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LIPID COMPOSITION OF SYMBIOSOMES FROM PEA ROOT NODULES

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Abstract—The lipid composition of several membrane fractions of pea (*Pisum sativum*) nodule and pea root plasma membrane (RPM) were analysed. The peribacteroid membrane (PBM) contained β-amyrin, a triterpenol that was not accumulated in the RPM. β-Amyrin was also found in the microsomal fraction of nodule cells (MFN), which mostly corresponded to plant cell endoplasmic reticulum vesicles. Δ^5-Avenasterol, a precursor of other phytosterols, was also accumulated in the PBM as observed in the MFN of pea nodule cells. Treatment of pea plants with a 5-day pulse of 10 μM γ-ketotriazole (an experimental herbicide) resulted in the accumulation of 14α-methyl sterols in the PBM, the MFN and the RPM. The amount of these sterols found in the RPM was *ca* five times less than in the PBM, suggesting that there was a requirement for large amounts of membrane material from sources other than plasma membrane to synthesize PBM. Phospholipid analysis revealed that both the PBM and the MFN had a greater concentration of phosphatidylcholine (PC) than phosphatidylethanolamine (PE). In the RPM, there was more PE than PC. Analysis of phospholipid-esterified fatty acid indicated that the PBM was more similar to the MFN than to the RPM.

INTRODUCTION

The last step in the establishment of a symbiosis between *Rhizobium* bacteria and leguminous plants, capable of fixing atmospheric nitrogen, involves the development of a symbiont organelle, the symbiosome [1, 2]. In pea plants, infection of polyploid cells occurs through infection threads. Once the bacteria reach the matrix of the infection droplet they differentiate into bacteroids and, by enclosing themselves in a plant derived membrane, referred to as peribacteroid membrane (PBM), *Rhizobium* enter the host plant cytosol [3].

The PBM behaves as a mosaic membrane and a number of hypotheses have arisen in the last few years about its origin [4–6]. There is some evidence from electron microscopy that the PBM shares specific characteristics with plant plasma membranes [7]. The use of enzyme markers, such as vanadate-inhibited and potassium-stimulated H^+-ATPase, have indicated that the PBM shares an ATPase enzyme similar to that found in plant plasma membranes [8, 9]. Other authors have found similarities between the PBM and the endomembrane system [5]. Brewin *et al.* [10] used antibodies raised against specific membrane proteins that recognized the PBM, the plasma membrane and the Golgi apparatus, but not endoplasmic reticulum (ER) membranes. Also, Katinakis and Verma [11] and Mellor *et al.* [12] obtained several antibodies that revealed the presence of specific PBM proteins (e.g. nodulin-24). However, the lipid composition of the PBM, in particular its fatty acid profile, appeared to be more related to the ER than to other nodule membranes [13]. Also, [^{14}C]CDP-choline was found to accumulate as phosphatidylcholine (PC) in the ER and the PBM [14]. Two isoenzymes of choline kinase have been identified in the ER and it was observed that choline kinase II was over-expressed when effective nodulation and complete development of the PBM occurred [15, 16]. On the other hand, Perotto *et al.* [17] described the disappearance of a glycosphingolipid from the PBM of pea nodules as it becomes differentiated in mature symbiosomes, whereas this glycolipid is always present in the plasma membrane of infected cells. Therefore, not only inclusion of new membrane components provided by endomembrane vesicles, but also changes in existing membrane components are needed to achieve the full development of the PBM.

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The bacteroid endocytosis process requires the delivery of large amounts of membrane material to enclose each bacteroid, since there is synchronous maturation of thousands of symbiosomes in the same infected cell [4]. This demand for new membrane components has been related to an over-expression of phosphatidylinositol-3-hydroxykinase in developing soybean nodules, an enzyme recently identified as part of a protein complex involved in signal transduction and vesicle targeting [18].

It is possible that the bacteroid contributes to PBM synthesis, as observed in the symbiosis between *Alnus rubra* and *Frankia* [19], where the bacteria provided specific bacterial lipids, triterpenoids of the hopanoid group, that were found in nodule tissue, but absent in host roots [20]. However, this symbiotic relationship is different from that between *Rhizobium* and legumes [21].

There is very little information about the lipid composition of the membranes involved in the symbiotic relationship between *Rhizobium* and legumes. In the present work, we determined the lipid composition of symbiosomes purified from mature pea root nodules and compared it with that of the plasma membrane of pea roots and the endomembrane system of pea nodule cells. The existence of a mechanism to select specific sterols in the synthesis of the PBM was assessed by a pulse-treatment with γ-ketotriazole, a xenobiotic that inhibits 14α-demethylation in phytosterol biosynthesis, leading to the accumulation of 14α-methyl sterols [22, 23].

RESULTS

Analysis of the sterols (as acetates) extracted from the symbiosome and PBM also revealed the presence of a triterpenol not detected in the root plasma membrane (RPM) (Table 1). This was identified as β-amyrin and accounted for *ca* 16% of the sterol-acetylated fraction. This compound was also identified in the microsomal fraction of nodule cells (MFN), but in lesser concentrations (Table 1). When a similar fraction prepared from free living bacteria was analysed, β-amyrin was not detected (data not shown).

The symbiosome lipid composition was compared with that of RPM. From the MFN, a two-phase system and a continuous sucrose density gradient were used to purify plasma membranes. In both cases, negligible amounts of material, which could be identified as plasma membrane, were recovered (<1% of the starting material). When the heterogenous MFN (11.3 ± 0.4 mg protein ml^{-1}) was separated on a continuous sucrose density gradient most of the material (3.8 ± 0.2 mg protein ml^{-1}) was recovered in the fraction corresponding to 25–30% sucrose. This fraction was identified as ER vesicles, confirmed by ATPase, NAD(P)H-cytochrome *c* reductase and cytochrome *c* oxidase marker enzyme assays (data not shown).

The sterol content of the PBM resembled that of the MFN more than the RPM (Table 1). Δ^5-Avenasterol, a precursor of stigmasterol and sitosterol, was found in the PBM and MFN, but not in the RPM. The specific content of sterols (μg mg^{-1} protein) was greater in RPM than in the PBM and MFN, because they contained larger amount of protein (*ca* 10 mg protein ml^{-1}; 20 times greater than RPM).

A 5-day treatment with 10 μM γ-ketotriazole resulted in the accumulation of 14α-methyl sterols, particularly obtusifoliol, in intact symbiosomes, MFN and RPM (Table 2). The appearance of 14α-methyl sterols was accompanied by a decline in the normal sterol content of these fractions, with stigmasterol and sitosterol being most affected, particularly in the MFN (Table 2). In intact symbiosomes and MFN, obtusifoliol represented *ca* 30% of the free sterol content, whereas in RPM it only accounted for 4.5%. These differences in 14α-methyl sterol accumulation suggest that the rate of incorporation of new material (possibly released from the endomembrane system) into the symbiosome was greater than that into RPM.

Analysis of phospholipids showed that the ratio of PC to phosphatidylethanolamine (PE) in the symbiosome, PBM and MFN was *ca* 2:1, but in RPM the PC/PE ratio was 1:2 (Table 3). Sphingolipids, phosphatidic acid (probably the result of some endogenous phospholipase activity), phosphatidylglycerol and diphosphatidylglycerol were found in all fractions, but were not quantified. The total content of phospholipids

Table 1. Free sterol and triterpenol content of symbiosomes (Sy), peribacteroid membrane (PBM), nodule microsomal fraction (MFN), root plasma membrane (RPM) (μg mg^{-1} protein) and two-day-old root tissue (RT) (μg g^{-1} root), plus standard error ($p < 0.05$). Results are the mean of 10 independent experiments. Relative sterol content (%) is shown in parentheses

	Sy	PBM	MFN	RPM	RT
Cholesterol	0.18±0.01 (7)	0.31±0.09 (6)	0.09±0.04 (2)	0.09±0.05 (<1)	5.30±0.31 (6)
Campesterol	0.08±0.03 (3)	0.16±0.08 (3)	0.21±0.06 (5)	4.65±0.23 (13)	13.03±0.25 (16)
Stigmasterol	1.09±0.07 (44)	2.49±0.10 (45)	2.25±0.19 (55)	21.69±0.15 (58)	16.51±0.18 (20)
Sitosterol	0.57±0.13 (23)	1.30±0.11 (24)	0.97±0.21 (24)	9.87±0.25 (27)	38.95±0.33 (49)
Δ^5-Avenasterol	0.17±0.05 (7)	0.45±0.09 (8)	0.45±0.12 (11)	n.d.	4.11±0.26 (5)
β-Amyrin	0.40±0.09 (16)	0.77±0.14 (14)	0.11±0.03 (3)	n.d.	1.01±0.35 (1)

n.d. = not detected.

Table 2. Free sterol and triterpenol content ($\mu g\ mg^{-1}$ protein) of intact symbiosomes (Sy), microsomal fraction of nodule cells (MFN) and root plasma membrane (RPM) of (A) control and (B) γ-ketotriazole treated plants. Results are the mean of three independent experiments, standard error ($p < 0.05$) lower than 5%. Relative sterol content (%) is shown in parentheses

	Sy		MFN		RPM	
	A	B	A	B	A	B
Cholesterol	0.08 (6.5)	0.05 (3.8)	0.07 (1.5)	0.08 (1.7)	2.1 (4.0)	2.1 (4.5)
Campesterol	0.04 (2.3)	0.03 (2.5)	0.25 (5.3)	0.09 (1.9)	7.3 (14.0)	6.3 (13.2)
Stigmasterol	0.53 (43.4)	0.38 (28.8)	2.38 (51.0)	1.49 (32.2)	24.6 (47.1)	20.2 (42.3)
Sitosterol	0.31 (25.4)	0.22 (16.7)	1.18 (25.3)	0.50 (10.8)	18.1 (34.9)	13.5 (28.3)
Δ^5-Avenasterol	0.10 (8.2)	0.04 (3.0)	0.68 (14.6)	0.20 (4.3)	n.d.	n.d.
Obtusifoliol	n.d.	0.41 (31.1)	n.d.	1.51 (32.6)	n.d.	3.1 (4.5)
Dihydroobtusifoliol	n.d.	0.05 (3.8)	n.d.	0.45 (9.7)	n.d.	1.9 (3.9)
14α-Methyl-Δ^8-ergostenol	n.d.	n.d.	n.d.	0.17 (3.8)	n.d.	0.6 (1.3)
β-Amyrin	0.17 (13.9)	0.14 (10.6)	0.11 (2.3)	0.14 (3.0)	n.d.	n.d.

n.d. = not detected.

Table 3. Phospholipid content ($\mu g\ mg^{-1}$ protein) of symbiosomes (Sy), peribacteroid membrane (PBM), nodule microsomal fraction of nodule cells (MFN), root plasmalemma (RPM) and naked bacteroids (NBA), plus standard error ($p < 0.05$). Results are the mean of nine independent experiments

	Sy	PBM	MFN	RPM	NBA
PE	0.27±0.06 (37.5)	0.23±0.05 (33.3)	0.37±0.08 (38.9)	0.34±0.09 (72.3)	0.19±0.06 (32.2)
PI	0.01±0.00 (1.4)	n.d.	0.04±0.03 (4.2)	n.d.	n.d.
PC	0.44±0.04 (61.1)	0.46±0.04 (66.6)	0.54±0.14 (56.8)	0.13±0.07 (27.7)	0.40±0.05 (67.8)
PC/PE	1.6	2.0	1.5	0.4	2.1
Sterol/phospholipid	3.5	7.9	4.3	77.2	n.d.

n.d. = not detected.
PI = phosphatidylinositol.

was of the same order in all fractions (*ca* 0.60 $\mu g\ mg^{-1}$ protein).

The esterified-fatty acid composition of symbiosomes and PBM was similar and both had an unsaturation ratio (UR) of *ca* 7.5 (Table 4). The composition of 18:*n* fatty acids of the MFN was similar to that of the PBM, but the proportion of 16:0 of MFN was greater and the proportion of 16:1 less, resulting in an UR of 3.3 (Table 4). RPM was different from PBM, where a small proportion of 17:1 was present, with a greater concentration of 16:0. The content of 18:0 was double that in PBM, and RPM had an UR of 2.6 compared with 7.5 for PBM (Table 4). The naked bacteroid fraction showed the greatest differences from the membrane fractions examined, with a large proportion of 18:0 (44.5%) and a UR of 1.2.

Table 4. Relative content (%) of esterified fatty acids of symbiosomes (Sy), peribacteroid membrane (PBM), microsomal fraction of nodule cells (MFN), root plasmalemma (RPM) and naked bacteroids (NBA), plus standard error ($p < 0.05$). Results are the mean of six independent experiments

	Sy	PBM	MFN	RPM	BNA
16:0	17.2±1.0	16.1±1.5	35.3±1.6	30.1±1.6	9.8±2.0
16:1	9.6±0.9	11.2±1.6	2.8±0.6	11.3±2.9	11.7±0.7
17:1	n.d.	n.d.	n.d.	2.7±0.6	n.d.
18:0	3.5±1.3	3.1±0.8	1.6±0.3	8.0±1.8	44.5±4.3
18:1	15.1±0.8	13.7±2.6	10.6±1.4	17.7±2.3	17.9±2.0
18:2	35.4±1.4	39.9±3.8	41.4±4.0	25.7±4.0	11.9±1.1
18:3	19.1±1.0	16.7±1.8	9.0±2.2	5.2±0.7	4.1±1.9
UR*	7.4	7.9	3.3	2.6	1.2

n.d. = not detected.
*Unsaturation ratio calculated from:

$$\frac{\Sigma\,[\text{unsaturated fatty acid}] \times \text{number double bonds}}{\Sigma\,[\text{saturated fatty acid}]}.$$

DISCUSSION

Phytosterols are synthesized from cycloartenol, which is derived from squalene-2,3-epoxide through the cycloartenol cyclase (E.C. 6.5.99.7; [24]). β-Amyrin is also synthesized from squalene-2,3-epoxide via β-amyrin cyclase (E.C. 5.4.99.8; [25, 26]). In pea seeds, β-amyrin cyclase activity was greatest just after germination, whereas cycloartenol cyclase was dominant several days later [27]. β-Amyrin cyclase was purified from the microsomal fraction of 2-day-old pea seedlings and it has been reported that this enzyme is located in the ER or Golgi [25–27]. Analysis of acetylated triterpenoid derivatives of germinating root tissue revealed the presence of small amounts of β-amyrin, indicating that this triterpenol is synthesized by the plant (Table 1). Furthermore, β-amyrin was not detected in the acetylated residue of free living bacteroids (data not shown). An unidentified acetylated compound ($[M]^+$ m/z 662) was detected, which did not correspond to hopanoids [19, 20, 28] or to other isoprenoids, such as α-tocopherolquinone and α-tocopherolquinol [29]. We are unable to find any such compounds in symbiosomes or PBM, suggesting that the bacteroid might not provide material to build the PBM. This is contrary to *A. rubra* nodules [21], where *Frankia* vesicle envelopes, obtained from these nodules, contained bacterial hopanoids [19, 20].

The role of β-amyrin in the PBM is not known. Sterols are known to stabilize the membrane bilayer structure by linking the fatty acid chains of phospholipids [30]. The maximum molecular length of β-amyrin was estimated to be *ca* 14.0 Å, which is shorter than, for instance, that of stigmasterol (17.5 Å) (both calculated using the software package Desktop Molecular Modeller 1.2). Therefore, the presence of this compound in the PBM may perturb its structure, which might be reflected in changes of membrane fluidity and integrity. Its presence may be the result of the large amount of membrane material needed to build up the bacteroid enclosing PBM [4, 18], as observed in germinating pea seeds, where there is a high rate of cellular division [27]. This notion is supported by the rapid accumulation of 14α-methyl sterols (*ca* 35% of total sterol content) found after five days of treatment with γ-ketotriazole (Table 2). The presence of these abnormal sterols also indicated that there was no specific selection of sterols to synthesize PBM. The sterol/phospholipid ratio in the RPM was 77.2, compared with 7.9 for PBM and 4.3 for MNF, suggesting that nodule membranes may have different physical characteristics, as compared with RPM (Table 3).

The sterol composition of PBM is also related to MFN. Δ^5-Avenasterol, the precursor of sitosterol and stigmasterol [23, 30], was not detected in RPM (Tables 1 and 2). The relationship between PBM and MFN is further supported by the results of five days' treatment with γ-ketotriazole, which resulted in the accumulation of 14α-methyl sterols in the PBM at a faster rate than that observed in the RPM (Table 2).

A similar PC/PE ratio of *ca* 1:2 found in RPM (Table 3) was observed in mung bean roots [31]. However, in pea plants, grown in the presence of NO_3^-, the PC/PE ratio of RPM was 2:1 (Hernández and Cooke, unpublished data). In bacteroids, a similar PC/PE ratio was observed, although an almost equal proportion of both phospholipids has been reported in free living *R. leguminosarum* [32]. This indicates that the lipid composition depends on the growing conditions of the plants, which has been suggested is related to environmental adaptation [33]. Soybean mutants lacking the PC kinase II isoenzyme expressed during PBM maturation, showed an incomplete development of PBM that was related to ineffective N_2-fixation [15, 16]. The accumulation of PC in the PBM might be of significance for the establishment of an effective symbiosis, although there was no evidence of greater accumulation of PC in soybean PBM compared to that of PE, and relative to other membrane fractions [14]. The lipid composition of plasma membrane-enriched fractions from soybean roots [34], indicated there was almost twice the amount of PC than PE, whereas in pea plasma membrane we found the opposite effect (Table 3).

The fatty acid composition of pea PBM (Table 4) corresponded with results reported for that from soybean, which was similar to the Golgi-ER fatty acid composition; other membrane fractions were not compared [35].

In conclusion, lipid analysis of membranes indicated similarities between PBM and MFN, of which a major portion corresponded to the endomembrane system. The presence of β-amyrin might be the result of a requirement for large amounts of new membrane material to enclose the bacteroids released into the plant cell cytosol, even though this may adversely affect the stability of the PBM. Moreover, the relatively large amounts of unsaturated fatty acids and the reduced sterol/phospholipid ratio may also result in a more fluid membrane. It is possible that an increase in membrane fluidity is required to achieve symbiosome development, as observed with bacteroids in soybean nodules [13, 36].

EXPERIMENTAL

Plant material. Pea seeds (*Pisum sativum* cv. Argona) were germinated and plants grown and inoculated with *R. leguminosarum* (biovar. *viciae* 3841) as previously described [37]. Plants were kept under glass (25° light and 18° dark) for 4–6 weeks. Four pots were sampled from each replicate and 7–10 g nodules and 15–20 g root free of nodules were collected. In pulse experiments addition of nutrient soln containing 10 μM γ-ketotriazole was done 5 days before harvest.

Preparation of symbiosomes and membrane fractions. Symbiosomes were isolated and purified from fresh nodules using the aq. two-phase polymer (APS) technique as described in ref. [38]. The final composition of the APS used was 6.3% (w/w) Dextran

T500, 6.3% (w/w) PEG 3500, 5 mM KCl, 350 mM mannitol and 5 mM KH_2PO_4 (pH 7.5). Samples were processed as previously described [38] and stored at −75°.

The supernatant of the nodule homogenate obtained after 10 000 *g* centrifugation was centrifuged again at 100 000 *g* for 30 min. The resulting pellet was resuspended in 2 ml storage buffer (5 mM PIPES, 350 mM mannitol, 5 mM EDTA, 5 mM EGTA, 10 mM NaF, at pH 6.5) and stored at −75°. This extract is referred to as the nodule cell microsomal fr. (MFN).

PBMs were prepd from symbiosomes by vigorously shaking them with a hypo-osmotic soln (storage buffer in absence of mannitol) for 10 min. The resulting suspension was centrifuged at 10 000 *g* for 15 min. Bacteroids and unbroken symbiosomes constituted the pellet that was diluted with 1.5 ml storage buffer and stored at −75°. The supernatant was further centrifuged at 100 000 *g* for 30 min to ppt. the PBM fr. The resulting pellet was resuspended in 1.5 ml storage buffer and stored at −75°.

RPMs were prepd from roots free of nodules, which were homogenized in extraction buffer (50 mM HEPES, 500 mM sucrose, 1 mM DTT, 5 mM ascorbic acid, 0.6% Polyclar AT PVPP, at pH 7.5). The homogenate was sieved (nylon cloth, 240 μm) and the filtrate centrifuged at 10 000 *g* for 15 min. The pellet was discarded and supernatant further centrifuged at 100 000 *g* for 30 min. The pellet was resuspended in 3 ml resuspension buffer (330 mM sucrose, 10 mM NaF, 5 mM K_2HPO_4/KH_2PO_4, at pH 7.8) and RPMs isolated by loading on to a APS having a final composition of 6.2% (w/w) Dextran T500, 6.2% (w/w) PEG, 330 mM sucrose, 5 mM K_2HPO_4 and 4 mM KCl (pH 7.5). The upper phase was further purified using a batch procedure and centrifuged at 100 000 *g* for 30 min. The resulting pellet was resuspended with storage buffer and stored at −75°.

Phospholipid and sterol analysis. Phospholipids and sterols were analysed as described in ref. [39]. $CHCl_3$–MeOH (1:2) (0.75 ml) was added to 0.25–0.50 ml sample fr. along with 2 μg β-cholestanol as int. standard. Sterol acetates were analysed by GC as described in ref. [22], using an SE 52-bonded capillary column coupled with a FID. Phospholipids were analysed by HPLC with an evaporative light-scattering detector using a modification of the method described in ref. [40]. HPLC-elution conditions were similar to those used in ref. [39].

Esterified fatty acid analysis. Me 17:0 (1 μg as int. standard) was added to 250 μl sample fr. A $CHCl_3$ extract was prepd as described above and 20 μl portions taken for esterified fatty acid determination by evapn to dryness under N_2, transmethylation with 500 μl 0.5 N NaOMe in dry MeOH and heating at 30° for 7 min. The resulting fatty acid Me esters were extracted and analysed by GC-FID as described in ref. [39].

Sterol content analysis of germinating root tissue. Pea seeds were germinated as described above. After 2 days growth, roots were sepd and *ca* 0.4 g homogenized with 4 ml MeOH–$CHCl_3$ (1:1). The homogenate was heated at 40° for 40 min and filtered; 0.5 ml H_2O was then added to separate the $CHCl_3$ and aq. phases. The $CHCl_3$ phase was removed and evapd under N_2 and the residue redissolved in 10 μl $CHCl_3$. An aliquot of 40 μl was taken for sterol analysis as described above.

Protein content. This was determined using the Bio-Rad Coomassie Blue assay reagent [41], with thyroglobulin as protein standard.

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