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MALIGNANT HUMAN BREAST AND OTHER TISSUES

CHARACTERIZATION OF THE INTERACTION BETWEEN HEPARIN AND
CHYMOTRYPSIN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

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<tr>
<td>A°</td>
<td>Angstrom</td>
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<tr>
<td>ANS</td>
<td>Anilinonaphthylene Sulfonate</td>
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<td>Asn</td>
<td>Asparagine</td>
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<td>AT</td>
<td>Antitrypsin</td>
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<td>ATEE</td>
<td>Acetyl-L-Tyrosine Ethyl Ester</td>
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<td>BHK</td>
<td>Baby Hamster Kidney</td>
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<td>BzAlaEE</td>
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<td>CNBr</td>
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<td>CRP</td>
<td>C-Reactive Protein</td>
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<td>CS-A</td>
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<td>GPANA</td>
<td>Glutaryl-L-Phenylalanine-p-Nitroanilide</td>
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<tr>
<td>GFNA</td>
<td>Glutaryl-L-Phenylalanine-β-Naphthylamide</td>
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<td>HA</td>
<td>Hyaluronic Acid</td>
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<tr>
<td>nm</td>
<td>Nanometer</td>
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<tr>
<td>NFGB</td>
<td>Nitrophenyl-p-Guanidinobenzoate</td>
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PAF Periodic Acid Formazan
PAS Periodic Acid Schiff's
PMB Phorbol-12-Myristate-13-Acetate
psi Pounds per Square inch
SA Sialic Acid
SDS Sodium Dodecyl Sulfate
SV Simian Virus
TAME Tosyl-L-Arginine Methyl Ester
TCA Trichloroacetic Acid
TEMED N,N,N',N' Tetramethylethylenediamine
TLCK Tosyl-L-Lysine Chloromethyl Ketone
TPCK Tosyl-L-Phenylalanine Chloromethyl Ketone
tyro Tyrosine
x g times gravity
INTRODUCTION

The knowledge of tissue protease inhibitors in normal and malignant tissues, their effect on neoplastic or transformed cells and in neoplastic conditions has expanded in the four years since this project began in mid 1972. The elevation of trypsin inhibitory activity in serum with neoplastic conditions was used for cancer screening (1, 2) until high levels of antitryptic activity were noted in non-neoplastic diseases (3). This phenomena has been suggested to be due to the release of protease inhibitors from neoplastic cells (4) but it is more likely due to the acute phase reaction causing increased synthesis of protease inhibitors such as $\alpha_1$-antitrypsin and $\alpha_1$-antichymotrypsin by the liver (5).

When this project was initiated early reports had appeared on the effects of proteinase inhibitors on the growth properties of transformed cells in culture (6) and their effects on tumorigenesis (7). Most of these experiments used the alkylating agents tosyl-L-lysine chloromethyl ketone (TLCK) and tosyl-L-phenylalanine chloromethyl ketone (TPCK) which inhibit trypsin and chymotrypsin, respectively. The interpretation of the results of these experiments has recently been questioned since TPCK and TLCK strongly inhibit cellular protein synthesis (8).
Preceding the beginning of this project, Brecher and co-workers reported the presence of tryptic and chymotryptic inhibitory activity in human astrocytoma (9), whole human brain (10, 11), glioma, ovarian carcinoma, malignant and adjacent normal breast and colon tissues (12). They observed that the brain protease inhibitors detected in whole brain were heat stable (10, 11) while an additional soluble heat labile inhibitor was detected in human astrocytoma (9). The major amount of the inhibitory activity in the non-brain tissues was heat-labile and nondialyzable (12).

The original purpose of this investigation was to isolate and characterize the chymotrypsin and trypsin inhibitor(s) present in malignant human breast and other tissues. Using affinity chromatography on Sepharose-chymotrypsin and Affi-Gel-chymotrypsin, a number of forms of \( \alpha_1 \)-antitrypsin have been isolated from malignant human breast tissue extracts, as well as, \( \alpha_1 \)-antichymotrypsin and \( \alpha_2 \)-macroglobulin and several low molecular weight inhibitors. With the addition of a preparative electrophoresis step, one form of \( \alpha_1 \)-antitrypsin has been purified to electrophoretic homogeneity. \( \alpha_1 \)-Acid glycoprotein, \( \alpha_2 \)-macroglobulin, antithrombin III and \( \alpha_1 \)-antitrypsin were detected immunologically in various malignant and adjacent normal tissues.

In contemplating possible inhibitors of chymotrypsin and trypsin which would bind to the Sepharose-chymotrypsin column, heparin, a mucopolysaccharide was considered since it had been reported to bind and inhibit proteolytic enzymes (13, 14, 15). Astrup et al. (16),
However, reported the inhibition observed by other workers was due to an impurity in the heparin preparations which was identified as a trypsin inhibitor. A complex between chymotrypsin and heparin was observed by electrophoretic means which retained hydrolytic activity towards glutaryl-L-phenylalanino-β-naphthylamide (GFNA). Heparin enhanced the enzymatic activity of chymotrypsin with the substrate glutaryl-L-phenylalanine-p-nitroanilide (GPANA). As a result of these studies, it was concluded that heparin was probably bound to the Sepharose-chymotrypsin column but was not present in one of the inhibitor peaks. Due to an interest in the chymotrypsin-heparin complex, this system was further studied. Ultraviolet and proflavin difference spectra, kinetic studies using GPANA and further electrophoretic studies both on basic and acidic polyacrylamide disc gels were used in the study of the heparin-chymotrypsin complex.
REFERENCES

HISTORICAL

Protease inhibitors present in the human body function in the regulation of the clotting, fibrinolysis, kallikrein and complement systems of plasma (1), the control of reactions involving macrophages (2), granulocytic (3) and neutrophilic (4) leukocytes, lymphocytes (5,6), the protection of the respiratory (3,7-9) and the reproductive tissues (10,11), the regulation of fertilization (10,11) and the regulation of the activation of pancreatic proteolytic enzymes (12-14). The plasma protease inhibitors include α₁-antitrypsin, α₁-antichymotrypsin, inter-α-trypsin inhibitor, α₁-macroglobulin, Cl inactivator, antithrombin III, plasminogen activator inhibitor and a high molecular weight thrombin inhibitor (15). Although α₁-acid glycoprotein is not normally considered as a protease inhibitor, it does inhibit several proteolytic enzymes (16,17).

The following review will be mainly directed towards human protease inhibitors.
A. INHIBITOR SPECIFICITY AND MECHANISM OF INHIBITION

Most proteinase inhibitors have a broad inhibitory capacity (List 1). However, some inhibitors inhibit only a narrow range of proteases. \(\alpha_1\)-Antichymotrypsin, which inhibits only a few proteases (18,19), and \(\alpha_2\)-macroglobulin, which inhibits a wide variety of proteinases (20), are representative of the two extremes in human plasma.

The number of molecules of protease molecules that can bind per inhibitor molecule varies from one inhibitor to another. The binding ratio reported for many of the plasma protease inhibitors varies as well from laboratory to laboratory (20,21). For some inhibitors which have been sequenced such as the Bowman-Birk soybean trypsin inhibitor, two inhibitory sites have been identified (22).

The general scheme for trypsin inhibition suggested by Laskowski, Jr. and co-workers (23) is the following:

\[ E + I \rightleftharpoons L \rightleftharpoons C \rightleftharpoons L^* \rightleftharpoons E + I^* \]

where \(E\) is the enzyme, \(I\) is the inhibitor in the native form and \(I^*\) in the modified form, \(L\) and \(L^*\) are the native and modified, loose, noncovalent inhibitor enzyme complexes, and \(C\) is the stable complex.

Most studies indicate that trypsin cleaves a single arginine or lysine residue located in a peptide loop formed by an intrachain disulfide bridge of soybean trypsin inhibitor during complex formation between the inhibitor and the enzyme (24,25). Laskowski, Jr. (26) suggests that the inhibitor is covalently bonded to the enzyme forming an acyl enzyme or tetrahedral intermediate in which
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Ct^-Antitrypsin
C Q.-Antichyrontrypsln
Antithrombiu III
Inter-n -Trypsin Inhibitor
LIW Plasma Inhibitor

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Cervical Mucus Inhibitor
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the active site serine of the trypsin molecule forms an ester bond with the carboxyl of the arginine or lysine of the inhibitor active site.

X-ray crystallographic analysis of the bovine trypsin-pancreatic trypsin inhibitor (Basic Kunitz) complex showed a tetrahedral adduct with a covalent bond linking the carbonyl carbon of lysine 15 of the inhibitor and the $\gamma$ oxygen of serine 183 of the enzyme to produce the stable complex, C(27). A similar tetrahedral adduct was found for the bovine trypsin-soybean trypsin inhibitor (Kunitz) complex by X-ray analysis in which a covalent linkage occurs between the carbonyl carbon and arginine 63 of the inhibitor and the $\gamma$ oxygen of serine 183 of the enzyme (28).

Based on in depth study of the X-ray crystallographic data and catalytic mechanism study data for inhibitor enzyme complexes, Tschesche and co-workers (29) have suggested two additional kinetic intermediates. These are an acyl intermediate, A and a second tetrahedral intermediate, $C^\star$, with the reactive site peptide bond hydrolyzed. The modified kinetic mechanism is the following:

$$E \rightleftharpoons I \rightleftharpoons L \rightleftharpoons A \rightleftharpoons C^\star \rightleftharpoons L^\star \rightleftharpoons T + I.$$  

The two new intermediates have not yet been kinetically identified but the other three can be identified for the bovine trypsin-soybean trypsin inhibitor complex (30). On the basis of these studies it has been suggested that cleavage of the inhibitor at the reactive site followed by the formation of a tetrahedral complex involving a covalent bond between the inhibitor and protease is the general mechanism of inhibition for all related complexes (30).
Feeney and co-workers (31), however, do not believe that peptide bond cleavage is necessary for inhibition of proteases. They believe that inhibition is the result of a large number of noncovalent forces between the protease and inhibitor. Support for this viewpoint comes from the observation of complex formation between active site modified proteases and protease inhibitors (32-37). The TLCK derivative of trypsin binds to chicken ovomucoid (32, 33) while the TPCK derivative binds turkey and pheasant ovomucoid and potato inhibitor (32). Anhydro-trypsin and anhydro-chymotrypsin, in which the active site serine is transformed into a dehydroalanine, interact with a wider range of inhibitors including the Kunitz and Kazal bovine pancreatic inhibitors (31, 34, 35). The modified proteases form strong complexes with the complex between lima bean inhibitor with the modified protease being bound stronger than the native protease (34). Methyl chymotrypsin can cause the dissociation of active chymotrypsin-ovomucoid complexes (36). The thermodynamic and kinetic parameters of the reaction between trypsin and pancreatic secretory trypsin inhibitor are only slightly altered by the substitution of anhydrotrypsin in the place of native trypsin (37).

1. $\alpha_1$-ACID GLYCOPROTEIN

$\alpha_1$-Acid glycoprotein inhibits elastase (16) and the conversion of prothrombin to thrombin (17).

2. $\alpha_1$-ANTITRYPSIN

$\alpha_1$-Antitrypsin inhibits a wide range of plasma, pancreatic and cellular enzymes. In plasma $\alpha_1$-antitrypsin inhibits kallikrein,
plasmin (1), in a time dependant manner (38, 39), and Hageman Factor (Factor XII) (38), thromboplastin antecedent (Factor XI) (40) and thrombin in a progressive manner (41, 42). α₁-Antitrypsin also inhibits pancreatic trypsin (21, 43, 44), chymotrypsin (21, 43, 44), elastase (45, 46) and kallikrein (47). It further inhibits cellular proteases such as polymorphonuclear leukocyte elastase and neutral proteinases (48-50), macrophage proteinases (51), granulocyte elastase and collagenase (52) and skin elastase (45, 46). Urokinase (53, 54) and renin (55, 56) are also inhibited by α₁-antitrypsin. In the reproductive system this inhibitor inhibits sperm acrosin in a progressive manner (57) and inhibits the rabbit blastocyte protease (19). α₁-Antitrypsin inhibits proteases from bacteria such as the alkaline protease from Bacillus subtilis (58) and a brinase from Aspergillus orajzæ (59, 60).

The binding ratio for proteases with α₁-antitrypsin is disputed among investigators. Both 1:1 and 2:1 complexes between chymotrypsin or trypsin with α₁-antitrypsin are reported (21, 44, 61). Johnson et al. (21) found that even in the presence of excess α₁-antitrypsin, two moles of trypsin are preferentially bound to 1 mole of α₁-antitrypsin. Urokinase forms both 1:1 and 2:1 complexes (53, 54) while elastase forms only 1:1 complexes (52) with this inhibitor.

The controlling factor in the rate of α₁-antitrypsin-trypsin or chymotrypsin reactions is an electrostatic attraction (44).

Cohen (62) has hypothesized that α₁-antitrypsin contains two inhibitor sites, one containing a positively charged residue and
the other containing an aromatic or leucine residue at the binding site for the active site of the proteases. In support of this hypothesis, his experiments have shown that trypsin and chymotrypsin compete for inhibitory sites; DFP-inactivated trypsin does not bind to the $\alpha_1$-antitrypsin molecule; and modification of the arginine residues prevents inhibition of trypsin but not chymotrypsin (62).

In contrast to these results of Cohen (62), Johnson and Travis (63) found that modification of the lysine residues of $\alpha_1$-antitrypsin resulted in the loss of inhibitor activity; however, modification of the arginine residues had no effect on the inhibitory activity. These later results are supported by the fact that the disulfide loop polypeptide, which probably contains the active site, contains lysine but no arginine (58). It is also high in leucine but does not contain proline which is often present near the inhibitory site (63). This loop does not contain a carbohydrate component (63).

A single peptide may be cleaved when proteases interact with $\alpha_1$-antitrypsin. Using a Sepharase-elastase column, Lo and Cohen (64) reported that a single peptide bond was cleaved during the elastase-$\alpha_1$-antitrypsin interaction.

A covalent linkage between $\alpha_1$-antitrypsin and proteases is suggested since the two molecules are generally not separated by SDS electrophoresis (63,65). Moroi and Yamasaki (65) detected crosslinking between bovine trypsin and $\alpha_1$-antitrypsin, suggesting an acyl bond between the carbonyl carbon of the inhibitor and the $\gamma$ oxygen of serine 183 of the trypsin molecule.
A bell shaped curve is observed for the association constant for the $\alpha_1$-antitrypsin-chymotrypsin interaction vs pH (44). A maximum is present at pH 7.5 indicating possibly a histidine at pH $\approx 7$ and an isoleucine at pH $\approx 8$ may be involved in an ionic interaction between the molecules (44).

In most cases dissociation of $\alpha_1$-antitrypsin-protease complexes results in the loss of both enzymatic and inhibitory activity as in the case of hydrazine (65) and guanidine (63). Hercz (66), however, has separated both $\alpha_1$-antitrypsin-trypsin and chymotrypsin complexes using 0.1% SDS and has recovered active chymotrypsin and trypsin. These results suggest possibly a covalent bond is not mandatory between the two molecules.

3. $\alpha_1$-ANTICHTYMOTRYPSIN

Chymotrypsin (18) and rabbit blastocyte protease are the only known proteases which are inhibited by $\alpha_1$-antichymotrypsin (19).

4. ANTITHROMBIN III

Antithrombin III inhibits the plasma enzymes, thrombin (42,67-73), plasmin (68,69), factor Xa (42,74-77), factor IXa (77), factor Xla, factor XIIa (78) and kallikrein (79,80). Thrombin and factor X are progressively inhibited by antithrombin III but in the presence of heparin are immediately inhibited (42) at a 50-100 fold increase in rate (70-73). If factor Xa is bound to factor V, antithrombin III has no effect on factor Xa (75). A 1:1 complex is formed between antithrombin III and factor Xa (76), plasmin and thrombin (68,69).
Kallikrein is inhibited by antithrombin III according to first order kinetics (79) and the rate of inhibition is doubled by the addition of heparin (79,80). Sperm acrosin is also inhibited progressively by antithrombin III (57).

The interaction between antithrombin III and thrombin is believed to occur between a reactive site serine of the enzyme and a reactive site arginine on the inhibitor (81). The antithrombin III-thrombin complex is stable in the presence of 8 M urea, 2% SDS, 0.1 M β-mercaptoethanol and 6 M guanidine hydrochloride suggesting a very stable bond (81).

5. HEPARIN

Heparin forms a complex with thrombin (82-87), chymotrypsin (88), trypsin (88,91), pepsin (92,93), plasmin (82,94), plasminogen (95) and collagenase (96). Early studies suggested that heparin inhibited proteolytic enzymes (88,91,97). However, purified inhibitor-free heparin preparations of heparin do not inhibit these enzymes (98).

Heparin binds tightly to thrombin outside the active site (77). Approximately 3-4 heparin molecules can bind to each thrombin molecule (77). In vivo studies of Smith and Craft (87) signify that a 1:1, thrombin:heparin complex is formed, followed by the binding of antithrombin III to the complex. The experiments of Machovich et al. (84) suggest that heparin binds to thrombin, inducing a conformational change which facilitates binding to antithrombin III. Modification of the arginine residues on thrombin
does not alter the thrombin proteolytic activity or the progressive inhibitory activity of antithrombin III but prevents the binding of heparin and the acceleration of thrombin inhibition. This implies that heparin binds to thrombin rather than antithrombin III (85).

In the traditional mechanism, heparin binds first to the antithrombin molecule effecting a conformational change which enhances the rate of inhibition (68, 81, 92). Guanidination of the ε-amino groups of lysine of antithrombin III with O-methylisourea prevents the binding of heparin to the inhibitor and prevents the increase in the rate of inhibition (81). Recently, Li et al. (86) have reported, using stop flow kinetic techniques that premixing of heparin with antithrombin III enhances the second order rate constant 200-400 fold for the thrombin-antithrombin III complex formation, while premixing of heparin and thrombin actually inhibits complex formation. From the experiments reported in the literature, the exact mechanism for the heparin-thrombin-antithrombin III interaction is not known.

Heparin interacts with mouse bone collagenase forming a strong ionic bond and enhances the enzymatic activity of collagenase (96).

6. OTHER THROMBIN INHIBITORS

Because of the problems separating antithrombin III from a second heparin cofactor inhibitor, antithrombin II, some workers believe there is only one molecule (80). Separation of the two antithrombins, however, has been reported by Ganrot (99) and Brigenshaw and Shanberge (100).
A larger molecular weight β-lipoprotein (1,000,000) immediately inhibits thrombin in contrast to antithrombin III (15,42). The interaction between this inhibitor and thrombin is prevented by heparin (42).

Four other types of antithrombins have been identified in plasma (101). Antithrombin I inhibits thrombin through the absorption of the enzyme by fibrin (102). Antithrombin IV is the antithrombin activity present in ether-treated plasma (103). Antithrombin V is a factor present in serum due to a defect in fibrin polymerization which is associated with multiple myeloma and rheumatoid arthritis. Antithrombin VI is a plasmin hydrolysis product from fibrinogen which inhibits thrombin (101).

7. INTER-α-TRYPSIN INHIBITOR AND RELATED INHIBITORS

Inter-α-trypsin inhibitor inhibits trypsin, chymotrypsin, plasmin (7-9), kallikrein (104,105), acrosin (57) and to some extent papain, bromelain and pronase (102). Human chymotrypsin and trypsin form unstable complexes with inter-α-trypsin inhibitor while bovine chymotrypsin or trypsin complexes with this inhibitor are much more stable (43). When inter-α-trypsin inhibitor reacts with plasmin (108), trypsin or kallikrein (104,105), two fragments are formed. These fragments also inhibit plasmin, trypsin and kallikrein. This is the first evidence for proinhibitors (105).

The two low molecular weight respiratory mucus inhibitors and the two low molecular weight urinary inhibitors, which are immunologically related to inter-α-trypsin inhibitor inhibit
kallikrein, chymotrypsin and trypsin (7-9). The respiratory mucus inhibitors additionally inhibit leukocyte proteases (7-9).

8. α₂-MACROGLOBULIN

α₂-Macroglobulin inhibits a wide range of endopeptidases including serine, thiol, carboxy and metal proteinases (20) plus carboxypeptidase, aspartate aminotransferase and phytohemagglutins (15). The serine proteases inhibited by α₂-macroglobulin include chymotrypsin (109,110), trypsin (110-112), plasmin (3,110, 113,114), thrombin (42,114,115), kallikrein (42,116), urokinase (119), elastase (3,46) and subtilopeptidase A and B (58) plus the bacterial neutral proteases from Staphylococcus aureus (49), Proteus vulgaris (49), Fusiformis nodosus (118) and Trechophyton mentagrophytes (119) as well as the mammalian neutral proteases from polymorphonuclear leukocytes of rabbit (120) and human and ovine testis (20). In addition brinase from Aspergillus oryzae is inhibited by α₂-macroglobulin (60). The thiol proteases, bromelain (20), ficin, papain (20) and cathepsin B-1 (121) are inhibited by this inhibitor. It also inhibits the carboxyl proteinase, cathepsin D (20) and the acid proteinase from Periplaneta sp. (122) and the metal proteases thermolysin, clostridiopeptidase A (122, 124) and collagenase (122-125).

The reactions between α₂-macroglobulin and trypsin, chymotrypsin and plasmin are immediate while the reaction with thrombin is slow (110).

The reported binding ratios between α₂-macroglobulin and
proteolytic enzymes vary considerably between laboratories.

Barrett and Starkey (20) report a 1:1 ratio with only one binding site for all proteinases using radioactive, labeled proteinases and active site titration experiments. In contrast to these experiments, Jacquot-Armand and Guinand (112) and Rinderknecht and Geokas (111) found that one mole of $\alpha_2$-macroglobulin can bind 2 moles of trypsin. Ohlsson and Olsson (52) found the same 2:1 ratio for the elastase: $\alpha_2$-macroglobulin complex. A 3:1 protease:inhibitor ratio was reported by Shigeharu et al. (126). Various reasons exist for the wide range of binding ratios based on isolation and experimental techniques. In some preparations, serum was used as the source of the $\alpha_2$-macroglobulin in which thrombin and plasmin were complexed with the inhibitor (114). The use of commercial enzymes also has led to inaccurate binding ratios due to inactive enzyme molecules (42).

The $\alpha_2$-macroglobulin-protease complex is unique due to the fact that the catalytic site of the enzymes remains active towards small substrates but is inhibited by large substrates (20). Trypsin acquires a thrombin-like specificity (111) and retains its activator activity towards chymotrypsinogen, trypsinogen (111) and plasminogen (127). Denatured casein, hemoglobin, fibrin, fibrinogen, angiotensin and lys-vasopressin as well as the synthetic substrates, benzoyl-L-arginine-p-nitroanilide, benzoyl-L-arginine ethyl ester and tosyl-L-arginine methyl ester are hydrolyzed by the trypsin-$\alpha_2$-macroglobulin complexes (20).
The nonpeptidase and exopeptidase reactions with $\alpha_2$-macroglobulin are reversible (15). In contrast, the reaction between this inhibitor and the proteases is an apparent irreversible type involving the active center since neither zymogens nor inactivated proteases react (20). The proteases can only be liberated by denaturation of the $\alpha_2$-macroglobulin molecule (20). Elastase inhibition is temporary since it cleaves $\alpha_2$-macroglobulin and escapes (46).

$\alpha_2$-Macroglobulin has a greater affinity for proteases than many other inhibitors and can remove proteases from other inhibitors such as $\alpha_1$-antitrypsin (128, 129) and soybean trypsin inhibitor (129).

Barrett and Starkey (20) propose a mechanism for the interaction of $\alpha_2$-macroglobulin with proteases in which the enzyme is trapped by the inhibitor rather than binding at a specific site. According to this mechanism, the protease (E) enters the macromolecule (M) forming a complex (EM) and then cleaves a sensitive bond forming a second intermediate (EM'). The cleavage causes a conformational change in the molecule trapping the enzyme forming the complex (EM*). This is a two stage mechanism represented by the following (20):

$$E + M \rightleftharpoons EM \rightarrow EM'$$

$$EM \rightleftharpoons EM^*$$

The trapped protease then cannot leave nor can a second protease molecule displace the first molecule. Only small molecules can diffuse into the macromolecular structure since steric hindrance prevents large molecules from diffusing to the active site of the protease.

A conformational change by $\alpha_2$-macroglobulin upon complex formation with proteases has been observed by electron microscopic techniques (130).
The observed increase in fluorescent yield of anilinonaphthalene sulfonate bound to $\alpha_2$-macroglobulin upon complex formation with trypsin also indicates that a conformational change occurs (131).

On the other hand, Steinbuch and Audran (42) believe that non-covalent hydrophobic binding is involved in the complex between proteases and $\alpha_2$-macroglobulin because the high esterase activity reflects a need for a fixed orientation of the enzyme rather than random trapping. The fact that even enzymes of a molecular weight of as high as 74,500 for plasmin or 108,000 for kallikrein are inhibited leads to problems in completely enclosing the enzyme in the $\alpha_2$-macroglobulin molecule of a molecular weight of only 8 times as great (42).

9. Cl-INACTIVATOR

The Cl-inactivator inhibits Factor XIIa (Hageman factor) Factor XIa, thrombin and plasmin (15,42,132-135), the Cl fragments, Clr and Cls of the complement system (42,134,136) and kallikrein (1,134). Thrombin (42) and kallikrein (134) are inhibited progressively by this inhibitor. The complex between Cl-inactivator and Cls and kallikrein are 1:1 complexes (136). These complexes are quite stable since they resist denaturing agents such as SDS and urea (136). Plasmin cleaves Cl-inactivator producing a fragment which inhibits plasmin but does not inhibit Cls (136).

10. INTER-\(\alpha\)-ANTIPLASMIN

Inter-$\alpha$-antiplasmin immediately inhibits plasmin and urokinase but has little or no inhibitory effect upon trypsin, elastase, thrombin or chymotrypsin (137).
11. PLASMINOGEN ACTIVATION INHIBITOR

The plasma α2-globulin plasminogen activation inhibitor inhibits plasminogen activation by inhibiting urokinase and streptokinase (138, 139).

12. SEMINAL PLASMA INHIBITORS

The 11,000 molecular weight inhibitor from human seminal plasma inhibits human and bovine trypsin, bovine chymotrypsin, human neutral leucocytic proteinases and human granulocytic elastase, whereas the 6,200 molecular weight inhibitor inhibits human and bovine trypsin and human and boar acrosin (140).

13. CERVICAL MUCUS INHIBITOR

The 11,500 molecular weight inhibitor which is similar to the corresponding inhibitor in seminal plasma inhibits trypsin, chymotrypsin and leukocyte proteases but not acrosin (141).

14. PANCREATIC INHIBITORS

The human pancreatic inhibitors isolated to date have been similar to the bovine Kazal acid secretory inhibitor (12-14). The 9,000 molecular weight Kazal inhibitor is a heat and acid stable molecule which selectively inhibits trypsin and thrombin (142). The human inhibitors inhibit human anionic and cationic trypsins as well as bovine and porcine trypsin (12-14). These inhibitors are weak inhibitors of porcine plasmin and bovine chymotrypsin (12-14), while human chymotrypsin is not inhibited (143).

Because of the homology between the human, porcine and bovine pancreatic secretory inhibitors (12), the human inhibitor probably in-
hibits trypsin by the same mechanism as the other mammalian pancreatic secretory inhibitors. The interaction between pancreatic trypsin inhibitor (Kazal) and trypsin results in temporary inhibition of the enzyme (144-146). The original mechanism proposed for this type of inhibitor by Laskowski, Sr. and Wu (144) was a two step procedure in which a trypsin-inhibitor complex formed, followed by a second trypsin molecule complexing with the first complex leading to the hydrolysis of the inhibitor. Subsequently a sequential mechanism has been suggested (145,146) in which trypsin cleaves the inhibitor between lysine 18 and isoleucine 19 followed by inhibition according to the scheme proposed by Laskowski, Jr. and co-workers (23), discussed at the beginning of Section A. The complex, then dissociates separating the enzyme and the modified inhibitor. The modified inhibitor is then susceptible to proteolytic attack by the free trypsin molecule. Hydrolysis of the arginine 44-glycine 45 bond causes inactivation of the inhibitor (145). Additional cleavages then occur at other susceptible bonds (145).

B. METHODS OF ISOLATION AND CHEMICAL AND PHYSICAL PROPERTIES OF PROTEASE INHIBITORS

Early methods of isolation of protease inhibitors relied on differences in solubilities of proteins and the differences in isoelectric points. Later methods take advantage of the charge variations between proteins by the use of ion exchange chromatography and the differences in molecular size by the use of gel filtration. Recently, procedures have been reported involving affinity chromatography which utilize specific interactions between a molecule bound
to a solid support and the desired component of a protein mixture.
With the development of solid supports and relatively stable ampholyte molecules, isoelectric focusing is being used as a preparative separation tool.

Most of the plasma protease inhibitors are glycoproteins with rather high molecular weights (1). Of the plasma inhibitors, the complete amino acid sequence of only \( \alpha_1 \)-acid glycoprotein is known (147). The amino acid sequences of some cyanogen bromide peptides of \( \alpha_1 \)-antitrypsin have been reported (148, 149). The only other human protease inhibitor, in which the amino acid sequence is known, is the pancreatic secretory inhibitor (12).

1. \( \alpha_1 \)-ACID GLYCOPROTEIN

Most isolation procedures take advantage of the precipitability of \( \alpha_1 \)-acid glycoprotein in ethanol, ammonium sulfate and Rivanol (150-152). Heide and Haupt (15) purified this protein with additional zone electrophoresis and perchloric acid precipitation steps.

\( \alpha_1 \)-Acid glycoprotein has a molecular weight of 44,100, a diffusion coefficient of 5.27, an intrinsic viscosity of 0.069, an extinction coefficient of 8.9 at 280 nm (153, 154) and an isoelectric point of 2.7 in 0.1 M phosphate buffer (155) or 1.82 upon electrophoresis (156). This glycoprotein is polymorphic, separating into as many as seven bands at pH 2.9 due to variations in sialic acid content (157, 158). Removal of sialic acid from this molecule raises the isoelectric point to 5 in sodium acetate buffer and decreases the electrophoretic mobility (159).
$\alpha_1$- Acid glycoprotein contains one polypeptide chain with no free N terminal amino acid and one mole of C terminal serine (160). The five heteropolysaccharide groups of the molecule are joined to the polypeptide through a 2-acetamido-N-1-(4'-L-aspartyl)-2-deoxy-D-glucopyranosylamine type linkage (147,161,162). This molecule has two disulfide bridges which are easily reduced and a third bridge which is "buried" and is essential for the three dimensional structure (163). Schmid and co-workers (147,164) have elucidated the amino acid sequence of $\alpha_1$-acid glycoprotein which contains 181 residues. A preliminary three dimensional model has also been described by this same group (165). At 21 positions, two different amino acids were found in various $\alpha_1$-acid glycoprotein molecules (151) which involve point mutations.

2. $\alpha_1$-ANTITRYPSIN

$\alpha_1$-Antitrypsin was first isolated by Bundy and Mehl (166) in a procedure using ammonium sulfate precipitation, chromatography on Dowex and zone electrophoresis. A few years later Schultze et al. (167) used additional Rivinol and methanol precipitation steps. Ion exchange chromatography on DEAE cellulose and QAE sephadex and gel filtration on Sephadex are also used for the isolation of $\alpha_1$-antitrypsin in addition to precipitation steps and preparative electrophoresis (168,170). Other methods utilize affinity chromatography to bind contaminants which are not easily separated from $\alpha_1$-antitrypsin (61,172-174). Affinity columns which have been used for this purpose include antialbumin-Sepharose (171), ConA-Sepharose (173), Sepharose-dextran (61,172) and Sepharose-antibody columns to $\beta$-lipoprotein, $\alpha_1$-antichymotrypsin, Gc globulins, haptoglobulins and fast $\alpha_2$-globulins (174).
Laurell et al. (175) have developed a method for isolation of \( \alpha_1 \)-antitrypsin using K type Bence Jones proteins linked to Sepharose in which the C terminal cysteine was converted to a mixed disulfide with 3 carboxy-4-nitrobenzenethiol (\( \text{Nbs}_2 \)). Due to the presence of a reactive thiol group on the \( \alpha_1 \)-antitrypsin molecule, this inhibitor binds preferentially to this affinity chromatography column. The \( \alpha_1 \)-antitrypsin can be removed with excess \( \text{Nbs}_2 \) (175). This procedure and several others, have been developed to retain the microheterogeneity of \( \alpha_1 \)-antitrypsin (171,175,176). Lebas et al. (176) report a unique one step preparative electrofocusing procedure for the isolation of \( \alpha_1 \)-antitrypsin.

\( \alpha_1 \)-Antitrypsin is a glycoprotein with a molecular weight of 54,000 (1). The reported physical, kinetic and thermodynamic data for this inhibitor are given in the following two tables.

### TABLE 1. PHYSICAL PROPERTIES OF \( \alpha_1 \)-ANTITRYPSIN

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Sedimentation Coefficient(s)</td>
<td>3.45 (169)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.41 (166)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.60 (61)</td>
<td></td>
</tr>
<tr>
<td>Diffusion coefficient</td>
<td>5.1 x 10^-7</td>
<td>(169)</td>
</tr>
<tr>
<td>Partial specific volume (cm²/gm)</td>
<td>0.726 (169)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.645 (166)</td>
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<tr>
<td>Molecular Weight Ultracentrifuge</td>
<td>59,000 (169)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45,000 (166)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>53,000 (61)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>54,000 (177)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>54,200 (161)</td>
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<tr>
<td>Residue Analysis</td>
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<td></td>
</tr>
<tr>
<td>pI</td>
<td>4.0 (166)</td>
<td></td>
</tr>
<tr>
<td>Extinction coefficient (1% 280nm)</td>
<td>5.0 (169)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.3 (60)</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 2. KINETIC AND THERMODYNAMIC DATA FOR $\alpha_1$-ANTITRYSIN (44)

<table>
<thead>
<tr>
<th></th>
<th>$k$ association</th>
<th>Order</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_1$-Antitrypsin-bovine trypsin</td>
<td>$3.5 \times 10^6$ l mol$^{-1}$ sec$^{-1}$</td>
<td>2nd</td>
</tr>
<tr>
<td>$\alpha_1$-Antitrypsin-bovine chymotrypsin</td>
<td>$3.1 \times 10^5$ l mol$^{-1}$ sec$^{-1}$</td>
<td>2nd</td>
</tr>
</tbody>
</table>

$\alpha_1$-Antitrypsin-chymotrypsin

$H^\circ$ association 22 kJ mol$^{-1}$

$G^\circ$ association -50 kJ mol$^{-1}$

$S^\circ$ association 240 J mol$^{-1}$ K$^{-1}$

25°C, pH 7.5, I= 0.19

$\alpha_1$-Antitrypsin is inactivated at high temperatures and at pH values below 6 (166). The molecule consists of one chain with one disulfide bridge (148,149). The amino terminal residue is glutamic acid (61,168,178) and the carboxy terminal residue is leucine (178). Four oligosaccharide units (Figure 1) are attached through asparagine to each $\alpha_1$-antitrypsin protein molecule (179). Sialic acid residues are found at the terminal positions of all branches of the carbohydrate unit (179).

Serum $\alpha_1$-antitrypsin is a highly polymorphic protein which separates under alkaline conditions into two bands. Under acidic conditions, near its isoelectric point, this protein separates into eight bands upon starch gel electrophoresis (180). Based on the mobility of these bands, twenty-six phenotypes of $\alpha_1$-antitrypsin have been identified (181).
Phenotype MM is the most common while PiS and PiZ are less common and are associated with low levels of α₁-antitrypsin in the deficiency state (182). Lebas et al. (176), using isoelectric focusing, separated four types of α₁-antitrypsin from the serum of an MM α₁-antitrypsin phenotype individual. These four types are believed to be conformational isomers (176).

One difference between normal M and deficiency types of α₁-antitrypsin such as Z is the sialic acid content (183-189). Microheterogeneity within MM and ZZ phenotypes may be additionally due to variation in the sialic acid content (184). At one time, the deficient state was believed to be due to a sialyltransferase deficiency (185). This, however, is not the case since α₁-antitrypsin deficient individuals with normal liver function have normal levels of sialyltransferase (186). This enzyme will transfer sialic acids as effectively to asialo ZZ α₁-antitrypsin as to asialo MM α₁-antitrypsin (186). Neither the ZZ α₁-antitrypsin nor the disialylated MM form show any loss in specific
inhibitory activity indicating that sialic acid is not required for inhibitory activity (187,190).

There must also be differences in the amino acid sequence between the MM and ZZ $\alpha_1$-antitrypsins since asialo MM and ZZ proteins do not have the same isoelectric point (184). Furthermore, isoelectric focusing of the cyanogen bromide fragments of these asialo proteins as well as the native proteins shows that the carbohydrates are located in two different CNBr fragments and that the ZZ protein has several amino acid substitutions (184). Chan and Rees (188) have observed that the ZZ $\alpha_1$-antitrypsin contains more arginine and glycine than the MM $\alpha_1$-antitrypsin. The ZZ form also lacks sialic acid and mannose. They propose that only 3 carbohydrate chains are present rather than the normal 4 carbohydrate chains (188). Yoshida et al. (189), using peptide analysis, noted that in addition to Pi ZZ $\alpha_1$-antitrypsin is deficient in sialic acid, two amino acid substitutions in the ZZ form. Lysine was substituted for a glutamic acid and glutamine was substituted for a second glutamic acid. Jeppsson (191) has also observed the substitution of glutamate for lysine.

3. $\alpha_1$-ANTICHYMOTRYSIN

$\alpha_1$-Antichymotrypsin has been isolated by precipitation with ammonium sulfate and Rivanol and preparative zone electrophoresis (192).

$\alpha_1$-Antichymotrypsin is a heat and acid labile glycoprotein with a molecular weight of 68,000. The sedimentation coefficient is 3.9 (193).

4. ANTITHROMBIN III

The traditional methods of isolation of antithrombin III involve ion exchange chromatography and gel filtration (68,194). One problem
with these procedures is that they give low yields of the inhibitor. Large quantities of antithrombin III can be isolated using a heparin-agarose affinity chromatography column (52, 195, 196). Miller-Anderson et al. (195) included additional ion exchange steps on DEAE Sephadex and gel filtration on Sephadex G200. The method of Thaler and Schmer (82) uses only the addition of a polyethylene glycol precipitation step.

Molecular weights of 65,000 (127) and 56,600 (197) for this inhibitor have been reported. The human antithrombin III is a single polypeptide chain with a 9% carbohydrate content (197). The amino terminal residue is histidine (195,197). The first seventeen residues of the N terminal end of antithrombin III have been sequenced (197).

5. HEPARIN

The general procedure most commonly used for purification of heparin involves an initial digestion with a proteolytic enzyme pronase (198) or papain (199,200) to release the mucopolysaccharide from the proteins attached. The heparin is then precipitated as a cetyltrimethylammonium complex followed by sodium chloride extraction and ethanol precipitation (201).

Heparin is a polydisperse molecule with molecular weights ranging from 3,000 to 37,500 (202). It is a polymer of D-glucuronic acid, L-iduronic acid and 2-amino-2-deoxy-D-glucose which contains an average of three sulfates attached per disaccharide unit (203). Silva and Dietrich (204) using heparinase digestion found 52% of the trisulfated disaccharide 0-(α-L-idoo-4-eneopyranosyluronic acid 2 sulfate)-(1→4)-2 sulfonamide-2-deoxy-D-glucose-6-sulfate plus 40% of a tetrasaccharide
which consists of the trisulfated disaccharide plus a disulfated disaccharide \( O-(\alpha \text{ D-glyco-4-enepyranosyluronic acid})-(1\rightarrow 4)-2\)-sulfoamide-2-deoxy-D-glucose-6-sulfate. They proposed the following as the major repeating unit for heparin (204):

![Repeating Structure of Heparin Diagram]

Figure 2. REPEATING STRUCTURE OF HEPARIN

X-ray crystallographic patterns suggest that structurally heparin is made up of repeating four monosaccharide units (205).

The heparin used as an anticoagulant only contains polysaccharide residues due to the method of isolation. The heparin found in the human body, however, is attached to a protein backbone (203). The linkage region between heparin and the protein backbone involves a 2 acetamido-2-deoxy-\( \alpha \)-D galactopyranosyl residue linked to a serine (203).

6. INTER-\( \alpha \)-TRYPSIN INHIBITOR

Heide et al. (206) used Rivanol precipitation, ammonium sulfate zone electrophoresis and gel filtration on Sephadex G 200 to isolate inter-\( \alpha \)-trypsin inhibitor. Steinbuch et al. (108) purified this in-
hibitor using EDTA solutions, ion exchange chromatography on DEAE cellulose, ethanol precipitation and gel filtration of Sephadex G 150.

Inter-\(\alpha\)-trypsin inhibitor has a molecular weight in the range of 140,000 (207) to 160,000 (192). The reported carbohydrate content varies from 9.1% (207) to 8.4% (192). The sedimentation coefficient of this protein is 6.4, and its extinction coefficient is 7.1 (206). Inter-\(\alpha\)-trypsin inhibitor can dissociate into a smaller molecular size \((s = 4.4)\) and an inhibitor peptide (206). Upon heating the protein to 56°C, aggregates form which have no immunological activity but which retain antiprotease activity (106).

The perchloric acid treated plasma supernatant fraction contains two low molecular weight inhibitors, 22,000 and 44,000, which are immunologically related to inter-\(\alpha\)-trypsin inhibitor (208). The two acid stable low molecular weight, 20,000 and 14,000, inhibitors are immunologically indistinguishable from inter-\(\alpha\)-trypsin inhibitor (21). Urine also contains a low molecular weight, 16,000, acid stable trypsin inhibitor (209) which is immunologically related to the plasma inhibitor (210). The mucus inhibitor differs in amino acid composition from the plasma and urinary inhibitors (207). Since both types of inhibitors are immunologically related to inter-\(\alpha\)-trypsin inhibitor they must be split from different parts of the molecule (207).

7. \(\alpha_2\)-MACROBLOBULIN

Early procedures used for purification of \(\alpha_2\)-macroglobulin involved ethanol precipitation, ultracentrifugation and ammonium sulfate precipitation plus zone electrophoresis (211,212). More recently Roberts et al. (213) isolated \(\alpha_2\)-macroglobulin by delipo-proteinization
using dextran sulfate plus MnCl₂, gel filtration on 6% agarose and Sephadex G 200. Song et al. (214) used polyethylene glycol fractionation and gel filtration on Sephadex 200 to purify this protein.

α₂-Macroglobulin is a high molecular weight plasma inhibitor in the range of 725,000 (215) - 820,000 (212) with a carbohydrate content of 7.7% (127) - 8.4% (216). The physical constants for this inhibitor are given in Table 3.

Table 3. PHYSICAL PROPERTIES OF α₂-MACROGLOBULIN

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>Molecular Weight</td>
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</tr>
<tr>
<td>Sedimentation Equilibrium</td>
<td>726,000</td>
<td>(213)</td>
</tr>
<tr>
<td></td>
<td>820,000</td>
<td>(212)</td>
</tr>
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<td></td>
<td>725,000</td>
<td>(215)</td>
</tr>
<tr>
<td>Sedimentation Coefficient</td>
<td>19.6</td>
<td>(212)</td>
</tr>
<tr>
<td>Diffusion Coefficient</td>
<td>2.41</td>
<td>(212)</td>
</tr>
<tr>
<td>Isoelectric Point</td>
<td>5.4</td>
<td>(212)</td>
</tr>
<tr>
<td>Extinction Coefficient 1% Sol 280nm</td>
<td>8.1</td>
<td>(217)</td>
</tr>
</tbody>
</table>

Various investigators (108, 130, 218) have noted that α₂-macroglobulin splits into two 380,000 molecular weight components upon treatment with 0.1% SDS. In the presence of β-mercaptoethanol, the 380,000 molecular weight components split further into 190,000 fragments (42). Trypsin, thrombin, kallikrein and plasmin cleave the quarter fragments into two 95,000 molecular weight fragments. These two fragments can be released by β-mercaptoethanol (103). Chymotrypsin, in contrast, causes the formation of 90,000 and 75,000 molecular weight derivatives, as well, indicating a second sensitive bond (218).
Harpel (218) suggests the following structure for α2-macroglobulin based on cleavage and denaturation studies:

\[
\begin{align*}
&\text{NH}_2 \quad \text{COOH} \quad \text{NH}_2 \quad \text{COOH} \quad \text{NH}_2 \quad \text{COOH} \\
&\quad \quad \text{S-S-} \quad \quad \text{S-S-} \quad \quad \text{S-S-} \quad \quad \text{S-S-} \\
\text{Trypsin} \quad \text{Plasmin} \quad \text{Thrombin} \quad \text{Kallikrein} \\
\text{NH}_2 \quad \text{COOH} \quad \text{NH}_2 \quad \text{COOH} \\
&\quad \quad \text{S-S-} \quad \quad \text{S-S-} \quad \quad \text{S-S-} \quad \quad \text{S-S-} \\
\end{align*}
\]

Figure 3. STRUCTURE OF α2-MACROGLOBULIN (218)

Removal of sialic acid from α2-macroglobulin does not alter the trypsin binding capacity of the inhibitor (219), again suggesting sialic acid is not involved in the inhibitory process.

Five different electrophoretic forms of α2-macroglobulin have been separated on polyacrylamide gels (217). Only the slower moving species have protease binding capacity. These, however, may be due to molecular alterations as a result of prolonged contact with Tris buffer (42) or other artifacts of the purification procedure. Galiango and Castillo (231), however, report three types of α2-macroglobulin which are believed to be genetic variants.
8. Cl INACTIVATOR

Cl inactivator is a 104,000 molecular weight glycoprotein with 34.7% carbohydrate content (127).

9. INTER-α-ANTIPRASEMIN

Gallimore (137) purified inter-α-antiplasmin by removal of euglobulins, ammonium sulfate precipitation, ion exchange chromatography on DEAE cellulose, gel filtration on Sephadex G 200 and G 75, isoelectric focusing and preparative agarose gel electrophoresis. The molecular weight of this inhibitor is 80,000 (137).

10. PLASMINOGEN ACTIVATOR INHIBITOR

The plasminogen activator inhibitor isolated by Hedner (133, 139) is an α2-globulin with a molecular weight of 75,000 and is acid and heat labile.

11. SEMINAL PLASMA INHIBITORS

The two acid stable seminal plasma protease inhibitors have been isolated by absorption onto SP Sephadex followed by elution with a sodium chloride gradient, affinity chromatography on trypsin-cellulose plus gel filtration on Sephadex G 75 and fractionation on SP Sephadex (140). They also have been purified by treatment of spermatozoa with detergent and Sephadex gel filtration (220).

The trypsin-chymotrypsin inhibitor has a molecular weight of 11,000 while the trypsin-acrosin inhibitor has a molecular weight of 6,200 (221, 222). The trypsin-chymotrypsin inhibitor may not have a specific trypsin amino acid residue since chemical modification of the
lysine and arginine residues did not prevent inhibition of trypsin (140).

The boar seminal acrosin inhibitor, which has been sequenced, has a high degree of homology with porcine secretory pancreatic inhibitor (11).

12. CERVICAL MUCUS INHIBITOR

Cervical mucus inhibitor is isolated by treating cervical mucus with perchloric acid followed by gel filtration on Sephadex G 50 at pH 2.2 (141). The 11,500 molecular weight acid stable inhibitor from human cervical mucus is very similar in amino acid composition to the corresponding molecular weight inhibitor in seminal plasma (141) and also cross-reacts immunologically with it (11).

13. PANCREATIC INHIBITOR

Greene et al. (12) have isolated human pancreatic secretory inhibitor from pancreatic juice using gel filtration on Sephadex G 75, ion exchange chromatography on DEAE cellulose and SP Sephadex. Five iso-inhibitors were isolated by this procedure (12).

Feinstein et al. (14) have isolated two similar inhibitors from human pancreas. A 45,000 x g supernatant was purified using gel filtration of Sephadex G 75, affinity chromatography on Sepharose-trypsin, and isoelectric focusing (14).

The human pancreatic secretory inhibitor from pancreatic juice contain fifty-six amino acids with three disulfide bonds (223). Greene and Bartelt have determined the amino acid sequence of this protein (228). In comparing the sequence of the human protein to that
of porcine and bovine pancreatic inhibitor, they concluded that the
differences in inhibitory activities towards different types of
proteases are due to amino acid substitutions near the reactive site
(224).

Both inhibitors isolated from human pancreas have similar amino
acid components as the pancreatic juice inhibitors and the bovine
Kazal inhibitor (14). They do, however, have fewer total residues.
One has 48 amino acid residues while the other has 39 amino acids (14).

C. DISTRIBUTION OF PROTEASE INHIBITORS

The plasma protease inhibitors in general are widely spread
throughout the body. Similarities between the inhibitors in the mucus
of the respiratory system, in the genital system, and the urinary
system suggest that these inhibitors may come from a common precursor.
In this section the normal distribution of the inhibitors will be dis­
cussed while the distribution under pathological conditions will be
discussed in the following section.

1. \( \alpha_1 \)-ACID GLYCOPROTEIN

The normal mean level of \( \alpha_1 \)-acid glycoprotein in serum is 0.90
mg/ml (225). This glycoprotein is also found in skin extracts (226-227),
edema fluids (228), urine (229), cerebrospinal fluid, saliva, bile,
bronchial secretions, human milk and colostrum, seminal fluid (225)
and synovial fluid (231).

2. \( \alpha_2 \)-ANTITRYPSIN

The normal serum mean level of \( \alpha_1 \)-antitrypsin is 2.2 mg/ml (232).
This inhibitor is additionally found in skin extracts (226,227), edema
fluids (228), urine (229), amnionic fluid (233), cerebrospinal fluid, saliva, bile, bronchial secretions, human milk and colostrum, seminal fluid (225), cervical mucus (234), lymph (235), synovial fluid (231) and aqueous humor (236). α₁-Antitrypsin is also present in alveolar macrophages (51), in the cytoplasm of human mast cells of skin and cervix uteri (237) and in the fallopian tubes (234). This inhibitor is also located in the pulmonary interstitium, within pinocytotic vesicles, in the endothelial and type I alveolar epithelial cells and within the alveolar lining (238). The liver contains an 18,000 molecular weight protein which cross reacts with α₁-antitrypsin (239).

3. α₁-ANTICHYMOTRYPSIN

The mean level of α₁-antichymotrypsin in human plasma is 0.45 mg/ml (127). It is also found in human seminal plasma, cervical mucus and the fallopian tubes (234).

4. ANTITHROMBIN III

Human serum contains a mean level of 0.23 mg/ml of antithrombin III (240). It is also found in cervical mucus (234).

5. HEPARIN

One major source of heparin is in mast cells (240) with the intestine (241), lung and skin as sources (242). Very little endogenous heparin is normally found in the blood.

6. INTER-α-TRYPSIN INHIBITOR

The normal range of inter-α-trypsin inhibitor content in normal plasma is 0.20 to 0.70 mg/ml (127). It is found in cervical mucus
The immunologically related inhibitors are found in the tissue or fluid in which the name implies such as plasma, urine and respiratory mucus (207).

7. \(\alpha_2\)-MACROGLOBULIN

The serum level of \(\alpha_2\)-macroglobulin varies between men and women and with age (243). Levels of this inhibitor are very high in umbilical cord blood and in young children (235,244). The level declines rapidly from age 15 to 20 and then slowly from age 30 to 40 (243). The mean level for adult nonpregnant females is 2.9 mg/ml and for adult males is 2.4 mg/ml (225).

\(\alpha_2\)-Macroglobulin is found in saliva (230), edema fluids (228), gastric juice (245,246), intestinal juice (230), sputum (230), cervical mucus (247, 248), synovial fluid (249), lymph (235) and bronchial mucus (250). It is also found in the parenchymal cells of the liver (251, 252), on the surface of lymphocytes (253) and in human platelet membrane and granule fractions (254). This inhibitor is additionally found on the luminal surface of endothelial cells of the arteries, veins and lymphatics (252). In the liver the endothelial cells of the sinusoids also have \(\alpha_2\)-macroglobulin on the luminal surfaces (252). In the kidney the luminal surface of the blood vessel endothelial cells but not the renal tubular epithelium have this inhibitor attached (252).

8. Cl-INACTIVATOR

The normal concentration range for Cl-inactivator in plasma is 0.15-0.35 mg/ml (127). It is also found in 5-10% of normal hepatic parenchymal cells (255) and in cervical mucus (234).
9. OTHER INHIBITORS

Inter-α'-plasmin inhibitor and the plasminogen activator inhibitor are found in plasma (15). The seminal plasma inhibitors and the cervical mucus inhibitor occur in the respective fluid and associated tissues (234). The acrosin inhibitor is found in testicular and epididymal tissues, spermatozoa and fluids (10). The Kazal type acidic secretory pancreatic inhibitor is present both in the pancreas (10) and the pancreatic juice (12).

D. SYNTHESIS AND DEGRADATION OF INHIBITORS

The plasma protease inhibitors are synthesized in the liver (230) while the pancreatic secretory inhibitor is synthesized by the pancreas (12) and the seminal plasma inhibitors and the cervical mucus inhibitors are synthesized by the endothelial cells of the respective organs (141). Embryonic hepatic tissues contain α₁-antitrypsin, Cl-inactivator, and α₂-macroglobulin at 29 days (256). Preliminary experiments by Tunstall and James (257) indicate that α₂-macroglobulin is synthesized by human lymphocytes.

The rate and mechanism of degradation of inhibitors varies from one inhibitor to another. In general, inhibitors complexed with proteases are degraded much faster than free inhibitors.

1. α₁-ACID GLYCOPROTEIN

The half-life for α₁-acid glycoprotein is 5.2 days in plasma with 11.5-14% of the body pool of the glycoprotein being degraded per day (258).
2. α₁-ANTITRYPSIN

Liebermann (259) reported α₁-antitrypsin is spontaneously degraded at a specific rate with abnormal α₁-antitrypsin molecules being degraded faster. After 6 hours only 45% of injected labeled α₁-antitrypsin was present in the vascular space. The half life for α₁-antitrypsin was 47 hours (260). After 5 minutes 50% of ¹³¹I trypsin-α₁-antitrypsin was still present in the circulatory system and completely removed in 3½ hours (128). ¹³¹I-Elastase-α₁-antitrypsin complexes were removed in 1-1½ hours (128). The radioactivity over the liver reached a maximum after 65-70 minutes with the ¹³¹I-trypsin-α₁-antitrypsin complex and after 45-60 minutes with the ¹³¹I-elastase-α₁-antitrypsin complex (128).

In the presence of excess protease, protease-α₁-antitrypsin complexes are readily degraded (61). The protease portion is degraded much more rapidly than the α₁-antitrypsin portion (61). With excess trypsin the α₁-antitrypsin complex is degraded progressively to 63,000, 54,000 and 50,000 molecular weight fragments (65).

The liver takes up MZ and ZZ antitrypsin in the same manner as asialo-antitrypsin and other asialo-glycoproteins into the Kupffer cells (261). Normal MM α₁-antitrypsin is not taken up by the same mechanism (261).

3. INTER-α-TRYPSIN INHIBITOR

After 3 hours, 45% of labeled injected inter-α-trypsin inhibitor and inter-α-trypsin inhibitor protease complexes remain in the vascular compartment. The half-life for this inhibitor and its complex with proteases is 40 hours (261).
4. $\alpha_2$-MACROGLOBULIN

After 1 hour in the circulatory system, 75% of injected labeled $\alpha_2$-macroglobulin-protease remained. After 2½ hours 60% $\alpha_2$-macroglobulin, 16% $\alpha_2$-macroglobulin-thrombin and 13% $\alpha_2$-macroglobulin-plasmin complexes remained (261). The half-life of $^{131}$I elastase-$\alpha_2$-macroglobulin complexes was 12 min. and of $^{131}$I trypsin-$\alpha_2$-macroglobulin complexes was 9-10 minutes (128).

In the dog the $^{131}$I-trypsin-$\alpha_2$-macroglobulin complexes are cleared from the circulatory system and deposited in the liver, spleen and bone marrow in cells of the reticulo-endothelial system (262). Proteinase-$\alpha_2$-macroglobulin complexes are taken up rapidly and selectively by rabbit alveolar and peritoneal macrophages but not by peripheral leukocytes (263). The complex first binds to the membrane which is dependant on the presence of Mg$^{++}$ or Ca$^{++}$ and is pH sensitive. The bound complex undergoes endocytosis which is inhibited by iodoacetamide. The complex is then hydrolyzed in a temperature dependant manner (264).

E. EVOLUTION OF INHIBITORS

There is quite a bit of homology among mammalian species inhibitors. The human, porcine, bovine and ovine pancreatic secretory inhibitor are identical in 37 of the 56 amino acid residues (223). The sulfhydryl bridges are in the same position in all three inhibitors (223).

Other proteins having structural homology to the pancreatic secretory trypsin inhibitor are the subtilisin inhibitor from Streptomyces albogriseolus, avian egg white trypsin inhibitor, acrosin inhibitor from boar seminal plasma and epidermal growth factor.
from mouse submaxillary gland (223). The Kunitz bovine pancreatic trypsin inhibitor has structural homology with turtle egg white inhibitor, bovine colostrum inhibitor, snail K inhibitor, Russell's viper venom inhibitor and toxin K and I inhibitors from black mamba venom.

There is evidence for gene elongation/multiplication of a precursor gene of a single inhibitory site inhibitor to produce two inhibitory site inhibitors (265). Examples of such inhibitors are the multisite inhibitors, lima bean proteinase inhibitor, soybean trypsin inhibitor and the avian ovomucoids (205).

A significant degree of homology is found between one 43 residue CNBr segment of $\alpha_i$-acid glycoprotein and the amino terminal end of the variable region of the K type L chain of human IgG as well as between the carboxy terminal end of $\alpha_i$-acid glycoprotein and the constant region of the H chain of IgG (147).

Immunologically human $\alpha_i$-antitrypsin is identical to the gorilla, chimpanzee, cerecophitecoidea and ceboidea (266). Human $\alpha_i$-antichymotrypsin and inter-$\alpha$-trypsin inhibitor are immunologically identical with the corresponding inhibitors in Gorilla gorilla and Pan traglagytes (266). Cl-inactivator is immunologically identical between man and anthropoid primates as the gibbon and only partially identical to the baboon (42).

Human $\alpha_2$-macroglobulin possesses 8 major antigenic determinants; of these 7 are present in the monkey, 5 in the goat, sheep, cow, donkey and zebra, 3 in the dog and cat, 2 in the pig and 1 in the guinea pig (267). Two protease inhibiting $\alpha_2$-macroglobulins are found in the dog (268) rabbit and rat (42).
F. PHYSIOLOGICAL FUNCTIONS OF INHIBITORS

The general function of protease inhibitors is to protect proteins from being broken down or activated in the case of zymogens. By inhibiting the enzymes in cascade type systems such as the clotting, kallikrein, complement and the renin-angiotensin systems, the inhibitors limit the activation of these systems.

α₂-Macroglobulin has several unique physiological roles as a result of the unusual types of reactions with proteases. It may function to protect proteases from inactivation by other protease inhibitors, and to convert proteases into peptidases which can cleave toxic peptides without affecting native proteins (269). Since the rate of turnover of α₂-macroglobulin-protease complexes is high, it also probably functions as a trap for enzymes leading to degradation (44,50).

1. BLOOD CLOTTING SYSTEM

Plasma proteinase inhibitors interact with the active forms of factors XII (Hageman Factor), XI (Plasma thromboplastic antecedent), X (Stuart factor) and II (Prothrombin) (42). The following figure shows the cascade effect of the clotting systems (42).

Factor XII is inhibited by α₁-antitrypsin and Cl-inactivator while factor XI is inhibited by these two inhibitors, as well as antithrombin III. Factor Xa and thrombin are inhibited progressively by antithrombin III but are inhibited instantaneously with the addition of heparin (42). Thrombin is additionally inhibited by β-lipoprotein, α₂-macroglobulin, Cl inactivator and α₁-antitrypsin (42). Factor VII is a glycoprotein which is inactivated by DFP (271) but is not in-
**INTRINSIC SYSTEM**

Hageman Factor (XII) → Contact → Active Hageman Factor

PTA(XI) → $\text{Ca}^{++}$ → Active PTA

PTC(IX) → $\text{Ca}^{++}$ → Active PTC

Antihemophilic Factor (VIII) → $\text{Ca}^{++}$ → AHF

Blood Convertin → Stuart Factor (X) → Active Stuart Factor

Proaccelerin (V) → Active Proaccelerin

Cephalin

Prothrombinase

Prothrombin (II) → Thrombin → Fibrinogen (I) → Fibrin

**EXTRINSIC SYSTEM**

Contact → Factor VII → Active Factor VII (Proconvertin)

Tissue Thromboplastin

Ca$^{++}$ → Tissue Convertin → Blood Convertin

Stuart Factor (X) → Active Stuart Factor

Proaccelerin (V) → Active Proaccelerin

Cephalin

Prothrombinase

Prothrombin (II) → Thrombin → Fibrinogen (I) → Fibrin

Figure 4: CLOTTING SYSTEM SCHEME (270).

Inhibited by antithrombin III (77). Factor IXa is also inhibited by antithrombin III (77). Heparin additionally interacts with factor XIII causing the loss of its fibrin stabilizing activity (272). It forms a complex with fibrinogen (273) and prevents the activation of factor VIII by factor IXa (274).
Antithrombin III may not be the major antithrombin in blood because no correlation is observed between the antithrombin III level and the antithrombin activity of plasmin (275).

The biological balance favors antiproteinases in this system but the thrombin-fibrin reaction occurs at much greater rates than the thrombin-inhibitor reaction (42).

2. FIBRINOLYSIS SYSTEM

The exact mechanism of fibrinolysis is not known. Two possible schemes are given below (276):

**SCHEME A**

- Fibrinogen
- Plasminogen → Thrombin → Fibrinogen
- Plasminogen → Fibrin → Plasmin → Soluble Fibrin Peptides

**SCHEME B**

- Fibrinogen
- Thrombin → Activator → Fibrin → Plasmin → Soluble Fibrin Peptides
- Plasminogen

Figure 5: FIBRINOLYTIC SYSTEM (276).

The activity of this system is controlled at two points. The plasminogen activator is inhibited by the plasmin activator inhibitor (138,139) and plasmin is inhibited by inter-α-plasmin inhibitor (137), α₁-antitrypsin (1), antithrombin III (68,69),
inter-α-trypsin inhibitor (7), α₂-macroglobulin (3) and Cl-inactivator (1). α₂-Macroglobulin is the most important plasmin inhibitor in plasma due to the high rate of inhibition (277).

Since kallikrein acts as a plasminogen activator (277), the inhibitors of the kallikrein system are also involved in the fibrinolysis system (Section F3).

3. KALLIKREIN SYSTEM

The general scheme for the kallikrein system is given below (42).

Bradykinin is an example of an active peptide formed by this system. Bradykinin in nanogram quantities causes hypotension and increased vascular permeability (42).

This system is inhibited at three levels. Hageman factor (XII) is inhibited by Cl-inactivator and α₁-antitrypsin (42). Kallikrein is inhibited by α₂-macroglobulin, Cl inactivator, antithrombin III (42), α₁-antitrypsin (38) and inter-α-trypsin inhibitor (104). The
inactivation of the active peptides by the active kininases is the third level of control (42). In plasmas with adequate protease inhibitors antithrombin III does not play a major role in the inhibition of kallikrein (66, 67).

4. RENIN ANGIOTENSINOGEN SYSTEM

The renin-angiotensinogen system is another cascade system which relies on proteolytic enzymes (42).

Angiotensinogen ($\alpha_2$-globulin) 
$\downarrow$ Renin
Angiotensin I (decapptide) 
$\downarrow$ converting enzyme
Angiotensin II (octapeptide) 
$\downarrow$ hepta-hexapeptide
$\downarrow$ Biologically and immunologically inactive products

Figure 7: RENIN-ANGIOTENSINOGEN SYSTEM

Renin is a tissue protease increasing during renal essential hypertension. The only serum protease inhibitor known to inhibit renin is $\alpha_1$-antitrypsin (55).

5. COMPLEMENT SYSTEM

In the classical scheme of the complement system Clr, Cls, C3-convertase, [a complex between C2a and C4b (C42)] and C3-dependent peptidase [a complex between C2a, C4b and C3b (C423)] possess proteolytic activity (42). The protein C3b inactivator is a trypsin-like enzyme which cleaves C3b into fragments C3c and
C3d (42). In the alternate complement pathway C3 proactivator convertase and C3 activator possess proteolytic activity (42).

The complement system is represented by the following scheme (42).

**ALTERNATE SYSTEM**

Activating substances
- F(ab')2
- Endotoxin
- Zymosan
- Inulin

Proenzyme → C3PA Convertase

**CLASSICAL SYSTEM**

Fc

\[
\text{IgG 1,2,3, IgM}
\]

Cl → Cl

\[
\text{Cl4,2} \rightarrow \text{Cl42}
\]

C3 Proactivator (C3PA)

CoF → Mg

Complex

CoF-3PA

C3 Activator

\[
\text{G3-9 Consumption} + \text{Biologically Active Products}
\]

Figure 8: COMPLEMENT SYSTEM (42)

Cl inactivator is the only inhibitor of these enzymes which inhibits only Cls and Clr (42). Heparin interacts with Cl inactivator potentiating the inhibitory activity of Cl inactivator (278). The total amount of this inhibitor would inhibit only about 1/3 of the possible Cls activity (42).
6. IMMUNE SYSTEM

Protease inhibitors interact with this system both through actions on the immune system cells and the soluble factors which control the actions of these cells. The complement system is part of the immune system.

The surface association of $\alpha_2$-macroglobulin with lymphocytes suggest that this inhibitor probably protects these cells and modulates the proteolytic reactions occurring on the surfaces (252). At physiological concentrations, $\alpha_2$-macroglobulin inhibits mitogenic and antigenic stimulation of lymphocytes; at lower concentrations, the inhibitor acts in synergy with the mitogen or the antigen (5). Soybean trypsin inhibitor inhibits transformation of leukocytes by mitogens (279).

$\alpha_2$-Macroglobulin inhibits the chemotactic activity of both kallikrein and plasminogen activator while Cl inactivator inhibits only the chemotactic activity of kallikrein (280). $\alpha_2$-Macroglobulin, $\gamma_1$-antitrypsin, Cl inhibitor, and antithrombin III interact with the macrophage membrane enhancing the activity of migratory inhibitory factor (2).

7. PANCREATIC SYSTEM

The secretory pancreatic inhibitors protect the pancreatic tissue from premature activation of the zymogens produced by the pancreas (14). The presence of these inhibitors in the pancreatic juice would also protect the enzyme precursors from being activated during the passage from the pancreas to the duodenum (12).
8. INTESTINAL TRACT

The intestinal mucosa of the small intestine does not contain inhibitors of chymotrypsin or trypsin (281). The duodenal and jejunal tissues, however, do contain low molecular weight acid stable inhibitors (282) which probably protect the cells from destruction when the pancreatic proteases bind to the mucosa in the active form (281). These proteases can then diffuse, as shown for rats, into the mesenteric vein (283). Both the colon tissue (282,284) and the mucosa of the colon contain (281) inhibitors of chymotrypsin and trypsin which protect the tissue from degradation. The intestinal juice additionally contains \( \alpha_2 \)-macroglobulin (230).

The plasma inhibitors, due to their ability to inhibit pancreatic proteases as trypsin, chymotrypsin, elastase and kallikrein, then protect plasma proteins from these proteases which may diffuse into the blood (43). Since the low molecular weight intestinal inhibitors also inhibit leukocyte proteases, they probably protect the intestinal wall from destruction during inflammatory reactions (282).

9. RESPIRATORY SYSTEM

The low molecular weight mucus inhibitors and \( \alpha_1 \)-antitrypsin function to protect the respiratory system from leukocytic proteases. In response to respiratory tract infections large numbers of leucocytes are mobilized (207). These cells in the extravascular compartment disintegrate rapidly releasing proteases which have an alkaline pH optimum (207). The proteases digest elastic lung tissues and basement membranes in vitro and are believed to be active mediators of inflammation (207).
The low molecular weight mucus inhibitors and \( \alpha'_1 \)-antitrypsin inhibitors have been identified bound to leukocyte proteases \((6, 285)\). \( \alpha'_1 \)-Antitrypsin and \( \alpha'_2 \)-macroglobulin protect the respiratory tract from the proteases of invading microorganisms \((285, 286)\).

10. REPRODUCTIVE SYSTEM

The trypsin-acrosin inhibitor in the male functions to protect the epididymal and testicular tissues and the proteins present in the seminal plasma from prematurely activated acrosin \((10)\). In the female the acrosin inhibitors, \( \alpha'_1 \)-antitrypsin, inter-\( \alpha \)-trypsin inhibitor and antithrombin III, are found in the cervical mucus. The levels of these inhibitors are quite low at ovulation and are highest near the menstrual period \((10)\). This indicates a protective function and a role in the fertilization process \((140)\) for these inhibitors.

The trypsin-chymotrypsin inhibitors of both seminal plasma and cervical mucus plus \( \alpha'_1 \)-antitrypsin protect the respective tissues and the spermatozoa from the proteases liberated under pathological conditions \((140, 141)\). The concentration of the cervical mucus trypsin-chymotrypsin inhibitor also varies during the ovulatory cycle being highest near the menstrual period. Levels are elevated during pregnancy with the peak towards the end of term \((141)\).

The antiprotease activity of human colostrum is mainly due to \( \alpha'_1 \)-antitrypsin in contrast to bovine and porcine colostrum which contain mainly low molecular weight inhibitors \((287)\). \( \alpha'_1 \)-Antitrypsin controls the pepsin-like and trypsin-like enzymes present in human colostrum \((288)\). The major purpose of the inhibitors in the bovine and porcine systems is to prevent the inactivation of immunoglobulins
present in the colostrum by the digestive enzymes of the newborn (289). In the human this function is not as vital because the colos­
trum is not the major source of immunoglobulins since the immunoglo­
bulins in the human can pass the placental blood barrier.

H. PATHOLOGICAL CONDITIONS INVOLVING PROTEASE INHIBITORS

1. ACUTE PHASE REACTION

In the acute phase reaction, the liver increases the production of a number of serum proteins in response to trauma such as pregnancy, injury, surgery, inflammation, infection, neoplastic growth or the administration of hormones (290). The protease inhibitors, \( \alpha \)-anti­trypsin, \( \alpha \)-antichymotrypsin and \( \alpha \)-acid glycoprotein, along with C-reactive protein (CRP), haptoglobin and fibrinogen increase most rapidly of the acute phase reactants (291). The increase in CRP, a phagocytosis promoting factor and \( \alpha \)-antichymotrypsin is much more rapid than the other proteins (291). The levels of these proteins are increased in cerebrospinal fluid as well as in serum (230). The reason for increases in protease inhibitors as part of the acute phase reaction has been suggested for prevention of damage after the release of lysosomal enzymes (292).

Following bone surgery, \( \alpha \)-antitrypsin, \( \alpha \)-antichymotrypsin and Cl-inactivator increased in the manner conventional to the acute phase reaction to trauma. Inter-\( \alpha \)-trypsin inhibitor and \( \alpha \)-macroglobulin decreased in the serum (293). In general the level of \( \alpha \)-antitrypsin in serum correlates with the severity of the disease (294).
2. STEROID TREATMENT AND PREGNANCY

Women on oral contraceptives have higher serum levels of \( \alpha_1 \)-antitrypsin than normal women (295), while the level in pregnancy increases to double the normal level at delivery (282). Antithrombin III levels decrease during progesterone-estrogen therapy (296, 297) and in the second half of pregnancy (194, 298). Combined estrogen-progesterone contraceptives raise \( \alpha_1 \)-macroglobulin serum levels, while with estrogen alone, the level increases. Progesterone alone had no effect (300). The Cl-inactivator level drops during pregnancy (301).

Anabolic steroids such as ethyloestrenol, norethandrolone, and methylandrostenediol, increase the level of \( \alpha_1 \)-antitrypsin and antithrombin III and decrease \( \alpha_2 \)-macroglobulin levels. In addition oxymethalone and stanozolal increase antithrombin III levels and decrease \( \alpha_2 \)-macroglobulin levels (302).

3. BLOOD CLOTTING DISORDERS

Elevated levels of antithrombin III causes a bleeding tendency (303), while the absence of this inhibitor may cause recurrent thromboses (303-306).

Heparin is given therapeutically as an exogenous anticoagulant (81).

4. RESPIRATORY DISTRESS SYNDROME

Serum protease inhibitors may be important in the prevention of the dissolution of the hyaline membranes, prevention of pulmonary vasoconstriction, protection of pulmonary tissue against leukocyte and
macrophage proteolytic enzymes and inhibition of vasoactive substances which may lead to the development of shock in babies (307). In idiopathic respiratory distress syndrome, low levels of $\alpha_1$-antitrypsin and $\alpha_1$-antichymotrypsin are observed in the serum of these babies (307). This may be due to the loss of $\alpha_1$-antitrypsin from the serum to the hyaline membranes since $\alpha_1$-antitrypsin is highly concentrated in the hyaline membrane in respiratory distress syndrome (294, 308). This condition is not related to the inheritable $\alpha_1$-antitrypsin deficiency since all babies tested had normal MM phenotypes (294).

5. $\alpha_1$-ANTITRYPSIN DEFICIENCY ASSOCIATED CONDITIONS

The hereditary deficiency of $\alpha_1$-antitrypsin was first reported in 1943 by Laurell and Eriksson (309). In the United States, 95% of white Americans and 98% of black Americans have the normal MM allele (310). The deficient Pi S and Pi Z alleles account for 3% and 1% of the total population (310). Twenty-six alleles have been identified (181).

Persons with $\alpha_1$-antitrypsin deficiency alleles are predisposed to lung diseases and liver diseases (311). In both of these diseases, an exogenous agent must initially injure the organ. The $\alpha_1$-antitrypsin deficient individual cannot defend against the injury leading to a chronic disease (311). Homozygote ZZ individuals respond very little to acute phase stimulation such as pregnancy and inflammation in comparison to homozygote MM individuals (182). This deficiency is associated with emphysema (312, 313), asthma (312), obstructive pulmonary
disease (313), bronchitis (314) and right heart failure (315, 316).

About 20-30% of infants with the ZZ \( \alpha_1 \)-antitrypsin phenotype develop hepatitis and cirrhosis (182). The adult liver disease state is correlated with the deficient states but it is not as striking as in the childhood disease (311,317,318).

In the \( \alpha_1 \)-antitrypsin deficient state and general liver disease as well as in malignant liver, \( \alpha_1 \)-antitrypsin accumulates in cytoplasmic globules in hepatocytes found within the smooth endoplasmic reticulum (318-321). The \( \alpha_1 \)-antitrypsin found in these liver inclusion bodies has a complete lack of sialic acid and a deficiency of other carbohydrate residues (183). This inhibitor is immunologically similar to the serum \( \alpha_1 \)-antitrypsin but has no trypsin inhibitory activity. These molecules tend to aggregate (38). The major defect in the \( \alpha_1 \)-antitrypsin deficient state may be in the polypeptide part of the molecule preventing addition of carbohydrates thereby preventing transport into the circulatory system (184).

6. RESPIRATORY DISEASES

In bronchial and nasal secretions of patients with bronchitis and other chronic respiratory diseases without \( \alpha_1 \)-antitrypsin deficiency, leukocyte proteases were found complexed with \( \alpha_1 \)-antitrypsin, the low molecular weight mucus inhibitors (285), \( \alpha_1 \)-antichymotrypsin and \( \alpha_2 \)-macroglobulin (286).

The etiology of emphysema in cadmium industry workers may
be related to the selective binding of cadmium to $\alpha_1$-antitrypsin in serum lowering the inhibitory capacity of serum (322).

7. CYSTIC FIBROSIS

The $\alpha_2$-macroglobulin present in the plasma of patients with cystic fibrosis has a lower affinity for trypsin, thrombin and papain than normal (323). Obligate heterozygotes for cystic fibrosis have intermediate levels of $\alpha_2$-macroglobulin (323).

8. EYE DISEASES

$\alpha_1$-Antitrypsin is a normal constituent of human tears while $\alpha_2$-macroglobulin is present only under pathological conditions. The $\alpha_1$-antitrypsin concentration in human tears is proportional to the severity of the eye inflammatory condition (324). This inhibitor inhibits the major collagenase in rabbit but not the major collagenase in human keratoplasty tissue (324). $\alpha_2$-Macroglobulin, present in the tears of patients with eye ulcerations (324, 325) does inhibit all 3 rabbit collagenases and the major human collagenase (324).

9. DIABETES

$\alpha_2$-Macroglobulin is elevated in diabetes and may prevent normal breakdown of capillary basement membrane by leukocyte neutral proteases since these membranes are thickened in diabetic microangiopathy (326).
10. PANCREATITIS

In this disease the pancreatic secretory inhibitor is not adequate to inhibit the uncontrolled activation of the pancreatic enzymes (52). $\alpha$-Antitrypsin and $\alpha_2$-macroglobulin are found complexed with trypsin in the ascites fluid, lymph and pancreatic-duodenal venous blood in experimental pancreatitis in dogs (327). $\alpha_2$-Macroglobulin which is complexed with a trypsin-like enzyme has also been purified from pleural fluids in patients with chronic pancreatitis (328).

11. APPENDICITIS PERITONITIS

$\alpha_2$-Macroglobulin as well as $\alpha$-antitrypsin is present in the peritoneal fluid of patients with diffuse appendicitis peritonitis complexed with granulocyte collagenase and elastase (52,327).

12. ARTHRITIS

Immunologically active $\alpha$-antitrypsin, $\alpha$-acid glycoprotein and $\alpha_2$-macroglobulin are found in synovial fluid (249). In patients with osteoarthritic and other joint diseases, the $\alpha_2$-macroglobulin is immunologically active but will not inhibit trypsin, chymotrypsin or elastase (249). Normal synovial fluid contains some inactive $\alpha_2$-macroglobulin, as well (231).

In vitro inhibition by $\alpha$-antitrypsin of the hydrolysis of chondromucoprotein from cartilage by lysosomal enzymes from polymorphonuclear leukocytes has been observed suggesting a role of protease inhibitors in controlling proteolysis in rheumatoid
arthritis (329). \(\alpha_1\)-Antitrypsin Pi types MZ and SZ are more prevalent in adults with rheumatoid arthritis than normal adults (330).

13. NEOPLASTIC DISEASES

a. INHIBITORS IN HUMAN NEOPLASIA

The levels of \(\alpha_1\)-antitrypsin, \(\alpha_1\)-antichymotrypsin and \(-\)acid glycoprotein, being acute phase reactants are elevated in neoplastic diseases (290). Several months after surgical removal of the neoplasms the serum inhibitor level returns to normal (331).

Patients with early breast cancer have just above normal serum \(\alpha_1\)-acid glycoprotein levels while advanced breast cancer patients have higher levels, and recurrent breast cancer and disseminated cancer patients have still higher levels (332). The level of this glycoprotein can be used to monitor progress in recovery and prognosis (331).

The \(\alpha_1\)-acid glycoprotein levels in gastric juice of patients with gastric cancer is nearly 31 times that normally found. Both the normal and an antigenically related form of \(\alpha_1\)-acid glycoprotein are observed (333). In alveolar cancer, the \(\alpha_1\)-acid glycoprotein level in sputum also increases (334).

The \(\alpha_2\_\text{macroglobulin} level in serum is elevated in Hodgkin's disease, reticulum cell sarcoma, and is lowered in acute myeloid leukemia. In chronic lymphatic leukemia, lymphosarcoma myeloma, melanoma, pemphigoid, dermatitis, herpeliformis, bronchogenic carcinoma and breast carcinoma, the level of \(\alpha_2\_\text{macroglobulin} is near normal (243).
Early experiments showed the presence of proteolytic inhibitors in malignant and adjacent normal breast and colon tissues and malignant ovary and glioma tissue (284). Since this time, α₁-antitrypsin has been reported in malignant lung (335,336) and stomach tissues (336) as well as in malignant and normal adjacent colon, anal and breast tissues (336) and endodermal sinus (yolk sac) tumors found in sacrococcygeal, testicle, ovary and pineal tissues (337). This inhibitor also is found in malignant and adjacent normal liver (338). In yolk sac tumors, a parallelism between elevations of α₁-antitrypsin and α₁-fetoprotein exists both in serum and in tissues (337). These proteins are found in the periodic acid Schiff's positive hyaline-like globules characteristic of yolk sac tumors (337). α₁-Antitrypsin can be used as a tumor protein marker (337).

α₁-Antichymotrypsin has been identified as a component of extracts of colonic carcinomas (339).

α₁-Acid glycoprotein is closely related to the occurrence of cell division in regenerating rat liver (340). Immunohistochemical studies show that this glycoprotein decreases in the cytoplasm of hepatocytes in hyperplastic nodules but increases in the cytoplasm of bile duct cells, oval cells, transition cells and cancer cells (341).

b. EFFECT OF HEPARIN ON NEOPLASIA

Heparin therapy decreases metastasis formation by preventing the attachment of tumor emboli in the capillary beds due to inhibition of fibrin production (342). Circulating
tumor cells stay in the blood longer increasing the probability of destruction as long as the mechanism for destroying tumors is not overwhelmed (342). Heparin also can stimulate the immune system by stimulation of phagocytosis, interferon induction and immunoadjuvant action (343). In patients with inoperable or metastatic carcinoma of the lung which had failed to respond to conventional therapy, the addition of heparin to the other chemotherapeutic drugs such as cyclophosphamide, 5-flurouracil, 6-thioguanine, methotrexate and vincristine, caused regression of the tumors (344).

I. EFFECTS OF PROTEASE INHIBITORS ON TRANSFORMED CELLS IN CULTURE AND EXPERIMENTAL TUMORS IN VIVO

In general, transformed cells in culture which are treated with protease inhibitors act more like normal cells than transformed cells and regain some of the phenotypic characteristics (345).

Cell proliferation of both normal and malignant cell types such as SV3T3, Py3T3, 3T3, 3T12, PyBHK and BHK, is decreased by protease inhibitors such as TPCK, TLCK, TAME, leupeptin, ovomucoid and bovine pancreatic inhibitor (346-350). The inhibitors inhibit cell growth at all cell stages (347). Ovomucoid, pancreatic inhibitor, soybean trypsin inhibitor, NFGB and TLCK alter the morphology of the cells giving them more normal characteristics (346,349-352). Soybean trypsin inhibitor, ovomucoid, NFGB and TLCK increase the adhesiveness of transformed cells and decrease hexose transport in these cells (350).
These effects are probably due to inhibition of the plasminogen activator and the plasmin produced by this enzyme by interacting with the plasminogen in the culture media (351, 353, 354). The plasminogen activator is present in both normal and transformed cells but is higher in transformed cells (354). Besides the plasminogen activator, another enzyme which is present in transformed cells may be inhibited by the inhibitors. This enzyme hydrolyzes fibrin without plasminogen activation (355).

Leupeptin, TAME, TPCK and TLCK inhibit chemically induced mouse skin tumorigenesis (356, 357). $\alpha_2$-Macroglobulin causes regression of DANA-435 tumors in rats (358).

J. METHODOLOGY

1. AFFINITY CHROMATOGRAPHY

Affinity chromatography is a technique used frequently to separate proteinases. Insolubilized enzymes have proven quite useful for the purification of proteinase inhibitors in a one or two step process. Fritz, Schultze, Neudecker and Werle (359) reported the isolation of the Kunitz inhibitor from crude extracts from bovine pancreas by the use of ethylene maleic anhydride bound to trypsin. The human secretory inhibitor has been isolated by Feinstein et al. (14) using Sepharose-trypsin. $\alpha_1$-Antitrypsin has been isolated using various affinity chromatography columns (61, 171-174).

In these affinity chromatography methods, the enzymes or other specific molecules were attached directly to cyanogen activated agarose (14, 61, 171-174).
Agarose consists of repeating units of D-galactose-3, 6-anhydro-L-galactose in double helixes between adjacent polysaccharide chains. During the activation of agarose with cyanogen bromide, it is postulated that the two chains of the double helix are linked by linear imidocarbonate groups which would account for the greater heat stability of CNBr-activated agarose over agarose (360).

![Structure of Activated Agarose](image)

**Figure 9: STRUCTURE OF ACTIVATED AGAROSE (361).**

The active site to which the enzyme or other molecule can be attached is the cyclic imidocarbonate end. This can react with an enzyme or other molecules to form three types of derivatives; N substituted imidocarbonate, N substituted carbamate and isourea derivatives (361).
Figure 10: AGAROSE CYANOCEN BROMIDE ACTIVATION PRODUCTS (361).

One problem in the synthesis of the Sepharose-enzyme complex has been in stabilizing the pH during the activation reaction using CNBr. In the methods reported by Porath, Axen and Ernbach (362) and by Guatrecasas and Anfinsen (363) the pH is maintained at 11 by the titration of NaOH as the activation reaction occurred. The need for titration can be avoided by the use of a concentrated buffer. Porath, Aspberg, Drevin and Axen (364) reported a method for the activation of Sepharose using 5 M phosphate buffer in the
Sepharose-CNBr activation reaction. A similar method reported by March, Parikh and Cuatrecasas (365) uses a 2 M solution of sodium carbonate and an acetonitrile solution of cyanogen bromide.

There are some inhibitors which are sterically hindered from binding to an enzyme attached directly to the agarose molecule due to the position of the enzyme on the double helix shape of the agarose molecule. By constructing an "arm", a carbon-carbon chain or a carbon-nitrogen chain, which acts as a spacer group between the agarose molecule and the enzyme, additional inhibitors can be purified by the technique of affinity chromatography. In the presence of spacer groups the support material has a higher binding capacity for the enzyme. Several derivatives of agarose which have been developed include acyl azide derivatives (366), w-aminoalkyl derivatives, bromoacetyl derivatives, succinylaminoethyl derivatives, tyrosyl derivatives (363,367,368) and aminoalkyl-N-hydroxysuccinimide ester derivatives (369).

The aminoalkyl-N-hydroxysuccinimide ester derivative is of particular interest because of its commercial availability, the ease of attachment of chymotrypsin to this derivative and the possibility of only a few side reactions in the chymotrypsin coupling step. The aminoalkyl-N-hydroxysuccinimide esters of agarose are normally prepared by reacting cyanogen activated agarose with the appropriate aminoalkyl molecule to give the desired "arm" (361) and then with succinic anhydride to form the succinylated derivative (369).
The succinylated derivative is then reacted with \( N,N' \) di-cyclohexylcarbodiimine and \( N \)-hydroxysuccinimide in dioxane to yield the active \( N \) hydroxysuccimide ester of agarose (369).

A \( N \)-hydroxysuccinimide derivative sold under the name Affi Gel-10 undergoes the last reaction given in Figure 11, in the presence of an enzyme (RNH2) (370).

The advantage of the spacer group between the enzyme and the helical agarose molecule can be visualized by comparing the last structure in Figure 11 with the structures of the three types of compounds (Figure 10) which result when the enzyme is coupled directly to the cyanogen bromide activated agarose molecule.

2. ULTRAFILTRATION

Ultrafiltration is one of many techniques which can be used for the concentration of biological solutions (371,372). The ultrafiltration process involves forcing the solvent through a semipermeable membrane under hydraulic pressure. The membranes used for ultrafiltration are permeable to solvents and small molecules but are impermeable to larger molecules. Since the size of the molecules held back depends upon the pore size of the ultrafiltration membrane, ultrafiltration can be used for partial purification as well as concentration (373,374). The pH and the ionic strength remains constant on both sides of the membrane because buffer salts are normally permeable (375). The permeability of molecules in solutions through a given membrane of a specific pore size depends upon the type of molecule;
Figure 11: Formation and Reaction of the N-Hydroxy-Succinimide Derivative of Agarose
ie. globular, branched or linear; its cross sectional diameter; the ionic strength of the solution and the pH of the solution. Since the diameter of proteins is very roughly proportional to the molecular weight, the manufacturers of ultrafiltration membranes often classify their products in terms of the molecular weight of the proteins each type of membrane will hold back; ie. an Amicon Diaflo XM 50 ultrafiltration membrane has a molecular weight cut off of 50,000. This is only a very rough approximation because the higher the charge on a molecule and the lower the ionic strength of the solution, the larger is the effective size of a molecule for a given molecular weight towards the membrane (375).

A comparison of four ultrafiltration membranes available from Amicon Corporation, Diaflo XM 50, PM 30, PM 10 and UM 2 is given in Table 4 (375). These membranes have a two part construction consisting of a thin (0.1-0.2 microns) layer of ultrafine pore material which functions as the molecular separation barrier and below a porous open celled foam which supports the skin without altering the flow resistance. Because the top layer is so thin, it is highly permeable to the solvent and is quite free from becoming plugged by the solute (375).

The molecular transport mechanism which separates the solute and solvent depends upon the pore size of the ultrafiltration membrane. In membranes of relative small pore sizes (molecular weight cut off 500-2000 ie. UM 2), the solvent and
### TABLE 4
COMPARISON OF PROPERTIES OF ULTRAFILTRATION MEMBRANES (375)

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Type of Material</th>
<th>Apparent Membrane Pore radius (Å)</th>
<th>Water Permeability at 100 psi (ml/cm²·min)</th>
<th>Nominal Membrane Compartment Molecular Weight Range</th>
<th>Molecules Retained (Examples)</th>
<th>Protein Retentivity % Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaflo XM 50</td>
<td>Amicon Anisotropic Substituted Olefin</td>
<td>33</td>
<td>0.7</td>
<td>50,000</td>
<td>Globular Proteins Alubmin 69,000 m.w.</td>
<td>Aldolase (142,000) 85</td>
</tr>
<tr>
<td>Diaflo PM 30</td>
<td>Amicon Anisotropic Aromatic Polymer</td>
<td>22</td>
<td>0.7</td>
<td>30,000</td>
<td>Peptain 35,000 m.w.</td>
<td>Albumin (67,000) 68</td>
</tr>
<tr>
<td>Diaflo PM 10</td>
<td>Amicon Anisotropic Aromatic Polymer</td>
<td>19</td>
<td>0.3</td>
<td>10,000</td>
<td>Cytochrome C 13,000 m.w.</td>
<td>Hemoglobin (64,500) 62</td>
</tr>
<tr>
<td>Diaflo UM 2</td>
<td>Amicon Anisotropic Polyelectrolyte Complex</td>
<td>12</td>
<td>0.1</td>
<td>2,000</td>
<td>Polyelectrolyte Complex</td>
<td>α-Chymotrypsin (24,500) 0</td>
</tr>
</tbody>
</table>

Molecules Retained:
- Globular Proteins: Alubmin 69,000 m.w.
- Branched Polysaccharides: Dextran 250 m.w.
- Linear Flexible Polymers: Polyethylene Glycol 20,000 m.w.
- Protein Retentivity % Retention:
  - Aldolase (142,000): 85%
  - Albumin (67,000): 68%
  - Hemoglobin (64,500): 62%
  - α-Chymotrypsin (24,500): 0%
  - Trypsin (20,000): 0%
  - Myoglobin (17,800): 0%
  - Cytochrome C (12,400): 0%
  - Cyanocobalamin (1355): 0%
solute both seem to migrate through the membrane by the process of dissolution and molecular diffusion (374). Solvent flux in this type of membrane is linear with applied pressure while solute flux remains constant. In other words, the solute retentiveness increases hyperbolically with pressure.

In membranes of relatively large open pores (molecular weight cut off greater than 5,000 i.e. PM 30, PM 10 and XM 50) separation occurs by a "molecular sieving" mechanism (381). The flux of the solvent through these membranes and the flux of the solute, which is small enough to permeate the pores of the membrane, increases with applied pressure. The retentiveness of the solute which is too large to penetrate the pores is virtually independent of pressure.

In both types of membranes flux reduction occurs due to concentration polarization which occurs on the upstream surface of the membrane in a highly concentrated solution (374,375). For solutions of low concentrations, the concentration polarization is negligible. The best way to prevent concentration polarization is to stir the solutions just above the membrane (376).

3. ANALYTICAL AND PREPARATIVE

POLYACRYLAMIDE DISC GEL ELECTROPHORESIS

Disc gel electrophoresis has been used to separate proteins giving an indication of the number of individual types of protein present (377,378). Proteins are separated according to differences in charge, molecular size and shape. The poly-
acrylamide gel acts as a molecular sieve. The size of the pores of the gel depends upon the degree of polymerization and of cross-linking which is a function of the concentration of monomer, acrylamide, and co-polymer, N,N' methylene-bis-acrylamide used. A catalyst-redox system is needed to provide free radicals for polymerization in this system. Ammonium persulfate plus N,N,N'N' tetramethylethlenediamine (TEMED) or riboflavin plus TEMED are used as catalysts (377).

Disc gel electrophoresis is distinguished from continuous gel electrophoresis by the fact that disc gel electrophoresis is a discontinuous separating system with respect to pH value, buffer composition and gel pore size. The high resolving power is due to the concentrating effect, the molecular sieving effect and the electrocharge effect (378).

In the Ornstein-Davis system (377,379) of disc gel electrophoresis there are three separate gels. The sample is mixed with the monomer and co-polymer before polymerization. When there is danger of denaturing the protein during polymerization, this sample gel, which acts only to hold the sample, can be replaced by a solution of 40% sucrose containing the sample. The stacking gel is a large pore (4%) gel in which the proteins are concentrated according to the Kohlrausch regulating function (386). The concentrated stack of proteins are separated according to their molecular weight, shape and charge in the separation gel.

The progress of the proteins through the gels can be monitored using a tracking dye such as bromophenol blue which
has a higher mobility than most proteins (378).

Proteins can be detected by scanning the gels spectrophotometrically at 280 nm or by the use of various dyes. Coomassie brilliant blue (381,382) dye which reacts with NH$_4^+$ and nonpolar groups in weakly acid medium, is the most sensitive protein stain available. Procion brilliant blue reacts with hydroxyl, amino, amide and peptide groups of proteins forming a covalent nearly irreversible protein bond (387). Because of this stable bond, Procion brilliant blue is the best stain for reproducibility and densitometric evaluation. The most frequently used protein stain is Amido Black 10 (377). A quick simple stain is a direct fluorescent labeling method using anilinonaphthylene sulfonate (ANS) (383). Minimal denaturization of enzyme or antigenic activity occurs with this staining method. It can also be used to detect proteins in gels which have been stained with periodic acid-Schiff's stain leaving the periodic acid-Schiff's stained bands visible (384).

Enzymatic activity can be visualized in gels by the use of enzymatic reactions which yield colored products. For proteolytic enzymes, the specific substrates most frequently employed yield β-napthylamine upon enzymatic cleavage. Since β-napthylamine is colorless, it is coupled to either Fast Garnet GBC (385) or tetrazotized ortho-dianisidine (Fast Blue) (386) for visualization. Glutaryl-L-phenylalanine-β-naphtylamide (GPANA) is usually used as the substrate for chymotrypsin-like enzymes (387).
Glycoproteins can be detected by periodic acid-Schiff's reagent (PAS) (381). This method is based upon two reactions. In the first reaction periodic acid reacts to cleave \( \alpha \)-glycols in the carbohydrate portion of the glycoprotein molecule to yield two aldehyde groups. The Schiff's reagent then reacts with the aldehyde groups to form a characteristic magenta color.

Using 6 mm glass tubes described by the Davis method (391), 10 to 200 \( \mu \)g of protein in a volume less than 0.05 ml is needed to detect most protein components. Using micro and ultramicro techniques, protein samples in the range of \( 10^{-6} \) to \( 10^{-12} \) grams have been separated (379). Grossbach (388) has used 25 \( \mu \)m i.d. glass capillaries to separate picogram quantities of protein. Burr (389) developed a technique by which relatively large samples of dilute solutions of protein can be separated on micro gels. Large volumes of sample can be used in this system because of the two part construction of the gel tube. The sample is contained in the top part of a conical tube. Below this gel is located the stacking gel which extends into the top of a capillary tube attached to the conical tube by means of silicone tubing. The 15\% acrylamide separation gel is in the bottom of the capillary tube.

Due to the excellent separation of proteins by polyacrylamide disc gel electrophoresis, in principle, this technique is ideal for the separation of proteins on a larger preparative scale. There are, however, technical problems in resolving large quantities of proteins on polyacrylamide gels
and quantitatively recovering the proteins (378). Some of the major problems include methods of sample loading, mechanical stability of the support material, dissipation of ohmic heating, maintenance of a uniform electrical field geometry, maintenance of a hydrostatic equilibrium, elution and the length of the separation time (378). Although many types of apparatus for preparative electrophoresis apparatus have been designed, the basic design of Jovin et al. (390), which is commercially available is most often used. This system includes a glass apparatus in which the cylindrical polyacrylamide gel is cooled on the inside by a cooling finger which extends the length of the gel and on the outside by a cooling collar. The elution chamber is relatively small with removal of the sample and elution buffer from the center of the bottom of the gel. Hydrostatic pressure is maintained by a leveling system. The system was designed for maximum electrical field uniformity by the use of two circular electrodes (390).

4. IMMUNOCHEMICAL METHODS

Immunochemistry provides relatively simple techniques for the identification of large molecules. By the use of antibodies to specific antigens, quantitative measurements can be made of the amount of either the antibody or antigen. Numerous techniques have been developed to simply visualize immunological reactions as well as to quantitate them (391).

The simplest method for visualizing the precipitin reaction involves a liquid media, in which the antigen solution
is placed over the antibody solution (391). The reaction can be quantitated by isolation of the precipitate, and, using the appropriate standards, measurement of the protein content. Additional information such as the specificity of the antibody, the relative identity of antigens or antibodies or the amount of antigen can be obtained by the use of antigen-antibody reactions in solid media such as agar or polyacrylamide gels.

The identity or partial identity of immunological determinates on two molecules can be determined using double immunodiffusion techniques. For this determination, the antisera to the antigen molecule is placed in a well equidistant from the two wells in which the test antigen solutions are placed (391). The solutions are allowed to diffuse through the support media towards each other. A precipitin line is formed if there is a reaction between the antigen and antibody. If the two antigens have a set of identical determinates with respect to the antibody used, the two precipitin lines coalesce (Figure 12a). When one antigen acts more fully with the antibody, i.e. has more antigenic determinants in common with the antibody than the other, a spur is formed indicating partial identity (Figure 12b). In the case where two spurs are formed (Figure 12c) the antigens share at least one common antigenic determinant but both have non identical determinants. If the two antigens contain no common antigenic determinates the two precipitin lines cross each other (Figure 12d) (391).
Figure 12: PRECIPITATION PATTERNS IN DOUBLE IMMUNODIFFUSION

One method for quantitation of the antigen in gels is radial immunodiffusion (391). In this method the antigen is deposited in a well in the gel. A ring of precipitate is formed as the antigen diffuses into the gel which contains the antibody. After 18 hours the diameter of the ring is proportional to the logarithm of the concentration of the antigen applied. After 48 hours the radius of the ring squared is proportional to the concentration of the antigen (391).

5. SPECTROSCOPIC METHODS

Ultraviolet spectroscopy can be used as a tool to follow reactions of proteins. Simple amides, peptides and amino acid polymers in the disordered configuration give an absorption band in the range of 180-190 nm. This band is believed to be due to II → II* molecular orbital transitions (392). Aromatic
chromophores exhibit bands due to II → II* transitions of electrons from the II aromatic system to unfilled II* electron orbitals. The three aromatic amino acids, phenylalanine, tyrosine and tryptophan exhibit two major absorption bands, a weak band at 260-280 nm and a strong band at 210-220 nm (393). Of these three amino acids, phenylalanine has the lowest extinction coefficient and absorbs at the lowest wavelength. The absorption just below 260 nm is due to a "forbidden" transition. When the electronic transition is coupled with various molecular vibrations so that the symmetry barrier is somewhat removed. In tyrosine, where a hydroxyl is substituted on the benzene ring, the symmetry is destroyed and the oscillator strength is increased. The absorption of this peak at 275 nm is seven times as great as that for phenylalanine (400). The shoulder at 280 nm of the tyrosine spectra is the only visible part of the fine structure of tyrosine in aqueous solution. Upon ionization of the phenolic hydrogen, the spectra of tyrosine is shifted to longer wavelengths and the absorptivity increases. Tryptophan exhibits the strongest absorption in the 270-290 nm region and some fine structure can be observed as a pronounced shoulder at 290 nm.

The lowest energy transitions in the ultraviolet spectra of amino acid containing disulfide bridges have been assigned to n→σ* transitions of electrons from a localized nonbonding orbital to a localized antibonding orbital. These transitions in cysteine, cystine and methionine are seen as absorption bands in the 215-180 region. These peaks tail into the 260-280 nm region.
The imidazole group of histidine exhibits a strong band in the region of 185-220 nm. This absorption in proteins is hid by the aromatic amino acids and the disulfide containing amino acids.

A carboxylate band is observed in the 180-200 nm region. This has been assigned to a $\pi \rightarrow \pi^*$ transition while a weaker carboxylate band at 210 nm has been assigned to a $n \rightarrow \pi^*$ transition (393).

Spectra of proteins in the range of 180-200 nm at the very least are quite complicated, however, quite useful as the absorption is quite sensitive to conformational change. In this region the experimental problems are great. In solution work, water itself absorbs in this area as well as oxygen. The best spectra in this region are obtained under vacuum conditions using thin films and an instrument designed for use in this region. For solution work at least the nitrogen should be excluded (393).

Two useful techniques applied to ultraviolet spectra have given useful results. These are spectrophotometric titration and difference spectra techniques. The spectra of proteins and amino acids differ at various pH values. These differences are useful in the study of proteins. Tyrosine, for example, is studied at 295 nm. This technique is often coupled with the use of difference spectra. The protein being studied is placed in both reference and sample cells under different conditions. The difference in the absorption of the protein under the two sets of conditions is recorded. Experimental details such as slit width,
adherence to Beer's law, concentration of the samples and cell match (394) must be carefully considered as well as the interpretation of the difference spectra.

Generally difference spectra of proteins arise from denaturation, proton dissociation or chemical modification (393). Reactions which result in a charge effect can be followed by changing the ionic strength and the pH (395). Inductive effects are seen only when the reacted moiety is within a few angstroms of the chromophore. These effects can be followed by using increasing concentrations of urea. Chemical modification can be of two types, either by the substitution directly on to the chromophore or by basic structural changes causing differences in solvation. The customary interpretation of difference spectral data of proteins is based on transfer of chromophores from the interior of the protein to an aqueous environment (396). Common types of difference spectra measure pH effects, solvent perturbation, thermal perturbation, concentration effects and changes due to molecular complexes. The three effects considered in the experimental section of this paper are pH effects, solvent effects and changes due to molecular complexes in the spectra of chymotrypsin, chymotrypsin plus heparin and heparin.

Molecular complexes cause deviation from the sum of the spectral contributions of the separate components causing a difference spectra. Bands which arise in spectral regions where neither component absorbs appreciably are charge transfer bands (399). The indole, phenol, benzene or disulfide chromophores
can react with the appropriate perturbant to form charge
transfer complexes (394). The absorption bands produced normally
absorb at longer wavelengths than either the donor or acceptor.
Other complexes are detected as small modifications of the max-
imum of absorption and oscillator strength in regions where one
component absorbs strongly while the other only absorbs weakly
(392). These complexes are very similar to those between solvent
and the solute. Spectral changes due to molecular aggregation
and conformational changes are observed in regions where both
components absorb.

The interaction of serine proteases with substrates and
inhibitors has been followed by the addition of proflavin
(399-402). The use of proflavin, 3,6 diaminoacridine, a com-
petitive inhibitor of chymotrypsin was first reported by Wallace
et al. (399). There is a 1:1 stoichiometry between chymotrypsin
and proflavin (398). The binding site for proflavin on chymo-
trypsin is the same as the binding site of the chymotrypsin sub-
strate acetyl-L-tyrosine ethyl ester (ATEE) since the enzyme-
competitive inhibitor dissociation constant is identical to the
dissociation constant of the enzyme-dye complex (398). A two
step mechanism has been reported for the reaction between pro-
flavin and chymotrypsin (401). This mechanism involves a bi-
molecular collision, followed by an isomerization of the complex.

Inhibitors of chymotrypsin including ovomucoid displace the
proflavin molecule from the chymotrypsin molecule (402). This
reaction and others at the active site can be followed by the
use of stopped flow kinetic techniques (398-401). Dissociation and rate constants have been measured as well as transient compounds (398,399) have been detected by the use of this technique. As a result of these experiments, substantial evidence has been obtained to support the hypothesis that conformation changes in the enzyme-substrate complex prior to catalysis is of major importance in the reaction (398-401). During acylation of the active site of chymotrypsin, the proflavin is ejected from the active site as a consequence of the reaction. The simplest explanation of this phenomenon is a change in conformation of the active site (398). Experiments using proflavin and chymotrypsin have probed into the character of the active site (398,402). Samakish et al. (402) concluded that the active site has a high polarity and has a high degree of structural mobility based on spectroscopic studies using various solutions of varying dielectric constants at 293° and 77° K.

Proflavin binds to molecules other than serine proteases such as DNA and mucopolysaccharides. The metachromatic reaction between mucopolysaccharides and proflavin depends upon the type of proflavin binding sites such as carboxyl or sulfate, their proximity and relative orientation on the mucopolysaccharide, the geometry and electronic properties of the dye and the solvation of the proflavin-mucopolysaccharide complex (404). Heparin has approximately 60 binding sites for dyes (405). The binding is nearly stoichiometric when the dye/site ratio is low (404).
REFERENCES


ISOLATION AND IDENTIFICATION OF $\alpha_1$-ANTITRYPSIN AS A COMPONENT OF MALIGNANT HUMAN BREAST AND OTHER TISSUES

SUMMARY

$\alpha_1$-Antitrypsin has been identified as a component of malignant and normal adjacent breast, colon and anal tissues, as well as, malignant lung, stomach and ileum tissues. Eleven peaks of anti-proteolytic activity have been separated from the 90,000 x g supernatant fraction of malignant and normal breast tissues by affinity chromatography on Sepharose-chymotrypsin and Affi-Gel 10-chymotrypsin. Eight of these peaks contain glycoproteins. $\alpha_1$-Antitrypsin is the major component in Peak #1 from the Affi-Gel 10-chymotrypsin column. The purification of the inhibitors, as judged by disc gel electrophoresis, is extensive. In some peaks only one or two protein bands are observed suggesting that Sepharose- or Affi-Gel 10-chymotrypsin might be used for the isolation of $\alpha_1$-antitrypsin and other inhibitors in preparative quantities.

INTRODUCTION

Proteases and proteolytic inhibitors are important in the control of malignant cells. Various reports cite elevated levels of chymotrypsin, trypsin and cathepsin-like enzymes in transformed cells (1-8). The proteolytic activity is believed to be related to several properties of transformed cells, including uncontrolled
proliferation and increased migration (4,5,7). They also are believed to play a role in metastases by decreasing the cohesiveness between cells in the primary tumor (9) and by breaking down the intracellular matrix which holds the cells together at the sites of metastases (10).

Cell growth can be controlled by the addition of protease inhibitors to cell cultures (11). Goetz (12) reported that pancreatic trypsin inhibitors promoted parallel alignment, increased adhesiveness and depressed proliferation of hamster tumor cells. Reich (5) reported that the fibrinolytic activity of transformed cells can be inhibited by nitrophenyl-p-guanidinobenzoate, protamine sulfate, soybean trypsin inhibitor, ε-aminocaproate and NaCl. Furthermore, the blood of animals infected with tumor cells inhibited the fibrinolytic activity (5). Ardenne and Chaplain (13) noted that tumor growth of leukemia cells (L 1210) and Dana 433 tumor cells of R-rats (Berlin Busch) was inhibited when the cells were incubated with \( \alpha_2 \)-macroglobulin, potato protease inhibitor, trasylol and the \( \alpha_2 \)-globulin fraction of blood (which includes \( \alpha_1 \)-antitrypsin). In vivo, \( \alpha_2 \)-macroglobulin caused regression or a decreased rate of growth of tumors in R-rats (13).

Wasilauskas and Brecher reported the presence of antiproteolytic activity in the 90,000 x g supernatant fraction of glioma, ovarian carcinoma, and normal and malignant breast and colon tissues (14). These fractions contained nondialyzable, heat labile inhibitors of trypsin and chymotryptic activity. These results were subsequently
confirmed by this author (appendix Table 12).

Colostrum, produced by lactating mammals, contains a number of inhibitors of trypsin and chymotrypsin (15,16). α₁-Antitrypsin has been identified as a component of the human secretion (15,17) while Laskowski et al. (16) have separated a number of distinct glycoprotein trypsin protease inhibitors in sow colostrum. α₁-Antitrypsin was first isolated from plasma by Schultze et al. (18) and by Bundy and Mehl (19). It has subsequently been reported to inhibit chymotrypsin, plasmin, elastase (21), pancreatic kallikrein (22,23) and thrombin (24) as well as trypsin. α₁-Antitrypsin has been noted in several secretory fluids (15,17, 25-29) as well as alveolar macrophages of lung (30) and mast cells of skin and cervix uteri (31).

This communication extends earlier findings (14) and reports the presence of α₁-antitrypsin in the 90,000 x g supernatant fractions of malignant human breast tissue of nonlactating women. This inhibitor and several others of chymotryptic and tryptic activity have been extensively purified from these tissue extracts by affinity chromatography on Sepharose-chymotrypsin or Affi-Gel 10-chymotrypsin. Immobilized chymotrypsin represents a new and efficient means of isolating the α₁-antitrypsin. A preliminary report has appeared elsewhere (32).

MATERIALS AND METHODS

Twice crystallized trypsin and three times crystallized α-chymotrypsin were obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Hammersten casein, benzoyl-L-tyrosine ethyl ester (BTEE) and hemoglobin were purchased from Schwartz/Mann Research
Laboratories, Orangeburg, New York. Bovine serum albumin, Cohn Fraction V, was obtained from Sigma Chemical Corporation, St. Louis, Missouri. Affi-Gel 10 was purchased from BioRad Laboratories, Richmond, California. Sepharose-chymotrypsin was obtained as a gift from Owens-Illinois, Toledo, Ohio. N,N,N',N'-Tetramethyl-ethylenediamine, acrylamide and N,N-methylene-bis-acrylamide were obtained from Eastman Kodak, Rochester, New York. Basic fuchsin and Coomassie brilliant blue R-250 were purchased from Fisher Scientific Company, Fairlawn, New Jersey and Colab Laboratories, Glenwood, Illinois, respectively. An α₁-Trypsin Inhibitor Quantitative Kit was purchased from Miles Laboratories, Kankakee, Illinois.

Samples of normal and malignant human breast were obtained after surgery and stored frozen until further use. The tissues were thawed and homogenized 1:9 in either 0.32 M sucrose solution or 0.1 M Tris buffer, pH 7.6, for two minutes using a Virtis homogenizer. The mixture was centrifuged at 20,000 x g for 30 minutes using a RC-2 Sorvall centrifuge. The 20,000 x g supernatant fraction was then centrifuged for 75 minutes at 90,000 x g using an L2-65B Beckman Ultracentrifuge. The 90,000 x g supernatant fraction was stored frozen in aliquots.

Levels of antitryptic and antichymotryptic activity were determined using the modifications of Wasilauskas and Brecher (14) of the caseinolytic assay of Kunitz (33). The protein content was determined by applying the procedure of Lowry et al. (34), utilizing bovine serum albumin, Cohn Fraction V as the protein standard.

Sepharose-chymotrypsin was prepared by the procedure of Porath et al. (35). The Sepharose-chymotrypsin preparation was washed
batchwise with portions of 0.1 M Tris buffer, pH 7.6, until no free chymotrypsin was present as indicated by the BTEE assay for chymotrypsin (36). The activity of the insolubilized chymotrypsin was determined to be 30 µg/ml at pH 7.6 by the casein assay for chymotryptic activity.

Chymotrypsin was coupled to the Affi-Gel 10 by shaking a mixture of 80 ml of 20 mg/ml chymotrypsin in 0.1 M phosphate buffer, pH 7.0 with four gm of Affi-Gel 10 at 4°C for 3.5 hours. The mixture was transferred to a 1.5 x 30 cm column and washed sequentially with 3 liters of 0.1 M phosphate buffer, pH 7.0, and 500 ml of 0.1 M Tris buffer, pH 7.6. The final activity of the column was determined to be 40 µg/ml at pH 7.6 using the casein assay for chymotryptic activity.

Charges varying from 9-168 ml of the 90,000 x g supernatant fraction of normal and malignant breast extracts were passed through either the Sepharose-chymotrypsin column or the Affi-Gel 10-chymotrypsin column. The columns were eluted sequentially using 0.1 M Tris buffer, pH 7.6, a 0.1 M to 0.3 M NaCl gradient in 0.1 M Tris buffer, pH 7.6, 0.1 M Tris buffer, pH 7.6, 1m M HCl and 0.1 M Tris buffer, pH 7.6. Typical volume ratios were 200: 1160: 220: 160 ml.

Peaks of antitryptic and antichymotryptic activity were pooled and concentrated by ultrafiltration using Amicon Diaflo PM 30 ultrafiltration membranes at 40 psi nitrogen pressure. The concentrated samples were further concentrated for electrophoresis and immunodiffusion experiments using Amicon B-15 miniconcentrators.

The proteins in the 90,000 x g supernatant fractions, the pooled column eluates, concentrated eluates and serum were separated either
by using the disc gel electrophoresis method of Davis (37) or the micro disc gel electrophoresis technique of Burr et al. (38). In both systems the glycine Tris buffer, pH 8.3, system of Davis was used in an ISCO Model 1270 electrophoresis apparatus at 4°C. The gels were stained for glycoprotein using the periodic acid Schiff's method of either Matthieu and Quarles (39) or Kapitany and Zebrowski (40). Protein was detected with Coomassie brilliant blue according to the procedure of Weber et al. (41).

Aliquots of 1 to 10 μl of the pooled inhibitory peaks from the Affi-Gel 10-chymotrypsin column were deposited on cellulose acetate strips and tested for glycoprotein content by application of the periodic acid Schiff's method (42).

Five μl samples of the concentrated, pooled peaks 1 to 11 from the Affi-Gel 10-chymotrypsin column, the 90,000 x g supernatant fraction of normal and/or malignant breast, colon, anal, ileum, stomach and lung tissues plus α₁-antitrypsin standards were placed on the radial immunodiffusion plates containing human plasma α₁-antitrypsin antisera. The α₁-antitrypsin content of these samples was determined using the method of Kueppers (43). The amount of blood in the breast tissue extracts was estimated by determining the hemoglobin content in the tissue extracts using a colorimetric oxy-hemoglobin method (44).

RESULTS

Figure 13 summarizes the purification and identification procedures which were employed. Figure 14 shows the inhibitory activity of the 90,000 x g supernatant fraction of normal and
LEGEND TO FIGURE 13:

SEQUENCE FOR PURIFICATION OF SOLUBLE PROTEASE INHIBITORS FROM NORMAL AND MALIGNANT HUMAN BREAST TISSUE

All steps were carried out at 0-4°C. The Sepharose-chymotrypsin and the Affi-Gel 10-chymotrypsin columns were eluted sequentially with 0.1 M Tris Buffer, pH 7.6; a 0.1 N to 0.3 M NaCl gradient in 0.1 M Tris Buffer, pH 7.6; 0.1 M Tris Buffer, pH 7.6; 1 mM HCl; and 0.1 M Tris Buffer, pH 7.6. Inhibitory activity towards trypsin and chymotrypsin were determined by the casein assay (14). A 30,000 molecular weight cut off membrane and a nitrogen pressure of 40 psi were used for ultrafiltration. Polyacrylamide disc gel electrophoresis was performed by the method of Davis (37). Radial immunodiffusion was used to detect the presence of α₁-antitrypsin (43).
**Figure 13**: SEQUENCE FOR PURIFICATION OF SOLUBLE PROTEASE INHIBITORS FROM NORMAL AND MALIGNANT HUMAN BREAST TISSUE.
LEGEND TO FIGURE 14:
INHIBITION OF TRYP TIC AND CHYMOTRYPTIC HYDROLYSIS OF CASEIN BY THE 90,000 x g SUPERNATANT FRACTION OF MALIGNANT AND ADJACENT NORMAL HUMAN BREAST TISSUE FROM PATIENT #8217

Aliquots of the 90,000 x g supernatant fraction of normal and malignant human breast tissue from patient #8217 containing 0.1 to 0.8 mg protein were added to 1.0 µg of chymotrypsin or trypsin in the casein assay (14) of proteolytic activity.

△, inhibition of chymotryptic activity by normal 90,000 x g supernatant fractions
○, inhibition of chymotryptic activity by malignant 90,000 x g supernatant fractions
▲, inhibition of tryptic activity by normal 90,000 x g supernatant fractions
●, inhibition of tryptic activity by malignant 90,000 x g supernatant fractions
Figure 14: Inhibition of Tryptic and Chymotryptic Hydrolysis of Casein by the 90,000 x g Supernatant Fraction of Malignant and Adjacent Normal Human Breast Tissue from Patient #8217
malignant breast tissue from patient #8217 towards the hydrolysis of casein by chymotrypsin and trypsin. These data are representative of the curves obtained for the 90,000 x g supernatant fractions of both normal and malignant breast tissue (Appendix Figures 40-42). Small variations between samples were observed probably depending upon the proportion of malignant cells and the efficiency of the homogenization process. Generally 0.1 mg of protein was required to give 50% inhibition of 1 µg of trypsin or chymotrypsin (Appendix Table 13). At high inhibitor concentrations, the mechanism of binding of the inhibitors to the enzymes may be altered, thereby accounting for a decrease in inhibitory activity.

Figure 15 represents a typical separation of the components found in malignant breast tissue extracts upon affinity chromatography on Sepharose-chymotrypsin (Appendix Figures 43-45). An aliquot of the 90,000 x g supernatant fraction of malignant breast tissue from patient #S73-1335 containing 120 mg protein was applied to the column. Eleven peaks of antichymotryptic and/or antitryptic activity are designated. Essentially all of the protein is located in the initial breakthrough peak with very low levels in the inhibitory peaks. A similar pattern is obtained in Figure 16 with Affi-Gel 10-chymotrypsin when an aliquot of the 90,000 x g preparation from patient #4150 containing 300 mg protein, was applied. Eleven peaks of antichymotryptic and antitryptic activity again were noted. Seven peaks of antichymotryptic activity were found in the NaCl gradient eluate. One peak of antichymotryptic and one peak of antitryptic activity were observed in the subsequent Tris buffer eluate. Two peaks of both antitryptic and antichymotryptic activity
LEGEND TO Figure 15:
SEPARATION OF THE CHYMOTRYPSIN AND TRYPsin INHIBITORS IN THE 90,000 \times g SUPERNATANT FRACTION OF MALIGNANT HUMAN BREAST TISSUE FROM PATIENT #S-73-1335 ON SEPHAROSE-CHYMOTRYPSIN

Sepharose-chymotrypsin was prepared by the procedure of Porath et al. (35). An aliquot, containing 120 mg protein, of the 90,000 \times g supernatant fraction of malignant breast tissue from patient #S-73-1335, was applied to the Sepharose-chymotrypsin column. The column was eluted sequentially with 100 ml 0.1 M Tris Buffer, pH 7.6; 200 ml of a 0.1 M to 0.3 M NaCl gradient in 0.1 M Tris Buffer, pH 7.6; 50 ml 0.1 M Tris Buffer, pH 7.6; 50 ml 1 M HCl; and 100 ml 0.1 M Tris buffer, pH 7.6. Antitryptic and antichymotryptic activity was determined by the casein assay (14) using 0.3 ml of column eluate and 0.5 \mu g enzyme. The protein content of the fractions was determined using the procedure of Lowry et al. (34).

- o, Antichymotryptic Activity
- ●, Antitryptic Activity
- △, Protein concentration
- ▲, Protein concentration at 280 nm
- ▼, 90,000 \times g supernatant fraction added
Figure 17: Polyacrylamide Disc Gel Electrophoresis of the 90,000 x g Supernatant Fraction of Malignant Human Breast Tissue, Purified Protease Inhibitor from Peak 1 and Serum
LEGEND TO FIGURE 16:

SEPARATION OF CHYMOTRYPSIN AND TRYPsin INHIBITORS IN THE 90,000 × g SUPERNATANT FRACTION OF MALIGNANT HUMAN BREAST TISSUE FROM PATIENT #4150 ON AFFI-GEL 10-CHYMOTRYPSIN

Affi-Gel 10 was reacted with chymotrypsin to form Affi-Gel 10-chymotrypsin. An aliquot containing 300 mg protein of the 90,000 × g supernatant of malignant breast tissue from patient #4150 was applied to the Affi-Gel 10-chymotrypsin column. The column was sequentially eluted with 200 ml of 0.1 M Tris Buffer, pH 7.6; 1160 ml of a 0.1 M to 0.3 M NaCl gradient in 0.1 M Tris Buffer, pH 7.6; 220 ml of 0.1 M Tris Buffer, pH 7.6; 220 ml of 1 M HCl; and 160 ml of 0.1 M Tris Buffer, pH 7.6. Antitryptic and antichymotryptic activity were determined by the casein assay using 0.2 ml of the column eluate and 0.5 µg enzyme. The protein content of the fractions was determined using the procedure of Lowry et al. (34) and by the absorbance at 280 nm.

- , Antichymotryptic Activity
- , Antitryptic activity
△, Protein concentration, Lowry Method
▲, Protein concentration at 280 nm
♀, Endogenous caseinolytic Activity
↓↓, 90,000 × g supernatant fraction added
Figure 15: Separation of the Chymotrypsin and Trypsin Inhibitors in the 90,000 x g Supernatant Fraction of Malignant Breast Tissue from Patient #S-73-1335 on Sepharose-Chymotrypsin
were seen in the Tris buffer eluate following the drop in pH. Consistently negligible amounts of protein were detected in the peaks after the initial breakthrough peak, suggesting a very high degree of purification of inhibitors. Very low levels of endogenous activity were seen in these eluates. Peaks 1 to 8 contained a glycoprotein component as detected with the periodic acid Schiff's method on cellulose acetate.

Preliminary electrophoresis experiments of the pooled fractions on the micro gels generally showed only one or two protein bands using 15% acrylamide gels (Appendix Figures 46, 47). The results of electrophoresis under the conditions of 7% acrylamide gels of Davis (37) are shown in Figure 17. Electrophoresis of the concentrated pooled fraction #1 yielded only two detectable protein bands both of which contained glycoproteins. The major band migrated to the same extent as $\alpha_1$-antitrypsin of serum. Peak 1 contained antitryptic and antichymotryptic activity. The major component of this peak was determined to be immunologically equivalent to plasma $\alpha_1$-antitrypsin by immunodiffusion. When the proteins of the 90,000 x g supernatant fraction were electrophoretically separated, bands which migrated to the same extent as those in serum were observed including bands in the regions of the proteolytic inhibitors, $\alpha_1$-antitrypsin and $\alpha_1$-acid glycoprotein.

Table 5 relates the levels of $\alpha_1$-antitrypsin found in the 90,000 x g supernatant fractions of normal and/or malignant human tissues (Appendix Figures 49, 50). The concentration of $\alpha_1$-antitrypsin in the tissue extracts from blood column 3 was calculated
LEGEND TO FIGURE 17:

POLYACRYLAMIDE DISC GEL ELECTROPHORESIS OF THE 90,000 x g SUPERNATANT FRACTION OF MALIGNANT HUMAN BREAST TISSUE, PURIFIED PROTEASE INHIBITOR FROM PEAK 1 AND SERUM

Polyacrylamide disc gel electrophoresis was performed by the method of Davis (37). The gels were stained with periodic acid Schiff's reagent for glycoprotein [1] and with Coomassie Brilliant Blue for protein [2]. A. Serum, B. 90,000 x g supernatant fraction from patient #4150, C. Peak 1 concentrated eluate from the Affi-Gel 10-chymotrypsin column.
Figure 16: Separation of Chymotrypsin and Trypsin Inhibitors in the 90,000 x g Supernatant Fraction of Malignant Human Breast Tissue from Patient #4150 on Affi-Gel 10-Chymotrypsin.
LEGEND TO TABLE 5:

$\alpha_1$-ANTITRYPSIN IN HUMAN TISSUE

The $\alpha_1$-antitrypsin concentration in the 90,000 x g supernatant fractions of normal and/or malignant human breast, colon, anal, ileum, lung and stomach was determined using the quantitative radial immunodiffusion technique (43). The hemoglobin content of the tissue extracts was determined by a colorimetric hemoglobin determination method (44). The amount of blood in the extracts was calculated using a value of 0.15 gm hemoglobin/ml serum. From this value the amount of $\alpha_1$-antitrypsin present due to blood was calculated using 4.4 mg $\alpha_1$-antitrypsin per ml of serum (43). (43, 45, 46) which is double the mean value for normal serum in order to account for the increased levels in patients with neoplastic diseases.
TABLE 5: $\alpha_1$-ANTITRYPSIN IN HUMAN TISSUE

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Tissue</th>
<th>$\alpha_1$-Antitrypsin/mg protein</th>
<th>Hemo- $\alpha_1$-Antitrypsin/mg protein</th>
<th>mg from blood protein (Estimated)</th>
<th>mg from blood protein (Corrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8217</td>
<td>Malignant Breast</td>
<td>3.0</td>
<td>4.7</td>
<td>1.3</td>
<td>1.8</td>
</tr>
<tr>
<td>5286</td>
<td>Malignant Breast</td>
<td>3.1</td>
<td>1.7</td>
<td>0.5</td>
<td>2.6</td>
</tr>
<tr>
<td>8994</td>
<td>Malignant Breast</td>
<td>3.2</td>
<td>3.5</td>
<td>1.1</td>
<td>2.1</td>
</tr>
<tr>
<td>S73-1335</td>
<td>Malignant Breast</td>
<td>2.6</td>
<td>1.2</td>
<td>0.4</td>
<td>2.2</td>
</tr>
<tr>
<td>4150</td>
<td>Malignant Breast</td>
<td>1.7</td>
<td>3.1</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>S73-2543</td>
<td>Normal Breast</td>
<td>1.6</td>
<td>3.9</td>
<td>1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>3120</td>
<td>Normal Colon</td>
<td>2.5</td>
<td>2.9</td>
<td>0.9</td>
<td>1.6</td>
</tr>
<tr>
<td>3120</td>
<td>Malignant Colon</td>
<td>1.8</td>
<td>2.0</td>
<td>0.6</td>
<td>1.2</td>
</tr>
<tr>
<td>M73-416</td>
<td>Normal Colon</td>
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<td>2.1</td>
<td>0.6</td>
<td>2.7</td>
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<tr>
<td>M73-416</td>
<td>Malignant Colon</td>
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<td>1.7</td>
<td>0.5</td>
<td>3.1</td>
</tr>
<tr>
<td>6161</td>
<td>Normal Colon</td>
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<td>2.8</td>
<td>0.8</td>
<td>2.6</td>
</tr>
<tr>
<td>6161</td>
<td>Malignant Colon</td>
<td>3.1</td>
<td>0.9</td>
<td>0.3</td>
<td>2.8</td>
</tr>
<tr>
<td>S73-06098</td>
<td>Normal Anal</td>
<td>2.2</td>
<td>2.0</td>
<td>0.6</td>
<td>1.6</td>
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<tr>
<td>S73-06098</td>
<td>Malignant Anal</td>
<td>1.5</td>
<td>2.2</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>M7342</td>
<td>Malignant Ileum</td>
<td>1.8</td>
<td>2.8</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>113097</td>
<td>Malignant Stomach</td>
<td>3.0</td>
<td>0.6</td>
<td>0.2</td>
<td>2.8</td>
</tr>
<tr>
<td>73-6430</td>
<td>Malignant Lung</td>
<td>4.4</td>
<td>4.6</td>
<td>1.4</td>
<td>3.0</td>
</tr>
</tbody>
</table>
from the hemoglobin content using 0.15 g/ml and 4.4 mg/ml as the concentration of hemoglobin (45) and $\alpha_1$-antitrypsin per ml of serum. The value for $\alpha_1$-antitrypsin was estimated by taking the mean value of $\alpha_1$-antitrypsin in normal humans (43, 44) which is also the standard value for Miles immunodiffusion plates used in this study, and doubling the value to account for the acute phase increase in $\alpha_1$-antitrypsin under neoplastic conditions (46, 47). The $\alpha_1$-antitrypsin content in the tissue column 4 was determined by subtracting the $\alpha_1$-antitrypsin due to blood from the total amount of $\alpha_1$-antitrypsin in the tissue extracts. These data indicate that $\alpha_1$-antitrypsin is a component of human breast, colon, anal, ileum, stomach and lung.

DISCUSSION

The 90,000 x g supernatant fractions of normal and malignant human breast tissue has considerable inhibitory activity towards chymotrypsin and trypsin. Similar inhibitory patterns were noted by Wasilauskas and Brecher (14). The decrease in inhibitory capacity at high 90,000 x g supernatant fraction concentrations in some of the casein assays for trypsin or chymotrypsin inhibition may be due to either a change in the binding mechanism to the enzyme or a change in proportion of the various inhibitors binding to the enzymes. It is possible that at low concentrations two molecules of enzyme are bound per inhibitor molecule while at higher concentrations only one molecule of enzyme is bound per inhibitor molecule. In the latter case possibly a greater proportion of free enzyme molecules may be available to hydrolyze casein than in the former case since
some inhibitors do bind two enzyme molecules such as $\alpha_1$-antitrypsin (48, 49) and possibly $\alpha_2$-macroglobulin (50-51). A more likely possibility would be a change in the proportion of enzyme molecules binding to $\alpha_2$-macroglobulin since bound trypsin or chymotrypsin complexes are still hydrolytically active towards small substrates such as partially degraded or denatured casein (52). Furthermore, $\alpha_2$-macroglobulin can displace other protease inhibitors such as $\alpha_1$-antitrypsin (53).

The isolation of chymotrypsin and trypsin inhibitors by affinity chromatography of the 90,000 x g extracts of human breast tissue on Sepharose-chymotrypsin or Affi-Gel 10-chymotrypsin represents a new, simple means of purifying and isolating $\alpha_1$-antitrypsin almost to electrophoretic homogeneity. This contrasts with the more complicated procedures recently described by Murthy and Hercz using Sepharose-concanavalin A and Myerowitz et al. (55) using Sepharose-antialbumin as one of the steps of multi-step purification procedures. The $\alpha_1$-antitrypsin isolated by these latter procedures and that reported herein have the same electrophoretic mobility as the major $\alpha_1$-antitrypsin band in serum in contrast to earlier purification methods, involving the application of low pH which irreversibly denatured the inhibitor causing an alteration in the electrophoretic mobility (56).

Ten additional peaks of antiproteolytic activity were obtained subsequent to the elution of the $\alpha_1$-antitrypsin peak in the procedure reported herein. The use of the NaCl salt gradient and the lowering of the pH with 1 mN HCl essentially removed all bound inhibitors as the columns were successfully used repeatedly (Appendix
Figures 43-45), in contrast with Sepharose-trypsin which binds $\alpha_1$-antitrypsin more tightly (57). Sepharose-chymotrypsin, in contrast to Sepharose-trypsin binds $\alpha_1$-antitrypsin and allows its displacement at relatively low salt concentrations. Sepharose-trypsin was originally believed to irreversibly bind $\alpha_1$-antitrypsin (57). The inhibitor has been displaced by the use of low pH (58) followed immediately by dialysis to prevent denaturation. The difference in binding properties of $\alpha_1$-antitrypsin towards Sepharose-chymotrypsin and Sepharose-trypsin is due to the fact that chymotrypsin and trypsin probably bind to two different sites on the $\alpha_1$-antitrypsin molecule (59).

Essentially the same type of chromatogram was obtained using both the Affi-Gel 10-chymotrypsin and Sepharose-chymotrypsin columns. It is tentatively suggested from the patterns of the elutes obtained that there are no steric problems in binding the inhibitors present in the breast tissue extracts to the Sepharose-chymotrypsin column.

The inhibitors are highly purified as evidenced by the fact that negligible amounts of protein are associated with the inhibitory peaks, and by the preliminary disc gel electrophoresis experiments on the 15% micro-polyacrylamide disc gels and the 7% standard gel for peak #1.

The proposed role of protease inhibitors is to control enzymatic action. The role of $\alpha_1$-antitrypsin and the other glycoprotein inhibitors found by Laskowski (60, 61) in colostrum is believed to protect the antibodies in the colostrum from proteolytic degradation in colostrum fluid and in the gastrointestinal tract.
(60-62). Fully active proteolytic enzymes have been found in mast cells of spleen and skin (63, 64), leukocytes (57) and transformed cells (1-7). The $\alpha_1$-antitrypsin located in mast cells of skin and cervix uteri is believed to control self-digestion of the mast cell (31). This inhibitor has also been found in complexes with the leucocyte protease, which has the same inhibitory profile as trypsin (57) and in respiratory secretions (65). Both mast cells and leukocytes are important in the defense system of the body.

Protease inhibitors may serve a further role, in addition to the inhibition of normal and neoplastic cell proliferation (5,12,67,68). As a result of observations of children after surgery, Lennert et al. (66) have suggested that $\alpha_1$-antitrypsin and $\alpha_2$-macroglobulin serve a role as wound healers. The increase in $\alpha_1$-antitrypsin in serum is probably due to an attempt by the body to control proteolytic activity. $\alpha_1$-Antitrypsin is one of the acute phase reactants produced by the liver in response to trauma such as inflammation, injury, surgery, infection, neoplastic growth, pregnancy or the administration of hormones (47). It has also been proposed that protease inhibitors in the plant kingdom promote wound healing (69,70).
REFERENCES


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IDENTIFICATION OF $\alpha_2$-MACROGLOBULIN, $\alpha_1$-ANTICHYMOTRYPSIN AND $\alpha_1$-ANTITRYPSIN AS COMPONENTS OF MALIGNANT HUMAN BREAST TISSUE USING SEPHAROSE-CHYMOTRYPSIN AFFINITY CHROMATOGRAPHY

SUMMARY

Antithrombin III, $\alpha_2$-macroglobulin, $\alpha_1$-acid glycoprotein and $\alpha_1$-antichymotrypsin, as well as $\alpha_1$-antitrypsin, were identified as components of the 90,000 x g supernatant fraction of malignant human breast tissue. Nine major peaks of chymotryptic and/or tryptic inhibitory activity were eluted from a preparative scale Sepharose-chymotrypsin column (1 liter) which had been charged with six grams of protein (90,000 x g supernatant fraction) and developed with 0.1 M Tris Buffer, pH 7.6, a 0.1 to 0.3 M NaCl gradient in 0.1 M Tris Buffer, pH 7.6, 0.1 M Tris buffer, pH 7.6, 1mM HCl and 0.1 M Tris Buffer pH 7.6. These nine major peaks were subdivided into twenty-five subpeaks and were then concentrated by ultrafiltration by first using a 30,000 molecular weight cut off membrane filter, followed by a 1,000 molecular weight cut off membrane filter. The 30,000 molecular weight concentrates of the first five major peaks contained $\alpha_1$-antitrypsin. The peak eluted when the pH of the eluant was lowered (Peak 8) contained $\alpha_1$-antitrypsin, $\alpha_2$-macroglobulin and $\alpha_1$-antichymotrypsin, in addition to endogenous caseinolytic activity. However, BTEE, TAMe and BzAlaEE were not hydrolyzed. The $\alpha_1$-antitrypsin in peak 8 was purified to electrophoretic homogeneity by a subsequent preparative electrophoresis step. Most of the 1,000 molecular weight concentrates of the second and third major peaks contained $\alpha_2$-macroglobulin.
weight filtrates also contain low molecular weight inhibitors of chymotrypsin and trypsin. In addition to these inhibitors, \( \alpha_1 \)-acid glycoprotein and antithrombin III, which did not bind to the column, were detected in the breakthrough peak. Affinity chromatography on the immobilized chymotrypsin column represents a means of separation of a variety of protease inhibitors from other cellular constituents in essentially one step.

INTRODUCTION

Proteases and their inhibitors may be important in neoplastic diseases (1-4). They function in the expression of some of the phenotypic characteristics of transformed cells (1). Various inhibitors of proteases are present in malignant and normal tissues (5-8). \( \alpha_1 \)-Antitrypsin is present in malignant and/or normal breast, colon, anal, ileum, stomach (5) and lung (5,6) and in yolk sac tumors (7). Due to the close correlation of the levels of \( \alpha_1 \)-fetoprotein and \( \alpha_1 \)-antitrypsin, \( \alpha_1 \)-antitrypsin may be useful as a tumor marker (7). \( \alpha_1 \)-Antichymotrypsin is a component of malignant colon extracts (8). Here we report the isolation of \( \alpha_1 \)-antichymotrypsin and \( \alpha_2 \)-macroglobulin and the purification of one form of \( \alpha_1 \)-antitrypsin to electrophoretic homogeneity from the 90,000 x g supernatant fraction of malignant breast tissue by a two-step process of affinity chromatography on Sepharose-chymotrypsin, followed by polyacrylamide gel electrophoresis.
MATERIALS

Three times crystallized α-chymotrypsin and twice crystallized trypsin were obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Sepharose 4B and purified agar were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey and Difco Laboratories, Detroit, Michigan respectively. Diaflo PM 30 and UM 2 ultrafiltration membranes were obtained from Amicon Corporation, Lexington, Mass. Electrophoresis grade acrylamide and N,N methylene-bis-acrylamide were purchased from Eastman Kodak, Rochester, N.Y. Nonspecific antisera to α₁-acid glycoprotein, α₁-antichymotrypsin, α₂-macroglobulin, antithrombin III and inter-α-trypsin inhibitor were obtained from Behring Diagnostics, Somerville, N.J. Radial immunodiffusion plates containing specific antisera to α₁-antitrypsin were purchased from Miles Laboratories, Kankakee, Illinois.

METHODS

Malignant human breast tissue was obtained after surgery and stored frozen until further use. A 314 gm sample of breast tissue from patient #73-3993 was homogenized 1:4 in 0.1 M Tris Buffer, pH 7.6, using a Waring blender at high speed for 1 min. The resulting homogenate was treated as previously reported (5) to obtain a 90,000 x g supernatant fraction.

One liter of Sepharose-chymotrypsin was prepared in three batches using the procedure of Porath et al. (9).
Sepharose-chymotrypsin was packed into a 53 cm x 5 cm column. To this column, a 1.2.1 aliquot of the 90,000 x g supernatant fraction of malignant breast tissue containing approximately 6 gm of protein was applied. The column was eluted sequentially with 3 liters of 0.1 M Tris Buffer, pH 7.6, 6.8 liters of a 0.1 M to 0.3 M NaCl gradient in 0.1 M Tris Buffer, pH 7.6, 3.88 liters of 0.1 M Tris Buffer, pH 7.6, 1.54 liters of 1 mN HCl and 4.06 liters of 0.1 M Tris Buffer, pH 7.6. The elution buffers were pumped through the column at 80 mg/hr, while 20 ml fractions were collected.

The chymotryptic and tryptic inhibitory activity of the fractions was determined using the modifications of Wasilauskas and Brecher (10) of the caseinolytic assay of Kunitz (11). The protein content was determined using the procedure of Lowry et al. (12).

The inhibitory activity of the fractions from the Sepharose-chymotrypsin column was conservatively divided into 25 subpeaks of activity and the fractions were pooled. These pooled fractions were concentrated by ultrafiltration using a PM 30 Amicon ultrafiltration membrane with a molecular cut off of 30,000 and a nitrogen pressure of 40 psi. The filtrate from this first concentration step was concentrated under the same conditions using a UM 2 Amicon ultrafiltration membrane with a molecular cut off of 1,000.

The endogenous esterolytic activities of the pooled, concentrated fractions were determined using benzoyl-L-tyrosine
ethyl ester (BTEE) as the substrate for chymotryptic-like activity and tosyl-L-arginine methyl ester (TAME) for tryptic-like activity by the methods of Hummel (13). Elastase-like activity was determined using benzoyl-L-alanine ethyl ester (BzAlaEE) in an assay similar to that for chymotryptic-like activity using BTEE.

The proteins present in the 30,000 molecular weight concentrate of peak 8 were separated by the preparative electrophoresis procedure of Jovin et al. (14) using a Buchler preparative electrophoresis apparatus and a polyacrylamide gel under basic conditions as the separation media.

Samples of the 90,000 x g supernatant fraction, the 30,000 and the 1,000 molecular weight concentrates and the fractions from preparative electrophoresis were subjected to analytic disc gel electrophoresis according to the procedure of Davis (15). Proteins were visualized on the gels using Coomassie brilliant blue (16).

The micro ring immuno test (17) and/or the microcoublediffusion technique in agar gel (18) were used to determine the presence or absence of $\alpha_1$-antitrypsin, $\alpha_2$-macroglobulin, $\alpha_1$-acid glycoprotein, $\alpha_2$-antichymotrypsin, antithrombin III and inter-$\alpha$-trypsin inhibitor in the 90,000 x g supernatant fraction, the concentrated fractions and the fractions from preparative electrophoresis. The $\alpha_1$-antitrypsin content of the isolated inhibitor fractions was determined using the radial immunodiffusion procedure of Mancini et al. (19).
One ml samples of the 1,000 molecular weight filtrates were taken to dryness and then hydrolyzed in 0.1 ml of 6 M HCl at 120°C for 24 hours in glass ampules sealed under nitrogen. The ampules were broken, taken to dryness and the residue was taken up in 0.1 ml of distilled H2O. The amino acid content was estimated using the quantitative ninhydrin method of Moore and Stein (20). Albumin was used as a standard.

RESULTS

Approximately 4 µl of the 90,000 x g supernatant fraction of malignant breast tissue was required to effect a 50% inhibition of 0.5 µg chymotrypsin, while 6 µl was needed for 50% inhibition of 0.5 µg trypsin (Appendix Figure 42). This indicates that enough inhibitory activity was placed on the Sepharose-chymotrypsin column to bind to 153 mg chymotrypsin.

The inhibitory activity toward the chymotryptic and tryptic hydrolysis of casein, the endogenous activity and the protein concentration of the eluted fractions from the Sepharose-chymotrypsin column separation of the proteins in the 90,000 x g supernatant fraction of malignant breast tissue are given in Figure 18. The eluted fractions were initially separated into 25 peaks. However, based upon analytical disc gel experiments and inhibitor identification for each peak the chromatographed peaks are better grouped into 9 major peaks. The separation observed here is very similar to that seen with small columns containing Sepharose-chymotrypsin or Affi Gel 10-chymotrypsin (5).
LEGEND TO FIGURE 18:

SEPARATION OF CHYMOTRYPSIN AND TRYPsin INHIBITORS FROM THE 90,000 x g SUPERNATANT FRACTION OF MALIGNANT BREAST TISSUE OF PATIENT #73-3993 ON SEPHAROSE-CHYMOTRYPSIN

A 1.22 liter aliquot of the 90,000 x g supernatant fraction of malignant human breast tissue from patient #73-3993 was applied to a Sepharose-chymotrypsin column. The column was eluted sequentially with 3 liters of 0.1 M Tris buffer, pH 7.6; 8 liters of a 0.1 M to 0.3 M NaCl gradient in 0.1 M Tris buffer, pH 7.6; 3.88 liters of 0.1 M Tris buffer, pH 7.6; 1.54 liters of 1 M HCl and 4.06 liters of 0.1 M Tris buffer, pH 7.6. Fractions of 20 ml were collected. Aliquots of the fractions were tested for endogenous proteolytic activity and for inhibitory activity toward chymotrypsin and trypsin using the caseinolytic activity assay for the enzymes. A ratio of 0.15 ml of the inhibitor solution of 0.5 μg enzyme was used. The protein concentration was determined by the procedure of Lowry et al. (12).

- Antichymotryptic Activity
- Antitryptic Activity
- Protein Concentration
- Endogenous Proteolytic Activity
Figure 18: Separation of Chymotrypsin and Trypsin Inhibitors from the 90,000 x g Supernatant Fraction of Malignant Breast Tissue of Patient #73-3993 on Sepharose-Chymotrypsin
(Figures 15, 16 plus Appendix Figures 43-45). The protein concentration associated with the inhibitor peaks is quite low indicating a high degree of purification.

Analytical polyacrylamide disc gel electrophoresis of the 30,000 and the 1,000 molecular weight concentrates indicated that few proteins were present in most of the isolated peaks. Figure 19 gives representative gels of the 30,000 molecular weight concentrates of the 9 major peaks (Appendix Figures 51-60).

The first fifteen pooled, concentrated subpeaks (major peaks 1-5) (Appendix Figures 61-66) which were eluted by the NaCl gradient contain $\alpha_1$-antitrypsin. Peak 8 contains $\alpha_1$-antitrypsin, $\alpha_2$-antichymotrypsin and $\alpha_2$-macroglobulin (Appendix Figures 66 & 67). In addition, the 90,000 x g supernatant fraction of malignant breast tissue contains $\alpha_1$-acid glycoprotein and antithrombin III (Appendix Figures 72 & 73). These two inhibitors, as well as some $\alpha_1$-macroglobulin and $\alpha_1$-antitrypsin, were found in the breakthrough peak. Inter-$\alpha$-trypsin inhibitor was not detected in the 90,000 x g supernatant fraction nor in the isolated inhibitor fractions. The $\alpha_1$-antichymotrypsin, antithrombin III, $\alpha_1$-macroglobulin and $\alpha_1$-acid glycoprotein present in the 90,000 x g supernatant fraction are immunologically equivalent to the corresponding plasma protease inhibitor (Appendix Figures 72-74). The $\alpha_1$-antitrypsin present in the 90,000 x g supernatant fraction gives two precipitin lines, one line of partial identity and a second of complete identity with plasma $\alpha_1$-antitrypsin (Appendix Figure 71). After passage of the 90,000 x g supernatant fraction through the Sepharose-chymotrypsin
LEGEND TO FIGURE 19:

POLYACRYLAMIDE DISC GEL ELECTROPHORESIS OF THE 30,000 MOLECULAR WEIGHT CONCENTRATES FROM THE SEPHAROSE-CHYMOTRYPSIN COLUMN ELUATES OF MALIGNANT BREAST TISSUE PROTEOLYTIC INHIBITORS

Nine major peaks of antichymotryptic and antitryptic activity eluted from the Sepharose-chymotrypsin column, were subdivided and pooled into 25 subpeaks. The 25 subpeaks were concentrated by ultrafiltration using a 30,000 molecular weight cut off membrane. Aliquots of 0.1 ml of the 30,000 molecular weight concentrates were electrophoresed on 7% basic polyacrylamide gels according to the procedure of Davis (15).

Gels Left to Right:

30,000 molecular weight concentrate of peak

1a
2b
4b
5d
6a
7c
8
9b
Figure 19: Polyacrylamide Disc Gel Electrophoresis of the 30,000 Molecular Weight Concentrates from the Sepharose-Chymotrypsin Column Eluates of Malignant Breast Tissue Proteolytic Inhibitors
column, only a reaction of partial identity was observed for the \( \alpha'_1 \)-antitrypsin (Appendix Figures 61-66). The \( \alpha'_2 \)-macroglobulin isolated in major peak 8 gave a reaction of identity with plasma \( \alpha'_2 \)-macroglobulin (Appendix Figure 67).

The levels of \( \alpha'_1 \)-antitrypsin in the 30,000 molecular weight concentrates are given in Table 6. The inhibitory activity of these fractions is quite low indicating that most of the \( \alpha'_1 \)-antitrypsin is inactive. Since the 6 major peaks containing \( \alpha'_1 \)-antitrypsin, peaks 1-5 and 8, show different \( R_f \) values for the protein component in the \( \alpha'_1 \)-antitrypsin migrating region (Figure 19) (Appendix Figures 51-54), these probably represent several forms of \( \alpha'_1 \)-antitrypsin.

Endogenous proteolytic activity towards casein was observed in the 30,000 molecular concentrations of major peaks 1-3 and 8 as given in Table 7. Peaks 1 and 2 contained esterolytic activity toward BTEE while none of the fractions contained activity towards TAMe or BzAlaEE. The protease is concentrated in major peaks 1 and 2 in the 30,000 molecular weight concentrate fraction only, with the exception of 2c, which suggests that the active protease has a molecular weight greater than 30,000. In peaks 3 and 8, the proteolytic activity, with the exception of 3a, was found in the 30,000 and 1,000 molecular weight concentrates, indicating that there are either two proteases present, or that the molecular weight of the protease is very close to 30,000. It is believed that the endogenous chymotryptic-like activity is a component of the 90,000 x g supernatant fraction since treatment of the Sepharose-
LEGEND TO TABLE 6:
THE $\alpha_1$-ANTITRYPSIN CONTENT OF THE POOLED CONCENTRATED FRACTIONS FROM THE SEPHAROSE-CHYMOTRYPSIN SEPARATION OF THE 90,000 x g SUPERNATANT FRACTION OF MALIGNANT BREAST TISSUE

20 µl aliquots of the pooled 30,000 molecular weight concentrate fractions from major peaks 1-3 and 8 were placed on radial immunodiffusion plates containing monospecific antisera to $\alpha_1$-antitrypsin. The concentration of $\alpha_1$-antitrypsin was determined using standard $\alpha_1$-antitrypsin solutions according to the procedure of Mancini et al. (19). The inhibitory activity of 0.15 ml of the pooled concentrated solutions towards 0.25 µg chymotrypsin or 0.25 µg trypsin or 0.35 µg trypsin was determined using a caseinolytic assay (10, 11).
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LEGEND TO TABLE 7:

ENDOGENOUS PROTEOLYTIC ACTIVITY OF THE POOLED 30,000 AND 1,000 MOLECULAR WEIGHT CONCENTRATE FRACTIONS FROM THE SEPHAROSE-CHYMOTRYPSIN COLUMN SEPARATION OF THE PROTEOLYTIC INHIBITORS IN MALIGNANT BREAST TISSUE

The twenty-five subpeaks of antichymotryptic and/or antitryptic activity obtained from the separation of the proteins present in the 90,000 x g supernatant fraction of human malignant breast tissue on a Sepharose-chymotypsin column, were concentrated by ultrafiltration using a 30,000 molecular weight cut off membrane filter. The filtrate from this first step was concentrated by a second ultrafiltration step using a 1,000 molecular weight cut off membrane. Both the 30,000 and the 1,000 molecular weight concentrates were additionally assayed for esterolytic activity using the BTEE assay for chymotryptic-like enzymes and the TAMe assay for tryp tic-like enzymes (14).
TABLE 7

ENDOGENOUS PROTEOLYTIC ACTIVITY OF THE POOLED 30,000 AND 1,000
MOLECULAR WEIGHT CONCENTRATE FRACTIONS FROM THE SEPHAROSE-
CHYMOTRYPSIN COLUMN SEPARATION OF THE PROTEOLYTIC INHIBITORS
IN MALIGNANT BREAST TISSUE

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<th>30,000 M.Wt.</th>
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<td>Esterolytic Activity</td>
<td>Equivalent µg</td>
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<td></td>
<td></td>
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<td>BTEE Chymotrypsin/ml</td>
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chymotrypsin column material with the solutions used to elute the column does not release free chymotrypsin (Appendix Table 17). Furthermore, chymotrypsin is not concentrated by the PM 30 ultrafiltration membranes (Table 4).

The 1,000 molecular weight filtrates contain considerable inhibitory activity towards chymotrypsin and/or trypsin. Levels of inhibitory activity as well as protein content are given in Table 8. These results suggest that some of the polypeptides may not contain tyrosine which is necessary for the Lowry assay (12), and further, that some of the inhibitory material may not be polypeptide in nature.

Preparative electrophoresis was used to isolate the inhibitors in peak 8 (Appendix Figure 68). Only $\alpha_1$-antitrypsin was detected in the fractions eluted from the preparative polyacrylamide electrophoresis gel (Appendix Figure 69). Neither $\alpha_2$-macroglobulin nor $\alpha_1$-antichymotrypsin were detected in the eluted fractions. It is quite possible that these proteins were hydrolyzed by the endogenous protease during the concentration stage of the preparative electrophoresis procedure.

Analytical disc gel electrophoresis on 5%, 7% and 10% gels indicated that the $\alpha_1$-antitrypsin isolated in the inhibitor fractions from the preparative electrophoresis column is homogeneous (Appendix Figure 70). Figure 20 gives the progressive purification patterns on 7% polyacrylamide gels. The $\alpha_1$-antitrypsin isolated gave a reaction of identity with plasma $\alpha_1$-antitrypsin in the macrodouble-immunodiffusion test (Appendix Figure 71). Possibly this final
LEGEND TO TABLE 8:

INHIBITORY ACTIVITY TOWARDS CHYMOTRYPSIN AND TRYPsin OF THE 1,000 MOLECULAR WEIGHT FILTRATES FROM THE SEPHAROSE-CHYMOTRYPSIN SEPARATION OF THE INHIBITORS PRESENT IN THE 90,000 x g SUPERNATANT FRACTION OF MALIGNANT BREAST TISSUE

The pooled fractions, containing inhibitory activity toward trypsin and chymotrypsin from the Sepharose-chymotrypsin column fractionation of malignant breast tissue inhibitors, were concentrated by ultrafiltration first using a 30,000 molecular cut off membrane filter and then a 1,000 molecular weight cut off membrane filter. The inhibitory activity of 0.15 ml of the 1,000 molecular weight filtrates towards 0.25 μg chymotrypsin or 0.25 μg trypsin was determined using a caseinolytic assay (10, 11). The protein content of the 1,000 molecular weight filtrate fractions was determined using the Lowry Folin Phenol method (12). Protein concentrations were also determined after hydrolysis of 1 ml samples of the filtrates at 120° for 24 hours in 6 M HCl in ampules sealed under nitrogen, by the quantitative ninhydrin method of Moore and Stein (20). Albumin was used as the standard reference protein for both protein assays.
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<td>43.9</td>
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LEGEND TO FIGURE 20:

ELECTROPHORESIS OF THE 90,000 x g SUPERNATANT FRACTION OF MALIGNANT BREAST TISSUE, PEAK 8 FROM SEPHAROSE-CHYMOTRYPSIN CHROMATOGRAPHY, AND α₁-ANTITRYPSIN PURIFIED BY PREPARATIVE ELECTROPHORESIS

One form of α₁-antitrypsin was purified from the 90,000 x g supernatant fraction of malignant breast tissue by an initial separation of the inhibitors on a Sepharose-Chymotrypsin column. Peak 8, which was eluted when the pH of the eluant was lowered, contained α₁-antitrypsin. This inhibitor from peak 8 was further purified to electrophoretic homogeneity by a subsequent preparative electrophoresis step. The lower band in all gels is the bromophenol blue tracking dye.

Gels Left to Right:

90,000 x g Supernatant Fraction of Malignant Breast
30,000 Molecular Weight Concentrate of Peak 8
α₁-Antitrypsin from Peak 8 purified by preparative electrophoresis
Figure 20: Electrophoresis of the 90,000 x g Supernatant Fraction of Malignant Breast Tissue, Peak 8, from Sepharose-Chymotrypsin Chromatography, and $\alpha_1$-Antitrypsin Purified by Preparative Electrophoresis
purification step separated the inhibitor from a protease or other tightly bound molecule which previously caused a reaction of partial identity with plasma $\alpha$,-antitrypsin. By this technique, 3.5 mg of $\alpha$,-antitrypsin was isolated. This form of $\alpha$,-antitrypsin represents approximately 2.2% of the total $\alpha$,-antitrypsin present in the breast tissue extracts.

DISCUSSION

Several inhibitors of chymotrypsin and trypsin are easily bound and separated by Sepharose-chymotrypsin affinity chromatography. One potential limitation with this general approach is that the immobilized active chymotrypsin may cause inactivation of the inhibitors during passage through the column. Future avenues for exploitation of this procedure might well utilize an immobilized active site-inhibited chymotrypsin. The low molecular weight inhibitor fractions were much more active than the macromolecular fractions from ultrafiltration.

At least six forms of $\alpha$,-antitrypsin which have various electrophoretic mobilities, were isolated from the breast tissue extracts using the Sepharose-chymotrypsin column. One or more forms are also present in the breakthrough peak which did not bind to the column. There may be several genetic distinct forms of $\alpha$,-antitrypsin present since 23 Pi alleles for human serum $\alpha$,-antitrypsin have been identified (21). One of the isolated forms of $\alpha$,-antitrypsin could be an oligomeric form since one has been identified electrophoretically in serum (22). The various forms might also be conformational isomers of one basic molecule.
similar to those identified in human serum by two dimensional immunoelectrofocusing (23). Complexes between proteases and \( \alpha_1 \)-antitrypsin are also possible as evidenced by various protease-\( \alpha_1 \)-antitrypsin complexes which have been isolated from human serum (24), ascitic fluids (25), synovial fluids (26), bronchial secretions (27) and cerebrospinal fluids (28). Neutral and acidic proteases are known to be present in malignant breast tissue from this study and other studies (3,4). Other molecules such as immunoglobulins and fibrinogen also bind to \( \alpha_1 \)-antitrypsin as found in the sera of patients with myeloma (29) and Bence Jones proteinemia (30).

The low molecular weight inhibitors contribute a major portion of the inhibitor activity of the pooled peaks from the Sepharose-chymotrypsin column (Appendix Table 18). These inhibitors may be proteolytic fragments from the Sepharose-chymotrypsin column, or, more likely, inhibitors which are present in the tissue extracts. Most of the proteolytic inhibitors isolated from plants and animals have been macromolecules with molecular weights of 50,000-900,000 for serum inhibitors, 10,000-20,000 for nasal and bronchial secretion inhibitors, and 4,000-6,500 for pancreatic inhibitors (31). In micro-organisms, however, small molecular weight inhibitors have been isolated (32). Leupeptin, antipain, chymostatin and elastin, which are polypeptide inhibitors of serine proteases have been isolated from actinomycyes culture filtrates (32). Similar inhibitors may possibly be present in the tissue extracts.
The presence of endogenous proteolytic activity in the isolated inhibitor peaks in excess of that present in the 90,000 x g supernatant fraction indicates that the proteases in the tissue extract are either bound to the inhibitors or are in a zymogen form, being activated by the Sepharose-chymotrypsin column. This proteolytic activity may be due to the presence of the chymotrypsin-like or trypsin-like mast cell proteases (33) or the neutral macrophage proteases such as elastase, collagenase or azocaseinase (34). The activity may also be due to intracellular proteolytic enzymes which are responsible for the initial inactivation of enzymes of neutral pH (35).
REFERENCES


IDENTIFICATION OF $\alpha_1$-ACID GLYCOPROTEIN, $\alpha_2$-MACROGLOBULIN AND ANTITHROMBIN III AS COMPONENTS OF NORMAL AND MALIGNANT HUMAN TISSUES

SUMMARY

$\alpha_1$-Acid glycoprotein, $\alpha_2$-macroglobulin, and antithrombin III have been identified, by immunological means, as components of the 90,000 x g supernatnat fraction of malignant and adjacent normal human breast, colon, and anal tissues, as well as malignant stomach and ileum. Malignant lung tissue only contained $\alpha_1$-acid glycoprotein. These protease inhibitors are immunologically equivalent to those present in human plasma.

INTRODUCTION

Proteases and their inhibitors are important in the proliferation of cells in culture (1). Higher levels of proteases are present in malignant tissues than in normal tissues (2,3). Administration of serum protease inhibitors has reduced the size of tumors in vivo (4). Serum levels of antiplasmin, antitrypsin and antichymotrypsin activities are elevated in neoplastic diseases (5). $\alpha_1$-Acid glycoprotein, although not normally considered as one of the plasma proteolytic inhibitors, inhibits elastase (6) and the conversion of prothrombin to thrombin (7). The level of this acute phase protein in plasma is a good index of the dissemination of breast cancer (8). It is present in the
cytoplasm of bile duct cells, oval cells, transitional cells and cancer cells of rat liver (9). Gastric juice of patients with gastric cancer, in contrast to normal controls, contains α₁-acid glycoprotein (10). α₂-Macroglobulin irreversibly inhibits a wide range of proteases (10). This inhibitor is effective in preventing the hydrolysis of large substrates but has little effect on the hydrolysis of small substrates (11). α₂-Macroglobulin functions as a carrier of proteases for the clearance of proteases from the circulatory system (11). The serum level of α₂-macroglobulin is elevated in lymphoid tissue neoplasia but is nearly normal in non-lymphoid tissue neoplasia (12). Antithrombin III is a slow and progressive inhibitor of thrombin, plasmin and factor Xa in the absence of heparin, while in the presence of heparin the rate of inhibition is greatly enhanced (13). This inhibitor also inhibits trypsin and acrosin from spermatozoa (13). Antithrombin III is found in plasma (13) and in cervical mucosa (14).

α₁-Antitrypsin has previously been reported to be present in various malignant and adjacent normal tissues (15,16,17). Due to the parallelism in the elevation of α₁-antitrypsin and α-fetoprotein, α₁-antitrypsin is a possible tumor protein marker (17). In this communication we report the presence of α₁-acid glycoprotein, α₂-macroglobulin and antithrombin III in various malignant and adjacent normal tissues.

MATERIALS AND METHODS

Immunospecific antisera and radial immunodiffusion plates for α₁-acid glycoprotein, α₂-macroglobulin and antithrombin III were
purchased from Behring Diagnostics, Somerville, N.J. Purified
agar was obtained from Difco Laboratories, Detroit, Mich. Minicon
concentrators were purchased from Amicon Corporation, Lexington,
Mass.

Samples of normal and/or malignant human breast, colon, ileum
stomach, anal and lung were obtained after surgery and stored
frozen until further use. The 90,000 x g supernatant fractions
were prepared in 0.1 M Tris Buffer, pH 7.6 as previously reported
(15).

The 90,000 x g supernatant fractions were concentrated from 10
to 30-gold using a Minicon concentrator. The protein content of
these concentrated fractions was determined using the procedure of
Lowry et al. (18). The amount of blood in the tissue extracts was
estimated by determining the amount of hemoglobin present using a
colorimetric oxyhemoglobin technique (19).

Agar gel double-immunodiffusion experiments were carried out
according to the Gelman microtechnique (20). Five μl of the con-
centrated 90,000 x g supernatnat fractions of the various tissues
and human plasma were applied to the outer wells of the agar gels
and were allowed to diffuse towards the antisera placed in the
center well.

Five μl samples of the concentrated tissue extracts were
placed in the wells of radial immunodiffusion plates which con-
tained antisera to human plasma α',-acid glycoprotein, α',-macro-
globulin or antithrombin III. The levels of the three proteins
were determined according to the procedure of Mancini et al. (21).
breast tissue using affinity chromatography on Sepharose-chymotrypsin or Affi-Gel 10-chymotrypsin columns. The columns were eluted sequentially with 0.1 M Tris buffer, pH 7.6, a 0.1 M to 0.3 M NaCl gradient in 0.1 M Tris buffer, pH 7.6, 0.1 M Tris buffer, pH 7.6, 1 mM HCl and 0.1 M Tris buffer, pH 7.6. Nine major peaks of antichymotryptic and/or antitryptic activity were detected in the column eluates. These major peaks were subdivided, pooled and concentrated by ultrafiltration first using a 30,000 molecular weight cut off membrane filter, followed by ultrafiltration using a 1,000 molecular weight cut off membrane filter. The first peak from the Affi-Gel 10-chymotrypsin column and the first five major peaks from the preparative Sepharose-chymotrypsin column (1 liter) contain $\alpha_1$-antitrypsin as the major component. Peak 8 from the Sepharose-chymotrypsin column, which was eluted when the pH of the eluant was lowered, contained $\alpha_2$-macroglobulin, $\alpha_1$-antichymotrypsin and $\alpha_1$-antitrypsin. Endogenous proteolytic activity was noted in major peaks 1, 2, 3 and 8 with casein substrate. The protease in major peaks 1 and 2 hydrolyzed the substrate Benzoyl-L-tyrosine ethyl ester while none of the proteases hydrolyzed tosyl-L-arginine methyl ester nor benzoyl-L-alanine ethyl ester. The $\alpha_1$-antitrypsin in peak 8 was purified to electrophoretic homogeneity by a subsequent preparative electrophoresis step. Most of the 1,000 molecular weight filtrates contained low molecular weight inhibitors of chymotrypsin and/or trypsin. In addition to these inhibitors, $\alpha_1$-acid glycoprotein and antithrombin III were detected in the breakthrough peak. Affinity chromatography on the immobilized chymotrypsin
represents a means of separation of a variety of protease inhibitors from other cellular constituents in essentially one step.

The interaction between heparin and chymotrypsin was studied using polyacrylamide disc gel electrophoresis, ultraviolet and proflavin difference spectra, and kinetic studies utilizing the chymotryptic substrate, glutaryl-L-phenylalanine-p-nitroanilide (GPANA). These studies determined that heparin forms a complex with chymotrypsin which is hydrolytically active towards GPANA and glutaryl-L-phenylalanine-3-naphthylamide (GPNA) at pH 7.6. The activity of chymotrypsin toward GPANA at pH 7.6 is enhanced in the presence of heparin. This mucopolysaccharide does not bind at the active site of the enzyme since proflavin is not displaced from the active site of chymotrypsin upon complex formation. The heparin-chymotrypsin complex migrates under basic polyacrylamide disc gel electrophoresis conditions to a position intermediate between heparin and free chymotrypsin. The complex is dissociable under acidic polyacrylamide gel electrophoresis conditions. It is estimated that 1 to 3 molecules of heparin can bind to each chymotrypsin molecule on the basis of electrophoretic information and enzymic activity data.
EXPERIMENTAL RESULTS

$\alpha_1$-Acid glycoprotein, $\alpha_2$-macroglobulin and antithrombin III were detected in malignant and adjacent normal breast, colon and anal tissues and in malignant stomach and ileum tissues using double immunodiffusion in agar gels (Appendix Figures 72-74 and 76-78). These experiments showed that these inhibitors were immunologically identical to those present in plasma. The inhibitors present in normal and malignant tissues were also immunologically equivalent. Only $\alpha_1$-acid glycoprotein was detected in the malignant lung tissue extract.

The amounts of $\alpha_1$-acid glycoprotein, $\alpha_2$-macroglobulin and antithrombin III present in the tissue extracts determined by radial immunodiffusion (Appendix Figures 79-84) are given in Tables 9, 10 and 11, respectively. The level of the inhibitors present in the extracts, due to "contamination" by the presence of blood, was calculated using 0.15 gm/ml as the hemoglobin concentration (22) and 1.80 mg/ml (2 x normal), 2.65 mg/ml and 0.23 mg/ml (23) as the concentrations of $\alpha_1$-acid glycoprotein, $\alpha_2$-macroglobulin and antithrombin III, respectively, per ml of blood. The level of $\alpha_1$-acid glycoprotein in the blood of cancer patients is approximately twice the normal level due to the acute phase reaction (24).

The actual level of the inhibitors in tissues varied depending upon which individual tissue sample was used. Due to the small number of samples, no statistical analysis could be made. The $\alpha_1$-acid glycoprotein content was higher in malignant than in adjacent normal breast tissues. In the colon extracts, the levels of the three inhibitors were very close in both types of tissues.
LEGEND TO TABLE 9:

α₁-ACID GLYCOPROTEIN IN HUMAN TISSUES

The α₁-acid glycoprotein concentration in the 90,000 x g supernatant fractions of normal and/or malignant breast, colon, anal, ileum, lung and stomach was determined using the quantitative radial immunodiffusion technique (24). The hemoglobin content of the tissue extracts was determined by a colorimetric hemoglobin determination method (19). The amount of blood in the tissue extracts was calculated using a value of 0.15 g hemoglobin/ml serum (22). From this value the amount of α₁-acid glycoprotein present due to blood was calculated using 1.80 mg/ml serum (23), which is double the mean value for normal serum in order to account for the increased levels in patients with neoplastic diseases.
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<th>α₁-Acid Glycoprotein</th>
<th>mg</th>
<th>α₁-Acid Glycoprotein</th>
<th>mg</th>
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<td>10.21 x 10⁻³</td>
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<td>35.68</td>
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</table>
LEGEND TO TABLE 10:

$\alpha_2$ -MACROGLOBULIN IN HUMAN TISSUE

The $\alpha_2$-macroglobulin concentration in the $90,000 \times g$ supernatant fractions of normal and/or malignant breast, colon, anal, ileum, lung and stomach was determined using the quantitative radial immunodiffusion technique (21). The hemoglobin content of the tissue extracts was determined by a colorimetric hemoglobin determination method. The amount of blood in the tissue extracts was calculated using a value of 0.15 g hemoglobin/ml serum (22). From this value the amount of $\alpha_2$-macroglobulin present due to blood was calculated using 2.65 mg/ml serum (23), which is the average of the mean value for men and women.
<table>
<thead>
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<th>Patient Number</th>
<th>Tissue</th>
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<th>Hemoglobin/ mg protein x 10^-1</th>
<th>$\alpha_2$-Macroglobulin/ mg protein (Estimated) x 10^3</th>
<th>$\alpha_2$-Macroglobulin/ mg protein (Corrected) x 10^3</th>
</tr>
</thead>
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<td>Normal Breast</td>
<td>9.62</td>
<td>1.21</td>
<td>2.13</td>
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<td>S73-06098</td>
<td>Malignant Anal</td>
<td>11.56</td>
<td>2.00</td>
<td>3.53</td>
<td>8.03</td>
</tr>
<tr>
<td>M73-3202</td>
<td>Malignant Ileum</td>
<td>14.34</td>
<td>2.03</td>
<td>3.58</td>
<td>10.76</td>
</tr>
<tr>
<td>73-6430</td>
<td>Malignant Lung</td>
<td>0</td>
<td>1.87</td>
<td>3.30</td>
<td>0</td>
</tr>
</tbody>
</table>
LEGEND TO TABLE II:

ANTITHROMBIN III IN HUMAN TISSUES

The antithrombin III concentration in the 90,000 x g supernatant fraction of normal and/or malignant breast, colon, anal, ileum, lung and stomach was determined using the quantitative radial immunodiffusion technique (21). The hemoglobin content of the tissue extracts was determined by a colorimetric hemoglobin determination method. The amount of blood in the tissue extracts was calculated using a value of 0.15 g hemoglobin/ml serum (22). From this value the amount of antithrombin III present due to blood was calculated using 0.23 mg/ml serum (23), which is the mean value.
<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Tissue</th>
<th>mg Antithrombin III/ mg protein</th>
<th>x 10^3</th>
<th>mg Hemoglobin/ mg protein</th>
<th>x 10^{-1}</th>
<th>mg Antithrombin III from blood protein (Estimated)</th>
<th>x 10^3</th>
<th>mg Antithrombin III/ mg protein (Corrected)</th>
<th>x 10^3</th>
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</thead>
<tbody>
<tr>
<td>6161A</td>
<td>Normal Breast</td>
<td>2.62</td>
<td>1.21</td>
<td>0.19</td>
<td></td>
<td>1.43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6161A</td>
<td>Malignant Breast</td>
<td>4.10</td>
<td>0.74</td>
<td>0.11</td>
<td></td>
<td>3.99</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>S73-2289</td>
<td>Malignant Breast</td>
<td>2.86</td>
<td>2.76</td>
<td>0.42</td>
<td></td>
<td>2.45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S73-2543</td>
<td>Normal Breast</td>
<td>7.43</td>
<td>6.08</td>
<td>0.93</td>
<td></td>
<td>6.50</td>
<td></td>
<td></td>
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<tr>
<td>S73-2543</td>
<td>Malignant Breast</td>
<td>3.04</td>
<td>1.22</td>
<td>0.19</td>
<td></td>
<td>2.85</td>
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<tr>
<td>M73-925</td>
<td>Normal Breast</td>
<td>3.83</td>
<td>1.32</td>
<td>0.20</td>
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<td>3.63</td>
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<tr>
<td>M73-925</td>
<td>Malignant Breast</td>
<td>5.72</td>
<td>1.76</td>
<td>0.27</td>
<td></td>
<td>5.45</td>
<td></td>
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<tr>
<td>113097</td>
<td>Malignant Stomach</td>
<td>2.88</td>
<td>0.54</td>
<td>0.08</td>
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<td>2.05</td>
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<tr>
<td>M73-31</td>
<td>Normal Colon</td>
<td>4.93</td>
<td>1.48</td>
<td>0.23</td>
<td></td>
<td>4.70</td>
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<td>M73-3201</td>
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<td>2.33</td>
<td>2.62</td>
<td>0.40</td>
<td></td>
<td>1.93</td>
<td></td>
<td></td>
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<tr>
<td>M73-3201</td>
<td>Malignant Colon</td>
<td>3.62</td>
<td>1.01</td>
<td>0.16</td>
<td></td>
<td>3.46</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>M73-416</td>
<td>Normal Colon</td>
<td>3.77</td>
<td>1.56</td>
<td>0.24</td>
<td></td>
<td>3.53</td>
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<tr>
<td>M73-416</td>
<td>Malignant Colon</td>
<td>2.74</td>
<td>1.44</td>
<td>0.22</td>
<td></td>
<td>2.52</td>
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<tr>
<td>S73-3120</td>
<td>Normal Colon</td>
<td>3.04</td>
<td>1.79</td>
<td>0.27</td>
<td></td>
<td>2.77</td>
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<tr>
<td>S73-3120</td>
<td>Malignant Colon</td>
<td>3.49</td>
<td>0.91</td>
<td>0.14</td>
<td></td>
<td>3.35</td>
<td></td>
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<tr>
<td>113020</td>
<td>Malignant Colon</td>
<td>3.09</td>
<td>0.99</td>
<td>0.15</td>
<td></td>
<td>2.94</td>
<td></td>
<td></td>
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<tr>
<td>S73-06098</td>
<td>Normal Anal</td>
<td>3.59</td>
<td>1.97</td>
<td>0.30</td>
<td></td>
<td>3.29</td>
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<tr>
<td>S73-06098</td>
<td>Malignant Anal</td>
<td>3.52</td>
<td>2.00</td>
<td>0.30</td>
<td></td>
<td>3.22</td>
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<td></td>
</tr>
<tr>
<td>M73-3202</td>
<td>Malignant Ileum</td>
<td>3.69</td>
<td>2.03</td>
<td>0.31</td>
<td></td>
<td>3.38</td>
<td></td>
<td></td>
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<td>73-6430</td>
<td>Malignant Lung</td>
<td>0</td>
<td>1.87</td>
<td>0.28</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
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</tbody>
</table>
This experiment confirmed the observation that the malignant lung tissue extract does not contain measurable amounts of $\alpha_2$-macroglobulin or antithrombin III. The level of $\alpha_1$-acid glycoprotein is much higher in the lung tissue extract than in other tissues.

**DISCUSSION**

The levels of inhibitors in normal and malignant colon are nearly the same probably because tissues which are considered microscopically adjacent normal are histochemically more like malignant than normal colon tissues (25). A higher level of protease inhibitor concentration would be expected in malignant tissues than in normal tissues in order for the cell to attempt to control the higher levels of proteolytic activity present in malignant tissues (2,3). It is possible that the levels of inhibitors reported here are much lower than actually are present in the cell since 0.1 M Tris Buffer, pH 7.6 may not have solubilized all of the inhibitors.

The presence of $\alpha_1$-acid glycoprotein, antithrombin III and $\alpha_2$-macroglobulin as well as $\alpha_1$-antitrypsin (15, 16) and $\alpha_1$-antichymotrypsin (26) in malignant and/or normal tissues stimulates speculation into the function of protease inhibitors in tissues. The main function probably is to prevent uncontrolled proteolysis within the tissues in a similar manner as observed in the circulatory system. Since only $\alpha_2$-macroglobulin has inhibitory activity toward acidic proteases (11), the major proteases inhibited by the inhibitors are probably neutral or basic extralysosomal proteases. These proteases are important in the initial degradation of cellular proteins, while the lysosomal proteases hydrolyze the partially
degraded proteins (26). These extralysosomal proteases are often very specific in action as in the case of myofibrillar protease (28), chromatin proteases (29,30) and the group specific proteases of skeletal and intestinal muscle, intestinal mucosa and liver, which hydrolyse almost exclusively pyridoxine dependant enzymes (31). Since these enzymes are inhibited by natural protease inhibitors such as soybean trypsin inhibitor (28,29,30), cellular proteolytic inhibitors likely play a role in regulating the synthesis and degradation of metabolic enzymes and structural components of the cell. α₂-Macroglubulin and similar inhibitors additionally may limit the activity of the intercellular proteases to small substrates in an analogous manner to that observed in plasma (11). Proteolytic inhibitors present in the tissue may also interact with the cell surface proteases (1) to regulate cell proliferation.
REFERENCES


CHARACTERIZATION OF THE INTERACTION BETWEEN CHYMOTRYPSIN AND HEPARIN

SUMMARY

Heparin forms a complex with chymotrypsin which is active towards glutaryl-L-phenylalanine-p-nitroanilide (GPANA) and glutaryl-L-phenylalanine-B-naphthylamide (GPNA) at pH 7.6. The activity of chymotrypsin towards GPANA at pH 7.6 is enhanced in the presence of heparin. Heparin does not bind at the active site of the enzyme since proflavin is not displaced from the active site of chymotrypsin upon complex formation. The heparin-chymotrypsin complex migrates under basic polyacrylamide disc gel electrophoresis conditions to a position intermediate between heparin and free chymotrypsin. The complex is dissociable under acidic polyacrylamide gel electrophoresis conditions. It is estimated that 1 to 3 molecules of heparin can bind to each chymotrypsin molecule on the basis of electrophoretic and enzymic activity data.

INTRODUCTION

Heparin forms a complex with proteases such as thrombin (1), plasmin (2), collagenase (3), pepsin, trypsin, and chymotrypsin (4,5). The nature of each of the products formed has been characterized to differing extents by various methods. Thus, chymotrypsin and heparin coprecipitate from a 1% aqueous solution at a pH of 8.4 and reach maximal precipitation rate of pH 5.75 (5). This complex has 130% of the original heparin activity as an
Van Haeringen (4) observed that the effect of heparin on the chymotryptic hydrolysis of casein and denatured hemoglobin depended upon the pH, the buffer and the substrate employed. He concluded that these effects were due to interactions between heparin and the substrate.

Heparin-thrombin (1,6) and heparin-plasmin (2) complexes have been studied most extensively due to their possible role in the control of blood coagulation. Complexes of heparin with active chymotrypsin- and trypsin-like molecules, are also important since these molecules are present in the intestine (7) and in mast cell granules (8-17). There is some evidence to suggest that heparin and chymotrypsin- and trypsin-like complexes are found in mast cell granules (9,13,14,17). Salt concentrations greater than 1 M are required to dissociate isolated heparin-protease complexes from mast cell granule homogenates (9,17). Although ultraviolet spectroscopy and proflavin difference spectra have been used extensively to study reactions of chymotrypsin (18,29), they have not previously been utilized to probe the interaction of chymotrypsin with heparin. Since proflavin binds to serine proteases at the active site with a 1:1 stoichiometry, it can be used as an active site probe (19). Proflavin, furthermore, binds to heparin (21), to a maximum of 60 molecules of proflavin per heparin molecule. It was therefore considered appropriate to study the nature of the interaction of chymotrypsin with heparin, using proflavin as a probe.

In this communication we report that heparin binds to chymotrypsin both under acidic and basic conditions to form a complex.
MATERIALS AND METHODS

Three times crystallized \( \alpha \)-chymotrypsin, (lot numbers CD16164, CDIOBK, CDI-3AB, CDI-0BK and CDI-2LX), was purchased from Worthington Biochemical Co., Freehold, New Jersey. Five times crystallized \( \alpha \)-chymotrypsin (lot no. CD\(_{640404}\)) was received as a gift from Worthington Biochemical Co. A molecular weight of 25,000 (18) was used for calculations involving chymotrypsin. Heparin isolated from porcine intestinal mucosa as the sodium salt was purchased from Sigma Chemical Co., St. Louis, Missouri. A molecular weight of 11,000 was employed for calculations involving heparin (23). Proflavin sulfate, GPNA and GPANA were obtained from Schwarz-Mann, Orangeburg, New York. The proflavin was used either without further purification or was re-crystallized from hot methanol. An extinction coefficient of 40,300 at 444 nm for proflavin in 0.01 M Tris Buffer, pH 7.6 is in good agreement with the reported values (19,20). Acrylamide and \( \text{N,N}' \) methylene-Bis-acrylamide were purchased from Eastman Organic, Rochester, New York.

The method of Davis (22) was used for basic polyacrylamide disc gel electrophoresis while the method of Reisfeld et al. (25) was used for acidic polyacrylamide disc gel electrophoresis. Pre-electrophoresis of the basic gels for 20 minutes at 5 mA/tube did not alter the electrophoretic pattern. The acidic gels were modified so that a large pore sample gel was cast between two small pore separation gels in order to observe the migration of heparin, which is negatively charged, and chymotrypsin, which is positively charged at pH 4.3. Samples of 0.030 to 1 mg of heparin and/or
chymotrypsin were applied to the gels in either a sample gel or in 40% sucrose.

The gels were treated with coomassie brilliant blue (26) to visualize protein, periodic acid Schiff's reagent (27) for glycoprotein, a 1% aqueous solution of toluidine blue for mucopolysaccharides, or GPNA in either 0.04 M or 0.1 M Tris Buffer, pH 7.6 and 0.14% fast garnet GBC to visualize the B-naphthylamine released upon enzymic activity (28).

The activity of the heparin-chymotrypsin complex was determined using the procedure of Erlanger et al. (29) using GPANA in 0.05 M Tris Buffer containing 0.02 M CaCl2 at pH 7.6 as the substrate.

Ultraviolet spectra of heparin, heparin plus chymotrypsin, chymotrypsin and heparin plus chymotrypsin vs chymotrypsin in 1 mM HCl and 0.10 M, 0.01 M and 0.001 M Tris Buffer, pH 7.6 were obtained using either a Beckman Acta M IV Spectrophotometer or a Cary 14 Recording Spectrophotometer. Visible and ultraviolet spectra of heparin, heparin plus chymotrypsin, and chymotrypsin were determined in the presence of 2.8 x 10^-5 M proflavin in 0.10 M, 0.01 M or 0.001 M Tris Buffer, pH 7.6 using a Beckman Acta M IV Spectrophotometer.

RESULTS

Figures 21-24 and 25-28 show the results of electrophoresis of heparin, heparin plus chymotrypsin and chymotrypsin on 7% basic and acidic polyacrylamide disc gels, respectively. The complex formed upon interaction between heparin and chymotrypsin migrates.
LEGEND TO FIGURES 21 - 24

ELECTROPHORESIS OF HEPARIN, HEPARIN PLUS CHYMOTRYPSIN, AND CHYMOTRYPSIN ON BASIC POLYACRYLAMIDE DISC GELS

Heparin, heparin plus chymotrypsin and chymotrypsin were electrophoresed on basic 7% polyacrylamide disc gels using the procedure of Davis (24). The gels were stained for protein using coomassie brilliant blue, for chymotryptic activity using GPNA followed by coupling to fast garnet GBC, for mucopolysaccharide using toluidine blue or for glycoprotein using the periodic acid Schiff's method.

**Figure 21:**
- **Stain:** coomassie brilliant blue
- Gels Left to Right
  - Heparin: 1 mg
  - Heparin: 0.033 mg plus Chymotrypsin: 0.075 mg
  - Chymotrypsin: 0.075 mg

**Figure 22:**
- **Stain:** GPNA
- Gels Left to Right
  - Heparin: 0.44 mg
  - Heparin: 0.44 mg plus Chymotrypsin: 1 mg
  - Chymotrypsin: 1 mg

**Figure 23:**
- **Stain:** toluidine blue
- Gels Left to Right
  - Heparin: 0.033 mg
  - Heparin: 0.033 mg plus Chymotrypsin: 0.075 mg
  - Chymotrypsin: 1 mg

**Figure 24:**
- **Stain:** periodic acid Schiff's
- Gels Left to Right
  - Heparin: 0.44 mg
  - Heparin: 0.44 mg plus Chymotrypsin: 1 mg
  - Chymotrypsin: 1 mg
Figure 21: Electrophoresis of Heparin, Heparin plus Chymotrypsin, and Chymotrypsin on Basic Polyacrylamide Disc Gels, Stain: Coomassie Brilliant Blue
Figure 22: Electrophoresis of Heparin, Heparin plus Chymotrypsin, and Chymotrypsin on Basic Polyacrylamide Disc Gels, Stain: GPNA
Figure 23: Electrophoresis of Heparin, Heparin plus Chymotrypsin and Chymotrypsin on Basic Polyacrylamide Disc Gels, Stain: Toluidine Blue
Figure 24: Electrophoresis of Heparin, Heparin plus Chymotrypsin, and Chymotrypsin on Basic Polyacrylamide Disc Gels, Stain: Periodic Acid Schiff's
LEGEND TO FIGURES 25 - 28:

ELECTROPHORESIS OF HEPARIN, HEPARIN PLUS CHYMOTRYPSIN AND CHYMOTRYPSIN ON ACIDIC POLYACRYLAMIDE DISC GELS

Heparin, heparin plus chymotrypsin and chymotrypsin were electrophoresed on split acidic 7% polyacrylamide disc gels using the procedure of Reisfeld et al. (25). The gels were stained for protein using coomassie brilliant blue, for chymotryptic activity using GPNA followed by coupling to fast garnet GBC, for mucopolysaccharide using toluidine blue or for glycoprotein using the period acid Schiff's method.

Figure 25: Stain: coomassie brilliant blue
Gels Left to Right
Heparin: 0.033 mg
Heparin: 0.033 mg plus Chymotrypsin: 0.075 mg
Chymotrypsin: 0.075 mg

Figure 26: Stain: GPNA
Gels Left to Right
Heparin: 0.44 mg*
Heparin: 0.44 mg plus Chymotrypsin: 1 mg
Chymotrypsin: 1 mg

Figure 27: Stain: toluidine blue
Gels Left to Right
Heparin: 0.033 mg
Heparin: 0.033 mg plus Chymotrypsin: 0.075 mg
Chymotrypsin: 0.075 mg

Figure 28: Stain: periodic acid Schiff's
Gels Left to Right
Heparin: 0.44 mg
Heparin: 0.44 mg plus Chymotrypsin: 1 mg
Chymotrypsin: 1 mg

* The color of the heparin "band" in Figure 26 does not reflect zymogen activity (it was not "orange").
Figure 25: Electrophoresis of Heparin, Heparin plus Chymotrypsin, and Chymotrypsin on Acidic Polyacrylamide Disc Gels, Stain: Coomassie Brilliant Blue
Figure 26: Electrophoresis of Heparin, Heparin plus Chymotrypsin, and Chymotrypsin on Acidic Polyacrylamide Disc Gels, Stain: CFNA
Figure 27: Electrophoresis of Heparin, Heparin plus Chymotrypsin, and Chymotrypsin on Acidic Polyacrylamide Disc Gels, Stain: Toluidine Blue
Figure 23: Electrophoresis of Heparin, Heparin plus Chymotrypsin, and Chymotrypsin on Acidic Polyacrylamide Disc Gels, Stain: Periodic Acid Schiff's
in the electrical field in basic gels at pH 8.3 to a position between that of chymotrypsin and heparin. Furthermore, the complex hydrolyzes GPNA to a greater extent than chymotrypsin alone. The complex migrates to a position with a greater Rf in 5% polyacrylamide gels (Appendix Figures 85 and 86) than in 7% polyacrylamide gels while it migrates to a position (Appendix Figure 87 and 88) with essentially the same Rf in 10% polyacrylamide gels as that in the 7% gels. Under acidic conditions an active complex forms in the pH 6.8 sample gel but never enters the pH 4.3 separation gel for electrophoresis. The complex dissociates and heparin migrates toward the anode while chymotrypsin migrates towards the cathode in the two separation gels.

All lots of 3 x crystallized and 5 x crystallized chymotrypsin formed complexes with heparin which gave the same electrophoretic pattern. Mucopolysaccharide staining material was found associated with the 3 x crystallized chymotrypsin lots but not with the 5 x crystallized chymotrypsin. This toluidine blue-positive material could not be separated from the chymotrypsin under basic electrophoresis conditions using 5,7, or 10% gels. Since a mucopolysaccharide stain is observed for 3 x crystallized chymotrypsin in polyacrylamide gels in which sucrose was eliminated, the observed mucopolysaccharide stain is not due to sucrose binding to the α-amino group of lysine. It may be an artifact of purification of the enzyme. The mucopolysaccharide material is separated from the active chymotrypsin under acidic electrophoresis conditions. The glycoprotein staining material is not separated under these conditions. Chymotrypsin under the electrophoretic conditions used, exhibits two active bands.
When solutions containing chymotrypsin and heparin at various molar ratios are electrophoresed on basic 7% polyacrylamide disc gels, all of the chymotrypsin complexes with heparin until the chymotrypsin:heparin ratio exceeds 1:1 (Figure 29). At higher ratios, increasing amount of free chymotrypsin are observed in the region where chymotrypsin alone migrates, indicating that one chymotrypsin molecule cannot interact with more than one heparin molecule. The electrophoretic experiments did not conclusively determine the maximum number of heparin molecules that could bind per chymotrypsin molecule due to the polydisperse nature of heparin.

Heparin stimulates the hydrolytic activity of chymotrypsin towards the substrate GPANA as given in Figure 30. A linear increase in activity is observed until the molar ratio of heparin to chymotrypsin of approximately 3:1 is reached. After this ratio is attained, no increase in chymotryptic activity is noted. This effect cannot be due to interaction of substrate molecules with heparin because the ratio of GPANA to heparin molecules is approximately 10^3:1. These results suggest that the maximum number of heparin molecules which can bind to one chymotrypsin molecule is three. Heparin alters the $K_m$ but not the $V_{max}$ of the hydrolysis reaction of GPANA by chymotrypsin as seen in Figure 31. Molar ratios of 1.4:1 and 2.4:1 were chosen so that the activity of chymotrypsin-heparin complexes would be different at the two levels of heparin. The calculated values for $V_{max}$, $K_m$ for chymotrypsin, $K_m'$ for chymotrypsin plus $2.27 \times 10^{-6}$ M heparin and $K_m''$ for chymotrypsin plus $3.79 \times 10^{-6}$ M heparin are $2.18 \times 10^{-16}$ M/min, $3.49 \times 10^{-4}$ M, $1.15 \times 10^{-4}$ M and $0.97 \times 10^{-4}$ M, respectively.
LEGEND TO FIGURE 29:

ELECTROPHORESIS OF HEPARIN PLUS CHYMOTRYPSIN USING VARIOUS HEPARIN: CHYMOTRYPSIN RATIOS

Various amounts of heparin and chymotrypsin were applied to basic polyacrylamide disc gels and electrophoresed according to the procedure of Davis (24). The gels were stained for protein using coomassie brilliant blue.

Gels Left to Right

- Heparin 30 μg: Chymotrypsin 20 μg
- Heparin 30 μg: Chymotrypsin 50 μg
- Heparin 33 μg: Chymotrypsin 75 μg
- Heparin 33 μg: Chymotrypsin 225 μg
Figure 29: Electrophoresis of Heparin plus Chymotrypsin Using Various Heparin : Chymotrypsin Ratios
LEGEND TO FIGURE 30:

INFLUENCE OF HEPARIN ON CHYMOTRYPTIC HYDROLYSIS OF GPANA

Various ratios of heparin and chymotrypsin in 1 mM HCl were incubated at 25°C for 10 minutes. Aliquots of these mixtures were allowed to hydrolyze $1 \times 10^{-3}$ M GPANA in a 0.05 M Tris buffer solution containing 0.02 M CaCl$_2$ according to the procedure of Erlanger et al. (29).

- $2.9 \times 10^{-7}$ M Chymotrypsin
- $4.3 \times 10^{-7}$ M Chymotrypsin
- $5.7 \times 10^{-7}$ M Chymotrypsin
- $8.6 \times 10^{-7}$ M Chymotrypsin
- $1.4 \times 10^{-6}$ M Chymotrypsin
Figure 30: Influence of Heparin on Chymotryptic Hydrolysis of GPANA

△ Optical Density at 410 nm
LEGEND TO FIGURE 31:

EADIE-HOFSTEE PLOT OF THE INFLUENCE OF HEPARIN ON CHYMOTRYPTIC HYDROLYSIS OF GLUTARYL-L-PHENYLALANINE-p-NITROANILIDE

Aliquots containing 30 µg and 50 µg of heparin in 1 mM HCl incubated for 10 minutes at 25ºC with 50 µg of chymotrypsin in 1 mM HCl. To 0.2 ml of these mixtures, 1.0 ml of 1 x 10⁻³ M, 0.75 x 10⁻³ M, 0.50 x 10⁻³ M or 0.25 x 10⁻³ M GPANA in 0.05 M Tris buffer containing 0.02 M CaCl₂ were added. After 10 minutes the reaction was terminated by addition of 0.2 ml of 30% acetic acid. The amount of p-nitroaniline released was determined spectrophotometrically at 410 nm.

- 1.68 x 10⁻⁶ M Chymotrypsin
- 1.68 x 10⁻⁶ M Chymotrypsin plus 2.27 x 10⁻⁶ M Heparin
- 1.68 x 10⁻⁶ M Chymotrypsin plus 3.79 x 10⁻⁶ M Heparin
Figure 31: Badie-Hofstee Plot of the Influence of Heparin on Chymotryptic Hydrolysis of Glutaryl-L-Phenylalanine-p-Nitroanilide
Additional kinetic constants can be calculated for the heparin-chymotrypsin complex by assuming this mucopolysaccharide functions kinetically according to the general scheme for non-essential activation given by the equations (30).

\[
\begin{align*}
K_S & \quad K_P \\
E + S & \rightleftharpoons ES \rightleftharpoons E + P \\
+ & + \\
K_A & \uparrow \\
EA + S & \rightleftharpoons ESA \rightleftharpoons EA + P
\end{align*}
\]

Values for \(\alpha\), \(\beta\), and \(K_A\) are 0.141, 1.03 and 4.79 \(\times 10^{-6}\) M, respectively. The calculated value for \(K_A\) indicates that chymotrypsin has a greater affinity for heparin than for the substrate GPANA. The enhancement of activity is mainly due to an increase in the rate of reaction of the ESA complex over the ES complex which shifts the equilibrium towards the ESA complex.

The ultraviolet spectra of heparin, heparin plus chymotrypsin and chymotrypsin in 1 mM HCl, pH 3 and in 0.01 M Tris Buffer, pH 7.6 are given in Figures 32 and 33 respectively. The spectral changes due to the heparin-chymotrypsin complex are much greater at pH 3 than at pH 7.6. At the higher pH, heparin only alters the chymotrypsin spectra in the 220-230 nm region while at pH 3, the aromatic region, 280-290, is also affected. These data suggest that the overall conformation of the chymotrypsin molecule is altered to a greater extent by heparin at the lower pH. Changes in the ionic strength from 0.1 M to 0.001 M Tris Buffer, pH 7.6
LEGEND TO FIGURE 32:
ULTRAVIOLET SPECTRA OF CHYMOTRYPSIN, HEPARIN, AND CHYMOTRYPSIN PLUS HEPARIN IN 1 mM HCl

The ultraviolet spectra of $4 \times 10^{-6}$ M chymotrypsin (0.1 mg/ml), $9 \times 10^{-6}$ M heparin (0.1 mg/ml) and $4 \times 10^{-6}$ M chymotrypsin plus $9 \times 10^{-6}$ M heparin in 1 mM HCl, pH 3 was determined using a Beckman Acta IV Spectrophotometer. The reference cuvette contained 1 mM HCl.

1. Heparin
2. Heparin plus chymotrypsin
3. Chymotrypsin
Figure 32: Ultraviolet Spectra of Chymotrypsin, Heparin and Chymotrypsin plus Heparin in 1 mN HCl
Legend to Figure 33:

Ultraviolet spectra of chymotrypsin, heparin, chymotrypsin plus heparin and chymotrypsin plus heparin against chymotrypsin in 0.01 M Tris buffer, pH 7.6 were scanned from 350 to 200 nm. The reference cuvette contained 0.01 M Tris buffer, pH 7.6.

1. Heparin
2. Chymotrypsin plus heparin
3. Chymotrypsin
4. Chymotrypsin plus heparin against chymotrypsin
Figure 33: Ultraviolet Spectra of Chymotrypsin, Heparin, Chymotrypsin plus Heparin and Chymotrypsin plus Heparin Against Chymotrypsin in 0.01 M Tris Buffer
alter the ultraviolet spectra of the heparin-chymotrypsin complex only to a small degree (Appendix Figures 89 and 90). The validity of the spectra was ascertained by Beer's Law plots (Appendix Figures 94-96).

A typical set of spectra showing the proflavin difference spectra of heparin, chymotrypsin, heparin plus chymotrypsin and heparin plus chymotrypsin vs heparin and vs chymotrypsin is given in Figure 3^4. The spectra for heparin has a maximum at 445 and an extinction coefficient of 20,800 which agrees quite well with the reported extinction coefficients of 21,000 (21) and 21,300 (1) where binding is approximately stoichiometric. The difference maximum and minimum of 465 nm and 430 nm respectively agrees with the reported spectra for chymotrypsin plus proflavin vs proflavin (19,20). There is a complex formed between heparin and chymotrypsin because the heparin plus chymotrypsin vs heparin or chymotrypsin spectra are not the same as the heparin or chymotrypsin spectra respectively. The proflavin molecule is not displaced from the active center when heparin complexes with chymotrypsin since the heparin plus chymotrypsin vs heparin difference spectra at 470 nm, the wavelength that characterizes the proflavin-chymotrypsin bond at the active site, is equal to or greater than chymotrypsin alone. Similar spectra are obtained when ratios of chymotrypsin over the range for 6.3:1 to 0.39:1 are used (Appendix Figures 98-101).

The effect on the proflavin difference spectra of the heparin chymotrypsin complex of increasing the chymotrypsin concentration
LEGEND TO FIGURE 34:

PROFLAVIN DIFFERENCE SPECTRA OF CHYMOTRYPSIN, CHYMOTRYPSIN PLUS HEPARIN, AND HEPARIN

Proflavin difference spectra of heparin, chymotrypsin plus heparin, chymotrypsin, chymotrypsin plus heparin against heparin, and chymotrypsin plus heparin against chymotrypsin were obtained in $2.8 \times 10^{-5}$ M proflavin dissolved in $0.01 \, M$ Tris buffer, pH 7.6. $1.5 \times 10^{-5}$ M chymotrypsin and/or $1.0 \times 10^{-5}$ M heparin were utilized. The reference cuvette contained $2.8 \times 10^{-5}$ M proflavin dissolved in $0.01 \, M$ Tris buffer, pH 7.6.

1. Heparin
2. Chymotrypsin plus heparin
3. Chymotrypsin
4. Heparin plus chymotrypsin against chymotrypsin
5. Heparin plus chymotrypsin against heparin
Figure 34: Proflavin Difference Spectra of Chymotrypsin, Chymotrypsin plus Heparin, and Heparin
of heparin and of increasing the heparin concentration on fixed concentration of chymotrypsin are given in Figures 35 and 36 respectively. The spectral change is much greater as chymotrypsin is added than when heparin is added. This may be due to the fact that the proflavin molecules are simply being rearranged between heparin molecules as the heparin is increased. However, as chymotrypsin is added the chymotrypsin is successfully competing for the proflavin molecules. Chymotrypsin, like thrombin and trypsin (1) attracts proflavin molecules from heparin when proflavin becomes limiting. At all ratios of chymotrypsin to heparin the heparin plus chymotrypsin difference spectra were not the same as the numerical addition of the chymotrypsin difference spectra plus the heparin difference spectra.

The effect of changing the ionic strength from 0.100 M to 0.010 M to 0.001 M on the proflavin difference spectra of heparin, heparin plus chymotrypsin and chymotrypsin is observed in Figures 37-39. The spectra at 0.010 M and 0.100 M Tris Buffer, pH 7.6 are essentially the same when the difference in magnitude of interaction between proflavin and heparin is considered. The difference is more pronounced in the 0.001 M Tris Buffer spectra as compared to the spectra at the other two buffer concentrations. Comparing the heparin plus chymotrypsin vs heparin difference spectra against the chymotrypsin difference spectra, the complex increases the absorbance at 470 nm in the 0.10 M and the 0.01 M while the absorbance is the same in the 0.001 M solution.
LEGEND TO FIGURE 35:

PROFLAVIN DIFFERENCE SPECTRA OF HEPARIN PLUS CHYMOTRYPSIN WITH INCREASING CHYMOTRYPSIN CONCENTRATION

Proflavin difference spectra of heparin plus chymotrypsin were obtained using 0.01 M Tris buffer, 2.8 x 10^{-5} M proflavin, pH 7.6 solutions containing 0 to 4.0 x 10^{-5} M chymotrypsin and 1.0 x 10^{-5} M heparin. The reference cuvette contained 2.8 x 10^{-5} M proflavin in 0.01 M Tris buffer, pH 7.6.

1. 1.0 x 10^{-5} M Heparin plus 0 chymotrypsin
2. Heparin plus 3.9 x 10^{-6} M chymotrypsin
3. Heparin plus 7.7 x 10^{-6} M chymotrypsin
4. Heparin plus 1.5 x 10^{-5} M chymotrypsin
5. Heparin plus 2.8 x 10^{-5} M chymotrypsin
6. Heparin plus 4.0 x 10^{-5} M chymotrypsin
Figure 35: Proflavin Difference Spectra of Heparin plus Chymotrypsin with Increasing Chymotrypsin Concentration
Proflavin difference spectra of heparin, chymotrypsin plus heparin and chymotrypsin were obtained using 0.01 M Tris buffer, 2.8 x 10^{-5} M proflavin, pH 7.6 solutions containing 1.0 x 10^{-5} M chymotrypsin and/or 4.4 x 10^{-6} to 4.3 x 10^{-5} M heparin. The reference cuvette contained 2.8 x 10^{-5} M proflavin in 0.01 M Tris buffer, pH 7.6.

1. Heparin
2. Chymotrypsin plus Heparin
3. Chymotrypsin
   a. 4.3 x 10^{-5} M Heparin
   b. 3.2 x 10^{-5} M Heparin
   c. 2.2 x 10^{-5} M Heparin
   d. 1.7 x 10^{-5} M Heparin
   e. 4.4 x 10^{-6} M Heparin
Figure 36: Proflavin Difference Spectra of Heparin, Chymotrypsin and Chymotrypsin plus Heparin with Increasing Heparin Concentration
PROFLAVIN DIFFERENCE SPECTRA OF CHYMOTRYPSIN, CHYMOTRYPSIN PLUS HEPARIN, AND HEPARIN AT VARIOUS TRIS BUFFER CONCENTRATIONS

The spectra of $1.1 \times 10^{-5}$ M heparin, $4.9 \times 10^{-6}$ M chymotrypsin, chymotrypsin plus heparin, chymotrypsin plus heparin against heparin and chymotrypsin plus heparin against chymotrypsin in $2.8 \times 10^{-5}$ M proflavin dissolved in 0.1, 0.01 and 0.001 M Tris buffer, pH 7.6 were scanned in the visible and ultraviolet ranges. The reference cuvettes contained $2.8 \times 10^{-5}$ M proflavin in 0.1, 0.01 and 0.001 M Tris buffer, pH 7.6.

1. Heparin
2. Chymotrypsin plus Heparin
3. Chymotrypsin
4. Chymotrypsin plus Heparin against Chymotrypsin
5. Chymotrypsin plus Heparin against Heparin

Figure 37: 0.1 M Tris buffer
Figure 38: 0.01 M Tris buffer
Figure 39: 0.001 M Tris buffer
Figure 37: Proflavin Difference Spectra of Chymotrypsin, Chymotrypsin plus Heparin, and Heparin at Various Tris Buffer Concentrations: 0.1 M
Figure 38: Proflavin Difference Spectra of Chymotrypsin, Chymotrypsin Plus Heparin, and Heparin at Various Tris Buffer Concentrations: 0.01 M
Figure 39: Proflavin Difference Spectra of Chymotrypsin, Chymotrypsin Plus Heparin, and Heparin at Various Tris Buffer Concentrations: 0.001 M
DISCUSSION

The site of attraction between chymotrypsin and heparin is not at the active site of chymotrypsin. The active site of the enzyme is available to cleave GPNA at pH 7.6 as seen in zymograms of the chymotrypsin-heparin complex in polyacrylamide disc gels. The proflavin spectra and the ultraviolet spectra also indicate the presence of a complex. The lack of displacement of proflavin from the active site of chymotrypsin upon addition of heparin is further supporting evidence indicating that interaction of heparin and chymotrypsin does not occur at the active site of chymotrypsin. Molecules which interact at the active site of serine proteases displace proflavin (1,20) resulting in a loss of absorption in the difference spectra at 470 nm. Additionally, the hydrolysis of GPANA by chymotrypsin is enhanced by heparin. The same type of enhancement has been reported for mouse bone collagenase-heparin complexes (3).

Determination of the stoichiometry of the interaction of chymotrypsin and heparin is complicated by the fact that heparin is polydisperse consisting of molecules varying in molecular weight from 3,000 to 37,000 (31). The electrophoresis data and the GPANA assay, however, suggest that one chymotrypsin molecule can interact with one, two or three heparin molecules. This complex formation range is the same as that observed for heparin and thrombin (1).

The bond between chymotrypsin and heparin must be quite different at pH 3 than at pH 7.6-8.3. The ultraviolet spectra indicates that heparin at pH 3 causes a greater absorption in the
tryptophan and tyrosine regions possibly by causing a conformation change in the molecule. Since this complex can be separated under electrophoretic conditions, an electrostatic interaction may be occurring. The complex formed under basic conditions does not separate upon electrophoretic separation conditions, indicating that the bond formed is stronger than under acid conditions. Since chymotrypsin undergoes major changes in conformation with accompanying changes in reactivity upon changes in pH (18), the complex between chymotrypsin and heparin at the two pH ranges may be quite different. The basic complex also appears to be held together by a very strong bond since changes in ionic strength from 0.001 M to 0.1 M Tris Buffer do not drastically alter the proflavin binding pattern. The complex has similarly been observed by these investigators in basic gels over a range of ionic strengths from 0.6 M to 0.76 M Tris Buffer.

Since chymotrypsin-heparin complexes can be formed, it is possible that heparin-protease complexes exist in mast cells. The heparin-chymotrypsin complex is probably also physiologically important in the small intestine since heparin is a component of the intestinal mucosa (32) being localized in the mast cells (33). The presence of heparin in the gastrointestinal system has been implicated in prolonged bleeding in the intestinal tract (34). Chymotrypsin-heparin complexes may be involved in the regulation of the turnover of the epithelial cells of the mucosa since active chymotrypsin molecules bind to the mucosa (35) and proteolytic enzymes react with cell membranes causing the release of cells in culture from density dependent growth inhibition (36,37).
REFERENCES


CONCLUDING DISCUSSION AND SUMMARY

The presence of proteolytic inhibitors in malignant and normal tissues and the interaction between mucopolysaccharides including heparin and chymotrypsin indicate that both types of molecules may play a role in the control of proteolytic reactions both in the cell and on the membrane surface. These interactions may be involved in the prevention of uncontrolled proliferation. Besides further discussion of the possible role of proteolytic inhibitors and heparin, some of the results obtained in these studies merit additional discussion.

In these studies bovine $\alpha$-chymotrypsin and trypsin were used with the inhibitors from human tissues and porcine intestinal mucosa heparin. Although bovine chymotrypsin and trypsin are homologous to the corresponding human pancreatic enzymes, they do react differently towards inhibitors such as pancreatic secretory inhibitor (1) and inter-$\alpha$-trypsin inhibitor (2). The human enzymes are in general more resistant to inhibition than are other mammalian proteolytic enzymes (3). Due to this difference in reactivity, it must be realized that the inhibitors isolated from the breast tissue extracts may not inhibit the human enzymes as effectively as they inhibit the bovine enzymes. The intracellular chymotrypsin-like and trypsin-like enzymes may likewise have very different inhibitor and substrate specificities. The extrapolation of in-
hibitory activity towards bovine pancreatic enzymes to intra-
cellular enzymes are at best speculatory.

The activity of the Sepharose-chymotrypsin and Affi-Gel
10-chymotrypsin columns as well as the presence of proteolytic
activity in the tissue extracts and the isolated inhibitor peaks
from the columns is a major consideration in the interpretation
of the data. If the mechanism for binding the macromolecular in-
hibitors to chymotrypsin indeed involves cleavage of the inhibitor
as believed by Laskowski Jr. and co-workers (4), then any in-
hibitors which bind to the chymotrypsin column would be altered.
If, however, the inhibitors only bind through ionic interactions
as proposed by Feeney and co-workers (5), the native inhibitors
can be eluted from the Sepharose-chymotrypsin columns. There is
some doubt that a covalent bond is formed between the inhibitors
and the immobilized chymotrypsin molecules since the inhibitors
can be removed from the columns by 1 mN HCl and the 0.1 M to 0.3
M NaCl gradient. These conditions are mild enough to prevent
denaturation of the inhibitors since even in the preparative
column the pH only dropped to 6.5 with the use of 1 mN HCl as a
eluant. The fact that the inhibitory activity of the large
molecular fractions is very low indicates that the inhibitors may
be altered by the immobilized enzyme or the endogenous proteases.
The low molecular weight inhibitors additionally may be proteolysis
products rather than normal components of the tissues. Further ex-
ploration of these inhibitors is needed to determine their source.
Even with the proteolysis problems, the affinity chromatography
method using immobilized chymotrypsin represents a quick, efficient, simple method of separation and identifying proteolytic inhibitors in the tissue extracts.

The use of radial immunodiffusion to quantitate inhibitors does not reflect the inhibitory capacity of the inhibitors measured. Both inactive and active inhibitors react with the antibodies to the inhibitors. Immunological methods do quantitate the total content of the individual inhibitors giving an indication of the inhibitory capacity before complex formation with enzymes.

Whenever synthetic substrates are used in place of natural ones, the results must be interpreted with respect to the substrate used. Synthetic substrates simplify the analysis of changes in the reactivity of enzymes. With the use of multisite natural substrates for proteolytic enzymes, changes in the enzyme molecule may be masked by reciprocal increases and decreases in the reactivity of the enzyme towards various portions of the protein due to its amino acid sequence.

In addition to the applications discussed in Chapter 6 and in Appendix F for mucopolysaccharides in general, heparin may function to protect chymotrypsin from inactivation by inhibitors over a short time scale. Heparin induces a conformational change in the enzyme which may decrease the rate of inactivation as proposed for the heparin-thrombin complex interaction with anti-thrombin III (6).

The interrelation of proteases and proteolytic to the neoplastic process is complicated and poorly understood. Some
roles of proteolytic inhibitors and heparin in this process were discussed in the historical section and previous chapters. If heparin, in general, binds to proteolytic enzymes causing a conformational change, it may enhance the proteolytic activity of the plasminogen activator and other tumor associated proteases. The major function of heparin, however, probably is to prevent fibrin formation needed for metastasis (7) through its interaction with circulating antithrombin III. It might also be important in binding to tissue antithrombin III to prevent formation of fibrin in the tissues.

In mast cells, heparin is bound by strong ionic bonds to proteins which have not been identified (8). High salt concentrations, 1-3 M are needed to dissociate the complexes (8). These possibly could be protease-heparin, inhibitor-heparin or protease-heparin-inhibitor complexes.

The acute phase reaction associated with neoplastic conditions which increases the antitryptic activity of serum and the levels of $\alpha_1$-antitrypsin, $\alpha_2$-antichymotrypsin and $\alpha_2$-acid glycoprotein (Historical Section) may inhibit the immune system to such an extent that this system is inadequate. This increase in serum proteolytic inhibitors and the presence of the tissue inhibitors would attempt to control the additional proteolytic activity associated with the neoplastic cells and the leukocytic proteases released by the immune system. The antiproteolytic activity might prevent the required activation of the lymphocytes, and subsequent activation of macrophages by lymphocytes, as well
as, inhibition of the complement system which are needed for immune surveillance. Infusions of proteolytic enzymes aids the return of immunological competence to cancer patients (9).

Intracellular protease inhibitors may possibly exert limited control the protease associated with neoplasia because of limited mobility, inadequate concentrations or lack of inhibitory activity towards these proteases. The latter reason may explain why the increased seru levels of inhibitory activity associated with this condition do not effectively control metastasis and membrane associated proteases. The inhibitors may be insufficient, also, to control the proteases which are involved in the vascularization of tumors. Fractions from cartilage, which are high in protease inhibitory activity, experimentally inhibit vascularization of tumors (10,11).

On the other hand, possible increases in certain intracellular proteolytic inhibitors may potentiate cell division by inhibition of enzymes which normally degrade histones or other DNA control proteins, the mitogenic enzymes, or abnormal proteins.

Proteases and inhibitors may also be mediators of the actions of hormones and carcinogens. The carcinogen, PMB (phorbol-12-myristate-13-acetate) induces increased levels of proteases in animals (12). The administration of steroid hormones alters the serum levels of proteolytic inhibitors (Historical section). These hormones also raise the protease levels in hormone-sensitive organs such as the uterus (12). These reactions may be important especially in hormone-related cancer such as breast and uterine cancer.
Future experiments in the area of tissue protease inhibitors could take a number of directions. The use of an inactivated chymotrypsin column for the isolation of these inhibitors should be explored to eliminate the possibility of cleavage of the inhibitors. An in depth study of the low molecular weight inhibitors including amino acid sequencing and inhibitor specificity determination is a logical sequel to this project. Both the inhibitors and the proteases in the malignant tissues should be purified and their interactions should be studied. Synthesis of specific inhibitors to the neoplastic related proteases may lead to a possible chemotherapeutic drug. Immunological studies on tissues combined with electron microscopic studies to determine the location of the protease inhibitors and heparin in the cell would be useful. Experiments to determine whether the macromolecular inhibitors are synthesized by the individual tissue or are synthesized by the liver and then endocytosed by the cell, could give additional useful information. The interaction of the malignant tissue inhibitors with macrophages and lymphocytes as well as the proteases found in these two immune system cells would be worthwhile. The interaction between the heparin-chymotrypsin complex and chymotrypsin inhibitors would be an interesting study, especially using stop flow kinetic methods.

In conclusion, the areas of intracellular proteolytic inhibitors and the modification of proteolytic reactions are exciting and expanding areas of research. The presence of protease inhibitors in the tissues indicates the importance of proteolytic
reactions and the control of those reactions. The understanding of these reactions may lead to control of various diseases such as neoplasia and arthritis.
REFERENCES

SUPPLEMENTARY TABLES AND FIGURES TO CHAPTER 3:

ISOLATION AND IDENTIFICATION OF $\alpha_1$-ANTITRYPsin AS A
COMPONENT OF MALIGNANT HUMAN BREAST AND OTHER TISSUES

The reference numbers refer to those in Chapter 3.
LEGEND TO TABLE 12:

THE EFFECT OF HEAT ON THE INHIBITION OF TRYPIC AND CHYMOTRYPTIC HYDROLYSIS OF CASEIN BY THE 90,000 x g SUPERNATANT FRACTION FROM HUMAN BREAST TISSUE

Samples of the 90,000 x g supernatant fraction of malignant and adjacent normal human breast tissue were either incubated at 4°C or at 100°C for 10 minutes. The inhibitory activity of the samples towards chymotrypsin and trypsin was determined using the caseinolytic assay of Kunitz (33) with the modifications of Wasilauskas and Brecher (14).
Table 12

THE EFFECT OF HEAT ON THE INHIBITION OF TRYPDIC
AND CHYMOTRYPTIC HYDROLYSIS OF CASEIN BY
THE 90,000 x g SUPERNATANT FRACTION OF HUMAN
BREAST TISSUE

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Patient Number</th>
<th>µg Protein</th>
<th>Tyrosine Liberated by 1 µg Trypsin (meq. x 10^4)</th>
<th>% Inhibition of 1 µg Trypsin</th>
<th>Tyrosine Liberated by 1 µg Chymotrypsin (meq. x 10^4)</th>
<th>% Inhibition of 1 µg Chymotrypsin</th>
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<tr>
<td>Normal 4°</td>
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<td>81</td>
<td>1.44</td>
<td>60.2</td>
<td>0.75</td>
<td>88.7</td>
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<td></td>
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<td>3.27</td>
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<td>10.4</td>
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<td>59.0</td>
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<td>71.8</td>
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<td>70.2</td>
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<td>5.78</td>
<td>19.1</td>
<td>7.69</td>
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INHIBITION OF TRYPtical AND CHYMOTRYPTIC HYDROLYSIS OF CASEIN BY THE 90,000 x g SUPERNATANT FRACTION OF NORMAL AND/OR MALIGNANT HUMAN BREAST TISSUE

Samples of normal and malignant breast tissue were homogenized 1:9 (w/v) with either cold 0.32 M sucrose or 0.1 M Tris buffer, pH 7.6 for 2 minutes in a Virtis Homogenizer with the exception of the tissue from patient #73-3993 which was homogenized 1:4. The mixture was centrifuged at 20,000 x g for 30 minutes using a RC 2 Sorvall centrifuge. The 20,000 x g supernatant fraction was then centrifuged for 75 minutes at 90,000 x g using a L2-65 B Beckman Ultracentrifuge. Aliquots of the 90,000 x g supernatant fraction were tested for antichymotryptic and antitryptic activity using the caseinolytic assay of Kunitz (34) with the modifications of Wasilauskas and Brecher (14).

Figure 40: INHIBITION OF CHYMOTRYPTIC HYDROLYSIS OF CASEIN BY THE 90,000 x g SUPERNATANT FRACTION OF MALIGNANT HUMAN BREAST TISSUE FROM PATIENT #373-1335.

Figure 41: INHIBITION OF TRYPtical HYDROLYSIS OF CASEIN BY THE 90,000 x g SUPERNATANT FRACTION OF MALIGNANT AND ADJACENT NORMAL HUMAN BREAST TISSUE FROM PATIENT #8994

Normal Tissue

Malignant Tissue
Figure 42: INHIBITION OF CHYMOTRYPTIC AND TRYPIC HYDROLYSIS OF CASEIN BY THE 90,000 × g SUPERNATANT FRACTION OF MALIGNANT HUMAN BREAST TISSUE FROM PATIENT #73-3993

▲ Chymotryptic Inhibitory Activity

● Tryptic Inhibitory Activity
Figure 40: Inhibition of Chymotryptic Hydrolysis of Casein by the 90,000 x g Supernatant Fraction of Malignant Human Breast Tissue from Patient #S73-1335
Figure 41: Inhibition of Tryptic Hydrolysis of Casein by the 90,000 x g Supernatant Fraction of Malignant and Adjacent Normal Human Breast Tissue from Patient #8994
Figure 42: Inhibition of Chymotryptic and Tryptic Hydrolysis of Casein by the 90,000 x g Supernatant Fraction of Malignant Human Breast Tissue from Patient #73-3993
LEGEND TO TABLE 13:
INHIBITION OF TRYPIC AND CHYMOTRYPTIC HYDROLYSIS OF CASEIN BY THE 90,000 x g SUPERNATANT FRACTION OF MALIGNANT AND AdjACENT NORMAL HUMAN BREAST TISSUE

Aliquots of the 90,000 x g supernatant fraction of malignant and adjacent normal human breast tissue were tested for antichymo-
tryptic and antitryptic activity using the Kunitz caseinolytic assay (33) with the modifications of Wasilauskas and Brecher (14). The protein content of the tissue extracts was determined using the Lowry Folin Phenol Assay (34).
Table 13

Inhibition of Tryptic and Chymotryptic Hydrolysis of Casein by the 90,000 x g Supernatant Fraction of Malignant and Adjacent Normal Human Breast Tissue

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Patient #</th>
<th>1 µg Trypsin</th>
<th>1 µg Chymotrypsin</th>
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<tbody>
<tr>
<td>Normal</td>
<td>8217</td>
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<td>0.043</td>
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<td>Malignant</td>
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<td>0.069</td>
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<td>0.110</td>
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<td>0.105</td>
</tr>
<tr>
<td>Normal</td>
<td>8994</td>
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<td>0.058</td>
</tr>
<tr>
<td>Malignant</td>
<td>8994</td>
<td>0.068</td>
<td>0.068</td>
</tr>
<tr>
<td>Malignant</td>
<td>4150</td>
<td>0.056</td>
<td>0.140</td>
</tr>
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</table>
LEGEND TO FIGURE 43:
SEPARATION OF THE CHYMOTRYPTIC INHIBITORS IN THE 90,000 x g SUPERNATANT FRACTION OF NORMAL BREAST TISSUE FROM PATIENT #8994 BY AFFINITY CHROMATOGRAPHY ON SEPHAROSE-CHYMOTRYPSIN

A 9 ml aliquot of the 90,000 x g supernatant fraction of normal human breast tissue from patient #8994 was placed on a 0.9 x 5.5 cm Sepharose-chymotrypsin column. The column was sequentially eluted with 50 ml of 0.1 M Tris buffer, pH 7.6, 125 ml of a 0.1 M to 0.3 M NaCl gradient in Tris buffer, pH 7.6, 25 ml of 0.1 M Tris buffer, pH 7.6 and 50 ml of 1 M HCl. Column fraction aliquots of 0.3 ml were used to determine the inhibitory activity towards 0.5 µg of chymotrypsin in the case-inolytic activity assay (14, 33). The protein concentration was determined by the absorbance of the column fractions at 280 nm. Bovine serum albumin was used as the standard.

○ Chymotrypsin Inhibitory Level
● Protein Concentration
Figure 43: Separation of the Chymotryptic Inhibitors in the 90,000 x g Supernatant Fraction of Normal Breast Tissue from Patient #8994 by Affinity Chromatography on Sepharose-Chymotrypsin
LEGEND TO FIGURES 44 and 45:
SEPARATION OF THE CHYMOTRYPTIC AND/OR TRYPTIC INHIBITORS PRESENT IN THE 90,000 x g SUPERNATANT FRACTION OF MALIGNANT BREAST TISSUE OF PATIENT #S73-1335 BY AFFINITY CHROMATOGRAPHY ON SEPHAROSE-CHYMOTRYPSIN

Two 35 ml aliquots of the 90,000 x g supernatant fraction of malignant breast tissue from patient #73-1335 were applied to a 0.9 x 5.5 cm Sepharose-chymotrypsin column. This column previously was used to separate the chymotrypsin inhibitors of normal breast tissue and then was extensively washed with 1 mM HCl and 0.1 M Tris buffer, pH 7.6. The same washing procedure was used on this column between the two aliquots of tissue extracts from patient #73-1335. The column was eluted sequentially with 120 ml of 0.1 M Tris buffer, pH 7.6; 450 ml of a 0.1 M to 0.3 M NaCl gradient in 0.1 M Tris buffer, pH 7.6; 50 ml of 0.1 M Tris buffer, pH 7.6; 100 ml of 1 mM HCl and 100 ml of 0.1 M Tris buffer, pH 7.6. The inhibitory activity of 0.3 ml aliquots of the fractions eluted from the column towards 0.5 µg of chymotryptin and/or trypsin were determined using a caseinolytic assay (14, 33). The protein concentration of the fractions was determined using the procedure of Lowry et al. (34).
LEGEND TO FIGURE 44:
SEPARATION OF THE CHYMOTRYPIC INHIBITORS PRESENT IN THE 90,000 x g SUPERNATANT FRACTION OF MALIGNANT TISSUE OF PATIENT #S73-1335 (BATCH 1), ON SEPHAROSE-CHYMOTRYPSIN

○ CHYMOTRYPIC INHIBITORY LEVEL
▲ PROTEIN CONCENTRATION

LEGEND TO FIGURE 45:
SEPARATION OF THE CHYMOTRYPIC AND TRYPTIC INHIBITORS PRESENT IN THE 90,000 x g SUPERNATANT FRACTION OF MALIGNANT BREAST TISSUE OF PATIENT #S73-1335 (BATCH 2), ON SEPHAROSE-CHYMOTRYPSIN

○ CHYMOTRYPIC INHIBITORY LEVEL
▲ TRYPTIC INHIBITORY LEVEL
○ PROTEIN CONCENTRATION
Figure 44: Separation of the Chymotryptic Inhibitors Present in the 90,000 x g Supernatant Fraction of Malignant Breast Tissue of Patient #973-1335, Batch 1, on Sepharose-Chymotrypsin
Figure 45: Separation of the Chymotryptic and Tryptic Inhibitors Present in the 90,000 x g Supernatant Fraction of Malignant Breast Tissue of Patient 873-1335, Batch 2, on Sepharose-Chymotrypsin
LEGEND TO TABLE 14:

PROTEIN CONCENTRATION AND CHYMOTRYPIC INHIBITORY ACTIVITY OF THE POOLED 50,000 MOLECULAR WEIGHT CONCENTRATES AND FILTRATES FROM THE SEPHAROSE-CHYMOTRYPSIN COLUMN SEPARATION OF PROTEINS IN MALIGNANT BREAST TISSUE EXTRACTS

The pooled peaks, of chymotryptic inhibitory activity from the Sepharose-chymotrypsin column separation of protein in the 90,000 x g supernatant fraction of malignant breast tissue from patient #S73-1335, were concentrated by ultrafiltration at 40 psi nitrogen using an Amicon Diaflo XM 50 membrane filter with a 50,000 molecular weight cut off. The inhibitory activity of 0.10 ml of the original solutions and the concentrates and 0.25 ml of the filtrates towards 0.25 μg of chymotrypsin was determined using the caseinolytic assay for chymotrypsin activity (14, 33). An estimate of the inhibitory level at 0.10 ml was made to compare the % inhibition of the filtrates with the pooled and concentrated fractions. The protein content of the solutions was determined by the method of Lowry et al. (34).
Table 14

PROTEIN CONCENTRATION AND INHIBITORY INHIBITORY ACTIVITY OF THE POOLED 50,000 MOLECULAR WEIGHT CONCENTRATES AND FILTRATES FROM THE SEPHAROSE-CHYMOTRYPSIN SEPARATION OF PROTEINS IN MALIGNANT BREAST TISSUE EXTRACTS

<table>
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<th>Pooled Fraction No.</th>
<th>Initial Vol. ml</th>
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<th>Volume Concentration Factor</th>
<th>Protein Concentration μg Protein/ml</th>
<th>Protein Concentration Factor</th>
<th>% Protein Retained above Filter</th>
<th>% Protein in Filtrate</th>
<th>% Protein Lost or Retained in Filter</th>
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LEGEND TO TABLE 15:
INHIBITORY ACTIVITY AND PROTEIN CONCENTRATION OF THE POOLED PEAKS, 30,000 MOLECULAR WEIGHT CONCENTRATES AND FILTRATES FROM THE AFFI-GEL 10-CHYMOTRYPSIN COLUMN SEPARATION OF MALIGNANT BREAST TISSUE EXTRACTS

The pooled peaks, of chymotryptic and/or tryptic inhibitory activity from the Affi-Gel 10-chymotrypsin column separation of the proteins in the 90,000 x g supernatant fraction of malignant breast tissue from patient #4150, were concentrated by ultrafiltration using 40 psi nitrogen and a PM 30 Amicon Diaflo ultrafiltration membrane filter with a 30,000 molecular cut off. The protein concentration and the inhibitory activity of the original pooled peaks, concentrates and filtrates were determined using the Lowry method (34) and the casein method for determination of the hydrolytic activity of chymotrypsin and trypsin (14, 33), respectively. A ratio of 0.25 \( \mu \)g enzyme to 0.1 ml of solution was used in all enzyme assays.
Table 15

INHIBITORY ACTIVITY AND PROTEIN CONCENTRATION OF THE POOLED PEAKS, 30,000 MOLAR WEIGHT CONCENTRATES AND FILTRATES FROM THE AFFI-GEL 10-CHYMOTRYPSIN COLUMN SEPARATION OF MALIGNANT BREAST TISSUE EXTRACTS

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<th>Pooled Fraction No.</th>
<th>Initial Vol. ml</th>
<th>Final Volume ml</th>
<th>Protein Concentration</th>
<th>% Protein Retained above Filter</th>
<th>Protein Lost or Retained in Filter</th>
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<td>Protein</td>
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<td>µg Enzyme Inhibited/mg Protein</td>
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LEGEND TO TABLE 16:

INHIBITORY ACTIVITY AND PROTEIN CONCENTRATION OF THE 1,000 MOLECULAR WEIGHT CONCENTRATES AND FILTRATES FROM THE AFFI-GEL 10-CHYMOTRYPSIN COLUMN SEPARATION OF MALIGNANT BREAST TISSUE EXTRACTS

The pooled peaks, of chymotryptic and/or tryptic inhibitory activity from the Affi-Gel 10-chymotrypsin column separation of the proteins in the 90,000 x g supernatant fraction of malignant breast tissue from patient #4150 were concentrated first by ultrafiltration using 40 psi nitrogen and a PM 30 Amicon Diaflo membrane filter with a molecular cut off of 30,000. The filtrates from this ultrafiltration step were further concentrated by ultrafiltration using a UM 2 Amicon Membrane Filter with a molecular cut off of 1,000. The protein concentration and the inhibitory activity of the 30,000 molecular weight filtrates, the 1,000 molecular weight concentrates and filtrates were determined using the Lowry method (34) and the casein method for determination of the hydrolytic activity of chymotrypsin and trypsin (14, 33), respectively. A ratio of 0.25 μg enzyme to 0.1 ml of solution was used in all enzyme assays.
Table 16

INHIBITORY ACTIVITY AND PROTEIN CONCENTRATION OF THE 1,000 MOLECULAR WEIGHT CONCENTRATES AND FILTRATES FROM THE AFFI-GEL 10-CHYMOTRYPSIN COLUMN SEPARATION OF MALIGNANT BREAST TISSUE EXTRACTS

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Table 16 (cont.)

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Table 16 (cont.)

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LEGEND TO FIGURE 46:

MICRODISC GEL ELECTROPHORESIS SEPARATION OF PROTEINS IN THE POOLED INHIBITOR PEAKS FROM THE AFFI-GEL 10-CHYMOTRYPSIN COLUMN

The micro disc gel electrophoresis technique of Burr (38) using 15% polyacrylamide gels was used to separate the proteins in 0.5 ml of the pooled peaks of antichymotryptic and antitryptic activity from the Affi-Gel 10-chymotrypsin column. The gels were stained for protein using coomassie brilliant blue stain (41). Bromophenol blue (BPB) was used as a tracking dye.
Figure 46: Microdisc Gel Electrophoresis Separation of Proteins in the Pooled Inhibitor Peaks from the Affi-Gel 10-Chymotrypsin Column
LEGEND TO FIGURE 47:

MICRODISC GEL ELECTROPHORESIS SEPARATION OF PROTEINS IN THE 30,000 MOLECULAR WEIGHT FILTRATES OF THE POOLED INHIBITOR PEAKS FROM ULTRAFILTRATION

The microdisc gel electrophoresis technique of Burr (38) on 15% polyacrylamide gels was used to separate the proteins in 0.5 ml of the 30,000 molecular weight filtrates of the pooled peaks of antichymotryptic and antitryptic activity from the Affigel 10-chymotrypsin column. The gels were stained for protein using coomassie brilliant blue stain (41). Bromophenol blue (BPB) was used as the tracking dye.
Figure 4: Microdisc Gel Electrophoresis Separation of Proteins in the 30,000 Molecular Weight Filtrates of the Pooled Inhibitor Peaks from Ultrafiltration
LEGEND TO FIGURE 48:

DISC GEL ELECTROPHORESIS SEPARATION OF THE PROTEINS IN THE 90,000 x g SUPERNATANT FRACTION OF MALIGNANT BREAST AND SERUM ON 5.25% POLYACRYLAMIDE GELS

Samples of 5 μl of the 90,000 x g supernatant fraction of malignant breast tissue from patient #4150 which had been concentrated using an Amicon miniconcentrator and serum were placed on 5.25% polyacrylamide disc gels. Electrophoresis was carried out using the procedure of Davis (37). The gels were stained with periodic acid Schiff's reagent (40) for glycoproteins and with coomassie brilliant blue for protein (41).

a. 90,000 x g Supernatant Fraction of Malignant Breast
b. Serum
   1. Coomassie Brilliant Blue for Protein
   2. Periodic Acid Schiff's for Glycoprotein
Figure 48: Disc Gel Electrophoresis Separation of the Proteins in the 90,000 × g Supernatant Fraction of Malignant Breast and Serum on 5.25% Polyacrylamide Gels
LEGEND TO FIGURE 49:

IMMUNODIFFUSION IDENTIFICATION OF $\alpha_1$-ANTITRYPSIN IN THE 90,000
x g SUPERNATANT FRACTION OF MALIGNANT HUMAN BREAST TISSUE AND IN
PEAK 1 CONCENTRATE

5 $\mu$l of serum (A-1), concentrated peak 1 eluate from the
Affi-Gel 10-chymotrypsin column (A-3) and concentrated samples
of the 90,000 x g supernatant fraction of malignant breast tissue
from patients #8217 (C-1), #5286 (C-2), #8994 (C-3), #5-73-1335
(C-4), #4150 (C-5), were applied to a Miles $\alpha_1$-Trypsin Inhibitor
Immunodiffusion Plate. Row B contains standard $\alpha_1$-antitrypsin
solutions.
Figure 49: Immunodiffusion Identification of $\alpha_1$-Antitrypsin in the 90,000 x g Supernatant Fraction of Malignant Human Breast Tissue and in Peak 1 Concentrate
LEGEND TO FIGURE 50:

IMMUNODIFFUSION IDENTIFICATION OF $\alpha_1$-ANTITRYPSIN IN THE 90,000 x $g$ SUPERNATANT FRACTION OF NORMAL AND MALIGNANT COLON, ANAL AND BREAST AND MALIGNANT LUNG, STOMACH AND ILEUM

5 $\mu$l of the concentrated 90,000 x $g$ supernatant fraction of normal breast tissue from patient #S73-2543 (A-1), normal and malignant colon tissues from patients #3120 (A-2, A-3, respectively), #M-73-416 (A-4, A-5, respectively) and #6161A (C-1, C-2, respectively), malignant ileum from patient #M-73-42 (A-6), malignant stomach from patient #113097 (C-3), malignant lung from patient #73-6430 (C-4) and normal and malignant anal from patient #S73-06098 (C-5, C-6, respectively) were applied to a Miles $\alpha_1$-Trypsin Inhibitor Immunodiffusion Plate. Standard $\alpha_1$-antitrypsin solutions were applied to row B. After an incubation period of 16 hours at room temperature, the size of the precipitin rings were measured and the plate was photographed.
Figure 50: Immunodiffusion Identification of $\alpha_1$-Antitrypsin in the 90,000 x g Supernatant Fraction of Normal and Malignant Colon, Anal and Breast and Malignant Lung, Stomach and Ileum
LEGEND TO FIGURES 51 - 55

POLYACRYLAMIDE DISC GEL ELECTROPHORESIS OF THE 30,000 MOLECULAR WEIGHT CONCENTRATES FROM THE SEPHAROSE-CHYMOTRYPSIN COLUMN ELUATES OF MALIGNANT BREAST TISSUE PROTEOLYTIC INHIBITORS

The nine major peaks of antichymotryptic and antitryptic activity eluted from the Sepharose-chymotrypsin column were subdivided and pooled into 25 subpeaks. The 25 subpeaks were concentrated by ultrafiltration using a 30,000 molecular weight cut off membrane. Aliquots of 0.1 ml of the 30,000 molecular weight concentrates were electrophorosed on 7% basic polyacrylamide gels according to the procedure of Davis (14).

Gels Left to Right

Figure 51: 1a, 1b, 2a, 2b, 2c
Figure 52: 3a, 3b, 3c, 3d, 3e
Figure 53: 4a, 4b, 5a, 5b, 5c
Figure 54: 5d, 6a, 6b, 7a, 7b
Figure 55: 7c, 8, 9a, 9b, 9c
Figure 51: Polyacrylamide Disc Gel Electrophoresis of the 30,000 Molecular Weight Concentrates from the Sepharose-Chymotrypsin Column Eluates of Malignant Breast Tissue Proteolytic Inhibitors: Peaks 1a, 1b, 2a, 2b & 2c
Figure 52: Polyacrylamide Disc Gel Electrophoresis of the 30,000 Molecular Weight Concentrates from the Sepharose-Chymotrypsin Column Eluates of Malignant Breast Tissue

Proteolytic Inhibitors: Peaks 3a, 3b, 3c, 3d & 3e
Figure 53: Polyacrylamide Disc Gel Electrophoresis of the 30,000 Molecular Weight Concentrates from the Sepharose-Chymotrypsin Column Eluates of Malignant Breast Tissue
Proteolytic Inhibitors: Peaks 4a, 4b, 5a, 5b & 5c
Figure 5: Polyacrylamide Disc Gel Electrophoresis of the 30,000 Molecular Weight Concentrates from the Sepharose-Chymotrypsin Column Eluates of Malignant Breast Tissue
Proteolytic Inhibitors: Peaks 5b, 6a, 6b, 7a & 7b
Figure 55: Polyacrylamide Disc Gel Electrophoresis of the 30,000 Molecular Weight Concentrates from the Sepharose-Chymotrypsin Column Eluates of Malignant Breast Tissue Proteolytic Inhibitors: 7c, 8, 9a, 9b & 9c
LEGEND TO FIGURES 56 - 60:

POLYACRYLAMIDE DISC GEL ELECTROPHORESIS OF THE 1,000 MOLECULAR WEIGHT CONCENTRATES FROM THE SEPHAROSE-CHYMOTRYPSIN COLUMN ELUATES OF MALIGNANT BREAST TISSUE PROTEOLYTIC INHIBITORS

The nine major peaks of antichymotryptic and antitryptic activity eluted from the Sepharose-chymotrypsin column were subdivided and pooled into 25 subpeaks. The 25 subpeaks were concentrated by ultrafiltration first using a 30,000 molecular weight cut off membrane. The filtrates from this first concentration step were then concentrated using a 1,000 molecular weight cut off membrane. Aliquots of 0.1 ml of the 1,000 molecular weight concentrates were electrophoresed on 7% polyacrylamide gels according to the procedure of Davis (14).

Gels left to Right:

Figure 56: 1a, 1b, 2a, 2b, 2c
Figure 57: 3a, 3b, 3c, 3d, 3e
Figure 58: 4a, 4b, 5a, 5b, 5c
Figure 59: 5d, 6a, 6b, 7a, 7b
Figure 60: 7c, 8, 9a, 9b, 9c
Figure 56: Polyacrylamide Disc Gel Electrophoresis of the 1,000 Molecular Weight Concentrates from the Sepharose-Chymotrypsin Column Eluates of Malignant Breast Tissue Proteolytic Inhibitors: Peaks 1a, 1b, 2a, 2b & 2c
Figure 57: Polyacrylamide Disc Gel Electrophoresis of the 1,000 Molecular Weight Concentrates from the Sepharose-Chymotrypsin Column Eluates of Malignant Breast Tissue

Proteolytic Inhibitors: Peaks 3a, 3b, 3c, 3d & 3e
Figure 58: Polyacrylamide Disc Gel Electrophoresis of the 1,000 Molecular Weight Concentrates from the Sepharose-Chymotrypsin Column Eluates of Malignant Breast Tissue

Proteolytic Inhibitors: Peaks 4a, 4b, 5a, 5b & 5c
Figure 59: Polyacrylamide Disc Gel Electrophoresis of the 1,000 Molecular Weight Concentrates from the Sepharose-Chymotrypsin Column Eluates of Malignant Breast Tissue
Proteolytic Inhibitors: Peaks 5a, 6a, 6b, 7a & 7b
Figure 60: Polyacrylamide Disc Gel Electrophoresis of the 1,000 Molecular Weight Concentrates from the Sepharose-Chymotrypsin Column Eluates of Malignant Breast Tissue
Proteolytic Inhibitors: Peaks 7c, 8, 9a, 9b & 9c
MICRODOUBLE IMMUNODIFFUSION IDENTIFICATION OF SERUM PROTEINASE INHIBITORS IN THE 30,000 MOLECULAR WEIGHT CONCENTRATES FROM THE SEPHAROSE-CHYMOTRYPSIN SEPARATION OF MALIGNANT BREAST TISSUE

Five μl aliquots, of the 30,000 molecular weight concentrates of the chymotryptic and/or tryptic inhibitory peaks from the Sepharose-chymotrypsin column separation of malignant breast tissue extracts, were applied to the outer wells of agar double-immunodiffusion plates. Monospecific antisera (Behring) to α₁-antitrypsin, α₂-macroglobulin, α₁-antichymotrypsin, α₁-acid glycoprotein or antithrombin III was placed in the center wells. The microdouble immunodiffusion technique in agar gels was used in these experiments (18).

a and b on all plates human plasma

Figures 61 - 66: Center well Antisera to α₁-Antitrypsin

Figure 61: 1. Peak 1b  2. Peak 1a  3. Peak 2b  4. Peak 2a
Figure 62: 1. Peak 3a  2. Peak 2c  3. Peak 3e  4. Peak 3b
Figure 63: 1. Peak 3d  2. Peak 3e  3. Peak 3b  4. Peak 4a
Figure 64: 1. Peak 5b  2. Peak 5a  3. Peak 5d  4. Peak 5c
Figure 65: 1. Peak 6b  2. Peak 6a  3. Peak 7b  4. Peak 7a
Figure 66: 1. Peak 8  2. Peak 7c  3. Peak 9b  4. Peak 9a
Figure 67: Center well Antisera to $\alpha_2$-Macroglobulin
1. Peak 8
2. Peak 8
3. 90,000 x g supernatant fraction
4. Peak 8
LEGEND TO TABLE 17:

CASEINOLYTIC ACTIVITY OF SOLUTIONS INCUBATED WITH SEPHAROSE-CHYMOTRYPSIN

A 42 mg sample of Sepharose-chymotrypsin was allowed to incubate at 4°C with 3 ml of 0.1 M Tris buffer, pH 7.6 for 30 minutes. The mixture was centrifuged at 27,000 x g for 15 minutes. The supernatant fraction was removed and saved. The same procedure was repeated sequentially using 0.1 M NaCl in 0.1 M Tris buffer, pH 7.6; 0.2 M NaCl in 0.1 M Tris buffer, pH 7.6; 0.3 M NaCl in 0.1 M Tris buffer, pH 7.6; 0.1 M Tris buffer, pH 7.6; 1mN HCl and 0.1 M Tris buffer, pH 7.6, to simulate the conditions used in the Sepharose-chymotrypsin column separation of the protease inhibitors in the 90,000 x g supernatant fraction of malignant breast tissue. Aliquots of 0.15 ml of the solutions were tested for hydrolytic activity towards casein using the caseinolytic assay of Kunitz (11) with the modifications of Wasilauskas and Brecher (10).
Table 17
Casinoletic Activity of Solutions Incubated with Sepharose-Chymotrypsin

<table>
<thead>
<tr>
<th>Caseinolytic Activity Equivalent μg Chymotrypsin/ml</th>
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<tbody>
<tr>
<td>0.1 M Tris Buffer, pH 7.6</td>
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<tr>
<td>0.1 M NaCl in 0.1 M Tris Buffer, pH 7.6</td>
</tr>
<tr>
<td>0.2 M NaCl in 0.1 M Tris Buffer, pH 7.6</td>
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<td>0.3 M NaCl in 0.1 M Tris Buffer, pH 7.6</td>
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<td>0.1 M Tris Buffer, pH 7.6</td>
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<td>1 mM HCl</td>
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<td>0.1 M Tris Buffer, pH 7.6</td>
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</table>
LEGEND TO FIGURE 68:

PROTEIN CONCENTRATION OF THE FRACTIONS FROM THE PREPARATIVE ELECTROPHORESIS SEPARATION OF THE PROTEINS IN THE 30,000 MOLECULAR WEIGHT CONCENTRATE OF PEAK 8

Approximately 2 mg of protein, in 27 ml of 0.1 M Tris buffer-0.022 M sucrose solution, pH 7.6 from the 30,000 molecular weight concentrate of peak 8 obtained from the Sepharose-chymotrypsin separation of the 90,000 x g supernatant fraction of malignant breast tissue, was applied to the top of the concentrating gel of a preparative polyacrylamide disc gel. Preparative electrophoresis was carried out according to the procedure of Jovin et al. (13) using a Buchler preparative electrophoresis apparatus and a 7% separating gel. Eluate fractions of 4.5 ml were collected. Aliquots of these fractions were assayed for protein content by the Lowry Folin Phenol method (12). α₁-Antitrypsin was detected in fractions 13-19 using a microdouble-immunodiffusion technique (18).
Figure 68: Protein Concentration of the Fractions from the Preparative Electrophoresis Separation of the Proteins in the 30,000 Molecular Weight Concentrate of Peak #8
Ten μl aliquots of the eluate fractions from preparative electrophoresis of the 30,000 molecular weight concentrate of peak 8 from the Sepharose-chymotrypsin column were placed in the outer wells of agar double immunodiffusion plates. Monospecific antisera (Behring) to α₁-antitrypsin was placed in the center well. The microdouble-immunodiffusion technique was used in this experiment.
Figure 69: Microdouble-Immunodiffusion Identification of $\alpha_1$-Antitrypsin in the Eluate Fractions from Preparative Electrophoresis of Peak #8 from the Sepharose-Chymotrypsin Column
LEGEND TO FIGURE 70:

DISC GEL ELECTROPHORESIS OF PURIFIED $\alpha_1$-ANTITRYPSIN

Approximately 7 $\mu$g of purified $\alpha_1$-antitrypsin was applied to 5, 7 and 10% polyacrylamide disc gels. The $\alpha_1$-antitrypsin was purified from the 90,000 x $g$ supernatant fraction of malignant human breast tissue by fractionation on a Sepharose-chymotrypsin affinity chromatography column followed by preparative electrophoresis. Electrophoresis was carried out according to the procedure of Davis (15). The gels were stained for protein using coomassie brilliant blue (16).

Gels Left to Right

5%
7%
10%
Figure 70: Disc Gel Electrophoresis of Purified $\alpha_1$-Antitrypsin
The nine major peaks of antichymotryptic and antitryptic activity eluted from the Sepharose-chymotrypsin column which was charged with the 90,000 x g supernatant fraction of malignant human breast tissue, were subdivided and pooled into 25 subpeaks. The 25 subpeaks were concentrated by ultrafiltration first using a 30,000 molecular weight cut off membrane. The filtrates from this first concentration step were then concentrated using a 1,000 molecular weight cut off membrane. Aliquots of 0.15 ml of the pooled fractions, concentrates and filtrates were assayed for antichymotryptic and antitryptic activity using the caseinolytic assay (10, 11) with 0.25 µg of the enzymes. The protein content of the solutions was determined using the procedure of Lowry et al. (12).
TABLE 18

INHIBITORY ACTIVITY OF THE POOLED CONCENTRATED FRACTIONS FROM
THE SEPARATION OF INHIBITORS OF CHYMOTRYPSIN AND TRYPsin IN
THE 90,000 x g SUPERNATANT FRACTION OF MALIGNANT BREAST TISSUE

THE SEPHAROSE-CHYMOTRYPSIN

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<th>% Inhibition Trypsin</th>
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APPENDIX SECTION C

SUPPLEMENTARY FIGURES TO CHAPTER 5:

IDENTIFICATION OF $\alpha_1$-ACID GLYCOPROTEIN, $\alpha_2$-MACROGLOBULIN AND ANTITHROMBIN III AS COMPONENTS OF NORMAL AND MALIGNANT HUMAN TISSUES

The reference numbers refer to those in Chapter 5.
LEGEND TO FIGURES 71 - 78

MICRODOUBLE IMMUNODIFFUSION IDENTIFICATION OF SERUM PROTEINASE INHIBITORS IN MALIGNANT TISSUE EXTRACTS AND PURIFIED SAMPLES

Five µl aliquots of human plasma and concentrated 90,000 x g supernatant fractions of normal and malignant human breast, colon, and anal, and malignant lung and stomach tissues were applied to the outer wells of agar double immunodiffusion plates. Monospecific antisera to \( \alpha_1 \)-antitrypsin, \( \alpha_2 \)-macroglobulin, \( \alpha_1 \)-antichymotrypsin, \( \alpha_1 \)-acid glycoprotein or antithrombin III was placed in the center wells. The microdouble-immunodiffusion technique of Gelman was used in these experiments (20).

\[ \begin{array}{c}
\text{a} \\
1 \circ \circ 3 \\
2 \circ \circ 4 \\
\text{b} \\
\end{array} \]

a and b on all plates human plasma

Figures 71 - 74
1. Malignant Breast #6161A
2. Normal Breast #6161A
3. Malignant Colon #M73-41
4. Normal Colon #M73-41

Figures 75 - 78
1. Malignant Lung #73-6430
2. Malignant Stomach #113097
3. Malignant Anal #S73-06098
4. Normal Anal #S73-06098

Figures 71 & 75 Antisera to \( \alpha_1 \)-Antitrypsin
Figures 72 & 76 Antisera to Antithrombin III
Figures 73 & 77 Antisera to \( \alpha_1 \)-Acid Glycoprotein
Figures 74 & 78 Antisera to \( \alpha_2 \)-Macroglobulin
LEGEND TO FIGURES 79 - 84:

RADIAL IMMUNODIFFUSION QUANTITATION OF THE $\alpha_1$-ACID GLYCOPROTEIN, $\alpha_2$-MACROGLOBULIN AND ANTITHROMBIN III CONTENT IN HUMAN TISSUE EXTRACTS

The $\alpha_1$-acid glycoprotein, $\alpha_2$-macroglobulin and antithrombin III content of the 90,000 x g supernatant fraction of malignant and/or normal adjacent breast, colon, anal, ileum, stomach and lung tissues were determined using the radial immunodiffusion procedure of Mancini et al. (21). Aliquots of 5 µl of the various 90,000 x g supernatant fractions were placed in the wells of the agar plates containing monospecific antisera to $\alpha_1$-acid glycoprotein, $\alpha_2$-macroglobulin or antithrombin III.

Figures 79 & 80 Antisera to $\alpha_2$-Macroglobulin
Figures 81 & 82 Antisera to Antithrombin III
Figures 83 & 84 Antisera to $\alpha_1$-Acid Glycoprotein

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<tr>
<td>3</td>
<td>M73-41</td>
<td>Malignant Colon</td>
<td></td>
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<tr>
<td>4</td>
<td>M73-416</td>
<td>Normal Colon</td>
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<td>5</td>
<td>M73-416</td>
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<td>6</td>
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<td>3130</td>
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<td>8</td>
<td>113020</td>
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<td>9</td>
<td>S73-06098</td>
<td>Normal Anal</td>
<td></td>
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<tr>
<td>10</td>
<td>S73-06098</td>
<td>Malignant Anal</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>M73-42</td>
<td>Malignant Ileum</td>
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</tr>
<tr>
<td>12</td>
<td>73-6430</td>
<td>Malignant Lung</td>
<td></td>
</tr>
</tbody>
</table>
Radial Immunodiffusion Quantitation of the $\alpha_2$-Macroglobulin Content in Human Tissue Extracts

Figure 79: Breast and Stomach

Figure 80: Colon, Anal, Ileum and Lung
Radial Immunodiffusion Quantitation of the Antithrombin III Content in Human Tissue Extracts:

Figure 81: Breast and Stomach

Figure 82: Colon, Anal, Ileum and Lung
Radial Immunodiffusion Quantitation of the α₁-Acid Glycoprotein Content in Human Tissue Extracts

Figure 83: Breast and Stomach

Figure 84: Colon, Anal, Ileum and Lung
APPENDIX SECTION D

SUPPLEMENTARY FIGURES TO CHAPTER 6:

CHARACTERIZATION OF THE INTERACTION BETWEEN CHYMOTRYPSIN AND HEPARIN

The reference numbers refer to those in Chapter 6.
LEGEND TO FIGURES 85 - 88:

ELECTROPHORESIS OF HEPARIN, HEPARIN PLUS CHYMOTRYPSIN AND CHYMOTRYPSIN ON BASIC 5% and 10% POLYACRYLAMIDE DISC GELS

Heparin, heparin plus chymotrypsin and chymotrypsin were electrophoresed on basic 5% and 10% polyacrylamide disc gels using the procedure of Davis (24). The gels were stained for protein using coomassie brilliant blue. They were stained for mucopolysaccharide with toluidine blue.

Figures 85 & 86:      5% Polyacrylamide Gels
Figures 87 & 88:      10% Polyacrylamide Gels
Figures 85 & 87:      Stain: Coomassie Brilliant Blue
                      Gels Left to Right
                      Heparin: 1 mg
                      Heparin: 0.033 mg plus Chymotrypsin: 0.075 mg
                      Chymotrypsin: 0.075 mg
Figures 86 & 88:      Stain: Toluidine Blue
                      Gels Left to Right
                      Heparin: 0.033 mg
                      Heparin: 0.033 mg plus Chymotrypsin: 0.075 mg
                      Chymotrypsin: 1 mg
Figure 85: Electrophoresis of Heparin, Heparin plus Chymotrypsin and Chymotrypsin on 5% Polyacrylamide Disc Gels; Coomassie Brilliant Blue
Figure 86: Electrophoresis of Heparin, Heparin plus Chymotrypsin and Chymotrypsin on 5% Polyacrylamide Disc Gels: Toluidine Blue
Figure 87: Electrophoresis of Heparin, Heparin plus Chymotrypsin and Chymotrypsin on 10% Polyacrylamide Disc Gels: Coomassie Brilliant Blue
Figure 88: Electrophoresis of Heparin, Heparin plus Chymotrypsin and Chymotrypsin on 10% Polyacrylamide Disc Gels: Toluidine Blue
LEGEND TO FIGURES 89 AND 90

ULTRAVIOLET SPECTRA OF CHYMOTRYPSIN, HEPARIN, CHYMOTRYPSIN PLUS
HEPARIN AND CHYMOTRYPSIN PLUS HEPARIN AGAINST CHYMOTRYPSIN IN
0.1 M AND 0.001 M TRIS BUFFER

Ultraviolet spectra of chymotrypsin, heparin, chymotrypsin
plus heparin and chymotrypsin plus heparin against chymotrypsin
in 0.001 M and 0.1 M Tris buffer, pH 7.6, were scanned from 350
to 200 nm.

Figure 89: 0.1 M Tris buffer, pH 7.6
Figure 90: 0.001 M Tris buffer, pH 7.6

A-Chymotrypsin plus Heparin against Chymotrypsin
B-Chymotrypsin plus Heparin
C-Chymotrypsin
D-Heparin
Figure 89: Ultraviolet Spectra of Chymotrypsin, Heparin, Chymotrypsin plus Heparin and Chymotrypsin plus Heparin Against Chymotrypsin in 0.1 M Tris Buffer
Figure 90: Ultraviolet Spectra of Chymotrypsin, Heparin, Chymotrypsin plus Heparin and Chymotrypsin plus Heparin Against Chymotrypsin in 0.001 M Tris Buffer
LEGEND TO FIGURE 91:

INFLUENCE OF TRIS BUFFER ON THE ABSORBANCE OF CHYMOTRYPSIN, HEPARIN AND CHYMOTRYPSIN PLUS HEPARIN

Ultraviolet spectra of chymotrypsin, heparin, chymotrypsin plus heparin and chymotrypsin plus heparin against chymotrypsin in 0.1 M, 0.01 M and 0.001 M Tris buffer, pH 7.6 were scanned from 350 to 200 nm. The absorbance of heparin, chymotrypsin and chymotrypsin plus heparin were plotted against Tris buffer concentration at 220, 230 and 240 nm.

○ 220, ● 230, ○ 240 nm Chymotrypsin
△ 220, ▲ 230, △ 240 nm Chymotrypsin plus Heparin
□ 220, ■ 230, □ 240 nm Heparin
Figure 91: Influence of Tris Buffer on the Absorbance of Chymotrypsin, Heparin and Chymotrypsin Plus Heparin
LEGEND TO FIGURE 92:

ULTRAVIOLET SPECTRA OF $4.0 \times 10^{-7}$ TO $2.5 \times 10^{-6}$ M CHYMOTRYPsin SOLUTIONS IN 1 mN HCl

The ultraviolet spectra of four levels of chymotrypsin in 1 mN HCl, pH 3 were scanned from 350 to 200 using a Cary 14 Recording Spectrophotometer

A. $4.0 \times 10^{-7}$ M Chymotrypsin (0.01 mg/ml)
B. $1.3 \times 10^{-6}$ M Chymotrypsin (0.033 mg/ml)
C. $1.9 \times 10^{-6}$ M Chymotrypsin (0.050 mg/ml)
D. $2.5 \times 10^{-6}$ M Chymotrypsin (0.066 mg/ml)
Figure 92: Ultraviolet Spectra of $4.0 \times 10^{-7}$ to $2.5 \times 10^{-6}$ M Chymotrypsin Solutions in 1 mN HCl
LEGEND TO FIGURE 93:

ULTRAVIOLET SPECTRA OF CHYMOTRYPSIN PLUS HEPARIN AT VARIOUS LEVELS
IN 1 mM HCl

The ultraviolet spectra of four levels of chymotrypsin plus heparin at a 1 to 3 molar ratio in 1 mM HCl, pH 3, were determined from 350 to 200 nm using a Cary 14 Recording Spectrophotometer.

A. \(3.9 \times 10^{-7} \text{ M} \) Chymotrypsin (0.01 mg/ml) plus \(8.9 \times 10^{-7} \text{ M} \) Heparin (0.01 mg/ml)

B. \(1.2 \times 10^{-6} \text{ M} \) Chymotrypsin (0.033 mg/ml) plus \(3.8 \times 10^{-6} \text{ M} \) Heparin (0.033 mg/ml)

C. \(1.8 \times 10^{-6} \text{ M} \) Chymotrypsin (0.05 mg/ml) plus \(4.1 \times 10^{-6} \text{ M} \) Heparin (0.05 mg/ml)

D. \(2.4 \times 10^{-6} \text{ M} \) Chymotrypsin (0.066 mg/ml) plus \(5.4 \times 10^{-6} \text{ M} \) Heparin (0.066 mg/ml)
Figure 93: Ultraviolet Spectra of Chymotrypsin plus Heparin at Various Levels in 1 mM HCl
LEGEND TO FIGURES 94 - 97:

BEER'S LAW PLOTS OF CHYMOTRYPSIN AND CHYMOTRYPSIN PLUS HEPARIN SPECTRA IN 1 mM HCl at 290, 280, 250 and 220 nm

The ultraviolet spectra of four levels of chymotrypsin and chymotrypsin plus heparin in a 1 to 3 molar ratio in 1 mM HCl, pH 3 were determined from 350 to 200 nm using a Cary 14 Recording Spectrophotometer. The absorbance vs concentration were plotted for selected wavelengths.

- Figure 94: 290 nm
- Figure 95: 280 nm
- Figure 96: 250 nm
- Figure 97: 220 nm

- Chymotrypsin
- Chymotrypsin plus Heparin
Figure 94: Beer's Law Plots of Chymotrypsin and Chymotrypsin plus Heparin in 1 mN HCl at 290 nm
Figure 95: Beer's Law Plots of Chymotrypsin and Chymotrypsin plus Heparin in 1 mM HCl at 280 nm
Figure 96: Beer's Law Plots of Chymotrypsin and Chymotrypsin plus Heparin in 1 m\text{N} HCl at 250 nm
Figure 97: Beer's Law Plots of Chymotrypsin and Chymotrypsin plus Heparin in 1 mM HCl at 220 nm
LEGEND TO FIGURES 98 - 101

PROFLAVIN DIFFERENCE SPECTRA OF HEPARIN PLUS CHYMOTRYPSIN, HEPARIN AND CHYMOTRYPSIN

Proflavin difference spectra of heparin, heparin plus chymotrypsin and chymotrypsin were obtained using 0.01 M Tris buffer 2.8 x 10^{-5} M proflavin, pH 7.6 solutions.

Figure 98: Heparin: 1.0 x 10^{-5} M
Chymotrypsin: 3.9 x 10^{-6} M

Figure 99: Heparin: 1.0 x 10^{-5} M
Chymotrypsin: 7.7 x 10^{-6} M

Figure 100: Heparin: 1.0 x 10^{-5} M
Chymotrypsin: 2.8 x 10^{-5} M

Figure 101: Heparin: 1.0 x 10^{-5} M
Chymotrypsin: 4.0 x 10^{-5} M

1. Heparin
2. Heparin plus chymotrypsin
3. Chymotrypsin
4. Heparin plus chymotrypsin against Chymotrypsin
5. Heparin plus chymotrypsin against Heparin
Figure 98: Proflavin Difference Spectra of Heparin plus Chymotrypsin, Heparin and Chymotrypsin
Heparin: $1.0 \times 10^{-5}$ M  Chymotrypsin $3.9 \times 10^{-6}$ M
Figure 99: Proflavin Difference Spectra of Heparin plus Chymotrypsin, Heparin and Chymotrypsin:
Heparin: $1.0 \times 10^{-5}$ M  Chymotrypsin: $7.7 \times 10^{-6}$ M
Figure 100: Proflavin Difference Spectra of Heparin plus Chymotrypsin, Heparin and Chymotrypsin
Heparin: $1.0 \times 10^{-5}$ M; Chymotrypsin: $2.8 \times 10^{-5}$ M
Figure 101: Proflavin Difference Spectra of Heparin plus Chymotrypsin, Heparin and Chymotrypsin:
Heparin: $1.0 \times 10^{-5}$ M;  Chymotrypsin: $4.0 \times 10^{-5}$ M
LEGEND TO FIGURE 102:

BEER'S LAW PLOT OF CHYMOTRYPSIN AT 470 and 430 nm AND CHYMOTRYPSIN PLUS HEPARIN AT 470 and 450 nm FROM THE PROFLAVIN DIFFERENCE SPECTRA

Proflavin difference spectra of heparin, chymotrypsin plus heparin, chymotrypsin, chymotrypsin plus heparin against heparin, and chymotrypsin plus heparin against chymotrypsin were obtained using 0.01 M Tris buffer, 3.0 x 10^-5 M proflavin solutions containing 3.9 x 10^-6 M to 4.0 x 10^-5 M chymotrypsin and/or 1.0 x 10^-5 M heparin. Absorbance against concentration were drawn for selected wavelengths.

○ Chymotrypsin 470 nm
● Chymotrypsin 430 nm
△ Chymotrypsin plus Heparin 470 nm
▲ Chymotrypsin plus Heparin 450 nm
Figure 102: Beer's Law of Chymotrypsin at 470 and 430 nm and Chymotrypsin plus Heparin at 470 and 450 nm from the Proflavin Difference Spectra
LEGEND TO FIGURE 103:

BEER'S LAW PLOT OF HEPARIN PLUS CHYMOTRYSIN AND HEPARIN PROFLAVIN
DIFFERENCE SPECTRA AT 470, 450 and 260 nm

Proflavin difference spectra of heparin chymotrypsin plus heparin and chymotrypsin were obtained using 0.01 M Tris buffer 3.0 x 10^{-5} M proflavin solutions containing 1.0 x 10^{-5} M chymotrypsin and/or 4.4 x 10^{-6} to 4.3 x 10^{-5} M heparin. Absorbance against concentration were plotted for selected wavelengths of the heparin and heparin plus chymotrypsin spectra.

- □ Chymotrypsin plus heparin ■ Heparin 470 nm
- △ Chymotrypsin plus heparin ▲ Heparin 450 nm
- ○ Chymotrypsin plus heparin ● Heparin 260 nm
Figure 103: Beer's Law Plot of Heparin plus Chymotrypsin and Heparin Proflavin Difference Spectra at 470, 450 and 260 nm
LEGEND TO FIGURES 104 - 107:

ABSORBANCE OF HEPARIN, CHYMOTRYPSIN AND CHYMOTRYPSIN PLUS HEPARIN IN THE PRESENCE OF PROFLAVIN IN RELATION TO TRIS BUFFER CONCENTRATION

The spectra of $1.1 \times 10^{-5}$ M heparin, $4.9 \times 10^{-5}$ M chymotrypsin, chymotrypsin plus heparin, chymotrypsin plus heparin against heparin and chymotrypsin plus heparin against chymotrypsin in $3 \times 10^{-5}$ M proflavin dissolved in 0.1, 0.01 and 0.001 M Tris Buffer, pH 7.6 were scanned in the visible and ultraviolet ranges. The absorbance for heparin, chymotrypsin and chymotrypsin plus heparin at 480, 470 and 450 nm were plotted against Tris buffer concentration and then the heparin absorbance at 450, 480 and 260 were replotted against log of Tris buffer concentration.

Figure 104: 480 nm
Figure 105: 470 nm
Figure 106: 450 nm
  ○ Chymotrypsin
  △ Chymotrypsin plus Heparin
  □ Heparin

Figure 107: Replot of Heparin absorbance vs log of Tris buffer concentration
  ○ 480 nm
  △ 450 nm
  □ 260 nm
Figure 104: Absorbance of Heparin, Chymotrypsin and Chymotrypsin plus Heparin in the Presence of Proflavin in Relation to Tris Buffer Concentration: 480 nm
Figure 105: Absorbance of Heparin, Chymotrypsin and Chymotrypsin plus Heparin in the Presence of Proflavin in Relation to Tris Buffer Concentration: 470 nm
Figure 106: Absorbance of Heparin, Chymotrypsin and Chymotrypsin plus Heparin in the Presence of Proflavin in Relation to Tris Buffer Concentration: 450 nm
Figure 107: Absorbance of Heparin, Chymotrypsin and Chymotrypsin plus Heparin in the Presence of Proflavin in Relation to Tris Buffer Concentration: Replot of Heparin Absorbance vs Log of Tris Buffer Concentration
APPENDIX E

INTERACTION OF MUCOPOLYSACCHARIDES WITH CHYMOTRYPSIN

FOREWORD

The data obtained in this section was obtained by Diane LaPointe who was trained and supervised in experimental techniques by me. The manuscript was written by me.

SYNOPSIS

Chondroitin sulfates A, B and C form complexes with chymotrypsin which enhance the hydrolytic activity of the enzyme towards glutaryl-L-phenylalanine-p-nitroanilide as much as 270%, as in the case of chondroitin sulfate A. Hyaluronic acid, a nonsulfated mucopolysaccharide, at a weight ratio of 6.67:1 to the enzyme, does not influence the rate of cleavage of the substrate by chymotrypsin. Proflavin difference spectra and ultraviolet spectra suggest that a complex is formed between chymotrypsin and hyaluronic acid, as well as chondroitin sulfates A, B and C. The proflavin molecule is not displaced from the active site of the enzyme upon association with the mucopolysaccharides. When the mucopolysaccharide-chymotrypsin complexes are electrophoresed on analytical polyacrylamide disc gels at pH 8.9, the complexes are visualized by toluidine blue (for mucopolysaccharide), coomassie brilliant blue (for protein) and by the zymogram technique (with glutaryl-L-phenylalanine-B-naphthylamide), at a position between that for chymotrypsin alone and that for the mucopolysaccharide alone.
The interaction between heparin and serine proteases such as thrombin (1-3) and plasmin (4) have been studied due to the probable role of these reactions in the blood clotting system. The complex between heparin and trypsin or chymotrypsin has been studied (5,6). These complexes have been probed for their possible use as fibrinolytic agents (7-10) and anti-inflammatory agents (11).

The interaction between proteolytic enzymes and other mucopolysaccharides has not been extensively studied. Chondroitin sulfate has been shown to inhibit pepsin under acidic conditions (12,13). This interaction probably protects the lining of the stomach since chondroitin sulfate A and other sulfated glycoproteins are secreted from the fundus (14). Under certain conditions, however, sulfated mucopolysaccharides have been shown to activate pepsinogen to pepsin (15). This interaction has been implicated in peptic ulcerogenesis (15).

Sulfated mucopolysaccharides have been detected in intestinal mucus (16) and the intestinal mucosa (16,17), being localized in the mucus secreting goblet cells (18) and mast cells (19). The interaction between sulfated mucopolysaccharides and pancreatic chymotrypsin and trypsin in the intestinal tract is probably important physiologically since these proteases bind to the intestinal mucosa (20), inactivate membrane-bound brush border enzymes (21), and pass through the intestinal mucosa into the mesenteric vein in an enzymatically and immunologically active form (22).
Hyaluronic acid (HA), chondroitin sulfate A (CS-A, chondroitin-4-sulfate), chondroitin sulfate B (CS-B, dermatan sulfate), chondroitin sulfate C (CS-C, chondroitin-6-sulfate and heparin represent a series of mucopolysaccharides which vary in the degree of sulfation and the relative concentrations of sugar residues. Hyaluronic acid, CS-A and CS-C contain glucuronic acid as the only uronic acid while CS-B and heparin contain iduronic acid as the major uronic acid and glucuronic acid as the minor uronic acid. HA contains non-sulfated N-acetylglucosamine while CS-A, CS-B and CS-C contain sulfated N-acetyl galactosamine residues. This series of mucopolysaccharides offers an opportunity to try to understand the binding between mucopolysaccharides and proteolytic enzymes.

The interaction between heparin and chymotrypsin has previously been studied (6) using proflavin difference spectroscopy, ultraviolet spectroscopy, electrophoresis and enzymatic studies. By the use of these techniques, a complex between heparin and chymotrypsin was detected which was enzymatically more active than chymotrypsin alone towards the synthetic substrates, glutaryl-L-phenylalanine-β-naphthylamide and glutaryl-L-phenylalanine-p-nitroanilide. Using these same methods, we report here that CS-A, CS-B, CS-C and hyaluronic acid form complexes with chymotrypsin which are similar to that formed between heparin and chymotrypsin. CS-A, CS-B and CS-C, like heparin (6), induce an enhancement of chymotryptic activity. Hyaluronic acid has no effect on the enzymatic activity of chymotrypsin. A preliminary report has appeared elsewhere (23).
MATERIALS AND METHODS

CS-A, CS-B, CS-C and HA from whale cartilage, pig skin, shark cartilage and vitreous humor, respectively, were obtained from Sigma Chemical Corporation, St. Louis, Mo. Electrophoresis grade acrylamide and N, N methylene-bis-acrylamide were purchased from Eastman Kodak, Rochester, N.Y. Proflavin sulfate, glutaryl-L-phenyl-alanine-p-nitroanilide (GPANA), glutaryl-L-phenylalanine-B-naphthylamide (GPNA) were obtained from Schwartz-Mann, Orangeburg, N.Y. Three times crystallized chymotrypsin was purchased from Worthington Biochemical Corporation, Freehold, N.J.

The enzymatic activity of chymotrypsin in the presence of the various mucopolysaccharides was determined using GPANA as the substrate according to the procedure of Erlanger et al (24). This assay was carried out using 0.05 M Tris Buffer, pH 7.6 containing 0.02 M CaCl₂ at 25°.

Ultraviolet spectra of chymotrypsin, the individual mucopolysaccharides, each mucopolysaccharide plus chymotrypsin, as well as, the difference spectra of the various mucopolysaccharides plus chymotrypsin vs the corresponding mucopolysaccharide, chymotrypsin or both, each in a separate cuvette at the appropriate concentrations, were obtained in 1 mM HCl and in 0.010 M Tris Buffer, pH 7.6 using a Beckman Acta IV Spectrophotometer. Proflavin spectra in the visible range were obtained using 3.0 x 10⁻⁵ M proflavin in 0.010 M Tris Buffer, pH 7.6 solutions.

Polyacrylamide disc gel electrophoresis was carried out under basic conditions according to the procedure of Davis (25). Protein
and mucopolysaccharide molecules were detected on the gels using coomassie brilliant blue (26) and toluidine blue (27), respectively. Chymotryptic activity was visualized using a two step procedure. The gels were first incubated at 37° for 2 hours in a solution prepared by dissolving GPNA first in methyl cellosolve, and then diluting the solution with 0.04 M Tris Buffer, pH 7.6. The ratio of GPNA:Tris Buffer:methyl cellosolve was 3 mg: 3 ml:3 ml. In the second step, the hydrolyzed naphthylamine was coupled to fast garnet GBC by placing the gels in 0.14% fast garnet GBC solution (in distilled water) for 1 hour.

RESULTS

Figures 108-110 and 111 show the influence of mucopolysaccharides on the chymotryptic hydrolysis of GPANA. CS-A, CS-B and CS-C act as non-essential activators of chymotrypsin while HA at weight ratios as high as 6.67:1 did not affect enzymatic activity. The CS-A-chymotrypsin complex and the CS-C-chymotrypsin complex act very similarly, kinetically, as expected since these molecules differ only in the position of the sulfate group on the N-acetylgalactosamine residues. The CS-B-chymotrypsin complex kinetically is quite distinct. The order of enhancement of chymotryptic activity is CS-A > CS-B > CS-C > HA.

Kinetic constants can be calculated for the CS-A, CS-B and CS-C plus chymotrypsin complexes by assuming the mucopolysaccharides function kinetically according to the general scheme for non-essential activation given by the equations (26).
LEGEND TO FIGURES 108 - 110:

INFLUENCE OF CHONDROITIN SULFATE A, B, AND C ON CHYMOTRYPIC HYDROLYSIS OF N-GLUTARYL-L-PHENYLALANINE-p-NITROANILIDE (GPANA)

One ml solutions containing chymotrypsin plus chondroitin sulfate A, B or C in 1 mM HCl were incubated at 25°C for 10 minutes. To 0.2 ml of these mixtures, 1.0 ml of 1.0 of 1.0 x 10⁻³ M GPANA in 0.05 M Tris Buffer, pH 7.6 containing 0.02 M CaCl₂ at 25°C was added. After 10 minutes, the reaction was terminated by the addition of 0.2 ml of 30% acetic acid. The amount of p-nitroaniline released was determined spectrophotometrically at 410 nm.

Figure 108:
- 50 µg Chymotrypsin
- 50 µg Chymotrypsin plus 140 µg Chondroitin Sulfate A
- 50 µg Chymotrypsin plus 200 µg Chondroitin Sulfate A

Figure 109:
- 50 µg Chymotrypsin
- 50 µg Chymotrypsin plus 200 µg Chondroitin Sulfate B
- 50 µg Chymotrypsin plus 140 µg Chondroitin Sulfate B

Figure 110:
- 50 µg Chymotrypsin
- 50 µg Chymotrypsin plus 250 µg Chondroitin Sulfate C
- 50 µg Chymotrypsin plus 200 µg Chondroitin Sulfate C
Figure 108: Influence of Chondroitin Sulfate A on Chymotryptic Hydrolysis of N-Glutaryl-L-Phenylalanine-p-Nitroanilide (GPANA)
Figure 109: Influence of Chondroitin Sulfate B on Chymotryptic Hydrolysis of N-Glutaryl-L-Phenylalanine-p-Nitroanilide (GPANA)
Figure 110: Influence of Chondroitin Sulfate C on Chymotryptic Hydrolysis of N-Glutaryl-Phenylalanine-p Nitroanilide (GPANA)
LEGEND TO FIGURE III:

EFFECT OF HYALURONIC ACID ON CHYMOTRYPIC HYDROLYSIS OF N-GLUTARYL-L-PHENYLALANINE-p-NITROANILIDE (GPANA)

Aliquots containing 1000, 700, 500 and 300 μg of hyaluronic acid in 1 mM HCl were incubated for 10 minutes at 25°C with 700 μg of chymotrypsin in 1 mM HCl. To 0.2 ml of these mixtures, 1.0 ml of 1.0 x 10^{-3} M GPANA in 0.05 M Tris Buffer, pH 7.6, containing 0.02 M CaCl₂ at 25°C were added. After 10 minutes the reaction was terminated by the addition of 0.2 ml of 30% acetic acid. The amount of p-nitroaniline released was determined spectrophotometrically at 410 nm.
Figure 111: Effect of Hyaluronic Acid on Chymotryptic Hydrolysis of N-Glutaryl-L-Phenylalanine-p-Nitroanilide (GPANA)
Values for $\alpha$, $\beta$, and $K_A$ are given in Table 19. The calculated values for $K_A$ indicate that chymotrypsin has a greater affinity for the mucopolysaccharides than for the substrate GPANA. The enhancement of activity is mainly due to an increase in the rate of reaction of the ESA complex over the ES complex which shifts the equilibrium towards the ESA complex. CS-A and CS-C shift the equilibrium mainly through the ES + $A^*\rightleftharpoons$ ESA pathway while CS-B shifts nearly equal amounts of the enzyme through the EA + S $\rightleftharpoons$ ESA and the ES + $A^*\rightleftharpoons$ ESA pathways.

Ultraviolet spectra of the mucopolysaccharides plus chymotrypsin in 1 mM HCl and 0.010 M Tris Buffer and given in Figures 112-115 and 116-119 respectively. In the presence of 1 mM HCl, all mucopolysaccharides, with the exception of CS-C, altered the absorption of the aromatic residues of chymotrypsin in the 270-290 nm region. However, no change in the 270-290 nm region was observed with 0.01 M Tris Buffer, pH 7.6. There was a greater effect on the spectra in the 220 nm region at the lower pH due to the complex between the mucopolysaccharides and chymotrypsin. These spectral changes, seen as a result of complex formation,
Values for $K_A$, $\alpha$, and $\beta$ were determined from Lineweaver-Burk plots of data gathered using the GPANA assay for chymotryptic activity of Erlanger et al. (24). The assays were carried out at 25°C in 0.05 M Tris-chloride buffer, pH 7.6, containing 0.02 M CaCl$_2$. The $K_S$ calculated for chymotrypsin was $4.0 \times 10^{-4}$ M.
Table 19

Kinetic Constants for the Hydrolysis of Glutaryl-L-Phenylalanine-p-Nitroanilide by Chondroitin Sulfate A, B and C and Heparin plus Chymotrypsin Complexes

<table>
<thead>
<tr>
<th>Mucopolyssacharide</th>
<th>$k_A \times 10^6 \text{ M}^{-1} \text{s}^{-1}$</th>
<th>$\alpha$</th>
<th>$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-A</td>
<td>1.06</td>
<td>0.318</td>
<td>1.98</td>
</tr>
<tr>
<td>CS-B</td>
<td>0.99</td>
<td>0.992</td>
<td>1.64</td>
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<tr>
<td>CS-C</td>
<td>1.18</td>
<td>0.475</td>
<td>1.21</td>
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<tr>
<td>Heparin (6)</td>
<td>4.79</td>
<td>0.141</td>
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</table>
LEGEND TO FIGURES 112 - 115:

ULTRAVIOLET SPECTRA OF CHONDROITIN SULFATE A, B, C, AND HYALURONIC ACID PLUS CHYMOTRYPSIN IN 1 mM HCl

Ultraviolet spectra of the mucopolysaccharides, the mucopolysaccharides plus chymotrypsin and chymotrypsin were determined in 1 mM HCl using a Beckman Acta M IV Spectrophotometer.

Figure 112:  
A. Chymotrypsin (100 µg/ml)  
B. Chondroitin Sulfate A (300 µg/ml)  
C. Chymotrypsin (100 µg/ml) plus Chondroitin Sulfate A (300 µg/ml)  
D. Chymotrypsin (1000 µg/ml) plus Chondroitin Sulfate A (300 µg/ml) vs Chymotrypsin (100 µg/ml)  
E. Chymotrypsin (100 µg/ml) plus Chondroitin Sulfate A (300 µg/ml) vs Chondroitin Sulfate A (300 µg/ml)  
F. Chymotrypsin (100 µg/ml) plus Chondroitin Sulfate A (300 µg/ml) vs Chymotrypsin (100 µg/ml) and Chondroitin Sulfate A (300 µg/ml)

Figure 113:  
A. Chymotrypsin (100 µg/ml)  
B. Chondroitin Sulfate B (300 µg/ml)  
C. Chymotrypsin (100 µg/ml) plus Chondroitin Sulfate B (300 µg/ml)  
D. Chymotrypsin (100 µg/ml) plus Chondroitin Sulfate B (300 µg/ml) vs Chymotrypsin (100 µg/ml)  
E. Chymotrypsin (100 µg/ml) plus Chondroitin Sulfate B (300 µg/ml) vs Chondroitin Sulfate B (300 µg/ml)  
F. Chymotrypsin (100 µg/ml) plus Chondroitin Sulfate B (300 µg/ml) vs Chymotrypsin (100 µg/ml) and Chondroitin Sulfate B (300 µg/ml)

Figure 114:  
A. Chymotrypsin (50 µg/ml)  
B. Chondroitin Sulfate C (300 µg/ml)  
C. Chymotrypsin (50 µg/ml) plus Chondroitin Sulfate C (300 µg/ml)  
D. Chymotrypsin (50 µg/ml) plus Chondroitin Sulfate C (300 µg/ml) vs Chymotrypsin (50 µg/ml)  
E. Chymotrypsin (50 µg/ml) plus Chondroitin Sulfate C (300 µg/ml) vs Chondroitin Sulfate C (300 µg/ml)  
F. Chymotrypsin (50 µg/ml) plus Chondroitin Sulfate C (300 µg/ml) vs Chymotrypsin (50 µg/ml) and Chondroitin Sulfate C (300 µg/ml)
Figure 115:  
A. Chymotrypsin (50 µg/ml)  
B. Hyaluronic Acid (200 µg/ml)  
C. Chymotrypsin (50 µg/ml) plus Hyaluronic Acid (200 µg/ml)  
D. Chymotrypsin (50 µg/ml) plus Hyaluronic Acid (200 µg/ml) vs Chymotrypsin (50 µg/ml)  
E. Chymotrypsin (50 µg/ml) plus Hyaluronic Acid (200 µg/ml) vs Hyaluronic Acid (200 µg/ml)  
F. Chymotrypsin (50 µg/ml) plus Hyaluronic Acid (200 µg/ml) vs Chymotrypsin (50 µg/ml) and Hyaluronic Acid (200 µg/ml)
Figure 112: Ultraviolet Spectra of Chondroitin Sulfate A plus Chymotrypsin in 1 mM HCl
Figure 113: Ultraviolet Spectra of Chondroitin Sulfate B plus Chymotrypsin in 1 mM HCl
Figure 114: Ultraviolet Spectra of Chondroitin Sulfate C plus Chymotrypsin in 1 mM HCl.
Figure 115: Ultraviolet Spectra of Hyaluronic Acid plus Chymotrypsin in 1 mM HCl
Legend to Figures 116-120

ULTRAVIOLET SPECTRA OF CHONDROITIN SULFATE A, B, C AND HYALURONIC ACID PLUS CHYMOTRYPsin IN 0.010 M TRIS BUFFER, pH 7.6.

Ultraviolet spectra of the mucopolysaccharides, the mucopolysaccharides plus chymotrypsin and chymotrypsin were determined in 0.01 M Tris Buffer, pH 7.6. The mucopolysaccharides and chymotrypsin were dissolved in 0.1 mM HCl and then diluted to 3 ml with 0.01 M Tris Buffer pH 7.6.

Figure 116: A. Chymotrypsin (100 µg/ml).
B. Chondroitin Sulfate A (300 µg/ml).
C. Chondroitin Sulfate A (300 µg/ml) plus chymotrypsin (100 µg/ml).
D. Chondroitin Sulfate A (300 µg/ml) plus Chymotrypsin (100 µg/ml) vs Chymotrypsin (100 µg/ml).
E. Chondroitin Sulfate A (300 µg/ml) plus Chymotrypsin (100 µg/ml) vs Chondroitin Sulfate A (300 µg/ml).
F. Chondroitin Sulfate A (300 µg/ml) plus Chymotrypsin (100 µg/ml) vs Chondroitin Sulfate A (300 µg/ml) and Chymotrypsin (100 µg/ml).

Figure 117: A. Chymotrypsin (100 µg/ml).
B. Chondroitin Sulfate B (300 µg/ml).
C. Chondroitin Sulfate B (300 µg/ml) plus Chymotrypsin (100 µg/ml).
D. Chondroitin Sulfate B (300 µg/ml) plus Chymotrypsin (100 µg/ml) vs Chymotrypsin (100 µg/ml).
E. Chondroitin Sulfate B (300 µg/ml) plus Chymotrypsin (100 µg/ml) vs Chondroitin Sulfate B (300 µg/ml).
F. Chondroitin Sulfate B (300 µg/ml) plus Chymotrypsin (100 µg/ml) vs Chondroitin Sulfate B (300 µg/ml) and Chymotrypsin (100 µg/ml).

Figure 118: A. Chymotrypsin (100 µg/ml).
B. Chondroitin Sulfate C (300 µg/ml).
C. Chondroitin Sulfate C (300 µg/ml) plus Chymotrypsin (100 µg/ml).
D. Chondroitin Sulfate C (300 µg/ml) plus Chymotrypsin (100 µg/ml) vs Chymotrypsin (100 µg/ml)
E. Chondroitin Sulfate C (300 µg/ml) plus Chymotrypsin (100 µg/ml) vs Chondroitin Sulfate C (300 µg/ml).
F. Chondroitin Sulfate C (300 µg/ml) plus Chymotrypsin (100 µg/ml) vs Chondroitin Sulfate C (300 µg/ml) and Chymotrypsin (100 µg/ml).
Figure 119:  
A. Chymotrypsin (50 µg/ml).
B. Hyaluronic Acid (200 µg/ml).
C. Hyaluronic Acid (200 µg/ml) plus Chymotrypsin (50 µg/ml).
D. Hyaluronic Acid (200 µg/ml) plus Chymotrypsin (50 µg/ml) vs Chymotrypsin (50 µg/ml).
E. Hyaluronic Acid (200 µg/ml) plus Chymotrypsin (50 µg/ml) vs Hyaluronic Acid (200 µg/ml).
F. Hyaluronic Acid (200 µg/ml) plus Chymotrypsin (50 µg/ml) vs Hyaluronic Acid (200 µg/ml) and Chymotrypsin (50 µg/ml).
Figure 116: Ultraviolet Spectra of Chondroitin Sulfate A in 0.01 M Tris Buffer, pH 7.6
Figure 117: Ultraviolet Spectra of Chondroitin Sulfate B in 0.010 M Tris Buffer, pH 7.6
Figure 118: Ultraviolet Spectra of Chondroitin Sulfate C in 0.01 M Tris Buffer, pH 7.6
Figure 119: Ultraviolet Spectra of Hyaluronic Acid in 0.010 M Tris Buffer, pH 7.6.
were not due mainly to sulfate interactions with the positively charged amino acid residues of chymotrypsin since the spectra of hyaluronic acid, which is not sulfated, plus chymotrypsin was nearly the same as the spectra for the sulfated mucopolysaccharide-chymotrypsin complexes.

Figures 120-123 shows the proflavin difference spectra of chymotrypsin plus the various mucopolysaccharides. The proflavin molecule was not removed from the active site of the chymotrypsin molecule upon complex formation with the chondroitin sulfate molecules or the hyaluronic acid molecule, since the absorbance at 465 nm, a wavelength which is characteristic of the proflavin chymotrypsin bond (29), was not diminished. It was enhanced. This indicated that the mucopolysaccharides were bound to the chymotrypsin molecule outside the active site.

Similar electrophoretic pattern: were seen when HA, CS-A, CS-B, and CS-C were electrophoresed with chymotrypsin. Figure 124 shows the electrophoretic pattern obtained for CS-A plus chymotrypsin. Complexes were formed between these sulfated mucopolysaccharides and chymotrypsin which migrated to positions intermediate to that of chymotrypsin and the individual mucopolysaccharides. The hydrolysis of GPANA by the complex was visually greater than that by chymotrypsin alone, thereby qualitatively confirming the stimulation of chymotryptic activity observed with GPANA.

DISCUSSION

CS-A, CS-B, CS-C and HA form complexes with chymotrypsin at pH 7.6 and 3.0. Ultraviolet and proflavin spectral studies and
Legend to Figures 120 - 123:

Flavin difference spectra of chondroitin sulfate A, B, C and hyaluronic acid plus chymotrypsin

Flavin difference spectra of the individual mucopolysaccharides, the individual mucopolysaccharides plus chymotrypsin and chymotrypsin were determined in 0.01 M Tris buffer, pH 7.6 containing 3.0 x 10^{-5} M proflavin.

Figure 120:
A. Chymotrypsin (133 µg/ml)
B. Chondroitin Sulfate A (400 µg/ml)
C. Chondroitin Sulfate A (400 µg/ml) plus Chymotrypsin (133 µg/ml)
D. Chondroitin Sulfate A (400 µg/ml) plus Chymotrypsin (133 µg/ml) vs Chymotrypsin (133 µg/ml)
E. Chondroitin Sulfate A (400 µg/ml) plus Chymotrypsin (133 µg/ml) vs Chondroitin Sulfate A (400 µg/ml)
F. Chondroitin Sulfate A (400 µg/ml) plus Chymotrypsin (133 µg/ml) vs Chondroitin Sulfate A (400 µg/ml) vs Chondroitin Sulfate A (400 µg/ml)

Figure 121:
A. Chymotrypsin (133 µg/ml)
B. Chondroitin Sulfate B (400 µg/ml)
C. Chondroitin Sulfate B (400 µg/ml) plus Chymotrypsin (133 µg/ml)
D. Chondroitin Sulfate B (400 µg/ml) plus Chymotrypsin (133 µg/ml) vs Chymotrypsin (133 µg/ml)
E. Chondroitin Sulfate B (400 µg/ml) plus Chymotrypsin (133 µg/ml) vs Chondroitin Sulfate B (400 µg/ml)
F. Chondroitin Sulfate B (400 µg/ml) plus Chymotrypsin (133 µg/ml) vs Chondroitin Sulfate B (400 µg/ml) vs Chondroitin Sulfate B (400 µg/ml)

Figure 122:
A. Chymotrypsin (133 µg/ml)
B. Chondroitin Sulfate C (400 µg/ml)
C. Chondroitin Sulfate C (400 µg/ml) plus Chymotrypsin (133 µg/ml)
D. Chondroitin Sulfate C (400 µg/ml) plus Chymotrypsin (133 µg/ml) vs Chymotrypsin (133 µg/ml)
E. Chondroitin Sulfate C (400 µg/ml) plus Chymotrypsin (133 µg/ml) vs Chondroitin Sulfate C (400 µg/ml)
F. Chondroitin Sulfate C (400 µg/ml) plus Chymotrypsin (133 µg/ml) vs Chondroitin Sulfate C (400 µg/ml) vs Chondroitin Sulfate C (400 µg/ml)
Figure 123:  
A. Chymotrypsin (133 µg/ml)  
B. Hyaluronic Acid (267 µg/ml)  
C. Hyaluronic Acid (267 µg/ml) plus Chymotrypsin (133 µg/ml)  
D. Hyaluronic Acid (267 µg/ml) plus Chymotrypsin (133 µg/ml) vs Chymotrypsin (267 µg/ml)  
E. Hyaluronic Acid (267 µg/ml) plus Chymotrypsin (133 µg/ml) vs Hyaluronic Acid (267 µg/ml)  
F. Hyaluronic Acid (267 µg/ml) plus Chymotrypsin (133 µg/ml) vs Hyaluronic Acid (267 µg/ml) and Chymotrypsin (133 µg/ml)
Figure 120: Proflavin Difference Spectra of Chondroitin Sulfate A plus Chymotrypsin
Figure 121: Proflavin Difference Spectra of Chondroitin Sulfate B plus Chymotrypsin
Figure 122: Proflavin Difference Spectra of Chondroitin Sulfate C plus Chymotrypsin
Figure 123: Proflavin Difference Spectra of Hyaluronic Acid plus Chymotrypsin
LEGEND TO FIGURE 124:

POLYACRYLAMIDE DISC GEL ELECTROPHORESIS OF CHONDROITIN SULFATE A, CHONDROITIN SULFATE A PLUS CHYMOTRYPSIN, AND CHYMOTRYPSIN ON 7% GELS

Polyacrylamide disc gel electrophoresis at pH 8.9 on 7% gels was carried out according to the procedure of Davis (25). Chondroitin Sulfate A, Chondroitin Sulfate A plus chymotrypsin, and chymotrypsin were placed on the gels, and the gels were electrophoresed at 2 mA per gel for 30 min., followed by 5 mA per gel for approximately 45 min. at 0-5°C. One set of the three gels was immediately placed in 12.5% TCA for one hr and then in coomassie brilliant blue stain for protein for 30 min. These gels were destained overnight in coomassie brilliant blue destaining solution. Another set of gels was examined for enzymic activity by placing it in glutaryl-L-phenylalanine-β-naphthylamide for 2 hours followed by Fast Garnet GBC for one hour, and destaining in 7% acetic acid overnight.

Left to Right:

Zymogram: 3 mg Chondroitin Sulfate A plus 1 mg Chymotrypsin
1 mg Chymotrypsin

Coomassie Brilliant Blue: 120 µg Chondroitin Sulfate A
120 µg Chondroitin Sulfate A plus 40 µg Chymotrypsin
40 µg Chymotrypsin
Figure 124: Polyacrylamide Disc Gel Electrophoresis of Chondroitin Sulfate A, Chondroitin Sulfate A plus Chymotrypsin, and Chymotrypsin on 7% Gels
enzymatic activity determinations using GPANA as a substrate, indicate that the conformation of the chymotrypsin molecule is altered progressively as the carboxyl to sulfate ratio on the mucopolysaccharide changes. The position of the sulfate group on the mucopolysaccharide is important because CS-A and CS-C, in which the sulfate groups are found at positions 4 and 6, respectively of the N-acetylgalactosamine residues, induce differing degrees of conformational change on the chymotrypsin molecule as observed spectrophotometrically. The sulfate group at position 4 is less available for binding as shown in binding studies using CS-A and CS-C with poly-L-lysine (30).

The presence of sulfate ions is not a major requirement for the formation of a complex between chymotrypsin and mucopolysaccharides since a complex is formed between chymotrypsin and HA which is spectrophotometrically very similar to the complexes between chymotrypsin and the sulfated mucopolysaccharides. The extent of interaction between chymotrypsin and the mucopolysaccharides does not increase with the number of sulfates nor the availability of the sulfate group. Conversely, it decreases.

In complex formation with poly-L-ornithine, poly-L-lysine and poly-L-arginine, Gelman and Blackwell (31), in contrast, found that the number of sulfate groups per disaccharide residue on mubopolysaccharides was of major importance. The strength of interaction of these polypeptides at pH 7.0 with the mucopolysaccharides increased according to the order HA < CS-A < heparin sulfate < CS-C < keratan sulfate < CS-B < heparin. These mucopolysaccharides were also found to induce different degrees of conformational change on the chymotrypsin molecule as observed spectrophotometrically.
polysaccharides contain approximately 0, 1.0, 1.0, 1.0, 1.0, 1.3, 2.3 sulfates per disaccharide, respectively (32). The extent of interaction between collagen and mucopolysaccharides at pH 3.9-4.4, however, is not simply a function of sulfate ion concentration (33). The order of increasing interaction between collagen and mucopolysaccharides, using circular dispersion techniques, was determined to be the following: CS-A < keratan sulfate < HA < CS-B < CS-C. The distribution of the charged residues on the collagen and the chymotrypsin molecules in relation to the positions of the sulfate and carboxyl ions and the conformation of the mucopolysaccharides likely play a greater role in determining the extent of interaction than the number of sulfate ions present.

The presence of sulfate groups appear, however, to be important in determining whether the enzymic activity of chymotrypsin is affected by the mucopolysaccharides. The sulfated mucopolysaccharides alter the rate of hydrolysis of GPANA by chymotrypsin, while hyaluronic acid does not alter the hydrolytic activity of chymotrypsin. CS-A, whose sulfate group is least accessible for interaction of the mucopolysaccharides studied, increased the enzymatic activity of chymotrypsin as much as 270% while heparin, which has an average of 2.3 sulfate groups per disaccharide, enhances the activity of chymotrypsin only 70%, at best (6).

The proflavin spectral studies indicate that the mucopolysaccharides bind at a site removed from the active site of chymotrypsin. The mucopolysaccharides might increase the enzyme activity of chymotrypsin in several ways:
a. By altering the equilibrium between active and inactive molecules of chymotrypsin. At pH 7.0, 85% of the chymotrypsin molecules are in the active conformation and 15% are in the inactive conformation (34). If the sulfated mucopolysaccharides were simply altering this equilibrium between active and inactive conformations, the maximum increase in activity would be 18% which is considerably below the 270% increase in activity noted for CS-A.

b. By altering the microenvironment around the chymotrypsin molecule making the active site more attractive to the substrate. The mucopolysaccharides may block some sites at which GPANA would interact with on the outside of the enzyme, leaving only the active site for the substrate molecule to bind. At pH 7.6, GPANA would be negatively charged, such that it would not interact with the mucopolysaccharide.

c. By altering the conformation of the active site of chymotrypsin. Both the proflavin and the ultraviolet spectra indicate that the mucopolysaccharides alter the conformation of the chymotrypsin. They probably change the conformation of the active site thereby facilitating increased interaction between the substrate and chymotrypsin.

The non-essential activation of chymotrypsin observed upon complex formation with the sulfated mucopolysaccharides most likely is due to a combination of these three factors, with c, being the major contributing factor.

Chymotrypsin probably interacts with the small intestine epithelial cells as a mucopolysaccharide-chymotrypsin complex.
This interaction may be important in control of the production of epithelial cells, since these cells have a high turnover rate (35) and proteolytic enzymes have been shown to be effective mitogens of normal cells in culture (36,37).
REFERENCES


APPENDIX F

METHODOLOGY EXPERIMENTATION

A number of experiments were performed to establish the appropriate methodology for obtaining information regarding enzyme and inhibitor characteristics. The results of these experiments are included in this section.

MATERIALS AND METHODS

Sepharose 4B, heparin and Diazo Blue B (O-dianisidine) were purchased from Sigma Chemical, St. Louis, Missouri. Proflavin and Hammerstein casein were obtained from Schwartz/Mann Orangeburg, New York. Acrylamide and N,N' methylene-Bis-acrylamide were purchased from Eastman Organic, Rochester, New York. Bovine salivary mucin, twice crystallized chymotrypsin were obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Five times crystallized α-chymotrypsin was a gift of Worthington Biochemical Corporation. α,-Trypsin Inhibitor Quantitative Kit was purchased from Miles Laboratories, Kankakee, Illinois. PM 30, XM 50 and UM 2 ultrafiltration membranes were obtained from Amicon Corporation, Lexington, Massachusetts.

Inhibitory activity towards chymotrypsin and trypsin was determined according to the procedure of Kunitz (1) with the modifications of Wasilauskas and Brecher (2). Ultrafiltration of test solutions was carried out using a milipore ultrafiltration apparatus or a similar one built at Bowling Green State University by Robert Frisch, 40 psi nitrogen and either XM 50, PM 30, or UM 2 Amicon membrane filters with molecular weight cut off points of
50,000, 30,000 and 1,000 respectively. The protein concentration of various solutions was determined using the method of Lowry et al. (3). Sepharose-chymotrypsin was prepared according to the procedure of Porath et al. (4) using a 5 M phosphate buffer solution during the cyanogen bromide activation step.

Disc gel electrophoresis on standard basic polyacrylamide disc gels was carried out by the procedure of Davis (5) while microgels were prepared by the method of Burr et al. (6). The modifications of this method are given in Figure 125. Coomassie brilliant blue (7) and 1-anilino-8-naphthalene sulfonate (ANS) (8) were used to detect protein on the gels. Glycoproteins were visualized by the periodic acid Schiff's reagent (PAS) using the method of Matthieu and Quarles (9) or Kapitany and Zebrowski (10) or by the periodic acid Formazan reaction (11). Toluidine blue (1% aqueous solution) was used to determine the position of the mucopolysaccharide components.

Ultraviolet and visible spectra were determined using a Beckman Acta IV spectrophotometer.

RESULTS AND DISCUSSION

Table 20 shows that the inhibitors present in the fractions from the Sepharose-chymotrypsin column separation of malignant breast tissue (Chapters 3 and 4) are more stable when stored frozen than kept at 4°C. Hence, all solutions were stored frozen until used.

It was determined by the casein assay, pH 7.6 that 800 μg of active chymotrypsin were bound per gram of Sepharose. 3.2 mg of
LEGEND TO FIGURE 125:

MICRO DISC GEL ELECTROPHORESIS APPARATUS

The basic apparatus design for micro disc gel electrophoresis of dilute protein solutions was reported by Burr (6). Pasteur pipets were adapted to take the place of Eppendorf pipet heads and #00 one hole rubber stoppers were used to adapt the modified pipets to the Isco Model 1270 Electrophoresis Apparatus. Polyethylene tubing was used in the place of silicone tubing to hold together the two parts of the apparatus.

1. Sample in 40% Sucrose or G-200 Sephadex
2. Stacking Gel
3. Separation Gel
4. Upper Buffer Chamber
5. Lower Buffer Chamber
6. Anode
7. Cathode
8. Modified Pasteur Pipet
9. Polyethylene Tubing
10. 1.5 mm id Capillary Tubing
11. Glass Plug
Figure 125: Microdisc Gel Electrophoresis Apparatus
LEGEND TO TABLE 20:
THE EFFECT OF STORAGE CONDITIONS ON THE CHYMOTRYPTIC INHIBITORY ACTIVITY OF POOLED FRACTIONS FROM THE SEPHAROSE-CHYMOTRYPSIN COLUMN SEPARATION OF MALIGNANT BREAST TISSUE EXTRACTS

The pooled fractions of inhibitory activity from the Sepharose-chymotrypsin column separation of the 90,000 x g supernatant fraction of malignant breast tissue from patient #73-1335 were divided into two portions. One portion was stored frozen and the second portion was stored at 4°C. Before storage and after two months the inhibitory activity towards chymotrypsin was determined using a casein assay (1, 2). At the time of storage a ratio of 0.3 ml of the pooled solutions to 0.5 µg chymotrypsin was used. After two months a ratio of 0.25 ml to 0.1 µg chymotrypsin was used in the casein assay.
Table 20

The Effect of Storage Conditions on the Chymotryptic Inhibitory Activity of Pooled Fractions from the Sepharose-Chymotrypsin Column Separation of Malignant Breast Tissue Extracts

| Pooled Fraction Number | % Inhibition at Time of Storage | % Inhibition After Two Months
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>4°C Frozen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.3</td>
<td>61.7 38.2</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>18.8 51.5</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>26.6 80.0</td>
</tr>
<tr>
<td>4</td>
<td>14.7</td>
<td>24.3 61.3</td>
</tr>
<tr>
<td>5</td>
<td>7.1</td>
<td>6.7 38.0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0 14.7</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>3.2 61.8</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>15.1 38.0</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>40.8 61.8</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>18.0 63.2</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>31.4 96.9</td>
</tr>
</tbody>
</table>
protein were detected bound per gram of Sepharose as measured by the Lowry assay (3). The activity of the immobilized enzyme over the pH range of 7.6-10.0 is given in Figure 126 showing maximum activity at pH 9.5.

The efficiency of the PM 50 ultrafiltration membranes towards hemoglobin (m.w. 64,500) was, on the average 80% (Table 21) which is in the same range as given in Table 4. One problem with ultrafiltration is the trapping of proteins by the membrane (Tables 14-16). In Table 22, the greater efficiency of 2% NaCO3 over 0.1 M Tris Buffer, pH 7.6, for releasing trapped protein is shown. The properties of 0.1 M Tris Buffer, pH 7.6 passed through UM 2 ultrafiltration membranes are given in Table 23. This experiment indicates there is some material released by the Tris Buffer solutions which reacts with the Lowry Folin phenol reagents even after soaking the membranes in distilled water. Substances such as hexose-amines, sucrose, most monosaccharides and high concentrations of Tris Buffer, interfere with the Lowry assay (12-15). There is essentially no inhibitory activity, however, released towards chymotrypsin or trypsin.

The separation of serum proteins using the basic polyacrylamide disc gel technique of Davis (5) is given in Figure 127. Coomassie brilliant blue is more efficient than the fluorescent stain, ANS. The advantage of the ANS stain over the coomassie brilliant blue stain is the ability to stain the gels first with periodic acid Schiff's reagent for glycoprotein and then stain with ANS for protein retaining the PAS color. The electrophoresis
LEGEND TO FIGURE 126:

pH DEPENDENCE OF THE HYDROLYTIC ACTIVITY OF SEPHAROSE-CHYMOTRYPSIN

The enzymatic activity of Sepharose-chymotrypsin, prepared by the procedure of Porath et al. (4), at various pH values from pH 7.6 to 10.0 in 0.1 M Tris buffer and 0.1 M Borate buffers using the modified caseinolytic assay of Kunitz (1, 2). The mass of Sepharose-chymotrypsin was determined by taking samples of Sepharose-chymotrypsin at each pH value to dryness and then weighing the samples.
Figure 126: pH Dependence of the Hydrolytic Activity of Sepharose-Chymotrypsin
LEGEND TO TABLE 21:

EFFICIENCY OF DIAFLO XM 50 ULTRAFILTRATION MEMBRANES USING HEMOGLOBIN SOLUTIONS

A solution of hemoglobin was prepared at a concentration of 4 mg/ml in distilled water. This solution was ultrafiltered using a Diaflo XM 50 ultrafiltration membrane at 40 psi nitrogen pressure. The concentration of hemoglobin in the original sample, concentrate and filtrate was determined spectrophotometrically at 500 nm.
<table>
<thead>
<tr>
<th>Filter</th>
<th>Initial Vol.</th>
<th>Final Vol.</th>
<th>Volume Concentration Factor</th>
<th>Hemoglobin Concentration mg/ml</th>
<th>Hemoglobin Concentration Factor</th>
<th>% Retention of Hemoglobin above the Filter</th>
<th>% Protein in Filtrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.3</td>
<td>1.7</td>
<td>6.6</td>
<td>4</td>
<td>22</td>
<td>5.5</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td>10.0</td>
<td>3.5</td>
<td>2.9</td>
<td>4</td>
<td>11</td>
<td>.10</td>
<td>85</td>
</tr>
</tbody>
</table>
LEGEND TO TABLE 22:
PROTEIN TRAPPED IN PM 30 ULTRATION MEMBRANES

Following ultrafiltration of four pooled fractions using Amicon Diaflo PM 30 ultrafiltration membranes, the membranes were placed in 3 ml of 0.1 M Tris buffer, pH 7.6 overnight. The membranes were then placed in 10 ml of a 2% Na₂CO₃ in 0.10 N NaOH solution. The protein concentration of each solution which the membranes were soaked in was determined using the Lowry method (3).
Table 22
Protein Trapped in PM 30 Ultrafiltration Membranes

<table>
<thead>
<tr>
<th>Solution Ultrafiltered</th>
<th>μg Protein on Filter Dissolved in 0.1 M Tris Buffer, pH 7.6</th>
<th>μg Protein on Filter Dissolved in 2% Na₂CO₃ in 0.1 N NaOH⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>
LEGEND TO TABLE 23:

PROPERTIES OF 0.1 M TRIS BUFFER, pH 7.6 PASSED THROUGH DIAFLO UM 2 ULTRAFILTER

Three UM 2 Diaflo ultrafiltration membranes were allowed to soak in distilled water for approximately 3 hours using 6 changes of water. The water was kept in motion using a magnetic stirring bar. A 10 ml aliquot of 0.1 M Tris buffer, pH 7.6 was forced through each filter in the ultrafiltration apparatus at 40 psi nitrogen pressure. The equivalent protein content was determined using the Lowry method (3) and the inhibitory activity towards tryptic and chymotryptic hydrolysis of casein (1, 2) was determined. A ratio of 0.1 ml 0.1 M Tris filtrate to 0.25 µg enzyme was used for the inhibition assay.
Table 23
Properties of 0.1 M Tris Buffer, pH 7.6 Passed Through Diaflo UM 2 Ultrafiltration Membranes

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Equivalent Protein Concentration μg/ml</th>
<th>INHIBITORY ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tyrosine % Liberated Inhibition towards</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chymotrypsin meq x 10⁴ /0.3 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trypsin % Liberated Inhibition towards</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>Tyrosine meq x 10⁴ /0.3 ml</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>3.33</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>4.07</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>3.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.23</td>
</tr>
</tbody>
</table>


LEGEND TO FIGURE 127:

SEPARATION OF SERUM PROTEINS BY DISC GEL ELECTROPHORESIS

Approximately 200 μg of serum proteins were separated by the analytical disc gel electrophoresis method of Davis (5). The gels were stained with either Coomassie Brilliant Blue stain (7) or with 1-Anilino-8-Naphthalene Sulfonate stain (8) for protein and by the periodic acid-Schiff's reagent (9) for glycoprotein. The separation of serum proteins by Davis (3) and the band assignments of Felgenhauer (15) are given for reference.

Gel 1 - Coomassie Brilliant Blue Stain for Protein
Gel 2 - 1-Anilino-8-Naphthalene Sulfonate Stain for Protein
Gel 3 - Periodic Acid-Schiff's Stain for Glycoprotein
Gel 4 - Davis Reference Gel
Gel 5 - Felgenhauer Band Assignments
Figure 127: Separation of Serum Proteins by Disc Gel Electrophoresis
technique of Davis (5) and the PAS reagent are applicable to the glycoprotein bovine salivary mucin as given in Figure 128. Approximately 10 μg of the glycoprotein are needed to visualize the glycoprotein using the PAS reagent.

The unique system of Burr (6) for the microgels allows the application of dilute solutions due to the use of the large concentration gel. Separation of the proteins in serum, however, is not as good (Figure 129). These gels are quite sensitive to the solvent in which the protein is dissolved in and the substance used to increase the density of the solution. Sucrose proved to be better than Sephadex for this purpose. These gels were very sensitive to ohmic heating due to the small diameter of the gels. The 15% polyacrylamide gels are much easier to remove from the capillary tubes than the 7% gels. These 15% gels do separate low molecular weight proteins quite well if the proteins are allowed to migrate until the tracking dye is very close to the bottom of the gel.

The proteins, in the standard α1-antitrypsin supplied with the Miles immunodiffusion kits for α1-antitrypsin were separated by standard disc gel electrophoresis as given in Figure 130. The standards contain numerous proteins and glycoproteins with the distinct absence of albumin.

In Figure 131 the polydisperse nature of heparin is evident due to the presence of mucopolysaccharide staining components over a wide range of Rf values with the major amount of the heparin molecules migrating near the bromophenol blue tracking dye. A
LEGEND TO FIGURE 128:

DISC GEL ELECTROPHORESIS OF BOVINE SALIVARY MUCIN

The proteins found in bovine salivary mucin obtained from Worthington Biochemicals, Freehold, New Jersey were separated using the alternate procedure of Davis (5) for disc gel electrophoresis. Samples containing 50 µg, 20 µg and 10 µg of bovine salivary mucin plus 40% sucrose were applied to the gels. One half of each gel was stained for protein using Coomassie Brilliant Blue stain (?) and the other half for glycoprotein using Periodic Acid-Schiff's stain (9).

Gel 1  50 µg Bovine Salivary Mucin
   a- Stained with Coomassie Brilliant Blue
   b- Stained with Periodic Acid Schiff's

Gel 2  20 µg Bovine Salivary Mucin
   a- Stained with Coomassie Brilliant Blue
   b- Stained with Periodic Acid Schiff's

Gel 3  10 µg Bovine Salivary Mucin
   a- Stained with Coomassie Brilliant Blue
   b- Stained with Periodic Acid Schiff's
Figure 128: Disc Gel Electrophoresis of Bovine Salivary Mucin
LEGEND TO FIGURE 129:

SEPARATION OF SERUM PROTEINS BY MICRO DISC GEL ELECTROPHORESIS

Serum proteins were separated using the micro disc gel electrophoresis technique of Burr (6). Both 7% and 15% acrylamide separation gels were used. Approximately 20 µg of serum proteins were applied to the polyacrylamide gels in 0.5 ml of a 0.1 M Tris buffer, pH 7.6, 40% sucrose solution, 0.5 ml of a 0.1 M Tris buffer G-200 Sephadex solution of 5 µl of 40% sucrose. The gels were stained first for glycoprotein using the Periodic Acid-Schiff's stain (9) and then for protein using Coomassie Brilliant Blue Stain (7).

Gel 1 20 µg Serum Proteins in 0.5 ml of 0.1 M Tris buffer pH 7.6, 40% Sucrose Solution

a- 7% Acrylamide Gel

1- Coomassie Brilliant Blue Stain for protein
2- Periodic Acid-Schiff's stain for glycoprotein

b- 15% Acrylamide Gel

1- Coomassie Brilliant Blue stain for protein
2- Periodic Acid-Schiff's stain for glycoprotein

Gel 2 20 µg Serum proteins in 0.5 ml of 0.1 M Tris buffer, pH 7.6, G-200 Sephadex Solution

a- 7% Acrylamide Gel

1- Coomassie Brilliant Blue Stain for Protein
2- Periodic Acid-Schiff's Stain for glycoprotein

b- 15% Acrylamide Gel

1- Coomassie Brilliant Blue stain for protein
2- Periodic Acid-Schiff's Stain for glycoprotein
Gel 3 20 μg Serum Proteins in 5 μl of 40% Sucrose

a- 7% Acrylamide Gel

1- Coomassie Brilliant Blue stain for protein
2- Periodic Acid-Schiff's stain for glycoprotein

b- 15% Acrylamide Gel

1- Coomassie Brilliant Blue Stain for protein
2- Periodic Acid-Schiff's stain for glycoprotein

BPB - Bromophenol Blue Tracking Dye Band Position
Figure 129: Separation of Serum Proteins by Micro Disc Gel Electrophoresis
LEGEND TO FIGURE 130:

DISC GEL ELECTROPHORESIS OF $\alpha_1$-ANTITRYPSIN STANDARD AND SERUM REFERENCE

Polyacrylamide disc gel electrophoresis was performed using the method of Davis (15) on 7% acrylamide gels. The gels were stained for glycoprotein using the periodic acid Schiff's method of Kapitany and Zebrowski (10) and then stained later for protein using coomassie brilliant blue.

A. $\alpha_1$-Antitrypsin Standard from Miles Laboratories
   $\alpha_1$-Trypsin Inhibitor Quantitative Kit

B. Serum standard
Figure 130: Disc Gel Electrophoresis of $\alpha$-Antitrypsin
Standard and Serum Reference
LEGEND TO FIGURE 131:

DISC GEL ELECTROPHORESIS OF HEPARIN UNDER VARIOUS CONDITIONS

Electrophoresis of samples containing 25 μg to 1 mg heparin was carried out in basic polyacrylamide disc gels using the procedure of Davis (5). Heparin was dissolved either in 40% sucrose or in 0.1 M Tris buffer, pH 7.6. The gels were stained with toluidine blue by one of three methods, periodic acid Schiff's (PAS) reagent or the periodic acid Formazan (PAF) reaction. The gels which were stained with toluidine blue were either (1) placed directly in an aqueous solution of toluidine blue, (2) placed in a 12% TCA solution for one hour before being placed in this solution or (3) were placed directly into an acetic acid solution containing toluidine blue.

1. 25 μg Heparin - 40% Sucrose-Toluidine Blue Method 1
2. 50 μg Heparin - 40% Sucrose-Toluidine Blue Method 1
3. 100 μg Heparin - 40% Sucrose-Toluidine Blue Method 1
4. 200 μg Heparin - 40% Sucrose-Toluidine Blue Method 1
5. 500 μg Heparin - 40% Sucrose-Toluidine Blue Method 1
6. 1 mg Heparin - 40% Sucrose-Toluidine Blue Method 1
7. 200 μg Heparin - 0.1 M Tris buffer-Toluidine Blue Method 1
8. 200 μg Heparin - 40% Sucrose-Toluidine Blue Method 2
9. 200 μg Heparin - 40% Sucrose-Toluidine Blue Method 3
10. 200 μg Heparin - 40% Sucrose-PAS
11. 200 μg Heparin - 0.1 M Tris buffer-PAS
12. 200 μg Heparin - 40% Sucrose-PAF
13. 1 mg Heparin - 40% Sucrose-PAF
Figure 131: Disc Gel Electrophoresis of Heparin Under Various Conditions
40% sucrose solution proved to be the best solvent for heparin. Essentially the same results were obtained when gels containing heparin were placed directly in the toluidine blue solution or were first placed in 12% TCA or a 7% acetic acid solution for an hour before staining with toluidine blue. The periodic acid Formazan method for glycoprotein staining is not as sensitive as the periodic acid Schiff's method for heparin. The method of Kapitany and Zebrowski (10) for the PAS glycoprotein stain is superior to the method of Matthieu and Quarles (9) because of the long oxidation, washing and staining periods in the later method permit heparin to diffuse form the gels. Polymerization of chymotrypsin in a sample gel at basic pH allowed autolysis to occur producing low molecular weight products as given in Figure 132. Polymerization of this enzyme in acid gels did not lead to autolysis or the loss of enzyme activity. All lots of the three times crystallized chymotrypsin contained toluidine blue staining components except for CDI-8LK. The five times crystallized enzyme did not have a toluidine blue staining component.

The silica cells used in the Beckman Acta IV spectrophotometer absorb light in the 240 to 190 nm region as shown in Figure 133. Of the four cells in this region 2 and 4 (Figure 134) are the best matched and were used in all subsequent experiments in the ultraviolet region. (The problems of the low wavelength ultraviolet region were discussed in the historical section). In the visible region all four cells were well matched as given in Figure 135. The proflavin spectra in Figure 136 agrees well with the published data (16,17). The two spectra in 0.1 M and 0.01 M Tris Buffer
LEGEND TO FIGURE 132:

ELECTROPHORETIC SEPARATION OF THE PROTEINS OF VARIOUS LOTS OF CHYMOTRYPSIN POLYMERIZED IN A SAMPLE GEL

One mg quantities of various lots of chymotrypsin were photopolymerized into sample gels. Electrophoresis was then carried out using the procedure of Davis (5). The gels were stained with toluidine blue for mucopolysaccharide, periodic acid Schiff's reagent for glycoprotein and coomassie brilliant blue for protein.

A. Worthington - Lot CDI-6164
B. Worthington - Lot CDI-81K
C. Worthington - Lot CDI-3AB
D. Worthington - Lot CDI-81K
E. Worthington - Lot CDI-640404
   1. Toluidine Blue
   2. Periodic Acid Schiff's
   3. Coomassie Brilliant Blue
Figure 132: Electrophoretic Separation of the Proteins of Various Lots of Chymotrypsin Polymerized in a Sample Gel
LEGEND TO FIGURE 133:

ABSORPTION OF ULTRAVIOLET LIGHT BY SILICA CELLS CONTAINING 1 mM HCl

Four matched 1 cm silica cells purchased from Fisher Scientific Company. 1 mM HCl was placed in each cell and the ultraviolet range from 350 to 180 nm was scanned at 1 nm/sec using a Beckman Acta IV spectrophotometer.

A. Cell 1
B. Cell 2
C. Cell 3
D. Cell 4
Figure 133: Absorption of Ultraviolet Light by Silica Cells Containing 1 mN HCl
LEGEND TO FIGURE 134:

DIFFERENCE SPECTRA OF SILICA CELLS CONTAINING 1 mM HCl

The difference spectra of three matched 1 cm silica cells containing 1 mM HCl were run to determine how well the cells matched. A Beckman Acta IV spectrophotometer was used.

1. Cell 2 vs Cell 4
2. Cell 2 vs Cell 1
3. Cell 1 vs Cell 2
Figure 134: Difference Spectra of Silica Cells Containing 1 mM HCl
LEGEND TO FIGURE 135:

0.1 M TRIS BUFFER vs 0.1 M TRIS BUFFER CHECK OF THE MATCH OF SILICA CELLS IN THE VISIBLE REGION

Three ml samples of 0.1 M Tris Buffer, pH 7.6 were added to the four 1 cm matched silica cells. Sequentially the spectra from 600 nm to 400 nm of the cells was scanned against one cell. The recorder was offset 0.1 absorbance unit for each scan.

1. Cell A vs Cell B
2. Cell A vs Cell C
3. Cell A vs Cell D
Figure 135: 0.1 M Tris Buffer vs 0.1 M Tris Buffer Check of the Match of Silica Cells in the Visible Region
LEGEND TO FIGURE 136:

SPECTRA OF PROFLAVIN IN 0.1 M, 0.01 M and 0.001 M TRIS BUFFER pH 7.6

The spectra of $3.0 \times 10^{-5}$ M proflavin in 0.1, 0.01 and 0.001 M Tris Buffer, pH 7.6 was scanned in the visible and ultraviolet ranges using a Beckman Acta IV Spectrophotometer.

A. 0.1 M Tris Buffer
B. 0.01 M Tris Buffer
C. 0.001 M Tris Buffer
Figure 136: Spectra of Proflavin in 0.1 M, 0.01 M and 0.001 M Tris Buffer pH 7.6
at pH 7.6 are very similar while the absorbance of proflavin increases in 0.001 M Tris Buffer.
REFERENCES

Isolation and Identification of α1-Antitrypsin as a Component of Normal and Malignant Human Breast and Other Tissues

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Proteases and inhibitors of proteolytic activity are believed to be important in the metabolism of malignant cells (1-12). Various reports cite elevated levels of chymotrypsin, trypsin, and cathepsin-like enzymes in transformed cells (1-6). The proteolytic activity is believed to be responsible for several properties of transformed cells, including uncontrolled proliferation and increased migration (3-5). Hydrolases also are believed to play a role in metastases by decreasing the cohesion/betwene cells in the primary tumor (9) and by breaking down the intracellular matrix which holds the cells together at the sites of metastases (10). Naturally occurring inhibitors of proteolytic activity are likewise observed in higher amounts in neoplastic tissues (13, 14) and in other conditions (13, 15-17). Naturally occurring and synthetic protease inhibitors have been reported to arrest cell growth of tumor cells and transformed cells (7, 8, 11, 12).

Wasilauskas and Brecher reported the presence of antiproteolytic activity in the 90,000 g supernatant fraction of glioma, ovarian carcinoma, and normal and malignant breast and colon tissues (18). These fractions contained nondialysable, heat labile inhibitors of trypsin and chymotryptic activity. This communication extends earlier findings (18) and reports the presence of α1-antitrypsin in the 90,000 g supernatant fractions of malignant and normal human breast tissue of nonlactating women as well as other tissues by radial immunodiffusion. This inhibitor and several other inhibitors of chymotrypsin and trypsin activity have been extensively purified from breast tissue extracts by affinity chromatography on Sepharose-chymotrypsin or Affi-Gel 10-chymotrypsin, representing a new and efficient means of isolating the α1-antitrypsin. A preliminary report has appeared elsewhere (19).

Materials and methods. Twice crystallized trypsin and three times crystallized α-chymotrypsin were obtained from Worthington Biochemical Corporation, Freehold, NJ. Hammersten casein, benzoyl-L-tyrosine ethyl ester (BTEE) and hemoglobin were purchased from Schwarz, Mann Research Laboratories, Orangeburg, NY. Bovine serum albumin, Cohn Fraction V and heparin containing 158 USP J-A units/mg were obtained from Sigma Chemical Corporation, St. Louis, MO. Affi-Gel 10 was purchased from BioRad Laboratories, Richmond, CA. Sepharose-chymotrypsin was obtained as a gift from Owens-Illinois, Toledo, OH. N, N', N''-Tetramethylethylenediamine, acrylamide and N,N'-methylenebis-acrylamide were obtained from Eastman Kodak, Rochester, NY. Basic fuchsin and Coomassie Brilliant Blue R-250 were purchased from Fisher Scientific Company, Fairlawn, NJ and Colab Laboratories, Glenwood, IL, respectively. An α1-Trypsin Inhibitor Quantitative Kit was purchased from Miles Laboratories, Kankakee, IL.

Samples of normal and or malignant human breast, colon, ileum, anal, lung, and stomach were obtained after surgery and stored frozen until further use. The tissues were thawed and homogenized 1:9 in either 0.32 M sucrose solution or 0.1 M Tris buffer, pH 7.6 for 2 min using a Virtis homogenizer. The mixture was centrifuged at 20,000g for 30 min using a RC-2 Sorvall centrifuge. The 20,000g supernatant fraction was then
centrifuged for 75 min at 90,000g using an L2-65B Beckman ultracentrifuge. The 90,000-
g supernatant fraction was stored frozen in
aliquots.
Levels of antitryptic and antichymotryptic
activity were determined using the modifica­
tions of Wasilauskas and Brecher (18) of the
caseinolytic assay of Kunitz (20). The pro­
tein content was determined by applying the
procedure of Lowry et al. (21), utilizing
bovine serum albumin, Cohn Fraction V as
the protein standard.
Sephrose-chymotrypsin was prepared by
the procedure of Porath et al. (22). The
activity of the active insolubilized chymo­
trypsin was determined to be 30 μg/ml at
pH 7.6 by the casein assay for chymotryptic
activity.
Chymotrypsin was coupled to the Affi-Gel
10 by shaking a mixture of 80 ml of 20 mg/ml
chymotrypsin in 0.1 M phosphate buffer,
pH 7.0 with 4 g of Affi-Gel 10 at 4° for 3.5 hr.
The mixture was transferred to a 1.5 x 30
cm column and washed sequentially with 3
liters of 0.1 M phosphate buffer, pH 7.0,
and 500 ml of 0.1 M Tris buffer, pH 7.6.
The final activity of the column was deter­
mained to be 40 μg/ml at pH 7.6 using the
casein assay for chymotryptic activity.
Charges of 90,000g supernatant fraction of
normal and malignant breast extracts were
passed through either the Sephafose-chymo­
trypsin column or the Affi-Gel 10-chymo-
trypsin column. The columns were eluted
sequentially using 0.1 M Tris buffer, pH
7.6; a 0.1 M to 0.3 M NaCl gradient in 0.1 M
Tris buffer, pH 7.6; 0.1 M Tris buffer, pH
7.6; 1 mM HCl, and 0.1 M Tris buffer, pH
7.6.
Peaks of antitryptic and antichymotryptic
activity were pooled and concentrated by
ultrafiltration using Amicon Diaflo PM
ultrafiltration membranes at 40 psi nitrogen
pressure. The 90,000g supernatant fractions
and the pooled concentrated column frac­
tions were separated by using the alternate
disc gel electrophoresis method of Davis
(23) or the micro disc gel electrophoresis
 technique of Burr et al. (24). In both systems
the glycine-Tris buffer, pH 8.3 system of
Davis was used in an ISCO Model 1270
electrophoresis apparatus at 4°. The gels
were stained for glycoprotein using the
periodic acid Schiff's method of either
Matthieu and Quarles (25) or Kapitany and
Zebrowski (26). Protein was detected with
Coomassie Brilliant Blue according to the
procedure of Weber et al. (27).
Aliquots of 1–10 μl of the pooled inhibi­
tory peaks from the Affi-Gel 10-chymotryp­
sin column were deposited on cellulose
acetate strips and tested for glycoprotein
content by application of the periodic acid
Schiff's method (28).
Five μl samples of the pooled concentrated
peaks from the Affi-Gel 10 column and the
90,000g supernatant fraction of normal and/
or malignant breast, column, anal, ileum,
stomach and lung tissue were placed on
radial immunodiffusion plates containing
human plasma α1-antitrypsin content by application of the periodic acid
Schiff's method (28).
Results. Figure 1 represents a typical
separation of the components found in
malignant breast upon affinity chromato­
graphy of 300 mg of protein from a 90,000g
extract on Affi-Gel 10-chymotrypsin. Eleven
peaks of antichymotryptic and antitryptic
activity were noted. Seven peaks of anti-
chymotryptic activity were found in the
NaCl gradient eluate, one peak of anti-
chymotryptic and one peak of antitryptic
activity were observed in the subsequent
Tris buffer eluate. Two peaks of both anti-
tryptic and antichymotryptic activity were
seen in the Tris buffer eluate following the
drop in pH. Consistently negligible amounts
of protein were detected in the peaks after
the initial breakthrough peak, suggesting a
very high degree of purification of inhibitors.
Very low levels of endogenous activity were
seen in these eluates. Peaks 1–8 contained a
glycoprotein component as detected with the
periodic acid Schiff's method on cellulose
acetate. Similar patterns of activity were ob-
Fig. 1. Separation of chymotrypsin and trypsin inhibitors in the 90,000g supernatant fraction of malignant human breast tissue from patient no. 4150 on Affi-Gel 10-chymotrypsin. Affi-Gel 10 was reacted with chymotrypsin to form Affi-Gel 10-chymotrypsin. An aliquot containing 300 mg protein of the 90,000g supernatant of malignant breast tissue from patient no. 4150 was applied to the Affi-Gel 10-chymotrypsin column. The column was sequentially eluted with 200 ml of 0.1 M Tris buffer, pH 7.6; 1160 ml of a 0.1 M-0.3 M NaCl gradient in 0.1 M Tris buffer, pH 7.6; 220 ml of 0.1 M Tris buffer, pH 7.6; 220 ml of 1 mM HCl; and 160 ml of 0.1 M Tris Buffer, pH 7.6. Antitryptic and antichymotryptic activity were determined by the casein assay (1) using 0.2 ml of the column eluate and 0.5 µg enzyme. O, Antichymotryptic Activity. •, Antitryptic activity. A, Protein concentration by method of Lowry et al. (21). A, Protein concentration at 280 nm. ▲, Endogenous caseinolytic Activity. ↓, 90,000g supernatant fraction added.

served with Sepharose-chymotrypsin columns. Electrophoresis of the pooled fractions on 15% polyacrylamide gel generally showed only one or two protein bands. Similar experiments on pooled peaks (peak 1) with 7% acrylamide gels of Davis (23) exhibited only two detectable protein bands both of which contained glycoproteins (Fig. 2). The major band migrated to the same extent as α₁-antitrypsin of serum. Peak 1 contained antitryptic and antichymotryptic activity. The major component of Peak 1 was determined to be immunologically equivalent to plasma α₁-antitrypsin by immunodiffusion. The 90,000g supernatant fraction also showed a band of comparable migration to that of α₁-acid glycoprotein of serum.

Table I relates the levels of α₁-antitrypsin found in the 90,000g supernatant fractions of normal and/or malignant human tissues. The concentration of α₁-antitrypsin in the tissue extracts from blood (Column 3) was calculated from the hemoglobin content using 0.15 gm/ml and 4.4 mg/ml as the concentration of hemoglobin (31) and α₁-antitrypsin per ml of serum. The value for α₁-antitrypsin was estimated by taking the mean value of α₁-antitrypsin in normal humans (29, 30), which is also the standard value for Miles immunodiffusion plates used in this study, and doubling the value to account for the acute phase increase (13, 33) in α₁-antitrypsin under neoplastic conditions. The α₁-antitrypsin content in the tissue (column 4) was determined by subtracting the α₁-antitrypsin due to blood from the total amount of α₁-antitrypsin in the tissue extracts.

Discussion. The isolation of chymotrypsin and trypsin inhibitors by affinity chromatography of the 90,000g extracts of human breast tissue on Sepharose-chymotrypsin or Affi-Gel 10-chymotrypsin represents a new, simple means of purifying and isolating α₁-antitrypsin almost to electrophoretic homogeneity, in contrast to the multistep procedures (34-36). Ten additional peaks of antiproteolytic activity were obtained subsequent to the elution of the α₁-antitrypsin.
Fig. 2. Polyacrylamide disc gel electrophoresis of the 90,000g supernatant fraction of malignant human breast tissue, purified protease inhibitor from Peak 1, serum. Polyacrylamide disc gel electrophoresis was performed by the method of Davis (23). The gels were stained with periodic acid Schiff's reagent for glycoprotein [1] and with Coomassie Brilliant Blue for protein [2]. A. Serum, B. 90,000g supernatant fraction from patient no. 4150, C. Peak 1 concentrated eluate from the Affi-Gel 10-chymotrypsin column.

Peak in the procedure reported herein. The use of the NaCl salt gradient and the lowering of the pH to three essentially removed all bound inhibitors as the columns were successfully used repeatedly in contrast with Sepharose-chymotrypsin which more tightly binds α1-antitrypsin (37).

The difference in binding properties of α1-antitrypsin towards Sepharose-chymotrypsin and Sepharose-trypsin is probably due to the fact that chymotrypsin and trypsin bind to two different sites on the α1-antitrypsin molecule (38). The inhibitors are highly purified as evidenced by the fact that negligible amounts of protein are associated with the inhibitory peaks, and by the preliminary disc gel electrophoresis experiments on the 15% acrylamide disc gels and the 7% Davis gel (23) for peak No. 1.

α1-Antitrypsin has been reported as a component of mast cells (39) and macrophages (40) which are contained in connective tissue (41). It would therefore be anticipated that α1-antitrypsin would be widely distributed, as indeed this report suggests and indeed confirms (39, 40, 42).

Protease inhibitors may serve a further role, in addition to the inhibition of normal and neoplastic cell proliferation (4, 7, 8, 11). As result of observations of children after surgery, Lennert et al. (43) have suggested that α1-antitrypsin and α₂-macroglobulin serve a role as wound healers. The increase in α1-antitrypsin in serum is probably due to an attempt by the body to control proteolytic activity. It is one of the acute phase reactants produced by the liver in response to trauma such as inflammation, injury, surgery, infection, neoplastic growth, pregnancy or administration of hormones (33). It has also been proposed that protease inhibitors in the plant kingdom promote wound healing (44, 45).

Summary. α1-Antitrypsin has been de-
TABLE I. \( \alpha_1 \)-Antitrypsin in Human Tissue.*

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Tissue</th>
<th>( \text{mg} \ \alpha_1 \text{-AT/mg protein} \times 10^2 )</th>
<th>( \text{mg} \ \text{Hb/mg protein} \times 10^1 )</th>
<th>( \text{mg} \ \alpha_1 \text{-AT/mg protein (estimated)} \times 10^3 )</th>
<th>( \text{mg} \ \alpha_1 \text{-AT/mg protein (corrected)} \times 10^3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>8217</td>
<td>Malignant breast</td>
<td>3.0</td>
<td>4.7</td>
<td>1.3</td>
<td>1.8</td>
</tr>
<tr>
<td>5286</td>
<td>Malignant breast</td>
<td>3.1</td>
<td>1.7</td>
<td>0.5</td>
<td>2.6</td>
</tr>
<tr>
<td>8994</td>
<td>Malignant breast</td>
<td>3.2</td>
<td>3.5</td>
<td>1.1</td>
<td>2.1</td>
</tr>
<tr>
<td>S73-1335</td>
<td>Malignant breast</td>
<td>2.6</td>
<td>1.2</td>
<td>0.4</td>
<td>2.2</td>
</tr>
<tr>
<td>7150</td>
<td>Malignant breast</td>
<td>1.7</td>
<td>3.1</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>S73-2543</td>
<td>Normal breast</td>
<td>1.6</td>
<td>3.9</td>
<td>1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>3120</td>
<td>Normal colon</td>
<td>2.5</td>
<td>2.9</td>
<td>0.9</td>
<td>1.6</td>
</tr>
<tr>
<td>3120</td>
<td>Malignant colon</td>
<td>1.8</td>
<td>2.0</td>
<td>0.6</td>
<td>1.2</td>
</tr>
<tr>
<td>M73-416</td>
<td>Normal colon</td>
<td>3.3</td>
<td>2.1</td>
<td>0.6</td>
<td>2.7</td>
</tr>
<tr>
<td>M73-416</td>
<td>Malignant colon</td>
<td>3.6</td>
<td>1.7</td>
<td>0.5</td>
<td>3.1</td>
</tr>
<tr>
<td>6161</td>
<td>Normal colon</td>
<td>4.2</td>
<td>2.8</td>
<td>0.8</td>
<td>2.6</td>
</tr>
<tr>
<td>6161</td>
<td>Malignant colon</td>
<td>3.1</td>
<td>0.9</td>
<td>0.3</td>
<td>2.8</td>
</tr>
<tr>
<td>S73-06098</td>
<td>Normal anal</td>
<td>2.2</td>
<td>2.0</td>
<td>0.6</td>
<td>1.6</td>
</tr>
<tr>
<td>S73-06098</td>
<td>Malignant anal</td>
<td>1.5</td>
<td>2.2</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>M7342</td>
<td>Malignant ileum</td>
<td>1.8</td>
<td>2.8</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>113097</td>
<td>Malignant stomach</td>
<td>3.0</td>
<td>0.6</td>
<td>0.2</td>
<td>2.8</td>
</tr>
<tr>
<td>73-6430</td>
<td>Malignant lung</td>
<td>4.4</td>
<td>4.6</td>
<td>1.4</td>
<td>3.0</td>
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</tbody>
</table>

The \( \alpha_1 \)-antitrypsin concentration in the 90,000 g supernatant fractions of normal and/or malignant human breast, colon, anal, ileum, lung and stomach was determined using the quantitative radial immunodiffusion technique (29). The hemoglobin content of the tissue extracts was determined by a colormetric hemoglobin determination method (30). The amount of blood in the extracts was calculated using a value of 0.15 g hemoglobin/ml serum (31). From this value the amount of \( \alpha_1 \)-antitrypsin present due to blood was calculated using 4.4 mg \( \alpha_1 \)-antitrypsin per ml of serum (29, 32, 33) which is double the mean value for normal serum in order to account for the increased levels in patients with neoplastic diseases.

The \( \alpha_1 \)-antitrypsin is bound to Sepharose or Affi-Gel 10, has been utilized to separate 11 peaks of antiproteolytic activity by affinity chromatography of normal and malignant human breast tissue extracts. Glycoproteins are associated with eight of the peaks. Peak 1 contains predominantly \( \alpha_1 \)-antitrypsin in addition to a minor component. The purification of the inhibitors, as judged by disc gel electrophoresis, is extensive. In some peaks, only one or two protein bands are observed, suggesting that affinity chromatography on Sepharose- or Affi-Gel 10-chymotrypsin might be used for the isolation of \( \alpha_1 \)-antitrypsin and other inhibitors in preparative amounts.

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