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The Existing Forms of Collagenase in the Human Uterine Cervix

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The existing forms of collagenase [EC 3.4.24.7] in the human uterine cervix were examined. The latent collagenase extracted by homogenization in 0.25% Triton X-100 containing 0.01 M $CaCl_2$ was indicated to be a complex of collagenase with α_2-macroglobulin by the behavior of the fraction of this enzyme before and after treatment with NaSCN on Sephadex G-150 column chromatography and an immunodiffusion method. The active collagenase was extracted by rehomogenization in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 M $CaCl_2$ from the insoluble residue at 0°C. Another latent collagenase was extracted from the insoluble fraction in the same buffer by heating at 60°C for 4 min and this enzyme was activated by 4-aminophenylmercuric acetate or trypsin. The molecular weights of the active and the latent forms were approximately 7.3×10^4 and 9.4×10^4, respectively. This indicates that the latency is due to the formation of a low molecular weight inhibitor enzyme complex.

These results clarified that the human uterine cervix contains three existing forms (α_2-macroglobulin complex, active form and low molecular weight inhibitor complex) of collagenase under these experimental conditions.

Collagenases from a wide variety of human tissues have been reported (*1-6*) and there is now substantial evidence to confirm the participation of these enzymes in collagen catabolism in both normal and pathological connective tissues. Recent studies on vertebrate collagenase demonstrated that the existing forms of collagenase can be grouped into four different types; 1) active enzymes (*7*), 2) complexes with α_2-macroglobulin (*8, 9*), 3) inactive forms with a low molecular weight inhibitor (*10, 11*), and 4) proenzymes (*12-14*). It is considered that the extracellular modulation in the catabolism of collagen is determined by the balance of various types of collagenase, but the precise role of each type of collagenase in degradation of collagen *in vivo* is still unclear.

Since it is important to obtain information on the physiological significance of latency in the regulation of collagenase activity in cervical tissue with special reference to cervical ripening, an investigation of the existing forms of collagenase in this tissue was attempted. This paper provides evidence showing that three different types of collagenase exist in the human uterine cervix.

Abbreviations: DNP-peptide, 2,4-dinitrophenyl-L-prolyl-L-glutaminyl-glycyl-L-isoleucyl-L-alanyl-glycyl-L-glutaminyl-D-arginine; α_2-M, α_2-macroglobulin; 4-APMA, 4-aminophenylmercuric acetate; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Tissue Sources—Uterine cervical tissue from women of child-bearing age was used and non-pregnant cervical tissue was obtained from patients

undergoing hysterectomy for fibromyoma not involving the cervix. The cervical epithelium and stroma were completely separated, and the stromatic tissues were rinsed with cold saline to remove the blood and mucus and stored at −20°C until use.

Reagents—Chemicals used in these experiments were obtained from the following sources: fluorescein isothiocyanate (Isomer I), 4-aminophenylmercuric acetate (4-APMA), and trypsin (bovine pancreas Type VII) from Sigma Chemical Company, U.S.A.; soybean trypsin inhibitor and anti-human α_2-macroglobulin serum (rabbit) from Miles Laboratories Ltd., U.S.A.; Sephadex G-150 from Pharmacia Fine Chemicals, Sweden; 2,4-dinitrophenyl-L-prolyl-L-glutaminyl-glycyl-L-isoleucyl-L-alanyl-glycyl-L-glutaminyl-D-arginine (DNP-peptide) from Protein Research Foundation, Japan. All other reagents used were commercially available and of analytical reagent grade.

Differential Extraction of Collagenase from Human Uterine Cervix—Collagenase was extracted from the cervical tissue by the method of Weeks *et al.* (*7*) with some modifications. The defrosted tissue was homogenized twice in 10 volumes of 0.25% Triton X-100 containing 0.01 M $CaCl_2$ in a VirTis 45 homogenizer at full speed for 30 s. The homogenates were centrifuged at $6,000 \times g$ for 20 min at 2°C. The supernatant was removed and named Fraction I. The pellet was rehomogenized for 30 s to the original volume of 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 M $CaCl_2$ ($CaCl_2$ buffer). After centrifuging at $10,000 \times g$ for 20 min, the supernatant was removed and named Fraction II. Then the pellet was resuspended in the same volume of the above buffer solution and the suspension was put in a 50 ml stainless steel centrifuge tube (10–15 ml/tube) and heated at 60°C for 4 min with manual agitation. The tube was chilled in ice-cold water and centrifuged at $10,000 \times g$ for 20 min at 2°C. The supernatant obtained was named Fraction III. Fractions II and III were dialyzed overnight against 40 mM Tris-HCl buffer, pH 7.6, containing 0.15 M NaCl to produce a final calcium concentration of 10 mM. Any precipitates formed were removed by centrifugation.

Collagenase Assay—Collagenase activity was measured by the method of Woessner (*12*) with slight modifications. The substrate used was fluorescein-labelled acid soluble collagen (F-collagen) prepared from rat tail tendon according to the method of Steven *et al.* (*15*). The substrate (2 mg) was incubated with 1 ml of enzyme preparation and 1 ml of 40 mM Tris-HCl buffer, pH 7.6, containing 10 mM $CaCl_2$ at 37°C. After 18 h, the insoluble collagen was removed by filtration, and the extinction of the filtrate was measured at 495 nm. Blanks were run with 10 mM EDTA in place of calcium. One unit of activity was calculated as the amount which released 1 nmol of fluorescein isothiocyanate during incubation under the above conditions.

Assay of Hydrolytic Activity against a Synthetic Substrate—DNP-peptide degrading activity was assayed by the method of Masui *et al.* (*16*). The peptide was dissolved in 50 mM Tris-HCl buffer, pH 7.6, containing 0.15 M NaCl, 5 mM $CaCl_2$, and 0.02% bovine serum albumin at a final concentration of 5×10^{-4} M. To 0.1 ml of the substrate was added 0.1 ml of the enzyme solution and the mixture was incubated at 37°C. After 2 h, the reaction was stopped by adding 0.5 ml of 1 M HCl. The DNP-peptide fragments released were extracted by vigorous shaking with 1 ml of ethyl acetate followed by centrifugation at $1,000 \times g$ at room temperature for 10 min to separate the two layers. The degree of hydrolysis was determined by measuring the absorbance of the organic layer at 365 nm. One unit of the activity was calculated as the amount which released 1 nmol of DNP-peptide fragment (DNP-Pro-Gln-Gly) per hour under the above conditions.

Detection of the Latent Collagenase—The latent collagenase was activated by 4-APMA, trypsin, and NaSCN according to the methods of Sellers *et al.* (*11*), Weeks *et al.* (*7*), and Abe and Nagai (*17*), respectively; a) with 4-APMA, the enzyme solution was incubated with 4-APMA at a final concentration of 1 mM for 60 min at 37°C; b) with trypsin, the sample (1 ml) was incubated with trypsin (50 μg) for 5 min at 37°C and then soybean trypsin inhibitor (180 μg) was added and the mixture was incubated for 5 min at 37°C; c) with NaSCN, the sample was dialyzed against 3 M NaSCN in 40 mM Tris-HCl buffer, pH 7.6, containing 10 mM $CaCl_2$ for 6 h at 4°C, and then dialyzed against 40 mM Tris-HCl buffer, pH 7.6, containing 10 mM $CaCl_2$ to remove NaSCN.

Preparation of Human α_2-M-Collagenase Complex—Human α_2-M was prepared by the method

of Woolley *et al.* (*2*), and the α_2-M-collagenase complex was prepared by reaction of a mixture of α_2-M and cervical collagenase at 37°C for 5 h. Proteinase inhibition activity of α_2-M was measured by the method of Sugiura *et al.* (*18*). One inhibition unit was defined as the amount of α_2-M which was required for 50% inhibition of 2 μg of trypsin. The α_2-M obtained had 12 units/mg, and 1 unit corresponds to the inhibition activity of collagenase (0.42 F-collagen unit).

Immunodiffusion Method—Immunodiffusion was performed according to the method of Ouchterlony (*19*), using anti-human α_2-M serum (rabbit) as an antibody.

Disc Gel Electrophoresis—Electrophoresis in 5% polyacrylamide gels containing 0.1% SDS (*20*) was carried out to examine the collagen degradation products. A mixture of collagen and enzyme was incubated at 25°C, the reaction stopped by the addition of EDTA, and then the reaction mixture was heated at 50°C for 30 min. After electrophoresis, gels were stained and destained according to the method of Woolley *et al.* (*3*).

Amino Acid Analysis of the DNP-Peptide Fragment—The action of cervical collagenase on a synthetic substrate was examined by amino acid analysis of the DNP-peptide fragment which was extracted in ethyl acetate. Incubation was carried out by the method described above and the cleaved peptide fragment containing a dinitrophenol group was extracted in ethyl acetate. The organic solvent layer was then dried off and the peptide fragment hydrolyzed under nitrogen gas in 6 M HCl at 110°C for 18 h and dried again. Amino acid analysis was done with a Hitachi model-835 amino acid analyzer.

RESULTS

Table I shows the degrading activities against F-collagen and DNP-peptide at each extraction step. In Fraction I there was no activity against F-collagen, but degrading activity against DNP-peptide existed. After treatment of Fraction I with 3 M NaSCN degrading activity against F-collagen appeared, but this activation did not occur with 4-APMA. Fraction II showed the degrading activities against these two substrates. On the other hand, Fraction III did not show any degrading activities against these two substrates, but after treatment of this fraction with 4-APMA or trypsin, degrading activities against both substrates appeared.

TABLE I. Distribution of the degrading activity of each fraction against F-collagen and DNP-peptide.

Tissue fraction	Treatment	Substrate	
		F-collagen	DNP-peptide
Fraction I	None	−	+
	4-APMA	−	+
	NaSCN	+	+
Fraction II	None	+	+
	4-APMA	+	+
Fraction III	None	−	−
	4-APMA	+	+
	Trypsin	+	+

+, Hydrolyzed; −, not hydrolyzed.

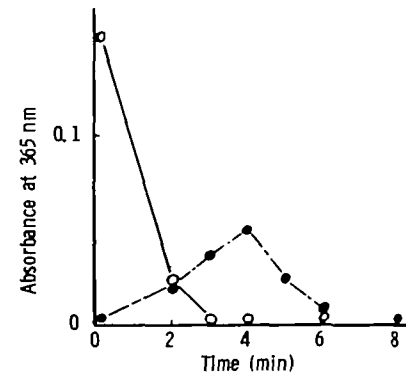

Fig. 1. Effect of temperature on the DNP-peptide degrading activity of Fraction II and on the extraction of Fraction III. Fraction II was treated at 60°C for each time and the remaining activity against DNP-peptide was assayed (○). The insoluble residue from Fraction II was resuspended in 0.1 M $CaCl_2$ buffer and heated at 60°C. Degrading activity of Fraction III against DNP-peptide was assayed after treatment with 4-APMA (●).

Figure 1 shows the effect of temperature on the activity degrading DNP-peptide of Fractions II and III. The activity of Fraction II disappeared on heating at 60°C for 3 min; however, the activity of Fraction III was maximum after heating at 60°C for 4 min. Furthermore, we also confirmed that the activity of Fraction III was maximal after treatment with a final concentration of 1 mM of 4-APMA for 60 min at 37°C (data not shown).

TABLE II. Effect of reagents on the activity of human cervical collagenase. The enzyme (6.14 U/ml) was treated with each reagent for 30 min at 37°C and then activity was assayed by using DNP-peptide as a substrate.

Reagent (5 mM)	Relative activity (%)
None	100
EDTA	0
1,10-Phenanthroline	13
L-Cysteine	0
N-Bromosuccinimide	59
Dithiothreitol	0
N-Ethylmaleimide	88
ICH_2COOH	40
Phenylmethylsulfonyl fluoride	96

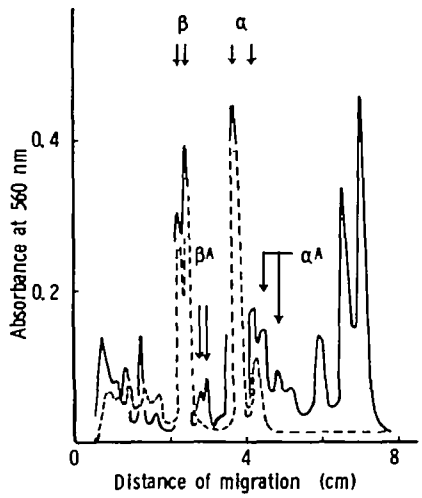

Fig. 2. Spectrophotometric scans of SDS-polyacrylamide gels of the reaction products of cervical collagenase and collagen in solution at 25°C. Incubation and electrophoresis were carried out by the methods described in the text. Undegraded rat tail tendon collagen (----), collagen plus enzyme (——).

The degrading activity against F-collagen was optimal in the pH range of 7.6–8.0 and decreased sharply above pH 8.5 or below pH 7.4.

The degrading activities against F-collagen and DNP-peptide had a correlation in our experimental system.

As shown in Table II, the degrading activity against DNP-peptide was inhibited by common

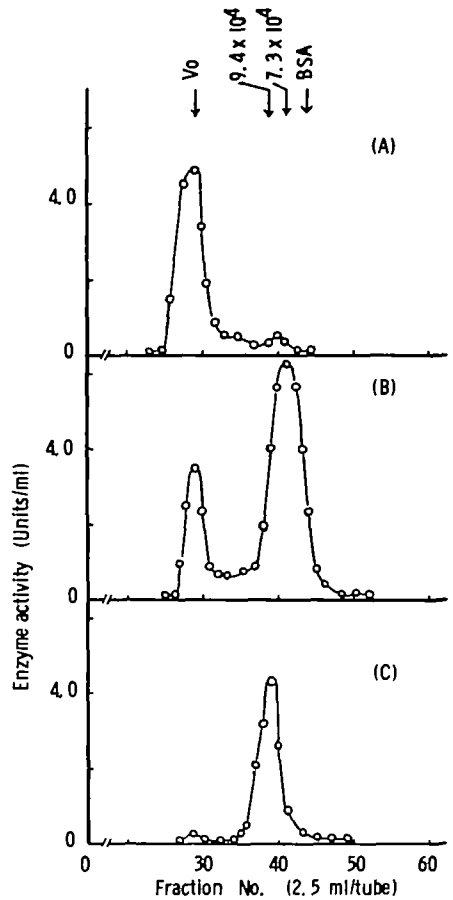

Fig. 3. Elution profiles of Fractions I, II, and III on Sephadex G-150 column chromatography. A column (1.7×65 cm) was eluted with 40 mM Tris-HCl buffer, pH 7.6, containing 0.15 M NaCl and 10 mM $CaCl_2$. Fractions I (A) and II (B) were applied to the column and eluted, and DNP-peptide degrading activity in each fraction was directly assayed. Fraction III (C) was assayed after treatment of each fraction with 4-APMA. Each Fraction was concentrated with polyethylene glycol 6,000 before being applied to the column. V_0; void volume of the column, BSA; bovine serum albumin.

vertebrate collagenase inhibitors. EDTA, 1,10-phenanthroline, L-cysteine, and dithiothreitol were shown to be strong inhibitors of this enzyme. *N*-Bromosuccinimide and iodoacetic acid partially inhibited the cervical collagenase but *N*-ethylmaleimide and phenylmethylsulfonyl fluoride had no inhibitory effects on this collagenase activity.

Figure 2 shows the SDS-polyacrylamide gel electrophoretic patterns of the collagen degradation

products. The characteristic products of the cleavage of native collagen, such as the βA and αA doublets (three-quarter-length fragments), can be seen below the β and α chains, respectively. However, because of the influence of the proteins in the enzyme solution, the bands corresponding to the one-quarter-length fragments (αB and βB) are not clearly visible.

On amino acid analysis of the DNP-peptide fragment which was extracted in ethyl acetate after enzymatic degradation, glutamic acid (11.0 nmol) and glycine (10.8 nmol) were found and proline (4.7 nmol) was also detected, since dinitrophenyl-proline was unstable under these conditions of hydrolysis (*21*). This clearly indicates that cervical collagenase cleaves DNP-peptide at the glycyl-isoleucine bond.

These results strongly suggested that degrading activities against DNP-peptide and F-collagen in the human uterine cervix were the activity of vertebrate collagenase.

The behavior of Fractions I, II, and III on Sephadex G-150 column chromatography was examined. In Fraction III, the degrading activity against DNP-peptide was assayed after treatment with 4-APMA. As shown in Fig. 3, a single peak at the void volume (latent collagenase I) and two peaks of DNP-peptide degrading activities (latent collagenase I and active collagenase) appeared on

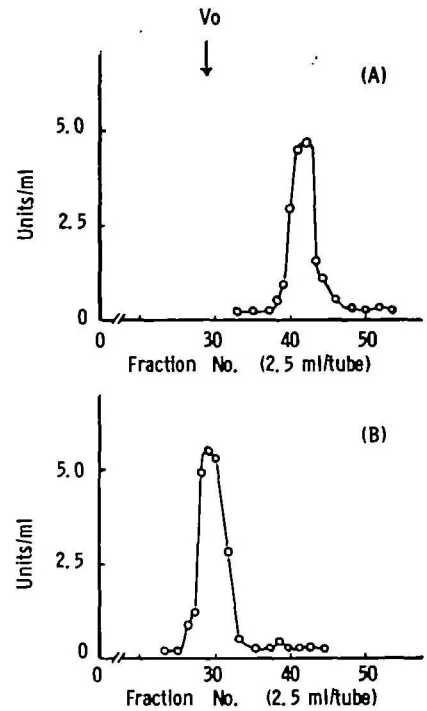

Fig. 4. Elution behavior on Sephadex G-150 column chromatography of the void volume activity of Fraction II after treatment with NaSCN and the second peak of Fraction II after treatment with α_2-M. (A), after treatment with NaSCN; (B), after treatment with α_2-M. Enzyme activity was determined by DNP-peptide assay.

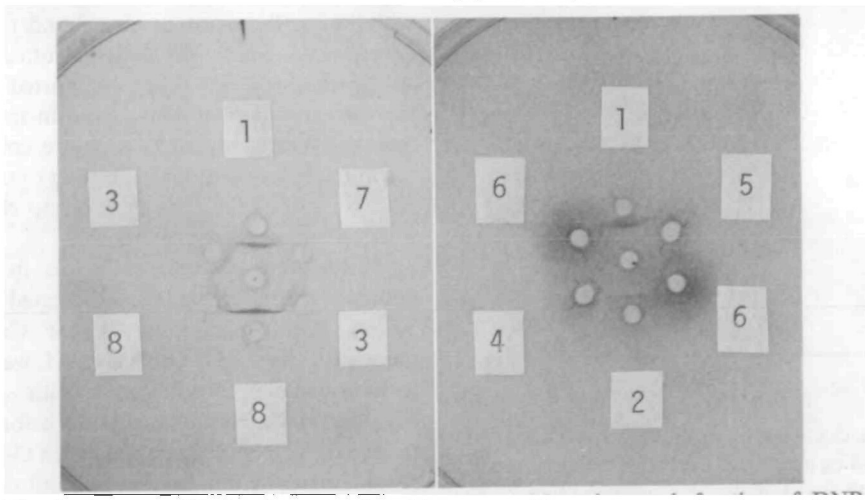

Fig. 5. Immunodiffusion in agar of anti-human α_2-M against each fraction of DNP-peptide degrading activity. Center well; anti-human α_2-M rabbit antibody. Well 1, human α_2-M; 2, Fraction I; 3, Fraction II; 4, Fraction III; 5, the second peak of Fraction II (Fig. 3B); 6, the first peak of Fraction II (Fig. 3B); 7, the peak of the activity in Fig. 4A; 8, the peak of the activity in Fig. 4B.

chromatographic profiles of Fractions I and II, respectively, and a single peak (latent collagenase II) eluted just before the second peak of Fraction II was obtained from Fraction III. Although the first peak (void volume) of Fraction II did not degrade F-collagen, the second peak of Fraction II and the peak of Fraction III after treatment with 4-APMA degraded F-collagen. By use of a calibrated Sephadex G-150 column we estimated the molecular weights of the active enzyme, the latent collagenase II and the activated enzyme from the human uterine cervix. The latent collagenase II had an apparent molecular weight of 9.4×10^4. Activation of the latent collagenase II by 4-APMA produced a decrease in molecular weight of 2.1×10^4. A molecular weight of 7.3×10^4 for the activated enzyme was in close agreement with the value for the active enzyme from Fraction II.

When the first peak of Fraction II was applied to a Sephadex G-150 column after treatment with 3 M NaSCN, the activity against DNP-peptide was eluted at the same position as the second peak (Fig. 4A) and then degrading activity against F-collagen also appeared. Furthermore, the second peak of Fraction II gave a peak in the void volume on chromatography after treatment with α_2-M at 37°C for 5 h (Fig. 4B) and degrading activity againt F-collagen disappeared.

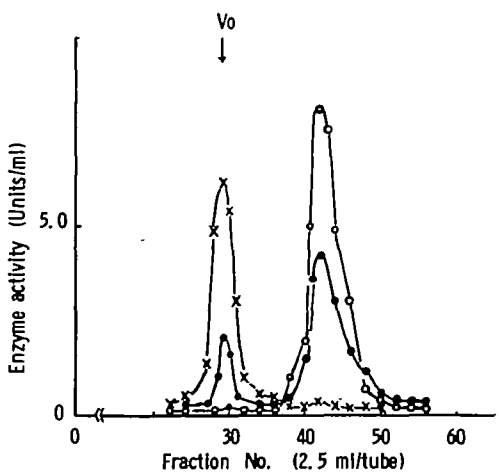

Fig. 6. Sephadex G-150 chromatography of DNP-peptide degrading activity of the active collagenase after treatment with α_2-M under various conditions. The second peak of Fraction II (5.2 F-collagen units) was treated with α_2-M (anti-collagenolytic activity, 22.4 units) at 0°C for 3 h (○), at 37°C for 2 h (●), and at 37°C for 5 h (×).

The reactivity of anti-human α_2-M against each fraction was investigated with an immunodiffusion method. The antigenicity of α_2-M in Fraction I and the first peak of Fraction II was detected, but not detected in Fraction III and the second peak of Fraction II (Fig. 5).

As shown in Fig. 6, the active collagenase was mixed with α_2-M under various conditions and the mixture was immediately applied to a column of Sephadex G-150. The peak of active collagenase did not move to the void volume at 0°C for 3 h. However, when the collagenase was incubated with α_2-M at 37°C for 5 h the enzyme moved to the void volume. The formation of the complex was incomplete at 37°C for 2 h. These facts were also confirmed from the inhibition experiment with human serum; about 6% and 30% the relative activities were shown by using F-collagen assay method, when the active collagenase was preincubated with fresh serum at 37°C for 5 h and 0°C for 3 h, respectively.

DISCUSSION

Recent reports indicated four different types of existing forms of vertebrate collagenase. However, systematic investigations on the existing forms in particular tissues have dealt almost exclusively with these collagenases produced under such special experimental conditions as tissue culture.

Recently, Weeks *et al.* (*7*) reported a procedure for the extraction of collagenase in involuting rat uterus. We attempted to separate collagenase in the human uterine cervix according to their method with some modifications in order to elucidate the existing forms of the enzyme.

The latent collagenase I was in the supernatant of the tissue homogenate treated with 0.25% Triton X-100 containing 0.01 M $CaCl_2$. Consequently, the latent collagenase I was indicated to be a complex of collagenase with α_2-M by the behavior of the resulting fraction before and after treatment with NaSCN on Sephadex G-150 column chromatography and an immunodiffusion method. The complex of collagenase with α_2-M was differentiated from active collagenase by the combined use of collagen and a synthetic substrate for the collagenase assay, since DNP-peptide was still

cleaved by the α_2-M-collagenase complex, but collagen was not, as pointed out by Hori and Nagai (*22*). We found a large amount of α_2-M-collagenase complex in Fraction I, but Weeks *et al.* (*7*) demonstrated that no appreciable amount of the complex was observed after treatment with KSCN in the case of involuting rat uterus. We also found that only a little α_2-M-collagenase complex was present in human uterine corpus (unpublished results). In order to examine the possibility that a complex of collagenase with α_2-M was artificially formed in the process of extraction, the active collagenase was treated with α_2-M under various conditions. Consequently we confirmed that an α_2-M-enzyme complex was not formed when cervical collagenase was incubated with α_2-M at 0°C for 3 h. A similar result was also obtained when fresh human serum was used instead of α_2-M. This indicated that the affinity of cervical collagenase with α_2-M was weaker compared to tadpole collagenase (*23*) and the formation of an α_2-M-enzyme complex (about 70% of the total activity) did not occur during extraction.

Furthermore, we could not find any activities against F-collagen in the supernatant of the extract in 0.1 M $CaCl_2$ buffer after heating at 60°C, although Weeks *et al.* (*7*) found the activities of both active and latent collagenase which were activated by trypsin. The active enzyme with the latent collagenase I was almost completely extracted by rehomogenization in 0.1 M $CaCl_2$ buffer from the insoluble residue without heating in order to separate the active enzyme from the latent collagenase II. The results are consistent with, but do not prove, the hypothesis that the active collagenase is associated with collagen fibers (*23*, *24*). The activity against DNP-peptide was completely lost on heating at 60°C, suggesting that the active collagenase and the latent collagenase I were heat-labile. Only the latent collagenase II was extracted from the insoluble residue by heating at 60°C for 4 min in 0.1 M $CaCl_2$ buffer. Exclusive chromatography on Sephadex G-150 gives an apparent molecular weight of approximately 9.4×10^4 for the latent collagenase II and 7.3×10^4 for the active form. The molecular weight of the active collagenase was different from that of other human collagenases; 38,000 in gastric mucosa (*2*), 40,000 in embryonic skin (*6*), skin fibroblasts (*25*), and gingiva (*26*), 60,000 in skin culture (*3*) and 64,000 in corneal fibroblasts (*5*). However, the molecular weight of the active cervical collagenase was similar to that of human leucocytes, 75,000 (*4*). The difference in molecular weight between the latent collagenase II and the active enzyme was approximately 2.1×10^4. Although we have not yet been able to isolate the fragment of low molecular weight from the latent collagenase II by the activation, it seems reasonable to conclude that the latent collagenase II exists as a non-covalently bound complex of the active collagenase and inhibitors, since the latent enzyme was converted into the active collagenase by 4-APMA as described by Sellers *et al.* (*11*). However, the existing ratio of the low molecular weight inhibitor enzyme complex activity to the total activity was under 10 per cent.

On the other hand, Woessner (*12*) described that the latent collagenase in rat uterus was activated by endogeneous serine proteinase and that this latent collagenase was a proenzyme of collagenase. More recently he also discussed the possibility that this latent form was a collagenase-inhibitor complex (*27*). However, the low molecular weight inhibitor collagenase complex obtained from human uterine cervix was not activated by a serine proteinase isolated from the same tissue (*28*).

Present studies clarify that there are three types of existing forms of collagenase in the human uterine cervix, though it is not clear that these results quantitatively reflect the true existing state of cervical collagenase *in vivo*.

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