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PHOSPHOLIPID COMPOSITION OF THE MEMBRANE OF LUTOIDS FROM *HEVEA BRASILIENSIS* LATEX

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Abstract—Intact lutoids were isolated from the latex of *Hevea brasiliensis* and purified on a sucrose density gradient. Lutoid membranes prepared by osmotic lysis are characterized by a high content of phosphatidic acid in which unsaturated and saturated fatty acids are present in equal proportions. Linolenic acid is absent. The results are discussed in relation to the biological role of lutoids in the latex vessels of *Hevea brasiliensis*.

INTRODUCTION

Natural *Hevea* latex is a complex cytoplasmic system in which rubber and non-rubber particles are dispersed in an aqueous phase called the cytoplasmic serum. Among the non-rubber components are the lutoids discovered by Homans and Van Gils [1] and these particles can be present in relatively large amounts. They possess a single membrane [2], are sensitive to the tonicity of suspension media [3,4] and are also able to accumulate various organic acids [5]. Their inner compartment contains a wide range of hydrolytic enzymes [6] and some of their enzymic properties are analogous to those of lysosomes of animal cells. For this reason they are generally considered as a type of phytolysosomes [2,5,6]. However, their biogenesis and metabolic role in rubber synthesis has so far remained highly speculative.

A biochemical study of the membrane of these particles which are found in a very peculiar biological context, the laticiferous cell which synthesizes rubber, may yield a clue to the specific role of these particles and add to the general knowledge of biological membranes in plants. In this paper, we report on the phospholipid and fatty acid composition of the lutoid membrane. The results are compared with those obtained from the study of the lipid composition of the membrane surrounding the rubber particles. Enzyme activities and electron transfer capacity of the lutoid membrane have been dealt with in another paper [7].

RESULTS

Purification of lutoids

In this work, where a rapid separation of the lutoid fraction was the main aim, only two fractions, a light one (from the 0.8/1.2 M interface) and a heavy one (from the 1.2/1.8 M interface) were obtained by centrifugation on sucrose density gradients. Lutoids, however, are rather heterogenous particles and more fractions can be obtained on more sophisticated gradients [7]. The heavy fraction is of a much larger volume and corresponds to the fraction whose electron transport capacity has been previously studied [7]. For this reason, all experiments described in this paper refer to this heavy fraction. Moreover, this fraction is not contaminated with mitochondria, microsomes or plastid-like Frey–Wyssling particles [6–8]. Parallel experiments have also shown that both lutoid fractions have very similar properties.

Phospholipid composition

The phospholipid content of lutoid particles is *ca* 370 μg phospholipid per mg protein. Compared with other subcellular membranes from plant cells, this value falls in the range of those found in microsomes or inner mitochondrial membranes [9]. It is much higher than the value for glyoxysomes [10] but noticeably lower than that of outer mitochondrial membranes [9].

The major spot by TLC of the phospholipid extract from lutoids corresponds with phosphatidic acid. This compound was confirmed by deacylation in an alkaline medium which yielded glycerophosphate. Two other components, which have not been identified, are also present. Although these unidentified phospholipids have R_f values expected for an artefact such as phosphatidyl ethanol, this has been ruled out on the basis of comparative experiments using boiling methanol or ethanol as inactivating agents for the lutoid membranes [11]. On a phosphorus basis, phosphatidic acid represents 82% of the total phospholipid fraction and the two other components only 18%.

TLC also showed that phosphatidyl choline and phosphatidyl ethanolamine, which are constituents of most biological membranes [9,12], or diphosphatidyl glycerol, a marker of inner mitochondrial membranes [9,13], are absent from the membranes. The absence of diphosphatidyl glycerol can be correlated with the absence of cytochrome $a + a_3$ in lutoid membranes [7], thus eliminating any major contamination of lutoid preparations by mitochondria [13]. This type of result was first observed on

fresh latex rapidly transported to Paris by air. Since the high content of phosphatidic acid could have been an artifact resulting from the action of phospholipase D [14] during air transport, it was decided to repeat these experiments in Africa immediately after tapping, the latex being collected in containers kept at 0°. These are the results presented in this paper and they are identical with those obtained with 24–36 hr-old latex. This indicates that phospholipase D is probably not responsible for the high phosphatidic acid content found in lutoid membranes, although phospholipase D activity has been located in the cytoplasmic serum [6], i.e. the cytosol phase of the latex, but not in the lutoid fraction. It should also be pointed out that phospholipase D activity in the serum is rather low [6] and that its optimum pH is 5, whereas in the latex pH it is *ca* 7.

In order to ensure that phospholipase D had only a negligible action during the time required for latex collection (*ca* 1 hr), parallel experiments were also run, under exactly the same set of experimental conditions, both on the phospholipid composition of lutoid membranes and on that of membranes surrounding the small rubber particles [15]. TLC clearly showed that both membranes have quite different phospholipid compositions. In particular, no phosphatidic acid is present in rubber particle membranes and phosphatidyl choline appears to be the major component of these membranes. Phosphatidyl ethanolamine also occurs in small amounts together with phosphatidylglycerol.

We therefore conclude that a qualitative difference exists between the lipids of the two types of membranes. It is difficult to imagine that phospholipase D can be responsible for the presence of phosphatidic acid in the membranes of the lutoids only, since both types of particles are in suspension in the same cytoplasmic serum. The comparison of the phospholipid compositions of both types of membrane clearly rules out the possibility of a selective destruction of phosphatidyl choline and phosphatidyl ethanolamine in lutoid membranes to yield the major component phosphatidic acid.

Fatty acid composition

GLC analysis of the fatty acids from phosphatidic acid extracted from lutoid membranes shows the following % composition: $C_{14:0}$, 1.6%, $C_{16:0}$, 20.5%, $C_{18:0}$, 25.3%, $C_{18:1}$, 13.8%, $C_{18:2}$, 38.8%, $C_{18:3}$, not detectable. Saturated and unsaturated fatty acids are present in equal proportions. The characteristic feature of this fatty acid composition is the absence of linolenic acid, an acid usually well represented in phospholipids from most other plant organelles [9,16].

DISCUSSION

This paper presents the results of an investigation on the nature of the lipid material in the membranes of lutoids from *Hevea brasiliensis*. Any major alteration of the phospholipid composition by phospholipases [14] were prevented by careful preparation of the latex, a rapid isolation-purification procedure together with rapid fixation of the lutoid membranes. The length of time for preparing purified lutoid membranes has been made as short as possible, but could not be reduced to less than 4 hr. Under these conditions, lutoid membranes were characterized by the absence of the nitrogen-containing phospholipids, phosphatidyl choline and phosphatidyl ethanolamine, and by the quasi-exclusive presence of phosphatidic acid (more than 80% of total phospholipids). Lutoid membranes are probably a rare case of biological membranes with such a predominent phospholipid component. Phosphatidyl choline, however, is well represented among the lipids of the membranes of rubber particles.

The fact that the lipid composition of the membrane surrounding the rubber particles markedly differs from that of the lutoids supports the evidence that phospholipase D cannot be responsible for the high phosphatidic acid content of the lutoid membranes. Indeed it would be difficult to explain why phospholipase D would specifically destroy the phospholipids of the lutoid particles and not those of the rubber particles which are in exactly the same environmental conditions within the latex.

The presence of phosphatidic acid in large amounts in lutoid membranes could explain the type of distribution of these organelles on density gradients [7] because the high electronegative charge observed on the lutoids [17] can be directly related to their high phosphatidic acid content. Charge interactions between electronegatively-charged particles (lutoids, rubber particles, etc...) within the cytoplasmic serum could play an important role in maintaining the latex stability or preventing its coagulation. Small, localized drops in the electronegative charge of the lutoids, by combination with cations [18] or by other mechanisms, could facilitate the aggregation of rubber particles on the lutoid surface. This could account for the apparent heterogeneity of the density of these particles and their loose distribution on density gradients as well as for the decrease in their density upon storage [7]. The high phosphatidic acid content of lutoid membranes is also probably related to the fact that these particles are able to accumulate various cations in their inner compartment [5]. The penetration of cations can be facilitated by binding first to the phosphatidic acid within the membranes, as has been suggested for calcium fixation by the plasma membrane of rat liver cells [18].

The relative abundance of saturated fatty acids, by comparison with other plant cell organelles [19,20], makes the lutoid membrane analogous to the outer membrane of plant mitochondria [9] or the plastid envelope [16]. The rigidity of these membranes [21] and the fragility of lutoids when they are exposed to hypotonic media [6] can be related to a lack of fluidity due to their relatively high content of saturated fatty acids.

In this work, the possibilities that phospholipase D could be responsible for the high phosphatidic acid content of the lutoid membranes has been eliminated as much as possible. We believe that this untypical membrane composition can truly be related to the particular environment of the lutoid particles. Lutoids are lysosome-like organelles found only in latex, in laticiferous cells or in latex vessels. It must also be stressed that the metabolic function of these cells is quite unique, being mainly oriented to the synthesis of rubber. Finally, even if the possibility of an artifact could not be entirely dismissed, it must be recalled that for practical purposes, in rubber production for instance, lutoids such as those whose phospholipid composition has been described in this paper are the ones that are actually found in fresh latex upon collection from the *Hevea* tree. That composi-

tion is undoubtedly of great importance to the physiology of the latex flow since it is closely related to the latex stability. A major difference between lutoids in freshly collected latex and lutoids *in situ* in the uninjured latex vessels seems highly improbable.

EXPERIMENTAL

Preparation of lutoid membranes. The latex of *Hevea brasiliensis* (clone PR 107) was collected at the I.R.C.A. plantation, Languédédou (Ivory Coast) in containers kept at 0° and treated within the following hr. In this study it was essential to operate as rapidly as possible in order to prevent phospholipid degradation. Moreoever, lutoids display a loose distribution [7] when centrifuged on a sucrose density gradient [6]. A simple separation of lutoids into two fractions was obtained by immediately centrifuging the collected latex, laid on top of 3 layers of 0.8, 1.2 and 1.8 M sucrose solns. An input of 15 ml of latex was used for each 60 ml-centrifuge tube. These tubes were centrifuged at 35000 *g* for 180 min. The fraction arrested at the interface 1.2/1.8 M was collected using a fraction collector and then disrupted by osmotic swelling [6]. Pure lutoid membranes were obtained as already described [7].

Preparation of rubber particles. Rubber particles were prepared by centrifuging pure latex at 35000 *g* for 30 min. The top pellet, which consists mainly of rubber particles with their surrounding membranes, was collected. After homogenization and washing in an appropriate vol of H_2O, the rubber particles were centrifuged again at 35000 *g* for 30 min. The top pellet was collected for analysis.

Phospholipid analysis. Following purification, lutoid membranes and rubber particles were inactivated in boiling EtOH for 10 min according to ref. [22]. Phospholipids were then extracted using the method of ref. [23], with the substitution of EtOH for MeOH in order to avoid the formation of phosphatidyl methanol [22]. The phospholipids were finally dissolved in $CHCl_3$. All these operations were carried out in Africa. The $CHCl_3$ extracts were then brought by air to Paris in ice-cold containers for further analysis. $CHCl_3$ extracts were chromatographed on Si gel columns. Neutral lipids and most of the free fatty acids were eluted with $CHCl_3$. The progressive elution of the various phospholipids was then carried out by elution with $CHCl_3$ containing increasing proportions of MeOH, according to ref. [24]. The eluates were then concentrated to dryness and the phospholipids dissolved in a small vol of $CHCl_3$. Phospholipids were identified by TLC [9]. Si gel was used as adsorbent and the solvent system was $CHCl_3$–MeOH–H_2O (65:25:4). Phospholipid spots were revealed by exposing the plates of OSO_4 vapours. Phospholipids were quantitatively determined in $CHCl_3$ solns or after elution from the plates by measuring their P contents according to ref. [25]. Deacylation in alkali was carried out according to ref. [26]. After deacylation, the phosphoric esters were chromatographed as described in ref. [22].

Fatty acid analysis. Following purification by Si gel TLC and elution, phospholipids were submitted to methanolysis using H_2SO_4–MeOH (0.1:4) for 90 min at 83°. Me esters were extracted with petrol and analyzed by GLC using a FID instrument and 2 m × 2 mm columns filled with 15% DEGS coated on Varaport 30. The column was maintained at 180° and detector and injector temps at 200° with N_2 at 12 ml/min. Standard mixtures of known fatty acid Me esters were used for comparison of Rt.

Protein determination. The protein contents of lutoid membrane preparations were determined by mineralization and Nesslerization.

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