Purification and Partial Characterization of Collagenolytic Enzymes from *Clostridium histolyticum**

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ABSTRACT: Among several collagenolytic enzymes from *Clostridium histolyticum*, three enzymes which are important in the digestion of so-called insoluble collagen were purified and their substrate specificities were studied. The three enzymes, designated as A-α, B-α, and B-β, were purified by precipitation with acetone and ammonium sulfate followed by chromatography on SE-cellulose and DEAE-cellulose. All appeared to be homogeneous by chromatography on DEAE-cellulose and were inactive against casein. A-α was active against 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-Arg and denatured collagen, but only weakly active against insoluble collagen. B-α and B-α hydrolyzed denatured collagen more rapidly than A-α, but were almost inactive against the above synthetic peptide and insoluble collagen. However, mixtures of B-α or B-β with A-α digested the insoluble collagen rapidly and almost completely.

The culture filtrate of *Clostridium histolyticum* was at one time thought to contain a single "collagenase" which specifically hydrolyzed collagens, azocoll, and certain synthetic peptides having the sequence X-Pro-Y-Gly-Pro- (or Hyp-) Z between an amino acid, Y, and glycine (*cf.* Mandl, 1961; Nordwig, 1962). However, the presence of more than one collagenolytic enzyme in the crude preparation was subsequently noted by Grant and Alburn (1959). Mandl *et al.* (1964), Yoshida and Noda (1965), and Harper *et al.* (1965), using chromatography on DEAE Sephadex or DEAE-cellulose, achieved the separation of two fractions having different specificities against the substrates mentioned above. Heyns and Legler (1960) and Strauch and Grassmann (1966) obtained preparations which were highly active against the peptides, and Schaub and Strauch (1965) noted the presence of a fraction which attacked collagen, but not the peptides.

As compared with crude preparations, however, the most highly purified fractions have not been very active against "insoluble collagen" (see below). It is significant in this regard that Mandl *et al.* (1964) observed a synergistic attack on collagen by two enzyme fractions. However, the components of the system were not at all clear, since the enzyme fractions were still heterogeneous.

The present work was initiated to obtain collagenolytic enzymes that would be highly active against insoluble collagen, but free from other enzymatic activities. Three such enzymes, designated as A-α, B-α, and B-β, have been purified and a preliminary characterization has been made of their mode of action and substrate specificities.

Since collagen is not a uniform compound and because there are several collagenolytic enzymes having different substrate specificities, it is important to note at the outset the particular preparations employed in this study. (1) So-called insoluble collagen purified from cattle Achilles tendon, as used by Grant and Alburn (1959) and Mandl *et al.* (1964), has been used throughout in this work and is simply referred to as collagen in this paper, unless it is necessary to specify otherwise. Although the material may be somewhat altered from its state *in vivo*, it is highly resistant to trypsin, with which the heat-denatured collagen (see below) is rapidly digested. This collagen preparation was highly resistant to the purified collagenolytic enzymes B-α and B-β, slowly attacked by A-α, and rapidly digested by a mixture of A-α and B-α, or A-α and B-β. (2) Two denatured collagen preparations, namely, the above-mentioned collagen after heat denaturation (this is termed heat denatured collagen) and azocoll, a hide powder coupled with azo-dye (Oakley *et al.*, 1946), have been used to detect and characterize B-α and B-β, as well as B-γ which has been partially purified. These enzymes, which enhance the apparent effect of A-α, have not hydrolyzed any other protein or synthetic substrates so far examined.

Materials and Methods

Several buffers were used repeatedly in the course of this work and are referred to as follows,[1,2] Tris A, 10 mM Tris-AcO–0.1 mM $Ca(AcO)_2$, (pH 7.5); Tris B, 20 mM Tris-AcO–0.1 mM $Ca(AcO)_2$ (pH 7.5); Tris C, 50 mM Tris-AcO–0.1 mM $Ca(AcO)_2$ (pH 7.5); pH 5.7

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[1] Abbreviation used: AcO, acetate.

[2] The pH values of the following solutions were adjusted by adding the components shown in parentheses, and the concentrations of the salts refer to ions that are written in italics: *Tris*-AcO (HAcO), *Tris*-Cl (HCl), sodium *3,3'-dimethylglutarate* (NaOH), *sodium barbital* (HCl), and NH_4AcO (NH_4OH).

buffer, 1 mM sodium 3,3′-dimethylglutarate–1 mM $Ca(AcO)_2$ (pH 5.7). For the precipitation of enzymes, acetone was purified by $KMnO_4$ treatment followed by distillation. A saturated solution of ammonium sulfate (enzyme grade from Nutritional Biochemicals Corp.) was prepared at 0° and adjusted to pH 7.0 at 0° using glass electrodes soaked in the solution. The pH of the solution was 5.9 when measured at room temperature after being diluted 50-fold with water. In preparation for chromatography, SE-cellulose (Cellex-SE, Bio-Rad, 0.2 mequiv/g) was purified as described by Peterson and Sober (1962) and equilibrated with the pH 5.7 buffer by the following procedures.[3] SE-cellulose in H^+ form was neutralized with 1 N NaOH, and for each volume of NaOH, one volume of 1 M $Ca(AcO)_2$ was added while stirring vigorously. The pH was adjusted to about 6 with 1 N acetic acid, and then to 5.7 with 0.1 M 3,3′-dimethylglutaric acid. The suspension was filtered, and the filter cake was suspended in pH 5.7 buffer. The last procedure was repeated once. High-capacity DEAE-cellulose (Cellex-D, Bio-Rad, 1.0 mequiv/g) was also purified as described by Peterson and Sober (1962), converted into the acetate, and equilibrated with Tris A. Sephadex G-25 (fine size) was obtained from Pharmacia, and treated as specified by the company.

Crude collagenase from *Cl. histolyticum* was purchased from Nutritional Biochemicals Corp. The preparation (lot no. 6109) had been obtained by a single precipitation from a culture filtrate with ammonium sulfate. Enzyme activities were assayed using the following substrates under the conditions described below. Undenatured collagen from cattle Achilles tendon (Calbiochem) was purified as described by Bergmann and Stein (1939) and Grant and Alburn (1959). Heat-denatured collagen was prepared by heating the above collagen at 75–80° for 15 min in the incubation medium described below. Azocoll, sodium caseinate, and 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-Arg were obtained from Calbiochem, Nutritional Biochemicals Corp., and Mann Research Laboratories, Inc., respectively. Undenatured or heat-denatured collagen (20 mg) was shaken for 1 hr at 37° with 1 ml of enzyme solution and 2 ml of 50 mM sodium barbital–0.15 M NaCl–0.15 mM $CaCl_2$ (pH 8.0). The reaction mixture was filtered after addition of 0.5 ml of ethanol (*cf.* Seifter and Gallop, 1962). The amount of peptides in the filtrate was assayed by the biuret method (Layne, 1957). In order to determine a small amount of the A enzyme (see below), the incubation medium containing native collagen was supplemented with an excess of a B enzyme (corresponding to more than 25 μg of the purified B-α) to enhance the apparent activity of the A enzyme. As is shown later, when the activity given by the B enzyme alone was subtracted, the result was proportional to the amount of the A enzyme. Azocoll (20 mg) was shaken for 20 min at 37° with 1 ml of enzyme solution and 2 ml of 0.1 M Tris-Cl–0.1 mM $CaCl_2$ (pH 7.5). The incubation mixture was filtered, and the peptides in the filtrate were assayed by measuring the absorbancy at 580 mμ. Sodium caseinate (2 mg in 1 ml of water) was incubated with 1 ml of enzyme solution and 1 ml of 50 mM sodium barbital (pH 7.5) for 30 min at 37°. Dichloroacetic acid (*cf.* Guidotti *et al.*, 1962) was added to make the final concentration 5%. The precipitate formed was filtered after 10 min, and the peptides in the filtrate were determined by the method of Lowry *et al.* (1951). Activity against the synthetic peptide was assayed by the colorimetric determination of ethyl acetate soluble 4-phenylazobenzyloxycarbonyl peptide as described by Wünsch and Heidrich (1963). In each case, one unit of enzyme activity was defined as the appearance of 1 mg of the reaction products/min in the filtrate prepared as described above, unless stated otherwise. Protein in an enzyme solution was determined either by the method of Lowry *et al.* (1951) (at purification steps 0–4) or from the optical density at 280 mμ (after step 5). Results of determinations of protein or peptide were calculated using crystalline bovine serum albumin as the standard. Free amino residues were determined by the ninhydrin method of Moore and Stein (1947), as modified by Spies (1957). The results were calculated using leucine as the standard. N-terminal amino acids of peptides were determined by the dinitrophenol (DNP) method, as described by Frankel-Conrat *et al.* (1955) using thin-

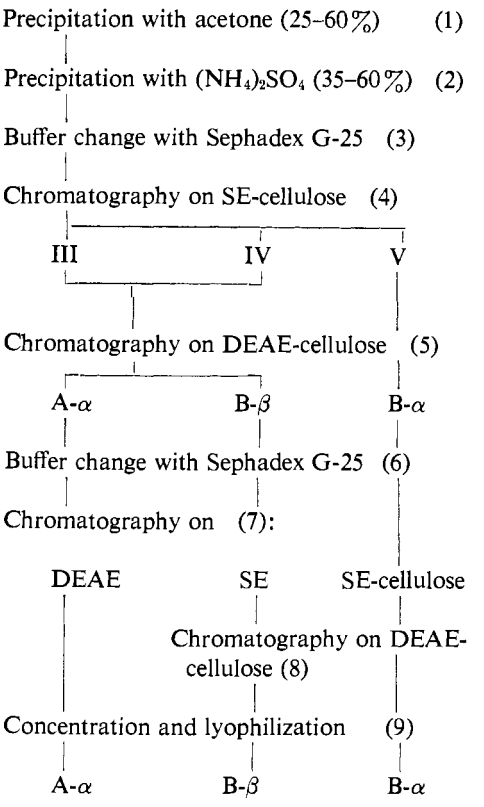

FIGURE 1: Steps in the purification of three collagenolytic enzymes from *Cl. histolyticum*.

[3] Recoveries of the enzymes (A-α, B-α, and B-β) from SE-cellulose were seriously affected by the pH and the concentration of calcium ion in the starting buffer solution. The best recoveries (75–100% in small scale stepwise tests) were obtained by using the pH 5.7 buffer, whereas the recoveries of the three enzymes were appreciably low when the pH was 5.5 or 5.9, and those of A-α and B-β (but not B-α) were only 30–40%, regardless of the pH, when the concentration of calcium acetate was 0.5 mM instead of 1.0 mM.

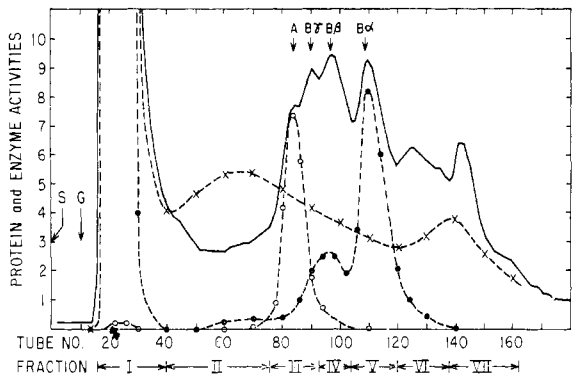

FIGURE 2: Chromatography of a partially purified enzyme preparation of SE-cellulose at step 4. Conditions for chromatography shown in Figures 2–7 are in the text. Curves are designated as follows: protein plus pigment, optical density at 280 mμ × 20 (——); activity against collagen, 0.9 unit/ml (O--O); activity against azocoll, 0.2 unit/ml (●--●); and activity against casein, 10 units/ml (x--x). Peaks of activities against azocoll and casein, and a peak of optical density at 280 mμ in fraction I are 24, 148, and over 60 units on the scale, respectively. The enzyme solution was applied to the column at S, and the gradient elution was started at G. The pH values in every 20th tubes from the 20th to the 160th were 5.7, 5.7, 6.0, 6.3, 6.6, 6.9, 7.1, and 7.3.

layer chromatography as described by Brenner *et al.* (1965). C-terminal amino acids were determined by the hydrazinolysis method of Akabori *et al.* (1953).

Purification of the Enzymes. A diagram of the purification procedures is shown in Figure 1. The purification was carried out at room temperature unless stated otherwise. As mentioned earlier, the A and B enzymes were assayed using collagen and azocoll, respectively, as substrates. The enzyme fractions were also assayed for their activities against casein which is generally accepted as a good substrate for the detection of proteolytic activities in crude collagenase (*e.g.*, Grant and Alburn, 1959; Mandl, 1961). Casein is also a substrate for so-called

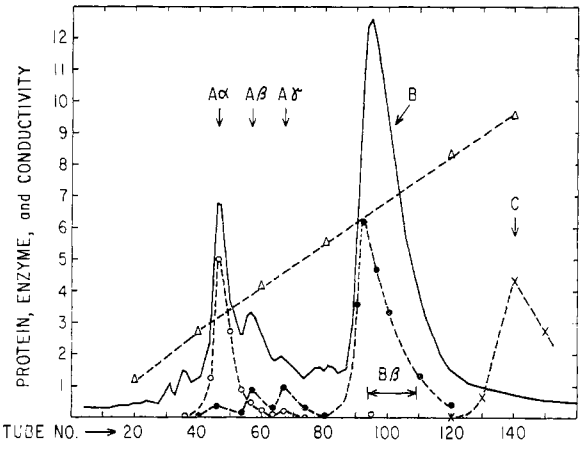

FIGURE 3: Chromatography of fractions III plus IV on high-capacity DEAE-cellulose at step 5. Curves are designated as follows: protein, optical density at 280 mμ × 20 (——); activity against collagen, 0.5 unit/ml (O--O); activity against azocoll, 0.25 unit/ml (●--●); activity against casein, 50 units/ml (x--x); and electrical conductivity, mmhos (△—△). Fractions A-α and B-β were obtained from tubes 45 to 53 and 94 to 109, respectively.

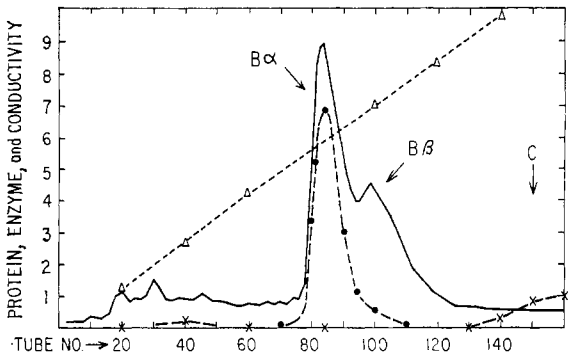

FIGURE 4: Chromatography of fraction V on high-capacity DEAE-cellulose at step 5. Legends used are same as those in Figure 3, except for activity against axocoll which is shown after being scaled down to 0.1 unit/ml. Fraction B-α was obtained from tubes 79 to 90.

amidase/esterase activity which is characteristic of trypsin (Yoshida and Noda, 1965; Labouesse and Gros, 1960; Strauch and Grassmann, 1966).

STEP 1. FRACTIONATION WITH ACETONE. The crude enzyme (1 g) was dissolved in 50 ml of 0.1 M Tris-Cl–1 mM $CaCl_2$ (pH 7.0) and adjusted to pH 7.0 ± 0.1 at 0° when necessary. The following operations at steps 1 and 2 were carried out at −5–0°. Cold acetone (17 ml) was added to the solution, and the mixture was immediately centrifuged at 12,000*g* for 10 min. The precipitate was discarded and 59 ml of cold acetone was added to the supernatant, and the mixture was immediately centrifuged as above.

STEP 2. FRACTIONATION WITH AMMONIUM SULFATE. The precipitate obtained at step 1 was immediately dissolved in 23.6 ml of cold 10 mM sodium barbital–0.1 mM $CaCl_2$ (pH 7.0) at 0°. To this solution, 13.5 ml of cold, saturated ammonium sulfate solution was added, and the mixture was centrifuged after 10 min. After discarding the precipitate, 25 ml of saturated ammonium sulfate solution was added to the supernatant, and the mixture was centifuged after 10 min.

STEP 3. BUFFER CHANGE. The precipitate obtained at step 2 was dissolved in 20 ml of the pH 5.7 buffer and was freed from ammonium sulfate by gel filtration with Sephadex G-25 (2.5 × 40 cm column) using the pH 5.7 buffer as the medium.

STEP 4. CHROMATOGRAPHY ON SE-CELLULOSE. The enzyme solution obtained at step 3 was adjusted to pH 5.7 ± 0.1 when necessary and applied to a column (2.0 × 30 cm) of SE-cellulose equilibrated with the pH 5.7 buffer.[3] The chromatography was carried out by gradually raising the pH of the medium by linearly increasing the concentration of Na^+ ion using 500 ml of the pH 5.7 buffer and 500 ml of freshly prepared 3 mM NaOH–1 mM 3,3′-dimethylgutaric acid–1 mM $Ca(AcO)_2$ as the limiting solutions (*cf.* Peterson and Sober, 1962). The flow rate was about 15 ml/hr per cm^2, and the effluent was collected in 5-ml portions. Fractions III plus IV and fraction V (Figure 2) were separately placed in flasks containing 0.2 M Tris-AcO (pH 8.0) to make the final Tris concentration 4 mM.

STEP 5. CHROMATOGRAPHY ON HIGH-CAPACITY DEAE-CELLULOSE. Fractions III plus IV and fraction V obtained

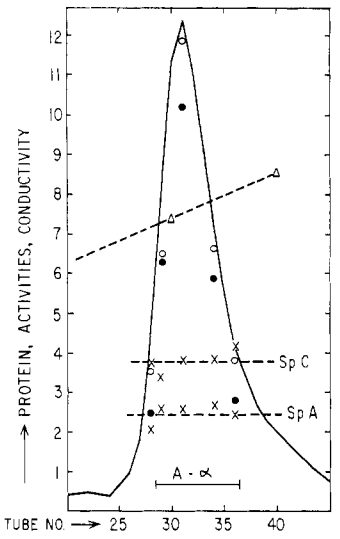

FIGURE 5: Chromatography of A-α on DEAE-cellulose at step 7. Curves are designated as follows: protein, optical density at 280 mμ × 40 (——); activity against collagen, units per milliliter (○); activity against azocoll, 5 units/ml (●); electrical conductivity, mmhos × 3 (△--△); specific activity against collagen, 0.1 unit/mg (x--x SpC); and specific activity against azocoll, 0.4 unit/mg (x--x SpA).

at step 4 were adjusted to pH 7.5 with 0.1 N acetic acid. Each solution was applied to a column (1.2 × 24 cm) of high-capacity DEAE-cellulose equilibrated with Tris A. The column was successively washed with 10 ml of Tris A and 50 ml of Tris B. The chromatography was carried out by linearly increasing the salt concentration of the medium using 300 ml of Tris B and 300 ml of 0.1 M Tris-AcO–0.15 M NaAcO–0.1 mM $Ca(AcO)_2$ (pH 7.5) as the limiting solutions. The flow rate was approximately 27 ml/hr per cm^2. The eluate was collected in 3-ml fractions, and fractions A-α (Figure 3), B-β (Figure 3), and B-α (Figure 4) were saved.

STEP 6. BUFFER CHANGES. At this and step 9, appreciable amounts of the enzymes were absorbed and destroyed by Sephadex unless the pH was 8.0 or above. Therefore, fraction A-α obtained at step 5 was adjusted to pH 8.0 with 1 M Tris-base and transferred into Tris A at pH 8.0 by gel filtration with Sephadex G-25 (2.0 × 40 cm). The eluate was adjusted to pH 7.5 with 1 N acetic acid. Likewise, fractions B-α and B-β were transferred into 1 mM sodium barbital–1 mM $Ca(AcO)_2$ (pH 8.3–8.5 when fresh) by gel filtration with Sephadex G-25 (2.5 × 40 cm). The eluate was adjusted to pH 5.7 with 0.1 M 3,3′-dimethylgutaric acid. Ionic composition of the resulting solution was practically the same as the pH 5.7 buffer used for chromatography on SE-cellulose. When the pH of the sodium barbital solution mentioned above was less than 8.0, 0.5 mM Na_2CO_3–1 mM $Ca(AcO)_2$ was added to make the pH 8.5.

STEP 7. SECOND CHROMATOGRAPHY. Two batches of fraction A-α obtained at step 6 were pooled and applied to a column (0.9 × 20 cm) of high-capacity DEAE-cellulose equilibrated with Tris A. The column was successively washed with 5 ml of Tris A and 30 ml of Tris B. The chromatography was carried out by linearly increasing the salt concentration of the medium using

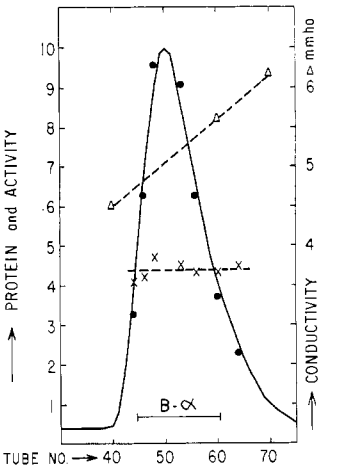

FIGURE 6: Chromatography of B-α on DEAE-cellulose at step 8. Curves are designated as follows: protein, optical density at 280 mμ × 40 (——); activity against azocoll, 0.2 unit/ml (●); and specific activity, 0.02 unit/mg (x--x).

150 ml of Tris B and 150 ml of 0.15 M Tris-AcO–0.1 mM $Ca(AcO)_2$ (pH 7.5) as the limiting solutions. The eluate was collected in 3-ml portions and fraction A-α (Figure 5) was saved. The B-α and B-β obtained at step 6 were separately applied to a column (1.2 × 24 cm) of SE-cellulose equilibrated with the pH 5.7 buffer. The chromatography was carried out by gradually increasing the pH of the medium by linearly increasing the concentration of Na^+ ion using 200 ml of the pH 5.7 buffer and 200 ml of 2.5 mM NaOH–1 mM 3,3′-dimethylglutaric acid–1mM $Ca(AcO)_2$ as the limiting solutions. The eluate was collected in 3-ml portions, and fractions B-α and B-β were obtained between the half-maxima of the corresponding protein peaks. To each pool, 0.2 M Tris-AcO (pH 8.0) was added to make the final Tris concentration 4 mM.

STEP 8. THIRD CHROMATOGRAPHY OF B-α AND B-β. Two batches each of fractions B-α and B-β obtained at step 7 were pooled. Each preparation, adjusted to pH

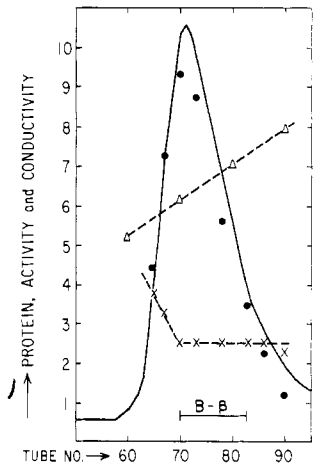

FIGURE 7: Chromatography of B-β on DEAE-cellulose at step 8. Curves are designated as follows: protein, optical density at 280 mμ × 40 (——); activity against azocoll, 1.5 units/ml (●); specific activity, 0.1 unit/mg (x--x); and electrical conductivity, mmhos (△--△).

TABLE I: Results of Purification of Collagenolytic Enzymes.[a]

Purification Step	Total Protein (mg)	Activities against Casein (unit)	Activities against Azocoll (unit)	Activities against Collagen (unit)	Specific Activity (unit/mg)
		A. Purification of A-α[e]			
0. Crude enzyme	1,070	2,620	29,600	743	0.695
1. Acetone	350	2,640	30,500	724	2.07
3. Sephadex	220	2,435	16,900	559	2.54
4. SE-cellulose (III + IV)	42.9	79.2	1,160	329[b]	7.68
5. DEAE-cellulose (A-α)	5.84	<0.04	28.1	164[b]	28.1
6. Sephadex	4.73			138[b]	29.2
7. DEAE-cellulose	2.53		15.8	96.7[b]	38.2
9. After Sephadex	1.93			55.5[b]	28.8
		B. Purification of B-α[f]			
0. Crude enzyme		Same as A			27.7
4. SE-cellulose (V)	24.7	42.1	2,620	15.7	106
5. DEAE-cellulose (B-α)	12.8	<0.04	1,990	5.8[c]	155
6. Sephadex	10.7		1,960		183
7. SE-cellulose	5.87		1,100		187
8. DEAE-cellulose	4.40		980		223
9. After Sephadex	2.82		630		223
		C. Purification of B-β[f]			
4. SE-cellulose (III + IV)		Same as A			27.7
5. DEAE-cellulose (B-β)	15.2	<0.04	451	5.8[c]	29.6
6. Sephadex	12.8		375		29.3
7. SE-cellulose	7.69		205		26.6
8. DEAE-cellulose	4.51		115		25.5
9. After Sephadex	3.38		86.4		25.6

[a] Mean values of four to six results are shown. [b] Activities were determined in the presence of excess B-α (*cf.* Methods). [c] Figures are not proportional to the enzyme concentration (*cf.* Figure 9). [d] The specific activity decreases as the strongly active B-α is removed. [e] Specific activity values as against collagen. [f] Specific activity values as against azocoll.

7.5 with 1 N acetic acid, was applied to a column (0.9 × 20 cm) of high-capacity DEAE-cellulose equilibrated with Tris A. The column was successively washed with 5 ml of Tris A and 30 ml of Tris C. The chromatography was carried out by linearly increasing the salt concentration of the medium using 150 ml of Tris C and 150 ml of 0.1 M Tris-AcO–0.1 M NaAcO–0.1 mM $Ca(AcO)_2$ (pH 7.5) as the limiting solutions. The eluate was collected in 3-ml portions, and fractions B-α (Figure 6) and B-β (Figure 7) were saved.

STEP 9. CONCENTRATION AND LYOPHILIZATION. Prior to lyophilization, the purified A-α, B-α, and B-β were concentrated, since they were senstitive to freezing and thawing as well as lyophilization unless the concentration was more than 0.5 mg of protein/ml. The enzymes were also sensitive to desalting by dialysis or gel filtration. Thus purified fractions from 4 g of the starting material were pooled and precipitated by dialyzing against three volumes of saturated ammonium sulfate solution at 0–5°. The precipitate was taken up in 1 ml of 0.1 M Tris-AcO (pH 8.0) (a part was insoluble) and transferred into 10 mM NH_4AcO (pH 8.0–8.5) by gel filtration using a small column (0.5 × 20 cm) of Sephadex G-25. The eluate was collected to make the protein concentration more than 0.5 mg/ml and lyophilized.

Results

Purification of the Enzymes. The results of the purification are summarized in Table I. A mixture of collagenolytic enzymes was purified approximately threefold by fractionation with acetone and ammonium sulfate followed by gel filtration at step 3. The enzymes were then separated into several fractions (A-α, B-α, B-β, etc.) by successive chromatography on SE-cellulose (step 4) and DEAE-cellulose (step 5). The fractions A-α, B-α, and B-β obtained at step 5 were free from any detectable activity against casein and from the brown pigments present in the crude preparation. In the subsequent steps, each enzyme fraction was further purified and lyophilized. The purification of A-α at step 7 was 55-fold but the extent of purification of B-α and B-β is unknown,

TABLE II: Activities of the Purified Enzymes against Various Substrates.

Enzyme Fractions	Specific Activities against Various Substrates				
	4-Phenylazo-benzyloxy-carbonyl-Pro-Leu-Gly-Pro-Arg[b]	Heat-Denatured Collagen			Undenatured Collagen[a]
		Ninhydrin Method[b]	Biuret Method[c]	Azocoll (*cf.* Table I)[c]	Biuret Method[c]
A-α	16.2	19.5	9.6	6.2	5.1
B-α	0.21	77.9	170	223	0.12
B-β	0.29	46.7	47.4	25.5	0.11

[a] Activity of A-α was assayed under the standard conditions (*cf.* Methods) in the absence of the B enzymes. Activities of the B enzymes were estimated from the results of 1- and 3-hr incubations, when the rate of reaction was constant after a small fraction of collagen was rapidly solubilized (*cf.* Figure 9). [b] In micromoles per minute per milligram o protein. [c] In milligrams per minute per milligram of protein.

since specific assay methods have not been established. Some more detailed considerations in the purification are described below.

Upon chromatography on SE-cellulose at step 4 (Figure 2) peaks designated at A, B-γ, B-β, and B-α were separated from the first and the third peaks of caseinolytic activities, though the separation of B-α from the third peak was incomplete. This third peak of caseinolytic activity coincided (unpublished data) with a large peak of so-called amidase esterase activity (Strauch and Grassmann, 1966) detected with benzoylarginine-β-naphthylamine, a substrate for trypsin.

The A peak and the B peaks were active against collagen and azocoll, respectively. Although the activity of B-γ could not be clearly distinguished, a peak was clearly detectable when the crude preparation was directly chromatographed on SE-cellulose (by the present procedures, a large part of B-γ remained in ammonium sulfate solution at step 2). B-β was more active than B-γ against azocoll, but the highest activity was given by B-α. Presence of three enzymes corresponding to the B enzymes has not been reported previously. Recoveries of A-α (see below), B-α, and B-β from SE-cellulose were peculiarly affected by the starting conditions for the chromatography, as noted elsewhere.[3]

When fractions III plus IV were chromatographed on high-capacity DEAE-cellulose at step 5 (Figure 3), A-α, which was active against collagen, was separated not only from the B peak but also from minor peaks including A-β and A-γ which were active against collagen and/or azocoll. Peaks at the region of the A enzymes were best separated by using solutions containing low concentration of calcium acetate (0.1 mM) and by stabilizing the pH at 7.5 by increasing the concentration of the buffer salt during the chromatography on high-capacity DEAE-cellulose, which gave better results than DEAE Sephadex. Separation of an enzyme corresponding to A-α from these minor peaks has not been reported previously. The B peak was mainly composed of B-β, but the left and right ends of the peak were contaminated with B-α and B-γ, respectively, as determined by the rechromatography on SE-cellulose. These peaks designed as A and B were inactive against casein (data for A-α and B-β are shown in Table I). A caseinolytic activity, corresponding to the second peak in Figure 2, was eluted subsequently.

Also at step 5 (Figure 4), B-α from fraction V in Figure 2 was partially separated from a protein, which was B-β. The B peaks were inactive against casein. Two peaks of caseinolytic enzymes were eluted at about tubes 40 and 160. Separate tests indicated that the first peak corresponded to the enzyme in fractions VI and VII of Figure 2. Note that the enzyme, if present, would contaminate A-α in Figure 3.

Fraction A-α at step 7 (Figure 5) appeared to be chromatographically homogeneous and the specific activities both against collagen and azocoll determined at various positions in the fraction seemed to be constant. Although a few irregular points are present in Figure 5, they may be the results of experimental error, as judged from the other data. Fractions B-α and B-β at step 8 (Figures 6 and 7) seemed to be chromatographically homogeneous and the specific activities against azocoll at various positions in each fraction appeared to be constant. The left half of the protein peak in Figure 7 may be contaminated with B-α,

Although one collagenase had been thought to dissociate into monomers (Harper *et al.*, 1965), all the six fractions designated as A and B appeared to have similar molecular weights, about 100,000, as judged by their elution volumes from long columns (0.9 $\times$ 150 cm) of Sephadex G-100 and G-200. Besides, there was no evidence of interconversion of the enzymes during the purification, as judged by the rechromatography.

Substrate Specificities of the Enzymes. EFFECTS ON THE SPECIFIC PEPTIDE. Among the purified enzymes, only A-α was highly active against a synthetic peptide which in the past was considered to be the specific substrate for collagenase as noted earlier (Table II, column 2). The specific activities of partially purified A-β, A-γ, and B-γ at step 5 were 6.9, 3.2, and 0.28 μmoles per min per mg, respectively. The specific activity of the starting

TABLE III: Solubilization of Collagen as Measured by Gravimetry.[a]

Enzyme	Collagen Not Solubilized (% of the original weight)				
	Heat-Denatured Collagen	Undenatured Collagen			
	60 min	30 min	60 min	120 min	180 min
No enzyme	106	101	97.4	100	100
A-α	24.5	83.9	61.4	31.4	26.8
B-α	17.2	98.4	100	96.0	97.4
B-β	21.8		99.6		97.9
A-α plus B-α		40.4	16.3	3.7	2.5
A-α plus B-β		51.7	17.7	5.9	5.1

[a] Collagen, either undenatured or heat denatured, was incubated under standard conditions (*cf.* Methods) with and without 90 μg of A-α, 205 μg of B-α, 200 μg of B-β, or their mixtures. The reaction mixture was filtered, the filter cake was washed, dried at room temperature, and weighed using the technics for micro-Carius (Pregl, 1949).

crude enzyme preparation was 0.27 μmole/min per mg. Thus, the purification of A-α was 60-fold (at step 7), which was comparable to the purification (55-fold) measured by activity against collagen (Table I). The optimum pH of A-α was between 7 and 9 (Figure 8). The single N-terminal amino acid generated by the hydrolysis was glycine. The N-terminal amino acid generated by hydrolysis with B-α or B-β was also glycine.

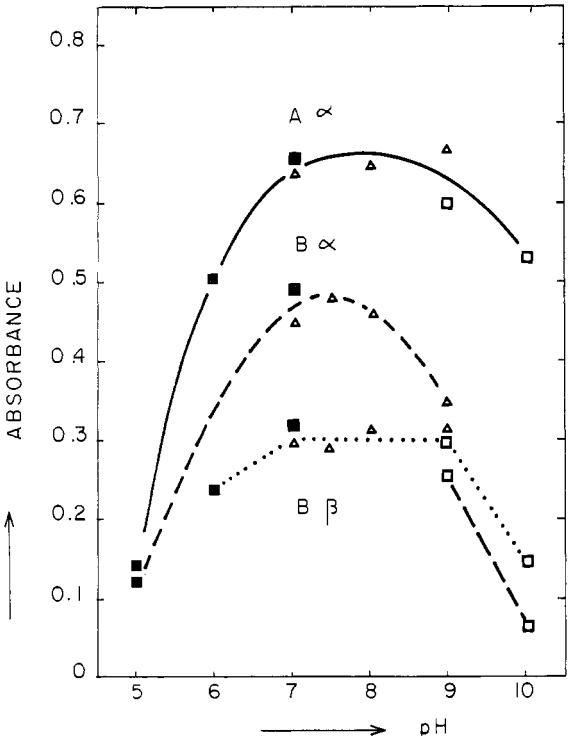

FIGURE 8: Effects of pH on enzyme activities. Activities of A-α and the B enzymes were assayed against 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-Arg and azocoll, respectively. Incubation media were 10 mM sodium 3,3'-dimethylglutarate (■) at pH 5–7, sodium barbital (△) at pH 7–9, and sodium glycine (□) at pH 9 and 10, all containing 0.1 mM $CaCl_2$. Each point in Figures 8–10 is the mean value of duplicate experiments.

EFFECTS ON HEAT-DENATURED COLLAGEN. In contrast to the results with the peptide, the enzymes A-α, B-α, and B-β rapidly hydrolyzed heat-denatured collagen as determined by the ninhydrin method (Table II, column 3). Thus, all these enzymes seemed to be proteases. It appeared that the enzymes might split heat-denatured collagen (still insoluble in the medium) into soluble peptides of different mean lengths, since ratios of the activities determined by the biuret method and by the ninhydrin method (Table II, columns 4 and 3) were quite different: 0.49, 2.20, and 1.00 mg per μmole of N terminals for A-α, B-α, and B-β, respectively. Nevertheless, no appreciable difference has been detected in either the N (mostly glycine) or C terminals (mostly glycine, alanine, and proline) of the peptides generated by hydrolysis with A-α, B-α, and B-β.

Specific activities of the enzymes against the heat-denatured collagen, as determined by the biuret method, were similar to those against azocoll (Table II, column 5). It seems probable, therefore, that these enzymes digest only the denatured collagen moiety in azocoll, as might be expected, since the enzymes were inactive against casein.

EFFECTS ON "UNDENATURED" COLLAGEN. Undenatured collagen was digested less rapidly by A-α than denatured collagens and very slowly by the B enzymes (Table II, column 6). Similar results were obtained by measuring the insoluble material gravimetrically (Table III). However, insoluble collagen was digested rapidly and almost completely by a synergistic attack of A-α and B-α, or A-α and B-β, as demonstrated by gravimetry (Table III) which is a direct measure of solubilization.

The synergism was also demonstrated using the biuret method for analysis as shown in Figure 9, where a small, fixed amount of A-α was mixed with different amounts of B-α and/or B-β. The activity of A-α alone (1.75 units) is shown by the point lying on the vertical

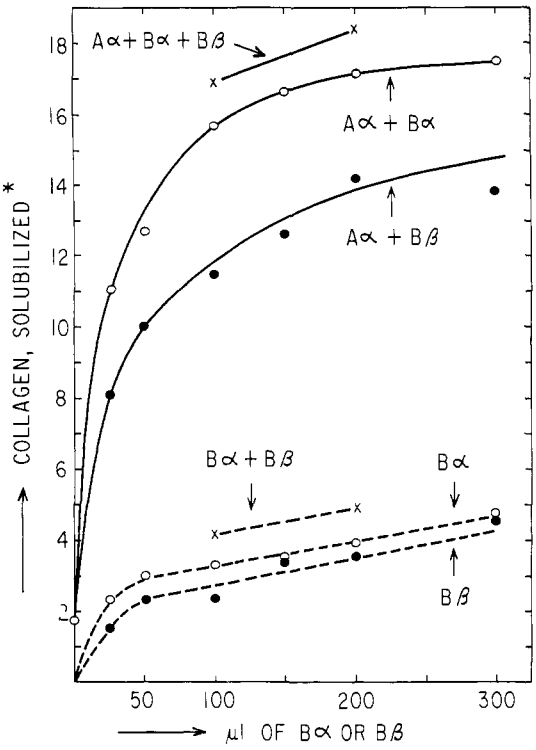

FIGURE 9: Solubilization of collagen with different amounts of B-α and B-β in the presence of limited amount of A-α. Collagen was incubated with indicated amounts of B-α (124 μg/ml), B-β (170 μg/ml), or their mixtures, in the presence and absence of 1.8 μg of A-α. The incubation was carried out under standard conditions for the assay of activity against collagen, and the amount of collagen solubilized in the filtrate was assayed by the biuret method. (*) unit on the scale is the optical density of the biuret test $\times$ 100.

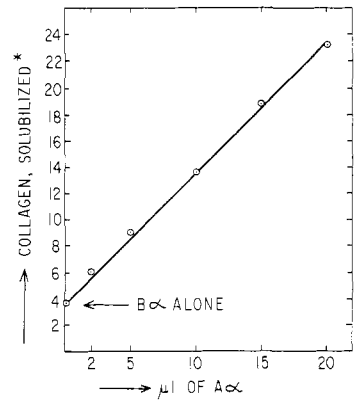

FIGURE 10: Solubilization of collagen with different amount of A-α in the presence of excess B-α. Collagen was incubated under standard conditions with indicated amounts of A-α (97 μg/ml) in the presence of 31.4 μg of B-α. Other conditions were the same as those in Figure 9.

ordinate where the amount of the B enzymes added was zero. The results (Figure 9) also indicated that (a) a small fraction of the collagen was easily solubilized by the B enzymes, (b) no synergistic effect was obtained with B-α and B-β, and (c) no increased synergism was seen when B-β was added to a mixture of A-α and excess B-α. In the presence of excess B-α the apparent activity of A-α was proportional to the amount of A-α (Figure 10).

The synergistic effect was not observed when one of the fractions was heated to 100° for 10 min or treated with 50% acetone at room temperature for 2 hr. Therefore, the rapid collagenolysis is most likely caused by two enzymes rather than stimulation of one enzyme by a cofactor of a small molecular weight in the other fraction. Presumably, the two enzymes involved are, in fact, the A-α and B-α, or B-β, in the respective fractions, since the fractions were free from other known enzymes. The role of the B enzymes in the synergistic reaction appears to be specific, since no synergism was observed when the B enzymes were replaced with either trypsin, or with fraction I in Figure 2.

Discussion

In earlier studies, a caseinolytic enzyme (seen in fractions VI and VII of Figure 2) may have contaminated preparations of collagenolytic enzymes. This enzyme was difficult to detect because it splits casein into large peptides which are mostly precipitated by trichloroacetic acid. In the present work, the caseinolytic activity was followed by using dichloroacetic acid, which precipitates only very large peptides (*cf.* Guidotti *et al.*, 1962), to remove unreacted casein.

Earlier preparations of collagenolytic enzymes have not been subjected to what appears to be the most critical test of purity. Multiplicity of the collagenolytic enzymes has been clearly demonstrated previously only by chromatography on DEAE-cellulose or DEAE Sephadex, and the best results (separation of the activities into two peaks both having at least one shoulder) have been obtained by gradient chromatography on DEAE Sephadex (Mandl *et al.*, 1964). Therefore, homogeneity of a fraction of the enzymes should be tested by gradient chromatography on DEAE Sephadex or DEAE-cellulose.

In the present work, the enzymes designated as A-α, B-α, and B-β were (a) freed from any detectable caseinolytic activity; (b) separated from other collagenolytic enzymes including A-β, A-γ, and B-γ; and (c) purified until they appeared to be homogeneous by gradient chromatography on high-capacity DEAE-cellulose which gave better resolution than DEAE Sephadex or medium-capacity DEAE-cellulose.

Judging from the substrate specificities and/or chromatographic characteristics, the A enzymes probably correspond to collagenase A, or A and B, of Grant and Alburn (1959), to the collagenase of Heyns and Legler (1960), to fraction I and a part of fraction II of Mandl *et al.* (1964), to collagenase A of Harper *et al.* (1965), to collagenase II of Yoshida and Noda (1965), and to a preparation of Strauch and Grassmann (1966). The last two preparations were reported to be homogeneous by certain criteria. However, the homogeneity of collagenase II of Yoshida and Noda (1965) is not conclusive, since collagenases II and I (see below) were obtained by stepwise chromatography on DEAE-cellulose under conditions that would cause division of the proteins in a partially purified preparation into only two fractions.

The identification of the fraction obtained by Strauch and Grassmann (1966) as a collagenolytic enzyme is not conclusive, since the activity against collagen was not reported. Although the fraction was active against a peptide which would be attacked by A-α, this peptide can be attacked by other enzymes (*e.g.*, the peptide shown in Table II was hydrolyzed at an appreciable rate by fraction I in Figure 2). Furthermore, chromatographic characteristics of the preparation on DEAE-cellulose or on DEAE Sephadex were not reported.

Based on the same criteria as above, the B enzymes probably correspond to collagenases B and C, or C alone, of Grant and Alburn (1959), to a part of fraction II of Mandl *et al.* (1964), and to collagenase I of Yoshida and Noda (1965). The last preparation was reported to be homogeneous by certain criteria, but as discussed above, the results are not conclusive. The data are not sufficient to correlate any of the present purified enzymes with the preparation of Seifter *et al.* (1959). All the present enzymes seem to have much larger molecular weights than collagenase B of Harper *et al.* (1965) and the tadpole enzyme of Nagai *et al.* (1966).

As mentioned earlier, Mandl *et al.* (1964) noted a rapid collagenolysis due to a synergistic attack by two heterogeneous fractions of the enzymes. In the present work, it was demonstrated that so-called insoluble collagen was solubilized rapidly and almost completely by a synergistic attack of purified A-α and B-α, or A-α and B-β. If it is assumed that indeed two enzymes are involved in the reaction, it can be postulated that A-α first attacks undenatured insoluble collagen at regions where the sequence is X-Pro-Y-Gly-Pro- (or Hyp-) Z as originally proposed by Nagai and Noda (1959) and Heyns and Legler (1959) for partially purified preparations. As this attack progresses, regions of the collagen molecules may be unfolded to expose bonds susceptible to attack by the B enzymes.

Substrate specificities of fractions similar to the B enzymes have been controversial (Mandl *et al.*, 1964; Yoshida and Noda, 1965; Harper *et al.*, 1965). The present results made it clear that both B-α and B-β are proteolytic enzymes which specifically and rapidly hydrolyze certain bonds in collagen molecules. The bonds split by the B enzymes seem to be characterized not only by the amino acids adjacent to these bonds but by other factors, possibly, as in the case of A-α, by the sequence of several amino acids near the bonds.

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