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Analysis of serum protease inhibitor activity by its application to the study of RBC membrane multi-proteins complex

Master Thesis

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Abstract

Protease inhibitors are enzymes that prevent the breakdown of important cellular proteins by proteases. Protease inhibitors can be both small molecules such as EDTA which is a metalloprotease inhibitor and large molecules such as alpha-1 antitrypsin. Most of the cellular protease inhibitors are proteins. Protease inhibitors are commonly used in proteomic analysis to preserve proteins from endogenous and exogenous proteolytic cleavage. A multitude of protease inhibitors are now commercially available that inhibit various protease classes including serine and cysteine proteases, amino peptidases, acid proteases and metalloproteases.

Most of these protease inhibitors are suitable for wide range of cellular protein analysis. Serum or plasma is known to contain a number of naturally occurring protease inhibitors including alpha -1 proteinase inhibitor (alpha-1 antitrypsin), antithrombin III, alpha -2 antiplasmin, alpha-1 antichymotrypsin, c1 inhibitor, alpha-2 macroglobulin, inter-alpha trypsin inhibitor, beta -1 anticollagenase and alpha-cysteine protease inhibitor. The effectiveness of these inhibitors for protecting plasma proteins from exogenous proteolytic digestion have shown that plasma protease inhibitors effectively inhibit trypsin (a serine protease), papain (a cysteine protease) , collagenase (a metalloprotease) and pepsin (an aspartate protease).

Inhibitory proportions of serum protease inhibitors have not found application in the cellular protein analysis. Cellular proteins do not carry out their functions alone; instead, they often act by participating in macromolecular multi-protein complexes which play essential roles in propagation and integration of cellular signals. Most of these multi-protein complexes have molecular weights above 400KD, while most of the serum protease inhibitors are of low molecular weight ranging from 10-200 KD.

In this project, we have compared serum protease inhibitory effect on exogenous commercial protease (i.e., trypsin) with endogenous protease by protection exerted on multi-protein complexes isolated from RBC membrane.

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Chapter 1
Introduction

1.1 Proteases and Protease Inhibitors

1.1.1 Protease enzymes

Proteases are hydrolases, which are the third class in enzyme classification [1]. They catalyze the cleavage of peptide bonds in peptides, polypeptides, and proteins through a hydrolytic mechanism. Many proteases catalyze the same general chemical reaction, similar to that shown in Figure1.1 [2]. Peptide bond hydrolysis is an addition-elimination reaction, with the protease acting as a nucleophile [3].

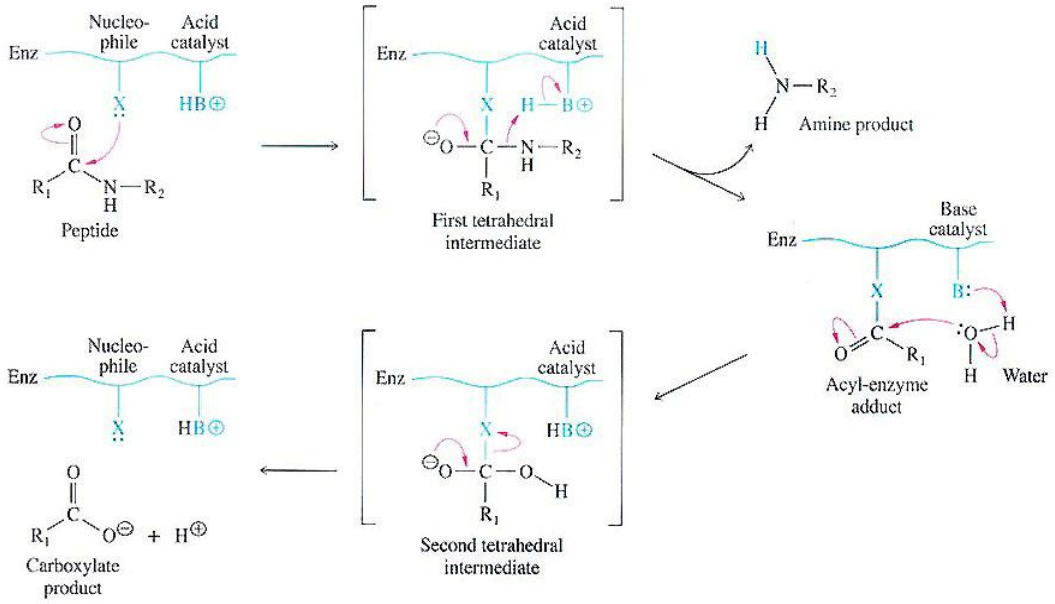

Figure 1.1: General mechanism for the enzymatic hydrolysis of a peptide substrate [4].

These enzymes are critical for cellular survival, by removing denatured proteins during stress conditions or proteins that have completed their functions. Various

proteases play distinct roles in the degradation of proteins, including proteinases that break down proteins and peptidases. Proteases are more stable in harsh conditions such as high heat [4]. Proteases are found in all living organisms and are vital in processes such as cellular growth and differentiation, cell death, etc. [5]. Proteases could be either intra- or extracellular [6]. The most prevalent intracellular proteases are aspartic (rennin), cysteine (cathepsin), and threonine (proteasome) proteases. Extracellular proteases are primarily serine proteases (elastases and blood proteases) and metalloproteases (matrix metalloproteases or MMP's), together with collagenases and gelatinases [5].
Proteases show specificity toward one or more peptide bonds, which is dependent on the neighboring amino acid residues [1, 7]. Proteases are also divided to two subclasses: exopeptidases and endopeptidase. Exopeptidases cleave at the carboxyl- or amino-terminus of a peptide chain and depending on the position of cleavage site can be divided into several subclasses. There are 6 different subclasses that cut carboxyl- or amino- terminus and, those that can cleave one, two, or three residues from target terminus [1, 7]. Table1.1 lists the different groups of exopeptidases [8]:

Table 1.1: Classification of exopeptidases [4]

Protease
Exoproteases
Aminopeptidases
Dipeptidyl peptidase
Tripeptidyl peptidase
Carboxypeptidase
Serine type protease
Metalloprotease
Cysteine type protease
Peptidyl dipeptidase
Omega peptidases

Proteases that can cleave through the interior of a polypeptide chain are termed endopeptidases. Endopeptidases generally cut the specific peptide substrates subsequent to interaction with the amino acid residues immediately adjacent to the cleavage site, and also with neighboring residues. Figure 2.1 shows a schematic representation of an endopeptidase active site bound to a polypeptide substrate. Cleavage of the substrate occurs at the bond between the two residues labeled P1 and P1'. The amino acid residues in all of the binding positions (P3-P3') affect on the rate of hydrolysis through two mechanisms: 1) steric hindrances and 2) the strength and stability of the binding between the enzyme and substrate [9].

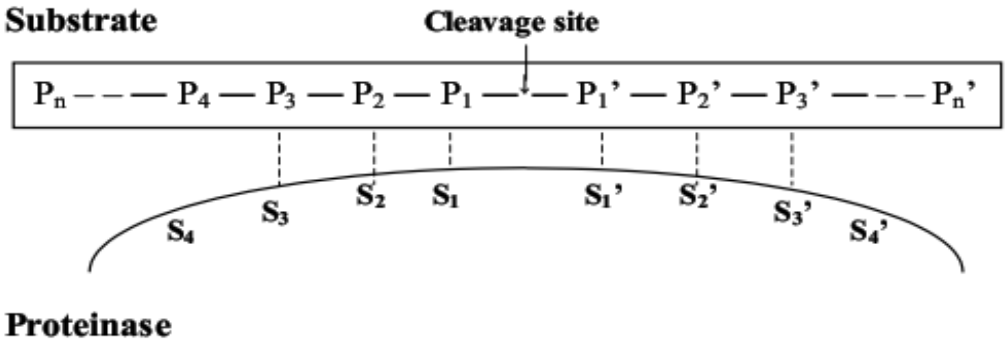

Figure 1.2: Schematic representation of the proteinase-substrate complex with six binding sites. Cleavage occurs between amino acid residues P1 and P1' [4].

These proteases are divided to several kinds based on the amino acid sequences of the active sites and the tertiary structures of the proteinases such as serine, cysteine, aspartic, metallo-, and unknown proteinases. Table 2.1 lists the distinguishing characteristics of these proteinases and provides examples of the most commonly known proteinases within each group. The serine proteinases are divided into 6 different clans. The cysteine proteinases and aspartic proteinases are divided into 20 and 3 families, respectively [10]. The metalloproteinases are divided into at least five distinct clans based on the metal binding motif present at the active site [11]. Serine proteases have serine and histidine, cysteine proteases have cysteine (thiol), metalloproteases have metal ions (e.g. $Zn2^{+}$, $Ca2^{+}$, $Mn2^{+}$) and aspartate proteases have an aspartic acid moiety in their active centers [12].

Table 1.2: Definitions of the protease groups [4]

Proteases	Definition
Serine proteinases	Ser and His residues in their active centers; mechanism of action involves formation of a covalent acyl-enzyme intermediate [8].
Cysteine (thiol) proteinases	Cys and His residues in the active site; mechanism of action involves formation of a covalent acyl-enzyme intermediate.
Aspartic proteinases	Use two acidic residues in the catalytic process; mechanism of action involves direct hydrolysis by water.
Metallo-proteinases	Use a metal ion and glutamic acid residue in the catalytic process; mechanism of action involves direct hydrolysis by water [13].

1.2.1 Protease inhibitors

Protease inhibitors are molecules that bind proteases and decrease their activity. Many drug molecules are protease inhibitors. Some of protease inhibitors are involved in the regulation of metabolism [14]. A very important set of inhibitors includes those natural and synthetic inhibitors that regulate the activity of thrombin. Thrombin, a serine protease in the chymotrypsin family, is a key participant in the cascade of events that occurs during coagulation through conversion of soluble fibrinogen to insoluble fibrin [15].

1.2.1.1 Reversible or Irreversible Inhibitors

Inhibitor binding is either reversible or irreversible. Irreversible inhibitors usually react with the protease and change it chemically. These inhibitors modify key amino acid residues needed for protease activity. In contrast, reversible inhibitors bind non-covalently and different types of inhibitions could be occurred depending on whether they bind the protease, the protease-substrate complex, or both [14].

1.2 Why is required to inhibit protease?

In biology and biochemistry, protease inhibitors are molecules that inhibit the function of proteases. Protease inhibitors are commonly used in proteomic analysis to preserve the protein population from endogenous and exogenous proteolytic cleavage. There are a multitude of protease inhibitors, which act upon the various protease classes, including serine proteases, cysteine proteases , aminopeptidases, acid proteases, and elastases[16]. Many naturally occurring protease inhibitors are proteins. Approximately 500-600 proteases have been known to exist in human and mouse genome [17]. Protease inhibitors classified by the type of protease that they inhibit and according to catalytic mechanism and active-site structure [4].

- Cysteine protease inhibitors (inhibit protease enzyme such as calpain, papain, cathepsin B, and cathepsin L)
- Serine protease inhibitors (inhibit protease enzyme such as trypsin, chymotrypsin, plasmin, urokinase, and kallikrein)
- Threonine protease inhibitors
- Aspartic protease inhibitors (inhibit protease enzyme such as pepsin, rennin, cathepsin D, chymosin, and protease B)
- Metalloprotease inhibitors

1.2.1 Broad spectrum protease inhibitor

For isolation and purification of proteins and enzymes of cell culture and tissue used of multitude of protease inhibitor combination is highly necessary to protect protein from various types of cellular proteases [12]. Most of the active biological protease inhibitors are protein of higher molecular weight, the combination of such protease inhibitor for the cellular protease inhibition is often not good for subsequence proteomic analysis. Therefore suitable protease inhibitor cocktails are very active for research. Now protease inhibitor cocktails are commercially available. In such cocktail, the inhibiters are genetically engineering peptide or small molecular weight below 10 kD. Therefore search commercial protease inhibitor cocktail greatly suitable for cellular proteomic analysis protein of molecular weight of above10 kD. Such commercial protease inhibitors are now widely used. In below paragraph I have discus some of the important commercial protease inhibitors and it cocktails.

1.2.2 Commercial protease inhibitor

The inhibitors vary in specificity for particular proteases and the rate at which they inactivate these proteases. [18]. Base on the structure of the inhibitor, the inhibitor could be small organic molecules or small peptide or protein.

1.2.3 Small organic molecule protease inhibitors

For example most of the irreversible and reversible inhibitors of cysteine proteases are the small organic molecule such as aldehydes, halomethyl ketones, acyloxymethyl ketones, diazomethyl ketones, and peptidyl sulphonium salts contain a carbonyl moiety that reacts with the thiol of the active-site cysteine residue [19]. The aldehyde inhibitors form only reversible complexes with cysteine proteinases and also inhibit serine proteinases [20].

1.2.4 Modified peptides and amino acid protease inhibitors

Such type of protease inhibitors (e.g., bestatin, and pepstatin A) or native peptides (e.g., aprotinin) serve as competitive reversible inhibitors, which bind to the active site of proteases but are not cleaved. Other reagents (e.g., sulfonyl fluoride derivatives) are competitive irreversible inhibitors, which covalently attach to critical amino acids in the active sites of proteases.

For examples antitrypsin, antithrombin III and CI inhibitor are members of the serine proteinase inhibitor superfamily. These inhibitors are involved in the physiological regulation of elastolysis, coagulation, fibrinolysis, kinin formation and complement activation, forming stable complexes with a range of activated serine proteinases. For antitrypsin the target proteinase is neutrophil elastase, and complex formation is accompanied by peptide-bond cleavage of the inhibitor at the Met358-Ser359 peptide bond within the reactive centre. Other proteinases can catalytically inactivate these inhibitors by cleaving between residues that are believed to lie on an exposed peptide loop and include the reactive site [16]. Peptidylchloromethanes similarly react with both serine proteinases and cysteine proteinases [20].

Now commercially available protease inhibitor such as Pefabloc SC which is the general inhibition of serine proteases widely used because of its high stability and irreversible inhibition mechanism, protein solutions are protected throughout total procedures, such as [21], extraction processes (from animal tissues or cells, plants, bacteria, yeast, and fungi), subsequent purification steps, sample storage conditions, downstream protein analysis, biochemical studies where proteins are required.

Now a complete Mixture of protease inhibitors in the form of solution or tablet that can stop a multitude of proteases, including serine proteases, cysteine proteases and metalloproteases are available. Use of such inhibitor cocktail in cellular extract preparation is now highly recommended. However different cell type has different requirement cellular protease inhibitors therefore it is highly recommend design particular inhibitor cocktail to fit the requirement particular cell type. Such designing require the understanding the type of protease inhibitors. Now the type of protease inhibitor they are specificity and purity commercially available, as show in (table 1.3):

Table 1.3: Classes of Protease Inhibitors commercial available [20]

General inhibitors			
Serine proteases	Cysteine proteases	Metallo proteases	Aspartic proteases
Antithrombin III Aprotinin 3,4-Dichloroisocoumarin APMSF Pefabloc SC and Pefabloc SC PLUS	E-64	EDTA-Na2 Phosphoramidon Bestatin (aminopeptidases) TIMP-2 (matrix metallo- proteinases)	Pepstatin
Leupeptin (inhibits serine and cystine proteases with trypsin-like specificity) PMSF and PMSF PLUS complete, EDTA-free Protease Inhibitor Cocktail Tablets			
complete Protease Inhibitor Cocktail Tablets			
α_2-Macroglobulin			

For optimal inhibition of metalloproteases, it is recommended to prepare protease inhibitor solution cocktails with buffers containing no divalent contains (e.g.Ca^{2+} ,Mg^{2+} or Mn^{2+}) [21]. Further different inhibitor protease can also combine in order to achieve particular purpose for preparation which can selected with table 1.4:

Table 1.4: Individual Protease Inhibitors [20]

Product	Description/Specifity of Inhibitor	Concentration Range
AEBSF-HCl	Irreversible inhibitor of Thrombin and other serine proteases. Inhibits by acylation of the active site of the enzyme.	0.1 - 2 µM

Amastatin-HCl	Non-toxic reversible metallo-protease inhibitor. Inhibits many membrane bound peptidases which are critical regulators of peptide hormones.	1 - 100 µM
ε-Aminocaproic acid	Highly active inhibitor of fibrinolysin and chymotrypsin.	1 - 20 µM 5
α_1-Antichymotypsin from human plasma	lycoprotein that inhibits chymotrypsin-like proteases by forming stable complexes.	Used at equimolar concentration
Antipain-HCL	Reversible inhibitor of serine and cysteine proteases. Inhibits papain and trypsin more specificly than leupeptin. Plasmin is inhibited only slightly.	1 - 100 µM
Antithrombin III from human plasma	Glycoprotein that plays a major role in controlling serine proteases in the blood clotting cascade. Inactivates above all thrombin by forming an extremely stable complex, an effect which is enhanced by heparin. Inhibits also other proteases of the coagulation cascade like plasmin, kallikrein, factor IXa, Xa, XIa and XIIa.	Used at equimolar concentrations
α_1-Antitrypsin from human plasma	Glycoprotein that is mainly involved in the control of neutrophil elastase activity. Inhibits most of other mammalian serine proteases but at a lower rate. Blocks the action of target enzymes by binding nearly irreversibly to their active site.	Used at equimolar concentra tions
Aprotinin (Trypsin inhibitor from bovine lung)	Basic single-chain polypeptide that inhibits numerous serine proteases by binding to the active site of the enzyme, form ing tight complexes. It inhibits above all plasmin, kallikrein,	In cell culture: 0.01 - 3 µg/ml; in other applications: 10 - 250 µg/ml

	trypsin, chymotrypsin and urokinase, but not carboxypeptidase A and B, papain, pepsin, subtilisin, thrombin and factor X. Used in cell culture to prevent proteolytic damage to cells and to extend lifetime of cells.	
Arphamenine A	Inhibitor of the metallo-protease Aminopeptidase B	0.006 µg/ml
Arphamenine B	Inhibitor of the metallo-protease Aminopeptidase B	0.002 µg/ml
Benzamidine-HCl	Potent inhibitor of thrombin and trypsin	0.1 - 50 µM
Bestatin-HC	Metalloprotease inhibitor with multipharmacological functions. Inhibits cell surface aminopeptidases (notably B) and leucine aminopeptidase. Inhibitor of leukotriene A4 hydrolase and of enkephalin degradation in cell preparations from brain. Has anti- carcinogenic and immunomodulating properties.	1 - 150 µM Mitogenic effects at nmolar concentrations
CA-074	nhibitor of Cathepsin B	0.01 - 1 µM
CA-074-Me	Proinhibitor for intracellular Cathepsin B. Membrane permeable analog of CA-074	1 µM
Calpain Inhibitor I	Tripeptide aldehyde. Specific inhibitor of the Ca2+-dependent cysteine protease calpain I and of cathepsin B and L.	1 - 50 µM
Calpain Inhibitor II	Tripeptide aldehyde. Specific inhibitor of the Ca2+-dependent cysteine protease calpain II and of cathepsin B and L.	1 -50 µM
Cathepsin Inhibitor Z-Phe-Gly-NHO- Bz-pMe	Specific inhibitor of Cathepsin B/L/S and Papain	0.15 - 16 µM
Chymostatin	Peptide-derived aldehyde . Reversible inhibitor of	10 - 100 µM

	chymotrypsin-like serine and some cysteine proteases	
DFP (Diisopropylfluoro phosphate)	A potent irreversible inhibitor of serine proteases and acetyl choline esterase. Highly toxic	10 - 100 µM
Diprotin A	Reversible inhibitor of the metalloprotease Dipeptidylaminopeptidase IV	2.2 µM
E-64	Non-competitive irreversible inhibitor of papain and other cysteine proteases. Forms a thioether bond with the sulfhydryl group in the active center of the enzyme. Useful for active site titration: one mole of E-64 inhibits one mole of protease.	1 - 10 µM
E-64d (EST)	Membrane-permeable analog of E-64c	1 µM
Ebelactone A	Non-toxic inhibitor for esterases, acylpeptide hydrolase, lipase and N-formylmethionine aminopeptidase	1 - 10 µM
Ebelactone B	Non-toxic inhibitor for esterase, lipase and N-formyl-methionine aminopeptidase. Inhibits also carboxypeptidase Y-like exopeptidase.	1 - 10 µM
EDTA-Na2	Reversible inhibitor of metalloproteases	1 - 10 µM
EGTA	Inhibits metalloproteases.. Reveals high selectivity for Ca2+ over Mg2+ ions.	1 - 10 µM
Elastatinal	Inhibitor of Elastase	0.21 µM
Leuhistin	Inhibitor of Aminopeptidase M	0.2 µg/ml