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PURIFICATION AND PARTIAL CHARACTERIZATION OF A COLLAGENOLYTIC ENZYME FROM *PSEUDOMONAS AERUGINOSA*

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Summary

A proteinase from *Pseudomonas aeruginosa* exhibiting collagenolytic activity was purified 1575-fold with a recovery of 24% by use of chemical and chromatographic technics. The enzyme preparation appeared to be homogeneous when subjected to chromatographic, electrophoretic and ultracentrifugational analyses. A standard state sedimentation coefficient of 2.10 S was calculated and further analyses indicated that the enzyme had a molecular weight of 17 500 and dimerizes under certain conditions to yield an apparent molecular weight of 34 000. In addition to insoluble collagen, the enzyme catalyzed the hydrolysis of congocoll, azocoll, soluble collagen and casein, but did not attack orcein-elastin, azoalbumin, *p*-toluene sulfonyl-L-arginine methyl ester, benzoyl-L-tyrosine ethyl ester, and the hexapeptide *N*-benzyloxycarbonyl-glycyl-L-prolylglycylglycyl-L-prolyl-L-alanine. Enzymatic activity against congocoll was 6-fold greater at pH 7.5 in Tris · HCl than in phosphate buffer at the same ionic strength. Cobalt, and to a lesser extent, Zn^{2+} appeared to activate the enzyme, especially in phosphate buffer. NaCN and *p*-chloromercuribenzoate did not appreciably inhibit enzyme activity, while $(NH_4)_2SO_4$, EDTA and cysteine displayed a significant inhibitory effect under certain conditions.

Introduction

Pseudomonas aeruginosa is an aerobic organism which produces several extracellular proteolytic enzymes. Previous studies indicate that one of the proteinases exhibits elastolytic activity [1] and is lethal and dermonecrotic for mice [2]. In addition to elastase, extensive collagen destruction of the cornea by *P. aeruginosa* [3] suggests that at least one of the proteinases also exhibit collagenolytic activity. Although collagenase production is well established in anaerobes, there is very little in the literature to indicate the production of this enzyme in aerobic organisms. In 1966, Schoelmann and Fisher [4] reported a

proteinase from *P. aeruginosa* which could hydrolyze a synthetic hexapeptide specific for detecting clostridial collagenase. On the other hand, Waldvogel and Swartz [5] reported that the *P. aeruginosa* collagenase was incapable of hydrolyzing native collagen (tropocollagen) and was therefore better named a peptidase. Their study also included a survey of 30 strains of *P. aeruginosa* tested for collagenolytic activity by the ability of growing cultures to clear an opaque collagen gel. However, none of the strains so tested could repeatedly clear the gel. In contrast, Adamcic and Clark [6] reported that several strains of *Pseudomonas* were capable of causing the release of large quantities of extractable proline from collagen-rich tissue as well as the release of ninhydrin-positive materials from soluble collagen. In view of such conflicting reports, the current study was undertaken. The design of the project was to develop a procedure to extensively purify an active proteinase from *P. aeruginosa* exhibiting collagenolytic activity. In addition, a partial characterization of the purified enzyme with regard to its *in vitro* properties is also described.

Materials and Methods

Chemicals. Unless otherwise indicated, all chemicals were analytical reagent grade commercial preparations.

The following materials were from commercial sources: tryptose agar and peptone from Difco Laboratories; trypticase soy broth from Baltimore Biological laboratories; QAE-Sephadex, blue dextran, and Sephadex G-150 from Pharmacia; aquacide II, congocoll, tropocollagen (calf skin), azocoll and azoalbumin from Calbiochem; bovine serum albumin, γ-globulin, ovalbumin, myoglobin, cytochrome *c* and *N*-benzyloxycarbonyl (Cbz)-glycyl-L-prolylglycylglycyl-L-prolyl-L-alanine from Mann Research Laboratories; insoluble collagen (bovine achilles tendon) and three times crystallized clostridial collagenase from Worthington Biochemical Company; Tris and two times crystallized trypsin from Nutritional Biochemicals Corporation; and pepsin from Pentex, Inc.

Bacterial strain. P. aeruginosa strain L-1 was isolated from the sputum of a 45-year-old, emphysemic, female who had *Pseudomonas* pneumonia. Stock cultures of this strain were maintained on slants of 4.1% tryptose agar media stored at 25° C and transferred every 30 days.

Enzyme harvest. Seed cultures were prepared by inoculating 50 ml of sterile media composed of 5% peptone and 0.25% trypticase soy broth with the washings from a stock culture slant. The broth cultures in 125-ml Erlenmeyer flasks were incubated at 37° C on a rotary shaker at 150 rev./min for 24 h. The contents of these growth flasks were then transferred to 2-l Erlenmeyer flasks containing 1 l of sterile peptone-trypticase soy broth and incubated as above.

After 24 h growth, the cultures were clarified by centrifugation at 5000 × *g* for 30 min in a Sorvall RC-2B refrigerated centrifuge at 4° C. All subsequent steps of the purification scheme were conducted at 4° C. Unless otherwise specified, Tris · HCl buffers refers to 0.05 M tris(hydroxymethyl)aminomethane prepared at 25° C to pH 7.5 and then used at various temperatures without correction for the resulting changes in ionic strength or pH.

Ethanol fractionation. The supernatant fluid was concentrated to one-third its volume in dialysis tubing against aquacide II. The resulting solution

was designated "crude concentrate." Absolute ethanol was added dropwise with stirring to the "crude concentrate" in amounts sufficient to give 65% concentration of ethanol. The resulting precipitate was allowed to settle overnight and was then removed by centrifugation at 48 000 × *g* for 20 min. This precipitate was dissolved in a minimum volume of Tris · HCl buffer, centrifuged again at 48 000 × *g* for 20 min to remove the (precipitated) material which remained insoluble, and then dialyzed against Tris · HCl buffer to which NaCl had been added to a concentration of 0.1 M.

Ion-exchange chromatography. The dialyzed solution was applied to a 2.5 × 50 cm column of diethyl-(2-hydroxypropyl) aminoethyl (QAE)-Sephadex A-50, which had been equilibrated in Tris · HCl buffer, containing 0.1 M NaCl. The column was then rinsed with 500 ml of this buffer followed by 500 ml of Tris · HCl containing 0.15 M NaCl. The absorbance of the eluent was continuously monitored at 280 and 254 nm and collected in 6.5-ml fractions.

Fractions containing the protein peak displaying major congocoll activity (see below) were pooled, concentrated against aquacide II, and dialyzed against Tris · HCl buffer to a final volume of less than 10 ml.

Gel filtration chromatography. The concentrated fraction from the ion-exchange chromatography was applied to a 2.5 × 100 cm column of Sephadex G-150 equilibrated in Tris · HCl buffer. The protein was eluted with 50 ml of the same buffer at a rate of 10 drops/min. The absorbance of the eluent was monitored as above and 5-ml fractions were collected. The fractions containing the protein peak displaying congocoll activity were pooled, concentrated, and dialyzed as above.

Congocoll hydrolysis. A modification of the assay described by Nelson et al. [7] was employed. Congocoll is a general proteolytic substrate, susceptible to hydrolysis by a number of proteases. 25 mg of congocoll in a 10-ml Erlenmeyer flask were hydrated with 5 ml of Tris · HCl buffer in a 37° C water bath for 5 min before addition of 0.1 ml of a suitable dilution of enzyme. The reaction mixture was shaken at 150 rev./min for 15 min and the reaction was stopped by the addition of 5 ml of 0.1 M NaOH. The insoluble substrate remaining was removed by filtration through a Millipore glass prefilter pad (No. AP250500) cut to fit micro-Buchner funnels. The absorbance of the filtrate was measured at 480 nm in a Beckman D-B spectrophotometer (1 cm light path) against the filtrate from a reaction flask containing 0.1 ml of enzyme solution which had been heat denatured at 100° C for 20 min. Units of enzymatic activity were defined as the change in absorbance of the filtrate at 480 nm after 15 min incubation.

Tropocollagen hydrolysis. The change in the flow rate of acid-soluble calf skin collagen (Calbiochem) with time in the presence of the enzyme was determined by a method modified from Seifter and Gallop [8]. Low shear Ostwald viscometers equipped with 2-ml bulbs and 10-cm capillaries with 80—100 s water calibration were filled with 5 ml of buffer and placed in a 20 ± 0.1° C water bath. Flow times for each viscometer were taken and the averages of three measurements were used to calculate the viscosity coefficient η_0. The reaction mixtures contained 3 ml of 0.2% collagen in 0.01 M acetic acid, 2 ml 0.1 M Tris · HCl buffer, pH 7.5, and 0.1 ml *Pseudomonas* collagenolytic enzyme containing 0.1 mg protein. For comparison, an identical amount of clos-

tridial collagenase was also assayed. The change in flow rate of each solution was determined every 5 min and the incubation time corresponding to the midpoint of the various flow determinations were recorded. One control viscometer containing no enzyme and one containing 100 μg of trypsin were simultaneously monitored. The relative viscosity (η_0) was calculated from the ratio of flow time of the reaction mixture (η) to the flow time of the buffer (η_0) for each viscometer and the log of specific viscosity (η/η_0-1) was plotted against the midpoint of the various flow determinations.

Insoluble collagen hydrolysis. A modification of the method of Mandl et al. [9] was used to measure hydrolysis of insoluble collagen. 25 mg of powered bovine Achilles tendon in a 10-ml Erlenmeyer flask were hydrated with 5 ml of Tris · HCl buffer for 5 min in a 37° C water bath before addition of 0.1 ml of enzyme solution. A small crystal of thymol was added to the flask to prevent microbial growth. The reaction mixture was shaken at 150 rev./min for up to 18 h and the reaction was stopped by the addition of 5 ml of 0.1 M HCl. The insoluble substrate remaining was removed by filtration and heat-denatured controls were conducted as described above. Filtrates of the reaction mixtures were assayed for the products of hydrolysis using trinitrobenzene sulfonic acid as described by Lin et al. [10]. Under these conditions, powdered bovine Achilles tendon liberates a small amount of soluble protein which may be sensitive to trypsin hydrolysis. However, the presence of 100 μg of trypsin could not be detected using this assay.

Azocoll hydrolysis. Azocoll is a general proteolytic substrate which was tested as described by Moore [11].

Hexapeptide hydrolysis. Hydrolysis of a synthetic hexapeptide *N*-Cbz-glycyl-L-prolylglycylglycyl-L-prolyl-L-alanine was quantified according to Grassmann and Nordwig [12] except trinitrobenzene sulfonic acid was used in place of ninhydrin.

Azoalbumin hydrolysis. Azoalbumin hydrolysis was measured by the method of Tomarelli et al. [13].

Casein hydrolysis. The modified casein digestion method of Kunitz was employed [14]. The filtrates were assayed for the relative concentration change in trichloroacetic acid-soluble protein components by the use of trinitrobenzene sulfonic acid.

Elastase assay. Powdered elastin impregnated with orcein was used for the colorimetric assay of Sachar et al. [15].

Chymotrypsin assay. Benzoyl-L-tyrosine ethyl ester (Bz-Tyr-OEt) was assayed spectrophotometrically at 256 nm in 0.08 M Tris · HCl, pH 7.8, according to the method of Hummel [16].

Trypsin assay. *p*-Toluenesulfonyl-L-arginine methyl ester (Tos-Arg-OMe) was assayed spectrophotometrically at 247 nm in 0.046 M Tris · HCl, pH 8.1, according to the method of Hummel [16].

Protein determination. Protein was determined by the microbiuret method of Koch and Putnam [17]. Bovine serum albumin was used as the protein standard.

Determination of free amino groups. Free amino groups were determined using the trinitrobenzene sulfonic acid technique of Lin et al. [10]. Leucine was used as the standard.

Inhibitor-activator studies. The effects of potential inhibitors and activators of the *Pseudomonas* enzyme were studied by mixing 0.1 ml of the enzyme solution with 0.1 ml of 50 mM solutions of the chemicals listed in Table IV and 0.8 ml of 0.1 M KH_2PO_4/Na_2HPO_4 (phosphate buffer), pH 7.0. The solutions were allowed to stand for 1 h at 25° C after which 0.1-ml quantities of each were subjected to the congocoll assay.

Molecular weight determination by Sephadex G-150. The molecular weight of the *Pseudomonas* enzyme was estimated by the method of Andrews [18] using a 2.5 × 85 cm column of Sephadex G-150 in Tris · HCl buffer. γ-Globulin, ovalbumin, myoglobin, and cytochrome *c*, all at 1 mg/3 ml and 3 ml of saturated blue dextran in Tris · HCl buffer were used as standards. 7-ml fractions were collected and the elution volume (V_e) for each standard was calculated.

Ultracentrifugation. The purified enzyme dissolved in 0.1 M potassium phosphate buffer, pH 7.0, was centrifuged at 25° C in a synthetic boundary, double-sector cell at 47 660 rev./min. An AN-D rotor was employed in a Beckman Model E ultracentrifuge. Photographs were taken of the resulting Schlieren patterns at various intervals up to 70 min and analyzed on a Nikon Model B microcomparator. The standard state sedimentation coefficient ($S^0_{20,w}$) was calculated in the conventional manner.

Electrophoresis in sodium dodecylsulfate-polyacrylamide gels. A modification of the electrophoretic method of Shapiro et al. [19] was employed to estimate the molecular weight of the enzyme. Gels and gel buffer were prepared as described by Weber and Osborn [20] except that the concentration of $(NH_4)_2S_2O_8$ solution was reduced to 10 mg/ml and the final concentration of *N,N,N',N'*-tetramethylethylenediamine was 0.1%. Gels were polymerized in 5 × 75 mm glass tubes. Ovalbumin, bovine serum albumin, myoglobin, and cytochrome *c*, and pepsin were used as standard molecular weight markers.

Polyacrylamide gel electrophoresis. Samples of the various fractions from the purification scheme were electrophoresed in polyacrylamide gels according to Davis [21].

Amino acid analysis. Samples of the purified enzyme were hydrolyzed in 6 M HCl in N_2-flushed, evacuated sealed tubes at 110° C for 22 and 118 h, respectively. Solutions were evaporated to dryness in vacuo in the presence of NaOH pellets, and were then redissolved in water for analysis on a Technicon amino acid analyzer as described by Spackman et al. [22]. Norleucine was added before hydrolysis to correct for manipulative losses and 2-amino-3-guanido-propionic acid was placed on the analyzer column to correct for variations in ninhydrin color yield.

Results

Values for a typical purification are summarized in Table I. For convenience, all fractions were monitored for enzyme activity by use of the congocoll assay [7] during the purification procedure. A 1575-fold increase in specific activity with a 24% recovery was obtained. The purified enzyme obtained from the post-Sephadex G-150 column was difficult to characterize due to its lability. The enzyme could not be stored in the refrigerator for prolonged

TABLE I

SUMMARY OF PURIFICATION OF *PSEUDOMONAS* COLLAGENOLYTIC ENZYME

Fraction	Volume (ml)	Congocoll (units/ml)	mg protein (ml)	Units/mg	Total units	Recovery (%)	Fold purification
Crude	3000	31	9	3.4	93000	100	—
Ethanol	20	4200	3.8	1105	84000	90	325
Post QAE-Sephadose	10	3650	1.5	2433	36500	39	715
Post-Sephadose G-150	6	3750	0.70	5357	22500	24	1575

periods without substantial loss in activity. In addition, lyophilization, or freezing of freshly purified preparations at −60° C resulted in an almost total loss of activity.

Fig. 1 shows the elution profile of a redissolved ethanol precipitate from a QAE-Sephadex column. Significant amounts of congocoll hydrolysis were achieved from samples eluting at 0.1 and 0.15 M NaCl gradient steps. However, hydrolysis of insoluble collagen and tropocollagen could be detected only in samples eluted by the 0.15 M NaCl step. This material was pooled, concentrated, dialyzed, and applied to a column of Sephadex G-150.

The post-void volume elution profile of the post-QAE fractions from a Sephadex G-150 column is presented in Fig. 2. Congocoll hydrolysis was demonstrated by fractions corresponding to a single protein peak eluting at about 150 ml.

This final fraction demonstrated a single band when electrophoresed on sodium dodecylsulfate gels. However, electrophoresis in Tris/glycine buffer, pH 8, according to the method of Davis [21], resulted in one major and one minor band. Both bands were capable of congocoll hydrolysis and when the

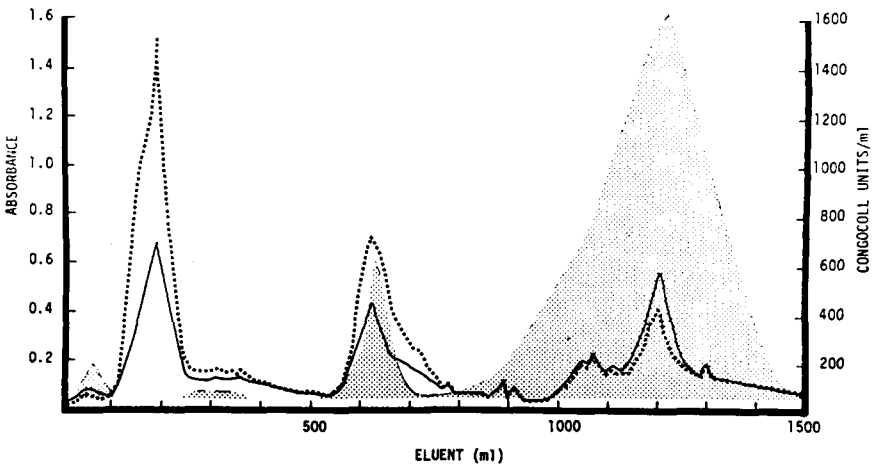

Fig. 1. Elution profile of redissolved ethanol precipitate from QAE-Sephadex column. 500 ml NaCl gradients of 0.05, 0.10, and 0.15 molar were employed. ———, 280 nm absorbance; - - - - - -, 254 nm absorbance; . . . . . ., congocoll activity.

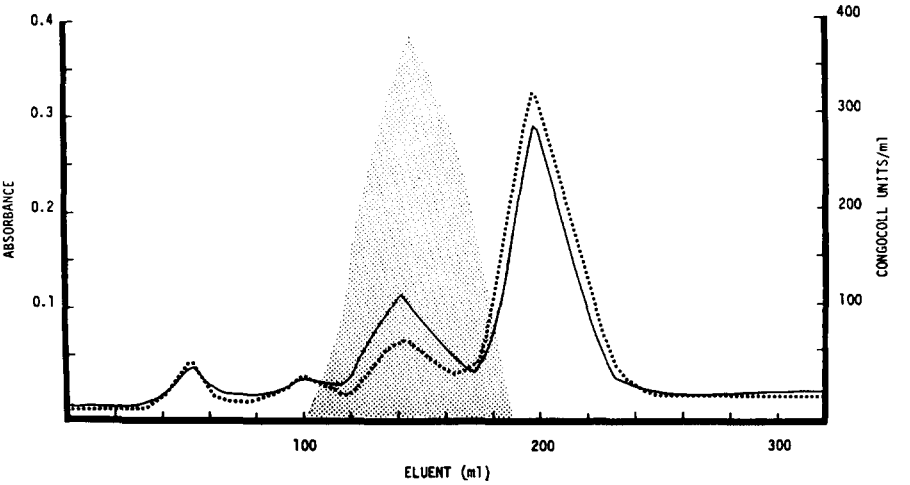

Fig. 2. Post-void volume elution profile of post-QAE fraction from Sephadex G-150 column. ———, 280 nm absorbance; - - - - - -, 254 nm absorbance; . . . . . ., congocoll activity.

area of the major band was cut from an unstained gel, macerated in buffer, and the eluting material re-electrophoresed, both the major and minor bands were again detected.

Sodium dodecylsulfate-acrylamide gel electrophoresis. Mobilities of standard proteins and of the *Pseudomonas* collagenolytic factor were determined and a semilogarithmic plot of these values was made. The results indicate a molecular weight for the enzyme of 34 000 ± 2000.

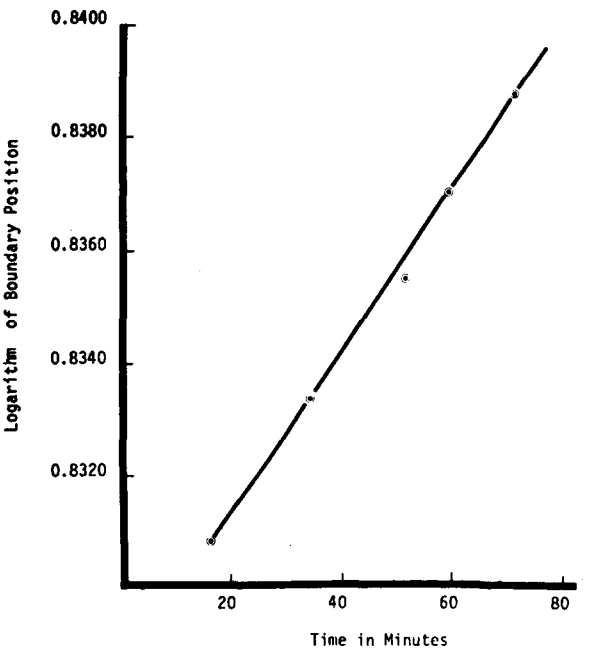

Fig. 3. Sedimentation coefficient determination of purified *P. aeruginosa* collagenolytic enzyme.

TABLE II

AMINO ACID ANALYSIS OF PURIFIED COLLAGENOLYTIC ENZYME

The first and second value for each amino acid represents values obtained at 22 and 118 h of hydrolysis, respectively.

Amino acid	Corrected μg	Corrected μM	Residues	Corrected residues
Aspartic acid	7.100	0.0563	11.7	12
	7.144	0.0562	11.5	
Threonine	2.676	0.0242	5.0	5
	2.451	0.0221	4.5	
Serine	3.167	0.0322	6.9	7
	2.374	0.0248	5.1	
Glutamic acid	4.197	0.0297	6.2	6
	3.900	0.0275	5.6	
Proline	2.011	0.0189	3.9	4
	2.110	0.0198	4.0	
Glycine	3.311	0.0530	11.0	11
	3.215	0.0513	10.5	
Alanine	2.785	0.0358	7.4	8
	2.776	0.0355	7.2	
Valine	2.509	0.0231	4.8	5
	2.507	0.0230	4.7	
Cysteine	0.547	0.0048	1.0	1
	0.551	0.0049	1.0	
Methionine	1.126	0.0078	1.6	2
	1.356	0.0094	1.9	
Isoleucine	1.329	0.0107	2.2	3
	1.513	0.0122	2.5	
Leucine	2.053	0.0166	3.4	4
	2.268	0.0182	3.7	
Tyrosine	4.768	0.0267	5.6	6
	5.025	0.0280	5.7	
Phenylalanine	2.553	0.0158	3.3	5
	3.351	0.0207	4.2	
Lysine	2.471	0.0176	3.7	4
	2.263	0.0161	3.3	
Histidine	1.409	0.0094	1.9	3
	1.692	0.0112	2.3	
Arginine	3.212	0.0188	3.9	4
	3.172	0.0185	3.8	

Molecular weight determination by Sephadex G-150. The void volume of the column (V_o) was determined by blue dextran to be 140 ml. The column bed was 85 cm high and 2.5 cm in diameter which corresponds to a total volume (V_t) of 1688 ml. A semi-logarithmic plot of K_{av} against the logarithm of molecular weight for each standard was made. The K_{av} calculated for the enzyme corresponded to a molecular weight of 17 500 ± 500.

Ultracentrifugation. The ultracentrifugation patterns of purified enzyme indicated a single, homogeneous peak without shoulders. From the data

TABLE III

SUBSTRATE SPECIFICITY OF *PSEUDOMONAS* COLLAGENOLYTIC ENZYME

Substrate*	Temperature (°C)	Time (min)	Activity	
Congocoll	37	15	7000	units/mg
Azure hide powder	37	15	1630	units/mg
Azocoll	37	15	720	units/mg
Azoalbumin	37	15	0	
Orcein elastin	37	15	0	
Insoluble collagen	37	1080	31	μmol leu/mg
Tropocollagen	20	30	152.8	units/mg
Hexapeptide	37	30	0	
Casein	37	20	35	μg protein/mg
Tos-Arg-OMe	37	30	0	
Bz-Tyr-OEt	37	30	0	

* See Materials and Methods section.

presented in Fig. 3, a value for the time rate of change of the logarithm of the boundary position of $1.527 \cdot 10^{-4}$ was calculated, resulting in an observed sedimentation coefficient of $2.35 \cdot 10^{-13}$.

A partial specific volume at 20°C of 0.749 was assumed for this protein, which resulted in a standard state sedimentation coefficient ($S^0_{20,w}$) of 2.10 S.

Amino acid analysis. The results of amino acid analysis are summarized in

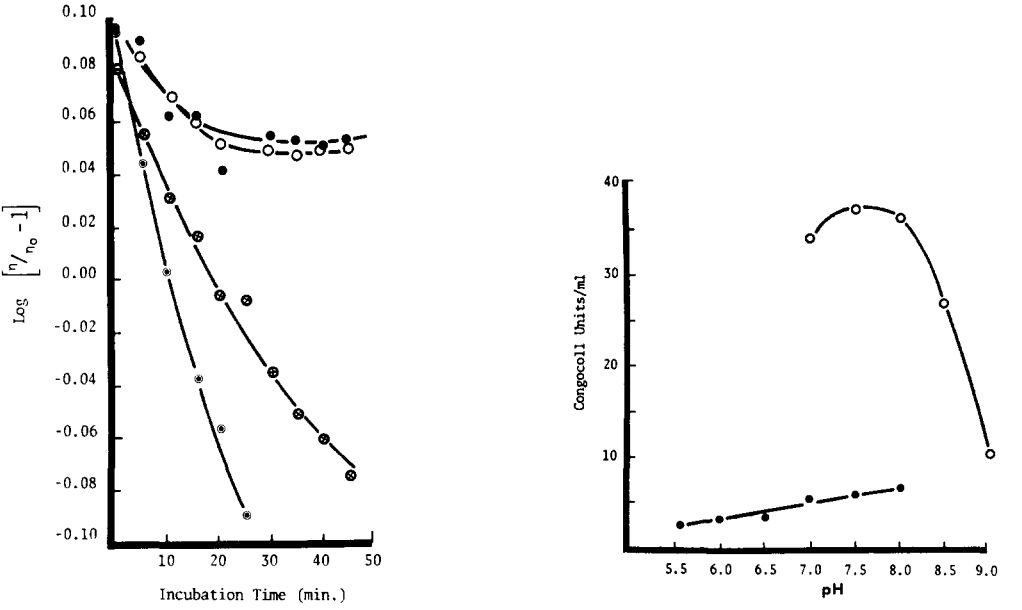

Fig. 4. Viscometric assay of tropocollagen hydrolysis by clostridial collagenase and *Pseudomonas* collagenolytic enzyme. •, control (no enzyme); ○, trypsin (0.1 mg protein); ⊗, *P. aeruginosa* collagenolytic enzyme (0.1 mg protein); ⊛, clostridial collagenase (0.1 mg protein).

Fig. 5. Effect of pH on congocoll assay. ○, Tris · HCl buffer, $I = 0.1$; •, phosphate buffer, $I = 0.1$.

Table II, which shows the average of values from analyses at 22 and 118 h of hydrolysis. Data for serine, threonine, and phenylalanine were extrapolated to zero time. Amide nitrogen and tryptophan were not determined. The number of residues is based upon one cysteine per molecule. This corresponds to a minimum molecular weight of 9900. If two cysteine residues per molecule are assumed, a molecular weight of 18 900 results.

Substrate specificity. The relative degrees of hydrolysis of several common substrates are presented in Table III. The reaction temperature and time of incubation vary among the assay systems according to the established convention for each substrate. Units of enzymatic activity are expressed in the manner employed by the originators of each assay. Due to the complex nature of the insoluble substrates, these various units are arbitrary and do not relate to each other in a quantitative manner.

The results of the viscometric assay of tropocollagen hydrolysis by clostridial collagenase and the *Pseudomonas* collagenolytic factor are presented in Fig. 4. Both reaction mixtures contained 100 μg of enzyme protein which resulted in values of 152.8 units/mg for the *Pseudomonas* enzyme and 276 units/mg for clostridial collagenase when calculated by the method of Seifter and Gallop [8]. Although a slight decrease in viscosity was observed in the control tube, it did not reflect a significant denaturation of the substrate due to its insensitivity to the commercial proteinase (trypsin control).

pH studies. The ability of purified enzyme to hydrolyze congocoll at different pH is presented in Fig. 5. Two buffer systems, phosphate and Tris · HCl, were employed at overlapping pH values.

Inhibitor-activator studies. The effects of potential inhibitors and activators of the enzyme are presented in Table IV. After exposing the purified enzyme to the various compounds at 25° C for 1 h the mixtures were assayed by congocoll hydrolysis in both Tris · HCl and phosphate buffers. The final concentration of these compounds in the reaction mixture was $5 \cdot 10^{-4}$ M. The values listed are the percent activity of enzyme solutions calculated after subtracting the absorbance of control blanks containing no enzyme.

TABLE IV

EFFECT OF VARIOUS COMPOUNDS ON THE HYDROLYSIS OF CONGOCOLL BY PURIFIED *PSEUDOMONAS* COLLAGENOLYTIC ENZYME

Compound*	Activity remaining phosphate buffer (%)	Activity remaining Tris · HCl buffer (%)
EDTA	27	21
Cysteine	8	0
NaCN	98	92
p-Chloromercuribenzoate	95	99
$MgCl_2$	78	96
$CaCl_2$	90	102
$FeCl_2$	6	93
$MnCl_2$	77	88
$CoCl_2$	150	119
$ZnCl_2$	117	100

* Final concentration in the reaction mixture was $5 \cdot 10^{-4}$ M.

TABLE V

EFFECT OF IONIC STRENGTH ON CONGOCOLL ASSAY

Assays performed as described in text. Activities given as $A_{480\,nm}$.

	Ionic strength				
	0	0.01	0.05	0.1	0.2
Heat-denatured enzyme control	0.95	0.44	0.12	0.10	0.05
Enzyme + reaction mixture	1.00	0.56	0.25	0.12	0.05

Ionic strength studies. The effects of altered ionic strengths (I) on congocoll hydrolysis by the purified enzyme are presented in Table V. 25 mg of congocoll were suspended in distilled water containing various concentrations of NaCl and the assay was conducted as described in Materials and Methods. The pH of all flasks at 37° C was 7.0 ± 0.2.

Discussion

It is now well established that *P. aeruginosa* is a highly proteolytic organism capable of producing several types of proteinases depending on cultural conditions [23]. The elaboration of either elastase or a collagenolytic enzyme suggests that the organism is potentially capable of attacking insoluble proteins in vivo and these proteinases may thus constitute potential virulence factors [2,3]. Also, the absence of elastase activity in both crude and purified collagenolytic preparations indicates the two enzymes to be separate and distinct entities. Conversely, no collagenolytic activity was detected in preparations exhibiting elastase activity. Substrate specificity studies as well as other data described herein indicate the previously described proteinase from *P. aeruginosa* capable of hydrolyzing a synthetic hexapeptide supposedly specific for clostridial collagenase [4] is also different from the enzyme described in this manuscript. The two enzymes can also be differentiated from one another on the basis of $(NH_4)_2SO_4$ sensitivity. Thus, Schoelmann and Fisher [4] were able to successfully employ $(NH_4)_2SO_4$ in the partial purification of their enzyme whereas in our hands collagenolytic activity was destroyed. The sensitivity of the enzyme to $(NH_4)_2SO_4$ is also consistent with the results of Liu and Hsieh [24] who found proteinase production inhibited by ammonium salts.

Characterization of the purified enzyme especially with reference to molecular weight studies was of particular interest. Estimation of molecular weight by sodium dodecylsulfate-acrylamide gel electrophoresis repeatedly yielded a value of 34 000 ± 2000 which was approximately twice the value obtained through gel filtration chromatography studies and was inconsistent with the $S^0_{20,w}$ of 2.10 calculated from analytical ultracentrifugation. A possible explanation for this unusual behavior may be derived from analysis of the electrophoretic patterns displayed by the molecular weight markers used to standardize the measurement. Examination of these gels (Fig. 3) reveals that even in the presence of sodium dodecylsulfate, bovine serum albumin and

ovalbumin both formed dimers and trimers. Systematic studies of these and other proteins [25] have demonstrated similar polymerizations, especially above pH 8. Reductive methylation of some proteins prior to electrophoresis has eliminated such patterns [9] but this has not yet been attempted with *Pseudomonas* collagenolytic enzyme. If dimerization was taking place in this enzyme preparation, it would also explain the occurrence of a major and minor band in the gels electrophoresed by the Davis technique [21].

Substrate specificity studies indicate that congocoll was the most sensitive of the collagen substrates tested for hydrolysis by the *P. aeruginosa* enzyme. Because of the high sensitivity of this assay and the comparative speed and economy, it was extensively employed in further enzymatic characterizations. However, it should be kept in mind that the impregnation of insoluble collagen with dyes (as in the preparation of congocoll, azure hide powder, and azocoll) results in as yet an undetermined amount of denaturation of the collagen matrix which renders it susceptable to hydrolytic cleavage by a variety of proteolytic enzymes. In addition, the structure and composition of insoluble collagens varies with the species, organ, and method of extraction. Consequently, it is the hydrolysis of tropocollagen by this enzyme which serves as the main enzymatic criterion for its classification as a collagenolytic enzyme. The integrity of the majority of the helical region of tropocollagen in this preparation is indicated by the relative stability of viscosity and the relative insensitivity to trypsin.

Significant hydrolysis of tropocollagen or insoluble collagen by the pre-column fractions could not be detected indicating that the enzyme concentrations were below the limits of sensitivities of these assays or that some inhibitory substance may have masked the activity.

The inability of the purified enzyme to hydrolyze the synthetic hexapeptide *N*-Cbz-glycyl-L-prolylglycylglycyl-L-prolyl-L-alanine indicates that the amino acid sequence of collagen specifying hydrolysis by clostridial collagenase is different than that for the enzyme described herein. Hydrolysis of casein is common to all *P. aeruginosa* proteinases, as is the lack of demonstrable esterase activity as estimated with *p*-toluenesulfonyl-L-arginine methyl ester or benzoyl-L-tyrosine ethyl ester [16].

The effect of cobalt and zinc on the activity of clostridiopeptidase has been the subject of investigation in several laboratories. Yagisawa et al. [27] studied the effects of several metal ions on the activity of clostridiopeptidase on a variety of synthetic substrates. They found inhibition by zinc in relatively high concentrations but also observed activation by cobalt. Takahashi and Seifter [28] also studied the effects of metals on this enzyme, however, employing collagen as the substrate. Their findings were that both cobalt and zinc activated clostridiopeptidase yielding 1.8 times the activity measured in a calcium-containing system. In 1972 Soru and Zaharia [29] added to the confusion about cobalt when they reported that clostridiopeptidase hydrolysis of the Cbz-hexapeptide was completely inhibited by this ion.

In general, analysis of data concerning the effects of various compounds on the hydrolysis of soluble and insoluble substrates should be conducted with great caution. As indicated by the articles cited above, this is especially true for collagenases and elastases due to the complex nature of their substrates, which

themselves may bind organic reagents and metal ions to specific sites and undergo conformational changes in tertiary structure.

The complex nature of such insoluble, structural proteins also complicates the detection and general characterization of enzymes capable of hydrolyzing them. For example, kinetic studies of this *Pseudomonas* enzyme are difficult until a simple, soluble substrate is discovered to parallel the Cbz-hexapeptide used with clostridiopeptidase or those of the *Pseudomonas* alkaline proteinase [30]. The inability of Waldvogel and Swartz [5] to detect collagenolytic activity from *P. aeruginosa* by the ability of growing cultures to clear an opaque collagen gel may also have been due to the complex nature of their substrate. These studies have indicated that *P. aeruginosa* collagenolytic enzyme has variable specific activity toward different types of collagen and that even with a single type of collagen substrate, the degree of hydrolysis can be greatly influenced by such factors as pH, type of buffer, ionic strength and presence of metal ions. Nevertheless, previous studies from our laboratory demonstrated that this purified enzyme caused massive destruction of collagen in vivo [31].

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