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MACROPHAGE ESTERASE: IDENTIFICATION, PURIFICATION AND PROPERTIES OF A CHYMOTRYPSIN-LIKE ESTERASE FROM LUNG THAT HYDROLYSES AND TRANSFERS NONPOLAR AMINO ACID ESTERS

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Summary

A chymotrypsin-like esterase was purified from beef lung. This lysosomal enzyme, not previously characterized, seemed to be composed of two or more forms with molecular weights of about 52 000. It hydrolysed *N*-benzoyl-DL-phenylalanine β-naphthol ester at acid and neutral pH; it polymerized L-phenylalanine methyl ester(Phe-OMe) at neutral pH; and it transferred the Phe-residue from Phe-OMe to hydroxylamine at neutral pH. Phenylmethanesulfonyl fluoride, an inhibitor of hydrolytic enzymes with serine in their catalytic site, inhibited this enzyme, but pepstatin, the cathepsin D (EC 3.4.4.23) inhibitor, did not. Sulfhydryl reagents were not required for activity. Macrophages, especially pulmonary alveolar macrophages, were a rich source of this esterase, so it is likely that the enzyme purified from lung came from its macrophages.

The esterase hydrolysed and transferred monoamino acid esters, especially those of the aromatic type. Cathepsin C, the dipeptidyl peptide hydrolase (EC 3.4.14.1), acted only on dipeptide esters and amides. Pancreatic chymotrypsin acted on both monoamino acid and dipeptide esters. The chymotrypsin-like esterase did not hydrolyse hemoglobin, casein, or plasma albumin. Thus its proteolytic activity, if present, must be limited to specific substrates, as yet unknown.

Abbreviations: Bz-Phe-ONap, *N*-benzoyl-DL-phenylalanine-β-naphthol ester; Phe-OMe, phenylalanine methyl ester. In this report, unless otherwise specified, chymotrypsin-like esterase refers to the esterase that both hydrolyses Bz-Phe-ONap and transfers the Phe-residue from Phe-OMe to hydroxylamine.

Introduction

The acid-acting proteinase of lung, identified many years ago by Nye [1] and Weiss [2], was partially purified and characterized by Dannenberg and Smith [3,4] and highly purified by Rojas-Espinosa et al. [5]. It resembles pepsin and cathepsin D [4—7] and seems to be the main proteinase of macrophages [5,7—11]. Both lung tissue and macrophages also contain a chymotrypsin-like enzyme [3,10] that hydrolyses *N*-acetyl-L-tyrosine ethyl ester [3] and *N*-benzoyl-DL-phenylalanine β-naphthol ester (Bz-Phe-ONap) [10].

Since both lung and macrophage homogenates polymerized aromatic amino acid esters [4], it was important to determine whether the hydrolytic and polymerizing (transferring) activities were due to the same or different enzymes. We therefore undertook a separation of these enzymes from beef lung, which is a tissue rich in macrophages, their probable source.

As mentioned above, the pepsin-like cathepsin D was first purified [3,5]. Then the chymotrypsin-like esterase was purified (this report) and found to consist of two distinct enzymes that hydrolysed Bz-Phe-ONap, only one of which had transferring and polymerizing activities. Further characterization of the latter revealed that it had monoamino acid specificity with a preference for aromatic amino acid esters. It therefore differs from cathepsin C, which is the dipeptidyl aminopeptidase I of MacDonald et al. [12] and the dipeptidyl transferase of Metrione et al. [13]. Thus the chymotrypsin-like esterase seems to be a monoamino acid esterase and transferase that has not previously been identified.

Materials and Methods

Source of enzyme

Fresh beef lungs were purchased from a local slaughter house, trimmed, cut into pieces, washed in distilled water, and frozen and stored at −70°C until used. After the lungs were thawed overnight at 4°C, an acetone-dried powder was prepared for the purification of both cathepsin D [5] and the chymotrypsin-like esterase (described below).

Peritoneal macrophages were obtained by injecting 35 ml of mineral oil intraperitoneally into rabbits and 5 days later collecting the resulting macrophages in citrated-saline solution (0.4% sodium citrate and 0.9% sodium chloride) [10,14]. The cell suspension was centrifuged, resuspended in citrated-saline, counted in a hemacytometer, and quickly frozen. After at least a week in the frozen state, it was thawed, homogenized with a serological pipette, diluted to the appropriate concentration with 0.9% saline, and used as a source of enzyme.

Pulmonary alveolar macrophages were obtained by perfusing fresh rabbit lungs four times intratracheally with 30 to 40 ml of citrated-saline solution [14,15]. Alveolar macrophages were collected, counted, frozen, stored and used as a source of enzyme in the manner just described.

Polymorphonuclear leukocytes were obtained from the peritoneal cavities of rabbits 18 h following the intraperitoneal injection of 0.1% glycogen (Nutritional Biochemical Corp.) in 0.9% NaCl solution [10,14,16]. The exudate was

collected 18 h later as just described for peritoneal macrophages.

Erythrocytes were collected from citrated rabbit blood, after repeated centrifugation with removal of the buffy coat of leukocytes [10]. They were suspended in 0.9% saline, counted, frozen, stored and used as a source of enzyme.

Hydrolysis of N-benzoyl-DL-phenylalanine β-naphthol ester

The method employed to assay the hydrolysis of Bz-Phe-ONap was that previously described [10] with slight modifications. The enzyme sample in 0.5 ml of 0.1 M sodium acetate buffer (pH 5.4) was mixed with 1.0 ml of freshly prepared substrate solution and incubated at 37°C for 30 min. (The substrate solution was made by mixing 8.0 mg of Bz-Phe-ONap in 4.0 ml of acetone with 76.0 ml of 0.1 M acetate buffer at pH 5.4 (see section on pH optimum below). After incubation a duplicate tube was removed to check the pH and the remaining tubes were transferred to an ice bath. Then 0.5 ml of cold Veronal buffer (0.05 M, pH 8.5) was added, followed by 0.2 ml of cold, freshly prepared NDBB reagent (40 mg of Naphthanil Diazo Blue B (Dajac Laboratories, Philadelphia, Pa. 19124) in 10 ml of cold water). After 3 min the reaction was stopped by adding 0.2 ml of 40% trichloroacetic acid. The color was then extracted by shaking with 2.0 ml of ethyl acetate. After centrifugation at 2000 rev./min for 5 min to clear the supernate, the absorbance of this red-purple supernate was read immediately at 540 nm in a Bausch and Lomb spectrophotometer against a blank prepared as described above with the enzyme solution replaced by distilled water.

One unit of activity with Bz-Phe-ONap as the substrate was defined as the quantity of chymotrypsin-like esterase that should cause an absorbance increase of 1.000. This is equivalent to 40 μg (0.28 μmol) of β-naphthol. All experiments were performed in the 0.100 to 0.300 range. Protein was determined by the method of Lowry et al. [17].

Reactions

The transfer reaction. The chymotrypsin-like esterase will catalyze (a) the formation of polyphenylalanine from phenylalanine methyl ester, and (b) the formation of phenylalanine hydroxamic acid from phenylalanine methyl ester in the presence of hydroxylamine. The hydroxamic acid will then react with $FeCl_3$ to produce the red-purple colored ferric hydroxamate.

The polymerization reaction. This reaction was used in a qualitative manner, noting the presence or absence of the polymer. The enzyme preparation (aqueous solution or in acrylamide gel) was incubated with 0.05 M Phe-OMe and 0.025 M β-mercaptoethylamine* for 30 min at 38°C. The precipitate in the gel was clearly visible in 10 min as a white precipitate (Fig. 4).

The hydroxylamine reaction. This reaction was modified from the method of Metrione et al. [13]. The following reagents were mixed in a test tube and incubated at 37°C: 0.1 ml of 2 M NH_2OH (freshly prepared from 4 M NH_2OH

* β-mercaptoethylamine was routinely added in the polymerization and hydroxylamine reactions, because cathepsin C (dipeptidyl peptide hydrolase) required SH-compounds for maximal activity. It turned out, however, that chymotrypsin-like esterase had no such requirement.

hydrochloride and adjusted to pH 6.8 with 4 M NaOH), 0.1 ml of 0.125 M β-mercaptoethylamine* (freshly prepared from a 0.5 M solution of the hydrochloride adjusted to pH 6.8 with 0.1 M NaOH), 0.1 ml of 0.25 M phenylalanine methyl ester hydrochloride (adjusted to pH 6.8 with 0.1 M NaOH) and 0.1 ml of water. After 3 min 0.1 ml of the enzyme solution was added and the reaction was stopped 30 min later by the addition of 0.5 ml of 20% trichloroacetic acid, followed by 0.5 ml of 5% $FeCl_3 \cdot 6\ H_2O$ in 0.1 M HCl. Finally, 0.5 ml of water was added and the tubes were centrifuged for 5 min at 2000 rev./min. The absorbance of the red-purple supernate was read at 510 nm in a Bausch and Lomb spectrophometer in cuvettes of 1.0 cm light path. (With crude preparations of lung and other organs, the color was more red-orange than red-purple.) Assay mixtures without enzyme and without substrate were included and the sum of their absorbances was subtracted from the absorbance of the full mixture.

One unit of activity with Phe-OMe as the substrate was defined as the quantity of chymotrypsin-like esterase that should cause an absorbance increase of 1.000. All experiments were performed in the 0.100 to 0.300 range. When 1.00 μmol of phenylalanine hydroxamate reacted with $FeCl_3$, an A of 0.300 was produced. A linear relationship held between the absorbance and the μmol of phenylalanine hydroxamate (Sigma Chemical Co.) up to an A of 0.600, which represented 2.0 μmol of the hydroxamate. The hydrolytic reaction with the naphthol substrate is 4 to 5 times more sensitive than the transamidation reaction with hydroxylamine.

Disc electrophoresis

A Buchler vertical gel electrophoresis apparatus (Polyanalyst, Catalog No. 3-1750, Buchler Instruments Division, 1327 Sixteenth St., Fort Lee, New Jersey 07024) was used with a modification of the method described by Campbell [18] and Gordon [19]. The samples, 0.1 to 0.2 ml, contained 40 to 200 μg of protein in 40% sucrose and tris(hydroxymethyl)aminomethane (0.01 M)/glycine (0.08 M) buffer (pH 8.2). They were electrophoresed at about 10°C on 7% acrylamide with a current of 3 mA per tube (about 300 V) for 90 min. The gels were stained for 10 min with 0.25% Coomassie Brilliant Blue R (Catalog No. B 0630, Sigma Chemical Co.) in water/methanol/acetic acid (5 : 5 : 1). The excess color was eluted with the same solvent without dye.

Phenylalanine hydroxamate formation could also be used to detect chymotrypsin-like esterase after disc electrophoresis. The gel containing the enzyme was incubated for 30 min with Phe-OMe, NH_2OH and β-mercaptoethylamine under the conditions described. The hydroxamate was detected by dipping the gels directly into the acidic $FeCl_3$ solution. The red-purple color appeared in a few seconds and faded gradually.

Isoelectric focusing in acrylamide gels

The Buchler vertical gel electrophoresis apparatus [20] was used with 1.45% H_3PO_4 as the anode solution and 2.0% monoethanolamine as the cathode solution.

Experimental Procedures and Results

Purification of chymotrypsin-like esterase (Table I)

Acetone-dried powder. Fresh beef lung (1 Kg) from the local slaughter house was blended at 4°C in a Waring blender with 2 litres of cold 50% acetone in water and centrifuged. The supernate was filtered through gauze and two volumes of 100% acetone (−20°C) were added. The sediment was centrifuged, washed several times with cold 100% acetone, and collected in a Buchner funnel. It was washed with acetone, acetone/ether and then ether, dried at room temperature and stored at 4°C.

Sephadex G-200 column. 3 g of the acetone-dried powder were stirred overnight at 4°C in 30 ml of 0.065 M sodium phosphate buffer (pH 6.8), and centrifuged at 7000 rev./min for 60 min (in a Sorvall rotor SM-24). The sediment was discarded and the supernate brought to 30 ml with the phosphate buffer. A 2 ml sample was saved for assay and the remaining 28 ml were fractionated on a Sephadex G-200 column (54 × 4.5 cm) equilibrated and eluted with the same phosphate buffer. 300 4-ml fractions were collected, and every other one was assayed for activity with the following substrates: Gly-Phe-NH_2 (with hydroxylamine) for dipeptidyl peptide hydrolase (cathepsin C) [12,13]; and Phe-OMe (with hydroxylamine) and Bz-Phe-ONap for the chymotrypsin-like esterase.

TABLE I

PURIFICATION OF CHYMOTRYPSIN-LIKE ESTERASE FROM BEEF LUNG

Purification step	Protein (mg)	Total [a] units	Specific [b] activity	Purification (fold)		Recovery %
Phe-OMe						
Aqueous extract			0.22 [c]		1.0 [d]	
I Acetone powder	1230.0	740	0.60	1.0 [e]	2.7	100
II Sephadex G-200	650.0	670	1.03	1.7	4.7	91
III DEAE-Cellulose	32.0	530	16.6	28.0	75.0	72
IV CM-Sephadex (sample at pH 6.5)	5.7	300	53.0	88.0	240.0	41
V CM-Sephadex (sample at pH 6.0)	0.88	220	250.0 [f]	420.0	1140.0	30
Bz-Phe-ONap						
Aqueous extract			1.0 [c]		1.0 [d]	
I Acetone powder	1230.0	3700	3.0	1.0 [e]	3.0	100
II Sephadex G-200	650.0	3600	5.5	1.8	5.5	97
III DEAE-Cellulose	32.0	2500	78.0	26.0	78.0	68
IV CM-Sephadex (sample at pH 6.5)	5.7	890	158.0	53.0	158.0	24
V CM-Sephadex (sample at pH 6.0)	0.88	890	1010.0	340.0	1010.0	24

[a] Assayed by the transfer reaction of hydroxylamine to L-phenylalanine methyl ester (see text).
[b] Units per mg of protein determined by the method of Lowry et al. [17].
[c] Average of three aqueous extracts that were different from those used to prepare the acetone powder.
[d] In relation to the aqueous extract.
[e] In relation to the acetone powder.
[f] This enzyme preparation lost specific activity on storage.

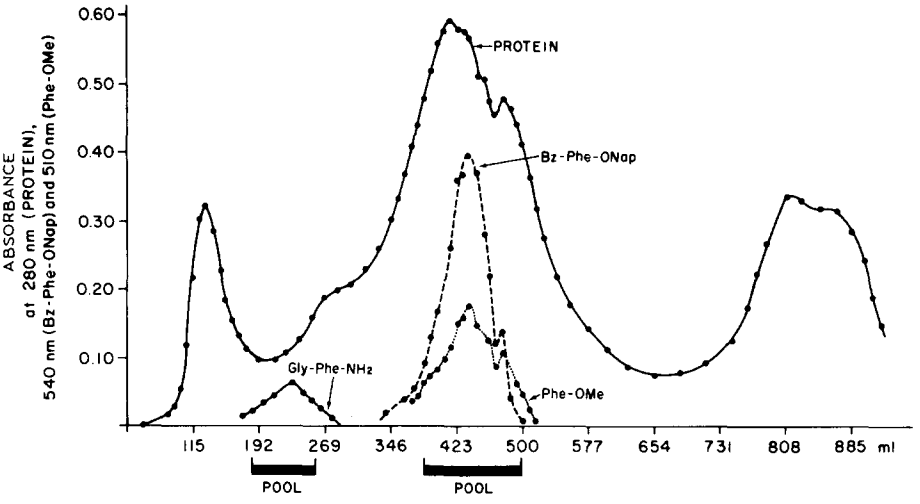


Fig. 1. Purification of chymotrypsin-like esterase on a Sephadex G-200 column in 0.065 M sodium phosphate buffer (pH 6.8). To obtain the absorbance (per ml) shown, the eluate was diluted 1 : 10 for protein, 1 : 200 for activity with Bz-Phe-ONap as substrate, 1 : 20 for activity with Phe-OMe, and 1 : 20 for activity with Gly-Phe-NH_2. In other words, the peaks at about 423 ml had absorbances of about 5.80, 78.0 and 3.40, respectively.

Three major protein peaks, measured by their absorbance at 280 nm, were obtained (Fig. 1). The first peak (in the void volume of 257 ml) was inactive except for traces of activity against Gly-Phe-NH_2. The Gly-Phe-NH_2 activity was eluted soon after the first peak, which is consistent with the molecular weight of about 200 000 found by McDonald [12] and Metrione et al. [13]. The second protein peak contained all of the activity against both Phe-OMe and Bz-Phe-ONap. The third protein peak was inactive.

DEAE-cellulose column. The fractions containing activity with Phe-OMe and Bz-Phe-ONap (about 120 ml) were pooled, dialyzed against 3 changes of 0.02 M Tris · HCl buffer (4000 ml each at pH 8.5), and the resulting 134 ml placed on a DEAE-cellulose column (2.5 × 45 cm) equilibrated with the same Tris · HCl buffer. The enzyme was then eluted with 0.02 M phosphate buffer (pH 7.5). 5-ml fractions were collected. Several peaks containing protein (i.e., absorbing at 280 nm) were obtained (Fig. 2), but the enzyme with activity on both Bz-Phe-ONap and Phe-OMe substrates was localized within the peak eluting between 364 and 468 ml of buffer.

An additional peak with activity on Bz-Phe-ONap, but not on Phe-OMe was retained in the column. It was eluted with a peak at 1217 ml of 0.02 M phosphate buffer (pH 6.8). Thus crude preparations of lung have at least two chymotrypsin-like esterases with activity on Bz-Phe-ONap: one which acts on both Bz-Phe-ONap and Phe-OMe, and one (not further purified or characterized), which only acts on Bz-Phe-ONap.

CM-Sephadex columns. The peak containing activity on both Bz-Phe-ONap and Phe-OMe was concentrated to one-third in a rotary evaporator (Büchi Rotavapor R/A, Brinkmann Instruments, Westbury, New York 11590), dialyzed against two changes of 0.03 M sodium acetate buffer (pH 6.0) (about

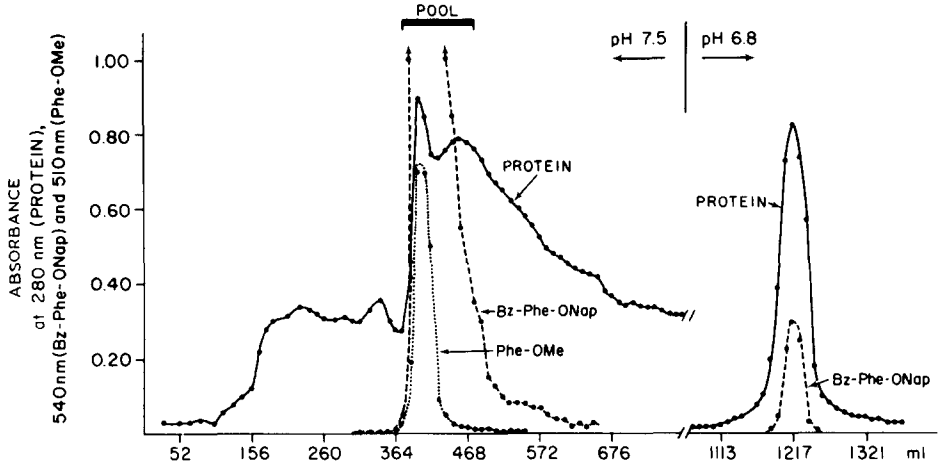


Fig. 2. Purification of chymotrypsin-like esterase on a DEAE-cellulose column. This enzyme (active on both Bz-Phe-ONap and Phe-OMe) was placed on the column, equilibrated with 0.02 M Tris · HCl buffer (pH 8.5). It was eluted with 360 to 570 ml of 0.02 M phosphate buffer (pH 7.5). After an additional 2 to 2.5 liters of this phosphate buffer, the esterase that was active only on Bz-Phe-ONap was eluted with 1160 to 1270 ml of 0.02 M phosphate buffer (pH 6.8). To obtain units per ml of enzyme activity the scale on the left ordinate should be multiplied by 10 for Bz-Phe-ONap and by 20 for Phe-OMe. When assays with Bz-Phe-ONap were repeated at an additional 1 : 5 dilution, a curve more similar to the Phe-OMe curve was obtained. For this Bz-Phe-ONap curve a factor of 50 would be required to obtain units per ml.

6 litres each) for only 20 h (which brought its pH to 6.5). (In later experiments we dialyzed the peak which contained activity against 3 changes of 0.03 M sodium acetate buffer (pH 6.4) for 48 to 72 h. This procedure also brought the pH to 6.5.) The esterase was then placed on a CM-Sephadex (C-50) column (2.0 X 17.5 cm), and fractions of 4 ml were collected. The pH 6.0 acetate buffer was used for equilibration and elution. The esterase came off of the column soon after most of the inert protein (Fig. 3A).

The active fractions were pooled, concentrated in a rotary evaporator, dialyzed for 18 h against 3 changes of 0.03 M acetate buffer (pH 6.0) and placed again on an identical CM-Sephadex column equilibrated as before with 0.03 M acetate buffer at pH 6.0 (Fig. 3B). The esterase was retained until a linear gradient of 0.03 to 0.3 M acetate buffer (pH 6.0) was started.

The only difference in the columns represented by Figs 3A and 3B was the pH of the enzyme solution placed on each. For Fig. 3A the enzyme solution was pH 6.5. For Fig. 3B it was pH 6.0. This difference is clearly demonstrated in Fig. 3C. Here the concentrated esterase from the DEAE-cellulose column was dialyzed for 3 days against 3 changes of 0.03 M acetate buffer (pH 6.0) to bring its pH to 6.0. When placed on the CM-Sephadex column, it was retained until it was eluted by the gradient as in Fig. 3B. Of interest is the fact that two peaks of enzyme activity appeared when the enzyme was eluted by the gradient (Figs. 3B and 3C) and only one peak (with a slight shoulder) when it was eluted from the column without a gradient (Fig. 3A).

Acrylamide gel electrophoresis. An extract of acetone powder (200 μg) (Step I) and the most purified esterase (40 μg) from the CM-Sephadex column (Step V) were placed on 7% acrylamide gel and electrophoresed (see Materials

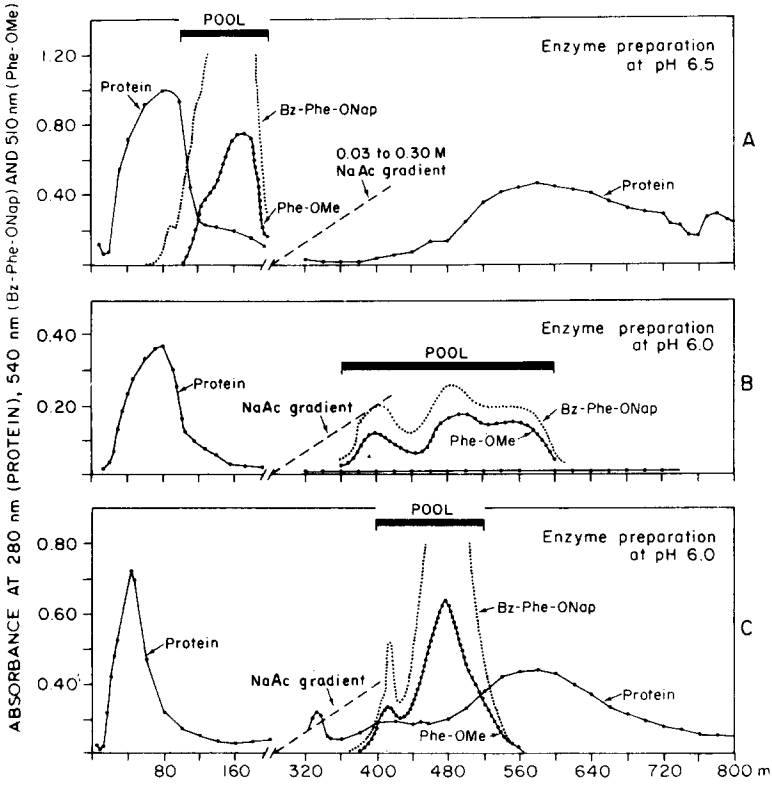


Fig. 3. Purification of chymotrypsin-like esterase on CM-Sephadex (C-50) columns at pH 6.0. In (A) the enzyme added to the column was at pH 6.5. In (B) the pooled peak from 3A at pH 6.0 was added to a similar column. In (C) the enzyme preparation is identical to that used in (A) except that it was brought to pH 6.0 before it was placed on the column. The break in the ordinate axis represents where the column was washed with 0.03 M sodium acetate buffer (pH 6.0) before a 1200 ml linear gradient (dashed lines) of 0.03 to 0.30 M sodium acetate buffer (pH 6.0) was begun. After the linear gradient, column (A) followed by (B) gave a rather complete separation of the chymotrypsin-like esterase from the inactive protein. The eluates were diluted 1 : 20 to assay the esterase activity with Bz-Phe-ONap and Phe-OMe as substrates. Protein was read at 280 nm on undiluted samples.

and Methods). The acetone powder showed many bands; whereas the purified preparation showed only two bands (Fig. 4). Both bands polymerized Phe-OMe suggesting that they were different forms of the same enzyme (Fig. 4). These results, along with those of isoelectric focusing (Fig. 5), suggest that the esterase had been highly purified.

Isoelectric focusing on acrylamide gel. The most purified esterase (4 μg) from the CM-Sephadex column (Step V) was placed on 4.6% acrylamide gel with 3% Ampholine (pH range 5 to 8) [20]. There were 0.20 ml of Ampholines pH 5 to 8 (LKB-Produkter AB, Sweden) in the ampholyte layer, and 0.10 ml of the enzyme (containing 4.0 μg protein) was applied below this layer. Electrofocusing was carried out in the cold (2°C) with a current of 1 mA per tube and maximal voltage of 400 V for 5 h. It took about 1.5 h to reach maximal voltage.

Fig. 5 diagrammatically depicts the results. Four protein bands lightly staining with Coomassie Blue and four bands showing polymerizing activity

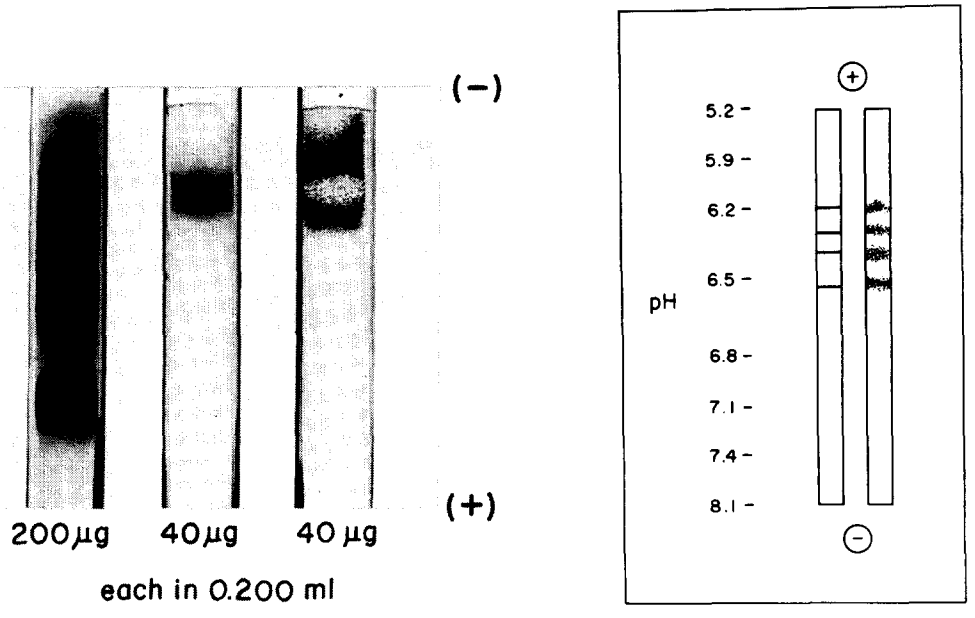


Fig. 4. Acrylamide gel electrophoresis of crude (step I) and purified (step V) chymotrypsin-like esterase. The purified enzyme (center and right) was electrophoresed in duplicate. One of these duplicates was stained with Coomassie Blue; the other was incubated with phenylalanine methyl ester for 30 min at pH 6.8. In 10 min a stable white precipitate (polyphenylalanine) formed in the gel exactly where the two protein bands were stained. By 30 min (our standard incubation time) this precipitate was the more diffuse type illustrated. It appears dark in this photograph because it was back-lighted.

Fig. 5. Isoelectric focusing of the purified chymotrypsin-like esterase (from step V) in acrylamide gel. Four active forms with isoelectric points of 6.54, 6.42, 6.33 and 6.20 were found. These stained with Coomassie Blue (left) and polymerized Phe-OMe (right).

with Phe-OMe (see Fig. 4) were consistently present. It seemed therefore that our highly purified esterase preparation was composed of 4 functional enzymes (with apparent isoelectric points of 6.54, 6.42, 6.33 and 6.20). No other protein contaminants were noted, but low amounts could be present and remain undetectable.

Properties of chymotrypsin-like esterase

Stability of the enzyme. Beyond the Sephadex G-200 step of purification, the esterase was irreversibly denatured when frozen slowly (in the freezer) in dilute buffers or normal saline solution. The addition of 15% sucrose (about 0.4 M) prevented this denaturation without interference with the enzyme assays or the determination of protein. Even so, non-rapid freezing and thawing or prolonged storage in the frozen state resulted in some loss of activity. The enzyme in the last step of purification (1140-fold) was extremely labile to dialysis against 0.9% NaCl. When the dialysis was performed for 48 h in an ice bath, 50% of its activity was lost.

Activity of the esterase as a function of enzyme concentration. The hydrolytic reaction with Bz-Phe-ONap as the substrate and the transfer reaction with Phe-OMe as the substrate showed no plateau as the enzyme concentration was increased. In fact the reverse phenomenon occurred (Fig. 6).

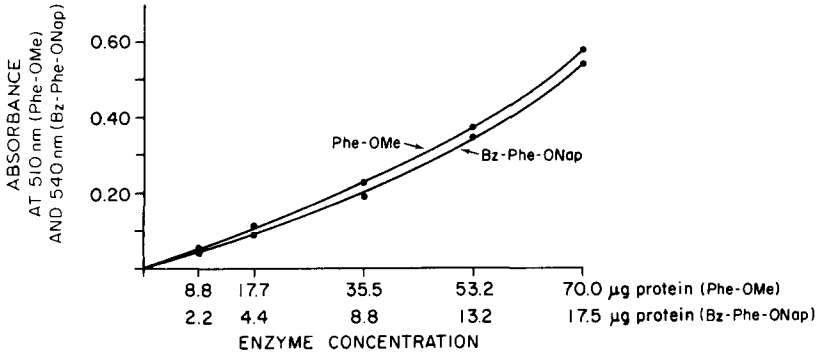


Fig. 6. Activity of chymotrypsin-like esterase as a function of enzyme concentration for both the transfer reaction (with L-phenylalanine methyl ester and hydroxylamine) and the hydrolytic reaction (with *N*-benzoyl-DL-phenylalanine β-naphthol ester). An esterase with a specific activity of 8.5 units per mg of protein with Phe-OMe as substrate was used. In order to produce equivalent optical densities, the Phe-OMe reaction required 4 times the amount of enzyme that the Bz-Phe-ONap reaction required.

pH optimum. For the hydrolytic reaction with Bz-Phe-ONap as the substrate, the chymotrypsin-like esterase of rabbit mononuclear exudate cells had a 5.4 pH optimum [10]. Therefore most of the hydrolyses of Bz-Phe-ONap by the beef lung esterase were performed at pH 5.4. The purified lung enzyme was found, however, to have a pH optimum around 6.8 with this substrate, but spontaneous hydrolysis of Bz-Phe-ONap occurs at 6.8, so the exact pH optimum was difficult to ascertain.

For the transfer reaction of the phenylalanine residue to NH_2OH, the pH optimum with the partially purified beef lung esterase (8.8 μg protein, 0.3 units of enzyme, specific activity 33.1) was between 6.82 and 6.85 (Fig. 7). Because of the 0.4 molarity of NH_2OH, 0.4 M acetate and Tris buffers were employed. (At this concentration, phosphate buffers interfered with the hydroxylamine reaction and could not be used.) At each pH, the reactions were run in triplicate, one tube being used solely for the pH determination.

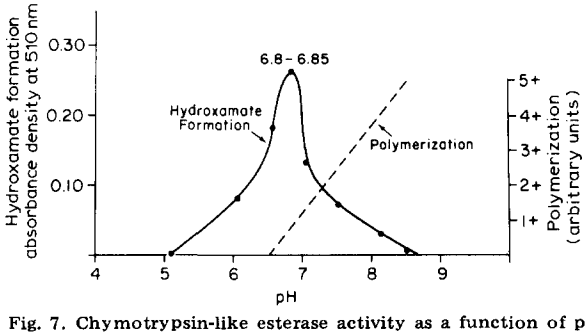


Fig. 7. Chymotrypsin-like esterase activity as a function of pH with L-phenylalanine methyl ester as the substrate. The amount of phenylalanine hydroxamate produced by this enzyme in the presence of hydroxylamine can be read on the left ordinate. ($FeCl_3$ reacts with the hydroxamate to produce a red-brown color.) The amount of Phe-OMe polymerized can be read on the right ordinate. Acetate buffer was used below pH 7.0 and Tris buffer above pH 7.0. The polymerization reaction occurred only above pH 6.5.

Molecular weight. The method of Andrews was used [21]. Sephadex G-200 was prepared in a 2 × 50 cm column and equilibrated with about 300 ml of 0.05 M Tris · HCl buffer (pH 7.5) containing 0.1 M KCl and 0.01% merthiolate. The column was calibrated with Blue Dextran, human γ-globulin (M_r = 160 000), ovalbumin (M_r = 45 000) and sperm whale myoglobin (M_r = 17 800). These proteins showed a good correlation between their molecular weight and their elution volume.

Two preparations of the esterase (specific activities of 36.4 and 24.1 (with Phe-OMe) containing 0.090 and 0.380 mg protein respectively) were placed at different times on the calibrated column. Each preparation left the column in the fraction eluting just before ovalbumin. If the esterase is a globular protein, its major component would therefore have a molecular weight of about 52 000.

Effect of possible inhibitors and activators. The esterase (spec. act. 39 units/mg with Phe-OMe as the substrate) was incubated with $CaCl_2 \cdot 2H_2O$, parachloromercuribenzoate, $Pb(NO_3)_2$, $FeNH_4(SO_4)_2 \cdot 12H_2O$, $CuCl_2 \cdot 2H_2O$, $HgCl_2$, $Mg(CH_3COO)_2 \cdot 4H_2O$, ethylenediaminetetraacetate or iodoacetate at 1.0 or 2.0 mM final concentration. None appreciably activated or inhibited this enzyme. Pepstatin: a pepsin [22,23] and cathepsin D [7,23–25] inhibitor at 10 μg/ml, Leupeptin: a plasmin, trypsin, papain and cathepsin B inhibitor [26–28] at 10 μg/ml, Antipain: a papain and trypsin inhibitor [28] at 10 μg/ml, and Chymostatin: a chymotrypsin inhibitor [29,30] at 20, 100 and 200 μg/ml also had no effect. Chymostatin (10 μg/ml) reduced the transferase activity of 0.18 units of pancreatic β-chymotrypsin (Sigma Chemical Co. (No. C4629)) to 30% of its original activity when assayed under these conditions. However, phenylmethanesulfonyl fluoride (0.4 mM) (an inhibitor of proteases and esterases with serine in their catalytic site [31]) was a highly effective inhibitor of the esterase. It inhibited the activity of the esterase 95%.

Each chemical was tested 2 to 4 times in tubes containing 0.34 units of enzyme, 0.025 M β-mercaptoethylamine, 0.40 M hydroxylamine and 0.05 M Phe-OMe in a final volume of 0.50 ml. Each chemical was preincubated with the enzyme (at 5 times the concentrations listed) for 10 to 15 min at 37°C and pH 6 to 7. Appropriate controls were included. Mercaptoethylamine was omitted when testing compounds affecting SH groups.

Hydrolysis of proteins. Highly purified esterase (1 to 10 μg from step V in Table I, spec. act. 134 units/mg protein) was incubated at 37°C with acid-denatured hemoglobin [5,32] urea-denatured hemoglobin [3,32], casein [33], or bovine plasma albumin [32] in 0.1 M buffer at pH 2.0 to 8.5 for 1 to 10 h. After incubation, the reaction mixtures were treated with 5 or 10% trichloroacetic acid, filtered (or centrifuged) and the filtrate (or supernate) analyzed for absorbance at 280 nm [3,5,32] or by the technique of Lowry et al. [17] (usually both methods were used). In no case was proteolytic activity observed.

Since the purified esterase did not hydrolyze the common protein substrates tested, a sample of this enzyme was tested by various investigators on the following reactions of proteins having a specific biological role: conversion of plasminogen to plasmin by Dr Saimon Gordon of Rockefeller University; activity of the guinea pig complement system and its C3 and C5 components by Dr Hyun Shin of Johns Hopkins University; activation of prekallikrein, plasminogen and Hageman Factor by Dr Henry Z. Movat of the University of

Toronto; hydrolysis of collagen and elastin by Dr Ines Mandl of Columbia University; and hydrolysis of the proteoglycan subunit of cartilage and the peptide substrates for chymotrypsin: Tyr-Gly-NH_2 and Cbz-Tyr-Gly-NH_2 by Dr J. Fred Woessner, Jr, of the University of Miami. In all cases, negative results were obtained. Thus if a protein substrate for the chymotrypsin-like esterase exists, it remains to be discovered.

Comparison of chymotrypsin-like esterase with pancreatic chymotrypsin and dipeptidyl peptide hydrolase (cathepsin C). The esterase polymerized aromatic monoamino acid esters and transferred their aromatic amino acid residue to hydroxylamine (Tables II and III). It had no action on monoamino acid amides or dipeptide esters. Dipeptidyl peptide hydrolase polymerized and

TABLE II

TRANSFERASE ACTIVITY OF CHYMOTRYPSIN-LIKE ESTERASE AND PANCREATIC CHYMOTRYPSIN WITH HYDROXYLAMINE AND VARIOUS AMINO ACID AND PEPTIDE ESTERS AND AMIDES

The incubation mixture contained 0.05 M substrate, 0.4 M NH_2OH, 0.025 M β-mercaptoethylamine and 0.33 units with Phe-OMe as the substrate for chymotrypsin-like esterase at purification steps II or V (1.5 μg protein for step V), or 0.26 units with Phe-OMe as the substrate for pancreatic chymotrypsin (1.5 μg protein).

Substrate [a]	Chymotrypsin-like esterase		Pancreatic chymotrypsin [b] (%)
	Enzyme at step II (%)	Enzyme at step I (%)	
Phe-OMe	100	100	100
Phe-OEt	64	63	72
Try-OMe	47	89	82
Tyr-OEt	36	41	185
Lys-OEt	8	0	0
Arg-OMe	0	0	0
Leu-OMe	0	0	0
Cys-OMe	—	20	0
DL-Asp$(OMe)_2$	—	16	0
Glu$(OEt)_2$	—	0	0
Glu-α-OMe	—	0	0
Phe-NH_2	0	0	0
Try-NH_2	—	0	0
Tyr-NH_2	—	0	0
Leu-NH_2	0	0	—
Lys-NH_2	0	0	0
Arg-NH_2	—	0	0
Gly-Phe-OMe	—	16	220
Gly-Lys-OMe	—	0	0
Gly-Phe-NH_2	0	0	20
Gly-Arg-NH_2	0	0	0
Leu-2-NNap	0	0	—

[a] The abbreviations are based on the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature listed in Vol. 2 of "Synthetic Peptides" [34] see also [35].

[b] Chymotrypsin, crystallized, salt free (Lot No. CD514-17) from Worthington Biochemical Corp., Freehold, N.J.

TABLE III

COMPARISON OF THE TRANSFERASE ACTIVITIES OF DIPEPTIDYL PEPTIDE HYDROLASE, PANCREATIC CHYMOTRYPSIN AND CHYMOTRYPSIN-LIKE ESTERASE WITH SELECTED PEPTIDE AND AMINO ACID ESTERS

The incubated mixture (0.50 ml) contained 0.05 M substrate, 0.40 M hydroxylamine, 0.025 M β-mercaptoethylamine, and 1.0 μg pancreatic chymotrypsin (see Table II) or 0.5 μg dipeptidyl peptide hydrolase or 1.5 μg of chymotrypsin-like esterase (0.33 units with Phe-OMe) at step V of purification. The dipeptidyl peptide hydrolase from rat liver was supplied by Dr. J. Ken McDonald, Ames Research Center, Moffett Field, Calif. Gly-Phe-OMe · HBr (Miles Laboratories, Inc., Elkhart, Ind.) and Gly-Lys-OMe · 2 HCl (Fox Chemical Co., Los Angeles, Calif.) were also supplied by Dr McDonald.

Enzyme	Gly-Phe-OMe		Gly-Lys-OMe		Phe-OMe	
	Spec. act. (units/mg)	Polymer formation	Spec. act. (units/mg)	Polymer formation	Spec. act. (units/mg)	Polymer formation
Dipeptidyl peptide hydrolase (Cathepsin C)	760	+	1960	—	0	—
Pancreatic chymotrypsin	220	+	0	—	100	—
Chymotrypsin-like esterase	35	—	0	—	220	+

transferred hydroxylamine to dipeptide esters including those containing a basic amino acid at the carboxyl end [12]. Pancreatic chymotrypsin was active with both amino acid and dipeptide esters, but not if they contained a basic amino acid at the carboxyl end (Tables II and III).

The esterase did not require an amino group in the transfer reaction with NH_2OH (Table IV). In fact, the reaction was more rapid without it. Pancreatic chymotrypsin did require an amino group, but the transfer of NH_2OH occurred readily if this group was acetylated (Table IV). With substrates blocked by the benzyloxycarbonyl group, neither the esterase nor pancreatic chymotrypsin was active.

The transfer activities of both enzymes with Phe-OMe and β-phenylpropionic acid as substrates at different pH's are presented in Table V. The pH optimum for each enzyme was around 6.8. The esterase functioned better than pancreatic chymotrypsin at a more alkaline pH (pH 7.7), and functioned worse at a more acid pH (pH 5.9). (Pancreatic chymotrypsin showed no activity with β-phenylpropionic acid.)

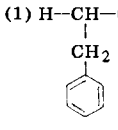
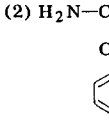
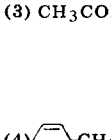
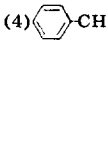
Trypsin (EC 3.4.4.4) (150 units per mg, catalog No. 3685, code TR, from Worthington Biochemical Corp., Freehold, New Jersey 07728) and partially purified beef lung cathepsin D (EC 3.4.4.23) (3.8 units per mg [5]) did not polymerize Phe-OMe or transfer its Phe-residue to hydroxylamine when 6.3 μg were used in our standard assay procedure.

The hydrolytic activities of the chymotrypsin-like esterase and pancreatic chymotrypsin at different pHs with Bz-Phe-ONap as the substrate are also presented in Table V. The esterase was quite active at pH 5.4 and pH 6.5, whereas the proteinase had lower activity with this substrate (one-tenth at pH 5.4 and one-sixth at pH 6.5). The pH optimum for each was around 6.5, but the proteinase retained only 46% of its activity at pH 5.4, whereas the esterase

TABLE IV

EFFECT OF THE AMINO GROUP ON THE TRANSFERASE ACTIVITY OF CHYMOTRYPSIN-LIKE ESTERASE AND PANCREATIC CHYMOTRYPSIN

The incubated mixture (0.50 ml) contained 0.05 M substrate, 0.40 M hydroxylamine, 0.025 M β-mercaptoethylamine, and 1.5 μg of chymotrypsin-like esterase (from step V, specific activity: 164 with Phe-OMe) or 1.5 μg of crystalline pancreatic chymotrypsin (see Table II).

Substrate[a]	Chymotrypsin-like esterase (1.5 μg)		Pancreatic chymotrypsin (1.5 μg)	
	Units[b]	Polymerization	Units[b]	Polymerization
(1) 	0.27 (233%)[c]	—	0.00 (0%)	—
(2) 	0.29 (100%)	+	0.20 (100%)	—
(3) 	0.07 (26%)	—	0.32 (160%)	—
(4) 	0.00 (0%)	—	0.00 (0%)	—

[a] (1) β-Phenylpropionic methyl ester, (2) L-phenylalanine methyl ester, (3) *N*-acetyl-L-phenylalanine methyl ester, (4) *N*-benzyloxycarbonyl-L-phenylalanine methyl ester.

[b] The percent of activity is in parentheses. For each enzyme the activity on Phe-OMe was taken as 100%. The units are absorbance units read at 510 nm.

[c] With β-phenylpropionic methyl ester, the assay was performed with 0.6 μg of esterase (instead of 1.5 μg) and the percent activity adjusted accordingly.

retained 81% of its activity at this acid pH. These differences in the hydrolytic activities of the two enzymes are probably related to the fact that the esterase is a tissue enzyme in lysosomes where an acid pH prevails; and the pancreatic proteinase is secreted into the intestinal tract where an alkaline pH pervades. (Since Bz-Phe-ONap undergoes spontaneous hydrolysis at alkaline pH, no pH higher than 6.5 was tested.)

Identification of chymotrypsin-like esterase in rabbit pulmonary alveolar macrophages, oil-induced peritoneal macrophages and glycogen-induced peritoneal polymorphonuclear leukocytes. These cells were collected as described in Materials and Methods, and total and differential cell counts were made. They were assayed with Bz-Phe-ONap and Phe-OMe (with NH_2OH) as substrates (Table VI). Per cell, alveolar macrophages were the richest source of the esterase. Peritoneal macrophages had about 40% the activity of alveolar macrophages, and polymorphonuclear leukocytes about 5% the activity of peritoneal macrophages. Erythrocytes had little or none.

TABLE V

COMPARISON OF THE ACTIVITIES OF CHYMOTRYPSIN-LIKE ESTERASE AND PANCREATIC CHYMOTRYPSIN ON L-PHENYLALANINE METHYL ESTER, β-PHENYLPROPIONIC METHYL ESTER (Ph-Prop-OMe) AND *N*-BENZOYL-DL-PHENYLALANINE-β-NAPHTHOL ESTER (B_Z-Phe-ONap) AT SEVERAL pH VALUES

Substrate	Reaction	Chymotrypsin-like esterase			Pancreatic chymotrypsin		
		pH 5.9	pH 6.8	pH 7.7	pH 5.9	pH 6.8	pH 7.7
Phe-OMe:	Specific activity	15	96	93	64	83	13
Transfer of	(%)[b]	(15)	(100)	(96)	(78)	(100)	(16)
NH_2OH[a]	Polymerization	—	+	++	—	—	—
Ph-Prop-OMe:	Specific activity	13	210	113	0	0	0
Transfer of	(%)[b]	(7)	(100)	(54)	(0)	(0)	(0)
NH_2OH[a]	Polymerization	—	—	—	—	—	—
		pH 5.4	pH 6.5		pH 5.4	pH 6.5	
Bz-Phe-ONap:	Specific activity	770	950		75	162	
Hydrolysis[c]	(%)[b]	(81)	(100)		(46)	(100)	

[a] For the hydroxylamine transfer reaction with Phe-OMe and Ph-Prop-OMe, the incubation mixture (0.5 ml) contained 2 μg of esterase (step V) or 2 μg of chymotrypsin, 0.025 M β-mercaptoethylamine, 0.40 M hydroxylamine, 0.018 M Phe-OMe or Ph-Prop-OMe, and 0.80 M acetate (pH 5.9) or 0.80 M Tris · HCl (pH 6.8 or 7.7) buffers. Ph-Prop-OMe, was assayed in 2% dimethyl formamide along with controls. These experiments were run in duplicate and the averages are listed.

[b] The figures in parentheses show the percent of activity of that observed at pH 6.8 or 6.5

[c] Bz-Phe-ONap hydrolysis was carried out as described in Materials and Methods: 0.25 μg of esterase and 1.0 μg of chymotrypsin w re used. At pH 6.5, 0.1 M Tris · HCl buffer was employed.

TABLE VI

ACTIVITY OF ALVEOLAR MACROPHAGES, PERITONEAL MACROPHAGES, POLYMORPHONUCLEAR LEUKOCYTES AND ERYTHROCYTES WITH *N*-BENZOYL-DL-PHENYLALANINE-β-NAPHTHOL ESTER (Bz-Phe-ONap) AND L-PHENYLALANINE METHYL ESTER AS SUBSTRATES FOR THE HYDROLYTIC AND TRANSFER REACTIONS RESPECTIVELY

Predominant cell type[a]	No. of rabbits	Differential cell count: AM, MN or PMN[a]	Bz-Phe-ONap as substrate Specific activity: Units per 10^6 cells[b]		Phe-OMe substrate Specific activity: Units with per 10^6 cells[b]	
			Uncorrected	Corrected[c]	Uncorrected	Corrected[c]
AM	4	99 ± 1[d]	5.7 ± 2.0[d]	5.7 ± 2.0[d]	0.115 ± 0.023[d]	0.117 ± 0.023[d]
MN	4	92 ± 10	2.2 ± 1.2	2.2 ± 1.1	0.039 ± 0.007	0.042 ± 0.005
PMN	4	87 ± 7	0.39 ± 0.23	0.11 ± 0.11	0.009 ± 0.002	0.003 ± 0.001
Erythrocytes	4		0.002 ± 0.001		0.002 ± 0.001	

[a] AM, pulmonary alveolar macrophages; Mn, oil-induced peritoneal macrophages; PMN, glycogen-induced peritoneal polymorphonuclear granulocytes. AM and MN include 0 to 5% small lymphocytes.

[b] Units are defined in Materials and Methods.

[c] Corrected: AM and MN for contaminating PMN; PMN for contaminating monocytes and lymphocytes.

[d] Average and its standard error:

$$\sqrt{\frac{\Sigma(X-\bar{X})^2}{n(n-1)}}$$

Discussion

The cathepsins. Much of the current knowledge on cathepsins has been reviewed in the book entitled "Tissue Proteases" edited by Barrett and Dingle [6]. Cathepsins, in general, are acid-acting exo- and endopeptidases of lysosomal origin. Cathepsin A (EC 3.4.2.-) is a carboxypeptidase. Cathepsin B (EC 3.4.4.-) resembles papain. Cathepsin C (EC 3.4.4.9) was originally thought to resemble chymotrypsin, but is now known to be an exopeptidase which splits dipeptides from the N-terminal end of protein molecules [12]. Cathepsins D (EC 3.4.4.23) and E (EC 3.4.4.-) are pepsin-like endopeptidases. Cathepsins A, D and E are unaffected by thiol reagents, but cathepsins B and C require them for maximal activity.

Chymotrypsin-like esterase and cathepsin C (dipeptidyl peptide hydrolase). The purified esterase, described in this report, did not fit into the known groups of cathepsins. While it had chymotrypsin-like properties, it was strikingly different from cathepsin C. The esterase hydrolyzed nonpolar amino acid esters as *N*-benzoyl-DL-phenylalanine β-naphthol ester, a substrate with a blocked amino group. It did not require thiol reagents nor metal activators. Similar to cathepsin C, the esterase had transfer activity, but mainly with monoamino acid esters (not amides) (Table II). In contrast, cathepsin C (dipeptidyl peptide hydrolase) [12,13,36,37] was active only on dipeptides (Table III), especially those containing arginine and lysine on the carboxyl side [12,36], and required a free NH_3^+ group, a thiol reagent and a halide (Cl^-) for maximal activity [12,36].

Chymotrypsin-like esterase and crystalline pancreatic chymotrypsin. The esterase was not specific for amino acid esters, as it transferred the β-phenylpropionic residue from its methyl ester to hydroxylamine (Tables IV and V). Pancreatic chymotrypsin did not do so. (β-phenylpropionic acid is phenylalanine without its NH_2-group.) The esterase had no apparent action on bovine hemoglobin, casein, or bovine serum albumin at pH 7.5, whereas the pancreatic proteinase readily hydrolyzed proteins at this pH [38]. Both enzymes transferred amino acid residues from their esters to hydroxylamine (Table II), but the esterase polymerized phenylalanine methyl ester, and the proteinase did not (Tables III and IV).

Chymotrypsin-like esterase and other esterases hydrolyzing N-benzoyl-phenylalanine β-naphthol ester. Bz-Phe-ONap could be hydrolyzed by esterases that have little if any transfer activity with phenylalanine methyl ester (unpublished work from our laboratory). Liver was the richest source of these other esterases (see [39]), whereas lung and macrophages (especially pulmonary macrophages) (Table VI) were a rich source of the chymotrypsin-like esterase that had both hydrolyzing and transferring activities. During the purification of this latter esterase on a DEAE-cellulose column (step III), a fraction that contained high esterase activity with Bz-Phe-ONap and little if any transfer activity with Phe-OMe was eluted after the peak containing the esterase with both activities (see Fig. 2). This finding further substantiated the existence of at least two enzymes that hydrolyzed Bz-Phe-ONap.

For the esterase that we purified, the ratio of the specific activities with

Bz-Phe-ONap and Phe-OMe as substrates was about 5 : 1 with beef lung (Table I) and about 25 : 1 with rabbit lung (unpublished work from our laboratory). Ratios of about 50 : 1 were found in preparations of rabbit pulmonary alveolar macrophages, peritoneal macrophages and peritoneal granulocytes (Table VI). The constancy of the ratio of 5 during the purification of beef lung esterase indicates that the preparations contained only small amounts of non-transferring esterase. Whether or not preparations of rabbit lung would similarly maintain a constant ratio during the purification of the transferring esterase remains to be determined.

Esterases resembling chymotrypsin-like esterase. Esterases somewhat similar to our purified beef lung esterase were found in rat mast cells [40—46] and rabbit [47,48] and human [49—51] granulocytes. The mast cell enzyme hydrolyzed esters of *N*-acetyl-L-tryptophan, *N*-acetyl-L-tyrosine and *N*-acetyl-L-phenylalanine. It was present in mast cell granules [43,46], was inhibited by organophosphonates [41], and hydrolyzed casein, bovine plasma albumin and insulin at pH 8 [43]. The granulocyte esterase hydrolyzed *N*-acetylphenylalanine β-naphthol ester [47—49], *N*-acetyltyrosine ethyl ester [50,51], casein [50], azocasein [50], fluorescein-labeled hemoglobin [49] and fluorescein-labeled fibrinogen [49]. Our purified beef lung esterase did not hydrolyze casein, hemoglobin or bovine plasma albumin.

The purified esterase of human granulocytes [49] and our beef lung esterase have molecular weights around 18 000 and 52 000, respectively and have different isoelectric points ([49,50] and Fig. 5). Multiple forms of each enzyme exist ([49,50] and Figs 4 and 5). Both esterases are inhibited by organophosphonates [47,48,51] or phenylmethanesulfonyl fluoride ([49] and this report), indicating that they have serine in their catalytic site. Neither rat mast cell esterase nor rabbit and human granulocyte esterase was tested for transferase activity [40—51].

Rabbit macrophages, especially pulmonary macrophages, contain 10 to 50 times more chymotrypsin-like esterase than rabbit granulocytes (Table VI). Because of the large content of this enzyme in macrophages, we feel that our purified beef esterase came from the macrophages present in lung tissue. Unfortunately, monocytes and macrophages were not evaluated in the studies on mast cells and granulocytes mentioned above.

Biological role of chymotrypsin-like esterase. The ability to polymerize amino acid amides or esters at neutral or alkaline pH has been called plastein formation [52,53] and seems to be a property of many proteases, e.g., pepsin [54], papain [55,56], pancreatic chymotrypsin [57—59] and cathepsin C [13,36,37]. Such polymer formation is probably a laboratory phenomenon and of little importance in living organisms, but the ability of the enzyme to transfer (and hydrolyze) single amino acids, like the ability of glycosyl transferases to transfer certain sugars, may affect cell membrane components that are important in cell function and recognition. Consistent with this possibility are the high concentrations of chymotrypsin-like esterase in the lysosomal fractions of lung and liver cells (unpublished experiments from our laboratory). Cell membrane receptors may be removed by pinocytosis (followed by fusion of the resulting vacuoles with lysosomes), and they may be added or replaced upon exocytosis of pinocytic or lysosomal vaculoles.

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