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Collagenase Enzymes from *Clostridium*: Characterization of Individual Enzymes†

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ABSTRACT: Four collagenases have been purified to apparent homogeneity from extracts of *Clostridium histolyticum* and partially characterized. The four purified enzymes are devoid of hydrolytic activity against casein and the synthetic substrate, benzoylarginine naphthylamide, but all retain activity against native collagen. The enzymes are initially separated by isoelectric focusing where three of the enzymes show distinct isoelectric points: collagenase I = 5.50, collagenase II = 5.65, and collagenases IIIa and IIIb = 5.90–6.00. Collagenases IIIa and IIIb can be subsequently separated on diethylaminoethylcellulose. The four purified enzymes show single bands upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Calibration of the molecular weights on the basis of migration distance shows a marked dependence on gel porosity. At high acrylamide concentration, collagenases I, II, and IIIa appear to converge to a limiting molecular weight ≃81 000, while collagenase IIIb has a distinctly lower value ≃72 000. The similarity between these molecular weight values and those derived from the sedimentation and diffusion coefficients of the native enzyme indicates that each collagenase is a single polypeptide chain. All of the collagenases have comparable catalytic activities against a series of natural and synthetic substrates and are immunologically cross-reactive. Although all four enzymes are evident upon initial electrofocusing of the crude extract, it is possible that the multiplicity of forms is, at least in part, a consequence of lysis following initial secretion from the cell.

Collagenolytic enzymes are among the many proteinases released extracellularly into the culture media of *Clostridium histolyticum.* One extensively studied collagenolytic enzyme, collagenase A, is known to have a high degree of substrate specificity, requiring the amino acid sequence Pro-*X*-Gly-Pro-*Y*, where *X* and *Y* can be any amino acid (Gallop et al., 1957; Harper and Kang, 1970; Seifter and Harper, 1971). The enzyme cleaves the bond between the *X* and Gly to release a Gly-NH_2 terminus. The strict substrate requirement of this enzyme and collagenases, in general, renders them powerful tools in the structural elucidation of collagen-like proteins (Seifter and Harper, 1971). However, purification of collagenolytic enzymes has been complicated by the presence in the starting materials of less specific proteases with physical and chemical characteristics similar to those of collagenases (Keller and Mandl, 1963; Kono, 1968; Mitchell, 1968; Peterkofsky and Diegelmann, 1971).

Two to six separate enzymes with collagenolytic activity have been noted in crude extracts and chromatographically

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purified fractions by a number of workers (Grant and Alburn, 1959; Noda, 1964; Mandl et al., 1964; Harper and Kang, 1970; Kono, 1968; Peterkofsky and Diegelmann, 1971; Kesselring et al., 1970). However, the relative abundance of the different collagenases in the crude enzyme preparations, their subunit molecular weights, composition, and the interrelationships among the collagenases have yet to be extensively examined. Moreover, to date, preparative work on the bacterial collagenases has been carried out at room temperature, conditions which may allow significant proteolysis of the collagenolytic enzymes by contaminating proteases. In this study, we present the purification to homogeneity and comparative characterization of four distinct collagenolytic enzymes.

Experimental Section

Materials

Crude collagenase (CLA class IV lot 44M 238; 175 U/mg) and trypsin (TRL grade) were purchased from Worthington Biochemical Corp. Benzoylarginine naphthylamide (BANA),[1] *N*-(1-naphthyl)ethylenediamine, *N*-ethylmaleimide (NEM), L-glycine, carbobenzoxyglycylprolylglycylglycylprolylalanine (cbzGPGGPA), and Coomasie brilliant blue were all from Sigma Chemical Co. Azocoll was from Calbiochem, La Jolla, Calif. Hydroxylapatite (Bio-Gel HTP 130-0420) was from Bio-Rad, Los Angeles, Calif. Sodium dodecyl sulfate was Sequanal grade of Pierce. Other chemicals were of reagent grade or the highest purity available.

Methods

Assay Procedures. Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Azocoll Hydrolysis. To assay enzyme activity against Azocoll, the method of Peterkofsky and Diegelmann (1971) was used.

Hydrolysis of the Synthetic Hexapeptide, cbzGPGGPA. The method of Grassmann and Nordwig (1960), as modified by Keller and Mandl (1963), was used. The total reaction volume was 250 μl of 50 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$ and 250 μg of substrate. Samples were incubated for 15 min and 40-μl aliquots were removed for determination of peptide bond hydrolysis using Rosen's (1957) ninhydrin assay. L-Glycine was used as a standard for the determination of the concentration of the N-terminal residues generated. Enzyme concentration was chosen so that the reaction course was linear at 15 min.

Hydrolysis of ^{14}C-Labeled Guinea Pig Skin Collagen. Preparation of ^{14}C-labeled reconstituted guinea pig skin collagen substrate and the assay for collagenolytic activity were carried out according to the procedures of Gross and Lapiere (1962), Lapiere and Gross (1963), and Nagai et al. (1966). The reaction mixture consisted of 0.79 mg of [^{14}C]collagen in 0.2 ml (sp act. 1.10 μCi/g of collagen), 0.02 ml of enzyme, 0.12 ml of 5 mM $CaCl_2$ in 50 mM Tris-HCl buffer, pH 7.4.

Before adding the buffer and enzyme, the collagen was incubated for 15–24 h at 37 °C to form a heat fibril gel (Gross and Kirk, 1958). The reaction mixture was then incubated for 20 min, at which time the nonsolubilized collagen was centrifuged and a 200-μl aliquot of supernatant was removed for counting in 10 ml of Bray's scintillation fluid with 4% Cab-O-Sil. A trypsin standard of 100 μg/ml of reaction mixture, which was routinely run to measure possible denaturation of the substrate, did not solubilize labeled collagen. Another control consisting of buffer in place of enzyme was also run with each assay.

Measurement of BANA Hydrolysis (Amidase/Esterase Activity). The method of Blackwood and Mandl (1961) was used. Absorbance readings were converted to μg of naphthylamine with the conversion factors given by Goldbarg and Rutenberg (1958).

Assay for Caseinolytic Activity. Casein (Matheson, Coleman and Bell) was titrated to pH 9.5 with dilute KOH and titrated back by dropwise addition of H_3PO_4 to pH 5.8. Caseinolytic activity was ascertained as described by Kono (1968). Protein concentration was measured on the filtrate by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Sodium Dodecyl Sulfate Disc Gel Electrophoresis. Polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulfate and 2-mercaptoethanol, was conducted in 8 × 0.5 cm tubes containing 5, 7.5, and 10% acrylamide gels according to the method of Weber et al. (1972). After reduction and solubilization, 2–11 μg of protein was placed on each gel. Gels were stained with Coomasie brilliant blue.

Density Gradient Centrifugation. Sedimentation in 5–20% sucrose gradients was carried out as described by Martin and Ames (1961) in the presence of 50 mM Tris-HCl, pH 7.5, and 5 mM $CaCl_2$ at 4 °C. β-Galactosidase, catalase, yeast alcohol dehydrogenase, and malate dehydrogenase were employed as standards.

Sephadex G-200 Gel Filtration. Sephadex G-200 gel filtration and estimation of diffusion coefficients were done according to Porath (1962) on an upward flow 60 × 2.5 cm column using the above buffer system. The temperature was 4 °C and the flow rate was adjusted to 6–10 ml/h. Standards for calibration of diffusion coefficients were blue dextran, catalase, lactate dehydrogenase, bovine serum albumin, malate dehydrogenase, human carbonic anhydrase, and [^3H]choline.

N-Terminal Analysis. Qualitative N-terminal analyses were carried out by the dansyl method described by Gray (1967).

Immunological Studies. Rabbits received 3 weekly injections in their footpads of 75 μg of collagenase IIIa mixed with complete Freund's adjuvant. They were bled by cardiac puncture 1 week after the last injection. Antisera development against crude and purified collagenase preparations was monitored on Ouchterlony immunodiffusion gels.

Results

Purification of Collagenases.

All preparative steps were performed at 4 °C. Five grams of crude Worthington CLS was dissolved in 250 ml of 1.0 mM sodium phosphate, pH 7.4, and to this was added 166.5 ml of a saturated solution of ammonium sulfate to achieve 40% saturation. The precipitate formed after 10 min was removed by centrifugation at 10 000*g* for 30 min. To the resulting supernatant (420 ml) was added 209 ml of a saturated solution of ammonium sulfate to achieve 60% saturation. The precipitate obtained after 10 min of standing was separated from the supernatant as described previously, dissolved in 60 ml of 1.0 mM sodium phosphate, pH 7.4, and dialyzed overnight against the same buffer.

DEAE-Cellulose Chromatography. The dialyzed solution (75 ml) was placed on a 2.0 × 20 cm DEAE-cellulose DE-52 column equilibrated with 1.0 mM potassium phosphate buffer,

[1] Abbreviations used are: BANA, benzoylarginine naphthylamide; cbzGPGGPA, carbobenzoxyglycylprolylglycylglycylprolylalanine; DDT, dithiothreitol; DEAE, diethylaminoethyl.

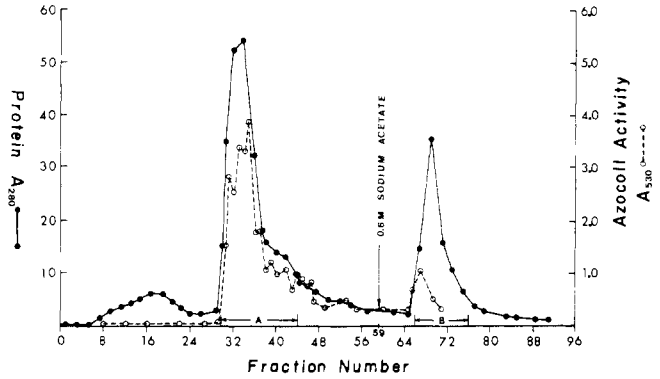

FIGURE 1: Initial chromatography of crude collagenase on a DEAE-cellulose DE-52 column. Crude collagenase (2.8 g) in 1 mM KPO_4, pH 7.4, after 60% $(NH_4)_2SO_4$ precipitation and subsequent dialysis, was layered on a 2.0 × 20 cm column at 4 °C. Protein peak A was eluted with 60 mM KPO_4, pH 7.4; peak B was eluted with 0.6 N NaOAc, pH 5.3. Six-milliliter fractions were collected. Curves are designated as follows: (●—●) A_{280nm}; (○—○) A_{530nm} which indicates activity against the synthetic substrate, Azocoll.

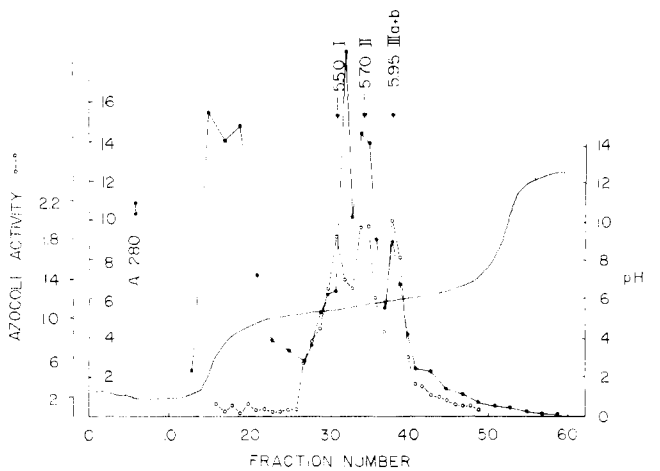

FIGURE 2: Initial preparative isoelectric focusing of the crude collagenases. Peak A (100 mg of protein) from the column of Figure 1 was subjected to electrofocusing for 60 h at 4 °C in a 110-ml column at a 400-V potential difference in the presence of pH 5–7 range ampholyte. Fractions of 1.8 ml were collected, and pH was measured at 4 °C with electrodes standarized at the same temperature. Curves are designated as follows: (●—●) A_{280nm}; (—) pH; (○—○) activity against Azocoll. Peaks corresponding to collagenases I, II, and III (a mixture of IIIa and IIIb) are indicated.

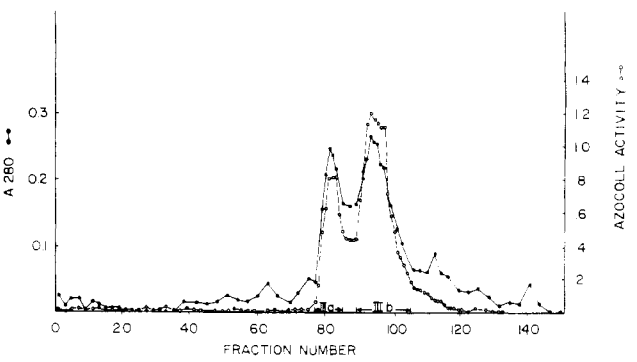

FIGURE 3: Separation of collagenases IIIa and IIIb. Sixteen milligrams of protein from peak III of the isoelectric focusing (Figure 3), subsequently dialyzed against 20 mM Tris-acetate, pH 7.5, 0.1 mM calcium acetate, were layered on a 1.5 × 15.0 cm DEAE-cellulose (DE 52) column equilibrated with the same buffer and eluted with the gradient as described in the text. Fractions of 1.5 ml were collected. Curves are designated as follows: (●—●) A_{280nm}; (○—○) A_{530nm} indicating activity against Azocoll.

pH 7.4 (Figure 1). The column was first eluted with 60 mM potassium phosphate, pH 7.4, to yield peak A, and subsequently with 0.6 M NaOAc, pH 5.3, to yield a second protein peak, B. Peak A had the major portion of the activity against Azocoll and was used in all subsequent collagenase purification steps. It should also contain protein(s) in common with that characterized earlier by Harper and Kang (1970).

Isoelectric Focusing. Peak A samples from DEAE-cellulose chromatography were concentrated on an Amicon filter system and subjected to isoelectric focusing on a 440 ml LKB 8101 column using a pH 5–7 ampholyte system. In the initial purification, the material was divided into three fractions and successively fractionated on 110-ml columns. Focusing in the electric field was carried out for 48–70 h at 4 °C with a 400-V potential difference and from 50 to 60 fractions were collected. The pattern obtained on the electrofocusing column showed three major peaks with activity towards Azocoll, which appear at pH values of 5.50, 5.65, and 5.90–6.00. These were designated as collagenases I, II, and III, respectively (Figure 2). Collagenase III actually consisted of collagenase IIIa and IIIb, which were not well resolved on the initial electrofocusing column.

Purification of Collagenases I and II. Peaks I and II when separately subjected to chromatography on hydroxylapatite columns as described by Mitchell (1968) did not produce a discrete separation from small quantities of the other collagenases, although the procedure afforded purification from other extraneous proteins. Final purification was achieved by an additional step of isoelectric focusing on a 110-ml column using the conditions described above.

Purification of Collagenases IIIa and IIIb. It was possible to separate these two proteins on DEAE-cellulose columns using conditions described by Kono (1968). In this case, 16 mg of protein from peak III of the electrofocusing column was dialyzed against the column buffer and put on a 1.5 × 15.0 cm DEAE-cellulose column equilibrated with 20 mM Tris-acetate, pH 7.5, containing 0.1 mM calcium acetate.

The protein was eluted with a linear gradient formed from 200-ml volumes of the above solution and a solution of 0.1 M Tris-acetate, 0.5 M sodium acetate, and 0.1 mM calcium acetate, pH 7.5. Two peaks, IIIa and IIIb, were obtained from such columns (Figure 3). Each peak consisted of a single protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, samples still exhibited a small amount of residual hydrolytic activity against BANA, which was removed with subsequent isoelectric focusing of the individual collagenases, IIIa and IIIb.

Characterization of Purified Collagenases

Enzymatic Activities. Activity of the various fractions against a reconstituted collagen, the synthetic hexapeptide, cbzGPGGPA, and Azocoll was monitored (Table I). The enzymatic activities toward BANA and casein, which should reflect the presence of contaminating proteolytic activity, were also tabulated. Collagenases II, IIIa, and IIIb showed 1.5–2.4-fold enhancement over the crude mixture in specific activity against native collagen. Collagenase I showed a decrease in specific activity. Purified fraction IIIa showed the greatest enhancement in specific activity against cbzGPGGPA, exhibiting a threefold increase over the crude material. Fractions I, II, and IIIb were enhanced 1.3, 1.4, and 1.6 times, respectively, in specific activity toward cbzGPGGPA, as compared

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

Preparation	mg of Protein Recovered	Specific Activities				
		Native Collagen[b]	Synthetic Peptide[c]	Azocoll[d]	BANA[e]	Casein[f]
Crude CLS	4800	3.29	0.43	9.27	0.0404	2.22
40–60% $(NH_4)_2SO_4$	2700	2.02	0.57	3.48	0.030	0.727
DEAE-cellulose I	1360	2.14	0.55	2.37	0.010	0.155
Isoelectric focusing						
I	156					
II	205					
IIIa,b	162					
Collagenase I	5.63	2.08	0.545	7.50	0.0	0.0013
Collagenase II	5.76	5.16	0.58	4.14	0.0	0.0013
Collagenase IIIa	1.44	4.94	1.37	3.96	0.0	0.009
Collagenase IIIb	2.40	7.72	0.67	2.23	0.0001	0.0013
Trypsin		0			0.037	8.68

[a] The overall purification scheme is described in Results. Following the electrofocusing, 78, 82, and 44 mg of peaks I, II, and III were employed in subsequent purification steps. Thus, overall recoveries for collagenases I, II, and III would be increased by factors of 2, 2.5, and 4, respectively. Since the collagenases exhibit synergistic activity with each other and with other proteases, it is not possible to calculate recoveries in the individual steps. [b] mg of collagen solubilized mg of protein^{-1} min^{-1}. [c] μmol of cbzCPGGPA hydrolyzed mg of protein^{-1} min^{-1}. [d] mg of Azocoll solubilized mg of protein^{-1} min^{-1}. [e] mg of 2-naphthylamine formed mg of protein^{-1} min^{-1}. [f] mg of casein solubilized mg of protein^{-1} min^{-1}.

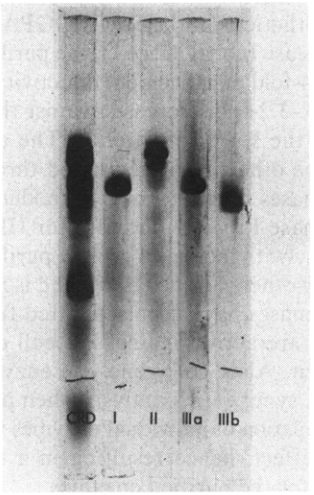

FIGURE 4: Polyacrylamide gel electrophoresis of purified collagenases in the presence of sodium dodecyl sulfate and 2-mercaptoethanol. Two to sixteen micrograms of protein was layered on each of the 0.5 × 8.0 cm, 7.5% polyacrylamide gels (Weber et al., 1972). CRD crude. Purified collagenases are labeled by Roman numbers, I to IIIb. Similar patterns were obtained when the collagenases were reduced by 1 mM DTT and subsequently alkylated after 30 min with 3 mM iodoacetic acid.

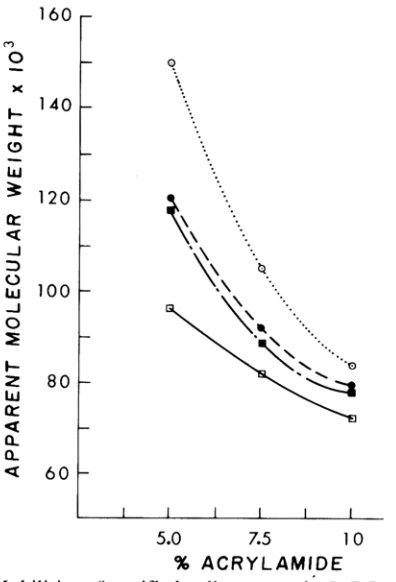

FIGURE 5: Mobilities of purified collagenases in 5, 7.5, and 10% polyacrylamide gels in the presence of sodium dodecyl sulfate and mercaptoethanol. Apparent molecular weights of collagenases I, II, IIIa, and IIIb, estimated from migration distances relative to the standard proteins (rabbit muscle phosphorylase, bovine serum albumin, catalase, and human carbonic anhydrase), are plotted vs. acrylamide gel concentration: (●—●) collagenase I; (○ - - - ○) collagenase II; (■—■) collagenase IIIa; (□—□) collagenase IIIb.

with the crude extract. Only an N-terminal glycine successively followed by proline and alanine was revealed with the sequential dansyl-Edman procedure on the hydrolyzed synthetic peptide.

Specific activities toward the partially denatured collagen derivative, Azocoll, decreased with the purification of the collagenases. Collagenase I showed the smallest decrease in specific activity, falling to only 80% of the crude material, while fractions II and IIIa showed 49% and fraction IIIb exhibited only 24% of the specific activity of the crude material. Purified collagenase fractions were essentially free of contaminating BANA hydrolyzing and caseinolytic activities.

Polyacrylamide Gel Electrophoresis. Following purification, the four collagenases appeared as single bands upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 4). Purification to apparent homogeneity can be achieved for the individual enzymes with the above fractionation scheme, although small amounts of contaminating protein were evident in the collagenase II preparation.

Subunit molecular weights ascertained from the calibration of the respective migration distances relative to standard proteins of known molecular weight yielded very different molecular weight values at each acrylamide concentration (Figure 5). This dependence on acrylamide concentration was most pronounced for collagenase II and less evident for IIIb. Deviations of this nature are characteristic of proteins pos-

TABLE II: Properties of Collagenolytic Enzymes.[a]

	Collagenase I	II	IIIa	IIIb
Sedimentation coefficient	5.4	5.4	5.4	4.3
Stokes radius	39.8	40.4	37.5	35.9
Frictional coefficient	1.34	1.36	1.30	1.35
Calculated mol wt	91 000	92 000	86 000	65 000
Subunit mol wt in sodium dodecyl sulfate	81 000	81 000	81 000	72 000
Isoelectric pH	5.50	5.65	5.90	6.00

[a] The sedimentation coefficients and Stokes radii were measured by sedimentation in 5–20% sucrose gradients and by upward flow Sephadex G-200 gel filtration, respectively, as described in the text. Molecular weights and frictional coefficients of the native proteins were calculated from the eq 1 and 2 under the Results using a value of 0.725 for $\bar{v}$. Subunit molecular weights and isoelectric pH's were determined as described in the text.

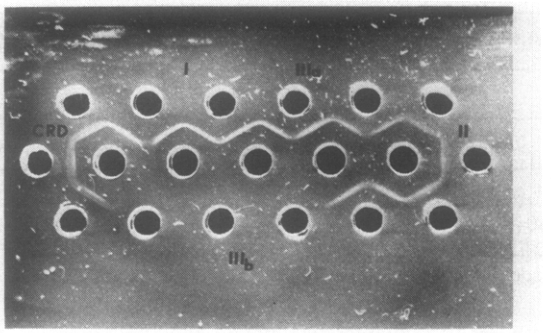

FIGURE 6: Immunological properties of collagenases. Antiserum was raised against collagenase IIIa. Several precipitin bands were formed against crude collagenase preparation. The antiserum also cross-reacted with collagenases I, II, and IIIb. The precipitin band against IIIb was evident but does not show well on the figure due to the low protein concentration. In the immunodiffusion studies, the serum was undiluted and the enzyme concentrations were between 50 and 220 μg/ml.

sessing a high carbohydrate content (Segrest et al., 1971; Schubert, 1970). For collagenases I, II, IIIa, a convergence in molecular weight is observed in 10% acrylamide (81 000), whereas the converging molecular weight for collagenase IIIb is somewhat lower (72 000).

Sedimentation and Hydrodynamic Behavior. Owing to limitations of available material, we have estimated the molecular weights of the collagenases from sedimentation coefficients and by ascertaining the Stokes radii from gel filtration measurements. Symmetrical peaks were observed upon density gradient centrifugation. For three of the collagenases the sedimentation coefficient was 5.4, while that of collagenase IIIb was 4.3 (Table II).

Upon gel filtration on an upward flow Sephadex G-200 column, the four collagenases eluted in similar but not identical positions. Exclusion behavior on gels provides a more accurate estimate of Stokes radius than of molecular weight (Siegel and Monty, 1966). Using the empirical relationship between the distribution coefficient and Stokes radius developed by Porath (1962), we have estimated the molecular weights, M, and frictional coefficients, f/f_0, for the four enzymes from the two formulas:

$$M = \frac{6\pi k\eta Nas}{1 - \bar{v}\rho}$$

$$\frac{f}{f_0} = \left(\frac{a}{(3\bar{v}M/4\pi N)}\right)^{1/3}$$

where a denotes the Stokes radius, k Boltzmann's constant, η the viscosity, N Avogadro's number, ρ the specific gravity, and $\bar{v}$ the partial molar volume (Table II).

Immunochemical Properties of the Collagenases. Rabbit antiserum directed against collagenase IIIa also cross-reacted with purified collagenases I, II, and IIIb to give single confluent precipitin bands on Ouchterlony double-diffusion plates (Figure 6). Multiple precipitin bands were formed against the crude-staining material. Anticollagenase antiserum, incubated with collagenase at room temperature for 15 min prior to addition of substrate, inhibited enzymatic activity against Azocoll between 50–80% for collagenases I, II, IIIa, and IIIb. Individual differences in antibody specificity have not been examined in detail.

Discussion

The purification scheme employed in this study yields four discrete collagenases, I, II, IIIa, and IIIb, in reasonable yields and free from BANA hydrolyzing and caseinolytic activities. The collagenases, as summarized in Table II, are distinguishable by their isoelectric points, molecular weights on sodium dodecyl sulfate gels, hydrodynamic properties, and their relative activities against reconstituted guinea pig skin collagen, the synthetic peptide, cbzGPGGPA, and Azocoll.

Except in one case (collagenase I), the purified collagenase displayed 1.5–2.4-fold enhancement of activity against native collagen and 1.3–3.2-fold increase against the hexapeptide, as compared to the starting material. The activity against Azocoll is, on the other hand, decreased throughout all the purified collagenases ranging from the moderate decrease of 19% for collagenase I to 76% decrease for IIIb. This decline in specific activity with increased protein purification has also been observed by others (Kono, 1968) and is not unexpected, since the collagenases have been separated from extraneous proteases which are active against Azocoll or partially hydrolyzed collagen. Also, collagenolytic enzymes have been shown to exhibit synergistic catalysis when present together (Kono, 1968). Isolation of individual enzymes would eliminate the synergistic effect, hence resulting in a lower apparent specific activity for the purified enzymes.

Grant and Alburn (1959) were the first to note the apparent heterogeneity of chromatographically purified collagenases. Subsequently, 2–6 enzymes have been noted by Noda (1964), Mandl et al. (1964), Harper et al. (1965), Harper and Kang (1970), Harper and Seifter (1974), Kono (1968), and Peterkofsky and Diegelmann (1971). Kesselring et al. (1970) detected three collagenolytic enzymes with isoelectric pH's 5.4, 5.8, and 6.1 that were partially separable from each other and from contaminating proteases. Although these workers did not further purify the collagenases, their fractionation closely resembles our collagenase separation, which yielded individual enzymes with isoelectric pH's of 5.50, 5.65, and 5.90–6.0.

Our findings reveal some distinct differences from previous studies where the enzyme characterization was less complete (Levdikova et al., 1963; 1964; Harper et al., 1965). It was proposed previously that collagenase A with a mass of 100 000 daltons was a tetramer composed of subunits with a molecular weight of 25 000. The collagenases in the present study, however, after reduction and subsequent alkylation, had subunit molecular weights between 72 000 and 81 000. These values were in agreement with those obtained from sedimentation and gel diffusion data on the undissociated enzyme (Table II). This indicates that the individual collagenases are,

in fact, single polypeptide chains and are not composed of individual subunits. While limited physical characterization of collagenases isolated earlier precludes relating the four enzymes we have isolated with those previously studied, the physical parameters of molecular weights, isoelectric points, sedimentation, and diffusion coefficients should enable one to identify individual collagenases isolated in subsequent preparations in relation to these defined physical properties.

The basis for the multiplicity of the collagenases will require a more detailed chemical characterization of the individual isozymes but the distinction in isoelectric points and subunit migration reflect discrete differences in primary structure. Nevertheless, similarity of the molecular weights and the immunological cross-reactivity suggest the possibility that one of the collagenases is the parent molecule and the remaining enzymes are derived from it as a consequence of removal of oligosaccharide residues and/or small peptide segments in the culture medium. It is also possible that a selective addition of carbohydrate occurs prior to secretion of the enzyme into the culture medium.

Isoelectric focusing at 4 °C of the extracellular extract from *Clostridium histolyticum* showed six peaks of collagenolytic activity, all of which possessed activity against native collagen (not shown). The fact that four of these peaks can be correlated with the purified collagenases suggests that the multiplicity of species arises prior to their purification. There appears to be an advantage to the multiplicity of the collagenases, since it has been shown by a number of workers that the collagenases work synergistically (Kono, 1968). Although the properties of the individual collagenases have not been examined in relation to the culture conditions, Takahashi and Seifter (1972) observed that the ratio of collagenase to general protease activity is increased if *Clostridium* is grown in a general reducing environment. Thus, it may prove informative to examine collagenase enzyme profiles following a variety of culture conditions.

A situation analogous to the multiple collagenases has been observed for the *endo*-1-4-β-glucanases from the fungus *Sportricum pulverulentum* (Eriksson and Pettersson, 1975). Five individual species were detected in the fungus crude culture filtrate. They have very similar amino acid compositions, molecular weights (28 500 to 37 000), and their carbohydrate contents range from 0 to 10.5%.

Although the precise structural relationships between the individual collagenases await a detailed analysis of their composition, the similarity in molecular weights, catalytic activity, and immunologic cross-reactivity suggests that the collagenases may exist as isozymes. The substrate specificity and the comparatively high turnover number of bacterial enzymes render them powerful tools in the structural analysis of collagen-like proteins (Reid et al., 1972). The use of completely purified collagenases of known physical and catalytic properties is critical to structural studies of potential substrates with more complex structures. We have recently employed these collagenases in the characterization of a tail unit on purified acetylcholinesterase, which appears to be associated with the ectolemma of the nerve synapse (Lwebuga-Mukasa et al., 1976).

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