# Purification and Separation of Individual Collagenases of *Clostridium histolyticum* Using Red Dye Ligand Chromatography†

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ABSTRACT: Six collagenases present in the culture filtrate of *Clostridium histolyticum* have been purified to homogeneity. Chromatography over hydroxylapatite, Sephacryl S-200, and L-arginine–Affi-Gel 202 removes the brown pigment and the great majority of the contaminating proteinases active against casein, benzoyl-L-arginine ethyl ester, and elastin. Reactive Red 120 dye ligand chromatography subdivides the collagenases, which have very similar physicochemical properties, among four fractions. The final purification is achieved by chromatography over DEAE-cellulose and SP-Sephadex. All six collagenases, designated $\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$, and $\zeta$ by the order of their purification, are highly active against collagen and devoid of other proteolytic activities. Each exhibits a single band on sodium dodecyl sulfate–polyacrylamide gels. Two distinct subspecies of the $\alpha$ and $\gamma$ enzymes have been isolated, which have the same molecular weight and activity but different isoelectric points. There is some less pronounced microheterogeneity for the other collagenases. On the basis of their activities toward native collagen and the synthetic peptide 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine (FALGPA), the six collagenases are divided into two classes. Class I collagenases ($\alpha$, $\beta$, and $\gamma$) have high collagenase activity and moderate FALGPA activity while the class II collagenases ($\delta$, $\epsilon$, and $\zeta$) have moderate collagenase and high FALGPA activities. The relationship between these six collagenases and others reported to have been isolated in the literature has also been examined.

The culture filtrate of *Clostridium histolyticum* contains a mixture of proteinases that is the most efficient known system for the degradation of connective tissue. The major constituents of the mixture are collagenases (EC 3.4.23.3),[1] which are enzymes that hydrolyze the triple helical region of collagen under physiological conditions (Seifter & Harper, 1971). In addition, the mixture also contains a brown pigment, an elastase (Sparrow & McQuade, 1973), nonspecific proteinases active against casein (Mandl et al., 1953), and enzymes that hydrolyze BAEE (Ogle & Tytell, 1953). There is evidence to suggest that the enzymes in this mixture act synergistically to degrade collagen (Mandl et al., 1964; Kono, 1968; Lwebuga-Mukasa et al., 1976; Van Wart & Bond, 1982). Thus, in order to gain a fundamental understanding of the mechanisms by which connective tissue is metabolized, it is important to study the roles of the individual enzymes.

Many reports concerning the purification of the collagenases secreted by *C. histolyticum* have appeared (Tytell & Hewson, 1950; Mandl et al., 1953, 1958, 1964; DeBellis et al., 1954; Gallop et al., 1957; Grant & Alburn, 1959; Seifter et al., 1959; Keller & Mandl, 1963; Levdikova et al., 1963; Schaub & Strauch, 1965; Yoshida & Noda, 1965; Strauch & Grassmann, 1966; Kono, 1968; Kesserling et al., 1970; Soru et al., 1970; Peterkofsky & Deigelmann, 1971; Lee-Own & Anderson, 1975; Lwebuga-Mukasa et al., 1976; Kula et al., 1976; Emod & Keil, 1977; Oppenheim & Franzblau, 1978; Emod et al., 1981), but there is no general consensus as to the number of enzymes produced and their properties. The present confusion is due, at least in part, to the fact that none of the previous studies have systematically purified all of the collagenases present in any given preparation to homogeneity by procedures that assay all chromatographic fractions simultaneously for both true collagenase activity as well as that of contaminants. Such studies have been hampered by the lack of sensitive, convenient assays for collagenase activity and by the fact that the individual collagenases have very similar physicochemical properties and are hard to separate by the commonly used procedures. As a result, there is considerable uncertainty regarding the properties of the individual enzymes and the relationship between them.

In this paper, we report the complete purification and separation of six collagenases that account for virtually all of the activity present in the starting preparation. In the accompanying papers (Bond & Van Wart, 1984a,b), the properties of these enzymes are presented, and the interrelationship between them is considered in detail. The purification has been made possible by the extensive use of two new collagenase assays (Van Wart & Steinbrink, 1981; Van Wart & Bond, 1982) and by our discovery that partial separation of the individual collagenases can be achieved by red dye ligand chromatography. On the basis of our findings, many of the conflicting reports concerning the multiplicity and properties of these collagenases can be condensed into a unified picture.

## Materials and Methods

*Materials.* Crude and partially purified collagenase preparations were purchased from various commercial suppliers, as listed in Table I. Hydroxylapaptite (Bio-Gel HT), Chelex 100, Affi-Gel 102, and Affi-Gel 202 were obtained from Bio-Rad Laboratories; Sephacryl S-200 (superfine), SP-Sephadex (C-50), Con A–Sepharose, Sepharose-4B, and AH-Sepharose 4B were from Pharmacia Fine Chemicals;

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[1] Abbreviations: collagenase, *Clostridium histolyticum* collagenase; FALGPP, 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-proline; FALGPA, 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine; PZ, *p*-phenylazobenzyloxycarbonyl; BAEE, benzoyl-L-arginine ethyl ester; [$^{14}CH_3$]-collagen, [*methyl*-$^{14}$C]collagen; [$^{14}CH_3$]casein, [*methyl*-$^{14}$C]casein; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; cpm, counts per minute; nkat, nanokatal.

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DEAE-cellulose (DE-52) was from Whatman, Inc.; Reactive Red 120–agarose was from Sigma Chemical Co.; *p*-(chloromercuri)benzoate–agarose, (6-aminocaproyl)-D-arginine–agarose, and soybean trypsin inhibitor–agarose were from Pierce Chemical Co.; a dye ligand chromatography kit, Minicon B-15 concentrators, and PM-10 membranes were from Amicon Corp. Acid-soluble calf skin collagen (type III), elastin (bovine neck ligament), bovine serum albumin, BAEE, and rivanol (6,9-diamino-2-ethoxyacridine lactate) were purchased from Sigma Chemical Co., and casein (Hammerstein) was from Chemalog.

*Synthesis of Immobilized Ligand Resins.* Various ligands were coupled to Affi-Gel 102, Affi-Gel 202, or AH-Sepharose 4B with the aid of 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (Van Wart & Lin, 1981). The immobilization of ligands on Sepharose 4B was achieved with cyanogen bromide as described by Cuatrecasas (1970).

*Substrate Syntheses.* FALGPA was synthesized as reported previously (Van Wart & Steinbrink, 1981). FALGPP was prepared by a parallel synthesis in which the *N*-hydroxysuccinimide ester of 2-furanacrylic acid was coupled to L-leucylglycyl-L-prolyl-L-proline. The concentration of the 2-furanacryloyl group was determined spectrophotometrically by using $\epsilon_{304}$ = 24 700 $M^{-1}$ $cm^{-1}$. Anal. $FA_{1.00}Leu_{0.98}Gly_{1.02}Pro_{2.08}$.

[$^{14}CH_3$]Collagen was prepared by reductive methylation of acid-soluble calf skin collagen with [$^{14}$C]formaldehyde (New England Nuclear) and sodium borohydride as described earlier (Van Wart & Steinbrink, 1981). [$^{14}CH_3$]Casein was prepared by the same procedure. The concentration of $^{14}$C in the radiolabeled collagen and casein was $2.0 \times 10^6$ and $3.3 \times 10^6$ cpm/mg of protein, respectively.

*Enzymatic Assays.* Collagenase activity was determined by measuring the hydrolysis of soluble [$^{14}CH_3$]collagen in 50 mM Tricine, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5 (Van Wart & Bond, 1982), in silanized glass tubes at 25 °C. Initial rates ($v_i$) are expressed as nanokatals (nanomoles per second), where a molecular weight of 300 000 has been used for collagen. The rate of hydrolysis of [$^{14}CH_3$]casein was determined by the same procedure except that (i) no $CaCl_2$ was added to the assay tubes, (ii) trichloroacetic acid (final concentration 10%) was used to precipitate undigested casein, and (iii) the substrate concentration was 50 μg/mL.

FALGPA and FALGPP assays were carried out spectrophotometrically at 23 °C in 50 mM Tricine, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, at a substrate concentration of 50 μM (Van Wart & Steinbrink, 1981). The hydrolysis of BAEE was measured at 25 °C in 25 mM Tricine, pH 7.6, containing 2.5 mM dithiothreitol after activation in this same buffer by following the absorbance change at 253 nm at a substrate concentration of 0.25 mM as described by Mitchell & Harrington (1968). Elastase activity was determined by measuring the initial rate of solubilization of a 1 mg/mL suspension of bovine neck ligament elastin in 50 mM Tricine, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5 at 35 °C. Aliquots of the suspension were removed as a function of time, and the concentration of solubilized material was determined fluorometrically after reaction with fluorescamine ($\lambda_{ex}$ = 390 nm; $\lambda_{em}$ = 480 nm).

Protein concentration of all fractions up to the red dye ligand column (Figure 1) was determined by using the Bio-Rad dye binding assay kit by the method of Bradford (1976) with crystalline bovine serum albumin as a standard. Since the ratio of $A_{280}$ to micrograms per milliliter of protein present became constant after the red dye ligand step, all subsequent concentrations were determined spectrophotometrically by assuming that $A_{280}$ = 1.0 corresponds to a concentration of 520 μg/mL.

*Chromatography.* All chromatographic columns were run at 4 °C with a controlled flow rate. Pooled fractions (indicated in the figures by upper-case letters) were concentrated by pressure ultrafiltration with PM-10 membranes and desalted by dialysis against metal-free buffer containing 1 mM $CaCl_2$. Up to the red dye ligand step, protein could be recovered by lyophilization. After this step, lyophilized powders did not redissolve well and enzymes were stored as concentrated solutions at −80 °C.

Since collagenases are metalloenzymes, metal-free conditions were maintained in all aspects of this work. Reagent-grade water (resistivity 18 M Ω $cm^{-1}$) was prepared with a Millipore Milli-Q system and used in all experiments. All solutions, salts, and substrates were freed from adventitious metal ions by extraction with dithizone in carbon tetrachloride or by passage over Chelex 100 resin.

Polyacrylamide gel electrophoresis and isoelectric focusing experiments were carried out as described in the accompanying paper (Bond & Van Wart, 1984a).

## Results

Collagenase preparations are available from several commercial sources. To determine the best starting material for the purification, the activities of a number of crude and partially purified preparations have been measured. The specific activities per mass of protein toward [$^{14}CH_3$]collagen, FALGPA, BAEE, and [$^{14}CH_3$]casein are listed in Table I. To assess the relative protein content of each preparation, the mass of protein per milligram of crude powder was also determined. These two sets of parameters give both the amount of each activity per mass of powder and the specific activities per mass of protein or powder.

The specific activities toward [$^{14}CH_3$]collagen and FALGPA and the ratio of these activities to those of contaminants vary widely between the different preparations. In general, however, samples with high collagenase activity also contain high activities toward the other substrates. Amongst the crude preparations, different lots of the Sigma Z-9999 enzyme consistently showed both the highest specific collagenase activity and the highest amount of activity per mass of crude powder. The partially purified samples all have high specific collagenase activities; however, only the product from Advanced Biofactures is substantially free of contaminants. Gel electrophoresis and isoelectric focusing experiments (not shown) indicate that the protein composition of the crude preparations is remarkably similar and, in some cases, almost identical. In particular, the gel electrophoresis patterns show that between all these preparations there are at least six discrete proteins in the $M_r$ 60 000–130 000 range in which the collagenases have previously been reported to lie. The only preparation that contained *all* six bands was the Sigma type Z-9999. Hence, this was chosen as the starting material for our purification.

The scheme used to purify and separate the collagenases is summarized in Figure 1. The upper-case letters pertain to pooled fractions identified in the elution profiles (Figures 2–7). Individual fractions from the columns were assayed for the relevant activities and selected fractions concentrated with Minicons and analyzed by gel electrophoresis and isoelectric focusing. On the basis of the results, fractions containing the collagenase activity (those listed below the vertical arrows, Figure 1) were pooled and applied to the next column. Multiple DEAE-cellulose and SP-Sephadex columns were run,

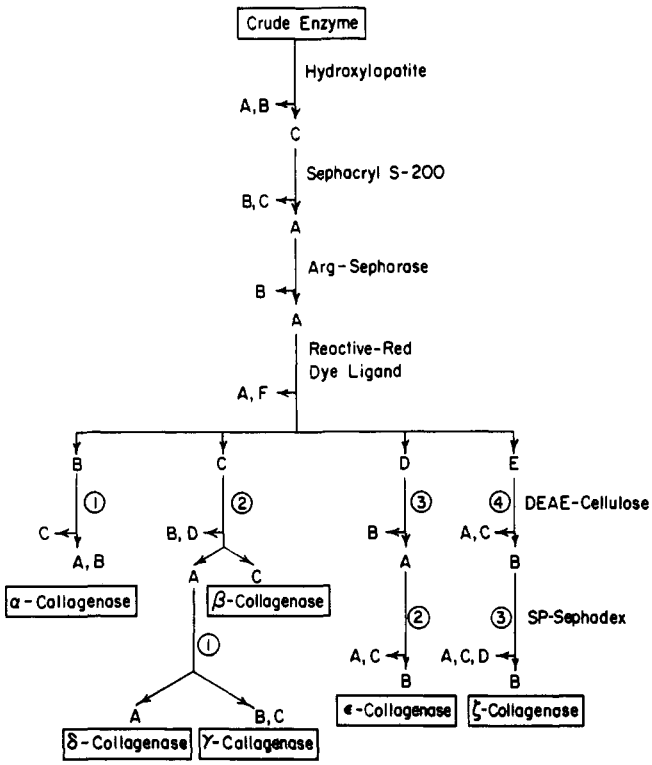


FIGURE 1: Summary of chromatographic steps in the purification and separation of collagenases. The letters A, B, etc. refer to pooled fractions from each column. Those listed next to a horizontal arrow were not studied further. The numbers next to the vertical arrows are for identification of the individual ion-exchange steps. These letters and numbers are used in Table II.

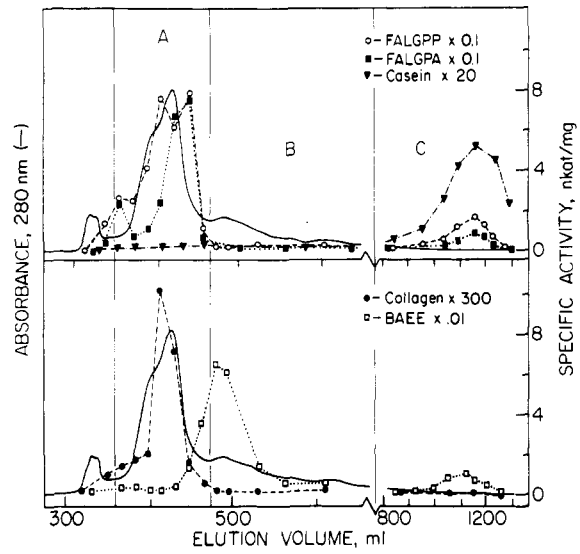


FIGURE 2: Gel filtration of fraction C from the hydroxylapatite column over Sephacryl S-200, superfine, at 4 °C. Enzyme (200 mg in 5 mL) was applied to the column (diameter 2.5 cm; height 190 cm) in 0.05 M phosphate buffer, pH 6.7, and eluted with the same buffer at a flow rate of 35 mL/h. The specific activities toward FALGPP, FALGPA, collagen, casein, and BAEE were determined after the individual chromatographic fractions were dialyzed against 5 mM Tricine, pH 7.5, to remove the phosphate.

and the numbers next to the arrows in Figure 1 are used to identify these.

The first step in the purification involves chromatography over hydroxylapatite as described by Porter et al. (1971). Sigma type Z-9999 collagenase, lot 81F-6819 (2.3 g of powder, 840 mg of protein), was dissolved in 60 mL of 0.05 M phosphate buffer, pH 6.7, and undissolved material removed by centrifugation. The sample was applied to the column, and

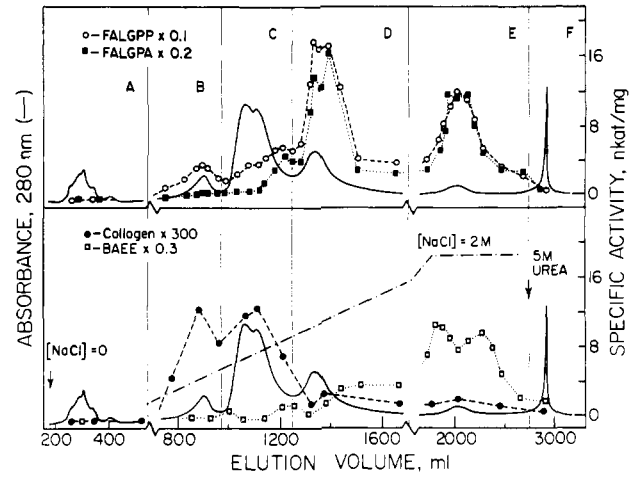


FIGURE 3: Chromatography of fraction A from the L-Arg-Affi-Gel 202 column over Reactive Red 120-agarose at 4 °C. Enzyme (130 mg in 4 mL) was applied to the column (diameter 2.5 cm; height 60 cm) in 5 mM Tris and 5 mM $CaCl_2$, pH 7.5, and eluted with this same buffer for 24 h. Elution was continued with a 2-L, 0–2 M NaCl gradient in this same buffer, followed by 5 M urea. The flow rate for all elutions was 40 mL/h.

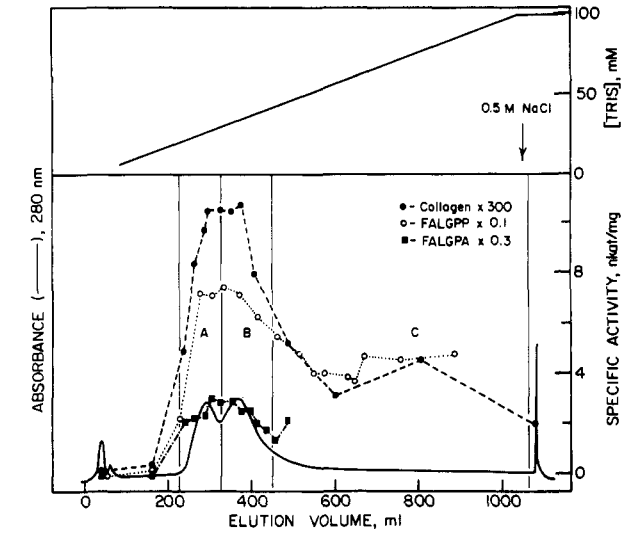


FIGURE 4: Chromatography of fraction B from the Reactive Red 120-agarose column over DEAE-cellulose at 4 °C. Enzyme (7.8 mg in 6 mL) was applied to the column (diameter 1.5 cm; height 4.2 cm), and elution was initiated with 1 mM Tris and 5 mM $CaCl_2$, pH 8. This was followed by a linear 1-L, 1–100 mM Tris gradient in this same buffer and, finally, 0.5 M NaCl. The flow rate for all elutions was 20 mL/h. The two lobes of the double peak in fraction A correspond to two forms of the same enzyme that were subsequently referred to as $\alpha_1$- and $\alpha_2$-collagenase, respectively.

elution was continued with the same buffer until the absorbance reached the base line to give fraction A (chromatograph not shown). The bound protein was eluted with a linear 0.05–0.4 M potassium phosphate gradient, pH 6.7, to give a shoulder (fraction B) and a major peak (fraction C). Fraction A contains the great majority of the pigment, 95% of the caseinase activity and 74% of the elastase activity. Fraction C contains 95% of the collagenase activity, but only 0.93, 1.4, and 37% of the caseinase, elastase, and BAEE activities, respectively (Table II). The specific collagenase activity, expressed as nanokatals per milligram of protein, increases 4-fold in this step. However, since substantial quantities of salts and pigment are removed, the specific activity per mass of sample (not shown in Table II) increases over 10-fold.

Fraction C was concentrated to 5 mL, dialyzed against 0.05 M phosphate buffer, pH 6.7, and chromatographed over Sephacryl S-200 in this same buffer to give the elution profile

Table I: Enzymatic Activities of Various Crude and Partially Purified Commercial Collagenase Preparations

| supplier | designation | lot | mg of protein/ mg of crude powder[a] | sp act. (nkat/mg of protein) [$^{14}CH_3$]-collagen | FAL-GPA | BAEE | [$^{14}CH_3$]-casein[b] |
|---|---|---|---|---|---|---|---|
| | | | Crude Preparations | | | | |
| Sigma Chemical Co. | type I | 121F-516 | 0.37 | 0.0029 | 2.8 | 540 | 1.9 |
| | type IA | 101F-6832 | 0.46 | 0.0013 | 1.0 | 25 | 0.27 |
| | type II | 91F-6812 | 0.26 | 0.00045 | 0.30 | 58 | 0.13 |
| | type IV | 42F-6838 | 0.43 | 0.0023 | 3.0 | 77 | 0.71 |
| | type V | 11F-6805 | 0.43 | 0.0033 | 2.7 | 160 | 0.54 |
| | Z-9999 | 81F-6819 | 0.37 | 0.0038 | 4.8 | 98 | 0.80 |
| Worthington Biochemical Corp. | type I | 42C008 | 0.46 | 0.0022 | 2.6 | 250 | 1.9 |
| | type II | W2H209 | 0.33 | 0.0023 | 3.2 | 650 | 2.0 |
| | type III | 41S130 | 0.36 | 0.0023 | 2.7 | 36 | 0.80 |
| | type IV | 41S123 | 0.74 | 0.0024 | 2.4 | 490 | 0.27 |
| Boehringer-Mannheim | none | 1451142 | 0.39 | 0.0023 | 2.3 | 440 | 1.1 |
| Advanced Biofactures Corp. | ABC-TD | 11-051082 | 0.23 | 0.0020 | 4.9 | 43 | 0.17 |
| | ABC-I | P-78R | 0.20 | 0.00060 | 0.65 | 100 | 0.13 |
| | ABC-II | P-79-02R | 0.17 | 0.0010 | 0.76 | 120 | 0.18 |
| Calbiochem-Behring Corp. | 234,222 | 103310 | 0.12 | 0.00065 | 1.2 | 130 | 0.16 |
| | 23,415 | 102364 | 0.20 | 0.0013 | 2.9 | 44 | 0.19 |
| | | | Partially Purified Preparations | | | | |
| Sigma Chemical Co. | type III | 61F-0566 | 0.90 | 0.0077 | 7.8 | 720 | 0.087 |
| | type VII | 111F-6831 | 0.77 | 0.0017 | 78 | 5.5 | 0.0011 |
| Worthington Biochemical Corp. | CLOSA | 51H319 | 0.49 | 0.00065 | 38 | 1.3 | 0.030 |
| | CLSPA | W2J452 | 0.72 | 0.012 | 11 | 72 | *b* |
| Advanced Biofactures Corp. | ABC-III | 186E | 0.11 | 0.020 | 19 | 3.0 | *b* |
| Calbiochem-Behring Corp. | 234,136 | 130075 | 0.15 | 0.0092 | 8.0 | 250 | 0.0075 |

[a] The milligrams of crude powder was determined by weighing the preparation, as received, while the milligrams of protein was determined by the dye binding assay. [b] sp act. is less than 0.0010 nkat/mg.

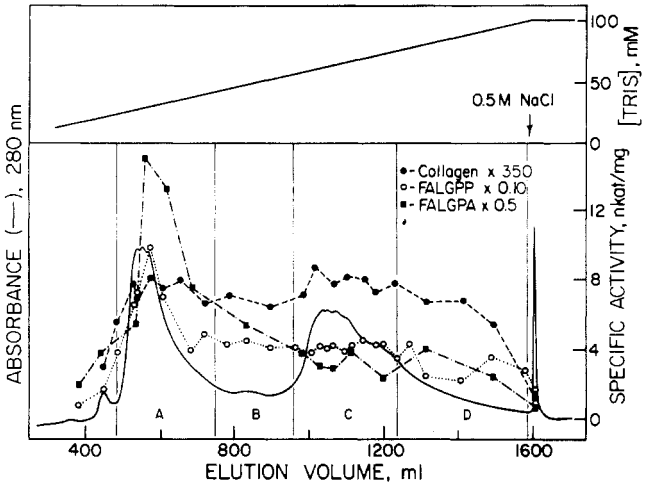


FIGURE 5: Chromatography of fraction C from the Reactive Red 120–agarose column over DEAE-cellulose at 4 °C. Enzyme (59 mg in 8 mL) was applied to the column (diameter 1.5 cm; height 10 cm) in 1 mM Tris and 5 mM $CaCl_2$, pH 8, and elution continued followed by a 1.5-L, 1–100 mM linear Tris gradient and finally 0.5 M NaCl, in this same buffer at a flow rate of 25 mL/h. The individual fractions under pooled fraction C all contained a single homogeneous protein band on sodium dodecyl sulfate–polyacrylamide gels and are subsequently referred to as $\beta$-collagenase.

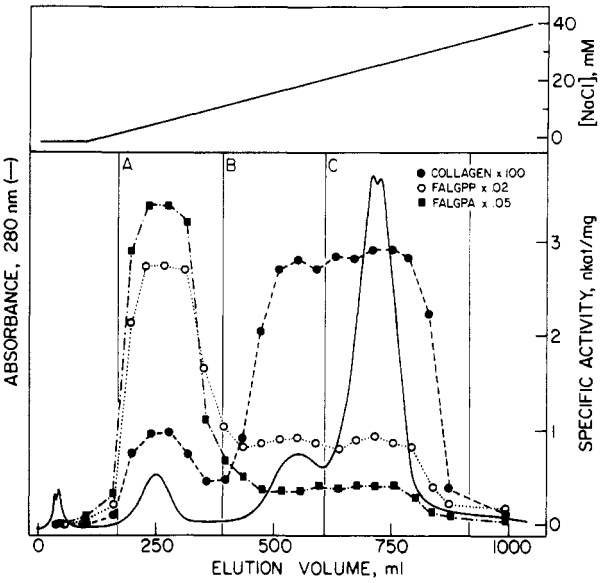


FIGURE 6: Chromatography of fraction A from DEAE-cellulose column 2 over SP-Sephadex at 4 °C. Enzyme (24 mg in 8 mL) was applied to the column (diameter 1.5 cm; height 10 cm) in 1 mM Mes and 2.5 mM $CaCl_2$, pH 6, and eluted with a 1.0-L, 0–40 mM NaCl gradient in this same buffer at a flow rate of 25 mL/h. Pooled fractions A–C are homogeneous and are subsequently referred to as $\delta$, $\gamma_1$, and $\gamma_2$-collagenases, respectively.

shown in Figure 2. There is a major peak that contains the bulk of the activity toward collagen, FALGPP, and FALGPA. This is almost, but not completely, separated from a peak of activity toward BAEE. The latter activity is usually attributed to clostripain, also called clostridiopeptidase B (Mitchell & Harrington, 1968). There is a third peak of activity toward all substrates tested except collagen. All of the collagenase activity from the major peak was pooled to give fraction A. This step increases the specific activity toward collagen from 0.015 to 0.017 nkat/mg, reduces the activities toward BAEE and casein over 5-fold, and eliminates the remaining elastase activity.

To separate the remaining BAEE activity from the collagenases, a series of possible affinity resins for these enzymes was investigated. Emod & Keil (1977) have reported that either soybean trypsin inhibitor or rivanol immobilized on agarose will bind the collagenase activity but not the BAEE activity. All attempts to reproduce their experiments failed, and collagenase was not found to have any affinity for these resins. (*p*-Aminophenyl)mercury–Sepharose 4B and [*p*-(chloromercuri)benzoyl]ethylenediamine–agarose also failed to separate the collagenase and BAEE activities. However,

Table II: Purification and Separation of Collagenases

| purification step[a] | collagenases present | protein (mg) | [$^{14}CH_3$]collagen sp act. (nkat/mg) | [$^{14}CH_3$]collagen yield (%) | FALGPP sp act. (nkat/mg) | FALGPP yield (%) | BAEE sp act. (nkat/mg) | BAEE yield (%) | [$^{14}CH_3$]casein sp act. (nkat/mg) | [$^{14}CH_3$]casein yield (%) | elastin sp act. (nkat/mg) | elastin yield (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| crude powder | all | 840 | 0.0038 | 100 | 8.5 | 100 | 98 | 100 | 0.80 | 100 | 0.0071 | 100 |
| hydroxylapatite, C | all | 200 | 0.015 | 95 | 34 | 96 | 150 | 37 | 0.031 | 0.93 | 0.00040 | 1.4 |
| Sephacryl S-200, A | all | 135 | 0.017 | 88 | 42 | 79 | 29 | 4.8 | 0.0067 | 0.14 | 0 | 0 |
| Arg–Affi-Gel 202, A | all | 130 | 0.018 | 86 | 43 | 78 | 3.4 | 0.54 | 0.0052 | 0.10 | | |
| Reactive Red | | | | | | | | | | | | |
| B | $\alpha$ | 7.8 | 0.019 | 6.4 | 31 | 4.4 | 0.81 | *b* | 0.0030 | *b* | | |
| C | $\beta$, $\gamma$, $\delta$ | 59 | 0.023 | 59 | 35 | 37 | 1.1 | 0.080 | 0.0013 | 0.012 | | |
| D | $\epsilon$ | 27 | 0.0069 | 8.0 | 99 | 48 | 2.9 | 0.095 | 0.0010 | *b* | | |
| E | $\zeta$ | 16 | 0.0057 | 3.8 | 67 | 19 | 20 | 0.38 | 0.0040 | *b* | | |
| DEAE-cellulose | | | | | | | | | | | | |
| 1A | $\alpha_1$ | 1.9 | 0.028 | 2.3 | 58 | 2.0 | 0.21 | *b* | *c* | *b* | | |
| 1B | $\alpha_2$ | 2.3 | 0.027 | 2.8 | 57 | 2.4 | 0.19 | *b* | *c* | *b* | | |
| DEAE-cellulose | | | | | | | | | | | | |
| 2A | $\gamma$, $\delta$ | 24 | 0.023 | 21 | 55 | 20 | 0.45 | 0.025 | *c* | *b* | | |
| 2C | $\beta$ | 15 | 0.023 | 15 | 44 | 12 | 0.33 | *b* | *c* | *b* | | |
| SP-Sephadex | | | | | | | | | | | | |
| 1A | $\delta$ | 0.87 | 0.0090 | 0.32 | 130 | 1.9 | 0.071 | *b* | *c* | *b* | | |
| 1B | $\gamma_1$ | 2.8 | 0.025 | 2.0 | 55 | 2.4 | 0.20 | *b* | *c* | *b* | | |
| 1C | $\gamma_2$ | 12 | 0.027 | 13 | 53 | 9.8 | 0.27 | *b* | *c* | *b* | | |
| DEAE-cellulose, 3A | $\epsilon$ | 11 | 0.0073 | 3.6 | 150 | 31 | 2.1 | *b* | *c* | *b* | | |
| SP-Sephadex, 2B | $\epsilon$ | 4.5 | 0.0084 | 1.6 | 210 | 16 | 0.56 | *b* | *c* | *b* | | |
| DEAE-cellulose, 4B | $\zeta$ | 3.4 | 0.0071 | 1.0 | 86 | 5.1 | 5.6 | *b* | *c* | *b* | | |
| SP-Sephadex, 3B | $\zeta$ | 2.1 | 0.0091 | 0.78 | 120 | 4.3 | 0.34 | *b* | *c* | *b* | | |

[a] The designation and flow of chromatographic fractions are shown in Figure 1. [b] A yield of less than 0.01%. [c] sp act. is less than 0.0010 nkat/mg.

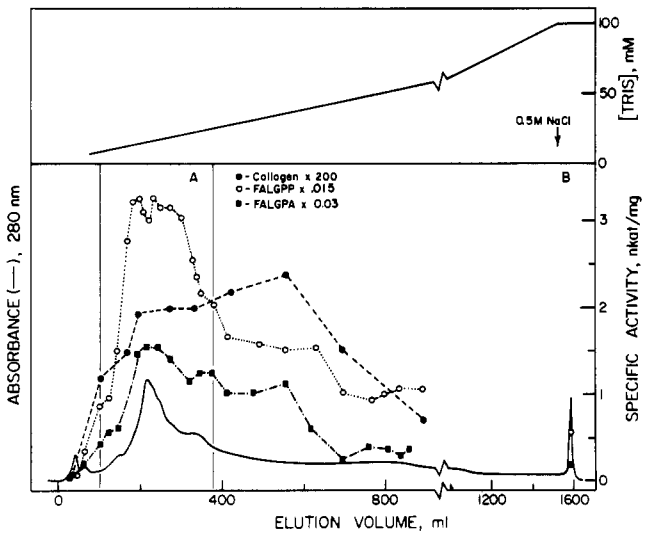


FIGURE 7: Chromatography of fraction D from the Reactive Red 120–agarose column over DEAE-cellulose at 4 °C. Enzyme (27 mg in 8 mL) was applied to the column (diameter 1.5 cm; height 4.2 cm) in 1 mM Tris and 5 mM $CaCl_2$, pH 8, and eluted with a 1.5-L, 1–100 mM Tris gradient in this same buffer, followed by 0.5 M NaCl, at a flow rate of 25 mL/h.

L-arginine–Affi-Gel 202 efficiently removes BAEE activity from the collagenase samples.

Fraction C from the Sephacryl S-200 column was concentrated to 35 mL and activated overnight at 4 °C by addition of 10 mg of dithiothreitol. Solid $NaH_2PO_4$ was added to a final concentration of 0.12 M, pH 6.7, and the enzyme was applied to the L-arginine–Affi-Gel 202 column. The column was washed with this buffer until the absorbance returned to the base line (fraction A), and the bound protein (fraction B) was removed by elution with the same buffer containing 0.6 M L-arginine. Fraction A contains 98% of the collagenase activity, and the specific activity toward BAEE is reduced from 29 to 3.4 nkat/mg. Interestingly, the remaining BAEE activity cannot be removed by a second round of purification over this resin, indicating that there are two distinct species present in the fraction usually called clostripain. The best results are obtained with a resin prepared by reaction of 5 mmol of L-arginine for each mmol of carboxyl groups on the Affi-Gel 202. A lower ratio significantly reduces the capacity of the resin. A higher ratio causes it to act as an anion-exchange resin, making it difficult to keep the collagenases ($pH_I$ <6.5) from binding nonspecifically.

Cumulatively, the first three steps in the purification remove all of the elastase activity and all but 0.54 and 0.10% of the BAEE and caseinase activities, respectively, while retaining 86% of the collagenase activity with a 4.7-fold purification (Table II). Sodium dodecyl sulfate gel electrophoresis of the L-arginine–Affi-Gel 202 fraction A shows six distinct bands in the $M_r$ 60000–130000 range. Subsequent steps were primarily designed to separate these six collagenases from each other. Samples of fraction A from the L-arginine–Affi-Gel 202 resin were applied to a series of ion-exchange columns, and elution with various salt and pH gradients was attempted. However, suitable separations of all the enzymes present could not be achieved. After screening a series of dye ligand columns, the Sigma Reactive Red 120 and Amicon Matrix Gel Red A resins (which contain the same ligand) were found to bind the collagenases. The key step in separation of the individual collagenases is achieved by use of the Reactive Red 120 resin.

Fraction A from the L-arginine–Affi-Gel 202 resin was concentrated to 4 mL, dialyzed against 5 mM Tris and 5 mM $CaCl_2$, pH 7.5, and applied to a reactive Red 120 column. A small peak (fraction A) with no activity eluted early, but the flow of buffer was continued for 24 h (Figure 3). The bound collagenases (fractions B–E) were eluted with a shallow 0–2 M NaCl gradient. If this gradient is initiated without the long preelution in salt-free buffer, the resolution of the fractions is diminished. Collectively, fractions B–E contain 90% of the collagenase activity applied to the column. The ratios of the specific activities toward collagen, FALGPA, and FALGPP vary widely for these four fractions (Figure 3, Table II). Electrophoresis shows that fractions B, D, and E contain a single major protein band while fraction C contains three bands. The final purification and separation of the collagenases from contaminants is achieved by a combination of cation- and anion-exchange chromatography.

Fraction B from the Reactive Red 120 column was concentrated, dialyzed against 1 mM Tris and 5 mM $CaCl_2$, pH 8, and applied to a DEAE-cellulose column. After elution of a small peak, the collagenase activity eluted as a double peak with a 1–100 mM Tris gradient (Figure 4). Fractions A and B, which correspond to the two lobes of the peak, were pooled and are referred to as $\alpha_1$- and $\alpha_2$-collagenase, respectively. All of the individual fractions in this doublet exhibit the same specific activity toward collagen, FALGPP, and FALGPA and run as an identical single band on sodium dodecyl sulfate gels. However, the two parts of the doublet focus with slightly different isoelectric points on isoelectric focusing gels (Bond & Van Wart, 1983a). Hence, $\alpha_1$- and $\alpha_2$-collagenases appear to be two subspecies of the same protein. Portions of these fractions were retained for further characterization, and the rest was pooled and subsequently referred to simply as $\alpha$-collagenase.

Fraction C from the Reactive Red 120 column was subjected to DEAE-chromatography by the same procedure as described above for fraction B. The elution profile shown in Figure 5 is divided into four pooled fractions (A–D). The collagenase activity profile across fractions A–C is almost flat. However, fraction A contains a spike of activity toward FALGPA and FALGPP, indicating the presence of at least two enzymes. Sodium dodecyl sulfate gel electrophoresis on individual fractions from this column confirmed that fraction A was a mixture of two proteins. Fraction C ran as one homogeneous band and is subsequently referred to as $\beta$-collagenase. The broad, asymmetric shape of the elution profile for $\beta$-collagenase is due to the presence of several minor subspecies that have slightly different isoelectric points, which are resolved on isoelectric focusing gels (Bond & Van Wart, 1984a). Fractions B and D, which together contain about 12% of the collagenase activity, both showed a broad distribution of weak bands and were not studied further.

Separation of the two collagenases present in fraction A of DEAE-cellulose column 2 was achieved by chromatography over SP-Sephadex. The sample was concentrated, dialyzed against 1 mM Mes and 5 mM $CaCl_2$, pH 6.0, applied to the SP-Sephadex column, and eluted with a 0–100 mM NaCl gradient in this same buffer. The elution profile (Figure 6) consists of a small peak (fraction A) with moderate collagenase and very high FALGPP and FALGPA activities and two peaks (fractions B and C) with high collagenase but markedly reduced FALGPA and FALGPP activities. These pooled fractions are referred to as $\delta$-, $\gamma_1$- and $\gamma_2$-collagenases, respectively. $\gamma_1$- and $\gamma_2$-collagenases have the same molecular weight but slightly different isoelectric points. The $\delta$- and $\gamma$-collagenases correspond to the two proteins observed in the previous step by gel electrophoresis.

DEAE-chromatography of Reactive Red 120 column fraction D produces an elution profile consisting of one major peak with a shoulder (Figure 7). Gel electrophoresis patterns of the individual fractions under this peak indicated that they were heterogeneous with one major and a few minor bands. Hence, fraction A from this column was concentrated, dialyzed against 1 mM Mes and 5 mM $CaCl_2$, pH 6, and subjected to chromatography over SP-Sephadex. Elution with a 0–30 mM NaCl gradient gave an elution profile with one large peak (fraction B, designated $\epsilon$-collagenase) whose central fractions are homogeneous by gel electrophoresis, have moderate collagenase and high FALGPA and FALGPP activities, and have only a trace of BAEE activity.

The collagenase present in Reactive Red 120 fraction E was similarly purified to homogeneity by sequential elution from DEAE-cellulose and SP-Sephadex columns. The Tris gradient from the DEAE-cellulose column gives one major peak (fraction B) with enriched activity toward collagen and FALGPP and reduced BAEE activity but which is not completely separated from a broad absorbance background due to other minor contaminants. After NaCl gradient elution of this fraction from SP-Sephadex, a single symmetrical peak with only a trace of BAEE activity that runs as a homogeneous band on polyacrylamide gels is obtained. This protein, which has moderate activity toward collagen and high activity toward FALGPP and FALGPA, is referred to as $\zeta$-collagenase.

This purification scheme yields six distinct collagenases, designated $\alpha$-$\zeta$, that are distinguished on the basis of their molecular weights and activities toward collagen, FALGPA, and FALGPP. All six enzymes are essentially devoid of activity against elastin, casein, and BAEE and are homogeneous by the criterion of sodium dodecyl sulfate–polyacrylamide electrophoresis. The physicochemical and enzymatic properties of these enzymes as well as the microheterogeneity revealed by electrofocusing experiments are considered in detail in the accompanying paper (Bond & Van Wart, 1984a).

To explore the relationship between the collagenases isolated in this study and those obtained from two procedures reported in the literature, two additional experiments were carried out. With fraction A from the L-arginine–Affi-Gel 202 resin as a starting material, the collagenases referred to as A and B (Harper et al., 1965; Seifter & Harper, 1970) and I and II (Yoshida & Noda, 1965) were isolated by chromatography over DEAE-cellulose as described by these authors. The elution profiles (not shown) were very similar to those reported, and individual chromatographic fractions were pooled according to the published procedures to give the enzymes nominally referred to as A, B, I, and II. The activities of these pooled fractions toward collagen and FALGPA were measured and the individual collagenases present in each sample determined by gel electrophoresis and isoelectric focusing analysis.

The results of these experiments are shown in Table III. The relative activities of each pair of enzymes toward collagen vs. the synthetic substrate (Cbz-Gly-Pro-Gly-Gly-Pro-Ala was used in the earlier studies rather than FALGPA) are in reasonably good agreement with those reported previously (Harper et al., 1965; Yoshida & Noda, 1965). All four enzymes have similar collagenase activities, but fractions A and II have substantially higher activities toward the synthetic substrate than their respective partners, B and I. The basis for this is evident from the electrophoretic and electrofocusing analysis of these species, which indicates that fractions B and

Table III: Possible Relationship between Collagenases Isolated in This Study and Other Preparations

| prepn[a] | ref | [$^{14}CH_3$]collagen sp act. (nkat/mg) | [$^{14}CH_3$]collagen % act. | FALGPA sp act. (nkat/mg) | FALGPA % act. | collagenases present[b] |
|---|---|---|---|---|---|---|
| collagenase A | *c* | 0.020 | 53 | 51 | 93 | $\alpha$, $\gamma$, $\delta$, $\epsilon$, $\zeta$ + trace of $\beta$ |
| collagenase B | *c* | 0.019 | 46 | 8.2 | 14 | $\beta$ + traces of $\alpha$, $\gamma$, $\delta$, $\epsilon$, $\zeta$ |
| collagenase I | *d* | 0.021 | 37 | 7.0 | 9.4 | $\beta$ + traces of $\alpha$, $\gamma$, $\delta$, $\epsilon$, $\zeta$ |
| collagenase II | *d* | 0.018 | 53 | 39 | 90 | $\alpha$, $\gamma$, $\delta$, $\epsilon$, $\zeta$ + traces of $\beta$ |

[a] The preparations indicated were isolated according to the procedures given in the references with fraction A from the Arg–Affi-Gel 202 column as the starting material. [b] Determined by sodium dodecyl sulfate–polyacrylamide electrophoresis and isoelectric focusing analysis. [c] Seifter & Harper (1970). [d] Yoshida & Noda (1965).

I contain essentially $\beta$-collagenase with traces of other species, while fractions A and II contain the great majority of the $\alpha$-, $\gamma$-, $\delta$-, $\epsilon$-, and $\zeta$-collagenases, with just a trace of $\beta$-collagenase. Thus, it is the presence in fractions A and II of one or all of the $\delta$-, $\epsilon$-, and $\zeta$-collagenases, which have a much higher activity toward FALGPA than the $\alpha$-, $\beta$-, or $\gamma$-collagenases, that is responsible for the relative activities observed.

## Discussion

The goal of this study was to purify to homogeneity the collagenases present in the culture filtrate of *C. histolyticum*. The complete purification reported here has been made possible by the use of new collagenase assays that are based on the hydrolysis of native collagen, FALGPP, and FALGPA. These assays have allowed us to investigate the effectiveness of a wide variety of chromatographic procedures, as well as to accurately quantitate the purifications achieved. Interestingly, since some of the collagenases have differential activities toward native collagen, FALGPP, and FALGPA, this has provided a sensitive means of detecting multiple collagenases that coelute from chromatographic columns (Figure 5).

The purification summarized in Figure 1 has been achieved in essentially two stages. Stage one consists of the first three steps, which are designed to remove the great majority of the pigment and contaminating proteinases that hydrolyze BAEE, casein, and elastin, with minimal loss of collagenase activity. Although the majority of the protein in crude commercial collagenase preparations is actually collagenase, the contaminating proteinases are highly active, and by the criterion of the amount of activity, they are actually more abundant than the collagenases (Table I). The hydroxylapatite step effectively removes almost all of the pigment, caseinase, and elastase activities, as well as 63% of the BAEE activity. Alternative methods that have been reported to remove the pigment include chromatography over SP-Sephadex (Lee-Own & Anderson, 1975; Oppenheim & Franzblau, 1978) or gel filtration (Seifter & Harper, 1970). We have found that SP-Sephadex chromatography is a poor choice for the separation of pigment from the collagenases. All of the collagenases are zinc metalloenzymes (Bond & Van Wart, 1984a) and are unstable below pH 6; thus, the SP-Sephadex column cannot be run at pH values below the isoelectric points of the collagenases, which are in the 5.35–6.20 range. As a result, a significant amount of collagenase activity coelutes with pigment at pH 6. Attempts to overcome this by running the column at lower pH values reduces the activity of the enzyme, presumably by loss of the catalytic zinc. Gel filtration of the crude enzyme does partially separate pigment from the collagenases, but the resolution is poor and far inferior to that achieved with the hydroxylapatite column.

The Sephacryl S-200 column in stage 1 exploits the differences in molecular weight between the collagenases and remaining contaminants. This has been used successfully by other workers (Oppenheim & Franzblau, 1978; Peterkofsky, 1982; Yoshida & Noda, 1965; Lee-Own & Anderson, 1975), and Figure 2 confirms that it substantially separates the collagenase and BAEE activities. Most of the remaining BAEE activity was removed by the L-arginine–Affi-Gel 202 affinity resin. This sequence of three steps in the first stage removes all the elastase, 99.9% of the caseinase, and 99.46% of the BAEE activities, while retaining 86% of the collagenase acxtivity. It should be noted that stage 1 removes at least two enzymes that cleave casein (hydroxylapatite fraction A and Sephacryl S-200 fraction C). In addition, there is evidence for three enzymes that cleave BAEE— one in Sephacryl S-200 fraction C and two in fraction B (one is removed from the L-arginine–Affi-Gel 202 resin and the other is not). Interestingly, there is a very small amount of an enzyme in Sephacryl S-200 fraction C that cleaves FALGPP and FALGPA but not collagen. The reason for the failure of the rivanol and soybean trypsin inhibitor columns to bind collagenase as described previously (Emod & Keil, 1977) is not obvious but is consistent with the fact that neither of these ligands inhibits these enzymes.

Stage 2 of the purification is designed primarily to separate the individual collagenases. All attempts to achieve complete separation of these enzymes by ion-exchange chromatography (Grant & Alburn, 1959; Mandl et al., 1964; Harper et al., 1965; Yoshida & Noda, 1965; Kono, 1968; Lee-Own & Anderson, 1975; Oppenheim & Franzblau, 1978) were only partially successful, probably because they have very similar isoelectric points (Bond & Van Wart, 1984a). Separation by preparative isoelectric focusing (Lwebuga-Mukasa et al., 1976) was also avoided for this reason, and also because of possible detrimental effects of ampholytes on the metal content of the enzymes. However, gradient elution from a red dye ligand column produces four fractions, three of which contain a single collagenase and another that contains the other three.

The combination of DEAE-cellulose and SP-Sephadex chromatography is capable of achieving the final purification and separation to give six collagenases with activities ranging from 0.0084 to 0.028 nkat/mg. The enzyme studied by us in our preliminary report (Bond et al., 1981) was $\beta$-collagenase, which is a major component of almost all commercial preparations. The six enzymes are completely homogeneous on sodium dodecyl sulfate–polyacrylamide gels (data shown in accompanying paper; Van Wart & Bond, 1984a), are devoid of caseinase and elastase activities, and contain only a trace of BAEE activity, which is extremely difficult to remove completely. While in principle these collagenases could have inherent residual activity toward casein (Gilles & Keil, 1976), none is indicated from our studies. The [$^{14}CH_3$]casein assay employed here is at least 1000 times more sensitive than that based on direct spectrophotometric or colorimetric analysis of the digestion products.

It is difficult to compare unambiguously the results of this study with others in the literature because of the uncertain

relationship between the starting materials. In principle, the collagenases studied by other workers could have been different enzymes from those isolated here due to the culturing of a different strain of the bacterium or to culturing under different conditions. The products sold by Sigma and Worthington are derived from the H4-M strain of *C. histolyticum*. Thus, all studies that used preparations from these suppliers or from this strain as starting materials should be directly comparable to that presented here. Moreover, since the electrophoretic profiles obtained for preparations from the other suppliers show few differences, they also probably contain the same collagenases.

Another problem encountered in making comparisons between studies is that numerous different methods and conditions have been used to measure the collagenase activity, few of which are directly comparable. The method employed in this study is convenient and accurate but is known to give an underestimate of the true collagenase activity because the step used to precipitate unhydrolyzed collagen also removes many of the large collagen fragments produced by the collagenases (Van Wart & Bond, 1982). This can be partially overcome by carrying out the assays in the presence of proteinases such as thermolysin that synergistically digest these large fragments so that they remain soluble after precipitation of the native collagen. This additional procedure was not used in this study because only relative collagenase activities were needed. The best means of comparing the activities of collagenases from different studies is by their action against PZ-Pro-Leu-Gly-Pro-D-Arg (Wunsch & Heidrich, 1963), since all such assays have been carried out under a single set of conditions. The activities of the $\delta$-, $\epsilon$-, and $\zeta$-collagenases toward this substrate, which are reported in the accompanying paper (Bond & Van Wart, 1984a), are significantly higher than reported (Kono, 1968; Emod et al., 1981) for any *Clostridial* collagenase.

The purification to homogeneity of six distinct collagenases with the properties described here and in the accompanying paper (Bond & Van Wart, 1984a) provides a basis for unifying many of the different reports in the literature concerning these enzymes. First, reports by various workers (Grant & Alburn, 1959; Mandl et al., 1964; Yoshida & Noda, 1965; Harper & Kang, 1970; Kono, 1968; Peterkofsky & Diegelman, 1971; Kesselring et al., 1970; Lwebuga-Mukasa et al., 1976) that there are multiple collagenases present in the culture filtrate of *C. histolyticum* are generally consistent with the present results. It is noteworthy that multiple collagenases have been observed in these studies even though some used fresh culture filtrate rather than commercial enzyme as the starting preparation. In fact, the number of enzymes detected generally correlates with how extensively the starting preparation was subjected to ion-exchange chromatography and the extent to which the various chromatographic fractions obtained were assayed. Of the recent studies in the literature, only that by Emod et al. (1981) has claimed the presence of a single collagenase in the culture filtrate. However, these authors made no attempt to purify all the collagenases present in their starting preparation. The enzyme isolated by these authors probably corresponds to either $\delta$-, $\epsilon$-, or $\zeta$-collagenase, though the physicochemical properties reported for their enzyme are only in fair agreement with those for ours (Bond & Van Wart, 1984a).

A second unifying observation that follows from this work is that, on the basis of their relative activities toward collagen vs. synthetic substrates, there appears to be two classes of collagenases. The $\alpha$-, $\beta$-, and $\gamma$-collagenases are highly active against collagen and moderately active against FALGPA while the $\delta$, $\epsilon$, and $\zeta$ enzymes are moderately active against collagen but highly active against FALGPA. These two sets of enzymes will be referred to as class I and class II collagenases, respectively. The presence of two distinct classes could account for the widely variable ratios of activities toward collagen and synthetic substrates variously reported for *Clostridial* collagenases if the enzymes isolated were actually mixtures of collagenases from the two classes. As a specific example, the data of Table III suggest a possible relationship between collagenases I and II (Seifter & Harper, 1970), A and B (Yoshida & Noda, 1965), and the enzymes isolated in this study, on the basis of the assumption that the starting material for the published procedures contained collagenases from both classes. While it cannot be stated with certainty which enzymes actually were present, the ratios of the activities toward collagen and the synthetic peptide reported by both sets of authors for their species would indicate that collagenases B and I contain predominantly $\beta$-collagenase, while collagenases A and II minimally contain some $\delta$-, $\epsilon$-, or $\zeta$-collagenase mixed with either $\alpha$- or $\gamma$- collagenase. This is consistent with the fact that all of the preparations listed in Table I contain at least three of these collagenases. Since collagenase A has been the most intensively studies *Clostridial* collagenase, especially with regard to substrate specificity, the possibility exists that this preparation was not homogeneous, and it may be necessary to reexamine its properties. It should be noted that some of the commercial preparations exhibited minor bands in the high molecular weight region that do not match the molecular weight of the collagenases isolated here, and it is possible that there are yet other collagenases present in these mixtures.

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**Registry No.** FALGPA, 78832-65-2; FALGPP, 89847-93-8; collagenase, 9001-12-1; 2-furanacrylic acid *N*-hydroxysuccinimide ester, 56186-54-0; L-leucylglycyl-L-prolyl-L-proline, 89827-23-6.

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# Characterization of the Individual Collagenases from *Clostridium histolyticum*†

Michael D. Bond and Harold E. Van Wart*

ABSTRACT: The six collagenases ($\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$, and $\zeta$) from *Clostridium histolyticum* isolated in the preceding paper [Bond, M. D., & Van Wart, H. E. (1984) *Biochemistry* (first paper of three in this issue)] have been characterized in detail. The molecular weights determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis range from 68 000 to 125 000. Isoelectric focusing experiments demonstrate that the isoelectric points of the collagenases are in the 5.35–6.20 range. These experiments also reveal that the subspecies of $\alpha$- and $\gamma$-collagenases ($\alpha_1$ vs. $\alpha_2$ and $\gamma_1$ vs. $\gamma_2$) have different isoelectric points but the same molecular weights. Microheterogeneity is also observed for the $\beta$- and $\epsilon$-collagenases. The amino acid compositions of all six collagenases have been determined, and analysis for neutral sugars and hexosamines shows that none of the enzymes have a significant carbohydrate content. Zinc and calcium are the only metals that copurify with the collagenases. The purified enzymes contain approximately 1 mol of zinc/mol of protein and a calcium content that varies from about 2 mol/mol for $\alpha$-collagenase to about 7 mol/mol for $\beta$-collagenase. All of the collagenases are 5–10 times more active against gelatin than collagen. The $\alpha$-, $\beta$-, and $\gamma$-collagenases are significantly less active toward the synthetic peptide substrates examined than the $\delta$-, $\epsilon$-, and $\zeta$-collagenases. This property, taken together with data on the stabilities and amino acid compositions of these enzymes, strongly supports their assignment to two distinct classes. This establishes clearly that *C. histolyticum* does, indeed, produce more than one different type of collagenase.

Over the last 30 years, numerous studies have been carried out in which one or more of the collagenases (EC 3.4.23.3)[1] from *Clostridium histolyticum* have been isolated [Bond & Van Wart (1984a) and references cited therein]. Most of what is presently known about *Clostridial* collagenases has come from the early pioneering studies of Mandl, Seifter, Harper, and their associates. Unfortunately, these enzymes have re-

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[1] Abbreviations: collagenase, *Clostridium histolyticum* collagenase; FALGPP, 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-proline; FALGPA, 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine; PZ, *p*-phenylazobenzyloxycarbonyl; [$^{14}CH_3$]collagen, [*methyl*-$^{14}$C]collagen; [$^{14}CH_3$]gelatin, [*methyl*-$^{14}$C]gelatin; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; cpm, counts per minute; nkat, nanokatal.

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