Differentiation of Staphylococcal and Micrococal Proteinases by Electrophoresis

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The electrophoretic separation of the proteinases produced by staphylococci and micrococci was studied in four buffers. The duration of electrophoresis was based on the migration of a marker dye for a predetermined distance. The migration distances of the enzymes and dye were measured, and enzyme-dye values were calculated. A comparison of enzyme-dye values showed that complete separation of eight serologically different proteinases did not occur in any one buffer; however, in most instances, their relative order of migration was the same in all buffers. Certain strains of Staphylococcus epidermidis produced two proteinases that were different serologically as well as electrophoretically. Staphylococcus aureus strains, on the other hand, produced up to four proteinases that were serologically the same. The proteinases of staphylococci and micrococcii can be best characterized by both electrophoretic and serological methods.

The serological differentiation of extracellular proteinases has been used for classifying organisms of the family Micrococcaceae (7, 13). Electrophoresis has been used for the separation and identification of various proteinases (9; O. Sandvik, Ph.D. thesis, Veterinary College of Norway, Oslo, 1962). Because eight serologically different proteinases of staphylococci and micrococci have been identified, we wanted to determine whether the enzymes also differed in their electrophoretic mobilities. The possibility existed that a preliminary identification of a proteinase could be made by electrophoresis and then confirmed with a specific antiserum. This procedure would obviate the need to test each proteinase with all eight antisera before an identification was made and thus conserve the antisera.

This report describes the separation of staphylococcal and micrococal proteinases by electrophoresis with different buffers and support media.

MATERIALS AND METHODS

Cultures. Fifteen cultures of Staphylococcus aureus, 67 of Staphylococcus epidermidis, and 11 of Micrococcus sp. were used in this study. All cultures were classified by the method of Baird-Parker (3) and by serological differentiation of their proteolytic enzymes (12, 13). Proteinase production by the different organisms was as follows: group A by S. aureus; groups B, C, D, F, and H by S. epidermidis; and groups E and G by Micrococcus sp. Five cultures classified as proteinase groups A, B, C, D, and E were obtained from Olav Sandvik, Veterinary College of Norway, Oslo, and the other cultures, including strains of groups F, G, and H, were isolated at the National Animal Disease Center, Ames, Iowa. All cultures were isolated from milk samples of infected bovine udders except six cultures of S. aureus; five of these six were of porcine origin obtained from Richard Shuman, and one was of avian origin obtained from Kenneth Heddleston of this Center.

Enzyme production. Heart infusion agar (Difco Laboratories, Detroit, Mich.) in petri dishes was covered with sterile dialysis membrane (4, 10). Each plate was inoculated with 0.5 ml of a 16- to 18-h brain heart infusion (Baltimore Biological Laboratories, Cockeysville, Md.) culture, which was spread over the surface of the membrane with a sterile glass spreader. After the culture was incubated at 30 C for 2 to 3 days in a candle jar, the growth on the membrane was washed off with 1.5 ml of phosphate-buffered 0.85% NaCl solution, pH 7.0. The cell suspension was centrifuged at 43,000 x g in a refrigerated centrifuge for 20 to 30 min, the supernatant fluid was harvested, and 1% aqueous thimerosal solution was added to give a final concentration of approximately 1:7,500. All solutions were tested for casein precipitating activity by the method of Sandvik (Ph.D. thesis, Veterinary College of Norway, Oslo, 1962) and stored at 4 C. A semisolid agar medium (Sandvik, Ph.D. thesis, Veterinary College of Norway, Oslo, 1962), N and M medium (11), and nutrient gelatin (Difco) were also used to produce enzymes.

Buffers. The following buffers at 0.05 M concentration were used in electrophoresis: barbitral B-1, pH 8.6 (Beckman Instruments, Inc., Fullerton, Calif.); tris(hydroxyethyl)aminomethane (Tris), pH 8.0 (mixture of Trizma-base and Trizma-hydrochloride, Sigma Chemical Co., St. Louis, Mo.); phosphate, pH
7.0 (5); and phosphate, pH 6.2 (Sandvik, Ph.D. thesis, Veterinary College of Norway, Oslo, 1962).

Electrophoresis of proteinases. Microelectrophoresis was carried out at 200 V in a Microzone cell (Beckman Instruments, Inc.). A pencil line was drawn across the cellulose acetate membrane at a distance of 43 mm from the center of the left-hand perforations. This distance, which became 45 mm after the membrane was wetted with buffer and stretched, represented 30 mm from the points of application. The volume of proteinase solutions applied to the membrane varied from 0.25 to 4.0 μl. Depending on the size of the zones of precipitation they produced in the casein precipitating test. Four proteinase solutions were applied to the membrane at alternate slots to prevent contact between enzyme samples. A 0.25-μl sample of RBY reference dye solution (Gelman Instrument Co., Ann Arbor, Mich.) was then applied at one of the unused peripheral slots. After about 25 min of electrophoresis, the lid was opened and the position of the dye on the membrane was observed. Electrophoresis was continued if the blue dye had not migrated to the pencil line.

The medium used to demonstrate proteinase activity was casein agar (Sandvik, Ph.D. thesis, Veterinary College of Norway, Oslo, 1962), which was modified to contain 2% agar (DL); 1% sodium caseinate (Eastman Kodak P914, Rochester, N.Y.); 0.01% thimerosal; and 0.08 M phosphate buffer, pH 5.7. The agar was melted in 0.01 M phosphate buffer. Sodium caseinate, as a 4% solution, pH 6.2, and thimerosal 1% were added to the melted agar immediately before pouring.

After electrophoresis, the membrane was placed on casein agar contained in a plate (12 by 18 cm) (Sandvik, Ph.D. thesis, Veterinary College of Norway, Oslo, 1962), which could accommodate up to three membranes. The lid was taped to the plate which was incubated at 37 C overnight. The membranes were then removed, and the spots of precipitation produced in the casein agar by the proteinases were recorded by making a contact print of the plate on a sheet (20.3 by 25.4 cm) of photographic enlarging paper. An enlarger was used as a light source. After the prints were processed, the center of each spot was determined and this point was used to measure the distance of proteinase migration. Because the dye sometimes migrated a few millimeters more or less than the desired 30 mm, an enzyme-dye distance (ED) value was calculated for each enzyme tested.

The Spinco model R cell (Beckman Instruments, Inc.) was also used with both thin paper and cellulose acetate strips because it permitted better separation of multiple enzymes produced by certain strains. Macroelectrophoresis was carried out in barbital buffer, pH 8.6, for 5 h at 200 V.

When multiple enzymes were observed, they were tested against the group antisera as follows: a specific antiserum was pipetted onto a filter paper strip (15 cm long and 2 mm wide) which had been previously placed on the surface of the caseinate agar. After incubation at room temperature for 2 to 4 h, the filter paper was removed and the paper or cellulose acetate strip containing the separated enzymes was placed on the agar over and parallel with the line of applied antiserum. After enzyme development, the strip was removed and the enzyme-antiserum reaction was observed (see Fig. 3). Two different antisera could be used for each electrophoresis strip.

Antisera. Antisera to the various proteinases were prepared as previously described (13). In addition, antisera to two S. aureus isoenzymes (1 and 2) were prepared by immunizing rabbits with enzyme solutions produced on cellophane-agar medium. Extremely small quantities of the solutions were injected intradermally at 4- to 7-day intervals until there was evidence of immunity to the staphylococcal nuclotoxins. Immunization was then continued by subcutaneous and intravenous injections of the enzyme solutions as described (13).

RESULTS

An example of the separation of proteinases by electrophoresis and their reactions on casein agar is illustrated in Fig. 1.

A comparison of the mean ED values for eight serologically different proteinases of staphylococci and micrococci when electrophoresis was carried out in four buffers is illustrated in Fig. 2. All enzymes migrated toward the anode except the group C proteinase in Tris, pH 8.0, and phosphate, pH 7.0, buffers. The relative order of electrophoretic mobilities of the eight proteinases based on the means of the ED ratio were the same in the different buffers, except for the group G enzyme in barbital buffer. However, when we consider the upper and lower confidence limits of the mean ED ratios, complete separation of all eight proteinases did not occur in any one buffer.

Some strains of S. epidermidis produced two proteinases (groups B and H) when grown on cellophane-agar medium, but not when grown

Fig. 1. Microelectrophoretic separations of four staphylococcal proteinases on cellulose acetate membranes in 0.05 M Tris buffer, pH 8.0, at 200 V after development of their reactions on casein agar. A, B, C, and D refer to the serological groups of the enzymes produced by strains of S. aureus (A) and S. epidermidis (B, C, and D). AP, Line of application.
in nutrient gelatin or N and M medium. Of the strains tested thus far, those that produced only group B proteinases were coagulase negative, whereas those that produced only group H proteinases were coagulase positive. However, some strains that produced both B and H proteinases were coagulase positive and others were coagulase negative. In tests for serological specificity, we found that the B and H enzymes were best separated electrophoretically on thin paper strips in barbital buffer in the model R apparatus. A comparison of the electrophoretic mobilities and serological reactions of groups B, H, and B-H proteinases is presented in Fig. 3.

**Fig. 2.** Mean ED values with upper and lower 95% confidence limits of eight serologically different proteinases (groups A to H) when microelectrophoresis was carried out on cellulose acetate membranes at 200 V in four buffers at 0.05 M. A to H, Serological groups of the enzymes. Numbers in parentheses are electrophoretic determinations for each enzyme.

**Fig. 3.** Macroelectrophoretic separation of S. epidermidis proteinases, groups B, H, and B-H, on thin paper strips in 0.05 M barbital buffer, pH 8.6, at 200 V. N, Neutralization of the enzymes by specific antiserums; Enz, proteinase group; Coag, coagulase reaction of the organisms that produced the enzymes; ap, line of application.
When both B and H proteinases were produced in combination by coagulase-positive or coagulase-negative strains, the H enzyme showed mobilities similar to those of the H enzyme produced individually by the coagulase-positive strains. As illustrated in Fig. 3, the B proteinase of the B–H combination migrated toward the cathode, whereas the B enzyme produced individually by the coagulase-negative strains migrated toward the anode.

In the Microzone apparatus, all of the B proteinases migrated toward the anode in barbital buffer; however, the mobilities of those produced in combination with the H proteinase were significantly slower ($P < 0.05$). In Tris buffer, the mobilities of the B proteinases, whether produced singly or in combination with H enzymes, were not significantly different. In phosphate buffer, pH 7.0, the mobilities of both the B and H proteinases of the B–H combination were significantly slower ($P < 0.01$) than those of the respective B–H enzymes produced individually. In addition, the B enzyme of the B–H combination migrated toward the cathode as illustrated in Fig. 3. In one instance, a coagulase-negative strain produced B and H enzymes with consistently faster migration rates toward the anode in barbital buffer than rates of the respective enzymes produced individually by coagulase-negative B strains and coagulase-positive H strains.

Four electrophoretically different group A proteinases produced on cellophane-agar medium by S. aureus strains could be separated only on cellulose acetate strips in the model R apparatus. In addition to the combination of proteinases present in two enzyme preparations (Fig. 4A and B), some strains produced only no. 1 or no. 2 or both no. 1 and no. 2 isoenzymes. All of the isoenzymes were neutralized by group A antisera only and appeared to be serologically homogeneous because antisera made from proteinase no. 1 or no. 2 neutralized all four.

The effect of medium on the number of proteinases produced was also demonstrated. When S. epidermidis strains that produced the B–H proteinases on the cellophane-agar medium were grown in nutrient gelatin, only the B enzyme could be demonstrated. When S. aureus strains that produced two and three electrophoretically different enzymes on the cellophane-agar medium were grown in nutrient gelatin, only one proteinase was demonstrated.

**DISCUSSION**

The results of this study indicate that some of the serologically different proteinases of staphylococci and micrococci show different electrophoretic mobilities, and that in most instances the relative order of enzyme migration was the
same in all four buffers. In general, the relative rates of migration for the proteinases were as follows: A or G > D > H, E, F, B > C. In some instances, electrophoresis of the proteinases in two buffers could be useful in identifying those enzymes showing similar migration rates. For example, in phosphate buffer, pH 7.0, the migration rates of group E and H proteinases were similar, whereas in barbital buffer the migration rate of group H proteinase was significantly faster than that of group E proteinase (Fig. 2). Simultaneously testing a known group E or H proteinase in barbital buffer with an unidentified enzyme that shows a similar ED value in phosphate buffer, pH 7.0, would assist in making an initial identification. The same procedure would also be applicable for group A and G proteinases. Final identification, however, would depend on neutralization of the proteinase with a specific antiserum.

Reproducibility of migration distances varied considerably when the proteinases were subjected to electrophoresis for a definite period of time, such as 30 min. The migration distances for any one proteinase varied between runs, although the different enzymes maintained their same positions relative to each other. Stevenson (14) used dye markers to assist in the calibration of the mobility of peptides during high voltage ionophoresis. Our using a marker dye to migrate a predetermined distance rather than using a definite period of time for electrophoresis proved to be equally valuable in comparing relative mobilities of the proteinases in different buffers.

A number of different organisms that produced more than one proteinase have been found (8; Sandvik, Ph.D. thesis, Veterinary College of Norway, Oslo, 1962). Dahle (8) found two proteinases of Aeromonas liquefaciens that were serologically different. In our study with certain strains of Staphylococcus epidermidis, we found two proteinases, B and H, that were not only serologically different, but also electrophoretically different. However, the four electrophoretically different isoenzymes of S. aureus appeared to be serologically the same. The reason for this result with the S. aureus strain may have been that the production of the antiserum against the no. 1 and no. 2 isoenzymes required long periods of inoculation, which might have caused the formation of cross-reacting antibodies.

The production of multiple enzymes was influenced by the medium in which the organisms were grown. Dahle (8) reported similar results for A. liquefaciens and indicated that the medium is important if an enzymoserological method is used to identify bacterial proteinases. Arvidson et al. (2) also showed that of three proteinases produced by a strain of Staphylococcus aureus one was not produced if yeast extract was omitted from the medium.

Sandvik (Ph.D. thesis, Veterinary College of Norway, Oslo, 1962) found that when Veronal buffer (pH 8.6) was used to separate serum proteins, subsequent testing for specific antibodies on casein agar (pH 6.2) required greater concentration of the developing proteinases, and the pH of the enzyme solutions had to be buffered between 5.0 and 5.5. By using casein agar at pH 5.7, we were able to compensate for the buffers of high pH carried over with the membrane.

Microelectrophoresis can assist in the initial screening of the proteinases before their final identification by the serological method. Macroelectrophoresis with the model R cell also provided evidence that certain strains of Staphylococcus aureus produced multiple proteinases. Sandvik (Ph.D. thesis, Veterinary College of Norway, Oslo, 1962) found only one fraction when the proteinase of a strain of Staphylococcus aureus was examined by paper electrophoresis. We too found only one fraction in some of the strains we examined. However, Vesterberg et al. (15) examined the proteolytic enzymes of a strain of S. aureus by isoelectric focusing and found that most of the activity was in three peaks, with isoelectric points at about pH 3.9, 5.0, and 9.7. These fractions might be analogous to the three isoenzymes present in preparation A, Fig. 4. Arvidson (1) also demonstrated three proteolytic enzymes produced by a strain of S. aureus; these could be differentiated by their rates of hydrolysis of casein and by their sensitivity to ethylenediaminetetraacetic acid and cysteine.

In previous reports (6, 7), the authors suggested that certain coagulase-positive staphylococci, which were nonpigmented and nonhemolytic, should be classified as S. epidermidis rather than S. aureus. This suggestion was based partly on the fact that S. epidermidis strains produced group H proteinase either alone or in combination with group B proteinase, whereas strains of S. aureus produced only group A proteinases (Fig. 3 and 4). In addition, other strains of S. epidermidis that had similar biochemical characteristics but were coagulase negative also produced group B proteinase (Fig. 3). Consequently, differentiation of the proteinases might be useful in the taxonomy of staphylococci and micrococci and thus aid in epidemiological and epizootiological studies of these organisms.
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