LATENT COLLAGENASE FROM HUMAN POLYMORPHONUCLEAR LEUCOCYTES AND ACTIVATION TO COLLAGENASE BY REMOVAL OF AN INHIBITOR

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Received 18 August 1980

1. Introduction

Human leucocytes contain several enzymes which participate in the degradation of collagen [1–3]. The cleavage of the tropocollagen molecule, (preferentially type I collagen), is initiated by a true collagenase splitting the collagen molecule into two distinct fragments [1]. Since the initial demonstration of collagenolytic activity in leucocytes [1], it has been shown that the enzyme exists in both active and latent forms in extracts of human leucocytes [2]. The latent collagenase can be activated by other active neutral proteases [4–5] and recently cathepsin G, a chymotrypsin-like enzyme, isolated from human leucocytes has been shown to be capable of activating the latent enzyme [7]. In previous years proteolytic enzymes have been used by many groups to activate the latent collagenases obtained from a variety of other tissues and a multistep scheme has been proposed for collagenase activation by limited proteolysis [8–11]. There also exists evidence to support the hypothesis that activation of the latent enzyme takes place through the removal of an inhibitor from an enzyme–inhibitor complex [2,5,6,11–13].

In this paper we wish to report on the isolation of a latent collagenase from human leucocytes and its activation to active collagenase by enzymically active and non-active agents, that remove an inhibitor protein (24 000 M_r) from the latent enzyme which is capable of inhibiting the active enzyme.

2. Materials and methods

Collagenolytic activity was measured by a method employing the use of 4-phenylspiro[furan-2(3 H),1-phthalan]3,3′-dione, (fluorescamine) (Sigma Chemical Co., St Louis, MO). Fluorescamine was used to measure the proteolytic activity of collagenase against neutral salt-soluble rat skin (NSS) collagen solution by the increase in fluorescence emission per unit time. NSS collagen solutions were prepared according to a modification of the method in [14]. Various amounts (10–100 μl) of latent collagenase extracts were added to 0.2% NSS collagen solutions (200 μl). The final reaction volume was made up to 400 μl by addition of either 0.1 M Tris/HCl; 50 mM $CaCl_2$ (pH 7.4) or by addition of one of the activators (0.1 mg/ml). On addition of latent collagenase extract to the collagen solution a 20 μl aliquot of the reaction mixture was taken. This measurement provided a zero time reading. After the incubation period (3 h at 30°C) was over a further 20 μl aliquot was taken. These aliquots were added to 0.2 M phosphate buffer at pH 6.8 (4.0 ml). Then fluorescamine reagent (2 ml, 0.1 mg/ml) in acetone was added to the phosphate buffer and the fluorescence emissions at 475 nm were measured employing an excitation wavelength of 390 nm on a JY 3 D spectrofluorimeter (Instruments SA GmbH). The difference between the zero time and the 3 h fluorescence emission readings gave a qualitative measurement of collagenase activity. The assay system was quantified by determining a calibration curve relating the fluorescence emission readings to an equivalent concentration of L-isoleucyl–L-alanylglycine ethyl ester (a personal gift from M/s G. Kay, The Queen's University of Belfast, N. Ireland). The solution was 100 μM (3.21 mg/l) in 0.2 M phosphate buffer (pH 6.8). It was calculated that 1.0 μM of amino groups formed by cleavage of the collagen molecule will react with fluorescamine to give 52.4 units of fluorescence. This fluorescence emission would be equivalent to the cleavage of 6.024×10^{17} bonds. Hence one may calculate the

number of bonds cleaved per unit increase in fluorescence as:

$$\frac{6.024 \times 10^{17}}{52.4} = 1.15 \times 10^{16} \text{ bonds}$$

Viscometric assay of extracts was carried out using an automatic viscometer (Macartney, Blumsom and Elmore, in preparation).

The preparation of leucocytes and granule extracts was based on a modification of the method in [3].

Activation of the latent collagenase with trypsin was done as in [8]. Activation with other proteolytic enzymes, the inhibited proteolytic enzymes (see table 1), the thiol blocking compounds cystine and glutathione insulin and the organomercurial compound *p*-chloromercuribenzoate was done with 10 μg activator/50 μl extract.

TLCK-trypsin was prepared as described [15] from pancreatic trypsin (Sigma Chemical Co., St Louis MO). DFP and PMSF inhibited cathepsin G were prepared by incubating cathepsin G (a gift from Bayer AG) with the corresponding inactivator at a ratio of enzyme:inhibitor of 1:1000 at 4°C for 4 h. All inhibited proteolytic enzymes were shown to possess no further proteolytic activity. Commercial preparations of mixtures of unresolved α, β, π as well as 90% β-trypsin and TLCK-trypsin were pre-incubated with cysteine for 1 h; collagenase was pretreated for 3 h with cysteine or reduced glutathione. 179,203-di-*S*-carboxymethyl-trypsinogen and 179,203-di-*S*-carboxymethyl-trypsin were kindly provided by A. Light, Purdue, Lafayette.

Partial purification of the latent collagenase and of the active enzyme was carried out on Sephadex G-75 Superfine (Deutsche Pharmacia GmbH). The gel filtration column was calibrated for molecular weight determination according to [16], with myoglobin (17.8×10^3); chymotrypsin (25×10^3); egg albumin (45×10^3); liver albumin (65×10^3); aldolase (147×10^3).

Table 1
Activation of the latent enzyme by enzymes, inhibited enzymes, cystine, oxidised glutathione and partly cysteine reduced proteins

Activator	Bonds cleaved	Relative % activation
Oxidised glutathione	2.795×10^{18}	100
Cystine	2.783×10^{18}	99.6
Insulin	2.348×10^{18}	84.0
Trypsinogen	2.043×10^{18}	73.1
TLCK–trypsin control	2.783×10^{18}	99.6
TLCK–trypsin cysteine treated	3.335×10^{17}	11.9
Trypsin–cysteine treated	3.795×10^{17}	13.5
β-Trypsin	9.200×10^{17}	32.9
di-*S*-carboxymethyl-trypsinogen	0	0
di-*S*-carboxymethyl-trypsin	3.450×10^{16}	1.2
Unactivated control[b]	2.875×10^{17}	10.3
Trasylol	0	0
Cathepsin G[a]	1.208×10^{18}	–
DFP–cathepsin G	9.660×10^{17}	100
PMSF–cathepsin G	8.860×10^{17}	92.0
Pancreas kallikrein	5.371×10^{17}	56.0
Trypsin control	8.050×10^{17}	83.0
Unactivated control[b]	2.530×10^{17}	26.0

[a] Due to the additional proteolytic activities of cathepsin G, or trypsin in the activation mixtures the collagenolytic activity of the latent enzyme after activation by inactive DFP–cathepsin G, or oxidised glutathione was chosen as 100% standard

[b] The latent enzyme preparations were contaminated with 10% and 26% active enzyme, respectively

Determination of the sulfhydryl group concentration was carried out by the method of Ellman [17] with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma Chemical Co., St Louis MO).

3. Results

The concentrated extracts from the nuclear and the crude granule preparations which showed significant collagenolytic activity were pooled and then subjected to gel filtration on a Sephadex G-75 Superfine column (fig.1), pre-calibrated with standard proteins (see section 2). Eluted fractions were collected in 10 ml aliquots and these were assayed for collagenolytic activity (fig.1). The latent enzyme eluted with a maximum activity at 80 ml corresponding to M_r ~80 000. A preparation of latent enzyme devoid of any collagenolytic activity could be collected by two (or more) repeated gel filtrations of the latent 80 000 M_r fraction. The active enzyme eluted with a maximum activity at 100 ml corresponding to M_r 65 000 and could be further activated by addition of zinc salt.

Activation of the crude enzyme concentrate by disulfide containing activators, prior to gel filtration, resulted in the collagenolytic activity being eluted at a molecular weight corresponding to M_r 60 000–65 000. Further activation produced no further significant increase in the collagenolytic activity (fig.2).

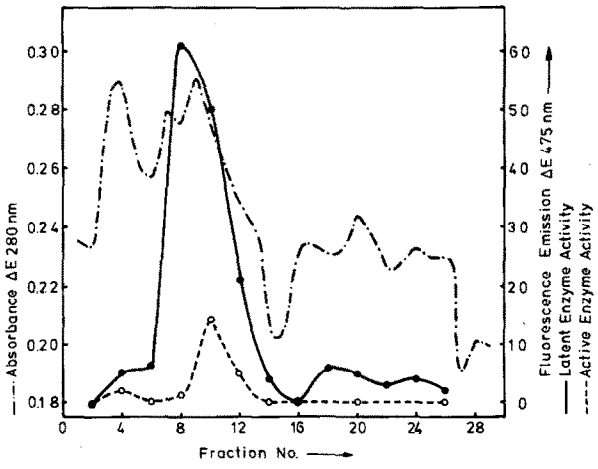


Fig.1. Effluent profile of latent human leucocyte collagenase on a column (1 cm × 90 cm) of Sephadex G-75 Superfine equilibrated and eluted with 0.1 M Tris/HCl (pH 7.4), 50 mM in $CaCl_2$. Flow rate 10 ml/h. Fraction volume 10 ml.

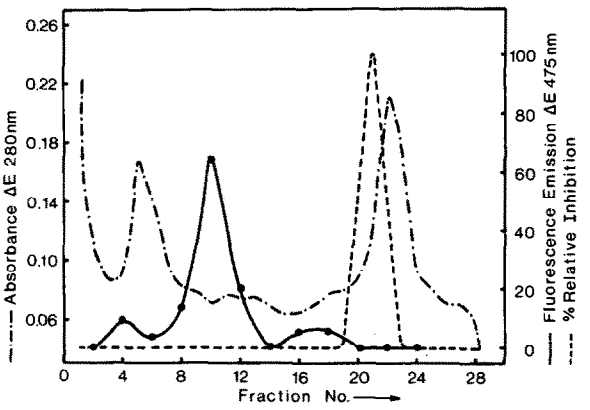


Fig.2. Effluent profile of cystine-activated human leucocyte collagenase from a column (1 cm × 90 cm) of Sephadex G-75 Superfine equilibrated and eluted with 0.1 M Tris/HCl (pH 7.4), 50 mM in $CaCl_2$. Flow rate 10 ml/h. Fraction volume 10 ml.

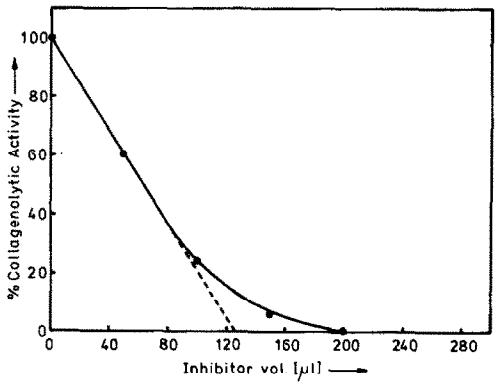


Fig.3. Titration curve of human leucocyte collagenase with human leucocyte collagenase inhibitor isolated from the latent enzyme (see fig.2). When 200 µl inhibitor (22.9 mg protein/ml) were added to 100 µl enzyme (7.2 mg protein/ml) the inhibitor was taken to represent 100% relative inhibition. For test conditions, see section 2.

A second fraction in the gel filtration corresponded to M_r 24 000 (fig.2). This inhibitor fraction was capable of completely inhibiting the collagenolytic activity when titrated with the 60 000–65 000 M_r enzyme fraction (fig.3). The inhibitor:enzyme molar combining ratio was estimated on the basis of protein determinations of the partially purified proteins (estimate 80–90% and 70–80% purity, respectively), assuming M_r 24 000 and 65 000 for the inhibitor and collagenase, respectively. This value varied between 1:1 and 1:2. The active collagenase obtained

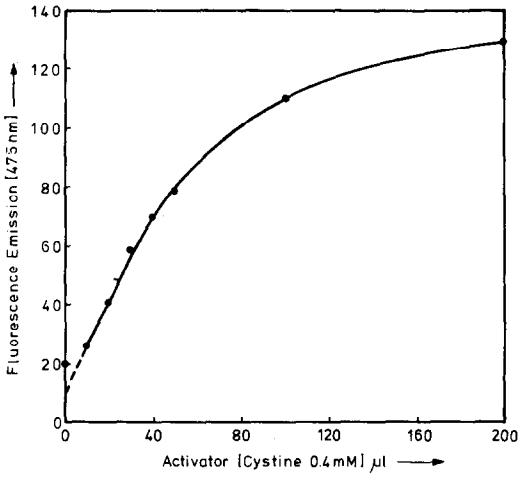


Fig.4. Activation curve of human leucocyte latent collagenase (100 µl) obtained by cystine (0.4 mM in 0.1 M Tris/HCl buffer (pH 7.4) and 50 mM in $CaCl_2$). Collagenolytic activity was determined by the fluorescamine method (see section 2).

was able to reduce the viscosity of a 0.2% NSS collagen solution by ~40%.

It was possible to activate collagenolytic activity in crude extracts using inhibited proteolytic enzymes, cystine, glutathione, insulin and *p*-chloromercuribenzoate (fig.4, table 1). No activation of the latent enzyme could be achieved with either elastase from pig pancreas or from human polymorphonuclear leucocytes, nor with commercial trypsin or TLCK-trypsin in both pretreated for 1 h with cysteine. Only slight activation occurred with 90% bovine β-trypsin. Neither could activation be achieved with 179,203-di-*S*-carboxymethyl-trypsinogen or 179,203-di-*S*-carboxymethyl-trypsin.

The activity of the active collagenase could be abolished by a 3 h pretreatment with either cysteine or reduced glutathione. All investigations on the inhibitor were performed with material isolated from partially purified latent enzyme (fig.1). The 80 000 M_r fraction was isolated, activated by cystine and subjected to a second gel filtration step to give active enzyme and the 24 000 low M_r fraction (fig.2) representing ~80% pure inhibitor. The inhibiting activity of the separated inhibitor could be abolished by a 1 h pretreatment with cystine, or oxidised glutathione (table 2).

Table 2
Inhibitor activity of 24 000 M_r inhibitor against active collagenase after 1 h preincubation of the inhibitor with oxidised glutathione

Tube contents	Bonds cleaved	Relative % Activation
Control	1.73×10^{18}	100
Inhibitor control	4.60×10^{17}	25[a]
Inhibitor + glutathione	1.81×10^{18}	100

[a] Amount of inhibitor added was insufficient to produce complete inhibition

The inhibitor was shown to contain a sulfhydryl group titratable with Ellman's reagent. From the protein concentration of the inhibitor (1.2×10^{-5} M, Folin determination) and the sulfhydryl group concentration (9.6×10^{-6} M, Ellman determination) the number of sulfhydryl groups per molecule of inhibitor was calculated to be nearly one on the basis of M_r 24 000 for the inhibitor. The inactivation of the inhibitor by cystine and/or oxidised glutathione can (easily) be explained by a thiol–disulfide interchange reaction (see scheme 1 and section 4).

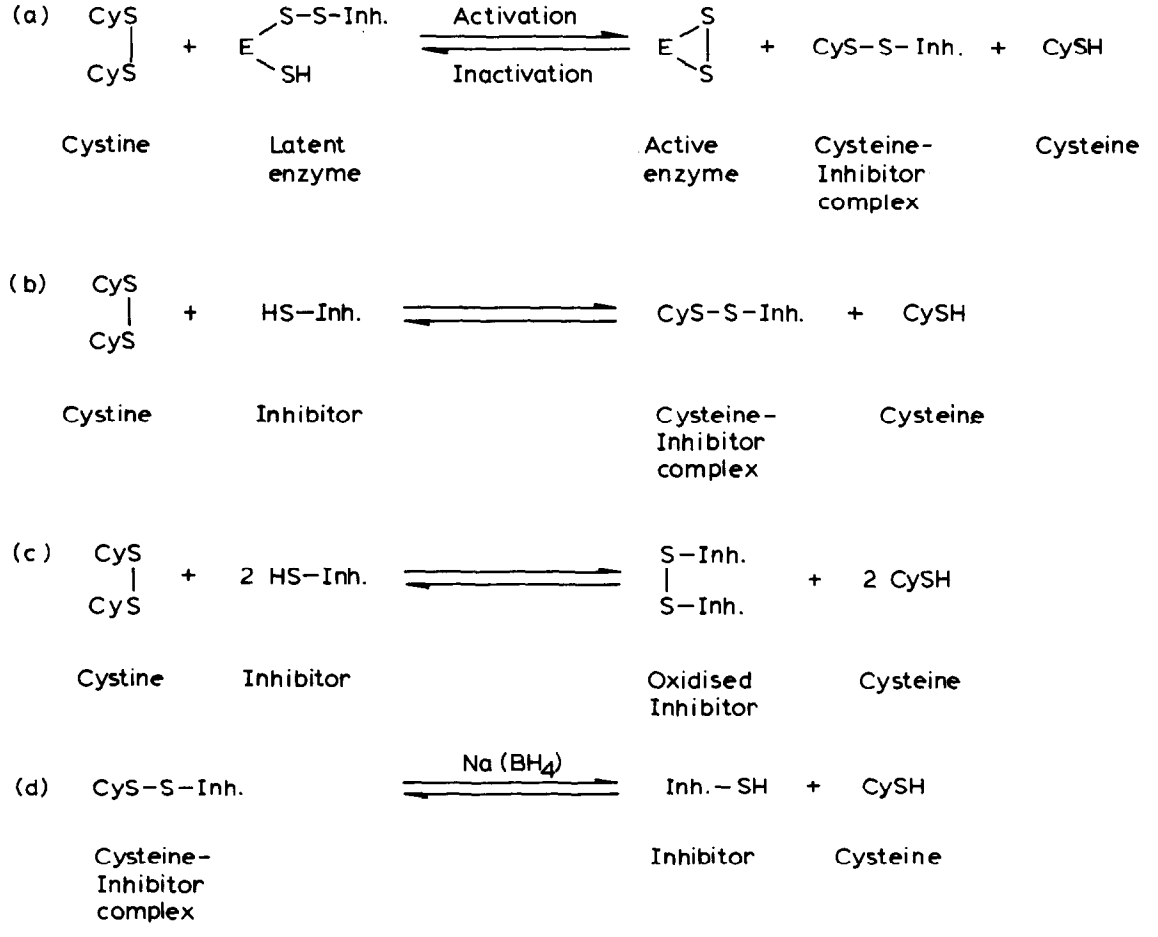
4. Discussion

The results indicate that a latent form of a true collagenase devoid of activity can be obtained from human polymorphonuclear (PMN) leucocytes. This latent collagenase can be activated by the activators discussed above (see table 1) to give the active form of the enzyme and an inhibitor separable by gel filtration. Partial inactivation of the collagenase by removal of the complex ligand zinc during this separation process can be reversed by incubation with zinc salts (table 1). In several of the PMN leucocyte preparations the active enzyme was also present in varying amounts. However this active portion could be completely removed by repeated gel filtration to yield only inactive latent enzyme. It could therefore be reasonably concluded that the observation of 'already active enzyme' in several of the crude PMN leucocyte preparations, prior to activation, is an artifactual result, since activation of a certain percentage of the latent enzyme may have taken place during the isolation procedure, e.g., via activation by cathepsin G demonstrated to be present in the extracts, or by oxidised glutathione present in the granules [18].

In contrast to other workers [2,19] our results indicate that the latent enzyme is present in the form of an enzyme–inhibitor complex, as has been previously suggested [20], and is not activated by a limited proteolytic process. The observed activation of the

latent enzyme seems to be due to removal of an inhibitor from the complex. The inhibitor can be isolated and contains an essential sulfhydryl group. It can be used in a titration experiment to abolish the collagenolytic activity and to restore the form of the inactive latent enzyme. This favours a mechanism of inhibition via formation of a disulfide bridge between enzyme and inhibitor. In this complex the active sulfhydryl group of the inhibitor may interact in a disulfide exchange reaction with one of the surface exposed disulfide bridges in the substrate binding pocket of the enzyme thus blocking the access of substrate. In reaction with trypsinogen the disulfide bridge Cys 179–Cys 203 in proximity to the reactive site [21] is the most favourable on the basis of the selective reduction experiments in [22]. This is supported by the failure to activate latent enzyme by trypsinogen preincubated with cysteine, or by 179,203-di-*S*-carboxymethyl-trypsinogen or by 179,203-di-*S*-carboxymethyl-trypsin and the fact that trypsin is inhibited after activation of the latent enzyme.

This mechanistic view is further strengthened by the findings that (a) the inhibitor contains one sulfhydryl group per molecule and could be inactivated by preincubation with oxidised glutathione and reactivated by treatment with sodium borohydride (scheme 1), and (b) that the protein activators (trypsininogen commercial and TLCK-trypsin) were unable to activate the latent enzyme after pretreatment with cysteine (table 1). The proposed mechanism is supported by the inactivation of the collagenase by pretreatment with either cysteine or reduced glutathione. The activation–inactivation mechanism is shown schematically below:



Activation-inactivation scheme

A similar mechanism of enzyme–inhibitor interaction has been proposed in [23] for the interaction of the basic bovine inhibitor (Kunitz, i.e., Trasylol®) and bovine trypsins, but that has been proven to be wrong on the basis of the X-ray crystallographic investigations of the complex [24]. In [25,26] a similar mechanism was proposed to explain the interaction of a trypsin-dependent neutral protease and its inhibitor present in malignant tissue.

It is also interesting to note that active, or inactive cathepsin G as well as oxidised glutathione and insulin activate latent collagenase effectively, a fact which may be of physiological significance in the cell or in inflamed tissue.

Acknowledgement

This work was supported by the Stiftung Volkswagenwerk, Hannover (I/35 191).

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