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# SERINE PROTEINASE ACTIVATION OF LATENT HUMAN SKIN COLLAGENASE

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SUMMARY: Latent and active collagenase were demonstrated following direct extraction from normal skin homogenates with 0.1M calcium chloride at 60°C. 83 % of the collagenase activity was in latent form and could be maximally activated with trypsin. Partial activation of the latent enzyme could also be demonstrated by incubation of the skin extract without added trypsin. This endogenous activation was inhibited by the addition of soya bean trypsin inhibitor, trasylol, di-isopropylphosphofluoridate and phenylmethanesulphonylfluoride, none of which inhibited collagenase directly. This suggests that the skin extracts contain a collagenase activating enzyme with the inhibition profile of a serine proteinase. A chymotryptic proteinase with a similar inhibition profile was extracted from normal human skin and partially purified. This enzyme activated fibroblast procollagenase derived from tissue culture of normal skin. The procollagenase was also partially activated by plasmin and chymotrypsin. This is the first demonstration of a collagenase activating enzyme in human skin and raises the possibility that collagenase activation by this mechanism may be responsible for collagen degradation in some disease processes. 

Collagen degradation in normal skin is initiated by a specific class of proteases, the collagenases (1). Recessive dystrophic epidermolysis bullosa (RDEB), a genodermatosis characterized by dermolytic blister formation in response to minor trouma, is characterized by an increased synthesis of an abberant collagenase enzyme which has been held responsible for the blister formation (2). Both these enzymes when secreted into the extracellular space are in an inactive state. The nature of the latency has been attributed by some workers to the secretion of the enzyme as a zymogen (3,4,5). The ability of organomercurials to activate collagenase has been cited as evidence that latent collagenase exists as an enzyme-inhibitor complex (5). In skin, recent evidence supports the zymogen theory of collagenase latency (5,7).

Abbreviations used: RDEB, recessive dystrophic epidermolysis bullosa; DFP, di-isopropylphosphofluridate; PMSF, phenylmethanesulphonylfluoride; TLCK, tosyllysine chloromethylketone; SBTI, soya bean trypsin inhibitor; ATEE, acetyltyrosine ethyl ester; TAME, tosylarginine methyl ester;. HEPES ,4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid .

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Collagenase from most tissues can be activated by many different proteinases including trypsin, plasmin (3,8), kallikrein (8) and canine mast cell proteinases (10). Experiments in this communication are part of a study in to the role of collagenase in blister formation in RDEB. In this paper we report a method for direct extraction of collagenase from normal skin extracts. The collagenase is both in latent and active state suggesting that there may be an enzymic endogenous activating process operating in skin extracts. In examining the nature of this activating process we report the partial purification of a serine proteinase extracted from normal skin and capable of activating normal fibroblast procollagenase.

## MATERIALS AND METHODS

Direct extraction of collagenase: Normal human skin, obtained at lipectomy was dissected free of subcutaneous fat and cut into small pieces , frozen in liquid $N_2$ and ground into a fine powder in a Wiley mill cooled with liquid $N_2$. This skin powder was extracted by the method developed for rat uterus (11). The skin powder was suspended in 10 vol of ice cold buffer 0.05 M Tris-HCl, pH7.6 with 0.01M $CaCl_2$, 0.154M NaCl and 0.25% Triton-X-100 and homogenised in a Teflon glass homogeniser for 20 sec so as to remove soluble protein and non-specific proteinases. This was followed by a second extraction with Tris buffered 0.1M $CaCl_2$ at 60°C for 4min which releases collagen bound collagenase together with its activating enzyme. The supernatant obtained at the end of this extraction contained both active and latent collagenase. All the above steps were carried out at 4°C.

Collagenase assay: Collagenase activity was measured using a modification of the assay method of Terato, et al. (12). The soluble [$^{14}$C] Collagen substrate was prepared by acetylation of pure calf skin collagen(Calbiochem) with [$^{14}$C] acetic anhydride(Amersham) (13). The [$^{14}$C] collagen used in assays of normal skin extracts had a specific activity of 20,000dpm/mg of collagen while in subsequent experiments with procollagenase derived from fibroblasts in culture we used [$^{14}$C] collagen of higher specific activity(410,000 dpm/mg) prepared by using pepsinized calf skin collagen (14). In a typical assay 200µL of activated enzyme in skin extract or culture media were added to 100µL of [$^{14}$C] -radiolabeled collagen solution in 0.05 M Tris-HCl buffer (pH 7.5), containing 5 mM $CaCl_2$ and 0.2M NaCl. The reaction mixture was incubated for 90 min at 35°C and stopped by adding 100µL of 0.4M EDTA solution containing 150µg of unlabeled collagen. The mixture was incubated for 30 min at 35°C and the uncleaved collage molecules were precipitated with 400µL of cooled dioxane/methanol (4/1 by vol). The mixture was centrifuged (12000xg,2min) and the radioactivity was measured in each 300µL aliquot of supernatant. Each assay contained a trypsin control of collagen denaturation in which substrate was incubated with 200µL of 0.02% trypsin (Worthington), and radioactivity obtained substracted from experimental values. One unit (U) of enzyme activity represents the cleavage of 1µg of native collagen per hour at 35°C and pH7.5.

Proteinase assay: Esterase activity was measured spectrophotometrically by the method of Schwert and Takenaka ,(15). Chymotryptic activity was measured using ATEE (Sigma) and TAME measured tryptic activity. One unit(U) of activity is that which hydrolized 1 micromole of substrate per min. at 25°C.

Inhibition studies: These were undertaken to determine the properties of collagenese activating enzyme in skin extracts . Inhibitors were tested by pre-incubating them with skin extracts. SBTI, Trasylol, PMSF, Ovoinhibitor,

Iodoacetate, DPF and Pepstatin were pre-incubated for 20 min. at 24°C. TLCK was allowed to react for 2 hours at 37°C. These inhibitors at the same concentration and under the same conditions were preincubated with the chymotryptic enzyme which was assayed using ATEE substrate.

Skin fibroblast procollagenase: Normal human skin fibroblasts were prepared from primary cultures of skin obtained at surgery. The cells were grown at 37°C in large plastic roller bottles in Dulbeccos modified Eagles media buffered with 0.03 M HEPES buffer pH 7.6 and containing 20% fetal calf serum. At visiual confluence the cells were put through several cycles of serum free medium for 24 hours(3) . The serum free medium containing the procollagenase was harvested, pooled, buffered with 0.05M Tris-HCl pH 7.5 and concentrated using the Amicon thin channel cell. The concentrated medium was stored at -20°C.

Human skin chymotryptic enzyme purification: Normal human skin obtained at lipectomy was frozen in liquid Nitrogen and ground into a fine powder. This powder was extracted by a modification of the method of Fracki and Hopsu-Havu (16). Skin powder (100g) was extracted with 900 ml of 20mM potassium phosphate pH 8 at 4°C for 60min, followed by centrifugation at 10,000g for 20min. The precipitate was extracted with 900ml 2MKl, 20mM potassium phosphate pH8 at 4°C for 60min followed by centrifugation at 10,000g for 30min. To the supernatant (880ml) was added 100mmole of calcium phosphate suspension. After stirring at 4°C for 60min, the filtrate was concentrated and applied to a column of calcium phosphate /sepharose and ATEE hydrolysing fractions were pooled, concentrated to 7.8ml by ultrafiltration on a PM10 membrane and applied to a column of G75 in 2MKCl, 10mMTris-HCl pH8 at 20ml/hr. Enzyme containing fractions were combined, concentrated to 2.2ml by ultrafiltration and applied to the affinity column (17) in 2MKCl, 10mMTris-HCl pH8 at 20ml/hr. The enzyme was not bound but only retarded on this column. Those fractions containing the most enzyme and least protein were pooled and comcentrated to 2ml by ultrafiltration.

Activation with Proteinase: Chymotryptic enzyme, plasmin and pancreatic chymotrypsin (Calbiochem) were incubated with fibroblast procollagenase for 45min at 35°C. The reaction was stopped with five fold excess of soya bean trypsin inhibitor and the amount of activated collagenase assayed.

Trypsin activation: A range of trypsin concentrations, usually from 2µg to 30µg per sample, was used to insure maximal collagenase activity was measured. Maximal activity of collagenase skin extract was achieved by reacting it with 25µg of trypsin for 10 min at 37°C. The reaction was stopped with 500µg of SBTI. Fibroblast procollagenase was maximally activated by 2µg of trypsin for 45min at 35°C and the reaction stopped by adding 5-fold molar excess of SBTI.

## RESULTS

Collagenase assays in extracts of two different skin samples weighing 30g and 50g (Table 1), demonstrate the presence of both active and latent forms of the enzyme as well as endogenous conversion of latent enzyme in the skin extracts. Maximal degradation of collagen is obtained in (a) by pretreatment of skin extracts with 25µg of trypsin at 37°C for 10 min followed by the addition of 500µg SBTI to quench the activity of the trypsin. In (b) assay of skin extact alone without pretreatment with trypsin, results in collagen degradation which is only 68% of the maximal amount. This result represent the

Table I COLLAGENASE ACTIVATION IN SKIN EXTRACTS

| Treatment | Collagenase Activity (U) in 0.2ml skin extract | | | Total |
|---|---|---|---|---|
| | Sample 1 | Sample 2 | Mean | Activity % |
| (a) Skin extract + trypsin + SBTI | 33.5±1.4 | 42.8±9.0 | 38.2 | 100 |
| (b) Skin extract + no addition | 20.8±3.4 | 29.4±1.6 | 25.1 | 66 |
| (c) Skin extract + SBTI | 6.2±1.5 | 6.8±2.1 | 6.5 | 17 |

Collagenase assay was performed on two normal skin samples 1 and 2. (a) After maximal activation with trypsin to determine total collagenase activity. (b) with no addition to determine the extent of endogenous activation during the 90min period of assay. (c) with excess of SBTI in the assay to measure the active collagenase in the extract. One unit (U) of collagenase activity represented cleavage 1µg collagen/hr. All assays were performed in quadruplicate.

collagen degredation by active collagenase together with the degradation of collagen by latent collagenase activated by an endogenous process over the 90 min incubation of the assay. In (c) SBTI is added to the skin extracts prior to assay, in the absence of trypsin pretreatment and the amount of collagen degradation is only 17% of the maximal collagen degradation. Thus there is an apparent inhibition of collagenase activity by SBTI. Since it is known that collagenase is not directly inhibited by SBTI(11,5), these results can be explained by the presence in the skin extracts of an enzymic activation process of latent collagenase the action of which is blocked by SBTI. The 17% of collagen degradation represents the amount of active collagenase already present in skin extracts.

The endogeous activation of collagenase was further studied by pretreatment of skin extracts with a series of known proteinase inhibitors in concentrations used by Woessner (1977) as outlined in METHODS. The degree of inhibition of collagen degradation by these inhibitors indicated their action on the endogenous activating enzyme. The effect of these ihibitors is assessed relative to the inhibition of collagen degredation produced by 125µg of SBTI taken as 100% of inhibition. The results in Table 2 suggest that the endogenous collagenase activator is a serine proteinase because it is completely inhibited by DFP and trasylol. PMSF, chloromercuribenzoate, and pepstatin are partial inhibitors while ovoinhibitor iodoacetae and TLCK show no inhibition.

To further characterise the proteinase activity in the skin extracts, esterase activity was measured with substrates ATEE and TAME. A low but

Table 2 INHIBITORS OF COLLAGENASE ACTIVATING ENZYME

| Inhibitor | Concentration mg/ml | Inhibition% | |
|---|---|---|---|
| | | Skin extract | chymotryptic enzyme |
| Soya-bean trypsin inhibitor(Kunitz) | 0.313 | 100 | 100 |
| Trasylol | 0.025 | 100 | 25 |
| Phenylmethanesulphonylfluoride | 0.5 | 72 | 100 |
| Di-ispropyl phosphofluoridate | 0.05 | 100 | 100 |
| p-chloromercuribenzoate | 0.05 | 86 | 50 |
| Ovoinhibitor | 0.1 | 0 | |
| Iodoacetate | 1.0 | 0 | |
| Tossyllysine chloromethylketone | 0.5 | 0 | |
| Pepstatin | 0.18 | 56 | 16 |

The inhibition studies with skin extract was performed as outlined in METHODS. The chymotryptic enzyme was preincubated with each inhibitor for 20 min at 24°C at the final concentration shown and the residual activity was measured against ATEE.

measurable rate (0.214μg/min/ml)was observed with the chymotryptic substrate ATEE but there was no activity with the tryptic substrate TAME. The chymotryptic proteinase was extracted from normal skin and purified 1400 fold following chromatography on calcium phosphate, sephadex G75 and by affinity chromatography. Table 2 shows that the enzymic activity of this proteinase against ATEE was completely inhibited by SBTI, PMSF and partially inhibited by trasylol chloromercuribenzoate and pepstatin. Procollagenase which was harvested from normal skin monolayer fibroblast culture was activated by a number proteinases. The data in Table 3 shows that the chymotryptic enzyme extracted from the skin markedly activated procollagenase if it was preincubated with that enzyme for 45min. at 35°C. Plasmin and pancreatic chymotrypsin activated procollagenase, under similar conditions only 8% and 25% respectively.

Table 3 PROTEINASE ACTIVATION OF LATENT FIBROBLAST COLLAGENASE

| Activating proteinases | Collagenase activity (U) |
|---|---|
| Buffer blank | 0 |
| Purified chymotryptic enzyme 9.4μg | 106.2±11.3 |
| Purified chymotryptic enzyme 3.2μg | 27.2±1.7 |
| Plasmin 29μg | 8.9±0.4 |
| Chymotrypsin 10μg | 17.1±0.6 |

Fibroblast procollagenase in 100μl of concentrated culture media was preincubated for 45 min at 35°C with 100μl of activating proteinase. The reaction was stopped by adding 30μg of SBTI. Collagenase was then assayed using soluble [$^{14}$C] collagen substrate as outlined in Methods. Values are given as mean +S.D. All assays were performed in triplicate.

## DISCUSSION

Collagenase has a crucial role in connective tissue metabolism of normal and RDEB skin, so that it is important to understand the mechanism of activation of the latent enzyme. The results with the direct extraction method reported in this paper suggest that the collagenase detected is that which is secreted into the extracellular space in latent form and becomes attached to its substrate the collagen fibres of the dermis. In this state the latent collagenase may become activated by a secondary proteinase. The inhibition by DFP and trasylol indicate that the activating enzyme is a serine proteinase. It has a number of similarities to the rat uterine proteinase (11) in being fully inhibited by SBTI, DFP and trasylol and partially inhibited by PMSF while TLCK, ovoinhibitor and iodoacetate have no effect. However it differs from that enzyme in being partially inhibited by chloromercuribenzoate and pepstatin. The finding of measurable levels of esterase activity in skin extracts with substrate ATEE suggests that it is a chymotrypsin-like enzyme. The chymotryptic proteinase which we extracted and purified from normal human skin had an inhibition profile similar to the endogenous activating enzyme detected in skin extracts. The inhibition pattern of this enzyme has been compared with those of a number of serine proteinases reported in the literature and it resembles "chymase", an enzyme secreted by mast cells (18). Proteinases which have previously been shown to activate latent collagenase are trypsin (3), plasmin (8), kallikrein (9), chymotrypsin (19), and canine mast cell enzyme (10). In this communication our data show the marked activation properties of human skin chymotrypsin on fibroblast procollagenase. The other two proteinases, plasmin and bovine chymotrypsin, which were reported to activate synovial procollagenase, showed little activation of skin fibroblast procollagenase. This is the first report of a proteinase extracted from the skin activating skin fibroblast procollagenase. The enzymic properties of the skin chymotryptic proteinase were similar to proteinases found in skin of patients with mastocytosis suggesting that it is a mast cell constituent (18). Briggamen et al. (20), reported that a similar chymotryptic enzyme caused separation at the lamina lucida of the

dermo-epidermal junction of skin, similar to the blister formation found in the junctional form of EB (21). In the dermolytic form of E.B. skin fragility is caused by excessive production of an abnormal collagenase which is secreted in latent form (2). Although our data on collagenase activation were obtained with normal skin, we have ( unpublished) evidence which shows that the chymotryptic enzyme was an activator of RDEB procollagenase . This information may be relevent for the elucidation of the pathophysiology and treatment of both the junctional and the dermolytic forms of the above disease.

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