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# A LATENT GELATIN SPECIFIC PROTEINASE OF HUMAN LEUCOCYTES AND ITS ACTIVATION

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## Summary

1. Gelatin specific proteinase (gelatinase) exists in human leucocytes extracts mainly in a latent form.
2. It is activated by different proteinases as well as by some chemicals (urea, NaSCN, $HgCl_2$).
3. Non-proteolytic activation of latent gelatinase and the decreasing of its molecular weight associated with it strongly suggests that it is an enzyme-inhibitor complex.

## Introduction

The human leucocytes contain at least two enzymes which participate in collagen degradation: collagenase [1] and gelatin specific proteinase (gelatinase) [2]. It has been demonstrated that these enzymes exist in extracts of human leucocytes in both active and latent forms [2,3]. The latent leucocyte enzymes are activated by different proteinases [4]. Recently, Kaczanowska et al. [5] found that latent collagenase and latent gelatinase are activated by chymotrypsin-like enzyme isolated from human leucocytes. However, the most important factor which controls extracellularly an activity of latent collagenolytic enzymes, especially in pathology, seems to be a specific activator in rheumatoid synovial fluids [3,4,6].

As in the other systems [7,8] it is not known whether the latent form of leucocyte gelatinase represents an enzyme-inhibitor complex or a zymogen. Previously we suggested [2,4] that trypsin activation of the latent gelatinase is a strong evidence for the existance of a zymogen which presumably would be activated as a consequence of limited proteolysis. On the other hand however, the reports on non-proteolytic activation of some latent tissue collagenses by

e.g. sodium thiocyanate [9—11] or the hiol-blocking reagents [4,12—14] suggest that activation can be a result of dissociation of the enzyme-inhibitor complex. The above controversy made us study the nature of the latent leucocyte gelatinase and its activation.

## Materials and Methods

*Enzyme preparation*

Leucocytes were obtained from heparinised whole blood of human donors as described by Kruze and Wojtecka [3]. The cells were suspended in 0.02 M Tris-HCl buffer, pH 7.5, containing 0.002 M $CaCl_2$ and disrupted by repeated freezing and thawing (6 times). After centrifuging at 10 000 × *g* for 30 min at 4°C the obtained supernatant was subjected to DEAE-Sephadex chromatography under the conditions used previously [2] for purifying the leucocyte gelatinase. Fractions containing enzyme activity were pooled concentrated with Aquacid II and applied on Sephadex G-200 column (1.6 × 75 cm) equilibrated with 1 M NaCl in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.005 M $CaCl_2$. Effluents were assayed for a latent gelatinase activity and active fractions were collected and concentrated with Aquacid II. Finally, all preparations of latent gelatinase were dialysed against 0.05 M Tris-HCl buffer, pH 7.5, containing 0.005 M $CaCl_2$ and 0.2 M NaCl and storen frozen.

Acid soluble calf skin collagen was isolated and purified by the method of Kang et al. [15]. Gelatin was prepared from collagen solution dialysed against the above buffer by its thermal denaturation at 45°C for 15 min.

*Enzyme assay*

Gelatinase activity was assayed by the method of Harris and Krane [16] except small modifications described previously [2]. The amount of hydroxyproline in peptides released from gelatin at 37°C and soluble in 15% cold trichloroacetic acid was taken as a measure of gelatinase activity. Content of the latent form of gelatinase was expressed as an increase of its proteolytic activity after enzymatic or non-enzymatic activation. One unit of gelatinase activity was defined as the amount of the enzyme which hydrolysed 1 μg of gelatin per minute at 37°C. One unit of latent gelatinase activity was the amount of the enzyme which after activation gave the activity of the one unit defined above. Specific activity refered to μg of gelatin hydrolysed per minute at 37°C per mg of protein.

Hydroxyproline concentration was determined according to the procedure of Stegemann and Stalder [17].

Protein concentration was measured by the method of Lowry et al. [18] using bovine serum albumin as a standard.

The specific activator was isolated from rheumatoid synovial fluids and purified as previously described [6].

Chymotrypsin-like enzyme was kindly provided by Dr. J. Kaczanowska of this laboratory. The enzyme was isolated from human leucocytes and purified by chromatography on CM-Sephadex, Fenylo-Sepharose and Sephadex G-75 (to be published).

Human γ-globulin was kindly offered by Dr. I. Buchowicz, Serum and Vaccine Manufacture, Warsaw.

The following commercially available products were used: bovine serum albumin, ovoalbumin, trypsin (Koch-Light); Triton X-100, iodo-acetic acid (BDH); Tris (Fluka A.G.); 5,5'-dithiobis(2-nitrobenzoic acid) (Sigma); *p*-chloromercuribenzoic acid sodium salt (Chemapol, Czechoslovakia); soyabean trypsin inhibitor (Mann. Res. Lab. Inc., U.S.A.); hydroxyproline (Nutritional Biochem. Corp., U.S.A.); DEAE-Sephadex A-50, Sephadex G-200 (Farmacia); Aquacid II (Calbiochem). All other reagents were Polish commercial products of analytical grade.

## Results

### 1. *Activation of latent leucocyte gelatinase by proteinases*

Under conditions applied for extraction gelatinase it remained almost entirely in its latent form (specific activity 29.1 ± 6.6 units per mg of protein). The partially purified gelatinase preparations contained no more than 3% of active enzyme when compared to the total activity unmasked by trypsin.

Activation of latent gelatinase can be achieved by its treatment with various proteolytic enzymes. We have found that chymotrypsin-like enzyme activated the latent gelatinase in an incubation time dependent manner (Fig. 1) like did trypsin and specific activator.

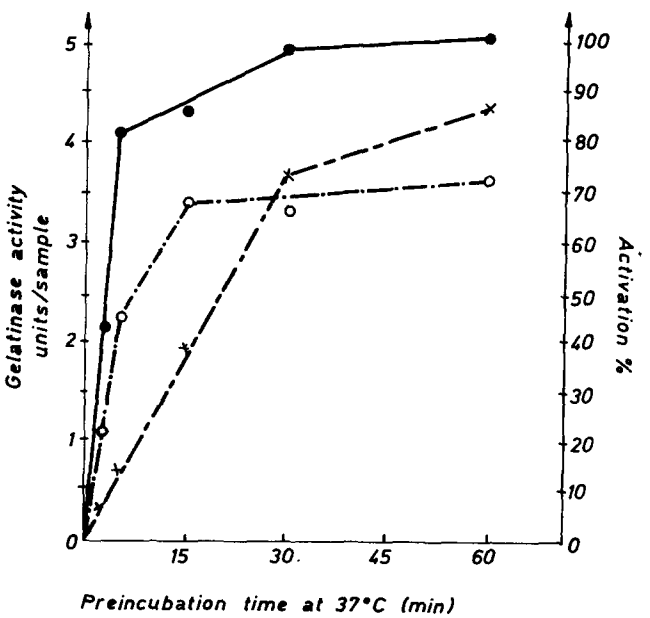


Fig. 1. Activation of latent gelatinase by specific activator, trypsin and chymotrypsin-like enzyme. A latent gelatinase preparation (9 μg) was preincubated at 37°C with 50 μg specific activator (●———●), 10 μg trypsin (X— · — · —X) or 50 μg chymotrypsin-like enzyme (○— · — · —○). The trypsin and chymotrypsin-like enzyme activation was stopped by adding excess of soyabean trypsin inhibitor (500 μg/ml final concentration). The samples were incubated with gelatin (3 mg in sample) at 37°C for 5 h. Resultant gelatinase activity was assayed as described under Materials and Methods. The activation with specific activator was defined as 100%.

TABLE I

EFFECT OF NON-ENZYMATIC TREATMENT ON ACTIVATION OF LATENT LEUCOCYTE GELATINASE

Aliquots of leucocyte gelatinase preparation (38 μg) were dialysed overnight against buffer (0.01 M Tris, pH 7.5, containing 0.005 M $CaCl_2$ and 0.2 M NaCl) or urea, NaSCN, NaCl, Triton X-100 in the buffer or against buffer of pH 10.0. Following removal of urea, NaSCN, Triton X-100, NaCl and buffer of pH 10.0 by exhaustive dialysis against the first buffer, each sample was assayed for gelatinase activity either directly or after trypsin activation.

| Gelatinase preparation dialysed versus | Activity of gelatinase (units/sample) | |
|---|---|---|
| | Trypsin unactivated | Trypsin activated |
| Buffer | 0.12 | 8.22 |
| 6 M urea | 1.58 | 6.76 |
| 3 M NaSCN | 1.66 | 4.78 |
| 3.5 M NaCl | 0.11 | 8.23 |
| 0.05 M glycine-NaOH buffer pH 10.0 | 0.11 | 8.52 |
| Triton X-100 (1 mg/ml) | 0.07 | 15.88 |

## 2. *Activation of latent leucocyte gelatinase by some chemicals*

It was found that a different degree of activation is obtained by treating the latent gelatinase by some chemicals (Tables I and II). As can be seen, gelatinase activity could be elicited by dialysis against 3 M NaSCN and 6 M urea (about

TABLE II

COMPARISON OF THE ACTIVATION OF LATENT GELATINASE BY THE THIOL-BLOCKING REAGENTS AND TRYPSIN

Latent gelatinase preparation (9 μg) was preincubated at room temperature for 30 min with the indicated amount of appropriate chemicals in 0.05 M Tris buffer, pH 7.5, containing 0.005 M $CaCl_2$ and 0.2 M NaCl. Aliquots of sample in 0.5 mM $HgCl_2$ were dialysed overnight against the buffer or against 0.5 mM $HgCl_2$ in the buffer. All samples were assayed for gelatinase activity either directly or after trypsin activation. The activation with trypsin was terminated by addition of soyabean inhibitor (500 μg/ml final concentration).

| | Reagents added (mM) | Activity of gelatinase (units/sample) | |
|---|---|---|---|
| | | Trypsin unactivated | Trypsin activated |
| Gelatinase preparation | — | 0.11 | 1.57 |
| Gelatinase preparation | | | |
| + $HgCl_2$ | 0.05 | 0.32 | 1.15 |
| | 0.10 | 1.10 | 0.37 |
| | 0.50 | 1.10 | 0.37 |
| | 1.00 | 0.92 | 0.57 |
| Gelatinase preparation | | | |
| + $HgCl_2$ then: | 0.50 | | |
| (a) dialysed versus buffer | | 0.27 | 1.11 |
| (b) dialysed versus $HgCl_2$ (0.5 mM) in buffer | | 1.02 | 0.15 |
| Gelatinase preparation | | | |
| + *p*-chloromercuribenzoate | 0.50 | 0.47 | — |
| Gelatinase preparation | | | |
| + 5,5′-dithiobis(2-nitrobenzoic acid) | 0.50 | 0.12 | — |
| Gelatinase preparation | | | |
| + iodo-acetic acid | 0.50 | 0.10 | — |

20% of that with trypsin). Inclusion of $HgCl_2$ (0.1—0.5 mM) in the gelatinase assay medium gave 70% of activation of the latent enzyme obtainable with trypsin, whereas *p*-chloromercuribenzoate (0.5 mM) activated only 30% of the latent gelatinase. Presence of $HgCl_2$ in the assay medium seems to be essential in this activation study since a removal of $Hg^{2+}$ by dialysis after preincubation of latent enzyme with $HgCl_2$ resulted in the decrease of gelatinase activity. Simultaneously, the increase in amount of latent gelatinase activable by trypsin was observed (Table II). Other chemicals tested 5,5'-dithiobis(2-nitrobenzoic acid), iodoacetic acid, buffer of pH 10.0 and 3.5 M NaCl, were without effect on activation. It is worth noticing that after dialysis of latent gelatinase against Triton X-100 about two-fold increase of trypsin-activable gelatinase was observed. The possible tendency of latent enzyme to interact with other proteins and capability of Triton X-100 to dissociate such complex could explain such result.

The disappearance of gelatinase activity during removal of $Hg^{2+}$ from assay medium and the relatively low activation produced by dialysis against 3 M

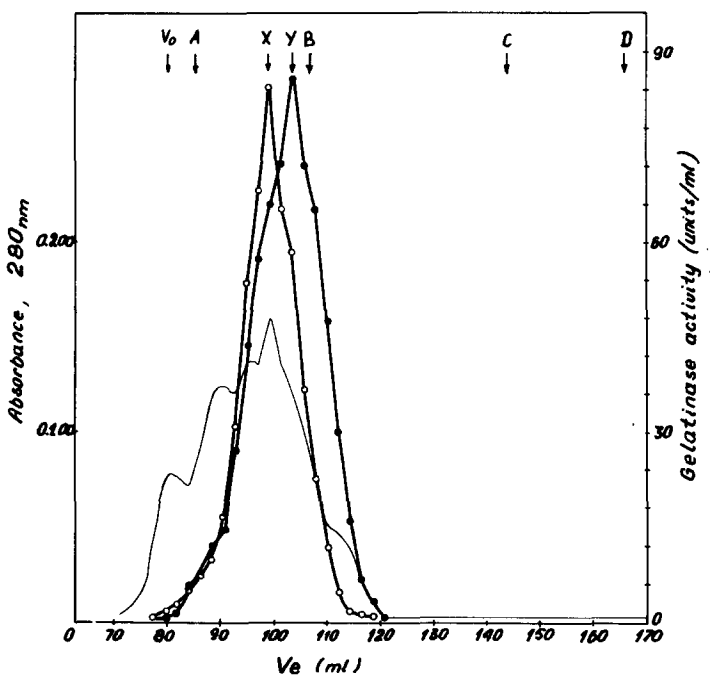


Fig. 2. Estimation of molecular weight of latent and active leucocyte gelatinase by gel filtration on Sephadex G-200. A latent gelatinase preparation (4.4 mg of protein) recovered from DEAE-Sephadex, A-A-50 was incubated with $HgCl_2$ (0.5 mM) for 30 min at room temperature and charged to a Sephadex G-200 column (1.6 × 100 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.005 M $CaCl_2$, 0.2 M NaCl and 0.0001 M $HgCl_2$. Fractions of 2.1 ml were collected at the flow rate of 7 ml/h and assayed for gelatinase activity either directly or after trypsin activation. The activation with trypsin was terminated by addition of soyabean trypsin inhibitor (500 μg/ml final concentration). ———, protein ($A_{280nm}$); •———•, active gelatinase activity; ○———○, latent gelatinase activity. A, B, C and D represent the $V_e$ values for the protein standards γ-globulin and its dimer, bovine serum albumin and ovo-albumin respectively. $V_0$, void volume. The mol. wt. of 175 000 for the latent gelatinase (X) and 156 000 for the active enzyme (Y) were calculated from a plot of $V_e/V_0$ against log(molecular weight) of the protein standards.

NaSCN and 6 M urea (followed by removal of these reagents by dialysis) can be explained by reversible binding of inhibitor(s) with already activated enzyme. To check this possibility we tried to separate the active enzyme from a hypothetical inhibitor using gel filtration. Chromatography of a sample containing latent gelatinase in 0.5 mM $HgCl_2$ on Sephadex G-200 resulted in producing an active enzyme with lower molecular weight (156 000). However, a latent enzyme was also found in higher molecular weight fractions (Fig. 2).

## Discussion

In 1974 Sopata and Dancewicz [2] have shown that apart of collagenase human leucocytes contain another collagenolytic enzyme, a gelatin specific proteinase, gelatinase. Gelatinase proceeds with the collagen degradation beyond the step catalyzed by tissue collagenases. Partially purified gelatin specific proteinase degrades thermally denatured collagen (gelatin) and Azocoll but to a small extent only. It does not degrade collagen, bovine nasal cartilage proteoglycans, casein, histones, haemoglobin, elastin and synthetic peptide substrate used in assay of bacterial collagenase [2,19]. Its properties make it similar to the enzyme produced by rabbit bone in culture [20] but it seems to be different from other neutral metallo-proteinases degraded gelatin [16,21].

Our results indicate that gelatinase exists in leucocytes extracts mainly in a latent form. The conditions which would favour the dissociation of an enzyme-inhibitor complex such as amonium sulfate fractionation, DEAE ion exchange chromatography and gel filtration, failed to activate the latent gelatinase [2]. Moreover, no active enzyme was found after Fenylo-Sepharose chromatography. However, the latent enzyme is activated by proteinases. Specific activator of rheumatoid synovial fluid, chymotrypsin-like enzyme of human leucocytes and pancreatic enzymes, trypsin, chymotrypsin, and elastases all of them were found to be the effective activators. The above indicates low specificity of activation obtained with proteinases. On the other hand, activation of the latent gelatinase is achieved in a non-proteolytic way using some chemicals. Dialysis of latent gelatinase against 3 M NaSCN or 6 M urea followed by removal of these agents by dialysis resulted in partial activation of the latent form. The latent gelatinase was also found to be activated by $HgCl_2$ and the activation was almost as effective as with trypsin treatment. This process depended on concentration of mercuric chloride with maximum of activation at 0.1—0.5 mM $HgCl_2$. It is worth noticing that the activation of latent gelatinase is reversed when $Hg^{2+}$ are removed by dialysis and that such inactive form is reactivated by, for example, trypsin. The effect of sodium thiocyanate, urea and mercuric chloride can be attributed to the dissociation of a gelatinase-inhibitor complex or may be a result of a conformational changes in the molecule of inactive enzyme. The results of the experiments illustrated in Fig. 2 speak in favour of the first explanation, since after treating the latent form with 0.5 mM $HgCl_2$ and gel filtration, the molecular weight of the latent enzyme estimated at 175 000, decreased to 156 000. Active gelatinase of about 150 000 molecular weight, was also found after trypsin or specific activator activation [22]. Thus, the difference in molecular weight between latent and active enzyme is approximately 20 000. After chromatography of a latent

gelatinase in 8 M urea on Sephadex G-200, a considerable increase of the amount of active enzyme (from 0 to 70%) with concomitant marked loss of latency of the enzyme was also observed. As yet however, we were not able to isolate an inhibitory factor which could be derived from latent gelatinase after its activation with $HgCl_2$ or urea. Sakamoto et al. [10] also failed to isolate an inhibitor after activating latent chick bone collagenase with 3 M NaI and 3 M NaSCN. On the other hand, Shinkai et al. [23] were able to isolate two collagenase inhibitors after treating the latent chick skin collagenase with 3 M NaI. Reynolds et al. [24] suggested lately that 4-amino-phenylmercuric acetate activates collagenase simply dissociating an enzyme-inhibitor complex. The authors showed that the activation of latent collagenases decreased their molecular weights as did trypsin activation. These observations are similar to those of Vater et al. [14]. Recently, Steven et al. [25] proposed a new interesting mechanism for activating a neutral protease by Hg ions. So, it is likely that convertion of the latent gelatinase to the active enzyme obtained with urea, NaSCN and mercuric compounds indicates that latent gelatinase present in human leucocytes extracts is an enzyme-inhibitor complex.

Our recent attempts to detect inhibitor(s) of leucocyte gelatinase have been successful (to be published). It was found that leucocytes extracts contained both an inhibitor of the urea-activated gelatinase as well as a latent form of the enzyme. The presence of excess amount of free inhibitor in leucocytes extracts suggests that it does not derive from zymogen, like in the case of pepsinogen activation [26].

In summary, it seem likely that latent gelatinase presents in leucocytes extracts is the enzyme-inhibitor complex rather that a true zymogen. It is also possible that latency of gelatinase may develop during enzyme preparation. One can assume that gelatinase is synthesized as an active enzyme and it can combine with inhibitor(s) present in the same cells. It is difficult, at the present moment, to exclude the possibility that gelatinase is synthesized as a zymogen which, after being activated binds with inhibitor. It remains to be found in what forms the gelatinase is produced by human leucocytes, stored within the cells and released into extracellular medium.

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