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Characteristics of Heat-Stable Extracellular Hemolysin from *Pseudomonas aeruginosa*

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Heat-stable hemolysin produced by the cellophane-agar plate method consisted of two major acidic glycolipids. One was composed of 2 mol of rhamnose and 2 mol of β-hydroxydecanoic acid, and the other was composed of 1 mol of rhamnose and 2 mol of β-hydroxydecanoic acid. The hemolysis-producing moiety of the hemolysin was shown to be the dimer of β-hydroxydecanoic acid contained in the glycolipid molecule.

Although it is difficult to obtain hemolysin from *Pseudomonas aeruginosa* grown in broth culture, Liu (6) reported in 1957 that hemolysin could be produced easily and rapidly by this organism when it was grown on cellophane sheets over nutrient-glucose agar plates. On the other hand, in 1949 Jarvis and Johnson (4) reported the production of a crystalline acidic glycolipid by *P. aeruginosa* in peptone-glycerol broth; this material had bacteriostatic activity against tubercle bacilli. This glycolipid consisted of 2 mol each of L-rhamnose and β-hydroxydecanoic acid (2-*o*-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate). The present report deals with the biochemical properties of hemolysin obtained from *P. aeruginosa* by the cellophane-agar plate technique (6) and the relationship of this hemolysin to the glycolipid obtained by the method of Jarvis and Johnson (4).

One strain of *P. aeruginosa* isolated from a patient with a urinary tract infection, strain 10-7113, was used in this study. The medium consisted of 0.8% nutrient broth (Difco Laboratories, Detroit, Mich.), 1% glucose, and 1.5% agar. An ordinary cellophane membrane was cut to fit a petri dish and sterilized with ethylene oxide gas. After the medium was poured in a petri dish and solidified, the cellophane membrane was placed thereon and stretched out aseptically. After the plate was kept overnight at 37°C, 2 or 3 drops of a broth culture of the organism were spread over the cellophane membrane with a sterile cotton swab and the plate was incubated at 37°C for 48 h. At the end of the incubation period, the bacterial lawn growing on the cellophane membrane was washed off with 3 to 5 ml of distilled water and centrifuged at 3,300 × *g* for 20 min to remove bacterial cells. The supernatant fluid possessed hemolytic activity amounting to 256 to 512 hemolytic units (HU) per ml. (The hemolytic activity was titrated by twofold dilution of the fluid in 0.5 ml of phosphate-buffered saline [pH 5.9]. A 0.5-ml portion of a 1% suspension of sheep erythrocytes which had been washed three times was added to each of the tubes containing the dilutions of the hemolysin, and the final reading of the hemolysis was made after 1 h of incubation at 37°C. The reciprocal of the highest dilution showing complete hemolysis was taken as the hemolytic units of the preparation.) These supernatant fluids were stored at −20°C for future use.

For biochemical analysis, the supernatant fluids were acidified to pH 2 with HCl and centrifuged at 30,000 × *g* for 30 min. The precipitate, which showed hemolytic activity, was dissolved in ethanol and centrifuged at 3,300 × *g* for 20 min to remove the insoluble materials. The soluble fraction was dried, dissolved in chloroform-methanol (2:1, vol/vol) and partitioned by the method of Folch et al. (3). Almost all of the hemolytic activities were found in the chloroform fraction.

Thin-layer chromatography (TLC) of the lipid extract using a silica gel H plate and a solvent system of chloroform-methanol-concentrated ammonia (65:35:5, vol/vol/vol) yielded two major spots (A and B; R_fs of 0.27 and 0.11, respectively), both of which were anthrone positive but ninhydrin and Dittmer reagent negative (Fig. 1, column 1). The two compounds were produced at almost the same ratio (A:B = ca. 1.6:1, wt/wt) on the cellophane-agar plates for 1 to 5 days. These glycolipids possessed approximately equal hemolytic activity.

To compare the hemolytic glycolipids with the rhamnolipid isolated from a peptone-glycerol medium culture of *P. aeruginosa*, the same strain of *P. aeruginosa* was grown in 4% peptone–3% glycerol broth at 30°C on a reciprocating shaker and cultures were harvested after 5 days by the method of Jarvis and Johnson (4). Then the lipid was extracted by the same procedure as in the case of the hemolysin separation. By TLC of this material, one major spot (B′) which had an R_f value similar to that of spot B of the hemolysin preparation was obtained (Fig. 1, column 2).

The three compounds (spot A, B, and B′ materials) were isolated by column chromatography on DEAE-cellulose (1) followed by preparative TLC using the same plate and solvent system as mentioned above. To identify the sugar components of the three purified glycolipids, the glycolipids were hydrolyzed with 1 N HCl in 50% dioxane for 2 h at reflux temperature and the water-soluble fraction obtained by acid hydrolysis was lyophilized after neutralization with Dowex-1:hydroxy-form resin. Then the hydrolyzed products were subjected to paper chromatography with two different solvent systems, *n*-butanol–pyridine–water (6:4:3, vol/vol/vol) and isopropanol-pyridine-water-acetic acid (8:8:4:1, vol/vol/vol/vol). By paper chromatography, the sugar components of all samples were identified as L-rhamnose (data not shown).

The sugar contents of spot A and B materials were

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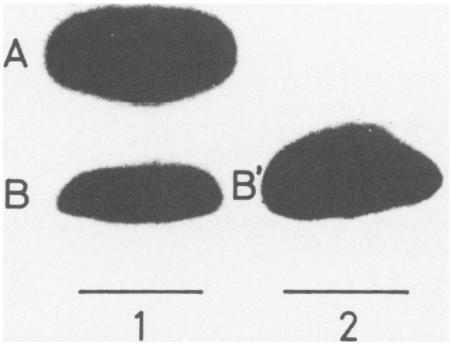

FIG. 1. Comparison of hemolysin (column 1) with rhamnolipid (column 2) from *P. aeruginosa* by TLC. The spots were developed by spraying with 50% sulfuric acid and heating at 120°C for 10 min. All spots were anthrone positive.

determined by the anthrone method to be 36.7 and 63.5%, respectively. The theoretical values, calculated on the basis of the fact that each rhamnolipid contains 1 or 2 mol of rhamnose, were 32.5 and 49.7%, respectively.

For identification of the fatty acid components of the three glycolipids, samples were esterified by the boron trifluoride-methanol method (8). Then gas chromatography was performed on a glass column (3 mm by 3 m) packed with 10% diethylene glycol succinate polyester on Chromosorb W. All samples showed five peaks, but by plotting of the carbon number and relative retention time of standard β-hydroxy fatty acids the main peak was shown to be 10:0 β-hydroxy fatty acid (data not shown).

Infrared spectrophotometry was done with 5% chloroform solutions of each glycolipid. Spot B material had twice the hydroxy band absorption of spot A material, but no qualitative differences between them were noted. The infrared spectrophotometric profile of spot B′ material was the same as that of spot B material.

Furthermore, hydrolysis of the glycolipids was performed to investigate the relationship between the hemolytic activity and the molecular composition of the ether-soluble fractions. The hydrolysates were monitored by TLC using the same plate and solvent system mentioned above. To cut mainly the glycoside linkage between rhamnose, rhamnose, and the fatty acid component, acid hydrolysis was performed. By acid hydrolysis of spot A or B material with 2 N HCl in a sealed ampoule at 100°C for 2 h, a major spot (C; R_f of 0.44) and a minor spot (D; R_f of 0.23) were detectable on TLC (Fig. 2, column 2). By more drastic acid hydrolysis using 1 N HCl in 50% dioxane for 2 h at reflux temperature, a major spot (D) and a minor spot (C) were obtained (Fig. 2, column 3). To cut mainly the ester linkage between fatty acids, alkaline hydrolysis using 1 N KOH at 50°C for 2 h was done. By alkaline hydrolysis of spot A material, two major spots (E [R_f of 0.08] and D) and a minor spot (C) appeared (Fig. 2, column 4). By alkaline hydrolysis of spot B material, two major spots (F [R_f of 0.04] and D) and a minor spot (C) appeared (Fig. 2, column 5). After alkaline hydrolysis of spot C material the appearance of spot D was noted (Fig. 2, column 6), whereas spot D material remained unchanged by this treatment. Spots A, B, E, and F were anthrone positive, but spots C and D were anthrone negative. On the basis of these results, the hydroxy group of β-hydroxydecanoic acid seemed to form an alkali-labile ester bond with the carboxyl group of another β-hydroxydecanoic acid, since the major spot, D, which seemed to be free β-hydroxydecanoic acid, was found by alkaline hydrolysis of spot A, B, and C materials. On the basis of the present findings and of the rhamnolipid structure proposed by Edward and Hayashi (2), the chemical structure of each spot compound was considered to be as follows: A, rhamnose-β-hydroxydecanoic acid-β-hydroxydecanoic acid; B, rhamnose-rhamnose-β-hydroxydecanoic acid-β-hydroxydecanoic acid; C, β-hydroxydecanoic acid-β-hydroxydecanoic acid; D, β-hydroxydecanoic acid; E, rhamnose-β-hydroxydecanoic acid; F, rhamnose-rhamnose-β-hydroxydecanoic acid.

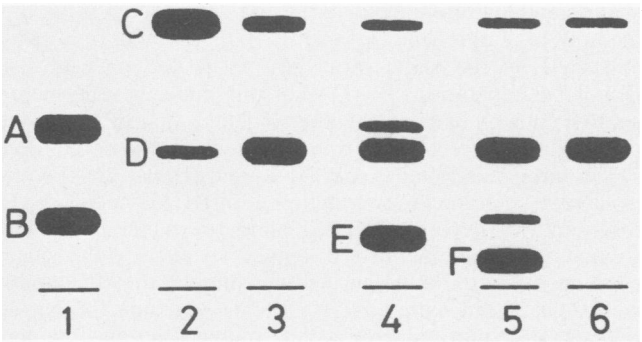

FIG. 2. Comparative illustration of various hydrolysates of hemolysin from *P. aeruginosa* by TLC. The spots were developed by spraying with 50% sulfuric acid and heating at 120°C for 10 min. Columns 1, hemolysin; 2, products of acid hydrolysis (2 N HCl at 100°C for 2 h) of hemolysin; 3, products of acid hydrolysis (1 N HCl in 50% dioxane for 2 h at reflux temperature) of hemolysin; 4, products of alkaline hydrolysis of spot A material; 5, products of alkaline hydrolysis of spot B material; 6, products of alkaline hydrolysis of spot C material. Spots A, B, E, and F were anthrone positive; spots C and D were anthrone negative.

The hemolytic titers of spot A, B, and C materials were 32 to 64 HU/mg, but those of spot D, E, and F materials were 1 to 2 HU/mg.

It is well known that some strains of *P. aeruginosa* produce heat-stable hemolysin, and a pathogenic role of hemolysin is suggested in ocular and pulmonary infections (7). From the experimental data, the hemolysin of *P. aeruginosa* obtained by the cellophane-agar plate technique was found to contain two acidic glycolipids, as suggested by Johnson and Boese-Marrazzo (5). One is identical to the rhamnolipid reported by Jarvis and Johnson (4), and the other is a rhamnolipid composed of 1 mol of rhamnose and 2 mol of β-hydroxydecanoic acid. It is obvious from our results that the hemolytic activity of this hemolysin is due to the dimer of β-hydroxydecanoic acid contained in the glycolipid molecule. The hemolytic mechanism of this hemolysin seems similar to some detergents, such as certain types of sodium salts of fatty acids.

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