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Partial Purification and Characterization of the Multiple Molecular Forms of Staphylococcal Clotting Activity (Coagulase)

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The clotting activity of *Staphylococcus aureus* strain 104 was purified 46,000-fold, but absolute purity was not achieved. Carbohydrate content of the purified material was not more than 5%. Elution of clotting activity from denaturing and nondenaturing polyacrylamide gels revealed the presence of four distinct molecular forms. Molecular weights of the forms were approximately 31,500, 34,800, 44,800, and 56,800 as determined by gel filtration in 8 M urea, by sodium dodecyl sulfate-urea polyacrylamide gel electrophoresis, and by calculation with determined values for the Stokes radius and sedimentation coefficient. Molecular weights determined on sodium dodecyl sulfate-urea gels were found to decrease as the gel concentration increased, suggesting that the amount of sodium dodecyl sulfate bound was less than normal. Estimated frictional ratios for the forms showed that they differ in shape from one another and that they are all highly asymmetrical. Each of the forms had an isoelectric point between pH 5.44 and 5.47 when focused in 6% polyacrylamide gels for 9 h; however, prolonged focusing altered the isoelectric point of the forms to within the range of pH 4.35 to 4.65. The multiple clotting forms were not artifacts of the purification procedure and did not appear to be products of the proteolytic degradation of a larger protein.

"Coagulase" has been reported as an extracellular protein of *Staphylococcus aureus* with the unique ability to activate the mammalian clotting mechanism in the presence of many anticoagulants (40). Numerous studies have characterized this protein, but few have agreed on its size. Molecular weight values have been reported ranging from 5,000 (25, 41) to 10,000 (41), 18,000 (48), 44,000 (10), 61,000 (4), and 90,000 (44). Serological studies have shown that there are several distinct antigens in this clotting activity (9), and that a single strain of *S. aureus* can produce more than one antigenic type (14). Miale et al. (23) reported that an alcohol-precipitated coagulase separated into five to eight bands of clotting activity by starch-gel electrophoresis; however, evidence was not presented to show that these bands were not an artifact of the precipitation procedure. Crude coagulase from a single strain of *S. aureus* has been separated into two fractions having clotting activity but with different isoelectrical points, one between pH 5.1 and 5.3 and one at pH 4.3 (M. C. Drummond, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, P263, p. 188). These data suggest either that coagulase may be a single protein which can exist in more than one molecular form due to aggregation or the presence of prosthetic groups such as carbohydrate, or that the term coagulase may actually represent a family of very different proteins that have a similar ability to stimulate clotting.

To examine this question of molecular diversity, we have attempted to purify and characterize the clotting activity of *S. aureus* strain 104. Very different molecular weights have been reported for the coagulase of this strain (4, 25, 41). The studies presented here show that there are four distinct molecular forms of the clotting activity of strain 104 which differ in size and shape but not in charge.

MATERIALS AND METHODS

Organism and growth conditions. *S. aureus* strain 104, which was originally isolated from a cutaneous abscess, was used throughout this study (40). Cells were grown at 37°C in brain heart infusion broth (Difco Laboratories) supplemented with trace metals as described previously (40, 42). For the production of coagulase, cells were grown in 3 liters of brain heart

infusion broth in a 6-liter flask for 6 to 7 days and were killed by the addition of merthiolate (Eli Lilly & Co.; 1:10,000). The culture supernatant, designated as crude coagulase, was obtained by centrifugation at 12,000 × *g* for 30 min and was stored at −20°C until needed; the cells were discarded.

Clotting assay. Coagulase activity was assayed by a modification of the method of Tager and Hales (42). Twofold serial dilutions of samples were made in 2% peptone (Sheffield Chemicals) in saline containing merthiolate (1:10,000). To each dilution (0.5 ml) was added an equal volume of a solution of citrated human plasma diluted 1:80 in 0.2% bovine fibrinogen (Rehais Chemical Co.) in saline. The test was incubated at 37°C for 16 to 20 h and then at 4°C for at least 1 h. The titer, in clotting units, was calculated to be double the dilution factor of the highest dilution to produce a solid clot. The plasma-fibrinogen solution was made fresh on the day of use and was kept in an ice bath. Human plasma was obtained as outdated blood bank plasma from the Red Cross and was stored at −20°C.

Enzyme assays. Bovine liver catalase (Worthington Diagnostics) was assayed by the method of Fukui et al. (12) at room temperature, with hydrogen peroxide as substrate. Staphylococcal acid phosphatase activity was assayed by a modification of the method of Barnes and Morris (3). The reaction mixture contained 2 ml of 0.1 M citrate buffer (pH 5.4), 0.1 ml of 0.3 M magnesium chloride, 0.5 ml of 0.5% *p*-nitrophenyl disodium phosphate (Sigma Chemical Co.), and 0.5 ml of enzyme. After incubation at 37°C for 10 min, the reaction was stopped by the addition of 1 ml of 2 N sodium hydroxide. The reaction tubes were centrifuged at 12,000 × *g* for 15 min to remove precipitated magnesium hydroxide, and absorbance of the supernatant was measured at 400 nm. Staphylococcal nuclease activity was determined by the procedure of Ryden et al. (33) with denatured calf thymus DNA (Worthington) as substrate. Staphylococcal protease activity was determined by the procedure of Ryden et al. (33). Hammarsten casein (Nutritional Biochemicals Corp.) was used as substrate, and absorbance was measured at 280 nm. Crystalline (2×) bovine pancreas trypsin (Worthington) was used as a control for the procedure. The limit of the sensitivity of this assay was about 0.1 μg protein of (2×) trypsin. Staphylococcal hemolysin activity was assayed by the procedure of Madoff and Weinstein (20), with rabbit erythrocytes. The hemolysin titer was calculated as double the dilution factor of the highest dilution showing 50% hemolysis.

Chemical assays. Protein was estimated with the Folin phenol reagent by the method of Lowry et al. (19). Absorbance was measured at 750 nm, and bovine serum albumin (Sigma) was used as the standard. Total carbohydrate was estimated by the phenol-sulfuric acid procedure of Dubois et al. (8). Absorbance was measured at 490 nm, and glucose was used as the standard. Reducing sugars were determined by the reduction of ferricyanide by the procedure of Park and Johnson ' .8), with glucose as the standard. Before this analysis, portions of each sample were hydrolyzed in 1 and 4 M hydrochloric acid at 100°C for 4 h (39).

Standard proteins. Purified proteins obtained from commercial sources (Sigma, Worthington) were used as standards in polyacrylamide gel electrophoresis and gel filtration procedures. The molecular weights and Stokes radii (centimeters × 10^7) of the proteins were: horse heart cytochrome *c*, 12,400, 1.69; horse heart myoglobin, 17,800, 1.97; soybean trypsin inhibitor, 21,500, 2.26; bovine pancreas trypsinogen, 23,700, 2.19; bovine pancreas α-chymotrypsinogen A, 25,000, 2.24; swine pepsinogen, 40,400, 2.85; ovalbumin, 45,000, 2.73; bovine serum albumin, 67,000, 3.55; rabbit muscle aldolase, 149,000, 4.59; and bovine liver catalase, 250,000, 5.20. Stokes radii for trypsinogen, pepsinogen, and aldolase were calculated from known physical parameters with the Stokes-Einstein equation as described by Siegel and Monty (36, 37).

Polacrylamide gel electrophoresis. Electrophoresis in nondenaturing gels (6 by 100 mm) containing 7.5% polyacrylamide was performed as described by Ornstein (26) and Davis (6). Electrophoresis in denaturing gels (6 by 100 mm) containing 7.5 or 10% polyacrylamide, 1% sodium dodecyl sulfate (SDS), and 5 M urea was performed in the alkaline gel system described by Laemmli (17). Samples for electrophoresis contained 100 to 400 μg of protein. When analyzed in denaturing gels, they were first denatured in 4% SDS containing 1% 2-mercaptoethanol and 8 M urea at 100°C for 2 min. Gels were stained with amido black (naphthol blue black; Eastman Kodak Co.). After electrophoresis, clotting activity was recovered from both denaturing and nondenaturing gels by slicing the gels into 2-mm fractions and incubating each fraction in 0.5 ml of peptone-saline at 4 or 22°C for 16 to 20 h. Each eluate was then assayed for clotting activity. Molecular weights were calculated from relative mobilities on denaturing gels as described previously (35).

Isoelectric focusing. Isoelectric focusing was conducted in 6% polyacrylamide gels (6 by 100 mm) containing 5% glycerol and 2% ampholytes (pH 3 to 10 Ampholines; LKB-Producter AB, Sweden) by a modification of the procedure of Righetti and Drysdale (30). Samples contained 200 to 400 μg of protein, and electrophoresis was conducted at 4°C for 9 or 17 h. Gels were sliced into 2-mm fractions which were incubated in 0.5 ml of normal saline at 4°C for 20 to 26 h. Each eluate was then assayed for pH and clotting activity.

Gel filtration. Molecular weight was estimated by gel filtration in 8 M urea as described by Davison (7). Samples were incubated in pH 7 phosphate-buffered saline (PBS) containing 8 M urea and 0.1 M 2-mercaptoethanol for 1 h at 22°C and were chromatographed on a 2.5- by 35.4-cm column of 6% agarose (Bio-Gel A-5m; Bio-Rad Laboratories) in the same solvent. The elution position of each standard protein was determined by protein analysis of the fractions after an initial 1:10 dilution of each fraction with PBS to eliminate interference from 2-mercaptoethanol. The elution position of coagulase was determined by the clotting assay. Elution data were analyzed by the method of Fish et al. (11).

Stokes radii were determined by gel filtration on a 2.5- by 95-cm column of Sephadex G-200 (Pharmacia Fine Chemicals) in PBS (pH 7.0) at 4°C (18, 29). Fractions were analyzed for either clotting activity or for protein when the standard proteins were used. The elution position of catalase was determined by the

catalase assay (12). Elution data were plotted and analyzed by the method of Reichert et al. (29). Molecular weights were also calculated from the G-200 elution data by the method of Andrews (1).

Sucrose gradient centrifugation. Sedimentation coefficients were determined on 5 to 20% linear sucrose gradients in PBS (pH 7.0) at 4°C by the method of Martin and Ames (21). Samples (0.5 ml) in PBS were layered onto 12-ml gradients which were centrifuged at 40,000 rpm for 20 h in an SW-41 rotor in a Beckman model L-2 ultracentrifuge. Horse heart myoglobin ($S_{20,w}$ = 2.04S; CRC Handbook of Biochemistry, Selected Data for Molecular Biology, 1968 edition) was included in each sample at a concentration of 1 mg/ml as an internal standard. Fractions (0.2 ml) were collected from the top of each gradient by pumping 60% sucrose in PBS into the bottom of the tube through a 20-gauge needle set in a rubber gasket in the barrel of a 12-ml disposable syringe. Each fraction was diluted by the addition of 0.8 ml of PBS before being assayed for clotting activity. Myoglobin concentration was measured by absorbance at 420 nm. Sedimentation coefficients were also calculated from these data with the Svedberg equation as described by Trautman and Cowan (45).

Purification of coagulase. Unless otherwise stated, all procedures were conducted at 4°C, and all buffers contained 0.02% sodium azide to prevent contamination. The culture supernatant of strain 104 was adjusted to pH 3.8 with 4 N HCl and allowed to stand overnight. The precipitate was collected by centrifugation at 8,500 × *g* for 30 min, washed once in ⅒ volume of 0.02 M pH 3.8 acetate buffer, dissolved in pH 8.0 PBS, and dialyzed against 20 volumes of pH 7.0 PBS for 18 h. Solid ammonium sulfate was added to 55% saturation, and the solution was stirred slowly for 16 to 20 h. The precipitate was collected by centrifugation (12,000 × *g* for 60 min), dissolved in pH 8.0 PBS, and dialyzed against two changes of 20 volumes of 0.05 M Tris-hydrochloride (pH 8.0). During this last dialysis, a fine precipitate formed which was removed by centrifugation. The precipitate contained no recoverable clotting activity.

Dialyzed coagulase was applied to a 5- by 37-cm column of DEAE-cellulose (DE52, Whatman) equilibrated with 0.5 M Tris-hydrochloride (pH 8.0). After washing the column with 1.5 column volumes of buffer, a linear gradient of 0 to 400 mM sodium chloride in buffer (7,000 ml total) was started. Fractions (20 ml) containing coagulase activity, which eluted as a single peak between 70 and 90 mM sodium chloride, were pooled, and coagulase was precipitated by the addition of solid ammonium sulfate to 85% saturation. The precipitate was dissolved in pH 7.0 PBS and dialyzed against two changes of 20 volumes of 0.02 M pH 6.8 phosphate buffer.

This material was applied to a 2.5- by 52-cm column of DEAE-cellulose equilibrated with 0.02 M phosphate buffer (pH 6.8). The column was washed with two column volumes of buffer and eluted with a 0 to 300 mM linear sodium chloride gradient in buffer (2,500 ml total). Fractions (10 ml) containing the clotting activity peak (about 90 mM sodium chloride) were pooled, and coagulase was precipitated with ammonium sulfate as before. The precipitate was dissolved in pH 7.0 PBS and dialyzed against two changes of 20 volumes of 0.01 M pH 6.8 phosphate buffer. A fine precipitate which formed during dialysis was removed by centrifugation and precipitate was dissolved in pH 7.0 PBS and dialyzed against two changes of 20 volumes of 0.01 M pH 6.8 phosphate buffer. A fine precipitate which formed during dialysis was removed by centrifugation and discarded.

The dialyzed coagulase was applied to a 5- by 19-cm column of hydroxyapatite (Bio-Gel HT; Bio-Rad Laboratories) equilibrated with 0.01 M phosphate buffer (pH 6.8). After a 2,000-ml buffer wash, a linear 10 to 200 mM phosphate gradient (pH 6.8, 7,000 ml total) was started. Fractions of 20 ml were collected; those containing clotting activity were pooled, and the activity was concentrated with ammonium sulfate. This material was dissolved in pH 7.0 PBS and applied to a 5- by 92-cm column of Sephadex G-200 (Pharmacia) the same buffer. Fractions of 20 ml were collected, and those containing clotting activity were pooled. The activity was concentrated with ammonium sulfate and dissolved in a small volume of pH 7.0 PBS.

RESULTS

Purification. The procedure summarized in Table 1 resulted in an almost 46,000-fold purification with an overall yield of 38%. An attempt was made to continue the purification by repeating the chromatography step on hydroxyapatite, but only 25% of the clotting activity was recovered, and the specific activity of the eluted material was less than 600,000 U/mg of protein. Analyses for other enzyme activites at each purification step (Table 1) showed that hemolysin and acid phosphatase were absent after DEAE-cellulose chromatography at pH 6.8; however, a trace of nuclease activity (0.001% of the starting material) was present in the G-200 material. Protease activity was not detected in the culture supernatant or in samples from each purification step, even after the incubation period with casein had been extended to 6 h. Electrophoresis of the G-200 step material on denaturing and nondenaturing polyacrylamide gels and elution of the clotting activity showed that four distinct forms of this activity were present (Fig. 1). A stained duplicate gel run under nondenaturing conditions showed that two protein bands were associated with the clotting activity peak closest to the cathode (form I, Fig. 1A). The second peak (form II) appeared to have only one stained band, as did the third peak (form III); however, no stained bands were associated with the fourth peak (form IV, Fig. 1A). The reason for this is not clear; all of these bands were somewhat diffuse and did not appear to stain well. At least two stained bands were not associated with any eluted clotting activity. Because of the unclear association of bands with eluted activity, no

TABLE 1. *Purification of clotting activity*

Step	Vol (ml)	Clotting activity (U/ml)	Protein (mg/ml)	Sp act (U/mg)	Purification (-fold)	Yield (%)
Culture supernatant	76,050	1.28×10^3	13.90	9.20×10^1	1	100
Acid precipitate	3,825	2.56×10^4	5.37	4.77×10^3	52	101
Ammonium sulfate precipitate	3,060	3.07×10^4	5.58	5.51×10^3	60	97
Tris-hydrochloride dialysis	3,050	3.07×10^4	4.41	6.97×10^3	76	96
DEAE-cellulose, pH 8.0	179	4.51×10^5	3.80	1.19×10^5	1,290	83
DEAE-cellulose, pH 6.8	30	1.97×10^6	9.23	2.13×10^5	2,320	61
Hydroxyapatite, pH 6.8	9	5.24×10^6	4.56	1.15×10^6	12,500	49
Sephadex G-200	7	5.24×10^6	1.24	4.23×10^6	46,000	38

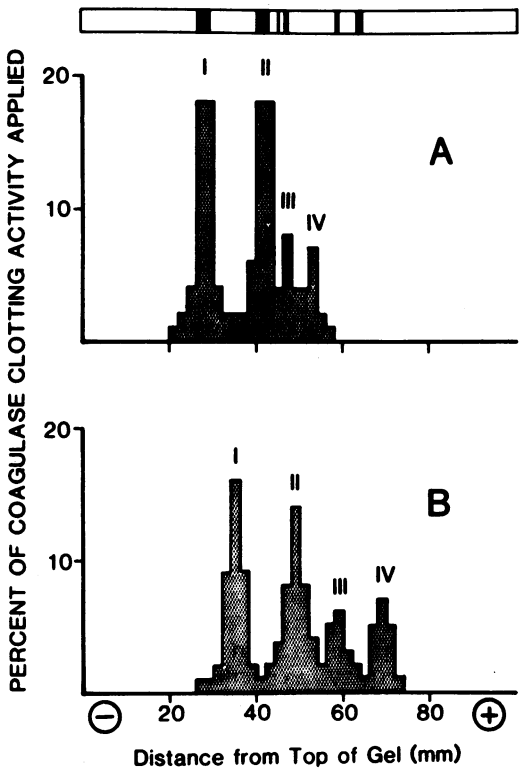

FIG. 1. *Polyacrylamide gel electrophoresis and elution of purified coagulase (G-200 step, Table 1) on 7.5% gels. Electrophoresis was from left to right. Clotting activity in each gel slice (2 mm) is shown as a percentage of the total activity that had been applied to the gel. A, Untreated sample run on a nondenaturing gel; total clotting activity recovered from the gel slices was 86%; a drawing of the pattern of bands found in a stained duplicate gel run under the same conditions is at the top of the figure. B, Denatured sample on an SDS-urea gel; recovery of clotting activity was 76%.*

attempt was made to estimate purity. Gels which had been run under denaturing conditions showed a similar pattern of stained bands associated with eluted clotting activity (Fig. 1B): however, the bands were slightly more diffuse than those seen in nondenaturing gels.

Chemical analysis of the G-200 step material (Table 1) revealed that its amino acid composition (24) was very similar to that reported by Bas et al. (reference 4, data not shown). Aspartic acid, glutamic acid, and lysine were the major amino acids present. A partial specific volume of 0.73 was calculated from these data by the method of Cohn and Edsall (5). This value was also obtained when the amino acid composition reported by Bas et al. (4) was used. A trace amount of one or more sugar amines was detected in all of the samples. Total carbohydrate was estimated to be 5%, and reducing sugars were estimated at 4.4%.

Distinct nature of the clotting activities. When each activity peak eluted from nondenaturing polyacrylamide gels was reelectrophoresed on either denaturing or nondenaturing gels, the distinctive mobility of that activity did not change. Activity peaks eluted from denaturing gels maintained their distinctive mobilities when reelectrophoresed under denaturing conditions. Pretreatment of purified coagulase with 1 M neutral hydroxylamine for 1 h at 22°C (27) did not alter the pattern of clotting activity eluted from either denaturing or nondenaturing gels from that shown in Fig. 1. Thus, the activity peaks were not aggregates held together by ester bonds (27, 31, 32).

Analysis on nondenaturing gels showed that all of the forms were present in the crude culture supernatant and in samples from all of the purification steps. Thus, the forms were not artifacts of the purification procedure. The multiple forms were also present in culture supernatants taken at intervals from the time that clotting activity first appeared. They were also present in crude lysates of cells lysed with lysostaphin (Mead Johnson Pharmaceutical Division) by the method of Tipper and Strominger (43). Also, protease activity was not detected in the culture supernatants, in the cell lysates, or in culture supernatants that had been concentrated 20-fold

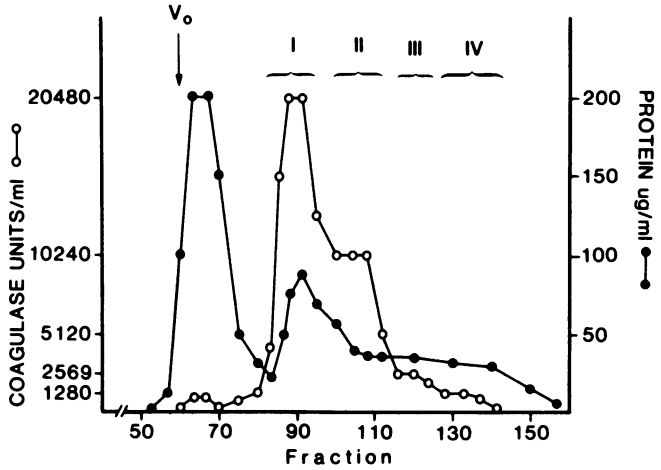

FIG. 2. *Elution of clotting activity from a 6- by 180-cm column of Sephadex G-200 at 4°C in PBS (pH 7.0). The sample volume was 50 ml, and elution was with ascending flow at 56 ml/h. Fraction volumes were 25 ml. Fractions containing clotting activity were pooled as shown by the brackets in the figure. The predominant clotting form found in each pool is shown above each bracket (Fig. 1). V_0 indicates the void volume of the column. Recovery of clotting activity was about 80%.*

by freeze-drying. These data, considered together, suggest that these clotting activity forms were not products of intracellular or extracellular degradataion of a larger protein.

Separation of the coagulase forms. As a means of aiding the characterization of these clotting activities, an attempt was made to separate them from each other. Because the amount of purified coagulase (G-200 material, Table 1) was too small to use as starting material, a second batch of crude coagulase was prepared as before. This batch was purified as shown in Table 1 up through the DEAE-cellulose (pH 8.0) step and was then chromatographed on a 6- by 180-cm column of Sephadex G-200 as in the purification procedure. The fractions of the resulting asymmetrical peak were pooled (Fig. 2) and concentrated with ammonium sulfate as before. The individual concentrates of each form were then rechromatographed on a smaller column (5 by 92 cm) of G-200 under the same conditions, concentrated, and dialyzed against pH 7.0 PBS. The resulting preparations of the forms were not absolutely pure, as was shown by the number of stained bands found on nondenaturing polyacrylamide gels; however, the elution of clotting activity from duplicate gels showed that 85 to 90% of the clotting activity that each preparation contained was of one clotting form only. Specific activities (clotting units per milligram of protein) of the preparations were: form I, 130,000; form II, 84,000; form III, 47,000; and form IV, 29,000. These preparations retained at least 90% of their original clotting activity during storage at −20°C for 9 months with repeated thawings.

Attempts were made to separate and purify the forms in one step by preparative polyacrylamide gel electrophoresis (15) of coagulase that had been purified through the DEAE-cellulose (pH 8.0) step. Four clotting activity peaks were recovered, but total recovery was never greater than 20%, and the recovered forms proved to be extremely labile when stored at 4°C. Almost all of the activity of each form was lost after storage for a few days at this temperature. This problem of instability made it necessary to use the semipurified preparations described above for the characterization studies.

Characterization of the separate forms. Molecular weights determined by chromatography on 6% agarose in 8 M urea and by electrophoresis on SDS-urea polyacrylamide gels are shown in Table 2. The molecular weights estimated by gel filtration in urea and on 10% polyacrylamide gels were in close agreement, but the values estimated on the polyacylamide gels depended on gel concentration. This behavior is typical of proteins which do not bind SDS in the same proportion as globular proteins (34). Molecular weights were also estimated by gel filtration on Sephadex G-200 under nondenaturing conditions (1). These results were substantially higher than those determined under denaturing conditions (Table 2), which suggested that the clotting forms were far more asymmetrical in shape or more hydrated than the proteins used as standards (2). The G-200 elution data were

TABLE 2. *Molecular weights of the clotting forms determined by different methods*

Clotting form	Molecular weight as determined by:				
	Gel filtration on Sephadex G-200 in PBS	Gel filtration on 6% agarose in 8 M urea	SDS-urea polyacrylamide gel electrophoresis		Calculation from Stokes radius and sedimentation data[a]
			7.5% gels	10% gels	
I	154,000	55,600	71,400	58,600	56,100
II	92,200	43,500	51,400	44,700	46,300
III	50,800	34,600	39,500	34,300	35,500
IV	43,400	31,800	31,600	29,600	33,200

[a] Calculated by the method of Siegel and Monty (36, 37), using a partial specific volume of 0.73 (based on amino acid composition) and an $S_{20,w}$ of 2.82S (Fig. 3) for each form.

also used to determine the Stokes radii of the forms (centimeters $\times 10^7$): form I, 4.75; form II, 3.93; form III, 3.01; and form IV, 2.81. These data were used with the determined sedimentation coefficients (Fig. 3) to calculate molecular weights by the Stokes-Einstein equation (36, 37). The resulting values (Table 2) were similar to those determined on agarose in urea and on 10% SDS-urea gels, which suggests that the true molecular weights are within the range of values determined by these three different methods. The means of these three different sets of values were 31,500, 34,800, 44,800, and 56,800.

In spite of differences in molecular weight, the estimated sedimentation coefficients of the forms were identical (Fig. 3). These results were unexpected; however, sedimentation can be affected by protein shape and hydration as well as by size (21). Frictional ratios were calculated from the Stokes radii and molecular weight data (36, 37): form I, 1.90; form II, 1.67; form III, 1.40; and form IV, 1.34. These values showed that none of the forms are shaped like a sphere (frictional ratio = 1.0), and that they differ from one another in shape. The sedimentation profiles of the forms in PBS-sucrose gradients were identical and included two unequal peaks (Fig. 3A); however, when 1% Triton X-100 (Sigma) was added, the smaller peak disappeared, and the larger peak increased slightly with no loss in the average recovery of clotting activity (Fig. 3B). Thus, the larger peak was considered to show the true sedimentation position of each form.

Isoelectric point. In previous studies, the isoelectric point of coagulase was reported in the range of pH 5.3 to 5.85 (10, 38) and pH 4.51 to 4.55 (4). In this study, the clotting forms were individually focused on 6% polyacrylamide gels in a pH 3 to 10 gradient for 9 h, and each form focused within pH 5.44 to 5.47 (form I in Fig. 4 is representative). When focusing was conducted

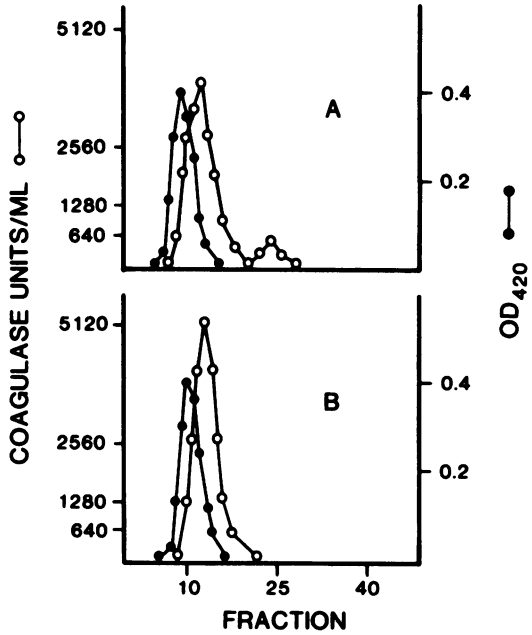

FIG. 3. *Sucrose gradient centrifugation of clotting form I (○) and myoglobin (●) in pH 7.0 PBS (A) and in PBS plus 1% Triton X-100 (B). The direction of centrifugation was from left to right. Fraction volumes were 0.2 ml. Data shown are the means of four separate experiments. The recovery of clotting activity averaged 84% with and without Triton X-100. The sedimentation patterns obtained with all four clotting forms were identical under these conditions. The patterns shown here with form I are representative of these data. Identical $S_{20,w}$ values of 2.82S were calculated for each of the forms (21, 45).*

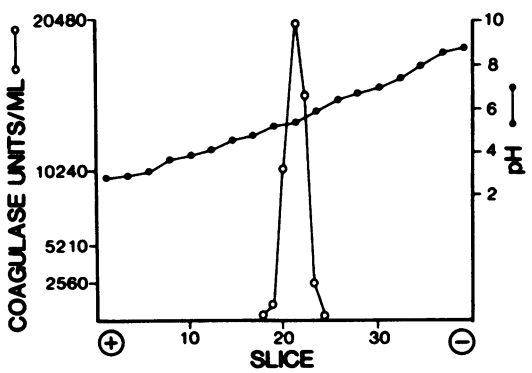

FIG. 4. *Isoelectric focusing of clotting form I for 9 h at 4°C. The recovery of clotting activity from this gel was 92%.*

for 17 h, however, each form focused as a single peak in the range of pH 4.35 to 4.65. Similar results were found when a pH 3 to 6 gradient was used (data not shown). This shift in isoelectric point during prolonged focusing was probably an artifact of the procedure (13), which could explain the discrepant reports of the isoelectric point of this protein.

DISCUSSION

The results of this study have shown that there are at least four distinct molecular forms of the clotting activity of *S. aureus* strain 104 which differ in shape and size with estimated molecular weights of 31,500, 34,800, 44,800, and 56,800 and which have a similar isoelectric point between pH 5.44 and 5.47. All of the forms were detected within the cell and in the extracellular medium during synthesis in the absence of detectable protease activity. Thus, the forms did not appear to be degradation products of a larger protein. Multiple clotting activities have also been detected in the culture fluids of two of five other strains of *S. aureus* (unpublished observations).

Establishment of the multiple molecular nature of coagulase helps explain why the reported physical characterization of this protein has yielded such diverse molecular weights (4, 10, 25, 41, 44, 48). Some of the discrepancies in these values may have been due to the characterization techniques that were used. As was found in this study, these clotting proteins are not spherical in shape. Such asymmetry can lead to problems in determining molecular weights by gel filtration when globular proteins are used as standards (2). The molecular weights determined by gel filtration in this study (Table 2) were clearly not comparable to the values obtained with the other methods. The aberrant behavior of the coagulase proteins on SDS-urea polyacrylamide gels suggests that less SDS was bound than expected, which would cause an overestimation of molecular weight (34). An inverse relationship of gel concentration to estimated molecular weight in SDS gels is typical of glycoproteins (34); however, the carbohydrate content of purified coagulase was estimated to be not more than 5%. It is not clear whether this amount would affect the electrophoretic behavior of these forms, or whether other types of prosthetic groups such as lipids or nucleic acids were responsible. The combination of denaturing and nondenaturing techniques provided similar molecular weight values for the impure forms, which suggests that these values are reasonably accurate. The use of a denaturing technique with an impure protein requires that the biological activity of that protein be recovered from the denaturant. Fortunately, clotting activity was readily recovered from SDS and urea by the simple process of diluting samples in peptone solution, as in the clotting assay. For most proteins, full recovery of the native state after SDS denaturation is seldom achieved, and then only after removal of the denaturant (47). Thus, coagulase may be unique in its reaction to denaturants. The results of this study did not answer the question of how chemically related these clotting activities are to each other. A more complete characterization of these proteins will have to wait until they have been separated and purified to homogeneity.

The purification of coagulase proved to be extremely difficult, partly because of problems with the stability of these activities. The degree of purification reported here is greater than any previously reported (Table 1). Bas et al. (4) reported a 35,700-fold purification of coagulase that resulted in 85% purity. The amino acid composition found in this study was similar to that reported by Bas et al. (4), which would support the view that the two preparations shared a similar degree of purification. The results of this study contrast with those of other studies which reported coagulase as a single polypeptide (4, 10, 25, 38, 48, 49); however, this may have been due to the use of other strains or other purification methods. Other *S. aureus* proteins such as lactate dehydrogeanse (16), protease (22, 46), α-hemolysin, deoxyribonuclease, and hyaluronidase (46) have been shown to exist in several molecular forms.

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