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Purification and Characterization of a Novel Isoform of Mast Cell Tryptase from Rat Tongue[1]

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Rat mast cell tryptase was purified to homogeneity from rat tongue by a series of standard chromatographic procedures. Since the enzyme gave band corresponding to molecular mass of 32-35 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis and exhibited a molecular mass of 135 kDa on gel filtration, it was presumed to be a noncovalently associated tetramer. The N-terminal amino acid sequence of 50 residues of the enzyme showed the highest degree of homology with the same region in mouse mast cell protease 7 (92%), and less homology to those of tryptases from man and dog, and peritoneal cells of rats and Mongolian gerbils. The inhibitor specificity of rat tongue tryptase was similar to that of rat peritoneal mast cell tryptase free from trypstatin: it was inhibited by α_1-antitrypsin, Kunitz-type soybean trypsin inhibitor and Bowman-Birk soybean trypsin inhibitor, but these inhibitors do not inhibit the tryptases from rat skin, human lung, and dog mast cells. Judging from these results, together with other enzymatic properties, the enzyme may be a novel isoform of tryptase in rat tongue. Analysis by differential staining with peroxidase-labeled lectins of the enzyme suggested that it has tri- and/or tetraantennary complex-type oligosaccharides containing a relatively high amount of sialic acid. The immunohistochemical distribution of this enzyme indicated that the reactive antigen was specific in connective tissue but not in mucosal mast cells.

Key words: glycosylation, mast cell, N-terminal amino acid sequence, rat tongue, tryptase.

Mast cells are classified into two subsets, mucosal and connective tissue mast cells (*1*), and secrete many mediators, such as histamine, proteoglycans and neutral proteases, after activation by either an IgE-dependent or IgE-independent agonist. Tryptase, accounting for the trypsin-like activity, is one of the principal neutral proteases and serves as a selective marker of mast cells (*2, 3*).

The considerable variation of tryptases in such properties as the amino acid sequence, charge, proteoglycan binding, glycosylation, catalytic efficiency, and regulated expression in mast cell subsets have been reported in man, mouse, and dog (*4, 5*). Rat tryptase has been purified from peritoneal mast cells (*6*) and skin (*7*), and they were reported to have different enzymatic properties. However, the diversity of the amino acid sequences of rat tryptases, and the functional differences of the isoforms and their cell-specific expression are poorly understood.

In this study, we purified a major isoform of mast cell tryptase from rat tongue and characterized it. Determination of its N-terminal amino acid sequence revealed that the sequence was closely related to those of tryptases reported but not identical. The N-terminal 50 residues analyzed were different from those of rat peritoneal mast cell tryptase, the sequence of which was deduced from the cDNA cloned by RT-PCR from peritoneal cells of Lewis rats infected with *Nippostrongylus brasiliensis* (*8*). Furthermore, the molecular mass and enzymatic properties of the purified enzyme were different from those of the tryptases from rat peritoneal mast cells and skin. Analyses by staining with peroxidase-labeled lectins, Con A, DSA, and WGA, of tryptases from various organs and species indicated that the glycosylation differed. Based on these results, we also discuss microenvironmental regulation of the expression and the post-transcriptional modification of mast cell tryptases in different organs.

MATERIALS AND METHODS

Materials—All MCA substrates were purchased from the Peptide Institute (Osaka). N-Glycosidase F was from Takara (Takara Shuzo, Kyoto). S-Sepharose, Blue-Sepharose CL-6B, and arginine-Sepharose 4B were from Pharmacia (Uppsala, Sweden). TSK G3000SW was from Toyo Soda (Tokyo). Protease inhibitors, chymostatin, pepstatin, antipain, E-64, and leupeptin, were from Sigma (Tokyo), and peroxidase-labeled Con A, DSA, and WGA from Honen (Tokyo). Tryptases from rat peritoneal mast cells and

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Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; Con A, concanavalin A; DSA, *Datura stramonium* agglutinin; WGA, *Triticum vulgaris* agglutinin; MCA, 4-methyl-coumaryl-7-amide; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; Boc, *N-tert*-butyloxycarbonyl; OBzl, benzyl; Suc, succinyl; Bz, benzoyl; DFP, diisopropylfluorophosphate.

human lung were purified to homogeneity by the methods described by Kido *et al.* (*6*), and Smith *et al.* (*9*), respectively.

Enzyme and Inhibitor Assays—Enzyme preparations were examined for amidolytic activity toward various synthetic peptides as follows: 20 μl of a test or diluted sample was mixed with 2.5 μl (20 mM) of an individual substrate in 477.5 μl of 100 mM Tris-HCl, pH 8.5, or at the indicated pH, in a quartz cuvette controlled thermostatically at 25°C. The reaction was started by adding the substrate, and the amount of 7-amino-4-methylcoumarin liberated from the substrate was determined fluorimetrically with excitation and emission wavelengths of 380 and 460 nm, respectively, with a Hitachi fluorescence spectrophotometer, 650-10 MS model. One unit of enzyme activity was defined as the amount degrading 1 μmol of substrate per min. For assaying of the effects of inhibitors, purified enzyme preparations were preincubated with various inhibitors at the concentrations shown in Table III for 5 min at 25°C, and then the residual activity was measured.

Protein Measurement—Protein concentrations were measured with bicinchoninic acid protein assay reagent (Pierce) (*10*).

Molecular Mass Determination—The molecular mass of the purified enzyme was determined on double-linked TSK G3000SW columns (7.5×600 mm each) equilibrated with 50 mM sodium acetate, pH 5.0, containing 0.2 M sodium sulfate. The system was calibrated with thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa).

Preparation of Antibodies—Antiserum against the purified tryptase from rat tongue was prepared by injecting the purified enzyme into rabbits (New Zealand White). For this, 100 μg of the purified enzyme in 1 ml of saline was emulsified with an equal volume of Freund's complete adjuvant and then injected subcutaneously. For 3 weeks later, the rabbits were given weekly subcutaneous booster injections of 100 μg of the protease in Freund's incomplete adjuvant for another 3 weeks, and were bled 10 days after the last injection.

N-Terminal Amino Acid Sequence Determination—The purified enzyme (12 μg) was reduced and *S*-pyridylethylated by the method of Hermodson *et al.* (*11*). The excess reagents used for *S*-alkylation were removed by reversed-phase HPLC on an Ultrapore C3 column (4.6×75 mm). Elution was performed at 1 ml/min with a linear gradient of 0–90% acetonitrile in 0.1% trifluoroacetic acid. The N-terminal amino acid sequence of the *S*-alkylated enzyme was determined with an Applied Biosystems 492 model gas-phase sequencer/140C HPLC system. The N-terminal amino acid sequence of the enzyme after electrophoretic transfer to a ProBrot membrane (Applied Biosystems, Foster City, CA) was also analyzed according to the manufacturer's instructions.

Electrophoresis and Western Immunoblotting—SDS-PAGE was carried out by the method of Laemmli (*12*) in a 10–20% gradient gel containing 0.1% SDS at room temperature. The gel was stained for protein with Coomassie Brilliant Blue R-250 or silver. The SDS-PAGE low range standards (Bio-Rad) used as molecular weight markers were phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14.4 kDa). For Western blot analysis, the enzyme preparation (2 μg) was subjected to SDS-PAGE, and then transferred electrophoretically to an Immobilon transfer membrane (Millipore): excess sites were blocked with 1% BSA. The membrane was then incubated overnight at 4°C with a 1:250 dilution of the antiserum against the purified enzyme. Bound antibodies were detected by the anti-rabbit alkaline phosphatase method (Stratagene) according to the manufacturer's instructions. For the detection of oligosaccharides in tryptases from various organs, the enzymes electrophoretically blotted onto Immobilon transfer membranes were incubated with 1:500 dilutions of various types of lectin-peroxidase at 4°C overnight. The bound lectins were detected with ECL Western blotting detection reagents (Amersham, Amersham, UK) according to the manufacturer's instructions.

Deglycosylation with N-Glycosidase F—*N*-Glycosidase F digestion was performed by the method of Tarentino *et al.* (*13*). The purified enzyme (0.5 to 1.0 μg) in 20 μl of 0.2 M sodium phosphate buffer, pH 8.6, containing 1% SDS and 10% 2-mercaptoethanol was denatured by boiling at 100°C for 5 min. The mixture was cooled to 37°C, and then incubated with *N*-glycosidase F, at concentrations of 10-25 mU/ml, and 1.4% Nonidet P40 at 37°C for 18 h. The reaction was stopped by adding an equal volume of 10% trichloroacetic acid and 5 mg BSA, as a carrier for acid precipitation, and standing at 0°C overnight. The enzyme was precipitated by centrifugation at 11,270×*g* for 15 min, and washed two times with 100 μl of diethyl ether, and then the precipitate was subjected to SDS-PAGE (10–20% gradient gel), followed by Western immunoblotting as described above.

Immunohistochemical Staining—The rat tongues and small intestines excised were fixed in 10% buffered formalin, pH 7.2, for one day at 4°C and then embedded in paraffin. The tongue sections were cut at 4 mm thickness and placed on glass slides coated with 1% aqueous gelatin. Immunohistochemical staining was performed by the avidin-biotin-peroxidase complex method (*14, 15*). The sections were deparaffinized and then soaked in 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature to block endogenous peroxidase activity. After hydration and rinsing in PBS, the sections were incubated with normal goat serum at 1:60 dilution for 20 min at room temperature to reduce nonspecific background staining, and then at 4°C overnight with anti-rat tongue mast cell tryptase antibodies at 1:800 dilution in PBS containing 0.1% BSA in a moist chamber. The sections were then rinsed with PBS and incubated for 50 min at room temperature in a 1:200 dilution of biotinylated goat anti-rabbit IgG (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). After rinsing in PBS, the sections were incubated for 60 min at room temperature with Avidin-biotin complex. They were then rinsed in PBS, and stained for 5 min with 50 mM Tris-HCl, pH 7.6, containing 0.1% 3,3′-diaminobenzidine tetrahydrochloride, 0.02% hydrogen peroxide, and 0.65 mg/ml of sodium azide. The sections were finally washed with PBS, counterstained for 10 min with 1% methyl green, dehydrated and then mounted. Controls were prepared in the same way, except that nonimmunized rabbit IgG was used instead of the primary antibodies.

RESULTS

Purification of Mast Cell Tryptase from Rat Tongue— There are large numbers of mast cells in the skin and tongue of rats and mice (*16-18*); both, like peritoneal mast cells, are good sources of connective tissue mast cell tryptase and chymase. We purified a tryptase from rat tongue as a source.

All purification procedures were performed at 0-4°C. Rat tongue tissue (300 g) was washed with saline, minced with scissors and then extracted by homogenization in 10 volumes of 0.1 M potassium phosphate buffer, pH 8.0, in a Polytron (Kinematica GmbH) for 10 min at 0°C, and the homogenate was centrifuged at 25,000×*g* for 20 min. The precipitate was extracted again by homogenization in the same buffer. The extracts were combined, brought to pH 4.5 by the drop-wise addition of 1 M acetic acid with stirring, and then centrifuged at 25,000×*g* for 20 min. The material hydrolyzing Boc-Phe-Ser-Arg-MCA was precipitated by the addition of $(NH_4)_2SO_4$ to 70% saturation and dissolved in a small volume of 50 mM sodium acetate buffer, pH 4.5 (buffer A), and then dialyzed against buffer A overnight. Insoluble material was removed by centrifugation and the supernatant was applied to a column (4× 22 cm) of S-Sepharose which had been previously equilibrated with buffer A. Fractions of eluate were monitored for absorbance at 280 nm. The column was washed with the same buffer until the absorbance of the eluate had returned to the base line, and then the enzyme was eluted with 2,000 ml of a linear gradient of 0-1.0 M NaCl (Fig. 1A). Fractions containing activity were combined, concentrated by Diaflow ultrafiltration on a YM-30 membrane (Amicon, Tokyo), and then passed through a Sephadex G-25 column (6.5×18

TABLE I. **Purification of mast cell tryptase from rat tongue.** The details of the purification procedure and assay are given under "MATERIALS AND METHODS." The enzyme was purified from 300 g of rat tongue.

Step	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Purification (-fold)	Yield (%)
Crude extract	2.1×10^4	2.4×10^4	1.1	1.0	100
Acid treatment	1.4×10^4	2.2×10^4	1.6	1.5	92
$(NH_4)_2SO_4$ fractionation	3.8×10^3	2.2×10^4	5.8	5.3	92
S-Sepharose	4.2×10^2	2.2×10^4	5.2×10	4.7×10	92
Blue-Sepharose CL-6B	7.1×10	1.3×10^4	1.8×10^2	1.6×10^2	54
Arginine-Sepharose 4B	1.0	5.5×10^3	5.5×10^3	5.0×10^3	23
HPLC TSK-G3000SW	2.6×10^{-1}	4.6×10^3	1.8×10^4	1.5×10^4	19

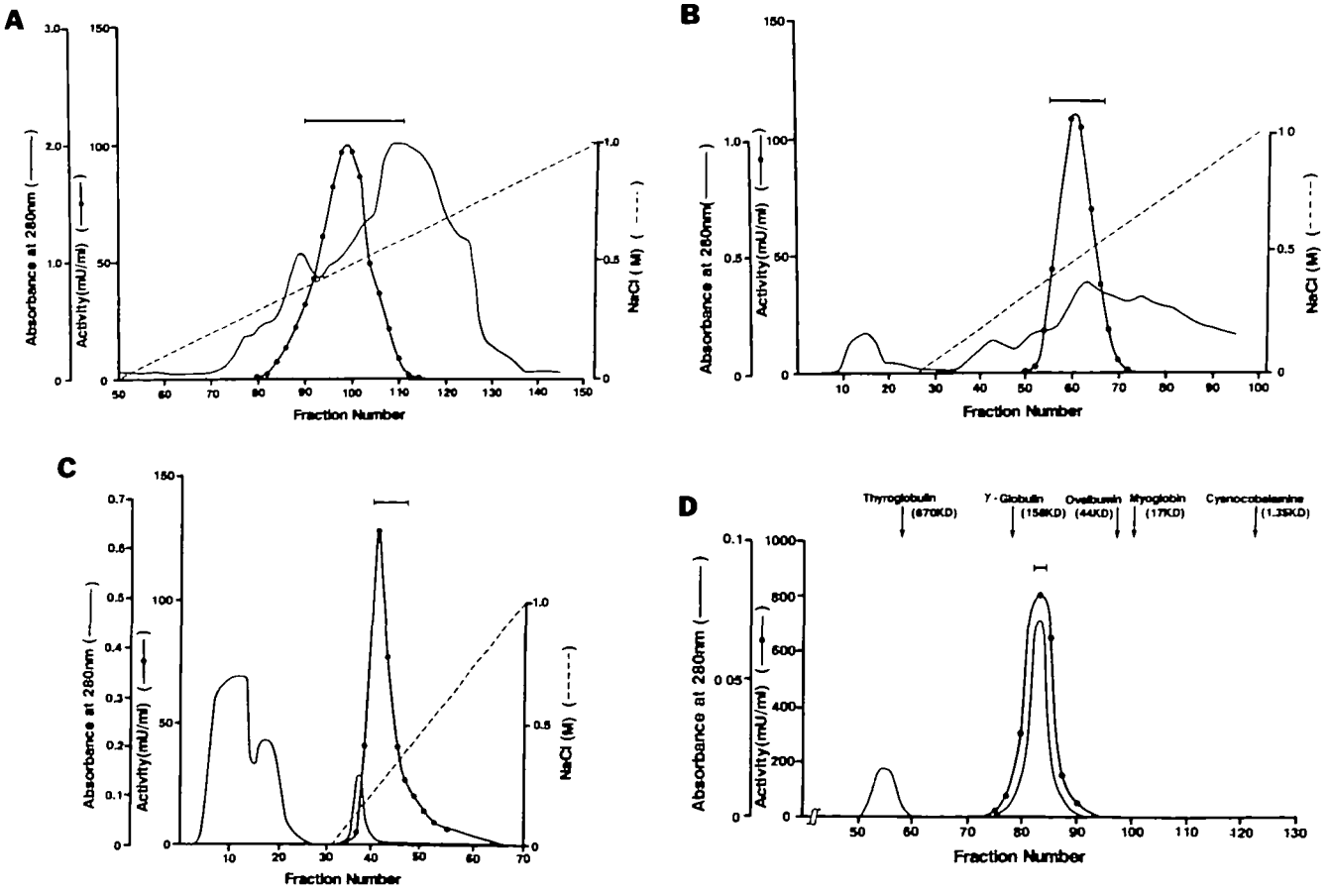

Fig. 1. **Column-chromatographic steps in the purification of the enzyme from rat tongue.** Details of the procedure are given in the text. (A) S-Sepharose. (B) Blue-Sepharose CL-6B. (C) Arginine-Sepharose 4B. (D) TSK G3000SW HPLC. Bars at the top indicate the fractions pooled. (—) Protein A_{280}; (····) gradient; (-●-) activity toward Boc-Phe-Ser-Arg-MCA.

cm) equilibrated with 20 mM sodium acetate buffer, pH 4.5, for desalting. The excluded fraction was applied to a Blue Sepharose CL-6B column (2.6×20.8 cm) previously equilibrated with 20 mM sodium acetate buffer, pH 4.5, and washed with the same buffer, and then the enzyme was eluted with a 1,000 ml linear gradient of 0-1.0 M NaCl (Fig. 1B). The active fractions eluted with 0.35-0.5 M NaCl were concentrated by ultrafiltration on a YM-30 membrane, passed through a Sephadex G-25 column (6.5×18 cm) equilibrated with 50 mM Tris-HCl buffer, pH 6.5, and then applied to an arginine-Sepharose 4B column (1.0×15 cm), which had been equilibrated with 50 mM Tris-HCl buffer, pH 6.5. The column was washed with the same buffer containing 10 mM $CaCl_2$ and then the enzyme was eluted with a 300 ml linear gradient of 0-1.0 M NaCl (Fig. 1C). The active fractions eluted with 0.15-0.35 M NaCl were combined, concentrated by ultrafiltration as described above, and then subjected to HPLC on double-linked TSK G3000SW columns (7.5×600 mm each) equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing 0.2 M sodium sulfate. The active fractions were eluted at the position corresponding to 135 kDa. The fractions containing activity were concentrated to about 500 μg/ml by ultrafiltration and stored at −80°C.

A summary of a representative purification of rat tongue tryptase is shown in Table I. The enzyme was purified approximately 1.5×10^4-fold from the crude extract. By this purification procedure, 0.3 mg of purified enzyme was obtained from 300 g of rat tongue tissue, the apparent yield of the enzyme being 19%. Although tryptase from rat peritoneal mast cells was purified with an associated protein trypstatin, no protein associated with the enzyme from rat tongue purified by the procedures described above was detected.

The purified enzyme from rat tongue exhibited an apparent molecular mass of 135 kDa on gel-permeation HPLC on a TSK G3000SW column (Fig. 1D), and gave diffuse band corresponding to molecular mass of about 32 to 35 kDa on SDS-PAGE under reducing (Fig. 2, lane 3) and non-reducing conditions (data not shown), suggesting that rat tongue tryptase is a tetramer of four glycosylated subunit proteins. In contrast, the molecular masses of the tryptases from rat peritoneal mast cells (lane 2) and human lung (lane 1), on SDS-PAGE, were 34 to 37 kDa and 33 to 36 kDa, respectively, being slightly different from that of rat tongue tryptase (Fig. 2).

Effect of pH on the Activity—Tongue tryptase showed maximal activity at pH 8.5 when the activity was measured with Boc-Phe-Ser-Arg-MCA as a substrate after preincubation of the enzyme in buffers of various pHs for 5 min (data not shown). Little activity was observed above pH 9.5 or below pH 6.0. The optimal pH was identical to that of the tryptase free from trypstatin from rat peritoneal mast cells (*6*), but was different from that of the tryptase from rat skin (*7*).

Substrate Specificity—Table II shows the relative rates of hydrolysis of various synthetic MCA substrates, all at 100 μM. Rat tongue tryptase preferentially hydrolyzed peptides with an arginine at the P_1 position [nomenclature of Schechter and Berger (*19*)], but little ones with other amino acids including lysine at the P_1 position. Of the compounds tested, Boc-Glu(OBzl)-Ala-Arg-MCA and Boc-Phe-Ser-Arg-MCA were the best substrates. The substrates with basic amino acids at positions P_1 and P_2 other than Boc-Gln-Arg-Arg-MCA were poor substrates for this enzyme, although these dibasic substrates are preferentially hydrolyzed by the tryptases from human and rat skin (*7, 20, 21*). Furthermore, the enzyme, unlike trypsin, scarcely hydrolyzed substrates with a single basic amino acid, such as Bz-Arg-MCA, suggesting that it requires more than a single amino acid residue for its hydrolytic activity.

Inhibitor Specificity—The effects of various protease inhibitors and other compounds on the activity of rat tongue tryptase are summarized in Table III. DFP at a concentration of 10 μM inhibited the activity, indicating that a serine group is necessary for the activity. Phenylmethylsulfonyl fluoride at 10 μM inhibited the activity little but at con-

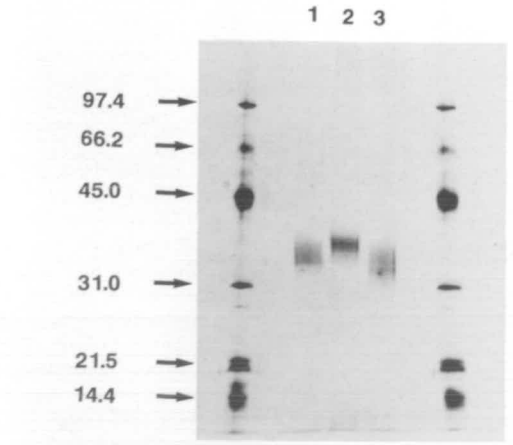

Fig. 2. **SDS-PAGE of the purified enzyme.** SDS-PAGE was carried out by the method of Laemmli (*12*) in a 10-20% gradient gel containing 0.1% SDS at room temperature. The gel was stained for protein with silver. Lane 1, human lung mast cell tryptase; lane 2, rat peritoneal mast cell tryptase; lane 3, the purified enzyme from rat tongue. Standard molecular weight markers are shown, with their molecular masses in kDa, on the left.

TABLE II. **Activities of the purified enzyme toward synthetic substrates.** Relative activities were calculated as percentages of the activity toward Boc-Phe-Ser-Arg-MCA.

Substrate[a]	Activity (mU/ml)	%
Boc-Gln-Gly-Arg-MCA	18.9	19.0
Boc-Leu-Gly-Arg-MCA	10.5	10.6
Boc-Leu-Arg-Arg-MCA	7.8	7.8
Boc-Leu-Lys-Arg-MCA	13.9	14.0
Boc-Gly-Arg-Arg-MCA	3.8	3.8
Boc-Glu-Lys-Lys-MCA	5.3	5.3
Boc-Glu(OBzl)-Gly-Arg-MCA	54.8	55.0
Boc-Glu(OBzl)-Ala-Arg-MCA	104.5	105.0
Boc-Phe-Ser-Arg-MCA	99.5	100.0
Boc-Gln-Arg-Arg-MCA	55.0	55.3
Boc-Val-Pro-Arg-MCA	64.0	64.3
Pro-Phe-Arg-MCA	0.0	0.0
Boc-Val-Leu-Lys-MCA	3.2	3.2
Boc-Ala-Gly-Pro-Arg-MCA	6.0	6.0
Boc-Ile-Glu-Gly-Arg-MCA	0.0	0.0
Glt-Gly-Arg-MCA	0.0	0.0
Z-Arg-Arg-MCA	4.5	4.5
Bz-Arg-MCA	2.6	2.6
Suc-Ala-Ala-Pro-Phe-MCA	3.8	3.8

[a]Boc, *N-tert*-butoxycarbonyl; Z, carbobenzoxyl; Bz, benzoyl; Bzl, benzyl; Suc, succinyl; Glt, glutaryl.

centrations over 1 mM significantly inhibited it (data not shown). The enzyme activity was markedly inhibited by all of the inhibitors of trypsin-type serine proteases tested, such as microbial inhibitors, leupeptin and antipain, and natural protein or peptide inhibitors, aprotinin, Bowman-Birk soybean trypsin-inhibitor, Kunitz-type soybean trypsin-inhibitor, and α_1-antitrypsin. These natural protein or peptide inhibitors, however, do not inhibit the tryptases from rat skin, various human organs and dog mast cells (*7, 9, 20, 22, 23*). Inhibitors of chymotrypsin-type serine, metallo, aspartic, and thiol proteases, and an inhibitor of elastase had no effect on the activity. Furthermore, the enzyme was not affected by $CaCl_2$ or NaCl, or by a chelating agent. Although human tryptase is significantly inhibited by 0.6 M NaCl (*9, 20, 23*), the enzyme from rat tongue was not inhibited by this concentration of NaCl (data not shown). These results indicate that the inhibitor specificity of rat tongue tryptase is similar to that of rat peritoneal mast cell tryptase free from trypstatin, but distinct from those of the tryptases from human, dog, and rat skin tryptase.

N-Terminal Amino Acid Sequence—The N-terminal 50 residues of *S*-alkylated tryptase (300 pmol) from rat tongue were analyzed and the alignment is shown in Table IV. The comparison with the sequences of human tryptase isoforms I, II/β, III, and α (*5, 24, 25*), dog tryptase (*26*), mouse tryptases 6 and 7 (also known as mouse mast cell proteases 1 and 2, respectively) (*27, 28*), and peritoneal mast cell tryptases of Lewis rats (*8*) and Mongolian gerbils (*29*) infected with *N. brasiliensis* is shown in Fig. 3. Although the protein band was diffuse (Fig. 2), a single

TABLE III. **Inhibitor sensitivity of the purified enzyme.** The purified enzyme was preincubated with each compound at the indicated concentration in 100 mM Tris-HCl, pH 8.5, for 5 min at 25°C. Then the activity toward Boc-Glu(OBzl)-Ala-Arg-MCA was assayed as described under "MATERIALS AND METHODS." Relative activities were calculated as percentages of that of the enzyme without an inhibitor.

Addition	Final concentration	Relative activity (%)
None		100.0
DFP	10 μM	9.8
Phenylmethylsulfonyl fluoride	10 μM	97.0
Leupeptin	10 μM	4.8
Aprotinin	10 μM	3.6
Antipain	10 μM	13.8
Bowman-Birk soybean trypsin inhibitor	10 μM	12.1
Kunitz-type soybean trypsin inhibitor	10 μM	11.1
α_1-Antitrypsin	10 μM	17.3
Chymostatin	10 μM	100.0
E-64c	10 μM	95.3
Pepstatin	10 μM	99.6
Elastatinal	10 μM	96.4
Phosphoramidon	10 μM	100.0
$CaCl_2$	1 mM	100.0
EDTA	1 mM	97.4
NaCl	1 mM	100.0

TABLE IV. **NH_2-terminal sequence of rat tongue mast cell tryptase.** Approximate yields of phenylthiohydantoin derivatives were calculated from peak areas on HPLC. The sample used was 300 pmol and repetitive yield was 93.6%.

Cycle No.	PTH amino acid	Yield (pmol)	Cycle No.	PTH amino acid	Yield (pmol)
1	Ile	143.7	26	Met	21.0
2	Val	108.3	27	His	8.7
3	Gly	117.0	28	Phe	14.4
4	Gly	106.2	29	Pec[a]	N.Q.[b]
5	Gln	102.6	30	Gly	9.0
6	Glu	88.5	31	Gly	18.0
7	Ala	99.0	32	Ser	9.0
8	Ser	39.6	33	Leu	24.9
9	Gly	64.5	34	Ile	9.3
10	Asn	83.7	35	His	5.4
11	Lys	122.7	36	Pro	5.7
12	Trp	40.5	37	Gln	13.2
13	Pro	52.5	38	Trp	2.1
14	Trp	18.6	39	Val	21.3
15	Gln	55.8	40	Leu	21.9
16	Val	71.7	41	Thr	6.3
17	Ser	23.7	42	Ala	4.5
18	Leu	49.2	43	Ala	5.4
19	Arg	40.5	44	His	5.1
20	Val	52.2	45	Pec[a]	N.Q.[b]
21	Asn	15.9	46	Val	19.8
22	Asp	30.6	47	Gly	8.1
23	Thr	19.5	48	Pro	3.6
24	Tyr	24.3	49	Asn	11.7
25	Trp	12.9	50	Lys	8.1

[a]*S*-pyridylethylcysteine, [b]not quantitated.

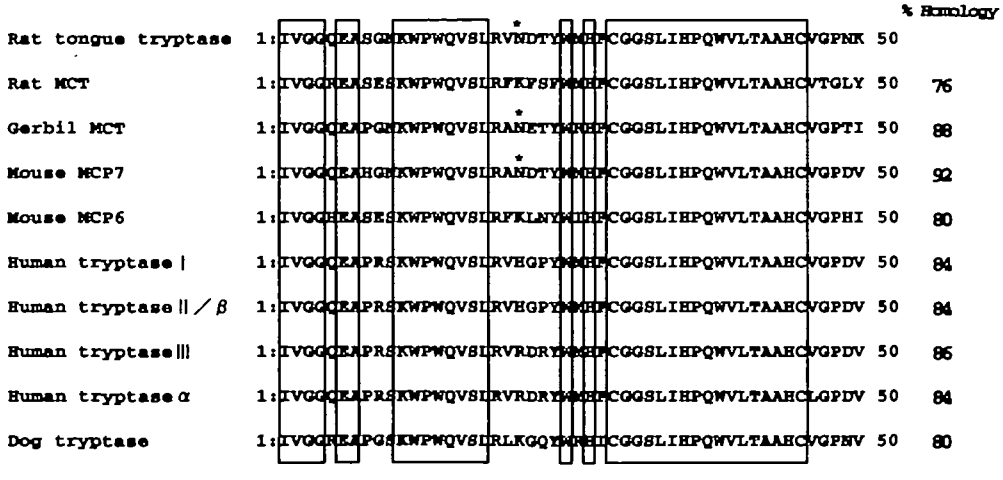

Fig. 3. **Comparison of the N-terminal amino acid sequence of rat tongue tryptase with those of other known tryptases.** The amino acid sequences of mast cell tryptases from peritoneal cells of Lewis rats (*8*) and Mongolian gerbils (*29*) infected with *Nippostrongylus brasiliensis*, mouse mast cell proteases 6 (*27*) and 7 (*28*), human tryptases I, II/β, III, and α (*5, 24, 25*), and dog tryptase (*26*). Identical amino acid residues among the ten sequences are boxed. Potential N-linked glycosylation sites, *. MCT, mast cell tryptase; MCP, mast cell protease.

amino acid sequence was detected without any contamination. Since no other tryptase was detected in rat tongue, the purified enzyme may be the major isoform of tryptase in rat tongue. The homology of the sequence of this enzyme was highest with that of mouse mast cell tryptase 7 (*28*), less homology being observed with the sequences of tryptases recently cloned by RT-PCR from peritoneal cells of rats and Mongolian gerbils (*8, 29*).

Analysis of Oligosaccharides—All characterized tryptases are known or predicted to have either one or two sites of *N*-linked glycosylation. On analysis of the N-terminal amino acid sequence of rat tongue tryptase, one predicted site of *N*-glycosilation was observed at Asn^{21} and the yield of the phenylthiohydantoin derivative was unexpectedly decreased (Table IV), suggesting that Asn^{21} is glycosylated. To characterize the carbohydrate side chains of the tryptase from rat tongue, differential staining of the enzyme on Immobilon membranes was carried out using various lectin-peroxidase reagents (Fig. 4). As a positive control, two glycoproteins (ovalbumin and transferrin) with known oligosaccharide chain structures and non-glycosylated protein BSA, as a negative control, were also analyzed. Since BSA was not stained with these lectin reagents, the bands observed in Fig. 4 do not reflect nonspecific interactions of lectins with non-glycoproteins. Rat tongue tryptase reacted with both DSA and WGA, but not with Con A, suggesting that it has a sugar chain with tri- and/or tetraantennary complex-type oligosaccharides containing a relatively high amount of sialic acid (*30-34*). The staining of rat peritoneal mast cell tryptase with DSA, but not with Con A or WGA, suggests that it has tri- and/or tetraantennary complex-type chains containing a low amount of sialic acid. On the other hand, human lung tryptase may be a glycoprotein mainly glycosylated with high-mannose, biantennary complex or hybrid type oligosaccharides and partly glycosylated with tri- and/or tetraantennary complex-type sugar chains, since it reacted with Con A, *i.e.* poorly with DSA and not with WGA (*35, 36*). These results indicate that the glycosylation pattern of tryptases varies among species and organs.

Treatment with N-Glycosidase F—In order to clarify whether or not the difference in molecular mass between tryptases from rat tongue and peritoneal mast cells, the tryptases were treated with *N*-glycosidase F, which cleaves high-mannose, and bi-, tri-, and tetraantennary chains from glycoproteins (*13*). Treatment of human lung tryptase with *N*-glycosidase F for comparison revealed the disappearance of a diffuse band and the appearance of a single major band corresponding to a molecular masses of 28 kDa, which corresponds to the theoretical molecular masses of the polypeptide backbones of α- and β-human tryptases (*5, 24, 25*) (Fig. 5A, lane 2). In contrast, treatment of the tryptases from rat tongue and peritoneal mast

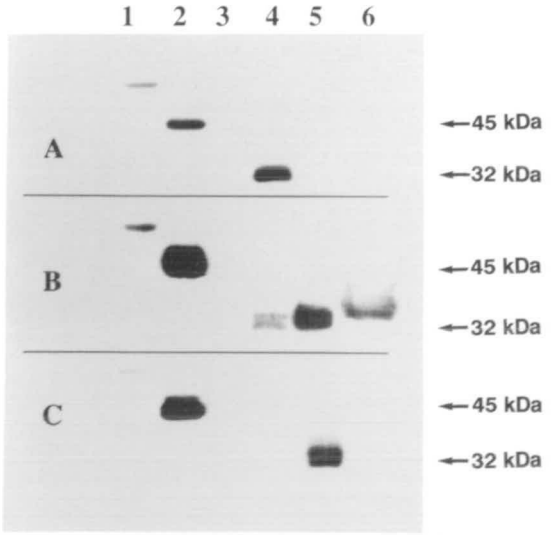

Fig. 4. **Detection of oligosaccharides with lectin-peroxidase reagents.** After SDS-PAGE, tryptases were electrophoretically transferred to an Immobilon transfer membrane and then binding of Con A (A), DSA (B), and WGA (C) to these proteins was visualized by staining with ECL reagents as described under "MATERIALS AND METHODS." Human lung tryptase (lane 4), rat tongue tryptase (lane 5), and rat peritoneal mast cell tryptase (lane 6), as well as transferin (lane 1) and ovalbumin (lane 2), as positive controls, and BSA (lane 3), as a negative control, were analyzed.

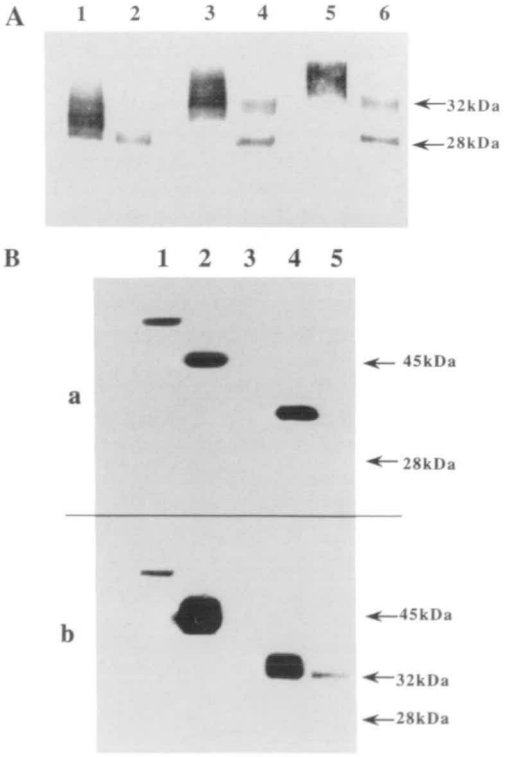

Fig. 5. **Western immunoblotting (A) and lectin-peroxidase staining (B) of tryptases before and after treatment with *N*-glycosidase F.** The deglycosylation of tryptases with *N*-glycosidase F was performed as described under "MATERIALS AND METHODS." A: Tryptases from human lung (lanes 1 and 2), rat tongue (lanes 3 and 4), and rat peritoneal mast cells (lanes 5 and 6), before (lanes 1, 3, and 5) and after (lanes 2, 4, and 6) treatment with *N*-glycosidase F, were subjected to SDS-PAGE, followed by Western immunoblotting with anti-rat tongue tryptase, which immunologically reacted with these tryptases, as described in the text. B: (a) Binding of Con A to transferin (lane 1), ovalbumin (lane 2), BSA (lane 3), and human lung tryptase before (lane 4) and after (lane 5) treatment with *N*-glycosidase F. (b) Binding of DSA to transferin (lane 1), ovalbumin (lane 2), BSA (lane 3), and rat tongue tryptase before (lane 4) and after (lane 5) treatment with *N*-glycosidase F. The detection of oligosaccharides with lectin-peroxidase reagents was performed as described under "MATERIALS AND METHODS."

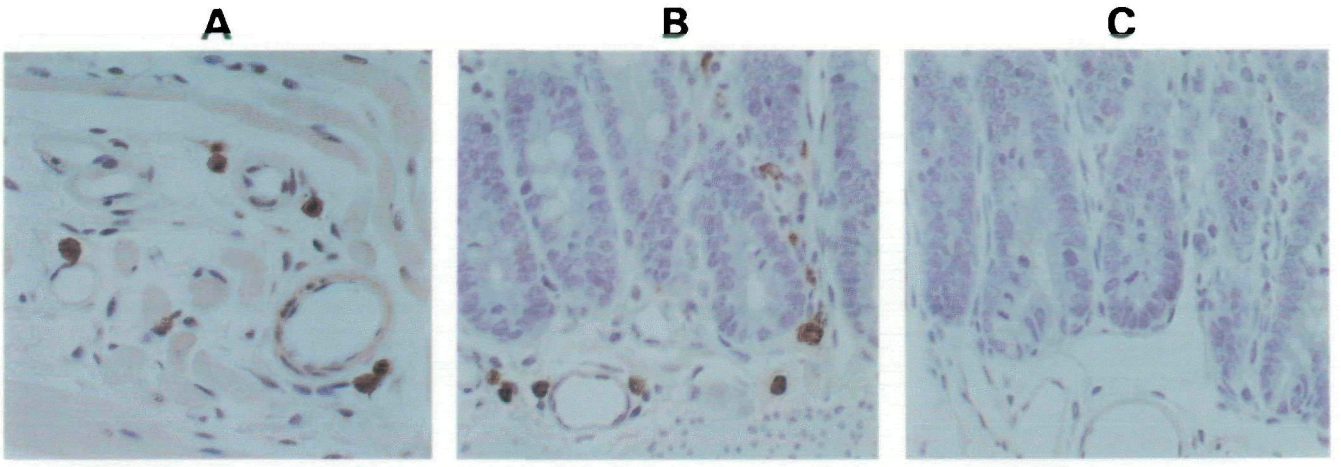

Fig. 6. **Immunohistochemical localization of the purified enzyme in rat tongue and small intestine.** Light micrographs show the immunohistochemical localization of the purified enzyme in rat tongue and small intestine. Positive staining with the antibody against rat tongue tryptase can be seen in connective tissue mast cells around blood vessels in rat tongue (A). Mucosal mast cells in the small intestine were specifically stained with anti-atypical chymase (also known as RMCP-II) (*39, 40*) (B), but not with the antibody against rat tongue tryptase (C). Magnification (A ×368, B ×368, C ×368).

cells with *N*-glycosidase F, respectively, generated two bands corresponding to molecular masses of 32 and 28 kDa. Similar results were obtained on deglycosylation with a double amount of *N*-glycosidase F and a double denaturation time prior to treatment with the enzyme (data not shown). After reaction with the enzyme, the 32 kDa band was stained with peroxidase-labeled DSA, but not the 28 kDa band (Fig. 5B, b, lane 5). These results indicated that the complex-type tri- and/or tetraantennary asparagine-linked glycans of rat tryptases are partly resistant to *N*-glycosidase F, although almost complete deglycosylation of the human lung tryptase was observed (Fig. 5B, a, lane 5). The previous reports support the efficiency of the cleavage by *N*-glycosidase F of complex-type tri- and/or tetra-antennary oligosaccharides in Fig. 5 (*13, 37*). The N-terminal 30 residues of the 32 and 28 kDa band materials from rat tongue and peritoneal mast cells analyzed showed that the sequence of each material was identical to that of rat tongue tryptase. Taken together, these results indicate that the 32 kDa protein of rat tryptases after deglycosylation may be a poor- or non-deglycosylated part of the enzyme, and the polypeptide backbones of the 32 and 28 kDa proteins may be identical. Furthermore, the N-terminal 30 residues of the rat peritoneal mast cell tryptase purified were not identical to the sequence deduced from cDNA of peritoneal cells of Lewis rats infected with *N. brasiliensis* (*8*).

Immunochemical and Immunohistochemical Studies—The specificity of the polyclonal antibody raised against the purified rat tongue tryptase was evaluated by Western immunoblotting. The antibody reacted with not only rat tongue tryptase but also the tryptases from rat peritoneal mast cells and human lung (data not shown). Similar results were obtained with a polyclonal antibody raised against the tryptase from rat peritoneal mast cells. The cross-reactivity may be supported by the high similarity of the amino acid sequences of these tryptases, as shown in Fig. 3. Both antibodies did not react with rat or porcine pancreatic trypsin.

A light micrograph showed that connective tissue mast cells around blood vessels in rat tongue were specifically stained with the antibody against rat tongue tryptase (Fig. 6A). On the other hand, no deposits of the reaction products were observed in mucosal mast cells in the small intestine (Fig. 6C), although mucosal mast cells were specifically identified on immunohistochemical staining with the antibody against atypical chymase (*38, 39*) (Fig. 6B). These results indicate that unlike human tryptase (*40, 41*), rat tongue tryptase is distributed only in connective tissue mast cells, but *i.e.*, it is not distributed or it is distributed in an undetectable level in mucosal mast cells of rat. These results are consistent with those of recent studies (*8, 41*).

DISCUSSION

The diversity of mast cell tryptases in man, dog, and mouse has been reported (*4*), but not clearly in rat to date. The different isoforms of tryptase in rat may be expressed in varying ratios in individual organs, although it still remains unclear whether these variations are functionally consequential. In this study we purified a novel isoform of mast cell tryptase from rat tongue which has different enzymatic properties (*23, 40*), such as molecular mass, substrate specificity, and inhibitor specificity, from the tryptases from rat peritoneal mast cells (*6*), rat skin (*7*), dog mastocytomas (*22*), and various human tissues (*2, 9, 20, 21*). The similarity of the sequence of the N-terminal 50 amino acid residues of rat tongue tryptase with that of mouse mast cell tryptase 7 was the highest, being higher than that with those of the tryptases from peritoneal cells of Lewis rats and Mongolian gerbils infected with *N. brasiliensis*. Although mouse mast cell tryptases 6 and 7, and peritoneal mast cell tryptases from Lewis rats and Mongolian gerbils have not been purified or characterized, the characteristics of mouse mast cell tryptase 7 may be similar to those of rat tongue tryptase. The N-terminal 25 amino acid residues of rat skin tryptase (*7*) were identical to those of rat tongue tryptase, but the inhibitor and substrate specificities, and the optimal pH were different. Overall, it is suggested that the tryptase purified from rat

tongue is a novel isoform of rat tryptase. Recently, we successfully cloned the cDNA encoding rat tongue tryptase and sequence analysis revealed that it is a novel isoform of rat tryptase (Takashima *et al.*, manuscript in preparation).

Rat tongue tryptase was purified free from trypstatin. Immunohistochemical studies, however, demonstrated that trypstatin is localized in the granules in rat connective tissue mast cells in various organs including tongue (H. Kido, unpublished results). These findings suggest that trypstatin is dissociated from rat tongue tryptase during the purification steps, probably at the S-Sepharose ion-exchange and/or Blue-Sepharose column chromatography steps.

The purified tryptase gave diffuse band corresponding to a molecular mass of about 32 to 35 kDa on SDS-PAGE, which is slightly different from that of the tryptase from rat peritoneal mast cells. However, treatment with *N*-glycosidase F of these tryptases gave deglycosylated 28 kDa and poor- or non-deglycosylated 32 kDa proteins. The incomplete access of *N*-glycosidase F together with the staining of these glycoproteins with peroxidase-labeled WGA and/or DSA suggests that the rat tryptases from tongue and peritoneal mast cells contain complex-type tri- and/or tetraantennary asparagine-linked glycans. The results of differential staining with various lectins of tryptases from human lung, rat tongue, and rat peritoneal mast cells suggest that tryptase is post-translationally modified through glycosylation in different manners, depending on the microenvironment of each organ, although it remains unclear whether this variation is functionally consequential.

Immunohistochemical studies involving polyclonal antibodies against the purified enzyme revealed immunoreactive deposits in the connective tissue mast cells of various organs but not in mucosal mast cells of rat. This is consistent with the data obtained in immunohistochemical studies with antibodies against tryptase from rat skin (*41*). However, the possibility that the expression of an another tryptase isoform in mucosal mast cells which is not cross-reactive with the antibody against this tryptase can not be completely ruled out.

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