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# COLLAGENASE PRODUCTION BY *ACHROMOBACTER IOPHAGUS*

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## Summary

*Achromobacter iophagus* produced collagenase (EC 3.4.24.3) when cultured aerobically in buffer containing 5% peptone. The bacterium is non-pathogenic and tests on rabbits indicated that the culture medium was atoxic. The collagenase, which hydrolyzed insoluble and soluble native collagen, was purified by $(NH_4)_2SO_4$ precipitation, starch block electrophoresis, and gel filtration. It was shown to be serologically distinct from *Clostridium histolyticum* collagenase and to have molecular weight and sedimentation coefficient values of approx. 112 000 and 5.3 S, respectively.

## Introduction

Collagenases (EC 3.4.24.3) are well established as laboratory tools for cell dispersion and for structural studies of collagen and collagen-like proteins. Moreover, they are important clinically, for they are effective in the treatment of various pathological conditions involving collagen-rich structures [1]. *Clostridium histolyticum* has been the best source of collagenase to date but, because of the lethal clostridial toxin, careful purification of the enzyme is necessary before it may be used clinically [2]. Furthermore, *Cl. histolyticum* produces collagenase only under anaerobic conditions [3] and requires a complex medium for good yields [4]. We now describe the isolation and partial characterization of a collagenase produced by *Achromobacter iophagus*, an aerobic non-pathogenic bacterium which seems highly suited to large scale production of collagenase for both experimental and clinical usage.

## Methods

### *Production and purification of collagenase*

The collagenase-producing strain of *A. iophagus* was isolated from cured hides and routinely maintained on a complex agar medium [5]. Sterile 250-ml

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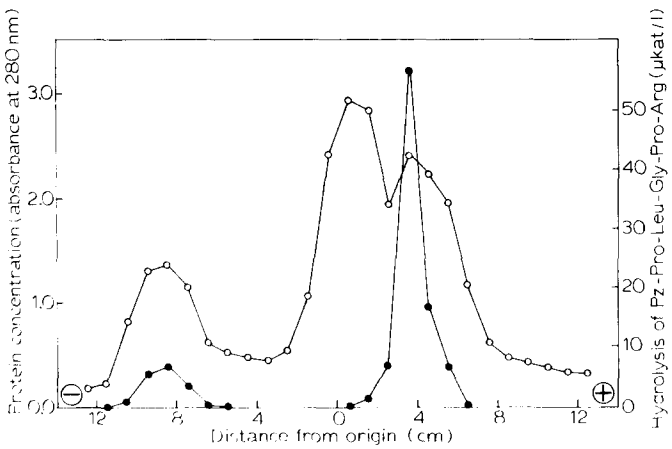


Fig. 1. Starch block zone electrophoresis of crude *A. iophagus* collagenase. A 40.0 × 2.5 × 1.0 cm block of washed potato starch was buffered with 32 mM barbital sodium/29 mM sodium acetate/2 mM $CaCl_2$ adjusted to pH 9.0 with normal HCl. Two hundred mg of crude collagenase were electrophoresed at 300 V and 6–8 mA for 24 h at 4°C, after which each 1.0 cm segment of the block was eluted with 5.0 ml of 0.1 M Tris · HCl/0.4 M NaCl/2 mM $CaCl_2$ (pH 7.6). o———o, protein concentration (absorbance at 280 nm); •———•, hydrolysis of Pz-Pro-Leu-Gly-Pro-Arg (μkat/l). Only those fractions indicated contained activity aginast Pz-Pro-Leu-Gly-Pro-Arg.

volumes of 0.1 M Tris · HCl/0.4 M NaCl/10 mM $CaCl_2$ (pH 7.6) supplemented with 5% (w/v) peptone were inoculated liberally from an overnight culture of the baterium. They were incubated at 30°C for 84 h with aeration by means of a vigorous stream of filtered air. After removing the cells by centrifugation at 27 000 × *g* for 10 min, the supernatant was brought to 60% satn with $(NH_4)_2SO_4$ and kept at 4°C for 24 h. The precipitate containing the collagenase was collected by centrifugation at 12 000 × *g* for 10 min, resuspended in and dialyzed against distilled water, and lyophilized.

Fractionation of the crude collagenase by starch block electrophoresis [6] resulted in the separation of two collagenase fractions (Fig. 1). Most of the collagenase migrated towards the anode, but a small portion of activity moved towards the cathode. The material recovered from each collagenase peak was pooled, dialyzed against distilled water, and lyophilized. Despite their opposite mobilities on the starch block, the collagenases from the two fractions behaved identically in gel filtration, polyacrylamide-gel disc electrophoresis, and serological comparisons by the Ouchterlony plate method; moreover, they had similar activities on phenylazobenzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-Lprolyl-D-arginine (Pz-Pro-Leu-Gly-Pro-Arg), insoluble collagen, and soluble collagen. Therefore, evidence for a multiplicity of forms of the enzyme was insufficient and the minor cathodically migrating collagenase was disregarded for the purposes of this paper. All further reference herein to *A. iophagus* collagenase indicates the enzyme which migrates towards the anode during the starch electrophoresis.

The collagenase preparation obtained by starch electrophoresis was purified by gel filtration through Sephadex G-200. Fractions with constant specific activity were pooled, dialyzed against distilled water, and lyophilized.

*Collagenase assays*

Three methods were used to assay collagenase activity:

*Hydrolysis of Pz-Pro-Leu-Gly-Pro-Arg (Fluka).* The method of Wünsch and Heidrich [7] was used, with activity being expressed in terms of katals or suitable multiples thereof (11.11 pkat = 1 unit according to Wünsch and Heidrich). Specific activity determinations were made using this substrate.

*Hydrolysis of insoluble collagen.* Bovine Achilles' tendon collagen (Boehringer) was used as the substrate. Hydrolysis was measured by following the release of ninhydrin-positive materials, expressed as μmol glycine equivalents per ml, using the procedure of Adamcic and Clark [8].

*Hydrolysis of soluble collagen.* Acid-soluble collagen was prepared by the method of Cooper and Davidson [9]. It was dissolved in 0.1 M Tris · HCl, 0.5 M $CaCl_2$ (pH 7.6) to a concentration of approx. 1 mg/ml and clarified by centrifuging at 105 000 × *g* for 1 h. Digestion of the soluble collagen was measured by determining the decrease in specific viscosity of the solution.

The native state of both the insoluble and the soluble collagen substrates was checked by treatment with pronase (Miles-Seravac).

*Protein determination*

Protein was measured by the method of Lowry et al. [10] with bovine albumin as standard. Protein elution profiles of the starch block and the gel columns were monitored at 280 and 230 nm, respectively.

*Analytical polyacrylamide-gel electrophoresis*

The method of Davis [11] was used with 5-mm diameter gels consisting of separation and spacer gels containing, respectively 10% (w/v) and 3% (w/v) of Cyanogum (British Drug Houses). Electrophoresis was performed at 100 V and 2 mA/gel for 10 min, followed by 200 V and 2—3 mA/gel for approx. 90 min. Preparation of samples and staining of gels with Amido Black was according to Maizel [12].

*Hydrolysis of casein*

The collagenase preparations were tested for caseinolytic activity by the method of Rippon [13]. Activity was expressed as mg protein released per mg of enzyme preparation per h.

*Serological comparison of collagenases*

Pure *A. iophagus* collagenase was treated with 0.2% formalin and used to immunize a male rabbit as follows: (1) 2 mg injected intravenously; (2) 1 week later, intramuscular injection of 4 mg in emulsion prepared with Freund's incomplete adjuvant; (3) 4 weeks later, 1 mg injected intravenously. The rabbit was bled 4 days after the final injection. The antiserum was tested for reactivity with *A. iophagus* collagenase and for cross-reactivity with *Cl. histolyticum* collagenase forms I and II (Koch-Light) using the plate method of Ouchterlony [14].

*Molecular weight estimation*

Two methods were used. The first involved electrophoresis in sodium

dodecylsulphate-polyacrylamide gel (4%) according to Maizel [12], using chymotrypsinogen A (25 700), ovalbumin (43 000), bovine albumin (68 000) and β-galactosidase (135 000) as markers. The second method was that of Andrews [15], involving gel filtration through columns of Sephadex G-200 (Pharmacia) measuring 2.5 cm × 94.0 cm and calibrated with cytochrome *c* (11 700), chymotrypsinogen A, ovalbumin, bovine albumin and catalase (195 000) [15].

*Sedimentation coefficient estimation*

The method of Martin and Ames [16] was used to estimate *s* for *A. iophagus* collagenase. Centrifugation was for 12 h at 36 000 rev./min in a Beckman SW50L rotor, using a linear 5—15% sucrose gradient with catalase (11.3 S) as the standard.

*Toxicity test*

As a preliminary test for the presence of a toxin, 2 ml aliquots of culture supernatant were injected sub-cutaneously into each of 3 rabbits.

## Results and Discussion

A typical purification is summarized in Table I, though yields varied from batch to batch and seemed highly dependent upon the particular lot of peptone used in the culture medium. Upon gel filtration of the material obtained by starch electrophoresis (Fig. 2), the collagenase was well separated from the other major protein peaks. Polyacrylamide-gel disc electrophoresis of the collagenase preparations after starch electrophoresis and gel filtration (Fig. 3) showed that the gel filtration was an efficient purification procedure.

Purified *A. iophagus* collagenase was shown to hydrolyze both insoluble and soluble collagen (Figs 4 and 5). This was a reliable indication of true collagenase activity, for pronase showed no significant activity against the insoluble collagen and produced only a slight reduction in the specific viscosity of the collagen solution. When serological comparisons were made, the *A. iophagus* collagenase recovered from starch electrophoresis reacted with the antiserum producing a single precipitin line, but neither of the *Cl. histolyticum* collagenases showed cross-reactivity.

TABLE I

TYPICAL PURIFICATION OF *A. IOPHAGUS* COLLAGENASE FROM A STARTING CULTURE VOLUME OF 3 l

| Preparation | Yield (mg) | Spec. act.* (μkat/kg) | Yield of activity* (%) | Casein hydrolysis ($mg \cdot mg^{-1} \cdot h^{-1}$) |
|---|---|---|---|---|
| Culture supernatant | 38480 | 122 | 100 | 0.02 |
| $(NH_4)_2SO_4$ | 558 | 5111 | 61 | 0.55 |
| Starch electrophoresis** | 213 | 9643 | 43 | 1.25 |
| Gel filtration | 10 | 107401 | 23 | 0.00 |

* Measured on Pz-Pro-Leu-Gly-Pro-Arg.
** Anodically migrating fraction only.

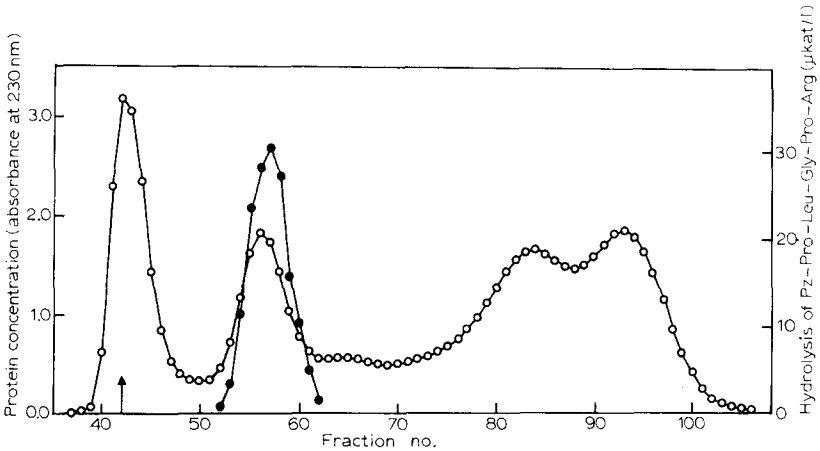


**Fig. 2. Gel filtration of *A. iophagus* collagenase. Elution of 80 mg of the preparation obtained by starch electrophoresis. The 2.5 × 93 cm column of Sephadex G-200 (Pharmacia; lot no. 9844) was prepared and eluted with 20 mM Tris · HCl/2 mM $CaCl_2$ (pH 7.6). The arrow indicates the void volume. A flow rate of 10 ml per h was maintained with a peristaltic pump and 5 ml fractions were collected. Tap water was circulated through the column jacket. ○———○ protein concentration (absorbance at 230 nm);●———●, hydrolysis of Pz-Pro-Leu-Gly-Pro-Arg (μkat/l). Only those fractions indicated contained activity against Pz-Pro-Leu-Gly-Pro-Arg.**

During electrophoresis in sodium dodecylsulphate-polyacrylamide gel, purified *A. iophagus* collagenase migrated as a single band of molecular weight 111 700 ± 800 S.D. (4). In the gel-filtration studies, the molecular weight of this collagenase was estimated to be 106 900 ± 1400 S.D. (3), and from the

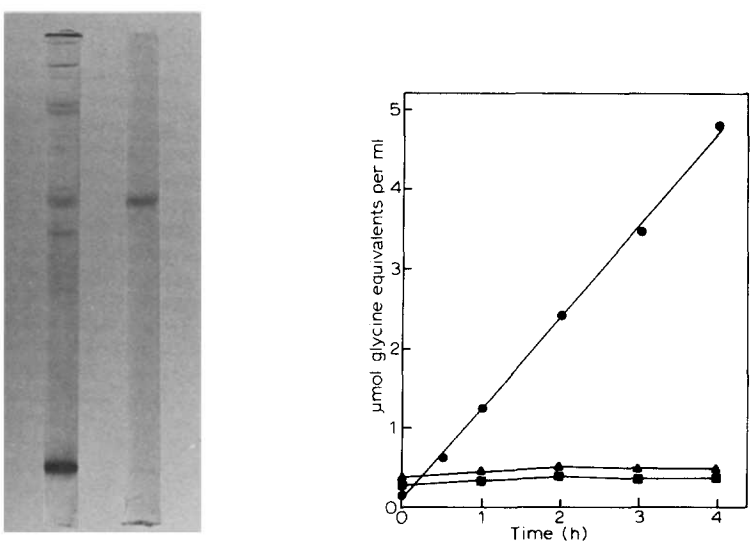


**Fig. 3. Analytical polyacrylamide gel electrophoresis of 200 μg of *A. iophagus* collagenase after starch block electrophoresis (left) and 15 μg after gel filtration (right). The method was that of Davis [11].**

**Fig. 4. Action of *A. iophagus* collagenase on insoluble collagen. Substrate suspensions consisted of 9 mg bovine Achilles' tendon collagen (Boehringer) in 3.0 ml of 0.1 M Tris · HCl/0.4 M NaCl/2 mM $CaCl_2$ (pH 7.6). The reaction was performed at 35° C. ●———●, collagenase (4444 pkat of activity on Pz-Pro-Leu-Gly-Pro-Arg); ▲———▲, pronase (enzyme : substrate 1 : 50); ■———■, enzyme-free control.**

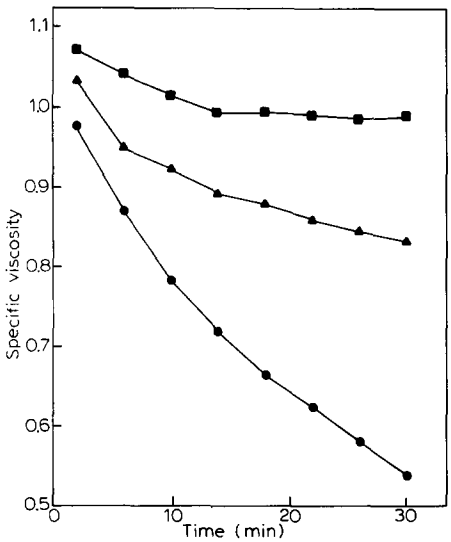


Fig. 5. Action of *A. iophagus* collagenase on soluble collagen. Reaction mixtures consisted of 6 ml of collagen solution (cf. text) mixed with 0.5 ml of enzyme solution. Specific viscosity was monitored at 20°C using a Cannon-Fenske viscometer, size 100. ●———●, collagenase (2089 pkat of activity on Pz-Pro-Leu-Gly-Pro-Arg); ▲———▲, pronase, (enzyme : substrate 1 : 30); ■———■, enzyme-free control.

sucrose gradient centrifugation a figure of 5.3 ± 0.1 S.D. (4) was calculated for its sedimentation coefficient. These data resemble those for one of the *Cl. histolyticum* collagenases, which has a molecular weight of approx. 100 000 [1] and a sedimentation coefficient of 5.3 S [6].

As the genus is non-pathogenic to warm-blooded animals [17], the production of a toxin by *A. iophagus* was considered unlikely. This assumption was supported by the absence of any ill-effects in the rabbits injected with culture supernatant. Following our preliminary experiments, full toxicological investigations carried out at the Institut Pasteur, Paris, confirmed that the crude enzyme preparation completely lacks any accompanying toxin. These results will be reported fully in a future publication from that institute.

The production of collagenase using *A. iophagus* instead of *Cl. histolyticum* has the advantages of aerobic culture, simple nutrient requirements and absence of toxicity. Compared with *Cl. histolyticum*, this strain of *A. iophagus* is therefore a potentially better source of collagenase for large scale production and clinical usage.

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## References


1 Mandl, I. (1972) in Collagenase (Mandl. I., ed.), pp. 1—16, Gordon and Breach, New York
2 Sizer, I.W. (1972) in Advances in Applied Microbiology (Perlman, D., ed.), Vol. 15, pp. 1—11, Academic Press, New York
3 Waldvogel, F.A. and Swartz, M.N. (1969) J. Bacteriol. 98, 662—667
4 MacLennan, J.D., Mandl, I. and Howes, E.L. (1953) J. Clin. Invest. 32, 1317—1322
5 Welton, R.L. and Woods, D.R. (1973) J. Gen. Microbiol. 75, 191—196
6 Yoshida, E. and Noda, H. (1965) Biochim. Biophys. Acta 105, 562—574
7 Wünsch, E. and Heidrich, H.G. (1963) Hoppe-Seyler's Z. physiol. Chem. 333, 149—151
8 Adamcic, M. and Clark, D.S. (1970) Can. J. Microbiol. 16, 709—712
9 Cooper, D.R. and Davidson, R.J. (1965) Biochem. J. 97, 139—147
10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
11 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
12 Maizel, J.V. (1971) in Methods in Virology (Maramorosch, K. and Koprowski, H., eds), Vol. 5, pp. 179—246, Academic Press, New York
13 Rippon, J.W. (1968) Biochim. Biophys. Acta 159, 147—152
14 Ouchterlony, O. (1958) in Progress in Allergy (Kallós, P., ed.), Vol. 5, pp. 1—78, Karger, New York
15 Andrews, P. (1965) Biochem. J. 96, 595—606
16 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372—1379
17 Skerman, V.B.D. (1967) in A Guide to the Identification of the Genera of Bacteria with Methods and Digest of Genetic Characteristics, 2nd edn, p. 144, Williams and Wilkins, Baltimore