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Purification and Characterization of Three Forms of Collagenase from *Clostridium histolyticum*†

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ABSTRACT: Three collagenases from *Clostridium histolyticum*, designated C_1, C_2, and C_3, with apparent molecular weights of 96 000, 92 000, and 76 000 were purified. Peptide maps of the enzymes prepared by digestion with *Staphylococcus aureus* V-8 protease were found to be similar. Cleavage of native C_1 with α-chymotrypsin or V-8 protease yielded C_2 and C_3. This suggested that proteolysis of the M_r 96 000 collagenase may have occurred in vivo, producing the other two lower molecular weight enzymes. Previously prepared antiserum directed against a form of the bacterial enzyme similar by molecular weight and charge to collagenase C_3 and Fab′ fragments generated from this antiserum inhibited the collagenolytic activity. C_1, C_2, and C_3 were immunologically identical by Ouchterlony double diffusion, and C_3 was able to compete with C_1 for the antiserum binding site. The ability of each enzyme to bind to antiserum raised against the bacterial collagenase supported the hypothesis that these three proteins were closely related. Zinc analyses of C_1 and C_3 resulted in a value of 1.14 mol of zinc/mol of C_1 and 0.82 mol of zinc/mol of C_3. C_1 did not contain carbohydrate as measured by gas–liquid chromatography or periodic acid–Schiff staining.

Collagenases are defined as endopeptidases which cleave the triple helical region of the collagen molecule (Gross et al., 1974). They can be obtained from a variety of animal tissues (Eisen et al., 1970; Harper, 1980), and lower organisms such as fungi (Hurion et al., 1977) or bacteria (Strauch, 1974; Keil, 1979).

The literature contains many reports on the purification of collagenase from *Clostridium histolyticum*. Peterkofsky & Diegelmann (1971) used gel filtration to purify the enzyme. A number of investigators separated collagenolytic activity from contaminating proteases and noted multiple forms: Lwebuga-Mukasa et al. (1976) employed isoelectric focusing to obtain four species with different isoelectric points; Kono (1968) used DEAE-cellulose[1] chromatography to generate three enzymes with different specific activities; Harper et al. (1965) separated two species of different molecular weight on DEAE-cellulose.

The purpose of this study was to determine if the multiple forms of the clostridial collagenases were derived from one polypeptide and could be generated by proteolysis. Rabbit antiserum directed against one form of the collagenase was used to elucidate the immunological similarity of three purified collagenases. The collagenases were analyzed for amino acid composition and zinc and carbohydrate content.

Materials and Methods

The source of crude *C. histolyticum* collagenase, class IV (lots 48B019, 48K181, and 40D208) or class III (lots 47D261

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[1] Abbreviations: DEAE, diethylaminoethyl; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; TPCK, tosylphenylalanine chloromethyl ketone, Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin.

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and 47M198), α-chymotrypsin, and trypsin was Worthington Biochemical Corp. *Staphylococcus aureus* V-8 protease and goat anti-rabbit Fab were from Miles Biochemicals. *Achromobacter iophagus* collagenase was purchased from Boehringer-Mannheim Biochemicals. *S. aureus* was a gift of Dr. I. Trowbridge (Salk Institute).

Protein Determinations. Protein was measured by the procedure of Lowry et al. (1951) with bovine serum albumin as the standard or by the method of Warburg & Christian (1941).

Assay for Collagen. The Bergman & Loxley (1963) hydroxyproline assay was employed to determine the concentration of collagen used in the collagenase assays as well as the quantity bound to the affinity resin.

Hydrolysis of [^{14}C]Glycine-Labeled Guinea Pig Skin Collagen. [^{14}C]Glycine-labeled collagen from guinea pig skin was prepared according to to the method of Gross & Lapiere (1962). Collagen (2000 cpm/0.100 mL or 0.2 mg) was preincubated at 37 °C for 15–24 h to form a heat fibril gel (Gross & Kirk, 1958). The reaction mixture consisted of 0.1 mL of fibrillar collagen, 0.005–0.1 mL of enzyme, and enough 50 mM Tris-HCl and 5 mM $CaCl_2$ (pH 7.4) buffer to bring the total volume to 0.3 mL. The assay tubes were incubated for 1 h at 37 °C in a water bath and then centrifuged for 5 min in a Beckman 152 microfuge at 15 000 rpm. A sample (0.2 mL) was taken from the supernatant fluids and placed in 5 mL of Biofluor (New England Nuclear) scintillation fluid, and the radioactivity was measured in a Beckman LS-3133T counter. Buffer and trypsin (10 μg) controls were included to measure the denaturation of the substrate, which amounted to 5–10% of the collagen.

Collagen–CH-Sepharose Preparation. Acid-extracted guinea pig skin collagen (40 mg), purified on DEAE-cellulose (Miller, 1971), was attached to 50 mL of Sepharose 4B via a spacer. The Sepharose was activated by CNBr and allowed to react with 15 g of ε-amino-*n*-caproic acid, and then the collagen attached by the method described by Cuatrecasas (1970). Between 0.3 and 0.7 mg of collagen was bound per mL of Sepharose 4B.

Protease and Inhibitor Assays. The protease activities of trypsin, chymotrypsin, *S. aureus* V-8 protease, and clostridial collagenase were measured according to a modified Kunitz (1947) assay. One milliliter of 0.6% Hammersten casein was mixed with 0.1 mL of an enzyme sample and incubated at 37 °C. The controls consisted of casein and 2 mL of 0.44 N trichloroacetic acid which were incubated under the same conditions. After 3 h all tubes were put in ice, and 2 mL of 0.44 N trichloroacetic acid was placed in each sample tube. The tubes were centrifuged at 27000*g* for 15 min at 0 °C, and the optical density of the fluids was measured with the control tubes as blanks.

Protease Cleavage of Collagenase C_1. Clostripain, α-chymotrypsin, TPCK–trypsin, and *S. aureus* V-8 protease were used to digest (2 h at 37 °C) collagenase C_1. Aliquots containing 6 μg of collagenase were removed from a tube which originally contained 40 μg of collagenase and protease, at 30-min intervals. For the digestion of collagenase, 7 μg of chymotrypsin, 8 μg of V-8 protease, 50 μg of clostripain, or 6 μg of TPCK–trypsin was employed, after which a 20-fold excess of SBTI and a 10-fold excess of PMSF, NEM, or SBTI, respectively, were added to inhibit the proteases. After protease inhibitors were added, 1- to 2-μg portions of collagenase were assayed for collagenolytic activity, and the remainder was analyzed by SDS–polyacrylamide gel electrophoresis. Alternatively, varying concentrations of V-8 protease were used to digest the collagenase. Tubes containing 6 μg of collagenase were incubated with 60, 120, 240, 480, or 960 ng of V-8 protease for 4 h at 37 °C. After the incubation, a 10-fold excess of PMSF was added to each tube on ice.

Sodium Dodecyl Sulfate (SDS) Disc Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Scans of the stained gels were made with a Gilford 2520 scanner at 550 nm. Molecular weight standards, RNA polymerase (39K, 155K, and 165K), unreduced and reduced γ-globulin (23.5K, 50K, and 150K), transferrin (85K), serum albumin (68K), and ovalbumin (43K), were electrophoresed on a 10% sDS–polyacrylamide slab gel with collagenases C_1, C_2, and C_3.

Peptide Mapping. *S. aureus* V-8 protease peptide maps of collagenase were prepared by the method of Cleveland et al. (1977) and included the modifications of Nathanson & Hall (1979).

Purification of Collagenase C_1, C_2, and C_3. The relative amount of each collagenase was determined as follows. Samples of crude collagenase were electrophoresed on a 10% SDS–polyacrylamide slab gel. After the gels were stained with Coomassie Blue, scans of the gel were performed on a Gilford 2520 scanner at 550 nm and the areas under each peak ascertained by cutting and weighing the paper.

All purification steps were performed at 4 °C. Ammonium sulfate fractionation between 35% and 60% and the first DEAE chromatography purification steps were performed according to Lwebuga-Mukasa et al. (1976). Gel filtration of the collagenase employed an Ultrogel AcA34 column (2.5 cm × 95 cm) to remove a low molecular weight brown pigment. Isoelectric focusing of the collagenolytically active peak from the Ultrogel AcA34 column was performed on a 110-mL column (LKB) as outlined by Lwebuga-Mukasa et al. (1976). At this step the purification method for collagenases C_1 and C_2 differs from that of C_3. For C_1 and C_2, the collagenase samples were dialyzed against 25 mM Tris-HCl and 2.5 mM $CaCl_2$ (pH 7.4) buffer before application to a collagen–CH-Sepharose (0.9 cm × 15 cm) column, which was equilibrated with 5 mM Tris-HCl and 0.5 mM $CaCl_2$ (pH 7.4). A 50 mM Tris-HCl and 5 mM $CaCl_2$ (pH 7.4) buffer and the same buffer including 1.5 M NaCl were used to elute proteins. For purification of collagenase C_3 a second DEAE-cellulose column (0.9 cm × 15 cm) was used (Kono, 1968) after the column was equilibrated with 20 mM Tris–acetate and 0.10 mM $CaOAc_2$ (pH 7.5). Then a sulfopropyl-Sephadex C-50 column (0.9 cm × 7.0 cm) was equilibrated with 1 mM succinate and 1 mM $CaOAc_2$ buffer at pH 5.7 (Lee-Own & Anderson, 1975), the active fractions from the DEAE column were applied, and a pH gradient between 5.7 and 8 in 1 mM succinate and 1 mM $CaAc_2$ was used to elute collagenase C_3.

Amino Acid Composition Analysis. The amino acid composition analysis of collagenase C_1 was performed by Barbara Cottrell in the laboratory of Dr. Russell Doolittle (University of California, San Diego). A Beckman Instruments 121 M analyzer and AA-20 resin were used. Samples were hydrolyzed in 6 N HCl for 24 h under nitrogen at 110 °C. Cysteine and tryptophan were not determined, and values for asparagine and glutamine are included in the aspartic acid and glutamic acid residue values, respectively. Norleucine was used as an internal standard.

Atomic Absorption Analyses. The analyses for the zinc content of purified collagenases C_1 and C_3 were carried out in the laboratory of Dr. John Leong (University of California, San Diego). A Varian AA-275 atomic absorption spectrophotometer with a graphite furnace was used with a Jarrell-

Ash zinc lamp. Collagenase C_1 was dialyzed against 1 mM Tris-HCl and 0.1 mM $CaCl_2$ buffer (pH 7.4), and collagenase C_3 was dialyzed against 1 mM Tris-HCl buffer (pH 8.0). They were then diluted 20-fold in water to 3.3 and 8.6 μg/mL, respectively. *A. iophagus* collagenase was dissolved in water at a concentration of 13.2 μg/mL.

Carbohydrate Analyses. Carbohydrate was visualized by the periodic acid–Schiff staining technique. The SDS–polyacrylamide gel slab was treated by the method of Fairbanks et al. (1971). The specificity was ascertained with the carbohydrate-free standards bovine serum albumin, concanavalin A, and α-chymotrypsin and the sensitivity determined by the carbohydrate standards transferrin and ovalbumin. The range of carbohydrate examined was between 0.06 and 0.75 μg per sample. Collagenase samples (23 and 45 μg) were applied to the SDS–polyacrylamide gel.

Gas chromatographic carbohydrate analysis was performed by Dr. Hud Freeze in the laboratory of Dr. Arnold Miller (University of California, San Diego). A Varian 3700 gas chromatograph was used with 3% SE-30 resin. Collagenase C_1 (260 μg or 2.6 nmol) was prepared and analyzed by the procedure as described by Clamp et al. (1972).

Immunochemical Studies. Rabbit antiserum directed against bacterial collagenase was prepared by Dr. Jamson Lwebuga-Mukasa in the laboratory of Dr. Palmer Taylor (University of California, San Diego). Briefly, 75 μg of purified collagenase IIIa and complete Freund's adjuvant were injected into rabbits every week for 3 weeks. The rabbits were bled 1 week after the last injection, and the serum was lyophilized and stored in the freezer until reconstituted with distilled water (Lwebuga-Mukasa et al., 1976). Rabbit antiserum was fractionated on a Ultrogel AcA34 (2.5 cm × 95 cm) column. The elution buffer was Tris–Saline (50 mM Tris-HCl, 5 mM $CaCl_2$, and 150 mM NaCl, pH 7.4) at a flow rate of 6.4 mL/h. The fractions which contained clostridial collagenase inhibitory activity were used for further studies. Alternatively, the immunoglobulin fraction was purified from rabbit antiserum by ammonium sulfate precipitation (Kendall, 1938). Fab′ fragments were prepared from the immunoglobulin fraction according to the method of Nisonoff et al. (1960).

Antiserum was incubated with collagenase overnight at 4 °C, and the immune complexes were precipitated with *S. aureus* by utilizing the method of Sefton et al. (1978). The immunoprecipitates and the supernatant fluids were analyzed by SDS–polyacrylamide gel electrophoresis.

Specificity of the antiserum was also ascertained by immunodiffusion. The wells in double diffusion plates containing 1.5% ionagar in 50 mM Tris-HCl (pH 8.0) and 0.1% sodium azide were filled with antiserum or collagenase and incubated at 4 °C. After 2 days the plates were washed 3–4 times in 0.15 M NaCl and then stained in 0.05% Coomassie Blue R-250 stain.

To ascertain inhibition, collagenase was mixed with the antiserum or Tris–saline buffer, incubated for 1 h at room temperature, and then assayed for collagenolytic activity. To analyze for competitive inhibition, collagenase fragment C_3 was preincubated overnight at 4 °C with the antiserum or buffer. Collagenase C_1 was added to the mixtures, incubated for 60 min at room temperature, and then assayed for collagenolytic activity.

Results

Purification of Collagenases C_1, C_2, and C_3. The relative amount of collagenase C_1, C_2, or C_3 to the total amount of crude protein varied with the preparation of collagenase type

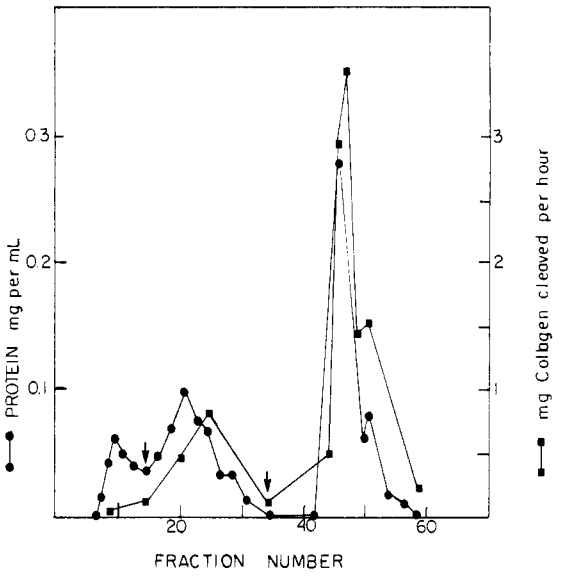

FIGURE 1: Collagen–CH-Sepharose chromatography. Collagenase (6 mg in 2 mL of buffer) was applied to a 0.9 cm × 10 cm column by using a flow rate of 10 mL/h. Elution was carried out with 50 mM Tris-HCl and 5 mM $CaCl_2$ (pH 7.4) buffer (first arrow) at a flow rate of 30 mL/h. The buffer was changed (second arrow) to 50 mM Tris-HCl, 5 mM $CaCl_2$, and 1.5 M NaCl (pH 7.4). Two-milliliter fractions were collected, and 0.03-mL aliquots were assayed for collagenolytic activity.

(Worthington nomenclature, III or IV). Collagenase C_1 ranged from 8% (type IV) to 38% (type III) of the total protein. Collagenase C_2 represented about 6% of the total protein for type III or IV. One preparation of collagenase (type IV) contained 9% collagenase C_3 while other samples did not contain appreciable levels of collagenase C_3.

Crude collagenase was subjected to ammonium sulfate fractionation, DEAE-cellulose chromatography, and a gel filtration column. No caseinolytic activity was detected in the collagenolytically active peak of the gel filtration column. After isoelectric focusing, collagen–CH-Sepharose chromatography was employed to separate two collagenases. The major peak of activity eluted in high salt (Figure 1). Sample fractions from the affinity column were electrophoresed on a SDS–polyacrylamide slab gel (Figure 2). An M_r 96 000 species of collagenase called C_1 (lane 12) was separated from the M_r 92 000 collagenase, C_2 (lane 5).

Another preparation (lot 40D208), which contained collagenase C_3, with a molecular weight of 76 000, was not subjected to isoelectric focusing or affinity chromatography but was applied to a second DEAE-cellulose column after the initial four purification steps. The active fractions from the DEAE-cellulose column were then prepared for sulfopropyl-Sephadex chromatography. Contaminating collagenases C_1 and C_2 eluted from the sulfopropyl-Sephadex column in the middle of the pH gradient, and collagenase C_3 eluted toward the end of the gradient. When necessary, a gel filtration column was employed to purify some lower molecular weight contaminants away from the M_r 76 000 fragment. A summary of the collagenases purification from the lot which contained C_3 is presented in Table I.

Collagenase Peptide Mapping. A peptide map illustrated the possible precursor–product relationship between collagenases C_1 and C_2. *S. aureus* V-8 protease produced C_2 from C_1 (Figure 3), and collagenases C_1 and C_2 were degraded into peptides which were identical with each other. Positions for uncleaved collagenases C_1 and C_2 were determined for this gradient system on another gel (data not shown). This same process was repeated on a preparation of collagenase in which

Table I: Purification of the Collagenases from *Clostridium histolyticum*

	mg of protein	units/mg[a]	total units	% yield	fold
(I) crude	2500	5	12500	100.0	
(II) ammonium sulfate	830	13	10790	86.32	2.6
(III) DEAE, peak A	310	17	5270	42.16	3.4
(IV) Ultrogel AcA34	100	38	3800	30.40	7.6
	Collagenase C_1 and C_2				
(V) isoelectric focusing	10	50	500	4.0	10
(VI) collagen–CH-Sepharose chromatography					
C_1	0.2	75	15	0.12	15
C_2	1.4	2	2.8	0.02	
	Collagenase C_3				
(V) DEAE, 2	54	40	2160	17.28	8.0
(VI) sulfopropyl-Sephadex					
C_3	3.4	8	27.2	0.22	1.6

[a] One unit of collagenase = the amount of enzyme required to cleave 1 mg of collagen/h. Specific activity = units per milligram of collagenase.

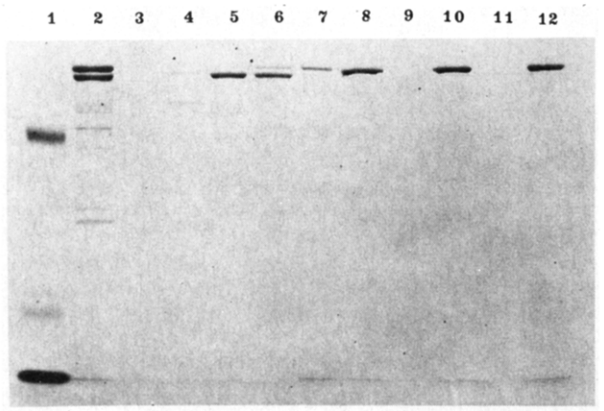

FIGURE 2: SDS–polyacrylamide gel electrophoresis of collagen–CH-Sepharose fractions. A sample of each fraction was boiled in SDS–polyacrylamide sample buffer and then applied to a 10% SDS–polyacrylamide gel. The lanes contained (1) bovine serum albumin and hemoglobin, (2) 14 μg of collagenase starting material, (3) 3 μg of fraction 10 (4) 2 μg of fraction 15, (5) 3.8 μg of fraction 21, (6) 3.2 μg of fraction 25, (7) 3.7 μg of fraction 45, (8) 4.8 μg of fraction 47, (9) blank, (10) 4.0 μg of fraction 49, (11) blank, and (12) 3.2 μg of fraction 51.

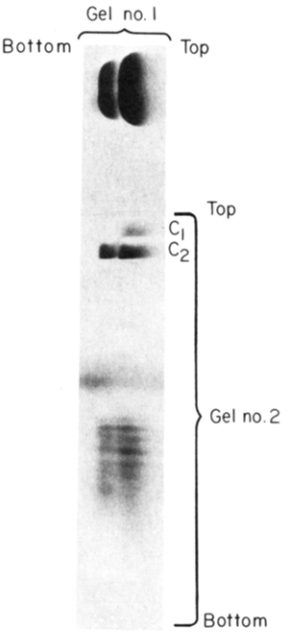

FIGURE 3: *S. aureus* V-8 protease peptide map of collagenases C_1 and C_2. A duplicate slice of a 7.5–15% SDS–polyacrylamide gradient gel containing collagenase (175 μg of the 35–60% pellet from ammonium sulfate fractionation) was placed on top of the stacking gel for this photo. The top of the slice and the collagenases C_1 and C_2 (C_1 and C_2, respectively) are indicated.

collagenase C_3 was prominent. The peptides generated from C_3 corresponded to the peptides generated from C_1 and C_2 proteins (data not shown).

Protease Digestions. Since the peptide mapping was in the presence of the detergent SDS, the relationship of collagenases C_1, C_2, and C_3 was also examined by cleaving active purified collagenase C_1 with chymotrypsin, trypsin, or *S. aureus* V-8 protease. Chymotrypsin generated C_2 from C_1 as well as other fragments and resulted in a loss of collagenolytic activity. The untreated collagenase cleaved 64% of the ^{14}C-labeled collagen in 2 h, whereas the chymotrypsin digest cleaved 22% of the collagen substrate in 2 h. After 30 min, chymotrypsin generated peptides with molecular weights between 25 000 and 70 000 (data not shown). In 60 min, an collagenase fragment the size of C_2 appeared as a should of C_1 along with an increase in the quantity of the peptides. Most of the peptides and collagenase C_1 were degraded to small molecular weight peptides after 90 min. Within 120 min, all of the detectable collagenases C_1 and C_2 were cleaved.

S. aureus V-8 protease produced collagenase fragments C_2 and C_3 from collagenase C_1. V-8 protease (60 ng) cleaved collagenase C_1 into a peptide with the molecular weight of collagenase C_3 and another small peptide (Figure 4, panel B). When the V-8 protease was increased to 120 (panel C) or 240 ng (panel D), a collagenase fragment the size of C_2 was also produced and appeared as a shoulder of collagenase C_1. Cleavage by 480 ng of V-8 protease (panel E) or 960 ng (panel F) resulted in a great reduction of collagenases C_1 and C_3. The collagenase activity decreased from an initial 88% of collagen cleaved in 2 h (panel A) to 51% of the collagen cleaved in 2 h after V-8 protease digestion (panel F). V-8 protease (640 ng) was not detected by the Coomassie Blue stain. Trypsin and clostripain caused a diminution in the collagenolytic activity of collagenase C_1; however, stable forms of collagenases C_2 and C_3 were not generated (data not shown).

Autolysis was also considered as a means of the fragment generation. Purified collagenase C_1 was incubated at 37 °C for the time periods of 0, 0.25, 1, and 3 days. No loss of collagenolytic activity was observed throughout the 3-day incubations, and SDS–polyacrylamide gel electrophoresis did not reveal any reduction of collagenase C_1 or appearance of other fragments.

Antiserum Cross-Reactivity. The immunological relationship of the three collagenases was studied with rabbit antiserum raised against a purified collagenase IIIa. The isoelectric point of collagenase IIIa is 5.9 (Lwebuga-Mukasa et al., 1976) which correlates with the behavior of collagenase

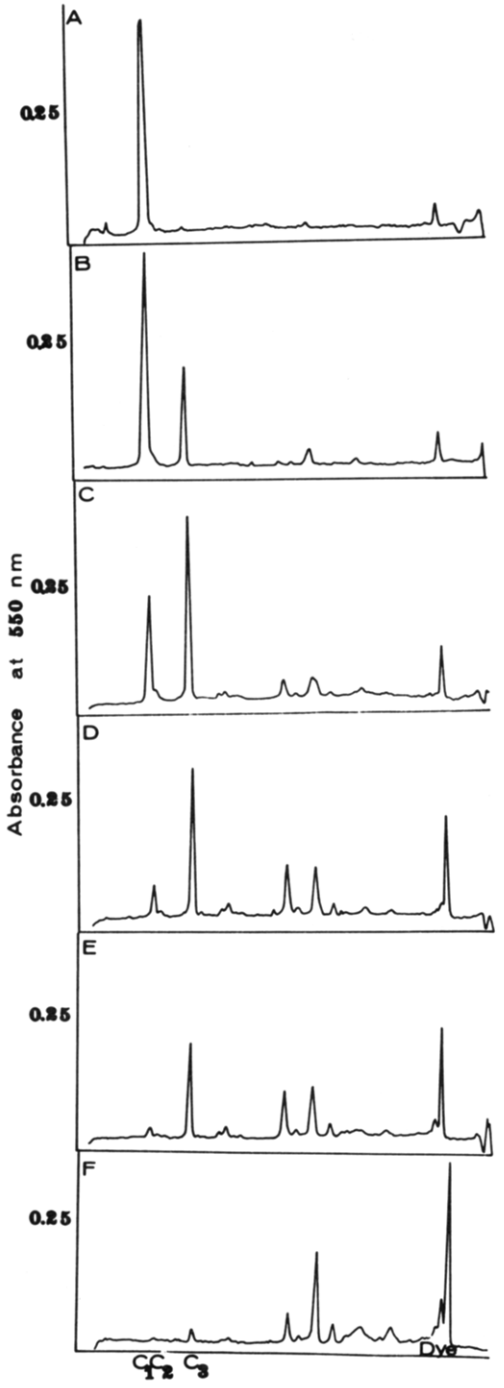

FIGURE 4: Scans of *S. aureus* V-8 protease cleavage products. After digestion of collagenase C_1 with varying amounts of V-8 protease, a sample of each was electrophoresed on a 10% SDS–polyacrylamide slab gel, stained with Coomassie Blue, and scanned at 550 nm with a Gilford 2520 gel scanner. (A) Collagenase C_1 (6 μg); (B) collagenase C_1 plus 0.06 μg of V-8 protease; (C) collagenase C_1 plus 0.12 μg of V-8 protease; (D) collagenase C_1 plus 0.24 μg of V-8 protease); (E) collagenase C_1 plus 0.48 μg of V-8 protease; (F) collagenase C_1 plus 0.96 μg of V-8 protease.

C_3 on sulfopropyl-Sephadex. The ionic character and similar molecular weights (78 000 and 76 000 on 10% SDS–polyacrylamide gels) of IIIa and C_3, respectively, indicate homology. The antibody did bind C_1 and C_2 as shown in Figure 5. *S. aureus* bacteria alone did not bind collagenase C_1 or C_2 (lane 9 and 10) but did precipitate the immunoglobulin (lane 2) and the immune complex formed from collagenases C_1 and C_2 and the immunoglobulin (lane 7). The C_3 fragment also immunoprecipitated with the antibody (lane 3) as shown in Figure 6, whereas normal rabbit serum did not (lane 5).

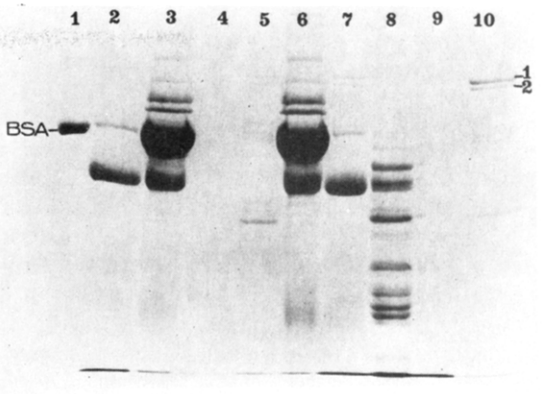

FIGURE 5: SDS–polyacrylamide gel electrophoresis of collagenase C_1 and C_2 immune complexes. The precipitates of collagenase and antiserum were analyzed by SDS–polyacrylamide gel electrophoresis. The lanes contained (1) bovine serum albumin (BSA), (2) pellet from *S. aureus* bacteria and antiserum alone, (3) 50 μg of the supernatant fluids from the pellet in lane 2, (4) SDS sample buffer wash of *S. aureus* bacteria, (5) 40 μg of crude collagenase, (6) 73 μg of the antiserum directed against the collagenase, (7) immunoprecipitate of *S. aureus* bacteria, antiserum, and collagenase, (8) 50 μg of the supernatant fluids from the precipitate in lane 7, (9) *S. aureus* bacteria and collagenase pellet, and (10) supernatant fluids of the pellet from lane 9.

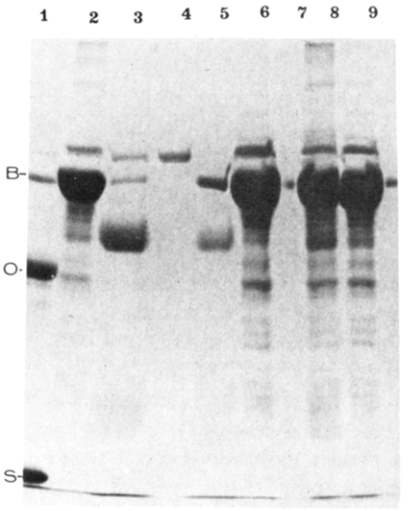

FIGURE 6: SDS (10%)–polyacrylamide gel electrophoresis of collagenase C_3 immunoprecipitates. The lanes contained (1) bovine serum albumin (B), ovalbumin (O), and SBT1 (S), (2) collagenase C_3 and rabbit antiserum immunoprecipitate supernatant fluids, (3) collagenase C_3, antiserum, and *S. aureus* precipitate, (4) 5 μg of collagenase C_3, (5) collagenase C_3, normal rabbit serum, and *S. aureus* pellet, (6) 30 μg of the supernatant fluids from the precipitate in lane 5, (7) a blank, (8) 28 μg of antiserum directed against the collagenase, and (9) 28 μg of normal rabbit serum.

In immunodiffusion plates collagenases C_1, C_2, and C_3 were precipitated by antiserum directed against the bacterial collagenase, and they gave a line of identity with each other (Figure 7). Normal rabbit serum did not precipitate with the collagenases (data not shown).

Antiserum Inhibition. Purified rabbit antiserum decreased the collagenolytic activity of crude or purified enzyme with increased antiserum concentration (data not shown). Nonprecipitating antibody fragments (Fab′), generated from normal rabbit serum and the antiserum by pepsin digestion, were also assayed for inhibitory activity of purified collagenase C_1. The results of the antibody inhibition are shown in Table II. Bovine serum albumin was included in the buffer to mimic any protein–protein interactions caused by addition of the serum to the clostridial collagenase. The Fab′ fragments did

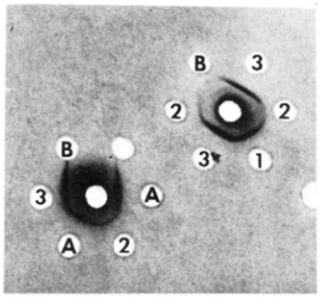

FIGURE 7: Immunodiffusion of antiserum and collagenase. The center wells contained 194 μg of antiserum directed against collagenase IIA. The outer wells contained either 3.3 μg of C_1 (1), 6.4 μg of C_2 (2), 6.6 μg of C_3 (3), 6.6 μg of a protein isolated from the collagenase preparation (B), or 11 μg of an ammonium sulfate fraction of the collagenase preparation (A) and are indicated by the parentheses.

Table II: Fab′ Inhibition of the Clostridial Collagenase

combination	% collagen cleaved/h[a]	% inhibition
clostridial collagenase (3 μg) and buffer	48	
clostridial collagenase (3 μg) and uncleaved antibody (275 μg)	6	88
clostridial collagenase (3 μg) and Fab′ fragments (312 μg)	4	93
clostridial collagenase (2 μg) and buffer with BSA (156 μg)	37	
clostridial collagenase (2 μg) and normal rabbit sera (141 μg)	75	0
clostridial collagenase (2 μg) and normal rabbit Fab′ (156 μg)	41	0

[a] Clostridial collagenase C_1 was combined with the preformed collagen fibril and antibody or antibody fragments in a volume of 0.2 mL at room temperature for 0.5 h. The collagenase was then assayed for collagenolytic activity at 37 °C for 1 h.

not precipitate the collagenase in the double-diffusion Ouchterlony plates (data not shown). To ensure that the binding of Fab′ to the enzyme was required for the Fab′ inhibition, the supernatant fluids of immunoprecipitates containing the Fab′ fragments, collagenase, goat anti-rabbit Fab′, and *S. aureus* bacteria were assayed for collagenolytic activity. The collagenolytic activity was much lower in the supernatant fluids of the antiserum–collagenase mixture than in the control normal rabbit Fab′ fragment mixture, 22 μg vs. 132 μg of collagen cleaved per h, which indicated that the anti-collagenase Fab′ did bind to the collagenase.

Collagenase fragment C_3 relieved the inhibitory activity of the antiserum on collagenase C_1 when added in excess amounts (data not shown).

Amino Acid Composition. The amino acid composition of collagenase C_1 is presented in Table III.

Atomic Absorption Spectroscopy. The zinc content of collagenases C_1, C_3, and *Achromobacter* collagenase was analyzed on an atomic absorption spectrophotometer, and the results are presented in Table IV. The clostridial collagenase contained approximately 1 mol of zinc/mol of collagenase C_1 or C_3. The *Achromobacter* collagenase also contained approximately 1 mol of zinc per molecular weight unit of 111 700.

Carbohydrate Analyses. Carbohydrate analysis was performed by staining a SDS–polyacrylamide slab gel with periodic acid–Schiff stain. Collagenase C_1 did not stain (data not shown). A 260-μg sample of collagenase C_1 was analyzed by gas–liquid chromatography, and only glucose was detected.

Discussion

Previously reported purifications on the clostridial enzyme isolated proteins with molecular weights between 72 000 and

Table III: Amino Acid Composition of Collagenase C_1

amino acid	residues/molecule[a] of C_1	amino acid	residues/molecule[a] of C_1
Asp	152[b]	Met	10
Thr	62	Ile	52
Ser	61	Leu	80
Glu	93	Tyr	32
Pro	23	Phe	54
Gly	90	His	15
Ala	58	Lys	93
Val	56	Arg	28

[a] Molecular weight of 96 000. [b] Averages of the duplicates were used for the calculations.

Table IV: Zinc Content

enzyme	absorbance[a]	mol of Zn/mol of enzyme[b]
collagenase C_1 (17 ng)	0.101 ± 0.016	1.14
collagenase C_3 (43 ng)	0.173 ● 0.003	0.82
Achromobacter collagenase (66 ng)	0.199 ● 0.025	0.92
zinc standard		
10 pg	0.087 ● 0.005	
25 pg	0.151 ● 0.007	
50 pg	0.259 ● 0.015	
dilute nitric acid (10^{-8} N)	0.022 ± 0.004	
dilute NaCl (2×10^{-5} M)	0.027 ● 0.006	

[a] Average of at least three determinations for the standards and at least nine for collagenases C_1 and C_3. [b] Molecular weights of 96 000, 76 000, and 111 700 were assumed for C_1, C_3, and *Achromobacter* collagenase, respectively.

112 000 (Mandl et al., 1964; Miyoshi & Rosenbloom, 1974; Oppenheim & Franzblau, 1978; Yoshida & Noda, 1965; Kono, 1968; Keil, 1979). To determine whether the multiplicity in molecular weight, isoelectric point, and collagenolytic activities was due to the proteolysis of a single gene product, we first purified three forms of the collagenase; C_1 with an apparent molecular weight of 96 000 as well as two other lower molecular weight fragments, C_2 and C_3. The purification procedure employed ammonium sulfate fractionation, DEAE-cellulose chromatography, gel filtration, isoelectric focusing, sulfopropyl-Sephadex chromatography, and affinity chromatography. C_3 was a fragment with a molecular weight of approximately 76 000 and was separated on sulfopropyl-Sephadex from the other proteins. The ammonium sulfate fractionation, which yielded a 2.6-fold purification, was similar to the 3-fold purification of Gallop et al. (1957). The final purification of collagenase C_1 was approximately 15-fold. Collagenase C_1 had a specific activity of 75 units/mg whereas collagenase fragments C_2 and C_3 were much less active: 2 and 8 units/mg, respectively.

Collagenase C_1 could be the parent protein of C_2 and C_3, since collagenase C_2 was produced from active collagenase C_1 by α-chymotrypsin cleavage and collagenases C_2 and C_3 were generated from C_1 with *S. aureus* V-8 protease. In the presence of SDS, collagenases C_1, C_2, and C_3 yielded similar peptide maps when digested with *S. aureus* V-8 protease. Production of C_3 and C_2 in vivo may not be due to sequential clipping of C_1 to yield C_2 and then C_3 but rather may be the result of one or two enzymes clipping different regions of C_1, one enzyme yielding a slightly higher specific activity enzyme (C_3) with a lower molecular weight than C_2 and the other yielding C_2 which is larger than C_3, but may have lost part of its active site or substrate binding region. There are proteases present in the collagenase preparations, which have been useful for tissue digestion (Worthington catalog) and apparently also cleave the collagenases to varying degrees as evidenced by the initial ratios of C_1, C_2, and C_3 to total crude

protein. The protease(s) responsible for the in vivo cleavage has (have) not been identified although clostripain can be eliminated, since clostripain, an enzyme produced by *C. histolyticum*, did not generate the C_2 and C_3 forms from purified collagenase C_1. Since the *A. iophagus* collagenase exhibits autolytic digestion at 4 °C (Keil-Dlouha, 1976), we considered this possibility for collagenase C_1 proteolysis; however, the collagenase was stable for 3 days at 37 °C.

Further evidence for some homology of these three enzymes comes from the antiserum data. Antiserum inhibited the collagenolytic activity of the clostridial collagenase (C_1) and also inhibited the enzyme activity in the nonprecipitating Fab' form. The clostridial collagenases C_1, C_2, and C_3 were precipitated by antiserum and gave a line of identify with each other when studied by double diffusion which indicated common antigenic sites.

The amino acid composition of collagenase C_1 (Table III) was similar to previously reported amino acid compositions (Keil, 1979; Yoshida & Noda, 1965).

The zinc content of the clostridial collagenase was approximately 1 mol of zinc/mol of collagenase C_1 or C_3. The zinc content for *A. iophagus* collagenase was close to 1 mol/mol of collagenase (Table IV), if a molecular weight of 111 700 was used, and confirmed Keil-Dlouha's (1976) colorimetric determination and a molecular weight of approximately 100 000 as originally found by Welton & Woods (1975).

Periodic acid–Schiff staining of the collagenase did not show the presence of carbohydrate. As little as 10 μg of ovalbumin or 0.4 μg of carbohydrate could be visualized by the stain. The method of gas–liquid chromatography was also used. Only glucose, a major contaminant in glycoprotein samples which have been purified by Sephadex or cellulose resins (Clamp et al., 1972), was detected by this carbohydrate analysis.

We have characterized and described a purification for three collagenases which may be used in clinical debridement (Howes, 1972) and can be employed as tools for collagen or collagen-like protein identification (Gottlieb et al., 1965; Garrels, 1979; Sage et al., 1980; Schubert & LaCorbiere, 1980).

Registry No. Collagenase, 9001-12-1.

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