PURIFICATION, STABILITY AND INHIBITION OF THE COLLAGENASE FROM *ACHROMOBACTER IOPHAGUS*

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Received 26 September 1975

1. Introduction

An aerobic microorganism, *Achromobacter iophagus,* which was isolated from cured hides and which is a source collagenolytic activity has been described in a series of papers [1–3]. The crude collagenase from *Achromobacter* is now available on a larger scale; this gave us an opportunity to study its further purification, as well as its enzymatic, pharmacological and immunogenic properties. In this paper purification is described leading to a collagenase of specific activity of 1587 nkat/mg, the highest activity obtained as yet for any collagenase. *p*-Chloromercuribenzoate removes most of the caseinolytic activity in the crude enzyme. EDTA, cysteine and histidine inhibit the enzyme, Dip-F, *N*-ethylmaleimide and ClHgBzOH are without effect. This localizes the enzyme as a metalloenzyme (EC 3.4.24).

2. Experimental

2.1. *Materials*

Crude collagenase from *Achromobacter iophagus* of spec. act. 170 nkat/mg was a gift from Institut Pasteur Production. Synthetic substrate 4-phenylazo-benzyloxycarbonyl-L-prolyl–L-leucyl–glycyl–L-prolyl–D-arginine dihydrate was purchased from Fluka. Casein (LAB) was a product of Merck. Calf skin collagen (acid soluble, C-1633) was purchased from Sigma, bovine Achilles tendon collagen (15414 SCAB) from Boehringer.

Abbreviations: Dip-F, diisopropyl-phosphofluoridate; ClHgBzOH, *p*-chloromercuribenzoate.

Diethylaminoethyl-cellulose (DE-32) Whatman and Sephadex G-100 fine from Pharmacia were used for colum chromatography. Diisopropyl-phosphofluoridate and p-chloromercuri-benzoate were purchased from Fluka, *N*-ethylmaleimide and trinitrobenzensulphonic acid from Sigma. Sodium dodecylsulphate (Sigma) was recristallized from ethanol.

2.2. *Enzymatic assays*

Collagenase assay with synthetic peptide as substrate was performed with 4-phenylazo-benzyloxycarbonyl-L-prolyl–L-leucyl–glycyl–L-prolyl–D-arginine dihydrate [4]. The buffer was made 3 mM in calcium chloride.

The routine evalutation of column chromatography samples was performed by incubating an aliquot of the fractions with insoluble collagen. The initial rate of increase of free α-amino groups was determined by colorimetry after reaction with trinitrobenzensulphonic acid [5].

For direct comparison of the final products of the purification steps with the collagenases obtained by Welton and Woods [3] we adopted the same condition as used in their study : 3 mg of insoluble collagen was incubated with the enzyme in 0.1 M Tris–HCl buffer pH 7.6 which contained 0.4 M NaCl and $2 \cdot 10^{-3}$ M $CaCl_2$ for 4 h at 37°C. The reaction was stopped by acidification. Ninhydrin colorimetry [6] was used and the results expressed in nmoles of glycine equivalents liberated per second (nkat) per mg.

For viscosimetric analysis the stock solution of native collagen [5] was diluted to 0.1% with 0.1 M Tris–HCl – 0.5 M $CaCl_2$ buffer (or with 0.1 M Tris–HCl – 0.5 M NaCl in inhibition tests), pH 7.0. Ostwald-type, low shear capillary viscosimeters according to

Seifter et al. [7] were used. The average elution time for water was between 16 and 25 sec at 25°C, specific viscosity of the diluted collagen solution was 3.3. The digestion mixture consisted of 1.9 ml of collagen solution and 0.1 ml of enzyme. The rate of decrease of specific viscosity at 25°C was followed within 60 min.

For the protease assay using casein as substrate one ml of enzyme solution was incubated with 1 ml of 1% casein solution in 0.1 M borate buffer at pH 7.6 and 37°C for 20 min. The reaction was stopped by addition of 3 ml of 5% trichloroacetic acid. After standing at 4°C for 16 h and centrifugation the optical density of the filtrate was evaluated at 280 nm. One unit is equal to a 280 nm absorbancy of 0.001 per min per μg [8].

2.3. *Purification of crude collagenase*

All steps were performed at 4°C. One g of lyophilized commercial enzyme was dissolved in 100 ml of 0.025 M Tris–HCl buffer pH 7.0 made 10^{-2} M in $CaCl_2$ and 10^{-4} M in ClHgBzOH. The insoluble fraction was removed by centrifugation and discarded, the supernatant applied to a column of DE-32 cellulose (30 × 2 cm) equilibrated with the same buffer. The flow rate was 12 ml per hour, 6 ml fractions were collected. A stepwise elution was made at pH 7.0 by subsequent application of three Tris–HCl buffers: 0.2 M–0.3 M–0.3 M made 0.7 M in NaCl. All buffers were 0.01 M in $CaCl_2$ and 10^{-4} in ClHgBzOH. In eluted fractions, the collagenolytic activity was tested on the synthetic peptide and insoluble collagen, the proteinase activity on casein. Pooled fractions were dialysed against 10^{-3} M $CaCl_2$ and lyophilized.

Twenty mg of lyophilized sample from the previous step was dissolved in 1 ml of 0.02 M $CaCl_2$ pH 7.0 and applied to a column (100 × 1 cm) of Sephadex G-100 fine equilibrated with the same solution. The flow rate was 14 ml/h. 2.8 ml fractions were collected. The same enzyme assays were used as previously.

2.4. *Inhibition and stability assays*

For the inhibition of caseinolytic activity in crude collagenase, the samples of enzyme (1.0 mg per ml of 0.1 M Tris–HCl – 0.01 M $CaCl_2$ buffer pH 7.0) were incubated with the inhibitor at 22°C for 2 h.

For the inhibition assays on the purified enzyme, the enzyme peak 3 from DE-cellulose, 0.6 mg per ml) was incubated in the appropriate buffer at 22°C under conditions described in table 3. In the experiments on the reactivation of the EDTA-inhibited collagenase, the last one was incubated for 1 h in the presence of 10^{-2} M $CaCl_2$. For the analytical evaluation by viscosimetry the buffer used was 0.1 M Tris–HCl – 0.5 M NaCl pH 7.0 (except if another pH is mentioned). For the peptide assay [4] veronal acetate buffer was used. All buffers contained the same final molarity of the inhibitor as the sample to be tested.

For stability measurements, solutions of purified collagenase (0.25 mg per ml) in veronal-acetate buffer made $3 \cdot 10^{-3}$ M in $CaCl_2$ adjusted to pH ranging from 4 to 10 were used. The decrease of activity was measured on the synthetic peptide as substrate.

2.5. *Disc electrophoresis*

Electrophoresis was performed on a Buchler apparatus with 12 cm long tubes according to Jovin et al. [9] or in the presence of sodium dodecyl-sulphate [10]. Fifty to 800 μg of the desalted and lyophilized protein were applied. In twin runs, one gel column was stained and scanned on a Vernon type PH 15 densitometer, the other was sectionned into uniform slices which were then eluted separately with 100 μl water at 4°C for 2 h. The activity of the eluates was assayed on the synthetic peptide as substrate.

3. Results

3.1. *Suppression of caseinolytic activity in crude collagenase*

The results summarized in table 1 show that the caseinolytic activity of crude collagenase is not influenc

Table 1
Effect of Dip-F and ClHgBzOH on caseinolytic and collagenolytic activity of collagenase

Inhibitor	Molar concentration	Casein (u/min/mg	Synth. peptide (nkat/mg)
None	–	$22 \cdot 10^{-2}$	170
Dip-F	10^{-4}	$22 \cdot 10^{-2}$	164
	10^{-3}	$21 \cdot 10^{-2}$	170
	10^{-2}	$23 \cdot 10^{-2}$	166
ClHgBzOH	10^{-4}	$1.86 \cdot 10^{-2}$	166
	10^{-3}	$1.85 \cdot 10^{-2}$	165

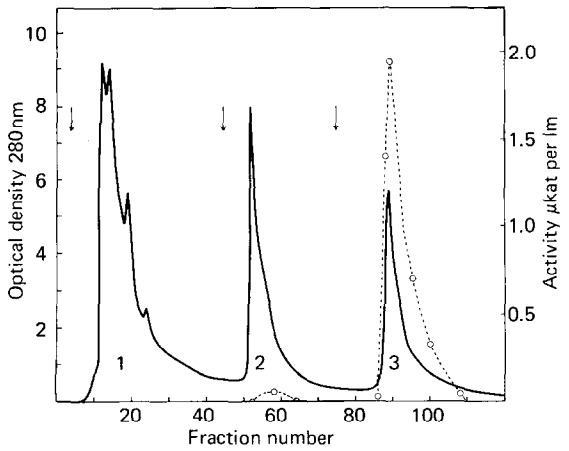

Fig.1. Chromatography of crude *Achromobacter* collagenase on DE-32 cellulose. (———) Protein plus pigment, optical density at 280 nm; (o--o) activity against synthetic peptide. Arrows mark the stepwise elution by buffers as described in Methods.

ed by Dip-F. On the other hand, preincubation of the enzyme with ClHgBzOH decreased the caseinolytic activity. Neither of the two reagents had any effect on the collagenolytic activity of the crude enzyme.

3.2. *Purification*

Chromatography of the crude enzyme on DEAE-cellulose (fig.1) resulted in the separation into three main peaks. The peak which emerged with the first buffer contained most of the absorbing material and caseinolytic activity of the crude sample. A slight collagenolytic activity was observed in this peak. Because its contribution to the whole eluted activity was negligible, its further study was not undertaken. Collagenolytic activity emerged in part with the second peak but mainly with the third peak. In fig.1 only the activity curve for the synthetic peptide is given but the assays on insoluble collagen and the synthetic peptide from several runs showed the same pattern and the peaks of activity coincided.

After dialysis and lyophilisation the pooled material from the second and the third peaks was applied independently to a column of Sephadex G-100 and the active material was lyophilized. Although low mol. wt impurities with a considerable absorption at 280 nm were removed by this procedure, the specific activity decreased.

The yields and activities of the products of purification are compared in table 2 with the product of purification by electrophoresis, described previously [3]. The viscosimetric assay with native collagen as substrate is presented in fig.2.

Densitometric pattern of the disc electrophoresis of the material recovered under peak 3 (table 2) is shown in fig.3. All major bands were active towards the synthetic substrate. After the chromatography of peak 3 on Sephadex G-100 additional bands of lower molecular weight were revealed by electrophoresis in the presence of sodium dodecylsulphate which shows a decomposition of the parent material.

3.3. *Effect of inhibitors and stability*

The results of inhibition assays on purified collagenase (peak 3, table 2) are summarized in table 3. The inhibition was complete in the presence of 10^{-2} M

Table 2
Recovery and specific activity resulting from purification of crude *Achromobacter* collagenase

Purification step	Weight (mg)	Specific activity: Synth. peptide (nkat/mg)	Specific activity: insoluble collagen (nkat/mg)	Specific activity: Casein (u/min/mg)	Recovery (%, peptide as substrate)
Crude	1000	170	43	$22 \cdot 10^{-2}$	100
DEAE, peak 2	64	55	20	$0.44 \cdot 10^{-2}$	2.1
DEAE, peak 3	95	1587	422	$0.55 \cdot 10^{-2}$	88.8
Peak 3 after G-100	78	1043	291	$0.40 \cdot 10^{-2}$	47.9
Welton and Woods, [3]		107	24		38.4
Aα (Kono, [12])		270			

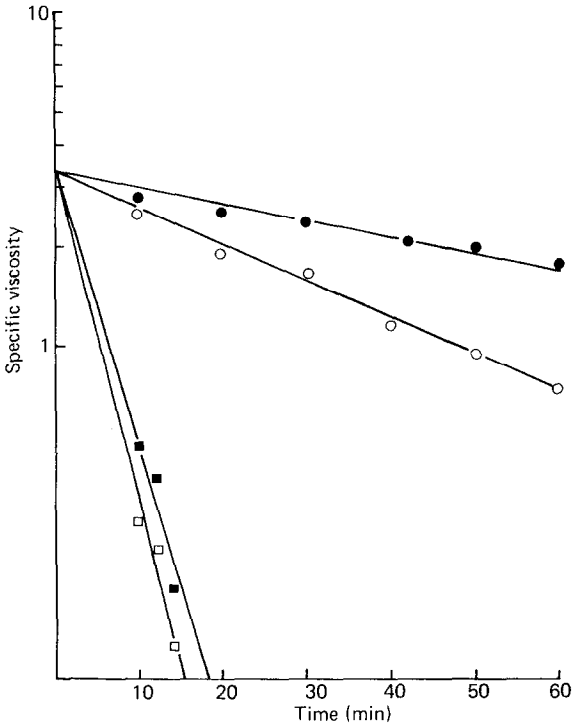

Fig.2. Viscosimetric assay of fractions resulting from purification of crude *Achromobacter* collagenase on native collagen as substrate. (○——○) Crude enzyme; peak 2 (●——●) and 3 (□——□) from DE-cellulose chromatography; (■——■) material recovered after Sephadex G-100 chromatography of peak 3.

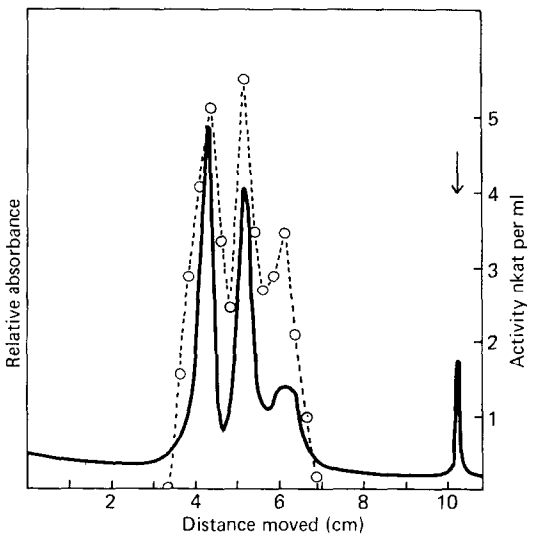

Fig.3. Disc electrophoresis of the peak 3 from DE-32 chromatography. The arrow marks the mobility of the dye; (——) scanning of colored bands; (○---○) activity of eluates from sliced gels.

EDTA. This inhibition could be partially reversed by addition of calcium ions. A complete inhibition of caseinolytic activity was also observed following EDTA treatment. On the other hand the powerful inhibitor of SH-enzymes, *N*-ethylmaleimide, influenced neither collagenolytic nor residual caseinolytic activity.

Table 3
Inhibition of purified collagenase from *Achromobacter iophagus*

Inhibitor	Molar concentration	pH	Incubation time (h)	Peptide (% of residual activity)	Collagen (Specific viscosity)
None				100	0.3
EDTA	10^{-2}	7.0	1	0	3.2
EDTA + $CaCl_2$	10^{-2}	7.0	1	n	1.33
Cysteine	10^{-2}	6.1	1	92	0.35
	10^{-2}	7.0	1	10.5	2.3
	10^{-2}	7.0	3	0	3.2
Histidine	10^{-1}	7.0	2	30	3.2
N-ethyl-maleimide	10^{-1}	7.0	1	104	n.d.

n.d. = not determined.
Specific viscosity was calculated on basis of the value determined at 30 min interval.
Collagen solution containing neither enzyme or inhibitor had a specific viscosity of 3.2

Incubation of the enzyme in 10^{-2} M cysteine solutions at pH 6.1 and room temperature for 1 h only slightly affected the collagenolytic activity. Under similar conditions but at pH 7.0 the activity decreased and within 3 h it was completely inactivated. The inhibition could not be reversed by calcium ions. Histidine at a concentration of 10^{-1} M completely inhibited the activity against native collagen, but 30% of the activity against the synthetic peptide still remained.

At neutral pH and 4°C, the activity of the purified enzyme did not change during dialysis or gel filtration in the presence of calcium ions at a concentration of 10^{-4} M; lyophilisation from such solutions results in a 30–40% loss of activity. Fig.4 shows the effect of pH on the activity over the range of pH 4–10. Incubation at pH 4 resulted in a sharp decrease of activity at 4°C. At pH 7 the activity decreased after a month of incubation at 4°C by only 7%.

4. Discussion

Purified collagenase from *Achromobacter iophagus*, obtained in this study, shows a much higher specific activity than any other collagenase yet described. It is fifteen times more active than the collagenase from the same source obtained in a previous study [3] and almost six times than the pure clostridiopeptidase from *Clostridium histolyticum* (Kono [12]). This can partly be ascribed to the already high activity of the crude enzyme, partly to the use of ClHgBzOH throughout the first purification step. The destructive action of other proteases present in the crude enzyme could thus be largely suppressed. Nevertheless, traces of non-specific proteolytic activity as assayed with casein as substrate remained in the collagenolytically active fractions during the purification. This activity can be inhibited by EDTA, simultaneously with the collagenolytic activity. A presence of a low caseinolytic activity in purified collagenase which could be removed by the inhibitors of collagenase, has been observed in the case of the enzymes from *Clostridium histolyticum* [11]. It is premature, however, to advance the hypothesis of caseinolytic action being inherent to collagenase before the fully homogeneous enzyme is available.

The lyophilized collagenase resulting from the chromatography on DE-cellulose still shows heterogeneity, but up to now, the attempt at further purification by gel filtration resulted in degradation and a drop in specific activity (table 2 and fig.2).

Inhibition studies show a close similarity of *Achromobacter* collagenase to that from *Clostridium histolyticum*. Both are inhibited by EDTA, cysteine and histidine. On the other hand they are influenced neither by ClHgBzOH nor Dip-F. This makes it plausible to advance the idea that *Achromobacter* collagenase belongs also to the group of hydrolytic enzymes containing metal in their active site.

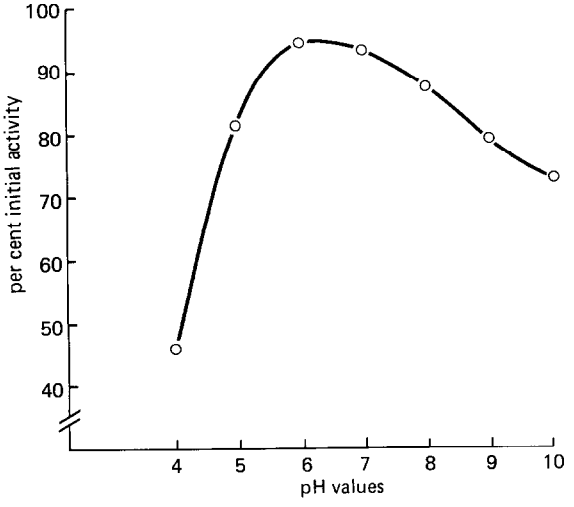

Fig.4. Loss of activity of *Achromobacter* collagenase at different pH values. Samples were stored 30 days at 4°C.

Acknowledgements

This work was supported by D.G.R.S.T. (project N° 72.7.0742). D.R. Woods acknowledges overseas bursories from the Ernest Oppenheimer Memorial Trust and the South African C.S.I.R. We thank Mr L. Bagilet for his skilled technical assistance.

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