EMULSIFIER OF *ARTHROBACTER* RAG-1: DETERMINATION OF EMULSIFIER-BOUND FATTY ACIDS

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1. Introduction

Growth of the hydrocarbon degrading bacterium, *Arthrobacter* sp. RAG-1, is accompanied by emulsification of the hydrocarbon substrate in the growth medium [1]. Emulsification is due to the production of an extracellular lipopolysaccharide with av. mol. wt 9.8×10^5 [2]. The major components of the polymeric emulsifier are D-galactosamine, an unidentified amino uronic acid, and fatty acids joined to the heteropolysaccharide backbone primarily through *O*-ester linkages [2]. The emulsifier-bound fatty acids appear to play an important role in the function of the polymer since:

(i) Removal of part of the fatty acids by mild alkaline hydrolysis caused a decrease in emulsifying activity;

(ii) Emulsifiers isolated from RAG-1 growing on hexadecane as the sole source of carbon and energy contained lower *O*-ester content and reduced specific activity [3].

In order for the *Arthrobacter* emulsifier to induce efficiently hydrocarbon in water emulsions, the hydrocarbon substrate must contain both aliphatic and cyclic components [4]. A better understanding of the structure–function relationships governing biologically produced emulsifiers requires a detailed characterization of the bioemulsifier. Here the major *O*-esters of the emulsifier are identified.

2. Experimental

Arthrobacter emulsifier was prepared from the culture supernatant fluid of ethanol-grown cells by the ammonium sulfate fractionation procedure as in [5]. Unless stated otherwise, experiments were performed with a single batch of emulsifying agent, referred to as A-4, prepared from 40 l culture fluid. Deproteinized emulsifier, A-4-W, was prepared from A-4 by the phenol method [6]. Emulsifier EF-RAG(HD)-W was described in [2]. Isolation of the mutant, *Arthrobacter* RAG-1-92-10, will be described elsewhere. Emulsifier isolated from the culture supernatant fluid of ethanol-grown cells of 92-10 is referred to as EF-RAG-92-10.

Fatty acid methyl ester standards were obtained from Packard Instr. Dodecanoic acid was obtained from Fluka Co. 3-Hydroxy fatty acids were obtained from D. Nelson. Methyl 2-hydroxydodecanoate was synthesized in the following manner: 1.6 g bromine (0.01 mol) in 5 ml benzene was added dropwise during 10 h to a solution of 2 g dodecanoic acid (0.01 mol) in 10 ml oxalyl chloride, under gentle reflux. After refluxing for an additional 10 h, excess oxalyl chloride and benzene were removed by distillation: the oily residue was poured on 50 ml absolute methanol, yielding methyl 2-bromododecanoate. The methyl 2-bromododecanoate and small amounts of unreacted methyl dodecanoate were dissolved in 25 ml of 90% formamide and the solution refluxed for 24 h. GLC analysis indicated that all the 2-bromoester were converted to the 2-hydroxyester. Upon addition of water, followed by extraction with ether and drying over $MgSO_4$, an etheral solution of methyl 2-hydroxydodecanoate, containing some methyl dodecanoate, was obtained.

Fatty acids were prepared from A-4 both by acid and base hydrolysis. For acid hydrolysis 1 g A-4 was hydrolyzed for 4 h in 100 ml 6 N HCl at 100°C under nitrogen. After cooling the fatty acids were extracted with ether (6 × 100 ml) and the ether solution dried over $MgSO_4$. Removal of the ether left 200 mg of an oil (20% yield) consisting primarily of a mixture of fatty acids and small quantities of ether-soluble polysaccharide degradation products.

Base hydrolysis of 1 g A-4 was performed in 400 ml 2.5% KOH in 90% methanol under reflux for 4 h. After removal of the methanol in vacuo, 500 ml water were added. The clear alkaline solution was washed 3 times with 150 ml ether, the ether discarded and the aqueous solution acidified to pH 2 with HCl. The acid solution was then extracted 5 times with 100 ml ether. The interphase that formed during each extraction was set aside. The combined interphase fractions were treated with acetone to precipitate protein and polysaccharide. After removal of the precipitate by filtration and the acetone by distillation in vacuo, the aqueous phase was again extracted with ether. The combined ether extracts were dried over $MgSO_4$. Removal of the ether left 130 mg (13% yield) of a mixture of fatty acids.

Fatty acid methyl esters were prepared by treating fatty acid mixtures (50 mg) dissolved in dry (KOH-treated) distilled ether (30 ml) with diazomethane. The diazomethane in ether solution was added slowly at room temperature until N_2 evolution ceased and the yellow colour persisted. After an additional 15 min at room temperature, the ether was removed by a stream of N_2.

Infrared, NMR and mass spectra were performed with Perkin-Elmer grating spectrometer, Model 337, Jeol JNM-C-60 HL spectrometer and Hitachi Perkin-Elmer RMU-6 instrument, electron energy 70 eV, respectively. Gas–liquid chromatography (GLC) was performed on a Packard Becker instrument, Model 417, equipped with a FID detector and recorded on a Packard Unicorder U-125. A 6 ft, 1/8 in diam. column filled with 15% DEGS on chrom-W 80/100 mesh was used. The following isothermal conditions were employed: column temperature, 170°C; injector and detector temperatures, 220°C; N_2 carrier, 14 p.s.i.; chart speed, 10 mm/min; fatty acid 1% in $CHCl_3$. Column chromatography was performed on a silica-gel Kieselgel Woelm 0.032–0.063 mm column, employing petroleum ether–ethyl acetate as solvents.

3. Results and discussion

Emulsifier A-4 contained 0.65 μmol *O*-bound ester/mg as determined by the hydroxamic acid test [2]. The dry weight of the mixture of fatty acids recovered from A-4 following alkaline hydrolysis, acidification and ether extraction was 13%. By combining the weight recovery of fatty acids and the *O*-bound ester content, an average equivalent weight of 200 for the fatty acids was determined. The infrared spectrum of the fatty acid mixture revealed the following absorption peaks:

$3610\ cm^{-1}$ (nonbonded O–H); $3500\ cm^{-1}$ (bonded O–H); $1705\ cm^{-1}$ (C=O); $1050\ cm^{-1}$ (C–OH).

The NMR spectrum in $CdCl_3$ indicated that the mixture consisted mainly of saturated and hydroxy-saturated fatty acids. From the ratio of methyl protons to total protons, an average chain length of C_{10}–C_{12} was estimated, in good agreement with the calculated equivalent weight of 200. Gas–liquid chromatography of the methyl esters of the fatty acid mixture led to the separation of 11 peaks (table 1).

Seven of the eleven fatty acid methyl esters were identified by comparing their retention volumes with standard mixtures of fatty acid methyl esters. Their identities were checked both on a DEGS column and a 10% SE-30 chromosorb-WHP 80–100 column. The retention volumes of the two major peaks, referred to as acids A and B, did not correspond to any of the

Table 1
Fatty acid methyl esters obtained from emulsifier A-4

Fatty acid methyl ester[a]			Relative retention volume
1.	Decanoic	C-10:0	0.17
2.	Dodecanoic	C-12:0	0.29
3.	Dodecanoic	C-12:1	0.34
4.	Unidentified		0.48
5.	Unidentified		0.61
6.	Hexadecanoic	C-16:0	1.00
7.	Hexadecenoic	C-16:1	1.14
8.	Acid A		1.30
9.	Acid B		1.69
10.	Octadecanoic	C-18:0	1.94
11.	Octadecenoic	C-18:1	2.16

[a] Fatty acid methyl esters were prepared from A-4 by alkaline hydrolysis, ether extraction and diazomethane treatment as in section 2

retention volumes of saturated and unsaturated fatty acid methyl esters from C_{10}–C_{21}. The retention volumes of the methyl esters of acids A and B on a SE-30 column were lower than on the more polar DEGS column, strongly suggesting that both acids A and B are hydroxy fatty acids.

Acid B was isolated from emulsifier A-4 following acid hydrolysis. Preliminary experiments had indicated that acid A was destroyed by the strong acid conditions, thereby simplifying the isolation of acid B. The mixture of fatty acid methyl esters (100 mg) was subjected to column chromatography on Kieselgel Woelm 0.032–0.063 mm using petroleum ether with increasing concentrations of ethyl acetate for development. The 20% v/v ethyl acetate fraction yielded pure acid B methyl ester (as observed by GLC). The structure assignment of acid B as methyl 3-hydroxydodecanoate was based on the following. The infrared spectrum in $CHCl_3$ showed adsorption maxima at:

3640 cm^{-1} (O–H); 1730 cm^{-1} (C=O); 1070 cm^{-1} (C–OH).

The NMR spectrum in $CdCl_3$ showed peaks at:

δ 0.85 (3 H–CH_3); 1.25 (16 H, 8 × CH_2); 2.40 (2 H, CH_2–$COOCH_3$); 3.58 (1 H, H–C–OH); 3.68 (3 H, $COOCH_3$); 3.80 (1 H, H–C–OH).

The mass spectrum showed no apparent peak at *m/e* 240; the strongest peak was at *m/e* 103, which is characteristic of methyl 3-hydroxyesters [7]. Finally, a small standard of 3-hydroxydodecanoic acid was methylated and shown to cochromatograph with acid B (on GLC). Acid A was identified as 2-hydroxydodecanoic acid by comparison with the compound synthesized by bromination and hydrolysis. When injected into the gas chromatograph, methyl 2-hydroxydodecanoate showed the same retention volume as that of acid A methyl ester. Furthermore, the synthetic 2-hydroxydodecanoate methyl ester cochromatographed with acid A methyl ester when injected together into the gas chromatograph.

The fatty acid compositions of 3 emulsifier preparations are shown in table 2. In addition to having different overall *O*-ester contents, the emulsifiers differed in the relative amounts of individual fatty acids. Emulsifier A-4-W, obtained from a wild-type culture grown with ethanol as the growth substrate, was the most active emulsifier, had the highest overall *O*-ester content (0.37 µmol/mg), and relatively high concentrations of 3-hydroxydodecanoic and dodecanoic acids. The C-12 acids represent 75% of the total *O*-esters of A-4-W. When the same strain was grown on hexadecane as a growth substrate, the resulting emulsifier EF-RAG(HD)-W, exhibited a much lower emulsifying activity (not shown), reduced total *O*-ester content (0.10 µmol/mg), and lower concentra-

Table 2
Estimation of the fatty acid compositions of *Arthrobacter* RAG-1 emulsifiers

Fatty acid[b]	Emulsifier[a] A-4-W (% total)	 EF-RAG(HD)-W (% total)	 EF-RAG-92-10 (% total)
C-10:0	11.4	16.1	8.5
C-12:0	23.0	16.9	14.2
C-12:1	2.4	3.4	1.5
C-16:0	0.7	trace	16.8
C-16:1	trace	trace	6.8
C-18:0	0.3	trace	4.0
C-18:1	trace	trace	9.6
3-Hydroxydodecanoic	39.5	22.4	9.9
2-Hydroxydodecanoic	10.5	18.6	10.0
Unidentified	12.0	22.0	18.2

[a] Emulsifiers A-4-W, EF-RAG(HD)-W and EF-RAG-92-10 contained 0.37 µmol/mg, 0.10 µmol/mg and 0.18 µmol/mg *O*-ester, respectively

[b] Fatty acid methyl esters were prepared by alkaline hydrolysis, ether extraction and diazomethane treatment as in section 2

tions of 3-hydroxydodecanoic and dodecanoic acids. Emulsifier isolated from EF-RAG-92-10 growing on ethanol had very low emulsifying activity, low *O*-ester content (0.18 μmol/mg) and greatly reduced quantities of dodecanoic and 3-hydroxydodecanoic acids. In fact, the C-12 acids of EF-RAG-92-10 represent only 34% of the total acids in the preparation. Although correlation is not proof, the data do suggest that both the total amounts and distributions of fatty acids in the emulsifier preparations play an important role in their emulsifying activities. At least quantitatively, 3-hydroxydodecanoic acid is the most important fatty acid in the active emulsifier preparations. In addition to the well characterized hydroxy fatty acids of *Pseudomonads* [8] and other Gram-negative bacterial lipopolysaccharides [9,10] have isolated from the culture fluids of a *Pseudomonas* sp., two complex glycolipids:

2-*O*-α-decenoyl-α-L-rhamnosyl-β-hydroxydecanoly-3-hydroxydecanoic acid

and

2-*O*-(2-*O*-α-decenoyl-α-L-rhamnosyl)-α-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoic acid.

These two glycolipids have been reported to be useful in activated sludge beds for the emulsification of hydrocarbons [11].

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