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THE ACTIVATION OF LATENT PIG SYNOVIAL COLLAGENASE

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Summary

Latent pig synovial collagenase (EC 3.4.24.7) can be activated by a variety of different treatments to give an active enzyme form of lower molecular weight which rapidly degrades collagen. Trypsin and plasmin effectively activated the latent collagenase whilst elastase and cathepsin G degraded most of the latent enzyme before it was activated. A number of mercurials were compared and maximum activation was achieved using 4-aminophenylmercuric acetate and phenylmercuric chloride. The latent collagenase bound to a mercurial-Sepharose column and was eluted in the active form with NaCl. The latent collagenase also activated spontaneously and the conditions which encouraged and prevented this activation were studied. High NaCl concentration, diisopropylphosphofluoridate, soybean trypsin inhibitor, low Zn^{2+} concentration and high and low pH all prevented the spontaneous activation of latent pig synovial collagenase.

Introduction

Collagen is the major protein found in connective tissues and in many pathological situations it is the enzymic breakdown of collagen which is responsible for irreversible connective tissue damage. Mammalian collagenase (EC 3.4.24.7), the enzyme responsible for the cleavage of collagen, is a metalloproteinase which is released from many of these tissues when they are placed in culture. Although this enzyme has been purified from many sources relatively little is known of its mechanism of action and properties. The enzyme is often secreted in a latent form and collagenase activity cannot be detected in culture medium of many connective tissues unless the medium is first treated with trypsin [1—5], chaotrophic agents [2,6—8] or mercurials [9—12]. In some situations the latent enzyme can activate spontaneously [4,8,11,13,14] either

in the culture medium or during subsequent purification steps. The activation of many collagenases is known to be accompanied by an apparent molecular weight change, for example the molecular weight of the latent rabbit bone collagenase changes from approx. 40—45 000 to 30 000 for the activated enzyme [9].

The exact nature of the different forms of collagenase and the factors which control the spontaneous activation are not known. It is likely that the appearance of collagenase as a latent complex represents a mechanism to control the extracellular activity of the enzyme. Thus it is important to understand how latent collagenase can be activated in order to know when this is likely to occur in vivo and how it can be prevented. In this study, we have cultured pig synovium and have investigated the different ways in which the latent collagenase, found in the culture medium, can be activated.

Materials

Chemicals were obtained from the following suppliers: 4-aminophenylmercuric acetate and 4-chloromercuribenzoate from Aldrich Chemical Co., Gillingham, Kent, U.K., mersalyl acid, 4-hydroxymercuriphenylsulphonic acid and diisopropylphosphofluoridate from Sigma (London) Chemical Co., Dorset, U.K., sodium tetrathionate from Pierce, Box 117, Rockford, IL 61105, U.S.A.; mercuric chloride, phenylmercuric chloride, 5,5′-dithiobis(2-nitrobenzoic acid) from BDH, Poole, Dorset, U.K.; Sepharose 6B from Pharmacia, Hounslow, Middlesex, U.K.; Ultrogel AcA-44 from LKB Instruments Ltd., Croydon, U.K. Human plasminogen was a gift from Dr. G. Salvesen, Strangeways Laboratory. Human elastase (EC 3.4.21.11) and cathepsin G (EC 3.4.21.20) were given by Dr. J. Saklatvala, Strangeways Laboratory. Streptokinase (kabikinase) (EC 2.4.21.7) Kabi vitrum Ltd., Ealing, London W5 2TH, U.K. was further purified to remove added human serum albumin by passing through a column (1 × 5 cm) of Cibacron Blue-Sepharose prepared as described by Travis and Parnell (1973) [15]. All other chemicals and biochemicals were commercially available analytical grade reagents.

Methods

Tissue culture. Synovial tissue was removed from the metacarpophalangeal joints of 5—7 month old pigs (70—95 kg) and cultured as described by Cawston and Tyler [16]. Approx. 75 mg synovial tissue were obtained from each joint.

Column chromatography. All buffers contained Brij 35 (0.05%) and either toluene (0.03%) or sodium azide (0.02%) as a preservative. Epoxy-activated-Sepharose was prepared by reacting Sepharose 6B with 1 : 4 butanediol/diglycidyl ether [17]. To prepare mercurial-Sepharose the damp dried epoxy-activated Sepharose (15 g) was then added to 4-aminophenylmercuric acetate (300 mg), dissolved in NaOH and diluted to 20 ml with 0.4 M Na_2CO_3 at pH 11.5. The mixture was placed in a roller rack at 40°C for 24 h. The mercurial-Sepharose was washed extensively to remove uncoupled 4-aminophenylmercuric acetate. Molecular weights were calculated using calibrated columns of Ultrogel AcA-44 as described by Sellers et al. [9].

Preparation of latent pig synovial collagenase. Pooled medium from days 1 and 2 (10.7 l) was thawed and taken to 20% saturation with $(NH_4)_2SO_4$ and left overnight at 2°C. After filtration through a Whatman 54 filter paper $(NH_4)_2SO_4$ was added to the filtrate to a final 80% saturation and the solution held at 2°C overnight. After centrifugation at 22 000 × *g* for 1 h at 0°C the pellet was suspended in 25 mM sodium cacodylate buffer, pH 7.2, containing 1 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij 35 and 0.02% NaN_3 and dialyzed against this buffer overnight. The concentrated medium was centrifuged at 40 000 × *g* for 1 h at 4°C and the pellet discarded. The supernatant (60 ml) was applied to a large column of Ultrogel AcA-44 (4.4 × 117 cm) equilibrated with the above buffer. The column was filtered by reverse flow at a flow rate of 30 ml/h and 11.0 ml fractions were collected. A single peak of enzymic activity was detected in the fractions after activation with 4-aminophenylmercuric acetate. No activity could be detected if the fractions were assayed without added mercurial. The fractions containing latent collagenase were pooled and stored at —20°C in 1-ml aliquots. This sample was used in all subsequent investigations of the activation of latent collagenase.

Collagenase assay and activation. [^{14}C]Acetylated collagen was used to measure collagenase activity in the diffuse fibril assay [18]. 1 unit collagenase digests 1 μg collagen fibrils 1 min at 37°C. Collagenase inhibitor was assayed as previously described [24].

Activation with mercurials and thiol reagents. All mercurials were dissolved at a concentration of 10 nM in 0.2 M NaOH and the pH adjusted to 10.0. Latent pig synovial collagenase, diluted in 25 mM sodium cacodylate, pH 7.5, containing 5 mM $CaCl_2$, 0.05% Brij 35 and 0.02% NaN_3 (80 μl), was incubated with 20 μl various 10 mM and 1 mM stock solutions of mercurials and thiol reagents. The samples were made up to 200 μl with 50 mM Tris-HCl buffer, pH 7.5, containing 15 mM $CaCl_2$ and then 100 μl [^{14}C]acetylated collagen was added. The samples were incubated at 37°C for 18 h before the undigested collagen fibrils were removed by centrifugation.

Activation with proteinases. Proteinases were incubated with latent pig synovial collagenase in 25 mM sodium cacodylate, pH 7.5, containing 0.05% Brij 35, 5 mM $CaCl_2$ and 0.02% NaN_3, for 15 min at 22°C at the concentrations shown. The reaction was stopped by the addition of diisopropylphosphofluoridata to 5 mM concentration or 5-fold excess of soybean trypsin inhibitor for the trypsin activation.

Preparation of plasmin. Streptokinase was added to plasminogen (1 : 100) and the mixture incubated for 20 min at 37°C and the plasmin generated was used immediately.

Results

Secretion of latent collagen by pig synovium. Pig synovial tissue was placed in culture and the medium changed every 2—3 days. No collagenase could be detected in culture medium when assayed in the absence of 4-aminophenylmercuric acetate. However large amounts of latent collagenase were shown to be present during the first six days when this reagent was added to the assays (Fig. 1). Subsequently these high levels fell quickly to less than 4 U/ml. An

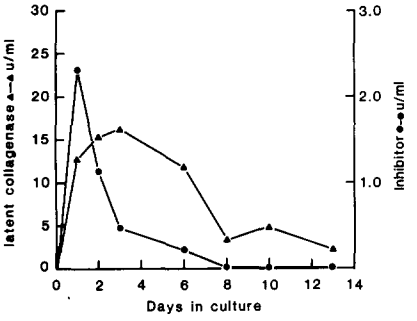


Fig. 1. Secretion of latent collagenase and inhibitor by pig synovium in tissue culture. Synovial tissue was cultured at 37°C as described by Cawston and Tyler [16]. The medium was changed daily for two days and then every 2—3 days until the latent collagenase levels fell to 1 U/ml (usually 14 days). The medium was assayed for latent collagenase and collagenase inhibitor.

inhibitor of collagenase was detected in the early days of culture (days 1—3). Days 1—2 were pooled and the latent collagenase partially purified by gel filtration (Methods). We investigated the activation of the latent collagenase using a variety of different treatments.

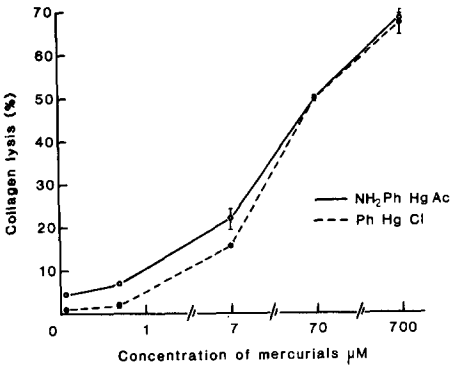
Activation of latent collagenase with mercurials. We compared the activation of latent collagenase achieved by a variety of mercurials and thiol reagents. Table I illustrates the results we obtained when latent collagenase was incubated with these reagents. The highest activation was found with 4-aminophenylmercuric acetate and phenylmercuric chloride. This activation was equal to that obtained with trypsin and no further activation was found when the two treatments were combined. The other reagents were not so effective

TABLE I

ACTIVATION OF LATENT PIG SYNOVIAL COLLAGENASE WITH VARIOUS MERCURIALS AND THIOL REAGENTS

Details of the activation are given in the Methods section. The results are expressed as the percentage of the latent collagenase activated in the presence of each reagent.

Reagent	Concentration (mM)	Latent collagenase activation
4-Aminophenylmercuric acetate	10	100
	1	71
4-Chloromercuribenzoate	10	29
	1	14
Mersalyl acid	10	32
	1	12
Sodium tetrathionate	10	7
	1	7
4-Hydroxymercuriphenylsulphonic acid	10	41
	1	10
Mercuric chloride	10	23
	1	30
Phenylmercuric chloride	10	97
	1	72
5,5′-dithiobis(2-nitrobenzoic acid)	10	16
	1	0



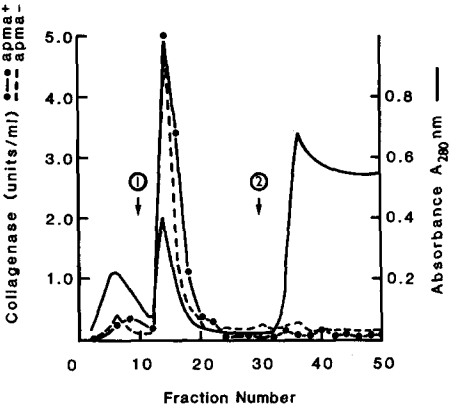


Fig. 2. Activation of latent pig synovial collagenase with increasing concentrations of 4-aminophenylmercuric acetate and phenylmercuric chloride. Latent pig synovial collagenase (0.1—0.2 units) was assayed in the presence of increasing concentrations of both phenylmercuric chloride (------) and 4-aminophenylmercuric acetate (———) in the diffuse fibril assay. Increasing collagen lysis occurs with increasing amounts of the two mercurials and reaches a maximum at a mercurial concentration of 700 μM.

Fig. 3. Activation of latent pig synovial collagenase with 4-aminophenylmercuric acetate-Sepharose. Latent pig synovial collagenase was thawed and dialysed against 25 mM sodium cacodylate, pH 6.5, containing 0.05% Brij 35, 1 mM $CaCl_2$ and 0.02% NaN_3. After 4 h the sample was loaded onto a column (0.9 × 2.0 cm) of 4-aminophenylmercuric acetate-Sepharose at a flow rate of 12.0 ml/h and 1.0-ml fractions were collected. The column was then eluted with the above buffer containing 200 mM NaCl, 1, and then with 5 mM cysteine in this buffer, 2.

although some activation was achieved with the 4-hydroxymercuriphenylsulphonic acid, mersalyl acid and 4-chloromercuribenzoate. Other thiol-reagents tested sodium tetrathionate and 5,5′-dithiobis(2-nitrobenzoic acid), did not activate the latent collagenase. Fig. 2 demonstrates the activation of latent enzyme by 4-aminophenylmercuric acetate and phenylmercuric chloride at lower concentrations. The activation reaches a maximum at 600—700 μM mercurial. No further activation could be achieved with higher concentrations of mercurial or trypsin. The plots for the two mercurials were very similar and 4-aminophenylmercuric acetate is used routinely because it is more soluble than phenylmercuric chloride.

Activation with 4-aminophenylmercuric acetate bound to Sepharose. The mechanism of the mercurial activation of latent collagenase is not known. We investigated the binding of latent pig synovial collagenase to 4-aminophenylmercuric acetate attached to Sepharose 4B. The latent enzyme was dialyzed against 25 mM sodium cacodylate buffer, pH 6.5, containing 0.05% Brij, 0.02% azide and 1 mM $CaCl_2$ and loaded onto a column (0.9 × 2.0 cm) of mercurial-Sepharose equilibrated with this buffer at room temperature. Most of the enzyme was activated by the mercurial column and could be eluted from the column by the addition of 200 mM NaCl (Fig. 3). The column was then eluted with 5 mM cysteine to remove any proteins still bound to the column. Incomplete inactivation of the latent enzyme was obtained when the column was eluted at 4°C. In a separate experiment (results not shown) the inhibitor of collagenase secreted in the first three days of culture was found to bind to the

column and was eluted, with good recovery, in the same way as the enzyme (Cawston, T.E., unpublished observations). However, no inhibitory activity was detected in any of the fractions eluted from the column loaded with the latent enzyme (Fig. 3).These results suggest that the interaction of mercurial with the latent enzyme is not just a simple removal of bound inhibitor to give the active form of the enzyme. The relatively weak binding of both the enzyme and inhibitor suggests that a mercurial-thiol interaction is not involved. However it is not possible using partially purified material to determine if a polypeptide is removed from the latent enzyme upon activation.

Activation with chaotrophic ions. Some workers have activated latent collagenase with sodium thiocyanate and sodium iodide [2,7,8,19]. Latent pig synovial collagenase was treated with 3 M sodium iodide overnight and the iodide was subsequently removed by dialysis against a buffer containing 1 M NaCl. When assayed 59% of the enzyme was active. No activation was obtained after treatment of latent collagenase with urea, sodium thiocyanate or guanidine hydrochloride and these reagents destroyed the enzyme activity.

Activation of latent collagenase with proteinases. Fig. 4 illustrates the activation of latent pig synovial collagenase when increasing amounts of trypsin were added at 22°C for 15 min. The latent collagenase was preincubated with trypsin prior to assay and the reaction stopped by the addition of excess soybean trypsin inhibitor. The concentration of trypsin shown is the concentration in the pre-incubation mixture. Trypsin at 1.5 μg/ml could completely activate the latent pig synovial collagenase.

Other proteinases were not so effective as trypsin at activating the latent collagenase. Fig. 5. shows the results we obtained when latent collagenase was treated with elastase, cathepsin G and plasmin for 15 min at 22°C. The reaction was stopped by the addition of 2 mM diisopropylphosphofluoridate and the collagenase activity then determined both with and without 4-aminophenylmercuric acetate, to determine the proportion of latent collagenase activated. Elastase activated the enzyme at high concentrations but at these levels some of the collagenase was degraded. At an elastase concentration of 500 μg/ml the collagenase was fully active but 80% of the enzyme originally present was destroyed. The results for cathepsin G were similar although less of the collagenase was degraded. Plasmin, however, even at concentrations of 500 μg/ml did not degrade the enzyme and 94 and 65% of the latent collagenase was activated at concentrations of 500 μg/ml and 50 μg/ml, respectively.

Spontaneous activation of latent collagenase. We found that partially purified latent pig synovial collagenase often activated spontaneously during purification procedures. Samples of the partially purified latent collagenase were routinely stored at −20°C in the buffer containing high salt (1 M), and under these conditions no spontaneous activation occurred. If the salt was removed and the enzyme stored at 4°C the latent enzyme slowly activated. The latent collagenase was thawed and dialysed against 25 mM sodium cacodylate buffer, pH 7.5, containing 0.05% Brij 35 and 0.02% sodium azide. Samples were removed at intervals and assayed to determine what proportion of the latent enzyme was active. After 48 h all of the latent enzyme had activated. We investigated this process to determine what factors prevented this spontaneous activation.

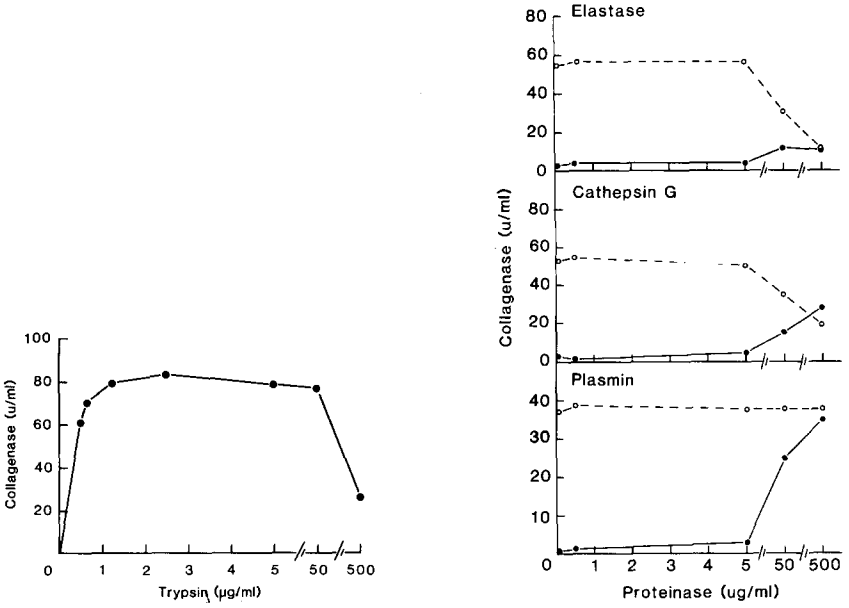


Fig. 4. Activation of latent pig synovial collagenase with increasing amounts of trypsin. Latent pig synovial collagenase (0.1—0.2 units) was added to trypsin at each concentration and incubated for 15 min at 22°C before the addition of excess soybean trypsin inhibitor. The samples were then assayed for collagenase activity.

Fig. 5. Activation of latent pig synovial collagenase with proteinases. Latent pig synovial collagenase (0.1—0.2 units) was treated with each proteinase for 15 min at 22°C at the concentrations shown. The reaction was stopped by adding diisopropylphosphofluoridate to 5 mM concentration. The collagenase was then assayed both with (○- - - - -○) and without (○———○) 4-aminophenylmercuric acetate, to determine the proportion of latent enzyme activated. The results show the amount of activated enzyme at each concentration of proteinase.

Latent collagenase was dialysed against buffers at various pH values, containing different amounts of NaCl and proteinase inhibitors and the proportion of active enzyme then measured after 60 h. Table II summarizes the results that we obtained. The spontaneous activation of the enzyme was prevented by a number of different reagents. Sodium chloride (0.1 M) prevented the activation as did buffers at pH 5.0 and 9.0, although 25% of the enzyme was destroyed after treatment at pH 5.0. Diisopropylphosphofluoridate (2 mM) prevented the enzyme activating. 1,10-phenanthroline (1 mM) destroyed most of the enzyme (80%) and only 50% of the enzyme remaining was active. Cysteine (10 mM) did not prevent the enzyme activating, whilst Zn^{2+} and Ni^{2+} (0.1 mM) both prevented the enzyme from activating spontaneously. Other metal ions were tested at this concentration and were found to follow the sequence $Zn^{2+} > Ni^{2+} > Cu^{2+} > Cd^{2+} > Mg^{2+} = Mn^{2+} > Co^{2+} > Ag^{2+} > Pb^{2+} > Hg^{2+}$ in their ability to prevent activation. The amount of active enzyme measured after incubation with these metal ions was 4, 13, 37, 43, 47, 47, 53, 62, 66 and 81%,

TABLE II

THE PREVENTION OF THE SPONTANEOUS ACTIVATION OF LATENT PIG SYNOVIAL COLLAGENASE

Samples of latent pig synovial collagenase (20 units), stored at −20°C in 25 mM sodium cacodylate buffer, pH 7.2, containing 10 mM $CaCl_2$, 1 M NaCl, 0.05% Brij 35 and 0.02% NaN_3 were thawed and dialysed at 4°C against the above buffer, for 60 h (control), and also against this buffer containing the reagents listed or at higher or lower pH. (Those samples containing inhibitors of collagenase were dialyzed into sodium cacodylate buffer containing 1 M salt to prevent any interference with the collagenase assay.) At the end of the dialysis the samples were assayed to determine what proportion of the latent enzyme had become active.

Sample	Active enzyme (%)
Control	100
pH 7.5 + 1 M NaCl	4
pH 7.5 + 0.1 M NaCl	3
pH 7.5 + 0.1 mM $ZnCl_2$	5
pH 7.5 + 0.1 mM $NiCl_2$	13
pH 7.5 + 2 mM DFP	3
pH 7.5 + 1 mM 1.10 Phenanthroline	50 *
pH 7.5 + 10 mM Cysteine	100
pH 5.0	2 **
pH 6.0	16
pH 9.0	1

* Only 18% of original activity remained.
** 75% of original activity remained.

respectively. Some loss of collagenase activity was observed with Cu^{2+} Pb^{2+} and Hg^{2+}.

Molecular weights of latent and active forms of collagenase. The molecular weights of the latent and active forms of collagenase were estimated by Ultrogel AcA-44 gel filtration (see Methods) and are shown in Table III. Two samples of collagenase were activated, the first with 4-aminophenylmercuric acetate and the second was allowed to activate spontaneously. For mercurial activation, latent enzyme was treated with 0.67 mM 4-aminophenylmercuric acetate for 4 h at 37°C. Spontaneous activation was for 60 h at 4°C. Both samples were fully activated prior to loading onto the column. A drop in molecular weight of approx. 10 000 occurred after activation by either method. The collagenase inhibitor had a molecular weight of 31 000.

TABLE III

MOLECULAR WEIGHT OF LATENT AND ACTIVE PIG SYNOVIAL COLLAGENASE

The molecular weight changes associated with the activation of latent enzyme. The molecular weights of the latent enzyme and of the enzyme after activation were calculated by gel filtration. Activation whether that achieved by proteinases or mercurials was always accompanied by a drop in molecular weight.

	Molecular weight
Latent enzyme	39 000
Spontaneously-activated enzyme	30 400
Mercurial-activated enzyme	26 600
Inhibitor	31 000

Discussion

Considerable controversy exists regarding the nature of latent collagenase and whilst some workers propose that the enzyme is secreted as a proenzyme others believe it consists of an enzyme-inhibitor complex. In this study we have investigated the activation of latent pig synovial collagenase using proteinases and other reagents in an attempt to understand how the activation of latent collagenase occurs in vivo.

A number of proteinases have been reported to activate latent collagenase and these include trypsin [1—4,7,8,20,21], plasmin [5,21], kallikrein [5], cathepsin B [5], papain [8,20], thermolysin [20], chymotrypsin [20] and bacterial fibrinolysin [8]. We chose three enzymes to study; elastase, cathepsin G and plasmin, which we thought could have a physiological role in the activation of latent collagenase. Plasmin was the only enzyme which activated the enzyme without destruction but more plasmin was required than trypsin to give the same activation unlike the findings of Werb et al. [21]. We have also found that plasma kallikrein activates latent collagenase (Nagase, H. and Cawston, T.E., unpublished observations). In vivo plasmin is the proteinase most likely to be involved in the activation of latent collagenase and plasminogen activator is often present in inflamed tissues [21].

An enhancement of collagenolytic activity after treatment with mercurials was first described by Lazarus et al. [22], in a manuscript describing human granulocyte collagenase. Subsequently Werb and Burleigh [23] used the same reagent *p*-chloromercuribenzoate to treat rabbit synovial cell culture medium and Sellers et al. [9] reported the superior properties of 4-aminophenylmercuric acetate and proposed that it was removing an inhibitor from the latent collagenase. Other workers have since reported activation with mercurials [10,11,25] but it is not clear why some mercurials activate latent collagenase whilst others do not. Differences also exist between the latent collagenases in this respect as human rheumatoid synovial collagenase is fully activated by mersalyl [11], whilst little activation is found when this reagent was added to latent pig synovial collagenase.

Several workers have described the spontaneous activation of latent collagenase [4,8,14,21] but the exact mechanism is not understood. All these studies found that incubation at 25°C for 24—48 h would activate the collagenase. Eeckhout and Vaes [8] have proposed that an activator exists in culture medium which must first be activated before, it, in turn, activates the latent collagenase. Stricklin et al. [4] found that the rate of activation varied from one crude culture medium to another. Storage of the purified procollagenase for several weeks at −20°C or at 37°C for 24 h completely activated the procollagenase although there was no corresponding drop in molecular weight. A similar observation was also made by Vater et al. [11].

We found a similar rate of spontaneous activation but there was little difference in rate between 4, 22 and 37°C once the high salt was removed. It is unlikely that this is an autoactivation as the rate of activation did not increase with time and was not increased by adding small amounts of activated enzyme or when small amounts of tissue inhibitor were added to the latent enzyme. Also chemical inhibitors of collagenase did not prevent the spontaneous activation.

The spontaneous activation of the pig synovial collagenase was prevented by a number of different reagents and conditions. The most effective of these were high salt, Zn^{2+}, diisopropylphosphofluoridate and soybean trypsin inhibitor. The exact mechanism of spontaneous activation was not clear although the inhibition by diisopropylphosphofluoridate, soybean trypsin inhibitor, Zn^{2+}, high and low pH were all consistant with the involvement of a serine proteinase. However, no serine proteinase could be detected in the latent enzyme preparation. After passage of the latent collagenase preparation through a column of both trasylol-Sepharose and soybean trypsin inhibitor-Sepharose the spontaneous activation still occurred (Cawstron, T.E., unpublished results).

The molecular weight data were consistant with that published previously [11,24] with a drop in molecular weight after activation with trypsin and mercurials. The spontaneously active enzyme had a similar molecular weight to the mercurial-activated enzyme. Some workers have reported that the spontaneously active enzyme has the same apparent molecular weight as the latent enzyme [11]. Pig spontaneously-active collagenase was often found in fractions eluted from gel filtration columns at the same elution volume as the latent enzyme but this was because the enzyme had activated after elution from the column. The enzyme eluted at the lower molecular weight if these fractions were then rechromatographed.

In this study we have investigated the activation of latent pig synovial collagenase by a number of different methods. Proteinases, chaotropic ions and mercurials were all effective activators and the latent enzyme activated spontaneously under certain conditions. However, the exact mechanisms responsible for activation in each case are not yet understood and it is still not clear if the latent enzyme is an enzyme-inhibitor complex. Further information will be obtained when both active and latent collagenase are purified and their interaction with purified inhibitor studied.

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