May 1985

Human Lung Tryptase Purification and Characterization

Timothy J. Smith
East Tennessee State University

Smith, Timothy J.

HUMAN LUNG TRYPTASE PURIFICATION AND CHARACTERIZATION

East Tennessee State University PH.D. 1985

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs or pages ✓
2. Colored illustrations, paper or print _____
3. Photographs with dark background ✓
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages ✓
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Dissertation contains pages with print at a slant, filmed as received _____
16. Other _____

_____

_____

HUMAN LUNG TRYPTASE

PURIFICATION AND CHARACTERIZATION

A Dissertation

Presented to

the Faculty of the Department of Biochemistry

Quillen-Dishner College of Medicine

East Tennessee State University

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Biomedical Sciences

---

by

Timothy J. Smith

May, 1985

APPROVAL

This is to certify that the Graduate Committee of

TIMOTHY J. SMITH

met on the

twenty-ninth day of March, 1985.

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council and the Associate Vice President for Research and Graduate Studies in partial fulfillment of the requirements for the degree Doctor of Philosophy in Biomedical Science.

David A. Johnson
Chairman, Graduate Committee

M. L. Entman

Cecile E. Hawkins

Richard A. Crofts
Associate Vice-President for the Research and Graduate Studies

Signed on behalf of
Graduate Council

Abstract

HUMAN LUNG TRYPTASE

PURIFICATION AND CHARACTERIZATION

by

Timothy J. Smith

Human lung tryptase (HLT), a mast cell derived trypsin-like enzyme, was isolated from whole human lung tissue obtained at autopsy. Increased yields from this purification process allowed extensive characterization of the enzyme. One of the critical steps in the purification scheme was the use of a linear heparin gradient to elute active material from cellulose phosphate. Gel filtration studies in 1.0 M NaCl yielded an apparent M_r of 135,000, and subsequent electrophoresis on sodium dodecyl sulfate-polyacrylamide gels demonstrated the presence of two active species with apparent M_r = 30,900 and 31,600. Enzymatic activity was sensitive to NaCl concentrations above 0.05 M and was only 50% in 0.15 M NaCl, decreasing to 18% in 0.6 M NaCl. The effects of synthetic and natural inhibitors were studied, confirming the enzyme's trypsin-like characteristics and demonstrating that naturally occurring serum inhibitors are incapable of diminishing its activity. A complete amino acid analysis showed a high tryptophan content. Antisera to human lung tryptase was generated, and the immunological identity of active fractions was investigated. The stability of HLT in various buffer systems was extensively studied, and 10 mM MES buffer, pH 6.1 appeared to provide the best conditions during extended storage and purification. The effect of heparin on the enzyme's activity using the synthetic substrates Z-Lys-SBzl was studied, and heparin concentrations of 10 micromolar stabilized HLT and allowed full expression of activity even at low ionic strengths. In the presence of heparin the enzyme retained full activity after 24 hours at 37°C, whereas in the absence of heparin, activity was lost after 30 min at this temperature. Heparin had a similar effect on HLT's ability to cleave natural substrates such as fibronectin. Assays comparing the activity of HLT on the substrates Z-Lys-SBzl and Z-Arg-SBzl were performed. The K_m and V_{max} of HLT for the above substrates were determined. The substrate 4-methylumbelliferyl-p-guanidinobenzoate (MUG-B) has been used to perform an active site titration on HLT, and a k_{cat} of 610/sec was calculated for the substrate Z-Arg-SBzl.

DEDICATION

to

Todd and Tadd

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to the faculty of the Department of Biochemistry for their instruction, patience, and friendship. I would like to thank the members of my committee Drs. David A. Johnson, M. Lou Ernst-Fonberg, Phillip R. Musich, Ellen Rasch and Arthur E. Hougland for their encouragement and suggestions during the preparation of this work. To Dr. Margaret Hougland a special thanks is extended for reviewing the manuscript. Lastly, I appreciate the assistance of Raymonde Cox in the preparation and typing of this dissertation.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

BZ-Arg-NA	Benzyl-Arg-p-nitroanilide
D-HLT	Deheparinized human lung tryptase
DIPF	Diisopropylphosphofluoridate
DTT	Dithiothreitol
[ES]	Enzyme substrate complex
HEPES	4-(2-hydroxethyl)-1-piperazineethanesulfonic acid
H-HLT	Heparinized human lung tryptase
HLT	Human lung tryptase
IF-A	Inflammatory factor of anaphylaxis
LPR	Late phase reaction
MES	2-(N-morpholino)ethane sulfonic acid
MU	Methyl coumarin
MUG-B	4-methylumbelliferyl-p-quanidino-benzoate
PMN	polymorphonuclear leukocyte
PN-I	Protease nexin I
p-CMB	p-chloromercuribenzoate
RMCP-I	Rat mast cell proteinase I
RMCP-II	Rat mast cell proteinase II
SDS	Sodium dodecyl sulfate
[S]	Substrate concentration
TAMe	Tosyl-L-Arginine methyl ester
TLCK	Tosyl-L-Lys-chloromethyl ketone
TPCK	Tosyl-L-Phe-chloromethyl ketone
Z-Lys-SBzl	CBZ-Lys-thiobenzyl ester
Z-Arg-SBzl	CBZ-Arg-thiobenzyl ester

CHAPTER 1

Introduction

Tissue proteinases may be involved in a variety of disease processes and biological functions including angiogenesis, fertilization, endocytosis, exocytosis, protein turnover, tissue degeneration, the immune response and inflammation (Woodbury and Neurath, 1980; Starkey, 1977). The role of the neutrophil proteinases, elastase and cathepsin G in diseases such as arthritis and emphysema has been well studied (reviewed by Starkey, 1977). However, the physiological role of some other tissue proteinases has not been so clearly elucidated. Such is the case with the trypsin-like tissue proteinases referred to as tryptases.

Glenner and Cohen (1960) were the first to recognize trypsin-like activity in human mast cells, and Johnson and Pascual (1978) noted tryptic activity in the high salt extracts of lung tissue. This activity seemed different from that of previously characterized tissue proteinases (Johnson, 1981). There have been reports of similar enzymes from skin (Fraki and Hopsu-Havu, 1975) and uterine cervix (Ito et al., 1980). It is now thought that these tryptases are all of mast cell origin and may represent the same enzyme (Tanaka et al., 1983).

Schwartz et al. (1981a) successfully purified what has been called human lung tryptase (HLT) from purified mast cell preparations. Unfortunately, general application of this method was hindered by the requirement for very fresh tissue, the isolation of mast cells and yields of only microgram quantities of pure enzyme. Preliminary

research in our laboratory had already indicated that by beginning with whole human lung obtained at autopsy, milligram quantities of HLT could be generated (Johnson, 1980 unpublished data), but complete purification of the enzyme was not yet possible. A continuation of efforts to purify larger quantities of enzyme subsequently resulted in a unique purification scheme, a complete characterization including physical and kinetic data, and raising antisera to HLT. Therefore, a major contribution to the understanding of mast cell function and tissue proteinases has been made.

In order to provide a foundation on which to base a discussion of human lung tryptase, a brief review of proteinases including definition, binding site specificity, limited proteolysis, types of proteinases, proteinase inhibitors and pathology has been included.

Definition of a Proteinase

Proteinases are enzymes capable of catalyzing the hydrolysis of peptide bonds (Figure 1). When these cleavages are internal in relation to the substrate, the proteinase is defined as an endopeptidase versus an exopeptidase which exerts its effect at a terminal peptide bond. An exopeptidase can be further defined by the position of the susceptible amide linkage as being either an aminopeptidase or carboxypeptidase (Figure 2).

Binding Site Specificity

Figures 1 and 2 are simplifications suitable only for demonstration of susceptible sites of cleavage by different groups of proteinases. The mechanism of action involved in the proteolytic digestion of

Figure 1. Hydrolysis of a peptide bond by a proteinase.

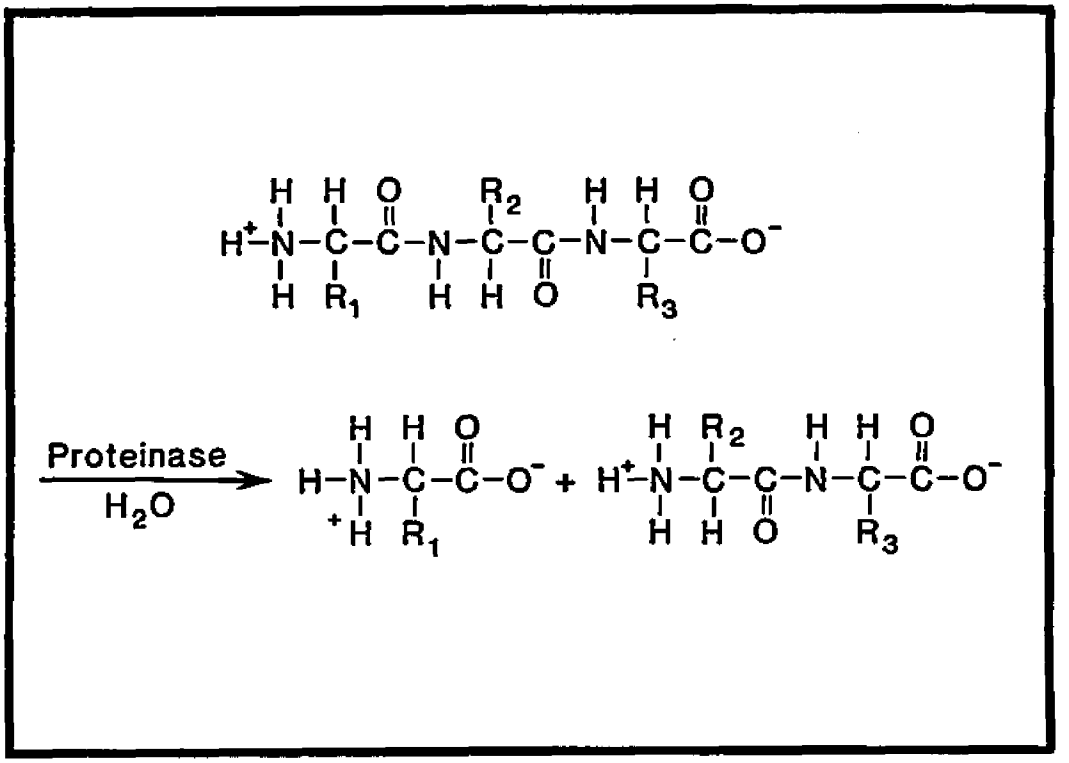
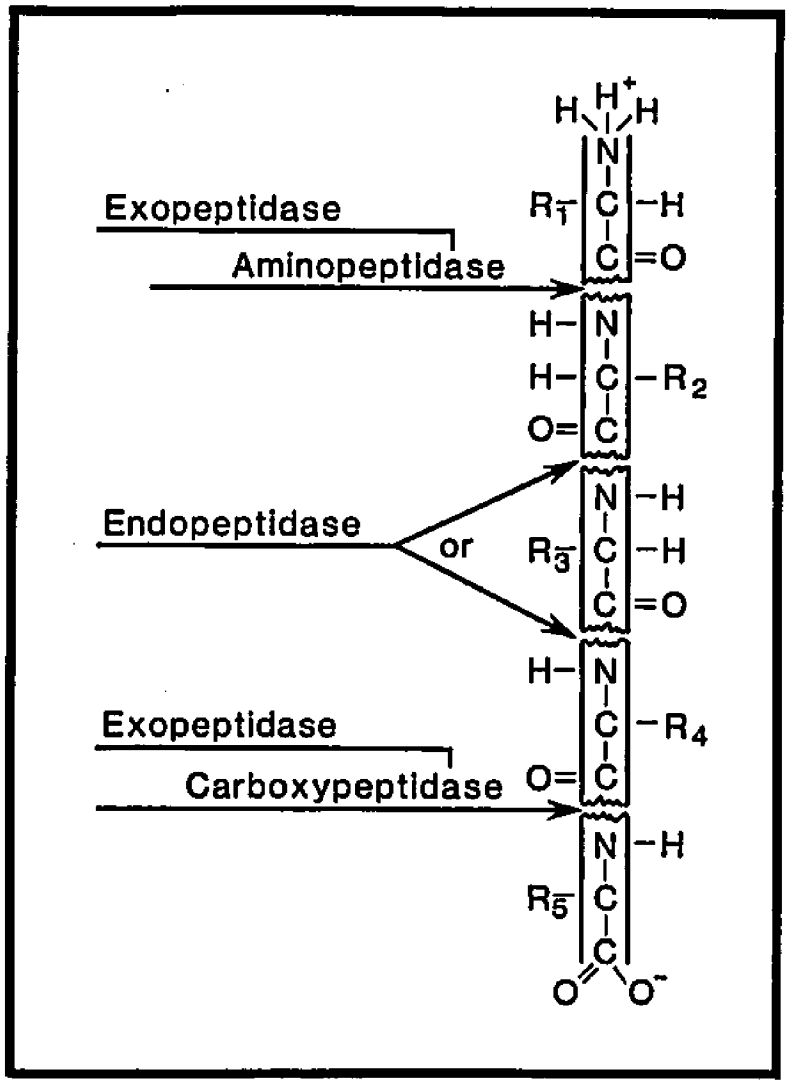

Proteinase
H_2O

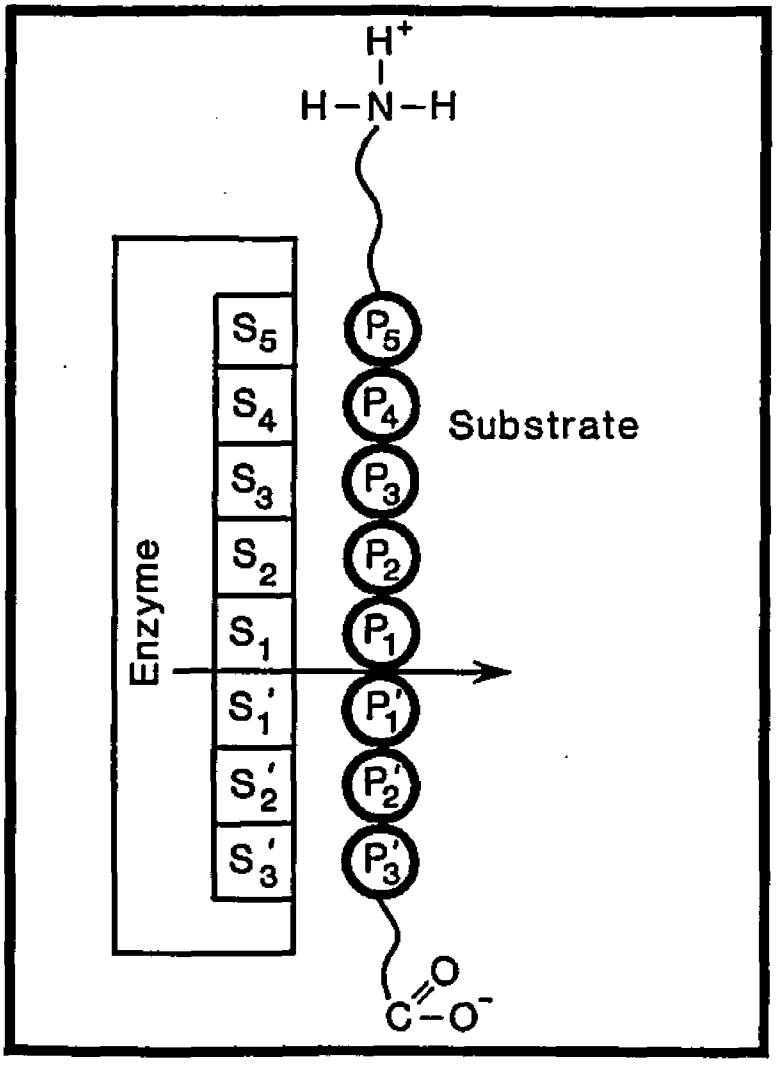
Figure 2. Schematic demonstration of the action of general groups of proteinases.


Exopeptidase
Aminopeptidase
Endopeptidase
or
Exopeptidase
Carboxypeptidase

polypeptides revolves around more than enzyme specificity for a single amino acid residue. While the action of proteinases results in cleavages of proteins at certain specific sites, other amino acids surrounding the susceptible bond also contribute to specificity. Joseph Fruton (1975) described proteolysis as "a multipoint cooperative process". By convention, the amino acid residue which contributes the carbonyl carbons to the peptide bond preferentially cleaved by a proteinase is called P1, and those amino acids to the left of P1 are termed P2, P3, P4, etc. The residues to the right of the "scissile bond" are designated P1', P2', P3', etc., and these amino acids are part of the "leaving group". Similarly, the enzyme contains a primary binding site (S1) and subsites to the left and right. The arrangement of these primary sites and subsites is represented in Figure 3. Substrates must enter a pocket where they are stabilized by noncovalent interactions. The arrangement of amino acids within the binding pocket determines the size and nature of the substrate to be accommodated. Since noncovalent forces are very weak, several interactions are needed in order to stabilize the enzyme-substrate complex ([E-S]), i.e. if amino acids at the primary binding site and those surrounding the P1 do not have the correct size, shape and charge, there will be no [E-S].

Some proteinases demonstrate a very narrow range of specificity. Thrombin, which is responsible for the conversion of fibrinogen to fibrin, is one such example. On the other hand, proteolytic enzymes such as pepsin or chymotrypsin show specificity for a wide range of substrates. Specificity rests within the binding site of the enzyme.

Figure 3. Arrangement of primary binding sites and subsites. Arrow indicates primary cleavage site. P_1-P'_1 cleavage is at the carboxyl group of P_1.


H+
H–N–H
Enzyme
S5
S4
S3
S2
S1
S1'
S2'
S3'
P5
P4
P3
P2
P1
P1'
P2'
P3'
Substrate
O
C–O-

More than one protein or peptide may possess the correct geometric complementarity necessary to act as a substrate (Walsh, 1979).

Limited Proteolysis

The action of proteolytic enzymes can be explained by discussing their specificity, but their biological function cannot be understood without a description of limited proteolysis. In biological systems, digestion by proteinases does not proceed randomly. There are only certain areas on the surface of proteins which contain sites where proteolytic cleavages can take place. "Accessibility" is the key word in an explanation of the nature of this phenomenon. Due to the tertiary structure of proteins, only certain peptide bonds are accessible (exposed) to the action of peptidases, and proteolysis is therefore limited (Neurath, 1975). Examples of this are seen when zymogen activation occurs due to the hydrolysis of specific peptide bonds. Consider the enzyme chymotrypsin. It is produced by pancreatic cells as the zymogen, chymotrypsinogen, and is secreted into the intestine. Once in the intestine it is converted to chymotrypsin by the action of trypsin. More specifically, trypsin, which has a preference for Lys-X or Arg-X residues, cleaves at only two such sites on chymotrypsinogen leading to the formation of active enzyme. Because of the tertiary structure of proteins, certain sites within a molecule may be more or less accessible to digestion by proteinases. Thus, the folding of the polypeptide chain can be viewed as an important control mechanism in limited proteolysis (Blackburn, 1976; Walsh, 1979). It was previously noted that chymotrypsin shows broad specificity. There are examples of

more specific limited proteolysis. Kallikriens, which convert kininogen to kinins, show such a high degree of specificity that they have little or no activity on other proteins. A similar situation is seen with the conversion of angiotensinogen to angiotensin I and II (Pisano, 1975). In addition, the blood clotting cascade involves a series of specific zymogen activations. The activated products of limited proteolysis serve to activate, in sequence, the next step in the cascade (Stryer, 1975).

As a research tool, the controlled digestion of native proteins has been used extensively to compare the action of different proteinases. Alternatively, a protein of interest could be subjected to denaturing conditions. This renders amino acids or sequences of amino acids which would normally be "hidden", susceptible to "complete proteolysis". Thus time and the sequence of amino acids around P1, not tertiary structure, become the factors controlling the rate of cleavage. Proteinases involved in the breakdown of dietary products and in intracellular protein catabolism offer examples of complete proteolysis in vivo. General proteolytic digestion results in the cleavage of all susceptible peptide bonds, whereas limited proteolysis leads to the hydrolysis of only accessible sites.

Proteinase Classification

In 1960, a convenient and abbreviated method for classifying proteases was proposed. While the classification neglected their biological function, it successfully grouped the proteinases into four distinct categories based on their catalytic mechanisms (Table I). This

was an improvement over nomenclature which until that point had been based entirely on origin or physiological role and substrate specificity (Walsh, 1975).

Serine proteinases

The serine proteinases, which are also referred to as hydroxyl or neutral proteinases, have pH optima around 7.0, and possess a reactive serine within their active site. Common features of this group of proteinases include the following: (1) inhibition by diisopropylphosphofluoridate (DIPF), (2) similarities in catalytic mechanism (formation of a tetrahedral intermediate), (3) secretion as zymogens, (4) amino acid sequence similarities, (5) endopeptidase activity and (6) presence of a "charge relay" system (Walsh, 1979; Fersht, 1977). Their crucial differences lie in their specificities, which are related to the sequence of amino acids in their binding sites (Walsh, 1979) and their individual biological functions.

The enzymes, trypsin, chymotrypsin, and elastase provide the best examples of this group of proteinases. Trypsin, which has a preference for the carboxyl side of Lys and Arg residues, contains an Asp at position 189 in the binding pocket. There is a Ser at position 189 within the binding pocket of chymotrypsin and elastase. The difference in specificity between chymotrypsin and elastase is related to the amino acids at positions 216 and 226. In elastase they are Val and Thr, respectively, so the preferred substrates contain small uncharged side chains. With chymotrypsin and trypsin, positions 216 and 226 are occupied by Gly residues. Thus, chymotrypsin can accommodate large

Table I

Classification of Proteinases

Class	Important Feature of Active Site	Inhibitor
Serine Proteinase	Serine	DIPF
Sulfhydryl Proteinase	Cysteine	Iodoacetate
Metalloproteinase	Zinc	o-phenanthroline
Acid proteinase	Aspartic Acid	Diazoketones

hydrophobic amino acids (Price and Stevens, 1982). Figure 4 demonstrates the differences in the binding pockets of these three proteinases.

With the serine proteinases, the serine residue at position 195 is especially reactive. Jansen et al. (1949) showed that when radiolabelled DIPF was incubated with chymotrypsin the enzyme was irreversibly inactivated by what appeared to be a covalent modification. It was later established that substrates could protect chymotrypsin from DIPF inactivation, implying that the modification was active site directed (Jansen et al., 1950). The structure of DIPF and the phosphorylation of the active site serine at position 195 is seen in Figure 5. The reason for the enzyme inactivation became apparent after extensive studies using x-ray crystallography revealed the presence of a "charge relay" system made up of three precisely arranged side chains (Asp 102, His 57, and Ser 195) in the protein structure (Blow et al., 1969). Figure 6 serves to illustrate the function of the "charge relay system" and the formation of the tetrahedral intermediate during catalysis.

Sulfhydryl proteinases

Cysteine or sulfhydryl proteinases contain a nucleophilic cysteine residue at their active site. The term thiol proteinase has also been used to describe these enzymes. They are endopeptidases, and an acylthioester intermediate is generated during catalysis. Cathepsin B is the best studied mammalian proteinase of the group; however, the ubiquity of this peptidase has been the cause of considerable difficulty

Figure 4. Important amino acid residues in the binding pockets of chymotrypsin, trypsin and elastase. Chymotrypsin contains Gly residues at positions 216 and 226 in order to accommodate large hydrophobic amino acids. In trypsin, position 189 is an Asp which preferentially binds Lys and Arg side chains. Due to position 216 and 226 being occupied by Val and Thr in elastase, only small uncharged side chains are bound.

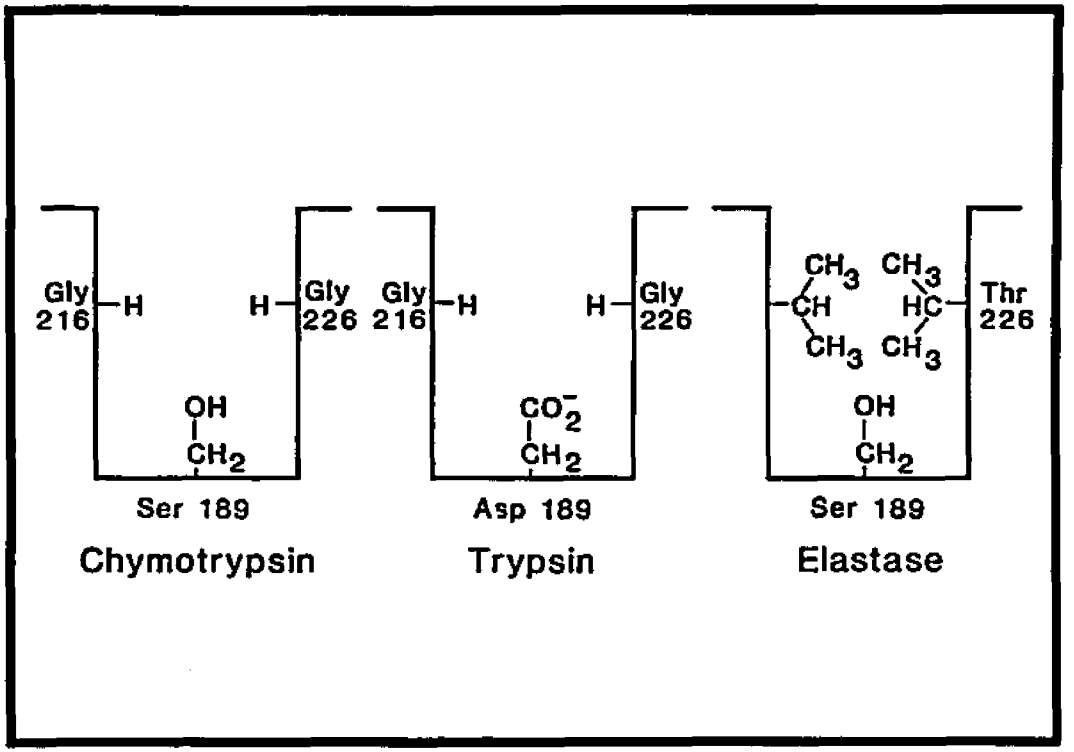

Gly 216
H
H
Gly 226
OH
CH2
Ser 189
Chymotrypsin
Gly 216
H
H
Gly 226
CO2
CH2
Asp 189
Trypsin
CH3
CH3
CH
HC
CH3
CH3
Thr 226
OH
CH2
Ser 189
Elastase

Figure 5. The action of DIPF on a serine proteinase leading to phosphorylation of the active site serine at position 195. DIPF reacts with the critical Ser residue to form an inactive diisopropylphosphoryl enzyme.

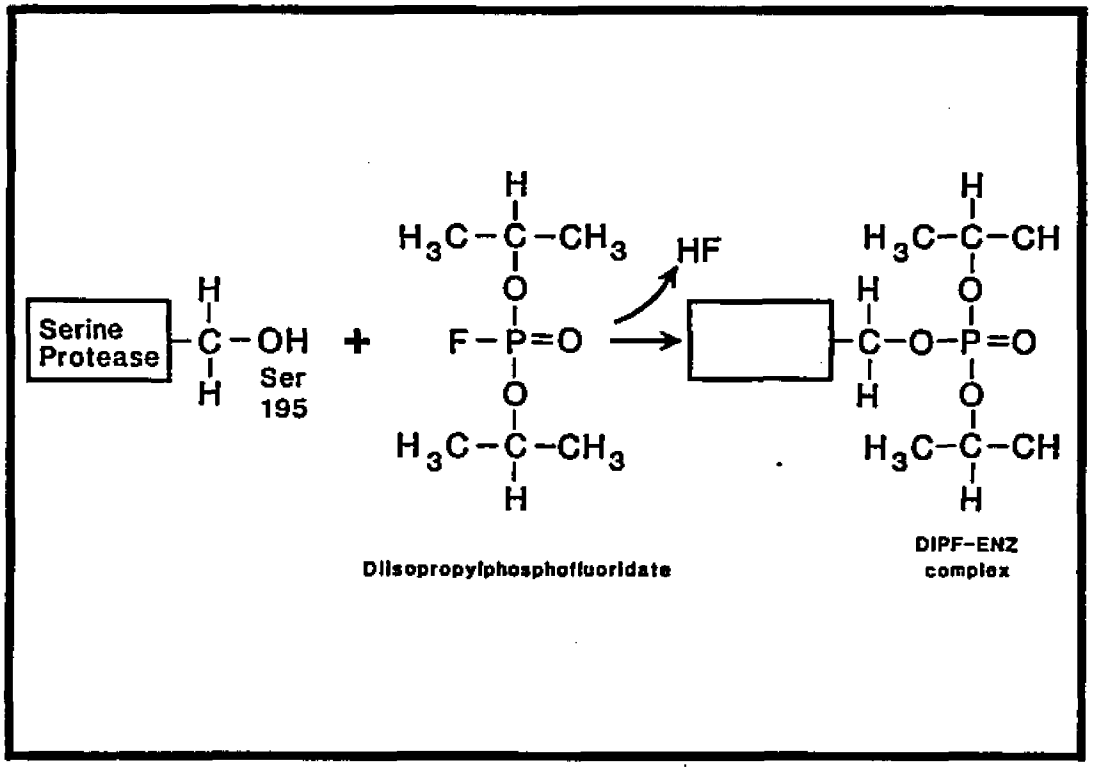
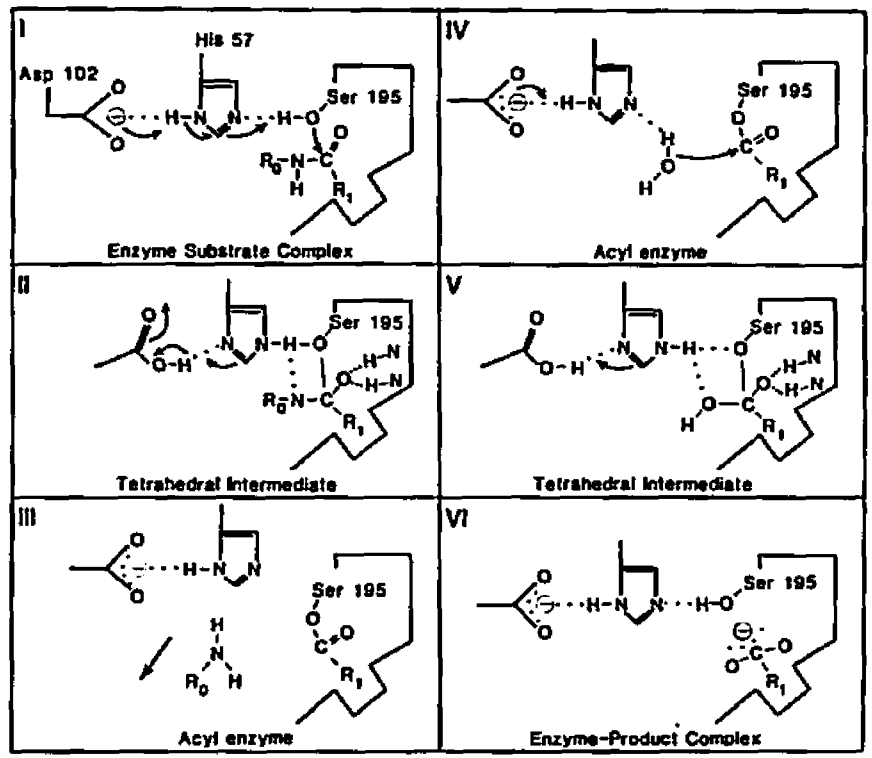

Serine Protease
OH
Ser 195
HF
Diisopropylphosphofluoridate
DIPF-ENZ complex

Figure 6. The mechanism of action for a serine proteinase. I. Catalysis begins with a nucleophilic attack of the Ser 195 hydroxyl at the carboxyl of the substrate. The charge relay system is made up of Asp 102, His 57 and Ser 195. General acid-base catalysis facilitates the attack. II. The oxyanion of the adduct is stabilized by hydrogen bonding to the amino group of Gly 193 and the Ser 195. III. The tetrahedral intermediate collapses via expulsion of the amine product which has become protonated from the N-3 of His 57 as it leaves. IV. Deacylation involves the reverse of acylation. An attacking water molecule becomes a strong nucleophile because of proton shuttling on the catalytic triad. V. There is a formation of a new tetrahedral intermediate due to proton transfer with the charge relay system. VI. Formation of the E-P complex, from which the product dissociates.


I
His 57
Asp 102
Ser 195
Enzyme Substrate Complex
IV
Ser 195
Acyl enzyme
II
Ser 195
Tetrahedral Intermediate
V
Ser 195
Tetrahedral Intermediate
III
Ser 195
Acyl enzyme
VI
Ser 195
Enzyme-Product Complex

in the study of tissue thiol proteinases in general. Slight variations in substrate susceptibility and isolation from many different tissues, often within the same species, has lead to confusion in the literature. "A major difficulty in the study of the tissue thiol proteinases is that of distinguishing them from each other" (Barrett, 1977).

There are important sulfhydryl proteinases from sources other than mammalian tissue. It is common practice to compare the mammalian and plant proteinases. There are many similarities in their activities and amino acid sequences, and enzymes such as papain, ficin, and bromelain are probably earlier representatives in the evolutionary pathway leading toward mammalian cysteine proteinases (Vavreinova and Turkova, 1975). For instance, the amino terminus of rat liver cathepsin B shows extensive homology with that of papain (Takio, 1980).

It seems certain that thiol proteinases are involved in intracellular protein catabolism. This is due to their presence within the lysosomes and the fact that there is a direct correlation between the rate of protein turnover and the activity of these enzymes (Barrett and Kirschke, 1980).

The classification of the proteinases in Table I shows that the sulfhydryl peptidases are inhibited by iodoacetamide, which reacts directly with the essential thiol group.

Metalloproteinases

These enzymes are classified into two separate categories, either metalloexopeptidases or metalloendopeptidases, and they are inhibited by o-phenanthroline, a zinc chelator. Both require stoichiometric

quantities of divalent cations for catalysis. Leucine aminopeptidase and carboxypeptidase A are perhaps the best examples of the exopeptidases of this category. Each is capable of sequential cleavage of amino acid residues beginning at their respective terminal sites.

Carboxypeptidase has been extensively studied. X-ray crystallography studies on the enzyme were completed in 1970 by Lipscomb et al., and a mechanism of action has been proposed (Blackburn, 1976; Walsh, 1979). Its rate of catalysis is enhanced if the terminal group is a large aromatic or aliphatic amino acid and the divalent cation involved in the mechanism is zinc. Leucine aminopeptidase also has a preference for aromatic amino acids. Very little is known about this enzyme aside from its requirement for zinc and its molecular weight of 300,000 daltons (DeLange and Smith, 1971).

Thermolysin, a metalloendopeptidase, has the same requirement for zinc, but it has a large hydrophobic pocket in its tertiary structure which is similar to that of chymotrypsin (Colman et al., 1972). Maximum catalysis is seen at acid pH; for this reason we might consider it to be an acid proteinase. Thermolysin raises an interesting point concerning the classification of proteolytic enzymes which was best described by Kenneth Walsh. "We must avoid over confidence. While we do recognize five fundamental sets of proteases and have inklings about their mechanisms, the list of sets is surely not complete and exceptions to the apparent sets are already known" (Walsh, 1975). Yet another example of inconsistency is seen with what have been referred to as the calcium activated proteinases. It appears that these may all be classified as

sulfhydryl proteinases due to the fact that they are inhibited by the common thiol proteinase inhibitors.

Acid Proteinases

The aspartyl proteinases are all inhibited by pepstatin which has little or no effect on other groups of proteinases (Knight and Barrett, 1976). Thus inhibition of a proteinase by pepstatin is indication that it is an acid proteinase. Pepsin, one of the better studied acid proteinases, was discovered in 1834. It is a gastric enzyme which functions to breakdown ingested protein and demonstrates its highest catalytic activity at pH 1-5. It is secreted by the gastric mucosal cells in zymogen form (pepsinogen) and is activated by autocatalytic limited proteolysis. The enzyme has endopeptidase activity and preferentially cleaves at aromatic and aliphatic amino acids (Fruton, 1971; Tang, 1971; Clement, 1973).

Proteinase Inhibitors

There are many synthetic inhibitors of the proteinases, some of which have been mentioned above. Although discussion of these inhibitors is not relative to the subject, it should be mentioned that the use of synthetic inhibitors to study proteinases has been extremely valuable, especially in comparing proteinases and elucidation of active site conformation.

In biological systems, proteinases are often found in close proximity to tissues which might be excellent substrates (i.e. they are not always compartmentalized assuring the "safety" of surrounding

Table II

Circulating Plasma Proteinase Inhibitors

Inhibitor	Concentration mg/dl
α_1Proteinase inhibitor	125 - 135
α_2Macroglobulin	190 - 320
α_1Antichymotrypsin	42 - 55
Inter-α-trypsin inhibitor	50 - 55
Antithrombin III	22 - 26
$\overline{C1}$ Inactivator	20 - 26
α_2 Antiplasmin	6 - 8

tissue). In order to control protein digestion, naturally occurring proteinase inhibitors have evolved. Table II contains a list of inhibitors found in human plasma and their approximate concentrations. Any absence or decrease in the amount of these important inhibitors can lead to random tissue destruction. Factors leading to an imbalance in the system of proteinases and their inhibitors and the complications which arise from such an imbalance are discussed below. For additional information on human plasma proteinase inhibitors the reader is referred to two recent reviews (Laskowski and Ikunoshin, 1980; Travis and Salvesen, 1983).

Pathology

The pathophysiology of proteinases is best understood if it is remembered that under normal conditions these enzymes remain either compartmentalized or controlled by the circulating plasma proteinase inhibitors. If a situation arises wherein compartmentalized proteinases are released or there is insufficient inhibitor for the control of proteolysis, tissue damage may ensue.

Above all other tissues and cells, the polymorphonuclear leukocyte (PMN) and its proteolytic enzymes have received the most attention in diseases involving uncontrolled proteolysis. This is not by chance. After all, it is this cell which is attracted via chemotactic stimuli to sites of inflammation and is sequestered in certain tissues with the physiological role of taking part in the inflammatory response.

The PMN is a granulated, phagocytic cell, and ingestion of material such as urate crystals, endotoxin, immune complexes and bacteria by the PMN have been shown to cause a "specific" release of serine proteinases

such as elastase and cathepsin G from the azurophilic granules (Movat et al., 1973; Oronsky et al., 1973). The release is specific in that there is no concomitant loss of cytoplasmic enzymes and the cells remain viable. Elastase and cathepsin G are capable of degrading a variety of tissues such as collagen, elastin, fibrin, proteoglycan and renal basement membrane (Janoff, 1972; Janoff and Blondin, 1973 and 1974). Starkey and Barrett (1976) showed that using cathepsin G and elastase, all of the major connective tissues could be hydrolyzed. Investigators speculate that several diseases involving chronic inflammation such as arthritis, emphysema and extensive wound healing are complicated by the release of proteolytic enzymes from the PMN (Weismann et al., 1971; Glynn, 1972; Simpson and Ross, 1972; Oronsky et al., 1973; Oronsky and Buermann, 1978).

In emphysema and respiratory distress syndrome of the newborn, a genetically transmitted deficiency of alpha-1-proteinase inhibitor leaves the neutral proteinases of sequestered lung PMN's free to degrade the alveolar wall (Eriksson and Berven, 1972; Evans et al., 1972). In other instances the amount of proteinase released from the PMN may be excessive and natural circulating inhibitors may not be present in sufficient concentrations to inhibit proteolysis. This may be at least part of the problem in chronic inflammatory diseases such as arthritis and gout. In either case, phagocytosis of immune complexes or other material leads to the release of proteolytic enzymes and a "self-propagation" of the disease process.

Tissue proteinases have also been implicated in degenerative diseases of the muscle such as muscular dystrophy and ischemia (Schwartz

and Bird, 1977). Some information regarding such an involvement seems quite convincing, since proteinases such as cathepsin B and D are known to be at higher levels in dystrophic muscle than in normal muscle (Spanier et al., 1977). McGowan et al. (1976) reported that leupeptin and pepstatin, inhibitors of these cathepsins, were able to inhibit the degeneration of muscle cells in culture.

Within recent years, the mast cell has been implicated in several diseases which are characterized by inflammation (reviewed in Wasserman, 1984). Tryptase, which is a trypsin-like enzyme, and chymase, a chymotrypsin-like serine proteinase, are associated with the mast cell granule and could be involved in inflammation and localized tissue destruction. Thus it seems quite possible that they could be involved in uncontrolled pathoproteolysis similar to cathepsin G or elastase.

In concluding this section on pathology, one observation would seem appropriate. By nature, proteinases are capable of digesting protein substrates, however, we must be cautious in assigning pathological roles to these enzymes without first investigating the feasibility of such an involvement. In our haste to assign such a function, it is possible to neglect understanding the proteinase in its normal physiological role.

The Proteolytic Enzymes of Mast Cells

Mast cells are large (10-30 μm), granule laden cells associated with the perivascular and perilymphatic spaces and are found in loose connective tissue and mucosal tissue in a variety of locations including skin, bladder, tongue, synovia, mesentery, and lung. The granules of the cell have a characteristic metachromasia when stained with basic dyes such as toluidine blue. This metachromatic staining has been

attributed to their proteoglycan content. Since the discovery of the mast cell in the 19th century, research has focused on the staining characteristics of the cell and its involvement in anaphylaxis. The membrane bound granules, which contain mediators of anaphylaxis such as biogenic amines, proteoglycans, neutral proteinases, acid hydrolases, oxidative enzymes, chemotactic factors, generated mediators of anaphylaxis and leukotrienes give the cell its true individuality. Degranulation of these cells produce the effects of immediate hypersensitivity and anaphylactic shock. The mechanism for this involvement in the allergic phenomenon has been firmly established. In brief, the mast cell contains on its surface specific receptors for IgE. After binding to these receptors via the Fc portion of the immunoglobulin, the antigen binding sites of two IgE molecules are dimerized by specific antigen. This dimerization leads to degranulation of the cell and the consequences of hypersensitivity or anaphylaxis. For an excellent review of the morphology and chemistry of the mast cell see Ishizaka (1984).

The morphology of mast cells may vary depending on the site of origin (Pearce et al., 1977), and evidence would indicate that there may be two populations of mast cells (Patterson et al., 1977; Woodbury et al., 1978; Seppa and Jarvinen, 1978; Woodbury and Neurath, 1978; Patterson and Suszko, 1980). These populations have been termed "typical" and "atypical" mast cells (Schwartz and Austen, 1981). In addition, mast cells contain neutral proteinases which show variation among and within species (Chiu and Lagunoff, 1972; Woodbury et al., 1981).

In rats, the predominant serine proteinase, comprising 50% of the granule protein and as much as 25% of the total cellular protein (Schwartz and Austen, 1981), is a chymotrypsin-like enzyme (chymase). chymotryptic activity was first reported in association with the rat mast cell in 1953 by a histochemical technique using 3-chloracetoxy-2-naphthoic acid anilide as substrate (Gomori, G., 1953). It is found bound to heparin, forming part of the granule matrix, and 1.0 M KCl is capable of eluting the chymase from other proteins (Lagunoff and Pritzel, 1976). Two forms of rat mast cell chymase have been found; each is associated with a particular type of tissue and mast cell type. Rat mast cell proteinase I (RMCP-I) is associated with connective tissue and is found predominantly in the typical mast cell. RMCP II is located primarily in mucosal tissue within the atypical mast cell (Woodbury et al., 1981). Although the amino acid sequences of the chymases are 75% homologous in their first 51 N-terminal amino acids, the two proteinases show no immunological cross reactivity (Woodbury et al., 1978), suggesting distinct genetic origins.

The biological function of rat mast cell chymase remains obscure. The enzyme is not in zymogen form inside the mast cell, but its strong binding to heparin (and possibly serotonin) and the internal pH of the mast cell granule may keep it relatively inactive (Yurt and Austen, 1977; Okuno-Kaneda et al., 1980). Once degranulation occurs, the heparin-chymase complex may persist, and the pH of the external medium may be more suitable for the complete expression of activity (Schwartz and Austen, 1984).

There has also been some tryptase activity associated with the mast cell of the rat. Tryptase is not considered a major component of this cell because the activity is at the lower limit of sensitivity using the TAMe-esterase assay (Schwartz and Austen, 1981). Trypsin-like activity in human lung mast cells on the other hand is at levels which compare to that of chymase activity in rats. Human lung tryptase has been estimated to comprise 23% of the total protein of the human mast cell. It is thought to exist as a tetramer with a molecular weight of 144,000 daltons, demonstrating two active subunits at 37,000 and 35,000 daltons. It is interesting to speculate that these active subunits may represent two tryptases analogous to the RMCP I and RMCP II of rat lung tissue. It seems likely that RMCP and tryptase are enzymes with similar functions from the mast cells of different species. In addition, it has been shown that tryptase has a strong affinity for heparin. The biological function of the mast cell tryptase is also unknown. Several pieces of interesting information have surfaced in recent years concerning both chymase and tryptase. It is possible that they play a role in the inflammatory response, but whether this role is intracellular, extracellular or both remains to be shown. Meier et al. (1983) demonstrated that a group of lung mast cell proteinases were capable of interacting with three proteins of the Hagamen Factor dependent pathways, prekallikrein, Hagamen Factor and kininogen. Schwartz et al. (1982) showed that mast cell tryptase could cleave C_3 to generate C_3a anaphylatoxin. Due to the amount of tryptase required for cleavage and the inability of the enzyme to cleave in the presence of

heparin, assigning this as a biological function of HLT seems inappropriate.

Dog mast cells contain tryptase, which has been shown to activate collagenase from fibroblasts (Birkedal-Hansen et al., 1976). Yoffe et al. (1984) demonstrated that collagenase production increased in rheumatoid synovial cells with the addition of dog mast cell extracts. Therefore, tryptase may be involved in pathoproteolysis. Supporting this, Vartio et al. (1981) demonstrated that rat mast cell chymase was capable of cleaving fibronectin in a fashion similar to cathepsin G. Fibronectin functions *in vivo* to promote cell adherence to the extracellular matrix (Klebe, 1974; Pearlstein, 1976; Pearlstein and Gold, 1978).

Mast cells are located in connective and mucosal tissue, at the ends of new blood vessels, around neural tissue and the airways of the respiratory system. Their numbers have been shown to increase during angiogenesis (Kessler et al., 1976) and inflammation in diseases such as arthritis (Sagreiya et al., 1983; Crisp et al., 1984; Godfrey et al., 1984; Wasserman, 1984). The possible role of mast cell tryptase in inflammation and angiogenesis will be discussed, however, tryptase could play a role in the inflammatory process similar to a degree equal that of elastase and cathepsin G.

Although the mast cell is not phagocytic, at least to the degree of the PMN, observed endocytosis using a variety of materials has shown that virtually all endocytized material becomes associated with the mast cell granule (Padewar, 1979). It has been shown that endocytized IgE is

transferred from the cytoplasmic surface to the granule in as little as one hour after passive sensitization of the cell (Halliwell, 1973). Active tryptase associated with the mast cell granule may function to either process or degrade endocytized proteins such as IgE.

Additional comments concerning the biological activity of tryptase will be reserved for the discussion, due to the fact that this research contributes information which is useful in understanding the enzyme's function. We have successfully purified HLT from whole lung tissue, and an extensive characterization has been made possible. Antibody to the purified enzyme was made to be used in the subcellular localization of the enzyme and the development of an immunoassay. In addition, the effects of HLT on natural substrates such as fibronectin and IgE and the role of heparin in stabilization of the enzyme have been studied. The goal of this research was to contribute information which might be valuable in understanding the function of the mast cell.

CHAPTER 2

MATERIALS

Octyl-Sepharose, cellulose phosphate, 2-(N-morpholino)ethane sulfonic acid (MES), molecular weight electrophoresis standards, Tris, Bz-DL-Arg-p-nitroanilide (Bz-Arg-NA), heparin, tosyl-L-arginine methyl ester, (TAMe), azocasein, benzamidine, 4-methylumbelliferyl p-quanidino-benzoate, glycine, 3,3'-diaminobenzidine, ammonium sulfate, soybean trypsin inhibitor, lima bean trypsin inhibitor, tosyl-L-Lys-chloromethyl ketone, (TLCK), tosyl-L-Phe-chloromethyl ketone (TPCK), ovoinhibitor, and p-chloromercuribenzoate (p-CMB) were obtained from Sigma Chemical Co. Diisopropylphosphofluoridate (DIPF) was the product of Aldrich Chemical Co. "Enlightin" and [^{3}H]-diisopropylphosphofluoridate were purchased from New England Nuclear. Acrylamide and other chemicals for electrophoresis were from Bio-Rad. Fractogel TSK-HW 55 was purchased from MCB. The standards for gel filtration molecular weight determination were from Pharmacia. Human alpha-1-proteinase inhibitor was prepared as previously described (Travis and Johnson, 1981). Human alpha-2-macroglobulin was a gift from Drs. J. Travis and G. Salveson, University of Georgia. Trasylol$^{(R)}$ bovine pancreatic trypsin inhibitor was a gift of Bayer A. G., West Germany. Bronchial inhibitor (bronchial leucocyte proteinase inhibitor) was isolated by the method of Smith and Johnson, 1985. Proteinase inhibitors originally isolated by H. Umezawa (Aoyagi and Umezawa, 1975) which included antipain, chymostatin, elastinal, leupeptin, pepstatin, and phosphoramidon were provided by the U.S.-Japan Cooperative Cancer Research Program through Dr. W. Troll,

N.Y.U. Proteinase Nexin I was from Dr. Joffe Bake, University of Kansas. Fibronectin was obtained from Calbiochem-Behring. All other chemicals were of reagent grade or equivalent quality.

CHAPTER 3

METHODS

Enzyme Assays

Activity was monitored using the substrate Benzyl-Arg-p-nitroanilide (Bz-Arg-NA) (Erlanger et al., 1961). One unit of activity was defined as the amount of enzyme which gave a change of 1.0 absorbance units/min at 410 nanometers. HLT, diluted as necessary, was added to 2.85 ml of 0.1 M Tris-HCl, 1 M glycerol, pH 8.0, and the assays were started with the addition of 50 microliters of 20 mM substrate in DMSO. After 30 min incubation at 37°C, the assays were stopped with the addition of 50 microliters of glacial acetic acid, and the absorbance was read on a Beckman Model 35 spectrophotometer. In order to compare specific activities, TAMe assays were performed as described by Schwartz et al. (1981a). Azocasein assays according to Barrett (1977) were used to test the effect of alpha-2-macroglobulin on the enzyme.

Stability studies and kinetic data were obtained using the substrates CBZ-Lys-thiobenzyl ester and CBZ-Arg-thiobenzyl ester. When saturating levels of substrate were indicated, the two substrates were dissolved in DMSO at [S] of 20 mM. One ml assays were typically performed by adding 20 microliters of substrate, 890 microliters of 0.1 M HEPES buffer which was 1.0 M in glycerol, 10% DMSO and pH 7.5, 80 microliters of 2 mM DTNB dissolved in the HEPES buffer and 10 microliters of enzyme diluted as necessary. The 410 nanometer absorbance change was monitored for 2 min on a Cary 219 Spectophotometer and converted to A_{410}/min.

The moles of enzyme active sites per mole of enzyme pooled from the cellulose phosphate peak were determined using the substrate 4-methylumbelliferyl-p-guanidinobenzoate (MUG-B) as described by Jameson et al. (1973). One ml assays were set up using 20 microliters of substrate, 50 to 150 microliters of enzyme, and 0.1 M barbiturate buffer, pH 8.3, added as necessary to give 1.0 ml. Blanks were included using 10 mM MES, pH 6.1, and 0.02% sodium azide in place of enzyme. Fluorescent activity was measured on a Gilson Spectro Glo Fluorometer using an excitation filter of 330-400 nanometers and an emission filter of 400 nanometers. The fluorometer was set to read 0.000 units with the barbiturate buffer and 1.000 with a 1.0 mM quinine sulfate standard in the same buffer. Enzyme activity was measured as micromolar 4-methyl coumarin generated by comparing fluorescent units generated to a standard curve of fluorescent units vs. micromolar 4-MU. The micromoles of 4-MU produced were equivalent to micromoles of HLT in the assay.

Lung Tissue

Lung tissue was obtained at autopsy. If the tissue was to be used immediately, it was refrigerated at 4°C. Material to be kept for future use was frozen at -20°C. Although some decrease in enzyme activity was noted with frozen as compared to unfrozen lung, freezing at this temperature provided a convenient method for storing large amounts of autopsied material for future use.

Enzyme Extraction

Unless specified all extraction steps were carried out at 4°C. Five hundred grams of human lung was cut into small pieces (1.0 cm^3) and

ground through a Climax meat grinder. A volume of water equivalent to the weight of the tissue was placed into a Waring blender, and the tissue was added. A short blending cycle of 15-20 sec was carried out at low speed. The homogenate was then centrifuged at 5°C, 20,000 x g for 20 min. Following centrifugation, the pellet was again placed into the blender and homogenized with 500 ml of 10 mM MES, pH 6.1, 0.15 M in NaCl, 0.02% sodium azide for 15 sec at low speed. This was followed by centrifugation as before. The blending and centrifugation steps were repeated a total of six times, until the supernatant fraction was free of hemoglobin and other soluble cellular components. The crude enzyme extract was prepared by homogenizing the pellet with 250 ml of 10 mM MES, pH 6.1, 2.0 M NaCl, 0.02% sodium azide at high speed for 1.0 min. Following homogenization, the preparation was centrifuged at 20,000 x g for 20 min. A total of three extractions were performed using the 2.0 M NaCl buffer. These supernatant fractions were pooled for further purification, and designated as "crude enzyme". The water and 0.15 M NaCl extracts contained blood and cellular proteins but only 13% of the total enzyme activity. Conversely, the 2.0 M NaCl extracts contained 87% of the extractable HLT activity but very little hemoglobin or other blood proteins.

Ammonium Sulfate Fractionation

Ammonium sulfate was slowly added to the pooled 2.0 M NaCl fractions at 4°C until a concentration of 2.0 M ammonium sulfate was reached, corresponding to approximately 45% saturation. The precipitated material was then removed by centrifugation at 5°C,

20,000 x g for 90 min. The clear supernatant was decanted and brought to room temperature before chromatography on octyl-Sepharose.

Octyl-Sepharose Chromatography

A 3.5 x 22.5 cm column of octyl-Sepharose was equilibrated with 10 mM MES, pH 6.1, 2.0 M NaCl, 0.02% sodium azide, 2.0 M ammonium sulfate. The material from ammonium sulfate fractionation (600 ml) was allowed to load by gravity onto the column at room temperature. Following this addition, the column was washed with equilibration buffer until the absorbance at 280 nanometers was less than 0.020. HLT was eluted from the column with 10 mM MES, pH 6.1, 0.02% sodium azide, 0.7 M NaCl, and 0.7 M ammonium sulfate. Fractions (9.0 ml) were collected at a flow rate of 2.0 ml min, and active fractions were pooled for subsequent purification.

Dialysis

Three hundred ml of pooled material from the Octyl-Sepharose column was concentrated and dialyzed on an Amicon PM-30 membrane, at 4°C with 10 mM MES, pH 6.1, 0.02% sodium azide, until the conductivity was equivalent to that of buffer containing 10 mM MES, pH 6.1, 0.02% sodium azide, and 0.3 M NaCl.

Cellulose Phosphate

Approximately 100 ml of dialyzed material was applied to a 1.5 x 9.8 cm column of cellulose phosphate equilibrated with 10 mM MES, pH 6.1, 0.3 M NaCl and 0.02% sodium azide. One ml fractions were collected at a flow rate of 10 ml/hr. Elution of the HLT was accomplished with a

heparin gradient (0 to 22 micromolar) in 10 mM MES, pH 6.1, 0.3 M NaCl, and 0.02% sodium azide.

Heparin-Sepharose

Heparin was efficiently removed from HLT by the use of a heparin-Sepharose column (1.5 x 15.0 cm) equilibrated with 10 mM MES, pH 6.1, 0.3 M NaCl, and 0.02% sodium azide. Material to be dehaparinized was added to the column and eluted with an NaCl gradient (0.3 to 1.0 M) in the above buffer. Four to ten ml of active HLT from the cellulose phosphate was commonly used for this column with excellent results. Active enzyme eluted from the heparin-Sepharose column was referred to as deheparinized HLT (D-HLT).

Electrophoresis

Electrophoretic analysis of reduced and unreduced SDS denatured protein was carried out on 1.5 mm, 12% polyacrylamide gels as described by Laemmli (1970), using a Bio-Rad Model 220 slab gel electrophoresis apparatus. A Bio-Rad 500 power supply was used starting at 15 mA (40 V) during stacking, changing to 25 mA constant amperage (70 V) after the tracking dye had entered the separation gel. Staining was accomplished with Coomassie brilliant blue in methanol, water and acetic acid (5:5:1). Destaining was performed using methanol, water and acetic acid in the same proportions.

M_r Determination

The apparent molecular weight of HLT was determined by chromatography on a 1.5 x 90 cm column of Fractogel TSK-HW 55 equilibrated with 10 mM MES, pH 6.1, 1.0 M NaCl, 0.02% sodium azide.

Fractions of 1.0 ml were collected at a flow rate of 15.0 ml/hr. The molecular weights of the standard proteins were plotted against their K_{avg}, and the elution position of HLT relative to the standards was used to estimate its apparent molecular weights. The proteins used to standardize the column were ferritin, aldolase and bovine serum albumin.

Log MW vs. R_f was used to ascertain the molecular weight of the two forms of the enzyme seen on 12% polyacrylamide electrophoresis gels. The protein standards used for these estimates were bovine serum albumin, ovalbumin, carbonic anhydrase, trypsinogen and trypsin inhibitor.

pH Optima

The pH optimum for HLT activity was determined by measuring the hydrolysis rate of Bz-Arg-NA in buffers of various pH. The buffers, all 50 mM, were: pH 3.0 and 4.0 sodium citrate; pH 5.0 sodium citrate-phosphate; pH 6.0 and 7.0 sodium phosphate; pH 7.0 and 7.5 Tris-phosphate; pH 8.0, 8.5, and 9.5 Tris-HCl; and pH 9.0 and 10.0 sodium carbonate. The effect of pH on HLT stability was determined by pre-incubating the enzyme in 0.25 ml of the buffers listed above for 18 hrs at 4°C prior to dilution with 2.7 ml of 0.1 M Tris-HCl, 1.0 M glycerol, pH 8.0 for assay with Bz-Arg-NA. Data were normalized with the most active samples being taken as 100% activity.

Salt Effects

The effect of salt concentration was investigated by assaying for Bz-Arg-NA activity in 0.1 M Tris-HCl buffer pH 8.0 containing 0.1 M, 0.15 M, 0.2 M, 0.3 M, 0.4 M and 0.6 M NaCl. The activity of the sample with no added NaCl was taken as 100% activity.

Amino Acid Analysis

Samples containing 0.0412 A_{280} units of protein each from the gel filtration peak, which was free of heparin, were hydrolyzed *in vacuo* for 12, 24, 48, and 72 hr with 6.0 N HCl at 110°C, followed by duplicate analyses. The values for serine and threonine were obtained by extropalation to zero time. The 72 hr values were taken for valine and isoleucine and the 24 and 48 hr data were averaged for the value of the other amino acids. The analysis of tryptophan was performed as described by Johnson (1983), after alkaline hydrolysis. Cysteine was determined as cysteic acid (Hirs, 1967). The extinction coefficient was calculated from the recovery of amino acids relative to the 280 nanometers absorbance of the sample.

Fluorography

Selected fractions from each side and the middle of the activity peak from cellulose phosphate elution were labeled with an excess of [^{3}H]-DIPF. Unbound label was removed by dialyses out and the protein was subjected to electrophoresis and stained as described above. The gel slab was submerged in "Enlightin" for 15 min and then dried under vacuum for approximately one hour. Positions of the standards were carefully marked with a pen containing [^{14}C]-toluene, and the dried slab

was placed against Kodak SB-5 film for 12 hr at -80°C. The developed film was compared with a Coomassie blue stained gel to demonstrate [^{3}H]-DIPF labeling of the stained protein bands. The apparent molecular weights of the HLT forms were estimated from a linear plot of log M_r for standard proteins vs. R_f values.

Adherence of HLT to Glass Surfaces

In order to determine if HLT adhered to glass, a dilution of active enzyme in 10 mM MES, pH 6.1, 0.1 M NaCl was placed into two sets of glass tubes. One set was mixed gently end-over-end in order to make certain that the contents of the tube came in contact with the glass surface. The other set was allowed to stand following gentle swirling. Samples were removed from the tubes at 5.0, 15.0, 20.0, and 30.0 min and assayed using the substrate Z-Lys-SBzl. The percent activity remaining was plotted against time. One hundred percent activity was assumed to be the activity of the enzyme dilution before it was added to the tubes. The identical experiment was performed using polyethylene tubes and glass tubes with 100 ul of 5.0% polyoxyethylene-23-lauryl ether (Brij 35), which is known to prevent protein adhesion to glass surfaces (Barrett, 1981).

Stabilization of HLT by Heparin

Increasing amounts of heparin were added to separate assays containing 25 micrograms (0.39 micromolar) deheparinized HLT (D-HLT) and incubations were carried out for 24 hrs at 37°C after which 20 microliters of the contents of each tube were assayed using Z-Lys-SBzl. Percent activity remaining in each assay was related to the A_{410}/min generated by a freshly made control containing D-HLT and 14.0 micromolar heparin.

Inactivation of H-HLT and D-HLT

Inactivation studies on both H-HLT and D-HLT were performed at 25°C and 37°C. Two tubes containing either H-HLT or D-HLT were incubated at each temperature. One tube in each set contained 7.0 micromolar heparin, and the other tube contained HLT with no heparin. Assays were performed on 20 microliter samples from each tube at 5, 20, 45 and 60 min using Z-Lys-SBzl substrate. One hundred percent activity was assumed to be the A_{410}/min of the sample with 7.0 micromolar heparin incubated at 25°C, since had been previously shown that 7.0 micromolar heparin would completely protect the enzyme at this temperature (see below).

Inhibition Assays

Inhibition of HLT was studied by pre-incubating various proteinase inhibitors with 8.0 micrograms of HLT for 15 min at room temperature in 1.0 ml of Tris-HCl buffer, pH 8.0. The samples were subsequently diluted to 2.85 ml and assayed using Bz-Arg-NA. Controls containing enzyme only were taken as 100% activity, and all other data were calculated relative to the controls. The inhibitors chosen and their concentrations are discussed in the results section.

Proteinase Nexin I

Inhibition studies were carried out by using increasing concentrations of PN-I against a fixed concentration of HLT. The enzyme (7.0 nM) was incubated 30 min with up to 188 nM PN-I in 0.1 M HEPES pH 7.5. After 30 min, assays were performed using the substrate Z-Lys-SBzl. The percent of activity remaining was based on a control containing only HLT.

Determination of K_m and V_{max}

The K_m and V_{max} for HLT using the substrates Z-Arg-SBzl and Z-Lys-SBzl were determined using a Lineweaver-Burke plot. Substrate concentrations ranged from 1.25 mM to 20 mM. Plots of 1/v vs. 1/[S] were then generated for the determination of K_m and V_{max}.

Purification of Fibronectin

Fibronectin was purified from human plasma by the method of Vuento and Vaheri (1979). While there were some contaminants present in our preparation, it was judged by electrophoresis to be of equal quality to commercially available fibronectin purchased from Calbiochem.

Fibronectin Digestion

Fibronectin (30 micrograms) was incubated in the presence of increasing concentrations of HLT (3.0 micrograms, 6.0 micrograms and 12 micrograms). Typical reaction mixtures contained 30 microliters of fibronectin, 50 to 200 microliters of HLT and sufficient 0.1 M Tris-HCl pH 8.1 to bring the total volume to 500 microliters. After incubation at 37°C for 24 hr, proteolysis was stopped with the addition of 50 microliters of 10% SDS. Samples were then brought to 1 ml total volume with the addition of 500 microliters of water and dialyzed against 3 changes of water at 4°C. After dialysis they were evaporated to dryness with a Buchler Evapomix. Laemmli sample buffer (50 microliters) and 10 microliters of 250 mM DTT were added and electrophoresis was carried out as described above.

Immunological procedures

Rabbit antisera to HLT was produced by intradermal injection of 200 micrograms of HLT suspended in Freund's complete adjuvant (1.0 ml total volume) at 10 sites on the animal's back. After two and four weeks the animal was given boosts containing 100 micrograms of HLT protein in Freund's incomplete adjuvant. Blood was obtained by cannulating the ear artery.

Immunodiffusion assays of the serum was performed according to Ouchterlony (1968). Each outside well contained 10 microliters of antigen. The center well contained 10 microliters of rabbit antisera to HLT. After incubation for 24 hrs at 25°C, the plate was washed in phosphate buffered saline, and precipitates were stained with Coomassie blue.

CHAPTER 4

RESULTS

Purification

Purification of HLT was performed on lung tissue which had been frozen and stored at -20°C. Storing lung tissue at this temperature had only a marginal effect on the total activity. All pulmonary tissue was judged to be grossly normal by the pathologist performing the autopsy, however, no distinction was made between smokers and nonsmokers.

Figure 7 shows the major steps in the purification scheme for HLT. The pH of 6.1 which is maintained throughout the purification procedure is the pH at which HLT is most stable and the internal pH of mast cell granules (Johnson et al., 1980). There is some solubilization of activity during the initial 0.15 M NaCl extraction steps, but the majority of the enzyme is not solubilized until the salt concentration is increased to 2.0 M.

Following the solublization of the enzyme, the extract was made 2.0 M in ammonium sulfate as required for hydrophobic chromatography on octyl-Sepharose (Fig. 8). A large amount of inactive material did not bind to the octyl-Sepharose and considerable lipid remained bound to the column after the elution of HLT. A significant amount of activity was lost using this particular column. Recently, we used a chloroform extraction as a substitute for octyl Sepharose chromatography. By using an equal volume of crude enzyme and cold chloroform, a clear extract was produced which gave a 97% yield. This chloroform extraction will require additional study but initial results are promising.

Figure 7. The major steps in the purification of human lung tryptase.

WHOLE HUMAN LUNG OBTAINED AT AUTOPSY

↓

HOMOGENIZATION IN H_2O

↓

HOMOGENIZATION IN 10 mM MES, pH 6.1,

0.15 M NaCl, 0.02% NaN_3 (X's 6)

↓

EXTRACTION WITH 10 mM MES, pH 6.1, 2 M NaCl,

0.02% NaN_3 (X's 3)

↓

AMMONIUM SULFATE FRACTIONATION

↓

OCTYL SEPHAROSE

↓

DIALYSIS

↓

CELLULOSE PHOSPHATE (HEPARIN ELUTION)

Figure 8. Elution pattern of 3.5 X 22.5 cm octyl-Sepharose column. Equilibration buffer 10 mM MES, pH 6.1, 2.0 m NaCl, 0.02% sodium azide, 2.0 m ammonium sulfate. Elution buffer same as above with 0.7 M NaCl and 0.7 M ammonium sulfate. Fractions of 9.0 ml were collected at a flow rate of 2.0 ml/min. ——, A_{280} concentration; o---o, activity. Between points A and B the column was washed with 2.0 L of equilibration buffer. Chromatography was performed at 25°C.

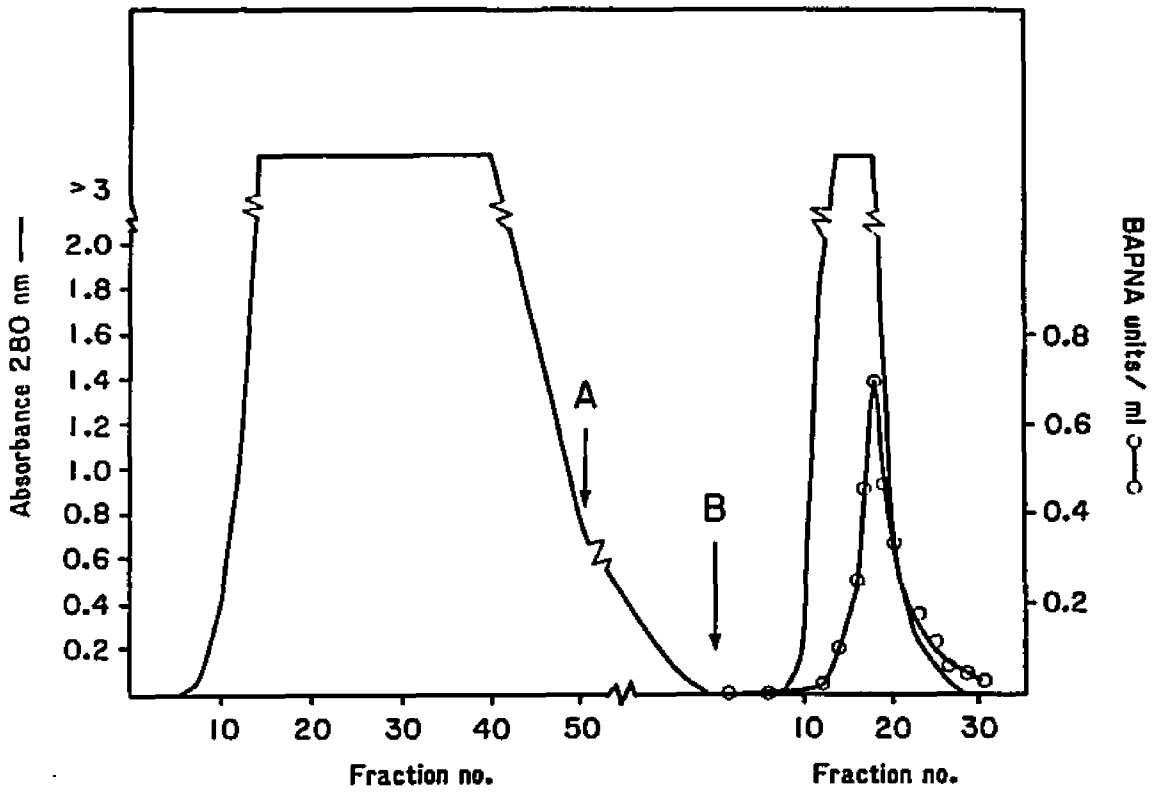
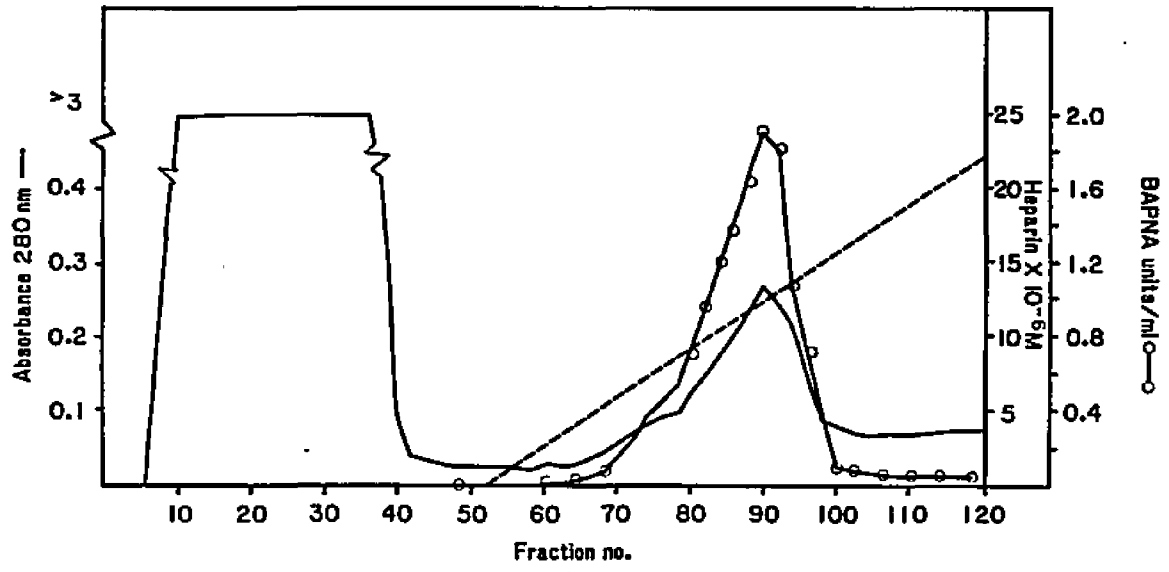

Absorbance 280 nm —
>3
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A
B
10
20
30
40
50
Fraction no.
10
20
30
Fraction no.
0.8
0.6
0.4
0.2
BAPNA units/ ml o—o

Figure 9. Elution pattern of cellulose phosphate column chromatography: —, A_{280}; o—o, activity; o---o, linear heparin gradient (0-22 micromolar, based on heparin MW of 10,000). Column was 1.5 X 9.8 cm, equilibrated with 10 mM MES, pH 6.1, 0.02% NaN_3, 0.3 M NaCl. Flow rate was 1 ml/min. Heparin gradient was made in above buffer. Chromatography was performed at 25°C.


Absorbance 280 nm
>3
0.4
0.3
0.2
0.1
25
20
15
10
5
Heparin X 10⁻⁶M
2.0
1.6
1.2
0.8
0.4
BAPNA units/ml
10
20
30
40
50
60
70
80
90
100
110
120
Fraction no.

Active fractions from the octyl-Sepharose were dialyzed to reduce the salt concentration to 0.3 M before applying to a column of cellulose phosphate (Fig. 9). Since considerable activity is lost by dialysis with membrane tubing, dialysis was performed using an Amicon concentrator with a PM-30 membrane. The material was repeatedly concentrated and diluted with 10 mM MES, pH 6.1, 0.02% NaN_3 until the conductivity matched that of 10 mM MES, pH 6.1, 0.3 M NaCl. This resulted in 70 to 80% recovery of activity. It is important to keep the enzyme in buffers of high ionic strength, because this reduces the chances of autolysis by suppressing HLT activity and decreases the tendency of the enzyme to bind to glass and plastics. The elution of HLT from cellulose phosphate with a heparin gradient demonstrates the high affinity of this enzyme for the acidic mucopolysaccharide. The HLT yield from the column was 15.8% with a concomitant 232-fold purification from the crude extract (Table III). From 500 g of lung, a total of 5.4 A_{280} units were obtained. This equals 1.92 mg of protein based on an extinction coefficient of 28.1 for a 1% solution of the HLT.

Electrophoretic Analysis and Molecular Weight Determination

Electrophoretic analysis of the enzyme preparation at each step in the purification is shown in Fig. 10. Results of reduced and unreduced samples of HLT from the cellulose phosphate reveal a single area of protein, suggesting a homogeneous preparation. Due to overload, the two molecular weight forms of HLT are not apparent on this gel, but gels with less protein as well as the fluorographs (Fig. 11) showed two bands. Additionally, some separation of the two molecular weight forms

TABLE III

Fractionation Data for Purification of HLT.

Activity based on assays with Bz-Arg-NA

Step	Vol ml.	Total A_{280}	Total Activity	Specific Activity	Purification	% Yield
Crude Enzyme	500	7950	215	0.027	1	100
Octyl-Sepharose	395	1059	51.9	0.049	1.8	14.1
Cellulose Phosphate	37	5.4	33.9	6.27	232.2	15.8

Figure 10. The results of 12% SDS-Polyacrylamide gel electrophoresis of HLT purification. Lane 1, crude enzyme; lane 2, octyl-Sepharose; lane 3, reduced HLT from pooled cellulose phosphate peak; lane 4, unreduced HLT from pooled cellulose phosphate peak; lane 5, standards. Standards are indicated by letters: A. bovine albumin (66,000); B. carbonic anhydrase (29,000); C. trypsin inhibitor (20,100); D. α-lactalbumin (14,200). Fifty micrograms of protein was loaded in each well. Gels were stained with 5:5:1 Coomassie blue in methanol, water and acetic acid.

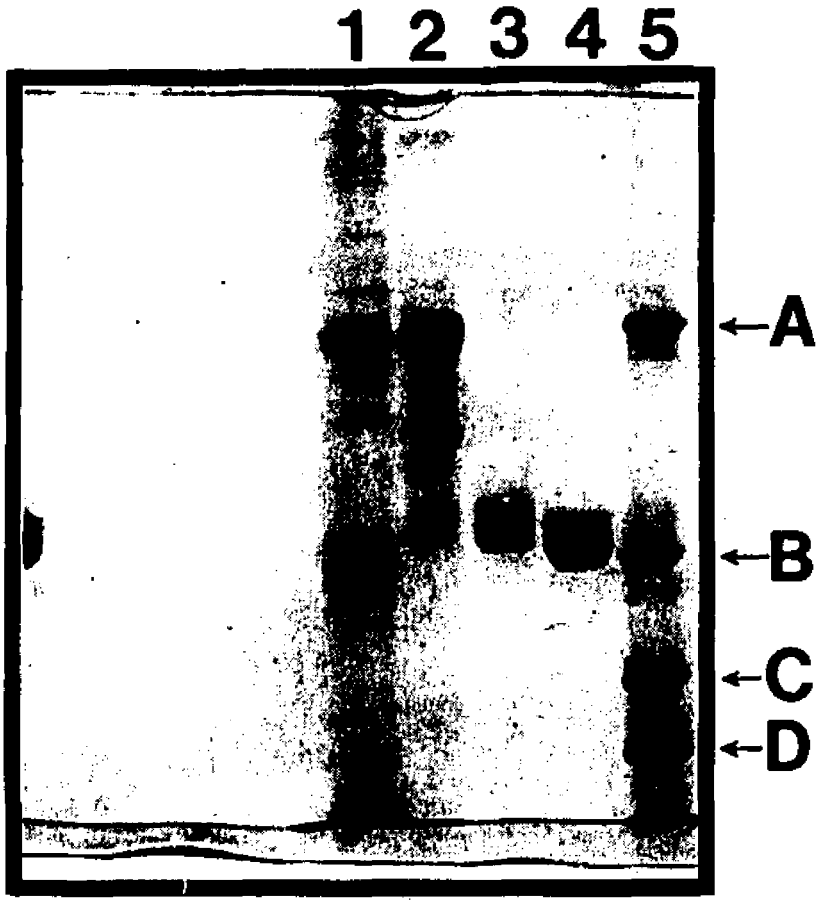
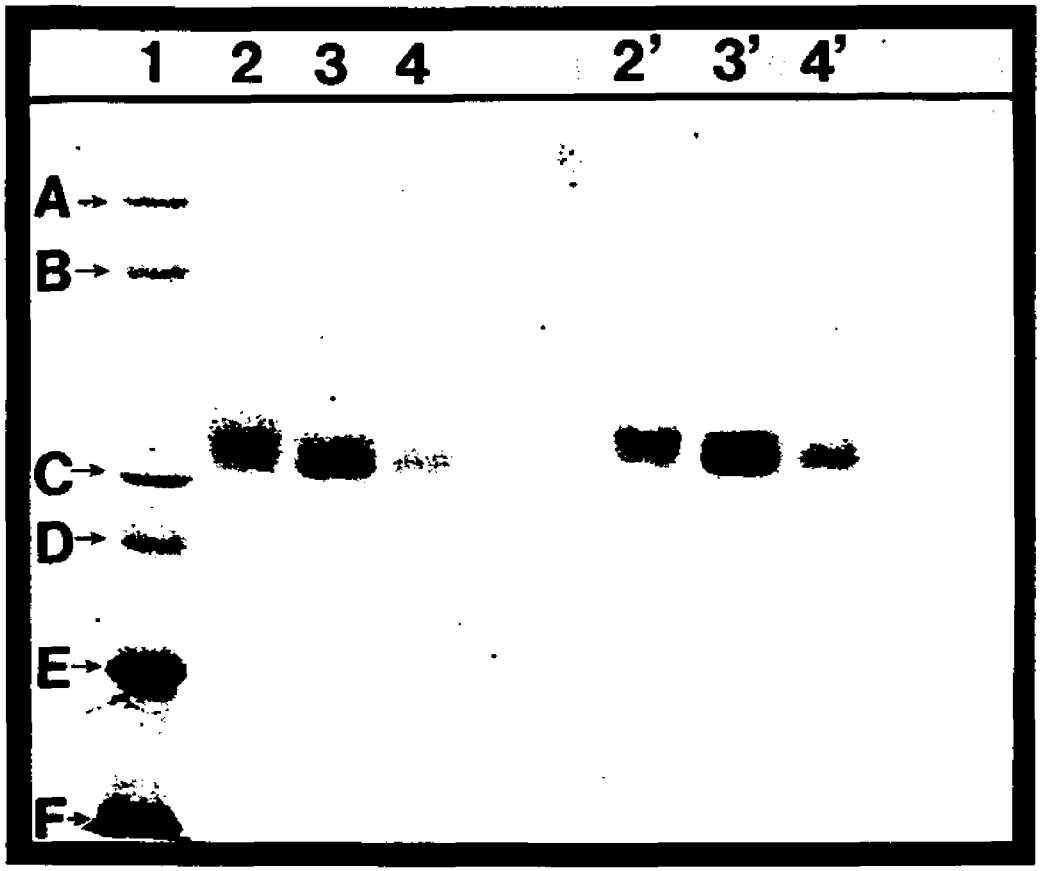

1 2 3 4 5
A
B
C
D

Figure 11. Results of 12% SDS-polyacrylamide gel electrophoresis (lanes 1, 2, 3, and 4) and fluorography (lane 2', 3', and 4') of peak and side fractions from cellulose phosphate. Lane 1, standards; lane 2 and 2', fraction #80; lane 3 and 3', fraction #85; lane 4 and 4', fraction #95. Standards, indicated by letters A to F, are bovine albumin (66,000), egg albumin (63,000), carbonic anhydrase (29,000), trypsinogen (23,000), trypsin inhibitor (20,100), and α-lactalbumin (14,200) respectively. Protein (30 micrograms) labeled with [^{3}H]-DIPF (1.9 x 10^{5}DPM) was loaded in lanes 2, 3, and 4, and lanes 2', 3', and 4' represent their respective fluorography after 12 hours at -80°C. The SDS-polyacrylamide gel electrophoresis was stained with 5:5:1 Coomassie blue in methanol, water and acetic acid.


1
2
3
4
2'
3'
4'
A
B
C
D
E
F

of HLT was accomplished during chromatography on cellulose phosphate. The front side of the cellulose phosphate activity peak was richer in the higher molecular weight form and the back side was enriched with respect to the lower molecular weight form. This separation is clearly visible in Fig. 11, lanes 2 and 4. Both species are active as evidenced by their reactivity with [^{3}H]-DIPF (Fig. 11, lanes 2' and 4'). Using log MW *vs.* R_f plots of standard proteins, the M_r values of the two species were determined to be 31,600 and 30,900 (Fig. 12).

The apparent molecular weight of HLT was estimated by gel filtration to be 135,000 daltons in buffers containing 1.0 M NaCl (Fig. 13). This apparent molecular weight may represent a tetramer, since two active species with molecular weight of 30,900 and 31,600 were observed upon SDS-polyacrylamide gel electrophoresis in the presence of reducing agents.

The percentage of active sites for the pooled cellulose phosphate peak was estimated to be 69% based on incorporation of [^{3}H]-DIPF. This is comparable to the value of 58% obtained by spectrofluorometric titration using MUG-B.

Amino Acid Analysis

The amino acid composition of HLT is given in Table IV along with that calculated from the data of Schwartz et al. (1981b) for their preparation of this enzyme from purified lung mast cells. These data, when tested for relatedness as described by Cornish-Bowden (1980) met the "strong test". The composition of anionic human trypsin (Mallory and Travis, 1973) was also compared to that of HLT, but this comparison

Figure. 12. Log MW *vs.* R_f for standards and HLT on 12% SDS polyacrylamide electrophoresis gels showing M_r of 31,600 and 30,900 for 2 species of HLT. Standards are A. bovine serum albumin (67,000), B. ovalbumin (45,000), C. carbonic anhydrase (29,000), D. trypsinogen (24,000), and E. trypsin inhibitor (20,100). Protein concentration was 20 µg/lane.

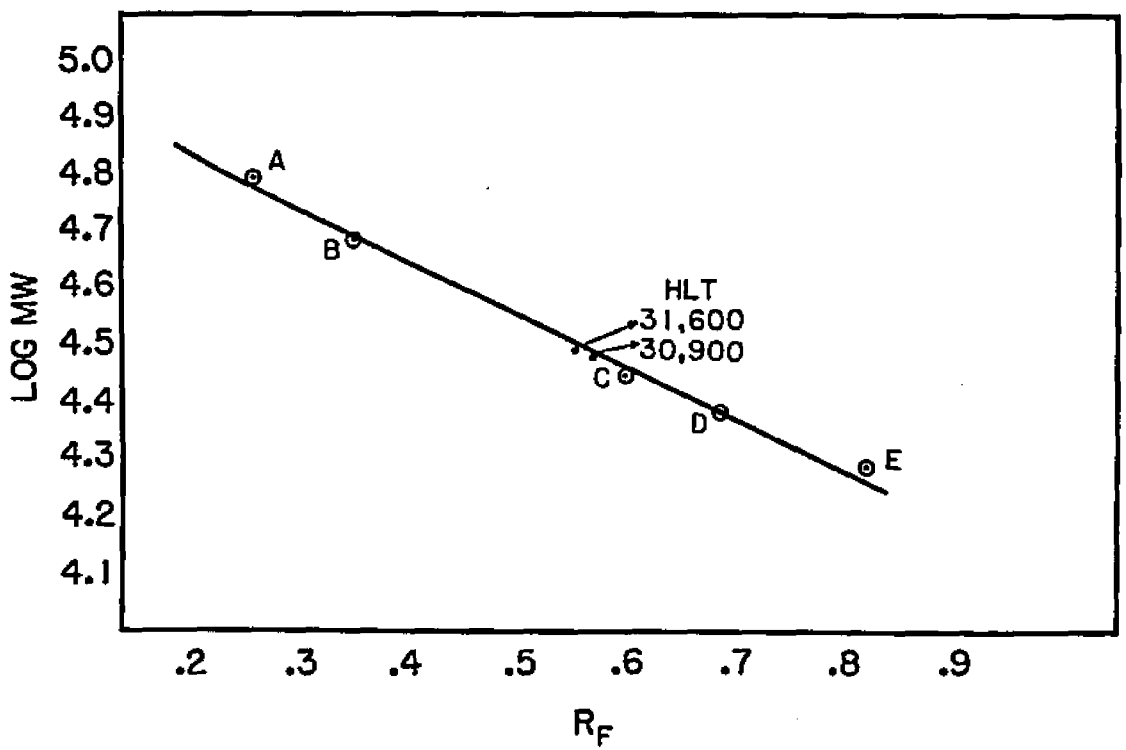

5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
LOG MW
A
B
HLT
31,600
30,900
C
D
E
.2
.3
.4
.5
.6
.7
.8
.9
R_F

Figure 13. M_r determination of HLT by gel filtration using Fractogel TSK-HW 55. Standards are A. ferritin (440,000), B. aldolase (158,000), and C. bovine serum albumin (66,000). Column size (1.5 X 90 cm). Buffer, 10 mM MES, pH 6.1, 0.02% sodium azide, 1.0 M NaCl. Fractions of 1.0 ml were collected at a flow rate of 15.0 ml/hr.

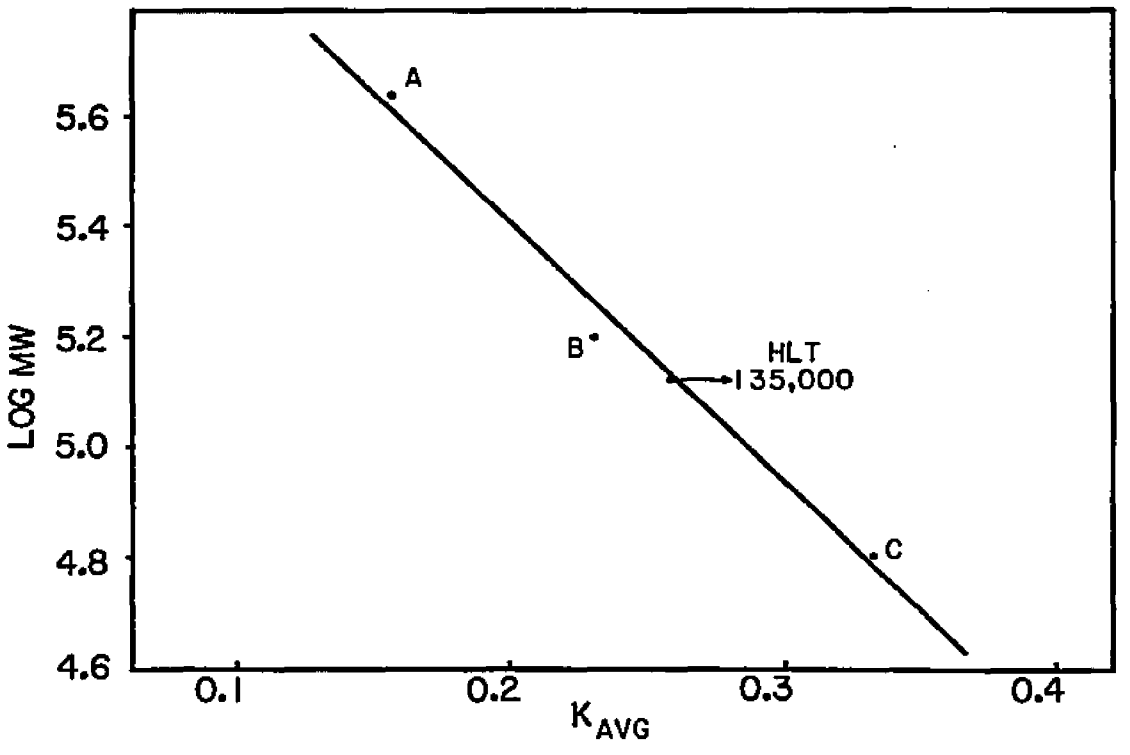

LOG MW
5.6
5.4
5.2
5.0
4.8
4.6
0.1
0.2
0.3
0.4
K_{AVG}
A
B
C
HLT
135,000

Table IV

Amino Acid Composition of HLT

Amino Acid	Value	HLT	Mast Cell Tryptase[a]	Human Anionic Trypsin[b]
Asp	25.05	25	25	21
Thr	12.63	13	11	20
Ser	13.31	13	19	24
Glu	25.74	26	30	21
Pro	25.62	27	24	9
Gly	26.60	27	30	20
Ala	16.34	16	17	13
Cys	8.60	9	1	8
Val	25.02	25	20	16
Met	4.10	4	3	1
Ile	11.94	12	8	12
Leu	25.66	26	21	12
Tyr	11.33	11	6	7
Phe	4.85	5	5	4
His	10.40	10	10	3
Lys	11.79	12	9	11
Arg	14.25	14	13	6
Trp	8.80	9	ND	3
	Total	284	252	211

[a]Data calculated from Schwartz et al. (1981a)

[b]From Malory and Travis (1973)

failed even the "weak test" for relatedness. The 280 nanometer extinction coefficient of 28.1 for a 1% protein solution was calculated from the recovery of amino acids.

pH and Salt Effects

HLT demonstrated best activity at pH 7.5 to 8.0 but was most stable at pH 5.5 - 6.1 (Fig. 14). As previously noted, high NaCl concentrations inhibited HLT activity and aided in stabilizing the enzyme during storage. At 0.15 M NaCl the enzyme was only 50% active (Fig. 15).

Adherence to Glass

The enzyme has a high affinity for glass surfaces. Polyethylene or polystyrene tubes and transfer devices and silicon treated glassware were found to be absolutely essential during the purification of HLT. As much as 50% of the activity for HLT could be lost in as little as 30 min if stored or assayed in glass. High salt concentrations were found to improve this situation somewhat.

Effects of Heparin on HLT

At 25°C the effect of heparin on HLT appeared to be dose dependent until a critical concentration was reached. At this point additional heparin failed to increase the activity of the enzyme. Deheparinized D-HLT at a concentration of 135 nanomolar required approximately 7.0 micromolar porcine heparin to reach a concentration where tryptase would remain 100% active during 1.0 hr incubations at 25°C in 0.1 M HEPES, pH 7.5 (Fig. 16). Assays using Z-Lys-SBzl were performed after the 1.0 hr preincubation as described previously. In order to see if

Figure 14. pH profile for HLT. The stability (---) plot is enzyme activity remaining after 18 hr at 4°C at the indicated pH. Assays were performed at pH 8.0, 25°C as described in Methods. The activity (——) plot is the activity of the enzyme assayed at the indicated pH. Bz-Arg-NA was the substrate.

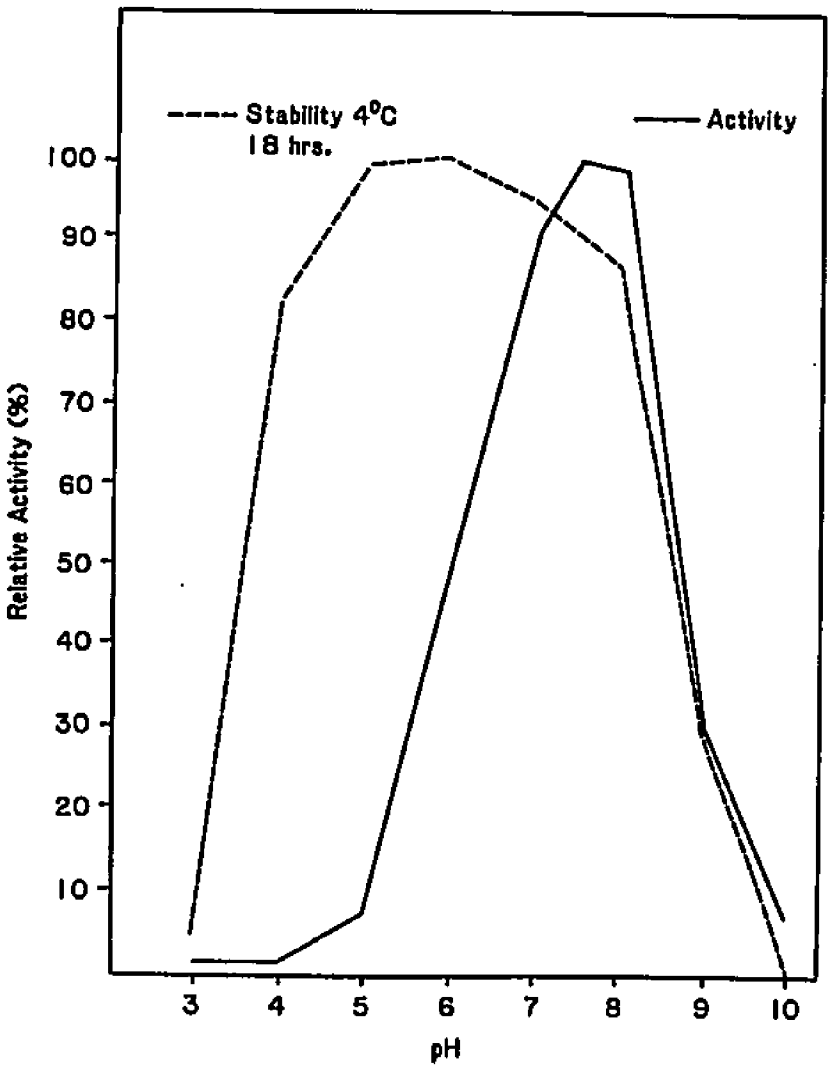
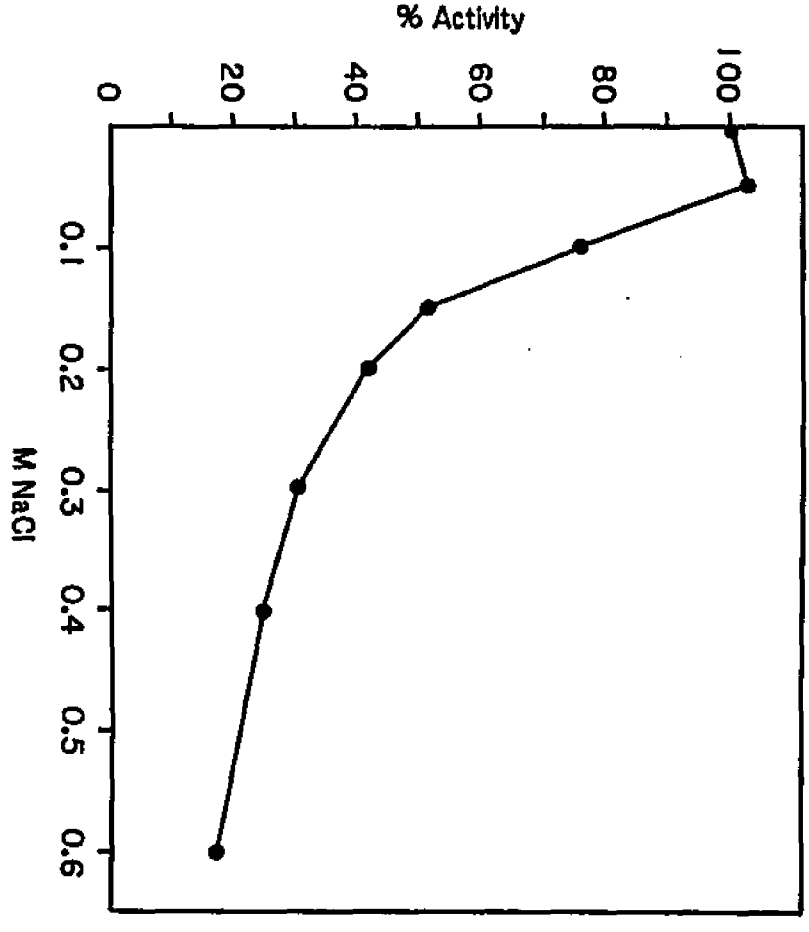

Stability 4°C
18 hrs.
Activity
Relative Activity (%)
100
90
80
70
60
50
40
30
20
10
3
4
5
6
7
8
9
10
pH

Figure 15. Effect of NaCl on human lung tryptase. Percent activity remaining vs. M NaCl. Activity was measured using the substrate Bz-Arg-NA at pH 8.0.


% Activity
0
20
40
60
80
100
M NaCl
0.1
0.2
0.3
0.4
0.5
0.6

the apparent stabilization was due totally to heparin, a total of four controls were performed with the experiment (Table V). An assay with fresh D-HLT was performed, representing 100% activity. Assays performed with 40 micromolar heparin yielded nearly 100% activity even after allowing the enzyme to preincubate 1.0 hr in the HEPES buffer. Another control in which 40 micromolar heparin was added after 30 min preincubation yielded only 81% of the initial activity. A final control containing no heparin showed only 42% activity relative to fresh D-HLT or D-HLT plus heparin at zero time after incubation for 1.0 hr.

An interesting observation was made when a solution of D-HLT containing 7.0 micromolar heparin and a solution containing only D-HLT were preincubated in 0.1 M HEPES, pH 7.5 (Fig. 17). Assays performed at 5.0, 20, 45, and 60 min demonstrated that D-HLT without heparin was rapidly inactivated at both 25 and 37°C and enzyme which had 7.0 micromolar heparin included in the solution was stable during these times. The inactivation at 25°C and 37°C did not fall below 58% and 40% of their initial values, respectively. In addition, subsequent studies performed under the same conditions for periods of up to 24 hours revealed that D-HLT activity never decreased below these values of 59 and 40 percent at 25°C and 37°C, respectively. Whether this was due to incomplete removal of heparin from the HLT by heparin-Sepharose chromatography or that one of the two forms of HLT was more heparin-dependent than the other is not clear.

Inhibitor Studies

Several inhibitors of proteolytic enzymes were tested for their effect on human lung tryptase activity, and the resulting data are

Figure 16. Stabilization of HLT by heparin. HLT (0.39 micromolar) was incubated with increasing concentrations of heparin up to 7.0 micromolar. Pre-incubations were carried out for 24 hrs at 25°C in 0.1 molar HEPES, pH 7.5. Assays were performed using the substrate Z-Lys-SBzl.

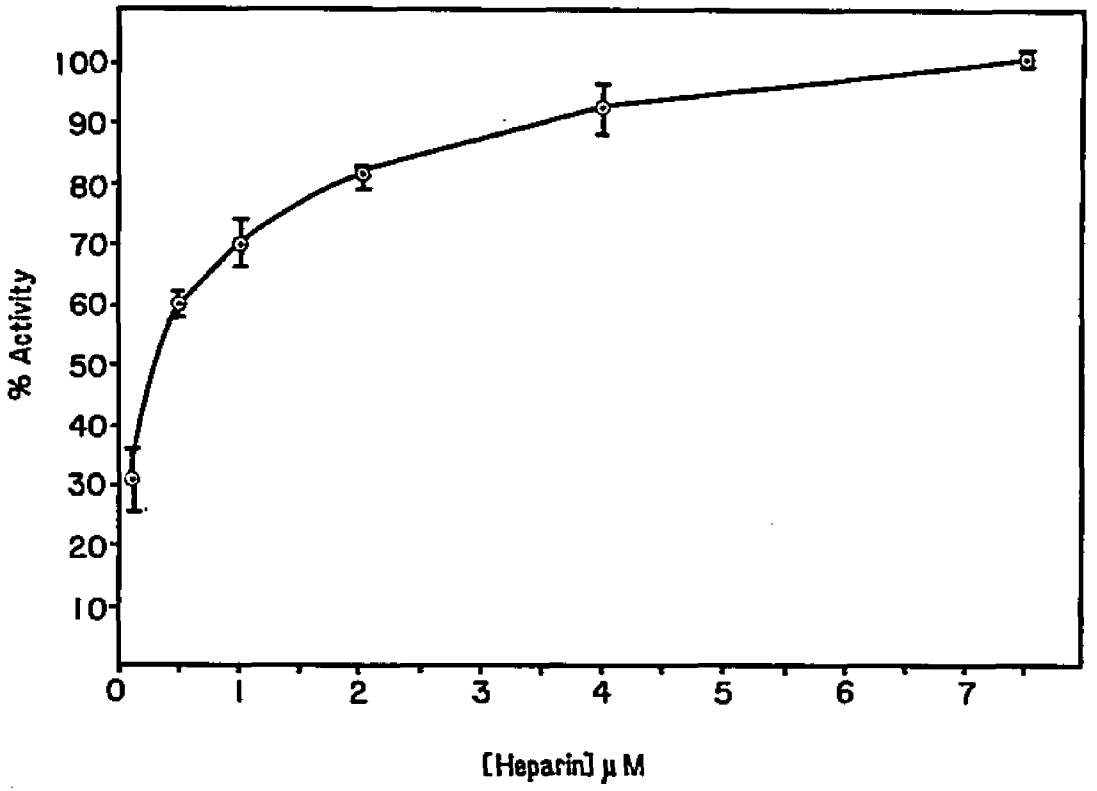
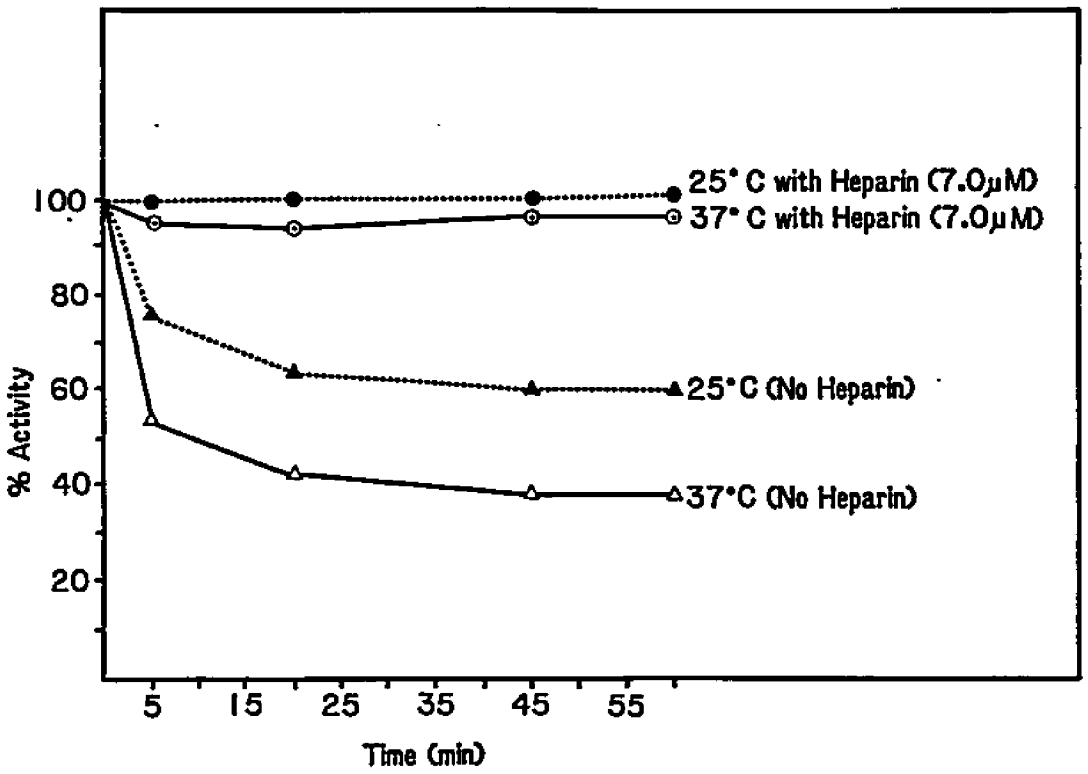

100
90
80
70
60
50
40
30
20
10
% Activity
0
1
2
3
4
5
6
7
[Heparin] μ M

TABLE V

Controls Performed With Heparin Stabilization Studies

Assay Conditions	% Activity
D-HLT + HEPES (assayed immediately)	100
D-HLT + HEPES + 40 μM Heparin, total incubation for 1.0 hr	100
D-HLT + HEPES + 40 μM Heparin added after 30 min, total incubation 1.0 hr	81
D-HLT + HEPES, total incubation 1.0 hr	42

Figure 17. Inactivation of D-HLT and stabilization with heparin. D-HLT (0.39 micromolar) was incubated with and without 7.0 micromolar heparin in 0.1 M HEPES, pH 7.5 for the indicated time periods. Assays were performed using the substrates Z-Lys-SBzl.


25° C with Heparin (7.0μM)
37° C with Heparin (7.0μM)
25°C (No Heparin)
37°C (No Heparin)
100
80
60
40
20
% Activity
5
15
25
35
45
55
Time (min)

presented in Table VI. HLT was inactivated by several inhibitors of trypsin: DIPF, TLCK, benzamidine, antipain and leupeptin. However, human serum which contains several trypsin inhibitors, as well as pure alpha-1-PI and alpha-2-macroglobulin, failed to inhibit in assays using either Bz-Arg-NA or azocasein. In addition, proteinase nexin I (PN-I), a cell secreted serine proteinase inhibitor, failed to inhibit HLT even when assay mixtures contained large excesses of PN-I. Neither of the metal ion chelators caused appreciable inactivation. Cysteine which activates thiol proteinases, and pCMB, an inhibitor of such enzymes, were also ineffectual. Calcium, an activator of some thiol proteinases, produced a 65% reduction in activity. Ovoinhibitor was the best protein inhibitor of human lung tryptase, causing a 50% reduction in activity.

Proteolysis of Fibronectin

Fibronectin obtained from the last step of purification demonstrated several lower molecular weight contaminants which were not removed for the digestion studies (Fig. 18). Electrophoretic analysis revealed that the fibronectin prepared in our laboratory was of equal quality to commercial preparations. Lane B of Figure 19 contained commercial fibronectin, and it demonstrated several minor contaminants. These contaminants were apparently due to proteolytic digestion of the 440,000 dalton protein. H-HLT digested native fibronectin into what appeared to be four major bands, but D-HLT did not cleave the protein to any significant degree. This is probably due to the instability of D-HLT during incubation and further substantiates the role of heparin in relation to the activity of the enzyme.

TABLE VI

Effect of Synthetic and Natural Inhibitors on HLT.

INHIBITOR	PRE-INCUBATION CONCENTRATION	% ACTIVITY*
None	0	100
DIPF	2 mM	0
TLCK	2 mM	7.9
TPCK	2 mM	70.4
Benzamidine	2 mM	4.5
EDTA	2 mM	87.5
1,10-phenanthroline	2 mM	80.9
p-CMB	2 mM	86.8
Cys	2 mM	95.7
Ca^{+2}	20 mM	35.1
Alpha-1-PI	0.1 mg/ml	100.5
Alpha-2-macroglobulin**	0.1 mg/ml	100
Bronchial Inhibitor	0.1 mg/ml	77.2
Human Plasma (EDTA)	2%	62.1
Human Plasma (Heparin)	2%	99.9
Trasylol	0.1 mg/ml	84.9
Ovoinhibitor	0.1 mg/ml	51.3
Soybean Trypsin Inhibitor	0.1 mg/ml	100.5

*Assays performed using Bz-Arg-NA

**Alpha-2-macroglobulin inhibition was assayed using azocasein as the substrate

TABLE VI

(Cont'd.)

Effect of Synthetic and Natural Inhibitors on HLT.

INHIBITOR	PRE-INCUBATION CONCENTRATION	% ACTIVITY*
Lima Bean Inhibitor	0.1 mg/ml	103.0
Ovomucoid	0.1 mg/ml	82.5
Antipain	0.1 mg/ml	0
Chymostatin	0.1 mg/ml	93.7
Elastinal	0.1 mg/ml	97.1
Leupeptin	0.1 mg/ml	0.9
Pepstatin	0.1 mg/ml	96.4
Phosphoramidon	0.1 mg/ml	93.2

*Assays performed using Bz-Arg-NA

**Alpha-2-macroglobulin inhibition was assayed using azocasein as the substrate

Figure 18. SDS polyacrylamide gel electrophoresis (6% gels) of selected fractions from the purification of fibronectin. Samples were reduced with DTT prior to electrophoresis. Arrow ➞ indicate fibronectin at M_r 220,000. Gels were stained using Coomassie blue in methanol, water and acetic acid 5:5:1. Thirty µg of protein was added to each well.

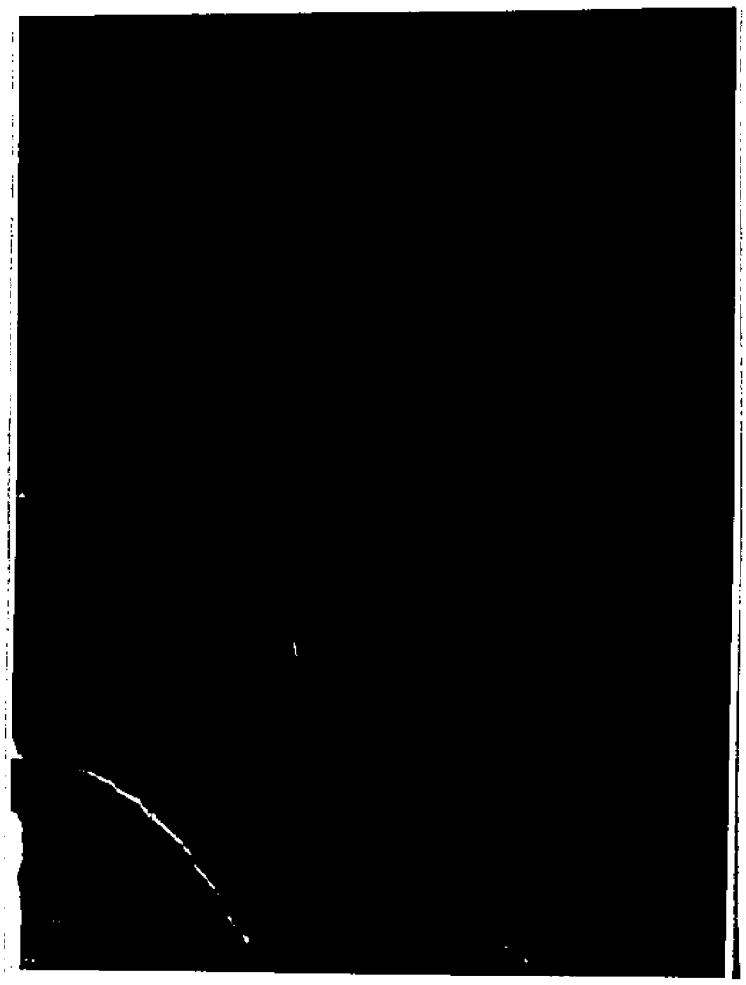
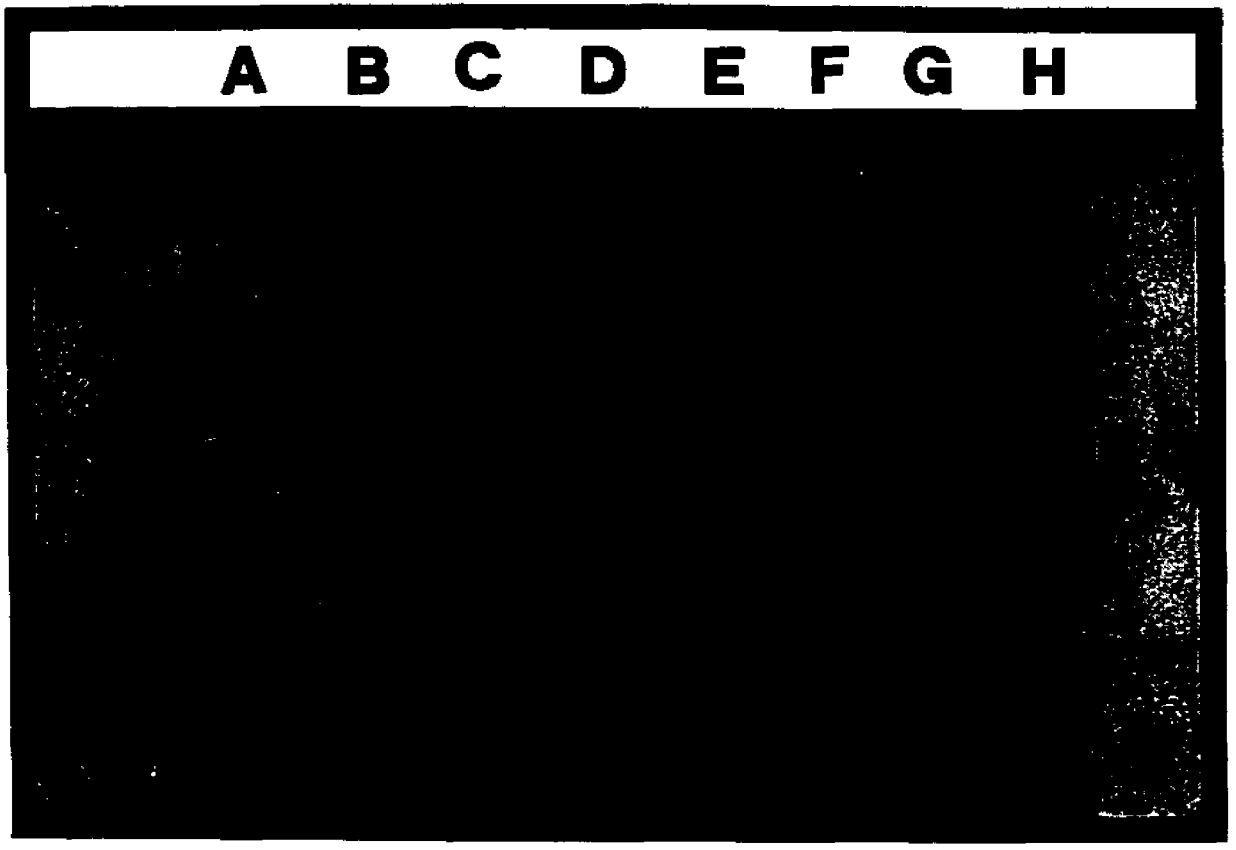


Figure 19. SDS electrophoresis of fibronectin and digestion products. Lane A contains myosin (200,000). Lane B contains commercial fibronectin. Lane C, D and E contain H-HLT:fibronectin 1:10, 1:20 and 1:40 respectively. Lane F, G and F contain the same ratio of D-HLT:fibronectin. Gels are 6% SDS polyacrylamide and samples were reduced. The standard in A is unreduced.


A B C D E F G H

Immunodiffusion

Two forms of human lung tryptase were compared by immunodiffusion of each enzyme form from the front of the cellulose phosphate peak and the back side of the peak against antisera raised against a mixture of forms (Fig. 20). A precipitin arc indicating identity was obtained. The predominant form in well A was the 31,600 M_r species while well E contained predominantly the 30,900 M_r species.

Enzyme Kinetics

The K_m, V_{max} and k_{cat} for H-HLT using the substrates Z-Lys-SBzl and Z-Arg-SBzl were determined. Tryptase apparently has a preference for the Arg containing substrate, since the k_{cat} for the Arg substrate was 610/sec and for the Lys substituted material was 210/sec. H-HLT used to determine these kinetic parameters was judged to be 43% active by titration with MUG-B. The results of all of the kinetic studies are indicated in Figure 21. Data obtained by using Eaddie-Hostee or Haynes plots were essentially the same as with the Lineweaver-Burke plots.

Figure 20. Ouchterlony reaction with anti-HLT vs. selected fractions from active peak off cellulose phosphate. Center well contained rabbit antisera to HLT and wells A to E were loaded with 10 microliters each of fractions 75, 80, 85, 90 and 95 respectively (see Fig. 9)

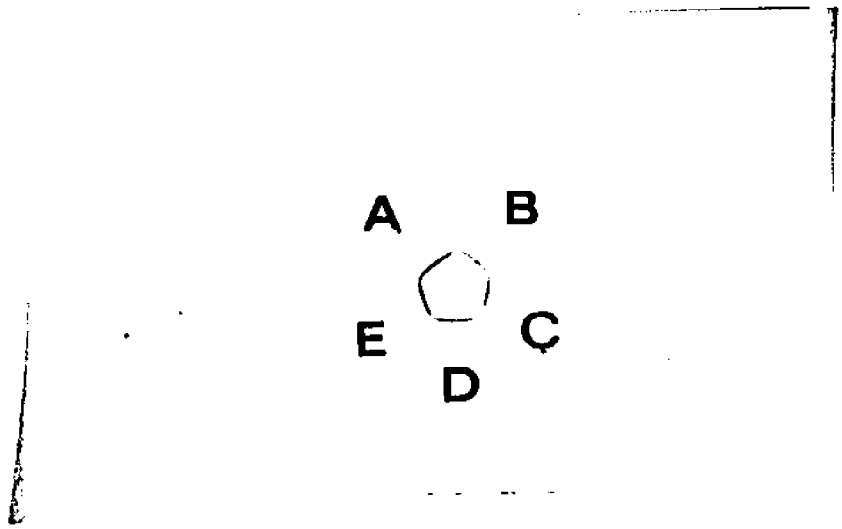
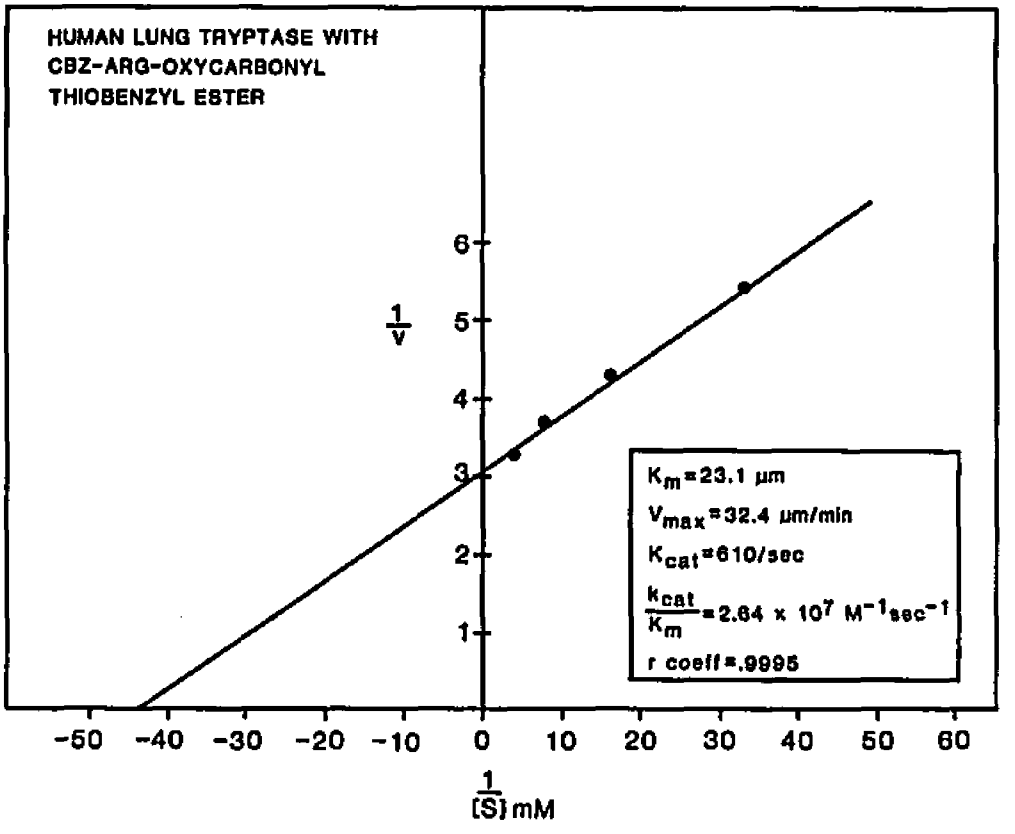
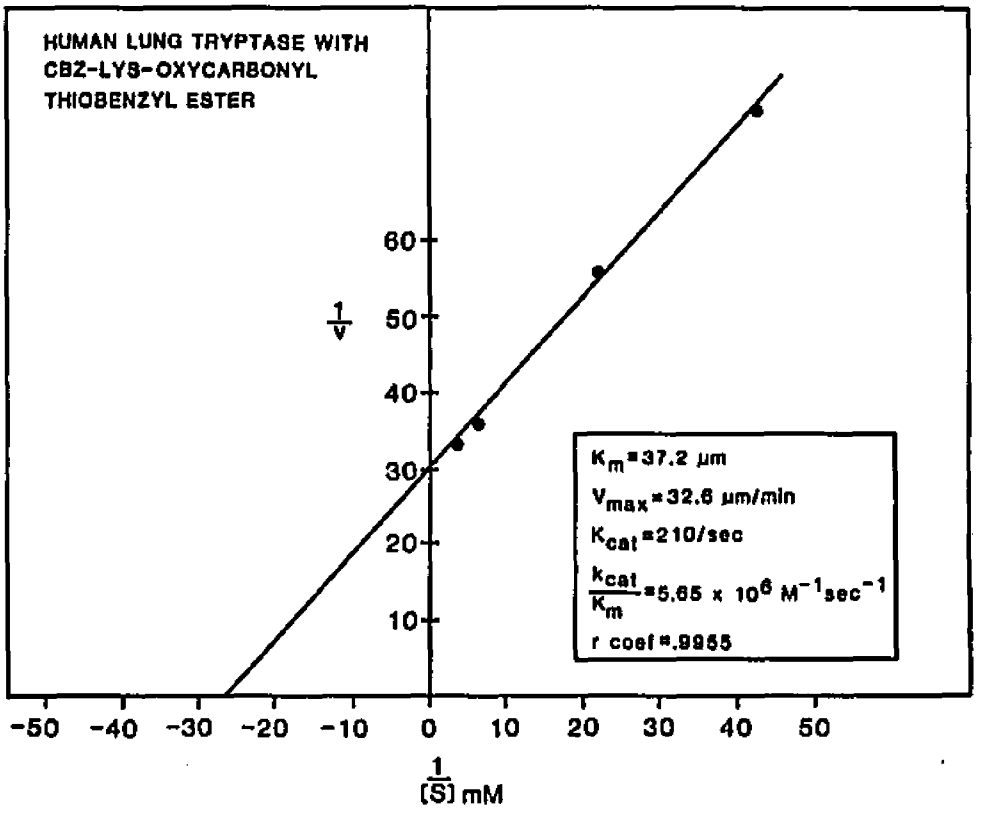

A
B
E
C
D

Figure 21. Lineweaver-Burke plots for HLT using the substrates Z-Lys-SBzl and Z-Arg-SBzl. Assays were performed at 25°C in the HEPES buffer pH 7.5 as described in Methods. Inserts contain essential kinetic data.


HUMAN LUNG TRYPTASE WITH
CBZ-ARG-OXYCARBONYL
THIOBENZYL ESTER
$\frac{1}{V}$
6
5
4
3
2
1
K_m=23.1 µm
V_{max}=32.4 µm/min
K_{cat}=610/sec
$\frac{k_{cat}}{K_m}$=2.64 x 10^7 $M^{-1}sec^{-1}$
r coeff=.9995
-50 -40 -30 -20 -10 0 10 20 30 40 50 60
$\frac{1}{[S]}$ mM


HUMAN LUNG TRYPTASE WITH
CBZ-LYS-OXYCARBONYL
THIOBENZYL ESTER
$\frac{1}{V}$
60
50
40
30
20
10
K_m=37.2 µm
V_{max}=32.6 µm/min
K_{cat}=210/sec
$\frac{k_{cat}}{K_m}$=5.65 x 10^6 $M^{-1}sec^{-1}$
r coef=.9955
-50 -40 -30 -20 -10 0 10 20 30 40 50
$\frac{1}{[S]}$ mM

CHAPTER 5

DISCUSSION

Nomenclature

The names chosen for tissue proteinases often vary and can cause considerable confusion. Initially most of these enzymes were called cathepsins, but additional information on their properties made this term somewhat obsolete. Tissue derived neutral serine proteinases with trypsin-like specificity have been called tryptase, while chymase has been used for chymotrypsin-like enzymes. Although we have called the enzyme in this study human lung tryptase, present data indicate that human lung mast cell tryptase may be a more appropriate name. Better terminology will probably result from future studies on the enzyme's cellular location and natural substrate specificity.

Purification

The isolation of HLT by the method described is superior to that outlined by Schwartz et al. (1981a) for several reasons. It is less complicated, employs a more available source, has higher yields and provides partial separation of the two enzyme forms. The purification of mast cells for enzyme extraction by previous methods is an involved process requiring considerable time, also the tissue used for such mast cell preparations must be obtained fresh from surgery. This presents an inconvenience, and due to the amount of tissue available, yields are much lower using the previously described procedure.

The use of a chloroform extraction following the 2.0 M NaCl solubilization of the enzyme may eventually replace the ammonium sulfate fractionation and chromatography on octyl-Sepharose. Initial studies using chloroform extraction suggest that it will simplify the process and raise yields to about 90%. There was a small amount of contaminant noted following cellulose phosphate chromatography when using chloroform extraction, but this should be a minor problem compared to the losses incurred with octyl-Sepharose chromatography.

The cellulose phosphate chromatography with subsequent elution by heparin gradient is an extremely powerful tool in the enzyme purification procedure. Nearly a 230-fold purification is realized through the use of this chromatography matrix. In excess of 1.0 gram of contaminating protein does not adhere to the column during loading, and elution with a porcine heparin gradient is both convenient and specific for the removal of HLT. Furthermore, elution with heparin provides a method whereby HLT is immediately stabilized. The H-HLT can be pooled and frozen for extended periods at -20°C without significant loss of activity.

The specific activity of purified HLT was 101 using the trypsin substrate called TAMe. This compared favorably to specific activities of 78 and 98 obtained by Schwartz et al. (1981a). These authors also reported only 38% activity in the enzyme following purification, based on [^{3}H]-DIPF incorporation. Similar studies with HLT from our laboratory revealed 69% activity. Using active site titration with the substrate MUG-B, we judged our preparation to be 58% and 43% active for separate preparations. That our enzyme should be more active can be

explained by the fact that the heparin from the porcine heparin gradient stabilizes the enzyme. The purification scheme of Schwartz et al. (1981a) involves the use of heparin-Sepharose chromatography, and this produces deheparinized HLT which is rapidly inactivated at room temperature.

Attempts to use anti-HLT affinity chromatography (data not shown) were largely unsuccessful; however, antibody affinity columns were not employed after the role of heparin in the purification was elucidated. Preliminary studies revealed that nearly 100% of the activity applied to the column would bind to the affinity matrix, but the harshness of the elution procedure would not allow an acceptable quantity of active enzyme to be eluted. We were able to remove a small amount of activity from anti-HLT-Sepharose using 3.0 M sodium thiocyanate in 5 mM phosphate buffer, pH 7.0. Considerable activity was lost, but whether this was due to the absence of heparin or to the concentration of NaCNS was never established. Tests were performed which demonstrated a decrease in activity of HLT with increasing concentrations of NaCNS, and control levels remained at 100%. However, since no heparin was added to the elution, it is difficult to say if the omission of the acid mucopolysaccharide may have resulted in a loss of activity. The use of antibody affinity chromatography in the purification of HLT should be investigated again in light of what we know concerning the enzyme's dependency on heparin.

Several buffering systems were investigated in conjunction with HLT in hopes that the best conditions possible might be maintained during purification and characterization. The 10 mM MES, pH 6.1, 0.02% sodium

azide with at least 0.3 M NaCl provides the best condition for isolation and storage of the enzyme. Either 0.1 M HEPES, pH 7.5 or 0.1 M Tris-HCl, pH 8.0, are suitable buffers to be employed while monitoring activity . Assays performed on material stored for 4.0 hr at 37°C showed that storage in MES buffer yielded between 15 and 25% more activity than enzyme stored in MOPS, TRIS, or NaH_2PO_4.

The tryptases of human tissues may all be of mast cell origin (Tanaka et al., 1983). Whether the same or similar purification procedure could be used to isolate tryptase from other sources would be of interest. At any rate, the use of whole tissue obtained at autopsy in preference to purified mast cell preparations has yielded more enzyme and lacks the inconvenience of obtaining fresh tissue from surgery.

This purification procedure has been performed approximately 12 times with similar results. Although the amounts of enzyme found varied only slightly, one lung yielded 10 times the normal level of activity. The reason for this increase could not be explained.

Amino Acid Analysis

Performing the amino acid analysis made it possible to compare the tryptase from whole human lung with that reported by Schwartz et al. (1981a), and it is clear from the data that the two enzymes are the same. The data that we provide are more accurate, because we were able to analyze samples hydrolyzed for various times and apply better methods for the analysis of cysteine and tryptophan. HLT did not appear to be related to human pancreatic trypsin which supports the suggestion of Woodbury et al. (1981) that the mammalian granulocyte serine proteinases form an evolutionarily distinct class of serine proteinases. The 280

nanometer extinction coefficient of 28.1 for a 1% solution may seem high, but this value is consistent with a high tryptophan content (9 residues/mol.)

Molecular Weight and Quarternary Structure

Apparent molecular weight values estimated by gel filtration and SDS polyacrylamide gel electrohporesis were slightly lower than reported by Schwartz et al. (1981a). Three determinations of molecular weight in our laboratory revealed a M_r of 135,000 daltons. The possibility that HLT exists as a tetramer is suggested by the separation of two forms with M_r = 30.9 and 31.6 kd. The partial separation of the two subunit forms on cellulose phosphate suggests the existence of two different tetramers, one containing small subunits and one large subunits. Gel filtration separation of two tetramers with an M_r difference of 2800 would be very difficult, explaining the observation of only one peak on gel filtration. Another explanation of these data is that the tetramer is an artifact resulting from the self-association of monomeric enzyme in 1.0 M NaCl. Since increasing amounts of NaCl reduce the activity of the enzyme, such an association seems possible. Gel filtration studies in low salt were not possible, because the enzyme binds to glass and plastics in the absence of salt. Lastly, there may be two species of mast cells in the humans similar to the "typical" and "atypical" mast cells of rats (Woodbury et al., 1981). Each of these mast cell types may contain a different species of tryptase just as the rat has two different chymases (RMCP I and RMCP II). Since lung contains both connective and mucosal tissue, the two human lung tryptase forms may be products of different cell types, making this an attractive explanation.

Inhibition

Inhibition studies clearly prove that HLT is a trypsin-like serine proteinase. Interestingly, whole plasma, pure alpha-1-proteinase inhibitor, and pure alpha-2-macroglobulin all failed to inhibit HLT. These data contradict the conclusion of Benitez-Bribiesca et al. (1973), who found alpha-1-proteinase inhibitor associated with mast cells and proposed regulation of mast cell tryptase by this inhibitor. The failure of plasma and pure alpha-2-macroglobulin to inhibit HLT suggests that the enzyme may be too large to be "trapped" by alpha-2-macroglobulin (Barrett and Starkey, 1973). These findings pose a question concerning the regulation of HLT activity. Although subcellular location of intracellular inhibitors may serve to control its activity within the cell, there seems to be no vascular inhibitor for controlling HLT, which has been shown to be released upon anaphylatic degranulation of mast cells (Schwartz et al., 1981b). Thus, it would appear that extracellular HLT may be free to attack tissue proteins or hydrolyze other substrates following mast cell degranulation.

Effects of NaCl Concentration on HLT

One distinguishing feature of HLT is decreased activity with increasing NaCl concentration, an effect also observed with a proteinase from human uterine cervix (Ito et al., 1980). In this respect HLT seems quite different from the rat mast cell chymases (Woodbury et al., 1981). Also, other proteinases such as the elastase from the granule of the polymorphonuclear leukocyte are assayed in buffers containing 1.0 M NaCl (Barrett, 1981).

The Role of Heparin

HLT's affinity for heparin not only provides a convenient tool for purification of the enzyme, but this association also creates an excellent method for stabilization of the enzyme while assaying for activity. While 7.0 micromolar heparin was necessary to protect and stabilize 0.39 micromolar HLT (nearly a 20-fold molar excess), it must be remembered that the concentration of heparin was calculated based on a M_r = 10,000 for commercial heparin from porcine intestinal mucosa. The same heparin sulfate is not found in humans and the molecular weight of heparin from different sources and species is known to be quite variable. Additionally, all heparin molecules do not bind to and activate antithrombin III and a similar situation may exist concerning HLT. Therefore, making any assumptions about the concentration of heparin involved in binding to HLT should be done with caution because of the problem in obtaining an exact molecular weight for heparin.

The mast cell granules are rich in heparin, and HLT has been localized HLT to these granules (Smith et al., 1984). It is likely that HLT exists in complex with the acid mucopolysaccharide *in vivo*. The present study substantiates the existence of such an association. If heparin is removed from purified HLT using heparin-Sepharose chromatography, the enzyme rapidly loses activity. Interestingly, this inactivation process does not go to completion. Approximately 40% of the enzyme activity remains even after extended incubations. It is possible that one of the subunits of HLT, either the 30.9 kd or the 31.6 kd species, may be more dependent on heparin for complete expression of activity. If this is true then only partial inactivation of D-HLT would

be possible. Another reason for this might be incomplete removal of heparin from H-HLT with heparin-Sepharose chromatography. In this case, residual heparin may be protecting the enzyme from complete inactivation.

Since 1.0 M NaCl is required to disrupt the binding of HLT to heparin one would expect that HLT released from degranulating mast cells would be bound to heparin which is released with HLT. The presence of heparin and the slightly acidic conditions of the mast cell granule may control and/or stabilize the enzyme in vivo. The complete inactivation of the enzyme by heparin and pH as proposed by Schwartz and Austen (1984) does not seem probable, since the enzyme is not in zymogen form and our studies indicate that HLT is 50% active at pH 6.1 in vitro. On the other hand, HLT has been shown to be released upon mast cell degranulation (Schwartz et al., 1981b). If the enzyme is complexed with heparin, then it may be active on surrounding tissue. The biological activity of HLT is not understood. There is some evidence to support the involvement of HLT in an intracellular role, but an equal amount of information would indicate that it functions extracellularly.

The absence of zymogen forms and the histochemical evidence of active enzyme within the cell argue that HLT is active inside the mast cell. If the physiological assignment of the enzyme is intracellular, then it seems possible that HLT might function in limited or complete proteolysis of endocytized material. If the proteolysis is limited, the enzyme might serve to create smaller peptides such as chemotactic factors which are active in anaphylaxis. Since most endocytized substances migrate to the mast cell granule, it is tempting to speculate

that HLT does have such a function. IgE is endocytized by the mast cell and can be found in contact with the mast cell granule in as little as 1.0 hr being endocytized. We have performed studies involving the digestion of IgE with HLT (Smith and Johnson, 1984), but found that HLT did not cleave the immunoglobulin, however, it may be possible that proteolysis did take place and was not readily apparent due to the type of electrophoretic analysis which was used. The question of whether HLT will cleave IgE or not deserves additional attention and experiments designed to look specifically for small peptides should be included. It has been shown that following mast cell degranulation there are two stages of pathology. First, the classic allergic reaction appears within minutes and subsides in 30-60 min. Four to eight hr later a second phase reaction which lasts for periods of up to 24 hr takes place. This has been termed late phase reaction (LPR) (Kaliner and Lemanske, 1984). LPR presents clinically as burning, ill-defined erythema and swelling and may be associated with the hyperactivity seen in the airways during hypersensitivity.

Part of the mast cell granule matrix contains inflammatory-provoking activity which is contained in the high salt extracts of mast cell granule preparations (Tannenbaum et al., 1980). The inflammatory factor of anaphylaxis (IF-A) is a 1400 dalton peptide (Oertel et al., 1980). HLT could be responsible for generating such a peptide from a larger precursor.

Heparinized HLT is capable of digesting fibronectin, whereas HLT with heparin removed is unable to cause significant proteolysis of the protein. This not only confirms heparin's involvement with HLT, but

also makes it tempting to speculate that degranulation of mast cells may release active enzyme capable of degrading surrounding tissue or protein. Since we have shown that HLT is not inhibited by any known plasma proteinase inhibitor, it is possible that active tryptase could be found within the interstitium and generalized or specific proteolysis might follow degranulation.

It has been shown that mast cells are found in increased numbers around new tumor sites before the in growth of new blood vessels (Kessler et al., 1976), and that angiogenesis can be stimulated by heparin (Taylor and Folkman, 1982). Interestingly, when heparin and cortisone are administered together blood vessel growth in new tumors stops (Folkman et al., 1983). The authors present an optimum heparin dose needed for this effect and suggest that heparin could function as both a promoter and inhibitor of angiogenesis. In the presence of cortisone only the latter function would be demonstrated. Heparin may be a stabilizing factor for HLT or another similar proteinase involved in tumor growth, since proteolysis is an essential part of establishing vasculature for new tumors.

Enzyme Kinetics

In comparing HLT's activity using two thioester substrates, Z-Lys-SBzl and Z-Arg-SBzl, it appears that the enzyme prefers the Arg substitution. A k_{cat}/K_m of 2.64×10^7 M^{-1} sec^{-1} makes the enzyme more active on this substrate than for any substrate thus far reported. Tanaka et al., (1983) tested 17 amino acid dipeptide thiolesters and 14 tripeptide 4-nitroanilide as substrates HLT. The authors reported k_{cat}/K_m ranges from 10^2 to 10^5, but these assays were performed on HLT

devoid of protecting levels of heparin. It is likely that the values quoted are considerably lower than what they actually would have been if H-HLT had been used. For this reason a k_{cat} = 610/sec and k_{cat}/K_m in the range of 10^7 may represent the most accurate kinetic data yet reported on the tryptase from lung mast cells. The best substrate for HLT is probably the one recommended by Tanaka et al. (1983), Z-Trp-Arg-SBzl. A re-investigation of K_m, k_{cat} and k_{cat}/K_m using this thioester substrate with H-HLT may be appropriate.

OVERVIEW

Since the discovery of the mast cell by Paul Erlich nearly 100 years ago, efforts to assign a biological function to the cell have eluded researchers. For nearly 75 years investigations centering on this fascinating cell were based largely on its staining characteristics. The discovery of the chymotrypsin-like activity of the rat mast cell by Gomori (1953) was a product of his work with different mast cell staining techniques. Subsequently, Mota and da Silva (1960) isolated rat peritoneal mast cells from sensitized animals, exposed the cells to antigen and witnessed degranulation, and Glenner and Cohen (1960) found trypsin-like activity associated with human mast cells, launching a new era of mast cell research. The literature reflects this new interest. Scores of articles have been published on the biochemistry of degranulation, the structure and function of the chemical mediators of mast cells and the possible function of this cell.

The work presented here offers a significant contribution to the understanding of the biochemistry of the mast cell. We have successfully isolated lung mast cell tryptase by a method which gives a superior yield to any other method used to date. This has given us the potential to carry out experiments on the enzyme such as a complete amino acid analysis, electrophoretic analysis, molecular weight determination, and other physical and kinetic studies.

Aside from the purification of human lung tryptase, data which are particularly useful in understanding mast cell function are the stabilization of the enzyme by heparin, the proteolytic digestion of fibronectin by H-HLT and the determination of k_{cat} for the enzyme. In addition, the antibody raised to HLT will be extremely valuable in the localization of the enzyme and in creating assays which are sensitive as well as specific for the measurement of HLT in tissues.

Future investigations will involve separating the two species of enzyme and generating antibody to each species. This may be used to determine whether or not two separate species of human lung tryptase exist within two different mast cell types, analogous to the situation with RMCPI and II in rat. When the two forms are successfully separated, comparisons involving their inactivation and dependency on heparin may be completed.

Plans also include the development of an enzyme-linked immunosorbent assay (ELISA) which will make it possible to monitor levels of HLT in various tissues. Testing serum or tissue levels for the presence of HLT during pathological conditions such as tumorogenesis and chronic inflammatory conditions would be of special interest. These

conditions are known to cause increases in the number of mast cells. Perhaps the circulating level of HLT would be increased, since there are no known serum inhibitors of the proteinase.

Tanaka et al. (1983) have provided extensive kinetic data on human lung tryptase using various substrates. These data may not be accurate, since the role of heparin in relation to HLT was not known at the time these studies were performed. Our kinetic data more accurately reflect the true activity of HLT. In addition, human lung tryptase may be found to be more closely related to the other tissue proteinases from a kinetic standpoint. For this reason kinetic studies using H-HLT are planned. The results of these studies will be very important in understanding the function of the mast cell tryptase.

While fibronectin was successfully cleaved by H-HLT, extensive studies such as those performed by Vartio et al. (1981) using the chymase from rat mast cells need to be performed. A comparison between digestion products from HLT proteolysis and those from chymase digestion can then be made. This would allow some comparison between species. Comparison with digestion products of trypsin would also be of value.

As with any newly isolated enzyme, there are countless experiments which would be of value in understanding the function of human lung tryptase. The amount of this enzyme, (nearly one-fourth of the total protein of the mast cell) tends to make one believe that its function is not of minor importance in the overall activity of the mast cell. Hopefully, the present work has laid a firm foundation for future studies on the function of HLT.

BIBLIOGRAPHY

BIBLIOGRAPHY

Aoyagi, T., and Umezawa, H. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D., and Shaw, E., eds) pp. 429-454, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Barrett, A. J. (1977) in Proteinases in Mammalian Cells and Tissues (Barrett, A. J., ed) pp. 181-208, North Holland Publishing Co., Amsterdam, New York, Oxford

Barrett, A. J. (1981) Methods Enzymol. 80, 581-588

Barrett, A. J. (1982) Personal Communication

Barrett, A. J., and Kirschke, H. (1980) in Methods Enzymol., 80 pp. 535-561, Academic Press, NY

Benitez-Bribiesca, L., Freyre, R., and De La Vega, G. (1973) Life Sci. 13, 631-638

Birkedal-Hansen, H., Cobb, C., Taylor, R., and Fuller, H. (1976) Biochem. Biophys ACTA 438, 273-286

Blackburn, S. (1976) in Enzyme Structure and Function pp. 11-96 Marcel Dekker, NY

Blow, D. M., Birktoff, J. J., and Hartley, B. S. (1969) Nature 221, 337-341

Clement, G. (1973) in Progress in Bioorganic Chemistry (Kaiser, T., and Kezdy, F., eds) 2 pp. 178, Wiley, NY

Chiu, H., and Lagunoff, D. (1972) Histochem. J. 4, 135-144

Colman, P. M., Jansoniums, J. N., and Matthews, B. W. (1972) J. Mol. Biol. 70, 701-710

Cornish-Bowden, A. (1980) Anal. Biochem. 105, 223-238

Crisp, A. J., Chapman, C. M., Kirkham, S. E., Schiller, A. L., and Krane, S. M. (1984) Arthritis and Rheum. 27, 845-851

De Lange, R., and Smith, E. L. (1971) in The Enzymes, 3rd Edition (Boyer, P., ed) 3, p. 81

Eriksson, S., and Berven, H. (1972) in Pulmonary Emphysema and Proteolysis (Mittman, C., ed) pp. 7-24, Academic Press, NY and London

Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961) Arch. Biochem. Biophys. 95, 271-278

Evans, H. E., Mandel, I., and Keller, S. (1972) in Pulmonary Emphysema and Proteolysis (Mittman, C., ed) pp. 91-99, Academic Press, NY and London

Fersht, A. R. (1977) in Enzyme Structure and Mechanisms pp. 302-325 W. H. Freeman and Co., San Francisco

Folkman, J., Langer, R., Linhardt, R. J., Haudenschild, C., and Taylor, S. (1983) Science 221, 719-725

Fraki, J. E., and Hopsu-Havu, U. K. (1975) Arch. Dermatol. Res. 253, 261

Fruton, J. (1971) in The Enzymes 3rd Edition (Boyer, P., ed) 3 pp. 179, Academic Press, NY

Fruton, J. S. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D. D., and Shaw, E., eds) p. 33, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Glenner, G. G., and Cohen, L. A. (1960) Nature (Lond.) 105, 846-847

Glynn, L. E. (1972) Ann. Rheum. Dis. 31, 412-420

Godfrey, H. P., Ilardi, C., Engber, W., and Graziano, F. M. (1984) Arth. Rheum. 27, 852-856

Gomori, G. (1953) J. Histochem. Cytochem. 1, 469-470

Halliwell, R. E. W. (1973) J. Immunol. 110, 422-430

Hirs, C. H. W. (1967) Methods Enzymol. 11, 59-62

Ishizaka, K. (1984) Mast Cell Activation and Mediator Release, in Progress in Allergy Vol. 34, Karger, Basel, Munchen, Paris, London, NY, Tokyo, Sydney

Ito, A., Ihara, H., and Mari, Y. (1980) Biochem. J. 185, 443

Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A., and Elmore, D. T. (1973) Biochem. J. 131, 107-117

Janoff, A. (1972) Amer. J. Path. 68, 579-592

Janoff, A., and Blondin, J. (1973) Lab. Invest. 29, 454-457

Janoff, A., and Blondin, J. (1974) Proc. Soc. Exp. Biol. Med. 145, 1427-1430

Jansen, E. F., Nutting, M. F., and Balls, A. K. (1949) J. Biol. Chem. 179, 189-195

Jansen, E. F., Nutting, M. F., and Balls, A. K. (1950) J. Biol. Chem. 185, 290-301

Johnson, D. A. (1980) unpublished results

Johnson, D. A. (1981) Fed. Proc. 40, 1717

Johnson, D. A. (1983) Anal. Biochem. 130, 475-480

Johnson, D. A., and Pascual, R. (1978) Fed. Proc. 37, 1437

Johnson, R. G., Curty, S. E., Fingerhood, B. J., and Scarpa, A. (1980) FEBS Lett. 120, 75-79

Kaliner, M., and Lemanske, R. (1984) Fed. Proc. 43, 2846-2851

Kaplan, P. D., Kuhn, C., and Pierce, J. A. (1973) J. Lab. Clin. Med. 82, 349-358

Kessler, D., Langer, R., Pless, N., and Folkman, J. (1976) Int. J. Cancer 18, 703

Klebe, R. J. (1974) Nature 250, 248-251

Knight, G. G., and Barrett, A. J. (1976) Biochem. J. 155, pp. 117-125

Laemmli, O. K. (1970) Nature (Lond.) 227, 680-685

Lagunoff, D., and Benditt, E. P. (1963) Ann. N. Y. Acad. Sci. 103, 185-198

Lagunoff, D., Pritzl, P. (1976) Arch. Biochem. Biophys. 173, 554-563

Laskowski, M., Jr., and Ikuroshin, K. (1980) Anal. Rev. Biochem. 49, 593-626

Lipscomb, W. N., Reeke, G. N., Hartsuck, J. A., Quiocho, F. A., and Bethge, P. H. (1970) Phil. Trans. Roy. Soc. 257, 170-179

Mallory, P. A. and Travis, J. (1973) Biochemistry 12, 2847-2851

McGowan, E. B., Shafig, S. A., and Stracher, A. (1976) Exp. Neurol. 50, 649-657

Meier, H. L., Kaplan, A. P., Lichtenstein, L. M., Revak, S., Cochrane, C. G., and Newball, H. H. (1983) J. Clin. Invest. 72, 574-581

Mittman, C. (1972) in Pulmonary Emphysema and Proteolysis (Mittman, C., ed.) pp. 1-7, Academic Press, NY and London

Mota, I. and Silva, W. D. da (1960) Nature 186, 245-248

Movat, H. Z., Steinberg, S. G., Habal, F. M., and Ranadive, H. S. (1973) Lab. Invest. 29, 669-684

Neurath, H. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D. D., and Shaw, E., eds) pp. 61-63, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Oertel, H. and Kaliner, M. (1981) J. Immunol. 127, 1398-1402

Oertel, H., Strassburger, J., Goldstein, R., Waxdal, M., and Kaliner, M. (1980) Fed. Proc. 39, 905-915

Okuno-Kaneda, S., Saito, T., Kawasaki, Y., Ichikawa, A., Tomita, K (1980) Biochem. Pharmac. 29, 1715-1722

Oronsky, A. L., and Buerman, C. W. (1978) in Neutral Proteases of Human Polymorphonuclear Leukocytes (Havemann, K., and Janoff, A., eds) pp. 361-371 Urban and Schwarzenberg Inc., Baltimore

Oronsky, A. L., Ignarro, L., and Perper, R. (1973) J. Exp. Med. 138, 461-472

Ouchterlony, O. (1968) Handbook of Immunodiffusion and Immunoelectrophoresis, Ann Arbor Science Publishers, Inc., Ann Arbor, MI

Padewar, J. (1979) in The Mast Cell: Its Role in Health and Disease (Pepy, S. J., Edwards, A. M., eds) Pittman, London, pp. 1-20

Patterson, R., McKenna, J. E., and Suszko, I. M. (1977) J. Clin. Invest. 59, 217-225

Patterson, R., Suszko, I. M. (1980) J. Allergy Clin. Immunol. 65, 278-284

Pearce, F. L., Behrendth, H., Blum, U., Poblete-Freund, G., Pult, P., Stang-Voss, C., and Schmutzler, W. (1977) Agents Action 7, 45-56

Pearlstein, E. (1976) Nature 262, 497-500

Pearlstein, E., and Gold, L. I. (1978) Ann. N. Y. Acad. Sci. 312, 278-292

Pisano, J. J. (1975) in Proteases and Biological Control (Reich, etc. p. 199

Price, N. C., and Stevens, L. (1982) in Fundamentals of Enzymology pp. 172-179, Oxford University Press, Oxford, NY

Sagreiya, K., Bhatia, A., and Ashokraj, G. (1983) Indian J. Pathol. Microbiol. 26, 111-115

Schmidt, W., Egbring, R., and Havemann, K. (1975) Thrombosis Res. 6, 315-326

Schwartz, L. B., and Austen, K. F. (1981) in Biochemistry of Acute Allergic Reactions, Alan R. Liss, Inc., NY pp. 103-121

Schwartz, L. B., and Austen, K. F. (1984) in Mast Cell Activation and Mediator Release (Ishizaka, K., ed) Progress in Allergy Vol. 34, Karger, Basel, Munchen, Paris, London, New York, Tokyo, Sydney

Schwartz, W. N., and Bird, J. W. (1977) Biochem. J. 167, 811-820

Schwartz, L., Lewis, R., and Austen, K. F. (1981a) J. Biol. Chem. 256, 11939-11946

Schwartz, L., Lewis, R., Seldin, D., and Austen, K. F. (1981b) J. Immunol. 126, 1290-1294

Schwartz, L. B. Riedel, C., Caulfield, J. P., Wasserman, S. I., and Austin, K. F. (1981c) J. Immunol. 126, 2071-2078

Schwartz, L. B., Schwartz, J. J., Vik, D., Fearon, D. T., and Austen, K. F. (1982) Fed. Proc. 41, 487

Seppa, H. E. J., and Jarvinen, M. (1978) J. Invest. Dermatol. 70, 84-89

Simpson, D. M., and Ross, R. (1972) J. Clin. Invest. 51, 2009-2023

Smith, C. E., and Johnson, D. A. (1985) Biochem. J. 225, 463-472

Smith, T. J., Hougland, M. W., and Johnson, D. A. (1984) J. Biol. Chem. 259, 11046-11051

Smith, T. J., and Johnson, D. A. (1984) Fed. Proc. 43, 1760

Spanier, A. M., Bird, J. W. C., and Triemer, R. E. (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. 36, 555-560

Starkey, P. M., and Barrett, A. J. (1976) Biochem. J. 155, 265-271

Starkey, P. M. (1977) in Proteases in Mammalian Cells and Tissues (Barrett, A. J. ed) pp. 57-87 North Holland Publishing Co., Amsterdam, NY, Oxford

Stryer, L. (1975) in Biochemistry p. 193, W. H. Freeman and Co., San Francisco

Takio, K. (1980) Biochem. Biophys. Res. Commun. 97, pp. 34-39

Tanaka, T., McRae, B. J., Kyukin, C., Cook, R., Fraki, J., Johnson, D. A., and Powers, J. C. (1983) J. Biol. Chem. 258, 13552-13557

Tannenbaum, S., Oertel, H., Henderson, W., Kaliner, M. (1980) J. Immunol. 125, 325-334

Tang, J. J. (1971) J. Biol. Chem. 246 pp. 4510-4515

Taylor, S., and Folkman, J. (1982) Nature (London) 297, 307-310

Travis, S., and Folkman, J. (1981) Methods Enzymol. 80, 754-765

Travis, J., and Salvesen, G. S. (1983) Ann. Rev. Biochem. 52, 655-709

Vartio, T., Seppa, H., and Veheri, A., (1981) J. Biol. Chem. 256, 471-477

Vuento, M., and Vaheri, A. (1979) Biochem. J. 183, 331-337

Vavreinova, S. and Turkova, J. (1975) Biochem. Biophys. ACTA 403, 506-513

Walsh, K. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D. D., and Shaw, E., eds) pp. 1-11, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Walsh, K. (1979) in Enzymatic Reaction Mechanisms pp. 53-107, W. H. Freeman and Co., San Francisco

Wasserman, S. I. (1984) Arth. Rheum. 27, 841-844

Weismann, G., Zurier, R. B., Spieler, P. J., and Goldstein, I. M. (1971) J. Exp. Med. 134, 149-165

Woodbury, R. G., and Neurath, H. (1978) Fed. Proc. 38, 834

Woodbury, R. G., and Neurath, H. (1980) FEBS Lett. 114, 189-196

Woodbury, R. G., Gruzenski, G. M., and Lagunoff, D. (1978) Proc. Natl. Acad. Sci. 75, No. 6, 2785-2789

Woodbury, R. G., Everitt, M. T., and Neurath H. (1981) Methods Enzymol. 80, 568-609

Yoffe, J. R., Taylor, D. J., and Wooley, D. E. (1984) Biochem. Biophys. Res. commun. 122, 270-276

Yurt, R., and Austen, K. F. (1977) J. Exp. Med. 146, 1405-1419

APPENDIX

VITA

TIMOTHY J. SMITH

1. Personal Data

Date of Birth:	April 6, 1946
Place of Birth:	Springfield, Ohio
Military Service:	United States Army, December 6, 1968, to September 11, 1970
Address:	1010 Buffalo Street, #5, Johnson City, TN 37601

2. Education

School	Location	Years	Degree
Southeastern High School	South Charleston, Ohio	1960-1964	
Milligan College	Milligan, Tennessee	1964-1968	B.A. (Biology)
East Tennessee State University	Johnson City Tennessee	1971-1973	M.S. (Microbiology)
East Tennessee State University	Johnson City, Tennessee	1980-1985	Ph.D. (Biomedical Sciences)

3. Professional Experience

Year	Position	Location	Supervisor
1968	Technician	Salem Medical Laboratory, Dayton, Ohio	Robert Green, MT (ASCP)
1969-1970	Clinical Microbiologist	149th General Hospital, Japan	Col. C.Greene, Ph.D. and Major H. Chin, M.D.
1970-1971	Supervisor Clinical Microbiology	USARJ, Camp Zama, Japan	Major S. Holley, M.D.
1970-1971	Teaching Assistant	East Tennessee State, University, Johnson City, Tennessee	Charles Clark, Ph.D., and William Gaby, Ph.D.

Year	Position	Location	Supervisor
1971-1972	Supervisor Clinical Microbiology	Bristol Memorial Hospital Bristol, Tennessee	Alvin Crawford, M.D.
1974-1979	Technical Director and Vice President	Englewood Medical Laboratory, Englewood, Florida	L.P. Weersoryia M.D.
1979-1980	Technical Director and President	Pathology and Nuclear Diagnostic Assays, Venice, Florida	L.P. Weersoryia M.D.
1981-present	Laboratory Supervisor	V.A. Medical Center, Mountain Home, Tennessee	Louis Boelin, M.D.
1983	Instructor	Milligan College, Milligan, Tennessee	Charles Gee, Ph.D.

4. Professional Registries and Licenses by Examination

American Society of Clinical Pathologists, M.T. 120014
American Medical Technologists, M.T. 020799
National Registry of Microbiologists, 1629
National Certification Agency for Medical Laboratory Personnel, C.S. 790143-5
Florida State Laboratory Registration, Director JC0010215

5. Organizational Affiliations

American Society of Clinical Pathologists
American Society of Microbiology
Sigma Xi Scientific Research Society

6. Publications

Smith, T.J. and Clark, C., Effects of L-ascorbic acid on diphtheria toxin, J. Nut. and Vitol. 2, 120 (1974)

Smith, T.J., and Johnson, D.A., Human lung tryptase is stabilized by heparin, Fed. Proc. 43, 1760 (1984)

Smith, T. J., Hougland, M. W., and Johnson, D. A., Human lung tryptase: purification and characterization, J. Biol. Chem. 259, 11046-11051 (1984)

7. Special Skills

Laboratory Administration
Teaching Biomedical Sciences
Research in Protein Chemistry