BBA 66431

NON-PANCREATIC PROTEASES OF THE CHYMOTRYPSIN FAMILY

II TWO PROTEASES FROM A MOUSE MAST CELL TUMOR

WILLIAM H VENSEL*, JANUSZ KOMENDER** AND ERIC A BARNARD***

Departments of Biochemistry and Biochemical Pharmacology, State University of New York, Buffalo, N Y 14214 (U S A)

(Received May 25th, 1971)

SUMMARY

A chymotrypsin-like protease has been prepared from the mouse P815Y mast-cell tumor It is similar in most respects to the first such enzyme purified from the normal peritoneal mast cells of the rat It, too, has a molecular weight (estimated by gel filtration) of about 25 000 The apparent molecular weight is lower, unless high salt concentrations are used to overcome affinity of this enzyme for the dextran or polyamide gel columns

The binding of indole, and the binding of potato chymotrypsin inhibitor I, are characteristically strong Specific alkylations and phosphorylation suggest a serine–histidine active center system The protein is basic, with strong affinity for the heparin of the mast cell granule High K^+ concentrations (>1 M), rather than Na^+, are needed to displace fully the enzyme from heparin in solution

A trypsin-like enzyme is also prominent in the mouse (but not the rat) cells The trypsin-like enzyme has a specificity and active center reactivity characteristic of pancreatic trypsin, and a molecular weight, estimated by gel filtration, of about 35 000

INTRODUCTION

As part of a study of the molecular evolution of chymotryptic enzymes, a chymotrypsin-like protease has been purified from the peritoneal mast cells of the rat[1] Another source is the rat thyroid, where a protease has been attributed to the mast cells[2] However, mast cells comprise only about 4% of the mammalian peritoneal population[3] and less than 1% of the thyroid cells[2] Mast cells may, on the other hand,

Abbreviations BTEE, benzoyl-L-tyrosine ethyl ester, DFP, diisopropylphosphorofluoridate, TAME, tosyl-L-arginine methyl ester, TLysCK, α-*N*-tosyl-L-lysine chloromethyl ketone, TLeuCK, *N*-tosyl-L-leucine chloromethyl ketone, TPCK, *N*-tosyl-L-phenyalanine chloromethyl ketone

* Present address Department of Biochemistry, University of Chicago, Ill , U S A

** On leave of absence from the Academy of Medicine, Warsaw, Poland (present address), while engaged in this work

*** Enquiries and reprint requests should be addressed to this author

be obtained in high concentrations from propagated mast cell tumors[3,4] The P815Y mastocytoma[4] possesses most of the characteristic features of mast cells, including the production of heparin, histamine and 5-hydroxytryptamine[5] We report herein on the preparation and characteristics of a chymotrypsin-like protease obtained from these tumor cells, and on a trypsin-like protease also present

MATERIALS AND METHODS

Unless otherwise specified, materials and methods were as described previously[1] α-*N*-Tosyl-L-lysine chloromethyl ketone (TLysCK), *N*-tosyl-L-leucine chloromethyl ketone (TLeuCK) and tryptophan substrates were obtained from Cyclo Chemical Corp TEAE-cellulose was Cellex-T (Bio-Rad Labs) and was prepared and used as recommended by the manufacturer Chymotrypsinogen A (6 × crystallized) and human serum albumin (crystallized, 100%) were from Mann, and bovine trypsinogen (crystallized) from Sigma Collagenase (from *Clostridium histolyticum*) was from Worthington (Purified), rat-tail collagen was a pure preparation kindly donated by Dr J Bello (Roswell Park Mem Institute, Buffalo) and elastin-orcein was from Sigma

Propagation of the tumor

The P815Y tumor was obtained from inoculated mice kindly supplied by Dr Glenn Fischer (Roger Williams General Hospital, Providence, Rhode Island) and maintained in our laboratory in the peritoneal cavities of female mice of the DBA/2 Ha strain The tumor was transferred by intraperitoneal injection of a suspension of the mastocytoma in sterile Hanks' medium 5–7 days later, the animals were killed and their peritoneal cavities washed either with sterile Hanks' solution (for inoculations) or with modified Tyrode's solution[6] (for homogenization and subsequent enzyme isolation)

Enzyme extraction and labeling

Homogenization of the washed mastocytoma cells was carried out in 0 25 M sucrose–0 01 M EDTA–0 01% Tween 80 solution, adjusted to pH 6 0 with HCl (5 ml of buffer per ml of packed cells), using a glass homogenizer with a driven teflon pestle For more than 5 ml of cells, a Virtis Model 45 homogenizer with a 125-ml chamber was used instead, at a setting of 55 Following a 10-min homogenization, the cell debris was collected at 900 × *g* (10 min), and re-homogenized, twice The combined supernates were centrifuged at 24 000 × *g* for 30 min, to yield the granule fraction, which could be stored frozen at −10°

The enzyme was extracted from the granules in 1 4 M KCl–0 05 M succinate–0 01 M EDTA solution (pH 6 0), either by stirring at room temperature for 30 min, or by using the glass/teflon homogenizer for 3 min, followed by centrifugation of the homogenate at 24 000 × *g* for 30 min After three homogenizations the supernates were combined

For labeling, [^{3}H]diisopropylphosphorofluoridate ([^{3}H]DFP) was added to a final concentration of 10^{-4} M in the sucrose–EDTA medium at pH 6 0 at 25°, either in the granule stage or after extraction of the enzyme The activity on BTEE was measured at intervals (and was seen to become extinguished) After 3 h, the DFP molarity was raised to 10^{-2} M by the addition of unlabeled DFP and incubation was

continued for another 3 h at 25°, for saturation and exchange The granules were then washed with 10^{-2} M unlabeled DFP in the same medium, and collected by centrifugation

Gel filtration

In the purification steps, the extracts were filtered in 5-ml batches on a column (usually 88 cm × 2 0 cm) of Sephadex G-100, at 4° Elution was by 0 05 M succinate–0 01 M EDTA–1 4 M KCl solution (pH 6 0) at the rate of 8 ml/cm² per h Desalting of each enzyme peak was effected on a column of Bio-Gel P-2 or Sephadex G-25, firstly in 50 mM HCl (ref 1) and subsequently in water

Other methods

Electrophoresis was conducted at room temperature in a Beckman Microzone apparatus with cellulose acetate membranes Staining of the strips for protein was with Buffalo Black NBR (Allied Chemical Corporation) in 10% trichloroacetic acid solution Collagenase activity was determined[7] by quantitative ninhydrin reaction of the products released from rat-tail collagen, with bacterial collagenase used as a reference Elastase was assayed by the elastin–orcein colorimetric method[8]

RESULTS

Isolation of proteases

Upon removal of the mastocytoma cells from the peritoneal cavity of the mouse, over 90% of the suspended cells were shown, upon toluidine blue staining[3], to be granulated mast cells Normal (non-malignant) mast cells, readily recognized by their cytology[3], comprised much less than 1% of the population Upon homogenization of these cells in 1 2 M NaCl (or KCl) medium to prepare the granule fraction, they released into solution all of their protease and benzoyl-L-tyrosine ethyl ester (BTEE) esterase activity On the other hand, homogenization in water or 0 05 or 0 2 M NaCl–0 01 M EDTA solution released about two-thirds of the protease activity, in contrast to the case of the rat normal mast cells[1] where the activity then remained in the granules The extracted protease was associated with soluble heparin Cell homogenization was, therefore, routinely performed in 0 25 M sucrose (which released only 4% of the BTEE–esterase from the granules), and the isolated granule fraction subsequently extracted in 1 4 M KCl medium

Alternatively, the enzyme was isolated similarly after inactivation by [^{3}H]-DFP This reaction could be conducted either on the granules or on the extracted enzyme in solution, both methods giving the same final result The extracts were fractionated by gel filtration on Sephadex G-100 While it was necessary, for separation to use an ionic strength > 1, the profile obtained was dependent upon the precise ionic medium used (Figs 1 and 2) Using the [^{3}H]DFP-inactivated material, extraction, of the granules in 1 1 M NaCl and filtration in that medium (Fig 1) resulted in a large peak (I) of labeled protein soon after the void volume, with a satellite (IA), and a smaller, more retarded peak (II) However, when the procedure was performed, instead, in 1 4 M NaCl medium, Peak II was increased at the expense of Peak I, and a further such redistribution occurred when the initial extract was gel-filtered in 1 4 M KCl medium, Peak II then being about 8 times larger (in ^{3}H content) than Peak

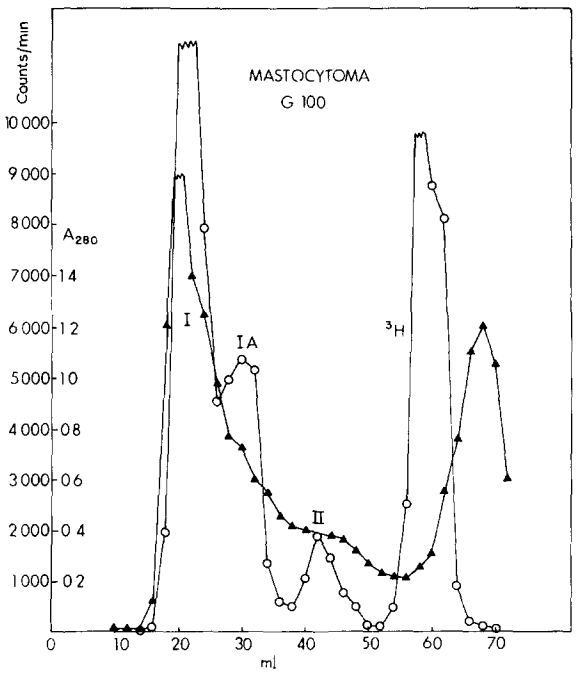

Fig 1 Gel filtration on a Sephadex G-100 column (1 cm × 75 cm) of [^{3}H]DFP-inhibited mastocytoma proteases, in 1 1 M NaCl (buffered with 0 05 sodium succinate–0 01 M EDTA (pH 6 0)) Fractions were sampled for protein ($A_{280\ nm}$, ▲), and for ^{3}H content (counts/min per ml, ○) The peaks centered near 60 and 70 ml contained no protein the former is attributed to [^{3}H]DFP hydrolysis products

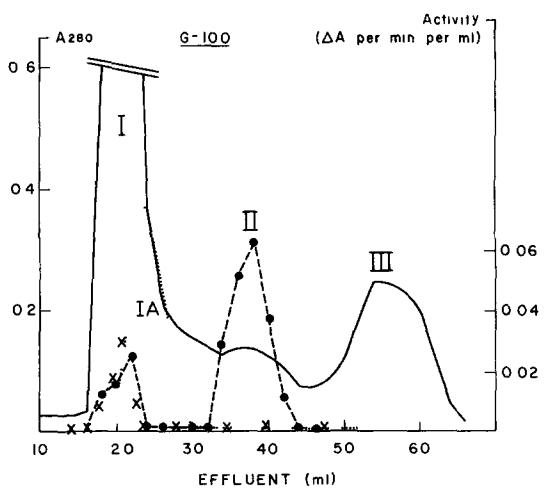

Fig 2 Fractionation of a non-inhibited enzyme preparation The Sephadex G-100 column (1 cm × 68 cm) was eluted with 1 4 M NaCl (buffered with 0 05 M dimethyl glutarate–0 01 M EDTA (pH 6 0)) at 4° The 2-ml fractions were sampled for $A_{280\ nm}$ reading, activity on BTEE (●——●, as $\Delta A_{256\ nm}$), activity on TAME (dotted line, as $\Delta A_{247\ nm}$) and heparin content (⁄, in units[1] on the left-hand scale) Peaks I, IA and II also contained (not shown) much protease activity (on casein as substrate[1]) Peak III did not contain material with a protein spectrum, but contains the low molecular weight components

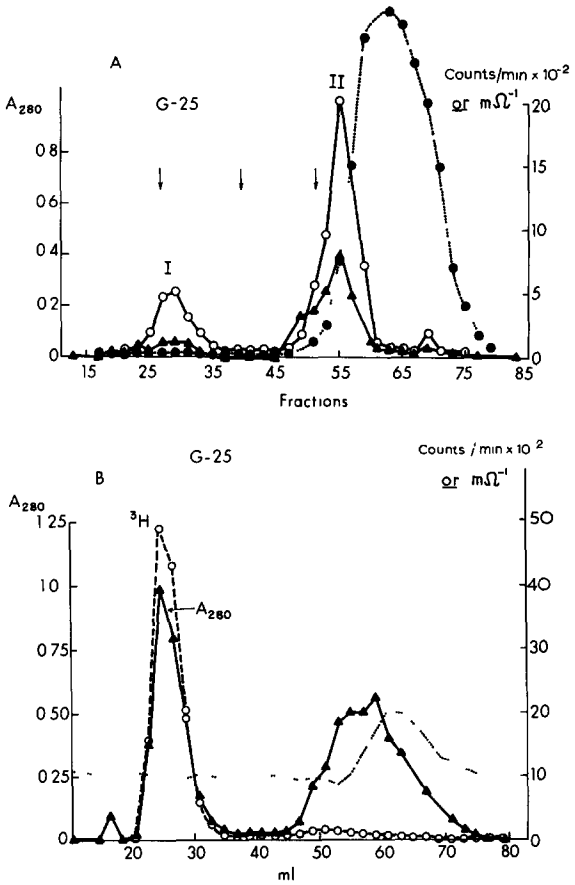

Fig 3 Gel filtration of material from the labeled Peak II obtained from separations as in Fig 1 Fractions were sampled for protein ($A_{280\ nm}$, ▲), counts/min per ml (○) and salt concentration measurement by conductivity (dotted line) (A) On a Sephadex G-25 column, in 1 0 mM HCl (B) On the same column as in A, but in 50 mM HCl When a similar BioGel P-2 column was used, in water, three labeled protein peaks were obtained at the positions of the arrows (in A), containing radioactivity in the ratio 1 4 6

I In each case refiltration of the material in the central fraction of Peak I resulted in two peaks in the positions of I and II

Gel filtration of the active extracts under identical conditions resulted in profiles equivalent to those obtained with the labeled protein, in each case (Fig 2) In all instances Peak I contained heparin (Fig 2) while Peak II did not Hence, in summary, Peak I contains a heparin-bound form of the DFP-reactive enzyme of Peak II This residual bound enzyme can be released from the heparin by a second treatment in high salt concentration (especially when K^+ is used)

Attempts to desalt Peak II (from the labeled enzyme preparation) by gel filtration on a Bio-Gel P-2 column in distilled water or on a Sephadex G-25 column in 1 0 mM HCl (Fig 3A) resulted in a minor Peak I which emerged at the void volume and a much larger ^3H-containing peak which overlapped the salt peak On the P-2 column, there was also a smaller labeled peak representing an intermediate retarda-

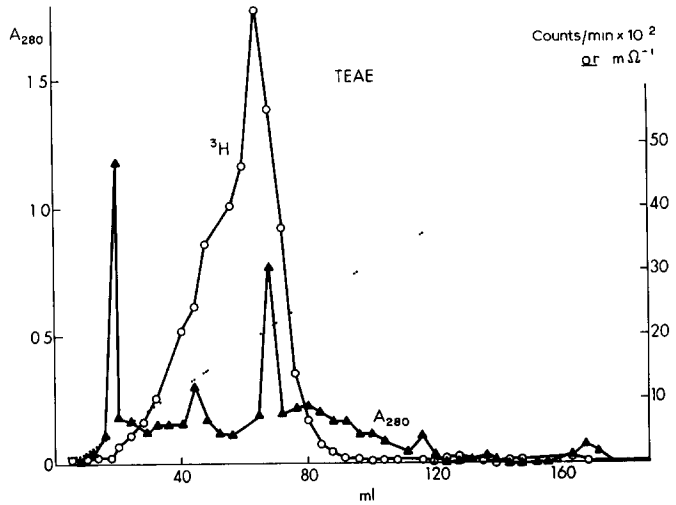

Fig 4 Chromatography on a TEAE-cellulose column (0 8 cm × 10 cm) at 4° The enzyme extract was first fractionated on G-100 as in Fig 2 the major labeled protein peak in the position of Peak II there was used, after dialysis ○—○, ^3H (counts/min per ml), dotted line, salt gradient, by conductivity readings, ▲, protein (by $A_{280\ nm}$) 150 ml were first collected during adsorption and washing (with one column volume of the equilibrating 20 mM Tris–HCl (pH 7 0) buffer) of the sample on the column the chromatography shown (with a gradient of 0–1 0 M KCl in the same buffer) was then commenced

tion Equivalent anomalous retardation was also found when the non-inhibited enzyme was refiltered similarly in distilled water on a P-6 gel column Refiltration of the radioactive Peak II on G-25 in 50 mM HCl, on the other hand, resulted in the radioactivity emerging from the column at the breakthrough position (Fig 3B), as expected for a protein on this gel

Ion-exchange cellulose chromatography

An improvement over the chromatographic results obtained[1] with the rat enzyme was found here when a purification step by gel filtration preceded chromatography on TEAE-cellulose (Fig 4) About 80% of the protein present emerged in the break-through fraction with less than 1% of the total radioactivity A gradient of KCl was then applied (Fig 4), when an unlabeled protein peak was followed by two labeled, overlapping peaks When the material in the combined labeled peaks was again gel-filtered on Sephadex G-100 in 1 4 M KCl medium, all of its radioactivity was present in two peaks corresponding to Peaks IA and II of Fig 2, in the respective proportions of the two labeled peaks of Fig 4 The results indicate that little, if any, of the label used in following the enzyme in the above-described experiments is attached to proteins other than the chymotrypsin-like and trypsin-like enzymes However, the yield of these enzymes in the TEAE-cellulose chromatography was only about 30% (by ^3H recovery)

A reasonable degree of purification for present purposes was, in practice, obtained by a series of gel filtrations (starting with Peak II from the crude extract) alternately in 1 4 M KCl solution and in low salt medium, as described for the rat mast cell chymotrypsin[1]

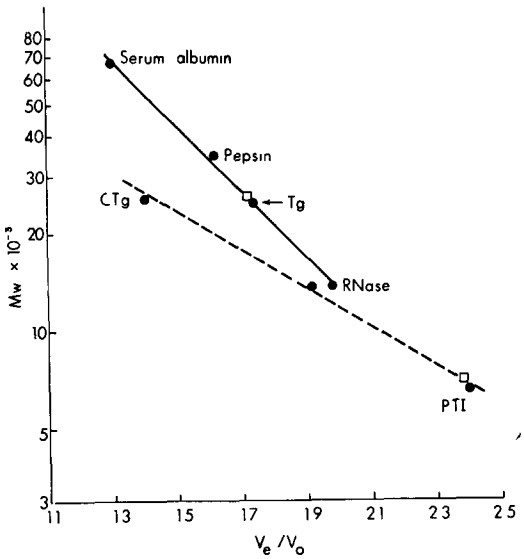

Fig 5 Molecular weight determination on Sephadex gel columns The calibration plot was constructed in each case using the data obtained on separate runs of each marker protein (●) shown (CTg = bovine chymotrypsinogen A, Tg = bovine trypsinogen, PTI = pancreatic trypsin inhibitor[20]) V_e = elution volume of the component studied, V_0 = void volume of the column (taken as the elution volume of Blue Dextran) Solid line experiments using G-100 gel (70 cm × 1 cm) in 1 4 M KCl medium (buffered by 0 05 M succinate–0 01 M EDTA, pH 6 0) Broken line experiments using G-75 gel (50 cm × 1 0 cm) in 0 1 M KCl medium (buffered by 0 02 M Tris–HCl–0 01 M EDTA, pH 7 5) V_e for the mastocytoma enzyme (□) was read in each case, by ^3H counting using the [^{3}H]DFP-inhibited enzyme, as well as by $A_{280\ nm}$ measurement in parallel experiments using the active enzyme, when V_e values identical to those shown for the labeled enzyme were obtained

Molecular weight

The purified preparation obtained was used for M_w determination[1,9] on a gel column (Fig 5) In 1 4 M KCl medium, a value of 26 000 was obtained, both for the active and the [^{3}H]DFP-inactivated enzyme On a gel column in 0 1 M KCl medium, on the other hand, a much smaller apparent M_w value was obtained (Fig 5), as found also[1] for the rat mast cell chymotrypsin in lower salt concentrations, due to binding to the gel column Intermediate apparent M_w values were found, as before[1], at intermediate salt concentrations (using a column of Agarose A–0 5 m gel in KCl solutions from 0 2 M concentration upwards), the constant value of M_w = 26 000 (±1000) being reached at and above 1 1 M concentration of KCl in the medium

Trypsin-like protease

On a G-100 column (Fig 2), a protease peak (IA) was eluted on the trailing side of Peak I Peak IA was characterized by its considerable activity on the trypsin substrate, tosyl-L-arginine methyl ester (TAME), and its total inactivity on the chymotrypsin substrate, BTEE It possessed protease activity on casein (Table I) at about the same ratio to its esterase activity as is found in the case of bovine pancreatic trypsin In the gel filtration of the labeled enzyme, a radioactive peak (enzymically inactive) was found (Fig 1) in the corresponding position The latter always remained

TABLE I

RELATIVE ACTIVITIES OF THE TRYPSIN-LIKE PROTEASE

The relative activities are expressed for each enzyme independently For conditions see Table II

Substrate	*Relative activity*	
	Peak IA enzyme	*Bovine trypsin*
TAME	1 00	1 00
BTEE	0 00	0 00
Casein	0 15*	0 12*

* Ratio of $\Delta A_{280\ nm}$ per 2 h in casein assay to $\Delta A_{247\ nm}$ per min in TAME assay

in the position of Peak IA, even after repeated filtration of that fraction in 1 4 M KCl medium, when it was completely separated from the heparin The position of this peak in the G-100 column effluent (in 1 4 M KCl) corresponded, by comparison with the calibration of Fig 5, to M_w about 35 000

Specificity

The heparin-free enzyme in Peak II (Fig 2), partly purified by further gel filtration (as noted above), was examined for activity on several substrates (Table II) It acts upon tyrosine and tryptophan esters, as well as on casein Activity on *N*-acetyl-tryptophan amide substrates was found to be, at the most, not greater than the relatively low activity of α-chymotrypsin thereon

The apparent Michaelis constant, K_m, for BTEE at pH 7 9, 25°, was determined as previously[1] for the rat enzyme and was found to be 6 10^{-4} M, a value close to that determined for bovine pancreatic α-chymotrypsin under the same conditions

TABLE II

SPECIFICITY OF THE CHYMOTRYPSIN-LIKE ENZYME

Measurements were made at 25 0 ± 0 2°, in 0 08 M Tris–0 10 M $CaCl_2$ (pH 7 8), except that casein was in 0 05 M phosphate (pH 7 6) Spectrophotometric recording was made of the absorbance change at 256 nm (BTEE), 247 nm (TAME), or 300 nm (tryptophan substrate) The activity on the substrate relative to that on BTEE is expressed (per min of reaction on each substrate) independently for the mastocytoma protease (MP) and for bovine α-chymotrypsin (CT) Reaction period, period over which average rate measurement was made Relative enzyme concentration, concentration of mastocytoma protease relative to the concentration used in the BTEE experiment The relative activity values are corrected for the concentration differences

Substrate	*Concentration*	*Reaction period*	*Relative enzyme concn*	*Relative activity on substrate*	
				MP	*CT*
BTEE	5 10^{-4} M	3 min	1	1 0	1 00
Acetyl-L-tryptophan methyl ester	5 10^{-4} M	10 min	5	0 15	0 43
TAME	10^{-4} M	20 min	5	0 00	0 00
Casein	0 5%	2 h	10	0 18*	0 28*

* Ratio of $\Delta A_{280\ nm}$ per 2 h in casein assay to $\Delta A_{256\ nm}$ per min in BTEE assay

In view of the possible role of the components of the mast cell granules in degradative attack on connective tissues in inflammatory proteases, the enzymes present in the total extract of the granules were examined for collagenase or elastase activity Using the elastin–orcein[8] or the rat-tail collagen solubilization[7] assay systems, no activity of either type was detectable in the conditions used, these, involving incubations of 24 h, were such that even a feeble activity would be detectable

Inhibitions

Indole is a strong competitive inhibitor of pancreatic α-chymotrypsin[10,11] Since the indole derivative, 5-hydroxytryptamine, is a physiologically active component of the granules of the P815Y mastocytoma[5], the effect of this compound and of indole on the chymotrypsin-like protease was examined 5-Hydroxytryptamine was found to be strongly inhibitory, but exact measurements were not made, since, in the assay system used (employing spectrophotometry in tyrosine or tryptophan ester solutions) the absorbance at the useful wavelengths even of concentrations as low as 1 10^{-4} M 5-hydroxytryptamine was so high as to decrease greatly the accuracy of the assay The absorbance at 300 nm of indole, however, is low enough to permit its

TABLE III

INHIBITIONS BY INDOLE AND BY CHYMOTRYPSIN INHIBITOR I FROM POTATOES

The activity is expressed as a percentage of that in a control sample in the absence of inhibitor MP = mastocytoma chymotrypsin-like protease (purified by TEAE-cellulose chromatography and gel filtrations) CT = bovine α-chymotrypsin, used in a similar experiment Inhibition by indole assays on acetyl-L-tryptophan methyl ester (5 10^{-4} M, pH 7 8, with 0 05 M $CaCl_2$ present, 25 0 ± 0 2°), recording at 300 nm over a 5-min period and plotting the initial velocity Potato inhibitor assays on BTEE in the same conditions Enzyme was either introduced (without preincubation) to start the assay, or was preincubated with inhibitor for the time shown, followed by substrate addition

Inhibitor	*Final concn (M)*	*Activity (% of control)*	
		MP	*CT*
Indole	0	100	100
	5 10^{-4}	76 5	80 3
	1 10^{-3}	70 6	75 9
	2 10^{-3}	29 4	48 9
Potato inhibitor	0	100	
	8 10^{-4}	0	
	3 2 10^{-4}	11	
	3 2 10^{-5}	25	
	3 2 10^{-5} (10 min)	0	
	1 5 10^{-5} (10 min)	16	
	0 7 10^{-5} (10 min)	35	

inhibition to be measured Such measurements (Table III) showed that indole is a slightly stronger inhibitor of this chymotrypsin-like enzyme than of pancreatic chymotrypsin Under similar conditions (at pH 7 6 instead of our 7 8) the pancreatic enzyme has a value of K_i for indole[11,12] of 6 10^{-4} M, a slightly smaller value is indicated for the mastocytoma enzyme

A highly effective inhibitor of pancreatic chymotrypsin has been purified from potatoes by BALLS AND RYAN[12] It does not inhibit the activity of trypsin on TAME (ref 13) A sample was kindly provided by Dr C A Ryan, and proved to be strongly inhibitory of the mastocytoma chymotrypsin-like enzyme (Table III) The reaction required up to 10 min for completion, at a concentration of about 10^{-5} M of the inhibitor

The irreversible inhibitors, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), TLeuCK, and TLysCK, rapidly alkylate, respectively, the active-center histidine residue from mammalian pancreatic chymotrypsin A, chymotrypsin C and trypsin[14–16] As expected, the mastocytoma chymotrypsin was not inhibited by TLysCK, but was inhibited, at about equal rates, by low concentrations of TPCK or TLeuCK (Fig 6) Although the rates observed are slower than those for bovine pancreatic chymotrypsin A, they are within the range of reactivity observed by MOCKEL AND BARNARD[17] for these phenylalanine- and leucine-based alkylating inhibitors acting on other vertebrate chymotrypsins

The trypsin-like enzyme of Peak IA (see above) was fully inhibited by TLysCK in an experiment parallel to that of Fig 6 It showed, as expected, no inhibition by TPCK in those conditions

It is evident from the labeling experiments (Fig 1) that the chymotrypsin-like protease is inhibited by DFP This was further examined the enzyme, when treated with 5 10^{-5} M DFP (pH 7 8, 25°) rapidly lost activity, after 45 min only 3% of the activity on BTEE remained This fast reaction rate is characteristic of an active center serine, although the rate found was approximately 3 times slower than that with bovine pancreatic α-chymotrypsin in the same conditions

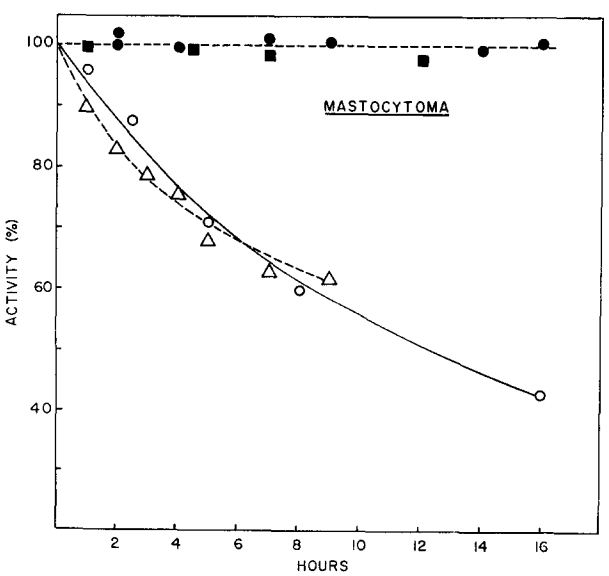

Fig 6 Inactivation of the purified chymotrypsin-like enzyme by specific alkylating agents Activity was measured (as a percentage of the initial activity) on BTEE, during incubation with 2 10^{-4} M TPCK (○—○), or 10^{-3} M TLeuCK (△ — — △), or 10^{-3} M TLysCK (■), or no inhibitor (●), all in 0 08 M Tris–0 02 M $CaCl_2$ (pH 7 2) in 3% (v/v) methanol, 25°

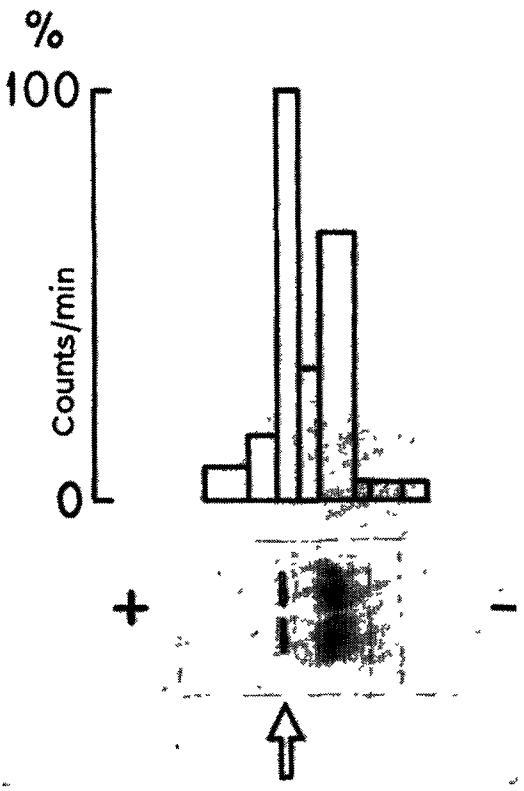

Fig 7 Electrophoresis on cellulose acetate of the [^{3}H]DFP-inactivated chymotrypsin-like enzyme, purified by three gel-filtration steps Run in 0 04 M Tris–HCl–0 001 M EDTA (pH 7 2), 4°, 20 min, 300 V, 3 mA The arrow shows the point of application After the staining for protein on one half of the membrane, the other half was cut into strips as indicated, for radioactivity measurement (^{3}H counts/min, shown on relative scale)

Stability

It can be seen from Fig 6 that the enzyme, when incubated at pH 7 8 at 25°, is stable for many hours This stability is greater than that shown by the rat mast cell protease[1] It was observed, however, that the trypsin-like enzyme (Peak IA, Fig 1C) was unstable in the active form, even at 4° (pH 6) about half of the activity on TAME was lost in one day, presumably due to self-digestion This might be due to the absence of calcium ions in the medium used in the purification, since calcium is commonly employed to stabilize pancreatic trypsins

Electrophoresis

In electrophoresis on cellulose acetate (Fig 7), part of the labeled chymotrypsin-like protein remained at the origin, while almost all of the remainder travelled in a band that was cationic at pH 7 2 and carried essentially all of the rest of the radioactivity Since the material remaining at the origin did not move under acidic or alkaline pH conditions, it is attributed to a partial denaturation due to the drying of the sample on the membrane (it being observed that freeze–drying inactivates this enzyme) If this is so, the undenatured enzyme comprises a single species

DISCUSSION

The mouse mastocytoma cells are seen to contain an enzyme which has the same characteristics as the enzyme studied[1] from normal mast cells and as pancreatic chymotrypsin, in terms of substrate specificity, specific active center inhibitions, and M_w Like the rat enzyme, it is bound in the granules of the mast cells, and is readily linked ionically to heparin in solution and, presumably, in the granules Potassium ions are much more effective than sodium in displacing it from heparin, for both the enzymes Hence, the tumor mast cells of the mouse synthesize an equivalent product The amount per cell, however, is reduced based upon mast cell counts, the content of enzyme (measured both by activity and by the number of molecules of [^{3}H]DFP reacted in the chymotrypsin fraction in each case) was, per mouse mastocytoma cell, only about 5% of that per rat mast cell The results are consistent with the known cytochemistry of this tumor mast cell It, too, contains many cytoplasmic granules, but these have much less capacity than in the normal mast cell to stain metachromatically with appropriate dyes, and they appear to contain considerably fewer sulfated groups of heparin[5] They also contain histamine and 5-hydroxytryptamine, but to a much lesser degree than normal mast cells[5] Production of the specific mast cell products is, therefore, being partly suppressed in this tumor, and this applies to the chymotrypsin-like protease, too

The position of elution of this enzyme on the Sephadex G-100 column used in the preparation (at high salt concentration) coincided with that of the rat enzyme, in agreement with the measurements of M_w The anomalous retardation of the mouse mastocytoma chymotrypsin on both dextran and polyamide gel columns, such that $>$ 1 M salt solution or 0 05 M HCl solution (Fig 3) is required for normal mobility thereon, was found similarly[1] with the rat mast cell chymotrypsin In the latter case, it was attributed[1] to its basicity, together with some other factor such as a concentration of aromatic groups Basicity of the mouse mastocytoma chymotrypsin has been demonstrated here by electrophoresis

The high affinity of the enzyme for indoles is an additional, interesting observation made on the mouse enzyme (and not yet tested in the rat) Since 5-hydroxytryptamine is an active agent stored in the mast cell[3], the presence of both it and this enzyme in the granule would provide a mutually inactivating system When the granules are released into a different milieu, liberation of both agents would occur, to promote inflammatory processes This would, indeed, be a reason for the specificity of the protease, if it has become adapted to provide an indole-binding site

The mast cell trypsin recognised here has no measurable counterpart in the normal mast cells from the rat[1,18] However, histochemical evidence, based on the use of benzoylarginine naphthylamide or a naphtholic ester of ε-amino caproate, has led to reports that a trypsin-like enzyme is present in human, canine and bovine mast cells and in a cat mastocytoma[19–22], as well as a histochemically detected chymotrypsin-like activity The separation obtained here shows that two distinct proteins are, in fact, involved Normal mast cells from mice without tumors have not been obtained in sufficient quantity for us to isolate their proteases, but cytochemical experiments have shown[23] that normal mast cells from the mouse peritoneum (in the same inbred strain of mice) appear to be essentially devoid of the tryptic activity, although they have the chymotryptic activity in abundance Investigation of the

differential synthesis of these two enzymes in the malignant growth of these cells is, therefore, indicated

ACKNOWLEDGEMENTS

This work was supported by Grant GM-16726 from the National Institutes of Health, U S Public Health Service, by Grant DRG-867 from the Damon Runyon Memorial Fund, and (for supporting services) N A S A Grant NGR33-015-002 We thank Dr Glenn Fischer for his great assistance in supplying mice bearing the P815Y tumor for our laboratory line, Dr C A Ryan (Washington State University, Pullman) for the gift of a pure specimen of potato chymotrypsin inhibitor I, and Dr M Laskowski, Sr (Roswell Park Memorial Institute, Buffalo) for the gift of pure pancreatic trypsin inhibitor

REFERENCES

1 J Kawiak, W H Vensel, J Komender and E A Barnard, *Biochim Biophys Acta*, 225 (1971) 172
2 I Pastan and S Almquist, *J Biol Chem*, 241 (1966) 5090
3 H Selye, *The Mast Cells*, Butterworths, Washington, 1965
4 T B Dunn and M Potter, *J Natl Cancer Inst*, 18 (1957) 587
5 D Glick, D von Redlich and B Diment, *Biochem Pharmacol*, 16 (1967) 553
6 S Norn, *Acta Pharmacol*, 25 (1967) 281
7 I Mandl, J D MacLennan and E L Howes, *J Clin Invest*, 32 (1953) 1323
8 L A Sachar, K K Winter, N Sicher and S Frankel, *Proc Soc Exp Biol Med*, 90 (1955) 323
9 P Andrews, *Biochem J*, 91 (1965) 222
10 H Huang and C Niemann, *J Am Chem Soc*, 75 (1953) 1395
11 H I Abrash and C Niemann, *Biochemistry*, 4 (1965) 99
12 A K Balls and C A Ryan, *J Biol Chem*, 238 (1963) 2976
13 C A Ryan, *Biochemistry*, 5 (1966) 1592
14 E B Ong, E Shaw and G Schoellman, *J Biol Chem* 240 (1965) 694
15 T Tobita and J E Folk, *Biochim Biophys Acta*, 147 (1967) 15
16 E Shaw, M Mares-Guia and W Cohen, *Biochemistry*, 4 (1965) 2219
17 W Mockel and E A Barnard, *Biochim Biophys Acta*, 141 (1969) 370
18 D Lagunoff and E P Benditt, *Ann N Y Acad Sci*, 103 (1963) 185
19 G G Glenner and L A Cohen, *Nature*, 185 (1960) 846
20 G G Glenner, V K Hopsu and L A Cohen, *J Histochem Cytochem*, 10 (1962) 109
21 D Lagunoff, E P Benditt and R Watts, *J Histochem Cytochem*, 10 (1962) 672
22 N Ende, Y Katayama and J V Auditore, *Nature*, 201 (1964) 1197
23 E A Barnard, *Int Rev Cytol*, 29 (1970) 213