DOKUZ EYLÜL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

REVERSIBLE IMMOBILIZATION OF CATALASE BY METAL CHELATE AFFINITY INTERACTION ON A NEW GENERATION MATRIX

by

Tülden KALBURCU

January, 2010

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M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled completed **REVERSIBLE IMMOBILIZATION OF CATALASE BY METAL CHELATE AFFINITY INTERACTION ON A NEW GENERATION MATRIX** by **TÜLDEN KALBURCU** under supervision of **ASSOC. PROF. NALAN TÜZMEN** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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REVERSIBLE IMMOBILIZATION OF CATALASE BY METAL CHELATE AFFINITY INTERACTION ON A NEW GENERATION MATRIX

ABSTRACT

p(AAm-AGE) cryogel was prepared by radical polymerization of acylamide and allyl glycidyl ether. Cibacron Blue F3GA is covalently attached on p(AAm-AGE) cryogel, via the reaction between the chloride groups of the reactive dyes and the epoxide groups of the AGE**.** Cibacron Blue F3GA attached p(AAm-AGE) cryogel was chelated with Fe^{3+} ions. p(AAm-AGE)-CB-Fe^{3+} cryogel was characterized by FTIR, SEM and swelling degree analysis. The IMAC cryogel carrying 25.8μmol Fe^{3+} ions was used in adsorption studies under different conditions (i.e., pH, protein initial concentration, flow rate, temperature and ionic strength). Maximum adsorption capacities were found to be 75.7 mg/g for p(AAm-AGE)-CB-Fe^{3+} cryogel and 60.6 mg/g for p(AAm-AGE)-CB cryogel, respectively, and the adsorbed amounts per unit mass of cryogel reached to a plateau value at about 1.5mg/mL at pH 6.0. K_m and V_{max} values were significantly affected by adsorption of catalase onto the p(AAm-AGE)-CB-Fe^{3+} cyyogel. The K_m values were found to be 0.73 g/L for the free catalase and 0.18 g/L for the immobilized catalase. The V_{max} value of free catalase ($2.0x10^3$ U/mg enzyme) was found to be lower than that of the immobilized catalase ($2.5x10^3$ U/mg enzyme). Activity of immobilized catalase was determined higher in a wider temperature range than the free enzyme. It was also observed that enzyme could be repeatedly adsorbed and desorbed on the p(AAm-AGE)-CB-Fe^{3+} cryogel.

Keywords: Catalase, Cryogel, Immobilized Metal-Chelate Affinity Chromatography, Dye-Ligand Affinity Chromatography, Enzyme Immobilization

YENİ NESİL MATRİKS ÜZERİNE METAL ŞETAL ETKİLEŞİMİ İLE KATALAZIN GERİ-DÖNÜŞÜMLÜ İMMOBİLİZASYONU

ÖZ

p(AAm-AGE) kriyojel, akrilamit ve allil glisidil eterin radikalik polimerizasyonu ile hazırlanmıştır. Cibacron Blue F3GA, p(AAm-AGE) kriyojel üzerine reaktif boyanın klor grupları ile allil glisidil eterin epoksit grupları arasındaki reaksiyon sonucu kovalent olarak bağlanmıştır. Cibacron Blue F3GA bağlanan p(AAm-AGE) kriyojel ile Fe^{3+} iyonları arasında şelat oluşturulmuştur. p(AAm-AGE)-CB-Fe^{3+} kriyojel, FTIR, SEM analizleri and şişme derecesinin belirlenmesi ile karakterize edilmiştir. 25,8µmol Fe^{3+} iyonları içeren IMAC kriyojel, farklı koşullarda (pH, başlangıç protein konsantrasyonu, sıcaklık, akış hızı ve iyonik şiddet) adsorpsiyon çalışmalarında kullanılmıştır. Maksimum adsorpsiyon kapasiteleri p(AAm-AGE)-CB ve p(AAm-AGE)-CB-Fe^{3+} kriyojelleri için sırayla 75,7 mg/g ve 60,6 mg/g olarak belirlenmiş ve g kriyojel başına adsorplanan miktar pH 6,0'da 1,5mg/mL derişimde doygunluk değerine ulaşmıştır. K_m and V_{max} değerlerinin p(AAm-AGE)-CB-Fe^{3+} kriyojel üzerine katalaz adsorpsiyonuyla belirgin olarak değiştiği belirlenmiştir. K_m değeri serbest katalaz için 0,73 g/L, immoblize katalaz için 0,18 g/L olarak belirlenmiştir. Serbest enzymin V_{max} değeri ($2.0x10^3$ U/mg enzyme) immobilize enziminkinden düşük ($2.5x10^3$ U/mg enzyme) bulunmuştur. İmmobilize katalazın serbest enzime göre daha geniş sıcaklık aralığında daha yüksek aktivite gösterdiği belirlenmiştir. Ayrıca, p(AAm-AGE)-CB-Fe^{3+} kriyojel üzerine enzimin defelarca adsorplanıp desorplanabildiği belirlenmiştir.

Anahtar Sözcükler: Katalaz, Kriyojel, İmmobilize Metal Şelat Afinite Kromatografi, Boya-Ligand Afinite Kromatografi, Enzim İmmobilizasyonu

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CHAPTER ONE

INTRODUCTION

1.1 Affinity Chromatography

Affinity sorption is already a well established method for identification, purification and separation of complex biomolecules. This may be achieved by a number of traditional techniques such as gel permeation chromatography, high performance liquid chromatography, chromatofocusing, electrophoresis, centrifugation, etc., in that the process relies on the differences in the physical properties (e.g., size, charge and hydrophobicity) of molecules to be treated. In contrast, affinity sorption techniques exploit the unique property of extremely specific biological recognition. This is due to the complementarity of surface geometry and special arrangement of the ligand and the binding site of the biomolecule. All biological processes depend on specific interactions between molecules. These interactions might occur between a protein and low molecular weight substances (e.g., between substrates or regulatory compounds and enzymes; between bioformative molecules-hormones, transmittors, etc., and receptors, and so on), but biospecific interactions occur even more often between two or several biopolymers, particularly proteins. Affinity chromatography enables the separation of almost any biomolecule on the basis of its biological function or individual chemical structure. Examples can be found from all areas of structural and physiological biochemistry, such as in multimolecular assemblies, effector-receptor interactions, DNA-protein interactions, and antigen-antibody binding.

Affinity chromatography owes its name to the exploitation of these various biological affinities for adsorption to a solid phase (Jonson, 1998; Wilcheck, 1984). One of the members of the pair in the interaction, the ligand, is immobilized on the solid phase, whereas the other, the counter ligand (most often a protein), is adsorbed from the extract

that is passing through the column. Examples of such affinity systems are listed in Table 1.1.

Affinity sorption requires that the compound to be isolated is capable of reversibly binding (i.e., sorption-elution) to a sorbent which consists of a complementary substance (i.e., the so-called ligand) immobilized on a suitable insoluble support, i.e., the so-called carrier.

Table 1.1 Examples of Biological Interactions Used in Affinity Chromatography.

Ligand	Counter ligand
Antibody	Antigen, virus, cell
Inhibitor	Enzyme (ligands are often substrate analogs or cofactor analogs)
Lectin	Polysaccharide, glycoprotein, cell surface receptor, membrane protein, cell
Nucleic acid	Nucleic acid binding protein (enzyme or histone)
Hormone, vitamin	Receptor, carrier protein
Sugar	Lectin, enzyme, or other sugar-binding protein

The term affinity chromatography has been given quite different connotations by different authors. Sometimes it is very broad, including all kinds of adsorption chromatographies based on nontraditional ligands, in the extreme all chromatographies except ion exchange. Often it is meant to include immobilized metal ion affinity chromatography (IMAC), covalent chromatography, hydrophobic interaction chromatography, and so on. In other cases it refers only to ligands based on biologically functional pairs, such as enzyme-inhibitor complexes. The term not only to include functional pairs but also the so-called biomimetic ligands, particularly dyes whose binding apparently often occurs to active sites of functional enzymes although the dye

molecules themselves of course do not exist in the functional context of the cell. Thus chromatography based on the formation of specific complexes such as enzyme-substrate, enzyme-inhibitor, etc., i.e on biological recognition, is termed bioaffinity or biospecific chromatography and the respective interaction-biospecific adsorption or bioaffinity (Porath, 1973). The original term "affinity chromatography" acquired a broader meaning also including hydrophobic chromatography, covalent chromatography, metal-chelate chromatography, chromatography on synthetic ligands, etc., i.e chromatography procedures based on different, less specific types of interaction. The broad scope of the various applications of affinity has generated the development of subspecialty techniques, many of which are now recognized by their own nomenclature. Table 1.2 summarizes some of these techniques. As can be seen from Table 1.2, some of these subcategories have become accepted useful techniques (Wilcheck, & Miron, 1999).

Table 1.2 Subcategories of affinity chromatography.

Affinity Chromatography	
	1. Hydrophobic Chromatography
	2. Immunoaffinity Chromatography
	3. Covalent AC
	4. Metal-Chelate AC
	5. Molecular Imprinting Affinity
	6. Membrane-Based AC
	7. Affinity Tails Chromatography
	8. Lectin Affinity
	9. Dye-Ligand AC
	10. Reseptor Affinity
	11. Weak AC
	12. Perfusion AC
	13. Thiophilic Chromatography
	14. High Performance AC
	15. Affinity Density Pertubation
	16. Library-Derived Affinity
	17. Affinity Partitioning
	18. Affinity Electrophoresis
	19. Affinity Capillary Electrophoresis
	20. Centrifuged AC
	21. Affinity Repulsion Chromatography

Principle of affinity chromatography is schematically shown in Figure 1.1. A wide variety of ligands may be covalently attached to an inert support matrix, and subsequently packed into a chromatographic column.

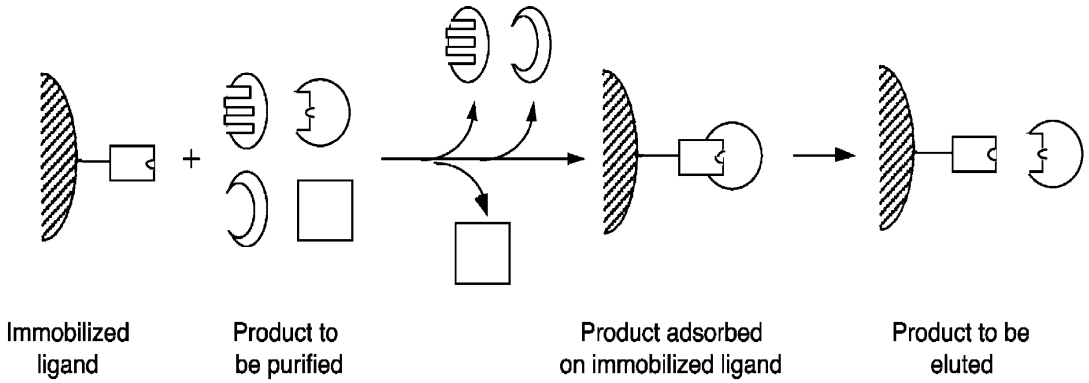


Figure 1.1 Principle of affinity chromatography

In such a system, only the protein molecules which selectively bind to the immobilized ligand will be retained on the column. Washing the column with a suitable buffer will flush out all unbound molecules. There are several techniques permit to desorb the product to be purified from the immobilized ligand.

Because affinity chromatography proper relies on the functional properties, active and inactive forms can often be separated. This is however, not unique to affinity methods. Covalent chromatography can do the same thing when the activity depends on a functional thiol group in the protein. By affinity elution, ion-exchange chromatography is also able to separate according to functional properties. These are, however, exceptions to what is a rule for the affinity methods.

Very often the use of affinity chromatography requires that the investigator synthesizes the adsorbent. The methods for doing this, are well worked out and are also easily adopted for those not skilled in synthetic organic chemistry. To further simplify the task, activated gel matrices ready for the reaction with a ligand are commerically

available. The immobilization of a ligand can, in the best cases, be a very simple affair. In addition, immobilizations are just as easy for proteins as for small molecules.

A property that needs special consideration is the association strength between ligand and counter ligand. If it is too weak there will be no adsorption, whereas if it is too strong it will be difficult to elute the protein adsorbed. It is always important to find conditions, such as pH, salt concentration, or inclusion of, for example, detergent or other substances, that promote the dissociation of the complex without destroying the active protein at the same time. It is often here that the major difficulties with affinity methods are encountered. Ligands can be extremely selective, but they may also be only group specific. The latter type includes glycoprotein-lectin interactions, several dye-enzyme interactions, and interactions with immobilized cofactors. However, these interactions have also proved to be extremely helpful in solving many separation problems. Good examples are ligands that are group selective against immunoglobulins (e.g., staphylococcal protein A or streptococcal protein G) (Janson, & Ryden, 1998).

1.1.1 Dye-Ligand Afinity Chromatography

In affinity chromatography a molecule having specific recognition capability (‘’ligand’’ or ‘’binder’’) is immobilized on a suitable insoluble support (‘’matrix’’ or ‘’carrier’’), which is usually a polymeric material in bead or membrane form. The molecule to be isolated (‘’analyte’’ or ‘’target’’) is selectively captured (‘’adsorbed’’) by the complementary ligand immobilized on the matrix by simply passing the solution containing the target through the chromatographic column under favorable conditions. The target molecules are then eluted (‘’desorbed’’) by using proper elutants under conditions favoring desorption, by adjusting the pH, ionic strength or temperature, using specific solvents or competitive free ligands, so that the interaction between the ligand and target is broken and the target molecules are obtained in a purified form. Since its first introduction (Cuatrecasas, Wilchek, & Anfinsen, 1968), thousands of different molecules (enzymes, antibodies, hormones, vitamins, receptors, many variety of other

proteins and glycoproteins, RNA, DNA, etc.), even bacteria, viruses, and cells have been separated or purified by affinity chromatography (Deutscher, 1990; Matejtschuk, 1997). A wide variety of functional molecules, including enzymes, coenzymes, cofactors, antibodies, amino acids, oligopeptides, proteins, nucleic acids, and oligonucleotides may be used as ligands in the design of novel sorbents. These ligands are extremely specific in most cases. However, they are expensive, due to high cost of production and/or extensive purification steps. In the process of the preparation of specific sorbents, it is difficult to immobilize certain ligands on the supporting matrix with retention of their original biological activity. Precautions are also required in their use (at sorption and elution steps) and storage. Dye-ligands have been considered as one of the important alternatives to natural counterparts for specific affinity chromatography to circumvent many of their drawbacks, mentioned above (Denizli, & Pişkin, 2001).

Dye-ligands are able to bind most types of proteins, especially enzymes, in some cases in a remarkably specific manner. They are commercially available, inexpensive, and can easily be immobilized, especially on matrices bearing hydroxyl groups (Denizli, & Pişkin, 2001), stable against biological and chemical attack, storage adsorbent without loss of activity, reusable: cleaning and sterilization, high capacity (Boyer, & Hsu, 1992). Although dyes are all synthetic in nature, they are still classified as affinity ligands because they interact with the active sites of many proteins by mimicking the structure of the substrates, cofactors, or binding agents for those proteins (Denizli, & Pişkin, 2001).

1.1.1.1 Reactive and Biomimetic Dyes

A number of textile dyes, known as reactive dyes, have been used for protein purification in dye-ligand affinity systems, since they bind a variety of proteins in a selective and reversible manner. Most of the reactive dyes used in dye-affinity systems consist of a chromophore (either azo dyes, anthraquinone, or phathalocyanine), linked to a reactive group often a mono- or dichlorotriazine ring. They also have sulfonic acid

groups to provide the desired solubility of the molecule in aqueous media. These groups are negatively charged at all pH values. Some dyes contain carboxyl, amino, chloride, or metal complexing groups; most contain nitrogen both in or outside on aromatic ring (Denizli, & Pişkin, 2001).

These dyes are prepared by the reaction of cyanuric chloric (Figure 1.2 a) with an amino-containing dye, thereby producing a dichlorotriazinyl dye, which corresponds to the Procion MX range of dyes produced by ICI (Figure 1.2 b). The triazine ring in these dyes contain two labile chlorine atoms which makes dyes of this type highly reactive. Subsequent reaction of these dyes with an amine or alcohol causes the replacement of one of the triazinyl chlorine atoms and produces a less reactive monochlorotriazine dye which corresponds to ICI's Procion H range and Ciba-Geigy's Cibacron range (Figure 1.2 c) (Boyer, & Hsu, 1992). The only difference betwen Cibacron and Procion H series are the position of sulfonate group on the aniline ring, which is in *ortho*-position on Cibacron, but in *meta*- or *para*-position in Procion H series (Denizli, & Pişkin, 2001).

Two dichlorotriazinyl molecules can be coupled with a bifunctional molecule (e.g., Diaminobenzene) to form bifunctional triazinyl dyes. An example is Procion H-E from ICI is shown in Figure 1.2 d. Some other examples of triazinyl dyes are monofluoro-triazinyl (Cibacron, Ciba-Geigy), trichloropyrimidnyl (Drimarene, Sandoz), and difluorochloropyrimidnyl (Lavafix, Bayer and Drimarene, Sandoz), which are shown in Figure 1.2 e–g, respectively. Note that, when the chloride atoms on the triazinyl ring are replaced with other groups, the reactivity of the dye is reduced, substantially. Dye-molecules having more chloride (or fluoride) atoms can easily react with the nucleophilic groups on the matrix at the ligand-immobilization step. The structure of several typical triazinyl dyes are shown in Figure 1.2 (Denizli, & Pişkin, 2001).

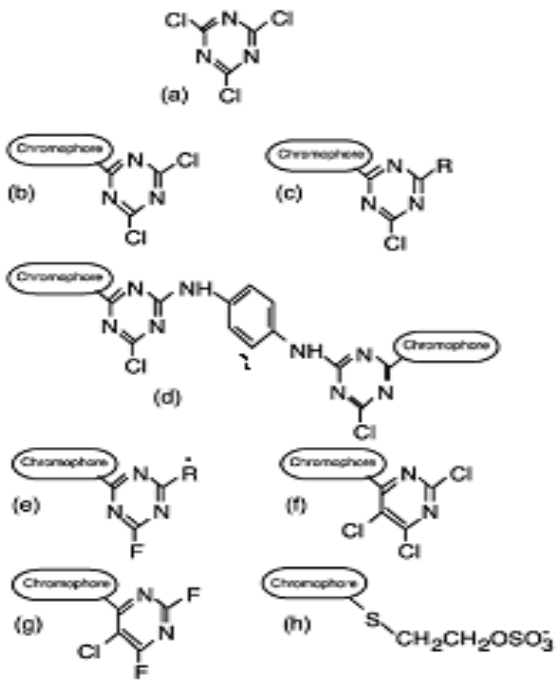


Figure 1.2 Structure of some of the reactive dye molecules; (a) cyanuric chloride; (b) Procion MX series (ICI); (c) Cibacron (Ciba-Geigy) and Procion H (ICI); (d) Procion H-E (ICI); (e) Monofluorotriazinyl, Cibacron, Ciba-Geigy; (f) Trichloropyrimidnyl, Drimarene, Sandoz; (g) Difluorochloro-pyrimidnyl, Levafix, Bayer and Drimarene, Sandoz; (h) Sulfatoethyl sulfone, Hoechst.

An important strategy is to tailor-make, or redesign the dye structure to improve the specificity of textile dyes for target proteins. This new type of ligand is called Abiomimetic dye B. It carries all the advantages of the parent (unmodified) dye including high specificity. This concept was first applied (Clonis, Stead, & Lowe, 1987) early in the 1980s and then successfully used by them and also by others for specific enzyme recovery, as recently reviewed (Clonis et al., 2000). The first biomimetic dye was prepared by linking benzamidine to the reactive chlorotriazine ring via a

diaminomethylbenzene group. It was used for the specific separation of trypsin from chymotrypsin (Denizli & Pişkin, 2001). Dye-ligands having two recognition moieties on the triazine ring were designed to isolate kallikrein from a crude pancreatic extract. By using biomimetic Cibacron Blue dye (phosphonated via a *p*-aminobenzyl ring), it was possible to purify alkaline phosphates from calf intestinal extract 280–330-fold in one chromatographic step after specific elution with inorganic phosphate. Developments in computational technology, especially in contemporary molecular modeling and bioinformatics, greatly improved the design of new series of biomimetic dye ligands.

A three-dimensional structural model of LDH as a guide, appropriate structure changes of the dye molecules have allowed a biomimetic design of the ligand to improve the purification of L-lactate dehydrogenase (Labrou, Eliopoulos, & Clonis, 1999). The terminal biomimetic moiety bears a carboxyl group or a ketoacid structure linked to the triazine ring, thus mimicking natural ligands of L-malate dehydrogenase and these dyes have shown high specificity in the affinity purification of this enzyme (Labrou, Eliopoulos, & Clonis, 1996). Ketoacid-group recognizing enzymes (i.e., formate dehydogenase, oxaloacetate decarboxylase and oxalate oxidase) were purified by using biomimetic ligands (mercaptopyruvic-, m-amino- benzoic-, and amino-ethyloxamic-biomimetic dyes) (Kotsira, 1997; Labrou, 1995; Labrou, 1999). Molecular modeling has recently been applied for the design of triazine non-dye ligands for Protein A, human IgG (Teng, Sproule, Husain, & Lowe, 2000) and insulin precursor (Sproule et al., 2000).

1.1.1.2 Immobilization of Dye Ligands

The most commonly used matrices for dye-ligand chromatography are gel filtration media including cross-linked agarose, cross-linked dextran, and beaded cellulose. Cross-linked agarose appears to be the best ‘’general purpose’’ matrix, due to its structural stability, flow properties, low incidence of non-spesific adsorption, and open pore structure which allows high protein binding capacity. The dye-matrices are typically prepared with the dye immobilized directly, rather than indirectly via a spacer arm,

because of significantly advantages in ligand leakage, capacity, and simplicity of immobilization (Denizli, & Pişkin, 2001).

There are many methods for immobilization of ligand molecules onto the support matrix. First of all immobilization should be attempted through the least critical region (not from the active site) of the ligand molecule, to ensure minimal interference on the specific interaction between the immobilized ligand and the target molecules. The active sites of biological molecules are often located deep within the three-dimensional structure of the molecule, which may cause an important steric hindrance between complementary ligand and target molecules (Figure 1.3). In these circumstances spacer arms, usually short alkyl chains, are frequently imposed between the matrix and the ligand to ensure their accessibility to the target (Denizli, & Pişkin, 2001).

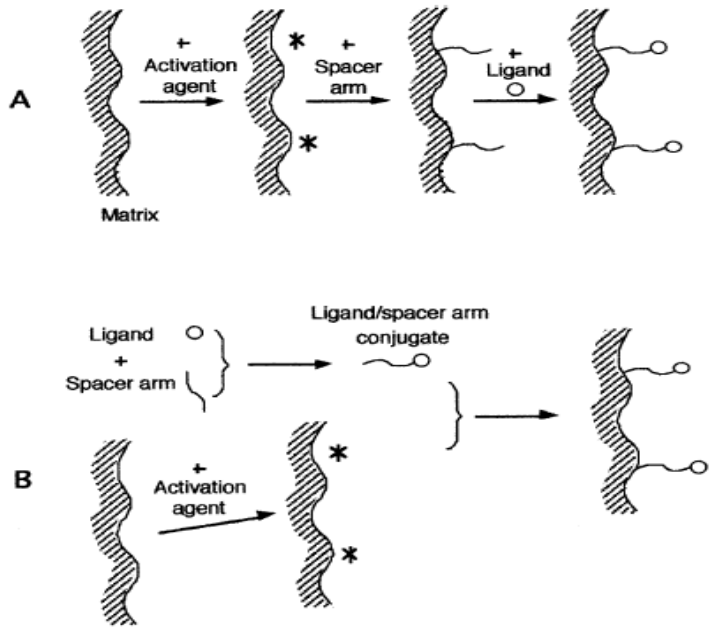


Figure 1.3 Strategies for coupling of ligand to the support matrix; (A) coupling through spacer arms; (B) coupling through spacer arm-ligand conjugates.

Many of the reactive dyes are immobilized onto matrix by direct reactions between the reactive groups (mainly hydroxyl groups) on the matrix and the dye molecules

(through chloride or fluoride atoms) on triazinyl groups. Nonreactive dyes can be coupled to the matrix by the usual activation procedures, and the subject has been extensively reviewed (Denizli, & Pişkin, 2001). Direct coupling of reactive triazinyl dyes to the matrices bearing hydroxyl groups is a simple, inexpensive and safe method (Baird, 1976; Clonis, 1986; Clonis, 1987; Hey, 1981). Coupling is achieved at alkaline conditions by nucleophilic substitution of hydroxyl groups with the reactive chlorine on the dye molecules.

1.1.1.3 Interactions Between Dye Ligands And Proteins

The binding site of a protein is a unique stereochemical arrangement of ionic, polar, and hydrophobic groups in its three-dimensional structure, and where the polypeptide chains probably exhibit greatest flexibility. The dye-ligand molecules participate in non-covalent interaction with the protein to achieve tight and specific binding. It has been shown in many kinetic studies that triazinyl dyes interact with an enzyme in a way involving the binding site (the substrate or coenzyme binding site, or the ‘’active site’’) for a natural biological ligand (NADH, NADPH, NAD^+, $NADP^+$, GTP, IMP, ATP, HMG-CoA, folate, etc.) of that enzyme so that this natural ligand cannot bind (Denizli, & Pişkin, 2001).

Triazine dyes, polysulphonated aromatic chromophores, mimic the naturally occuring heterocycles such as nucleotide mono-, di-, and triphosphates, NAD, NADH, flavins, acethyl-CoA and folic acid and inactivate typical nucleotide-dependent enzymes with different efficacy (Kaminska, Dzieciol, Koscielak, & Triazine, 1999). Thus, they can be used as affinity ligands for glycosyltransferases. Several spectrophotometric techniques including UV visible, FTIR, NMR, ESR, and circular dichroism, have been utilized to explain dye protein interactions, the existence of competitive ligands (e.g., substrates and coenzymes) and perturbing solutes (e.g., salts and organic solvents) (Federici, 1985; Lascu, 1984; Skotland, 1981; Subramanian, 1984). These studies have revealed that confirmation of both the dye and enzyme is important, and the interactions might be a

mixture of electrostatic and hydrophobic forces, and also at discrete sites rather than in an indiscriminate fashion. Interactions of the parent dyes (especially Cibacron Blue F3G-A) and their analogs with several oxidoreductases, phosphokinases, and ATPases have been investigated (Denizli, & Pişkin, 2001). These studies have shown that both the anthraquinone and the adjacent benzene sulfonate rings on these dyes are important in binding to the enzymes. They do bind to the enzyme molecules at a similar position and in a way similar to the AMP moiety of the coenzyme. Molecular models have shown a rough resemblance between Cibacron Blue F3G-A and NAD^+, but the most important similarities are with the planar ring structure and the negative charge groups. It has been shown by X-ray crystallography that this blue dye binds to liver alcohol dehydrogenase at an NAD^+ site, with correspondences of the adenine and ribose rings but not the nicotinamide. Thus, it was proposed that the dye is an analog of ADP-ribose, and it interacts with the ‘’nucleotide fold’’ found in AMP, IMP, ATP, NAD^+, $NADP^+$, and CTP binding sites of the corresponding enzymes. Cibacron Blue F3G-A have been an ideal dye-ligand for especially nucleotide-binding proteins. The interaction between the dye ligand and proteins can be concluded as follows: Dye molecules mimic natural ligands, and bind some protein molecules very specifically at their active points. However, under same conditions all proteins can be adsorbed onto dye-ligand affinity sorbents, which means that these ligands provide numerous opportunities for other interactions with other parts of the proteins. Most proteins are bound nonspecifically by complex combination of electrostatic, hydrophobic, hydrogen bonding, and charge-transfer interactions, all of which are possible considering the structural nature of the dyes (Denizli, & Pişkin, 2001).

1.1.2 Immobilized Metal Chelate Affinty Chromatography

Immobilized Metal Ion Affinity Chromatography, IMAC, is a separation technique that uses covalently bound chelating compounds on solid chromatographic supports to entrap metal ions, which serve as affinity ligands for various proteins, making use of coordinative binding of some amino acid residues exposed on the surface (Gaberg-Porekar, & Menart, 2001).

IMAC, was introduced by Porath and coworkers (Porath, Carlsson, Olsson, & Belfrage, 1975) in 1975 under the name of Metal Chelate Affinity Chromatography. In this short publication, the authors described the use of immobilized zinc and copper metal ions for the fractionation of proteins from human serum.

IMAC utilizes the differential affinity of proteins for immobilized metal ions to effect their separation. This differential affinity derives from the coordination bonds formed between metal ions and certain amino acid side chains exposed on the surface of the protein molecules. Since the interaction between the immobilized metal ions and the side chains of amino acids has a readily reversible character, it can be utilized for adsorption and then be disrupted using mild (i.e., non-denaturing) conditions (Chaga, 2001).

On the other hand, IMAC holds a number of advantages over biospecific affinity chromatographic techniques, which have a similar order of affinity constants and exploit affinities between enzymes and their cofactors or inhibitors, receptors and their ligands or between antigens and antibodies. The benefits of IMAC-ligand stability, high protein loading, rapid purification, mild elution conditions, simple regeneration and low cost (Arnold, 1991) -are decisive when developing large-scale purification procedures for industrial applications. Initially, IMAC techniques were used for separating proteins and peptides with naturally present, exposed histidine residues, which are primarily responsible for binding to immobilized metal ions (Gaberg-Porekar, & Menart, 2001).

Table 1.3 presents a comparison between IMAC and a number of other adsorption principles. This shows that IMAC occupies a space between "true affinity" chromatography and other adsorption principles, and so complements them rather well.

Table 1.3 Comparison of IMAC with other adsorption principles

Property	**IMAC**	**Affinity**	**IEC**	**HIC**
Capacity	High (Medium)	Low	High	High (Medium)
Recovery	High	Medium	High	Medium
Loading	Mild	Mild	Mild	Sometimes harsh
Eluation	Mild	Harsh	Mild	Mild
Regeneration	Complete	Incomplete	Complete	Incomplete
Selectivity	Medium-high	High	Low-medium	Low-medium
Cost	Low	High	Low	Low

1.1.2.1 Mechanism, Ligands, Ions, and Techniques

In IMAC the adsorption of proteins is based on the coordination between an immobilized metal ion and electron donor groups from the protein surface. Figure 1.4 illustrates protein binding to a metal-chelated affinity support. Most commonly used are the transition-metal ions Cu(II), Ni(II), Zn(II), Co(II), Fe(III), which are electron-pair acceptors and can be considered as Lewis acids. Electron-donor atoms (N, S, O) present in the chelating compounds that are attached to the chromatographic support are capable of coordinating metal ions and forming metal chelates, which can be bidentate, tridentate, etc., depending on the number of occupied coordination bonds. The remaining metal coordination sites are normally occupied by water molecules and can be exchanged with suitable electron-donor groups from the protein.

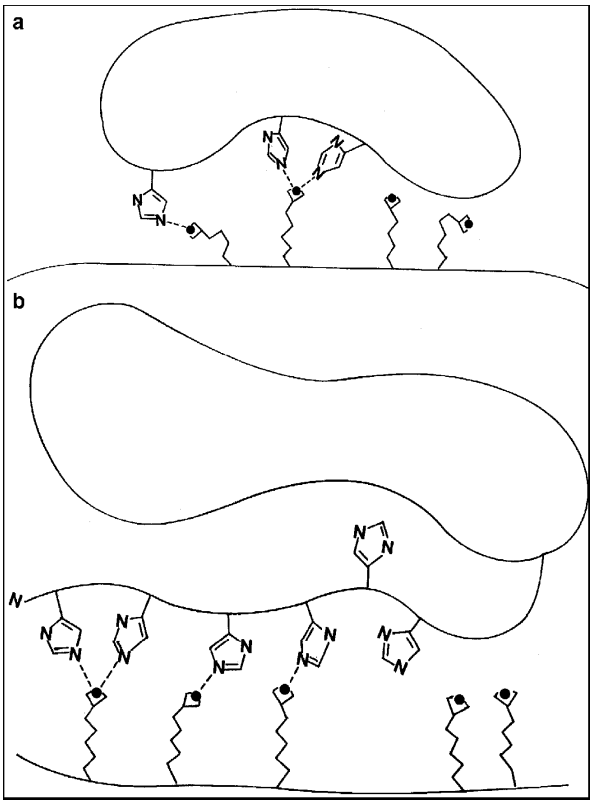


Figure 1.4 Schematic illustration of the protein binding to a metal-chelated affinity support. Strong binding of a protein onto the IMAC matrix is achieved predominately by multi-point attachment of native or engineered surface histidines (a), or by histidine tag (b) added to the N- or C-terminus of the protein.

In addition to the amino terminus, some amino acids are especially suitable for binding due to electron donor atoms in their side chains. Although many residues, such as Glu, Asp, Tyr, Cys, His, Arg, Lys and Met, can participate in binding, the actual protein retention in IMAC is based primarily on the availability of histidyl residues. Free cysteines that could also contribute to binding to chelated metal ions are rarely available in the appropriate, reduced state. However, aromatic side chains of Trp, Phe and Tyr appear to contribute to retention, if they are in the vicinity of accessible histidine residues (Arnold, 1991; Sulkowski, 1989). Adsorption of a protein to the IMAC support

is performed at a pH at which imidazole nitrogens in histidyl residues are in the nonprotonated form, normally in neutral or slightly basic medium. Usually relatively high-ionic-strength buffers (containing 0.1 to 1.0 M NaCl) are used to reduce nonspecific electrostatic interactions, while the buffer itself should not coordinatively bind to the chelated metal ion. Elution of the target protein is achieved by protonation, ligand exchange or extraction of the metal ion by a stronger chelator, like EDTA. Elution buffers with lower pH or lowering pH gradients are widely used for elution of the target protein. However, for proteins sensitive to low pH, ligand exchange, e.g., with imidazole, at nearly neutral pH is more favorable. In this case, the IMAC columns must be saturated and equilibrated with imidazole prior to chromatographic separation to avoid the pH drop caused by the imidazole proton pump effect (Sulkowski, 1996; Sulkowski, 1996). Application of a strong chelating agent, such as EDTA, also results in elution of the bound proteins, although the binding properties are also destroyed and the column must be recharged with metal ions prior to the next separation (Gaberg-Porekar, & Menart, 2001).

Selectivity in protein separation can be effected through various approaches: by choice of the metal ligand, through variation of the structure of the chelating compound, by variation of the spacer arms, ligand density, concentration of salts and competing agents, etc. For example, in the case of human growth hormone, reduction of ligand IDA–Cu(II) density on chelating sorbent resulted in higher protein purity and increased yield (Liesiene et al., 1997). The apparent affinity of a protein for a metal chelate depends strongly on the metal ion involved in coordination. In the case of the iminodiacetic acid (IDA) chelator, the affinities of many retained proteins and their respective retention times are in the following order: Cu(II) > Ni(II) > Zn(II) ≥ Co(II) (Gaberg-Porekar, & Menart, 2001).

Some chelating compounds used in IMAC are listed in Table 1.4. IDA is by far the most widely used chelating compound. It is commercially available from many producers, although in the past several years, other chelators have also been tried for

immobilization to support particles. In general, tetradentate ligands, such as NTA and CM-Asp., have higher affinities for metal ions than the tridentate chelator IDA, but they exhibit lower protein binding due to the loss of one coordination site. This is even more pronounced in a pentadentate TED chelating ligand, where in an octahedral arrangement around a divalent metal ion only one coordination site is left for protein binding (Gaberg-Porekar, & Menart, 2001).

Table 1.4 Some chelating compounds in use for immobilization in IMAC

Chelating Compound	Coordination	Metal Ions
Aminohydroxamic acid	Bidente	Fe(III)
Salicylaldehyde	Bidente	Cu(II)
8-Hydroxy-quinoline (8-HQ)	Bidente	Al(III), Fe(III), Yb(III)
Iminodiasetic acid (IDA)	Tridente	Cu(II), Zn(II), Ni(II), Co(II)
Dipicolylamine (DPA)	Tridente	Zn(II), Ni(II)
Ortho-phosphoserine (OPS)	Tridente	Fe(III), Al(III), Ca(II), Yb(III)
N-(2-pyridylmethyl)-aminoacetate	Tridente	Cu(II)
2,6-Diaminomethylpyridine	Tridente	Cu(II)
Nitrilotriacetic acid (NTA)	Tetradentate	Ni(II)
Carboxymethylated aspartic acid (CM-Asp)	Tetradentate	Ca(II), Co(II)
N,*N*,*N*'-tris(carboxymethyl) ethylenediamine (TED)	Pentadentate	Cu(II), Zn(II)

Figure 1.5 illustrates putative structures of metal ion complexes and most popular chelators.

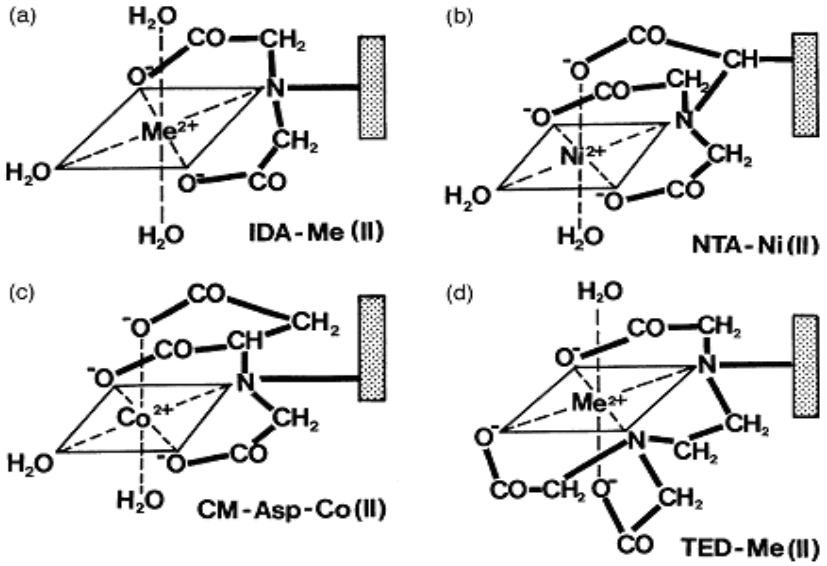


Figure 1.5 Putative structures of some representative chelators in complex with usually used metal ions: IDA–Me(II), NTA–Ni(II), CM–Asp–Co(II), TED–Me(II). Me(II) stands for Cu(II), Ni(II), Zn(II) or Co(II).

In Figure 1.5, spacers to the solid support are not specified, but may vary in length and chemical structure, which also affects chromatographic behavior. Water molecules can be replaced by other ligands, usually histidines exposed on the protein surface. This represents the major binding interaction of the protein towards the IMAC matrix, provided that unspecific, residual interactions, e.g., ionic or hydrophobic, are minimized by selection of appropriate matrix material and buffer composition (Gaberg-Porekar, & Menart, 2001).

1.1.2.2 Metal Ion Affinities and Mechanisms

Pearson (Pearson, 1973) postulated a classification system for IMAC that metal ions can be divided into three categories (hard, intermediate and soft) based on their preferential reactivity towards nucleophiles. To the group of hard metal ions belong Fe^{3+}, Ca^{2+} and Al^{3+}, which show a preference for oxygen. Soft metal ions such as Cu^{+}, Hg^{2+}, Ag^{+}, etc., prefer sulfur. Intermediate metal ions (Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}) coordinate

nitrogen, oxygen and sulfur. The strength of binding of a protein to an immobilized metal ion adsorbent can be a function of multipoint interactions of this type. Carboxylic amino acids as well as occasionally available tyrosine or phosphorylated side chains of serine, threonine can also act as targets for hard metal ions. The selectivity of hard and intermediate metal ions is very different. At the pH for their optimal adsorption (acidic pH or neutral pH, respectively) hard and intermediate metal ions interact with different side chains on the protein surfaces. The distinction between the two types has been exploited to achieve impressive separations and even single-step purifications of proteins from complex biological mixtures (Chaga, 2001).

Based on whether one uses hard or intermediate immobilized metal ions (Pearson, 1973) there will be different adsorption selectivities when applying the same sample to the columns. For example, immobilized Fe^{3+} would adsorb a distinct profile of proteins at acidic pH from that which would be adsorbed to immobilized Cu^{2+} at neutral pH. The selectivity of the metal ions is also known to change under different environmental conditions. For example, intermediate metal ions start exhibiting affinity for the amino group at the N-terminus of peptides and proteins, as well as the peptide bonds at pH values greater than 8 (Hansen, 1992; Andersson & Sulkowski, 1992).

Studies determined that the type of matrix (silica or agarose) used for immobilization of the chelating ligands (and consequently the metal ions) did not play a significant role on the selectivity or capacity of the IMAC adsorbents. Some early attempts were also made to utilize IMAC for separation of mono-di- and oligo-nucleotides. These studies determined that purines possess the highest affinity for intermediate metal ions. IMAC has seen extensive use in the successful purification of proteins from complex biological samples (Chaga, 2001). Among these were purification procedures for interferon, nucleoside diphosphatase, trypsin inhibitors, superoxide dismutase, acid protease, serine carboxypeptidase, glycogen phosphorylase, lactate dehydrogenase, etc. The broad range of conditions under which IMAC can be carried out have also proved useful in the isolation of proteins from organisms that live in extreme natural environments. For

example, immobilized Fe^{3+} ions have been used at high salt concentrations (1.5–3 M NaCl) for the purification of proteins from halophilic microorganisms (Chaga, 1993).

1.1.2.3 Adsorbent Maturation (Charging With Metal Ion, Removal of Excess Metal Ion, Equilibration of the Adsorbent)

There are a number of ways to charge, wash and equilibrate IMAC adsorbents, and currently there is no protocol that is accepted as a standard procedure. Charging can be performed, among other ways, in buffer, deionized water, and weak acid. The removal of excessive amounts of metal ion is also performed under a variety of conditions, such as washing in the presence of glycine, Tris buffer, deionized water, weak acid, low concentrations of imidazole, etc. One of the most important steps—equilibration—can be carried out with a large number of buffering substances, among which are sodium phosphate, Tris, and MOPS for intermediate metal ions; and sodium acetate, MES, and PIPES for hard metal ions. Use of Tris buffers with intermediate metal ions results in weak but definite leakage of the metal ions and decreased adsorbent capacity due to the weak coordination of the metal ions with the buffering substance itself. In spite of this, there are numerous applications in which Tris has been used and it is even recommended by some manufacturers (Chaga, 2001).

1.1.2.4 Sample Requirements

While ion exchange chromatography (IEC) and hydrophobic interaction chromatography (HIC) can be influenced by factors that are more readily analyzed and controlled, such as ionic strength and pH, IMAC can be influenced by additional factors, such as the presence of low and/or high molecular weight chelators in the sample which are difficult to account for in advance. In this respect, IMAC requirements are definitely as “strict” as those of other affinity methods. Most significant is the requirement for the complete absence of strong chelators, such as EDTA, in the loading step. Depending on

the type of sample which is applied, there are four possible outcomes from an IMAC experiment (Chaga, 2001).

1. The protein of interest might not coordinate with the immobilized metal ion and so will pass through the column without retardation (negative adsorption)
2. The protein of interest might coordinate and remove the metal ion from the chelating ligand and pass through the column without retention (referred to as metal ion transfer or MIT (Sulkowski, 1989).
3. The protein of interest might coordinate with and bind to the immobilized metal ion.
4. The protein of interest can also be applied in the presence of metal ions and might be retained on a metal-free column—so-called "reverse IMAC" (Porath, 1997).

Hutchens and Yip (Hutchens, & Yip, 1990) have shown that the presence of free metal ions in the liquid phase does not necessarily have a detrimental effect on the retention of peptides and proteins on immobilized intermediate metal ions. Similarly, Chaga et al., have demonstrated the purification of calcium binding proteins on Eu3q-TED in the presence of a significant concentration of Ca^{2+} in the loading buffer (Chaga, 1996). It was suggested that in this case Ca^{2+} ions might have a structure forming function (resulting in exposure of metal binding sites for Eu^{3+} on the surface of the target protein molecules). While direct adsorption of the target protein is desired in most cases, negative adsorption can be useful in some applications (Chaga, 2001).

1.1.2.5. Mode of operation (Batch, Gravity, Low Pressure, Medium&High Pressure)

The mode of operation will influence both the capacity and the selectivity of the adsorbent. Applications in packed chromatographic beds designed for adsorbents that can withstand elevated flow rates and pressures will ensure better reproducibility, higher yields and better selectivity. Such steps, however, might not be necessary at the beginning of the purification scheme, where the main requirement is speed. The use of IMAC as a first capture step is simplified at present by the availability of large bead size

chelating adsorbents from Amersham Pharmacia Biotech, Sterogene and Clontech. This makes possible the use of expanded bed or batch mode IMAC of crude samples from supernatants or cell lysates in very short times (Chaga, 2001).

1.1.2.6 Regeneration of the adsorbents

IMAC adsorbents have excellent regeneration properties. The chelated metal ion can be removed almost quantitatively by strong chelators, and then the resin can undergo very stringent washing and sterilization procedures which are impossible with most affinity adsorbents. (Chaga, 2001).

1.1.2.7 Advantages and Disadvanges of IMAC

The production of pure, biologically active proteins involves denaturation and refolding, renaturation, which is classically accomplished by the low-efficiency techniques of dialysis or dilution. IMAC has the advantage of enabling histidine-tagged proteins to be separated efficiently in the presence of denaturing concentrations of urea or guanidine–HCl as well as a large number of non-ionic detergents, making it extremely useful in the initial steps of purification immediately after the extraction/isolation procedure. Additionally affinity tagging by consecutive histidines offers the possibility of efficient purification and refolding in a single IMAC step (Gaberg-Porekar, & Menart, 2001).

Several amino acids, especially histidine, lysine, cysteine, proline, arginine, and methionine, are susceptible to metal-catalyzed oxidation reactions that produce highly reactive radical intermediates which can damage a variety of proteins (Krishnamurthy, Madurawe, Bush, & Lumpkin, 1995). Taking into account that metal chelates as well as Cu(II) ions themselves can be used for the site-specific cleavage of proteins (Cheng, 1994; Humphreys, 1999; Rana, 1994), it is not surprising that destruction of amino acid side chains and cleavage of the protein backbone can also be provoked during IMAC

chromatography. In such cases, the replacement of high redox-active Cu(II) with a less active metal ion, such as Zn(II), may prevent, or at least minimize, protein damage. The majority of routine IMAC separations are carried out under aerobic, mildly oxidative conditions, due to oxygen dissolved in the sample and buffers. Potential damage to proteins, caused by reactive oxygen species or metal-catalyzed reactions inside the IMAC column, has not been studied enough. In experiments under forced conditions, e.g., when hydrogen peroxide or ascorbate—and especially a combination of both—were added to elution buffers, a significant loss of protein activity was demonstrated on Cu(II)-IDA columns (Krishnamurthy et al., 1995).

1.1.2.8 Applications of IMAC

Many reports on IMAC used for purifying pharmaceutically interesting proteins, such as interferons, vaccines and antibodies, have been published but relatively few data exist on actual large-scale purifications of pharmaceutical proteins. On the other hand, IMAC offers all possibilities for large-scale purification of many industrial enzymes as well as proteins for research in genetics, molecular biology, and biochemistry. There are many more reports on the application of His6 tag for IMAC isolation of potential therapeutics. However, IMAC technology should be further improved with respect to metal-ion leakage, dynamic capacity, reproducibility, etc. We can conclude that there are many attempts to use IMAC matrices for large-scale isolation of biopharmaceuticals, but many of them are still in the trial phase, or else the data are not accessible to the public (Gaberg-Porekar, & Menart, 2001).

Expanded-bed adsorption (EBA) techniques constitute another broad field of IMAC application and require additional properties of column matrix, e.g., higher particle density and high resistance to harsh conditions during column cleaning or sanitization. Expanded-bed techniques are less attractive on a small, laboratory scale but potentially highly advantageous at an industrial scale. Downstream processing procedures from unclarified *E. coli* or yeast homogenates are being developed for native (Willoughby,

1999; Clemmitt, 2000) as well as histidine-tagged proteins (Clemmitt, & Chase, 2000). Generally, recoveries over 80% of the protein were achieved in successful cases, but at least two major weak features must be further improved: low dynamic capacity and efficiency of Clean In Place (CIP) procedures for eliminating contaminants. Elimination of centrifugation and filtration in large industrial-scale isolations is a major driving force for the introduction of EBA in the isolation of therapeutic proteins. The combination of IMAC and EBA techniques should provide a unique approach to simplifying the whole downstream process, reduce the number of steps and start-up investment, and thus make the purification more economical.

IMAC has become very popular, especially for scientific or research work on a laboratory scale. Commercial cloning vectors, containing sequences that encode histidine tags, are available as well as antibodies for specific detection of His-tagged proteins (Lindner et al., 1997). For rapid high-throughput laboratory detection or purification of His6-tagged recombinant proteins from cleared lysates on (Nieba et al., 1997) well plates, different systems were developed. In proteins containing no histidines, which bind to IMAC at high pH due to the accessible N-terminal a-amino group, this type of chromatography can be used to reveal modifications of the N-terminal (Arnold, 1991). IMAC has also been used for affinity purification of nonprotein molecules, such as DNA, by employing an affinity tag of six successive 6-histaminylpurine residues, urea which mediate selective adsorption to Ni–NTA chelate resin (Min, & Verdine, 1996). Native DNA binds weakly to IMAC matrices, while RNA and oligonucleo tides bind strongly due to accessible aromatic nitrogens in the bases (Murphy, White, & Willson, 2000). In the last decade, some non-chromatographic techniques have appeared, such as Metal-Affinity Precipitation of proteins with attached histidine affinity tails through formation of a metal chelate complex, e.g., with $EGTA(Zn)_2$ (Lilius, Persson, Bulow, & Mosbach, 1991) or with new Cu(II)-loaded copolymers (Mattiasson, Kumar, & Galaev, 1998). Immobilized-Metal-Ion Affinity Partitioning is another related technique for preparative extraction of proteins based on different content and distribution of histidine residues. Immobilized-Metal-Ion Affinity

Electrophoresis on, e.g., PEG–IDA–Cu II in agarose gels (Goubran-Botros, Nanak, Abdul, Birkenmeir, & Vijayalakshmi, 1992) and Immobilized-Metal-Ion Affinity Capillary Electrophoresis with soluble polymer-supported ligands (Haupt, Roy, & Vijayalakshmi, 1996) are examples of further applications of the same basic principle, although none of these techniques has become as popular as IMAC itself.

1.1 Polymeric Gels and Cryogels

Polymeric gels have applications in many different areas of biotechnology including use as chromatographic materials, carriers for the immobilization of molecules and cells, matrices for electrophoresis and immunodiffusion, and as a gel basis for solid cultural media. A variety of problems associated with using polymer gels, as well as the broad range of biological objects encountered, lead to new, often contradictory, requirements for the gels. These requirements stimulate the development and commercialization of new gel materials for biological applications.

Polymeric materials combined under the name ‘gels’ are the systems ‘polymer – immobilized (solvate) solvent’, in which macromolecules connected via non-fluctuating bonds form a 3D-network (i.e. via the bonds that, to a large extent, remain unchanged with time). The gel morphology (homo- or heterophase) is determined by the method of gel preparation, and the nature of the bonds is determined by the chemical structure of the polymers (Table 1.5). The role of the solvent immobilized within a 3D-polymer network in gels is crucial because the solvent does not allow the formation of a compact polymer mass, preventing the collapse of the system. Gels are physical objects that can withstand considerable reversible deformation without flowing or destruction. According to the nature of intermolecular bonds in the junctions of polymer network, gels can be divided into two groups: chemical and physical gels.

Table 1.5. Classification of polymeric gels and gel formation processes

Polymeric gels			
Type	**Physicochemical causes of gel-formation**	**Examples**	**Comments**
Chemotropic gels	Intermolecular chemical bonds resulting in 3D covalent network	– Polyacrylamide gels; – Ion-exchange resins based on polystyrene or polyacrylate matrices; – Cross-linked dextran gels known as Sephadexes, etc.	This is a large group of gels; the gel-formation occurs during branched polymerization of monomeric precursors, or during covalent cross-linking of polymeric precursors. The gels are widely used in biotechnology.
Ionotropic gels	Ion-exchange reactions giving rise to stable intermolecular ionic (salt) bonds	– Gels based on polyelectrolyte complexes like alginate-polylysine or chitosan-polyphosphate mixed matrices, etc.	These gels are stable in media of definite composition but could easily be dissolved by changing, for example, the pH or ionic composition in outer liquid medium. The gels are used as carriers of immobilized (encapsulated) microbial, plant and animal cells.
Chelatotropic gels	Chelating reactions giving rise to stable intermolecular coordination bonds	Gels formed on addition of multivalent strongly coordinating metal ions (e.g., Cu(II) or Co(II)) to chitosan solutions or Cr(III) ions to carboxymethyl cellulose solution or alginate gels cross-linked by, for example, calcium ions.	Ca-alginategels are used for cell immobilization, other chelatropic gels have not yet found biotechnological applications.
Solvotropic (or solvatotropic) gels	Gelation due to the changes of solvent composition	The gels formed as a result of so-called coacervation phenomenon; such type of gelation is an intermediate stage in the processes of wet-formation of films and fibers, for example, from cellulose nitrates or cellulose acetates.	Gels are formed, when a non-solvent is added to the polymer solution thus reducing the polymer affinity to the medium and promoting non-covalent polymer–polymer interactions. No documented biotechnological application.
Thermotropic gels	Gelation caused by heating of an initial polymer system	– Gels of hydrophobically modified hydroxyethyl cellulose; – Ovalbumin and egg white gels.	Intermolecular hydrophobic interactions have a significant role in the gel-formation.
Psychrotropic (from ψιχρια – psychria – chill) gels	Gelation caused by chilling (no freezing) of initial polymer system	– Gelatine gels; – Starch gels; – Agarose and agar–agar gels; – Carrageenan gels.	Psychrotropic gels are well known; they are related to the physical ones. The gels are widely used in biotechnology as solid media for cell cultivation, as chromatographic materials or as electrophoresis matrices.
Cryotropic (from κριοσ – kryos – frost, ice) gels (**cryogels**)	Gelation induced by freezing of an initial system.		

One of the new types of polymer gels with considerable potential in biotechnology is ‘cryogels’ (from the Greek krios (kryos) meaning frost or ice) (Lozinksky, 2002). Cryogels are gel matrices that are formed in moderately frozen solutions of monomeric or polymeric precursors (Lozinsky et al., 2003). Cryogels were first reported, 40 years ago and their properties, which are rather unusual for polymer gels, soon attracted attention. The biomedical and biotechnological potential of these materials has now been recognized (Lozinsky et al., 2003).

Cryotropic gelation produces polymeric materials with essentially different morphology compared with gels obtained in non-frozen systems. Cryogels could be of any chemical type- covalent, ionic or non-covalent. Obviously, only the precursors of heat induced (thermotropic) gels cannot be used for the preparation of cryogels. With some exceptions, freeze-dried polymeric materials soaked in solvent (in which the polymer swells without dissolution) can be considered as materials with macro and microstructure similar to that of cryogels. The solvent freezing followed by the sublimation of solvent crystals (ice in case of aqueous systems) forms a system of interconnected pores in the polymeric material. However, no gel formation takes place per se in unfrozen liquid microphase. Freeze-dried materials can be produced only as relatively thin objects, for example, films, plates or small beads. The production of freeze-dried cylinders or thick blocks is impractical from a technical point of view. On the contrary, cryogels can be formed in any desirable shape, for example, blocks, cylinders, tubes, granules and disks. Moreover, the production of cryogels is simpler than production of freeze-dried materials because solvent removal under reduced pressure is not necessary. A system of large interconnected pores is a main characteristic feature of cryogels; some cryogels possess spongy morphology. The pore system in such sponge-like gels ensures unhindered convectional transport of solutes within the cryogels, contrary to diffusion of solutes in traditional homophase gels. The size of macropores within cryogels varies from tens or even hundreds to only a few micrometers. The interconnected system of large pores makes various cryogels promising materials for the production of new chromatographic matrices tailormade for the separation of biological nano- and microparticles (plasmids, viruses, cell organelles and even intact cells), and also for the implementation as carriers for immobilization of molecules and cells (Lozinsky et al., 2003).

1.2.1 Cryotropic Gel Formation

Among the polymeric materials utilized in bioseparation, gel matrices are the most widely used, for instance, as carriers in chromatography, media for electrophoresis, isotachophoresis and isoelectric focusing, as media for immunodiffusion assays, etc. Gels present structured *polymer-immobilized solvent* systems in which macromolecules form a three-dimensional network fixed by relatively stable, temporally non-fluctuating bonds.

There are two main ways of producing gels (Figure 1.6).

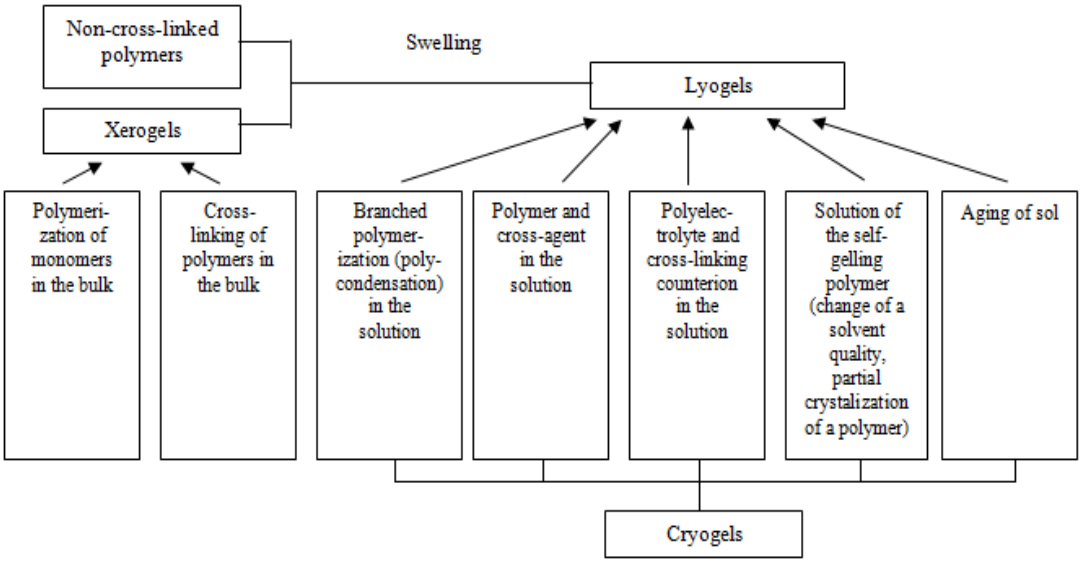


Figure 1.6 Classification of gelling systems (re-drawn from lozinsky, 1994a).

The first is via limited swelling of a non-crosslinked polymer (a block, a film, a powder or fibres) or via swelling (maximal at equilibrium) of a xerogel (a polymer network produced by chemical synthesis without solvent or by drying a lyogel). The second is via formation in a liquid system. This is the most common method employed. In this case, the initial system consists of either a solution of monomers in which gelation takes place as a result of branched polymerization, or a solution of polymer in

which gel formation is the result of chemical cross-linking, self-gelation upon the change of thermodynamic quality of the solvent, or phase transition of sol into a gel. Gels can be divided into covalently cross-linked, ionotropic gels where the macromolecules are bound by electrostatic interactions, and physical gels, where the macromolecules are bound by hydrophobic interactions and hydrogen bonds. Aging of sols usually produces heterophase gels with complicated morphology.

Cryotropic gel formation can take place (i) during freezing, e.g., in the case of gelatinized starch pastes (Richter, Augustat, & Schierbaum, 1969) or aqueous solution of locust bean gum (Bringham, 1994; Lozinsky, 2000e; Tanaka, 1998) and results in thermoreversible physical cryogels; (ii) during storage of the samples in the frozen state mainly as chemically cross-linked cryogels (Lozinsky, 1982a, b, 1998a; Rogozhin, 1982); (iii) when thawing frozen samples, which is typical for cryotropic formation of gels from aqueous poly(vinyl alcohol) (PVA) solutions (Damshkaln, 1999; Domotenko, 1988; Lozinsky, 1998b; Lozinsky, 2000).

The essential feature of cryogelation is crystallization of the solvent (Figure 1.7), which distinguishes cryogelation from the *cooling-induced gelation*, where gelation takes place upon decreasing the temperature e.g., the gelation of gelatin, or agar-agar solutions, which proceeds without any phase transition of the solvent. The latter gels can obviously be termed *psyhrotropic gels*.

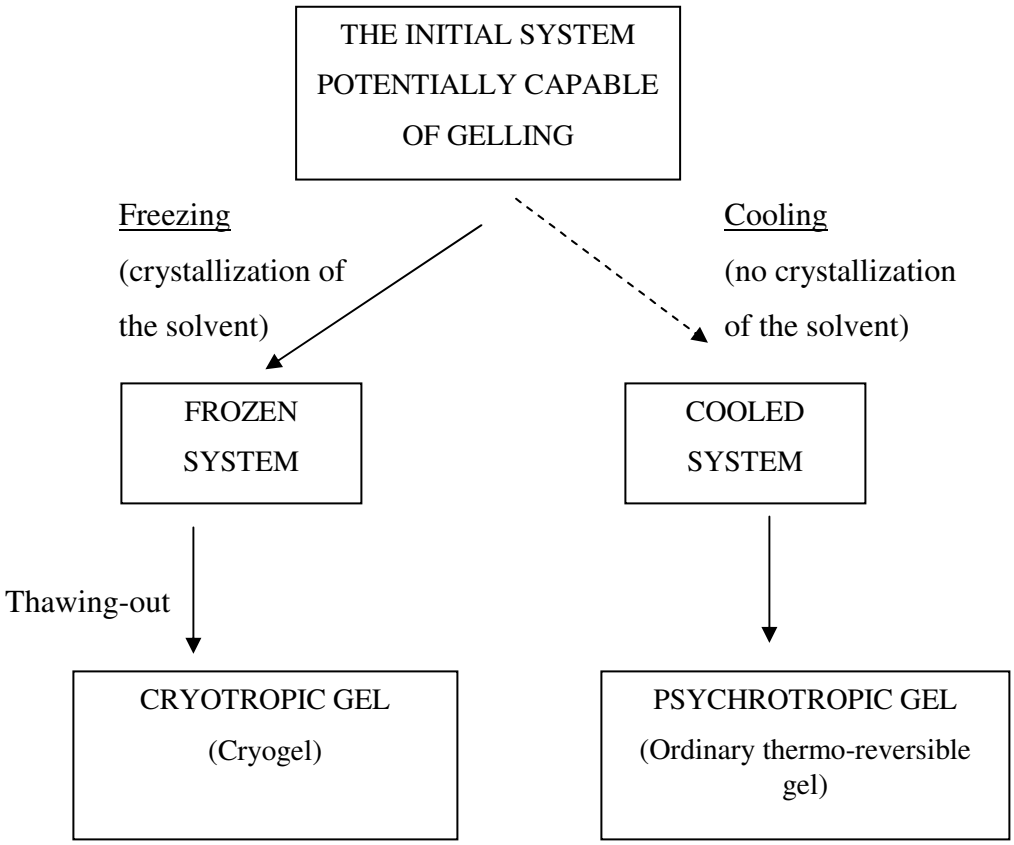


Figure 1.7. Distinctions between chilling-induced and freezing-induced gelation (re-drawn from Lozinsky, 1994a)

Gel formation in frozen media has a variety of peculiar features distinguishing it from gel formation in liquid solvents. Cryogels are formed at significantly lower concentrations of both polymer and cross-linking agent than those in gels formed at room temperature (Table 1.6).

Table 1.6 Traditional (25^0C) and cryotropic (-8^0C) production of chitosan gels by cross-linking with glutaraldehyde (Lozinsky et al., 1982b)

Ratio NH_2/CHO (mol/mol)[a]	Gel-fraction yield (%)					
	Chitosan concentration					
	1.25%		2.50%		5.00%	
	Traditional gel	Cryogel	Traditional gel	Cryogel	Traditional gel	Cryogel
60:1	–[b]	52	–	54	6.9	41
30:1	–	65	–	72	39	68
15:1	–	82	19	82	76	71
7.5:1	–	69	57	82	78	76
3.75:1	–	67	78	72	84	78

[a]Molar ratio between amino groups in chitosan and aldehyde groups of the cross-linking agent.
[b]No gelation occurred.

In other words, the critical concentration of gel formation (CCG) is considerably reduced during gelation in a frozen system. The decrease in the CCG is a characteristic feature of cryotropic processes, whether gelation takes place as the result of covalent or physical cross-linking of macromolecules, or as the result of network synthesis from the monomer precursors (Lozinsky, 1998a).

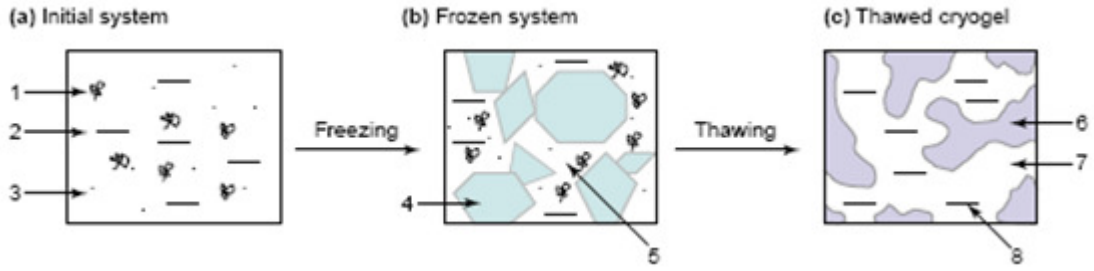
Traditional chitosan gels cross-linked with glutaraldehyde are typical monophase systems. In a swollen state they are transparent and rather brittle, especially at high degrees of cross-linking. All the solvent in the equilibrium swollen gel is bound by the polymer network. It is impossible to squeeze the solvent mechanically out of the gel without causing gel destruction. Conversely, cryogels formed from exactly the same substances are heterophase, spongelike, non-transparent materials. The total volume of the liquid inside the cryogel consists of two fractions, solvent bound by the polymer network and capillary bound solvent. The latter can easily be removed from the gel mechanically (squeezed out) even under rather small compression. Capillary-bound solvent constitutes a major part of the total solvent in cryogel, up to hundreds of mL water per g of cross-linked chitosan, while the polymer network binds only a few mL per g of cryogel. In traditional cross-linked chitosan gel, the total volume of the sample is determined only by the amount of polymer network-bound water.

Cryotropic gelation in polymerizing systems will be considered using the example of the well-known radical copolymerization of acrylamide (AAm) and *N*,*N*'-methylene-*bis*-acrylamide (BisAAm) initiated by the redox pair persulphate–tertiary amine. The kinetics of poly(acrylamide) (PAAm) cryogel formation, their macro- and microstruscture have been studied in detail (Belavtseva, 1984; Gusev, 1993; Lozinsky et al., 1983, 1984b, 1986a, 1989a; Tighe, 1991).

In pioneer studies, Butler and Bruice (Butler, 1964; Bruice, 1964) and later Pincock (Pincock, 1969; Pincock, 1966) hypothesized that in moderately-frozen solutions, part of the solvent is still unfrozen (the so-called *unfrozen liquid microphase*). Dissolved substances concentrate in these regions of non-frozen solvent (so-called *cryoconcentration*) allowing chemical reactions to proceed although the whole sample appears to be a solid block. Detailed experimental proof of the existence of non-frozen liquid microphase, as well as detailed kinetic and thermodynamic analyses of the reactions in the microphase, has been provided by Sergeev & Batyuk (Sergeev, 1976, 1978). It is clear now, that at moderately low temperatures, a macroscopically solid system consists of two phases, a polycrystalline phase of frozen pure solvent, and the above mentioned unfrozen liquid microphase containing nearly all dissolved components present in the initial solution. As the volume of the microphase is significantly less than the volume of initial solution, pronounced concentration of the dissolved substances takes place. High concentrations of dissolved substances in the microphase accelerate chemical reactions, and they can proceed even faster than reactions in a homogeneous solution above freezing point, despite the fact that the temperature is lower.

When the initial solution contains low-molecular weight, or macromolecular gel precursors, moderate freezing promotes cryotropic gel formation (Figure 1.8), which takes place at significantly lower initial concentrations of gelling agents than gelation in a liquid sample. Thus, the decrease in the CCG typical for cryogelation is due to the cryoconcentration of gel precursors.

In general, an initial system (A in Figure 1.8) could include any of the gel-forming systems indicated in Figure a. It is critical that the gelation rate is not too high, otherwise sufficient gelation will take place already in the liquid sample before it freezes. The final product in this case has a combined morphology, consisting partially of that of a traditional gel (probably destroyed in some way by frozen solvent) and that of a cryogel formed from a part of the polymer, which remained non-gelled till the sample is frozen. The frozen system is heterogeneous (B in Figure 1.8) and consists of solid phase (crystals of frozen solvent) and unfrozen liquid microphase.



1. Macromolecules in a solution
2. Solvent
3. Low-molecular solutes
4. Polycrystals of frozen solvent
5. Unfrozen liquid microphase
6. Polymeric framework of a cryogel
7. Macropores
8. Solvent

Figure 1.8 Schematic presentation of cryotropic gelation in polymer systems (re-drawn from Lozinsky, 1994a).

The volume of the microphase depends on the nature of the solvent, the initial concentration of dissolved substances, the thermal history of the sample during freezing, the presence of soluble and insoluble admixtures etc. Unfrozen microphase deliberately is presented in Figure 1.8 on a scale not reflecting the real ratio between frozen and non-frozen parts. The latter usually constitutes 0.1–10% of the total sample (Gusev, 1990, 1993; Konstantinova, 1997; Lozinsky, 1989b, 2000a; Mikhalev, 1991). One should take

into account the fact that, at such high concentration factors, dissolved substances start to precipitate out of the liquid microphase due to limited solubility. On the other hand, if the dissolved substances are consumed in a chemical reaction, e.g., gel formation, precipitated substances redissolve in the microphase. This has been seen to happen, for example, during the formation of PAAm cryogels by *in situ* NMR monitoring of the process (Gusev et al., 1993). After thawing of the frozen sample, the gel formed has a macroporous structure (C in Figure 1.8). Crystals of the frozen solvent play the role of pore-forming agent, or porogen. Melting of these crystals leaves cavities in the cryogel, which became filled with liquid solvent. Surface tension at the interface of the gel and the liquid causes the shape of the initially sharply angled cavities to become rounded. Together with macropores in between polymeric walls, the latter have micropores of their own between macromolecules forming these walls. Heterophase and heteroporous (a combination of macro- and micropores) morphology of cryogels endows them with a unique combination of physical properties.

Concentrated polymer solutions are prone to overcooling i.e., the temperature should be low enough to ensure freezing. At the thawing stage, the slower the thawing the longer the system is maintained at the temperature interval favourable for cryogelation. When thawing is too fast, only few intermolecular contacts are formed because of a relatively low mobility of macromolecules in a highly viscous liquid microphase (Gusev, 1990; Lozinsky, 1998b, Lozinsky, 2000b; Mikhalev, 1991).

1.2.2 General Properties of Polymeric Cyrogels

The most attractive feature of polymeric cryogels from the bioseparation view point is the combination of macropores formed by the crystals of frozen solvent and micropores in between polymer macromolecules forming the walls of macropores. It should be emphasized, that macropores in cryogels are not closed as in foam-like polymers e.g., foam rubber, but interconnected. This pore morphology appears in cryogels due to the fact that crystals of the freezing solvent grow until they meet the

faucets of other crystals. Thus, the polycrystalline phase in the frozen sample is a continuous one and after thawing it generates a system of interconnected pores.

1.2.3 Cryogels in Bioseparation

A crucial element of modern process biotechnology is the separation and purification of the target product from a fermentation broth or cell rupture supernatant (Lozinsky et al., 2003). The continuous demand for increasingly pure biologically active preparations (low-molecular-weight compounds, biopolymers like proteins, DNA, viruses, cellular organelles and whole cells) requires rapid improvement of existing polymeric materials used in bioseparation and the development of new materials (Lozinsky, 2001). Traditional packed-bed chromatography with immobile stationary phase, despite its elegance and high resolving power, has a major limitation: incapability of processing particulate-containing fluids, for example, cell suspensions or non-clarified crude cell homogenates. Particulate material is trapped between the beads of the chromatographic carrier resulting in increased flow resistance of the column and complete blockage of the flow. To address this drawback, expanded-bed chromatography has been proposed (Bioseparation, 1999). However, despite all its advantages, expanded bed chromatography requires a special type of columns and equipment and cannot be fitted in traditional packed bed chromatographic systems. It is attractive to have a packed-bed chromatographic carrier with pores large enough to accommodate cell debris and even the whole cells without being blocked. The porosity of cryogels makes them appropriate candidates as the basis for such supermacroporous chromatographic materials (Lozinsky et al., 2003). It is sufficient to mention the commercial introduction of high performance ion exchangers for amino acid analysis; molecular sieves, like Sephadex, for size-exclusion chromatography; electrophoresis in poly(acrylamide) (PAAm) gels; micro- and ultrafiltration membranes. These polymeric materials allowed rapid development in the isolation and purification of individual proteins and nucleic acids, as well as elucidation of their structure and function. Hence, the introduction of novel polymeric materials with new, sometimes unusual, properties, is of great interest in various areas of

biotechnology. As is often the case, it is difficult to foresee strictly defined areas in which the application of these new materials will be most beneficial (Lozinsky, 2001).

The advent of new polymeric materials has had a revolutionary effect in applied biochemistry. For instance, a continuous chromatographic column based on cryoPAAG with grafted metal chelating iminodiacetic ligands has been prepared (Arvidsson, Plieva, Lozinsky, Galaev, & Mattiasson, 2003). Owing to supermacroporosity and interconnected pore-structure, such a chromatographic matrix has a very low flow resistance. Water passes freely through the 4cm high column at linear flow rate (volumetric flow rate divided by cross-section area of the column) of ≈750–2000 cm h^{-1} at a pressure of ≈0.01 MPa. For comparison, HPLC operates at flow rates of 300–1700 cm h^{-1} at excessive pressures of 2–10 Mpa (Chase, 1994) and expanded bed chromatography at flow rates of 200–400 cm h^{-1} at excessive pressure ≈0.01 MPa (Arvidsson et al., 2003). Thus, it is reasonable to assume that continuous supermacroporous beds produced by the cryotropic gelation would allow chromatographic process at flow rates comparable with those in HPLC and exceeding those used in expanded bed chromatography, while using only minimal pressures typical for low-pressure protein chromatography. Owing to the large pore size in such cryogels, crude homogenate can be processed directly with no need for the clarification. Owing to the low diffusivity of nanoparticles, and even more so for cells, an efficient convectional transport within the pores of the matrix is needed for chromatographic separation of these objects within a reasonable time. Pore size in traditional chromatographic adsorbents is usually so that 95% of convective flow takes place in the liquid in between the beads of the chromatographic matrix. Even in the ideal case of most densely packed spheres, the interparticle volume is, 27% of the total column volume –in practice it is higher owing to the irregularities of size and shape of the beads. To improve the convectional transport, the columns with large pore size, as well as minimal dead volume, are required. The enforced convectional transport is realized in monolithic columns (Josic, 2001; Rodrigues, 1997; Tennikova, 2000).

Biomolecules has been based on the existence of highly efficient techniques for isolation and purification of molecular entities with molecular weights ≈106 Da. However, the purification of objects, often combined under the name of biological 'nanoparticles' (such as plasmids, cell organelles, viruses, protein inclusion bodies and macromolecular assemblies) still remain a challenge. Large particle sizes (20–300 nm), low diffusion rates and complex molecular surfaces distinguish such objects from soluble macromolecules (commonly ,10 nm). Macroporous inorganic chromatographic matrices, similar to porous glasses, seem to meet these requirements. They are available with a variety of pore sizes in the micrometer range, mechanically stable and sustain thermal sterilization. Although the use of these matrices for virus-specific immunosorbents has been reported (Njayou, & Quash, 1991) the brittleness, pronounced non-specific sorption and chemical instability in alkaline media limit their application.

Cryogels present a very interesting chromatographic material allowing the direct separation of proteins from unprocessed crude extracts or even from fermentation broth in the case of extracellulary expressed proteins. Cryogel-based chromatographic materials open a whole new area of bioseparation, in which bioparticles, similar to viruses, microbial cells and even mammalian cells, are isolated and separated in chromatographic mode.

It is evident that the use of cryogels as matrices of immobilized biopolymers (enzymes, polysaccharides, nucleic acids) is reasonable in cases where unique properties of cryogels give better results than traditional gel carriers. For instance, the biocatalyst produced by the immobilization of enzymes in the macropores of cryogels allows processing of macromolecular substrates because they diffuse undisturbed in the macropores (Kokufuta, 1997; Kumakura, 1997). Supermacroporous continuous beds, such as cryogel columns developed for the separation of bioparticles, are perfectly suited for the plug-flow bioreactors. The ability of macroporous PVA cryogels to retain water strongly turned out to be useful for the immobilized biocatalysts functioning in non-aqueous media. The cryogel matrix provides the water needed for enzyme to catalyze

the reaction in organic media (Belokon, 2000; Belyaeva, 2001; Filippova, 2001; Plieva, 2000).

Given that the walls of macropores in cryogels consist of highly concentrated microporous gel (Lozinsky, 2002), the diffusion of macromolecules in the pore walls is limited, if at all possible. The biopolymers could be immobilized in cryogels either via entrapment within the walls or via the attachment to the inner surface of the macropores. The former example is the immobilisation in spongy cryogels produced by the radiation cryopolymerisation of certain acrylate monomers in the presence of biopolymers to be entrapped (Kumakura, 1997, 2000). Enzymes immobilized in this way are separated from the outer medium by a dense polymer network and hence are accessible only for low-molecularweight substrates. However, the diffusion time of the substrates in pore walls is short owing to the small width of the walls.

Different studies have been done using cryogelic matrixes for purification, recognition of proteins and immobilization of enzymes (Arvidsson, 2002; Babac, 2006; Baydemir, 2009; Belyaeva, 2005; Bereli, 2008; Demiryas, 2007; Filippova, 2001; Mojovic, 1998; Plieva, 2000; Yılmaz, 2009).

1.3 Catalase

Catalase (EC 1.11.1.6), present in the peroxisomes of nearly all aerobic cells, serves to protect the cell from the toxic effects of hydrogen peroxide by catalyzing its decomposition into molecular oxygen and water without the production of free radicals. It can be found in human tissues with its highest activity in the erythrocytes, liver and kidney (Aebi, 1974), while its activity is very low in human serum (Goth, 1982). Catalase has one of the highest turnover rates of all enzymes; one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen per second. The optimum pH for catalase is approximately 7, while the optimum temperature varies

by species. It works at an optimum temperature of 37^0C, which is approximately the temperature of the human body.

Catalase is inhibited by acetate, formate, fluoride, azide and cyanide; for association, and dissociation velocities (Nicholls, 1963). Other substances combining at the sixth coordination site on catalase haematin are NH_3, NH_2OH, and N_2N_4. the catalase-azide compound reacts with peroxide to give a ferrous derivative which combines with CO. for inhibition of catalase by aminotriazole and its analogs (Nicholls, 1963). Urea and benzoate may cause denaturation of the enzyme. Catalase is inactivated (Tarhan, 1991), by freezing and thawing. Freedrying modifies the the enzyme, but retains activity. The 622 nm band shifts to the red and the iron becımes reducible by dithionite. At alkaline pH (> 10) catalase breaks up into 4 subuints with los of activity. In 5 M guanidine-HCl, or in 8 M urea, or at pH 3 the catalase molecule dissociates into 2 subunits only, whereas all other denaturating conditions used give rise to four subunits.

The protein exists as a dumbbell-shaped tetramer of four identical subunits (220,000 to 350,000 kD), each over 500 amino acids long. Each monomer contains a heme prosthetic group at the catalytic center to react with the hydrogen peroxide. Catalase monomers from certain species (e.g. cow) also contain one tightly bound NADP per subunit. This NADP may serve to protect the enzyme from oxidation by its H_2O_2 substrate.

Catalase contains 4 haematin gorups, which correspond to a haematin content of 1.1 % and an iron content of 0.09% (Aebi, 1974). Protohaematin IX, the prosthetic group of all known catalases, is loosely bound to the protein and can be split off by acid acetone. Catalase seems to have a labile tetriary structure. Removal of the prosthetic group by acidacetone gives onuly a denaturated apoenzyme. The iron in the catalases is trivalent. It can not be reduced to the ferrous state by e.g. dithionite (Akertek, 1994).

Catalase was one of the first enzymes to be purified to homogeneity, and has been the subject of intense study. The enzyme is among the most efficient known, with rates approaching 200,000 catalytic events/second/subunit (near the diffusion-controlled limit). Catalase structure from many different species has been studied by X-ray diffraction. Although it is clear that all catalases share a general structure, some differ in the number and identity of domains.

Catalase was first noticed as a substance in 1811 when Louis Jacques Thénard, who discovered H_2O_2 (hydrogen peroxide), suggested that its breakdown is caused by a substance. In 1900 Oscar Loew was the first to give it the name catalase, and found its presence in many plants and animals. In 1937 catalase from beef liver was crystallised by James B. Sumner and the molecular weight worked out in 1938. In 1969 the amino acid sequence of bovine catalase was worked out. Then in 1981, the 3D structure of the protein was revealed.

The reaction is believed to occur in two stages:

$H_2O_2 + Fe(III)\text{-}E \rightarrow H_2O + O{=}Fe(IV)\text{-}E(.+)$
$H_2O_2 + O{=}Fe(IV)\text{-}E(.+) \rightarrow H_2O + Fe(III)\text{-}E + O_2$

Here Fe(III)-E represents the iron centre of the heme group attached to the enzyme. Fe(IV)-E(.+) is a mesomeric form of Fe(V)-E, meaning that iron is not completely oxidized to +V but receives some "supporting electron" from the heme ligand. This heme has to be drawn then als radical cation (.+).

As hydrogen peroxide enters the active site, it interacts with the amino acids Asn147 (asparagine at position 147) and His74, causing a proton (hydrogen ion) to transfer between the oxygen atoms. The free oxygen atom coordinates, freeing the newly-formed water molecule and Fe(IV)=O. Fe(IV)=O reacts with a second hydrogen peroxide molecule to reform Fe(III)-E and produce water and oxygen. The reactivity of the iron

center may be improved by the presence of the phenolate ligand of Tyr357 in the fifth iron ligand, which can assist in the oxidation of the Fe(III) to Fe(IV). The efficiency of the reaction may also be improved by the interactions of His74 and Asn147 with reaction intermediates. In general, the rate of the reaction can be determined by the Michaelis-Menten equation.

Catalase can also oxidize different toxins, such as formaldehyde, formic acid, and alcohols. In doing so, it uses hydrogen peroxide according to the following reaction:

$$H_2O_2 + H_2R \rightarrow 2H_2O + R$$

Catalase present many industrial aplications, namely:
(a) Degradation of hydrogen peroxide after textile bleaching in order to prevent problems in subsequent dyeing.
(b) In the dairy industry, elimination of the H_2O_2 added to sterilize milk.
(c) Coupled with oxidases, prevention of the inactivation of the oxidases by the deleterious action of a high concentration of peroxide.
(d) Coupled with oxidases, prevention of side reactions caused by the H_2O_2 that can destroy the target product (Betancor et al., 2003).

Catalase is used in the food industry for removing hydrogen peroxide from milk prior to cheese production. Another use is in food wrappers, where it prevents food from oxidizing. Catalase is also used in the textile industry, removing hydrogen peroxide from fabrics to make sure the material is peroxide-free. A minor use is in contact lens hygiene -a few lens- cleaning products disinfect the lens using a hydrogen peroxide solution; a solution containing catalase is then used to decompose the hydrogen peroxide before the lens is used again. Recently, catalase has also begun to be used in the aesthetics industry. Several mask treatments combine the enzyme with hydrogen peroxide on the face with the intent of increasing cellular oxygenation in the upper layers of the epidermis.

1.3.1 Studies of Catalase Immobilization

Immobilized enzymes can offer many advantages over their soluble forms, making this a topic of active research in the area of biotechnology. There are various methods for enzyme immobilization. Immobilized catalase has useful applications in food inustry in the removal of excess hydrogen peroxide fron food products after cold pasteurization and in the analytical fields as a component of hydrogen peroxide and glucose biosenser systems.

Sarı et al., have been studied catalase immobilization by metal chelate affinity interaction on a magnetic metal-chelate adsorbent utilizing 2-hydroxyethyl methacrylate (HEMA) and *N*-methacryloyl-(L)-cysteine methyl ester (MAC). The Fe^{3+} loading was 12.7μmol/g of support. Fe^{3+}-chelated poly(magnetic beads were used in the immobilization of catalase in batch system. The maximum catalase immobilization capacity of the magpoly(HEMA-MAC)-Fe^{3+} beads was observed to be 192 mg/g at pH 5.5. The *K*m value for immobilized catalase [mag-poly(HEMA-MAC)-Fe^{3+}] (32.6 mM) was found higher than that for free catalase (22.8 mM). It was determined that immobilized catalase exhibits enhanced stability in reaction conditions over a wide pH range (pH 5.5-7.0) and retains an activity of 76% after 10 successive batch reactions, demonstrating the usefulness of the enzyme loaded magnetic beads in biocatalytic applications. The optimum temperature for the immobilized preparation of mag-poly(HEMA-MAC)-Fe^{3+}-catalase at 45 °C was found 5 °C higher than that of the free enzyme at 40 °C (Sarı, Akgöl, Karataş, & Denizli, 2006).

In the study of Akgöl & Denizli, a novel metal-chelate adsorbent utilizing *N*-methacryloyl-(l)-histidine methyl ester (MAH) as a metal-chelating ligand was prepared by suspension polymerization of ethylene glycol dimethacrylate (EGDMA), and MAH. Fe^{3+} ions were chelated on the magnetic beads with an average size of 150-250μm. The maximum catalase adsorption capacity of the mag-poly(EGDMA–MAH)–Fe^{3+} beads was observed as 83.2 mg/g at pH 7.0. The *K*m valuesfor immobilized catalase (mag-

poly(EGDMA–MAH)–Fe^{3+}) (20.5 mM) was determined higher than that of free enzyme (16.5 mM). Storage stability was found to increase with immobilization. It was observed that enzyme could be repeatedly adsorbed and desorbed without significant loss in adsorption capacity or enzyme activity (Akgöl & Denizli, 2004).

Arıca et al., have been studied with dye-ligand and metal-chelate poly(2-hydroxyethylmethacrylate) membranes for affinity seperation of glucose oxidase (GOD), catalase and bovine serum albumin (BSA). Cibacron Blue F3GA was covalently immobilized onto poly(2-hydroxyethyl methacrylate) (pHEMA) membranes via the nucleophilic reaction between the chloride of its triazine ring and the hydroxyl group of pHEMA. Fe^{3+} ions were complexed by chelation with immobilized Cibacron Blue F3GA molecules by changing the concentration of Fe^{3+} ions and pH of the reaction medium. The adsorption capacities of membranes with or without Fe^{3+} were determined by changing the that pH and the concentration of the proteins in the adsorption medium. It was observed that the maximum adsorption capacities of Fe^{3+}-complexed membranes for GOD, BSA and catalase were greater than unterated membranes (Arıca, Testereci, & Denizli, 1998).

In the study of Öztürk et al., bentonite-cysteine (Bent-Cys) microcomposite affinity sorbents were used for reversible immobilization of catalase. Pseudo-biospecific affinity ligand l-cysteine was covalently binded onto the bentonite structures for specific adsorption. The maximum catalase adsorption capacity of the Bent–Cys microcomposite affinity sorbents was determined as 175mg/g while the non-specific catalase adsorption onto the bentonite was determined very low (about 2.7mg/g). A degrease in the activity yield was determined with the increase of the enzyme loading. It was observed that there was a significant change between *V*max value of the free catalase and *V*max value of the adsorbed catalase on the Bent–Cys microcomposite affinity sorbents. The *K*m value of the immobilized enzyme was higher 1.1 times than that of the free enzyme. Optimum operational temperature was 10 ^{0}C higher than that of the free enzyme and was significantly broader (Öztürk, Tabak, Akgöl, & Denizli, 2008).

Kubal, & D'Souza have studied immobilization of catalase by entrapment method. An immobilized preparation of whole cell-based catalase was obtained by cross-linking the yeast cells permeabilized with toluene in hen egg white using glutaraldehyde. Optimal preparations were obtained when cross-linking was carried out for 2 h at 4 ^{0}C. Both the free and immobilized cells were exhibited pH optima at 7.0. ıt eas observed that immobilization was found to enhance the thermostability and immobilized cell preparation can be stored at 4 ^{0}C for about 40 days retaining over 90% of the original activity. Immobilized cells could be reused for the removal of H_2O_2 from milk. (Kubal, & D'Souza, 2004).

Catalase was immobilized for different aims. Costa et al., have immobilized catalase for treatment of textile bleaching effluents (Costa et al. 2001). Fernández-Lafuente et al., have studied coimmobilization of D-amino acid oxidase and catalase for transformation of D-aminoacids into α-keto acids (Fernández-Lafuente, Rodriguez, & Guisán, 1998). Betancor et al., have prepared a stable biocatalyst of catalase (Betancor et al., 2003). Yoshimoto et al., have been studied stabilization of quaternary structure and activity of catalase through encapsulation method (Yoshimoto, Sakamoto, Yoshimoto, Kuboi, & Nakao, 2007). Wan et al., have studied catalase immobilizadtion on electrospun nanofibers (Wan, Ke, Wu, & Xu, 2007). Chen et al., have developed catalase biosensor for nitrite determination (Chen, Moustry, Chen, & Cosnier, 2007). Salimi et al., have studied electrochemirty and electroactivty of immobilized catalase (Salimi, Sharifi, Noorbakhsh, & Soltanian, 2006).

CHAPTER TWO

EXPERIMENTAL METHODS AND MATERIALS

2.1 Materials

Acrylamide (AAm, more than 99.9% pure, electrophoresis reagent), allyl glycidyl ether (AGE, 99%), N,N'-methylene-bis(acrylamide) (MBAAm), ammonium persulfate (APS) and N,N,N',N'-tetramethylene diamine (TEMED, more than 99%) were supplied by Sigma (St Louis, USA). Catalase (E.C.1.11.1.6) was purchased from Sigma. All other chemicals were of the highest purity commercially available and were used without further purification. All water used in the experiments was purified using a All water used in the adsorption experiments was purified using a Millipore S.A.S 67120 Molsheim-France facility whose quality management system is approved by an accredited registering body to the ISO 9001. Before use the laboratory glassware was rinsed with deionised water and dried in a dust-free environment.

2.2 Production of p(AAm-AGE) Cryogel

Production of p(AAm-AGE) monolithic cryogel was performed using the Arvidsson et al's procedure by modification (Arvidsson et al., 2002). Allyl glycidyl ether (Allyl-2,3-epoxypropylether- AGE, % 99) was selected in order to insert reactive epoxy groups in the cryogel. Shortly, monomers (1.3068g AAm, 218µL AGE (0.97g/mL)) were dissolved in 30 mL deionized water. Total concentration of monomers was 6% (w/v). The cryogel was produced by radical polymerization initiated by TEMED (24µL) and APS (14.4mg). After adding APS the solution was cooled in an ice bath for 15 min. TEMED was added and reaction mixture was stirred for 30 min. Then, reaction mixture was poured into plastic syringe (4mL) with closed outled at the bottom. The

polymerization solution in the plastic syringe was frozen at -12^{0}C for 24h and then thawed at room temperature. After washing with 200 ml of water, the cryogel was stored in buffer containing 0.02% sodium azide at 4 ^{0}C until use.

2.3 Cibacron Blue F3GA Immobilization

p(AAm-AGE) monolithic cryogels were washed with 2L water to remove unreacted monomers. Cibacron Blue F3GA immobilization was performed using the book of immobilized affinity ligand techinques (Hermanson, Mallia & Smith, 1992). p(AAm-AGE) cryogels were bottled up into Cibacron Blue F3GA solution (100mg Cibacron Blue F3GA dissolved in 30mL deionized water) and shaked at 150rpm for 30min at 60^{0}C. 1.5g NaCl was added to reaction mixture and shaked for 1h at 60^{0}C. Then, temperature of reaction was increased to 70^{0}C and 0.15g Na_2CO_3 added to reaction mixture. Reaction was continued for 2h at 70^{0}C. Reaction mixture was cooled to room temperature and cryogels were washed with water until washings are colourless. Finally, cryogels were washed with 1.0M NaCl and water. Cibacron Blue F3GA immobilized cryogels were stored in 0.02% sodium azide at 4^{0}C.

2.4 Incorporation of Fe^{3+} Ions

Chelates of Fe^{3+} ions with Cibacron Blue F3GA immobilized p(AAm-AGE) cryogels were carried out in a recirculating system with 50mL of aqueous solutions containing 100ppm Fe^{3+} solution. Fe^{3+} solution was prepared in universal buffer at pH 5 which was the optimum pH for Fe^{3+} chelate formation at room temperature [$Fe(NO)_3 \cdot 9\ H_2O$ was used as the source of Fe^{3+} ions]. Cibacron Blue F3GA immobilized p(AAm-AGE) cryogels were conditoned with pH 5 universal buffer before Fe^{3+} immobilization. Then, Fe^{3+} solution was passed through the cyrogels for 7 hours. Fe^{3+} ion concentrations in initial solution and at equlibrium were determined with Perkin-Elmer AA 700 atomic absorption spectrophotometer. Fe^{3+} leakage from p(AAM-AGE)-CB-Fe cyrogels was

investigated as a function of medium pH (5.0-8.0) and also in the catalase desorption medium containing 0.2 M NaSCN.

2.5 Characterization of Cryogels

The swelling degree of the cryogels (S) was determined. Cryogels were dried to constant mass at vacuum oven at 55^0C and 100 mbar and masses of dried cryogels were determined ($m_{dry\ gel}$). The dried cryogels were bottled up to 50mL ionized water and masses of swollen cryogels were determined regularly for 24h period ($m_{wet\ gel}$).

The swelling degree was calculated as:

$S = (m_{wet\ gel} - m_{dry\ gel}) / m_{dry\ gel}$

Percentage of porosity and porosity for macropores were also calculated.

% porosity = [(m swollen gel - m water bound)/m swollen gel] x 100

% porosity for macropores = [(m swollen gel - m squeezed gel)/m swollen gel] x 100

The morphology of a cross section of the dried cryogel was investigated by scanning electron microscope (SEM). The sample was fixed in 2.5% gluaraldehyde in 0.15 M sodium cacodylate buffer overnight, post-fixed in 1% osmium tetroxide for 1 h. Then the sample was dehydrated stepwise in ethanol and transferred to a critical point drier temperated to $+10^0$C where the ethanol was changed for liquid carbon dioxide as transitional fluid. The temperature was then raised to $+40^0$C and the pressure to ca. 100 bar. Liquid CO_2 was transformed directly to gas uniformly throughout the whole sample without heat of vaporization or surface tension forces causing damage. Release of the pressure at a constant temperature of $+40^0$C resulted in dried cryogel sample. Finally, it was coated with gold-palladium (40: 60) and examined using a JEOL JSM 5600 scanning electron microscope (Tokyo, Japan).

FTIR spectra of the p(AAm-AGE), p(AAm-AGE)-CB and p(AAm-AGE)-CB-Fe^{3+} cryogel were obtained by using a FTIR spectro-photometer (FTIR 8000 Series, Shimadzu, Japan). The dry cryogel (about 0.1 g) was thoroughly mixed with KBr (0.1 g, IR Grade, Merck, Germany), and pressed into a tablet, and the spectrum was then recorded.

2.6 Chromatographic Procedures

2.6.1 Catalase Adsorption Studies

The adsorption experiments were conducted for 30min which was the equalibrium period for the adsorption of catalase at room temperature. Initial and final protein concentrations were determined by UV-spectrophotometer (Schimadzu 1601, Japan). The amount of adsorbed catalase was calculated as

$$Q = \frac{(C_0 - C)\,V}{m}$$

where Q is the amount of catalase adsorbed on a unit mass of the dry cryogels (mg/g); Co and C are the concentrations of catalase in the initial solution and in the aqueous phase after treatment for certain period of time, respectively (mg/mL); V is the volume of the aqueous phase (mL); and m is the mass of the dry cryogels used in adsorption (g).

Catalase adsorption to the matrix was studied at various pH values, either in acetate buffer (0.05 M, pH 4.0-5.0) or in phosphate buffer (0.05 M, pH 6.0-8.0). The initial catalase concentration was 0.5 mg/mL in the corresponding buffer. To optimize conditions, different catalase concentrations in the medium (0.1-1.5 mg/mL) were studied at optimum pH value. To determine the effect of temperature on adsorption capacities, adsorption studies were performed between 4-45^{0}C. Effect of ionic strengh on adsorption capasity was studied in the range between 0.05M and 1.0M NaCl. Flow rate was also studied and value was varied in the range 0.3-2.0 mL/min.

2.7 Activity Assays of Free and Immobilized Catalase

Catalase activity was determined spectrophotometrically, by direct measurement of the decrease in the absorbance of hydrogen peroxide at 240 nm due to its decomposition by the enzyme. Hydrogen peroxide solutions (10mM) were used to determine the activity of both the free and the immobilized enzyme. The decrease in absorbance at 240 nm was recorded for 1 min that catalase activity was complied with first order reaction kinetic. The rate of change in the absorbance ($\Delta A240$ min^{-1}) was calculated from the initial linear portion with the help of the calibration curve (the absorbance of hydrogen peroxide solutions of various concentrations (1–30mM) at 240 nm). One unit of activity is defined as the decomposition of 1μmol hydrogen peroxide per min at 25^0C and pH 7.0. 1mL 10mM H_2O_2 solution was reacted with 10μL free catalase (0.5mg/mL) and the decrease in the absorbance at 240nm was followed for 1min to determine the activity of free catalase. 10mM H_2O_2 solution was passed through the catalase immobilized cyrogels to determine the activity of immobilized enzyme. The absorbance of the H_2O_2 solution, recovered from cryogels, was determined and the immobilized catalase activity was calculated. These activity assays were carried out over the pH range of 4.0–8.0 and temperature range of 5–45 ^{0}C to determine the pH and temperature profiles for the free and immobilized enzyme. The effect of substrate concentration was tested in the 5–30mM H_2O_2 concentrations range. Km and Vmax values of the enzyme were determined by measuring initial rates of the reaction with H_2O_2 (10 mM) in phosphate buffer (50 mM, pH 6.0) at 25 ^{o}C.

2.8 Storage Stability

The activities of free and immobilized catalase (0.5 mg/mL) in phosphate buffer (50 mM, pH 7.0) were measured every day periodically as mentioned above. At the end of activity assay period, the enzyme-immobilized cryogels were washed with 0.05 M, pH 7.0 phosphate buffer for three times.

2.9 Desorption of Catalase from Cryogels and Repeated Use

Catalase desorption from cryogels was carried out with 0.2 M NaSCN solution. The desorption ratio was calculated from the amount of catalase adsorbed on the cryogels and the final protein concentration in the desorption medium.

$$\text{Desorption Ratio (\%)} = \frac{\text{amount of catalase desorbed}}{\text{amount of catalase adsorbed}} \times 100$$

The cryogels were washed with deionized water for a while and conducted with buffer used in adsorption and were then reused in enzyme adsorption.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1. Characterization of p(AAm-AGE)-CB-Fe^{3+} Cryogel

A supermacroporous cryogel was produced by copolymerization in the frozen state of monomers, acrylamide (AAm) and allyl glycidyl ether (AGE) with N,N'-methylene-bis(acrylamide) (MBAAm) as a cross-linker in the presence of amonium persulfate (APS)/N,N,N',N'-tetramethylene diamine (TEMED) as initiator/activator pair. The functional epoxy groups on the surface in the pores of the cryogels allowed their modification with the ligand, Cibacron Blue F3GA. The scanning electron micrograph of the internal structure of the poly (AAm-AGE)-CB-Fe^{3+} cryogel is shown in Figure 3.1. p(AAm-AGE)-CB-Fe^{3+} cryogel produced in such a way have non-porous and thin polymer walls, large continuous inter-connected pores (10-100 μm in diameter, supermacro-porous) that provide channels for the mobile phase to flow through. Pore size of the matrix is much larger than the size of the protein molecules, allowing them to pass easily. Size of catalase is 7 nm × 8 nm × 10 nm, and a relative molecular weight is 240,000. As a result of the convective flow of the solution through the pores, the mass transfer resistance is practically negligible. The equilibrium swelling degree of the p(AAm-AGE)-CB-Fe cryogel was 21.8 g H_2O/g cryogel. p(AAm-AGE)-CB-Fe cryogel is opaque, sponge like and elastic. This cryogel can be easily compressed by hand to remove water accumulated inside the pores. When the compressed piece of cryogel was submerged in water, it soaked in water and within 1-2 s restored its original size and shape. Percentage of porosity and porosity for macropores were also determined as 4.4 % and 77.2%, respectively.

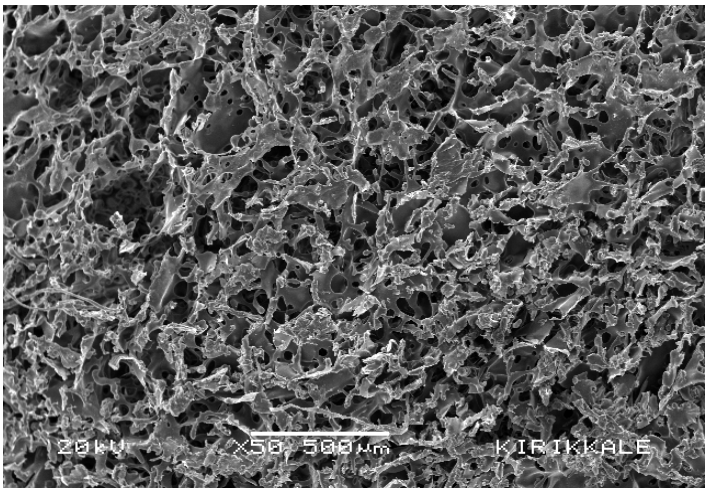


Figure 3. 1 Scanning electron micrograph of the inner part of the p(AAm-AGE)-CB-Fe^{3+} cryogel matrix.

The schematic diagram for the production of p(AAm-AGE) cryogel and immobilization of Cibacron Blue F3GA molecules through the p(AAm-AGE) cryogel was given in Figure 3.2.

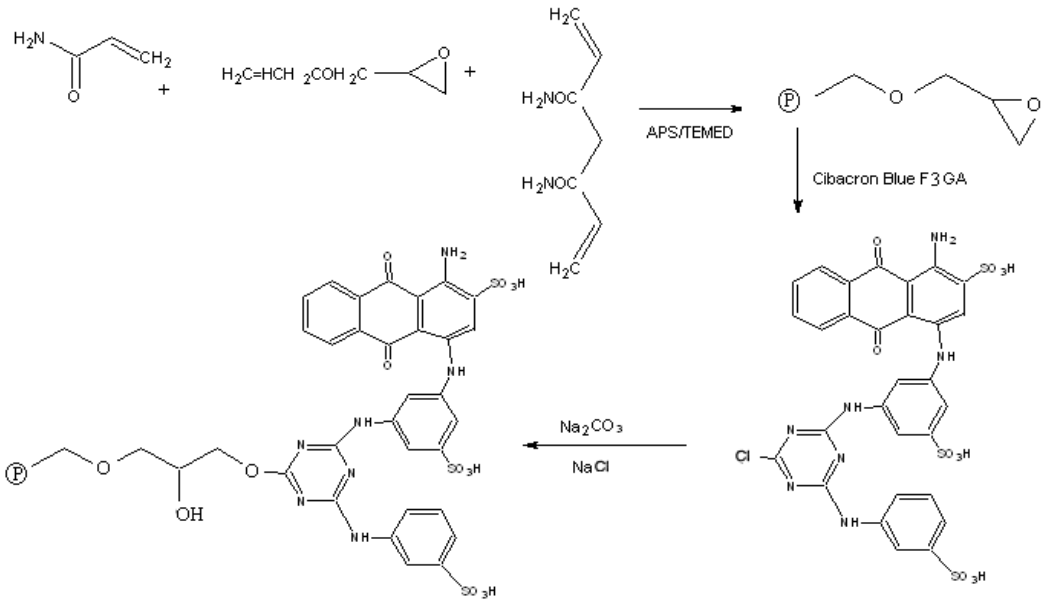


Figure 3.2 Schematic diagram for the production of p(AAm-AGE) cryogel and immobilization of dye molecules through the p(AAm-AGE) cryogel.

Cibacron Blue F3GA was used as the dye-ligand. Then, Fe^{3+} ions covalently incorporated onto matrix. The incorporation of Cibacron Blue F3GA was found to be 168.2 µmol/g cryogel. The studies of Cibacron Blue F3GA leakage from the p(AAM-AGE) cryogel showed that there was no dye leakage in any medium used throughout this study. Maximum Fe^{3+} loading was found to be 25.8 µmol/g cryogel. The number of locations of surface-exposed electron-donating amine groups and their ability to coordinate with chelated metal ions dictate the adsorption of proteins on metal-immobilized adsorbents.

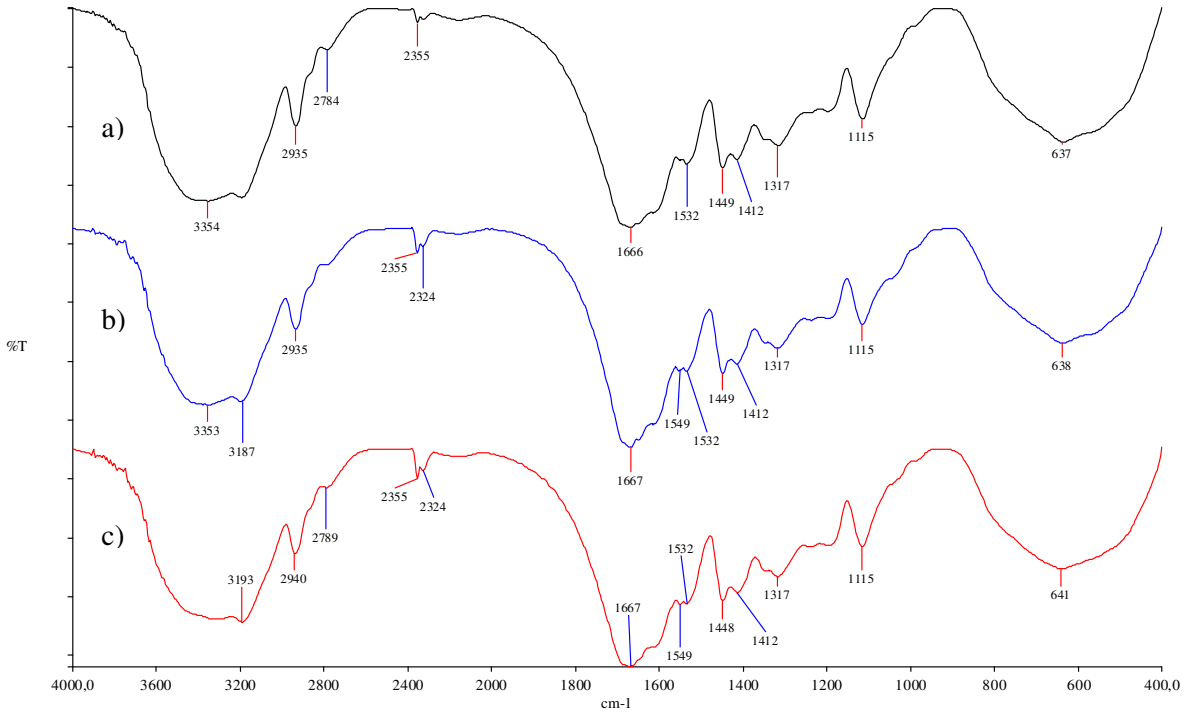


Figure 3.3 FTIR spectrum of a) p(AAm-AGE) b) p(AAm-AGE)-CB c) p(AAm-AGE)-CB-Fe^{3+} cryogels.

Cibacron Blue F3GA is covalently immobilized on p(AAm-AGE) cryogel, via the reaction between the chloride groups of the reactive dyes and the epoxide groups of the AGE**.** The FTIR bands observed around 1549 cm^{-1} was assigned to N-H bending (Figure 3.3). The FTIR bands observed around 1400 cm^{-1} and 2900 cm^{-1} were assigned to bending of C-H and streching of C-H, respectively. It should be noted that the bending band of C-H

in epoxide groups of AGE observed 2784 cm^{-1} was absent in figure 3.3.b which shows FTIR spectrum of p(AAm-AGE)-CB. The band observed at 3500 cm^{-1} was assigned to the -OH functional group. After Cibacron Blue F3GA attachment, the intensity of the -OH band increases due to NH stretching. The band observed at 3187 cm^{-1} indicates also streching of N-H groups of the dye molecule. The shift observed at the band around 3200 cm^{-1} might be due to the chelation between $–NH_2$ groups of the dye and Fe^{3+} ions. These bands show the attachment of Cibacron Blue F3GA within the p(AAm-AGE) cryogel. The visual observations (the colour of the cryogel) ensured attachment of dye molecules**.**

3.2 Optimization of Catalase Adsorption

3.2.1 Effect of Contact Time

Effect of contact time on catalase adsorption onto p(AAm-AGE)-CB-Fe^{3+} cryogel is shown in figure 3.4. Adsorption increased with time and enzyme adsorption reached a plateau at 60min which shows the saturation.

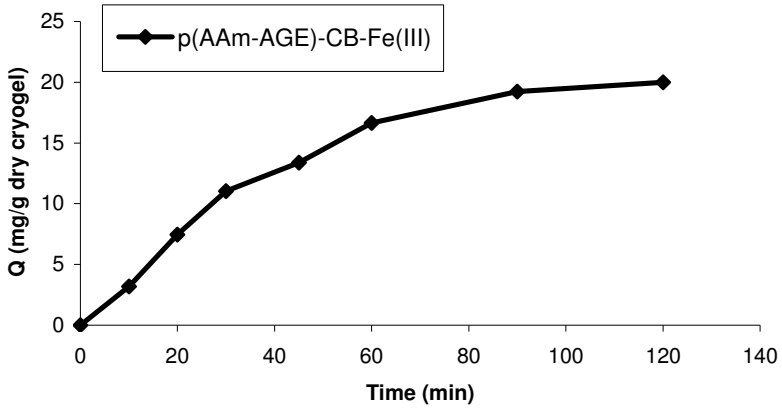


Figure 3.4 Effect of contact time on the catalase adsorption:
pH: 6.0, Catalase concentration: 0.5mg/mL, Flow Rate: 0.5mL/min, T: 25^0C

3.2.2 Effect of pH

The effect of pH on the adsorption of catalase onto p(AAm-AGE)-CB-Fe^{3+} cryogel was studied in the pH range 4.0- 8.0 and the effects of pH on adsorption are presented in Figure 3.4. The variation in adsorption capacities was marginal in nötral and basic conditions (pH 7-8). Nevertheless, increase in adsorption capacities was quite significant for increasing pH in the range of pH 4.0-6.0. Suggested by Sharma and Agarwal, the optimal pH for adsorption largely depends upon the nature of the metal ion, the chelating ligand, and the three dimentional structure of the protein. Protons affect the net charge on the immobilized metal ion as well as the protein. The operational pH range is usually between pH 5.0-8.0. For immobilized Cu^{2+}, Ni^{2+}, Co^{2+}, and Zn^{2+}, which are directed chiefly toward accessible histidine residues on the proteins, the optimal adsorption occurs in the pH range 6.0-8.0. However, for hard metal ions, such as Ca^{2+}, and Fe^{3+}, maximal adsorption occurs in the lower pH range (5.0-6.0) (Sharma, & Agarwal, 2001). In this study, catalase adsorption increased with increased pH from 4.0-6.0 and then decreased when the pH was raised further. The reason of these observation can be explained as follows: The partial deprotonation of the exposed histidine residue of the protein would be promoted by increasing pH in the range 4.0-6.0, which would increase the possibility of spesific binding with immobilized cationic iron ions (Wu, Suen, Chen, & Tzeng, 2003). The maximum adsorption of catalase was found close to its p*I* 6.4, it was slightly shifted (0.4 pH unit) to rather acidic pH value. The reason of decreasing adsorption capacity at alkaline pH can be explained like that at a more alkaline range, cordinations with amino functional groups are favored, thus decreasing selectivity (Ueda, Gout, & Morganti, 2003). Also at alkaline pH, surface of enzyme becomes cationic, so electrostatic repulsions between enzyme and Fe^{3+} ions on matrix increase. Thus, adsorption capacity of matrix decreases.

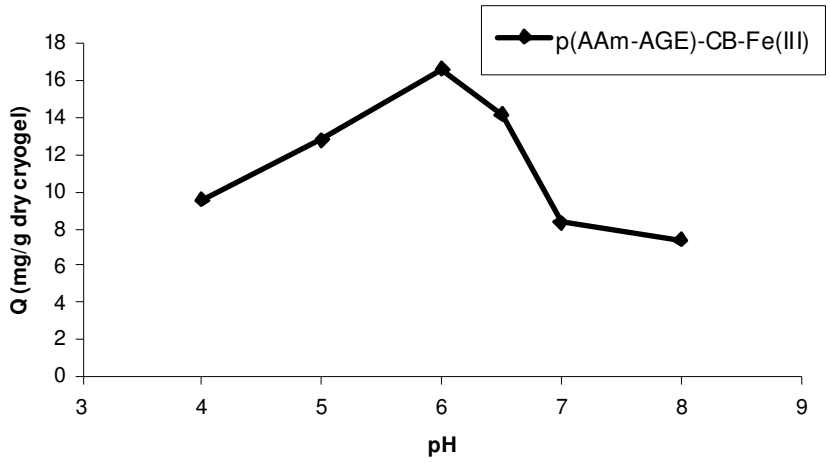


Figure 3.5 Effect of medium pH on the catalase adsorption:
Catalase concentration: 0.5mg/mL, Flow Rate: 0.5mL/min, T: 25^0C
Contact time: 60min

3.2.3 Effect of Initial Catalase Concentration

Figure 3.6 shows the effect of initial catalase concentrations on adsorption. It was observed that the amount of adsorbed protein was increased with the initial protein concentration. Maximum adsorption capacities were found to be 75.7 mg/g for p(AAm-AGE)-CB-Fe^{3+} cryogel and 60.6 mg/g for p(AAm-AGE)-CB cryogel, respectively, and the adsorbed amounts per unit mass of cryogel reached to a plateau value at about 1.5mg/mL. It should be also noted that negligible amounts of catalase adsorbed non-specifically on the plain p(AAm-AGE) (3.0 mg/g for catalase). It is clear that this increase is due to ternary complex formation between triazine dye, Fe^{3+} ions and protein molecules. The binding of proteins to transition metals occurs via the electron donating side chains of residues such as histidine and cysteine, which substitue water molecules coordinated to the metal. The number of binding sites on the protein (histidine and cysteine residues), the chelate-polymer structure and pH influence metal affinity protein

precipitation (Denizli, Köktürk, Salih, Kozluca, & Pişkin, 1997). Catalase is an iron containig metalloenzyme and this may cause a spesific interaction either the iron ions on the cryogel through the iron binding sites of the catalase. This high spesific interaction may lead to an increase in the Qmax value (about 25%) of the Fe^{3+}-attached p(AAm-AGE)-CB cryogel.

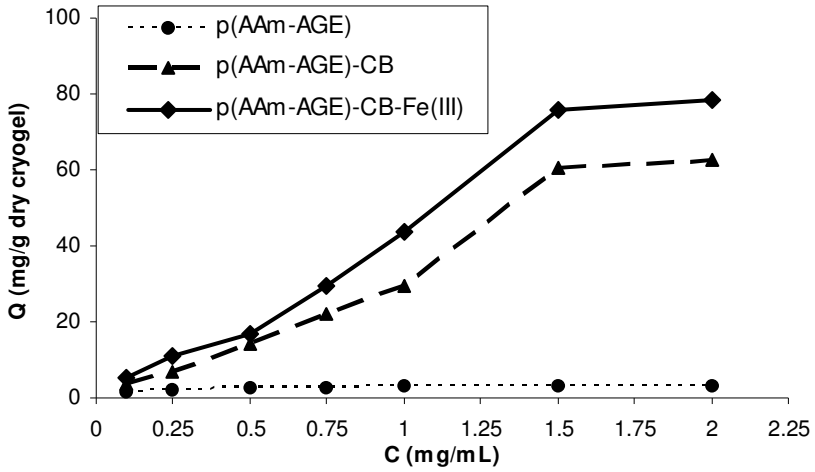


Figure 3.6 Effect of initial catalase concentration on catalase adsorption: pH: 6.0, Contact time: 60min, Flow Rate: 0.5mL/min, T: 25^0C

3.2.4 Effect of Temperature

Effect of temperature on catalase adsorption was studied in the range of 4-45 ^{0}C. Catalase adsorption capacity of the p(AAm-AGE)-CB-Fe^{3+} cryogel was significantly increased with increasing temperature but it sharply decreased over 25^0C. As seen in figure 3.7, from 4 to 25^0C, the adsorption capacity of cryogel increased about 95%. This may due to that Van der Waals attraction forces increase with increasing temperature. It is believed that protein adsorption on IMA gels is a cumulative effect of electrostatic (or ionic), hydrophobic, and/or donor–acceptor (coordination) interactions (Ueda et al., 2003). The reason of the decrease over 25^0C may be reduction of interactions between enzyme and Fe^{3+} ions and sterical hinderance.

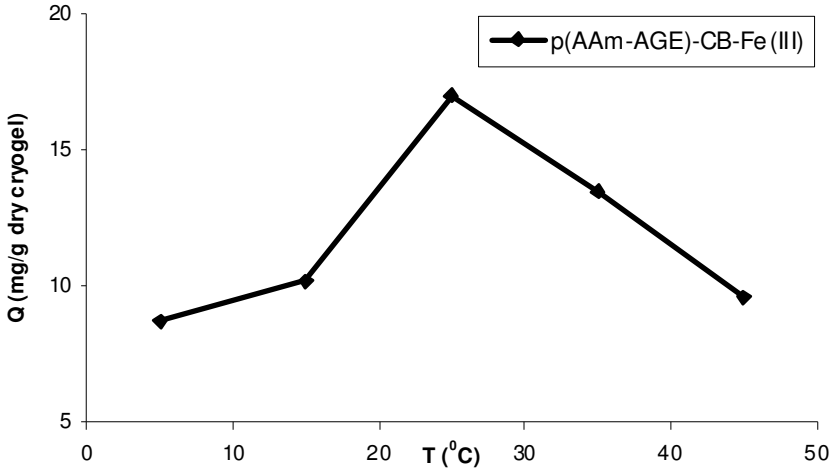


Figure 3.7 Effect of temperature on catalase adsorption:
pH: 6.0, Catalase concentration: 0.5mg/mL, Contact time: 60min,
Flow Rate: 0.5mL/min,

3.2.5 Effect of Flow Rate

The adsorption capacities at different flow-rates are given in figure 3.8. Results showed that the catalase adsorption capacity of the p(AAm-AGE)-CB-Fe^{3+} cryogel decreased significantly from 16.6 to 10.8 mg/g with the increasing flow-rate from 0.5 to 2.0 mL/min. This is due to decrease in contact time between the catalase molecules and the p(AAm-AGE)-CB-Fe^{3+} cryogel at higher flow-rates. These results are also in agreement with those referred to the literature (Yılmaz et al., 2009). When the flow-rate decreases, the contact time in the column is longer. Thus, catalase molecules have more time to diffuse to the pore walls of cryogel, hence a better adsorption capacity is obtained.

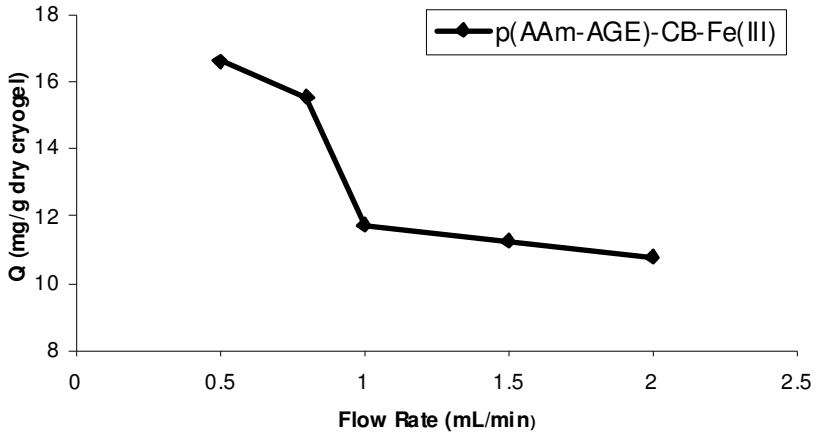


Figure 3.8 Effect of flow rate on catalase adsorption:
pH: 6.0, Catalase concentration: 0.5mg/mL, Contact time: 60min, T: 25^0C

3.2.6 Effect of Ionic Strength

Effect of ionic strength on catalase adsorption is given in figure 3.9. As shown in figure 3.9, the amount of catalase adsorbed onto p(AAm-AGE)-CB decreased about 18.6 % as the NaCl concentration changes from 0.05 to 1.0 M. Increasing the NaCl concentration could promote the adsorption of the dye molecules to the polymer surface by hydrophobic interaction. Moreover, the hydrophobic interactions between the immobilized dye molecules themselves would also become strong, because it has been observed that the salt addition to a dye solution caused the stacking of the free dye molecules. Thus, the numbers of the immobilized dye molecules accessible to catalase would decrease as the ionic strength increased, and the adsorption of the catalase to immobilized dye became difficult (Demiryas et al., 2007). Conversely, catalase adsorption onto p(AAm-AGE)-CB-Fe^{3+} cryogel increased with increasing ionic strength from 0.05M to 0.1M NaCl. Higher protein adsorption capacities under low ionic strength conditions may be due to nonspecific adsorption and hence, signify the role of amino acid residues other than histidine, cysteine, and tryptophan that are usually considered responsible for immobilized metal affinity phenomenon. This observation also hints at the weak ion-exchange properties shown by chelating gels. Although charging the gels

with metal ions drastically alters their adsorption characteristics, they still function as weak ion-exchangers under low ionic strength conditions, but with altered charge characteristics Such behavior has normally been noticed for IMA gels loaded with hard metal ions such as Fe^{3+}, Al^{3+}, Ca^{2+}, Yb^{3+} (Sharma, & Agarwal, 2001). The effect of electrolytes on protein retention is releated to the affinity of metal ion for its solvated water molecules. Weakening of the forces between the metal ions and water molecules by increasing the ionic strength of the buffers, favors the protein adsorption processes. The decrease of adsorption capacity observed above 0.5M NaCl salt concentration may be due to that an increase in NaCl concentration result in the reduction of electrostatic interactions (Yavuz, & Denizli, 2004).

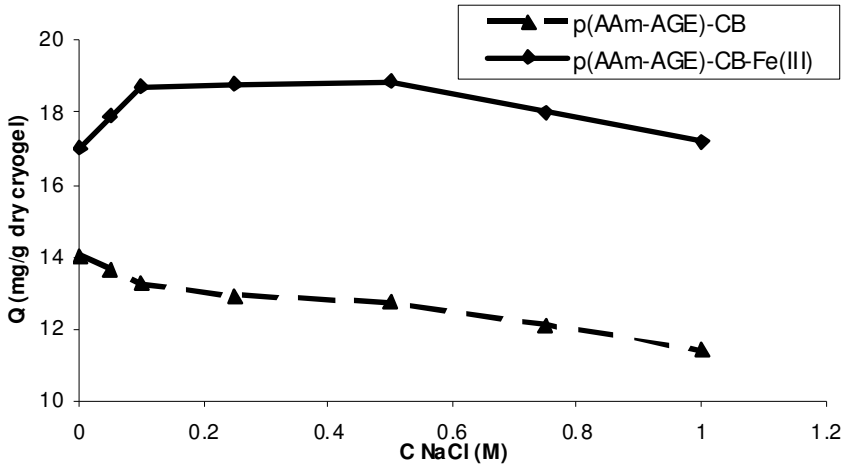


Figure 3.9 Effect of ionic strengh on catalase adsorption:
pH: 6.0, Catalase concentration: 0.25mg/mL, Flow rate: 0.5mL/min
Contact time: 60min, T: 25^0C

3.3 Desorption Studies and Repeated Use

Desorption of catalase from the p(AAm-AGE)-CB-Fe^{3+}cryogel was also carried out in continuous system. p(AAm-AGE)-CB-Fe^{3+}-CAT preparation was placed within the desorption medium containig 0.2M NaSCN at room temperature for 2h. It was then repeatedly used in adsorption. It should be noted that there was no Cibacron Blue F3GA and Fe^{3+} release in this case.

In order to show the reusability of the p(AAm-AGE)-CB-Fe^{3+} cryogel, the adsorption-desorption cycle was repeated five times using the same cryogel. There was no remarkable reduce in the adsorption capacity of the cryogel (Figure 3.10). This result showed that Fe^{3+} incorporated p(AAm-AGE)-CB cryogel can be repeatedly used in enzyme immobilization without detectable losses in its initial adsorption capacity.

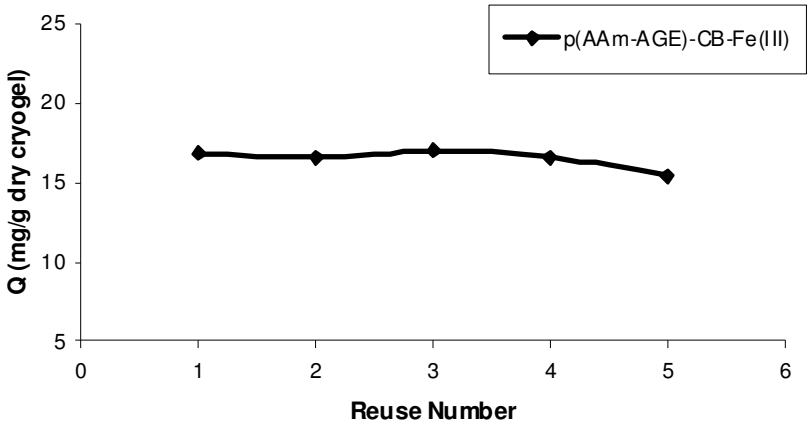


Figure 3.10 Repeated use of p(AAm-AGE)-CB-Fe^{3+} cryogel
pH: 6.0, Catalase concentration: 0.5mg/mL, Flow rate: 0.5mL/min
Contact time: 60min, T: 25^0C

3.4 Kinetic Parameters

Kinetic parameters, namely, the Michaelis constant (K_m) and V_{max} for free and immobilized catalase were determined using H_2O_2 as the substrate with Lineweaver-Burk plots. V_{max} defines the highest possible rate when the enzyme is saturated with substrate, so this parameter reflects the intrinsic characteristics of the adsorbed enzyme, although it can be affected by diffusion constraints. K_m is defined as the substrate concentration that gives a reaction rate of $1/2V_{max}$. This parameter reflects the effective characteristics of the enzyme and depends on both partition and diffusion effects (Kara, Osman, Yavuz, Beşirli, & Denizli, 2005). In our study, as expected, the K_m and V_{max} values were

significantly affected by adsorption of catalase onto the p(AAm-AGE)-CB-Fe^{3+} cyyogel. The K_m values were found to be 0.73 g/L for the free catalase and 0.18 g/L for the immobilized catalase, i.e., there was an approximately 4-fold decrease in the K_m value for the adsorbed enzyme. The V_{max} value of free catalase ($2.0x10^3$ U/mg enzyme) was found to be lower than that of the immobilized catalase ($2.5x10^3$ U/mg enzyme). The Km value is known as the affinity of the enzymes to substrate (Park, Haam, Jang, Ahn, & Kim, 2005) and the lower values of Km emphasize the higher affinity between enzymes and substrates. The results have shown that the affinity of the catalase to its substrate was increased by immobilization. The change in the affinity of the enzyme to its substrate is probably caused by structural changes in the enzyme introduced by the immobilization procedure. The V_{max} value of immobilized catalase was found higher than that of free catalase. The reason of this increase might be attributed to the conformational changes on the enzyme surface.

3.5 Effect of Temperature and pH on the Catalytic Activity

The temperature dependence of the activities of free and immobilized catalase was studied in 50 mM phosphate buffer (pH 7.0) in temperature range 4–45^0C and temperature profiles of free and immobilized catalase are shown in Figure 3.11. The behavior of the activity with temperature was as expected: increased temperature caused an increased activity, up to optimum reaction temperature and then overlapped with the deactivation of the enzyme in a reversible or irreversible ways or both. As a result, the activity increased to a peak and then fell down. Optimum temperature was found at about 37^0C for free catalase and immobilized catalase. In general, the effects of changes in temperature on the rates of enzyme catalyzed reactions don't provide much information on the mechanism of catalysis. However, these effects could be important in indicating structural changes in enzymes (Akgöl, &Denizli, 2004). Immobilized catalase showed higher activity in a wider temperature range than the free enzyme. The conformational flexibility of the enzyme might be affected by immobilization.

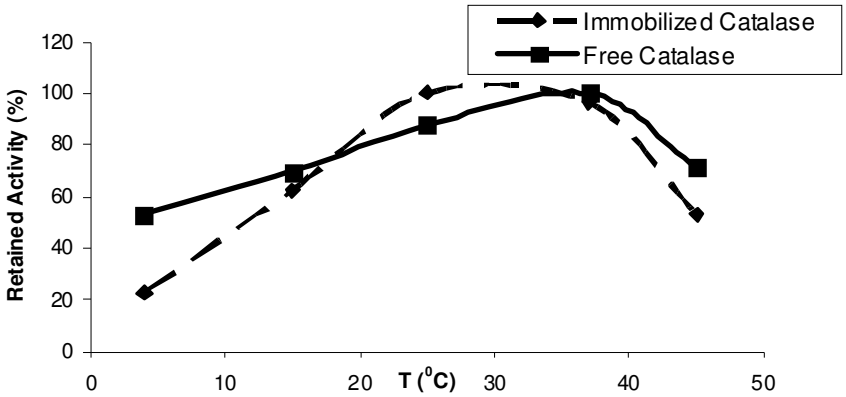


Figure 3.11. Temperature profiles of free and immobilized catalase; catalase concentration: 0.5 mg/mL, pH:7.0

The pH effect on the activity of free and immobilized catalase for hydrogen peroxide degradation was studied at various pHs at 25 ^{0}C. The reactions were carried out in acetate and phosphate buffers and the results are presented in Figure 3.12. The pH optima of free and immobilized catalase were 7.0 and 6.5, respectively. There was a shift of 0.5 pH value towards the acidic side resulting from immobilization of catalase on p(AAm-AGE)-CB-Fe^{3+} cryogel. A shift in enzyme activity upon immobilization towards acidic or basic direction is natural since the microenvironment of the free and immobilized enzyme is quite different. The pH profile of the immobilized catalase was much broader than that of the free enzyme, which means that this method preserves the enzyme activity in a wider pH range. Similar observations, for immobilization of catalase and other enzymes have been reported by other researchers (Çetinus, 2003; Sarı, 2006). This may also be due to secondary interactions (e.g., ionic and polar interactions, hydrogen bonding) between the enzyme and the p(AAm-AGE)-CB-Fe^{3+} cryogel. This indicates that sensitivity to pH is reduced as a result of immobilizaiton.

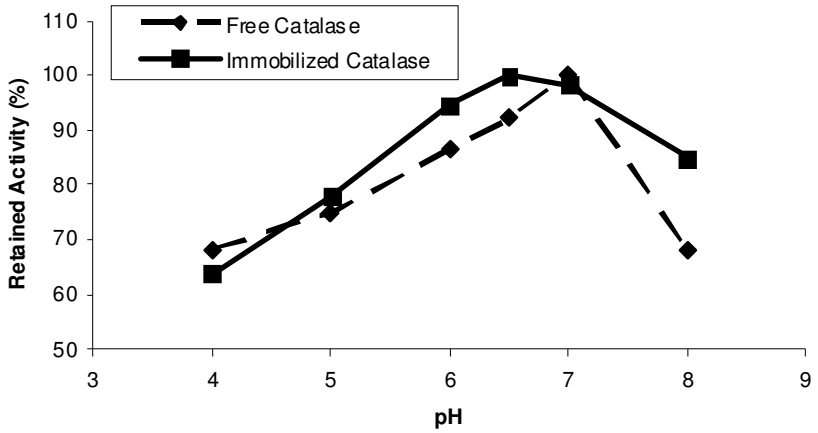


Figure 3.12 pH profiles of free and immobilzed catalase;
Catalase concentration: 0.5 mg/mL; T: 25^0C.

3.6 Thermal Stability of Catalase

The effect of temperature on the stability of the free and immobilized catalase is shown in figure 3.13. The activities of free and immobilized enzymes did not significantly changed in the temperature range of 15-37^0C. At 45^0C, the free catalase lost all its initial activity after a 6 h of heat treatment, whereas the immobilized catalase showed significant resistance to termal inactivation (retaining about 20% of its initial activity after the same period). These results suggest that termostability of immobilized catalase becomes higher at higher temperature. If the heat stability of enzymes increased upon immobilization the potential applications of enzymes would be extended.

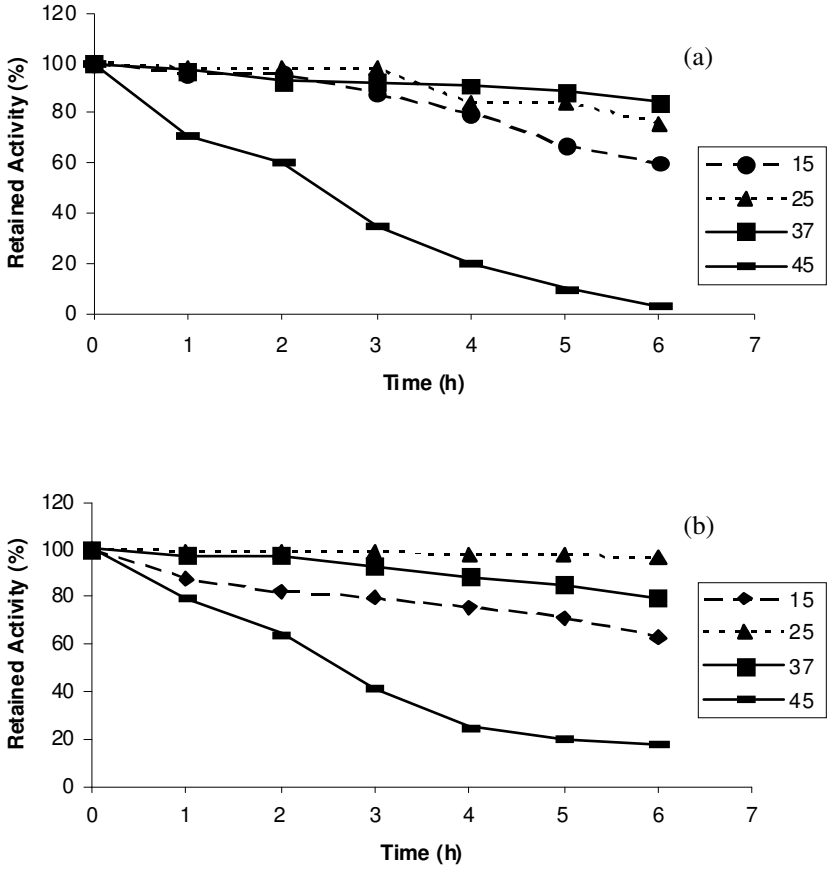


Figure 3.13 Thermal stability of free (a) and immobilized catalase (b)
catalase concentration: 0.5 mg/mL; pH:7.0

3.7 Storage Stability

Free and immobilized catalase preparations were stored in a phosphate buffer (50mM pH 7.0) at 4^0C and the activity measurement were carried out for a period of 20 days (Figure 3.14). The free enzyme lost all of its activity within 20 days whereas the immobilized enzyme lost about 30 % of its activity during the same period. The decrease in activity was explained as a time-dependent natural loss in enzyme activity and this was prevented to significant degree by immobilization.

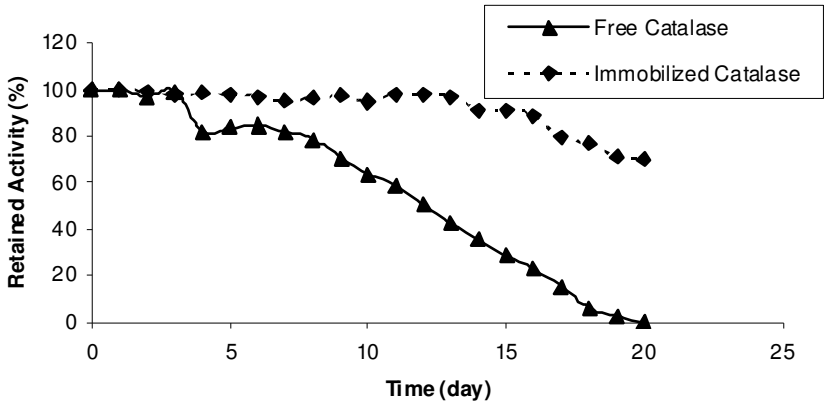


Figure 3.13 Storage stability of free and immobilized catalase

Catalase concentration: 0.5 mg/mL; pH:7.0

CHAPTER FOUR

CONCLUSIONS

The time consuming and high cost of metal-chelating procedure has inspired a search for suitable low-cost sorbents. The main advantage of immobilized metal affinity chromatography (IMAC) consists in its simplicity, universality, stability and cheapness of the chelating supports (Ueda, 2003; Altintas, 2006). Catalase has been successfully immobilized onto p(AAm-AGE)-CB-Fe^{3+} cryogel. The immobilized catalase preparation retains much of its activity over wider ranges pH than does the free form, which means that this method preserves the enzyme activity in a wider pH range. The optimum temperature profile of the immobilized enzyme was not drastically modified upon immobilization. Optimum temperature was found at about 37^0C for free catalase and immobilized catalase and also immobilized catalase showed higher activity in a wider temperature range than the free enzyme. The reusability and storage stability of the immobilized catalase presented in this work may provide economic advantages for large-scale biotechnological applications.

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