Effect of the Hemolysin of *Pseudomonas aeruginosa* on Phosphatides and on Phospholipase c Activity

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ABSTRACT

The hemolysin of *Pseudomonas aeruginosa* was found to function as a detergent in solubilizing various phosphatides. Incorporation of the hemolysin into reaction mixtures containing phosphatides and phospholipase c significantly increased the rates of enzyme activities. Stimulation of enzyme activity was most likely due to improved dispersion of the substrates.

Production of hemolytic substance by *Pseudomonas aeruginosa* was first described by Bullock and Hunter (2) and was confirmed by Landsteiner and Raubilschek (8). The latter workers pointed out that this hemolytic substance was soluble in lipid solvents and, therefore, was quite dissimilar to other bacterial hemolysins which are usually protein in nature. In 1949, Jarvis and Johnson (7) described production by *P. aeruginosa* of a crystalline acidic glycolipid consisting of two moles of L-rhamnose and 1-β-hydroxydecanoic acid. This observation was confirmed by Hauser and Karnovsky (5). Although the hemolytic activities of these products were not examined, the identity of this glycolipid with the hemolysin described by earlier investigators was pointed out by Sierra (14).

It was found in this laboratory (9) that active hemolysin of *P. aeruginosa* could be produced rapidly on a plate covered with cellophane. Nutritional requirements for the production of the hemolysin were similar to the requirements for the production of phospholipase c (11), although there was no question that the two substances were distinct entities (12). In the titration of phospholipase c activity with egg yolk solution, we have noted peculiar effects of the hemolysin on the reaction. When the crude preparation of phospholipase c containing hemolysin was serially diluted, the tubes containing a high concentration of the enzyme and hemolysin often failed to show turbidity of the yolk, and typical reactions appeared only on further dilution. However, phospholipase c activity could be demonstrated readily in such tubes by the appearance of acid-soluble phosphate or choline. Inhibition of turbidity development in the egg yolk suspension was not observed if the phos-

pholipase c was previously separated from the hemolysin. This finding suggested that the hemolysin may solubilize the lipids which had become insoluble after the destruction of the lecithin by phospholipase c. The present communication described the detergent-like activity of the hemolysin.

MATERIALS AND METHODS

Preparation of the culture fluid of P. aeruginosa. Two strains of *P. aeruginosa* were used in this study. PA-103 produces only phospholipase c, and PA-7 produces both phospholipase c and hemolysin (12). The organisms were grown in a synthetic liquid medium containing 0.29% DL-glutamic acid, 0.13% DL-alanine, 0.01% CaCl$_2$, 0.01% MgSO$_4$, 0.01% ZnSO$_4$, 0.61% glucose, and 0.43% NaCl. To this basic medium was added 3% (v/v) of the dialysate of Brain Heart Infusion (Difco) prepared as follows: 74 g of Brain Heart Infusion powder was suspended in 200 ml of water, and the suspension was dialyzed against 4.0 liters of deionized water at 5 C overnight. The dialysate contained 0.42 mg of inorganic phosphate and 0.17 mg of ester-linked phosphate per ml. The medium was adjusted to pH 7.0 with concentrated NaOH. A 250-ml amount of the medium was placed in a 1,000-ml flask and inoculated with 1.0 ml of an 18-hr-old broth culture of the organism. Incubation followed at 30 C for 24 hr in a Psycrotherm incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 180 rev/min.

Purification of the hemolysin of PA-7. The culture fluid of PA-7 was 80% saturated with ammonium sulfate after adjusting the fluid to pH 4.5 with glacial acetic acid. The mixture was allowed to stand at room temperature until the cells and insoluble substances rose to the surface. It was then filtered through Whatman no. 541 paper and washed with cold acetone until the filtrate was clear. The filtrate containing the hemolysin was dialyzed against running tap water overnight to remove the acetone. The insoluble sub-

stances precipitated during the dialysis were eliminated by decantation. The clear fluid was adjusted to a pH of 8 to 8.5 with concentrated NaOH and was treated with anionic ion-exchange resin (AG 1 × 2, Cl-type, Bio-Rad Laboratories, Richmond, Calif.) by a batch method. In this procedure, the hemolysin was absorbed on the resin. The hemolysin was further purified by column chromatography as follows: the resin was packed in a suitable column and was washed with deionized water and with 0.25 N NaOH. The hemolysin was eluted with 1 N NaOH in 50% ethyl alcohol. The eluate was dialyzed against deionized water at 4 C for 2 days. A white crystalline-like substance was precipitated gradually and collected by centrifugation.

Preparation of phospholipase c. Phospholipase c of PA-7 was present in the precipitate after the extraction of hemolysin in cold acetone. The precipitate was suspended in water, was dialyzed against deionized water at 4 C overnight, and was centrifuged at 8,000 rev/min for 40 min. The supernatant fluid was fractionated with ammonium sulfate. The activity of phospholipase c was precipitated at 30 to 50% saturation, completely free from the hemolysin. Phospholipase c of PA-103 was also separated by the same procedure.

Phospholipase c of *Bacillus cereus* was prepared in the same medium as used for *P. aeruginosa* except for the addition of 0.5% phosphatidylserine. Incubation was carried out at 37 C for 24 hr as before. Partial purification of this enzyme was accomplished by the following method: the supernatant fluid culture was added to 4 volumes of cold ethyl alcohol; the precipitate was collected with centrifugation, was resuspended in cold alcohol, and was centrifuged again to remove completely the phosphatidylserine. The precipitate was dissolved in 0.02 M tris(hydroxymethyl)-aminomethane chloride buffer (pH 7.2) and was dialyzed against the buffer overnight. This solution was fractionated with ammonium sulfate and the activity present in the fraction precipitated at 20 to 50% saturation.

Phospholipase c of *Clostridium welchii* was purchased from Worthington Biochemical Corp., Freehold, N.J. and was used after suitable dilution.

Titration of hemolysin. The estimation of the hemolytic units (HU) was done according to the method described previously (10).

Dispersion of lecithin by the hemolysin. Dispersion of lecithin by the hemolysin was demonstrated through diminution of the turbidity of lecithin sol. The latter was prepared as follows: small portions of alcoholic lecithin solution were added to water, and the nonaqueous solvent was subsequently removed by boiling. The turbidity was read at 500 mμ in a Beckman DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) and was expressed as the percentage of the original value of the lecithin sol.

Assay of phospholipase c. To determine enzymatic activity, the amount of acid-soluble phosphate was measured. The liberation of inorganic phosphate from the acid-soluble phosphate was accomplished by the method of Horecker et al. (6).

Choline determination. Choline determination was done according to the method of Appleton et al. (1).

RESULTS

Chromatography of hemolysin. Chromatography of the hemolysin is shown in Fig. 1. The turbidity of each fraction was read at 500 mμ after the addition of 2.0 ml of 1.5 N acetic acid to a 1.0-ml portion of each fraction. Fractions 12, 13, and 14 were combined and placed in a cellophane bag and then were dialyzed against deionized water. Crystalline-like hemolysin was found to contain 32 HU/mg.

Effect of the hemolysin on the lecithin sol. The lecithin sol possessed considerable turbidity; the addition of hemolysin to the sol produced a clear solution. This decrease in turbidity was probably due to further dispersion of lecithin particles. (Fig. 2).

Effect of the hemolysin on phospholipase c activity. Cultures of both PA-7 and PA-103 showed almost the same phospholipase c titer by use of the egg yolk titration method (10). However, the initial enzymatic velocities were quite different if the cultures were incubated with synthetic L-α-lecithin as shown in Fig. 3.

The difference was ascribed to hemolysin which is present in the preparation of PA-7 but not in that of PA-103. As shown in Fig. 4, the addition of hemolysin to the PA-103 preparation or to hemolysin-free PA-7 enzyme stimulated enzyme activity.

Effect of the hemolysin on other phospholipase c. In this experiment, phospholipase c of *B. cereus* and *C. welchii* were used. The addition of the hemolysin to incubation mixtures stimulated the rate of hydrolysis of synthetic lecithin just as it did the enzyme of *P. aeruginosa* (Fig. 5).
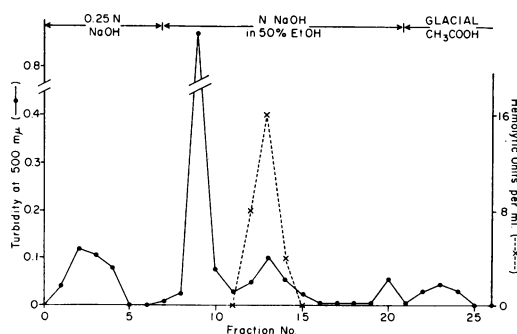
Effect of the hemolysin on the opalesence forma-

FIG. 1. *Chromatography of the hemolysin of Pseudomonas aeruginosa. Column was 2.5 × 15 cm, the resin was AG 1 × 2, Cl-type, and each fraction equalled 5 ml*
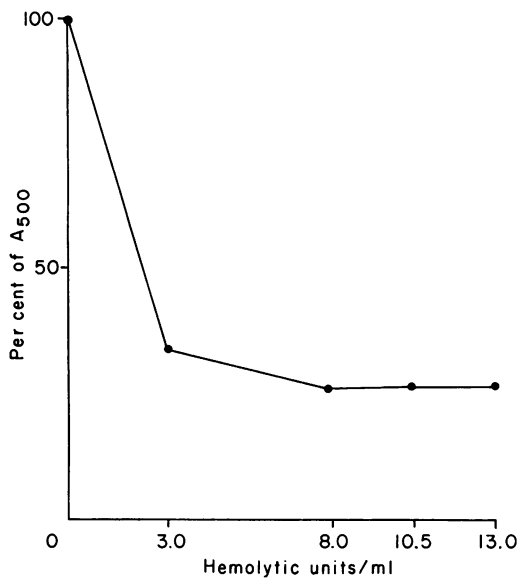
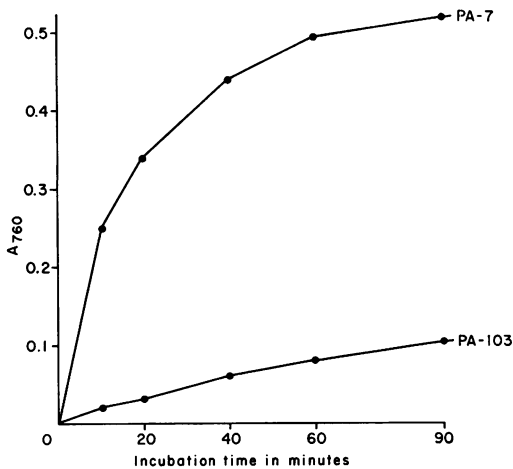FIG. 2. *Effect of the hemolysin on the turbidity of ecithin sol. Concentration of lecithin = 3 mM.*

FIG. 3. *Phospholipase c activity of different strains of Pseudomonas aeruginosa. Substrate: synthetic 5 mM L-α-lecithin.*

FIG. 4. *Stimulation of phospholipase c activity by the hemolysin. Substrate: synthetic 5 mM L-α-lecithin.*
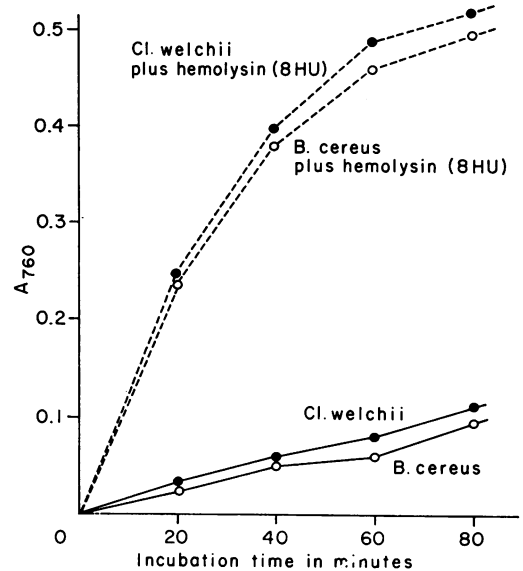
FIG. 5. *Effect of the hemolysin on phospholipase c activities. Substrate: synthetic 5 mM L-α-lecithin.*

tion in egg yolk solution. The addition of the hemolysin to the egg yolk reaction mixture inhibited the appearance of turbidity caused by phospholipase c (Fig. 6). The turbidity of the reaction mixture containing 5 HU/ml was almost the same as that of the reference. However, the amount of liberated choline did not change. This result indicated that the enzymatic reaction itself in the egg yolk solution was not affected by the presence of the hemolysin.
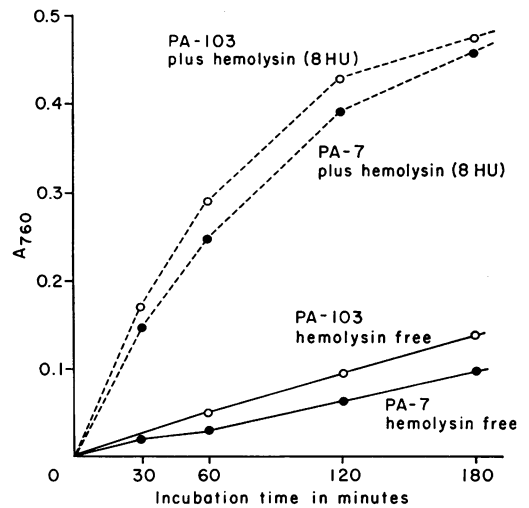
DISCUSSION

The roles of the hemolysin of *P. aeruginosa* in the physiology and pathogenesis of the species have been a subject of speculation since its presence was demonstrated in the culture. Most of the experimental data, however, would seem to suggest that the hemolysin by itself is relatively nontoxic (10) and that the amount of hemolysin produced in serum was so small that its toxicity would be negligible (12).
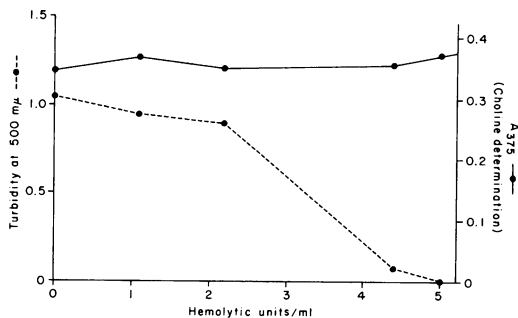
FIG. 6. *Effect of the hemolysin on the egg yolk reaction. Egg yolk concentration = 1.25%, alkaline phosphatase concentration = 0.2 unit per ml. Phospholipase c was a hemolysin-free PA-7 enzyme and incubation was at 37 C for 4 hr.*

In a study of the nutritional requirements of *P. aeruginosa* for the production of the hemolysin, of phospholipase c, and of proteinase (11), it was found that the requirements for hemolysin and for phospholipase c formation were the same and that the two substances were produced together; the conditions for proteinase production were different. These observations suggested the possibility that the hemolysin may in some ways cooperate with the phospholipase c. Inhibition of the appearance of turbidity in the egg yolk suspension treated with phospholipase c seemed, in the beginning, to suggest that hemolysin inhibited phospholipase c activity. However, this suspicion was dispelled by the demonstration that liberation of phosphate and choline in the clear egg yolk solution was actually occurring as rapidly in the presence of the hemolysin as in its absence. The phenomenon was eventually explained by the finding that the hemolysin exhibited detergent-like effects in solubilizing various types of lipids and phosphatides.

It is interesting to note that, in a medium containing a very low concentration of phosphate and a high concentration of glucose, *P. aeruginosa* produced hemolysin, phospholipase c, and an alkaline phosphatase (12). All of these substances probably play some role in the liberation of phosphate from phosphatides. It may be that the hemolysin solubilizes the phosphatides and that the phospholipase c hydrolyzes the phosphatides, thus liberating phosphorylcholine which, in turn, is hydrolyzed by the phosphatase. This biological system for liberation of phosphate appears much more efficient than the method in which the phosphatides are dispersed by ultrasonic treatment and the phosphate is then extracted by wet ashing at high temperature.

The finding that hemolysin of *P. aeruginosa* solubilizes phosphatides in aqueous medium and

enhances the activity of phospholipase c may have some significance in the pathogenesis of the organism. As described previously (11), production of the hemolysin by this organism in normal sera of animals is negligible. However, in diabetic human sera, about 4 units of hemolysin per ml will be produced by most strains of *P. aeruginosa*. This amount of hemolysin does not exhibit significant toxicity itself (12), but, as shown in the present study, it is sufficient to enhance considerably the activity of phospholipase c, a major factor in the production of skin lesions characteristic of this species (12).

There are other bacterial hemolysins which are soluble in lipid solvents and are, therefore, detergent-like in their characteristics. The best example is probably the δ-hemolysin of staphylococci (13). It is well-known (4) that δ-hemolysin does potentiate the hemolytic activity of the β-hemolysin of the same organism. It has been suggested recently (3) that β-hemolysin of staphylococci is identical with sphingomyelinase and lysophospholipase of this organism. If this is true, the mechanism of potentiation between the δ-hemolysin and the β-hemolysin of staphylococci must be very similar to that of the hemolysin of *P. aeruginosa* and its phospholipase c.

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