Procollagenase activator produced by rabbit uterine cervical fibroblasts

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Culture medium from rabbit uterine cervical fibroblasts contained a procollagenase and a neutral proproteinase which acts as a procollagenase activator. These two proenzymes have been purified by a combination of ion-exchange, affinity and gel chromatographies. The purified neutral proproteinase showed M_r 60000 with sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This neutral proproteinase was activated by trypsin, 4-aminophenylmercuric acetate (APMA) and plasmin, and the active species of the proteinase had M_r 53000 when activated by APMA; kallikrein and urokinase did not activate this proproteinase. The purified neutral proteinase was inhibited by EDTA, 1,10-phenanthroline and rabbit plasma, but not by serine proteinase inhibitors, suggesting that this proteinase is a metal-dependent proteinase. The purified enzyme could also degrade gelatin, casein, proteoglycan and type IV procollagen. The purified procollagenase had M_r 55000 and was activated by trypsin, APMA and the active neutral proteinase. These activations were accompanied by decrease in M_r , and the activated species had an M_r which was approx. 10000 less than that of the procollagenase. In particular, procollagenase activation with neutral proteinase depended on incubation time and proteolytic activity of proteinase. These results indicate that activation of procollagenase by the rabbit uterine neutral proteinase is related to limited proteolysis in the procollagenase molecule.

INTRODUCTION

It is well known that the predominant macromolecular components of connective tissue are collagen and proteoglycan. Mammalian collagenases, which have been detected and well characterized in various tissues and in their culture systems, can specifically cleave the interstitial collagens (types I, II and III), and contribute to collagenolysis in normal and/or pathological tissues (Harris & Krane, 1974; Woessner, 1980; Harris *et al.*, 1984).

It has been reported that collagenolysis is regulated at various steps from biosynthesis of procollagenase to inhibition of active collagenase. Inhibitory mechanisms of collagenase activity have been made relatively clear: active collagenase is inhibited by plasma inhibitors such as α_2 -M (Eisen *et al.*, 1970; Abe & Nagai, 1973; Nagai, 1973) and β_1 -AC (Woolley *et al.*, 1976). Several investigators have demonstrated TIMP in cultures of explants and connective tissue cells (Murphy *et al.*, 1981, 1985a; Sakyo *et al.*, 1986b). An interesting report that β_1 -AC and TIMP are the same protein has been made by Welgus & Stricklin (1983). At present, the theory that TIMP contributes more extensively to the regulation of collagenolysis in connective tissues than the above inhibitors in plasma is generally accepted.

However, the activation mechanisms of procollagenase are poorly understood. Certainly, it is well recognized that collagenase is activated by organic mercurial compounds such as APMA (Cawston *et al.*, 1981a;

Stricklin *et al.*, 1983) or by limited proteolysis with trypsin (Vaes, 1972; Bauer *et al.*, 1975; Stricklin *et al.*, 1983) *in vitro*. Other proteinases such as lysosomal cathepsin B (Eeckhout & Vaes, 1977), plasma kallikrein (Nagase *et al.*, 1982), mast cell proteinase (Birkedal-Hansen *et al.*, 1976) and plasmin (Werb *et al.*, 1977) also have the capability of activating certain mammalian procollagenases *in vitro*. However, the physiological significance of these proteinases in the activation of procollagenase is still unknown. Vater *et al.* (1983) have demonstrated that rabbit synovial fibroblasts produced an endogenous activator of procollagenase in a latent form *in vitro*, and this activator also possessed proteolytic activity against numerous non-collagenous proteins. Tyree *et al.* (1981) have reported another activation mechanism of procollagenase by an activator derived from human skin or rat uterus.

The report that rabbit synovial fibroblasts synthesize a regulatory protein of procollagenase production (Brinckerhoff *et al.*, 1985) is of interest from the viewpoint of regulatory mechanisms of procollagenase biosynthesis.

We have reported the changes in collagen concentration and collagenase and other proteinase activities concerning uterine cervical ripening and dilatation (Ito *et al.*, 1979; Kitamura *et al.*, 1979, 1980a,b; Ito *et al.*, 1981; Sakyo *et al.*, 1983, 1986a). During the course of the above studies, we have found that rabbit uterine cervical neutral proteinase, which acts as an endogenous procollagenase activator, was secreted as a proenzyme

Abbreviations used: α_2 -M, α_2 -macroglobulin; β_1 -AC, β_1 -anti-collagenase; TIMP, tissue inhibitor of metalloproteinase; APMA, 4-aminophenylmercuric acetate; MEM, Eagle's minimum essential medium; FCS, fetal calf serum; BSA, bovine serum albumin; Dip-F, diisopropyl fluorophosphate; DNP-peptide, 2,4-dinitrophenyl-L-Pro-L-Gln-Gly-L-Ile-L-Ala-Gly-L-Gln-D-Arg.

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together with procollagenase, and the mechanism of procollagenase activation is different from that of the above activators (Vater *et al.*, 1983; Tyree *et al.*, 1981). In this report, we describe the purification and characterization of the uterine cervical neutral proteinase together with procollagenase, and also discuss the activation mechanism of procollagenase by this neutral proteinase in the rabbit uterine cervix.

EXPERIMENTAL

Materials and culture instruments

Tissue culture plasticware was purchased from Corning Glass Works, Corning, NY, U.S.A. The following reagents were commercially obtained: MEM from Grand Island Biological Co., Grand Island, NY, U.S.A.; FCS from Boehringer-Mannheim G.m.b.H., Mannheim, W. Germany; CM-52 cellulose and DE-52 cellulose from Whatman, Maidstone, U.K.; Sephadex G-100 (fine), Chelating-Sepharose 6B and Heparin-Sepharose 6B from Pharmacia, Uppsala, Sweden; BSA (fraction V), trypsin (bovine pancreas, type III), soya-bean trypsin inhibitor (type II-S), APMA, kallikrein (porcine pancreas), plasmin (porcine blood), α -chymotrypsinogen A (bovine pancreas, type II), Dip-F and phenylmethane sulphonyl fluoride from Sigma Chemical Co., St. Louis, MO, U.S.A.; egg albumin (4 $\times$, crystallized) from ICN Pharmaceuticals, Cleveland, OH, U.S.A.; urokinase from Fujisawa Pharmaceutical Co., Osaka, Japan; Hammersten casein from E. Merck, Darmstadt, W. Germany; DNP-peptide from Protein Research Foundation, Osaka, Japan; [1- 14 C]acetic anhydride (20 mCi/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K.

The type IV procollagen from EHS sarcoma was kindly provided by Dr. K. Kimata, Department of Chemistry, Faculty of Science, Nagoya University.

Other reagents used were of analytical reagent grade.

Cell culture

Rabbit uterine cervical fibroblasts were harvested from Nippon white rabbits (3.5–4.2 kg body wt.) with 23 days of gestational age and maintained in culture of MEM supplemented with 10% FCS as described by Sakyo *et al.* (1986a). Culture medium was changed every 2 days after confluence. Crude culture medium from day 2 through to day 20 was adjusted to pH 7.5 with 0.05 vol. of 1 M-Tris-HCl/0.2 M-CaCl₂/3 M-NaCl, pH 7.5, and was stored at –20 °C until required for enzyme purification.

Enzyme assays

Collagenase activity was determined by the fibril assay (Nagai & Hori, 1972) using [14 C]acetylated collagen (designated as [14 C]collagen), which was prepared by the method of Cawston & Barrett (1979) from guinea-pig back skin collagen. One unit of the enzyme hydrolyses 1 μ g of reconstituted collagen fibril/min at 37 °C.

Gelatinolytic activity was measured by the method of Harris & Krane (1972), with slight modifications as described in a previous paper (Ito *et al.*, 1981). One unit of the gelatinase activity hydrolyses 1 μ g of gelatin/min under the conditions used.

DNP-peptide-degrading activity was assayed by the method of Masui *et al.* (1977). Enzymic cleavage sites of this peptide were estimated as described in a previous paper (Kitamura *et al.*, 1980b).

Caseinolytic activity was determined by degradation of 2% (w/v) casein as a substrate by the method of Anson (1938).

Latent forms of collagenase and neutral proteinase were activated by the incubation with either trypsin or APMA. Standard conditions used for trypsin treatment were as follows. Samples were incubated with 0.5 vol. of trypsin (10 μ g/ml in 50 mM-Tris-HCl/10 mM-CaCl₂/150 mM-NaCl, pH 7.5) for 10 min at 25 °C, and then another 0.5 vol. of soya-bean trypsin inhibitor (50 μ g/ml in the same buffer) was added and further incubated for 10 min at 37 °C. Alternatively, samples were incubated with APMA at a final concentration of 0.25–1.0 mM in the same buffer for 30 min at 37 °C.

Enzyme purification

All chromatographic procedures were performed at 4 °C and all buffers contained 0.05% (w/v) Brij 35 and 0.02% (w/v) NaN₃.

Crude culture medium was concentrated by addition of solid (NH₄)₂SO₄ at 60% saturation. After centrifugation at 20000 *g* for 20 min at 4 °C, the precipitated protein was dissolved in and dialysed against 25 mM-Tris-HCl/10 mM-CaCl₂, pH 7.5. The dialysate was centrifuged to remove the insoluble materials and the supernatant was chromatographed on a column (3.5 cm $\times$ 10 cm) of CM-52 cellulose equilibrated with the same buffer. The column was washed until the *A*₂₈₀ reached base-line, and the bound protein was eluted with a linear gradient of NaCl (0–0.4 M) in the same buffer. Flow rate was 12 ml/h and 4.5 ml fractions were collected.

The fraction containing neutral proteinase, as determined by [14 C]gelatin degradation, from CM-52 cellulose was dialysed against 10 mM-Tris-HCl/5 mM-CaCl₂, pH 7.5, and was applied to a DE-52 cellulose column (2.0 cm $\times$ 25 cm) equilibrated with the same buffer. The bound protein was eluted with a linear gradient of NaCl (0–0.3 M) in the same buffer. Flow rate was 12 ml/h and 3.6 ml fractions were collected.

The neutral proteinase pool from DE-52 cellulose chromatography was concentrated with an Amicon PM-30 membrane and was applied to a zinc-chelate Sepharose column (2.0 cm $\times$ 6.0 cm) previously equilibrated with 50 mM-Tris-acetate/1 mM-CaCl₂/800 mM-NaCl, pH 7.0. Enzyme protein adsorbed on the column was eluted with a linear gradient of pH (7.0–5.5) in the same buffer. Flow rate was 12 ml/h and 2.9 ml fractions were collected.

Pooled neutral proteinase fraction from the zinc-chelate Sepharose chromatography was concentrated with an Amicon PM-30 membrane and applied to a Sephadex G-100 column (1.7 cm $\times$ 70 cm) previously equilibrated with 50 mM-Tris-HCl/10 mM-CaCl₂/150 mM-NaCl, pH 7.5. Flow rate was 12 ml/h and 2.5 ml fractions were collected. The gelatinolytic activity purified by this series of chromatographies was stored at –20 °C until use.

Purification of collagenase fraction obtained from the above CM-52 cellulose was independently performed by chromatographies on Sephadex G-100 and after zinc-chelate Sepharose column chromatography as described above. Collagenase activity pools obtained by zinc-chelate Sepharose column chromatography were concentrated by using Amicon PM-10 membrane and applied to a heparin-Sepharose column (1.2 cm $\times$ 5.0 cm) previously equilibrated with 50 mM-Tris-HCl/5 mM-CaCl₂/

50 mM-NaCl, pH 7.4. Adsorbed protein in this column was eluted with a linear gradient of NaCl (50–500 mM) in the same buffer. Flow rate was 12 ml/h and 2.4 ml fractions were collected. Purified collagenase was also stored at -20°C until use.

SDS/polyacrylamide-gel electrophoresis

This was performed by the method of Laemmli (1970) using 7.5, 10.0 or 12.5% (w/v) polyacrylamide slab gel in 25 mM-Tris-HCl/192 mM-glycine/0.1% (w/v) SDS, pH 8.3, under reducing conditions. The gels were stained with 0.2% (w/v) Coomassie Brilliant Blue in 45% methanol/9.2% acetic acid (v/v).

Degradation of type IV collagen

Degradation of type IV procollagen by purified neutral proteinase was examined as follows. Portions of enzyme (25 μl) were incubated with 54.5 μg of type IV collagen in 200 μl of 50 mM-Tris-HCl/10 mM- CaCl_2 /150 mM-NaCl, pH 7.4, for 12 h at 37°C . The reaction products were analysed by SDS/polyacrylamide-gel electrophoresis as described above.

RESULTS

Purification of collagenase and neutral proteinase

In a previous paper we have reported that rabbit uterine cervix fibroblasts produced much collagenase and neutral proteinase as proenzymes in the presence of FCS, respectively (Sakyo *et al.*, 1986a). Accordingly, the neutral proteinase was distinguished from the collagenase by monitoring its gelatinolytic activity in the culture medium, and it was purified by the chromatographic techniques described in the Experimental section. Both proenzymes in the crude and $(\text{NH}_4)_2\text{SO}_4$ -fractionated culture media were apparently activated only by excess amounts of trypsin, but not by APMA, because their activated forms were inevitably present together with α_2 -M and β_1 -AC (TIMP) derived from FCS.

When the $(\text{NH}_4)_2\text{SO}_4$ -fractionated culture medium was chromatographed on CM-52 cellulose, neutral proproteinase was passed through the column with almost all FCS-derived proteins, and procollagenase was eluted with 0.1 M-NaCl in the starting buffer (Fig. 1). Under the above conditions, the neutral proproteinase was completely separated from the procollagenase. Secondly, the pooled gelatinolytic activity from CM-52 cellulose was further chromatographed on DE-52 cellulose. By utilizing this chromatographic technique, the neutral proproteinase was separated into two parts: i.e., the major activity was passed through the column with a small amount of protein and the minor one was adsorbed with almost all protein derived from FCS. In the following procedures, only neutral proproteinase in the flow-through fraction was purified. Further purification of both neutral proproteinase and procollagenase were performed by the chromatographic procedures.

Tables 1 and 2 summarize the purification and activity recovery for procollagenase and neutral proproteinase throughout a typical purification procedure as described in the Experimental section. All enzyme units, except for the starting material, were calculated after the treatment of samples with APMA. Purified procollagenase had a specific activity of 4663 units/mg, and a 540-fold increase in this activity was obtained as compared with the starting material. The final preparation of neutral proteinase had a specific activity of 135 units/mg and was purified about 50-fold over the crude culture medium. We usually observed that 20–50% of the proproteinase was converted into the active form by the purification procedures.

To assess the purity of both enzymes, procollagenase and neutral proproteinase obtained from each final purification step were applied to an SDS/polyacrylamide slab gel (Fig. 2). In the presence of 2-mercaptoethanol, the purified procollagenase migrated as a single protein band and no other contaminant protein bands were shown, except for a small amount of auto-activated

Fig. 1. CM-52 cellulose ion-exchange chromatography of rabbit uterine cervical neutral proteinase and collagenase

The $(\text{NH}_4)_2\text{SO}_4$ -fractionated culture medium was applied to a CM-52 cellulose column (3.5 cm $\times$ 10 cm) equilibrated with 25 mM-Tris-HCl/10 mM- CaCl_2 /0.02% (w/v) NaN_3 /0.05% (w/v) Brij 35, pH 7.5. The adsorbed protein was eluted with a linear salt gradient (0–0.4 M-NaCl), and the column eluate was monitored for protein (A_{280} ; ○), collagenase (□) and neutral proteinase (△).

Table 1. Purification of rabbit uterine procollagenase

Aliquots of eluate pools, except for the starting material, were activated by incubation with 1 mM-APMA for 30 min at 37 °C and were assayed for the degradation of [¹⁴C]collagen.

Step	Total activity (units)	Specific activity* (units/mg)	Recovery (%)
Crude culture medium†	19950	8.6	100
CM-52 cellulose	1447	831	7.2
Sephadex G-100	1500	1111	7.5
Zinc-chelate	1100	3055	5.5
Sepharose			
Heparin-Sepharose	695	4663	3.4

* Protein concentration was determined by measurement of A_{280} ; $A_{280}^{1\%}$ is calculated as 10.0.

† Starting material volume was 2500 ml of culture medium and activity was determined after incubation with 0.8 mg/ml trypsin for 10 min at 25 °C, followed by 4.0 mg/ml soya-bean trypsin inhibitor for 10 min at 37 °C.

Table 2. Purification of rabbit uterine cervical neutral proproteinase

Aliquots of eluate pools, except for the starting material, were activated by incubation with 1 mM-APMA for 30 min at 37 °C and were assayed for the degradation of [¹⁴C]gelatin.

Step	Total activity* (units)	Specific activity† (units/mg)	Recovery (%)
Crude culture medium‡	7931	2.9	100
CM-52 cellulose	6398	2.6	81
DE-52 cellulose	2607	34	32
Zinc-chelate	216	50	2.7
Sepharose			
Sephadex G-100	135	135	1.7

* Neutral proteinase units were expressed as gelatinase units, which were defined as μ g of gelatin degraded/min at 37 °C.

† Protein concentration was determined by measurement of A_{280} ; $A_{280}^{1\%}$ is calculated as 10.0.

‡ Activity was measured after the trypsin treatment as described in Table 1.

material as shown in Fig. 2, lane 2. In the case of the neutral proteinase (Fig. 2, lane 6), however, both proproteinase (upper) and its active form (lower) were found in the electrophoretic pattern, but no other protein bands could be seen.

Characterization of neutral proteinase

The purified neutral proteinase was used to determine substrate specificity. This proteinase degraded gelatin, but was different from the gelatin specific proteinase found in the rabbit uterine myometrium (Sakyo *et al.*, 1983). This neutral proteinase also hydrolysed casein,

Fig. 2. SDS/polyacrylamide-gel electrophoresis of rabbit uterine cervical collagenase and neutral proteinase

Purified preparation of procollagenase ($A_{280} = 0.100$) or neutral proproteinase ($A_{280} = 0.103$) was run on a 12.5% polyacrylamide slab gel under reducing conditions, and the gel was then stained with Coomassie Brilliant Blue. Lanes 1, 5 and 8: protein standards [bovine serum albumin (BSA), egg albumin (EA), α -chymotrypsinogen A (CTN)]. Lane 2: procollagenase. Lane 3: procollagenase (75 μ l) incubated with 0.5 mM-APMA (25 μ l) for 30 min at 37 °C. Lane 4: procollagenase (75 μ l) incubated with auto-activated neutral proteinase (25 μ l, 5.6 units of gelatinolytic activity) for 12 h at 37 °C. Lane 5: neutral proproteinase (upper) and auto-activated proteinase (lower). Lane 6: neutral proproteinase (75 μ l) incubated with 0.5 mM-APMA (25 μ l) for 30 min at 37 °C.

and 1 unit of the caseinolytic activity was equivalent to 0.61 μ g of trypsin. This enzyme could cleave DNP-peptide, and the specificity of cleavage site (Gly-Ile bond) was the same as that for the uterine cervix (Kitamura *et al.*, 1980b) and other mammalian collagenases (Masui *et al.*, 1977; Miller *et al.*, 1976; Kobayashi & Nagai, 1978) and that also reported for the human skin gelatinase (Seltzer *et al.*, 1981). In addition, this neutral proteinase had an activity against proteoglycan of bovine nasal cartilage embedded in polyacrylamide-gel beads (data not shown). As this enzyme had gelatinolytic activity, it was examined to see whether the enzyme was capable of degrading type IV procollagen or not. As shown in Fig. 3, this proteinase could degrade type IV procollagen without a specific cleavage site, as compared with type IV collagen specific collagenase from certain tumours (Salo *et al.*, 1983; Fessler *et al.*, 1984).

Inhibitors of neutral proteinase

Degradation of [¹⁴C]gelatin substrate by neutral proteinase was totally inhibited by EDTA (disodium salt) and 1,10-phenanthroline and significantly inhibited by L-cysteine and dithiothreitol, but not by serine proteinase inhibitors such as phenylmethanesulphonyl fluoride, Dip-F and soya-bean trypsin inhibitor. *N*-Ethylmaleimide and *p*-chloromercuribenzoate also had no effect on the activity. The inhibition by rabbit plasma is probably due to the formation of α_2 -M- and/or β_1 -AC-enzyme complex, and re-activation of these inactive forms with APMA was impossible (results not shown). As shown in Table 3, the sensitivity of the neutral proteinase to the various reagents was almost the same as that of the collagenase.

Fig. 3. Degradation of type IV procollagen by neutral proteinase

Type IV procollagen (54.5 μ g in 200 μ l of 50 mM-Tris-HCl/10 mM-CaCl₂/150 mM-NaCl, pH 7.4) from EHS sarcoma was incubated alone (lane 1) or with purified neutral proteinase (25 μ l, 4.2 units) at 37 °C for 12 h (lane 2). Reaction products were analysed on a 10% polyacrylamide slab gel under reducing conditions, and the gel was stained with Coomassie Brilliant Blue. Type I collagen from guinea-pig back skin was used as a marker of M_r (lane 3).

Table 3. Inhibition of purified collagenase and neutral proteinase

Each reagent, in 50 mM-Tris-HCl/10 mM-CaCl₂/150 mM-NaCl, pH 7.5, was preincubated with the enzyme preparations for 30 min at 37 °C before the assay at the final concentration shown, and the residual activity was measured against [¹⁴C]collagen or [¹⁴C]gelatin, respectively. All values are means of duplicate samples. N.D., not determined.

Reagent	Final concn. (mM)	Relative activity (%)	
		Collagenase	Neutral proteinase
Control	—	100	100
EDTA-Na ₂	2	0	0
1,10-Phenanthroline	2	0	0
L-Cysteine	2	5	30
Dithiothreitol	2	30	7
N-Ethylmaleimide	2	88	97
p-Chloromercuribenzoate	1	112	97
Phenylmethanesulphonyl fluoride	2	80	82
Dip-F	0.01	N.D.	96
Soya-bean trypsin inhibitor	2 (mg/ml)	108	83
Rabbit plasma	5% (v/v)	10	2

Procollagenase activation by purified neutral proteinase

As shown in Table 4, the purified auto-activated neutral proteinase could activate procollagenase as well as APMA and trypsin. We examined the relationship between concentration of this proteinase (procollagenase activator) against procollagenase and the appearance of collagenase activity. When various amounts of the neutral proteinase were incubated with a constant

Table 4. Activation of procollagenase and neutral proproteinase

Procollagenase preparations ($A_{280} = 0.100$) were incubated with (1) buffer (50 mM-Tris-HCl/10 mM-CaCl₂/150 mM-NaCl, pH 7.5) for 30 min at 37 °C, (2) 1 mM-APMA for 30 min at 37 °C, (3) 10 μ g/ml trypsin for 10 min at 25 °C, followed by 50 μ g/ml soya-bean trypsin inhibitor for 10 min at 37 °C, or (4) neutral proteinase ($A_{280} = 0.103$, gelatinase activity of the fully auto-activated form of which was 5.6 units) for 16 h at 37 °C, [¹⁴C]collagen degrading activity was determined during a 2 h fibril assay. Samples of neutral proproteinase ($A_{280}/\text{ml} = 0.012$) were assayed for gelatinolytic activity after treatment with (1) the buffer, (2) 0.25 mM-APMA for 30 min at 37 °C or (3) 10 μ g/ml trypsin for 10 min at 25 °C, followed by 50 μ g/ml soya-bean trypsin inhibitor for 10 min at 37 °C. All values are means of duplicate samples. N.D., not determined.

Reagents	Procollagenase (units)	Neutral proproteinase (units)
Buffer	0.0	0.4
APMA	34.9	4.2
Trypsin	43.3	4.1
Activator	57.5	N.D.

amount of purified procollagenase for 18 h at 37 °C, the increase in collagenolytic activity was parallel with the increase of neutral proteinase concentration and the activation with saturable, as shown in Fig. 4. In this experiment, the maximum activity of collagenase obtained by the treatment with neutral proteinase was 1.2-fold, as compared with that obtained with APMA. When the neutral proproteinase was used instead of the active enzyme, negligible collagenolytic activity appeared, suggesting that this procollagenase activation was dependent on the proteolytic activity of neutral proteinase.

The time-dependent nature of the procollagenase activation by the treatment with neutral proteinase was further examined. When procollagenase and the active neutral proteinase, which was sufficient to activate procollagenase completely, were incubated together (Fig. 5), the rate of procollagenase activation depended on the incubation time. Under these circumstances, the maximum activity of the collagenase was observed at the 12 h incubation, and the apparent activity was retained after 24 h incubation.

Changes in M_r values of enzymes

To analyse the effect of activation on M_r values of procollagenase and neutral proproteinase, both enzymes were electrophoresed on an SDS/polyacrylamide slab gel under reducing conditions (Fig. 2).

Although the purified neutral proproteinase with M_r 60000 (Fig. 2, lane 6, upper) coexisted with an almost equal amount of auto-activated form (lane 6, lower), it was converted into an active form with M_r 53000 after the activation with APMA (lane 7). Procollagenase was also shown as a single band with M_r 55000, slightly lower than that of the neutral proproteinase (lane 2). However, by the treatment with APMA under the same conditions, active collagenase was found to exist as doublet forms:

Fig. 4. Effect of neutral proteinase concentration on procollagenase activation

Samples of the purified and fully auto-activated neutral proteinase ($A_{280} = 0.343$) were diluted with 50 mM-Tris-HCl/10 mM- CaCl_2 /150 mM-NaCl, pH 7.5, to obtain sample solutions containing 0.125, 0.25, 0.5, 1.0 and 2.0 units/ml. Each solution was incubated with an equal volume of purified procollagenase ($A_{280} = 0.400$) for 18 h at 37 °C. Reaction mixtures were then assayed for [^{14}C]collagen-degrading activity for 1 h at 37 °C. Collagenase units were calculated after subtraction of procollagenase or neutral proteinase blank.

Fig. 5. Effect of incubation time on procollagenase activation

Sample solutions containing a constant amount of procollagenase ($A_{280} = 0.400$) were incubated with an equal volume of neutral proteinase, at incubation times ranging from 1 to 24 h. Reaction mixtures were then assayed for [^{14}C]collagen-degrading activity for 1 h at 37 °C. The purified neutral proteinase ($A_{280} = 0.343$) was the same preparation as that described in Fig. 4, and was used at a concentration of 4 units/ml. Collagenase units were calculated after subtraction of procollagenase or neutral proteinase blank.

the major protein band and the minor one had M_r 48000 and 46000, respectively (lane 3). Accordingly, both procollagenase and neutral proproteinase decreased approx. 10000 in M_r after the treatment APMA. When the procollagenase was incubated with the active form of neutral proteinase for 12 h at 37 °C,

procollagenase was converted into an active form with M_r 46000, and had the same M_r as that of the lower- M_r band with APMA treatment.

However, in the case of activation of procollagenase with trypsin, it was difficult to confirm the change in M_r of this enzyme with SDS/polyacrylamide-gel electrophoresis, as the trypsin-soya-bean trypsin inhibitor complex was found to interfere in detection of the enzyme protein bands. Therefore, we examined the change in M_r by utilizing Sephadex G-100 column chromatography. Both procollagenase and the activated collagenase with trypsin were eluted in the fractions equivalent to M_r 49000 and 41000, respectively. On the other hand, the active collagenase obtained by treatment with APMA was eluted in a single peak corresponding to M_r 44000 with a shoulder of M_r 41000. When APMA-treated collagenase was further incubated with trypsin, the M_r of 44000 was completely changed to M_r 41000. These results indicated that the lower M_r of active enzyme was an essential form. The differences in M_r between SDS/polyacrylamide-gel electrophoresis and column chromatography have been observed, but the degree of decrease in M_r by two methods was very similar. The above phenomena might be due to the different behaviour of the enzymes on the polyacrylamide gel and the column.

Endogenous activator of neutral proproteinase

In the rabbit uterus system, procollagenase was effectually activated by the endogenous neutral proteinase as described above. This proteinase, however, was synthesized as a proenzyme and the activation was accomplished by its proteolytic activity. Therefore, we took an interest in the true activator of this proproteinase *in vivo*. The activator of neutral proproteinase has been examined by use of several commercial proteinases which might be called physiological activators. Urokinase (50, 100 and 300 units/ml), kallikrein (0.05, 0.5 and 1.0 unit/ml) and plasmin (0.05, 0.1 and 0.2 unit/ml) were preincubated with an equal volume of purified neutral proproteinase ($A_{280} = 0.205$) for 24 h at 37 °C. After termination of the reaction by addition of Dip-F, all preparations were assayed for proteolytic activity against [^{14}C]gelatin. Only plasmin, which might play an important role as an activator *in vivo*, displayed a concentration-dependent activation of the neutral proproteinase, and no activation effects were observed by treatments with kallikrein and urokinase.

DISCUSSION

We have demonstrated that rabbit uterine cervical fibroblasts produce a neutral proteinase which acts as a procollagenase activator together with procollagenase. The purified neutral proteinase occurred in a latent form, as well as the collagenase, and its M_r was slightly higher than that of collagenase. Activation of this proteinase could be achieved at any time during the purification processes by the use of trypsin and APMA, and activated species had an M_r which was approx. 10000 less than that of the latent form. As with the collagenase, the activity of neutral proteinase could be inhibited by the chelators such as EDTA and 1,10-phenanthroline and rabbit plasma, but not by serine proteinase inhibitors, suggesting that the neutral proteinase is the metal-dependent proteinase.

The purified neutral proteinase degraded gelatin, casein, DNP-peptide, proteoglycan and type IV procollagen, but we do not yet know the most suitable substrate for this enzyme purification. Therefore, the final preparation of this proteinase had a relatively low specific activity when gelatinolytic activity was used as an indicator in the calculation of the purification factor for neutral proproteinase. However, no contaminant protein in the final preparation was detected except for auto-activated species by the SDS/polyacrylamide-gel electrophoresis. Thus, it has been considered that this neutral proteinase was satisfactorily purified by the purification techniques described in the Experimental section, and we had no objection to using this preparation in the subsequent experiments. In the previous study, Sakyo *et al.* (1983) extracted a gelatinase from the rabbit uterine myometrium, and characterized its enzymic properties in detail, but our purified neutral proteinase could be distinguished clearly from their gelatinase in the respect of the substrate specificities.

It is generally accepted that connective tissue cells appear to secrete three neutral proteinases active against collagen, gelatin and casein. Rabbit uterine fibroblasts, however, might produce only two enzymes, procollagenase and gelatinase (procollagenase activator), because the caseinolytic activity in the culture media was less than the two activities above and both this activity and gelatinolytic activity were detected identically during the purification procedures (data not shown).

Many investigators have reported that the procollagenase was activated with trypsin or organic mercurial compounds (Murphy *et al.*, 1985a; Cawston *et al.*, 1981a; Stricklin *et al.*, 1983; Vaes, 1972; Bauer *et al.*, 1975; Eeckhout & Vaes, 1977; Nagase *et al.*, 1982), and Stricklin *et al.* (1983) have demonstrated the activation mechanisms of procollagenase with the above agents by using human skin fibroblast procollagenase. The procollagenase was converted into an active species by the limited proteolysis with trypsin, and the decrease in M_r of approx. 10000 was observed. In treatment with APMA, the same authors described that procollagenase activation was performed by two steps, i.e., the human skin procollagenase was fully activated by a conformational change in the molecule without loss of M_r . After a variable lag phase, however, the fully active collagenase underwent autocatalysis, and species with lowered M_r were produced. It has been reported that endogenous procollagenase activators were isolated from the culture medium of rabbit synovium (Vater *et al.*, 1983), human skin and rat uterus (Tyree *et al.*, 1981), and some activation mechanisms of procollagenase were suggested in each system. Vater *et al.* (1983) have established an activation model in which rabbit synovial procollagenase is subsequently achieved only by an activator. In the absence of the activator, trypsin and APMA generated only a lower M_r form of inactive species of procollagenase. Thus, it is suggested that both agents are capable only of converting the pro-activator to an active species, and the activation of procollagenase is performed by the activated activator. It was also proposed that the activation of procollagenase by the treatment with trypsin or APMA (Cawston *et al.*, 1981a; Vaes, 1972; Bauer *et al.*, 1975; Eeckhout & Vaes, 1977) resulted from contamination by a small amount of the activator in the procollagenase preparation used. On the other hand,

Tyree *et al.* (1981) reported a stoichiometric mechanism of procollagenase activation, i.e., the activation of procollagenase with human skin or rat uterus activator was independent of its proteolytic activity, and the activation did not lead to an apparent decrease in M_r of procollagenase. This is interesting, because the activators which possess some proteolytic activities did not induce any apparent changes in the procollagenase molecule during the activation processes (Vater *et al.*, 1983; Tyree *et al.*, 1981).

In this paper we demonstrate that rabbit uterine cervical procollagenase could be activated with trypsin, APMA and active species of neutral proteinase, respectively. Our results for the procollagenase activation obtained with trypsin and APMA were almost the same as those of Stricklin *et al.* (1983). In spite of the fact that our procollagenase was purified to the electrophoretic homogeneity shown in SDS/polyacrylamide-gel electrophoresis, it could degrade gelatin at a very low rate. However, Welgus *et al.* (1982, 1985) have reported that rat uterus collagenase could possess potential activity against gelatin. Accordingly, we concluded that the gelatinolytic activity in our purified collagenase was not due to the contaminated activator, and that trypsin or APMA is an activatable agent of cervical procollagenase itself. The procollagenase activation with the neutral proteinase was accompanied by an apparent decrease in M_r , and the activation depended upon the amount of the proteinase and activation period. These results suggest that procollagenase activation with the neutral proteinase is due to the proteolytic activity of the proteinase, and this activation mechanism is different from the mechanisms proposed by Vater *et al.* (1983) and Tyree *et al.* (1981).

Our purified procollagenase activator also degraded some protein components of connective tissues as described above. A group of neutral metalloproteinases, such as so-called elastase (Mainardi *et al.*, 1980a,b), gelatinase (Fessler *et al.*, 1984; Sopota & Danciewicz, 1974; Hibbs *et al.*, 1985; Murphy *et al.*, 1985b) and proteoglycanase (Cawston *et al.*, 1981b; Galloway *et al.*, 1983), secreted co-ordinately with collagenase have been reported as accelerators of degradation of interstitial collagens and other extracellular matrix components. Thus, the rabbit uterine cervical neutral proteinase may play an important role in collagenolysis, with its abilities of activating procollagenase and degrading connective tissue components, during cervical ripening and dilatation. However, we do not yet know the principal role of this neutral proteinase.

It is interesting that the activator synthesized as a proenzyme was activated by the plasmin, which is known as a procollagenase activator (Werb *et al.*, 1977), but the detailed mechanisms of biosynthesis and physiological activation of this proactivator are still unclear, and further investigations are needed.

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