ISOLATION AND PARTIAL CHARACTERIZATION OF A NOVEL GLYCOLIPID FROM AN EXTREMELY THERMOPHILIC BACTERIUM

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Summary: A glycolipid was isolated from an extreme thermophile, Flavobact.thermophilum HB8 as a major lipid component. It contained galactose, glucose, glucosamine, glycerol, fatty acid esters, fatty acid amide in a molar ratio of 2:1:1:1:2:1. The fatty acid amide was in the form of N-(15-methyl hexadecanoyl)glucosaminide. On the basis of methylation studies this glycolipid most likely has one of the following structures: Gal(f)(1→2)Gal(p)(1→6)GlcN(iso-C17)(1→2)Glc(p)-diglyceride, or Gal(f)(1→2)Gal(p)(1→2)Glc(p)(1→6)GlcN(iso-C17)-diglyceride.

Introduction: An extreme thermophile, Flavobacterium thermophilum HB8(sp.n.) has been isolated from a hot spring and its maximum growth temperature was estimated to be 85°C(1), which is probably the highest one hitherto reported. The glycolytic enzymes of this bacterium were found to be extremely thermostable and some of them exhibited allosteric nature(2,3,4). The thermostability of ribosome and tRNA of this strain has also been studied(1). These findings suggest that the ability to grow at such a high temperature is due to the inherent thermostability of the individual cellular components, but the biological mechanisms which enable the bacterium to prefer

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Abbreviations: Gal(f):galactofuranoside, Glc(p):glucopyranoside, GlcN(iso-C17):N-(15-methyl hexadecanoyl)glucosaminide, iso-C15: 13-methyl tetradecanoic acid, TLC:thin layer chromatography, GLC:gas-liquid chromatography, GL:glycolipid.

the high temperature has not been fully understood yet. Studies on the cell membrane components rather than the soluble inner cell constituents may be the another approach to solve the secret of thermophilicity. The spheroplast membrane of the medium thermophile, Bacillus stearothermophilus is stable at its optimum temperature(55°-65°C), without any stabilizing reagents(6). Certain membrane-bound enzyme of the thermophile may be stabilized by the association with the cell membrane(7). We have initiated an investigation on thermostability and thermophilicity of the cell membrane, from the study of individual lipid component of the bacteria. The present paper describes the isolation and characterization of a novel glycolipid which was found as a major lipid in the extreme thermophile.

Materials and Methods: F.thermophilum HB8 was aerobically grown at 75°C in a medium consisting of 0.8%(w/v) polypeptone, 0.4% yeast extract and 0.2% NaCl. Cells were harvested at the late exponential stage. After the cells were washed twice with 0.9% saline the lipids were extracted with 20 vols.(w/v) of $CHCl_3$-Methanol(2/1, v/v) with stirring overnight. Residue was further extracted with the same solvent mixture in Soxhlet apparatus. The extracts were combined and partitioned with 0.2 vols.(v/v) of 0.2% $BaCl_2$ and the lower organic phase was evaporated to dryness(8). The crude lipids were treated with 30 vols.(w/v) of acetone and fractionated to soluble and insoluble portions. The acetone-soluble yellow pigments (carotenoids) were separated from the insoluble lipids. The acetone-insoluble polar lipid fraction was subjected to silicic acid column chromatography, first with Kantokagaku silica gel(100mesh, Kantokagaku Co.) and 2nd with Silic AR CC-7(200-325 mesh, Mallinckrodt Co. Ltd.) and finally to DEAE-cellulose(acetate form) column chromatography(9). The above eluates were dried and dissolved in

warm methanol and the white crystalline glycolipid(GL) was obtained on cooling. The pure GL was then characterized by chemical, physical and enzymatic analyses. The amounts of fatty acids were determined by GLC(25% ethylene glycol succinate on Gas-Chrom CLZ by Shimadzu GC-5A) using palmitic acid as an internal standard.

Results and Discussion: 635g of wet packed cells of F.thermophilum were harvested from 132 liter of culture medium. Crude lipids (10.9g) were separated to acetone-soluble lipids(mainly pigments, 670mg) and 8.3g of acetone-insoluble lipids. On TLC of the acetone-insoluble fraction, the major spot was strongly positive with anthrone reagent. And two phosphatide spots and another faint glycolipid spot were found with an acidic solvent system. The major glycolipid(GL) was eluted with 72-64% $CHCl_3$ in methanol from Kantokagaku silica gel and with 78-70% $CHCl_3$ in methanol from Silic AR CC-7 column. By eluting with $CHCl_3$-methanol(7/3,v/v) from DEAE-cellulose column chromatography, the last trace of phospholipids were entirely eliminated and the pure GL(460mg) was obtained. The GL showed a single spot on TLC under neutral, acidic or alkaline solvent systems. The Rf values of the GL and the reference substances are listed in Table 1.

On TLC the spot of GL was positively detected with anthrone-sulfuric acid(11) and periodate-Schiff(12) spray, but it gave negative reactions with molybdate for phosphorous(13) and ninhydrin for free amino groups.

The analytical data (Table 2) indicates the GL contains 2 moles of galactose,2 equivalents of ester and one mole each of glucose, glucosamine and glycerol. Standard solution of galactose/glucose (2/1,w/w) was used for the determination of hexose by anthrone-sulfuric acid(11). Hexose content was 36.2% of the GL. Glucosamine

Table 1. Analytical Data of the Glycolipid

Determinations	Ester	Glycerol	Gal	Glc	GlcN
Colorimetric					
Ester*	1.98				
Glucosamine					0.90
Hexose			3.00		
Enzymatic		1.09	2.00	1.00	
Gaschromatographic					
8% Tripalmitin(190→210°C)			2.02	1.00	0.82
3.8% UC-W(166→245°C)			1.98	1.00	0.80
7% Ucon(isothermal)			1.95	1.00	

* Determined by the method of Snyder and Stephens(14).

Enzymatic glucose, galactose and glycerol determinations were performed by the reduction of NAD or NADP measured at 340nm, using glucose-6-phosphate-dehydrogenase(16), galactose dehydrogenase (17) and glycerol-3-phosphate-dehydrogenase(18) respectively,after glucose and glycerol were treated with kinases.

GLC were performed as the trimethylsilyl derivatives after the methanolysis(0.8N methanolic HCl, 80°C, 24 hrs.) and N-acetylation(acetic anhydride with Ag_2CO_3)(19).

Table 2. The Rf values of the GL and the reference substances

Solvent systems	GL	(DDG*)	(TDG**)
$CHCl_3$-Methanol-H_2O(65:35:8)	0.79	(0.89)	(0.70)
$CHCl_3$-Methanol-Acetic acid-H_2O(65:25:10:8)	0.30	(0.52)	(0.27)
$CHCl_3$-Methanol-7% NH_4OH(65:35:8)	0.71	(0.76)	(0.65)
$CHCl_3$-Methanol-Acetone(85:25:15)	0.16	(0.36)	(0.12)

* Kojibiosyl diglyceride(10), ** Triglucosyl diglyceride(10).
TLC plates(10 x 10cm, 0.25mm thick) were activated at 110°C, 3 hrs. before use.

was found to be 13.5% of the GL by Elson-Morgan reaction(15).

Ester (1740cm^{-1}) and amide (1545, 1640cm^{-1}) linkages were found on infrared spectrum of the GL(Fig.1).

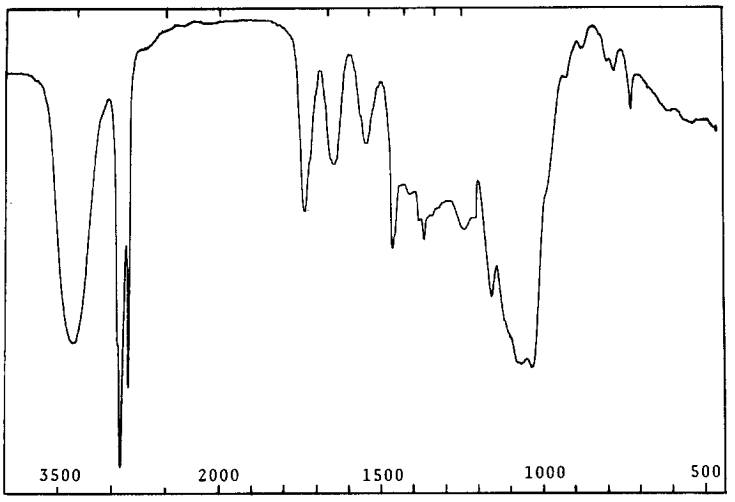

Fig.1 Infrared spectrum of the glycolipid from F.thermophilum.

Mild alkaline hydrolysis(0.2N NaOH, 37°C, 2 hrs.) liberated two moles of esterified fatty acids(mainly iso-C15 and iso-C17 acid). And a strong acid treatment(3% methanolic HCl, 100°C, 3 hrs.) cleaved further one mole of iso-C17 acid with disappearence of amide absorptions on the infrared spectrum. This finding indicates that the third iso-C17 acid was linked with the amino group of the glucosamine moiety.

The sugar sequences were studied by the partial acid hydrolysis or the enzymatic hydrolysis of the GL and it was found that the non-reducing end was a galactose and that the penultimate sugar was also galactose, which was linked to the next sugar by an α-linkage. GLC(5% neopentyl glycol succinate on Gas-Chrom CL7 at 160°C), after the permethylation(20) and the methanolysis indicated the presence of methyl-2,3,5,6-tetra-O-methyl galactofuranoside, methyl-3,4,6-tri-O-methyl glucopyranoside, and methyl-3,4,6-tri-O-methyl-galactopyranoside. The presence of methyl-3,4-di-O-methyl glucosaminide was demonstrated by GLC(3% OV-1 on Celite 545,135→160°C)

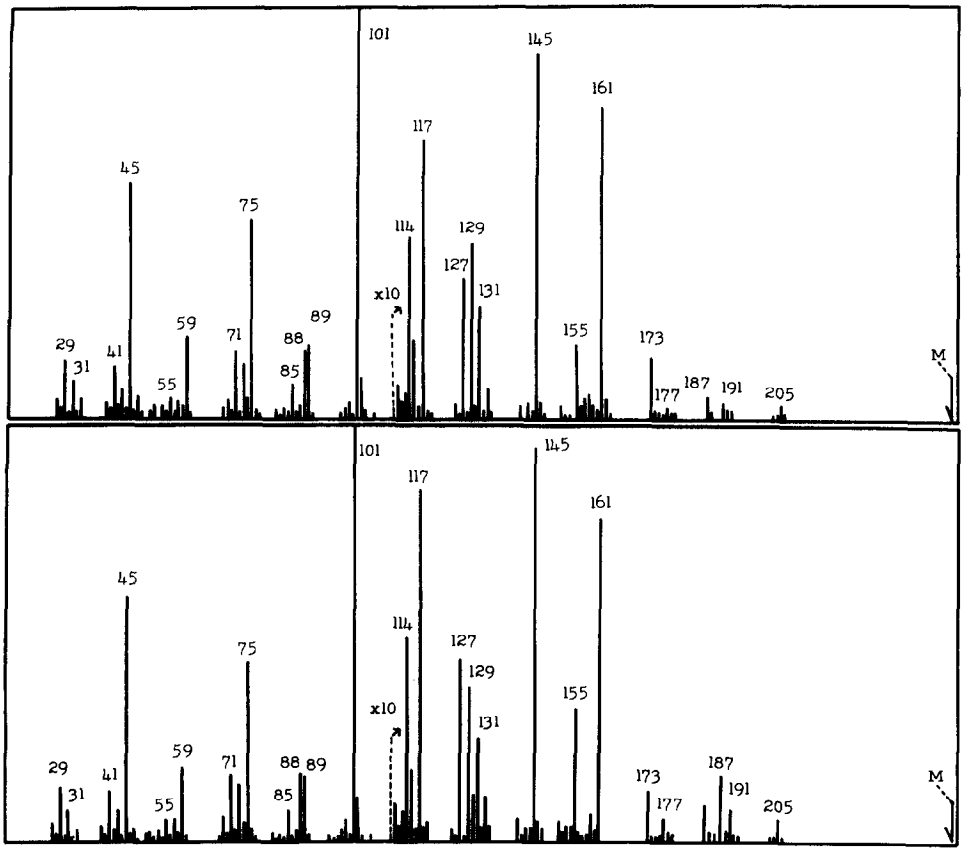

Fig.2 GC-Mass spectra of the authentic standard of methyl-2,3,5,6-tetra-O-methyl-β-D-galactofuranoside(top) and that obtained by the permethylation of the GL(bottom).

after N-acetylation and trimethylsilylation of the methanolysate.

Methyl 2,3,5,6-tetra-O-methyl galactofuranoside was determined not only by the retention time on GLC but also by GC-Mass spectrum using Shimadzu-LKB GC-Mass spectrometer(Fig.2). From the above findings, two possible structures for the GL are proposed: Gal(f)(1→2)Gal(p)(1→2)Glc(p)(1→6)GlcN(iso-C17)-diglyceride or Gal(f)(1→2)Gal(p)(1→6)GlcN(iso-C17)(1→2)Glc(p)-diglyceride.

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