Purification and Properties of the Cathepsin D Type Proteinase from Beef and Rabbit Lung and Its Identification in Macrophages

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The acid-acting proteinase, cathepsin D (EC 3.4.4.23), was purified from extracts of homogenized rabbit lung and beef lung by autolysis at acid pH, acetone and ammonium sulfate fractionation, column chromatography, and isoelectric focusing. Four isoenzymes were obtained from each source. With acid hemoglobin as the substrate, the proteinase from rabbit lung had a pH optimum of 3.0 and that from beef lung had a pH optimum of 3.6. Their activity was not affected by thiol reagents or by Fe²⁺, Mn²⁺, or Mg²⁺. One isoenzyme from rabbit lung was used to immunize a goat, and one from beef lung was used to immunize a rabbit. In immunoelectrophoresis, each resulting antisera formed a single precipitin line with its homologous enzyme. They cross-reacted with the other three isoenzymes from the same species, but not with any isoenzyme from the other species. At high concentrations, each antisera completely inhibited the proteolytic activity of its homologous enzyme. The antisera against rabbit lung cathepsin D also inhibited the proteolytic activity of rabbit peritoneal and pulmonary macrophages. In limited quantities, this antisera has now been made commercially available and is being used with labeled antibody techniques to identify under a microscope the presence of cathepsin D in macrophages and to study its role in the pathogenesis of tuberculosis.

The lung not only is an organ of respiration, but also a major defense organ, disposing of infectious and other inhaled agents. A substantial part of the lung parenchyma consists of alveolar macrophages, which often accomplish these defense functions by means of hydrolytic enzymes.

In 1955, we partially purified from beef lung a proteinase having a pH optimum between 3 and 4, with hemoglobin as the substrate (12, 13). It hydrolyzed the B chain of insulin at the same sites as pepsin (13) and cathepsin D (7, 19, 21, 47), namely adjacent to the aromatic, long chain aliphatic, and dicarboxylic amino acids. Although cathepsin D has also been purified from liver (4, 5), spleen (19, 21, 33, J. B. Ferguson, J. R. Andrews, I. M. Voinick, and J. S. Fruton, Biochemistry, in press), and uterus (47), its complete purification from lung has not been previously accomplished. This report describes such a purification from both rabbit and beef lungs. The cathepsin D probably comes from the macrophages that lung contains.

Antibodies to the purified rabbit and beef proteinases were produced in goats and rabbits, respectively. These antibodies were used (i) to confirm the purity of each enzyme by immunoelectrophoresis, (ii) to inhibit both the lung and macrophage enzymes, and (iii) to confirm the presence of this proteinase in macrophages by a labeled antibody histochemical technique (cf. 15, 32, 43). The latter is described in another report (O. Rojas-Espinosa, A. M. Dannenberg, Jr., L. Sternberger, and T. Tsuda, Amer. J. Pathol., in press), which evaluates the role of this cathepsin D in the pathogenesis of tuberculosis.

MATERIALS AND METHODS

Sources of enzyme. Frozen rabbit lungs, type 3, were purchased from Pel-Freez Biologicals Inc., Rogers, Ark. Fresh beef lungs were purchased from a local slaughter house. All lungs had been trimmed and washed. Granulomatous rabbit lungs were produced in 25 animals by the intravenous injection of 0.3 ml of heat-killed, lyophilized tubercle bacilli (BCG) (1.0
mg/ml) in Aquaphor-Marcol 52 (1:4) (27, 30). Fourteen days later, these lungs, containing multiple confluent granulomata, were removed, trimmed, washed in distilled water, and frozen at −20°C for several weeks.

Rabbit peritoneal macrophages (MN) were obtained by injecting 35 ml of mineral oil intraperitoneally into rabbits and, 4 to 6 days later, collecting the MN in citrated saline solution (10, 11). The cell suspension was centrifuged, suspended in citrated saline, counted in a hemacytometer, and quickly frozen at −60°C. Differential counts showed 80 to 90% macrophages (10, 11). After at least a week in the frozen state, the cell suspension was thawed, homogenized with a serological pipette, diluted to the appropriate concentration with 0.9% saline, and used as a source of proteinase.

Rabbit pulmonary alveolar macrophages (AM) were obtained by perfusing fresh rabbit lungs four times intratracheally with 30 to 40 ml of citrated saline solution (11, 29). The differential counts showed 98 to 100% AM (11). The AM were counted, frozen, stored, and used as a source of proteinase in the manner just described.

Activity of purified proteinase with acid hemoglobin as the substrate. Macrophage and lung cathepsin D activity was assayed with hemoglobin as the substrate by a method (10, 12, 25) adapted from that of Anson (1). Bovine hemoglobin substrate powder (4.0 g) (Worthington Biochemical Corp., Freehold, N.J.) was dissolved in 100 ml of 0.05 M HCl. The resulting 4.0% solution had a pH of 3.2. No buffer was added. The enzyme and substrate solutions (each 1.3 ml) were mixed and incubated in a water bath at 38°C. At 0 h and 1 h, 1.0-ml samples were removed, mixed with 1.0 ml of 5% trichloroacetic acid, and centrifuged. A 1.0 ml portion of supernatant fluid was diluted with 2.0 ml of distilled water, and the optical density (OD) was read at 280 nm in 1-cm quartz cuvettes. The difference in OD between the 0-h and 1-h samples was linearly proportional to proteolytic activity up to an OD of about 0.350. A 0.10 unit of proteolytic activity was defined as that quantity of enzyme that caused a difference of OD of 0.100 under these conditions. The specific activity of the enzyme preparation was defined as the total units of activity per milligram of protein determined by the Lowry procedure (22).

Isoelectric focusing. A modification of the original technique of Svensson (40) was used. A 110-ml column (no. 8102, LKB-Produkter AB, S-161 25 Bromma 1, Sweden) was prepared by adding 20 ml of 2% monoethanolamine in 60% sucrose to the bottom, the cathode end. Next was added a 100-ml linear sucrose gradient, from 50% sucrose (containing 1% ampholytes, pH 5 to 8) to distilled water (containing the partially purified enzyme in 1% ampholytes, pH 5 to 8). Finally, 10 ml of 2.9% phosphoric acid solution in water (i.e., 2.9% of the 85.5% commercial solution) was added on top, the anode end. The current was applied at 300 V for 24 h and then at 1,200 V for the next 48 h. The column was cooled to 4°C by water from a constant-temperature bath. The gradient was removed by pumping distilled water into the top of the column at a rate of about 1 ml/min. Fractions (6 ml) were collected and analyzed for OD at 280 nm, for pH, and for cathepsin D activity.

**Disk electrophoresis.** A Buchler vertical gel electrophoresis apparatus (Polyanalyst, no. 3-1750, Buchler Instruments Div., Fort Lee, N.J.) was used with a modification of the method described by Campbell (9) and Gordon (16). The samples, 0.1 to 0.2 ml, contained about 200 μg of protein in 40% sucrose and tris(hydroxymethyl)aminomethane (0.01 M)-glycine (0.08 M) buffer (pH 8.2). They were subjected to electrophoresis on 7% acrylamide at 3 mA per tube for 90 min. The gels were stained for 10 min with 0.25% Coomassie blue R (no. B 0630, Sigma Chemical Co. St. Louis, Mo.) in water-methanol-acetic acid (5:5:1). The excess color was eluted with the same solvent without dye.

**Immunization of goats with purified rabbit lung cathepsin D.** High-titer goat anti-rabbit cathepsin D was produced by Cappell Laboratories, Inc., Downingtown, Pa., in the following manner. Our most highly purified cathepsin D preparations, namely the pH 6.6 isoenzyme (from normal rabbit lungs [I] and from granulomatous rabbit lungs [II]), were shipped to Cappell Laboratories in a frozen state. A 9-ml portion of preparation I, containing 0.22 mg of protein per ml of 0.9% NaCl, was emulsified with 9 ml of complete Freund adjuvant (Cappell lot 6001) and injected subcutaneously (s.c.) into a healthy goat in multiple sites. Every 2 weeks thereafter, for a 10-week period, 2.5 ml of the same preparation was emulsified with 2.5 ml of Freund incomplete adjuvant (Cappell Lot 6030) and injected s.c. The goat was bled weekly (150 ml) from 4 weeks on and exsanguinated at 12 weeks.

A second healthy goat was similarly immunized with preparation II containing 0.13 mg of protein per ml. A 12-ml portion (instead of 18 ml) was injected initially, and three (instead of five) biweekly booster injections (5 ml each) were made. The goat was bled weekly from 3 weeks on and exsanguinated at 10 weeks.

Samples of these antisera are still available from Cappell Laboratories, as we did not purchase their complete supply.

**Immunization of a rabbit with purified beef lung cathepsin D.** A 2-ml portion of our most purified beef cathepsin D preparation (containing 0.056 mg of protein per ml of 0.9% NaCl) was emulsified with 2 ml of complete Freund adjuvant (3119-60-5 Adjuvant, complete with H3Ra, Difco Laboratories, Detroit, Mich.) and injected into a 4-kg New Zealand rabbit in 40 intradermal sites. Each site thereafter, for a 10-week period, 2.5 ml of the same preparation was emulsified with 2.5 ml of Freund incomplete adjuvant (Cappell Lot 6030) and injected s.c. The goat was bled weekly from 4 weeks on and exsanguinated at 10 weeks.

The rabbit was injected again, s.c. in several sites, with antigen every 2 weeks. The first booster injection was similar in composition to the initial injection. Each subsequent booster injection consisted of 2.0 ml of the antigen (in saline solution) previously heated for 30 min at 65°C to allow its aggregation, and no adjuvants were employed. A good antibody response was observed after the third booster injection, and the rabbit was exsanguinated 10 days after the fourth booster injection.

**Immunoelectrophoresis.** A modification of the method described by Grabar (17) was employed (see also 91). Microscope slides were coated with 2.5 ml of 1% Special Agar Noble (no. B-142, Difco) or 1% Agarose (no. A 6877, Sigma Chemical Co.) in 0.05 M barbitol buffer, pH 8.2. Two small holes, made beside
the central slot in the agar, were filled three times with the antigens at the concentrations described in the two preceding sections. Electrophoresis was then carried out in a Lucite cell (no. 3-1021, Buchler Instruments Div.) filled with the same buffer at a constant current of 7 mA per slide. Electrophoresis was allowed to proceed until the tracking dye (0.1% bromophenol blue added after the antigen) reached the edge of the plate (about 90 min). The central slots were then filled with antiserum to purified lung cathepsin D, and the resulting precipitin lines were allowed to develop for 24 h at room temperature. The slides were then dialyzed against 0.9% NaCl solution, stained with Coomassie brilliant blue R as in disk electrophoresis, eluted in solvent, blotted with filter paper, and then dehydrated further in an incubator at 37°C for 2 to 6 h.

RESULTS

Purification of the cathepsin D type proteinase of rabbit lung. This acid-acting proteinase was purified by a modification of the Barrett method for the purification of rabbit liver cathepsin D (4, 5).

Normal rabbit lungs (1,500 g) were homogenized in a Waring blender for 5 min with 1% n-butanol in 1% NaCl (1200 ml) at 2 to 4°C. The resultant suspension was diluted to 4,000 ml with the saline-butanol and centrifuged at 7,000 rpm (8,000 × g) for 30 min at 2°C. The pH of the supernatant fluid (a fine suspension) was adjusted to 3.6 with 5.0 M sodium formate buffer, pH 3.0, and the preparation was incubated at 37°C overnight to permit autolysis to occur. The suspension was centrifuged at 7,000 rpm for 30 min at 2°C, the precipitate was discarded, and the clear brown supernatant fluid was diluted to 4,000 ml with distilled water (DW).

When larger amounts of lung were homogenized as above, the protein in the clear brown supernatant fluid was first concentrated by precipitation with (NH₄)₂SO₄ at 90% saturation, dissolved in a minimum amount of DW, and dialyzed against 0.05 M sodium citrate buffer, pH 3.5, until the sulfate ions were absent when tested with BaCl₂. Its volume was then brought to 4,000 ml with DW.

The solution was cooled to 2°C in an ice bath. Then, 3,600 ml of chilled (10°C) anhydrous acetone (sodium sulfate dried) was added over a period of 10 min with continuous stirring. The stirring was continued for an additional 1 to 2 h in the cold. The fine precipitate was removed by centrifugation (7,000 rpm for 30 min at 2°C), and the supernatant fluid was treated as before with an additional 3,600 ml of chilled anhydrous acetone. The resulting suspension was left overnight at 2 to 4°C.

After centrifugation (7,000 rpm for 30 min at 2°C), the precipitate was suspended in a minimum volume of 0.002 M Tris-hydrochloride buffer, pH 9.0, and dialyzed against four to six changes of the 0.002 M Tris-hydrochloride buffer (each 12,000 ml).

The enzyme preparation was then applied to a column of DEAE-cellulose equilibrated with 0.0144 M Tris-hydrochloride buffer (pH 7.8), in a ratio of two volumes of enzyme to 1 volume of packed diethylaminoethyl (DEAE)-cellulose bed. The column was then washed with ten volumes of the buffer, and the enzyme was eluted with ten volumes of a linear gradient of 0 to 0.2 M NaCl in the Tris-hydrochloride buffer, pH 7.8. Fractions of about 13.5 ml were collected and tested for enzymatic activity. Fractions with high specific activity were pooled and dialyzed for 36 h against three or four changes of 0.03 M sodium acetate buffer, pH 4.75, (Fig. 1).

The enzyme preparation was then applied to a CM-Sephadex C-50 column equilibrated with 0.03 M acetate buffer, pH 4.75, in the ratio of three volumes of enzyme to one volume of packed CM-Sephadex. The enzyme was then eluted with pH 5.5 sodium acetate buffer (rif/0.2). Fractions of 12 ml were collected and tested for protein and enzyme activity (Fig. 2). The fractions with high specific activity were pooled and dialyzed against 1% glycerol in water. In subsequent experiments, dialysis was made against 0.3% glycerol.

The dialyzed enzyme was placed in an isoelectric focusing column containing a sucrose gradient with ampholites, pH 5 to 8, (see Materials and Methods). The fractions from this column were analyzed, and the peaks of proteinase activity were pooled. There were four

![Fig. 1. Distribution of rabbit lung cathepsin D in fractions eluted from a DEAE-cellulose (DE-52) column by a linear gradient of 0 to 0.2 M NaCl in Tris-hydrochloride buffer. The proteinase activity of cathepsin D (represented by the OD increment at 280 nm) was determined with hemoglobin as the substrate at pH 3.2. The total protein present was considered proportional to the OD of the fractions at 280 nm. The shaded area indicates the fractions pooled for the CM-Sephadex column.](http://iai.asm.org/download)
active fractions at pH 5.8, 6.2, 6.6 and 6.8, respectively (Fig. 3), which probably represent different isoenzymes (5). The pH 6.6 isoenzyme was used to immunize the goat.

Compared with the original saline homogenate, there was approximately 150-fold purification of rabbit lung cathepsin D with 0.7 to 3% yield (Table 1). Figure 4 shows the results of electrophoresis on acrylamide gel during these various stages of purification. Note that the sample used to immunize a goat showed one main band on gel electrophoresis and one very minor band, which was, however, not detected by immunoelectrophoresis. The latter is probably the tail of the pH 6.8 isoenzyme, which did not fully separate from the pH 6.6 isoenzyme. The broad band near the positive end of the gels is probably an isoenzyme plus considerable inactive protein.

The purification procedure just outlined was repeated by using 25 rabbit lungs (1,500 g) made granulomatus by the intravenous injection of lyophilized heat-killed BCG in oil. Table 1 summarizes the results. The specific activity of the original homogenate of granulomatus lungs was about twice that of normal lungs. The purification steps were similar, and the end

<table>
<thead>
<tr>
<th>Step</th>
<th>Specific activity (units/mg of protein)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
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</thead>
<tbody>
<tr>
<td>Normal lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I Initial homogenate</td>
<td>0.17</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>II Autolysis</td>
<td>0.18</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>III Acetone</td>
<td>2.1</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>IV DEAE Cellulose</td>
<td>4.5</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>V CM Sephadex</td>
<td>10.8</td>
<td>64</td>
<td>1</td>
</tr>
<tr>
<td>VI Isoelectric focusing</td>
<td>27.0*</td>
<td>160*</td>
<td>0.7*</td>
</tr>
<tr>
<td>Granulomatus lung</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>I Initial homogenate</td>
<td>0.46</td>
<td>1</td>
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<td>41</td>
</tr>
<tr>
<td>III Acetone</td>
<td>4.7</td>
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<td>13</td>
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<td>IV DEAE Cellulose</td>
<td>12.0</td>
<td>26</td>
<td>8</td>
</tr>
<tr>
<td>V CM Sephadex</td>
<td>37.0</td>
<td>80</td>
<td>5</td>
</tr>
<tr>
<td>VI Isoelectric focusing</td>
<td>66.0*</td>
<td>144*</td>
<td>3*</td>
</tr>
</tbody>
</table>

*See text.
*A unit of proteolytic activity is the amount that should produce (if linearity held) an OD difference of 1.000 under conditions described in Materials and Methods.
*Value for the isoenzyme used to immunize a goat.
*Sum of all four isoenzymes.

![Figure 2](http://iai.asm.org/)

**Fig. 2.** Distribution of rabbit lung cathepsin D in fractions eluted from a CM-Sephadex (C-50) column by acetate buffer. The shaded area indicates the fractions pooled for isoelectric focusing. (See Fig. 1 for other details.)

![Figure 3](http://iai.asm.org/)

**Fig. 3.** Distribution of rabbit lung cathepsin D after isoelectric focusing in a sucrose gradient containing pH 5 to 8 ampholytes. The shaded area indicates the fractions pooled for immunizing a goat.
product apparently was identical. The pH 6.6 isoenzyme was also used to immunize a goat.

**Purification of the cathepsin D type proteinase of beef lung.** This acid-acting proteinase was purified by continuing the method described by Dannenberg and Smith (12) through additional steps. Fresh beef lung (2,000 g) was blended at 4 C in a Waring blender with 4,000 ml of cold (–10 C) 50% acetone in water. (Note that a typographical error was present in the original directions (12): 50%, not 100%, acetone must be used). The blended mixture was centrifuged, and the supernatant fluid was filtered through gauze. Two volumes of cold 100% acetone (–20 C) were added, and the resulting suspension was left overnight in the cold room (2 to 4 C). The sediment was mixed with three or four volumes of cold 100% acetone and centrifuged. The precipitate, mixed with three times its volume of 100% acetone, was centrifuged again. The sediment was collected as a paste and stored in a desiccator at –80 C. It did not appear necessary to form an acetone dried powder as previously described (12).

About 40 g of the paste was mixed with 1,000 ml of DW and centrifuged. Fe(NH4)2(SO4)2 was added to the supernatant fluid to a final concentration of 0.004 M, and the pH was adjusted to 3.0 with 0.1 M HCl. After the addition of citrate buffer (1.0 M, pH 3.0) (1 ml for each 24 ml), the suspension was allowed to stand for 3 to 7 days in the cold (2 to 4 C).

The precipitate was discarded, and the supernatant fluid was further purified by a 0.45 to 0.90 saturated ammonium sulfate cut. After dialysis against 0.004 M Fe(NH4)2(SO4)2, the proteinase was concentrated fourfold in a lyophilizer.

The partially purified cathepsin was then chromatographed on CM-Sephadex (C-50) columns containing 0.05 M sodium phosphate buffer, pH 7.4, and eluted with the same buffer. The most active fractions leaving the columns were pooled and placed in the isoelectric focusing apparatus. Similar to the rabbit cathepsin, four active isoenzymes were found (Fig. 5). They had isoelectric points at pH 5.75, 6.4, 6.6, and 7.0. The pH 7.0 isoenzyme was used to immunize a rabbit.

Compared with a saline homogenate of whole beef lung, there was a 93-fold purification of beef lung cathepsin D with a 24% yield (Table 2). The sample used to immunize the rabbit had only one band in acrylamide gel electrophoresis (Fig. 4).

**Immunoelectrophoresis.** The purified rabbit and beef lung cathepsin D (used to immunize the goat and rabbit, respectively) showed a single precipitin line in immunoelectrophoresis with their respective antisera (Fig. 6), indicating a high degree of purity. The purified cathepsin D from both normal and granulomatous rabbit lungs showed an identical single precipitin line with antibody against the normal rabbit lung cathepsin D. The antibody prepared against rabbit lung cathepsin D showed no line with purified beef lung cathepsin D. Conversely, the antibody against beef lung cathepsin D showed no line with purified rabbit lung cathepsin D. Similar results were observed by the Barrett group (5) when antibodies to purified chicken, human, and rabbit liver cathepsin D were compared. Sheep antibody against purified rabbit liver cathepsin D, supplied by Barrett (5, 15), showed one precipitin line with our purified rabbit lung cathepsin D, but no line with our purified beef preparation. Thus, antibodies to these purified proteinases are specific for the species of origin, but nonspecific for the organ of origin.

The undiluted antisera against the pH 6.6
rabbit isoenzyme and the pH 7.0 beef isoenzyme precipitated in capillary tubes the four isoenzymes of their own species. They did not precipitate the isoenzymes of the other species. These results were confirmed in immunoelectrophoresis with the four rabbit isoenzymes and three of the beef isoenzymes. (Our supply of the pH 5.8 beef isoenzyme had been exhausted.)

**Specificity of antiserum.** Goat antiserum against purified rabbit lung cathepsin D (0.1 ml) was incubated with the purified rabbit enzyme (0.1 ml) in 0.9% NaCl in small test tubes (10 by 50 mm) for 30 min at 37 C, chilled in an ice bath for 10 min, and centrifuged in the cold to remove the precipitate. The supernatant fluids (0.1 ml) were assayed for protease activity at pH 3.2 with hemoglobin as the substrate. Similar procedures were performed with homogenates of rabbit pulmonary AM and rabbit oil-induced MN. Also, rabbit antiserum against purified beef lung cathepsin D was tested against the purified beef enzyme. The results are listed in Table 3. All of the protease activity of these preparations was removed by undiluted goat antiserum against rabbit cathepsin D (listed as 1:2 final concentration). More dilute antiserum was less effective. About 75% of the protease activity of purified beef lung cathepsin D was removed by its specific antiserum.

The antiserum against rabbit cathepsin D only partially inhibited the proteolytic activity of rabbit AM and MN when the enzyme-antibody precipitate was not removed: a 1:2 dilution of the antiserum inhibited 88% of the AM and MN activity, and a 1:4 dilution of the antiserum inhibited 50% of their activity. Evidently at pH 3.2, the antiserum only partially blocks the enzyme’s active site or readily dissociates from it.

**Characterization of purified rabbit and beef lung cathepsin D.** These studies employed rabbit and beef cathepsin D purified through the CM-Sephadex step. The rabbit protease had a pH optimum of 3.0, and the beef protease had an optimum of 3.6 (Fig. 7).

<table>
<thead>
<tr>
<th>Source of proteinase</th>
<th>Dilution of antibody (after mixing with enzyme) and percentage of original proteolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rabbit lung cathepsin (0.30 units)</strong></td>
<td>1:2 0%</td>
</tr>
<tr>
<td></td>
<td>1:4 10</td>
</tr>
<tr>
<td></td>
<td>1:8 54</td>
</tr>
<tr>
<td></td>
<td>1:16 76</td>
</tr>
<tr>
<td></td>
<td>1:32 100</td>
</tr>
<tr>
<td><strong>Rabbit pulmonary macrophages</strong></td>
<td>1:2 0%</td>
</tr>
<tr>
<td>(0.6 x 10^6 cells 0.30 units)</td>
<td>1:4 4</td>
</tr>
<tr>
<td></td>
<td>1:8 34</td>
</tr>
<tr>
<td></td>
<td>1:16 44</td>
</tr>
<tr>
<td></td>
<td>1:32 100</td>
</tr>
<tr>
<td><strong>Beef lung cathepsin (0.30 units)</strong></td>
<td>1:2 26%</td>
</tr>
<tr>
<td></td>
<td>1:4 65</td>
</tr>
<tr>
<td></td>
<td>1:8 82</td>
</tr>
<tr>
<td></td>
<td>1:16 91</td>
</tr>
<tr>
<td></td>
<td>1:32 100</td>
</tr>
<tr>
<td><strong>Rabbit peritoneal macrophages</strong></td>
<td>1:2 1%</td>
</tr>
<tr>
<td>(5 x 10^6 cells 0.36 units)</td>
<td>1:4 24</td>
</tr>
<tr>
<td></td>
<td>1:8 40</td>
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<td></td>
<td>1:16 58</td>
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<td></td>
<td>1:32 93</td>
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</table>

* Antibody was mixed with its respective enzyme preparation for 15 min at 37 C and centrifuged. The proteolytic activity remaining in the supernatant fluid is listed (see Text). Antirabbit cathepsin D antibody (produced in goats) was mixed with the rabbit enzyme preparations; antirabbit cathepsin D antibody (produced in a rabbit) was mixed with the beef enzyme preparation.

* See Table 1.

* The results listed represent an average of four preparations.
The proteolytic activity of both enzymes was a linear function of enzyme concentration up to an OD increment of about 0.350. Then there was a gradual decrease in slope.

Various compounds were tested for the ability to inhibit or activate these cathepsins (see 25). At twice the final concentration, they were preincubated with the enzyme (0.3 units) for 15 min at 37°C before the hemoglobin substrate was added. With both proteinases, cysteine had no effect, nor did any of the following compounds: iodoacetamide, p-chloromercuribenzoate, disodium ethylenediaminetetraacetate, Fe (NH₄)₂(SO₄)₂, Mg (CH₃COO)₂, and MnCl₂. The concentrations of each were 0.0005 and 0.005 M after the addition of substrate. Pb(NO₃)₂ and HgCl₂ (both 0.005 M) inhibited the rabbit enzyme about 15%. HgCl₂ inhibited the beef enzyme about 20%.

Sodium Pepstatin, a highly specific cathepsin D inhibitor (2, 3, 6, 18, 20, 25, 28, 39, 41, 46), inhibited the beef and rabbit lung proteinases 100% at 0.05 μM concentration and about 50% at 0.005 μM (25). The beef enzyme (specific activity of 1.43 units per mg of protein) had been purified through the CM-Sepahex step. The rabbit enzyme was more crude (specific activity 0.54). A CM-Sepahex purified rabbit proteinase (specific activity 10.8) was also tested and found to be inhibited by one-tenth of the above Pepstatin concentrations. The proteolytic activity of rabbit pulmonary and peritoneal macrophages was inhibited 100% by 0.05 μM Pepstatin and about 50% by 0.005 μM Pepstatin (25).

**Acid proteinase activity of rabbit and beef organs.** Beef and rabbit liver, spleen, lung, and kidney were assayed for proteinase activity at pH 3.2. Four animals of each species were evaluated. A 1-g portion of each organ was homogenized with five times its weight of DW in a chilled Potter-Elvehjem homogenizer. The cells were further disrupted by three cycles of freezing and thawing in a dry ice-acetone bath and a 37°C water bath, respectively. The suspensions were then centrifuged at 4°C for 30 min at 3,000 rpm (2225 g). The supernatant fluids were diluted 10-fold with 0.05 M sodium citrate buffer, pH 3.2, and their proteinase activity was determined. The method of Lowry was used to determine their protein concentration.

Table 4 shows the results. The extractable proteinase per gram of tissue was highest for spleen, as was the specific activity. Rabbit lung extracts had the next highest specific activity. Extracts of rabbit liver and kidney showed less specific activity than extracts of beef liver and kidney. At present, we have no truly satisfactory explanations of these findings.

**DISCUSSION**

Cathepsin D is a major lysosomal enzyme of many cells: macrophages, lymphocytes (8), fibroblasts, epithelial and synovial cells, chondrocytes, and liver parenchymal cells (see 32). Cells, like macrophages, that specialize in digestive functions are particularly rich in this acid-acting proteinase (10, 31, 32; O. Rojas-Espinosa, A. M. Dannenberg, Jr., L. Sternberger, and T. Tsuda, Amer. J. Pathol., in press). A brief discussion of how this proteinase hydrolyzes its substrates appears in our review of inhibitors of pepsin and cathepsin D (25).

Woessner (unpublished data) tested our partially purified beef lung cathepsin D (specific activity 2.6) on the hexapeptide Glu-Ala-Leu-Tyr-Leu-Val and found that it split the Leu-Tyr and Tyr-Leu bonds. With Phe-Phe-NH₂, the split occurred at the Phe-Phe bond and with Phe-Tyr-NH₂ at the Phe-Tyr bond (unpubl).

**Table 4. Acid proteinase activity of rabbit and beef organs**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Rabbit</th>
<th>Beef</th>
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<tr>
<td></td>
<td>Extractable proteinase per gm of tissue (units)^a</td>
<td>Specific activity of extract per gm of tissue (units)</td>
</tr>
<tr>
<td>Lung</td>
<td>11.7 ± 3.3</td>
<td>0.20 ± 0.07</td>
</tr>
<tr>
<td>Liver</td>
<td>10.1 ± 2.1</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Spleen</td>
<td>16.4 ± 1.2</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>Kidney</td>
<td>5.1 ± 0.2</td>
<td>0.07 ± 0.01</td>
</tr>
</tbody>
</table>

^a The means (and their standard errors) of results from at least four animals are listed.

^b See Table 1.
lished data). Cleavage of the hexapeptide and Phe-Tyr-NH₂ was blocked by 2 μg of Pepstatin.

Partially purified preparations of beef lung cathepsin D polymerized aromatic amino acid esters (13). Purified beef lung cathepsin D did not (O. Rojas-Espinosa, P. Arce-Paredes, A. M. Dannenberg, Jr., and R. L. Kamenetz, manuscript in preparation). In fact, the polymerizing activity of the impure preparations was found to be associated with the chymotrypsin-like protease that hydrolyzed N-benzoyl-D,L-phenylanine-β-naphthol ester (10, 12). Purification and characterization of this new enzyme (a monoaminopeptidase) will be the subject of another report (O. Rojas-Espinosa et al., manuscript in preparation).

The lung cathepsin D described herein probably comes from the lung macrophages for the following reasons: (i) the lungs are rich in macrophages; (ii) labeled-antibody and substrate film techniques showed that lung macrophages contain more cathepsin D than any other cell present (O. Rojas-Espinosa et al., Amer. J. Pathol., in press, cf. 32); (iii) granulomatous lungs contained more cathepsin D and more macrophages (Table 1); (iv) the pH optima of lung proteinase and macrophage proteinase are identical (10, 12); (v) Pepstatin, a highly specific cathepsin D inhibitor (2, 3, 6, 18, 20, 25, 28, 39, 41, 46), readily inhibits the proteinase from both sources (25); and (vi) the antibody to purified lung cathepsin D also inhibits macrophage cathepsin D (Table 3).

The cathepsin D present in macrophages throughout the body undoubtedly has many biological functions. It aids these cells in digesting the bacteria, fungi, and viruses that they ingest. It also aids them in digesting other proteins, e.g., the hemoglobin from effete erythrocytes and many antigens. In the latter case, the antigenic dose is thereby regulated to avoid an excess that might induce tolerance.

Split-protein products released from macrophages probably contribute to the vasodilation and exudation of the inflammatory response (26, 38, 45). C5a can be produced from the C5 component of complement by lung and macrophage cathepsin D (36). This fragment is an extremely active chemotactic factor for both PMN and MN (35, 36, 37, 42). Cathepsin D should therefore increase the number of cells (and their proteases) at the site of chronic inflammation. Because it can also digest cartilage in vitro (14, 15, 44, 46), it may play a role in the development of rheumatoid arthritis. Macrophage cathepsin D may also be a mediator of the fibrotic response, because macrophage infiltration frequently precedes fibroblast infiltration.

Studies in our laboratory (T. Tsuda, A. M. Dannenberg, Jr., M. Ando, and O. Rojas-Espinosa, manuscript in preparation) and Yamamura's (25, cf. 48) indicate that macrophage cathepsin D is involved in the liquefaction of the caseous focus in tuberculosis. Liquefacton results in tremendous extracellular multiplication of tubercle bacilli, with frequent development of antibiotic-resistant mutants and spread of the disease throughout the bronchial tree and to other people (23, 24, 34). It is theoretically possible that the use of effective protease (and other) inhibitors might arrest the process of liquefaction and aid in the control of tuberculosis.

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