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Chromatographic resolution of partially perdeuterated diglucosyldiacylglycerols from *Acholeplasma laidlawii*

Magnus H.J. Bergqvist[1]

Department of Analytical Chemistry, Stockholm University, Stockholm, Sweden

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Abstract

Diglucosyldiacylglycerol from *Acholeplasma laidlawii* was isolated by adsorption chromatography and subsequently subjected to molecular species analysis by reversed-phase high-performance liquid chromatography. Twenty-three molecular species were resolved. The bacterium was grown in a medium supplemented with a mixture of oleic acid and perdeuterated palmitic acid, and incorporation of perdeuterated acyl moieties occurred in several molecular species. Short and odd-numbered acyl chains were found in combinations with palmitic acid, perdeuterated palmitic acid and oleic acid. Also, molecular species differing only by perdeuterated and protonated acyl chain content were resolved chromatographically. Separation between diglucosyldiacylglycerol and digalactosyldiacylglycerol isolated from a plant source was achieved with adsorption chromatography. The polarity of diglucosyldiacylglycerol as compared to digalactosyldiacylglycerol is discussed.

Keywords: *Acholeplasma laidlawii*; Diglucosyldiacylglycerols; Glycerols; Glucolipids; Lipids

1. Introduction

The bacterium *Acholeplasma laidlawii* strain A-EF22 synthesizes several glucolipids, such as 1,2-diacyl-3-O-[α-D-glucopyranosyl-(1→2)-O-α-D-glucopyranosyl]-*sn*-glycerol (DGlcDAG). The structure of this lipid (Fig. 1) was determined by ^{1}H NMR and ^{13}C NMR [1]. By cultivating the organism in a medium containing perdeuterated palmitic acid ([$^{2}H_{31}$]16:0; 16:0-d_{31}), incorporation of this fatty acid into DGlcDAG was achieved. This lipid preparation can then be used in mixtures of membrane glucolipids for experiments concerning, for example, orientational order and dynamics of the acyl chains in lipid bilayers [2].

Separation of polar molecular species is normally done by high-performance liquid chromatography in the reversed-phase mode (RP-HPLC). Each molecu-

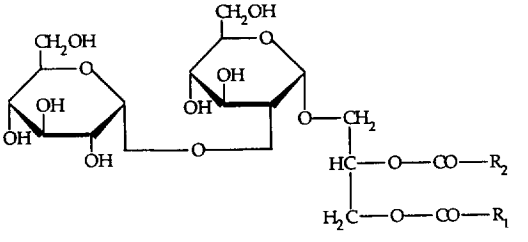

Fig. 1. Structure of DGlcDAG. –CO–R_1 and –CO–R_2 denote any acyl group.

[1]Address till October 1996: Banting and Best Department of Medical Research, University of Toronto, Toronto M5G 1L6, Canada.

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lar species can be given a partition number, PN, defined as $CN-2n$, where CN is the number of carbon atoms in the acyl moieties and n is the number of double bonds in the acyl chains [3]. In RP-HPLC the elution order of molecular species is in the order of increasing PN. Solvent systems with a relatively high polarity compared with, for example, solvent systems for triacylglycerols are needed to achieve separation of molecular species of intact polar lipids. The eluent systems are therefore often based on alcohols and water. The alcohol and water systems elute molecular species with the same PN in the order of increasing unsaturation [4–11]. Intact polar molecular species have also been successfully separated by alcohol–water eluent systems containing silver ions [12]. Polar lipids are often derivatized to increase detectability [5,13–19] or to decrease the polarity of the compound, thus obtaining higher resolution [13]. These more apolar derivatives are frequently used with acetonitrile solvent systems, thereby eluting the molecular species with decreasing unsaturation among molecular species having the same PN. This elution behaviour gives characteristic PN groupings of molecular species. The resolving strength of the alcohol–water solvent systems seems to be stronger than that of the acetonitrile systems, and polyunsaturated molecular species may overlap between PN groupings in the former [20,21].

The separation of DGlcDAG molecular species has not been described previously although several papers have described the separation of digalactosyldiacylglycerol (DGalDAG) molecular species. The alcohol–water eluent system described in the present study has previously been used for the separation of DGalDAG molecular species [22]. However, the eluent system can be used to determine the molecular species composition of DGlcDAG, although retention time shifts are observed when compared to DGalDAG. Also, molecular species with perdeuterated acyl chains incorporated were separated from their protonated analogues by the same technique. The retention time shifts indicate different polarities for the two glycoglycerolipids. The different polarity characteristics of the two lipid classes were confirmed by adsorption chromatography on HPLC; a comparison which, to the author's knowledge, has not been described previously.

2. Experimental

2.1. Chemicals and reagents

Fatty acid standards were from Sigma (St. Louis, MO, USA) and from Larodan (Malmö, Sweden). Acetone, methanol, heptane, butanol, tetrahydrofuran and isooctane were from Merck (Darmstadt, Germany), and hexane and 2-propanol were from Riedel-de Haën (Seelze, Germany). All solvents were of HPLC grade. Water was ultrapurified with Nanopure equipment (Barnstead, Dubuque, IA, USA).

2.1.1. Growth of A. laidlawii

A. laidlawii strain A-EF22 was grown statically in 15 l of the medium described by Eriksson et al. [2]. The medium was supplemented with 100 μM oleic acid (18:1c) and 25 μM perdeuterated palmitic acid (16:0-d_{31}). These two fatty acids were biosynthetically incorporated, without being elongated or broken down, into all the membrane lipids of the bacterium. However, the bovine serum albumin, fraction V (Boeringer Mannheim, Mannheim, Germany), used in this growth medium was not completely lipid depleted. The organism was consequently capable of de novo synthesis of saturated fatty acids from the primers acetate (to make even-chain acids) and propionate or valerate (to make odd-chain acids) [23,24]. These fatty acids were then also incorporated into the membrane lipids. Growth and harvest of the cells were performed as described by Eriksson et al. [2].

2.1.2. Purification of DGlcDAG

Lipid extraction of the cells and purification of DGlcDAG utilising a combination of Sephadex gel, silicic acid column and thin-layer chromatography were performed according to a procedure described by Eriksson et al. [2].

2.2. Methods

2.2.1. Reversed-phase high-performance liquid chromatography

RP-HPLC of the DGlcDAGs was conducted on a Kromasil C_{18} column, 250×4.6 mm, particle size 5

μm, pore size 100 Å, from Eka Nobel (Bohus, Sweden). The HPLC system consisted of two Shimadzu LC-6A pumps (Kyoto, Japan) integrated with a Shimadzu SCL-6B system controller. The column was thermostated with a water bath at 55°C to lower the system back-pressure. Detection was done with a light-scattering detector from S.E-.D.E.R.E. (Sedex 45, Vitry sur Seine, France). The nebulizer pressure was set to 1.5 bar and the evaporation chamber temperature was set to 65°C. Elution was done utilising a gradient over 40 min from acetone–2-propanol–methanol–heptane–water (5:38:28:2:27, v/v) to (5:47:37:2:9, v/v) as described earlier [22].

2.2.2. Adsorption chromatography

Separation of lipid classes was done with a HPLC pump from LDC (Model CM 4000, Milton Roy, Riviera Beach, FL, USA) connected to a polar diol phase column (LiChrospher, 250×4.6 mm, 5 μm, Merck) which was thermostated to 75°C. The eluent was a gradient from 100% A (hexane–2-propanol–butanol–tetrahydrofuran–isooctane–water, 64:20:6:-4.5:4.5:1, v/v) to 100% B (2-propanol–butanol–tetrahydrofuran–isooctane–water, 75:6:4.5:4.5:10, v/v) over 25 min as described by Arnoldsson and Kaufmann [25]. An ammonium acetate (NH_4Ac) salt concentration of 180 mg/ml solvent mixture was held constant throughout the elution. Detection was as for the RP-HPLC system but with a nebulizer pressure set to 1.6 bar and the evaporation chamber temperature set to 97°C.

2.2.3. Gas chromatography

The total fatty acid profile as well as profiles for the fractions taken from the RP-HPLC experiment were determined by converting the fatty acids to their methyl esters by an alkaline methanolysis procedure described by Olsson et al. [26]. The GC system consisted of a Varian 3500 equipped with a temperature programmable rotary valve on-column injector (Walnut Creek, CA, USA). The analytical column was a DB-WAX, 30 m×0.25 mm I.D. from J&W (Folsom, CA, USA). A retention gap was installed between the injector and the analytical column. The temperature was held at 130°C for 2 min, then increased by 50°C/min to 150°C and finally increased by 3.3°C/min to 235°C where it was maintained for 5 min. Helium was used as the carrier gas and was set to 2.3 ml/min. Detection was made by a flame ionisation detection (FID) at 250°C. The detection signals were monitored by the Gynkosoft Chromatography Data System (Gynkotek, Germering b. München, Germany).

2.2.4. Liquid chromatography–mass spectrometry.

Molecular species were separated on RP-HPLC and introduced via a particle beam interface to the mass spectrometer as described by Nilsson and Liljenberg [27]. The particle beam interface settings were adjusted to obtain optimal mass transfer.

3. Results and discussion

In the present study a solvent system based on alcohols and water was used to separate diglucosyldiacylglycerol molecular species. Fractions representing one or more molecular species were collected, as shown in Table 1, and analysed as their fatty acid methyl esters (FAMEs) by means of gas chromatography. Tentative identification was done by comparing the retention times to FAME standards. 16:0-d_{31} elutes ca. 0.5 min ahead of 16:0 on the DB-WAX column under the described conditions.

In Fig. 2, twenty-three peaks representing one or several diglucosyldiacylglycerol molecular species are separated. The fatty acids 12:0, 13:0, 14:0, 15:0, 16:0-d_{31}, 16:0 and 18:1 form molecular species combinations with 16:0-d_{31}, 16:0 and 18:1. About 85% of the DGluDAG consists of 18:1/18:1, 18:1/16:0 and 18:1/16:0-d_{31} molecular species combinations. Due to the presence of odd-numbered acyl chains the resolution between molecular species is quite small. However, 14:0/18:1 elutes after 14:0/16:0-d_{31} and 14:0/16:0 and the same behaviour is observed for the 15:0 and the 16:0 combinations, respectively. Furthermore, a separation is observed between the 16:0-d_{31}/18:1 (peak 14) and 16:0/18:1 (peak 15) combinations. The molecular species with the perdeuterated acyl chain elutes ahead of the corresponding protonated molecular species. Although fractionated together, separation of the 16:0-d_{31} and 16:0 combinations is observed between

Table 1
Fatty acids and molecular species from *Acholeplasma laidlawii* DGlcDAG

Fraction	Peak	Fatty acids [area (%)]											Area (%)	Tentative molecular species	PN
		10:0	12:0	13:0	14:0	15:0	16:0-d_{31}	16:0	17:0	18:1	18:2	20:1			
Total[a]		0.2	0.7	1.0	5.2	3.3	14.0	14.2	1.1	57.0	0.3	2.1			
1	1	9.3	17.7	2.6	9.0	3.4	14.2	11.0		21.1		11.6	0.3	14:0/14:0	28
	1													12:0/16:0-d_{31}	28
	1													12:0/18:1	28
	1													12:0/18:1	28
	1													10:0/20:1	28
2	2			26.2	12.1	11.6	18.2	16.4		10.9			0.2	14:0/15:0	29
	2													13:0/16:0-d_{31}	29
	3												0.4	13:0/16:0	29
	4												0.1	13:0/18:1	29
3	5				43.8	1.9	26.8	21.1		2.1			1.8	14:0/16:0-d_{31}	30
	6												1.2	14:0/16:0	30
4	7				45.2					54.8			2.1	14:0/18:1	30
5	8				9.9	27.2	17.8	19.5	3.0	14.1	6.0		0.2	15:0/16:0-d_{31}	31
	9												0.4	15:0/16:0	31
	9													17:0/18:2	31
6	10					16.1	41.9	20.4		20.5			2.1	15:0/18:1	31
	11												1.7	16:0-d_{31}/16:0-d_{31}	32
	12												1.3	16:0/16:0-d_{31}	32
	13												0.4	16:0/16:0	32
7	14						41.5	6.7		50.9			15.2	18:1/16:0-d_{31}	32
8	15						8.7	38.6		52.8			22.6	18:1/16:0	32
9	16									98.5			47.6	18:1/18:1	32
10	17							1.9	25.2	72.9			0.2	16:0/17:0	33
	18												0.6	18:1/17:0	33
n.f.[b]	19												0.1	17:0/17:0	34
	20												0.2	18:0/16:0-d_{31}	34
	21												0.2	18:0/16:0	34
	22												0.3	18:0/18:1	34
	23												1.1	20:1/18:1	34

[a] Values are given in area (%), discrepancies are noted between the observed amounts of fatty acids by GC and the calculated amounts from HPLC, probably due to different detection modes.
[b] Not fractionated.

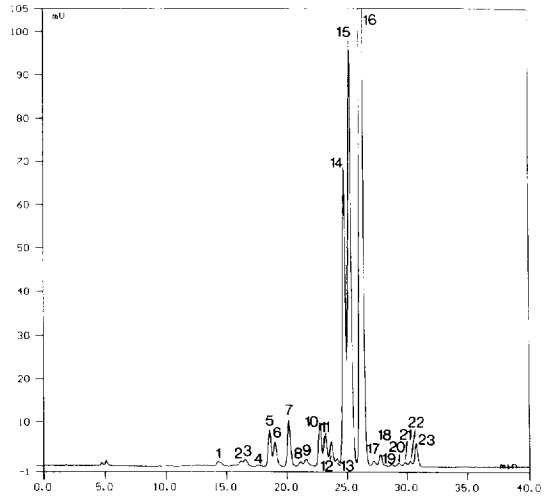

Fig. 2. Reversed-phase HPLC chromatogram of *Acholeplasma laidlawii* molecular species. Fractions representing one or more molecular species were collected as indicated in Table 1.

peaks 2 and 3, peaks 5 and 6, peaks 8 and 9 and peaks 11, 12 and 13.

The last five components were not fractionated and subjected to gas chromatography. Instead, a curve was fitted from molecular species combinations of 18:1 and several saturated fatty acids and their respective retention times (Fig. 3). Extrapolation of the curve gave a retention time of 30.4 min for the 18:0/18:1 combination. This retention time

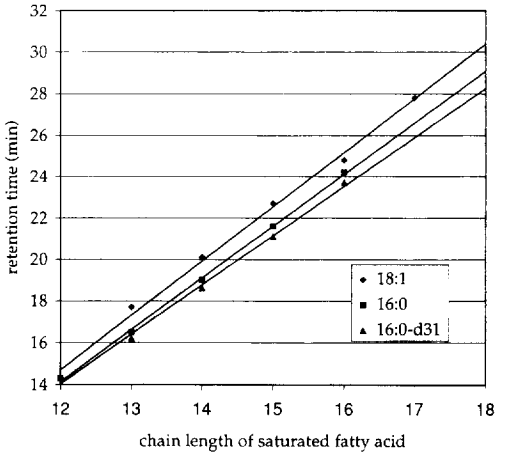

Fig. 3. Relationship between molecular species combinations containing the fatty acids 18:1, 16:0 and 16:0-d_{31} and retention time.

was compared with the retention times of the peaks shown in Fig. 2. Peak 22 had a retention time at 30.36 min, thereby being tentatively identified as the 18:0/18:1 combination. The 20:1/18:1 combination, if present, should elute just after 18:0/18:1, suggesting peak 23 as the 20:1/18:1 component.

The RP-HPLC system was used in conjunction with a mass spectrometer equipped with a particle beam interface. The identification of molecular species is facilitated by comparing fragment ions, e.g. diglyceride, monoglyceride and carbonyl ions (Table 2). For example, combinations of molecular species containing 16:0-d_{31} produce ions that are 31 m/z higher than the ions of molecular species containing 16:0 and are therefore readily recognised.

A comparison of the retention times of molecular species from 1,2-diacyl-3-O-[α-D-galactopyranosyl-(1→6)-O-β-D-galactopyranosyl]-*sn*-glycerol (DGalDAG) isolated from oats was done [22]. The 16:0/18:1 and 18:1/18:1 molecular species from DGlcDAG eluted several minutes after the corresponding 16:0/18:1 and 18:1/18:1 molecular species from DGalDAG. This elution behaviour indicates that DGlcDAG is less polar than DGalDAG.

Chromatography of the two glycoglycerolipid classes on a polar diol-phase column confirmed the observation from the RP-HPLC experiment. DGlcDAG eluted several minutes ahead of DGalDAG (Fig. 4). The conformations of the hydroxyl groups in the two glycoglycerolipids are different. The hydroxyl groups in DGlcDAG are all equatorial, whilst the epimeric DGalDAG has axial hydroxyl groups in the C-4 positions [28]. The availability of the hydroxyl groups for interactions with the polar stationary phase is thus enhanced for DGalDAG with a higher retention time as a result. The two glycoglycerolipids also differ in their anomeric configuration, α-, α- and β-, α- configurations for DGlcDAG and DGalDAG, respectively. The carbohydrate moieties may thus have different orientations in the elution media with a concomitant retention behaviour for the glycoglycerolipids.

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Table 2
Observed mass spectrometry fragmentation of DGlcDAG molecular species from *Acholeplasma laidlawii*

Peak no.	*m/z* of fragment ion					Proposed R_1CO/R_2CO[b]
	$[M-DGlc]^+$	$[M-DGlc-R_1CO]^+$	$[M-DGlc-R_2CO]^+$	$[R_1CO]^+$	$[R_2CO]^+$	
5	554	344	285	211	270	14:0/16:0-d_{31}
6	523	313	285	211	239	14:0/16:0
7	549	339	285	211	265	14:0/18:1
8	568	n.d.[a]	299	225	270	15:0/16:0-d_{31}
9	537	313	299	225	n.d.	15:0/16:0
10	563	339	299	225	265	15:0/18:1
11	609	344	344	270	270	16:0-d_{31}/16:0-d_{31}
12	582	344	313	239	270	16:0/16:0-d_{31}
13	551	313	313	239	239	16:0/16:0
14	608	339	344	270	265	16:0-d_{31}/18:1
15	577	339	313	239	265	16:0/18:1
16	603	339	339	265	265	18:1/18:1
18	591	339	327	253	265	17:0/18:1

[a] Not detected.
[b] The *sn*-positions of R_1 and R_2 are not determined.

discussions and for supplying the *Acholeplasma laidlawii* lipid preparation and to Mr. Ralf Nilsson, Department of Plant Physiology, University of Göteborg for the collection and interpretation of mass spectrometric data. Financial support from the Swedish Council for Forestry and Agricultural Research (SJFR) is also acknowledged.

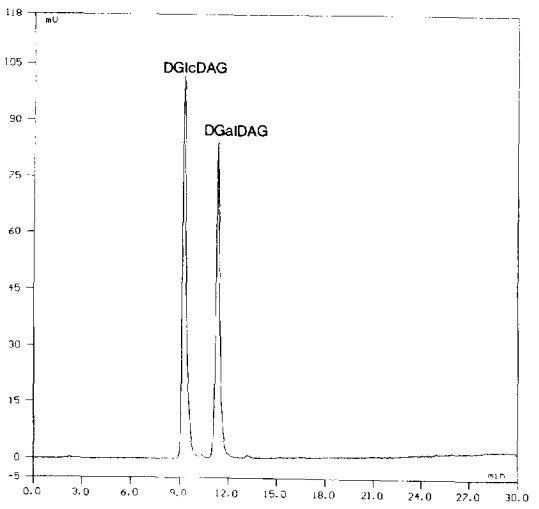

Fig. 4. Adsorption HPLC chromatogram of DGlcDAG from *Acholeplasma laidlawii* and DGalDAG from oats.

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