ACTIVATION OF LATENT COLLAGENASE FROM POLYMORPHONUCLEAR LEUKOCYTES BY CATHEPSIN G

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Cathepsin G was isolated from human polymorphonuclear leukocytes and purified by affinity chromatography on Antilysin-Sepharose column. Purified enzyme activated latent collagenase isolated from leukocytes. Activation at 36°C was maximal after 30 min incubation. Inhibitors of cathepsin G — soya-bean trypsin inhibitor, diisopropyl phosphofluoridate and Antilysin were active in inhibiting the activation of latent collagenase by cathepsin G.

Neutral proteases as collagenase, cathepsin G and elastase are major constituents of cytoplasmatic granules of polymorphonuclear leukocytes. On encountering bacteria, urate crystals, immune complexes or activated complement factors the leukocytes tend to release the contents of their cytoplasmatic granules extracellularly. Presumably, release also occurs at the end of the very short life-span of the cells. The release of these proteases into extracellular space leads to the degradation of important macromolecular components of connective tissue as collagen, proteoglycans and elastine.

While cathepsin G is released from leukocytes as an active enzyme the leukocyte collagenase is secreted in latent form[1]. Many of the mammalian collagenases are released as a latent precursor from the synthetizing cells. Latent collagenases are found in mouse bone explants[2], beef gingiva[3], embryonic human skin explants[4], involuting rat uterus[5], *etc.* The problem whether the latent precursor is the zymogen of the enzyme or a complex enzyme-inhibitor has not yet been solved. Kruze and Wojtecka[1] who isolated the latent collagenase from human leukocytes suggest that it is a zymogen of the enzyme.

Latent collagenase relased by mouse bone explants in culture is *in vitro* activated[6] by trypsin, plasmin, kallikrein and cathepsin B. It remains to be shown whether these proteases act directly on the latent collagenase or their effect goes through the "proactivator" as suggested[6]. Activation of latent collagenase through limited proteolysis by trypsin was used by several authors for the preparation of an active enzyme from latent precursor[3,5,7].

The aim of our study was to find whether the intracellular serine protease cathepsin G which is released from polymorphonuclear leukocytes at the same time with latent collagenase could activate this inactive precursor resulting in the formation of an active enzyme.

EXPERIMENTAL

Isolation of polymorphonuclear leukocytes. Leukocytes were prepared from fresh blood samples of healthy adult volunteers. The blood was anticoagulated with sodium citrate and was mixed with solution of 6% dextran T 500 in isotonic sodium chloride in ratio 5 volumes of blood and 1 volume of dextran solution. The supernatant containing leukocytes was decanted and centrifuged at 900*g* for 5 min. The cell pellet was resuspended in saline and the remaining red cells removed by brief hypotonic hemolysis. The leukocytes after washing with saline were collected and homogenized in 0·01M-Tris-HCl containing 0·005M-$CaCl_2$ and 0·15M-NaCl (pH 7·5). The homogenate was frozen and thawed (5times) and centrifuged at 12000*g* for 20 min. The supernatant was used as a source of latent collagenase (extract I). The resulting pellet containing the cathepsin G activity was resuspended in 0·05M-Tris-HCl buffer (pH 7·5) containing 1M-NaCl, repeatedly homogenized and centrifuged. The supernatant was used as a source of cathepsin G (extract II).

Purification of collagenase was performed by gel filtration on Sephadex G-200 followed by gel filtration on Sephadex G-100 by the method of Kruze and Wojtecka[1]. The columns were equilibrated with 0·01M-Tris-HCl buffer (pH 7·5) containing 0·15M-NaCl and 0·005M-$CaCl_2$. The fractions with collagenase activity were concentrated using Polyaethyleneglykol 20000 (Serva). The content of latent collagenase in collagenase fractions was 80—90%.

Removal of active collagenase. Active collagenase was removed by the incubation of collagen fibrils with solution of latent collagenase and active collagenase at low temperature[8]. Collagen fibrils were gently stirred for 2 h at 10°C with collagenases in 0·01M-Tris-HCl buffer (pH 7·5) containing 0·2M-NaCl and 0·01M-$CaCl_2$. The ratio of suspended collagen fibrils to protein in solution was 5 to 1 (w/w).

Purification of cathepsin G. The extract II from leukocytes in 0·05M-Tris-HCl buffer (pH 7·5) with 1M-NaCl was used as a source of cathepsin G. The extract was dialysed against large volume of 0·05M-Tris-buffer (pH 7·5). Cathepsin G was precipitated and after centrifugation the sediment was solubilized in 0·05M-Tris-HCl buffer (pH 8) containing 0·4M-NaCl, and applied to a column of Sepharose-Antilysin. The preparation of Sepharose-Antilysin and purification of cathepsin G by affinity chromatography were carried out by the method of Baugh and Travis[9]. Antilysin, a polyvalent inhibitor of proteases, was isolated from bovine lungs and prepared[10] by Spofa, Czechoslovakia. Antilysin could replaces Trasylol in affinity chromatography.

Collagenase activity was determined using [^{14}C]glycine labelled collagen fibrils. The acid-extracted radioactive collagen ($15 \cdot 10^3$ dpm/mg) was prepared by the method of Lazarus and coworkers[11] and purified as described[12]. The reconstituted [^{14}C]glycine labelled collagen fibrils were prepared by heating solutions of collagen (3 mg per ml) containing 0·05M-Tris-HCl (pH 7·5) with 0·2M-NaCl at 36°C overnight, followed by centrigugation at 10000*g* for 5 min. The reconstituted fibrils were incubated with trypsin to check that the collagen had not been denaturated during preparation and with clostridial collagenase to measure total fibril lysis.

Collagenase assay was performed by incubation of collagen fibrils with collagenase in appropriate buffer (pH 7·5) at 36°C for 15 h. After incubation to measure collagenolysis, the reaction mixtures were centrifuged (10000*g* 10 min) and the radioactivity released was measured in 0·1 ml of supernatant[13]. Latent collagenase was quantitated as the activity difference between trypsin activated and unactivated aliquots. Activation was achieved according to the method of Vaes[2] by 10 min incubation at room temperature with 1 μg trypsin (Serva) per 2 units of latent collagenase, followed by the addition of a 10fold excess of soya-bean trypsin inhibitor (Serva). The inhibitor was allowed to react for 10 min with trypsin before the collagen fibrils were added.

One unit of collagenase was defined as the activity which solubilized 1 μg collagen fibrils per 1 min at 36°C. One unit of latent collagenase was defined as the amount of an enzyme which gave rise to 1 unit of active collagenase when activated as described.

Cathepsin G activity was measured against N-acetyl-tyrosine ethyl ester by a method of Rybák and coworkers[14]. The splitting of the ester was determined from the amount of N-acetyl-tyrosine set free in the reaction. The released N-acetyl-tyrosine as well as the unchanged ester were coupled with the diazonium salt of *p*-nitroanilide. The diazo compound of the ester can be extracted by ethyl acetate whereas diazo compound of N-acetyl-tyrosine remains almost completely in the aqueous phase and can be determined by measuring the absorbance of the water solution at 500 nm. Enzyme assay was carried out in a mixture (1 ml) containing 0·2 ml of 0·5M-Tris-HCl (pH 7·5), 0·5 ml of 0·05M-Tris-HCl buffer containing 1M-NaCl, 0·1 ml of an enzyme solution and 0·2 ml of 0·025M N-acetyl-tyrosine ethyl ester in dimethyl sulphoxide. Incubation lasted 20 min at 37°C. The units of cathepsin G activity were expressed as nkat (nanokatal is defined as the activity of an enzyme hydrolyzing 1 nmol of the substrate per second). Proteolytic activity of cathepsin G was determined by hydrolyzing casein[15,16]. Concentration of protein in enzyme extracts was determined by the method of Lowry and coworkers[17].

Activity of elastase was estimated on succinyl-alanyl-alanyl-alanine *p*-nitroanilide by the method of Kasafírek and coworkers[16].

Activation of latent collagenase with cathepsin G. The reaction mixture contained 1 unit of latent collagenase in 0·01M-Tris-HCl buffer containing 0·2M-NaCl and 0·01M-$CaCl_2$ (0·3 ml), 0·32 nkat of cathepsin G in 0·01M-Tris-HCl buffer containing 1M-NaCl and 0·005M-$MgCl_2$ (0·4 ml). The activation was carried out at 36°C. The reaction was stopped by adding soya-bean trypsin inhibitor (250 μg/20 μl). Collagenase activity was determined by adding radioactive collagen fibrils (total radioactivity 7 200 c.p.m. per 600 μg of collagen) followed by incubation at 36°C for 15 h. Collagen was determined by the method of Stegemann and coworkers[18].

Inhibition of collagenase activation. Cathepsin G was preincubated with Antilysin and soya-bean trypsin inhibitor for 10 min at room temperature before the addition of latent collagenase. Using diisopropyl phosphorofluoridate cathepsin G was preincubated at 4°C for 8 h. The fluoridate was dissolved in 2-propanol.

Acrylamide gel electrophoresis was performed on 15% gels at pH 4·3 and 4°C according to Reisfeld and coworkers[19]. The gels for protein were stained with Coomassie brillant blue[20]. Elution of protein from 2-mm slices of gels was performed with 0·5 ml of 0·1% Triton X-100 in 0·1M-Tris-HCl (buffer (pH 7·5). The eluates were dialyzed against the elution buffer and subsequently assayed for the esterase and proteolytic activity.

RESULTS AND DISCUSSION

Purification and Assay of Cathepsin G

Purification of cathepsin G on Antilysin-Sepharose column is shown in Fig. 1. Elution of cathepsin G was accomplished by developing the affinity column with 0·05M sodium acetate and 0·4M-NaCl at pH 5. First fractions of cathepsin G contained small amounts of elastase activity. The bulk of elastase activity together with collagenase remained in the extract I. The absence of elastase could be explained by purifica-

tion, dialysis and following sedimentation of extract II. The specific activity of cathepsin G increased after affinity chromatography 32times.

Polyacrylamide gel electrophoresis of pooled cathepsin G fractions is shown in Fig. 2. Electrophoresis toward the cathode (pH 4·3) gave three clearly visible bands of the same mobility as lysozyme. Proteins isolated from these bands had enzymatic activity against N-acetyl-tyrosine ethyl ester. The slow-moving band without any chymotryptic activity (which represents[21,22] inactivated enzyme) was also present.

Starkey and Barrett[22] who correlated the catalytic, electrophoretic and immunological properties of cathepsin G extracted from human spleen with chymotrypsin-like enzyme isolated by Rindler–Ludwig and Braunstein[21] and by Gerber and coworkers[23] from human neutrophile leukocytes could show that these enzymes are identical and that the enzyme from human neutrophils is after all cathepsin G. The

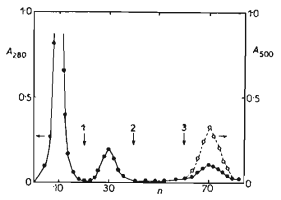

FIG. 1

Chromatography of Human Leukocyte Extract II on Antilysin-Sepharose

The column (2 × 8 cm) was equilibrated with 0·05M-Tris-HCl and 0·1M-NaCl (pH 8·0). After sample application (52 mg protein) the column was washed with starting buffer until the absorbance at 280 nm was less than 0·020, followed by 0·05M-Tris-HCl with 0·4M-NaCl (*1*) and 0·05M sodium phosphate with 0·4M-NaCl (pH 6·5) (*2*). Cathepsin G was eluated with 0·05M sodium acetate with 0·4M-NaCl (pH 5·0) (*3*). ● Protein concentration measured at 280 nm; ○ cathepsin G esterase activity assayed against N-acetyl-tyrosine ethyl ester measured at 500 nm.

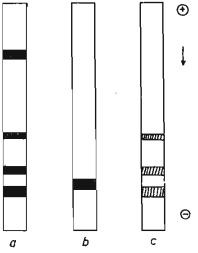

FIG. 2

Gel Electrophoresis of Cathepsin G from Polymorphonuclear Leukocytes

Samples *a* and *c* represent cathepsin G (20 μg), sample *b* lysozyme (20 μg). Gels *a* and *b* were stained for protein whereas gel *c* was used for the determination of cathepsin G esterase activity.

inability to detect the activity of cathepsin G from human spleen against N-acetyl-tyrosine ethyl ester can be explained[24] by the relative insensitivity of the used spectrophotometric assay. We have used for the detection of cathepsin G activity the method[14] which has been more sensitive allowing to detect cathepsin G in amount 0·002 nkat. Cathepsin G could be determined by this method also in crude leukocytar extracts.

Activation of Latent Collagenase by Cathepsin G

The course of activation of latent leukocyte collagenase is demonstrated in Fig. 3. The activation reached its maximum after 30 min of incubation and after 2 h fell down slowly. Higher temperature (36°C) and longer period of incubation which were necessary for the activation document the lesser sensitivity of the latent collagenase against cathepsin G in correlation with trypsin and plasmin.

Activation of latent collagenase by cathepsin G should be inhibited by soya-bean trypsin inhibitor, diisopropyl phosphorofluoridate and Antilysin. These substances are active inhibitors of cathepsin G and therefore inhibited also the activation of latent collagenase through this enzyme. They had no inhibitory effect on the activity of leukocytar collagenase which had been previously activated by trypsin.

Activation of latent leukocyte collagenase by cathepsin G could play an important role in rheumatoid arthritis where the inflammed joint is infiltrated by a large amount of polymorphonuclear leukocytes. Collagen of articular cartilage is arranged in the form of insoluble collagen fibrils which are entangled in the fibrils of proteoglycans. Cathepsin G from polymorphonuclear leukocytes together with elastase may participate[25] not only on the degradation of proteoglycans but also on the depolymerization of collagen fibrils. Monomeric collagen is subsequently cleaved by neutral

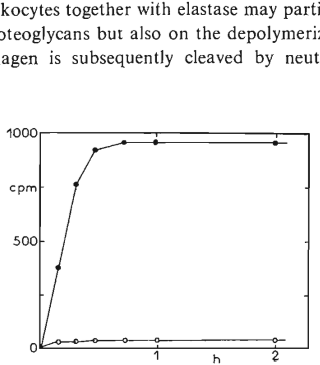

FIG. 3

Time Course of Activation of Latent Collagenase from Polymorphonuclear Leukocytes with Cathepsin G

Aliquots of 1 unit of latent collagenase were incubated at 36°C with 32 nkat of cathepsin G. The activation (●) was stopped by soya-bean trypsin inhibitor (300 μg). Controls (○) were incubated with premixed cathepsin G and inhibitor. Resultant collagenase activity was measured in radioassay (15 h). The ordinate gives the radioactivity of released collagen in 0·1 ml of supernatant and the abscissa the incubation time of latent collagenase with cathepsin G.

collagenase. Cathepsin G could in this stage promote the degradation of collagen and destruction of articular cartilage as an activator of latent collagenase.

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