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Relationship between emulsifying activity and carbohydrate backbone structure of emulsan from Acinetobacter calcoaceticus RAG-1

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Various emulsan samples with the different degrees of branching of the carbohydrate backbone were obtained from Acinetobacter calcoaceticus under different culture conditions. The emulsifying activity of emulsan had a linear correlation to the branching degrees of the carbohydrate backbone ($r^2 = 0.930$) suggesting that the structure of carbohydrate backbone was an important factor influencing emulsifying activity.

Introduction

Emulsan is a well-known lipopolysaccharide biosurfactant produced by *Acinetobacter calcoaceticus* RAG-1 and its molecular weight is about 1,000 kDa in average. Emulsan is composed of carbohydrate backbone as its hydrophilic portion and a fatty acid as its hydrophobic portion, the latter is attached to the carbohydrate backbone via ester and amide bonds (Gutnick, 1987). It is a strong emulsion stabilizer rather than an interfacial tension reducer (Hayes *et al.*, 1986). Since an emulsion once formed can be stable and will exhibit the very high affinity for oil and water interface, a low concentration of emulsan is required (Zosim *et al.*, 1982). Owing to these properties, emulsan can commercially be applied to emulsion stabilization, heavy oil transportation, and solubilization (Gutnick *et al.*, 1991). Emulsan, however, has a disadvantage that its composition and properties can be varied with the culture conditions of *A. calcoaceticus* (Sar and Rosenberg, 1983). By the reason of this, it is important to elucidate the factors influencing its emulsifying activity.

In this article, the emulsifying activity of emulsan from *Acinetobacter calcoaceticus* RAG-1 is investigated and the relationship between the carbohydrate backbone structure and its emulsifying activity is described.

Materials and methods

Microorganism and culture conditions

Acinetobactor calcoaceticus RAG-1 (ATCC 31012) was grown on 10 g carbon source (ethanol or various fatty acids), 2 g urea, 16.9 g K_2HPO_4, 7.3 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 1.08 mg $CaCl_2.2H_2O$, 1.87 mg $CuSO_4.5H_2O$, 1.81 mg $FeSO_4.7H_2O$, 1.78 mg $MnSO_4.4H_2O$, 1.27 mg $ZnSO_4.7H_2O$, 2.36 mg $CoCl_2.6H_2O$, and 2.10 mg Na_2MoO_4. The strain was inoculated to the 50 ml medium in a 250 m*l* Erlenmeyer flask at 30°C, 250rpm, for 24 h. The resultant culture of 5 m*l* was transferred to the 500 m*l* medium in a 2.8 *l* Furnback flask. The culture was incubated in a NBS-rotary shaking incubator at 30°C, 250 rpm for 72 h. The initial pH of the culture was 7.3.

Isolation and purification of emulsan

Cells were removed from the culture broth by centrifugation. Ammonium sulfate, 137 g, was added to the supernatant solution of 500 m*l* which was precipitated during overnight in a cold room of 4°C. The precipitate was dialyzed with a dialysis tubing (MW cut off: 12,000–13,000) and the residue was freeze-dried. The freeze-dried sample was extracted with diethyl ether in a Sozhlet apparatus and dried again. the emulsan obtained was used for the structural analysis (Kaflan, *et al.*, 1982).

Measurement of emulsifying activity and fatty acid content

0.1 m*l* test oil (hexadecane:2-methylnaphthalane = 1:1, v.v) and 1 mg emulsan sample were dispersed 7.5 m*l* TM buffer (20 mM Tris-HC1, pH 7.2, 10 mM $MgSO_4.7H_2O$) in a 125 m*l* Erlenmeyer flask (Zukerberg *et al.*, 1979). The mixture was reciprocally shaken at

30°C for 1 h (150 strokes/min) and was used for the measurement. The emulsifying activity was determined by measuring the increased in optical density of 600 nm. One unit of emulsifying was defined as one absorbence in a spectrometer. In addition, the fatty acid content of emulsan was determined by GLC after hydrolysis, extraction, and methylation of one (Belsky *et al.*, 1979).

Determination of branching degree of emulsan

To determine the branching degree of carbohydrate backbone in emulsan, the free hydroxyl or amide group should be methylated. Methylation of emulsan was carried out using methyl tri-fluoromethanesulfonate with 2,6-di-(*tert*-butyl)pyridine and trimethyl phosphate under nitrogen gas (Prehm, 1980). The methylated emulsan was subsequently hydrolyzed, reduced, and acetylized according to the method of conventional analysis (Stellener *et al.*, 1973). The terminal carbohydrate of emulsan was modified to tetramethyl-diacetyl-alditol, the branched carbohydrate to dimethyl-tetraacetyl-alditol, and the non-terminal non-branched carbohydrate to trimethyl-triacetyl-alditol (Fig. 1). The partially methylated and the acetylated monosaccharides were obtained by GLC and GC-MS. The branching degree was calculated as a peak area ratio of dimethyl-tetraacetyl alditol of branched carbohydrate (B) to trimethyl-triacetyl alditol of non-terminal non-branched carbohydrate (C).

Results and discussion

Various emulsan samples of different branching degrees and emulsifying activities were obtained under various culture conditons such as different temperatures, shaking conditons, concentrations of inhibitor (cerulenin), addition of fatty acids at 24 h, and culture times (Table 1). The branching degree of emulsan was estimated by the ratio of branched carbohydrate of emulsan

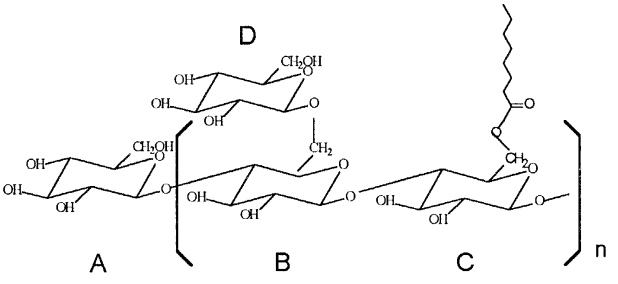

Figure 1 Schematic structure of emulsan A, D: Terminal carbohydrate was modified to tetramethyl-diacetyl alditol; B: Branched carbohydrate was modified to dimethyl-tetraacetyl alditol; C: Non-terminal non-branched carbohydrate was modified to trimethyl-triacetyl alditol; n = 1000–1500.

Table 1 Branching degree and emulsifying activity of emulsan under various culture conditions

Culture condition	Branching degree (arbitrary unit)	Emulsifying activity (unit/mg)
10 g oleic acid/l, 20°C	0.82	1.42
10 g oleic acid/l, 25°C	0.48	1.20
10 g oleic acid/l, 30°C	0.70	1.77
10 g ethanol/l, 30°C, urea deficiency	0.44	0.94
10 g ethanol/l, flask with rubber stopper (dissolved oxygen limitation)	0.11	0.25
10 g ethanol/l, round flask	0.98	2
10 g ethanol/l, baffled flask	0.23	0.58
10 g ethanol/l, dilution rate = 0.05 h^{-1}	0.75	1.40
10 g ethanol/l, dilution rate = 0.10 h^{-1}	0.96	1.96
10 g ethanol/l, dilution rate = 0.15 h^{-1}	0.87	1.56
10 g ethanol/l, dilution rate = 0.20 h^{-1}	0.23	0.22
10 g ethanol/l, 0.5 mg cerulenin/l	0.70	1.77
10 g ethanol/l, 1 mg cerulenin/l	1.50	2.89
10 g ethanol/l, 2 mg cerulenin/l	0.50	1.40
8 g ethanol/l, 0.3 mg cerulenin/l at 24 h 2 g pentanoic acid methyl ester/l at 24 h	1.55	2.50
8 g ethanol/l, 0.3 mg cerulenin/l at 24 h 2 g myristic acid methyl ester/l at 24 h	0.32	0.76
8 g ethanol/l, 0.3 mg cerulenin/l at 24 h 2 g stearic acid methyl ester/l at 24 h	0.92	1.90
10 g ethanol/l, culture time = 54 h	0.48	1.20
10 g ethanol/l, culture time = 72 h	1.11	2.29

to the non-terminal, non-branched ones. Five samples containing approximately the same content of fatty acid were selected to measure the emulsifying activity. As shown in Fig. 2, the emulsifying activity increased with increasing the branching degree even though the emulsan had the same content of fatty acid. This result suggested that the higher branching degree exhibited the higher emulsifying activity.

To investigate the relationship between the structure of carbohydrate backbone and the emulsifying activity of *Acinetobacter calcoaceticus* RAG-1 emulsan, the emulsifying activity against the branching degree was plotted (Fig. 3). The ussed emulsan exhibited various branching degrees and different fatty acid contents. The emulsifying activity of emulsan (Y) was closely related to its branching degree (X) via the correlation of $Y = 2.0\ X$, $r^2 = 0.930$. This expression is generally valid for a variety of emulsan produced from different culture conditions and suggests that the structure of the

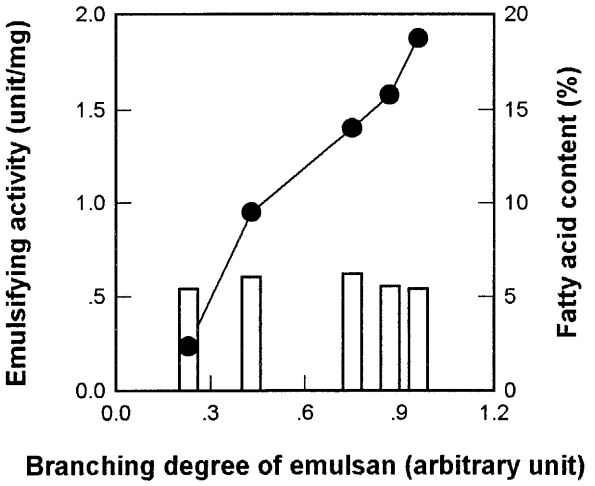

Figure 2 Relationship between branching degree and emulsifying activity of emulsan with approximately the same content of fatty acid. Emulsifying activity (●) and fatty acid content (□).

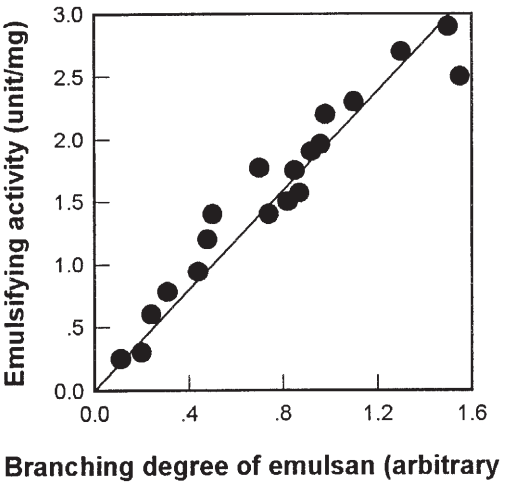

Figure 3 Emulsifying activity as a function of branching degree of emulsan.

carbohydrate backbone in emulsan determines its emulsifying activity.

It was previously reported that emulsan was a good emulsion stabilizer rather than a good emulsifier due to its branching arm of backbone in addition to its polymeric nature (Hayes *et al.*, 1986). Once the emulsion

In conclusion, the emulsifying activity of emulsan can be predicted by estimating its branching degree of carbohydrate backbone because it increases with increasing the branching degree.

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