RAT PERITONEAL MAST CELL CARBOXYPEPTIDASE: LOCALIZATION, PURIFICATION, AND ENZYMATIC PROPERTIES

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Received 6 December 1979

1. Introduction

Mast cells are secretory cells distributed throughout mammalian connective tissues. Activation of rat mast cells with secretogogues results in the release by mature cells of dense, metachromatic secretory granules, which contain large amounts of histamine, serotonin, heparin, and a variety of proteins including a protease with chymotrypsin-like specificity [1]. Recently the presence of a carboxypeptidase was reported in mast cells and mast cell containing tissues [2–4]. The carboxypeptidase hydrolyzed an ester substrate, hippuryl-L-β-phenylactate [5], and a number of dipeptides with carboxyl terminal aromatic residues, e.g., Z-AlaPhe [2]. High ionic strength was required for solublization and the enzyme was inhibited by *o*-phenanthroline [2,5] and mercurials [2]. Estimates of molecular weight ranged from 34 500 [2] to 43 000 [4]. The enzyme was stable prior to extraction [5], but after extraction it was rapidly degraded by the mast cell chymotrypsin [2]. In general, these reports suggested that the enzyme was similar to bovine carboxypeptidase A, but neither the precise subcellular location nor the extent of similarity to bovine carboxypeptidase A, were convincingly documented. We now present data concerning the site of carboxypeptidase storage in mast cells, the isolation of the enzyme by affinity chromatography, and a comparison of several structural and enzymatic properties with bovine carboxypeptidases A and B.

Abbreviations: DFP diisopropylphosphorofluoridate; MES, 2-(*n*-morpholine) ethanesulfonic acid; Tris–HCl, tris-(hydroxymethyl)aminomethane hydrochloride; Z, benzyloxycarbonyl; SDS, sodium dodecyl sulfate

2. Materials and methods

Sprague-Dawley rats were obtained from Tyler Labs (Bellevue, WA). Potato carboxypeptidase inhibitor [6] and carboxypeptidase inhibitor–agarose [7] were generous gifts of Dr G. Michael Hass.

Enzymatic activity was determined spectrophotometrically using 1 mM BzGlyPhe as substrate for carboxypeptidase A [8] and 1 mM BzGlyArg for carboxypeptidase B [9]. Substrate (3 ml) in 0.05 M Tris–HCl containing 0.5 M NaCl (pH 7.5) was added to the enzyme (10–100 μl) and the initial rate of reaction determined from the A_{254} increase. Esterase activity was measured in a similar manner using hippuryl-L-β-phenyllactate (BzGlyOPhe) as substrate [10]. The hydrolysis rates of other *N*-acylated dipeptides were determined by monitoring the A_{224} decrease [11]. Activity of the chymotrypsin-like protease was assayed with 1 mM BzTyrOEt as in [12]. For measurements of the pH dependence of enzymatic activity, MES buffer was used at pH 5–7, phosphate buffer at pH 6–8, Tris–HCl at pH 7–9, ammonia at pH 8–10 and glycine/NaOH at pH 9–11.

Mast cells were isolated from peritoneal lavage fluid by sedimentation through 30% albumin. Secretory granules were separated from degranulated and partially degranulated cells by differential centrifugation after agitation of purified cells in distilled water [13].

SDS–polyacrylamide gel electrophoresis was done in the presence of 8 M urea as in [12]. Amino acid analyses were done on a Durrum amino acid analyzer (D-500) by the methods in [14,15].

3. Results

3.1. *Localization of carboxypeptidase activity*

3.1.1. Assay of peritoneal lavage fluid

To collect cells and cellular debris, peritoneal lavage fluid was centrifuged at 2300 × *g* for 15 min. The precipitate was frozen and thawed through 6 cycles to release the cellular contents. The soluble material was removed by centrifugation at 2300 × *g* and the precipitate was extracted with 0.8 M potassium phosphate, 2% protamine sulfate (pH 8.0). Fractions from each step were assayed for carboxypeptidase A and B activity. No hydrolysis of BzGlyArg was noted in any of the samples. Significant levels of hydrolysis of BzGlyPhe were found only in the 0.8 M phosphate extract.

3.1.2. Localization to mast cell secretory granules

Mast cells and mast cell secretory granules were prepared as in section 2. Carboxypeptidase activity (65%) was found in the fraction containing purified mast cell granules. The remaining activity (35%) was located in the pellet of partially degranulated cells (av. 3 determinations). The proportion of the carboxypeptidase in the fractions was essentially identical to the proportion of the granule associated chymotrypsin-like protease measured in each fraction.

3.1.3. Inhibition of mast cell proteases

In order to verify that the carboxypeptidase and chymotrypsin-like activities are not catalyzed by the same enzyme, the effects of a variety of specific inhibitors were investigated. Carboxypeptidase activity was inhibited by either *o*-phenanthroline or by the carboxypeptidase inhibitor from potatoes [16], while 0.1 mM DFP or lima bean trypsin inhibitor had no effect. In contrast, the chymotrypsin-like protease was not inhibited by *o*-phenanthroline or the potato carboxypeptidase inhibitor, but was completely inhibited by 0.1 mM DFP and lima bean trypsin inhibitor. These results strongly suggest that the 2 protease activities are due to 2 separate enzymes.

3.2. *Purification of mast cell carboxypeptidase*

3.2.1. Affinity chromatography on potato carboxypeptidase inhibitor–agarose

The column (1 × 5.5 cm) was equilibrated with 100 ml 0.8 M potassium phosphate containing 1 mg/ml bovine serum albumin (pH 8.0). Peritoneal cell extract in 0.8 M potassium phosphate containing 2% protamine sulfate (pH 8.0) was applied to the column followed by 20 ml equilibration buffer containing 1 mM phenylmethanesulfonyl fluoride and 0.1 mg/ml lima bean trypsin inhibitor. The column was washed with 2 vol. 0.1 M $NaHCO_3$ (pH 8.0). Carboxypeptidase activity was found in fractions eluted with 0.1 M Na_2CO_3 containing 0.5 M NaCl (pH 11.4) [11]. Active fractions were pooled and adjusted to pH 8.0 to stabilize the activity.

A summary of the purification is shown in table 1. The final yield of active carboxypeptidase was 39% with a 23-fold purification based on specific activity. The enzyme lost activity rapidly ($t_{1/2}$ = 1 h) under the elution conditions (pH 11.4) and losses were minimized by collecting 1 ml fractions in tubes containing 1 ml 0.2 M Tris–HCl (pH 7.5). For subsequent studies, the protein could be concentrated by precipitation with acetone (80%) to yield an easily solublized material. Treatment of the enzyme with trichloroacetic acid produced an oily precipitate which was soluble only in 90% formic acid (60°C).

Table 1
Purification of mast cell carboxypeptidase from the peritoneal cells of 100 rats

Purification step	Vol. (ml)	Total prot. (mg)	Total act. (units)[a]	Spec. act. (units/mg)	Recovery (%)	Purification (-fold)
Extract	20	245	81.3	0.33	100	1
Affinity column eluate	10	4.18	31.5	7.54	39	23

[a] One carboxypeptidase unit is defined as the amount of enzyme required to hydrolyze 1 μmol BzGlyPhe/min at 25°C under the conditions described

Table 2
Amino acid compositions of rat peritoneal mast cell carboxypeptidase and bovine pancreatic carboxypeptidases

Amino acid	Mast cell carboxypeptidase		Carboxypeptidase A	Carboxypeptidase B
		mol amino acid	mol amino acid	mol amino acid
	(g/100 g protein)	mol protein	mol protein	mol protein
Alanine	(3.8)	17	21	22
Arginine	(6.9)	16	11	13
Aspartic/NH_2	(10.0)	30	29	28
Glutamic/NH_2	(6.5)	18	25	25
Glycine	(3.6)	19	23	22
Half-cystine[a]	(1.4)	5	2	7
Histidine	(3.5)	9	8	7
Isoleucine	(6.5)	20	21	16
Leucine[b]	(7.0)	21	23	21
Lysine	(9.7)	27	15	17
Methionine	(2.6)	7	3	6
Phenylalanine	(5.6)	13	16	12
Proline	(4.3)	15	10	12
Serine[c]	(7.4)	28	32	27
Threonine[c]	(5.7)	19	26	27
Tryptophan[d]	(4.6)	9	7	8
Tyrosine	(6.6)	14	19	22
Valine	(4.4)	15	16	14
Total residues		306	307	306

[a] Determined as cysteic acid after performic acid oxidation
[b] Value after 96 h hydrolysis
[c] Value after extrapolation to zero time of hydrolysis
[d] Determined on the amino acid analyzer after alkaline starch hydrolysis [12]

3.2.2. *Characterization of purified mast cell carboxypeptidase*

The purity of the purified mast cell carboxypeptidase was examined by electrophoresis in SDS–8 M urea in the presence of 2-mercaptoethanol. A single band was observed corresponding in mobility to app. mol. wt 35 000 for the reduced enzyme. In the absence of reducing agent, the enzyme displayed an increased mobility and a second minor band was visible with app. mol. wt 63 000.

The amino acid composition of purified mast cell carboxypeptidase is given in table 2. When compared with bovine carboxypeptidases A and B the mast cell enzyme contains a higher proportion of basic residues than either bovine enzyme with an overall composition midway between the two. The mast cell enzyme appears to contain an odd number of half-cystine residues, analogous to bovine carboxypeptidase B.

3.3. *Enzymatic properties*

3.3.1. Kinetics of ester and peptide hydrolysis

Specificity studies, presented in table 3, using a series of N-substituted dipeptides indicate that the mast cell carboxypeptidase resembles in its action bovine carboxypeptidase A. In most cases the catalytic efficiencies (k_{cat}/K_m) of the two enzymes toward a given substrate differ by a factor of <2. The best substrate examined as noted [2] is Z-AlaPhe. Comparison of the rates of hydrolysis of BzGlyPhe and Z-GlyPhe shows a reduction in k_{cat} with a minimal change in K_m for the mast cell enzyme, while in the case of bovine carboxypeptidase A, K_m is increased 2-fold with only a minor increase in k_{cat}. Neither enzyme hydrolyzed the substrate containing a penultimate prolyl residue. Excess substrate inhibition was observed for the hydrolysis of BzGlyOPhe by the

Table 3
Comparison of the kinetic parameters of the hydrolysis of various substrates by mast cell carboxypeptidase and bovine carboxypeptidase A

Substrate[a]	Mast cell carboxypeptidase			Bovine carboxypeptidase A		
	K_m (10^3 M)	k_{cat} (s^{-1})	k_{cat}/K_m	K_m (10^3 M)	k_{cat} (s^{-1})	k_{cat}/K_m
BzGlyPhe[b]	0.45	16	36	0.83	45	54
Z-GlyPhe[c]	0.31	4.2	14	1.8	52	29
Z-AlaPhe[c]	0.14	89	640	0.38	79	210
Z-ProPhe[c]	No hydrolysis			No hydrolysis		
Z-PheLeu[c]	0.23	66	290	0.24	113	470
Hippuryl-L-β-phenyllactic acid[b]	0.062	454	7300	0.13	466	3600

[a] 2.4–9.7 × 10^{-4} M substrate in 0.05 M Tris–HCl, 0.5 M NaCl (pH 7.5)
[b] Monitored spectrophotometrically at 254 nm
[c] Monitored spectrophotometrically at 224 nm

mast cell carboxypeptidase. This finding is similar to that in [7] for the bovine enzyme.

3.2.2. *pH dependence of peptide and ester hydrolysis*

Figure 1 shows the pH dependency of hydrolysis of BzGlyPhe and BzGlyOPhe. The peptidase activity shows a relatively sharp maximum at pH 8.0 whereas esterase activity has a broader profile with a plateau (>90% optimal activity) over pH 7.0–8.5.

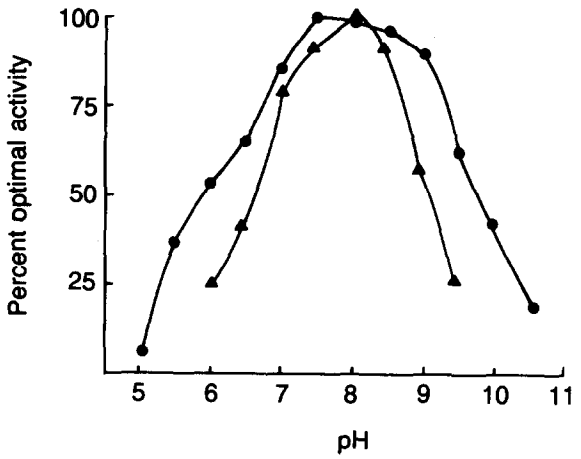

Fig.1. pH Dependence of peptidase and esterase activities of rat mast cell carboxypeptidase. Activities are expressed as % of the maximal rates of hydrolysis. (▲——▲) Peptidase activity determined with BzGlyPhe; (●——●) esterase activity observed with BzGlyOPhe. At pH >9.5 corrections for base-catalyzed hydrolysis of the ester substrate were applied.

4. Discussion

The purification described here is both simple and efficient. Starting with frozen peritoneal cells, only 1 day is required to produce mg quantities of enzyme in high yield. Although analytical gel electrophoresis shows a second, minor band in samples of non-reduced enzyme, the loss of this band after reduction suggests that oxidation of the free sulfhydryl groups on 2 molecules of carboxypeptidase may be occurring. Formation of intrachain disulfide bonds would result in dimers of 60 000–70 000 mol. wt.

Rat peritoneal mast cell carboxypeptidase resembles the bovine pancreatic carboxypeptidases in both chemical and enzymatic properties. The kinetic parameters, K_m and k_{cat}, of the mast cell enzyme for several characteristic substrates are nearly identical with those of bovine carboxypeptidase A. The enzymes have similar pH optima, in the alkaline region, for peptide and ester hydrolysis [17]. The proteases are inactivated by the metal-chelating agent *o*-phenanthroline and by potato carboxypeptidase inhibitor. In addition their molecular weights are essentially the same, i.e., 35 000, and their amino acid compositions show distinct similarities.

Although the mast cell enzyme contains at least 1 free sulfhydryl residue, there is no convincing evidence that such a residue is part of the active site as suggested in [2]. Both bovine carboxypeptidase A which contains no sulfhydryl group [18,19] and carboxypeptidase B which contains 1 [20] are

inactivated by mercury and by a variety of mercurials. The crystal structures of these enzymes [21,22] reveal no evidence for the presence of a sulfhydryl group in the active site or for its role in the enzymatic mechanism. In fact, the inhibition of the mast cell carboxypeptidase by both *o*-phenanthroline and the carboxypeptidase inhibitor from potatoes fits the criteria of a zinc metalloexopeptidase [23].

At present little is known concerning the physiological roles of the mast cell carboxypeptidase and mast cell chymotrypsin. Because the 2 enzymes have complementary specificities, they could function in tandem, as proposed for lysosmal carboxypeptidase A/cathepsin D [24], pancreatic carboxypeptidase A/chymotrypsin, and pancreatic carboxypeptidase B/trypsin. However, the true physiological substrate of the mast cell carboxypeptidase remains unidentified.

Acknowledgements

The authors wish to thank Dr G. Michael Hass for his generous gifts of potato carboxypeptidase inhibitor and potato carboxypeptidase inhibitor–agarose. We thank Drs David Lagunoff and Kenneth A. Walsh for valuable discussion, and Mr Richard Granberg for expert technical assistance. This work has been supported by the National Institutes of Health (GM-15731).

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