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Isoelectric Analysis of Cytolytic Bacterial Proteins

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In this note, we describe the application of isoelectric focusing to the characterization of two cytolytic proteins, the α-toxin or phospholipase C of *Clostridium perfringens* (clostridial PLC) and cereolysin. We used a 110-ml electrolysis column (LKB Instruments, Rockville, Md.), the design of which was given by O. Vesterberg et al. (Biochem. Biophys. Acta **133**:435, 1967), who also summarized the theoretical and practical aspects of the method. A detailed study of the electrophoretic behavior of staphylococcal α-toxin in *p*H gradients was carried out by T. Wadström (*in preparation*); he separated and characterized several varieties of staphylococcal α-toxin as to *p*I value (isoelectric *p*H) and hemolytic activity.

Apparently, clostridial PLC has not been obtained in highly purified form, except possibly by A. D. Bangham and R. M. C. Dawson (Biochim. Biophys. Acta **59**:103, 1962), who separated it by electrophoresis in a glycerol density gradient. With a dried concentrate prepared from a culture filtrate of *C. perfringens* Type A, kindly supplied by R. O. Thomson (Wellcome Research Laboratories, Beckenham, Kent, England), and with the same conditions (without a *p*H gradient) used by Bangham and Dawson, but substituting sucrose for glycerol, we obtained (Fig. 1) a single peak of phospholipase activity, as measured by the capacity of fractions to cause turbidity in diluted egg yolk.

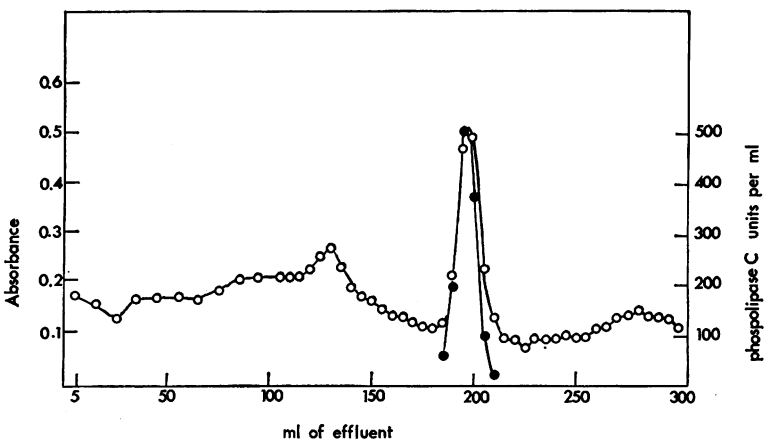

FIG. 1. *Density gradient electrophoresis of Clostridium perfringens α-toxin at pH 6.8. The sample consisted of 25 mg of toxin dissolved in 2.75 ml of 0.005* M *dimethyl glutaric acid-NH_4OH buffer (pH 6.8), in which 50 mg of glycine and sufficient sucrose were dissolved to bring the density to that of the gradient at the 220-ml level where the sample was introduced. Voltage and current were 300 v and 8 ma for 21 hr. Anode to left. Symbols: (○) absorbance at 280 mμ; (●) units of phospholipase C per milliliter, as estimated by capacity of fractions to cause turbidity of diluted egg yolk. A unit of phospholipase C is that dilution 1 ml of which produces an optical density of 1.0 at 520 mμ, 15 min after mixing with 1 ml of egg yolk reagent prepared according to D. J. Kushner (J. Bacteriol.* **73**:*297, 1957). Reaction mixtures were incubated at 37 C and optical densities were read in a Zeiss PMQ II spectrophotometer. The toxin diluent was 0.05* M *dimethyl glutaric acid-NaOH buffer (pH 6.8) containing 0.1% bovine serum albumin.*

Another portion of the same dried concentrate was subjected to isoelectric focusing (Fig. 2). In contrast to the single peak obtained without a *p*H gradient, two peaks of phospholipase activity coincident with two peaks of hemolytic activity and of protein (280-mμ absorbance) were evident, one of *p*I 5.2 and one of *p*I 5.5. We recovered 64% of the input enzyme activity. The ratio of hemolytic to enzymatic activity of all fractions was constant within the limits of error of the assay methods. When passed through a column of Sephadex G-75 Superfine (Pharmacia, Uppsala, Sweden), fractions 7 and 9 showed identical

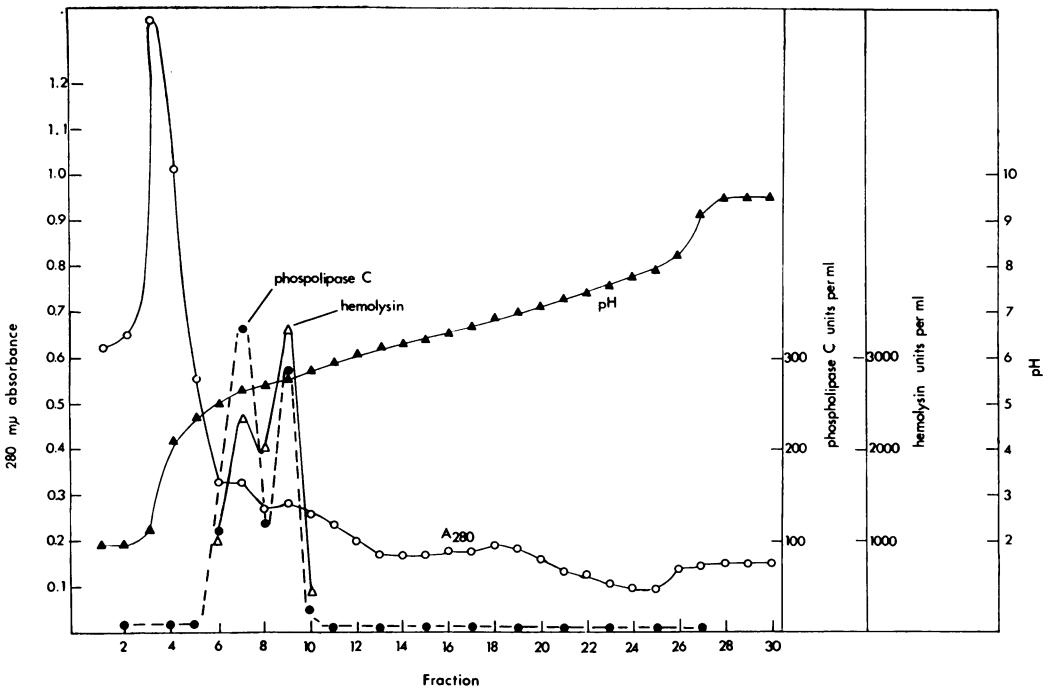

FIG. 2. *Isoelectric focusing of Clostridium perfringens α-toxin. The gradient was prepared from a less dense solution consisting of 51 ml of water, 4 ml of 1% ampholine, pH 5 to 8 (LKB Instruments, Rockville, Md.), and 15 mg of toxin, and a more dense solution consisting of 37 ml of water, 8.5 ml of 8% ampholine (pH 5 to 8), and 25 g of sucrose. Focusing was done at about 4 C for 42 hr, with a final potential of 900 v. Fractions of 4 ml were assayed for 280-mμ absorbance* (○), *phospholipase C activity* (●) *as in Fig. 1, and hemolytic activity* (△); *the pH* (▲) *was measured. A unit of hemolytic activity is that dilution 1 ml of which releases half of the hemoglobin from 1 ml of 0.7%* (*v/v*) *washed sheep erythrocytes in 30 min at 37 C followed by 30 min in an ice bath. The diluent was 0.15* M *NaCl and 0.005* M *calcium acetate.*

effluent volumes with respect to both hemolytic and enzymatic activity and with respect to each other, indicating that the molecular sizes of the two electrophoretic varieties of clostridial PLC are very similar and that the two kinds of activities were not separated under the conditions of gel filtration employed.

Cereolysin, a hemolytic growth product of *Bacillus cereus* (A. W. Bernheimer and P. Grushoff, J. Gen. Microbiol. **46**:143, 1967; J. Bacteriol. **93**:1541, 1967) which closely resembles streptolysin O, was prepared through stage 4 of the method described earlier (A. W. Bernheimer and P. Grushoff, J. Gen. Microbiol. **46**:143, 1967). It was subjected to isoelectric focusing (Fig. 3) which showed it to have a *p*I of 6.5. About 70% of the input activity was recovered. When recycled in a narrower *p*H range, most of the activity disappeared but that which remained again focused at *p*H 6.5.

Isoelectric focusing of samples of streptolysin S and streptolysin O was also attempted. The former failed to separate from the bulk of the polynucleotide, much of which is known to be inactive. Most of the streptolysin O (hemolytic) activity remained associated with a large amount of 280-mμ absorbing material which migrated close to the anode in a *p*H 6 to 8 gradient, but a very small peak of activity focused at *p*H 6.5 and this may represent the true *p*I of free streptolysin O.

We conclude that the α-toxin or phospholipase C of *C. perfringens* consists of two molecular species differing slightly in charge (*p*I 5.2 and 5.5) but otherwise very similar; in contrast, cereolysin behaves as a homogeneous protein with respect to charge (*p*I 6.5). In addition to the analytical information provided, the method of isoelectric focusing may prove useful for the purification of some toxic bacterial proteins.

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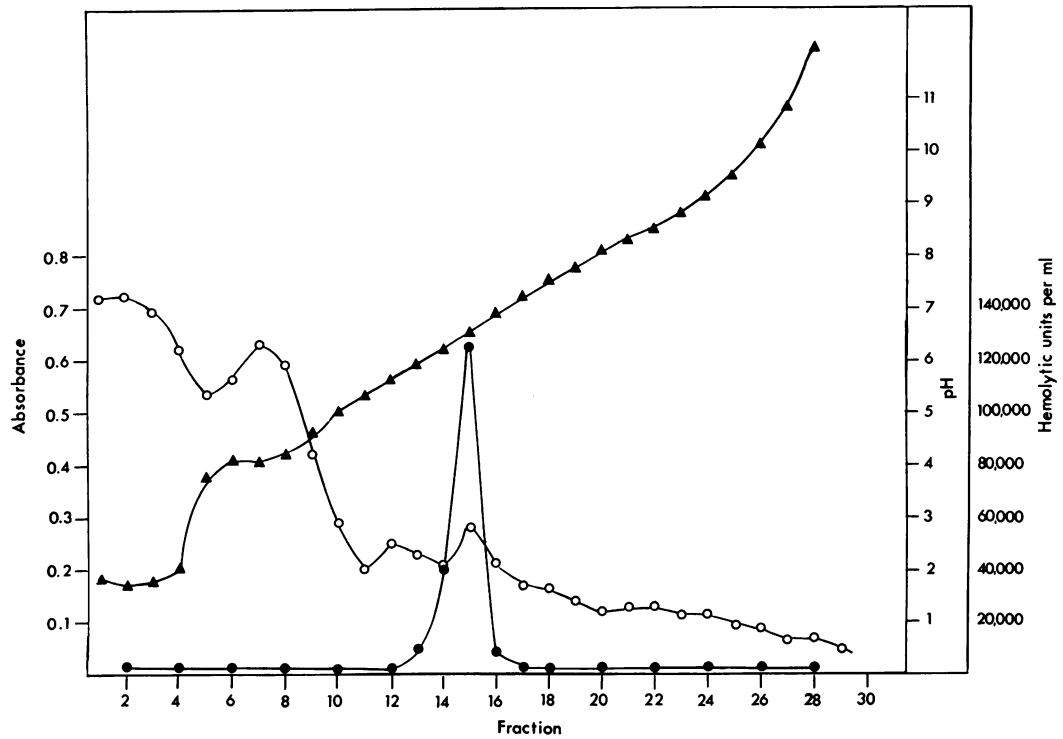

FIG. 3. *Isoelectric focusing of cereolysin. The gradient was prepared in a manner similar to that of Fig. 2, but ampholine (pH 3 to 10) was employed. A sample containing 1.8 × 10^6 hemolysin units and 12.2 absorption units (280 mμ) was incorporated into the less dense solution. Focusing was done at about 4 C for 44 hr, with a final potential of 600 v. Fractions of 4 ml were examined for 280-mμ absorbance (○), hemolytic activity (●), and pH (▲). Hemolytic activity was measured as described by A. W. Bernheimer and P. Grushoff (J. Gen. Microbiol.* **46**:*143, 1967).*