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An Exocellular Protein from the Oil-Degrading Microbe *Acinetobacter venetianus* RAG-1 Enhances the Emulsifying Activity of the Polymeric Bioemulsifier Emulsan

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The oil-degrading microorganism *Acinetobacter venetianus* RAG-1 produces an extracellular polyanionic, heteropolysaccharide bioemulsifier termed emulsan. Emulsan forms and stabilizes oil-water emulsions with a variety of hydrophobic substrates. Removal of the protein fraction yields a product, apoemulsan, which exhibits much lower emulsifying activity on hydrophobic substrates such as *n*-hexadecane. One of the key proteins associated with the emulsan complex is a cell surface esterase. The esterase (molecular mass, 34.5 kDa) was cloned and overexpressed in *Escherichia coli* BL21(DE3) behind the phage T7 promoter with the His tag system. After overexpression, about 80 to 90% of the protein was found in inclusion bodies. The overexpressed esterase was recovered from the inclusion bodies by solubilization with deoxycholate and, after slow dialysis, was purified by metal chelation affinity chromatography. Mixtures containing apoemulsan and either the catalytically active soluble form of the recombinant esterase isolated from cell extracts or the solubilized inactive form of the enzyme recovered from the inclusion bodies formed stable oil-water emulsions with very hydrophobic substrates such as hexadecane under conditions in which emulsan itself was ineffective. Similarly, a series of esterase-defective mutants were generated by site-directed mutagenesis, cloned, and overexpressed in *E. coli*. Mutant proteins defective in catalytic activity as well as others apparently affected in protein conformation were also active in enhancing the apoemulsan-mediated emulsifying activity. Other proteins, including a His-tagged overexpressed esterase from the related organism *Acinetobacter calcoaceticus* BD4, showed no enhancement.

Biosurfactants have been used in a variety of industrial and environmental applications (11, 13, 42). Low-molecular-mass biosurfactants such as glycolipids (15, 21) and lipopeptides (23, 44) generally act as detergents, lowering interfacial tension at liquid-liquid or liquid-solid interfaces (3, 15, 22, 33). In addition, microbes have been shown to produce higher-molecular-mass bioemulsifiers, which also stabilize oil-water and water-oil emulsions (9, 10, 17). In this regard, hydrocarbon-degrading *Acinetobacter* species frequently produce extracellular emulsifying agents, generally consisting of high-molecular-mass polysaccharides associated with proteins. Some examples include emulsan (47), the exopolysaccharide from *Acinetobacter calcoaceticus* BD4 (24), and alasan, produced by *Acinetobacter radioresistens* K53 (31).

The most extensively studied of these polymeric complexes is emulsan, an amphipathic extracellular polyanionic bioemulsifier produced by *Acinetobacter venetianus* RAG-1 (16, 43). Purified deproteinized emulsan (apoemulsan, 10^3 kDa) consists of D-galactosamine, L-galactosamine uronic acid (pKa, 3.05), and a diamino, 2-desoxy *n*-acetylglucosamine (47). The amphipathic properties of this biopolymer are due in part to the presence of fatty acids covalently bound to the polysaccharide backbone in both ester and amide linkages (5). In addition, when recovered from the growth medium, crude emulsan contains a complex consisting of about 10 to 20% protein, which also contributes to its amphipathicity and to the hydrocarbon substrate specificity (39, 46). For example, as a protein-polysaccharide complex, emulsan is active primarily with a mixture of aliphatic and aromatic hydrocarbons (35), although it does show low activity with purely aliphatic substrates such as hexadecane. Removal of the emulsan-associated proteins generated a product, termed apoemulsan, which retained many of the physical properties of emulsan itself, exhibiting partial emulsifying activity with relatively polar hydrocarbon substrates. However, emulsification of very hydrophobic hydrocarbons by apoemulsan was severely reduced (47). Part of this activity could be recovered by the addition of larger amounts of the crude denatured protein (45).

While the genes responsible for the biosynthesis and control of the emulsan heteropolysaccharide have recently been cloned and sequenced and shown to exist within a single gene cluster termed the *wee* regulon (30), little is known about the protein component(s) of the emulsan complex. Emulsan release from the bacterial cell surface is apparently mediated by the action of a cell surface esterase, which is one of the key components in the active emulsan-protein complex (39). This esterase, encoded by the *est* gene (909 bp) has been cloned, sequenced, and overexpressed in *Escherichia coli* (1). The facts that the exocellular esterase is involved in emulsan release, has been shown to deacetylate the biopolymer (38), and has been found associated with the extracellular emulsan complex suggest that its interaction with apoemulsan might affect the emul-

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sifying activity of the biopolymer. In this report, we demonstrate that the entire protein fraction of the emulsan complex can be replaced with either a recombinant form of the wild-type esterase or a series of mutant esterases defective in catalytic activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *A. venetianus* RAG-1 was initially mistakenly identified as an *Arthrobacter* species (34) but was subsequently placed in the genus *Acinetobacter*, which at that time consisted of a single species, *A. calcoaceticus* (4). The organism was assigned to the species *A. lwoffii* at the suggestion of management of the American Type Culture Collection (ATCC), where the strain was deposited and referred to as *Acinetobacter* species strain ATCC 31012. Recently the strain was placed within the species *A. venetianus* (43) and will subsequently be referred to as *A. venetianus* RAG-1.

A. venetianus RAG-1 was grown in a minimal ethanol salts medium (ETMS) containing (per liter) 22.2 g of $K_2HPO_4 \cdot 3H_2O$; 7.26 g of KH_2PO_4; 4 g of $(NH4)_2SO_4$; 0.2 g of $MgSO_4 \cdot 7H_2O$; and 25 ml of absolute ethanol. For preparation of ETMS plates, the liquid medium was solidified with 2% agar (Difco). Growth was carried out as described previously (39). Cultures were grown in an incubated gyratory shaker (New Brunswick model G-25) at 30°C and 250 rpm. Inocula were prepared from overnight cultures and diluted 1:20 into fresh ETMS. *E. coli* strains were routinely prepared on LB medium as described previously (36). *E. coli* BL21(DE3, pLysS) containing the *est* gene from *A. venetianus* RAG-1 cloned in plasmids pET-11c and pET-14b (see below) was grown at 37°C in liquid LB medium supplemented with 200 μg of ampicillin and 34 μg of chloramphenicol per ml at 37°C to an A_{600} of 0.6 to 1.0. At this point, the cells were induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM for 2 h in order to obtain optimal yields of recombinant esterase in the cell extract. For obtaining maximum levels of esterase in inclusion bodies, the induction period was extended to 3 h.

Chemicals, reagents, and hydrophobic substrates for emulsification. All chemicals were of the highest purity available. Bacto-yeast extract, Bacto-tryptone, and Bacto-agar were obtained from Difco; ampicillin, chloramphenicol, *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate, *p*-nitrophenyl caproate, RNase, DNase, lysozyme, sodium deoxycholate, sodium dodecyl sulfate (SDS), 4-chloronaphthol, bovine serum albumin, IPTG, and Coomassie blue G-250 were from Sigma; while proteinase K, NaCl, ethanol, $(NH_4)_2SO_4$, potassium salts, $MgSO_4$, and imidazole were from Merck. Acrylamide and *N,N′*-methylenebisacrylamide were supplied by BDH. Primers were prepared by Gibco, and DNA polymerase used in PCR amplification was purchased from Takara. Hydrocarbon substrates used in this work included mineral oil obtained from Sigma; immersion oil from Carl Zeiss; soy oil from Milimor; and petroleum refinery sludge, crude oil, and diesel oil obtained from the Haifa Refinery, Haifa, Israel. Anthracene, 2-methylnaphthalene, pyrene, squalene, hexadecane, octadecane, eicosane, and tetracosane were obtained from Merck. Dicyclohexane and fluoranthene were obtained from Aldrich, while heptadecane was obtained from Fluka.

Plasmid construction. The pET system (Novagen) was used for overexpression of parental and mutant esterases. Plasmids were constructed by ligation of the *Kpn*I-*Nde*I-*Bam*HI fragments of the *A. venetianus* RAG-1 *est* gene by PCR amplification with the following oligonucleotide primers: pESTAL-11c (5′-GC GC*GGTACCCATATG*AAATTTGGTACT-3′; the introduced 5′ *Kpn*I-*Nde*I site is in italics) and pESTAL-14b (5′-CGC*GGATCC*AGGTTAGTCTAGATC-3′; the introduced 3′ *Bam*HI site is in italics). Plasmid pRA17 (1) served as the template for PCR amplification.

Site-directed mutagenesis. Site-directed mutagenesis was carried out by two methods: M13-derived mutagenesis with either the Sculptor in vitro mutagenesis system of Amersham Life Science or primer-mediated mutagenesis with PCR. The former procedure is based on the phosphorothioate technique (37) and was carried out as described previously the system manual. The latter procedure involves mutagenesis of PCR fragments via mismatched primer and was performed as described previously (20). Primers used for site-directed mutagenesis of *A. venetianus* RAG-1 esterase and for subsequent sequence analysis of all mutated fragments were $His^{79}Gly$ (5′-GATCTTTCATATTGGTGGCGGTGC-3′), $His^{79}Ala$ (5′-GATCTTTCATATTGCTGGCGGTGC-3′), $Asp^{110}Gly$ (5′-G CAAGAGGGTAACCCACATGAATC-3′), $Ser^{149}Gly$ (5′-CAGGTGATGGAT GTGGTGCCAATCTC-3′), $Ser^{149}Ala$ (5′-CAGGTGATGCATGTGGTGCCA ATCTC-3′), $Asp^{196}Gly$ (5′-CAGAAGCACGGTGCATTGTTATCG-3′), Asp^{196} Ala (5′-CAGAAGCACGCTGCATTGTTATCG-3′), $Glu^{244}Gly$ (5′-CGGTTCT AAAGGGATTCTGCTGGATG-3′), $Glu^{244}Ala$ (5′-CGGTTCTAAAGCGATT CTGCTGGATG-3′), $His^{274}Gly$ (5′-CGGGAATGTGGGGTAACTTCCAAAT G-3′), and $His^{274}Ala$ (5′-CGGGAATGTGGGCTAACTTCCAAATG-3′).

Plasmids were transformed into *E. coli* TG-1 competent cells prepared as described before (18). All additional recombinant DNA techniques were performed as described previously (36).

Esterase activity assay and protein quantification. Esterase activity was assayed by following the hydrolysis of *p*-nitrophenylacetate to yield *p*-nitrophenol as previously described (39). One unit of esterase activity is defined as the formation of 1 nmol of *p*-nitrophenol per minute, and the specific activity is given in units per milligram pf protein. Protein was estimated as described previously (8), with bovine serum albumin as a standard.

Cell extract preparation. Induced cells (see above) from 800-ml cultures were harvested by centrifugation at 5,000 × *g* for 5 min at 4°C. Supernatant fluid was discarded, and the cell pellet was resuspended in 32 ml of ice-cold binding buffer (5 mM imidazole) and sonicated on ice until the sample was no longer viscous. The cell lysate was centrifuged at 12,000 × *g* for 30 min to remove debris and insoluble proteins (inclusion bodies). Cell supernatant fluid obtained was filtered through a 0.2-μm-pore-size membrane to prevent clogging of column resin.

Ni column chromatography. Purification of His-tagged recombinant protein was performed as described previously the Novagen system manual. After the cell extract was loaded on the column, a flow of 10 column volumes per hour was applied for efficient purification. The column was washed with 10 volumes of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl [pH 7.9]), followed by washing with 6 volumes of washing buffer (containing 30 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl[pH 7.9]). The bound recombinant esterases were eluted with 6 volumes of elution buffer (1 M imidazole, 500 mM NaCl, and 20 mM Tris-HCl [pH 7.9]).

Solubilization of inclusion bodies. The pellet remaining from the cell extract preparation containing the inclusion bodies (formed during plasmid induction) was washed twice with 50 mM Tris-HCl buffer (pH 8.0) and centrifuged at 4°C and 12,000 × *g* for 30 min. The pellet was resuspended in the same buffer solution, and lysozyme (50 μg/ml), RNase (30 μg/ml), and DNase type I (30 μg/ml) were subsequently added. The suspension was incubated for 60 min at 37°C and centrifuged at 12,000 × *g* for 30 min at 4°C. The last pellet was washed twice with sterile saline. Finally, the pellet was resuspended in 2% (wt/vol) sodium deoxycholate in 50 mM Tris-HCl (pH 8.5) for 60 min. The solution was slowly dialyzed against a solution containing 50 mM Tris-HCl (pH 7.8) at 4°C for 96 h (fresh buffer was replaced twice daily) and then centrifuged at 12,000 × *g* at 4°C for 30 min. Protein was determined as described previously (8) with bovine serum albumin as the standard protein and then stored in aliquots at −20°C.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (27) in a Hoffer apparatus. Protein bands were stained with Coomassie brilliant blue G-250. Molecular mass standards were obtained from Bio-Rad.

Western blot analysis. Protein and polysaccharide extract samples were subjected to electrophoresis in SDS-polyacrylamide gels (12.5%). The gels were then electroblotted onto nitrocellulose filters with transfer buffer (19). The filters were washed with phosphate-buffered saline (PBS), blocked with 1% milk for 3 h at 30°C, and reacted with antibodies against recombinant esterase, followed by reaction with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Jackson Laboratories). The antibody binding was detected by precipitation of 4-chloronaphthol.

Preparation of antibodies. Two rabbits were injected subcutaneously (for primary immunization) with 0.5 mg/ml of a mixture of recombinant esterase dissolved in PBS and Freund's complete adjuvant (Sigma). Two booster immunizations were continued with antigen in PBS mixed with Freund's incomplete adjuvant at 4-week intervals. Rabbits were bled 14 days following the immunizations, and blood was allowed to stand for 4 h at room temperature and 4°C overnight. After removal of clots and debris by centrifugation for 20 min at 3,000 rpm, the serum was assayed by enzyme-linked immunosorbent assay and stored at −20°C. The antiserum preparation did not cross-react with either bovine serum albumin or lysozyme. Titers were determined monthly, and the last blood sample was drawn 6 months after the initial immunization.

Emulsan preparation. Cell-free supernatant of an *A. venetianus* RAG-1 stationary-phase culture was obtained by centrifugation at 10,000 × *g* for 10 min and 4°C in a Sorvall refrigerated centrifuge (model RC 5). Crude emulsan was precipitated from the supernatant fluid in the presence of ammonium sulfate (60% saturation), followed by exhaustive dialysis against 1,000 volumes of distilled water for 24 h (fresh water was replaced twice). The polymer was stored as a lyophilized powder at room temperature.

Preparation of apoemulsan. A stock 1-mg/ml solution of apoemulsan was prepared by incubation of the lyophilized emulsan with proteinase K (at a final concentration of 10 μg/ml) for 60 min at 45°C. After exhaustive dialysis against

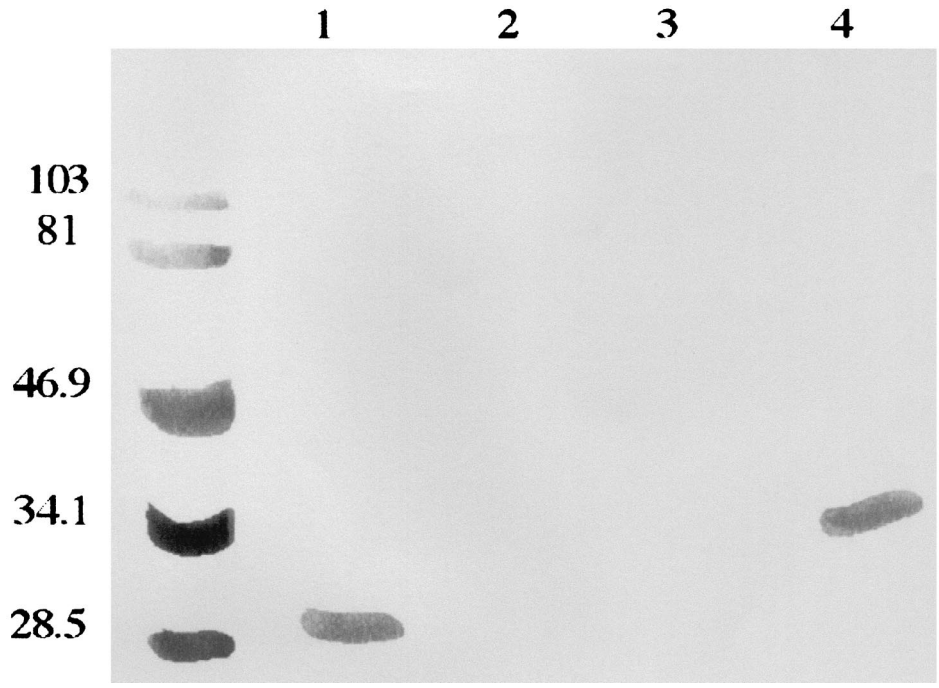

FIG. 1. Western immunoblot analysis with polyclonal antibodies raised against recombinant, His-tagged esterase produced in *E. coli* BL21(pESTAL-14b). Proteins were separated by SDS–12% PAGE, blotted on nitrocellulose binding matrix, and 4-chloronaphthol immunostained. Lanes: 1, cell-free emulsan; 2, cell-free *Acinetobacter* BD4 exopolysaccharide; 3, colanic acid, prepared as described previously (32); 4, recombinant RAG-1 esterase.

1,000 volumes of distilled water for 24 h (fresh water was replaced twice), the apoemulsan solution was stored at −20°C for subsequent use. Apoemulsan preparations contained less than 0.5% protein.

Emulsifying activity. An aliquot of hydrocarbon substrate (typically 80 to 100 mg) was added to 7.5 ml of buffer containing 20 mM Tris-HCl (pH 7.8), 10 mM $MgSO_4$, and either 60 or 100 μg of emulsan or apoemulsan, depending on the hydrophobic substrate used. After reciprocal shaking at 30°C for 1 h, the turbidity of the oil-in-water emulsion was measured in a Klett-Summerson colorimeter fitted with a green filter (model 800-3). Before assaying, samples were diluted with water so that Klett readings were always in the range from 75 to 200 Klett units. One unit of emulsifying activity was defined as the amount of emulsan or apoemulsan giving rise to an emulsion turbidity of 100 Klett units in the standard assay. Specific emulsifying activity is given in units per milligram. Purified emulsan preparations generally exhibited between 150 to 300 U per mg of biopolymer. When apoemulsan was assayed in the presence of proteins, specific activity is given as units per milligram of bioemulsifier per milligram of protein.

In addition to the esterase preparations, other proteins used included a recombinant form of the esterase from *A. calcoaceticus* BD4 (prepared previously by Y. Berdichevsky [unpublished data]); pancreatic lipase, pig esterase, bovine serum albumin, lysozyme, and DNase, all from Sigma; dockerin, kindly provided by R. Lamed; and isopenicillin synthase, kindly provided by G. Cohen. All the proteins were dissolved in the same buffer used for emulsification assays at a concentration of 1 mg/ml, aliquotted, and stored at −20°C. In the case of recombinant esterases, protein concentrations were adjusted to a concentration of 1 mg/ml with the dialysis buffer, aliquotted, and stored at −20°C.

Emulsion stability. Emulsions of hexadecane in water were formed with apoemulsan in the presence of increasing amounts of His-tagged recombinant esterase solubilized from inclusion bodies. After formation, the emulsions were allowed to stand at room temperature without mixing, and the turbidity was recorded after 24 h. The stability was calculated as the percent decrease in the turbidity of the emulsion after 24 h with no shaking.

RESULTS

Overexpression of RAG-1 esterase in *E. coli*. *est* gene overexpression was carried out in *E. coli* strain BL21(DE-3, pLysS) carrying either plasmid pESTAL-14b or plasmid pESTAL-11c. Plasmid pESTAL-14b was used for overproducing the His-tagged recombinant esterase. After addition of IPTG to a growing culture, the induced cells were harvested. Whole-cell suspensions as well as sonicated cell extracts were assayed for esterase activity and subjected to SDS-PAGE (Materials and Methods). Esterase activities of about 2,500 U/mg of protein were detected in IPTG-induced whole cells as well as in cell extracts of induced strains carrying either of the two plasmids. In contrast, uninduced cells and cells of strain *E. coli* BL21 carrying the original plasmids lacking the insert were almost completely devoid of esterase activity (<20 U/mg of protein). When subjected to SDS-PAGE, a band corresponding to a molecular mass of 34.5 kDa was found in the cell extract as well as the insoluble fraction (inclusion bodies) in IPTG-induced cells harboring pESTAL-14b (recombinant His-tagged esterase). No such bands were found in uninduced cells from the same strains (not shown). Induced cells carrying pESTAL-11c yielded a band with a molecular mass of about 32.5 kDa lacking the His tag and thrombin site.

Identity of the esterase was confirmed by Western immunoblot analysis with the wild-type RAG-1 esterase as a positive control (Fig. 1). Interestingly, two different bands were observed in the Western blots, both of which reacted with the antibody against the RAG-1 esterase. One of the bands was the His-tagged intact recombinant esterase produced in strain BL21, corresponding to a molecular mass of 34.5 kDa (lane 4). The second band (lane 1) was the esterase band normally present in the crude cell-free emulsan preparation from RAG-1, which was smaller by about 5 kDa, apparently owing

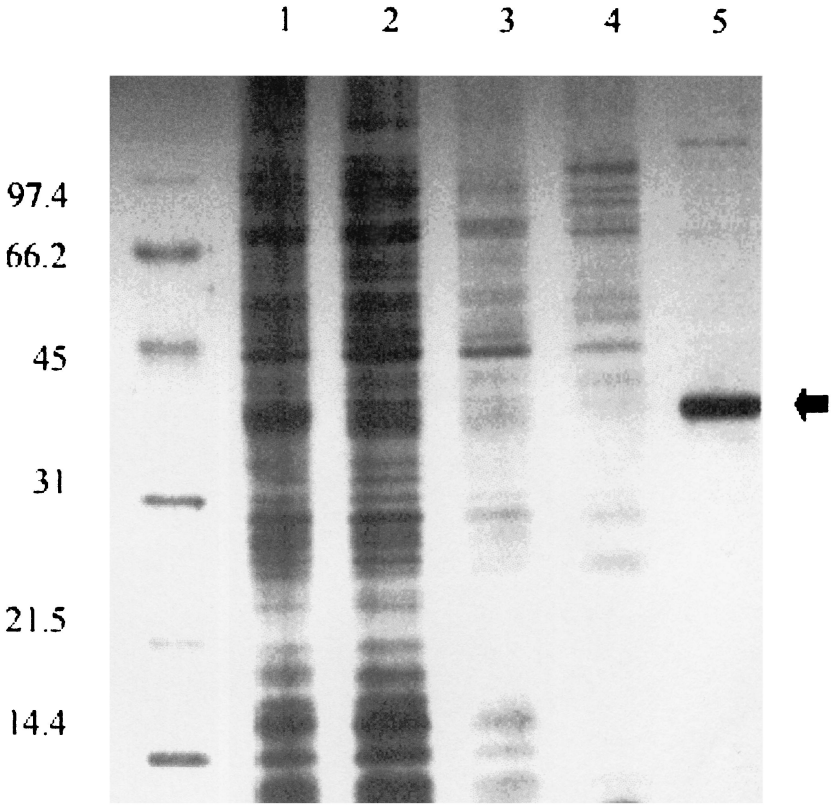

FIG. 2. Purification of overexpressed RAG-1 esterase by metal chelation affinity chromatography. The soluble recombinant RAG-1 esterase prepared from induced *E. coli* strain BL21(pESTAL-14b) was purified with Novagen His-Bind resin. Aliquots of column fractions were separated by SDS–12% PAGE and stained with Coomassie blue. Lanes: 1, start material; 2, column flowthrough; 3, wash with 5 mM imidazole; 4, wash with 80 mM imidazole; 5, elution with 1 M imidazole.

to cleavage of the signal peptide in the protein produced in RAG-1 and to the absence of the His tag fragment on the N terminus.

The majority of overexpressed esterase protein appeared in inclusion bodies (not shown). About 20% of the esterase protein was estimated to be present in a soluble, catalytically active form, which was subsequently purified from cell extracts by affinity chromatography on Ni columns (Fig. 2). The specific activity of the enzyme in the crude extract was 2,340 U/mg of protein, while purified esterase specific activity was 50,200 U/mg of protein, with a yield of 55.5%. In addition, a procedure for solubilizing the esterase protein present in inclusion bodies (as described in Materials and Methods) yielded a soluble form of the enzyme that lacked catalytic activity.

Site-directed mutagenesis of recombinant esterase from RAG-1. The amino acid sequence deduced from the open reading frame of the *est* gene showed homology to several proteins from the PIR and Swiss-Prot libraries, as determined with the FASTA sequence comparison (2). The homologous proteins were primarily esterases and lipases. The conserved sequence GDSCG in the RAG-1 esterase corresponds to the G-X_1-S-X_2-G box found in lipases and esterases as well as in serine proteases (7). The serine in this sequence is probably the nucleophilic residue in the catalytic triad, consisting of an additional histidine and aspartate or glutamate (6). The RAG-1 esterase also contains the sequence ^{79}HGGAF, corresponding to the sequence HGGGF found in almost all lipases and esterases. In addition to these two conserved motifs, ^{110}D, ^{196}D, ^{244}E, and ^{274}H were also conserved (1).

Since each of the amino acids mentioned above could be part of the catalytic triad serine-histidine-aspartate/glutamate, they were systematically replaced by site-directed mutagenesis with either glycine or alanine (Materials and Methods). The mutant proteins were overexpressed and subsequently analyzed for esterase activity. With one exception, mutant ^{110}D-G, none of the glycine- or alanine-substituted mutant proteins showed any esterolytic activity in either whole cells or cell extracts. Moreover, as with the parental overexpressed recombinant enzyme, the mutant proteins were found primarily in inclusion bodies (not shown). These aggregates of mutant proteins were subsequently solubilized with detergent and tested for emulsification enhancement (Table 1).

Enhancement of emulsifying activity of apoemulsan by recombinant wild-type and mutant esterases. According to previous reports, the hydrocarbon substrate specificity of emulsan requires that the emulsified substrate contain a mixture of both aromatic and aliphatic residues (35). Moreover, while some small residual activity towards hexadecane alone was present in crude emulsan preparations, the deproteinized form of emulsan, apoemulsan, completely lacked activity towards the hydrophobic substrate. Consistent with these results, addition of protein to apoemulsan resulted in emulsifying activity toward

TABLE 1. Effect of different proteins and mutant esterases on enhancement of apoemulsan emulsifying activity[a]

Protein	Avg emulsifying activity (U/mg of apoemulsan/mg of protein) (SD)
Recombinant esterase derivatives	
Wild type	300 (45)
$H^{79}A$	230 (35)
$H^{79}G$	170 (25)
$D^{110}G$	66 (10)
$S^{149}A$	240 (40)
$S^{149}G$	140 (25)
$D^{196}A$	100 (15)
$D^{196}G$	410 (65)
$E^{244}A$	250 (40)
$E^{244}G$	460 (75)
$H^{274}A$	230 (35)
$H^{274}G$	150 (25)
Other proteins	
Pancreatic lipase	52 (10)
Pig esterase	38 (10)
BD4 esterase	10
Lysozyme	80 (15)
Dockerin	0
Isopenicillin synthase	0
DNase	0
Bovine serum albumin	31

[a] Emulsification assays were carried out as described in Materials and Methods. One milligram of each protein was added to the reaction along with 0.1 mg of apoemulsan and 0.1 ml of hexadecane. The results are the averages of three independent assays. Numbers in parentheses are the standard deviations.

the aliphatic hydrophobic substrate hexadecane (Fig. 3). Maximal emulsifying activity of about 300 U/ml was obtained in the presence of 1 mg of the catalytically active recombinant esterase (specific esterolytic activity of 7,190 U/mg of protein). This corresponds to a 30-fold enhancement compared with emulsions generated by apoemulsan in the absence of protein. In the absence of apoemulsan, no emulsification was associated with either the overexpressed active enzyme or the solubilized inactive inclusion bodies. Emulsion stabilities of 64, 85, and 98% were found for emulsions formed with apoemulsan in the presence of 100, 300, and 750 μg of His-tagged recombinant esterase, respectively (see Materials and Methods).

All of the mutant proteins showed emulsification enhancement (Table 1). It is of interest that two of the mutant preparations were even more effective at enhancing emulsification than the wild type; mutant esterases $D^{196}G$ and $E^{244}G$ showed enhanced emulsification activities of 410 and 460 U/mg, respectively. In contrast, lower values were observed with mutants $D^{110}G$ and $D^{196}A$. It should be noted that the enhancement by mutant esterase preparations was the same regardless of whether the mutant proteins were solubilized from inclusion bodies or isolated in the soluble form (not shown). The mutants could be divided into two principal groups, according to the level of enhancement. The first group produced esterases that enhanced the emulsifying activity of the apoemulsan to about 230 U of esterase per mg and included mutants $H^{79}A$, $S^{149}A$, $E^{244}A$, and $H^{274}A$. The second group produced esterases that enhanced the emulsifying activity of the apoemulsan to a lower level (about 60 to 150 U of esterase per mg of protein) and included mutants $H^{79}G$, $D^{110}G$, $S^{149}G$, $D^{196}A$, and $H^{274}G$.

Specificity of protein requirement. Extracellular esterases from *A. venetianus* RAG-1 and *A. calcoaceticus* BD4 and BD413 are most likely members of the α/β-hydrolase family of proteins (2, 26). In order to determine whether enhancement of the apoemulsan-emulsifying activity was related in some way to this structural characteristic, other proteins belonging to the

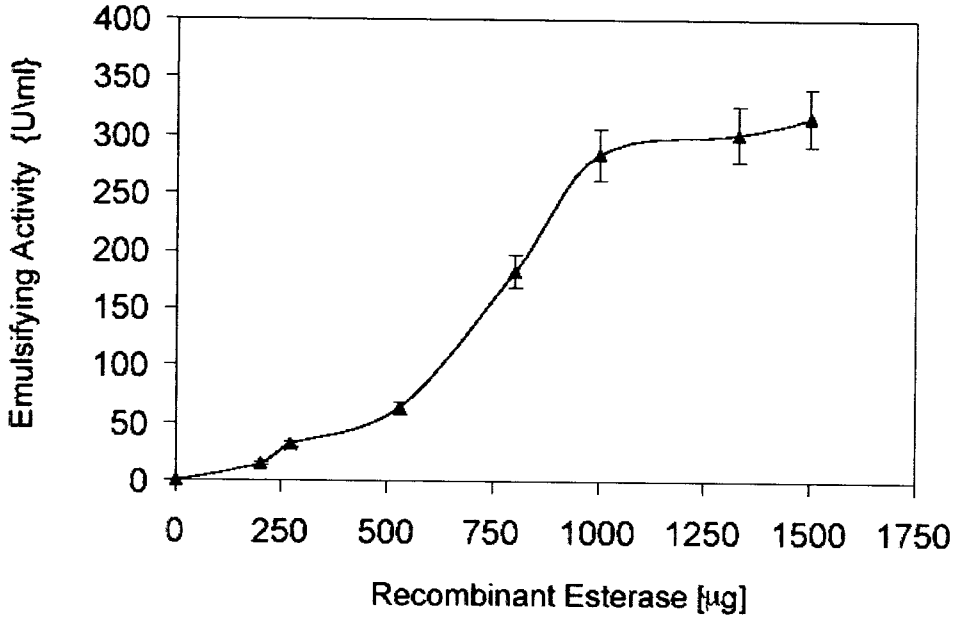

FIG. 3. Enhancement of emulsifying activity of apoemulsan with recombinant active RAG-1 esterase. Emulsions were prepared as described in Materials and Methods with increasing amounts of recombinant active esterase whose specific activity was 7,190 U/mg of protein in the presence of 0.1 mg of apoemulsan and 0.1 ml of hexadecane.

TABLE 2. Enhancement of apoemulsan activity on different hydrophobic substrates by recombinant esterase.

Hydrophobic substrate	Avg emulsifying activity (SD)	
	Emulsan (U/mg)	Apoemulsan + esterase (U/mg of apoemulsan/mg of esterase)
Anthracene	0	970 (170)
Crude oil	1,100 (140)	4,900 (800)
Dicyclohexane	310 (50)	2,800 (500)
Diesel oil	730 (130)	4,200 (700)
Eicosane	0	1,800 (260)
Fluoranthene	0	590 (150)
Heptadecane	120 (25)	2,400 (380)
Immersion oil	75 (15)	780 (100)
2-Methylnaphthalene	0	2,000 (400)
Mineral oil	450 (80)	3,000 (400)
Octadecane	0	2,200 (320)
Petroleum refinery sludge	580 (95)	1,200 (220)
Pyrene	0	420 (75)
Soya oil	0	1,300 (190)
Squalene	0	600 (150)
n-Tetracosane	0	510 (75)

[a] Emulsification assays were carried out as described in Materials and Methods; 1 mg of recombinant esterase was added to the reaction along with 0.1 mg of apoemulsan or emulsan and 0.1 ml of hydrophobic substrate. Results are presented as the averages of three independent assays. Numbers in parentheses are the standard deviations. Solid substrates were dissolved in acetone and added to the emulsification assay from a 1-mg/ml stock solution.

α/β-hydrolase family as well as unrelated proteins solubilized from inclusion bodies were examined for their ability to enhance emulsification of hexadecane by apoemulsan in water. Three α/β-hydrolases, including a His-tagged overexpressed form of the recombinant BD4 esterase from *A. calcoaceticus* BD4, pancreatic lipase, and pig esterase, were tested along with two unrelated proteins (both solubilized from inclusion bodies as described in Materials and Methods), dockerin and isopenicillin synthase. Dockerin serves as a docking surface for assembly of the multienzyme complex, the cellulosome, responsible for cellulose hydrolysis (28, 29). Bovine serum albumin was tested because of its stabilizing properties for oil-in-water emulsions (12), while lysozyme was assayed because of its polysaccharide-binding capacity. DNase served as a negative control.

It is clear from the results presented in Table 1 that only the recombinant RAG-1 esterase showed any appreciable enhancement of apoemulsan-mediated hexadecane emulsification. The only other protein that showed any enhancement was lysozyme, which gave only slight emulsification. Despite the structural similarities between the esterase from *A. venetianus* RAG-1 and the esterase encoded by the *estA* gene from *A. calcoaceticus* BD4 (2, 26), only the RAG-1 product was active in enhancing emulsification. However, the RAG-1 esterase was active in enhancing the emulsification activity of the capsular polysaccharide from *A. calcoaceticus* BD4 (not shown; H. Bach, unpublished data).

Hydrocarbon substrate specificity. In addition to the emulsification of hexadecane, the apoemulsan recombinant-esterase mixture was investigated for emulsification of a wide range of pure and crude oil products from a variety of sources, and the activities were compared with that of fully proteinated emulsan (Table 2). The esterase-apoemulsan mixture emulsified all of the substrates, although the emulsification activities varied with the different hydrophobic substrates. The most effective substrates for emulsification were the petroleum-based crude oils, while vegetable-based oils such as soy oil and microscope immersion oil were less effectively emulsified. When aromatic substrates were assayed, the emulsifying activity appeared to differ depending on the number of rings. For example, for a two-ring molecule such as 2-methylnaphthalene, an emulsifying activity of almost 2,000 U/mg of apoemulsan per mg of esterase was observed. With anthracene (three rings), only half of the activity obtained with a two-ring molecule was observed, while for a four-ring molecule such as pyrene, an emulsifying activity of only 420 U/mg of apoemulsan per mg of esterase was obtained. In the case of aliphatic hydrocarbons, the emulsifying activity appeared to depend to some extent on the number of carbon atoms in the hydrocarbon chain, decreasing from 2,400 U/mg of apoemulsan/mg of esterase in the case of heptadecane to only about 500 U/mg of apoemulsan/mg of esterase in the case of tetracosane (C-24). In all cases, apoemulsan alone was inactive. It is also of interest that the esterase-apoemulsan complex was more active than the original crude emulsan preparation from which the apoemulsan was prepared.

DISCUSSION

Many *Acinetobacter* strains produce polymeric bioemulsifiers, generally consisting of complexes of polysaccharides and proteins (25). The best characterized of these bioemulsifying complexes is emulsan, which is produced by the organism *A. venetianus* RAG-1. In this case, both an amphipathic heteropolysaccharide and a complex of associated proteins are required for optimal emulsifying activity towards a variety of hydrocarbon substrates (35). Recently, the genes encoding the biosynthetic enzymes responsible for the synthesis of the polysaccharide component, apoemulsan, were cloned, sequenced, and localized to a 27-kbp cluster, termed *wee*, in the *A. venetianus* RAG-1 chromosome (30). Mutants deleted for several of these genes were defective in emulsification, indicating that the polysaccharide is absolutely essential for extracellular emulsifying activity.

Here we show that the protein in the emulsan complex can be replaced with a His-tagged recombinant extracellular esterase overexpressed in *E. coli.* The enzyme has previously been shown both to bind to and partially deesterify the apoemulsan biopolymer (39).

Several microbial proteins have been shown to play a role in emulsification (39). In some cases, such as the protein-polysaccharide complex of *A. calcoaceticus* BD4, both fractions are absolutely required for emulsification (25), while in others the protein itself is the active emulsifying agent (41). In contrast to this OmpA-like protein in alasan (40, 41), the protein in emulsan is not an active bioemulsifier by itself but rather enhances the activity of the polysaccharide component, apoemulsan. The activity of apoemulsan in the absence of protein is due to the presence of the fatty acids which contribute to the amphipathicity of the apoemulsan polymer. This feature of apoemulsan is presumably the reason that emulsification of more polar hydrocarbon mixtures occurs in the absence of the protein fraction. For less polar hydrocarbons, such as hexadecane, the fatty

acid composition is insufficient to bring about emulsification. Results presented here with mutant proteins demonstrated that catalytic activity was not an essential element in the enhancement of emulsification. In this regard, an approach to engineering emulsan derivatives by modifying fatty acid composition during fermentation has been reported (14).

Apparently another domain(s) of the protein is involved in mediating the enhancing activity. In this regard, although the esterase has been implicated as a member of the α/β-hydrolase family (2), other proteins from this family, including the cloned esterase (EstA) from *A. calcoaceticus* BD413 (26), were inactive in this process. Efforts are currently in progress to identify the portion of the esterase protein that is responsible for emulsification enhancement.

Previous results had shown that emulsification of gas-oil by an apoemulsan preparation could be enhanced by about 50% by a crude denatured protein preparation obtained after hot phenol treatment of crude emulsan (47). Some nonspecific enhancement was also observed with several other proteins. It should be noted, however, that in the present work a different substrate for emulsification was used. Emulsification of hexadecane, a substrate normally not emulsified very well even by crude emulsan itself, was enhanced some 30-fold in the presence of recombinant esterase. In this regard, it was of interest that the esterase-apoemulsan complex was actually more effective in emulsifying a variety of hydrophobic substrates that are normally not emulsified by crude emulsan itself. This gain of function may be due to the presence of larger amounts of recombinant esterase in these experiments. In addition, it may be that the protein mixture associated with emulsan contains inhibitory proteins which could affect the activity towards many of these substrates. Preliminary evidence suggests that crude emulsan preparations contain such proteins, and efforts to isolate and characterize them are currently in progress (R. Avigad, unpublished data).

The results presented here suggest that with the recombinant protein, a more versatile emulsifier complex can be generated with a broader substrate range than normally observed for emulsan and possibly for other bioemulsifiers as well. The ability to produce the protein and its derivatives in *E. coli* in high quantities together with the existing fermentation technology available for production of the emulsan biopolymer in commercial quantities suggests the possibility of producing new bioemulsifier formulations based on well-defined protein (or perhaps peptide)-polysaccharide interactions.

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