INVESTIGATIONS IN ORIGINS OF ACTIVITY OF MOLECULARLY IMPRINTED POLYMER MIMICS OF CHYMOTRYPSIN

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BY

BHALCHANDRA SHRIPAD LELE (M.Sc.)

POLYMER SCIENCE AND ENGINEERING GROUP
CHEMICAL ENGINEERING DIVISION
NATIONAL CHEMICAL LABORATORY
PUNE 411008
INDIA

JUNE 1997

DEDICATED TO MY PARENTS
AND
TO THE MEMORIES OF MY GRANDMOTHER

CERTIFICATE

Certified that the work incorporated in the thesis entitled **"Investigations In Origins Of Activity Of Molecularly Imprinted Polymer Mimics Of Chymotrypsin"** submitted by **Mr. B.S. Lele**, was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

National Chemical Laboratory.

Pune 411008.

Dr. R. A. Mashelkar

(Research Guide)

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TABLE OF CONTENTS

List of figures

List of tables

ABSTRACT

Chapter 1 : Introduction and Literature Survey

In this chapter, a brief description of the objectives of research in mimicking enzymatic activity is given. Research efforts in this area are broadly classified into two classes, synthetic chemistry and polymer chemistry. Various approaches developed in enzyme mimics in both the categories have been sighted with few important examples. In polymers, attention has been particularly given to molecularly imprinted crosslinked polymers. Various approaches developed in MIPs which facilitate achieving active site orientation as that of enzymes are described and research efforts in hydrolytic enzyme mimics by MIPs are sighted. Also, various applications of MIPs and relevant examples are described. Lastly, results of research work in our group in the area of enzyme mimics by surface imprinting have been described.

Chapter 2 : Objectives and scope of the work

Objectives in undertaking the present investigation in view of prior results obtained using surface imprinting technique in our group earlier have been summarized. The scope of the work is mainly confined to the investigations of factors that govern the activity of mimics and methodologies to enhance the same.

Chapter 3 : Surface imprinting of coordination complex of functional monomers and metal ion leading to catalytic triad : Concept generalization

A novel polymeric mimic comprising N-methacryloyl derivatives of serine, aspartic acid and histidine was synthesized by grafting Co (II) coordinated monomers - template assembly onto a microporous support. Cooperative effect amongst functional groups was demonstrated by comparing the activity of polymers which contained either imidazole or hydroxyl and imidazole groups with the polymer which contained all the three functional groups viz. hydroxyl, carboxyl and imidazole. Ability of the imprinted mimic to recognize the substrate was demonstrated.

Chapter 4 : Enhancing the activity of polymeric mimics of chymotrypsin : Structure - property correlation.

In this chapter, various polymeric mimics with different functional monomers bearing hydroxyl, carboxyl and imidazole groups were synthesized and hydrolytic activity of these mimics was evaluated. It is shown that k_{cat} of mimics increased with increase in the nucleophilicity of hydroxyl bearing monomer as well as by the cooperative action of carboxyl bearing monomer. K_m of the mimics was found to be governed by surface area of the support. Supports with low surface area exhibited high K_m values. The kinetic trends observed for the mimics were shown to be similar to those of immobilized enzymes.

Chapter 5 : Reactivity of the mimic as a function of substrate structure and support composition.

The effect of substrate structure and the support composition on hydrolytic activity was studied in this chapter. It is shown that k_{cat} of mimics decreased with increase

in the hydrophobicity of N-acyl substituent in the structure of substrates (N-acyl tyrosine para nitrophenyl esters). The mimics grafted on hydrophilic support exhibited unproductive binding for hydrophobic N-acyl substituent as in the case of chymotrypsin. The mimic grafted on hydrophobic support did not exhibited nonproductive binding. Also the activity of mimic was found to be enhanced by the use of hydrophobic support.

Chapter 6 : Summary and Conclusions

In this chapter all the results of present investigation have been summarized which highlight the factors that govern the catalytic activity of mimics.

Chapter 6 : Suggestions for future work

Based on the results obtained from this study, some suggestions towards the design and synthesis of more efficient mimics have been made in this chapter.

Chapter 1

Literature survey

1.1.0 Introduction

Molecular recognition forms the basis of a number of biochemical interactions such as antibody - antigen, drug - receptor and enzyme - substrate interactions. Of these, enzyme - substrate interactions are of particular interest because enzymatic reactions have crucial role in metabolism and their use as catalysts is of practical interest. Enzymes are catalytic proteins or polypeptides which recognize certain substrates and catalyze reactions on them because of the shape, size and charge complementarity of their binding sites or pockets for particular substrates. Binding sites are situated near active sites of enzymes. These sites orient the substrate in favorable conformation and the functional amino acids in active sites bring about the chemical transformations of substrates. Thus, basic features of any enzymatic reaction are **1.** Shape and size complementarity of active site of enzyme for a particular substrate which induces binding of substrate with the enzyme to form the enzyme - substrate complex. **2.** Cooperative action amongst the functional amino acids in the active site which brings about the reaction to break the complex into products and enzyme. (Schowen 1988).

Enzymes are widely used as catalyst in variety of bio- organic and synthetic organic reactions. They catalyze these reactions under mild conditions with high levels of specificity and enantioselectivity. Use of enzymes is also beset with problems such as instability of enzymes at high temperature and pH, denaturation on solvent and reagent action, loss in activity on repeated use, high cost etc. (Wiseman & Dalton 1987).

Therefore synthetic materials which can mimick enzymatic reactions and overcome problems described above are desirable. Apart from practical applications, studies in enzyme mimics also lead to better understanding of mechanistic aspects of enzymatic activity. Accordingly, research in enzyme mimics has been is pursued in various branches of chemistry such as synthetic, polymer and biochemistry. Various efforts are reported to induce these features of enzymes in synthetic materials. Most of the research work in enzyme mimics is concentrated mainly on mimicking hydrolytic enzymes (serine protease) such as chymotrypsin, alkaline phosphatase, ribonuclease etc. since the structures and mechanism of their activity are well known.

A detailed review of the efforts in mimicking chymotrypsin activity should cover thorough information on chymotrypsin itself and the synthetic mimics that have been developed so far. But as the scope of the present work is mainly focused on molecularly imprinted polymer mimics of chymotrypsin, the present literature survey includes the following .

The literature survey starts with a brief section on native chymotrypsin in which description of its active site, mechanism of the hydrolytic activity is given.. This is followed by a section on kinetics of enzyme catalyzed reactions wherein Michaelis Menten kinetics is discussed in particular. Previous efforts in hydrolytic enzyme mimics have been classified into two broad categories viz. synthetic supramolecular materials and polymeric materials. The first category comprises brief review on various efforts reported so far is done. In the second category efforts in both soluble and crosslinked polymeric enzyme mimics of hydrolytic enzymes which include various methodologies and relevant

examples that have been reported so far are reviewed. The literature survey ends with concluding remarks which summarize the past efforts and indicates the need for further investigations that are undertaken in the present work.

1.2.0 Chymotrypsin, its structure, specificity and mechanism of hydrolytic activity

Chymotrypsin is formed in the pancreas in the form of its precursor chymotrypsinogen which eventually breaks to set free chymotrypsin. There are three forms of chymotrypsin of which structure and mechanism of α chymotrypsin is well known. The tertiary structure of chymotrypsin comprises of three chains which are stabilized by hydrogen bonds and interconnected with three disulfide bonds. The binding site of chymotrypsin for peptidic substrates is on the enzyme's surface and has an area of approximately 25^{0} A. The primary binding site (S1) possesses a precisely shaped deep hydrophobic pocket near the catalytic groups into which hydrophobic side chain of amino acid whose amide bond is hydrolyzed is fitted. Because of this hydrophobic binding pocket chymotrypsin exhibits specificity for substrate which contain hydrophobic amino acids such as tyrosine and phenyl alanine. Further, key amino acid residues in the substrate structure R1, R2 and R3 bind to the complementary binding sites of the enzyme p1, p2 and p3 respectively (for more details see chapter 5). Due to such perfect fit, substrate is favorably oriented for hydrolysis.

The active site of chymotrypsin is formed by three amino acids namely serine (195), histidine (57) and aspartic acid (102). These amino acids form a catalytic triad called as "charge relay system". The hydroxyl group of serine forms a hydrogen bond with the nitrogen atom of imidazole group of histidine. Another nitrogen atom of the

imidazole group forms hydrogen bond with the carboxyl group of aspartic acid. Thus charge may be displaced along these bonds. Another important amino acid isoleucine (16) forms ionic bond with aspartic acid (194) and provides exact orientation for the catalytically active serine (195) hydroxyl group. During the hydrolysis, serine hydroxyl group attacks the carbonyl group of amide bond in the substrate to form - O - acylated enzyme and releases alcohol or amine depending upon the ester or amide hydrolyzed. Then the water molecule attacks the acyl enzyme to set free enzyme and the acid residue of the substrate.

Thus hydrolysis of substrate catalyzed by chymotrypsin can be described by the following equation

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.S \xrightarrow{k_2} E.P + P1$$

$$E.P \xrightarrow{k_3} E + P2.$$

wherein, E.S is enzyme - substrate complex, E.P is acyl enzyme, P1 and P2 are hydrolysis products , k_2 is rate constant for acylation and k_3 is rate constant for deacylation. The above equation can be reformulated as

$$K_m = K_s \, . \, k_3 / k_2 + k_3$$

wherein, K_m is Michaelis constant which reflects the strength of the complex E.S, and K_s is dissociation constant of the complex E.S.

$K_s = k_{-1} / k_1$

$k_{cat} = k_2 . k_3 / k_2 + k_3$

wherein, k_{cat} is the catalytic constant for the enzyme.

The ratio of k_2 and k_3 is different for esters and amides. In case of ester hydrolysis rate determining step is deacylation while in case of amide hydrolysis rate determining step is acylation. Since it is difficult to generate individual values of k_2 and k_3 from the observed hydrolysis, data for the kinetic constants for enzyme catalyzed hydrolysis reaction are generated by plotting the velocity of the reaction and the substrate concentration in various types of plots described below.

1.2.1 Kinetics of enzyme catalyzed reactions

Enzymes bring about reactions on their substrates in a peculiar way. When excess of substrate is processed by enzyme, in the initial stages the reaction rate is very high which then subsides due to the saturation of enzyme active sites with substrate. Thus initial burst followed by saturation of activity is a typical feature of any enzyme catalyzed reaction. Michaelis - Menten formulated a kinetic model for enzymatic reaction. According to this model, in any enzymatic reaction, enzyme first forms a complex with its substrate, an enzyme - substrate complex E.S. This is followed by conversion of substrate into products and dissociation of the complex into enzyme and products. Michaelis - Menten derived a kinetic equation for this as follows,

$v = V_{max} . [S] / K_m + [S]$

wherein, v = initial velocity of the reaction, $[S]$ = substrate concentration, V_{max} = maximum velocity of the reaction and K_m = Michealis - Menten constant.

Value of the constant K_m reflects the strength of the enzyme - substrate complex, in other words, affinity of enzyme for a particular substrate. Lower the value of K_m stronger is the enzyme substrate complex. Generally, with increased strength of the complex higher catalytic activity is exhibited by enzyme.

1.2.2 Lineweaver - Burk plot

The above kinetic equation was reformulated by Lineweaver - Burk into a double reciprocal equation as follows,

$1/v = K_m / V_{max} . 1 / [S] + 1 / V_{max} .$

When a plot of $1 / v$ vs. $1 / [S]$ is plotted which is called as Lineweaver - Burk plot, it gives a straight line with intercept on y axis equal to $1 / V_{max}$ and intercept on x axis equal to - K_m. Thus the plot gives the values of V_{max} and K_m. The value of catalytic constant k_{cat} can be calculated from V_{max} value as follows,

$k_{cat} = V_{max} / [E]$

wherein [E] is concentration of the enzyme.

Finally, efficacy of enzyme in catalyzing the reaction can be calculated as,

$K_r = k_{cat} / K_m.$

Thus k_{cat}, K_m and K_r are important kinetic constants which quantify the performance of enzyme as catalyst. Above described kinetic equations are also applied to synthetic enzyme mimics in order to evaluate their activity (Fife 1994). Kinetic constants for the polymer mimics synthesized and studied in this work are calculated using above equations.

Lineweaver - Burk plots have been criticized by some critics mainly because small errors in determination of velocity of reaction (v) get magnified when the reciprocal $1 / v$ are plotted. Therefore several other plots are also used to plot the data.

1.2.3 Hanes - Woolf plot

The Lineweaver - Burk equation is rearranged to yield the linear equation for Hanes - Woolf plot as follows.

$$1/v = K_m / V_{max} \cdot 1 / [S] + 1 / V_{max} .$$

multiplying both the sides of the equation by [S] :

$$[S] / v = [S] . K_m / V_{max} . 1/ [S] + K_m / V_{max}.$$

or, $$[S] / v = 1/ V_{max} . [S] + K_m / V_{max}$$

Thus a straight line equation is obtained when [S] / v versus [S] (Hanes - Woolf plot) is plotted. The intercept on y axis gives the value of K_m / V_{max} and the slope of the line gives reciprocal $1/ V_{max}$.

1.2.4 Woolf - Augustinsson - Hofstee plot

In this plot, the basic velocity equation of Michaelis - Menten is divided by [S] in numerator and the denominator to get

$v = V_{max} / (K_m / [S] + 1)$

$V_{max} = v. K_m / [S] + v$

or, $v = (-K_m. v / [S]) + V_{max.}$

Thus a straight line equation is obtained when v versus v / [S] is plotted (Woolf - Augustinsson - Hofstee plot). The slope of the line is equal to $-K_m$ and the intercept on v axis equal to V_{max}.

1.2.5 Edie - Scatchard plot

When the Michaelis - Menten equation is rearranged as described above we get

$V_{max} = v. K_m / [S] + v$

Dividing both the sides of equation by K_m we get

$v / [S] = -1/ K_m . v + V_{max} / K_m.$

Thus plot of v / [S] versus v is linear with slope of $-K_m$ and intercept of V_{max} / K_m on v/ [S].

As described above, data of the velocity of enzyme catalyzed reaction can be plotted in various ways to obtain values of kinetic constants k_{cat}, K_m, V_{max} and K_r. In the case of native enzyme as the reaction rates too high to monitor exactly, there are chances

of errors in determining the *v*. But in case of synthetic mimics, specially crosslinked heterogeneous mimics, reaction velocity could be easily monitored. Therefore we have used Lineweaver - Burk plots to quantify the results of ester hydrolyses catalyzed by polymer mimics.

In the next section various efforts from different branches of chemistry towards developing synthetic enzyme mimics surveyed. Research in enzyme mimics is broadly classified into two categories. **1.** Enzyme mimics based on supramolecular assemblies. & **2.** Enzyme mimics based on synthetic polymers. In following sections efforts in both areas are reviewed.

1.3.0 Enzyme mimics based on supramolecular assemblies

Research in this category mainly involves design and synthesis of simple molecules, supramolecular assemblies and peptides. Various approaches that have been developed to synthesize molecules capable of substrate binding and reaction, are further divided into sub - categories as cyclodextrins, cyclic peptides, catalytic antibodies, organometallic complexes, cyclophanes and crown ethers.

1.3.1 Cyclodextrins

In one of the earlier approaches, Rogers and Bruice (1974) synthesized a model for chymotrypsin comprising 6 and 8 membered rings based on 4(5) methyl -5(4) dimethyl derivatives of O- acetyl esters of substituted 2(2' hydroxyphenyl) imidazoles. It was found that these models catalyzed the intramolecular hydrolysis via imidazoyl acid and base catalysis assisted by water molecule attack at the ester bond. In this case, no O -

N acyl transfer was observed as in the case of the enzyme. Bender et al (1984) synthesized a model of chymotrypsin comprising 7 and 10 membered rings based on bicyclo (2,2,1) heptane derivative, (endo, endo, -5(2-(2-carboxyphenyl)-4(5)imidazoyl) bicyclo (2,2,1) hept-2-yl trans - cinnamate. This model catalyzed intramolecular cinnamate ester hydrolysis through charge relay system similar to that of chymotrypsin. After this, concerted efforts of Bender & coworkers in cyclodextrins resulted into cyclodextrin based mimic of chymotrypsin which catalyzed hydrolysis of t-butyl phenyl acetate and exhibited enzyme like features such as complete turnover, burst kinetics etc. (Mallick et al 1987). This mimic was synthesized by reacting beta cyclodextrin, 2,3 epoxide, with O -(4(5)- mercaptomethyl-4(5) methyl imidazol-2-yl) benzoic acid. Thus β cyclodextrin containing hydroxyl groups was derivatized with imidazole and carboxyl moieties so as to mimick the catalytic triad of chymotrypsin. Schematic representation of catalytic activity of this mimic is shown in Figures 1.1a and 1.1b.

Cyclodextrins are hydrophobic cyclic molecules with varying ring sizes like α, β, γ cyclodextrins which have capacity of substrate binding (depending on the size of the cavity) as that of enzymes. Hydroxyl groups present on cyclodextrin can be derivatized into different catalytically active functional groups. Breslow (1990) employed this feature of cyclodextrins, to synthesize mimics of other enzymes such as ribonuclease, thyamine phosphatase, pyridoxal / pyridoxamine coenzyme etc. These efforts are summarized below.

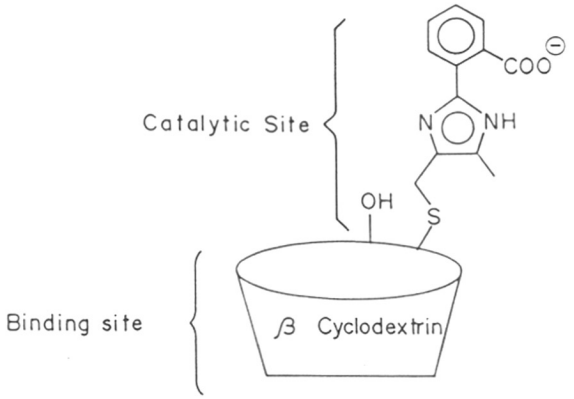


FIG. 1.1a : CYCLODEXTRIN BASED MIMIC OF CHYMOTRYPSIN DEVELOPED BY BENDER et al.

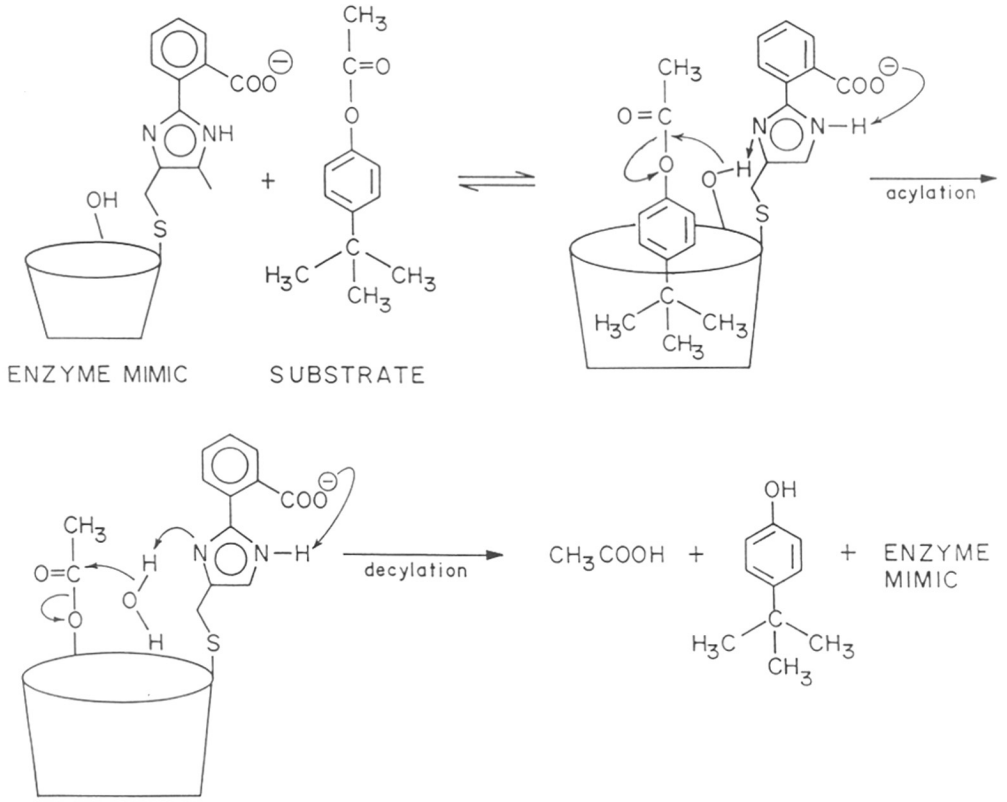


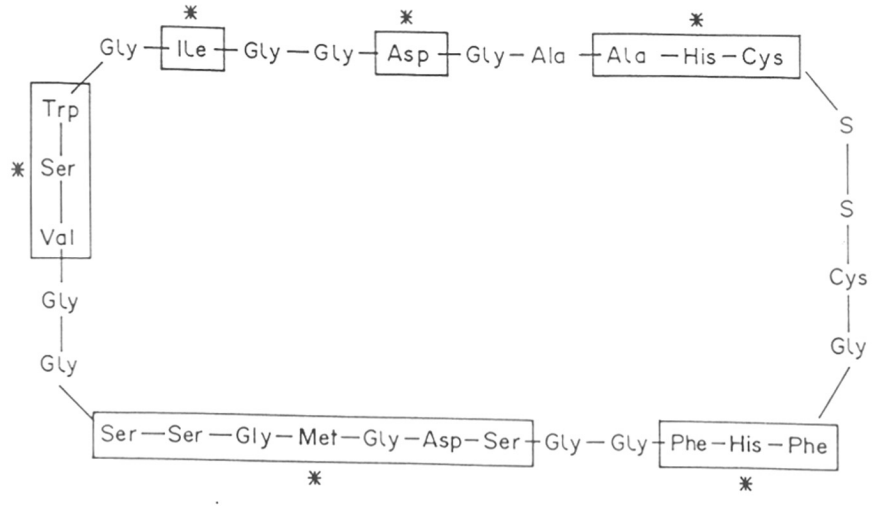
FIG. 1.1b : MECHANISM OF THE CATALYTIC ACTIVITY OF CYCLODEXTRIN BASED CHYMOTRYPSIN MIMIC.

β cyclodextrinylbisimidazole exhibited catalytic hydrolysis of cyclic phosphate ester of t-butyl catechol, and thus ribonuclease activity (Breslow et al 1978). Thiazolium linked gamma cyclodextrin bound two benzaldehyde molecules and also catalyzed benzoin condensation (Breslow & Kool 1988). Beta cyclodextrin with covalently linked pyridoxamine group catalyzed transamination of alpha keto acid into amino acids (Breslow et al 1980).

In summary, cyclodextrin based mimics, due to their ability of substrate binding and catalytic activity like enzymes are of particular interest for synthetic chemists for developing various enzyme mimics. But the main drawback of these materials is the size of the cyclodextrin cavity. Substrates which are bulky in size cannot easily fit into cavity of these materials. For bulky substrates to fit in, coupling of multiple cyclodextrin moieties is required.

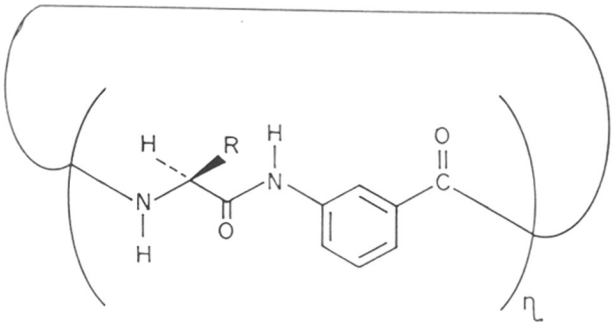
1.3.2 Cyclic peptides

In this approach, cyclic polypeptides which contain active site amino acid sequences of enzymes are synthesized. Such peptides, because of their cyclic structures simulate active site orientation of enzyme and are therefore catalytically active. Attasi & Manshouri (1993) synthesized mimics of chymotrypsin and trypsin by employing surface simulation technique in peptide synthesis. Active site amino acid sequences of chymotrypsin and trypsin alongwith glycine spacers were cyclized through disulfide bridge (Figure 1.2a). Pepzymes (Chpepzyme & Trpepzyme) so synthesized , contained active site amino acid sequences of chymotrypsin and trypsin respectively. These mimics



*ACTIVE SITE AMINO ACID SEQUENCES OF CHYMOTRYPSIN

FIG.1.2a CHYMOTRYPSIN PEPZYME SYNTHESIZED BY SURFACE SIMULATION. OF PEPTIDES.



Cyclo $(-AA-Aba-)_n$ AA = AMINO ACID

Aba = 3 AMINO BENZOIC ACID

e.g. CYCLO (–Se–Aba–His–Aba–Asp–Aba–Ala–Aba–)

FIG.1.2b : CYCLIC PEPTIDES CONTAINING NON-NATURAL AMINO ACID AS BUILDING BLOCKS.

exhibited hydrolytic activity equal to that of enzymes against benzoyl tyrosine ethyl ester (BTEE) and tosyl arginyl methyl ester (TAME), specific substrates for chymotrypsin and trypsin respectively. In another example of cyclic peptides, Ishid et al (1995) synthesized a series of cyclic peptides containing 3 amino benzoic acid as spacer for cyclization (Figure 1.2b). Cyclic peptides comprising serine, aspartic acid, histidine and 3 amino benzoic acid exhibited hydrolytic activity against 4 nitrophenyl acetate. Although the pepzymes synthesized by this approach are significantly active, they are not stable at high temperature and did tend to lose the activity (Attasi & Manshouri 1993). Also the catalytic activity of the pepzymes is not always reproducible (Corey and Phillips 1994).

1.3.3 Catalytic antibodies

Generating antibodies in presence of a particular hapten results in affinity and specificity of that antibody towards substrates which are structurally similar to the hapten used. Because of this, antibodies have capacity to catalyze certain reactions. Shokat et al (1989) reported dehydrofluorination of 4 fluoro, 4 (p-nitrophenyl) butane 2-one, catalyzed by antibody generated against a hapten (Figure 1.3). Carboxylate anion of Aspartate / glutamate residue of this antibody was involved in elimination step. The antibody exhibited typical characteristics of enzyme catalyzed reactions such as turnover, competitive inhibition, substrate specificity and inactivation on blocking the carboxylate group. Antibodies are also reported to catalyze reactions such as acyl transfer (Pollock et al 1986), carbon - carbon bond cleavage (Jackson et al 1988, Hilvert et al 1988) and carbon - carbon bond formation (Cochran et al 1988). Thus, catalytic antibodies are

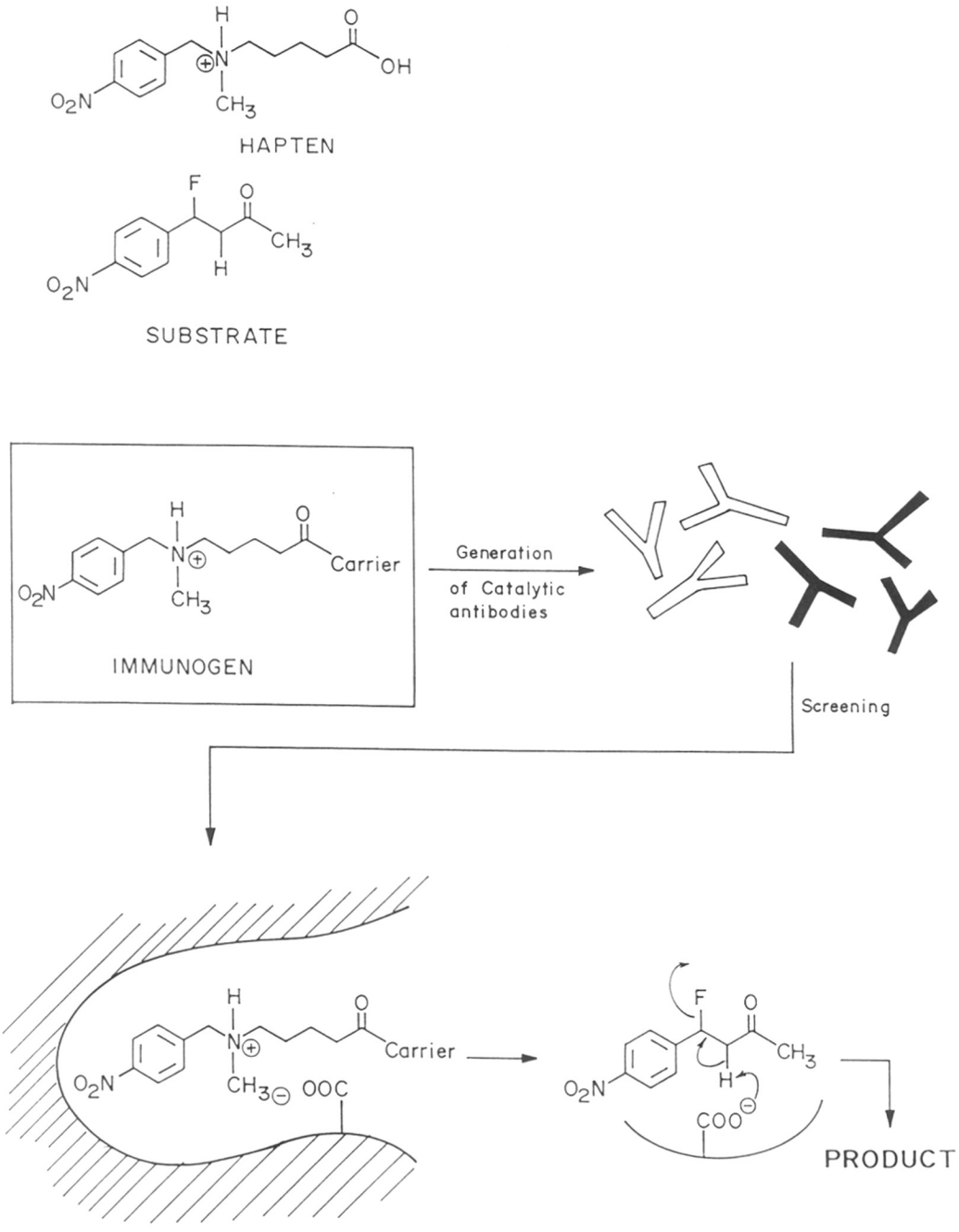


FIG. 1.3 : GENERATION OF CATALYTIC ANTIBODY AND ITS USE AS DESIGNED CATALYST.

regarded as potential enzyme mimics and extensive research is in progress in this area (Lerner et al 1991).

1.3.4 Organometallic complexes

Certain hydrolytic enzymes such as zinc proteases require metal ion for their catalytic activity. Extensive work has been done on the synthesis of organometallic complexes which can mimick the hydrolytic activity of enzymes. These efforts are summarized as follows.

Chin & Jubian (1989) synthesized copper (II) complex of dipyridylamine, (2,2/ dipyridylamine Cu $(OH_2)_2)^{+2}$ which catalyzed hydrolysis of inactive ester, methyl acetate at pH 7.0 and temperature 25°C. Mechanism of this catalysis was reported to be coordination of ester to the copper followed by intramolecular metal hydroxide attack on the coordinated ester (Figure 1.4). Copper (II) 1,4,7 triazacyclononane dichloride (Cu (9) ane N_3) Cl_2 synthesized by Hegg and Burstyn (1995) catalyzed hydrolysis of both dipeptides and polypeptides e.g. bovine serum albumin under physiological conditions. Hydrolytic activity of alkaline phosphatase mimicked by copper (II) chloride complexes was reported by Young et al (1995). In this, Cu (II) N-(2-hydroxyethyl) bis (pyridylmethyl) amine dichloride (1) & Cu (II) N-(3-hydroxypropyl) bis (pyridylmethyl) amine dichloride (2) exhibited catalytic cleavage of 2,4 dinitrophenyl phosphate. It was shown that in case of (1), ester cleavage takes place by hydrolysis whereas in case of (2) it takes place by transesterification. A dinuclear copper (II) complex based on 1,4,7 triazacyclononane orthamide was synthesized by Chin & Young (1995) which catalyzed hydrolysis of RNA, thus exhibited RNAase activity.

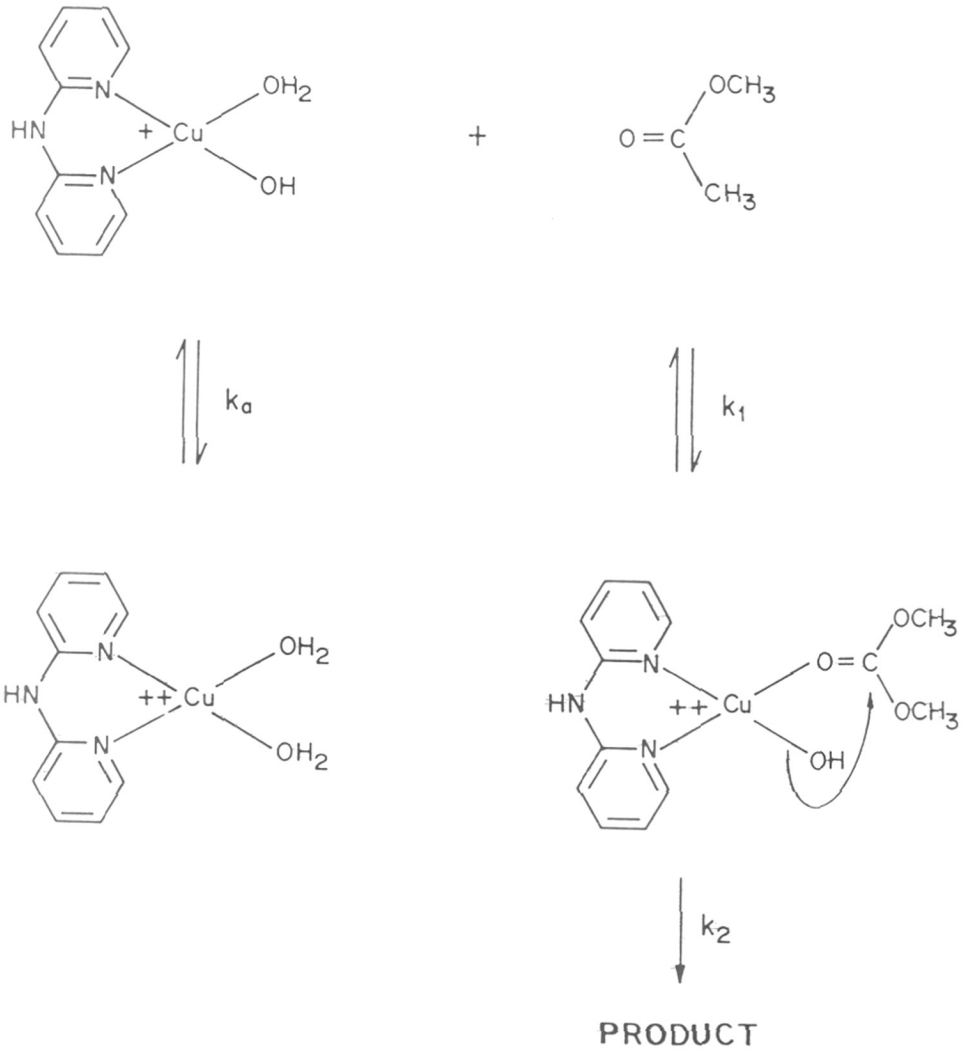


FIG. 1.4 : ORGANOMETALLIC COMPLEX CATALYZED HYDROLYSIS OF ESTER BY COORDINATION OF ESTER TO METAL ION AND INTRAMOLECULAR METAL HYDROXIDE ATTACK.

Zinc (II) containing complexes which mimick activity of alkaline phosphatase and other Zn (II) enzymes are also reported. A zinc (II) macrocyclic tetramine complex based on 1,4,7,10 tetraazacyclododecane ligand catalyzed hydrolysis of bis (2,4 dinitrophenyl) phosphate. This hydrolysis was shown to be a two step reaction. Pendant hydroxyl group was initially phosphorylated and then Zn (II) bound water brought about intramolecular hydrolysis (Koike et al 1995, Kimura et al 1995). Also dimeric zinc complexes based on 1,4,7 triazacyclononane & 1,5,9 triazacyclotetradecane were examined as catalyst for phosphate ester hydrolysis. These studies showed that one Zn coordinates with phosphate group while the other delivers a nucleophile oxide anion (Chapman and Breslow 1995). A mechanistic study in Cu(II) catalyzed hydrolysis of esters and amides by copper (II) complexes is recently reported by Mortellaro et al (1995) in which nucleophilic metal hydroxide catalyzed hydrolysis was demonstrated. In brief, these complexes have shown significant activities and more intensive research is being pursued on this subject.

1.3.5 Crown ethers and Cyclophanes

Macrocyclic polyethers and aza - polyethers have ability to bind with substrates because of their cyclic structures. Introduction of functional groups into these assemblies makes them catalytically active. Tetrafunctional aza - polyether derivatized with four cysteine molecules exhibited substrate binding and esterase activity for p- nitrophenyl esters of amino acids. It exhibited substrate specificity and rate enhancement for bound glycine dipeptide ester (Lehn & Sirlin 1978).

Cyclophane based mimics of serine transacylases were reported by Cram et al (1986) in which, variety of imidazole containing cyclophanes were synthesized. These mimics catalyzed acyl transfer reaction for substrate L- alanyl para nitrophenyl ester salt from substrate molecule to imidazole.

These supramolecular assemblies are mainly investigated for binding studies of various metal ions and substrates. These are potential carriers of ions across membranes and their use as enzyme mimics is limited (Lehn 1985), (Hosseini 1983, 1989).

Various examples sighted above show that a variety of novel approaches have been developed in synthetic chemistry to mimick enzymatic activities. Some of these approaches like catalytic antibodies, organometallic complexes and pepzymes have achieved significant catalytic activities and these could be considered as potential enzyme mimics. Hitherto, use of these materials is associated with the practical problems such as recovery of the catalysts, their compatibility with different solvent systems, stability of catalysts and general applicability to wide range of substrates. Polymers on the other hand are more convenient as catalysts. Due to stability of polymers under harsh conditions, compatibility with solvents, relatively simple methods of synthesis and easy recovery of catalysts, polymeric materials are preferred over synthetic molecules as catalysts (Ford 1986, Sherrington 1988). Intensive research efforts are reported in polymer science towards the design and synthesis of polymeric enzyme mimics.

Enzyme mimics based on synthetic polymers

Research in polymers is broadly classified into two categories viz. Soluble polymers and Crosslinked polymeric networks.

1.4.0 Soluble polymers as enzyme mimics

Results from the structure - property relationship in the synthetic polymers and enzymes, enabled polymer chemists to begin exploration of synthetic polymers as hydrolytic enzymes in 1950 s. Strauss et al (1954, 1961)) reported the ability of poly (1-alkyl-4-vinylpyridinium)s to form self organized micelles when dissolved in water. Klotz et al (1969) also observed similar behavior of partially laurylated polyethyleneimines when dissolved in water. Okubo and Ise (1973) studied the effect of poly (1-alkyl-4-vinylpyridinium)s on OH^- catalyzed hydrolysis of para nitrophenyl esters of ethanoic to dodecanoic acids. It was observed that the rate of reaction increased, as the hydrophobicity of alkyl group in the polymer and that in the substrate increased. This indicated that the hydrolysis of esters is catalyzed by poly (1-alkyl-4-vinylpyridinium)s by forming polymer - substrate complex similar to enzyme - substrate complex.

Overberger et al (1967, 1965, 1976) investigated soluble polymer poly (4(5) vinyl imidazole) as mimic of serine protease (mainly chymotrypsin). Various aspects of polymer catalyzed hydrolysis of esters such as effects of pH, solvent, temperature, substrate structure, monomer structures etc. were elucidated by Overberger and coworkers which are summarized as follows.

Polymeric mimics which carry charge complementary to that of charged substrate were examined by Overberger et al (1965). These investigations showed that polycationic poly (4(5) vinyl imidazole) exhibited preference for anionic substrate 3 nitro 4 acetoxy benzoate anion. Effects of hydrophobic and electrostatic interactions were also investigated by Overberger et al (1973). These studies demonstrated the presence of hydrophobic interactions in catalytic hydrolysis, as was evident by catalyst - substrate binding. Multifunctional polymeric mimics containing imidazole and hydroxyl groups synthesized by Overberger et al (1967) exhibited cooperative effect as that of triad in serine protease. Monomers 4 vinyl phenol and 4(5) vinyl imidazole were copolymerized to yield a bifunctional copolymer poly (4 vinyl phenol - co - 4(5) vinyl imidazole). This polymer was ten times more effective than poly (4(5) vinyl imidazole) in catalyzing hydrolysis 3 nitro 4 acetoxy p-nitrophenyl benzoate.

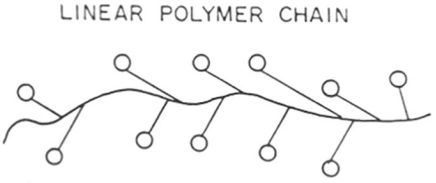
Kunitake and Okahata (1976) parallely investigated soluble polymeric enzyme mimics. Kunitake et al (1969) demonstrated inhibition of hydrolytic activity of copolymers of N- vinyl imidazole and N- vinyl pyrrolidone by benzyl alcohol, 2,4 dinitrophenol, dioxane etc. It indicated that the hydrophobic nature of catalytic site induced substrate binding to the polymeric mimic. Continued efforts in increasing the hydrolytic activity of polymeric mimics were reported by Kunitake et al (1976). In this, copolymers containing N-phenyl hydroxamate and imidazole units were synthesized which exhibited typical burst kinetics in hydrolysis of excess of p-nitro phenyl acetate. It was shown that hydrolysis occurred through acylation and deacylation steps similar to that in the case of enzymes.

Letsinger et al (1962, 1965) also reported work on linear polymeric mimics. They investigated partially protonated poly (4(5) vinyl imidazole) as catalyst for its electrostatic interactions with charged substrates. In the investigations regarding charge complementarity and structure of functional monomers, Morawetz et al (1968) synthesized a polycation poly (1-vinyl-3-methylimidazolium iodide) (PVMI), which was found to enhance solvolytic rates of negatively charged substrates 4 acetoxy 3 nitrobenzoic acid & 4 acetoxy 3 nitrobenzene sulfonate. This polycation did not exhibit any rate enhancement in solvolysis of uncharged substrates viz. p-nitrophenyl acetate and p-nitrophenyl haxanoate. Copolymers of VMI containing 86 mole % of vinyl alcohol did not exhibit rate enhancement, whereas copolymer of VMI containing 63 mole % of p-vinyl phenol exhibited a two fold rate enhancement due to high concentration of phenoxide nucleophile.

Kiefer et al (1972) showed Michaelis Menten kinetics for partially laurylated polyethylene imine derivatized with imidazole groups. This polymer catalyzed hydrolysis of 4 nitrocatechol sulfate, which followed Michaelis Menten kinetics. Arai et al (1979) synthesized a series of block copolymers of vinyl alcohol & styrene sulfonic acid. The polymers in which vinyl alcohol content was very high, exhibited Michaelis Menten kinetics in hydrolyzing dextrin substrate at 70°C, whereas, copolymers in which vinyl alcohol content was less, exhibited only second order kinetics. Cho et al (1982) investigated stereoselective hydrolysis of optically active esters by optically active polymers. They found that solvolysis catalyzed by poly (N-methacryloyl L-histidine) showed no difference in the rates of solvolysis of D & L N-methoxycarbonyl- phenyl

alanyl-p-nitrophenyl esters. But the effect of hydrophobic interactions on stereoselectivity was found in solvolysis catalyzed by N-octanoyl L-histidine.

Recent investigations in this area are more focused on the use of functionalized 4 (dialkyl amino) pyridines (DAAP). Klotz et al (1982) synthesized a series of poly ethyleneimines derivatized with dialkylamino pyridines which exhibited high catalytic activity in hydrolysis of p-nitro phenyl caproate. Vaidya and Mathias (1986) reported homopolymer of 4 N, N diallylamino pyridine which catalyzed hydrolysis of p-nitro phenyl esters of various fatty acids. The polymer exhibited Michaelis Menten kinetics with the formation of acyl pyridinium intermediate. Fife et al (1990, 1991,1994) synthesized oligomer containing alternate DAAP and bistrimethyleneamino-disiloxane units which exhibited enzyme like activity in solvolysis of para nitro phenyl esters of fatty acids. Fife et al (1996) also provided a kinetic evidence for "acyl-enzyme" in poly (DAAP) catalyzed hydrolysis of para nitrophenyl ester of fatty acids, wherein "burst kinetics" were observed when stoichiometric amounts of catalyst and substrate were used. Sutton et al (1990) reported that the hydrolytic activity of oligomer of DAAP and trimethyleneamino disiloxanes compared favorably with that of cholesterol esterase in the hydrolysis of p-nitrophenyl esters of ethanoic to dodecanoic acids. The oligomer also exhibited enzyme characteristics such as turnover, substrate specificity and competitive inhibition by fatty acids. The oligomer of DAAP and bis trimethyleneamino disiloxane units reported by Menger et al (1968) exhibited an unusual order of reactivity. The hydrolysis of para nitrophenyl dodecanoic acid, catalyzed by the oligomer was conducted in media containing methanol, water and a fatty alcohol. It was found that alcoholysis is



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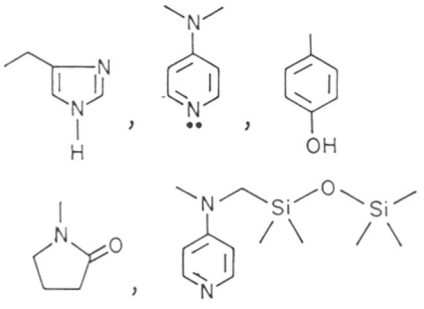
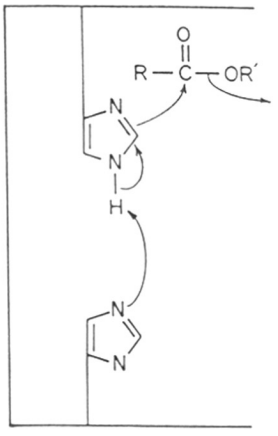
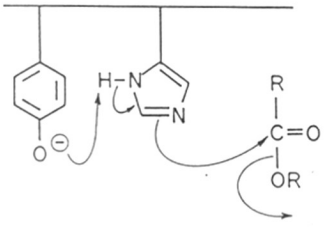
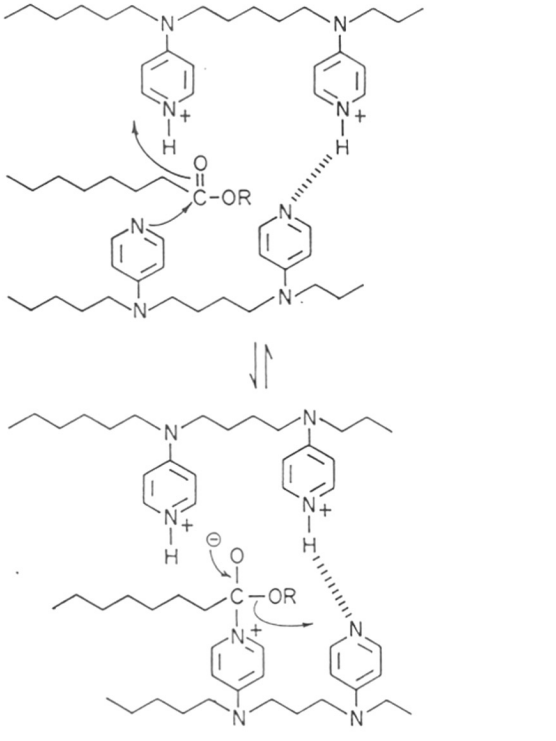




HYDROLYSIS MECHANISM IN
MONOFUNCTIONAL POLYMERS



HYDROLYSIS MECHANISM IN
MULTIFUNCTIONAL POLYMERS.



HYDROLYSIS MECHANISM IN DAAP POLYMERS

FIG 1·5 : VARIOUS LINEAR POLYMERIC MIMICS FOR HYDROLYTIC ENZYMES

predominant rather than methanolysis. Hydrolytic activities of various linear polymers is schematically shown in Figure 1.5.

Thus results from the research on linear polymers demonstrated that polymers can mimick the enzyme activity. These have elucidated the mechanistic aspects, factors determining the substrate binding and the effect of various functional groups on the activity of mimics. Thus the work on linear soluble polymers serves as basic framework for any further studies in polymeric systems.

Soluble polymeric mimics mainly rely on self coiling of polymer chain around the substrate and nucleophilic catalysis wherein, substrate binding is governed by either hydrophobicity or the complementary charges. In soluble polymers, specific cavity in the polymer structure which is complementary to the shape and size of incoming substrate as in the case of enzymes is absent. Also the specific mechanism to induce cooperative action of multiple functional groups like enzymes is lacking in many cases.

1.5.0 Molecularly imprinted crosslinked polymeric networks

Molecular imprinting is the present state of the art technology which overcomes the limitations mentioned above. Its basic principle could be summarized as,

1. Preorganization of functional monomers around the template molecule which resembles the shape and the size of the substrate molecule, by different types of interactions such as covalent, noncovalent and coordination interactions.

2. Polymerization of such assembly with excess of crossilnker and subsequent removal of template so as to retain the specific orientation of functional groups around the cavity created by the template molecule of its size and shape.

3. Application of moleculary imprinted polymers (MIP) containing functional groups which provide binding interactions as separation materials and those which contain catalytically active functional groups as catalysts or enzyme mimics. (Figure 1.6)

Basic framework of this subject is established mainly from the efforts of Wulff (1995), Mosbach (1996), Shea (1994), Sherrington (1995), and Dhal et al (1991). Wulff and coworkers have pioneered the approach of covalent imprinting in developing MIPs for separation materials such as HPLC stationary phases for racemic resolution of sugars and carbohydrates etc. Mosbach and coworkers have investigated noncovalent interactions between the template and functional or binding monomers so as to develop MIPs for chiral resolution of peptides and drugs, and enzyme mimics. Shea and coworkers have concentrated mainly on designer catalyst based on MIPs, while Dhal and coworkers have investigated metal ion coordination interactions for synthesizing MIPs. Sherrington and coworkers have pioneered the novel application of molecularly imprinted polymers. They have developed molecularly imprinted polymeric optical sensors.

In the following sections methods of achieving molecular imprinting are discussed.

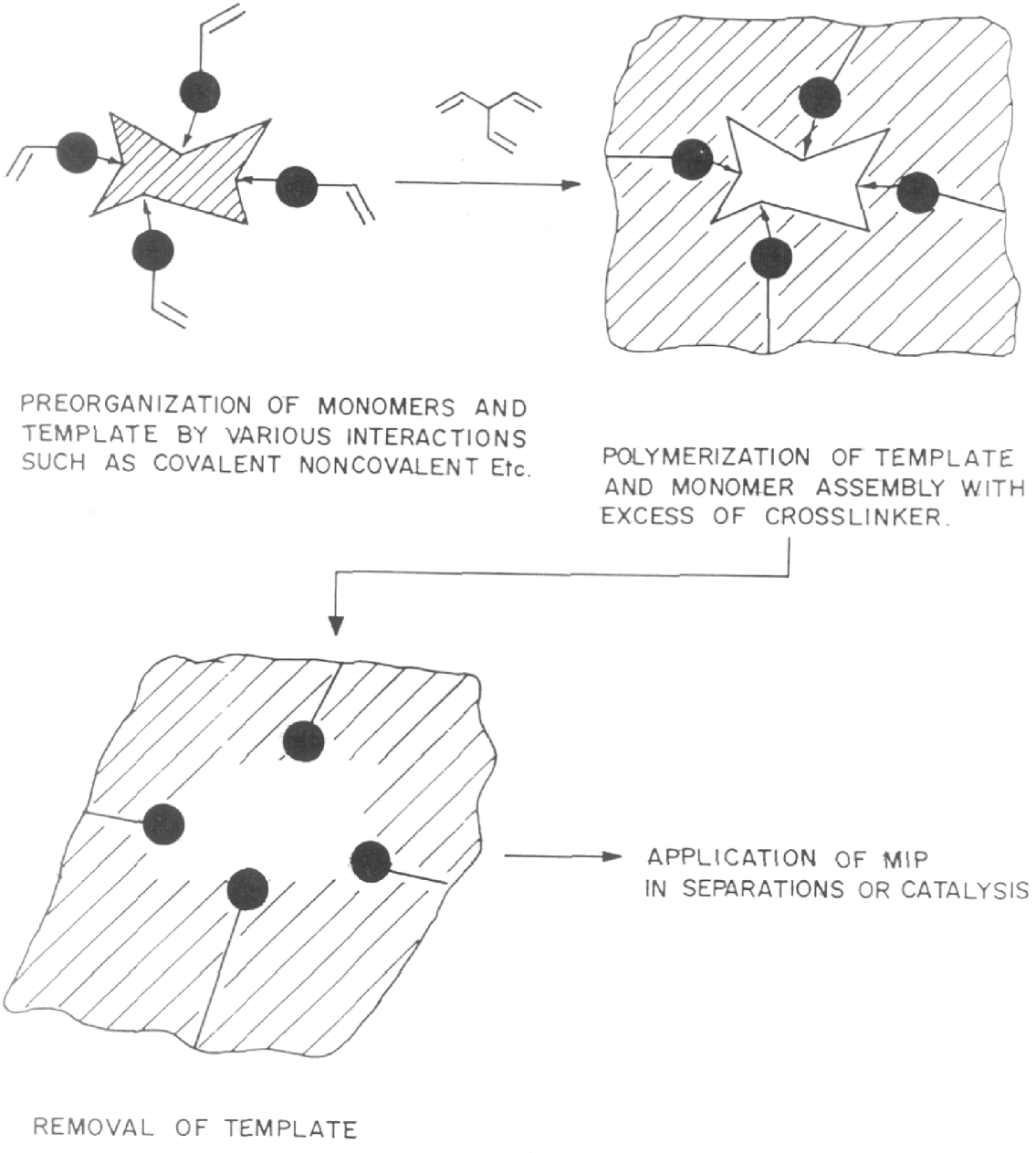


FIG. 1·6 : GENERAL SCHEME FOR SYNTHESIS OF MOLECULARLY IMPRINTED POLYMERS.

Methods of achieving molecular imprinting

1.5.1 Covalent interactions

In this approach, in order to form shape and size selective cavity around the functional monomers in polymeric matrix, the functional monomer is covalently linked via ester bond to the template molecule being imprinted. Then polymerization of monomers - template assembly with excess of crosslinker is performed and the template is split off the polymer by hydrolysis. The cavity that is formed by this method is much more specific than that is formed by noncovalent or metal ion coordination interactions. Therefore MIPs synthesized from covalent interactions are more suitable for racemic resolution. But the application of this approach has a drawback in that extensive use of synthetic chemistry is involved to synthesize monomers - template assembly.

Wulff and Sarahan (1972) developed the concept of molecular imprinting in crosslinked polymers using polymerizable template - monomer assembly. A template molecule α D- mannopyranoside was esterified with para vinylphenyl boronic acid. Thus template was covelently bound to functional monomer. Polymerization of this assembly, with divinylbenzene yielded a highly crosslinked polymer with boronate ester covalently linked to the polymer. Template molecule was then removed from the network by hydrolysis of the ester. The free boronic acid groups in the polymer exhibited specific binding for the template used in imprinting.

The template used was optically active. Verification of the correct structure of the cavity was made by equillibriating the polymers with racemates of α D mannopyranoside.

Polymers exhibited the selective sorption of the imprinted enantiomer with separation factor α varying from 1.2 to 6.0 depending upon the experimental conditions. It was shown that upto 70 percent *ee* enriched D- α-D mannopyranoside can be obtained in a batch process (Wulff 1995). Extensive research efforts on the covalent imprinting approach have been reported by Wulff and coworkers (1985,1995) which elucidated various parameters that enhanced the performance of MIPs as HPLC stationary phases (Figure 1.7).

1.5.2 Noncovalent interactions

In this approach the template molecule and the binding monomer are dissolved in suitable solvent in which weak interactions such as hydrogen bonding, ionic bonding and hydrophobic interactions are favored amongst the template and monomers. Crosslinker is then added to this solution and polymerization is conducted. The polymers so synthesized are subsequently worked up to elute out the template. Due to the above mentioned interactions amongst monomers and template, a cavity which is complementary to the size and shape of the template molecule is created around the functional monomers in the polymer. The most important advantage of this methodology is its general applicability because of which it is employed in synthesizing separation material for drugs, peptides, metal ions, sugars, nucleosides etc.

Ashardy and Mosbach (1981) developed the concept of noncovalent interactions for creating specific cavity in polymeric networks. Various crosslinked polymers containing methyl methacrylate (binding monomer) and N,N methylene bis acrylamide or N,N 1,4 diphenylenediacrylamide (crosslinker) were synthesized in presence of

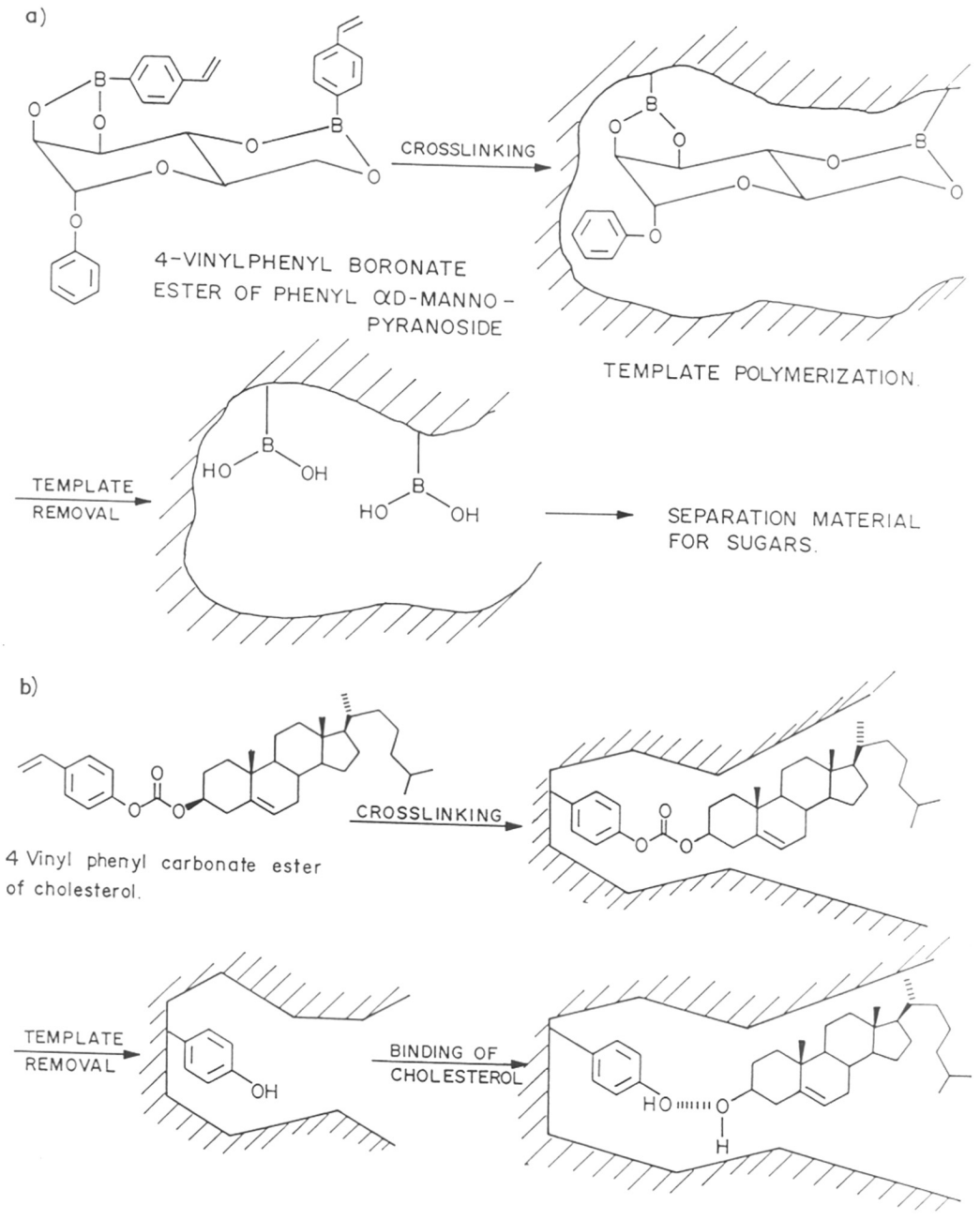


FIG. 1·7 : COVALENT IMPRINTING FOR SYNTHESIS OF MIPS.
a) SEPARATION MATERIAL FOR SUGARS b) SEPARATION MATERIAL FOR CHOLESTROL.

rhodaniline blue and safranine. After removal of dyes the polymers exhibited selective binding for the dye which was present in the polymerization mixture.

As described above, noncovalent interactions rely mainly on hydrogen bonding and ionic bonding interactions between template and the binding monomers (Figures 1.8a and 1.8b). Many investigations that revealed the factors governing these interactions are reported by Mosbach et al (1988) using N-phenyl alanine anilide as template and methacrylic acid as binding monomer. Ramstrom et al (1993) employed two chemically distinct monomers viz. basic 2 vinyl pyridine and acidic methacrylic acid (MAA) in the same polymer network. Dansyl phenyl alanine which is capable of exhibiting hydrogen bonding interactions with metacrylic acid and ionic interactions with 2 vinyl pyridine was used as template molecule. The template - monomer assembly was polymerized with excess of crosslinker ethylene glycol dimethacrylate (EGDMA) to synthesize the imprinted polymers. Bifunctional MIPs so synthesized were employed as stationary phases in HPLC columns. It was found that bifunctional MIP always exhibited higher separation factor α than those exhibited by monofunctional MIPs containing either methacrylic acid or 2 vinyl pyridine. Mosbach and coworkers further extended this approach in developing various separation materials for drugs and peptides (Ekberg & Mosbach 1989, Mosbach et al 1994, 1996, 1991). Noncovalent interactions were also extended by Shea and coworkers in developing catalysts, and separation materials (1993).

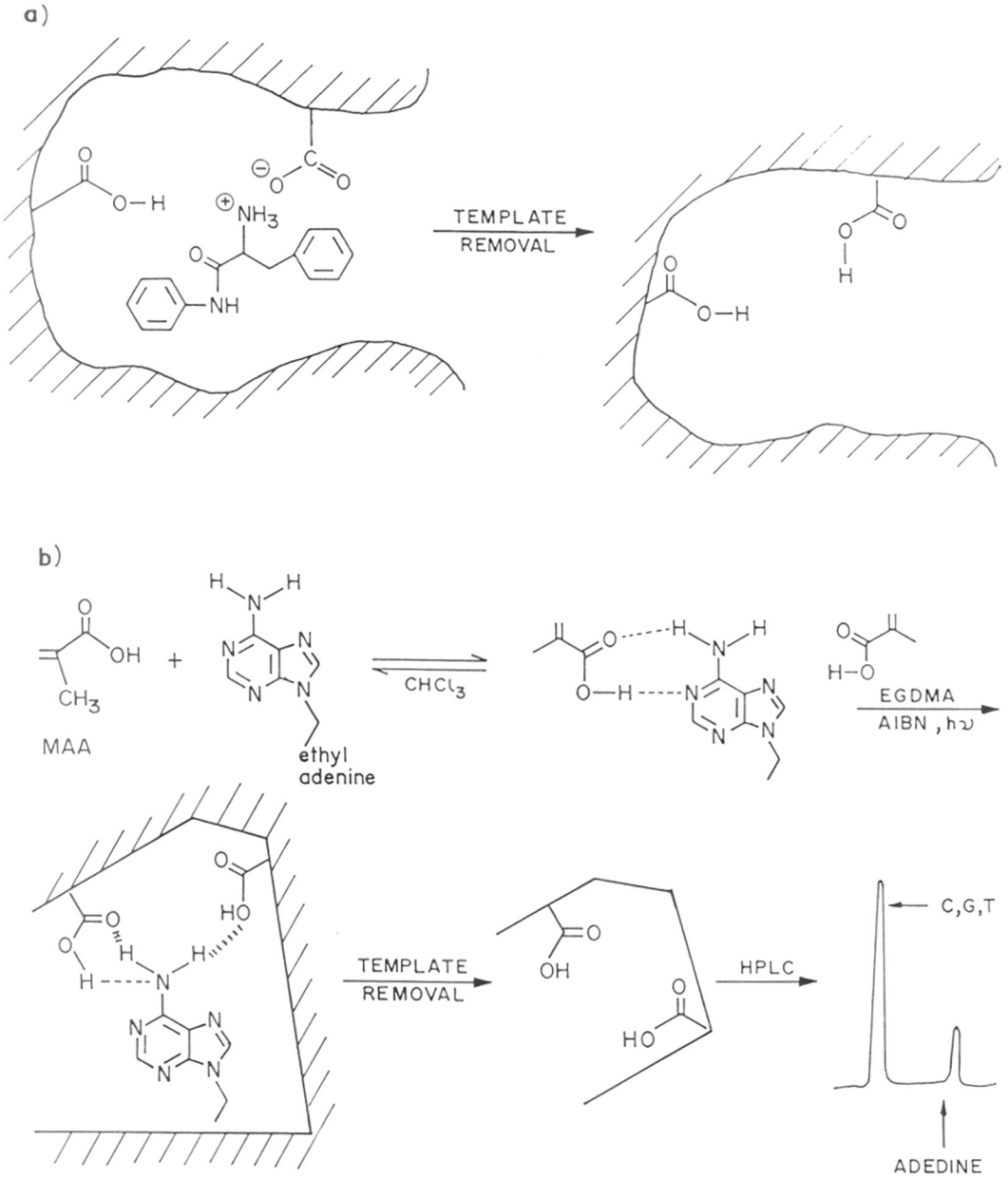


FIG. 1·8a : NONCOVALENT IMPRINTING BY IONIC INTERACTIONS

1·8b : NONCOVALENT IMPRINTING BY HYDROGEN BONDING INTERACTIONS.

1.5.3 Metal ion coordination interactions

This approach is slightly different than the two described above. In this case functional monomers and the template molecule are complexed with transition metal ion such as Co(II), Cu(II)etc. Due to this complexation monomers and template are oriented close to one another around the metal ion in such a way that cooperative effect as in the case of enzymes is possible in many cases (Belokon et al 1980, 1982, Mosbach et al 1987). The advantage of this approach over covalent and noncovalent interactions is in orienting large number of functional monomers close to one another with greater simplicity.

Fujii et al (1985) used metal ion coordination for achieving molecular imprinting. 4-(p-vinyl benzyloxy) salicylaldehyde was complexed with Co(II) and N-benzyl D-valine. This complex was polymerized with styrene and divinyl benzene and then subsequently worked up to remove valine template. The polymer containing cobalt complex exhibited an exceptionally high separation factor of 682 in the resolution of racemic mixture of the template molecule (Figure 1.9a). Molecular imprinting using metal ion coordination was recently reported by Dhal et al (1991, 1992) in which coordination complex of copper (II) with (N-4(vinylbenzyl) imino) diacetic acid and various substituted bisimidazoles was used. This complex was polymerized with EGDMA. The polymers obtained after removal of template and copper were reloaded with copper. Copper loaded polymers exhibited selective binding for templates used in the formation of coordination complex (Figure 1.9b). This approach was further extended by Dhal et al (1995) in synthesizing polymeric support by grafting copper (II) (N-4 -vinylbenzylimino) diacetic acid onto

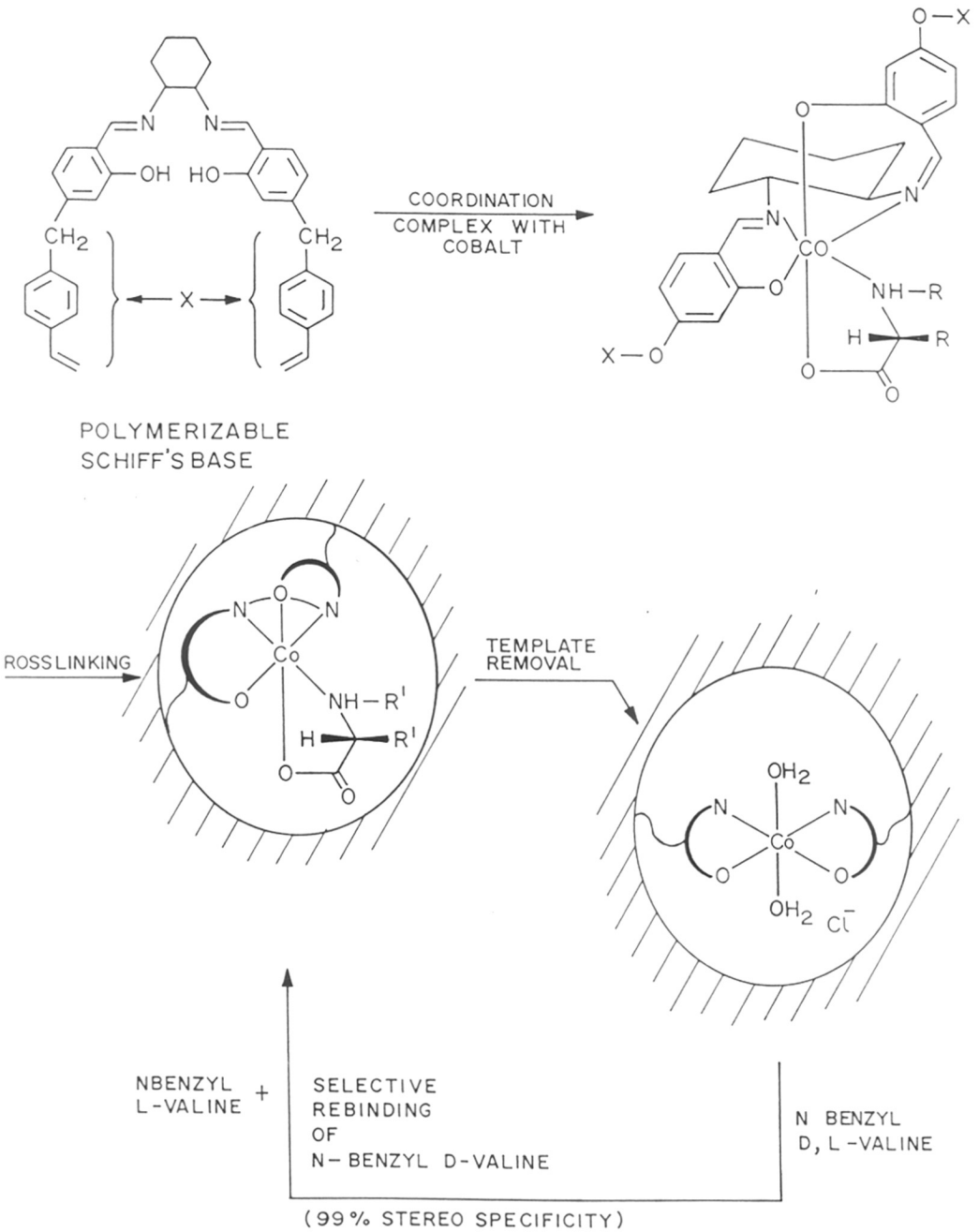


FIG. 1·9 a) RACEMIC RESOLUTION OF AMINO ACIDS BY METAL ION COORIDINATION.

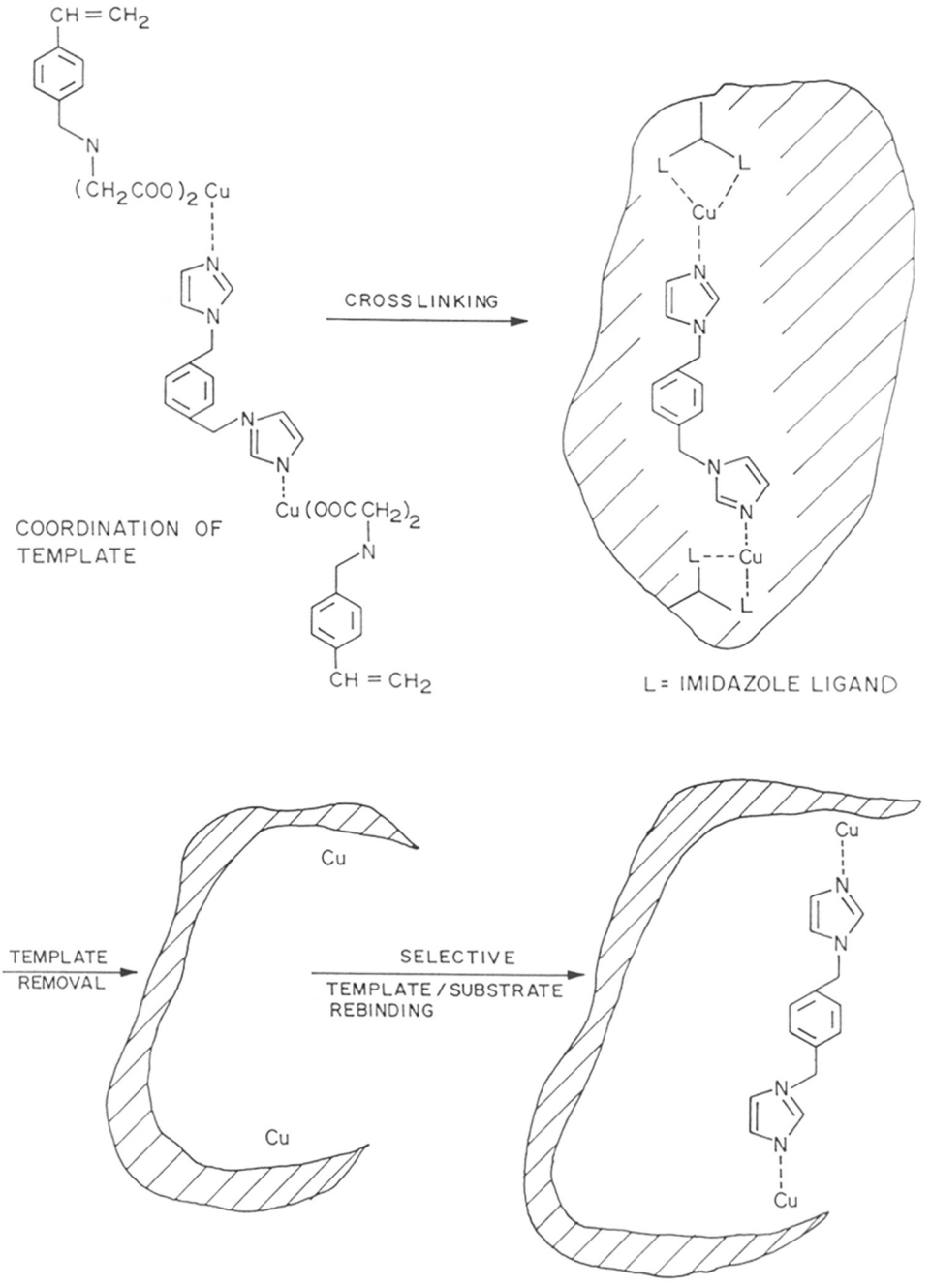


FIG. 1·9 b) MOLECULAR IMPRINTING THROUGH METAL ION COORDINATION OF TEMPLATE / SUBSTRATE.

surface of microporous poly (trimethylol propane trimethacrylate) (poly (TRIM)) support which also exhibited selective rebinding. This technique has a significant advantage over highly crosslinked MIPs. In this case the functional monomers are present on the surface of the support, therefore the problem of slow adsorption and desorption of substrates experienced by highly crosslinked networks, is circumvented. The technique has been termed as **"surface grafting".**

Above three are the important methods of achieving molecular imprinting in crosslinked polymers. Numerous examples of applications of MIPs as separation materials have been reported which could be summarized into following categories with relevant examples.

1.6.0 Application of MIPs in separations

1.6.1 Proteins and peptides

Glad et al (1985) have employed organic silanes as supports for chromatography. In this, covalent imprinting of the substrate was achieved by using silanes derivatized with 3 amino benzoic acid. This silane was reacted with protein transferrin and crosslinker bis (2 hydroxyethyl) aminopropyl triethoxysilane. The silane polymer was coated on silica. Stationary phase so synthesized, exhibited specificity in binding the imprinted protein. Venton & Gudipati (1995) synthesized polysiloxanes copolymers from 3 aminopropyl triethoxysilane & tetraethyl orthosilicate in presence of urease and bovine serum albumin (BSA). Polymers showed specific binding for the imprint used in their preparation. Urease imprinted polymer bound 90% urease and BSA imprinted polymer

bound 60% BSA in a batch process. Racemic resolution of dipeptide cbz-L-ala-L-ala-Ome was reported by Kempe et al (1995) using MIP based on trimethylol propane trimethacrylate (crosslinker) and methacrylic acid (binding monomer). The polymer was synthesized by noncovalent imprinting of cbz-L-ala-L-ala-Ome. HPLC column packed with this polymer exhibited resolution factor of 4.50 for cbz-L-ala-L-ala-Ome and 1.71 for cbz-D-ala-D-ala-Ome.

1.6.2 Sugars, carbohydrates and nucleosides

Investigations in the origins of selectivity for covalently imprinted MIPs were reported by Wulff et al (1991). In this work, 4 vinyl phenyl boronate ester of 6-O-benzyl α D-galactopyranose and 1-O-benzyl beta D-fructopyranose were synthesized as polymerizable template - monomer assembly. The assemblies were polymerized with EGDMA crosslinker and templates were split off to yield MIPs. It was found that D & L imprinted polymers exhibited high selectivities for D & L galactose respectively from its racemic mixture. But more importantly, polymer imprinted with D galactose showed selective adsorption of L-fructose from its racemate ; which indicated that orientation of functional groups within the cavities is more important than shape of the cavity. Anomeric and epimeric discrimination in carbohydrate resolution has been demonstrated by Mayes et al (1994). MIPs based on MAA and EGDMA were synthesized by noncovalent imprinting of para nitrophenyl alpha D- galactoside. HPLC columns packed with the polymers gave baseline separation for α and ß anomers of the imprinted molecule. Similarly a high epimeric selectivity was also exhibited by these MIPs towards their imprints.

Shea et al (1993) reported the use of MIPs in separations of biologically important nucleosides. A polymeric network based on MAA, N,N',3 phenylenebis (2-methyl-2-propenamide) and EGDMA was synthesized in presence of 9-ethyladenine (template for adenine). The polymer loaded in HPLC column exhibited strong affinity for adenine and its derivatives.

1.6.3 Drugs

Fischer et al (1991) synthesized a polymeric network containing itaconic acid as functional monomer for the first time in presence of a template S (-) Timolol. HPLC column packed with these polymers gave high degree of selectivity for S (-) Timolol when racemic mixture of Timolol was eluted.

Application of MIPs as a substitute for antibody in drug assay in human serum was shown by Vlatakis et al (1993). Theophylline and diazepam imprinted polymers based on MAA & EGDMA were used for binding these drugs from human serum. The assay employed determination of inhibition of radiolabelled ligand binding by the serum analyte. The results showed that both MIPs specifically bound their own substrates. Theophylline imprinted polymer bound 14-224 micromoles of theophylline and diazepam imprinted polymer bound 0.44 to 28 micromole, both in the range of therapeutic monitoring of drug.

1.6.4 Dyes

Silica derivatized with methacrylate groups i.e. methacrylate silica was employed by Norlow et al (1984) in resolution of different dyes. Methacrylate silica was polymerized with methyl methacrylate, & N,N methylene bis acrylamide in presence of Rhodaniline blue and Safaranine-O for noncovalent imprinting. Substrate selective binding was exhibited by these polymers in which Safaranine-O printed polymer gave selectivity factor R = 0.78 and Rhodaniline blue printed polymer gave selectivity factor R = 1.00.

1.6.5 Metal ions

Nishide & Tsuchida (1976) synthesized crosslinked polymers of poly (4-vinyl pyridine) & 1,4 dibromobutane in presence of Cu^{+2} ions which selectively sorbed Cu^{+2} ions because of the imprints of copper formed during crosslinking. Ion exchange resins for selective metal ion separation were synthesized by Kunchen & Schram (1988) in which polymerizable copper (II) complexes of tetrakis (μ- methacrylato -O-O) bis [L / copper II] (L = H_2O, pyridine, vinyl pyridine) were polymerized with EGDMA. Resins thus obtained were tested for their Cu (II) selectivity. Effective capacity of copper imprinted resins was highest (45 micromole /g) in comparison with other metal ions such as Zn (II) 10.3, Cd (II) 7.4 and Pb (II) 15.5 micromole /g.

1.7.0 MIPs as sensors

MIPs have potential applications in sensor materials. Especially biomimetic sensors based on MIPs would be a better alternative where biosensors cannot work owing

to their instability under harsh conditions. Optical sensor based on molecularly imprinted polymeric monolith was reported for the first time by Sherrington et al (1996). The monolith was synthesized using Michler's ketone (4,4' bis (dimethylamino) benzophenone) as template molecule, 2 acryl amido 2 methyl propane sulfonic acid (AMPS) as functional monomer and trimethylolpropane trimethacrylate (TRIM) as crosslinker. When the monolith was irradiated with linearly polarized light the template molecule was covalently linked with the network by free radical reaction to yield a transparent anisotropic monolith. Dichroism exhibited by the monolith was shown to originate from the photogenerated decomposition of the Michler's ketone template. That the monoliths are indeed molecularly imprinted was shown by competitive binding studies. It was also shown that this approach could be extended to photoinactive templates by equilibrating polymers with photoactive molecules after removal of template. But the polymers were found to be shape and size selective but not necessarily for the imprinted template, due to the lack of sufficient interactions between the binding sites and the template.

Strategies to enhance these binding interactions are also reported by Sherrington et al (1993). In this case molecular imprinting of flat polycondensed aromatic molecules in macroporous polymers was reported. In this work a methodology was developed which enables MIPs to selectively sorb flat polycondensed aromatic molecules and assay them quantitatively via linearly polarized infra red spectroscopy. In this work 2,6 diamino anthraquinone (DAAQ) was noncovalently bound to acidic functional monomers such as methacrylic acid and 2 acrylamido 2 methyl propane sulfonic acid (AMPS). The

monomers - template assembly was polymerized with excess of crosslinker to obtain the imprinted polymers. It was found that the polymers containing weakly acidic binding sites of monomers like methacrylic acid or acrylic acid did not exhibit shape selective sorption of DAAQ. But the polymers containing strongly acidic binding sites of AMPS indeed exhibited selective sorption of DAAQ as compared to nontemplated polymers.

Use of MIPs as biomimetic sensors has also been reported. Kritz et al (1995) demonstrated fiber optic detection of dansyl phenyl alanine (fluoroscent labeling of amino acid) by MIP. The polymer was synthesized using dansyl phenyl alanine (template), MAA , 2 vinyl pyridine (binding monomers) and EGDMA (crosslinker). Similar example of sensor exhibiting potentiometric detection of phenyl alanine anilide in a flow through column electrode has been reported by Andersson et al (1990).

MIPs as catalysts and enzyme mimics

Research in molecularly imprinted enzyme mimics and catalysts have achieved significant progress over the past few year. As stated above the work in this area is classified into two categories.

1.8.0 MIPs as designer catalysts

One of the obvious applications of MIPs is catalysis. Prearranged functional groups of MIPs with specific cavity for individual substrate, have enabled researchers to synthesize and apply a variety of designer catalysts in catalyzing various reactions.

First efforts in designer catalysts were reported by Shea et al (1978).in which optically active cyclopropane formation through MIP was achieved. In this work, benzyl

alcohol groups (preoorganized by forming ester with trans 1,2 cyclobutane dicarboxylic acid) present in the styrene / DVB network were reacted with fumaryl chloride. Insertion of methylene group across this double bond to form cyclopropane ring was achieved by (dimethyl amino) methyl- phenyl- oxosulfonium reagent. 1,2 cyclopropane dicarboxylic acid thus formed was freed from the polymer support by hydrolysis and recovered as methyl diester. This product showed 0.05 % e,e enantiomeric excess because of chiral environment of reactive groups in the support.

Damen & Neckers (1980) demonstrated the use of molecularly imprinted designer catalyst in selective synthesis of anomers of truxilic acid which were used for imprinting the catalyst. For this, α, β and δ bisvinyl phenyl truxinate esters were synthesized as polymerizable template - monomer assembly. The assemblies were polymerized with DVB and the templates were subsequently removed by hydrolysis. Thus cavities complementary to α, β and δ truxinic acid were created. These MIPs were reacted with cinnamoyl chloride and irradiated with uv light to induce dimerization. The dimers (truixinic acids) were then split off polymers by hydrolysis. The polymer imprinted with β truxinic acid gave 53 % β truxinic acid and the polymer imprinted with δ truxinic acid gave 52.7 % δ truxinic acid.

Wulff et al (1989) were successful in achieving asymmetric synthesis of α amino acids with 36% enantiomeric excess using molecularly imprinted catalyst. The template molecule L-DOPA was bound to 4 vinyl salicylaldehyde and 4 vinyl phenyl boronic acid to obtain polymerizable template - monomer assembly. After its polymerization with DVB and template splitting, glycine was bound to the polymer. It was then treated with

lithium diisopropylamide base and the enolate anion with chiral environment so obtained was reacted with 4 methyl benzyl bromide. Subsequently the amino acid formed was split off the polymer. High percentage of e,e form of L-methyl phenyl alanine was obtained in the product. Similarly the reaction of glycine enolate with acetaldehyde resulted into 35% enantiomeric excess of threo diastereomer of L-threonine (Figure 1.10b).

A remarkable stereoselectivity in hydride reduction of steroidal ketone was demonstrated by Bystrom et al (1993). Acrylate ester of steroid 17 -ol was polymerized with DVB and template was freed by hydrolysis. Carboxyl group in the polymer was then reduced to alcohol and converted into hydride using $LiAlH_4$. This catalyst was then treated with steroid 1 -17 dione to get exclusive reduction at C_{17} carbonyl group in which 70 : 30 distribution of β and α alcohol was obtained.

Morihara et al (1988) employed molecular imprinting (footprint) to kieselgel. They reacted kieselgel with aluminum chloride ($AlCl_3$) in presence of N-benzoyl benzene sulfonamide (template for imprinting benzoic anhydride). As a result of this, the gel was functionalized with Al^{+3} ions with an imprint for substrate benzoic anhydride. This molecularly imprinted Lewis acid efficiently catalyzed butanolysis of benzoic anhydride at 50° C. Imprinting effect was evident from the low K_m values for imprinted catalysts as compared to those for non imprinted catalysts. This work was further extended to synthesize catalysts imprinted with various templates. These catalysts exhibited competitive inhibition for the templates used in their synthesis.

MIP catalyzed dehydrofluorination with activity comparable to that of catalytic antibodies was achieved by Shea et al (1994) using N-(2 amionethyl) methacrylamide as functional monomer. In this, benzyl malonic acid was used as template to orient the functional monomer and then it was crosslinked with EGDMA. Subsequent removal of template afforded catalyst which catalyzed dehydrofluorination of 4-fluoro-4(nitrophenyl)butane 2-one (Figure 1.10a). It exhibited catalytic turnover when excess of substrate was used. It also exhibited competitive inhibition for the template used in the imprinting.

Efendiev (1994) reported use of metal ion and template imprinting in polymers so as to synthesize designed oxidation catalysts. Crosslinked copolymers of diethyl vinyl phosphonate and acrylic acid were prearranged with cobalt and used as catalyst in oxidations of xylene into toluic acid. It was found that prearranged catalyst afforded 82 percent yield of p-toluic acid whereas conventional cobalt benzoate catalyst afforded yield of 63 percent.

1.9.0 Enzyme mimics

Different approaches developed towards this end have been classified by Mosbach et al (1996) into following categories. **1.** Transition state analogue based mimics. **2.** Coenzyme analogues based mimics. **3.** Bait & switch type mimics.

1.9.1 Transition state analogues based mimics

In this approach the template molecule which can represent the transition state in ester hydrolysis of substrate is used as an imprint. Functional monomers are oriented

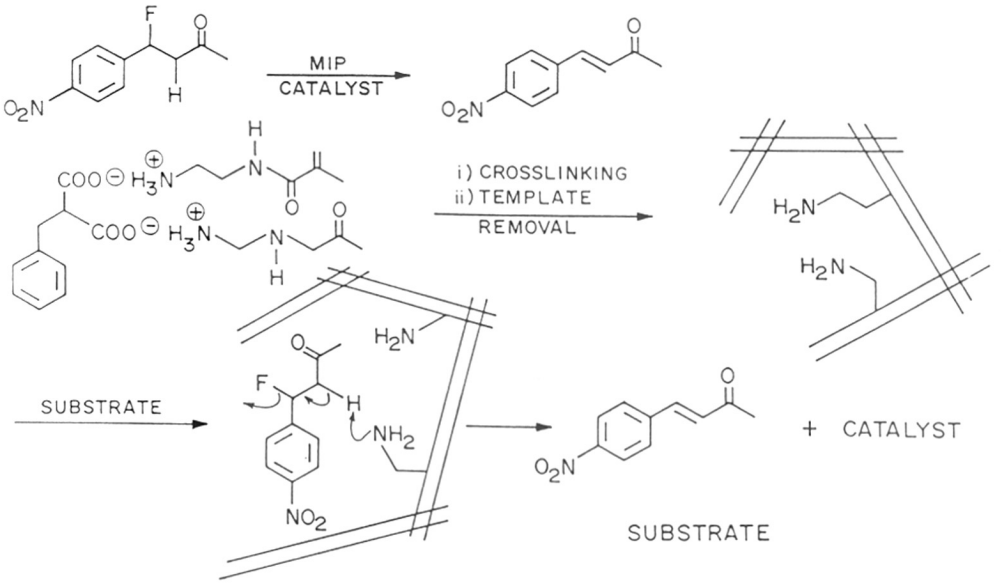


FIG. 1·10 a) MIP CATALYZED DEHYDROFLUORINATION (DESIGNER CATALYST.)

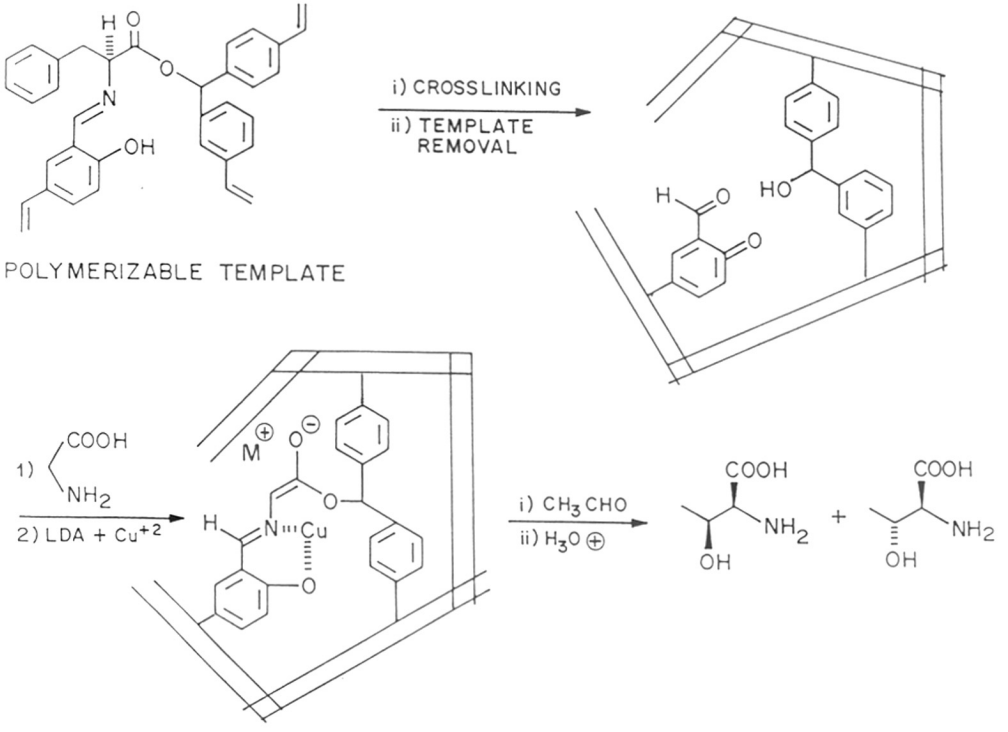


FIG. 1·10 b) ASYMMETRIC SYNTHESIS OF AMINO ACIDS BY MIP CATALYST.

around such a transition state analogue and polymerized to obtain an enzyme mimic. Robinson & Mosbach (1989) synthesized a mimic of hydrolytic enzyme by polymerizing 4(5) vinylimidazole and 1,4 dibromobutane (crosslinker) in presence of p-nitro phenyl methyl phosphonate (a transition state analogue for hydrolysis of p-nitro phenyl acetate) and cobalt for creating binding sites for functional groups. The polymer obtained after work up was 60% more active in hydrolyzing the substrate than the control polymer. The polymer also exhibited inhibition of activity in presence of template molecule. More recently Ohkubo et al (1995) reported hydrolytically active mimic based on methyl N-acryloyl histidinate. Polymer was imprinted with N-(N-benzyloxycarbonyl L-leucinoyl) anthranilic acid which represented transition state in hydrolysis of N-benzyloxycarbonyl L-leucinyl p-nitrophenyl ester. High catalytic activity of imprinted mimic was accompanied by lower energy of activation as compared to that of control polymer.

Enantioselective hydrolysis of ester by mimic imprinted with transition state analogue was reported by Sellergren et al (1994). In this, polymerizable template - monomer assembly (4 vinyl- 2 imidazoyl- phenyl ester of N-t-boc D Phenyl alaninyl ethyl phosphonate) was polymerized with MAA (co- monomer) and EGDMA (crosslinker). The template which represented the transition state of hydrolysis of N-t-boc D-Phe-ala-PNP ester was split off. The resulting mimic containing phenol - imidazole catalytic group exhibited faster rate of hydrolysis for N-t-boc D-Phe-ala-PNP than that for N-t-boc L-Phe-ala-PNP. Also the mimic imprinted for N-t-boc L-Phe-ala-PNP exhibited faster hydrolysis of N-t-boc L-Phe-ala-PNP than that of N-t-boc D-Phe-ala-PNP.

1.9.2 Coenzyme analogues based mimics

In this approach coenzyme substrate analogue is imprinted with the polymers so as to obtain a mimic of coenzyme. Andersson & Mosbach (1989) synthesized such a mimic for the first time. N-pyridoxal- L-phenyl alanine anilide was used as template for imprinting the Schiff's base between pyridoxal and phenyl alanine anilide. The mimic was synthesized by noncovalent imprinting of template with methacrylic acid and polymerizing the monomers-template assembly with EGDMA. Thus the polymer was imprinted with N-pyridoxal L-phenyl alanine anilide. The polymeric mimic so synthesized, exhibited eight fold rate enhancement in catalyzing condensation reaction between pyridoxal phosphate and phenyl alanine.

1.9.3 Bait and switch based mimics

In this category, examples of mimics synthesized from metal ion coordination of monomers are prominent. In this approach, functional monomers are complexed with transition metal ion and the complex is polymerized with crosslinker. Subsequent removal of metal ion directs the functional groups in vicinity of one another due to which cooperative action of functional groups as that of enzymes can be obtained. Leonhardt & Mosbach (1987) synthesized a hydrolytically active mimic which exhibited catalytic turnover as in the case of hydrolytic enzyme. A pseudotetrahedral complex of Co (II) with two molecules of 4(5) vinylimidazole and a template molecule was polymerized with divinylbenzene and subsequently worked up to remove metal ion. The mimic so synthesized, exhibited higher rate of hydrolysis of imprinted substrate with a turnover

when excess of substrate was used. This work was further extended by Robinson & Mosbach (1989) for transition state analogues.

A biomimetic MIP of pyridoxal enzyme was synthesized by Belokon et al (1980, 1982). Stiochiometric amounts of 5 methacryloylaminosalicylidene & N^{e} -methacryloyl (S) lysine were reacted with copper acetate to give a complex N^{x}-5 methacryloylaminosalicylideno - N^{e} methacryloyl- (S) lysinato copper (II). This was copolymerized with acrylamide and N N' methylenebisacrylamide. The polymer mimic obtained after work up exhibited nucleophilic catalysis by pendant alpha amino group of lysine in the mimic to form the semicarbazone derivative between salicylaldehyde moiety of the mimic and semicarbazide. The pyridoxal mimic also exhibited formation of internal aldimine Schiff's base because of the vicinity amongst amino groups of lysine and aldehyde group of salicylaldehyde, due complexation with copper (Figure 1.11).

More recently, this approach was used by Karamalkar et al (1996) from our group in synthesizing a catalytically active hydrogel. Co (II) coordinated assembly consisting 2 hydroxyethyl methacrylate (HEMA), methacrylic acid (MAA) and N-methacryloyl L-histidine (MA-histidine) and template isobutyryl 6 amino caproyl phenyl alanyl 2 amino pyridinamide (IBA-6ACA-Phe ala-2AP) was prepared and it was polymerized with HEMA and EGDMA to yield hydrogel. Template & metal ion were then removed and the substrate methacryloyl 6 amino caproyl phenyl alanyl para nitro phenyl ester (MA-6ACA-Phe ala-PNP) was sorbed into hydrogel. The imprinted gel exhibited high catalytic activity in hydrolyzing the substrate than the gel which was synthesized in the absence of cobalt.

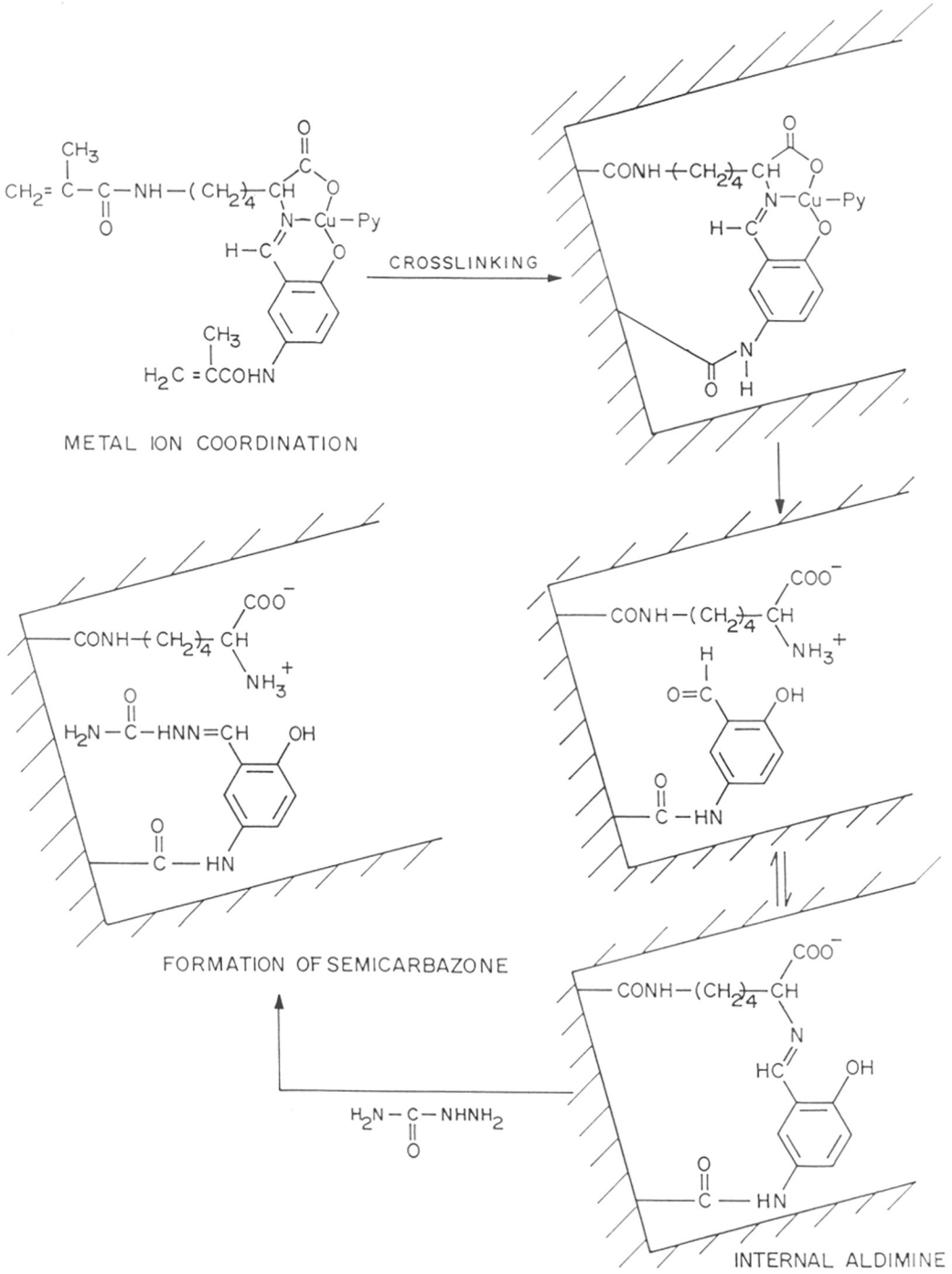


FIG. 1.11 : PYRIDOXAL ENZYME MIMIC FROM METAL ION COORDINATION IN MIP.

This work was further extended to synthesize an enzyme mimic of chymotrypsin.(Karamalkar et al 1996). Co(II) coordinated monomers-template assembly of HEMA, MAA, MA-histidine (representing hydroxyl, carboxyl and imidazole groups of serine, aspartic acid and histidine respectively) and template IBA-6ACA-Phe ala-2AP was grafted onto poly (glycidyl methacrylate - co - ethylene glycol dimethacrylate) support and the support was subsequently worked up. The mimic so synthesized exhibited hydrolysis of substrate MA-6ACA-Phe ala-PNP with mechanistic similarity to chymotrypsin (Figure 1.12). The mimic lost activity when treated with tosyl phenyl alanine chloromethyl ketone (TPCK), a known inihibitor of chymotrypsin. The activity was also lost when hydroxyl group of HEMA was acetylated. This indicated that the origin of hydrolytic activity of the mimic could be in cooperative effect amongst the three monomers as in the case of chymotrypsin. The mimic also exhibited desired properties such as efficient repeated use and stability at high temperature.

1.10.0 Concluding remarks

Various attempts towards development of enzyme mimics have shown that it is possible to replicate enzymatic features by synthetic materials. From the extensive efforts in synthetic chemistry materials such as organometallic complexes, cyclic peptides and catalytic antibodies have emerged as potential materials for enzyme mimics. In order to mimick hydrolytic enzymes such as chymotrypsin, hydroxyl, carboxyl and imidazole bearing moieties have been brought close to one another (cyclodextrins and cyclic peptides). The materials so synthesized have exhibited chymotrypsin like activity with mechanistic similarity. But these efforts have had limited success e.g. while cyclodextrin

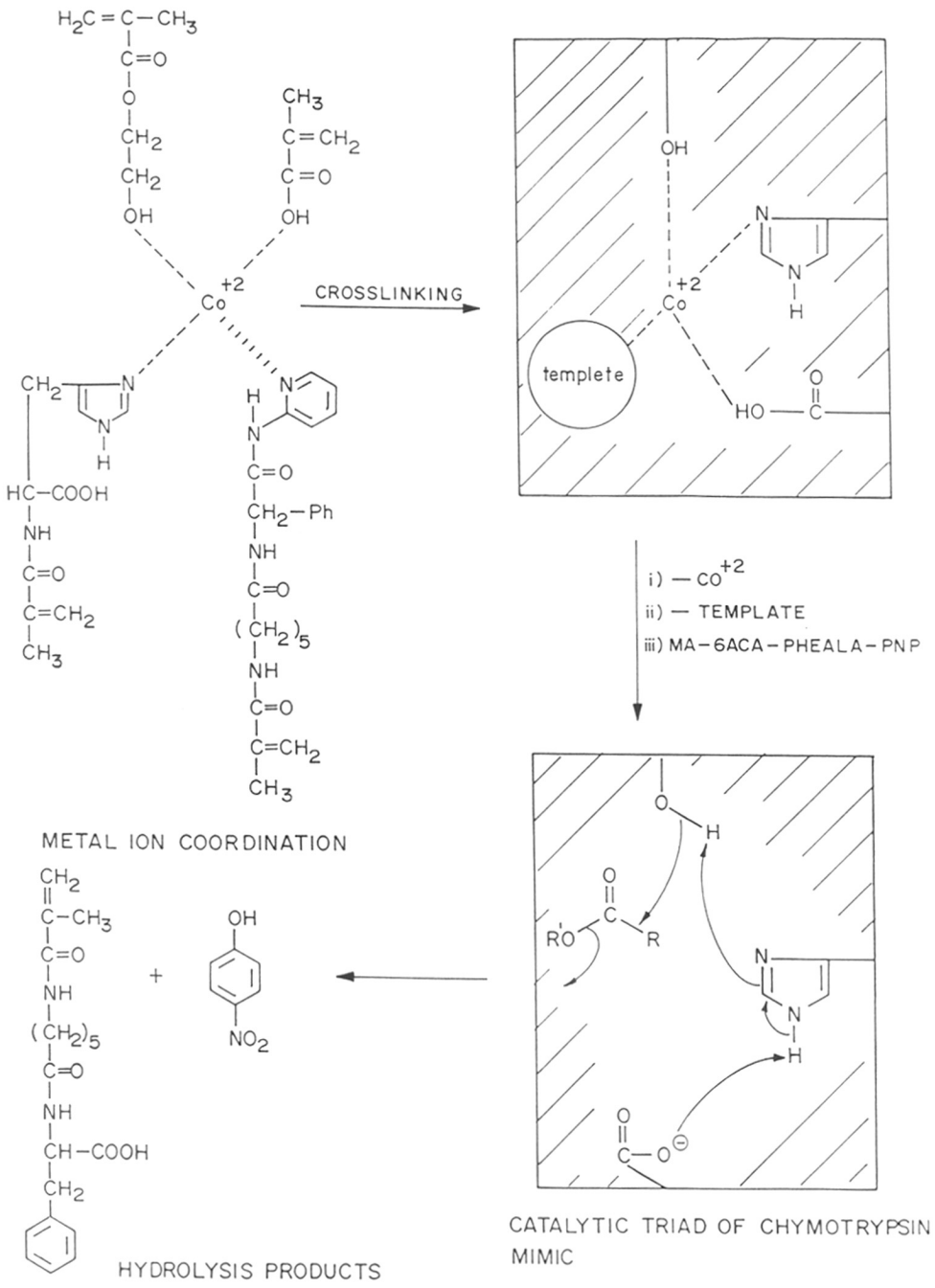


FIG. 1.12 : SURFACE IMPRINTED CHYMOTRYPSIN MIMIC FROM METAL ION COORDINATION.

based mimics required highly basic conditions to catalyze the hydrolysis of substrate (D;Souza et al 1987), cyclic peptides exhibited denaturation of the activity at ambient temperatures of 40^0C. Cyclic peptides were found to be stable only at lower temperatures (Attasi and Manshouri 1993). Also hydrolytic activity of cyclic pepzymes were not always reproducible (Corey et al 1994). As compared to these delicate supramolecular assemblies polymeric materials are found to be better choice due to their ability withstand harsh conditions.

Linear soluble polymers containing multiple nucleophilic groups did exhibit some enzyme like features in their hydrolytic activity but they always lacked the ability to recognize the substrate. Molecular imprinting in crosslinked polymeric networks renders them with the ability of recognizing substrate as in the case of enzymes. Metal ion coordination of monomers and template provides both, substrate recognition and cooperative effect amongst functional groups. Therefore MIPs based on metal ion coordination interactions are suitable candidates for synthesizing enzyme mimics. MIP based mimics of hydrolytic enzymes reported by Mosbach et al (1987), Sellergren et al (1994), Ohkubo et al (1995), Robinson and Mosbach (1989) were hydrolytically active and capable of exhibiting higher rates of hydrolysis for imprinted substrate. Thus these efforts demonstrated the substrate recognition. But these mimics could not demonstrate cooperative effect amongst hydroxyl, carboxyl and imidazole groups as in the case of chymotrypsin because these were based on only imidazole bearing monomer. Karamalkar et al (1996) from our group reported chymotrypsin mimic which was comprised of hydroxyl, carboxyl and imidazole bearing monomers as in the case of

chymotrypsin. The mimic exhibited mechanistic similarity with chymotrypsin but the cooperative effect was not demonstrated. Also the substrate that was employed in the work was not a specific substrate for chymotrypsin.

But the results reported by Karamalkar et al (1996) clearly indicate the potential of the approach of grafting metal ion - monomer complex to synthesize chymotrypsin mimics capable of exhibiting substrate recognition as well as cooperative action. In order to validate the concept proposed by Karamalkar et al (1996) it is necessary to take up systematic investigations of the approach. The investigations should be focused on demonstrating the cooperative effect and substrate recognition unequivocally. The polymeric system should be developed which is relevant to the native enzyme with respect to the functional monomers and substrate used. The investigations should also reveal various parameters that govern the hydrolytic activity of the mimics.

Chapter 2

Objectives and scope of the work

2.0.0 Objectives and scope of the work

It can be seen from various efforts to design and synthesize enzyme mimics, especially chymotrypsin, sighted in chapter 1 that the metal ion coordination of functional monomers offers a much simpler alternative to orient bring functional groups constituting the triad close to one another and also orient the substrate towards it as compared to other synthetic methodologies. Immobilization of the orientation so achieved by crosslinking results in the placement of the functional groups which could be expected to lead to cooperative action similar to that in the case of chymotrypsin and related enzymes. Molecularly imprinted polymer mimic of chymotrypsin reported by Karamalkar et al (1996) clearly indicates the potentials of this approach for the design and synthesis of efficient mimics. Further investigations in this area are therefore needed to validate the approach and to build on it. The present investigation has been undertaken with the following objectives.

1. To explore the possibility of generalizing and validating the concept of surface grafting of metal ion - functional monomers complex for the synthesis of polymeric mimics exhibiting cooperative action as in chymotrypsin.

2. To investigate various parameters such as the type of functional monomers, carrier support and substrate structure that govern the catalytic activity of mimics, as to elucidate the origins of activity of molecularly imprinted polymeric mimics and to compare the same with those in chymotrypsin.

3. To evaluate hydrolytic activity of various polymeric mimics so synthesized, in terms of the Michaelis - Menten constants k_{cat} and K_m and correlate these constants with the structural attributes of the mimics mentioned above.

4. Finally based on the results we wish to evolve the strategies for the design and synthesis of surface imprinted chymotrypsin mimics which would exhibit enhanced catalytic activity, than those observed so far.

Chapter 3

Surface imprinting of coordination complex of functional monomers and metal ion leading to catalytic triad : Concept generalization

3.1.0 Introduction

Research in the design of synthetic enzyme mimics has spanned over three decades. These studies are expected to improve understanding of the mechanism of enzymatic reactions and lead to mimics, which will overcome the limitations of natural enzymes, viz. denaturation in organic media, difficulties in isolation, loss of activity on recycling etc. (Wiseman, 1987). Besides, additional features such as photoswitchability, thermal and pH switchability can be incorporated in these mimics. Most of the research efforts in this area have focused on chymotrypsin since the nature of the active site and mechanism of its hydrolytic action are quite well understood (Bender et al 1961 and Kunitake et al 1976). Briefly, the active site of chymotrypsin comprises hydroxyl group of serine (195), carboxyl group of aspartic acid (102) and imidazole group of histidine (57). The three are brought in close vicinity of one another as a result of the specific conformation acquired by chymotrypsin due to chain folding. The nucleophilicity of serine hydroxyl group is enhanced by the cooperative action within the three groups. The triad is buried in a hydrophobic pocket. As a result, chymotrypsin specifically hydrolyzes esters and amides comprising phenyl alanine or tyrosine residues next to the carbonyl group in the ester or amide (Bender, 1960, Bender and Ke'zdy 1964).

Past efforts to synthesize chymotrypsin mimics can be classified into three categories. **1.** Soluble, vinyl polymers comprising neighboring functional groups. **2.** Functionalized cyclodextrin derivatives. **3.** Cyclic peptides having chymotrypsin like activity. These efforts are briefly summarized below. Overberger et al (1967) synthesized

a wide range of vinyl copolymers containing hydroxyl and imidazole pendant groups. In the copolymer comprising 4(5) vinyl imidazole and 4 vinyl phenol, a cooperative effect between the two monomers which enhanced nucleophilicity of hydroxyl group was noted.(Overberger et al 1967). A similar effect in the copolymer of N- methyl acrylohydroxamic acid and 4(5) vinyl imidazole was reported by Kunitake et al.(1976). The field has been reviewed earlier by Kunitake and Okahata (1976) and recently by Fife (1995). In this approach however, there is no specific mechanism whereby the functional groups are brought in the vicinity of one another. Also there is no element of substrate recognition.

Functionalized cyclodextrins were investigated as chymotrypsin mimics by Bender et al. (1985). O- (4,(5)- mercaptomethyl - 4(5)- methyl imidazole - 2- yl) benzoate was linked to secondary site of cycloheptaamylose (ß cyclodextrin). While the mimic exhibited chymotrypsin like activity in the hydrolysis of t-butyl phenyl acetate, highly basic conditions (pH 10) were required for the efficient hydrolysis (D'Souza et al 1987)

Hahn et al (1990) designed four short parallel peptides bearing serine, aspartic acid and histidine residues at the end of chains. Features such as cooperative effect and substrate binding were incorporated in the peptides, which eventually resulted in 1 % of the activity of chymotrypsin in the hydrolysis of N- acetyl tyrosine ethyl ester. Attasi and Manshouri (1993) designed cyclic peptides based enzyme mimics of chymotrypsin and trypsin which exhibited hydrolytic activity almost equal to that of chymotrypsin in the hydrolysis of N-benzoyl tyrosyl ethyl ester. But the results need further validation owing

to the irreproducibility of the results by other workers (Corey et al 1994). These approaches demonstrate that any effort to design chymotrypsin mimic has to include a mechanism whereby the functional groups constituting the triad are brought into the vicinity of one another and a feature whereby the triad would selectively recognize hydrophobic amino acid such as phenyl alanine and / or tyrosine. The past efforts seem to have had modest success in this direction.

Functional monomers can be brought in close proximity of one another by forming metal ion coordination complexes prior to polymerization. The proximity so achieved can be retained by crosslinking even after the removal of the metal ion. Thus, Belokon et al (1982) copolymerized copper complex, N^{α} 5 methacryloyl amino salicylidine N^{ϵ} methacryloyl (S) lysinato pyridine Cu(II) with acrylamide and methylenebisacrylamide to synthesize a pyridoxal enzyme mimic. The approach still lacks a mechanism for substrate recognition. Molecular imprinting of polymers provides a means of substrate recognition which has been explored for applications in separations and catalysis (Wulff 1995, Sherrington et al 1995, Mosbach et al 1996, Shea et al 1994). Leonhardt and Mosbach (1987) polymerized Co (II) complex of 4(5) vinyl imidazole and t-butyloxycarbonyl- leucylpicolylamide (used for creation of substrate recognition site) with excess of crosslinker. The crosslinked polymer exhibited some features of enzyme catalyzed reactions in the hydrolysis of t-butyloxycarbonyl-leucyl para nitro phenyl ester. This work was further extended to imprint imidazole containing polymers with transition state analogues (Robinson et al 1989, Ohukubo et al 1994[a,b,c]) But as the polymers contained only one group i.e. imidazole, multifunctional cooperative action could not be

demonstrated. Besides, crosslinked polymers synthesized by bulk polymerization with excess of crosslinker exhibit limited access and slow adsorption and desorption kinetics of substrate (Wulff 1990, Wulff et al 1990). To overcome this limitation, a new technique in which metal ion coordinated monomers are grafted onto the surface of microporous support termed as **" surface grafting"** has been exploited (Dhal et al 1995).

In our group, the potentials of molecularly imprinted polymers in controlled release and as enzyme mimics are being explored (Karamalkar et al 1996[a,b],1997). The work in this chapter has been undertaken to validate the generic nature of the concept which would lead to a chymotrypsin mimic. In this chapter we describe the chymotrypsin mimic synthesized by surface grafting Co (II) coordinated monomers-template assembly comprising N-methacryloyl L-serine, N-methacryloyl L-aspartic acid, N-methacryloyl L-histidine and N-nicotinoyl tyrosyl benzyl ester (template) onto crosslinked poly (glycidyl methacrylate - co - ethylene glycol dimethacrylate) beads. In order to demonstrate the cooperative effect amongst functional groups of the mimic, a series of surface grafted polymers comprising one, two or all the three functional monomers onto the support have been synthesized and evaluated. Also, the ability of the mimic, resulting from molecular imprinting, to recognize the substrate is demonstrated.

3.2.0 Experimental section

3.2.1 Materials

Methacrylic acid (MA), dicyclohexyl carbodiimide (DCC), glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EGDMA), were from Aldrich. N-

benzyloxycarbonyl L-Tyrosine (N-cbz-Tyr) was from sigma. These chemicals were used as received. All chemicals used, other than listed above were from local suppliers. These were purified prior to use following the standard procedures (Perrin et al 1981).

3.2.2 Instrumentation and analyses

Melting points were recorded on Mettler melting point apparatus. Infrared spectra were recorded on Perkin - Elmer 1600 FT-IR spectrophotometer. ^{1}H spectra were recorded on Varian spectrometer operating at 200 MHz.. Electronic absorption spectroscopic measurements were carried out on Shimadzu UV 240 spectrophotometer. Surface areas and pore volume measurements were made on Quanta-chrome Autoscan porosimeter. ESR spectra were recorded at 298 K on Bruker spectrometer operating at 9.73 Hz using ST 8484 cavity.

3.2.3 Synthesis of methacryloyl chloride

In a 100 ml capacity round bottom flask, 24 ml methacrylic acid (0.3 M), 3 g of hydroquinone and few drops of dimethyl formamide were placed. To this solution, 23 ml $SOCl_2$ (0.32 M) was added dropwise over 1 hr at room temperature. The reaction mixture was heated at 60°C for 5 to 6 hrs. It was then allowed to cool and left overnight at room temperature. 1 g of hydroquinone was added to the reaction mixture and the acid chloride was distilled off at 92°C. Yield 60 percent.

3.2.4 Synthesis of N-methacryloyl L-histidine (MA-histidine)

N-methacryloyl L-histidine was synthesized according to the following procedure (Okuda et al 1987). In a 100 ml beaker equipped with pH electrode, 3.1 g L-histidine

(0.02 M), 20 ml distilled water, 0.8 g NaOH (0.02 M) were placed to give clear solution (pH 12). The solution was stirred with magnetic needle at 5 to 10°C (ice water bath). To this solution, 1.95 ml methacryloyl chloride (0.02 M) dissolved in 3 ml dichloromethane was added dropwise over 1.5 to 2 hrs. After half of the addition of acid chloride was completed, pH of the reaction mixture dropped to 7 - 7.5. pH of the solution was then maintained between 7.5 to 8 by the addition of NaOH solution (0.8 g NaOH dissolved in 5 ml water). The reaction mixture was then extracted with 20 ml ether to remove dichloromethane and unreacted acid chloride. Clear aqueous solution was then acidified to pH 5 - 5.5 with conc. HCl. 1 ml of this solution was taken in a stoppered test tube and 20 ml of acetone was added to it and mixed well to precipitate out white sticky material. It was triturated with 3 ml methanol. (This procedure was repeatedly used to extract the product from reaction mixture). Methanol solution was filtered to remove salts and clear methanol solution was added to excess of acetone (750 ml) to reprecipitate the product. After two reprecipitations, white low melting hygroscopic solid was obtained. Yield 50 percent.

IR (methanol)

1612 cm^{-1} (methacryl double bond), 1651 cm^{-1} (amide carbonyl), 1705 cm^{-1} (acid carbonyl).

^{1}H NMR (D_2O)

1.8 - 1.9 3H singlet (-C = C-CH_3), 3.2 - 3.3 2H doublet (-C-CH_2-imidazole), 4.4 1H triplet (-CH-COOH), 5.3 - 5.4 1H singlet (-CH_a = C-), 5.6 - 5.7 1H singlet (-CH_b = C-),

7.2 1H singlet (ring proton of imidazole at C_5), 8.4 - 8.5 1H singlet (ring proton of imidazole at C_2).

3.2.5 Synthesis of N-methacryloyl L-serine (MA-serine)

N-methacryloyl L-serine was synthesized and purified according to the same procedure followed in the synthesis of MA-histidine using 0.02 M L-serine. Yield 31 percent.

IR (methanol)

1600 cm^{-1} (methacryl double bond), 1651 cm^{-1} (amide carbonyl), 1700 cm^{-1} (acid carbonyl).

^1H NMR (D_2O)

2.1 3H singlet (-C = C- CH_3), 3.6 - 3.7 2H doublet (-CH_2-OH), 4.3 - 4.4 1H triplet (-CH-COOH), 5.5 - 5.6 1H singlet (-CH_a = C-), 5.7 - 5.85 1H singlet (-CH_b = C-).

3.2.6 Synthesis of N-methacryloyl L-aspartic acid (MA-aspartic acid)

N-methacryloyl L-aspartic acid was synthesized and purified according to the same procedure followed in the synthesis of MA-histidine using 0.02 M L-aspartic acid. In this case an extra mole of NaOH is required to neutralize ß carboxyl group of aspartic acid. Yield 35 percent.

IR (methanol)

1606 cm^{-1} (methacryl double bond), 1651 cm^{-1} (amide carbonyl), 1700 cm^{-1} (acid carbonyl).

^{1}H NMR (D_2O)

2.0 3H singlet (-C = C-CH_3), 3.4 - 3.5 2H doublet (-CH_2-COOH), 5.5 1H singlet (-CH_a = C-), 5.6 1H singlet (-CH_b = C-).

3.2.7 Synthesis of substrate, N-benzyloxycarbonyl L-tyrosyl- para nitrophenyl ester (N-cbz-Tyr-PNP)

In a 250 ml capacity round bottom flask, 3.15 g N-cbz-Tyr (0.01 M), 1.39 g para nitro phenol (0.01 M) were dissolved in 30 ml ethyl acetate and stirred at room temperature. To this solution 2.12 g DCC (0.01 M) dissolved in 30 ml ethyl acetate was added in one portion. Dicyclohexyl urea (DCU) formed within few minutes. The reaction mixture was stirred overnight. DCU was filtered and the clear filtrate was concentrated to half its original volume. This solution was added to excess of petroleum ether (700 ml) under stirring to yield white powdery material, which was washed with petroleum ether and dried. It was then purified by two reprecipitations from acetone into petroleum ether. Yield 90 percent.

Melting point 165 - 166°C.

IR (nujol)

3583 cm^{-1} (aromatic -OH stretching), 3342 cm^{-1} (-NH stretching), 1752 cm^{-1} (ester carbonyl), 1648 cm^{-1} (amide carbonyl), 1522 cm^{-1} (aromatic -CH bending).

^{1}H NMR (acetone d_6)

2.66 2H singlet (-O-CH_2-Ph of benzyloxy), 3.22 2H doublet (-CH_2-Ph of Tyrosine), 4.44 1H triplet (-CH-COOR of Tyrosine), 6.55 5H multiplet (ring protons of benzyloxy -O-CH_2-Ph), 6.66 4H multiplet (ring protons of Tyrosine), 7.33 multiplet (ring protons of para nitrophenyl ring).

3.2.8 Synthesis of tyrosyl benzyl ester

The tyrosyl benzyl ester was synthesized according to following procedure (Zerwas et al 1957, Miller et al 1952). In a 500 ml capacity round bottom flask 9.05 g tyrosine (0.05 M) and 10 g p-toluene sulfonic acid monohydrate (0.05 M) were placed. To this, 100 ml benzyl alcohol and 50 ml benzene were added. This suspension was then refluxed using Dean - Stark trap till azeotropic removal of water was completed (2.5 to 3 hrs). Clear solution thus formed, was allowed to cool down. In order to precipitate out the product, 500 ml diethyl ether was added and stored in refrigerator overnight. White precipitate of tyrosyl benzyl ester p-toluene sulfonate was isolated by filtration and dried. 15 g of this product was obtained. It was then dissolved in 40 ml dimethyl formamide and 3.33 ml triethyl amine was added to it. The reaction mixture was stirred overnight and poured into 1 liter ice cold water to precipitate the crude product. Tyrosyl benzyl ester was isolated and purified by reprecipitation from acetone into petroleum ether. Yield 60 percent.

Melting point 120^{0}C.

3.2.9 Synthesis of template, N-nicotinoyl tyrosyl benzyl ester

3.08 g tyrosyl benzyl ester (0.017 M) and 2.12 g nicotinic acid (0.017 M) were suspended in 50 ml ethyl acetate at 0^0C. 3.54 g DCC (0.017 M) dissolved in 30 ml ethyl acetate was added to above reaction mixture in a single portion and stirred at 0^0C for one hr and then at 25^0C overnight. It was then filtered to remove dicyclohexyl urea and clear solution was concentrated in vacuo to yield crude solid which was purified on silica gel column using 30 / 70 acetone / petroleum ether mixture as mobile phase. Yield 65 percent.

Melting point 125 - 127°C.

IR (nujol)

3450 cm^{-1} (-OH stretching), 3300 cm^{-1} (-NH stretching), 1750 cm^{-1} (ester carbonyl),1640 cm^{-1} (amide carbonyl), 1620 cm^{-1} (-C = N of nicotinoyl ring), 1520 cm^{-1} (aromatic -CH bending).

^{1}H NMR (acetone d_6)

2.8 2H singlet (-O-CH_2-Ph), 3.2 2H doublet (-CH_2-Ph of Tyrosine), 4.8 1H triplet (-CH-CONHR of Tyrosine), 6.7 2H double doublet (Tyrosine ring protons), 6.9 2H double doublet (Tyrosine ring protons), 7.0 - 7.2 5H multiplet (ring protons of -O-CH_2-Ph), 7.5 4H multiplet (nicotinoyl ring protons).

3.2.10 Synthesis of crosslinked polymer support, Poly (glycidyl methacrylate - co - ethylene glycol dimethacrylate) (poly (GMA-EGDMA))

The polymer support was synthesized according to the following procedure (Svec et al 1975). In a three neck 500 ml round bottom flask, 180 ml water and 0.6 g poly (vinyl pyrrolidone) (MW 60,000) were placed and stirred with an overhead constant speed stirrer at 700 rpm. The contents of the flask were heated to 75°C and nitrogen was purged into it for half an hour to expel out air and dissolved oxygen. Purging was then stopped and from a dropping funnel an organic phase containing 5.7 g ethylene glycol dimethacrylate (EGDMA), 2.45 g glycidyl methacrylate (GMA), 10 ml cyclohexanol, 80 mg azobis isobutyronitrile (AIBN) was added in dropwise manner into aqueous phase being stirred at 700 rpm and at 75°C. Addition was over in about 5 minutes. Polymerization was carried out for 6 hours. Stirring was stopped and contents of the flask were poured in a beaker. The beads formed were allowed to settle. Solution was decanted off and beads were washed with methanol and water and dried in vacuum oven at room temperature. Dry beads when sieved gave following size distribution.

< 37 microns 1.0 g, 37 -45 microns 1.2 g, 45 -75 microns 2.5 g, >75 microns 2.0 g.

For all enzyme mimics synthesized by surface grafting, beads in the range of 45 to 75 microns were used. For a given set of enzyme mimics care was taken to use the support material from the same suspension polymerization experiment in order to avoid complications arising from differences in surface area, pore size and morphology of the support.

3.2.11 Preparation and Electron Spin Resonance (ESR) characterization of Co(II) coordinated monomers-template assemblies

Monomers, template and $CoCl_2.6H_2O$ were dissolved in methanol and the solution was stirred under nitrogen for 1 hour. The solution was then evaporated in presence of poly (GMA-EGDMA) beads to sorb the Co(II) coordinated monomers-template assemblies onto the support. Various compositions of the assemblies with sequential addition of monomers to $CoCl_2.6H_2O$ were prepared as listed in Table 3.1. ESR spectra of these assemblies (sorbed on the support) were recorded on spectrometer using ST 8484 cavity.

3.2.12 Synthesis of polymer mimic of chymotrypsin (P3)

0.33 g (0.00150 M) MA-histidine, 0.26 g (0.00150 M) MA-serine, 0.30 g (0.00150 M) MA-aspartic acid, 0.564 g (0.00150 M) N-nicotinoyl tyrosyl benzyl ester (template), 0.357 g (0.00150 M) $CoCl_2.6H_2O$ were dissolved in 60 ml methanol. The solution was stirred for 1 hr under nitrogen purging. To this clear purple blue solution, 0.09 g EGDMA (10 percent of the wt. of functional monomers) and 35 mg AIBN (3 percent of the wt. of polymerizable monomers) were added. poly (GMA-EGDMA) support 0.98 g (1 : 1 weight ratio of support : monomers) was added into this solution and methanol was evaporated in vacuo to adsorb the Co (II) coordinated assembly onto the support. This purple blue mass was maintained in vacuum oven at 75°C for polymerization. In the first five hrs 50 mm Hg vacuum was applied. Polymerization was continued for 24 hrs. After this, the material was allowed to cool. It was washed thrice using a mixture comprising 95 ml methanol and 5 ml of 35 percent HCl till all the cobalt,

template and ungrafted monomers were leached out. (This was monitored by the absorbance of this elute at 700 nm). This material was then washed with water and phosphate buffer and dried. All polymers **P1** to **P6** were synthesized according to this general procedure. The details are listed in Table 3.2.

3.2.13 Estimation of total amino acids in mimics

In order to establish the levels of grafting and estimate the functional groups per unit weight of poly (GMA-EGDMA) beads, we estimated the amount of total amino acids in mimics. A typical estimation procedure is described below. 0.1 g of polymeric mimic was suspended in 10 ml 0.2 N HCl and this suspension was heated to reflux under stirring for 24 hrs to hydrolyze amino acids from methacryl backbone. The suspension was allowed to cool down to room temperature and pH was adjusted to 5.5 by adding 1 N NaOH. Suspension was filtered to remove polymer support and clear solution was used for Ninhydrin test (Jayaraman 1985). 1 ml of above hydrolysate was diluted with 4 ml water and 1 ml Ninhydrin reagent was added. (To prepare the reagent, 2 g Ninhydrin was dissolved in 25 ml acetone and 25 ml acetate buffer was added to it). This solution was heated in a test tube to 90°C in water bath for half an hour and then cooled to room temperature. The purple color developed in the solution. 1 ml 50 percent aqueous ethanol was added and absorbance was recorded on spectrophotometer at 550 nm. This absorbance value was used to estimate the amount of total amino acids i.e. total functional groups in polymeric mimic using relevant standard calibration plot as reference.

3.2.14 Evaluation of hydrolytic activity

In a 25 ml jacketed reactor 50 mg polymeric enzyme mimic was placed. 10 ml of 40 : 60 acetonitrile : phosphate buffer (0.05 M pH 7.8) was added. This suspension was stirred with magnetic needle at 37^0C. A predetermined amount of substrate N-cbz-Tyr-PNP was dissolved in 1 ml acetonitrile and added while stirring. Hydrolysis was followed by monitoring absorbance of released para nitrophenol at 400 nm. For mimics **P1**, **P2** and **P3** fifty fold molar excess of N-cbz-Tyr-PNP (over the functional groups in the mimic) was used and the hydrolysis was monitored for 60 minutes.

Hydrolytic activity of the mimics **P4**, **P5** and **P6** was evaluated in a similar manner in the framework of Michaelis - Menten kinetics. In this case a range of substrate concentrations between 50 to 150 fold molar excess was used and initial rates of hydrolysis (v_{obs}) were monitored to draw Lineweaver - Burk plots.

3.3.0 Results and Discussion

Earlier, Karamalkar et al (1996[b]) reported molecularly imprinted, hydrolytically active hydrogel as well as surface grafted polymeric mimic of chymotrypsin which exhibited hydrolytic activity similar to chymoptrypsin. These results encouraged us to investigate this approach further. We, therefore have extended the investigations to explore if a generalized methodology to synthesize polymeric mimics of chymotrypsin can be established. In the present work we selected functional monomers bearing functionalities present in the active site of chymotrypsin. In order to highlight the cooperative activity amongst the functional groups of the polymer mimic, a series of

polymers was synthesized and evaluated in which one, two or all three functional monomers were grafted onto the polymer support. Additionally, the role of template in enhancing the substrate binding is demonstrated.

3.3.1 Methodology for the synthesis of chymotrypsin mimic

Our concept of chymotrypsin mimic comprising surface grafting of preorganized monomers-template assembly on the microporous support can be summarized as follows.

1. Coordination of functional monomers bearing hydroxyl, carboxyl and imidazole groups with Co(II) to bring the three in the vicinity of one another. **2.** Coordination of template to Co(II) so that the catalytic groups are imprinted for substrate specificity. **3.** Surface grafting Co(II) coordinated monomers-template assembly onto poly (GMA-EGDMA) beads along with EGDMA so that the proximity among functional monomers achieved by complexation is retained after polymerization. **4.** Removal of Co(II), unpolymerized monomers and template from the beads. **5.** Hydrolysis of "chymotrypsin specific substrate" by the mimic and evaluation of Michaelis - Menten constant. These steps are schematically represented in Figure 3.1.

3.3.2 Choice of functional monomers, substrate and template

Since the active site of chymotrypsin comprises serine, aspartic acid and histidine, first step to synthesize chymotrypsin mimic would be to incorporate these amino acids in polymers. Efforts to incorporate these amino acids in cyclic peptides have been recently reported (Attasi and Manshouri 1993, Ishid et al 1995). We synthesized MA-histidine, MA-aspartic acid, MA-serine by reaction between methacryloyl chloride and L-histidine,

L-aspartic acid and L-serine respectively, under Schotten Baumann conditions (Okuda et al 1987).

Enzyme activities are generally highly substrate specific. Chymotrypsin exhibits specificity for hydrophobic amino acids like tyrosine and phenyl alanine which are accommodated in its hydrophobic pocket. Substrate N-cbz-Tyr-PNP is widely used as "typical substrate" to evaluate chymotrypsin activity (Bender et al 1961). We therefore selected N-cbz-Tyr-PNP as substrate to evaluate the efficacy of the polymer mimic.

In order to induce specificity for this substrate, a template molecule structurally similar to N-cbz-Tyr-PNP and which can form a complex with cobalt is needed. The template used in this work was N-nicotinoyl- tyrosyl benzyl ester. Structures of monomers, substrate and template are shown in Figure 3.2. It can be seen from Figure 3.2 that structures of substrate and template contain similar phenyl moieties at N and O terminals of tyrosine.

3.3.3 ESR characterization of Co(II) coordinated monomers-template assemblies

In order to achieve cooperative action amongst functional groups, they should be brought in the vicinity of one another. Cobalt can form coordination complexes with many ligands. Characterization of such complexes by ESR spectroscopy has been reported by Dhal et al (1992). In the present case, for characterization of coordination of monomers and template with Co(II), these were dissolved in methanol containing $CoCl_2.6H_2O$ and the assembly was adsorbed on poly (GMA-EGDMA) support. Various Co (II) coordinated monomers-template assemblies were prepared as listed in Table 3.1.

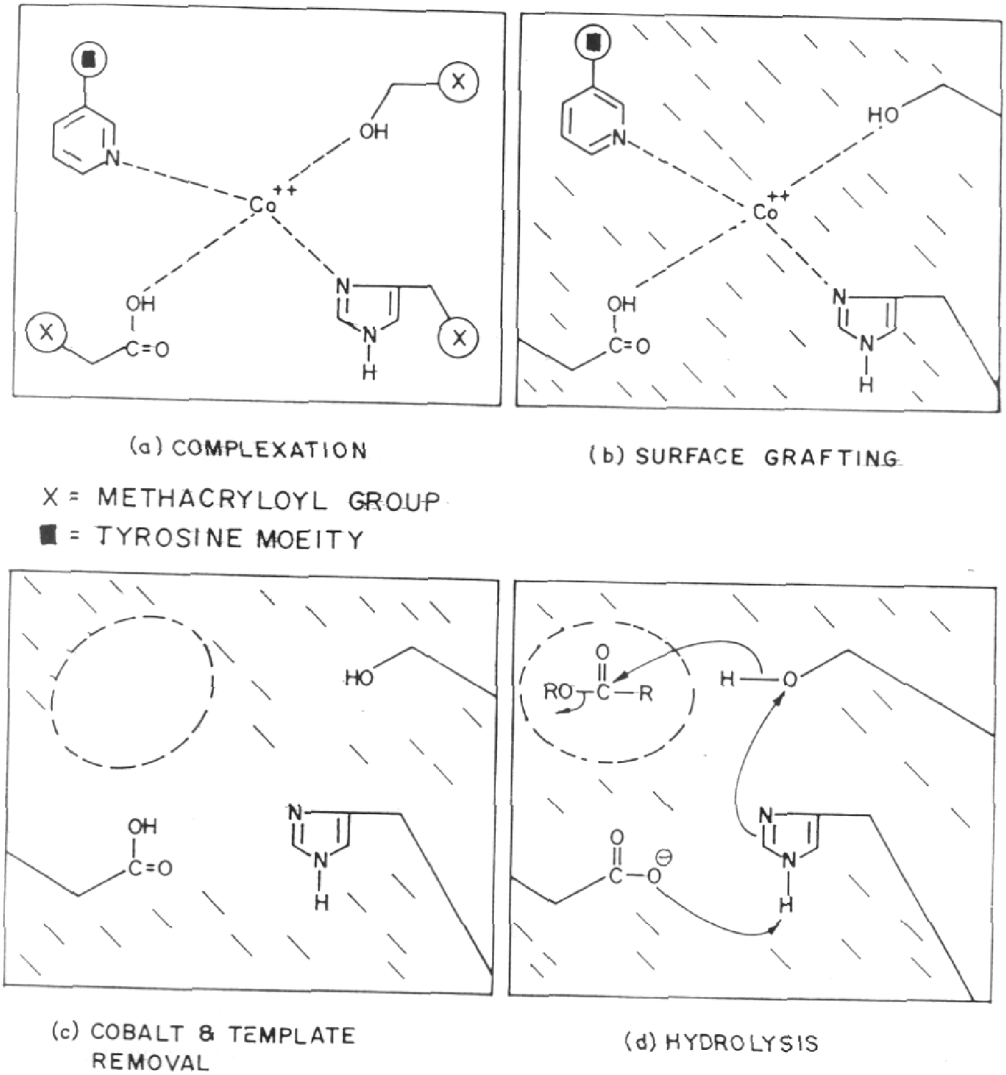


FIG 3·1 : SCHEMATIC REPRESENTATION OF SYNTHETIC METHODOLOGY.

ESR spectra of these assemblies were recorded and $g_{\parallel}$ values were calculated in each case (Table 3.1).

Sequential addition of monomers and template to $CoCl_2.6H_2O$ caused a decrease in $g_{\parallel}$ value of Co(II) from 2.199 to 1.829 (Table 3.1). This was also accompanied by change in the color of the Co(II) coordinated assemblies from pink to deep blue. ESR spectrum of the assembly comprising stoichiometric amounts of Co (II), template N-nicotinoyl tyrosyl benzyl ester and the monomers MA-serine, MA-aspartic acid, MA-histidine in the ratio 1:1:1:1 shows a sharp peak at 4000 gauss (Figure 3.3). This confirms the coordination of monomers and template with Co(II) and the formation of polymerizable Co(II) coordinated monomers-template assembly comprising stoichiometric amounts of each of the monomers. concentration. These results are in agreement with the trends reported by Dhal et al (1992) and the trends reported by Karamalkar et al(1996[b]). As discussed by Belokon et al (1982) polymerization of this Co (II) coordinated assembly would result in a polymer comprising the three monomers in the vicinity of one another.

3.3.4 Synthesis and characterization of surface grafted mimics

Crosslinked poly (GMA-EGDMA) beads were used as carrier support to graft the metal coordinated assembly comprising the monomers and the template. For the grafting, presence of unsaturation in the carrier support is essential (Dhal et al 1995). This was achieved by reducing suspension polymerization time for poly (GMA-EGDMA) beads from 8 hours reported in the literature to 6 hours. Presence of methacryl double bonds on poly (GMA-EGDMA) beads was confirmed from FT-IR spectrum of blank poly (GMA-

Table 3.1

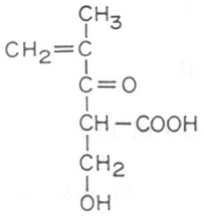
ESR characterization of Co(II) coordinated monomers- template assembly

Entry	Description of the complex	g_{II}
1	(0.0005 M) $CoCl_2$. $6H_2O$	2.199
2	(0.0005 M) $CoCl_2$. $6H_2O$ + (0.0005 M) MA-serine	2.1106
3	(0.0005 M) $CoCl_2$. $6H_2O$ + (0.0005 M)MA-serine + (0.0005 M) MA-aspartic acid	2.0598
4	(0.0005 M) $CoCl_2$. $6H_2O$ + (0.0005 M) MA-serine + (0.0005 M) MA-aspartic acid + (0.0005 M) MA-histidine	2.0005
5	(0.0005 M)$CoCl_2$. $6H_2O$ + (0.0005 M) MA-serine + (0.0005 M) MA-aspartic acid + (0.0005 M) MA-histidine + (0.0005 M) Template	1.8294

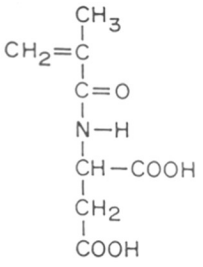
Co(II) coordinated assemblies of above stoichiometry were adsorbed on poly (GMA-EGDMA) support and spectra were recorded under following conditions.

Temperature 298 K, Microwave 9.73 Hz, Cavity ST 8424, Frequency 100 K Hz, Field modulation intensity 1.25 Gpp, Field intensity : Mild range 2600 G, Scan range 5000 G, Scan range 200 sec.

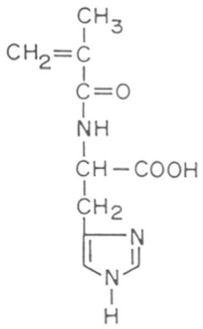
(a) FUNCTIONAL MONOMERS



MA-SERINE

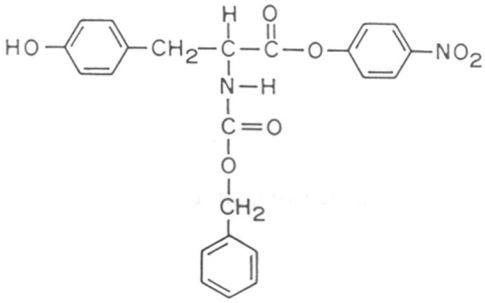


MA-ASPARTIC ACID



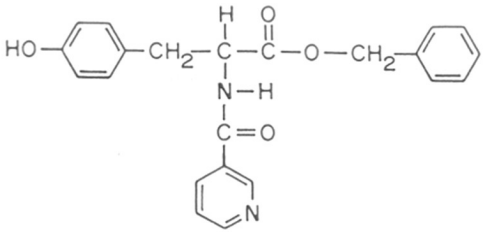
MA-HISTIDINE

(b) SUBSTRATE



N-cbz-Tyr-PNP

(c) TEMPLATE



N-NICOTINOYL-Tyr-BENZ

FIG. 3·2- STRUCTURES OF FUNCTIONAL MONOMERS, SUBSTRATE AND TEMPLATE.

EGDMA) beads (Figure 3.4(a)), which shows a peak at 1620 cm^{-1} attributed to vinyl unsaturation. Co(II) coordinated monomers-template assembly of MA-serine, MA-aspartic acid, MA-histidine and N-nicotinoyl tyrosyl benzyl ester alongwith EGDMA and AIBN was adsorbed on poly (GMA-EGDMA) beads. Vacuum was applied to evaporate off the solvent and ensure enhanced sorption of the assembly on the surface of the polymer matrix. Polymerization was carried out for 24 hours at 75°C. The beads were then washed with acidified methanol to elute out unreacted monomers, cobalt and template. Complete elution of cobalt was confirmed by monitoring the absorbance of the elute at 700 nm. The beads were then washed with water, phosphate buffer and dried under vacuum. Grafting of monomers was confirmed by FT-IR analysis of the beads (Figure 3.4(b)), in which, peak at 1620 cm^{-1} is absent since the vinyl unsaturation in the polymer matrix is opened up during polymerization. Also a small shoulder at 1650 cm^{-1} is seen which corresponds to amide bonds of grafted monomers. Grafting of the assembly also resulted in decrease in the surface area of poly (GMA-EGDMA) beads. (Mimics **P4**, **P5**, **P6** in Table 3.2) Other experimental details of feed compositions for all the mimics are summarized in Table 3.2.

3.3.5 Polymerization of metal coordinated monomers

Belokon et al (1982) discussed the polymerization of metal coordinated assembly of monomers. It was observed that copolymerization of the copper complex, N^{α} 5 methacryloyl amino salicylidine N^{ε} methacryloyl (S) lysinato pyridine Cu (II) with

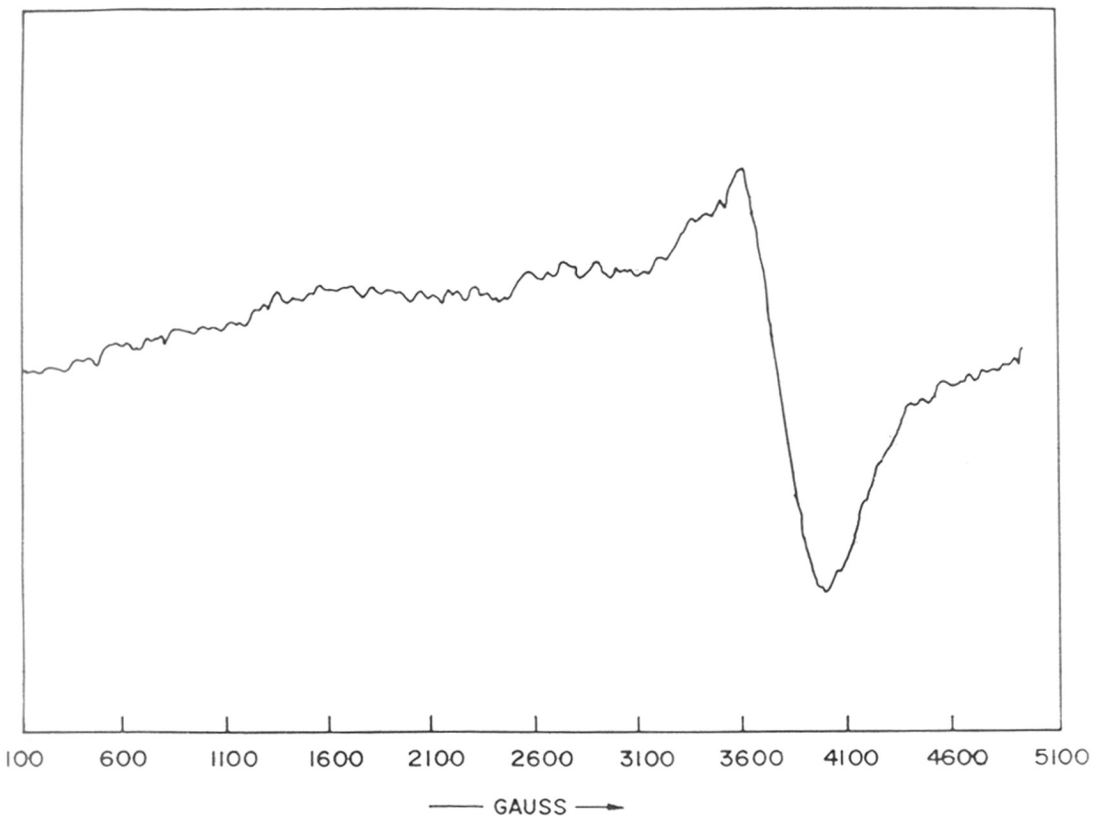


FIG. 3·3 - ESR SPECTRUM OF CO(II) COORDINATED MONOMERS - TEMPLATE ASSEMBLY OF MA-SERINE MA-ASPARTIC ACID MA-HISTIDINE AND N- NICOTINOYL TYR-BENZYL ESTER.

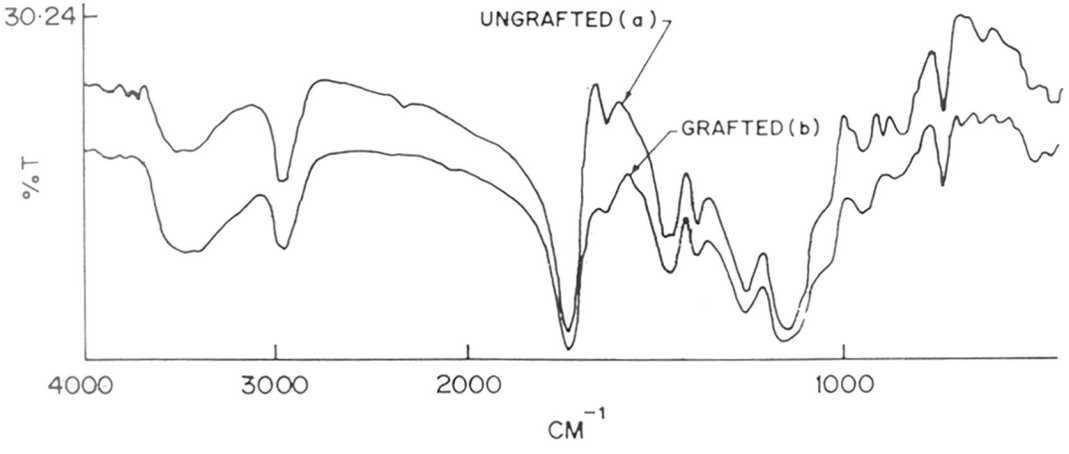


FIG. 3·4:- FT - IR SPECTRUM OF BLANK AND GRAFTED POLY (GMA - EGDMA) BEADS.

Table 3.2. Data for feed of monomers & hydrolytic activity of mimics P1 to P6.

No.	1	2	3	4	5	6	7
Polymer	Blank	P1	P2	P3	P4	P5	P6
MA-serine (M)	-	-	0.00150	0.00150	0.00150	0.00150	0.00150
MA-aspartic acid (M)	-	-	-	0.00150	0.00150	0.00150	0.00150
MA-histidine (M)	-	0.00150	0.00150	0.00150	0.00150	0.00150	0.00150
Template (M)	-	0.00150	0.00150	0.00150	-	-	0.00150
$CoCl_2.6H_2O$ (M)	-	0.00150	0.00150	0.00150	-	0.00150	0.00150
poly(GMA-EGDMA) (g)	0.98	0.36	0.65	0.98	0.98	0.98	0.98
Functional grs./ 50 mg (M)	-	$1.68 * 10^{-6}$	$3.01 * 10^{-6}$	$1.53 * 10^{-6}$	nd	$1.02 * 10^{-6}$	$1.8 * 10^{-6}$
Surface area (m^2/g)	49.63	nd	nd	nd	17.42	29.22	16.01
Pore volume (cm^3/g)	0.14	nd	nd	nd	0.07	0.09	0.04
k_{cat} (sec^{-1})	-	nd	nd	nd	No activity	0.22	0.18
K_m (M)	-	nd	nd	nd	No activity	$1.17*10^{-3}$	$5.29 * 10^{-4}$
k_{cat} / K_m ($sec^{-1} M^{-1}$)	-	nd	nd	nd	No activity	196	347

Foot note for Table 3.2.

* nd = not determined.* In all mimics **P1** to **P6** the weight ratio of poly (GMA-EGDMA) beads to monomers is 1:1.* Feeds of all assemblies **P1** to **P6** contain EGDMA (10 % of the wt. of monomers) and AIBN (3 % of the wt of monomers). * All mimics are synthesized according to the procedure described in the experimental section with above feed compositions. * Estimation of functional groups is done by Ninhydrin test according to the procedure described in experimental section on the hydrolysates obtained for each mimic.

acrylamide resulted in block structures in which monomers complexed with Cu(II) were placed in juxtaposition along the polymer chain. It was shown that the rate of intracyclopolymerization of Cu(II) complex was higher than its copolymerization with acrylamide. From these results it was concluded that monomers coordinated with metal ion have tendency to undergo polymerization as monomeric complex rather than random copolymer (Belokon et al 1982). Similar trend has been reported by Karamalkar et al (1996[b]). It was shown that copolymerization of HEMA, MAA and MA-histidine coordinated with Co(II), resulted in the copolymer in which the three monomers are so placed along the polymer chain that they could be again brought close to each other to form complex with Co(II). Due to the different monomer reactivity ratios of the three monomers, such placement of monomers was not observed when copolymerization was carried out in the absence of Co(II) (Karamalkar et al 1996[b]). This indicated that monomers bearing hydroxyl, carboxyl and imidazole groups when coordinated with Co(II), undergo polymerization as monomeric Co(II) complex and not the random copolymer of the three monomers. In the present work, sharp peak in the ESR spectrum of the Co (II) coordinated assembly of stoichiometric amounts of MA-serine, MA-aspartic acid, MA-histidine and N-nicotinoyl tyrosyl benzyl ester shown in Figure 3.3 confirm this. Therefore we believe that in the present case polymerization of the Co (II) coordinated assembly results in grafting of the three monomers MA-serine, MA-aspartic acid and MA-histidine in 1 : 1 : 1 molar ratio on the support.

3.3.6 Estimation of functional groups in mimics

In order to estimate the grafting of Co (II) coordinated assembly on the support, mimics were refluxed in HCl to hydrolyze the amino acids from the methacryl backbone. Quantitative Ninhydrin test was performed on the hydrolysate to estimate the amount of total amino acids in the hydrolysate (Jayaraman 1985). The absorbance of intense purple coloration was measured on spectrophotometer at 550 nm. From this absorbance, the amount of **total amino acids** in the hydrolysate i.e. the amount of **total functional groups** in the mimics was estimated using standard calibration plot of absorbance versus. concentration for Ninhydrin test of serine, aspartic acid and histidine composition in 1:1:1 ratio. The amount of each functional monomer per unit weight of the support was estimated as one third the total amino acids estimated. As noted above, this assumes that the three monomers when coordinated with Co (II) , are always polymerized in the ratio 1:1:1 which is justified in the view of results reported by Belokon et al (1982) and Karamalkar et al.(1996[b]) The amount of functional groups in **P3**, **P5**, **P6** was estimated as above and the amount of functional groups in **P1** and **P2** was estimated using calibration plots for histidine and serine- histidine composition respectively.

3.3.7 Choice of polymer mimics to demonstrate chymotrypsin like activity

In order to demonstrate that the mimic synthesized in this study exhibits cooperative action amongst hydroxyl, carboxyl and imidazole groups present on the polymer support and recognizes the imprinted substrate as in the case of chymotrypsin, we synthesized mimics **P1** to **P6**. Data for feed composition of all Co(II) coordinated

assemblies, estimated amounts of functional groups in the resulting mimics and their hydrolysis kinetics are listed in Table 3.2. A brief description of polymers is given below.

Mimic **P1** consisted only MA-histidine as the functional monomer coordinated with Co (II) alongwith the template. In the case of **P2**, MA-histidine, MA-serine and the template were coordinated with Co(II). In **P3** all the three monomers, MA-serine, MA-aspartic acid, MA-histidine and the template were coordinated with Co(II). While in **P4,** all the three monomers were grafted on poly (GMA-EGDMA) beads without coordinating with Co(II) and in the absence of template. In case of mimic **P5** , the three monomers were coordinated with Co (II) but the template N-nicotinoyl tyrosyl benzyl ester was not incorporated in the coordinated assembly. The methodology for the synthesis of mimic **P6** was similar to that for **P3.**

3.3.8 Hydrolytic activity of mimics P1 to P6

As described in the experimental section, course of hydrolysis was monitored for **P1**, **P2**, **P3** for duration of 60 minutes. A plot of percent hydrolysis versus. time for **P1**, **P2** and **P3** is shown in Figure 3.5. 50 fold molar excess of substrate over the amount of functional groups was used.

The mimic **P1** consists of only MA-histidine as functional monomer. Hydrolysis of N-cbz-Tyr-PNP is catalyzed by the nucleophilic imidazole group of MA-histidine and it follows first order kinetics without any burst (Figure 3.5, (curve a)). According to Michaelis - Menten kinetics, when enzyme catalyzes hydrolysis of large excess of

substrate, it exhibits initial burst kinetics resulting into rapid increase in percent hydrolysis followed by saturation of its activity due to excess substrate (Bailey and Ollis 1986). It can be seen from Figure 3.5 (curve a), that burst and the saturation both are not exhibited by **P1**.

The mimic **P2** contains MA-serine and MA-histidine i.e. hydroxyl and imidazole groups are in the vicinity of one another as a result of coordination with Co (II) prior to polymerization. This situation is similar to soluble copolymer of 4(5) vinyl imidazole and N-methyl acrylohydroxamic acid reported by Kunitake et al (1976). in which imidazole and hydroxyl groups are present on the polymer chain. As shown in Figure 3.5 (curve b), **P2** exhibits initial burst but not saturation. Thus **P2** is a bifunctional catalyst comprising hydroxyl and imidazole groups of serine and histidine present on the polymer support, wherein nucleophilicity of the attacking hydroxyl group is enhanced by the imidazole group in its vicinity. This observation is consistent with the results reported by Kunitake et al (1976) for copolymer of 4(5) vinyl imidazole and N-methyl acrylohydroxamic acid.

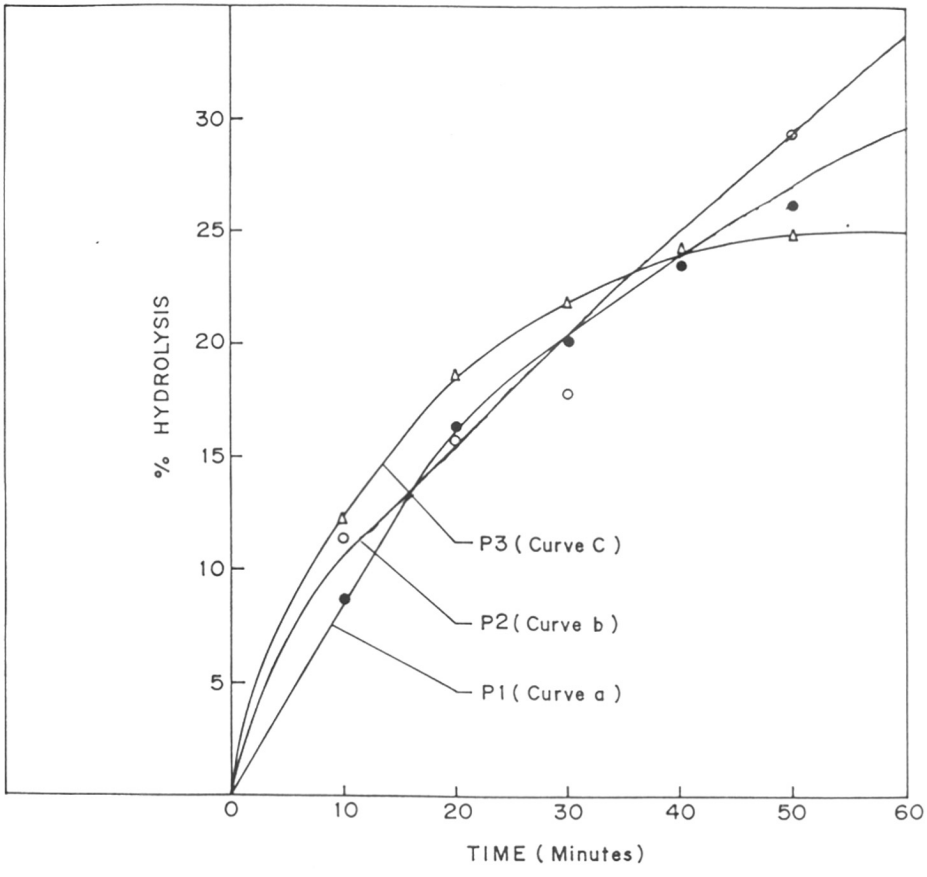
P3 consists of all the three monomers MA- serine, MA-aspartic acid and MA-histidine. Thus catalytically active hydroxyl, carboxyl and imidazole groups are brought in the vicinity of one another as a result of coordination with Co(II) prior to the synthesis of the mimic. Therefore **P3** is expected to exhibit enzyme like hydrolysis. As shown in Figure 3.5 (curve c) **P3** exhibits burst kinetics for hydrolysis of N-cbz-Tyr-PNP (upto 30 minutes) followed by saturation of its catalytic activity. Curve c in Figure 3.5 represents a typical plot of turnover versus time for an enzyme catalyzed reaction under the excess substrate condition. It shows that mimic **P3** does not behave merely like nucleophilic

catalyst but exhibits multifunctional catalysis similar to chymotrypsin wherein cooperative action amongst hydroxyl, carboxyl and imidazole groups is involved. This cooperative effect in **P3** results in the enhancement of the nucleophilicity of serine hydroxyl group due to carboxyl and imidazole groups in its vicinity.

Enhancement in the nucleophilicity of hydroxyl group in the polymeric mimic by carboxyl group is more clearly demonstrated in chapter 4. It is shown that the catalytic activity of the mimic comprising monomers HEMA, N-methacryloyl ß alanine and MA-histidine was four times higher than that of the mimic comprising monomers HEMA, methacrylic acid and MA-histidine. Thus for same nucleophilic hydroxyl group of monomer HEMA, catalytic activity of the mimic was enhanced when carboxyl group from N-methacryloyl ß alanine was incorporated into the mimic.

The catalyst to substrate ratio in the experiment is 1 : 50. Within the time span of the analysis, the mimic **P3** hydrolyzed twelve times the molar excess of substrate over the functional groups in the mimic (Figure 3.5 (curve c)). In fact this turnover has also been demonstrated in the Lineweaver - Burk plots for mimics **P5** and **P6** in which upto 150 fold molar excess of substrate is used (Figures 3.6 and 3.7). This shows that chymotrypsin mimic **P3** exhibits catalytic turnover under excess substrate conditions and also the saturation of its activity due to excess of substrate.

In order to emphasize the role of metal ion coordination and the presence of template in the coordination step, prior to polymerization, we synthesized mimics **P4-P6.**



[Catalyst] : [Substrate] ratio 1 : 50

FIG. 3.5 - PLOT OF PERCENT HYDROLYSIS vs TIME

POLYMER	FUNCTIONAL GROUPS
P1	HISTIDINE
P2	HISTIDINE SERINE
P3	HISTIDINE SERINE ASPARTIC ACID

Thus mimic **P4** was synthesized by grafting all the three monomers MA-serine, MA-aspartic acid and MA-histidine on poly (GMA-EGDMA) beads without Co(II) complexation. This leads to random distribution of monomers on the support since the coordination with Co (II) was omitted. As the functional groups in **P4** are not in the vicinity of one another, **P4** is catalytically inactive in the hydrolysis of N-cbz-Tyr-PNP.

The mimic **P5** comprised all the three monomers brought in the vicinity of one another by coordination with Co(II). However, the template N-nicotinoyl tyrosyl benzyl ester is not a part of the Co (II) coordinated assembly. Methodology for the synthesis of the mimic **P6** is same as that for **P3**. Hydrolytic activity of **P5** and **P6** was evaluated in the framework of Michaelis - Menten kinetics. For this, 4 to 5 substrate concentrations in the range of 50 to 150 fold molar excess were used in each set of the experiments and initial rates of hydrolysis were monitored. From these, the initial velocities of the observed hydrolysis v_{obs} were calculated. The plots of $1/v_{obs}$ versus. $1/s$ (s = initial substrate concentration) for **P5** and **P6** are shown in Figures 3.6 and 3.7 respectively. Values of V_{max} and K_m were obtained from plots and the value of k_{cat} was calculated from ratio V_{max} / [functional groups in mimics]. Kinetic data are listed in Table 3.2. Surprisingly, k_{cat} of **P5** is somewhat higher (0.22 sec^{-1}) than that for **P6** (0.18 sec^{-1}) (the reasons for which are not clear).

In Michaelis - Menten kinetic K_m represents the affinity of enzyme for a particular substrate. Lower the value of K_m higher is the affinity (Bailey and Ollis 1986). In the present case K_m represents affinity of functional groups of the mimics for substrate N-cbz-Tyr-PNP. K_m value for **P5** is almost two times higher than K_m value for **P6** (Table

3.2). Thus although the functional groups in **P5** and **P6** are identical and have been preorganized by coordination with Co (II), the mimic **P6** exhibits higher affinity for substrate N-cbz-Tyr-PNP (K_m = 5.29 * 10^{-4} M). This is because the functional groups in the mimic **P6** are imprinted with the template since the template is incorporated in the Co (II) coordinated assembly for **P6** prior to polymerization. Since the template is not incorporated in the Co (II) coordinated assembly for **P5**, it lacks affinity for the substrate. Thus **P5** exhibits almost two fold lower affinity for the substrate (K_m = 1.17 * 10^{-3} M) as compared to **P6**.

Similar trends for higher substrate affinity for esterification catalysts have been reported by Morihara et al (1988). The ratio V_{max} / K_m reflects the substrate specificity of enzyme. As a result of imprinting, **P6** exhibits three fold higher substrate specificity for N-cbz-Tyr-PNP (V_{max} / K_m = 6.0 * 10^{-4} sec^{-1}) than that of **P5** (V_{max} / K_m = 1.8 * 10^{-4} sec^{-1}). This shows that chymotrypsin mimic synthesized by surface grafting of Co(II) coordinated monomers-template assembly are molecularly imprinted and they have ability to recognize the substrate specifically. Due to imprinting effect, catalytic efficiency of **P6** (k_{cat} / K_m = 347 sec^{-1} M^{-1}) is almost two fold higher than that of **P5** (k_{cat} / K_m = 196 sec^{-1} M^{-1}). It can be seen from the results of hydrolytic activity for **P1** to **P6** that each step in the synthetic methodology of surface grafting plays a role in enhancing the hydrolytic activity of resulting polymer mimic. The results show that cooperative action amongst hydroxyl, carboxyl and imidazole groups on the polymer support, similar to

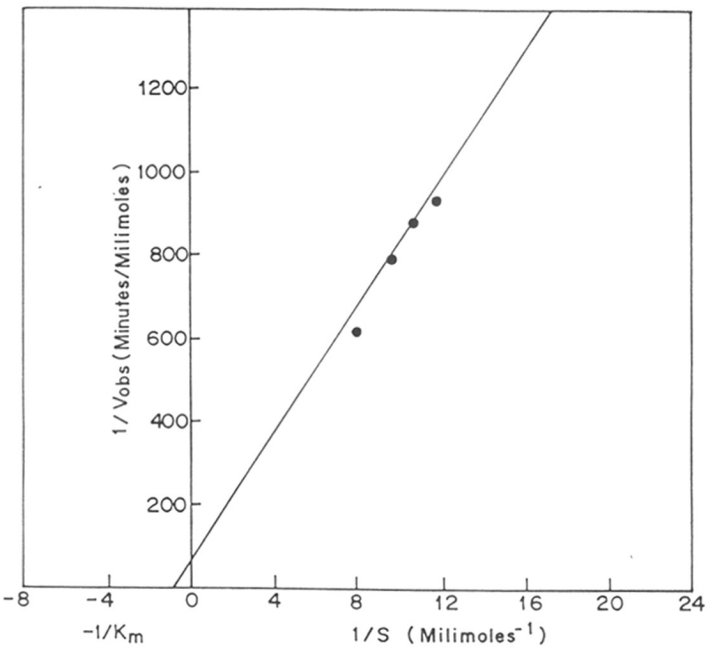


FIG. 3·6: LINEWEAVER-BURK PLOT FOR HYDROLYSIS OF N-cbz-Tyr-PNP CATALYZED BY NOT IMPRINTED P5 AT 37°C AND pH 7·8. THE ASSAY EMPLOYED 50 mg P5 WHICH CONTAINED 0·00102 MILIMOLES OF ACTIVE GROUPS MEASUREMENTS WERE DONE IN TRIPLICATES AT VARIOUS SUBSTRATE CONCENTRATIONS RANGING FROM 0·08224 MILIMOLES TO 0·1233 MILIMOLES.

Kcat = 0·22 Sec^{-1} Km = $1·17 \times 10^{-3}$ M Kr = 196 Sec^{-1} M^{-1}

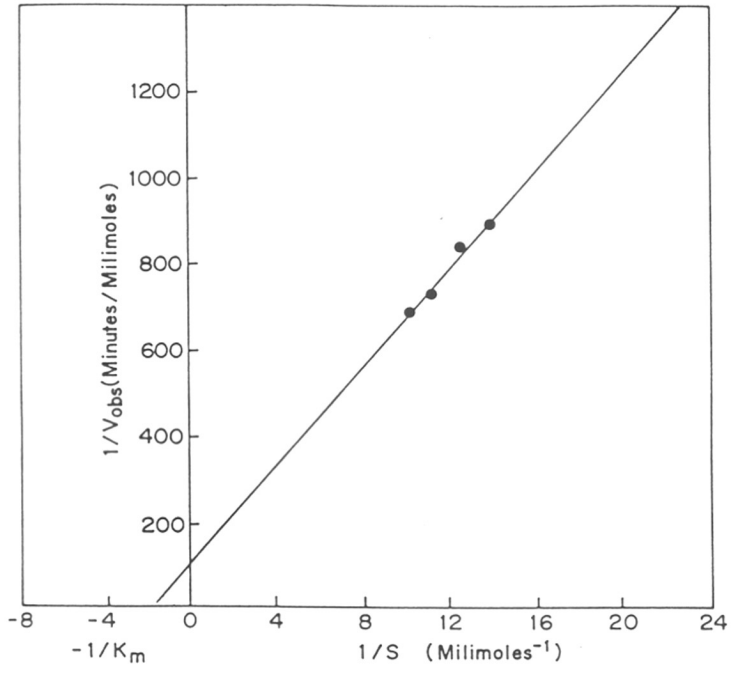


FIG. 3·7 - LINEWEAVER - BURK PLOT FOR HYDROLYSIS OF N-Cbz-Tyr-PNP CATALYZED BY IMPRINED P6 AT 37°C AND pH 7·8 THE ASSAY. EMPLOYED 50 mg P6 WHICH CONTAINED 0·00181 MILIMOLES OF ACTIVE GROUPS. MEASUREMENTS WERE DONE IN TRIPLICATES AT VARIOUS SUBSTRATE CONCENTRATIONS RANGING FROM 0·0724 MILIMOLES TO 0·1086 MILIMOLES.

K_{cat} = 0·18 Sec^{-1} Km = 5·299 x 10^{-4}M Kr = 347 Sec^{-1} M^{-1}

chymotrypsin, could be achieved when functional monomers on the polymer support are placed in the vicinity of one another by metal complexation. Additionally, substrate specificity similar to enzymes could also be incorporated in the surface grafted mimics by molecular imprinting of template during the metal coordination step.

3.4.0 Conclusions

A chymotrypsin mimic was synthesized by surface grafting Co(II) coordinated monomers-template assembly comprising MA-serine, MA-aspartic acid, MA-histidine and template N-nicotinoyl tyrosyl benzyl ester. Chymotrypsin mimic so synthesized exhibited cooperative action amongst the hydroxyl, carboxyl and imidazole groups of monomers grafted on the support, in the hydrolysis of substrate N-cbz-tyr-PNP. Polymer containing only histidine group exhibited nucleophilic catalysis without burst and saturation kinetics. Polymer containing hydroxyl and imidazole groups exhibited initial burst hydrolysis but no saturation. Only the polymer containing all the three functional groups viz. hydroxyl, carboxyl and imidazole groups exhibited burst kinetics and then saturation of hydrolytic activity. The three functional groups in the polymer mimic thus mimick the catalytic activity of chymotrypsin. The hydrolysis obeyed Michaelis - Menten kinetics. The mimic imprinted with the template exhibited two fold higher substrate affinity than the mimic not imprinted with the template. The mimic synthesized in the absence of Co (II) was hydrolytically inactive. In summary, this work demonstrates generic nature of the methodology which comprises surface grafting metal ion

coordinated monomers-template assembly to synthesize hydrolytically active chymotrypsin mimics.

Native chymotrypsin exhibits very high hydrolytic activity in hydrolysis of N-cbz-Tyr-PNP (k_{cat} = > 300 sec^{-1}, K_m = 3.25 * 10^{-5} M, k_{cat} / K_m = > 9.2 * 10^6 sec^{-1}, M^{-1}) since it exhibits specificity for the substrate (Bender et al 1961). Although the hydrolytic activity of the mimic reported herein is much lower than that of chymotrypsin, it demonstrates the role of molecular imparting technique in achieving enzyme like activity. Enhancement in the activity resulting from the choice of appropriate functional monomers and the carrier support are discussed in the subsequent chapters.

Chapter 4

Enhancing the catalytic activity of the mimic : Structure - property correlation

4.1.0 Introduction

In chapter 3 we described synthesis and evaluation of chymotrypsin mimic synthesized by grafting Co(II) coordinated monomers-template assembly comprising N-methacryloyl L-serine, N-methacryloyl L-aspartic acid and N-methacryloyl L-histidine onto crosslinked support poly (glycidyl methacrylate -co- ethylene glycol dimethacrylate). The hydrolytic activity of the mimic was shown to result from cooperative effect amongst hydroxyl, carboxyl and imidazole groups of monomers grafted on the support. As mentioned in objectives of work, after validation of the synthetic methodology, further work was focused mainly on elucidating various parameters that govern the hydrolytic activity of surface imprinted chymotrypsin mimics. In this chapter we describe the effect of two parameters namely choice of functional monomers and surface area of the carrier support on hydrolytic activity of chymotrypsin mimics.

Polymeric systems have an advantage in that a variety of functional monomers can be incorporated to mimick the triad. Exhaustive investigations have been reported on the relationship between functional monomers and activity of soluble polymers synthesized to mimick hydrolase enzymes (Kunitake et al 1976[a,b], Letsinger et al 1965, Overberger et al 1965, 1967, 1969, Strauss et al 1954, Klotz et al 1969). The cooperative bifunctional catalysis between hydroxyl and imidazole groups has been exhibited by copolymers poly (4 vinyl phenol - co - 4 (5) vinyl imidazole) and poly (N-methyl acrylohydroxamic acid - co - 4 (5) vinyl imidazole) (Overberger et al 1967, Kunitake et al 1976[b]).

The concept proposed in chapter 3 can be extended to a variety of functional monomers. In this chapter we report a series of chymotrypsin mimics synthesized by grafting Co(II) coordinated monomers-template assemblies of different functional monomers bearing hydroxyl, carboxyl and imidazole groups onto surface of poly (glycidyl methacrylate - co - ethylene glycol dimethacrylate). Hydrolytic activity of mimics is evaluated in the framework of Michaelis - Menten kinetics. The activity of the mimics is shown to increase with the nucleophilicity of the hydroxyl group as well as due to cooperative effect amongst the monomers grafted onto the support. The trends in the kinetic constants k_{cat} and K_m are shown to be similar to those for immobilized enzymes.

4.2.0 Experimental section

4.2.1 Materials

Methacrylic acid (MA), dicyclohexyl carbodiimide (DCC), glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EGDMA), 4-acetoxy styrene were from Aldrich. N-benzyloxycarbonyl L-Tyrosine (N-cbz-Tyr) was from sigma. These chemicals were used as received. All other chemicals were from local suppliers. These were purified prior to use following the standard procedures (Perrin et al 1981).

4.2.2 Instrumentation and analyses

Melting points were recorded on Mettler melting point apparatus. Infrared spectra were recorded on Perkin - Elmer 1600 FT-IR spectrophotometer. ^{1}H spectra were recorded on Varian spectrometer operating at 200 MHz. Electronic absorption spectroscopic measurements were carried out on Shimadzu UV 240 spectrophotometer.

Surface areas and pore volume measurements were made on Quanta-chrome Autoscan porosimeter. ESR spectra were recorded at 298 K on Bruker spectrometer operating at 9.73 Hz using ST 8484 cavity.

4.2.3 Synthesis of functional monomers

N-methacryloyl L-serine (MA-serine), N-methacryloyl L-aspartic acid, N-methacryloyl L-histidine (MA-histidine), N-methacryloyl L-cysteine (MA-cysteine), N-methacryloyl ß alanine (MA-ß alanine) were synthesized by reacting methacryloyl chloride and respective amino acid under Schotten Baumann conditions as reported in chapter 3.

N- methacryloyl L-cysteine (MA-cysteine)

IR (methnol)

1600 cm^{-1} (methacryl double bond), 1650 cm^{-1} (amide carbonyl), 1700 cm^{-1} (acid carbonyl).

^{1}H NMR (D_2O)

1.9 3H singlet (-C = C-CH_3), 3.4 2H doublet (-CH_2-SH), 5.5 1H singlet (-CH_a = C-), 5.8 1H singlet (-CH_b = C-).

N-methacryloyl ß alanine (MA-ß alanine)

IR (methanol)

1620 cm^{-1} (methacryl double bond), 1660 cm^{-1} (amide carbonyl), 1720 cm^{-1} (acid carbonyl).

^{1}H NMR (D_2O)

1.85 3H singlet (-C = C-CH_3), 2.4 2H triplet (-C-CH_2-NH-), 3.5 2H triplet (-C-CH_2-COOH), 5.4 1H singlet (-CH_a = C-), 5.6 1H singlet (-CH_b = C-).

4.2.4 Synthesis of 4-Vinyl phenol

4- vinyl phenol was synthesized as follows (Corson et al 1958). In a 250 ml round bottom flask, 16.2 g 4-acetoxy styrene (0.1 M) dissolved in 40 ml methanol was placed and stirred at 5 to 10°C (ice water bath). To this, 13.8 g KOH (0.25 M) dissolved in 140 ml water was added in a single portion. The reaction mixture was stirred at 5 to 10°C until homogeneous (1hr). Then it was acidified with conc. HCl and the precipitated product was extracted in ethyl acetate (3 * 100 ml). Organic phase was dried on anhydrous sodium sulfate and concentrated in vacuo to viscous mass. This was dissolved in 200 ml methanol and treated with charcoal. It was then filtered and concentrated in vacuo and the viscous mass thus obtained was poured in 1litre water under stirring with occasional scratching to yield white solid product. It was isolated and purified by two reprecipitations from methanol into water. Yield 60 percent.

Melting point 120°C.

IR (nujol)

3396 cm^{-1} (-OH stretching), 1612 cm^{-1} (-CH = CH_2), 1510 cm^{-1} (aromatic -CH bending).

^{1}H NMR (DMSO d_6)

2.5 1H triplet (-CH = C-), 3.5 2H doublet (-CH_2 = C-), 6.5 4H broad singlet (aromatic protons), 9.0 1H singlet (phenolic -OH).

4.2.5 Synthesis of substrate

N-benzyloxy carbonyl tyrosyl para nitro phenyl ester (N-cbz-Tyr-PNP) was synthesized according to procedure reported in chapter 3.

4.2.6 Synthesis of template

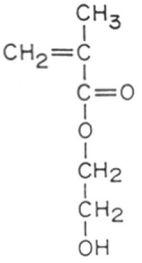
N-nicotinoyl tyrosyl benzyl ester was synthesized according to the procedure reported in chapter 3.

Structures of all functional monomers, substrate, template are shown in Figure 4.1.

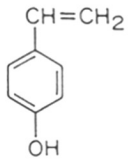
4.2.7 Synthesis of poly (glycidyl methacrylate - co - ethylene glycol dimethacrylate) (poly (GMA-EGDMA)) support

Beads of poly (GMA-EGDMA) were synthesized by suspension polymerization of glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EGDMA) as reported in chapter 3. Beads in the range of 45 to 75 microns were used for surface grafting. For a given set of mimics care was taken to use beads from the same suspension polymerization experiment in order to avoid complications arising from differences in surface area, pore size and morphology of support. Thus mimics **P1** to **P5** were

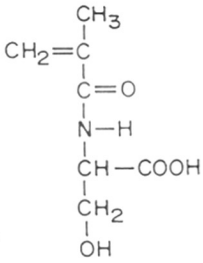
(a) HYDROXYL / SULPHHYDRYL MONOMERS



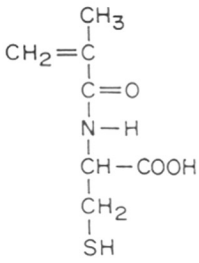
HEMA



4 VINYL PHENOL

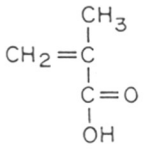


MA-SERINE

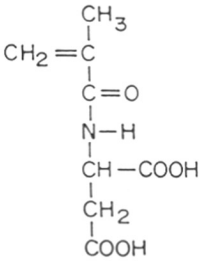


MA-CYSTEINE

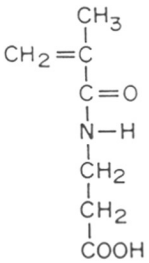
(b) CARBOXYL MONOMERS



MAA

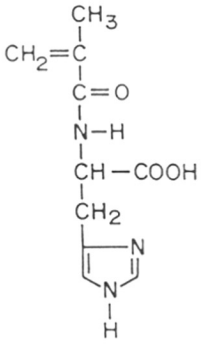


MA-ASPARTIC ACID



MA-β ALANINE

(c) IMIDAZOLE MONOMER



MA-HISTIDINE

FIG. 4·1 - STRUCTURES OF FUNCTIONAL MONOMERS.

synthesized with support from one batch and mimics **P6** to **P9** were synthesized with support from another.

4.2.8 Preparation and Electron Spin Resonance (ESR) characterization of Co (II) coordinated monomers-template assemblies

Monomers, template and $CoCl_2.6H_2O$ were dissolved in methanol and the solution was stirred under nitrogen for 1 hour. Methanol was then evaporated under vacuum in the presence of poly (GMA-EGDMA) beads to sorb the Co(II) coordinated monomers-template assemblies onto the support. The assemblies prepared are described in Tables 4.1 to 4.5. ESR spectra of the assemblies sorbed on the support were recorded using ST 8484 cavity.

4.2.9 Synthesis of mimics

Mimics were synthesized by grafting Co (II) coordinated monomers-template assemblies comprising different monomers bearing hydroxyl, carboxyl, imidazole groups and template N-nicotinoyl tyrosyl benzyl ester, on poly (GMA-EGDMA) beads. The assemblies alongwith EGDMA, AIBN were adsorbed on poly (GMA-EGDMA) beads and polymerized at 75°C for 24 hours and worked up as reported in chapter 3. The feed compositions of the assemblies and other relevant data for mimics **P1** to **P5** are listed in Table 4.6. Also, data for mimics **P6** to **P9** are listed in Table 4.7.

4.2.10 Estimation of the total amino acids and the extent of grafting in mimics

In order to estimate the functional groups per unit weight of the support and overall percentage of the grafting, we estimated total amino acids in mimics. The mimics

were refluxed in HCl to hydrolyze the grafted amino acids from the methacryl backbone. Quantitative Ninhydrin tests on the hydrolysates so obtained were carried out as reported in chapter 3. The intense purple coloration developed due to the tests was measured on spectrophotometer at 550 nm. These absorbance values were used to estimate the amount of total amino acids i.e. the amount of total functional groups in the mimics, using relevant standard calibration plot as reference. Overall percentage grafting was calculated from the amounts of functional groups in mimics and those in the feed of assemblies.

4.2.11 Evaluation of hydrolytic activity

Hydrolytic activity of all mimics towards the substrate N-cbz-Tyr-PNP was evaluated in the framework of Michaelis - Menten kinetics. 50 mg polymeric mimic was placed in a jacketed reactor. 10 ml of 40 : 60 acetonitrile : phosphate buffer (0.05 M, pH 7.8) was added. The suspension was stirred with magnetic needle at 37^0C. A predetermined amount of substrate was dissolved in 1 ml acetonitrile and added while stirring. Hydrolysis was followed by monitoring absorbance of para nitro phenol released, at 400 nm. A range of substrate concentrations between 50 to 150 fold molar excess over the functional groups in mimics was used and initial rates of hydrolysis (v_{obs}) were monitored to draw Lineweaver - Burk plots.

4.3.0 Results and Discussion

In the preceding chapter we demonstrated that the nucleophilicity of hydroxyl group is enhanced by the presence of carboxyl and imidazole groups in its vicinity. In this chapter we describe chymotrypsin like activity of molecularly imprinted polymer mimics

comprising a wide range of functional monomers. The nucleophilicity of serine hydroxyl group is varied by the choice of hydroxyl bearing monomers as well as by the choice of the carboxyl bearing monomers.

4.3.1 ESR characterization of Co (II) coordinated monomers-template assemblies

Characterization of such monomers-template assemblies by ESR spectroscopy has been reported by Dhal et al (1992). Coordination of monomeric ligand to metal ion is confirmed by the decrease in the $g_{\parallel}$ value of the metal ion (Dhal et al 1992). In the present case, ability of all monomers to form coordination complex with Co(II) was characterized by ESR spectroscopy. The assemblies were prepared in which monomers were sequentially added to Co(II) (Tables 4.1 to 4.5). ESR spectra of all assemblies were recorded and $g_{\parallel}$ values calculated in each case. It can be seen from data listed in Tables 4.1 to 4.5, that in each case sequential addition of monomers and template to $CoCl_2.6H_2O$ leads to significant decrease in $g_{\parallel}$ value of cobalt. This indicates the coordination of all monomeric ligands with cobalt. This also indicates that the three monomers will be placed in the vicinity of one another to give cooperative action in the resulting mimics.

4.3.2 Synthesis and characterization of mimics

Mimics were synthesized by adsorbing Co(II) coordinated monomers-template assemblies alongwith EGDMA and AIBN on poly (GMA-EGDMA) beads. The assemblies were polymerized at 75°C for 24 hours, then worked up and characterized by FT-IR and surface area measurements as reported in chapter 3. Data for the feed composition of the assemblies and for the mimics are listed in Tables 4.6 and 4.7.

Table 4.1

Coordination of monomeric ligands and template to $CoCl_2.6H_2O$ for HEMA, MAA, MA-histidine (PI)

Entry	Description of complex	g_{II}
1	(0.0005M) $CoCl_2.6H_2O$	2.199
2	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) HEMA	2.1390
3	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) HEMA + (0.0005M) MAA	2.0140
4	(0.0005 M) $CoCl_2.6H_2O$ + (0.0005M) HEMA + (0.0005M) MAA + (0.0005M) MA-histidine	1.9445
5	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) HEMA + (0.0005M) MAA + (0.0005M) MA-histidine + (0.0005M) Template	1.9984

Table 4.2

Coordination of monomeric ligands and template to $CoCl_2.6H_2O$ for 4-Vinyl phenol, MAA, MA-histidine (P2)

Entry	Description of complex	g_{II}
1	(0.0005M) $CoCl_2.6H_2O$	2.45
2	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) 4-Vinyl phenol	2.4530
3	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) 4-Vinyl phenol + (0.0005M) MAA	2.3759
4	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) 4-Vinyl phenol + (0.0005M) MAA + (0.0005M) MA-histidine	2.3443
5	(0.0005M) $CoCl_2.6H_2O$ +(0.0005M) 4-Vinyl phenol + (0.0005M) MAA + (0.0005M) MA-histidine + (0.0005M) Template	2.2862

Table 4.3

Coordination of monomeric ligands and template to $CoCl_2.6H_2O$ for MA-serine, MA-aspartic acid, MA-histidine (P3)

Entry	Description of complex	$g_{\parallel}$
1	(0.0005M) $CoCl_2.6H_2O$	2.199
2	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) MA-serine	2.1106
3	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) MA-serine + (0.0005M) MA-aspartic acid	2.0598
4	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) MA-serine + (0.0005M) MA-aspartic acid + (0.0005M) MA-histidine	2.0005
5	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) MA-serine + (0.0005M) MA-aspartic acid + (0.0005M) MA-histidine + (0.0005M) Template	1.8294

Table 4.4

Coordination of monomeric ligands and template to $CoCl_2.6H_2O$ for MA-cysteine, MA-aspartic acid, MA-histidine (P4)

Entry	Description of complex	$g_{\|\|}$
1	(0.0005M) $CoCl_2.6H_2O$	2.199
2	(0.0005m) $CoCl_2.6H_2O$ + (0.0005M) MA-cysteine	2.0371
3	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) MA-cysteine + (0.0005M) MA-aspartic acid	2.0276
4	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) MA-cysteine + (0.0005M) MA-aspartic acid + (0.0005M) MA-histidine	1.9651
5	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) MA-cysteine + (0.0005M) MA-aspartic acid + (0.0005M) MA-histidine + (0.0005M) Template	1.9542

Table 4.5

Coordination of monomeric ligands and template to $CoCl_2.6H_2O$ for HEMA, MA- ß alanine, MA-histidine (P5)

Entry	Description of complex	g_{II}
1	(0.0005M) $CoCl_2.6H_2O$	2.199
2	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) HEMA	2.0371
3	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) HEMA + (0.0005M) MA-ß alanine	1.9701
4	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) HEMA + (0.0005M) MA-ß alanine + (0.0005M) MA-histidine	1.9026
5	(0.0005M) $CoCl_2.6H_2O$ + (0.0005M) HEMA + (0.0005M) MA-ß alanine + (0.0005M) MA-histidine + (0.0005M) Template	1.8981

Co (II) coordinated assemblies of above mentioned stoichiometry were adsorbed on 0.5 g GMA-EGDMA support and spectra were recorded under following conditions.

Temperature 298 K, Microwave frequency 9.72 to 9.74GHz, Cavity ST 8424, Frequency 100K Hz, Field modulation intensity 1.25 Gpp, Field intensity (mild range 2600G) (scan range 5000G), scan time 200sec.

4.3.3 Estimation of functional groups and extent of grafting in mimics

As discussed in chapter 3, estimation of functional groups in the mimics is carried out assuming that the polymerization of the Co (II) coordinated assembly comprising hydroxyl, carboxyl and imidazole monomers results in the grafting of the three monomers in 1:1:1 molar ratio on the support. Thus estimation of the total amino acids i.e. functional groups, from the absorbance of purple coloration of the hydrolysates, was done using relevant calibration plot depending upon the functional monomers in the mimics.

A standard calibration plot of absorbance versus. concentration for Ninhydrin test of serine, aspartic acid, histidine composition in 1:1:1 ratio was used in the estimations of mimics **P3** and **P6-P9**. A similar plot for the composition cysteine, aspartic acid and histidine was used for **P4**. Since the monomers HEMA, MAA and 4 vinyl phenol do not give Ninhydrin test, the absorbance versus. concentration plot for histidine was used in the estimations of **P1** and **P2**. For the estimation of **P5**, the calibration plot for histidine and ß alanine composition in 1:1 ratio was used.

The amount of each functional monomer per unit weight of the support was estimated as one third the total amino acids estimated. As noted above, this assumes that the three monomers when coordinated with Co (II), are always polymerized in the ratio 1:1:1, which is justified in view of the results reported by Belokon et al (1982) and Karamalkar et al (1996[b]). Ratios of crosslinker, initiator and weights of monomers to support were kept constant for **P1** to **P5** (Table 4.6). Also the polymerization conditions

Table 4.6

Data for feed of monomers and hydrolytic activity of mimics P1 to P5.

No.	1	2	3	4	5	6
Polymer	Blank	**P1**	**P2**	**P3**	**P4**	**P5**
Hydroxyl monomer (M)	-	HEMA, (0.0015)	4 vinyl phenol, (0.0015)	MA-serine, (0.0015)	MA-cysteine, (0.00069)	HEMA, (0.0015)
Carboxyl monomer (M)	-	MAA, (0.0015)	MAA, (0.0015)	MA-aspartic acid, (0.0015)	MA-aspartic acid, (0.00069)	MA-ß alanine. (0.0015)
Imidazole monomer (M)	-	MA-histidine, (0.0015)	MA-histidine, (0.0015)	MA-histidine, (0.0015)	MA-histidine, (0.00069)	MA-histidine. (0.0015)
Template (M)	-	0.0015	0.0015	0.0015	0.00069	0.0015
$CoCl_2.6H_2O$ (M)	-	0.0015	0.0015	0.0015	0.00069	0.0015
poly (GMA-EGDMA) (g)	0.8	1.34	1.17	1.8	0.85	1.53
Functional grs./ 50mg(M)	-	$2.05 * 10^{-6}$	$2.86 * 10^{-6}$	$1.23 * 10^{-6}$	$1.05 * 10^{-6}$	$1.11 * 10^{-6}$
Percentage grafting	-	5.64	9.66	4.57	4.00	3.5
Surface area (m^2/g)	49.63	37.51	28.43	41.43	45.60	29.35
Pore volume (cm^3/g)	0.1427	0.0997	0.0784	0.1169	0.1037	0.0784
k_{cat} (sec^{-1})	-	0.06	0.15	0.12	0.21	0.25
K_m (M)	-	$5.64 * 10^{-4}$	$1.28 * 10^{-3}$	$5.26 * 10^{-4}$	$7.39 * 10^{-4}$	$1.47 * 10^{-3}$
k_{cat} / K_m ($sec^{-1} M^{-1}$)	-	116	121	245	286	170

Foot note for Table 4.6

* In all mimics ratio of wt. of poly (GMA-EGDMA) beads to wt. of monomers is 1 : 1.82. * Feed of all assemblies contain EGDMA (10 % of the wt. of monomers) and AIBN (3 % of the wt. of monomers). * All mimics are synthesized according to procedure described in chapter 3. * Estimation of functional groups and percentage grafting is done by Ninhydrin test on hydrolysate obtained for each mimic.

were identical. Yet, levels of grafting obtained varied significantly. Higher levels of percentage grafting viz.5.64 and 9.66 were observed in **P1** and **P2** than in the case of **P3**, **P4** and **P5** (4.57, 4.0, 3.5 respectively). Co (II) coordinated monomers-template assemblies constituting **P1** and **P2** contain hydrophobic monomers such as 4 vinyl phenol and methacrylic acid, while those constituting **P3**, **P4**, **P5** contain hydrophilic monomers like MA-serine, MA-aspartic acid, MA-cysteine and MA-ß alanine. MA-histidine and template N-nicotinoyl tyrosyl benzyl ester are incorporated in the assemblies of all mimics. Thus with increase in the hydrophobicity of monomers, the hydrophobic character of the Co(II) coordinated assembly increases which results in efficient grafting of the assembly onto poly (GMA-EGDMA) beads. We observed a similar trend for templates in the work described later in chapter 5 wherein grafting level of hydrophilic monomers viz. MA-serine, MA-aspartic acid and MA-histidine increased with increase in the hydrophobicity of template molecule in the Co(II) coordinated assembly being grafted. This could be attributed to enhanced hydrophobic interactions of the assemblies with the supports.

4.3.4 Choice of functional monomers

Different monomers bearing hydroxyl, sulphhydryl, carboxyl and imidazole groups were selected and incorporated into the mimics so as to study the effect of functional monomers on the hydrolytic activity of mimics. Rationale in the selection of these monomers is summarized below.

P1. This mimic consists of Co(II) coordinated assembly of HEMA, MAA, MA-histidine grafted on poly (GMA-EGDMA) beads. Thus serine hydroxyl group is represented by HEMA, aspartyl carboxyl group by MAA and histidyl imidazole group by MA-histidine. Synthesis of this mimic was reported earlier but its catalytic activity was not evaluated against N-cbz-Tyr-PNP Karamalkar et al 1996[c]).

P2. In this mimic Co(II) coordinated assembly of 4 vinyl phenol, MAA, MA-histidine is used. In comparison to **P1** which contains aliphatic hydroxyl group from HEMA, the phenolic hydroxyl group of 4 vinyl phenol in **P2** is more nucleophilic. Incorporation of 4 vinyl phenol as a comonomer with 4(5) vinyl imidazole is reported to result in higher hydrolytic activity of poly (4 vinyl phenol -co - 4(5) vinyl imidazole) (Kunitake et al 1976[b]). In the present case too, we expect the same effect.

P3. The mimic **P3** contains the same functional amino acids as those in chymotrypsin viz. MA-serine, MA-aspartic acid and MA-histidine. Origins of hydrolytic activity of this mimic have been discussed in chapter 3. It is included here for the sake of comparison with other polymers.

P4. The constituents of the mimic **P4** are same as those of **P3** except that MA-serine is replaced by MA-cysteine. The imidazole and carboxyl moieties in **P4** are kept same as in **P3** and the hydroxyl group is replaced by sulphhydryl group. Since -SH group is more nucleophilic than -OH group, **P4** is expected to be more active than **P3**.

P5. The mimic **P5** is consists of Co(II) coordinated assembly of HEMA, MA-ß alanine, MA-histidine. Functional monomers in **P5** differ from those in **P1** only in that

methacrylic acid is replaced by MA-ß alanine. The methylene spacers in the monomer MA-ß alanine are expected to place carboxyl group in the monomer closer to hydroxyl and imidazole groups. Besides, there are no free side chains in their structures. This mimic is therefore expected to exhibit chymotrypsin like activity more closely.

4.3.5 Hydrolytic activity of mimics

Hydrolytic activity of all mimics in the hydrolysis of N-cbz-Tyr-PNP was evaluated in the framework of Michaelis - Menten kinetics. Lineweaver - Burk plots for **P1** to **P5** are shown in Figures 4.2 to 4.6 respectively. Kinetic data for **P1** to **P5** are listed in Table 6. The values of V_{max} and K_m were obtained from the plots and the values of k_{cat} were obtained from the equation $k_{cat} = V_{max}$ / [Functional grs. in mimics].

4.3.6 Factors governing k_{cat}

As seen from the Table 4.6, k_{cat} for **P1** is 0.06 sec^{-1} which is lowest among all five. It is the base reference with which the hydrolytic activity of other mimics is compared. **P2** contains phenolic -OH group from 4 vinyl phenol which is more nucleophilic than aliphatic -OH group of HEMA. As expected, k_{cat} value for **P2** is two fold higher (0.15 sec^{-1}) than that for **P1** (0.06 sec^{-1}) and is in agreement with the observation that soluble copolymer (poly 4 vinyl phenol -co - 4 (5) vinyl imidazole) exhibited higher hydrolytic activity than poly (4 (5) vinyl imidazole) (Overberger et al 1967). This indicates that in crosslinked polymers too, hydrolysis takes place by similar cooperative action. Value of k_{cat} for **P3** is 0.12 sec^{-1}, which is higher than k_{cat} of **P1** (0.06 sec^{-1}) but lower than that for **P2** (0.15 sec^{-1}). In **P4**, hydroxyl group is replaced by

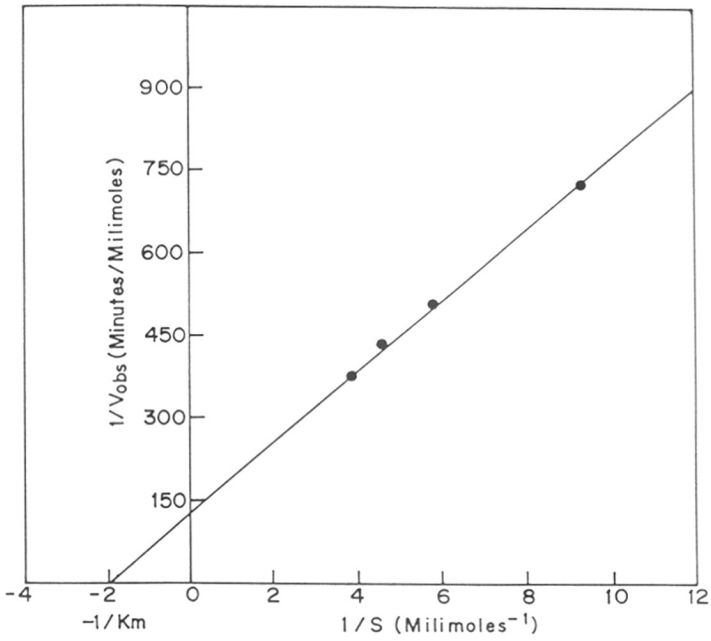


FIG. 4·2: LINEWEAVER–BURK PLOT FOR HYDROLYSIS OF N-cbz-Tyr-PNP CATALYZED BY P1 AT 37°C AND pH 7·8. THE ASSAY EMPLOYED 50mg P1, WHICH CONTAINED 0·00205 MILIMOLES OF ACTIVE GROUPS. SUBSTRATE CONCENTRATION WAS VARIED BETWEEN 0·1066 MILIMOLES TO 0·3218 MILIMOLES.

Kcat = 0·063 Sec^{-1} Km = $5·64 \times 10^{-4}$ M Kr = 116 $Sec^{-1} M^{-1}$

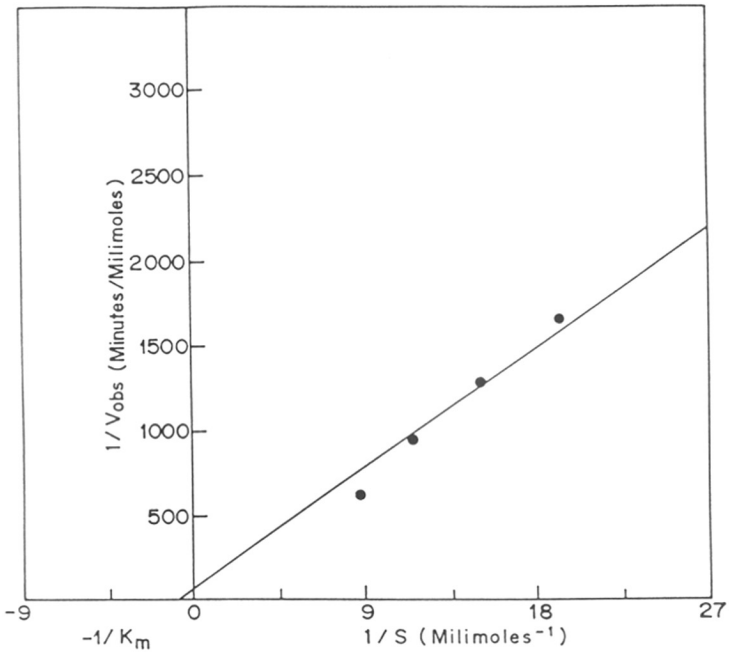


FIG. 4·3 : LINEWEAVER-BURK PLOT FOR HYDROLYSIS OF N-cbz-Tyr-PNP CATALYZED BY P2 AT 37°C AND pH 7·8. THE ASSAY EMPLOYED 50 mg P2 WHICH CONTAINED 0·00286 MILIMOLES OF ACTIVE GROUPS. SUBSTRATE CONCENTRATION WAS VARIED BETWEEN 0·0143 MILIMOLES TO 0·1144 MILIMOLES.

Kcat = 0·15 Sec^{-1} Km = 1·28 x 10^{-3} M Kr = 121 Sec^{-1} M^{-1}

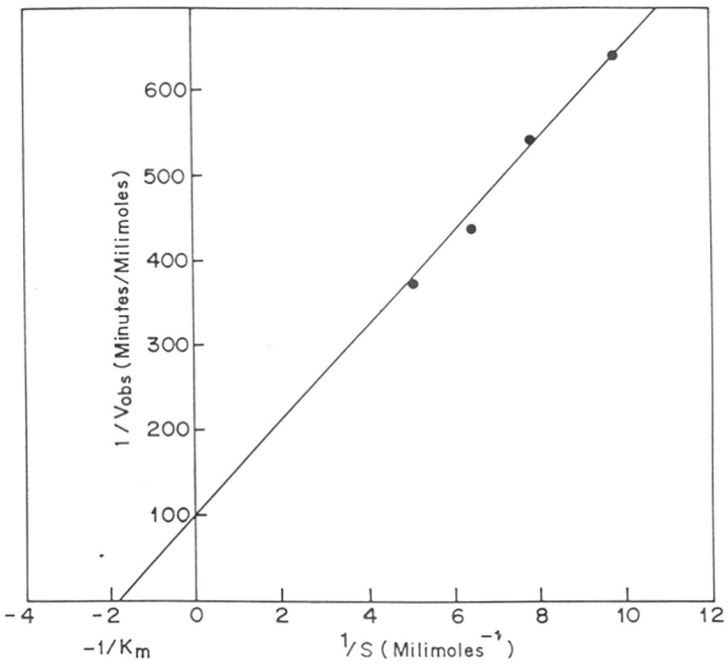


FIG. 4·4 : LINEWEAVER-BURK PLOT FOR HYDROLYSIS OF N-cbz-Tyr-PNP CATALYZED BY P3 AT 37°C AND pH 7·8. THE ASSAY EMPLOYED 50 mg P3 WHICH CONTAINED. 0·00123 MILIMOLES OF ACTIVE GROUPS. SUBSTRATE CONCENTRATION WAS VARIED BETWEEN 0·06396 MILIMOLES TO 0·1931 MILIMOLES.

Kcat = 0·12 Sec^{-1} Km = $5·26 \times 10^{-4}$ M Kr = 245 Sec^{-1} M^{-1}

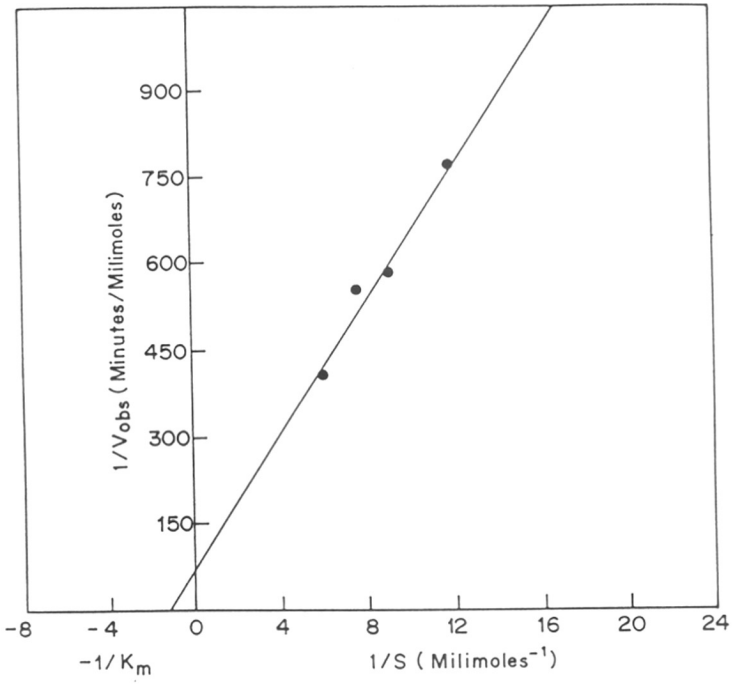


FIG 4.5 : LINEWEAVER-BURK PLOT FOR HYDROLYSIS OF N-cbz-Tyr-PNP CATALYZED BY P4 AT 37°C AND pH 7·8. THE ASSAY EMPLOYED 50 mg P4 WHICH CONTAIND 0·00105 MILIMOLES OF ACTIVE GROUPS. SUBSTRATE CONCENTRATION WAS VARIED BETWEEN 0·0548 MILIMOLES TO 0·184 MILIMOLES.

Kcat = 0·21 Sec^{-1} Km = $7{\cdot}39 \times 10^{-4}$ M Kr = 286 Sec^{-1} M^{-1}

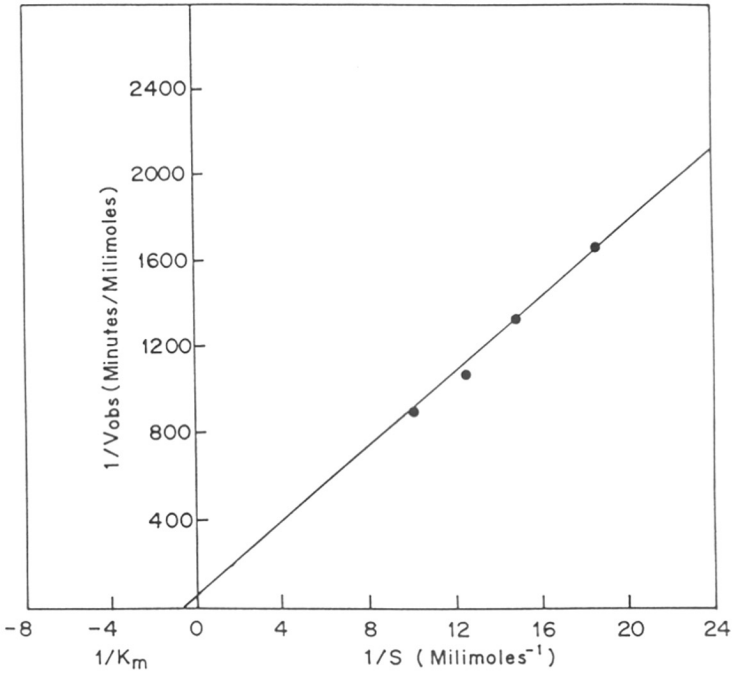
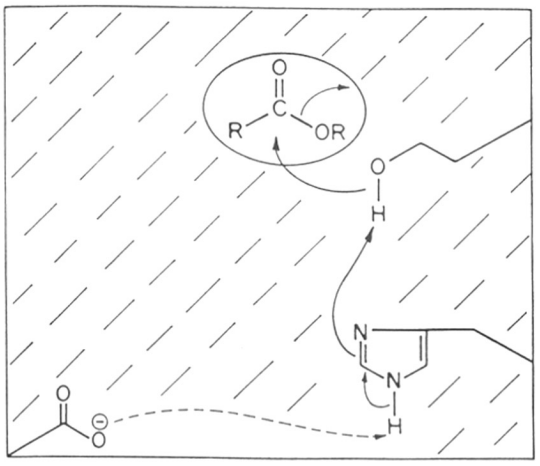


FIG. 4·6 : LINEWEAVER–BURK PLOT FOR HYDROLYSIS OF N-cbz-Tyr-PNP CATALYZED BY P5 AT 37°C AND pH 7·8. THE ASSAY EMPLOYED 50 mg P5 WHICH CONTAINED 0·00111 MILIMOLES OF ACTIVE GROUPS. SUBSTRATE CONCENTRATION WAS VARIED BETWEEN 0·0333 MILIMOLES TO 0·100 MILIMOLES.

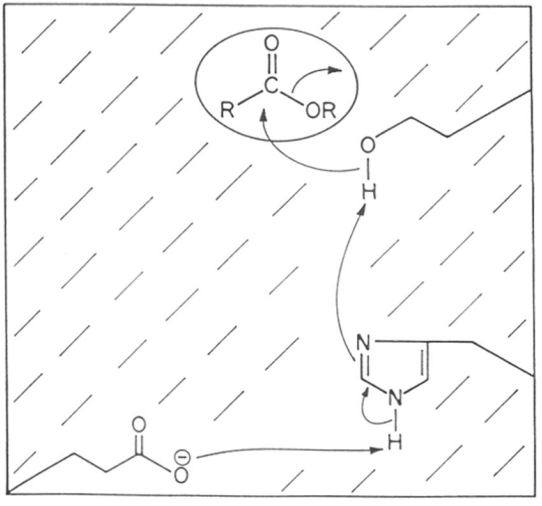
Kcat = 0·25 Sec^{-1} Km = 1·47 x 10^{-3} M Kr = 170 Sec^{-1} M^{-1}

sulphhydryl group of cysteine. Since -SH group is more nucleophilic than -OH group, higher catalytic activity is anticipated for **P4**. This is borne out by enhanced value of k_{cat} for **P4** (0.21 sec^{-1}) This is consistent with the trend of higher nucleophilicity observed for **P2**. Here, it may be noted that the imidazole functionality MA-histidine is same in all mimics. Higher k_{cat} values for both **P2** and **P4** than **P1** and **P3** indicate that hydrolytic activity of mimics can be enhanced by incorporating increasingly nucleophilic monomers as serine moiety.

The cooperative action within functional monomers grafted on the support which results in higher catalytic activity of hydroxyl group is more clearly demonstrated in the case of mimic **P5**. Nucleophilic monomers HEMA and MA-histidine are same in both **P1** and **P5**. The carboxyl functionality in **P5** is represented by MA-ß alanine whereas that in **P1** it is represented by methacrylic acid. Due to the presence of two methylene groups in ß alanine, its carboxyl group is expected to be closer to hydroxyl and imidazole groups of HEMA and MA-histidine. As a result, cooperative action among hydroxyl, carboxyl and imidazole groups takes place more efficiently which renders the hydroxyl group in **P5** more nucleophilic and results in highest k_{cat} value within the series viz. 0.25 sec^{-1}. A schematic representation of this effect is shown in Figure 4.7. Thus for same nucleophilic monomer HEMA, a four fold increase in catalytic activity is obtained by replacing methacrylic acid by MA-ß alanine. This indicates that the origin of higher catalytic activity of the mimic **P5** indeed lies in cooperative effect within hydroxyl, carboxyl and imidazole functional monomers as in the case of chymotrypsin. However, the degree of enhancement is not as high as in chymotrypsin, since the mimic contains the three



(a) Schematic representation of cooperative action in P1.



(b) Schematic representation of cooperative action in P5.

FIG. 4·7 - SCHEMATIC REPRESENTATION OF SPACER EFFECT OF METHYENE GROUPS OF β ALANINE IN P5.

functional groups brought in vicinity of one another merely by complexation with cobalt but it lacks the conformational specificity of chymotrypsin. Perhaps the groups all need to be much closer.

4.3.7 Factors governing K_m.

Michaelis - constant K_m represents the affinity of the enzyme for the substrate. Low value of K_m indicates high affinity. In this work K_m indicates the affinity of functional groups of mimics for substrate N-cbz-Tyr-PNP. K_m values for **P2** and **P5** are almost two times higher than K_m values for **P1**, **P3** and **P4** (Table 4.6).This indicates that functional groups in **P2** and **P5** have lower affinity for the substrate than those in **P1**, **P3** and **P4**. Although mimics **P1** to **P5** are synthesized from the same batch of poly (GMA-EGDMA) beads (49.63 m^2/g), surface areas of **P2** and **P5** are 28.43 m^2/g and 29.35 m^2/g respectively, while those for the mimics **P1**, **P3** and **P4** are 37.51 m^2/g, 41.43 m^2/g and 45.60 m^2/g respectively (Table 4.6). It therefore appears that mimics with lower surface areas and porosities exhibit high K_m values. This is consistent with the trends reported by Revillion et al (1989) and Guyot et al (1991/1992) for poly (styrene - co -divinyl benzene) alkylation catalysts, wherein decrease in surface area results in decrease in substrate accessibility. However also wish to point out that there is no direct relationship between k_{cat} and K_m of enzymes and this especially holds for immobilized enzymes.

The polymer mimics discussed in this work can be looked upon as enzyme active sites immobilized on heterogeneous supports. Immobilization of enzyme within the microcapsule is reported to result in higher K_m values as a result of diffusional

limitations experienced by the substrate during transfer from bulk of the solution into the microcapsule in which the enzyme is immobilized (Artmanis et al 1984, O'Grady et al 1981, Moo-Young et al 1972). Although the K_m value for enzymes immobilized in microcapsules is high, the reaction velocity is also high (Boguslasky et al 1974, Dean et al 1977). In the hydrolysis of N-acetyl tyrosyl ethyl ester (ATEE) by sepharose immobilized chymotrypsin, Kasche and Bergwall (1974) reported k_{cat} = 150 sec^{-1} and K_m = 1.0 * 10^{-2} M while corresponding values for free chymotrypsin were 130 sec^{-1} and 2.0 * 10^{-3} M respectively. Thus the parameters V_{max} and K_m were independent of each other. The trends indicate that for immobilized enzymes, higher k_{cat} values could be obtained despite high values of K_m. Higher values of k_{cat} as well as K_m for mimics **P2** and **P5** can be similarly explained.

Although the trends in k_{cat} and K_m observed for **P1** to **P5** are similar to those observed for immobilized enzymes, the functional constituents of each mimic are not the same. In order to unequivocally establish the results, we synthesized four mimics **P6** to **P9** in which the same set of functional monomers (MA-serine, MA-aspartic acid, MA-histidine) with increasing loading of functional groups on the support were used. k_{cat} and K_m values for these mimics were obtained for hydrolysis of N-cbz-Tyr-PNP and the similarity in trends of k_{cat} and K_m for these mimics and immobilized enzymes is demonstrated in the following sections.

4.3.8 Synthesis of polymer mimics P6 -P9

Increasing amount of monomers can be grafted on crosslinked support as long as the double bonds on the support are available for grafting (Dhal et al 1995). We have utilized this feature to synthesize mimics **P6** to **P9**. As listed in Table 4.7 the wt. ratio of Co(II) coordinated assembly (of MA-serine, MA-aspartic acid, MA-histidine and template) to poly (GMA-EGDMA) beads was decreased from 1 : 1.82 to 1:1. The mimics were synthesized following the same procedure as that for mimics **P1** to **P5**. Data for feed of assemblies and mimics are listed in Table 4.7. A series of mimics **P6** to **P9** is obtained with increase in loading of functional groups from 6.56 * 10^{-7} M / 50 mg of beads to 1.28 * 10^{-6} M / 50 mg of beads. Surface area of mimics **P6** to **P9** decreased from 22.75 m^2 /g to 14.07 m^2 /g as the amount of functional groups on the support increased. The support material from the same batch was used for synthesis of **P6** to **P9**.

4.3.9 Hydrolytic activity of mimics P6 to P9

Hydrolytic activity of mimics **P6** to **P9** for hydrolysis of N-cbz-Tyr-PNP was evaluated in the framework of Michaelis - Menten kinetics. Lineweaver - Burk plots of $1/v_{obs}$ versus. $1/s$ were plotted for (Figures 4.8 to 4.11 respectively) and values of V_{max}, k_{cat} and K_m were obtained from the plots. Kinetic data listed in Table 4.7 show that K_m value increased with decrease in surface area. This is consistent with the trends observed earlier for variation of K_m for the series **P1** to **P5**.

V_{max} values for mimics in the series **P6** - **P9** increased from 4.59 * 10^{-8} M / sec to 2.30 * 10^{-7} M / sec (Table 4.7). Thus with increase in the loading of functional groups

Table 4.7

Data for feed monomers and hydrolytic activity of mimics P6 to P9

No.	1	2	3	4	5
Polymer	Blank	**P6**	**P7**	**P8**	**P9**
MA-serine (M)	-	0.0015	0.00175	0.002	0.0025
MA-aspartic acid (M)	-	0.0015	0.00175	0.002	0.0025
MA-histidine (M)	-	0.0015	0.00175	0.002	0.0025
Template (M)	-	0.0015	0.00175	0.002	0.0025
$CoCl_2.6H_2O$ (M)	-	0.0015	0.00175	0.002	0.0025
poly (GMA-EGDMA) (g)	1.8	1.8	1.8	1.8	1.8
Wt. ratio of poly (GMA-EGDMA) to monomers	-	1.82 : 1	1.57 : 1	1.37 : 1	1.09 : 1
Functional grs. per 50 mg (M)	-	$6.56 * 10^{-7}$	$6.98 * 10^{-7}$	$8.26 * 10^{-7}$	$1.28 * 10^{-6}$
Surface area (m^2/g)	44.40	22.75	21.50	17.65	14.07
Pore volume (cm^3/g)	0.1240	0.0734	0.0605	0.0649	0.0452
V_{max} (M/sec)	-	$4.59 * 10^{-8}$	$8.37 * 10^{-8}$	$1.23 * 10^{-7}$	$2.30 * 10^{-7}$
k_{cat} (sec^{-1})	-	0.07	0.12	0.15	0.18
K_m (M)	-	$1.35 * 10^{-4}$	$3.13 * 10^{-4}$	$7.69 * 10^{-4}$	$1.42 * 10^{-3}$
k_{cat} / K_m (sec^{-1}, M^{-1})	-	588	383	305	243

Foot note for Table 4.7

* Feed of all assemblies contain EGDMA (10 % of the wt. of monomers) and AIBN (3 % of the wt. of monomers). * All mimics are synthesized according to procedure reported in chapter 3. * Estimation of functional groups is done by Ninhydrin test on the hydrolysate obtained for each mimic.

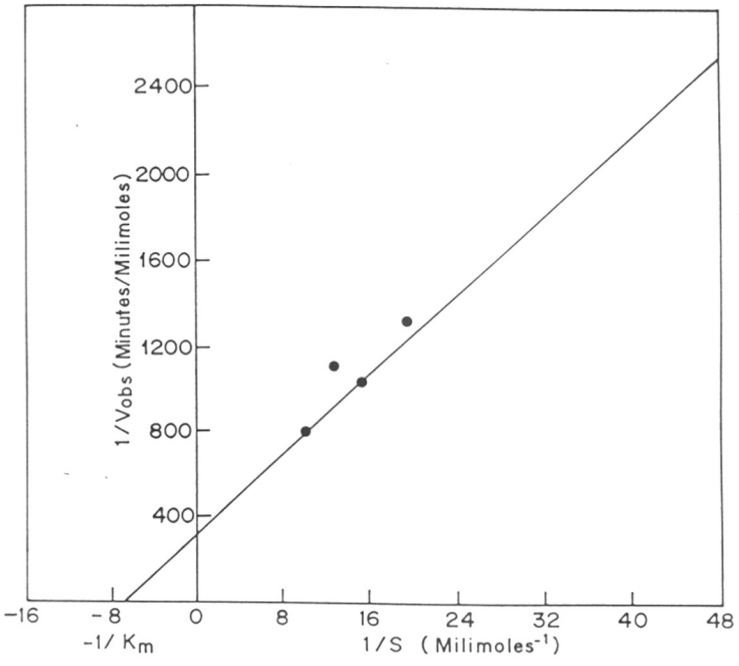


FIG. 4·8 : LINEWEAVER-BURK PLOT FOR HYDROLYSIS OF N-cbz-Tyr-PNP CATALYZED BY P6 AT 37°C AND pH 7·8 ASSAY EMPLOYED 50 mg P6 WHICH CONTAINED 0·000656 MILIMOLES OF ACTIVE GROUPS. MEASUREMENTS WERE DONE IN TRIPLICATES AT VARIOUS SUBSTRATE CONCENTRATIONS RANGING FROM 0·0325 MILIMOLES TO 0·0975 MILIMOLES.

Kcat = 0·07 Sec^{-1} Km = $1·35 \times 10^{-4}$ M Kr = 588 Sec^{-1} M^{-1}

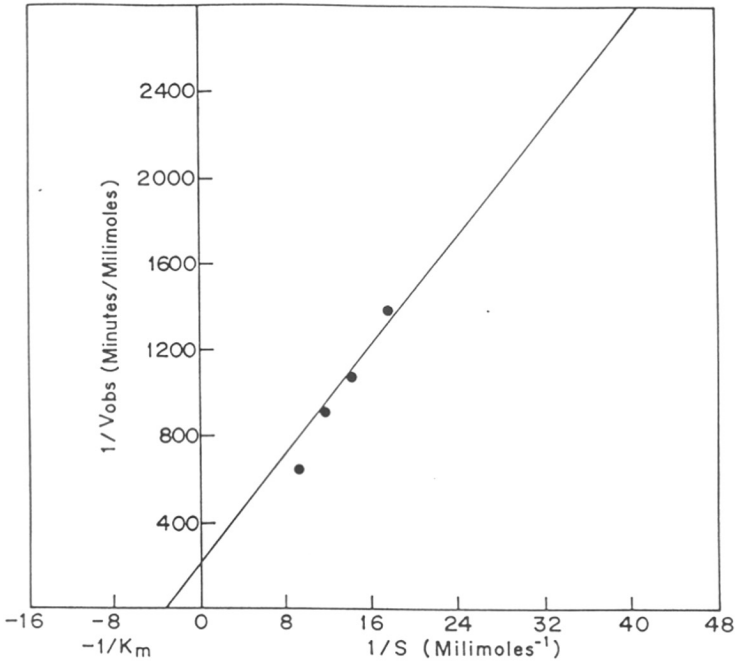


FIG. 4·9 : LINEWEAVER – BURK PLOT FOR HYDROLYSIS OF N-cbz-Tyr-PNP CATALYZED BY P7 AT 37°C AND pH 7·8. ASSAY EMPLOYED 50 mg P7 WHICH CONTAINED 0·000698 MILIMOLES OF ACTIVE GROUPS. MEASUREMENTS WERE DONE IN TRIPLICATES AT VARIOUS SUBSTRATE CONCENTRATIONS RANGING FROM 0·0357 MILIMOLES TO 0·107 MILIMOLES.

Kcat = 0·12 Sec^{-1} Km = $3·13 \times 10^{-4}$ M Kr = 383 Sec^{-1} M^{-1}

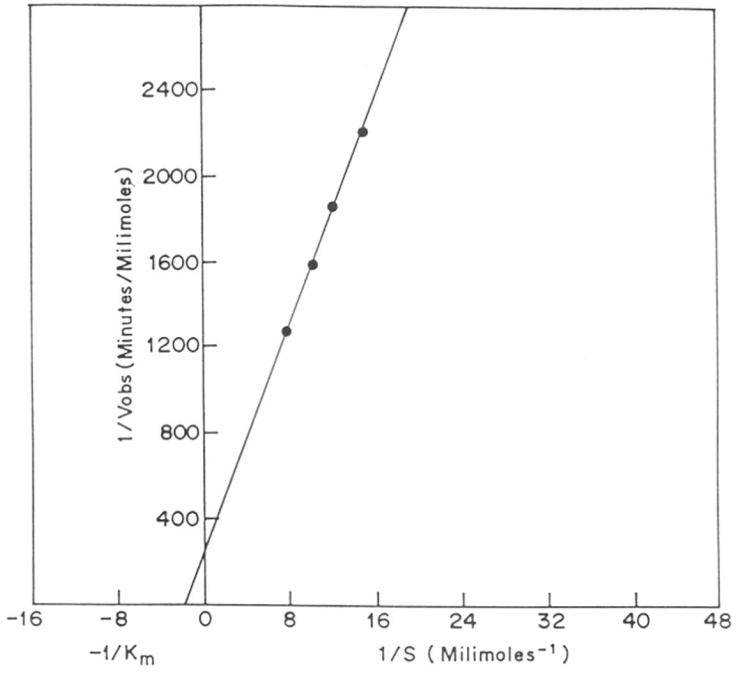


FIG. 4.10: LINEWEAVER-BURK PLOT FOR HYDROLYSIS OF N-cbz-Tyr-PNP CATALYZED BY P8 AT 37°C AND pH 7·8. ASSAY EMPLOYED 50mg P8 WHICH CONTAINED 0·000826 MILIMOLES OF ACTIVE GROUPS. MEASUREMENTS WERE DONE IN TRIPLICATES AT VARIOUS SUBSTRATE CONCENTRATIONS RANGING FROM 0·0415 MILIMOLES TO 0·124 MILIMOLES.

Kcat = 0·15 Sec^{-1} Km = $7{\cdot}69 \times 10^{-4}$ M Kr = 305 $Sec^{-1} M^{-1}$

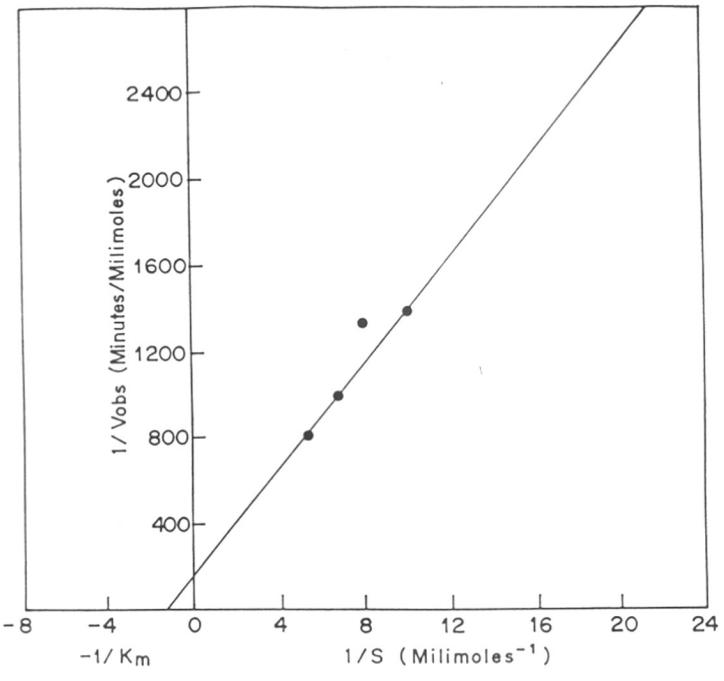


FIG. 4.11 : LINEWEAVER-BURK PLOT FOR HYDROLYSIS OF N-cbz-Tyr-PNP CATALYZED BY P9 AT 37°C, pH 7·8 ASSAY EMPLOYED 50mg P9 WHICH CONTAINED 0·00128 MILIMOLES OF ACTIVE GROUPS. MEASUREMENTS WERE DONE IN TRIPLICATES AT VARIOUS SUBSTRATE CONCENTRATIONS RANGING FROM 0·0635 MILIMOLES TO 0·190 MILIMOLES.

Kcat = 0·18 Sec^{-1} Km = $1·42 \times 10^{-3}$ M Kr = 243 Sec^{-1} M^{-1}

per unit weight of the support, velocity of the reaction increased, which is similar to high rates of reaction for immobilized enzymes. In the same series value of k_{cat} increased from values of **P6** to **P9** increased from 0.07 sec^{-1} to 0.18 sec^{-1} while corresponding values for K_m also increased from 1.35 * 10^{-4} M to 1.42 * 10^{-2} M. Thus in the series too, increase in values of k_{cat} is accompanied by increasing values of K_m as in the case of immobilized enzymes.

k_{cat} / K_m values of **P6** to **P9** decreased from 588 sec^{-1} M^{-1} to 243 sec^{-1} M^{-1} as a result of increased K_m values. Thus catalytic efficacy of mimics decreased with decrease in surface area of supports. Alternatively, this indicates that the efficacy of mimics could be improved using polymer supports with high surface area for grafting.

4.4.0 Conclusions

A series of polymeric mimics exhibiting chymotrypsin like hydrolytic activity was synthesized by grafting Co (II) coordinated assemblies of functional monomers bearing various hydroxyl, carboxyl and imidazole groups onto microporous support. Hydrolytic activity of these mimics towards the hydrolysis of N-cbz-Tyr-PNP was evaluated. The values of k_{cat} for these mimics were enhanced increasing nucleophilicity of monomers mimicking serine hydroxyl group. But highest k_{cat} was obtained for **P5** with carboxyl group from ß alanine. As compared to the carboxyl groups from methacrylic acid in **P1**, carboxyl groups from ß alanine are closer to hydroxyl and imidazole groups. This resulted in efficient cooperative action amongst the functional groups in **P5**. This indicates that closer simulations of natural triad of chymotrypsin will

result in further enhancement in catalytic activity of mimics. Values of K_m for mimics increased with decrease in surface area. This suggests that K_m value of mimics can be lowered using supports with high surface area. Mimics exhibited high k_{cat} values despite high K_m values. These kinetic trends were found to be similar to those observed for immobilized enzymes. In summary, k_{cat} of chymotrypsin mimics is governed by nucleophilicity of functional monomers as well as cooperative effect of carboxyl groups. K_m of the mimics is governed by surface area of the support and improvement in K_m (low K_m) can be achieved using supports with very high surface area.

Chapter 5

Reactivity of the mimic as a function of substrate structure and support composition

5.1.0 Introduction

In chapter 4 we described hydrolytically active polymeric mimics of chymotrypsin synthesized by grafting Co (II) coordinated assembly of different monomers bearing hydroxyl, carboxyl and imidazole groups and a template onto surface of microporous support. It was shown that k_{cat} of the mimics is governed by functional monomers and K_m is governed by surface area of the carrier support.

In chymotrypsin, the active site is situated in a hydrophobic environment. Its hydrolytic activity towards a particular substrate is governed by the structure of the substrate and the type and the extent of its binding with the active site (Berezin et al 1971). In analogy with chymotrypsin, the hydrolytic activity of chymotrypsin mimics is also expected to depend on the structure of the substrate and the type of the substrate binding with the support material.

Microenvironmental effects on reactivity in polymer supported catalysts have been reported. Deratani et al (1987) reported high acylation efficacy of (dialkyl amino pyridine) groups bound to polystyrene supports when degree of functionalization was less than 50 %. This was attributed to the hydrophobic environment around (dialkyl amino) pyridine groups. Yaroslavsky et al (1970) reported a study on halogenation of cumene by polymer supported reagent. It was observed that poly (N-bromo maleimide) favored dehydrobromination, an undesirable side reaction. On the other hand, poly (N-bromomaleimide -co -styrene) was more efficient in producing brominated cumene (Yaroslavsky et al 1970). Recently Alaxandratos and Miller (1996) reported a study on microenvironmental effects in diphenyl phosphine reagent supported on polystyrene. It

was shown that due to enhanced hydrophobic effects, the rates of Mitsunobu reaction increased with decrease in loading of diphenyl phosphine groups on polystyrene support. These studies indicate that the hydrophobic environment of support enhances the substrate binding and therefore the catalytic activity of polymer supported reagent.

In this chapter, we describe the effect of polymer support composition of chymotrypsin mimic on substrate binding and hydrolytic activity for a series of para nitro phenyl esters. Thus the other two important parameters viz. substrate structure and support composition that would govern hydrolytic activity of mimics are studied in this work.

Mimics were synthesized by coordinating Co(II) with of N-methacryloyl L-serine, N-methacryloyl L-aspartic acid, N-methacryloyl L-histidine and templates and grafting the assemblies on hydrophilic and hydrophobic supports. The influence of substrate structure and hydrophobicity of the support, on the hydrolytic activity is shown to be similar to that in the case of chymotrypsin for hydrophilic support. For hydrophobic support, enhanced binding and higher activity was observed.

5.2.0 Experimental section

5.2.1 Materials

Dicyclohexyl carbodiimide (DCC), glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EGDMA), 1-hydroxy benzotriazole were from Aldrich. N-acetyl-tyrosine and N-benzyloxycarbonyl L-Tyrosine (N-cbz-tyrosine) were from Sigma. All other

chemicals were from local suppliers and were purified prior use, following the standard procedures (Perrin 1981).

5.2.2 Instrumentation and analyses

All characterizations and measurements procedures were same as reported in chapters 3 and 4.

5.2.3 Synthesis of functional monomers

Functional monomers N-methacryloyl L-serine (MA-serine), N-methacryloyl L-aspartic acid (MA-aspartic acid), N-methacryloyl L-histidine (MA-histidine) were synthesized by reaction between methacryloyl chloride and the respective amino acid as reported in chapter 3..

Synthesis of N-acyl tyrosines

5.2.4 Synthesis of N-benzoyl Tyrosine

4.63 g Tyrosyl methyl ester . Hydrochloride (0.02 M) (synthesized according to reported procedure (Huang et al 1979)) was suspended in 80 ml tetrahydrofuran at $0^{o}C$. To this suspension, 6.3 ml triethylamine (0.044 M) was added and the suspension was stirred with magnetic needle. To this suspension, 2.34 ml benzoyl chloride (0.02 M) dissolved in 5 ml tetrahydrofuran was added dropwise over 1hr. The reaction mixture was stirred at room temperature for 12 hrs and filtered to remove salts. Filtrate was concentrated in vacuo and poured into 500 ml cold water. Oily material separated which solidified into white product upon scratching. The product was isolated and dried.

Completion of the reaction was confirmed from ester and amide peaks in IR spectrum of this product (N-benzoyl tyrosyl methyl ester). 4.5 g N-benzoyl- tyrosyl methyl ester (0.015 M) was dissolved in 30 ml methanol. To this solution 1.2 g NaOH dissolved in 30 ml water was added and the reaction mixture was stirred at 25°C for two hrs. Then it was acidified to pH 4 to 4.5 and methanol was evaporated in vacuo. Aqueous solution obtained was cooled down and acidified to pH 2 and stored in refrigerator for 1hr. White sticky material separated which was extracted into 2 * 200 ml of ethyl acetate and dried over sodium sulfate. This solution was concentrated in vacuo to yield viscous mass which was poured into 250 ml petroleum ether and scratched to yield white powder. The product was purified by reprecipitation from acetone into petroleum ether. Yield 70 percent.

Melting point 162°C.

IR (nujol)

3400 cm^{-1} (-OH stretching), 3180 cm^{-1} (-NH stretching), 1709 cm^{-1} (acid carbonyl), 1633 cm^{-1} (amide carbonyl), 1575 cm^{-1} (aromatic -CH bending).

^{1}H NMR (DMSO d_6)

3.0 2H doublet (-CH_2-Ph of Tyrosine), 4.5 1H triplet (-CH-COOH of Tyrosine), 6.6 2H double doublet (Tyrosine ring protons), 7.1 2H double doublet (Tyrosine ring protons), 7.5 3H multiplet (Benzoyl ring protons), 7.8 2H double doublet (Benzoyl ring protons), 8.6 1H doublet (-NH proton), 9.25 1H singlet (-OH proton).

5.2.5 Synthesis of N-butyryl tyrosine

13.55 g Tyrosyl benzyl ester (0.05 M) (synthesized as reported in chapter 3) and 4 ml pyridine (0.05 M) were dissolved in 100 ml tetrahydrofuran and stirred at 0°C. To this solution, 4.8 g n-butyryl chloride (0.05 M) was added dropwise over 1.5 hrs. Then the reaction mixture was stirred at room temperature for 12 hrs. It was then filtered to remove salts and filtrate was concentrated in vacuo to give viscous oil. This oil was poured into 1000 ml cold water and scratched to yield white solid product. It was isolated and dried. Completion of the reaction was confirmed from ester and amide peaks in the IR spectrum of the product (N-butyryl tyrosyl benzyl ester). The product was used without further purification.

5.2.6 Catalytic deblocking of N-butyryl tyrosyl benzyl ester

Deblocking was performed as follows (Beig et al 1985). 13.42 g N-butyryl tyrosyl benzyl ester was dissolved in 70 ml methanol. To this solution 1.342 g 10 percent Palladium.Charcoal (Pd.C) and 15 g ammonium formate were added. The reaction mixture was refluxed under stirring at 65°C for two hrs. Then it was cooled to room temperature and filtered. Filtrate was added slowly to 1000 ml acetone under stirring. White product precipitated. It was collected and dried. It was purified by two reprecipitations from methanol into acetone. Yield 50 percent.

Melting point 153°C.

IR (nujol)

3530 cm^{-1} (-OH stretching), 3317 cm^{-1} (-NH stretching), 1711 cm^{-1} (acid carbonyl), 1632 cm^{-1} (amide carbonyl), 1567 cm^{-1} (aromatic -CH bending).

^1H NMR (DMSO d_6)

0.75 3H triplet (-CH_3 at C_4 of butyryl group), 1.4 2H multiplet (-CH_2 at C_3 of butyryl group), 2.0 2H triplet (-CH_2 at C_2 of butyryl group), 3.0 2H doublet (-CH_2-Ph of Tyrosine), 3.9 1H triplet (-CH-COOH of Tyrosine), 6.6 2H double doublet (Tyrosine ring protons), 6.9 2H double doublet (Tyrosine ring protons).

5.2.7 Synthesis of N-dodecanoyl tyrosine

13.55 g tyrosyl benzyl ester (0.05 M) and 4 ml pyridine (0.05 M) were dissolved in 100 ml tetrahydrofuran and stirred at 0°C. To this solution, 10.09 g n-dodecanoyl chloride (0.05 M) was added dropwise over 1.5 hrs. Then the reaction mixture was stirred at room temperature for 12 hrs. It was then filtered to remove salts and filtrate was concentrated in vacuo to give viscous oil. This oil was poured into 1000 ml cold water and scratched to yield white solid product. It was isolated and dried. Completion of the reaction was confirmed from ester and amide peaks in the IR spectrum of the product (N-dodecanoyl tyrosyl benzyl ester). The product was used without further purification.

5.2.8 Catalytic deblocking of N-dodecanoyl tyrosyl benzyl ester

Deblocking was performed as follows (Beig et al 1985). 21.68 g N-dodecanoyl tyrosyl benzyl ester was dissolved in 150 ml methanol. To this solution 2.168 g 10 %

percent Palladium.Charcoal (Pd.C) and 15 g ammonium formate were added. The reaction mixture was refluxed under stirring at 65°C for two hours. Then it was cooled to room temperature and filtered. Filtrate was added slowly to 1000 ml acetone under stirring. White product precipitated. It was collected and dried. It was purified by two reprecipitations from methanol into acetone. Yield 65 percent.

Melting point 104°C.

IR (nujol)

3355 cm^{-1} (-OH stretching), 3317 cm^{-1} (-NH stretching), 1710 cm^{-1} (acid carbonyl), 1625 cm^{-1} (amide carbonyl), 1571 cm^{-1} (aromatic -CH bending).

^{1}H NMR (DMSO d_6)

0.75 3H triplet (-CH_3 at C_{12} of dodecanoyl group), 1.2 16H broad singlet (methylene groups of C_3 to C10 of dodecanoyl group), 1.4 2H sextet (-CH_2 at C_{11} of dodecanoyl group), 2.0 2H triplet (-CH_2 at C_2 of dodecanoyl group), 2.75 2H doublet (-CH_2-Ph of Tyrosine), 2.9 1H triplet (-CH-COOH of Tyrosine), 6.5 2H double doublet (Tyrosine ring protons), 6.9 2H double doublet (Tyrosine ring protons), 7.4 1H doublet (-NH proton).

Synthesis of templates

5.2.9 Synthesis of N-acetyl-tyrosyl- 2 amino pyridinamide (N-acetyl-Tyr-2AP)

In a 250 ml capacity round bottom flask, 4.46 g N-acetyl tyrosine (0.02 M) and 1.88 g 2 amino pyridine (0.02 M) were suspended in 60 ml ethyl acetate. The suspension was stirred at room temperature with magnetic needle. To this suspension, 4.12 g DCC

(0.02 M) dissolved in 40 ml ethyl acetate was added in one single portion. Reaction mixture was stirred for 2 days at room temperature. It was then filtered to remove dicyclohexyl urea (DCU) formed. Clear filtrate was concentrated in vacuo to yield a crude product which was purified on silica gel column using 30 / 70 acetone / petroleum ether as mobile phase. Yield 50 percent.

Melting point 152 - 153°C

IR (nujol)

3342 cm^{-1} (-OH stretching), 3270 cm^{-1} (-NH stretching), 1654 cm^{-1} (amide carbonyl), 1541 cm^{-1} (aromatic -CH bending).

^{1}H NMR ($CDCl_3$)

2.1 3H singlet (O = C-CH_3), 2.9 - 3.0 2H doublet (-CH_2-Ph of Tyrosine), 4.65 1H triplet (-CH-CONHR of Tyrosine), 6.5 - 6.7 4H triplet (Tyrosine ring protons), 6.8 - 7.0 4H triplet (2 amino pyridine ring protons).

5.2.10 Synthesis of N-butyryl-tyrosyl- 2 amino pyridinamide (N-butyryl-Tyr-2AP)

5.02 g N-butyryl - tyrosine (0.02 M) and 1.88 g 2 amino pyridine (0.02 M) were suspended in 70 ml ethyl acetate and stirred at room temperature. To this suspension, 4.12 g DCC (0.02 M) dissolved in 50 ml ethyl acetate was added in one single portion. The reaction mixture was stirred for two days. Then it was filtered to remove DCU. Filtrate was concentrated in vacuo and crude product obtained was purified on silica gel column using 30 / 70 acetone / petroleum ether as mobile phase. Yield 40 percent.

Melting point 126^0C.

IR (nujol)

3259 cm^{-1} (-OH stretching), 3100 cm^{-1} (-NH stretching), 1643 cm^{-1} (amide carbonyl), 1516 cm^{-1} (aromatic -CH bending).

^{1}H NMR ($CDCl_3$)

1.8 3H triplet (-CH_3 at C_4 of butyryl group), 2.0 2H multiplet (-CH_2 at C_3 of butyryl group), 2.9 2H triplet (-CH_2 at C_2 of butyryl group), 3.5 2H doublet (-CH_2-Ph of Tyrosine), 4.1 1H triplet (-CH-CONHR of Tyrosine), 6.8 2H double doublet (Tyrosine ring protons), 7.0 2H double doublet (Tyrosine ring protons), 7.5 4H multiplet (2 amino pyridine ring protons).

5.2.11. Synthesis of N-dodecanoyl-tyrosyl- 2 amino pyridinamide (N-dodecanoyl-Tyr-2AP)

3.63 g N-dodecanoyl tyrosine (0.01 M), 1.35 g 1-hydroxy benzotriazole (0.01 M) and 0.94 g 2 amino pyridine (0.01 M) were dissolved in 30 ml dimethyl formamide. The solution was stirred at 5 to 10^oC in ice water bath. 2.21 g DCC (0.01 M) dissolved in 10 ml dimethyl formamide was added in one single portion to above solution. Reaction mixture was stirred for 1 hr. at 5 to 10^oC and 12 hrs at room temperature. Then it was filtered and filtrate was poured slowly into 1000 ml ice cold water under stirring. White product precipitated out. It was purified by two reprecipitations from dimethyl formamide into water. Yield 72 percent.

Melting point 167^{0}C.

IR (nujol)

3383 cm^{-1} (-OH stretching), 3316 cm^{-1} (-NH stretching), 1666 cm^{-1} (amide carbonyl), 1642 cm^{-1} (amide carbonyl), 1610 cm^{-1} (-C =N - of 2 amino pyridine ring), 1532 cm^{-1} (aromatic -CH bending).

^{1}H NMR (DMSO d_6)

0.75 3H triplet (-CH_3- at C_{12} of dodecanoyl group), 1.1 - 1.3 16H broad singlet (C_3 to C_{10} methylene groups of dodecanoyl chain), 1.4 2H sextet (-CH_2 at C_{11} of dodecanoyl group), 2.0 2H triplet (-CH_2 at C_2 of dodecanoyl group), 2.9 2H doublet (-CH_2-Ph of Tyrosine), 4.3 1H triplet (-CH-CONHR of Tyrosine), 6.6 2H double doublet (Tyrosine ring protons), 7.0 2H double doublet (Tyrosine ring protons), 7.3 4H multiplet (2 amino pyridine ring protons), 7.6 1H doublet (-NH proton), 9.1 1H singlet (-OH proton).

5.2.12 Synthesis of N-nicotinoyl-tyrosyl benzyl ester (N-nicotinoyl-Tyr-benz)

Synthesized according to the procedure reported in chapter 3.

Synthesis of substrates

5.2.13 Synthesis of N-acetyl-tyrosyl- para nitrophenyl ester (N-acetyl-Tyr-PNP)

In a 100 ml capacity round bottom flask, 1.32 g p-nitro phenol (0.0094 M) and 2.11 g N-acetyl tyrosine (0.0094 M) were suspended in 20 ml ethyl acetate and the suspension was stirred at 5 to 10^{0}C in ice water bath. To this, 1.95 g DCC (0.0094 M) dissolved in 15 ml ethyl acetate was added in a single portion. The reaction mixture was

stirred for 1 hr at 5 to 10°C and 12 hrs. at room temperature. It was then filtered to remove DCU. Filtrate was concentrated and the crude product was purified on silica gel column using 30 / 70 acetone / petroleum ether mobile phase. The pure product was obtained as viscous liquid. Yield 80 percent.

IR (nujol)

3370 cm^{-1} (-OH stretching), 1748 cm^{-1} (ester carbonyl), 1651 cm^{-1} (amide carbonyl), 1519 cm^{-1} (aromatic -CH bending).

^{1}H NMR (acetone d_6)

3.0 3H singlet (O = C-CH_3), 3.5 2H doublet (-CH_2-Ph of Tyrosine), 4.5 1H triplet (-CH-COOR of Tyrosine), 7.2 4H multiplet (Tyrosine ring protons), 7.5 4H multiplet (p-nitrophenyl ring protons).

5.2.14 Synthesis of N-butyryl-tyrosyl- para nitrophenyl ester (N-butyryl-Tyr-PNP)

5.02 g N-butyryl tyrosine (0.02 M) and 2.78 g p-nitrophenol (0.02 M) were suspended in 80 ml ethyl acetate and stirred at 5 to 10°C. To this suspension, 4.12 g DCC (0.02 M) dissolved in 40 ml ethyl acetate was added in a single portion. The reaction mixture was stirred at 1 hr at 5 to 10°C and 12 hrs at room temperature. It was then filtered to remove DCU and filtrate was concentrated in vacuo. The crude product obtained was purified on silica gel column using 30 / 70 acetone / petroleum ether as mobile phase. Yield 50 percent.

Melting point 147°C.

IR (nujol)

3400 cm^{-1} (-OH stretching), 3100 cm^{-1} (-NH stretching), 1740 cm^{-1} (ester carbonyl), 1630 cm^{-1} (amide carbonyl), 1500 cm^{-1} (aromatic -CH bending).

^{1}H NMR (acetone d_6)

0.9 3H triplet (-CH_3 at C_4 of butyryl group), 1.8 - 1.9 2H multiplet (-CH_2 at C_3 of butyryl group), 3.0 2H triplet (-CH_2 at C_2 of butyryl group), 3.5 2H doublet (-CH_2-Ph of Tyrosine), 4.5 1H triplet (-CH-COOR of Tyrosine), 7.3 - 7.5 multiplet 4H (Tyrosine ring protons), 7.7 - 7.9 multiplet 4H (Tyrosine ring protons).

5.2.15 Synthesis of N-dodecanoyl-tyrosyl- para nitrophenyl ester (N-dodecanoyl-Tyr-PNP)

3.63 g N-dodecanoyl tyrosine (0.01 M), 1.39 g p-nitrophenol (0.01 M) and 1.35 g 1-hydroxy benzotriazole (0.01 M) were dissolved in 30 ml dimethyl formamide. This solution was stirred at 5 to 10°C in ice water bath. To this solution 2.06 g DCC (0.01 M) dissolved in 10 ml dimethyl formamide was added in a single portion. Reaction mixture was stirred for 1 hr at 5 to 10°C and 12 hrs at room temperature. Then it was filtered to remove DCU and filtrate was slowly poured into 1000 ml ice cold water under stirring. White product precipitated out. It was isolated and purified by two reprecipitations from dimethyl formamide into water. Yield 80 percent.

Melting point 236°C.

IR (nujol)

3384 cm^{-1} (-OH stretching), 3316 cm^{-1} (-NH stretching), 1750 cm^{-1} (ester carbonyl), 1641 cm^{-1} (amide carbonyl), 1522 cm^{-1} (aromatic -CH bending).

^{1}H NMR (DMSO d_6)

0.7 3H triplet (-CH_3 at C_{12} of dodecanoyl group), 1.2 16H broad singlet (C_3 to C_{10} methylene groups of dodecanoyl chain), 1.4 2H multiplet (-CH_2 at C_{11} of dodecanoyl group), 2.1 2H triplet (-CH_2 at C_2 of dodecanoyl group), 3.5 2H doublet (-CH_2-Ph of Tyrosine), 4.3 1H triplet (-CH-COOR of Tyrosine), 6.6 2H double doublet (Tyrosine ring protons), 7.0 2H double doublet (Tyrosine ring protons), 7.25 4H multiplet (p-nitrophenyl ring protons), 7.8 1H doublet (-NH proton), 9.1 singlet (-OH proton).

5.2.16 Synthesis of N-benzoyl-tyrosyl- para nitrophenyl ester (N-benzoyl-Tyr-PNP)

5.70 g N-benzoyl tyrosine (0.02 M) and 2.78 g p-nitrophenol (0.02 M) were dissolved in 100 ml ethyl acetate. The solution was stirred at room temperature. To this, 4.12 g DCC (0.02 M) dissolved in 50 ml ethyl acetate was added in a single portion. The reaction mixture was stirred at room temperature for 12 hrs. Then it was filtered to remove DCU. Filtrate was concentrated to yield crude product which was purified on silica gel column using 30 / 70 acetone / petroleum ether as mobile phase. Yield 70 percent.

Melting point 230°C.

IR (nujol)

3561 cm^{-1} (-OH stretching), 3399 cm^{-1} (-NH stretching), 1734 cm^{-1} (ester carbonyl), 1684 cm^{-1} (amide carbonyl), 1560 cm^{-1} (aromatic -CH bending).

^{1}H NMR (acetone d_6)

3.4 2H doublet (-CH_2-Ph of Tyrosine), 5.0 1H triplet (-CH-COOR of Tyrosine), 6.8 2H double doublet (Tyrosine ring protons), 7.25 2H double doublet (Tyrosine ring protons), 7.4 2H double doublet (Benzoyl ring protons), 7.5 3H multiplet (Benzoyl ring protons), 7.9 2H double doublet (p-nitrophenyl ring protons), 8.3 2H double doublet (p-nitrophenyl ring protons).

Synthesis of supports

5.2.17 Synthesis of Phenyl methacrylate

9.5 g phenol (0.1 M) and 17 ml triethylamine (0.12 M) were dissolved in 100 ml benzene and stirred at 0°C. To this solution, 9.75 ml methacryloyl chloride (0.1 M) dissolved in 10 ml benzene was added dropwise over 1.5 hours at 0°C. Then the reaction mixture was stirred at room temperature for 12 hours. It was then filtered to remove salts. Benzene solution was washed with 2 * 100 ml 3 percent NaOH solution to remove unreacted phenol and acid chloride. Then it was washed with water and dried over sodium sulfate. Benzene was evaporated in vacuo to give crude product. It was purified on silica gel column using 10 / 90 ethyl acetate / petroleum ether as mobile phase. Yield 50 percent.

IR (neat)

1750 cm^{-1} (ester carbonyl), 1620 cm^{-1} (methacryl double bond), 1500 cm^{-1} (aromatic -CH bending).

^1H NMR ($CDCl_3$)

2.0 3H singlet (-C = C-CH_3), 5.7 1H singlet (-CH_a = C-), 6.2 1H singlet (-CH_b = C-), 7 - 7.2 4H multiplet (aromatic ring protons).

5.2.18 Synthesis of hydrolyzed poly (glycidyl methacrylate - co - ethylene glycol dimethacrylate) (hydrolyzed poly (GMA-EGDMA))

This was synthesized according to the following modification in the reported procedure (Svec et al 1975). In a three neck round bottom flask of 500 ml capacity, 180 ml water and 0.6 g poly (vinyl pyrrolidone) (MW 60000) were placed. This solution was stirred with an overhead constant speed stirrer at 700 rpm. Contents of the flask were heated to 75°C and nitrogen gas was purged inside for half an hour to expel out air and dissolved oxygen. Then purging was stopped and organic phase containing 4.8 g GMA, 3.2 g EGDMA, 10 ml cyclohexanol and 80 mg azobis isobutyronitrile (AIBN) was added dropwise to aqueous phase at 75°C stirring at 700 rpm. This addition was completed in five minutes. Then polymerization was allowed to proceed 4 hrs. After this, contents of the flask were poured in beaker and beads formed were allowed to settle. The supernatant solution was decanted off and beads were washed with methanol to remove any unreacted monomers. Beads were then successively washed with methanol and water and dried.

Hydrolysis of epoxide ring was done according to the following modification in reported procedure (Smigol et al 1993). 5 g beads were suspended in 50 ml 0.1 M H_2SO_4 and stirred at 60°C for 10 hrs to ensure complete hydrolysis of epoxide ring (as monitored by absence of peak at around 950 - 990 cm^{-1} in the IR spectrum of beads). After this beads were filtered off, washed with water and dried. Dry beads were sieved from standard test sieves and only the beads within 45 to 75 microns range were selected for grafting of mimic.

5.2.19 Synthesis of poly (Phenyl methacrylate - co- ethylene glycol dimethacrylate) poly (PHMA-EGDMA)

An organic phase containing 4.8 g phenyl methacrylate, 3.2 g EGDMA, 10 ml cyclohexanol and 100 mg AIBN was added dropwise to the aqueous phase comprising 180 ml water, 0.6 g poly (vinyl pyrrolidone) stirring at 700 rpm at 80°C. Polymerization was carried out for four hours in similar manner as described above. Worked up beads were sieved and beads in the range of 75 to 150 microns were selected for grafting.

5.2.20 Preparation of Co (II) coordinated monomers-template assemblies

Co(II) coordinated monomers-template assemblies of MA-serine, MA-aspartic acid, MA-histidine with 4 different templates were prepared by dissolving monomers, template and $CoCl_2.6H_2O$ in methanol. The methanol solution was stirred under Nitrogen for 1 hour and methanol was evaporated in vacuo in presence of support beads to sorb the assemblies on the support . Data for the assemblies prepared are listed in Tables 5.1 and 5.2.

Synthesis of mimics

5.2.21 Synthesis of mimics P-A to P-D (Hydrophilic support)

Four Co(II) coordinated assemblies were prepared for four different substrates as above and sorbed on hydrolyzed poly(GMA-EGDMA) beads alongwith EGDMA and AIBN. The assemblies were polymerized at 75°C for 24 hours and mimics were worked up as reported in chapter 3. Data for feed composition and loading on the mimics **P-A** to **P-D** are listed in Table 5.1.

5.2.22 Synthesis of mimics P-E to P-H (Hydrophobic support)

Four Co(II) coordinated assemblies were prepared for four different substrates and sorbed on poly (PHMA-EGDMA) beads alongwith EGDMA and AIBN. The assemblies were polymerized at 75°C for 24 hours and mimics were worked up as reported in chapter 3. Data for feed composition and loading on mimics **P-E** to **P-H** are listed in Table 5.2.

5.2.23 Estimation of total amino acids and percentage grafting in mimics

The mimics were refluxed in HCl for 24 hours to hydrolyze grafted amino acids from methacryl backbone. Quantitative Ninhydrin tests of hydrolysates so obtained were carried out and the amount of total amino acids i.e. the amount of total functional groups in hydrolysates were estimated using the standard calibration plot of absorbance vs. concentration for Ninhydrin test of serine, aspartic acid and histidine composition in 1 : 1: 1 ratio. Overall percentage grafting was calculated from the amount of functional groups in feeds and those in the mimics.

5.2.24 Evaluation of hydrolytic activity of mimics

Activity of mimics **P-A** and **P-C** was evaluated for hydrolysis of N-acetyl-Tyr-PNP and N-benzoyl-Tyr-PNP respectively, in the framework of Michaelis - Menten kinetics as reported in chapters 3 and 4. Hydrolytic activity of mimics **P-B** and **P-D** was evaluated against N-butyryl-Tyr-PNP and N-dodecanoyl Tyr-PNP respectively.

Similarly activity of mimics **P-E** and **P-G** was evaluated for hydrolysis of N-acetyl-Tyr-PNP and N-benzoyl-Tyr-PNP respectively, in the framework of Michaelis - Menten kinetics and hydrolytic activity of **P-F** and **P-H** was evaluated against N-butyryl-Tyr-PNP and N-dodecanoyl Tyr-PNP respectively.

5.3.0 Results and Discussion

In recent years interest in the investigations of microenvironmetal effects in polymer supports has been growing since polymer supported reagents, catalysts and stationary phases are finding ever increasing applications. The effect of nature of the groups that surround the functional groups in supported catalysts are termed as microenvironmetal effects by Morawetz (1979). These effects significantly influence substrate binding. In the following sections we show that the effects of substituent in the substrate on the hydrolytic activity of the mimic are similar to those in case of chymotrypsin. Further, for a given substrate, the hydrolytic activity of a mimic can be enhanced by selecting a hydrophobic support.

5.3.1 Substrate binding and reactivity in natural chymotrypsin

Hydrolytic activity of natural chymotrypsin is governed by the structure of the substrate and its effective binding onto the active site. Berezin et al (1971) have reported effect of substrate structure on the hydrolytic activity of chymotrypsin. N-acyl tyrosine esters represented by the general formula **R1CO. NHCH(R2)COO.R3** were used as substrates, wherein, R1 is N-acyl substituent, R2 is tyrosine ring and R3 is the substitution on the ester group (Figure 5.1). The active site of chymotrypsin has three binding loci p1, p2 and p3 complementary to R1, R2 and R3 groups of the substrate respectively. Binding of the substrate with the active site such that R1 binds to p1, R2 binds to p3 and R3 binds to p3, results in "productive binding" and the hydrolysis of the substrate takes place efficiently. Hydrophobicity of the locus p2 is higher than the hydrophobicity of the locus p1. As a result, with increase in the hydrophobicity of R1, its binding with p2 increases. Such R1 to p2 binding results in "nonproductive binding". It was observed that with increase in the hydrophobicity of N- acyl substituent R1, nonproductive (wrong way) binding of substrate with the active site increased which resulted in low k_{cat} value as well as low K_m value. (**"Better the binding , poorer the reaction"**). When R1 is hydrophilic, productive binding takes place which results in high k_{cat} , but K_m is also high as reported by Berezin et al (1971) for native chymotrypsin. Thus, for a given substrate, chymotrypsin offers a specific binding pattern.

5.3.2 Substrate binding and reactivity in polymeric mimics

In case of chymotrypsin, binding patterns are governed by the hydrophobicities at p1, p2 and p3. In the case of polymeric mimics of chymotrypsin, there are no well defined

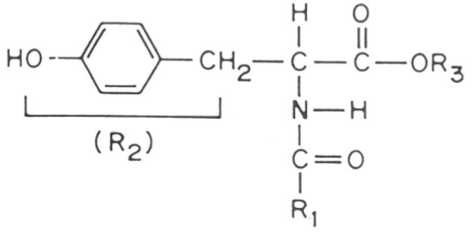


FIG. 5·1 : GENERAL STRUCTURE OF A STANDARD SUBSTRATE.

subsites which will preferentially bind the groups R1, R2 and R3 on the substrate. However, the overall microenvironment around the functional groups constituting the mimic can be varied by varying the composition of the microporous support on which the mimic is grafted. Substrate binding can also be varied by changing the substituents R1, R2 and R3 on the substrate. Especially, hydrophobicity of R1 affects the reactivity in two ways, firstly it governs the binding as described above, secondly it also causes increased steric hindrance for the approach of serine hydroxyl group towards the ester group in substrate (Berezin et al 1971).

With this background, objectives of the present work were set as follows, **1.** To study the effect of R1 substituent in substrate structure on reactivity of mimics. **2.** To demonstrate that for appropriate support composition of the mimic, trends in k_{cat} and K_m for productive and nonproductive binding are similar to those reported for chymotrypsin. **3.** To explore the possibility of overcoming unproductive binding for substrate with hydrophobic R1, by the choice of proper support composition.

5.3.3 Choice of substrate and templates

For the present study on substrate structure and reactivity, we have chosen N-acyl tyrosyl para nitro phenyl esters as substrates. Four substrates were selected and synthesized viz. N-acetyl tyrosyl para nitro phenyl ester (N-acetyl-Tyr-PNP), N-butyryl tyrosyl para nitro phenyl ester (N-butyryl-Tyr-PNP), N-benzoyl tyrosyl para nitro phenyl ester (N-benzoyl-Tyr-PNP), N-dodecanoyl tyrosyl para nitro phenyl ester (N-dodecanoyl-

Tyr-PNP). Thus, hydrophobicity of N- acyl substituent increased from N-acetyl to N-dodecanoyl keeping R2 and R3 same. Structures of substrates are shown in Figure 5.2.

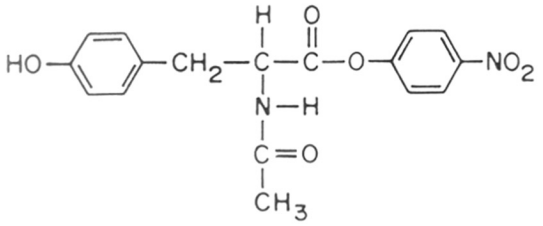
In order to imprint substrate specificity in polymers, template molecule which are structurally similar to substrate are necessary. The templates should also have ability to form complex with Co(II). 2 amino pyridyl or N-nicotinoyl derivatives of N-acyl tyrosines were synthesized as templates. The four templates synthesized were N-acetyl tyrosyl 2 amino pyridinamide (N-acetyl-Tyr-2AP), N-bytyryl tyrosyl 2 amino pyridinamide (N-butyryl-Tyr-2AP), N-nicotinoyl tyrosyl benzyl ester (N-nicotinoyl-Tyr-Benz). Nitrogen from pyridine rings of these templates form complex with Co(II). Structures of templates are shown in Figure 5.3.

5.3.4 Choice of functional monomers

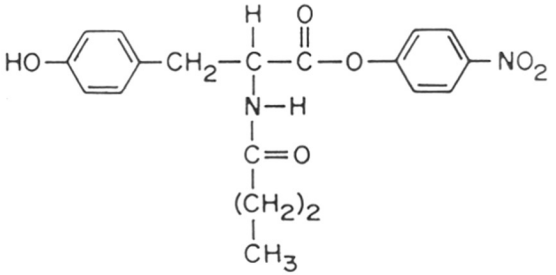
In chapter 4 we described various functional monomers that can be incorporated in mimics. In the present case we particularly selected functional monomers MA-serine, MA-aspartic acid and MA-histidine. These monomers contain amino acids present in native chymotrypsin and we wanted to compare binding patterns of mimics with those of chymotrypsin.

5.3.5 Choice of polymer supports

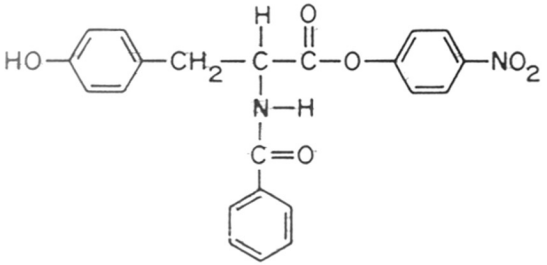
It is difficult to induce and quantify well defined hydrophobic sites in polymer support as in the case of chymotrypsin. Therefore two polymeric supports, one hydrophilic and the other hydrophobic, were selected. In chapter 4 we have already shown the effect of surface area of support on the substrate binding i.e. on K_m values. Thus, in



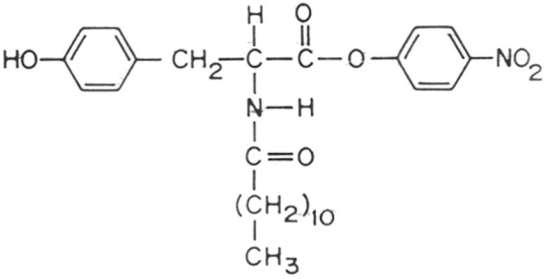
N-acetyl-Tyr-PNP



N-butyryl-Tyr-PNP

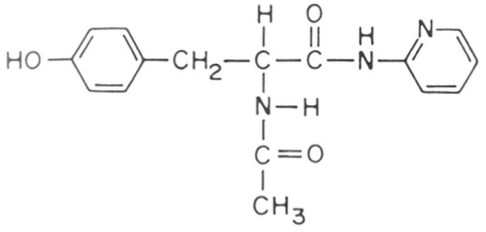


N-benzoyl-Tyr-PNP

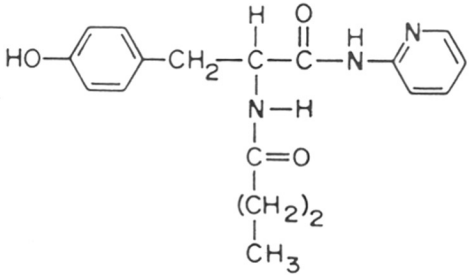


N-dodecanoyl-Tyr-PNP

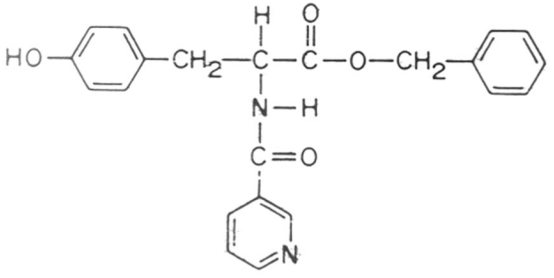
FIG. 5·2 : STRUCTURES OF SUBSTRATES



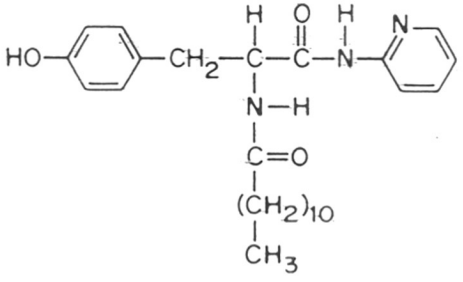
N-acetyl-Tyr-2AP



N-butyryl-Tyr-2AP



N-nicotinoyl-Tyr-Benz



N-dodecanoyl-Tyr-2AP

FIG. 5.3 : STRUCTURES OF TEMPLATES.

order to unequivocally establish the effect of support composition on K_m values, supports with minimal surface area need to be used in the present case. Additionally, supports also need to contain higher percentage of hydrophilic or hydrophobic comonomer than that of the crosslinker in order to achieve desired hydrophilicity or hydrophobicity in the support.

Chemical modification of poly (GMA-EGDMA) supports and its effect on chromatographic elution of proteins has been reported by Smigol et al (1993). It was found that acidic hydrolysis of epoxide ring of GMA in the support to vicinal diols renders the support hydrophilic. Further, acetalization of vicinal diols with benzaldehyde converts the support from hydrophilic one to hydrophobic one. It was shown that hydrophilic support so synthesized exhibited lower retention time for proteins as compared to hydrophobic supports. In the present case, we selected hydrolyzed poly (GMA-EGDMA) support for hydrophilic support composition. For hydrophobic support composition,rather than acetalization of diols, we opted to work with poly (phenyl methacrylate - co- ethylene glycol dimethacrylate) (poly (PHMA-EGDMA)). Phenyl methacrylate is known to enhance hydrophobicity of supports (Narasimhaswamy et al 1991).

Synthesis of supports

5.3.6 Hydrophilic support (hydrolyzed poly (GMA-EGDMA))

A composition containing 60 % GMA and 40 % EGDMA was polymerized according to the procedure described in experimental section. Polymerization time was

reduced to 4 hours from 8 to 10 hours reported in the literature so that complete crosslinking does not take place (Svec et al 1975). This ensured presence of unpolymerized methacryl double bonds for grafting Co(II) coordinated monomeric assembly, as well as low surface area due to lack of complete crosslinking. Epoxide ring of GMA in the support so synthesized was hydrolyzed in acidic medium to induce hydrophilicity. Surface area of this blank support was 28.86 m^2 /g.

5.3.7 Hydrophobic support (poly (PHMA-EGDMA))

A composition of 60 % phenyl methacrylate and 40 % EGDMA was polymerized according to procedure described in experimental section . In this case too, polymerization time was restricted to 4 hours for the reasons described above. Surface area of this blank support was 14.64 m^2/g.

5.3.8 Mimics on hydrophilic support (P-A to P-D)

Mimics were synthesized by adsorbing Co(II) coordinated assemblies on hydrolyzed poly (GMA-EGDMA) beads alongwith EGDMA and AIBN. The assemblies were polymerized at 75°C for 24 hours and the mimics were worked up according to procedure described in chapter 3. Estimation of functional groups per unit weight of the support and overall percentage grafting was carried out by acid hydrolysis of polymers and quantitative Ninhydrin test on the hydrolysates recovered as reported in chapter 3. Data for the feed compositions of assemblies and for the mimics **P-A** to **P-D** are summarized Table 5.1. Polymerization conditions and the ratios of crosslinker, initiator, and wt. ratio of support to monomer remaining the same, percentage grafting increased

as the hydrophobicity of R1 substituent in template increased from **P-A** to **P-D** (Table 5.1). This indicates that as the hydrophobicity of template increased, hydrophobic character of the Co (II) coordinated monomeric assemblies increased which resulted in efficient grafting of the assemblies onto the support. This behavior could be attributed to increased hydrophobic interactions between the support and the assemblies sorbed onto it. These results are in agreement with the trends observed in chapter 4 in which for the same template N-nicotinoyl tyrosyl benzyl ester in Co(II) coordinated assembly, percentage grafting level increased with increase in the hydrophobicity of functional monomers. e.g. grafting levels for the assemblies containing methacrylic acid, 4 vinyl phenol were higher than for those containing MA-serine, MA-histidine, MA-aspartic acid.

5.3.9 Mimics on hydrophobic support (P-E to P-H)

Mimics were synthesized by adsorbing Co(II) coordinated assemblies on poly (PHMA-EGDMA) beads alongwith EGDMA and AIBN and polymerizing at 75°C for 24 hours. The mimics were worked up and functional groups estimated as described earlier. Data for feed compositions of assemblies and for mimics listed in Table 5.2 illustrate that for the mimics **P-E** to **P-G** also, increase in the hydrophobicity of R1 substituent in the template leads to higher levels of grafting. But **P-H** exhibited lower grafting level. The reason for this behavior is not clear.

In chapter 4 we reported the effect of surface area of the support on K_m. It was shown that for a series of mimics comprising MA-serine, MA-aspartic acid and MA-

Table 5.1

Data for feed of monomers and grafting levels in mimics P-A to P-D.

No.	1	2	3	4	5
Polymer	Blank support	**P-A**	**P-B**	**P-C**	**P-D**
MA-serine (M)	-	0.00125	0.00125	0.00125	0.00125
MA-aspartic acid (M)	-	0.00125	0.00125	0.00125	0.00125
MA-histidine (M)	-	0.00125	0.00125	0.00125	0.00125
Template (M)	-	N-acetyl-Tyr-2AP, (0.00125)	N-butyryl-Tyr-2AP, (0.00125)	N-nicotinoyl-Tyr-Benz, (0.00125)	N-dodecanoyl-Tyr-2AP, (0.00125)
$CoCl_2.6H_2O$	-	0.00125	0.00125	0.00125	0.00125
Hydrolyzed poly(GMA-EGDMA)(g)	0.825	0.825	0.825	0.825	0.825
Functional gr. per 5o mg (M)	-	$6.04 * 10^{-7}$	$1.25 * 10^{-6}$	$1.74 * 10^{-6}$	$2.58 * 10^{-6}$
Percentage grafting	-	1.59	3.18	4.58	6.79
Surface area (m^2/g)	28.86	11.17	nd	0.03	nd
Pore volume (cm^3/g)	0.0575	0.0221	nd	0.0064	nd

Foot note to Table 5.1

* nd = not determined. * In all mimics **P-A** to **P-D** ratio of wt. of hydrolyzed poly (GMA-EGDMA) beads to wt. of monomers is 1 :1. * Feed of all assemblies **P-A** to **P-D** contain EGDMA (10 % of the wt. of monomers) and AIBN (3% of the wt. of monomers). *All mimics are synthesized as per procedure reported in chapter 3. Estimation of functional groups in mimics is done by Ninhydrin test on the hydrolysates and percentage grafting is calculated from the estimated amount of functional groups in mimics and that in the feed.

Table 5.2

Data for feed of monomers and grafting levels in mimics P-E to P-H

No.	1	2	3	4	5
Polymer	Blank support	**P-E**	**P-F**	**P-G**	**P-H**
MA-serine (M)	-	0.00125	0.00125	0.00125	0.00125
MA-aspartic acid (M)	-	0.00125	0.00125	0.00125	0.00125
MA-histidine (M)	-	0.00125	0.00125	0.00125	0.00125
Template (M)	-	N-acetyl-Tyr-2AP, (0.00125)	N-butyryl-Tyr-2AP, (0.00125)	N-nicotinoyl-Tyr-Benz, (0.00125)	N-dodecanoyl-Tyr-2AP, (0.00125)
$CoCl_2.6H_2O$ (M)	-	0.00125	0.00125	0.00125	0.00125
poly (PHMA-EGDMA) (g)	0.825	0.825	0.825	0.825	0.825
Functional grs per 50 mg (M)	-	$1.55 * 10^{-7}$	$1.67 * 10^{-6}$	$5.19 * 10^{-6}$	$3.77 * 10^{-7}$
Percentage grafting	-	0.4	1.36	4.39	0.99
Surface area (m^2/g)	14.64	0.13	nd	4.75	nd
Pore volume (cm^3/g)	0.0381	0.0241	nd	0.0265	nd

Foot note for Table 5.2

* nd = not determined. * In all mimics **P-E** to **P-H** ratio of wt. of hydrolyzed poly (GMA-EGDMA) beads to wt. of monomers is 1 :1. * Feed of all assemblies **P-E** to **P-H** contain EGDMA (10 % of the wt. of monomers) and AIBN (3% of the wt. of monomers). *All mimics are synthesized as per procedure reported in chapter 3. Estimation of functional groups in mimics is done by Ninhydrin test on the hydrolysates and percentage grafting is calculated from the estimated amount of functional groups in mimics and that in feed .

histidine, as the surface area decreased from 22.75 m^2/g to 14.07 m^2/g, K_m value increased from 1.35 * 10^{-4} M to 1.42 * 10^{-3} M [1]. Thus in order to minimize the effect of surface area in improving substrate binding and to highlight only the effects of support compositions on K_m, it was necessary to work with mimics having low surface areas. It can be seen from Tables 5.1 and 5.2 that the surface areas of mimics are very low. At these low values of surface area we expect K_m values to reflect only the effects of hydrophilicity or hydrophobicity of support.

5.3.10 Evaluation of hydrolytic activity of mimics

Hydrolytic activity of molecularly imprinted mimics against the respective substrates was evaluated in the framework of Michaelis - Menten kinetics. Typical Lineweaver - Burk plots are shown in Figures 5.4, 5.5 and 5.6, 5.7. Data for kinetic constants are summarized in Tables 5.3 and 5.4.

5.3.11 Effect of N-acyl substituent on reactivity

As described earlier, with increase in the hydrophobicity of N-acyl substituent, its steric hindrance for the approach of attacking nucleophile towards ester group increases leading to decreased reactivity (Berezin et al 1971). In accordance with this trend, we observed decrease in k_{cat} for the mimics on both hydrophilic and hydrophobic supports with increase in the hydrophobicity of N-acyl substituent (Tables 5.3 and 5.4). Moreover, N-butyryl and N-dodecanoyl substituents offered such a high steric hindrance that the mimics were not able to hydrolyze substrates N-butyryl-Tyr-PNP and N-dodecanoyl-Tyr-PNP respectively. Thus the effect of N-acyl substituent R1 in the substrate structure, on

the reactivity of mimics is similar to that in enzyme chymotrypsin. In fact the steric hindrance exhibited by dodecanoyl group in substrate N-dodecanoyl-Tyr-PNP was so high that it was not hydrolyzed even by strong alkali such as NaOH or KOH. Kinetics for substrates N-acetyl-Tyr-PNP and N-benzoyl-Tyr-PNP are discussed in following sections.

5.3.12 Hydrolytic activity of mimics on hydrophilic support

Mimic **P-A** is imprinted for N-acetyl -Tyr-PNP and mimic **P-C** is imprinted for N-benzoyl-Tyr-PNP. Thus hydrolytic activity of **P-A** is evaluated against N-acetyl-Tyr-PNP and hydrolytic activity of **P-C** is evaluated against N-benzoyl-Tyr-PNP. Typical Lineweaver - Burk plots are shown in Figures 5.3 and 5.4. Kinetic data are listed in Table 5.3.

The hydrophilic support chosen was hydrolyzed poly (GMA-EGDMA). k_{cat} for **P-A** (substrate N-acetyl-Tyr-PNP) is 0.45 sec^{-1} and k_{cat} for **P-C** (substrate N-benzoyl-Tyr-PNP) is 0.11 sec^{-1} respectively. Thus a four fold decrease in k_{cat} is observed as the hydrophobicity of N-acyl substituent R1, in the substrate structure. This is due to increased steric hindrance caused by N-benzoyl group than that of N-acetyl group for the approach of serine hydroxyl in mimics towards the ester group in the substrate. K_m value for **P-A** and **P-C** are $3.33 * 10^{-4}$ M and $1.14 * 10^{-4}$ M respectively (Table 5.3).

Thus improved substrate binding is exhibited by hydrophilic binding sites of hydrolyzed poly (GMA-EGDMA) in mimic **P-C** for substrate N-benzoyl-Tyr-PNP. It is to be noted here that the mimics **P-A** to **P-D** are synthesized using the support material from same batch in order to avoid complications arising from differences in the surface

area, pore volume and morphology of the support. Surface area of **P-A** (11.17 m^2/g) is more than that of **P-C** (0.03 m^2/g) due to the differences in the grafting levels of their assemblies. In chapter 4 we have seen that with decrease in the surface area of the support, K_m values for mimics increase . Thus based on surface area considerations alone, K_m value for **P-A** should have been lower (strong binding) than that for **P-C**. However K_m value exhibited by **P-C** for N-benzoyl-Tyr-PNP is almost one third than that exhibited by **P-A** for N-acetyl-Tyr-PNP, indicating improved binding.

Thus with increase in hydrophobicity of R1 substituent, the binding of substrate with mimic has increased while its catalytic activity has decreased (low k_{cat} as well as low K_m). This trend is the same as **"better the binding poorer the reaction"** observed in case of chymotrypsin for hydrophobic R1 substituent in the substrate structure (Berezin et al 1971). In other words, mimic **P-C** grafted on hydrophilic support has exhibited nonproductive binding with increase in hydrophobicity of R1 substituent. We believe that the site for nonproductive binding could be methyl group -C-CH_3 next to hydrophilic vicinal diols, since methyl groups are known to contribute to hydrophobic interactions in monomers such as methacrylic acid and GMA. Schematic representation of these binding sites is shown in Figure 5.8.

For the substrate with hydrophilic R1 substituent i.e. N-acetyl-Tyr-PNP mimic **P-A** exhibited high k_{cat} as well as high K_m values as in the case of chymotrypsin. Thus the trends in variation of k_{cat} and K_m for chymotrypsin mimic grafted on hydrophilic support are similar to those observed for native chymotrypsin (Berezin et al 1971).

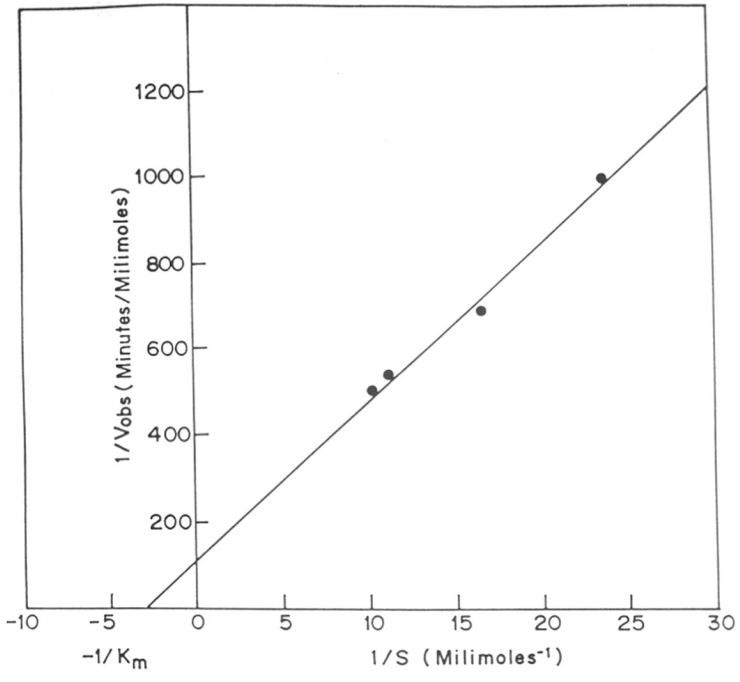


FIG.5·4 : LINEWEAVER-BURK PLOT FOR HYDROLYSIS OF N-ACETYL-Tyr-PNP CATALYZED BY P-A (HYDROPHILIC SUPPORT) AT 37°C AND pH 7,8 THE ASSAY EMPLOYED 25 mg P-A WHICH CONTAINED 0·000302 MILIMOLES OF ACTIVE GROUPS. SUBSTRATE CONCENTRATION WAS VARIED BETWEEN 0·0322 MILIMOLES TO 0·0959 MILIMOLES.

Kcat = 0·45 sec^{-1} Km = $3{\cdot}33 \times 10^{-4}$ M Kr = 1350 sec^{-1} M^{-1}

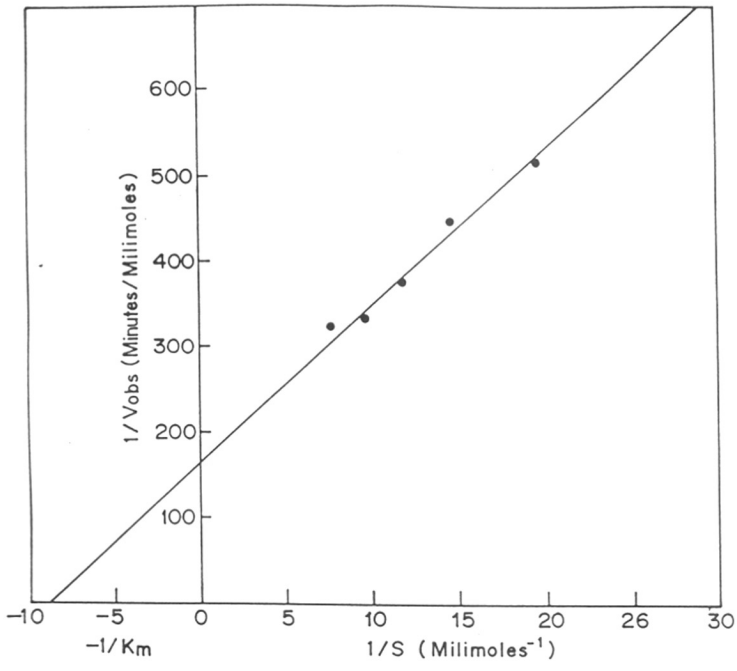


FIG. 5·5 : LINEWEAVER-BURK PLOT FOR HYDROLYSIS OF N-BENZOYL-TYR-PNP CATALYZED BY P-C (HYDROPHILIC SUPPORT) AT 37°C AND pH 7·8. THE ASSAY EMPLOYED 25 mg P-C WHICH CONTAINED 0·00087 MILIMOLES OF ACTIVE GROUPS. SUBSTRATE CONCENTRATION WAS VARIED BETWEEN 0·0522 MILIMOLES TO 0·1305 MILIMOLES.

Kcat = 0·11 Sec^{-1} Km = $1·14 \times 10^{-4}$ M Kr = 1018 Sec^{-1} M^{-1}

Table 5.3

Kinetic constants for P-A to P-D (mimics on hydrophilic support)

Entry	Polymer	Substrate	k_{cat} ,sec^{-1}	K_m , M	k_{cat} / K_m,$sec^{-1} M^{-1}$
1	**P-A**	N-acetyl-Tyr-PNP	0.45	$3.33 * 10^{-4}$	1350
2	**P-C**	N-benzoyl-Tyr-PNP	0.11	$1.14 * 10^{-4}$	1018
3	**P-B**	N-butyryl-Tyr-PNP	no activity	-	-
4	**P-D**	N-dodecanoyl-Tyr-PNP	no activity	-	-

5.3.13 Hydrolytic activity of mimics on hydrophobic support

Polymer **P-E** is imprinted for N-acetyl-Tyr-PNP and **P-G** is imprinted for N-benzoyl-tyr-PNP. Thus hydrolytic activity of **P-E** is evaluated against N-acetyl-Tyr-PNP and hydrolytic activity of **P-G** is evaluated against N-benzoyl-Tyr-PNP. Typical Lineweaver - Burk plots are shown Figures 5.6 and 5.7. Kinetic data are listed in Table 5.4.

The choice of the hydrophobic support was made in order to provide enhanced substrate binding and also different environment for binding sites than that of chymotrypsin. k_{cat} for **P-E** (substrate, N-acetyl-Tyr-PNP) is 0.33 sec^{-1} while that for **P-G** (substrate, N-benzoyl-Tyr-PNP) is 0.26 sec^{-1}. Thus in the case of mimics grafted on hydrophobic support too, the effect of steric hindrance of hydrophobic R1 substituent is observed as discussed above. K_m value for **P-E** and **P-G** are $1.0 * 10^{-4}$ M and $2.0 * 10^{-4}$ M respectively (Table 5.4). Thus the choice of hydrophobic support has resulted in improved binding (low K_m value) for N-acetyl-Tyr-PNP in comparison to that of N-benzoyl-Tyr-PNP. This improved binding is also accompanied by higher reactivity, as higher k_{cat} value for mimic **P-E** (0.33 sec^{-1}) than that for mimic **P-G** (0.26 sec^{-1}) is observed. Thus in the case of hydrophobic support the trend of reactivity is **"better the binding, better the reaction",** which is exactly opposite to that observed in case of hydrophilic support. In other words, fo substrate N-benzoyl-Tyr-PNP wherein R1 substituent is hydrophobic, improved but nonproductive binding (low K_m value) as in the case of mimic **P-C** (hydrophilic support) is not observed. While the effects of substrate structure on the

activity of mimic on a hydrophilic support are same as in native chymotrypsin, the same mimic on a hydrophobic support shows enhanced k_{cat} and lower K_m .

Binding sites in support poly (PHMA-EGDMA) consist of phenyl methacrylate moieties which contain both hydrophobic methyl and phenyl groups whereas, the binding sites in support hydrolyzed poly (GMA-EGDMA) consist of hydrophilic vicinal diol groups as well as hydrophobic methyl groups (Figure 5.8). Since the support poly (PHMA-EGDMA) does not contain binding sites differing in hydrophobicity, it does not exhibit nonproductive binding for the substrate N-benzoyl Tyr-PNP. This latitude to manipulate the hydrophobicity of binding sites p1, p2 p3 is not available in case of natural chymotrypsin. Schematic representation of hydrophobic binding sites in poly (PHMA-EGDMA) is shown Figure 5.8.

The effect of hydrophobicity of the support is also reflected in the k_{cat} value for P-G for the substrate N-benzoyl-Tyr-PNP. Mimic **P-G** with hydrophobic binding sites exhibited k_{cat} 0.26 sec^{-1}, whereas mimic **P-C** with hydrophilic binding sites exhibited k_{cat} 0.11 sec^{-1} for the hydrolysis of substrate N-benzoyl-Tyr-PNP. This three fold increase in k_{cat} for **P-G** in comparison to that of **P-C** can be attributed to the enhanced hydrophobic interactions between the support and the substrate.

5.3.14 Enhanced activity due to the hydrophobic support

Data for the kinetic constants of both sets of mimics are summarized in Table 5.5. It is seen that k_{cat} / K_m values which are measures of efficacy of catalysts are higher for mimics **P-E** and **P-G** (3308 $sec^{-1} M^{-1}$, 1338 $sec^{-1} M^{-1}$ respectively) than those for **P-A** and

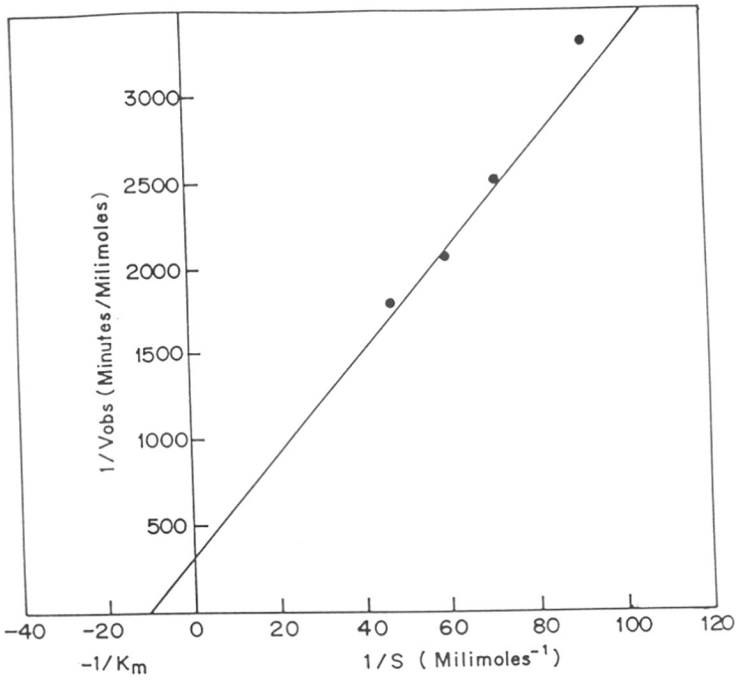


FIG. 5·6: LINEWEAVER-BURK PLOT FOR HYDROLYSIS OF N-ACETYL-Tyr-PNP CATALYZED BY P-E (HYDROPHOBIC SUPPORT) AT 37°C AND pH 7.8. THE ASSAY EMPLOYED 50 mg P-E WHICH CONTAIND 0·000155 MILIMOLES OF ACTIVE GROUPS. SUBSTRATE CONCENTRATION WAS VARIED BETWEEN 0·00775 MILIMOLES TO 0·0217 MILIMOLES.

Kcat = 0·33 Sec^{-1} Km = $1·0 \times 10^{-4}$ M Kr = 3308 Sec^{-1} M^{-1}

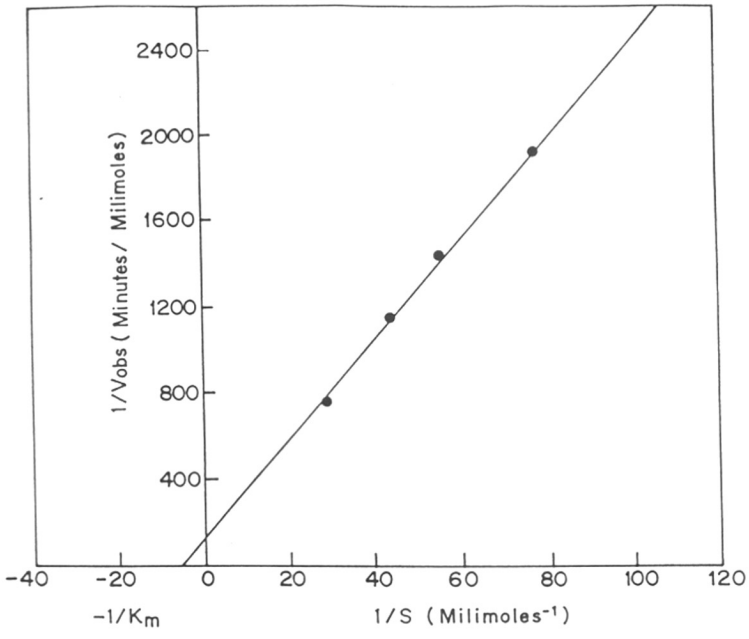


FIG. 5·7: LINEWEAVER-BURK PLOT FOR HYDROLYSIS OF N-BENZOYL-TYR-PNP CATALYZED BY P-G (HYDROPHOBIC SUPPORT) AT 37°C AND pH 7.8. THE ASSAY EMPLOYED 50 mg P-G WHICH CONTAINED 0·00519 MILIMOLES OF ACTIVE GROUPS. SUBSTRATE CONCENTRATION WAS VARIED BETWEEN 0·0129 MILIMOLES TO 0·03633 MILIMOLES.

K_{cat} = 0·26 Sec^{-1} Km = 2·0 x 10^{-4} M Kr = 1338 $Sec^{-1} M^{-1}$

Table 5.4

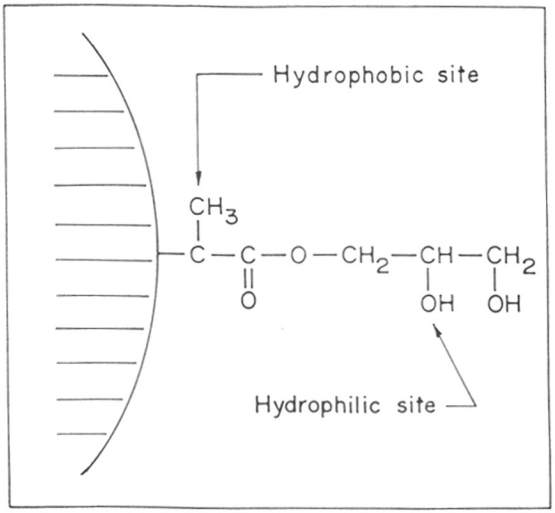
Kinetic constants for P-E to P-H (mimics on hydrophobic support)

Entry	Polymer	Substrate	k_{cat}, sec^{-1}	K_m, M	k_{cat} / K_m, $sec^{-1} M^{-1}$
1	**P-E**	N-acetyl-tyr-PNP	0.33	$1.0 * 10^{-4}$	3308
2	**P-G**	N-benzoyl-tyr-PNP	0.26	$2.0 * 10^{-4}$	1338
3	**P-F**	N-butyryl-Tyr-PNP	no activity	-	-
4	**P-H**	N-dodecanoyl-Tyr-PNP	no activity	-	-

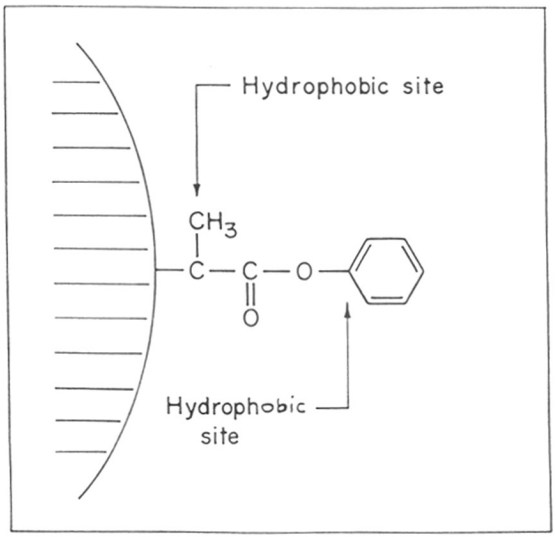
Table 5.5

Comparison of k_{cat} / K_m values of mimics on hydrophilic and hydrophobic supports.

Entry	Polymer	Support	Substrate	k_{cat} / K_m, sec^{-1} M^{-1}
1	**P-A**	Hydrophilic	N-acetyl-Tyr-PNP	1350
2	**P-E**	Hydrophobic	N-acetyl-Tyr-PNP	3308
3	**P-C**	Hydrophilic	N-benzoyl-Tyr-PNP	1018
4	**P-G**	Hydrophobic	N-benzoyl-Tyr-PNP	1338



(a) Binding sites in hydrolyzed poly(GMA-EGDMA)



(b) Binding sites in poly(PHMA-EGDMA)

FIG. 5·8 : SCHEMATIC REPRESENTATION OF BINDING SITES IN HYDROPHILIC AND HYDROPHOBIC SUPPORTS.

P-C (1350 sec^{-1} M^{-1}, 1018 sec^{-1} M^{-1} respectively). Increase in the activity of the mimic grafted on hydrophobic support is due to the increased hydrophobic interactions between substrate and the support. Thus the choice of appropriate support provides additional means of enhancing the activity of the mimic towards a given substrate.

5.4.0 Conclusions

This work describes the effect of substrate structure and support composition on the catalytic activity of chymotrypsin mimics. For both mimic and chymotrypsin, hydrophobicity of substituent in R1 position increases the steric hindrance for the approach of serine nucleophile towards esters group of substrate and decreases the reactivity. Nonproductive binding (low k_{cat} as well as low K_m) was exhibited by the mimic grafted on the hydrophilic support for substrate containing hydrophobic N-acyl substituent. It has been shown that in the case of the mimic, the choice of hydrophobic support helps overcome the unproductive binding. The mimic grafted on hydrophobic support did not exhibit nonproductive binding for substrate with hydrophobic N-acyl substituent. The choice of hydrophobic support also resulted in increased activity of the mimic as compared to hydrophilic support. Thus, in addition to the choice of functional monomers and surface area of support, support composition offers an additional means of improving the hydrolytic activity of mimics towards standard substrates.

Chapter 6

Conclusions and suggestions for future work

6.1.0 Summary and Conclusions

Various methodologies for the design and synthesis of mimics of hydrolytic enzymes have emerged. Linear soluble polymers exhibiting bifunctional catalysis have been investigated as chymotrypsin mimics. Since these polymers lacked specific mechanism for multifunctional cooperative action and substrate recognition, they had limited success as enzyme mimics. Molecularly imprinted polymers have potentials to overcome the limitations mentioned above. In our own group, Karamalkar et al (1996). reported synthesis of chymotrypsin mimic by grafting Co (II) coordinated assembly of HEMA, MAA, MA-histidine and template was grafted onto poly (GMA - EGDMA) support. This mimic exhibited typical features of chymotrypsin such as turnover, TPCK inhibition and inactivation on acylation of HEMA hydroxyl group.

The objectives of the present investigation were a) to validate the methodology proposed earlier b) to elucidate various parameters that govern the activity of the mimic and c) to correlate the kinetic constants of the mimic with these parameters and evolve the strategies that would enhance the hydrolytic activity of the mimic.

The key conclusions arrived at from present investigations are as follows

- Complexation of hydroxyl, carboxyl and imidazole bearing monomers with Co (II) brings the three close to one another which leads to cooperative effect amongst them as in the case of native chymotrypsin. Incorporation of template in the Co (II) coordinated assembly during polymerization renders the mimic the ability to

recognize the substrate. The mimic synthesized in the absence of cobalt is hydrolytically inactive (Chapter 3)

- k_{cat} of mimics can be enhanced by incorporating increasingly nucleophilic monomers representing serine hydroxyl group as well as by efficient cooperative effect of carboxyl bearing monomer. e.g. the mimic comprising functional monomers HEMA, MAA and MA-histidine exhibited k_{cat} 0.06 sec^{-1}, but replacement of MAA by MA- β alanine in the same mimic resulted four fold enhancement in k_{cat} of the mimic (k_{cat} 0.25 sec^{-1}) (Chapter 4)

- K_m can be improved by using supports with high surface area (Chapter 4).

- k_{cat} of the mimic also depends on the structure of the substrate. As the hydrophobicity of N-acyl substituent in the N-acyl tyrosine esters increase, catalytic activity of the mimic decreased . e.g. k_{cat} for N-acetyl tyrosine para nitrophenyl ester was 0.45 sec^{-1} and 0.33 sec^{-1}, while for N-benzoyl tyrosine para nitro phenyl ester, k_{cat} was 0.11 sec^{-1} and 0.26 sec^{-1} , for mimics on hydrophilic and hydrophobic supports respectively (Chapter 5).

- Composition of the support on which the mimic is grafted plays an important role in substrate binding. Hydrophobic supports exhibit stronger substrate binding and reactivity than hydrophilic support (Chapter 5).

6.2.0 Suggestions for future work

1). All serine protease enzymes depend upon the serine hydroxyl group for their catalytic activity. But due to the secondary structures, they exhibit specificity for different substrates. Thus one particular enzyme cannot act on variety of substrates. Molecularly imprinted polymer mimics of chymotrypsin reported in this work have an advantage of tailoring substrate specificity by merely incorporating an appropriate template during polymerization. Detail study of catalytic activity of such mimics against various substrates is worthwhile as it would offer simpler alternative for site directed mutagenesis in enzymes.

2) Key to orient functional monomers close to one another is formation of coordination complex of monomers with the transition metal ion. Stability constants of these coordination complexes will depend upon the choice of the metal ion. Therefore it would be interesting to study the effect of metal ions on the strength of the complexes and correlate with the activity of resulting mimics. Investigations with metal ions such as Fe, Ni, Cu, Zn can be taken up in this regard.

3) Structural characterization of the metal ion coordinated assembly of monomers and template to reveal the distances of the functional group from one another and the metal ion will be desirable. Correlation of these distances with the activity would lead to strategies to enhance the mimic activity further.

4) Activity of chymotrypsin is known to be affected in organic solvents such as dimethyl formamide, dimethyl sufoxide etc. Polymer mimic of chymotrypsin synthesized in this

study is expected to be stable against the action of various solvents. Hence a study on the effect of solvent on the activity of the mimic and native chymotrypsin is desirable as it would demonstrate advantages of polymeric mimic over the native enzyme.

5) Chymotrypsin and most of the other serine proteases catalyze hydrolysis of L- amino acid esters and amides since the active sites of enzymes also contain L-amino acids. Enzymes which catalyze hydrolysis of D-amino acid esters are very few. Such enzymes are of practical use . e.g. D- phenyl glycine, an important amino acid for penicillins is isolated by reactive separation of ester of racemic mixture. N-methacryloyl derivatives of D- serine, D-aspartic acid and D-histidine can be incorporated in the mimics and reactive separation of phenyl glycine can be attempted. Thus one can induce specificity for D as well as L substrates in the mimics.

We also wish to suggest that the synthetic methodology used in the present work can be extended to develop highly efficient designer catalyst as follows-

6) Taking into account high hydrolytic activity of organometallic complexes, investigations combining molecular imprinting and surface grafting of such organometallic complexes are worthwhile. e.g. Cu (II) complex of 2,2' dipyridyl amine has been reported to exhibit hydrolysis of inactive ester methyl acetate at 25^0C and at 7.0 pH. Vinyl derivative of Cu (II) complex of 2,2' dipyridyl amine can be grafted on polymer support in the presence of template molecule in order to induce substrate specificity in it. Cu (II) complexes immobilized on polymer support in this way would led

to novel heterogeneous catalysts which have advantages of easy recovery and repeated use.

7) Metal ion coordination of functional monomers can also be used to develop highly active designer catalysts. e.g. monomers containing Lewis acid groups such as styrene sulfonic acid, 2, acryl amido, 2 methyl propane sulfonic acid (AMPS) can be oriented into "active sites" by metal ion coordination and grafted on the support. Catalysts so synthesized may offer advantages like reduction in reaction temperature and time over the commonly used catalysts in esterification reactions.

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CURRICULUM VITAE

Name : Bhalchandra Shripad Lele.

Address : National Chemical Laboratory,
Chemical Engineering Division,
Polymer Science and Engineering Unit,
Pune 41108, INDIA.

Permanent address : 1387, Shukrawar Peth,
Bajirao Road,
Pune 411002, INDIA.

Date of Birth : 30 th September 1968.

Educational Qualifications :

Examination /Degree	Year	University / Institution.	Percentage marks	Class	Subjects
S.S.C.	1984	S.S.C. Board, Pune	84	First with Distinction	Science, Mathematics, English, History, Geography.
H.S.C.	1986	H.S.C. Board, Pune	72	First with Distinction	Physics, chemistry, Biology, English, Geography.
B.Sc.	1989	University of Pune	68	First	Physics, Chemistry, Geography.
M.Sc.	1991	University of Pune	64	First	Organic Chemistry, Inorganic Chemistry, Physical Chemistry.
Ph.D.	1997	University of Pune		Thesis *	Enzyme mimics, Molecular imprinting.

* Thesis entitled "Investigations in origins of activity of molecularly imprinted polymer mimics of chymotrypsin" is submitted to the University of Pune.

Scholarships and Awards :

- National loan merit scholarship by Government of Maharashtra in 1984.
- Qualified for the award of Junior Research Fellowship in the National Eligibility Test (NET) held by CSIR-UGC in 1992.
- Junior Research Fellowship by CSIR 1993 - 1995.
- Senior Research Fellowship by CSIR 1995 - 1998.

Industrial Experience :

Worked as trainee chemist in Chemical Division of THERMAX LTD. from January 1992 to January 1993. The work involved,

- Synthesis and characterization of various water soluble polyelectrolytes such as poly (acrylic acid), poly (acrylamide), poly (dimethyl diallyl ammonium chloride). Evaluation of performance of these polymers as flocculating agents and dispersing agents in lab as well as field trials.
- Synthesis of biocidal polymers based on epichlorohydrin and dimethyl amine. Synthesis of various quaternary ammonium salts as biocides. Synthesis of monomers such as dimethyl diallyl ammonium chloride required for polyelectrolytes.
- Development of formulations of water treatment chemicals based on polyelectrolytes and various sequestering agents and evaluation of their performance such as scale inhibitor for strontium sulfate, calcium chloride etc.
- The job also involved miscellaneous work in oil field chemicals such as synthesis of acrylate and methacrylate esters of fatty alcohols.

Research Experience :

Joined National Chemical Laboratory in January 1993 as junior research fellow for Ph.D. under the guidance of Dr. R.A. Mashelkar.

- **Biodegradable polymers based on α amino acids.**
- The work in this area is particularly on L-Tyrosine based poly (iminocarbonates) which involved,

- Synthesis of various N and O protected tyrosine derivatives, dipeptides, diphenols and dicyanates. Purification of these compounds by column chromatography. Synthesis of poly (iminocarbonates) by polycondensation of diphenols and dicyanates. Preparation of melt pressed discs of these polymers with physically dispersed model dyes and the release studies of such devices.

Ph.D. Thesis work

Investigations in origins of activity of molecularly imprinted polymer mimics of chymotrypsin :

- This work was under taken in view of elucidating various factors that govern the origins of hydrolytic activity of surface grafted polymer mimics of enzyme chymotrypsin.

- In order to mimick catalytic triad of Serine, Aspartic acid and Histidine in chymotrypsin, various methacryl functional monomers bearing hydroxyl, carboxyl and imidazole groups were synthesized. The monomers were coordinated with Co(II) in order to orient them close to one another. An appropriate template molecule which resembles the structure of the substrate to be hydrolyzed was also coordinated with Co(II). The Co(II) complexes were characterized by ESR spectroscopy. The complexes were grafted on crosslinked support beads of poly (glycidyl methacrylate - co -ethylene glycol dimethacrylate) bearing residual double bonds. The polymers obtained after removal of cobalt, template and ungrafted monomers were found to be hydrolytically active against para nitro phenyl esters of N-acyl tyrosines.

- Various experiments were performed which demonstrated that the hydrolytic activity originates from cooperative action amongst hydroxyl, carboxyl and imidazole monomers present on the support beads. Imprinting effect was exhibited by the mimics with higher specificity for the imprinted substrate. These chymotrypsin mimics exhibited catalytic turnover under excess substrate conditions and saturation kinetics with initial burst, thus obeyed Michaelis Menten kinetics.

- Structure property relationship between functional monomers and hydrolytic activity was studied. The results of these studies showed that highly nucleophilic monomers with appropriate cooperative action leads to enhancement in the activity. It was found that Michaelis constant K_m of these polymers depend on the surface area of the support. With decrease in surface area, the value of K_m increased, indicating increased restrictions for the free access of substrate molecules towards the active groups inside the polymer matrix of support. Moreover, k_{cat} and K_m trends of mimics were found to be similar to those of immobilized chymotrypsin.

- Microenvironmental effects of support composition on the activity of mimics were studied as a function of substrate structure. Two support materials were synthesized for this study, one was hydrophilic and the other was hydrophobic. Para - nitro phenyl esters of tyrosine with increasing hydrophobicity of N- acyl substituent were synthesized. Two sets of mimics (with N-methacryloyl derivatives of serine, aspartic acid and histidine as functional monomers) were synthesized by grafting Co(II) complexes on both the supports. Hydrolytic activity of these mimics was evaluated as a function of substrate structure. It was found that k_{cat} of mimics decreased with increase in the hydrophobicity of N- acyl substituent. The mimic grafted on the hydrophilic support exhibited "nonproductive binding" for hydrophobic substrate, which is similar to native chymotrypsin. The mimic grafted on hydrophobic support did not exhibited nonproductive binding for hydrophobic substrate. It was also found that mimics on hydrophobic support exhibited higher hydrolytic activity than mimics on hydrophilic support.

- Thus various factors that govern the activity of surface grafted chymotrypsin mimics were elucidated and a framework was developed for further research in this area.

List of publications.

1) B.S.Lele, M.G.Kulkarni, R.A.Mashelkar.
Molecularly imprinted polymer mimics of chymotrypsin (I) : Cooperative effects and substrate specificity. Macromolecules (Communicated).

2) B.S.Lele, M.G.Kulkarni, R.A.Mashelkar.
Molecularly imprinted polymer mimics of chymotrypsin (II) : Functional monomers and hydrolytic activity. Macromolecules (Communicated).

3) B.S.Lele, M.G.Kulkarni, R.A.Mashelkar.
Surface grafted and molecularly imprinted polymer mimics of chymotrypsin (III) : Substrate binding and the support choice. Macromolecules (Communicated).