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Purification and Characterization of Lymphocyte Chymase I, a 
Granzyme Implicated in Perforin-Mediated Lysis

Susan L. Woodard,* Stephanie A. Fraser,‡ Ulrike Winkler,* Delwin S. Jackson,§ Chih-Min Kam,§ James C. Powers,§ and Dorothy Hudig2*†

One mechanism of killing by cytotoxic lymphocytes involves the exocytosis of specialized granules. The released granules contain perforin, which assembles into pores in the membranes of cells targeted for death. Serine proteases termed granzymes are present in the cytotoxic granules and include several chymases (with chymotrypsin-like specificity of cleavage). One chymase is selectively reactive with an inhibitor, Biotinyl-Aca-Aca-Phe-Leu-PheP2(OPh)2, that blocks perforin lysis. We report the purification and characterization of this chymase, lymphocyte chymase I, from rat natural killer cell (RNK)-16 granules. Lymphocyte chymase I is 30 kDa with a pH 7.5 to 9 optimum and primary substrate preference for tryptophan, a preference distinct from rat mast cell chymases. This chymase also reacts with other selective serine protease inhibitors that block perforin pore formation. It elutes by Cu2+--immobilized metal affinity chromatography with other granzymes and has the N-terminal protein sequence conserved among granzymes. Chymase I reduces pore formation when preincubated with perforin at 37°C. In contrast, addition of the chymase without preincubation had little effect on lysis. It should be noted that the perforin preparation contained sufficient residual chymase activity to support lysis. Thus, the reduction of lysis may represent an effect of excess prolytic chymase I or a means to limit perforin lysis of bystander cells. In contrast, other chymases and granzyme K were without effect when added to perforin during similar preincubation. Identification of the natural substrate of chymase I will help resolve how it regulates perforin-mediated pore formation. The Journal of Immunology, 1998, 160: 4988–4993.

There are multiple pathways that cytotoxic lymphocytes utilize in the killing of target cells, including the Fas-ligand-Fas pathway (1) and the granule exocytosis pathway (2, 3; also reviewed in Ref. 4). The granule exocytosis pathway utilizes perforin and serine proteases of the granules (granzymes) to kill target cells. The trypsin granzymes A and K and the Asp-ase granzyme B mediate cytotoxic activity through their ability to induce DNA fragmentation after entering cells through perforin pores (5–9). Another granzyme, a chymase (with chymotrypsin-like specificity of cleavage), has a role in perforin pore formation (10, 11).

Lymphocyte granzyme B has been difficult to purify to date. The enzyme activity has been detected in cytotoxic rat (10) and human (M. Poe, unpublished results) lymphocytes. From computer models of granzyme B, serine protease active sites, mouse granzyme genes D, E, F, and G, rat granzyme P7, and human granzyme H are predicted to be chymases (Refs. 12–14, respectively). To date, the only chymase purified from cytolytic granules has been a carboxypeptidase, cathepsin A-like protective protein (15). The abundance of the mRNA for granzymes D, E, and F suggests that these granzymes may be expressed at 100-fold lower concentra-

Materials and Methods

Purification of chymase I

Chymase I was purified from RNK-16 cell granules by size exclusion and cation exchange chromatography. Isolation of RNK-16 granules. RNK-16 cells (18) were grown as ascites cells in F344 rats. Our RNK-16 subline has retained granule lytic activity while the live cells no longer bind to or kill YAC-1 target cells. To obtain the granules, the cells were disrupted by nitrogen cavitation, and the lysate was centrifuged over 54% Percoll (Sigma Chemical, St. Louis, MO). Granules were isolated from the dense portion of the gradient as previously described (17). The granule pool was made 1 M NaCl and then freeze thawed three times to disrupt the granule membranes. The granule extract was stored at −20°C. The yield was approximately 0.5 mg granules per billion RNK-16 cells.

Superdex 200 size exclusion chromatography. Twenty milliliters of granule extract were concentrated approximately fivefold by centrifugation with Centriprep 10 concentrators (Amicon, Beverly, MA) before loading 3 ml onto a 2.6 × 70-cm Superdex 200 (Pharmacia Fine Chemicals, Piscataway, NJ) column. The running buffer was 20 mM HEPES, 1 M NaCl, 10% betaine, 0.1 M EGTA, and 0.05% NaN3, Aprotinin (6.5 kDa), carbonate

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5 Abbreviations used in this paper: 6(OPh)2, phosphonate phenylester; Aca, 6-aminocaproic acid; Bi, biotinyl; Boc, butyloxycarbonyl; IMAC, immobilized metal affinity chromatography; MES-2-morpholinoethanesulfonic acid; RNK, rat NK cell; SBzl, thienobenzyl; Suc, succinyl; Z, benzoyloxycarbonyl; DCI, 3,4-dichloroisocoumarin.
anhydride (29 kDa), BSA (66 kDa), and IgG (150 kDa) were used for size calibration. The void volume was determined using blue dextran (2000 kDa).

**MonoS cation exchange chromatography.** The leading one-third of the SD200 low m.w. chymase peak was omitted from the pool loaded onto an HR5/5 MonoS fast protein liquid chromatography (FPLC) column (Phar- macia) because these fractions contained granzyme B that would copurify with chymase I on MonoS. The SD200 chymase pool was diluted with an equal volume of NaCl-free buffer (125 mM MES, 0.15 mM EGTA, 15% betaine, pH 5.9). Pure ethylene glycol (enzyme grade, Fisher Biotech, Fair Lawn, NJ) was added to make the chymase solution 0.4 M NaCl, 20% ethylene glycol, 50 mM MES, 10% betaine, 0.05% NaN₃, and 0.1 mM EGTA, pH 6.0. The diluted sample was then in MonoS running buffer. At MonoS running buffer B was similar to buffer A but with 1 M NaCl. The sample was loaded and washed in 0.4 M NaCl (60% A, 40% B). A linear gradient was then run from 40% to 100% B. In additional experiments not illustrated, chymase I was isolated using immobilized metal affinity chromatography (IMAC) with Cu⁺⁺ bound to Poros IMAC beads (PerSeptive Biosystems, Cambridge, MA) (19).

**Chymase detection**

**Substrate hydrolysis.** Rates of hydrolysis of peptide thiobenzyl substrates were measured using Ellman’s reagent, diithiothreitol (2-nitrobenzoic acid) (1.2 mM in assay), to monitor the production of HSBzl. The extinction coefficient for the colored thiol product at 410 nm is 13,600 M⁻¹ cm⁻¹ (20). Rates were measured in esterase buffer (0.1 M HEPES, 0.5 M NaCl, pH 7.5) at 25°C. For assaying activity from column runs, 10 to 100 μl of each fraction were assayed in a total volume of 200 μl in 96-well microtiter plates using a Molecular Devices kinetics microplate reader (Palo Alto, CA). Rates were determined in duplicate and averaged. The substrate Suc-Phe-Leu-Phe-SBzl (Bachem Bioscience, King of Prussia, PA) was used for the chymase assays, except where indicated in Table II. Boc-Lys-SBzl (Sigma, Bloomington, IN); PMSF and Tosyl-Phe-CH₂Cl (Sigma); 3,4-dichloroisocoumarin (Boehringer Mannheim, Indianapolis, IN); PMSF and Tosyl-Phe-CH₂Cl (Sigma); Z-Gly-Leu-Phe-CH₂Cl (ESP), 2-(Z-NH(CH₂)₂CO-NH)C₆H₄SO₂F (24), Bi-Aca-Aca-Phe-Leu-Phe(OPh)₂ (25), and FITC-Ala-Ala-Met(OPh)₂ (25) were synthesized in the laboratory of J.C.P.

**Protein characterization**

**Protein determinations.** Protein assays were done with bicinchoninic acid (BCA) (23) (Pierce, Rockford, IL) with crystalline BSA as a standard. Gel electrophoresis. Samples were run on SDS-PAGE under reducing conditions using 12% MiniProtean gels (Bio-Rad Laboratories, Richmond, CA). Samples were run on SDS-PAGE under reducing conditions using 12% MiniProtean gels (Bio-Rad Laboratories, Richmond, CA). Protein sequencing was performed on an Applied Biosystems (Foster City, CA) Procise 494 gas phase sequencer by Dr. Matthew Williamson (University of California at San Diego, San Diego, CA). The protein was bound to ProBlott (Applied Biosystems) polyvinylidene difluoride (PVDF) membrane for sequencing.

**Determination of pH optimum**

Hydrolysis rates of Suc-Phe-Leu-Phe-SBzl were measured in Good’s buffer solutions containing 0.1 M MES (pH 5.58, 5.87, 6.12, and 6.43), 0.1 M PIPES (pH 6.44, 6.83, and 7.23), 0.1 M HEPES (pH 7.09, 7.50, 7.75, and 7.94) or 0.1 M TAPS (pH 7.94, 8.33, 8.69, and 9.10) and 0.75 M NaCl in the presence of 1.2 mM Ellman’s reagent. The data were fit using nonlinear least squares algorithm to an equation of the form y = ax³ + bx + c.

**Chymase inhibition**

Chymase isolated after SD200 was treated with 0.1 mM inhibitors for 20 min at room temperature in esterase buffer and then immediately assayed. The inhibitors were dissolved at 20 to 40 mM in anhydrous DMSO and stored at −20°C. Controls were treated with DMSO alone. The inhibitors came from the following sources: 3,4-dichloroisocoumarin (Boehringer Mannheim, Indianapolis, IN); PMSF and Tosyl-Phe-CH₂Cl (Sigma); Z-Gly-Leu-Phe-CH₂Cl (ESP), 2-(Z-NH(CH₂)₂CO-NH)C₆H₄SO₂F (24), Bi-Aca-Aca-Phe-Leu-Phe(OPh)₂ (25), Suc-Phe-Leu-Phe(OPh)₂ (25), and FITC-Ala-Ala-Met(OPh)₂ (25) were synthesized in the laboratory of J.C.P.

**Chymase effects on perforin lysis**

Purified chymase I (or other granzymes) and isolated perforin were incubated together at 37°C for 30 min and then diluted before hemolytic assays. Perforin was prepared by Cu⁺⁺-IMAC (Poros resin; PerSeptive Biosystems) (19), followed by phenyl-Superose (Pharmacia) hydrophobic interaction chromatography (26). In detail, 50 ml of perforin solution was mixed with 100 ml of chymase I, dilutions of chymase I in MonoS buffer, or MonoS buffer as a control and incubated. Then 850 ml of salt-free 10 mM HEPES pH 7.5 buffer was added (to bring the solution to physiological salt), and the solution was diluted with HEPES-buffered saline (10 mM HEPES, pH 7.5, 0.15 M NaCl) with 10 μg/ml BSA (crystalline; U.S. Bio- logic, Cleveland, OH, No. 10856). In some experiments, the preincubation was omitted. Lysis was measured using five twofold serial dilutions of perforin (starting with a 1/20 final dilution) with rabbit RBC. The amount of perforin needed to lyse 50% of the erythrocytes was defined as 1 LU and calculated by linear regression of the log of the volume of perforin assayed vs lysis. Lysis of treated perforin was compared on the basis of LU per milliliter of perforin.

**Results**

**Chymase I purification**

Chymase I, the granzyme preferentially reactive with Bi-Aca-Aca-Phe-Leu-Phe(OPh)₂, was purified from RNK-16 granule extracts with approximately 30% recovery and 500-fold enrichment (Table I). The initial separation, by Superdex 200 size exclusion (Fig. 1A), was selected because the step was compatible with the high salt (>0.4 M NaCl) needed to stabilize the chymase activity. The high salt was also needed to separate the chymase from the high Mr proteoglycan (27) that complexes with granzymes (28) and interferes with their purification. Size exclusion also depleted most of the tryptases (GrA and GrK) and the Asp-ase (GrB) from the chymase peaks (Fig. 1B). The majority of granule protein (as indicated by the OD₂₈₀) eluted with the void volume where the high Mr proteoglycan also eluted (not indicated). There were four chymase peaks with apparent molecular masses of 600, 190, 88, and 14 kDa. The peak with the lowest apparent Mr was the most reactive with the biotinylated inhibitor (>99% inactivation after treatment with 0.1 mM for 10 min) and remained inhibited after dialysis. The first peak (600 kDa) was 28% inhibited and the second (190 kDa) peak of chymase was ~68% inhibited when 50 μM inhibitor was present in the enzyme assays. The third peak of chymase was unaffected. Neither the first or second chymase peak was inhibited after dialysis (or labeled with biotin detectable by SDS-PAGE). These data are consistent with unfavorable positioning of the inhibitors’ reactive phosphonate with these chymases, despite ability of the peptide inhibitor to compete with the peptide thioester substrate in the assays.

The net recovery of total chymase after sizing was close to the initial activity. From this recovery, we assumed that the initial amount of chymase I in the granules was proportional to the ~10% fraction appearing in the lowest Mr peak. We used the activity of

<table>
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<td>1570</td>
<td>196000</td>
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a Initial chymase based on the 9.5% relative abundance observed after the SD200 column.
b Recovery assumed to be equivalent for all four chymases.
this peak as 100% for our determination of the relative enrichment and recovery of chymase I (Table I). MonoS cation exchange chromatography was used as the second step of purification. The ion exchange depleted the contaminant tryptase GrK from the chymase (Fig. 2). The leading SD200 fractions of chymase I were excluded from further purification because they contained Asp-ase, GrB, which would copurify with chymase I by MonoS chromatography (not illustrated). Chymase I eluted from MonoS at ~650 mM NaCl. The overall enzymatic yield of chymase I after purification was ~30%. The protein yield was 8 μg from 13.7 mg of starting protein. This recovery indicates that the maximal abundance of chymase I protein within the granule extract is less than 0.2% of the total protein, based on calculations assuming 30% recovery of the protein (rather than better than 30% recovery, which could be the case if some of the enzyme lost activity).

Purified chymase I appears homogeneous after SDS PAGE. It was detected as a single silver-stained protein (Fig. 3A) and as a single biotinylated protein after reaction with the selective inhibitor (Fig. 3B). Chymase I has a molecular mass of ~30 kDa by SDS PAGE (Fig. 3), which indicated that it was nonspecifically retained on the SD200 column (where it eluted with 15-kDa proteins, Fig. 3A, lane 2). Purified chymase from as many as 3 × 10^{10} RNK-16 cells was sequenced. The limited N-terminal amino acid sequence that we obtained was Ile-(Ile/Leu)-Gly and is similar to the N-terminal Ile-Ile-Gly-Gly sequence common among the granzymes (29). In addition, the chymases eluted with GrB, GrA, GrK, and the other granzymes in a single sharp peak when a Cu^{2+}-IMAC column with an imidazole gradient was used to separate granzymes from other granule proteins (19). Chymase I could be separated from the other granzymes afterward by SD200 chromatography. However, the chymase recovery was lower than with the procedure of Table I.
Enzymatic properties of chymase I

Chymase I has a broad pH optimum between pH 7.5 and 9 (Fig. 4), which is the pH optimum characteristic of serine-dependent proteases. When the optimal pH was determined for unfractionated chymases within the granule extract, the maximum activity toward the same substrate was at pH 7.5 (data not shown), indicating that chymase I (optimal at pH 8.7) is different from the predominant chymases. Chymase I demonstrated a marked preference for tryptophan at the P1 amino acid of substrates (see Ref. 30 for substrate nomenclature) when used to hydrolyse several Boc-Ala-Ala-[P1=X]-SBzl substrates (Table II). Comparisons were made at 90 mM substrate concentration. Ninety micromolar concentrations of substrate were employed to facilitate detection of the low hydrolysis rates of Leu and Ser P1 substrates. Hydrolysis of the Trp P1 substrate was more than twofold faster than hydrolysis of the Phe P1 substrate. It is noteworthy that there was no detectable hydrolysis of the Tyr P1 substrate by chymase I while this substrate was concurrently hydrolysed by the unfractionated granule extract. Km values were not determined due to the low availability of chymase I. The turnover rate for the Suc-Phe-Leu-Phe-SBzl substrate at the solubility limit of 28 μM was 4.5 pmols/s whereas the rate for Boc-Ala-Ala-Trp-SBzl at 90 μM was 7.5 pmols/s, both with 97 ng of chymase I, indicating that the commercial reagent was a reasonably sensitive substrate despite its lack of an optimal P1 amino acid. These substrates have also been used to indicate that chymase I is unique. Ratios of enzyme activities of this peak toward Suc-Phe-Leu-Phe-SBzl vs Boc-Ala-Ala-Tyr-SBzl substrates indicate that it (chymase I) is distinct; the other chymases hydrolysed Boc-Ala-Ala-Tyr-SBzl (not indicated).

Biologic properties of chymase I

A biotinylated serine protease inhibitor, Bi-Aca-Aca-Phe-Leu-Phe(OPh)2, inactivates perforin-mediated lysis (17). A chymase reactive with this inhibitor copurified with perforin on SD200. It was detectable when the ~70-kDa perforin fraction was treated with the inhibitor and then highly concentrated before SDS-PAGE and protein blotting. (See the biotinylated 30-kDa protein of Fig. 3B, lane 1). Purified chymase I also reacted with the biotinylated inhibitor (Fig. 3B, lane 3) and has the same Mr as the perforin-associated chymase. An additional ~45-kDa band visible in Figure 3B, lanes 1 and 2, is an endogenous avidin-binding protein found with the granule proteins. So far we have been unable to purify perforin to homogeneity and retain lytic activity. For this reason, we supplemented highly enriched (and still lytic) perforin with purified chymase I to determine its effects on perforin lysis. When perforin was preincubated with chymase I at 37°C (without calcium), there was a dose-dependent loss of lytic activity (Fig. 5). Without the preincubation, there was no effect on perforin lysis when identical amounts of chymase I were added in similar protocols. Because calcium will inactivate perforin during preincubation, there are two other differences in these protocols: the presence or absence of calcium and the availability of a membrane to accept the pore. Pretreatment of perforin with GrK or the two other chymase peaks separated by SD200 (at similar mOD min⁻¹ ml⁻¹ of enzyme activity) was without effect on lysis. Addition of human neutrophil cathepsin G, another “chymase,” was also without effect. It should be noted that these preparations of perforin contain ~1 mOD min⁻¹ of chymase per 1000 lytic units of perforin (~100 mOD min⁻¹ per mg of perforin) (31).
CHYMASE I, A GRANZYME IMPLICATED IN PERFORIN-MEDIATED LYSIS

We have purified a new rat chymase. We believe that it is the first lymphocyte chymase granzyme purified and characterized enzymatically. Chymase I has many properties typical of a serine protease of the chymotrypsinogen subfamily. The molecular mass of 30 kDa suggests that there are 1 or 2 glycosylation sites on a 30-kDa chymase participates in perforin-mediated cytotoxicity. For this interpretation to be valid, chymase I would have to have a human species equivalent. Granzyme H (14, 36), so far found only in humans, is a potential species homologue for equivalent function because granzyme H contains a large, apparently uncharged, substrate-binding pocket that is able to accommodate aromatic amino acids. The other human granzymes (A, B, K, and M) lack chymase activity (J.C.P., unpublished data).

FIGURE 5. Supplementation of perforin with chymase I. Highly enriched perforin was incubated with the indicated concentrations of purified chymase I for 30 min at 37°C, diluted, and then assayed using further twofold dilutions for lysis of RBC. Specifically, the indicated concentrations (mOD min^-1 ml^-1) of chymase I (or buffer control) was added to a final volume of 0.15 ml with purified perforin and incubated (see Materials and Methods) before 20- to 80-fold dilutions in the lytic assays. The perforin preparation contained traces of other proteins, including perforin enhancing protein (PEPr) (26) and calreticulin (Ref. 40 and our unpublished results).

Discussion

We have purified a new rat chymase. We believe that it is the first lymphocyte chymase granzyme purified and characterized enzymatically. Chymase I has many properties typical of a serine protease of the chymotrypsinogen subfamily. The molecular mass of 30 kDa suggests that there are 1 or 2 glycosylation sites on a chymotrypsin-like structure that lacks additional domains. (The kringle structures of many blood coagulation proteases are examples of additional domains.) The pH optimum is also typical. Chymase I was inhibited by both DCI (32) and PMSF, “general” serine protease inhibitors that react with almost all serine proteases. The limited N-terminal amino acid sequence and its elution from Cu2+ -IMAC suggest that chymase I is likely to be a granzyme.

A simple interpretation is that this 30-kDa chymase participates in perforin-mediated cytotoxicity. For this interpretation to be valid, chymase I would have to have a human species equivalent. Granzyme H (14, 36), so far found only in humans, is a potential species homologue for equivalent function because granzyme H contains a large, apparently uncharged, substrate-binding pocket that is able to accommodate aromatic amino acids. The other human granzymes (A, B, K, and M) lack chymase activity (J.C.P., unpublished data).

We also found that preincubation of enriched perforin with purified chymase I depressed lytic activity. The experimental conditions are compatible with premature proteolysis of a perforin-associated granule protein by chymase I. Under calcium- and membrane-free conditions, the nascent product may have assumed a nonfunctional and irreversible conformation that will not enhance lysis. Lysis was reduced as a consequence when calcium and red cells are provided. Because the perforin contains only residual chymases, the enzyme supplementation (to physiologic ratios) may be required to produce its effects during preincubation.

The reduction of lysis could be interpreted as counter to a positive regulatory role for chymase I in lysis. A down-regulatory role for chymase I would place it as an intragranular protein to limit lysis of “by-stander” cells that are not targeted for lysis and add chymase I to the plasma proteins protein S (vitronectin) (37) and apolipoprotein B (38, 39), which reduce perforin’s lytic activity. However, we suggest that the issue of up- vs down-regulation of lysis remains open. Chymase I definitely reacts with a protein important to lysis. This interaction appears specific because the other endogenous chymases lacked an effect on lysis under the same conditions.

The specificity of chymase I is also reflected by the enzymatic and the genetic variability among the chymase granzymes. Chymase I prefers Trp P1 while the two other rat lymphocyte chymases that are in separate SD200 peaks prefer Phe to Trp at P1 (S.A.F., unpublished results). The rodent granzymes predicted to encode chymases differ in the genetic regions that specify their substrate binding sites (12). At this time, perforin and perforin enhancing protein (PEPr; Ref. 26) are two proteins that participate
in lysis that are potential natural substrates to examine for selective hydrolysis by lymphocyte chymase I.

In summary, we have purified and characterized a native lymphocyte chymase. Our data suggest that it (chymase I) may have a role in perforin lysis. At present, its absolute requirement for lysis is unresolved. The low abundance of this granzyme within granules indicates that recombiant expression of the rat chymase granzymes would greatly benefit future studies.

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