found in spleen and rheumatoid synovial membrane were surprisingly large in view of the scarcity of neutrophils in these tissues.[10,23] The elastase of macrophages is a metalloproteinase with quite different properties from the neutrophil enzyme. Leukocyte elastase is certainly not confined to humans, however; elastases have been detected in the polymorphonuclear cells of dog,[45] horse,[46] pig,[47] and rabbit.[48]

[46] A. Koj, J. Chudzik, and A. Dubin, *Biochem. J.* **153**, 397 (1976).
[47] M. Kopitar and D. Lebez, *Eur. J. Biochem.* **56**, 571 (1975).
[48] T. G. Cotter and G. B. Robinson, *Biochim. Biophys. Acta* **615**, 414 (1980).

[45] Mast Cell Proteases

By RICHARD G. WOODBURY, MICHAEL T. EVERITT, and HANS NEURATH

Mast cells are widely distributed in the connective tissue of most vertebrates and play a central role in the early stages of inflammation. It is generally thought that these cells help in the defense against parasites.[1]

Mast cells contain dense secretory granules that, in most animal species, including humans, contain histamine, heparin, and several proteins.[2] A considerable amount of the protein of the mast cell granules of many species examined consists of serine proteases, which differ in substrate specificity from one species to another.[3] Since the activity of the mast cell granules can be detected in tissue slices using histochemical ester substrates,[3] it appears that the proteases are present in the granules in active form rather than as zymogens. Rats, as well as several other mammals examined, possess two types of mast cells. One of these, referred to as the *normal mast cell,* is found in most loose connective tissues and in the peritoneal cavity. The other one, known as the *atypical mast cell,* is present exclusively in mucosal tissues and hence is sometimes referred to as the mucosal mast cell.[4]

Although it is questionable whether normal and atypical mast cells are histologically related to each other,[5,6] it is clear that the secretory granules

[1] A. Sher, *Nature (London)* **263**, 334 (1976).
[2] D. Lagunoff and P. Pritzl, *Arch. Biochem. Biophys.* **173**, 554 (1976).
[3] H. Chiu and D. Lagunoff, *Histochem. J.* **4**, 135 (1972).
[4] R. Veilleux, *Histochemie* **34**, 157 (1973).
[5] H. R. P. Miller and R. Walshaw, *Am. J. Pathol.* **69**, 195 (1972).
[6] E. J. Ruitenberg and A. Elgersma, *Nature (London)* **264**, 258 (1976).

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of each cell type contain a similar yet distinct serine protease with chymotrypsin-like esterase specificity.[7,8] In order to distinguish these enzymes from each other, that from rat normal mast cells will be called rat mast cell protease I (RMCP I) and that from atypical mast cells, rat mast cell protease II (RMCP II).

Katunuma and co-workers[9] examined both enzymes and, without a knowledge of the cellular localization of these proteases, had designated them "group-specific" intracellular proteases.

In addition to the chymotrypsin-like serine protease, normal mast cells obtained from rat peritoneum also contain an enzyme with carboxypeptidase A-like activity.[10,11]

I. Serine Protease of Peritoneal Mast Cells

Assay

Reagents

1.07 m*M* benzoyl tyrosine ethyl ester (BzTyrOEt) in 50% methanol
80 m*M* Tris-HCl buffer (pH 7.8) containing 100 m*M* $CaCl_2$

Procedure. This assay, which measures chymotrypsin-like esterase activity, was first described by Hummel.[12] Substrate (1.4 ml) is mixed with 1.5 ml of buffer and 0.1 ml of dilute enzyme preparation (1–10 μg) is added. The absorption change at 256 nm is recorded for about 5 min.

An enzyme unit is defined as an amount of enzyme activity that results in the hydrolysis of 1 μmol of substrate per minute at pH 7.8, 25°C.

Purification Procedure

Mast cells are collected from Sprague-Dawley rats (250–400 g) by washing the peritoneal cavities with 10 ml of ice-cold phosphate-buffered saline solution at pH 7.2 (4.1 m*M* Na_2HPO_4–2.1 m*M* KH_2PO_4–154 m*M* NaCl–2.17 m*M* KCl–0.68 m*M* $CaCl_2$). All subsequent steps are carried out at 4°C. Mast cells of the pooled peritoneal washes generally represent

[7] D. Lagunoff and E. P. Benditt, *Ann. N.Y. Acad. Sci.* **103**, 185 (1963).
[8] R. G. Woodbury, G. M. Gruzenski, and D. Lagunoff, *Proc. Natl. Acad. Sci. U. S. A.* **75**, 2785 (1978).
[9] N. Katunuma, E. Kominami, K. Kobayashi, Y. Banno, K. Suzuki, K. Chichibu, Y. Hamaguchi, and T. Katsunuma, *Eur. J. Biochem.* **52**, 35 (1975).
[10] R. Haas, P. C. Heinrich, and D. Sasse, *FEBS Lett.* **103**, 168 (1979).
[11] M. T. Everitt and H. Neurath, *FEBS Lett.* **110**, 292 (1980).
[12] B. C. W. Hummel, *Can. J. Biochem. Physiol.* **37**, 1393 (1959).

less than 5% of the total cell population. If desired, a preparation can be obtained that contains more than 95% mast cells by sedimentation of the cells through an isotonic solution of bovine serum albumin.[2] However, for the purification of the mast cell protease this enrichment step is unnecessary and usually results in some loss of material. The peritoneal washes are combined and the cells pelleted by centrifugation at 2300 *g* for 15 min. The cell pellet is resuspended in 20 ml of the phosphate-buffered saline solution, and this suspension is quickly frozen and thawed through six cycles. The mast cell granules released by cell lysis are collected along with cellular debris by centrifugation at 27,000 *g* for 20 min. The protease is fully active while it is associated with the granules. The pellet, containing granules, is extracted three times with 20 ml of a solution of 0.8 *M* potassium phosphate–2% protamine sulfate (pH 8.0). The extracts containing solubilized mast cell protease are pooled (60 ml) and centrifuged at 27,000 *g* for 20 min.

Affinity-Adsorption Chromatography. Hen ovoinhibitor is coupled to CNBr-activated Sepharose CL-6B by the method described by Marsh *et al.*[13] A small column (1 × 4 cm) of the Sepharose-coupled ovoinhibitor is equilibrated with a solution of 0.8 *M* potassium phosphate containing 1 mg/ml bovine serum albumin. The protease solution is passed slowly through the column. Approximately 5-ml fractions are collected at a flow rate of 20 ml/hr. The column is washed with equilibration buffer until no material that absorbs at 280 nm is eluted. The protease is desorbed from the ovoinhibitor–Sepharose with 25 m*M* formic acid containing 1 m*M* EDTA–1 mg/ml bovine serum albumin–20% glycerol (pH 3.0). At this point the flow rate is reduced to 10 ml/hr and 1-ml fractions are collected in tubes containing 1 ml of 0.2 *M* ammonium bicarbonate–1 mg/ml bovine serum albumin (pH 8.6). The eluate is monitored at 280 nm and the peak fractions are assayed for esterase activity using BzTyrOEt as substrate. The contents of fractions containing enzyme activity are pooled.

Adsorption to Barium Sulfate. The protease solution obtained by the affinity-adsorption step is diluted 2-fold with distilled water. Two grams of X-ray grade barium sulfate (Matheson, Coleman and Bell) is added slowly to the protease solution with constant stirring. After 15 min, the suspension is centrifuged at 2500 *g* for 10 min and the pellet is washed twice with 20 ml of 10 m*M* Tris-HCl buffer (pH 8.0) to remove the albumin. The protease is solubilized by washing the barium sulfate pellet with 5 ml of 10 m*M* Tris-HCl buffer (pH 8.0) containing 1.0 *M* NaCl. The purified protease is stored at −20°C. A summary of a typical purification of the protease from 100 rats is given in Table I.

Comment. During the purification of the mast cell protease, it is important to maintain the solutions at relatively high ionic strength in order to

[13] S. C. Marsh, I. Parikh, and P. Cuatrecasas, *Anal. Biochem.* **60**, 149 (1974).

TABLE I
PURIFICATION OF PERITONEAL MAST CELL PROTEASE[a]

Step	Volume (ml)	Total activity (BzTyrOEt units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification factor (-fold)
Crude extract supernatant	56	527	330	1.6	100	1
Affinity chromatography on ovoinhibitor–agarose	20	321	—	—	61	—
Barium sulfate adsorption	15	232	9.1	25.5[b]	44	15

[a] From the peritoneal cells of 100 rats (250 g).
[b] The preparation is known to contain approximately 50% inactive enzyme.

prevent the enzyme from adsorbing to surfaces. In solutions of low ionic strength, or in the absence of detergent, the protease adsorbs to glass, dialysis tubing, Sephadex, agar, polyacrylamide, and agarose.

Other Purification Procedures. The protease from rat peritoneal mast cells (also known as chymase) has been purified in small quantities by Yurt and Austen[14] using a procedure that includes ion-exchange chromatography on Dowex-1, gel filtration, and affinity chromatography on D-tryptophan methyl ester coupled to Sepharose.

Mast cell protease, which at the time was known as group-specific protease, was isolated by Katunuma *et al.*[9] from rat skeletal muscle and liver. This rather lengthy procedure yields small quantities of homogeneous protease with a high specific activity.

Properties

Purity. The preparation of mast cell protease (RMCP I) is homogeneous according to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate and 8 *M* urea. Also, amino-terminal sequence analysis of the protease preparation indicates the presence of a single polypeptide chain.

Stability. At pH 7–8, the solubilized protease is quite labile even at 4°C, and most activity is lost in a few days due to autolysis. For this reason, the purification must be accomplished quickly to ensure a reasonable yield of active protease. At −20°C the protease is relatively stable for several months. The protease can be freeze-dried and stored at −20°C with little loss in activity. Alternatively, the enzyme can be adsorbed to barium sulfate and stored at 4°C without appreciable autolysis.

Specific Activity. Although the protease prepared as described is homogeneous, it was found that approximately 55% of the enzyme could not bind to potato chymotrypsin inhibitor immobilized on agarose. The nonbinding material also lacked esterase activity. Since the binding and nonbinding fractions of protease have identical amino acid compositions and molecular weights, it appears that a significant amount of the protease is irreversibly inactivated, most likely when it is exposed to the 25 m*M* formic acid solution during the affinity-chromatography step. In fact, nearly 40% of the initial esterase activity is lost during this manipulation. However, for structural studies this is of little consequence and for enzymatic studies one may readily calculate the proportion of active protease on the basis of a specific activity of fully native enzyme of 58 BzTyrOEt units/mg of enzyme.[15]

[14] R. W. Yurt and K. F. Austen, *J. Exp. Med.* **146,** 1405 (1977).
[15] M. T. Everitt and H. Neurath, *Biochimie* **61,** 653 (1979).

TABLE II
AMINO ACID COMPOSITION[a] OF RAT MAST CELL PROTEASE (RMCP I)

Amino acid	g/100 g protein	Residues/molecule
Aspartic acid	7.4	17
Threonine[b]	6.5	16
Serine[b]	4.2	12
Glutamic acid	9.2	19
Proline	5.6	15
Glycine	5.4	21
Alanine	4.3	14
Half-cystine[c]	3.1	8
Valine	8.6	22
Methionine	2.5	5
Isoleucine[d]	5.5	13
Leucine[d]	5.1	12
Tyrosine	4.2	7
Phenylalanine	3.8	7
Histidine	4.5	9
Lysine	11.2	23
Arginine	7.0	12
Tryptophan[e]	1.2	2
Total residues		234
Molecular weight (composition)		25,800
Molecular weight (SDS electrophoresis)		26,000

[a] Duplicate samples were analyzed after 24, 48, 72, and 96 hr of hydrolysis in 6 *N* HCl at 110°C.
[b] Determined by extrapolation to zero-hydrolysis time.
[c] Determined as cysteic acid after performic acid oxidation.
[d] Values were obtained from analysis of 96-hr hydrolysates.
[e] Determined after alkaline hydrolysis (48 hr) of the protease.

Physical and Chemical Properties. The physical, chemical, and enzymatic properties of the protease obtained from the total cell population of the peritoneal washings are identical to those of the enzyme obtained from enriched preparations containing more than 95% mast cells. The purified protease migrates as a single polypeptide chain during electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate and 8 *M* urea, with or without the addition of 2-mercaptoethanol. The apparent molecular weight of the protease is 26,000. In the absence of urea, however, the apparent molecular weight of the protein is significantly higher and the protein migrates in a relatively broad band.

The amino acid composition (Table II) of RMCP I indicates that the protein has a relatively high content of basic residues (19%). This is con-

sistent with the observation that the isoelectric point of the protease is at pH 9.5. The tendency of the protease to adsorb to surfaces when in solutions of low ionic strength may be explained by its highly basic nature. It has also been proposed that the relatively high content of lysine in RMCP I allows the enzyme to form strong complexes with the highly acidic heparin molecules within the mast cell granules.[16]

The amino-terminal sequence of the first 52 residues of RMCP I is known.[17] Approximately 40% of these residues are identical to those of bovine chymotrypsin A, indicating a homologous relationship between this enzyme and the mammalian serine proteases. A high degree of sequence identity (greater than 85%) is observed when the amino-terminal region of RMCP I is compared to those of the protease from rat atypical mast cells, RMCP II,[18] and human neutrophil cathepsin G[19] (Fig. 1). This sequence similarity, in addition to numerous similarities in chemical and enzymatic properties,[20] suggests that the mammalian granulocyte serine proteases may have followed a common evolutionary pathway, which has led to a distinct class of serine proteases, in the sense that the pancreatic serine proteases comprise a group that is different from the blood-coagulation enzymes.

Substrate Specificity. RMCP I has chymotrypsin-like esterase specificity. As already mentioned, the specific activity of this enzyme is 58 units/mg protein[15] using BzTyrOEt as substrate. The protease activity of RMCP I is, however, relatively low.[9] When peptides, such as glucagon and the oxidized form of the β-chain of insulin, are used as substrates, the protease preferentially cleaves peptide bonds on the carboxyl side of tyrosine and phenylalanine.[21,22] The overall protease specificity of RMCP I resembles that of human cathepsin G more nearly than that of chymotrypsin. The chemical, physical, and enzymatic similarities between the mast cell protease and cathepsin G have been discussed by Starkey.[23] However, a recent detailed investigation of the substrate specificities of both of these proteases toward 4-nitroanilide peptides clearly indicates

[16] R. G. Woodbury and H. Neurath, *FEBS Lett.* **114**, 189 (1980).
[17] R. G. Woodbury, M. Everitt, Y. Sanada, N. Katunuma, D. Lagunoff, and H. Neurath, *Proc. Natl. Acad. Sci. U. S. A.* **75**, 5311 (1978).
[18] R. G. Woodbury, N. Katunuma, K. Kobayashi, K. Titani, and H. Neurath, *Biochemistry* **17**, 811 (1978).
[19] J. Travis, personal communication.
[20] R. G. Woodbury and H. Neurath, *Proc. Life Sci.* in press (1981).
[21] K. Kobayashi, Y. Sanada, and N. Katunuma, *J. Biochem. (Tokyo)* **84**, 477 (1978).
[22] M. T. Everitt and H. Neurath, *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **38**, 834 (1979).
[23] P. M. Starkey, *in* "Proteinases in Mammalian Cells and Tissues" (A. J. Barrett, ed.), p. 57. North-Holland Publ., Amsterdam, 1977.

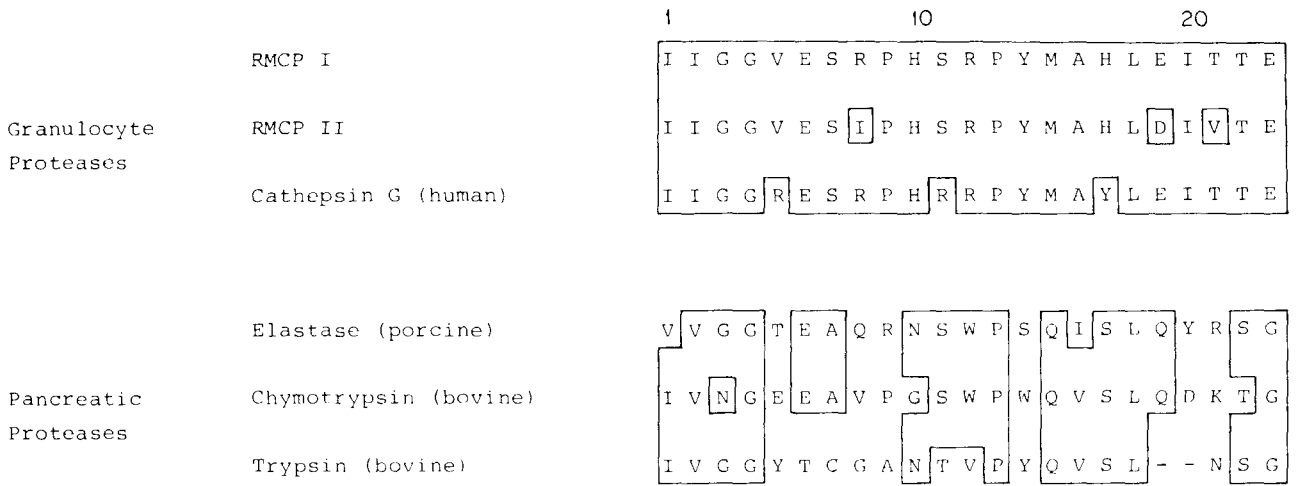

FIG. 1. The amino-terminal sequences of the granulocyte serine proteases rat mast cell protease I (RMCP I), rat mast cell protease II (RMCP II), and human neutrophil cathepsin G. A comparison of the amino-terminal sequences of these granulocyte enzymes to those of the pancreatic serine proteases elastase (porcine), chymotrypsin (bovine), and trypsin (bovine) illustrates the highly conserved structures of the granulocyte proteases, suggesting these enzymes have followed a common evolutionary pathway.

that the enzymes are quite different.[24] In this study it was observed that the best substrates of RMCP I contained hydrophobic residues not only at the primary cleavage site but also at neighboring positions that interact with the extended binding regions of the protease. The best substrate tested, succinyl-Phe-Leu-Phe-4-nitroanilide, was observed to have a k_{cat}/K_m of approximately $10^6\ M^{-1}\ sec^{-1}$, one of the highest specificity constants observed for any protease. It was also observed that, unlike other serine proteases such as chymotrypsin and cathepsin G, RMCP I will readily hydrolyze substrates that contain a prolyl residue at the P_3 subsite.

Inhibitors. RMCP I is inhibited by diisopropyl phosphorofluoridate at a rate (second-order rate constant k_2 = 61 liters mol^{-1} min^{-1}) which is considerably slower than that of bovine chymotrypsin (k_2 = 17,300 liters mol^{-1} min^{-1}).[15] The protease is also slowly inhibited by N^{α}-tosyl-L-phenylalanine chloromethyl ketone, and more readily so by benzoyloxycarbonyl-L-phenylalanine bromomethyl ketone. Since peptide 4-nitroanilides containing a proline at the P_3 subsite are good substrates for RMCP I, Yoshida and co-workers[24] examined the inhibitory activity of chloromethyl ketone peptides, which also contained a proline residue at the P_3 subsite. It was observed that succinyl-prolyl-leucyl-phenylalanine chloromethyl ketone was not only an excellent inhibitor of the enzyme, but also was relatively selective in its action, since it inhibited RMCP I at a rate approximately 10 times greater than that of chymotrypsin.

In addition to hen ovoinhibitor and potato chymotrypsin inhibitor I, soybean- and lima bean-trypsin inhibitors also are effective inhibitors of RMCP I. Pancreatic basic trypsin inhibitor (Kunitz) and ovomucoid, as well as cysteine, fail to inhibit the protease.

Physiological Role

Although the chymotrypsin-like protease (chymase) of rat peritoneal mast cells has been studied for over 20 years, the function of the enzyme remains obscure. Very likely it acts at some level in the inflammatory reactions associated with mast cell degranulation. There is fragmentary evidence suggesting the involvement of mast cell proteases in chemotaxis,[25] in degradation of connective tissue proteoglycans,[26] in

[24] N. Yoshida, M. T. Everitt, R. G. Woodbury, H. Neurath, and J. C. Powers, *Biochemistry* **19**, 5799 (1980).

[25] V. B. Hatcher, G. S. Lazarus, N. Levine, P. G. Burke, and F. J. Yost, Jr., *Biochim. Biophys. Acta* **483**, 160 (1977).

[26] H. Seppä, K. Väänänen, and K. Korhonen, *Acta Histochem.* **64**, 64 (1979).

selective degradation of basement membrane collagen (type IV),[27] as well as in the promotion of vascular permeability.[28]

Alternatively, the action of the protease following mast cell degranulation may be viewed as a pathological function, unrelated to the role of the enzyme in the normal physiological state. If mast cells function in the maintenance of homeostasis of the microenvironment without undergoing degranulation, as has been suggested,[29,30] then conceivably the protease may also have an intracellular function. Consistent with this hypothesis is the observation that the enzyme is fully active both as an esterase and as a protease when bound to the granule matrix heparin.[3,14]

II. Serine Protease of Atypical Mast Cells

Assays

Method A

Principle. This protease was first detected in rat small intestine by Katunuma and co-workers[9] by its ability to inactivate the apo form of ornithine aminotransferase, OAT (EC 2.6.1.13). In the early stages of the purification scheme this assay is much more sensitive and reliable than assays based on the esterase activity of the protease.

Reagents

Ornithine aminotransferase is isolated from rat livers and purified as described by Peraino *et al.*[31]

Apo ornithine aminotransferase is obtained by the method of Katunuma *et al.*[9]

50 m*M* potassium phosphate buffer, pH 8.0

0.1 m*M* pyridoxal phosphate in 50 m*M* potassium phosphate, pH 8.0

Procedure. A volume (10–100 μl) of dilute protease solution is added to 0.1 mg of apo ornithine aminotransferase in 50 m*M* potassium phosphate, pH 8.0 (stock solution contains 1 mg OAT/ml buffer). The volume is increased to 0.3 ml by the addition of 50 m*M* potassium phosphate, pH 8.0. Individual assays are carried out in duplicate for 0, 5, 15, and 30 min

[27] H. Sage, R. G. Woodbury, and P. Borstein, *J. Biol. Chem.* **254**, 9893 (1979).
[28] H. Seppä, *Inflammation* **4**, 1 (1980).
[29] K. F. Austen, *J. Immunol.* **121**, 793 (1978).
[30] J. Padawer, *Immunol. Ser.* **7**, 301 (1978).
[31] C. Peraino, L. G. Bunville, and T. N. Tahmisian, *J. Biol. Chem.* **244**, 2241 (1969).

at 37°C. Proteolytic assays are also performed using the holo form of ornithine aminotransferase as controls. At the appropriate time, each incubated solution is diluted 10-fold with 50 m*M* potassium phosphate buffer (pH 8.0) containing 0.1 m*M* pyridoxal phosphate, and is further incubated at 4°C for 30 min to allow any remaining apo ornithine aminotransferase to be converted to the holo form. Ornithine aminotransferase activity is measured by the method of Jenkins and Tsai.[32]

An enzyme unit is defined as the amount of enzyme that inactivates 50% of the ornithine aminotransferase activity in 30 min under the conditions described.

Method B

This assay measures the hydrolysis of BzTyrOEt by the method of Hummel,[12] as described earlier in this chapter.

Purification Procedure

Small intestines are removed from 50 Sprague-Dawley rats (250 g) and cut open lengthwise. The intestines are rinsed thoroughly with several changes of ice-cold 0.15 *M* NaCl (pH 7.2), gently scraped to remove the mucus-rich upper layer of mucosa, and then minced into small pieces. All subsequent steps are performed at 4°C. The minced intestines are homogenized in a Waring blender at moderate speed for 1–2 min in 3 volumes of 0.15 *M* KCl containing 5 m*M* benzamidine. Benzamidine is added to inhibit the activity of a small amount of a trypsin-like protease, which has also been observed by others[33] in homogenates of rat intestine. The crude homogenate is centrifuged at 15,000 *g* for 10 min and solid $(NH_4)_2SO_4$ is added to the supernatant containing the protease to give a solution that is 65% saturated. After 30 min, the precipitated material is collected by centrifugation at 15,000 *g* for 15 min and the pellet is resuspended in 200 ml of 0.15 *M* KCl containing 5 m*M* benzamidine. The suspension is centrifuged at 15,000 *g* for 30 min. Acetone (−20°C) is slowly added to the supernatant to give a concentration of 30% (v/v). The precipitate is removed by centrifugation at 15,000 *g* for 5 min and the supernatant is adjusted to a concentration of 55% acetone. The resulting precipitate is resuspended in 50 ml of 0.1 *M* Tris-HCl buffer, pH 7.8. The protease solution is incubated for 1 hr at 4°C with N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (1 m*M*) and N^{α}-*p*-tosyl-phenylalanine chloromethyl ketone (1 m*M*), and sodium chloride (1 *M*) is then added.

[32] W. T. Jenkins and H. Tsai, this series, Vol. 17A, p. 281.
[33] R. J. Beynon and J. Kay, *Biochem. J.* **173**, 291 (1978).

Affinity-Adsorption Chromatography. Subunit C of potato chymotrypsin inhibitor that is purified by the method of Melville and Ryan[34] is coupled to Sepharose-4B as described by Marsh *et al.*[13] The inhibitor–Sepharose matrix is equilibrated with 0.1 *M* Tris-HCl buffer (pH 7.8) containing 1 *M* NaCl, and the protease-containing solution is passed slowly through the affinity-adsorption column (1.5 × 10 cm). The column contents are washed with approximately 5 bed volumes of the equilibration buffer and then with several volumes of distilled water. The protease is desorbed from the affinity adsorbant with 0.1 *M* acetic acid. 1-ml fractions are collected and the eluate is monitored at 280 nm. Peak fractions are assayed for protease and esterase activity and the contents of the appropriate fractions are pooled. This material is immediately dialyzed against 0.05 *M* Tris-HCl buffer (pH 7.8) or freeze-dried for storage at −20°C. A typical purification (Table III) of RMCP II from 50 small intestines results in about 25 mg of enzyme with more than 50% overall yield.[35]

Comments. It is essential to wash the column of Sepharose-coupled inhibitor free of the Tris buffer and NaCl, since in the presence of acetic acid, much of the desorbed protease precipitates with complete loss of activity.

The small amount of protease with trypsin-like specificity that is present in the homogenates of small intestine copurifies with RMCP II during the affinity-adsorption step. The presence of just a few micrograms of this protease results in substantial inactivation of RMCP II. The addition of N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (1 m*M*) to the protease solution prior to the affinity-adsorption step serves to inhibit the contaminating protease and prevent its binding to the affinity resin.

Other Purification Procedures. This protease was first purified by Katunuma and co-workers,[9] at which time it was referred to as a group-specific protease. Although this procedure is considerably more time consuming and results in a lower overall yield of enzyme than the procedure outlined here, it does result in a homogeneous stable product with excellent activity.

Properties

Purity. Amino-terminal sequence analysis of the preparation of protease indicates that it consists of a single polypeptide chain. The preparation is also homogeneous according to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate. The physical, chemical, en-

[34] J. C. Melville and C. A. Ryan, *J. Biol. Chem.* **247**, 3445 (1972).
[35] R. G. Woodbury and H. Neurath, *Biochemistry* **17**, 4298 (1978).

TABLE III
PURIFICATION OF THE PROTEASE (RMCP II) OF RAT ATYPICAL MAST CELLS[a]

Step	Total activity[b] (protease units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification factor (-fold)
Crude homogenate (supernatant)	3830	4366	0.9	100	1.0
Ammonium sulfate precipitation	2930	2264	1.4	77	1.6
Acetone precipitation	2210	590	3.8	58	4.2
Affinity adsorption	2000	25	80	52	89

[a] From the small intestines of 50 rats (250 g).
[b] Determined by measuring the ability of the protease to inactivate apo ornithine amino transferase.

zymatic, and immunological properties of RMCP II are identical to those of the protease isolated by the procedure of Katunuma *et al.*[9]

Stability. The purified protease is remarkably stable even when stored in solution at neutral pH for several weeks at 4°C, indicating the lack of significant autolysis.

Specific Activity. The activity of the purified protease (80 units/mg of protease), as measured by its ability to inactivate apo ornithine aminotransferase, is as high as that of enzyme prepared by the method of Katunuma *et al.*[9] (78 units/mg protease). Complete inactivation of the purified protease with ^{14}C-labeled diisopropyl phosphorofluoridate resulted in the incorporation of 1 mol of the ^{14}C-labeled diisopropylphosphoryl group per mole of protein, indicating that all of the protease molecules in this preparation had functional catalytic sites.[35] As measured by the hydrolysis of BzTyrOEt, the esterase activity of the homogeneous enzyme is 8.5 units/mg enzyme.

Physical and Chemical Properties. As in the case of RMCP I, RMCP II is extracted from tissue in a fully active form and thus seems to lack a zymogen precursor. The protease consists of a single polypeptide chain with a molecular weight of approximately 25,000 on the basis of the results of polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate. The molecular weight of the protease determined from its amino acid sequence is 24,655.[18] Table IV compares the amino acid composition obtained after acid hydrolysis of the enzyme to that calculated from the amino acid sequence. Since the enzyme has 25 basic residues and only 21 acidic ones, it is somewhat basic at neutral pH, but not as basic as RMCP I. The major structural features of RMCP II are as follows:

1. Approximately 35% sequence identity when compared to bovine chymotrypsin A.
2. The presence of the residues of the charge-relay system (Ser, Asp, His) of the active site.
3. An alanyl residue at the primary substrate binding site, identified as Asp^{177} in trypsin and Ser^{189} in chymotrypsin. Thus the primary binding site of RMCP II is likely to be more nonpolar when compared to those of the known serine proteases.
4. An arrangement of the three disulfide bonds of RMCP II that is unique among the known serine proteases.

Clearly, the structural data indicate a homologous relationship between RMCP II and the mammalian serine proteases. At the same time, there are structural features that distinguish this protease from other serine proteases. The significance of the various structural features of the protease with respect to enzymatic activity has been discussed previously.[18,20,24]

TABLE IV
AMINO ACID COMPOSITION[a] OF RAT ATYPICAL MAST CELL PROTEASE (RMCP II)

Amino acid	Moles of amino acid/mole of protein	
	After hydrolysis	From sequence analysis
Aspartic acid	14.0	9
Asparagine	—	5
Threonine[b]	12.5	13
Serine[b]	13.0	13
Glutamic acid	17.2	12
Glutamine	—	5
Proline	14.8	15
Glycine	18.4	18
Alanine	16.0	16
Half-cystine[c]	5.8	6
Valine[d]	21.6	22
Methionine	4.7	5
Isoleucine[d]	16.8	18
Leucine[d]	15.6	16
Tyrosine	8.8	9
Phenylalanine	6.1	6
Histidine	8.8	9
Lysine	12.7	13
Arginine	12.0	12
Tryptophan[e]	1.7	2
Total residues	223	224
Molecular weight	24,522	24,655

[a] Duplicate samples were analyzed after 24, 48, 72, and 96 hr hydrolysis in 6 *N* HCl at 110°C.
[b] Determined by extrapolation to zero-hydrolysis time.
[c] Half-cystine determined as *S*-pyridyl-ethylcysteine.
[d] Values were obtained from analysis of 96-hr hydrolysates.
[e] Determined after 24 hr hydrolysis in 4 *N* methanesulfonic acid at 110°C.

A comparison of the amino acid sequence of RMCP II to those of other serine proteases has provided evidence suggesting that the ancestral protease molecule of RMCP II may have evolved some time after that of trypsin but before those of chymotrypsin and elastase.[18] This may be true also for other granulocyte serine proteases, since human neutrophil cathepsin G, RMCPI, and RMCP II show a remarkable degree of amino-terminal sequence identity (Fig. 1). If the degree of sequence identity is maintained throughout the remaining structures of RMCP I and cathepsin G, such homology would provide evidence that the granulocyte serine

proteases have a common evolutionary pathway and together form a characteristic and distinct class of serine proteases.[20]

Substrate Specificity. The chymotrypsin-like esterase activity of RMCP II is significantly lower than that of commercially available (Worthington Biochemical Corporation) bovine chymotrypsin (8.5 BzTyrOEt units/mg for RMCP II as compared to 45 BzTyrOEt units/mg for bovine chymotrypsin). The protease activity of RMCP II is much lower than those of chymotrypsin and RMCP I, particularly when native proteins are used as substrates.[9,36]

Using peptide hormones as substrates, it was found that the protease specificity of RMCP II was more restrictive than that of bovine chymotrypsin.[37] The rat protease preferentially cleaved peptide bonds between hydrophobic residues. This observation is consistent with that of Yoshida *et al.*,[24] who found that the preferred 4-nitroanilide peptide substrates contained hydrophobic residues at both the primary and secondary subsites. The best substrates tested are succinyl-Phe-Pro-Phe-4-nitroanilide and succinyl-Phe-Leu-Phe-4-nitroanilide (k_{cat}/K_m = 36,000 and 30,000 M^{-1} sec^{-1}, respectively). These two substrates are also optimal for RMCP I. This similarity in substrate specificity together with the observation that RMCP II, as well as RMCP I, act on substrates containing a prolyl residue at the P_3 subsite, suggests that these two rat proteases have similar physiological functions. The major enzymatic differences between these proteases are: (1) a shift in optimal activity of RMCP II from pH 8.0 to pH 6.6 toward substrates containing a negatively charged group at the P_4 subsite; and (2) the observation that for each substrate, the catalytic rate of RMCP I is about 50-fold greater than that of RMCP II.[24] The structural features of the proteases that may explain these enzymatic differences have been discussed previously.[16,20]

Inhibitors. In contrast to RMCP I, RMCP II is not inhibited by N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone. The protease is effectively inhibited by extended peptide chloromethyl ketones that contain, in addition to phenylalanine at the primary binding site, hydrophobic residues at one or more subsites.[24] The structures of inhibitors of this type are apparently analogous to those of preferred substrates. Since the substrate specificity of RMCP II (and RMCP I) shows substantive differences compared to those of other chymotrypsin-like serine proteases (e.g., cathepsin G and pancreatic chymotrypsin), it is likely that peptide chloromethyl ketone inhibitors can be designed that should prove uniquely selective for RMCP II.

[36] R. G. Woodbury, unpublished observation.

[37] K. Kobayashi and N. Katunuma, *J. Biochem.* (*Tokyo*) **84**, 65 (1978).

RMCP II is completely inhibited within 15 min by a 2- to 3-fold molar excess of potato chymotrypsin inhibitor, α_1-antitrypsin, hen ovoinhibitor, or lima bean trypsin inhibitor. Soybean trypsin inhibitor inactivates only 70% of the protease activity after 30 min. No inhibition of RMCP II is observed by pancreatic trypsin inhibitor.[35] The inhibition of RMCP II by various proteinase inhibitors is very similar to that observed for RMCP I,[15] again suggesting that the catalytic sites of the two proteases have similar extended binding regions.

Physiological Role

The *in vivo* function of RMCP II is unknown. The atypical (mucosal) mast cells containing the protease have long been recognized to function at some level in the expulsion of intestinal parasites.[38] On the basis of association, one may speculate that the protease also is involved in the expulsion process, perhaps by acting directly on the parasite or by mediating some other function of the mast cell. It is possible that the protease does not promote the general or widespread degradation of connective tissue proteins because those native proteins that have been examined were found to be resistant to the action of RMCP II even when high levels of enzyme are used.[9,36] Since the best synthetic substrates of RMCP II are those containing extended hydrophobic structures,[24] the resistance of native proteins to the action of the protease may be related to the fact that such hydrophobic regions are likely to be restricted to the interior of protein molecules and thus inaccessible to proteolytic attack. Such considerations suggest that the physiological action of RMCP II is highly selective and, perhaps limited to unique sites of the protein substrate.

III. Carboxypeptidase of Peritoneal Mast Cells

Assay Method

Reagents

Benzoyl-glycyl-phenylalanine (BzGlyPhe)
Benzoyl-glycyl-arginine (BzGlyArg)
50 m*M* Tris-HCl buffer, pH 7.5

Procedure. The assay for carboxypeptidase A activity is based on the method of Folk and Shirmer[39] using BzGlyPhe as substrate. Carboxypep-

[38] H. R. P. Miller and W. F. H. Jarrett, *Immunology* **20**, 277 (1971).
[39] J. E. Folk and E. W. Shirmer, *J. Biol. Chem.* **238**, 3884 (1963).

tidase B activity is measured using BzGlyArg as substrate.[40] Enzyme activities are determined spectrophotometrically as follows: extracts (10–100 μl) containing enzyme are added to 3 ml of substrate (1 m*M*) dissolved in 50 m*M* Tris-HCl buffer (pH 7.5). The initial rate of reaction is determined from the change in absorption at 254 nm.

A carboxypeptidase unit is defined as the amount of enzyme that hydrolyzes 1 μmol of substrate per min at 25°C under the conditions described.

Purification Procedure

Mast cells are obtained from Sprague-Dawley rats by washing the peritoneal cavity as described earlier for the preparation of the serine protease RMCP I. The cells are isolated by sedimentation through 30% albumin. The cell pellet consisting of at least 95% mast cells is resuspended in distilled water. Cell lysis releases the secretory granules, which are then purified by differential centrifugation.[2] Extraction of the granules with 0.8 *M* potassium phosphate containing 2% protamine sulfate (pH 8.0) solubilizes carboxypeptidase activity hydrolyzing BzGlyPhe, but not BzGlyArg.

In order to purify substantial amounts of mast cell carboxypeptidase,[11] the peritoneal cavities of 100 rats are lavaged as previously described in this chapter. The pooled washes are centrifuged at 2300 *g* for 15 min in order to collect cells and cellular debris. The pellet is resuspended in distilled water and the solution is frozen and thawed through six cycles to release mast cell granules. This suspension is centrifuged at 2300 *g* for 15 min and the pellet containing the granule-associated carboxypeptidase is extracted with 20 ml of 0.8 *M* potassium phosphate containing 2% protamine sulfate (pH 8.0).

Affinity-Adsorption Chromatography. Potato carboxypeptidase inhibitor is coupled to agarose.[13] A column (1 × 5.5 cm) of this affinity adsorbant is equilibrated with 0.8 *M* potassium phosphate containing 1 mg/ml bovine serum albumin (pH 8.0). The extract of granules containing solubilized carboxypeptidase A activity is passed slowly through the column. The contents of the column are washed with 20 ml of the equilibration buffer containing 1 m*M* phenylmethanesulfonyl fluoride and 0.1 mg/ml lima bean trypsin inhibitor. After washing the column with 2 volumes of 0.1 *M* $NaHCO_3$ (pH 8.0), the carboxypeptidase is eluted with 0.1 *M* Na_2CO_3 containing 0.5 *M* NaCl (pH 11.4). One-milliliter fractions are collected in tubes containing 1 ml of 0.2 *M* Tris-HCl buffer (pH 7.5).

[40] E. Wintersberger, D. J. Cox, and H. Neurath, *Biochemistry* 1, 1069 (1962).

TABLE V
PURIFICATION OF MAST CELL CARBOXYPEPTIDASE A[a]

Step	Volume (ml)	Total activity (BzGlyPhe units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification factor (-fold)
Crude extract	20	81.3	245	0.33	100	1
Affinity-adsorption chromatography	10	31.5	4.18	7.54	39	23

[a] From the peritoneal cells of 100 rats.

Fractions containing significant carboxypeptidase activity are pooled. A typical purification protocol of the preparation of the enzyme from 100 rats (Table V) yields a preparation containing approximately 4 mg of protein and about 40% of the initial carboxypeptidase A activity present in the extract of granules.

Properties

Purity. The preparation of mast cell carboxypeptidase by this procedure is homogeneous on the basis of polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate, 8 *M* urea, and 2-mercaptoethanol. Also, amino-terminal sequence analysis indicates that the protein contains a single polypeptide chain.[41]

Stability. Preparations of homogeneous carboxypeptidase are relatively stable at 4°C or lower temperatures. However, during the purification, the enzyme is rapidly degraded by the mast cell serine protease (RMCP I) unless preventive measures are taken. Thus it is essential to carry out all steps quickly at 4°C and to add an inhibitor of RMCP I, e.g., lima bean-trypsin inhibitor.

Specific Activity. The purified mast cell carboxypeptidase A has a specific activity of 7.5 BzGlyPhe units/mg enzyme, which is significantly lower than that of commercially obtained (Worthington Biochemical Corporation) bovine pancreatic carboxypeptidase A (approximately 35 units/mg enzyme). The affinity-adsorption step results in a 60% loss of the original enzyme activity perhaps due, in part, to a rapid loss ($t_{1/2}$ = 1 hr) of activity when the enzyme is in the elution buffer at pH 11.4.[11] It is therefore possible that the enzyme preparation consists of both active and inactive molecules of carboxypeptidase.

Physical and Chemical Properties. The enzyme migrates during electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate and 2-mercaptoethanol as a single band. The apparent molecular weight is approximately 35,000.

Comparison of the amino acid composition of mast cell carboxypeptidase A and bovine carboxypeptidases A and B (Table VI) indicates a general similarity of the mast cell enzyme to both bovine enzymes. Despite its carboxypeptidase A specificity, however, the mast cell enzyme resembles more closely bovine carboxypeptidase B in its half-cystine and methionine content. In fact, the odd number of half-cystine residues of the mast cell enzyme suggests that it contains a free sulfhydryl group, which is analogous to bovine carboxypeptidase B, but unlike bovine carboxypeptidase A. A major chemical difference between the mast cell and

[11] M. T. Everitt, Doctoral Dissertation, University of Washington, Seattle (1980).

TABLE VI
AMINO ACID COMPOSITIONS[a] OF RAT MAST CELL CARBOXYPEPTIDASE AND BOVINE PANCREATIC CARBOXYPEPTIDASES

Amino acid	Mast cell carboxypeptidase	Bovine enzymes	
		Carboxypeptidase A	Carboxypeptidase B
Aspartic acid	30	29	28
Threonine[b]	19	26	27
Serine[b]	28	32	27
Glutamic acid	18	25	25
Proline	15	10	12
Glycine	19	23	22
Alanine	17	21	22
Half-cystine[c]	5	2	7
Valine	15	16	14
Methionine	7	3	6
Isoleucine[d]	20	21	16
Leucine[d]	21	23	21
Tyrosine	14	19	22
Phenylalanine	13	16	12
Histidine	9	8	7
Lysine	27	15	17
Arginine	16	11	13
Tryptophan[e]	9	7	8
Total residues	306	307	306

[a] Duplicate samples were analyzed after 24, 48, 72, and 96 hr of hydrolysis in 6 *N* HCl at 110°C.
[b] Determined by extrapolation to zero-hydrolysis time.
[c] Determined as cysteic acid after performic acid oxidation.
[d] Values were obtained from analysis of 96-hr hydrolysates.
[e] Determined after alkaline hydrolysis (48 hr).

bovine carboxypeptidases is the presence of nearly twice as much lysine in the mast cell enzyme, which may be involved in the binding to the highly acidic heparin and may account for the necessity of using solutions of high ionic strength to extract the enzyme from mast cell granules.

Immunofluorescent localization studies using specific antisera directed toward mast cell carboxypeptidase, with the total cell population of peritoneal washes show positive staining only of mast cell granules.[41] Similar studies using this antiserum indicated no fluorescence associated with the atypical mast cells of the small intestine.

Substrate Specificity. Purified mast cell carboxypeptidase hydrolyzes BzGlyPhe, but not BzGlyArg; hence its designation as a carboxypep-

tidase A. In a more detailed examination of the substrate specificity of the mast cell enzyme, it was observed that for several dipeptide substrates the kinetic parameters were similar to those of bovine carboxypeptidase A.[11]

Inhibitors. Mast cell carboxypeptidase is inhibited by 1,10-phenanthroline and by mercurials, as well as by potato carboxypeptidase inhibitor, suggesting that, in analogy with the pancreatic carboxypeptidases, the mast cell enzyme is also a metalloenzyme. Diisopropyl phosphorofluoridate, phenylmethanesulfonyl fluoride, and lima bean trypsin inhibitor are without effect.

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[46] γ-Subunit of Mouse Submaxillary Gland 7 S Nerve Growth Factor: An Endopeptidase of the Serine Family

By KENNETH A. THOMAS and RALPH A. BRADSHAW

Introduction

Nerve growth factor (NGF) is a hormone-like protein that is required for both maintenance of, and neurite outgrowth from, sympathetic and certain sensory neurons.[1] Cells from tissues that are innervated by NGF-responsive neurons *in vivo* secrete this substance in culture,[2] and it is presumed that these tissues are the physiologically relevant sources of the factor for its endocrine activities *in vivo*.[3] In addition, a number of exocrine tissues and their secretions—including the submaxillary gland[4] and saliva of male mice[5,6]; the prostate and semen of ox, guinea pig, sheep, and goats[7,8]; and the venom of poisonous land snakes[9]—have very high

[1] R. Levi-Montalcini and P. U. Angeletti, *Physiol. Rev.* **48**, 534 (1968).
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[8] G. P. Harper, *Abstr. 7th Meet., Int. Soc. Neurochem.* p. 9 (1979).
[9] R. A. Hogue-Angeletti and R. A. Bradshaw, *Handb. Exp. Pharmacol.* [N.S.] **52**, 276 (1977).

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