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ORIGINAL ARTICLE

Glycoprotein emulsifiers from two marine *Halomonas* species: chemical and physical characterization

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Abstract

Aims: To partially purify and characterize bioemulsifiers produced by two new marine *Halomonas* species, TG39 and TG67, and to compare their emulsifying activities with those of commercial emulsifiers.

Methods and Results: The production of emulsifiers HE39 and HE67 was achieved from glucose-supplemented marine broth, and recovered by cell removal, concentration by ultrafiltration, precipitation with salt and ethanol, and lyophilization. Purification and chemical analysis revealed both emulsifiers to be glycoproteins of high molecular weight with a notably high content of protein and uronic acids. Physical characterization showed both glycoproteins to effectively emulsify a wide range of food oils under both neutral and acidic pH conditions and withstand acid and high temperature treatment.

Conclusions: The emulsifying activities of these two new glycoprotein emulsifiers were comparable and, under certain conditions, superior to those produced by commercial emulsifiers tested (xanthan gum, gum arabic and lecithin). They show the highest reported emulsifying activities derived from a *Halomonas* species.

Significance and Impact of the Study: These strains, and the emulsifiers produced, appear to be promising candidates for further development in applications requiring emulsifiers that are natural and compatible to the existing commercial emulsifiers.

Introduction

Surface-active agents are amphipathic molecules (i.e. consist of both hydrophobic and hydrophilic domains) that are essential components in a wide range of products and applications (Desai and Banat 1997; Banat *et al.* 2000). A large proportion of the total global production of these compounds, which exceeds three million tonnes per annum, is based almost entirely on organochemical synthesis from hydrocarbons (Banat *et al.* 2000). This is problematic because they are derived from a nonrenewable resource, and there are concerns about their environmental impact and potential health risks. Generally, synthetically-derived products are often associated with higher toxicities, poorer biodegradability and lower functional diversity as compared with their biologically-derived counterparts (Desai and Banat 1997). Conversely, the products of biological origin, biosurfactants and bioemulsifiers, have received increasing interest, mainly fuelled by changing government legislation requiring a shift towards industrial use of renewable and less toxic compounds, and an increasing consumer demand for natural and 'environment-friendly' ingredients (Weiner 1997; Banat *et al.* 2000). Microbial production of surface-active agents offers a sustainable and potentially cost-effective alternative to chemical synthesis (Rosenberg 1993; Shepherd *et al.* 1995; Weiner 1997).

One important class of natural surface-active agents with significant industrial application is biopolymeric emulsifiers, such as the bioemulsans (Rosenberg and Ron 1997). These are high molecular weight compounds with proven large-scale industrial production and commercial applicability, and are particularly attractive because of the

relative ease by which they can gain health clearance for use in the food, cosmetic or pharmaceutical sector (Hasenhuettle and Hartel 1997; Garti 1999). Their high molecular weight is suggested to endow them with structural and functional heterogeneity, possibly because of a high number of reactive groups per molecule, which allows them to adhere well to oil droplets during emulsification and provide steric stability to the emulsions formed (Rosenberg and Ron 1997). Such interactions play a significant role in the formation, structure and stabilization of emulsions, particularly in processed foods (Garti and Leser 1999; Bach and Gutnick 2005). As a result of modern developments in industrial processing technologies, there is an increasing demand for new types of biopolymers with novel or enhanced functionalities. New microbial isolates, particularly of a marine origin, offer a relatively underexploited resource (Weiner 1997).

In this report, we describe the identification and partial purification of two novel high molecular weight glycoprotein emulsifiers produced by two marine halomonads and characterize some of their chemical and physical properties. These emulsifiers appear to be the most effective emulsifying and stabilizing agents isolated from a *Halomonas* species to date, and are comparable to commercially available hydrocolloid emulsifiers.

Materials and methods

Isolation, growth, identification and screening

Bacteria were isolated on solid synthetic seawater medium (SSW) (Passeri *et al.* 1992) supplemented with NH_4NO_3 as the nitrogen source. Isolation was performed by spread-plating ten-fold serial dilutions of marine water samples onto SSW, with *n*-hexadecane supplied via the vapour phase as the sole carbon source. Colonies were grown in the dark at 28°C for 3 weeks. Isolates displaying distinct colony morphologies were streaked onto ZM/10 agar and stored at −80°C in ZM/1 broth supplemented with 20% glycerol. Both ZM/10 and ZM/1 media were prepared as previously described by Green *et al.* (2004).

Two bacterial isolates, strains TG39 and TG67, were selected because of their production of high emulsifying activity during growth in ZM/1 broth amended with glucose at 1% (w/v) concentration. During growth, samples were taken periodically for emulsification and tensiometric assays. Both whole-broth cultures and cell-free supernatants (13 000 ***g***; 10 min) were assayed. Growth was monitored by spectrophotometric measurement (540 nm) of whole culture broth using a Nicolet Evolution 300 spectrophotometer (Thermo Electron Corporation, USA). Growth experiments were repeated at least three times, and all analyses were performed in triplicate. Strains TG39 and TG67 were identified by DNA sequencing of their 16S rRNA genes amplified by PCR using oligonucleotide primers 27f and 1492r (Weisburg *et al.* 1991) as described by Green *et al.* (2004).

Production and extraction of emulsifiers

TG39 and TG67 were grown in 13 2-l Erlenmeyer flasks containing 770 ml of ZM/1 medium amended with glucose (1% w/v) and incubated (28°C; 150 rev min^{-1}) for 35–40 h at which point the cultures were pooled together. The emulsifying fraction was then separated from the biomass by cross-flow filtration (0·2 μm; Schleicher & Schuell, Dassel, Germany). The cell-free permeate was then passed through a 100 kDa membrane cassette (Schleicher & Schuell) and the retentate dialysed with approx. 5 l of distilled water. The emulsifiers were then precipitated using KCl (7·5% w/v) and 3–4 volumes of cold 99% ethanol. The precipitate was allowed to settle overnight at 10°C, recovered by centrifugation, and subsequently dialysed against distilled water and lyophilized. The resultant dried material was used in all subsequent chemical and physical characterization experiments.

Emulsification assays

Emulsifier production was determined using a modified version of the method described by Cooper and Goldenberg (1987). Samples of culture broth, with cells removed (13 000 ***g***; 5 min), were mixed with an equal volume of *n*-hexadecane in acid-washed (0·1 N HCl) screw-cap glass tubes (100 × 13 mm), manually shaken (15 s) and vortexed at 2200 rev m^{-1} (15 s) to homogeneity, left to stand for 10 min, shaken as before, and the height of the emulsion layer (EI_{24}) measured after allowing the mixture to stand for 24 h at 21°C. The same assay was used to measure the EI_{24} produced by solutions of the extracted emulsifiers (HE39 and HE67) against *n*-hexadecane.

The ability of the extracted emulsifiers to form oil-in-water emulsions was based on a modified version of the method described by Cirigliano and Carman (1984). For this, a 2·5-ml aqueous solution of the test emulsifier (0·02% w/v) was mixed with 0·4 ml of the test oil in the same way as described previously. The solution was allowed to stand for 10 min prior to measuring the turbidity of the bottom aqueous layer at 540 nm, and every 10 min thereafter for up to 60 min. The log of these values was plotted against time, and the slope of the curve generated was expressed as the decay constant, K_d. This term describes the stability of the emulsions formed, as previously described by Cirigliano and Carman (1985). A final absorbance reading was recorded after allowing the emulsion to stand undisturbed at room temperature for 24 h, and this value was expressed as

the emulsifying activity (A_{540}) for that emulsion. All K_d and A_{540} values were expressed as the average from triplicate experiments. Emulsifiers xanthan gum, gum arabic (Sigma) and lecithin (Centrolex, Central Soya Co., USA) were used as commercial controls. Activities were compared under neutral (0·1 mol l^{-1} phosphate buffered saline, pH 7·5) and acidic (0·1 mol l^{-1} sodium acetate buffer, pH 3·5) conditions. The effect of hydrolysing conditions on emulsifying activity was evaluated by subjecting the emulsifiers to 100°C in 0·1 N HCl for 60 min.

Tensiometry

Tensiometry was performed using a Nima DST-9005 tensiometer (Nima Technology Ltd, UK) by the Wilhelmy plate method to initially screen bacterial isolates for surfactant production. The du Noüy ring method was used to measure surfactant production by strains TG39 and TG67 during growth in liquid medium and to determine whether the emulsifiers could reduce the surface tension of water at different concentrations.

Chemical analysis

For determination of the monosaccharide composition in the emulsifying extracts HE39 and HE67, three equal aliquots (10 μl) of 1% (w/v) solutions of the isolated extracts were dissolved in 500 μl of 2 mol l^{-1} trifluoroacetic acid (TFA) and hydrolysed (100°C for 4 h). The samples were then prepared for analysis by high performance anion exchange chromatography (HPAEC) using a Dionex Carbopac PA-20 column (Dionex Ltd, UK) and quantified using external standards. The individual amounts of monosaccharides were used to calculate the total carbohydrate content.

For determination of amino acid composition, acid hydrolysis and derivatization was performed on three equal amounts (100 μl) of the emulsifier extracts. Samples were hydrolysed in 1 ml of acid (6 N HCl; 112°C for 22 h) and then prepared for analysis using a Waters Alliance HPLC system (Waters Ltd, UK) equipped with a Zorbax XDB C18 reverse phase column (Agilent Technologies, Germany). Quantification was performed using external standardization with amino acid standard mixtures. The individual amounts of amino acids were used to calculate the total protein content.

Fatty acids were extracted from three equal amounts of the emulsifying extracts and analysed by gas chromatography (GC), as described previously (Cook *et al.* 2000).

Nuclear magnetic resonance analysis

Approximately 5 mg of the emulsifying extracts HE39 and HE67 were lyophilized twice from D_2O 99·9% (Apollo Scientific, Stockport, UK) and then taken up in 0·7 ml of the same solvent with the addition of 1 μl of a 2% solution of acetone in D_2O as an internal reference. Proton nuclear magnetic resonance (NMR) spectra were recorded at 60°C using a Varian Inova 500 MHz spectrometer (Varian Ltd, UK).

High-performance liquid chromatography

To evaluate the purity of the HE39 and HE67 extracts and to obtain an estimate on the molecular mass (M_r) of their constituent components that confer these extracts with emulsifying activity, high performance size-exclusion chromatography (HPSEC) was performed with an Agilent 1100 chromatograph (Agilent Technologies) equipped with a refractometer and diode-array UV detector. A Waters Ultrahydrogel 2000 column (7·8 × 300 mm; Waters Ltd) was used at 30°C. The eluent was 0·1 mol l^{-1} $NaNO_3$ (pH 7) and the flow rate was 0·6 ml min^{-1}. Dextran standards of M_r range 12 000–1 800 000 Da were used to calibrate the column for molecular weight estimation.

Results

Isolation and strain characterization

During screening for bioemulsifier and biosurfactant production by marine bacterial isolates, two strains (TG39 and TG67) were selected for their ability to produce high emulsification activities against hexadecane oil. Each isolate grew well in both ZM/10 and ZM/1 medium alone, but the highest emulsification values (EI_{24} = 60%) were obtained from spent culture broth during growth in ZM/1 amended with glucose. Strains TG39 and TG67 were Gram-negative, heterotrophic organisms that produced circular, convex and creamy colonies on solid medium with a sticky to ropy texture in aged colonies. The 16S rDNA sequencing revealed both strains to be members of the genus *Halomonas*. GenBank accession numbers for TG39 and TG67 are DQ994160 and DQ994161, respectively.

Growth and emulsifier production

The growth characteristics and emulsifier production by TG39 in ZM/1 broth amended with glucose is shown in Fig. 1a. Intense growth commenced soon after inoculation, coinciding with an increase in emulsification and a moderate reduction in the surface tension of the cell-free culture broth. A marked increase in the pH of the medium occurred at 12 h, reaching a maximum pH value of 7·8 by 18 h, then dropping slightly to 7·5 and increasing again to 7·8 within the next few hours. A gradual decrease

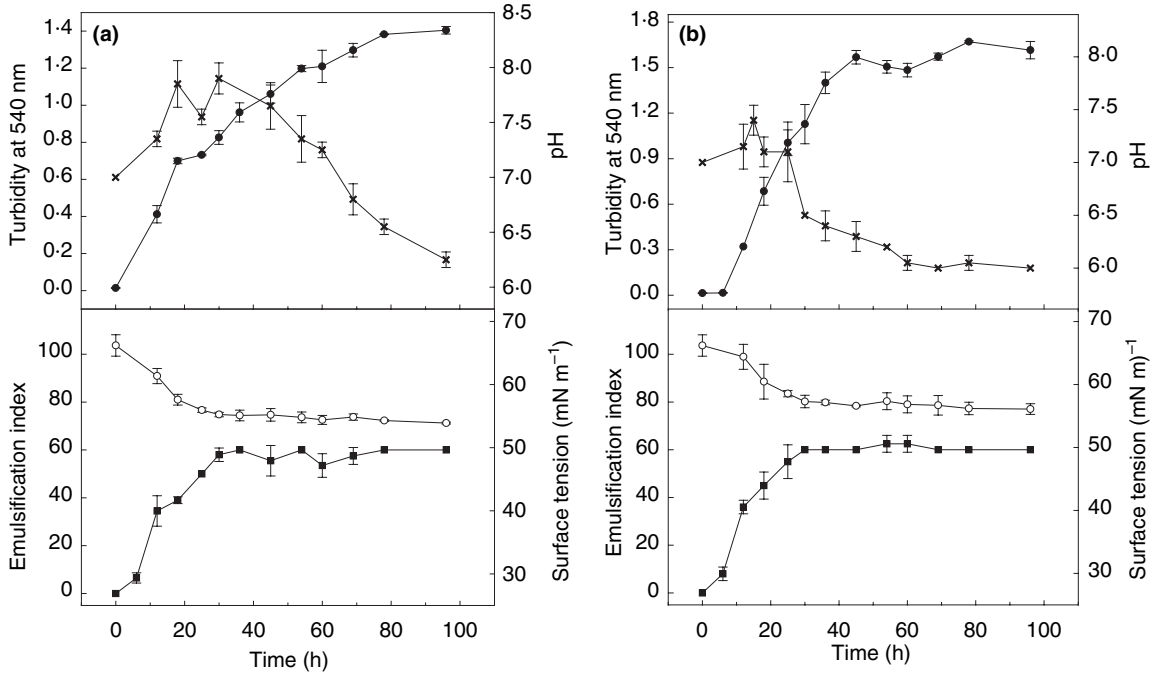

Figure 1 Growth and emulsifier production by *Halomonas* sp. TG39 (a) and TG67 (b) in ZM/1 broth amended with 1% (w/v) glucose. Emulsification index and surface tension values were derived from cell-free culture broth after removal of the cells by centrifugation. (●) Cell turbidity; (×) pH; (○) surface tension; (■) emulsification index. Error bars represent the standard deviation of at least three independent measurements from different culture flasks.

in the pH occurred after 30 h, reaching a value of 6·2 by 98 h. High emulsification values (EI = 50%) were measured in the cell-free culture broth at 25 h, which increased to 60% after 30 h by the mid-phase of intense growth. Even after several months of allowing these emulsions to stand unshaken at room temperature, they remained completely stable without any signs of coalescence (results not shown). By 36 h, the surface tension of cell-free culture broth had decreased from an initial value of 67·4 mN m^{-1} to 54·5 mN m^{-1}, but remained relatively constant thereafter.

The growth characteristics and emulsifier production of TG67 in ZM/1 broth amended with glucose is shown in Fig. 1b. Intense growth commenced within 10 h of inoculation, which was coupled to emulsification and a lowering in the surface tension of the cell-free culture broth. There was also a slight increase in the pH from an initial value of 7·0 to 7·4 after 18 h. The pH then gradually decreased to 6·0 by 60 h, at which point it remained relatively constant thereafter. High emulsification values were measured after 30 h (EI = 60%). The emulsions formed using cell-free broth of TG67 remained stable indefinitely (>6 months) without coalescing (results not shown). After 30 h, the surface tension of the cell-free broth had lowered from an initial value of 67·4 mN m^{-1} to 56·6 mN m^{-1}, and reached its lowest value of 55·5 mN m^{-1} after 70 h.

From an initial volume of 10 l ZM/1 broth amended with 1% glucose and grown for approx. 35–40 h, the average dry-weight yield of the emulsifying extracts HE39 and HE67 was 1·31 ± 0·74 g and 0·28 ± 0·06 g, respectively. This yield is equivalent to 131·0 ± 0·07 mg l^{-1} and 28·0 ± 0·006 mg l^{-1} of HE39 and HE67, respectively.

Chemical composition and molecular mass

The total carbohydrate content of HE39 and HE67, relative to the total dry weight of extracted polymer, was 17·3 ± 1·0% and 22·7 ± 0·8%, respectively (Table 1). The major monosaccharides identified in HE39 were rhamnose (31·7 ± 2·1%), glucuronic acid (27·9 ± 1·9%) and galactose (15·3 ± 0·5%). Galactosamine, glucose, glucosamine, mannose, xylose and galacturonic acid were each present at less than 10%, and together they contributed about 25% to the total carbohydrate content. For HE67, the major monosaccharides detected were glucuronic acid (58·8 ± 0·4%), glucosamine (10·9 ± 0·1%) and mannose (11·5 ± 0·5%), while rhamnose, galactose, galactosamine, glucose, muramic acid and galacturonic acid were each present at less than 10% of the total monosaccharide

Table 1 Monosaccharide analysis of emulsifiers HE39 and HE67

	Mean mol% composition in*	
Component	HE39	HE67
Rha	31·7 ± 2·1	3·9 ± 0·1
Fuc	ND	ND
Gal	15·2 ± 0·5	1·6 ± 0·0
GalN	2·5 ± 0·1	4·1 ± 0·2
Glc	8·0 ± 0·7	6·8 ± 0·4
GlcN	7·7 ± 0·1	10·9 ± 0·1
Man	3·3 ± 0·3	11·5 ± 0·5
Xyl	0·8 ± 1·1	ND
Mur	ND	0·1 ± 0·1
GalA	2·9 ± 0·2	2·2 ± 0·1
GlcA	27·9 ± 1·9	58·8 ± 0·4
Total (%)†	17·3 ± 1·0	22·7 ± 0·8

N-acetylglucosamine and *N*-acetylgalactosamine are de-*N*-acetylated during the acid hydrolysis and are detected as glucosamine and galactosamine.

ND, not detected; Rha, rhamnose; Fuc, fucose; Gal, galactose; GalN, galactosamine; Glc, glucose; GlcN, glucosamine; Man, mannose; Xyl, xylose; Mur, muramic acid; GalA, galacturonic acid; GlcA, glucuronic acid.

*Values are the mean of triplicate samples ± SD.

†Total % values are expressed as the mean percentage of total dry weight of the polymer from triplicate determinations.

Table 2 Amino acid composition from acid hydrolysis of the extracellular emulsifiers HE39 and HE67 produced by *Halomonas* sp. TG39 and TG67

	*Mean mol per cent composition in	
Component	HE39	HE67
Asp	11·6 ± 0·2	16·9 ± 0·4
Glu	11·7 ± 0·4	11·1 ± 0·2
Ser	5·2 ± 0·1	6·6 ± 0·0
Gly	11·2 ± 0·2	10·7 ± 0·1
His	1·7 ± 0·0	0·7 ± 0·0
Thr	6·8 ± 0·1	7·8 ± 0·2
Arg	5·3 ± 0·1	2·9 ± 0·1
Ala	10·6 ± 0·1	12·0 ± 0·1
Pro	4·0 ± 0·0	2·8 ± 0·1
Tyr	2·5 ± 0·1	2·7 ± 0·3
Cys	0·5 ± 0·0	0·1 ± 0·2
Val	7·1 ± 0·1	7·2 ± 0·2
Met	1·0 ± 0·1	0·2 ± 0·4
Ile	5·0 ± 0·0	4·4 ± 0·1
Leu	7·5 ± 0·1	7·5 ± 0·1
Lys	4·9 ± 0·1	2·6 ± 0·3
Phe	3·4 ± 0·1	3·7 ± 0·1
Total (%)†	26·6 ± 1·0	40·5 ± 1·6

*Values are the mean of triplicate samples ± SD.

†Total % values are expressed as the mean percentage of total dry weight of the polymer from triplicate determinations.

composition, and together they contributed only 18% to the total carbohydrate content.

The total amino acid content of HE39 and HE67, relative to the total dry weight of extracted polymer, was 26·6 ± 1·0% and 40·5 ± 1·6%, respectively (Table 2). Amino acid analysis of hydrolysed samples identified the presence of four major amino acids in both emulsifiers – aspartic acid, glutamic acid, glycine and alanine, which in total contributed 45·1% and 50·7% to the total amino acid content of HE39 and HE67, respectively. The percentage contribution of polar amino acids to the total amino acid content in both HE39 and HE67 was approximately the same at 61·5%, whereas that of hydrophobic nonpolar amino acids was about 38·0%. Lipid analysis did not identify any fatty acids in HE39 and HE67.

Figure 2(a and b) shows expansions of the proton NMR spectra acquired from both extracts. Both spectra are in agreement with a glycoprotein composition for these compounds, with strong peaks from the glycan component predominating. Detailed analysis of the anomeric proton (H1) region (4·4–5·5 ppm) and the ring proton (H2–H5) region was not possible on these complex samples, but some well-resolved features of the spectra offer corroboration of the monosaccharide analysis. An intense peak at 1·30 ppm in the spectrum of HE39 can be assigned to the methyl H6 protons of rhamnose; the equivalent peak in the spectrum of HE67 (1·28 ppm) is much less intense, as would be expected for the rhamnose content of both samples listed in Table 1. Intense signals at 2·03 ppm in the spectra of both HE39 and HE67 are consistent with the presence of acetyl methyl groups of *N*-acetylglucosamine and *N*-acetylgalactosamine, thus indicating that at least some of the amino sugars are *N*-acetylated. In the spectra of HE39, the relatively large signals with chemical shifts at 1·46, 3·64 and 3·75 ppm are attributable to low molecular weight impurities.

During chromatography of the HE39 extract on a calibrated Ultrahydrogel 2000 column, three UV_{280} peaks were eluted at retention times 10·0 (peak 1), 16·4 (peak 2) and 18·6 min (peak 3), each with a corresponding Refractive index (RI) signal (Fig. 3a). Fractions of these peaks were taken to identify the component responsible for emulsification in the HE39 extract. Because of poor separation of peaks 2 and 3, and even after numerous attempts to alter the chromatography conditions and testing different columns, they were recovered together as a single fraction. Peak 1, and peaks 2 and 3 combined, tested positive for emulsification activity against hexadecane. Using a plot of log M_r of standards *vs* retention time, we estimated the relative molecular mass of the eluted components in these peaks to be >2000 kDa, approx. 150 kDa and approx. 16·5 kDa, respectively. Chromatography of

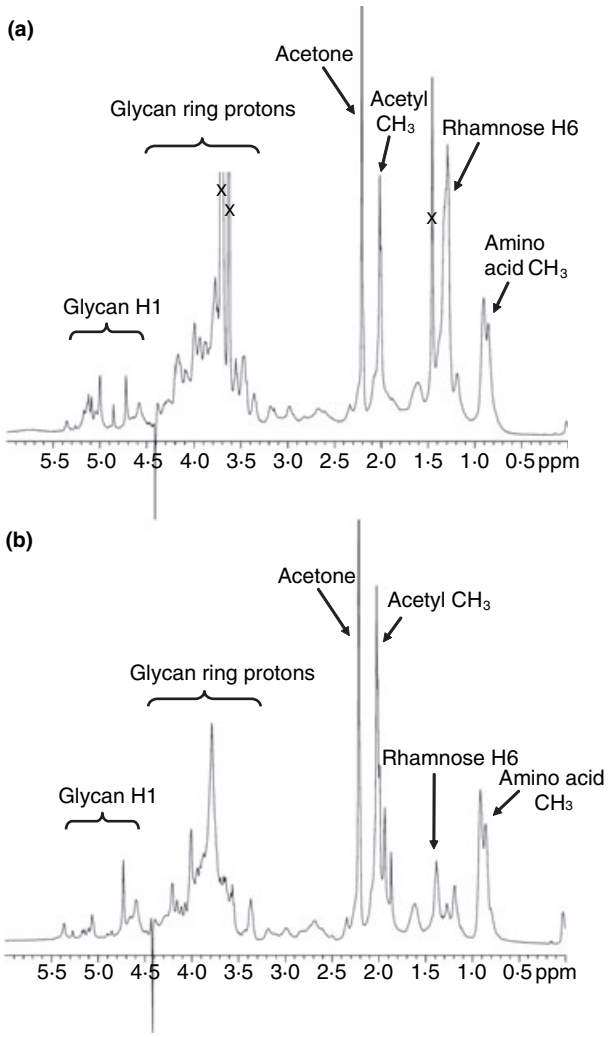

Figure 2 Proton NMR spectra acquired for the extracts HE39 (a) and HE67 (b) at 500 MHz, 60°C in D_2O. Assignments for some prominent signals in the spectrum are indicated; the remaining, unlabelled peaks arise from the protein component. Acetone is present as an internal standard (2·218 ppm); peaks marked X are attributable to low molecular-weight compounds, possibly including ethanol.

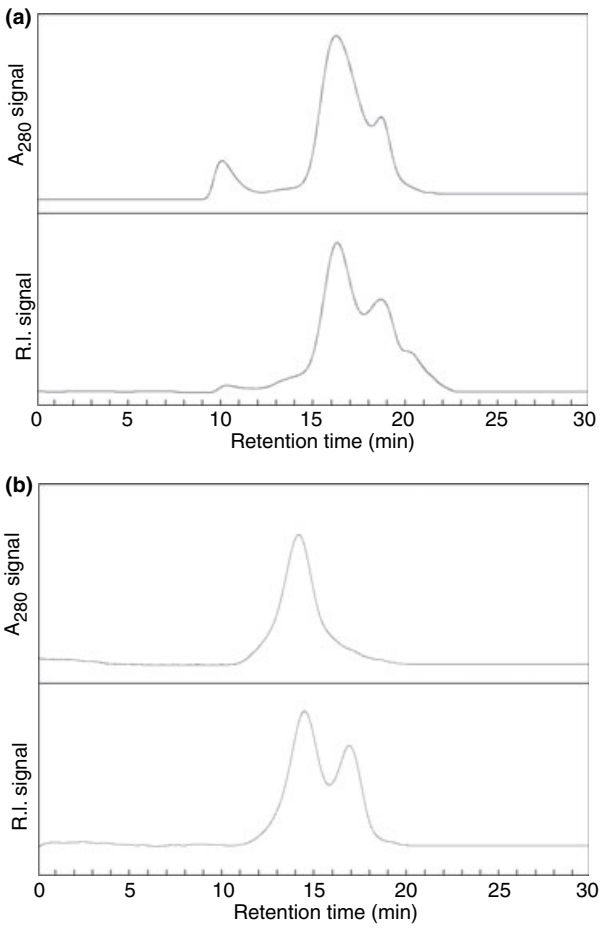

Figure 3 High-performance liquid chromatography of the HE39 (a) and HE67 (b) extracts on an Ultrahydrogel 2000 size-exclusion column. Refractive index (RI); absorbance at 280 nm (A_{280}).

the HE67 extract revealed two RI signals (Fig. 3b), which eluted at 16·0 and 18·6 min. The first of these peaks had a corresponding UV peak with an absorption maximum at 280 nm, to which all the emulsifying activity was found to be associated. The relative M_r of this peak was estimated to be approx. 1300 kDa. The second RI peak was estimated to be approx. 56 kDa and had no emulsifying activity when tested against *n*-hexadecane.

Emulsification of different oils

Figure 4a shows the measured emulsifying activities of different emulsifiers under neutral pH conditions when tested against four different food oils. Table 3 shows the corresponding decay constants (K_d) calculated for each of these emulsions, in which a higher value denotes greater stability. Against all oils, lecithin had the highest emulsifying activities and produced the most stable emulsions. Compared with the other two commercial emulsifiers (xanthan gum and gum arabic), HE39 and HE67 produced higher activities against all the oils, with the exception of the marginally lower activity by HE67 against sunflower oil. The stability of emulsions was highest using HE39, while HE67 produced higher or comparable K_d values. Figure 4b shows the effect of acidic conditions (pH 3·5) on the activities of different emulsifiers against the four food oils, and Table 3 shows the corresponding stability constants. Overall, HE39 produced higher emulsifying activities under acidic conditions than did HE67 and compared well with all three commercial emulsifiers. The stabilities of the emulsions produced by HE39 were also comparable to those of the three commercial emulsi-

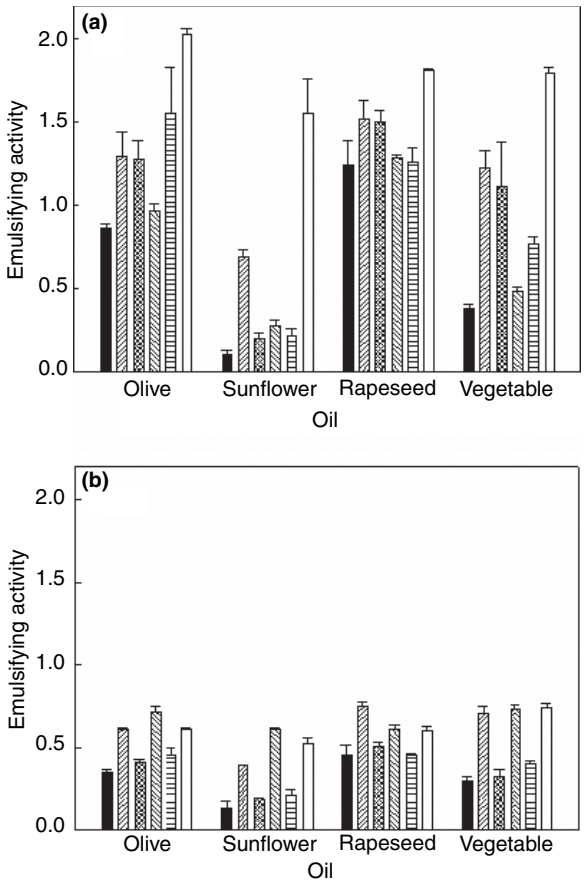

Figure 4 Emulsifying activity at pH 7·5 (a) and pH 3·5 (b) for O/W emulsions prepared using HE39 and HE67 compared with commercial emulsifiers. (■) Untreated control; (▨) HE39; (▩) HE67; (▧) xanthan gum; (▤) gum arabic; (□) lecithin. Error bars represent the standard deviation of independent measurements from three different emulsions.

fiers, although HE67 also gave high K_d values, particularly against sunflower and rapeseed oil.

When tested against *n*-hexadecane, both HE39 and HE67 (at 0·02% w/v in 0·1 mol l^{-1} PBS, pH 7·5) produced EI_{24} values of 60%. These emulsions remained stable and showed no signs of droplet coalescence after standing at 21 ± 2°C for several months.

Effect of acid and heat on emulsifying activity

The effect of heat treatment (100°C), at neutral pH or in 0·1 N HCl, on the emulsifying activity of HE39 and HE67 is shown in Table 4. With both emulsifiers, acidic conditions produced lower emulsifying activity (<45%) as compared with emulsification at neutral pH. HE39 produced its highest activity (1·35) when heated for 5 min at neutral pH. Further heating caused a slight reduction in its activity, which remained relatively constant at 1·25. Acid and heat treatment, however, caused HE39's activity to decrease from 0·47 to 0·25 within the first 15 min, followed by an increase in activity to 0·42 after 30 min, and then it dropped to its lowest after 60 min. The effect of heat treatment on HE67 caused its emulsifying activity at neutral pH to drop from 0·90 to 0·75 within the first 5 min, which gradually increased thereafter to 1·08 after

Table 3 Stability (K_d) of the emulsions formed by emulsifiers HE39 and HE67 against different food oils at pH 7·5 and 3·5, compared with commercial emulsifiers

	Decay constant, K_d ($-\chi^{-1} \pm 10^{-2}$)*			
Emulsifier	Olive	Sunflower	Rapeseed	Vegetable
At pH 7·5				
None	6·5	1·3	6·5	2·9
HE39	7·1	3·3	7·0	7·8
HE67	6·4	1·8	7·8	4·7
Xanthan gum	2·5	2·6	3·6	1·9
Gum arabic	6·5	1·8	5·1	3·5
Lecithin	17·9	13·4	14·7	12·5
At pH 3·5				
None	2·7	1·6	2·1	1·2
HE39	4·0	2·8	4·3	3·9
HE67	4·7	22·2	11·8	8·7
Xanthan gum	5·4	4·1	3·4	4·9
Gum arabic	3·4	1·7	2·2	2·4
Lecithin	7·1	3·4	5·3	5·6

*Values are the mean of triplicate samples; maximal deviation ≤5% between triplicates.

Table 4 Effect of heat and acid treatment on the emulsifying activity of HE39 and HE67

	Emulsifying activity (OD_{540})*			Relative activity†		
Treatment at 100°C (min)	None	HE39	HE67	None	HE39	HE67
At pH 7·5						
0	0·84	1·14	0·90	1·0	1·36	1·07
5		1·35	0·75		1·61	0·89
15		1·25	0·81		1·49	0·96
30		1·25	1·00		1·49	1·19
60		1·26	1·08		1·50	1·29
In 0·1 N HCl						
0	0·08	0·47	0·14	1·0	5·88	1·75
5		0·28	0·18		3·50	2·25
15		0·25	0·13		3·13	1·63
30		0·42	0·09		5·25	1·13
60		0·21	0·35		2·63	4·38

*Values are the mean of triplicate samples measured after allowing the emulsions of olive oil and test emulsifier to stand for 24 h at room temperature; maximal deviation ≤5% between triplicates.
†Relative activity is the activity compared with untreated control.

60 min. Under acidic conditions, heating HE67 for 5 min increased its emulsifying activity from 0·14 to 0·18, followed by a gradual decrease to 0·09 after a total of 30 min of heating. Further heating, however, increased its activity to a maximum of 0·35 after 60 min. Overall, HE39 produced higher activities than HE67 under both neutral and acidic pH, with the exception of the higher activity produced by HE67 at 60 min in the acid treatment. The measured relative activities for both HE39 and HE67, which compared the fold-increase in activity to the control (no emulsifier), were higher under acidic conditions (Table 4).

Surface tension

The HE39 and HE67 extracts were dissolved in water at concentrations ranging from 0·01% to 0·2% (w/v). These emulsifiers did not reduce the surface tension of water (72·1 mN m^{-1} at 21°C) at any of the concentrations examined.

Discussion

There has been an increase in the number of reports on exopolysaccharides produced by members of the genus *Halomonas* with interesting emulsifying and/or rheological properties (Calvo *et al.* 1998, 2002; Martinez-Checa *et al.* 2002; Pepi *et al.* 2005). In this report, we describe the partial purification and chemical and physical characterization of two extracellular water-soluble emulsifying agents, HE39 and HE67, produced by two marine *Halomonas* species, TG39 and TG67.

During the growth in ZM/1 broth amended with glucose, emulsifying activity was found tightly coupled with intense growth, and both strains partially reduced the surface tension of the culture medium to intermediate values of approx. 55·0 mN m^{-1}. Bacteria that produce surface-active agents are sometimes categorized based on their production of low molecular-weight surfactants, which lower the interfacial and/or surface tension of liquids, or those that produce high molecular-weight polymers that primarily act to form stable emulsions (Rosenberg and Ron 1999). Strains TG39 and TG67, like a few other bacteria (Rapp *et al.* 1979; Neufeld and Zajic 1984), appear to produce both types, although it is their emulsifiers that are of particular interest.

The partially purified emulsifying components, HE39 and HE67, were found to be readily extractable from the spent cell-free medium by solvent precipitation, and the recovered material could be used to produce stable emulsions with hexadecane and various food oils. Because of the inability of HE39 and HE67 to alter the surface tension of water (72·1 mN m^{-1}), these polymer extracts are, therefore, primarily emulsifying agents. The measured decrease in surface tension, recorded during the growth of TG39 and TG67 in ZM/1 broth amended with glucose, can be attributed to some other component possessing surfactant properties that was not recovered in the HE39 and HE67 extracts using our method of extraction.

Chemical characterization, using monosaccharide, amino acid and proton NMR analyses, identified both HE39 and HE67 to be glycoproteins composed of polysaccharide with a high uronic acid content and a relatively high proportion of protein (Table 2). Size-exclusion chromatography provided further confirmation of their glycoprotein composition, as shown by a close coupling between the RI and UV_{280} signals in most of the peaks resolved. Although further work is required to obtain an accurate molecular weight determination, such as by mass spectrometry or light scattering, the apparent molecular weight of the major contributing emulsifying component in HE39 and HE67 was estimated at >2000 and 1300 kDa, respectively. The lower molecular-weight components (i.e. <100 kDa) that were identified in each of these extracts were not expected, because during extraction the cell-free spent medium had been subjected to ultrafiltration through a 100 kDa molecular weight cut-off membrane to remove the lower molecular-weight fraction. Preliminary work to investigate this suggests that they may have become associated with the higher molecular weight fraction because of decreased ionic strength when distilled water was used during ultrafiltration of the spent medium prior to precipitation with ethanol.

An interesting finding was that the carbohydrate component of both emulsifiers had a relatively high uronic acid content of 30·8% and 61·0% for HE39 and HE67, respectively. This was contributed by the high levels of galacturonic and glucuronic acids in both emulsifiers. These values are similar to other reports describing extracellular polysaccharides (EPS) produced by marine bacteria that may contain anywhere between 20% and 50% uronic acids of total carbohydrate content (Kennedy and Sutherland 1987; Manusco Nichols *et al.* 2005). In comparison, polymeric emulsifiers extracted from saline-soil derived *Halomonas maura* (Arias *et al.* 2003) and those from strains of *Halomonas eurihalina* (Calvo *et al.* 2002; Martinez-Checa *et al.* 2002) contained significantly lower levels of uronic acids (1·5–8·1%). In addition, compositional analysis of a glycolipid emulsifier produced by an Antarctic psychrotrophic halomonad, *Halomonas* sp. ANT-3b, did not identify any uronic acids (Pepi *et al.* 2005).

With the recent increase in the number of reports describing new types of soluble emulsifying agents derived from members of the genus *Halomonas*, the emulsifying

activities reported in this study, for HE39 and HE67, appear comparatively higher. For example, different emulsifying extracts from *H. eurihalina* strain H-28 isolated from cells grown in the presence of nine different substrates were reported to produce anywhere between 13% and 53% of emulsifying activity (EI_{24}) against *n*-hexadecane (Martinez-Checa *et al.* 2002). Similarly, various emulsifying agents extracted from four other strains of this species grown on different substrates were found to produce activities in the range of 27% to 56% against *n*-hexadecane (Calvo *et al.* 2002). In both these reports, the described emulsifiers also produced similar emulsifying activities against other aliphatics, aromatics and petroleum fractions. Using the same emulsification assay (Cooper and Goldenberg 1987) employed in these reports, we showed our HE39 and HE67 emulsifiers to produce 60% activity when tested against hexadecane, and the emulsions formed remained stable for months without showing signs of droplet coalescence. However, Mauran, an exopolysaccharide produced by *Halomonas maura*, was reported to produce up to 78% activity against hexadecane (Bouchotroch *et al.* 2000). It is notable that high emulsifying activities produced by HE39 and HE67 were achieved using relatively low concentration (0·02%), which was approx. 25–50 times less than that in the other reports (Bouchotroch *et al.* 2000; Calvo *et al.* 2002; Martinez-Checa *et al.* 2002). This apparently 'high yield value' is a potentially useful property both in terms of process economics and potential biotechnological application (Sanderson 1990).

With a few exceptions, acidic conditions appear to have an overall inhibitory effect on the emulsifying activity of HE39, HE67 and the commercial emulsifiers tested (Fig 4), as indicated by the significantly reduced activities produced at pH 3·5 as compared with that at neutral pH. Although further work will be needed to properly understand these effects, Rosenberg *et al.* (1979) observed a similar effect with emulsan and offered the suggestion that protonation of carboxyl groups on the fatty acids of oils and on the emulsifiers may effectively reduce their emulsifying potential. Overall, the emulsifying performance and stabilizing effects of both HE39 and HE67, particularly the former, compared well with those of the commercial emulsifiers (gum arabic, xanthan gum and lecithin). The same was also true under neutral pH conditions, although lecithin was observed to be the better emulsifier. Interestingly, HE67's stabilizing effect under acidic conditions was disproportional to its emulsifying activity, producing quite stable emulsions with reduced emulsifying activity at pH 3·5, particularly against rapeseed and sunflower oils. Thus, it may be suggested that HE67 is a better stabilizer than emulsifier of food oils, a property that more closely reflects hydrocolloid stabilizers (Garti and Leser 1999). With all the emulsions formed using HE39 and HE67, an observed decrease in the turbidity of aqueous phases was because of phase separation (i.e. 'creaming') rather than a result of droplet coalescence, because the 'cream' layer formed could be re-dispersed back into the aqueous layer. Hence, the retention of oil droplets in the aqueous phase is likely to be proportional to density differences between the two phases (Rosenberg *et al.* 1979). The emulsifying activities observed in the untreated controls (i.e. no emulsifier added) could be explained by the intrinsic presence of free fatty acids in the oils (Ma and Hanna 1999). When compared with the control without any added emulsifier, heat and acid treatment (100°C; 0·1 N HCl) appeared to increase the relative activity of HE67 (Table 4). This mechanism, referred to as heat activation of polymeric emulsifiers, was first described by Navon-Venezia *et al.* (1995) using alasan from *Acinetobacter radioresistens*. This treatment may have induced the release of a higher number of emulsifying moieties from these biopolymers that would contribute to their increased emulsifying capacity.

The polysaccharide component of HE39 and HE67 may have a functional role in emulsion stabilization, as observed with some galactomannans (Coia and Stauffer 1987; Garti and Reichman 1994) and xanthan gum (Ikegami *et al.* 1992). According to Tolstogusov, the adsorption to and stabilization of oil droplets can be mediated by carboxylate and methoxycarbonyl groups of polyuronates (Kaplan *et al.* 1987; Tolstogusov 1991, 1994). For instance, polysaccharide fractions of pectins have been isolated exhibiting excellent emulsifying activity, and this property has been attributed to their high uronic acid content (44–60%) as compared with less active commercial pectins with lower levels of these acids (Dea and Madden 1986). The presence of 6-deoxyhexoses or increased substitution by acetyl moieties can render polysaccharide compounds quite lipophilic (Dea and Madden 1986; Graber *et al.* 1988). This characteristic could also help contribute to the amphipathic nature of these polymeric emulsifiers, especially HE39, as it was found to contain a high rhamnose content (31·7%) from the monosaccharide analysis and confirmed by proton NMR. On the contrary, the active mechanism of steric emulsion stabilization could be conferred by the proteinaceous component of HE39 and HE67, which was found to be considerably higher in content as compared with that in polymeric emulsifiers from other halomonads (Calvo *et al.* 2002; Martinez-Checa *et al.* 2002; Arias *et al.* 2003). This has been shown to be the case with gum arabic (Williams *et al.* 1990), emulsan (Kaplan *et al.* 1987) and other microbially-derived polysaccharides (Garti and Leser 1999). Proteins adsorb to oil droplets by way of their hydrophobic moieties, penetrating into the oil to become

solvated and act as anchoring points. As no lipids were detected in either HE39 or HE67, the amphipathic nature of both emulsifiers may be contributed by their relatively high nonpolar amino acid content (approx. 38%). This nonpolar amino acid fraction may play a complementary role to the lipid component of lipid-containing amphipathic molecules, thereby permitting HE39 and HE67 to interact with different oil substrates. Although proteins are known for their surfactancy (Patel and Fry 1987), the protein in these extracts did not exhibit this characteristic because no significant reduction to the surface tension of water (72·1 mN m^{-1}) was measured in solutions containing various concentrations of these extracts. While experiments are planned, including 2-D NMR and partial amino acid sequence determination, to be used to better define the functional role of the polysaccharide and protein components of HE39 and HE67, at present we can infer that these glycoprotein emulsifiers represent a new type of halomonad-derived polymers endowed with high concentrations of uronic acids and protein, and exhibit excellent emulsifying and stabilizing properties.

Interestingly, although the HE39 and HE67 emulsifiers are composed of relatively high molecular-weight components, their ability to form stable emulsions occurs without significantly contributing to the viscosity of the continuous phase (data not shown). The same has been observed with other high molecular-weight emulsifiers such as some galactomannan gums (Coia and Stauffer 1987; Garti and Reichman 1994) and gum arabic (Williams *et al.* 1990). The mechanism of forming low-viscosity emulsions may be similar to that of gum arabic, as ascribed to its highly branched and globular arabinogalactan-protein complex (Williams *et al.* 1990).

When compared with lecithin, HE39 and HE67 produced emulsions that were overall more stable under the acidic conditions tested (pH 3·5). In contrast to polymeric emulsifiers, lecithins are low molecular-weight phospholipid emulsifiers that are essentially hydrophobic molecules, yet they suffer from limited functionality in many food products subjected to modern food processing conditions (Shepherd *et al.* 1995). For this reason, they are generally used at quite high concentrations to obtain the desired levels of functionality, and are thus limited to use in complex foods such as margarine, bread, ice-cream and chocolate (Garti 1999). The type of lecithin used in this study, Centrolex, is a derivative of crude lecithin, yielding a more expensive oil-free product with enhanced solubility and improved emulsifying properties. For this reason, it was used here as a 'gold standard' to which HE39 and HE67 could be compared. The reduced stabilizing performance of lecithin observed at pH 3·5 is in agreement with results reported by Yamamoto and Araki (1997), who tested the ability of crude and pure lecithins to stabilize emulsions of different oils with β-lactoglobulin, and observed lecithin to decrease the stability of emulsions formed under acidic conditions (pH 3–4·5). The higher stabilizing properties of HE39 and HE67, as compared with that of lecithin, may be attributable to their ability to remain soluble and the resistance of their protein component to aggregate under the low pH conditions tested, which may be closer to their isolelectric point. Further research towards developing new glycoprotein emulsifiers, particularly for applications that require low pH conditions, would be a viable proposition in light of these promising results.

The emulsification and stabilizing properties of these extracellular glycoprotein emulsifiers suggests that they may have potential commercial applications. These novel bioemulsifiers may find many uses as natural polymeric emulsifiers and as a replacement to currently-used emulsifiers that are limited by reduced functionality and/or yield values. Interestingly, as these biopolymers contain high concentrations of charged components, notably uronic acids, they may also be useful for bioremediation of toxic metals, as has been described for other compounds with similar composition (Sutherland 1994; de Philipps and Vincenzini 1998). Further work is already planned to optimize the production of these glycoprotein emulsifiers and characterize their performance and functionality (particularly at industrial-scale) before firm conclusions on their commercial value can be made.

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