A Novel Purification Method of Sterols and Steryl Glucoside from Plasmodia of a True Slime Mold, *Physarum Polycephalum*

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Abstract

A novel purification method of sterols and steryl glucoside from *Physarum polycephalum* was developed. Sterols and steryl glucoside were purified to apparent homogeneity from the plasmodia of *Physarum*, by a combination of organic solvent extraction, and column chromatographies on DEAE-cellulose, Iatrobeads, and Capcell Pak C18 (HPLC). HPLC on Capcell Pak C18 was very efficient for the separation of three species of *Physarum* sterols, which could not have been easily separated.

Introduction

A true slime mold, *Physarum polycephalum*, has diploid and haploid stages in its life cycle (1–3). Haploid myxoamoebae recognize different mating types and fuse each other to form diploid zygotes, and in this phenomenon, mannosyl glycoprotein(s) located on the cell surface are considered to play important role(s) (4). Diploid cells grow as multinuclear plasmodia and they fuse each other without any artificial treatment. During the course of differentiation, changes in membrane characteristics might occur.

Recently, we found the expression of a poriferasterol monoglucoside associated with the differentiation from myxoamoebae to plasmodia in the wild strain of *Physarum polycephalum* (5). This substance was not detected in the myxoamoebae, but after conjugation, it appeared and increased. And the enzyme, UDP-glucose: poriferasterol glucosyltransferase, which transfer a glucose moiety from sugar nucleotide, UDP-glucose, to sterol, was also found to be expressed in the course of differentiation of wild-type *Physarum polycephalum* (6).

A mating-less mutant, *Colonia* strain, differentiates from myxoamoebae into plasmodia without conjugation. In this process, no change of nuclear DNA occurs. Using the cells of this mutant, we have found the existence of poriferasterol monoglucoside and its synthesizing enzyme in both stages, myxoamoebae, and plasmodia (7).

These findings suggest that the sterol glycosylation may have some important roles in the membrane function, and that *Physarum* cells may be utilized as a powerful tool to investigate the biological significance of the glycosylation of membrane sterol(s).

On the other hand, we examined the sterol contents of myxoamoebae and plasmodia, and reported that they contained poriferasterol, Δ^5-ergosterol and 22-dihydroporiferasterol with the molar ratio of 80 : 15 : 5 in the myxoamoebae and 47 : 21 : 32 in the plasmodia, respectively (8).

In order to know the roles of sterol glucoside in the membrane function, it is an efficient way to get pure sterols and steryl glucoside from *Physarum* cells and to examine the ability of membrane fusion, glucose uptake, and so on, using liposomes constituted with these substances. Still now, no effective purification method for such sterols and steryl glucoside has reported, and it has been very difficult to get them for detailed analyses of the function of these substances. We tried and succeeded to purify these substances before, and structural studies were performed (5, 8), but the method was not so efficient and amounts obtained by this procedure were not sufficient for further investigation. In this report, we show the novel and effective purification method of these sterols and steryl glucoside to apparent homogeneity from the plasmodia of *Physarum polycephalum* using high performance liquid chromatography.

Experimental Procedures

Plasmodia of *Physarum polycephalum* were cultured in a sterile semi-defined culture medium by the method of Daniel and Rusch (9), and they were harvested and washed several times with distilled water to remove extracellular slime polysaccharide and used for the preparation of sterols and steryl glucoside.

Extraction and separation of the sterols and steryl glucoside were performed as follows. Washed plasmodia (10 g) were homogenized in 2 vols of a mixture of chloroform-methanol (1 : 1), sonicated and centrifuged. The supernatant was pooled, and to the precipitate 5 vols of chloroform-methanol (2 : 1) were added, sonicated, and centrifuged in the same way. Extraction with chloroform-methanol (2 : 1) was repeated 5 times and the combined supernatant was concentrated to dryness, and partitioned by the

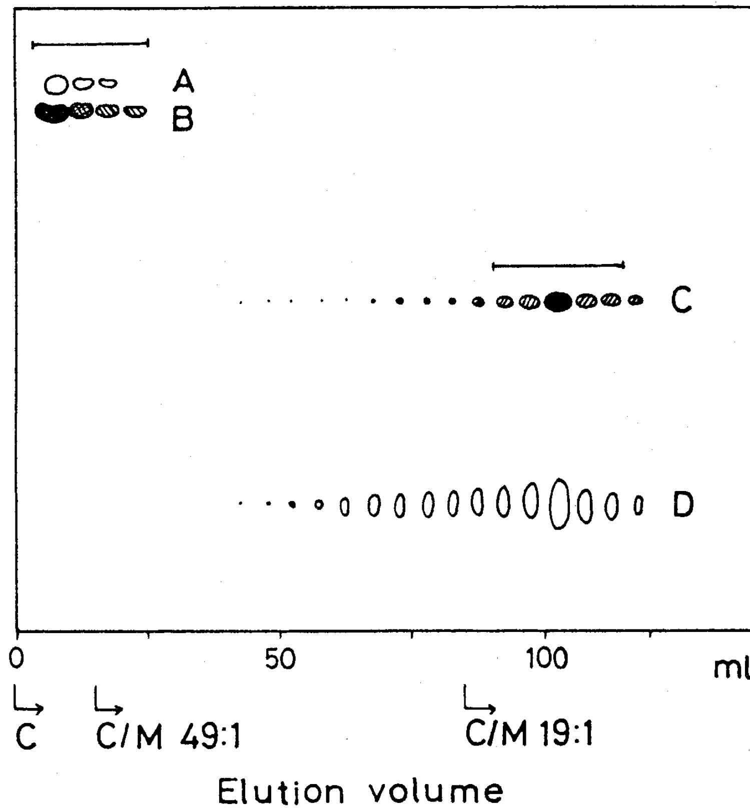

Fig. 1. Elution profile of the crude lipids on a DEAE-cellulose column. TLC was developed in the solvent system I. Spots designated as A and D were visualized as yellow ones, B as red spots and C as purple spots. The fractions under the each bar were pooled for further purification.

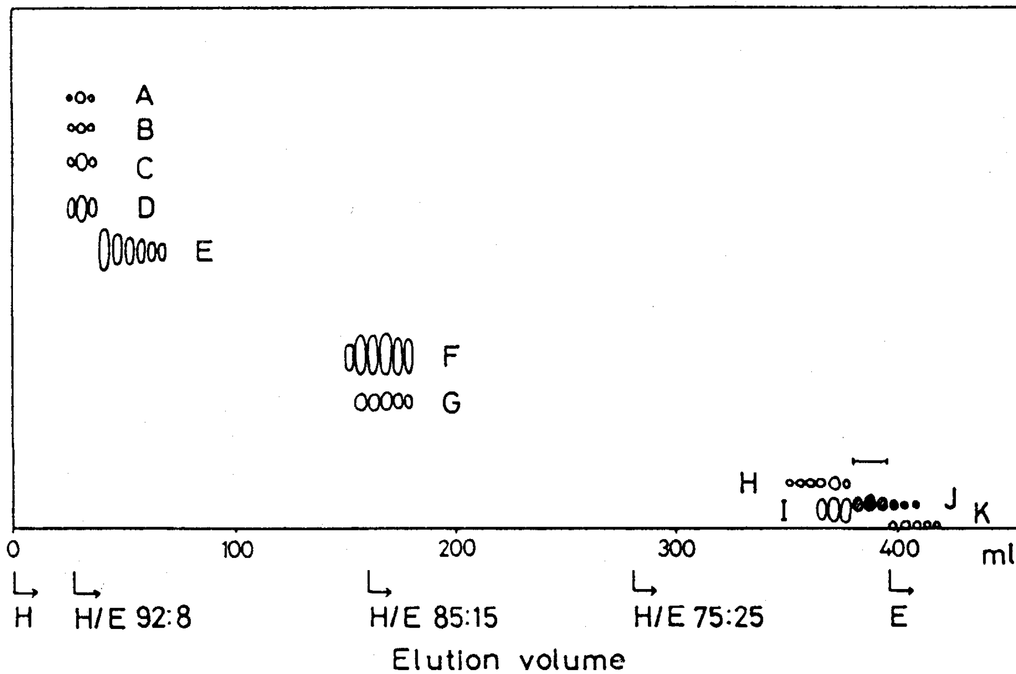

Fig. 2. Iatrobeads column chromatography of the sterol mixture. TLC was developed in the solvent system II. Spots designated as A–I and K were visualized as yellow ones and J as red spots. Fractions under the bar were pooled for the next step.

method of Bligh and Dyer (10). The partitioning was repeated and the combined supernatant was washed with distilled water, then evaporated to dryness. The crude lipid fraction (57 mg) was dissolved in 1 ml of chloroform and applied to a column (1.2×15 cm) of DEAE-cellulose (acetate form, Pharmacia Fine Chemicals) equilibrated with chloroform, and eluted with 4 vols of chloroform-methanol (49 : 1) and 4 vols of chloroform-methanol (19 : 1), successively. Unadsorbed fractions containing sterols and adsorbed fractions containing steryl glucoside were pooled separately for further purification (Fig. 1).

Unadsorbed sterols were dried (26.4 mg) and dissolved in 0.5 ml of hexane, and applied to a column (1.2×24 cm) of silica beads, Iatrobeads (Iatron, Tokyo) equilibrated with hexane, then eluted with 5 vols each of 92 : 8, 85 : 15, and 75 : 25 mixtures of hexane-ether, successively, and finally with ether. Sterols were eluted with hexane-ether (75 : 25) and fractions containing sterols were pooled (Fig. 2) and evaporated to dryness. Sterol

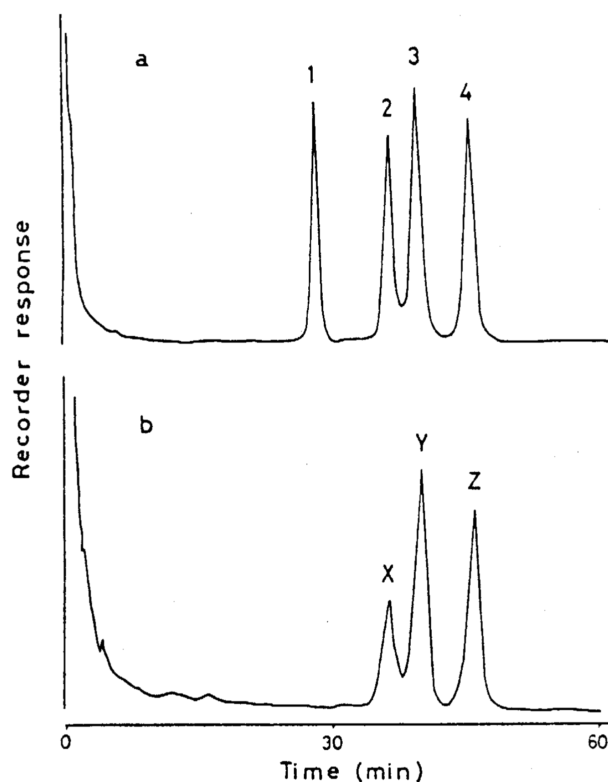

Fig. 3. Gas liquid chromatogram of *O*-trimethylsilyl derivatives from (a) standard sterols and (b) sterol mixture after Iatrobeads column chromatography. Peaks correspond to the following sterols: cholesterol, 1; Δ^5-ergosterol (C-24 epimer of campesterol), 2; poriferasterol, 3; 22-dihydroporiferasterol (C-24 epimer of sitosterol), 4. Peak X corresponds to Δ^5-ergosterol, peak Y to poriferasterol and peak Z to 22-dihydroporiferasterol.

mixture (7.1 mg), which contained three kinds of sterols as shown in Fig. 3, was then dissolved in a small portion of methanol, and at each time 200 μg each of the sterol mixture were separated with high performance liquid chromatography on a column of Capcell Pak C18 AG120 S-5 μm (10× 250 mm, Shiseido Company, Ltd., Tokyo) equipped with Gilson HPLC Model (pump, Model 302; manometric module, Model 802C; dynamic mixture, Model 811; UV-meter, Model 1001; system controller, MS-1-Mark II). Elution solvent was 100% methanol and a flow rate was 4 ml/min. Wave length for the detection of sterols was 205 nm (Fig. 4). This chromatographic run was repeated 35 times to separate all samples containing three kinds of sterols.

Further purification of poriferasterol monoglucoside was performed by a column chromatography on Iatrobeads (1.2×24 cm) equilibrated with chloroform. Evaporated sample was dissolved in a small portion of chloroform-methanol (5: 1) and applied to the column, and eluted with chloroform-methanol, 10: 0, 9: 1 and 8: 2, successively. The steryl glucoside was eluted at the end of the second solvent (9: 1) elution.

If necessary, radioactive substances were able to be obtained as follows.

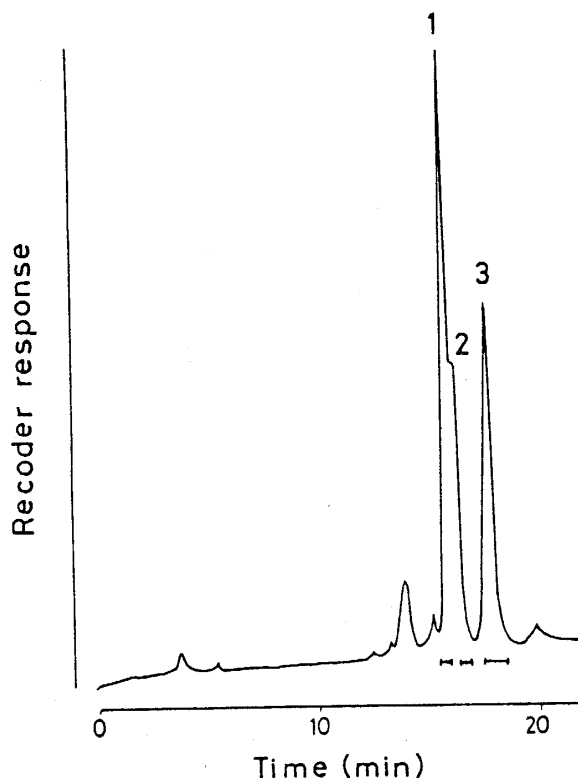

Fig. 4. High performance liquid chromatography of the sterol mixture on a column of Capcell Pak C18. Fractions corresponding to peaks 1-3 (under the bars) were pooled separately, and analyzed by GLC (see Fig. 5).

Physarum plasmodia were cultured in the semi-defined medium (200 ml) containing 1 mCi of [U-^{14}C] acetate (56 mCi/mmol, Amersham) and isolation was carried out as described above.

Gas liquid chromatography (GLC) was performed with the trimethylsilylated sterols on a column of 3% OV-101 (Shimadzu, Kyoto) at 250°C. For the steryl glucoside, the sample was methanolyzed in 5% HCl-methanol and the resulting methyl glucoside and sterol were trimethylsilylated and analyzed by GLC on the same column (5).

Thin layer chromatography (TLC) was carried out with high performance TLC plates of silica gel 60 (Merck, Darmstadt) in the solvent system I or II : I, chloroform-methanol-acetone-acetic acid-water (10 : 2 : 4 : 2 : 1) ; II, hexane-ether (85 : 15). Plates were first exposed to I_2 vapor and then separated sterols and steryl glucoside were visualized with 0.2% orcinol in 2N H_2SO_4 followed by heating. Sterols and steryl glucoside appear as red spots and as purple spot, respectively.

Results and Discussion

Figure 5 shows GLC profiles of purified sterols after HPLC separation. Peak 1 from HPLC corresponds to poriferasterol, peak 2 to Δ^5-ergosterol and peak 3 to 22-dihydroporiferasterol, respectively (8). These results suggest that three kinds of sterols were purified to apparent homogeneity from the plasmodia of *Physarum polycephalum*.

A typical recovery of sterols and steryl glucoside was summarized in Table I. From 10 g of plasmodia, 3.2 mg of poriferasterol, 1.4 mg of Δ^5-ergosterol and 1.5 mg of 22-dihydroporiferasterol were obtained. Comparing to these values, the amounts of them those we had obtained before were 1.6 mg of poriferasterol, 0.2 mg of Δ^5-ergosterol and 0.4 mg of 22-dihydroporiferasterol, respectively, from 10 g of the plasmodia, according to the procedure which was used by many investigators for a purification of plant sterols (8). So, the method described in this manuscript is very effective to get a good deal of sterols in homogeneous states. A pure steryl glucoside was also obtained after Iatrobeads colum chromatography with the amount of 0.32 mg.

Table I. Typical recovery of sterols and steryl glucoside

	mg obtained/10 g of plasmodia
Δ^5-ergosterol	1.4
poriferasterol	3.2
22-dihydroporiferasterol	1.5
poriferasterol monoglucoside	0.32

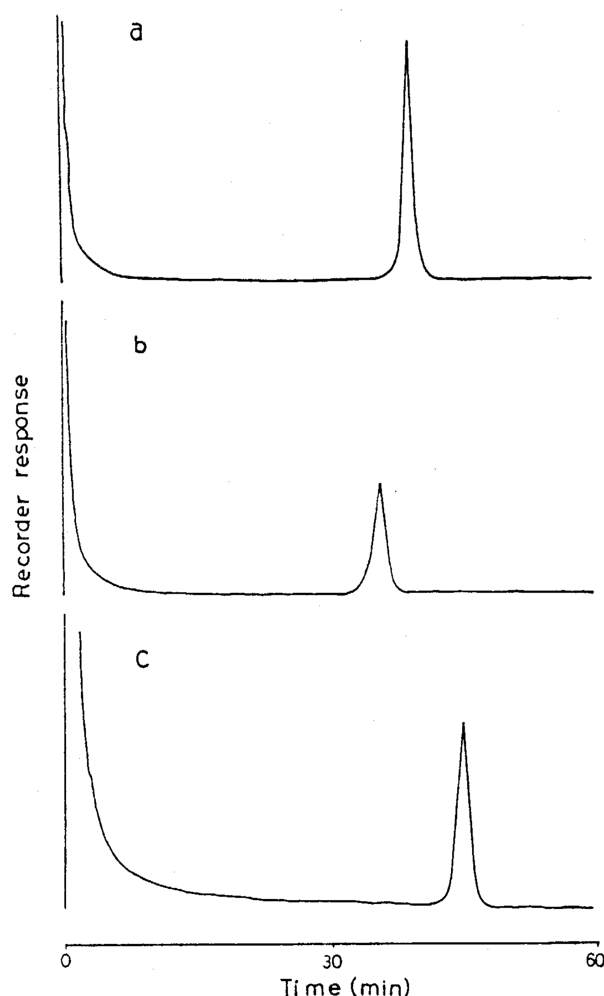

Fig. 5. Gas liquid chromatogram of *O*-trimethylsilyl derivatives from HPCL-purified sterols: peak 1, a; peak 2, b; and peak 3, c.

Characteristics of these sterols and steryl glucoside were examined by gas liquid chromatography-mass spectrometry (GC/MS), fast atom bombardment mass spectrometry (FAB/MS), 400 MHz nuclear magnetic resonance (NMR) spectometry as reported before (5, 8). These sterols from *Physarum* cells are unusual ones, but the epimers of them have been observed as popular sterols in the higher plant kingdom. Steryl glucosides have been also widely observed in the higher plant kingdom, and many investigators have suggested that they act as metabolically active components of the plant membrane structure (11), intercellular transporters of sterols (12), or glucose carriers through cell membranes (13, 14). But definite evidences for the suggestions have not yet been presented. Now, we may have sufficient amounts of pure substances to investigate the physiological roles of these substances *in vitro*.

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