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ACTIVATION OF FIBROBLAST PROCOLLAGENASE BY MAST CELL PROTEASES

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Summary

Proteases capable of activating procollagenase from gingiva and from fibroblast and macrophage monolayer cultures were harvested from homogenates of canine tumor mast cells. The mast cell proteases lysed casein and Azocoll but not native collagen. In low salt concentrations the enzymes existed as high molecular weight complexes, which were dissociated by increasing the salt concentration above 1.0 M (NaCl, KCl). Gel filtration in 1.4 M KCl separated the protease activity into three peaks, all of which activated procollagenase. Two of the enzymes showed substrate specificities (hydrolysis of *p*-tosyl-L-arginine methyl ester and benzoyl-tyrosine ethyl ester) and reactive center reactivities similar to pancreatic trypsin and chymotrypsin. Based on gel filtration, apparent molecular weights of 160 000 (*p*-tosyl-L-arginine methyl ester esterase), 90 000 (main procollagenase activator) and 36 000 benzoyl-tyrosine ethyl ester esterase) were determined. Activation of procollagenase resulted in a 18—20 000 decrease of the molecular weight. The activation was directly related to the amount of activator added within certain limits. Further addition of activator resulted in proteolytic inactivation of collagenase.

Introduction

Collagenases (EC 3.4.24.3) are enzymes capable of cleaving the helical portion of native collagen molecules in a characteristic manner. A series of studies have shown that animal collagenase may exist in a latent form [1—6]. More-

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over, data have recently been presented which indicate that collagenase is latent at the time of release from the cells and that the enzyme is synthesized or stored intracellularly as an inactive precursor, procollagenase [7]. Most of these procollagenases are activated by trypsin.

Proteases resembling pancreatic trypsin and chymotrypsin have been demonstrated in granules of normal and tumor mast cells from a number of species [8—10]. These enzymes have optimal proteolytic activity at neutral or slightly alkaline pH and hydrolyze certain ester substrates also hydrolyzed by trypsin and chymotrypsin. Moreover, a synergy between collagenolysis of rat mesenterium and mast cell products was recently revealed [11,12]. Consequently, the question arose whether mast cell proteases may participate in the activation of procollagenase and in this way serve as a factor in the local regulation of collagen breakdown. The present study shows that proteases harvested from canine tumor mast cells indeed activate procollagenase from gingival, fibroblast and marcrophage cultures.

Material and Methods

Preparation of mast cell proteases

A mastocytoma was excised from a dog submitted for euthanasia during the terminal stage of the disease. Tyrode solution was repeatedly forced through the tumor tissue with needle and syringe yielding a suspension of 6.3×10^8 cells. The remaining tissue was minced with scissors and stirred in Tyrode solution at 4°C for 30 min. The supernatant containing $7.9 \cdot 10^8$ cells was harvested after sedimentation of the tissue. Toluidine blue and hematoxylin-eosin-stained smears revealed that more than 95% of the cells were mast cells. The majority of these were immature and only 5—10% showed distinct metachromasia. Part of the cell suspension was used for cell cultures and maintained and subcultured for month as previously described [13]. Approx. 10^9 cells were used for preparation of proteases. Cell fractionation procedures were monitored by phase contrast microscopy. The cells were suspended in Tyrode solution and disrupted by sonication for 3 min at room temperature. Unbroken cells and nuclei were removed by centrifugation at $480 \times g$ for 10 min. The supernatant was then frozen and thawed in 10 cycles and centrifuged at $48\,000 \times g$ for 20 min to remove unbroken granules and membranes. The supernatant ("granule fraction") was dialyzed against 0.2 M NaCl in Tris/$CaCl_2$ buffer (0.03 M Tris/HCl, 5 mM $CaCl_2$, pH 7.4) and stored frozen. A total of 19.8 mg protein was obtained in this fraction. The pellets from the first and second centrifugation were extracted overnight at 4°C with 1.4 M KCl in Tris/$CaCl_2$ buffer and stored frozen.

Protease activity was also extracted directly from the residual tumor mass. The tissue was resuspended in 200 ml 1.4 M KCl in Tris/$CaCl_2$ buffer and homogenized in an ice-cooled Polytron homogenizer for 3 periods of 0.5 min each. The homogenate was then extracted twice for 30 min with the same buffer and the supernatants ($48\,000 \times g$, 20 min) were combined and dialyzed against 0.2 M NaCl in Tris/$CaCl_2$ buffer. To remove the heparin and nucleic acids present in these salt extracts, protamine sulfate, 10 mg/ml, (Sigma) was added slowly and dropwise during stirring. The heavy white precipitate which

formed immediately upon addition of protamine sulfate was harvested by centrifugation and this procedure was repeated until no more precipitate formed. The supernatant was then dialyzed against 0.2 M NaCl in Tris/$CaCl_2$ buffer, and stored frozen.

Preparation of collagenase

Procollagenase and collagenase were prepared from cultures of whole gingiva, of gingival fibroblasts, and of alveolar macrophages as detailed elsewhere [7,14, 15]. The culture media were harvested every second day and either dialyzed against distilled water and lyophilized or fractionated by ammonium sulfate precipitation [16]. All preparations were finally dialyzed against Tris/$CaCl_2$ buffer containing 0.2 M NaCl and stored frozen.

The media protein from the cell and tissue cultures usually contained both latent and manifest collagenase activity [7]. Samples used in the present study either contained only latent or only active collagenase when harvested from the cultures, or were prepared from samples containing both. Procollagenase was prepared from such mixtures by incubation with purified bovine α_2-macroglobulin followed by chromatographic separation of the latent collagenase from the collagenase-α_2-macroglobulin complex [7]. Manifest collagenase was prepared by gel filtration of L-tosylamide-2-phenylethylchloromethyl ketone (Tos-PheCH_2Cl)-trypsin-activated media protein (see below).

Preparation of α_2-macroglobulin

Bovine α_2-macroglobulin was prepared from whole serum by ammonium sulfate fractionation, ion-exchange chromatography and gel filtration as previously described [17]. When tested on Azocoll, 1 mg α_2-macroglobulin from this preparation inhibited 22 μg Tos-PheCH_2Cl-trypsin (Worthington).

Assay procedures

Collagenase activity was assayed essentially as described by Robertson et al. [18]. [^{14}C]Glycine-labelled collagen produced by chick calvaria in culture [19] was mixed with acid-soluble, rat-skin collagen [20] at a concentration of 5 mg/ml. This mixture was dialyzed against 0.2 M NaCl in Tris/$CaCl_2$ buffer in the cold and gelled at 37°C in 200-μl aliquots. Assay mixtures were made up to a total volume of 500 μl with the same buffer and incubated at 36.5°C for from 2—20 h depending on the level of activity. In some instances 100 μg bovine serum albumin were added as a protective protein, since collagenase in dilute solutions showed a marked affinity to glass surfaces. The assay was terminated by filtering the reaction mixtures through Unichem filter samplers (Scientific Products) and the release of labelled, soluble peptides was measured in a liquid scintillation counter. One unit of collagenase was defined as the activity which solubilized 1 μg collagen gel per min at 36.5°C. Controls included substrate gels incubated with buffer or clostridial collagenase under identical conditions. Procollagenase was assayed as previously described [7] after activation with Tos-PheCH_2Cl-trypsin for 10 min at room temperature followed by addition of a 10—15 fold molar excess of soybean trypsin inhibitor. One unit of procollagenase was defined as the amount of zymogen which gave rise to one unit of collagenase when activated with trypsin.

The content of procollagenase activator was measured by preincubating the latent enzymes with aliquots of the mast cell preparations for from 1 to 8 h and measuring resultant collagenase activity in radioassay. Controls included procollagenase preincubated for the same period of time with buffer.

Esterase activity was determined by a modification of the method described by Hummel [21] using *p*-tosyl-L-arginine methyl ester (Tos-Arg-OMe) and benzoyl-tyrosine ethyl ester (Bz-Tyr-OEt) as substrates. Incubation was with 1 mM substrate solutions at pH 7.4 and 22°C in Tris/$CaCl_2$ buffer containing 0.2 M NaCl and in the case of Bz-Tyr-OEt also containing 23% w/w methanol. Hydrolysis of the substrates was monitored spectrophotometrically at 247 and 256 nm. One unit was defined as the esterase activity which hydrolyzed 1 μmol substrate per min at pH 7.4 and 22°C.

Neutral protease activity was measured at pH 7.4 using Azocoll and casein as substrates. Azocoll was dispensed at 5 mg/ml in 0.2 M NaCl in Tris/$CaCl_2$ buffer. Assay mixtures consisted of 500 μl substrate and 200 μl enzyme solution. Incubation was at 36.5°C for from 1 to 6 h depending on the level of activity. Controls included substrate incubated with buffer under identical condition. The assay was terminated by addition of 3.0 ml buffer and filtering the reaction mixture through Unichem filter samplers. Hydrolytic release of soluble peptides was determined spectrophotometrically at 528 nm. Substrate for assay of caseinolytic activity was prepared from crude technical casein (Sigma). This product was dissolved at 10 mg/ml in 0.2 M NaCl in Tris/$CaCl_2$ buffer and precipitated by addition of 2 vol. of 5% trichloroacetic acid. The precipitate was harvested by centrifugation and dissolved in, and dialyzed against, distilled water and lyophilized. The dry powder was dissolved at 60°C in Tris/$CaCl_2$ buffer containing 0.2 M NaCl at a concentration of 10 mg/ml and pH was adjusted to 7.4 with 1.0 M NaOH. Assay mixtures consisted of 500 μl substrate and 200 μl enzyme solution. Incubation was at 36.5°C for from 1 to 6 h. Release of trichloroacetic acid-soluble peptides was measured either directly at 280 nm or at 520 nm after reaction with ninhydrin in a Technicon autoanalyzer. One unit of protease activity hydrolyzed 1 mg casein per h to trichloroacetic acid-soluble peptides.

Protein concentration was determined by the method of Lowry et al. [22] using bovine serum albumin as a standard and carbazole assay for uronic acid [23] was employed for detection of heparin.

Chromatography

Gel filtration was performed on Sephadex G-100 (1.6 × 92 cm) and Sephadex G-150 (1.6 × 94 cm) columns equilibrated with 0.2 M NaCl or 1.4 M KCl in Tris/$CaCl_2$ buffer. The columns were eluted at 10—20 ml/h at room temperature and 3—5-ml fractions were collected at 4°C and assayed for enzyme and activator activities as described. The columns were calibrated for molecular weight determination using blue dextran, aldolase (158 000), bovine serum albumin (69 000), ovalbumin (45 000), chymotrysinogen (25 000) and ribonuclease (13 700) as standards. Molecular weights of mast cell components and collagenases were determined from activity elution curves based on the method of Andrews [24].

Ion-exchange chromatography was performed on a 0.4 × 8 cm column of

DEAE-cellulose equilibrated with 0.025 M NaCl in Tris/$CaCl_2$ buffer. Elution was at 10 ml/h with a 50 ml linear salt gradient from 0.025 NaCl to 1.4 M KCl in the same buffer. Fractions of 1—2 ml were collected in the cold and assayed for collagenase activator, protease and esterase activities.

Inhibition of mast cell proteases

The effect of enzyme inhibitors on the mast cell enzymes was tested using the assay procedures described. The inhibitors were preincubated with the mast cell preparations for 4—6 h and resultant activity was measured and compared to uninhibited controls. Synthetic inhibitors included the Dip-F substitute, phenylmethylsulfonylfluoride (Pms—F), *N*-tosyl-L-lysine chloromethyl ketone (Tos-LysCH_2Cl), Tos-PheCH_2Cl, benzamidine/HCl, 6-aminohexanoic acid, EDTA and 1,10-phenanthroline. Protein protease inhibitors were soybean trypsin inhibitor, α_1-antitrypsin (Worthington) and α_2-macroglobulin prepared as described.

Results

Homogenates of mast cells lysed casein, Azocoll and the ester substrates Tos-Arg-OMe and Bz-Tyr-OEt at pH 7.4. All of these activities were confined essentially to the granule fraction of the cell preparations (Table I).

Neither this fraction nor the crude homogenate showed lytic activity on native collagen fibrils, but when incubated with procollagenase from different sources, it caused the activation of these latent enzymes (Table II, Fig. 1). The mast cell proteases were also harvested from salt extracts of the minced tumor tissue. Such extracts, however, contained considerable amounts of heparin and nucleic acids. These were largely removed by precipitation with the basic polypeptide protamine, but part of the esterase and protease activities coprecipitated with the polyanions (Table III).

Activation of fibroblast procollagenase by mast cell proteases was accompanied by a 19 000 decrease of the molecular weight (Fig. 2). Two samples of fibroblast procollagenase were chromatographed on a Sephadex G-150 column with and without prior incubation with the mast cell protease preparation. The unactivated sample eluted as entirely latent collagenase with an apparent mo-

TABLE I

DISTRIBUTION OF ESTERASE ACTIVITIES IN MAST CELL FRACTIONS

Approx. 10^9 tumor mast cells were disrupted by sonication and centrifuged at 480 × *g*. The supernatant containing the granules was frozen and thawed and centrifuged at 48 000 × *g*. The pellet fractions were redispersed and assayed for esterase activities together with the supernatants and freshly prepared solutions of trypsin (Worthington, TRTPCK) and α-chymotrypsin (Worthington, CDI).

Fraction	Tos-Arg-OMe esterase (units)	Bz-Tyr-OEt esterase (units)
Pellet, 480 × *g*	4.9	1.2
Supernatant, 480 × *g*	15.5	3.4
Pellet, 48 000 × *g*	0.3	0.1
Supernatant, 48 000 × *g*	11.8	2.6
Trypsin, 1 mg	68.0	—
α-Chymotrypsin, 1 mg	—	18.8

TABLE II

ACTIVATION OF GINGIVAL AND MACROPHAGE COLLAGENASE BY MAST CELL PROTEASES

Aliquots of latent macrophage (0.7 unit) and gingival collagenase (0.5 unit) were preincubated at room temperature with mast cell granule fraction (MGF) for 1 h or with trypsin for 10 min. Trypsin activation was stopped with soybean inhibitor (30 μg), while no attempt was made to stop the mast cell granule fraction activation. Resultant collagenase activity was measured in radioassay. Total substrate: 1995 cpm.

Collagenase preparation	Activator	Collagenase activity (^{14}C cpm)
Macrophage	None	20
	Trypsin, 2 μg	1275
	MGF, 52 μg	751
Gingival	None	6
	Trypsin, 2 μg	1035
	MGF, 52 μg	882
None	MGF, 52 μg	60

lecular weight of 79 000, while the activated enzyme eluted at a slightly larger volume corresponding to a molecular weight of 60 000.

Fractionation of the granule extract by gel filtration in salt concentrations of 0.2 M NaCl or less revealed that all of the procollagenase activator, protease and esterase activities existed in the form of high molecular weight complexes which were excluded on the Sephadex G-150 column (Fig. 3). Chromatography at salt concentrations above 1.0 M, however, dissociated these aggregates (Fig. 4). The esterase activity was separated into two peaks corresponding to molecular weights of 160 000 (Tos-Arg-OMe esterase) and 36 000 (Bz-Tyr-OEt esterase).

Protease activity eluted as one major and two minor peaks with identical profiles on casein and on Azocoll. The major peak (III) cochromatographed with the Bz-Tyr-OEt esterase and one of the minor peaks (I) with the Tos-Arg-

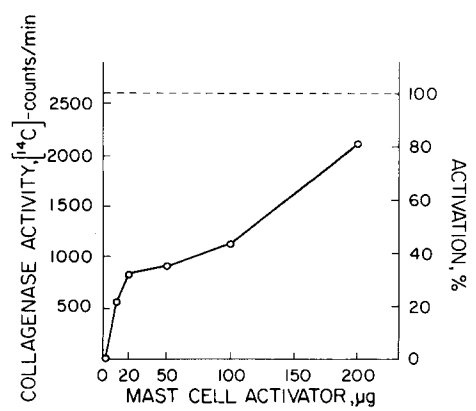

Fig. 1. Activation of procollagenase with mast cell proteases. Aliquots of 0.7 units of gingival procollagenase were incubated for 1 h at 37°C with increasing amounts of the mast cell granule fraction. Resultant collagenase activity (o———o) was measured in radioassay. The activation obtained with trypsin was defined as 100% (- - - - -). Total counts of substrate: 2930 cpm.

TABLE III

FRACTIONAL PRECIPITATION OF TUMOR SALT EXTRACT WITH PROTAMINE SULFATE

Tumor salt extract corresponding to approx. 85 units of caseinolytic activity was dialyzed against 0.2 M NaCl in Tris/$CaCl_2$ buffer and cleared by centrifugation (crude extract). Protamine sulfate was added in drops to the supernatant until no more precipitate formed. After renewed centrifugation the supernatant was decanted (protamine supernatant) and the pellet redissolved in 1.4 M KCl in Tris/$CaCl_2$ buffer (protamine pellet). Each fraction was assayed for protease and esterase activity as described in Materials and Methods.

Fraction	Enzyme activity (units)		
	Tos-Arg-OMe	Bz-Tyr-OEt	Casein
Crude extract	6.2	3.4	83.4
Protamine pellet	0.8	0.5	26.0
Protamine supernatant	8.4	2.4	88.4

OMe esterase. Procollagenase activator eluted over a broad area with minor peaks corresponding to the esterases. The major activator peak, however, eluted between the esterases (II) at the same position as the third and minor protease peak (Fig. 4, arrow) in the molecular weight range of 80—100 000. From this chromatogram, fractions corresponding to the three protease peaks (I, II and III) were isolated. Each of these preparations were capable of activating fibroblast procollagenase (Fig. 5). The activation was dose dependent (Figs. 1 and 5)

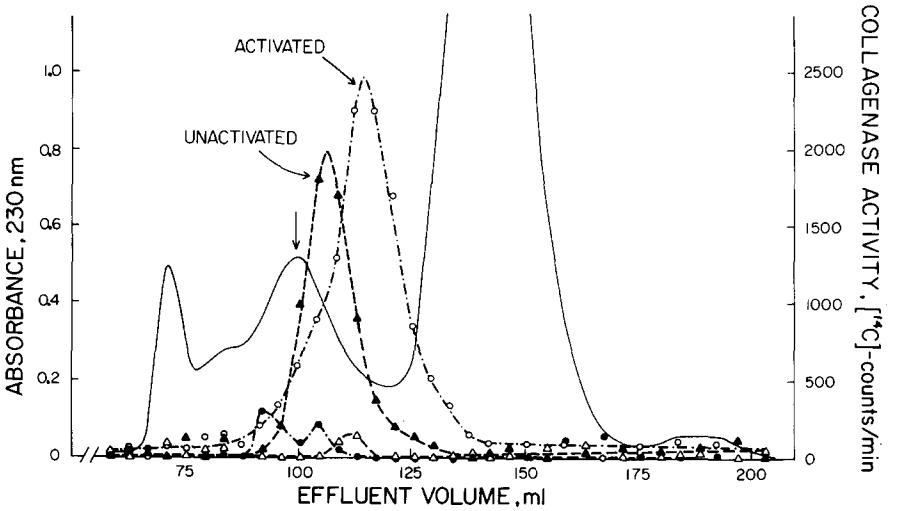

Fig. 2. Gel filtration of activated and unactivated procollagenase. A sample containing 400 units of fibroblast procollagenase was divided into two halves. One half was incubated for 2 h at 37°C with 60 μg mast cell protein (1.5 units of caseinolytic activity) prepared by salt extraction and protamine sulfate precipitation. After addition of 10 mg soybean inhibitor the sample was chromatographed on a 1.6 × 94 cm column of Sephadex G-150. Elution was at 20 ml/h with 0.2 M NaCl in Tris/$CaCl_2$ buffer. Elution of ultraviolet-absorbing material was monitored at 230 nm (———) and fractions of 4 ml were collected and assayed for latent (●— · —●) and manifest (○— · —○) collagenase activity. The control sample was preincubated with buffer, chromatographed on the same column and eluate fractions were assayed for latent (▲- - - - -▲) and manifest (△- - - - -△) collagenase activity. The protein peak eluting ahead of the collagenase activity (arrow) and the soybean inhibitor served as internal controls for the comparative molecular weight determination. Total counts of substrate: 2930 cpm.

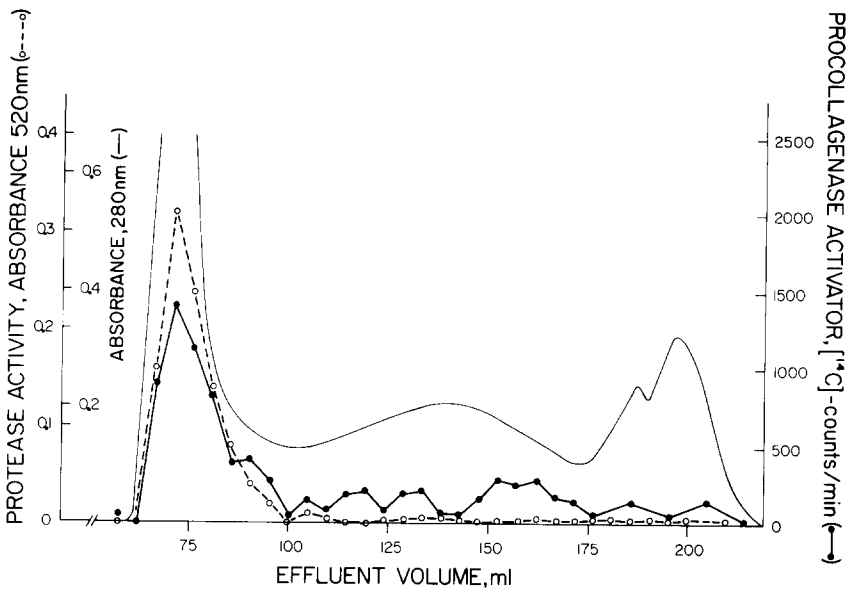

Fig. 3. Gel filtration of mast cell proteases in 0.2 M NaCl. The mast cell granule fraction was dialyzed against 0.2 M NaCl in Tris/$CaCl_2$ buffer and chromatographed on a 1.6 × 94 cm column of Sephadex G-150 in the same buffer. Elution was at 18 ml/h and fractions of 5 ml were collected in the cold and assayed for procollagenase activator (•———•) using fibroblast procollagenase as substrate and protease activity (o- - - - - -o) using casein as substrate. Elution of ultraviolet-absorbing material was monitored at 280 nm (———). Total counts of substrate: 2130 cpm.

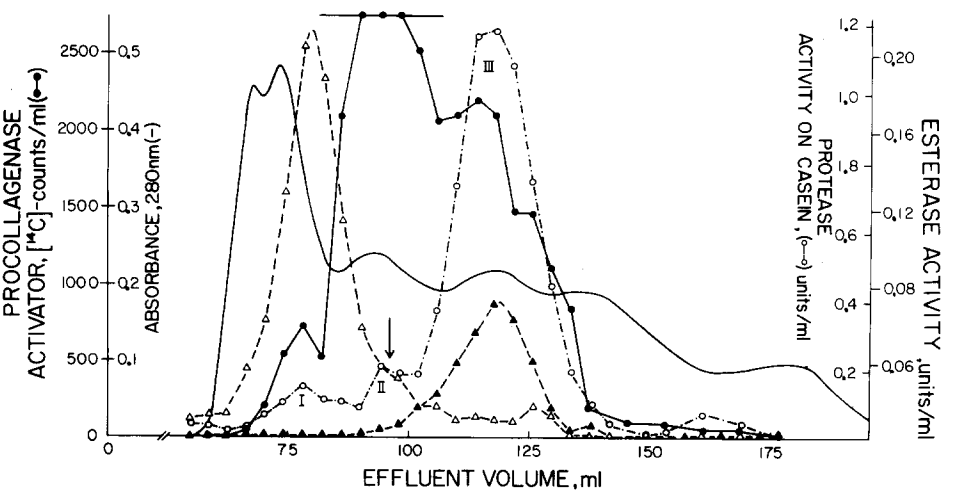

Fig. 4. Gel filtration of mast cell proteases in 1.4 M KCl. The mast cell granule fraction was dialyzed against 1.4 M KCl in Tris/$CaCl_2$ buffer and chromatographed on a 1.6 × 92 cm column of Sephadex G-100 in the same buffer. Elution was at 20 ml/h and fractions of 4 ml were collected in the cold and assayed for procollagenase activator (•———•) using fibroblast procollagenase as substrate, protease activity (o—·—o) using casein as substrate and esterase activity using Tos-Arg-OMe (Δ- - - - -Δ) and Bz-Tyr-OEt (▲- - - - -▲) as substrates. Elution of ultraviolet-absorbing material was monitored at 280 nm (———). Total counts of substrate: 2740 cpm.

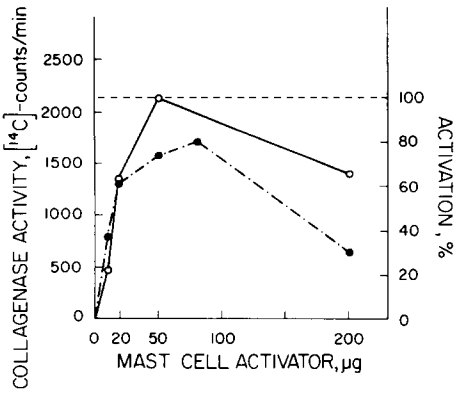

Fig. 5. Activation of procollagenase with mast cell esterases. Aliquots of 2 units of fibroblast procollagenase were incubated for 4 h at 37° C with increasing amounts of the Tos-Arg-OMe (o———o) and Bz-Tyr-OEt (•— · —•) esterases from peaks I and III of Fig. 4. Resultant collagenase activity was measured in radioassay. The activation obtained with trypsin was defined as 100% (- - - - -). Total counts of substrate: 2740 cpm.

and increased with increasing amount of activator added, until maximally the same level was reached as with trypsin. Further addition of activator resulted in a decrease of collagenase activity.

The collagenase activator from peak II of Fig. 4 was further purified by DEAE-cellulose chromatography in Tris/$CaCl_2$ buffer at pH 7.4. During dialysis against the start buffer containing 0.025 M NaCl a notable precipitate formed, which when redissolved in 1.4 M KCl in Tris/$CaCl_2$ buffer proved to contain part of the esterase activity. The major part of the protein which remained in solution, including the procollagenase activator, esterase and protease activities, were absorbed on the column and eluted with a steep, linear salt gradient from 0.025 M to 1.4 M KCl. The activator eluted as a double peak in the range from 0.2 to 0.6 M KCl together with the major part of the proteases and esterases (Fig. 6). A minor peak of activator eluted at an even higher salt concentration (0.8 M). As judged from the carbazole assay, none of these fractions contained heparin.

Studies on the inhibition of protease and esterase activities are summarized in Table IV. The Tos-Arg-OMe esterase was inhibited by Tos-LysCH_2Cl and the Bz-Tyr-OEt esterase by Tos-PheCH_2Cl. Both were inhibited by Pms-F. The protein protease inhibitors had little effect when tested on the ester substrates. Soybean trypsin inhibitor, however, was partly effective towards the Bz-Tyr-OEt esterase but not at all towards the Tos-Arg-OMe esterase. In accordance with its behavior towards other neutral proteases [25], α_2-macroglobulin inhibited proteolytic activity on high molecular weight substrates, but not esterase activity on the low molecular substrates. Both of the esterases exhibited metal requirement as indicated by the complete inhibition by EDTA and by 1,10 phenanthroline. The study of the effect of inhibitors on the procollagenase activator met with the problem that several of these compounds also inhibit collagenase activity (EDTA, 1,10 phenanthroline, α_2-macroglobulin). The collagenase activator harvested from the peak II of Fig. 4, however, clearly lost its ability to activate procollagenase when incubated with Pms-F (Fig. 7) which

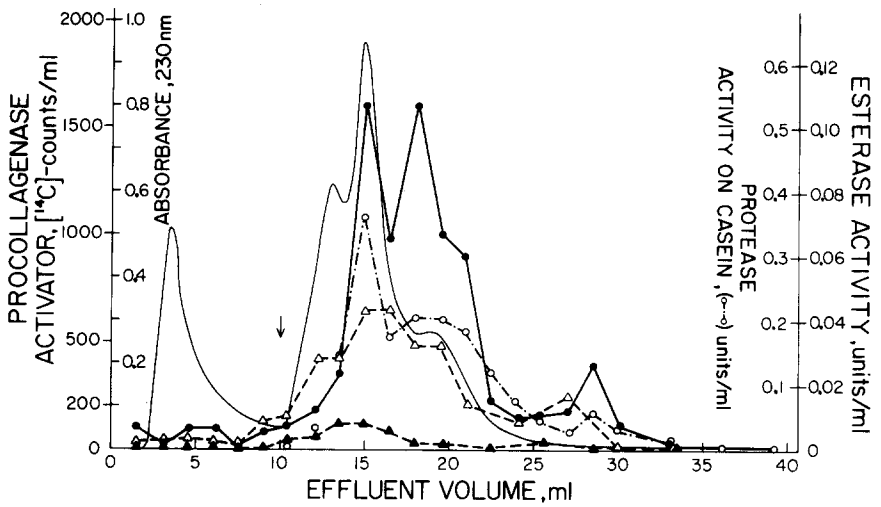

Fig. 6. DEAE-cellulose chromatography of procollagenase activator. The procollagenase activator from peak II of fig. 4 was dialyzed against 0.025 M NaCl in Tris/$CaCl_2$ buffer and applied to a 0.4 × 8 cm column of DEAE-cellulose equilibrated with the same buffer. Absorbed material was eluted at 10 ml/h with a 50 ml linear salt gradient from 0.025 M NaCl (arrow) to 1.4 M KCl in Tris/$CaCl_2$ buffer. Fractions of 1.5 ml were collected in the cold and assayed for procollagenase activator (●———●) using fibroblast procollagenase as substrate, protease activity (○— · —○) using casein as substrate and esterase activity using Tos-Arg-OMe (△- - - - -△) and Bz-Tyr-OEt (▲- - - - -▲) as substrates. Elution of ultraviolet-absorbing material was monitored at 230 nm. Total counts of collagenase substrate: 2145 cpm.

does not inhibit active collagenase (Birkedal-Hansen, H., Taylor, R.E. and Fullmer, H.M., unpublished). Moreover, partial inhibition (20–50%) of the Bz-Tyr-OEt esterase on procollagenase was achieved with soybean trypsin inhibitor.

In crude preparation the procollagenase activating activity was relatively heat stable. It was inactivated by heating to 90°C for 10 min but not by heating to 60°C for 30 min (Table V). However, after gel filtration, half of the activity

TABLE IV

INHIBITION OF MAST CELL PROTEASES

Aliquots of the mast cell granule fraction were incubated with inhibitors for 4 h (Azocoll) or 6 h (ester substrates) and resultant activity was determined as described in Materials and Methods.

Inhibitor	Concentration	Percentage inhibition		
		Substrate: Tos-Arg-OMe	Bz-Tyr-OEt	Azocoll
Tos-PheCH_2Cl	10^{-3} M	16	69	65
Tos-LysCH_2Cl	10^{-3} M	91	7	22
Pms-F	$5 \cdot 10^{-3}$ M	81	92	90
6-Aminohexanoic Acid	10^{-1} M	4	0	—
Benzamidine/HCl	10^{-2} M	100	52	39
α_2-Macroglobulin	800 μg/ml	0	0	79
α_1-Antitrypsin	400 μg/ml	0	4	45
Soybean inhibitor	400 μg/ml	0	66	54
EDTA	10^{-2} M	82	84	51
1,10-Phenanthroline	$5 \cdot 10^{-3}$ M	100	100	40

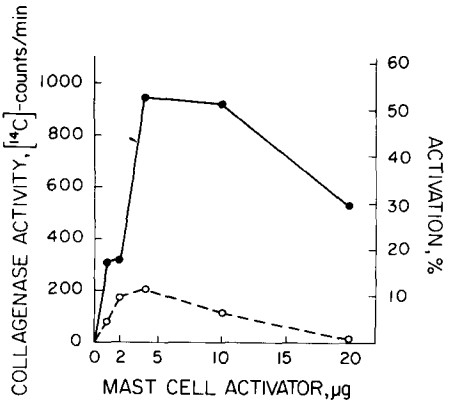

Fig. 7. Inhibition of procollagenase activator from mast cells. Procollagenase activator from peak II of fig. 4 was incubated for 4 h at room temperature with a 5×10^{-3} M solution of Pms-F or with 0.2 M NaCl in Tris/$CaCl_2$ buffer. Inhibition was measured by incubating aliquots of fibroblast procollagenase with increasing amounts of Pms-F-treated (o- - - - -o) or untreated activator (●———●) and measuring resultant collagenase activity in radioassay. The activation obtained with trypsin was defined as 100%. Total counts of substrate: 2145 cpm.

(peak II) was lost at 60°C (30 min). Following further purification by DEAE-cellulose chromatography, the procollagenase activator, protease and esterase activities were unstable and lost within a few days when stored in the cold.

Since earlier results have indicated that mast cell proteases may occur in a complex with heparin, the effect of this and two other biological polyanions (DNA and RNA) on procollagenase was investigated. In concentrations from 1 μg to 1 mg/ml none of these substances activated procollagenase.

Previous reports of a synergy between collagenase activity and mast cell factors have emphasized the ability of mast cell products to prevent inhibition of collagenase by serum α_2-macroglobulin [11,12]. It was thus studied whether the present mast cell proteases had a similar effect. Table VI shows that the in-

TABLE V

HEAT INACTIVATION OF PROCOLLAGENASE ACTIVATOR FROM CANINE MAST CELLS

Aliquots of one unit of fibroblast procollagenase were preincubated for 1 h at room temperature with control and heat treated (60°, 30 min or 90°C, 10 min) mast cell preparations. These were either crude mast cell granule fraction (MGF) or material purified by gel filtration (peak II of Fig. 4). Resultant collagenase activity of test and control samples was measured in radioassay. Trypsin activation was stopped with 20 μg soybean trypsin inhibitor. Total counts of substrate: 2532 cpm.

Activator	Pretreatment of activator	Collagenase activity (^{14}C cpm)
None		16
Trypsin, 2 μg		2159
Crude MGF, 200 μg	None	1240
	60°C, 30 min	1301
	90°C, 10 min	22
Purified MGF, 20 μg	None	1713
	60°C, 30 min	914
	90°C, 10 min	217

TABLE VI

INACTIVATION OF α_2-MACROGLOBULIN BY MAST CELL PROTEASES

Aliquots of one unit of active bovine gingival collagenase were incubated for 1 h at room temperature with purified bovine α_2-macroglobulin or with α_2-macroglobulin inactivated with crude mast cell granule fraction (MGF). Resultant collagenase activity was measured in radioassay. Total counts of substrate: 2130 cpm.

Sample	Collagenase activity (^{14}C cpm)	% activity
Collagenase + Buffer	1394	100
+ α_2-macroglobulin (30 μg)	554	40
+ α_2-macroglobulin (30 μg) preincubated with MGF (60 μg)	1448	106

hibitory effect of α_2-macroglobulin towards collagenase was lost when the globulin was preincubated with the granule extract. Saturation of α_2-macroglobulin with one protease preventing the subsequent binding of others has previously been found in similar systems [25].

Discussion

The data show that mast cell proteases are capable of activating procollagenase in a manner which resembles in certain respects the activation of gingival and fibroblast procollagenase with trypsin [7,14]. Like trypsin, the mast cell proteases lowered the molecular weight of collagenase by approx. 15—20 000. On the other hand, the activated enzyme proved to be a much better substrate for mast cell protesaes than for trypsin [7] as judged from the progressive loss or collagenase activity with increasing amounts of activator added.

The data suggest that besides the Tos-Arg-OMe and Bz-Tyr-OEt esterase even a third proteolytic activator was present in the homogenates of canine mastocytoma cells. This study, however, did not permit a detailed characterization of the third activator. The esterases resemble the trypsin and chymotrypsin like enzymes described in mast cells from other species [8,10]. While the Bz-Tyr-OEt esterase is common to mast cells of all species studied, the Tos-Arg-OMe esterase is of a more restricted distribution [8].

The proteases existed in the preparations as high molecular weight complexes. Their dissociation in elevated salt concentrations is suggestive of ionic enzyme-heparin complexes [9,10]. A number of studies have shown that such complexes represent the in vivo storage of the enzymes [9,26,27] and not preparational artefacts. It has been reported that mouse bone collagenase activity is enhanced by heparin [28]. However, it is unlikely that the activation of procollagenase in this study is caused by heparin, directly or indirectly. Heparin as well as other biological polyanions did not activate procollagenase when tested over a wide range of concentrations. Moreover, protease and activator activities were retained after successive purification steps which eliminated heparin from the preparations.

There is accumulating evidence that a series of proteases may activate procollagenase [3,29]. Those identified include the pancreatic enzymes trypsin,

chymotrypsin and elastase, the serum proteases plasmin and kallikrein and the tissue protease cathepsin B.

The present study adds to this list the neutral proteases of mast cells. That activation may be achieved by any of a multitude of enzymes with different substrate specificities would suggest either than procollagenase contains a propeptide region with several bonds susceptible to proteolytic attack, or, as contended by Vaes [30], that one or more steps are intercalated between the action of these proteases and the activation process proper. An answer to this question, however, must await a detailed definition of each single component of the procollagenase activating system.

The biological function of the mast cell remains enigmatic. The identification of granula proteases has suggested a role in local, extracellular proteolysis. Calculations based on normal mast cells have revealed that 10—20% of the granula content [8] or 10% of the cell dry weight [31] is accounted for by these enzymes. It is thus evident that substantial amounts of protease activity are released in connection with granule discharge. Since this occurs as a result of specific stimulation or unspecific environmental changes, it is possible that mast cells may contribute to the local regulation of collagen catabolism.

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References

1 Harper, E., Bloch, K.J. and Gross, J. (1971) Biochemistry 10, 3035—3041
2 Harper, E. and Gross, J. (1972) Biochem. Biophys. Res. Commun. 48, 1147—1152
3 Vaes, G. (1972) Biochem. J. 126, 275—289
4 Vaes, G. (1972) FEBS Lett. 28, 198—200
5 Kruze, D. and Wojtecka, E. (1972) Biochim. Biophys. Acta 285, 436—446
6 Oronsky, A.L., Perper, R.J. and Schroder, H.C. (1973) Nature 246, 417—419
7 Birkedal-Hansen, H., Cobb, C.M., Taylor, R.E. and Fullmer, H.M. (1976) Biochim. Biophys. Acta, in press
8 Lagunoff, D. (1968) Biochem. Pharmacol. Suppl. 221—227
9 Kawiak, J., Vensel, W.H., Kommender, J. and Barnard, E.A. (1971) Biochim. Biophys. Acta 235, 172—187
10 Vensel, W.H., Kommender, J. and Barnard, E.A. (1971) Biochim. Biophys. Acta 250, 395—407
11 Taylor, A.C. (1971) J. Dent. Res. 50, 1301—1306
12 Simpson, J.W. and Taylor, A.C. (1974) Proc. Soc. Exp. Biol. Med. 145, 42—47
13 Cobb, C.M., Birkedal-Hansen, H. and Denys, F. (1975) J. Oral Pathol., in press
14 Birkedal-Hansen, H., Cobb, C.M., Taylor, R.E. and Fullmer, H.M. (1976a) Arch. Oral Biol., in press
15 Birkedal-Hansen, H., Cobb, C.M., Taylor, R.E. and Fullmer, H.M. (1976b) Arch. Oral Biol., in press
16 Fullmer, H.M., Gibson, W.A., Lazarus, G.S., Bladen, H.A. and Whedon, K.A. (1969) J. Dent. Res. 48, 646—651
17 Birkedal-Hansen, H., Cobb, C.M., Taylor, R.E. and Fullmer, H.M. (1974) J. Oral Pathol. 3, 284—290
18 Robertson, P.B., Taylor, R.E. and Fullmer, H.M. (1972) Clin. Chim. Acta 42, 43—45
19 Siegel, R.C. and Martin, G.R. (1970) J. Biol. Chem. 245, 1653—1658
20 Bornstein, P. and Piez, K.A. (1966) Biochemistry 5, 1393—1399
21 Hummel, B.C.W. (1959) Can. J. Biochem. Physiol. 37, 1393—1399
22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) 193, 265—275
23 Bitter, T. and Muir, H.M. (1962) Anal. Biochem. 4, 330—334
24 Andrews, P. (1965) Biochem. J. 96, 595—606
25 Barret, A.J. and Starkey, P.M. (1973) Biochem. J. 133, 709—724

26 Lagunoff, D., Philips, M.T., Iseri, O.A. and Benditt, E.P. (1964) Lab. Invest. 13, 1331—1344
27 Budd, G.C., Darzynkiewicz, Z. and Barnard, E.A. (1967) Nature 213, 1202—1203
28 Sakamoto, S., Goldhaber, P. and Glimcher, M.J. (1973) Calcif. Tiss. Res. 12, 247—258
29 Vaes, G. and Eeckhout, Y. (1975) in Protides of Biological Fluids (Peeters, H., ed.), Vol. 22, pp. 397, Pergamon Press, Oxford
30 Vaes, G. and Eeckhout, Y. (1975) in Dynamics of Connective Tissue Macromolecules (Burleigh, P.M. C. and Poole, A.R., eds.), pp. 129—146, North-Holland Publishing Company, Amsterdam
31 Darzynkiewicz, Z. and Barnard, E.A. (1967) Nature 213, 1198—1202