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OCCURRENCE OF STERYL GLYCOSIDES AND ACYLATED STERYL GLYCOSIDES IN SOME MARINE ALGAE

ROBERT DUPERON, MARTINE THIERSAULT and PAULETTE DUPERON

Department of Plant Physiology, Faculty of Sciences, University of Tours, Parc de Grand Mont, 37200 Tours, France

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Key Word Index—*Ulva gigantea; Cladophora rupestris*; *Fucus vesiculosus*; *Ascophyllum nodosum*; *Rhodymenia palmata*; *Porphyridium* sp.; Chlorophyta; Pheophyta; Rhodophyta; algae; sterols; steryl glycosides; acylated steryl glycosides.

Abstract—There is some controversy concerning the presence of steryl glycosides and acylated steryl glycosides in eucaryotic algae. These two classes of sterol compounds were investigated in species belonging to the three major groups of eucaryotic algae: green algae (*Ulva gigantea*, *Cladophora rupestris*), brown algae (*Fucus vesiculosus*, *Ascophyllum nodosum*) and red algae (*Rhodymenia palmata*, *Porphyridium* sp.). All these algae contain both steryl glycosides and acylated steryl glycosides. The sterol components of these compounds vary according to the alga but they are always the same as the free sterols of the alga in question. The most common sugar moiety is glucose. In the acylated steryl glycosides, the fatty acid is mainly palmitic acid. The percentage of these compounds (as a percentage of the total sterol content) is often low.

INTRODUCTION

It is now recognized that steryl glycosides (SG) and acylated steryl glycosides (ASG) are nearly always present in higher plants [1–7] along with free sterols (FS) and esterified sterols (ES). In these plants the glycosidic compounds appear to behave, like the FS, as components of cell membranes [1, 7–11], whereas the intracellular localization of the ES is much less clearly defined [7–9].

The existence of FS and ES has also been well-established in lower plants; however, the presence of steryl glycosides in these plants is sometimes questioned. According to Lewin [12], eucaryotic algae, in particular, do not appear to contain steryl glycosides. Moreover, *Chlorella* and *Scenedesmus* (Chlorophyta) do not seem to contain any UDPG sterol glucosyltransferase, the enzyme responsible for the synthesis of these glucosides [13]. However, on the contrary, a UDPG sterol glucosyltransferase, does seem to be active in *Prototheca zopfii* (Chlorophyta) which suggested the possible existence of these glucosides in this alga [14]. In a preliminary study we also succeeded in identifying these SG in *Ulva gigantea* (Chlorophyta) [15].

Faced with these contradictory results we have investigated the SG and ASG in green, red and brown algae. Accordingly, the following algae were studied. Chlorophyta: *Ulva gigantea* (Kützing) Bliding, *Cladophora rupestris* (Linné) Kützing; Rhodophyta: *Palmaria palmata* (Linné) O. Kuntze = *Rhodymenia palmata*, *Porphyridium* sp.; Pheophyta: *Fucus vesiculosus* (Linné), *Ascophyllum nodosum* (Linné) Le Jolis.

RESULTS

The results obtained after purification of the total lipid extract and estimation by GC (Table 1) show that, in the algae studied, the total sterol content (% dry wt) is

Table 1. Sterol content of algae

Alga	Month of collection	TS (% dry wt)	Composition (% of TS) FS	ES	SG	ASG
Fucus vesiculosus	June	0.3	98	1.5	0.25	0.25
	January	0.23	95	4.3	0.4	0.3
Ascophyllum nodosum	October	0.04	77	13	6.5	3.5
Ulva gigantea	April	0.08	96.5	0.5	1.5	1.5
Cladophora rupestris	October	0.03	95	2.6	1.5	0.9
Porphyridium sp.	Culture on sterile mineral medium	0.2	75	14	4	7
Palmaria palmata = *Rhodymenia palmata*	October	0.04	91	5	2.5	1.5

TS, Total sterols; FS, free sterols; SG, sterols from steryl glycosides; ASG, sterols from acylated steryl glycosides; ES, sterols from esters.

Table 2. Nature and percentage of principal sterols in each class of sterol compounds

Alga	Month of collection	FS		ES		SG		ASG	
Fucus vesiculosus	October	Fucosterol	99	Fucosterol	91	Fucosterol	75	Fucosterol	78
		Cholesterol	1	Cholesterol	7	Cholesterol	19	Cholesterol	16
	January	Fucosterol	99	Fucosterol	92	Fucosterol	67	Fucosterol	67
		Cholesterol	1	Cholesterol	8	Cholesterol	28	Cholesterol	28
Ascophyllum nodosum	October	Fucosterol	93	Fucosterol	92	Fucosterol	91	Fucosterol	57
		Cholesterol	7	Cholesterol	5	Cholesterol	5	Cholesterol	38
Ulva gigantea	April	Isofucosterol	93	Cholesterol	52	Isofucosterol	65	Isofucosterol	54
		Cholesterol	4	Isofucosterol	38	Cholesterol	30	Cholesterol	36
Cladophora rupestris	October	Clionasterol*	54	24-Methylene-cholesterol	39	Clionasterol	45	Clionasterol	51
		24-Methylene-cholesterol	42	Cholesterol	34	24-Methylene-cholesterol	33	24-Methylene-cholesterol	34
		Cholesterol	4	Clionasterol	27	Cholesterol	17	Cholesterol	12
Porphyridium sp.	Culture in sterile mineral medium	*trans*-22-Dehydro-cholesterol	82	Cholesterol	60	Cholesterol	32	Cholesterol	30
		Cholesterol	10	Clionasterol	18	Clionasterol	18	Sterol 'X'	30
		Clionasterol	5	*trans*-22-Dehydro-cholesterol	6	Sterol 'X'	18	Clionasterol	16
						trans-22-Dehydro-cholesterol?		*trans*-22-Dehydro-cholesterol?	
Palmaria palmata	October	Desmosterol	94	Desmosterol	58	Desmosterol	50	Cholesterol	65
		Cholesterol	6	Cholesterol	41	Cholesterol	36	Desmosterol	15

Key as for Table 1. Results expressed as a percentage of total sterols in every class. Minor sterols are not indicated and some sterols were not clearly identified, e.g. some sterols from the glycosides of *Porphyridium* sp.

*C-24 substituted sterols from algae are assumed to have the β-configuration [22].

extremely variable. In some cases (*Ascophyllum*, *Cladophora*, *Palmaria*) the total sterol content is somewhat lower than is usual for higher plants (e.g. in seeds it is from 0.05 to 1%) [3]. However, in *Fucus* and *Porphyridium* this content is relatively high.

In each of the six species, FS exist in a much greater amount than sterol conjugates. The presence of ES, which has already been noted in algae [16–18], remains constant. Our investigations also clearly indicated the existence of SG and ASG. However, the proportion of these glycosidic forms is low, especially in *Fucus vesiculosus*.

In most cases, the glycosides contain the same sterols as those present as free or esterified forms (Table 2). Thus, in *Palmaria palmata* desmosterol and cholesterol, which are abundant in the free or esterified forms [19–21], are also present in the SG and ASG forms (Table 2). However, the relative proportions of the various sterols from the glycosides may be different from the proportions encountered in the free or esterified forms. On the other hand, in each of the species examined, the glycosides were examined on TLC using a developing solvent able to show the number of sugar molecules bound to the sterol. The algal glycosides contained only a single sugar molecule. This fact was confirmed in the case of SG isolated from *Ulva gigantea*, which was purified and then hydrolysed. The sugar–sterol ratio was found to be 1.02. (see Experimental)

In all the algae analysed, the sugar present is essentially glucose. It is possible that rhamnose and fucose are occasionally present but this remains to be confirmed. These observations concern both SG and ASG. In the latter, the fatty acid attached to the sugar is predominantly palmitic acid. Occasionally, small quantities of oleic and myristic acids are also present.

DISCUSSION

Many previous studies have shown the variable nature of sterols in algae. Most of these studies were carried out after saponification of the total lipid extract, and thus identify only the sterols in the FS and ES. In the algae studied in this paper, the nature of the sterols is usually the same as that described in previous studies [21]. However, whereas Patterson [22] found 28-isofucosterol, 24-methylene cholesterol and cholesterol in *Cladophora flexuosa*, we found, in *Cladophora rupestris*, clionasterol, 24-methylene cholesterol and cholesterol. The latter sterols had already been found in *Cladophora echinus* [23]. For *Porphyridium* sp. our results on the nature of the sterols were the same as those described by Beastall *et al.* [24].

Whereas the nature of FS and ES has often been described the problem of the presence or absence of SG and ASG has never been fully reported. Our research shows that all the algae studied contain small quantities of SG and ASG, generally in the form of monoglucosides. However, as the total lipid extract (after extraction by chloroform–methanol) contains many other substances (numerous pigments, neutral lipids, polar lipids, hydrocarbons, etc.) it is difficult to detect such small quantities of glycosidic forms without numerous purification processes. First, a succession of chromatographic separations, gave fractions which were still impure, one containing the SG, the other the ASG. These two fractions were then purified further by various methods to eliminate certain unwanted substances (e.g. glycolipids). The SG and ASG can be characterized only after these operations. There were inevitable losses due to the multiplicity of purification processes necessary to obtain the steryl glycosides. Therefore, the data concerning the content of SG and the ASG (Table 1) can be only considered as approximate and are probably slightly lower than the real contents.

Our results showing the occurrence of the SG and the ASG in algae add weight to those of Hopp *et al.* [14] which indicated the presence of a UDPG glucosylsterol-transferase in *Prototheca* (Chlorophyta).

In our work a comparison of the percentages of total sterols (% dry wt) shows important differences from one species to another, e.g. *Fucus vesiculosus* and *Porphyridium* sp. seem to be richer in sterols than *Ascophyllum nodosum*, *Cladophora rupestris* and *Palmaria palmata*. However, to draw such a comparison in this paper would be risky as the algae were not all collected at the same time and, according to some authors, the sterol content varies with the season [25, 26]. Nevertheless, *Fucus vesiculosus*, which was collected at two different times of the year for this study, showed only a very small difference in sterol content (Table 1).

The presence of SG and of ASG in algae raises the question of their intracellular localization in these plants. Is this localization the same as that found in higher plants, i.e. essentially in the membranes? Further research in this area is warranted which may yield information on the still unknown significance of these substances in plants.

EXPERIMENTAL

Materials. Algae (except *Porphyridium* sp.) were collected and identified by the Station Biologique de Roscoff, 29211 Roscoff, France. *Porphyridium* sp. was cultured in sterile mineral medium [27], in the laboratory of the 'Physiologie Cellulaire Végétale', Université Paris-Sud, 91400 Orsay, France.

Extraction and separation of sterol compounds. After collecting and homogenizing the algae, the total lipids were extracted by $CHCl_3$–MeOH (2:1). The extract was subjected to prep. TLC (Si gel, 2 mm) with two successive developments in the same direction: (1) Et_2O–C_6H_6–EtOH–HOAc (40:48:4:0.5) and (2) hexane–Et_2O (94:6). The four bands corresponding to ES, FS, ASG and SG were located by lateral migration of standards detected by the $SbCl_3$ reagent. The bands were scraped off, and the sterol compounds recovered by Et_2O (ES, FS) or $CHCl_3$–MeOH (2:1) (SG, ASG).

Purification of fractions, identification and quantitative estimation of sterol compounds. ES and FS fractions were purified by CC on Al_2O_3 (Woelm neutral Brockman activity, grade III) using increasing concns of Et_2O in petrol. The ES were saponified (8% KOH in MeOH under N_2). Both classes of sterols were purified by TLC, then identified and quantitatively estimated by GC (as free forms or as TMSi derivatives) on a glass column (3 m × 2 mm) packed with 2% OV 17 on DMCS Chromosorb W 100–120 mesh, isothermal 239°, N_2 at 30 ml/min.

For purification of the SG fraction the crude fraction, obtained above which contained numerous contaminating compounds, was chromatographed by TLC with triple development in the same direction, using the first solvent mixture described above. The band corresponding to SG was treated with $CHCl_3$–MeOH (2:1). The residue after evaporation was gently saponified (2.4% KOH soln in MeOH at 37°, 30 min and under N_2) to remove some of the saponifiable contaminating substances (e.g. different glycolipids) without destroying SG [28]. After this operation the fraction was rechromatographed by TLC with the developing solvent $CHCl_3$–MeOH–H_2O (65:25:4) [30]. This chromatography gave a clear separation of mono-, di-, tri-, etc. glycosyl

sterols, and moreover, several alkali-stable glycolipids can be separated from SG. Generally, in algae, only one spot of SG was detected, which corresponded to monoglycosides. After elution of SG, the concd soln gave a positive reaction with the Liebermann reagent indicating the presence of a sterol and with anthrone reagent revealing the presence of a sugar. It gave a negative reaction with ninhydrin. The residue was subjected to mild hydrolysis (1% H_2SO_4 in EtOH at 85° for 3 hr under N_2). The sterols freed from the glycosides were extracted with petrol and hydrolysis was repeated twice to increase the yield, while avoiding sterol destruction or isomerization (some sterols such as fucosterol or isofucosterol, are unstable in acid media). However, the sterol yield obtained by this method did not reach 100%.

Sterols were identified and estimated by GC as described above. Sugars obtained by hydrolysis were also identified, as TMSi derivatives, by GC on a column (3 m × 2 mm) packed with 15% Carbowax 20 M on Gas Chrom Z, isothermal 130°, N_2 at 60 ml/min, or a column (2 m × 2 mm) packed with 4% SE 30, on DMCS Chromosorb W, isothermal 150°, N_2 at 80 ml/min.

Pure glycosides were isolated from the extract from *Ulva gigantea*. After the various purifications described above, two complementary purifications were carried out by TLC ($CHCl_3$–MeOH, 9:1); glycosides were eluted with EtOH. The glycosides were crystallized in aq. pyridine. After mild hydrolysis (see above) the free sterols were analysed by GC/MS. It appeared that 28-isofucosterol represented *ca* 85% of the total sterols in these purified glycosides. The MS data were similar to those reported by Gibbons *et al.* [29] for this sterol. GC/MS, 70 eV, *m/z* (rel. int.): 412 $[M]^+$ (7), 397 $[M - Me]^+$ (2), 394 $[M - H_2O]^+$ (1), 379 $[M - Me - H_2O]^+$ (2), 314 [M – part of side chain $C_7H_{14}]^+$ (100), 299 $[M - C_7H_{14} - Me]^+$ (23), 296 $[M - C_7H_{14} - H_2O]^+$ (14), 281 $[M - C_7H_{14} - Me - H_2O]^+$ (26), 271 [M – side chain $-2H]^+$ (11). The sugar moiety liberated by hydrolysis was glucose (identified by GC). The quantitative determination of this sugar was made by an enzymatic method. Glucose was phosphorylated by hexokinase and ATP; the resulting glucose-6-P was oxidized by glucose-6-P dehydrogenase in the presence of NADP. NADPH was determined by spectrophotometric measurement. The glucose–sterol ratio was 1.02. This result showed that the isolated SG were monoglucosides, essentially 28-isofucosterol monoglucoside.

For purification of the ASG fraction, after the initial TLC, the crude fraction (less charged than the SG fraction) was rechromatographed with triple development in the same direction by the first solvent mixture described above. The band of ASG was treated with $CHCl_3$–MeOH (2:1). After concn, the residue gave a positive reaction with the Liebermann reagent (sterol) and with anthrone reagent (sugar). A mild saponification (see above) converted ASG into SG (this transformation was checked by co-chromatography with SG standard). The SG from ESG were purified by TLC [30] as above for SG. Acid hydrolysis, sugar and sterol identification and estimation were carried out as for SG. Fatty acids obtained by saponification were purified by TLC (Et_2O–C_6H_6–EtOH–HOAC, 40:48:4:0.5). After elution by Et_2O, fatty acids were methylated and identified by GC (1.5 m × 2 mm column packed with 10% DEGS on DMCS Chromosorb W temp. programmed from 110° to 150° at 1°/min, N_2 at 50 ml/min).

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