The extraction of a neutral metalloproteinase from the involuting rat uterus, and its action on cartilage proteoglycan

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1. Homogenates of rat uteri removed 1 and 2 days *post partum* were centrifuged at 6000g. Both pellets and supernatants degraded Azocoll, a general proteinase substrate, at pH 7.5. More than 80% of the total activity was in the pellet fraction. 2. Part of the pellet activity was in a latent form. Trypsin and 4-aminophenylmercuric acetate (a thiol-blocking agent) both activated this latent form, indicating that it is an enzyme-inhibitor complex. An endogenous serine proteinase activated part of the latent enzyme during the assay. 3. The enzyme activity was low before parturition and after involution; it was highest during the first 2 days *post partum*, when the largest losses of uterine wet weight and matrix macromolecules occur. 4. Up to 70% of the enzyme in the pellets was extracted by heating at 60°C for 4 min in 0.1 M-CaCl₂/0.05 M-Tris/HCl, pH 7.5. Approx. 30% of the extracted enzyme was still latent. 5. The extracted enzyme was a metalloproteinase, since it was inhibited completely by 1,10-phenanthroline, but not by inhibitors of thiol or serine proteinases. 6. The enzyme was further purified 15–30-fold by gel chromatography and precipitation with (NH₄)₂SO₄. The apparent molecular weight, estimated by gel filtration, was 24 000 for the latent form and 12 000 for the active form. The pH optimum was 7–7.5. 7. The enzyme also degraded cartilage proteoglycan. This activity was studied by viscometry and the products were analysed by analytical ultracentrifugation. The major product had a mol.wt. of approx. 100 000. The sites of cleavage were in the protein core, since no free oligosaccharides were detected. 8. This neutral metalloproteinase is distinct from uterine collagenase and from a uterine metal-dependent endopeptidase that hydrolyses a heptapeptide related to collagen.

Few representatives of the metalloproteinase class of proteolytic enzymes have been found in animal tissues. The first of this group was collagenase (Gross & Lapière, 1962). Articular cartilage contains neutral metalloproteinases capable of degrading proteoglycans (Sapolsky *et al.*, 1976). Several cells and tissues have been reported to produce metalloenzymes that degrade gelatin (Harris & Krane, 1972; Sopata & Dancewicz, 1974; Werb & Reynolds, 1974; Sellers *et al.*, 1978), elastin (Werb & Gordon, 1975) and proteoglycans (Werb & Reynolds, 1974; Werb & Gordon, 1975; Sellers *et al.*, 1978; Vaes *et al.*, 1978; Hauser & Vaes, 1978; Werb *et al.*, 1978). So far, this class of proteinases appears to have a special ability to degrade the extracellular components of the connective-tissue matrix. However, there has been little evidence that

correlates metalloproteinase activity with the processes of tissue matrix degradation in a given connective tissue.

The involuting rat uterus is the site of the fastest non-pathological resorption of a connective tissue known (Harkness & Moralee, 1956; Montfort & Pérez-Tamayo, 1961; Woessner, 1962). As such it is an ideal system for the study of enzymes that may participate in the resorption of the major matrix macromolecules: collagen, proteoglycans and elastin. The rate of collagen breakdown during the involution of the rat uterus *post partum* is closely correlated with the total activity of collagenase (Woessner, 1979a). This temporal correlation strongly supports the hypothesis that collagenase participates in collagen breakdown *in vivo*. Collagenase is specific for collagen (Harris & Cartwright, 1977), so it follows that there must be additional proteinases for the resorption of the other uterine matrix

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proteins. In the present paper we report the identification and extraction of a second neutral metalloproteinase of the involuting rat uterus. This proteinase degrades Azocoll and cartilage proteoglycan.

Materials and methods

Materials

The sources of most chemicals used in this study have been described previously (Woessner, 1977, 1979a,b). Azocoll was from Calbiochem, La Jolla, CA, U.S.A. Bovine nasal cartilage was obtained frozen from Pelfreeze, Rogers, AR 72756, U.S.A. Ultrogel AcA 54 was supplied by L.K.B., Rockville, MD 20852, U.S.A. Spectrapor dialysis membrane, molecular-weight cut-off 3500, was obtained from Spectrum Medical Industries, Los Angeles, CA 90054, U.S.A.

Animals

Pregnant rats were obtained several days before parturition from Sprague-Dawley, Madison, WI, U.S.A. They were maintained in a room with controlled light and fed on Purina Rat Chow and water *ad libitum*. The time of parturition was noted and the young were allowed to suckle. The rats were killed at various stages before and after parturition. The uteri were removed, dissected free of mesentery and nodules at the implantation sites, weighed, and frozen at -20°C in sealed containers until used.

Preparation and fractionation of uterine homogenates

Uteri were thawed at room temperature, minced finely with scissors and homogenized in 10 vol. of ice-cold 0.01 M-CaCl₂ containing 0.25% (v/v) Triton X-100. The homogenates were centrifuged at 2°C for 20 min at 6000g to obtain a pellet and a supernatant. The pellet was resuspended in a volume of 0.05 M-Tris/HCl (pH 7.5)/0.1 M-CaCl₂ equal to that of the original homogenate, and NaN₃ was added to a concentration of 0.02% (w/v). Collagenase and neutral proteinase were extracted from the pellet by heating to 60°C for 4 min with constant shaking. The extracted enzymes were separated from the residue as a supernatant after centrifugation for 20 min at 2°C at 10000g. Additional collagenase and neutral proteinase were extracted from the residual pellet by repeating the extraction procedure.

Further purification of neutral proteinase was achieved by concentrating the extract 5-fold by ultrafiltration on an Amicon PM-10 membrane (Lexington, MA 02173, U.S.A.) and chromatographing the concentrated extract on a column of Sephadex G-150 (90 cm $\times$ 2.6 cm) equilibrated and eluted with 0.05 M-Tris/HCl (pH 7.5)/0.2 M-NaCl/0.01 M-CaCl₂/0.02% (w/v) NaN₃. Fractions con-

taining neutral proteinase activity were pooled and solid (NH₄)₂SO₄ was added slowly with stirring to 60% saturation. The precipitate was collected by centrifugation at 40000g for 30 min, dissolved in a maximum of 5 ml of 0.05 M-Tris/HCl (pH 7.5)/0.2 M-NaCl/0.01 M-CaCl₂/0.02% (w/v) NaN₃ and dialysed in Spectrapor tubing no. 3 (mol.wt. cut-off 3500) against 100 vol. of the same buffer to give the partially purified enzyme preparation with a specific activity 15–30 times that of the enzyme in the extract.

Enzyme assays

(a) *Azocoll digestion*. Samples of resuspended pellets, uterine supernatant, uterine extracts, column fractions or partially purified neutral proteinase were made to 2 ml with 0.05 M-Tris/HCl (pH 7.5)/0.2 M-NaCl/0.01 M-CaCl₂/1% (v/v) butan-1-ol. Azocoll (0.5 ml) was added as a suspension (4 mg/ml) in the same buffer supplemented with 0.05% (w/v) Brij-35 to minimize sticking of substrate to the walls of the assay tube. Incubations were at 37°C with constant shaking to ensure thorough mixing. The small amount of enzyme activity present in each assay required incubations of 17–20 h in order to demonstrate sufficient substrate lysis. At the end of the assay, tubes containing samples of uterine pellet were centrifuged for 15 min at 40000g to sediment the pellet and undegraded substrate. The supernatant was decanted and the A_{520} was measured. Incubations made with enzyme from other fractions were terminated by directly decanting the supernatant from the undegraded substrate. All the assays were linear with respect to enzyme concentration provided that no more than 50% of the substrate was hydrolysed. Total lysis of 2 mg of Azocoll gave $A_{520} = 0.42$. A unit of Azocoll-degrading activity was defined as the hydrolysis of 1 μg of Azocoll/min under the assay conditions.

(b) *Fluorescein-collagen digestion*. The assay was based on the method of Steven *et al.* (1975), as described by Woessner (1977). Fluorescein-labelled insoluble collagen from rat tail tendon (2 mg) was incubated with shaking at 37°C . Incubations of 18–20 h were required to demonstrate sufficient substrate lysis, owing to the small amount of enzyme activity present in the assays. The volume of the incubation was made up to 1 ml with 0.05 M-Tris/HCl/0.2 M-NaCl/0.01 M-CaCl₂/0.02% (w/v) NaN₃.

(c) *Cartilage proteoglycan digestion*. The assay method was that of Nagase (1977) derived from the bead assay of Dingle *et al.* (1977). Enzyme was incubated with 4 mg of polyacrylamide beads containing proteoglycan subunit from bovine nasal cartilage. The total volume was 200 μl , the pH was 7.5, and the assay incubation was for 2 h. At the end of this period, 0.5 ml of deionized water was added.

The samples were chilled and centrifuged at 10000g for 5 min in a Beckman model B microfuge. Then 200 μ l of supernatant was removed for the determination of uronic acid by the method of Bitter & Muir (1962). Digestion was quantified as A_{530} . Blanks were prepared by incubating enzyme preparation in the absence of beads to measure endogenous uronic acid, or by incubating beads with buffer alone to measure the solubilization of uronic acid from the beads or by boiling enzyme (100°C for 15 min) before assay. The assay was linear with respect to enzyme concentration provided that no more than 10% of the total uronate in the beads was released (Nagase, 1977).

Determination of molecular weights by gel chromatography

All estimates of apparent molecular weights were made with columns of Ultrogel AcA 54 (90 cm $\times$ 1.5 cm). Flow rates were 12–15 ml/h and the columns were eluted with 0.05 M-Tris/HCl (pH 7.5)/0.2 M-NaCl/0.01 M-CaCl₂ containing either 1% (v/v) butan-1-ol or 0.02% (w/v) NaN₃ as preservative; 2.5–3.0 ml fractions were collected. Columns were calibrated by the method of Andrews (1964), with bovine serum albumin (68 000), ovalbumin (42 000), carbonic anhydrase (29 000), soya-bean trypsin inhibitor (22 500) and cytochrome c (12 500) as standards.

Trypsin activation of neutral proteinase

Samples (0.2–0.25 ml) of resuspended uterine pellet or supernatant or extract were incubated in a final volume of 2 ml with 0–80 μ g of trypsin and 1.7 ml of 0.05 M-Tris/HCl (pH 7.5)/0.2 M-NaCl/0.01 M-CaCl₂/1% (v/v) butan-1-ol. Incubations were for 3 min at 37°C. Reactions were terminated by the addition of a 4-fold weight excess of soya-bean trypsin inhibitor. Enzyme activity was then measured with Azocoll as substrate. The optimum amount of trypsin was 25–30 μ g/0.25 ml of pellet, supernatant or extract.

Characterization of proteoglycan digestion products

Proteoglycan subunit was extracted from bovine nasal cartilage by the method of Hascall & Sajdera (1969). Guanidine hydrochloride extracts were filtered through glass wool and adjusted to a density of 1.56 g/ml by the addition of solid CsCl. A single dissociative centrifugation was performed for 48 h at 34 000 rev./min in a Spinco model L centrifuge rotor no. 40 at 20°C. The bottom two-fifths of each gradient was collected, dialysed exhaustively against water and freeze-dried. The final product had a uronic acid content of 24% by weight.

Samples of proteoglycan subunit were suspended in 0.15 M-KCl and allowed to disperse overnight at

2°C to give solutions containing 12 mg of proteoglycan/ml. Proteoglycan solution (0.4 ml; final concn. 4 mg/ml) was mixed with 0.8 ml (14 units) of partially purified enzyme (trypsin-activated) or with 0.05 M-Tris/HCl (pH 7.5)/0.2 M-NaCl/0.01 M-CaCl₂/0.02% (w/v) NaN₃ to give 1.2 ml of the following samples: neutral proteinase, neutral proteinase plus 2 mM-1,10-phenanthroline, and buffer containing 30 μ g of trypsin and 120 μ g of soya-bean trypsin inhibitor. Samples (1 ml) were placed in Ostwald viscometers and suspended in a 37°C bath immediately after adding the enzyme. Viscosity measurements were performed as soon as possible and then at intervals up to 5 h.

At the end of the viscosity study, the samples were removed from the viscometers for study in the Beckman model E analytical ultracentrifuge. For comparison, 4 mg of proteoglycan was exhaustively digested with papain (0.1 mg, 1 h at 37°C) in an incubation mixture (final volume 1 ml) containing 0.1 M-KCl/1 mM-dithiothreitol/1 mM-EDTA. Two double-sector schlieren cells were used to hold 0.4 ml of each of the four samples so that all the samples could be examined simultaneously. The samples were centrifuged at 60 000 rev./min, 20°C, and photographs were taken at appropriate intervals. Sedimentation coefficients were calculated by the program of Trautman (1966). Measurements were made at three initial concentrations, 4, 2 and 1 mg/ml. This permitted the extrapolation of sedimentation coefficients to zero concentration as described by Rosenberg *et al.* (1970). The measured sedimentation coefficients for the various concentrations of undegraded proteoglycan subunit were almost identical with those given by Rosenberg *et al.* (1970).

Results

Neutral proteinase activity in fractions of the involuting rat uterus

The most dramatic losses in wet weight and matrix components of the involuting uterus occur in the first 2 days after parturition (Woessner, 1962; Grant, 1965). Accordingly, homogenates of rat uteri prepared from pools of tissue removed 1 day and 2 days *post partum* were fractionated into supernatants and pellets as described in the Materials and methods section. The fractions were assayed for their ability to degrade Azocoll, a general proteinase substrate, at pH 7.5 (Table 1). When untreated supernatant fractions were assayed, enzyme activity was barely detectable (0.9 unit/g wet wt. of tissue). However, equivalent amounts of the resuspended uterine pellet preparation hydrolysed the substrate extensively (26.3 units/g wet wt. of tissue). Surprisingly, the assay of enzyme activity in the uterine pellets with the insoluble but not endogenous

Table 1. *Neutral proteinase activity in fractions of uterine homogenates*

Homogenates of uteri removed 1 day and 2 days *post partum* were prepared and fractionated as described in the Materials and methods section; 0.5 g of tissue (finely minced) was used in each case. Preincubations were made either with trypsin (25 μ g/0.2 ml of fraction; 3 min at 37°C) or with an equal volume of buffer in a final volume of 2 ml. Soya-bean trypsin inhibitor (100 μ g) was added to the tubes containing trypsin. Other incubations received an equal volume of buffer. All tubes were left for 10 min at room temperature before assay. Results are expressed as units/g of wet tissue, $\pm$ S.E.M. for six observations. Alternatively, results were expressed as a percentage of the maximum enzyme activity.

Pretreatment	Azocoll degradation (units/g wet wt. of tissue)		Relative digestion of Azocoll	
	Pellet	Supernatant	Pellet	Supernatant
Trypsin, then soya-bean trypsin inhibitor	37.8 $\pm$ 1.6	7.6 $\pm$ 1.7	100	20.1
Buffer only	26.3 $\pm$ 2.6	0.9 $\pm$ 0.7	69.6	2.4

substrate, Azocoll, was linear with respect to enzyme concentration up to 50% hydrolysis of the substrate (results not shown).

In the case of collagenase, the other metalloproteinase previously studied in the rat uterus, most of the enzyme activity was latent (Woessner, 1977, 1979a), but the latent enzyme could be activated by trypsin (Woessner, 1977). Similar results were obtained for the Azocoll-degrading activity of the pellets (Table 1). A brief treatment of the pellets with trypsin, followed by inhibition of the trypsin with a 4-fold weight excess of soya-bean trypsin inhibitor (see the Materials and methods section), resulted in an additional 20–30% digestion of Azocoll by the pellets. In the supernatant there was a more striking activation, but the activity in the supernatant was only about 17% of the total activity in the uterus. Because more than 80% of the Azocoll-degrading activity was found in the pellet fraction, subsequent studies were performed with this fraction of the uterine homogenates.

Effect of proteinase inhibitors on Azocoll degradation by resuspended pellet fractions

Resuspended pellets treated with trypsin, then with soya-bean trypsin inhibitor and assayed without inhibitors, had an average of 37.2 units of enzyme activity/g wet wt. of tissue (Table 2). In the presence of the metal-chelating agents 1,10-phenanthroline (final concn. 2 mM) and EDTA (20 mM) 63% of the enzyme activity was inhibited. Greater inhibition of enzyme activity was seen in the presence of 8 mM-1,10-phenanthroline (70% inhibition) and 50 mM-EDTA (75% inhibition). Incubation with phenylmethanesulphonyl fluoride (2 mM), an inhibitor of serine proteinases, and with 4-aminophenylmercuric acetate (0.5 mM), an inhibitor of thiol proteinases, produced no significant inhibition of Azocoll degradation. The majority of

Azocoll degradation appears to be due to metalloproteinase activity.

Untreated pellets, i.e. pellets not incubated with trypsin, had an average of 24.9 units of enzyme activity/g wet wt. of tissue. This activity was blocked by more than 70% by 1,10-phenanthroline (2 mM) and by EDTA (20 mM), suggesting that the vast majority of the enzyme activity was metalloproteinase in nature. However, Azocoll degradation by untreated pellets was also inhibited 64% by phenylmethanesulphonyl fluoride (2 mM) and 48% by soya-bean trypsin inhibitor (40 μ g/ml), inhibitors of serine proteinases. It seems unlikely that 16 units of enzyme activity (24.9 minus 8.9) are due to the direct action of a serine proteinase on Azocoll, since all but 6 units are sensitive to metal-chelating agents. An alternative interpretation is that a large proportion of the Azocoll degradation is due to metalloproteinase activity that exists predominantly in a latent form. As with uterine collagenase (Woessner, 1977), a serine proteinase present in the uterine pellet converts the latent enzyme into an active enzyme during the assay. In the presence of phenylmethanesulphonyl fluoride or soya-bean trypsin inhibitor the serine proteinase is inhibited; therefore the metalloproteinase remains latent and does not degrade the Azocoll. That fraction of enzyme activity remaining after incubation of the untreated pellets with phenylmethanesulphonyl fluoride or soya-bean trypsin inhibitor is the metalloproteinase already active at the time of homogenization of the uterus. This view is supported by the observation that the enzyme active in the presence of soya-bean trypsin inhibitor is almost completely inhibited by the metal-chelating agents. Furthermore, 4-aminophenylmercuric acetate (0.5 mM) markedly enhanced Azocoll degradation by pellets that were not trypsin-treated. The effect was almost as great as that produced by trypsin. The

Table 2. *Effect of potential inhibitors on the neutral proteinase activity in pellets of uterine homogenates*

Resuspended pellet preparations were made from uteri removed 1 day *post partum*. Samples (0.2 ml) were pretreated as described in Table 1 and incubated for 1 h at 25°C with the additions indicated. Controls were done by using an equal volume of the appropriate solvent in place of the inhibitor. The maximum inhibition by any of the solvents alone was 10%. Assay conditions were as described in the Materials and methods section and in Table 1. Results are expressed as units of enzyme/g wet wt. of tissue or as percentage inhibition or activation of the enzyme compared with the trypsin-activated or untreated control. Correction was made for the inhibition of enzyme by the action of inhibitor solvents. The results are the means of three observations in each case. Abbreviation: n.d., not determined.

	Trypsin-treated pellet		Untreated pellet	
	(units)	(% inhibition)	(units)	(% inhibition)
None	37.2*	0	24.9	0
1,10-Phenanthroline				
0.2 mM	17.1	54	n.d.	n.d.
2.0 mM	13.8	63	6.7	73
8.0 mM	11.2	70	n.d.	n.d.
EDTA				
20.0 mM	13.8	63	6.0	76
50.0 mM	9.3	75	n.d.	n.d.
Phenylmethanesulphonyl fluoride (2.0 mM)	36.5	2	8.9	64
Soya-bean trypsin inhibitor (40 µg/ml)	n.d.	n.d.	13.0	48
Soya-bean trypsin inhibitor (40 µg/ml)	n.d.	n.d.	2.6	90
+ 1,10-phenanthroline (2.0 mM)				
Soya-bean trypsin inhibitor (40 µg/ml)	n.d.	n.d.	2.6	90
+ EDTA (20.0 mM)				
4-Aminophenylmercuric acetate (0.5 mM)	37.9	+2†	33.9	+36†
4-Aminophenylmercuric acetate (0.5 mM)	n.d.	n.d.	4.6	87
+ 1,10-phenanthroline (2.0 mM)				

* Excess soya-bean trypsin inhibitor was present at a concentration of 0.03 µg/ml.

† Activation of Azocoll degradation.

addition of 1,10-phenanthroline (2 mM) decreased this activity by 85%. 4-Aminophenylmercuric acetate and trypsin probably activate a latent metalloproteinase. Since 4-aminophenylmercuric acetate did not enhance Azocoll degradation by trypsin-treated pellets, it is likely that a single enzyme is being activated by the two agents.

Neutral proteinase activity in uteri removed at different stages

Uteri were removed from rats at five different stages (Table 3). There was little change in the wet weight of uteri in the 2 days before parturition. In the 24 h after parturition the wet weight of the uteri was halved, and by 5 days *post partum* the average weight of the uteri was only 16% of the weight of the uteri at parturition (Table 3). The maximum weight loss was in the 24 h immediately after parturition. These results agree well with previous measurements of uterine weight loss during involution (Woessner, 1962, 1969, 1979a).

Uterine homogenates were prepared and fractionated as described in the Materials and methods section. The supernatant fractions were discarded. This decision was based on the distribution of neutral proteinase activity in the uteri at 1 day and 2 days *post partum* (Table 1), where, at the time of

maximum resorption, 80% of the total Azocoll-degrading activity was found in the pellet fraction. In these experiments all the pellet preparations were pretreated with trypsin to obtain maximum activity. When Azocoll degradation was determined per g wet wt. of tissue (0.2 ml of pellet preparation was equivalent to 20 mg wet wt. of uterus), the highest concentration of neutral proteinase activity was found at 1 and 2 days *post partum* (Table 3). When calculated as units/uterus, however, the uterine content of neutral proteinase was maximum at 1 day *post partum*, and it was high (86% of the maximum) at parturition. There was a good correlation between the content of Azocoll-degrading neutral proteinase in the uterine pellets and changes in the wet weight of the uterus.

Extraction and characterization of neutral proteinase from the involuting rat uterus

Further study of the neutral proteinase activity required its solubilization from the uterine pellet. The method developed by Weeks *et al.* (1976) for extracting collagenase was tested on uteri removed 1 day *post partum* (see the Materials and methods section). By this method 55–60% of the Azocoll-degrading activity was solubilized, and up to 70% of the total activity was extracted if the

Table 3. *Neutral proteinase activity in pellets made from uteri at different stages of parturition*

Samples (0.2 ml) of resuspended pellet prepared from uteri of rats at different stages were pretreated with trypsin as described in Table 1 and assayed with Azocoll substrate (see the Materials and methods section). The weight of each uterus was recorded before homogenization. Results are expressed as units of enzyme activity/g wet wt. of tissue or as units/uterus, $\pm$ s.e.m. of four observations in each case.

State of uterus	Wet wt. (g)	Enzyme activity (Azocoll units)	
		(units/g wet wt. of uterus)	(units/uterus)
Pre partum*	2.82 ± 0.25	6.10 ± 1.70	16.0 ± 3.0
Parturient	2.85 ± 0.22	14.00 ± 0.18	40.8 ± 7.8
Post partum			
1 day	1.43 ± 0.08	32.70 ± 2.70	47.3 ± 6.0
2 days	0.85 ± 0.04	31.70 ± 1.92	26.8 ± 0.68
5 days	0.46 ± 0.05	14.10 ± 2.50	6.9 ± 1.9

* Uteri were removed approx. 24 h before parturition.

Fig. 1. *Chromatography of uterine extracts on Sephadex G-150*

An extract (110 ml) of the pellet fraction from 10 g of uterus *post partum* was concentrated to 25 ml by ultrafiltration on an Amicon PM-10 membrane. The concentrated extract was clarified by centrifugation at 10000 *g* for 20 min and applied to a column of Sephadex G-150 (90 cm $\times$ 2.6 cm) equilibrated with 0.05 M-Tris/HCl (pH 7.5)/0.2 M-NaCl/0.01 M-CaCl₂/0.02% (w/v) NaN₃. The column was eluted by gravity at a flow rate of 30 ml/h with the same buffer; 6.5 ml fractions were collected. Samples (1 ml) of alternate fractions were assayed with or without trypsin activation. Half of the sample (0.5 ml) was assayed for neutral proteinase activity with Azocoll as substrate. The remaining sample (0.5 ml) was assayed for the ability to degrade fluorescein-collagen as described in the Materials and methods section. Fractions pretreated with trypsin and then soya-bean trypsin inhibitor: ●, neutral proteinase; △, collagenase. Fractions pretreated with buffer: ○, neutral proteinase (A₅₂₀); —, protein (A₂₈₀). The horizontal bar indicates fractions containing neutral proteinase activity that were pooled for (NH₄)₂SO₄ precipitation.

procedure was repeated. Measurements of enzyme recovery were valid because, when extracted enzyme was assayed in the presence of uterine pellets, the Azocoll degradation was equal to the sum of the enzyme activities in both the pellet and extract (determined independently). Without activation, the

Azocoll-degrading activity of the extract was only 60–65% of that estimated after activation by trypsin. Optimum conditions for trypsin activation of extract neutral proteinase were 30 μ g of trypsin/ml of extract for 3 min at 37°C, followed by 120 μ g of soya-bean trypsin inhibitor/ml of extract.

Extracts prepared from uteri 1 day *post partum* were concentrated 4–5-fold by ultrafiltration through Amicon PM-10 membranes, and the concentrated enzyme was applied to a column of Sephadex G-150. Fractions were assayed for neutral proteinase activity with Azocoll as substrate and for collagenolytic activity with fluorescein-labelled rat tail-tendon collagen (Fig. 1). When fractions were activated with trypsin before assay, the major part of the Azocoll-degrading activity was eluted as a broad peak clearly separated from, and retarded with respect to, the uterine collagenase characterized by Woessner (1977). Similar results were obtained if the fractions were assayed in the presence of 4-amino-phenylmercuric acetate (0.5 mM). We pooled the fractions in this major peak and recovered 70–80% of the Azocoll-degrading activity applied to the column. This activity was inhibited 100% by 1,10-phenanthroline (2 mM). An additional 10–15% of the Azocoll-degrading activity was observed as a small peak that emerged ahead of the main peak and was slightly retarded with respect to collagenase; no attempt was made to characterize this proteinase. When column fractions were assayed without activation, a single broad peak of Azocoll degra-

dation was seen (Fig. 1). This peak was lower and skewed to the right compared with the peak seen when fractions were activated before assay. This indicates that the active enzyme is retarded in comparison with enzyme that was latent at the time of chromatography. A profile identical with the above was obtained if fractions were assayed in the presence of soya-bean trypsin inhibitor (50 µg/ml; results not shown). The active enzyme was inhibited more than 90% by 1,10-phenanthroline (2 mM).

We determined the apparent molecular weights of the latent and active neutral proteinase after further purification of the pool (see Fig. 1) from Sephadex G-150 chromatography by $(\text{NH}_4)_2\text{SO}_4$ precipitation. The protein precipitated at 60% saturation was redissolved in column buffer and applied to a calibrated column of Ultrogel AcA 54 (see the Materials and methods section). The apparent molecular weights of the latent and active neutral proteinase were 24 000 and 12 000 respectively. Trypsin treatment of the enzyme preparation before chromatography resulted in a complete conversion of the latent neutral proteinase into active neutral proteinase, with an apparent loss of mol.wt. of 12 000. The conversion of the latent neutral pro-

Fig. 2. Influence of pH on the degradation of Azocoll by uterine extracts (a) and partially purified enzyme preparation (b). Uterine extract and partially purified enzyme preparation were incubated with trypsin (30 µg/ml of sample; 3 min, 37°C) followed by soya-bean trypsin inhibitor (120 µg/ml; 10 min, 25°C) to activate maximally the uterine proteinase. Samples of extract (0.25 ml) or partially purified enzyme (0.035 ml) were then made to 0.25 ml, where necessary, with 0.05 M-Tris/HCl (pH 7.5)/0.1 M-CaCl₂. Assay buffer (2 ml) containing 0.06 M of the appropriate buffer at the required pH and also 0.25 M-NaCl, 0.01 M-CaCl₂ and 1% (v/v) butan-1-ol was added and the incubation was begun by adding 0.5 ml of Azocoll suspension [4 mg/ml in water containing 0.05% (w/v) Brij-35]. Controls were made without enzyme preparation. Incubations were for 17 h at 37°C with constant shaking. The pH of the incubation was determined at the end of the assay when incubations were cooled to 25°C. Results are the mean of at least two independent experiments in each case and are expressed as a percentage of the maximum degradation of Azocoll in each experiment. The buffer systems used were: ●, sodium acetate; ○, Tris/acetate; ▲, Tris/HCl; △, glycine/NaOH.

teinase into active neutral proteinase suggests that the latent and active neutral proteinases are different forms of the same enzyme.

pH optimum of uterine neutral proteinase

The effect of pH on the digestion of Azocoll by both uterine extracts and partially purified neutral proteinase was investigated in the range pH 3.5–10.5 (Fig. 2). The proteinase was first activated maximally with trypsin treatment at pH 7.5 before the addition of assay buffer at the appropriate pH. There was some Azocoll digestion at pH 4.5, possibly

owing to acid cathepsin activity; however, the major digestion of Azocoll (>90%) was seen in the range pH 6.5–8.0. In Tris/HCl buffer, the pH optimum was 7.5, although there was still appreciable activity in both the extract and partially purified enzyme preparation at pH 6.5 and 8.

Action of uterine neutral proteinase on cartilage proteoglycan

We investigated the ability of the partially purified uterine neutral proteinase to degrade bovine nasal proteoglycan entrapped in polyacrylamide beads, using a modification (Nagase, 1977) of the assay described by Dingle *et al.* (1977). The neutral proteinase was incubated with trypsin to obtain maximum activity and then assayed with Azocoll and with proteoglycan beads. The enzyme degraded both Azocoll and cartilage proteoglycan. One Azocoll unit of neutral proteinase degraded between 2 and 4 μ g of cartilage proteoglycan/min. The degradation of both substrates was inhibited completely by 1,10-phenanthroline (2 mM), but was not inhibited significantly by phenylmethanesulphonyl fluoride (2 mM) or soya-bean trypsin inhibitor (1 mg/ml). These results suggest that the degradation of the cartilage proteoglycan was also due to the action of metalloproteinase. The enzyme activity responsible for the degradation of Azocoll and cartilage proteoglycan co-chromatographed on Ultrogel AcA 54 (Fig. 3), confirming that the same or very similar enzymes degrade both Azocoll and proteoglycan.

The action of the partially purified trypsin-activated metalloproteinase on cartilage proteoglycan was also studied by viscometry and analytical ultracentrifugation. The viscometric studies (Fig. 4) show that the enzyme produced a rapid decline in viscosity of the proteoglycan, approaching an asymptotic limit of about 35% of the initial specific viscosity. 1,10-Phenanthroline blocked most of this change in viscosity. This inhibitory effect was made clearer when the data were plotted as a second-order reaction (Fig. 4). The second-order rate constant (the slope of the line) was decreased 97% by 1,10-phenanthroline. There was a slow degradation of proteoglycan in the assay buffer, presumably owing to endogenous proteinase activity.

The digestion products resulting after 5 h of incubation in the experiment of Fig. 4 were compared with unincubated proteoglycan and with papain-digested proteoglycan in the analytical ultracentrifuge. These samples are illustrated in Fig. 5. Peak 1 is undegraded proteoglycan, with an apparent sedimentation coefficient of 7.0S. Analysis at lower concentrations enabled us to make an extrapolation to zero concentration, which gave a value of $s_{20,w}^0 = 19.8S$. Peak 2 is the enzyme-digestion product, with an apparent $s = 2.6S$. Extra-

Fig. 3. Degradation of Azocoll and cartilage proteoglycan by uterine neutral proteinase

A sample (5 ml) of partially purified uterine neutral proteinase was chromatographed on a calibrated column of Ultrogel AcA54 as described in the Materials and methods section. Fractions were activated with trypsin (30 μ g of trypsin/ml of fraction; 3 min, 37°C) followed by soya-bean trypsin inhibitor (120 μ g/ml of fraction). A sample of each fraction (0.5 ml) was assayed for the ability to degrade Azocoll and another sample of the same fraction (0.2 ml) was assayed for the ability to degrade cartilage proteoglycan. ●, Azocoll degradation (A_{520}); ▲, degradation of cartilage proteoglycan (A_{530}); —, protein (A_{280}); V_0 , void volume; V_t , total volume of column.

Fig. 4. Changes in the viscosity of cartilage proteoglycan incubated with trypsin-activated partially purified uterine metalloproteinase

Mixtures were set up as described in the Materials and methods section and incubated for 5 h at 37°C. Results are expressed as a percentage of initial specific viscosity at each time point (curved lines): ▲, proteoglycan + enzyme; ●, proteoglycan + enzyme + 1,10-phenanthroline (2 mM); and ○, proteoglycan blank. The same results are also plotted as a second-order kinetic function $(\eta_0 - \eta_t)/(\eta_t - \eta_\infty)$ where η_0 = initial viscosity, η_t = viscosity at time t and η_∞ = final viscosity (Woessner, 1973). These data are shown as straight lines with the same symbols.

polation to zero concentration gave $s_{20,w}^0 = 3.8S$. On the basis of an equation derived by Wasteson (1971), this would correspond to a product of mol.wt. 100 000–110 000. This result is only approximate, since it involves extending the equation beyond the range tested experimentally by Wasteson (1971). Papain (peak 4) gave an apparent $s = 1.7S$; extrapolation to zero concentration was not done. The incubation of proteoglycan with inhibited enzyme gave a partial degradation product (peak 3) with $s = 5.3S$ and an extrapolated value of $s_{20,w}^0 = 17.5S$. Analysis of the distribution function of sedimentation coefficients [g(s) function] (cf. Hascall & Sajdera, 1970) indicates a considerable broadening of this peak, suggesting that a range of intermediate-size products was produced. This would reflect the action of enzyme that was not completely inhibited or the action of trace enzyme impurities.

A sample (0.1 ml) of the incubation mixture containing the products of enzyme digestion of cartilage proteoglycan was adjusted to 1 ml with

Fig. 5. Schlieren patterns of undegraded and degraded proteoglycan subunit

Undegraded and enzyme-digested proteoglycan subunit preparations were analysed in the analytical ultracentrifuge as described in the Materials and methods section. (a): 1, proteoglycan + enzyme; 2, proteoglycan (not incubated). (b): 3, proteoglycan + enzyme + 1,10-phenanthroline; 4, proteoglycan + papain. Sedimentation was from right to left.

distilled water and dialysed overnight in Spectrapor tubing (mol.wt. cut-off 3500) against 10 vol. of distilled water. More than 95% of the uronic acid in the proteoglycan was retained under these conditions, whereas over 80% of an equivalent amount of glucuronic acid was lost from the dialysis bag under identical conditions. These data are further evidence that the enzyme is a proteinase as opposed to a polysaccharidase.

Discussion

Our data show the presence of a novel metalloproteinase activity in extracts of the involuting rat uterus. Molecular-weight determinations show that the enzyme exists in both latent and active forms. Activation of the latent form of the metalloproteinase by trypsin leads to an apparent 12 000 decrease in mol.wt. from 24 000 to 12 000. These values must be viewed with caution, however, because of the small amount of enzyme present and its possible interaction with the gel-chromatography matrix under the conditions used. The occurrence of latent and active forms of the enzyme is a point of similarity to uterine collagenase; however, the metalloproteinase can be clearly distinguished from uterine collagenase by its lower molecular weight and its relative inability to solubilize native insoluble collagen. The proteinase can also be distinguished from a uterine metalloproteinase that hydrolyses a

heptapeptide related to collagen, since the latter has a higher molecular weight and does not show latency (Woessner, 1979b).

The results obtained previously with rat uterine pellet preparations strongly suggest that collagenase is activatable by a serine proteinase present in the uterine pellet (Woessner, 1977). The present studies support the idea that the uterine neutral metalloproteinase is also activated by the same or a similar serine proteinase. The non-enzymic activation of the uterine metalloproteinase by 4-aminophenylmercuric acetate and our recent finding (A. Sellers & J. F. Woessner, unpublished work) that uterine collagenase is partially activated by this compound make it likely that these latent enzymes, like other latent metalloproteinases (Reynolds *et al.*, 1977; Sellers *et al.*, 1978; Vater *et al.*, 1979), are enzyme-inhibitor complexes.

The partially purified neutral proteinase degrades Azocoll and cartilage proteoglycan. Both actions are completely inhibited by 1,10-phenanthroline and are not affected by phenylmethanesulphonyl fluoride or soya-bean trypsin inhibitor. Since proteoglycan-degrading activity and Azocoll-degrading activity also co-chromatograph on Ultrogel AcA 54, the degradation of each substrate is due to the same or similar metalloproteinases. The fragments of proteoglycan produced by action of the enzyme do not diffuse through a membrane with an exclusion limit of 3500 daltons, proving that oligosaccharides are not generated, so that the enzyme is presumably an endopeptidase. Analysis of the fragments by ultracentrifugation shows that, under the conditions of our experiments, the apparent molecular weight of the fragments is larger than that of free chondroitin sulphate chains generated by the action of papain. It is not possible to say conclusively that these fragments, whose approx. mol.wt. is 100 000, represent the limit of enzyme action; however, the apparent homogeneity of the fragments as judged by their narrow size distribution in the ultracentrifuge suggest that these fragments are the major product of the action of the enzyme. After 5 h of incubation with the enzyme (14 units), no undegraded proteoglycan could be detected, and in that time 4 mg of the substrate was hydrolysed.

There are similarities between the rat uterine metalloproteinase and other proteoglycan-degrading metalloproteinases extracted from cartilage (Sapolsky *et al.*, 1976) and found in the medium of cultures of rabbit macrophages (Horwitz *et al.*, 1976; Hauser & Vaes, 1978), rabbit bone (Sellers *et al.*, 1978), mouse bone (Vaes *et al.*, 1978) and rabbit synovial fibroblasts (Werb & Reynolds, 1974; Werb *et al.*, 1978). The apparent molecular weights of these enzymes do, however, vary and not all of the enzymes have been shown to have latent forms. The greatest similarity to the uterine metalloproteinase is

found with the metalloproteinases from rabbit macrophages (Horwitz *et al.*, 1976; Hauser & Vaes, 1978). This may be significant in view of the large increase in macrophage-like cells in the uterus at parturition (Tansey & Padykula, 1978).

Our observations on the involuting uterus add weight to the idea that metalloproteinases play a significant role in the resorption of connective tissues. Like uterine collagenase (Woessner, 1979a), neutral proteinase activity in uterine pellets was highest in uteri removed 1 day *post partum*. In contrast with uterine collagenase, the uterine content of neutral proteinase was also high immediately after parturition. However, although collagen loss from the involuting uterus does not become significant until 12 h after parturition (Woessner, 1962; Grant, 1965), there is a rapid loss of uterine proteoglycan immediately *post partum* (Grant, 1965). Uterine neutral proteinase activity is temporally correlated with loss of uterine proteoglycans and also with the loss in wet weight of the tissue.

The uterine metalloproteinase represents only the second instance of a metalloproteinase that has been extracted from a mammalian connective tissue and correlated with processes of matrix breakdown. It is attractive to postulate that this neutral metalloproteinase and uterine collagenase (also a metalloproteinase) are both produced by the same cell type and are designed to degrade connective-tissue components during uterine involution. It is noteworthy that many of the reports of metalloproteinases in the literature describe the same time course of production and release for all the metalloproteinases, including collagenase, from a particular tissue or cell population. The simultaneous synthesis and release of these proteinases and their collective abilities to degrade collagen, gelatin and proteoglycan make it attractive to propose that they form a 'battery' of enzymes released by certain cells subjected to certain stimuli to resorb connective-tissue matrices.

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