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Human neutrophil collagenase cleaves α_1-antitrypsin

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Inactivation of the plasma serine-proteinase inhibitor α_1-antitrypsin (α_1-AT) by neutrophil metalloproteinases has been reported [Vissers, George, Bathurst, Brennan & Winterbourn (1987) Fed. Proc. Fed. Am. Soc. Exp. Biol. **46**, 1390a; (1988) J. Clin. Invest. **82**, 706–711; Desrochers & Weiss (1988) J. Clin. Invest. **81**, 1646–1650]. To identify the enzyme responsible, supernatant from neutrophils stimulated with phorbol 12-myristate 13-acetate was subjected to preparative SDS/PAGE, both with and without activation of latent metalloproteinases with $HgCl_2$. The lanes were subsequently sliced into pieces, the slices incubated with equimolar amounts of type I collagen and α_1-AT in the presence of $HgCl_2$, and the reaction products separated by SDS/PAGE. With the latent supernatant, the characteristic collagen-cleavage products and cleaved α_1-AT were present in the same slices, corresponding to an M_r of 80000–85000. On treatment with $HgCl_2$ both degradative activities underwent the same molecular-mass shift to a position corresponding to M_r 60000–65000. Western blots of neutrophil supernatants, using a polyclonal antibody to purified collagenase, showed M_r values of 83000 for the latent enzyme and 63000 for the $HgCl_2$-activated enzyme. Neutrophil collagenase was purified to homogeneity and shown also to exist in a second latent form with M_r 70000. When activated to the M_r-63000 form by $HgCl_2$ and incubated with equimolar amounts of collagen and α_1-AT, collagenase cleaved α_1-AT at almost twice the rate at which collagen was cleaved. α_1-AT cleavage was inhibited by 1,10-phenanthroline and by high concentrations of collagen. That the purified collagenase did not contain a contaminant proteinase such as stromelysin was indicated by inability of the preparation to cleave casein. Taken together these results lead us to conclude that neutrophil collagenase is capable of degrading α_1-AT. Neutrophil gelatinase also cleaved α_1-AT, but cleavage was slow when compared with its activity against gelatin.

INTRODUCTION

Human polymorphonuclear leucocytes (neutrophils) contain the metalloproteinase collagenase, which is known to degrade the native collagens types I, II and III, and gelatinase, which has a greater specificity for denatured collagens (Woolley, 1984; Birkedal-Hansen, 1987). Both metalloenzymes are latent when released from the neutrophil and can be activated by either limited proteolysis or incubation with mercurial compounds (Hibbs *et al.*, 1985; Hasty *et al.*, 1986). Whereas collagenase and gelatinase can degrade the major components of connective tissue and have been postulated to aid in the movement of neutrophils through extracellular matrix (Marchesi, 1970), their function in the neutrophil is poorly understood.

We have recently shown that a metalloproteinase activity released from stimulated neutrophils catalytically inactivates the plasma serine-proteinase inhibitor, α_1-antitrypsin (α_1-AT) (Vissers *et al.*, 1987, 1988). Similar findings have been reported by Desrochers & Weiss (1988). Inactivation, measured as loss of elastase-inhibitory capacity, was accompanied by cleavage of an M_r-4000 peptide from the *C*-terminus of α_1-AT (Vissers *et al.*, 1988). Partially purified neutrophil collagenase and gelatinase cleaved α_1-AT, although the gelatinase preparation showed much less activity. In each case the cleavage site was close to the reactive centre, at a Phe–Leu bond between positions P^6 and P^7 (Vissers *et al.*, 1988).

Since collagenase is known to cleave only Gly–Ile or Gly–Leu bonds on triple-helical collagen, it was thought that the Phe–Leu cleavage in α_1-AT was most likely to be due to another metalloenzyme. However, we were unable to identify this putative enzyme. In the present study, by determining the size and the activation properties of the cleaving enzyme, we demonstrate that neutrophil collagenase is primarily responsible for this activity.

MATERIALS AND METHODS

DEAE-Sepharose, Sephadex G-100, and Ficoll 400 were from Pharmacia Fine Chemicals, Uppsala, Sweden; α-casein was from Dr. L. Creamer, N.Z. Dairy Research Institute, Palmerston North, New Zealand; all other specialist chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A.

α_1-AT was purified from human plasma by thiol–disulphide interchange (Laurell *et al.*, 1975). It was 70% active against porcine pancreas elastase, indicating that 30% was oxidized.

Collagenase was purified from homogenized neutrophils in the presence of phenylmethanesulphonyl fluoride (PMSF) and benzamidine by successive column-chromatography steps using DEAE-Sepharose, Zn^{2+}-chelate–Sepharose (Sundberg & Porath, 1974; Porath *et al.*, 1975), Cibacron Blue–Sepharose (Böhme *et al.*, 1972), Sephadex G-100 and gelatin–Sepharose (Hibbs *et al.*, 1985).

Polyclonal antibodies against purified neutrophil collagenase were raised in a rabbit and purified by $(NH_4)_2SO_4$ precipitation and ion-exchange chromatography on DEAE-Sepharose (Tijssen, 1985). The antibody preparation completely inhibited neutrophil collagenase activity against soluble type I collagen and showed no cross-reactivity with neutrophil gelatinase in double-diffusion experiments (results not shown).

Preparation of neutrophils and neutrophil supernatant

Human neutrophils were prepared from the peripheral blood of healthy donors by centrifugation through Ficoll–Hypaque, dextran sedimentation and hypo-osmotic lysis of contaminating

Abbreviations used: α_1-AT, α_1-antitrypsin; PBS, phosphate-buffered saline (150 mM-NaCl/10 mM-sodium phosphate, pH 7.4); PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulphonyl fluoride.

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red cells (Boyum, 1968). The cells were suspended at 2×10^7/ml in 10 mM-sodium phosphate buffer, pH 7.4, containing 150 mM-NaCl (PBS), 1 mM-$CaCl_2$, 0.5 mM-$MgCl_2$ and glucose (1 mg/ml) and were stimulated at 37 °C with phorbol 12-myristate 13-acetate (PMA) (0.1 μg/ml). After 10 min the neutrophils were pelleted by centrifugation at 1000 *g* for 5 min. The supernatant, which contained released gelatinase and collagenase, was collected, 2 mM-PMSF added, dialysed overnight against 20 mM-Tris/HCl (pH 7.5)/5 mM-$CaCl_2$/50 μM-$ZnCl_2$/0.02% NaN_3 and then concentrated 50-fold in an Amicon Centricon 30 microconcentrator.

Activation of metalloproteinases

Latent metalloproteinase activity in neutrophil supernatant was activated by incubation at 37 °C either (i) for 20 min with 5 mM-$HgCl_2$ or (ii) with trypsin (10 μg/200 μl of neutrophil supernatant) at 37 °C. The trypsin concentration was determined by active-site titration using *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride (Chase & Shaw, 1970). Samples were taken at various times and the trypsin reaction terminated by the addition of 5-fold excess of soya-bean trypsin inhibitor and leaving the mixture for 5 min at 25 °C. Conversion into the active form was analysed in Western blots and by estimating enzyme activity by incubation with either collagen or gelatin.

Enzyme assays

Collagenase. Collagenase activity was assessed as specific cleavage of soluble type I calf skin collagen at 25 °C. Appearance of the β^A and α^A fragments was monitored by SDS/PAGE.

Gelatinase. Gelatinase activity was measured using type I collagen which was heat-denatured at 60 °C for 20 min immediately before addition to the reaction mixture. The assay was performed at 37 °C and the reaction products revealed after SDS/PAGE.

Simultaneous assay for collagen or gelatin and α_1-AT cleavage. Equimolar amounts of α_1-AT (18 μg) and collagen or gelatin (90 μg) were incubated with either 0.5 μg of purified collagenase or 50 μl of supernatant from 5×10^7 cells in 120 μl of 20 mM-Tris/HCl (pH 7.5)/5 mM-$CaCl_2$/50 μM-$ZnCl_2$/0.02% NaN_3 (final volume) for 0–160 min. The collagen concentration of 2.5 μM was higher than the reported K_m for neutrophil collagenase and collagen (Hasty *et al.*, 1987), ensuring that collagen was cleaved at near maximum rate. Collagenolytic activity was measured at 25 °C and gelatinolytic activity at 37 °C. The reaction was terminated at intervals by heating with EDTA/SDS/mercaptoethanol, and the reaction mixtures were separated by SDS/PAGE. The gels were scanned to estimate the amounts of collagen and α_1-AT cleaved. The percentage of cleaved collagen was quantified by measuring the formation of single cleavage α^A products using the equation:

$$\text{Degradation (\%)} = \frac{\frac{4}{3}[\alpha^A]}{\frac{4}{3}[\alpha^A]+[\alpha]} \quad \text{(Welgus } et\ al.\text{, 1981)}$$

Localization of collagenase- and α_1-AT-cleaving activity in preparative polyacrylamide gels

Supernatant from 1.5×10^7 neutrophils stimulated with PMA was subjected to preparative SDS/PAGE on a 12.5% (w/v) polyacrylamide gel, both with and without activation of latent metalloproteinases with $HgCl_2$. Samples were not heated before electrophoresis (Heussen & Dowdle, 1980). The gel was washed three times in 20 mM-Tris/HCl (pH 7.5)/5 mM-$CaCl_2$/50 μM-$ZnCl_2$/2.5% Triton X-100/0.02% NaN_3 for 15 min, and once in the buffer without Triton X-100, and each lane was sliced into 2 mm pieces (Hasty *et al.*, 1986). Slices were incubated with 60 μg of collagen and 12 μg of α_1-AT in 100 mM-Tris/HCl (pH 7.5)/5 mM-$CaCl_2$/50 μM-$ZnCl_2$/0.5 mM-$HgCl_2$/0.02% NaN_3, in a total volume of 100 μl for 48 h at 25 °C. The reaction mixtures were then separated by SDS/PAGE.

Electrophoresis

SDS/PAGE was performed by the method of Laemmli (1970) in a continuous slab-gel system (Studier, 1973) using a 9% (w/v) gel for separation of α_1-AT, and collagen or gelatin, and a 12.5% gel for neutrophil supernatant. Samples were incubated for 2 min at 90 °C with EDTA/SDS/mercaptoethanol before loading. Gels were stained either with silver (Heukeshoven & Dernick, 1985) or with picrate/Coomassie Blue (Stephano *et al.*, 1986).

Immunoblotting

Samples to be analysed by immunoblotting were separated by SDS/PAGE and then electrophoretically transferred to nitrocellulose (150 mA for 1 h) (Burnette, 1981). After the excess protein-binding sites were saturated with 3% defatted powdered milk in 50 mM-Tris/HCl (pH 7.9)/150 mM-NaCl/0.05% Tween 20 (solution A), for 30 min at 20 °C, the nitrocellulose was incubated with antibody (0.1 mg/ml in solution A) for 16 h at 20 °C. After washing with solution A, the membrane was incubated with goat anti-rabbit antibody–alkaline phosphatase conjugate (diluted 1:5000) in solution A for 1.5 h at 20 °C. After washing, the immunoblots were revealed by the addition of Nitro Blue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate in 100 mM-Tris/HCl (pH 9.5)/100 mM-NaCl/5 mM-$MgCl_2$. Molecular-mass markers were revealed after blotting on separate tracks with Amido Black in methanol/acetic acid/water (4:1:5, by vol.).

RESULTS

To identify the enzymes responsible for cleaving α_1-AT, we made use of our observation (see below) that the latent metalloproteinases released from neutrophils undergo a decrease in size on activation. Our approach to showing whether collagenase cleaves α_1-AT was to determine whether the M_r shift in collagenase activity is identical with the shift in activity against α_1-AT. It was first necessary to characterize the M_r values of latent and active collagenase.

Determination of the M_r values of latent and active collagenase from neutrophil supernatant

Western blots of supernatant from PMA-stimulated cells with a polyclonal antibody raised against neutrophil collagenase showed one band of M_r 83000 (Fig. 1). Activation with $HgCl_2$ resulted in an M_r shift to 63000 within 5 min, and no further changes were seen in up to 160 min. A M_r-20000 single cleavage product as a result of the M_r shift was not detectable. Full activity assessed by type I collagen cleavage was measured after 5 min (results not shown). By contrast, when trypsin was used as an activating agent, the M_r was reduced to 70000 within the first 5 min (Fig. 1), and up to 160 min incubation was necessary to convert collagenase into the M_r-63000 form. No collagenase activity could be measured until 160 min, indicating that the M_r-63000 form, but not the M_r-70000 form, was active.

These results show that collagenase in PMA-stimulated neutrophil supernatant is latent, with M_r 83000, and upon activation, either by $HgCl_2$ or trypsin, is converted into an active, M_r 63000, form.

Cleavage of collagen and α_1-AT by media from PMA-stimulated neutrophils

To identify the M_r of the enzyme responsible for cleaving

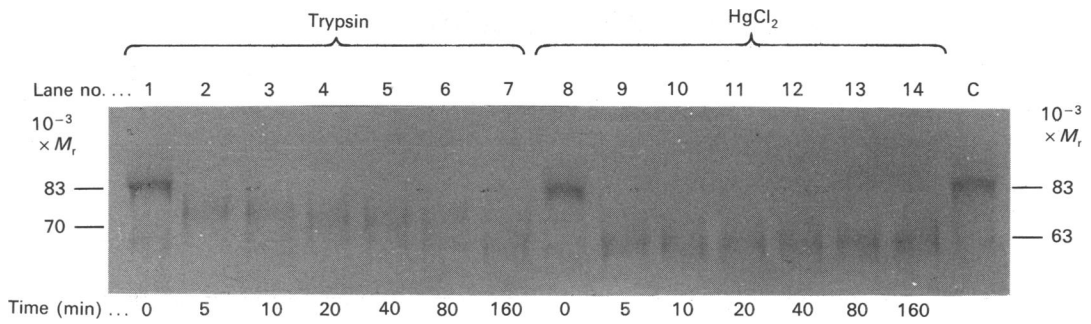


Fig. 1. Immunoblot of neutrophil supernatant

Supernatant from PMA-stimulated neutrophils was concentrated 50-fold. One half of the sample was activated with trypsin at 37 °C (lanes 1–7), and the other with 5 mM-$HgCl_2$ (lanes 8–14). Aliquots were taken at 0, 5, 10, 20, 40, 80 and 160 min, and the reaction stopped as described in the Materials and methods section. Untreated supernatant was used as control (C). Samples were electrophoresed on an SDS/12.5%-polyacrylamide gel, transferred to nitrocellulose and allowed to react with rabbit polyclonal antibody to human neutrophil collagenase, followed by goat anti-rabbit antibody conjugated to alkaline phosphatase. M_r values were determined by comparison with markers, as in Fig. 3.

α_1-AT, $HgCl_2$-activated and untreated samples from PMA-stimulated cells were subjected to SDS/PAGE without prior heat denaturation. The tracks were then sliced horizontally into 2 mm pieces and incubated with equimolar amounts of type I collagen and α_1-AT in the presence of $HgCl_2$. The reaction products were separated by SDS/PAGE to determine which slices contained collagenolytic and α_1-AT-cleaving activity.

As shown in Fig. 2(*a*), the untreated supernatant gave three areas of activity against α_1-AT corresponding to M_r values of 92000, 80000–85000 and 60000–65000. The lanes containing the latter two areas of activity showed parallel cleavage of collagen to its characteristic β^A and α^A products. These correspond to the M_r values of latent and active collagenase in Fig. 1. Collagenolytic activity between M_r 60000 and 65000 in Fig. 2(*a*) reflects collagenase which was activated during the experimental procedure. With the $HgCl_2$-activated supernatant the collagenolytic and the α_1-AT-cleaving activities were detected at a position corresponding to M_r 60000–65000 (Fig. 2*b*). No activity was seen between M_r 80000 and 85000. These results indicate that one of the proteinases responsible for cleaving α_1-AT has the same M_r in its latent form, and shows the same M_r reduction during activation as does collagenase.

The area of cleavage of α_1-AT at M_r 92000 was not associated with any collagenolytic activity (Fig. 2*a*). This M_r is in agreement with that reported for neutrophil gelatinase (Sopata, 1982; Hibbs *et al.*, 1985; Vissers *et al.*, 1988). This activity underwent a slight M_r shift during activation with $HgCl_2$ (Fig. 2*b*).

Cleavage of collagen and α_1-AT by purified neutrophil collagenase

Latent neutrophil collagenase was purified to homogeneity and shown to have an M_r of 70000 (Fig. 3). This form was probably a result of limited proteolysis during enzyme purification of the M_r-83000 latent collagenase seen in the neutrophil supernatant, and it showed no detectable gelatinase activity. When incubated with 5 mM-$HgCl_2$ at 37 °C for 20 min the latent M_r-70000 enzyme was converted into the active M_r-63000 form (results not shown). This preparation was incubated with equimolar amounts of type I collagen and α_1-AT at 25 °C for 0–160 min. Subsequent analysis of the samples by SDS/PAGE showed time-dependent cleavage of the collagen to its specific cleavage products and cleavage of α_1-AT. Comparison of the percentage of cleavage of α_1-AT and collagen, obtained by scanning the gel (Fig. 4), showed that, in the first 20 min of incubation, collagenase cleaved α_1-AT at almost twice the rate at which it cleaved collagen. The maximal amount of α_1-AT cleavage

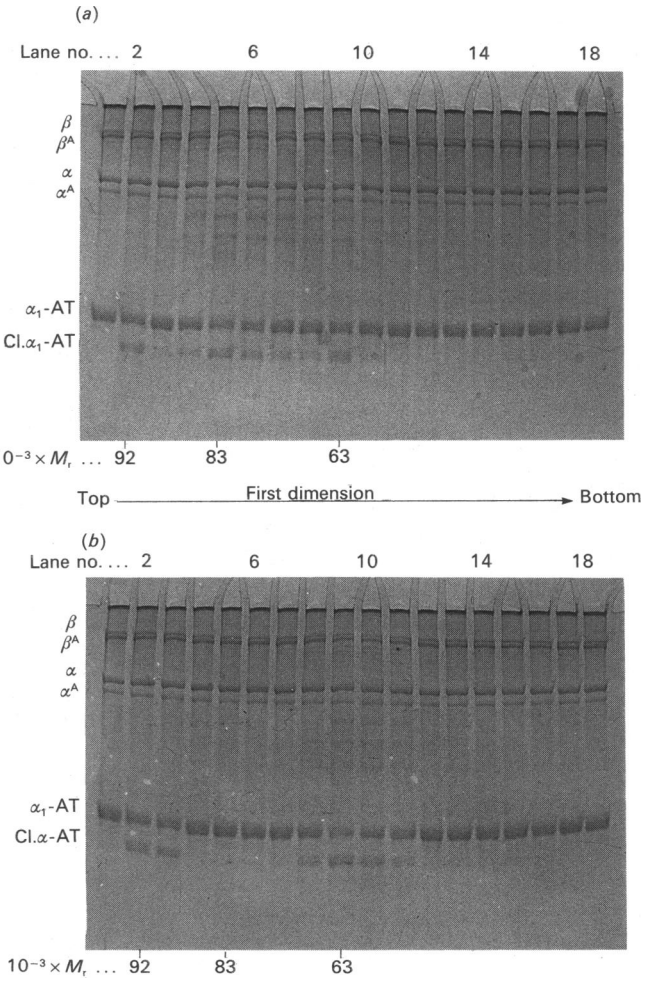


Fig. 2. Collagenase activity and α_1-AT-cleaving activity of neutrophil supernatant

Supernatant from PMA-stimulated neutrophils was electrophoresed (*a*) before and (*b*) after activation of latent metalloproteinases with $HgCl_2$. The lanes were sliced into 2 mm pieces and each piece incubated with equimolar amounts of collagen and α_1-AT at 25 °C for 48 h in the presence of $HgCl_2$. The reaction products were separated on SDS/9%-polyacrylamide gels. First dimension top–bottom is left to right in this Figure. The positions of bands were determined by comparison with M_r markers. Abbreviation: Cl., cleaved.

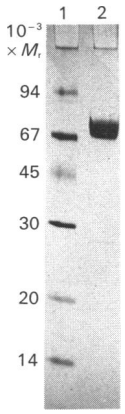
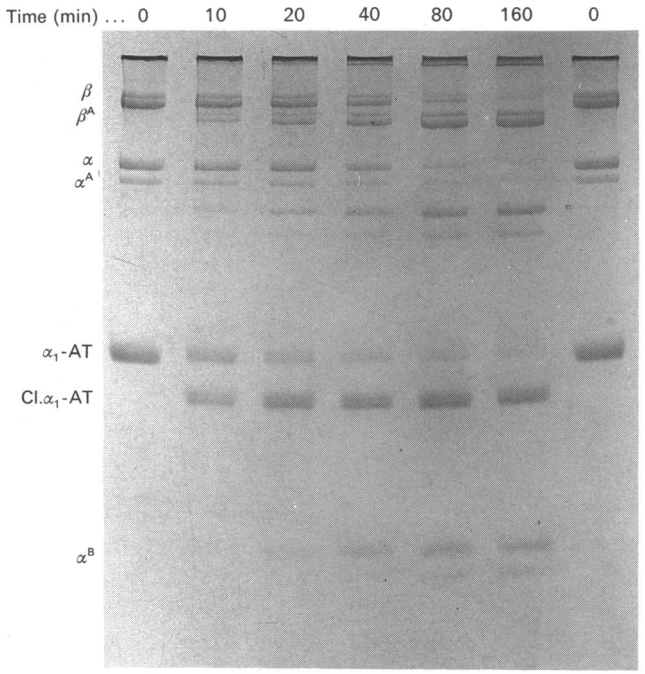


Fig. 3. SDS/PAGE of purified latent neutrophil collagenase

A 9% gel containing mercaptoethanol and silver-stained is shown: lane 1, M_r markers in descending order [phosphorylase *b* (94000), BSA (67000), ovalbumin (45000), carbonic anhydrase (30000), soya-bean trypsin inhibitor (20000) and α-lactalbumin (14000)]; lane 2, collagenase.



Substrate	Time (min) ...	Cleavage (%) 0	10	20	40	80	160
Collagen		0	27	38	51	70	80
α_1-AT		0	51	63	72	77	79

Fig. 4. Enzyme activity of purified neutrophil collagenase

Purified neutrophil collagenase was activated with 5 mM-$HgCl_2$ at 37 °C for 20 min before the addition of equimolar amounts of collagen and α_1-AT. The reaction was terminated at the times indicated by heating a sample for 2 min at 90 °C in the presence of EDTA, SDS and mercaptoethanol before SDS/PAGE. Abbreviation: Cl., cleaved.

corresponds to the approx. 70% native α_1-AT in our preparation. This represents maximum activity, since oxidized α_1-AT is cleaved only very slowly by neutrophil metalloenzymes (Vissers *et al.*, 1988). Both the collagenolytic and α_1-AT-cleaving activity were inhibited by 1 mM-1,10-phenanthroline (results not shown).

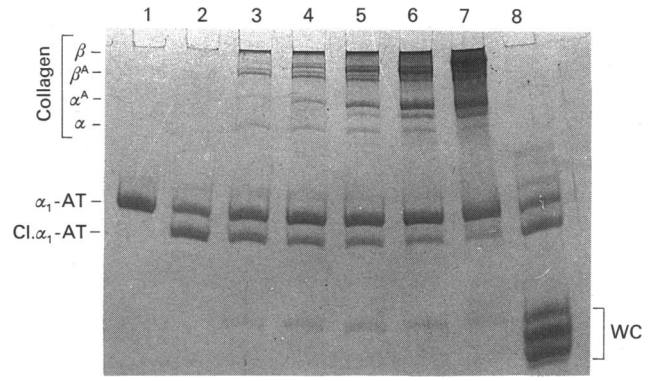


Fig. 5. Cleavage of α_1-AT and increasing amounts of collagen by neutrophil collagenase

Purified and activated neutrophil collagenase was incubated with a substrate mixture containing constant amounts of α_1-AT (2 μM) and increasing amounts of collagen (0.5–8.3 μM). The molar ratio of collagen to α_1-AT was: lane 2, no collagen added; lane 3, 0.25:1; lane 4, 0.5:1; lane 5, 1:1; lane 6, 2:1; lane 7, 4:1. In lane 8, the amount of collagen added in lane 7 was replaced by casein. Lane 1 contains α_1-AT and buffer only. The reaction was stopped after incubation for 18 h at 25 °C, and the reaction products were separated by SDS/10%-PAGE. Abbreviations: Cl., cleaved; WC, whole casein.

Cleavage of collagen and α_1-AT by neutrophil collagenase was repeated with a constant concentration of α_1-AT (2 μM) and increasing concentrations of type I collagen (0.5–8.3 μM) (Fig. 5). Increasing the concentration of collagen progressively reduced α_1-AT cleavage indicating that collagen competes for the α_1-AT-cleaving activity. α_1-AT cleavage was not affected when casein as a control for non-specific protein binding was added instead of collagen (Fig. 5).

However, increasing the α_1-AT concentration from 2 μM to 170 μM at constant collagen concentrations (1 μM) did not decrease the amount of collagen cleavage (results not shown). In this concentration range, in the presence or absence of collagen, saturation of the enzyme with α_1-AT was not apparent. α_1-AT concentrations above 170 μM (10 mg/ml) could not be used because of solubility problems (results not shown).

Activity of the collagenase preparation towards α-casein

Cleavage of α_1-AT by the matrix metalloproteinase stromelysin, isolated from rabbit synovial fibroblasts, has been reported by Chin *et al.* (1985). Stromelysin co-migrates with collagenase in standard chromatographic runs and degrades many of the proteins of the extracellular matrix. Of the substrates tested, stromelysin preferentially degraded the α-chain of casein, but did not degrade type I collagen (Chin *et al.*, 1985).

To rule out the possibility that α_1-AT degradation by our purified collagenase preparation was due to an as yet unknown stromelysin-like neutrophil enzyme, we investigated the caseinolytic activity of the collagenase.

The experiment was carried out with purified collagenase at an enzyme/substrate ratio of 1:200 (w/w) with either bovine milk α-casein or α_1-AT at 25 °C. After 6 h, 50% of the α_1-AT was cleaved, but no α-casein was degraded (Fig. 6). A stromelysin-like enzyme would have degraded α-casein completely in this period of time (Chin *et al.*, 1985) and therefore cannot account for the α_1-AT-cleaving activity.

Comparative cleavage of α_1-AT and gelatin by gelatinase in neutrophil supernatant

The comparative cleavage of α_1-AT and gelatin by neutrophil

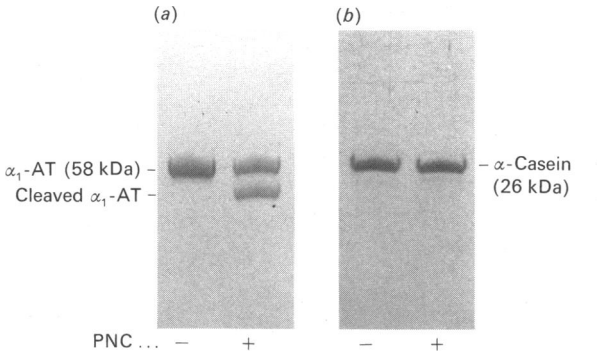


Fig. 6. Comparison of the cleavage of α_1-AT and α-casein by neutrophil collagenase

Substrates were incubated separately at 25 °C with (+) or without (−) purified neutrophil collagenase (PNC) for 6 h at an enzyme/substrate ratio of 1:200 (w/w). The reaction mixtures were then separated by SDS/PAGE on (*a*) 10% gels for α_1-AT and (*b*) 14% gels for casein.

gelatinase was assessed by using a cell supernatant in which the gelatinase was activated by a short incubation with trypsin but in which collagenase remained latent. Treatment of the supernatant with trypsin for periods up to 1 h gave no degradation of collagen (Fig. 7*a*), but activated gelatinase, as shown by the degradation of gelatin (Fig. 7*b*). This lack of activation of collagenase is consistent with the lack of formation of the M_r-63000 band during this period (Fig. 1*a*). Under conditions in which we observed complete gelatin degradation, no cleavage of α_1-AT was seen (Fig. 7*b*). This indicates that gelatinase is less active towards α_1-AT than it is for gelatin, and will not attack α_1-AT in the presence of gelatin. Although no α_1-AT degradation was seen after 5 min incubation (Fig. 7*b*), after 18 h cleavage of α_1-AT was apparent (Fig. 7*a*). Since there was no accompanying collagen degradation in the sample, the α_1-AT cleavage was most likely due to gelatinase. Consistent with this, the amount of cleavage of α_1-AT at each point in Fig. 7(*a*) reflects closely the amount of active gelatinase in the supernatant (Fig. 7*b*).

As shown in Fig. 7(*a*), incubation of the neutrophil supernatant with $HgCl_2$ activated collagenase and fully converted into the M_r-63000 form (Fig. 1). However, $HgCl_2$ caused less activation of gelatinase activity than did exposure to trypsin for 20 min or longer (Fig. 7*b*). Therefore the α_1-AT degradation by the $HgCl_2$-activated supernatant (Fig. 7*a*) was due to the action of partly activated gelatinase and completely activated collagenase.

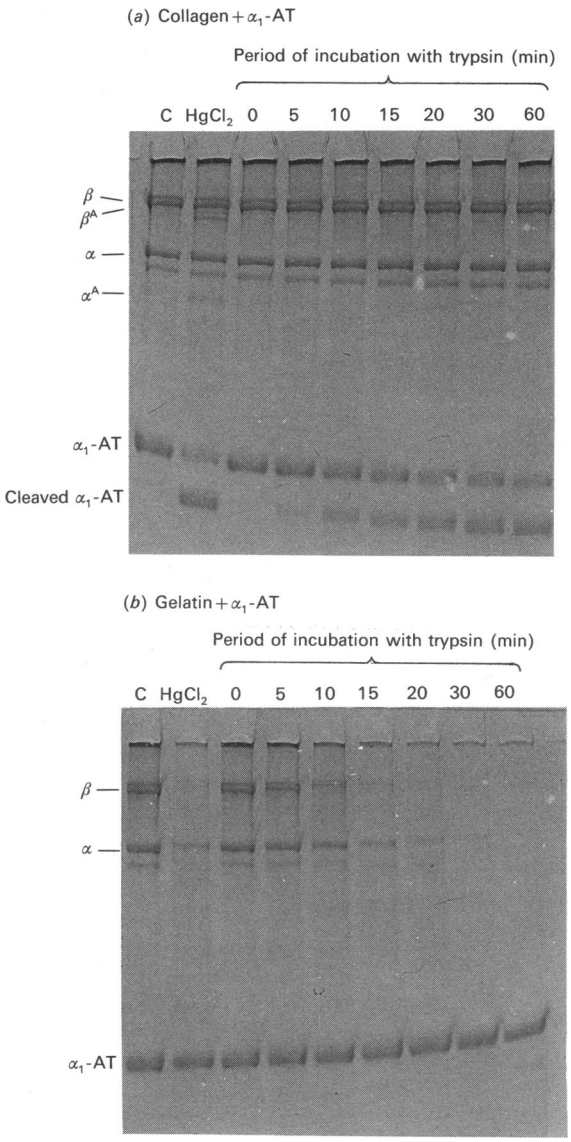


Fig. 7. Comparative activity of gelatinase in neutrophil supernatant against gelatin or α_1-AT

Supernatant from PMA-stimulated neutrophils was incubated either with $HgCl_2$ for 60 min or trypsin at 37 °C for the times indicated. Subsequently the samples were incubated (*a*) for comparative degradation of collagen and α_1-AT with equimolar amounts of both substrates for 18 h at 25 °C or (*b*) for comparative cleavage of gelatin and α_1-AT with equimolar amounts of both substrates 5 min at 37 °C. The control (C) was incubated without supernatant. The reaction products were separated by SDS/9%-PAGE.

DISCUSSION

We have demonstrated that both collagenase and gelatinase from human neutrophils have the capacity to cleave α_1-AT. Using preparative SDS/PAGE we have demonstrated that α_1-AT-cleaving activity in neutrophil supernatant coincides with the M_r for latent collagenase and undergoes an identical change in M_r on activation. We have also shown that α_1-AT is cleaved simultaneously with collagen by purified collagenase. This activity was inhibited by chelating agents. The collagenase purification included a gelatin–Sepharose column, which removes all traces of gelatinase, and the preparation was shown to contain no gelatinase activity. It was also free of caseinolytic activity, which rules out contamination with an as yet unknown stromelysin-like enzyme that could conceivably exist in the neutrophil.

Further support for cleavage of both substrates being due to the same enzyme was the progressive inhibition of α_1-AT degradation by increasing collagen concentrations. The converse inhibition of collagen degradation by α_1-AT was not observed, but this can be explained by a very high K_m for α_1-AT and the inability to saturate the enzyme with achievable inhibitor concentrations.

In our previous paper we showed that cleavage occurred at a Phe–Leu bond on α_1-AT and was the same cleavage site for purified gelatinase and partially purified collagenase, which lacked any gelatinolytic activity (Vissers *et al.*, 1988). Since this is a cleavage site unknown for neutrophil collagenase, which has been thought to be a highly specific enzyme, it then seemed unlikely that collagenase was capable of cleaving α_1-AT. This, together with some doubt as to the purity of our collagenase preparation, led us to conclude that another enzyme was re-

sponsible for α_1-AT cleavage. Our present results, however, make the existence of another α_1-AT-cleaving enzyme extremely unlikely and indicate a new and unusual activity for neutrophil collagenase.

When purified collagenase was incubated with equimolar amounts of soluble type I calf skin collagen and α_1-AT, it cleaved α_1-AT at almost twice the rate of collagen cleavage. This suggests that α_1-AT can compete with collagen as a substrate for collagenase, and that this reaction may be significant *in vivo*. Preliminary investigations on the rate of α_1-AT cleavage by collagenase showed that the enzyme is not saturated at 170 μM-α_1-AT in solution, a value much higher than the reported K_m for collagen, 0.6–1.8 μM (Hasty *et al.*, 1987). Therefore the preference for α_1-AT under physiological conditions may be even greater than we observed.

We also demonstrated α_1-AT cleavage in supernatants in which gelatinase, but not collagenase, was active, and showed that this activity corresponds to the M_r for gelatinase. Gelatin, however, was a much better substrate for gelatinase than was α_1-AT.

We previously showed α_1-AT-cleaving activity in the supernatant of neutrophils stimulated with fMet-Leu-Phe that released gelatinase but no detectable collagenase (Vissers *et al.*, 1988). Although we then had doubts whether this was due to gelatinase, we now consider that we may have underestimated gelatinase activity against α_1-AT, and that it was responsible for the cleavage observed. However, when neutrophils release collagenase and gelatinase, both contribute to α_1-AT cleavage.

The ability of neutrophil collagenase to cleave peptide bonds other than Gly–Leu or Gly–Ile is not a unique observation for collagenases. Sottrup-Jensen & Birkedal-Hansen (1989) have reported that human fibroblast collagenase cleaves the 'bait' regions of human and rat α-macroglobulins several orders of magnitude faster than collagen chains. The cleavage sites include Ala–Leu and Ala–Met in rat α_1-macroglobulin, and His–Leu and Phe–Leu in rat α_2-macroglobulin. Our results indicate that neutrophil collagenase must not be considered as specific for collagen and that the susceptibility to this proteinase of other serpins (serine-proteinase inhibitors) and other compounds of connective tissue should be examined.

Our results also provide information on the mechanism of activation of neutrophil collagenase. They show an M_r of 83000 for the latent collagenase released from PMA-stimulated neutrophils, which had decreased to M_r 70000 during purification. We observed that collagenase underwent an M_r decrease when activated by either $HgCl_2$ or trypsin. This could be explained by a mechanism similar to that proposed by Grant *et al.* (1987) and Okada *et al.* (1988) for skin- or rheumatoid-synovial-fibroblast collagenase, in which the activator promotes autolytic cleavage to the active enzyme.

An M_r difference of approx. 20000 for latent and active neutrophil collagenase has previously been observed by Hasty *et al.* (1986) and Sorsa (1987). We report a third form of collagenase with an intermediate M_r. Although there are similarities between the three studies, a comparison between the different latent and active forms of collagenase is difficult to make. Our results also demonstrate a difference in the relative abilities of $HgCl_2$ and trypsin to activate gelatinase and collagenase, with collagenase showing surprising resistance to activation by trypsin.

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