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A LATENT COLLAGENASE FROM RHEUMATOID SYNOVIAL FLUID

PURIFICATION AND PARTIAL CHARACTERIZATION

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Summary

1. A latent collagenase (EC 3.4.24.3) has been isolated from rheumatoid synovial fluids and purified by $(NH_4)_2SO_4$ precipitation and column chromatography, utilising Sephadex G-150, DEAE-Sephadex A-50 and Sephadex G-100 superfine grade.

2. The final preparation activated by trypsin (EC 3.4.21.4) had a specific activity against thermally reconstituted collagen fibrils of 259 μg collagen degraded/min per mg enzyme protein, representing a nearly 800-fold increase over that of the original rheumatoid synovial fluid.

3. The latent collagenase preparation can be activated by trypsin and to some extent by $HgCl_2$ but not by 3 M NaSCN, 3.5 M NaCl, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) or *p*-chloromercuribenzoate.

4. Inhibition studies and the acrylamide gel electrophoretic pattern of collagen degradation products showed that the trypsin-activated enzyme has the essential features of a neutral collagenase.

5. The molecular weights, determined by calibrated gel filtration, were 52 000 and 43 000 for the latent and the activated enzyme, respectively.

6. The nature of the latency of synovial fluid collagenase is discussed.

Introduction

The key role in the breakdown of joint tissue collagen in rheumatoid arthritis is ascribed to the action of specific collagenases (EC 3.4.24.3) [1]. The main contribution to the enzymatic attack upon collagenous structures seems

Abbreviation: DFP, diisopropyl phosphorofluoridate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

to come from the synovial membrane collagenase [2,3] and collagenase released from the polymorphonuclear leucocytes infiltrated into the joint cavity [4]. As in other tissues, these collagenases exist in both active and latent forms [5—7]. The latter may be activated by enzymatic or chemical treatment [8—12]. The nature of the latent form of collagenase has not yet been unequivocally demonstrated. It is considered to be a zymogen or an enzyme-inhibitor complex. It is possible that there exist in rheumatoid synovial fluid these two types of the latent form of collagenase.

Recently, it has been shown that, apart from the active collagenases [13,14], synovial fluid contains two different inactive forms of the enzyme which can be activated by treatment with 3 M NaSCN [15,16]. One of them is a high molecular weight collagenase-α_2-macroglobulin complex, the other is believed to be a complex of collagenase with a much lower weight inhibitor. This inhibitor is unidentified.

The paper describes the purification and some properties of the latent collagenase isolated from rheumatoid synovial fluid. This purified latent enzyme is not activated by NaSCN, only partly by $HgCl_2$ and fully activated by trypsin.

Materials and Methods

Synovial fluid. Synovial fluid was aspirated from knee joints of patients with rheumatoid arthritis; it was centrifuged at 4°C for 30 min at 10 000 × *g* and kept frozen until assayed.

Collagen. Acid-soluble collagen was extracted from minced calf skin with 0.5 M acetic acid and purified by the method of Kang et al. [17]. For assays, collagen solution (about 0.2%), obtained by dissolving lyophilized collagen in cold 0.05% acetic acid, was dialysed against 0.05 M Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl and 0.005 M $CaCl_2$ (Tris/NaCl/$CaCl_2$ buffer) and then centrifuged at 4°C for 1 h at 10 000 × *g*.

Collagenase assay. Collagenase activity was measured by determination of hydroxyproline-containing peptides released from trypsin-resistant, reconstituted collagen fibrils. A typical reaction mixture consists of collagen fibrils (4 mg collagen) which had been allowed to gel for 6 h at 37°C, 0—100 µl enzyme solution made up to a total volume of 3 ml with Tris/NaCl/$CaCl_2$ buffer. The mixture was incubated for 18 h at 37°C and then filtered to remove insoluble collagen fibrils. Aliquots of the filtrates were hydrolysed in 6 M HCl and their hydroxyproline contents were determined.

Latent collagenase was activated with trypsin. The concentration of trypsin and duration of incubation required for maximal activation of the latent enzyme was established in preliminary experiments. Generally, a 20 min incubation at room temperature with 2—5 µg/ml trypsin was used for activation of the purified latent collagenase. For the activation of the latent collagenase in synovial fluid or in $(NH_4)_2SO_4$ fraction samples, a higher concentration of trypsin was used (50—100 µg trypsin/ml). After incubation, soybean trypsin inhibitor (5-fold excess) or DFP (2 mM final concentration) was added and incubated for 10 min prior to the collagenase assay. The quantification of the latent form was based on its maximal activation determined with varying amounts of trypsin for each batch of enzyme preparation. 1 unit of collagenase

activity was defined as the amount of enzyme which solubilized 1 μg collagen gel per min in the assay system. 1 unit of latent collagenase was defined as the amount of the latent form, which gave 1 unit of collagenase activity when activated as described. Specific activity refers to μg of collagen hydrolysed per min at 37°C per mg of enzyme protein. All determinations of collagenase activity were made under conditions assuring linearity of the assay system.

Molecular weight estimation. The apparent molecular weight of the latent and trypsin-activated enzyme was estimated by gel filtration at 4°C in a column (75 × 1.7 cm) of Sephadex G-100 (superfine grade). The column was calibrated with protein standards: bovine serum albumin, egg albumin, α-chymotrypsinogen A and cytochrome *c*, and the void-volume value was determined with Blue Dextran solution. The eluting buffer was 0.05 M Tris-HCl, pH 7.5, containing 0.005 M $CaCl_2$ and 1 M NaCl. Fractions (2.2 ml) were collected at a rate of 5 ml/h. Each fraction of the column eluate was assayed for latent or active-collagenase activity. Absorbance at 280 nm was also measured.

Viscometry. Viscosity measurements were carried out at 25°C using an Ostwald type viscometer. Trypsin-activated or latent collagenase was added to the reaction mixture, giving a final concentration of 0.67 mg of collagen/ml in 0.05 M Tris-HCl buffer, pH 7.5, 0.35 M NaCl and 0.005 M $CaCl_2$.

Purification of latent collagenase. All procedures described were carried out at 4—6°C. Synovial fluids containing more than 15 units of latent collagenase/ml were pooled, pretreated with hyaluronidase (40 ml of synovial fluid and 11 mg of hyaluronidase were incubated for 16 h at 4°C) and then centrifuged at 10 000 × *g* for 10 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to 0.5 saturation and the suspension was allowed to stand for 1 h in an ice-bath; the precipitate, collected by centrifugation at 10 000 × *g* for 15 min, was dissolved in a small volume of Tris/NaCl/$CaCl_2$ buffer, dialysed against 0.05 M Tris-HCl buffer, pH 7.5, containing 1 M NaCl and 0.005 M $CaCl_2$ and filtered through a column of Sephadex G-150 (75 × 4.5 cm) equilibrated with the buffer at a flow rate of 20 ml/h. The fractions containing the latent collagenase (trypsin activatable) were pooled and concentrated using Aquacide. The concentrated sample was dialysed against 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 0.005 M $CaCl_2$ and applied to DEAE-Sephadex A-50 ion-exchange resin column (20 × 1.5 cm) equilibrated with the dialysis buffer. A linear gradient (0.1—0.5 M NaCl) was applied with a buffer flow rate of 15 ml/h. The enzyme was not retarded on the column under conditions employed. Fractions containing the latent collagenase were pooled and concentrated using Aquacide. For further purification, the latent enzyme was applied to a column (75 × 1.7 cm) of Sephadex G-100 superfine grade. The equilibration and elution buffer was 0.05 M Tris-HCl, pH 7.5, containing 1 M NaCl, 0.005 M $CaCl_2$ and 20% poly(ethylene glycol). The enzyme sample was eluted at a flow rate of 5 ml/h. The effluent fractions containing the latent enzyme were pooled and stored at −20°C.

Disc gel electrophoresis. Enzyme preparations were examined by disc gel electrophoresis in 7.5% polyacrylamide gel containing sodium dodecyl sulphate [18].

For analysis of collagen degradation products polyacrylamide gel electrophoresis was performed according to Nagai et al. [19].

Other methods. Hydroxyproline was determined by the method of Stegemann and Stalder [20] and protein by the method of Lowry et al. [21].

Chemicals. The following commercially available products were used: trypsin, DFP, bovine serum albumin, ovalbumin, dithiothreitol (Koch-Light); soybean trypsin inhibitor (Mann Research Laboratories Inc., U.S.A.); Sephadex G-100, Sephadex G-150, DEAE-Sephadex A-50, Blue Dextran (Pharmacia); *p*-chloromercuribenzoic acid sodium salt (Chemapol, Czechoslovakia); 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma); α-chymotrypsinogen A, cytochrome *c*, Tris (Fluka A.G.); Aquacid II (Calbiochem); cysteine, bovine testis hyaluronidase (Reanal, Hungary); hydroxyproline (Nutritional Biochemical Corp., U.S.A.). Other reagents were Polish commerical products of analytical grade.

Results

Purification of latent synovial fluid collagenase

The latent collagenase was purified from the rheumatoid synovial fluid by $(NH_4)_2SO_4$ precipitation and a series of chromatographic steps on Sephadex G-150, DEAE-Sephadex A-50 and Sephadex G-100, superfine grade. Poly(ethylene glycol) and 1 M NaCl used in the gel filtration steps seem to stabilize the latent enzyme. Their omission caused a sharp decrease in activity of latent collagenase. The purification procedure is summarized in Table I. The final preparation had a specific activity of 259 units per mg of enzyme protein. It represents a 761-fold increase over that of the synovial fluid used for preparation of the latent enzyme. The final yield of the enzyme was small. It amounted to about 9% recovery of the initial enzyme activity. The purified latent collagenase preparations contained no more than 2% of the active enzyme when compared to the total activity unmasked by trypsin. These preparations were unstable, storage at −20°C for about 10 days decreased the latent collagenase activity by about 70% and it could not be detected as an active collagenase.

The final preparation of the latent collagenase does not yet seem to be homogeneous and still shows several protein bands on polyacrylamide gel electrophoresis.

TABLE I

PURIFICATION OF LATENT COLLAGENASE FROM SYNOVIAL FLUID

Purification step	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Increase in specific activity (fold)	Enzyme recovery (%)
Synovial fluid	1 814	0.34	630.2	1	100
$(NH_4)_2SO_4$ precipitate	1 000	0.53	538.7	1.5	85
Sephadex G-150	56	4.96	278.4	14.5	44
DEAE-Sephadex A-50	2.4	61.56	147.7	181.0	23
Sephadex G-100 (superfine grade)	0.23	259.02	59.5	761.8	9

Activation of latent collagenase by trypsin

Synovial fluids from patients with rheumatoid arthritis showed, in the majority of samples tested, very low collagenolytic activity. However, this activity increased considerably upon treatment of these fluids with trypsin. Trypsin also activated the purified latent synovial fluid collagenase. Activation is a time- and a concentration-dependent process (Fig. 1). Excess of trypsin caused a decrease of collagenase activity, suggesting proteolytic degradation of the active enzyme.

Trypsin activation of the latent synovial fluid collagenase was accompanied by a decrease in molecular weight of about 9000. The unactivated sample was eluted as entirely latent collagenase with an apparent molecular weight of 52000, while the activated enzyme was eluted at a slightly larger volume corresponding to a molecular weight of 43000 (Fig. 2). Since the amount of protein applied to the column was very small, the absorbance of the effluents at 280 nm was too low to include it in Fig. 2.

Viscosity measurements demonstrated that activation of latent synovial fluid collagenase by trypsin resulted in an approx. 30% decrease in viscosity of collagen solution during 6 h of incubation. The pattern of collagen degradation products resulting from the action of trypsin-activated collagenase was similar to that generally obtained with tissue collagenases. The trypsin-activated collagenase was inhibited almost completely by EDTA, dithiothreitol and 2% human serum, whereas cysteine inhibited it by about 40% (data not shown).

Attempts to activate latent collagenase by chemicals

Preliminary studies were undertaken to activate the latent synovial fluid collagenase by chemical treatment. As seen in Table II, collagenase remained latent after dialysis against 3 M NaSCN or 3.5 M NaCl, whereas 8 M urea and

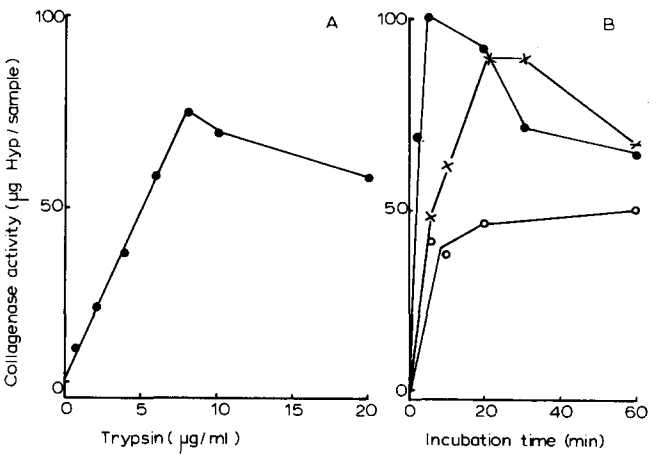

Fig. 1. Activation of purified latent synovial fluid collagenase with trypsin: A, as a function of trypsin concentration and B, as a function of time. Conditions of the assay were as follows: A, latent collagenase preparation (1 µg) was incubated in 1 ml total volume, with various amounts of trypsin, for 20 min at room temperature. B, latent collagenase preparation (3 µg) was incubated in 1 ml total volume with trypsin (o———o, 1 µg; X———X, 3 µg; •———•, 10 µg) for various time intervals. Details of the assay system are described in Materials and Methods. Hyp, hydroxyproline.

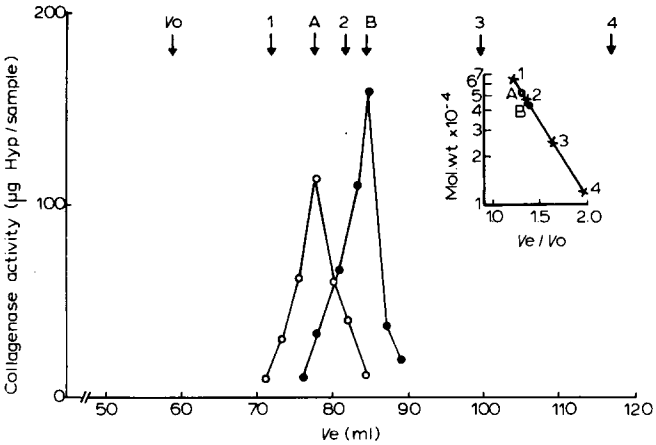

Fig. 2. Estimation of molecular weight of latent and trypsin-activated synovial fluid collagenase by molecular sieving on Sephadex G-100 superfine. A latent collagenase preparation (250 µg) recovered from Sephadex G-100 superfine was activated with trypsin (20 µg/ml). The activation was terminated by addition of DFP (2 mM final concentration) and the enzyme sample was subsequently rerun through the column equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, containing 1 M NaCl and 0.01 M $CaCl_2$. Fractions of 2.2 ml were collected at a flow rate of 5 ml/h and assayed for latent or active collagenase. ○———○, latent collagenase; ●———●, active collagenase; 1, 2, 3, 4, A and B represent the elution volume values (V_e) for bovine serum albumin, ovalbumin, α-chymotrypsinogen A, cytochrome *c*, latent and active collagenase, respectively; V_0, void volume. The molecular weights of 52 000 for the latent collagenase (A) and 43 000 for the active enzyme (B) were calculated from a plot of V_e/V_0 against log(molecular weight) of the protein standards (insert). Hyp, hydroxyproline.

buffer, pH 2.5, almost completely destroyed the enzyme activity.

Among thiol-blocking reagents, only mercuric chloride activated the latent enzyme. The activation was about 50% that obtained with trypsin. DTNB and *p*-chloromercuribenzoate had an insignificant effect on the latent form of the synovial fluid enzyme (Table III).

TABLE II

EFFECT OF NON-ENZYMATIC TREATMENT ON ACTIVATION OF LATENT SYNOVIAL FLUID COLLAGENASE

Latent collagenase preparation (10 µg) was dialysed against Tris/NaCl/$CaCl_2$ buffer for 16 h at 4°C or urea, NaCl, NaSCN in the buffer or against buffer of pH 2.5. Following removal of the chemicals by exhaustive dialysis against Tris/NaCl/$CaCl_2$ buffer, each sample was assayed for collagenase activity either directly or after trypsin activation. Hyp, hydroxyproline

Dialysed against	Collagenase activity (µg Hyp/sample)	
	Direct assay	Assay after trypsin activation
Buffer	12.6	96.0
3 M NaSCN	9.3	102.0
3.5 M NaCl	8.4	103.8
8 M urea	7.8	14.4
0.05 M glycine/CH_3COOH buffer, pH 2.5	3.0	0.6

TABLE III

EFFECT OF THIOL-BLOCKING REAGENTS ON ACTIVATION OF LATENT SYNOVIAL FLUID COLLAGENASE

The reagents were dissolved in 0.1 M Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl and 0.005 M $CaCl_2$ and incubated with latent collagenase (2.5 μg) and collagen fibrils for 18 h at 37°C. Hyp, hydroxyproline

Reagent added	Concentration in assay (mM)	Increment of collagenase activity (μg Hyp/sample)
DTNB	1.0	7.4
	2.0	10.2
p-chloromercuribenzoate	1.0	4.8
	2.0	0.0
$HgCl_2$	0.1	41.4
	0.2	43.8
	0.5	23.4
Trypsin	2 μg/ml	80.0

Discussion

The present studies demonstrated that rheumatoid synovial fluid contains latent collagenase which can be activated by trypsin but not by NaSCN. This latent enzyme was isolated and partially characterized.

Purification of the latent collagenase was about 800-fold, this being the highest reported value so far for synovial fluid collagenase. A high activity of the latent collagenase in the starting material was necessary for such high purification. Upon activation with trypsin the purified preparation had a specific activity against reconstituted collagen fibrils of 259 μg collagen degraded/min/mg enzyme protein. These data are similar to those obtained by Wooley et al. [22] for active synovium collagenase from culture medium.

The apparent molecular weight of the latent enzyme was 52 000 whilst that of the active form resulting from trypsin activation was about 43 000. A similar molecular size of latent enzyme was observed in synovial fluids from patients with osteoarthritis [16] and in the culture media from rheumatoid synovial cells [12], embryonic human skin [23] and chick bones [24].

The origin of the latent collagenase in synovial fluids is unknown. It may be released from polymorphonuclear leucocytes or produced by synovium. The latter is more probable if one considers the molecular weight reported to be 49 000 for latent collagenase produced by synovial cells in the culture [12]. However, the molecular weight (33 000) of the active collagenase isolated from the culture medium of rheumatoid synovium [22] as well as from synovial fluids (50 000 and 30 000 for collagenase 'A' and 'B', respectively [13]) differs from that of the present trypsin-activated collagenase. One of the reasons for this may be a different activating factor operating in vivo.

The trypsin-activated enzyme from rheumatoid synovial fluid has the essential features of a neutral collagenase. It was always completely inhibited by human serum, EDTA and dithiothreitol and partly by cysteine. The activated enzyme was not inhibited by the soybean trypsin inhibitor of DFP, used to stop the tryptic activation of latent collagenase. Trypsin-activated collagenase

degraded collagen in solution in a manner characteristic for tissue collagenase.

The nature of the latency of synovial fluid collagenase is not fully understood. It may be activated by trypsin and partially by $HgCl_2$. NaSCN, DTNB, *p*-chloromercuribenzoate and 3.5 M NaCl or $(NH_4)_2SO_2$ precipitation, molecular sieve in 1 M NaCl and chromatography on DEAE-Sephadex failed to activate the latent enzyme. It remained latent after chromatography on Fenylo-Sepharose (data not shown). It is possible that it represents a true zymogen similar to procollagenase found in culture media of human skin and bovine gingiva fibroblasts [25,26] or mouse bone explants [27] which are activated by limited proteolysis.

I have not checked all chemicals reported recently as agents activating latent collagenolytic enzymes [12,23,28—30]. However, the failure of those methods indicated above to activate the latent synovial fluid collagenase described in this paper indicates against it being an easily dissociable enzyme-inhibitor complex.

Activation of the purified preparation of the latent enzyme could not be achieved by treatment with 3 M NaSCN, which is at variance with the observation of Nagai et al. [16]. However, they used a crude latent synovial fluid collagenase for activation. It is possible, therefore, that the latent collagenase described in this paper represents a different species from that found by Nagai et al. [16], or NaSCN activates a procollagenase proactivating enzyme system which is removed in the following purification.

The physiological role and the fate of the latent synovial fluid collagenase remain obscure at present. It can be assumed, however, that activation of latent enzymes is an important regulatory mechanism of collagenolytic activity in vivo.

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