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PROPERTIES OF PROTEASE IN MAST CELL GRANULES*

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Abstract—A neutral protease partially purified from rat peritoneal mast cells closely resembled α-chymotrypsin in pH optimum and in susceptibility to several inhibitors including chymostatin, but the two enzymes were quite different in some respects. Mast cell protease was a more basic protein than α-chymotrypsin as indicated by their isoelectric points (9.3 and 8.5, respectively). In contrast to α-chymotrypsin, which consisted of three different subunits, mast cell protease was apparently composed of a single polypeptide chain of molecular weight 27,000 as estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. Mast cell heparin ionically linked to mast cell protease and suppressed the activity of the enzyme toward *N*-benzoyl-L-tyrosine ethyl ester and casein by 35 and 75 per cent, respectively, but it did not affect α-chymotrypsin in a medium containing 50 mM $CaCl_2$. Porcine intestinal mucosa heparin also showed similar inhibitory effects. The inhibitory action of heparin was more evident in low salt medium. Serotonin (0.25 mM), but not histamine, produced an 80 per cent inhibition of mast cell protease (2 μg) activity toward casein in the presence of heparin (2.5 μg). Zn^{2+} (0.008–0.2 mM) also suppressed the protease activity by 40–90 per cent in the presence of heparin.

Mast cells are known to contain an alkaline protease, in addition to heparin, histamine, serotonin and other active agents, in their granules [1–10]. The protease was partially purified from thyroidal [6] and peritoneal [10] mast cells of rats, and the functional resemblance of this enzyme to pancreatic chymotrypsin was reported. To study the activity control mechanism of this protease, which is apparently present in an active form in mast cell granules [2, 7, 8], we partially purified the enzyme from rat peritoneal mast cells, and examined its properties and interaction with other granular components. We found that mast cell protease was a basic protein, consisting of a single peptide chain, and that its proteolytic activity was suppressed significantly by binding to heparin, especially in the presence of Zn^{2+} or serotonin.

EXPERIMENTAL PROCEDURES

Isolation of mast cell granules

Mast cells were obtained from the peritoneal cavity fluids of normal male Wistar rats (10- to 13-weeks-old), weighing 250–300 g, and purified by Ficoll density gradient centrifugation as described previously [11]. Mast cell granules were then isolated, according to the method of Lagunoff [12], from the cells disrupted by vigorously mixing in ice-cold distilled water ($0.2–1 \times 10^7$ cells/ml) on a Vortex-type mixer ten times for a period of 15 sec each. The suspension was chilled for 15 sec between mixings. Granules were then isolated by centrifuging the suspension at 2700 *g* for 15 min after the initial centrifugation at 110 *g* for 10 min at 4°.

Enzyme assays

Protease activity in fractions of water-disrupted mast cells was determined using 0.4% casein as substrate in 0.1 M borate buffer (pH 8.0) [13]. The activity of partially purified enzyme preparations was measured using *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as substrate [13]. The standard assay mixture (0.8 ml) contained 40 mM Tris–HCl (pH 7.8), 0.5 mM BTEE (dissolved in 50% methanol; final methanol concentration, 2.5%), and 50 mM $CaCl_2$. (Mast cell protease was less responsive than α-chymotrypsin to Ca^{2+}, being stimulated by 18 and 65 per cent over the controls, respectively, by 50 mM $CaCl_2$. However, $CaCl_2$ was included in the standard assay mixture.) The effect of heparin on protease activity was studied in a low salt assay mixture containing 1 mM Tris–HCl (pH 7.8) and no $CaCl_2$. The reaction was initiated by the addition of BTEE or enzyme at 37°, and hydrolysis of substrate was measured by absorbance at 256 nm. *N*-Acetyl-β-glucosaminidase was assayed fluorometrically, according to the method of Leaback and Walker [14], using methyl umbelliferyl *N*-acetyl-β-glucosamine as substrate.

One enzyme activity unit is defined as the amount of enzyme which catalyzes the hydrolysis of 1 μmole of substrate per min under the assay conditions. When casein was used as substrate, one unit of enzyme caused an increase of absorbance of 1.0 per min at 280 nm. Protein was determined either using Folin–Ciocalteu reagent or from the absorbance at 280 nm as described by Layne [15].

Purification of mast cell protease and heparin

Mast cell protease was purified by a slight modification of the method of Kawiak *et al.* [10]. Granules obtained from 3.6×10^7 mast cells were extracted

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with 2 M NaCl (0.8 ml), left for 30 min at 4°, and then centrifuged at 12,000 *g* for 10 min. The supernatant fraction, which contained practically all the protease activity and heparin of the granules, was applied to a column (1.5 × 90 cm) of Sephadex G-100 which had been equilibrated with 10 mM Tris–HCl (pH 8.0) containing 1.5 M NaCl. To achieve a good separation of the protease from other components, the column was eluted with the same buffered NaCl solution, first at a flow rate of 10 ml/hr for 2 hr, and then at a reduced flow rate of 4 ml/hr, collecting 1-ml fractions. Heparin was eluted close to the void volume of the column, followed by a second protein peak which contained the protease activity. Combined protease fractions were concentrated by dialyzing in a collodion bag (Sartorious Membrane Filter) against 10 mM Tris–HCl (pH 8.0).

Combined heparin fractions (12 ml) were dialyzed against distilled water overnight, condensed to about 2 ml on a rotatory evaporator, and thoroughly mixed with 10% cetylpyridinium chloride (50 μl). After incubation at 37° for 1 hr, the reaction mixture was centrifuged at 2000 *g* for 10 min. The precipitate obtained was dissolved in 2 M NaCl (0.5 ml), mixed with 4 vol. of ethanol, and left at 4° overnight. After centrifugation at 2000 *g* for 10 min, the precipitate was dissolved in water (0.5 ml), and the treatment with cetylpyridinium chloride and ethanol was repeated once again as described above.

Heparin was further purified on DEAE-cellulose by applying the method of Horner [16] for preparation of rat skin heparin. The aqueous solution of heparin was applied to a column (1.6 × 10 cm) of DEAE-cellulose which had been equilibrated with 0.3 M NaCl adjusted to pH 2.5 with 3 N HCl. The column was eluted with a 400-ml linear NaCl gradient (from 0.3 to 1.8 M, pH 2.5) at a flow rate of 16 ml/hr. Fractions of 4 ml were collected into tubes which contained 1 ml of 1 M phosphate buffer (pH 7.0) to prevent decomposition of heparin at lower pH levels. Combined heparin fractions were dialyzed against water, condensed on a rotatory evaporator, and further purified on a column (1.5 × 80 cm) of Sepharose 4B pre-equilibrated with 10 mM Tris–HCl (pH 7.5) containing 1 M NaCl. The column was eluted with the same buffer at a flow rate of 8 ml/hr, collecting 2-ml fractions. Mast cell heparin was only partly included in Sepharose 4B, as reported by Yurt *et al.* [17], indicating its macromolecular nature [18]. Combined heparin fractions were dialyzed against water, evaporated to dryness on a rotatory evaporator or lyophilized, and stored at −20°. Heparin was measured by uronic acid content estimated by the method of Bitter and Muir [19]. Commercial pig intestinal mucosa heparin (Sigma) was purified on DEAE-cellulose at pH 2.5 as described above [16], and used as standard.

Preparation of mast cell protease–heparin complex

Purified mast cell protease (150 μg) was incubated with purified heparin (80 μg) in 1 ml of 10 mM Tris–HCl (pH 8.0) containing 0.05 M NaCl at 4° for 2 hr, and then filtered through a columm (1.5 × 90 cm) of Sephadex G-100 pre-equilibrated with the same buffer containing 0.05 M NaCl. Almost all the enzyme activity was eluted as the protease–heparin complex with 10 mM Tris–HCl (pH 8.0)–0.05 M NaCl close to the void volume. When re-chromatographed on the same column with the same buffer, the complex was eluted again as a single peak close to the void volume with about 90 per cent recovery of both the enzyme activity and heparin.

Preparation of [^{3}H]*diisopropylphosphoryl protease*

Purified mast cell protease (1.53 mg protein) in 10 mM Tris–HCl (pH 8.0) was incubated with [1,3-^{3}H]diisopropylfluorophosphate (780 nmoles, 2.9 Ci/mmole, Amersham, U.K.) in propyleneglycol at room temperature for 3 hr. The reaction mixture was thoroughly dialyzed against the same buffer at 4° with several changes of the buffer, and then centrifuged at 10,000 *g* for 15 min. The supernatant fraction was used as ^{3}H-labeled protease (7700 c.p.m./μg of protein).

Electrophoresis

Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn [20]. Protein solution in 10 mM phosphate buffer (pH 7.0) containing 1% SDS and 1% 2-mercaptoethanol was heated at 100° for 3 min. Electrophoresis was performed at a current of 6 mA/tube, and protein was stained with coomassie brilliant blue. Bovine serum albumin (68,000), beef liver catalase (58,000), ovalbumin (43,000), pepsin (35,000), trypsin (23,000) and egg white lysozyme (14,300) were used as reference proteins.

Gel electrofocusing

Isoelectric focusing was performed on polyacrylamide gels containing pH 3–10 ampholine in glass tubes (0.4 × 7 cm) according to the method of Wrigley [21]. At the end of electrophoresis, one gel was sliced into 2-mm pieces, soaked in 1.2 ml water at room temperature for 1 hr, and the pH of the water extract of each piece was measured. Another identical gel was stained with amido black for protein after removal of ampholine.

Materials

Diisopropylfluorophosphate, phenylmethylsulfonyl fluoride, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone, bovine pancreas α-chymotrypsin (3 × crystallized), beef liver catalase, trypsin (2 × crystallized), pepsin (2 × crystallized) and egg white lysozyme were obtained from Sigma, St. Louis, MO; bovine serum albumin from Armour. Kankakee, IL; ovalbumin (5 × crystallized) from Nutritional Biochemicals, Cleveland, OH; Ficoll, Sephadex G-100, and Sepharose 4B from Pharmacia, Uppsala, Sweden; casein according to Hammersten from Merck, Darmstadt, F.R.G.; and ampholine from LKB, Stockholm, Sweden. Various protease inhibitors of microbial origin, chymostatin, pepstatin, leupeptin, antipain, phosphoramidon and elastinal [22] were gifts from Dr. K. Aoyagi, Institute of Microbial Chemistry, Tokyo, Japan. Other chemicals of reagent grade were commercial products.

RESULTS

Purification of mast cell protease

The effects of NaCl concentration on extraction

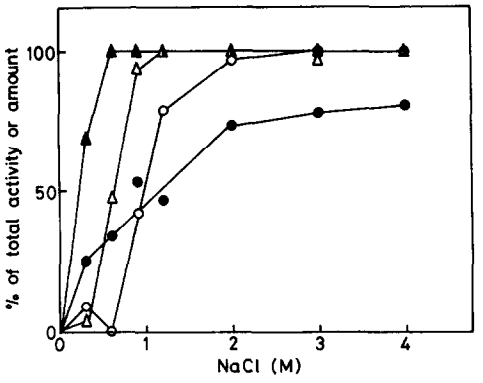

Fig. 1. Effect of NaCl concentration on the extraction of mast cell granular components. Granules obtained from 10^6 mast cells were suspended in 1 ml each of various concentrations of NaCl, left at 4° for 15 min, and then centrifuged at 41,000 *g* for 60 min. Both the supernatant fraction and the precipitate were assayed for protein, heparin, and enzyme activities as described under Experimental Procedures. All values (of supernatant) were expressed as percentage of the total (supernatant + precipitate) enzyme activity or the total amount of protein and heparin. Key: *N*-Acetyl-β-glucosaminidase (▲), protease (△), heparin (○) and protein (●).

of granular components of mast cell are shown in Fig. 1. *N*-Acetyl-β-glucosaminidase activity [23, 24] was readily extracted with 0.6 M NaCl at 4° in 30 min, protease with 1.2 M NaCl and heparin with 2 M NaCl. About 15% of the granular proteins remained insoluble even after extraction with 4 M NaCl. Histamine was readily released with 0.01 M NaCl (not shown). Therefore, the protease was purified from a 2 M NaCl extract of granules as summarized in Table 1. Through these purification stages, the ratio of the activity on BTEE and casein was not altered significantly.

Properties of mast cell protease

The purified mast cell protease moved as a single protein band on SDS-polyacrylamide gel electrophoresis (Fig. 2A). In contrast, bovine pancreatic α-chymotrypsin, which consists of three different subunits [25], showed two protein bands (Fig. 2B), since the smallest subunit (thirteen amino acid residues) was not retained by the gel as reported by Weber and Osborn [20]. From a semilogarithmic

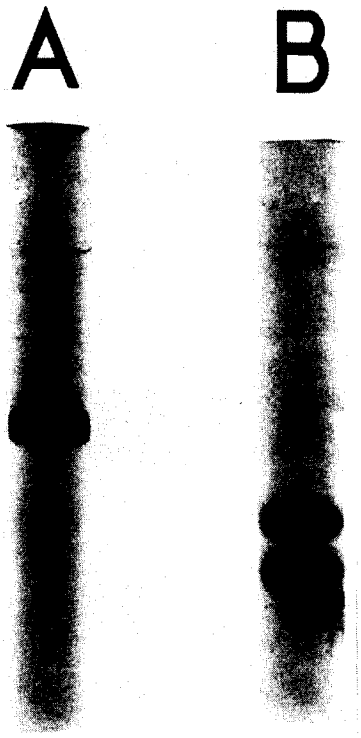

Fig. 2. SDS-polyacrylamide gel electrophoresis of (A) purified mast cell protease (20 μg) and (B) bovine pancreatic α-chymotrypsin (20 μg).

plot of relative mobilities of purified protease and reference proteins on SDS-polyacrylamide gel electrophoresis (not shown), a molecular weight of 27,000 was obtained for the protease.

Figure 3 shows the pH profile of mast cell protease and α-chymotrypsin on isoelectric focusing. The estimated isoelectric points for the protease and α-chymotrypsin were 9.3 and 8.5, respectively. The value of 8.5 for α-chymotrypsin was very close to that (8.1–8.6) reported by Laskowski [26]. Mast cell protease was apparently a more basic protein than α-chymotrypsin.

Mast cell protease exhibited optimal activity on BTEE in the pH range 7.8–8.2 (pH profile not shown). Substrate (BTEE)-saturation curves at pH 7.8 are shown in Fig. 4. From the Lineweaver–Burk plot, the K_m value for BTEE was estimated to be 1.9×10^{-4} M (Fig. 4 inset).

Table 1. Purification of mast cell protease*

Stage	Protein (mg)	Protease activity on Casein: Total units	Casein: Specific activity (units/mg)	BTEE: Total units	BTEE: Specific activity (units/mg)
Mast cells	2.7	0.32	0.11	7.9	2.9
Granules	2.3	0.96	0.41	19.5	8.5
2 M NaCl extract	1.4	2.14	1.52	38.8	27.7
Sephadex G-100	1.3	2.62	2.02	53.0	40.7

* As the starting material, 3.6×10^7 mast cells were used.

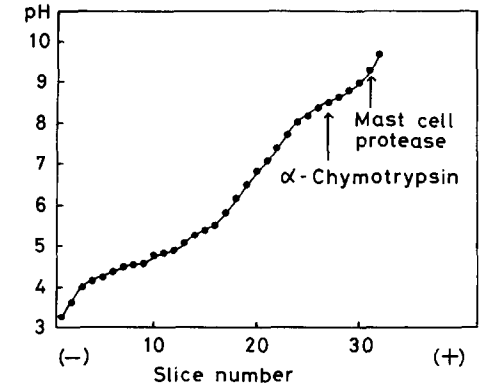

Fig. 3. Isoelectric focusing of purified mast cells protease (46 μg) and α-chymotrypsin (50 μg) on polyacrylamide gels containing pH 3–10 ampholine.

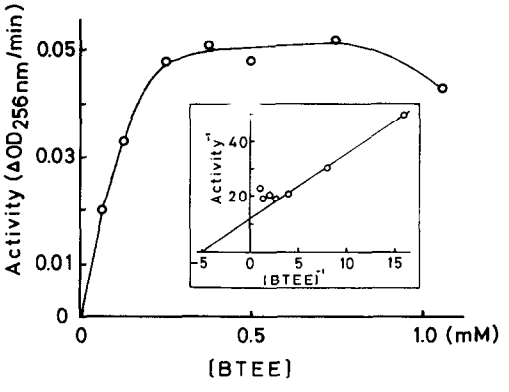

Fig. 4. Relationship between substrate concentration and protease activity. Protease activity on BTEE was assayed with 2.5 μg of the purified enzyme as described under Experimental Procedures. The inset shows the double reciprocal plot of the data.

Effect of protease inhibitors

Of several protease inhibitors of microbial origin [20] tested, only chymostatin, a potent inhibitor of α-chymotrypsin, strongly inhibited mast cell protease (Table 2). Other chymotrypsin inhibitors, diisopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF) and L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK) were also inhibitory, but *p*-chloromercuribenzoate (PCMB) and EDTA were ineffective. The time course and dose dependency of the effects of chymostatin, PMSF and TPCK (Figs. 5A and B; 5C and D; and 5E and F) indicate that, although mast cell protease was slightly less sensitive to these inhibitors than α-chymotrypsin, the two enzymes probably have similar active center(s).

Inhibition of mast cell protease by heparin, serotonin and Zn^{2+}

Heparin. When [^{3}H]diisopropylphosphoryl protease was centrifuged with mast cell heparin (MC heparin) on a linear sucrose gradient (from 5 to 20%, w/v), a significant amount of ^{3}H-labeled protease was sedimented with MC heparin (Fig. 6), indicating a protease–heparin complex formation. However, this co-sedimentation was not observed in the presence of 2 M NaCl.

Table 3 shows that MC heparin (2 μg) inhibited the activity of mast cell protease (2 μg) on BTEE and casein by about 35 and 75 per cent, respectively, when assayed in the standard assay mixture containing 50 mM $CaCl_2$. However, MC heparin did not affect the activity of α-chymotrypsin. Porcine intestinal mucosa heparin (PM heparin) also inhibited the activity of mast cell protease on BTEE and significantly suppressed that on casein, but did not affect the activity of α-chymotrypsin. Heparin was more inhibitory in a low salt medium. In 1 mM Tris–HCl (pH 7.8), both MC heparin and PM heparin (0.4 μg) inhibited the activity of mast cell protease (2 μg) on BTEE by 70 and 50 per cent, respectively.

Table 2. Effects of various inhibitors on mast cell protease and α-chymotrypsin*

	Inhibitor	Concn	Mast cell protease	α-Chymotrypsin
		(μg/ml)	(Per cent activity)	
(1)	Chymostatin	12.5	0	0
		0.15	23	2
	Pepstatin	12.5	99	97
	Leupeptin	12.5	99	99
	Antipain	12.5	110	76
	Phosphoramidon	12.5	112	99
	Elastinal	12.5	109	98
		(mM)		
(2)	PCMB	0.1	99	96
	DFP	1.0	40	2
	PMSF	0.25	0	0
		0.05	24	1
	TPCK	0.2	29	4

* The standard assay mixture (0.76 ml), containing 2 μg of purified mast cell protease or α-chymotrypsin without BTEE, was preincubated with various inhibitors at 30° for 20 min (group 1) or 30 min (group 2) and then the reaction was initiated by the addition of 0.04 ml of 10 mM BTEE.

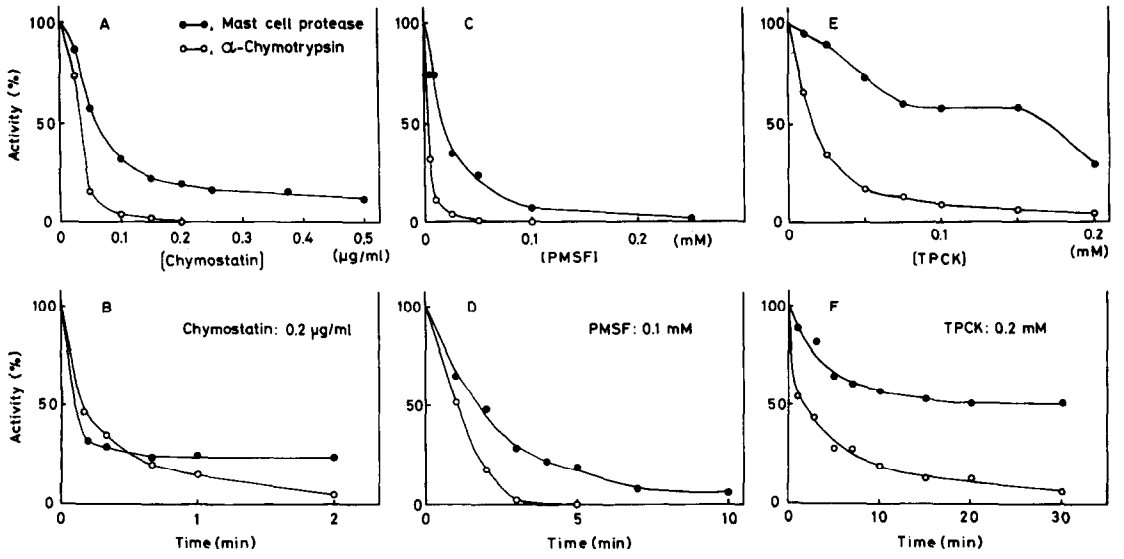

Fig. 5. Effects of chymostatin, PMSF and TPCK on mast cell protease and α-chymotrypsin. The dose response was determined from the initial rate of the reaction measured as described under Experimental Procedures using purified mast cell protease (●, 2 μg) and α-chymotrypsin (○, 2 μg). Standard assay mixtures (0.76 ml) minus BTEE were preincubated with various concentrations of (A) chymostatin, (C) PMSF, and (E) TPCK at 30° for 20 min. Then the reaction was initiated by the addition of 10 mM BTEE (0.04 ml). The time course was determined by using the lowest dose that produced the largest response. Standard assay mixtures (0.76 ml) minus BTEE were preincubated with (B) chymostatin (0.2 μg/ml), (D) 0.1 mM PMSF and (F) 0.2 mM TPCK at 30° for various intervals. Then the reaction was initiated by the addition of 10 mM BTEE (0.04 ml).

Furthermore, heparins also became inhibitory to the activity of α-chymotrypsin on BTEE.

Gel filtration of the protease–heparin complex (10.8 units/mg of protein on BTEE) on Sephadex G-100 in 10 mM Tris–HCl (pH 8.0) containing 2 M NaCl resulted in the elution of heparin (89 per cent) at the void volume and the protein (75 per cent) with the protease activity (16.5 units/mg) at the apparent molecular weight of 25,000 (not shown).

Serotonin. High doses of serotonin, but not of histamine, were known to inhibit the activity of mast cell protease on casein (a 50 per cent inhibition by 3 mM serotonin) [27]. Table 4 shows that serotonin, but not histamine, at a concentration of 0.25 mM, which was only slightly (20 per cent) inhibitory, produced about 80 per cent inhibition of the activity of mast cell protease on casein in the presence of heparin. Serotonin was also inhibitory to the mast cell protease–heparin complex, suppressing the activity of the complex by 85 per cent which was about 55 per cent as active as the free enzyme. In contrast, serotonin with or without heparin did not significantly affect the activity of α-chymotrypsin.

Zn^{2+}. Increasing concentrations of Zn^{2+} (from 0.008 to 0.2 mM) progressively inhibited the activity of mast cell protease on casein (Table 5). Among other divalent metal ions tested, Cu^{2+} and Co^{2+} (1 mM) were slightly inhibitory, but Mn^{2+} and Mg^{2+} were without effect. Furthermore, lower concentrations (0.008 and 0.04 mM) of Zn^{2+} became more inhibitory to mast cell protease in the presence of heparin.

Table 3. Effect of heparin on the activity of mast cell protease and α-chymotrypsin*

		Relative protease activity (%) on			
		BTEE		Casein	
Addition	Concn (μg)	Mast cell protease	α-Chymotrypsin	Mast cells protease	α-Chymotrypsin
None (control)		100	100	100	100
Mast cell heparin	2	66	101	26	104
	0.4†	30	50		
Pig intestinal mucosa heparin	2	79	100	28	90
	0.4†	49	47		

* Protease activity was assayed in the standard assay mixture containing 50 mM $CaCl_2$, 0.5 mM BTEE or 0.4% casein, and 2 μg each of enzyme and heparin as described under Experimental Procedures.

† Protease activity on BTEE was assayed in 1 mM Tris–HCl (pH 7.8) without Ca^{2+} instead of the standard assay mixture.

Table 4. Effects of histamine and serotonin on the inhibitory action of heparin on mast cell protease*

Addition	Mast cell protease (2 μg)	Mast cell protease–heparin complex (2.5 μg)	α-Chymotrypsin (1 μg)
		(Per cent activity)	
None (control)	100	100†	100
Histamine (0.25 mM)	100	100	100
Serotonin (0.25 mM)	80	15	88
Heparin (2.5 μg)	68	65	85
+ serotonin (0.25 mM)	18	13	80
+ histamine (0.25 mM)	67	64	88

* Protease activity was assayed in the standard assay mixture containing 0.4% casein as substrate.
† Specific activities (units/mg): mast cell protease, 2.02; and mast cell protease–heparin complex, 1.13.

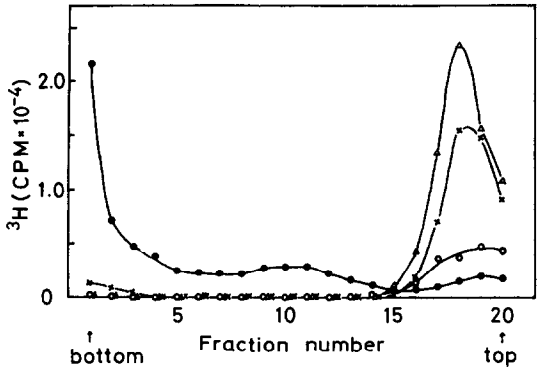

Fig. 6. Interaction of mast cell heparin with mast cell protease. [^{3}H]Diisopropylphosphoryl protease (8.3 μg, 64,000 c.p.m.) with or without mast cell heparin (15 μg) in 10 mM Tris–HCl (pH 8.0, 0.11 ml) was layered on 4.2 ml of a 5–20% sucrose gradient. Centrifugation was carried out for 15 hr at 100,000 *g* and 4°. After the run, 10-drop fractions were collected from the bottom of the tube into counting vials for radioactivity measurements. Key: [^{3}H]protease (○), [^{3}H]protease centrifuged together with mast cell heparin (●), [^{3}H]protease and mast cell heparin centrifuged in sucrose gradient containing 2 M NaCl (△), and [^{3}H]protease alone centrifuged in sucrose gradient containing 2 M NaCl (×).

Effect of Ca^{2+} *on the inhibitory action of heparin*

With increasing concentrations of several cations, MC heparin became less inhibitory to mast cell protease. In the presence of 0.25 M Ca^{2+} or Mg^{2+}, heparin was no longer inhibitory (Fig. 7). Na^+ or K^+ was less effective, requiring a 1 M concentration to prevent the inhibitory action of heparin. In contrast, when Ca^{2+} was added to an MC heparin–protease mixture in 1 mM Tris–HCl (pH 7.8), where the enzyme activity was inhibited by 70 per cent, Ca^{2+} at a concentration as low as 30 mM (in contrast to 0.25 M in Fig. 7) was effective in restoring the full enzyme activity (Fig. 8).

DISCUSSION

In the present study, we partially purified a chymotrypsin-like protease from rat peritoneal mast cells, and examined some of its properties. Our results were mostly consistent with those reported by other investigators on the extractability from granules with high ionic strength, pH optimum, molecular weight, and effects of various inhibitors [1, 2, 6, 9, 10, 27, 28]. However, the K_m value for BTEE (0.9 mM) (Fig. 4) of our preparation was somewhat lower than those reported by Pastan and Almqvist

Table 5. Effects of Zn^{2+} and other metal ions on the inhibitory action of heparin on mast cell protease*

Metal ions	Concn (mM)	Mast cell protease (2 μg)		α-Chymotrypsin (1.5 μg)	
		−Heparin	+Heparin (2.5 μg)	−Heparin	+Heparin (2.5 μg)
			(Per cent activity)		
None		100	65	100	96
Zn^{2+}	0.008	95	58	100	94
	0.04	80	45	100	92
	0.2	11	10	97	86
Cu^{2+}	1.0	92		100	
Co^{2+}	1.0	88		100	
Mn^{2+}	1.0	110		100	
Mg^{2+}	1.0	100		100	

* Protease activity was assayed in the standard assay mixture containing 0.4% casein as substrate.

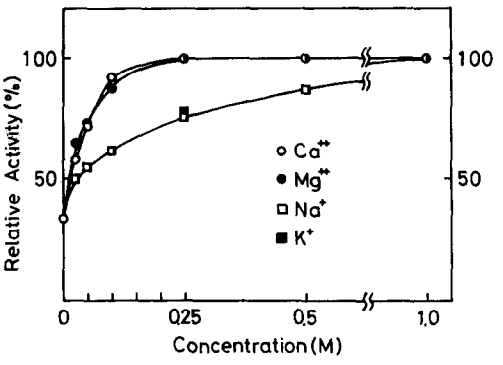

Fig. 7. Effects of Ca^{2+} and other cations on the inhibitory action of heparin. Assay mixtures (0.76 ml) contained 1 mM Tris–HCl (pH 7.8), mast cell protease (0.25 μg), mast cell heparin (0.3 μg) and various concentrations of Mg^{2+} (●), Ca^{2+} (○), Na^+ (□) or K^+ (■). After a 1-min preincubation at 30°, the reaction was initiated by the addition of 10 mM BTEE (0.04 ml).

[6] (3.8 mM), and by Kawiak *et al.* [10] (1.1 mM). The reason for this difference is not clear yet. The molecular weight (27,000) of our preparation estimated by SDS-polyacrylamide gel electrophoresis was similar to those (23,000–29,000) reported by other investigators using various methods [6, 10, 24, 27]. However, much lower molecular weights, 7000 [10] and 5600 [29] based on ultracentrifugation conducted in 0.1 M NaCl and Sephadex gel filtration performed in 0.1 M KCl, respectively, were also reported for the enzyme. Apparently mast cell protease behaved anomalously in the low salt medium, as suggested by Kawiak *et al.* [10].

Although a close resemblance of mast cell protease and α-chymotrypsin was further confirmed by the inhibitory action of chymostatin (Table 2), the two enzymes were different in several respects. First, mast cell protease was a more basic protein than bovine α-chymotrypsin (isoelectric points = 9.3 and 8.5, respectively) (Fig. 3). Second, in contrast to

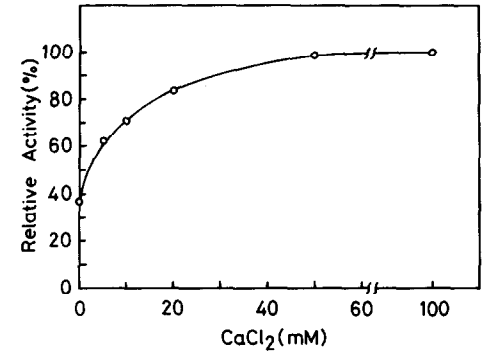

Fig. 8. Effect of Ca^{2+} on protease activity inhibited by heparin. Assay mixtures (0.66 ml) containing 1 mM Tris–HCl (pH 7.8), mast cell protease (2.5 μg) and mast cell heparin (0.3 μg) were preincubated at 30° for 1 min. Then various concentrations of $CaCl_2$ (0.1 ml) were added to the mixture. After incubation at 30° for another 1 min, the reaction was initiated by the addition of 10 mM BTEE (0.04 ml).

α-chymotrypsin, which is composed of three different polypeptide subunits [25], mast cell protease consisted of a single polypeptide chain (Fig. 2). In this respect, studies on the primary protein structure of the enzyme are desirable. Third, both MC heparin and PM heparin apparently ionically linked to mast cell protease (Figs. 6–8, Tables 3 and 4) and significantly suppressed the enzyme activity in the presence of 50 mM $CaCl_2$, whereas the heparin did not affect the activity of α-chymotrypsin (Table 3). The inhibitory effect of heparin became more potent in a low salt medium (Ca^{2+}-free, 1 mM Tris–HCl) (Fig. 7, Table 3), and this inhibitory effect was abolished in the presence of Ca^{2+} or by the addition of Ca^{2+} to the heparin-inhibited protease (Figs. 7 and 8). Furthermore, the protease–heparin complex was also dissociated in 2 M NaCl, releasing the free enzyme. These results indicate that interaction between mast cell protease and heparin is of an ionic nature and controlled by the intracellular ionic strength.

In contrast to our findings, Davies *et al.* [30] suggested that heparin increased the histone-digesting activity of a neutral protease of polymorphonuclear leukocytes by preventing the enzyme from binding to the cytosolic inhibitor. The presence of activity regulators other than heparin in intra- and extragranular fractions of mast cells awaits further investigations. Recently, Everitt and Neurath [31] reported that the MC heparin–mast cell protease complex was only 60 per cent as active as the free enzyme on ornithine aminotransferase apoenzyme as substrate, but the complex formation did not affect the kinetic properties of the enzyme when the substrate was BTEE. In our case, heparin was inhibitory to the enzyme activity toward both BTEE and casein. The reason for this difference is not clear yet. Fourth, in contrast to α-chymotrypsin, mast cell protease was more susceptible to the inhibition induced by serotonin or Zn^{2+} in the presence or absence of heparin (Tables 4 and 5). A strong inhibition (70 per cent) of the activity of mastocytoma cell protease by indole (20 mM) [28], and a 50 per cent inhibition of the activity of mast cell protease by serotonin (3 mM) [27] were also reported. According to Yurt and Austen [27], the molar content of serotonin is similar to that of protease in rat mast cells. Although appreciable molar excess of serotonin was employed to inhibit the protease in the presence of heparin (Table 4), our results indicate that serotonin may interact with the heparin–protease complex to regulate the enzyme activity in mast cell granules *in vivo*. However, serotonin is not associated with mast cells in animals other than the rat and mouse [32]. Therefore, it is not clear yet whether serotonin acts as a physiological regulator of the mast cell protease activity in all animals. As to the storage of histamine and serotonin, Uvnäs and his co-workers [33, 34] suggested the ionic linkage of both amines to free carboxyl groups of protein in the heparin–protein complex in granular matrix. It is not clear at present whether a basic protein (mol. wt = 5600) isolated from rat mast cell granules by Bergqvist *et al.* [29] or the protease is involved in the heparin–protein complex formation. Although a fair amount of Zn^{2+} is present in rat mast cells (4.1 nmoles/10^6 cells) [29],

the function of Zn^{2+} was discussed mostly for its effect on histamine-binding to granules [29] and to heparin [35]. Significant inhibition of mast cell protease activity by Zn^{2+} in the presence of heparin indicates a possible role of Zn^{2+} in the regulation of the enzyme activity.

Neutral proteases were also found in mouse mastocytoma P-815 cells [27], human seminal plasma [36], human granulocytes [37–39], rabbit granulocytes [30], human purulent sputum [40], rat peritoneal macrophages [41], human spleen [42–44], human skin [45], and liver, skeletal muscle and small intestine of rats [46]. Their roles in a variety of physiologic and pathologic events have been suggested, such as digestion of connective tissue components during inflammation [47], chronic obstructive pulmonary disease (emphysema) [40] by granulocyte proteases, and myofibrillar protein degradation in muscular dystrophy by serine protease in muscle [48]. Although a chymotrypsin-like protease in mast cells was fairly well characterized, its physiologic role has not been fully elucidated yet, and certainly requires further investigation.

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