Pseudomonas protease. Purification, partial characterization, and its effect on collagen, proteoglycan, and rabbit corneas

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The extracellular protease of a virulent strain of Pseudomonas aeruginosa was purified by DEAE-cellulose chromatography in two steps. SDS-polyacrylamide gel electrophoresis of the purified enzyme revealed a single band, and the enzyme was shown to be the major component of the bacterial filtrate. The protease was fully inhibited by Na2 EDTA, 1,10-phenanthroline, L-cysteine and Zn2+ ions but was insensitive to diisopropylphosphofluoridate. The elastase substrates orcein-elastin and acetyl-L-alanyl-L-alanyl-L-alanine-methyl ester were degraded by the enzyme. The protease activity toward soluble and insoluble collagen was found to be limited to the telopeptide region of the collagen molecule. With soluble collagen, conversion of the β and γ chains into monomeric α chains was observed. About 60% of the total proteoglycans and 1.5% of the total collagen were solubilized from rabbit corneas following incubation with the enzyme, and the solubilized products were nondialyzable. It was concluded that the purified protease has little or no collagenolytic activity and that dissolution of the cornea by Pseudomonas protease infection results essentially from the degradation of the protein backbone of the corneal proteoglycans.

Key words: Pseudomonas aeruginosa, Pseudomonas protease, Pseudomonas elastase, corneal ulcers, corneal proteoglycans, corneal collagen.

Infections of the cornea with Pseudomonas aeruginosa are among the most destructive bacterial diseases of the human cornea. Typically, a severe central corneal ulcer develops, which is characterized by a rapid dissolution of the corneal stroma. The ulcerations frequently progress to perforations if not treated promptly and intensively.

This rapid corneal destruction has been attributed by Fisher and Allen2,3 to an extensive degradation of corneal collagen by an extracellular Pseudomonas collagenase. It has been recently shown by Brown, Bloomfield, and Tam4 that although the...
cornea-damaging enzyme produced by *P. aeruginosa* is proteolytic in nature, it does not exhibit collagenolytic activity. In addition, they demonstrated that the enzyme was capable of degrading proteoglycans in vitro. Kreger and Griffin have also shown that the enzyme does not have collagenolytic activity. More recently histochemical and ultrastructural studies of rabbit corneas infected with *P. aeruginosa* indicated that proteoglycans rather than collagen were lost from those corneas. The conclusion of the last three studies was that the rapidly progressing ulcers produced by *P. aeruginosa* are the result of an enzymatic degradation of the stromal proteoglycans. However, this conclusion was based on indirect evidence, and the exact nature of the biochemical changes exerted on the cornea by the *Pseudomonas* enzyme has not yet been well defined. In addition, the enzyme had not been purified completely, and only limited information was available with regard to its enzymatic properties.

This paper presents the purification and further characterization of the enzyme and its effects on collagen, proteoglycans, and the corneal stroma.

**Materials and methods**

**Bacterial strain and cultivation conditions.** A strain of *P. aeruginosa* isolated from a human corneal ulcer was used throughout this work. Stock cultures were maintained on nutrient agar slants (Micro-Media, Pittsburgh, Pa.) at 4° C. and retransferred every 3 months. Tryptic soy broth without dextrose (Difco Laboratories, Detroit, Mich.) was used for cultivation of bacteria. The bacteria were removed from the growth medium by centrifugation (Model RC-2-B, Ivan Sorvall, Inc., Newton, Conn.) at 16,000 × g for 20 minutes, followed by filtration through a Millipore filter (0.45 μ). Solid ammonium sulfate (Schwarz/Mann, Orangeburg, N. Y., ultra pure) and added to the clear supernatant to 90% saturation, and the precipitate formed after standing overnight at 4° C. was collected by centrifugation (16,000 × g, 40 minutes). The precipitate was dissolved in approximately 500 ml. of 0.01M Tris-HCl, pH 7.5, containing 0.5 mM CaCl₂ and dialyzed extensively against the same buffer. The dialyzed solution was lyophilized, and the dried powder (about 3 gm.) was stored at -20° C.

**Protease purification.** The crude powder (1.2 gm.) was dissolved in 60 ml. of Tris buffer (0.02M Tris-HCl, pH 7.5, containing 0.5 mM CaCl₂) and dialyzed against the same buffer. The dialyzed solution was applied to a diethylaminoethyl (DEAE)-cellulose (DE-52, Whatman, Maidstone, U. K.) column (5 by 27 cm.) equilibrated and eluted with Tris buffer at a flow rate of 90 to 95 ml./hr. Fractions of 12 ml. were collected. The active fractions (Fig. 1) were pooled, concentrated approximately 100-fold by ultrafiltration (Diaflo, UM 10 membrane), and dialyzed against Tris buffer. The concentrated pool was divided into two equal fractions. Each was rechromatographed separately on a second DEAE-cellulose column (3 by 27 cm.), equilibrated and eluted as before, but at a flow rate of 34 to 38 ml./hr. and with the collection of 4 to 6 ml. fractions. The active fractions (Fig. 2) were pooled, concentrated by ultrafiltration to an approximate protein concentration of 2.5 mg./ml., and dialyzed against Tris buffer. The dialyzed enzyme solution was stored in small vials at -20° C. All the above operations were performed at 4° C.

**Protease assay.** Protease activity was assayed with azocasein (Sigma Chemical Co., St. Louis, Mo.) used as the substrate. The reaction solution (1 ml.) consisted of 750 μl of azocasein solution (4 mg./ml. in 0.05M Tris-HCl, pH 7.5), 220 μl of 0.05M Tris-HCl, pH 7.5, and 30 μl of enzyme solution (0.005 to 0.02 unit). This solution was incubated at 37° C. for 10 minutes, and the reaction stopped by adding 0.5 ml. of 10% trichloroacetic acid. The precipitate formed after standing at room temperature for 30 minutes was removed by centrifugation (Sorvall RC2-B, 16000 × g, 30 minutes), and the absorbance of the supernatant at 400 nm. was measured with a Beckman DB-G spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.). The reaction rate was linear up to an OD increase of 0.25 unit. The above pH and temperature of the reaction were selected because they were found to be optimal conditions for maximum activity. One
unit of activity was defined as the amount of enzyme that produces an OD increase of 1 unit per minute under the assay conditions.

**Preparation of fluorescein-bound insoluble collagens.** Insoluble bovine tendon collagen was prepared by Dr. I. Freeman of this laboratory. Reaction of the collagen with fluorescein isothiocyanate (Eastman Kodak Co., Rochester, N. Y.) was performed according to the method of Steven, Torre-Blanco, and Hunter. The amount of fluorescein bound was derived from the absorbance at 495 nm. of a fluorescein-labeled collagen solution obtained by total digestion with papain, with the use of the molar extinction coefficient E₄₀₅ = 4.25 x 10⁴.

**Salt-soluble collagen preparation.** Salt-soluble collagen was purified from fetal calf skin (Pel-Freez Bio-Animals, Inc., Rogers, Ark.) by the method of Jackson and Cleary. The purified collagen was lyophilized and stored at -20° C. Its purity was confirmed by SDS polyacrylamide gel electrophoresis.

**Digestion of fluorescein-bound insoluble collagens with proteases.** Approximately 6 mg. of the insoluble collagen were weighed out and suspended in 0.45 ml. of 0.05M Tris buffer, pH 7.5, 5 mM in CaCl₂. Enzyme solution or buffer (50 μl) was added to each suspension and incubated at 37° C. with constant shaking for 5 to 23 hours. The reaction was stopped by centrifugation (Sorvall RC2-B, 16,000 x g, 30 minutes), and the absorbance of the supernatant at 495 nm. was measured to determine the amount of fluorescein liberated by the enzyme. Samples from each supernatant were also analyzed for hydroxyproline. The following enzyme solutions were used: *Pseudomonas* protease (2.5 mg./ml.), trypsin (Sigma Chemical Co.; 1 mg./ml.), pancreatic elastase (Sigma; 1 mg./ml.), and bacterial collagenase (approximately 1 mg./ml., purified and used according to the method of Peterkosky and Diegelman).

**Digestion of proteoglycan with the enzyme.** The proteoglycan subunit derived from bovine nasal septum (a gift from Dr. F. Kieras of the Rockefeller University, New York, N. Y.) was incubated with the enzyme at 37° C. for 64 hr. The final concentrations in the reaction solution (1.25 ml.) were 5.6 mg./ml. proteoglycan, 18 units/ml. protease, 0.05M Tris-HCl, pH 7.5, and 0.0008% sodium azide. The reaction was stopped by the addition of 0.1M ethylenediamine tetracetic acid (EDTA) to a final concentration of 1 mM. The reaction solutions, as well as control solutions which were incubated in absence of either enzyme or proteoglycan, were analyzed for sugar reducing ends and for free amino groups.

**Digestion of rabbit corneas with *Pseudomonas* protease.** The epithelium, Descemet's membrane, and endothelium of 80 rabbit corneas (Pel-Freez Bio-Animals, Inc.) were removed by scraping. The stromas obtained were washed with 0.05M Tris-HCl, pH 7.5, divided into two equal groups, and cut into small pieces (about 2 by 2 mm.). These pieces of tissue were further ground by a stainless steel mill with liquid nitrogen, and the powder obtained from each group was suspended in 7 ml. of the above buffer supplemented with penicillin and streptomycin sulfate (0.063 and 0.1 mg./ml., respectively). Purified protease (150 μl containing 450 μg of the enzyme) was added to one of the suspensions, and Tris-buffer (150 μl) was added to the other suspension for control. Both were incubated at 20° ± 1° C. with constant shaking for 15 hours. A second aliquot of either enzyme solution or buffer (100 μl) was then added as before, and incubation continued for an additional 5 hours. The suspensions were then centrifuged (Sorvall RC2-B, 12,000 x g, 30 minutes), and the precipitated tissue was washed once with 0.05M Tris-HCl and incubated at 70° C. for 5 hours to produce a homogeneous suspension, from which aliquots were taken for further analyses.

**Tests for elastolytic activity.** The enzyme (2 units/ml.) was incubated with orcein-elasticin (Sigma, 10 mg./ml.) in 0.05M Tris-HCl, pH 8, at 37° C. with constant shaking for 16 hours. The suspension was centrifuged, and absorbance of the supernatant at 950 nm. was measured. The synthetic substrate acetyl-L-alanyl-L-alanyl-L-alanine-methyl ester (Ac-Alaa-OMe) (Sigma; 5 mM in 0.05M Tris-HCl, pH 7.5) was incubated with the enzyme (0.1 units/ml.) at 37° C. for 3 hours. The reaction solution was analyzed by thin-layer chromatography.

**Viscometry.** Viscosity measurements were carried out at 25° C. in a Cannon-Ubbelohde semimicro type viscometer, with a water flow time of 68 seconds. Reactions were started by adding 50 μl of enzyme solution (1 mg./ml. of either *Pseudomonas* protease, trypsin, pancreatic elastase, or bacterial collagenase) to 950 μl of the collagen solution (0.8 mg./ml. in 0.05M Tris-HCl, pH 7.5, 0.5M NaCl, and 5 mM CaCl₂) and were followed for about 3 hours. Sodium dodecylsulfate (SDS)—polyacrylamide gel electrophoresis. Bio-phore gels (7.5%, Bio-Rad Laboratories, Richmond, Calif.) were pre-equilibrated and used with Bio-Rad Tris-acetate-SDS buffer, pH 6.4, as recommended by the...
Table I. Purification of Pseudomonas protease

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Activity (units/ml)</th>
<th>Total activity (units)</th>
<th>Recovery (%)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (units/mg)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude: 90% (NH₄)₂SO₄</td>
<td>60</td>
<td>71.5</td>
<td>4,290</td>
<td>100</td>
<td>4.1</td>
<td>17.5</td>
<td>1.0</td>
</tr>
<tr>
<td>DEAE-cellulose*</td>
<td>13.8</td>
<td>124.5</td>
<td>1,718</td>
<td>40</td>
<td>4.8</td>
<td>26.2</td>
<td>1.5</td>
</tr>
<tr>
<td>DEAE-cellulose*,†</td>
<td>14.6</td>
<td>81</td>
<td>1,180</td>
<td>28</td>
<td>2.4</td>
<td>33.8</td>
<td>1.9</td>
</tr>
</tbody>
</table>

The table summarizes data obtained by processing 1.2 gm. of the crude enzyme, corresponding to 2.5 liters of the original bacterial filtrate.

*Values obtained after concentration by ultrafiltration.
†The enzyme solution obtained after the first DEAE-cellulose step was divided into two equal fractions which were further processed separately. Volume, total activity, and recovery are the sum, but all the other data are the averages of the values obtained in the two runs.

Results

Purification of the protease. The crude protease preparation, obtained by ammonium sulfate precipitation of the growth medium, was purified by DEAE-cellulose chromatography (Fig. 1), followed by a rechromatography step using similar conditions as before.

Thin-layer chromatography (TLC). TLC on cellulose plates (Eastman Kodak Co.) was performed with a mixture of n-butanol-acetic acid-water (4:1:1, v/v) used as the solvent. Spots were detected by spraying the plates with 0.5% ninhydrin in acetone-water-pyridine (100:5:0.4, v/v) and heating them to 70° C. for 15 minutes.

Analytical methods. Hydroxyproline was measured after hydrolysis in 6N HCl at 100° C. for 7 hours, with the Elson-Morgan reaction as modified by Boas. Sugar-reducing ends were assayed by the method of Park and Johnson. Amino terminal ends were determined by the ninhydrin method of Rosen, and protein concentrations were determined according to Lowry et al., with bovine serum albumin as the standard.
Fig. 2. DEAE-cellulose rechromatography of the pooled active fractions from the first DEAE-cellulose step (see Fig. 1). The column (3 by 26.5 cm.) was equilibrated and eluted with 0.02M Tris-HCl, pH 7.5, containing 0.5 mM CaCl₂ at a flow rate of 38 ml./hr. Five milliliters of the same buffer containing 700 units of enzyme were applied, and 5.5 ml. fractions were collected.

...conditions (Fig. 2). Under the conditions stated, the enzyme was retarded but not adsorbed to the column (Fig. 1). The purified enzyme was obtained at 28% yield and with a purification factor as low as 2 (Table I). The progress of purification, as followed by SDS-polyacrylamide gel electrophoresis is shown in Fig. 3, from which it is evident that the enzyme is the major protein component of the bacterial filtrate. It is also seen that the purified preparation consists of a single band (Fig. 3, C), suggesting homogeneity of the preparation.

**Effect of metallic cations and enzyme inhibitors on the protease activity.** The enzyme (0.02 units in 0.25 ml. of 0.05M Tris-HCl, pH 7.5) was preincubated with each of the compounds tested (Table II) at room temperature for 30 minutes. Enzymatic reactions were initiated (after the solutions were heated to 37° C.) by adding 0.75 ml. of azocasein solution (pre-equilibrated to 37° C.) and were completed and analyzed as detailed in Methods. It was found (Table II) that Ca²⁺ and Mg²⁺ at 0.625 mM had no effect on the enzyme activity. Mn²⁺, Co²⁺, and Fe³⁺ at the same concentration partially inhibited the enzyme (causing 30%, 50%, and 75% inhibition, respectively) whereas Cu²⁺ and Zn²⁺ fully inhibited the enzyme. Na₂ EDTA (10 mM), 1,10-orthophenanthroline (1 mM), and L-cysteine (1.25 mM) completely inhibited the enzyme, but diisopropylphosphofluoridate (DFP) (20 mM) had no effect on activity.

**Elastolytic activity of the protease.** The absorbance at 590 nm of the supernatant of an orcein-elastin suspension increased 15-fold following incubation with the protease, indicating enzymatic hydrolysis of elastin. The synthetic elastase substrate Ac-Ala₃-OMe was also hydrolyzed by the protease. TLC of the reaction solution revealed that only the first peptide bond was cleaved, yielding acetyl-L-alanine and L-alanyl-L-alanine-methyl ester.

**Effect of Pseudomonas protease on soluble collagen.** The effect of *Pseudomonas* protease on soluble collagen was examined by following the change in viscosity of a collagen solution upon incubation with the
Pseudomonas protease

Table II. Effect of metallic cations and common enzymatic inhibitors on the activity of Pseudomonas protease

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration* (mM)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>0.625</td>
<td>100</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.625</td>
<td>103</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>0.625</td>
<td>73</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>0.625</td>
<td>53</td>
</tr>
<tr>
<td>FeCl₂</td>
<td>0.625</td>
<td>24</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>0.625</td>
<td>2</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0.625</td>
<td>0</td>
</tr>
<tr>
<td>Na₂ EDTA</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>1,10-orthophenanthroline</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>1.25</td>
<td>0</td>
</tr>
<tr>
<td>DFP</td>
<td>20</td>
<td>100</td>
</tr>
</tbody>
</table>

*Concentration in the reaction solution. For all experiments the enzyme was preincubated (30 minutes at room temperature) with the compound tested at a 4-fold concentration.

more intense. Only limited degradation of the α chains took place.

Effect of Pseudomonas protease on insoluble collagen. The effect of Pseudomonas protease on insoluble collagen was compared to that of trypsin, elastase, and bacterial collagenase. Fluorescein bound insoluble bovine tendon collagen was incubated with each of the enzymes for 5, 8, and 23 hours, and the amounts of fluorescein and hydroxyproline solubilized were determined. The values obtained after 8 and 23 hours were practically the same, and those obtained after 23 hours of incubation are presented in Table III. The Pseudomonas protease, like trypsin, solubilized approximately two out of the five fluorescein residues bound to the molecule, but only one residue was solubilized by elastase. As expected, all of the five fluorescein residues were solubilized by the bacterial collagenase. Similarly, limited solubilization of hydroxyproline (which did not exceed 25% of the total hydroxyproline content) was observed with the proteases, and all of the hydroxyproline was solubilized by the collagenase.

Effect of Pseudomonas protease on proteoglycans. No increase in the level of sugar reducing ends was detected after incubation of the proteoglycan subunit with the purified protease. On the other
**Fig. 4.** Effect of *Pseudomonas* protease on the viscosity of a collagen solution at 25°C, as compared to that of other proteases. Reactions were performed in 0.05M Tris-HCl, pH 7.5, containing 0.5M NaCl and 5 mM CaCl₂. Collagen concentration was 0.8 mg./ml., and all enzymes were used at 50 µg/ml.

hand, 20-fold increase in the ninhydrin color yield was found, indicating an extensive breakdown of the polypeptide portion of the proteoglycan molecule.

**Effect of *Pseudomonas* protease on rabbit corneas.** The ability of the *Pseudomonas* protease to solubilize collagen and proteoglycans from the cornea was examined by incubating small slices of rabbit corneas with the enzyme at 20°C, and analyzing the supernatant for hydroxyproline, glucuronic acid, and hexosamines (markers for collagen and proteoglycans). As seen in Table IV, only 1.5% of the total collagen was released in the presence of the enzyme, whereas approximately 60% of the total proteoglycans were solubilized under the same conditions. Of the solubilized markers, 80% to 90% were nondialyzable, indicating a relatively high molecular weight for the reaction products. Similar results were obtained when the incubation was performed at 37°C, except that the amount of solubilized hydroxyproline found in the high molecular weight fraction of the supernatant was reduced to 10%.

**Discussion**

The properties of an extracellular protease isolated from a virulent strain of *P. aeruginosa* were investigated in this work.

In contrast to earlier studies reporting the production of more than one protease by a single strain of *Pseudomonas*, we were not able to detect more than one proteolytic species in the bacterial filtrate. As judged from the electrophoretic patterns of the crude filtrate and of the puri-
fied enzyme (Fig. 3), the protease seems to be the major component of the filtrate. The relatively high contents of the enzyme in the bacterial filtrate suggests the high destructive potential of the strain studied.

Since some strains of *P. aeruginosa* were reported to produce proteases with elastolytic activity, we examined the effect of our preparation on orcein-elastin and on the synthetic substrate Ac-Ala<sub>3</sub>-OMe which is considered as a specific substrate of pancreatic elastase. Both substrates were degraded by the enzyme, indicating that it has elastolytic activity. Pancreatic elastase cleaves the ester bond of the compound Ac-Ala<sub>3</sub>-OMe, whereas the *Pseudomonas* enzyme acts on the first peptide bond of this compound. Although the two enzymes differ in their action on this particular substrate, they resemble each other in their ability to cleave bonds involving alanine residues. Pancreatic elastase has been shown to preferentially attack peptide bonds involving the carboxylic group of alanine in both, insulin B chain, and the S peptide of RNase, and the *Pseudomonas* enzyme was found by us to hydrolyze N-blocked tri-alanine peptides.

An extracellular elastase from *Pseudomonas aeruginosa* has been described before by Morihara et al. Our enzyme is similar to their elastase in being fully inhibited by the chelating agents, Na<sub>2</sub>EDTA and 1,10-orthophenanthroline. In addition, both are inhibited by Zn<sup>2+</sup> ions and are DFP resistant. The two enzymes are however not identical, since they differ in their molecular weights and in their sensitivity to cysteine. The protease described in this work is completely inhibited by 1.25 mM of cysteine, but the elastase retains 70% of its activity at this concentration of cysteine, and the molecular weight of our enzyme is 33,000, as opposed to the value of 39,000 reported for the elastase.

The reduction in viscosity of a collagen solution induced by *Pseudomonas* protease was somewhat higher than that observed with trypsin and elastase but did not exceed 30% (Fig. 4). This reduction in viscosity is within the range reported for other proteases (40% reduction in viscosity was observed with pronase) and is smaller than that caused by both mammalian (50% to 60%) and clostridial collagenase (90% to 100%). Such a limited decrease in viscosity of a collagen solution is typically observed with the class of proteases which is referred to as “general proteases” and which includes enzymes such as trypsin, elastase, and chymotrypsin. Their activity toward collagen is limited to the nonhelical, telopeptide region of the collagen molecule, in which most of the intermolecular and intramolecular cross-links of the molecule occur. Treatment of collagen with enzymes of this type results

### Table III. Effect of Pseudomonas protease on fluorescein-bound insoluble bovine tendon collagen as compared to that of some other proteases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fluorescein solubilized (mol./mol. collagen)&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Hydroxyproline solubilized (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas protease</td>
<td>2.2</td>
<td>26</td>
</tr>
<tr>
<td>Trypsin</td>
<td>1.6</td>
<td>14</td>
</tr>
<tr>
<td>Elastase</td>
<td>0.9</td>
<td>ND</td>
</tr>
<tr>
<td>Collagenase</td>
<td>4.8</td>
<td>108</td>
</tr>
<tr>
<td>Clostridium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papain†</td>
<td>5.3</td>
<td>100</td>
</tr>
</tbody>
</table>

ND = not determined.

*<sup>*</sup>Mol. wt. 270,000 was assumed for collagen and Eₜₒₓ = 4.25 × 10<sup>4</sup> (ref. 8) was used for calculating the fluorescein contents. Results are the average of 3-6 experiments.

†The papain digest was performed at 60° C, yielding the total contents of fluorescein in the collagen molecule.

### Table IV. Solubilization of rabbit corneal collagen (as hydroxyproline) and proteoglycans (as glucuronic acid and hexosamines) by *Pseudomonas* protease

<table>
<thead>
<tr>
<th></th>
<th>Amount solubilized (%)</th>
<th>% in high mol. wt.* fraction of solubilized material</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C †</td>
<td>E †</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>0.7</td>
<td>1.5</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>23.9</td>
<td>55.2</td>
</tr>
<tr>
<td>Hexosamines</td>
<td>23.2</td>
<td>61.8</td>
</tr>
</tbody>
</table>

<sup>*</sup>Nondialyzable.

†C = control; E = enzyme digest.
in the conversion of the \( \gamma \) and \( \beta \) species (trimers and dimers) of collagen to the \( \alpha \) chains (monomers).\(^{24}\) Electrophoretic analysis of the reaction solution of soluble collagen and the *Pseudomonas* protease (Fig. 5) revealed the conversion of the \( \gamma \) and \( \beta \) species into the \( \alpha \) (or altered \( \alpha \)) chains, indicating that *Pseudomonas* protease acts on soluble collagen in the same mode as the general proteases.

Fluorescein-labeled insoluble collagen was used by Steven, Torre-Bianco, and Hunter\(^7\) as a substrate to distinguish between protease and collagenase activity. This method is particularly sensitive because of the typical fluorescence and absorbance of the fluorescein residues and their distribution in the molecule (about 40\% of the label is found in the telopeptide region\(^7\)). Using this method, we have demonstrated that about 40\% of the total fluorescein bound to the collagen molecule was solubilized by *Pseudomonas* protease and by trypsin and that approximately 20\% was released by elastase. All of the bound fluorescein was solubilized by the bacterial collagenase (Table III). This indicates that the action of the *Pseudomonas* protease on insoluble collagen, like that of trypsin and elastase, is limited to the non-helical parts of the collagen molecule. The amount of hydroxyproline solubilized by the *Pseudomonas* protease was higher than that released by trypsin and by elastase (Table III), indicating that this protease reaches bonds further toward the helical region of the collagen molecule than the other proteases. However, the extent of hydroxyproline solubilization by the enzyme did not exceed 25\% of the total hydroxyproline content. It is therefore concluded that although the *Pseudomonas* enzyme may cleave bonds located closer to the helical portion of the collagen molecule than either trypsin or elastase, it is not a specific collagenase.

The potential of the *Pseudomonas* protease to solubilize collagen and proteoglycans from the cornea was studied at 20\° C. in order to prevent denaturation of collagen. Under these conditions, approximately 60\% of the total proteoglycans and only 1.5\% of the total collagen was solubilized (Table IV). The major fraction of the hydroxyproline solubilized from the cornea under these conditions was of relatively high molecular weight. This indicates that the helical portion of the solubilized collagen was not attacked by the enzyme, which is in good agreement with the results obtained with pure collagen. The majority of the solubilized glucuronic acid and hexosamines was also found to be of high molecular weight, indicating that the polysaccharide chains of the solubilized proteoglycans remained intact, which is compatible with the results observed using the pure proteoglycan as the substrate.

The amounts of corneal collagen and proteoglycan solubilized by the enzyme at 37\° C. were practically the same as at 20\° C. It is therefore believed that similar changes are exerted by the enzyme on corneas in vivo.

The negligible amounts of collagen solubilized from the cornea by the *Pseudomonas* protease, as opposed to the large extent of proteoglycan solubilization, provide direct evidence that the corneal melting by *Pseudomonas* protease results from an extensive breakdown of corneal proteoglycans and not from direct attack upon corneal collagen fibrils.

We wish to thank Dr. Robert Church for helpful discussions during the course of this work.

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