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Transition-State Analog Affinity Purification of Proteases

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"TRANSITION-STATE ANALOG" AFFINITY PURIFICATION OF PROTEASES

by

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This work is dedicated to my family

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VITA

This author, Arunbhai Haribhai Patel, is the son of Haribhai Mathurbhai Patel and Kapilaben Patel. He was born August 15, 1949, in Kayavarohan, Gujarat, India.

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PUBLICATIONS

Publications

Vinson, J.A., Patel,D.D.,and Patel, A.H. (1977)
Detection of tetrahydrocannabinol in blood and serum using a fluorescent derivative and thin-layer chromatography.
Anal. Chem. 49(1), 163.

Arun H. Patel and Richard M. Schultz (1982)
Transition-state affinity purification of proteases; the preparation of an argininal affinity resin for the selective binding of trypsin-like proteases.
Biochem. Biophy. Res. Comm. 104(1), 181

Arun H.Patel, Ahmed Ahsan, B. Suthar and R.M. Schultz (1983)
Transition-state affinity chromatography of trypsin-like proteinases with dipeptidyl argininal ligands.
Biochim. Biophys. Acta. 748, 321-330.

Richard M. Schultz, Arun H. Patel, Ahmad Ahsan (1983)
Covalent transition-state affinity chromatography of Trypsin-like proteinases.
Proceedings of the Fifth International Symposium on Affinity Chromatography and Biological recognition, New York.
Academic Press Inc., 475.

J.A. Vinson and A.H. Patel (1984)
Detection and quantitation of tetrahydrocannabinol in serum using thin-layer chromatography and fluorometry.
J. Chromat. 307, 493-499.

Abstracts

Vinson, J.A., Patel, A.H. - Feb. 1976
Determination and quantitation of tetrahydrocannabinol in blood using fluorometry.
Annual meeting of Academy of Forensic Sciences, Washington D.C.

Vinson, J.A. and Patel, A.H.- Spring 1976
Quantitation of riboflavin in milk by column chromatography and fluorescence.
The Annual Meeting of The American Chemical Society, New York.

Richard M. Schultz and Arun H. Patel - June 1982
Transition-state affinity purification of proteases; The preparation and binding properties of dipeptidyl argininal affinity resins for the purification of proteases.

The Gorden Research Conference on Proteolytic Enzymes and their Inhibitors.

H.L. Messmore, B. Griffin, J. Corey, A. Patel - July 1985
Effect of affinity for antithrombin III on aggregation of platelets by heparin in the presence of serum from patients with heparin induced thrombocytopenia.
10th Congress of The International Society on Thrombosis and Hemostasis.

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ABBREVIATIONS USED IN THIS DISSERTATION

Argal	Arginine aldehyde
Arg-Sc	Arginine semicarbazone
BAEE	N-benzoyl-arginine ethyl ester
BzTyrEE	N-benzoyl-tyrosine ethyl ester
Cbz	Carbobenzoxy
EDAC	N-ethyl N'-(3-dimethylaminopropyl)carbodiimide
Et	Ethyl
FPA	Fibrinopeptide-A
FPB	Fibrinopeptide-B
HOAc	Acetic acid
ME	Methyl ester
Pheal	Phenylalanine aldehyde
pNA	para-nitroaniline
TAME	Tosyl-arginine methyl ester
TMOFB	Trimethyl oxonium tetrafluoro borate

Chapter 1

INTRODUCTION

1A. Transition-state analog theory

Collision theory calculates the probability for formation of an activated chemical state using ordinary statistical mechanics. This probability for formation of an activated state multiplied by the rate of decomposition gives the rate of a chemical reaction (Eyring, 1935). The theory need not consider the size or the geometrical shape of the colliding molecules. A more sophisticated treatment to the structure-reactivity relationship in chemical reaction rate is given in the transition state theory. The two salient differences of this theory from the "collision theory" are that the way in which the reactants collide is totally ignored, and only two physical states considered are the ground state and the most activated-state on the reaction pathway. In this dissertation the combination of the transition state theory and knowledge of mechanism of protease catalysis will result in development of a powerful technical tool, an affinity chromatography ligand for the selective purification of proteolytic enzymes.

Originally Pauling (1948) proposed that an enzyme has a configuration complimentary to the substrate in its activated state, and as a result will have higher affinity to the substrate's activated state (transition state) than to its ground state. This prediction of higher

affinity for the transition state comes from a straight forward argument made from the scheme depicted in Fig.1. A unimolecular conversion of a reactant, S, to a product, P, will occur by the reaction pathway with the lowest energy barrier. The structure of the highest energy on this lowest energy pathway is designated the transition state, $S^{\#}$.

The top of Figure 1 shows the reaction of the substrate, S, reacting to product, P, in a reaction not catalyzed by enzyme, E. The bottom of the Figure-1 shows the same reaction catalyzed by enzyme, E, through transition state, $ES^{\#}$. Accordingly K_S is the equilibrium constant for association of substrate with the enzyme; $K^{\#}_N$ and $K^{\#}_E$, the equilibrium constants for formation of the nonenzymic and enzymic transition states, $S^{\#}$ and $ES^{\#}$, respectively; and K_T the equilibrium association constant for association of the substrate in its transition state configuration to the enzyme. Based on the definitions for the respective equilibrium constants in Fig-1, it is easily shown that they are related by equation-1.

$$\frac{K_T}{K_S} = \frac{K^{\#}_E}{K^{\#}_N} \qquad (1)$$

In the transition state chemical bonds are in the process of being made and broken. The rate at which the transition state decomposes is the same as the vibrational frequency v of the bond which is being broken. The rate of reaction is therefore obtained from the transition

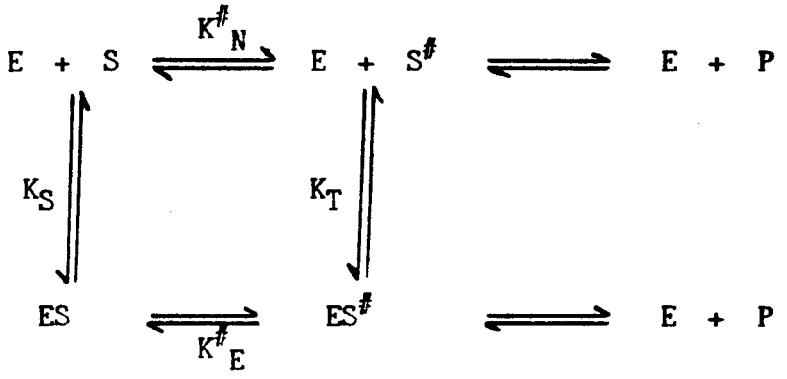


Figure 1: Thermodynamic schematic representation of the "Transition-state Theory" as applied to enzymatic catalysis. See text for detail.

state theory (Lienhard, 1973) by multiplying the concentration of the transition state by the rate constant for decomposition. Based on a derivation from quantum theory (E=hv) and classical physics (E=kT) the first order rate equation will be as shown by equation-2.

$$\frac{d(P)}{dt} = \frac{kT}{h}(S^{\#}) = \frac{kT}{h}K^{\#}_{N}(S) = k_n(S) \qquad (2)$$

where k_n is the first order rate constant for non-enzymic reaction, k the Boltzman constant, T, the absolute temperature and h the Plank's constant. A similar equation can be written for k_e and $K^{\#}$ or $ES^{\#}$ in the enzymic pathway. Combining equations (1) and (2) gives equation (3).

$$\frac{K_S}{K_T} = \frac{K^{\#}_{E}}{K^{\#}_{N}} = \frac{k_e}{k_n} \qquad (3)$$

where k_e/k_n is the ratio of the first order rate constant for the enzymic and non-enzymic reaction. It is commonly believed that the ratio k_e/k_n is at least 10^8 (Schultz and Cherva, 1975). Transition state theory gives no information about the extent of similarity in structure between the transition state of the non-enzymic reaction, $S^{\#}$, and the substrate portion of the transition state of the enzymic reaction. However, regardless of the extent of similarity, equation (3) may be valid (Lienhard, 1973).

Equation (3) indicates that the equilibrium association constant

for binding of the transition state $S^{\#}$ to the enzyme, K_T, will be at least 8 orders of magnitude larger than the association constant for the binding of substrate itself, K_S. Generally the value 10^8 is not observed, but many examples have been shown of a higher affinity for transition-state analogs to an enzyme active site than for an enzyme's substrates (Table-1) (Lienhard, 1973; Wolfenden, 1972; Wolfenden, 1976; Schray and Klinman, 1974; Lindquist, 1975).

The purification of proteolytic enzymes from complex biological systems is often difficult, especially when the concentration of the enzyme is low and multiple enzymes with an identical primary binding site specificity are present. Pancreatic proteases were the first proteinases purified in the late 20's and 30's by Sumner and Howell. The major method of purification was by fractional crystallization. In these cases the starting protease had to be present in concentrations from milligram to gram quantities. Current conventional chromatographic techniques primarily utilize the principals of gel filtration and ion-exchange chromatography. These methods are non-specific and tedious. A more specific form for the purification of proteases is affinity chromatography. The ligands used in affinity resins for protease purification are general inhibitors, cofactors or substrate analogs. More recently antibodies to a particular protease enzyme have been successfully employed in purification of proteases. In many cases the small inhibitors or substrate analogs do not have high binding constants or high selectivity with the protease.In dilute solutions of enzyme, the concentration of enzyme is often too low to bind in

Table 1

COMPARISON OF PROPOSED TRANSITION STATE ANALOG INHIBITON CONSTANTS (K_I) WITH RESPECTIVE SUBSTRATE MICHAELIS CONSTANTS (K_m) TO SELECTED HYDROLASE ENZYMES

Enzyme	Inhibitor, K_I, or Substrate, K_m, M	K_I/K_m
Lysozyme	(N-acetylgalactoseamine)$_4$-lactone, 3×10^{-7} (N-acetylgalactoseamine)$_4$, 1×10^{-5}	10^2
-Glucosidase (yeast)	1-aminoglucoside, 2.3×10^{-6} p-nitrophenyl-glucoside, 1.4×10^{-4}	10^2
-Glucosidase (yeast)	1-aminogalactoside, 1.6×10^{-4} p-nitrophenyl-galactoside, 1.7×10^{-2}	10^2
Carboxypeptidase-A	L-benzylsuccinate, 4.5×10^{-7} Cbz-L-Phe, 8×10^{-4}	10^3
Elastase	N-Acetyl-Pro-Ala-Pro-alaninal, 8×10^{-7} N-Acetyl-Pro-Ala-Pro-AlaOMe, 1.5×10^{-3}	10^5
Papain	N-Acetyl-Phe-Glycinal, 4.6×10^{-8} N-Acetyl-Phe-GlyOMe, 3.2×10^{-5}	10^3
Subtilisin	Benzeneboronic acid, 8×10^{-4} N-Acetyl-TyrOEt, 9.0×10^{-2}	10^2
Amidase	Acetaldehyde-NH_3, 1.6×10^{-5} Acetamide, 8.3×10^{-4}	10^2

W.P. Kennedy. 1980.

considerable yield. Antibodies have been used as ligands to alleviate the problem of low binding constant. However, antibodies are difficult to produce. More importantly, often severe conditions are required to dissociate the protease from the antibody, which may result in the irreversible denaturation of the protease (Scouten, 1974).

1B. Peptide aldehydes as ligand in affinity resins.

In the case of the serine proteases, the mechanism of substrate catalysis has been shown to go through an acylserine intermediate (ES') in a scheme depicted by equation 4; where ES is the Michaelis complex, k_2 the first order rate constant for formation of the acylserine intermediate from ES, k_3 the pseudo first order rate constant for hydrolysis of the acylserine intermediate, P_1 the first product of the reaction (amine or alcohol), and P_2 the second (acid) product of the reaction (Bender and Kezdy, 1964; Henderson, 1970).

$$E + S \overset{K_s}{\rightleftharpoons} ES \overset{k_2}{\rightleftharpoons} ES' + P_1 \overset{k_3}{\underset{H_2O}{\rightleftharpoons}} E + P_2 \qquad (4)$$

A non-covalent intermediate is formed first between substrate and enzyme followed by the attack of the hydroxyl of the serine active site on the substrate to give an acylserine intermediate, releasing the amine or alcohol (step k_2). The acylenzyme is then hydrolyzed to form the enzyme and second product (step k_3). The transition state configuration for the hydrolyzable peptide (or ester) carbonyl carbon in steps k_2 and

k_3 is thought to be tetrahedral (sp^3) (see fig-3) (Kraut, 1969; Roburtus et al., 1972). X-ray crystallographic studies of the enzyme-substrate binding complex of various polypeptides to subtilisin BPN' using a difference-Fourier method and model building experiments suggest that the carbonyl carbon assumes a tetrahedral conformation in the transition state, as this results in more favourable contacts between the substrate specificity residue P_1 and the enzyme. The enzyme active site can form four hydrogen bonds to stabilize the transition-state and thereby lower the free energy of activation for reaction (Robertus et al., 1972). In addition, structural data indicate the binding of leaving groups at P_1' and hydrogen bonds to the carbonyl group of the scissile bond, are better oriented when the carbonyl carbon becomes tetrahedral (Blow et al., 1974). Accordingly, substrate analogs that place the proper charge distribution and stereochemistry around the carbonyl carbon, that is an sp^3 configuration in the catalytic site, may mimic features of the transition state configuration and will be expected to associate with extremely high affinity (Wolfenden, 1969).

Peptidyl aldehyde substrate analogs may present an sp^3 C-1 configuration to the active site of protease enzymes when associated as the hydrated adduct IB or as a covalent hemiacetal IIA or IIB (Fig.2), and thus may be predicted to associate with high affinity as a transition state analog. In agreement with the prediction, it has been found that specifically designed natural and synthetic peptide aldehydes associate 10^2 to 10^4 times better to serine proteases than their analogous substrates (Kennedy and Schultz, 1979; Aoyagi and Umezawa,

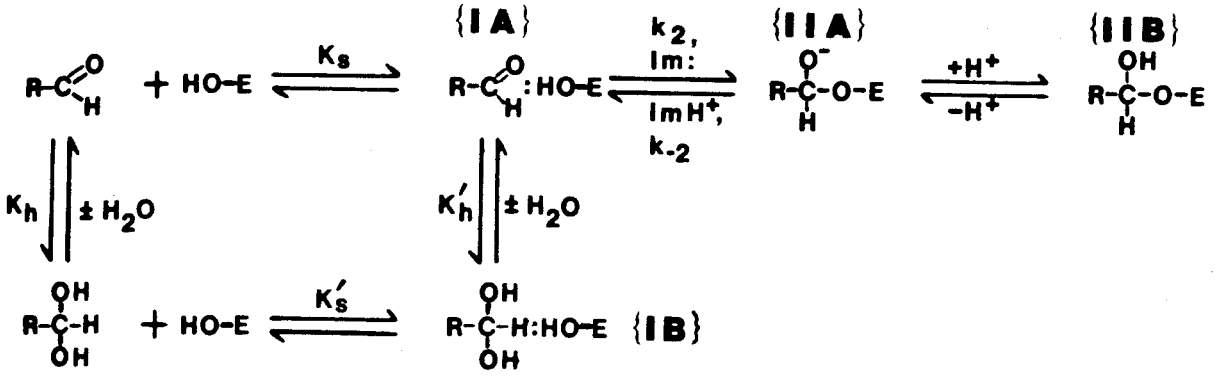


Figure 2. Scheme for association of peptide aldehyde with the active site of SERINE proteases: IA and IB are Michaelis complexs with the C-1 carbonyl carbon of the aldehyde in sp^2 and sp^3 configurations, respectively; IIA and IIB are hemiacetal adducts with the active site Ser-195 of the enzyme. In the hemiacetal adducts IIA and IIB the C-1 carbonyl carbon of the aldehyde also has a sp^3 configuration as in the transition state of the substrate reactions catalysed the protease enzyme.

1975; Thompson, 1973; Westerick and Wolfenden, 1972; Frankfater and Kuppy, 1981).

In future discussions the nomenclature introduced by Schechter and Burger (1967) will be used as follows: the substrates are lined up with the enzyme in such a way that the hydrolyzing CO-NH group occupies the catalytic site; the amino acid residues of the substrate each occupy adjacent subsites in the enzyme, those towards the NH_2-terminal occupy subsites S_1, S_2, S_3, etc., those towards the COOH-terminal of the substrate occupy subsites S'_1, S'_2, S'_3 etc of the enzyme. The positions of the residues in the substrates are numbered P_1, P_2,P_3 or P'_1, P'_2, P'_3 according to the subsites they occupy.

Umezawa initiated the study of peptide aldehydes with the discovery of the leupeptins from Actinomycetes culture medium. The leupeptins are inhibitors of trypsin-like (proteases specific to arginine or lysine in the primary binding site, P_1) proteases (Aoyagi et al., 1969). These inhibitors were shown to be peptide aldehydes in which the carboxylic group is reduced to an aldehydic group. These were the first examples of peptide aldehyde competitive inhibitors of serine proteases. Aldehyde inhibitors are turned over by protease enzymes in a similar way to normal substrates (Kennedy and Schultz, 1979). They reversibly form an acyl serine type adduct, which decomposes to a product that is the same as starting substrate (Kennedy and Schultz,1979). This assures a constant concentration of the peptide aldehyde inhibitor in solutions of inhibitor and enzyme. If a peptide aldehyde is used as a ligand in affinity resin it may therefore be

expected that this resin could bind serine proteases effectively by forming a reversible covalent adduct with the protease of high affinity. Many interesting serine proteases such as these involved in thrombolytic and fibrinolytic processes have a primary specificity for arginine. Argininal affinity resin should bind these serine proteases containing a primary site (P_1) specificity for arginine.

Currently utilized protease affinity resins for arginine specific proteases utilize ligands such as benzamidine and lysine with binding constants to protease enzymes of $5x10^{-4}$ M to $1x10^{-2}$ M (Holmberg et al., 1976). In contrast the peptidyl aldehyde ligands have affinity constant 2 to 7 orders of magnitudes higher than these commonly utilized ligands. Because of the higher affinity of the peptidyl aldehyde ligand, dilute proteases may be purified with significantly higher yields.

Investigations on the secondary specificity of trypsin-like proteases show that variation of the amino acid sequence N-terminal to the P_1 residue in the substrate can lead to the ability of a substrate to be hydrolyzed selectively by a particular plasma protease within a group of plasma proteases. Accordingly, the peptide p-nitroanilide H-D-Phe-Pro-Arg-pNA is selective substrate of thrombin. It may be suggested that D-Phe-Pro-Argal would be a selective inhibitor of thrombin (Bajusz et al., 1978). While the benzamidine and lysine sepharoses do not show any dramatic specificity or selectivity between different plasma proteases with trypsin-like specificity, the selective affinity of the peptidyl aldehydes can be used for the purification of an individual specific protease within a biological pathway. Thus X-Y-

Argininal affinity resins can be designed for the purification of a particular protease among a group of proteases with a primary site specificity towards arginine.

1C Goals of this Dissertation

The major objective of my research is to exploit the thermodynamic predictions of the transition-state theory for the practical purpose of protease purification utilizing highly selective and specific peptidyl aldehydes. The application of this technique will have a general usefulness in the isolation and purification of biologically important proteases. In this Disertation I will:

(1) Synthesize tripeptidyl aldehyde ligand affinity resins of the general formula R-X-Y-Argininal where R- is the resin matrix and X-Y is a dipeptide. Methods are developed to bind and dissociate protease enzymes to these resins and selectively separate a particular trypsin-like protease.

(2) Show progress towards development of a develop general procedure for the purification of proteases in which the substrate of the protease is attached by its N-terminal residue to an inert support, treated with a biological solution containing the protease activity to be purified, and the COOH-terminal of the peptide fragment chemically modified to a peptidyl aldehyde to generate the transition state analog inhibitor of the protease of interest. This affinity resin is evaluated for the selective purification of the protease that was used to generate the acid ligand product. By this procedure, an unknown protease can be

purified from complex biological systems based solely on the availability of its substrate.

In this research the examples of thrombin and urokinase are used to demonstrate what I view as general procedures of protease purification. It is hoped that establishing of the general procedure will allow other workers to utilize these techniques in their own work, with their protease of interest.

Chapter 2

REVIEW OF THE RELATED LITERATURE

2A. Protease purification and mechanism of action of serine proteases.

The mechanism of action for enzymes was proposed even before it was known that enzymes are proteins. For example important kinetic studies on cell free extracts of enzymes were carried out by Buchne in 1897. It wasn't until 1935 that the first enzyme was crystallized by Northrop and Kunitz, producing enough pure enzyme to prove that the enzymes were proteins (Northrop and Kunitz, 1939). Purification by crystallization is useful only when the protease is present in large quantity. When the quantity of enzyme is low and the matrix from which the protease is to be purified is complex, chromatographic techniques become essential. For the purification of bovine pancreatic trypsin, Chao (1965) used CM-cellulose in citrate buffer at pH 4.8. Three years later Schroder (1968) used sulfonyl Sephadex to separate five different active forms of trypsin. In the last twenty five years, since its introduction by Porath (1959), gel-filtration has occupied a key position in the purification of hundreds of enzymes. A comprehensive review of literature on the use of gel-filtration and ion-exchange chromatographic techniques by various researchers is beyond the scope of this dissertation.

Serine proteases are a class of proteases containing a uniquely reactive serine residue that is primarily responsible for their

catalytic activity. A general criterion of the serine proteases class is that they react irreversibly with organophosphates such as diisopropyl fluorophosphate (Jansen, 1949) with complete loss of its proteolytic activity. The pH maxima of most extracellular serine proteases is around 7.8 with a group of pKa = 6.8 controlling activity. Modification studies using N-tosyl-L-phenylalanine chloromethyl ketone by Schollmann and Shaw (1963) indicated that the group of pK_a 6.8 is a histidine. The X-ray studies (Blow and Steiz, 1970) showed that His-57 (chymotrypsin numbering) is within H-bonding distance of the active site serine-195. A third important group shown by X-ray crystallography studies to be present at the active site of serine proteases is belived to be an aspartic acid residue (Asp-102).

By early 1960's utilizing innovative kinetic and analytical techniques, the basic features of the mechanism of action of serine proteases on amide and ester substrates was elucidated (Bender et al., 1964; Zerner and Bender, 1964; Kezdy et al., 1964; Mc conn et al., 1971; Fersht, 1971; Fastrez et al., 1971; Mackenzi, et al., 1984; Nakagawa and Umeyama, 1984). The details of the evidences in support of the mechanism have been thoroughly reviewed (Bender and Kezdy, 1965). The accepted reaction sequence for serine proteases is as shown in Fig.3 (Kraut, 1977).

The initial step in the reaction pathway is the formation of a reversible non-covalent intermediate (the Michaelis complex) with dissociation constant, K_s, between substrate and protease. In a second step, nucleophilic attack at the substrate scissile carbonyl carbon by

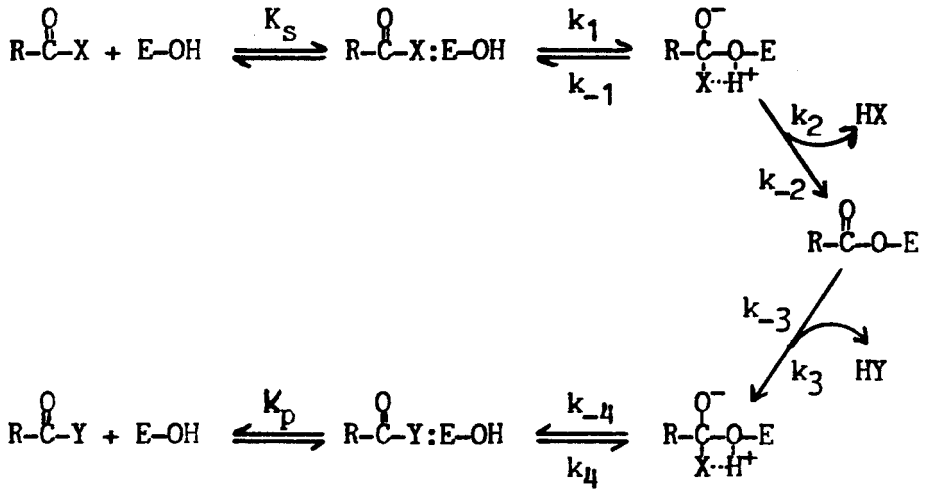


Figure-3. The reaction sequence for serine proteases.

(Kraut, 1977).

an active site serine γ-OH residue results in the formation of an acyl-enzyme intermediate concomitant with the release of an alcohol or amine product. In the third step (step k_3), water acts as the nucleophile in the hydrolysis of the acyl enzyme intermediate, regenerating the free enzyme and releasing the acid product.

The focal point of the future discussion will be the carbonyl carbon (C_1) of the substrate undergoing hydrolysis. As we shall see there is considerable evidence for the occurrence of a tetrahedral intermediate in serine protease-catalyzed hydrolysis. Earlier it was shown that non-enzymatic acyl substitution reactions with carbon-oxygen fission generally occur through the formation of an acyl-nucleophile adduct in which the carbonyl carbon assumes a tetrahedral configuration (Bender, 1960). From the kinetic study of chymotrypsin catalyzed hydrolysis of p-nitrophenyl thiolacetate and p-nitroanilide, Frankfater and Kezdy suggested of necessitating the existence of an intermediate in which the carbonyl carbon most likely to assume a tetrahedral configuration (Frankfater and Kezdy, 1969). It was also suggested by Caplow (1969) that the carbonyl carbon of the substrate assumes sp^3 configuration in proposed transition state similar in structure to the putative tetrahedral intermediate, during formation of acyl enzyme intermediate. X-ray crystallography with difference-fourier calculations and model building to the active site also suggest that the carbonyl carbon assumes a tetrahedral configuration (Kraut, 1977; Robertus, 1972). X-ray diffraction studies at high resolution, 1.8 A^o, of Streptomyces griseus with a peptide aldehyde also showed the existence

of tetrahedral conformation for the aldehyde in the active site, supporting the stability of the sp^3 conformation in substrate hydrolysis (James et al., 1980).

In addition to the X-ray crystallography, spectrophotometric techniques such as laser Raman spectroscopy and ^{13}C NMR has provided evidence for formation of tetrahedral conformation. Hess and Seybert (1975) using chymotrypsin and phenylethane boronic acid with laser Raman spectroscopy showed a band at 684 cm^{-1} present in the spectra of the crystalline enzyme inhibitor complex, which indicates a tetrahedral configuration for the boronic acid in the enzyme. Additional evidence for formation of a tetrahedral intermediate in protease enzymes with transition-state analog inhibitors will be reviewed in the transition state analog section.

2B. Inhibitors of Serine Proteases

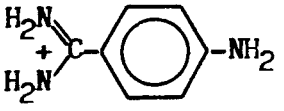
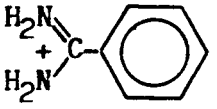
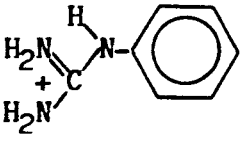
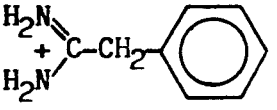
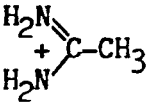
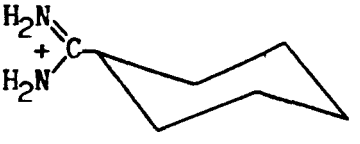
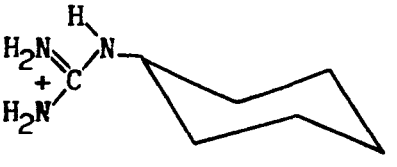
The knowledge of the primary specificity for a protease in its hydrolysis of peptide bonds provides the starting point for the development of substrate related synthetic inhibitors. Specificity is primarily determined by the side chain of the amino acid contributing the carbonyl function to the bond to be split. Chymotrypsin requires an aromatic or bulky nonpolar side chain, in P_1 while trypsin requires a lysine or arginine residue in P_1. X-ray studies have shown that these different substrate specificities arise from structural changes in the enzyme binding site. In trypsin-like proteases, one residue in the binding pocket is an aspartate. This aspartate can form a strong

electrostatic bond with a positively charged lysine or arginine side chain of a substrate. Accordingly non-substrate compounds that similarly contain a positively charged group, either amidinium or guanidinium will be expected to bind preferentially trypsin-like proteases at the active site and hence act as competitive inhibitors. Thus Guia and Shaw (1965) showed that benzamidine, p-aminobenzamidine, phenylguanidine, cyclohexylcarboxamidine, cyclohexylguanidine, 2-phenylacetamidine and acetamidine are competitive inhibitors of trypsin (Table 2).

Benzamidine and p-aminobenzamidine were the first examples of the most potent small molecular competitive inhibitors of trypsin. They are approximately the same size as the side chain of lysine or arginine. Many other compounds with a positively charged group and a hydrophobic moiety have also been found to possess an inhibitory effect. These are alkyl and aryl guanidines, amidine and agmatine derivatives, benzoyl and tosyl arginine, aliphatic and aromatic amines, and p- or ω-amino carboxylic acids (Keil, 1971). The reversible binding property of these small inhibitors have been exploited to map the spatial arrangements at the specificity site of the proteases. Table-3 shows the K_i values of variety of low molecular weight compounds containing guanidino group for thrombin (Geratz and Tidwell, 1977). From the data it is evident that the ring system other than benzene achieve an even tighter fit with the enzyme, most likely due to improved hydrophobic interaction. The introduction of a pyruvic acid group in the <u>para</u> position to the amino side chain augmented the inhibitory activity not only in thrombin but also with plasmin, trypsin and enterokinase (Geratz and Tidwell, 1977).

Table 2

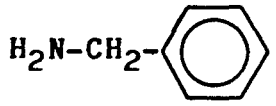
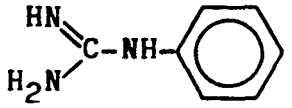
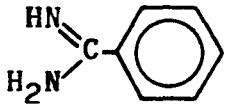
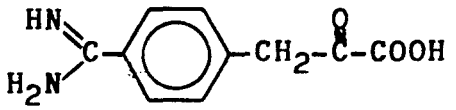
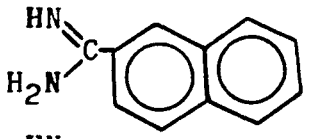
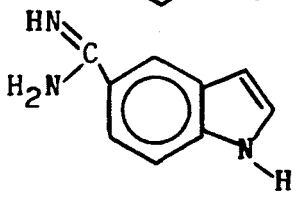
Competitive inhibitors of trypsin at pH 8.15 and 15 °C

Inhibitor	K_I, M
	8.25×10^{-6}
	1.8×10^{-5}
	7.25×10^{-5}
	1.51×10^{-2}
	3.65×10^{-2}
	4.27×10^{-4}
	1.54×10^{-3}

Guia and Shaw (1965)

Table-3

Thrombin inhibitors restricted to the specificity pocket of the enzyme

Compound	Structure	K_i, M
Benzylamine		12.3×10^{-3}
Phenylguanidine		9.0×10^{-3}
Benzamidine		2.2×10^{-4}
p-Aminophenyl-pyruvic acid		6.5×10^{-6}
β-Napthamidine		8.5×10^{-5}
5-Amidinoindole		7.7×10^{-6}

Geraz *et al.*, (1977).

Initially the development of more active inhibitors directed towards trypsin-like proteases beyond primary specificity pocket was chiefly a empirical process. It consisted of the attachment of a number of different side chains to the starter compound which in most cases were represented by benzamidine. The substituents chosen were aliphatic, aromatic, arylaliphatic, bicyclic or heterocyclic in nature.

Even though substituted benzamidines can differentiate between two different enzymes from different specificity classes, like chymotrypsin and trypsin, they will fail to differentiate the enzymes in the same class, for example between thrombin and trypsin. Rather than an empirical process for designing low molecular weight inhibitors, the use of small peptides and peptide derivatives is more logical. To obtain additional direct information about the binding sites of proteases outside of the primary binding site, reactive polypeptide inhibitors were designed. For example Schollmann and Shaw (1962) in developing chloromethyl ketone inhibitors combined N-tosyl-L-Phe to confer affinity to the primary active center and halomethyl ketone grouping to achieve irreversible alkylation of active site histidine. It was predicted that the increasing the peptide length to dipeptide and tripeptides can lead to higher specificity. Morihara and Oak (1970) showed that on incubation of two different chloromethyl ketone derivatives of peptide substrates, CBZ-L-Ala-L-Phe and CBZ-L-Ala-Gly-L-Phe with subtilisin, a much higher rate of inactivation of the enzyme was observed compared to chloromethyl ketone of CBZ-L-Phe. During the same period Powers and Wilcox (1970) reported the synthesis of specific peptide chloromethyl

ketones for chymotrypsin and showed an inhibition of crystal growth. Thompson and Blout (1973) also showed that three different peptide chloromethyl ketones differ markedly in their ability to inactivate the elastase with the longer peptides reacting more rapidly. Thompson and Blout (1970) reported large increase in the esterase, amidase and peptidase activities of elastase $4x10^6$ fold on increasing the length of peptide substrate from one to five amino acids N-terminal from the scissile bond. Too much increase in the length of the peptide also could be counter productive in case of inappropriate folding of the peptide. Table 4 shows binding constants of natural and synthetic peptides to thrombin. Scheraga (1977), and Blomback et al. (1977) have shown that while large segments of the residues P_{16} through P'_{35} of the fibrinogen are required for high peptidase activity by thrombin, the equilibrium binding constants (K_m or K_s) for small peptide segments or synthetic peptide analogs of fibrinopeptide-A may be as good or better than that for the natural substrate fibrinogen (Table-4). From the binding constants it could be seen that small peptides up to penta peptide inhibitors bind better than its natural substrate and CNBr digestion products.

Plasma proteases involved in coagulation processes and fibrinolytic processes like thrombin, urokinase, kallikrein etc. have a primary specificity for arginine, and are therefore grouped in the class of trypsin-like proteases. Investigations on the secondary site specificities of these trypsin-like proteases show that oligopeptide substrates having the appropriate amino acid sequence N-terminal to the

Table 4
Binding constants of natural and
synthetic polypeptides to thrombin

Polypeptide	K_I, M
Fibrinogen (natural substrate)	1.2×10^{-5}[a], 3×10^{-6}[b]
CNBr-A( ) (human)[c]	4.7×10^{-5}
CNBr-B( ) (bovine)[d]	1.9×10^{-4}
Synthetic peptide chloromethylketones:[e]	
Gly-Val-ArgCH_2Cl	1.3×10^{-5}
Val-Pro-ArgCH_2Cl	6.7×10^{-7}
Phe-Ala-ArgCH_2Cl	2.9×10^{-6}
Phe-Ala-LysCH_2Cl	3.7×10^{-5}
H-D-Pro-Phe-Arg-OH	1×10^{-6}

[a] At pH-9 from K_m in which rate of fibrinogen conversion to fibrin followed by light scattering technique. [b] At pH-7.4 from K_m in which rate of fibrin conversion followed by appearance of fibrinopeptides by R.I.A. [c] Residues 1 through 51 in fibrinogen A-chain. [d] Residues 1 through 115 in fibrinogen B-chain. [e] Determined from K_s in the study of rates of thrombin modification by chloromethylketones.

Richard M. Schultz N.I.H grant application (HL24910).

P_1 residue could be selectively hydrolyzed by a particular plasma protease within a mixture of related enzymes (Fareed et al., 1981; Cleason and Aurell, 1981). Table-5 shows specific activity of various plasma proteases against various synthetic chromogenic substrates. Because these are trypsin-like proteases, the presence of either arginine or lysine in the P_1 position is essential for primary specificity. Variation of the amino acid sequence of residues in positions P_2 through P_4 in small peptide substrates leads to the ability to selectively react with one of the plasma proteases in the presence of other trypsin-like plasma proteases.

The design of small peptide inhibitors thus relies on the knowledge of the required amino acid sequence around the scissile bond (Kettner et al., 1980). Hegeman and Scheraga studied the sequence around the Arg-Gly bond in fibrinogen hydrolysis by thrombin to define the required structural elements for the optimum thrombin-fibrinogen interaction. Blomback et al. (1977) also independently came to a common conclusion that amino acids 1 to 51 in A chain contain all the necessary elements for optimal interaction. To construct a 51 amino acid residue long peptide inhibitor could be a very difficult task. The approach taken by Thompson and Blout (1973b) seems to be a more practical approach. They synthesized a number of peptide amides for elastase mimicing the natural substrate elastin, and measured kinetic parameters for the elastase catalyzed hydrolysis of dipeptides to pentapeptides. By comparing K_i values they identified S_3 as the subsite unable to bind proline. The contributions from subsites S_5 to S_2 towards interaction between enzyme

Table 5

Relative activities of trypsin-like proteases towards different substrates

$-P_3-P_2-P_1-$	Relative activity of the enzyme					
	Thrombin	Factor Xa	Plasma Kallikrein	Glandular Kallikrein	Plasmin	Urokinase
Bz-Phe-Val-Arg-pNA	0.140	0.002	0.004	0.001	0.002	0.001
Bz-Ile-Glu-Gly-Arg-pNA	0.005	0.200	0.005	0.003	0.006	0.007
D-Phe-Pip-Arg-pNA	0.480	0.008	0.007	0.004	0.002	0.002
D-Val-Leu-Lys-pNA	0.002	0.001	0.007	0.020	0.040	0.000
D-Val-Leu-Arg-pNA	0.005	0.001	0.006	0.130	0.002	0.001
D-Pro-Phe-Arg-pNA	0.030	0.040	0.190	0.100	0.040	0.000
L-Glu-Gly-Arg-pNA	0.620	0.003	0.004	0.001	0.001	0.001	0.040

A.B Kabi, Blood product division, Sweden.

and substrate was shown to be sufficient for practical purposes, and interactions beyond S_5 could be safely ignored for the design of a peptide inhibitor. In case of thrombin-fibrinogen system, important contribution to binding are made by residues relatively far from the scissile bond within the structure such as the Phe in P_9 and Asp in P_{10} positions. When the substrate is folded in a helical structure, the ninth position N-terminal becomes close to the S_3 position (Marsh et al., 1982; 1983). Accordingly, the peptide aldehyde H-D-Phe-Pro-Argal in which a hydrophobic residue at position 3 in a D conformation, is found to be able to mimic the Phe in position 9 of the normal substrate and to be a selective and potent tripeptide inhibitor of thrombin (Bajusz et al., 1978). This peptide inhibitor is thus designed on the basis of one of the products of thrombin hydrolysis, namely the N-terminal segment of substrate (P_2 in eq. 4).

Design of the peptide inhibitors based on a substrate which spans the active site on both directions i.e. N-terminal and C-terminal of the scissile bond, have also been investigated (Szelke et al., 1982). A strategy involved in designing these inhibitors is to reduce the succeptible amide bond to the $-CH_2-NH-$ moiety. Since the protease can not hydrolyze $-CH_2-NH-$ bond, the peptide inhibitor is termed as non-hydrolyzable isosteric peptide inhibitor. For example a number of such inhibitors for renin are synthesized by Szelke et al. (1982). The inhibitor designed in this way H-Pro-His-Pro-Phe-His-Leu-^{R}-Val-Ile-His-Lys-OH is 30,000 times more active than the human octapeptide from which it was derived. It is argued that the greatly enhanced renin inhibitory

activity of angiotensinogen analog bearing a reduced peptide bond is due to the change of a configuration at the scissile bond. The $-CH_2-NH-$ moiety is isosteric with the tetrahedral transition state formed during amide hydrolysis and is therefore bound to the enzyme more tightly than the substrate. It is further argued that because transition state isosters bind to the enzyme so much more tightly, the effect of structural variations present in substrates of different species is magnified, resulting in the large difference in binding to human compared with dog renin (Szelke et al., 1982).

A potential transition state analog for serine proteases could be designed by considering the charge distribution and stereochemistry around C-1 of an inhibitor which should give a resemblance to the proposed tetrahedral transition-state. The peptide acids generated by protease catalyzed hydrolysis of proteins are generally good inhibitors of the enzyme that produced them, but the analog which mimics relevant features of the transition state complex will form an even stronger complex with the enzyme. A species I (fig-4) similar to transition state in step-2 of fig-3, has been proposed by Jencks (1969) and Thompson (1973) to be intermediate in the hydrolysis reaction catalyzed by several proteolytic enzymes, e.g. the serine and cysteine proteases. This suggests that the functional group which will provide tetrahedral geometry and suitable charge distribution upon binding to the protease's active site is expected to be a strong inhibitor for serine and cysteine proteases.

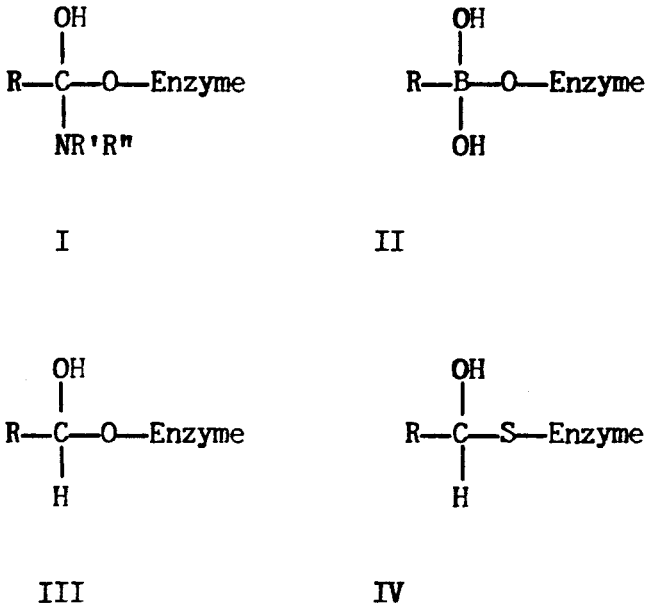


Figure-4. Tetrahedral intermediates of serine and cysteine proteases. (See text for detail)

2C. Transition state analogs of serine and cysteine proteases

Initially alkyl boronic acids, which readily form tetrahedral addition compounds, Fig. 4, were proposed as transition state analogs for chymotrypsin (Koehler and Lienhard, 1971). The use of boronic acid group as an inhibitor, that binds strongly but yet could be removed easily by a slight change in pH, was initiated by Berezin.

The tetrahedral intermediate will be highly unstable by virtue of good leaving groups on the carbonyl carbon. It will collapse easily to give either the acyl enzyme or the substrate. In case of alkyl boronic acid as a substrate, the ionized alkyl boronic acid (species II in fig-4) will be relatively stable. The support to this argument comes from the experimental observation that the benzene boronic acid (K_I=1.96x10^{-4} M) associates stronger to chymotrypsin than to its substrate analog, benzylalcohol (K_I=1.0x10^{-2}M). X-ray crystallography of benzene boronic acid-subtilisin complex the showed formation of a tetrahedral structure for benzene boronic acid, which formed a covalent bond between boron and alcoholic oxygen of serine residue of subtilisin (Mathews, 1975). Spectrophotometric determinations revealed a two step mechanism, in which a fast bimolecular association process is followed by a slower unimolecular isomerization process representing covalent bond formation (Nakatani et al., 1977).

X-ray crystallography of the complex formed between subtilisin and L-Phe-L-Ala-L-Lys chloromethyl ketone at 2.5 A^{o} showed that it also formed a stable hemiacetal in which carbonyl carbon atom was tetrahedrally coordinated (Poulous et al., 1976; Robertus et al., 1972).

X-ray investigation at 2.8A° of the binding of papain to some peptide chloromethyl ketone inhibitors, containing L-Phenylalanine at P_2 provided informative structural data about enzyme substrate interaction. Electron density difference maps of the papain-inhibitor complex revealed that the carbonyl oxygen of the P_1 residue is near two potential hydrogen donating groups in the active site of the enzyme. The carbonyl oxygen of the P_1 residue, carrying a formal negative charge in the tetrahedral intermediate, may be stabilized by formation of two hydrogen bonds with the backbone NH of cysteine-25 and the NH_2-group of glutamine-19 (Drenth, 1976). Thus the C_1 carbonyl carbon of peptide halomethyl ketones, upon associating with serine and cysteine proteases, appears to assume a tetrahedral structure in its stable inhibitor complex. Since these polypeptide halomethyl ketones inactivate serine and cysteine proteases by alkylating an active site histidine, the binding is irreversible and the utility of these inhibitors as ligands in affinity chromatography for purification of proteases is non-practical. This leaves alkyl boronic acids or peptide aldehydes as possible alternatives for ligands in affinity chromatography.

It is clear that derivatives of peptide acids in which the carboxylic acid group has been replaced by an aldehydic group are more potent inhibitors of certain proteases than are the original acids. This phenomenon was first discovered by Kawamura and Umezawa (1969) while screening filtrates of Actinomycetes for naturally occurring protease inhibitors. It was also independently discovered by Westerick and Wolfenden (1972) and by Thompson (1973) in a search for transition state

analogs for cysteine and serine proteases. Aldehydes are unique among carbonyl carbon in preferring to exist as tetrahedral addition complexes. The hemiacetal (species III) is relatively stable. There are clear analogies between the tetrahedral species I, II and IV. Hemiacetals and hemithioacetals might therefore be expected to be good transition state analogs for those proteases which react through a tetrahedral intermediate or transition-state of general structure I. These hemiacetals or hemithioacetals should be easily generated from the corresponding aldehyde and the enzyme.

It is of interest to find out whether aldehydes are bound by enzymes as covalent adducts or not. Alternatively inhibition could be by a non-covalently associated aldehyde hydrate. Using a cross-saturation technique in nuclear magnetic resonance spectroscopy, Bendall et al. (1977) provided the first direct evidence for formation of hemithioacetal, formed from the aldehyde and the active-site thiol group in papain. It was also shown that the aldehyde, and not the hydrated form, is the true inhibitor. Lewis and Wolfenden (1977) made use of effect of deuterium substitution on the equilibrium addition of nucleophiles to aldehydes to discriminate between noncovalent and covalent associations of aldehydes to papain. The secondary isotope effects on K_i can theoretically distinquish between covalent and noncovalent binding of aldehyde. The results suggested a thiohemiacetal is formed at the active site, indicating a covalent binding.

Because of the strong nucleophilic character of the active site serine residue in serine proteases, formation of a stable covalent

complex between the enzyme and the aldehyde was considered to be very likely (Thompson, 1973). Since the formation of aldehyde hydrates and hemiacetals are subject to general-base catalysis, the His-residue in the active site may, a priori be expected to facilitate formation of a hemiacetal by removal of a proton from the serine. Aldehydes exist predominantly as hydrates in aqueous solution. The hydrates, which are gemdiols, can bind to proteases non-covalently. However, it was not thought that the hydrate would have a high affinity even though they present a tetrahedral C_1 carbon to the enzyme as Ac-Pro-Ala-Pro-Alaninal binds 1000 times stronger than Ac-Pro-Ala-Pro-Alaninol to elastase at pH-7.0 (Thompson, 1973). Since it was believed that the non-covalent binding of the aldehyde hydrate could not strongly bind to elastase, Thompson (1973) suggested that it is not the hydrate but the unhydrated form of the aldehyde that binds to the enzyme.

Additional evidence to support binding of the free aldehyde and not the hydrate also comes from NMR. A plot of the corrected line-width of the CH proton of the aldehyde and the hydrated aldehyde for the chymotrypsin-inhibitor (Bz-Pheal) ratio at pD 6.5 showed that the aldehyde and not the hydrated aldehyde is in equilibrium with the inhibitor on the enzyme (Chen et al., 1979). In ^{19}F NMR spectra of DL-Ac-Pheal there are two signals assigned to the aldehyde. The smaller signal at -37.6 ppm is assigned to the free aldehyde and the larger signal at -38.4 ppm to the hydrate. Upon addition of chymotrypsin, two new signals appeared at -33.8 and -37.1 ppm. The percentage of the total ^{19}F signal area corresponding to the -33.8 ppm signal increases with

increasing E/I ratios. Concomitantly the free aldehyde signal at -37.6 ppm decreases dramatically in intensity relative to the hydrate signal, leading to the same conclusion reached before by Chen et al. (Gorenstein and Shah, 1982).

As the unhydrated aldehyde is the species that is primarily associated, the true K_I for aldehyde association is equal to $K_{I(obs)}/(1+K_h)$, where K_h is the hydration association constant in water.

Although 1 M formaldehyde irreversibly denatures thrombin, simple aldehydes such as formaldehyde and acetaldehyde have no effect at micromolar concentration. This suggests that the inhibition of peptidyl aldehydes are unlikely to be a non-specific reaction of the aldehyde with the enzyme (Westerik and Wolfenden, 1974).

The support for association for the aldehyde analog to chymotrypsin as a hemiacetal (IIA or IIB in fig 2) comes from the observation of association of aldehyde analog of the nonspecific substrate hydrocinamide to chymotrypsin by a cross-saturation NMR technique (Low and Nurse, 1977). This was further supported by similar double resonance experiments but with specific substrate analog, Bz-Pheal, with chymotrypsin (Chen et al., 1979). The chemical shifts of hemiacetal are most clearly defined under conditions of slow exchange on the NMR time scale with the bound aldehyde (EI), which is known from the observed line broadening to be in fast exchange with the free aldehyde. In the case of the chymotrypsin and Ac-Pheal, maximum cross-saturation is observed at the chemical shift of 5.36 ppm. This is the region in which maximum cross-saturation is expected to be observed for the proton of

the hemiacetal formed between the aldehyde and the active site serine residue of chymotrypsin. The chemical shifts and signal line widths for all protons of both Ac-Pheal and Bz-Pheal are essentially unaffected by added (methyl-histidinyl-57)- chymotrypsin. The lack of clear evidences for chemical exchange or cross-saturation in (methyl-histidinyl-57)-chymotrypsin is due to tighter binding of aldehyde to the enzyme and hence a slower dissociation rate which will fall within the NMR slow exchange limit (Chen et al., 1979).

Cross-saturation NMR studies of N-trifluoroacetyl-L-Ala-L-Pheal with chymotrypsin showed broadening of free aldehyde resonance upon addition of chymotrypsin (Gorenstein and Shah, 1982). The detailed analysis identified the enzyme bound hemiacetal as the species which was in slow exchange with the free aldehyde. From the line broadening dependency with pD, it is suggested that a single group on the free enzyme (pK_a=7.9) control the appearance of an alkaline form of the hemiacetal (Wyeth et al., 1980). Fluorine NMR spectra of N-Ac-DL-p-fluoro-Pheal in the presence of chymotrypsin showed separate signals for the hemiacetal complex, the bound aldehyde along with the free aldehyde, and the free hydrate (Gorenstein and Shah, 1982; Shah and Gorenstein, 1983).

The pH dependency of $K_{i(obs)}$ for Bz-Pheal binding to native chymotrypsin between pH-3 and pH-8 exhibit a lack of a significant pH-dependency. This lack of pH dependency suggests that the basic form of charge relay system, which probably participates in the transition state of substrate catalysis (Kraut, 1977), is not required for the high

affinity of the aldehyde Bz-Pheal to chymotrypsin. This result correlates with the high affinity observed for Bz-Pheal to (methyl-histidinyl-57)-chymotrypsin, in which the charge-relay system is modified by methylation of His-57 instead of protonation at pH<7 (Kennedy and Schultz, 1979). Chen et al. (1979) also found a poor affinity between Bz-Pheal and (dehydroalaninyl-195)-chymotrypsin by proflavin displacement. This is because of the lack of ability of Ser-195 to form covalent hemiacetal adduct between Bz-Pheal and Ser-195. In addition the observation that the alcohol derivative (Bz-Pheol), which contains an sp^3 configuration about its C-1 carbon and thus can only associate non-covalently, does not have a high affinity to native chymotrypsin suggests the formation of a covalent hemiacetal adduct for the tightly bound aldehyde (Kennedy and Schultz, 1979).

A reasonable explanation of the pH independence of $K_{i(obs)}$ for Bz-Pheal association is that a two proton transfer occurs on Bz-Pheal association leading to the protonated tetrahedral complex IIB (Fig.2) as the major association species, rather than the anion species IIA. The simplest form of reaction sequence between an aldehyde and a serine protease could be depicted as in equation 5.

$$E + \text{Aldehyde}_{(\text{unhydrated})} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} IA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} IIA \text{ and or } IIB \quad ....(5)$$

where IA, IIA and IIB are species shown in fig-2. Step k_2 may be depicted as a two step proton transfer process in which the His-57 imidazole first abstracts the proton from the r-O of serine-195

generating the γ-O^- nucleophile, and in a second step the solution donates a proton to the oxygen anion on C-1 of the aldehyde resulting in the capture of the stable neutral tetrahedral adduct IIB. As the reverse reaction would be specific base-general acid catalysis and it is kinetically indistinguishable from general base catalysis, both k_2 and k_{-2} have the formate $k_x = k_{(lim)}/(1+H^+/K_a)$. The denominator term cancels out in the ratio k_2/k_{-2} and thus $K_{I(obs)}$ shows no dependency on hydrogen ion concentration. The small variation in $K_{I(obs)}$ with pH may be simply due to small increases in K_s with decreasing pH.

Alternatively, a two proton transfer can occur in a concerted rather than in a stepwise fashion. In a concerted process the His-57 in step k_2 acts both to abstract the -OH proton from the serine and simultaneously donate that proton to the developing oxygen anion of incipient IIA, generating IIB in a single step. In any case, both steps k_2 and k_{-2} require the basic form of the His-57, and the ratio k_2/k_{-2} and thus $K_{I(obs)}$ show no clear pH dependency (Kennedy and Schultz, 1979).

James et al. (1980) described a structure of the microbial enzyme Streptomyces griseus protease-A in its native conformation at 1.8 A^o, and in complexes with Ac-Pro-Ala-Pro-Phe-OH, Ac-Pro-Ala-Pro-Try-OH and Ac-Pro-Ala-Pro-Pheal, at pH 4.1. The resultant electron density maps showed that the peptide aldehyde forms a covalent hemiacetal bond with Ser-195. The distance from the carbonyl carbon of the aldehyde to the γ-O of Ser-195 being 1.73 A^o. The corresponding distances in the protease-peptide complexes are 2.58 A^o and 2.66 ^oA, and continuous electron

density from γ-O to the carbonyl carbon atom of the peptides is absent.

Papain, a cysteine protease has similarities in mechanism to serine proteases in association of peptide aldehydes. Aldehydes, structurally related to the acyl portion of the substrates, are extremely potent competitive inhibitors of papain at pH- 5.5 and 25° C, approximately 1 mole of acetyl-L-phenylalanyl amino acetaldehyde binds per mole of papain, with $K_i = 4.6 * 10^{-8}$ M (Westerik and Wolfenden, 1972). Also it is shown that aldehydes protect the enzyme against inactivation by N-ethylmalemide. It is clear that the aldehydes form stable thiohemiacetals which are similar to the hemiacetals formed in serine proteases.

Thus the reaction of aldehydes with papain occurs in two steps as shown in equation 6 (Mattis et al., 1977),

$$E + A \overset{K_s}{\rightleftharpoons} EA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EA' \qquad \text{.....(6)}$$

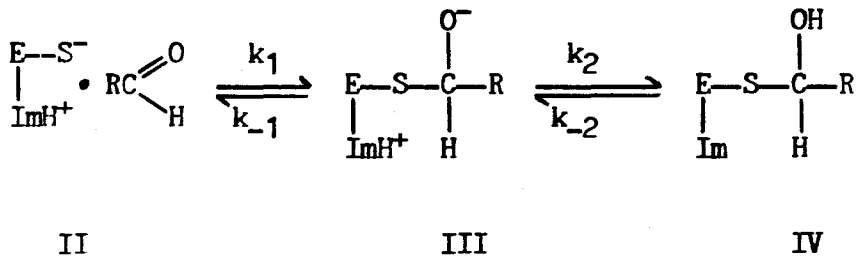
where EA is the Michaelis complex and EA' is suggested to be a covalent, tetrahedral hemithioacetal with the cysteine-25 sulfhydryl. Frankfater and Kuppy (1981) studied the association of Ac-Phe-Glycinal with papain in the pH range 3.5-9.5 at 25° C using intrinsic papain fluorescence to measure aldehyde binding. The limiting values of K_i and the second order association rate constant k_2/K_s, corrected for hydration of Ac-Phe-Glycinal in solution, are 9.05×10^{-10} M and 4.95×10^{7} $M^{-1}s^{-1}$, respectively. The solvent deuterium isotope effect on the rate of association is 0.77, indicative of the participation of the thiolate-

imidazolium ion pair. The mechanism of aldehyde association shown in fig. 5 with papain is proposed. Since it is likely the structure IV is the predominant form of the hemiacetal in solution, a proton transfer must occur in subsequent steps following formation of the Michaelis complex. The value of <3 for the pKa of His-159 in papain-aldehyde adduct may reflect a direct interaction between the N-1 of His-159 and the hydroxyl group of the hemithioacetal. It is argued that the hemithioacetal may be about 10^3 times more stable in papain than in solution. The wide range and diagnostic power of ^{13}C NMR made possible signal assignments for the aldehyde inhibitor-papain complex. Two distinct hemithioacetal forms were observed which can be titrated separately, one of which is more stable than the other at pH 7.0. They appear to be equal amounts of both distereoisomers formed by the creation of the new assymetric carbon generated on hemithioacetal formation. One diastereoisomer bound in a nonproductive mode is in rapid equilibrium with the Michaelis complex (ES) while the other distereoisomer in a productive mode is in slow equilibrium with the Michaelis complex (ES) (see Fig. 6) (Gamcsik et al., 1983).

Thus the formation of a covalent hemiacetal or hemithioacetal adduct between a peptide aldehyde and serine or cysteine protease is supported by various techniques such as stopped-flow spectrophotometry, proton NMR, ^{13}C NMR, fluorine NMR, and X-ray crystallography.

2D. Peptide aldehydes as inhibitors of proteases

As it was noted previously, Umezawa initiated the study of peptide



II III IV

Figure-5. Formation of hemithioacetal by addition of the Cys-25 thiolate of the Papain to the aldehyde carbonyl of peptide aldehyde inhibitors.

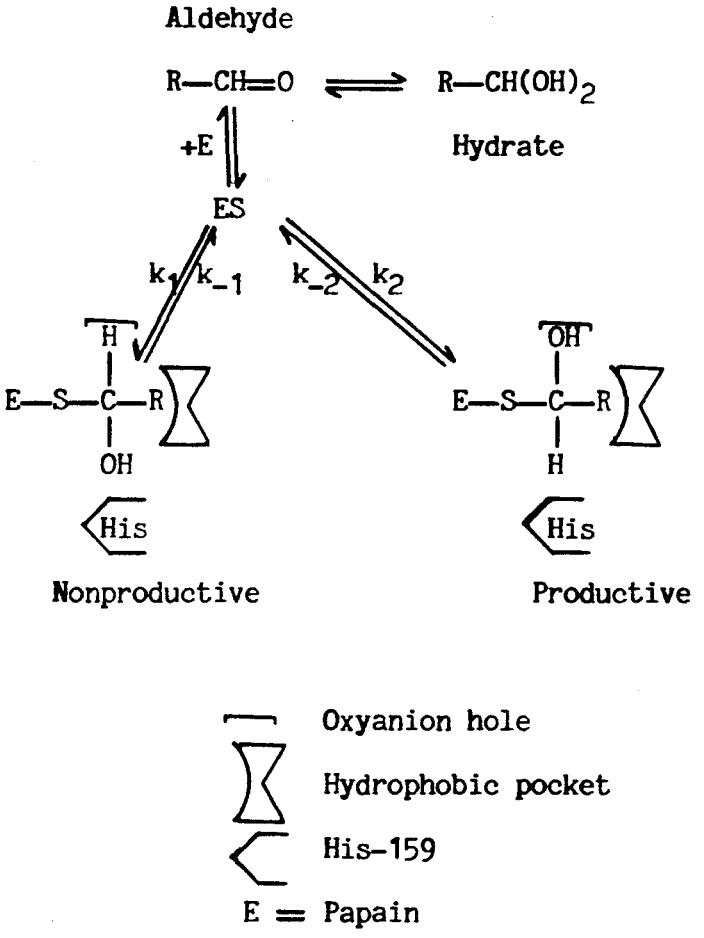
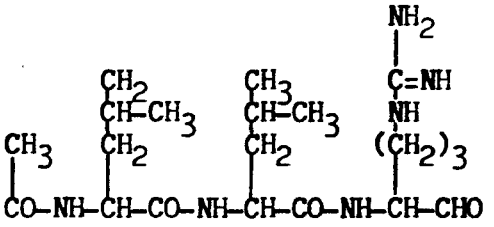


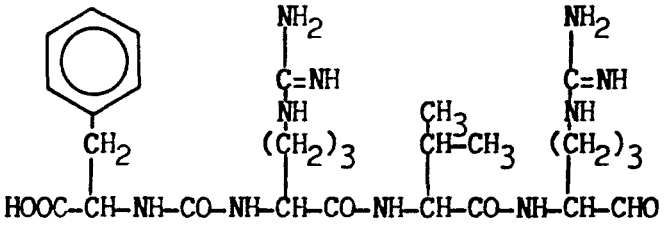
Figure-6. Model of association of aldehyde to papain.

aldehydes produced by microorganisms as inhibitors of protease enzymes in 1965. Since than nearly 50 inhibitors of various mammalian enzymes have been identified in microbial culture filtrates. Their structures have been elucidated and most of them have been chemically synthesized (Umezawa, 1982). Leupeptin, which inhibited plasmin, trypsin, pepsin and cathepsin B, was discovered by testing for plasmin inhibiting activity in Streptomyces culture filtrates (Aoyagi et al., 1969). Two leupeptins were isolated, and their structures were determined to be acetyl or propionyl-L-Leu-L-Leu-L-Argal (Kawamura et al., 1969). Later a number of other leupeptins were identified which contained L-Ile or L-Val instead of L-Leu. NMR studies of leupeptins in aqueous solution indicates that most of leupeptin exists as the hydrated and the hydroxypiperidine forms (Maeda et al., 1971; Schultz and Kozlowski, unpublished data). While leupeptin inhibits trypsin like proteases, it does not inhibit chymotrypsin. The aldehyde group in the argininal moiety is an absolute requirement for inhibiting activity. If it is oxidized to acid or reduced to alcohol, the compound's inhibitory activity disappears.

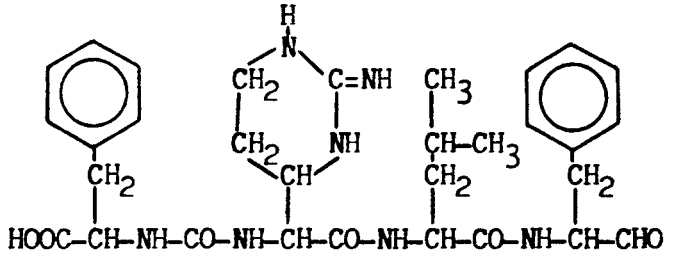
Along with leupeptins other important peptide aldehydes of the microbial origin are antipain, chymostatin and elastinal. Fig 7 shows the chemical structures of these inhibitors. Antipain inhibits papain, trypsin, cathepsin B; chymostatin inhibits chymotrypsin like proteases; and elastinal inhibits pancreatic elastase. Table 5 shows the inhibition constants of leupeptin, elastinal and pepstatin against trypsin, elastase and pepsin, respectively. The biosynthetic pathway of the leupeptins have been elucidated from Actinomycetes and the three



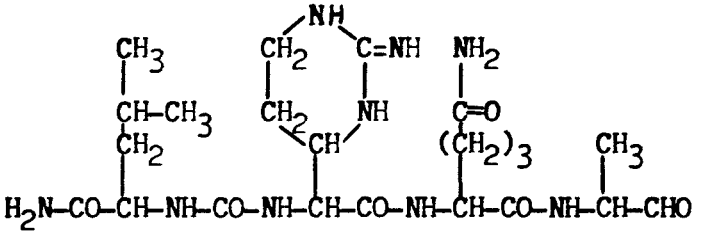
LEUPEPTIN



ANTIPAIN



PAPSTATIN



ELASTINAL

Figure 7. Chemical structures of protease-inhibitors of microbial origin.

Table 6

Kinetic constants of endopeptidase inhibitors

Inhibitor	Enzyme	Substrate	K_m ($\times 10^4$ M)	K_I ($\times 10^7$ M)	Type of inhibition
Leupeptin	Trypsin	TAME	7.1	3.4	Competitive
		BAEE	4.8	1.3	Competitive
Elastinal	Elastase	Ac-$(Ala)_3$-NA	3.2	2.4	Competitive
Pepstatin	Pepsin	Phe-Gly-His-Phe (NO_2)-Phe-Ala Phe-ME	0.4	0.001	Competitive

Umezawa et al., (1983).

important enzymes of the pathway have been isolated. These enzymes are leucine acetyl transferase, leupeptin acid synthetase, and leupeptin acid reductase. The membrane bound leupeptin acid reductase requires ATP and NADH (Umezawa, 1983).

Even though the physiological function of leupeptins and other inhibitors of microbial origin is not clearly known, the possible use of these inhibitors as therapeutic agents has received considerable attention. In addition, these enzyme inhibitors could be useful in the analysis of biological functions and disease processes. Biological activities of leupeptins have been investigated by Aoyagi et al., (1969). Oral administration of leupeptin exhibited an anti-inflammatory effect in edema. Leupeptin also inhibited coagulation in humans and rabbits, but not in dogs and rats. These inhibitors are relatively non toxic (Libby and Goldberg, 1978; Sher et al., 1981). Intra-venous injection of 250 mg/kg and intraperitoneal injection of 1000 mg/kg of antipain caused no deaths in mice. In the case of leupeptin, HD_{50} to rats and rabbits are as follows: rats 125 mg/kg by intravenous injection, >4000 mg/kg by subcutaneous injection and oral administration; rabbits 35mg/kg by intravenous injection, 300mg/kg subcutaneous injections, >1500 mg/kg by oral administration (Aoyagi et al., 1969). The use of leupeptin as possible therapeutic agent in muscular dystrophy, cancer metastasis and number of other diseases have been investigated in animal models (Umezawa,1983; Umezawa,1979). The exceptionally good inhibition constants of these microbial peptide inhibitors have generated a good amount of interest for use in the

inhibition of the important physiological proteases.

Specificity toward a particular enzyme may be obtained by varying amino acid sequence in P_2, P_3 and P_4 position of inhibitors in a C-terminal aldehydic peptide similar in structure to the naturally occurring peptide aldehydes. A number of peptide aldehydes targeted toward specific enzymes have been synthesized and tested for their inhibitory properties. Galpin et al., (1984) synthesized protease inhibitors with the general structure Z-Arg-X-Pheal where X= Leu, Ile or Val. These compounds showed strong inhibitory activity towards chymotrypsin whereas the semicarbazide derivatives of the compounds and showed considerably reduced activity. Renin catalyzes hydrolytic release of angiotensin I from the N-terminal end of angiotensinogen. From prior knowledge of the amino acid sequence around the scissile bond in Szelke's (1982) experiments on angiotensinogen, Kokub et al., (1984) postulated that small peptides of the angiotensin I sequence with leucinal at its C-terminal might inhibit renin. Three different compounds were synthesized and the results obtained show that these aldehyde compounds are highly selective and species specific inhibitors for human and monkey renins. Fehrenz et al., (1984) synthesized a series of tripeptide aldehydes of the type P-A-Y-Xal where P is a α-NH_2 protecting group, A is an aromatic residue, Xal is either leucinal or phenylalaninal and Y is a variable residue. The result showed that these peptide aldehydes are also very specific and potent inhibitors of renin.

Acrosin is an important intracellular endopeptidase of spermatozoa that is exposed to the cell surface at the time of gamete fusion. The

proteinase assists the penetration of spermatozoon through the outer investment of the ovum by proteolytic degradation of the zona pellucida, a glycoprotein layer surrounding the oocyte. The presence of natural and synthetic acrosin inhibitors effectively prevents zona penetration and hence fertilization *in vitro* and *in vivo* (McRoric and Williams, 1974). Consequently, synthesis of acrosin directed inhibitors is highly desirable in view of their potential contraceptive action. Khatri and Stammer (1980) synthesized small N-phosphorylated peptide aldehydes analogous to both leupeptin and phosphoramidon. As phosphoramiradon is an inhibitor of acrolysin, combination of these two structures in to one molecule produced a compound inhibitory to both the enzymes. Borin *et al.*, (1981) synthesized a series of leupeptin analogs R-Lel-Leu-Argininal with variable N-terminal substituents. These modified leupeptins proved to be strong competitive inhibitors of the acrosin from mammalian spermatozoa with inhibition constants in the range of 4.7×10^{-7}M (R=H) to 9.7×10^{-7}M (R=t-Boc). Preliminary data in this study indicated that Boc-leupeptin and F_3Ac-Leupeptin derivatives do not exhibit higher toxicity than the leupeptin.

Specific peptide aldehyde inhibitors have been targeted for a number of enzymes involved in coagulation and fibrinolytic processes. In case of thrombin, the structure of these inhibitors correspond to the thrombin sensitive region of its clotting factor substrates. Bajusz *et al.* (1978) synthesized a series of tripeptide aldehydes, X-Pro-Argal and X-Val-Argal. The molecular excess of inhibitors to enzyme required for doubling the clotting time of fibrinogen in plasma was found to range

over five orders of magnitudes, 6.3 times for D-Phe-Pro-Argal to 45950 times for Gly-Val-Argal. From this observation of D-Phe-Pro-Argal being a potent inhibitor of thrombin, Tremoli et al., (1981,1984) showed that it significantly prolonged the human whole blood recalcification time in vitro and in vivo, as well as plasma thrombin, partial thromboplastin and prothrombin times. In vivo experiments showed a 90% reduction in experimental thrombosis in rats at 6mg/kg intravenous administration. A comparison of the inhibiting activity of Boc-D-Phe-Pro-Argal and D-Phe-Pro-Argal towards thrombin and plasmin indicates that the Boc-derivative inhibits both plasmin and thrombin while the free peptide aldehyde has practically no effect on plasmin (Bajusz et al., 1981).

2E. Affinity chromatography

Thirty years after its introduction (Lerman, 1953), affinity chromatography. (a method of purification based on affinity between two molecules) has become a major means for the purification of biologically active molecules. The purification of proteins by conventional procedures is frequently laborious and incomplete, and the yield are often low. Enzyme purification based on a highly specific biological property - strong reversible association with specific substrates or inhibitors - has received major attention (Cuatrecasa, Wilchek and Anfinsen, 1968). Despite the enormous expansion of the field and many enzymes that have been purified, the directions for the investigation of methods have not changed markedley. The component attached to the solid matrix support is called a ligand. The attachment may be made by

physical adsorption or by a covalent bond. The covalent attachment is far superior and should be used whenever possible to prevent leakage of the ligands. In affinity chromatography of an enzyme, the enzyme to be purified is passed through a column containing a crossed linked polymer or gel to which a specific inhibitor, cofactor, substrate analog or an antibody specific to the enzyme has been covalently attached. All proteins, without substantial affinity for the bond ligand will pass directly through the column, where as the enzyme that recognizes the ligand will be retarded or stay bound, depending upon its affinity constant. Elution of the bound enzyme is readily achieved by changing such parameters as ionic strength, pH or by addition of a competitive inhibitor in the eluting solution. In this dissertation we introduce additional method for removing a bound enzyme by reversible chemical modification of the ligand thereby, effectively reducing the concentration of active ligand (Patel and Schultz,1982; Patel et al., 1983).

The success of the technique depends largely on mimicking the interaction between the two components that occurs when both compounds are in free solution. The successful application of the method requires that the adsorbent have a number of favourable characteristics. The unsubstituted matrix or gel should show minimal interaction with proteins, in general, both before and after coupling to the specific binding groups. It also must form a loose, porous network that permit easy entry and exit of macromolecules and have favourable flow properties. The chemical structure of the support must permit the

convenient and extensive attachment of ligands under relatively mild conditions, and through chemical bonds that are stable to the conditions of adsorption and elution. Finally, the ligand which is critical in the interaction must be sufficiently distant from the solid matrix to minimize steric interference with the binding process. This could be achieved through a spacer arm between ligand and matrix.

Enzymes represent the largest group of macromolecules purified by affinity chromatography. The purification of enzymes by affinity chromatography can be divided mainly in to three categories. The first category includes ligands designed for the purification of a wide range of enzymes by using general ligands like cofactors. The most commonly used cofactors are NAD, NADH, ATP and Co-A or their derivatives. The second category includes ligands directed at enzymes of narrow specificity, i.e. enzymes purified with the aid of specific inhibitors or substrates. In this case the specificity is much higher for the enzyme for which the column was tailored. At times, specific columns serve also for the purification of a family of enzymes having similar specificity. e.g. benzamidine-Sepharose for trypsin like proteases. The third category includes ligands designed on a knowledge of a part of the three dimensional structure of the enzyme to be purified. For this method either polyclonal or monoclonal antibodies are used as ligands. Unless there is cross-reactivity, these affinity resins are highly selective for a particular enzyme. In any case the degree of complementarity between interacting molecular surfaces or regions of the constituents of a complex determines the magnitude of attraction, and

thus the strength of a complex.

An agarose is the predominant choice as an innert matrix in affinity chromatography of enzymes, and cyanogen bromide as an activating agent. Fig 8 shows mechanism of activation of Sepharose by CNBr and subsequent coupling of the ligand. Detailed analytical data of freshly activated Sepharose showed that 60–85% of the resins total coupling capacity is due to the formation of the cyanate esters (cyanate ester 80%, imidocarbamates 20%) (Kohn, 1981). Once the mechanism of activation was established, it opened up new ways to develop more efficient activation procedures and these are extensively reviewed by Wilcheck et al., (1984). Among these, organic sulfonyl chlorides provide a coupling procedure which give a high yield of stable linkages at neutral pH. Organic sulfonyl chlorides such as p-toluene sulfonyl chloride (tosyl chloride) and 2,2,2-trifluoroethane sulfonyl chloride (tresyl chloride), can be used to convert hydroxy groups into good leaving group (sulfonates) that, on reaction with nucleophiles, allow stable linkages to be formed between the nucleophile and the initial hydroxyl group carrying carbon. These reagents appear to be suitable for the immobilization of enzymes and affinity ligands to support bearing a hydroxyl group, such as agarose, cellulose, glycophase glass and others. The activation and coupling of the ligands to the support are though to involve the following steps (Nilsson and Mosbach, 1984).

Activation:

$$\text{Support-CH}_2\text{OH} + \text{RSO}_2\text{Cl} \longrightarrow \text{Support-CH}_2\text{OSO}_2\text{-R}$$

Coupling: e.g. NH_2- nucleophile

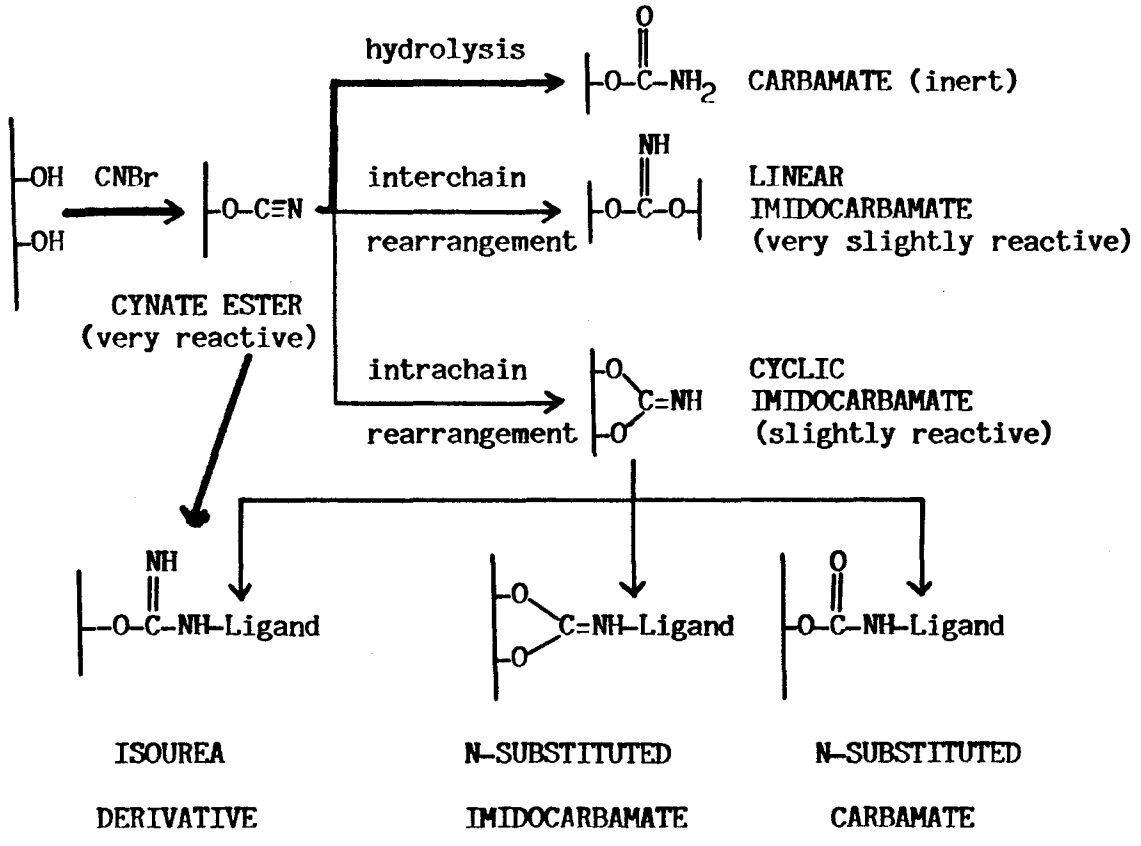


Figure-8. Mechanism of activation of Sepharose by CNBr and subsequent coupling of ligand. Heavy lines indicate major reaction pathways.

(Wilchek et al., 1984)

$$\text{Support-}CH_2OSO_2\text{-R} + H_2N\text{-ligand} \longrightarrow \text{Support-}CH_2\text{-NH-ligand}.$$

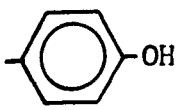
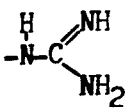
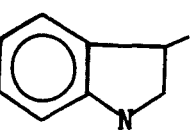
The influence of R group on the relative reactivity of sulfonates with organic molecules containing hydroxyl groups was found to be 1:100:4000 with R= $-C_6H_4CH_3$, $-CH_2CF_3$ and $-CF_3$ respectively. Thiols and primary amino groups are the most reactive nucleophiles with sulfonate esters on gels. Thiols have the highest reactivity. Imidazole and tyrosine hydroxyl groups can also displace the esters. This broad range of reactive nucleophiles can be a great advantage when a very tight binding between protein and support is desire, i.e. for stabilization or freezing of the protein conformation.

Among the synthetic carriers, hydrophylic cross-linked polyacrylic polymers are of great interest. The distinct advantages of these polymers are the far superior mechanical stability compared to agaroses and their innertness to microbial degradation. In addition they have a higher stability towards acids, bases and organic solvents. The degree of porosity and chemical composition can be achieved by selective co-polymerization or by chemical modification of preformed polymers. Various synthetic polymers based on acryamide or acrylate and their use to immobilize enzymes have been reviewed by Mosbach (1977).

Certain amino acid residues in proteins are amenable to covalent reaction for immobilization of proteins or peptides on innert supports. Table 7 shows eleven amino acids whose residues could be considered reactive enough to participate in covalent coupling to a carrier. Of the amino acids listed in Table 7, the most frequently used functional groups are $-NH_2$, -SH and -COOH.

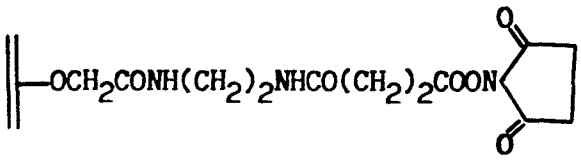
Table 7

Reactive residues of proteins

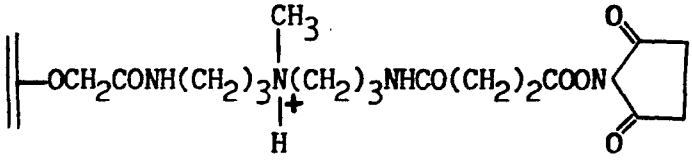
$-NH_2$	-Amino of Lysine and N-terminus amino group
-SH	Sulfhydryl of cysteine
-COOH	Carboxyl of Aspartate and Glutamate and C-terminus carboxyl groups
	Phenolic of Tyrosine
	Guanidino of Arginine
	Imidazole of Histidine
—S–S—	Disulfide of Cysteine
	Indole of Tryptophan
CH_3–S—	Thioether of Methionine
$-CH_2$–OH	Hydroxyl of Serine and Threonine

The most widely used procedure for covalent coupling of proteins to a support has been the one devised by Axen et al., (1967). The polysaccharide (Sepharose) is activated with cyanogen bromide and the activated sepharose is coupled to an amino group on the protein to yield the immobilized product. Currently a number of sepharose derivatives with desired spacer arm and appropriate functional group to covalently bind to specific ligands are commercially available. The spacer arms ranges from four carbon atom to fifteen carbon atoms long, predominantly either with amino or carboxylic terminal. The reactive derivatives of spacer arm equipped sepharoses are also commercially available. Among these derivatives, N-hydroxy succinamide ester activated sepharoses are very useful for rapid, high efficiency coupling for all ligands that have a primary amino group.

Affigel-10 and Affigel-15 (Bio Rad) are N-hydroxy succinimide esters of a derivatized cross-linked beaded agarose gel support. Both couple to ligands containing primary amino group spontaneously in aqueous or non-aqueous solutions. Figure 9 shows the chemical structures of Affigel-10, Affigel-15, and the coupling reaction of the Affigels with the ligand containing a free amino groups. Affigel-10, which contains a neutral 10-atom spacer arm, has been used to couple a wide variety of ligands in affinity chromatography. Affigel-15 contains a cationic charge in its 15 atom spacer arm, which significantly enhances coupling efficiency for acidic proteins at physiological pH. The N-hydroxy succinamide active ester method is highly selective for primary amino groups of ligands between pH-6.5 to 8.5. Proteins can be coupled



Affi-Gel 10



Affi-Gel 15

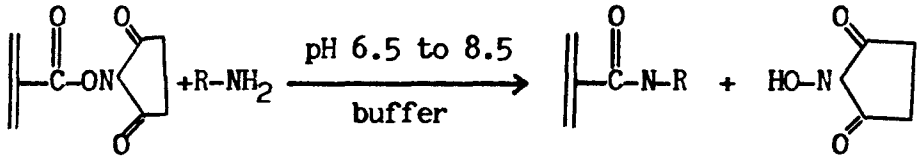


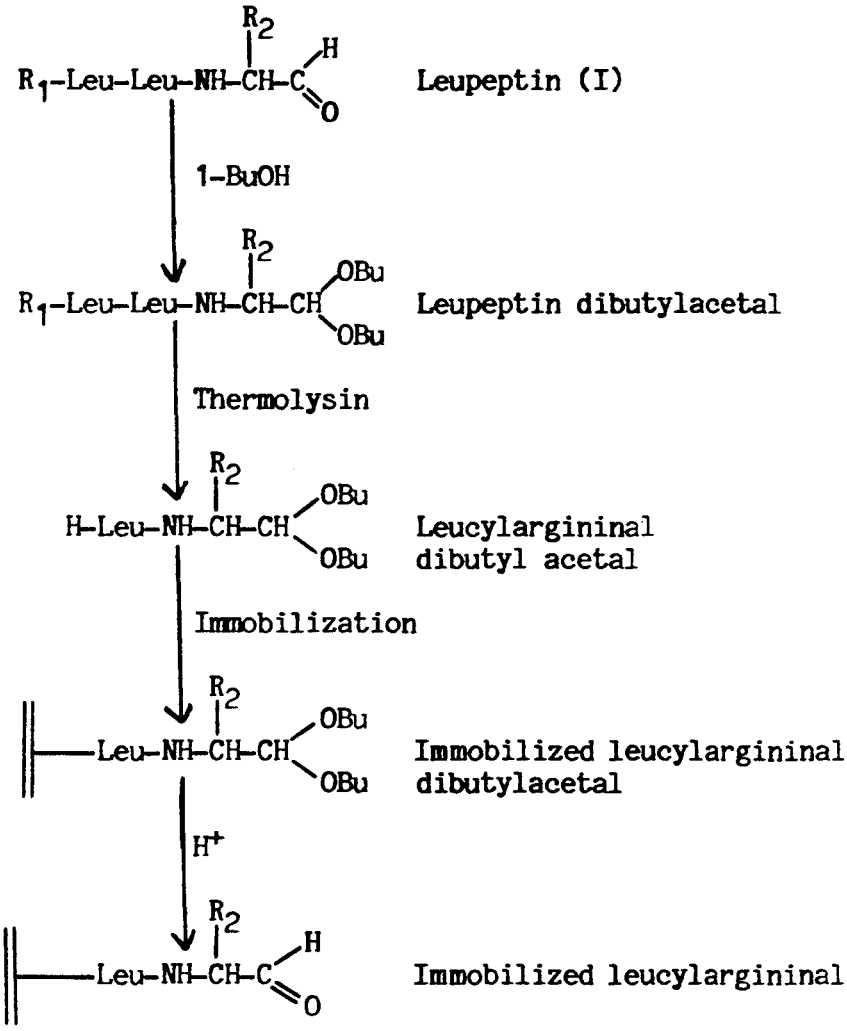
Figure-9. Coupling reaction of Affi-Gels 10 and 15 with ligand containing free amino groups.

within four hours at 4°C. Since the coupling is through an amide bond and the spacer arm is linked through an ether bound to the matrix, an excellent resistance to urea, guanidine.HCl, heat, solvents, acids and bases (pH 2 to 11) virtually eliminates ligand leakage during storage and use (Conn et al., 1981). Both Affigel-10 and Affigel-15 are used during this investigation to prepare affinity resins. The exact coupling strategy and conditions will be discussed in a later section.

Initially lysine and benzamidine sepharose affinity resins were prepared for purification of trypsin like proteases. A typical procedure involved activation of sepharose with CNBr and coupling of either lysine or p-amino benzamidine with or without a spacer arm (Hatton and Recoeczi, 1976). The solution containing enzyme is charged onto a column of benzamidine Sepharose in low ionic strength (50 mM NaCl buffer) and subsequently washed with higher ionic strength NaCl-buffer in order to wash off nonspecifically bound proteins. Elution of the enzyme is done under competitive conditions, i.e. the eluent contains either p-amino benzamidine or other protease inhibitor (Anderson et al., 1977).

In affinity chromatography of trypsin like proteases, the positively charged aminidinium or guanidinium group is required for primary specificity. These groups must be excluded from the covalent bonding of ligand to matrix. In the case of peptidyl aldehydes as ligands, the aldehydic group is also essential for the formation of a reversible covalent bond with the protease. This leaves only the N-terminal α-NH_2 group for covalent bonding with the matrix. Since the N-terminal α-amino group is acetylated by either acetyl or propionyl group

in the leupeptins, in order to use leupeptin as a ligand for affinity adsorbents, appropriate modification is required. Ishii and Kasai (1980) used thermolysin to hydrolyze the bond between two leucines in leupeptin to expose an α-amino group. Figure 10 shows the outlines of the preparation of the affinity resin. The aldehyde group was protected as dibutyl acetal and deprotected after immobilization. As expected, these affinity resins showed strong affinity for trypsin-like proteases. In addition, the resins completely lost their binding ability, if the aldehyde group was converted to the corresponding alcohol by treatment with $NaBH_4$. The argininal resin bound enzymes like Streptomyces griseus trypsin, plasmin, kallikrein, urokinase and clostripain. Ishii could not elute the enzymes by the conventional methods, such as by an elevation of ionic strength, lowering of pH, or addition of denaturents. Successful elution was achieved only by the addition of 0.02 mM leupeptin to the eluting buffer at very slow rate, after incubating overnight. The eluted enzyme was not active because it was complexed with leupeptin and the activity was only restored after inactivating leupeptin by $NaBH_4$ (2mM). To regain enzyme activity it was important that the enzyme tolerate such reductive treatment. Nishikata et al., (1981) prepared Sepharose-Gly-Gly-Argal, and showed it to be a strong affinity resin for bovine trypsin. The enzyme was eluted with 8 M urea or 1mM leupeptin in buffer at a flow rate of 6 ml/hr. While tosyl-lysine chloromethyl ketone-trypsin and (dehydroalanine-195)-trypsin failed to bind the resin, carbomoyl methylated (His-46) trypsin bound to the resin and could be eluted with 50mM HCl/1M NaCl.



R_1: Acetyl or propionyl

R_2: $-(CH_2)_3-NH-C(=NH)-NH_2$

Figure-10. Preparation of affinity resin using Leu-Argininal as ligand.

Ishii et al., (1980).

Anderson and Wolfenden (1980) have described a technique they named "transition state affinity jump" chromatography. In this procedure, a double selection method for isolation of active enzyme, a substrate analog is used as a ligand and a transition- state analog is placed in the solution of eluent. Thus a mixture of native chymotrypsin and dehydroalanine-(195)-chymotrypsin was applied to a column containing D-tryptophan methyl ester as an affinity ligand. While both forms of chymotrypsin were retained, catalytically active enzyme selectively eluted with the peptide aldehyde chymostatin leaving catalytically inactive dehydroalanine-chymotrypsin bound to the column.

Nishikata (1983) also prepared an affinity resin using Gly-Gly-Pheal which is a synthetic analog of chymostatin as a transition -state analog for chymotrypsin. Bovine chymotrypsin and Streptomyces griseus protease B showed strong affinity toward the adsorbent. The dissociation constant, K_d, of the enzyme-immobilized ligand complexes were determined by frontal gel chromatography. The value of dissociation constants varied from 13×10^{-6}M at pH-6.5 to 1.7×10^{-6}M at pH-8.2 for chymotrypsin. The author tried to validate the results by comparing the dissociation constant obtained with Ac-Val-Val-Pheal at pH 7.7 (3.8×10^{-6}M), and that of Ac-Pro-Ala-Pro-Pheal to be 3×10^{-6}M at pH-8.0, with the argument that Ac-Val-Val-Pheal and the ligand Gly-Gly-Pheal have similar structures. The difference in size and hydrophobic nature of side chains of glycine and valine should be pointed out here, and it could be very significant in terms of interaction with the enzyme in S_2 and S_3 subsites.

Proposed transition state analogs other than peptide aldehydes also

have been used as affinity ligands. Pohl et al., (1984) used Val-D-Leu-Pro-Phe-Phe-Val-D-Leu as an affinity ligand, which is a transition-state analog inhibitor of aspartate proteinases of the pepsin type. The affinity adsorbent bound human, porcine and chicken pepsin, human gastrin and bovine cathepsin D over the pH range 2 to 5. The elution of enzyme was achieved by a low ionic strength buffer at higher pH containing 20% dioxane, in yields exceeding 70%. The resin however, failed to separate human pepsin from human gastrin. A more specific inhibitor like H-77 (D-His-Pro-Phe-His-Leu-^{R}- Leu-Val-Tyr, where R is a reduced isosteric bond -CH_2-NH-) of acid-proteases, was used as ligand for purification of human kidney renin by McIntire et al., (1983). With this affinity adsorbent, human renin was isolated from crude extract of kidney cortex with 5500 fold purification and 76% yield. The purified enzyme was free of non-specific acid-proteases and was stable at pH 6.8 and -20^{o}C over a period of several weeks.

2F. Plasminogen activator

The examples of plasminogen activator and thrombin are selected in this dissertation to develop affinity adsorbents. Both of these enzymes are trypsin-like proteases and have important roles in fibrinolytic and coagulation processes. Both plasminogen activator and thrombin resemble trypsin and plasmin in their activities towards synthetic substrates, but there are differences in their relative activities which depend upon the particular substrate on which they act (Table 4). However,

plasminogen activator and thrombin are much more selective in their specificity toward proteins. There are several different types of plasminogen activators, including tissue activator of plasminogen, vascular plasminogen activator, blood plasminogen activator and urokinase. Tissue activator of plasminogen may be identical or similar to blood plasminogen activator, but different from urokinase which is isolated from urine or kidney cell cultures. Tissue plasminogen activators are serine proteases with a molecular weight of approximately 66,000 and composed of one polypeptide chain. By limited proteolytic action, this one-chain activator is converted in to a two-chain activator linked by disulfide bonds. Both the one chain and two chain plasminogen activator have similar activity in clot lysing systems, but different specificities toward synthetic substrates (Wallen *et al.*, 1981). Urinary plasminogen activator was described in 1951 by Williams and in the following year was named urokinase. There are two forms of urokinase, one with M_w=54,000 and a smaller form with M_w=34,000. Both high and low molecular weight urokinase behave similarly in an *in vitro* whole blood thrombolytic perfusion system (Greenwald and Chandler, 1983). It has been shown that urokinase is much more selective than plasmin in its action on proteins, plasminogen being the only substrate against which urokinase shows activity. Robins *et al.*, (1967) showed that during the activation of plasminogen to plasmin, a single bond Arg^{560}-Val^{561} is cleaved by urokinase.

The mechanism of proteolysis of a single-chain plasminogen molecule to two-chain disulfide-linked plasmin molecule by urokinase is by the

following steps (Violand and Castellino, 1976; Summaria et al., 1975). In the first step urokinase catalyzes cleavage of Arg^{650}-Val^{561} peptide bond forming two chains linked through a disulfide bond. In the second step the Lys^{76}-Lys^{77} peptide bond is hydrolyzed by autolytic cleavage. With this hydrolysis, a whole 1 to 77 N-terminal peptide is removed. The resultant plasmin molecule with M_w=75,000 to 81,000 (Robbins and Summaria, 1976), has a broad trypsin-like specificity towards proteins.

In normal cells plasminogen activator synthesis can be modulated by physiological concentrations of hormones or other biologically active compounds (Reich, 1978; Weinstein, 1978). Transformation to neoplastic cells may be correlated with increased plasminogen activator synthesis. This circumstantial association has been explored by the researchers who are interested in the biology of the malignant cells. Most studies indicate increased synthesis of plasminogen activator in malignant cells compared to normal controls. Because of plasminogen activator's association with neoplasia, important purification techniques from culture media will be reviewed.

Goldfarb and Quigley (1980) isolated plasminogen activator (urokinase) from culture supernatents of Rous sarcoma virus transformed chick embryo fibroblasts treated with the tumor promoter phorbol-12-myristate-13-acetate (PMA) by multiple affinity chromatography. Plasminogen activator was adsorbed on to p-aminobenzamidine-CH Sepharose-B at pH 7.0. Following adsorption the resin was washed with buffer containing Triton X-100. The enzyme was eluted with buffer containing 0.5 M benzamidine at pH 4.0. All the fractions were dialyzed

to remove benzamidine and the fractions containing enzyme were pooled and re-adsorbed on benzamidine adsorbent at pH 7.0. The enzyme was eluted and fractions were dialyzed to remove benzamidine as previously described. Active fractions, partially purified and concentrated by two rounds of affinity chromatography were subjected to a molecular sieving gel (G-100 Sephadex) at 4°C. Plasminogen activator containing fractions from gel-filtration were dialyzed and applied to cation exchange resin (sulfopropyl-Sephadex C-25) at 4°C. The enzyme was eluted by stepwise increase of ammonium sulfate concentration and each fraction was assayed for protein and enzyme concentration. The final yield of enzyme was 43%. The enzyme activity profile demonstrated a major plasminogen activator activity of 46,000-48,000 daltons and a minor activity of 23,000-26,000 daltons.

Dano et al., (1980) and Maroti et al., (1982) also reported purification procedure of plasminogen activator from mouse cells transformed by an oncogenic viruses using an affinity adsorbent containing p-aminobenzamidine as the ligand. In the procedure, serum free media was brought to pH 5.5 and passed over p-aminobenzamidine-aminocaproyl-Sepharose. After washing the column with 0.5 M NaCl, the proteins were eluted with buffer containing 0.9 M benzamidine at pH 7.4. The benzamidine was removed by dialysis against 10 mM Tris-HCl, and pH 7.4/1 mM zinc acetate. The proteins were recovered as precipitates by centrifugation at 12,000xg for 30 min. The proteins were extracted into 100 mM glycine-HCl, pH 2.2/1 mM zinc acetate. The clear solution was put over a QAE-Sephadex A-50 column to further purify. The fractions

containing enzyme activity were subjected to preparative gel electrophoresis followed by elution with buffer and then dialysis. These types of procedures are laborious and low yielding.

Since benzamidine-Sepharose affinity is a general purpose affinity adsorbent for trypsin-like proteases, more specific systems for purification of urokinase were exploited using one of the properties of urokinase, which is to bind to the fibrin clot. The gel-like consistency of fibrin is not practical for the purpose of purification, but fibrin deposited on celite was found suitable for affinity chromatography (Husain et al., 1981). The procedure involves adsorption of vascular plasminogen activator from plasma onto a fibrin/celite matrix, and the elution of plasminogen activator with a buffer containing 0.2 M arginine. The fractions containing active protease were subject to gel-filtration through a Sephadex G-100 column. This high fibrin affinity plasminogen activator had a low specific activity of 500 U/mg protein, compared to tissue or urine plasminogen activators (100,000 to 200,000 U/mg).

Immunological reactivity is highly specific and the binding constants are very high between antigen and antibody. Wallen et al., (1983) reported a three step purification procedure combining the principles of immunospecificity, affinity for arginine and gel-filtration,in the purification of melanoma cell tissue type plasminogen activator. The first step was the adsorption onto an antibody-resin. The second step involved chromatography over arginine-sepharose. The third step was a gel-filtration on Sephadex G-150. The enzyme was eluted off

the antibody column with a strong denaturant, 3M KSCN, guanidinium chloride (0.3 M).

A slight variation of the same procedure was utilized by Booyse et al. (1984) in the purification of urokinase type plasminogen activator from cultured human endothelial cells. In their procedure, the enzyme was isolated first by p-aminobenzamidine agarose affinity chromatography followed by gel-filtration on Sephacryl-200. The final step involved adsorption on anti-urokinase IgG-Sepharose CL-4B. The elution of the enzyme was accomplished with 4 M Guanidine-HCl at pH 6.5 buffer containing Triton X-100. The overall yield was 49.6% with specific activity 94,000 U/mg protein.

Proteases are commonly synthesized in inactive proenzyme forms and are activated by a modulator or by another protease. Anderson et al., (1984) showed that tissue-type plasminogen activator from human melanoma cells is synthesized in an inactive proenzyme form with a single chain (M_w-66,000). A single step purification procedure using monoclonal antibody affinity resin is described. Salerno et al., (1984) reported the identification of the single-chain prourokinase precursor with monoclonal antibodies from rabbit reticulocyte cell free system. This prourokinase appears to be the precursor of both the A and B chains of human urinary urokinase.

Human tissue type plasminogen activator has been obtained by expression of its cloned gene in a mammalian cell system. Thus, plasminogen activator is currently synthesized by recombinant DNA technology for commercial purposes and an easy purification technique is

highly desirable.Highly purified plasminogen activator promises practical approach for coronary thrombosis in patients with acute myocardial infarction (Van De Werf et al., 1984).

Tripeptide substrates for plasminogen activators shows its specificity for synthetic peptide sequences. Val-Gly-Arg and Glu-Gly-Arg tripeptides are specific toward both tissue-type plasminogen activator and urokinase. In addition to these two tripeptides, Gly-Gly-Arg and p-Glu-Gly-Arg also show a high specificity towards both types of plasminogen activators (Cleason and Aurell, 1981). The aldehyde derivative of these tripeptides should also be highly specific and potent inhibitors of plasminogen activator. These tripeptide aldehydes could be used as ligands for preparation of affinity adsorbent for a single step purification plasminogen activators. Among all the tripeptide aldehydes, Gly-Gly-Argal would be the easiest to synthesize. The urokinase purified using this affinity adsorbent will be expected to be free from other contaminating proteases and proteins.

2G. Thrombin

Thrombin is a proteolytic enzyme homologous to trypsin, chymotrypsin and elastase with M_w-34,000. The biology and chemistry of thrombin has been extensively studied over the last fifty years. Major attention has been paid to the kinetics and the inhibition of the proteolytic action of thrombin on fibrinogen. Thrombin is the enzyme involved in the final step of the coagulation of mammalian blood. In this step four Arg-Gly bonds are cleaved releasing four fibrinopeptides. While it is a highly specific protease, it has a wide specificity

towards small synthetic substrates. It is clear that thrombin exists in multiple covalent forms which have different specific activities towards fibrinogen and ester substrates. These forms are referred to as α-thrombin, β-thrombin (fig.11). β-thrombin is formed from α-thrombin by the extensive degradation of the A chain and excision of a small fragment containing carbohydrate, from B-chain. This results in a three-chain structure. γ-Thrombin arises from further degradation of the B_2 chain. Fig 11 shows structures of bovine α, β and γ-thrombin (Lundbald, 1976). The relative activities of α-thrombin and β-thrombin toward fibrinogen is 21.6 to 1.0 indicating a major contribution of the α-form towards clotting activity. However, their relative activity toward the small synthetic substrates Bz-ArgNaN is only 5.25 to 1.0 (Workman and Lundblad, 1977).

Even though there are a number of known protein substrates for thrombin, the kinetics and chemistry of fibrinogen-fibrin conversion by thrombin is the most extensively studied. Fig 12 shows a primary structure model of human fibrinogen. In the Figure, the chains have been aligned according to homology, the N-termini pointing towards the middle. The connecting links represent disulfide bridges, the arrows point to the thrombin cleavage sites and the diamonds point to carbohydrate side chains. Fibrinopeptides A and B are peptide fragments released from amino-terminal portions of fibrinogen upon thrombin catalyzed gelation. The physiological role of these peptides in the protein is to prevent spontaneous polymerization of the fibrinogens. The amino acid sequence and length of fibrinopeptides A and B is species

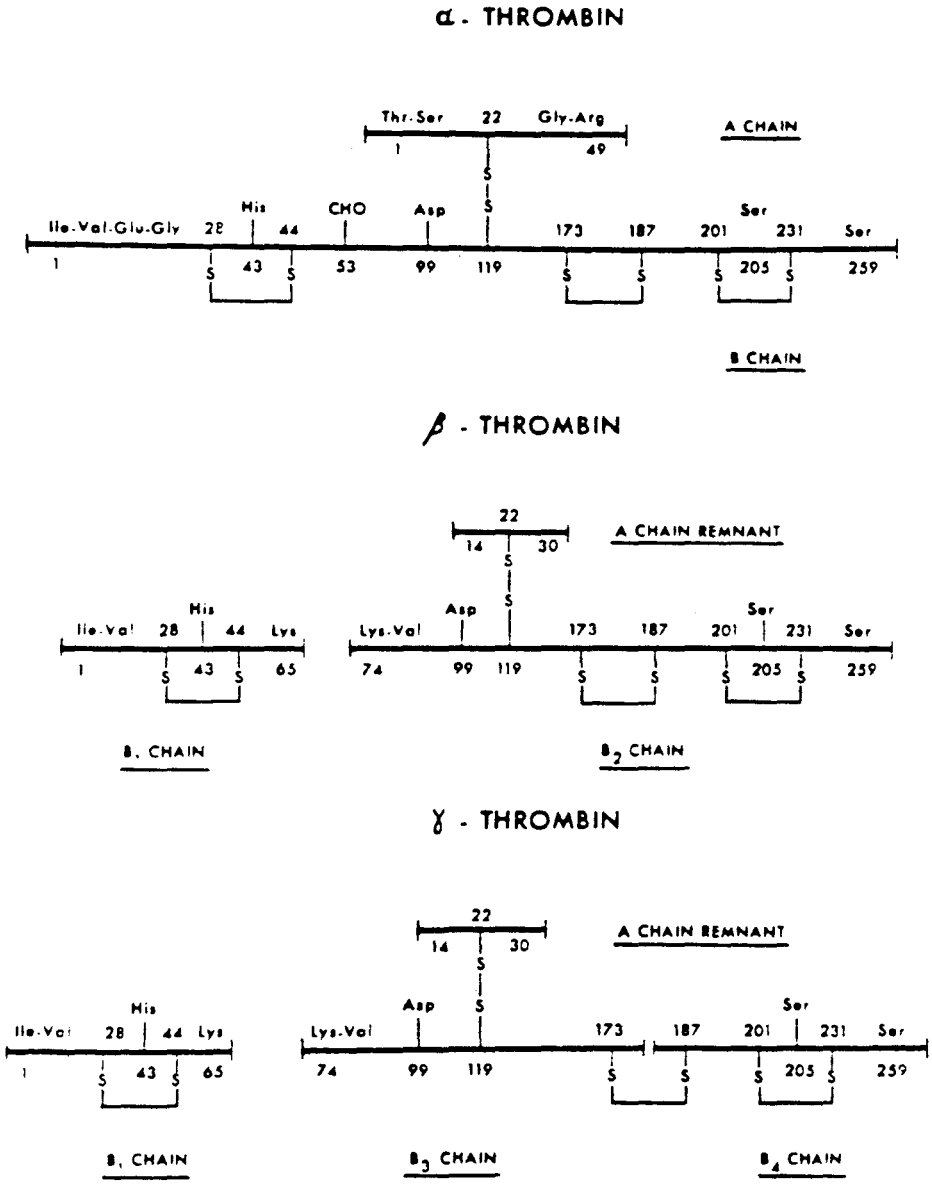


Figure 11. Diagrammatic structures of bovine thrombins.

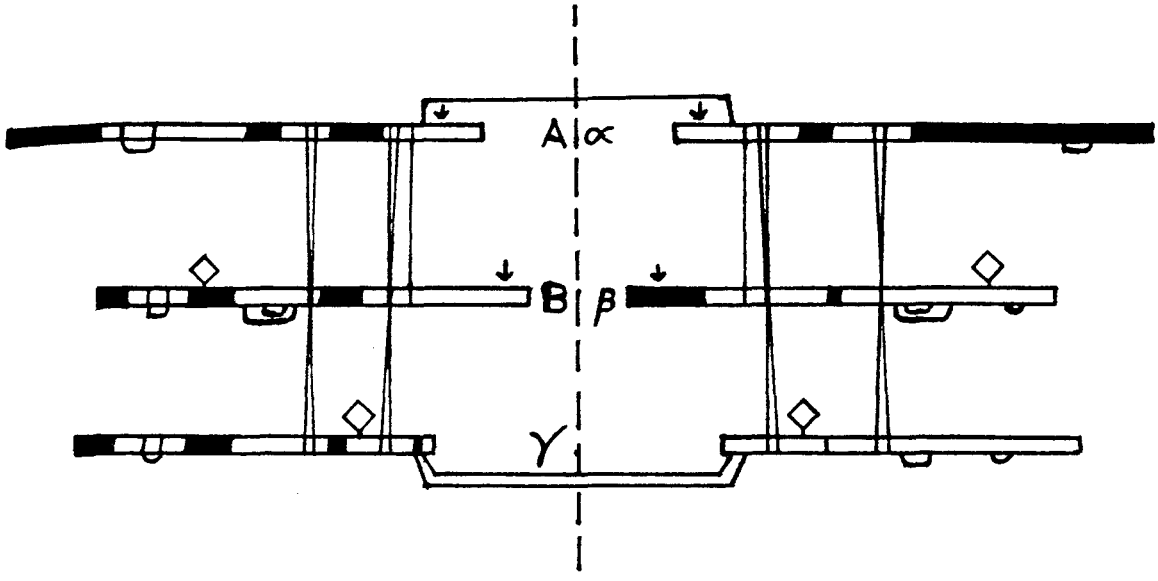


Fig. 12. Structure of Fibrinogen

dependent, indicating they can serve as markers for studying phylogenic relationships and the evolutionary process of primates on a molecular level (Nakamura et al., 1983). Fig 13 shows the amino acid sequences of human and bovine fibrinopeptide-A (FPA) and fibrinopeptide-B (FPB). The kinetics of the released of the fibrinopeptides show an initial and rapid released of FPA, followed after a lag phase by the release of FPB. The A and B chains behave independently in their competition for thrombin. Phosphorylation of the Ser-3 of the A chain appears to increase the rate of its release, due to enhanced binding of thrombin (Hanna and Scheraga, 1984).

Initially thrombin was purified by ion-exchange chromatography. Baughman and Waugh (1967) used a combination of DEAE-Cellulose chromatography, phosphocellulose chromatography and gel-filtration to obtain a preparation with a specific activity of 2100 NIH U/mg for bovine thrombin. Affinity chromatography for purification of thrombin using p-aminobenzamidine as a ligand has been reported (Schmer, 1972; Hixon, 1973). The benzamidine agarose is a potent affinity adsorbent for thrombin. From crude thrombin, a 16-fold purification was obtained. The preparation also contained small amounts of plasmin and had to be further purified on SE-sephadex column to obtained 2000 NIH U/mg -α- thrombin with an overall yield of 50% to 60%. Electrophoresis showed a major and a minor band on a SDS-gel. In the elution of thrombin from the benzamidine resin, 0.05M p-amino-benzamidine in Tris-KCl buffer at pH-8.0 was utilized (Hixon and Nishikawa, 1973).

Heparin acts as an anti-coagulant on binding to thrombin at a site

Fibrinopeptide-A (FPA)

Human:- Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-GLy-Val-Arg.

Bovine:- Glu-Asp-Gly-Ser-Asp-Pro-Pro-Ser-Gly-Asp-Phe-Leu-Thr-Glu-Gly-Gly-Gly-Val-Arg.

Fibrinopeptide-B (FPB)

Human:- p-Glu-Gly-Val-Asn-Asp-Asn-Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg.

Bovine:- pcA-Phe-Pro-Thr-Asp-Tyr-Asp-Glu-Gly-Gln-Asp-Asp-Arg-Pro-Lys-Val-Gly-Leu-Gly-Ala-Arg.

Figure-13. Amino acid sequence of fibrinopeptides.

other than the catalytic site. This property of heparin was exploited for preparation of an affinity adsorbent for thrombin (Miller-Anderson, 1980; Funahashi, 1982). Three different types of heparin-Sepharoses can be prepared. First, by the coupling of carboxyl groups of heparin with amino-Sepharose. Secondly by the coupling amino groups of heparin with carboxyl-Sepharose, and, thirdly, by the reductive amination of heparin and amino-Sepharose with sodium cyanoborohydride. Among these three types of heparin resins, the third type was found to be the most efficient affinity adsorbent for thrombin and anti-thrombin III (Funahashi, 1982).

Miller-Anderson (1980) combined all three techniques, ion-exchange chromatography, gel-filtration and affinity chromatography to prepare a human thrombin standard. In the procedure, a prothrombin complex was activated and purified on a CG-50 ion-exchange column followed by chromatography on SP-Sephadex and then chromatography on heparin-Sepharose. The resulting thrombin had a specific activity of 2400 to 2850 IU/mg.

These methods are lengthy and if used alone, are not exclusive for thrombin. Heparin has affinity not only for thrombin, but also for antithrombin III, lipoprotein lipase, collagenase, DNA polymerase and number of other biomolecules. Benzamidine-Sepharoses are specific for all trypsin-like proteases and bind thrombin, urokinase, plasmin, trypsin, etc. A single step purification affinity adsorbent could be prepared using a specific active site inhibitor of thrombin as a ligand. As discussed previously Phe-Pro-Argal offers the required

characteristics for this purpose. Accordingly, in this dissertation an Affi-gel-Phe-Pro-Argal resin will be prepared and tested for its utility as an affinity adsorbent for the purification of thrombin.

The amino acids in fibrinopeptides from many species differ in the P_2 and P_3 positions. The Phe at position P_9 is conserved, suggesting the importance of Phe in P_9 position for thrombin action. Marsh and Scheraga (1982) synthesized the fibrinopeptide sequences Ac-Phe-Leu-Ala-Glu-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$ (F-6) and Ac-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-$NHCH_3$(F-7) and the rates of hydrolysis of Arg-Gly bond in these peptides by thrombin were measured. The rate for the Phe containing peptide F-6 was found to be significantly larger than that for F-7. Indeed, F-7 is such a poor substrate that it was not possible to obtained Michaelis-Menten parameters. This and previous work demonstrate that Phe-Leu at position P_9-P_8 is very important for thrombin binding to its substrate. The work was extended further to investigate the importance of Asp at the P_{10} position, which indicates that Asp(P_{10}) is also important for thrombin fibrinogen interactions (Marsh and Scheraga, 1983). On the basis of these observations and high affinity of Phe-Pro-Arg for thrombin, a hairpin-like structure in the NH_2 terminal of the A chain of fibrinogen is proposed. Additional bends present at Pro-Arg (P'_2-P'_3) and possibly between P_4 and P_8 bring the Phe(P_9) close to Val-Arg-Gly. In other words Phe can occupy S_3 position in thrombin.

This leads to a conclusion that in the design of a small specific inhibitor of a protease, the selection of amino acid sequence may not

necessarily have to be based upon substrate sequence. There is one consolation to this, that is, there are only 20 or so common amino acids. However, the permutations of these could become very difficult when dealing with an unknown protease. This problem could be solved by using the natural substrate of the protease. This plan of attack is explained in a later section.

2H. Peptide synthesis

This rather narrow area of bio-organic chemistry has received considerable attention. It is impossible to review all the methodologies to synthesize peptides, therefore only cases pertaining to this research will be discussed. The formation of the peptide bond requires activation of the carboxyl group of an amino acid. The reactive intermediate of the amino acid then reacts with α-NH_2 of another amino acid to form the peptide bond. The reversible protection of the amino group and of the carboxylic group which were not meant to be part of the amide bond, remained problematic for a long time. A major break through toward the solution of this problem was the discovery of the carbobenzoxy group. This protecting group could be easily removed by catalytic hydrogenation at room temperature and ordinary pressure, and generates relatively harmless by products, toluene and carbon dioxide. Since the guanidino group of arginine is a nucleophile, it should blocked to prevent undesirable reactions. There are three amino groups in the guanidine which is a single, monoacidic cation. The basicity of the guanidino group in arginine is extreme, and it remains protected under the usual conditions of peptide synthesis by simple solution protonation. In this Dissertation, I have NO_2 protection of the guanidino group to promote the solubility of Arginine peptides in tetrahydrofuran (THF). This NO_2 protection can be removed under similar conditions to remove the carbobenzoxy group, the only difference is the requirement for acid and a longer reaction time. During deprotection of the NO_2, special care

needs to be taken against the reductive saturation of aromatic nucleui and other unsaturated bonds (Galpin et al., 1984). Other problems with guanidino protection with NO_2 are reviewed in detail by Bodanszky (1984).

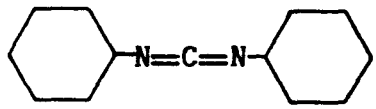
The aldehyde function is reactive towards even weak nucleophiles, and has to be protected from participating in undesirable nucleophilic reactions. This protection should be easily removable under mild conditions to regenerate the aldehydic function. There are number of ways to accomplish this goal. Without going into detail, two distinct alternatives are either to prepare the acetal or the semicarbazone derivative. Both methods are easy, but acetals are acid labile and during removal of the NO_2 protection of the guanidino group, the acidic conditions may also remove the acetal aldehyde protection. Semicarbazones are relatively stable to acids. Also it is advantageous because of the low solubility of the semicarbazone products, which will help in crystallization of the aldehyde derivative.

The carbonyl group can be activated in a number of ways. One of the most popular methods is the conversion of protected amino acids to reactive esters, Z-NH-CHR-COOR', where R' is an electron-withdrawing group. From numerous active esters in the literature, N-hydroxy succinamide esters were selected herein (Anderson, 1963, 1964) because of the water solubility of the products of the hydrolysis. The N-hydroxy succinamide esters are crystalline, stable compounds which have excellent reactivity in aminolysis reactions. The synthesis of N-hydroxy succinamide esters are not different from the formation of a peptide

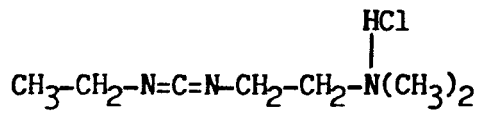
bond. The N-protected amino acid is allowed to react with N-hydroxy succinamide in presence of an acyl transfer reagent, for example a carbodiimide (Bodanszky, 1957).

In the case of utilization of a carbodiimide catalyst, the activation of carboxyl group occurs through its addition to N=C double bond of the carbodiimide. Fig. 14 shows the most commonly used carbodiimides. Dicyclohexyl carbodiimide (DCC) was introduced by Sheehan and Hess (1955). The water soluble variant of DCC is N-ethyl-N-(3-dimethylaminopropyl) carbodiimide (EDC). There are other water soluble variants, but EDC seems to give better coupling yields (Bodanszky, 1984). Fig. 15 shows a carbodiimide mediated peptide bond formation. The by product of the coupling reaction is an isourea. In the case of DCC the N,N'-dicyclohexyl urea is insoluble in most organic solvents and removed by filtration. A water soluble carbodiimide is advantageous in certain cases because of the solubility of the urea by product. This is particularly useful when the desired product is linked to an insoluble support.

Proteolytic enzymes also can be used as coupling reagents by thermodynamically or kinetically controlled enzyme catalyzed aminolysis of specific acids or esters. In recent years protease catalyzed peptide synthesis has undergone intensive development. Enzymic synthesis processes a number of advantages over chemical synthesis. Since it does not require an activation of a carboxyl group, functional side groups amino acids do not have to be protected. In addition, these reactions are stereospecific and undesirable racemization does not occur. Several

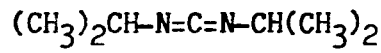


Dicyclohexylcarbodiimide



N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide

(water soluble carbodiimide)



diisopropylcarbodiimide

Figure-14. Structures of carbodiimides.

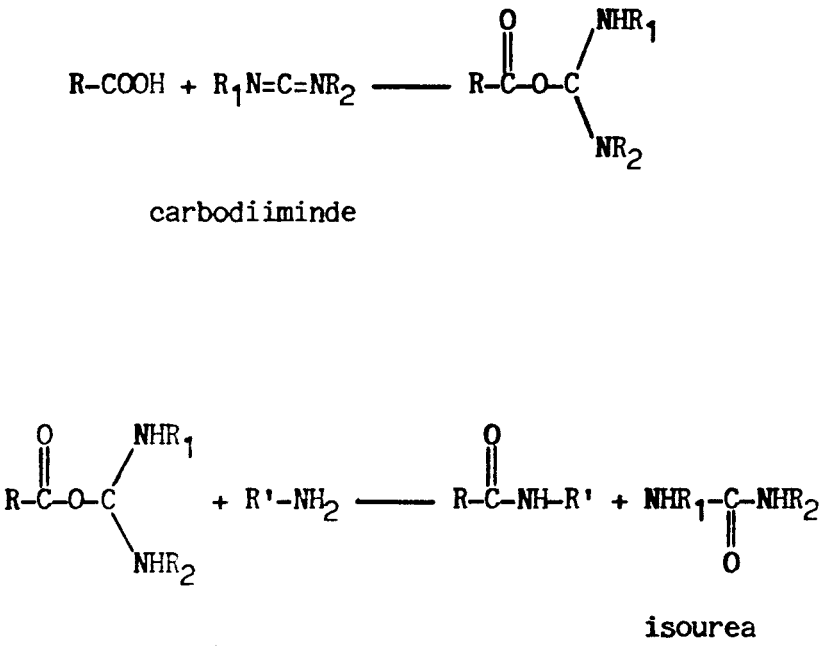


Figure-15. Carbodiimide coupling reaction.

papers devoted to successful synthesis of various peptides using chymotrypsin, papain, subtilisin, trypsin, carboxypeptidase and other proteases have been published (Mitin, 1984).

Conditions have to be worked out to shift the equilibrium in such a manner that the enzyme remains catalytically active and peptide bond synthesis is favoured rather than peptide bond hydrolysis. Three different techniques have been applied to achieve this goal, mass action (Sealock and Laskowski, 1969), change in pH (Mitin et al., 1984) and addition of an organic co-solvent to change the equlibrium in favor of the peptide synthesis(Homanberg and Laskowski, 1978; 1979).

In the first technique, a des-arg modified protein was reacted with large quantities of arginine, trypsin and carboxypeptidase-B. This procedure succeeded in adding the initially removed Arg residue back to the des-Arg protein. In the second technique, Mitin et al. (1984) synthesized peptides in the presence of papain at pH 8 to 9 using acylamino acid alkyl esters as starting substrates and peptide nucleophiles (amides or tert-butylesters of amino acids as well as peptides) as amino group donating compounds. In the third technique (Sealock and Laskowski, 1969), the pH dependence of K_{syn} in water and in 60% (v/v) triethylene glycol was varied and conditions found that thermodynamically favored peptide bond synthesis in the chymotrypsin mediated peptide bond formation between Cbz-Try and Glu-NH_2. In the study of the resynthesis of the peptide bond between bovine pancreatic ribonuclease S and S peptide by subtilisin in water, the maximal amount of synthesis was only 4.3%. By increasing the glycerol concentration to

90% (v/v) glycerol, the glycerol perturbed the equlibrium between synthesis and hydrolysis so that at equlibrium, 50% of the RNAase was in the natural covalent form.

2I. Rational for utilization of fibrinogen-thrombin system for the development of a general method for protease affinity chromatography

The example of thrombin was selected to demonstrate a general method of affinity chromatography herein because the chemistry of fibrinogen-thrombin system is known. In addition to this fibrinopeptides are commercially available and the potential problems of side chain COO^- groups modification could be studied. Since the amino terminal residue of FPB is p-Glu, it is not available to form a linkage between FPB and the resin matrix. This leaves FPA as the possible choice for immobilization on the resin. The FPA is immobilized rather than fibrinogen for two important reasons. First, the activation of immobilized fibrinogen by thrombin will lead to formation of a large clot entrapping all the matrix beads. Secondly, since fibrinogen is a very large molecule (MW= 340,000), a high ligand concentration can not be achieved due to molecular weight differences between the by product fibrin and desired product (FPA). However, a very high concentration could be achieved on the matrix if FPA is added directly to the resin.

During the coupling of ArgSc to des-ArgFPA, the side chain COO^- groups of glutamate and aspartate have the potential to be modified by the reaction. This problem could be remedied by either the selective

reversible modification of these acid groups or by the use of an enzyme reagent to form the peptide bond with a specificity to the C-terminal carboxylic group. Both approaches are attractive and will be later discussed.

Fibrinopeptide-A will be covalently joined to an affinity resin by one of the standard procedures previously discussed. This resin containing fibrinopeptide-A will be treated with carboxypeptidase-B to remove the terminal arginine residue. Carboxypeptidase-B is commercially available in high purity and exhibits preference in the removal of basic amino acids like arginine and lysine. The release of free arginine from the peptide can be directly followed by the Sakaguchi test (Messineo, 1966). Following removal of the COOH-terminal arginine, Arg-Sc (argininal semicarbazone) (Patel and Schultz 1983) will be added to the desarginine peptide. The simplest way to do this is by a chemical means utilizing a water soluble carbodiimide to catalyze peptide bond formation.

An apparent difficulty with this scheme is that the addition of the Arg-Sc will not only be to the desired COOH-terminal group of the des-Arg FPA, but also to the Glu and Asp carboxylic acid side chain groups. Whether the effect of these added Arginals make any difference in the binding of thrombin to the affinity resin remains to be seen. However, the nonspecific addition of Arg-Sc could be avoided by revesibly modifying COOH side chain groups or using proteases as reagent for peptide bond formation as discussed previously. In the reversible modification approach, methyl ester protection of these COOH groups is

very attractive. This protection could be obtained using triethyl oxonium fluoroborate (Merwein reagent) in aqueous solution (Hamada and Yonemitsu, 1971; Paterson and Knowles, 1972; Osbahr, 1982). The methyl esters could be easily hydrolyzed at pH 12 within a short period of time after the C-terminal arginine is converted to argininal. A number of different techniques can then be used to substitute the terminal arginine by Arg-Sc.

Chapter III

MATERIALS AND METHODS

3A. General Materials and Sources

Unless otherwise stated the chemical reagents utilized in this work were reagent grade or better. Reagents were generally purchased through the Eastman Chemical Company, Scientific Products (S/P), J.T. Baker Chemical Company or from the Malincrodt Chemical Works. Special instruments utilized in this work are indicated at the appropriate places.

Specific mixtures of developers (CH_3OH:$CHCl_3$:15:90 and others as indicated) were formulated to develope thin layer chromatography (TLC) plates were prepared each day and were used only once. Specific spray reagents were prepared intermitantly as per requirements to maintain optimum sensitivity. Appropriate buffers were freshly prepared and stored at 4 ^{o}C. Affinity resins were also stored in CH_3:H_2O/50:50 at -20 ^{o}C and equilibrited with appropriate buffer overnight before use. The substrate solutions were prepared according to required concentrations and stored at -20 ^{o}C in 2 ml aliquot. Enzyme solutions also were prepared according to their desired concentrations and stored at -20 ^{o}C in appropriate aliquots.

Because of its higher sensitivity of silica gel plates Bakerflex IB2F (fluorescent type) from J.T. Baker Chemicals towards the Sakaguchi

test, IB2F plates were used where the Sakaguchi test was performed. If a Sakaguchi test was not required, silica gel plates GF from Analtech (cat# 02521) were used.

Bovine fibrinogen type IV containing approximatly 40% buffer salts (sodium citrate/sodium chloride::0.02:0.85) was purchased from Sigma chemicals (cat# F-4753) and was used without further purification.

Human fibrinogen containing approximatly the same buffer salts as bovine fibrinogen was purchased from Sigma Chemical (cat# F-3254) and used without further purification. Bovine thrombin was purchased from Miles Lab. (85 NIH units/mg protein, Lot# 82-03615), bovine trypsin from Millipore Corp. (LOt# 3703 TRL 39N993P), and human urokinase from Abbott Laboratories (Abbokinase, Lot# 19-022-AF, 150,000-200,000 I.U/mg protein).

The following are the spray reagents for identification of different functional groups in peptide derivatives.

Dinitrophenylhydrazine (DNP) test: To identify aldehydes, aldehyde hydrates or semicarbazones on TLC, the developed and dried silica gel plates were initially sprayed with a solution of dinitrophenylhydrazine (DNP) (Aldrich) in ethanol containing 1% concentrated HCl and dried at 80 ^{o}C in an oven for 10 min. To enhance the sensitivity, the plates were subsequently sprayed with a solution of 0.2% potassium ferricyanide (Fisher) in 2 N HCl which upon drying destroy the yellow color of unreacted DNP and the aldehyde or semicarbazone are amplified by their yellow color.

Sakaguchi test: The chromatogram is sprayed with a mixture of a 16%

urea solution followed by 0.2% ethanolic α-naphthol solution. The plate is dried at 80 °C for 10 minutes and subsequently sprayed with a solution of 3.3 ml bromine in 500 ml 5% NaOH solution.

Ninhydrin test: 100 mg of ninhydrin was dissolved in 100 ml of acetone. After spraying the chromatogram with the ninhydrin solution, a pink to purple color appears upon heating at 80 °C for 10 min.

Iodine chamber: A few crystals of iodine are kept in an air tight wide-mouth glass jar and a dried chromatogram is allowed to react with the iodine vapor for 10 min.

Schiff's reagent: 1 gm of basic fuchsin (Sigma Chemicals) is added to 200 ml of distilled water at 90 °C and stirred to disolve. The solution is cooled to 50 °C, filtered, and 20 ml of 1 N HCl added. The solution is further cooled to 25 °C, 1 gm of sodium bisulfite added, and the solution kept for 18-20 hours in the dark. To the straw colored solution, a few grains of activated charcoal is added to decolorized the solution. The reagent is stored at 4 °C in a tightly stoppered brown bottle. The solution is discarded when a pink color appears.

3B. Synthesis of Cbz-(NO_2)-L-Arginine Semicarbazone (Cbz-NO_2-Arg-SC

I have published the synthesis of this compound (Patel and Schultz, 1982). To a solution of 2.02 gm (5.72 mMoles) of Cbz-NO_2-Arg in 15 ml of anhydrous tetrahydrofuran (THF)(Malincrodt) under nitrogen was added 1.0 gm of 1,1'-carbonyl diimidazole (Sigma). The solution was stirred at 10

^{o}C untill all the solids dissolved and then cooled to -75 ^{o}C (dry-ice acetone bath). To the solution was added 0.84 gm of $LiAlH_4$ in 17 ml of THF over 30 minutes and the mixture stirred for 3 hr at -75 ^{o}C under N_2. Unreacted $LiAlH_4$ was decomposed by the addition of 50 ml cold 2N HCl to pH-3.0. The reaction was extracted 3 times with $CHCl_3$, the $CHCl_3$ extract washed with H_2O, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under vacuum on a rotary evaporator. The white residue (impured Cbz-NO_2-Argal) was dissolved in 80 ml of 70% ethanol to which 1.20 gm of semicarbazide.HCl and 1.84 gm of sodium acetate were added. The solution was warmed briefly to 60 ^{o}C and the solvent evaporated under vacuum. The residue was purified on a silica gel column eluted with 10% CH_3OH in $CHCl_3$, yielding 1.5 gm (66.4% yield) of Cbz-NO_2-Arg-Sc, crystallized from absolute ethanol, mp. 105-108 ^{o}C (uncorrected) (Lit. 107-109 ^{o}C), TLC over silica gel GF plates gave a single spot of R_f=0.19 in 90:15::$CHCl_3$:CH_3OH.

3C. Removal of CBz and NO_2 protection from CBz-NO_2-Arg-Sc.

Cbz-NO_2-Arg-Sc (50 mg) was dissolved in 20 ml of CH_3OH. To the solution was added 5 mg of palladium black (Aldrich Chemical Co), 5 ml of H_2O and 0.2 ml glacial acetic acid. The mixture was placed in a Parr hydrogenator, reduced for one hour at room temperature and 5 lb/in^2 of hydrogen gas pressure. The mixture was then subjected to centrifugation to remove the catalyst. The catalyst was washed twice with 5 ml portions of CH_3OH to recover adsorbed Arg-Sc from the catalyst. The washings and supernatent were combined and evaporated at 30 ^{o}C to drynes under vacuum

in a rotary evaporator. To the concentrated Arg-Sc was added 10 ml of H_2O and then the solution was evaporated (to dryness to remove traces of acetic acid). The residual Arg-Sc was used in experiments without any further purification.

3D. Synthesis of Cbz-NO_2-Arginine diacetal (Cbz-NO_2-Arg-OCH_3))

A solution of 2.02 gm (5.72 mMoles) of CBz-NO_2-Arg in 15 ml of anhydrous THF under nitrogen was stirred at 10 °C until all the solids dissolved, and then cooled to -75°C (dry-ice acetone bath). To the solution was added 0.48 gm of $LiAlH_4$ in 17 ml of THF over 30 minutes and the mixture stirred for 3 hrs at the same temperature under N_2. Unreacted $LiAlH_4$ was decomposed by the addition of 50 ml of cold 2 N HCl to pH 3.0. The reaction was extracted three times with $CHCl_3$, the $CHCl_3$ extract washed with H_2O, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under vacuum on a rotary evaporator. The white residue was dissolved in 20 ml CH_3OH and to the solution was added 4 gm of sodium bisufite in 20 ml of water. The solution was stirred at room temperature for 20 minutes. The pH of the solution was adjusted to 6.8 to 7.0 with sodium carbonate and let it stirred for an additional 20 minutes. The solution was washed three times with 50 ml $CHCl_3$. The pH of the solution was adjusted to 9 to 10 and was evaporated under vacuum on a rotary evaporator to 20 ml. The solution was extracted three times with $CHCl_3$, the $CHCl_3$ extract washed with H_2O, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness under vacuum on a rotary evaporator, yielding 1.21 gm Cbz-NO_2-Argal (70% yield). The aldehyde was

dissolved in 100 ml of absolute methanol and 200 µl of concentrated HCl added in 50 µl portions with stirring, and the reaction mixture stirred for 10 minutes. The Cbz and NO_2 protections were removed by catalytic hydrogenation as described before and was used without any further purification.

3E. Activation of Enzacryl AH

Enzacryl AH (100 mg) was placed into a test tube and stirred in 10 ml of 2 N HCl at 0-5°C. To the mixture, 4 ml of 4% ice-cold sodium nitrite was added with stirring, the mixture stirred for 15 minutes, and the resulting diazo-enzacryl AH washed four times with 5 ml portions of borate buffer (pH-9.2). The resin was added to a solution of 28 mg of Arg-Sc in 5ml borate bufer/10 ml methanol and stirred over night. The next day the resin was collected on a sintered glass funnel and washed with 0.01 N borate solution. Excess unreacted active groups were neutralized by stirring the resin in 1 M ethanol amine (pH 9) for one hour.

3F. Synthesis of Affigel-Argal Resin.

Cbz-NO_2-Arg-Sc (50 mg) was reduced as described above and the resultant Arg-Sc dissolved in 4:1/ CH_3OH:phosphate buffer (0.025 M sodium phosphate, pH 7.5), and the solution cooled to 4 °C. Affigel-10 (7 ml) (Bio-Rad Corp.) was collected in the sintered glass funnel and washed three times with 7 ml portions each of isopropanol. The washed gel was added to a pre-cooled Arg-Sc solution, shaken over night at 4 °C, the

resin washed with $CH_3OH:H_2O$ (4:1) to remove excess Arg-Sc, the resin added to 10 ml of 1 M ethanol amine at pH 8.0, stirred for two hours, the resin collected and washed with H_2O. To remove the semicarbazone protection, the resin was added to a solution of $CH_3OH:CH_3COOH:HCHO$ (5:1:1) and stirred overnight at room temperature. The Affi-Argal resin was collected on a funnel, washed with H_2O, and equilibrated in the protease binding buffer prior to the use.

3G. Synthesis of Model Ethanolamine Resin

To 10 ml of Affigel-10 was added 10 ml of 1 M of ethanol amine at pH-8.0, the mixture mixed for mixed for 3 hrs at room temperature, the resin collected on a funnel and washed with cold H_2O. The resin was stored as described previously.

3H. General Procedure For The Synthesis of R-(X-Y-Arginal) Resins

The affinity resins of general formula R-(X-Y-Arginal) were prepared by dissolving 50 mg of the respective dipeptide -X-Y- (-X-Y-= Phe-Ala, Phe-Pro, or Gly-Gly) in a solution of methanol/buffer (0.025M sodium phosphate at pH-7.5), cooling to 4 ^oC, adding 5 ml of washed affigel-10 and the mixture shaken over night in an inverting shaker at 4 ^oC . The resin was collected on a sintered glass funnel, washed with methanol/water, the resin added to 10 ml 1 M ethanol amine (pH-8.0) to block any unreacted N-hydroxy succinimide ester groups, the mixture shaken for 5 hrs, the resin collected on a funnel and washed with water. The R-(X-Y) resin was then added to a 4:1 methanol/water solution

containing 50 mg of Arg-Sc and a 5-10 fold molar excess of water soluble carbodiimide (EDAC, Pierce Chemical Co), the solution brought to pH 4.5, and mixed in an inverting shaker over night at room temperature. The resin was collected on a sintered glass funnel and washed with 4:1 methanol/water, the washed resin added to a solution of 3:1:1 methanol/ acetic acid/ formaldehyde (37%), and mixed overnight in an inverting shaker to remove the semicarbazone protection and generate the free argininal. The resin was collected on a sintered glass funnel, washed with water and equilibrated with buffer prior to use.

3I. Chemical Analysis of Resins

1. Chemical analysis for guanidino function

Chemical analysis for guanidino function in resins and solutions was carried out similar to a method described by Messineo (1966) with modifications described: The reagents were prepared in the following manner:

Reagent A:- Four hundred mg of KI was dissolved in 100 ml of deionized water.

Reagent B:- To 100 ml of 2 M KOH was added 2 g of potassium sodium tartarate, followed by 100 mg of 2,4-dichloro-1-naphthol (Eastman Chem. Co.). To the solution, 180 ml of 95% ethanol was added. The solution was allowed to stand overnight before use.

Reagent C:- Twenty ml of commercial NaOCl (approximate concentration 4-6%) was diluted with 80 ml of deionized water.

For qualitative and quantitative analysis of guanidino function,

the reagents were used in the following manner: To the resin or the sample solution in acidic medium (pH 1 to 2) containing 1 to 5 ug of arginine, 0.3 ml of reagent A and 1.0 ml of reagent B were added in this order. After standing for few minutes at room temperature, 0.2 ml of reagent of reagent C was added. After 10 minutes the absorbance was measured against a blank at 520 nm. One or two standards were used each time to assure accuracy for variations in different batches of reagents.

2. Aldehyde concentration in the resin.

Quantitative analysis for the aldehyde concentration in the resin was based on an analytical procedure previously described by Ulbrich and Schellenberger (1979) (Patel and Schultz (1982)) as follows. To 0.1 ml of Argal-resin was added a saturated solution of p-phenyl-azoaniline in ethanol and acetic acid (10:1), the mixture incubated for 1 hr, and the resin washed with ethanol until the eluent is colorless. The resin is added to 1 ml of 8:1:1 methanol:acetic acid:salicyl aldehyde and shaken for 30 min, the supernatent collected, and the procedure repeated 2 more times with the same solvent. The supernatents are combined, made up to 5.0 ml, and the absorbance read at 405 nm with respect to a solution obtained by treatment of model ethanol amine-resin in the same way. The excitation coefficent for the Schiff adduct of p-phenyl-azoaniline and salicyl-aldehyde was determined on a sample of this product independently synthesized by procedure previously described (Patel and Schultz, 1982).

3J. General procedure

1. Preparation of fibrinopeptides:

To the solution of 0.9% NaCl was added 1 g of either human or bovine fibrinogen and was stirred to dissolve at pH 8.5. To the solution, 20 units of purified thrombin and 0.5 ml of 2.5 M $CaCl_2$ was added, the fibrin clott removed as it is formed by a glass rod, and the stirring continue for six hours. The solution was filtered through Whatman #1 filter paper followed by filtration through a PM-10 membrane filter (Millipore Corp.) under 20 atm. N_2 pressure. The filtrate was evaporated at 30 ^{o}C under reduced pressure to dryness in a rotary evaporator. The residue was extracted with 20 ml mixture of methanol/water (50:50) and the supernatant evaporated to dryness. The residue was now extracted in to 10 ml CH_3OH/H_2O (75:25) and the supernatent evaporated to dryness. The residue was reconstituted in 2.5 ml 0.9% NaCl and loaded on a G-25 superfine Sephadex column (2.5x10 cm). The column was eluted with the same NaCl solution at flow rate of 1 ml/min. The peaks determined at 280 nm were collected

2. Synthesis of Affigel-fibrinopeptide-A resin (Affi-FPA)

Fibrinopeptide-A was dissolved in 4 ml of CH_3OH/0.05 M imidazole (75:25), adjusted to pH 7.4, and cooled to 4 ^{o}C. Affigel-15 (7 ml) (Bio-Rad Corp.) was collected in sintered glass funnel and washed three times with 7 ml portions of isopropanol followed by a 10 ml wash of CH_3OH/H_2O (75:25). Washed gel was added to pre-cooled FPA solution, shaken for two hours at 4 ^{o}C, to the slury was added 5 ml of 1 M ethanolamine (pH 8.0),

shaken for 4 hrs at room temperature, the resin collected, and washed with H_2O.

3. Synthesis of Affigel-Fibrinopeptide-A-Argal resin (Affi-FPA-Argal)

Direct substitution: Affi-FPA and Arg-Sc were prepared as described previously. To the solution of Arg-Sc in 10 ml CH_3OH/0.1 N $NaHCO_3$ (5/5), in a screw cap test tube Affigel-FPA was added. To the mixture, 0.2 ml of carboxypeptidase-B (Type I -DFP, Sigma Chemicals, cat# C 7261, 2 units/ml in saline) was added and the solution shaken overnight at 37 ^oC. Similar reactions were carried out in variable relative amounts of methanol and buffer, or with ethylene glycol or other organic solvents in place of methanol.

Chemical method: Affi-FPA and Arg-Sc were prepared as described previously Affi-FPA was suspended in 4 ml of 1 M ethanol amine at pH 8.0 and 200 mg of water soluble carbodiimide (EDAC) was added, reacted overnight at room temperature, the resin collected on sintered glass funnel and washed three times with water. The resin then was suspended in 4 ml of 0.05 M Tris buffer pH 8.1 and reacted with 0.05 mg trypsin (Sigma Chemicals, 2X crystallized), or with 100 units of bovine thrombin (Miles Laboratories, cat# 82-036-1, Lot# 45) for one hour at room temperature. To remove the terminal arginine, Affi-FPA was suspended in 0.1 M $NaHCO_3$ and 0.2 ml of carboxypeptidase-B (2 U/ml in saline) was added and reacted for variable lengths of time (30 min to overnight) at room temperature or 37 ^oC. Affi-desarginine FPA was collected in a sintered glass funnel and washed three times with normal saline. The resin was then added to a 4:1 methanol/water solution containing

arginine semicarbazone and 200 mg EDAC, the solution brought to pH 4.5, and mixed in an inverting shaker overnight at room temperature. The resin was collected on a sintered glass funnel and washed with 4:1 methanol/water, the washed resin added to a solution of 3:1:1 methanol/acetic acid/formaldehyde (37%), and mixed overnight in an inverting shaker to remove the semicarbazone group and generate the free argininal. The resin was collected on a sintered glass funnel, washed with water and eqilibrated with buffer prior to use.

In addition to the ethanol amine protection for side chain COO^- groups, $-OCH_3$ protection was also attempted. To 1 mg of human fibrinopeptide-A in 2 ml of methanol/water (3:1) was added 120 ul of trimethyloxyfluoroborate (TMOFB) $(CH_3)_3O(BF_4)$ in CH_3CN (100 mg/ml) in 10 ul fractions during 30 minutes. During addition of the reagent, the pH was kept between 4.5 to 5.5 with the addition of 0.5 M NaOH. The pH was adjusted to 7.0, stirred for 30 minutes at room temperature, and cooled down to 4 ^oC. Affigel-15 (7 ml) was washed with isopropanol as described before, and reacted with above solution for 60 minutes at 4 ^oC, 3 ml of 1 M ethanolamine added, and mixing was continued for an additional one hour at room temperature. The resin was collected on a sintered glass funnel and washed with water. The gel was added to 4 ml of 0.025 M imidazole containing 0.2M NaCl, 0.025M $CaCl_2$, and 0.01% carbowax-20M at pH-7.4. To the mixture 10 units of thrombin were added and reacted for one hour at room temperature. The gel was collected on a sintered glass funnel and washed with saline. The terminal arginine was removed with carboxypeptidase-B as described previously Arg-Sc was added

back to the Affi-desarginine-FPA using water soluble carbodiimide as described previously. Semicarbazone proction was removed and the resin equilibrated with buffer prior to use.

Enzymatic synthesis: In this approach papain was used as the reagent to form the argininal resin peptide bond at pH 9.2. Affi-desArg-FPA was prepared as described previously. The resin was suspended in 3.5 ml potassium phosphate buffer containing 0.2 M dithiothreitol, 60 mg of Arg-Sc, and 0.2M EDTA at pH-9.2 and 0.5 ml CH_3OH was added to it. To the mixture, 200 ul of papain (Sigma Chemical, Cat# P-3125, 2X crystallized, 22 mg protein/ml, 22 U/mg protein) was added, the pH adjusted to 9.0, and the reaction stirred at room temperature. for 15 minutes. The resin was colected on a sintered glass funnel and washed with water. Semicarbazone protection was removed, and the resin equlibrated with water prior to use.

3K General procedure for the binding and elution of proteinases

1. Column chromatography: The equilibrated 3 ml of resin in an appropriate buffer was placed in a small polypropylene column attached to an ultraviolate detector (280 nm) with fraction collector. The protease solution (1 ml) was diluted with 1.5 ml of the appropriate buffer and placed onto the resin. The resin was washed with 7.5 ml of initial buffer. The resin was washed with 10 ml aliquots of the same buffer, but with a higher ionic strength due to the presence of an increasing concentration of NaCl in the buffer. Each of the 10 ml

aliquots were analyzed for absorption at 280 nm, for protein concentration, and enzymatic activity with an appropriate substrate. In some cases smaller fractions were obtained in order to better analyze the pattern of proteases.

2. Enzyme assays: Chymotrypsin and trypsin were assayed with BzTyrEE and TosylArgEE respectively, according to the methods described in Worthington enzyme catalog (Worthington enzymes, 1977).

Bovine thrombin and human urokinase were routinely assayed with peptide p-nitroanilide substrates in which the rate of appearance of p-nitroaniline product was determined at 405 nm with a Perkin Elmer spectrophotometer (Model# 320). The composition of the reaction solutions were as follows.

Urokinase: at 37 ^{o}C

0.100 ml $1x10^{-3}$ M p-Glu-Gly-Arg-pNA (Kabi Group Inc, S-2444)

0.8 ml 0.05 M Tris buffer pH 8.4

0.2 ml protease test solution

Thrombin: at 25 ^{o}C

0.05 ml $1x10^{-3}$ M D-Phe-Pip-Arg-pNA (Kabi Group Inc. S-2238)

0.95 ml 0.05 M Tris buffer pH 8.4

0.020 ml protease test solution

In some experiments thrombin activity was also measured with the substrate fibrinogen which measures mainly the activity of the alpha form of the thrombin (Fenton, 1981). In this assay, a stock fibrinogen solution (50 mg fibrinogen in 25 ml 0.05 Tris containing 3 mM EDTA, pH

7.4) was filtered and adjusted to 1.0 absorption units at 280 nm. This solution (200 µl) was placed into a Fibrometer (B.B.L., Bectin Dickinson and Co), 50 ul of Tris buffer pH 8.1 added, followed by 50 ul of the test thrombin solution in the eluting buffer from the affinity resin, which always contained 25 mM $CaCl_2$. The time interval for formation of fibrin clot was correlated with a standard curve of thrombin units *vs.* cloting time, prepared previously under identical conditions.

Chapter IV

RESULTS

4A Synthesis of Cbz-NO_2-Arg-Sc

Because of the difficulty of direct reduction of carboxylic acids to aldehydes, a number of activated carboxylic acid derivatives such as acid halides, t-amides esters and lactones have been utilized as substrates for reduction. The problem is to stop the reduction at the aldehydic stage because the aldehyde once formed is easily further reduced to the alcohol derivative. Alternatively, alcohols can be oxidized to aldehydes by number of oxidative procedures. Thompson (1973) carried out the oxidation of the alcohols by the dicyclohexyl carbodiimide-dimethyl sulfoxide reagent, first introduced by Pfiztner and Moffatt. Oxidation with pyridinium dichromate seems to cause considerable racemization, but Hamada and Shiori (1982) reported oxidation of N-protected β-amino alcohols by the use of a combination sulfur dioxide-pyridine complex and dimethyl sulfoxide in the presence of triethyl amine to give highly optically active α-amino aldehydes. Anderson and Wolfenden (1982) reported the use of horse liver alcohol dehydrogenase which shows a broad specificity for its alcohol substrates in oxidation of β-amino alcohols to α-amino aldehydes.

Despite of difficulty in the direct reduction of carboxylic acids to aldehydes, a few methods have been reported that utilized strong reducing agents like lithium methyl amine, bis(N-methyl-piperidynyl)

aluminum hydride, thexylborane or grignard reagents catalyzed by dichloro-bis(cyclopentadienyl) titanium. These methods lack general applicability and chemoselectivity because of severe reaction conditions. Fujisawa et al., (1983) introduced a simple and chemoselective procedure using N,N-dimethyl chloromethylenimium chloride and $LiAlH(OBu^t)_3$ in a one pot operation. When strong reducing agents like $LiAlH_4$ or $LiAl(OBu^t)_3$ are used, the reduction could be limited to the aldehydic stage by decreasing temperature below -50 °C.

Arg-Sc was synthesized by reducing the carbonyl diimidazole ester of Cbz-(NO_2)-Arg with $LiAlH_4$ at -70 °C. Because of unfavourable solubility of Cbz-Arg at -70 °C in THF, the guanidino group was protected with the NO_2 group solely for its the favourable solubility. The aldehyde function could be protected either by a forming bisulfite adduct or by Schiff's base formation. Since strong amine nucleophile forms a stable Schiff's base, semicarbazide, a strong nucleophile, was selected as a derivatizing agent. Use of a stronger nucleophile than semicarbazide could present a problem for the regeneration of the aldehydic function. The optimum conditions for acid catalyzed semicarbazone formation is around pH 4.0, where the carbonyl oxygen is partially protonated and the concentration of unprotonated primary amine is present in low but appreciable amount. Sodium acetate provides a conjugate weak acid to provide desirable conditions for the optimum acid-catalyzed formation of semicarbazone.

The degree of racemization during silica gel chromatography varies with the structure of the Cbz-α-aminoaldehyde. Longer retention times

appear to cause more racemization. The semicarbazone derivatives, in which keto-enol tautomerism is suppressed, showed little racemization during chromatography (Ito et al., 1975). In the case of Cbz-Argal, racemization is limited because of the contribution of its non-carbonyl carbinolamine structure. From the above two arguments, it is safe to assume that purification of Cbz-NO_2-Arg-Sc during silicagel chromatography will lead to little, if any racemization.

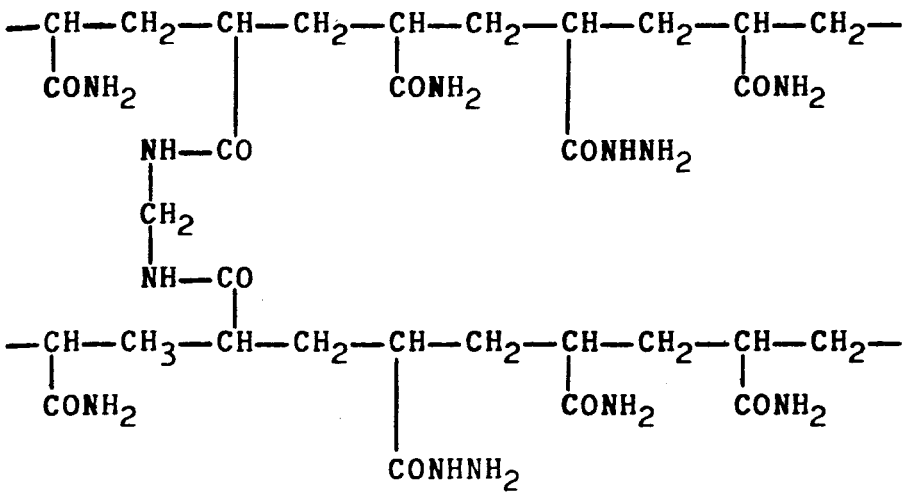
4B. Removal of Cbz and NO_2 group protection

The Cbz and NO_2 protecting groups of Cbz-NO_2-Arg-Sc are removed by catalytic hydrogenation over palladium black (Aldrich Chemical Co Cat# 20583-4). The hydrogenolysis was carefully monitored from 2 hrs to overnight for complete removal of Cbz and NO_2 groups (time utilized depended upon the activity of the palladium catalyst). Prolonged hydrogenolysis produced a side product which was Sakaguchi positive and DNP negative. Galpin et al., (1984) showed by NMR that some reduction of the carbon-nitrogen double bond in the semicarbazone derivatives may occur during hydrogenolysis. To minimize this reduction of the semicarbazone double bond, the hydrogen pressure was reduced from 25 lb/in^2 to 5 lb/in^2 when highly active catalyst was used. The reaction was monitored by the Sakaguchi test and the reaction was stopped at after a plateau at A_{520} was achieved in the Sakaguchi test.

4C Enzacryl AH

Enzacryl AH (Koch-light Laboratories Ltd.) is a hydrophilic copolymer containing acyl hydrazide groups as potentially active side chains for ligand binding. Fig.16 shows the structural features and outlin the coupling procedure used with the Enzacryl AH resin. In the procedure, the polymer is activated by the action of dilute nitrous acid which converts the acid hydrazides to reactive acid azide groups. Primary amino groups will react with the azides at mild conditions (pH 8.0 to 9.5 and 0-5 ^{o}C) to form an amide bond. Accordingly, Arg-Sc in borate buffer was reacted with the azide resin to obtain Enzacryl AH-Arg-Sc. Since this resin is stable to acids and organic solvents, regeneration of aldehydic function on argininal was achieved by treatment of resin with CH_3OH/ HCl (2 N)/HCHO (10:2:2).

The association of trypsin and chymotrypsin was studied by loading a mixture of trypsin and chymotrypsin onto a small column (0.9 cm x 5 cm) in 0.05 M phosphate buffer. The resin was then washed with 2 ml portions of same buffer. Table 8 shows enzyme activities in each fraction. It is evident from the results that the resin failed to bind either trypsin or chymotrypsin. It was expected that trypsin would bind to the column, while chymotrypsin would not show any affinity towards the resin. The retention of some enzyme activity on the column was nonspecific, and most of the enzyme activity could be easily washed out with the initial buffer. Failure to show any specificity towards trypsin by the resin led to the search for a different type of resin.



Major structural features of Enzacryl AH.

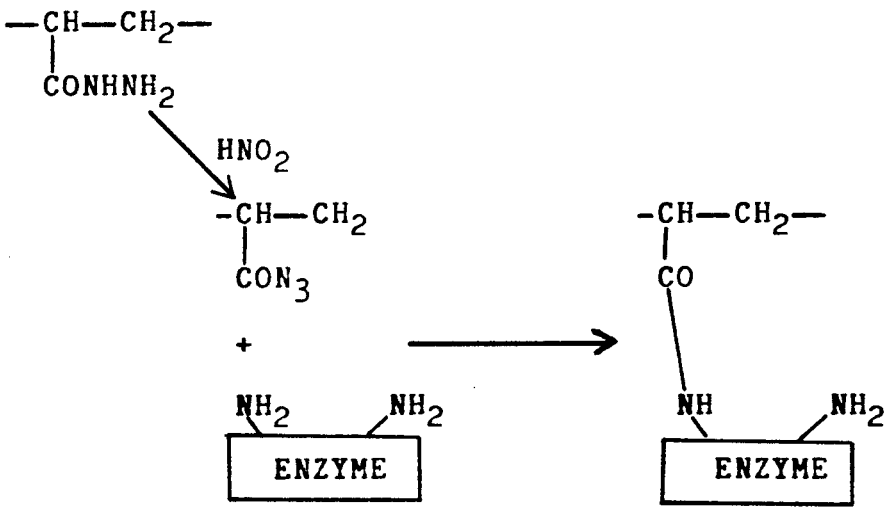


Figure-16. Coupling procedure with Enzacryl AH.

Table 8

Relative enzyme activities in 10 ml fractions of column effluents.

Resin: Enzacryl AH-Argal

Enzyme: Mixture of 0.5 mg Trypsin and 0.5 mg Chymotrypsin.

Buffer: 0.05 M Phosphate buffer containing 0.1 M NaCl.

Eluting buffer	% Enzyme activity	
	Trypsin	Chymotrypsin
Unbound fraction (Initial)	72	65
First wash	—	—
Second wash	5	9.5
1 M NaCl in buffer wash	9	1.8

4D Affigel-Argal resin

The procedure utilized for the synthesis of Affigel-Argal resin is outline in Fig.17. The coupling reaction was monitored by the Sakaguchi test before and after reaction of Arg-Sc with the Affigel. Affigel (2.5 ml) is capable of coupling 30 μmoles of Arg-Sc. The resin was also qualitatively tested for presence of guanidino group in the same fashion as for the Argal in solution. In a positive guanidino test, the resin turns a pink color.

The semicarbazone protection group of the Arg-Sc ligand was removed by treating the resin with CH_3OH:acetic acid:formaldehyde (7:1:2) overnight. In this reaction the semicarbazide is transfered from Arg-Sc to formaldehyde in a reaction catalyzed by acetic acid. The formation of aldehyde in this reaction is qualitatively followed by reaction with 2,4-dinitrophenyl hydrazine, which gives a yellow color on the resin. Alternatively, quantitative analysis of the aldehyde concentration is carried out by reaction of the Argal-resin with excess p-phenyl-azoaniline, which forms a Schiff base with the aldehyde groups (Fig.18). After washing the excess p-phenyl azoaniline from the resin, the modified resin is treated with salicyl aldehyde, resulting in the transfer of p-phenyl-azoaniline from Argal-resin to salicyl aldehyde. This adduct is eluted from the resin and quantitated by its absorbance at 405 nm. A comparison of the absorption obtained from the p-phenyl azoaniline salicyl aldehyde adduct from the Argal resin compared to that obtained with an ethanol amine resin blank showed a minimum of 0.1 μmoles/ml of aldehyde in the Argal-resin.

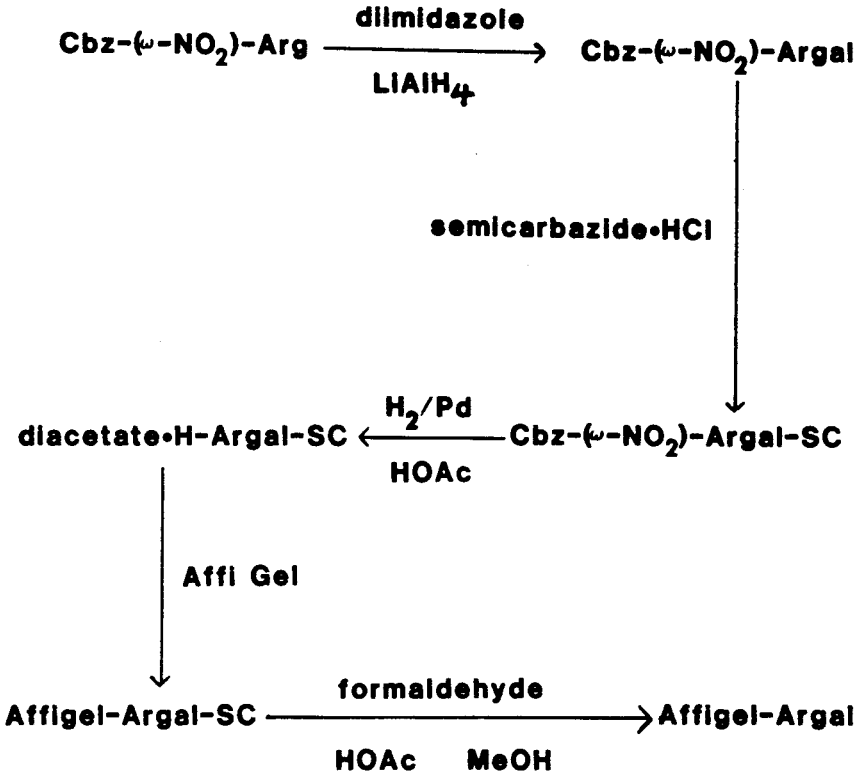


Figure-17. Synthetic scheme utilized in the synthesis of Argininal liganded affinity resin.

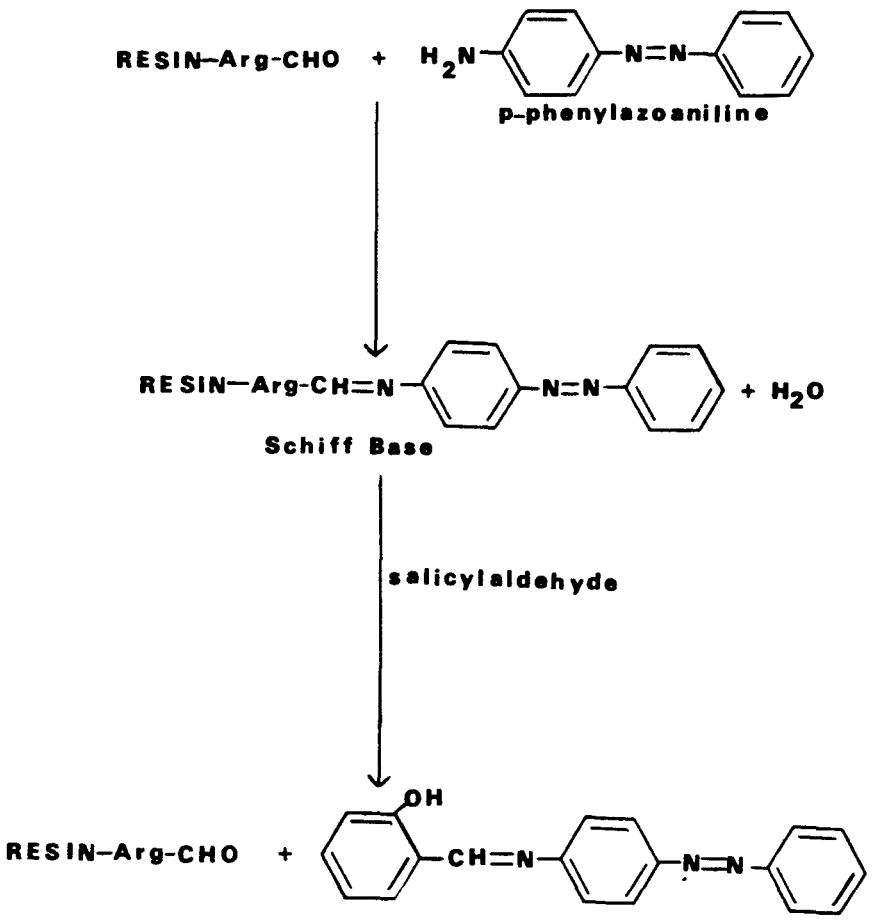


Figure-18. Scheme utilized to analyze Argininal-Resin for aldehyde concentration.

4E Association of trypsin and chymotrypsin to Affi-Argal resin

In a typical experiment, to 3 ml of Affi-Argal resin in 0.025 M sodium phosphate (pH 6.0) in a small column (0.9 x 5 cm) was added a mixture of 0.5 mg chymotrypsin and 0.5 mg of trypsin. When the resin was washed with pH 6.0 buffer, in the first 10 ml eluted 100% of the chymotrypsin activity placed on the column. The trypsin activity remained bound to the resin even after exhaustive washing. The elution of trypsin activity was not possible even with 2 N NaCl or at pH values between 2.0 and 6.0 (Table 9). However, essentially all the trypsin activity was eluted from Affi-Argal resin after incubation of the resin with 0.1 N semicarbazide and 0.1 N sodium acetate (pH 3.8) for 15 to 30 min, and then eluting the trypsin in 10 ml of same reagent.

Control experiments were carried out utilizing Affi-Arg-Sc and Affi-ethanol amine resins. To the Affi-ethanol amine resin no affinity of either chymotrypsin or trypsin was observed in pH-6.0 (0.025M sodium phosphate) buffer. The trypsin associated with the Affi-Arg-Sc resin at pH 6.0 (0.025M sodium phosphate) buffer, but was elute in 1N NaCl phosphate buffer at pH-6.0.

4F Affigel-X-Y-Argal resin

1. Synthesis: The procedure utilized for the synthesis of dipeptidyl Argal-liganded resin is outlined in Fig.19. The resin matrix containing a ten atom diaminoethyl spacer arms activated as N-hydroxy-succinimide esters was first covalently linked to dipeptides -X-Y- (where -X-Y-=Phe-

Table 9

Relative enzyme activities in 10 ml fractions of column effluents.

Resin: Affi-Gel—Argal

Enzyme: Mixture of 0.5 mg Trypsin and 0.5 mg Chymotrypsin

Buffer: 0.025 M sodium phosphate pH 6.0

Eluting buffer	% Enzyme activity	
	Trypsin	Chymotrypsin
Initial buffer (unbound)	0	90
0.2 M NaCl in buffer wash	0	5
2 M NaCl in buffer wash	0	0
10 ml Saline (between pH-2.0 and 6.0)	0	0
0.1 N Semicarbazide in 0.1 N NaOAc pH-3.8	100	0

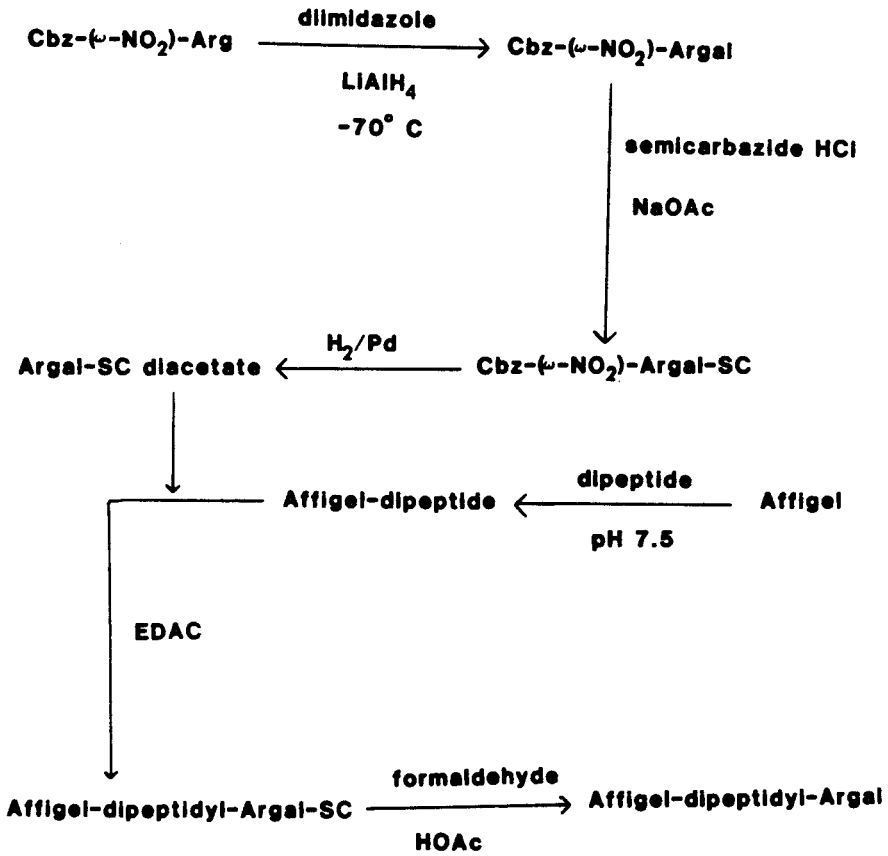


Figure-19. Synthetic scheme utilized in the synthesis of dipeptidyl-Argininal liganded affinity resin.

Pro, Phe-Ala or Gly-Gly), and then the carboxy terminal of -X-Y- joined to Arg-Sc in the presence of a water soluble carbodiimide. It was found easier to synthesize the tripeptide aldehyde ligand on the resin in a two-step, solid phase procedure than to synthesize in solution the complete tripeptide aldehyde and then attach it to the resin. The tripeptide synthesis on the resin is N-terminal to C-terminal, which is opposite from the commonly utilized Merrifield solid phase peptide synthesis. A disadvantage of our N-terminal to C-terminal synthetic procedure is that the amino acid Y in the Affi-X-Y resin may be expected to racemize during its joining to Arg-Sc. However, the affinity of the liganded resins to their selective proteinase was observed to be sufficient, and the racemization of the amino acid residue in Y, may therefore, not be a serious disadvantage. Furthermore, in the examples studied in this work, the Y residue was frequently Gly or Pro, which either does not racemize or racemizes with difficulty.

The semicarbazone protecting groups in the Affi-X-Y-Arg-Sc resins were removed by incubating the semicarbazone derivatized resins in 3:1:1 methanol/acetic acid/formaldehyde (37%) overnight. Our procedure successfully substitutes a weak acid for the commonly utilized 0.5-2.0 M HCl as the acid catalyst in previous reported procedures (Nishikata et al, 1981; Ishii and Kasai, 1981). All resins gave a positive test when tested by the Sakaguchi test, as described previously, and gave a minimal aldehyde content of between 0.07 and 0.1 umole/ml of resin. This analytical procedure for quantitation of aldehyde function may significantly under-estimate the aldehyde content as the yield of the

aldehyde chromophore adduct in the analysis is probably less than 100% (Patel and Schultz, 1982).

2. General procedure for the binding and elution of proteases: The conditions for binding and elution from Affi-X-Y-Argal resins (-X-Y-= Phe-Ala, Phe-Pro, Gly-Gly) of the serine proteinases trypsin, thrombin and urokinase was shown in this work. These enzymes have an identical primary site specificity toward arginine, but differing secondary site specificities towards residues in substrates N-terminal to the amino acid containing the scissile peptide bond in their substrates.

The higher ionic strength buffers decreases the the strength of non-covalent ionic interaction and may be expected to elute proteinases only bound electrostatically to the resin. For example, proteinases can often be remove from affinity resins containing lysine, benzamidine or protease inhibitor ligands in buffers of high ionic strength. However, in all cases in which the peptidyl argininal showed a high specificity to a particular protease (i.e., Gly-Gly-Argal for urokinase and Phe-Pro-Argal for thrombin), the protease could not be removed with high ionic strength buffers. Tables 10, 11 and 12 shows protease activities in eluted fraction for the resins Affi-Phe-Ala-Argal, Affi-Phe-Pro-Argal and Affi-Gly-Gly-Argal, respectively.

In the case where concentration of salt failed to elute the protease (urokinase from Affi-Gly-Gly-Argal and thrombin from Affi-Phe-Pro-Argal), the tightly bound protease could be removed in 0.1 M semicarbazide containing 0.1 M NaOAc, 0.025 M $CaCl_2$ and 0.01 % Carbowax

20M. Carbowax 20M was included in the buffer to minimize non-specific adsorption of protease molecules to glass and other surfaces. Semicarbazide reacts with the argininal aldehyde ligands, forming semicarbazone derivatives of the aldehyde groups, and thus acts to decrease the affinity of the protease to the resin by decreasing the concentration of free aldehyde in the resin.

3. The example of thrombin: The thrombin utilized in this study is a crude commercial preparation from fresh bovine plasma, in which the proenzyme has been partially purified by ion-exchange precipitated with other contaminants, and then activated by bovine brain thromboplastin and calcium. As such it represents a crude preparation of 85 NIH units/mg protein specific activity, that serves as a starting preparation for further purification. Purified bovine thrombin has a reported specific activity of about 2100 NIH units/mg protein (Lundblad et al., 1976).

The sequence Phe-Ala-Argal may be expected to have a selective affinity to thrombin, based on the observed selective reactivity of the chloromethyl ketone derivatives of this sequence towards thrombin (Kettner and Shaw, 1979). Therefore, Affi-Gel-Phe-Ala-Argal resin was prepared for the study of thrombin binding. Following the application of between 20 to 200 units of impure thrombin in sodium phosphate buffer (pH 6.0) onto a 3 ml of Affi-Phe-Ala-Argal resin, approximately 72% of the enzyme activity bound to the resin. The bound thrombin activity was eluted in succeeding 10 ml washes of 0.2 and 1.0M NaCl in phosphate buffer (pH 6.0) and in a 10 ml semicarbazide containing buffer (Table

Table 10

Relative enzyme activities in 10 ml fractions of column effluents.

Resin: Affi-Gel—Phe-Ala-Argal

Enzyme: Trypsin and Thrombin (separate experiments)

Buffer: 0.025 M sodium phosphate pH 6.0

Eluting buffer	% Enzyme activity	
	Trypsin	Thrombin
Initial buffer (unbound)	0	37±8
0.2 M NaCl in buffer wash	-	47±4
1 M NaCl in buffer wash	87	37±13
0.1 M Semicarbazide in 0.1 M NaOAc pH 5.0	0	21±6

10). However, almost all the 280 nm absorbing protein (over 99%) was eluted in the initial 10 ml buffer, thus giving a dramatic purification of the thrombin in these later fractions. The thrombin eluted in the later fractions had a specific activity of equal to was at least 2100 NIH units/mg protein, which is the specific activity reported for purified bovine thrombin.

While thrombin was purified to a high specific activity on the Affi-Phe-Ala-Argal resin, the ability to elute a significant amount of thrombin activity in the 0.2 M and 1.0 M NaCl buffers indicates that thrombin binding is, to a large extent, electrostatic. However, when the ligand sequence was changed to Phe-Pro-Argal only 9+2 % of the thrombin could be eluted with 0.2 M NaCl. The remaining activity apparently bound strongly, and was only eluted in a semicarbazide containing buffer after incubation of the resin with semicarbazide for 30-60 minutes to dissociate the tightly bound thrombin (Table 11). The higher affinity of thrombin towards Affi-Phe-Pro-Argal is in agreement with the observed better affinity of thrombin for this sequence in substrate specificity and peptide inhibitor studies (Cleason and Aurell, 1981). In contrast to the high affinity shown by the thrombin to the Phe-Pro-Argal resin, thrombin had no affinity towards the Affi-Gly-Gly-Argal resin (Table 12).

4. The example of urokinase: The sequence Gly-Gly-ArgOH has a selective affinity towards urokinase and plasminogen activators, as this sequence has been utilized for the selective assay of these enzymes (Bigbee et al., 1978). On the application of 500-1000 (I.U.) of urokinase through 3 ml of a Affi-Gly-Gly-Argal resin, the urokinase activity bound tightly

Table 11

Relative enzyme activities in 10 ml fractions of column effluents.

Resin: Affi-Gel—Phe-Pro-Argal

Enzyme: Trypsin, Urokinase and Thrombin (separate experiments)

Buffer: 0.025 M Imidazole containing 0.025 M $CaCl_2$, 0.01% Carbowax 20M pH 7.0

Eluting buffer	% Enzyme activities		
	Trypsin	Urokinase	Thrombin
Initial buffer (unbound)	0	100	0
0.2 M NaCl in buffer wash	3	0	9±2
1.0 M NaCl in buffer wash	0	0	0
0.1 M Semicarbazide containing 0.1 M NaOAc, 0.025 M $CaCl_2$, 0.01 % Carbowax 20M pH 5.0	10	0	86±3

Table 12

Relative enzyme activities in 10 ml fractions of column effluents.

Resin: Affi-Gel—Gly-Gly-Argal.

Enzyme: Trypsin, Urokinase and Thrombin (separate experiments)

Buffer: 0.025 M Imidazole containing 0.025 M $CaCl_2$,
0.01 % Carbowax 20M pH 7.0

Eluting buffer	% Enzyme activity		
	Trypsin	Urokinase	Thrombin
Initial buffer (unbound)	0	0	88±6
0.2 M NaCl in buffer wash	3	-	22±6
1.0 M NaCl in buffer wash	10	13	3
0.1 M Semicarbazide containing 0.1 M NaOAc, 0.025 M $CaCl_2$, 0.01 % Carbowax 20M pH 5.0	102	71-82	0

in the presence of 1.0 M NaCl, and less than 10% of applied activity was eluted in semicarbazide buffer under standard conditions. Even washing with 0.1 N HCl and 1.0 M NaCl removed only 30-35% of the bound activity. However, the addition of both calcium and carbowax 20M (0.01%) to the eluting semicarbazide and preincubating the resin with the buffer for 30-60 min prior to elution increased the yield of eluted urokinase. In this way 86+3% of urokinase was eluted with sodium acetate buffer in 0.025 M $CaCl_2$, 0.1 M semicarbazide and 0.01 % carbowax at pH 6.0 (Table 12).

5. Separation of urokinase from thrombin on Affi-X-Y-Argal resins.

Where as urokinase bound strongly to Affi-Gly-Gly-Argal resins, it was not retarded by the Affi-Phe-Pro-Argal resins, which bound thrombin strongly. In turn, thrombin had a poor affinity to Affi-Gly-Gly-Argal, which bound tightly to Affi-Phe-Pro-Argal. Accordingly, the proteinases could be separated from one another on either of these resins, as shown in Figures 20 and 21. In Fig.20, the elution of a mixture of 83 units of thrombin and 500 units of urokinase over Affi-Phe-Pro-Argal is shown. The two proteases were dissolved in 4 ml of the imidazole buffer (pH 7.0), as described previously, and incubated with the resin with gentle stirring for 15 minutes. The resin was washed with the original buffer followed by 10 ml same buffer containing 1.0 M NaCl. All the urokinase activity and a small amount of the thrombin activity was eluted in these initial fractions. The buffer was then changed to a semicarbazide buffer (0.1 M semicarbazide, 0.1 M NaOAc, 0.025 M $CaCl_2$ and 0.01 % carbowax

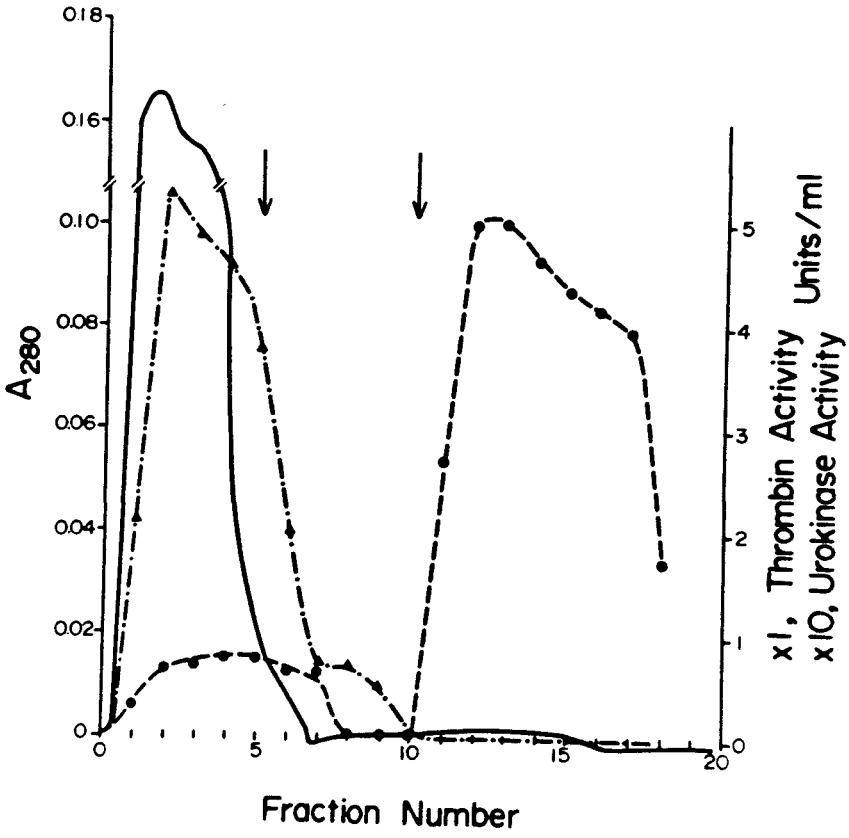


Figure-20. Separation of α-thrombin and urokinase on an **R-(Phe-Pro-Argal)** resin.

———, protein absorption (280nm) (left axis)

▲-·-▲, urokinase activity (right axis)

●---●, -thrombin activity (right axis)

First arrow shows position of the addition of the 1.0M NaCl to the buffer. Second arrow shows position of the change to buffer containing 0.1M semicarbazide, 0.1M sodium acetate, 0.025M $CaCl_2$ and 0.01% Carbowax (pH-6.0).

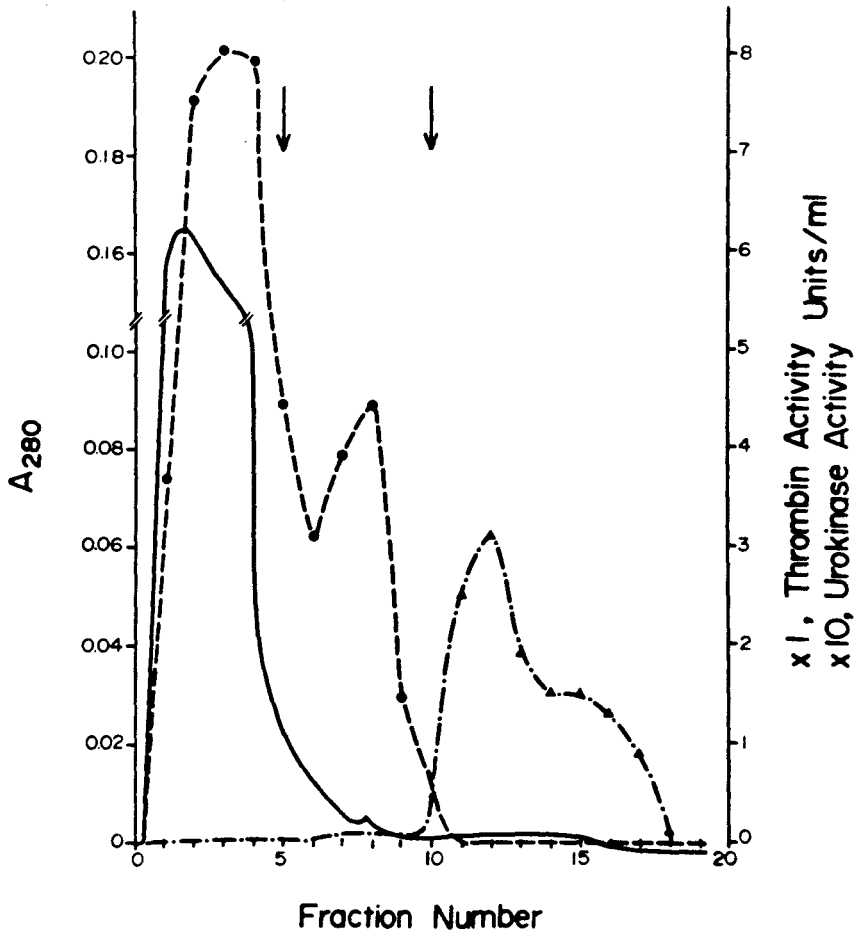


Figure-21. Separation of α-thrombin and urokinase on an R-(Gly-Gly-Argal) resin.

———, protein absorption (280 nm) (left axis)

▲-·-▲, urokinase activity (right axis)

●---●, -thrombin activity (right axis)

First arrow shows position of the addition of 1.0M NaCl to the buffer. Second arrow shows pos ition of change to 0.1M Semicarbazide, 0.1M sodium acetate, 0.025M $CaCl_2$ and 0.01% Carbowax (pH-6.0).

20M, pH 6.0), which eluted the tightly bound thrombin activity separated from the urokinase activity. In Fig.21 is shown the same experiment, but over the Affi-Gly-Gly-Argal resin. On this later resin, the thrombin activity is eluted initially, followed by the elution of the purified urokinase activity in the semicarbazide buffer.

4G Reproducibility of affinity chromatography results and regeneration of the resins

The results reported in Tables 9,10,11 and 12 are for multiple runs on a single resin preparation of any one kind of resin, that was continually regenerated after treatment with semicarbazide eluting buffers by overnight incubation in methanol/acetic acid / formaldehyde at room temperature. Some of the resins have been regenerated in this way for more than 40 experiments, without any apparent loss of their protease binding ability. In fact, over time the binding to a particular resin by a protease appears to get better, rather than poorer, perhaps due to a close packing of the resin with use.

Results between different resins preparations of the same sequence showed a greater variability (10-15%) in each of the initially eluted percentages over the standard deviation for repeated experiments over a single preparation of a resin. Accordingly, the procedure for derivatization is not systemized to yield a consistent titer of ligand between different preparation of the same sequence. This is perhaps due to variation of potency of the catalyst used in hydrogenolysis. In later stages of this work, this factor was minimized by the careful monitoring of the hydrogenolysis reaction with the Sakaguchi test, as described

previously. However, whereas differences exist between different preparations of the same Affi-X-Y-Argal sequence resin, they all show analogous behavior. Thus, all preparations of Affi-Gly-Gly-Argal showed a high affinity toward urokinase; and all Affi-Phe-Pro-Argal resins showed a relatively high affinity toward thrombin and no affinity toward urokinase.

4H Example of a General Procedure for Purification of Proteases Based on Modification of Their Natural Hydrolysis Product

1. Preparation of fibrinopeptides: Since fibrinopeptides-A constitutes less than 1% of the fibrinogen molecule, large amount of fibrinogen was utilized to get appreciable amount of fibrinopeptide-A. Fibrinogen (1 gm) (Sigma Chemical Co.) was used without further purification. Protein impurities in commercial preparation with larger than 5000 MW would be removed during membrane filtration and smaller contaminants will be removed during gel-filtration. The enzyme thrombin releases both fibrinopeptide A and B from fibrinogen while the enzyme reptilase exclusively releases the fibrinopeptide-A. The presence of fibrinopeptide-B will not contribute to the coupling on the resin as its NH_2-terminal group is blocked and there are no lysines in its sequence therefore there is no functional amino group in FP-B to join to resin matrix. The stoichiometry of thrombin induced release of fibrinopeptides from fibrinogen theoretically release 5.4 mg (3.52 micro moles) fibrinopeptids-A from 1 gm of fibrinogen. The Sakaguchi test showed the presence of 5.1 to 5.7 mg of fibrinopeptide A and B combined in

$CH_3OH:H_2O$ (75:25) extracts, which is about 50% of the theoretical yield. The fibrinopeptide fraction from the gel-filtration indicated the presence of 3.9 mg (36%) of fibrinogen. Under these circumstances of low yields and a long procedure, it was decided to purchase fibrinopeptide-A from Sigma Chemicals for further experiments.

2. Synthesis of Affi-FPA: Because fibrinopeptide-A is a highly acidic protein (pI about 3), Affigel-15 which contain a cationic charge in the spacer arm, has a significantly higher coupling efficiency than Affigel-10 as shown in the experiment described below. Out of 2 mgs (1.3 uM) of fibrinopeptide-A (from Sigma Chemicals), 1.63 mg was coupled to 5 ml of Affigel-10 in CH_3OH/ imidazole (0.05 M at pH 7.5) / 2.5 M $CaCl_2$ (7.5/2.5/0.3) overnight at room temperature. In absence of Ca^{+2}, the coupling of FPA with Affigel-10 was negligible under identical conditions. When Affigel-15 was used, almost all fibrinopeptide-A was coupled at 4^oC in one hour. Since Affigel-15 has 15 umoles /ml coupling capacity, unreacted active groups were coupled with ethanolamine by adding 3 ml 1 M ethanol amine after the fibrinopeptide-A coupling was completed, and letting it react at room temperature for four hours. After the reactions were completed, the resin gave a strong positive Sakaguchi test, indicating the presence of the arginine containing FPA on the resin.

4I Substitution of terminal Arginine by Argal

1. Direct substitution: The synthesis of Affi-FPA-Argal was carried out in three different ways. In the first approach Arg-Sc was directly

substituted for arginine using carboxypeptidase-B as a transacylation reagent and Arg-Sc as a nucleophile. In the second approach, arginine was removed by carboxypeptidase-B and Arg-Sc was attached to desarginine-FPA using a chemical method such as an active ester procedure or a carbodiimide coupling procedure, with or without blocking the side chain COO^- groups of glutamates and aspartates. In the third approach, the Affi-desArg-FPA was prepared followed by addition of Arg-Sc at the COO^- terminal using an enzyme as tool to from a peptide bond.

The procedures utilized for the preparation of Affi-FPA-Argal using carboxypeptidase-B are outline in Fig.22. It is difficult to monitor the substitution reaction because of presence of large excess of Arg-Sc. Since there is even exchange of guanidino group, the qualitative test for guanidino groups on resin should remain the same. This leaves aldehydic functional group as the only means to follow the substitution reaction. Affigel is an agarose and gives some background towards tests of aldehyde function, and since the concentration of exchanged Arg-Sc is very low, an accurate estimation is extremely difficult. Despite this difficulty, the qualitative test of resin with DNP indicated the presence of a small amount of Arg-Sc.

Carboxypeptidase-B (CPB) catalyzes hydrolysis of the basic amino acids lysine, arginine and ornithine from the C-terminal position in polypeptides. Because of its high specificity for basic amino acids and relatively very low activity towards other amino acids, it may be possible to carry out trans-peptidation using CPB to exchange arginine selectively. Since the free COO^- end group is required for CPB action,

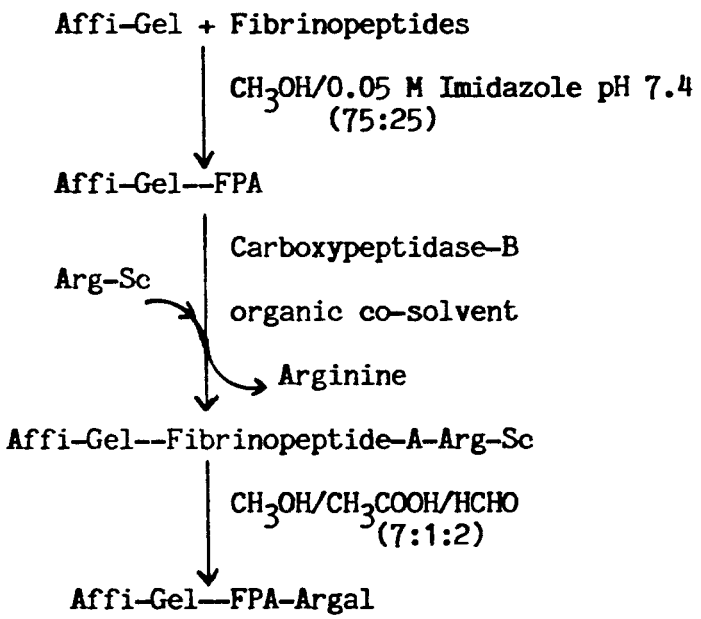


Figure-22. Synthetic scheme utilized in the synthesis of a FPA-Argal liganded affinity resin using carboxypeptidase-B and the Arg-Sc as a nucleophile. **(Direct substitution)**

the substituted FPA-Arg-Sc will not be a substrate for CPB and the FPA-Arg-Sc product, once formed, should be stable in the presence of CPB. Sealock and Laskowski, 1969) utilized the same strategy to substitute lysine for terminal Arg-64 in soybean trypsin inhibitor by CPB using a large amount of trypsin as the product trapping reagent. Along with trans peptidation, hydrolysis also occurs during the reaction because of the competition of solvent water as a nucleophile. This competition could be minimized by a large excess of Arg-Sc and the use of organic solvent which inverses the equilibrium towards peptide bond formation. The concentration of Arg-Sc is controlled by limited however, by the solubility of the semicarbazone derivative, leaving organic solvent as the major factor to promote peptide bond formation. Table 13 shows four different combinations of organic solvents utilized for preparation of Affi-FPA-Argal and the results observed for the binding of thrombin to the product resin. The activities of unbound fractions (from 33 to 51 %) indicate a lack of strong affinity of the column towards thrombin. Mixed solvents using 1,4-butanediol or methanol produced better results compared to ethylene glycol or ethylene glycol monomethyl ether. Thrombin was retarded to a certain extent in all columns prepared by this approach, but the binding characteristics of the columns were far below expected levels.

2. Chemical Modifications: The procedures utilized for the preparation of Affi-FPA-Argal using chemical modification are outlined in Fig.23. The side chain COO^- groups were blocked with ethanolamine using a water soluble carbodiimide as a coupling agent, to prevent the

Table 13

Relative enzyme activities in 10 ml fractions of column effluents.

Resin: Affi-Gel—FPA-Arg̃al (prepared by direct substitution using Carboxypeptidase-B)

Enzyme: Thrombin

Buffer: 0.025 M Cacodylate containing 0.025 M $CaCl_2$, 0.01% Carbowax 20M pH 6.0

Eluting buffer	% Enzyme activities			
	CH_3OH/ 0.1 N $NaHCO_3$ (1:1) overnight	E tylene glycol 100% overnight	Ethylene glycol 100% 6 hours	1,4-butanediol 0.1 M $NaHCO_3$ (2:1) overnight
Initial buffer (unbound)	51	73	77	33
0.2 M NaCl in buffer wash	51	10	10	-
1.0 M NaCl in buffer wash	0	0	-	25
0.1 M Semicarbazide containing 0.1 M NaOAc, 0.025 M $CaCl_2$, 0.01 % Carbowax 20M, pH 6.0	0	-	-	0

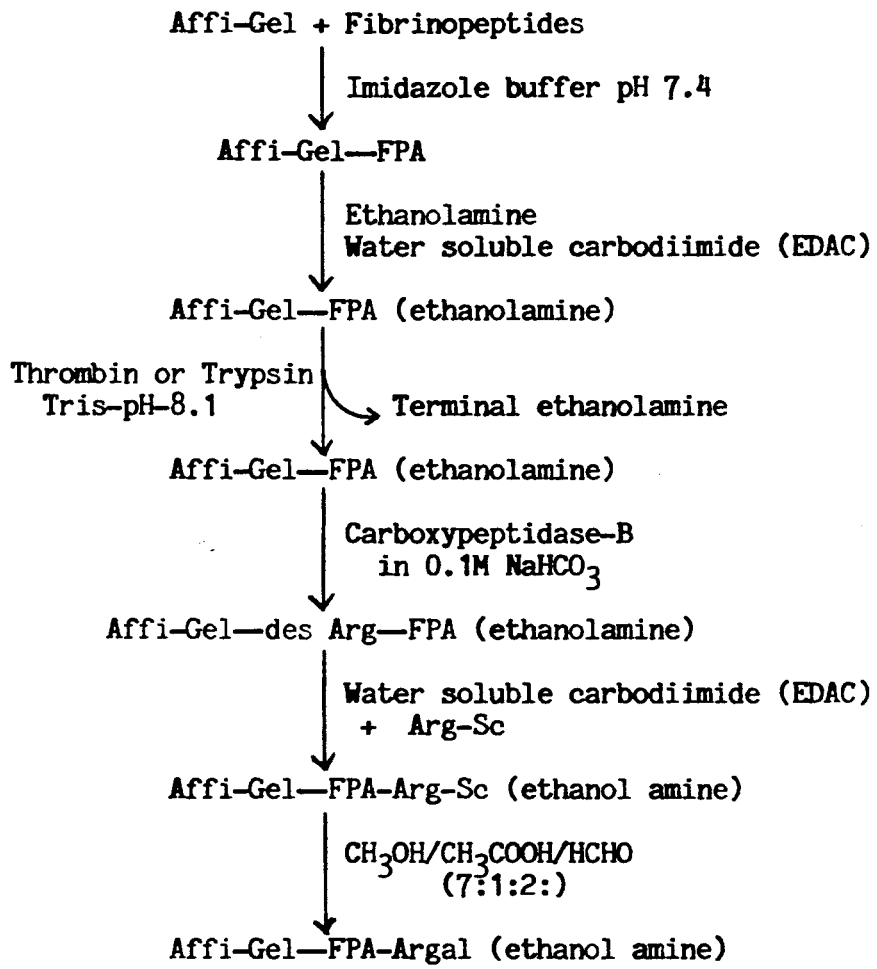


Figure-23. Synthetic scheme utilized in the synthesis of a FPA-Argal liganded affinity resin. (Chemical method)

coupling of Arg-Sc with side chain COO^- groups. This procedure produces an amide bond formed between the COO^- and the NH_2 of ethanolamine. The COOH-terminal ethanolamine was removed by treating the resin with trypsin which specifically hydrolyzes only the C-terminal Arginine-NH-CH_2-CH_2OH bond generating a free COO^- terminal. This COO^- group is crucial for CPB action in the removal of the COOH-terminal arginine from fibrinopeptide-A and forming desArg-FPA. After a number of trials for various lengths of time and different temperatures, it was concluded that 40 minutes at room temperature is sufficient to remove most of terminal arginine. The maximum amount of arginine released from the Affi-FPA was 0.428 umoles (0.66 mg out of 1 mg total on the resin) in the first 30 minutes of reaction. This quantitation of released arginine is lower than its actual value because of interference from buffer components during the Sakaguchi test for arginine. A qualitative test for arginine on the resin after CPB treatment showed a very low amount of residual arginine on the column. After 30 min of of reaction, the residual arginine could not be remove even after 24 hr reaction at 37°C. However the exposure of resin to CPB of more than 40 minutes at room temperature could remove other terminal amino acids, producing heterogeneous ligands on the resin.

The binding studies of thrombin with the resin were carried out in a similar fashion as with the Affi-Phe-Pro-Argal resin. Table 14 shows thrombin activities in various fractions of eluents during different stages of preparation of the Affi-FPA-Argal resin. It could be seen that while the virgin fibrinopeptide-A has some affinity towards thrombin, the

Table 14

Relative enzyme activities in 10 ml fractions of column effluents.

Resin: Affi-Gel—FPA at various stages.

Buffer: 0.025 M Imidazole, 0.025 M $CaCl_2$, 0.01% Carbowax pH 7.0

Enzyme: Thrombin

Eluting buffer	% Enzyme activity		
	Affi-FPA	Affi-FPA Ethanol amine	Affi-FPA-Argal Ethanol amine
Initial buffer (unbound)	68	90	4
0.2 M NaCl in buffer wash	14	6	11
1.0 M NaCl in buffer wash	0	0	0
0.1 M Semicarbazide containing 0.1 M NaOAc, 0.025 M $CaCl_2$, 0.01 % Carbowax 20M, pH 5.5 (overnight incubation)	-	-	2
50 mM Benzamidine in buffer	-	-	0
3 M KSCN	-	-	0

ethanolamine treated fibrinopeptide-A does not have any affinity towards thrombin and the ethanolamine treated fibrinopeptide-A-Argal prepared by chemical method has high affinity towards thrombin. All efforts to elute thrombin from the column failed including 3 M KSCN. It should be noted here that this particular resin was reacted overnight with CPB to remove terminal arginine,and only 30 minutes is required to remove all the releasable terminal arginine. However, when only 40 minutes are allowed for removal of terminal arginine to assure maximum homogeniety of the peptide ligand, and COO^- side chain groups are not blocked with ethanolamine, lower affinity of the column to thrombin was observed (Table 15).

An alternative to ethanol amine as a protecting group for COO^- side chains is the use of esters. These esters could be easily hydrolyzed under basic conditions. Methyl esters are an attractive alternate to ethanolamine groups which could be hydrolyzed easily above pH 10.5. Proteins may be sensitive to the acidic conditions of the standard esterification procedure which utilizes dry HCl in absolute methanol. In addition to denaturation, there is always a danger of O-alkylation and N-alkylation reactions. It would be prefered to carry out the methylation in an aqueous medium. However, the main difficulty in such a procedure, is the competition of water with the protein for the reagent. Trimethyloxonium tetra fluoro borate (TMOFB) was introduced by Meerwein to selectively esterify carboxylic acids in aqueous solutions. This reagent has been used to specifically modify the free carboxyl groups in proteins at pH 4 to 5 (Osbahr, 1982; Peterson and Knowles, 1972; Hamada

Table 15

Relative enzyme activities in 10 ml fractions of column effluents.

Resin: Affi-Gel—FPA-Argal (prepared by chemical method)

Enzyme: Thrombin

Buffer: 0.025 M Imidazole containing 0.025 M $CaCl_2$, 0.01 % Carbowax 20M. pH 7.0

Eluting buffer	% Enzyme activity Affi—FPA-Argal
Initial buffer (unbound)	0
0.2 M NaCl in buffer wash	70
1.0 M NaCl in buffer wash	10

Table 16

Relative enzyme activities in 10 ml fractions of column effluents.

Resin: Affi-Gel—FPA-Argal (prepared with $-OCH_3$ protection)

Enzyme: Thrombin

Buffer: 0.025 M Imidazole containing 0.025 M $CaCl_2$, 0.01 % Carbowax 20M. pH 7.0

Eluting buffer	% Thrombin activity
Initial buffer (unbound)	64
0.2 M NaCl in buffer wash	27
1.0 M NaCl in buffer wash	n.d.

and Yonemitsu, 1971). The half life of TMOFB in water at pH 4 to 5 is 10 minutes. This is sufficient time to selectively modify the free COO^- groups in proteins in aqueous conditions.

Fibrinopeptide-A was modified with TMOFB as described in the Methods Section and was reacted with Affigel. In the procedure a 100-fold excess of reagent was dissolved in to dry acetonitrile and then added slowly to the aqueous peptide solution. In order to achieve maximum efficiency, the pH was controlled between 4.5 and 5.5 using 0.5 M NaOH. This modified fibrinopeptide-A was reacted with Affigel-15, as described previously. In case of ethanol amine protection, the modification is carried out after FPA was attached to the resin. This was not done in esterification because maximum concentration could be obtain in solution rather than in immobilized form on the resin during its reaction with TMOFB. The terminal arginine methyl ester was selectively hydrolyzed using thrombin or trypsin. The terminal arginine was removed by action of CPB and Arg-Sc was added using EDAC at pH 4.5 where the esters are stable. The methyl ester protection of side chain COO^- groups was removed at room temperature at pH 11.0. Table 16 shows thrombin binding of different modified forms of the resin during the synthesis of Affi-FPA-Argal using the methyl ester protection method. From the Table 16 it is evident that the resin prepared in this way lacks, a strong affinity towards thrombin.

3. Enzymatic synthesis: The procedure utilized for the preparation of Affi-FPA-Argal using papain as reagent to form a peptide bond is outlined in Fig.23. In this approach the Affi-desArg-FPA resin was

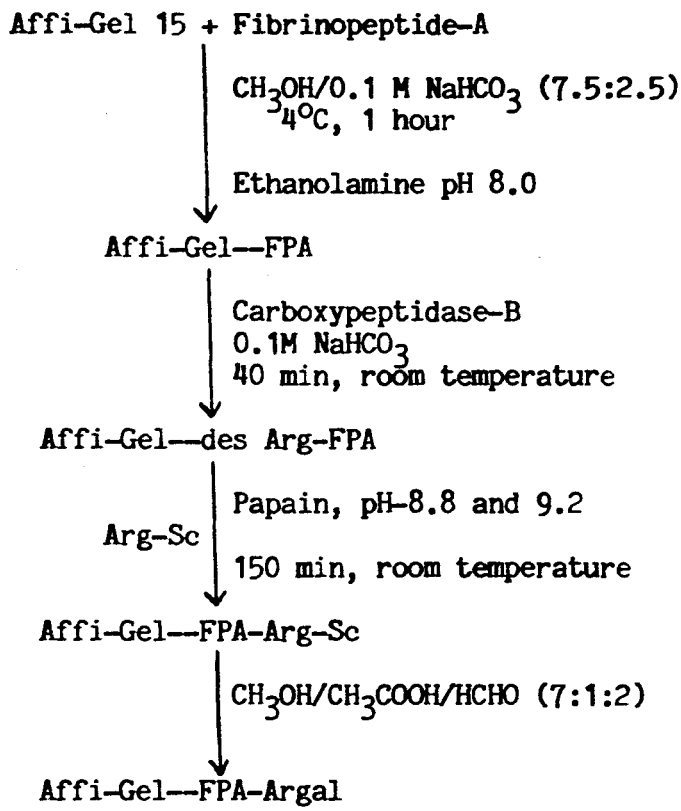


Figure-24. Synthetic scheme utilized in the synthesis of a FPA-Argal liganded affinity resin. (Enzymatic method)

prepared as usual and the Arg-Sc added to the desarginine fibrinopeptide ligand using papain, as described by Mitin et al. (1984). In this method, a low affinity between thrombin and Affi-FPA-Argal (Table 17) was observed. Qualitative test for presence of arginine showed very little arginine on the column by the the Sakaguchi test.

4J. Separation of urokinase from thrombin on Affi-FPA-Argal column:

Since both proteinases has different specificity, urokinase should not show an affinity towards Affi-FPA-Argal resins. Even though appreciable affinity was not observed in any of the preparations, the resin prepared without protecting side chain COO^- groups (chemical method) showed some affinity towards thrombin. This preparation was utilized to try to separate urokinase and thrombin from a mixture. Table 18 shows the activities of thrombin and urokinase in different fractions of elution of a 84 units of thrombin and 500 units of urokinase over Affi-FPA-Argal column. The procedure was same as described for the separation of two proteases over Affi-Phe-Pro-Argal column. The resin showed slightly greater affinity towards thrombin than to urokinase, but the difference is too small for practical purposes.

Table 17

Relative enzyme activities in 10 ml fractions of column effluents.

Resin: Affi-Gel--FPA-Argal (prepared by enzymatic method)

Enzyme: Thrombin

Buffer: 0.025 M Imidazole containing 0.025 M $CaCl_2$, 0.01 % Carbowax 20M. pH 7.0

Eluting buffer	% Thrombin activity
Initial buffer (unbound)	20
0.2 M NaCl in buffer wash	65
1.0 M NaCl in buffer wash	n.d.

Table 18

Relative enzyme activities in 10 ml fractions of column effluents.

Resin: Affi-Gel—FPA-Argal (prepared by chemical method)

Enzyme: Mixture of Thrombin and Urokinase.

Buffer: 0.025 M Imidazole containing 0.025 M $CaCl_2$, 0.01 % Carbowax 20M. pH 7.0

Eluting buffer	% Enzyme activity	
	Thrombin	Urokinase
Initial buffer (unbound)	17	62
Additional 10 ml of initial buffer wash	1	2
0.2 M NaCl in buffer wash	55	36

CHAPTER V

DISCUSSION

5A. Preparation of affinity resins

1. Argal as ligand: Argal resins could be synthesized either by immobilization of arginine onto the resin followed by reduction of COO^- group of arginine to an aldehydic group or by reducing the arginine COO^- prior to immobilization. There are a number of technical difficulties in the first approach such as possible side reactions with the matrix and the physical separation of side products or solid reagent catalysts. The second approach appears technically more feasible and cleaner. It also allows characterization of ligand prior to its immobilizationan on the resin.

Since the work was started in 1979, a number of new syntheses have been reported for α-aminoaldehydes from amino acids and alcohols. The chemo selective reduction of acids to aldehydes (Fujisawa et al.,. 1983) and the oxidation of N-protected β-amino alcohols using a combination of sulfur trioxide-pyridine complex and DMSO in the presence of triethyl amine to optically active α-amino aldehydes (Hamada and Shoiri, 1982) are good examples of new synthetic procedures. These techniques allow preparation of optically active α-amino aldehydes from most of the amino acids and the preparation of peptide aldehydes to be used as specific inhibitors.

In this work preparation of affinity resin containing α-amino argininals as a ligands have been established. Possibilities still to be explored include the use of phenylalaninal as a ligand in affinity resin for the purification of proteases with chymotrypsin-like specificity.

In preparation of affinity resins the spacer arm plays very important role in binding efficiency. When $(Gly)_n$-Tyr-NH_2 was used as ligand to bind chymotrypsin, the resin did not show affinity when n=0. The resin only showed affinity with n=3 or 4 (Blumberg and Katchalski-Katzier, 1979). This type of steric effect of the resin in preventing association of the enzyme where the ligand is bound directly to the resin may be one of the reasons for the failure to of the Enzacryl-Argininal resin to bind trypsin, as no spacer arm group was present. In addition to the length of the spacer arm, the ionic character of the spacer arm is also important. This is demonstrated by the increased efficiency of coupling of the negatively charged fibrinopeptide-A with Affigel-15 containing a positively charged spacer arm, with respect to its binding to the Affigel-10.

The Sakaguchi test for guanidino group is convenient for qualitative and quantitative determinations of arginine. The extreme ease and speed of the test is useful in monitoring reactions involving guanidino group. During the removal of the NO_2 guanidino protection with hydrogenolysis it was important to remove the NO_2 protection and limit any undesirable reduction of the C=N Schiff's base double bond. The Sakaguchi test was necessary in monitoring this deprotection reaction. In addition to the guanidino group monitoring, aldehydic assays were

also crucial during hydrogenolysis. Preliminary work indicated that this could be easily accomplished spectrophotometrically using pararosaniline. Quantitation of aldehydic function on the resin may not be accurate because the reaction of p-phenyl azoaniline with aldehyde may not go to completion and its subsequent transfer to salicyl aldehyde might also fall short of complete reaction. This test establishes a minimum value for the presence of aldehydic group. Affi-Argal showed minimum of 0.1 μm/ml of aldehyde by this test. Considering a 1:1 stoichiometry between trypsin and Argal, the resin could bind 2.5 mg of trypsin per ml of resin. The theoretical capacity of the resin is 375 mg of trypsin per ml of resin based on 15 μm/ml of resin. However, it is impossible to achieve this binding capacity due to steric reasons. At our minimal capacity of 2.5 mg trypsin/ml resin, 3 ml of column can purify significant quantities of protease in pure form. In comparison, a 3 ml gel-filtration or ion-exchange column could only obtain a small amount of pure enzyme.

5B Procedure for purification of serine or cysteine proteases utilizing the acid product of a protease reaction

A number of biological events are controlled by proteolytic action. In many instances the product of a biologically important proteolytic action is known and well characterized, but the protease itself has not been defined or characterized. For example during the induction of lytic growth of the Escherichia Coli prophage lambda, the C1-repressor protein is cleaved. The hydrolysis products of this reaction are well

characterized (Roberts and Devoret, 1983). Rec-A protein is belived to play an important role in this process. The proteolytic cleavage occurs between alanine and glycine in comparable positions of the four polypeptides of repressor protein. Affinity resins could be prepared for purification of the protease responsible for the proteolytic process in similar fashion as described for fibrinogen-thrombin reaction. This could be done by immobilizing repressor on a resin and cleaving the repressor with a cell lysate solution containing the protease, followed by the conversion of the newly formed COOH-terminal alanine to alaninal. A presupposition here is that the protease is a serine or cystine protease.

The example of fibrinogen-thrombin is described in this work. Immobilization of neutral proteins or peptides do not present a major problem with most of the matrices, but highly basic or acidic proteins or peptides need a careful selection of the matrix or spacer arm. Immobilization of the highly acidic fibrinopeptide-A was very efficient with Affigel-15, which contains a positively charged spacer arm. With the Affigel-10, which contains a neutral spacer arm, appreciable coupling was obtained only after addition of Ca^{+2} to the coupling solution. Monitoring the coupling reaction with Sakaguchi test provides both qualitative and quantitative results. Since most proteins contain arginine, this test could be adopted for monitoring coupling reactions between proteins and matrix. Large proteins may not require a spacer arm for efficient binding, if the protease binding site in the protein substrate is reasonably far away from the matrix.

5C Modification of COOH-terminal peptidyl amino acid to an aldehydic function.

In the biosynthesis of leupeptin, the leupeptin acid is reduced to leupeptin by leupeptin acid reductase (Umezawa and Aoyagi, 1983). This membrane bound enzyme could perhaps be used to reduce terminal COO^- groups. However, since the enzyme is membrane bound, it is difficult to use in in the laboratory preparation of Argininal. In addition it appears specific to the Leu-Leu-Arg sequence. The chemical approaches taken in this work for substituting the terminal amino acid by the corresponding amino aldehyde are more practical and general.

The direct substitution of Arg-Sc using carboxypeptidase-B seemed to be a better alternate than to use carboxypeptidase-Y or other carboxypeptidases. Since the rate of removal of a terminal amino acid by carboxypeptidase-B is much higher for basic amino acids, there is a much higher probability of obtaining a homogeneous ligand. The penultimate amino acid in fibrinopeptide-A is valine and carboxypeptidase-B has a low activity towards valine. The use of carboxypeptidase-Y in trans peptidation reactions produced complex mixtures of products (Breddam *et al.*, 1981). In addition to the carboxypeptidase activity, carboxypeptidase-Y has an amidase activity, which resulted in the subsequent formation of secondary products.

Addition of organic co-solvents or change in pH or ionic strength of the buffer changes the equilibrium constant between hydrolysis and amidation. Addition of methanol or 1,4-butanediol to the reaction media

thus alters the equilibrium catalyzed by carboxypeptidase-B in favour of transpeptidation.

The resin Affi-FPA-Argal produced by carboxypeptiase B with added Arg-Sc nucleophile in 1,4-butanediol showed some affinity towards thrombin. This affinity is not high enough to be useful, but the low activity could be solely due to a low total concentration of the desirable ligand, FPA-Argal. The DNP test on the resin showed the presence of very little aldehydic function.

Affi-FPA-Argal resins prepared by chemical procedures showed a higher affinity towards thrombin (Tables 14,15). Although the pK_a's of side chain COO^- and terminal COO^- groups are expected to be different, it is difficult to join Arg-Sc on just the terminal COO^- selectively. This could be accomplished only by first blocking the side chain COO^- groups by methylation or with ethanolamine. Blocking the COO^- sidechain groups by methylation results in decrease solubility of the protein, but since the reaction was carried out in 75% methanol, this appears not to pose a problem.

After modification of all the free carboxyls by methylation, the terminal COO^- was generated by the treatment with trypsin or thrombin, both enzymes serve the same purpose. In case of an unknown protease the biological solution containing the protease could be used to generate the terminal COO^- group.

To remove the terminal amino acid, carboxypeptidase proteases are an excellent reagents. An appropriate carboxypeptidase has to be selected for efficient removal of just the terminal amino acid. The

time, concentration and temperature conditions need to be carefully worked out. In the case of carboxypeptidase-B, the high selectivity for basic amino acids provides a very convenient method of selectively removing the terminal arginine. Also the reaction is easy to monitor with the Sakaguchi test. However, small residual amount of arginine remained on the resin, even after overnight treatment at 37 ^{o}C. The arginine remained perhaps because some arginine may not be accessible to CPB due to steric reasons. The work presented establishes the procedure to remove terminal arginine and also a method for monitoring the reaction is described.

After addition of Arg-Sc to the des-Arginine (γ and ϵ-methyl)-FPA, the removal of the methyl group protection of from the side chain COO^{-} groups is important in order to restore the substrate to its natural form. Ethanolamine protection is not possible to selectively remove. The methyl esters are labile at basic conditions and treatment with sodium carbonate at pH 11 may be safe and effective for most protein substrates. Proteins and peptides can usualy survive this basic conditions for a short time without denaturation.

Enzymatic synthesis, contrary to chemical synthesis, does nor require a special activation of the carboxyl group. Also a consequence of this specificity functional side groups of amino acids do not have to be protected. The additional advantages of enzymic synthesis are stereo specificity and milder reaction conditions. Since the penultimate amino acid in fibrinopeptide-A is valine, neither trypsin nor chymotrypsin have a primary specificity for the terminal amino acid and hence can not

be utilized as reagents.

In thermolysin catalyzed peptide bond synthesis, the enzyme exhibits preference for a hydrophobic amino acid as the donor of the carboxyl group for the newly formed bond (Wayne and Fruton, 1983). The relative rate of the peptide bond synthesis with Z-Val-OH was observed to be 0.03 % compared to Z-Phe-OH. This indicates that thermolysin is not a good choice for formation of a Val-Arg-Sc bond.

Papain has broad primary specificity towards substrates and therefore was selected as a possible reagent to form the Val-ArgSc bond. The kinetic approach described by Mitin et al., (1984) was used at pH 9.2. This pH value was selected to minimize hydrolysis of fibrinopeptide-A and to maximized formation of the peptide bond between desarginine fibrinopeptide-A and Arg-Sc. The peptidase activity of papain above pH 8.0 is negligible, but the synthesis activity at this pH is high. However, the Sakaguchi test on the resin Affi-FPA-Argal prepared by this method showed very little argininal present on the resin. In the pilot experiments by MItin to establish the reaction conditions, protected amino acid methyl esters and dipeptide methyl esters were used(Mitin et al., 1983). In addition, Mitin found the rate of synthesis is generally inversly proportional to the length of COO^- donating peptide. With respect to the amine component, dipeptides are better nucleophiles than mono amino acids or other small amines (Mitin et al., 1983; Bajkowski and Frankfater, 1983; Petkove and Stoineva, 1984). DesargFPA is 15 amino acids long and therefore the rate of synthesis may be expected to be slow, and the nucleophile Arg-Sc is

only a mono amino acid making it disadvantageous from both sides.

Among all the three different approaches to substitute ArgSc for arginine in Affi-FPA, the chemical synthetic approach showed some success, with a minimum of 66% removal of terminal arginine by CPB followed by high degree of addition of Arg-Sc to the desArg ligand. The total concentration of the FPA on the resin is 0.6 nanamoles/ml, and after chemical modification, the concentration of FPA-Argal could be as much as 0.4 nanamoles/ml. This is a low concentration compare to tripeptide aldehyde resins (0.1 to 0.21 micromoles). It translates into a capacity of 0.034 mg (75 units) of total thrombin binding capacity. This capacity of the resin could be increase by increasing the ligand concentration.

Nishikata et al., (1981) and Ishii and Kasai (1981) have previously reported the preparation of Leu-Argal and Gly-Gly-Argal affinity resins for purification of Streptomyces grieses trypsin and bovine trypsin. In their work, strong acid was used to decomposed a semicarbazone derivatized resin to free aldehyde and either strong acid (50 mM HCl) or $1x10^{-4}$ M leupeptin was used to elute the tightly bound trypsins from the resins. In the leupeptin containing buffers, the dissociated enzyme is eluted as an inhibited complex with the leupeptin inhibitor. The leupeptin then needs to be dialyzed or destroyed by $NaBH_4$ reduction before the appearance of catalytic activity in the eluted fractions.

In this work, it was found that treatment of our resins with a strong acid was destructive of the trypsin binding properties to the resin. However, the removal of the semicarbazone protection in acetic

acid/methanol/formaldehyde, as described, was found to be non-destructive as repeated treatments with this reagent did not have any detrimental effect on proteinase affinity. Furthermore, the removal of tightly bound proteases with semicarbazide containing buffers rather than buffer containing proteinase inhibitors has the advantage of not requiring additional steps before the generation of a catalytic activity in the eluted enzyme fractions. Nonspecific adsorption of enzyme on the resin and the glassware was prevented by including a surfactant, carbowax-20M, in the buffer.

5D Association of proteases to affinity resins.

As argued in Chapter 2, the the peptidyl aldehyde ligands associate serine proteases by forming a reversible covalent hemiacetal with the active site serine, as shown in Fig.2, where E-OH is the active site serine γ-OH of the enzyme and RCH=O the peptidyl aldehyde. The rate of hemiacetal formation has been shown in the serine proteinase chymotrypsin to be general base-specific acid-catalyzed, as depicted in Fig.2 (Kennedy and Schultz, 1977) and the hemiacetal (IIB in Fig.2) has a similarity to the tetrahedral intermediate and/or transition state formed during substrate catalysis (Kennedy and Schultz, 1977; Thompson, 1973; Chen et al., 1979; Thompson and Bauer, 1979). The free energy for aldehyde association is much greater in the enzyme active site (approximatly 2.8 to 3.0 Kcal/mole) for aldehydes with a specific P_1 side chain than for their amide substrate analogs (Thompson and Bauer, 1979). Based on this value, peptide aldehyde will associate about 1000

fold strongly to the enzyme than the same peptide without the aldehydic group. From X-ray crystallography, non-covalent complexes between serine proteases and specific ligands are characterized by low internal mobility, and covalent reactions within these complexes should therefore occur with abnormally favourable entropies of reaction (James et al., 1980). In the case of a protease catalyzed substrate hydrolysis, the transition-state is a high energy ester with a pyramidal carbonyl carbon atom and with the carbonyl oxygen remaining in the strongly polarizing electrostatic field of the oxyanion hole. Contrary to this, in case of peptide aldehydes, the hemiacetal is a low energy complex with a protonated oxygen in the oxanion hole (productive mode in Fig 6) or the aldehydic C_1 hydrogen in the oxyanion hole (non-productive mode in Fig 6).

The high affinity of the aldehyde ligands to serine proteases is supported by the affinity of the Affi-Argal resin to trypsin, which is significantly greater than to an affinity resin in which trypsin is associated non-covalently. This is shown by the ability to elute trypsin from the non-covalent affinity resins such as lysine or benzamidine liganded resins by high salt and/or low pH, where as the Affi-Argal resin binds trypsin irreversibly under these conditions. In addition to the aldehydic function, the importance of side chain interactions is clearly demonstrated by the preferential affinity to trypsin over chymotrypsin, which clearly shows that the guanidinium group is also required for the selectivity and specificity of binding.

The Affi-Argal resin serves as a model for the preparation of

highly effective affinity resins using specific peptidyl aldehyde ligands. The tighter binding to the Argal ligand than to non-covalent binding resins show that the Argal resin can be used for the affinity purification or inhibition of proteases at low protease concentrations. In addition, as peptidyl aldehydes do not show any significant pH dependency between pH 3 and 8 for association of proteases, affinity purification of proteases can be carried out at pH<7.0 at which pH values most proteases are catalytically inactive and, thus, autocatalytic degradation will not be of consequence.

By keeping Argal in the terminal position and extending the ligand towards the N-terminal, the specificity of the ligand was increased to discriminate between proteases in the same class. The combination of secondary specificity with the aldehydic function makes these ligands highly specific and potent inhibitors. Thus, the peptide aldehyde D-Phe-Pro-Argal has shown to have a binding constant towards thrombin of between 4.5×10^{-7} and 7.5×10^{-8}M (Bajusz et al., 1978; Fareed et al.,1981). As peptide argininals in water are in equilibrium with its aldehyde hydrate and with a tautomeric carbinolamine structure formed by the reversible addition of the guanidino nitrogen to the aldehyde function, the concentration of free aldehyde available to participate in the equilibrium with the enzyme has been shown by NMR to be 1% of the total concentration of the peptide in aqueous solution (Kozlowski and Schultz, unpublished results). Accordingly, based on the concentration of free aldehyde, the true K_i for the binding of D-Phe-Pro-Argal is in the range of 10^{-11}M. The binding constant to L-Phe-Pro-Argal is

estimated to be 100 fold poorer than for D-Phe-Pro-Argal to thrombin (Bajusz et al.,1978). Thus, the corrected binding constant to the Phe-Pro-Argal ligands in Affi-Phe-Pro-Argal can be estimated to be near 10^{-8} to 10^{-10} M. Similarly the estimated binding constant of Affi-Gly-Gly-Argal is expected to be near 10^{-7} to 10^{-8} M (Ahsan, Pagast and Schultz, unpublished results). These low values of K_i for the binding of specific peptidyl argininals to proteinases approach those binding constants observed between an antibody and an antigen. Even lower values of K_i than to the serine proteinases can be calculated for peptidyl aldehyde binding to cysteine proteases (Westerick and Wolfenden, 1972; Frankfater and Kuppy, 1981). Accordingly, the peptidyl aldehyde liganded resins will have an extremely high affinity to both serine and cysteine proteases and can potentially be utilized to purify proteinases present in very low concentrations in biological fluids.

The ability to bind a particular proteinase by variation of X-Y represents an amplification of normal peptide binding specificity similar to that previously observed for peptide aldehyde inhibitors of chymotrypsin, elastase and papain (Thompson, 1973; Kennedy and Schultz, 1979; Thompson and Bauer,1979; Wolfenden, 1972). For example, in the case of chymotrypsin, an increased affinity of approximately 140-fold can be calculated for the binding of the specific aldehyde Bz-L-Pheal over that of the non-specific aldehyde hydrocinnamaldehyde (after correction for hydration), whereas the respective K_s values for the substrates Bz-L-Phe-NH_2 and hydrocinnamide differ by a factor of only two (Kennedy and Schultz, 1979; Schultz and Cheerva, 1975). This

amplification of normal differences in binding affinities for specific aldehyde ligands over non or less-specific aldehyde ligands is due to an increse in the ratio of k_2/k_{-2} in fig-2, and may reflect the greater complementarity of the more specific analog to the transition state configuration of the enzyme.

I had a limited success with fibrinopeptide Argininal resins. The tighter binding was observed in a case in which overnight reaction with carboxypeptidase-B was carried out to prepare desArg-FPA on the resin. In this case the exact nature of the ligand is unknown, but irreversible binding was observed. In other preparations, where the nature of ligand could be predicted to be FPA-Argal with a certain certainty, the affinity is poor for thrombin. In our prediction of a high affinity for the ligand, we are assuming that the conformation of product (P_2) is retained, as in the parent protein molecule, even after proteolytic cleavage and also after its immobilization on the matrix. Pentamer to decamer peptides form inter molecular β-structure in the solid state and in some solvents; and an eicosapeptide exhibits a high percentage of α-helical structure both in the solid state and in solution (Toniolo et al, 1984). X-ray analysis of a linear hexa and octa peptides suggests formation of a hairpin shaped conformation stabilized by multiple intra-molecular H-bonds (Bavaso et al., 1982; Lagant et al., 1984). However the immobilized fibrinopeptide-A-Argal may assume a stable conformation which could be different from fibrinopeptide-A portion of fibrinogen. In addition after proteolytic cleavage, conformation changes often occur in the protein around the proteolytic cleavage site. For example, a

monoclonal antibody study shows that the amino terminus of the $A\alpha$-chain in human fibrin exhibits a different conformation than in human fibrinogen (Hui and Haber, 1983). If the proteolytic cleavage occurs between two domains of a large protein the conformational change may not be drastic. Thus the example of fibrinopeptide-A-thrombin could be a poor choice to demonstrate the idea of a general procedure to prepare affinity resins using aldehyde transition state analog. Other cases,such as the plasminogen-plasminogen activator system, might prove to be more successful.

As many trypsin-like proteinases often coexist in a particular biological compartment or pathway (Reich et al., 1975), it is of importance to be able to separate a particular trypsin-like proteinase from other proteinases of similar primary binding site specificity for arginine or lysine. This work shows the ability of the R-(X-Y-Argal) resins to separate a particular serine proteinases (thrombin or urokinase) from a solution containing a mixture of two proteinases of identical primary binding specificity for arginine. Accordingly, from an a prioi knowledge of an arginine specific proteinase's secondary binding site specificity, a R-X-Y-Argal sequence can be designed to selectively separate a proteinase of interest.

SUMMARY

The purification of proteolytic enzymes from biological systems is often difficult, especially when the concentration of the enzyme is low and multiple enzymes with an identical primary specificity are present. One of the more promising techniques for protease purification is affinity chromatography. I have synthesized a new family of affinity chromatography ligands consisting of peptides containing the aldehyde analog of arginine in the C-terminal position. Upon association with the serine protease, such peptide aldehydes mimic features of the transition-state configuration of substrates during hydrolysis and hence will be expected to associate with extremly high affinity and specificity as predicted for transition-state analogs.

The aldehyde analog of arginine was synthesized by reduction of Cbz-NO_2-Arginine with $LiAlH_4$ in tetrahydrofuran in presence of 1,1'-carbonyldiimidazole at -70 ^{o}C. The aldehydic function was protected by forming a semicarbazone derivative, which served as a stable intermediate. Cbz and NO_2 protections were removed by catalytic hydrogenation in the presence of palladium black. Arginine semicarbazone was immobilized on Affi-Gel-10 through a stable amide bond. The resin showed extremly high affinity toward trypsin and no affinity towards chymotrypsin. The trypsin activity remained bound to the resin even after exhaustive washing.

To exploit secondary specificity of proteases, affinity resins of the general formula R-X-Y-Arginal (where R- is resin matrix and -X-Y- is a dipeptide) were synthesized. The tripeptide synthesis on the resin was N-terminal to C-terminal, which is opposite from the commonly utilized Marrifield solid phase peptide synthesis. The conditions for binding and elution from Affi-X-Y-Arginal resins (-X-Y- = Phe-Pro, Phe-Ala and Gly-Gly) of the serine proteases trypsin, thrombin and urokinase are described in this work. In all cases in which the peptidyl argininal showed a high specificity to a particular protease (i.e., Gly-Gly-Arginal for urokinase and Phe-Pro-Arginal for thrombin), the protease could not be removed with high ionic strength buffers. Urokinase and thrombin could be separated from one another and purified by either an Affi-Phe-Pro-Arginal or an Affi-Gly-Gly Arginal affinity column. A method to elute the tightly bound proteases in an active form was established using 0.1 M semicarbazide containing 0.1 M sodium acetate, 0.025 M $CaCl_2$ and 0.01 % Carbowax 20M. The resin could be regenerated repeatedly without loss of its protease binding capacity by overnight treatment with methanol/ acetic acid/ formaldehyde.The concentration of Arginine aldehyde was determined by p-aminoazoaniline and salicyaldehyde system and showed a minimum of 0.1 micromole/ ml of resin. Based on this value, the protease binding capacity of the resin is 2.5 mg of trypsin-like protease/ ml of resin.

A general procedure for purification of proteases in which acid product of a specific substrate of the the protease is attached by its N-terminal residue to an inert support and the COOH-terminal amino acid

modified to a peptide aldehyde to generate the transition-state analog inhibitor of the protease of interest is descried. A particular case of fibrinopeptide-A to purify thrombin was investigated with limited success, perhaps due to very low concentration of the ligand on the affinity resin. Three different approaches to substitute the terminal arginine in fibrinopeptide-A by Arginine semicarbazone were investigated including (i) direct substitution, (ii) chemical method and (iii) enzymatic method.

These transition-state affinity purification technique should have a general usefulness in the isolation and purification of biologically important proteases for therapeutic use in coagulation disorders.

REFERENCES

Anderson, G., Zimmerman, J., Callahan, F. (1963). J. Amer. Chem. Soc. 85, 3039. "N-hydroxy succinamide esters in peptide synthesis."

Anderson, G., Zimmerman, J., Callahan, F. (1964) J. Amer. Chem. Soc. 86, 1839-1842. "The use of esters of N-hydroxy succinamide in peptide synthesis."

Anderson, L., Walsh, K. and Neurath, H. (1977) Biochemistry 16, 3354-3360. "Bovine enterokinase, purification, specificity and some molecular properties."

Anderson, L. and Wolfenden, R. (1980) J. Biol. Chem. 255, 11106- 11107. "Transition-state affinity jump chromatography."

Anderson, L. and Wolfenden, R. (1982) Anal. Biochem. 214, 150- 157. "General method of α-aminoaldehydes synthesis using alcohol dehydrogenase."

Anderson, P.A., Nielsen, L., Grondahl-Hansen, J., Skriver, L., Zeuthen, J., Stephens, R. and Dano, K. (1984) EMBO. J. 3, 51- 56. "Inactive proenzyme to tissue-type plasminogen activator from human melonama cells, identified after affinity purification with a monoclonal antibody."

Aoyagi, T., Miyata, S., Nabo, M., Kojima, E., Matsuzaki, M., Ishizuka, M., Takeuchi, T. and Umezawa, H. (1969) J. Antibio. (Tokyo) 22, 558-

568. "Biological activities of leupeptins."

Aoyagi, T. and Umezawa, H. in Reich, E., Rifkin, D.B. and Shaw, E. (eds) (1975). "Proteases and biological control." Cold Sprin Harbor Press, Cold Spring Harbor, New York, p. 429.

Axen, R., Porath, J. and Ernback, S. (1967) Nature (London) 214, 1302-1304. "Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides."

Bajkowski, A. and Frankfater, A. (1983) J. Biol. Chem. 258, 1645- 1649. "Steady-state kinetic evidence for an acyl enzyme intermediate in reactions catalyzed by bovine spleen cathepsin-B."

Bajusz, S., Barabas, E., Talnay, P., Szelle, E.and Bagdy, D. (1978). Int. J. Peptide Protein Res. 12, 217-221. "Inhibition of thrombin and trypsin by peptide aldehydes."

Bajusz, S., Szell, E., Barabas, E. and Bagdy, D. (1981) in the Proceeding of the 7^{th} American Peptide Symposium. (Rich, D. and Gross, E., eds). Pierce Chem. Co. (publ.) Rockford, Ill.

Baughmann, D. and Waugh, D. (1967) J. Biol. Chem. 242, 5252-5259 "The bovine thrombin, purification and certain properties."

Bendall, M.R., Cartwright, I.L., Clark, P., Low, G. and Nurse, D. (1977) Eur. J. Biochem. 79, 201-209. "Inhibition of papain by N-Acyl-aminoacetaldehydes and N-Acyl-aminopropanones."

Bender, M.L., (1960) Chem. Rev. 60, 53-113. Mechanism of catalysis of nucleophillic reactions of carboxylic acid derivatives."

Bender, M.L. and Kezdy, F.J. (1964) J. Amer. Chem. Soc. 86, 3704- 3714. "The current status of the α-chymotrypsin mechanism."

Bender, M.L. and Kezdy, F.J. (1965) Ann. Rev. Biochem. 34, 49-76. "Mechanism of action of proteolytic enzymes."

Bigbee, W., Weintraub, H.and Jensen, R. (1978) Anal. Biochem. 88, 114-122. "Sensitive fluorescence assays for urokinase using synthetic peptide 4-methoxy-β-napthylamide substrates."

Blackburn, S. (1977) Enzyme structure and function. Pub: Marcel Dekker Inc. New York. chapter 1.

Blomback, B., Hussel, B., Hogg, D. and Cleason, G. (1977). in chemistry and Biology of Thrombin. Lundblad, R.L., Fenton, J.W. and Mann, K.G. (eds.): Ann Arbor Science (pbl.) Ann Arbor, MI. pp. 275-290.

Blow, D. and Steitz (1970). Ann. Rev. of Biochemistry 39, 63-100. "X-ray diffraction studies of enzymes."

Blow, D.M., Janine, J., Sweet, R.M.(1974) Nature, 249, 54-57. "Model of action of soybaen trypsin inhibitor (Kunitz) as a model for specific protein-protein interactions."

Blumberg, S., Katchalski-Katzier, E. (1979) Biochemistry, 18, 2126-2133. "Inhibition of chymotrypsin by D-tryptophane amide covalently bound to macromolecular carriers: Specific, Steric and electrostatic effects."

Bodanszky, M., du Vigneaud, V. (1959) J. Amer. Chem. Soc. 81, 5688-5691. " A method of synthesis of long peptide chains using a synthesis of oxytocin as an example."

Bodanszky, M. (1984). in Principles of Peptide Synthesis. Springer-Verlag, New York, p. 137.

Borin, G., Cheesa, G., Cavaggion, G., Marchiori, F. and Mulleer-

Esterl, W.(1981) Hopp. Z. Physiol. Chem. 362, 1435-1445. "Synthesis of leupeptins and inhibition of proteinases."

Booyse, F., Osikowicz, G., Feder,S., and Scheinbuks, J. (1984) J. Biol. Chem. 259, 7198-7205. "Isolation and characterization of a urokinase type plasminogen activator from cultured human endothelial cells indistinguishable from urinary urokinase."

Breddam, K., Widmer, F. and Johnson, J. (1981) Carlsberg Res. Comm. 46, 361-372. "Carboxy peptidase-Y catalyzed C-terminal modification in the B-chain of proinsulin."

Caplow, M. (1969) J. Amer. Chem. Soc. 91, 3639-3645. "Chymotrypsin catalysis. Evidence for a new intermediate."

Chao, L., and Liner, I.E. (1965) Biochem. Biophys. Acta. 96, 508- 516. "Auto activation of porcine trypsinogen in the presence and absence of calcium."

Chen, R., Gorenstein, D., Kennedy, W.P., Low, G., Nurse, D. and Schultz, R.M. (1979) Biochemistry, 18, 921-926. "Evidence for hemiacetal formation between N-acetyl-Pheal and chymotrypsin by cross saturation NMR."

Cleason, G. and Aurell, L. (1981) Ann. N.Y. Acad. Sci. 370, 798- 811. "Small synthetic peptides with affinity for proteases in coagualtion and fibrinolysis: An overview."

Conn, D.M., Smith, R. and Rogers, D. (1981) J. Biol. Chem. 256, 1098-1100. "Stimulation of pituitary gonadotropin release does not require internalization of gonadotropin-releasing hormone."

Cuatrecasas, P., Wilchek, M. and Anfinsen, C. (1968). Proc. Natl.

Acad. Sci., U.S.A., 61, 636-643. "Selective enzyme purification by affinity chromatography."

Dano, K., Moller, V., Ossowski, L. and NIelson, L. (1980). Biochem. Biophys. Acta. 613, 543-555. "Purification and characterization of a plasminogen activator from mouse cells transformed by an oncogenic viruses."

Delbaer, L.T.J., Hutcheon, W.L.B., James, M.N.G. and Theissen, W.E. (1975) Nature Lond. 257, 758-762. "Tertiary structural differences between microbial serine proteases and pancreatic serine enzymes."

Drenth, J., Kalk, K. and Swen, H.M. (1976) Biochemistry 15 3731- 3738. "Binding of chloromethyl ketone substrate analoges to crystalline papain."

Erying, H. (1935). J. Chem. Physics. 3 107-115. "The activated complex in chemical reaction."

Fareed, J., Messmore, H.,Kindel, G. and Balis, J. (1981). Ann. N.Y. Acad. Sci. 370, 765-784. "Inhibition of serine proteases by low molecular weight peptides and their derivatives."

Fastrez, J., and Fersht, A. (1971). Biochemistry. 12, 1067-1074. "Mechanism of chymotrypsin. structure, reactivity and non- productive binding relationships."

Fehrentz, J., Heitz, A., Castro, B., Cazaubon, C. and Nisato, D. (1984) FEBS Leters, 167, 273-276. "Aldehydic peptides inhibiting renin."

Fersht, A., Blow, D.M. and Fastrez, J. (1971) Biochemistry, 12, 2025-2041. Demonstration of the acyl-enzyme mechanism for the hydrolysis

of peptides and anilides by chymotrypsin."

Frankfater, A. and Kezdy, F.J. (1971) J. Amer. Chem. Soc. 93, 4039-4043. "Kinetics of hydrolysis of p-nitrophenyl thiolate by chymotrypsin."

Frankfater, A. and Kuppy, T. (1981) Biochemistry, 20, 5517-5523. "Mechanism of association N-Acetyl-Phe-Glycinal to Papain."

Fujisawa, T., Mori, T., Tsuge, S., Sato, T. (1983) Tetrahedron Lett. 24, 1543-1546. "Direct and chemoselective conversion of carboxylic acids into aldehydes."

Funahashi, M. Matsumoto, I. and Seno, N. (1982) Anal. Biochem. 126, 414-421. "Preparation of three types of heparin-sepharoses and their binding activities to thrombin and antithrombin III."

Galpin, I., Wilby, A., Place, G. and Beynon, R. (1984) Int. J. Peptide Protein Res. 23, 477-486. "Synthetic analogs of the proteinase inhibitor; chymostatin."

Geratz, J.D. and Tidwell, R.R. (1977) in Chemistry and biology of thrombin. Ann Arbor Science (eds Lundblad R.L., Fenton, J.W. and Mann, K.G.). pp. 179.

Golfarb, R. and Quigley, J. (1980) Biochemistry 24, 5463-5471. "Purification of plasminogen activator from Rous sarcoma viruses transformed chick embryo fibroblasts treated with the tumor promoter PMA."

Gorenstein, D. and Shah, D. (1982) Biochemistry 21, 4679-4686. "Proton and Fluorine nuclear magnetic resonance spectroscopic observation of hemiacetal formation between N-Acyl-p- fluorophenyl alaninals and

chymotrypsin."

Greenwald, S. and Chandler, A. (1983) Thrombosis Res. 31, 799- 806. "Comparative study of thrombosis by high and low molecular weight urokinase in an in vitro perfusion system."

Guia, M. and Shaw, E. (1965) J. Biol. Chem. 240, 1597. "Studies on the active center of trypsin."

Hageman, T. and Scheraga, H.A. (1974) Arch. Biochem. Biophys. 164, 707-715. "Mechanism of action of thrombin on fibrinogen."

Hamada, T. and Yonemitsu, O. (1969) Chem. Pharm. Bull. 19, 1444- 1449. "Esterification of model peptides in aqueous solution."

Hamada, T. and Shioiri, T. (1982) Chem. Pharm. Bull. 30, 1921- 1924. "A practical method for the preparation of optically active N-protected -amino aldehydes and peptide aldehydes."

Handerson, R.L. (1970) J. Mol. Biol. 54, 341-354. "Structure of crystalline chymotrypsin. The structure of indoleacryloyl-α-chymotrypsin and its relevance to the hydrolytic mechanism of the enzyme."

Hanna, L.,Scheraga. H., Francis, C. and Marder, V. (1984) Biochemistry 23, 4689-4687. "Comparison of structures of various human fibrinogens and a derivative thereof by a study of the kinetics of release of fibrinopeptides."

Hess, G. and Seybert, D. (1975) Science, 189, 384-386. "Tetrahedral intermediate in a specific chymotrypsin inhibitor complex dected by laser Raman spectroscopy."

Hixon, H. and Nishikawa, A. (1973) Arch. Biochem. Biophys. 154, 501-

509. "Affinity chromatography: purification of bovine trypsin and thrombin."

Holmberg, L., Blaelh, B. and Astedt, B. (1976) Biochem. Biophys. Acta. 445, 215-222. "Purification of urokinase by affinity chromatography."

Homandberg, G., Mattis, J. and Laskowski, M. (1978) Biochemistry 17, 5220-5227. "Synthesis of peptide bonds by proteinases. Addition of organic cosolvants shifts peptide bond equilibria toward synthesis."

Homandberg, G. and Laskowski, M. (1979) Biochemistry 18, 586-592. "Enzymatic synthesis of the hydrolyzed peptide bond in Ribonuclease S."

Ishii, S. and Kasai, K. (1980) Methods in enzymology 80, 842- 848. "Affinity methods using argininal derivatives."

Ito, A., Takahashi, R. and Baba, Y. (1975) Chem. Pharm. Bull. 23, 3081-3087. "A new method to synthesize α-amino aldehydes."

Jansen, E.F., Nutting, M.D.F., Jang, R. and Balls, A.K. (1949) J. Biol. Chem. 179, 189-199. "Inhibition of the proteinases and easterase activities of trypsin and chymotrypsin by diisopropyl fluoro phosphate."

Jencks, W.P. (1969). Catalysis in Chemistry and Enzymology, New York, N.Y., Mcgraw Hill, p.225.

Kabi Products for research and diagnostic use (1979), Stockholm, sweden p.18,

Kawamura, K., Kondo, S., Meada, K. and Umezawa, H. (1969) Chem. Pharm. Bull. 17, 1902-1909. "Structure and synthesis of leupeptins pr-LL

and Ac-LL."

Keil, B. (1971) in The Enzymes. (Boyer ed.) Academic Press, New York, (ed. Boyer) p.274.

Kennedy, W.P. and Schultz, R.M. (1979) Biochemistry 18, 349-356. "Mechanism of association of a specific aldehyde transition- state analog to the active site of chymotrypsin."

Kettner, C. and Shaw, E. (1979) Biochem. Biophys. Acta. 569, 31- 40. "The succeptibility of urokinase to affinity labelling by peptides of arginine chloromethyl ketones."

Kettner, C., Mirabelli, C., Pierce, J. and Shaw, E. (1980) Arch. Biochem. Biophys. 202, 420-430. "Active site mapping of human and rat urinary kallikreins by peptidyl chloromethyl ketones."

Kezdy, F.J., Clement, G.E. and Bender, M.L. (1964) J. Amer. Chem. Soc. 86, 3690-3698. "The observation of acyl-enzyme intermediates in the α-chymotrypsin catalyzed reactions of N——acetyl-L-tryptophane derivatives at low pH."

Khatri, N.H. and Stammer, C. (1980) Biochem. Biophys. Res. Comm. 96, 163-167. "The synthesis of some N-phosphoryl dipeptide aldehydes."

Koelher, K. and Lienhard, G.E. (1971) Biochemistry 10, 2477-2483. "2-Phenylethaneboronic acid, a possible transition state analog for chymotrypsin."

Kohn, J. and Wichek, M. (1981). Anal. Biochem. 115, 375-382. "Procedures for the analysis of cyanogen bromide-activated sepharoses or sephadex by quantitative determination of cynate esters and imidocarbamates."

Kokubu, T., Hiwada, K., Sato, Y., Iwata, T., Imamura, Y., Matsueda, R., Yabe, Y., Kogen, H., Yamazaki, M., Ijama, Y. and Baba, Y. (1984). Biochem. Biophys. Res. Comm. 118, 929-933. "Highly potent and specific inhibitors of human renin."

Kondo, S., Kawamura, K., Iwanaga, J., Hamada, M., Aoyagi, T., Maeda, K., Takeuchi, T. and Umezawa, H. (1969) Chem. Pharm. Bull. 17, 1896-1901. "Isolation and characterization of leupeptins produced by actinomycetes."

Kraut, J. (1977) Ann. Rev. Biochem. 46, 331-358. "Serine proteases; structure and mechanism of catalysis."

Lerman, L.S. (1953) Proc. Natl. Acad. Sci. 39, 232-236. "A biochemically specific method for enzyme isolation."

Lewis, C.A. and Wolfenden, R. (1977) Biochemistry. 16, 4890-4895. "Thiohemiacetal formation by inhibitory aldehydes at the active site of papain."

Libby, P. and Goldberg, A.L. (1978) Science 199, 534-536. "Leupeptin, a protease inhibitor, decreases protein degradation in normal and diseased muscles."

Linehard, G.E. (1973) Science 180, 149-154. "Enzymatic catalysis and transition-state theory."

Lindquist, R.N. in E.J. Ariens (ed), "Drug desin" Vol.V, Academic press, New York, 1975. Chapter-2.

Lowe, G. and Yuthavong, Y. (1971) Biochem. J. 124, 107-115. "Kinetic specificity in papain catalyzed hydrolysis."

Lowe, G. and Nurse, D. (1977) J. Chem. Soc. Chem. Comm. 22, 815- 817.

"Evidence for hemiacetal between α-chymotrypsin and hydrocinnamaldehyde by cross-saturation NMR."

Maeda, K., Kawamura, K., Kondo, S., Aoyagi, T., Takeuchi, T. and Umezawa, H. (1971) J. Antibiot. 24, 402-404. "The structure and activity of leupeptins and related analogs."

Maroti, K., Belin, D. and Strickland, S. (1982) Developmental Biology 90, 154-159. "The production of distinct forms of plasminogen activator by mouse embryonic cells."

Marsh, H.C., Meinwald, Y.C., Lee, S. and Scheraga, H. (1982) Biochemistry, 21, 6167-6171. "Mechanism of action of thrombin on fibrinogen. Direct evidence for the involvement of phenylalnine at position P_9."

Marsh, H., Meinwald, Y.C., Thanhauser, T. and Scheraga, H. (1983) Biochemistry, 21, 4170-4174. "Mechanism of action of thrombin on fibrinogen. Kinetic evidence for involvement of Asp at position P_{10}."

Mathews, D.A., Richard, J.J., Birktoft, S.T. and kraut, J. (1975) J. Biol. Chem. 250, 7120-7126. "X-ray crystallographic study of boronic acid adducts with subtilisin BPN' ".

Matis J., Hanes, J.B. and Fruton, J.S.(1977) J. Biol. Chem. 252, 6776-6782. "Interaction of papain with derivatives of phenylalaninylglycinal."

McConn, J., Ku, E., Himoe, A., Brandt, K.G. and Hess, G.P. (1971) J. Biol. Chem. 246, 2918-2925. Determination of pre-steady state parameters for specific substrates by stopped-flow techniques."

McIntire, G., Leckie, B., Hallett, A. and Szelke, M. (1983) Biochem. J. 211, 519-522. "Purification of human renin by affinity chromatography using a new peptide inhibitor of renin, H.77 (D-His-Pro-Phe-His-Leu-^{R}-Leu-Val-Tyr)."

McRorie, R.A. and Williams, W.L. (1974) Ann. Rev. Biochem. 43, 777-803. "Biochemistry of mamalian fertilization."

Messineo, L. (1966) Arch. Biochem. Biophys. 117, 534-540. "Modification of the Sakaguchi reaction: Spectrophotometric determination of arginine in proteins without previous hydrolysis."

Miller-Anderson, M.,Gaffney, P., Seghtchain, M. (1980) Thrombosis Res. 20, 109-122. "Preparation and stability of a highly purified thrombin standard."

Mitin, Yu., Zapevalova, N. and Gorbunova, E. (1984) Int. J. Pepetide Protein Res. 23, 528-534. "Peptide synthesis catalyzed by papain at alkaline pH values."

Morihara, K. and Oka, T. (1970) Arch. Biochem. Biophys. 138, 526- 531. "Subtilisin BPN: inactivation by chloromethyl ketone derivatives of peptide substrates."

Nakamura, S., Takenaka, O., Takahashi, K., (1983) J. Biochem. 94, 1973-1978. "Fibrinopeptides A and B of baboons: Their amino acid sequences and evolutionary rates and a molecular phylogeny for the baboons."

Osbahr, A. (1982) Thromb. Heamosta. 48, 226-231. "Esterification of the carboxyl groups in fibrinogen by the application of a highly specific methylating agent".

Patel, A.H. and Schultz, R.M. (1982) Biochem. Biophys. Res. Comm. 104

181-186. "Transition-state affinity purification of proteases; the preparation of an argininal affinity resin for the selective binding of trypsin like proteases."

Patel. A.H., Ahsan. A., Suthar, B.P. and Schultz, R.M. (1983) Biochem. Biophys. Acta. 748, 321-330. "Transition state affinity chromatography of trypsin like proteases with dipeptidyl argininal ligands."

Peterson, A., Knowles, J. (1972) Eur. J. Bichem. 51, 510-517. "The number of catalytically esential carboxyl groups in pepsin."

Pauling, L. (1948) American Scientist 36, 58. "Chemical achievement and hope for the future."

Petkov, D. and Stoineva, Iv. (1984) Biochem. Biophys. Res. Comm. 118, 317-323. "Nucleophile specificity in chymotrypsin peptide synthesis."

Phillipp, M. and Bender, M.L. (1970) Proc. Natl. Acad. Sci. U.S.A., 68, 478-480. "Inhibition of serine proteases by ary boronic acids."

Pohl, J., Zaoral, M., Jindra, A. and Kostka, V. (1984) Anal. Biochem. 139, 265-271. "Purification of pepsins and cathepsin D by affinity chromatography on sepharose 4B with an immobilized synthetic inhibitor."

Porth, J., Flodin, P. (1959) Science 183, 1657-1659. "Gel-filtration: A method for desalting and group separation."

Paulos, T.L., Alden, R.A., Freer, S.T., Birktoft, J.J. and Kraut, J. (1976) J. Biol. Chem. 251, 1097-1103. "Polypeptide helomethyl ketones bind to serine proteases as analogs of the tetrahedral intermediate."

Powers, J.C. and Wlcox, P.E. (1970) J. Amer. Chem. Soc. 92, 1782-1783. "Design and synthesis of inhibitors for crystallographic studies on

the active site of chymotrypsin."

Reich, E. (1978) Activation of plasminogen: in (Ruddon, R.W. ed). Biological Markers of Neoplasia: Basic and Applied aspects, The Netherlands: Elsevier/North Holland. pp. 491-500.

Rich, D.H., Bernatowicz, M.S. and Schmidt, P. (1982) J. Amer. Chem. Soc. 104, 3535-3536. "Direct ^{13}C NMR evidence for a tetrahedral intermediate in the binding of a pepstatin analogue to porcine."

Robertus, J.D.,Alden, R.A., Birktsft, J.J. and Kraut, J.J. (1972a) Biochemistry 11 2439-2449. "An X-ray crystallographic study of the binding of peptide chloromethyl ketone inhibitors to subtilisin BPN."

Robertus, J.D., Kraut, J., Alden, R.A. and Birkoft, J.J. (1972b) Biochemistry, 11, 4293-4303. "Subtilisin; a stereo chemical mechanism involving transition state stabilization."

Roberts, J. and Devorate, R. (1983) Lysogenic induction, in Lambda II, (Hendrix, R.W., Roberts, J.W., Stahl, F.W. and Weiberg, R.A. ed), Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y. pp. 123-144.

Robins, K., Summaria, L., Hsieh, B. and Shah, K. (1967), J. Biol. Chem. 242 2333-2342. "The peptide chains of human plasmin. Mechanism of action of human plasminogen to plasmin."

Robins, K. and Summaria, L. (1976) Methods in Enzymology 45, 257-273. "Plasminogen and Plasmin."

Salerno, G.,Verda, P., Nolli, M., Corti, A. and Blasi, F. (1984) Proc. Natl. Acad. Sci. U.S.A., 81, 110-114. "Monoclonal antibodies to human urokinase identify the single chain pro-urokinase precursor."

Schechter, I. and Berger, A. (1967) Bioch. Biophys. Res. Comm. 27, 157-

162. "On the size of the active site in protease papain."

Schmer, G., (1972) Hopp Z. Phys. Chem. 353, 810-814. "The purification of bovine thrombin by affinity chromatography on benzamidine-agarose."

Schollman, G. and Shaw, E. (1963) Biochemistry 2, 252-255. "Direct evidence for the presence of histidine in the active center of chymotrypsin."

Schray, K. and Klinman, J.P. (1974) Biochem. Biophys. Res. Comm. 57, 641-648. "The magnitude of transition-state analog binding constants."

Schroeder, D.D. and Shaw, E. (1968) J. Biol. Chem. 243, 2943-2949. "Chromatography of trypsin abd its derivatives."

Scouten, W.H. (1981) in Affinity Chromatography, (Sairam, M.R., Clark, W.C., Chung, D., Porath, J. and Li, C.H. eds) J.Wiley and sons (publ) New York, pp.281.

Sealock, R. and Laskowski, M. (1969) Biochemistry 8, 3703-3710. "Enzymatic replacement of the Arginyl by a Lysyl residue in the reactive site of soybean trypsin inhibitor."

Shah, D. and Gorenstein, D. (1983) Biochemistry 22 6069-6101. "Fluorine nuclear magnetic resonance spectroscopy of transition state analogue complexes of the D and L enentiomers of N-Acetyl-p-fluorophenylalaninal and α-chymotrypsin."

Sher, J.H., Stracher, A., Shafiq, S.A. and Hardy-stashin, J. (1981) Proc. Natl. Acad. Sci. U.S.A., 78, 7742-7744. "Successful treatment of murine muscular dystrophy with the proteinase inhibitor leupeptin."

Szelke, M., Leckie, B., Hallette, A., Jones, D., Sueiras, J., Atrash, B., and Lever, A.F. (1982) Nature 299, 555-557. "Potent new inhibitors

of human renin."

Thompson, R.C. and Blout, E. (1970) Proc. Natl. Acad. Sci. U.S.A., 67, 1734-1740. "Evidence for an extended active center in elastase."

Thompson, R.C. (1973a) Biochemistry 12, 44-51. "Use of peptide aldehydes to generate transition state analogs of elastase."

Thompson, R.C. and Blout, E. (1973b) Biochemistry 12, 51-57. "Dependence of kinetic parameters for elastase catalysed amide hydrolysis on the length of peptide substrates."

Thompson, R.C. and Bauer, C. (1979) Biochemistry 18, 1552-1558. "Reaction of peptide aldehydes with serine proteases. Implications for the entropy changes associated with enzymatic catalysis."

Trimoli, E., Morazzoni, G., Maderna, P., Colli, S. and Paoletti, R. (1981) Thrombosis Res. 23, 549-553. "Studies on the antithrombotic action of Boc-D-Phe-Pro-Argal (GYKI 14, 451)."

Tremoli, E., Colli, S. and Paoletti, R. (1984) Adv. Exp. Med. Biol. 164, 187-192. "GKI 14, 451, A synthetic tripeptide inhibitor of thrombin."

Umezawa, H. (1979) GANN Monograph on Cancer Research 24, 159-178. "Protease inhibitors produced by microorganisms."

Umezawa, H. (1982) Ann. Rev. Microbiol. 36, 75-99. "Low molecular weight enzyme inhibitors of microbial origin."

Umezawa, H. (1983) in Proteinase inhibitors- medical and biological aspects. (Katunuma, N., Umezawa, H. and Holzer, H. eds.) Japan Scientific Socities Press Tokyo. pp.4.

Vande De Werf, F., Bergman, S., Fox, K., Hoyng, F., Sbel, B. and Collen, D. (1984) Circulation 69, 605-610. "Coronary thrombosis with

intravenously administered human tissue-type plasminogen activator produced by recombinent DnA."

Violand, B. and Castellino, F. (1976) J. Biol. Chem. 251, 3906- 3912. "Mechanism of the urokinase-catalysed activation of human plsdminohrn."

Wallen, P., Randy, M., Bergsdorf, N. and Kok, P. (1981) Prog. Chem. Fibrinolysis Thrombosis 5, "Two forms of tissue plasminogen activator and their enzymatic properties."

Wallen, P., Pohl, G., Bergsdorf, n., Randy, M. and Jornvall (1983) Eur. j. Biochem. 132, 681-686. "Purification and characterization of a melonoma cell plasminogen activator."

Wayne, S. and Fruton, J. (1983) Proc. Natl. Acad. Sci. U.S.A., 0, 3241-3244. Thermolysis catalyzed peptide bond synthesis."

Weinstein, I., Wigler, M., Yamasaki, H., Lee, L., Fisher, P.B. and Mufson, A. (1978) in Biological Markers of Neoplacia; Basic and applied aspects (Ruddon, R.W. ed) A regulation of the expression of certain biological markers of neoplasia, The Netherlands: Elsevier/North Holland. pp. 451-471.

Westerick, J. and Wolfenden, R. (1972) J. Biol. Chem. 247, 8195-8197. "Aldehydes as inhibitors of papain."

Westerick, J. and Wolfenden, R. (1974) J. Biol. Chem. "Aspartic-β-semialdehyde: A potent inhibitor of Escherichia Coli L-asparginase."

Wicheck, M, Miron, T. and Kohn, J. (1984) Methods in Enzymology. 104, 3-55. "Affinity chromotography."

Wolfenden, R. (1969) Nature 223, 704-705. "Transition state analogs

for enzyme catalysis."

Wolfenden, R. (1972) Accts. Chem. Res. 5, 10-18. "Analog approaches to the structure of the transition state in enzymatic reactions."

Wolfenden, R. (1976) Ann. Rev. Biophys. Bioeng. 5, 271-306. "Transition state analog inhibitors and enzymatic catalysis."

Workman, E., Uhteg, C., Kingdon, H. and Lundblad R. (1977) in Chemistry and biology of thrombin. (Lundblad, R. Fenton, J. and Mann, K. ed). Ann Arbor Science, Ann Arbor, Mich. pp.23.

Worthington Enzymes, enzyme reagents and related biochemicals (1977) Worthington Biochemical Corporation, Freehold, N.J. 07728.

Wyeth, P., Shasrma, R.P. and Akhtar, M. (1980) Eur. J. Biochem. 105, 581-585." A proton magnetic resonance study of N-trifluoroacetyl-L-alanyl-L-phenylalaninal binding to chymotrypsin."

Zedrner, B. and BEnder, M.L. (1964) J. Amer. Chem. Soc. 86, 3669-3674. "The kinetic consequences of the acyl enzyme mechanism for the reactions of specific substrates with chymotrypsin."

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The final copies have been examined by the director of the Dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the Dissertation is now given final approval by the committee with reference to content and form.

The Dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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