PREPARATIVE PURIFICATION OF THE RAT MAST CELL CHYMASE
Characterization and Interaction with Granule Components*

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A chymotrypsinlike enzyme was localized to rat mast cell granules by histochemical techniques 25 yr ago (1) and was subsequently further demonstrated by the cleavage of synthetic substrates with whole cells (2), cell extracts (3), and granule preparations (4–6). The enzyme, termed "chymase" (3), was purified to a specific activity of approximately 60 U/mg from rat thyroid (7) and peritoneal mast cells (8) and was shown to have an apparent molecular weight of 23,000 (8) to 29,000 (5) and to be cationic based upon its electrophoretic mobility at alkaline pH (4, 6). The synthetic substrate specificity was similar to that of pancreatic α-chymotrypsin and the active site reactivity with irreversible inhibitors indicated the presence of serine and histidine residues (8).

The development of a preparative method for the isolation of the rat mast cell chymase has now permitted its purification to homogeneity, as assessed by analytical techniques, and the demonstration of a single cationic polypeptide chain composition. The activity of the chymase in the mast cell granule is partially masked by its binding to the macromolecular proteoglycan heparin (9, 10), with which it is released by immunologic activation of the isolated rat mast cell (10, 11). Further, the active site of soluble chymase is inhibited by 5-hydroxytryptamine (5-HT)1 but not by histamine. Thus, the expression of the proteolytic activity of mast cell granule chymase is regulated by two additional mediator components of the mast cell granule.

Materials and Methods
α-Chymotrypsin, three times crystallized, N-benzoyl-L-tyrosine ethyl ester (BTEE), D-tryptophan methyl ester, histamine, free base and dihydrochloride, 5-hydroxytryptamine hydrochloride, and L-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK) (Sigma Chemical Co., St. Louis, Mo.); casein, Hammersten quality and N-α-tosyl-L lysine chloromethane ketone (TLCK) HCl (ICN Pharmaceuticals Inc., Cleveland, Ohio); porcine intestinal heparin, 130 U/ml and sucrose-

* Supported by grants AI-07722, AI-10356, AM-05577, HL-17382, and RR-05669 from the National Institutes of Health.
† Postdoctoral trainee supported by training grant AM-07031 from the National Institutes of Health.

1 Abbreviations used in this paper: BTEE, N-benzoyl-L-tyrosine ethyl ester; DFP, diisopropylfluorophosphate; 5-HT, 5-hydroxytryptamine; SDS, sodium dodecyl sulfate; TGD, Tyrode's buffer containing 0.1% gelatin and 10 μg/ml deoxyribonuclease; TLCK, N-α-tosyl-L lysine chloromethane ketone; TPCK, L-tosylamide-2-phenyl-ethylchloromethyl ketone.
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Ultra pure (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.); deoxyribonuclease (Calbiochem, San Diego, Calif.); Aquasol, carrier free \( ^{35} \text{S} \) sulfate as \( \text{H}_2\text{SO}_4 \), and \( ^{35} \text{S} \) (New England Nuclear, Boston, Mass.); Metrizamide, analytical grade (Accurate Chemical & Scientific Corp., Hicksville, N.Y.); Sephadex G-75, Sepharose 4B, and activated CH-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.); Dowex 1-X2, chloride form, 100–200 mesh and polyacrylamide (Bio-Rad Laboratories, Richmond, Calif.); 2-iodoacetamide and \( -\)mercaptoethanol (Eastman Kodak Co., Rochester, N.Y.); cellulose nitrate tubes number 331101 (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.); PM-10 ultrafiltration membranes (Amicon Corp., Lexington, Mass.); and Sprague-Dawley rats (Gofmoor Farms, Southborough, Mass.) were obtained as noted.

Affinity chromatography gel was prepared by mixing 26.7 mg of n-tryptophan methyl ester in 6 ml of 0.1 M NaHCO\(_3\), with 1 g of activated CH-Sepharose according to the instructions of the manufacturer (Pharmacia Fine Chemicals). Analytical acid disc gel electrophoresis, pH 4.3, and alkaline disc gel electrophoresis were performed as described (12). Electrophoresis in 6 cm 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) (13) utilized molecular weight standards of ovalbumin (43,000), chymotrypsinogen A (25,700), \( \alpha \)-chymotrypsin (approximately 24,000), and ribonuclease A (12,500); the chymase and each marker protein were alkylated by incubation for 1 h at 37\(^\circ\)C in 0.05 M iodoacetamide in the presence of 8 M urea and 1% SDS immediately before application to the gels (14). The chymase and \( \alpha \)-chymotrypsin were also reduced by 0.01 M \( -\)mercaptoethanol in the presence of 8 M urea and 1% SDS and then alkylated and run in the same way. The SDS and alkaline and acid disc gels were stained with 0.1% Coomassie Blue dye for protein (13).

Six sucrose density gradients of 12 ml each were formed on a 1-ml cushion of 60% sucrose in 0.0075 M Tris, pH 7.4 in 9/16-inch diameter cellulose nitrate tubes by mixing equal volumes of 5 and 30% sucrose in the same buffer in a Buchler gradient maker (Buchler Instruments Div. Searle Analytic Inc., Fort Lee, N.J.). The gradients were kept on ice for 30 min and the samples were layered on the gradient surface. The gradients were centrifuged in a Beckman L2-65B ultracentrifuge in a SW40 Ti rotor (Beckman Instruments) at 210,000 g at the bottom of the gradient for 5 h or at 3,000 g at the bottom of the gradient for 1 h. The samples were collected from the bottom with a Beckman fractionator.

Isolation of Rat Mast Cells. The cells of 100 male and female Sprague-Dawley rats, each weighing 200–300 g, were collected by lavage of the peritoneal and thoracic cavities of each rat with 20 ml Tyrode’s buffer containing 0.1% gelatin and 50 \( \mu \)g/ml commercial heparin. Mast cells were isolated as previously described (11, 15) by centrifugation through 22.5% wt/vol Metrizamide. The mast cell purity, determined by a differential count of the nucleated cells in smears stained with toluidine blue, was 90–95% with a yield of approximately 10\( ^6 \) mast cells per rat (11).

Labeling and Isolation of Rat Mast Cell Heparin. Mast cells, isolated as described above, were radiolabeled in vivo by mixing \( 4 \times 10^7 \) mast cells in 1.0 ml of Tyrode’s buffer containing 0.1% gelatin and 10 \( \mu \)g/ml deoxyribonuclease (TGD) with 10 mCi \( ^{35} \text{S} \) sulfate in 1.0 ml of Hank’s balanced salt solution and Eagle’s basal medium (9). After incubation for 4 h at 37\(^\circ\)C, the cells were washed six times by suspending with TGD and sedimenting at 400 g for 5 min at room temperature. The cells were frozen and thawed six times, and the solution was brought to 1 M with NaCl and incubated for 30 min at room temperature. The mixture was centrifuged at 400 g for 5 min to remove cellular debris, and the supernate was applied to a 1 × 15-cm column of Dowex 1 equilibrated in 1 M NaCl. The column was eluted with 1, 3, and 4 M NaCl at 15 ml/h (16). Fractions were assayed for heparin content by metachromasia with Azure A (11, 17) and for \( ^{35} \text{S} \) with a Searle Mark III liquid scintillation spectrometer. The 3 M eluate fractions containing the heparin (9) were pooled, dialyzed against distilled water, lyophilized, and applied in a 1-ml vol of 2 M NaCl to a 1 × 50-cm column of Sepharose 4B equilibrated in 2 M NaCl. The column was eluted with 2 M NaCl at a flow rate of 4 ml/h, and 1-ml fractions were assessed for \( ^{35} \text{S} \) and heparin by metachromasia. The \( ^{35} \text{S} \)heparin cochromatographed with the chemically defined heparin, thereby giving a broad peak with a molecular weight of approximately 750,000, as previously reported for heparin labeled for 4 h in vitro (9) with the same markers. The \( ^{35} \text{S} \)heparin was pooled, dialyzed against distilled water, lyophilized, and stored at \(-70^\circ\)C.

Assay of the Chymase. The chymase was measured by its cleavage of the specific chymotrypsin synthetic ester substrate BTEE (18) or of \( ^{125} \text{I} \)-labeled casein. The BTEE assay was performed as described (19) by measurement of the time-related linear increase in absorbance at 256 nm in a Beckman DU spectrophotometer, and the change in absorbance per minute was converted to
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micromoles of substrate cleaved per minute. The assay detected 2 μg of commercial α-chymotrypsin which had an activity of 45 U/mg; a unit is arbitrarily defined as the amount of enzyme required to cleave 1.0 μmol BTEE/min at 25°C.

500 μg of casein was labeled with 99 μCi 125I by the insolubilized lactoperoxidase method (20), and the mixture was filtered on a 0.7 × 50-cm column of Sephadex G-50 equilibrated in 0.0075 M Tris, pH 7.8, containing 0.15 M NaCl. 1.5-ml fractions were collected, and the 125I-labeled material that filtered in the void volume of the column was pooled and dialyzed for 1 h against 5 liters of column buffer. The 125I-label in a fraction of the dialyzed material was 96% precipitated by exposure for 20 min at room temperature to 5% trichloroacetic acid followed by centrifugation at 8,000 g, or 4 min in a Beckman microcentrifuge (Beckman Instruments, Wilmington, Mass.).

The specific activity of the labeled casein was approximately 40 Ci/mmol. The caseinolytic assay was carried out according to the method of Kunitz as described by Laskowski (21) for unlabeled substrate. 25 μl of 125I-casein, 4,000-5,000 cpm, was added to 1.5-ml microcentrifuge tubes containing 25 μl of 0.08 M Tris, pH 7.8 with 0.02 M CaCl₂. The mixture was brought to a final volume of 100 μl with either 0.15 saline or enzyme portions in 0.15 M saline, and the proteolytic reaction was carried out for 20 min at 37°C. The reaction was terminated by the sequential addition of 0.4 ml of 0.625% unlabeled casein which had been adjusted to pH 9.0 with 1 M NaOH and 0.6 ml of 5% trichloroacetic acid. After 20 min at room temperature, this mixture was centrifuged at 8,000 g for 4 min, and the unprecipitated 125I was measured by counting the supernate in a gamma spectrometer. 125I-labeled fragments of casein were released in a linear relationship to the dose of α-chymotrypsin in the range between 2 and 10 ng. The chymase was quantitated from a standard curve generated with α-chymotrypsin.

Protein was determined by measurement of the optical density at 280 nm and was converted to micrograms from a standard curve relating known amounts of α-chymotrypsin to its optical density at 280 nm. One optical density unit equaled 0.490 mg of α-chymotrypsin as previously reported (22).

Results

Isolation of the Chymase. Purified rat mast cells were suspended in 40 ml of 0.0075 M Tris, pH 7.4 containing 0.01 M NaCl, 8 × 10⁻⁴ M MgCl₂ and 10 μg/ml deoxyribonuclease at a concentration of 4.25 × 10⁶ cells/ml. The cells were frozen and thawed six times, and the cellular debris was sedimented at 400 g for 10 min at room temperature. The supernate was decanted and centrifuged at 3,000 g for 20 min at 4°C to obtain a granule pellet (6, 11) and granule exchange supernate. The granule exchange supernate was made 1 M with respect to NaCl, and the granule pellet and the cellular debris were each brought to a 5-ml vol of 1.0 M NaCl. After 30 min of incubation at room temperature, each of the subcellular fractions was directly assayed for esterase activity and for protein content. Chymotryptic activity was not detected in the granule exchange supernate which contained 31.6 mg protein. The cellular debris, which is known to retain membrane adherent granules (23), and the granule pellet fraction contained a combined chymase activity of 63.4 U and 42.8 mg of protein.

The chymase-containing fractions were pooled and adjusted to a final concentration of 0.01 M Tris, pH 9.0 and 0.01% gelatin in 1 M NaCl. The sample was applied to a 1 × 20-cm column of Dowex 1 equilibrated in 0.01 M Tris, pH 9.0 containing 0.01% gelatin and 1 M NaCl and eluted with this buffer; and 10 4-ml fractions were collected. The column was further eluted with 20 ml of 3 M NaCl and 20 ml of 4 M NaCl (Fig. 1). Protein and esterase activity eluted with the starting buffer, whereas heparin eluted in the 3 M NaCl. Minimal amounts of protein and no heparin or chymase were found in the 4 M NaCl eluate.

The chymotryptic activity in the Dowex 1 eluate, 4-16 ml, was pooled and
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Fig. 1. Dowex 1 chromatography of rat mast cell granule and granule-membrane fractions treated with 1 M NaCl. The width of a bar is equal to a fraction volume. Fractions were assayed for chymase with BTEE as substrate, for heparin by metachromasia with Azure A, and for protein by absorbance at 280 nm.

Concentrated to 1.1 ml by ultrafiltration on a PM-10 Amicon membrane, and a 1-ml sample was applied to a 1 × 45-cm column of Sephadex G-75 equilibrated at 4°C in 0.01 M Tris, pH 7.8 containing 1.2 M KCl (8) and 0.01% gelatin. The sample was filtered in the same buffer, and 0.6-ml fractions were collected at a flow rate of 2 ml/h. The chymotryptic activity (Fig. 2) filtered in the same position as chymotrypsinogen A at an apparent molecular weight of 25,000, while more than 50% of the protein filtered at a higher molecular weight. Fractions 27 through 34 were pooled and dialyzed against 5 liters of 0.05 M Tris, pH 8.0 at 4°C for 1 h. The sample was applied over 30 min at room temperature to a 300-μl column of d-tryptophan methyl ester coupled to CH-Sepharose equilibrated in 0.05 M Tris, pH 8.0. The column was eluted with the equilibrating buffer until the optical density of the eluate fractions was zero, and the chymase was then eluted according to the methods for α-chymotrypsin (24) with 0.1 M acetic acid, pH 3.0. The acetic acid-containing fractions were placed on ice, and each was adjusted to pH 7.0 by the addition of 75 μl of 1 M Tris. The initial eluate contained a broad peak of protein devoid of esterolytic activity (Fig. 3), whereas the acetic acid eluate contained a coincident peak of protein and chymotryptic activity. The specific activity of the affinity chromatography-purified material was 67.8 U/mg of protein and represented a 78-fold purification with an overall yield of 23% relative to the activity in the solubilized cellular debris and granule pellet. The single fraction containing the chymase was dialyzed against 5 liters of 0.0075 M Tris, pH 7.8 at 4°C for 1 h and stored at 4°C in a polycarbonate tube. The specific activity of two other
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Blue Ovalbumin Chymotrypsinogen A Tritiated Dextran 43,000 25,700 Water

Fig. 2. Sephadex G-75 gel filtration of chymase-containing fractions obtained from Dowex 1. The filtration characteristics of the standards were determined in separate analyses. Fractions were assayed for protein (●●) and chymase activity (○○).

purified preparations was 45.1 and 62.4 U/mg protein, and the percent yields were 12 and 25%, respectively.

Chymase Purity and Function. The purity of the isolated enzyme was assessed by electrophoresis of 10-μg samples in replicate acid disc gels. One gel was stained, and a replicate gel was sliced and each section eluted for 48 h at 4°C in 200 μl of 0.05 M Tris, pH 8.0 containing 0.5 M NaCl and 0.01% gelatin. 15% of the activity applied was eluted in the area corresponding to the stained protein band, whereas <1% was recovered at the cathode in the absence of a stained band in the region of the dye front (Fig. 4). Analytic alkaline disc gel electrophoresis of 10 μg of purified chymase demonstrated no stainable protein bands, thereby indicating that the charge of the enzyme was such that it did not enter the gel.
10 μg of purified chymase and 10 μg of α-chymotrypsin were each alkylated, reduced, or reduced and alkylated and then applied to SDS analytical electrophoresis gels. The alkylated chymase presented a single stained band (Fig. 5 A) with a mobility similar to that of alkylated α-chymotrypsin (Fig. 5 B). Based on the position of the marker proteins ovalbumin, chymotrypsinogen A, and ribonuclease A, the molecular weight of chymase, like that of the α-chymotrypsin, is apparently 25,000. Exposure to conditions of reduction (Fig. 5 C) or reduction and alkylation (Fig. 5 E) did not change the mobility of chymase; whereas α-chymotrypsin, after reduction (Fig. 5 D) or reduction and alkylation (Fig. 5 F), migrated as two bands as previously reported for this enzyme (13).

The $K_m$ and $V_{max}$ were determined from the rate of hydrolysis of BTEE at six substrate concentrations between $5.35 \times 10^{-5}$ M and $1.07 \times 10^{-3}$ M by the purified chymase. A double reciprocal plot of $1/v$ vs. $1/s$ was linear with a correlation coefficient of $r = 0.999$ and gave a $K_m$ of $1.56 \times 10^{-3}$ M and a $V_{max}$ of 67.8 μmol/min per mg. In the same experiment α-chymotrypsin had a $K_m$ of $1.17 \times 10^{-3}$ and a $V_{max}$ of 46.4 μmol/min per mg.

Inhibitors of Purified Chymase. 5 μg of the purified chymase and 5 μg of α-chymotrypsin were each incubated in $2 \times 10^{-4}$ M TPCK, $1 \times 10^{-3}$ M TLCK, or solvent for the inhibitors in a volume of 75 μl at 24°C; the solvent consisted...
of 0.03 M Tris, pH 7.8, 0.03 M CaCl₂, and 5% methanol. The esterase activity was assessed after 1, 2, 4, and 8 h of incubation. In the absence of inhibitor there was no loss of enzyme activity through the first 4 h and a 30 and 10% reduction by 8 h for chymase and α-chymotrypsin, respectively. TLCK produced no change in activity of either α-chymotrypsin or chymase as compared to enzyme incubated in solvent buffer alone. In contrast, in the presence of...
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Fig. 6. The inhibition of the proteolytic activity of the purified chymase by 5-HT. The mean value for the duplicate points is plotted and the bars express the range of the duplicates.

TPCK, both enzymes showed a time-dependent loss of activity, with \( \alpha \)-chymotrypsin being completely inhibited at 2 h and chymase at 8 h.

Because indoles have been shown to inhibit \( \alpha \)-chymotrypsin (25) and the chymotrypsinlike enzyme of the mouse mastocytoma (26), and 5-HT appears to compete with diisopropylfluorophosphate (DFP) for a binding site in the rat mast cell granule (27), the effect of 5-HT on the purified rat mast cell chymase was investigated. 5 ng of purified chymase was preincubated for 1 h at room temperature with buffer or varying concentrations of 5-HT HCl in a final volume of 75 \( \mu \)l containing 0.03 M Tris, pH 7.8, and 0.005 M CaCl\(_2\). Duplicate samples were assessed for proteolytic activity on the \(^{125}\)I-casein substrate. A dose-dependent inhibition of the chymase (Fig. 6) was observed from \( 10^{-3} \) to \( 10^{-2} \) M 5-HT with a 50% inhibitory dose of \( 3 \times 10^{-3} \) M.

Because 5-HT and histamine have been shown to compete for binding sites on the rat mast cell granule (23) that are presumed to be carboxyl residues on the granular protein (28, 29), the effect of histamine on the 5-HT-mediated inhibition of the chymase was determined. Histamine was prepared by titrating 0.9 M histamine base in distilled water with 0.9 M histamine hydrochloride in distilled water to a pH of 7.8. 5 ng of purified chymase were preincubated for 1 h at room temperature with buffer alone, 300 mM histamine, increasing concentrations of 5-HT, and increasing concentrations of 5-HT in the presence of histamine in a final volume of 75 \( \mu \)l containing 0.03 M Tris, pH 7.8, and 0.005 M CaCl\(_2\). Duplicate samples were assessed for proteolytic activity on the \(^{125}\)I-casein substrate. The dose-related inhibition of chymase was again observed with 5-HT alone (Fig. 7). 5-HT at 3.75 mM gave 55% inhibition, whereas 300 mM histamine produced only 20% inhibition. When histamine and 5-HT were introduced together, the inhibition of chymase was approximately the same as that observed with 5-HT alone.

Interaction of Rat Mast Cell Heparin with Purified Chymase. Previous experiments in which isolated rat mast cell granules were solubilized and chymase activity and \(^{35}\)S]heparin then assessed suggested a molar ratio of
chymase to macromolecular heparin in native granules of 40 to 1 (10). Purified chymase and purified [35S]heparin of 750,000 mol wt were mixed in various ratios, and complex formation was assessed by ultracentrifugation of the mixture through sucrose density gradients. In each experiment 4,800 cpm (0.78 μg) [35S]heparin in a 10-μl vol was added to varying amounts of the purified enzyme to give a final volume of 30 μl in 0.0075 M Tris, pH 7.4. Each mixture was incubated on ice for 1 h and applied to a 13-ml sucrose gradient. In a typical experiment 0.33, 0.44, and 1.1 μg of chymase, respectively, were added to [35S]heparin to give molar ratios of approximately 12:1, 16:1, and 40:1. These mixtures, [35S]heparin alone, 1.1 μg chymase alone, and isolated rat mast cell granules containing approximately 1.5 μg chymase, were centrifuged at 210,000 g at the bottom of the gradient at 2°C for 5 h. After 200-μl portions had been collected until each tube was empty, the bottom portion of each tube was cut off and washed with 200 μl of 0.05 M Tris, pH 7.4 containing 0.5 M NaCl. Alternate samples of the gradient, a portion of the wash fluid from the bottom of the tube, and the bottom portion of the tube itself were suspended in scintillation fluid and counted for [35S]heparin. In addition the 200-μl gradient fractions of the chymase alone, the isolated granules, and the 1:40 molar mixture of heparin and chymase were assayed for chymase content by diluting each sample to 1:10 with physiologic saline containing 0.01% gelatin and measuring proteolytic activity with 125I-casein. The chymase alone stayed at the top of the gradient (Fig. 8 A) but only 10% of the activity applied was recovered. [35S]heparin alone (Fig. 8 B) moved slightly into the gradient and 70% of the activity applied was recovered. At a 1:12 ratio 34% (Fig. 8 C) and at a 1:16 ratio 40% (Fig. 8 D) of the [35S]heparin recovered sedimented to the bottom of the tube, and the overall recovery in these experiments was approximately 70%. In the 1:40 mixture all of the [35S]heparin recovered sedimented to the bottom of the tube with a total recovery of 45%, representing a combination of the counts in the wash fluid and those remaining on the bottom of the tube; the wash fluid from the bottom of the tube also contained 0.22 μg of proteolytic...
activity (Fig. 8 E). The isolated granules were localized only to the bottom of the tube by recovery of 0.155 μg of proteolytic activity.

Because the proteolytic activity of the granule-bound chymase is not fully expressed until the granule is solubilized (10), portions of the natural granules or the 1:40 mixture of native heparin and purified chymase which had sedimented to the bottom of the tubes were solubilized in a final concentration of 1 M NaCl for 30 min at room temperature. The solubilized samples were then diluted with 0.01% gelatin in distilled water so that the final molarity was 0.15 M NaCl. Unsolubilized samples were also diluted for assay with 0.01% gelatin.
in 0.15 M NaCl. The proteolytic activity of the isolated granules increased from 0.155 to 0.494 μg with solubilization, thereby reflecting an overall recovery of 30%. The sedimenting complex formed at the 1:40 ratio exhibited a 2.8-fold increase in activity from 0.22 to 0.617 μg with solubilization, indicating a better than 50% overall recovery of the chymase. Based upon the 0.24 μg of heparin in the same sample, the molar ratio of heparin to chymase was 1:66 in the fully sedimented complex formed in the 1:40 original reaction mixture.

Under the same conditions as in the above experiment, neither heparin alone nor heparin with chymase in a 1:8 ratio moved into gradients farther than the heparin depicted in Figure 8 B. At higher ratios of 1:80 and 1:160 essentially all of the [35S]heparin recovered sedimented to the bottom of the centrifuge tubes with an overall recovery in the 70% range. In a different experiment with centrifugation at only 3,000 g at the bottom of the gradient for 1 h, which represents conditions utilized to sediment granules in aqueous solution, [35S]heparin alone or in combination with chymase in 1:8, 1:12, or 1:16 ratios showed no sedimentation. In contrast, a mixture of 1:40 resulted in sedimentation to the bottom of all the heparin recovered; recovery of [35S]heparin was 69%.

Discussion

A rat mast cell chymotrypsinlike enzyme (chymase) has been preparatively purified to homogeneity in order to define its physical and functional interaction with other mast cell granule constituents. Inasmuch as the chymase was known to be granule associated (4–6), the starting material for isolation utilized granule-rich subcellular fractions solubilized in 1 M NaCl to dissociate the chymase from macromolecular anionic heparin. The chymase alone, with almost all of the protein, was then separated from the heparin, which is the predominant rat mast cell proteoglycan (9), by Dowex 1 anion exchange chromatography (Fig. 1). Sephadex G-75 gel filtration separated the chymase from much of the protein but did not yield a distinct protein peak in the area of chymase activity (Fig. 2). Binding of the chymase by a n-tryptophan methyl ester affinity column and elution at low pH yielded concordance between a protein peak and chymotryptic activity (Fig. 3). The purified chymase gave a single stained protein band on acid disc gel electrophoresis which corresponded in position to the region in which function was recovered by elution from a replicate gel (Fig. 4). Electrophoresis in SDS after alkylation, reduction, or both reduction and alkylation (Fig. 5) also revealed a single protein. The specific activity of the purified mast cell chymase of 67.8, 62.4, and 45.1 U/mg in three preparations is comparable to that obtained by other procedures in which homogeneity of the protein was either not established (7, 8) or was achieved by autodigestion before a final gel filtration step (5).

The molecular weight of mast cell chymase of approximately 25,000 by gel filtration on Sephadex G-75 in the presence of 1.2 M KCl and 0.01% gelatin is the same as that observed by others (8) with gel filtration under similar buffer conditions. Much lower molecular weights of 5,600 by sucrose density ultracentrifugation (6) and 7,000 by gel filtration (8) were obtained with buffers of low ionicity. The validity of the 25,000 mol wt figure is established by the identical
positions of mast cell chymase and α-chymotrypsin after alkylation and electrophoresis in SDS gels (Fig. 5) and is in agreement with the figure obtained by others (5) with the same technique. That mast cell chymase is cationic had been appreciated by its mobility at alkaline pH and is compatible with the amino acid composition of the DFP-treated major granule protein (6). The cationic nature of the purified chymase is apparent from its mobility on acid disc gel electrophoresis (Fig. 4) and its failure to enter the gel on analytic alkaline disc gel electrophoresis. Although the mast cell chymase has a size and charge comparable to α-chymotrypsin, it differs from α-chymotrypsin by not revealing two chains when analyzed on SDS gel electrophoresis after reduction or reduction and alkylation.

The \( K_m \) of the purified chymase for BTEE of \( 1.56 \times 10^{-3} \) is comparable to the value of \( 1.1 \times 10^{-3} \) M observed with a different preparation (8). The purified chymase was inhibited by TPCK and not by TLCK, and the earlier demonstration of both TPCK and DFP (8) inhibition is good evidence that its active center is similar to that of α-chymotrypsin. α-Chymotrypsin is reversibly inhibited by indoles, which belong to a family of monofunctional chymotrypsin inhibitors (25), and preliminary experiments indicated that 5-HT (serotonin) inhibited the action of α-chymotrypsin on \(^{125}\)I-casein. 5-HT produced a dose-dependent inhibition of the proteolytic activity of the isolated rat mast cell chymase (Fig. 6) with a 50% inhibitory dose of \( 3 \times 10^{-3} \) M. Histamine was not significantly inhibitory at 100 times the 50% inhibitory dose of 5-HT and did not increase or decrease inhibition when introduced in the presence of increasing doses of 5-HT (Fig. 7). Based on the rat mast cell content of 1 \( \mu \)g of 5-HT/10\(^8\) cells (30) and the rat mast cell granule volume of 300 \( \mu \)m\(^3\) (31), the calculated granule 5-HT \textit{in situ} molarity of \( 1.89 \times 10^{-2} \) M is the concentration at which 97% inhibition of chymase is seen (Fig. 6).

Previous experiments in which rat mast cell granules were solubilized and assessed for micrograms of chymase by using both the amino acid ester and the casein substrates, and for micrograms of heparin by metachromasia with Azure A revealed a weight ratio of approximately 1.3 to 1 (10). This value fell within the total granule protein to heparin weight ratio of 2:1 as reported by others (4, 28) and was compatible with the finding that the solubilized granule contained a major protein considered to be chymase and two less prominent proteins (6). The weight ratio of chymase to macromolecular heparin observed for the isolated granules would give a molar ratio of 40:1. When purified chymase and macromolecular heparin were mixed at this ratio and centrifuged on sucrose density gradients of 5-30% at 3,000 \( g \) at the bottom of the gradient for 1 h, a complex sedimented as assessed by following the radiolabeled heparin; there was no sedimentation when lower ratios of 1:8, 1:12, and 1:16 were employed to form the reaction mixture. When sucrose density gradient ultracentrifugation was extended for 5 h at 200,000 \( g \) at the gradient bottom, 34% of the recovered \(^{35}S\)heparin in the 1:12 mixture, 40% in the 1:16 mixture, and 100% in the 1:40 mixture sedimented to the bottom (Fig. 8). Under these conditions, heparin and chymase alone each remained at the top of the gradient. Solubilization of the material washed from the bottom of the gradient tube in the 1:40 reaction mixture gave 55% recovery of chymase (0.617 \( \mu \)g),
which, together with the 33% recovery of [35S]heparin (0.257 µg), yields a molar ratio of 1:66. The chymase activity sedimented and recovered from the 1:40 mixture compares favorably with the 30% recovery of chymase from the isolated granules sedimented under the same conditions. Further, the fact that solubilization of the sedimented complex and isolated granules gave a 2.8-fold and threefold increment in chymase activity, respectively, argues that masking of the active site was similar in the two circumstances. All the data taken together for isolated granules and experimental chymase-heparin complexes are compatible with a molar ratio in the range of 40–60:1. Based on a heparin content of approximately 100 µg/10^6 cells (16), a histamine content of 10 µg/10^6 cells (32), and a 5-HT content of 1 µg/10^6 cells (30), the approximate molar ratio of heparin:chymase:histamine:5-HT is 1:40–66:1,352:42. The calculation that the molar content of 5-HT is similar to that of chymase, together with the finding that 5-HT inhibits chymase function, introduces the possibility that at least one site of 5-HT binding in the granule is different from that of histamine. Histamine does not inhibit the chymase, is present in appreciable molar excess over 5-HT and chymase, and binds more readily than 5-HT to isolated discharged granules (28).

The similarity between the rat chymase with respect to molecular weight, chemical inhibition, and substrate specificity and the α-chymotrypsinlike enzyme in human peripheral polymorphonuclear leukocytes (33, 34) is noteworthy because the latter generates chemotactic activity from serum and C3 and C5 proteins (35), exhibits bactericidal activity (36), enhances phagocytosis (37) and the platelet release reaction (38), and degrades rabbit articular cartilage (39). Such a potentiality for the rat mast cell chymase would be regulated by two other constituents of the granule. Chymase bound in natural, discharged granules exhibits only a fraction of the proteolytic activity uncovered by solubilization. Whether 5-HT together with heparin completely masks the active site in a truly native granule is unknown. Should solubilization occur by mechanisms yet to be defined, 5-HT could function as a reversible active site inhibitor.

Summary

The rat mast cell granule chymotrypsinlike enzyme was purified to homogeneity from 1 M NaCl solubilized membrane and granule-rich fractions of concentrated rat peritoneal mast cells by a preparative technique utilizing chromatography on Dowex 1, filtration on Sephadex G-75, and affinity chromatography with D-tryptophan methyl ester. Acid disk gel electrophoresis of the purified chymase disclosed a single stained band with activity being eluted from a replicate sliced gel in the same region. SDS-polyacrylamide gel electrophoresis of purified protein gave a single stained band that did not change in position with reduction and alkylation. Mast cell chymase is thus a cationic protein of 25,000 mol wt composed of a single polypeptide chain. The apparent $K_m$ of the chymase for BTEE was $1.5 \times 10^{-3}$ M and the $V_{max}$ was 67.8 µmol/min per mg. The enzyme was inhibited by TPCK and not by TLCK. The chymase complexed with native macromolecular rat mast cell heparin in molar ratios of 12:1 and 16:1, and complete heparin uptake occurred at a 40:1 ratio of chymase
to heparin. Chymase activity was partially masked by combination with heparin in the isolated granule or experimental chymase-heparin complex, and soluble purified chymase was inhibited by concentrations of 5-HT comparable to those present in mast cells. It is therefore possible that the active site of chymase in the mast cell granule is largely masked by the combined effects of macromolecular heparin and 5-HT.

The skilled assistance of Ms. Judith Litvin is gratefully acknowledged.

Received for publication 25 July 1977.

References