugations at high speed were performed in a Spinco L2-65B ultracentrifuge using rotors SW 25.2 and SW 41 for large and small volumes, respectively, in appropriate cellulose nitrate tubes. SW 41 gradients with a total volume of 11 ml were fractionated with the aid of an Isco fractionator model 640 into 0.6 ml fractions.

For isolation of microsomes protoplasts were prepared with cellulysin. The protoplast suspension was forced through a nylon net and the resulting homogenate centrifuged at $3300 \times g$ on a sucrose gradient exactly as described before [5]. The super-

natant loading zone including the microsomal band was removed with a pasteur pipette, diluted with 5 volumes of gradient buffer and centrifugated for 90 min at $110000 \times g$ to give sedimented microsomes.

Preparation of protoplasts

Primary leaves of *Avena sativa* L. cv. Gelbhafer-Flämingskrone were harvested after growth for seven days under conditions as described before [6] and cut with razor blades into pieces of about 0.5 cm length. Leaf pieces (20–30 g) were placed into a petri dish (25 cm diameter) with 150 ml of enzyme solution [7] containing Pectolyase Y-23 (0.05%, w/v), Cellulase Onozuka R-10 (0.6%, w/v) and 0.6 M sorbitol adjusted to pH 5.5 with KOH. Protoplasts were released by incubation at room temperature for 18 h, freed from undigested material by filtration through a net of stainless steel and washed twice by sedimentation from 0.6 M sorbitol in 5 mM Mes/KOH pH 5.6 at $100 \times g$ for 5 min.

Isolation of vacuoles

Sedimented protoplasts were resuspended in hypotonic shock buffer (50 mM Hepes/Tris pH 7.5; 80 ml per 50–80 mg chlorophyll) and kept for 15 min at 0 °C. The resulting organelle suspension was placed on Ficoll gradients (10 ml 0.5% on top of 10 ml 7% in 0.5 M sorbitol and 50 mM Hepes/Tris pH 7.5) and centrifuged for 30 min at $40000 \times g$ in a SW 25.2 rotor. The vacuoles banding at the 0.5%/7% interface (Fig. 1 a) were removed with a pasteur pipette, mixed with about half of the resulting suspension volume with Ficoll solution (20%, w/v, in 0.5 M sorbitol and 50 mM Hepes/Tris pH 7.5) and filled into centrifuge tubes [8] which had the form of ampoules (thicker part 2.5 × 9.5 cm, constricted part 1 × 2 cm). The ampoules were filled exactly to the beginning of the constricted zone into which 0.5 ml of diluted Ficoll solution (3%, w/v, in shock buffer) was placed. Centrifugation at $1400 \times g$ for 60 min in a swing out rotor (Hettich/Rotixa K centrifuge) resulted in flotation of the vacuoles into the overlay (Fig. 1 b).

Isolation of vacuolar membranes

After centrifugation the overlay was removed, diluted with 4 ml of shock buffer and sucked up and down several times into a syringe through a narrow needle. The resulting suspension was placed on a stepped sucrose gradient (3 ml 20%, w/v, sucrose on top of 2 ml 30% sucrose in 5 mM Hepes/Tris pH 7.5) and centrifuged in a SW 41 rotor for 60 min at $110000 \times g$. The two membrane bands (Fig. 1 c) at the two interfaces (0/20% and 20/30%) were removed and combined, whereas the greenish sediment was discarded.

This suspension was diluted with 10 volumes of shock buffer, sedimented for 30 min at $110000 \times g$ and resuspended in 1 ml of 5 mM Hepes/Tris pH 7.5. For measurement of isopycnic density this suspension was placed on a continuous sucrose gradient (10–60%) and centrifuged for 18 h at $100000 \times g$ in a SW 41 rotor. To determine enzymatic activities the membrane suspension was placed on a stepped sucrose gradient (5 ml 20% on top of 5 ml 40%) and centrifuged for 18 h at $100000 \times g$ (Fig. 1 d). For lipid analyses the combined tonoplast bands from the first sucrose gradient were diluted with 2 volumes of gradient buffer, placed on a stepped sucrose gradient (1 ml 10% on top of 2 ml 30%) and sedimented onto the 30% sucrose cushion by centrifugation for 30 min at $110000 \times g$ (Fig. 1 e). Per day, leaf pieces from usually 12 petri dishes were carried through the isolation procedure, giving 200–400 µg of tonoplast membrane protein.

Analysis of lipids

Tonoplast bands were removed from the gradient and an aliquot was used for protein determination. After dilution with water, lipids were extracted twice with $CHCl_3$/MeOH 2/1 as described before [9]. Tonoplast extracts from five days equivalent to 1–2 mg of protein had to be combined for a complete phospho- and glycolipid analysis. Phospholipids were determined exactly as before [5]. Glycolipids were separated by a separate TLC run in $CHCl_3$/MeOH/H_2O 75/25/1, located by spraying with anilinonaphthalene sulfonate (0.5% in MeOH) and quantified with anthrone solution as described before [10] based on calibration curves obtained with simultaneously chromatographed standards covering the range of 50–250 µg. Because of its low level, SQD was determined after combination of the spots from two TLC plates. All standard lipids were available from previous investigations [9] with the

exception of cerebroside, which was a commercial sample of bovine origin.

Fatty acid methyl esters were prepared by methanolysis from chromatographically separated compounds and analyzed by GLC [9]. Hydroxylated fatty acid methyl esters from cerebroside were separated from nonhydroxylated ones by TLC in hexane/diethyl ether/HAc 50/40/0.5. The identities of chromatographically assigned cerebroside, SG and ASG were confirmed by mass spectrometry of acetylated and/or methylated derivatives repurified in diethyl ether/petroleum ether 2/1. Acid hydrolysis of cerebroside, SG and ASG for subsequent identification of sugar components by TLC followed established procedures [11]. The positional distribution of fatty acids was determined as before [9].

Assays of enzymatic activities

References for the following assays have been given before [5, 6]: Catalase E.C. 1.11.1.6, cytochrome C oxidase E.C. 1.9.3.1, glyceraldehydephosphate dehydrogenases E.C. 1.2.1.12 and E.C. 1.2.1.13, NAD(P)H-dependent cytochrome C reductases E.C. 1.6.2.2 and E.C. 1.6.2.4, formation of AGD from tritiated MGD, UDPG: sterol glucosyltransferase, formation of galactolipids from UDP-[^{14}C]Gal and acyl-CoA thioesterase E.C. 3.1.2.2 with [^{14}C]palmitoyl-CoA. Discontinuous assays based on radioactive substrates were used to measure glucan synthetase II with [^{14}C]UDPG [12] and CDP-choline: diacylglycerol phosphocholinetransferase E.C. 2.7.8.2 with CDP-[^{14}C]choline [13]. Activities of ATPase E.C. 3.6.1.3 [14], IDPase E.C. 3.6.1.6 [15], α-mannosidase E.C. 3.2.1.24, β-glucosidase E.C. 3.2.1.21, phosphodiesterase E.C. 3.1.4.1 and acid phosphatase E.C. 3.1.3.2 were determined by discontinuous colorimetric assays [16]. The intactness of protoplast plasmamembranes was calculated from the activity of UDPG pyrophosphorylase E.C. 2.7.7.9 [17] before and after addition of Triton X-100 (0.1% final concentration) to the assay mixture.

Results and Discussion

Isolation of vacuolar membranes

Tonoplasts were isolated from oat primary leaves *via* protoplasts and vacuoles by application and modification of previously successful methods [18].

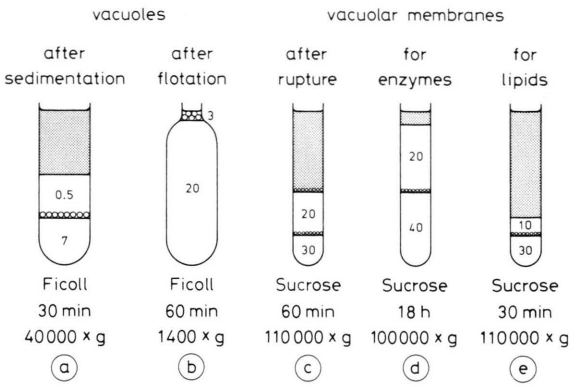

Fig. 1. Centrifugation scheme and gradients used for isolation of vacuoles and vacuolar membranes. Vacuoles were released from protoplasts by osmotic shock and purified by sedimentation onto a Ficoll cushion (a) and subsequent flotation into a hypotonic medium (b). After osmotic and mechanical disruption of vacuoles the two membrane bands recovered from a stepped sucrose gradient (c) were combined and further purified by recentrifugation on a second sucrose gradient for subsequent measurement of enzymatic activities (d) or lipid extraction (e). Numbers in gradient layers represent concentrations in % (w/v) of gradient media given below each tube. Centrifugation times and forces are also shown, details are given in the experimental section.

In short, vacuoles were released by osmotic shock from protoplasts, separated by sedimentation and flotation in different gradients and ruptured to yield two tonoplast membrane bands which were finally purified on sucrose gradients (Fig. 1).

Sufficient quantities of protoplasts were obtained by cutting oat leaves into small pieces followed by incubation overnight with pectolyase and cellulase [7]. This avoids the time-consuming step of peeling oat leaves as used before [6]. The intactness of protoplasts was in the order of 90% based on the activity of the cytoplasmic enzyme UDPG pyrophosphorylase [19] in protoplast suspensions before and after addition of Triton X-100. This assay [17] depends on substrates and auxiliary enzymes which can reach the cytoplasmic enzyme only after destruction of the plasmamembrane by Triton X-100 which has no effect on the enzymatic activity itself.

Incubation for 15 min in the hypotonic shock buffer resulted in protoplast rupture and release of vacuoles and other organelles. Vacuoles were separated by centrifugation through a 0.5% Ficoll layer and collected on a 7% Ficoll cushion (Fig. 1 a). The centrifugational force of $40000 \times g$ at this step

removes most of the chloroplasts and mitochondria which sediment at the bottom of the tubes, whereas the majority of the soluble cellular contents is separated from the vacuolar band by the 0.5% Ficoll layer.

The vacuolar fraction recovered from the Ficoll cushion was diluted and centrifuged in specially designed ampoules [8] (Fig. 1 b). This resulted in flotation for additional purification and concomitant concentration of vacuoles in a small volume of hypotonic medium. Microscopic inspection of this concentrated suspension showed the presence of a few protoplasts, although their number was very small compared to the number of vacuoles. Examination of lipid extracts from vacuoles at this stage of purification showed severe contamination by chloroplast lipids as indicated by the green colour of the extract. Although vacuoles of comparable purity are useful for functional studies, they cannot be used for an analysis of tonoplast lipids, since the few chloroplasts present contribute a substantial proportion of membranes and lipids.

Therefore, vacuoles were disrupted mechanically in hypotonic buffer and the tonoplast fractions purified by further centrifugation steps on sucrose gradients. The first gradient separated most of the soluble vacuolar contents from two membrane bands (Fig. 1 c). They contained about equal proportions of material and very similar lipid mixtures (see below). It has been observed before that breakage of vacuoles produces several populations of tonoplast vesicles [18]. At this step residual chloroplasts, thylakoids and mitochondria present in the vacuolar fraction before breakage are removed. During centrifugation at $110000 \times g$ they pass through the cushion of 30% sucrose (0.99 M, density 1.13) used to collect the lower of the two tonoplasts vesicle bands (Fig. 1 c). Accordingly, we consistently observed a small greenish pellet after this centrifugation.

The two membrane fractions were combined, diluted and directly or after resedimentation and resuspension, centrifuged through a sucrose layer and collected on a denser cushion (Fig. 1 d, e). At high concentration the resulting tonoplast band had a brownish colour. Centrifugation of this band for 18 h at $100000 \times g$ in a linear sucrose gradient gave a somewhat diffuse band centered at a density of 1.13. For similar preparations from *Nicotiana* and *Hippeastrum*, densities of 1.08–1.12 have been reported [16, 18, 20].

Purity and enzymatic activities of vacuolar membranes

The purity of the membrane fraction obtained from vacuoles was examined by measuring markers of other compartments [21]. Chlorophyll (as measured in concentrated lipid extracts), cytochrome C oxidase, NADH-dependent glyceraldehydephosphate dehydrogenases and flavonoids could not be detected in this fraction. This excludes contamination by thylakoids, inner mitochondrial membranes and soluble components from cytoplasm, stroma or cell sap. Catalase activity, a marker for leaf peroxisomes, was detectable in the final tonoplast preparations to an extent of 0.5–1% of the total cellular

Table I. Enzymatic activities found in vacuolar and microsomal membranes isolated via protoplasts from oat mesophyll. Both membrane systems were free from chlorophyll. Specific activities of the first three enzymes are given in pmol/min/mg protein, those of the others in nmol/min/mg protein. n. d. = not detected.

Enzyme	Specific activity in	
	vacuolar membranes	microsomal membranes
CDP-choline: diacylglycerol phosphocholinetransferase	43	510
UDPG: sterol glucosyltransferase	92	490
UDP-Gal: lipid acceptor galactosyltransferase	256	300
Acyl-CoA thioesterase	1.6	4.9
Formation of acylgalactosyl diacylglycerol	7.0	9.6
NADH-cytochrome C-reductase	68	83
NADPH-cytochrome C-reductase	n. d.	25
ATPase	200	201
IDPase	n. d.	38
Glucan synthetase II	5.8	2.9

activity taking into account a 4% recovery of vacuoles (based on flavonoid content in protoplasts and vacuoles, respectively; data not shown). Therefore, severe contamination from this organelle can also be excluded. Galactolipid labelling from UDP-[^{14}C]Gal served as marker for chloroplast envelopes [22]. Lipid labelling from this sugar nucleotide in tonoplast fractions was consistently observed (Table I). The specific activity of this enzyme in tonoplast, microsomal and envelope membranes was 0.3, 0.3 and 7.0 nmol/min/mg protein. Therefore, contamination by envelope membranes was indicated.

Several "microsomal" enzymes were demonstrated in vacuolar membranes. To this group of enzymes belong antimycin – insensitive NADH-cytochrome C reductase, phosphocholinetransferase and UDPG: sterol glucosyltransferase (Table I). From these only NADH-cytochrome C reductase has occasionally been detected in vacuoles or tonoplast membranes [23–26]. But all three have also been found in other membrane systems such as plasmalemma, endoplasmic reticulum, glyoxysomal, mitochondrial, oil droplet or Golgi membranes [21]. Therefore, none of the activities found in vacuolar membranes (Table I) can serve as specific marker for tonoplasts. On the other hand, we did not detect NADPH-cytochrome C reductase or IDPase in the tonoplast fraction which represent the only significant differences between the two membrane systems described in Table I. The absence of these two activities erxcludes a contamination by endoplasmic reticulum or Golgi membranes [21]. The specific activity of glucan synthetase II was higher in vacuolar than in microsomal membranes (Table I) which would indicate an enrichement of plasmalemma [12, 21], provided that the activity is exclusively localized in this membrane type.

In confirmation and extension of previous experiments with intact vacuoles [27–30] we detected ATPase activity in the purified tonoplast fraction (Table I). It had a specific activity of about 200 nmol/min/mg measured under conditions recommended for exclusion of phosphatase activity [14]. 100 µM molybdate did not inhibit this membrane-bound ATPase [14], whereas omission of K^+ from the assay reduced the activity to 80% and omission of K^+ and Mg^{2+} left only 50% of the original activity. The purified tonoplasts had only very low acid phosphatase activity (< 5 nmol/min/mg) which therefore could not interfere significantly with the ATPase assays. On the other hand, acid phosphatase was readily detected in the fraction of flotated vacuoles. After disruption of vacuoles and centrifugation on the first sucrose gradient (Fig. 1 c), the acid phosphatase was recovered in the supernatant fractions, thereby indicating that it is a soluble protein of the cell sap and confirming previous results [16, 30, 31]. Our attempts to detect other hydrolytic enzymes in the vacuoles, such as α-mannosidase, β-glucosidase or phosphodiesterase, where without success.

Lipid composition of vacuolar membranes

The lipid mixture extracted from tonoplasts is very heterogenous and contains about twice as much glycolipids as phospholipids (Table II). The phospholipid mixture resembles that from beet root tonoplasts [3], although the proportion of PG is higher and PA was not detected in our preparation. Tonoplasts from beet root and oat mesophyll differ from membranes of lutoids in *Hevea* latex [32], in which PA is the major phospholipid (82%). The fatty acid composition of oat tonoplast phospholipids agrees closely with that of phospholipids from other cellular membranes (Table III and ref. [5]). An important observation is the presence of 3-*trans*-hexadecenoic acid in PG from tonoplasts, since this fatty acid should be confined to chloroplast PG [4]. Since chlorophyll was not detected in tonoplast lipids, we assume that PG with 3-*trans*-hexadecenoic acid is due to contamination by chloroplast envelopes as also indicated by the presence of galactosyltransferase activity.

Table II. Lipid composition in weight % of tonoplasts and chloroplast envelopes isolated from oat mesophyll protoplasts. n. d. = not detected.

Lipid	Tonoplast	Envelope
MGD	10.2	37.3
DGD	16.7	37.6
SQD	1.7	5.5
SG	7.8	n. d.
ASG	13.2	n. d.
CER	19.6	n. d.
PC	15.4	13.5
PG	6.4	4.9
PI	2.8	1.1
PE	6.4	trace

Table III. Fatty acid composition (weight %) of lipids from tonoplasts (tpl), chloroplast envelopes (env) and thylakoids (thy) isolated from oat mesophyll protoplasts. Fatty acids are characterized by numbers of carbon atoms and double bonds. 16:1 = *trans*-3-hexadecenoic acid. ASG contained additional stearic (13.4%) and myristic acid (7.7%).

		Fatty acids				
Lipid	Membrane	16:0	16:1	18:1	18:2	18:3
MGD	tpl	3.6	–	1.7	9.7	83.6
	env	0.4	–	–	11.0	88.6
	thy	1.2	–	0.6	8.9	93.3
DGD	tpl	20.4	–	5.8	16.3	57.4
	env	16.1	–	3.2	8.8	71.9
	thy	10.3	–	1.9	5.2	82.6
SQD	tpl	35.4	–	–	–	64.6
	env	23.0	–	–	10.1	67.9
	thy	29.1	–	1.7	8.7	61.1
PC	tpl	30.6	–	–	31.5	37.9
	env	22.7	–	4.3	47.1	25.9
	thy	25.4	–	3.3	41.8	30.0
PG	tpl	37.0	11.5	–	16.6	35.0
	env	34.3	10.9	–	12.7	42.1
	thy	22.2	25.7	2.2	8.2	41.6
PI	tpl	44.0	–	–	24.0	31.0
	env	45.6	–	–	23.6	30.8
	thy	36.9	–	–	27.1	34.2
PE	tpl	29.4	–	–	40.3	30.3
ASG	tpl	52.4	–	17.9	7.3	1.3

A cerebroside accounts for about 20% of all tonoplast lipids and therefore represents the predominating lipid component. Its structure was confirmed by mass and NMR spectroscopy. Acid hydrolysis indicated that the sugar present was glucose and that the amide linked fatty acids were almost exclusively hydroxy fatty acids in agreement with previous studies on leaf cerebrosides [33, 34].

The "microsomal" lipids SG and ASG (both structures confirmed by mass spectrometry) represent additional prominent compounds of tonoplast lipids. In previous studies on subcellular localization these compounds have been found in many membrane systems with the exception of thylakoids. The fatty acids of ASG are characterized by the predominance of saturated fatty acids. Sugar analysis of both glycolipids revealed only glucose.

Another important group of lipids in the membrane fraction isolated from vacuoles are glycosyl diacylglycerols such as MGD, DGD and SQD. As discussed above for PG, we have to assume that these lipids are due to contamination by chloroplast envelopes. Electron microscopic pictures (J. P. Carde, personal communication) suggest that an intimate association between tonoplast and envelope

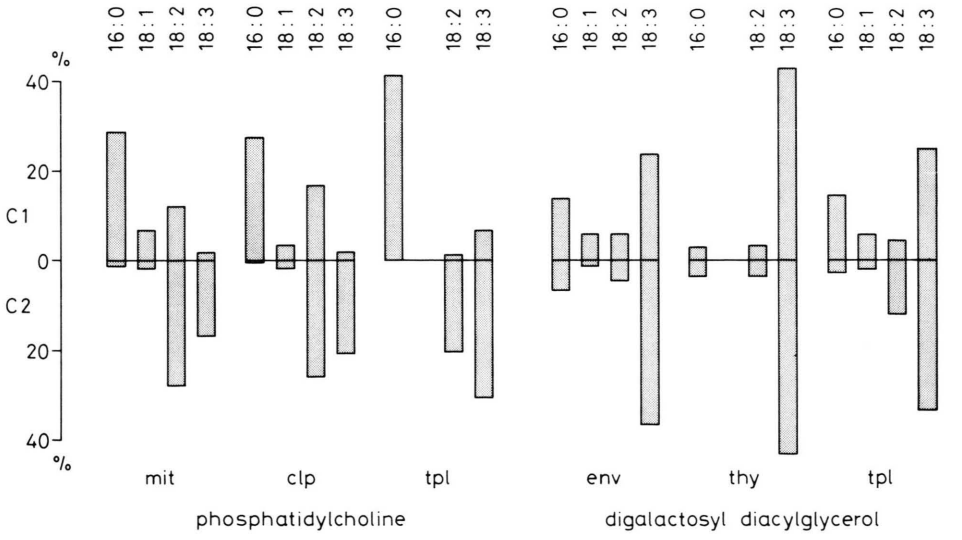

Fig. 2. Positional distribution of fatty acids in PC and DGD of different subcellular origin. C1 and C2 denote the *sn*-1 and *sn*-2 positions at the glycerol backbone of these lipids. Fatty acids are characterized by numbers of carbon atoms and double bonds. mit = mitochondria, clp = chloroplast, env = chloroplast envelope, thy = thylakoid, tpl = tonoplast.

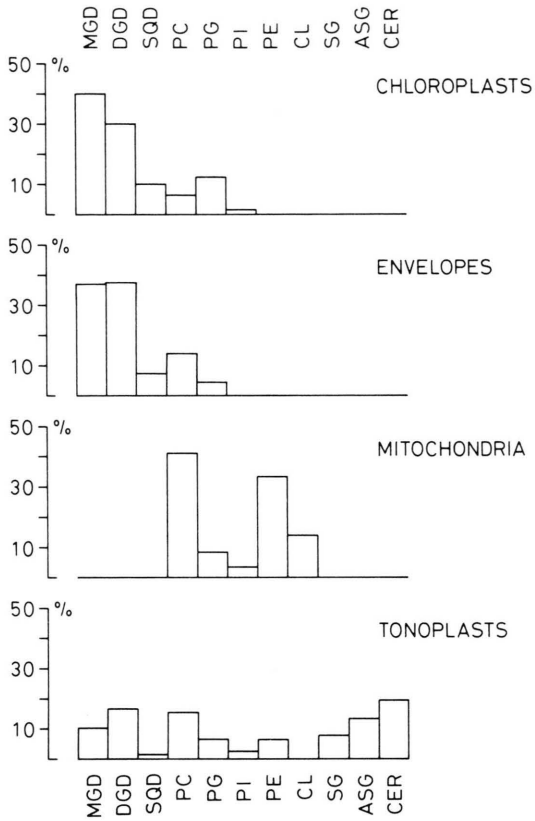

Fig. 3. Lipid composition in weight % of membrane systems from oat mesophyll cells. Mitochondria [5] and tonoplasts were isolated *via* protoplasts. Chloroplasts were prepared by mechanical homogenisation of leaves and purified on sucrose gradients. Envelopes were isolated from these chloroplasts according to ref. [22].

may already exist in intact cells due to numerous envelope extrusions surrounded by the tonoplast membrane.

The fatty acid composition of individual lipids present in the tonoplast can be compared with corresponding data from envelope, thylakoid and mitochondrial membranes of the same cells (Table III and ref. [5]). Glycolipids show a slight enrichement of palmitic acid in tonoplast and envelope membranes as compared to thylakoids. Positional analyses demonstrated that this fatty acid is localized at the C-1 position of PC (Fig. 2) and PE [5] irrespective of their intracellular origin, whereas PG and DGD carry also a small, but definite proportion at C-2 (Fig. 2).

The lipid mixtures of different membranes isolated so far from oat mesophyll cells are shown in Fig. 3 to demonstrate the exceptional profile of tonoplast lipids. Whether the high proportion of SG, ASG and cerebroside has a functional or ontogenetic/phylogenetic basis is unknown. A presently accepted view of vacuole formation from specialized endoplasmic reticulum membranes [35] would suggest that other membranes of similar origin could have a similar set of glycolipids.

Acknowledgements

The financial support by the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

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