Structures of Two Water Soluble Hemolysins Isolated from the Green Alga *Ulva pertusa*[†]

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Received May 29, 1975

Two water soluble hemolysins isolated from the green alga *Ulva pertusa* have been identified as 1'-*O*-palmitoyl-3'-*O*-(6-*O*- α -D-galactopyranosyl- β -D-galactopyranosyl)-glycerol (1) and 1'-*O*-palmitoyl-3'-*O*-(6-sulfo-*O*- α -D-quinovopyranosyl)-glycerol (2).

In our previous paper,¹⁾ we reported that two water soluble hemolysins, substances I and II, were isolated from the green alga *Ulva pertusa*. The two compounds were similar to each other in biological, chemical and physical properties, and were supposed to be glycolipids.

Substance I, C₃₁H₅₈O₁₄, showed IR absorption at 3400, 1730, 1150, 1070, and 1030 cm⁻¹, indicative of galactosyl glycerides.^{2,3)} On hydrolysis with 1 N hydrochloric acid it yielded glycerol, galactose and palmitic acid in the molar ratio 1:2:1. Deacylation of I with 0.1 N potassium hydroxide at 37°C afforded an equimolar mixture of palmitic acid and 6-*O*- α -D-galactopyranosyl- β -D-galactopyranosyl glycerol. The latter compound had mobilities identical with those of known 6- α -D-galactopyranosyl- β -D-galactopyranosyl-*sn*-glycerol in paper chromatography.^{4~6)} These results indicated that substance I is monopalmitoyl 6-*O*- α -D-galactopyranosyl- β -D-galactopyranosyl glycerol.

The position of attachment of palmitic acid was confirmed by the mass and NMR spectra of the trimethylsilyl (TMS) derivative. The mass spectrum with prominent ion peaks at *m/e* 401, 385, 371, 269, and 239 indicated that the 2-position of glycerol is not substituted. These ions may be interpreted by fragmentation scheme 3.⁷⁾ The mass spectrum of the acetate also supported this struc-

ture. Since the TMS derivative gave no ions beyond *m/e* 700 by the direct inlet method, GC-MS analysis using a short glass column was attempted. A single peak with a small shoulder was obtained in gas chromatography, and ion peaks as high as *m/e* 991 were observed. Beside the peaks mentioned above, prominent ion peaks at *m/e* 204 (base), 217, 241, 271, 281, 309, 319, 341, 361, and 475

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were observed. These peaks were supposed to be fragment ions derived from the sugar moiety.^{8,9)} An intense peak at m/e 761 may be attributable to fragment ion (4). Peaks at m/e 991 ($M^+ - 239$) and 829 ($M^+ - 401$) were also recognized, though no parent ion was obtained.

The NMR spectrum of the TMS derivative in deuteriochloroform gave further information on the unsubstituted 2-position of glycerol moiety. One terminal methyl of the palmitoyl group appeared as a triplet at δ 0.79, methylene protons at δ 1.14 (singlet) and α -methylene protons at δ 2.25 (triplet). Complex signals from δ 3.2 to δ 4.3 and an α -anomer proton¹⁰⁾ of the galactosyl group at δ 4.66 (doublet, $J \approx 3$ Hz) were observed. Absence of low field signals strongly indicated that the 2-position of glycerol is not conju-

gated.¹¹⁾ These values also coincided well with those reported for synthetic 1'-O-palmitoyl-3'-O-(6-O- α -D-galactopyranosyl- β -D-galactopyranosyl)-*sn*-glycerol.¹²⁾ Structure (1) is therefore assigned to substance I.

Substance II, $\text{C}_{25}\text{H}_{47}\text{O}_{11}\text{SK}$, gave no sulfuric acid upon hydrolysis. Its IR spectrum exhibited absorption bands at 3400, 1730, 1170, 1150, 1060, 1035, and 771 cm^{-1} , indicative of 6-sulfoquinovosyl glycerides.^{2,13)} Strong bands at 1170, 1060, 1035, and 771 cm^{-1} suggested the presence of a sulfonyl group.¹⁴⁾ With anthrone reagent the compound produced a blue color with λ_{max} 592 nm, characteristic of 6-sulfoquinovose.¹⁵⁾

Acid hydrolysis of substance II with 1 N hydrochloric acid afforded glycerol, 6-sulfoquinovose and palmitic acid in the molar ratio 1:1:1. Upon mild alkaline hydrolysis with 0.1 N potassium hydroxide it released an equimolar mixture of palmitic acid and 6-sulfo- α -D-quinovopyranosyl glycerol. The latter compound showed mobilities quite similar to those of known 6-sulfo- α -D-quinovopyranosyl-*sn*-glycerol.^{5,16)} These results led us to assume that substance II has the structure of monopalmitoyl 6-sulfo- α -D-quinovopyranosyl glycerol.

The mass spectrum of the TMS derivative indicated that the 2-position of glycerol is not involved in ester linkage, as in the case of substance I. In GC-MS analysis, the TMS derivative exhibited a single peak and prominent ion peaks at m/e 204 (base), 371, 385, 430, 485, 574, and 705, though no parent ion peak was observed. These fragment ions may be assigned as shown in (5). Intense ion peaks at m/e 574 and 430 may be attributable to (6) and (7), respectively.

The NMR spectrum of the TMS derivative in deuteriochloroform gave further confirmation for the 2-position of the glycerol moiety. One terminal methyl of the palmitoyl moiety was observed at δ 0.78 (triplet), a large methylene signal at δ 1.17 (singlet), and an α -methylene signal at δ 2.20 (triplet). An α -anomer proton¹⁰⁾ of the sugar moiety appeared as a doublet at δ 4.74 ($J \approx 3$ Hz). Lack of low field signals

indicated the absence of substitution at the 2-position of glycerol.¹¹ These data allow us to conclude that substance II has structure (2).

To our knowledge, this is the first time that a lysogalactolipid and lysosulfolipid were isolated pure, while their presence had been postulated for many years.^{4,17,18} It is very interesting that only palmitic acid was obtained on hydrolysis, since every glycolipid isolated so far released a mixture of fatty acids.¹⁹ We previously isolated palmitic acid as the fat soluble hemolysin, which is the predominant fatty acid in the alga.¹ It is most likely that palmitic acid plays a unique role in the physiology of the green alga *U. pertusa*.

EXPERIMENTAL

IR spectra were measured with a JASCO IR-S spectrometer. NMR spectra were recorded on a JEOL-JNM-4H spectrometer and mass spectra on a Hitachi RMU-6L mass spectrometer. GC-MS analyses were carried out with an LKB 9000 GC-MS system. Gas liquid chromatography (GLC) was performed on a Shimadzu GC-5A instrument equipped with hydrogen flame ionization detector.

Fatty acids were derivatized to their methyl esters by the method of Horning *et al.*²⁰ and analyzed on glass columns (3 mm × 2 m) packed with 5% SE-30 on Shimalite W (60/80) and with 20% DEGS on Shimalite W (60/80). The former column was operated by linear programming at 4°C/min from 100 to 230°C, and the latter isothermally at 190°C.

Trimethylsilyl ethers (TMS) for GLC analyses were prepared by the method of Sweeley *et al.*²¹ using dimethylformamide (DMF), *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS). GLC analyses of the acid hydrolysis products were carried out on a glass column (3 mm × 2 m) packed with 3% OV-1 on Shimalite W (60/80). The column temperature was maintained at 70°C for 5 min and programmed at 10°C/min to 180°C then at 4°C/min to 250°C.²² Ethylene glycol was used as the internal standard and peak areas were measured by triangulation. The TMS derivatives of intact hemolysins were analyzed on a 1 m column packed with 1.5% OV-1 on Shimalite W (60/80). The TMS derivatives for measurement of NMR spectra were prepared according to Kamerling *et al.*¹⁰ and the spectra were taken in deuteriochloroform with tetramethylsilane as the internal standard.

Thin-layer chromatography was carried out on silica gel H (Merck) with benzene-acetone (4: 1) (solvent *a*)

and *n*-hexane-diethyl ether-acetic acid (70: 30: 1) (solvent *b*). For detection of components plates were sprayed with 50% sulfuric acid and 10% phosphomolybdic acid. Paper chromatography was performed by the ascending technique with Toyo No. 51 paper. Solvents used were 2-propanol-pyridine-water-acetic acid (8: 8: 4: 1) (solvent *c*), 1-butanol-pyridine-water (6: 4: 3) (solvent *d*), 1-butanol-propionic acid-water (142: 71: 100) (solvent *e*), phenol-water (100: 40, v/v) (solvent *f*), phenol-water (100: 40, w/w) (solvent *g*), and water-saturated 2,4,6-trimethylpyridine (solvent *h*). The components were detected by spraying with *p*-anisidine-HCl, aniline phthalate, diphenylamine, and periodate-benzidine reagents.

Isolation of substances I and II

Isolation and some properties of the hemolysins were reported in the previous paper.¹

TMS derivative of substance I, colorless gum, NMR: δ 0.79 (triplet), 1.14 (singlet), 2.25 (triplet), 3.2~4.3 (complex signals), 4.66 (doublet, $J \approx 3$ Hz); MS: *m/e* 117, 129, 149, 167, 239 (base), 257, 269, 281, 313, 341, 369, 371, 385, 401, 415, 549, 625.

Acetate of substance I, mp 56~57°C, MS: *m/e* 117, 129, 171, 239 (base), 269, 313, 331, 341, 355, 381, 523, 551, 579.

TMS derivative of substance II, colorless gum, NMR: δ 0.78 (triplet), 1.17 (singlet), 2.20 (triplet), 3.0~4.2 (complex signals), 4.74 (doublet, $J \approx 3$ Hz); MS: *m/e* 117, 171, 185, 239 (base), 257, 269, 311, 331, 341, 355, 371, 385, 401, 429, 465, 550, 625.

Acid hydrolysis of substance I

A 2 mg portion of substance I dissolved in 1 N hydrochloric acid (1 ml) was heated at 100°C for 2 hr. The reaction mixture was extracted with diethyl ether after addition of water. The aqueous layer was freed from hydrochloric acid by repeated evaporation and examined by paper chromatography. The presence of glycerol and galactose was recognized as shown in Table I.

The ether extract gave only one spot on silica gel H layers, when developed with solvents *a* and *b*. GLC analysis of the methyl ester showed the presence of palmitic acid. The residue was recrystallized from 80% ethanol and obtained as white scales with mp 63~63.5°C, coinciding with an authentic sample of palmitic acid in IR and mass spectra.

TABLE I. *R_f* VALUES OF THE WATER SOLUBLE PRODUCTS OBTAINED BY ACID HYDROLYSIS OF SUBSTANCE I

Solvent	Glycerol	Products	Galactose
<i>c</i>	0.78	0.78, 0.54	0.54
<i>d</i>	0.58	0.58, 0.33	0.33
<i>e</i>	0.47	0.48, 0.21	0.21
<i>f</i>	0.63	0.63, 0.36	0.36

TABLE II. DETERMINATION OF THE ACID HYDROLYZATES BY GAS LIQUID CHROMATOGRAPHY

Sample	Retention time (min)	Molar ratio
Glycerol	13.6	
Galactose	23.0, 23.6, 24.5	
Palmitic acid	25.6	
Hydrolyzates	13.6	1
	22.9, 23.6, 24.4	1.8
	25.6	0.9

Determination of the acid hydrolysis products

One milligram of substance I was hydrolyzed and freed from hydrochloric acid. To the residue were added DMF (100 μ l), BSTFA (40 μ l) and TMCS (20 μ l), and the mixture was heated at 80°C for 10 min. One μ l of the reaction mixture was subjected to GLC analysis (Table II).

Deacylation of substance I

To 25.8 mg of substance I dissolved in 1 ml of water was added 1 ml of 0.2 N potassium hydroxide, and the solution was kept at 37°C for 24 hr. The reaction mixture was acidified with cation resin, AG 50W-X8 (H⁺ form) and extracted with diethyl ether. On evaporation to dryness, the aqueous layer afforded colorless solids (14.1 mg). Paper chromatographic analysis with solvents *c* (*R_f* 0.11), *d* (0.12), *e* (0.51), and *h* (*R_{galactose}* 0.40) indicated the presence of 6-O- α -D-galactopyranosyl- β -D-galactopyranosyl glycerol.⁴⁻⁶⁾

The ether extract gave colorless scales (11.6 mg) upon evaporation. The residue was recrystallized from 80% ethanol to afford colorless scales with mp 63~63.5°C (Found: C, 75.00; H, 12.31. C₁₆H₃₂O₂ requires C, 74.94; H, 12.58%). This compound was identical with palmitic acid in GLC, IR and mass spectra.

GC-MS analysis of the TMS derivative of substance I

One milligram of substance I was trimethylsilylated with BSTFA (40 μ l) and TMCS (20 μ l) in DMF (100 μ l) and analyzed by GLC. The column temperature was maintained at 270°C for 1 min and then programmed at 4°C/min to 330°C. A single peak with a small shoulder was obtained. For GC-MS analysis, the temperature programming was set at 2°C/min from 290 to 300°C. MS: *m/e* 204 (base), 217, 231, 243, 271, 281, 305, 341, 361, 385, 401, 451, 731, 761, 829, 991.

Acid hydrolysis of substance II

Two milligrams was hydrolyzed in 1 ml of 1 N hydrochloric acid in the same manner as described for substance I. The aqueous layer was freed from the acid and analyzed by paper chromatography. As shown in Table III, the presence of glycerol and 6-sulfoquinovose was recognized. The latter compound

TABLE III. *R_f* VALUES OF THE WATER SOLUBLE PRODUCTS OBTAINED BY ACID HYDROLYSIS OF SUBSTANCE II

Solvent	Glycerol	Products	6-Sulfo-quinovose
<i>c</i>	0.78	0.78, 0.20	0.20
<i>d</i>	0.58	0.58, 0.08	0.08
<i>e</i>	0.48	0.48, 0.08	0.08
<i>g</i>	0.70	0.70, 0.12	0.12

coincided well with an authentic specimen synthesized by the method of Miyano and Benson.²⁸⁾

The ether extract afforded white solids, identical with an authentic specimen of palmitic acid in GLC, IR and mass spectra.

Determination of the acid hydrolysis products

One milligram of the compound was hydrolyzed and the trimethylsilylated mixture was analyzed by GLC in the same manner mentioned above. It was found that glycerol, 6-sulfoquinovose and palmitic acid were present in the molar ratio 1:1:1.

Mild alkaline hydrolysis of substance II

To a 51.2 mg portion dissolved in 2.5 ml of water was added 2.5 ml of 0.2 N potassium hydroxide. The mixture was kept at 37°C for 24 hr and 31.3 mg of colorless solids was obtained as the water soluble product after usual work-up. Paper chromatography with solvents *c* (*R_f* 0.19), *e* (0.08) and *g* (0.20) suggested the presence of 6-sulfo-O- α -D-quinovopyranosyl glycerol.^{5,16)}

The ether soluble fraction upon evaporation gave white scales (19.1 mg) identical with palmitic acid in mp, combustion analysis, IR and mass spectra.

GC-MS analysis of the TMS derivative of substance II

The TMS derivative of substance II was prepared by the routine method and analyzed on a 1 m glass column of 1.5% OV-1 with temperature programming: at 2°C/min from 270°C. Only one peak appeared and showed ion peaks at *m/e* 204 (base), 217, 271, 281, 313, 339, 361, 371, 385, 401, 430, 485, 574, 576, 647, and 705.

Acknowledgements. This work was supported by the fund from the Ministry of Education. We are grateful to Professor Tamao Yoshida, Tokyo University of Fisheries for GC-MS analyses and to Dr. Kazuyuki Aizawa of this University for measurement of IR, NMR and mass spectra. Thanks are also due to Professor Paul J. Scheuer, Department of Chemistry, University of Hawaii at Manoa, who kindly read our manuscript.

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