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Tryptase in Rat Mast Cells: Properties and Inhibition by Various Inhibitors in Comparison with Chymase

Mutumi MURAMATU, Tadashi ITOH, Masao TAKEI and Koichi ENDO

Faculty of Pharmacy, Tokushima Bunri University

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Summary: Previous studies with *trans*-4-(guanidinomethyl)cyclohexanecarboxylic acid 4-*tert*-butylphenyl ester (GMCHA-OPhBu^t), a trypsin inhibitor, strongly suggested the involvement of a trypsin-like protease in histamine release from mast cells induced by various secretagogues (Takei, M., Matumoto, T., Endo, K. & Muramatu, M. (1988) *Agents and Actions*, in press; Takei, M., Matumoto, T., Ito, T., Endo, K. & Muramatu, M.; Takei, M., Matumoto, T., Endo, K. & Muramatu, M. and Takei, M., Matumoto, T., Urashima, H., Endo, K. & Muramatu, M., unpublished results). Two serine proteases, chymase (Benditt, E.F. & Arase, M. (1959) *J. Exp. Med.* **110**, 451–460) and tryptase Kido, H., Fukusen, N. & Katunuma, N. (1985) *Arch. Biochem. Biophys.* **239**, 436–443) were demonstrated in rat peritoneal mast cells. Both enzymes were purified and the effects of inhibitors for trypsin and chymotrypsin on these proteases were examined. The trypsin-like protease was found in saline extract and purified by successive chromatographies on Sephadex G-100 and DEAE-cellulose columns. The molecular mass of this protease was apparently 120 000 Da. This protease showed maximal activity at pH 7.1 and was named pH 7 tryptase. Chymase was obtained from 1.5M NaCl extract. pH 7 Tryptase markedly hydrolysed Boc-Phe-Ser-Arg-NH-Mec and Boc-Val-Pro-Arg-NH-Mec among the various substrates containing arginyl and lysyl bonds but did not cleave Tos-Arg-OMe. Tos-Lys-CH_2Cl and diisopropylfluorophosphate strongly inhibited this protease. Various inhibitors for trypsin inhibited pH 7 tryptase, and those for chymotrypsin inhibited chymase. Among the esters of GMCHA examined, GMCHA-OPhBu^t most strongly and competitively inhibited pH 7 tryptase but it had no effect on chymase.

Tryptase in Mastzellen der Ratte: Eigenschaften und Hemmbarkeit durch verschiedene Inhibitoren im Vergleich zur Chymase

Zusammenfassung: Frühere Untersuchungen mit *trans*-4-(Guanidinomethyl)cyclohexancarbonsäure-4-*tert*-butylester (GMCHA-OPhBu^t), einem Trypsininhibitor, machten die Beteiligung einer trypsinähnlichen Protease bei der durch verschiedene Sekretagoge induzierten Histamin-

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Enzyme:
Chymase (EC 3.4.21.39).

Abbreviations:
NH-Mec: 7-amido-4-methylcoumarin; Boc: *tert*-butyloxycarbonyl; Cbz: benzyloxycarbonyl; Suc: 3-carboxypropanoyl; Glt: 4-carboxybutyryl; Ac-Tyr-OEt: acetyl-L-tyrosine ethyl ester; Tos-Arg-OMe: tosyl-L-arginine methyl ester; STI: soybean trypsin inhibitor; LTI: lima bean trypsin inhibitor; Tos-Lys-CH_2Cl: 4-tosyl-L-lysylchloromethane; Tos-Phe-CH_2Cl: tosyl-L-phenylalanylchloromethane; iPr_2*P*-F: diisopropylfluorophosphate; GMCHA: *trans*-4-(guanidinomethyl)cyclohexanecarboxylic acid; GMCHA-OPhBu^t: 4-*tert*-butylphenyl ester of GMCHA; GMCHA-OPh: phenyl ester of GMCHA; GMCHA-OPhZ:2-benzyloxycarbonylphenyl ester of GMCHA; Me_2SO: dimethylsulfoxide; NPGB: 4-nitrophenyl 4-guanidinobenzoate; I_{50}: inhibitor concentration for 50% inhibition; Hepes: 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.

freisetzung aus Mastzellen sehr wahrscheinlich (Takei, M., Matumoto, T., Endo, K. & Muramatu, M. (1988) *Agents and Actions*, in press, sowie Takei, M., Matumoto, T., Ito, T., Endo, K. & Muramatu M.; Takei, M., Matumoto, T., Endo, K. & Muramatu, M. und Takei, M., Matumoto, T., Urashima, H., Endo, K. & Muramatu, M., unveröffentlicht). Zwei Serinproteasen, Chymase (Benditt, E.F. & Arase, M. (1959) *J. Exp. Med.* **110**, 451–460) und Tryptase (Kido, H., Fukusen, N. & Katunuma, N. (1985) *Arch. Biochem. Biophys.* **239**, 436–443) waren in peritonalen Mastzellen der Ratte nachgewiesen worden. Beide Enzyme wurden gereinigt und die Wirkung von Trypsin- und Chymotrypsininhibitoren auf diese Proteasen wurde untersucht. Die trypsinähnliche Protease wurde mit physiologischer Kochsalzlösung extrahiert und durch Chromatographie an Sephadex G-100 sowie DEAE-Cellulose gereinigt. Die scheinbare molekulare Masse dieser Protease betrug 120 000 Da. Die Protease zeigte maximale Aktivität bei pH 7.1 und wurde pH-7-Tryptase genannt. Chymase wurde durch Extraktion mit 1.5M NaCl erhalten. Von verschiedenen Substraten mit Arginyl- und Lysylbindungen wurden durch pH-7-Tryptase besonders Boc-Phe-Ser-Arg-NH-Mec und Boc-Val-Pro-Arg-NH-Mec, aber nicht Tos-Arg-OMe gespalten. Tos-Lys-CH_2Cl und Diisopropylfluorophosphat hemmten diese Protease stark. pH-7-Tryptase wurde durch verschiedene Trypsininhibitoren und Chymase durch Chymotrypsininhibitoren gehemmt. Von den untersuchten GMCHA-Estern hemmte GMCHA-OPhBu^t die pH-7-Tryptase am stärksten und kompetitiv, hatte aber keine Wirkung auf Chymase.

Key words: Mast cells, tryptase, chymase, preparation, inhibition.

The participation of a serine protease in histamine release has been strongly suspected since Benditt and Arase found a chymotrypsin-like protease in rat mast cells[1]. Austen and Brocklehurst[2] reported that the anaphylactic release of histamine is prevented by substrates and inhibitors for chymotrypsin but not by those for trypsin. These observations suggested that the activation of a chymotrypsin-like enzyme is necessary for the anaphylactic release of histamine. Later, Ishizaka reported that substrates and inhibitors for trypsin and chymotrypsin including diisopropylfluorophosphate inhibit phospholipid methylation and the increase in cAMP induced by bridging of IgE receptors on mast cells[3,4]. Muramatu et al.[5] observed a strong inhibitory effect of *trans*-4-(guanidinomethyl)cyclohexanecarboxylic acid 4-*tert*-butylphenyl ester (GMCHA-OPhBu^t), a synthetic inhibitor of trypsin, on histamine release from rat mast cells. Recently, Takei et al. reported the strong inhibitory effects of GMCHA-OPhBu^t on histamine release from mast cells[6], increase in cAMP*, phospholipid methylation** and $Ca^{2\oplus}$ uptake***, induced by various secretagogues, such as antigen, anti-IgE, concanavalin A and A23187. The effects of GMCHA-OPhBu^t on these biochemical events were the strongest (I_{50} of the order 10^{-6}M) among the various inhibitors tested. These results suggest the involvement of a trypsin-like protease in histamine release and are therefore in contrast to the above observations of Austen and Brocklehurst[2] and Ishizaka[3]. To solve these discrepancies, we examined the effects of various protease inhibitors including some esters of *trans*-4-(guanidinomethyl)cyclohexanecarboxylic acid (GMCHA) on tryptase and on chymase.

Chymase was purified from rat mast cells by Yurt and Austen[7], Everitt and Neurath[8], and Okuno-Kaneda et al.[9]. Tryptase was first purified by Schwartz et al.[10] from human pulmonary mast cells, and recently, Kido et al.[11] purified a tryptase from rat peritoneal mast cells. Tryptase purified by them showed maximal activity above pH 8.0.

In this paper, we report the purification of a tryptase with an optimal pH of 7.1 from rat mast cells, and designate it pH 7 tryptase, and we present data of inhibition experiments with this protease and chymase. A subsequent paper describes the possible involvement of pH 7 tryptase in activation of adenylate cyclase by antigen, anti-IgE and concanavalin A.

* Takei, M., Matumoto, T., Ito, T., Endo, K. & Muramatu, M., unpublished results.

** Takei, M., Matumoto, T., Endo, K. & Muramatu, M., unpublished results.

*** Takei, M., Matumoto, T., Urashima, H., Endo, K. & Muramatu, M., unpublished results.

Materials and Methods

Materials

4-Methylcoumarin-7-amides of Boc-L-valyl-L-prolyl-L-arginine, Boc-L-leucyl-glycyl-L-arginine, Boc-L-leucyl-

L-seryl-L-threonyl-L-arginine, Boc-L-phenylalanyl-L-seryl-L-arginine, glutarylglycyl-L-arginine, 3-carboxypropanoyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine, L-prolyl-L-phenylalanyl-L-arginine, 3-carboxypropanoyl-L-alanyl-L-prolyl-L-alanine, Boc-L-glutamyl-L-lysyl-L-lysine, N^{α}-benzoyl-L-arginine, Boc-L-valyl-L-leucyl-L-lysine, 3-carboxypropanoyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine, L-arginine and L-leucine, N^{α}-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt), N^{α}-tosyl-L-arginine methyl ester hydrochloride (Tos-Arg-OMe), antipain, leupeptin and chymostatin were from the Peptide Institute Inc., Minoh-shi, Osaka. 4-Nitrophenyl 4-guanidinobenzoate (NPGB), benzyloxycarbonylglycycl-L-phenylalanine, lima bean trypsin inhibitor (LTI), and soybean trypsin inhibitor (Kunitz) (STI) were from Sigma Chem. Co., St. Louis, Mo. *N*-Tosyl-L-lysyl chloromethane hydrochloride (Tos-Lys-CH_2Cl) and *N*-tosyl-L-phenylalanyl-chloromethane (Tos-Phe-CH_2Cl) were from Nakarai Chem. Ltd., Kyoto. Benzamidine was from Tokyo Chem. Ind. Ltd., Tokyo. Indol and dimethylsulfoxide (Me_2SO) were from Wako Pure Chem. Ind., Osaka. *trans*-4-(Guanidinomethyl)cyclohexanecarboxylic acid phenyl ester (GMCHA-OPh), 2-benzyloxycarbonyl-phenyl ester (GMCHA-OPhCbz.) and 4-*tert*-butyl-phenyl ester (GMCHA-OPhBu^t) were obtained from Nippon Chemiphar Co., Tokyo, as their hydrochlorides. Sephadex G-100 and Percoll were from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-Cellulose (Brown) was from Seikagaku Kogyo Co., Tokyo.

Preparation of mast cells

Peritoneal cells were obtained from 20 male Wistar rats weighing 250 to 300 g as described by Saeki[12], and mast cells were purified by the method of Németh and Röhlich with Percoll[13] but with 0.15M NaCl instead of Hepes buffer. The purity of the mast cell suspension (2×10^7 cells) was above 90%.

Assay of protease activity

In the purification experiments trypsin-like protease activity was assayed with Boc-Val-Pro-Arg-NH-Mec in 0.1M phosphate buffer, pH 7.1. Mixtures of 1 m*l* of the above buffer containing various amounts of protease with 0.5 m*l* of the substrate solution were incubated at 37 °C. After 30 min, 1 m*l* of 30% acetic acid was added, and 7-amino-4-methylcoumarin released was determined fluorometrically as described by Kanaoka et al.[14]. The final concentration of substrate was 2.9×10^{-4}M. Chymotrypsin-like protease activity was assayed with Suc-Leu-Leu-Val-Tyr-NH-Mec in 0.1M borate buffer, pH 8.0, containing 0.15M NaCl as above. The final concentration of substrate was 2×10^{-4}M. One unit (U) of trypsin- or chymotrypsin-like protease activity was defined as an amount required to hydrolyze 1 μmol of the substrate per min under the above conditions. The assay of hydrolytic activity of tryptase on various fluorogenic substrates was carried out as above except for final substrate concentration, which was for each substrate 33μM. Tos-Arg-OMe- and Ac-Tyr-OEt-hydrolytic activities were determined by Hesterin's method[15] after incubation for 30 min at 37 °C with 15mM substrate at pH 7.1 and 8.0, respectively.

Preparation of saline extract and 1.5M NaCl extract

Purified mast cells (2×10^7 cells) from 20 rats were suspended in 2 m*l* of 0.15M NaCl, freeze-thawed three times, and centrifuged at 60000 x *g* for 15 min at 5 °C. The precipitate was washed twice by suspending in 5 m*l* of 0.15M NaCl and by centrifuging the suspension as above. The supernatants were combined (12 m*l*) and named "saline extract".

The precipitate was homogenized with 4 m*l* of 1.5M NaCl and centrifuged at 60000 x *g* for 15 min at 5 °C. Then the precipitate was washed twice with 4 m*l* of 1.5M NaCl as above. The supernatants were combined (12 m*l*) and named "1.5M NaCl extract". The precipitate was suspended in 4 m*l* of 1.5M NaCl and named "insoluble fraction".

Gel filtration of the saline extract and 1.5M NaCl extract

The saline extract (12 m*l*) was adjusted to a final concentration of 1.5M NaCl by adding 0.95 g of NaCl, and subjected to gel filtration on a Sephadex G-100 column, 3.2 x 72 cm, equilibrated with 1.5M NaCl. The flow rate was 10 ml/h, and fractions of 5 m*l* were collected (see Fig. 2a). Fractions No. 32 to 51 were combined and concentrated to 30 m*l* with an Amicon YM 5 membrane for protein assay (see Table 2a).

The 1.5M NaCl extract (12 m*l*) was subjected to gel filtration on a Sephadex G-100 column as above (see Fig. 2b).

DEAE-Cellulose column chromatography

Fractions No. 32 to 51 obtained by gel filtration of the saline extract were combined, concentrated as described above, and dialysed against 0.01M phosphate buffer, pH 7.4. The dialysate was applied to a DEAE-cellulose column, 1.2 x 4 cm, equilibrated with 0.01M phosphate buffer, pH 7.4, and washed with 50 m*l* of the same buffer. Adsorbed materials were eluted with a linear gradient formed from 130 m*l* of the same buffer and 130 m*l* of the buffer containing 0.5M NaCl (see Fig. 3).

Inhibition of protease activity

Mixtures of 1 m*l* of the above buffer containing 2 μU of protease and various amounts of inhibitor were incubated with 0.5 m*l* of the substrate, and activity was assayed as described above. Final concentration of the substrate was 33μM. When high-molecular-mass inhibitors, such as STI and LTI, were used, the protease was incubated with various amounts of inhibitor for 5 min at 25 °C, then 0.5 m*l* of the substrate was added, and incubation was continued at 37 °C for 30 min. The inhibition constant K_i was determined from a Lineweaver-Burk plot[16].

Inhibition of protease by iPr_2P-F and Tos-Lys-CH_2Cl

A mixture of 1 m*l* of 0.1M phosphate buffer, pH 7.4, containing 370 μU of tryptase and 1mM of Tos-Lys-CH_2Cl or iPr_2P-F was incubated at 37 °C. At intervals 10 μl of samples of the mixture were drawn, and mixed with 1.49 m*l* of 0.1M phosphate buffer, pH 7.1, containing 33μM Boc-Val-Pro-Arg-NH-Mec and incubated for 30 min at 37 °C to determine the remaining activity.

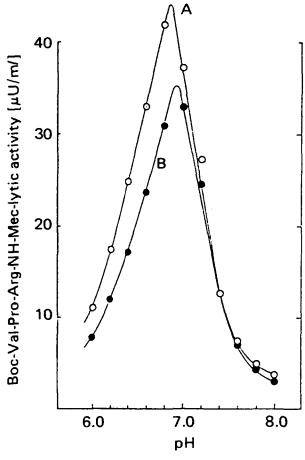

Fig. 1. Effects of pH on trypsin-like protease activity of the saline extract with and without preincubation.

The saline extract was prepared as described in the text. Curve A: activity of trypsin-like protease examined at various pH values in 0.1M phosphate buffer by simultaneous addition of saline extract and substrate. Curve B: activity of trypsin-like protease examined after preincubation at 16 °C for 10 min at various pH values in 0.1M phosphate buffer. Boc-Val-Pro-Arg-NH-Mec was used as a substrate.

Protein assay

Protein was determined from the absorbance at 280 nm or by Lowry's method[17] with bovine serum albumin as a standard.

Results

Trypsin-like and chymotrypsin-like protease activities of the saline extract, 1.5M NaCl extract and insoluble fraction

The trypsin-like and chymotrypsin-like activities of the extracts and insoluble fraction were examined with Boc-Val-Pro-Arg-NH-Mec and Suc-Leu-Leu-Val-Tyr-NH-Mec, respectively. High trypsin-like (151 mU) and very low chymotrypsin-like activities (1.35 mU) were found in the saline extract. As reported also by others, high chymotrypsin-like activity (chymase activity) is present in the 1.5M NaCl extract, 57.3 mU of Suc-Leu-Leu-Val-Tyr-NH-Mec-hydrolytic activity corresponded to 254 mmol of Ac-Tyr-OEt-hydrolyzing activity per min. Trypsin-like activity (17.5 mU) was also found in the 1.5M NaCl extract. Both activities were very low in the insoluble fraction.

Effect of pH on trypsin-like protease of the saline extract

The activity of the trypsin-like protease of the saline exteract was examined at various pH values in 0.1M phosphate buffer, and results are shown in Fig. 1. A sharp activity profile was observed with maximal activity at pH 7 (see curve A).

The pH-activity relationship of the trypsin-like protease after preincubation in buffer of various pH values at 16 °C for 10 min is shown in Fig. 1, curve B. Comparison of curves A and B shows that this protease was considerably labile at all pH values examined except pH 7.4.

The trypsin-like protease of the saline extract was most stable in 1.5M NaCl, and showed no change in activity during storage in ice for several months.

Gel filtration of the saline extract and 1.5M NaCl extract

Fig. 2a shows the results of elution from a Sephadex G-100 column at 10 ml/h. High trypsin-like activity was eluted rapidly, followed by low chymotrypsin-like activity. The apparent molecular masses of these proteases were estimated by the method of Andrews[18] to be about 120 000 and 25 000, respectively. The elution profile of protein is not shown in Fig. 2a, because of its very low content. Fractions No. 32 to 51 were combined and concentrated to 30 ml with an Amicon YM 5 membrane for protein determination (see Table 2a).

Fig. 2b shows the results of gel filtration of the 1.5M NaCl extract on a Sephadex G-100 column. The elution positions of trypsin- and chymotrypsin-like activities were the same as those observed with the saline extract, but the chymotrypsin-like activity was higher than the trypsin-like activity. When the flow rate was more than 10 ml/h, the chymotrypsin-like protease was eluted close to the void volume. The chymotrypsin-like protease corresponded to chymase as described by Okuno-Kaneda et al.[9]. The chymase fraction (fractions No. 68 to 80) were combined and used in the following inhibition experiments without further purification.

Fractions No. 33 to 50 were combined and the properties of the enzyme were examined without further purification. Comparing the elution position from the Sephadex G-100 column, the pH-activity, profile, and the results of kinetic and inhibition experiments, the trypsin-like protease in the 1.5M NaCl extract proved to be identical with that of the saline extract (see below). This trypsin-like protease was named tryptase.

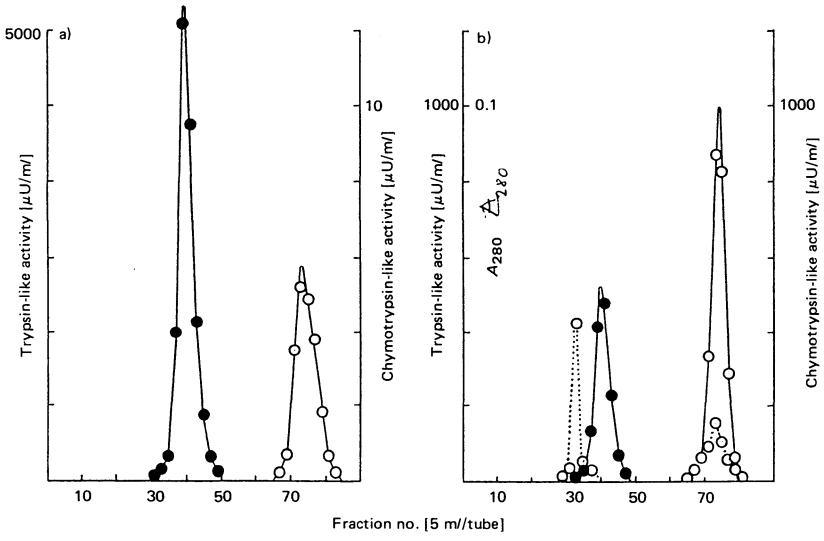

Fig. 2. Gel filtration of the saline extract and 1.5M NaCl extract on a Sephadex G-100 column.

a) A mixture of 12 m*l* of saline extract, prepared as described in the text, and 0.95 g of NaCl (final concentration, 1.5M) was subjected to gel filtration on a Sephadex G-100 column, 3.2 x 72 cm, equilibrated with 1.5M NaCl. The flow rate was 10 m*l*/h, and fractions of 5 m*l* were collected. ●: Trypsin-like activity; ○: chymotrypsin-like activity. b) A volume of 12 m*l* of the 1.5M NaCl extract was subjected to gel filtration as described in a). ·····○·····: absorbance at 280 nm; –●–: trypsin-like activity; –○–: chymotrypsin-like activity. Trypsin- and chymotrypsin-like activities were determined with Boc-Val-Pro-Arg-NH-Mec and Suc-Leu-Leu-Val-Tyr-NH-Mec as substrates, respectively.

DEAE-Cellulose column chromatography

Fractions No. 32 to 51 obtained by gel filtration of the saline extract were subjected to a DEAE-cellulose column chromatography. Fig. 3 shows the results. A single sharp peak of tryptase activity was obtained. The elution profile of protein is not shown in Fig. 3, because of its low content.

A summary of the purification profile for tryptase of the saline extract from 20 rats is shown in Table 1a. The recovery was 40% with an about 30-fold increase in specific activity. This protease preparation was used in subsequent experiments. The results of gel filtration of tryptase from the 1.5M NaCl extract are summarized in Table 1b. The recovery was 87% with a 20-fold increase in specific activity. This protease was used in the following experiments without further purification.

Effect of pH on purified tryptase

The effect of pH on the purified tryptase from the saline extract was examined in 0.1M phosphate buffer, and the results are shown in Fig. 4. A sharp activity profile was observed with maximal activity at pH 7 (Fig. 4). These results differ from those reported by Kido et al.[11] and Schwartz et al.[10].

The pH-activity relationship of tryptase after preincubation in buffer of various pH values at 20 °C for 10 min was examined, however, enzyme activity was not changed during preincubation. Similar results were obtained with tryptase from the 1.5M NaCl extract (data not shown). Thus this tryptase in these fractions was named pH 7 tryptase. However, for sake of brevity, "tryptase" is used for pH 7 tryptase in this paper.

Substrate specificity of tryptase

Hydrolytic activity of tryptase purified from the saline extract on various fluorogenic substrates was examined in 0.1M phosphate buffer, pH 7.1, containing 33μM of each substrate. The tryptase clearly hydrolysed trypsin-specific substrates, and among them markedly Boc-Phe-Ser--Arg-NH-Mec and Boc-Val-Pro-Arg-NH-Mec (Table 2). Boc-Leu-Ser-Thr-Arg-NH-Mec, Boc--Leu-Gly-Arg-NH-Mec and Boc-Glu-Lys-Lys--NH-Mec were also hydrolysed by tryptase, however, clearly more slowly than Boc-Val-Pro--Arg-NH-Mec.

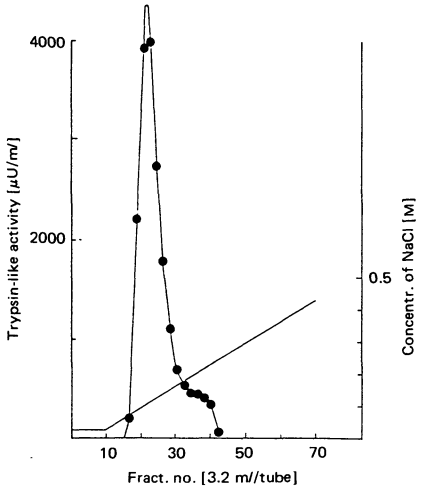

Fig. 3. DEAE-Cellulose column chromatography of trypsin-like protease-containing fractions No. 32 to 51 from the Sephadex G-100 column.

Fractions No. 32 to 51 of Fig. 2a were combined, concentrated to 30 ml with an Amicon YM 5 membrane, and dialysed against 0.01M phosphate buffer, pH 7.4. The dialysate was applied to a DEAE-cellulose column, 1.24 x 4 cm, equilibrated with 0.01M phosphate buffer, pH 7.4, and washed with 50 ml of the same buffer. Adsorbed materials were eluted with a linear gradient formed from 130 ml of the same buffer and 130 ml of the buffer containing 0.5M NaCl. The flow rate was 0.25 ml/min and fractions of 3.2 ml were collected. ●: Trypsin-like activity, ——: concentration of NaCl. Boc-Val-Pro-Arg-NH-Mec was used as a substrate.

Table 1.

a) Purification of the trypsin-like protease from the saline extract.

	Protein [mg]	Protease activity [mU]	Specific activity [mU/mg]	Recovery [%]
Saline extract	1.56	151	96.5	100
Sephadex G-100	0.21	132	619.7	87.4
DEAE-cellulose	0.02	60.9	3045	40.3

b) Purification of the trypsin-like protease from the 1.5M NaCl extract.

	Protein [mg]	Protease activity [mU]	Specific activity [mU/mg]	Recovery [%]
1.5M NaCl extract	2.45	17.5	7.1	100
Sephadex G-100	0.1	15.2	155.1	86.9

Other fluorogenic substrates containing an arginine or lysine residue in P_1 position, such as Glt-Gly-Arg-NH-Mec, Pro-Phe-Arg-NH-Mec, Arg-NH-Mec, Bz-Arg-NH-Mec and Boc-Val-Leu--Lys-NH-Mec, were very slowly hydrolysed by this protease. These results suggest that tryptase requires certain amino-acid sequence upstream the P_1 residue for maximal activity.

Fluorogenic substrates for chymotrypsin, for elastase and that for leucine aminopeptidase were practically not hydrolysed by tryptase.

Similar results were obtained with tryptase from the 1.5M NaCl extract (data not shown).

Inhibition of tryptase by Tos-Lys-CH_2Cl and iPr_2P-F

iPr_2*P*-F rapidly inhibited tryptase during incubation, and the effect was almost completed after 10 min (Fig. 5). Similar results were obtained with tryptase from the 1.5M NaCl extract (data not shown).

Tryptase was time-dependently inhibited by Tos-Lys-CH_2Cl, and its activity decreased to 15% of the original value after 30 min incubation (Fig. 5), while tryptase activity was not affected during preincubation without Tos-Lys-

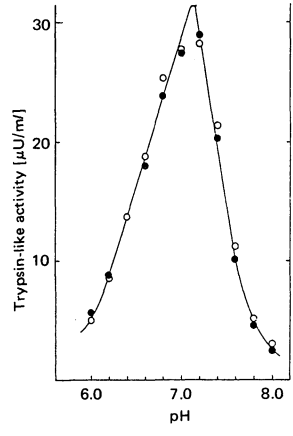

Fig. 4. Effect of pH on the activity of purified trypsin-like protease.

The activity of purified trypsin-like protease with or without preincubation was examined. ●: activity of trypsin-like protease examined at various pH values in 0.1M phosphate buffer by simultaneous addition of the protease and substrate. ○: activity of trypsin-like protease examined after preincubation at 20 °C for 10 min at various pH values in 0.1M phosphate buffer. Boc-Val-Pro-Arg-NH-Mec was used as a substrate.

Table 2. Hydrolytic activity of tryptase on various fluorogenic substrates.

	Relative activity [%]
Boc-Val-Pro-Arg-NH-Mec	100
Boc-Leu-Gly-Arg-NH-Mec	14.2
Boc-Leu-Ser-Thr-Arg-NH-Mec	17.9
Glt-Gly-Arg-NH-Mec	1.9
Boc-Phe-Ser-Arg-NH-Mec	112.3
Suc-Ala-Ala-Pro-Phe-NH-Mec	1.0
Pro-Phe-Arg-NH-Mec	1.3
Suc-Ala-Pro-Ala-NH-Mec	0.8
Leu-NH-Mec	0.2
Boc-Glu-Lys-Lys-NH-Mec	9.2
Arg-NH-Mec	0.5
Bz-Arg-Mec	3.3
Boc-Val-Leu-Lys-NH-Mec	3.8
Suc-Leu-Leu-Val-Tyr-NH-Mec	0

-CH_2Cl. Similar results were obtained with tryptase from the 1.5M NaCl extract (data not shown).

These results indicated that tryptase is a serine protease.

Inhibition of tryptase and chymase by various trypsin and chymotrypsin inhibitors

The effects of various trypsin and chymotrypsin inhibitors on tryptase and chymase were examined to characterize both proteases in more detail.

The inhibitory effects on tryptase were examined using Boc-Val-Pro-Arg-NH-Mec as a substrate in 0.1M phosphate buffer, pH 7.1. The effects on chymase were estimated using Suc--Leu-Leu-Val-Tyr-NH-Mec as a substrate in 0.1M borate buffer, pH 8.0, containing 0.15M NaCl. Final concentration of each substrate was 33μM. Results are given in Table 3.

Benzamidine, a trypsin inhibitor, competitively inhibited tryptase, however, it was not inhibitory on chymase. NPGB, like an active site titrant[19], strongly inhibited both tryptase and chymase. Antipain strongly and competitively inhibited tryptase ($K_i = 9.5 \times 10^{-7}$M) but not chymase (even above 1mM). Leupeptin strongly and competitively inhibited tryptase ($K_i = 1.5 \times 10^{-6}$M), it inhibited chymase only weakly ($I_{50} = 258\mu$M) and noncompetitively.

Indol noncompetitively inhibited chymase ($I_{50} = 680\mu$M) and was not inhibitory on tryptase. Cbz-Gly-Phe competitively inhibited chymase ($K_i = 2.6 \times 10^{-4}$M). Chymostatin extensively and competitively inhibited chymase ($I_{50} = 27.5$nM, $K_i = 9.1 \times 10^{-9}$M).

Lima bean trypsin inhibitor and soybean trypsin inhibitor strongly inhibited chymase, however, their effects on tryptase were lower than on chymase or not inhibitory on tryptase even above 5×10^{-5}M.

Among GMCHA esters examined, 4-*tert*-butylphenyl ester was strongly and competitively inhibitory on tryptase ($K_i = 1.3 \times 10^{-5}$M) but not inhibitory on chymase (even above 1mM). Phenyl and 2-benzyloxycarbonylphenyl esters were more weakly inhibitory for tryptase than 4-*tert*-butylphenyl ester and 2-benzyloxycarbonylphenyl ester was also inhibitory on chymase. These esters were not inhibitory on histamine release.

Discussion

Participation of a cellular protease on histamine release from mast cells was suggested from inhibition experiments with various protease inhibitors[1–3]. However, the role of this protease in histamine release is not clear, and information on the nature of the protease in mast cells

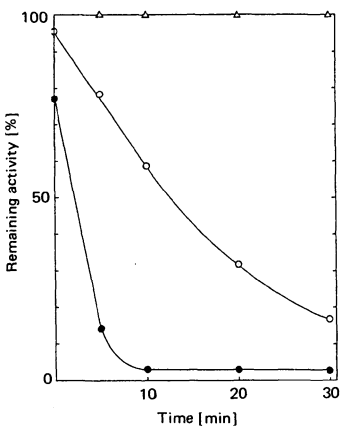

Fig. 5. Effects of Tos-Lys-CH_2Cl and iPr_2P-F on pH 7 tryptase purified from the saline extract.

A mixture of 1 ml of 0.1M phosphate buffer, pH 7.4, containing 370 μU of pH 7 tryptase and 10 μl of 100mM Tos-Lys-CH_2Cl or iPr_2P-F, dissolved in Me_2SO, was incubated at 26 °C. In a control sample Tos-Lys-CH_2Cl or iPr_2P-F was replaced by 10 μl of Me_2SO. At 5–10-min intervals 10-μl samples of the mixture were mixed with 1.49 ml of 0.1M phosphate buffer, pH 7.1, containing 2.9×10^{-4}M Boc-Val-Pro--Arg-NH-Mec and incubated for 30 min at 37 °C, and the remaining activity was determined. ○: effect of Tos-Lys-CH_2Cl; ●: effect of iPr_2P-F; △: without inhibitor.

Table 3. Inhibitory effects of substrates and inhibitors on tryptase and chymase.
N.I.; not inhibitory at 1mM, non comp.; non competitive.

	Tryptase		Chymase	
	I_{50} [μM]	K_i [M]	I_{50} [μM]	K_i [M]
Benzamidine	30	3.3×10^{-5}	N.I.	
Tos-Lys-CH_2Cl	270		N.I.	
NPGB	7.4×10^{-7}		6.3	
Antipain	0.93	9.5×10^{-7}	N.I.	
Leupeptin	1.4	1.5×10^{-6}	258	non. comp.
Indol	N.I.		680	non. comp.
Cbz-Gly-Phe	N.I.		480	2.6×10^{-4}
Tos-Phe-CH_2Cl	400		240	
Chymostatin	N.I.		2.8×10^{-4}	9.1×10^{-9}
Lima bean	2.6		1.8×10^{-2}	
Soybean	N.I.		3.4×10^{-2}	
GMCHA-OPhBu	7	1.3×10^{-5}	N.I.	
GMCHA-OPh	52.5	8.4×10^{-5}	N.I.	
GMCHA-OPhZ	200	1.9×10^{-4}	550	non comp.

has been obtained only recently. Benditt and Arase[1] found a chymotrypsin-like protease in mast cells, and named it chymase[20]. This chymase was later purified and characterized by many workers[7–9].

Schwartz et al.[21] found a Tos-Arg-OMe-hydrolytic serine protease in human pulmonary mast cells, and named it tryptase[10]. Smith et al. also purified a tryptase from human lung and characterized it[22]. These tryptases showed maximal activity at about pH 8.0. Recently, Kido et al.[11] found a tryptase and an inhibitor of it, called trypstatin, in rat mast cells, and purified and characterized the protease.

The trypsin-like protease purified by us and named pH 7 tryptase differs from the tryptases described as judged by the following criteria. Schwartz et al.[10] extracted Tos-Arg-OMe-hydrolytic tryptase by sonication in Tris buffer, pH 7.8, containing 1M NaCl. Such an extraction procedure using high salt concentration is similar to that used for chymase from rat mast cells. However, in the present work the protease was extracted by repeated freeze-thawing of the cells in saline because sonication resulted in lower recovery of activity (data not shown). The purified protease which proved to be very stable in saline had a pH optimum of 7.1, and its activity at pH 8.0 was only about one tenth of its maximal activity (see Fig. 4). This pH 7 tryptase did not hydrolyze Tos-Arg-OMe. Kido et al.[11] reported that the purified tryptase had maximal activity at pH 8.5, and that the enzyme was associated with an inhibitor, trypstatin in mast cells. The complex showed maximal activity at pH 7.5 when preincubated in buffers of various pH values for 5 min, however, without preincubation it showed maximal activity at pH 8.0 to 9.0. Then, possible contamination with trypstatin was considered in our preparation. However, as seen in Figs. 1 and 4, neither the saline extract nor the purified preparation showed a difference in the optimum pH with and without preincubation in buffers of various pH values for 10 min at 16 °C. These results, which differ from those of Kido et al., exclude the possibility of contamination of the preparation with trypstatin.

Tryptase was found in both the saline extract and 1.5M NaCl extract, and the activities in both extracts were concluded to be due to the same protease from the results of gel filtration, pH activity experiments, enzyme kinetics and inhibition experiments. Thus, the tryptase may be present in a soluble form in the cytosol and in a form bound to granules or membranes of mast cells. Further experiments are required to clarify its cellular distribution.

Tryptase seems to preferably attack arginyl and lysyl bonds, however, it seems to require specific amino-acid sequence around the sensitive arginyl or lysyl bond for maximal activity (Table 2).

Presumably, it may attack (a) specific protein component(s) in mast cells or serve to activate (a) proenzyme(s) during histamine secretion (see below).

Ishizaka[3,4] speculated that a protease(s) might participate in activation of methyltransferase and increase in cAMP in IgE-mediated histamine release from inhibition experiments with substrates and inhibitors for chymotrypsin and trypsin including iPr_2P-F. Tryptase was strongly and rapidly inhibited by iPr_2P-F as well as

chymase[1]. These results indicate that tryptase would be a candidate for the protease and that it would participate in the various events in histamine release. Tryptase was strongly and specifically inhibited by GMCHA-OPhBu^t. If tryptase participated in the histamine release from mast cells induced by various secretagogues, GMCHA-OPhBu^t could be used to probe the mechanism of histamine secretion.

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Mutumi Muramatu, Tadashi Itoh, Masao Takei and Koichi Endo Yamashiro-cho, Tokushima-shi, Tokushima 770, Japan.