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ISOLATION AND IDENTIFICATION OF STEROLS FROM SUBCELLULAR FRACTIONS OF BEAN LEAVES (*Phaseolus vulgaris*)

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SUMMARY

Chloroplasts, mitochondria and microsomes were isolated from a cell-free extract of green and etiolated leaves of bean, by differential and sucrose or ficoll gradient centrifugation. The sterols and sterol esters of whole leaves and subcellular fractions were compared.

The following sterols were identified from the leaves: cholesterol (cholest-5-en-3β-ol), Δ^7-cholestenol (cholest-7-en-3β-ol), campesterol (cholest-5-en-(24*R*)-24-methyl-3β-ol), stigmasterol (stigmast-5,Z-22-dien-3β-ol), sitosterol (stigmast-5-en-3β-ol) and isofucosterol (stigmast-5,Z-24(28)-dien-3β-ol). These sterols are present in all subcellular fractions but their concentrations differ significantly; in particular the chloroplasts and the mitochondria are much richer in cholesterol (24 %) compared to the leaves (1 %).

The amount of sterols per mg of proteins in the fractions varied as follows: chloroplasts < mitochondria < endoplasmic reticulum. Sterol esters were generally enriched in cholesterol (25–75 %) and poor in stigmasterol (10 %).

These results are discussed in terms of the role of the sterols in the structure and function of plant cell membranes.

INTRODUCTION

The ubiquitous presence of sterols in eucaryotes raises the problem of their biological significance. In animals cholesterol is a constituent of cellular membranes[1]. It is thought that cholesterol probably plays an important role in the structure (rigidity)[2] and the function (permeability)[3] of these membranes. The presence of sterols in higher plants has been known for many years but to our knowledge their role has never been clearly defined. The presence of sterols in subcellular fractions from higher plants has been reported recently[4,5]. However, it seems to us that these studies are not conclusive in showing the presence of sterols in membrane fractions clearly defined morphologically and biochemically.

Extraction of plant tissues generally gives a mixture of sterols (campesterol,

Abbreviations: cholesterol, cholest-5-en-3β-ol; Δ^7-cholestenol, cholest-7-en-3β-ol; campesterol, cholest-5-en-(24*R*)-24-methyl-3β-ol; stigmasterol, stigmast-5,Z-22-dien-3β-ol; sitosterol, stigmast-5-en-3β-ol; isofucosterol, stigmast-5,Z-24(28)-dien-3β-ol.

stigmasterol, sitosterol...), whereas in animals essentially one sterol is found (cholesterol). The problem thus arises in plants: is the mixture of sterols found in a plant a reflection of the character of the cellular membranes or simply a reflection of the extraction techniques used, whereby particular sterols associated specifically with a particular membrane are mixed together.

To reply to this question, we prepared subcellular fractions in a high state of purity, clearly defined morphologically and biochemically. We then carried out quantitative and qualitative comparison of the sterols and sterol esters of these fractions.

MATERIALS AND METHODS

Bean plants (*Phaseolus vulgaris*, var. Saxe) were grown on sterile vermiculite in a temperature-controlled greenhouse at 25 °C with a photoperiod of 18 h for the green plants and in total darkness for the etiolated plants. The leaves of 10-days-old plants were harvested (4.5 g dry weight for the green leaves and 2.1 g dry weight for the etiolated leaves), chopped with a razor blade and homogenized in a Moulinex mixer at low speed by ten bursts, each of 10 s duration, in the presence of twice the weight of the following medium: 0.5 M mannitol, 0.1 M Tris–HCl (pH 7.6) 1 mM EDTA, and 5 mM cysteine, 10 mM potassium pyrophosphate (only for green leaves). The extract was filtered through six layers of muslin and the filtrate passed through nylon ("Blutex", Tripette et Renaud 50 μ). An initial centrifugation at 750 $\times$ g for 10 min gave a pellet of crude chloroplasts (C_1) and a supernatant. This was centrifuged at 6000 $\times$ g for 10 min giving a crude mitochondrial pellet (C_6) and a second supernatant which was further centrifuged at 120000 $\times$ g for 60 min to give a microsomal pellet (C_{100}). The pellet C_1 was purified on a continuous gradient of 10–18 % "Ficoll", 0.5 M saccharose, 10 mM Tris–HCl (pH 7.5), by centrifugation at 2300 $\times$ g (SW 25-1) for 30 min. Two layers were removed from the gradient corresponding to broken chloroplasts (higher layer C_{1a}) and purified chloroplasts (lower layer C_{1b}). The C_6 pellet was purified on a discontinuous gradient of saccharose comprised of six layers each of 4 ml (0.8, 1, 1.2, 1.4, 1.6 and 1.8 M), by centrifugation at 32 500 $\times$ g (SW 25-1) for 1 h. Under these conditions, two principal layers obtained corresponding to swollen mitochondria C_{6a} (at a density corresponding to 1.4 M) and intact mitochondria C_{6b} (1.6 M). The C_{100} pellet was resuspended in the following medium: 2.2 M saccharose, 2 mM Mg^{2+}, 2 mM mercaptoethanol, 20 mM Tris–HCl (pH 7.7). 2 ml were placed on the bottom of a centrifuge tube and covered with 8 ml of a similar medium containing 1.64 M saccharose. This gradient was centrifuged at 120000 $\times$ g (SW-41) for 16 h (ref. 6). This treatment gave three layers, C_{100-1}, C_{100-2} and C_{100-3} in order of increasing density. All fractions obtained were resuspended in the following medium: 0.5 M saccharose, phosphate (pH 7.4) for the chloroplasts, 0.5 M saccharose, Tris–HCl (pH 7.7) for the mitochondria and Tris–HCl (pH 7.7) *plus* 2 mM Mg^{2+} for the microsomes. All these operations were carried out at 0–4 °C.

Morphological integrity of these fractions was controlled by electron microscopy and will be the object of a further publication. The biochemical purity of these fractions was controlled by the use of the following marker enzymes: Hill reactions for the chloroplasts, succinate dehydrogenase (EC 1.2.99.1) for the mitochondria, glucose-6-phosphatase (EC 3.1.3.9), NADPH–cytochrome *c* reductase

(EC 1.6.2.1) and 5'-nucleotidase (EC 3.1.3.5) for the microsomes (M. Ferne, P. Benveniste, C. Gigot and M. A. Hartman, unpublished).

The subcellular fractions were lyophilized then extracted once with acetone and three times with chloroform–methanol (2:1, v/v). The extracts were bulked together, evaporated to dryness and chromatographed on silicagel thin layers (Merck F 254, 0.25 mm) using for solvent system benzene–ether (4:1, v/v). Two bands were scraped off the plates corresponding to sterol esters (R_F 0.9) and sterols (R_F 0.35). Sterol esters were hydrolyzed by refluxing in methanol–water (9:1, v/v) containing 6 % KOH for 2 h under argon and the nonsaponifiable lipids extracted with light petroleum (b.p. 40–60 °C) and chromatographed on silicagel thin layers with chloroform as eluant (2 migrations). The free sterol fraction was treated similarly to remove chlorophyll. Sterols were analyzed by gas–liquid chromatography on a Varian Aerograph 1740 chromatograph equipped with a flame ionisation detector, and a column OV-17 1 % at 255 °C and sterol acetates on OV-17 1 % at 270 °C, XE-60 1 % at 240 °C and SE-30 1 % at 270 °C. Quantitative determinations were made by measuring peak area compared to that of an internal standard of *n*-dotriacontane (n-$C_{32}H_{66}$). Sterol acetates were further separated by $AgNO_3$ thin-layer chromatography with cyclohexane–benzene (8:2, v/v) as eluent. Isofucosterol acetate (R_F 0.35) was eluted and analysed by gas–liquid chromatography and mass spectrometry (Thomson–Houston THN 208).

RESULTS

The obtention of pure mitochondria from green plants is difficult because of contamination by grana and other chloroplastidic fragments. For this reason we used dark grown plants containing only etioplasts. We have shown that mitochondrial and microsomial fractions thus obtained are much purer. From electron microscopic studies it seemed that the chloroplast fraction C_{1b} was more than 90 % pure; the chloroplasts had lost their external membrane and the stroma but their internal membrane structure was preserved. The mitochondrial fraction C_{6b} was more than 90 % pure and presented a classical morphological aspect: presence of a double membrane and many cristae. The microsomal fraction C_{100} was similar to analogous fractions obtained from animal tissues[6]: presence of smooth vesicles, rough vesicles and free ribosomes. The subfractions obtained have been observed (unpublished): C_{100-1} consists of smooth vesicles, C_{100-2} of rough vesicles and free ribosomes and C_{100-3}, which, although not yet identified with accuracy, contains vesicles probably originating from etioplasts and nuclear fragments (chromatin). The distribution of enzymatic activities was in agreement with these morphological observations.

The results of the comparative analysis of free sterols are shown in Table I. In contrast to barley[7], the green and etiolated leaves of bean have a very similar sterol composition with, however, enrichment in cholesterol in etiolated tissues. This enrichment in cholesterol was found in all subcellular fractions obtained from etiolated leaves accompanied by a drop in stigmasterol content.

The crude fractions C_1, C_6 and C_{100} have a sterol composition similar to that of whole leaves suggesting that a comparison at this level of purification is not significant. However after purification significant differences appeared, principally in the case of the fractions C_{1b}, C_{6b} and C_{100-3}. We noted an important enrichment in cholesterol

and a drop in stigmasterol and, it seems, campesterol. The isofucosterol appears as a significant constituent of the subcellular fractions and in particular for the etiolated tissues. The quantitative analysis showed that the level of sterols in chloroplasts is lower than that in mitochondria and particularly than that in the microsomes. These observations are in agreement with those already published on maize[4] and tobacco[5]. Furthermore the significant decrease of sterol concentration of the submicrosomal fraction C_{100-1} compared to C_{100} might be interpreted by postulating the existence of two pools of sterols, one being a constituent of the membranes, the other associated with the sites of sterol biosynthesis, this latter being eliminated in the course of the purification on the gradient. The figure for the fraction C_{100-2} is probably not representative of the sterol content of the membranes since this fraction is very rich in ribosomes, which probably do not contain sterols. The high percentage of cholesterol in fraction C_{100-3} might be attributed to the presence of vesicles from etioplasts.

The comparison of sterol esters shows profound differences from that of free sterols (Table II). The most outstanding difference is the high level of cholesterol and the low level of stigmasterol. This result is similar to that previously found with maize[4]. One notes also a high level of isofucosterol. From a quantitative point of view the amounts of sterol esters present are much less than those of the free sterols in

TABLE I

RELATIVE PERCENTAGE AMOUNTS OF FREE STEROLS IN SUBCELLULAR FRACTIONS FROM GREEN AND ETIOLATED BEAN LEAVES

Percentages were determined by measuring gas–liquid chromatographic peak areas as described in Materials and Methods. Standard error approx. 5% (two experiments).

Sterols	*Fraction*								
	Whole leaves	C_1	C_{1b}	C_6	C_{6b}	C_{100}	C_{100-1}	C_{100-2}	C_{100-3}
Green bean leaves									
Cholesterol	1	9	24	—	—	1	2	6	26
Δ^7-Cholestenol	—	2	1	—	—	—	1	—	1
Campesterol	6	7	5	—	—	8	6	4	2
Stigmasterol	34	32	22	—	—	33	31	31	17
Sitosterol	57	48	48	—	—	53	52	56	46
Isofucosterol	2	2	Trace	—	—	5	8	3	8
μg/mg protein **	0.5 *	0.1	0.5	—	—	12	4.8	1.2	0.4
Etiolated bean leaves									
Cholesterol	6	—	—	6	27	6	11	15	24
Δ^7-Cholestenol	—	—	—	2	3	2	4	3	2
Campesterol	7	—	—	8	5	8	9	4	3
Stigmasterol	31	—	—	27	17	23	19	15	14
Sitosterol	53	—	—	50	37	55	51	53	47
Isofucosterol	3	—	—	7	11	6	6	10	10
μg/mg protein **	0.7 *	—	—	1.3	1.5	9.2	5.9	4.3	3.0

* μg/mg dry weight for this value.

** These values are essentially relative because they do not take into account the losses during the extraction processes.

the whole leaves and the crude fractions C_1, C_6 and C_{100} (ref. 4). In contrast, at the level of purified membrane fractions, with the exception of C_{100-1} levels of sterol esters are similar to those of free sterols.

TABLE II

RELATIVE PERCENTAGE AMOUNTS OF STEROL ESTERS IN SUBCELLULAR FRACTIONS FROM GREEN AND ETIOLATED BEAN LEAVES

Percentages were determined by measuring gas–liquid chromatographic peak areas as described in Materials and Methods. Standard error approx. 5% (two experiments).

Sterols	*Fraction*								
	Whole leaves	C_1	C_{1b}	C_6	C_{6b}	C_{100}	C_{100-1}	C_{100-2}	C_{100-3}
Green bean leaves									
Cholesterol	1	70	33	—	—	28	56	18	42
Δ^7-Cholestenol	—	3	5	—	—	3	5	4	6
Campesterol	7	2	2	—	—	1	2	1	2
Stigmasterol	28	7	8	—	—	10	6	11	10
Sitosterol	51	14	31	—	—	39	22	43	28
Isofucosterol	13	4	21	—	—	19	9	23	12
μg/mg protein **	0.6*	0.02	0.6	—	—	0.2	0.1	0.4	0.7
Etiolated bean leaves									
Cholesterol	23	—	—	16	26	34	Trace	33	29
Δ^7-Cholestenol	—	—	—	4	4	3	Trace	3	3
Campesterol	6	—	—	6	2	1	Trace	2	2
Stigmasterol	4	—	—	26	11	10	Trace	10	9
Sitosterol	41	—	—	43	40	35	Trace	37	40
Isofucosterol	26	—	—	5	17	17	Trace	15	17
μg/mg protein **	—	—	—	1.3	1.8	0.8	Trace	2.0	2.9

* μg/mg dry weight for this value.

** These values are essentially relative because they do not take into account the losses during the extraction processes.

DISCUSSION

The results presented show that all the subcellular fractions studied contain sterols. It seems to us important to distinguish between mitochondrial and chloroplastidic fractions on the one hand and the microsomal and submicrosomal fractions on the other: the former consist of organelles having a membrane system relatively complex and diversified while the latter consist of simple membrane structures coming from the fragmentation of the endoplasmic reticulum. From the point of view of the concentration of sterols in the subcellular fractions we find considerable differences. It cannot be excluded that a further fragmentation of the chloroplasts and mitochondria (extraction of grana and cristae) would show even greater differences. With reference to this point, it has been shown that the internal membranes of rat liver mitochondria do not contain sterols whereas the external membranes contain significant quantities[1].

Furthermore the extraction of subcellular fractions leads to a mixture of sterols and sterol esters of which the composition is different from that of an extraction of

whole leaves. These results show also that no sterol among those found in the leaf is associated specifically to a particular type of membrane, at least at the level of our studies; here again it is not excluded that a further fragmentation of the organelles would show such a specificity.

The mesophyll of leaves is characterised by the presence of a large number of chloroplasts which represent an important percentage of the total membranes. The fact that one finds there a high percentage of cholesterol (24 %) compared to whole leaves (1 %) seems to us particularly significant and confirms the uniqueness of the membrane of chloroplasts from the point of view of sterol composition. Studies carried out on other lipids (phospholipids[8], galactolipids[9]) lead to identical conclusions. Similarly the high level (26 %) of cholesterol in mitochondria confirms the particular character of the membranes from the point of view of sterols. If one compares the high percentage of cholesterol in the chloroplasts and mitochondria to the low percentage of whole leaves, one is lead to the conclusion that other membrane fractions contain very small percentages of cholesterol. Among these latter can be included fraction C_{100-1} (2 % cholesterol) and also probably fractions that were not isolated (plasmalemma). A similar explanation can be put forward to explain the composition of sterol esters in green leaves (Table II). The high percentages of cholesterol esters contained in the subcellular fractions (20–70 %) relative to whole leaves (1 %) could be explained by the presence in green leaves of other subcellular fractions (lipid bodies) which could be rich in sterol esters but poor in cholesterol

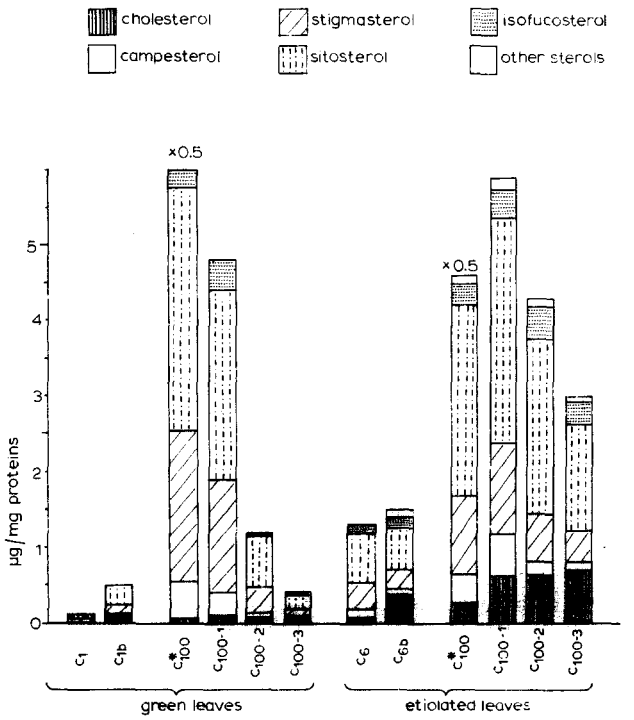

Fig. 1. Concentration and composition of sterols in each subcellular fraction from green and etiolated bean leaves. Amounts were determined as in Material and Methods.

* These areas have been reduced 2 times.

esters. In the same way one could explain the fact that the subcellular fractions of green leaves are relatively low in stigmasterol whereas the whole leaves are relatively high (28 %). Further to this point, the mitochondrial fractions (C_6 and C_{6b}) obtained from green leaves have been analysed and have a sterol and sterol ester composition similar to that of C_1, but are not shown in Tables I and II because they were contaminated by chloroplast fragments.

The presence of sterols in all the subcellular fractions studied poses the problem of their role in the structure and function of higher-plant membranes. It has been suggested that cholesterol may have a "condensing" effect and increase the rigidity of the membrane[1] and also that it may play a role in its permeability[3,10].

Extending this idea to higher plants there may be nuances in the role of each sterol. Our results can be interpreted in this sense: all the sterols found here are constituents of membranes but cholesterol plays a more particular role in membrane permeability according to recent studies[10]. However Fig. 1 shows a certain constancy in the concentration of cholesterol in the membranes (particularly evident in green tissues) and thus it may be the changing concentration of the other sterols in the membrane that modifies this role played by cholesterol and this may be particularly reflected in chloroplasts and mitochondria.

The quantity of sterol esters found in fractions C_{1b} and C_{6b} is relatively important and raises the problem of their role. Several hypothesis could be put forward: these sterol esters may be included in lipid bodies which can be seen in chloroplasts and which may constitute a sterol reserve. Also the association of sterol esters to the membranes cannot be excluded. However, this latter hypothesis poses problems if one considers the classical ideas whereby the 3β-hydroxyl group plays a role in the molecular association between the sterol and the phospholipids[11]. For this reason we intend to investigate the validity of this possibility by isolating and studying the different membrane systems of chloroplast and mitochondria.

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