Human mast cell tryptase isoforms: separation and examination of substrate-specificity differences

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Tryptases are trypsin-like enzymes found in mast cell granules that appear to exist as tetramers. These enzymes are not controlled by blood plasma proteinase inhibitors and only cleave a few physiological substrates *in vitro*, including high-molecular-mass kininogen (HMMK) and vasoactive intestinal peptide (VIP). Purified human lung mast cell tryptase (HLT) contained two bands of approx. molecular mass 29 and 33 kDa on SDS/PAGE. These two forms of HLT have been separated by chromatography on a cellulose phosphate column, with the high-molecular-mass form (high-HLT) being eluted with 10 μ M heparin and the low-molecular-mass form (low-HLT) subsequently eluted with 1 M NaCl. Removal of asparagine-linked carbohydrate caused both isoforms to run as single sharp bands on SDS/PAGE, differing slightly in molecular mass. Separation

of these two isoforms of tryptase shows that tetramers consist of four homologous subunits rather than mixtures of the two isoforms. Using HMMK and VIP as substrates, these two forms of HLT were found to differ with regard to specificity and rate of cleavage. High-HLT initially cleaved HMMK at Arg-431 within the C-terminal anionic binding region of the molecule, whereas low-HLT cleaved HMMK simultaneously at multiple sites within the C-terminal portion of the molecule. On the basis of HPLC peptide mapping, each isoform also cleaved VIP at different sites. Comparison of cleavage rates based on the active-site concentrations of titrated isoforms showed that low-HLT cleaved HMMK more rapidly than did high-HLT. These two isoforms may represent different gene products or they may result from post-translational modification.

INTRODUCTION

Tryptases are trypsin-like serine proteases found in the cytoplasmic granules of mast cells that cleave peptide substrates on the carboxyl side of lysine and arginine residues, but differ from trypsin in that they have little or no activity on denatured proteins such as casein and are resistant to inhibition by natural inhibitors of trypsin. On mast cell degranulation, tryptase is released along with other mediators into the extracellular milieu (Schwartz et al., 1981a). The functions of tryptase after its release from the cell are not clearly understood, but studies *in vitro* have shown that it cleaves several biologically significant molecules, including high-molecular-mass kininogen [HMMK (Maier et al., 1983)] and vasoactive intestinal peptide [VIP (Caughey et al., 1988; Tam and Caughey, 1990)].

Human mast cell tryptases have been isolated from lung (Smith et al., 1984; Schwartz et al., 1981b), skin (Harvima et al., 1988), pituitary (Cromlish et al., 1987) and a human mastocytoma cell line (Butterfield et al., 1990). In all these cases the purified enzyme migrated as two major bands on SDS/PAGE with molecular mass differing by about 4 kDa. In addition, purified bovine mast cell tryptase also yielded two bands on SDS/PAGE (Fiorucci et al., 1992). These findings have led to speculation that the tetramer observed on gel filtration is comprised of two different subunits represented by the two species seen on SDS/PAGE.

Although two different tryptase cDNAs have been cloned from a human lung tissue cDNA library (Miller et al., 1989, 1990) and two additional tryptase cDNAs were found in a human skin cDNA library (Vanderslice et al., 1990), there is no information on possible functional differences between the vari-

ous forms of tryptase. Previous work from this laboratory has shown that the two major SDS/PAGE bands could be partially separated and they were shown to be immunologically cross-reactive (Smith et al., 1984). We have now isolated these two forms of tryptase, which are designated high-HLT (high-molecular-mass human lung tryptase) and low-HLT (low-molecular-mass human lung tryptase). These tryptase isoforms are shown to cleave natural substrates HMMK and VIP differently at different rates.

EXPERIMENTAL

Materials

Frozen human lung tissue was obtained through the National Disease Research Interchange (Philadelphia, PA, U.S.A.). Cellulose phosphate (fibrous form), cetylpyridinium chloride, heparin (porcine; ammonium salt), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), *N*-carbobenzoxyl-Lys-thiobenzyl ester (Cbz-Lys-SBzl), *N*-carbobenzoxyl-Phe-Arg-7-amidino-4-aminomethylcoumarin (CBZ-Phe-Arg-NMec), *N*-carbobenzoxyl-Arg-Arg-7-amidino-4-aminomethylcoumarin (CBZ-Arg-Arg-NMec), Brilliant Blue G Colloidal Concentrate, trichloroacetic acid and fish gelatin were purchased from Sigma. Mes, NaCl, NaN₃, glycerol, Hepes, Brij 35, Me₂SO, enzyme-grade sodium acetate trihydrate and HPLC-grade acetonitrile were products of Fisher Scientific. Other materials and their suppliers were as follows: enzyme-grade (NH₄)₂SO₄ from ICN Biomedicals; HPLC-grade trifluoroacetic acid (TFA) from Pierce Chemical Co.; Toyopearl Butyl 650-M hydrophobic interaction chromatography column material from TosoHaas; VIP from Bachem; Nucleosil C₁₈ HPLC column (glass-lined; 30 nm pore; 4 mm $\times$ 250 mm) from SGE;

Abbreviations used: HMMK, high-molecular-mass kininogen; high-HLT, high-molecular-mass tryptase; low-HLT, low-molecular-mass tryptase; VIP, vasoactive intestinal peptide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); Me₂SO, dimethyl sulphoxide; Cbz, carbobenzoxy; SBzl, thiobenzyl ester; NMec, 7-amidino-4-aminomethylcoumarin; PVDF, poly(vinylidene difluoride); MUGB, 4-methylumbelliferyl-*p*-guanidinobenzoate hydrochloride; TFA, trifluoroacetic acid; TTBS, Tween Tris-buffered saline.

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Immobilon-P poly(vinylidene difluoride) (PVDF membrane) from Millipore; PVDF-Plus Transfer Membrane from MSI; Protein G Gold kit from Bio-Rad; *N*-Glycanase from Genzyme. Monoclonal antibodies to the light chain of HMMK were kindly provided by Dr. A. Schmaier (University of Michigan, Ann Arbor, MI, U.S.A.). Fresh-frozen human blood plasma for the purification of HMMK (Johnson et al., 1987) was obtained from the American Red Cross. All other reagents were ACS grade unless otherwise specified.

Assay of trypsin activity

Enzyme activity was monitored using the substrate Cbz-Lys-SBzl, in the presence of DTNB for detection of the benzoyl thiol product. One unit of activity was defined as the amount of enzyme needed to produce an absorbance change of 1.0/min at 410 nm. Specific activity refers to units per A_{280} of the sample. An appropriate amount of enzyme was added to assay buffer (0.1 M Hepes, 10 μ M heparin, 10% glycerol, 0.05% Brij 35, 0.02% NaN_3 , pH 7.5) containing 1 mM DTNB to a total volume of 980 μ l. Assays were started by adding 20 μ l of 20 mM Cbz-Lys-SBzl in Me_2SO to a final volume of 1 ml (final substrate concentration equalled 0.4 mM) and the change in A_{410} /min was monitored for 3 min at room temperature. The fluorescent substrates CBZ-Phe-Arg-NMec and CBZ-Arg-Arg-NMec were used at a concentration of 5 μ M to compare to isoforms. For these assays the fluorimeter was standardized with 0.5 μ M 7-amino-4-aminomethylcoumarin in the assay buffer described above, using excitation and emission wavelengths of 380 nm and 460 nm respectively (Zimmerman et al., 1976). Finally, active-site titrations were performed on each trypsin isoform using 4-methylumbelliferyl-*p*-guanidinobenzoate hydrochloride (MUGB) as reported by Jameson et al. (1973).

Isolation of trypsin isoforms

Frozen human lung tissue (500 g) was cut into small pieces and disrupted in a Waring blender with 500 ml of cold distilled water for 10–15 s. The homogenate was centrifuged at 25400 *g* for 20 min at 5 °C and the supernatant was discarded. The blending and centrifuging process was repeated using cold 20 mM Mes with 0.15 M NaCl and 0.02% NaN_3 at pH 6.1 in place of distilled water until supernatant fractions were visibly free of haemoglobin, usually five or six cycles. Trypsin activity was extracted from the tissue by repeating the blending and centrifuging process four times using 500 ml of cold 20 mM Mes, pH 6.1, containing 2 M NaCl and 0.02% NaN_3 each time. The viscosity of the crude extract was reduced by adding an equal volume of 2% cetylpyridinium chloride in 10 mM Mes/0.02% NaN_3 pH 6.1, and incubating at room temperature for 30 min followed by centrifugation at 25400 *g* (5 °C) for 60 min. The extract was then made 2 M in $(\text{NH}_4)_2\text{SO}_4$, centrifuged as above, and mixed with 50 ml of Toyopearl Butyl 650-M column material equilibrated with 10 mM Mes, pH 6.1, containing 0.5 M NaCl, 2 M $(\text{NH}_4)_2\text{SO}_4$, 20% glycerol and 0.02% NaN_3 . After incubation at room temperature for 45 min, the mixture was poured into a chromatography tube (2.5 cm $\times$ 12 cm) and washed extensively with equilibration buffer. All of the trypsin activity bound to the column material and was subsequently eluted with a 250 ml linear gradient of decreasing ionic strength from 2.0 to 0 M $(\text{NH}_4)_2\text{SO}_4$; 2 ml fractions were collected at 1 min intervals. Active fractions were pooled, made 0.05% in Brij 35, and dialysed against 4 litres of 10 mM Mes, pH 6.1, containing 0.3 M NaCl and 0.02% NaN_3 for 3 h at 4 °C, followed by dialysis against 4 litres of the same buffer containing 20% glycerol for 3 h. This pool was then loaded on to a 20 ml cellulose phosphate

column equilibrated with the last dialysis buffer. The column was washed with equilibration buffer, followed by elution of high-HLT with 10 μ M heparin in the same buffer. Low-HLT was then eluted with 1 M NaCl in equilibration buffer. The overall recovery of activity was usually 25%, with the high-HLT and low-HLT fractions accounting for approx. 80% and 20% respectively of the total activity.

Cleavage of HMMK

Reactions were performed at room temperature in 1 ml of assay buffer containing 200 μ g (1.7 nmol) of HMMK and the appropriate amount of enzyme. Preliminary experiments were performed to determine the relative amount of each trypsin form needed for approximately equal rates of cleavage. HMMK (200 μ g, 1750 pmol) was incubated with high-HLT (1 μ g, 34.7 pmol of active enzyme) or low-HLT (0.0014 μ g, 1.31 pmol of active enzyme) in a total volume of 1.5 ml in assay buffer at room temperature. The molar ratios of active enzyme to substrate (E/S) were 1:52 for high-HLT and 1:1336 for low-HLT. Aliquots of 40 μ g of HMMK were withdrawn at 0, 5, 10, 20, 40 and 60 min, and the reaction was immediately terminated by precipitation of the proteins with 20% trichloroacetic acid, followed by centrifugation and washing of the precipitates twice with 700 μ l of cold 10% trichloroacetic acid and twice with 700 μ l of cold acetone, before drying at room temperature. Samples equivalent to 5 μ g of HMMK were run in each lane of the gels in Figure 2. Cleavage rates were determined by densitometric measurement of the HMMK band on gels. Samples for the Western blot shown in Figure 3, were similarly prepared except that the reaction was stopped by precipitation with 9 vol. of ice-cold ethanol. Precipitates were collected by centrifugation [12000 *g* (14000 rev./min); 4 °C; 5 min] after 12 h incubation at –20 °C and were dried at room temperature.

Electrophoresis and Western-blot analysis

SDS/PAGE analysis of cleavage products was carried out under reducing conditions on 1.5 mm-thick polyacrylamide gels as described by Bury (1981) and fixed and stained with colloidal Coomassie Brilliant Blue according to the manufacturer's protocol. Electrophoresis of proteins to be transferred was carried out as above except that the gel thickness was reduced to 1.0 mm. Transfer was carried out for 40 min in a Bio-Rad Transblot Semi-Dry Transfer Cell using 48 mM Tris, pH 9.2, containing 39 mM glycine and 20% methanol as the transfer buffer. Membranes were blocked with 3% fish gelatin for 1 h and exposed to primary antibody for 15 h with gentle shaking at room temperature. The primary antibody solution consisted of a 1:1000 dilution of a monoclonal antibody to the C-terminal region of HMMK in 20 mM Tris, pH 7.4, containing 500 mM NaCl, 0.05% Tween 20 and 0.02% NaN_3 (TTBS) with 1% gelatin. Protein G Gold was used to detect bound IgG and further developed using Bio-Rad's silver enhancement procedure.

Deglycosylation of trypsin isoforms

High-HLT (20 μ g) and low-HLT (20 μ g) were precipitated with trichloroacetic acid and resolubilized in 10 μ l of 20 mM Tris, pH 7.5, containing 0.5% SDS, 50 mM 2-mercaptoethanol and 0.02% NaN_3 , followed by heating in a boiling-water bath for 5 min. Then 5 μ l of 7.5% Nonidet P40, 1.2 μ l of *N*-Glycanase (9.1 μ g/ml) and 13.8 μ l of water were added to each sample followed by incubation at 43 °C for 21 h.

Sequencing of HMMK fragment

HMMK (40 μ g, 350 pmol) was incubated with either 7 pmol of active high-HLT (E/S molar ratio of 1:50) or 0.26 pmol of active low-HLT (E/S of 1:1346 molar ratio) in assay buffer for 10 min at room temperature (cleavage corresponds to the 10 min time point in Figure 2) followed by precipitation with cold trichloroacetic acid as described. Fragments were separated by SDS/PAGE on a 6% gel and electroblotted on to Immobilon-P. The membranes were lightly stained with 0.1% Coomassie Blue R-250 in 50% methanol, 10% acetic acid and then dried. Amino acid sequencing of the excised bands was performed by the Molecular Biology Core facility in the Biochemistry Department at Bowman Gray School of Medicine (Winston-Salem, NC, U.S.A.).

VIP cleavage

Trypsin cleavage of VIP (30 nmol) was performed at room temperature in 500 μ l of assay buffer with 24.7 pmol of active isozyme. The molar ratios of trypsin isoform to substrate were 1:17340 in both cases. Equal aliquots were withdrawn from the reaction mixtures at 5, 10, 20, 30 and 60 min and 5 μ l of acetic acid was added to stop the reaction, followed by the addition of 130 μ l of 0.1% TFA in water (HPLC buffer A). The samples were then filtered and chromatographed on a C18 column (4 mm $\times$ 250 mm); elution was with a 10–40% linear gradient from buffer A to buffer B (0.1% TFA in acetonitrile) over 20 min at a 0.5 ml/min flow rate. Peptides were detected by monitoring absorbance at 210 nm.

RESULTS

Purification of trypsin from human lung tissue produced two pools of activity with molecular masses of approx. 33 and 29 kDa on SDS/PAGE (lanes A and C respectively in Figure 1). High-HLT (33 kDa) was eluted from the cellulose phosphate column with heparin, but elution of low-HLT (29 kDa) from this column required 1 M NaCl. Low-HLT yields were only 20% of the high-HLT yields. Some low-HLT preparations contained minor contaminants co-eluted from the cellulose phosphate column, requiring rechromatography for final purification. Active-site titration of the two trypsin isoforms with MUGB

Figure 1 SDS/PAGE (10% gels) of high-HLT and low-HLT before and after deglycosylation

Lane A, high-HLT (10 μ g); lane B, deglycosylated high-HLT (10 μ g); lane C, low-HLT (10 μ g); lane D, deglycosylated low-HLT (10 μ g). Molecular-mass markers (lanes marked Std) are phosphorylase *b* (100 kDa), transferrin (78 kDa), BSA (68 kDa), IgG (50 kDa), carbonic anhydrase (29 kDa), soya-bean trypsin inhibitor (21 kDa), cytochrome *c* (12.75 kDa) and Trasylol (6.5 kDa).

Figure 2 Time-course SDS/PAGE (6% gels) of HMMK and products after cleavage with high-HLT and low-HLT

Lanes are labelled with the length of the cleavage reaction in minutes. (a) HMMK cleaved with high-HLT (E/S, 1:50); (b) HMMK cleaved with low-HLT (E/S, 1:1346). Molecular-mass markers are the same as in Figure 1.

Figure 3 Western blot of HMMK light-chain-cleavage products with high-HLT and low-HLT

Cleavage was for 20 min with E/S ratios as in Figure 2 followed by reduced SDS/PAGE (8% gels). Lane A, HMMK with high-HLT; lane B, HMMK with low-HLT. A monoclonal antibody to the light chain of HMMK was used as the primary antibody. Antibody binding was visualized using Protein G Gold and silver enhancement.

showed that high-HLT was 95% active, whereas low-HLT was only 15% active. Deglycosylation of the two isoforms resulted in sharper bands and increased mobilities of each form on SDS/PAGE (Figure 1, lanes B and D), but did not totally eliminate the size differences between the isoforms.

HMMK consists of heavy chain, composed primarily of three cysteine proteinase inhibitor domains, a region coding for bradykinin and a histidine-rich light chain that includes the procoagulant domain (Figure 4). Cleavage of HMMK by high-HLT (Figure 2a) or low-HLT (Figure 2b) produced different

Figure 4 Schematic representation of HMMK showing the primary cleavage site for high-HLT

HMMK (40 μ g, 350 pmol) was cleaved with 7 pmol of high-HLT (E/S of 1:50) for 10 min and 8 μ g of the sample was electrophoresed in each of five lanes of an SDS/6% polyacrylamide gel and transferred to an Immobilon-P membrane. Limited N-terminal sequence analysis of the 46 kDa fragment from this cleavage yielded Asp-Gln-Gly-Xaa-Gly-Xaa-Gln. This is the only Asp-Gln sequence present in the HMMK molecule.

fragmentation patterns on SDS/PAGE. The amounts of each isoform were adjusted in these experiments to result in similar rates of cleavage. Several smaller fragments generated by low-HLT failed to be precipitated with 20% trichloroacetic acid used to prepare the samples for Figure 2, but were subsequently

detected when ethanol was used for precipitation. This observation is consistent with the small fragments being from the C-terminal region which is rich in basic amino acid residues, explaining their failure to be precipitated with trichloroacetic acid. High-HLT cleavage of HMMK resulted initially in two major bands of molecular mass 65 and 46 kDa, with the 46 kDa fragment disappearing by 40 min (Figure 2a). In contrast, low-HLT cleavage of HMMK produced a minor band of molecular mass 100 kDa and two major bands of molecular mass 75 and 65 kDa. By 40 min, only the 65 kDa fragment was visible in Figure 2(b). Western-blot analysis of the fragmentation products with each isoform was performed after ethanol precipitation to ensure recovery of the C-terminal fragments, and a monoclonal antibody specific for the light chain of HMMK was used to identify these fragments. As shown in Figure 3, a single 46 kDa fragment produced by the action of high-HLT on HMMK reacted strongly with the antibody, whereas low-HLT produced multiple fragments of less than 50 kDa that reacted only weakly with the C-terminal-specific antibody, indicating numerous non-specific cleavages in the HMMK light chain by low-HLT.

Limited N-terminal sequencing of the 46 kDa high-HLT HMMK-cleavage fragment yielded Asp-Gln-Gly-Xaa-Gly-Xaa-Gln, corresponding to HMMK residues Asp⁴³²-Gln-Gly-His-Gly-His-Gln⁴³⁸ in the light chain of HMMK (Figure 4). Thus the initial cleavage site of high-HLT in HMMK is between Arg-431 and Asp-432. Attempts to sequence the 75 and 65 kDa bands produced by the low-HLT reaction were unsuccessful. Presumably they contain the N-terminus of HMMK, which is unavailable to Edman degradation because of the presence of an N-terminal 5-oxoproline (Kellermann et al., 1986). A primary

Figure 5 Reversed-phase HPLC of VIP-cleavage products generated by high-HLT and low-HLT

Cleavage reactions were performed at room temperature as described in the Experimental section. The chromatograms produced by each isoform are offset and overlaid at each time point for comparison. The analysed samples contained 20 μ g (6 nmol) of native VIP and 0.35 pmol of active sites of the appropriate trypsin isoform, resulting in E/S molar ratios of 1:17340 at room temperature. Unchanged VIP was eluted as a single sharp peak at 29 min (results not shown). A large void-volume peak present in each chromatogram is not shown.

Table 1 Cleavage rate of HMMK and VIP by high-HLT and low-HLT isoforms

Data are in mol of substrate cleaved per mol of active tryptase isoform.

Time (min)	Cleavage rate			
	HMMK		VIP	
	High-HLT	Low-HLT	High-HLT	Low-HLT
0	0	0	0	0
5	10.9	482	1020	2040
10	23.7	852	1818	4140
20	40.6	1183	3822	4980

Table 2 High-HLT and low-HLT hydrolysis rates with synthetic substrates

Isoform	Rate of hydrolysis (mol hydrolysed/min per mol of active enzyme)		
	Z-Phe-Arg-NMec	Z-Arg-Arg-NMec	Ratio
High-HLT	0.25	0.16	0.64
Low-HLT	1.26	2.53	2.0

site of HMMK cleavage by low-HLT could not be determined because of the multiple C-terminal fragments produced.

To determine whether the substrate-specificity differences observed with HMMK cleavage by the tryptase isoforms extended to other substrates, cleavage of VIP was also examined. VIP cleavage by each of the two tryptase isoforms produced different chromatographic profiles when analysed by C18 reversed-phase HPLC (Figure 5). Unchanged VIP was eluted as a single sharp peak at 29 min (results not shown). Low-HLT produced peaks at 23 and 28 min that were essentially non-existent in the high-HLT digests, whereas high-HLT produced major peaks at 24 and 27 min. Both low-HLT and high-HLT generated peptides eluted at 24 min. Although both chromatographic profiles changed as the reactions proceeded to completion, they never became identical.

A comparison of cleavage rates with natural substrates, HMMK and VIP, is given in Table 1. Rates of HMMK cleavage were determined by densitometric scanning of the HMMK band in photographic negatives of the electrophoresis gels in Figure 2. At 5, 10 and 20 min low-HLT cleaved HMMK 44, 36 and 29 times faster than did high-HLT at the corresponding time points. VIP-cleavage rates were measured by following the decreasing areas of the VIP peaks in the chromatograms shown in Figure 5. Low-HLT cleaved VIP 2, 2.3 and 1.3 times faster than did high-HLT at the 5, 10 and 20 min time points. There was too little VIP remaining at 30 min to quantify. In addition, these data show that VIP is a better substrate than is HMMK. Approx. 50 % of the VIP was cleaved by high-HLT in 20 min and by low-HLT in 10 min at E/S molar ratios of 1:17000. In contrast, approx. 50 % cleavage of HMMK (10 min) required E/S molar ratios of 1:50 for high-HLT and 1:1300 for low-HLT.

The synthetic substrates Z-Phe-Arg-NMec and Z-Arg-Arg-NMec were also used to compare specificity and hydrolysis rates of the tryptase isoforms. As shown in Table 2, high-HLT catalysed the hydrolysis of Z-Phe-Arg-NMec at a higher rate than Z-Arg-Arg-NMec, whereas low-HLT cleaved Z-Arg-Arg-

NMec faster than Z-Phe-Arg-NMec. The hydrolysis rates with Z-Arg-Arg-NMec divided by the rates with Z-Phe-Arg-NMec were 0.64 and 2.0 for high-HLT and low-HLT respectively.

DISCUSSION

Previous preparations of human mast cell tryptase have contained two bands in the 29–35 kDa range differing by 2–5 kDa on SDS/PAGE. Because gel-filtration chromatography of these tryptase preparations yielded estimated molecular sizes of 130–144 kDa (Schwartz et al., 1981b; Smith et al., 1984), it has been assumed that tryptase exists as a tetramer containing two slightly different active subunits. However, our separation of the two active forms of tryptase implies that the tetramer is composed of homologous subunits.

In addition, high-HLT was eluted from a cellulose phosphate column with heparin, whereas the elution of low-HLT required 1 M NaCl. Thus low-HLT appears to have a lower affinity for heparin, relative to high-HLT, providing additional evidence of structural differences in the two isoforms. It has been previously reported that the activity of human tryptase is dependent on its interaction with heparin (Smith and Johnson, 1984; Schwartz et al., 1990). However, the apparently lower affinity for heparin exhibited by low-HLT indicates that the activity of this isoform may not be stabilized by heparin. Low-HLT, by failing to form complexes with heparin, may diffuse through the tissues surrounding the site of mast cell degranulation to a much greater extent than high-HLT. Le Trong et al. (1987) presented data suggesting that the specificity of rat chymase, another neutral serine protease found in the granules of connective-tissue mast cells, may vary according to the presence or absence of heparin. Therefore the differences in substrate specificity reported here may be related to differences in affinity for heparin between the two tryptase isoforms.

Two tryptases, designated tryptase- α (Miller et al., 1989) and tryptase- β (Miller et al., 1990), have been cloned from human lung cDNAs. Tryptase- α was also cloned from the Mono Mac 6 cell line (Huang et al., 1993). The derived amino acid sequences of tryptase- α contained two putative glycosylation sites per subunit. Although tryptase- β was the same length and 95 % identical in sequence with tryptase- α , tryptase- β contained only one putative glycosylation site. Deglycosylation of the isoforms led to sharper bands on SDS/PAGE, indicating that both isoforms were glycosylated. In addition, the calculated core protein molecular mass for tryptase- α is 27655 Da, whereas that of tryptase- β is slightly lower at 27457 Da. Our finding that deglycosylated high-HLT is slightly larger than deglycosylated low-HLT (Figure 1, lanes B and D) is consistent with the possibility that high-HLT and low-HLT correspond to tryptase- α and tryptase- β respectively. However, the high degree of sequence identity in the tryptases and the small yields of low-HLT have prevented us from performing the peptide mapping and sequencing studies needed to correlate each isoform with a known cDNA-derived amino acid sequence.

HMMK is a blood plasma protein that participates in coagulation (Movat, 1979) and releases bradykinin on cleavage by kallikrein (Webster, 1970). Human lung tryptase was reported to destroy the coagulation function of HMMK without releasing bradykinin, supporting a possible role for tryptase in anticoagulation (Maier et al., 1983). VIP is a 28-amino acid neuropeptide that acts as a potent relaxant of human bronchial and vascular smooth muscle (Barnes and Dixon, 1984; Morice et al., 1984). Tam and Caughey (1990) found that tryptase made multiple cleavages in VIP, possibly resulting in a local deficiency of the bronchial relaxant activity of VIP, which may be a

contributing factor to asthma-like symptoms (Said, 1984). Our results confirm the reports that HMMK (Maier et al., 1983) and VIP (Tam and Caughey, 1990) are substrates for human tryptase *in vitro* and show that the activities of high-HLT and low-HLT with these two natural substrates differ in rate and cleavage-site specificity.

Low-HLT cleaved HMMK approx. 30 times faster than high-HLT, when compared on the basis of the number of active sites (Table 1). In addition, high-HLT initially cleaved HMMK at a single site in the light chain, whereas low-HLT cleaved the HMMK light chain into several fragments. However, VIP under these assay conditions was a better substrate for tryptase than was HMMK, a finding consistent with the suggestion that loop structures in tryptases adjacent to the active site may sterically hinder larger molecules (substrates or inhibitors) from interacting with the active site (Johnson and Barton, 1992). A secondary structure of the light chain of HMMK predicted by Lottspeich et al. (1985) shows that the region of the molecule containing the high-HLT cleavage site is a large random-coil loop that would be accessible to proteolytic cleavage. In addition, this region of the molecule has been shown to be responsible for binding to anionic surfaces (DeLa Cadena and Colman, 1992) and must be on the surface of the HMMK molecule.

Limited N-terminal sequencing of the 46 kDa fragment of HMMK generated by high-HLT showed that the major cleavage site is between Arg-431 and Asp-432 of the mature molecule [Arg⁴⁴⁹-Asp⁴⁵⁰ of prekininogen (Takagaki et al., 1985)], which is in the histidine-glycine-rich procoagulant region of domain 5 of HMMK (Kunapuli et al., 1993). This finding explains the mechanism by which tryptase destroys the ability of HMMK to participate in the coagulation cascade (Maier et al., 1983). Low-HLT produced cleavage products of molecular mass 75 and 65 kDa, but not the 46 kDa fragment produced by high-HLT. Western blotting with antibodies specific for the C-terminal (light chain) region of HMMK showed reaction with the 46 kDa fragment produced by high-HLT and with several smaller fragments produced by low-HLT. Because low-HLT simultaneously cleaved HMMK at several positions in the C-terminal region, determination of a primary site of cleavage was not possible.

The chromatographic profiles from VIP digestion with high-HLT as compared with low-HLT (Figure 5) show that this substrate is also cleaved differently by the two isoforms. However, they cleaved VIP at comparable rates, in contrast with the 30-fold difference in cleavage rates observed with HMMK. In addition, the use of dipeptide synthetic substrates provides evidence for differences in specificity, with high-HLT being more active on the Phe-Arg substrate, and low-HLT being more active on the Arg-Arg substrate. Although the noted differences in specificity and cleavage rates suggest different functions *in vivo*, this has yet to be determined.

Taken together these findings demonstrate that there are at least two distinct tryptase isoforms in human lung tissue differing in cleavage-site specificity and reaction rate with both natural and synthetic substrates. Although the tryptase isoforms described appear to represent different gene products, it is possible

that they results from other modifications, such as proteolysis at the C-terminus. It should be pointed out that the natural substrates used in these studies may not be the most significant *in vivo*, and, irrespective of their origins, the two tryptase isoforms may serve different functions *in vivo*.

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