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Production of Collagenase Inhibitor by the Growth Cartilage of Embryonic Chick Bone: Isolation and Partial Characterization

NATSUO YASUI[1], HISAE HORI[2] and YUTAKA NAGAI[2]

[1] Department of Orthopaedic Surgery, Osaka University Medical School, Fukushima-ku, Osaka, Osaka 553, Japan and [2] Department of Tissue Physiology, Medical Research Institute, Tokyo Medical and Dental University, Kandasurugadai, Chiyoda-ku, Tokyo 101, Japan

Abstract

Production of collagenase and collagenase inhibitors by the explants of epiphyseal, metaphyseal and diaphyseal regions of embryonic chick limbs during development has been investigated. Collagenase-inhibitory activity was first detected in the culture medium of the diaphyseal region of limbs at stage 36 where cartilage matrix erosion began to occur. However, neither active nor latent collagenase was detected in the media of any regions examined. At stage 38 and later, the maximum production of the inhibitory activity was observed in the explants of metaphyseal region (growth cartilage), while collagenase production was only in the diaphyseal region.

Two collagenase inhibitors were isolated and purified approximately 150 fold from the culture medium of stage 38 and 43 metaphyseal regions of limbs by anion- and cation-exchange chromatography followed by gel filtration. The inhibitors are cationic proteins with the same molecular weight of approximately 25,000, but slight difference in molecular charge. They are heat-stable and inhibit collagenases from tadpole skin, chick bone and skin and human granulocytes and gelatinases from human granulocytes and chick skin as well as trypsin, but not *Clostridium histolyticum* collagenase.

A possible function of the inhibitors in multistep regulations of the collagen-degrading enzyme system at the region of osteo-chondral transition is discussed.

Key words: Chick embryo, collagenase, growth cartilage, protease-inhibitor

Introduction*

During the embryonic limb development, the skeleton is first formed in cartilage models which are destined to be resorbed and replaced by bone (Bloom and Fawcett, 1968). In the process of endochondral ossification, active transition of matrix from cartilage to bone is accompanied by degradation (of type II collagen) and synthesis of (type I) collagen, which is the major macromolecular component of extracellular matrix (Lutfi, 1971; Linsenmayer et al., 1973; von der Mark et al., 1976). A possible mechanism for selective degradation of type II collagen at the proximate region of resorbing cartilage and newly forming osteoid tissue was reported by demonstrating the modulation of type II collagen degradation by collagenase by the presence of other type(s) of collagen (Hayashi et al., 1980a; Hayashi et al., 1980b). However, regulation mechanisms of collagenase activity during limb development remain obscure at present.

This paper reports the production of collagenase and its inhibitor by the explants of epiphyseal, metaphyseal and diaphyseal regions of embryonic chick limbs during development. Inhibitory effect of partially purified collagenase inhibitor on other neutral proteases is also described.

Materials

DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH (Peptide P) was obtained from the Protein Research Foundation, Minoh, Osaka. Tosylphenylchloromethylketone-treated trypsin (317 U/mg) was purchased from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.; *Clostridium histolyticum* collagenase (1000 Mandl U/mg) from Seikagaku Kogyo Co., Ltd., Tokyo; urokinase (2000 U/mg) from Midori-Juji Co., Ltd., Osaka; chymotrypsin (salt-free) from Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A. Swine pancreatic elastase (crystallized four times) from Eisai Co., Ltd., Tokyo; tosyl-L-arginine methylester from Fluka AG, Buchs, Switzerland; tosyllysylchloromethylketone (TLCK) from Sigma Chemical Co., St. Louis Missouri, U.S.A and Ham's F-12 (modified, lot No. FP1083003) from Flow Laboratories, Rockville, Maryland, U.S.A. All other chemicals used were of analytical grade. Collagenase preparations from tadpole skin, chick skin, human granulocyte were obtained, as reported previously (Hori and Nagai, 1979; Kawamoto and Nagai, 1976; Kobayashi and Nagai, 1978). Human granulocyte and chick skin gelatinases were prepared as described previously (Kobayashi and Nagai, 1978; Sunada and Nagai, 1980). Plasminogen was prepared from human plasma by lysine-Sepharose chromatography according to the methods of Deutsch and Mertz (1970) and Shiba (1976).

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* *Abbreviations:* Peptide P, dinitrophenyl-L-prolyl-L-glutaminylglycyl-L-isoleucyl-L-alanylglycyl-L-glutaminyl-D-arginine; SDS, sodium dodecylsulfate; TLCK, tosyllysylchloromethylketone; TPCK, tosylphenylchloromethylketone, Tris-NaCl-$CaCl_2$, 0.05 M Tris-HCl, pH 7.6, containing 0.15 M NaCl and 5 mM $CaCl_2$.

Methods

Tissue Culture

All procedures for tissue culture were carried out under sterile conditions. White Leghorn chick embryos were staged according to Hamburger and Hamilton (1951). The tibiae and femur from stage 33–43 chick embryos were resected, freed of the adherent connective tissue and further resected into two or three anatomical regions, i.e., epiphyseal, metaphyseal and diaphyseal regions. Like regions were combined, sliced into small pieces (2 × 2 × 2 mm) and 0.15 g of each explant was spread upon a filter paper disc floating in 2 ml of modified Ham's F-12 medium supplemented with sodium ascorbate (50 μg/ml) (Kawamoto and Nagai, 1976) in a 60 mm Petri dish. These were cultured at 37 °C in a humidified incubator under 5 % CO_2 – 95 % air, changing the medium every second day. For preparation of collagenase and collagenase inhibitor, stage 43 chick embryos were used to obtain larger quantities of the materials. Explants of 0.5 g tissues were cultured in a 100 mm Petri dish containing 9 ml of medium. The harvested media were centrifuged at 6000 × g for 20 min to remove any floating tissue debris and stored at –20 °C until used.

Isolation of Chick Bone Collagenase

All procedures for enzyme purification were carried out at 4–10 °C. The cultured media, about 200 ml, of the diaphyseal region of stage 43 embryonic chick bones were treated with 3 M NaI (Shinkai and Nagai, 1977) to activate latent collagenase, then concentrated to 4 ml by ultrafiltration through a Diaflo UM-10 membrane under a pressure of 1.5 kg/cm² of N_2 gas. The resulting media was centrifuged at 100,000 × g for 30 min, then applied on a Sephadex G-75 superfine column (2.0 × 81 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.6, containing 0.15 M NaCl and 5 mM $CaCl_2$ (Tris-NaCl-$CaCl_2$). Aliquots of the effluent fractions were assayed for collagenase activity.

Purification of Collagenase Inhibitor

All procedures for the purification of collagenase inhibitor were carried out at 4–10 °C. Collagenase inhibitor was purified from the cultured media of the explants of the metaphyseal region of stage 43 embryonic chick bones. The harvested cultured media were dialyzed against 0.1 M Tris-HCl buffer, pH 8.0, containing 2 M urea, and centrifuged at 100,000 × g for 30 min. The supernatant, 690 ml, was applied on a DEAE-cellulose (DE-32) column (2.6 × 25 cm) equilibrated with the same buffer. The column was washed and two step elutions were carried out, the first with 0.1 M NaCl and then with 2.0 M NaCl. Aliquots of the effluent fractions were dialyzed against Tris-NaCl-$CaCl_2$ buffer and assayed for collagenase inhibitory activity using purified tadpole enzyme (37 munits) (Hori and Nagai, 1979). The break-through fraction, the fractions containing inhibitory activity, were combined, concentrated to 118 ml with an Amicon Diaflo UM-10 membrane, dialyzed against 0.02 M Acetate buffer, pH 5.4, and applied on a Whatman CM-32 column (1.5 × 35.5 cm). The column was washed with 113 ml of the starting buffer and a 600 ml-linear gradient of 0 to 1.0 M NaCl

in the same buffer was used to elute the inhibitor. The fractions containing collagenase inhibitory activity (peak A, tube numbers 42–44 and peak B, tube numbers 46–50) were pooled separately, dialyzed against Tris-NaCl-$CaCl_2$ buffer containing 0.02 % NaN_3, concentrated to 4 ml with an Amicon Diaflo UM-10 membrane, then chromatographed on a Sephadex G-75 superfine column (2.0 × 81 cm) equilibrated with Tris-NaCl-$CaCl_2$ buffer. Aliquots (0.1 ml) of the effluent fractions were assayed for collagenase inhibitory activity.

Assay of Collagenase Activity

For collagenase assays the cultured medium was dialyzed against 3 M NaI in Tris-NaCl-$CaCl_2$ buffer for 16 h to activate latent enzyme, then extensively dialyzed against Tris-NaCl-$CaCl_2$ buffer without NaI. Collagenase activity was determined by measuring the release of soluble radioactivity from ^{14}C-labeled reconstituted guinea-pig skin collagen fibrils (gel method, Nagai and Hori, 1972) or the release of radioactivity into the supernatant of 50 % dioxane after enzyme digestion of ^{14}C-labeled soluble collagen at 35 °C (solution method, Terato et al., 1976). One unit of collagenase activity is defined as the amount of protein necessary to degrade 1 μg collagen per minute under the conditions employed.

Assays of Peptidase and Gelatinolytic Activities

Peptidase activity against Peptide P was assayed, as described previously (Masui et al., 1977; Hori and Nagai, 1979). Gelatinolytic activity was assayed using ^{3}H-labeled heat-denatured polymeric collagen (gelatin) as a substrate (Sunada and Nagai, 1980). The method is based on enzyme digestion of the gelatin at neutral pH and 37 °C and selective extraction of the digestion products into 67 % dioxane for determination of the radioactivity released. One unit of gelatinolytic activity is defined as the amount of protein degrading 1 μg of gelatin per minute under the conditions employed.

Determination of Protease-inhibiting Activity

Protease-inhibiting activity of the cultured media of embryonic chick bones or their chromatographed fractions was determined by measuring the depression of enzyme activity of various proteases against ^{14}C-labeled collagen (Nagai and Hori, 1972; Terato et al., 1976), ^{3}H-labeled heat-denatured polymeric collagen (Sunada and Nagai, 1980) or Peptide P (Masui et al., 1977; Hori and Nagai, 1979). One inhibition unit (IU) is expressed as the amount of protein required for 50 % inhibition of 2 units of collagenase by the gel method.

Electrophoresis

Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis was carried out using a 6 % gel in Tris-glycine buffer, pH 8.3, containing 0.1 % SDS (Hayashi and Nagai, 1979).

Results

Production of Collagenase and Collagenase Inhibitor by Embryonic Chick Limb Explants during Development

Light microscopic observations on the developmental changes of embryonic chick limbs showed that by stage 33 most of the skeleton was composed of cartilage destined to be absorbed and replaced by bone. Around stage 36 the hypertrophic cartilage matrix of diaphysis began to disintegrate, was invaded by small blood vessels and was ensheathed in a thin collar of perichondral bone. By stage 38 marrow cavity of the shaft has expanded a great deal and active transition of matrix from cartilage to bone occurred in the metaphyseal region (Fig. 1, bottom).

To elucidate whether the transition resulted from the manifestation of collagenase activity during this morphogenesis, the culture media of the explants

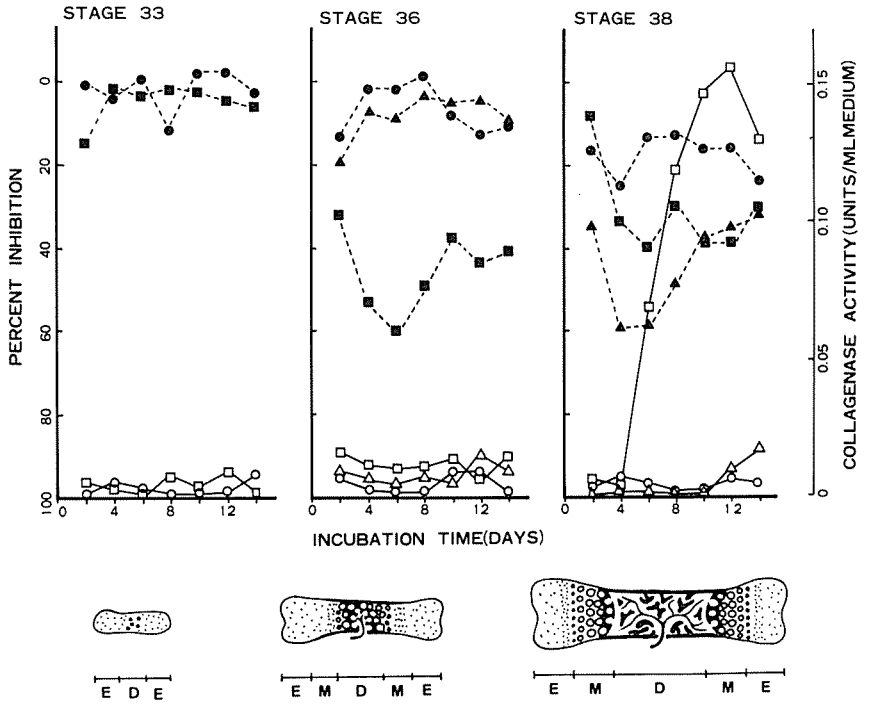

Fig. 1. Production of collagenase and collagenase inhibitor by the explants of different anatomical regions of embryonic chick limbs at three different stages. Culture medium was refreshed every second day. Collagenase activity in a series of the cultured media was assayed after 3 M NaI treatment to activate latent enzyme. Epiphyseal (○), metaphyseal (△) and diaphyseal (□) regions of limbs. Collagenase inhibitory activity was assayed by incubating tadpole collagenase (37 munits/ml) with 0.1 ml of cultured medium after dialysis against Tris-NaCl-$CaCl_2$ buffer. Epiphyseal (●), metaphyseal (▲) and diaphyseal (■) regions.

of different anatomical regions of developing limbs were assayed for collagenase and its inhibitory activities (Fig. 1). By stage 36 no or little collagenase activity was detected within the period of culture (14 days) of any explants. At stage 38 collagenase activity emerged only in the culture medium of the explants of the diaphyseal region after 6 days in culture. The diaphyseal region at this stage consists mainly of new bone matrix and bone marrow cells. The enzyme activity became maximum at 12th day in culture and declined thereafter. Culture media from other two anatomical regions at this stage, epiphysis and metaphysis, did not show any detectable collagenase activity during culture period. On the other hand, collagenase-inhibitory activity was first detected in the culture medium of the diaphyseal region of limbs at stage 36, where cartilage matrix erosion began to occur. By stage 38 the inhibitor was released into culture medium more or less by the explants of all anatomical regions examined. At this stage the zone of matrix transition from cartilage to bone migrated to the metaphyseal region, producing the largest amount of collagenase inhibitor. The production of collagenase and its inhibitor at stage 43 was quite similar to that by explants at stage 38 (data not shown). The collagenase inhibitor released from the metaphyseal region retained its full inhibitory activity even after NaI treatment.

Isolation and Purification of Collagenase Inhibitor

Collagenase inhibitor could be highly purified from the culture medium of the explants of stage 38–43 metaphyseal (growth cartilage) regions using anion- and cation-exchange chromatography followed by gel filtration. On DE-32 column chromatography, most of collagenase inhibitory activity was recovered in the break-through fraction under the conditions described in "METHODS". The fractions eluted at either 0.1 M NaCl or 2.0 M NaCl showed no detectable inhibitory activity even after 10 fold concentration. Apparent recovery of the inhibitory activity in the break-through fraction was as high as 100 % (Table 1).

Table 1. Purification and recovery of the collagenase inhibitor from the explants of the metaphyseal region of embryonic chick bones at stage 43

Fractionation	Total volume (ml)	Total OD_{280}	Collagenase inhibitory activity			
			Units (IU)	Specific activity	Purification	Recovery (%)
Culture medium	690	89.7	91.9	1.02	1	100
DE-32 chromatography	920	36.8	112.3	3.05	3.0	122
CM-32 chromatography	168	7.18	37.1	5.17	5.1	40.3
Gel chromatography A	23	0.302	15.7	52.0	51.0	17.1
Gel chromatography B	29	0.400	19.3	48.3	47.3	21.0

Figure 2 shows further purification of the inhibitor on a CM-32 column. Two major inhibitor peaks eluted at 0.22 M NaCl and 0.29 M NaCl concentrations were obtained. Although a small inhibitory activity was obtained at 0.13 M NaCl effluent fraction, no further characterization was performed with this fraction because of the limited amount of the material. The two major inhibitor frac-

tions (A and B) were pooled separately, dialyzed, concentrated and applied on a Sephadex G-75 superfine column (Fig. 3). Either inhibitor was recovered at the same effluent fraction as a sharp peak corresponding to a molecular weight of

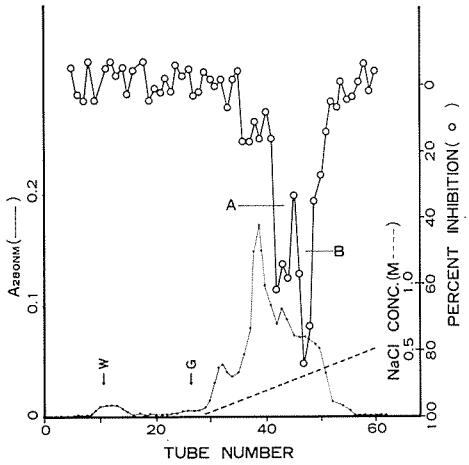

Fig. 2. CM-cellulose column chromatography of growth cartilage collagenase inhibitor. Cultured medium containing collagenase inhibitory activity from the metaphyseal region of stage 43 embryonic chich limbs was first chromatographed on a DE-32 column as described under "METHODS". The break-through fraction containing collagenase inhibitory activity was concentrated, dialyzed against 0.02 M acetate buffer, pH 5.4, and chromatographed on a CM-32 column (1.5 × 35.5 cm) equilibrated with the same buffer. The column was washed until the absorbance at 280 nm reduced to base line. Elution was accomplished by applying a 600 ml linear gradient of 0 to 1.0 M NaCl. Effluent fractions of 10.5 ml were collected at a flow rate of 16 ml/h. G, gradient and W, washing.

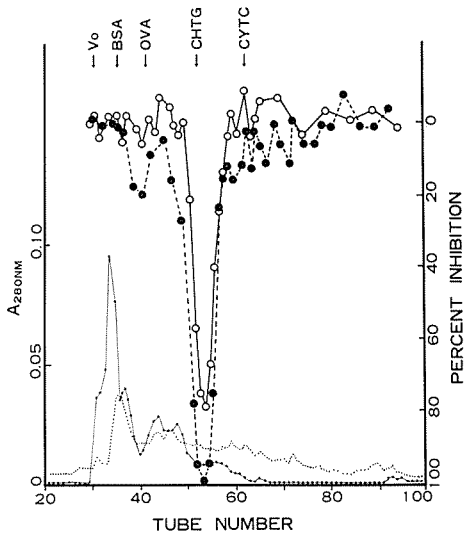

Fig. 3. Sephadex G-75 superfine chromatography of growth cartilage collagenase inhibitor. Effluent fractions containing collagenase inhibitor from a CM-32 column (fraction A, tube numbers 41–44, and fraction B, tube numbers 46–50 in Fig. 2) were pooled separately, dialyzed against Tris-NaCl-$CaCl_2$ buffer, concentrated and applied on a Sephadex G-75 superfine column (2.0 × 81 cm) equilibrated with the same buffer. Effluent fractions of 2.9 ml were collected at a flow rate of 7 ml/h. Aliquots (0.1 ml each) of the effluent fractions were incubated with tadpole collagenase (90.6 munits for sample A, ○——○ or 40.0 munits for sample B, ●——●). A_{280} nm; ——, A and-----, B. Vo, void volume; BSA, bovine serum albumin; OVA, ovalbumin; CHTG, chymotrypsinogen and CYTC, cytochrome C.

approximately 25,000. The specific activity of the inhibitors recovered at the peak fractions (tube numbers 54 for A and 53 for B) were 143.8 IU and 148.0 IU, respectively. This indicates that both inhibitors were purified approximately 150 fold, though the average specific activity of the inhibitor containing fractions was about 50 IU (Table 1). Therefore, the peak fractions were employed for the following experiments.

Kinetic Studies of Collagenase Inhibitor

Using purified collagenase inhibitor (tube number 53 of B in Fig. 3) and tadpole collagenase (Hori and Nagai, 1979), effect of inhibitor concentrations on the rate of collagenase inhibition was studied by the gel method. As shown in Figure 4, addition of the inhibitor to purified collagenase resulted in a dose-dependent

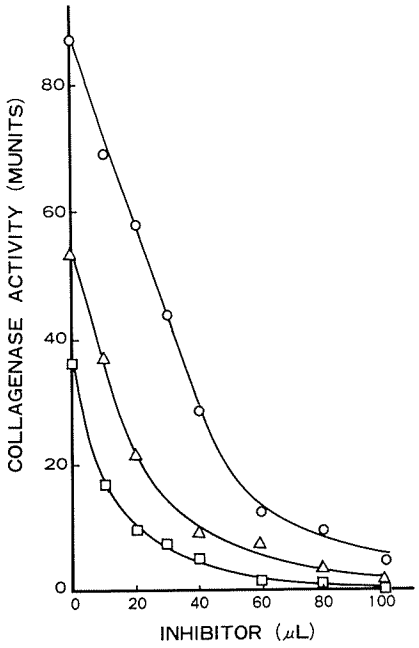

Fig. 4. Changes of collagenase activity as a function of inhibitor concentration. In a series of small test tubes containing 0.4 ml of ^{14}C-labeled collagen gels, various amounts of purified growth cartilage inhibitor (tube No. 53 of sample B in Fig 3) was added, then enzyme reaction was started at 37 °C by adding 36 munits (□), 54 munits (△) or 90 munits (○) of tadpole collagenase.

inhibition of enzyme activity and the degree of inhibition was inversely proportional to enzyme concentration. However, with increasing inhibitor concentrations, no longer proportional inhibition was observed and a large excess of the inhibitor was required to inhibit the enzyme completely.

Time course of inhibition of tadpole collagenase by the inhibitor is shown in Figure 5. To a fixed amount of collagenase, increasing amounts of inhibitor were added and hydrolysis of reconstituted collagen fibrils was measured at selected periods. The hydrolysis was linear up to 24 h with or without inhibitor and the percentage of inhibition at individual inhibitor concentrations was nearly constant throughout the assay period, indicating that both enzyme and inhibitor retained their activities during the period of the assay (48 h).

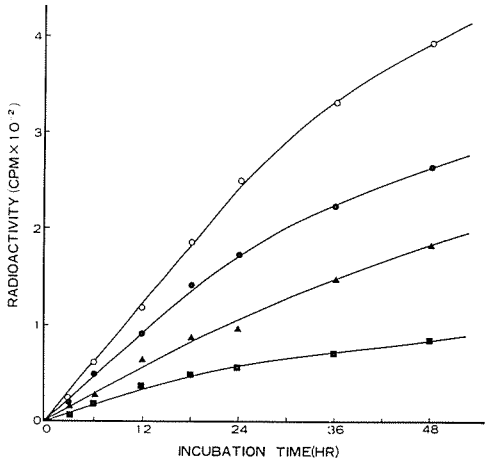

Fig. 5. Kinetic analysis of collagenase activity in the presence of growth cartilage inhibitor with various concentrations. In a series of small test tubes containing 0.4 ml of ^{14}C-labeled collagen gels, 0 μl (○), 25 μl (●), 50 μl (▲) and 100 μl (■) of the inhibitor was added and total volume was adjusted to 0.5 ml by adding Tris-NaCl-$CaCl_2$ buffer. Then, enzyme reaction was started by adding 0.1 ml of tadpole collagenase (108 munits) at 37 °C.

Action of Inhibitor on Collagenase and Other Neutral Proteases from Various Sources

Action of an inhibitor on various collagenases and other neutral proteases was examined using collagen and gelatin as substrates. As summarized in Table 2,

Table 2. Action of the collagenase inhibitor on collagenases from various tissues. The indicated amounts of various collagenases were incubated with 600 μg of ^{14}C-labeled collagen fibrils in a final volume of 600 μl (Tris-NaCl-$CaCl_2$ buffer) at 37 °C for 30–50 h with or without added inhibitor (107 mIU, 0.1 ml). When soluble collagen was used as a substrate, the enzymes were incubated with 400 μg of ^{14}C-labeled soluble collagen in a final volume of 400 μl at 35 °C for 28 h. After termination of the enzyme reaction by adding 20 μl of 80 mM o-phenanthroline, an equal volume of dioxane was added to the reaction mixture and the radioactivity in the supernatant was determined with a Beckman LS-9000 liquid scintillation system

Enzyme source	Amount (ng)	Collagenase activity against Collagen fibrils: Control (munits)	+ Inhibitor (munits)	Inhibition (%)	Soluble collagen: Control (munits)	+ Inhibitor (munits)	Inhibition (%)
Tadpole skin[1]	3.8	32	9	72	61	34	44
	4.4	37	2	95*	–	–	–
Chick bone[2]	10 μl	75	7.5	90	91	15	84
Chick skin[3]	100 μl	21	9.2	56*	–	–	–
Human granulocyte[4]	25 μl	36	8.5	77*	–	–	–
Cl. histolyticum	5	37	37	0	–	–	–

[1] 8400 units/mg protein (*Hori* and *Nagai*, 1979).
[2] 4.93 units/OD_{280} nm.
[3] 3.70 units/OD_{280} nm.
[4] 5.90 units/OD_{280} nm.
* 164 mIU inhibitor was employed.

the inhibitor blocked the action of collagenase from tadpole skin, chick bone and skin as well as human granulocytes, but not bacterial collagenase. The most potent inhibition was observed with chick bone collagenase using either collagen fibrils or soluble collagen as substrates. The blockage of the action of collagenase was confirmed by the detection of diminished degradation products (α^A) of collagen by the presence of inhibitor (Fig. 6). The ability of the inhibitors (A

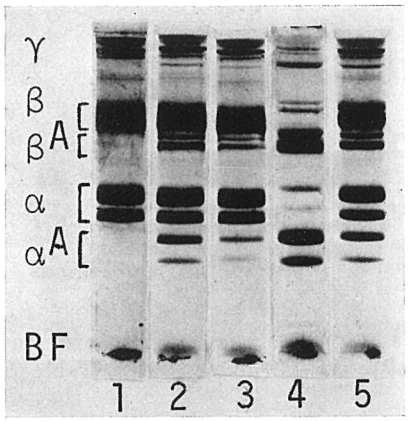

Fig. 6. Comparison of hydrolysis of native collagen by collagenase with or without added inhibitor. Acid soluble guinea pig skin collagen, 80 μg, was incubated with 70 mU tadpole collagenase or 30 mU chick bone collagenase in 100 μl of Tris-NaCl-$CaCl_2$ buffer containing 0.25 M D-glucose in the absence or presence of 40.8 mIU inhibitor for 60 h at 20 °C (2 and 3) or 26 °C (1, 4 and 5). After termination of the enzyme reaction by adding 20 μl of 0.2 M EDTA, SDS-polyacrylamide gel electrophoresis was performed at pH 8.3. BF, buffer front. 1, buffer; 2, tadpole collagenase; 3, tadpole collagenase with inhibitor; 4, chick bone collagenase and 5, chick bone collagenase with inhibitor.

and B) to inhibit neutral proteases other than collagenase was further investigated using ^{3}H-labeled heat-denatured polymeric collagen as a substrate (Table 3). Both preparations of inhibitor blocked the gelatinolytic activity of tadpole collagenase, chick skin gelatinase, and human granulocyte gelatinase, while that of bacterial collagenase was unaffected by the inhibitor. No other proteases examined were affected by the inhibitor, with the exception that tryptic activity against gelatin was blocked by the inhibitor. A slight inhibition of plasmin was ascribed to the inhibitory effect of inhibitor on urokinase which was added to the plasmin preparation to activate plasminogen (Table 3). In other experiments, purified tadpole collagenase (37 mU and 54 mU) was incubated with Peptide P (final 2.5×10^{-4} M) for 44 h at 37 °C in the absence or presence of inhibitor (164 mIU). The inhibitor blocked the peptidase activity by 94 %.

Stability of Collagenase Inhibitor

The collagenase inhibitor was heat stable. About 90 % of its inhibitory activity was retained after treatment at 90 °C for 5 min. Sensitivity of the inhibitor to protease was also examined. Since the inhibitor was found to block the tryptic activity against gelatin (Table 3), the inhibitor was preincubated with various amounts of chymotrypsin. After destroying chymotryptic activity with chymostatin, the reaction mixture was assayed for remaining inhibitory activity. As shown in Table 4, the inhibitor was sensitive to chymotrypsin, indicating that the inhibitory activity is strongly associated with a protein component.

Table 3. Action of collagenase inhibitor on neutral proteases. The indicated amounts of various collagenases and neutral proteases were incubated with 200 μg of ^{3}H-labeled heat-denatured polymeric collagen in a final volume of 200 μl (Tris-NaCl-$CaCl_2$ buffer) at 37 °C for 1–43 h with or without added inhibitor (53 mIU, 50 μl). After termination of the enzyme reaction by adding two volumes of dioxane, the radioactivity in the supernatant was determined (average of duplicate experiments)

Enzyme source	Amount (ng)	Gelatinolytic activity Control (munits)	+ Inhibitor (munits)	Inhibition (%)
Collagenase				
tadpole skin	3.2	8.4	0*	100
Cl. histolyticum	0.5	79	82*	0
	5.0	788	775	1
	5.0[5]*	834	958	0
Gelatinase				
chick skin[1]	50 μl	24	4	85
human granulocyte[2]	5 μl	73	35*	52
Trypsin	0.4	225	20	91
	2.0	1360	564*	59
Chymotrypsin[3]	5.0	27	37	0
	10.0	62	59	4
Elastase, pancreatic	5.0	14	13*	6
Plasmin[4]	10 μl	22	19	12
	20 μl	37	33	10

[1] 46.2 units/OD_{280} nm using gelatin as a substrate (*Sunda* and *Nagai*, 1980).
[2] 15.0 units/OD_{280} nm using gelatin as a substrate (*Kobayashi* and *Nagai*, 1978).
[3] Pretreated with 2.5×10^{-4} M TLCK for 1 h at room temperature.
[4] 6.7 μmol/min · mg protein using tosyl-L-arginine methylester as a substrate (after activation with urokinase at 37 °C for 15 min).
[5] Pretreated with 31 mM N-ethylmaleimide.
* 82 mIU inhibitor was employed.

Table 4. Effect of chymotrypsin on the collagenase inhibitor, 107 mIU/100 μl of collagenase inhibitor was preincubated with various concentrations of TLCK-treated chymotrypsin for 1.5 h at 37 °C. Chymotryptic activity was destroyed by adding chymostatin (10 μg/10 μl), then 15 μl of 27 mU tadpole collagenase was added and the final volume was adjusted to 200 μl with Tris-NaCl-$CaCl_2$ buffer. 190 μl of the mixture was assayed for collagenase activity using ^{14}C-labeled collagen fibrils as a substrate

Con. of chymotrypsin during preincubation (ng)	Inhibitor (mIU)	Enzyme activity (mU)	Inhibition (%)
0	0	25.9	–
0	107	9.8	62
10	107	12.3	53
100	107	12.7	51
1000	107	16.3	37

Discussion

It has generally been understood that extracellular degradation of collagen fibers is initiated through the action of specific collagenase. As shown in Figure 1, cartilaginous explants produce neither active nor latent collagenase during culture period, regardless of the stages examined, while the explants of bony tissue including marrow cells begin to produce latent collagenase at around stage 38 and later. It is possible that, during matrix transition from cartilage to bone, the degradation of cartilage matrix collagen depends on collagenase activity produced by invading bone marrow-derived cells. The collagenase was found as an inactive form in culture media which could be easily activated by simple dialysis against 3 M NaI in the cold. This suggests that the latent form of the enzyme is an enzyme-inhibitor complex and dissociated into active enzyme and a dialyzable component which has inhibitory activity. The results concur well with previous reports by Shinkai and Nagai (1977). On the other hand, during development, production of non-dialyzable collagenase inhibitory activity, distinct from the dialyzable inhibitor, precedes the production of latent collagenase. The collagenase inhibitory activity is most remarkable at the site of active matrix transition. These results suggest that collagenase activity at the region of osteo-chondral transition is regulated possibly by multistep process involving conversion of latent collagenase to active form as well as modification of the resultant enzyme activity by some other regulating principle(s) such as inhibitors reported here. Since degradation of cartilage matrix must proceed in parallel with deposition of osteoid matrix, such multi-step process would provide for discrete control of collagenolytic activity and allow collagen resorption only under those conditions in which active collagenase exceeds local inhibitory capacity.

The collagenase inhibitors (A and B) produced by chick growth cartilage are cationic proteins with the same molecular weight of approximately 25,000 but slight difference in molecular charge. The growth cartilage inhibitor differs in apparent molecular weight from serum inhibitors (Nagai, 1973; Werb et al., 1974; Woolley et al., 1976) and from cartilage anti-invasion factor (Kuettner et al., 1976). The inhibitor has much higher collagenase inhibiting capacity than lysozyme (Sakamoto et al., 1974). It seems quite similar to inhibitors from rabbit cranial bone (Sellers and Reynolds, 1977), human skin fibroblasts (Welgus et al., 1979) and human tendon (Vater et al., 1979). The inhibitor is heat-stable, but sensitive to chymotrypsin. It blocks the action of collagenase on both reconstituted collagen fibrils and collagen in solution. Although kinetic studies on the inhibition of collagenase have difficulties mainly because of restricted range of adequate substrate concentration, the dose-response curve of purified inhibitor and collagenase (Fig. 4) suggests that the inhibition is stoichiometric. Moreover, a fairly good linearity with time course of the action of inhibitor is obtained during the assay period.

The inhibitor has a broad specificity against neutral proteases as summarized in Tables 2 and 3. It blocks the activity of collagenases from tadpole skin, chick bone and skin and from human granulocytes. Human granulocyte and chick skin gelatinases which may participate in the post-collagenase catabolism of collagen (Sunada and Nagai, 1980) are also inhibited by the inhibitor, as reported (Sellers et al., 1979). It is of interest that the inhibitor blocks tryptic activity against gelatin, when assayed at enzyme concentrations similar to collagenase, but not

chymotryptic activity. The results described above suggest that the inhibitors reported here may participate in multi-functional regulations of the collagen-degrading enzyme system. In addition, the findings of the maximum production of inhibitors at the growth plate may support a hypothesis that the inhibitors are functioning at the proximate region of selective cartilage resorption and newly forming osteoid tissue where degenerating hypertrophic chondroblasts synthesize both type I and II collagens (Gay et al., 1976; von der Mark, 1977) and the degradation rate of type II collagen is increased by the presence of other types of collagen (Hayashi et al., 1980b).

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Dr. Yutaka Nagai, Department of Tissue Physiology, Medical Research Institute, Tokyo Medical and Dental University, Kandasurugadai, Chiyoda-ku, Tokyo 101, Japan.