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Purification of an Atypical Mast Cell Protease and Its Levels in Developing Rats†

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ABSTRACT: A chymotrypsin-like serine protease produced by atypical mast cells in gut mucosa of rats was purified by a method involving affinity adsorption chromatography on potato chymotrypsin inhibitor I immobilized on Sepharose. The protease was shown by chemical, physical, enzymatic, and immunologic criteria to be identical with a protease isolated from small intestine by a method previously described (Katunuma, N., et al. (1975) *Eur. J. Biochem. 52*, 37–50). The levels of the protease in small intestine were determined in developing rats by a radial immunodiffusion assay. Protease levels were relatively low in suckling rats, but rapidly increased approximately fivefold 2 to 3 weeks after weaning and thereafter decreased to adult levels. The atypical mast cell population of small intestine followed a similar pattern. There was a maturation of existing cells as well as an increase in their numbers prior to the period of rapid protease production. A dramatic increase in the immunoglobulin A levels coincident with the increase in both protease and mast cell contents of the small intestine suggests that the enzyme and the cell may be involved in the first active immune response at the mucosal surface. The levels of ornithine aminotransferase, purported to be a substrate of the protease in vivo, were disproportionately low compared with those of the protease in adult rats and showed no correlation to the protease levels in developing rats. These observations are inconsistent with the concept that the protease initiates the degradation of several intracellular pyridoxal phosphate dependent enzymes in vivo.

Katunuma & co-workers (1971) isolated from rat small intestine a chymotrypsin-like serine protease of restricted specificity and proposed that this protease initiated the degradation of several intracellular pyridoxal phosphate dependent enzymes such as ornithine aminotransferase (OAT[1]) (Katunuma et al., 1971, 1975). The apo forms of these enzymes are susceptible to the action of the protease, but not the holoenzymes nor the apo forms of enzymes requiring other cofactors. Because of its limited and apparently selective action, the protease was termed "group-specific protease".

Recently, it was determined by immunofluorescent methods that the protease is located in "atypical" mast cells of the intestinal mucosa (Woodbury et al., 1978a). Such cells, containing the protease, also were observed beneath the epithelium of the bronchioles in lung. The enzyme was shown to be similar to, but distinct from, the previously known chymotrypsin-like protease of rat peritoneal mast cells (Benditt & Arase, 1959). To distinguish it from the newly discovered enzyme, the latter protease is designated rat mast cell protease I (RMCP I), and the present one rat mast cell protease II (RMCP II).

The cell-specific location of RMCP II suggests that it is a secretory enzyme. In light of this, it is appropriate to reconsider the proposal that the protease plays a role in the degradation of intracellular enzymes. In the current study, we have examined the quantitative relationship of the protease to its putative substrate, ornithine aminotransferase in both adult and developing rats. In addition, a new method employing affinity adsorption chromatography is described which permits rapid and efficient purification of the protease.

Experimental Section

Materials

A small amount (10 mg) of the protease was prepared from the thoroughly washed small intestines of 50 adult, female, outbred white rats (Tyler Laboratory, Bellevue, Wash.) as previously described (Katunuma et al., 1975) with the exceptions that benzamidine (5 mM) was added to all buffers and DEAE-Sephacel was substituted for DEAE-cellulose.

Ornithine aminotransferase was purified from the livers of 50 rats maintained on a high protein (60% casein) diet for 7 days (Peraino et al., 1969).

Chicken ovoinhibitor and human α_1-antitrypsin were gifts from H. Ako (University of Hawaii). Soybean, pancreatic and

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[1] Abbreviations used: OAT, ornithine aminotransferase; RMCP I, rat mast cell protease I; RMCP II, rat mast cell protease II; P.I.-1, potato inhibitor 1; Tos-Lys-CH_2Cl, *N*-α-*p*-tosyl-1-lysine chloromethyl ketone; Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; TosArgOMe, tosylarginine methyl ester; BzTyrOEt, benzoyltyrosine ethyl ester; DFP, diisopropyl phosphorofluoridate; DIP, diisopropylphosphoryl; Pth, phenylthiohydantoin.

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lima bean trypsin inhibitors (crystalline) were obtained from Worthington Biochemical Corp. Subunit C of potato chymotrypsin inhibitor I (P.I.-1) was purified as described by Melville & Ryan (1972).

Anti-RMCP II sera were prepared by injecting into rabbits (New Zealand Whites) at 4-week intervals, 50–100 μg of the protease purified by the method of Katunuma et al. (1975) following an initial intramuscular inoculation of antigen with complete Freund's adjuvant. The specificity of the antisera was determined by immunodiffusion tests. Rabbit anti-rat IgA serum was purchased from Miles Laboratories.

Tosyl arginine methyl ester (TosArgOMe) and benzoyltyrosine ethyl ester (BzTyrOEt) were from Worthington Biochemical Corp. *N*-α-*p*-Tosyl-L-lysine chloromethyl ketone (Tos-LysCH_2Cl) and L-1-tosylamido-2-phenylethyl chloromethyl ketone (Tos-PheCH_2Cl) were obtained from Sigma Chemical Co. [^{14}C]Diisopropyl phosphorofluoridate (DFP) was purchased from New England Nuclear.

All other reagents were of the highest grade available from commercial sources.

Methods

The apo form of OAT was prepared by dialyzing a solution of purified enzyme (10 mg/mL) against several changes of ice-cold 50 mM ornithine, pH 7.6, followed by further dialysis against several changes of ice-cold 50 mM potassium phosphate buffer, pH 6.6 (Katunuma et al., 1975). Typical preparations contained less than 5% residual holoenzyme. Complete OAT activity was regenerated by adding an excess of pyridoxal phosphate to the apoenzyme. Apo-OAT was stored at −20 °C for over 2 months without significant loss of recoverable OAT activity.

The inactivation of apo-OAT by RMCP II was ascertained by the method of Katunuma et al. (1975). A unit of protease activity is defined as the amount of RMCP II which will produce a 50% decrease in OAT activity (0.1 mg of OAT) in 30 min at 37 °C relative to a control assay containing protease plus holo-OAT. Ornithine aminotransferase activity, after reconstituting any remaining native enzyme, was measured by the method of Jenkins & Tsai (1970).

The relative effectiveness of several protease inhibitors toward RMCP II was examined by adding them (two- to threefold molar excess each) to the protease solution (1–2 units) 15 min prior to the OAT inactivation assay. Remaining OAT activities were determined after 15 and 30 min.

Subunit C of potato chymotrypsin inhibitor I was covalently coupled to Sepharose 4B as described by Cuatrecasas (1970). Twenty milliliters of the affinity resin bound approximately 30 mg of bovine α-chymotrypsin.

To prepare RMCP II for affinity adsorption chromatography, the homogenate of 50 washed small intestines was carried through the acetone fractionation step as described by Katunuma et al. (1975). Benzamidine (5 mM) was included in all buffers to inhibit trypsin if present. Trypsin and chymotrypsin activities were measured according to the methods of Hummel (1959).

The partially purified protease in 50–75 mL of 0.1 M Tris-HCl buffer, pH 7.8, containing 1 M NaCl was incubated with 1 mM Tos-PheCH_2Cl and 1 mM Tos-LysCH_2Cl for 1 h at room temperature and then passed over a 20-mL column of P.I.-1 immobilized on Sepharose. The column contents were washed with several bed volumes of the initial buffer, followed by several volumes of distilled water. The protease was desorbed from the resin with either 0.1 M acetic acid or 1 mM HCl. The eluate was monitored at 280 nm. Peak fractions were assayed for enzyme activity and the appropriate pools were made. Pools were immediately dialyzed against 0.05 M Tris-HCl buffer, pH 7.8, or lyophilized. If the protease binding capacity of the column was exceeded, the unbound enzyme was rechromatographed.

Amino acid analyses were performed on a Durrum Model D-500 amino acid analyzer. Duplicate samples were hydrolyzed in 6 N HCl at 110 °C for 24 h. Tryptophan and half-cystine were not determined.

Automatic sequence analyses were performed on a Beckman Sequencer Model 980B by the method of Edman & Begg (1967) as modified by Hermodson et al. (1972). Pth-amino acids were identified by gas chromatography.

Gel electrophoresis in the presence of sodium dodecyl sulfate and dithiothreitol was carried out by the method of Weber & Osborn (1969).

Double immunodiffusion was performed to determine if the protease isolated by affinity adsorption chromatography cross-reacted with antiserum directed toward protease prepared as described by Katunuma et al. (1975).

To determine the levels of intestinal protease and OAT in developing rats, segments (20 cm) were removed from the midsection of small intestines from freshly killed rats (1 to 16 weeks old). The entire small intestine was used in the case of very young rats. Intestines were cut open longitudinally, washed thoroughly with ice-cold 0.15 M NaCl, gently blotted on clean paper towels to remove excess fluid, weighed, and homogenized in 3 volumes of ice-cold 0.15 M KCl containing 0.1 mM pyridoxal phosphate. Levels of RMCP II were determined by radial immunodiffusion assay. Optimal assay conditions were obtained by adding 0.2 mL of anti-RMCP II serum to 9 mL of warm (55–60 °C) liquid agar (1%) in 0.1 M potassium phosphate buffer, pH 7.8, containing 0.01% sodium azide. An assay gel (85 × 75 × 1 mm) was prepared from this solution containing 30 sample holes (3 mm in diameter). Samples (5 μL) of tissue homogenates were applied in duplicate as were appropriate standards of purified protease. Precipitin rings were allowed to develop overnight at room temperature and their diameters were determined to within 0.1 mm. Similarly, the relative levels of IgA were estimated in the homogenates. Activities and concentrations of OAT were measured as indicated by Jenkins & Tsai (1970). Protein concentration was determined by the method of Lowry et al. (1951).

A piece (5 mm) of small intestine from each rat was fixed in ice-cold Carnoy's fluid (Lillie & Fullmer, 1976) for 1 h, dehydrated in cold ethanol overnight, embedded in paraffin, and sectioned (4 μm thick). Sections were rehydrated and stained with alcian blue and safranin (Enerbäck, 1966) to visualize "atypical" mast cells. The stained sections were examined with an Olympus Vanox microscope and the average mast cell population per intestinal villous-crypt unit (Miller & Jarrett, 1971) was determined for a minimum of 30 units for each age group.

In another study 3 adult female rats (16 weeks) were killed, their intestines were removed and thoroughly washed, and each was cut into 10 equal segments. Segments from similar regions were weighed, pooled, and homogenized in 3 volumes of ice-cold 0.15 M KCl containing 0.1 mM pyridoxal phosphate. The homogenates were assayed for protease (radial immunodiffusion) and OAT activity.

Results

Inhibition of RMCP II. To determine which protease inhibitors would be appropriate in preparing an affinity adsorption resin, the effectiveness of several inhibitors toward RMCP II, obtained by the method of Katunuma et al. (1975),

TABLE I: Relative Effectiveness of Inhibitors toward RMCP II.[a]

inhibitor	% inhibition 15 min	30 min
potato chymotrypsin inhibitor I (PI-1)	100	100
α_1-antitrypsin	99	100
lima bean inhibitor	98	100
ovoinhibitor (chicken)	97	83
soybean trypsin inhibitor	86	70
pancreatic trypsin inhibitor	0	0
Tos-PheCH$_2$Cl (mM)	0	0
Tos-LysCH$_2$Cl (mM)	0	0
benzamidine (5 mM)	0	0
DFP (0.5 mM)	100	100

[a] The effectiveness of inhibitors toward RMCP II was determined by preincubating them (two- to threefold molar excess) with protease for 15 min prior to carrying out the OAT-inactivation assay. Remaining OAT activities were measured after 15 and 30 min in the presence of inhibitor/protease mixtures.

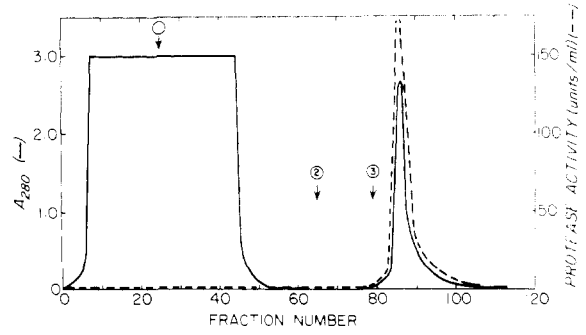

FIGURE 1: Affinity adsorption chromatography of RMCP II on potato inhibitor I immobilized on Sepharose 4B. The crude homogenate of the small intestines from 50 adult rats was carried through the acetone fractionation step as described by Katunuma et al. (1975). The column contained 20 mL of the affinity resin. (1) Elution of nonbinding material with 0.1 M Tris-HCl buffer, pH 7.8, containing 1 M NaCl; (2) column was eluted with distilled water to remove buffer and salt; (3) elution of bound material with 0.1 M acetic acid (1 mM HCl can also be used). Fractions (2.0 mL) were monitored at 280 nm for protein (—) and for proteolytic activity toward apo-OAT (- - -). Peak fractions of the material eluted by 0.1 M acetic acid had similar specific activities and were pooled.

was evaluated (Table I). Potato chymotrypsin inhibitor, α_1-antitrypsin, and lima bean trypsin inhibitor completely inhibited the action of the protease toward apo-OAT. Ovoinhibitor initially inhibited nearly completely proteolysis but as the assay continued there was a significant loss in OAT activity. Soybean trypsin inhibitor was even less effective, consistent with the observation of Katunuma et al. (1975). Apparently the complex of this inhibitor, and to a lesser extent that of the ovoinhibitor, with RMCP II dissociated at a significant rate. Pancreatic trypsin inhibitor had no effect toward the protease. [^{14}C]DFP (0.5 mM) completely inhibited 2 units of protease within 15 min. The molar ratio of DIP/protease was 1.0 using a molar extinction coefficient for RMCP II of $E_{280}^{1\%} = 11.0$ as calculated from a molecular weight of 24 655 (Woodbury et al., 1978b) and protein concentrations determined by the method of Lowry et al. (1951). Both Tos-PheCH$_2$Cl and Tos-LysCH$_2$Cl were ineffective as inhibitors of protease activity as was benzamidine. Titration of the protease with P.I.-1 or α_1-antitrypsin indicated that 1 mol of inhibitory sites bound 1 mol of RMCP II. No attempt was made to determine the K_i of inhibitors toward RMCP II.

Affinity Adsorption Chromatography of RMCP II. Although trypsin activity (using TosArgOMe as substrate) could not be detected in crude homogenates of small intestine, activity (representing 2 to 5 μg trypsin) frequently was observed after the ammonium sulfate precipitation or acetone fractionation steps. For this reason, tissue was homogenized in the presence of 5 mM benzamidine. Trypsin, if present, copurified with the intestinal protease through the affinity adsorption step. In order to inhibit any trypsin or chymotrypsin, Tos-LysCH$_2$Cl (1 mM) and Tos-PheCH$_2$Cl (1 mM) were added to the preparation prior to its passage over the affinity resin. It was important that all trypsin activity be inhibited since a few micrograms of this enzyme rapidly inactivated purified RMCP II. Further, trypsin mimicked the "group-specific" action of the intestinal protease by inactivating apo-OAT but not holo-OAT. Since the specific activity of trypsin toward apo-OAT was much greater than that of RMCP II, trace amounts of contaminating trypsin resulted in significant overestimation of RMCP II levels in partially purified preparations. Bovine chymotrypsin also was found to inactivate apo-OAT at a much faster rate than holo-OAT.

The elution pattern resulting from the affinity adsorption chromatography of partially purified protease is shown in Figure 1. Usually none of the protease was eluted by the Tris-HCl buffer. At least 90% of the protease applied to the column was eluted by 0.1 M acetic acid or more gradually by 1 mM HCl. Since the desorbed protease precipitated at acidic pH in the presence of NaCl, it was necessary to wash the column free of buffer and salt before collecting the protease. To regenerate the column the remaining protease (approximately 5–10% of the total) was eluted in denatured form by applying 7 M urea in 0.1 M formic acid. The column was then washed with Tris-HCl buffer, pH 7.8, containing 1 M NaCl.

The specific activity of the protease was essentially the same in all peak fractions and that of the pooled material (80 units/mg of protein) was in good agreement with that reported by Katunuma et al. (1975). The results of a typical preparation are summarized in Table II, together with a similar summary reported in part by Katunuma et al. (1975). If the intestines were not stripped of the upper half of the mucosa, the amount of protease obtained was even greater. Gentle stripping of this layer, however, removes much of the thick mucus which, when present, makes the purification more difficult.

Twenty milliliters of freshly prepared affinity adsorption resin completely adsorbed 25–30 mg of protease. After repeated use (5–10 preparations), the binding capacity gradually decreased to less than half this level. In addition, small amounts of contaminating proteins were adsorbed by the older resin.

A portion of the pooled, lyophilized protease obtained by the affinity adsorption procedure was subjected to gel electrophoresis in the presence of sodium dodecyl sulfate and dithiothreitol. Most preparations contained only a single component with a mobility identical with that of RMCP II. Occasionally, small amounts (5–10% of the total) of material of lower molecular weight were observed, probably due to autodigestion of some enzyme susceptible to denaturation. If the pH of the protease solution was not adjusted to neutrality, much of the protease underwent autodigestion within a few weeks at 4 °C. Otherwise, RMCP II solutions were stable at 4 °C for several months provided microbial growth was prevented.

No tryptic or chymotryptic-like activity inhibitable by Tos-PheCH$_2$Cl was detected in purified RMCP II preparations.

The relative amino acid content (Table III) of RMCP II prepared by the present method is nearly identical with that of protease prepared by the method of Katunuma et al. (1975).

TABLE II: Purification of RMCP II by Affinity Adsorption Chromatography.[a]

purification step	total protein (mg)	protease units	units/mg of protein	% yield
crude homogenate	4366 (8889)	3830 (21 250)	0.9 (2.4)	100 (100)
ammonium sulfate (65%)	2264 (4419)	2930 (34 000)	1.4 (7.7)	77 (160)
acetone (55%)	590 (1572)	2210 (29 460)	3.8 (18.7)	58 (139)
affinity adsorption	25	2000	80	52
(DEAE-cellulose)	(236)	(8824)	(37.4)	(42)
(CM-Sephadex)	(112)	(7855)	(70.0)	(37)
(Sephadex G-75)	(95)	(7125)	(75.0)	(34)
(crystallization)			(78.0)	

[a] Values in parentheses are from Katunuma et al. (1975). Protease isolated by the affinity adsorption step was obtained from the intestines of 50 normal white female rats (250 g). No information was given with respect to the number and age of rats used by Katunuma and co-workers except that the rats were maintained on a protein-free diet for 1 week.

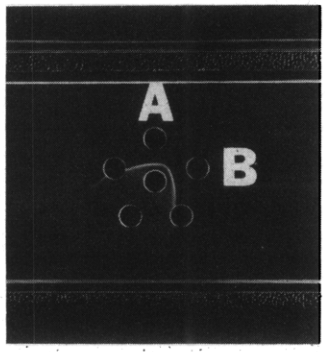

FIGURE 2: Immunodiffusion test for cross-reactivity between antiserum directed toward protease prepared by the method of Katunuma et al. (1975) and protease prepared by the affinity adsorption method. Center well contained 5 μL of antiserum; A contained 5 μL of protease (0.1 mg/mL) isolated by the method of Katunuma et al. (1975); B contained 5 μL of protease (0.1 mg/mL) prepared by affinity adsorption chromatography.

TABLE III: Relative Amino Acid Content[a] of RMCP II Prepared by Affinity Adsorption Chromatography.

amino acid	RMCP II (affinity)[b]	RMCP II (conventional)[c]
Asp	13.4	14.0
Thr	11.7	12.4
Ser	10.8	10.8
Glu	15.4	16.0
Pro	14.5	15.0
Gly	16.9	18.4
Ala	16.3	16.0
Val	20.6	21.0
Met	5.1	4.6
Ile	14.9	15.6
Leu	15.6	16.4
Tyr	9.5	8.8
Phe	6.0	6.0
His	8.8	9.0
Lys	12.8	13.0
Arg	12.2	12.0

[a] Duplicate samples were hydrolyzed for 24 h in 6 N HCl at 110 °C. Half-cystine and tryptophan were not determined. Amino acid compositions are given as residues per molecule, calculated for a molecular weight of 25 000. [b] (Affinity) refers to protease purified by affinity adsorption chromatography. [c] (Conventional) refers to protease isolated by the method of Katunuma et al. (1975).

The amino-terminal sequences of proteases prepared by the two methods are also identical, consisting of Ile-Ile-Gly-Gly-Val-Glu-Ser-Ile-Pro-His in the first ten positions. No contaminating sequences were observed in either preparation.

Double immunodiffusion tests for cross-reactivity with antiserum toward intestinal protease isolated by the method of Katunuma et al. (1975) indicated that the proteases purified by the two methods were immunologically identical (Figure 2).

Distribution of RMCP II and OAT in Small Intestines. The levels of protease and OAT along the length of small intestine are shown in Figure 3. Both enzyme levels were fairly constant throughout the tissue except that RMCP II level decreased about 20% in the most distally located segments. At peak concentration of OAT (segment 3), the RMCP II to OAT molar ratio was 32. Ornithine aminotransferase in homogenates was completely in the holo form since addition of pyridoxal phosphate (0.1 mM) did not increase the apparent activity. Thus, the low OAT activity relative to RMCP II levels was not due to inactivation of OAT by RMCP II. Katunuma & Kominami (1977) reported that endogenous OAT activity in homogenates of intestine was very slowly (half-life = 5 h) depleted at 37 °C by a serine protease, which was probably RMCP II. The amount of RMCP II detected by radial immunodiffusion in all cases was consistent with that determined by enzymatic assay.

Levels of RMCP II and OAT in Developing Rats. When OAT activities were determined in the mid region of small intestines of 1–16 week old female rats (5–8 animals in each age group), the enzyme level was relatively high in 1 and 2 week old rats, but thereafter dropped precipitously (Figure 4a). After 4 weeks the OAT level reached the adult level (approximately 0.1 mg of enzyme/g of tissue) and remained constant. In contrast, RMCP II levels were low in young rats (0.2–0.25 mg of protease/g of tissue), but approximately 2 weeks after weaning (21 days after birth) increased rapidly to about 1.4 mg of enzyme/g of tissue. The high level was maintained for several weeks and then gradually fell to adult levels (0.5 mg of protease/g of tissue). Several observations should be noted. First, OAT activity decreased sharply about 1 to 2 weeks before there was a significant increase in RMCP II levels. In fact, both enzymes were nearly at their lowest concentrations after 4 weeks. Second, the fall in RMCP II after 8 weeks to adult levels had no effect on OAT activity. Finally, at maximum RMCP II concentration, the RMCP II to OAT molar ratio was 100 to 1. Soluble protein (16–20 mg of protein/mL of homogenate supernatant fluid) was quite constant for each age. Thus, the observed changes in enzyme levels were not due to variable tissue homogenization.

Since the intestinal protease was shown to be located in atypical mast cells (Woodbury et al., 1978a), the number of

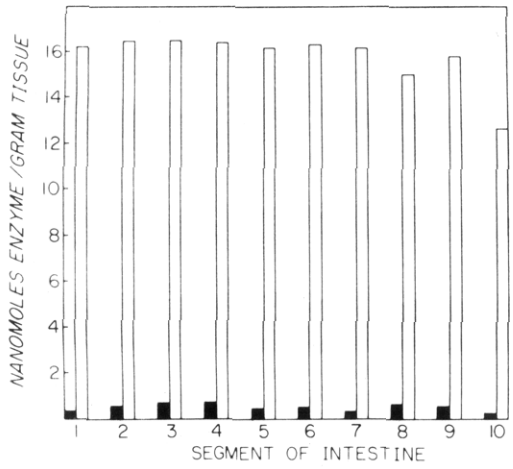

FIGURE 3: Distribution of RMCP II (□) and OAT (■) along the length of small intestine. Segments of equal length were removed sequentially from proximal to distal small intestine (left to right). Similarly located segments of three intestines were washed, pooled, and homogenized. Levels of RMCP II were determined by single radial immunodiffusion assay and activities and concentrations of OAT were measured by the method of Jenkins & Tsai (1970). Concentrations are in nmol of enzyme/g of tissue.

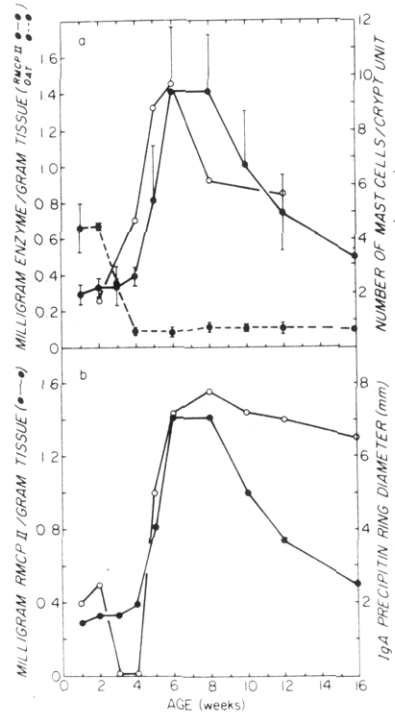

FIGURE 4: The levels of RMCP II in developing rats. (a) Levels of RMCP II (mg of enzyme/g of tissue) (●—●) compared with those of OAT (● - - - ●). The mid region of small intestines of 1–16 week old female rats (5–8 animals in each age group) was used. Standard deviations in enzyme levels are indicated by bars (I). Weaning of rats occurred on the 21st day after birth. The average number of mast cells (○—○) per intestinal villous-crypt unit (from a minimum of 30 units) for each group was determined after sections of fixed tissue were stained with alcian blue–safranin. (b) Levels of RMCP II (from a) compared with those of immunoglobulin A (○—○). The levels of both proteins were determined by single radial immunodiffusion assay, but, due to the lack of an appropriate standard of known IgA concentration, the immunoglobulin levels are given in relative (precipitin ring diameter) concentrations.

these cells was expected to increase in proportion to the protease levels in developing rats. This pattern was in fact observed (Figure 4a). The mast cell population doubled between weeks 4 and 6, the period of greatest increase in RMPC II levels,

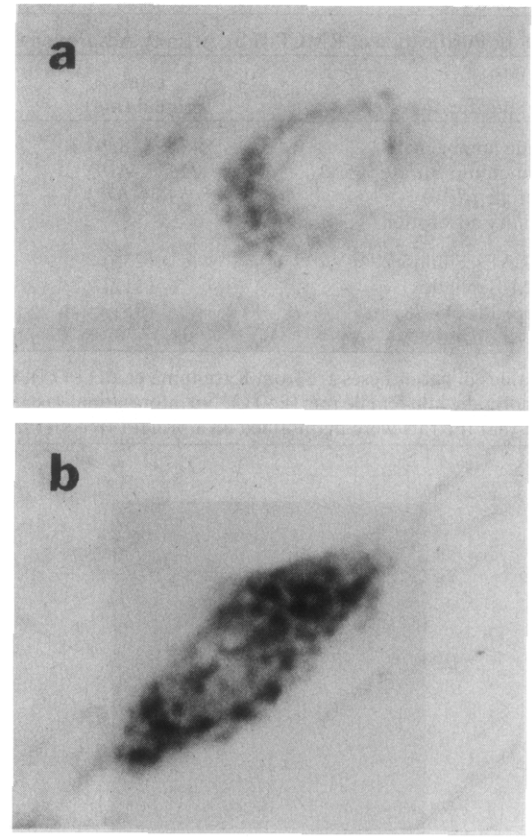

FIGURE 5: Maturation of atypical mast cells in small intestine during rat development. (a) Representative mast cell in gut lamina propria of 3 week old animal showing a few weakly stained granules. (b) Typical cell observed in 6 week old rat containing numerous intensely stained granules. Sections were stained in alcian blue–safranin. Magnification: ×1000.

remained elevated for several weeks, and then fell in a manner corresponding to the protease level. Closer examination of these patterns revealed that, whereas the mast cell population increased twofold between weeks 4 and 6, the protease level increased fourfold. Accompanying the increase in mast cell numbers at 5 weeks was a maturation of existing cells. In the typical cell observed in young rats (Figure 5a), the cytoplasm contained very few weakly stained alcian blue-positive granules. In contrast, the cells observed in 5 week old rats (Figure 5b), and thereafter, contained many intensely stained secretory granules typical of mature mast cells.

Relationship of RMCP II to IgA in the Intestines of Developing Rats. Comparison of IgA levels with RMCP II concentrations in developing rats indicates that IgA and RMCP II follow similar patterns (Figure 4b). IgA levels were very low in young rats, suggesting that the immunoglobulin was of maternal origin. After weaning there was a rapid increase in IgA levels which corresponded closely to the rise in RMCP II levels. As observed for RMCP II, there appeared to be a peak level of IgA between 5 and 8 weeks.

Discussion

The serine protease purified from rat small intestine by the affinity adsorption method described herein is identical in chemical, physical, enzymatic, and immunologic properties with that purified by the method of Katunuma et al. (1975). With the new method, the time required to purify 25 mg (1 μmol) of protease from 50 rat intestines was reduced from about 2 weeks to just 2 days. At the same time the overall yield increased from 34% to 52%. The protease isolated by the affinity method is comparable in purity to that isolated by the

procedure of Katunuma et al. (1975) as judged by gel electrophoresis in the presence of sodium dodecyl sulfate and dithiothreitol, its specific activity in the OAT-inactivating assay, its incorporation of 1 mol of [^{14}C]DFP/mol of protein, its amino acid composition, and the results of amino-terminal sequence analysis which indicated that at least 95% of the total protein consisted of the protease. Subunit C of potato inhibitor I, used in preparing the affinity resin, was effective, stable, and readily obtained. Other protease inhibitors, e.g., ovoinhibitor and soybean trypsin and lima bean trypsin inhibitors, might prove equally effective.

The amount of RMCP II in rat intestine is remarkably high. A single intestine (approximately 6 g) from an adult rat contains at least 3 mg of protease. It is estimated that in 6 to 8 week old rats nearly 1% (by weight) of the total and 3% of the soluble protein of small intestine consists of RMCP II. In light of these figures, one may expect that the concentration of the protease within the mast cell is exceedingly high. Unquestionably, RMCP II must be a major if not the major protein product of atypical mast cells.

Although the apparent OAT levels are low compared with those of RMCP II (Figure 3), they are actually higher in small intestine than in any other rat tissue including liver (Herzfeld & Raper, 1976). Thus, the putative substrate attains its highest concentration in tissue containing by far the highest level of the protease purported to initiate degradation of the enzyme. Further, the high molar ratio of protease to substrate (100:1) in 6 to 8 week old rats (Figure 4a) is greater still since the actual substrate, apo-OAT, was shown to be virtually absent in crude homogenates. Thus, the protease is present in far greater quantity with respect to its substrate than the catalytic amounts normally expected of an enzyme.

The disproportionate amount of RMCP II with respect to OAT activity in adult rats and the apparent absence of any correlation of the levels of these enzymes in developing rats (Figure 4a) would appear to be inconsistent with the concept that OAT is a physiological substrate of the protease.

What then is the function of RMCP II? Considering the amount of the protease, one is tempted to speculate that its substrate also is present in high concentration in gut mucosa or perhaps that the resulting proteolysis is extensive, involving several substrates. Ultimately, however, its function must be related to that of atypical mast cells. The current view is that these cells in mammals (including man) are involved in the local immune response at mucosal surfaces, particularly in intestinal parasite infections (Miller & Jarrett, 1971; Ogilvie & Jones, 1973; Ruitenberg & Elgersma, 1976). Miller & Jarrett (1971) observed that these cells in gut increased in numbers at least tenfold at the time when rats spontaneously expelled nematodes from this site. Recent evidence suggests that the appearance and proliferation of atypical mast cells, but not of normal mast cells, are dependent on T-lymphocytes (Ruitenberg & Elgersma, 1976; Burnet, 1977). Indeed, Kitamura et al. (1977) have shown that atypical mast cells of mouse small intestine, but not normal mast cells, arise from precursor cells derived from bone marrow. These observations coupled with the well-established morphologic (Enerbäck & Lundin, 1974) and histologic (Enerbäck, 1966; Miller & Walshaw, 1972) differences between atypical and normal mast cells of rat strongly suggest that the cells are distinct types. The indication that the atypical cell originates from bone marrow suggests that this cell may be related more to the blood basophilic leukocyte than to mast cells.

In the current study it was observed that a rapid rise in mast cell number and protease content of the gut normally occurs during rat development (Figure 4a). This rise is coincident with the first active immune response of the gut as indicated by a similar rise in IgA levels (Figure 4b). Immunoglobulin A is found almost exclusively at mucosal surfaces and in their secretions and is considered to be the major immunoglobulin at these sites (Tomasi et al., 1965). The temporal relationship of atypical mast cells and IgA suggests (and is consistent with the observations in parasite infected animals) that the cells function in the immune response of the gut, and that the appearance and maturation of these cells is T-cell dependent.

It appears that atypical mast cells, and perhaps the protease(s) they contain, may function in the immune or inflammatory response at mucosal surfaces. The level at which they function in this process remains unknown, but since the mucosal surfaces of the gut and respiratory tracts are sites prone to immediate-type hypersensitivity further research in this area seems justified.

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Human Plasma P Component: Isolation and Characterization[†,‡]

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ABSTRACT: A calcium-binding glycoprotein was isolated from human plasma by barium citrate adsorption-elution, DEAE-Sephadex chromatography, and chromatography on heparin-agarose in the presence of calcium. The preparation was homogeneous by disc gel electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoelectrophoresis, and amino-terminal sequence to 30 residues. The subunit molecular weight estimate on sodium dodecyl sulfate-10% polyacrylamide gels was 25 000. Electrophoretic migration at pH 8.6 was with α-globulins when run in EDTA, but in calcium there was little, if any, migration from the origin. Amino acid analysis revealed two half-cystine residues, which formed an intrachain disulfide bond, a single methionine residue, and no γ-carboxyglutamic acid. The protein contained 11.2% carbohydrate consisting of 5.6% neutral sugars, 3.0% glucosamine and 2.6% sialic acid. Two fragments were isolated following cyanogen bromide digestion and gel filtration. The half-cystine residues and carbohydrate were present in the amino-terminal, larger fragment. Compared with vitamin K dependent proteins, the intact protein bound less tightly to DEAE and more tightly to heparin-agarose. The subunit size, composition, and amino-terminal sequence for this calcium-binding plasma protein are the same as described for P component purified from human serum and the sequence is nearly identical with that of P component from amyloid tissues. The amino-terminal region of P component from human plasma is homologous with the corresponding portion of C-reactive protein as 18 of the first 30 amino acid residues are identical; size and composition of cyanogen bromide fragments indicate internal homology as well. Of residues 8–12, four are identical with those in a five residue segment near the amino terminus of the Fc fragment of the γ_1-immunoglobulin chain. Plasma P component, however, failed to cross-react with either anti-C-reactive protein or anti-Fc fragment antibodies. A monospecific rabbit antibody to plasma P component was used in a quantitative immunoelectrophoretic system. The plasma level of a pool of normal donors was 44 μg per mL. Plasma P component was identical with a soluble fraction of P component from the tissue of a patient with amyloidosis when analyzed by immunodiffusion and tandem crossed immunoelectrophoresis. Subunit migration was identical in sodium dodecyl sulfate gel electrophoresis indicating that the plasma and tissue-derived forms have the same molecular size.

Amyloid is a pathological, proteinaceous substance which is associated with organ dysfunction. Extracts contain a fibrillar protein (either AA or immunoglobulin types, Benditt & Eriksen, 1971; Glenner et al., 1971) and a more soluble "P component" (Cathcart et al., 1965). The latter is so named because of a characteristic pentameric structure on electron micrographs (Bladen et al., 1966). Although the amount in saline tissue extracts from different patients varies, the presence of P component is independent of fibrillar type.

P component is also present in normal serum where it migrates as an α-globulin (Cathcart et al., 1967; Binette et al., 1974). When purified from normal human serum by gel filtration and preparative gel electrophoresis, P component was found by Binette et al. (1974) to be immunologically identical to an α_1-glycoprotein purified on carboxymethylcellulose by Haupt et al. (1972). Purification from serum by affinity chromatography using an insolubilized antibody to tissue P component has also been achieved (Benson et al., 1976).

In fractionating the subcomponents of the first component of complement (by affinity chromatography on insolubilized IgG with calcium), P component was copurified and thought to be a 4th subcomponent, called C1t (Assimeh et al., 1974). Although highly purified, complement activity of these preparations was due to contaminant protein (Painter, 1977). The preparation, however, contained pentameric structures on electron microscopy (Pinteric et al., 1976) and was immunologically identical to tissue P component and to serum preparations obtained by adsorption to unsubstituted agarose in the presence of calcium (Pepys et al., 1977a).

P component subunits, which are noncovalently bound, have reported molecular weights of 23 000 (Pinteric et al., 1976) or 36 000 (Benson et al., 1976). In the latter study a similar amino acid composition between tissue and serum P component preparations was found, although no amino-terminal sequence could be obtained from their serum fractions. In contrast, Osmand et al. (1977) reported a single amino-terminal sequence from the serum P component preparation (C1t) which suggests the presence of identical subunits. Homology with the C-reactive protein was also noted.

During the course of purifying vitamin K dependent proteins from human plasma, a calcium-binding glycoprotein which

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