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**Human lung mast cell tryptase isozymes: Separation and
examination of structural and functional differences**

Little, Susan S., Ph.D.

East Tennessee State University, 1993

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

HUMAN LUNG MAST CELL TRYPTASE ISOZYMES: SEPARATION
AND EXAMINATION OF STRUCTURAL AND FUNCTIONAL DIFFERENCES

A Dissertation
Presented to
the Faculty of the Department of Biochemistry
James H. Quillen College of Medicine
East Tennessee State University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Biomedical Sciences

by
Susan S. Little
May 1993

APPROVAL

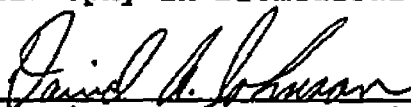
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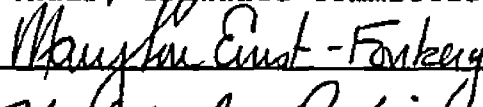
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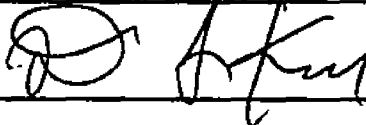


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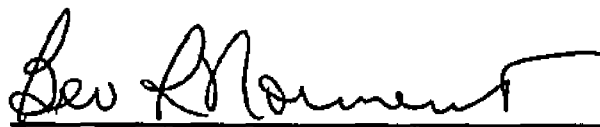








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Associate Vice-President for
Research and Dean, School of
Graduate Studies

ABSTRACT

HUMAN MAST CELL TRYPTASE ISOZYMES: SEPARATION AND EXAMINATION OF STRUCTURAL AND FUNCTIONAL DIFFERENCES

by

Susan S. Little

Tryptases are trypsin-like enzymes found in mast cell granules. Although *in vivo* substrates have not been positively identified, tryptases cleave a limited number of potential physiological substrates *in vitro*, including high molecular weight kininogen (HMWK) and vasoactive intestinal peptide (VIP). Purified human lung mast cell tryptase (HLT) apparently exists as a tetramer with an M_r of 135-144 kDa by gel filtration, whereas SDS-PAGE yielded two bands of M_r 29 kDa and 33 kDa. Tryptases are resistant to inhibition by most natural trypsin inhibitors and display some affinity for heparin. The existence of tryptase isozymes has been implied from the cloning of two tryptase cDNAs from human lung tissue, but distinct isozymes have not been isolated and characterized.

This knowledge gap has been filled by isolating and characterizing two electrophoretically different forms of human lung mast cell tryptase, designated high-HLT (high molecular weight HLT) and low-HLT (low molecular weight HLT). These two forms of HLT have been separated by chromatography on a cellulose phosphate column, with the high M_r form eluting with 10 μ M heparin and the low M_r form subsequently eluting with 1 M NaCl. Using HMWK 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 HMWK at a single Arg residue, whereas low-HLT cleaved HMWK simultaneously at multiple sites. Both isozymes cleaved VIP at multiple sites, but differed with regard to the preferential site of cleavage. Low-HLT was, on an active site basis, 25 and 2 times more active than high-HLT on HMWK and VIP, respectively. In addition, gel filtration of the isozymes yielded M_s of 125 kDa for high-HLT and 28 kDa for low-HLT, indicating tetrameric and monomeric quaternary structures, respectively. Both isozymes were inhibited by human secretory leukocyte proteinase inhibitor (SLPI), but not by other trypsin inhibitors tested. This work provides the first evidence for the existence of distinct tryptase isozymes, with supposedly different *in vivo* functions, and identification of an inhibitor that may control tryptase activity *in vivo*.

DEDICATION

This is dedicated with love, respect and gratitude to my parents, Charlie and Betty Little, who have taught me the truly valuable lessons in life and have instilled in me the value of integrity, humility and quality of character, for which I strive. Their example of Christ-like love, discipline, sacrifice, and commitment continues to amaze and inspire me. Without their continuous encouragement and support, and the safe haven of a loving home to occasionally escape to, the completion of this degree would not have been possible.

This is also dedicated to my sisters and brothers, Kathy, David, Michael, Margaret, Steven, and Carol, whose love and encouragement have always been of inexpressible value and without whose friendship my life would be a lot less rich.

To my dear friends: Whitney Clower, whose friendship and encouragement, not to mention food, many times provided the strength I needed to make it through another day; Robbie Waites (and her family), whose friendship and insightful pep talks helped keep me on track; and Pat Hackett, who provided me with friendship, encouragement and a desperately needed job.

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CONTENTS

	Page
APPROVAL	ii
ABSTRACT	iii
DEDICATION	iv
ACKNOWLEDGMENTS	v
LIST OF FIGURES	x
LIST OF ABBREVIATIONS	xii
CHAPTER	
1. INTRODUCTION	1
Mast Cells	3
Source and Distribution	3
Morphology	3
IgG Receptors and Degranulation	4
Mast Cell Mediators	7
Rapidly Dissociated Preformed Mediators	7
Histamine	7
Chemotactic Factors	9
Acid Hydrolases	9
Newly Generated Mediators	10
Prostaglandins and Thromboxanes	10
SRS-A, HETE, HPETE and PAF	10
Membrane-bound Preformed Mediators	11
Heparin	11
Arylsulfatase B	12
Carboxypeptidase	12

CHAPTER	Page
Chymase	12
Tryptase	14
Mast Cell Heterogeneity	19
Pathogenesis of Mast Cells	22
Project Objectives and Overview	22
2. MATERIALS AND METHODS	24
Materials	24
Tryptase Activity Assay	25
Tryptase Purification	27
Electrophoresis	29
Electroelution of Proteins from SDS-PAGE	29
Active Site Titration	30
Deglycosylation of Tryptase Isozymes	31
Western Blot Analysis	32
Gel Filtration	33
Inhibitor Assays Using Cbz-Lys-SBzl as the Substrate	35
SLPI, Ecotin and ASTI	35
APPI and I α I	35
Arg ¹⁵ Glu ⁵² aprotinin	36
CMTI-III	36
α_2 M and α_1 I ₃ Activity Assay	37
Inhibitor Assays Using HMWK as the Substrate	39
α_2 M and α_1 I ₃	39
Arg ¹⁵ Glu ⁵² aprotinin	40

CHAPTER	Page
APPI, IαI, ASTI and SLPI	41
CMTI-III	42
Comparison of Inhibitory Effects of SLPI on High-HLT and Low-HLT	43
Tryptase Cleavage of Laminin, Fibronectin and Fibrinogen	44
Carboxymethyl-papain Column Preparation . . .	46
Kininogen Purification	47
HMWK Cleavage	49
Sequencing of HMWK Fragment	50
VIP Cleavage	51
Amino Acid Analysis of VIP Cleavage Products .	51
3. RESULTS	53
Purification and Separation of the Two Tryptase Isozymes	53
Structural Analyses of the Two Isozymes . . .	56
Deglycosylation of High-HLT and Low-HLT . .	56
Gel Filtration	59
Inhibition Studies of High-HLT and Low-HLT Using Several Trypsin Inhibitors	62
Testing of Several Biological Molecules as Tryptase Substrates	72
Investigation of Laminin, Fibrinogen and Fibronectin as Tryptase Substrates	72
Cleavage of HMWK by High-HLT and Low-HLT . .	75
Cleavage of VIP by High-HLT and Low-HLT . .	80
Amino Acid Analysis of VIP Cleavage Products .	85
Attempted Peptide Mapping of the Tryptase Isozymes	85

CHAPTER	Page
4. DISCUSSION	90
Purification and Separation of the Tryptase Isozymes	90
Structural Characterization of the Isozymes. .	91
Functional Differences of the Tryptase Isozymes	96
BIBLIOGRAPHY	106
APPENDIX	124
VITA	131

LIST OF FIGURES

FIGURE	Page
1. Schematic illustration of selected mast cell secretagogues	5
2. Sequence alignments used for modelling the mast cell tryptases	20
3. SDS-PAGE of High-HLT and Low-HLT Isozymes	54
4. SDS-PAGE (10%) of the Progressive Steps of Purification of High-HLT and Low-HLT	58
5. A) SDS-PAGE (10%) of High-HLT and Low-HLT Deglycosylated Following Denaturation and Reduction	60
B) SDS-PAGE (10%) of High-HLT Deglycosylated Without Prior Denaturation and Reduction	61
6. A) Chromatogram of a Mixture of High-HLT and Low-HLT on a Sephacryl S200-HR Gel Filtration Column with MW Standards Overlay	63
B) Chromatogram of a Mixture of High-HLT and Low-HLT on a Sephacryl S200-HR Gel Filtration Column with Activity Overlay	64
7. SDS-PAGE (10%) of Peaks Collected from Chromatography of High-HLT and Low-HLT Mixture on Gel Filtration Column	65
8. Western Blot and Coomassie Stained Membrane of Gel Filtration Peaks	66
9. SDS-PAGE (8%) of the Inhibitory Effects of SLPI on High-HLT and Low-HLT	71
10. SDS-PAGE (7%) of the Inhibitory Effects of Arg ¹⁵ Glu ³³ aprotinin on High-HLT and Trypsin at pH 7.5 and 9.1	73
11. A) Time Course SDS-PAGE (6%) with Reduction of HMWK and Products after Cleavage with High-HLT	77
B) Time Course SDS-PAGE (6%) with Reduction of HMWK and Products after Cleavage with Low-HLT	78

FIGURE	Page
12. Western Blot of HMWK and Cleavage Products with High-HLT and Low-HLT	79
13. Schematic Representation of HMWK Showing the Primary Cleavage Site by High-HLT	81
14. A) Reverse-phase HPLC Chromatogram of VIP . . .	82
B & C) Reverse-phase HPLC Chromatogram of the Initial Cleavage Products Generated by the Activity of High-HLT and Low-HLT	83
D & E) Reverse-phase HPLC Chromatogram of the Final Cleavage Products Generated by the Activity of High-HLT and Low-HLT	84
15. Sequence of VIP Showing the Proposed Initial Cleavage Sites by Low-HLT and High-HLT	88
16. Amino Terminal Sequence Alignment of Trypsins, Trypsin, and Chymotrypsin	93
17. A) Space Filling Model of HST-1 Showing the Major Insertions Relative to Trypsin and the Active Site	94
B) Space Filling Model of HST-1 Showing the Major Insertions Relative to Trypsin and Substrate Binding Pocket	95

LIST OF ABBREVIATIONS

α_1 -AC	alpha-1-antichymotrypsin
α_1 I ₃	rat alpha-1-inhibitor-3
α_2 M	bovine alpha-2-macroglobulin
α_1 -PI	alpha-1-proteinase inhibitor
AB	assay buffer
AMC	7-amino-4-methyl coumarin
APPI	Alzheimer's amyloid precursor protein inhibitor
ASTI	Ascaris suum trypsin inhibitor
BCIP	5-bromo-4-chloro-3-indolyl phosphate
β -ME	beta-mercaptoethanol
BPTI	bovine pancreatic trypsin inhibitor
BSA	bovine serum albumin
Cbz	carbobenzoxy
CGRP	calcitonin gene-related peptide
CM	carboxymethyl
CMTI-III	<i>Curcubita maxima</i> trypsin inhibitor III
CPC	cetylpyridinium chloride
dH ₂ O	deionized water
DMSO	dimethyl sulfoxide
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
ECF	eosinophil chemotactic factor
EDTA	ethylene diamine tetraacetic acid
EtOH	ethanol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HETE	monohydroxyeicosatetraenoic acids

HIC	hydrophobic interaction chromatography
high-HLT	high molecular weight human lung tryptase
HMWK	high molecular weight kininogen
HPETE	hydroperoxyeicosatetraenoic acids
HPLC	high performance liquid chromatography
HST	human skin tryptase
I α I	inter-alpha-trypsin inhibitor
IgG	immunoglobulin G
LMWK	low molecular weight kininogen
low-HLT	low molecular weight human lung tryptase
LT	leukotrienes
MC _T	mast cells containing tryptase but no chymase
MC _{TC}	mast cells containing tryptase and chymase
MES	2-(4-morpholino)-ethane sulfonic acid
MeOH	methanol
MUGB	4-methylumbelliferyl p-guanidinobenzoate hydrochloride
MWCO	molecular weight cutoff
NBT	nitro blue tetrazolium
NCF	neutrophil chemotactic factor
NP-40	Nonidet P-40
PAF	platelet activating factor
PAGE	polyacrylamide gel electrophoresis
PG	prostaglandins
PITC	phenylisothiocyanate
PMSF	phenylmethylsulfonyl fluoride

PVDF	polyvinylidene difluoride
RMCP I	rat mast cell protease I
RMCP II	rat mast cell protease II
RT	room temperature
SBTI	soybean trypsin inhibitor
SBzl	thiobenzyl ester
SDS	sodium dodecyl sulfate
SLPI	secretory leukocyte proteinase inhibitor
SRS-A	slow-reacting substance of anaphylaxis
TCA	trichloroacetic acid
TEA	triethylamine
TFA	trifluoroacetic acid
VIP	vasoactive intestinal peptide
V ₀	void volume

CHAPTER 1

INTRODUCTION

Over three decades ago, a tryptic-like activity was discovered in mast cells by Glenner and Cohen (1960) using histochemical techniques. This activity, subsequently named tryptase (Lagunoff and Benditt, 1963), has become of increasing interest due to the ubiquitous nature and biochemical activity of mast cells. Tryptase is a trypsin-like serine protease found in the cytoplasmic granules of most mammalian mast cells and comprises 23% of the total cellular protein of human mast cells (Schwartz et al., 1981a). Tryptases cleave peptide substrates on the carboxyl side of Lys and Arg residues but differ from trypsin in that they have little or no activity on denatured proteins such as casein. Tryptases are stored and released as active enzymes (Glenner and Cohen, 1960; Alter et al., 1987), which are stabilized by heparin, a component of mast cell granules (Smith and Johnson, 1984; Schwartz and Bradford, 1986; Alter et al., 1987). Histamine, another major component of mast cells, has been used as a specific marker of mast cell activation. However, histamine is also found in basophils (White et al., 1987), and is cleared more rapidly from the site of release than is tryptase (Schwartz et al., 1987a). Tryptase has been localized specifically to mast cells (Schwartz, 1985; Craig et al., 1986; Schwartz et al., 1985

and 1987b) and remains in the vicinity of release longer than histamine (Schwartz et al., 1987a) and therefore has been proposed as a more specific marker of mast cell activation.

Upon mast cell degranulation, tryptase is released along with other mediators into the extracellular milieu (Schwartz et al., 1981a). The function(s) and regulation of tryptase after its release from the cell are not clearly understood, but in vitro studies have shown that tryptase cleaves several biologically significant molecules. These include complement C3 (Schwartz et al., 1983), fibrinogen (Schwartz et al., 1985), vasoactive intestinal peptide (VIP) (Caughey et al., 1988b; Tam and Caughey, 1990), and high molecular weight kininogen (HMWK) (Maier et al., 1983).

Human lung tissue has served as the primary source of mast cell tryptase (Smith et al., 1984; Schwartz et al., 1981a), but other sources include skin (Harvima et al., 1988b), pituitary (Cromlish et al., 1987) and a human mast cell line (Butterfield et al., 1990). In all these cases the purified enzyme migrated as two major bands on SDS-PAGE with M_r differing by about 4,000. Interestingly, purified bovine mast cell tryptase also yielded two bands upon SDS-PAGE (Fiorucci et al., 1992). These findings have led to speculation that the tetramer observed upon gel filtration is comprised of two subunits represented by the two species seen on SDS-PAGE.

Mast Cells

Source and Distribution

Mast cells are derived from bone marrow stem cells, migrate through the bloodstream in an immature form and into tissues where they differentiate and mature (Barrett and Metcalfe, 1987). It is thought that all mast cell subsets differentiate from the same precursor cells (Barrett and Metcalfe, 1987), but that basophils develop from a distinct precursor (Ishizaki et al., 1989). Mast cells are widely distributed in the body (Metcalfe et al., 1981) and are most numerous at the host-environment interface, such as in the intestinal tract, skin and lungs (Malone and Metcalfe, 1988), but are also found along blood vessels and nerves, and throughout connective and lymphoid tissues (Metcalfe et al., 1981; Friedman and Kaliner, 1987; Bienenstock et al., 1987; Stead et al., 1987; Bienenstock et al., 1991). In the lung, mast cells are most concentrated around the trachea, beneath the pleura, and in the connective tissue around small airways (Friedman and Kaliner, 1987), although about half of the total number of lung mast cells are found in peripheral lung tissue (Friedman and Kaliner, 1987).

Morphology

Mast cells are ovoid or irregularly elongated cells of about 10-30 μm in diameter with ovoid or round nuclei (Metcalfe et al., 1984). The cytoplasm is packed with

densely-staining granules of 0.1-0.4 μm diameter (Metcalf et al., 1984), that display a crystalline structure appearing as whorls, scrolls, and lattices (Caulfield et al., 1980).

IgE Receptors and Degranulation

The biological activities associated with mast cells are the result of the effects of mediators that are released into the extracellular milieu upon mast cell activation. The complex process of mast cell activation culminates in the exocytosis of the cytoplasmic granule contents, including histamine and tryptase, as well as the production and release of newly formed mediators. Mast cells have on their surfaces receptors that bind to the Fc portion of immunoglobulin E (IgE) molecules (Marom and Casale, 1983). There are $1-5 \times 10^5$ receptors/mast cell and about 10% of these receptors are normally occupied at any given time (Metcalf et al., 1981). Mast cells are most often activated by crosslinking (or bridging) of two IgE molecules by antigen, which initiates a cascade of events involving movement of calcium into the cell, activation of a cell membrane associated serine esterase, glycolysis, calcium liberation, microtubule assembly, and sequential exocytosis of the granules (Kaliner, 1977). However, mast cells can be induced to degranulate in response to other stimuli. As shown in Figure 1 (Friedman and Kaliner, 1987), a number of

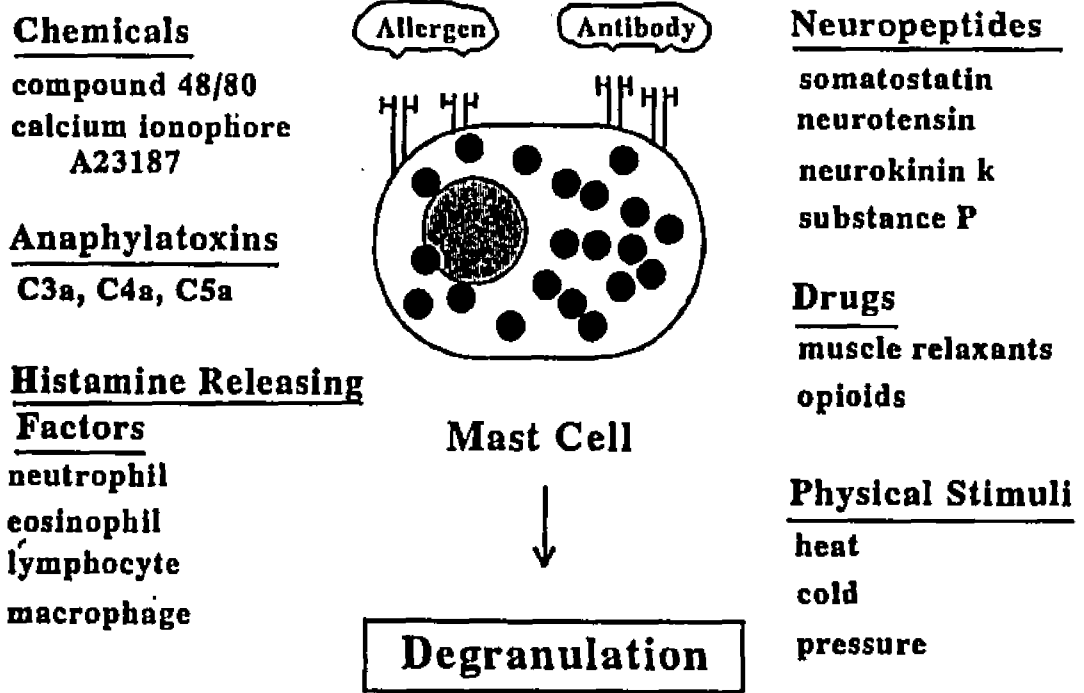


Figure 1. Schematic illustration of selected mast cell secretagogues. This figure was adopted with modifications from Friedman and Kaliner (1987).

physiological stimuli, such as neuropeptides, anaphylatoxins, and histamine-releasing factors from other cell types, as well as non-endogenous compounds, such as chemicals and drugs, can cause mast cell degranulation. Even physical stimuli, such as heat, cold and pressure, can invoke a mast cell response.

Morphologically, degranulation proceeds through many stages including swelling of the granules, decrease in the amount of stainable matrix material, disappearance of crystalline gratings and lattices, increased visualization of scroll patterns, and the appearance of reticular, particulate, or flocculent constituents (Caulfield et al., 1980; Dvorak et al., 1985). The bridging of IgE receptors on rat mast cells leads to phospholipid methylation which induces, among other things, Ca^{+2} influx and histamine release (Ishizaka et al., 1981). There have been two modes of release of granule mediators observed: 1) granular membranes fuse with other granular membranes and with the cellular membrane, forming large channels through which the mediators exit the cell (Trotter and Orr, 1973; Trotter and Orr, 1974; Hashimoto et al., 1966; Kobayasi and Asboe-Hansen, 1969; Orr, 1977; Behrendt et al., 1978); or, 2) no large channels form, with the speculation that transient, small pores allow mediators to escape (Galli et al., 1984; Berlin and Enerback, 1984; Fernandez et al., 1984). Degranulation may not result in extrusion of all

granules/granule contents and is not thought to be cytotoxic (Friedman and Kaliner, 1985; Friedman et al., 1986; Dvorak et al., 1985).

Mast Cell Mediators

Three types of mediators have been identified in mast cells: 1) those that are preformed and rapidly dissociate from the cell upon degranulation; 2) those newly generated upon mast cell activation; and 3) those that are preformed but do not readily dissociate from the granule. These mediators are summarized in Table 1.

Rapidly Dissociated Preformed Mediators

Histamine. Histamine (5[2-aminoethyl]-imidazole) is stored in the granules of human mast cells ($5 \mu\text{g}/10^6$ cells) in association with heparin proteoglycan, and in human basophils ($1 \mu\text{g}/10^6$ cells) in association with chondroitin-4 sulfates, comprising 5-10% of the human mast cell granule by weight (White et al., 1987; Wasserman, 1983). Histamine diffuses through the tissues rapidly after release and is detectable in the bloodstream within minutes of mast cell activation (White et al., 1987). Histamine exerts its effects through binding to two types of cell surface receptors, H_1 and H_2 . Binding of histamine to H_1 receptors can cause a variety of effects including smooth muscle contraction, increased vascular permeability, an increase in

Table 1. Mast Cell Mediators^{*}****Rapidly Dissociated Preformed Mediators**

Histamine

Eosinophil chemotactic factor

Neutrophil chemotactic factor

Exoglycosidases (β -hexosaminidase, β -glucuronidase, β -D-galactosidase^{*})

Arylsulfatase A

Kininogenase^{**}Serotonin^{*}**Newly Generated Mediators**

SRS-A: Leukotrienes C, D, and E

Prostaglandins and thromboxanes

HETEs

HPETEs

Platelet activating factor

Prostaglandin generating factor

Membrane-bound Preformed Mediators

Heparin

Neutral proteases: Tryptase and Chymase

Arylsulfatase B

Inflammatory factors^{*}Peroxide^{*}Superoxide dismutase^{*}

^{*} Demonstrated in nonhuman mast cells. ^{**} Kininogenase has been determined to be tryptase (Proud et al., 1988; Walls et al., 1992). ^{***} Compiled from Marom and Casale, 1983, and Friedman and Kaliner, 1987.

cGMP, prostaglandin generation, pruritus, pulmonary vasoconstriction, tachycardia, increased nasal mucus production and activation of airway vagal afferent nerves (White et al., 1987; Wasserman, 1983). Binding of histamine to H₂ receptors is responsible for an additional group of effects including an increase in vascular permeability, gastric acid secretion, airway mucus production, and cAMP; stimulation of suppressor T-cells; inhibition of skin and basophil histamine release and neutrophil chemotaxis and enzyme release; and bronchodilatation and esophageal contraction (White et al., 1987; Wasserman, 1983). Human diseases associated with histamine release and binding include asthma, anaphylaxis, urticaria and rhinitis (Wasserman, 1983).

Chemotactic Factors. Eosinophil chemotactic factors (ECF), both high and low molecular weight, serve to attract eosinophils and increase eosinophil receptors for complement C4 and C3b. Neutrophil chemotactic factors (NCF) attract neutrophils and then deactivate them to further chemotactic activation by other cells (Wasserman, 1983). There are several other less well characterized chemotactic factors in mast cells that affect the migration of T and B lymphocytes and basophils (Wasserman, 1983).

Acid hydrolases. Acid hydrolases are located primarily in lysosomes and function in degradation. Beta-

hexosaminidase removes terminal glucosamine residues from carbohydrates; β -glucuronidase removes terminal β -glucuronate residues; and arylsulfatases hydrolyze aromatic sulfate esters (Wasserman, 1983).

Newly Generated Mediators

Mediators derived from arachidonic acid include slow-reacting substances of anaphylaxis (SRS-A), prostaglandins (PGs), monohydroxyeicosatetraenoic acids (HETEs), hydroperoxyeicosatetraenoic acids (HPETEs), thromboxanes (TXs), and platelet activating factor (PAF).

Prostaglandins and Thromboxanes. PGs and TXs are derived from arachidonic acid via the cyclooxygenase pathway. Chemically, they are C-20 polyunsaturated hydroxyacids with a cyclopentane or oxane ring and they function to increase cAMP and cGMP, attract eosinophils and neutrophils, and aggregate platelets.

SRS-A, HETE, HPETE and PAF. SRS-A, HETE and HPETE are derived from arachidonic acid via the lipoxygenase pathway and PAF is an ether analog of phosphatidyl choline. SRS-A is made up of the leukotrienes (LT) C, D, and E (6-sulfido-ether linked conjugated trienes). The LTs affect mucus secretion, while the HETEs and HPETEs affect mediator release, increase vascular permeability and vasodilatation, inhibit T cell secretion of lymphokines, and inhibit lymphokine transformation and lymphocyte-mediated

cytotoxicity (Marom and Casale, 1983). PAF is the most potent platelet-aggregating compound known and can cause a wide variety of other effects such as augmenting neutrophil migration, enzyme release and oxidative metabolism (Wasserman, 1983).

Membrane-bound Preformed Mediators

The third type of mediators are not readily dissociated from the granule membrane following degranulation and may require high ionic strength for separation. These include heparin, arylsulfatase B and the neutral proteases carboxypeptidase, chymase and tryptase.

Heparin. Heparin is a proteoglycan with highly sulfated glycosaminoglycan side chains that give it a high negative charge density (Wasserman, 1983). Human lung and skin mast cells contain about 5 μg of heparin (MW 60 kDa) per 10^6 cells (Metcalfe et al., 1979; Metcalfe et al., 1980; Wasserman, 1983). Some of the activities attributed to human heparin include binding histamine, inhibiting complement activation, acting as an anticoagulant, binding platelet factor 4 and binding and stabilizing the activity of tryptase (Wasserman and Austen, 1977; Schwartz et al., 1981b; Smith et al., 1984; Wasserman, 1983). Rat serosal mast cells contain about 25 μg of heparin proteoglycan (MW 750 kDa) per 10^6 cells, which can bind histamine and inhibit complement activity (Yurt et al., 1977; Weiler et al.,

1978).

Arylsulfatase B. Arylsulfatase B is a glycoprotein that hydrolyzes aromatic sulfate esters and is present in rat mast cells and human pulmonary mast cells (Schechter et al., 1983).

Carboxypeptidase. Carboxypeptidases isolated from mouse (Serafin et al., 1987), rat (Everitt and Neurath, 1980), and human (Goldstein et al., 1987; Goldstein et al., 1989) mast cells are zinc-dependent metalloexopeptidases (Reynolds et al., 1989). There are two classes of mast cell carboxypeptidases (MC-CP), designated carboxypeptidase A (MC-CPA) and carboxypeptidase B (MC-CPB), that exhibit a substrate specificity for carboxy-terminal aliphatic/aromatic amino acids and basic amino acids, respectively (Reynolds et al., 1989). An MC-CPA has been isolated from a subset of human mast cells that contain both of the other mast cell neutral proteases, chymase and tryptase (Irani et al., 1991). The mature protein contains 308 amino acids, has a MW of 36.1 kDa, carries a positive charge of 16 at neutral pH, and functions to remove carboxy-terminal Phe and Leu residues from substrates (Reynolds et al., 1989; Irani et al., 1991).

Chymase. Chymases and tryptases are the two major neutral serine proteases found in the cytoplasmic granules of mast cells. Chymases are chymotrypsin-like enzymes that

cleave target proteins on the carboxyl side of hydrophobic residues, more specifically Tyr and Phe residues (Craig and Schwartz, 1989). They have been isolated from several species including rodent (Woodbury and Neurath, 1980), sheep (Huntley et al., 1986), dog (Caughey et al., 1988a) and human (Schechter et al., 1983). The enzyme is a monomer of 30 kDa and, like tryptase, is stored in its active form in the secretory granules, probably bound to heparin (Schechter et al., 1983; Johnson, et al., 1986; Wintroub et al., 1986; Sayama et al., 1987). Unlike tryptase, however, chymase is inhibited by the plasma proteinase inhibitors alpha-1-proteinase inhibitor (α_1 -PI) and alpha-1-antichymotrypsin (α_1 -AC) (Schechter et al., 1989). Human mast cell chymase is much more abundant in skin mast cells than in lung mast cells (Schechter et al., 1983; Schechter et al., 1986). Human skin chymase is similar to RMCP I (rat chymase) and dog skin chymase in its activity on synthetic substrates (Powers et al., 1985). Chymases have been observed to convert angiotensin I to angiotensin II (a vasoconstrictor) in vitro and to inactivate bradykinin and kallidin, two vasodilators, suggesting a role in initiating or perpetuating vasoconstriction (Reilly et al., 1982; Reilly et al., 1985; Wintroub et al., 1984). Chymase also cleaves VIP (a bronchodilator), substance P (a potent bronchoconstrictor and mast cell activator), and calcitonin gene-related peptide (CGRP) (a potent vasodilator and

bronchoconstrictor) (Brain et al., 1985; Palmer et al., 1987; Franconi et al., 1989), further indicating a role in the control of vasoactive peptides (Brain and Williams, 1988; Caughey et al., 1988b). Data has also been produced indicating that chymase is a very potent secretagogue for airway serous cells (Sommerhoff et al., 1989), supporting a role for chymase in airway hypersecretion.

Tryptase. Tryptases have been isolated from a number of species, including human (Schwartz et al., 1981a; Smith et al., 1984; Cromlish et al., 1987; Harvima et al., 1988b), dog (Caughey et al., 1987; Schechter et al., 1988), rat (Kido et al., 1985a) and bovine (Fiorucci et al., 1992). Although tryptases in general are 40% identical to trypsin in amino acid sequence, they differ from trypsin and each other in several ways. Unlike trypsin, tryptases are not inhibited by plasma proteinase inhibitors, such as α_1 -PI and alpha-2-macroglobulin (α_2 M), or other trypsin inhibitors, like bovine pancreatic trypsin inhibitor (BPTI), soybean trypsin inhibitor (SBTI) and lima bean trypsin inhibitor (LBTI) (Schwartz et al., 1981b; Smith et al., 1984; Cromlish et al., 1987). Whereas trypsin cleaves proteins such as casein and albumin, tryptases are not active on most proteins. Also unlike trypsin, human tryptases, both lung (Schwartz et al., 1981a; Smith et al., 1984) and skin (Harvima et al., 1988a), have been reported to exist as

tetramers, bind heparin, and show reduced activity with increasing NaCl concentrations. Dog tryptase (Caughey et al., 1987; Schechter et al., 1988), rat tryptase (Kido et al., 1985a; Braganza and Simmons, 1991), and bovine tryptase (Fiorucci et al., 1992) displayed similar, although not identical, characteristics. Dog tryptase was inhibited by BPTI; rat tryptase did not bind heparin and was inhibited by several of the low molecular weight trypsin inhibitors, specifically α_1 -PI, SBTI, BPTI, and trypstatin, an endogenous inhibitor co-localized in rat mast cell granules (Kido et al., 1988); and bovine tryptase formed oligomers of 360 kDa (subunit sizes of 39 and 41 kDa) and was inhibited by BPTI, which is actually made in mast cells (Fritz et al., 1979).

While tryptase is a poor protease in that there are very few known natural substrates, there are several molecules that are known to be cleaved by tryptase. Of particular note are vasoactive intestinal peptide (VIP) (Caughey et al., 1988b; Tam and Caughey, 1990), and high molecular weight kininogen (HMWK) (Maier et al., 1983).

HMWK is a plasma protein that participates in coagulation by contributing to the activation of Factor XII at the beginning of the intrinsic pathway (Movat, 1979). Cleavage of HMWK by kallikrein releases bradykinin, a nonapeptide, causing pain, increased vascular permeability, and bronchoconstriction (Webster, 1970). Human lung

tryptase destroyed the procoagulation function of HMWK, which supports a possible role for tryptase in anticoagulation, but did not release bradykinin (Maier et al., 1983). While it was likely that tryptase inactivated the procoagulant function of HMWK via cleavage in the C-terminal region, the exact cleavage site was not determined.

VIP is a 28 amino acid neuropeptide that acts as a potent relaxant of human bronchial and vascular smooth muscle (Barnes et al., 1984; Morice et al., 1984). Mast cells in the lung are in close proximity to nerves controlling bronchial muscle tone (Stead et al., 1987), which led Tam and Caughey (1990) to examine the effect of human lung tryptase on VIP. They found that tryptase degrades VIP by cleaving it in several positions, possibly resulting in a local deficiency of VIP's bronchial relaxant activity, which may be a contributing factor to asthma-like symptoms (Said, 1984).

Complement C3, another reported tryptase substrate, is cleaved to produce the anaphylatoxin C3a, which causes smooth muscle contraction and increased vascular permeability (Schwartz et al., 1983). Fibrinogen, which in vivo is a substrate for thrombin and a precursor to fibrin in the blood coagulation cascade, lost all clotting ability after being cleaved by tryptase in vitro (Schwartz et al., 1985). These data suggest possible tryptase involvement in anticoagulation, the immediate hypersensitivity response,

and bronchial smooth muscle contraction in asthma-like conditions.

Other substrates cleaved by tryptases include pro-atrial natriuretic factor (pro-ANF) (Imada et al., 1987 and 1988; Seidah et al., 1986; Proctor et al., 1991; Wypij and Harris, 1988) to produce ANF, a potent diuretic, natriuretic and hypotensive (DeBold, 1985); peptide histidine-methionine (PHM), a potent bronchodilating peptide, and CGRP, a potent vasodilator and bronchoconstrictor (Tam and Caughey, 1990); kinetensin, a peptide isolated from pepsin-treated human plasma that shares homology with neurotensin and angiotensin (Mogard et al., 1986; Goldstein et al., 1991); prothrombin to thrombin (Kido et al., 1985b; Dietze et al., 1990); and synovial procollagenase and/or pro-stromelysin (Gruber et al., 1988; Gruber et al., 1989) to produce stromelysin and collagenase, which degrade proteoglycans, fibronectin, laminin, type IV and type IX collagen, and gelatin (Sellers et al., 1978; Harris et al., 1984; Okada et al., 1986). Johnson and Cawston (1985) reported that tryptase was not a direct activator of procollagenase. Gruber et al. (1988) later reported that tryptase was an activator of procollagenase, but subsequently (Gruber et al., 1989) reported that tryptase only activated prostromelysin which then activated procollagenase, confirming the original data of Johnson and Cawston. These data suggest that tryptase could be involved in the control of fluid volume and blood

pressure control (Wypij and Harris, 1988), bronchial tone (Tam and Caughey, 1990) and contribute to matrix degradation in rheumatoid arthritis (Gruber et al., 1989). There is a conflicting report about the ability of human tryptase to cleave prothrombin (Harvima et al., 1989), leaving doubt as to its potential procoagulant activity. Most of these substrate studies were performed in vitro and ,therefore, their in vivo significance is unclear.

To investigate the underlying structural features of tryptase responsible for the tendency to form tetramers/oligomers and for the seeming unavailability of the active site for interaction with protein substrates and inhibitors, Johnson and Barton (1992) performed computer molecular modelling studies based on the structure of bovine trypsin. Their data showed two large insertions on either side of the substrate binding pocket that may limit access of many substrates and inhibitors to the active site. Additionally, two hydrophobic patches unique to the tryptases were apparent on the models and may contribute to tetramer formation and/or interaction with other hydrophobic molecules such as membranes.

In experimental studies previously performed, the human lung mast cell tryptase preparations used may have contained one or more isozymic forms. Two different tryptase cDNAs have been cloned from a human lung tissue library (Miller et al., 1989; Miller et al., 1990) and three from a human skin

cDNA library (Vanderslice et al., 1990), one of which was identical to one of the lung cDNAs. A dog tryptase has been cloned from dog mastocytoma cells (Vanderslice et al., 1989) and two mouse tryptases have been cloned from a mouse mast cell line (Chu 1990; Reynolds et al., 1991; McNeil et al., 1992). The amino acid sequences derived from these cDNAs are shown aligned with trypsin and chymotrypsin in Figure 2. The coding regions of all of the tryptases are 244 or 245 amino acids in length with one or two putative N-linked glycosylation sites (Asn-Xaa-Thr/Ser). The human tryptases show >90% sequence identity to each other and 73-78% identity to dog tryptase whereas the two mouse tryptases share only 70% identity to each other. A high degree of homology between all the tryptases in an 11 amino acid propeptide is also observed. The propeptide is unusual in that it ends in a Gly residue, which is unique among the serine proteases and suggests a novel mode of activation (Vanderslice, 1989). All of the tryptases have an Asp residue in the proposed base of the substrate binding pocket, conferring the preference of tryptase for substrates having Lys or Arg at the P1 position. Multiple cDNAs isolated from humans and evidence of multiple tryptase genes (Vanderslice, 1990) suggest the existence of multiple active forms of the enzyme.

Mast Cell Heterogeneity

It should be noted that not all mast cells produce

equivalent amounts of the various mediators, even within the same individual. For example, mast cells from human lung released a much higher amount of histamine and leukotrienes D₄ and E₄ than those from human foreskin (Church et al., 1989). Varying amounts of the neutral proteases tryptase and chymase have led to a proposed system of nomenclature for human mast cells based on the presence or absence of these two enzymes. Mast cells containing tryptase but no chymase have been labeled T mast cells (MC_T), while mast cells containing both tryptase and chymase have been labeled TC mast cells (MC_{TC}) (Irani et al., 1986). Ninety percent of the total number of mast cells found in human pulmonary tissue and 98% in intestinal mucosa were the T type, whereas the TC type accounted for 88% of mast cells in human skin and 87% in intestinal submucosa (Irani et al., 1986). It has been determined that T mast cells contain approximately 10.8 pg of tryptase per cell while TC mast cells contain approximately 35 pg of tryptase and 4.5 pg of chymase per cell (Schwartz et al., 1987c).

Rat mast cells have been categorized as either mucosal or connective tissue type based primarily on location and the type of chymotrypsin-like enzyme present. Connective tissue type mast cells contain a chymase called rat mast cell protease I (RMCP I) (Yurt and Austen, 1977; Seppa and Jarvinen, 1978; Woodbury et al., 1978a) and mucosal type mast cells contain a chymase called RMCP II (Woodbury et

al., 1978b). The physiological significance of mast cell heterogeneity between tissues is not clearly understood.

Pathogenesis of Mast Cells

Mast cells are thought to participate in normal homeostasis by contributing to: resistance to parasitic infestation (Miller and Jarrett, 1971; Haig et al., 1984; Capron et al., 1978), wound healing (Matsuda and Kitamura, 1981), angiogenesis (Azezthan et al., 1980; Kessler et al., 1976), and tumor immunity (Henderson et al., 1981; Farram and Nelson, 1980). They have also been implicated in a number of pathological conditions, including anaphylaxis (Austen, 1974), asthma (Crisp et al., 1984; Godfrey et al., 1984), inflammatory bowel disease (Dvorak et al., 1980; Barrett and Metcalfe, 1984), and rheumatoid arthritis (Wasserman, 1984). The symptoms in these disease states range from acute and life-threatening to chronic and debilitating. It is apparent from the above information that the basic biochemical functions of mast cells and their mediators in health and disease are varied and complex, and an understanding of them is crucial in the quest to maintain or restore health.

Project Objectives and Overview

In spite of the knowledge of multiple cDNAs and genes for human tryptase, there is no information concerning possible functional differences between the various forms of

tryptase, neither has it been possible to correlate different electrophoretic forms with cDNA sequences. This project was designed to fill these knowledge gaps by isolating human tryptase isozymes, which could then be compared with regard to structure and function.

Two forms of the human lung tryptase, differing in molecular weight, were partially separated by chromatographic techniques and were shown in this laboratory to be independently active (Smith et al., 1984). Starting with human lung tissue, two forms of active tryptase have been isolated, which are designated high-HLT (high molecular weight human lung tryptase) and low-HLT (low molecular weight human lung tryptase). These two isozymes have been examined, and in some cases compared, with respect to their structural and functional characteristics in an attempt to confirm the existence of two independently active tryptases in human lung, to partially define those differences, and to determine their relationship to the previously discussed cDNAs cloned from human lung mast cells.

CHAPTER 2
MATERIALS AND METHODS

Materials

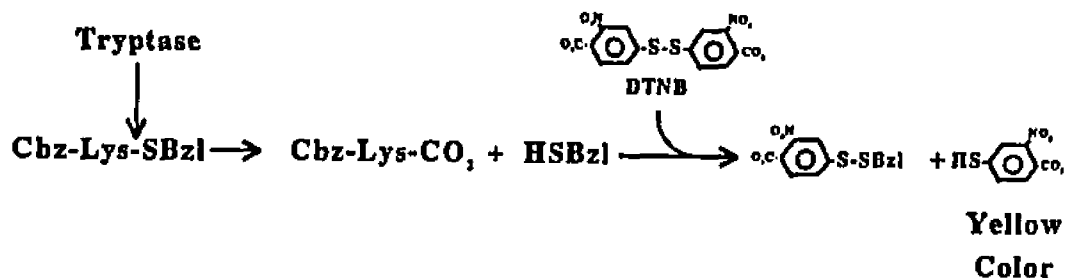
Frozen human lung tissue was procured through the National Disease Research Interchange. Cellulose phosphate (fibrous form), cetylpyridinium chloride, porcine heparin (ammonium salt, average MW = 10,000), DTNB, Cbz-Lys-SBzl, Brilliant Blue G Colloidal Concentrate, fish gelatin, trichoroacetic acid, TFA, and Amino Acid Standard solution (#A9781) were purchased from Sigma Chemical Company. MES, NaCl, NaN₃, glycerol, HEPES, Brij 35, DMSO, HNO₃, enzyme grade sodium acetate trihydrate, HPLC grade acetonitrile were products of Fisher Scientific. Enzyme grade ammonium sulfate was purchased from ICN Biomedicals. Spectra/Por standard dialysis tubing (4.5 cm; 12,000-14,000 MWCO) was a product of Spectrum Medical Industries, Inc. Absolute (200 proof) ethanol was a product of AAPER Alcohol & Chemical Co. HPLC grade triethylamine was purchased from Pierce Chemical Co. Copper Stain & Destain Kit was purchased from Bio-Rad Laboratories. Toyopearl Butyl 650-M hydrophobic interaction chromatography column material was purchased from TosoHaas. Sephacryl S200-HR gel filtration column material was purchased from Pharmacia. VIP was purchased from Bachem, Inc. Recombinant N-Glycanase was purchased from Genzyme. A glass-lined, 300A pore, Nucleosil C₁₈, HPLC column (4mm x

250mm) was purchased from SGE. Pico-tag C₁₈ column and Immobilon-P PVDF membrane were purchased from Millipore and PVDF-Plus Transfer Membrane was obtained from MSI. NH₄acetate, purchased from Mallinckrodt, was kindly provided by Dr. M.-L. Ernst-Fonberg. SLPI was kindly provided by Dr. R. Thompson (Synergen, Boulder, CO). Arg¹⁵Glu⁵²aprotinin was graciously provided by Dr. H. Fritz (Univ. of Munich, Munich, Germany). ASTI was the kind gift of Dr. R. Peanasky (Univ. of S. Dakota, Vermillion, SD). IαI was the kind gift of Dr. J. Enghild (Duke Univ., Durham, NC). APPI was kindly provided by Dr. T. Hynes (Genentech, Inc., San Francisco, CA). Ecotin was the kind gift of Dr. M. McGrath (Univ. of California at San Francisco, SF, CA). CMTI-III was kindly provided by Dr. C. McWherter (Monsanto, St. Louis, MO). Protein G Gold Immun-blot Assay Kit, purchased from Bio-Rad, was kindly provided by Dr. J. Suttles. Monoclonal antibodies to the light chain of HMWK were graciously furnished by Dr. A. Schmeier (University of Michigan, Ann Arbor). Laminin, plasminogen-free fibrinogen, and fibronectin were the kind gifts of Dr. S. Stack (Duke University, Durham, NC). Fresh frozen human blood plasma was obtained from the American Red Cross. All other reagents were ACS grade unless otherwise specified.

Tryptase Activity Assay

Enzyme activity was monitored using the substrate Cbz-

Lys-SBzl, which when hydrolyzed produces a free thiol that reacts with DTNB to produce a yellow color. The chemical reaction involved is shown below:



One unit of activity was defined as the amount of enzyme needed to produce an increase in absorbance at 410 nm (A_{410}) of 1.0/minute. Specific activity was defined as the increase in A_{410} /minute/ml divided by the A_{280} . An appropriate amount of enzyme was added to 0.1 M HEPES, 10 μ M heparin (100 μ g/ml), 10% glycerol, 0.05% Brij 35, 0.02% NaN_3 , pH 7.5 (assay buffer) containing 1 mM DTNB for a total volume of 980 μ l. Assays were started by adding 20 μ l of 20 mM Cbz-Lys-SBzl in DMSO for a final volume of 1 ml (final substrate concentration equaled 0.4 mM) and the change in A_{410} /minute was monitored for 3 minutes at room temperature in a Beckman DU-30 spectrophotometer using the kinetics data program on the Beckman Kinetics cartridge. Chromatography fractions were assayed using a microtiter plate reader and 96-well microtiter plates with the assay incubation mixture reduced

proportionately to 200 μ l.

Tryptase Purification

Frozen human lung tissue (500 g) was cut into small pieces and disrupted in a Waring blender with 500 ml of cold distilled H₂O for 10-15 seconds. The homogenate was centrifuged at 25,400 x g for 20 min. in a Beckman J-21C centrifuge 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% NaN₃, at pH 6.1 in place of dH₂O until supernatant fractions were visibly free of hemoglobin. Tryptase activity was extracted from the tissue by repeating the blending and centrifuging process four times using 500 ml of cold 20 mM MES 2 M NaCl 0.02% NaN₃, pH 6.1 each time. The viscosity of the crude extract was reduced by removing the lipid present; this involved adding an equal volume of 2% cetylpyridinium chloride in 10mM MES 0.02% NaN₃, pH 6.1 and incubating at room temperature for 30 minutes followed by centrifugation at 25,400 x g (5°C) for 60 minutes. The extract was then made 2 M in (NH₄)₂SO₄, centrifuged as above, and filtered through glass wool. This filtrate was mixed with 50 ml of Toyopearl Butyl 650-M column material equilibrated with 10 mM MES, 0.5 M NaCl, 2 M (NH₄)₂SO₄, 20% glycerol, 0.02% NaN₃, pH 6.1 and incubated at room temperature for 45 minutes. This mixture was centrifuged at 5860 x g (5°C) and the supernatant containing

unbound proteins was decanted and tested for activity. The column material with bound trypsin was collected by centrifugation, the slurry was poured into a chromatography tube (2.2 x 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 0.0 M $(\text{NH}_4)_2\text{SO}_4$, collecting 2 ml fractions at one minute intervals. The active fractions were pooled, made 0.05% in Brij 35, and dialyzed against 4 liters of 10 mM MES, 0.3 M NaCl, 0.02% NaN_3 , pH 6.1 for 3 hours at 4°C, followed by dialysis against 4 liters of the same buffer containing 20% glycerol for 3 hours. This pool was then loaded onto a 20 ml cellulose phosphate column equilibrated with the last dialysis buffer. After washing with equilibration buffer, one of two elution profiles was used. Either high-HLT was eluted first with 10 μM heparin in equilibration buffer, followed by elution of low-HLT with 1 M NaCl in equilibration buffer, or a gradient of 0.3 M to 1.5 M NaCl and 0 to 50 μM heparin in equilibration buffer was used to elute both isozymes, with the high-HLT eluting first. The total gradient volume was 150 ml and 1 ml fractions were collected at 1 ml/minute for both elution profiles. The overall recovery of activity was usually about 25% with the high M_r fraction representing approximately 85% of the total and the low M_r form accounted for 15% of the activity on

Cbz-Lys-SBz1. The active fractions from each peak were pooled and analyzed by SDS-PAGE.

Electrophoresis

SDS-PAGE analysis of proteins or cleavage products was carried out under reducing conditions on 1.5 mm thick 6%, 8% or 10% polyacrylamide gels as described by Bury (1981). Proteins were stacked at 15 mA and the current was increased to 20 mA after the dye front entered the separating gel. The gels were placed in MetOH/acetic acid/H₂O (40%/7%/53% v/v) to fix the proteins in the gels, stained with colloidal Coomassie Brilliant Blue and destained with MetOH/acetic acid/H₂O (25%/10%/65% v/v) for 30 seconds followed by MetOH/H₂O (25%/75% v/v) until the background stain disappeared.

Electroelution of Proteins from SDS-PAGE

High-HLT and low-HLT were electroeluted from SDS-PAGE gel slices for the purpose of performing a Western blot. High-HLT and low-HLT (240 µg of each) were TCA precipitated and electrophoresed as described above. High-HLT was electrophoresed on two gels, loading 20 µg per lane in 12 total lanes. After electrophoresis, the gels were stained with copper stain, which stains the gel a light blue while leaving the protein bands unstained, and the bands containing high-HLT were cut out of the gels, destained, and loaded in an IBI, Model UEA, electroelution apparatus. The

protein was electroeluted from the gel slices into 7.5 M NH_4 acetate at 50 V for 2½ hours. The NH_4 acetate solution containing the high-HLT was removed from the apparatus, separated into 6 microcentrifuge tubes and the protein was precipitated with TCA as described previously. The procedure was repeated with low-HLT. It was estimated that each tube contained approximately 20 μg of protein.

Active Site Titration

Active site titrations were performed on each trypsin isozyme using the titrant 4-methylumbelliferyl p-guanidinobenzoate hydrochloride (MUGB), which reacts with the active site serine of trypsin-like enzymes (Jameson et al., 1973). The excitation and emission wavelengths were 365 nm and 445 nm, respectively, and known concentrations of the fluorescent product, 7-amino-4-methyl coumarin (AMC), were used as standards. A stock solution of 0.1 M AMC in DMSO was diluted 1:100 with 0.1 M HEPES, 10 μM heparin, 10% glycerol, 0.05% Brij, 0.02 NaN_3 , pH 7.5 to produce a 1 nmol/ml solution. The fluorimeter was calibrated to zero against 500 pmol of substrate in 1 ml of assay buffer. A solution containing 100 pmol/ml of AMC in assay buffer was used to set the fluorimeter manually to a value of 100. Standards of 10, 20, 40, 60, and 80 pmol in a final volume of 1 ml were read and the fluorescence values were plotted against the molar values to produce a standard line. Five

hundred pmol of substrate and a range of 20-200 pmol of enzyme was used per assay in a final volume of 1 ml.

Deglycosylation of Tryptase Isozymes

Two aliquots of 20 μg each of high-HLT and low-HLT were TCA precipitated in 1.5 ml microcentrifuge tubes by adding TCA to each sample for a final concentration of 20% and incubating the samples on ice for 1 hour, followed by centrifugation and washing of the precipitates twice with 700 μl of cold 10% TCA and twice with 700 μl of cold acetone and drying at room temperature. The precipitated protein was resolubilized by adding 10 μl of 20 mM Tris pH 7.5 containing 0.5% SDS, 50 mM β -ME and 0.02% NaN₃, and heating at 95°C for 5 minutes. To one tube of each isozyme, 5 μl of 7.5% NP-40 was added along with 1.2 μl of N-Glycanase enzyme (9.1 $\mu\text{g}/\text{ml}$) and 13.8 μl of dH₂O was added to all four tubes. The samples were incubated at 43°C for 21 hours followed by the addition of 25 μl SDS ammediol sample buffer containing 1% β -ME and electrophoresis as described previously using 25 μl of each sample, a 10% 1.5 mm gel, and colloidal Coomassie Brilliant blue stain.

High-HLT was also deglycosylated without denaturation and reduction to determine if the activity was reduced as a result of deglycosylation. One hundred μl (4.8 μg) of high-HLT was incubated with 2 μl of N-Glycanase at 43°C for 21 hours. A control of 100 μl of high-HLT with no N-Glycanase

was also incubated as above. After cooling, the control, the treated sample, and the high-HLT pool before incubation at 43°C were assayed using Cbz-Lys-SBzl as the substrate. Fifty μ l of the control and the treated sample were then TCA precipitated, electrophoresed on a 10% 1.0 mm SDS gel and stained with silver stain.

Western Blot Analysis

Proteins were electroblotted onto either Immobilon-P or PVDF-Plus membrane. Electrophoresis of proteins to be transferred was carried out as above except that the gel thickness was reduced to 1.0 mm. The gels were subsequently equilibrated in 48 mM Tris, 39 mM glycine, 20% MeOH pH 9.2 and the proteins were transferred to the membrane at 15 V constant voltage for 40 minutes in a Bio-Rad Transblot SD Semi-Dry Transfer Cell. The membranes were blocked with 3% fish gelatin or BSA for one hour and exposed to primary antibody for 15 hours with gentle shaking at room temperature. The primary antibody solution for analysis of high- HLT or low-HLT consisted of a 1:2000 (v/v) dilution of rabbit anti-HLT antibody in TBS (20 mM Tris 500 mM NaCl 0.02% NaN₃, pH 7.5). The secondary antibody solution consisted of a 1:2000 (v/v) of goat anti-rabbit IgG antibody conjugated with alkaline-phosphatase in TBS. The reactive proteins were visualized by development of the alkaline-phosphatase with NBT/BCIP. The primary antibody solution

for analysis of the HMWK cleavage products consisted of a 1:1000 (v/v) dilution of a monoclonal antibody to the carboxy terminal region of HMWK in TTBS (20mM Tris 500mM NaCl 0.05% Tween-20 0.02% NaN₃, pH 7.5) with 1% gelatin. The membranes were then exposed to Protein G Gold solution (a 1:25 dilution of the stock solution provided in 20 mM citrate pH 5.5 containing 500 mM NaCl, 0.1% BSA, 0.05% Tween-20, 0.02% NaN₃, and 0.4% fish gelatin) to detect bound IgG and further developed using Bio-Rad's silver enhancement procedure. This involved incubating the membrane in citrate buffer (0.2 M citrate, 75 mM sodium citrate pH 3.7) for five minutes followed by incubation for 5-15 minutes in enhancement solution (a mixture of 0.11 g of silver lactate dissolved in 10 ml of dH₂O with 0.85 g of hydroquinone dissolved in 90 ml of citrate buffer) in the dark. The enhancement reaction was stopped by rinsing the membrane with dH₂O and the silver was fixed on the membrane by rinsing with a 1:10 dilution of the fixing solution in dH₂O for five minutes.

Gel Filtration

Sephacryl S200-HR column material (155 ml) was washed with dH₂O, equilibrated with 10 mM MES, 1.5 M NaCl, 20% glycerol, 0.02% NaN₃, pH 6.1, and poured into a 1.5 x 88 cm glass chromatography tube. The void volume (V₀) was determined to be 60 ml by applying 500 µl of a solution of

blue dextran (MW = 2000 kDa) with an A_{280} of 0.5 to the column, eluting at 0.5 ml/minute. To calibrate the column further, additional standards solutions were made up as follows: IgG (3 mg/ml, A_{280} = 2.2, MW = 150 kDa); BSA (4 mg/ml, A_{280} = 5.0, MW = 67 kDa); and chymotrypsinogen A (4 mg/ml, A_{280} = 15, MW = 25 kDa). IgG (500 μ l) was loaded on the column and eluted with a flow rate of 0.5 ml/minute. Fifty ml of buffer was collected into a graduated cylinder before collecting 70 fractions. The A_{280} of each fraction was read and plotted on the Y-axis vs. the fraction number on the X-axis. The chromatography and plotting described above was repeated using BSA as the standard and again using chymotrypsinogen A (except that 250 μ l was loaded). Then new solutions of the three standards were prepared with concentrations of 8 mg/ml, 250 μ l of each standard was mixed and the mixture was chromatographed as above. A solution of trypsin containing both high-HLT and low-HLT (A_{280} = 0.226, approximate total protein = 1.45 mg) was concentrated from 18 ml to 1.5 ml in an Amicon pressure cell using a 10,000 MWCO (YM-10) membrane and chromatographed on the standardized column. The absorbance of the fractions was read at 280 nm and a microtiter plate assay of the fractions was performed as previously described. The peaks of activity were pooled separately and analyzed by SDS-PAGE and Western blotting.

Inhibitor Assays Using Cbz-Lys-SBzl as the Substrate

The inhibitors tested for activity against high-HLT using Cbz-Lys-SBzl as the substrate were recombinant human Alzheimer's amyloid precursor protein inhibitor (APPI), human inter- α -trypsin inhibitor (I α I), recombinant bovine Arg¹⁵Glu⁵²aprotinin (also known as bovine pancreatic trypsin inhibitor or Trasylol), recombinant human secretory leukocyte proteinase inhibitor (SLPI), *Ascaris suum* trypsin inhibitor (ASTI), and ecotin (a trypsin inhibitor from *E. coli*). *Curcubita maxima* trypsin inhibitor-III (CMTI-III; a trypsin inhibitor from squash seed) was tested with high-HLT and low-HLT.

SLPI, Ecotin, and ASTI. Fifty μ l of high-HLT (37 μ g/ml; 57.5 pmol) and 575 pmol of inhibitor (a 1:10 enzyme:inhibitor molar ratio) was added to 100 μ l of assay buffer (0.1 M HEPES pH 7.5 containing 0.05% Brij 35 and 0.02% NaN₃) in a 1 ml cuvette and incubated at room temperature for 10 minutes, 20 minutes or 60 minutes. Remaining trypsin activity was assayed using the Cbz-Lys-SBzl substrate as previously described. Controls were exact duplicates of the samples except that no inhibitor was added.

APPI and I α I. Assays were performed in 1 ml spectrophotometric cuvettes at room temperature using a 1:100 enzyme:inhibitor molar ratio. Inhibitors (APPI, 18.4

$\mu\text{l} = 2.88 \text{ nmol} = 18.4 \mu\text{g}$; or I α I, $170 \mu\text{l} = 2.88 \text{ nmol} = 634 \mu\text{g}$) were added to $200 \mu\text{l}$ of assay buffer (0.1 M HEPES pH 7.5 containing $10 \mu\text{M}$ heparin, 10 % glycerol, 0.05% Brij 35, and 0.02% NaN_3) containing $25 \mu\text{l}$ of high-HLT (28.75 pmol ; $0.93 \mu\text{g}$) and incubated at room temperature for 3 hours. A control of $25 \mu\text{l}$ of high-HLT in $200 \mu\text{l}$ of assay buffer was also incubated as above. Remaining trypsin activity was assayed with Cbz-Lys-SBzl substrate as previously described. This experiment was repeated using $400 \mu\text{l}$ of assay buffer and 1.44 nmol of each inhibitor and 14.38 pmol of high-HLT. Also included were assays using 0.1 M Tris buffer pH 8.0, 8.5, and 8.8. The incubation and assay conditions were identical to the ones described above.

Arg¹⁵Glu⁵² aprotinin. This procedure was performed essentially as above using $200 \mu\text{l}$ of assay buffer (0.1 M HEPES pH 7.5 containing $10 \mu\text{M}$ heparin, 10 % glycerol, 0.05% Brij 35, and 0.02% NaN_3), $25 \mu\text{l}$ of high-HLT (28.6 pmol ; 915 ng), and $18.7 \mu\text{l}$ of Arg¹⁵Glu⁵² aprotinin (2.88 nmol ; $18.7 \mu\text{g}$). A control was performed using $200 \mu\text{l}$ of assay buffer and $25 \mu\text{l}$ of high-HLT. The samples were incubated at room temperature for 10 minutes, followed by an activity assay using Cbz-Lys-SBzl as described above.

CMTI-III. This procedure was performed as above using $350 \mu\text{l}$ of the above assay buffer, 140 pmol of CMTI-III ($4.6 \mu\text{l}$ of a $100 \mu\text{g/ml}$ solution; 460 ng) and 3 pmol of active

low-HLT (20 pmol of protein; \approx a 1:50 molar E:I ratio based on active sites of low-HLT). A control was performed using 350 μ l of assay buffer and 1.4 pmol of low-HLT. The samples were incubated at room temperature for four hours, followed by an activity assay using Cbz-Lys-SBzl as described above. This experiment was repeated using 350 μ l of HEPES assay buffer at pH 7.5, or Tris assay buffer at pH's 8.0 or 8.5 per sample. Low-HLT (20 pmol of protein) and CMTI-III (2 nmol) (a 1:100 molar E:I based on protein concentration or a 1:667 E:I based on active sites of low-HLT) were added to each sample cuvette and incubated and assayed as above. Identical assays were also performed using 100 μ l of pH 8.0 assay buffer, 20 pmol of low-HLT or 5 μ l of high-HLT (concentration not recorded), and 2 nmol of CMTI-III.

α_2 M and α_1 I₃ Activity Assay

Bovine alpha-2-macroglobulin (α_2 M) (9.8 mg/ml = 13.5 nmol/ml) that had been stored at -80°C for two years was diluted 1:1 with 0.1 M Tris pH 8.0 (assay buffer). Porcine trypsin (100 μ l of a 2 nmol/ml solution = 0.2 nmol) was used per reaction. A 6% solution (w/v) of azocasein in dH_2O was used as the substrate due to the unique mechanism of action of α_2 M (refer to Results). A series of reactions were set up in 1.5 ml microcentrifuge tubes as follows:

- 1) blank = 100 μ l azocasein + 900 μ l AB (assay buffer)
- 2) 100 μ l trypsin + 800 μ l AB

- 3) " + 790 μ l AB + 10 μ l α_2 M (\approx 0.07 nmol)
- 4) " + 780 μ l AB + 20 μ l "
- 5) " + 770 μ l AB + 30 μ l "
- 6) " + 760 μ l AB + 40 μ l "
- 7) " + 750 μ l AB + 50 μ l "
- 8) " + 725 μ l AB + 75 μ l "
- 9) " + 700 μ l AB + 100 μ l " (\approx 0.67 nmol)

The reaction was started by the addition of 100 μ l of 6% azocasein to each tube, followed by incubation at room temperature for 20 minutes. The reaction was stopped and the undigested azocasein and other proteins were precipitated by the addition of 200 μ l of 20% TCA to each tube. The samples were centrifuged for 5 minutes and the absorbance of each supernatant was read at 366 nm. The basis of this assay is that uncleaved proteins are more readily precipitated than small cleavage products. Therefore, the control (sample 2), containing only trypsin and azocasein, would theoretically have the largest amount of protein (cleaved) left in the supernatant after TCA precipitation and centrifugation and, consequently, would retain the highest absorbance at 366 nm. As increasing amounts of inhibitor were added, the A_{366} of the samples should decrease due to the decreased amount of enzyme available to digest the azocasein. Sample 2 (no inhibitor) was designated as producing 100% activity based on the absorbance relative to the blank and the absorbances of

samples 3 - 9 were used to determine the percent of activity relative to sample 2. The percent activity (Y-axis) was plotted against the μl of $\alpha_2\text{M}$ (X-axis) to determine the relative activity of the $\alpha_2\text{M}$.

The above assay was repeated using rat alpha-1-inhibitor-3 ($\alpha_1\text{I}_3$) (6.4 mg/ml = 33.7 nmol/ml) as the inhibitor. The stock solution was diluted 1:5 with assay buffer and the amounts used were 10, 20, 30, 40, 50, 75, 100, 150 μl (≈ 0.07 nmol - 1.0 nmol). The amount of assay buffer used per reaction was adjusted accordingly to produce a final reaction volume of 1 ml.

Inhibitor Assays Using HMWK as the Substrate

The inhibitors tested for activity against the tryptase isozymes using HMWK as the substrate were bovine $\alpha_2\text{M}$, rat $\alpha_1\text{I}_3$ (the rat equivalent to human $\alpha_2\text{M}$), APPI, I α I, ASTI, SLPI and CMTI-III.

$\alpha_2\text{M}$ and $\alpha_1\text{I}_3$. HMWK was used as the substrate for these inhibitory assays of tryptase and trypsin was used as a control enzyme. High-HLT (0.88 pmol of tetrameric enzyme, 3.52 pmol of active sites; 2.64 μl of a 46.5 $\mu\text{g}/\text{ml}$ solution) was added to each of three 1.5 ml microcentrifuge tubes containing 240 μl of assay buffer (0.1 M HEPES pH 7.5 containing 0.05% Brij 35 and 0.02% NaN_3). To one of the tubes, 2.6 μl (2.6 μg , 3.51 pmol) of $\alpha_2\text{M}$ was added and to another tube 2.64 μl (1.34 μg , 3.51 pmol) of $\alpha_1\text{I}_3$ was added

(a 1:4 molar ratio of E:I based on a tetrameric enzyme or a 1:1 based on trypsin active sites) and all three tubes were incubated at room temperature for 30 minutes. Trypsin was added to three additional tubes containing 240 μ l of assay buffer and the same amount of α_2 M and α_1 I₃ as above was added to two of these tubes and incubated as above. After 30 minutes, 258 μ l (50 μ g, 438 pmol; a 1:125 molar ratio of E:S based on number of active sites of enzyme) of HMWK was added to each tube and 130 μ l (25 μ g, 219 pmol) of HMWK was added to a seventh tube for a control and all the tubes were incubated at 37°C for 20 minutes. The reaction was stopped by the addition of cold 40% TCA for a final concentration of 20%. After centrifugation, the precipitates were washed with cold 10% TCA and acetone and dried. The samples were solubilized with 50 μ l of Tris-HCl sample buffer containing 5% β -ME and heated at 95°C for 10 minutes and cooled. Twenty μ l (20 μ g) of each sample were analyzed on a 7% SDS-PAGE gel using the ammonium buffer system and the proteins were visualized with colloidal Coomassie Brilliant Blue stain.

Arg¹⁵Glu⁵²aprotinin. Trypsin (3.52 pmol) or high-HLT (3.52 pmol) and inhibitor (352 pmol) were added to each of seven 1.5 ml microcentrifuge tubes containing 400 μ l of reaction buffer of either pH 7.5 or pH 9.1 (0.1 M HEPES pH 7.5 containing 10 μ M heparin, 10 % glycerol, 0.05% Brij 35,

0.02% NaN₃ or 0.1 M Tris pH 9.1 containing the same additives) as listed below:

- 1) pH 7.5 + HMWK
- 2) " + " + trypsin (3.52 pmol)
- 3) " + " + " + inhibitor
(352 pmol)
- 4) " + " + tryptase (3.52 pmol)
- 5) " + " + " + "
- 6) pH 9.1 + " + "
- 7) " + " + " + "

The E:I ratio was 1:200 and the E:S ratio was 1:50. After incubating the enzyme and inhibitor at room temperature for 30 minutes, HMWK (20 µg; 176 pmol) was added to each tube and an additional 60 minute incubation was allowed at room temperature. The reactions were stopped by TCA precipitation and the samples were prepared and electrophoresed on a 7%, 1.5 mm gel as previously described.

APPI, IαI, ASTI and SLPI. HMWK (20 µg; 176 pmol) was put into each of six 1.5 ml microcentrifuge tubes containing 400 µl of reaction buffer (0.1 M Tris pH 8.5 containing 10 µM heparin, 10 % glycerol, 0.05% Brij 35 and 0.02% NaN₃). High-HLT (3.52 pmol; 113 ng) was added to each of five of the tubes, and 352 pmol of APPI, IαI, ASTI or SLPI were added to four of the tubes containing the high-HLT (for a 1:100 molar ratio of E:I). All 6 tubes were incubated at

room temperature for 30 minutes. HMWK (176 pmol) was then added to all tubes containing high-HLT (for a 1:50 molar ratio of E:S) and the samples were incubated at room temperature for 60 minutes. The samples were then precipitated with TCA as previously described and were analyzed by SDS-PAGE. A 7% 1.0 mm gel was used and the samples were visualized using colloidal Coomassie Brilliant blue stain.

CMTI-III. Low-HLT (2.22 pmol of protein or 0.33 pmol of active sites; a 1:100 E:I ratio based on protein concentration or a 1:667 ratio based on number of active sites of low-HLT) or high-HLT (2.12 μ l, concentration not recorded) was incubated with CMTI-III (222 pmol) in 1.5 ml microcentrifuge tubes containing 315 μ l of assay buffer (0.1 M HEPES pH 7.5 containing 10 μ M heparin, 20% glycerol, 0.05% Brij 35, 0.02% NaN₃) for 4 hours at room temperature. HMWK (443 pmol; 50 μ g; a 1:200 E:S ratio based on protein concentration or a 1:1342 E:S ratio based on number of low-HLT active sites) was added to each tube and the mixtures were incubated for another 30 minutes at room temperature. Each sample mixture was then divided into 3 tubes containing 146 μ l each and the proteins were precipitated by the addition of 1314 μ l of ice cold absolute EtOH to each tube and storage at -20°C overnight. A control containing 126 μ l of assay buffer, 0.89 pmol of low-HLT, and 20 μ g of HMWK was

prepared in the same way and precipitated in a single tube using 1350 μ l of cold EtOH. The precipitated proteins were collected by centrifugation, the supernatant was discarded, and the precipitates were dried at room temperature. The samples were solubilized with 10 μ l of 1X SDS ammediol sample buffer containing 1% β -ME, the control with 30 μ l of the same sample buffer and 50 μ l of sample buffer were added to 10 μ l of MW standards. All tubes were heated to 95°C for 10 minutes and cooled. The samples and standards were electrophoresed on an 8% 1.5mm SDS-polyacrylamide gel as previously described and stained with colloidal Coomassie Brilliant blue.

Comparison of Inhibitory Effects of SLPI on High-HLT and Low-HLT

In this assay, the inhibitory activity of SLPI on high-HLT and low-HLT cleavage of HMWK was compared. A 1:50 E:S molar ratio (\approx a 1:53 ratio for high-HLT and a 1:331 ratio for low-HLT based on the number of active sites) and a 1:100 E:I molar ratio (\approx a 1:105 ratio and a 1:662 ratio for high-HLT and low-HLT, respectively, based on the number of active sites) was used. Five 1.5 ml microcentrifuge tubes each containing 400 μ l of assay buffer (0.1 M HEPES pH 7.5 containing 10 μ M heparin, 10% glycerol, 0.05% Brij 35 and 0.02% NaN_3) were incubated at room temperature for 30 minutes after the following additions:

- 1) no addition
- 2) high-HLT (3.51 pmol or 3.33 pmol of active sites)
- 3) high-HLT (3.51 pmol) and SLPI (351 pmol)
- 4) low-HLT (3.51 pmol or 0.53 pmol of active sites)
- 5) low-HLT (3.51 pmol) and SLPI (351 pmol)

HMWK (175.5 pmol) was added to all 5 tubes followed by a second incubation for 60 minutes. The reactions were stopped by TCA precipitation as previously described and the samples were analyzed by SDS-PAGE using an 8% 1.5 mm gel. The proteins were visualized using colloidal Coomassie Brilliant blue stain.

Tryptase Cleavage of Laminin, Fibrinogen, and Fibronectin

Several naturally occurring proteins were tested as or confirmed as substrates for one or both of the tryptase isozymes. These included fibrinogen (Schwartz et al., 1985), HMWK (Maier et al., 1983), and VIP (Tam and Caughey, 1990), which had been reported in the literature as tryptase substrates, and laminin and fibronectin, which had not been previously tested. Cleavage of HMWK and VIP by high-HLT and low-HLT is described in another section. In this experiment, laminin, fibrinogen, or fibronectin (20 μ g) were tested as substrates for high-HLT. Laminin contains 3 chains with a combined MW of approximately 1000 kDa. The stock solution contained 680 μ g/ml (680 pmol/ml); therefore, 20 pmol equaled 20 μ g. Fibrinogen has a MW of 340 kDa. The stock solution contained 1 mg/ml (2.94 nmol/ml); therefore,

58.8 pmol equaled 20 μ g. Fibronectin contains 2 chains with a combined MW of 500 kDa. The stock solution contained 600 μ g/ml (1.2 nmol/ml); therefore, 40 pmol equaled 20 μ g. A 1:200 E:S molar ratio was used for each substrate. Six 1.5 ml microcentrifuge tubes containing 200 μ l of assay buffer (0.1 M HEPES pH 7.5 containing 10 μ M heparin, 10% glycerol, 0.05% Brij 35, and 0.02% NaN₃) and the following amounts of substrate and enzyme were incubated at room temperature for 30 minutes:

- 1) 20 pmol of laminin
- 2) 20 pmol of laminin and 0.1 pmol of high-HLT
- 3) 58.8 pmol of fibrinogen
- 4) 58.8 pmol of fibrinogen and 0.294 pmol of high-HLT
- 5) 40 pmol of fibronectin
- 6) 40 pmol of fibronectin and 0.2 pmol of high-HLT

The reactions were stopped and the proteins precipitated using cold 40% TCA as described under HMWK Cleavage. Thirty μ l of 1X SDS ammediol sample buffer containing 1% β -ME was added to the dried samples and 20 μ l of the same sample buffer was added to 10 μ l of MW standards followed by heating at 95°C for 5 minutes and cooling. Twenty μ l of each sample were loaded on a 7% 1.0 mm gel and the samples and standards were electrophoresed as described previously and visualized with colloidal Coomassie Brilliant blue. A second gel, a Bio-Rad prepoured 4-20% polyacrylamide gel, was run using the remainder of the samples and additional

standards. A Tris-glycine running buffer was used (25 mM Tris, 208 mM glycine, 0.5% SDS pH 8.3) and the gel was run at a constant voltage of 200 V. The proteins were visualized using the Bio-Rad Silver Stain kit.

Carboxymethyl-papain Column Preparation

CM-papain column material was prepared by a modification of a previously published method (Johnson et al., 1987). One hundred ml of SpectraGel A6 agarose column material was washed with cold 1.5 M Na_2CO_3 and incubated with 25 g of CNBr dissolved in 25 ml of acetonitrile at room temperature with stirring in a fume hood until the mixture appeared uniform. The column material was then washed thoroughly on a Buchner funnel with cold dH_2O followed by 0.1 M Na_2HPO_4 pH 7.8 (coupling buffer). Fifty mg of papain (twice crystallized, Na acetate suspension) dissolved in 30 ml of coupling buffer was incubated with the column material overnight at 4°C. The mixture was warmed to room temperature and 2 ml of ethanolamine was added (to block unreacted CNBr coupling groups) and allowed to react for 30 minutes, followed by washing the column material with one liter of 0.2 M Na acetate 0.5 M NaCl pH 4.5 and one liter of 0.05 M Tris. The A_{280} of the initial wash solution was checked and compared to the unreacted papain solution to determine the amount of papain coupled to the column material (23 mg). The column material was washed with 0.2 M

Tris pH 8.5 and suspended in 100 ml of the same buffer. Papain was activated with β -ME at a final concentration of 1 mM, which assures a free active site thiol group, followed by the addition of 27.9 mg of ICH_2COOH (dissolved in 1 ml of 1 M NaOH) for a final concentration of 1.5 mM and incubation at room temperature for 15 minutes to carboxymethylate the cysteine residues present in the papain. The column material was washed thoroughly with dH_2O and then with 50 mM Na_2HPO_4 , 0.02% NaN_3 , pH 7.5.

Kininogen Purification

HMWK was purified from frozen human plasma by a previously published method (Johnson et al., 1987). Approximately 200 ml of plasma was thawed by immersing the bag in lukewarm water and the plasma was transferred to a plastic beaker. Inhibitors were immediately added to prohibit plasma proteases from degrading the kininogens. The inhibitors added were 10 mM benzamidine HCl (inhibits trypsin-like serine proteinases), 40 $\mu\text{g}/\text{ml}$ polybrene (blocks negatively charged surfaces), 2 mM EDTA (inhibits metalloproteinases by chelating metal ions), 0.2 mM PMSF (irreversibly inhibits serine proteases by binding to the active site serine), and 0.2 mg/ml SBTI (inhibits serine proteinases). After adding NaCl to a final concentration of 2 M, the plasma was centrifuged at $16,300 \times g$ (5°C) for 30 minutes and the supernatant was incubated with 78 ml of

carboxymethyl-papain Sepharose column material equilibrated with 50 mM Na_2HPO_4 2 M NaCl pH 7.5 containing 1 mM benzamidine, 40 $\mu\text{g/ml}$ polybrene, 2 mM EDTA, 0.2 mM PMSF, 0.02% NaN_3 in a plastic flask for 45 minutes at room temperature. The column material was collected on a Buchner funnel, washed with equilibration buffer and poured into a siliconized glass chromatography tube. The column was further washed with equilibration buffer until the A_{280} fell below 0.01 followed by washing with 100 ml of 50 mM Na_2HPO_4 pH 7.5 containing 2 mM EDTA, 0.1 mM PMSF, 0.02% NaN_3 . The kininogens were eluted from the column with 50 mM Na_2HPO_4 pH 11.5 containing 2 mM EDTA and 0.02% NaN_3 , and 3 ml fractions were collected at 2 minute intervals into plastic tubes containing 125 μl of 1 M Na acetate pH 4.4 containing 4 mM PMSF to achieve a final concentration of 0.2 mM PMSF and a pH of 5.5. The absorbance of the fractions was read at 280 nm and the appropriate fractions were pooled (50 ml) and concentrated in an Amicon pressure cell using a 30,000 MW cutoff membrane (YM-30) to 20 ml and the sample was diluted to 50 ml with 10 mM Na acetate pH 5.5 containing 1 mM benzamidine and 0.2 mM PMSF and the sample was concentrated to 6 ml. Three ml of the sample were loaded on a Mono Q 5/5 HPLC column equilibrated in 10 mM Na acetate pH 5.5 at 0.5 ml/minute and the column was washed with equilibration buffer at 1.0 ml/minute until the A_{280} reached zero. LMWK was eluted with a NaCl gradient of 0-0.25 M over 30 minutes

followed by HMWK elution with a 0.25-0.5 M NaCl gradient over 5 minutes. The remaining 3 ml of the concentrated sample from the carboxymethyl-papain Sepharose column was chromatographed on the Mono Q column in the same way. The fractions corresponding to the A_{280} peak were pooled and analyzed by SDS-PAGE with reduction. The HMWK pool produced a major band with an M_r of 114 kDa as expected for intact HMWK and a contaminating band of M_r 55 kDa and LMWK appeared as a 67 kDa band. The contaminant co-purified with HMWK, even in the presence of 5 M urea, and most likely was the amino terminal region of HMWK resulting from autolysis by kallikrein during preparation and storage of the blood plasma (Kato et al., 1981). This band did not react with antibodies specific for the carboxy terminal portion of HMWK.

HMWK Cleavage

All cleavage reactions were performed in 1.5 ml microcentrifuge tubes at room temperature. To 1000 μ l of reaction buffer (0.1 M HEPES 10 μ M heparin 10% glycerol 0.05% Brij 0.02% NaN_3 , pH 7.5), 1.74 nmol (443 μ g/ml) of HMWK and the appropriate amount of enzyme was added. A 1:50 molar ratio (enzyme:substrate) was used for high-HLT while a 1:1300 molar ratio was used for low-HLT, based on the number of active sites of tryptase. These enzyme-substrate ratios, which were based on preliminary experiments, yielded similar

cleavage rates. Aliquots of twenty μg of HMWK were withdrawn at each of five time points, 5, 10, 20, 40, and 60 minutes, and the reaction was immediately terminated by precipitating the proteins with either TCA or ethanol. TCA precipitation, used for the samples on the gels shown in Figure 2, involved adding TCA to each sample for a final concentration of 20% and incubating the samples on ice for 1 hour, followed by centrifugation and washing of the precipitates twice with 700 μl of cold 10% TCA and twice with 700 μl of cold acetone and drying at room temperature. The TCA precipitation protocol used initially did not recover some smaller fragments that were subsequently detected when ethanol precipitation was used. Ethanol precipitation, used for the samples on the Western blot shown in Figure 3, involved adding nine volumes of ice cold absolute ethanol to each sample followed by incubation for 12 hours at -20°C . Precipitates were collected by centrifugation at 14,000 rpm (4°C) for 5 minutes and were dried at room temperature.

Sequencing of HMWK Fragment

HMWK (40 μg ; 350pmol) was incubated with either 7 pmol of high-HLT (E:S of 1:50) or 1.76 pmol of low-HLT (E:S of 1:200 based on protein concentration or 1:1326 based on number of active sites) in assay buffer for 10 minutes at room temperature (cleavage corresponds to the 10 minute time period in Figure 2) followed by precipitation with cold

trichloroacetic acid as described under HMWK Cleavage. Eight μg of the sample were loaded in each of five lanes of a 6% SDS-PAGE gel and then electrophoresed and electroblotted onto Immobilon-P as described under Electrophoresis and Western Blot Analysis. The membranes were lightly stained with 0.1% Coomassie R-250 in 50% MeOH 10% acetic acid (v/v), dried, and sent to Dr. Mark Lively at Bowman Gray School of Medicine (Winston-Salem, NC) for amino acid sequencing.

VIP Cleavage

Cleavage of VIP by the tryptase isozymes was performed in 1.5 ml microcentrifuge tubes at room temperature. Thirty nmoles of VIP were added to 500 μl of reaction buffer and 24.7 pmoles of either high-HLT or low-HLT active sites (E:S of 1:17000). Equal aliquots were withdrawn from the reaction mixture at 5, 10, 20, 30, and 60 minutes and 5 μl of acetic acid was added to each aliquot to stop the reaction, followed by the addition of 130 μl of 0.1% TFA (HPLC buffer A). The samples were then filtered and applied to a C_{18} column at 0.5 ml/minute. The column was eluted at 0.5 ml/minute with a 10-40% gradient of buffer B (0.1% TFA in acetonitrile) over 20 minute and the absorbance was monitored at 210 nm.

Amino Acid Analysis of VIP Cleavage Products

The peak at 24.34 minutes from the high-HLT cleavage of

VIP and the peak at 28.54 minutes from the low-HLT cleavage of VIP were collected in separate microcentrifuge tubes. The samples were transferred to HNO₃-cleaned 6 x 50 mm glass tubes and dried in a SpeedVac for 45 minutes, then hydrolyzed in an evacuated vial with 6 N HCl at 110°C for 23 hours. Hydrolyzed samples and amino acid standards (500 pmol) were dried as before and PITC derivatized according to the Pharmacia protocol. This involved the addition of 10 µl of MeOH:TEA:H₂O (2:2:1) to each tube and drying followed by addition of 20 µl of MeOH:H₂O:TEA:PITC (7:1:1:1) to each tube and drying. Immediately before use, samples or standards were solubilized in 25 µl of HPLC buffer A (940 ml of 140 mM sodium acetate trihydrate containing 0.05% v/v triethylamine mixed with 60 ml of acetonitrile) and chromatographed on a Waters Pico-Tag column at 1 ml/minute with a 0-46% buffer B (60% acetonitrile in H₂O) convex gradient (curve #5) over 15 minutes. Quantification of the amino acids was accomplished with Waters Maxima software on an IBM/AT computer.

CHAPTER 3

RESULTS

Purification and Separation of the Two Tryptase Isozymes

Purification of tryptase from human lung tissue produced two pools of activity with M_r values of approximately 29 kDa and 33 kDa (Figure 3). The 29 kDa pool (low-HLT) was obtained in small quantities (15% of high-HLT) and was more difficult to purify. A variety of columns and loading/elution conditions were tried with the goal of improving isozyme separation, purity and yield over previous purification protocols, with little success. A Dowex-1X column was used to reduce the viscosity of the crude extract. Dowex binds heparin and other negatively charged molecules while allowing tryptase to be collected in the flowthrough. The recovery of activity was 56% and the viscosity was only slightly reduced. This pool was dialyzed and chromatographed on a heparin-Sepharose column and eluted with increasing salt. The binding capacity of the heparin-Sepharose was low and about 50% of the activity was detected in the flowthrough. Two peaks of activity eluted from the column but both contained numerous proteins (Figure 1 in the Appendix). An affinity column, p-aminobenzamidine agarose, was also tried but also exhibited low binding capacity. The CPC extract was loaded on at pH 7.5, the pH at which tryptase is most active but less stable than at

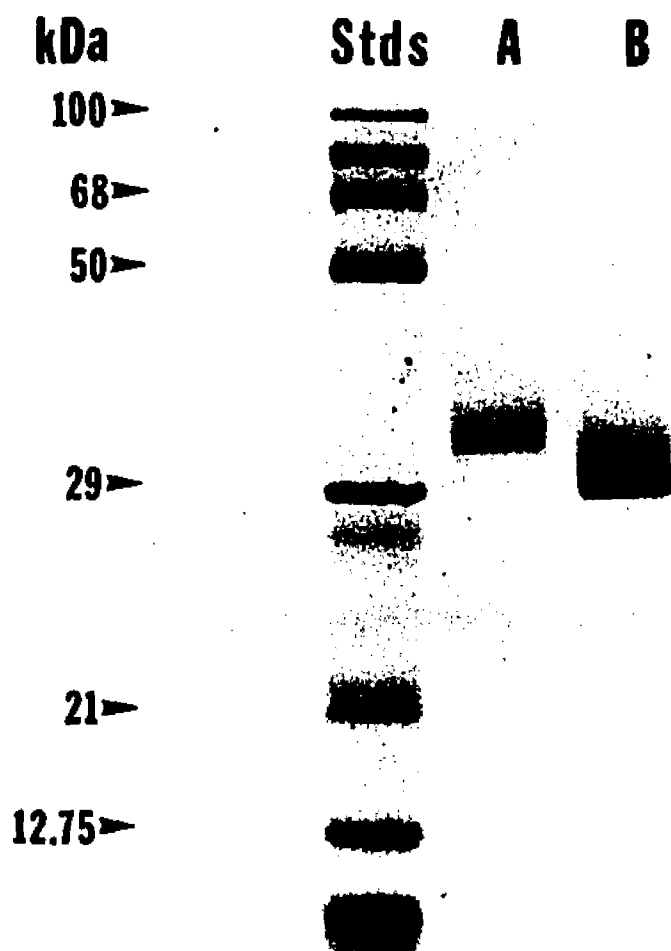


Figure 3. SDS-PAGE of High-HLT and Low-HLT Isozymes. Lane A, high-HLT (10 μ g); Lane B, low-HLT (10 μ g). Samples and standards were denatured and reduced before electrophoresis on a 10% gel. Molecular weight markers were, as indicated, phosphorylase B (100 kDa), transferrin (78 kDa), BSA (68 kDa), IgG (50 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa), cytochrome c (12.75 kDa), and Trasylol (6.5 kDa).

pH 6.1, and eluted with pH 4.5 buffer or 1 M NaCl buffer. The yields of protein as detected by A_{280} were very low as were the yields of activity ($\leq 10\%$). The sulfopropyl-type cation exchange columns Spectra Gel SP and Mono S HPLC were tried because the cation exchanger cellulose-phosphate bound trypsin very well. However, the binding capacities of these columns were lower than cellulose-phosphate and the NaCl in the sample had to be reduced to 0.2 M or less for binding to occur. Eventually, the best purification scheme found utilized CPC treatment of crude extracts followed by chromatography on butyl hydrophobic interaction chromatography (HIC) and cellulose-phosphate columns.

Both trypsin isozymes, along with many other proteins, bound to the butyl HIC column in 2 M $(\text{NH}_4)_2\text{SO}_4$ and eluted together with a decreasing $(\text{NH}_4)_2\text{SO}_4$ gradient. Elimination of the $(\text{NH}_4)_2\text{SO}_4$ and reduction of the NaCl concentration of the butyl pool by dialysis against MES buffer containing 0.3 M NaCl allowed subsequent binding of both isozymes to cellulose-phosphate column material. High-HLT eluted from the cellulose-phosphate column with 10 μM heparin whereas low-HLT did not elute with heparin but required 1 M NaCl for elution. Alternately, a gradient of 0-50 μM heparin and 0.3 M-1.5 M NaCl dissociated both isozymes from the cellulose-phosphate column, with high-HLT eluting first. SDS-PAGE analysis showed that some low-HLT preparations contained minor contaminants, that coeluted from the cellulose-

phosphate column, which required re-chromatography to remove. The presence of trypsin throughout the purification procedure was monitored by a chromogenic activity assay using the synthetic trypsin substrate Cbz-Lys-SBzl. The overall recovery of activity was usually about 25% with the high M_r fraction representing approximately 85% of the total activity and the low M_r form accounting for 15% of the activity on Cbz-Lys-SBzl. A representative purification table is shown in Table 2, and Figure 4 shows the stages of purification on SDS-PAGE. Active site titration of the two trypsin isozymes with MUGB showed that the high-HLT was 95% active, whereas the low-HLT was only 15% active.

Structural Analyses of the Two Isozymes

Deglycosylation of High-HLT and Low-HLT

Deglycosylation of the two isozymes was attempted in order to get an indication of the carbohydrate content in relation to the M_r and to determine if the primary difference in size between the isozymes was due to glycosylation. Treating both forms of trypsin that had been TCA precipitated, dissolved in SDS buffer and reduced with DTT, with N-Glycanase (a recombinant form of endoglycosidase-F), yielded much sharper bands on SDS-PAGE, but only high-HLT decreased noticeably in M_r (an approximate decrease of 1-2 kDa). Low-HLT remained at the same relative

Table 2. Purification of High-HLT and Low-HLT from 500 g of Human Lung Tissue. A representative purification table for the purification of high-HLT and low-HLT shows an ultimate combined recovery of activity of 25%, with high-HLT representing 84% of the total activity. The Activity is expressed as the increase in absorbance at 410 nm/minute/ml. The Specific Activity is defined as the Activity/ A_{210} . The Fold Purification is based on the increase in Specific Activity and the Percent Recovery is based on the Total Activity in each pool (volume x activity) compared to the crude extract.

	<u>Volume (ml)</u>	<u>Total</u> <u>A_{210}</u>	<u>Total</u> <u>Activity</u>	<u>Specific</u> <u>Activity</u>	<u>Fold</u> <u>Purification</u>	<u>Percent</u> <u>Recovery</u>
Crude lung extract	460	5750	15272	2.7	1.0	100
CPC/(NH ₄) ₂ SO ₄ extract	975	3023	19403	6.4	2.4	127
Butyl pool	110	275	13090	47.6	18	86
Cellulose-phosphate						
Heparin fraction	22	7.0	3212	456	169	21
NaCl fraction	12.5	1.5	375	250	93	4

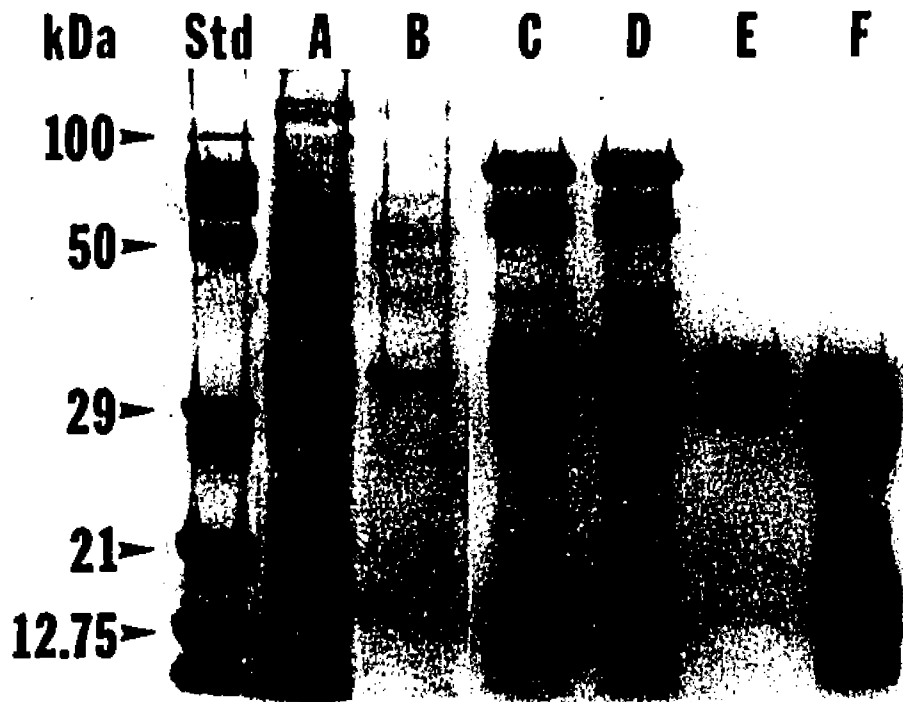


Figure 4. SDB-PAGE (10%) of the Progressive Steps of Purification of High-HLT and Low-HLT. Lane A, crude lung extract (20 μ l); Lane B, activity pool from butyl column before dialysis (10 μ g), Lane C, activity pool from butyl column after dialysis (10 μ g); Lane D, activity pool from the heparin elution of the cellulose-phosphate column (10 μ g); and Lane E, activity pool from the NaCl elution of the cellulose-phosphate column (10 μ g). The samples and MW standards were denatured and reduced before electrophoresis. The gel was stained with colloidal Coomassie blue.

MW but the profile of the band changed significantly (Figure 5A). High-HLT was also treated with N-Glycanase without first TCA precipitating the enzyme and without denaturation and reduction with SDS and β -ME, respectively, in order to test the effect of deglycosylation on the activity of high-HLT. SDS-PAGE analysis of this sample (Figure 5B) shows only a slight reduction in M_r . The results of an activity assay of 5 μ l of the sample and the controls are presented below:

<u>Sample</u>	<u>ΔA_{410}/minute</u>	<u>% Activity</u>
Before treatment	0.2277	100
Control	0.1574	69
Treated sample	0.1752	77

Both the control and the N-Glycanase-treated sample lost 25-30% of the activity in the original sample, presumably due to the incubation at 43°C for 21 hours. However, there is no significant difference in activity level between the control and the N-Glycanase-treated high-HLT.

Gel Filtration. Another structural study carried out was gel filtration to determine any differences in quaternary structure between the two tryptase isozymes. After calibrating a Sephacryl S-200HR column with Blue dextran (MW = 2000 kDa; to determine the V_0), IgG (MW = 150 kDa), BSA (MW = 67 kDa), and chymotrypsinogen A (MW = 25

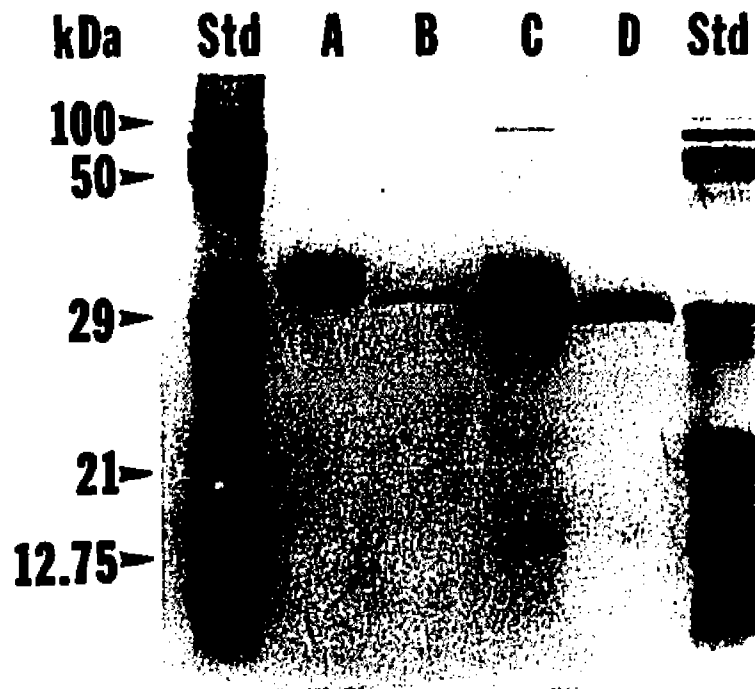


Figure 5 (A). SDS-PAGE (10%) of High-HLT and Low-HLT Deglycosylated Following Denaturation and Reduction. Lane A, high-HLT; Lane B, high-HLT treated (deglycosylated) with N-Glycanase; Lane C, low-HLT; and Lane D, low-HLT treated with N-Glycanase. Samples were denatured with SDS and reduced with β -ME before N-Glycanase treatment. Each sample lane contained approximately 10 μ g of protein. Colloidal Coomassie blue stain was used. The samples and MW standards in A and B were reduced before electrophoresis.

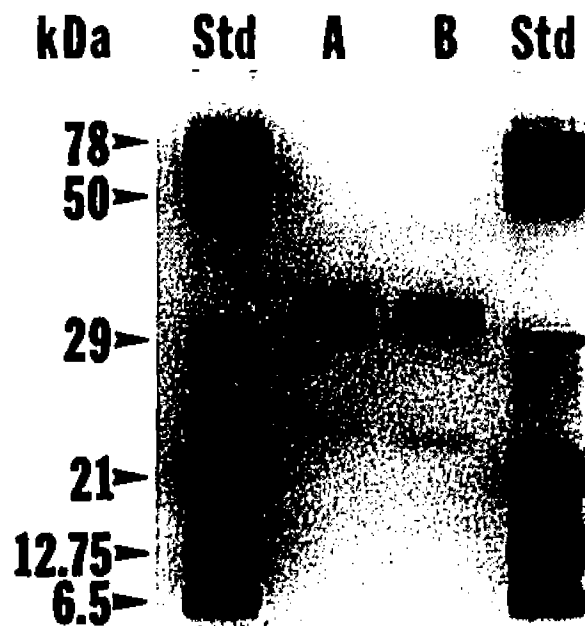


Figure 5 (B). SDS-PAGE (10%) of High-HLT Deglycosylated Without Prior Denaturation and Reduction. Lane A, high-HLT (2.5 μ g); Lane B, high-HLT (2.5 μ g) treated with N-Glycanase. The sample was not denatured or reduced before deglycosylation. The proteins were visualized using silver stain.

kDa), a mixture of high-HLT and low-HLT was chromatographed on the column. The A_{280} of the fractions were read and the values were plotted vs. the fraction number, yielding, as shown in Figure 6A, three distinct absorbance peaks with M_r of approximately 125 kDa, 55 kDa and 28 kDa. An overlay of the A_{280} of the fractions and the corresponding activities on Cbz-Lys-SBzl showed that the peaks of activity corresponded exactly with the A_{280} peaks (Figure 6B). SDS-PAGE analysis of the three peaks of activity showed that the first and second peaks contained high-HLT and the third peak contained low-HLT (Figure 7). These data indicated that high-HLT may exist primarily as a tetramer, with a M_r of about 125 kDa, while low-HLT may exist as a monomer, with a M_r of about 28 kDa. There may also be a dimeric form (M_r of 55 kDa) containing two heterologous subunits. Western blot analysis of these pools was somewhat inconclusive. The two high M_r pools migrated out of the gel onto PVDF or nitrocellulose membrane and reacted with the polyclonal anti-HLT antibody (Figure 8). The low M_r pool, however, did not migrate out of the gel under various transfer conditions and therefore was not immunologically analyzed.

Inhibition Studies of High-HLT and Low-HLT Using Several Trypsin Inhibitors

Two types of inhibitor assays were done, one using the small synthetic substrate Cbz-Lys-SBzl and one using the

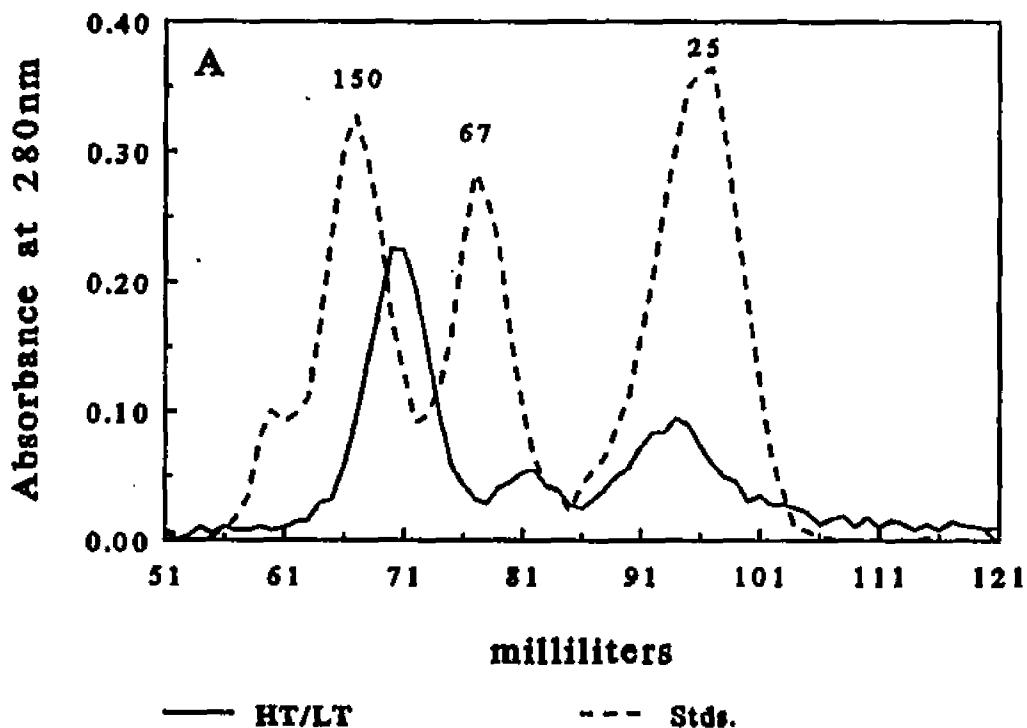


Figure 6 (A). Chromatogram of a Mixture of High-HLT and Low-HLT on a Sephacryl S200-HR Gel Filtration Column with MW Standards. A standard solution of 2 mg each of IgG (MW = 150 kDa), BSA (MW = 67 kDa) and chymotrypsinogen A (MW = 25 kDa) was chromatographed on a 1.5 x 88 cm gel filtration column at 0.5 ml/min. and the A_{280} of each fraction was read and graphed. The graph was overlaid on a chromatogram of the A_{280} of fractions collected after a mixture of high- and low-HLT (1-1.5 mg of total protein) was applied to the column. The V_0 had previously been determined to be 60 ml by chromatography of 500 μ l of a saturated solution of blue dextran (MW = 2000 kDa).

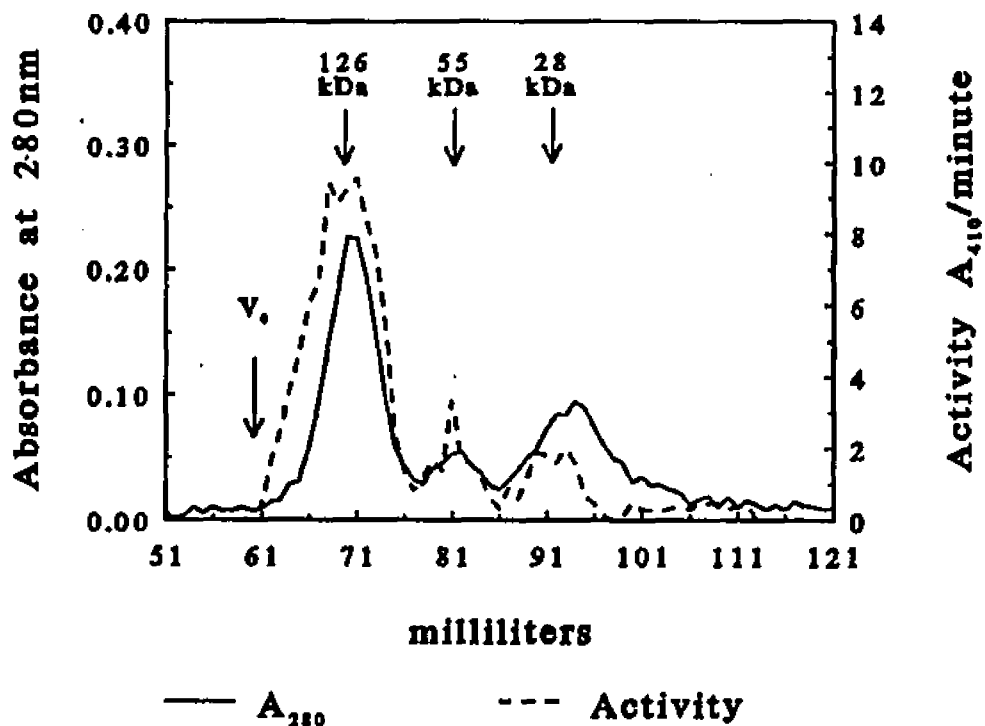


Figure 6 (B). Chromatogram of a Mixture of High-HLT and Low-HLT on a Sephacryl S200-HR Gel Filtration Column with Activity Overlay. The activities of the high- and low-HLT fractions were measured by microtiter plate assay using Cbz-Lys-SBzl as the substrate and the A_{410}/minute was graphed over the A_{280} of the fractions.

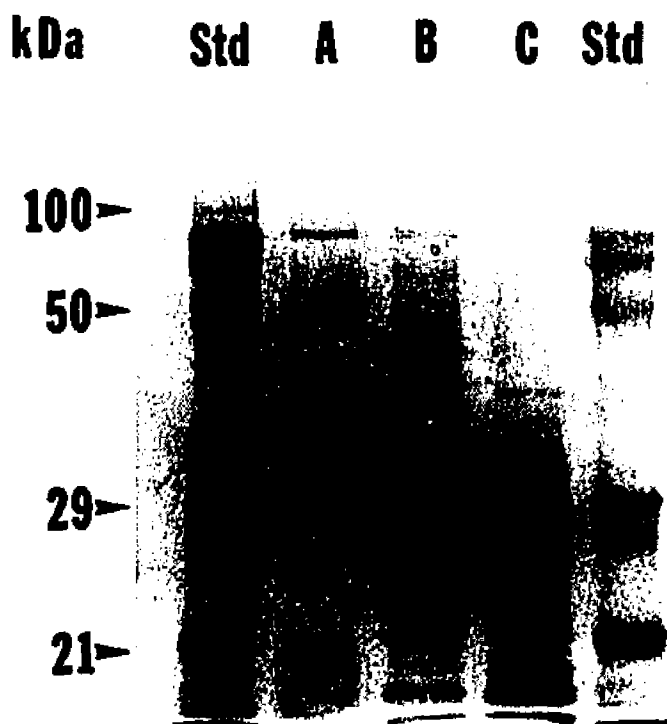


Figure 7. SDS-PAGE (10%) of Peaks Collected from Chromatography of High-HLT and Low-HLT Mixture on Gel Filtration Column. Lane A, Pooled fractions 62-74 (10 μ g); Lane B, pooled fractions 78-84 (10 μ g); Lane C, pooled fractions 89-91 (10 μ g). The samples and MW standards were reduced before electrophoresis and were visualized with colloidal Coomassie blue.

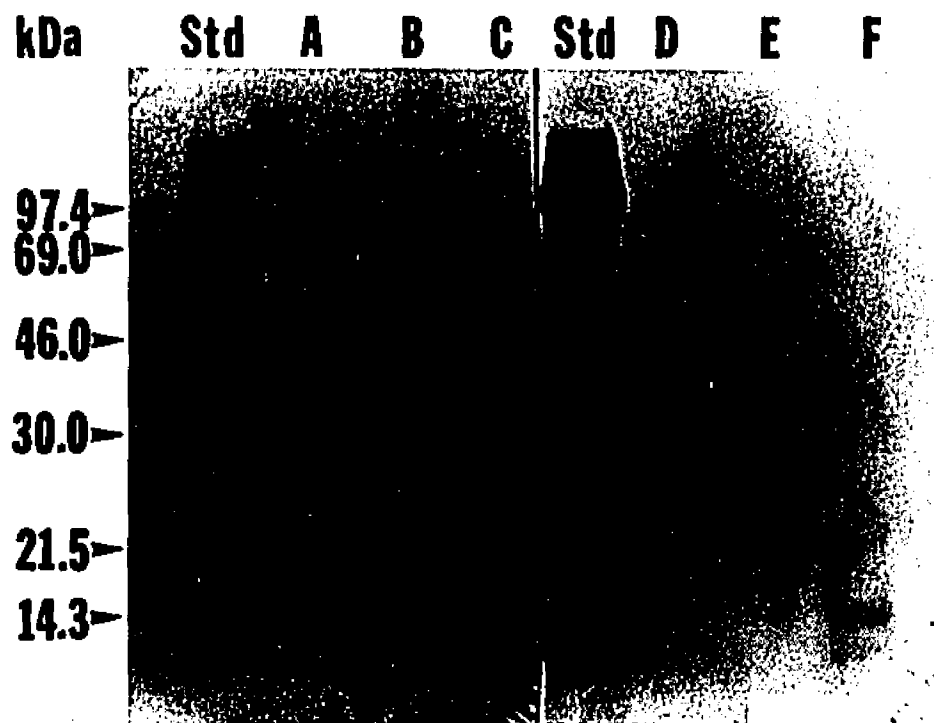


Figure 8. Western Blot and Coomassie Stained Membrane Gel Filtration Peaks. Lane A, Pooled fractions 62-74 (0.5 μ g); Lane B, pooled fractions 78-84 (5 μ g); Lane C, pooled fractions 80-91 (5 μ g). The proteins were electrophoresed on a 10% SDS-PAGE gel with reduction, transferred to a PVDF membrane, blocked with BSA, incubated with 1:2000 rabbit anti-HLT antibody, washed and incubated with 1:2000 goat anti-rabbit IgG antibody conjugated with alkaline phosphatase, and developed with NBT/BCIP. Lanes D, E, and F were duplicates of lanes A, B, and C, respectively. The samples were electrophoresed as above and the protein was transferred to PVDF membrane and visualized by staining with 0.1% Coomassie R-250 in 25% MeOH/10% oAc (v/v).

large protein substrate HMWK. The inhibitors tested using Cbz-Lys-SBzl as the tryptase substrate were SLPI, ecotin, ASTI, APPI, I α I, CMTI-III, and Arg¹⁵Glu⁵²aprotinin. The assays with SLPI, ecotin and ASTI were done using a 1:10 E:I ratio based on protein concentration; the assays with APPI, I α I and Arg¹⁵Glu⁵²aprotinin were done using a 1:100 E:I ratio based on protein concentration; and the assays with CMTI-III were done using a 1:50 E:I based on the number of active sites of enzyme. The results of the individual assays with the corresponding controls, summarized in Table 3, show that none of the inhibitors demonstrated significant inhibition of tryptase.

The second type of assay, using HMWK as the substrate, produced results showing that one inhibitor, bovine α_2 M, may inhibit high-HLT (it was not tried with low-HLT), and one other inhibitor, SLPI, showed definite inhibition of both isozymes. The other inhibitors tested included rat α_1 I₃, Arg¹⁵Glu⁵²aprotinin, APPI, I α I, ASTI, and CMTI-III. Assays with α_2 M and α_1 I₃ used E:I molar ratios of one, based on the number of active sites of tryptase; assays with Arg¹⁵Glu⁵²aprotinin, APPI, I α I, ASTI, SLPI, and CMTI-III were performed at an E:I ratio of 1:100, based on protein concentration.

Previous studies had indicated that human tryptase was not inhibited by human α_2 M nor did tryptase cleave the bait

Table 3. Inhibition Assays of High-HLT Using Various Trypsin Inhibitors. All assays were performed at room temperature using Cbz-Lys-SBzl as the substrate. Each set of assays was set up separately as described in Materials and Methods. An E:I ratio of 1:100 was used for the trypsin controls and incubated for 20 minutes before addition of the substrate.

Control - Test of Inhibitor Activity on Trypsin

	<u>ΔA_{410}/minute</u>	<u>% Activity</u>
Porcine Trypsin Control	0.4088	100
" + APPI	0.0068	1.7
" + I α I	0.0446	11
" + Arg ¹⁵ Glu ⁵² aprotinin	0.0030	≤ 1
" + ASTI	0.0267	6.5
" + SLPI	0.2226	54

	<u>10 minutes</u>		<u>60 minutes</u>	
	<u>ΔA_{410}/min.</u>	<u>% Act.</u>	<u>ΔA_{410}/min.</u>	<u>% Act.</u>
Tryptase Control	0.4820	100	0.3655	100
SLPI	0.4092	85	0.2962	81
ASTI	0.4457	92	0.3443	94
Ecotin	0.4165	86	0.3246	89

Table 3. (cont.)

	<u>pH 7.5⁺</u>		<u>pH 8.0⁺⁺</u>		<u>pH 8.5⁺⁺</u>	
	<u>ΔA_{410}/minute</u>	<u>% Activity</u>	<u>ΔA_{410}/minute</u>	<u>% Activity</u>	<u>ΔA_{410}/minute</u>	<u>% Activity</u>
Tryptase Control	0.4451	100	0.4338	100	0.4256	100
APPI	0.4271	96	0.4309	99	0.4148	97
I α I	0.4538	102	0.4462	103	0.4267	100
			0.4506	104	0.4662	109
			0.4221	97	0.4288	101
Arg ¹⁵ Glu ²² aprotinin			0.4230	98	0.4381	103

*Porcine trypsin is not as well inhibited by SLPI as bovine trypsin (Smith and Johnson, 1985).

**Times refer to the length of incubation of enzyme (E) and inhibitor (I) before addition of substrate. An E:I ratio of 1:10 was used for these assays.

*E:I ratios of 1:100; 3 hour incubation of E and I before addition of substrate.

**E:I ratios of 1:100; 20 minute incubation of E and I before addition of substrate.

region (D. A. Johnson and T. J. Smith, unpublished data). A limited experiment using bovine α_2M and high-HLT was inconclusive (see Figure 2 in the Appendix). The cleavage of HMWK was used as the control in this experiment, but was not carried out to completion; therefore, analysis of the inhibition of cleavage in the sample lanes was difficult. However, there was a discernable difference between the control and the sample containing the inhibitor, α_2M , suggesting some degree of inhibition of high-HLT by α_2M . Additional experiments using high-HLT and low-HLT (independently) are needed to determine more definitively the extent of inhibition, if any, of the two tryptase isozymes by α_2M .

Inhibition of human mast cell tryptase by SLPI had been previously suggested (Fritz, 1988), but no supportive data was provided. Therefore, SLPI was tested as an inhibitor of both human tryptase isozymes. SLPI inhibition of high-HLT and low-HLT using HMWK as the substrate is shown in Figure 9. The cleavage of HMWK was allowed to proceed for 60 minutes (presumably to completion) and, although over 6 times the number of active sites of high-HLT as low-HLT were used, only the low-HLT control cleaved all of the substrate. Subsequently, it was found that low-HLT was 25 times more active on HMWK than high-HLT. Low-HLT also appeared to be more thoroughly inhibited by SLPI than high-HLT; however, a six-fold higher E:I ratio was used for low-HLT, based on the

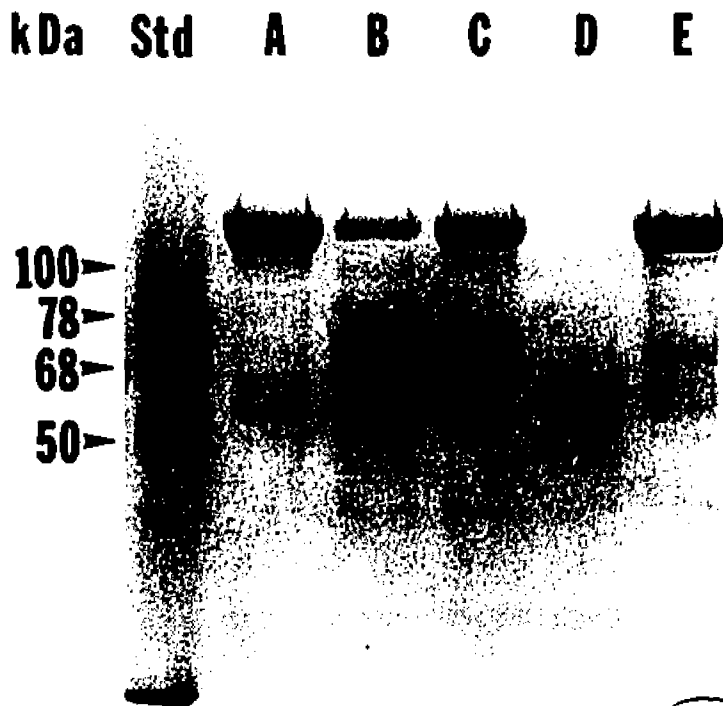


Figure 9. SDS-PAGE (8%) of the Inhibitory Effects of SLPI on High-HLT and Low-HLT. Lane A, HMWK (20 μ g, 175.5 pmol); Lane B, HMWK and high-HLT (3.51 pmol); Lane C, HMWK, high-HLT and SLPI (351 pmol); Lane D, HMWK and low-HLT; Lane E, HMWK, low-HLT and SLPI. The cleavages represented in lanes B and D were allowed to proceed for 60 minutes at RT before being TCA precipitated for analysis. The inhibitory effects shown in lanes C and E were the result of incubation of the enzyme (HLT) and inhibitor (SLPI) at RT for 30 minutes followed by addition of substrate (HMWK) for 60 minutes before TCA precipitation. The samples and MW standards were reduced before electrophoresis and were visualized with colloidal Coomassie blue.

number of active sites, because the experiment was performed with equal amounts of enzyme protein prior to active site titration. Therefore, although a comparison of inhibitory activity of SLPI on high-HLT and low-HLT was not performed, it was clear that both isozymes were inhibited by SLPI.

Arg¹⁵Glu⁵²aprotinin had been reported to inhibit tryptase in the degranulation supernatants of human tonsillar mast cells (Dietze et al., 1990); therefore, we tested this modified trypsin inhibitor as an inhibitor of purified tryptase. As shown in Figure 10, no inhibition of high-HLT by Arg¹⁵Glu⁵²aprotinin was seen at pH 7.5 or 9.1, while the activity of trypsin on HMWK was completely inhibited by Arg¹⁵Glu⁵²aprotinin at pH 7.5.

Testing of Several Biological Molecules as Tryptase

Substrates

Investigation of Laminin, Fibrinogen, and Fibronectin as Tryptase Substrates. Laminin, fibrinogen, and fibronectin are all fibrous proteins or precursors of fibrous proteins. Fibrinogen is involved in the later stages of the blood coagulation cascade and is converted to fibrin, which aggregates to form a thrombus, upon cleavage by thrombin. Both fibrinogen and fibronectin are involved in the enhancement of blood clotting, whereas both fibronectin and laminin are classified as adhesive proteins involved in cell adhesion to the extracellular matrix (Hynes, 1985). Also,

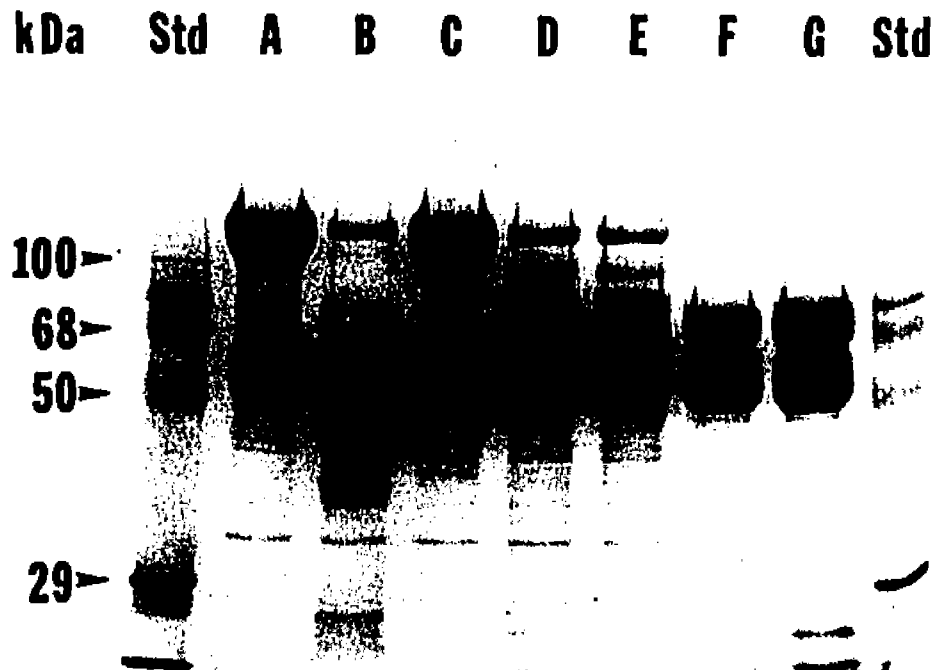


Figure 10. SDS-PAGE (7%) of the Inhibitory Effects of Arg¹⁵Glu⁵²aprotinin on High-HLT and Trypsin at pH 7.5 and 9.1. Lane A, HMWK (20 μ g, 176 pmol); Lane B, HMWK and trypsin (0.88 pmol) at pH 7.5; Lane C, HMWK, trypsin and Arg¹⁵Glu⁵²aprotinin (88 pmol) at pH 7.5; Lane D, HMWK and high-HLT (1.76 pmol) at pH 7.5; Lane E, HMWK, high-HLT and Arg¹⁵Glu⁵²aprotinin (176 pmol) at pH 7.5; Lane F, HMWK and high-HLT at pH 9.1; Lane G, HMWK, high-HLT and Arg¹⁵Glu⁵²aprotinin at pH 9.1. Inhibitor and enzyme in lanes C, E and G were incubated at RT for 20 minutes before addition of substrate and a second incubation at RT for 30 minutes followed by TCA precipitation. Samples and MW standards were reduced before electrophoresis were visualized with colloidal Coomassie blue.

both laminin and fibronectin contain relatively high numbers of Lys and Arg residues (Timpl et al., 1979), making them good possible candidates as tryptase substrates. Tryptase in the presence of heparin had been reported to cleave both the α and β chains of fibrinogen (Schwartz et al., 1985) and fibronectin had been shown to be cleaved by tryptase in the presence of heparin (Smith, 1985). Therefore, laminin, fibronectin, and fibrinogen were all exposed to high-HLT and analyzed by SDS-PAGE with reduction. The results of these experiments were not definitive due to the poor quality of the electrophoresis gels, which are only presented in the Appendix. The data (Figure 3A in the Appendix) indicate that fibrinogen was cleaved by high-HLT, as evidenced by the appearance of a new band at approximately M_r 65 kDa in the tryptase-treated sample. This agrees with the initial results obtained by Schwartz et al. (1985), which showed the production of a band of M_r 65 kDa after a brief exposure of fibrinogen to tryptase, and the disappearance of that band and concurrent appearance of smaller bands following prolonged exposure. While no cleavage of either laminin or fibronectin was apparent in Figure 3A, a difference in banding pattern between the tryptase-treated and untreated fibronectin samples was detectable in Figure 3B. This gradient gel ran and stained poorly, but still clearly showed an additional band of about M_r 45 kDa in lane 7 when compared to lane 6. Again, no cleavage of laminin was

detectable. Additional analysis of the effect of high-HLT on laminin and fibronectin is needed to confirm the above results, and investigation of the effects of low-HLT on all three proteins is also necessary.

Cleavage of HMWK with High-HLT and Low-HLT

HMWK is a plasma protein which consists of a heavy chain, composed primarily of three cysteine proteinase inhibitor domains, and a light chain, containing a histidine-rich region that includes the procoagulant activity, separated by bradykinin (refer to Figure 13). Although the three-dimensional structure of HMWK is unknown, it seems likely that the highly charged light chain assumes a conformational structure that makes it readily available for interaction with other molecules, as it has been shown that this part of the molecule is responsible for binding to anionic surfaces (DeLa Cadena and Colman, 1992). Cleavage of HMWK by high-HLT or low-HLT produced different fragmentation patterns on SDS-PAGE (Figure 11). Several smaller fragments generated by low-HLT failed to precipitate with 20% TCA, but were subsequently detected by using concentrated samples and performing SDS-PAGE without precipitating the samples (Figure 4 in the Appendix). Some of these smaller fragments were also detected using ethanol instead of TCA to precipitate the samples (data not shown). This observation is consistent with the small fragments being from the carboxy terminal region that is rich in basic

amino acid residues, and therefore may be less likely to precipitate in an acidic environment. High-HLT cleavage of HMWK resulted initially in two major bands of M_r 65 kDa and 46 kDa, with the 46 kDa fragment disappearing by 40 minutes (Figure 11A). In contrast, low-HLT cleavage of HMWK produced a minor band of M_r 100 kDa and two major bands of M_r 75 kDa and 65 kDa. By 40 minutes, only the 65 kDa fragment was visible in Figure 11B. Western blot analysis of these products was performed after ethanol precipitation using a monoclonal antibody to the light chain of HMWK to determine which were the carboxy terminal fragments for sequencing. As shown in Figure 12, a single 46 kDa fragment produced by the action of high-HLT on HMWK reacted strongly with the antibody, whereas low-HLT produced multiple fragments that reacted only weakly with the C-terminal antibody, including many fragments of less than 50 kDa, indicating numerous nonspecific cleavages in the HMWK light chain by low-HLT.

Limited sequencing of several cleavage fragments was performed by Mark Lively at Bowman Gray School of Medicine in Winston-Salem, NC, using an Applied Biosystems gas phase sequencer. In order to obtain a better understanding of the equipment and procedures used in automated sequencing and analysis, I went to Bowman Gray and observed the sequencing of these fragments. The results of sequencing the 46 kDa fragment from the high-HLT reaction indicated an amino terminal sequence of Asp-Gln-Gly-Xaa-Gly-Xaa-Gln,

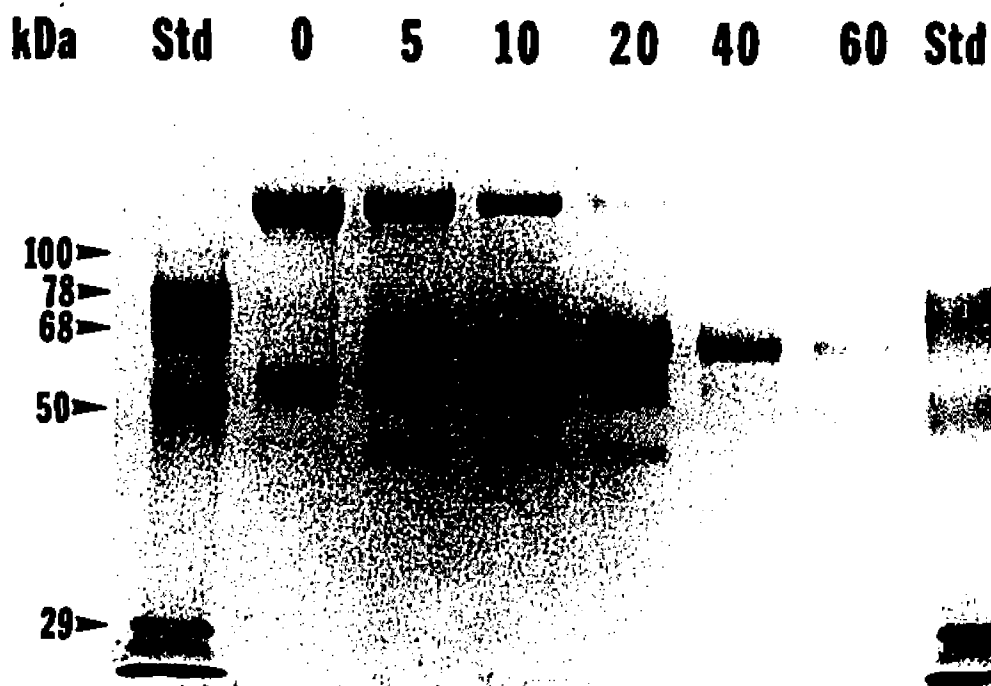


Figure 11. Time Course SDS-PAGE of HMWK and Products after Cleavage with High-HLT and Low-HLT. Lanes are labeled with the length of cleavage reaction in minutes, which was stopped by TCA precipitation. Samples of 5 μ g were loaded in each well of the 6% SDS-PAGE gel with reduction and the bands were visualized with Coomassie Blue staining. **A**, HMWK (5 μ g, 219 pmol) cleaved with high-HLT (4.4 pmol; 1:50 E:S based on protein concentration) ; **B**, HMWK cleaved with low-HLT (1.1 pmol; 1:200 E:S based on protein concentration) . Molecular weight markers are the same as in Figure 3.

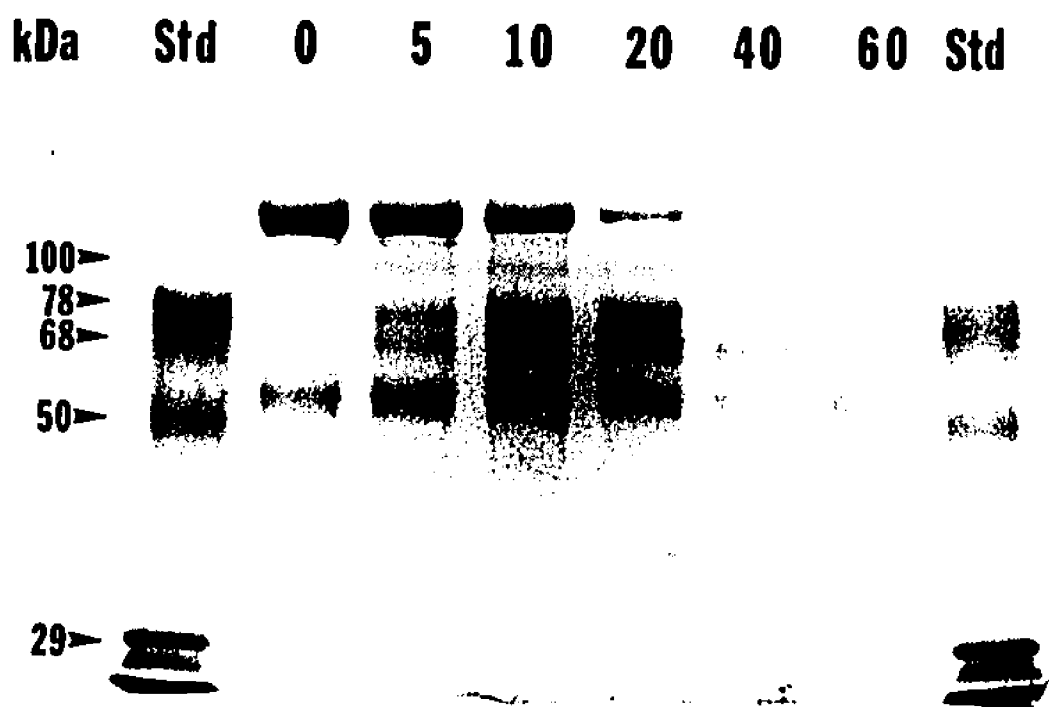


Figure 11 (B).

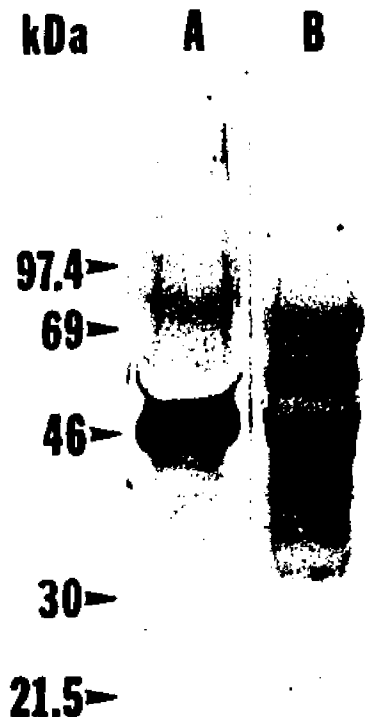


Figure 12. Western Blot of HMWK and Cleavage Products with High-HLT and Low-HLT. Lane A, HMWK and cleavage products (20 μ g, 174 pmol) after a 20 minute cleavage with high-HLT (3.48 pmol; 1:50 E:S based on protein concentration); Lane B, HMWK and cleavage products (20 μ g, 174 pmol) after a 20 minute cleavage with low-HLT (0.87 pmol; 1:200 E:S based on protein concentration). Reactions were stopped and products were collected by ethanol precipitation. The samples and MW standards were reduced, electrophoresed on an 8% SDS-PAGE gel and transferred to a PVDF blotting membrane. A monoclonal antibody to the light chain (C-terminus) of HMWK was used as the primary antibody. Antibody binding was visualized using Protein G Gold and silver enhancement.

corresponding to HMWK residues Asp₄₃₂-Gln-Gly-His-Gly-His-Gln₄₃₈ in the light chain of HMWK (Figure 13). Thus, high-HLT cleaves HMWK between Arg₄₃₁ and Asp₄₃₂ (or Arg₄₄₉ and Asp₄₅₀ of prekininogen; Takagaki et al., 1985). Attempts to sequence the 75 kDa and 65 kDa bands produced by the low-HLT reaction were unsuccessful. Presumably they contain the amino terminus of HMWK, which is unavailable to Edman degradation due to the presence of an amino terminal 5-oxoproline (Kellermann et al., 1986). Due to the multiple C-terminal fragments produced by low-HLT, determination of a primary cleavage site was not possible.

Cleavage of VIP by High-HLT and Low-HLT

VIP cleavage by each of the two tryptase isozymes produced different chromatographic profiles when analyzed by C₁₈ reverse-phase HPLC. As shown in Figure 14, the heights of the residual VIP peaks were approximately the same at 10 minutes with low-HLT (Figure 14C) and at 20 minutes with high-HLT (Figure 14B). The enzyme/substrate ratios (based on the number of tryptase active sites) were the same in these experiments, indicating that low-HLT cleaved VIP at twice the rate of high-HLT. The initial major cleavage product resulting from cleavage by high-HLT eluted at 24.4 minutes and the initial major cleavage product resulting from cleavage by low-HLT eluted at 28.4 minutes, relative to native VIP at 29.1 minutes (Figure 14A). Although both

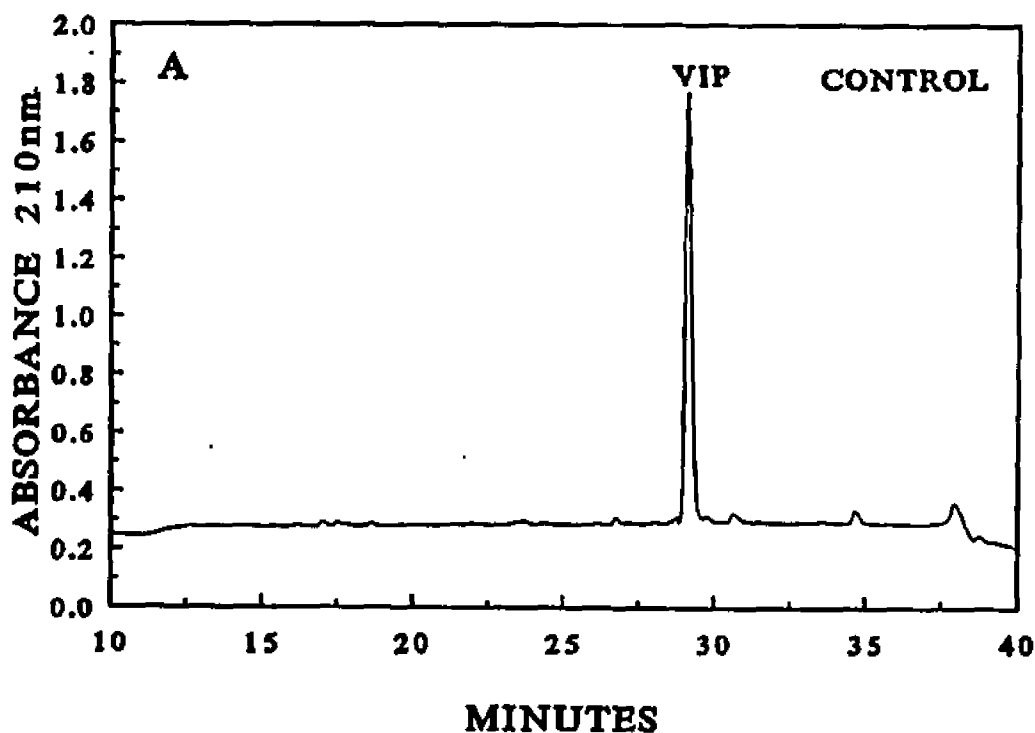


Figure 14. Reverse-phase HPLC chromatograms of VIP and the cleavage products generated by the activity of high-HLT and low-HLT. A, native VIP (20 μ g or 6 nmol); B, VIP (20 μ g) after exposure to 0.346 pmol of active high-HLT (1:17340 E:S) for 20 minutes; C, VIP (20 μ g) after exposure to 0.346 pmol of active low-HLT for 10 minutes; D, VIP (20 μ g) after exposure to 0.346 pmol of active high-HLT (1:17340 E:S) for 60 minutes; and E, VIP (20 μ g) after exposure to 0.346 pmol of active low-HLT for 60 minutes. Five μ l of glacial acetic acid and 130 μ l of 0.1% TFA were added to each sample before chromatography on a C18 column and elution with a 10-40% acetonitrile linear gradient.

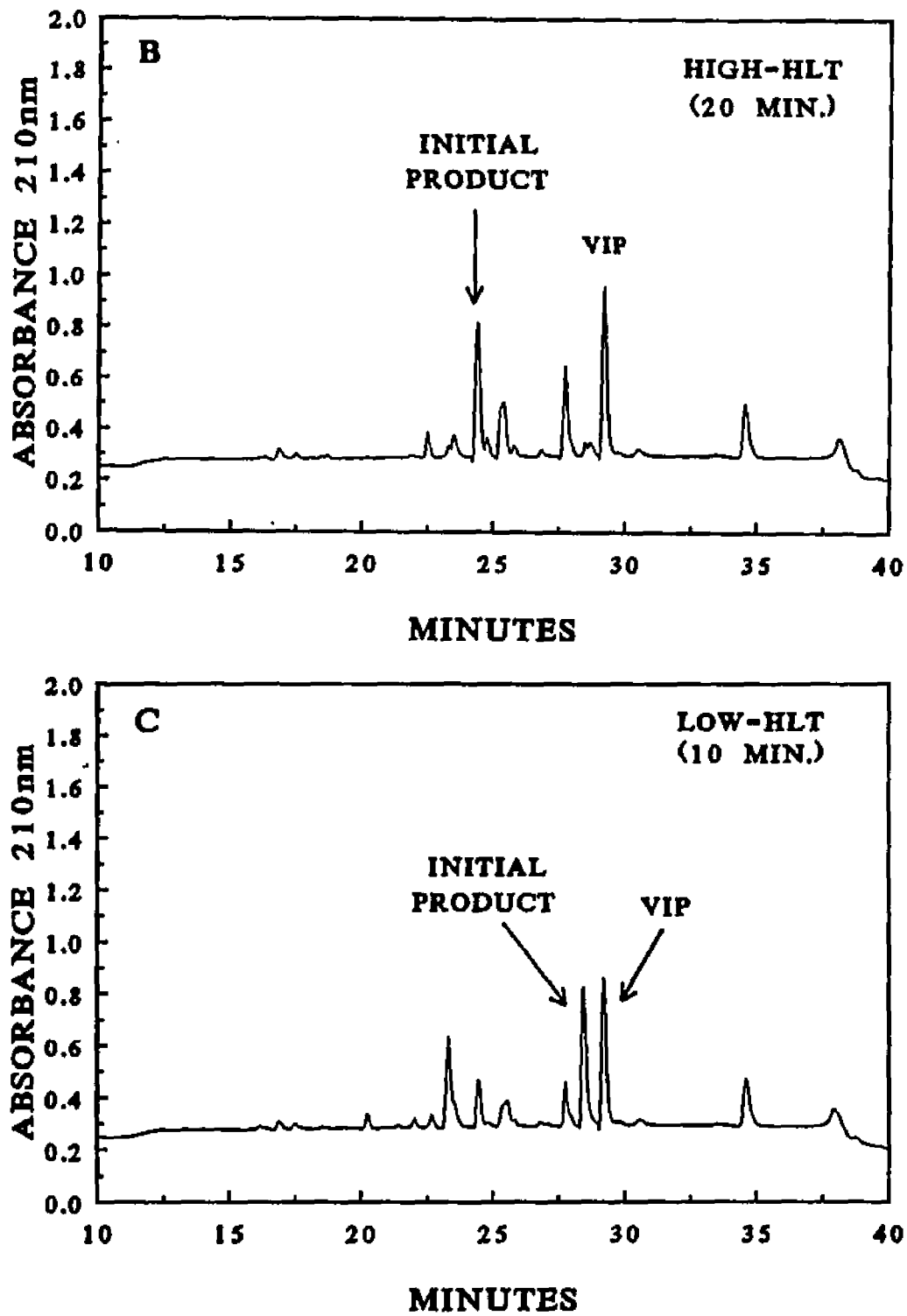


Figure 14 (B & C).

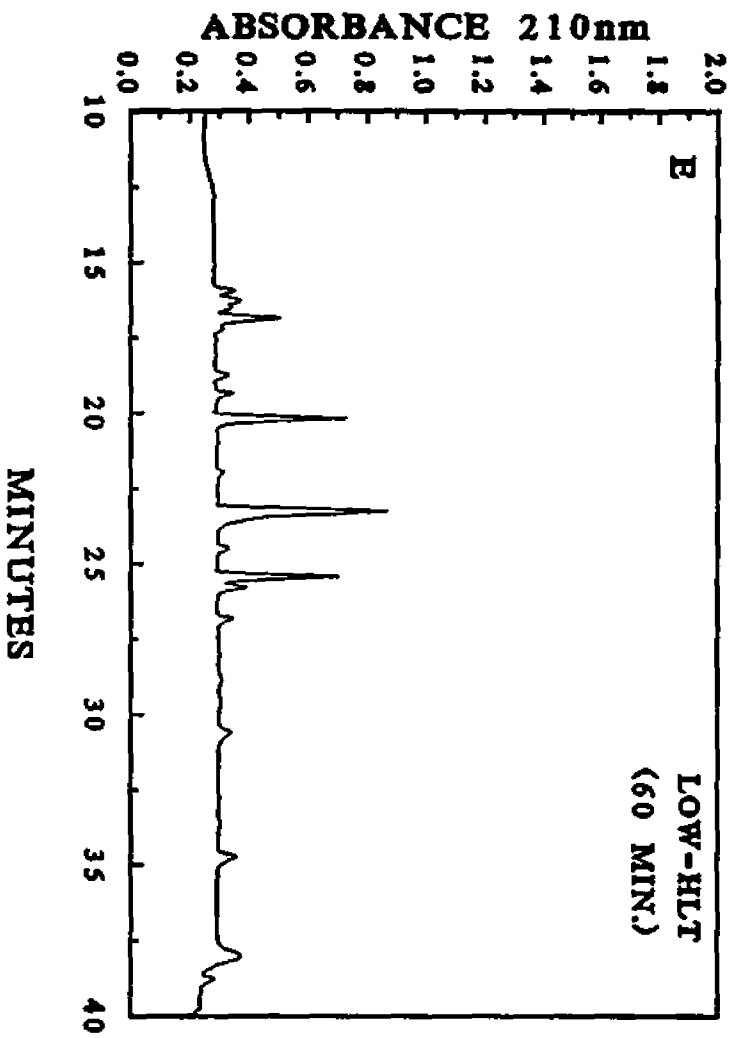
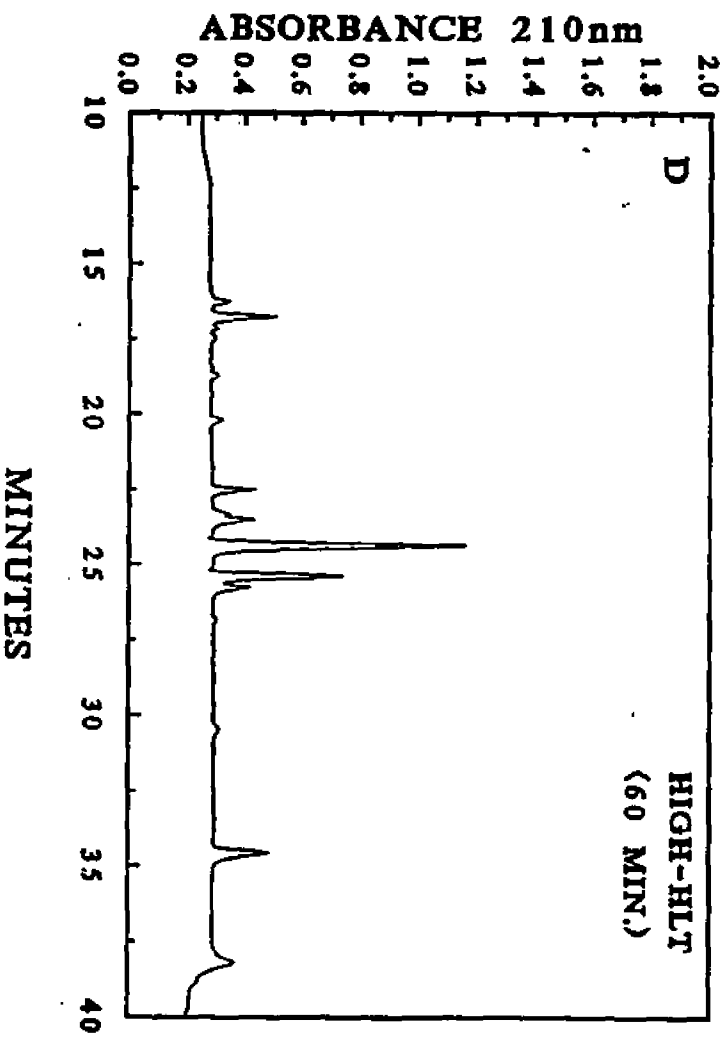


Figure 14 (D & E).

chromatographic profiles changed as the reactions proceeded to completion, they never became identical (Figure 14D & E), further indicating a difference in substrate specificity.

Amino Acid Analysis of VIP Cleavage Products

The initial major cleavage product of the activity of each of the tryptase isozymes on VIP was collected and amino acid analysis was performed. The results, shown in Table 4, suggest that the primary cleavage site for high-HLT is Arg₁₄, which agrees with the findings of Tam and Caughey (1990); and, although the amino acid analysis results were not as clear for low-HLT, the data suggest that the primary cleavage site for low-HLT is Arg₁₂. These data correspond to fragments of 1-14 for the high-HLT product and 13-28 for the low-HLT product. Figure 15 shows the sequence of VIP and the proposed cleavage sites for high-HLT and low-HLT.

Attempted Peptide Mapping of the Tryptase Isozymes

Peptide mapping of the two tryptase isozymes would provide some very important information for determining whether these isozymes are the gene products of any of the cDNAs cloned from human mast cells. Peptide mapping involves cleaving a protein at certain points and performing limited sequencing of the peptides produced. Peptide mapping of the tryptase isozymes was attempted using Lys-C (endoproteinase Lys-C from Promega), which cleaves proteins on the carboxyl side of Lys residues, and CNBr, which

Table 1. Amino Acid Analysis of the Initial Products 86
Resulting from the Cleavage of VIP with High-HLT and Low-HLT. The peaks corresponding to the two initial products (see Figure 5B and 5C) resulting from the cleavage of VIP with each of the tryptase isozymes were hydrolyzed, PITC derivatized, and analyzed using the Waters Pico-Tag method. An amino acid with a theoretical yield of one was chosen to represent an actual yield of one and all other pmol values were divided by that pmol value to determine their actual yields. The theoretical yields represent VIP residues 1-14 corresponding to the high-HLT initial product and VIP residues 13-28 corresponding to the low-HLT initial product.

High-HLT Initial Peak

	<u>p moles</u>	<u>Actual Yield</u>	<u>Theoretical Yield</u>
Asp	32	2.9	3
Glu	2	0.2	0
Ser	18	1.6	1
Gly	4	0.4	0
His	26	2.4	1
Arg	23	2.1	2
Thr	13	1.2	2
Ala	13	1.2	1
Tyr	11	1.0	1
Val	9	0.8	1
Met	1	0.1	0
Cys & Ile	6	0.5	0
Leu	13	1.2	1
Phe	10	0.9	1
Lys	1	0.1	0

Table 1. Amino Acid Analysis of the Initial Products 87
Resulting from the Cleavage of VIP with High-HLT and Low-
HLT. (Continued)

	<u>Low-HLT Initial Peak</u>		
	<u>p moles</u>	<u>Actual</u> <u>Yield</u>	<u>Theoretical</u> <u>Yield</u>
Asp	26	3.7	2
Glu	6	0.9	1
Ser	4	0.6	1
Gly	2	0.3	0
His	2	0.3	0
Arg	7	1.0	1
Thr	6	0.9	0
Ala	8	1.1	1
Tyr	10	1.4	1
Val	3	0.4	1
Met	4	0.6	1
Cys & Ile	8	1.1	1
Leu	15	2.1	3
Phe	2	0.3	0
Lys	15	2.1	3

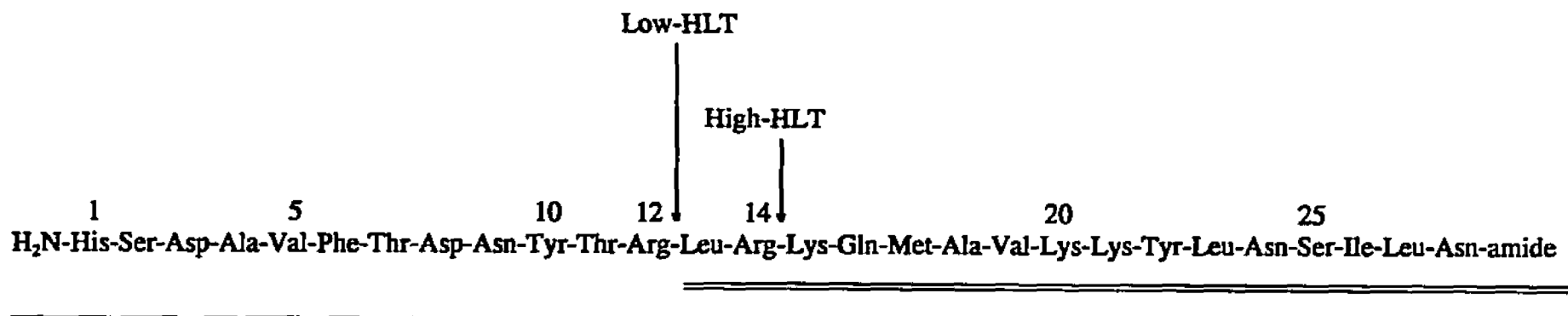


Figure 15. Sequence of VIP Showing the Proposed Cleavage Sites by Low-HLT and High-HLT. Results of amino acid analysis indicated that the initial product of the cleavage of VIP by low-HLT was residues 13-28 and the initial product of the cleavage of VIP by high-HLT was residues 1-14.

chemically severs proteins at Met residues. These means of cleavage were chosen to try to compare the two isozymes to the amino acid sequences encoded by the α and β tryptase cDNAs cloned from lung mast cells, which exhibit differences in the number of these two amino acids. Several months were spent on this set of experiments with no significant results. However, it was determined that tryptase is apparently resistant to cleavage by Lys-C, as evidenced by a lack of decrease in M_r of tryptase on SDS-PAGE after exposure to Lys-C. Intact tryptase completely disappears on SDS-PAGE after exposure to CNBr, but successful chromatography of the products on reverse-phase HPLC was never achieved. C_8 and C_{18} columns were tried with a variety of gradients and two different buffer systems (0.1% TFA/acetonitrile buffer system and 6 mM HCl/acetonitrile buffer system) without any positive results.

CHAPTER 4

DISCUSSION

Purification and Separation of the Tryptase Isozymes

Purification and separation of the tryptases proved to be somewhat difficult to achieve. The main problems encountered were low yields (an average of 25% total recovery of activity) and unequal amounts of the two isozymes (about 5 times more high-HLT than low-HLT based on protein concentration; about 32 times more high- than low-HLT based on number of active sites). Low-HLT was also more difficult to purify to homogeneity, nearly always containing some low M_r contaminants that were difficult to remove and frequently containing a very small amount of high-HLT. Cetylpyridinium chloride treatment of crude extract greatly reduced the viscosity of the extract by precipitating mucopolysaccharides and nucleic acids. Chromatography on butyl HIC followed by cellulose-phosphate ion exchange chromatography produced the highest yields and most consistent separation of the isozymes. However, an inhibitor column, such as SLPI or Arg¹⁵Glu⁵²aprotinin, may improve enzyme purity and yield. Elution of high-HLT from cellulose-phosphate with heparin suggests that high-HLT has a high affinity for heparin, whereas low-HLT required high salt for elution, suggestive of low heparin affinity.

Chromatography of crude extracts on heparin-Sepharose with Western blot detection has shown that only high-HLT binds to heparin (Burnette-Vick, unpublished data).

Structural Characterization of the Isozymes

Previous preparations of human mast cell tryptase have contained two bands in the 29-35 kDa range differing by 2-5 kDa upon SDS-PAGE. Because gel filtration chromatography of these tryptase preparations in the past yielded estimated molecular sizes of 135-144 kDa (Schwartz et al., 1981a; Smith et al., 1984), it was assumed that tryptase was a tetramer containing two slightly different active subunits. Assuming that tryptase actually exists as a tetramer in vivo, separation of the two active forms of tryptase implies that the tetramer is composed of homologous subunits, rather than a mixture of the two bands seen on SDS-PAGE. Gel filtration of the two forms of tryptase separated in this lab has indicated that high-HLT probably exists as a tetramer (approximate M_r of 125 kDa) whereas low-HLT may exist as a monomer (approximate M_r of 28 kDa).

Low-HLT appears to have a lower affinity for heparin, relative to high-HLT, providing additional evidence of structural differences in the two forms. Rat tryptase does not bind heparin (Braganza and Simmons, 1991) and it has been shown that mouse tryptase also lacks this affinity (Johnson and Burnette-Vick, unpublished data). Heparin

binding motifs with the sequences BBXB and BBXXB have been noted (where B is Lys or Arg; Blankenship et al., 1990) and it has hypothesized that R¹⁹-V-R²¹-D-R²³ represents a new heparin binding motif of the sequence BXBXB (D. Johnson, unpublished data). This sequence is found in HST-3 and HLT- α , but is missing from HST-1 and HLT- β /HST-2 (Figure 16).

Molecular modeling of the tryptases has shown the presence of two insertions not present in trypsin, one on either side of the active site (Figure 17), that may restrict access to the active site by bulky substrates or inhibitors, while allowing small molecules or loop structures to reach the active site (Johnson and Barton, 1992). Figure 17A shows a space filling model of human skin tryptase 1 with the two insertions shown in orange and red and the active site Ser and His in green and yellow, respectively. Another angle of this molecule is shown in Figure 17B which exposes the Asp residue at the bottom of the S₁ substrate binding pocket. This may explain in large part the high degree of substrate specificity and resistance to most inhibitors displayed by the tryptases. This information will be useful in future investigations of the interaction of tryptase with inhibitors such as SLPI and substrates such as HMWK and VIP.

Another interesting observation was that there was a difference between the two isozymes in their migration out

	1	11	21	31	Heparin Binding
HST-1					?
HST-2/HLT-β					?
HST-3					?
HLT-α					?
DMCT					?
MMCT-1					No
MMCT-2					No
Trypsin					No
Chymotrypsin					No

Figure 16. Amino terminal sequence alignment of the mast cell tryptases showing the sequences of the tryptases in comparison to those of trypsin and chymotrypsin. HST-1, human skin tryptase 1; HST-2/HLT- β , human skin tryptase 2/human lung tryptase beta; HST3, human skin tryptase 3, HLT- α , human lung tryptase alpha; MMCT-1, mouse mast cell tryptase 1; MMCT-2, mouse mast cell tryptase 2; DMCT, dog mast cell tryptase; trypsin, bovine trypsin, chymotrypsin, bovine chymotrypsin. The arginine and lysine residues of the potential heparin binding motif site are in bold print. The putative Asn-linked glycosylation site in MMCT-2 is in bold lower case print.

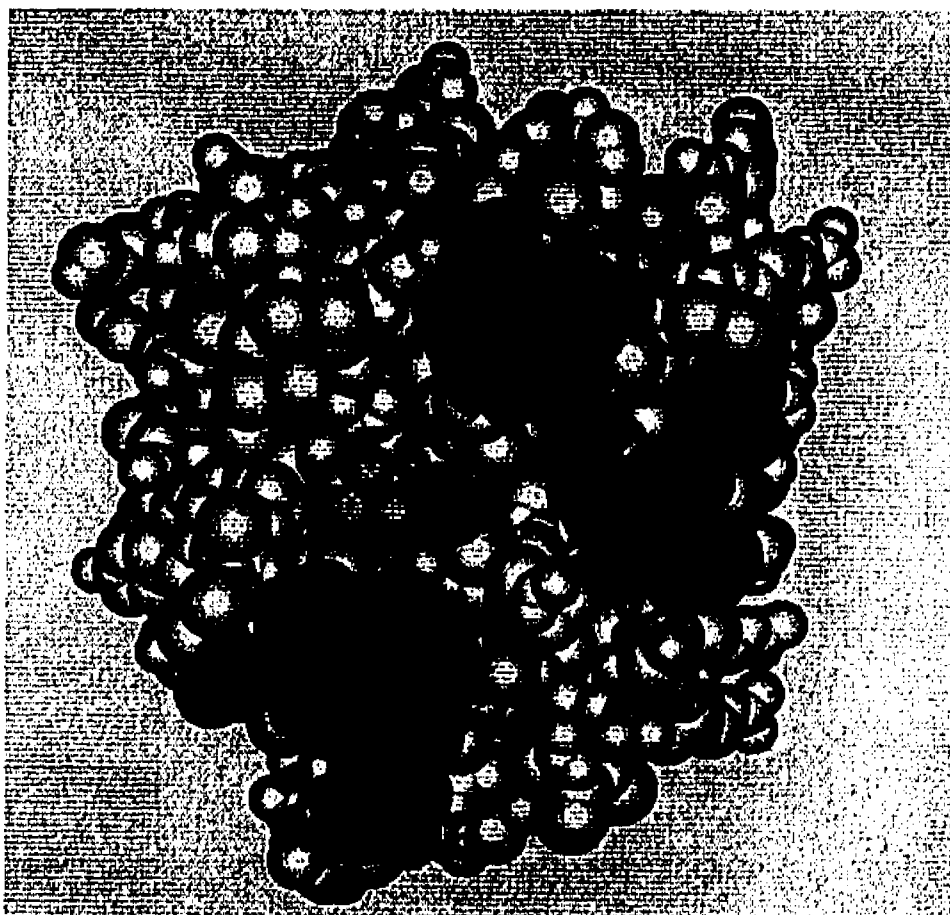


Figure 17 A. Space filling model of HST-1 (Johnson and Barton, 1992) constructed on the crystal structure coordinates of trypsin. The active site Ser is colored green and the active site His residue is yellow. The smaller insertion of four amino acids (residues 20 through 26) is colored orange and the larger insertion (residues 164 through 174) is colored deep red. Also, the proline-rich hydrophobic region of tryptase (residues 139 through 145) is colored purple. The nitrogen of the putative Asn-linked glycosylation site (residue 203) is colored blue/green and is just visible at the top of the model.

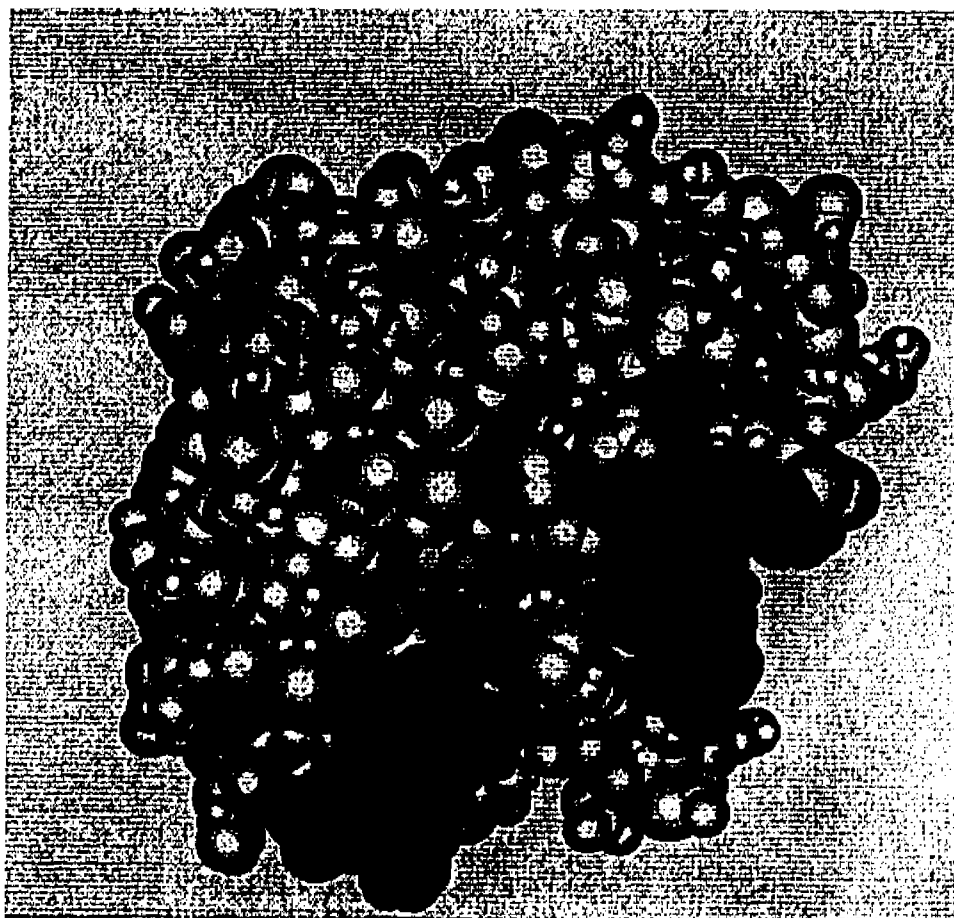


Figure 17 B. Another view of the space filling model of human skin tryptase 1 with insertion loops colored orange (20-26) and deep red (164-174) as in Figure 17 A. This view shows Asp 188 at the bottom of the S₁ substrate binding pocket colored dark purple. The active site Ser and His residues are green and yellow, respectively. The proline-rich region and the Asn glycosylation site are colored purple and blue/green, respectively, as in Figure 17 A.

of gels onto PVDF and nitrocellulose blotting membranes. The reason underlying this difference has not been elucidated, but suggests further structural differences. Isoelectric focusing studies on the enzymes might produce data pertinent to this discrepancy.

Vanderslice et al. (1990) presented evidence supporting the existence of multiple tryptase genes, and it is possible that high-HLT and low-HLT correspond to the gene products of the two tryptase sequences that have been derived from human lung cDNAs, tryptase- α and tryptase- β (Miller et al., 1989 and 1990). Tryptase- α is 244 amino acids in length with two putative glycosylation sites per subunit, whereas tryptase- β is 245 amino acids in length with only one putative glycosylation site. Since tryptase- α with two glycosylation sites would probably have a higher M_r than tryptase- β , high-HLT may correspond to α while low-HLT may correspond to β . Confirmation of this must await further structural characterization of the two isozymes.

Functional Differences of the Tryptase Isozymes

It has been previously reported that the activity of human tryptase is dependent on its interaction with heparin (Smith et al., 1984; Schwartz et al., 1990). However, if, as preliminary data suggest, low-HLT exists as a monomer and has a low affinity for heparin, its activity may be regulated in an entirely different manner from high-HLT. No

endogenous inhibitors of trypsin have been reported; therefore, a number of trypsin inhibitors were tested for activity against trypsin. The inhibitors tested differed with respect to size, source and P1 amino acid residue, which makes elucidation of the characteristic(s) responsible for trypsin inhibition more difficult. None of the inhibitors with an Arg residue in the active site inhibited trypsin, whereas SLPI, which contains a Leu in the active site, inhibited both trypsin isozymes.

All of the inhibitors tested are known to inhibit trypsin and some are known to inhibit other proteases as well. Ecotin, a trypsin inhibitor isolated from *E. coli*, contains 142 amino acids, has a subunit MW of 17 kDa and is active as a dimer (McGrath et al., 1991). In addition to trypsin, ecotin also inhibits chymotrypsin and neutrophil elastase, with Met^H in the P1 position of the inhibitory site (McGrath et al., 1991). This active site residue is somewhat unusual for trypsin and elastase inhibitors, which normally have Lys or Arg and Leu or Val, respectively, in their active site (McGrath et al., 1991). However, α_1 -proteinase inhibitor, a plasma protein that inhibits neutrophil elastase, has a Met in its active site and also inhibits trypsin and chymotrypsin (Johnson and Travis, 1978), but does not inhibit trypsin.

SLPI is a monomeric protein containing 107 amino acid residues with a MW of 11,726 Da. It is found in various

mucous-containing body fluids and also exhibits strong inhibitory effects on several proteases, including bovine trypsin and chymotrypsin, leukocyte elastase, cathepsin G, and chymase (Fritz, 1988; Van-Seuningen and Davril, 1991). Its activity against porcine trypsin, however, is reduced compared to bovine trypsin (Smith and Johnson, 1985). The active inhibitory site has been determined to be Leu⁷² for all proteases that interact with it (Eisenberg et al., 1990; Van-Seuningen and Davril, 1991), similar to the peculiar inhibitory activity of ecotin.

ASTI is isolated from the porcine roundworm *Ascaris suum* and has a MW of 6880 Da (Peanasky et al., 1987). APPI is part of the Alzheimer's amyloid β -protein precursor and blocks proteases that cleave at basic residues (Hynes et al., 1990). It has a MW of 6416 Da, is 58 amino acids in length, with an Arg¹⁵ P₁ active site (Hynes et al., 1990).

I α I is a large protein complex found in human plasma that is made up of three chains--two heavy chains of approximately MW 66 kDa each, designated as subunits β and γ , and a light (α) chain of MW 46 kDa that contains the inhibitory activity (Gebhard et al., 1990; Potempa et al., 1989; Salier, 1990). The light chain, also known as bikunin or HI30, has two active inhibitory sites, one with a P₁ Arg and one with a P₁ Met, and inhibits trypsin, chymotrypsin, neutrophil elastase, plasmin, and acrosin (Potempa et al., 1989; Salier, 1990). The function of the β and γ subunits

is unknown (Gebhard et al., 1990).

CMTI-III is a 29 amino acid protein of MW 3268 Da that is found in pumpkin (*Curcubita maxima*) seed extracts (Hojima et al., 1982; McWherter et al., 1989). It has an Arg in the active site and inhibits Hageman factor fraction (β -factor XIIa) and bovine trypsin (Hojima et al., 1982).

Recombinant Arg¹⁵Glu³²aprotinin is a genetically modified form of aprotinin (Auerswald et al., 1988) or bovine pancreatic trypsin inhibitor (BPTI), which normally contains a Lys at position 15, the P1 residue of the active site (Scott et al., 1987). BPTI inhibits trypsin, plasmin, kallikrein, and chymotrypsin (Auerswald et al., 1988). Arg¹⁵Glu³²aprotinin inhibited human plasmin, bovine α -chymotrypsin, porcine pancreatic kallikrein, human plasma kallikrein, human urinary kallikrein and human anionic and cationic trypsin (Auerswald et al., 1988).

α_2 M is a large glycoprotein (subunit MW \approx 180 kDa) that usually exists as a tetramer and inhibits a wide range of plasma proteinases (Arakawa et al., 1989). The mechanism of inhibition by α_2 M is one of steric hindrance of the proteinase after cleavage of a bait region in the α_2 M molecule by the proteinase which triggers a major conformational change and results in the "trapping" of the proteinase (Arakawa et al., 1989; Sottrup-Jensen et al., 1989). The "trapped" proteinase is then inaccessible to large substrates but may still interact with very small

substrates (Sottrup-Jensen et al., 1989).

$\alpha_1\text{I}_3$, a rat plasma proteinase inhibitor, is similar to $\alpha_2\text{M}$ except that it is a monomer of MW 190 kDa (Lonberg-Holm et al., 1987) and has a less efficient trapping mechanism that is dependent on the ability of $\alpha_1\text{I}_3$ to form covalent crosslinks with proteinase Lys residues (Enghild et al., 1989).

The results of the inhibitor assays showed that both trypsin isozymes were inhibited by SLPI but were resistant to inhibition by all other trypsin inhibitors tested. This may be due, at least in part, to specific structural characteristics of the isozymes that restrict the activity of most inhibitors, such as the loop structures (discussed previously) located on either side of the active site. These data indicate that there may be other endogenous inhibitors of trypsin, in addition to heparin, that have not been examined.

The two trypsin isozymes were also shown to differ with respect to substrate specificity and rate of activity on two protein substrates, HMWK and VIP. Although low-HLT cleaved HMWK and VIP at 25 and 2 times as fast as high-HLT, respectively, low-HLT preparations were only 15% active compared with 95% active high-HLT preparations; thus, low-HLT appears to be less stable than high-HLT. Low-HLT may, however, diffuse through the tissues surrounding the site of mast cell degranulation to a much greater extent than high-

HLT, giving it a broader range of activity. Le Trong et al. (1987) presented data suggesting that the specificity of chymase may vary according to the presence or absence of heparin. Therefore, the difference in substrate specificity reported on here may be directly related to the difference in affinity for heparin between the two tryptase isozymes.

Our results confirm the reports that HMWK (Maier et al., 1983) and VIP (Tam and Caughey, 1990) are substrates for human tryptase in vitro and show that both human lung tryptase isozymes degrade these substrates. Further, high-HLT and low-HLT differ with respect to the rates and the primary sites of cleavage on these two natural substrates.

With regard to their activity on HMWK, low-HLT cleaved HMWK approximately 25 times as fast as high-HLT, when compared on the basis of the number of active sites. In addition, high-HLT cleaved HMWK at a single site in the light chain, compared with low-HLT, which cleaved the light chain into several fragments. Similarly, low-HLT cleaved VIP approximately twice as fast as high-HLT, with the generation of different cleavage products as monitored by HPLC. However, the small VIP molecule appears to be a better substrate for tryptase than the large HMWK molecule, since the E:S ratio required for cleavage was much smaller for VIP (1:17,340 based on the number of active sites of tryptase) than for HMWK (1:52 with high-HLT and 1:1333 with low-HLT). Based on the previously discussed molecular

modeling studies of tryptase, it has been suggested that loop structures adjacent to the active site may provide steric hindrance for larger molecules (substrates or inhibitors), making interaction with the active site more difficult (Johnson and Barton, 1992).

Attempts to determine the primary cleavage site resulting from the activity of each isozyme on HMWK were partially successful. High-HLT produced only two major cleavage products of 65 kDa and 46 kDa. Limited amino-terminal sequencing of the 46 kDa fragment showed that the major cleavage site is immediately carboxyl to Arg₄₃₁, which is in the histidine-glycine-rich procoagulant region of the light chain of HMWK. This supports previous reports that tryptase destroys the ability of HMWK to participate in the coagulation cascade (Maier et al., 1983) and the cleavage site is within the recently identified Zn⁺² binding region of HMWK (DeLa Cadena and Colman, 1992) which is in domain 5 containing the anionic surface binding subdomain (Kunapuli et al., 1993). A predicted secondary structure of the light chain of HMWK by Lottspeich et al. (1985) shows this region of the molecule as a large loop made up of random coil, which is additional evidence for the availability of sites in this region to proteolytic cleavage. Low-HLT produced cleavage products of M_r 75 kDa and 65 kDa, but lacked the 46 kDa fragment produced by high-HLT. Western blotting with antibodies specific for the C-terminal (light chain) region

of HMWK reacted with only the 46 kDa fragment produced by high-HLT and with several smaller fragments produced by low-HLT. Attempts to sequence the two low-HLT generated fragments of M_r 75 kDa and 65 kDa were unsuccessful, presumably due to the blocked amino terminus of HMWK. This suggests that these two fragments contain the original amino terminus of the molecule. Because low-HLT simultaneously cleaved HMWK at several positions in the C-terminal region, location of these cleavage positions was not possible.

As previously discussed, prostromelysin (also called pro-matrix metalloproteinase-3, MMP-3) has also been reported as a tryptase substrate (Gruber et al., 1988 and 1989). Tryptase presumably cleaved proMMP-3 to MMP-3 which then cleaved procollagenase to collagenase. However, preliminary data resulting from a collaboration with H. Nagase (Univ. of Kansas, Lawrence, KS) has indicated that high-HLT does not cleave proMMP-3, but that low-HLT does. In addition, a collaboration with S. Stack (Duke Univ., Durham, NC) has produced data indicating that high-HLT cleaves plasminogen activator and plasmin. Plasmin is involved in the dissolution of blood clots by breaking down fibrin, the major constituent of clots. The events leading to clot breakdown involve activation of single-chain plasminogen activator to two-chain plasminogen activator, which converts plasminogen to plasmin. Plasmin has the capability of activating more plasminogen activator in a

feedback amplification. High-HLT has now been shown to produce plasmin both directly by cleaving plasminogen to plasmin and indirectly by cleaving single-chain plasminogen activator to produce two-chain plasminogen activator in vitro. These data suggest a possible role of tryptase in clot dissolution in addition to its potential role in anticoagulation. However, the in vivo significance of these reactions is unclear.

Taken together these findings clearly demonstrate that there are at least two distinct tryptase isozymes in human lung tissue that differ in reaction rate and cleavage site specificity with two natural substrates. As presented above, there is also evidence that they may differ in several other respects, such as heparin binding and quaternary structure. The implications of these data include possible differences in primary structure, radius of activity and physiological substrates (function), regulation, carbohydrate content, and even secondary and tertiary structure. This view differs from the generally accepted hypothesis that tryptase exists only as a heterotetramer in association with heparin, and expands the study of tryptase to include isozymic forms. A modified purification procedure must be used in order to retain low-HLT and to separate the isozymes. The possible existence of endogenous inhibitors and/or other forms of regulation of tryptase other than heparin binding should also be

investigated further.

The physiological significance of the existence of these two tryptase isozymes in vivo is not obvious. Both isozymes would presumably have the capability to inactivate HMWK and VIP upon release from mast cells. It is still unclear, however, whether one isozyme actually has a more specific function than the other. There is notable variability in the amount of tryptase isolated from lung tissue obtained at autopsy, but whether this variability is due to individual differences or other variables, such as time of death relative to when the tissue was frozen, remains to be seen. Both low and high-HLT have been observed in all our preparations. It seems likely that tryptase is of physiological importance given the ubiquitous distribution of mast cells in the body and the large amount of tryptase present in all mast cells. Mast cells are also known to participate in certain pathological conditions, thereby implicating tryptase as a contributing factor in these conditions. This points to tryptase as a potential target of preventive or therapeutic drug therapy. The elucidation of the function(s) and regulation of tryptase and the corresponding mechanisms, therefore, has potentially important medical benefits.

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APPENDIX

APPENDIX

The Appendix contains data that was meaningful but not of high enough quality to be included in the body of the dissertation. These data were included so that people continuing the tryptase project in the future would have all data resulting from this project in a collective and organized form. The data herein are referred to in the body of the text and are further explained in the figure legends accompanying each figure.

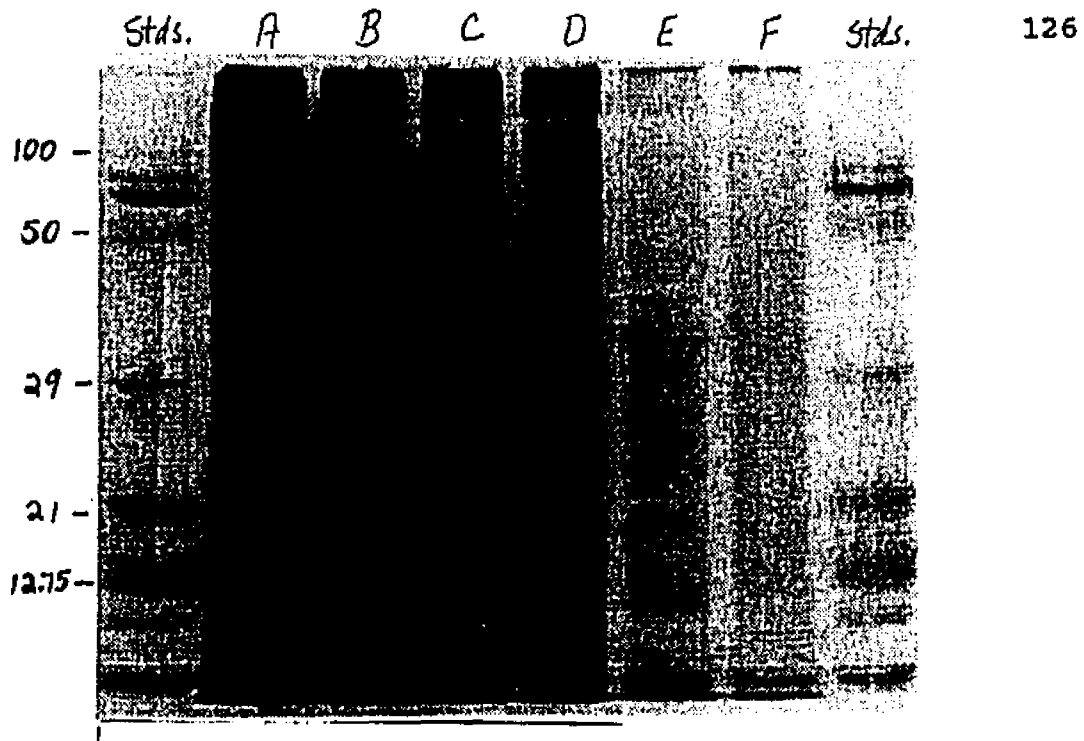


Figure 1. SDS-PAGE (10%) with Reduction of Pools from the Dowex and Heparin-Sepharose Columns. Lane A, crude lung extract (25 μ l); Lane B, Dowex flowthrough (25 μ l); Lane C, Dowex pool after dialysis (25 μ l); Lane D, flowthrough from heparin-Sepharose column (25 μ l); Lane E, first activity peak from heparin-Sepharose (5 μ g); Lane F, second activity peak from heparin-Sepharose (5 μ g). Samples were TCA precipitated and resuspended in sample buffer before electrophoresis. The outside lanes contain MW markers: phosphorylase B (100 kDa), transferrin (78 kDa), BSA (68 kDa), IgG (50 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa), cytochrome c (12.75 kDa), and Trasylol (6.5 kDa). Protein was visualized with silver stain.

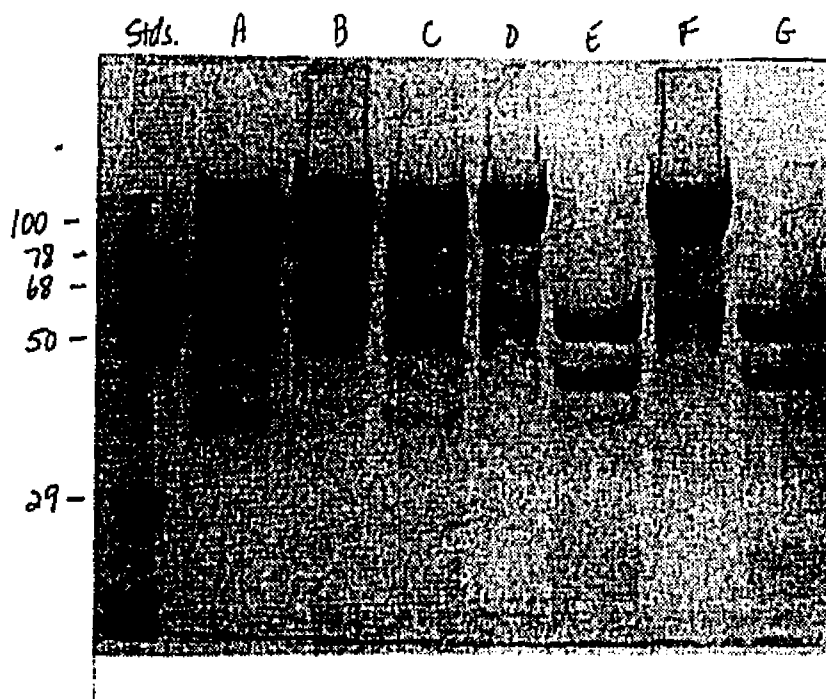


Figure 2. SDS-PAGE (7%) with Reduction of the Inhibitory Effects of α_2M and α_1I_3 on High-HLT and Trypsin. Lane A, HMWK (20 μ g; 438 pmol) and high-HLT (3.5 pmol of active sites); Lane B, HMWK, high-HLT and α_2M (3.5 pmol); Lane C, HMWK, high-HLT and α_1I_3 (3.5 μ g); Lane D, HMWK (10 μ g); Lane E, HMWK and trypsin (0.88 pmol); Lane F, HMWK, trypsin and α_2M ; Lane G, HMWK, trypsin and α_1I_3 . The enzyme (high-HLT or trypsin) and inhibitor was incubated at RT for 30 minutes before addition of HMWK, which was incubated at 37°C for 20 minutes before TCA precipitation and gel analysis. The MW standards in the outside lanes are the same as in Figure 1. Protein was visualized with colloidal Coomassie blue.

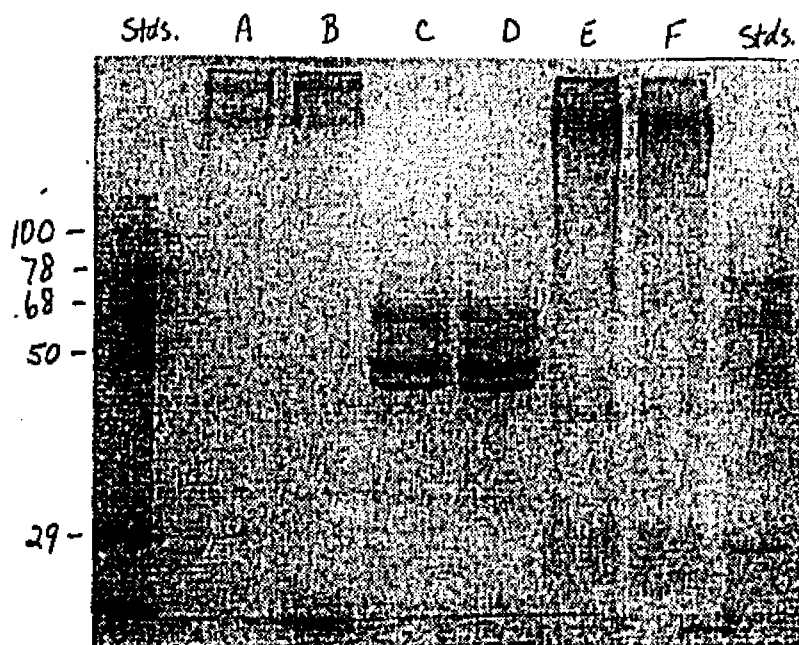


Figure 3-A. SDS-PAGE (7%) with Reduction of Laminin, Fibrinogen and Fibronectin after Exposure to High-HLT. Lane A, laminin (13.4 μg , 13.4 pmol); Lane B, laminin and high-HLT (0.07 pmol); Lane C, fibrinogen (13.4 μg , 39.4 pmol); Lane D, fibrinogen and high-HLT (0.20 pmol); Lane E, fibronectin (13.4 μg , 26.8 pmol); Lane F, fibronectin and high-HLT (0.13 pmol). The E:S ratio in each case was 1:200 based on protein concentration. MW standards are the same as in Figure 1. Proteins were visualized with colloidal Coomassie blue.

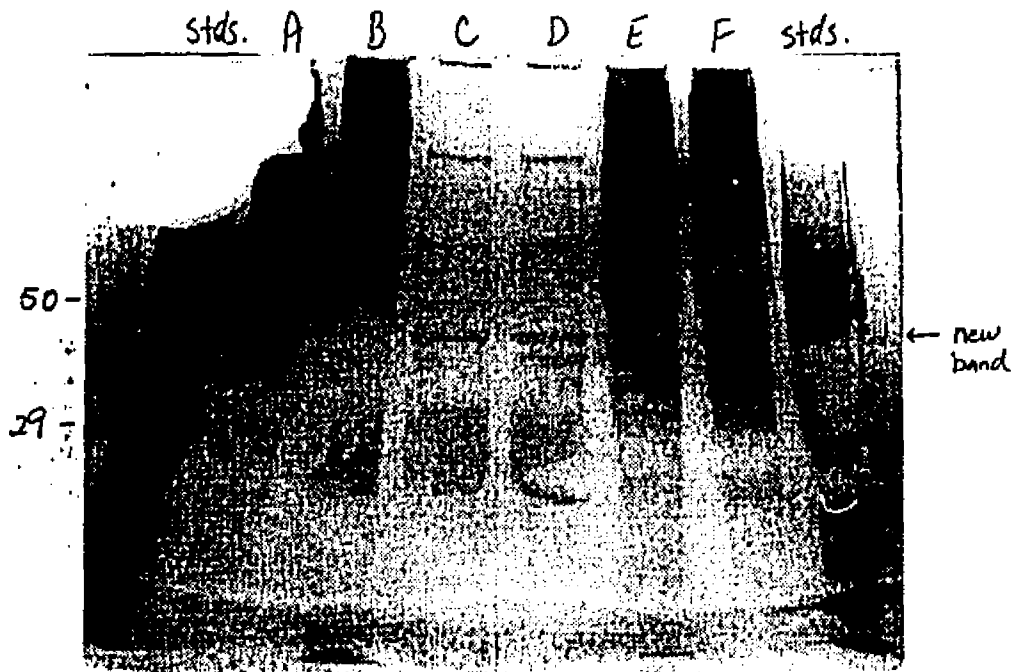


Figure 3-B. SDS-PAGE (4-20%) with Reduction of Laminin, Fibrinogen and Fibronectin after Exposure to High-HLT. This gel was a duplicate of Figure 3-A except that half the amount of protein was loaded in each lane and the gel was developed with silver stain.

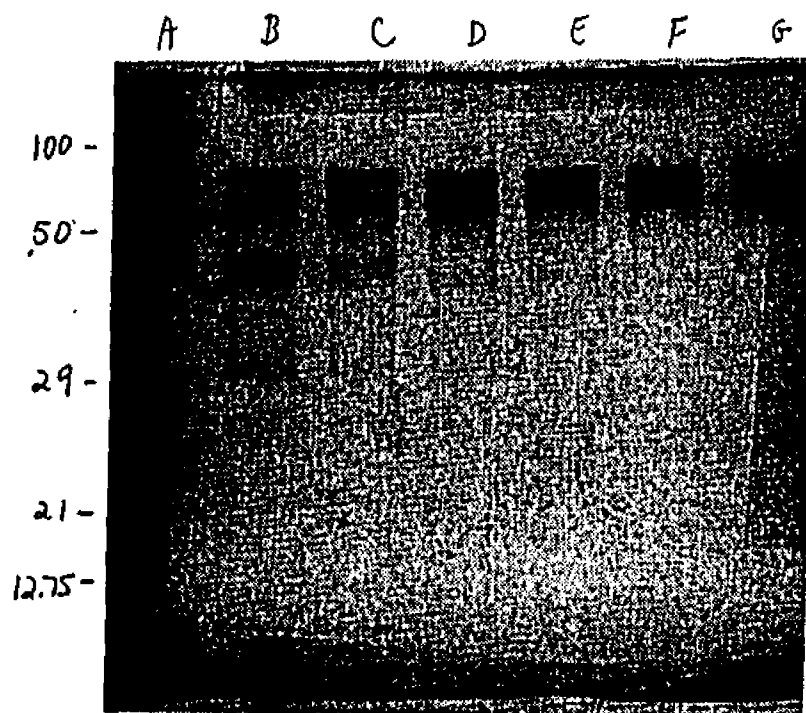


Figure 4-A. SDS-PAGE (10%) with Reduction of the Cleavage of HMWK by High-HLT Without Precipitation Prior to Electrophoresis. Lane A, HMWK at zero minutes (2 μ g, 17.8 pmol); Lane B, HMWK and high-HLT (0.04 pmol) after a 5 minute incubation at RT; Lane C, 10 minutes; Lane D, after 20 minutes; Lane E, 40 minutes; Lane F, after 60 minutes; Lane G, after 90 minutes. The E:S ratio was 1:50 based on protein concentration. MW standards were as in Figure 1 and proteins were visualized with silver stain.

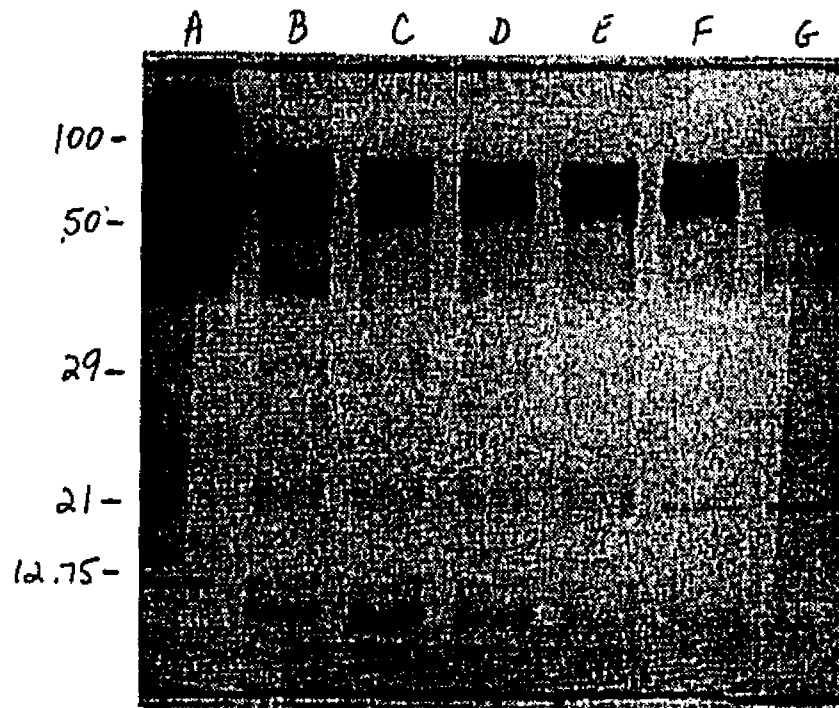


Figure 4-B. SDS-PAGE (10%) with Reduction of the Cleavage of HMWK by Low-HLT Without Precipitation Prior to Electrophoresis. This gel was performed as in Figure 4-A except that 0.01 pmol of low-HLT was used per sample instead of 0.04 pmol of high-HLT and Lane A contained HMWK after 90 minutes at RT, pH 7.5.

VITA

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- Little, S. S. and D. A. Johnson. Human Mast Cell Tryptases: Structure and Specificity. James H. Quillen College of Medicine 7th Annual Research Forum, East Tennessee State University, Johnson City, TN, April 4-5, 1991
- Little, S. S. and D. A. Johnson. Human Mast Cell Tryptases: Structure and Specificity. Federation of American Societies for Experimental Biology 75th Annual Meeting, Atlanta, GA, April 21-25, 1991, Abstract # 466
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