A Latent Form of Collagenase in the Involuting Rat Uterus and its Activation by a Serine Proteinase

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1. The involuting rat uterus displays an extremely rapid breakdown of collagen. Collagenase activity can be assayed directly in the insoluble 6000g pellet of uterine homogenates. At 1 day *post partum*, about 85% of this collagenase activity is in a latent form. 2. This latent form can be activated by trypsin or by a serine proteinase present in the uterine pellets. 3. The activating enzyme of the tissue is inhibited by a wide spectrum of trypsin inhibitors, including Trasylol, soya-bean and lima-bean trypsin inhibitors, snail inhibitor and di-isopropyl phosphorofluoridate. Partial inhibition is produced by benzamidine, phenylmethanesulphonyl fluoride, ϵ -aminohexanoate, leupeptin, antipain and α_1 -antitrypsin. Ovomucoid, 7-amino-1-chloro-3-tosylamido-1-heptan-2-one and 1-chloro-4-phenyl-3-(*N*-benzyloxy-carbonyl)amino-L-butan-2-one are not inhibitory. 4. Extraction of uterine pellets with 0.1M-CaCl₂ at 60°C releases both latent and active collagenase. Exclusion chromatography on Sephadex G-100 gives an apparent molecular weight of approx. 77000 for the latent form and 66000 for the active form. The latent form is suggested to be a zymogen of collagenase.

The metabolic breakdown of collagen poses a problem to the animal organism, since the collagen fibres lie in the extracellular matrix of the tissues. The cells responsible for collagen degradation must secrete proteolytic enzymes into the extracellular space, where these enzymes may escape cellular control and produce excessive digestion of proteins. It is likely that a series of control mechanisms would have evolved to govern this process. It was shown by Gross & Lapierre (1962) that collagen breakdown is initiated by a specific collagenase. We now know that this enzyme is controlled in part by its specificity, which is limited almost exclusively to collagen (Gross *et al.*, 1974), and by various inhibitors present in serum and tissues (Werb *et al.*, 1974; Woolley *et al.*, 1975; McCroskery *et al.*, 1975).

One might also expect that, as with many other secreted proteinases, collagenase would be synthesized in a zymogen form. The first evidence for such a zymogen form was obtained in cultures of resorbing tadpole tail by Harper *et al.* (1971): a zymogen of mol.wt. 120000 was converted by a factor in the culture medium, but not by trypsin, into an active form of mol.wt. about 105000. Since this original observation, there have been a number of reports of inactive or latent forms of collagenase in cultures of various tissues. In most cases it has been possible to activate these collagenases with trypsin (Vaes, 1972;

Hook *et al.*, 1973; Oronsky *et al.*, 1973; Birkedal-Hansen *et al.*, 1975a; Bauer *et al.*, 1975). Activating enzymes have also been detected in rheumatoid synovial fluid (Kruze & Wojtecka, 1972; Oronsky *et al.*, 1973; Wize *et al.*, 1975), dental plaque (Robertson *et al.*, 1974) and mast cells (Birkedal-Hansen *et al.*, 1975b). Although it is difficult to determine whether activation of collagenase in these cases is due to the destruction or competitive binding of an endogenous collagenase inhibitor or to the cleavage of a zymogen form to yield active enzyme, the latter interpretation is favoured by most authors.

Most of the reports of latent collagenase are based on findings in tissue culture. However, the present paper describes an activatable form of collagenase detected in tissue homogenates. In this work the involuting rat uterus was used, since this organ displays extremely rapid collagen breakdown. Collagenase activity can be measured directly in tissue homogenates; it is found in the insoluble fraction, possibly bound to its collagen substrate (Ryan & Woessner, 1971). Subsequent studies (Woessner & Ryan, 1973; Weeks *et al.*, 1976) have shown that this collagenase activity can be extracted and that it possesses the same characteristics as the collagenase obtained from cultures of tissue fragments from involuting uterus (Jeffrey & Gross, 1970). Efforts to enhance the recovery of collagenase in tissue

homogenates by adding trypsin to bind possible inhibitors led to the present findings. Preliminary notice of this work has been presented in abstract form (Woessner, 1975, 1976).

Materials and Methods

Uterine-pellet assay

Pregnant rats were obtained, several days before parturition, from Sprague-Dawley, Madison, WI, U.S.A. Preliminary studies indicated that collagenase activity was maximal at 1 day *post partum* (Ryan & Woessner, 1974). Accordingly, rats were killed at this time. The uteri were removed, dissected free of mesentery and nodules at the implantation sites and frozen until used (within 1–6 days). Collagenase was assayed by the method of Ryan & Woessner (1971), with slight modification. The uteri were minced and homogenized in 10 vol. of ice-cold 0.01M-CaCl₂ containing 0.25% (v/v) Triton X-100. The homogenates were centrifuged for 20 min to obtain a 6000g pellet. This pellet was resuspended to the original volume in 0.04M-Tris/HCl/0.15M-NaCl/0.01M-CaCl₂, pH 7.4, containing antibiotics. During 18 h incubation at 37°C, collagenase acted on the uterine collagen in the pellet to release soluble hydroxyproline-containing peptides. These were separated from insoluble collagen by centrifugation of the digests at 4°C for 30 min at 30000g. The resulting supernatants and pellets were separately hydrolysed in 6M-HCl and assayed for hydroxyproline (Woessner, 1961). Blanks contained 0.01M-EDTA in place of CaCl₂. A unit of collagenase is defined as the amount of enzyme digesting 1 µg of collagen in 1 h (assuming that collagen contains 13.4% hydroxyproline).

Fluorescent collagen assay for extracted collagenase

Collagenase activity was extracted from the 6000g pellets by a method previously outlined (Weeks *et al.*, 1976). This consists of heating at 60°C for 4 min in 10 vol. of 0.1M-CaCl₂/0.04M-Tris/HCl, pH 7.5, followed by centrifugation at 10000g for 20 min at 4°C. A rapid assay for soluble collagenase was based on the method of Steven *et al.* (1975). Fluorescein-labelled insoluble rat tail tendon collagen (2mg) was incubated for 18 h, at 37°C, with 0.2 ml of enzyme preparation and 0.8 ml of 0.04M-Tris/HCl/0.01M-CaCl₂, pH 7.5. The insoluble collagen was removed by centrifugation at low speed in a bench-top centrifuge, and the supernatant was diluted 1:10 in water for assay in a Turner 110 fluorimeter (G. K. Turner Associates, Palo Alto, CA, U.S.A.), by using appropriate filters for excitation at 490 nm and emission at 520 nm. Blanks were of two types: 0.01M-EDTA in place of Ca²⁺, or enzyme omitted. Total fluorescence of the collagen preparation was determined after exhaustive digestion with papain. With this information, the arbitrary fluorescence units of

the fluorimeter could be converted into µg of collagen digested. Again, a unit of collagenase is defined as the amount of enzyme digesting 1 µg of collagen in 1 h.

Inhibitor studies

Enzymes, proteins and inhibitors were obtained as follows: bovine trypsin, hog pancreatic elastase, soya-bean trypsin inhibitor (Kunitz), human α₁-antitrypsin, clostridiopeptidase A, chymotrypsinogen A, ovomucoid from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; lima-bean trypsin inhibitor, ovalbumin, bovine serum albumin, di-isopropyl phosphorofluoridate, fluorescein isothiocyanate from Sigma Chemical Co., St. Louis, MO, U.S.A.; phenyl-methanesulphonyl fluoride from Calbiochem, La Jolla, CA, U.S.A.; TosLysCH₂Cl, * ZPheCH₂Cl from Fox Chemical Corp., Los Angeles, CA, U.S.A.; soya-bean trypsin inhibitor type II (Bowman-Birk) from Miles Laboratories, Elkhart, IN, U.S.A. Pepstatin, antipain and leupeptin were generous gifts from Dr. H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan; *Helix pomatia* trypsin inhibitor was from Dr. H. Tschesche, Technical University, Munich, W. Germany; *Achatina fulica* haemolymph was from A. Kareem, University of Miami; transferrin was from Dr. K. Brew, University of Miami; Ac(Ala)₄CH₂Cl was from Dr. J. C. Powers, Georgia Institute of Technology, Atlanta, GA, U.S.A.; Trasylol was from Dr. K. Kuettner, Rush Medical Center, Chicago, IL, U.S.A.

Inhibitors were tested in the pellet assay system. They were normally added to the resuspended pellets at 4°C 10 min before the incubation at 37°C. Phenyl-methanesulphonyl fluoride and ZPheCH₂Cl were added as solutions in 5 µl of dimethyl sulphoxide. TosLysCH₂Cl and ZPheCH₂Cl were allowed to react for 2 h, 37°C, pH 6.9 (during which time less than 5% of the total digestion occurred), before adjustment to pH 7.4 and final incubation for the assay.

Results

Trypsin activation of uterine pellets

The work of Sakamoto *et al.* (1972) suggested that trypsin might be used to bind trypsin inhibitors such as α₂-macroglobulin, thereby preventing these from inhibiting collagenase. This approach was taken in the assay of collagenase in insoluble pellets of uterine homogenates (pellet assay), and trypsin was found to enhance the activity of collagenase. Maximum activity was obtained by activating with 100 µg of trypsin/2 ml of resuspended pellet for 3 min at 37°C. The trypsin digestion was then stopped by adding

* Abbreviations: TosLysCH₂Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one; ZPheCH₂Cl, 1-chloro-4-phenyl-3-(*N*-benzyloxycarbonyl)amino-L-butan-2-one; Ac, acetyl; QAE; diethyl-(2-hydroxypropyl)aminoethyl.

a 4-fold molar excess (360 μ g) of Kunitz soya-bean trypsin inhibitor before incubation for measurement of the collagenase activity. Trypsin activation required a finite time inversely proportional to the trypsin concentration; e.g. the same activation was obtained with 100 μ g of trypsin for 3 min or 5 μ g for 60 min.

To validate the comparison of assays with and without trypsin it was necessary to show the linearity of the assay under the two conditions (Fig. 1). It had previously been shown that the pellet assay was linear with time to 48 h (Ryan & Woessner, 1971). The present data re-confirm the linearity to 24 h and show that trypsin treatment does not affect the linearity of the subsequent assay.

Table 1(a) summarizes a series of experiments on trypsin activation of uterine pellets. The collagenase activity in the pellets was enhanced an average of 30% by the trypsin treatments. However, a peculiar effect was noted in controls to which Kunitz soya-bean trypsin inhibitor was added without the prior addition of trypsin. In this case, collagenase activity was inhibited by 80% compared with untreated pellets. This was surprising, since soya-bean trypsin inhibitor is not an inhibitor of collagenase; this was shown by adding the compound to trypsin-activated pellets in molar ratios ranging from 1 to 4 with no resultant change in collagenase activity. It was concluded that the uterine pellets contained a proteinase capable of activating uterine collagenase and that this activating proteinase was blocked by Kunitz soya-bean trypsin inhibitor. This conclusion is further supported by evidence given below.

The activation of collagenase by trypsin showed a

certain degree of specificity. It was not possible to activate the enzyme by the use of chymotrypsin or pancreatic elastase under equivalent conditions of time and concentration. Trypsin had only a slight effect on uterine collagen during the 3 min exposure; it produced no further release of hydroxyproline after the addition of Kunitz soya-bean trypsin inhibitor. The inhibition of trypsin after the activation step

Fig. 1. Linearity of the pellet assay for collagenase activity. Collagenase activity was measured in the insoluble 6000g pellets obtained from homogenates of 1-day-post-partum rat uteri as described in the Materials and Methods section. Fourteen incubation mixtures were prepared for each line and pairs were removed at random from the 37°C bath at intervals from 0 to 24 h for analysis of collagen digestion. ●, Pellets treated with trypsin for 3 min to activate collagenase; ○, pellets not treated with trypsin.

Table 1. Activation of uterine collagenase by trypsin

In (a) collagenase was assayed by its action on uterine collagen contained in the 6000g pellet. Tissue from one uterus was assayed under three sets of conditions; the activity in the tube treated with trypsin was set equal to 100 and the other digestions were calculated relative to this. This was necessitated by the biological variability among uteri. The average digestion corresponding to 100 relative units was 205 μ g of hydroxyproline released from 890 μ g total in the pellet, or 23% (range: 14–30%). This is equivalent to 85 collagenase units/200mg of wet tissue. Values are given as means $\pm$ s.d. In (b) collagenase was extracted from uterine pellets at 60°C and assayed on fluorescein-labelled polymeric rat tail tendon collagen. The average digestion corresponding to 100 relative units was 17%. This was obtained by activating 0.2 ml of uterine extract with 10 μ g of trypsin as in the pellet assay. This activity is equivalent to 48 collagenase units/200mg of wet tissue. The trypsin inhibitor in (a) and (b) was Kunitz soya-bean trypsin inhibitor.

	Relative digestion	No. of samples
(a) Collagenase in uterine pellet		
Pellet + trypsin + trypsin inhibitor	100	
Pellet + no addition	77 $\pm$ 7	13
Pellet + trypsin inhibitor	15 $\pm$ 12	9
(b) Extracted enzyme assayed on fluorescein-labelled collagen		
Extract + trypsin + trypsin inhibitor	100	
Extract + no addition	52 $\pm$ 12	21
Extract + trypsin inhibitor	34 $\pm$ 11	21

could also be accomplished by adding excess of TosLysCH₂Cl (3mM) or phenylmethanesulphonyl fluoride (0.3mM). If no inhibitor was added after the trypsin-activation step, the digestion was equal to the sum of the separate actions of collagenase and trypsin (with EDTA added), indicating that active collagenase is not susceptible to trypsin digestion under the assay conditions.

Collagenase activator of the uterus

The first group of experiments indicated that 360 μ g of Kunitz soya-bean trypsin inhibitor/2ml of resuspended uterine pellet blocked an endogenous proteinase. Further studies showed that this amount gave the maximum inhibition possible, and that with smaller amounts inhibition diminished. There did not seem to be sufficient proteinase in the pellets to give maximal activation of the collagenase under the assay conditions.

The activation of uterine collagenase by the endogenous proteinase is a time-dependent process requiring 6h to reach 90% of the maximum value (Fig. 2). There is no evidence for a lag period in this activation process, since a finite time is required for

Fig. 2. Activation of latent collagenase by endogenous proteinase

Pellets from homogenates of 1-day-post-partum uteri were incubated for 24h according to the normal pellet-assay method (see the Materials and Methods section). At various time-intervals incubation tubes were opened and 360 μ g of Kunitz soya-bean trypsin inhibitor was added. Incubation was then resumed for the full 24h period. Beyond 6h the addition of trypsin inhibitor had very little effect, indicating that the collagenase had become fully active.

Table 2. Inhibitors of collagenase activation in the rat uterus-pellet assay

The uterine-pellet assay was performed, with no additions, to measure collagenase activated by the endogenous activating enzyme. The decrease in this activity produced by 360 μ g of Kunitz soya-bean trypsin inhibitor was taken as 100% inhibition.

Inhibitor	Amount		Inhibition (%)
	(μ g/2ml)	(mM)	
Trasylol	45	0.0035	100*
Soya-bean trypsin inhibitor (Kunitz)	360	0.008	100*
<i>Helix pomatia</i> trypsin inhibitor	200	0.012	100*
Lima-bean trypsin inhibitor	360	0.018	100*
Soya-bean trypsin inhibitor (Bowman-Birk)	360	0.022	100
Di-isopropyl phosphorofluoridate	90	0.24	100
Benzamidine	1000	3.2	88*
ϵ -Aminohexanoate	50000	190	86*
Phenylmethanesulphonyl fluoride	1000	2.9	84*
<i>Achatina fulica</i> haemolymph	(50 μ l)	—	71
Whole fresh egg white	(10 μ l)	—	60*
Leupeptin	100	0.11	60*
Tosylarginine methyl ester	1000	2.7	60
Dithiothreitol	30	0.10	56†
Human α_1 -antitrypsin	1000	0.012	40*
Antipain	100	0.08	31
Ovomucoid	360	0.006	8
Ac(Ala) ₄ CH ₂ Cl	1000	2.7	8
TosLysCH ₂ Cl	1000	2.7	0*
ZPheCH ₂ Cl	1000	2.7	0
Iodoacetate	1000	5.4	0
Iodoacetamide	40	0.41	0
Pepstatin	100	0.15	0*
p-Chloromercuribenzoate	100	2.8	0*

* Indicates experiments in which there was no inhibition of collagenase as tested by the addition of inhibitor to pellets fully activated by trypsin.

† 22% inhibition when added to trypsin-activated pellets.

activations. The lower curve in Fig. 1 should show an acceleration during the first 6h. In fact, some of the early time-points do fall below the line in Fig. 1. However, a detailed analysis of this early period is difficult, since it involves measurements with large standard deviations.

Inhibition studies were undertaken to determine the properties of the collagenase-activating enzyme (Table 2). Inhibition by di-isopropyl phosphorofluoridate indicates that the uterine enzyme is probably a serine proteinase, whereas the failure of iodoacetate, iodoacetamide and pepstatin to inhibit indicate that it is probably not a thiol or carboxyl proteinase. Four other trypsin inhibitors appear to work as effectively as di-isopropyl phosphorofluoridate and Kunitz soya-bean trypsin inhibitor in inhibiting the enzyme, namely Trasylol, *Helix pomatia* inhibitor, lima-bean inhibitor and Bowman-Birk soya-bean inhibitor. Partial inhibition is shown by benzamidine, ϵ -aminohexanoate, phenylmethanesulphonyl fluoride, *Achatina fulica* inhibitor, whole egg white, tosylarginine methyl ester, leupeptin and antipain. Dithiothreitol is partially inhibitory at 0.1 mM; however, study of this compound is complicated by the fact that it is also inhibitory to active collagenase. Minimal or no inhibition is found with ovomucoid, TosLysCH₂Cl, ZPheCH₂Cl or Ac-(Ala)₄CH₂Cl, although the first two compounds are effective against trypsin.

Extraction of collagenase from uterine pellets

The study of collagenase activation in uterine pellets is difficult because there is no way to vary the ratio of enzyme to substrate and the identification of separate components of the system must be based on indirect measurements. A method had previously been developed to extract active collagenase from the insoluble pellets (Weeks *et al.*, 1976). When this method was applied to the present problem, the results in Table 1(b) were obtained. About 65% of the total assayable collagenase of the uterus is extracted (Weeks *et al.*, 1976). Of the amount extracted 34% is already active and Kunitz soya-bean trypsin inhibitor does not inhibit it. A small amount of activating enzyme is carried along in the extracts, causing the activity of collagenase to rise during the assay incubation to 52% of the total amount extracted. Trypsin activation produces a further doubling of the collagenase activity.

The assay using fluorescein-labelled collagen was used in these studies in order to have a rapid method for the screening of large numbers of fractions from chromatographic columns. Since part of the fluorescein is attached to telopeptide portions of the polymeric collagen (Steven *et al.*, 1975), this substrate is susceptible to digestion by non-specific proteinases in addition to collagenase. It was therefore necessary to show that the digestion in the present study was due

to collagenase. It has been shown previously that, when the pellet assay is performed, more than 95% of the collagen solubilized is recovered as fragments of less than 100000 mol.wt. (Woessner & Ryan, 1973). This establishes the uterine activity as being due to collagenase activity, not to an activity on telopeptides. Uterine extracts made at 60°C have been shown to digest native collagen molecules to the typical 75%/25% fragments (Weeks *et al.*, 1976). When the extracts are applied to uterine polymeric collagen, they digest collagen at the rate of approx. 55 μ g of collagen/h per 200mg of wet tissue extracted. When applied to fluorescein-labelled rat tail tendon polymeric collagen, they digest at the closely comparable rate of 48 μ g of collagen/h per 200mg of tissue extracted (Table 1b). Further, the activity requires Ca²⁺, is completely inhibited by EDTA, is activated by trypsin and emerges as a single peak from Sephadex G-150 columns (see below). For these reasons it is concluded that the activity measured in uterine extracts represents true collagenase activity. Non-specific proteinase activity that may be present in the uterus has presumably been discarded with the supernatant at the time at which the uterus was first homogenized. The linearity of the assay with fluorescein-labelled collagen when applied to uterine extracts is illustrated in Fig. 3.

Fig. 3. Linearity of the collagenase assay with fluorescein-labelled collagen as substrate

Fluorescein-labelled collagen (2mg) was assayed with collagenase extracted at 60°C from a 1-day post-partum rat uterus. The assay procedure is outlined in the Materials and Methods section. Various amounts of extract were tested in triplicate; the final assay volume was 1 ml. Complete digestion of the substrate with papain yielded 360 fluorescence units; therefore the assay is shown to be linear with amounts of enzyme up to 30% digestion of the substrate.

Exclusion chromatography of extracted collagenase

Extracts of uterine pellets were assayed for latent and active collagenase and then passed through a column of Sephadex G-150 (Fig. 4). Assay of the effluent in the presence of Kunitz soya-bean trypsin inhibitor showed a small peak of fully active collagenase centring around fraction 43. Assay with no additions revealed a much larger peak of activity centred on the same fraction. This activity is attributed to latent collagenase that has been activated by the uterine proteinase present in the same region. Activation of the fractions by trypsin gave a much larger peak of collagenase, with a midpoint near fraction 40. This suggests that the activating enzyme does not underlie the entire peak of latent collagenase, but is retarded somewhat with respect to it.

Self-activation of the extracted collagenase was quite variable, indicating that extraction of the activating enzyme was not consistent. In some cases the extracts became active on storage for several days in the cold; others became active only after concentration by ultrafiltration followed by incubation at 37°C for 18 h. Self-activation eventually yields the same collagenase activity that can be produced by trypsin activation. Trypsin activation is a time-

Fig. 4. Chromatography of uterine extracts on Sephadex G-150

A column (2.6 cm × 90 cm) of Sephadex G-150 was equilibrated at 4°C with 0.04 M-Tris/HCl buffer, pH 7.5, containing 0.15 M-NaCl and 0.01 M-CaCl₂. Extracts of 6000g pellets of rat uterine homogenates were prepared at 60°C and chilled; 36 ml of extract was applied to the column and eluted with the same buffer at a rate of 20 ml/h. Fractions (6 ml) were collected and assayed on fluorescein-labelled collagen in three different ways. (A) Activation by 10 µg of trypsin/ml of eluate for 3 min, followed by 36 µg of Kunitz soya-bean trypsin inhibitor; (B) no addition; (C) addition of 36 µg of Kunitz soya-bean trypsin inhibitor only. The total activity applied was 860 units (calculated as fully activated) and recovery was 470 units (55%). —, Protein (*A*₂₈₀); ●, collagen digestion (fluorescence units).

Fig. 5. Activation of extracted latent collagenase by trypsin. Uterine extracts were first treated with Kunitz soya-bean trypsin inhibitor (100 µg/ml) and then passed through Sephadex G-150 columns as described in Fig. 4, to obtain a partially purified latent enzyme. An amount of latent enzyme corresponding to 72 fluorescence units when fully activated was incubated in 1 ml total volume with trypsin (●, 0.5 µg; ○, 0.25 µg) for various time-intervals to activate the latent enzyme. Kunitz soya-bean trypsin inhibitor was then added in 20-fold excess and the mixture incubated for 20 min at 25°C. At this point 2 mg of fluorescein-labelled collagen was added and the normal fluorescence assay was performed.

Fig. 6. Behaviour of latent and active collagenase on chromatography on Sephadex G-150

Conditions were as for Fig. 4 except that the column length was 82 cm. (a) Extract of 1-day-post-partum uterus treated with Kunitz soya-bean trypsin inhibitor to prevent activation of the collagenase (fractions activated by trypsin); (b) extract activated by incubation overnight at 37°C before chromatography; (c) extract activated by 3 min exposure to trypsin (100 µg/ml).

Fig. 7. Molecular-weight determination by molecular sieving on Sephadex G-100

A column (2.6 cm $\times$ 88 cm) of Sephadex G-100 was equilibrated at 4°C with 0.04 M-Tris/HCl/0.01 M-CaCl₂/0.4 M-NaCl, pH 7.5. Enzyme samples and protein markers were applied in a volume of 15 ml (determined by the detection limits for the enzyme). Elution was with the same buffer by gravity flow and fractions (4 ml) were collected. Inactive collagenase was obtained by adding Kunitz soya-bean trypsin inhibitor to 60°C extracts of uterus to bind activating enzyme. After chromatography, the inactive enzyme (A) was activated by 3 min treatment with 5 μ g of trypsin/ml; 18 μ g of trypsin inhibitor was added before assay with fluorescein-labelled collagen. Active collagenase (B) was obtained either by trypsin activation of extracts before application to the column or by incubation at 37°C for 18 h to allow activation by the endogenous activating enzyme. The eluted fractions were then assayed in the presence of trypsin inhibitor. Each point was determined in duplicate (four points for B). K_{av} is calculated as $(V_e - V_0)/(V_t - V_0)$, where V_e is elution volume of protein, V_0 is the void volume determined with Blue Dextran and V_t is the total column bed volume determined with Dnp-lysine. Markers are: 1, clostridiopeptidase A; 2, transferrin; 3, bovine serum albumin; 4, hexokinase subunit; 5, ovalbumin; 6, chymotrypsinogen.

dependent process for the extracts, just as for the pellets. The time-curves in Fig. 5 illustrate this point.

If extracts are treated with Kunitz soya-bean trypsin inhibitor, then passed through the Sephadex G-150 column, self-activation does not occur and a peak of inactive collagenase is obtained which can be measured only after trypsin activation (Fig. 6a). On the other hand, extracts that have undergone self-activation (Fig. 6b) or have been activated by trypsin (Fig. 6c) give an active peak of collagenase which is retarded by Sephadex G-150 and emerges about 15 ml later than the inactive peak.

A more detailed study of molecular weights was done on a carefully calibrated Sephadex G-100 column (Fig. 7). In this experiment the latent collagenase emerged ahead of active collagenase at a position corresponding to approx. 77 000 mol.wt. The size of active collagenase was determined in two ways. First, the enzyme was fully activated with trypsin before it was applied to the column. Secondly, the extract was allowed to undergo self-activation by the endogenous proteinase. Complete activation was obtained by incubating the extract for 18 h at 37°C before chromatography. Both methods of activation gave identical results on the Sephadex G-100 column, corresponding to a molecular weight of approx. 60 000.

Discussion

The uterus collagenase may now be added to the growing list (see the introduction) of collagenases that can be activated by trypsin treatment. An important question that has not been completely resolved for any collagenase, including the uterine enzyme, is whether the latent form is a zymogen of collagenase or a collagenase-inhibitor complex. The evidence to date supports, but does not prove, the hypothesis that the uterine enzyme is a zymogen form. Several collagenase-inhibitor complexes are known, including those involving α_2 -macroglobulin (Abe & Nagai, 1972) and a serum inhibitor of about 40 000 mol.wt. (Woolley *et al.*, 1975). The molecular weight of the uterine latent collagenase is not compatible with that expected for a macroglobulin-enzyme complex, nor can activity be recovered with KCNS treatment. The weight change of 17 000 daltons that accompanies activation does not accord with an inhibitor of mol.wt. 40 000. The soluble fraction of uterine homogenates inhibits the activation step, but does not inhibit the collagenase. Therefore no excess of presumptive collagenase inhibitor can be detected in the uterus. The latent form cannot be activated by heparin, *p*-chloromercuribenzoate or iodoacetate or by chromatography on CM-, DEAE- or QAE-Sephadex.

The uterine collagenase zymogen appears to be identical with the fibroblast procollagenase described by Birkedal-Hansen *et al.* (1976). This enzyme is secreted by cultured fibroblasts as a 78 000-mol.wt. form which is activated by trypsin to a 60 000-mol.wt. collagenase. Evidence is presented for the direct secretion of the zymogen form as opposed to the formation of an enzyme-inhibitor complex after synthesis of the enzyme.

It is believed that both the latent and the active forms of collagenase in the uterus may be extracellular, since experiments designed to recover intracellular collagenase by homogenization in 0.25 M-sucrose and differential centrifugation have

not yielded detectable activity in particulate or soluble fractions. Rather the activity is associated with the cell-debris-fibre fraction and can be recovered by sedimentation at 1g. It is tempting to speculate that a latent form of collagenase is secreted by the cells, becomes deposited on the collagen fibres, and there awaits final activation by a second proteinase. This would provide a mechanism of storing large amounts of collagenase in preparation for the extremely rapid and extensive (90%) breakdown of collagen during uterine involution. At the moment, evidence supporting this hypothesis is largely circumstantial. Collagenase and collagen both occur in an insoluble residue after homogenization, and extracted collagen often has collagenase activity associated with it (Pardo & Perez-Tamayo, 1975). However, such an association may not occur until the tissue is homogenized.

The activating enzyme is probably a serine proteinase. It has a number of similarities to trypsin, but also several significant differences in that trypsin inhibitors such as TosLysCH₂Cl and ovomucoid are not effective. The inhibition pattern of this enzyme has been compared with patterns of 24 known mammalian serine proteinases described in the literature. The uterine enzyme appears to be distinct from all except clotting Factor X_A (Fujikawa *et al.*, 1972). A definitive identification of the enzyme must await further purification. Trypsin-like activity has previously been reported for human uterine tissue by Rybak *et al.* (1974) and Notides *et al.* (1973), but Kunitz soya-bean trypsin inhibitor did not inhibit this activity. Katz *et al.* (1976) have detected an insoluble oestrogen-dependent proteinase of the rat uterus which is partially inhibited by di-isopropyl phosphorofluoridate, leupeptin and antipain. There are insufficient data to decide whether this enzyme corresponds to the collagenase-activating enzyme. Such a correspondence would be of considerable interest in view of the oestrogen-dependence of uterine collagenase activity (Ryan & Woessner, 1974).

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