Purification and Properties of a Mast Cell Protease

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SUMMARY

A mast cell protease has been purified over 260-fold from rat thyroid homogenates. The purified enzyme hydrolyzes denatured thyroglobulin, denatured hemoglobin, and fibrinogen. It also rapidly hydrolyzes the ester substrates, benzoyltyrosine ethyl ester, benzoylphenylalanine ethyl ester, and acetyltyrosine ethyl ester. The pH optimum for thyroglobulin and benzoyltyrosine ethyl ester hydrolysis is 7.8. The enzyme is inhibited by diisopropyl fluorophosphate, diphenylcarbamyl chloride and L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone. The inhibition by diisopropylfluorophosphate, diphenylcarbamyl chloride and L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone is 23,000. Thus, the purified enzyme resembles chymotrypsin A in many of its properties.

In the thyroid, thyroxine and triiodothyronine are stored in peptide linkage in the protein thyroglobulin. For release of these into the circulation, peptide bonds must be hydrolyzed. It has recently been reported that rat thyroid extracts contain a protease that hydrolyzes thyroglobulin at pH 6 to 10 (1, 2). Previously described thyroid proteases are principally active below pH 6 (3). Although the neutral protease is the most active protease present in rat thyroid homogenates, it does not appear to participate in thyroglobulin hydrolysis in vivo as much as it is located in mast cells of the rat thyroid and thyroglobulin hydrolysis appears to occur in acinar cells (4). This paper reports on the purification and properties of the neutral protease. The purified enzyme resembles chymotrypsin A in many of its properties. The mast cell protease of the thyroid is also very similar to or identical with an enzyme identified in mast cells obtained from the peritoneal cavity of the rat (5).

MATERIALS AND METHODS

N-Benzoylarginine methyl and ethyl esters, BTE and N-acyetyltyrosine ethyl ester were purchased from Mann. diisopropylfluorophosphate, TCA, trichloroacetic acid, and diisopropylfluorophosphate, diphenylcarbamyl chloride and L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone were purchased from Sigma. Iodoacetamide and N-ethylmaleimide were purchased from Sigma. Iodacetate, recrystallized as the acid from petroleum ether, was a gift of Dr. J. Wolff (National Institutes of Health). Pepsinogen and ribonuclease were purchased from Worthington. Frozen rat thyroids were obtained from Pentex.

Proteolytic activity was measured by using 3H-thyroglobulin as the substrate under standard assay conditions. Each assay tube contained, in a total volume of 0.05 ml, 123 μg of 3H-thyroglobulin, 0.1 M ethanolamine, 0.05 M mercaptoethanol, and 0.5 M KCl. The pH was 8.2. When reduced thyroglobulin was the substrate, mercaptoethanol was omitted. Samples were incubated at 37°C for 30 or 60 min, and the incubations were arrested by the addition of 3 volumes of cold 10% TCA. The samples were centrifuged, and an aliquot of the supernatant was removed for measurement of radioactivity. Activity is expressed as the calculated micrograms of thyroglobulin found to be soluble in 7.5% TCA per 1 hour of incubation. The preparation of dog 3H-thyroglobulin, its chemical reduction, and other details of the assay were previously described (2).

Hydrolysis of BTE, N-benzoylphenylalanine ethyl ester, N-benzoylarginine methyl ester and DFP were gifts of Dr. J. Gladner (National Institutes of Health), and all the other ester substrates were gifts of Dr. J. Folk (National Institutes of Health). Diphenylcarbamyl chloride was purchased from Eastman and L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone from Calbiochem. Iodoacetamide and N-ethylmaleimide were purchased from Sigma. Iodacetate, recrystallized as the acid from petroleum ether, was a gift of Dr. J. Wolff (National Institutes of Health). Pepsinogen and ribonuclease were purchased from Worthington. Frozen rat thyroids were obtained from Pentex.

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ethanol, and 1
37,000
was established by measurement of the absorbance at 280 rnp
were sampled in 0.2-ml aliquots. The position of the markers
291 hours in a Spinco model L centrifuge at 4°. The gradients
sucrose were prepared in Lusteroid centrifuge tubes. The
sucrose contained 0.01
of Martin and Ames (10) was used. Linear gradients of 5 to 20%
activity as described under "Materials and Methods." 

Density Gradient Centrifugation—The sucrose gradient method
of Martin and Ames (10) was used. Linear gradients of 5 to 20%
sucrose were prepared in Lusteroid centrifuge tubes. The sucrose contained 0.01 M Tris buffer (pH 7.5 or 8.7), and applied to a DEAE-cellulose column (1 X 10 cm) equilibrated in 0.01 M Tris (pH 7.5 or 8.7). The enzyme emerged between the salt and the unretarded peak (Fig. 1). The active fractions were pooled, dialyzed against 0.01 M Tris (pH 7.5 or 8.7), and applied to a DEAE-cellulose column (1 X 10 cm) equilibrated in 0.01 M Tris at either pH 7.5 or 8.7. The enzyme was eluted with a linear gradient of KCl made by introducing 75 ml of 0 M KCl in 0.01 M Tris (pH 7.5 or 8.7) into 75 ml of 0.01 M Tris (pH 7.6 or 8.7). Because of the small amounts of protein remaining at this stage, the enzyme was collected in siliconized tubes. A column that was run at pH 8.7 (Fig. 2) was selected in order to compare the elution of protein and ester-hydrolyzing activities (see below). However, the purification of the protease was better at pH 7.6 where the enzyme was separated from the two adjoining protein peaks (Fig. 2). Data on the purification are summarized in Table I. The final purification is probably much greater than 200-fold, since peptidases, active at neutral pH (3), increase the conversion of 125I-thyroglobulin to TCA-soluble material in assays with crude enzyme.

The A260:A280 of the active fraction was >1 at all stages of purification suggesting contamination with RNA. The enzyme, when kept frozen in liquid nitrogen, did not lose appreciable activity over 1 year of storage. Attempts at further purification by rechromatography on DEAE-cellulose resulted in intolerable losses in activity. These losses were probably due to the small amount of protein present. Since the thyroids from 500 rats were needed to achieve the present level of purification, it was not possible to use more tissue. Unfortunately, the thyroids of various larger animals examined do not contain the enzyme in significant quantities (2). The enzyme does appear to be present in rat skin and tongue, and these appear to be useful sources for further purification.

RESULTS

Substrate Specificity—The enzyme hydrolyzed thyroglobulin, denatured hemoglobin, and fibrinogen. The product of the fibrinogen digestion did not clot spontaneously nor would it clot on the addition of thrombin.2 Of a number of synthetic substrates tested, benzoyltyrosine ethyl ester, benzoylphenylalanine ethyl ester, and acetyltyrosine ethyl ester were rapidly hydrolyzed (Table II). Acetyltryptophan ethyl ester, benzoylleucine ethyl ester, and benzoylmethionine ethyl ester were hydrolyzed more slowly. Hydrolysis of three trypsin substrates, benzoylarginine ethyl ester, benzoylarginine methyl ester, and p-toluenesulfonfyl-L-arginine methyl ester, was not detected.

Kinetics—The rate of hydrolysis of thyroglobulin and of all of the various ester substrates was directly proportional to enzyme concentration (Fig. 3). The Kₘ for benzoyltyrosine ethyl ester,

2 The studies with fibrinogen and thrombin were kindly performed by Dr. Jules Gladner, of the National Institutes of Health.
TUBE NUMBER

Fig. 2. Copurification of protease and esterase on DEAE-cellulose. Sephadex G-100-purified enzyme (68.5 ml), dialyzed to achieve a pH of 8.6 and a conductivity of 4.7 mohms⁻¹, was applied to a DEAE-cellulose column (1 × 6 cm) and eluted with a gradient of KCl. Samples (3.5 ml) were collected and analyzed for proteolytic and esterolytic activity as described in "Materials and Methods." Esterase activity is micromoles of BTE hydrolyzed per min.

### Table I

**Enzyme purification**

Specific activity is defined as the micrograms of thyroglobulin soluble in TCA per hour per mg of enzyme protein under standard assay conditions. The thyroids from 500 rats were used in this preparation.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total volume</th>
<th>Protease activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Relative purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole homogenate</td>
<td>47</td>
<td>100</td>
<td>325</td>
<td>0.19</td>
<td>1</td>
</tr>
<tr>
<td>Particle suspension</td>
<td>17</td>
<td>102</td>
<td>122</td>
<td>0.83</td>
<td>4.4</td>
</tr>
<tr>
<td>Soluble (1 m KCl)</td>
<td>15</td>
<td>78</td>
<td>75</td>
<td>1.04</td>
<td>5.5</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>187</td>
<td>106</td>
<td>17</td>
<td>6.2</td>
<td>33</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>30</td>
<td>60</td>
<td>1.2</td>
<td>50.0</td>
<td>260</td>
</tr>
</tbody>
</table>

### Table II

**Rate of hydrolysis of various ester substrates**

Each cuvette contained 1.3 µg of DEAE-cellulose-purified enzyme in a total volume of 0.2 ml. Other assay conditions are described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Ester substrate</th>
<th>Rate of hydrolysis (µmoles/ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoyltyrosine ethyl ester</td>
<td>21.0</td>
</tr>
<tr>
<td>Benzoylphenylalanine ethyl ester</td>
<td>18.0</td>
</tr>
<tr>
<td>Benzyllleucine ethyl ester</td>
<td>0.6</td>
</tr>
<tr>
<td>Benzoylvaline ethyl ester</td>
<td>0.0</td>
</tr>
<tr>
<td>Benzoylhistidine ethyl ester</td>
<td>0.0</td>
</tr>
<tr>
<td>Benzoylmethionine ethyl ester</td>
<td>0.6</td>
</tr>
<tr>
<td>Benzoylthreonine ethyl ester</td>
<td>0.0</td>
</tr>
<tr>
<td>Acetyltyrosine ethyl ester</td>
<td>9.0</td>
</tr>
<tr>
<td>Acetyltryptophan ethyl ester</td>
<td>0.4</td>
</tr>
<tr>
<td>p-Toluenesulfonyl-L-arginine ethyl ester</td>
<td>0.2</td>
</tr>
<tr>
<td>Benzyloarginine methyl ester</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Fig. 3. Rate of hydrolysis of [³H]-thyroglobulin and BTE by DEAE-cellulose-purified enzyme as a function of enzyme concentration. The assay conditions are described in "Materials and Methods."

Identity of Proteolytic and Esterolytic Activities—To show that the enzyme hydrolyzing thyroglobulin and the one hydrolyzing BTE were identical, we performed assays for both substrates over a 200-fold purification. The results of parallel assays during Sephadex G-100 filtration and DEAE-cellulose chromatography are shown in Figs. 1 and 2. No separation of activities was detected. Recoveries of both activities were about 60%.
I. Pastan and X. Almqvist 5093

PH FIG. 4. Effect of pH on enzyme activity. O-0, hydrolysis of 125I-thyroglobulin; C-0, hydrolysis of BTE. The assay conditions are described in “Materials and Methods” except that 0.1 M sodium citrate buffer was used for pH 5.5 to 6.9, 0.1 M Tris-HCl for pH 7.3 to 8.8, and 0.1 M triethylamine-HCl for pH 8.9 to 9.8.

Optimal pH—The effect of pH was examined for both the protein substrate thyroglobulin and the synthetic substrate BTE. The optimal pH for hydrolysis of both substrates was about 7.8 (Fig. 4).

Role of Mercaptoethanol—As with the crude particulate enzyme, where it was shown that the role of mercaptoethanol was to reduce the thyroglobulin (2), the omission of mercaptoethanol decreased the rate of hydrolysis of native thyroglobulin by 80 to 95%. When reduced thyroglobulin was used as a substrate, the dependence on mercaptoethanol was abolished. Further evidence that the effect of the mercaptoethanol was not on the enzyme itself was the finding that 10⁻⁵ M mercaptoethanol had no effect on the rate of hydrolysis of BTE.

Dependence on KCl—The addition of 0.4 to 0.5 M KCl approximately doubles the rate of thyroglobulin hydrolysis. Since this effect occurs in the presence or absence of mercaptoethanol, it is not an effect of KCl on the reduction of thyroglobulin by mercaptoethanol. However, the dependence on KCl is not as striking as with the particulate enzyme, for which the reaction is 95% inhibited by omission of the salt (2). The rate of hydrolysis of BTE is increased 30% in 1 M KCl.

Effect of Inhibitors—Exposure of the enzyme to 10⁻³ M DFP decreases its activity towards thyroglobulin. Iodoacetate and N-ethylmaleimide are not inhibitory (Table III). DFP inhibition was also noted with the BTE assay. Inactivation at 0°C occurred at a DFP concentration of 10⁻⁴ M (Fig. 5). No inactivation occurred at 10⁻⁴ M DFP at 0°C, although rapid inactivation occurred at 23°C. It is unlikely that the DFP inactivation is due to the presence of the sulfhydryl poison which has been found in some preparations of DFP (12) since (a) mercaptoethanol afforded no protection to DFP inactivation, and (b) 10⁻³ M iodoacetate or N-ethylmaleimide caused minimal or insignificant inactivation.

Diphenylcarbamyl chloride is also an effective inhibitor (Fig. 6). Indole, which prevents the inhibition of chymotrypsin by diphenylcarbamyl chloride (13), also prevents the inhibition of the mast cell protease. Another inhibitor of chymotrypsin, L- (1-tosylamido-2-phenyl)ethyl chloromethyl ketone, also inhibits the mast cell enzyme. Both diphenylcarbamyl chloride and L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone were effective inhibitors when tested at 23°C, but the same concentrations were not inhibitory when incubated with the enzyme for 60 min at 0°C.

Effect of Chelating Agents and Metal—There was no inhibition of thyroglobulin hydrolysis by 10⁻² M EDTA, α,α'-dipyridyl, cupferron, or 1,10-phenanthroline. In fact, EDTA seemed to stabilize the purified enzyme when it was kept at 0°C for 6 hours.

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Table III

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Temperature</th>
<th>Thyroglobulin hydrolyzed</th>
<th>μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>DFP (10⁻³ M)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>DFP (10⁻⁴ M)</td>
<td></td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>Experiment B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Iodoacetate (10⁻³ M)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>N-Ethylmaleimide (10⁻³ M)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 5. Inhibitors of BTE hydrolysis. DEAE-cellulose-purified enzyme, 15 μg, was incubated with the inhibitor at 2°C for the indicated times at pH 7.8 (0.05 M Tris). Total volume, 1.0 ml. Aliquots were removed for assays. O-0, control; Δ-Δ, N-ethylmaleimide (10⁻³ M); O-O, iodoacetate (10⁻³ M); △-△, DFP (10⁻³ M).
ethyl ester as well as some other ester substrates of chymotrypsin. Throughout a 260-fold purification, purified by filtration on Sephadex G-100 followed by DEAE-

These extracts also catalyze the hydrolysis of acetyltyrosine is estimated to be 2.5 S. The molecular weight is therefore Table IV. The sedimentation coefficient of the thyroidal enzyme the protease with that of pepsinogen and ribonuclease is shown in 

Metal ions on BTE hydrolysis was not examined. No alteration 
in activity was observed with Ca++ or Mg++. The effect of 
metal ions on BTE hydrolysis was not examined. 

**Molecular Weight**—A comparison of the sedimentation rate of 
the protease with that of pepsinogen and ribonuclease is shown in 
Table IV. The sedimentation coefficient of the thyroidal enzyme is estimated to be 2.5 S. The molecular weight is therefore about 23,000. 

**DISCUSSION**

It has been previously shown that unfractionated extracts of 
mast cell granules contain a protease which is inhibited by DFP. 
These extracts also catalyze the hydrolysis of acetyltyrosine 
ethyl ester as well as some other ester substrates of chymotrypsin (5). The esterolytic activity is also inhibited by DFP. However, it has not been proved that the two activities reside in a single enzyme, since purification of the enzyme had not been achieved. In the studies reported here, the protease has been purified by filtration on Sephadex G-100 followed by DEAE-

cellulose chromatography. Throughout a 260-fold purification, 
the two activities were inseparable. Furthermore, no other enzyme capable of hydrolyzing thyroglobulin or BTE at pH 7.8 was detected. The proteolytic and esterolytic activity of the purified enzyme were both inhibited by DFP and relatively unaffected by iodoacetate and N-ethylmaleimide. Thus, a single enzyme appears to account for both the proteolytic and esterolytic activity.

The purified enzyme has a number of additional properties in common with chymotrypsin. Both are inhibited by diphenyl-
carbamyl chloride and the inhibition is prevented by indole. Both are also inhibited by L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone. The molecular weight of both is about 23,000. The substrate specificity of the mast cell protease resembles porcine and bovine chymotrypsin A more than that of chymotrypsin B or C, since it hydrolyzes BTE more rapidly than either benzoylleucine or benzoylmethionine ethyl ester (5). In preliminary studies, no evidence has been obtained for an in 

The mast cell protease was readily extracted from subcellular 
particles by treatment with 1 M KCl. However, further purification was not readily achieved, for when the 1 M KCl extract was dialyzed to lower the ionic strength, a precipitate formed and enzymatic activity was lost. Only a small portion of that activity could be recovered by redissolving the precipitate in 1 M KCl. Since the enzyme could not be transferred to solutions of low ionic strength, purification by ion exchange or adsorption chromatography could not be achieved. Ammonium sulfate fractionation was also not useful. However, after the protease had been separated from other high and low molecular weight substances by filtration on Sephadex G-100 equilibrated in 1 M KCl, it could be dialyzed against 0.01 M Tris (pH 7.5 or pH 8.7) without precipitate formation or appreciable loss of activity. Therefore, precipitation in solutions of low ionic strength is not an intrinsic property of enzyme itself. Preliminary experiments have shown that the protease combines with a substance present in a less retarded fraction from Sephadex G-100. 

**Acknowledgments**—We are indebted to Dr. Jules Gladshe for performing the fibrinogen hydrolysis studies, and to Miss Carol Oulahan for her expert technical assistance. 

**REFERENCES**

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