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Protease Production by *Streptococcus sanguis* Associated with Subacute Bacterial Endocarditis

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A viridans streptococcus (*Streptococcus sanguis* biotype II) isolated from the blood of a patient with subacute bacterial endocarditis was examined for protease production. In broth culture, extracellular proteolytic enzymes were not produced by this organism until after the early exponential phase of growth, with maximal protease production occurring during the stationary phase. Four distinct proteases were isolated and purified from the supernatant fluids of stationary-phase cultures, employing a combination of ion-exchange column chromatography, gel filtration column chromatography, and polyacrylamide gel electrophoresis. All four proteases could be eluted from a diethylaminoethyl cellulose column at a sodium chloride gradient concentration of 0.25 M but were separable by gel filtration chromatography on a Sephadex G-100 column. They varied in molecular weights as determined by gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis from approximately 13,000 to 230,000. All four proteases had pH optima of between 8.0 and 9.0, and two of the proteases were active against casein, human serum albumin, and gelatin but were not active against elastin and collagen. The remaining two proteases were able to degrade only casein and gelatin. These results show that *S. sanguis* is able to excrete maximal levels of potentially destructive enzymes when the organisms are not actively multiplying. This finding may explain some of the damage caused in heart tissue by these organisms during subacute bacterial endocarditis.

The viridans streptococci form the single most prevalent group of bacteria causing subacute bacterial endocarditis (SBE) (13). Among the alpha-hemolytic streptococci, *Streptococcus sanguis* has been shown to be the causative agent in SBE between 30 and 40% of the time (2, 28). The factors which enable this normally avirulent organism to cause SBE are unknown despite intensive investigation directed toward elucidating the pathogenic mechanism(s). Several workers (9, 30) have suggested that viridans streptococcal invasion of the bloodstream and the resultant infection of the endocardium is most probably due to a combination of bacterial virulence factors and altered host resistance capabilities.

Although the mechanism which allows viridans streptococci to infect endocardial tissue is poorly defined, even less is understood about the process by which these organisms are capable of destroying the underlying heart tissue. The observation that there is often extensive valvular damage in the immediate vicinity of the infected endocardial vegetation would seem to imply the presence of diffusible toxic bacterial products or inflammatory responses or both. However, Durack (3) rarely observed granulocytes in the immediate vicinity of bacterial colonies, suggesting that valvular damage due to an inflammatory response is unlikely. The role of toxic bacterial products in bacterial endocarditis was investigated by Gutschik et al. (10). These authors demonstrated in a rabbit model of bacterial endocarditis that proteolytic strains of *Streptococcus faecalis* resulted in a shorter survival time, a higher incidence of bacteremia, and a larger number of kidney infarcts as compared with rabbits infected with non-proteolytic strains.

It has recently been shown in two laboratories that strains of *S. sanguis* are capable of producing proteolytic enzymes (16, 24). In one of these studies, the two strains of *S. sanguis* that were examined were isolated from confirmed cases of SBE, and each strain was shown to be capable of producing more than one species of proteolytic enzyme (24). In light of the fact that extracellular protease production by *S. sanguis* could contribute to the cardiac tissue damage observed in SBE, the production and characteristics of these enzymes were examined in this study.

MATERIALS AND METHODS

Bacterium. *S. sanguis* biotype II (no. 381-81) was isolated from a confirmed case of SBE and kindly supplied by R. R. Facklam, Centers for Disease Control, Atlanta, Ga. Cultures were stored frozen at −70°C in Todd-Hewitt Broth (Difco Laboratories, Detroit, Mich.). For routine use, frozen cultures were thawed and incubated for 24 h at 37°C on tryptic soy agar plus 5% sheep erythrocytes (Austin Biologicals, Austin, Tex.). The stock cultures used to inoculate the blood agar plates were only one transfer away from the original frozen culture. Colonies from the blood agar were used to initiate cultures in the chemically defined medium described below.

Medium and growth conditions. A chemically defined medium (FMC), as previously described by Terleckyj et al. (26), was used to grow *S. sanguis*. Briefly, this medium contains amino acids, vitamins, purine and pyrimidine bases, minerals, 2% glucose, and 0.019 M sodium carbonate at an initial pH of 7.0. All experiments were performed with static aerobic cultures grown at 37°C in a circulating water bath. Starter culture inocula were grown to the mid-exponential phase of growth (0.23 mg/ml, dry weight) in the chemically defined medium in volumes that were approximately 1/10 of the final culture volume. Growth was measured turbidimetrically at 675 nm in a Bausch and Lomb Spectronic 20. The value obtained was multiplied by 1,000 and converted to adjusted optical density (AOD) units so that the values would be in accordance with Beer's law and be proportional to bacterial mass (27). One AOD unit was equivalent to 0.39 μg of cells (dry weight) per ml (24). When cultures were ready for harvest, they were immediately chilled in an ice bath, a final AOD reading was made, and the culture volume was determined. Cells were removed by centrifugation at 14,300 × *g* in a Beckman J2-21 refrigerated centrifuge at 4°C for 30 min. The supernatant fluids were then dialyzed for 3 days against 10 mM Tris-hydrochloride buffer (pH 8.0) containing 0.02% (wt/vol) sodium azide (2 liters of supernatant fluid per 16 liters of buffer) with daily buffer changes and then lyophilized to dryness.

Effect of growth phase on protease production. *S. sanguis* II was grown in 10 liters of FMC. Two-liter aliquots were withdrawn at several time points during the growth phase (lag [AOD = 101], early exponential [AOD = 311], late exponential [AOD = 700], and stationary [AOD = 1,400] phases). Samples were immediately chilled on ice, and the broth cultures were centrifuged, lyophilized, and treated as described above. The lyophilized supernatant fluids were dissolved in 2 ml of 0.01 M Tris-hydrochloride buffer (pH 8.0) containing 0.02% sodium azide and dialyzed overnight against 2 liters of the same buffer. This material was then placed on a DEAE-Sephacel (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) column (2.5 by 20 cm) which had been equilibrated overnight at 4°C with the same 0.01 M Tris-hydrochloride (pH 8.0) buffer. The column was washed twice with the starting buffer, and the adsorbing material was eluted with a linear NaCl (0 to 0.5 M) gradient in the starting buffer. The eluting peaks were monitored at 280 nm, employing a flow-through 2138 Uvicord S monitor (LKB Instruments, Inc., Rockville, Md.) and recorded by a 6520-5 Chopper-Bar six-channel recorder (LKB Instruments). Fractions of 100 drops (approximately 4.2 ml) were collected. The peaks were then pooled, dialyzed against the starting Tris-hydrochloride buffer, concentrated by lyophilization, brought up to 1 ml with the starting buffer, and dialyzed against 2 liters of the same buffer. Protease activity of the various peaks was tested by the horse hide powder blue assay as described below (21).

Assays. Protein concentrations were measured by the procedure of Lowry et al. (19). Several assays were used to quantitate proteolytic activity of the test samples. To test for nonspecific proteolytic activity of the column eluate, the procedure of Rinderknecht et al. (21) was employed. Briefly, scintillation vials containing 20 mg of horse hide powder blue substrate (Calbiochem-Behring Corp., La Jolla, Calif.) in 0.01 M phosphate buffer (pH 8.0 at 37°C) with 0.02% sodium azide were inoculated with 50 to 100 μl of the enzyme sample, and the final volume was brought to 2.5 ml. These mixtures were incubated for 6 to 9 h on a horizontal rotator (100 rpm) at 37°C before assaying. For pH optima determinations of the purified proteases, a pH range from 6.0 to 10.0 was examined. The protease assay employed was the horse hide powder blue procedure described above, except the pH was changed appropriately. A 0.01 M phosphate buffer was used to obtain pH values in the range of 6.0 to 8.0, whereas a 0.01 M Tris-hydrochloride buffer was employed with pH values between 8.5 and 10.0. Assays were performed in duplicate, and the reactions were terminated by filtration with a Millipore filtration apparatus (qualitative filter paper grade 613; Scientific Products Div., McGaw Park, Ill.) which removed any remaining insoluble substrate. Enzyme preparations inactivated by heating at 100°C for 5 min were used as blanks for each sample. The filtrate was read at 595 nm (21), and the protease activity was reported as units per minute per milligram of protein. One unit was defined as an optical density increase of 0.001 above the background value.

The method of Lin et al. (18) was employed to determine the specificity of each of the proteases. Reductively methylated casein (α-casein; Sigma Chemical Co., St. Louis, Mo.), gelatin (swine skin, type I; Sigma Chemical Co.), human serum albumin (HSA) (crystallized; Sigma Chemical Co.), elastin (bovine neck ligament; Sigma Chemical Co.), and collagen (bovine achilles tendon; Sigma Chemical Co.) were employed as substrates. Tubes containing 1.0 mg of the dimethylated substrate (casein, gelatin, HSA, elastin, or collagen), 10 mM Tris-hydrochloride buffer (pH 7.7 at 37°C), 20 mM $CaCl_2$, 0.02% sodium azide, and 20 to 50 μl of sample in a final volume of 1.0 ml were incubated in a shaking water bath at 37°C and 200 rpm for 22 h. All assays were performed in duplicate, using two different concentrations (20 and 50 μl) of enzyme, and the reaction was terminated by heating the mixture for 2 min at 100°C. The contents of each tube were then quantitatively assayed colorimetrically for the presence of new terminal amino groups, using the trinitrobenzene sulfonic acid reagent. Each enzyme reaction mixture was compared for absorbancy at 340 nm with a blank containing all of the reagents of the assay, including enzyme which had been heat inactivated for 5 min at 100°C. Tubes that did not show a linear increase in activity with increasing concentrations of enzyme under saturating substrate conditions

(absorbancy at 340 nm of up to 0.400) were considered negative. Specific activities were calculated by using the molar extinction coefficient of 1.3×10^{-4} M^{-1} cm^{-1} and expressed as nanomoles of peptide bonds cleaved per milligram of protein per hour (18). Elastase activity was also checked by the procedure of Sachar et al. (22) as modified by Johnson et al. (12), using orcein-elastin (Sigma Chemical Co.) as substrate. The reaction mixture consisted of 20 mg of orcein-elastin and 50 μl of the protease being tested in a total volume of 2.5 ml of 0.01 M Tris-hydrochloride containing 0.02% sodium azide (pH 8.0). These assays were performed in scintillation vials incubated at 37°C and rotated at 200 rpm for 12 h. After removal of nonhydrolyzed substrate by filtration, the absorbancy of the supernatants was measured at 590 nm. Assays were run in duplicate and compared with blank tubes containing all of the reagents of the assay, including enzyme which had been heat inactivated for 5 min at 100°C. The assay for immunoglobulin A (IgA) protease was performed as follows. Various dilutions of the enzyme were incubated at 37°C for 8 and 16 h in 100 μl of 0.01 M Tris-hydrochloride buffer (pH 8.0) containing 100 μg of human fraction IgA (Cappel Laboratories, Cochranville, Pa.). The enzyme reaction was stopped by adding 10 μl of 0.2 M EDTA, mixing, and cooling to 4°C. Double-diffusion analysis in gels of samples of the incubation medium against an antiserum against human IgA was performed (20) to evaluate the extent of the digestion.

Purification of the extracellular proteases of *S. sanguis* II strain 381-81. The extracellular proteases were obtained from a stationary-phase culture (AOD = 1,400) of *S. sanguis* II grown in 10 liters of FMC. The supernatant fluid was collected and treated as described above, up to and including ion-exchange chromatography on DEAE-Sephacel. The peaks eluting from DEAE-Sephacel which possessed proteolytic activity after concentration by lyophilization and dialysis against the starting 0.01 M Tris-hydrochloride buffer were then applied to an ascending flow column (2.6 by 90 cm) of Sephadex G-100 (Pharmacia Fine Chemicals) equilibrated with 0.01 M Tris-hydrochloride buffer–0.02% sodium azide (pH 8.0) at 4°C. One hundred drop fractions were collected (approximately 4.6 ml), and protein peaks were monitored and recorded at 280 nm. Protein-containing peaks were pooled, concentrated by lyophilization, and brought up to 2 ml with 0.01 M Tris-hydrochloride buffer (pH 8.0) and dialyzed against 2 liters of the same buffer at 4°C. Peaks were tested for protease activity by the horse hide powder blue assay of Rinderknecht et al. (21). The protease-containing peaks were then subjected to polyacrylamide gel electrophoresis (PAGE) on 7% acrylamide gels by the procedure of Davis (1). Samples containing 100 μg of protein in a maximum volume of 100 μl were mixed with 20 μl of 0.2% bromophenol blue and 100 μl of unpolymerized stacking gel, layered under buffer onto a stacking gel, and electrophoresed at 2 to 3 mA per gel at 0 to 4°C for 2 h. One gel was immediately fixed and stained for 2 h at 25°C in 0.2% Coomassie brilliant blue R in methanol-acetic acid-water (5:1:5) and destained by diffusion in a solution containing 5% (vol/vol) methanol and 7.5% (vol/vol) acetic acid. For localization of protease activity, the other gels were immediately sliced (0.5-cm sections), and each fraction was mashed and eluted at 4°C for 48 h with 1 ml of 0.01 M Tris-hydrochloride buffer–0.02% sodium azide (pH 8.0). The gel residue was then removed, and the eluate was dialyzed against 200 volumes of 0.01 M Tris-hydrochloride buffer–0.02% sodium azide (pH 8.0) for 24 h and then examined for protease activity by the procedure of Rinderknecht et al. (21). Analytical PAGE was also performed during the various stages of protease purification in exactly the same manner, except only 20 to 30 μg of protein was placed on these gels.

Molecular-weight estimations. (i) SDS-PAGE. After elution from 7% polyacrylamide analytical gels, the purified protease preparations and reference proteins were subjected to sodium dodecyl sulfate (SDS)-PAGE, using the discontinuous system described by Laemmli (17). Samples were dissociated and reduced in 0.01 M Tris-hydrochloride buffer (pH 8.0) containing 1% 2-mercaptoethanol and 1% SDS for 2 min at 100°C and directly loaded onto a 12% acrylamide separation gel with a stacking gel composed of 5% acrylamide. Standard proteins with molecular weights ranging from 14,400 to 94,000 or from 18,500 to 330,000 (Pharmacia Fine Chemicals) were run simultaneously with the purified protease preparations. Plots of percent migration of standards and unknown protein samples versus $\log_{10}$ molecular weights were prepared, and unknown molecular weights were estimated by interpolation (29).

(ii) Gel filtration on Sephadex G-100. The gel filtration column used to obtain purified extracellular proteases was calibrated with proteins of known molecular weights (Pharmacia Fine Chemicals). Elution volumes were determined for blue dextran (molecular weight, 2×10^6), aldolase (molecular weight, 145,000), bovine serum albumin (molecular weight, 67,000), ovalbumin (molecular weight, 43,000), chymotrypsinogen A (molecular weight, 25,000), and RNase (molecular weight, 13,700) by recording the volume at which these proteins or dextran displayed maximal absorbance at 280 nm. The molecular weights of the purified proteases were then estimated by interpolation from a graph of K_{av} values versus the $\log_{10}$ molecular weights, utilizing the K_{av} values of the standard proteins and the peaks containing protease activity (6).

RESULTS

Effect of growth phase on protease production. When the samples from the various phases of growth were chromatographed on DEAE-Sephacel (Fig. 1A–D) and monitored at 280 nm, six major peaks were obtained. The amount of 280-nm absorbing material produced by *S. sanguis* increased as these organisms progressed from the lag phase to the stationary phase of growth. However, the organisms began producing protease only after they had left the early exponential phase of growth.

Protease production occurred only in the late-exponential and stationary phases of growth (Fig. 1A–D). Protease production was not evident before this time, and all proteolytic activity eluted from the DEAE-Sephacel column at an NaCl gradient concentration of 0.25 M. The amount of proteolytic activity associated with

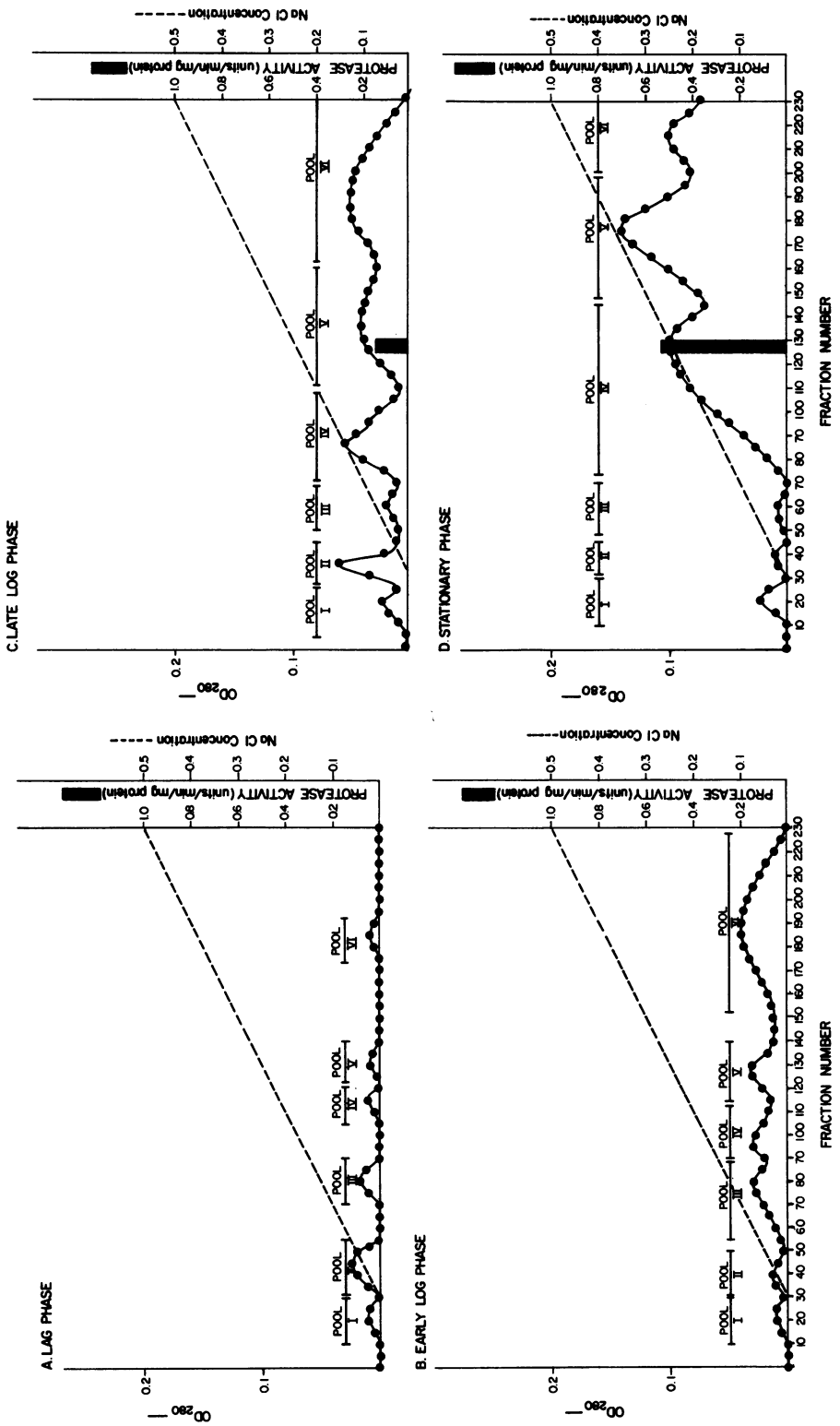

FIG. 1. DEAE-Sephacel elution profiles of 280-nm absorbing material from supernatant fluids (2 liters) of *S. sanguis* 11. Samples were obtained during (A) lag, (B) early exponential, (C) late exponential, and (D) stationary phases of growth. The peaks were eluted with an increasing NaCl gradient from 0 to 0.5 M in 0.01 M Tris-hydrochloride buffer (pH 8.0). All peaks were assayed for protease activity by the method of Rinderknecht et al. (21).

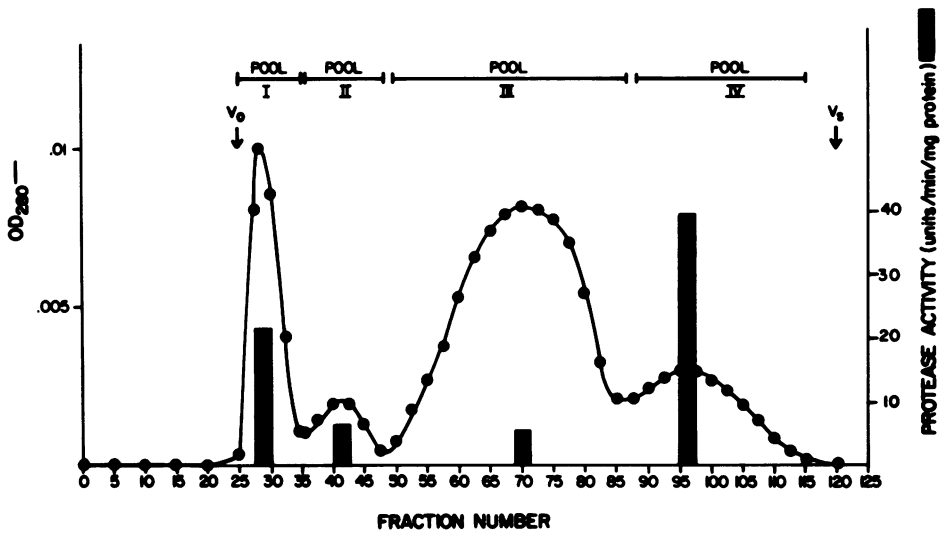

FIG. 2. Sephadex G-100 elution profile of 280-nm absorbing material from DEAE-Sephacel pool IV obtained from the supernatant fluids (10 liters) of a stationary-phase culture of *S. sanguis* II. The peaks were eluted with 0.01 M Tris-hydrochloride buffer (pH 8.0). All peaks were assayed for protease activity by the method of Rinderknecht et al. (21).

the late-exponential-phase cells was 0.15 U of protease activity per min/mg of protein, whereas the amount produced by stationary-phase cells was 0.53 U/min per mg of protein. It thus appears that *S. sanguis* II releases the majority of its proteolytic enzymes in the stationary phase of growth. No IgA protease activity was detected in the supernatant fluids of this organism.

Purification of the extracellular proteases of *S. sanguis* II strain 381-81. Once it was established that the stationary phase of growth occurred when *S. sanguis* II released the majority of its proteolytic enzymes, the supernatant fluids of stationary-phase cells were employed for protease purification. Ten liters of cells grown to the stationary phase in FMC (AOD = 1,400) were centrifuged, dialyzed, and concentrated by lyophilization. This unfractionated preparation had very little protease activity (<0.02 U/min per mg of protein). It was then applied to a DEAE-Sephacel column, and the elution profile obtained was similar to that shown in Fig. 1D. All of the protease activity produced by the 10-liter stationary-phase culture eluted from the ion-exchange column at an ionic strength of 0.25 M NaCl. The protease activity associated with this peak was 0.68 U/min per mg of protein, which is in close agreement with the protease activity (0.53 U/min per mg of protein) seen in the same peak from the 2-liter stationary-phase culture (Fig. 1D).

To further examine the nature of this proteolytic activity, pool IV was dialyzed against 0.01 M Tris-hydrochloride buffer (pH 8.0) to remove the NaCl and then concentrated by lyophilization. This material was then brought to 5 ml in the starting Tris-hydrochloride buffer and applied to an upward-flow Sephadex G-100 column at 4°C. The peaks were then eluted with 0.01 M Tris-hydrochloride buffer–0.02% sodium azide (pH 8.0); the elution profile is shown in Fig. 2. Pool IV from DEAE-Sephacel was separated into four distinct peaks, as monitored by absorbancy at 280 nm, when it was fractionated on Sephadex G-100, and each of the four peaks possessed proteolytic activity against the horse hide powder blue substrate. Pool I (protease I) from Sephadex G-100 possessed 21.5 U of protease activity per min/mg of protein, whereas pools II, III, and IV (proteases II, III, and IV) possessed 6.75, 5.25, and 39.70 U/min per mg of protein, respectively. It thus appeared that *S. sanguis* II is capable of producing at least four different molecular species of proteolytic enzymes.

When the four protease preparations obtained by gel filtration chromatography were examined by PAGE, a number of different bands were observed in all samples but pool IV (Fig. 3). Gel 1 in this figure represents the unfractionated extracellular material produced by *S. sanguis* II. As expected, a large number of extracellular proteins are produced by this organism during the stationary phase of growth. Gel 2 represents the electrophoretic separation of the material found in pool IV, which eluted from DEAE-Sephacel at an NaCl concentration of 0.25 M

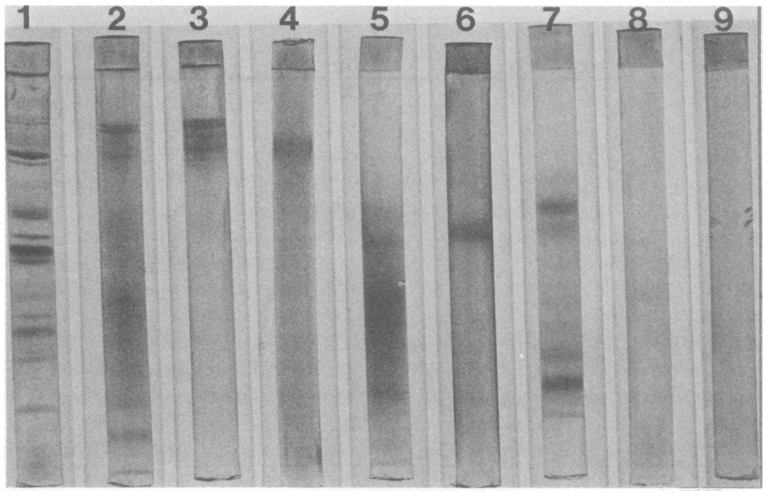

FIG. 3. Disk PAGE of extracellular material and protease preparations elaborated by stationary-phase cells of *S. sanguis* II. Gel 1, unfractionated supernatant fluids; gel 2, pool IV from DEAE-Sephacel; gels 3, 5, 7, and 9, peaks I, II, III, and IV, respectively, from Sephadex G-100; gel 4, protease purified from Sephadex G-100 peak I by PAGE; gel 6, protease isolated from G-100 peak II by PAGE; gel 8, protease isolated from G-100 peak III by PAGE. Each sample contained 20 to 30 μg of protein and was run at a pH of 8.3. The gels were stained for protein-containing components with Coomassie brilliant blue.

NaCl. This gel shows that a large number of contaminating proteins were removed by the ion-exchange purification step. Gels 3, 5, 7, and 9 represent the PAGE separation of peaks I, II, III, and IV from Sephadex G-100 gel filtration, respectively (Fig. 2). The four preparations all contained numerous protein components, except for peak IV. The electrophoretic separation of the proteins present in peak IV indicated that all of the Coomassie blue-stainable material remained in the stacking gel.

To purify the proteases from Sephadex G-100 pools I, II, and III to homogeneity, the preparations were run in PAGE. Approximately 100 μg of protein was applied to each disk gel, and after the electrophoretic separation was complete, the gels were sliced into 0.5-cm pieces and the proteins were eluted from the slices. The eluate from each slice was tested for protease activity, and the active fractions were pooled and analyzed on disk polyacrylamide gels for purity. Gel 4 in Fig. 3 represents the PAGE-purified protease from Sephadex G-100 pool I, whereas gels 6 and 8 represent the PAGE-purified proteases from Sephadex G-100 pools II and III, respectively. The PAGE-purified protease from Sephadex G-100 pool I possessed 50.43 U of protease activity per min/mg of protein, whereas the PAGE-purified proteases from Sephadex G-100 pools II and III possessed 30.16 and 51.38 U of protease activity per min/mg of protein, respectively. When an attempt was made to localize the protease activity associated with pool IV from Sephadex G-100 (gel 9 in Fig. 3), it was observed that all of the protease activity was found in the stacking gel. It thus appears that the protease associated with pool IV from the Sephadex G-100 column will not migrate into the running gel under the conditions employed.

Molecular weights and pH optima of the extracellular proteases of *S. sanguis* II. The molecular weights of the four proteases, which were determined by gel filtration chromatography on Sephadex G-100, were found to be 48,000, 30,000, and 13,000 for proteases II, III, and IV, respectively (Table 1). The molecular weight of protease I could not be determined by chromatography on Sephadex G-100, as it eluted in the void volume. However, when protease I was subjected to SDS-PAGE, a band was obtained that corresponded to a molecular weight of approximately 230,000. SDS-PAGE indicated molecular weights for proteases II, III, and IV of 47,500, 27,500, and 18,000, respectively (Table 1).

The results of the pH optimum determinations performed on the four purified protease preparations are also shown in Table 1. Proteases I and III both possessed a pH optimum of 9.0, whereas proteases II and IV possessed pH optima of 8.0 and 8.5, respectively.

Substrate specificity of four extracellular proteases produced by *S. sanguis* II. The activity of the four streptococcal proteases against five protein substrates was examined (Table 2). None of the extracellular proteases were active against collagen, elastin, or orcein-elastin (data not shown), whereas all four were capable of hydrolyzing casein and gelatin. Protease I was active against

TABLE 1. pH optima and molecular weights of four proteases produced by stationary-phase cells of *S. sanguis* II

Protease	pH optimum	Mol wt as determined by:	
		SDS-PAGE[a]	Chromatography[b]
I	9.0	230,000	ND[c]
II	8.0	47,500	48,000
III	9.0	27,500	30,000
IV	8.5	18,000	13,000

[a] Determined as described by Laemmli (17).
[b] Determined on Sephadex G-100.
[c] ND, Not determined; protease I eluted from Sephadex G-100 in the void volume of the column.

gelatin and HSA and extremely active against casein (1,160.88 nmol of peptide bonds cleaved per mg of protein per h). Proteases II and III, although not capable of hydrolyzing HSA, did possess moderate activity against gelatin and casein. Protease IV, on the other hand, was the most active of the proteases against HSA (469.90 nmol of peptide bonds cleaved per mg of protein per h) and possessed moderate activity against gelatin (265.78 nmol of peptide bonds cleaved per mg of protein per h) and casein (584.72 nmol of peptide bonds cleaved per mg of protein per h).

DISCUSSION

The data presented in this study indicate that *S. sanguis* II has the ability to elaborate several proteolytic enzymes during the exponential and stationary phases of growth. These data also demonstrate that this viridans streptococcus, isolated from a confirmed case of SBE, produces more protease activity during its stationary growth phase than any other phase of growth. For example, Fig. 1 shows that stationary-phase cells produce about four times as much protease as do cells which are undergoing balanced growth in the exponential phase. This could be of considerable importance, because Durack and co-workers (4, 5) have shown that, in an experimental rabbit model of SBE, the majority of the infecting organisms reach a stage where they cease growing approximately 48 h after initial infection. These authors attributed this cessation of growth to the very large population density (up to 10^9 organisms per g of tissue) found in these vegetations. Hooke and Sande (11) have also shown that the vast majority of viridans streptococci in endocardial vegetations have entered the stationary phase of growth. These authors demonstrated that such a state of reduced growth is actually reached by these organisms in the fibrin-covered vegetation. By studying the eradication of a viridans streptococcus from the endocardial vegetations of nonwarfarinized and warfarinized rabbits, the authors showed that animals that receive the anticoagulant do not form vegetations and respond to penicillin treatment in only 3 days. On the other hand, a 7-day course of penicillin therapy is necessary to completely eradicate the same organism from the endocardial vegetations of animals that do not receive the anticoagulant. These authors concluded that the reduced ability of penicillin to remove viridans streptococci from the heart valves of rabbits is probably related to the large numbers of nongrowing bacteria located deep within the lesion. Direct demonstration of the reduced metabolic capability of these organisms in the endocardial vegetations associated with SBE was reported by Durack et al. (5). These investigators, using a labeled metabolite (L-alanine) and autoradiography, demonstrated that the viable bacteria located deep within the lesion are metabolically less active than the organisms located at the periphery of the endocardial vegetation. These results suggest that the vast majority of organisms in an endocardial vegetation (and also those in the closest proximity to cardiac tissue) are in the stationary phase of growth.

The purification scheme employed (Fig. 1–3) allows for the purification of the four proteases in a relatively simple manner. The utilization of a defined medium increases the ease of purification, because there is no need to worry about removal of proteins and high-molecular-weight carbohydrates that are introduced when organisms are grown in a complex medium. Although all four proteases eluted from the DEAE-Sephacel column at the same NaCl concentration, they appear to be distinct proteases. For example, each one possessed a different molecular weight (Fig. 2 and Table 1), and each purified protease possessed a different electrophoretic mobility when subjected to PAGE. They do, however, appear to have similar pH optima (8.0 to 9.0) (Table 1). The significance of these pH optima in SBE is unknown, since the pH in the immediate proximity of the endocardial vegetation has never been measured.

The substrate specificity of the proteases (Table 2) provides additional evidence that all four represent distinct molecular species. Although none of the four proteases attacked elastin or collagen, they all possessed activity against gelatin and casein. Proteases I and IV were active against HSA, whereas proteases II and III were inactive against this protein. Thus, considerable variations were observed in the activities of the enzymes against the substrates. Additionally, IgA protease production by this strain of *S. sanguis* II could not be demonstrated. This is not necessarily surprising in light of a study by

TABLE 2. Hydrolysis of various proteins by the four extracellular proteases of *S. sanguis* II

Protease[a]	Activity (nmol of peptide bonds cleaved per mg of protein per h) against[b]:				
	Gelatin	HSA	Casein	Elastin	Collagen
I	276.31	80.59	1,160.88	ND[c]	ND
II	196.07	ND	144.03	ND	ND
III	103.70	ND	232.44	ND	ND
IV	365.78	469.90	584.72	ND	ND

[a] Proteases I, II, III, and IV refer to the first, second, third, and fourth proteases eluting from the Sephadex G-100 column, respectively.

[b] All substrates were dimethylated as described by Lin et al. (18). *S. sanguis* II was grown to the stationary phase. Activity was determined by the method of Lin et al. (18) with *N,N*-dimethylated proteins as substrate. All assays were performed in duplicate with inactivated enzyme blanks substracted as described in the text.

[c] ND, Not detected.

Genco et al. (7), who demonstrated that of the 21 *S. sanguis* strains that they examined, only about half were able to produce an IgA protease.

The concept that *S. sanguis* produces several different proteases has support in two earlier studies (16, 24). Straus et al. (24) showed that two biotypes of *S. sanguis* (I and II) each produced at least two proteases separable by gel filtration chromatography on Sephadex G-100. In a more recent study, in which protease production by *S. sanguis* was examined, Labib et al. (16) observed multiple peaks of proteolytic activity after column chromatography of stationary-phase cultures. In addition, the degree of proteolytic activity against casein differed from one chromatographically obtained pool to another, and the effects of various inhibitors on the protease pools obtained by ion-exchange chromatography were different. Thus, there can be little doubt that *S. sanguis* is capable of producing several different species of proteolytic enzymes.

It is also apparent from this study that fractionation of the extracellular material produced by *S. sanguis* is essential before proteolytic activity can be detected. This phenomenon has been observed in other species of streptococci (25). Although the nature of this protease inhibition in concentrated supernatant fluids is unknown, diluting crude mixtures of inactive proteases has previously been shown to allow for the expression of protease activity (14). It is unclear whether fractionation removes a natural protease inhibitor or whether the original concentrated supernatant contains so much extracellular protein that protease expression is difficult to measure because the protease is attacking streptococcal protein as well as the test substrate.

Proteolytic destruction of endocardial tissue in SBE caused by viridans streptococci should remain a candidate to explain the tissue destruction (valvular and nonvalvular) seen in this disease process. Although the proteases produced by *S. sanguis* do not appear to degrade elastin and collagen (the major proteins found in valvular tissue), this does not mean that they could not be responsible for some of the other tissue damage seen in SBE. The destructive effects on nonvalvular material produced by this infection include erosive aneurysms of the sinus of valsalva and rupture of papillary muscle and of the interventricular septum (15). Also, of course, myocarditis is still an important complication of endocarditis. Although its exact pathogenesis is still unknown, one of the present theories includes damage produced by microbial toxins (15). As stated previously, the studies of Gutschik et al. (10) clearly show that, in bacterial endocarditis caused by *S. faecalis*, the proteolytic strains produced more severe cardiac infections, as evidenced by shorter survival times, higher numbers of bacteria in the blood, and larger numbers of kidney infarcts than did the non-proteolytic strains. A possible reason for increased protease production by a microorganism during the stationary phase of growth has been suggested by Gnosspelius (8) in a paper on protease production by the myxobacterium *Myxococcus virescens*. In that study, the author suggested that an organism may produce proteases to supply itself with additional amino acids by hydrolyzing proteins of host origin. This could occur in streptococcal SBE, because the organisms have entered a nutritionally deprived state (24) and the proteolytic digestion of host tissue would be one way to supply the organisms with the additional amino acids they require to survive.

In conclusion, these studies have confirmed previous studies showing that *S. sanguis* is capable of producing several different proteolytic enzymes (16, 24). The data in this study also indicate that maximum protease production occurs during the stationary phase of growth. This may be of considerable importance in viridans streptococcal SBE, because the majority of the organisms in the endocardial vegetation appear

to be in the nonreproducing state (5). Histopathological study of the endocardial lesion in SBE demonstrates a slow, progressive destruction of tissue (23), possibly owing to proteases produced by these organisms. Slow, progressive tissue destruction seen in these infections may occur because of the time required for the organisms to attain sufficiently large populations to produce enough protease to cause tissue damage.

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