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Research Article

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Lipid-protein microdomains in tonoplast of *Beta vulgaris* L.: comparison between the results obtained by detergent and detergent-free isolation techniques

Beta vulgaris L tonoplastındaki lipid-protein mikro alanları: Deterjanlı ve deterjansız izolasyon tekniklerinin elde edilen sonuçlarla karşılaştırılması

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Abstract

Objective: The membranes of plant and animal cells contain lipid-protein microdomains (LPMs) in their structure. We aimed to compare biochemical and biophysical characteristics of LPMs isolated by the two techniques from beet root tonoplast.

Methods: For the purposes of comparison the biochemical characteristics of LPMs the content of protein (Bradford’s method), the content of lipids (gravimetric method) were assessed. For the purpose of separating different kinds of lipids, one-dimensional thin layer chromatography (TLC), two-dimensional TLC and different specific systems of solvents were used. The content of fatty acids and sterols was determined by chromatography-mass spectrometry and gas-liquid chromatography-mass spectrometry correspondingly. In order to determine the orderings of the lipids in LPMs the laser scanning confocal fluorescence microscopy was used.

Results: Significant differences were revealed as a result of investigation of biochemical and biophysical characteristics of LPMs. The total content of proteins was substantially higher (4 times as large) in LPMs identified by the detergent-free technique than in LPMs identified by the detergent technique. The orderings of lipids in LPMs isolated by detergent-free technique was substantially lower than that of LPMs isolated by detergent technique.

Conclusion: These results may suggest an existence of several types of LPMs in tonoplast.

Keywords: Tonoplast; Lipid-protein microdomains; Detergent technique; Detergent-free technique; Gravimetric method.

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belirledi. LPM’lerde lipiderin sıralamasını belirlemek için, lazer tarama konfokal floresan mikroskobu kullanılmıştır.

**Bulgular:** LPM’lerin biyokimyasal ve biyofiziksel özelliklerinin araştırılması sonucunda belirgin farklılıklar ortaya çıkmıştır. Deterjan içermeyen teknikle tanımlanan LPM’lerde, deterjan tekniği ile tanımlanan LPM’lerden çok proteinlerin toplam içeriği önemli derecede yükselti (4 kat daha büyük). Deterjansız teknikle izole edilen LPM’lerdeki lipiderin düzenleniliği, deterjan tekniği ile izole edilen LPM’lerden önemli derecede düştüktür.

**Sonuç:** Tonoplastta bu sonuçlar çeşitli LPM türleri bulunduğunu düşünülebilir.

**Anahtar Kelimeler:** Tonoplast; Lipid-protein mikro alanları; Deterjan tekniği; Deterjan içermeyen teknik; Gravimetrik yöntem.

**Introduction**

According to the contemporary understanding, membranes of plant and animal cells contain lipid-protein microdomains (LPMs) in their structure, these LPMs being constructed of definite proteins surrounded with lipids of definite species. Thus far, microdomains [qualified as detergent insoluble microdomains (DIMs)] have been identified in the plasma membrane [1, 2], in the Golgi complex membrane [3], in endoplasmatic reticulum and mitochondria membranes [4]. Several attempts of identification of DIMs in plant cell organelle membranes were in recent years conducted mainly on plasmalemma [5]. The presence of DIMs in plant cell vacular membranes was proved in [6] and confirmed in [7]. Furthermore, a specific lipid-protein content of such DIMs was identified.

During the recent 15 years, identification of LPMs was bound up with the characteristics by which LPMs differ from the local environment, i.e. with structural, biochemical, biophysical and functional characteristics. As far as structural characteristics are concerned, majority of the authors understand LPMs as nano-dimensional (10–500 nm) non-homogeneous structural components, in which definite proteins are surrounded with a mass rich in lipids of definite species [8, 9]. Detergent insoluble microdomains have specific contents of both lipids and proteins. It is known that the content of DIMs in membranes of animal cells is characterized by excess of definite lipid species, mainly, glycolipids, sphingolipids, in particular, sphingomyelin [9, 10] and various species of sterols [9]. It has been also noted that the content of DIMs of plant cell membranes are characterized by the excess, mainly, of sphingolipids, free sterols [11] and ceramides [12, 13].

Protein contents of DIMs isolated from cell membranes of many different organisms also vary substantially [11, 14]. For example, the protein component of DIMs isolated from various organelle membranes of plant cells may be represented by aquaporins, ATPases, caveolae and also G-proteins and protein kinases.

In contemporary biology, it has become normal to feel free to discuss functions of LPMs. LPMs are assumed to be involved in such processes of cellular metabolism as exo- and endocytosis, processes of apical transition of substances to the plasma membrane and inside it [3], transition and supply of proteins to the membrane’s surface [9, 15].

On the initial stage of the epoch of investigations bound up with identification of LPMs, many specialists used the detergent technique for isolation of membrane fractions assumed to contain the microdomains. They assumed that – in the process of detergent isolation – LPMs occur in a so-called detergent-insoluble (detergent-resistant) membrane fraction (DIMF), in the process of fractionation in the density gradient (normally, in the sucrose density gradient). The LPMs obtained by this technique were qualified as lipid-protein detergent-insoluble (or detergent-resistant) membrane microdomains (DIMs, or DRMs) and conventionally called the lipid rafts. This technique has a number of shortcomings. The certain shortcomings of the detergent technique suggested the idea that plausibility in the interpretation of the research results bound up with identification of LPMs by the detergent technique should be questioned. So, it was necessary to propose another technique of isolation. Many researchers began to use the detergent-free technique for isolation of LPMs [16, 17], which was considered to be more adequate. Some of the specialists found the detergent-free technique preferable.

Note, isolation of LPMs in plant cell membranes by the detergent-free technique as well as the comparison of potentials of the two techniques have never been conducted before. In the present paper, we describe an attempt of such san investigation.

Our hypothesis states that the detergent technique does not ensure reliable isolation of DIMFs and hence plausible identification of LPMs, which would not represent artefacts. So, it is reasonable to speak only about the type of LPMs identified by the detergent-free technique, which is more adequate.

The objectives of the present investigation presumed: (i) experiments on tonoplast of Beta vulgaris L. roots by
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Materials and methods

Preparation of tonoplast

Our experiments were conducted on tonoplast of Beta vulgaris L. beet roots, variety Bordeaux (the seeds being obtained from Sibirskii Sad, Inc., Novosibirsk, Russia, and planted on the experimental field of Siberian Institute of Plant Physiology and Biochemistry, Siberian Branch of RAS). The tonoplast from the beet root tissues was isolated and purified with the aid of a technique described in [18]. The technique of tonoplast isolation can be found in [6].

Isolation of LPMs from the tonoplast by the detergent technique

The scheme for isolation of LPMs from tonoplast by the detergent technique is shown in Figure 1A, isolation of detergent-insoluble membrane fractions (DIMFs) from tonoplast presumed flotation in the discontinuous sucrose density gradient. Tonoplast fractions were solubilized during 30 min in 1% Triton X-100 at 4°C, the detergent to protein ratio being 8 : 1 (w/w). The tonoplast fraction (0.6 mg) was solubilized in a shaker with 0.5 mL buffer containing 1% Triton X-100, 50 mM Tris-HCl, 3 mM EDTA, 300 mM sucrose, 1 mM PMSF, pH 7.5 during 30 min at 4°C. Next, the suspension obtained was placed onto the bottom of the centrifuge test tube containing the step-wise sucrose density gradient of 60%, 45%, 35%, 25%

Figure 1: The scheme of flotation to the end of isolating LPMs from red beet root tonoplast by detergent (A) and detergent-free (B) techniques.
and 15%. After that, the procedure of ultracentrifuging in Sorvall Discovery 90SE (Japan) during 18 h at 200,000 g followed at 4°C. In the process of centrifuging the tonoplast fractions underwent flotation through the sucrose density gradient. One fraction (further called DIMF) for further experiments was taken from the interval of 25% of sucrose (the density being 1.083 g cm⁻³) – the opalescence zone.

**Isolation of LPMs from the tonoplast by the detergent-free technique**

The scheme for isolation of LPMs from tonoplast by the detergent-free technique is shown in Figure 1B. Isolation of nondetergent-isolated membrane fractions (NIMFs) from tonoplast by the detergent-free technique also presumed flotation in the discontinuous sucrose density gradient. The tonoplast fraction (0.6 mg) was solubilized in a shaker with 0.5 mL buffer containing 20 mM Tris-HCl, 1 mM MgCl₂, 1 mM CaCl₂, 300 mM sucrose, 0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 1 mg mL⁻¹ aprotinin, 10 μM bestatin, 3 μM E-64, 10 mg mL⁻¹ leupeptin, 2 μM pepstatin, pH 7.8 during 30 min at 4°C.

According to the simplified technique for preparing detergent-free lipid microdomains proposed in [16], the suspension obtained after shaking was passed through the syringe to the end of complete decomposition of the mass of membranes. Next, the suspension containing the decomposed membranes was placed onto the bottom of the centrifuge test tube containing the stepwise sucrose density gradient of 60%, 45%, 35%, 25% and 15%. After that, the procedure of ultracentrifuging in Sorvall Discovery 90SE (Japan) during 18 h at 200,000 g followed at 4°C. As a result of centrifuging, the tonoplast fractions underwent flotation through the sucrose density gradient. One fraction (called NIMF) for further experiments was taken from the interval of 25%-30% of sucrose (the density being 1.083–1.096 g cm⁻³) – the opalescence zone. NIMFs were further washed in 2 mL of the buffer to remove residual sucrose.

**Obtaining the biochemical characteristics of the tonoplast fraction and the isolated microdomains**

The mass of proteins in the tonoplast fraction and in the fractions supposed to contain microdomains isolated by detergent and detergent-free techniques (DIMFs and NIMFs, respectively) was determined by Bradford's method [19] with the use of bovine serum albumin as the method's standard. The mass of lipids in the tonoplast fraction and in the fractions supposed to contain microdomains (DIMFs and NIMFs) was determined by the well-known Bligh and Dyer technique [20]. The content of lipids was determined by the gravimetric method [21].

The DIMFs, NIMFs and the tonoplast fraction underwent the process of lipid extraction according to Bligh's technique. For the purpose of separating different kinds of lipids, one-dimensional thin layer chromatography (TLC), two-dimensional TLC and different specific systems of solvents were used.

The techniques of lipid separation and identification were as described in [6].

**Application of chromatography-mass spectrometry in analysis of the fractions.** In course of our experiments, the lipids extracted from the DIMFs, NIMFs and from the tonoplast fraction were methylated (according to [22]) and analyzed with the aid of the chromatography-mass spectrometer 5973 N/6890N MSD 6890N (Agilent Technology, USA) as described in [6].

**Application of gas-liquid chromatography-mass spectrometry for finding the content of sterols in the fractions.** The chromatography mass spectrometer GC-MS 7000/7890A Triple Quad, Agilent Technologies (USA) was used. The volume of the sample was 0.02 μL. In the process of separation, we used the capillary column HP-5MS (30 m × 0.250 mm × 0.50 μm), Agilent Technologies (USA) and the following parameters of chromatography: stationary phase, 5% phenyl-methyl polysyloxane, the temperature in the evaporator, 250°C; the temperature of the ion source, 230°C; the temperature of the detector, 150°C; the mobile (non-stationary) phase – helium; the gas flow rate, 1 mL min⁻¹; separation of the flows, 5 : 1. Analysis was conducted in the separate ions mode (SIM).

To the end of identification of phytosterols we compared their holding times and the times of holding the standards) and used the libraries of mass spectra NIST08 and WILEY7.
Biophysical characteristic of the tonoplast fraction and the isolated microdomains

In order to determine the phase state of lipid in tonoplast vesicles and the fractions of DIMF and NIMF were observed with the confocal luminescent scanning laser microscope (from now on – a confocal microscope) MicroTime 200 (PicoQuant GmbH, Berlin, Germany) allowing a picosecond time resolution, and using the lipophilic probe laurdan (Sigma-Aldrich) as marker. Laurdan was solubilized in methanol (the concentration of the stock solution being 1 mM). We added laurdan to the suspension of tonoplast vesicles and the fractions of DIMF and NIMF separated from the sucrose density gradients until obtaining the final concentration of 10 μM. The scrutinized objects were incubated with laurdan at 20 ± 2°C for 10 min. These preparations were then analyzed with the confocal microscope. The size of each confocal microscope’s image (snapshot) was 500 × 500 pixels (1 pixel corresponding to 0.16 μm). GP values were computed for each pixel of the images. We have computed not less than 1000 and 300 pixels for each image of membranes of tonoplast vesicles and fractions (DIMFs or NIMFs), respectively.

In case of laurdan use, we used two channels for confocal measuring the generalized polarization (GP) values. The band passes of these two channels were: (i) 470–530 nm and (ii) 400–460 nm. The GP values were computed for each pixel of the image obtained for tonoplast vesicle fraction or for the fractions of DIMF and NIMF. We have computed GP values for not less than 1000 and 300 pixels for each image of membranes of tonoplast vesicles and fractions (DIMFs or NIMFs), respectively. The criterion of taking some or another pixel of the image into consideration was its brightness, which reflected the number of photons reaching the channel’s sensor. The critical value of pixel brightness was the brightness corresponding to the number of photons more than 10. This means that only those pixels were considered, which were fluorescing and giving more than 10 photons. Five to 10 snapshots were taken for each of the variants.

Statistical analysis

The data are given in mean values ± SD each time for at least three independent experiments from three different biological samples. Comparisons were performed using Mann-Whitney U-test (p < 0.01 was considered as statistically valid).

Results

The first stage of our investigation presumed experiments with determination of the total content of proteins and lipids in the microdomains identified, respectively, with the aid of the detergent technique (DIMFs) and the detergent-free technique (NIMFs) (Figure 2). These contents were computed with respect to those in the tonoplast fraction, as the check samples.

As obvious from Figure 2, the total content of lipids in NIMFs identified by the detergent-free technique was 1.2 times larger than in DIMFs identified by the detergent technique. Meanwhile, the total content of proteins was substantially higher (4 times as large) in NIMFs identified by the detergent-free technique than in DIMFs identified by the detergent technique. Consequently, if we presume the presence of only one type of LPMs in the vacuolar membrane, such a difference has to be explained by the influence of incubation with the detergent, when a substantial part of proteins in the membrane undergoes solubilization and as a result of super-centrifugation in the sucrose gradient turns out to occur outside the opalescence zone, which we relate with the presence of LPMs. The quantitative data related to the protein content in the fractions of sucrose density gradient are given in the histogram of Figure 3.

The contents of polar lipids in the tonoplast fraction and in the LPMs identified by the detergent technique (DIMFs) and by the detergent-free technique (NIMFs) is shown in Figure 4. As obvious from Figure 4, phosphatidilinositol (PI) and phosphatidiglyceride (PG) are absent in the list of polar lipids of NIMFs and DIMFs.

![Figure 2](image-url)  
Figure 2: The total contents of lipids and proteins in the fraction type identified by the detergent technique (DIMFs) and in the fraction type identified by the detergent-free technique (NIMFs). These contents were computed with respect to those in the tonoplast fraction, as the check samples. *Significant at p < 0.01(Mann-Whitney U-test).
According to the literary data, phosphatidic acid (PA), whose level in membranes is only 1–2 per cent of the total content of phospholipids, is said to play a definite role in lipid metabolism of plant cells, in connection with the processes of synthesis of phospho- and glycolipids [23]. Furthermore, PA is considered to be a secondary messenger of lipid nature, which signals about some damages, water, salt or oxidative stresses and possibly about other processes in plant cells [24]. In our investigation, PA has been found in tonoplast in very small amounts. It has been revealed in some larger amounts only in NIMFs (detergent-free technique) (Figure 4).

Application of the detergent technique reveals a relative excess in sphingolipids (SLs) in DIMFs with respect to their content in tonoplast (the indicator, which is presently considered to be the main biochemical criterion for identifying LPMs). Meanwhile, application of the detergent-free technique proves that, vice versa, the fraction of SLs in NIMFs is almost twice lower than the content of SLs in tonoplast.

The detergent technique does not reveal (possibly, it solubilizes) phosphatidylethanolamin (PE) in DIMFs, meanwhile, it allows one to reveal the presence of such glycolglycerolipids as MGDG and DGDG. As known from the literature, the content of MGDG and DGDG is especially high in chloroplasts [25]. However, high content of these lipids is not characteristic of the majority of plant membranes. Although, according to [8], the content of MGDG in tonoplast isolated from ethiolated mung beans (Vigna radiata L.) and the content of DGDG are, respectively, 5 and 4.2 times as large as in the plasma membrane. In our case, the content of MGDG and DGDG in DIMFs identified by the detergent technique is approximately twice as low to compare with the results obtained by the detergent-free technique.

The contents of neutral lipids in the tonoplast fraction and in the LPMs identified by the detergent technique (DIMFs) and by the detergent-free technique (NIMFs) is shown in Figure 5. This Figure, first of all, attracts attention to the contents of free sterols (FS), free fatty acids (FFA) and free hydrocarbons (FHC). Meanwhile, the following facts are obvious.

The presence of alkylglycerides (ADGs) is hardly ever a criterion for identification of LPMs, because these compounds are absent in the list of neutral lipids in DIMFs and NIMFs obtained by the two techniques.

The detergent technique does not reveal (possibly, it solubilizes) also such neutral lipids as methyl ketone

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**Figure 3:** The protein content in fractions of the sucrose density gradient.

Ten fractions obtained with the aid of either detergent or detergent-free techniques were collected from top to bottom of the flask (15% sucrose – fractions 1–2; 25% – fractions 3–4; 35% – fractions 5–6; 45% – fractions 7–8; 60% – fractions 9–10). Data were from three biological replicates ± SD. *Significant at p < 0.01 (Mann-Whitney U-test).

**Figure 4:** The detailed contents of polar lipids in the tonoplast fraction and in the LPMs identified by the detergent technique (DIMFs) and by the detergent-free technique (NIMFs).

X, Unidentified lipids; SLs, sphingolipids; PI, phosphatidilinositol; PC, phosphatidicholine; PA, phosphatic acid; PE, phosphatidylethanolamin; PG, phosphatidylglyceride; MGDG, monogalactosyldiglyceride; DGDG, digalactosyldiglyceride. *Significant at p < 0.01 (Mann-Whitney U-test).

**Figure 5:** The detailed contents of neutral lipids in tonoplast fraction and in the LPMs identified by the detergent technique (DIMFs) and by the detergent-free technique (NIMFs).

MG, Monogalactaslyde; FS, free sterols; FFA, free fatty acids; TAG, triacylglyceride; MK, methyle ketone; MEFA, methyl esters of fatty acids; ADG, alkylglycerides; SE, sterol esters; FHC, free hydrocarbons. *Significant at p < 0.01 (Mann-Whitney U-test).
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(MK), methyl ethers of fatty acids (MEFA) and sterol ethers (SEs).

The results of detergent and detergent-free techniques taken in comparison have proved the following. The excess content of free sterols revealed by the detergent technique (and considered with respect to that in tonoplast) (see Figure 5), the excess content of free hydrocarbons (whose fraction in case of the detergent technique of isolation turns out to be 3.5 times as large as that in tonoplast and larger than the concentration registered by the detergent-free technique) (Figure 5), as well as the total content of fatty acids (FAs) in DIMFs (see also Figure 6) – cannot be considered as plausible criteria good for identification of DIMFs. It is very likely that an excess in contents of these compounds is just the consequence of applying the detergent technique of isolation. The comparison of the data related to the total content of saturated fatty acids and the total content of unsaturated fatty acids (Figure 6) speaks in favor of the detergent-free technique.

Some experts consider the content of unsaturated fatty acids in lipids of LPMs as an important characteristic good for identification of LPMs [26]. The method of chromatography mass spectrometry was applied to obtain the comparative characteristic of the content of unsaturated fatty acids in lipids of LPMs for detergent and detergent-free techniques. The total amounts of saturated and unsaturated fatty acids are shown in Figure 6. The detailed contents of fatty acids in the lipids of the tonoplast fraction, as well as in the LPMs identified by the detergent technique (DIMFs) and by the detergent-free technique (NIMFs), are given in Figure 7.

In course of analysis, we marked the relative predominance (in the mass of fatty acids) of such saturated fatty acid as palmitic acid (C16:0), and such unsaturated fatty acids as oleic acid (C18:1) and linoleic acid (C18:2).

The result of quantitative analysis of sterols in membrane fractions under scrutiny are shown in Figure 8. Plausible differences in the sterol content are obvious. It is obvious that concentration of cholesterol, α-tocopherol and stigmasterol are not indicative. The content of β-sitosterol in NIMFs was corresponding to that in tonoplast. Meanwhile, the content of β-sitosterol in DIMFs was twice as large.

In order to characterize the orderedness of the lipids in LPMs, experiments with the aid of a laser scanning confocal fluorescence microscope (from now on – a confocal microscope) were conducted with the use of laurdan. Application of this probe was conditioned by the assumption that the position of its emission maxima is determined by the relation between liquid-disordered (Ld) and...
liquid-ordered (Lo) phase domains in the membrane. This relation is normally accessed via the value of generalized polarization (GP) of probe fluorescence. When the lipids are in the gel phase, the laurdan emission maximum is registered at 440 nm, and when lipids are in the liquid-crystalline phase, the emission maximum corresponds to 490 nm [27]. On the basis of the laurdan emission intensity in different spectrum parts it is possible to compute the GP value, which reflects the membrane’s phase state, while using the equation [28]. GP may assume the values from −1 to +1. In the case of Ld and Lo membrane domains, this value varies from 0.05 to 0.25, and for denser membrane domains – from 0.25 to 0.55 [29].

In our experiments conducted with the aid of a confocal microscope, series of images of both vacuolar membranes and fractions of LPMs (in both cases stained with laurdan) isolated by the two techniques have been obtained. The computation of GP values for each of the image pixels has been fulfilled (Figure 9).

In the series of our experiments, the GP value for the scrutinized vacuolar membrane was −0.007 ± 0.033, what indicated to its liquid crystalline phase state (Figure 9). In the case of DIMFs, the GP value was 0.425 ± 0.048, and this gave evidence that there were highly ordered (dense) LPMs in these fractions. The GP value characteristic of NIMFs was higher than that of tonoplast, but at the same time, it was substantially lower than that of DIMFs and made 0.088 ± 0.018. The latter indicated to the fact that orderedness of lipids in NIMFs (isolated by detergent-free technique) was substantially lower than that of DIMFs (isolated by detergent technique).

**Discussion**

The definition of microdomains proposed by L. Pike states that these are “small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes” [30]. But the data available could allow one to discuss only their size and the biochemical enrichment. The concept, which allowed K. Simons and E. Ikonen to identify LPMs in the membranes [31], was grounded on the idea of local membrane’s enrichment with sterols and sphingolipids. Obviously, the idea of enrichment itself was underinvestigated, and the criterion of enrichment was never correctly verified. Of course, a simple statistically valid difference in concentration is one, but the threshold, beginning from which it is possible to speak about presence / absence of plausible sterol and sphingolipid enrichment in LPMs, is something
absolutely different. What (and how large) is the value of relative excess in the content of sterols and sphingolipids in DIMFs with respect to the rest part of the membrane, beginning from which it is already possible to speak about the enrichment (5%, 15%, 25% or more)? In essence, this is the issue of existence (or absence) of a really plausible technique for identification of LPMs.

Furthermore, another problem bound up with plausible identification of LPMs in membranes is the imperfect character of the detergent technique of isolation: many researchers state that this technique provokes artefacts [32].

Meanwhile, despite the deficit of reliable results, the specialists dared to discuss the aspects of microdomains’ formation, stability, protein-protein, lipid-protein interactions and even the functions. With time, this approach had inevitably led to the idea of diversity of microdomains [33] and to the idea of existence of non-rafts microdomains [34].

A substantial aspect bound up with the issue of plausible identification of LPMs in vacuolar membranes is the issue of biophysical characteristics of the microdomains isolated by the most gentle technique. That is why, after isolating DIMFs from tonoplast [6], we have proceeded to isolating LPMs by a detergent-free (softer) technique, what, as expected, has given us the possibility to avoid artifacts bound up with the use of Triton X-100 and obtain LPMs, which have no substantial differences from those expected to be located in DIMFs isolated from tonoplast. The results obtained give evidence of significant differences between DIMFs and NIMFs with respect to biochemical and biophysical characteristics. The comparison of the results of application of detergent and detergent-free techniques (under similar experimental conditions) has given the following important results.

On the stage of isolating LPMs with the use of detergent-free technique, the opalescence zone, which, as we supposed, contained LPMs, was registered at the same location as in case of isolating DIMFs. As far as determination of the total fractional content lipids and proteins is concerned, the detergent technique has induced the loss of 3/4 proteins and over 11% of lipids contained in tonoplast. The total content of lipids in LPMs isolated by the two techniques was practically the same. The differences in the quantitative content of proteins were expected.

Significant differences were revealed as a result of investigation of biophysical characteristics of LPMs. For example, orderedness of lipids in LPMs isolated by the two techniques, which was assessed via GP values, obviously differed. So, despite the fact that values of GP for the LPMs isolated without detergents were 12.6 times as large as those of tonoplast (0.088 and 0.007, respectively), these values were at the same time 4.8 times lower than for DIMFs (0.425 and 0.088, respectively).

A definite lipid content is an important characteristic of LPMs. After revealing the lipid content it has become obvious that in case of detergent-free isolation we have to do with the structures, which significantly differ from those present in the opalescence zone when Triton X-100 is used. This difference relates mainly the content of sterols and sphingolipids. For example, the concentration of sterols in DIMFs was 1198 ng/100 mg of lipids, and the concentration of sterols in NIMFs was 663.9 ng/100 mg of lipids. The differences in the content of sphingolipids was ever larger: in case of DIMFs, sphingolipids made 64.7% of the total content of polar lipids, while in case of NIMFs, sphingolipids made only 19.1%. Also we registered a relatively smaller content of saturated fatty acids in NIMFs with respect to DIMFs.

When discussing the results obtained for polar lipids in DIMFs, it is worth to note that the detergent technique: (i) does not reveal phosphatidylethanolamin (PE); (ii) reveals the presence of such glycerolipids as MGDG and DGDG in DIMFs; (iii) reveals a relative excess in sphingolipids (SLs) in DIMFs with respect to their content in tonoplast (the indicator, which is presently considered to be the main biochemical criterion for identifying LPMs).

Note, application of the detergent-free technique proves that, vice versa, the fraction of SLs in NIMFs is almost twice as low as the content of SLs in tonoplast. The content of MGDG and DGDG identified by the detergent technique is approximately twice as low as the results obtained by the detergent-free technique.

When discussing the results obtained for neutral lipids in DIMFs, it is worth to note that the detergent technique does not reveal the presence of methyl ketones (MK), methyl ethers of fatty acids (MEFA), sterol ethers (SEs) and alkylglycerides (ADGs). These compounds are absent in the list of neutral lipids in DIMFs and NIMFs obtained by the two techniques.

The excess content of free sterols revealed by the detergent technique (and considered with respect to that in tonoplast), the excess content of free hydrocarbons and also the total content of fatty acids are not plausible criteria good for identification of DIMFs. The excess in contents of these compounds is the consequence of applying the detergent technique of isolation. The comparison of the data related to the total content of saturated fatty acids and the total content of unsaturated fatty acids speaks in favor of the detergent-free technique.

In conclusion, there are already many publications in which it has been shown that there may simultaneously
exists several types of LPMs in membranes [35, 36]. And despite the fact that these results have been obtained for the animal and yeast membranes, it cannot be excluded that plant vacuolar membranes may also contain several types of LPMs. We propose the existence at least two types of LPMs in vacuolar membrane.

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