Partial Purification and Characterization of a Neutral Protease Which Cleaves Type IV Collagen†

Lance A. Liotta,* Karl Tryggvason, Spiridione Garbisa, Pamela Gehron Robey, and Shigeto Abe

ABSTRACT: A neutral protease has been extracted from the media of cultured metastatic tumor cells and purified approximately 1000 times after sequential ammonium sulfate fractionization, concanavalin A column chromatography, and molecular sieve chromatography. The protease has an apparent molecular weight of 70 000–80 000, is inactive at acid pH, requires trypsin activation, and is inhibited by ethylenediaminetetraacetic acid but not by phenylmethanesulfonyl fluoride, *N*-ethylmaleimide, or soybean trypsin inhibitor. The enzyme produces specific cleavage products for both chains of pro type IV collagen isolated without pepsinization and apparently cleaves at one point in a major pepsin-extracted chain of placenta type IV collagen. The partially purified enzyme fails to significantly degrade other collagens or fibronectin under digestion conditions in which specific reaction products are produced for type IV collagen. The existence of this enzyme is significant since previously described animal collagenases fail to degrade type IV collagen. Such a type IV specific collagenase could play a role in tumor invasion and may be secreted by other cells such as endothelial cells, epithelial cells, and immune cells.

Tumor cells penetrate basement membranes during many of the successive stages of the metastatic process. The transition from in situ to invasive carcinoma is accompanied by dissolution of the underlying epithelial basement membrane (Ozello, 1959; Rubio & Biberfeld, 1975). Local dissolution of the basement membrane has been observed in association with tumor cells entering or exiting vascular channels (Vlaeminck et al., 1972) or invading myocytes (Babai, 1976), but the nature of this process at the molecular level has not been clarified. In order to explore possible biochemical mechanisms involved in this process, we have studied metastatic tumors for the presence of an enzyme activity which degrades type IV collagen, which is a major structural component of basement membranes. Such an activity has been identified in cultures of a highly metastatic murine tumor (Liotta et al., 1979a). The crude medium activity preferentially digested type IV collagen at neutral pH, had low activity against collagen types I, II, and III, and also failed to degrade type V (αA and αB) collagen.

The triple-helical domain of the collagen molecule of types I, II, III, and V (αA and αB) is resistant to most proteases, but specific enzymes, which degrade native collagen, have been described in mammalian tissues (Gross, 1974; Horwitz et al., 1977; McCroskery et al., 1975; Woolley et al., 1978). These collagenases have been shown to degrade types I, II, and III but not type IV (Woolley et al., 1978; Liotta et al., 1979a,b; Timpl et al., 1979; Crouch et al., 1980). In contrast, native type IV collagen can be at least partially degraded by proteases like pepsin, trypsin, and chymotrypsin after reduction and alkylation of disulfide bonds (Timpl et al., 1978; Crouch et al., 1980). As it was not completely understood whether or not the breakdown of type IV collagen by the metastatic tumor enzyme (Liotta et al., 1979a) was due to a specific type IV collagen selective enzyme or to a less specific proteolysis, we decided to further purify this activity. In the present study, we report partial purification and characterization of the type IV collagenolytic activity.

Materials and Methods

Preparation of Substrates. [^{14}C]Proline-labeled type IV collagen was prepared in organ cultures of EHS sarcoma essentially as described elsewhere (Tryggvason et al., 1980). The basement membrane producing tumors were grown in C57BL/6J mice as reported previously (Orkin et al., 1977) and harvested after 2 weeks of growth. The excised tissue was minced, washed with PBS,[1] and preincubated for 30 min in a proline-free Dulbecco–Vogt medium containing 20% dialyzed fetal calf serum, 75 μg/mL ascorbate (Sigma), and 50 μg/mL β-aminoproprionitrile fumarate (Aldrich Chemical Co.) in a moist atmosphere containing 5% CO_2 and 95% air. After the preincubation period, [^{14}C]proline (>285 mCi/mmol, Amersham) was added to a final concentration of 5 μCi/mL and the incubation continued for 5 h. The tissue was homogenized (1 mL of tissue/9 mL of solution) in ice-cold 0.5 M acetic acid containing 20 mM EDTA (Sigma) and 8 mM MalNEt (Sigma) and extracted overnight in the same solution. The mixture was then clarified and the supernatant precipitated with 1.71 M NaCl, pH 3.0. The precipitate formed was redissolved in 0.5 M acetic acid, clarified, and dialyzed against 0.05 M Tris-HCl and 0.5 M NaCl, pH 7.4, and the small amount of precipitated material was removed by centrifugation. The supernatant was then dialyzed against 0.05 M Tris-HCl and 1.71 M NaCl, pH 7.4, and the precipitate formed was redissolved in 0.5 M acetic acid and dialyzed against the enzyme reaction buffer (0.05 M Tris-HCl, pH 7.6, containing 0.2 M NaCl and 5 mM $CaCl_2$) and stored frozen until use. Each substrate preparation was studied for purity on polyacrylamide slab gels by autoradiography.

Type I collagen was prepared from guinea pig skin by the method of Nagai et al. (1966), and type III collagen was purified from fetal calf skin by the method of Epstein (1974). Fibronectin was purified by gelatin column chromatography

† From the Laboratories of Pathophysiology and Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205 (L.A.L. and S.G.), and the Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205 (K.T., P.G.R., and S.A.).
Received March 14, 1980.

[1] Abbreviations used: PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; MalNEt, *N*-ethylmaleimide; Cl_3CCOOH, trichloroacetic acid; TA, tannic acid; SBTI, soybean trypsin inhibitor; ME, β-mercaptoethanol; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride.

from the media of cultures of human fibroblasts (Hopper et al., 1976; Engvall & Ruoslahti, 1977).

Assays for Type IV Collagenolytic Activity. For the standard assay of collagenase activity, 400 μL of the enzyme solution was activated with 100 μL of trypsin (100 μg/mL, 3 times crystallized; Sigma) at 37 °C for 4 min followed by addition of 100 μL of soybean trypsin inhibitor (500 μg/mL, Sigma). The substrate, 2000 cpm, was added in 50 μL of reaction buffer, the final reaction volume being 650 μL. The reaction was carried out for 4 h at 37 °C and stopped by addition of 20 μL of bovine serum albumin (1 mg/mL) and 100 μL of a solution of 10% trichloroacetic acid (Cl_3CCOOH) and 0.5% tannic acid (TA) to a final concentration of 2% Cl_3CCOOH and 0.1% TA. The mixture was incubated for 30 min at 4 °C and centrifuged at 5000*g* for 15 min at 4 °C to remove undigested material. Aliquots of 400 μL of the supernatant containing digested products were dissolved in 10 mL of Aquasol (Amersham) and counted for radioactivity in a Beckman scintillation counter.

In each experiment series, controls were carried out with bacterial collagenase to achieve the maximum degradation level, negative control was used without enzyme, trypsin and soybean inhibitor were used alone to assure that all trypsin was inhibited, and chymotrypsin was used to assay for nonspecific activity. Samples from various experiments were examined by slab polyacrylamide gel electrophoresis to study the reliability of the assay.

Purification of Type IV Collagenolytic Activity. Ammonium sulfate fractionation was conducted as follows: Serum-free media from cultures of the highly metastatic murine PMT sarcoma were harvested as described previously (Liotta et al., (1980a). The media proteins were directly precipitated by slowly adding ammonium sulfate to a saturation of 25% at 4 °C. The precipitate was collected by centrifugation at 27 000*g* for 60 min, and solid ammonium sulfate was similarly added to the supernatant to achieve a final saturation of 50%. The 25–50% ammonium sulfate precipitate was collected by centrifugation at 27 000*g* for 60 min and dialyzed against the enzyme reaction buffer.

Concanavalin A–agarose and gel filtration chromatograph procedures were the following: The sample (10–20 mL) was passed through a 10-mL concanavalin A–agarose column (Con A–Sepharose, Pharmacia) which was equilibrated at 4 °C in 0.05 M Tris-HCl and 10 mM $CaCl_2$, pH 7.4. The flow rate was about 0.5 column volume/h. After the column was washed with the enzyme buffer, the enzyme was eluted with 0.3 M methyl α-D-mannoside. Fractions of 4 mL were collected and assayed for enzyme activity, and the absorbance was measured at 230 nm. The fractions with the highest specific activity were pooled and precipitated with 50% ammonium sulfate as described above. The precipitate was dissolved in 2 mL of the enzyme buffer and dialyzed against the same buffer overnight with two changes. The sample was then passed over a 1.6 × 95 cm Sephadex G-200 column (Pharmacia) equilibrated with the enzyme buffer at 4 °C with a flow rate of 0.1 mL/min. Fractions of 2 mL were collected, and fractions containing enzyme activity were pooled and precipitated with 50% ammonium sulfate as above. The precipitate was dissolved in the collagenase assay buffer and dialyzed against the same buffer. This nonactivated enzyme sample was stored frozen at −70 °C.

Polyacrylamide Slab Gel Electrophoresis and Autoradiography. Polyacrylamide slab gel electrophoresis was usually performed with a Tris–glycine buffer system containing sodium dodecyl sulfate as described by Laemmli (1970) with 5% separating gels and 3% stacking gels. Urea (0.5 M) was included in the solutions to improve separation of the collagen chains. The gels were stained with Coomassie Brilliant Blue, and labeled bands on the gels were detected by autoradiography. Acid gel electrophoresis of pepsin-solubilized type IV collagen from placenta was carried out as described elsewhere (Nagai et al., 1964).

Table I: Assay for Type IV Collagenolytic Activity

treatment	radioactivity in supernate cpm	% of total
bacterial collagenase	3010	100
partially purified tumor enzyme (trypsin activated)	2880	96
partially purified tumor enzyme (unactivated)	846	28
no enzyme	50	1
MalNEt (30 mM) + tumor enzyme	2820	94
PMSF (30 mM) + tumor enzyme	2810	93
cysteine (30 mM) + tumor enzyme	780	26
chymotrypsin alone (1.0 μg/mL)	300	10
EDTA (30 mM) + tumor enzyme	190	7
EDTA (30 mM) + cysteine (30 mM) + tumor enzyme	105	5

Bacterial Collagenase Digestion. Digestion with highly purified bacterial collagenase was carried out in the presence of MalNEt according to Peterkofsky & Diegelmann (1971).

Results

Collagenase Assay. The assay used in the present study utilizes biosynthetically [^{14}C]proline-labeled type IV collagen instead of chemically ^{3}H-labeled type IV collagen previously used (Liotta et al., 1979a). Each preparation of the ^{14}C-labeled type IV collagen prepared from EHS sarcoma tissue was studied on polyacrylamide gels and shown to contain two pure type IV procollagen polypeptide chains, 185K and 170K molecular weight, as described elsewhere for type IV collagen synthesized by EHS tumor sarcoma (Tryggvason et al., 1980), parietal yolk sac (Clark & Kefalides, 1978; C. C. Clark and K. A. Kefalides, unpublished experiments), amniotic fluid cells (Crouch et al., 1980), cultured mammary gland ducts and alveoli (Liotta et al., 1979c), and cultured human placenta villi (J. M. Foidart, unpublished experiments). The optimal concentration of trichloroacetic acid/tannic acid for the precipitation of undegraded type IV collagen was studied on both native and denatured substrates. Native type IV substrate was all precipitable by 2.0% Cl_3CCOOH–0.1% TA whereas the denatured substrate was precipitated by 4.0% Cl_3CCOOH–0.2% TA. A concentration of 2.0% Cl_3CCOOH–0.1% TA was used in the standard assay. Trypsin (1 μg/mL, 2 h, 26 °C) degraded 14% of the substrate. No radioactive counts were released from the substrate when trypsin plus SBTI alone were used in the reaction, indicating that the SBTI concentration used during enzyme activation completely inhibited any proteolytic activity of the trypsin. Chymotrypsin released less than 10% of the substrate counts (Table I). Nonactivated enzyme ("type IV collagenolytic activity") had about 25% of the activity of the same amount of trypsin-activated enzyme. Under the standard conditions, the rate of reaction was linear until 55–60% of the substrate had been degraded, and in this range the activity was linearly related to enzyme concentration.

Purification of Type IV Collagenolytic Activity. It has been shown that many intracellular enzymes of collagen biosynthesis

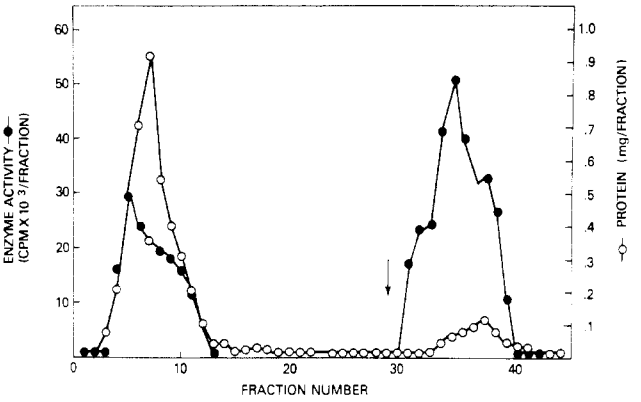

FIGURE 1: Chromatography of type IV collagenolytic activity on a 10-mL concanavalin A–agarose column. Serum-free media from 5-day cultures of PMT invasive tumor cells was precipitated with ammonium sulfate, and 20 mL of sample was applied with a flow rate of 0.5 column volume/h. The column was washed first with column buffer and then eluted in the same buffer containing 0.3 M methyl α-D-mannoside. Fractions of 2 mL were collected. Elution was initiated at the fraction indicated by the arrow.

Table II: Partial Purification of Type IV Collagenolytic Activity[a]

enzyme fraction	total protein (μg)	total act. (cpm)	sp act. (cpm/μg)	recovery	purification
crude	195200	75 000	0.3	1.00	1
25–50% $(NH_4)_2SO_4$	7440	65 250	8.7	0.87	23
concanavalin A bound fraction	265	25 100	94.7	0.33	240
molecular sieve	42	16 080	382.9	0.21	1007

[a] Activity is expressed in cpm of substrate degraded under digestion conditions with excess substrate such that there was a linear relationship between enzyme concentration and cpm present in the supernate. Protein concentration was measured by a modification of the method of Lowry et al. (1951) with bovine serum albumin as standard.

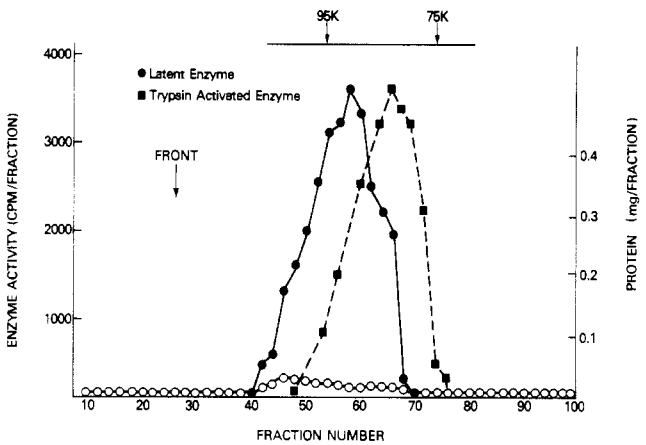

FIGURE 2: Gel filtration of type IV collagenolytic activity on a 1.6 × 95 cm Sephadex G-200 column at 4 °C with a flow rate of 0.1 mL/min. Fractions of 2 mL were collected. Standardization of the gel filtration column with globular proteins indicated that enzyme in the major peak was about 70 000–80 000 daltons.

Table III: Selective Digestion of Type IV Collagen in a Mixture of Labeled Collagens I, III, and IV by the Type IV Collagenolytic Activity[a]

	scan density units in collagen bands		
enzyme treatment	type IV	α1, α2 type I	α type III
none	171	339	194
type IV collagenolytic act.	5	330	186
bacterial collagenase	0	1	0

[a] Mixtures of collagens I, III, and IV labeled with [^{14}C]proline were incubated with activated enzyme for 6 h at 37 °C. The reaction products were subjected to polyacrylamide gel electrophoresis in $NaDodSO_4$. Fluorograms prepared from the gels were scanned with a densitometric scanner containing a peak integrator (Helena Lab.).

(Rhyanen, 1976; Anttinen et al., 1978; Risteli, 1978; Tryggvason et al., 1979) as well as procollagen *N*-peptidase and certain mammalian collagenases (Dr. J. J. Jeffrey, unpublished experiments) bind to concanavalin A–agarose columns, indicating their glycoprotein nature. It was therefore presumed that the enzyme studied here might have a similar nature. As shown in Figure 1, most of the tumor type IV collagenolytic activity became bound to the concanavalin A–agarose column and could be eluted with buffer containing 0.3 M methyl α-D-mannoside. The enzyme recovered from the column was concentrated by ammonium sulfate precipitation, redissolved in the enzyme buffer, and then passed over a molecular sieve column. The enzyme activity eluted from the column as a solitary peak (Figure 2). As shown in Table II, the purification procedure yielded almost 1000-fold purification over the crude media activity.

Properties of the Enzyme Protein. The molecular weight of the purified latent enzyme was about 70–80K as determined by gel filtration with globular proteins as standards. When the enzyme was run on the molecular sieve after trypsin activation, the molecular weight decreased to about 60K (Figure 4). The partially purified enzyme obtained from the molecular sieve was studied for its pH requirements as well as for its sensitivity to a variety of protease inhibitors. The enzyme activity was irreversibly abolished by boiling for 5 min or by exposure to acid pH. Maximum enzyme activity was present at neutral pH. The enzyme activity was reduced by 90% at pH 5.0. As shown in Table II, metal chelating agents abolished the activity, but inhibitors of thiol and serine proteases did not.

Substrate Specificity. To study the substrate specificity of the partially purified enzyme, its proteolytic activity on the other collagen types as well as noncollagenous proteins was tested. The enzyme cleaved both chains of type IV collagen but failed to significantly degrade collagens I and III (Table III). After digestion, the two chains of type IV collagen were equally degraded, giving rise to four fragments with molecular weights of 135K, 120K, 60K, and 50K (Figure 3). All of these fragments were attacked by bacterial collagenase, indicating their collagenous nature. The enzyme did not significantly degrade fibronectin under the same digestion conditions (Figure 4). Digestion of pepsinized placenta collagen containing two major chains (95K and 200K), kindly supplied by Dr. Glanville (Glanville et al., 1979), resulted in cleavage of the 95K component but not the 200K component (Figure 5). The 95K component and not the larger molecular weight component is reported to be a major portion of one of the chains of type IV collagen (Glanville et al., 1979).

Discussion

Heterogeneity among collagenases with respect to substrate specificity has been established. Horwitz et al. (1977) demonstrated that human leukocytes attacked collagen type I preferentially in comparison to type III collagen while enzyme from human fibroblasts and rabbit macrophages attacked the two substrates at equal rates. Woolley et al. (1978) as well as Liotta et al. (1979a) reported that human skin collagenase

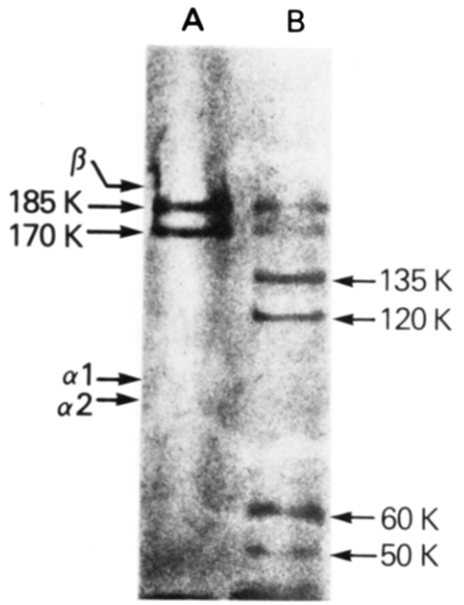

FIGURE 3: Polyacrylamide gel electrophoresis in $NaDodSO_4$ of reaction products of type IV collagen incubated with activated type IV collagenolytic protease at 27 °C for 24 h at pH 7.6 in the presence of 5 mM phenylmethanesulfonyl fluoride. (A) Control substrate; (B) reaction products. All reaction products required reduction with dithiothreitol to migrate completely in the gel. By densitometric scanning, 94% of the substrate was degraded. These products are different from 175K, 110K, 74K, 48K, and 20K digestion products of this substrate produced by pepsin (Tryggvason et al., 1980).

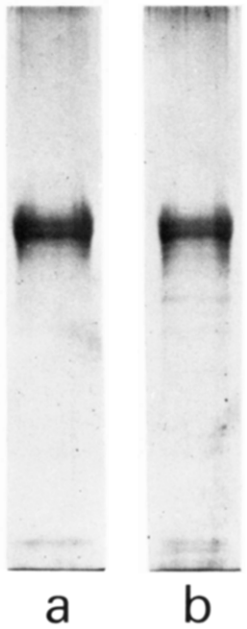

FIGURE 4: Polyacrylamide gel electrophoresis in $NaDodSO_4$ of fibronectin treated with the enzyme under conditions identical with those described for Figure 3: (a) control substrate; (b) undigested substrate after enzyme treatment. By densitometric scanning, the fibronectin bands in (b) are reduced by only 12% as compared to (a).

fails to degrade type IV collagen when types I, II, and III are readily cleaved. The present study demonstrates that the previously described type IV collagenolytic activity secreted by metastatic murine tumor cells selectively cleaves type IV collagen but not types I and III after partial purification of the enzyme activity.

The [^{14}C]proline-labeled biosynthetically prepared type IV collagen used in this study has several advantages over the sodium [^{3}H]borohydride-labeled type IV collagen used previously (Liotta et al., 1979a). The ^{14}C-labeled substrate is easy

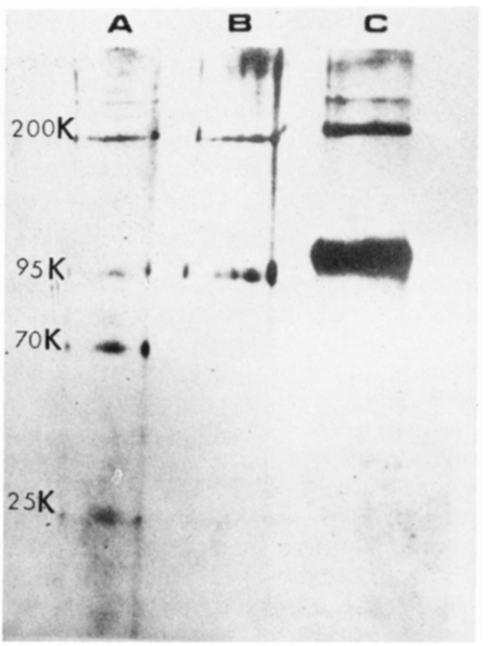

FIGURE 5: Polyacrylamide gel electrophoresis at acid pH (Nagai et al., 1964) of reaction products of placenta collagen (provided by Dr. Glanville) incubated with activated enzyme in the presence of 5 mM PMSF at 26 °C, pH 7.6, for 36 h. The native α-size (95K) placenta type IV collagen chain is selectively cleaved with 70K and 25K cleavage products produced. The β-sized placenta collagen (200K) is not of type IV origin. (A) Placenta collagen plus enzyme; (B) placenta collagen control; (C) type I collagen standard.

to prepare in EHS tumor cultures. It contains only two high molecular weight type IV collagen chains synthesized by various basement membrane tissues (Crouch et al., 1980; Liotta et al., 1979; Tryggvason et al., 1980) while the previous substrate which was extracted from lathyritic EHS tumor prior to labeling contained cross-linked components of the two chains which caused difficulties in interpretation of results. Furthermore, the labeling procedure with sodium [^{3}H]borohydride may cause some degradation of the protein; the specific activity of the material varies, and the substrate cannot be used for more than 3–5 months following labeling since the assay background increases with time due to migration of the tritium to the surrounding phase.

After digestion of the substrate with tumor type IV collagenolytic activity, the 185K- and 170K-dalton chains [pro α1 (IV) and pro α2 (IV)] were degraded to four fragments which is consistent with a single cleavage site in both chains. The large fragment of each chain is two-thirds the size of the original chain. Kinetic digestion studies and staphylococcal V8 protease mapping of the fragments cut from the dried gels (data not shown) indicated that the two low molecular weight fragments (60K and 50K) are derived from separate chains and therefore analogous to the TC^{B} fragments of type I collagen produced by mammalian collagenase. These fragments were all attacked by bacterial collagenase, indicating that they have a major collagenous composition and, consequently, the enzyme is not merely cleaving off noncollagenous extensions of the type IV procollagen molecule. Further evidence in favor of a major cleavage site within the collagenous portion of the molecule was shown by cleavage of pepsinized type IV collagen from human placenta. Thus, the enzyme probably has a cleavage site within a major pepsin-resistant fragment of the molecule.

The type IV collagenolytic activity was purified about 1000-fold and partially characterized. The apparent high

purification relative to the crude media was due in part to the high protein content of the crude media. Molecular sieve column chromatography of the latent enzyme resulted in a single peak with a molecular weight of aproximately 70–80K. After trypsin activation, the molecular weight was reduced by about 10K. This is similar to molecular weights of several other mammalian collagenases (Birkedal et al., 1976; Stricklin et al., 1977; Gillet et al., 1977). Similar to other collagenases, its activity was inhibited by EDTA, indicating a requirement for divalent metal ions. The effect of pH on enzyme activity indicated maximum activity at neutral pH with no activity in the range that acid cathepsins are active.

The present results support the existence of a collagenase activity which preferentially recognizes type IV collagen over the other collagen types. The molecular weight of this enzyme and its susceptibility to inhibitors make it distinct from mast cell protease (Crouch et al., 1980) and other serine proteases. The size and the fragments produced from type IV collagen by the enzyme are different from those produced by pepsin or chymotrypsin. The cleavage products are consistent with a single cleavage site in each pro type IV collagen chain [pro α1 (IV) and pro α2 (IV)] and in the placenta 95K chain. It has been deduced that the 95K chain of placenta type IV collagen is derived from the pro α1 (IV) chain (Crouch et al., 1980). If it is assumed that the cleavage site in both components is the same, then the position of the 95K chain along the length of the pro α1 (IV) can be evaluated. The 95K chain, therefore, may exist in the middle region of the pro α1 (IV) molecule.

The identification of the present enzyme suggests that there may exist a physiologically significant class of metalloenzymes with different substrate specificities for each collagen type. Metalloenzymes selective for type V collagen (αA, αB) have also been identified (Liotta et al., 1980b). Type IV collagen degrading activity has been detected in a series of metastatic tumors, in cultured endothelial cells, in leukocytes, and in involuting mammary epithelium (Liotta et al., 1979c, 1980a). An enzyme or enzymes similar to the one described in the present report may participate in basement membrane turnover in a variety of normal as well as pathological conditions.

Added in Proof

Mainardi et al. (1980) recently reported digestion of type IV collagen by leukocyte elastase.

Acknowledgments

We thank Dr. George R. Martin for his many helpful suggestions. We are grateful for the excellent technical assistance of Karen Kniska.

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