# SPECIFICITY OF COLLAGENASE FROM *ACHROMOBACTER IOPHAGUS*

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## 1. Introduction

Many enzymes which have been originally described as collagenases, were shown later to be either proteases or peptidases of broad or different specificity. A true collagenase degrades in the helical regions of the native collagen preferentially the bond Y–Gly. The observations that an enzyme degrades synthetic peptide of a composition similar to sequences existing in collagen or that it degrades collagen are not sufficient evidence that it is a collagenase.

A new collagenolytic enzyme, synthesized by a non-pathogenic, aerobic strain of *Achromobacter iophagus* has been first described by Woods and Welton [1,2]. Recently this enzyme has been obtained in the homogeneous state on a preparative scale in our laboratory [3,4]. Its enzymatic activity using both collagen and synthetic substrates is many times higher than the purest samples of the collagenases from *Clostridium histolyticum*.

Both theoretical and practical interest in this highly active, non-toxic enzyme prompted us to examine its specificity, namely in comparison with the widely studied *Clostridium* collagenase. According to the generally accepted scheme, the *Clostridium* enzyme cleaves in the helical regions of native collagen predominantly the bond Y–Gly in sequences of the type –Pro–Y–Gly–Pro, where Y is most frequently a neutral amino acid [5,6]. The enzyme cleaves readily this kind of bond also in many synthetic peptidic substrates [7,8]. The interpretation of the results is often handicapped by the complex nature of the collagenase samples used for the studies: chromatographically purified commercial collagenases are heterogeneous, and further purifications undertaken by different authors yielded varying number of subfractions of different degrees of purity [9–14].

In our study we did not want to try to resolve the problem of the heterogeneity of the *Clostridium* enzyme; it served us mainly as reference and therefore we used the purified commercial enzyme; on the other hand the *Achromobacter* collagenase was homogeneous. The mode of action of the two collagenases, *Achromobacter* and *Clostridium* on a synthetic substrate and on native collagen have been determined by amino acid analysis, viscosimetry, electrophoresis, ORD measurements and Edman degradation.

## 2. Experimental

### 2.1. *Material and methods*

Crude collagenase from *Achromobacter iophagus* was a gift from Institut Pasteur Production. Homogeneous *Achromobacter* collagenase of spec. act. 1.58 μkat per mg on the synthetic substrate has been obtained by chromatography on DE-32 cellulose and Sephadex G-100 as described [3,4]. Collagenase from *Clostridium histolyticum* type III fraction A, lot CO 255 was a product from Sigma. 4-Phenylazo-Cbz–Pro–Leu–Gly–Pro–D–Arg dihydrate was purchased from Fluka. Calif., skin collagen (acid-soluble, C-1633) from Sigma.

### 2.2. *Cleavage of the synthetic substrate*

0.4 mg of 4-phenylazo-Cbz–Pro–Leu–Gly–Pro–D–Arg dihydrate [15] was dissolved in 0.4 ml of acetate-veronal buffer and after addition of 0.3 μg of *Achromobacter* collagenase in 100 μl of 0.3 M Tris–HCl, 0.01 M $CaCl_2$, 1 M NaCl, (pH 7) the mixture was incubated 15 min at 37°C. The aqueous phase was

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extracted by 5 ml of ethylacetate and the two phases were evaporated to dryness. After acid hydrolysis (6 N HCl 105°C for 20 hr) the amino acid content was determined on a Beckmann Multichrome amino acid analyzer.

2.3. *Viscosimetry*

Stock solution of calf-skin collagen (3 mg in 1 ml of 0.4 M NaCl) was diluted to 0.15% with 0.05 M Tris buffer made 0.002 M in $CaCl_2$, pH 7.4. Ostwald-type, low sheer capillary viscosimeters had an average elution time for water between 16 and 25 sec at 25°C, specific viscosity of the diluted collagen solution was 4.4. The rate of decrease of specific viscosity of the digestion mixtures (1.9 ml of collagen and 0.1 ml of enzyme solution) at 25°C as followed within 90 min.

2.4. *Disc electrophoresis*

Electrophoreses were performed on a Buchler apparatus with 12 cm long tubes [16]. 75 μg of digestion mixtures were applied. The conditions of the digestion were the same as for the viscosimetric assay. The optical density of the separated bands stained by Coomassie blue was scanned on a Vernon type PH 15 densitometer.

2.5. *Optical rotatory dispersion*

Native collagen exhibits a large negative Cotton effect with a minimum around 210 nm [17]. The magnitude of the negative extremum is strongly diminished by heat denaturation or by enzymatic degradation (fig.3). The assay used in this study was based on recording the disappearance of the negative rotation of digestion mixtures at 215 nm.

To a solution of 3 mg of collagen in 3 ml of 0.1 M Tris buffer pH 7.2 made 1 mM in $CaCl_2$ and 0.4 M in NaCl was added 10–50 μl of the enzyme solution and the decrease of negative rotation at 215 nm was followed in a recording polarimeter SICA (France) during 60 min 25°C; full scale of the recorder corresponded to 0.5°.

2.6. *Edman degradation*

12 mg of collagen in 4 ml of 0.4 M $(NH_4)_2CO_3$ buffer pH 7.8 was digested after the addition of 120 μg of *Clostridium* collagenase or 12 μg of *Achromobacter* collagenase at 25°C for 60 min. The digestion was stopped by short heating at 100°C and the digestion mixture was subjected to a manual Edman degradation [18] using DMAA or Quadrol as buffers. The phenylthiohydantoins were evaluated by thin layer and gas chromatography.

## 3. Results and discussion

The comparison of the specificity of the *Achromobacter* and *Clostridium* collagenase by viscosimetric and electrophoretic techniques gave us only limited information. Fig.1 shows that the drop of viscosity of collagen solutions is not typical for the action of collagenases; proteinases as trypsin or pronase degrading the collagen in non-helical regions can give a similar effect. Although the electrophoretic pattern of the products obtained from collagen by the action of the two enzymes differs quantitatively (fig.2), its complexity does not allow any clear conclusion.

On the other hand ORD measurements of the negative Cotton effect (fig.3) gave a direct evidence that both collagenases cleave preferentially the helical re-

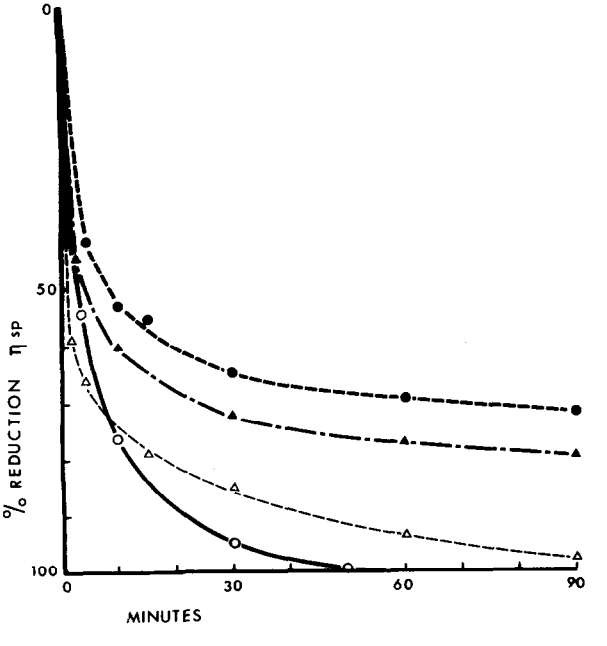


Fig.1. Effect of various enzymes on viscosity, (○—○) Collagenase from *Achromobacter iophagus*, collagen/enzyme ratio 1000:1; (△– –△) Collagenase from *Clostridium histolyticum*, collagen/enzyme ratio 100:1; (●– –●) Pronase, collagen/enzyme ratio 10:1; (▲– –▲) β Trypsin, collagen/enzyme ratio 10:1.

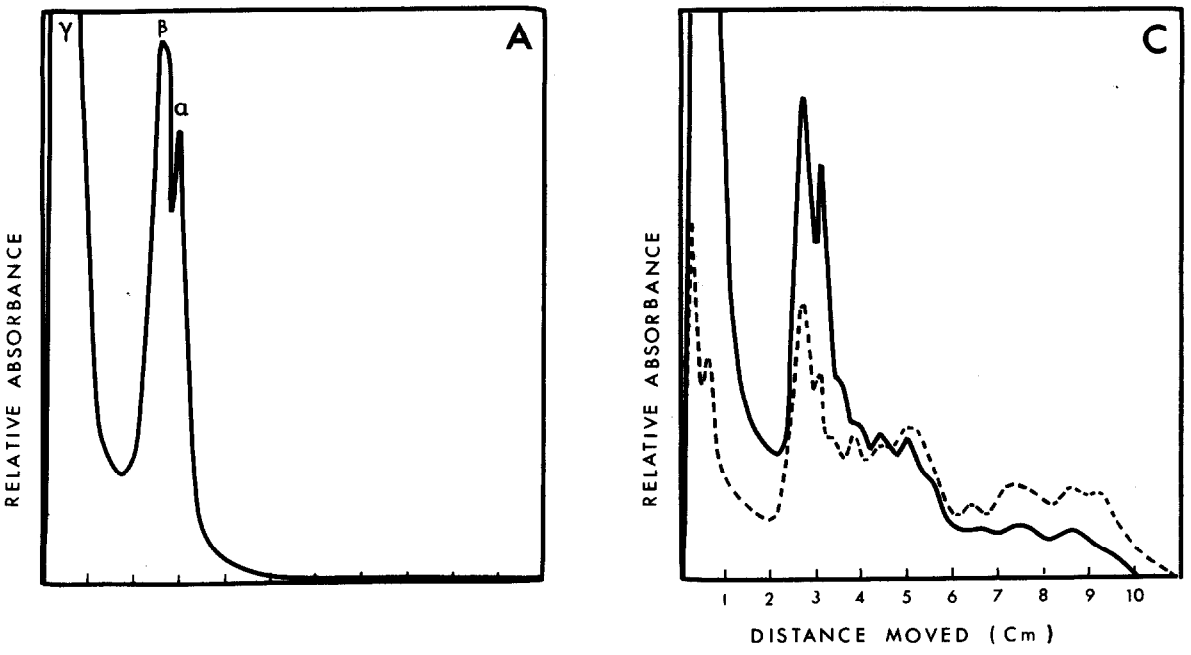


Fig.2. Polyacrylamide SDS gel electrophoresis of collagen treated with collagenases from *Clostridium histolyticum* or *Achromobacter iophagus*. (A) control digestion without enzyme; (B) digestion by *Clostridium histolyticum* för 5 min (——) and 20 min (– – – –); (C) digestion by *Achromobacter iophagus* for 5 min (——) and 20 min (– – – –).

gions in native collagen. The two other proteases compared, β-trypsin and pronase, even in relatively high concentrations which caused a considerable decrease of viscosity (fig.1), did not change the negative Cotton effect of collagen solutions at all. This technique proves to be a very selective assay for collagenase action; it has been applied successfully in another study in our laboratory [4].

Edman degradation of the products of collagen breakdown has shown that both enzymes cleave exclusively the bond to which glycine contributes with its amino group. However, the substrate affinity of the two enzymes differs as regards the amino acid residue on the carboxyl side of glycine (table 1). *Achromobacter* collagenase cleaves faster the bond Y–Gly–Ala than Y–Gly–Pro. The contrary is true for *Clostridium* collagenase. These results become more significant, if we compare the relative occurrence of these sequences the primary structure of the collagen used as substrate: only 60 Y–Gly–Ala as compared with 116 Y–Gly–Pro in the same chain. This seems to indicate that *Achromobacter* collagenase has a clear preferential affinity for the sequence Y–Gly–Ala.

Comparative study on synthetic peptides should bring additional information in this respect. In the present study we have compared the action of the two

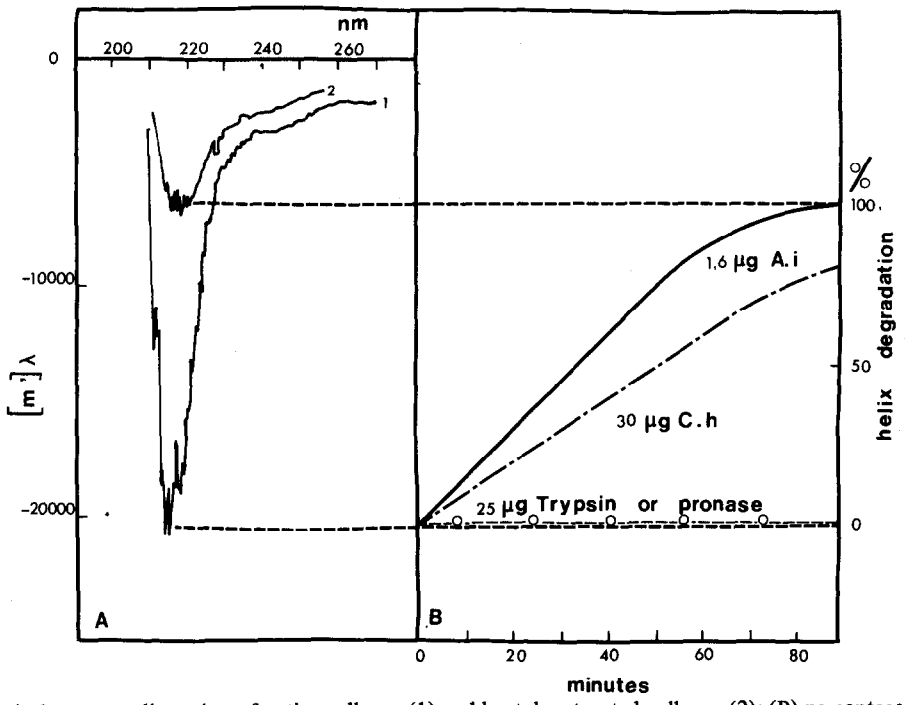


Fig.3. (A) optical rotatory dispersion of native collagen (1) and heat denaturated collagen (2); (B) percentage of loss of the negative Cotton effect at 215 nm. Collagen treated with *Achromobacter iophagus* (A.i.) (——), *Clostridium histolyticum* (C.h.) (– – –) and trypsin or pronase (— — — —).

collagenases only on one synthetic peptide, which has been used as substrate for the routine assay of *Clostridium* collagenase, 4-phenylazo-Cbz–Pro–Leu–Gly–Pro–D–Arg [15]. After the separation and hydrolysis of the two products of cleavage obtained by *Achromobacter* collagenase, the amino acid analysis gave only proline and leucine (1:1) in the organic phase, and glycine, proline and arginine (1:1:1) in the aqueous phase. *Achromobacter* collagenase splits therefore in this peptide the same bond Leu–Gly as does the *Clostridium* enzyme; this substrate can therefore be used for the quantitative assay of *Achromobacter* collagenase without modification.

| | | % PTH found after each step | |
|---|---|---|---|
| | PTH. | Achromobacter iophagus | Clostridium histolyticum |
| Step 1 | Gly | 100 | 100 |
| Step 2 | Pro | 25-40 | 55-65 |
| | Ala | 40-60 | 25-35 |
| | Leu | 15 | 4-6 |
| | Phe | 5 | |

Table 1 – Edman degradation of collagenase from *Achromobacter iophagus* (A.i.) and *Clostridium histolyticum* (C.h.) digest. Collagen/A.i. ratio 1000:1 and collagen/C.h. ratio 100:1.

Even in the highly purified *Achromobacter* collagenase we found traces of caseinolytic activity [3]. The evidence that this activity corresponds to the specificity of collagenase will be published elsewhere.

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