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Improved Method for the Isolation of Biosurfactant Glycolipids from *Rhodococcus* sp. Strain H13A

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An improved method for the isolation of the biosurfactant glycolipids from *Rhodococcus* sp. strain H13A by using XM 50 diafiltration and isopropanol precipitation was devised. This procedure was advantageous since it removes protein coisolated when the glycolipids are obtained by organic extraction and silicic acid chromatography. The protein apparently does not contribute any biosurfactant characteristics to the glycolipids. The deacylated glycolipid backbone included only a disaccharide.

Rhodococcus sp. strain H13A (previously named *Arthrobacter* sp. strain H13A) degrades hexadecane and produces exocellular glycolipids, one or more of which are biosurfactants. The glycolipids are isolated from culture supernatant by extraction with ethyl acetate-methanol and silicic acid chromatography (5). Analysis of the glycolipid fraction from the silicic acid column by thin-layer chromatography (TLC) reveals one major component (~90%) and several minor components. This isolation procedure results in protein being coisolated with the glycolipids (11). The protein was believed to be integral to the biosurfactant. This work describes an improved method for isolating the biosurfactant glycolipids by using diafiltration and isopropanol precipitation. This method removes the protein coisolated by organic extraction and silicic acid chromatography with no loss of biosurfactant capacity from the glycolipids.

Rhodococcus sp. strain H13A was grown on *n*-hexadecane as previously described (5). Cultures were centrifuged at 5,000 × *g* for 20 min. The supernatant was filtered through a 1.2-μm-pore-size Millipore filter by vacuum filtration and concentrated by evaporation in a rotoevaporator. This filtrate (50 ml) was fractionated by diafiltration with 2 liters of 50 mM phosphate buffer (pH 7) on a 43-mm-diameter XM 50 membrane filter from Amicon (Danver, Mass.). The diafiltration equipment was obtained from Amicon and included an ultrafiltration device with a connecting reservoir that added solvent to the retentate as the filtrate was removed. The biosurfactant was detected in the retentate (11 ml). The retentate was precipitated with isopropanol overnight at 8°C to remove carbohydrate (retentate:isopropanol ratio, 1/2). The precipitated solution was centrifuged at 3,000 × *g* for 10 min and decanted. The supernatant, containing the biosurfactant, was rotoevaporated to dryness and resuspended in 50 mM phosphate buffer (pH 7.0) to 11.6 ml. The biosurfactant glycolipids were also isolated by organic extraction and silicic acid chromatography for comparison (5, 12).

Interfacial tension (IFT) was measured by the drop-weight method against dodecane (6). Total carbohydrate was measured by the anthrone method (3); glucose was used as the standard. Protein was estimated by the method of Lowry et al. (8) by using bovine serum albumin as the standard. TLC was performed with Redi Plates Silica Gel G from Fisher Scientific Co. (Pittsburgh, Pa.). The solvent system used to analyze glycolipids was chloroform–methanol–5 N NH_4OH (65/30/5, vol/vol/vol) (9). The solvent system used to analyze the deacylated glycolipid backbone was *n*-propanol–ethyl acetate–water (65/10/25, vol/vol/vol) (9). Plates were analyzed for the presence of carbohydrate by being sprayed with a solution of 200 mg of orcinol in 100 ml of 75% sulfuric acid, followed by heating at 105°C for 10 min (4). Ninhydrin analysis for detection of amino acids and proteins was performed by spraying of TLC plates with a solution containing 0.2% ninhydrin and 2% pyridine in acetone, followed by heating at 105°C for 2 to 3 min (9). All chemicals were of analytical grade.

The biosurfactant glycolipids were saponified with 0.2 N NaOH for 30 min at 80°C, acidified to pH 7, and extracted with diethyl ether twice to remove the fatty acid (5). The aqueous fraction was rotoevaporated to dryness, and the deacylated glycolipid backbone was redissolved in a known volume of 100 mM phosphate buffer (pH 7). The backbone was applied to a P-2 Bio-Gel column (2.75 cm [internal diameter] by 100 cm) (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 100 mM phosphate buffer (pH 7) to estimate the size.

The characteristics of fractions of the biosurfactant glycolipids isolated by diafiltration from *Rhodococcus* sp. strain H13A are listed in Table 1. Steps 1 to 3 are the biosurfactant glycolipids obtained by organic extraction and silicic acid chromatography (step 1) and subsequently diafiltered on an XM 50 membrane (steps 2 and 3). The biosurfactant remained in the retentate, as indicated by the IFT values, whereas coisolated protein or amino acids were extruded into the filtrate. Steps 4 to 8 are the glycolipids obtained from culture supernatant by using only diafiltration and isopropanol precipitation. Steps 5 and 6 indicate that protein was extruded through the XM 50 membrane, although the biosurfactant glycolipids remained in the retentate. Isopropanol precipitation of the diafiltration retentate removed the remaining protein and carbohydrate (steps 7 and 8). The biosurfactant remained in the supernatant, as indicated by the IFT values.

Figure 1 is a TLC plate developed with the solvent system to chromatograph glycolipids. Lanes 1 through 8 correspond to steps 1 through 8, respectively, of Table 1. The isolation of the glycolipids by organic extraction and silicic acid chromatography (lane 1), compared with isolation by diafiltration and isopropanol precipitation (lanes 2, 5, and 7), did not result in the loss of an orcinol-positive spots. Carbohy-

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TABLE 1. Isolation of biosurfactant glycolipids from *Rhodococcus* sp. strain H13A by XM 50 diafiltration and isopropanol precipitation

Procedure and step	IFT (mN/m)	Anthrone (mg)	Protein (mg)
Organic extraction			
1. Glycolipids[a]	~1.0	14.28	8.4
2. XM 50 retentate	1.0	9.25	0.0
3. XM 50 filtrate	4.4	0.67	7.4
Culture supernatant			
4. Supernatant[b]	4.4	25.0	16.5
5. XM 50 retentate[c]	<1.0	15.4	1.4
6. XM 50 filtrate	9.2	9.35	13.7
7. Isopropanol supernatant	~1.0	8.93	0.0
8. Isopropanol precipitate	13.0	4.4	1.05

[a] Obtained by organic extraction and silicic acid chromatography. Samples were dried under N_2 and redissolved in 12 ml of 50 mM phosphate buffer (pH 7) prior to diafiltration (steps 2 and 3).
[b] After vacuum filtration and rotoevaporation.
[c] Precipitated with isopropanol (steps 7 and 8).

drate, which develops at the origin of each lane if present, was apparently removed by isopropanol precipitation since it was absent in lane 7.

Staining the TLC plate with ninhydrin, as in Fig. 1 (data not shown), indicated no protein or amino acids in lanes 2 or 7, which is consistent with the Lowry protein analysis in Table 1. The critical micelle concentration curves of the biosurfactant glycolipids isolated by either organic extraction or diafiltration were identical (data not shown). Addition of concentrated filtrate to the retentate of the XM 50 diafiltration did not lower the IFT of the retentate. Addition of resuspended precipitate to the supernatant from the isopropanol precipitation did not lower the IFT of the supernatant.

The deacylated glycolipid backbone was developed on a TLC plate with *n*-propanol–ethyl acetate–water (data not shown). Regardless of the concentration of the backbone applied to the plate, only one orcinol-positive spot developed, moving to the same R_f point as authentic disaccharide (trehalose) (5). The deacylated backbone eluted as a single peak from the standardized P-2 Bio-Gel column at a position consistent with a disaccharide, as determined by anthrone

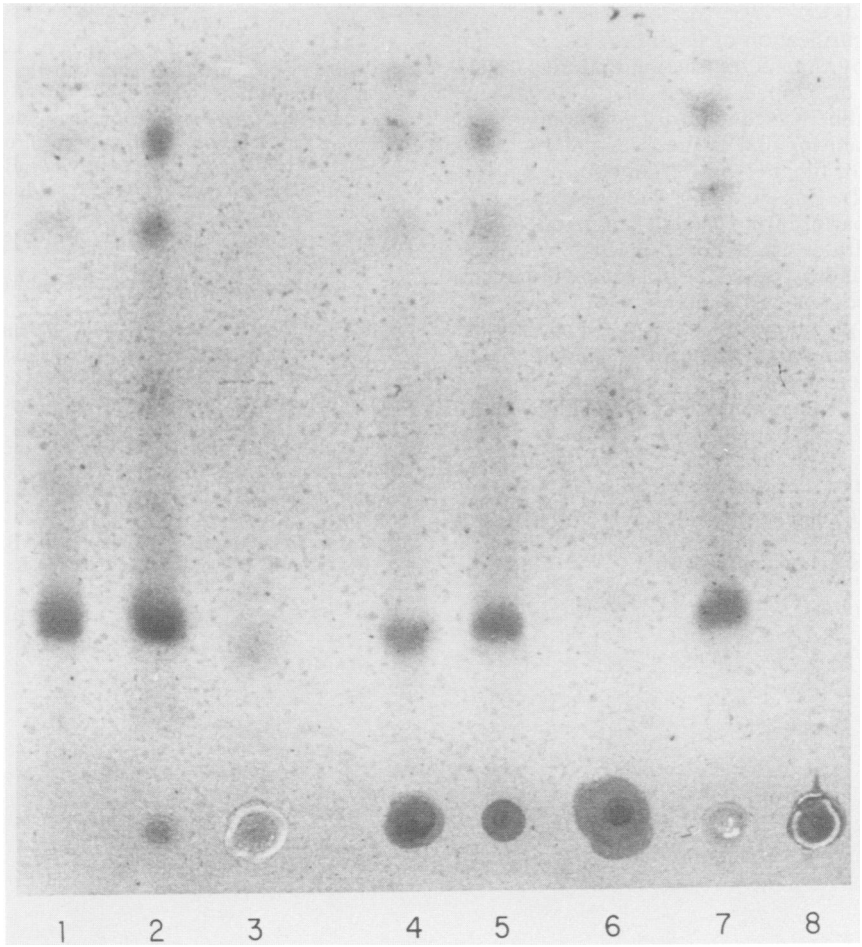

FIG. 1. TLC of biosurfactant glycolipids obtained from *Rhodococcus* sp. strain H13A. Lanes 1 to 8 correspond to steps 1 to 8, respectively, of Table 1. Chromatography was conducted with chloroform-methanol-hydroxylamine (65/30/5, vol/vol/vol). Each lane was spotted with 10 to 100 μl to reflect approximately 25 μg of anthrone in each lane. The TLC plate was analyzed by being sprayed with a solution of 200 mg of orcinol in 100 ml of 75% sulfuric acid, followed by heating at 105°C for 10 min. Carbohydrate remains at the origin. Glycolipids are chromatographed with the solvent front.

analysis. Apparently only disaccharide composes the glycolipid backbone of the biosurfactant. Detailed chemical and physical characteristics of the biosurfactant glycolipids from *Rhocococcus* sp. strain H13A will be published subsequently (William Finnerty, personal communication).

Prior to diafiltration or saponification, the glycolipids obtained by organic extraction and silicic acid chromatography were dried under a stream of N_2 to remove the organic solvent and were then suspended in 12 ml of 50 mM phosphate buffer (pH 7) (IFT = 1.96 mN/m). The disaccharide concentration of the resuspended glycolipids was 4.05 μmol/ml. (Anthrone estimation was adjusted to reflect a disaccharide. Trehalose was used for comparison to ensure accuracy.) The protein concentration of the resuspended glycolipids was 0.082 mg of amino acid residue per ml or 0.68 μmol of amino acid residue per ml, assuming 120 as the average M_r. The disaccharide-to-amino acid ratio was not sufficient to account for even 1 μmol of amino acid per micromole of glycolipid backbone.

The biosurfactant glycolipids from *Rhodococcus* sp. strain H13A apparently are not novel (14). Diglycosyl glycolipids are the most typical glycolipids found in microorganisms. Trehalose-containing glycolipids are common in exocellular glycolipids involved in the uptake of hydrophobic substrates. Protein is not a component of any other reported biosurfactant glycolipids.

The procedure for purification of neutral lipids, glycolipids, and polar lipids by organic extraction and silicic acid chromatography was developed during the 1960s. These techniques are still routinely used to purify lipid components from a variety of organisms and tissues, including actinomycetes (1), maize (10), fungi (7), rat brain (13), and pheochromocytoma cells (2). Consequently, the use of diafiltration is a significant departure from established practices. All proteins and carbohydrates are removed by diafiltration on an XM 50 membrane and by isopropanol precipitation. The isolation procedure does not result in any loss of biosurfactant capacity of the glycolipids. The XM 50 membrane apparently retains glycolipids, presumably allowing low-molecular-weight protein to be extruded. The isopropanol precipitation removes the remaining protein. Although the complete specificity of such membranes is beyond the scope of this communication, given the simplicity of this technique it may prove adaptable to separate or isolate lipids, glycolipids, or membrane components. The method also affords the capacity to purify or concentrate lipids, glycolipids, or membrane components anaerobically.

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