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NON-PANCREATIC PROTEASES OF THE CHYMOTRYPSIN FAMILY

I. A CHYMOTRYPSIN-LIKE PROTEASE FROM RAT MAST CELLS

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SUMMARY

A protease has been purified from the isolated granules of mast cells from the peritoneal cavity of the rat. It resembles pancreatic chymotrypsin in most functional aspects, and has (by gel filtration measurement) the same molecular weight. It is active on tyrosine esters, but not arginine esters, and has a pH optimum and Michaelis constant in the hydrolysis of benzoyl-L-tyrosine ethyl ester (BTEE) that are very similar to those for bovine α-chymotrypsin. It exhibits the active-center-directed reactivities that are associated with the serine and with the histidine residues in chymotrypsin. There is evidence to suggest that it exists in the mast cell not as a zymogen, but as the active enzyme, bound to heparin.

This enzyme exhibits an exceptional affinity for Sephadex or Bio-Gel gels, and is strongly retarded in filtration on these in neutral 0.1 M salt solutions. This leads to large errors in molecular weight estimation, which are removed when the filtration is conducted in 1.4 M KCl solution. The origin of such anomalies on the gel columns is discussed.

INTRODUCTION

Pancreatic chymotrypsins have been studied from a number of vertebrate species and are, by direct or indirect evidence, homologous[1–6]. Molecular evolutionary information can be derived by examining such a series of proteins, but could be considerably extended if a series of non-pancreatic proteases in vertebrates could be shown to be structurally related to the pancreatic set. The pancreatic chymotrypsins and trypsins from the vertebrates are extracellular, single-chain, disulfide-

Abbreviations: BTEE, benzoyl-L-tyrosine ethyl ester; DFP, diisopropylphosphorofluoridate; TAME, α-*N*-tosylarginine methyl ester; TPCK, L-1-tosylamidophenylethylchloromethyl ketone.

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bridged proteins having a serine–histidine active center[2–7], whereas little is known of the intracellular proteases of vertebrate tissues. A relationship between one line of the latter and the extra-cellular pancreatic chymotrypsin line would be of distinct evolutionary interest.

The mast cells, which are part of the connective tissue system of vertebrates, appear to offer a useful case for such an investigation. There is cytochemical evidence[8,9] for the existence in rat mast cells of an enzyme with activity on amide and ester substrates resembling that of chymotrypsin, and such an esterase and protease activity has been found by assay of saline extracts of mast cells or mast cell granules[10,11] or tissue rich in mast cells[12,13]. The determination of the number of such esterase molecules within each cell[14], as well as the confirmation[15] of the location of these sites within the characteristic granules of the mast cells, has been accomplished by the use of intact mast cells which had been incubated with isotopic diisopropylphosphorofluoridate (DFP). In addition to its susceptibility to DFP, the enzyme has, on the basis of substrate specificity studies on rat mast cells or their extracts[9–11,13] a remarkable resemblance to pancreatic α-chymotrypsin. The present report deals with the isolation of a chymotrypsin-like enzyme from rat mast cells and on some of its enzymic characteristics.

Further, we may note that, in view of the abundance of mast cells in the mammalian body and the apparent involvement of their granules in allergenic and inflammatory responses[16–18], it is of interest to have biochemical information on the individual proteases in these granules, to consider a possible role for these in attacking tissue components.

MATERIALS AND METHODS

Materials

Benzoyl-L-tyrosine ethyl ester (BTEE) and α-*N*-tosylarginine methyl ester (TAME) (M.A. grade) were from Mann, L-1-tosylamidophenylethyl chloromethyl ketone (TPCK) (Grade I) from Cyclo Chemical Corp., Tween 80 from General Biochemicals, and deoxyribonuclease I (1575 units/mg, with negligible chymotryptic activity) from Worthington. [^{32}P]DFP and [^{3}H]DFP were from Amersham Searle, Chicago; they were stored in anhydrous propane-1,2-diol at −20°, and were placed in aqueous media only at the time of use. All other materials were of analytical grade, or were as specified elsewhere[1,2,19]. For the gel filtration molecular weight standards, homogeneous pancreatic ribonuclease A was prepared[19], porcine pepsin and bovine pancreatic α-chymotrypsin (thrice crystallized, 45 units/mg, used also for standardizing assays on BTEE and casein) were from Worthington and the basic trypsin inhibitor from bovine pancreas was a pure specimen[20] kindly donated by Dr. M. Laskowski, Sr. Sephadex (Pharmacia), and Bio-Gel and Agarose (Bio-Rad), gels were pre-equilibrated for 2 days at room temperature with the buffer to be used, after removal of all fines, and the gel columns were poured and used at 4°.

Isolation of mast cell granules

Rats (Holzmann strain, males) weighing 200–300 g, were lightly anesthetized with ethyl ether and 7–8 ml of sterile Hank's solution (Medium 199-IX, Grand Island Biol. Co.) was then introduced through a small incision into the peritoneal cavity.

After massage for 1 min, the suspension of peritoneal cells was withdrawn, and was kept at 0° while others were collected. Suturing (with rapid healing) and later re-use of the same donors was usually performed.

All the peritoneal cells obtained on one occasion (usually from 30–40 donors) were at once collected by sedimentation at 900 × *g* for 10 min. This and all the preparative procedures were conducted at 0–4°. The pellet of cells was resuspended in 5 vol. of 0.25 M sucrose containing EDTA (0.01 M) and Tween 80 detergent (0.01%), adjusted with NaOH to pH 6.0. This suspension was homogenized (in a Potter homogenizer with mechanically driven teflon plunger at a speed chosen so as not to break the cell nuclei) for 10 min. By centrifugation at 900 × *g* for 5–10 min, a sediment was removed that contained (when examined by light or electronmicroscopy: see below) some residual intact cells, fragments of cytoplasm, all of the cell nuclei, and some clumped granules; the free mast cell granules and some other particulate material remained in suspension. The supernatant fraction was separated, and the pellet re-homogenized in 2 vol. of the same medium and re-centrifuged. This latter process was repeated four further times, until the monitoring of samples with toluidine blue (see below) showed that the release of mast cell granules into the supernatant was ceasing. Pooling of the supernatant fractions provided a suspension containing the majority of the mast cell granules. These granules were sedimented at 10 000 × *g* for 30 min, re-washed similarly, and then used for the preparation of the enzyme. The residual supernatant at this final stage also exhibited some enzymic activity on BTEE, indicating an extraction of a small fraction of the enzyme.

Activity determinations

Chymotryptic activity was determined[1] using BTEE ($5 \cdot 10^{-4}$ M) as substrate, but with the $CaCl_2$ concentration 0.025 M and methanol 15%. The initial rate of hydrolysis is expressed in units of the change in $A_{256\ m\mu}$/min, at pH 7.9 and 25.0°. If $CaCl_2$ was omitted and 0.001 M EDTA added, essentially the same activity was recorded. Tryptic activity was determined[1] on TAME as substrate, reading $A_{247\ m\mu}$ similarly. For protease activity, a version[1] of the Kunitz assay on casein was used, and expressed in units of the change in $A_{280\ m\mu}$/min, similarly. The casein substrate for these assays was pretreated[21] to remove all ribonuclease activity, which would interfere at the level of crude extracts.

Identification of mast cell granules and counting of mast cells

For cell counting (by hemocytometer) and for routine examination during the cell fractionation steps, a specimen was treated in suspension with a 0.1% solution of toluidine blue in 4% formalin–80% ethanol and examined by light microscopy for the characteristic metachromatic[16] staining. For the specimens identified in the electron microscope, a sample of the preparation of mast cell granules was fixed in suspension in 4% glutaraldehyde in collidine buffer (pH 7.0, 4°, 2 h) and processed for electron microscopy[15]. The electron microscopy was kindly performed by Dr. R. Zobel.

Other methods

Protein concentrations were determined either (on a relative basis) by spectrophotometry at 280 mμ with correction (using $A_{260\ m\mu}$) for any nucleotide material

present[22], or by the method of LOWRY *et al.*[23] as modified by LAYNE[22]. Heparin was determined by measurement of uronic acid[24], and was expressed in units of the final absorbance at 530 mμ in the assay conditions[24], read against an appropriate blank. Radioactivity determinations were by liquid scintillation counting[19], adding, where necessary for solubility, 1 ml of Soluene (Packard Instruments, Inc.) and 50 μl concentrated HCl to the vial; efficiency of counting was determined on each ^{3}H-containing sample by subsequent addition of a [^{3}H]toluene internal standard.

Extraction of isotopically labeled enzyme

Cells, immediately after collection from the peritoneum and washing in Hank's medium, were incubated in suspension in that medium ($2 \cdot 10^6$ cells/ml) with $1 \cdot 10^{-4}$ M [^{32}P]DFP (12 mC/mmole) or [^{3}H]DFP (4.8 C/mmole) at pH 7.6 and 25° for 45 min. After three washings, each in 15 ml of the medium, the cells were treated similarly (1 h) with $1 \cdot 10^{-2}$ M unlabeled DFP, for full exchange of unreacted isotope. After re-washing in Hank's medium, the mast cell granules were isolated and extracted(in 1.2 M NaCl at pH 6.4) as described below.

RESULTS

Isolation of the protease

Isolation of the enzyme from separated mast cells. Mast cells were separated from other cells present in the peritoneal fluid by fractionation in a sucrose density gradient, following the procedure of PADAWER AND GORDON[25]. A fraction containing about 80% (by differential cell count) of mast cells was separated thus, compared to the 4% in the initial peritoneal fluid samples. These mast cells were homogenized gently in the equilibrating sucrose solution (density 1.200) at 4°. In confirmation of the reports noted above, the mast cell homogenates contained a high degree of enzymic activity on BTEE as substrate.

In initial experiments the homogenate was extracted at 4° in 1 M NaCl (in 0.05 M dimethylglutarate buffer (pH 6.0)) or in 0.5 M NaCNS, and was centrifuged (at 900 × *g*, 30 min) to give a solution containing the BTEE esterase activity. Alternatively, homogenization was continued further, when the cell nuclei were broken: clumped material was sedimented at 200 × *g*, and the enzyme in the granules (which were in the supernate) was solubilized in the NaCl or NaCNS media. These procedures followed the method of LAGUNOFF AND BENDITT[11]. Such solutions were not satisfactory for initiating the purification procedure, however, since they contained a large amount of DNA, as shown by their high viscosity and 260-mμ absorbance. This DNA interfered in the assay procedure, where a turbidity formed, attributed to precipitation of DNA-containing material at the lowered ionic strength: this phenomenon was prevented by prior incubation of the extract with deoxyribonuclease. Similarly, when an extract was made (in 0.5 M NaCNS–0.2 M Tris (pH 6.5)) of the nuclear fraction obtained by homogenization of mast cells in water, a solution with distinct BTEE esterase activity was obtained. This activity was increased by 45% after the solution was treated with deoxyribonuclease (0.1 mg/ml, 60 min, 35°). Hence it appears that problems will arise in the purification due to binding of the mast cell protease to DNA when this is present, as from broken cell nuclei. It had previously been noted that, when such a crude extract of mast cell

granules was dialyzed against water, most of the chymotryptic activity was in a precipitate[11,13]; we have noticed comparable effects, and believe that they are due to complexing of the protein with DNA. Care was, therefore, taken in the preparative methods to avoid disruption of cell nuclei, and EDTA was added to the media to minimize DNA release.

In the enzyme isolation method, the homogenate prepared from the purified mast cells was fractionated: the nuclei and cell debris were removed by centrifugation at 900 × *g*, and the mast cell granules were then obtained by sedimentation at

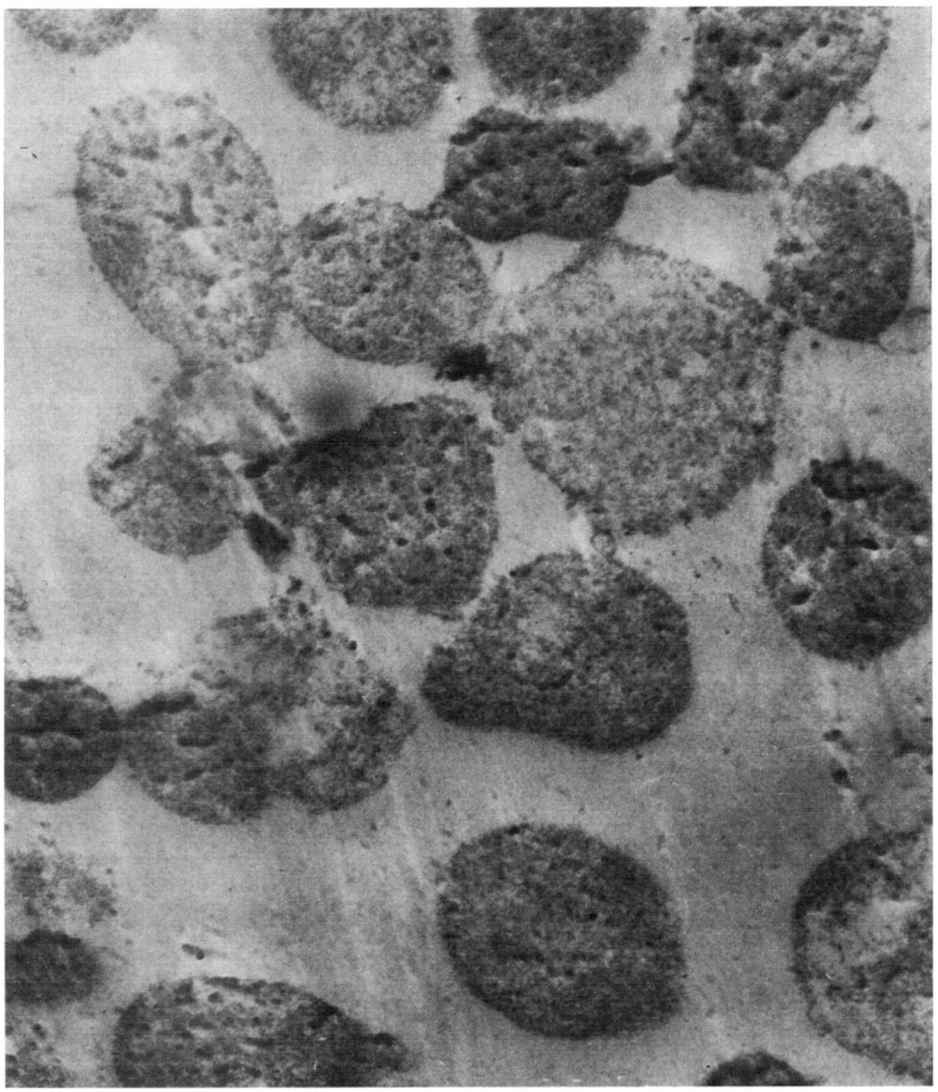
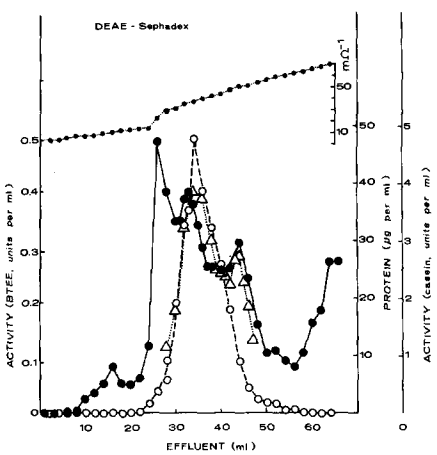


Fig. 1. The purified mast cell granule fraction. Electron micrograph showing a field typical of the whole specimen. Essentially only intact granules were present, with a few membraneous fragments. Fixation was in glutaraldehyde followed by osmic acid (× 23 200).

10 000 × *g* (30 min). Examination of this fraction in the electron microscope showed (Fig. 1) that it consisted essentially of clean mast cell granules; mitochondria and nuclei were absent, but a few adhering membranes were seen. These granules retained their characteristic metachromatic[16] staining when tested with toluidine blue.

These mast cell granules, suspended in the sucrose medium, were placed directly on a column of DEAE-Sephadex A-25, which was eluted with a salt gradient (Fig. 2). A large inactive protein peak was eluted first, while a peak with high activity on BTEE was obtained at about 0.6 M NaCl. Uronic acid estimation of heparin showed none was detectable in this latter peak; protease activity was found in two peaks, one of which coincided with the BTEE esterase peak (Fig. 2). This chromatography served to demonstrate that a chymotrypsin-like enzyme, free of heparin, can be isolated in one peak from intact mast cell granules, but it was not used routinely for preparation of the enzyme, due to considerable losses of the enzymic activity applied. Such losses were found whenever the enzyme was separated on DEAE-Sephadex, or on DEAE-cellulose (Whatman micro-granular DE-52) which gave results similar to those of Fig. 2; a number of such attempts were made with salt elution either in a gradient or in steps (and using either whole granules or a 1.2 M NaCl homogenate of them) with similar losses in all cases.

It should be noted that the granule fraction (Fig. 1) obtained in this procedure



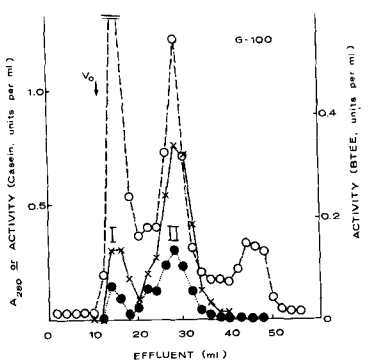


Fig. 2. Chromatography on a DEAE-Sephadex (0.9 cm × 75 cm) column of the eluate from the mast cell granules placed thereon. The medium was 0.05 M dimethylglutarate–0.01 M EDTA (pH 6.0) and a gradient from 0 to 3 M NaCl in the same medium was applied (shown by the conductivity readings, upper curve and scale). Fractions (1 ml) were sampled for protein[23] (●), protease activity on casein (△·····△), and activity on BTEE (○– – –○). Heparin was also present (not shown), in the first large protein peak only.

Fig. 3. Gel filtration of the mast cell granule extract on a Sephadex G-100 (0.9 cm × 65 cm) column. The medium was 1.2 M NaCl–0.05 M dimethylglutarate–0.01 M EDTA (pH 6.0). Fractions (2 ml) were sampled for protein (by $A_{280\ m\mu}$, ○– – –○), protease activity on casein (●·····●), and activity on BTEE (×—×). No activity on TAME was detectable anywhere in such chromatograms. Heparin was present in a peak coinciding with the protease Peak I, at a level of 200 units/unit of protease activity on casein; heparin was absent in Peak II, and present (at one-quarter its concentration in Peak I) in the enzymically inactive peak centered at 45 ml. V_0 denotes the void volume of the column. For further purification of the main Peak II material, the fractions at 25–32 ml were used.

carried the bulk of the BTEE esterase activity (Table I). The nuclear and supernatant fractions carried smaller amounts of activity, probably originating in some diffusion of enzyme from the granules. The relative activity in the supernatant fraction is actually less than that which is apparent, since the activity present in the granules was measured on a suspension of the latter, which leads to an underestimate of enzymic content.

Isolation of the enzyme from the peritoneal cell suspension. The procedure of first isolating the mast cell fraction from the peritoneal cell suspension limited greatly the amount of starting material that could be obtained for the preparation of enzyme, and that procedure was dispensed with once it was demonstrated that the same heparin-free enzymic peak could also be obtained from the whole peritoneal cell suspension.

The whole peritoneal cell suspension was used directly to prepare a granule fraction, as described in MATERIALS AND METHODS. This fraction contained a large percentage of mast cell granules, as shown by staining with toluidine blue. The isolated granules were extracted with a series of NaCl solutions to solubilize the chymotrypsin-like enzyme. At pH 6 (at 4°) extraction became maximal at 1.2 M NaCl concentration, as observed by LAGUNOFF[26]. In the preparative procedure, therefore, the granules were extracted with either 1.4 M KCl or 1.2 M NaCl in the same buffer (containing 0.01 M EDTA), with shaking or stirring for 30 min at 4°. After centrifugation as before, the supernate was filtered on a column of Sephadex G-100 which was equilibrated in and eluted by the 1.2 M NaCl medium. Two main peaks containing both protease and BTEE esterase activity were consistently observed (Fig. 3). Heparin was found in the first peak and in an additional peak after the protein peaks. Hence, some heparin is released in 1.2 M NaCl solution, while some remains in a protein–heparin complex which contains some of the mast cell protease (Peak I). When Peak I was re-filtered in the 1.2 M NaCl medium as in Fig. 3 (or better still, using 1.2 M KCl) about 80% of its BTEE esterase activity was now separated in the Peak II position, while none of the heparin moved there, but all of the latter traveled in Peak I with the residual bound enzyme.

Peak II (Fig. 3) appears to contain the chymotrypsin-like enzyme in a heparin-free form. This peak was collected and re-filtered in 0.1 M salt solution through a second gel column of smaller pore size (Sephadex G-25 or Bio-Gel P-2), and yielded the same result in either case. A small initial protein peak without enzymic activity was followed by an unusually retarded protein peak which had all of the protease and esterase activities that had been applied to the column. This peak emerged at a position just ahead of, and overlapping, the eluted excess inorganic salt (as shown by conductivity readings).

The latter protein peak was collected, and concentrated by ultracentrifugation at 105 000 × *g* for 72 h at 5° in a plastic tube. The layered solutions were rapidly frozen, and the lower one-fifth of the tube was cut off. When thawed, this fraction was found to contain all of the BTEE esterase activity present. Alternatively, cautious rotary evaporation was used for routine concentration of the enzyme. For the best preparation used, the filtration on Sephadex G-100 was repeated, followed by the filtration on Sephadex G-25 in 0.1 M NaCl, followed by concentration and dialysis against 0.1 M NaCl solution. Some data on the purification are noted in Table I. The proteolytic activity on casein remained identically associated with the

TABLE I

PURIFICATION OF THE MAST CELL CHYMOTRYPSIN

The activity is in units of μmoles BTEE hydrolyzed per min at 25° and pH 7.9. The protein concentration was determined[23] using bovine pancreatic chymotrypsin, arbitrarily, as a standard.

Stage	*Activity on BTEE (units)*	*Specific activity (units/mg protein)*
Mast cells, whole homogenate (5 ml)	74.3	0.85
Cell fractions		
(1) Nuclei + unbroken cells	13.5	0.17
(2) Granules	33.7	5.65
(3) Supernatant	11.0	1.11
Purification of Fraction 2		
DEAE-Sephadex, 2nd peak		33.3
or Sephadex G-100, Peak II		20.0
Sephadex G-100, Peak II (re-filtered) → Sephadex G-25 in 0.1 M NaCl		⩾60*

* Uncertain due to small amount of protein in the final preparation.

BTEE esterase activity in the purified material, when re-filtered on Sephadex G-100 or re-chromatographed on DEAE-Sephadex as above.

When the mast cells that had been separated from other cells on the sucrose gradient (see above) were used instead to prepare the pure granule fraction, results were obtained in the subsequent enzyme purification that were identical to those shown in Fig. 2 (if DEAE-Sephadex chromatography were used) or to those of Fig. 3 (if the Sephadex G-100 procedure was used). This confirmed that the contaminating cells in the peritoneal suspension were not the source of an appreciable part of Peak II; after this check was made, the cell separation step was eliminated.

Purification of isotopically-labeled enzyme. Mast cells were reacted in the intact state (*in vitro*) with [^{32}P]DFP or [^{3}H]DFP, given adequate washes (including an exchange with a 100-fold excess of unlabeled DFP), and used for enzyme preparation

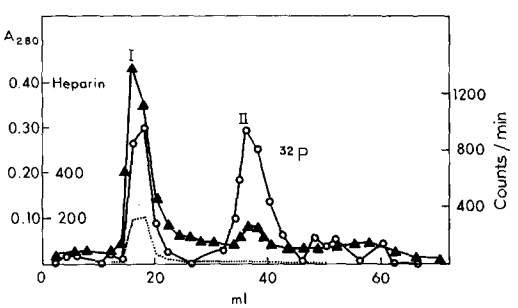


Fig. 4. Gel filtration on a Sephadex G-100 column of the [^{32}P]DFP-inhibited mast cell enzyme preparation, in the 1.2 M NaCl medium as used in Fig. 3. Fractions were sampled for protein (by $A_{280\ m\mu}$, ▲—▲), heparin (in arbitrary units; · · · · · ·) and radioactivity (counts/min per ml, ○—○). The minor peaks after Peak II were shown, on analysis, to contain no protein.

by a procedure identical to that described above. All activity on BTEE was abolished in the extract after the labeled DFP treatment. On filtration on Sephadex G-100 and scintillation counting of the effluent (Fig. 4), with either type of labeling a profile was obtained similar to that found previously (Fig. 3) for the enzymic activity; a well-labeled protein peak was eluted at a position exactly equivalent to that previously occupied by the BTEE esterase Peak II. In repeat experiments using 1.2 M KCl in the eluant, this salt was found to be much more effective than 1.2 M NaCl in releasing the labeled chymotrypsin from its complex with heparin (present in Peak I).

Material from such a ^{3}H-labeled peak corresponding to the chymotrypsin-like enzyme was filtered on a gel of smaller pore size, Bio-Gel P-2, in water for de-salting (Fig. 5). A labeled, salt-free protein peak was eluted near the void volume, as expected for the inactivated chymotrypsin-like enzyme, but a further, much larger, labeled protein peak was anomalously retarded, being centered only a little ahead of the salt peak. However, when the ^{3}H-labeled enzyme fraction (Peak II) from the Sepha-

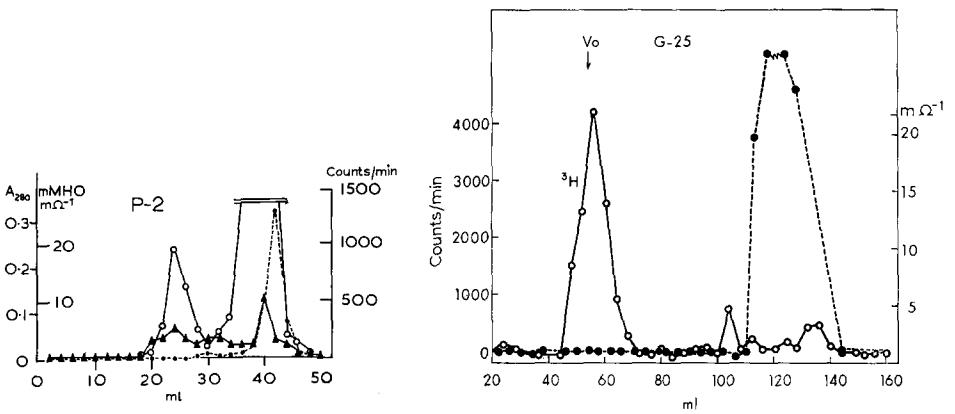


Fig. 5. Gel filtration on Bio-Gel P-2 in water, of material from a ^{3}H-labeled Peak II obtained as in Fig. 4. Fractions were sampled for $A_{280\ m\mu}$ reading (▲—▲), ^{3}H counts/min (○—○) and salt content (by conductivity reading, ●- - - - -●).

Fig. 6. Gel filtration on Sephadex G-25 in 0.05 M HCl of a sample parallel to that used in Fig. 5. Details as for Fig. 5. Protein was present in the first peak only.

dex G-100 column was run, instead, on a Sephadex G-25 (or a Bio-Gel P-2) gel column equilibrated and eluted with 0.05 M HCl, a different profile was obtained (Fig. 6). In this medium, the enzyme was eluted entirely at the void volume and was all salt-free. Sephadex G-25 and Bio-Gel P-2 gels could be used interchangeably in each of these two experiments, without change in the results. Only the salt-free peak material was used in the further studies. If necessary, it could be obtained in pure form by a chromatography on DEAE-Sephadex as described above (Fig. 2), followed by a similar desalting in 0.05 M HCl of the labeled peak and removal of the HCl by dialysis or freeze-drying.

Properties of the purified enzyme

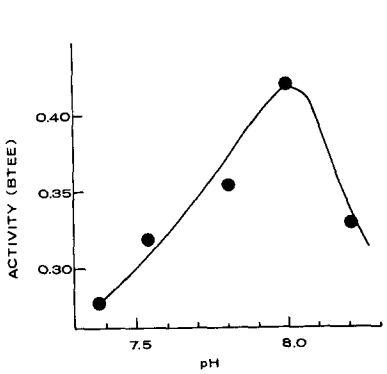
Enzyme activity. The active enzyme as prepared by this gel filtration method had a high esterase activity on BTEE (Table I) and protease activity on casein; these

two activities were always parallel in the peaks of this enzyme obtained in the various separations described above, and it is concluded that, as with pancreatic chymotrypsin, they are intrinsic properties of one enzyme molecule. The purest preparation of the enzyme is obtained by a combination of the DEAE-Sephadex chromatographic and Sephadex G-100 gel filtration procedures. It has no detectable tryptic activity as measured[1] on TAME. The activity on casein was 21% of that of bovine pancreatic α-chymotrypsin, when equal amounts (as measured by the activity on BTEE at pH 7.9) of the pancreatic enzyme and the mast cell chymotrypsin were compared.

It is not yet been feasible to determine very accurately the specific enzymic activity, for comparison with pancreatic chymotrypsin, due to the very small amounts of enzyme prepared so far in the purest state. From estimates made on the basis of the incorporation of DFP (Fig. 5), as a measure of the number of active centers present, and the equivalent activity on BTEE, the specific activity on that substrate is of the same order of magnitude as that of bovine α-chymotrypsin. When the refiltered preparation was analyzed using bovine chymotrypsin as a standard for the determination[23] of protein content, a specific activity on BTEE of the order of 60 units/mg was obtained (Table I). Commercial thrice crystallized bovine α-chymotrypsin had a value of 45 units/mg in the same conditions.

The pH dependence of activity on BTEE was determined (Fig. 7) in conditions where the enzyme is essentially saturated with substrate. An optimum at pH 8.0 was found. The apparent Michaelis constant, K_m, was determined (at pH 7.9, 25°) in the Tris–$CaCl_2$ assay medium, from a plot of $v/[S]$ against $[S]$, where $[S]$, the substrate concentration, varied from $6 \cdot 10^{-5}$ to $1.1 \cdot 10^{-3}$ M. A line obtained by least squares fitting gave a value for K_m of $1.1 \cdot 10^{-3}$ M.

Stability. The enzyme slowly loses activity at 25° at pH 7–8 (Fig. 8, broken line); about 30% of the activity remains after 12 h. This loss is presumed to be due to self-digestion; it was not prevented by the presence of $2 \cdot 10^{-2}$ M $CaCl_2$. At pH 6.0 and 4°, the enzyme is stable for at least 2 days, and manipulations of it were, therefore, performed in or near these conditions.



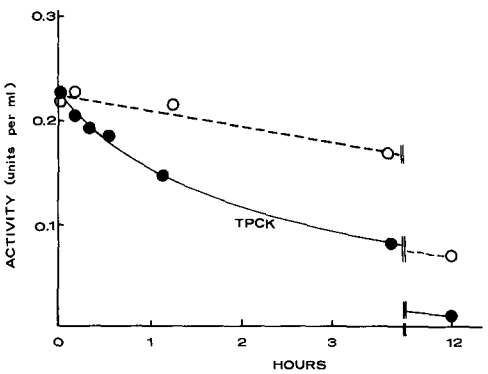


Fig. 7. Activity of the purified enzyme on $5 \cdot 10^{-4}$ M BTEE (in units/100-μl sample) in the standard assay medium adjusted to the pH values as shown.

Fig. 8. Inactivation of the purified mast cell chymotrypsin by $4 \cdot 10^{-4}$ M TPCK, in 0.05 M Tris–HCl–0.02 M $CaCl_2$ (pH 7.8) in 1% (by vol.)methanol, 25 ± 0.1°. ●, activity on BTEE; ○, activity after incubation in identical medium without TPCK.

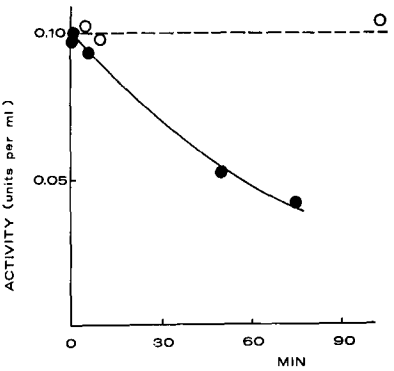


Fig. 9. Inactivation of the purified mast cell chymotrypsin by $1.0 \cdot 10^{-4}$ M DFP. The medium was 0.05 M dimethylglutarate (pH 6.0) (containing an unknown amount of NaCl) and 2.5% (by vol.) propane-1,2-diol, at 25 ± 0.1°. ●, activity on BTEE; ○, activity in incubation in identical medium without DFP.

Active center reactivities. DFP was applied at $1 \cdot 10^{-4}$ M concentration, at pH 6.0 and 25°. Enzymic activity was irreversibly lost (Fig. 9), with a half-time of 57 min for the inactivation. The same concentration of DFP was observed to inactivate faster at higher pH values in the range 6–8, but kinetics were not determined due to the concurrent spontaneous loss of the activity above about pH 7.

The specific chymotrypsin inhibitor, TPCK, which alkylates one active center histidine in the pancreatic enzyme[6,27] was applied at $4 \cdot 10^{-4}$ M concentration (Fig. 8).

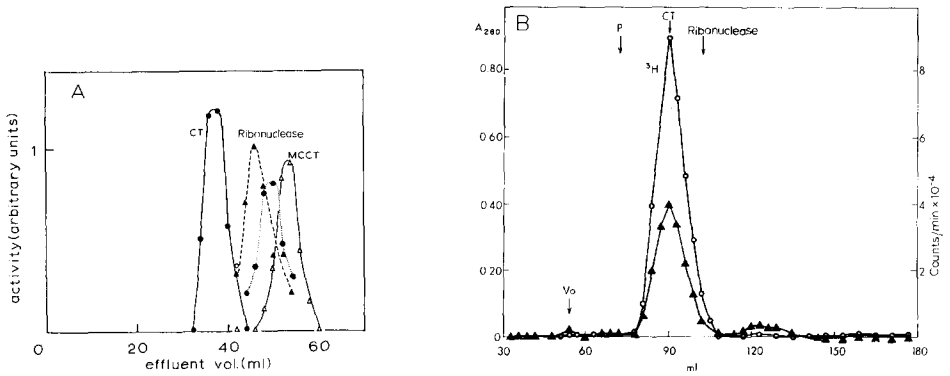


Fig. 10. Molecular weight determinations on Sephadex G-100 columns. A. In 0.1 M KCl medium. Included for calibration are bovine α-chymotrypsin (CT, ●—●) measured by activity on BTEE, ribonuclease A (▲) measured by activity on RNA[1] and pancreatic trypsin inhibitor (●·····●) measured by its inhibition[20] of the activity of bovine trypsin on TAME. The mast cell chymotrypsin (MCCT) was measured by BTEE activity (△) in a separate run on the same column. All activities are normalized to a relative value of 1, as shown. V_0, the void volume, was 18 ml (determined by elution of blue dextran), and V_i, the included volume, was 65 ml (determined by elution of phenol red). B. In 1.4 M NaCl medium. The purified [^{3}H]DFP-inactivated mast cell chymotrypsin was used. ○, radioactivity; ▲, protein (as $A_{280\ m\mu}$). Calibration employed bovine α-chymotrypsin (CT), ribonuclease A, and pepsin (P): their positions (centers of the peaks, in separate runs) are shown by the arrows. V_0 denotes the void volume (the column being larger than that used in A). For both columns, the medium used was buffered with 0.05 M dimethylglutarate (pH 6.0).

Correction must be applied for the considerable spontaneous inactivation at the pH used (Fig. 8). However, the inactivation by TPCK is highly significant, since curves such as that shown in Fig. 8 were consistently obtained in a set of similar experiments at pH 7.1 and 7.8; after correction for the spontaneous activity decay, the inactivation by TPCK reached on average 60% (at pH 7.1) and 90% (at pH 7.8), in 12 h at 25°.

Molecular weight. As noted during the purification, the enzyme was retarded considerably even on gels of very low theoretical exclusion limit, Sephadex G-25 or Bio-Gel P-2 (Figs. 5 and 6), when these columns were eluted with distilled water, but not when dilute HCl was used. Further, when the enzyme was gel-filtered on a calibrated[28] Sephadex G-100 gel column in 0.1 M KCl at pH 6 (Fig. 10A), the protein was eluted from the column just prior to the salt peak, and appeared to be smaller than a marker of molecular weight 6513, the pancreatic basic trypsin inhibitor[20]. In contrast, on a similar gel column in 1.4 M KCl the enzyme had an elution volume identical to that of bovine pancreatic α-chymotrypsin (Fig. 10B), and now emerged well before the trypsin inhibitor marker. The same results in each case were obtained in these KCl media when the buffer present was changed to 0.02 M Tris–HCl (pH 7.6).

Gel filtration in dilute HCl was also examined, in an experiment parallel to that of Fig. 10A, but using Sephadex G-50 in 0.05 M HCl. On this column (void volume 29 ml, included volume 81 ml) a linear calibration plot was again obtained, with ribonuclease and glucagon falling at their expected positions. DFP-inactivated bovine α-chymotrypsin[1] was eluted at 35 ml, and the purified [^{3}H]DFP-inactivated mast cell chymotrypsin was eluted at the same point in a parallel run. Hence, its molecular weight is not distinguishably different, within the accuracy of the gel filtration method, from that of bovine chymotrypsin. In a confirmation using another gel type, Agarose, a calibrated column of Bio-Gel A-0.5m in 1.4 M KCl was operated (at pH 7.6) in an experiment replicating that of Fig. 10B. The purified [^{3}H]DFP-inactivated mast cell chymotrypsin had an elution volume of 1.85 relative to the void volume for the column, while bovine pancreatic trypsinogen yielded a value of 1.95.

It was concluded (see DISCUSSION) that the behavior in the low-salt media was anomalous and that the true molecular weight of the protease is very close to that of bovine pancreatic chymotrypsin.

DISCUSSION

This protease is of especial interest for at least two reasons. Firstly, it shows a remarkable similarity to pancreatic chymotrypsin in several properties, including its active center reactivity. This encourages an examination for structural homology. Secondly, the presence in the mast cell granules of very large amounts of a specific protease would indicate a role for it, as has been suggested[14], in the functional activity of these cells; we may plausibly suppose that degradation of some target proteins occurs by the action of this enzyme, upon the release of the granules from a stimulated mast cell. It has been shown[14], by direct measurement of the number of DFP-reactive sites in the mast cells, that there are close to $8 \cdot 10^8$ molecules of such enzyme per rat mast cell. Hence, it is, indeed, a major storage product of these cells. The data presented here show that the chymotrypsin-like enzyme accounts for much of the total protease activity of the granule and for almost all of the activity on BTEE

and the reactivity towards DFP. We have extended the existing evidence[9–11,13] by showing that, in a purer preparation, a protease activity remains associated with very high activity on a chymotrypsin substrate, BTEE. The pH dependence of activity and the value of the apparent K_m for BTEE are also compatible with an active center similar to that of pancreatic chymotrypsin.

The reactivity with DFP is of the type that in other cases is due to a serine in the active center[4]. The reaction was fairly slow, but this is ascribed to the pH of 6.0 used (to avoid self-digestion): with pancreatic chymotrypsin the DFP reaction is also much slower at pH 6 than at pH 7–8. DFP reacted with the mast cell protease *in situ* in the intact cell, and after washing of those cells all of the chymotryptic activity was found to be inhibited. Entire granules (Fig. 1) from non-inhibited cells are active enzymically. These observations all indicate that the active center of this enzyme is readily available in the intracellular state, so that it does not exist as a zymogen comparable to pancreatic chymotrypsinogen. Since it is bound in the heparin granule (this work and ref. 15) it is proposed that the enzyme molecule is kept innocuous within the mast cell by this immobilization within the granule. This removes the need for an inactive precursor form.

The reaction with TPCK is of very great specificity for the chymotrypsin active center[2,27]. The combined occurrence of the TPCK and DFP reactions on the mast cell protease is good evidence that its active center is similar to that of pancreatic chymotrypsin. This conclusion needs confirmation by isolation of peptides containing an alkylated histidine and a phosphorylated serine, respectively.

The rate with TPCK here is much slower (about 40 times) than that[27] of bovine pancreatic α-chymotrypsin. It is still, however, a greatly facilitated alkylation compared to the negligible rate of reaction of this agent with normal groups in proteins. The reaction rates of TPCK with a series of vertebrate pancreatic chymotrypsins have been compared[2,29], and cover a wide range. Some of those rates are as slow as, or even slower than, that exhibited by the mast cell chymotrypsin. Hence, the comparison with the bovine enzyme as standard is an arbitrary one. The differences in TPCK alkylation rates of the pancreatic chymotrypsins are associated with quantitative differences in their specificity[2].

The retardation observed on gel columns in low or moderate salt concentrations is highly anomalous. This behavior is such that consecutive gel filtrations in media of low and high ionic strength lead to a high degree of purification of the enzyme. The anomalous retardation initially led us to assign an molecular weight of about 7000 to this enzyme, as noted in an abstract[30]. The behavior in sedimentation equilibrium also showed[30] an anomalously low apparent molecular weight; this is attributed to non-ideality in these conditions, an effect which has been found with other proteins in certain conditions, *e.g.* glyceraldehyde phosphate dehydrogenase in 1.3 M phosphate solution[31] or yeast hexokinase in acid[32], where apparent molecular weights down to about one quarter of the true value are registered in sedimentation equilibrium.

Both dextran and polyacrylamide gels showed strong retardation of this enzyme. This type of effect might be thought to be due to a non-specific adsorption of the protein to the gel, as has been found with amino acids and peptides having predominantly aromatic side chains[33–36], but its contribution in the case of an entire protein molecule is unknown. However, with aromatic peptides such as hexaphenyl-

alanine, this effect[36] was marked on Sephadex G-10, less on Sephadex G-25 and absent on Sephadex G-50, and was not removed in 1 M acetic acid, whereas the present anomaly was strong even on Sephadex G-100, and was removed in dilute acid.

Another possible cause of the anomaly would be the tendency[28,33] of basic proteins to bind to the acidic groups present as by-products in the dextran gel. This binding has generally been overcome by the use of dilute salt solutions, about 0.1 M ionic strength[28,37,38]. The polyacrylamide Bio-Gel is stated by the manufacturer[39] to be almost devoid of acidic groups, but binding of basic materials to it has been noted in practice. Even 1 M acetic acid was inadequate[34] as an eluant to overcome all of the retardation of arginine on Bio-Gel P-2. Proteins generally behave normally, however, on Bio-Gel[34,39], and gel filtration of proteins on Sephadex or Bio-Gel columns has been assumed to be without error from adsorption if a medium such as 0.1 M KCl or a dilute buffer solution is employed[28,37,39]. This clearly does not apply to the mast cell chymotrypsin, for which (Figs. 6 and 10, and Table II) either an ionic strength above 1 M or a very low pH (0.05 M HCl) are needed to suppress the binding to Sephadex or Bio-Gel columns.

When gel columns are operated at high ionic strength, the elution volume of a given molecule is changed due to the effect of the salt on the pore size of the gel.

TABLE II

GEL FILTRATION OF MAST CELL CHYMOTRYPSIN AND OTHER PROTEINS IN DIFFERING SALT CONCENTRATIONS

K_{av} is the relative elution volume of the center of the peak of the protein, corrected for the change of bed volume of the gel due to the salt concentration[40]. Sephadex G-75 was employed.

Protein	K_{av}			*Mol. wt.*
	0.1 M KCl	*0.6 M KCl*	*1.1 M KCl*	
Pepsin	0.17	0.18	0.23	35 000
Trypsinogen	0.29	0.30	0.32	24 500
Ribonuclease	0.55	0.49	0.46	13 800
Mast cell chymotrypsin	1.04 (M_{app} 100–1000)*	0.36 (M_{app} 20 000)*	0.30	25 000**

* Apparent molecular weight.
** Molecular weight obtained from the K_{av} value in 1.1 M KCl medium.

It is preferable in such a case to compare not the elution volumes, V_e, which will vary with the salt concentration, but the fractional volume available, K_{av}, as employed by LAURENT AND KILLANDER[40]:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_0 is the void volume and V_t the total bed volume. It has been shown[40] with many proteins on Sephadex columns that K_{av} values are a reliable guide to the molecular radii.

Elution, void and total bed volumes were determined here, and the corrected elution volumes, expressed as K_{av}, were found to remain almost constant (Table II), for proteins which are eluted normally (according to the molecular size) on gels at

lower (approx. 0.1 M) salt concentrations. These proteins can be used as standards at any ionic strength. Pancreatic ribonuclease showed a slight decrease in K_{av} with increase of ionic strength, attributed to adsorption due to its basicity. The mast cell chymotrypsin showed a large effect in the same direction (Table II), but this did not change further above 1.1 M KCl concentration. The best estimate of its molecular weight from gel filtration data is, therefore, that found in the latter medium, 25 000. Since the same elution volume was recorded for the active chymotrypsin and for the labeled form prepared after DFP inactivation at the intact cell level, it is concluded that this value represents the molecular weight of the native protease, without self-digestion.

The isolated protein has been observed to be cationic in cellulose acetate electrophoresis at pH 7–9, but insufficient amounts of completely pure material have yet been obtained for a determination of its isoionic point. Since ribonuclease (isoionic point 9.6) or lysozyme (isoionic point 11.0) or (Fig. 10A) the pancreatic trypsin inhibitor[20] (isoelectric point in similar conditions, 10.8), do not show such a strong binding, the basicity of the protease would have to be exceptional to account for its behavior; perhaps basicity is combined with some unusual conformation or concentration of aromatic groups. It is noteworthy, in any event, that a protein may possess properties such that a determination of its molecular weight, either by gel filtration using standard[28] conditions or by sedimentation equilibrium, gives a completely misleading result, unless data over a wide range of ionic strengths are analyzed.

The mast cell protease also interacts ionically with heparin or DNA. There seems to be a slow equilibrium between bound and unbound enzyme as shown by the two peaks obtained when protein is present (Fig. 3). Pancreatic chymotrypsin, on the other hand, shows only a slight affinity for heparin[26]. The affinity of the mast cell protease for heparin, and the need for the use of 1.2 M salt solution for release from that complex, are in accord with the interpretation[14] that this enzyme is bound ionically to heparin in its intracellular situation in the mast cell granules. It is interesting that K^+ is more effective than Na^+ in dissociating this complex.

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