Purification and Characterization of Liposan, a Bioemulsifier from *Candida lipolytica*†

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The inducible water-soluble bioemulsifier liposan (M. C. Cirigliano and G. M. Carman, Appl. Environ. Microbiol. 48:747–750, 1984) was purified from the yeast *Candida lipolytica*. The purification procedure included repeated solvent extractions of a concentrated culture filtrate and Affi-Gel concanavalin A affinity chromatography. The procedure yielded a preparation containing a major band (Mr = 27,600) which stained for protein and carbohydrate upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Liposan is composed of approximately 83% carbohydrate and 17% protein. Acid and enzymatic digestions of the emulsifier revealed that the carbohydrate portion is a heteropolysaccharide consisting of glucose, galactose, galactosamine, and galacturonic acid. Liposan effected and stabilized oil-in-water emulsions with a variety of commercial vegetable oils. Emulsification and stabilization properties of liposan were compared to those of a number of commercial emulsifiers and stabilizers.

In recent years there has been a growing interest in the isolation and identification of new microbial polysaccharides and surfactants that might have application in enhanced oil recovery processes (5, 9, 22, 24). The possibility of discovering a new microbial gum such as xanthan (2) or gellan (4) or a unique bioemulsifier like emulsan (27, 29, 30) that possesses novel properties allowing its use as a gelling agent, emulsifier, stabilizer, flocculant, lubricant, or dispersing agent has encouraged this interest (4, 5).

The yeast *Candida lipolytica* grows on agar plates containing a variety of water-immiscible carbon substrates (7). When grown in liquid medium containing hexadecane, *C. lipolytica* produces substances which reduce the interfacial tension in the medium (26, 28). *C. lipolytica* produces an inducible extracellular emulsification activity when grown with a number of water-immiscible carbon substrates (8). Emulsification activity is not produced by the organism when grown with glucose as the carbon source (8). A water-soluble emulsifying agent, which we call liposan, was partially purified from the cell filtrate of hexadecane-supplemented cultures of *C. lipolytica* (8). The partially purified preparation of liposan effects stable oil-in-water emulsions with a number of water-immiscible compounds (8). In this report we describe the purification of liposan to apparent homogeneity and the characterization of some of its chemical and physical properties. We also compared the emulsification and stabilizing properties of liposan to those of commercial emulsifiers and stabilizers.

**MATERIALS AND METHODS**

**Growth conditions.** *C. lipolytica* ATCC 8662 was obtained from the American Type Culture Collection. Stock cultures of the organism were maintained on yeast mold agar slants and transferred once a month. A 300-ml batch of YNB medium (0.6% yeast nitrogen base, pH 5.0) supplemented with 1% hexadecane was inoculated with 100 CFU of a 72-h culture of *C. lipolytica* grown in yeast mold broth per ml. The culture was incubated for 130 h at 27°C on a rotary shaker at 220 rpm.

**Purification of liposan.** The culture was refrigerated for 24 h at 4°C to solidify remaining hexadecane and to effect yeast settling. The following steps were performed at 25°C unless otherwise stated. The culture was filtered through a Schleicher & Schuell grade 588 fluted filter, followed by filtration through a 0.45-μm-pore-size membrane (Millipore Corp.). The cell-free filtrate (250 ml) was concentrated to 50 ml by pervaporation in dialysis tubing at 4°C. The concentrated filtrate was extracted three times with 500 ml of chloroform-methanol (2:1 [vol/vol]) as previously described (8). The white precipitate that formed after the third extraction was collected on Whatman no. 42 filter paper and air dried (8). A 4-mg sample (based on carbohydrate) of the white precipitate was dissolved in 2 ml of 20 mM Tris hydrochloride buffer (pH 7.4) containing 0.5 M NaCl and applied to an Affi-Gel concanavalin A (ConA) column (12.5 by 145 mm) equilibrated with the same buffer. The Affi-Gel ConA column was washed with 7 column volumes of elution buffer. The column was then saturated with 100 mM sodium acetate elution buffer (pH 3.6) and incubated overnight. Liposan was then eluted from the column with elution buffer at a flow rate of 10 ml/h. Fractions (3 ml) were collected and assayed for emulsification activity, carbohydrate, and protein.

**Electrophoresis.** Electrophoresis under nondenaturing conditions was performed in 7.7% polyacrylamide gel tubes (5 by 60 mm) as described by Davis (12). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in 10% polyacrylamide gel tubes (5 by 60 mm) as described by Weber and Osborn (33). Gels were stained for protein with Coomassie blue (33) and for carbohydrate with periodic acid-Schiff reagent (14).

**Bio-Gel A-1.5m column chromatography.** A sample of purified liposan (containing 50 μg of carbohydrate) was applied to a Bio-Gel A-1.5m column (2 by 42 cm) equilibrated and eluted with 100 mM potassium phosphate buffer (pH 7.2). Fractions (2 ml) were collected at a flow rate of 12 ml/h and analyzed for emulsification activity, carbohydrate, and protein.

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Assay of emulsification activity. Liposan emulsification activity was measured as previously described (8). Briefly, liposan was diluted with 100 mM sodium acetate buffer (pH 3.0) to a final volume of 4 ml and placed in a screw-capped test tube (15 by 125 mm); 1 ml of hexadecane (750 mg) was added, the tube was capped, and the mixture was shaken for 2 min at 25°C at a rate of 220 strokes per min (stroke length, 3 cm). The resulting uniform emulsion was allowed to sit for 10 min, after which its absorbance was measured at 540 nm. Liposan was omitted from the blank. One unit of emulsification activity was defined as that amount of emulsifier that effected an emulsion with an absorbance at 540 nm of 1.0.

Acid and enzymatic digestion of liposan. Purified liposan was subjected to acid hydrolysis with 4 N hydrochloric acid for 18 h and with 4 N sulfuric acid for 4 h as described by Keleti and Lederer (20). Liposan was enzymatically digested with beta-galactosidase, hemicellulase, hyaluronidase, neuraminidase, and pectinase under the conditions suggested by the supplier. The products of the acid and enzymatic digestions were subjected to ascending paper chromatography with sugar standards, using Whatman no. 1 filter paper in a solvent system containing n-butanol-pyridine-water (6:4:3 [vol/vol/vol]). The standards were arabinose, ribose, xylose, galactose, glucose, mannose, rhamnose, glucosamine, galactosamine, glucuronic acid, and galacturonic acid. Chromatograms were visualized with ninhydrin (amino sugars), alkaline silver nitrate (reducing sugars), and naphthoresorcinol (uronic acids) as previously described (20).

Analytical methods. Protein was determined by the method of Bradford (6), with bovine serum albumin as the standard. Carbohydrate was determined by the phenol-sulfuric acid procedure (20), using dextran as the standard. Fatty acids were analyzed by gas chromatography (8). Surface tension measurements were carried out as previously described (1).

Materials. All chemicals were reagent grade. Growth media and casein were purchased from Difco Laboratories. Affi-Gel ConA, Bio-Gel A-1.5m, bovine serum albumin, electrophoresis reagents, and molecular weight standards were purchased from Bio-Rad Laboratories. Dextran, sugars, polysaccharide digestive enzymes, Triton X-100, and Tween 20 were obtained from Sigma Chemical Co. Tween 80 was supplied by Atlas. Gum arabic and gum ghatti were purchased from TIC Gums Inc. Pectin and carageenin were obtained from Hercules Inc. Alginate was supplied by Kelco. Gelatin was obtained from Kind and Knox Inc. Hydroxypropylmethylcellulose was from Dow Chemical Co. Hexadecane was purchased from Eastman Kodak Co. Pure vegetable oils (with no added preservatives) were obtained from T. J. Lipton Co. Free fatty acids were removed from vegetable oils as previously described (7).

RESULTS

Purification of liposan. Liposan was partially purified from the culture filtrate of C. lipolytica grown in hexadecane-supplemented medium as previously described (8). The liposan precipitate contained both carbohydrate and protein and no fatty acids as previously described (8). About 53% of the emulsification activity that was present in the culture filtrate was recovered in the precipitate. The remaining emulsification activity was recovered in the organic solvent fraction and was not investigated in our studies. A portion of the liposan precipitate (containing 4 U of activity) was further purified by Affi-Gel ConA column chromatography (Fig. 1). About 38% of the liposan activity that was applied to the ConA column was eluted from the column after the
addition of the acetate buffer. Purified liposan contained approximately 83% carbohydrate and 17% protein. A summary of a purification for liposan is presented in Table 1. The purification procedure yielded essentially a homogeneous protein preparation as evidenced by polyacrylamide gel electrophoresis under nondenaturing conditions and in the presence of sodium dodecyl sulfate (Fig. 2). The apparent subunit molecular weight of the liposan protein was estimated to be 27,600 (Fig. 2). When a sodium dodecyl sulfate-polyacrylamide gel was stained with periodic acid-Schiff reagent, a diffuse carbohydrate band was observed with the same relative mobility as the stained protein band. When a sample of purified liposan was applied to a Bio-Gel A-1.5m gel filtration column, the carbohydrate and protein peaks were coincident with the emulsification activity peak.

**Acid and enzymatic digestions of liposan.** To characterize the carbohydrate associated with the liposan, the purified preparation was subjected to a series of acid and enzymatic digestions, followed by the identification of the products by paper chromatography (Table 2). When liposan was subjected to an 18-h digestion with 4 N hydrochloric acid at 100°C, glucose, galactose, galactosamine, and galacturonic acid were identified. Treatment with 4 N sulfuric acid for 4 h at 100°C, resulted in the identification of galactose. Treatment with beta-galactosidase, which is specific for terminal nonreducing beta-D-galactose residues, resulted in the identification of galactose and galacturonic acid. Hemicellulase, which catalyzes the hydrolysis of D-galactose from hemicellulose, hydrolyzed galactose and galactosamine from liposan. Treatment with hyaluronidase, which hydrolyzes beta-1,4 linkages between N-acetyl galactose and N-acetyl galactosamine and glucuronic acid, resulted in the identification of galactose. Neuraminidase and pectinase were not active toward liposan.

**Emulsification and stabilization properties.** Partially purified liposan has been shown to effect stable oil-in-water emulsions with a number of hydrocarbons (8). In this study

![Image](http://aem.asm.org/Downloadedfromhttp://aem.asm.org)
TABLE 3. Stabilization of vegetable oil emulsions by liposan

<table>
<thead>
<tr>
<th>Vegetable oil</th>
<th>Decay constant (Kd) (10^-3)</th>
<th>Difference</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Without liposan</td>
<td>With liposan</td>
</tr>
<tr>
<td>Cottonseed</td>
<td>-8.6</td>
<td>-2.6</td>
</tr>
<tr>
<td>Corn</td>
<td>-7.7</td>
<td>-2.6</td>
</tr>
<tr>
<td>Soybean</td>
<td>-7.3</td>
<td>-2.3</td>
</tr>
<tr>
<td>Peanut</td>
<td>-8.2</td>
<td>-3.3</td>
</tr>
<tr>
<td>Olive</td>
<td>-5.4</td>
<td>-2.7</td>
</tr>
<tr>
<td>Safflower</td>
<td>-6.6</td>
<td>-4.3</td>
</tr>
<tr>
<td>Sunflower</td>
<td>-6.2</td>
<td>-4.1</td>
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</table>

* The emulsification assay was performed in the absence and presence of liposan (0.8 mg based on carbohydrate) as described in the text, except that the indicated vegetable oils (750 mg) were substituted for hexadecane. After the initial 10-min holding period, absorbance readings were taken every 10 min for 50 min. The log of the absorbance was then plotted versus time, and the slope (decay constant, Kd) of the line was calculated.

we examined the ability of partially purified liposan to emulsify and stabilize oil-in-water emulsions using commercial preparations of vegetable oils. The emulsification and stabilization of a soybean emulsion in the absence and presence of liposan is shown in Fig. 3. The presence of liposan in the soybean emulsion reduced the rate of emulsion decay over a 50-min time period. The slope of the emulsion decay plot was used as a decay constant, Kd, to describe the stabilization ability of liposan. Emulsion decay plots were constructed for a number of vegetable oil emulsions in the absence and presence of liposan and the Kd were calculated (Table 3). The smaller the Kd, the greater the stability. All of the oils examined showed a reduction in emulsion decay in the presence of liposan. By taking the difference in Kd for the decay plots with and without liposan, the relative stabilizing effect on each oil emulsion was compared. The greater the difference in the Kd, the more effective liposan was as a stabilizer. The greatest stabilizing effect by liposan was for the cottonseed, corn, soybean, and peanut oil emulsions.

Several commercial emulsifiers-stabilizers were examined for their emulsification and stabilization properties, using hexadecane as the water-immiscible oil. These agents are ranked in Table 4 according to their emulsification and stabilization abilities. Of the thirteen agents examined, liposan ranked sixth in its effectiveness as an emulsifier and eighth as a stabilizer.

**Surface tension.** Partially purified liposan was dissolved in aqueous solutions at concentrations ranging from 0.002 to 0.2% (wt/vol). The 0.2% solution of liposan is the concentration routinely used in the emulsification assay (8). Liposan did not significantly reduce the surface tension of water (72.8 dyn/cm at 23°C) at any of the concentrations examined.

**DISCUSSION**

The extracellular water-soluble emulsifier liposan is produced by *C. lipo-lytica* during the latter stages of fermentation when grown with hexadecane as the carbon substrate (8). In this communication we describe purification of liposan by repeated chloroform-methanol-water phase partitions of the culture filtrate and affinity chromatography with Affi-Gel ConA. Electrophoresis of the Affi-Gel ConA fraction under nondenaturing conditions and in the presence of sodium dodecyl sulfate indicated that liposan was purified to apparent homogeneity with respect to protein. When the polyacrylamide gel was stained for carbohydrate with periodic acid-Schiff reagent, a diffuse band was present at the same Mr as the stained protein band. The association of the carbohydrate peak with the protein peak after Bio-Gel A-1.5m chromatography further indicated that liposan was a glycoprotein. Purified liposan was composed of approximately 83% carbohydrate and 17% protein. The sugar content of liposan is similar to that of the emulsifier emulsan, which also contains glucose, galactosamine, and a uronic acid in its carbohydrate moiety (36). Where extracellular bioemulsifiers of microbial origin have been characterized chemically, most have been determined to be either glycolipid (9, 10, 16, 18, 19, 22, 32) or lipoprotein (3, 17) in nature, or consist of protein, carbohydrate, and lipid (31, 35, 36). Microbial- and plant-derived gums (2, 15, 25) as well as some plant and animal proteins (11, 21, 23, 34) have also been found to possess emulsification abilities. The association of protein with gellan gum, xanthan gum, and other microbial-derived polysaccharides has been reported (4, 5). Whether liposan requires both protein and carbohydrate moieties for emulsification activity is currently under investigation in our laboratory.

We examined the ability of liposan to emulsify and stabilize a number of commercial vegetable oils. Owing to the potential feasibility of commercial production, we used partially purified liposan (material that precipitated after solvent extractions; 8) in our studies. The commercial vegetable oils did form emulsions with water in the absence of liposan; however, these emulsions rapidly separated after 10 min. The addition of liposan to the emulsions stabilized them over the 50-min time period examined. Liposan was most effective in stabilizing cottonseed oil, corn oil, soybean oil, and peanut oil emulsions. Although we did remove free fatty acids from the commercial vegetable oils, commercial oils do contain trace amounts of monoglycerides and diglycerides (13) which could account for the emulsification in the absence of liposan.

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**LITERATURE CITED**