

SUPERMACROPOROUS HYDROPHOBIC AFFINITY SORBENTS FOR PROTEIN CHROMATOGRAPHY

PROTEİN KROMATOĞRAFI İÇİN SÜPERMAKROGÖZENEKLİ HİDROFOBİK AFİNİTE SORBENTLER

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ÖZ

Hidrofobik etkileşim kromatografisi; proteinlerin yapısında bulunan polar olmayan bölgeler ile hidrofobik ligandlar arasında gerçekleşen hidrofobik etkileşimleri temel alan bir protein saflaştırma tekniğidir. Bu teknik proteinlerin yüzeylerinin farklı hidrofobik karakterlere sahip olması temeline dayanır. Bu çalışmada, adsorbent olarak poli(2-hidroksietil metakrilat-metakriloltriptofan), poli(HEMA-MATrp) kriyojeli hazırlanmıştır. MATrp monomeri, ligand olarak yapısına girdiği poli(HEMA) ya hidrofobik özellik kazandırır. Poli(HEMA) ise temel taşıyıcı katı destek olarak seçilmiştir. 60–100 µm gözenek büyüklüğüne sahip süpermakrogözenekli hidrofobik poli(HEMA-MATrp) kriyojeli sulu dağıtma ortamında, HEMA ve MATrp monomerlerinin serbest radikal polimerizasyonu ile üretilmiştir. Süpermakrogözenekli hidrofobik kriyojel 50 m²/g spesifik yüzey alanına sahip olup; şişme deneyleri, yüzey alanı, gözenek boyut analizi, Fourier Transform Infrared Spektroskopisi (FTIR), Nükleer Magnetik Rezonans (NMR), Taramalı Elektron Mikroskopisi (SEM), ile karakterize edilmiştir. Denge şişme oranı poli(HEMA-MATrp) için 6.99 g H₂O/g kriyojel iken poli(HEMA) için 7.18 g H₂O/g' dır. Bu polimerler sulu çözeltiden ve yumurta akından lizozim adsorpsiyonu için kullanılmıştır. Adsorpsiyon deneyleri, farklı ortam koşullarında sürekli kolon sisteminde incelenmiştir (pH, akış hızı, protein konsantrasyonu, sıcaklık, tuz etkisi). Poli(HEMA-MATrp) ve poli(HEMA) kriyojellerin lizozim adsorpsiyon kapasitesi sırasıyla 44.68 mg/g ve 2.9 mg/g olarak bulunmuştur. Poli(HEMA-MATrp) kriyojeli yumurta akından lizozim saflaştırılması için kullanılmış ve adsorpsiyon kapasitesi 12.9 mg/g olarak bulunmuştur. Desorpsiyon için etilen glikol kullanılmış ve %91 geri kazanım sağlanmıştır. Adsorpsiyon–desorpsiyon döngüsü 5 kez tekrarlanmış ve adsorpsiyon kapasitesinde önemli bir değişim gözlenmemiştir. Fluorimetrik ölçümler sulu çözeltideki ve yumurta akındaki lizozim için yapılmıştır. Lizozimin denaturasyonu 560 nm deki sinyalin şiddetindeki artışla tespit edilmiştir. Yumurta akındaki lizozim hızlı protein sıvı kromatografisi ile

ayrılmıştır. Lizozimin saflığı SDS-PAGE elektroforez yöntemiyle belirlenmiştir. Isısal kararlılığı tespit etmek için termogravimetrik analiz (TGA) yapılmıştır.

ANAHTAR KELİMELELER: Protein Saflaştırılması, Hidrofobik Etkileşim Kromatografisi, Lizozim, Kriyojel.

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ABSTRACT

Hydrophobic interaction chromatography (HIC) takes advantage of the hydrophobicity of proteins promoting its separation on the basis of hydrophobic interactions between hydrophobic ligands and non-polar regions on the surface of the proteins. HIC is assumed to separate proteins by the differences in their surface hydrophobic character. In this study, poly(2-hydroxyethyl methacrylate) poly(HEMA) cryogel containing 2-methacryloyltryptophan (MATrp) ligand, as a comonomer, providing hydrophobic functionality to the adsorbent was prepared. We selected poly(HEMA) as the basic solid matrix. Supermacroporous hydrophobic cryogel with 60-100 μm pore size were obtained by free radical polymerization of HEMA and MATrp, conducted in an aqueous dispersion medium. Supermacroporous hydrophobic cryogel had a specific surface area of 50 m^2/g and were characterized by means of swelling studies, surface area measurement, pore size analysis, Fourier Transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR), Scanning Electron Microscope (SEM) and Thermogravimetric analysis (TGA). The equilibrium swelling ratio of the cryogel is 6.99 g $\text{H}_2\text{O}/\text{g}$ for poly(HEMA-MATrp) and 7.18 g $\text{H}_2\text{O}/\text{g}$ cryogel for poly(HEMA). These polymers were used for the adsorption of lysozyme from the aqueous solution and chicken egg white. Adsorption experiments were investigated under different conditions in a continuous system (i.e., medium pH, flow rate, protein concentration, temperature, salt type). Lysozyme adsorption capacity of poly(HEMA-MATrp) and poly(HEMA) cryogels were estimated as 44.68 mg/g and 2.9 mg/g respectively. Poly(HEMA-MATrp) cryogel was used for the purification of lysozyme from chicken egg white and adsorption capacity was estimated as 12.9 mg/g. Ethylene glycol was used for desorption of lysozyme. The adsorbed lysozyme was desorbed with 91% recovery. It was observed that lysozyme after 5 adsorption-desorption cycle, poly(HEMA-MATrp) cryogel can be used without significant loss in lysozyme adsorption capacity. Fluorimetric measurements were done for both aqueous solution of lysozyme and egg white

lysozyme. Intensity increasing at 560 nm showed us denaturation of lysozyme. Fast protein liquid chromatography was used for separation of egg white lysozyme. Purity of lysozyme was estimated by using SDS-PAGE. Thermostability of cryogel was observed with thermogravimetric analysis.

KEYWORDS: Protein Purification, Hydrophobic Interaction Chromatography, Lysozyme, Cryogel.

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To the memory of my dear father
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1. INTRODUCTION

The development of techniques and methods for the separation and purification of proteins has been essential for many of the recent advancements in biotechnology research. The purity of a protein is a pre-requisite for its structure and function studies or its potential application. A wide variety of protein purification techniques are available today, however, different types of chromatography have become dominant due to their high resolving power. In gel filtration chromatography, dye-affinity chromatography, ion-exchange chromatography, affinity chromatography and hydrophobic interaction chromatography (HIC), protein separation is dependent on their biological and physico-chemical properties; molecular size, net charge, biospecific characteristics and hydrophobicity, respectively (Kennedy, 1991; Garcia and Pires, 1993). Affinity chromatography is already a well-established method for the identification, isolation, separation and purification of biomolecules and based on highly specific molecular recognition (Scouten, 1981; Scopes, 1982; Turkova, 1993). In this method, a molecule having specific recognition capability is immobilized on a suitable insoluble support, which is usually a polymeric material in bead or membrane form. The molecule to be isolated is selectively captured by the complementary ligand immobilized on the matrix by simply passing the solution containing the target through the chromatographic column or in a batch system under favorable conditions. The target molecules are then eluted using proper elutants under conditions favoring desorption, adjusting the pH, ionic strength or temperature, using specific solvents or competitive free ligands, so that the interaction between the ligand and the target is broken and the target molecules are obtained in a purified form (Turkova, 1993). Since its first introduction, thousands of different molecules (enzymes, antibodies, hormones, vitamins, receptors, many of variety of other proteins and glycoproteins, RNA, DNA, etc. (Deustscher, 1990; Scouten, 1981; Matejtschuck, 1997). Dyes have been incorporated with a variety of carrier systems and have been used for separation of biomolecules from different sources (Klinkman, 1989; Ray, 1984; Denizli, 1995 (a,b,c); Kayırhan-Denizli, 2001; Odabaşı and Denizli, 2001).

Although ion Exchange chromatography (IEC) remains the most widely used chromatographic method for large-scale separations, Hydrophobic interaction chromatography (HIC) is also widely used. HIC is usually assumed to separate proteins by the differences in their surface hydrophobic character (Fausnaugh and Regnier, 1986).

Hydrophobic interaction chromatography (HIC) takes advantage of the hydrophobicity of proteins promoting its separation on the basis of hydrophobic interactions between immobilized hydrophobic ligands and non-polar regions on the surface of the proteins. The adsorption increases with high salt concentration in the mobile phase and the elution is achieved by decreasing the salt concentration of the eluent (Melander and Horvath, 1977; Fausnaugh and Regnier, 1986; Roe, 1989). Therefore, the term of "salt-promoted adsorption" could be used for this type of chromatography (Porath, 1986). The different types of elution conditions can be used for purification of complex mixtures of proteins that would be difficult to separate using other chromatographic techniques. In fact, HIC has been successfully used for separation purposes as it displays binding characteristics complementary to other protein chromatographic techniques (Janson and Ryden, 1993). During the last years, HIC has been studied by many researchers and today it is an established and powerful bioseparation technique in laboratory-scale, as well as in industrial-scale purification of proteins (Wu and Karger, 1996; Grund, 1998). The development of a large variety of stationary phases for HIC has promoted a wide range of HIC applications in the purification of biomolecules, like serum proteins, nuclear proteins, hormones, recombinant proteins and enzymes (Comings, 1979; Hrkal and Rejnkova, 1982; Lefort and Ferrara, 1986; Angelova, 1997; Queiroz, 1999).

Hydrophobicity is the repulsion between a non-polar compound and polar environment such as water. This term is also been used to refer to particular molecular models, generally involving the ordering of water molecules around non-polar solutes (Dill, 1990). If non-polar compounds are put into water they spontaneously associate due to hydrophobic interactions. An increase in entropy ($\Delta S > 0$) is observed, resulting from a displacement of the ordered water molecules

around the non associated hydrophobic groups to the more unstructured bulk water. This implies a negative change in free energy ($\Delta G < 0$) and a thermodynamically favorable process. In other words, the interaction between two or more hydrophobic molecules in aqueous solutions proceeds spontaneously and is mainly determined by the change in entropy (Lewin, 1974; Jennissen, 1976; Ochoa, 1978; Tanford, 1980; Shansky, 1990).

A lot of different type of hydrophobic molecules which have side chains of non-polar amino acids such as alanine, methionine, tryptophan and phenylalanine on their surface can be used as a ligand in HIC. These pseudospecific ligands have low binding constants (10^{-4} - 10^{-6} M⁻¹) and consequently, belong to weak affinity ligands family. Nevertheless, they can exhibit selectivity resulting from the cumulative effects of multiple weak binding events as; electrostatic, hydrophobic, hydrogen binding and van der Waals interactions with fast kinetics (Pitiot, 2000). Recently small amino acid molecules as pseudospecific ligands may hold certain advantages for industrial bioaffinity separations since they are not likely to cause an immune response in case of leakage into the product. These ligands are also much more stable than protein ligands because they don't require tertiary structure for maintaining biological activity (Huang and Carbonell, 1999). They offer additional advantages over biological ligands in terms of economy, ease of immobilization, and high adsorption capacity. L-Phenylalanine has been used as a pseudospecific hydrophobic ligand for the isolation of tRNA ligase (Forrester and Hancock, 1973) and chorismate mutase/prephenate dehydratase (Gething and Davidson, 1976), purification of human IgG from serum (Doellgast and Plaut, 1976).

Lysozyme is an enzyme that destroys bacterial cell walls. It does so by hydrolyzing the β (1→4) glycosidic linkages from N-acetylmuramic acid (NAM or MurNac) to N-acetylglucosamine (NAG or GlcNac) those which are cell wall peptidoglycans. It likewise hydrolyzes β (1→4)-linked poly(NAG), a cell wall of fungi as well as the major component of the exoskeletons of insects and crustaceans. Lysozyme occurs widely in the cells and secretions of vertebrates, where it may function as a

bactericidal agent or help dispose of bacteria after they have been killed by other means.

Lysozyme is a commercially valuable enzyme, and its common applications are (1) as a cell-disrupting agent for the extraction of bacterial intracellular products, (2) as an antibacterial agent in ophthalmologic preparations, (3) as a food additive in milk products, and (4) as a drug for the treatment of ulcers and infections (Ghosh, 2000 (a,b)). The potential for its use as an anticancer drug has been demonstrated by animal and in vitro cell culture experiments (Das, 1992). Lysozyme has also been used in cancer chemotherapy (Cartei, 1991). In a recent article, it has been reported that lysozyme can be used to increase the production of immunoglobulin by hybridoma technology (Murakami et al., 1997).

The aim of this study is to prepare a tryptophan containing hydrophobic sorbent, for selective and efficient separation of lysozyme from aqueous and egg white solutions. The present work was organized in three main parts. In the first part of the study; a new tryptophan containing comonomer 2-methacryloyltryptophan (MATrp) was synthesized by reacting methacryloyl chloride with L-tryptophan. Then, poly(2-hydroxyethyl methacrylate) [poly(HEMA)] having methacryloyl tryptophan (MATrp) ligand as a comonomer, providing hydrophobic functionality to the adsorbent were obtained by free radical polymerization of HEMA and MATrp conducted in an aqueous dispersion medium.

The main criteria of selection of poly(HEMA) as the basic component are its inertness, mechanical strength, chemical and biological stability and well tolerability. Cryogel was characterized using, pore size analysis, Fourier Transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR), elemental analysis, Scanning Electron Microscope (SEM) and swelling test. Lysozyme adsorption properties of these adsorbents from aqueous solutions were investigated at different pH's and different salt types in a continuous system. The last part of the study, desorption of lysozyme and reusability of these pseudospecific hydrophobic affinity adsorbents were tested. This novel approach for the preparation of affinity matrices has many advantages over conventional

techniques; those need the activation of the matrix for ligand immobilization (Bueno et al., 1996). In this procedure, MATrp comonomer acts as the ligand, and there is no need to activate the matrix for the ligand immobilization. MATrp monomer is polymerized with HEMA and no leakage of the ligand is possible.

2. GENERAL INFORMATION

2.1. Bioaffinity Chromatography

Bioaffinity chromatography has the highest selectivity among affinity techniques. 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 substance (e.g., between substrates or regularity compounds and enzymes; and so on) but biospecific interactions occur even more often between two or several biopolymers, particularly proteins. The interaction of an affinity ligand with a biomolecule increases with the degree of complementarity of the interacting groups.

Therefore substrate analogues, competitive inhibitors, allosteric inducers and coenzymes are the most suitable bioaffinity ligands for enzymes. Examples of biological interactions used in affinity chromatography are listed in Table 2.1.

Table 2.1. 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 (Porath et al., 1973; O'Carra et al., 1973) and the respective interaction-biospecific adsorption or bioaffinity (O'Carra, 1974; Barry and O'Carra, 1973). The original term "affinity chromatography" acquired a broader meaning also including hydrophobic chromatography (Shaltiel, 1974), covalent chromatography (Blumberg and Strominger, 1974), 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 2.2 summarizes some of these techniques. As can be seen from Table 2.2, some of these subcategories have become accepted useful techniques (Wilcheck and Miron, 1999).

Table 2.2. 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.	Receptor Affinity
	11.	Weak AC
	12.	Perfusion AC
	13.	Thiophilic Chromatography
	14.	High Performance AC
	15.	Affinity Density Perturbation
	16.	Library-Derived Affinity
	17.	Affinity Partitioning
	18.	Affinity Electrophoresis
	19.	Affinity Capillary Electrophoresis
	20.	Centrifuged AC
	21.	Affinity Repulsion Chromatography

Schematic representation of the main steps in affinity chromatography is shown in Figure 2.1. Affinity chromatography demonstrated in this figure is based on the simple principle that every biomolecule usually recognize another natural or artificial molecule. A wide variety of ligands may be covalently attached to an inert support matrix, and subsequently packed into a chromatographic column.

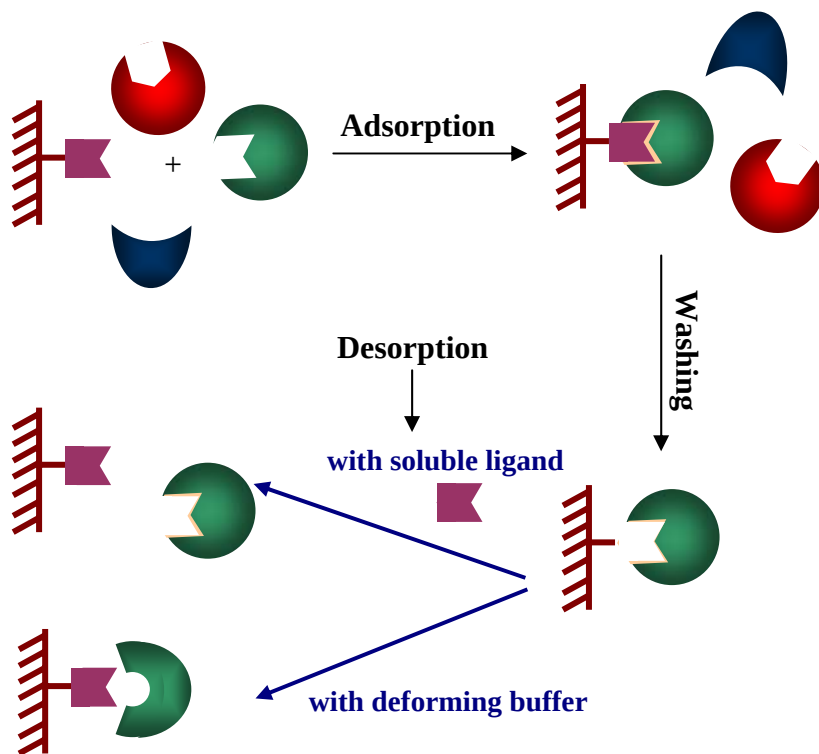


Figure 2.1. Schematic representation of the main steps in affinity chromatography.

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.

Affinity chromatography has proved to be of great value also in the fractionation of nucleic acids, where complementary base sequences can be used as ligands, and in the separation of cells, where cell surface receptors are the basis of the affinity. Its main use has, however, been in the context of protein purification.

A field that has been so successful that it is often treated separately is affinity based on antigen-antibody interactions, called immunosorption. Sometimes this is the only available route to the purification of a protein and is especially attractive when there is a suitable monoclonal antibody at hand.

Very often the use of affinity chromatography requires that the investigator synthesizes the adsorbent. The methods for doing this, which are described later, 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 commercially 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 counterligand. 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 and Ryden, 1998).

2.2. Design of an Affinity Chromatography Process

Each protein separation process must be individually optimised and no general rules can predict the factors and parameters for a specific separation. However, some general factors that influence the design of an affinity chromatography process and its incorporation in a final purification protocol are well known (Labrou

and Clonis, 1994; Lowe, 2001). These factors are mainly determined by the nature and quality of desired product and its intended use (Lowe et al., 2001; Sadana and Beelaram, 1994; Narayanan, 1994).

For example proteins for industrial use need not always be absolutely pure whereas therapeutic proteins need to be extremely pure (>99,9%) to minimise the risk of side effects or immunogenic response (Lowe, 2001; Turkova, 1999).

Also, the desired amount of the final product must be defined as it may influence the process. For example, a final dose of pharmaceutical is not known until the end of clinical trials (Sadana et al., 1994).

Certain factors determine the success of an affinity chromatographic step. Some of them are;

- 1) the ligand selectivity,
- 2) recovery,
- 3) through put,
- 4) reproducibility,
- 5) stability, maintenance and economy

The selectivity of the ligand perhaps is the most important parameter. Recovery is determined by the amount of biological active protein that is eluted after the end of the process, and throughput is among the several factors that influence dramatically the cost effectiveness of the process. Reproducibility must be determined in order for the process get validation. Differences in purity should be taken into consideration before a purification method is incorporated in large scale protocol. Ligand stability is also an important factor which determines the durability of the column itself and the cost of the process (Lowe, 2001). Downstream processing costs can be minimal in the case of industrial enzymes, because the purity requirements are low. In contrast, the purity of pharmaceutical proteins, especially those to be administered should be very high (99,9%) .

In such cases, purification and recovery costs can account for as much as 80% of

the total manufactured cost. Product cost increases with the number of steps involved in the process, not only because of the capital and operating costs but also because the overall yield decreases with the number of steps.

2.2.1. Affinity Interactions

The chemistry of biological interactions involves a variety of mostly non-covalent contacts between reactive groups in the receptor and ligand. Affinity reactions between biological molecules are regulated by different types of interactions which depend on their spatial configuration, their charge, their hydrophobicity, etc. These various forces may be complementary and additive, their sum determining the dissociation constant (k_d), or affinity of the binding such that, $k_d = [A][B]/[AB]$ where A is for instance, an antibody, B is an antigen and AB is the complex formed between them.

Binding affinity is also influenced by solid support characteristics and the activity and functional availability of the solid phase-bound protein (Cuatrecasas, 1972) such that the K_d for affinity chromatography varies between 10^{-3} and 10^{-7} M (1985). Both the chemical and mechanical properties of the solid phase are important in this regard. Non-specific interactions between the solid phase and ligand are inconsistent and unpredictable and therefore complicate elution profiles (Fleminger et al., 1990).

On the other hand the physical structure of the solid phase polymer may sterically hinder effective interaction between free ligand and immobilized protein even at high k_d values. Both of these problems can be significantly reduced by prudent use of inert spacer arms (Soltys and Etzel, 2000; Scouten, 1981).

Ultimately, the k_d for a particular biological interaction can only be used as a guide to developing an appropriate elution system. For very high affinities (10^{-8} M) such as hormone-receptor, high affinity monoclonal antibody-hapten and immobilized dye ligand interactions (Cochet et al., 1994).

The elution buffer may need to be very stringent. This can lead to partial denaturation (Narhi et al., 1997) and loss of activity of the released target protein. Alternatively, weak interactions may result in lack of binding specificity and low purification factors, although this can also be used to advantage.

2.2.2. Choice of Matrix

As in all adsorption chromatography, an adsorbent with a large surface area per unit column volume is desirable to maximize the capacity of the affinity adsorbent. Hydrophilic gels with a high surface-to-volume ratio are very stable as matrices. For affinity chromatography applications, the ideal gel material should meet the following characteristics.

1. Macroporous to accommodate the free interaction of large molecular weight proteins with ligands that could themselves be proteins or other macromolecules.
2. Hydrophilic and neutral to prevent the proteins from interacting nonspecifically with the gel matrix itself.
3. Contain functional groups to allow derivatization by a wide variety of chemical reactions.
4. Chemically stable to withstand harsh conditions during derivatization, regeneration, and maintenance.
5. Physically stable to withstand hydrodynamic stress in packed beds and, when applicable, sterilization by autoclaving.
6. Readily available at low cost to facilitate industrial applications.

These characteristics point to gels based on polymers highly substituted with alcohol hydroxyls and thus to polysaccharides. Among the latter, the spontaneously gel-forming galactan agarose indeed possesses most of the characteristics of an ideal matrix for affinity chromatography. The major weakness of native agarose is its chemical and physical instability, which, however, has been largely compensated for by chemical cross-linking of the physically cross-linked so-called junction zones in the agarose gel structure. Ever since its introduction in 1968 by

Cuatrecasas, Wilchek, and Anfinsen, 4% agarose has been the most popular matrix for affinity chromatography (Cuatrecasas, 1972; Wilchek and Anfinsen, 1968). A contributing reason for this popularity, in addition to the advantageous matrix properties as such, is that there were early simple and convenient coupling methods developed for agarose and even commercially available.

Less frequently used gel matrices for affinity chromatography are cellulose, cross-linked dextran, polyacrilamid, and silica. To this group also belong the potentially interesting and commercially available matrices made of mixtures of polyacrylamide and agarose (Ultrogel) and polymerized tris (hydroxymethyl) acrilamid (Trisacrl, Biosepra, France). Gels based on cross-linked dextran and polyacrilamid both suffer from the serious disadvantage of having too small pore diameters, which become still smaller after derivatization with affinity ligands. Also, fewer methods are available for immobilization on polyacrilamide.

In traditional low-pressure affinity chromatography systems, beads with a diameter of approximately 100 μm are usually standard. However, beads with diameters in the range 5 to 30 μm are used in so-called high-performance liquid affinity chromatography (HPLAC). In this variety of affinity chromatography, higher pressure drops are often required, which also means a demand for higher gel rigidity. This is why the first HPLAC applications were based on modified and derivatized porous silica. The major reason for using smaller particles is to increase the chromatographic efficiency by decreasing the diffusion path lengths and increasing the interphase area between the stationary and the mobile liquids. An alternative to silica for HPLC applications, which has also proven useful in HPLAC, is small-diameter agarose beads. Synthetic organic polymers, highly substituted with alcohol by hydroxyls and with adequate porosity and rigidity, should also present interesting matrices. Some of these are now commercially available (Janson and Ryden, 1998).

2.2.3. Choice of Ligand

The rapid growth of bioinformatics and molecular docking techniques and the introduction of combinatorial methods of systematic generation and screening of

large numbers of novel compounds, has made feasible the rapid and efficient generation of ligands for affinity chromatography (Spalding, 1991; Labrou, 2002; Clonis, 1990). A good affinity ligand should possess the following characteristics:

1. The ligand must be able to form reversible complexes with the protein to be isolated or separated.
2. The specificity must be appropriate for the planned application.
3. The complex constant should be high enough for the formation of stable complexes or to give sufficient retardation in the chromatographic procedure.
4. It should be easy to dissociate the complex by a simple change in the medium, without irreversibly affecting the protein to be isolated or the ligand.
5. It should have chemical properties that allow easy immobilization to a matrix.

For any particular protein intended to be purified by affinity chromatography, there is often a choice of several different ligands. In addition to the obvious choice of using a monoclonal antibody of appropriate affinity, which is generally applicable to all immunogenic solutes, one may look for components of naturally occurring biospecific pairs such as enzyme-substrate (analogs), enzyme-cofactor and enzyme-inhibitor complexes. For glycoproteins, there is the possibility of using immobilized carbohydrates. Considerable interest has been focused immobilized biomimetic dyes, which show a wide variety of specificities applicable to several groups of enzymes, plasma proteins, and other proteins such as interferons.

2.2.3.1. Classification of Ligands

Generally ligands may be classified as either monospecific or groupspecific, each of which in turn may be into low molecular weight or macromolecular.

2.2.3.1.1. Monospecific Low Molecular Weight Ligands

This group includes ligands such as steroid hormones, vitamins and certain

enzyme inhibitors. The term monospecific refers to the fact that these ligands bind to a single or a very small number of proteins in any particular cell extract or body fluid. Despite the high specificity, nonspecific adsorption may occur. This can be due to interaction with the ligand or with residues from the immobilization reaction or the spacer arm. One way to cope this problem is to make a second adsorbent lacking only the ligand itself and allow to the desorbed material from the ligand-containing adsorbent to pass this under identical conditions (Kula et al., 1979).

2.2.3.1.2. Group-Specific Low Molecular Weight Ligands

This is the largest group of ligands containing a wide variety of enzyme, cofactor and their analogs. This group also includes biomimetic dyes, boronic acid derivatives, and a number of amino acids and vitamins. Despite its relatively broad specificity, very high purification factors may be obtained using specific elution protocols with either soluble cofactors or by ternary complex formation using a combination of cofactor and substrate. Alternatively, when the ligand-enzyme association with a soluble cofactor may result in adequate separation. Ligands of a high molecular weight type (e.g., proteins) with a large number of suitable functional groups can normally be immobilized without adversely influencing structure or function. In low molecular weight ligands the coupling of course results in a relatively large change in the molecule. If the affinity interaction decreases, a chemical modification of the ligand may be necessary to provide it with an appropriate functional group for immobilization.

The functional group used should permit the formation of a stable covalent bond so that the ligand is not released from the matrix. This is particularly important for small ligands where "single-point attachment" is often the case. For proteins, "multipoint attachment" between ligand and matrix is rather common. In such affinity adsorbents, the stability of each individual bond is less critical.

It is, of course, also essential that the ligand remains intact during the immobilization procedure and that it is sufficiently stable to allow the planned affinity chromatography to be carried out.

This might be a problem when proteins are coupled at high pH. It is essential that the ligand reagent is as pure as possible and, in particular, does not contain substances with functional groups that can react competitively in the immobilization. Proteins should be subjected to gel filtration to remove low molecular weight substances such as ammonium sulfate (Janson and Ryden, 1998).

2.2.4. Choice of Spacer Arm

Occasionally, an affinity adsorbent might show poor function due to low steric availability of the ligand. This rarely happens with high molecular weight ligands but may occur with low molecular weight ligands.

The use of a "spacer arm" in many cases solves this problem. Commonly used spacer arms are aliphatic, linear hydrocarbon chains with two functional groups located at each end of the chain. One of the groups (often a primary amine, $-\text{NH}_2$) is attached to the matrix, whereas the group at the other end is selected on the basis of the ligand to be bound. The latter group, which is also called the terminal group, is usually a carboxyl ($-\text{COOH}$) or amino group, ($-\text{NH}_2$). The most common spacer arms are 6-aminohexanoic acid [$\text{H}_2\text{N}-(\text{CH}_2)_5-\text{COOH}$], hexaethylene diamine [$\text{H}_2\text{N}-(\text{CH}_2)_6-\text{NH}_2$], and 1,7-diamino-4-azooheptane (3,3-diamino dipropylamine) (Cuatrecasas, 1970, Lowe et al., 1974)

It is important to consider the chemical nature of the spacer arm and particularly its hydrophilic or hydrophobic character. Hydrophobic arms have the advantage of being available and relatively simple compounds, but may create non-specific interactions. The most encountered hydrophilic spacer arms are generally synthetic polypeptides but they may induce non-specific ionic interactions.

Longer spacer arms can be introduced by first immobilizing a spacer arm with a terminal primary amine and then increasing the length of the arm by reaction with succinic anhydride (Cuatrecasas, 1970) but a drawback with the hydrophobic arms, especially the longer ones, is that they can give rise to unwanted nonspecific interactions. Polypeptides, particularly glycine oligomers, are examples of

hydrophilic spacers. These, however, might bind proteins by nonspecific interactions (Lowe, 1974)

The essential purpose of the use of spacer arm is to increase sterical approach between the immobilized ligand and the product to be separated. The length of the spacer arm is thus an important parameter which must be decided according to each case.

If short spacer arms do not systematically eliminate steric effects, long spacers may favor non-specific adsorptions, particularly when the hydrocarbon chain has a hydrophobic character.

2.2.5. Immobilization Techniques

There are many methods for immobilization of ligand molecules onto the support matrix, in which usually several intermediate steps are followed (Turkova, 1993; Matejtschuk, 1997). The immobilization procedure consist of three steps;

- 1- Activation of the matrix to make it reactive toward the functional group of the ligand,
- 2- Coupling of the ligand,
- 3- Deactivation or blocking of residual active groups by a large excess of a suitable low molecular weight substance such as ethanolamine.

The main points for a successful ligand immobilization are given. Note that the correct choice of coupling method and conditions depends on both the matrix and the ligand.

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 (Denizli et. al., 2001). The activation normally consists of the introduction of an electrophilic group into the matrix. This group later reacts with nucleophilic groups, such as NH_2 (amino), $-\text{SH}$ (thiol), and OH (hydroxyl) in the ligand.

Alternatively, a matrix with nucleophilic groups can be used to immobilize a ligand containing an electrophilic group, although such an approach is less common. The activated structure is sometimes stable enough for the activated matrix to be isolated and stored until the coupling of ligand is performed. In other cases the coupling procedure has to be performed immediately after activation.

In the following, we will describe a few of them and the most representative. The most frequently used method is the cyanogen bromide-activation of agarose (activated resin is commercially available), which leads to a highly reactive cyanate ester. Subsequent coupling of ligands to the activated matrix results in an isourea linkage. Despite the popularity of this method, the isourea linkage of the ligand causes several problems during the purification procedure, including nonspecific binding due to charge and leakage of ligand due to instability of the isourea bond.

Since the early days of affinity chromatography, active esters, in particular N-hydroxy-succinimide (NHS) esters, have also been used for immobilizing ligands. The preparation of active esters requires a matrix that contains carboxylic groups. Such matrices can be easily obtained from agarose by activation of the hydroxyl groups with different reagents, including cyanogen bromide, activated carbonates, etc. (Figure 2.2), and successive reaction with ω -amino acids of different sizes depending on the length of spacer arm required. The NHS ester is then prepared by mixing the carboxylic matrix with dicyclohexylcarbodiimide and NHS. Due to a stability problem (Wilchek and Miron, 1987), a new method was introduced based on N',N',N',N'-tetramethyl (succinimido) uronium tetrafluoroborate.

The covalent attachment of ligands to such activated carriers proceeds through the production of stable amide bonds.

Another method for activating polysaccharides is use of N,N-disuccinimidyl carbonate (DSC), which forms highly reactive carbonate derivatives with polymers containing hydroxyl groups. These derivatives react nucleophiles under mild, physiological conditions (pH 7.4), and the procedure results in a stable carbamate linkage of the ligand coupled to the carrier.

The immobilization of different ligands (e.g., enzymes, enzyme inhibitors, antigens and antibodies) to activated carbonate carriers has been achieved, together with excellent maintenance of biological activity of the proteins (Wilchek and Miron, 1999).

It must be remembered that in certain cases, high degree of substitution may favor non-specific adsorptions. The multipoint binding of an antigen molecule results in changes in its conformation that leads to weakening of the interactions between the antigen and the active site of the antibody.

Since affinity involves the adsorption of one molecule of product to be separated by one molecule of immobilized ligand, the ratio of their molecular weights is of primary importance for gel adsorption capacity. The degree of immobilization must take into account; the ligand size, the ligand specificity and the gel porosity. If the ligand is a low molecular weight compound and the product to be separated is a macromolecule, the degree of substitution may be very low. In this case, the ratio of molecular weights is reflected by a high capacity, providing that the method of immobilizing the ligand and the porosity of the gel were well chosen.

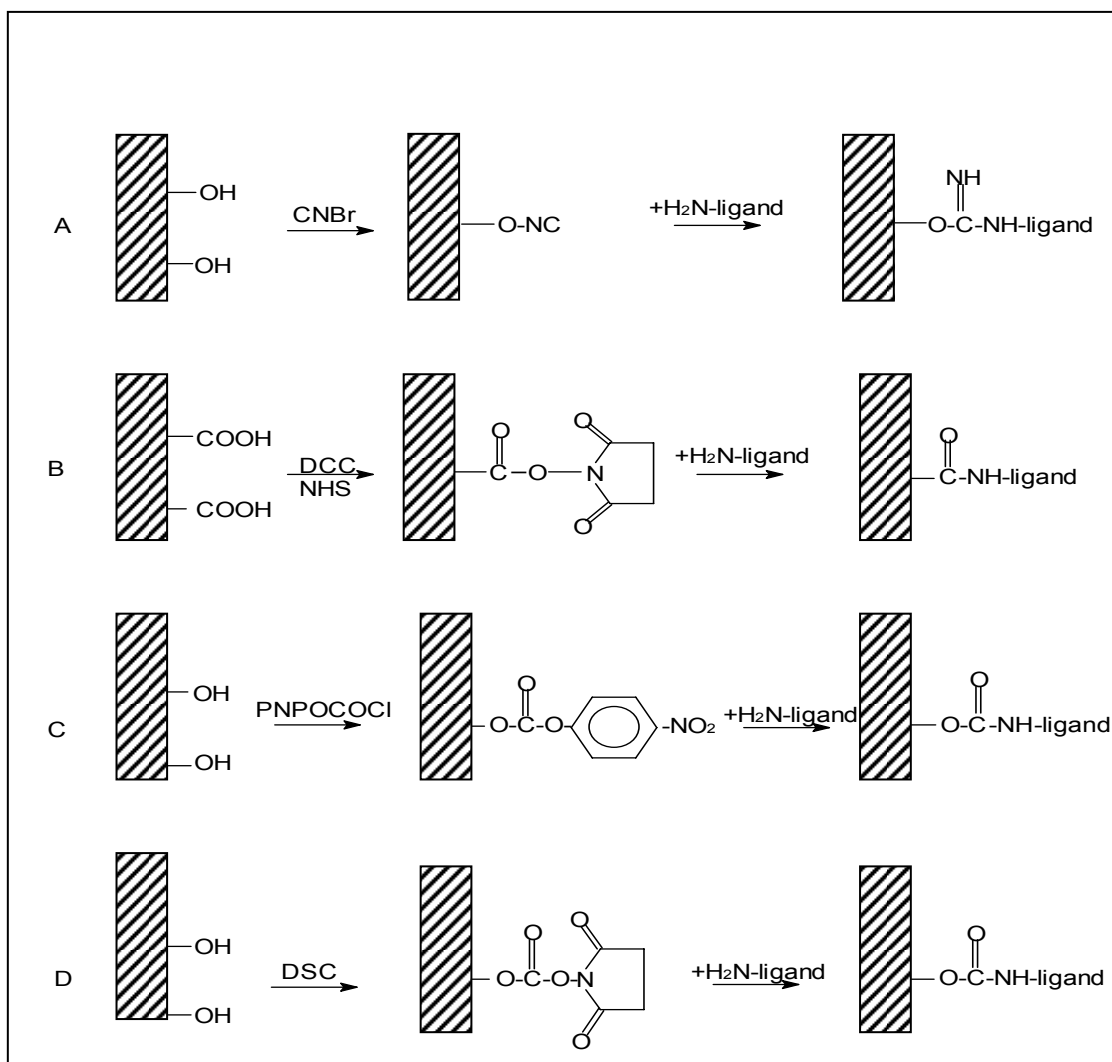


Figure 2.2. Immobilization of ligands to different activated carriers:

(A) cyanate ester, obtained by activation with cyanogen bromide, leading to an isourea derivative; (B) N-hydroxysuccinimide ester yielding an amide derivative; (C) and (D) p-nitro-phenyl and N-hydroxysuccinimide carbonate obtained by activation with p-nitro phenylchloroformate or DSC, giving carbamate derivatives been achieved, together with excellent maintenance of biological activity of the proteins (Wilchek and Miron, 1999).

The degree of immobilization depends on activation and coupling procedures:

- 1- quantity of activating agent,
- 2- reaction time,
- 3- pH of the medium,

4- reaction temperature,

5- ligand concentration.

2.3. Hydrophobic Interaction Chromatography

A wide variety of protein purification techniques are available today, however, different types of chromatography have become dominant due to their high resolving power. In gel filtration chromatography, ion-exchange chromatography, affinity chromatography and hydrophobic interaction chromatography (HIC), the proteins separation is dependent on their biological and physico-chemical properties: molecular size, net charge, biospecific characteristics and hydrophobicity, respectively (Kennedy, 1990; Garcia and Pires, 1993).

HIC takes advantage of the hydrophobicity of proteins promoting its separation on the basis of hydrophobic interactions between immobilized hydrophobic ligands and non-polar regions on the surface of proteins. The adsorption increases with high salt concentration in the mobile phase and the elution is achieved by decreasing the salt concentration of the eluent (Melander and Horvath, 1977; Fausnaugh and Regnier, 1986; Roe, 1989). Therefore, the term “salt-promoted adsorption” could be used for this type of chromatography (Porath, 1986). The different types of elution conditions can be used for purification of complex mixtures of proteins that would be difficult to separate using other chromatographic techniques.

In fact, HIC has been successfully used for separation purposes as it displays binding characteristics complementary to other protein chromatographic techniques (Janson and Ryden, 1993). Figure 2.3. shows schematic representation of HIC.

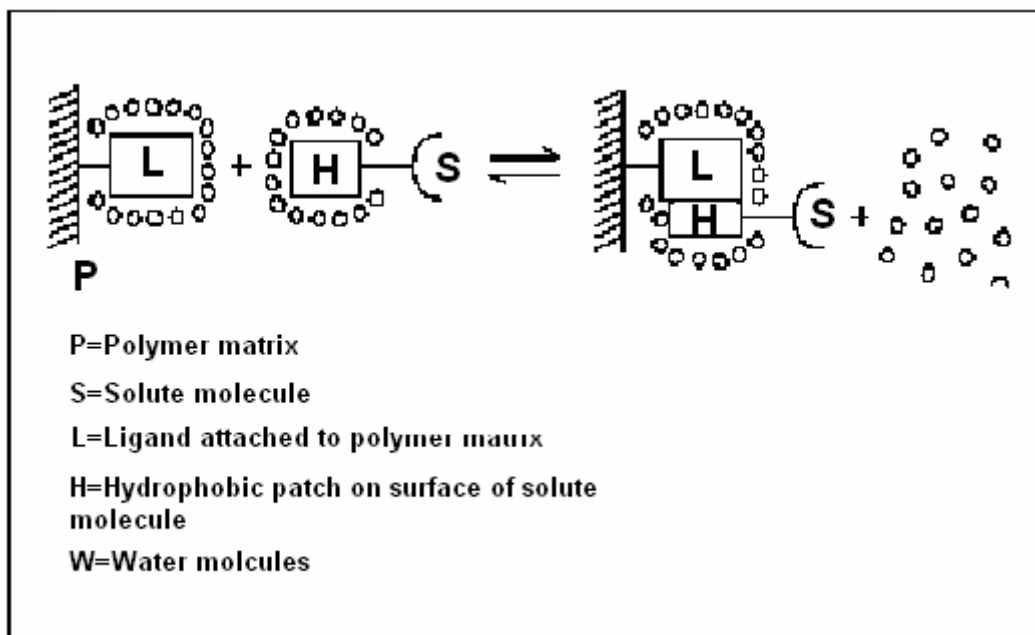


Figure 2.3. Schematic representation of HIC.

Van Oss et al. (Van Oss et al., 1986) proposed that the van der Waals forces are the major contributing factor to the hydrophobic interactions (interfacial forces) despite the complex mechanism involved. Thus, the structural damage to the biomolecules is minimum and its biological activity is maintained using HIC, due to a weaker interaction than affinity, ion-exchange or reversed phase chromatography (RPC) (Fausnaugh et al., 1984; Regnier, 1987). HIC is an alternative way of exploiting the hydrophobic properties of proteins, working in a more polar and less denaturing environment than RPC, since this technique requires the use of non-polar solvents for the protein elution due to strong binding to adsorbent (El Rassi, 1996). During the last years, HIC has been developed by many researchers and today is an established and powerful bioseparation technique in laboratory-scale, as well as in industrial-scale purification of proteins (Wu and Karger, 1996; Grund, 1998). The development of a large variety of stationary phases for HIC has promoted a wide range of HIC applications in the purification of biomolecules, like serum proteins, nuclear proteins, hormones, recombinant proteins and enzymes (Commings et al., 1979; Hrkal and Rejnkova, 1982; Lefort and Ferrara, 1986; Angelova et al., 1997; Queiroz et al., 1999).

2.3.1. Protein Hydrophobicity and Hydrophobic Interactions

Hydrophobicity is the repulsion between a nonpolar compound and a polar environment such as water. This term is also been used to refer to particular molecular models, generally involving the ordering of water molecules around non-polar solutes (Dill, 1990a). When a non-polar compound is inserted into water, an overall increase in the degree of order of water molecules surrounding the hydrophobic group is observed with a decrease in entropy ($\Delta S < 0$). Since the enthalpy change (ΔH) is small compared with the $T\Delta S$ value, there is a positive change in free energy ($\Delta G > 0$), according to equation (2.1):

$$\Delta G = \Delta H - T\Delta S \quad (2.1)$$

Therefore, this process does not occur spontaneously because it is thermodynamically unfavorable. On the other hand, if non-polar compounds are put into water they spontaneously associate due to hydrophobic interactions. An increase in entropy ($\Delta S > 0$) is observed, resulting from a displacement of the ordered water molecules around the non associated hydrophobic groups to the more unstructured bulk water. This implies a negative change in free energy ($\Delta G < 0$) and a thermodynamically favorable process. In other words, the interaction between two or more hydrophobic molecules in aqueous solutions proceeds spontaneously and is mainly determined by the change in entropy (Lewin, 1974; Jennissen, 1976; Ochoa, 1978; Tanford, 1980; Shansky et al., 1990). In fact, the heat of adsorption data obtained for bovine serum albumin in HIC, under linear isotherm conditions is also driven by entropy changes (Esquibel-King et al., 1999).

Two approaches have been used for the estimation of the hydrophobicity of amino acids. The first is based on direct measurements of the solubilities of individual amino acids in water and organic solvent (Tanford, 1962). A hydrophobicity scale for the different amino acids has been constructed on the basis of free energy of transfer for the amino acids from ethanol or dioxane to water.

The second approach is on the empirical inspection of known protein structures (Rose et al., 1985). Here, several hydrophobicity scales are based on, for example,

the environment of the different amino acids, the fraction of amino acids that is buried in the protein, a side chain interaction parameter, or a fractional accessibility to the surrounding solvent of the different residue. For a comparative study of hydrophobicity scale published (Cornette et al., 1987).

Table 2.3. is a comparison of two scale of hydrophobicity, the first based on amino acid solubility (Tanford, 1962) and the second based on the fraction of amino acids buried within proteins (Wertz et al., 1978) (average values obtained from 20 proteins with known structures). Some differences are striking. Proline is a rather hydrophobic amino acid, but its secondary structure-breaking properties make it appear in bends typically on the surface of proteins. Lysine is also classified as a rather hydrophobic residue in the scale based on solubility studies, although it is the most exposed of the amino acids. The four methylene groups of the lysine side chain disfavor the solubility of this amino acid in water whereas the amino group with its hydrogen-binding capability has a strong tendency to be exposed on the surface of proteins.

Hydrophobic interactions have a great importance in the biological systems. They are the dominant force in protein folding and structure stabilization (Privalov and Gill, 1988; Dill, 1990a; Murphy et al., 1990; Makhatafze and Privalov, 1995) and play an important role in other biological processes like antibody-antigen reactions (Dandliker et al., 1967; Van Oss et al., 1986), enzyme-substrate reactions and the maintenance of the lipid bilayer structure of biological membranes (Tanford, 1973). These processes are driven by hydrophobic effect and according to thermodynamic analysis of calorimetric data they are accompanied by significant entropy and heat capacity changes at room temperature (Vailaya and Horvath, 1996).

The importance of the hydrophobic interactions in protein folding was first introduced by Kauzmann (Kauzman, 1959). After that, many works based on spectroscopic and high resolution differential scanning calorimetry experiments (Privalov and Gill, 1988; Dill, 1990b), crystal structures of proteins (Chothia, 1976), computer simulations (Novotny et al., 1988; Baumann et al., 1989) and the influence of different salt species on protein stability (Arakawa and Timasheff,

1984), continue to support the importance of the hydrophobic interactions. Despite the fact that internal hydrophobic core is considered an important factor in determining the native structure of a protein, it was shown that non-polar amino acids (e.g. phenylalanine, tryptophan, and valine) occur frequently on the surface of the proteins, such as lysozyme or serum albumin (Klotz, 1970; Hofstee, 1975; Meirovitch and Scheraga, 1980).

The thermodynamic analysis of this chromatographic method yielded essentially the same results as those obtained with other processes based on the hydrophobic interactions (Vailaya and Horvath, 1996). HIC also offers an alternative approach to calorimetry in the study of the hydrophobic effect (Haidacher et al., 1996).

Table 2.3. Hydrophobicity scales for amino acids

Scale I (kcal/mol)		Scale II (Fraction Buried)	
Trp	3.77	Phe	0.87
Ile	3.15	Trp	0.86
Phe	2.87	Cys	0.83
Pro	2.77	Ile	0.79
Tyr	2.67	Leu	0.77
Leu	2.17	Met	0.76
Val	1.87	Val	0.72
Met	1.67	His	0.70
Lys	1.64	Try	0.64
Cys	1.52	Ala	0.52
Ala	0.87	Ser	0.49
His	0.87	Arg	0.49
Arg	0.85	Asn	0.42
Glu	0.67	Gly	0.41
Asp	0.66	Thr	0.38
Gly	0.10	Glu	0.38
Asn	0.09	Asp	0.37
Ser	0.07	Pro	0.35
Thr	0.07	Gln	0.35
Gln	0.00	Lys	0.31

Scale I is based on the solubility of the different amino acids expressed as free energy of transfer for the amino acids from ethanol or dioxane to water. Scale II is based on the fraction of the number of the different amino acids buried within proteins (average values obtained from 20 proteins with known structures.)

2.3.2. Factors Affecting Hydrophobic Interaction Chromatography

To obtain success in a chromatographic process for a specific application, two major elements have to be considered a stationary phase and a fluid mobile phase.

2.3.2.1. Stationary Phase

The various kinds of stationary phases could differ on the type of ligand, the ligand chain length, ligand density and on the type of matrix or support.

The most widely used ligands for HIC are linear chain alkanes with or without a terminal amino group. Phenyl (and other aromatic groups) is also used as a ligand with good results due to mixed hydrophobic and aromatic (π - π) interactions. At a constant degree of substitution on the matrix the *n*-alkane ligands constitute a homologous series in a hydrophobicity scale (Tanford, 1972):

methyl < ethyl < propyl < butyl < pentyl < hexyl < heptyl < octyl

The hydrophobicity and the strength of interaction increase with the increase in *n*-alkyl chain length but the adsorption selectivity may decrease.

The relation between ligand density and protein retention is apparently dependent upon the size of the protein, although the protein surface hydrophobicity should be considered to be the most affecting factor (Fausnaugh et. al., 1984). An increase in the degree of substitution of immobilized ligand leads to an increase in protein binding capacities of HIC gels, due to the higher probability of forming multi-point attachment between the protein and the ligand (Hjerten et. al., 1974). Sometimes, in these cases, it can be difficult to elute the bound protein without denaturation,

due to the use of harsh elution conditions (organic solvents, detergents or chaotropic agents). Ligands with intermediate hydrophobic character could be more useful since they provide moderate binding strength and an elution by simply decreasing the salt concentration of the eluent. Jennissen and Heilmeyer (Jennissen and Heilmeyer, 1975) presented evidence that the adsorption of proteins to hydrophobic agaroses occurs at a critical alkyl-group density and is a function of the hydrophobicity of the supports. Lin et al. (Lin et al., 2000) found that the major contributor to the increase of adsorption enthalpies with ligand density was the heat required for the dehydration process of the protein and the adsorbent, despite the play of hydrophobic interactions, as well as structural rearrangement of the protein in all process. Therefore, the ligand density is an important tool that can be manipulated to obtain distinct types of stationary phases with different selectivity and binding capacities for a desired protein separation. Figure 2.4 shows the schematic structures of some ligands for HIC.

The most widely used supports are hydrophilic carbohydrates (e.g. cross-linked agarose), silica or synthetic copolymer materials. Using the same type of ligand, the selectivity of the stationary phases can change in function of the different type of supports.

2.3.2.2. Mobile Phase

The protein retention in HIC depends not only on the stationary phase but also on characteristics of the mobile phase, such as the type and concentration of the salt, pH, temperature and additives.

The effect of salt composition on the protein retention follows the order of the salts in the lyotropic series for the precipitation of proteins or for their positive influence in increasing the molal surface tension of water (Melander and Horvath, 1977; Melander et al., 1984). Salts such as sodium, potassium or ammonium sulfates are the most effective to promote ligand protein interactions, due to the higher “salting out” or molal surface tension increment effects. However, magnesium sulphate and magnesium chloride do not enhance the protein retention despite the fact that they

increase the surface tension of water, as the above-mentioned salts (Table 2.4). This fact implies that the effect of salt composition on protein retention is a very complex phenomenon. It appears to include other factors than surface tension of the solution, namely specific interactions between the protein and the salt ions, which may or may not change the protein structure and the hydration of the protein (Arakawa and Timasheff, 1984; Fausnaugh and Regnier, 1986). A change of the salt type in the eluent results in significant alterations not only in overall retention of the proteins, as well as in the selectivity of the separations (Rippel and Szepesy, 1994).

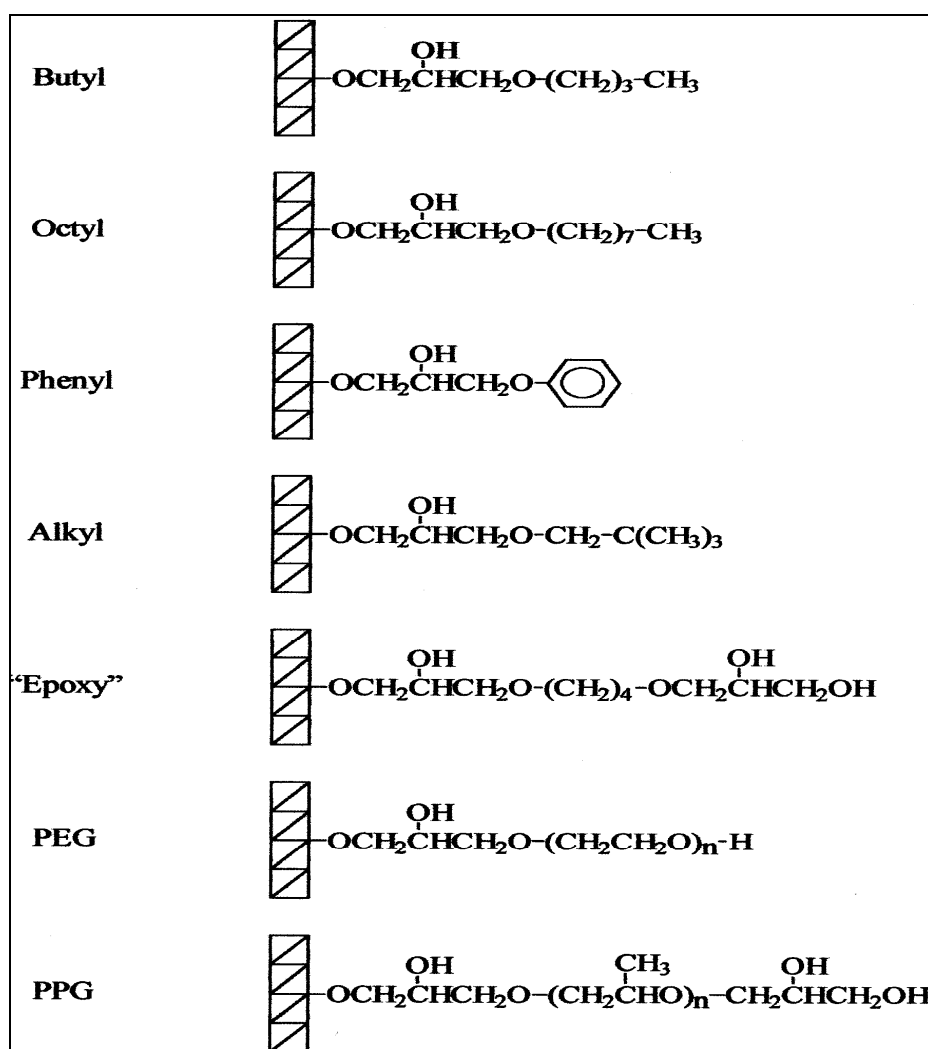


Figure 2.4. The schematic structures of some ligands for HIC.

Table 2.4. Molal surface tension (σ) of various salts (Melander and Horvath, 1977).

Salt	$\Sigma \times 10^3$ (dyn.g/cm.mol)
KClO ₄	1.40
NaCl	1.64
Na ₂ HPO ₄	2.02
MgSO ₄	2.10
(NH ₄) ₂ SO ₄	2.16
K ₂ SO ₄	2.58
Na ₂ SO ₄	2.73
MgCl ₂	3.16

The concentration of salt strongly influences the selectivity in protein adsorption and this influence is different and dependent both on the stationary phase and the buffer salts (Oscarsson and Karsnas, 1998). In HIC, the use of high salt concentration on the equilibration buffer and sample solution promotes the ligand–protein interactions and consequently the protein retention. The amount of the bound protein increases almost linearly with the enhancement in ionic strength and continues to increase in an exponential manner at still higher concentration. The adsorbed proteins are eluted by stepwise or gradient elution at decreasing salt concentration in the eluent. The neutral salts used in HIC must be highly soluble to avoid salt precipitation when they are added in high concentration to the eluent to drive the hydrophobic interaction. The viscosity, UV transparency and stability at alkaline pH values are other important factors for choosing the neutral salts (Narhi et al., 1989).

The pH of mobile phase can be an important factor that affects the protein retention in HIC (Hofstee, 1973; Strop et al., 1983; Sanz et al., 1998). Usually, an increase in the pH value (up to 9–10) decreases the hydrophobic interaction between proteins and the hydrophobic ligands, due to the increased hydrophilicity promoted by the change in the charge of the protein (Hjerten, 1973; Porath et al., 1973). On the

other hand, a decrease in pH results in an apparent increase in hydrophobic interactions. However, Hjerten et al. (Hjerten et al., 1986a) have observed that basic proteins, like lysozyme and cytochrome-c, bind weakly to the hydrophobic ligands at lower pH. Also, they have found that the retention of proteins changed more drastically at pH values above 8.5 and or below 5 than in the pH range 5–8.5. Since each protein could have different behavior in distinct pH values, this parameter should be used in the optimization of protein separation by HIC.

In HIC, increasing the temperature enhances protein retention and lowering the temperature generally promotes the protein elution (Hjerten et al., 1974). In fact, the HIC is an entropy-driven process, as earlier mentioned, where the Gibbs free energy is giving according to the equation (2.1). However, an opposite behavior can occur on protein retention, due to temperature effect on the conformational state of different proteins and on their solubilities in aqueous solutions. Even though the role of temperature in HIC is not so simple, this parameter can be used to achieve weaker interaction and promote elution and separation of proteins under mild conditions without denaturation (El Rassi, 1996).

Certain additives can be used in HIC, not only to improve protein solubility or to modify protein conformation, but also to promote the elution of the bound proteins. The most widely used are water-miscible alcohols (e.g. ethanol and ethylene glycol), detergents (e.g. Triton X-100) and aqueous solutions of chaotropic salts. The non-polar parts of alcohols and detergents achieve the displacement of bound proteins due to the competition for the ligands on HIC stationary phase. When ionic detergents are bound to HIC media the separation mode is a mixed ion-exchange hydrophobic interaction process, due to the presence of the charged group of the detergent (Janson and Ryden, 1993). Chaotropic salts promote desorption of the proteins by affecting the ordered structure of water and or of the bound proteins. The latter can be denatured or inactivated by high concentration of additives; therefore, its use in elution is usually considered a last resort, when other milder conditions do not promote protein recovery. However, if strongly hydrophobic proteins bind to the stationary phase, additives can be used in cleaning up HIC columns.

2.3.3. Examples of Stationary Phases Used in HIC

The first type of hydrophobic stationary phases derived from coupling various non-polar amino acids to an inert support or matrix (Rimerman and Hatfield, 1973). Agarose was the support most widely used, but cellulose, dextran and silica have also been employed.

Initially, the stationary phases used in HIC exhibit both hydrophobic and ionic character (Erel et al., 1972; Yon, 1972; Hofstee, 1973) resulting from the coupling of aliphatic and aromatic amines to cross linked agarose (Sephacrose) by cyanogen bromide method (Axen et al., 1967). Thus, the chromatographic fractionation with these stationary phases was due to both electrostatic and hydrophobic interactions with proteins.

Porath et al. (Porath et al., 1973), Hjerten et al. (Hjerten et al., 1974) were the first to synthesize charge-free hydrophobic adsorbents. Hjerten used a coupling method based on glycidyl ether (with an epoxide, oxirane functional group) that today is widely used for production of the commercially available gels octyl- and phenyl-Sepharose. The gels produced by this process were neutral and exhibited mainly hydrophobic interactions with the proteins of human plasma and of *Trichoderma* Giride crude extract (Hjerten et al., 1974).

A free charge gel was also synthesized by Maisano et al. (1985) by activation of agarose with a bisepoxide (1,4-butanediol diglycidyl ether) and then coupled with an alkyl-mercaptan. Another coupling method is based on activation of the agarose with γ -glucydoxypropyltrimethoxysilane in water and immobilization performed with alcohol (Hjerten et al., 1986 a, b). These two last methods produce charge free gels with spacer arms that make the ligands more accessible for the protein. However, it is important that the spacer arm does not interfere in the adsorption process either by electrostatic or hydrophobic interactions.

The polysaccharide gels (e.g. agarose) are the supports most widely used in traditional low-pressure HIC, but several rigid micro particulate stationary phases

based on organic or inorganic supports, that can be used at high pressure HIC, have been introduced. Thus, silica and polymeric supports were derivatized through their hydroxyl groups with butyl, phenyl and polyether functions. Kato et al. (Kato et al., 1983, 1986) synthesized resinbased weakly hydrophobic supports for high pressure, which are commercially available under the trade name of TSK gel Phenyl-5PW and TSK gel Ether-5PW. Gooding et al. (Gooding et al., 1984) have produced silica based stationary phases containing methyl, hydroxypropyl, propyl, benzyl or pentyl groups. Also, poly(alkylaspartamide) (Alpert, 1986) and acylated polyamine (Berkowitz and Henry, 1987) silica matrices have been effective in HIC protein separations.

Hjerten et al. (Hjerten et al., 1986a) introduced a rigid highly cross-linked agarose for use in HIC of proteins under HPLC conditions. Also, rigid materials (Separon-HEMA) that are copolymers of 2-hydroxyethyl methacrylate and ethylene dimethacrylate were used in HIC-HPLC (Kleinmann et al., 1989; Smídl et al., 1990). Macroporous agarose beads were converted into non-porous particles by shrinkage and cross-linking in organic solvents using butanediol diglycidyl ether as cross-linker (Hjerten and Liao, 1988; Hjerten et al., 1991). These beads are stable up to pH 14 contrary to silica based materials and can be used for high performance HIC without derivatization.

Kato et al. (Kato et al., 1989) have developed a stationary phase commercially available under the tradename TSKgel Butyl-NPR which is useful for rapid separation of proteins with high resolution by HIC. It was prepared by chemically bonding butyl groups on the surface of non-porous spherical synthetic hydrophilic resin of 2.5 mm particle diameter.

Recently, a new type of agarose material called superporous agarose was used as a support for HIC with good results in the separation of a protein mixture at a much higher flow velocity than the homogeneous bead column. This support has beads with large connecting flow pores in addition to their normal diffusion pores (Gustavsson et al., 1999).

2.3.4. Selected Applications

HIC takes advantage of the hydrophobicity of proteins and is a versatile liquid chromatography technique, being a logical part of any rational purification strategy, often in combination with other chromatographic techniques. HIC has been used in the purification of a great variety of proteins.

The outcome of a protein purification procedure is obviously dependent on the choice of separation techniques. The order in which the different techniques are combined is also of great importance. For instance, HIC could be used after a precipitation step (Stracke et al., 1992), which often comes at the beginning of a downstream process, or in combination with gel filtration (Tomaz and Queiroz, 1999) or with ion-exchange chromatography (Unge et al., 1990; Roder et al., 2000; Wenk and Kruij, 2000). Negative side-effects have also been noted. The binding is, in many cases, too strong to be useful in a chromatographic process and may be practically irreversible. In order to elute a strongly bound protein, organic solvents, detergents or chaotropic agents may be required (Taipa et al., 1994), which may lead to protein denaturation. In this respect, the use of some mild hydrophobic ligands appears to be a promising alternative, since this would permit an adequate binding strength without the above-mentioned drawbacks. The purification of plasminogen activator from human myocardial tissue is a good example of current applications of HIC in producing biotechnological products (Sofer, 1986). In the plasminogen activator purification procedure, the HIC step (octyl-agarose column) was introduced after tissue extraction and precipitation with ammonium sulphate and was followed by affinity chromatography and gel filtration steps. Some other applications that demonstrate how HIC can be integrated into a large scale downstream process are the production of recombinant factor VIII or the purification of annexin V, an anticoagulant protein, produced by recombinant DNA technology in *E. coli* as an intracellular product. The potential of HIC has been also demonstrated for other biomolecules, such as nucleic acids. HIC has been used in order to take advantage of the more hydrophobic character of single stranded nucleic acid impurities, when compared with double stranded plasmid DNA (Diogo et al., 1999b, 2000a). Even when we scaled-up (60-fold) the HIC

purification of a plasmid DNA vaccine against rabies, no loss in purity and only marginal losses in yield were obtained (Diago et al., 2000b).

2.4. Lysozyme

Lysozyme (N-acetylmuramide glyconohydrolase) is commonly known to be present in places such as human bodies, animals, plants, microbiology or chicken egg white. It mainly attacks the peptidoglycan in the cell walls of gram-positive bacteria, but more or less, of the gram-negative bacteria. It is extensively applied in physic and pharmaceutics technology as a drug to treat ulcer or infection and as a potent antibacterial agent in milk products. It is also applied in farming and bioengineering (Wang and Che, 2005; Diluzio, 1979; Wu et al., 2002; Rong et al., 1999). Lysozyme is one of the best characterized hydrolases. It cleaves $\beta(1\rightarrow4)$ glycosidic linkages of N-aceylglucosamine (NAG or GlcNac) homopolymer and N-aceylglucosamine-N-acetylmuramic acid (GlcNac-MurNac) heteropolymer, which causes lysis of the bacteria containing these polymers in the cell wall. It likewise hydrolyzes $\beta(1\rightarrow4)$ -linked poly (NAG), a cell wall of fungi as well as the major component of the exoskeletons of insects and crustaceans. Lysozyme occurs widely in the cells and secretions of vertebrates, where it may function as a bactericidal agent or help dispose of bacteria after they have been killed by other means.

Lysozymes have been classified into three distinct forms by their amino acid sequences and tertiary structures, namely, C-type (Canfield, 1963; Jolles et al., 1963; Blake et al., 1965) T₄-type (Inoue et al., 1970; Matthews and Remington, 1974), and G-type (Grutter et al., 1979; Simpson et al., 1980; Weaver et al., 1985). More recently, a novel member of lysozyme, i-type or invertebrate lysozyme is of much interest due to differences in primary structure from other reported lysozymes, with different numbers of cysteine residues and lack of conservation of the second catalytic amino acid (the active aspartate) (Bachali et al., 2004). Invertebrate lysozyme was first reported in the starfish by Jolles and Jolles (Jolles and Jolles, 1975). This type of enzyme has been characterized in marine bivalves, earthworm, leech, starfish and nematode (Jolles et al., 1996; Ito et al., 1999; Nilsen et al.,

1999; Olsen et al., 2003; Bachali et al., 2004). The i-type lysozyme showed activity towards the bacteria in the digestive system.

Hen egg white (HEW) lysozyme is the most widely studied species of lysozyme and is one of the mechanistically best understood enzymes. It is a rather small protein (14.6 kD) whose single polypeptide chain consists of 129 amino acid residues and is internally cross-linked by four disulfide bonds. Lysozyme catalyzes the hydrolysis of its substrate at a rate that is $\sim 10^8$ fold greater than that of the uncatalyzed reaction. It was discovered by Alexander Fleming in 1922. As early as 1921 Fleming announced that he had found a "remarkable bacteriolytic element" present in many tissues and secretions which was able to interfere with the growth of some specific bacterial colonies.

The term "lysozyme" (or rather lysozymes considering their ubiquity and their various structural differences) refers to an enzyme with well-defined hydrolasic activity. In nature, there are types of lysozyme with different characteristics according to their origin. The nature has provided hen egg-white with high content of lysozyme, as protection of yolk integrity, making the albumen the preferred raw material for its industrial production. Figure 2.5.a and Figure 2.5.b show the lysozyme extracted from hen egg-white consisting of 129 amino acid residues. The four disulfide bridges among the eight cysteine residues are essential for the lysozyme activity.

In mammals, lysozyme has been isolated from nasal secretions, saliva, tears, intestines, urine and milk. It lyses certain bacteria by hydrolyzing the linkages between the muramic acid and N-acetylglucosamine of the mucopolysaccharides which are present in the bacterial cell wall. It has a large application area, used in medicine as a bacteriolytic agent, wound therapeutic agent, and antibiotic activator.



Figure 2.5.a. The lysozyme extracted from hen egg-white consisting of 129 amino acid residues.

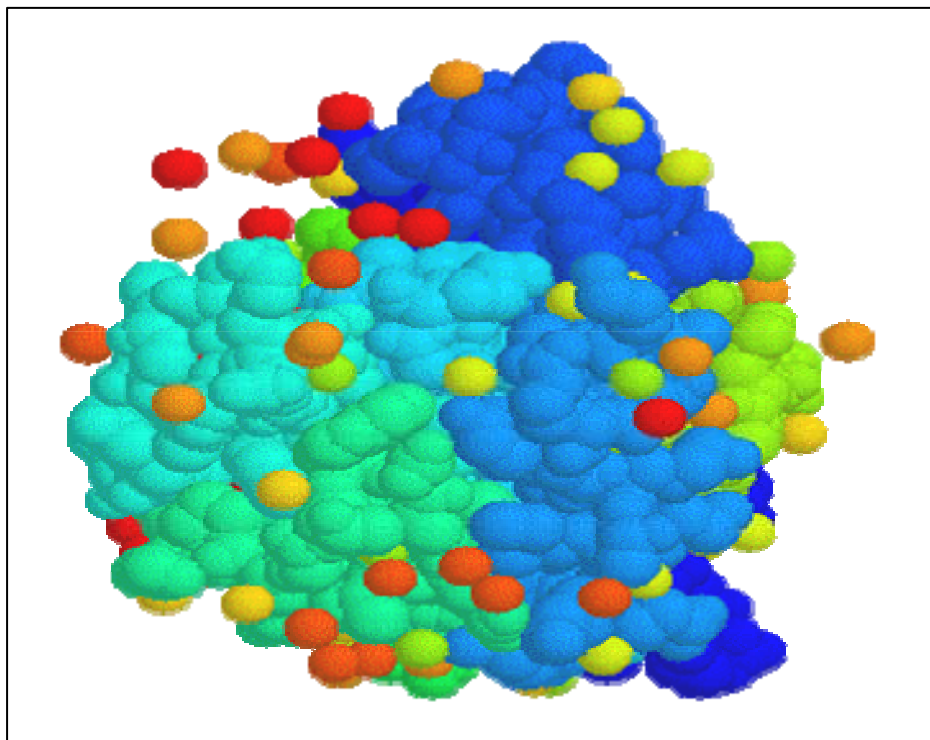


Figure 2.5.b. The lysozyme extracted from hen egg-white consisting of 129 amino acid residues.

2.5. Polymerization Techniques

The main polymerization techniques are bulk, solution, suspension and emulsion polymerization. Plasma polymerization and gas phase polymerization are other possibilities for polymerization. To produce spherical beads, emulsion or suspension or dispersion polymerization is performed. The size of resulting beads is less than 1 μm and ranges from 100 μm to 5 mm, if emulsion or suspension technique is followed, respectively. Suspension polymerization proceeds by dispersing the monomer in water with a suspending agent and then converting the dispersed droplets into polymer by reaction with an initiator that is normally soluble in the monomer.

The monomer droplets are usually more finely dispersed in emulsion polymerization, with diameters ranging from a few microns to $>10\ \mu\text{m}$. Unlike suspension polymerization, the emulsion process does not simply involve polymerization of the monomer droplets. Nucleation and growth of smaller particles is a significant mechanism in emulsion polymerization (Billmeyer, 1971).

2.5.1. Free Radical Polymerization Technique

Free radical polymerization is the most widespread method of polymerization of vinylic monomers. Free radical polymerizations are chain reactions in which every polymer chain grows by addition of a monomer to the terminal free radical reactive site called “active center”. The addition of the monomer to this site induces the transfer of the active center to the newly created chain end. Free radical polymerization is characterized by many attractive features, such as applicability for a wide range of polymerizable groups including styrenic, vinylic, acrylic and methacrylic derivatives, as well as tolerance to many solvents, small amounts of impurities and many functional groups present in the monomers. However, classical free radical polymerization has some limitations, inherent to its mechanism. In particular, it is difficult to control molar masses and polydispersities as well as to introduce defined end-groups, or to prepare special macromolecular architectures such as block copolymers. In order to overcome these limitations,

new strategies in free radical polymerization have emerged recently, often referred to as “controlled” free radical polymerization (Fischer, 2001).

Characteristically, these methods use reagents that transform reversibly the propagating radicals into a dormant species. So in average, the polymer chains grow simultaneously, and no more one after the other. Thus, chain growth and monomer consumption takes place at a comparable rate. Processes that have received most attention are: i) nitroxide-mediated radical polymerization (NMRP) (Georges, 1993; Hawker et al., 2001), ii) Atom Transfer Radical Polymerization (ATRP) (Matyjaszewski, 2000; Kamigaito et al., 2001) catalysed by transition metal complexes, and more recently iii) polymerization via Reversible Addition-Fragmentation chain Transfer (RAFT) (Le et al., 1998; Rizzardo et al., 1999; Moad et al., 2000) or Macromolecular Design via Interchange of Xanthates (MADIX) (Charmot et al. 1999, Destarac et al., 2002). In the case of xanthates are used as chain transfer agents. Within these methods, the first two ones are based on reversibly blocking the growing polymer radical, whereas the last one is based on degenerative chain transfer (Jagur and Grodzinski, 2001, Matyjaszewski, 2003). The RAFT process seems to be well adapted to numerous classes of monomers, solvents (a priori, this process is also compatible with water), functional moieties and to a large temperature window. It enables in many systems the control of the molar mass and of the polydispersities, and allows to prepare complex architectures e.g stars (Mayadunne et al., 2002; Barner et al., 2003) and comb-like graft block copolymers (Quinn et al., 2002). In order to understand the advantages of the RAFT process on a classical free radical polymerization we will describe the mechanism of free radical polymerization for comparison.

2.5.2. Stages of Free Radical Polymerization

The mechanism of the free radical polymerization (Chiefari et al., 1998; Hawthorne et al., 1999) can be divided into three distinct stages referenced as initiation, propagation and termination. Initiation: The initiation step is considered to involve two reactions creating the free radical active center. The first event is the production of free radicals. This can be done by several mechanisms such as

thermal, redox or photochemical reactions. The most common case is the homolytic dissociation of an initiator I to yield two radicals $R_1^\bullet$ often referred as primary radical. The subsequent event is the addition of this radical to a monomer molecule M producing the initial propagating species $P_1^\bullet$.

Equation (2.2; 2.3):



Since the formation of $R_1^\bullet$ proceeds more slowly than the reaction of $R_1^\bullet$ with monomer, the first step is rate-determining, so that V_i can be neglected. Propagation: Propagation consists in the successive addition of a large number of monomer molecules M to the primary radical $P_n^\bullet$. Each rapid sequential addition of a monomer molecule creates a new radical $P_{n+1}^\bullet$ that is chemically identical to the previous one, except that it is larger by one monomer unit. The successive additions are represented in a general equation by: Equation (2.4):

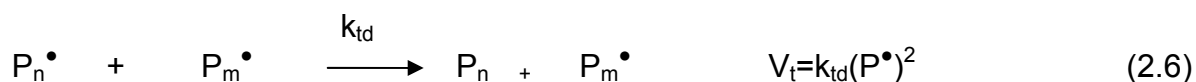


where k_p is the constant rate for propagation and $[P^\bullet] = \sum_i [P_i^\bullet]$. Usually, the propagation takes place rapidly. The value of k_p for most monomers is in the range 10^2 - $10^4 \text{ l}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$. Typically, the time required for the addition of a monomer to a growing chain is about a millisecond. In fact, thousands of addition can occur within few seconds. Termination: In this step, the growth of the polymer chain is terminated irreversibly. The two most common mechanisms for termination involve bimolecular reactions. The first one, combination, is the reaction between two growing polymer chains leading to the annihilation of the radical active centers and

the formation of a single polymer chain. Termination by combination is represented by the equation: Equation (2.5):



where k_{tc} is the rate constant for combination. The second one, is the termination via disproportionation. A hydrogen atom is abstracted from a growing chain and transferred to another one. This reaction leads to two terminated polymer chains, one with an unsaturated end-group and the other one with a saturated end-group. Equation (2.6):



This equation represents the termination by disproportionation where k_{td} is the rate constant. Because in a conventional free radical polymerization, combination and disproportionation prevail, the overall rate constant for termination for which the mode of termination is not specified can be approximated as $k_t = k_{tc} + k_{td}$. Typical rate constant values for termination are in the range of 10^6 - $10^8 \text{ l} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$, i.e. they are very high. The termination step leads unavoidably to *dead polymer*: the growth of the polymer chain is terminated and the active centers are irreversibly annihilated. This implies that it is impossible to form block copolymers by adding a new monomer and re-activating the polymerization system.

2.6. Cryogel

At present, polymeric gels have application 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. One of the new types of polymer gels with considerable potential in biotechnology is 'cryogels' (from the greek kryos meaning frost or ice) (Lozinsky, 2002). Cryogels were first reported nearly 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, 2002; Nambu, 1990; Kaetsu, 1993; Suzuki, and Hirasa, 1993; Lozinsky, 1998; Lozinsky and Plieva 1998; Hassan and Peppas 1998; Lozinsky et al., 2001).

Conventional packed-bed columns possess some inherent limitations such as the slow diffusional mass transfer and the large void volume between the beads (McCoy et al., 1996). Although some new stationary phases such as the non-porous polymeric beads (Denizli et al., 2000) and perfusion chromatography packings are designed to resolve these problems, these limitations cannot be overcome in essence (Özkara et al., 2003). Recently, cryogel materials are considered as a novel generation of stationary phases in the separation science because of their easy preparations, excellent flow properties and high performances compared to conventional beads for the separation of biomolecules (Lozinsky et al., 2003; Arvidsson et al., 2003; Lozinsky et al., 2002; Arvidsson, et al., 2002; Babaç et al., 2006; Dainiak et al., 2006; Hanora et al., 2006).

Porous cryogels are a very good alternative to protein separation with many advantages. Several potential advantages of cryogels are large pores, short diffusion path, low pressure drop and very short residence time for both adsorption and elution.

Cryogels are gel matrices that are formed in moderately frozen solutions of monomeric or polymeric precursors. Cryogels typically have interconnected macropores (or supermacropores), allowing unhindered diffusion of solutes of practically any size, as well as mass transport of nano and even microparticles. The unique structure of cryogels, in combination with their osmotic, chemical and

mechanical stability, makes them attractive matrices for chromatography of biological nanoparticles (plasmids, viruses, cell organelles) and even whole cells. Polymeric cryogels are efficient carriers for the immobilization of biomolecules and cells. Cryogels are formed as a result of cryogenic treatment (freezing, storage in the frozen state for a definite time and defrosting) of low or highmolecular weight precursors, as well as colloid systems all capable of gelling.

Production is carried out in partially frozen reaction system where ice crystals perform as pore-forming material (porogen) and the gelation proceeds in non-frozen microphase of the apparently frozen reaction system. Figure 2.6.a shows cryogel formation.

When gelation in microphase is completed, melting of the reaction system results in a system of large pores (the space previously occupied by ice crystals) surrounded by walls of dense hydrogel formed in the unfrozen microphase. In Figure 2.6.b different forms of cryogels can be seen. SEM photographs of some cryogels can be seen in Figure 2.6.c.

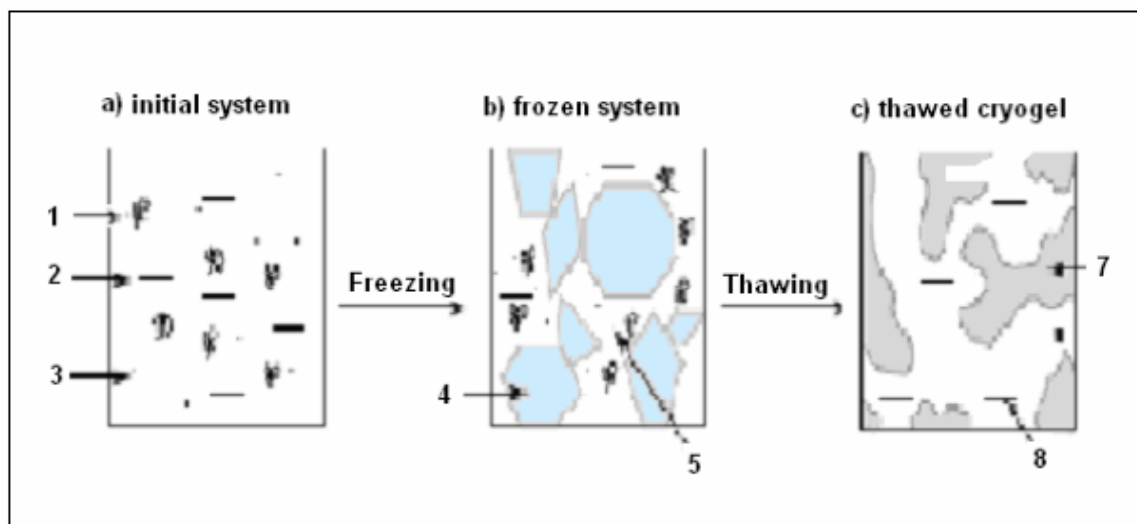


Figure 2.6.a. Cryogel formation.

- 1)Macromolecules 2) Solution 3) Solvent 4) Polycrystals of freezed solvent
5)Unfreezed solvent 6) Cryogel 7) Macropores 8) Solvent.

Cryogels are formed in different forms; Beads, monolithic columns, disks.



Figure 2.6.b. Different forms of cryogel.

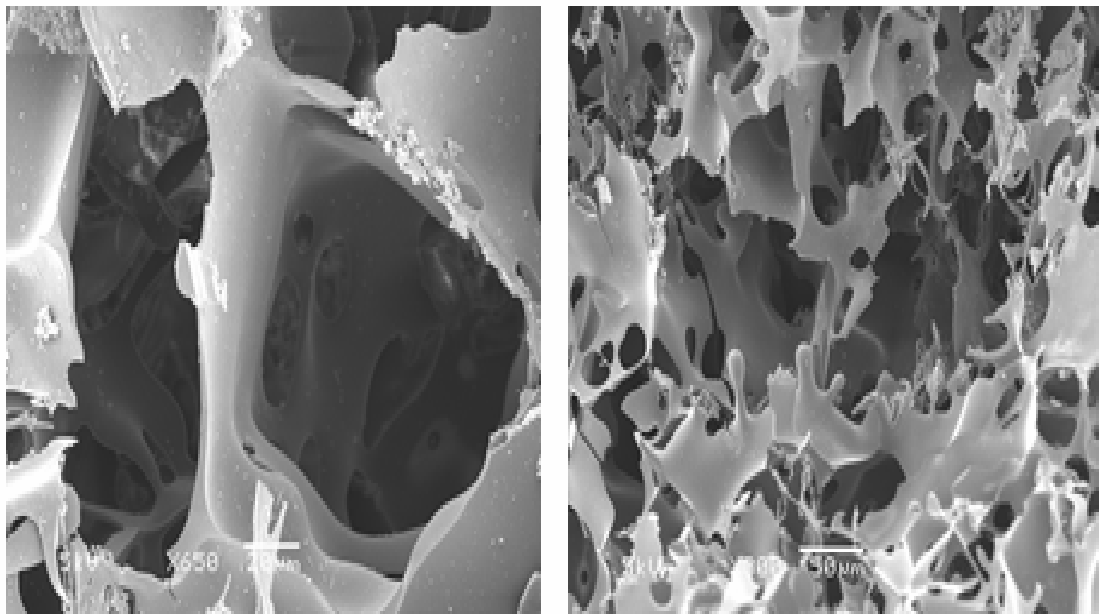


Figure 2.6.c. SEM micrographs of cryogels (Galaev, 2003).

3. EXPERIMENTAL

3.1. Materials

2-Hydroxyethyl methacrylate (HEMA) was supplied by Fluka A.G. (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor, and stored at 4°C until use. L-tryptophan and methacryloyl chloride were supplied by Sigma (St. Louis, MO) and used as received. N,N'-methylene-bis(acrylamide) (MBAAm) and ammonium persulfate (APS) were supplied by Sigma (St. Louis, USA). N,N,N',N'-tetramethylene diamine (TEMED) was obtained from Fluka A.G. (Buchs, Switzerland). Wide range molecular weight marker supplied by Sigma (St. Louis, MO), Eggs were purchased from market. All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany) unless otherwise noted. All water used in the adsorption experiments was purified using a Barnstead (Dubuque, IA) ROpure LP[®] reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure[®] organic/colloid removal and ion exchange packed-bed system. Laboratory glassware was kept overnight in a 5% nitric acid solution. Before use the glassware was rinsed with deionised water and dried in a dust-free environment.

3.2. Preparation of Cryogel

3.2.1. Synthesis of Methacryloyltryptophan (MATrp) Monomer

For the synthesis of methacryloyltryptophan (MATrp), the following experimental procedure was applied: 5.0 g of L-tryptophan and 0.2 g of hydroquinone were dissolved in 100 mL of CH₂Cl₂ solution. This solution was cooled down to 0°C. 12.74 g triethylamine was added to the solution. 5.0 mL of methacryloyl chloride was poured slowly into this solution under nitrogen atmosphere and then this solution was stirred magnetically at room temperature for 2 hour. At the end of this chemical reaction period, unreacted methacryloyl chloride was extracted with 10%

NaOH. Aqueous phase was evaporated in a rotary evaporator. The residue (i.e., MATrp) was dissolved in ethanol.

3.2.2. Preparation of Poly(HEMA-MATrp) Cryogel

The macroporous affinity cryogel was prepared as; HEMA (1.3 mL) and MATrp (20 mg) were dissolved in 5.0 mL deionized water. MBAAm (0.283 g) was dissolved in 10 mL deionized water. Second solution was mixed with previous one. The cryogel was then produced by free radical polymerization initiated by TEMED (25 μ L) and APS (20 mg). After adding APS (1% (w/v) of the total monomers) the solution was cooled in an ice bath for 2-3 min. TEMED (1% (w/v) of the total monomers) was added and the reaction mixture was stirred for 1 min. Then, the reaction mixture was poured into plastic syringe (5 mL, id. 0.8 cm) with closed outlet at the bottom. The polymerization solution in the syringe was frozen at -16°C for 24 hour 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°C until use. To see the effect of monomer concentration to the pore size, two different poly(HEMA-MATrp) cryogel were synthesized. Table 3.1. illustrates the recipe to obtain cryogels.

Table 3.1. Monomer ratio of MATrp to total volume.

Polymer	HEMA (mL)	MATrp (μ L)	TEMED (μ L)	MBAA (g)	APS (mg)	Total volume (mL)	Molarity of MATrp (M)
Polymer I	1.3	56.8	25	0.283	20	10	$7.35 \cdot 10^{-3}$
Polymer II	1.3	56.8	25	0.283	20	15	$4.90 \cdot 10^{-3}$

Schematic representation of polymerization system used in the preparation of poly(HEMA-MATrp) cryogel is shown in Figure 3.1.

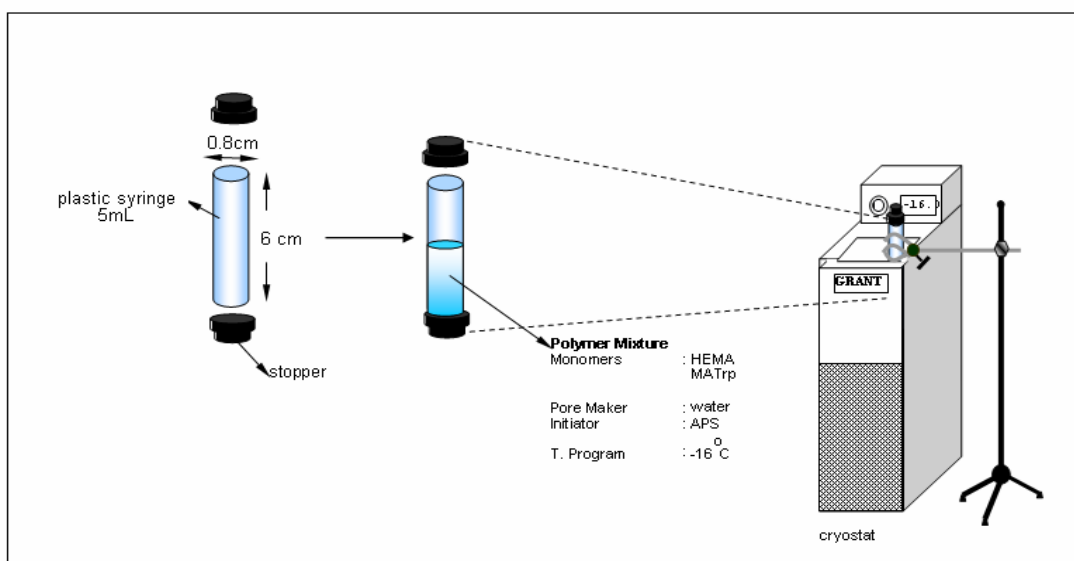


Figure 3.1. Schematic representation of polymerization system used in the preparation of poly(HEMA-MATrp) cryogel.

3.3. Characterization of Poly(HEMA-MATrp) Cryogel

3.3.1. Surface Area Measurements

Porosity of the poly(HEMA-MATrp) cryogel was measured by the nitrogen sorption technique, performed on Flowsorb II, (Micromeritics Instrument Corporation, Norcross, USA).

The surface area of the poly(HEMA-MATrp) cryogel was determined in BET isotherm of nitrogen with an ASAP2000 instrument (USA, Micromeritics). Pore volumes and average pore diameter greater than 20 Å were determined by mercury porosimeter up to 2000 kg/cm² using a Carlo Erba model 200.

3.3.2. Surface Morphology

The surface morphology of the poly(HEMA-MATrp) cryogel was examined using scanning electron microscopy (SEM). The sample was fixed in 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer overnight, post-fixed in 1% osmium tetroxide for

1.0 h. Then the sample was dehydrated stepwise in ethanol and transferred to a critical point drier tempered to +10°C where the ethanol was changed for liquid carbon dioxide as transitional fluid. The temperature was then raised to +40°C and the pressure to ca. 100 bar. Liquid CO₂ 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°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).

3.3.3. Swelling Test

Water uptake ratios of the poly(HEMA) and poly(HEMA-MATrp) cryogel were determined in distilled water. The experiment was conducted as follows: initially dry cryogel were carefully weighed (167.7 mg) before being placed in a 40 mL vial containing distilled water. The vial was put into an isothermal water bath with a fixed temperature (25±0.5°C) for 2 h. The sample was taken out from the water, wiped using a filter paper, and weighed. In different times the weight of cryogel was recorded. After 24 hour the last weight of cryogel was recorded. The weight ratio of dry and wet samples was recorded. The water content of the Poly(HEMA) and poly(HEMA-MATrp) cryogel was calculated by using the following expression;

$$\text{Water uptake ratio \%} = [(W_s - W_o) / W_o] \times 100 \quad (3.1)$$

W_o and W_s are the weights of cryogel before and after uptake of water, respectively.

The total volume of macropores in the swollen cryogel was roughly estimated as follows. The weight of the sample ($m_{\text{squeezed gel}}$) was determined after squeezing the free water from the swollen gel matrix. For porosity measurement, cryogel was swelled in 40mL water and then squeezed. The porosity was calculated as follows;

$$\text{Porosity \%} = (m_{\text{swollen}} - m_{\text{squeezed}}) / m_{\text{swollen}} \times 100 \quad (3.2)$$

3.3.4. FTIR Studies

The characteristic functional groups of the MATrp and poly(HEMA-MATrp) cryogel were analyzed by using a Fourier Transform infrared spectrophotometer (FTIR, 8000 Series, Shimadzu, Japan). The samples were prepared by mixing with approximately 100 mg of dry, powdered KBr (0.1 g, IR Grade, Merck, Germany), and pressed into a pellet form. The FTIR spectrum was then recorded.

3.3.5. ^1H -NMR and ^{13}C -NMR Studies

A JEOL GX-400 instrument (300 MHz) was used to acquire ^1H - and ^{13}C -NMR spectra of monomer solutions in CDCl_3 . The residual non-deuterated solvent (CHCl_3) served as an internal reference. Chemical shifts are reported in ppm (δ) downfield relative to CHCl_3 .

Variable contact time ^{13}C CP/MAS spectra were recorded at room temperature at 150.90 MHz on a Bruker AV-600. The samples were spun at 8 kHz, and 1000–1800 scans were accumulated. The contact time varied from 0.01 to 11 ms. For the dry samples, the tablets (received from Labopharm) were crushed before the NMR experiments. For the hydrated samples, tablets were crushed and the powder was swollen in distilled water at 37 °C for 48 h.

^{13}C -NMR spectrum of poly(HEMA-MATrp) cryogel was taken as a solid state NMR. Bruker AVANCE instrument was used for solid state NMR. For solid samples MAS (Magic angle spinning) and CP (cross polarization) methods were used.

3.3.6. Thermogravimetric Analysis

Thermogravimetric analysis or TGA is an analytical technique used to determine a material's thermal stability and its fraction of volatile components by monitoring the weight change that occurs as a specimen is heated. The measurement is normally carried out in air or in inert atmosphere, such as Helium or Argon, and the weight

is recorded as a function of increasing temperature. Sometimes the measurement, is performed in a lean oxygen atmosphere (1 to 5% O₂ in N₂ or He) to slow down oxidation. TGA was used to determine degradation temperatures, absorbed moisture content of materials, the level of inorganic and organic components in materials, decomposition points of explosives, and solvent residues. For Thermogravimetric Analysis DTG 60 Shimadzu Model was used. TGA curve of the sample recorded at a heating rate of 10 °C/min. in nitrogen atmosphere.

3.4. Lysozyme Adsorption-Desorption Studies

3.4.1. Adsorption of Lysozyme from Aqueous Solution

The lysozyme adsorption studies were carried out in a recirculating system equipped with a water jacket for temperature control. Figure 3.2 shows used column system for adsorption experiments. The cryogel was washed with 30 mL of water and then equilibrated with 50 mM phosphate buffer containing 0.1 M (NH₄)₂SO₄ (pH 6.0). Then, the prepared lysozyme solution (1.0 mg/mL, 30 mL, pH 6.0 containing 0.1 M (NH₄)₂SO₄ salt) was pumped through the column under recirculation for 2.0 hour. The adsorption was followed by monitoring the decrease in UV absorbance at 280 nm. Effects of lysozyme concentration, flow rate, pH of the medium, temperature and salt type on the adsorption capacity were studied.

To observe the effect of the initial concentration of lysozyme on adsorption capacity, it was changed between 0.3-2.0 mg/mL.

To estimate the effect of flow rate, different flow rates were investigated for 0.5 mg/mL lysozyme solution. Lysozyme solution was prepared in 50 mM phosphate buffer containing 0.1 M (NH₄)₂SO₄ (pH 6.0, 30 mL) and pumped through the column under recirculation for 2.0 hour at different flow rates; 0.5, 1.0, 1.5, 2.0 mL/minute.

To determine the effect of pH on the adsorption capacity, pH of the solution was changed between 4.0 and 12.0.

To observe the effects of the temperature on the adsorption capacity, adsorption studies were carried out at 4°C, 25°C, 37°C and to observe the effects of salt type firstly; the adsorption experiments were carried out in salt free solutions and in solutions containing Na₂SO₄, (NH₄)₂SO₄ and NaCl. The concentration of salts were 0.1 M. The amount of adsorbed lysozyme was calculated as:

$$q = [(C_0 - C) V] / m \quad (3.3)$$

Here, q is the amount of lysozyme adsorbed onto unit mass of cryogel (mg/g); C_0 and C are the concentrations of lysozyme 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 cryogel used (g). Each experiment was performed twice for quality control and statistical calculations.

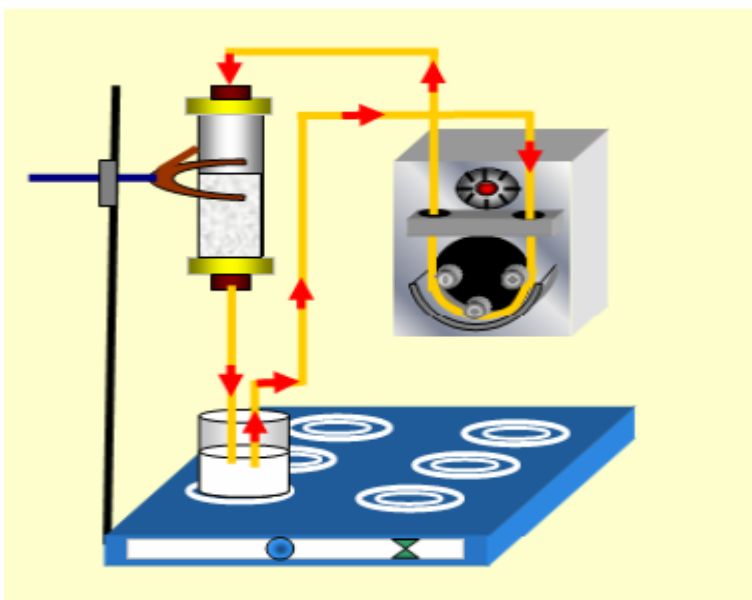


Figure 3.2. Continuous system used for adsorption experiments.

3.4.2. Adsorption of Lysozyme from Chicken Egg white

The content of lysozyme in chicken egg white is about 3.4% (Huopalahti et al., 2007). The classical lysozyme purification method required several steps, such as precipitation, centrifugation and affinity adsorption (Altıntaş and Denizli, 2006). In this study, lysozyme purification from egg white was studied in a column system.

To estimate adsorption capacity of poly(HEMA-MATrp) cryogel for the egg white lysozyme, chicken egg white separated and diluted with pH 6.0 phosphate buffer solution (1:10). Prepared solution (30 mL) was pumped through poly(HEMA-MATrp) cryogel for 2.0 hour. The adsorption was followed by monitoring the decrease in UV absorbance at 280 nm.

The activity of desorbed lysozyme was determined spectrophotometrically at 620 nm, the decrease in the turbidity of culture of *Micrococcus lysodeikticus* cells suspended in phosphate buffer (0.1 M, pH 6.0) was followed for 5.0 minute after addition of lysozyme. One unit lysozyme activity was defined as the amount of enzyme causing a decrease of 0.001 optical density value per minute at 25°C and pH 6.0.

3.4.3. Desorption and Reusability of Adsorbent

The desorption of the adsorbed lysozyme from the poly(HEMA-MATrp) cryogel was studied in continuous experimental setup. Lysozyme adsorbed poly(HEMA-MATrp) cryogel were circulated with 0.5 M ethylene glycol solution for 2.0 hour at room temperature. The final lysozyme concentration in the desorption medium was determined by Bradford method at 595 nm. The desorption ratio for lysozyme was calculated with the following expression:

$$\text{Desorption ratio (\%)} = \frac{\text{Amount of lysozyme released}}{\text{Amount of lysozyme adsorbed}} \times 100 \quad (3.4)$$

In order to show the reusability of cryogel, adsorption-desorption cycle was repeated five times by using the same cryogel.

3.5. Fluorimetric Measurement

In order to determine whether is there any denaturation in lysozyme structure, fluorimetric measurements were taken with a Shimadzu spectrofluorimeter (Tokyo, Japan) with 1-cm quartz cells. Monochromatic readings were taken from a digital display with a 0.25-s time constant and with a 5-nm band width on the excitation side and a 5-nm band width on the emission side. The initial calibration was carried out with a standard solution of lysozyme in phosphate- buffered saline with a 280-nm fluorescence excitation wavelength and a 340-nm emission wavelength (Öncel et al. 2005).

3.5.1. Fluorimetric Measurements for Aqueous Solution of Lysozyme

1.0 mg/mL lysozyme solution including 0.1 M $(\text{NH}_4)_2\text{SO}_4$ salt was prepared in pH 6.0 phosphate buffer solution (30 mL). From this solution 5.0 μL sample was taken and diluted to 5.0 mL with pH 6.0 phosphate buffer solution and fluorimetric observation was done. Result was recorded as initial. Lysozyme solution was passed through cryogel column for 2.0 hour. Then, adsorbed lysozyme was desorbed by using 0.5 M 30 mL ethylene glycol solution. Again fluorimetric measurement was done and recorded as desorbed. For denaturation, lysozyme solution was boiled for 1.0 hour and sonicated for 15 minute. Fluorimetric spectrum was taken.

3.5.2. Fluorimetric Measurements for Lysozyme of Egg

Before applying fluorimetric measurements, proteins other than lysozyme were removed with saturated $(\text{NH}_4)_2\text{SO}_4$ solution. 10 mL of egg white was diluted to 50 mL with pH 6.0 buffer. By taking 5.0 mL of this solution, egg white solution was again diluted to 50 mL. From this solution 5.0 μL sample was taken and diluted to 5.0 mL with pH 6.0 phosphate buffer solution and fluorimetric observation was

performed. Result was recorded as initial. Prepared lysozyme solution was passed through cryogel column for 2.0 hour. For desorbed and denatured lysozyme fluorimetric measurement was done as mentioned above. For denaturation heating treatment was performed.

3.6. Fast Protein Liquid Chromatography

Fast protein liquid chromatography, usually referred to as FPLC, is a form of column chromatography, used to separate or purify proteins from complex mixtures by using ion chromatography. Columns used with an FPLC can separate macromolecules based on size, charge distribution, hydrophobicity or biorecognition.

Chicken egg white lysozyme and the other proteins were separated by using FPLC system. Chicken egg white was separated from fresh egg and diluted (1:4) with phosphate buffer (50 mM, pH 6.0). The diluted egg-white was homogenised in an ice bath and centrifuged at 4°C, at 10.000 rpm for 30 min. To increase the intensity of the peak for lysozyme, the supernatant of this solution was processed with saturated $(\text{NH}_4)_2\text{SO}_4$ solution to remove proteins with high molecular weight like albumin.

FPLC separation was performed using an AKTA-FPLC (Amersham Bioscience, Uppsala, Sweden) system equipped monitor, INV-907 injection valve and Frac920 fraction collector. Separation was carried out at GE Healthcare column (10/10, 19-5001-01) that packed with poly(HEMA-MATrp) cryogel. FPLC mobile phases A and B were prepared using 50 mM phosphate buffer solution containing 0.1 M $(\text{NH}_4)_2\text{SO}_4$ (pH 6.0) and ethylene glycol (0-0.5 M). The chromatographic separation was performed using a linear gradient at 2.0 mL/min flow rate. After a 9.0 min starting period with 100% mobile phase A, a linear gradient started from 0% B to 100% B in 3.0 min, continued with 4.0 min 100% eluent B and finished last 4 min 100% buffer A. All buffers and protein solutions were filtered before use. 30 mL protein mixture was applied to the column. Absorbance was monitored at 280 nm.

The separation was performed at room temperature. KBr was used as the void marker. Capacity factor (k') and separation factor (α) were calculated as;

$$k' = (t_R - t_0) / t_0 \quad (3.5)$$

$$\alpha = k'_2 / k'_1 \quad (3.6)$$

where t is the retention time of the protein and t_0 is the retention time of the void marker. The resolution (R_s) and theoretical plate numbers (N) were calculated using the following equations;

$$N = 5.54 (t_R / w_{0.5})^2 \quad (3.7)$$

$$R_s = (t_{R,2} - t_{R,1}) / (w_2 + w_1) \quad (3.8)$$

Where $w_{0.5}$ is the peak height at the corresponding peak height fraction, $t_{R,1}$ and $t_{R,2}$ are the retention times of two adjacent peaks, w_1 and w_2 are the widths of the two adjacent peaks at the baseline.

3.7. Electrophoretic Separation

Electrophoresis is a separation technique that is based on the the mobility of ions in an electric field. Positively charged ions migrate towards a negative electrode and negatively-charged ions migrate toward a positive electrode. Ions have different migration rates depending on their total charge, size, and shape, and can therefore be separated. Since molecules of a variety of shapes and sizes, they had to be done so that the proteins no longer have any secondary, tertiary or quaternary structure (i.e. we want them to have the same linear shape). Not only the mass but also the shape of a protein will determine how well it can move through and environment. So all proteins were converted to the same shape. SDS was used for making all proteins negative.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out 10% acrylamide separating gel (in 1.5 M Tris-HCl pH 8.8) and a 5% acrylamide stacking gel (in 1.0 M Tris-HCl pH 6.8) containing 10% SDS. 3.0 mg/mL lysozyme solution

was prepared (30 mL) and recirculated for 2.0 hour. Initial, and desorbed samples (15 μ L,) were taken and mixed with 15 μ L bromophenol (0.002% bromophenol blue in 50% aqueous glycerol containing 1% mercaptoethanol). This sample was runned through Tris-glycine buffer at pH 8.3 containing 0.1% SDS. Electrophoresis was run at a current of 16 mA for 1.0 hour and 30 mA for 5 hour. These currents were used for 5% stacking and 10% separating gel respectively. After electrophoresis, the gels were stained with 0.25% (w/v) Coomassie Brilliant R 250 in acetic acid-methanol-water (1:5:5, v/v/v) and destained in ethanol-acetic acid-water (1:4:6, v/v/v).

3.7.1. Electrophoretic Separation of Lysozyme from Aqueous Solutions

3.0 mg/mL of lysozyme solution was prepared in pH 6.0 buffer solution as 30 mL. 15 μ L of this solution and 15 μ L bromophenol blue solution were mixed and poured to the well of the gel. After electrophoresis, separated lysozyme was visualized with coommasie blue and recorded as initial.

The cryogel was washed with 30 mL of water and then equilibrated with 50 mM phosphate buffer containing 0.1 M $(\text{NH}_4)_2\text{SO}_4$ (pH 6.0). Then, the prepared lysozyme solution containing 0.1M $(\text{NH}_4)_2\text{SO}_4$ salt (30 mL of the aqueous lysozyme solution in pH 6.0 as a 3.0 mg/mL) was pumped through the column under recirculation for 2.0 hour. After this period, adsorbed lysozyme was desorbed with 0.5 M ethylene glycol and 15 μ L of this solution was used for electrophoretic mobilization and recorded as final.

3.7.2. Electrophoretic separation of Lysozyme from Egg White

The content of lysozyme in chicken egg white is about 3.4%. Chicken egg white was separated from fresh egg and diluted (1:10) with phosphate buffer (50 mM, pH 6.0). The diluted egg white was homogenised in an ice bath and centrifuged at 4°C, at 10.000 rpm for 30 min. The supernatant of this solution was processed with saturated $(\text{NH}_4)_2\text{SO}_4$ solution to remove proteins with high molecular weight like

albumin. Same procedure was applied for egg white solution as mentioned for aqueous lysozyme solutions.

4. RESULTS AND DISCUSSION

Lysozyme is found in a variety of vertebrate cells and secretions, such as spleen, milk, tears and egg white. Lysozyme lyses certain bacteria by hydrolysing the β -linkages between the muramic acid and N-acetylglucosamine of mucopolysaccharides which are present in the bacterial cell wall. Its common applications are as a cell disrupting agent for extraction of bacterial intracellular products, as an antibacterial agent in ophthalmologic preparations, as a food additive in milk products and as a drug for treatment of ulcers and infections (Ghosh et al., 2000). The potential for its use as an anticancer drug has been demonstrated by animal and in-vitro cell culture experiments (Das and Banerjee, 1992). Lysozyme has also been used in cancer chemotherapy. The studies conducted so far show that it induces the activity of phagocytizing cells, influences immunological processes by stimulating immunoglobulin synthesis, promotes interferon synthesis and modulates tumour necrosis factor generation (Sugahara, et al., 2000). High purity requirements for both natural and therapeutic proteins along with the commercial pressures to reduce processing costs have stimulated more efficient, simple and relatively less expensive separation techniques for lysozyme production in the recent years (Murakami et al., 1997). The large scale applications require more efficient and cost effective techniques for its isolation (Odabaşı and Denizli, 2004).

Aminoacid based ligands may hold certain advantages for industrial bioaffinity separations since they are not likely to cause an immune response in case of leakage into the product. These ligands are also much more stable than protein ligands because they don't require tertiary structure for maintaining biological activities. They can be manufactured aseptically in large quantities under GMP (good manufacturing practices) conditions, and at significantly lower cost (Huang and Carbonell, 1999).

In this thesis, a novel MATrp containing hydrophobic affinity adsorbent poly(HEMA-MATrp) cryogel was prepared for efficient separation of lysozyme from aqueous solutions. The main criteria of selection of poly(HEMA) as the basic component is

due to its inertness, mechanical strength, chemical and biological stability (Denizli and Pişkin, 1995). In this procedure, MATrp comonomer acts as a hydrophobic ligand, and there is no need to activate the matrix for the ligand immobilization. MATrp monomer was polymerized with HEMA and no leakage of the ligand was observed. Figure 4.1 represents cross-section photographs of cryogels:

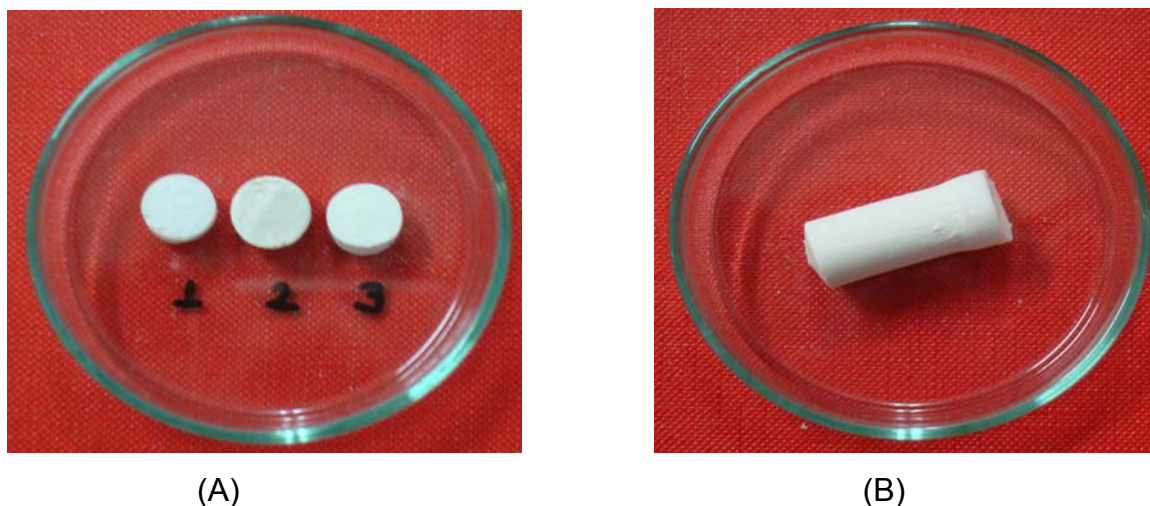


Figure 4.1. Photographs of cryogels; (A) represent cross-section photographs of cryogels: 1) stand for poly(HEMA), 2) poly(HEMA-MATrp) polymer I 3) poly(HEMA-MATrp), polymer II (B) represent block cryogel of poly(HEMA-MATrp), polymer II.

4.1. Characterization of poly(HEMA-MATrp) Cryogel

4.1.1. Surface Area Measurements Surface Morphology

The specific surface area of the poly(HEMA-MATrp) cryogel was found to be 50 m²/g polymer by the multipoint BET method. According to the BJH method, the pore diameter of the poly(HEMA-MATrp) cryogel changed between 20 and 245 Å and average pore diameter is 34.6 Å. This indicated that the poly(HEMA-MATrp) cryogel has macropores. This pore diameter is possibly in the range of diffusion of lysozyme molecules. The molecular size, diameter and weight (M_w) of lysozyme are 0.30-0.30-0.45 Å, 27.3 Å and 14.6 kDa, respectively. On the basis of these data, it was concluded that the poly(HEMA-MATrp) cryogel has effective pore volume and dimensions for lysozyme molecules.

The surface area of the poly(HEMA-MATrp) cryogel was found to be 50 m²/g. An advantage of cryogels is that they have large, highly interconnected pores. Although this provides cryogels with low back pressures but it also gives them much lower surface areas compared to other chromatographic supports. This in turn can result in small amounts of immobilized ligand and low sample capacities when using cryogels in affinity separations (Arvidsson, et al., 2003).

Table 4.1. Surface area measurements of poly(HEMA-MATrp) cryogel.

Polymer	Surface Area (BET) ^a (m ² /g)	Total Pore Volume ^b (mL/g)	Average Pore Diameter ^c (Å)
poly(HEMA-MATrp)	50	0.173	34.6

a. Determined using multipoint BET method

b. BJH cumulative desorption pore volume of pores between 20 and 245 Å

c. BJH desorption average pore diameter of pores between 20 and 245 Å

4.1.2. Surface Morphology

The surface morphology of poly(HEMA-MATrp) cryogel was exemplified by the scanning electron micrographs in Figure 4.2. SEM micrographs were taken for two type monomer percent.

Polymer II was used for experiments, because as can be seen from Figure 4.2.a, polymer I cryogel has not large pores because ratio of monomers to the total volume were so high. In Figure 4.2.a, it can be seen neglectable pores in polymer I. Cryogel structure of polymer I was highly stiff. Pores are formed, when the molarity of MATrp decreased from 7.35.10⁻³ M to 4.90.10⁻³ M. As clearly seen from Figure 4.2.b, the polymeric cryogel has macropores which formed during the polymerization procedure. The presence of pores within the cryogel interior is clearly seen in this photograph. It can be concluded that the poly(HEMA-MATrp) cryogel has a macroporous interior, in the dry state. These pores reduce diffusional resistance of lysozyme molecules and facilitate mass transfer because of high pore size. Poly(HEMA-MATrp) cryogel produced in such a way have large continuous

interconnected pores (60–100 μm in diameter, supermacroporous) 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. This also provides higher lysozyme adsorption capacity.

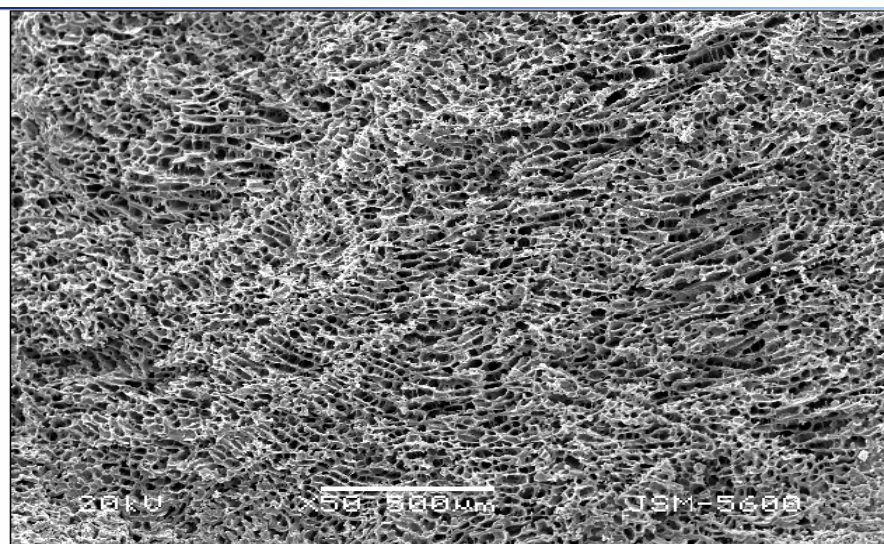


Figure 4.2.a. SEM micrograph of poly(HEMA-MATrp) cryogel, Polymer I.

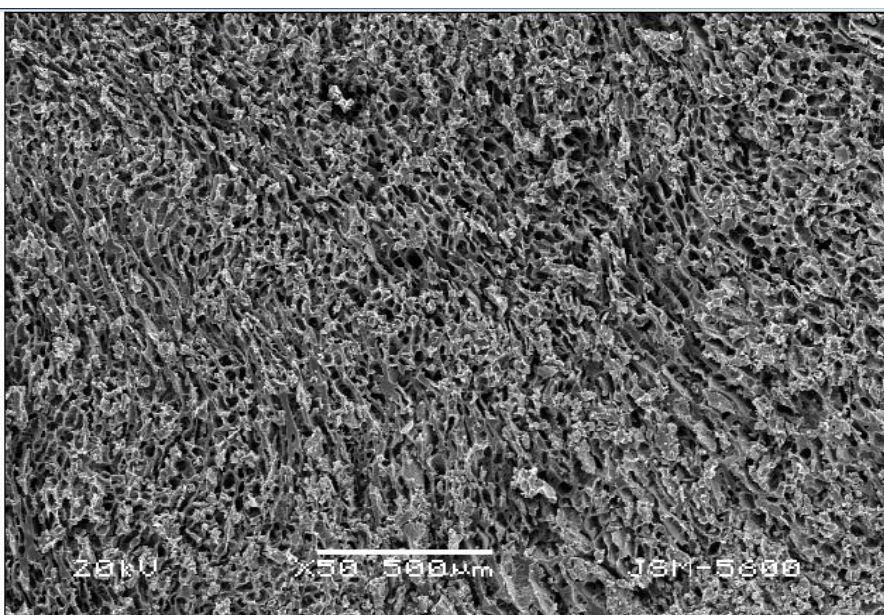


Figure 4.2.b. SEM micrograph of poly(HEMA-MATrp) cryogel, Polymer II.

4.1.3. Swelling Test

The poly(HEMA-MATrp) cryogel is cross-linked. Cryogel does not dissolve in aqueous media, but swell depending on the degree of crosslinking and on the

hydrophilicity of the matrix. The equilibrium swelling ratio of the cryogel used in this study is 6.99 g H₂O/g for poly(HEMA-MATrp) and 7.18 g H₂O/g for poly(HEMA). Water uptake ratio of the poly(HEMA-MATrp) cryogel is smaller than poly(HEMA) cryogel. It was expected to be higher water uptake value for poly(HEMA-MATrp) cryogel. Because copolymerizing MATrp with HEMA could effectively decrease the molecular weight of the resulting polymer and reduce the crystallinity of the structure. Therefore, the water molecules penetrate into the entanglement polymer chains more easily, resulting in an improvement of polymer water uptake behavior in aqueous solutions. But hydrophobicity of MATrp is enormous and in fact fact increasing hydrophobicity means decreasing water uptake. Hydrophobicity effect is dominant in water uptake. Figure 4.3 shows swelling degree of Poly(HEMA-MATrp) cryogel with time.

Porosity of poly(HEMA-MATrp) cryogel was estimated. For estimation, firstly cryogel was swollen and then squeezed. Porosity in water was found 69% and in carbontetrachloride as 81%.

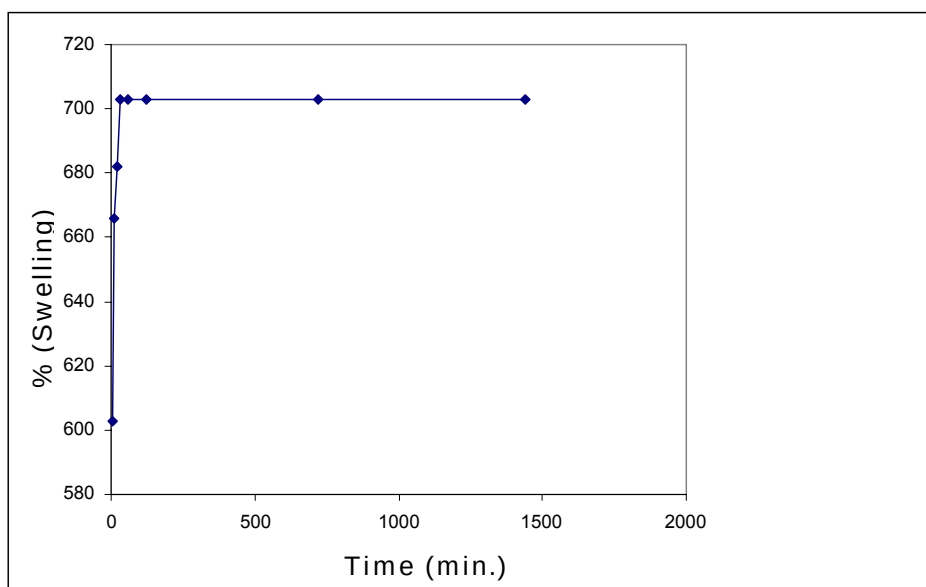


Figure 4.3. % Swelling of poly(HEMA-MATrp).

4.1.4. FTIR Studies

As mentioned before, MATrp was selected as the comonomer and hydrophobic ligand for the selective separation of lysozyme from aqueous solutions. In the first step, MATrp was synthesized from L-tryptophan and methacryloyl chloride. Then MATrp was incorporated into the poly(HEMA). The molecular formula of newly synthesized MATrp comonomer was shown in Figure 4.4. Figure 4.5 shows molecular formula of poly(HEMA-MATrp) cryogel.

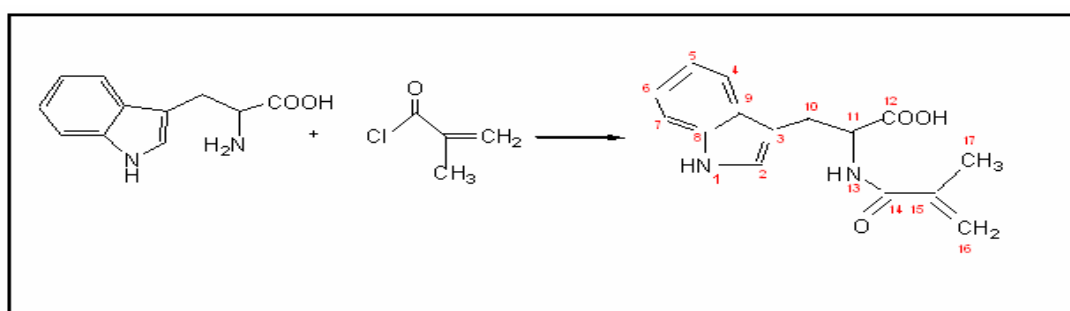


Figure 4.4. Molecular formula of MATrp comonomer.

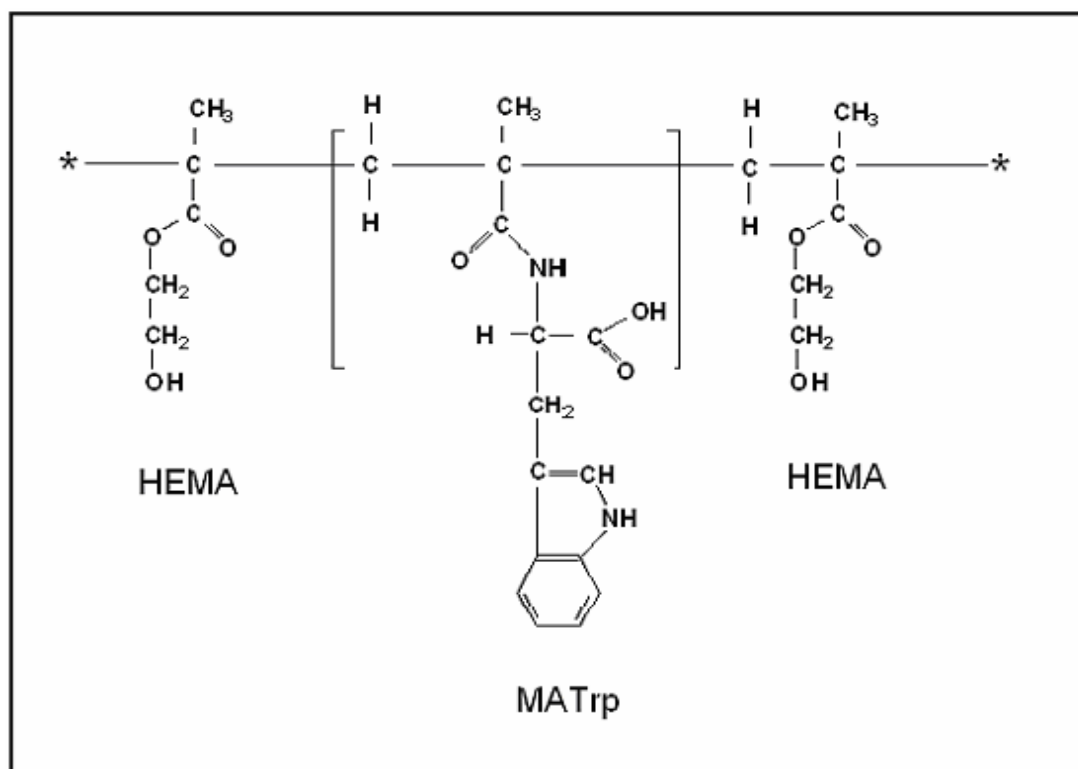


Figure 4.5. Molecular formula of poly(HEMA-MATrp) cryogel.

Figure 4.6 shows FTIR spectrum of MATrp. From the spectrum, absorption bands were observed as NH stretching (3379 cm^{-1}), CH stretching (aromatic, $3100\text{--}3000\text{ cm}^{-1}$), CH stretching (alkyl, 2978 cm^{-1}), broad band for OH group of carboxylic acid ($3000\text{--}3400\text{ cm}^{-1}$), stretching of C=O of acid (1728 cm^{-1}), stretching of C=O of amide (1657 cm^{-1}), C=C stretching (1621 cm^{-1}), NH bending (1522 cm^{-1}), C-N stretching (aromatic, $1458\text{--}1374\text{ cm}^{-1}$) and C=O bending of acid ($1347\text{--}1198\text{ cm}^{-1}$).

Molecular formula of poly(HEMA-MATrp) cryogel can be seen in Figure 4.5 and to show the incorporation of MATrp within the polymeric structure, FTIR spectra of the HEMA monomer and poly(HEMA-MATrp) cryogel were taken. Figure 4.7 and Figure 4.8 show FTIR spectra of the HEMA monomer and poly(HEMA-MATrp) cryogel respectively. As seen in Figure 4.7, HEMA has absorption bands as 3420 cm^{-1} (-OH stretching), 2954 cm^{-1} (CH alkyl stretching), 1718 cm^{-1} (C=O stretching), and 1633 cm^{-1} (C=C stretching). Figure 4.8 shows FTIR spectrum of macroporous affinity Poly(HEMA-MATrp) cryogel. As seen in Figure, the spectrum has some bands related to MATrp monomer as following, 1533 cm^{-1} (NH bending), $1454\text{--}1391\text{ cm}^{-1}$ (CN aromatic), and 1657 cm^{-1} (C=O amide), respectively. The result shows that functional monomer, MATrp, was incorporated in to the bulk structure of poly(HEMA) cryogel.

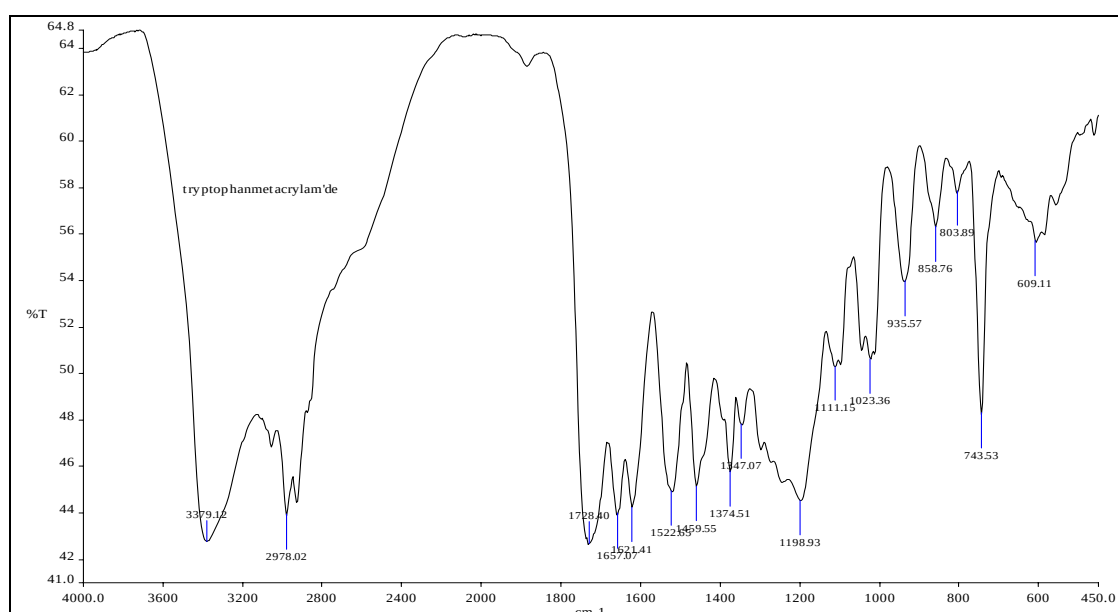


Figure 4.6. FTIR spectrum of MATrp.

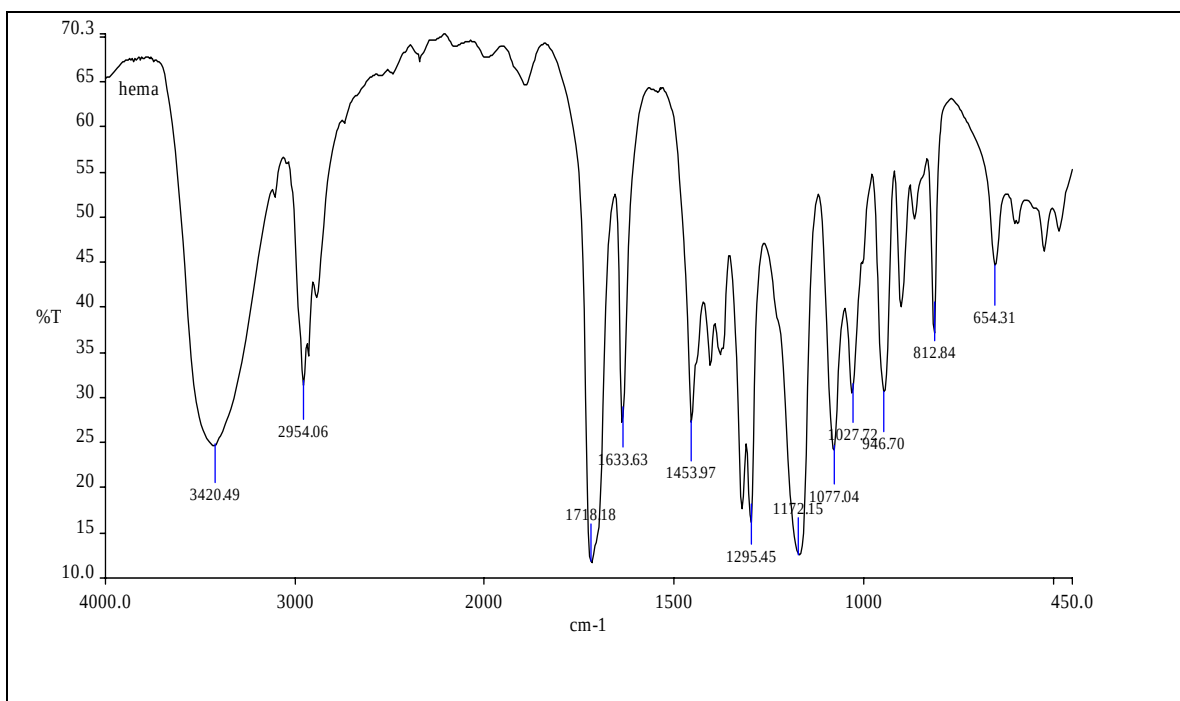


Figure 4.7. FTIR spectrum of HEMA.

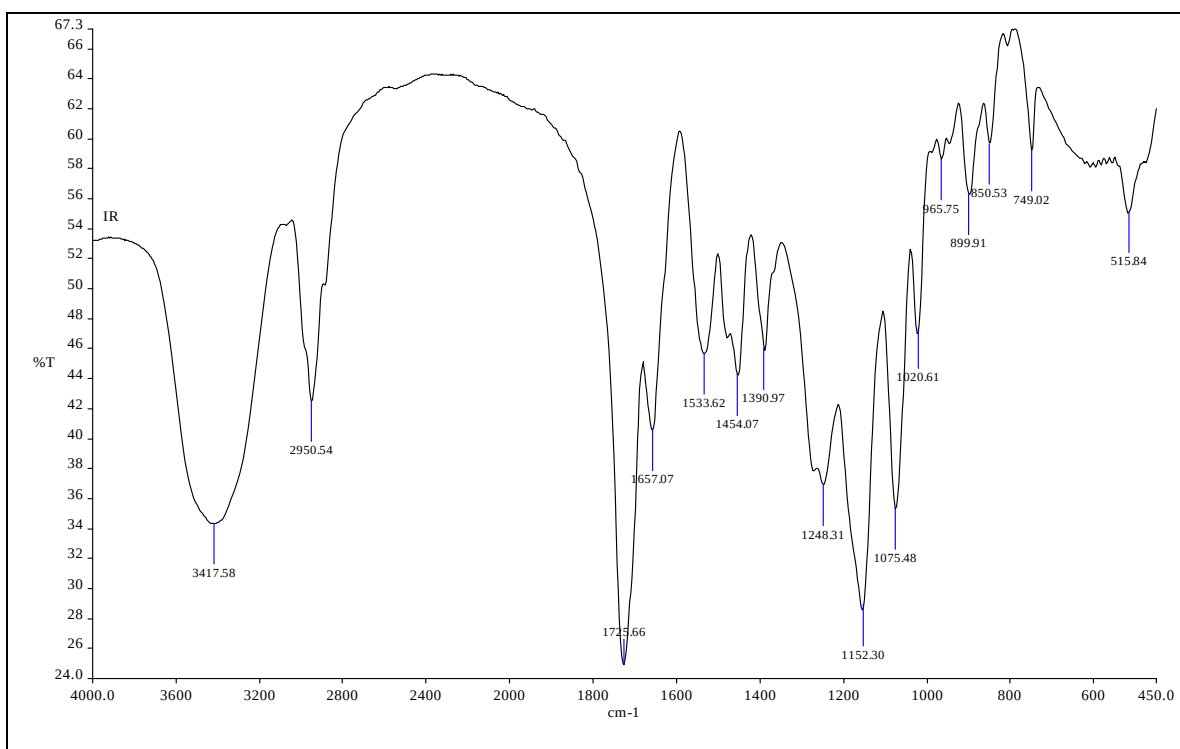


Figure 4.8. FTIR spectra of poly(HEMA-MATrp) cryogel.

4.1.5. NMR Studies

4.1.5.1 ^1H -NMR Studies

^1H -NMR was used to determine the synthesis of MATrp structure. Figure 4.9 shows the ^1H -NMR spectrum of MATrp. ^1H -NMR spectrum was shown to indicate the characteristic peaks from the groups in MATrp monomer.

Peaks in ^1H -NMR spectrum were observed as 3H, $^{17}\text{CH}_3$ 1.80 (s), 2H, $^{10}\text{CH}_2$ 3.42-3.36 (q), 1H, ^{11}CH 4.50-4.51 (m), 1H, $^{16}\text{CH}_2$ 5.33 (s), 1H, $^{16}\text{CH}_2$ 5.64 (s), 1H, ^{13}NH (amid) 7.15 (d), 5H, $^{4,5,6,7,2}\text{indol}$ 7.15-7.57 (m), 1H, ^1NH (indol) 8.25(d), 1H, ^{12}OH (acid) 11.1 (s). Respected atoms were also showed as superscript.

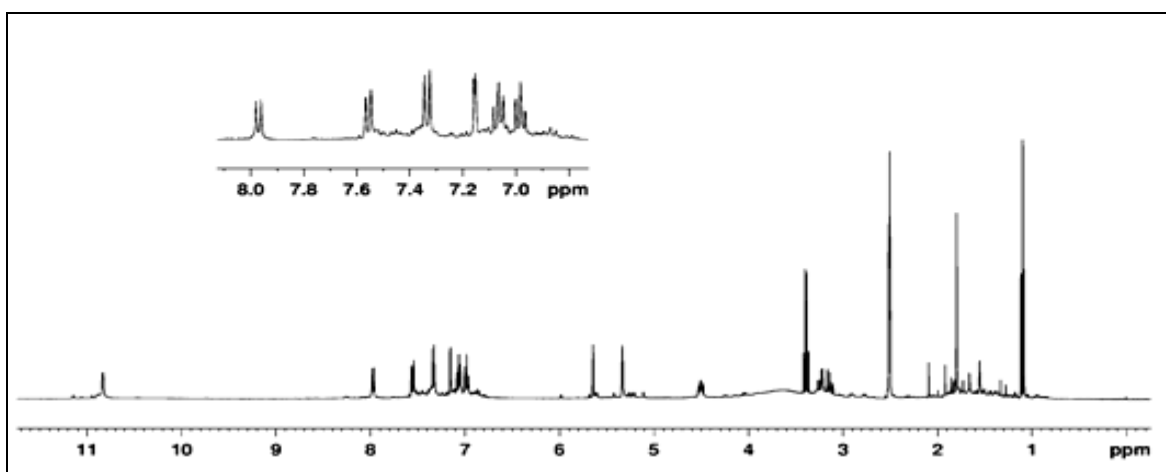


Figure 4.9. ^1H -NMR spectrum of MATrp monomer.

4.1.5.2. ^{13}C -NMR Studies

Peaks in ^{13}C -NMR spectrum were observed as CH_3 , 18.9 ppm; CH , 53.8 ppm; CH_2 , 65.4 ppm; C (vinyl), 110.7 ppm; CH (indole), 111.8 ppm; CH (benzene ring), 118.6 ppm; CH (benzene ring), 118.8 ppm; CH_2 (vinyl), 120.0 ppm; CH (benzene ring), 121.37 ppm; CH (benzene ring), 124.0 ppm; C (indole), 127.6 ppm; C

(benzene ring), 136.6 ppm; CH (benzene ring), 139.9 ppm; C=O (amide), 167.9 ppm and C=O (acid), 173.9 ppm, respectively. Peaks can be seen in Figure 4.10 for MATrp monomer.

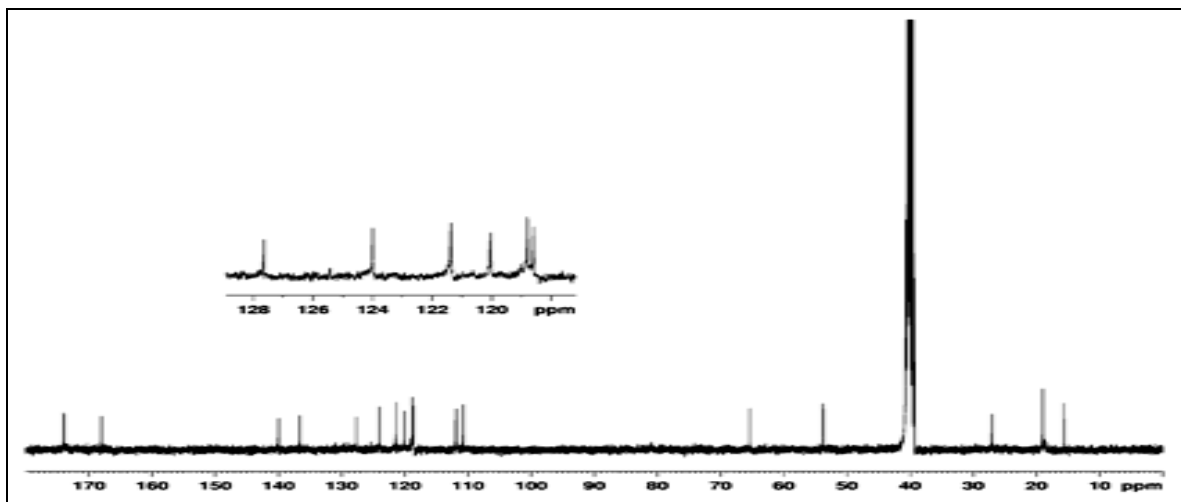


Figure 4.10. ^{13}C -NMR spectrum of MATrp monomer.

As seen in Figure 4.11, ester carbons which came from HEMA monomer, amide carbons which came from MATrp monomer were observed around 205 and 193 ppm respectively. Peaks belong to the indole groups of MATrp monomer were also observed around 110 and 155 ppm. The peaks were shown on the figure.

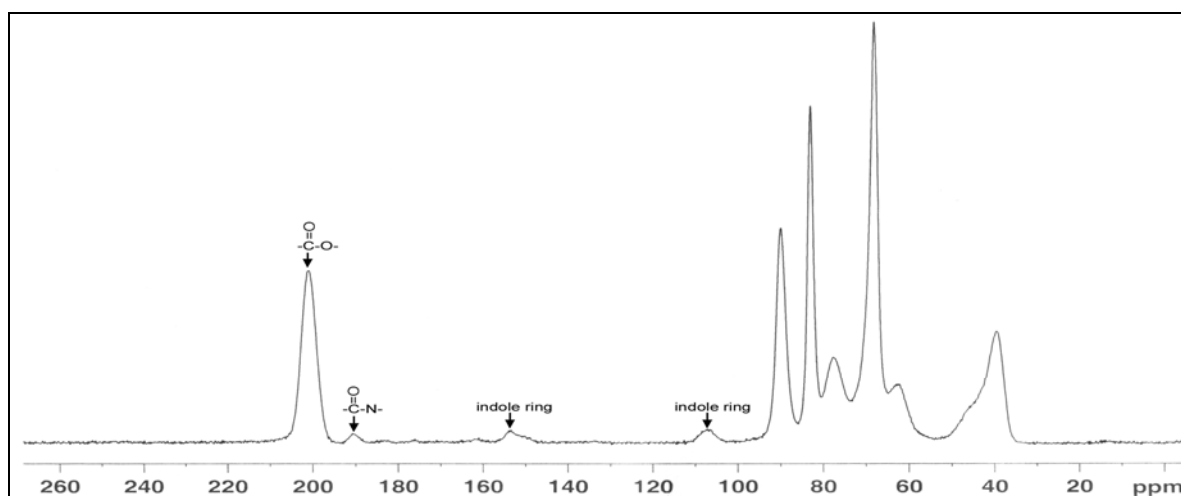


Figure 4.11 Solid state ^{13}C -NMR spectrum of macroporous poly(HEMA-MATrp) affinity cryogel.

4.1.6. Thermogravimetric Analysis of Cryogel

To estimate decomposition temperature and thermomechanical stability of poly(HEMA-MATrp) cryogel, thermogravimetric analysis (TGA) was done.

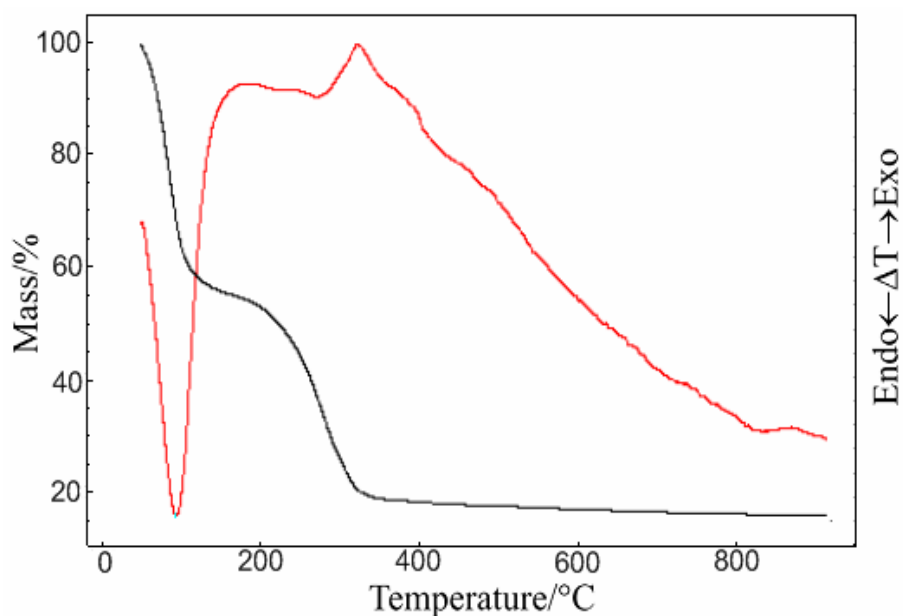


Figure 4.12. TGA curve of MATrp monomer recorded at 10°C/min. in nitrogen atmosphere.

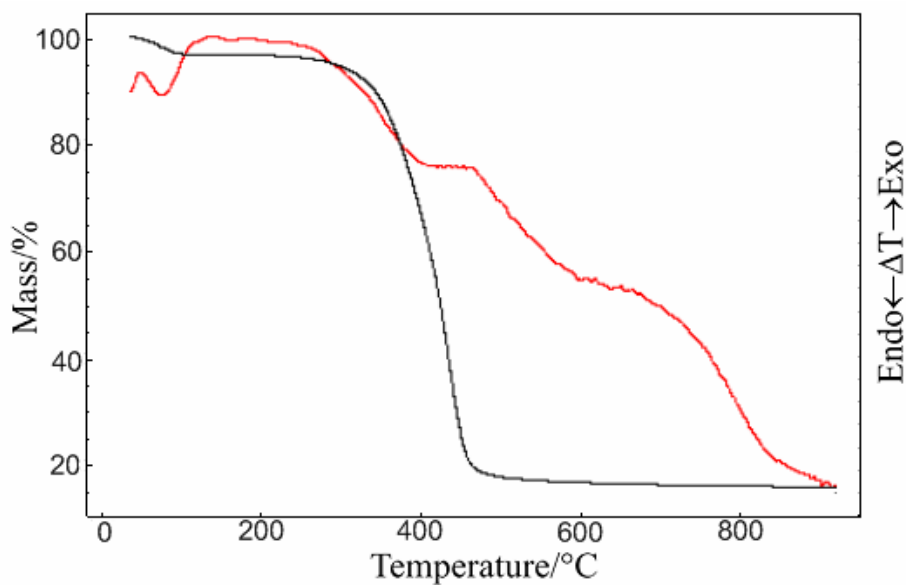


Figure 4.13. TGA curve of poly(HEMA) cryogel recorded at 10°C/min. in nitrogen atmosphere.

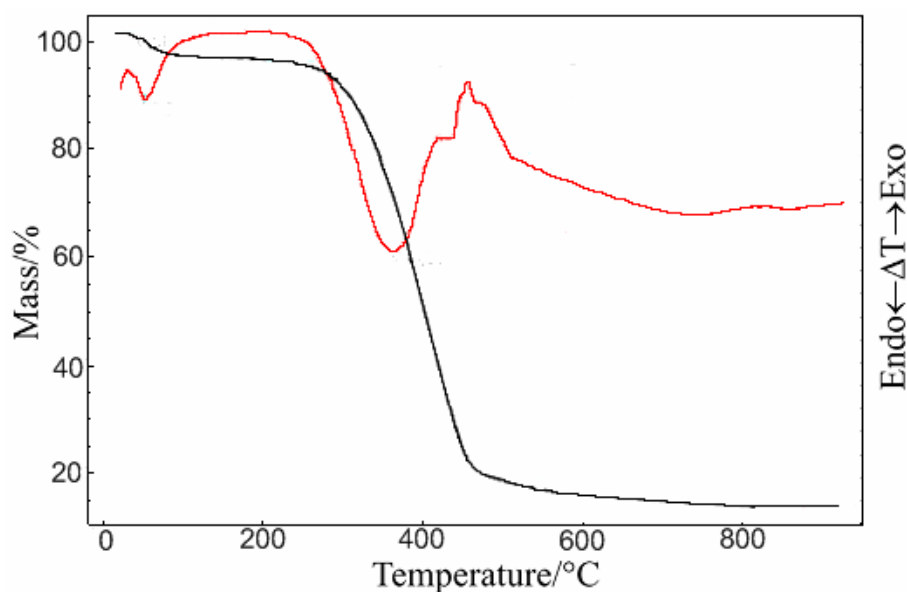


Figure 4.14. TGA curve of poly(HEMA-MATrp) cryogel recorded at 10°C/min. in nitrogen atmosphere.

Figure 4.14 shows weight losses of poly(HEMA-MATrp) cryogel due to increasing of temperature up to 900 °C. The negative first derivative of the weight loss at curve at 53.34°C displays peaks corresponding to the loss of adsorbed water. Weight loss was recorded as 5.51%. Second negative weight loss curve at 352.5°C belongs to the maximum decomposition rate of poly(HEMA-MATrp) cryogel. Decomposition starts nearly from 225°C and continuous until 500°C. Weight loss for this range is %89.9. All of the decomposition steps have endothermic peaks.

As can be easily seen decomposition of poly(HEMA-MATrp) cryogel is performed in one stage show us MATrp is coordinated to HEMA totally. There is no unreacted MATrp.

As can be seen from Figure 4.13 decomposition temperature of poly(HEMA) cryogel starts nearly from 250°C and continuous until 450°C. Introducing of comonomer MATrp to the HEMA monomer increased total thermal stability of pHEMA cryogel. Because decomposition temperature of poly(HEMA-MATrp) cryogel increase to 500°C. Decomposition starting temperature of p(HEMA-MATrp) cryogel at 225°C is less than for poly(HEMA) cryogel. This may be due to

decomposition temperature of MATrp monomer which starts nearly 90°C (Figure 4.12). Weight loss curve at 400.43°C belongs to the maximum decomposition rate of poly(HEMA) cryogel. Maximum decomposition rate temperature decrease for poly(HEMA-MATrp) cryogel.

Polymers are thermally stable only below a certain limiting temperature range usually from 250°C to 350°C (for flexible aliphatic polymers) or from 350°C to 550°C (for rigid aromatic polymers).

4.2. Adsorption of Lysozyme from Aqueous Solutions

4.2.1. Effect of pH

Figure 4.15 shows lysozyme adsorption capacity at different pH values and 0.1 M $(\text{NH}_4)_2\text{SO}_4$ salt content. pH ranges were 4.0-5.0 for acetate 6.0-8.0 for phosphate and 9.0-12.0 for carbonate buffers. Maximum adsorption was observed at pH 6.0. The amount of adsorbed lysozyme was 46.84 mg/g polymer. At pH values lower and higher than pH 6.0, the adsorbed amount of lysozyme drastically decreased. Because ammonium sulfate is unstable under basic conditions, the pH should be below 8.0 (Michael, K., 2000) and lysozyme activity is highest from pH 3.5 to pH 7.0. Maximum adsorption of lysozyme at pH 6.0 may be due to optimal activity of enzyme in this range.

The isoelectronic (pI) value of lysozyme is 11.2, and second maximum peak capacity was observed at pH 10.0 indicating that the binding of the lysozyme on the poly(HEMA-MATrp) cryogel should involve hydrophobic interactions with a high percentage according to the other interactions. The fact that, the net charge of a protein is zero at its isoelectronic point does not mean that it is devoid of charge. Depending on its amino acid composition, a protein can have several charged groups at the pI, the spatial arrangement of which is a function of primary, secondary, tertiary and quaternary structure. Therefore, the interaction of a protein with an affinity system may not occur at its pI value.

The lysozyme molecule has one histidine, four aspartic acid, two glutamic acid and two tyrosine residues (on the basis of its surface accessible residues). These amino acid side chain groups of the lysozyme molecules could cause other type of interactions with low percentage according to the hydrophobic interactions.

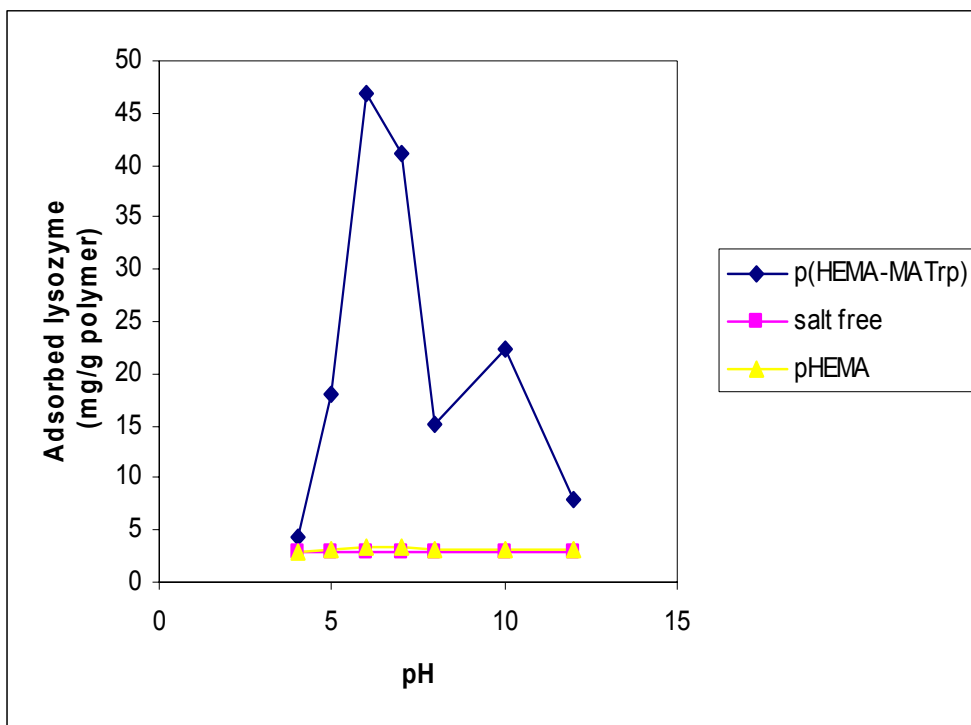


Figure 4.15. Effect of pH on lysozyme adsorption capacity of poly(HEMA-MATrp) and poly(HEMA) cryogel; lysozyme concentration: 1.0 mg/mL in 50 mM buffer containing 0.1 M $(\text{NH}_4)_2\text{SO}_4$; flow rate: 1.0 mL/min.; recirculation time: 2 h.; T: 25°C.

A point worth noting that, there was not significant lysozyme adsorption capacity (3.4 mg/g polymer) until salt used (1.0 mg/mL in 0.1 M $(\text{NH}_4)_2\text{SO}_4$ pH 6.0 phosphate buffer). Adding salt $(\text{NH}_4)_2\text{SO}_4$ into adsorption media, a significant increase (46.84 mg/g) in adsorption capacity was observed. This result was not a surprise, because, the adsorption increases with high salt concentrations in the mobile phase in hydrophobic interaction chromatography (HIC). Ammonium sulphate is effective to promote ligand interactions, due to the higher “salting out” or molal surface tension increment effects. Ammonium sulphate also stabilizes proteins. So as a result of using an HIC column you can expect your protein to be

in its most stable form. And there was negligible lysozyme adsorption onto the poly(HEMA) cryogel which was about 2.9 mg/g for phosphate buffer (pH 6.0). There are no reactive binding groups or sites onto poly(HEMA) cryogel, which interact with lysozyme molecules. Hence the nonspecific adsorption may be due to diffusion of lysozyme molecules into the swollen cryogel and weak interactions (van der Waals interaction and hydrogen binding) between lysozyme and hydroxyl groups on the surface of poly(HEMA) cryogel.

4.2.2. Effect of Initial Concentration of Lysozyme

In order to observe the effect of the initial lysozyme concentrations to the maximum adsorption capacity the concentrations of lysozyme were changed between 0.3-2.0 mg/mL. Figure 4.16 shows the effect of initial lysozyme concentrations to the adsorption capacity.

The amount of lysozyme adsorbed per unit mass of the affinity cryogel was increased first with the initial concentration of lysozyme then reached a plateau value for 1.0 mg/mL lysozyme concentration, which represents saturation of the active adsorption sites (which are available and accessible for lysozyme) on the affinity cryogel. It becomes constant when the protein concentration is higher greater than 1.0 mg/mL.

Maximum adsorption capacity was found to be (44.68 mg/g) cryogel at 25°C. This increase in the lysozyme adsorption capacity may have resulted from hydrophobic interactions caused by the MATrp monomer and by hydrophobic amino acids on the lysozyme molecules. It should be mentioned that MATrp is hydrophobic overall, and it prefers to interact with hydrophobic groups in lysozyme structure.

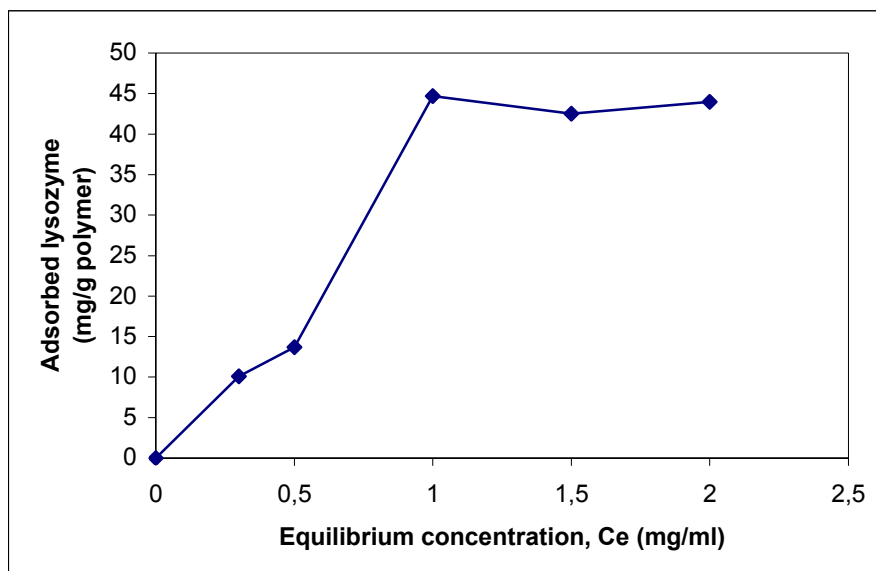


Figure 4.16. Effect of equilibrium lysozyme concentration on adsorption capacity of poly(HEMA-MATrp) cryogel: lysozyme in 50 mM phosphate buffer, pH 6.0 containing 0.1 M $(\text{NH}_4)_2\text{SO}_4$; flow rate: 1.0 mL/min., recirculation time: 2 h.; T: 25°C.

4.2.3. Effect of Flow Rate to The Adsorption of Lysozyme

The adsorption amounts of lysozyme at different flow-rates are given in Figure 4.17. Results show that the lysozyme adsorption capacity onto the affinity cryogel decreases when the flow-rate through the column increases.

The adsorption capacity decreased significantly from 58.38 mg/g to 22.34 mg/g cryogel with the increase of the flow-rate from 0.5 mL/min to 2.0 mL/min. An increase in the flow-rate reduces the solution volume treated efficiently until breakthrough point and therefore decreases the service time of cryogel column. This is due to decrease in contact time between the lysozyme molecules and the affinity cryogel at higher flow-rates. When the flow-rate decreases the contact time in the column is longer. Thus, lysozyme molecules have more time to diffuse to the pore walls of cryogel and to bind to the binding sites of adsorbent, hence a better adsorption capacity is obtained.

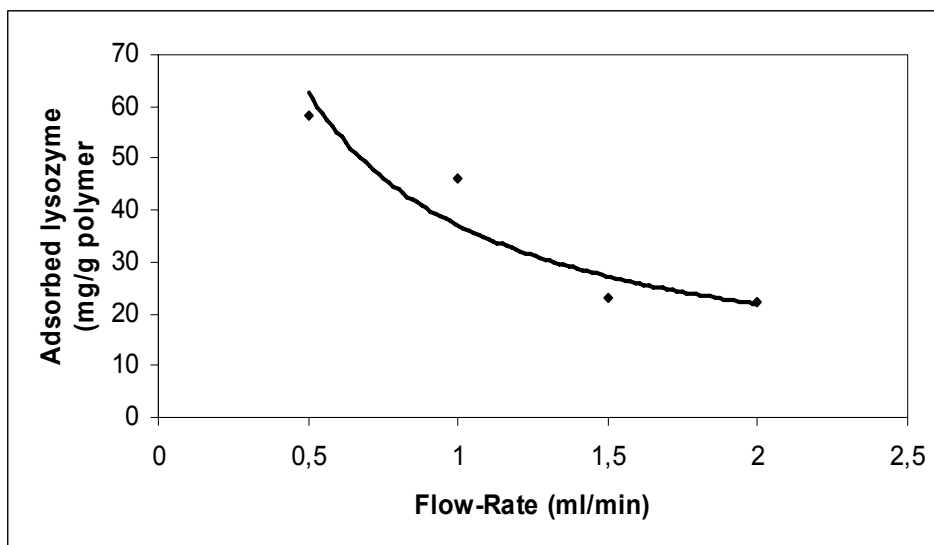


Figure 4.17. Effect of flow rate on lysozyme adsorption capacity of poly(HEMA-MATrp) cryogel; lysozyme concentration: 1.0 mg/mL in 50 mM pH 6.0 phosphate buffer containing 0.1 M $(\text{NH}_4)_2\text{SO}_4$; recirculation time: 2 h.; T: 25°C.

4.2.4. Effect of Temperature to the Adsorption of Lysozyme

Effect of temperature on lysozyme adsorption were studied in the range of 4.0-37°C. Effect of temperature on the adsorption of lysozyme on poly(HEMA-MATrp) cryogel was presented in Figure 4.18. As shown in the Figure 4.18 adsorption of lysozyme on the poly(HEMA-MATrp) cryogel was significantly increased with increasing temperature. From 4°C to 37°C, the adsorption capacity of the cryogel increased about 62% for the poly(HEMA-MATrp) cryogel. Lysozyme was adsorbed 19.46 mg/g, 43.24 mg/g, 51.89 mg/g respectively for 4°C, 25°C and 37°C.

In hydrophobic interactions chromatography (HIC), increasing the temperature enhances protein retention and lowering the temperature generally promotes the protein elution. In fact, the hydrophobic interactions chromatography (HIC), is an entropy-driven process. Since ΔH may be a small positive or negative value, ΔG is controlled by a positive entropy change and thus increases with temperature. Van der Waals attraction forces, which operate in hydrophobic interactions increase with increasing temperature.

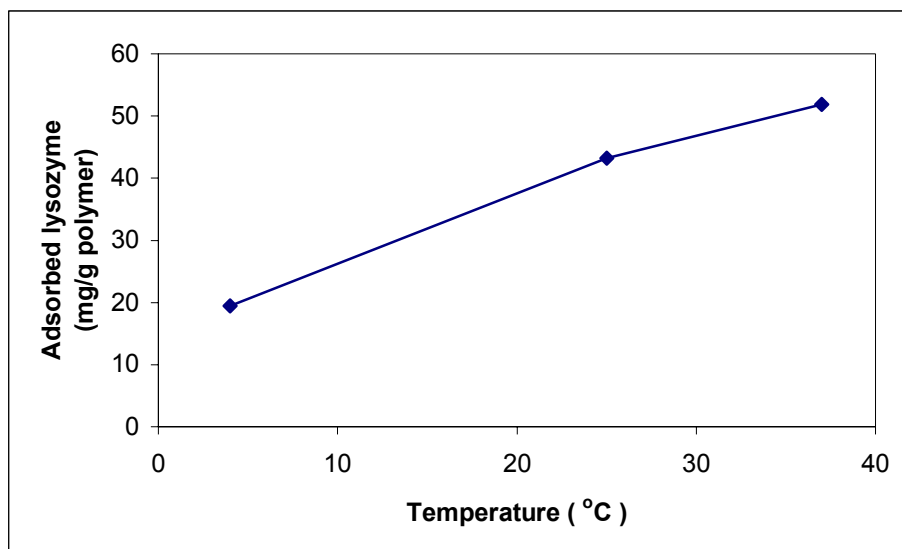
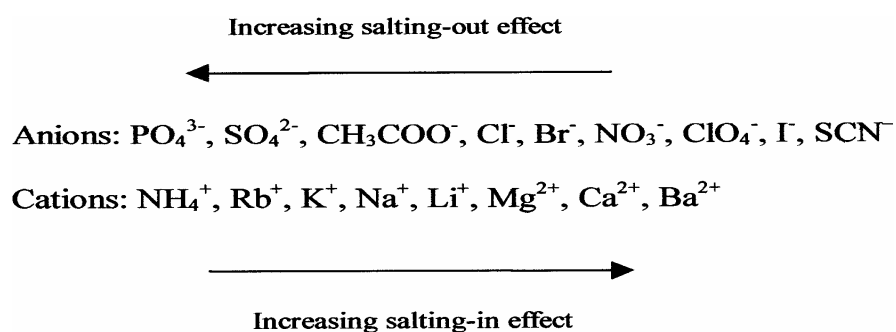


Figure 4.18. Effect of temperature on lysozyme adsorption of poly(HEMA-MATrp) cryogel: lysozyme concentration: 1.0 mg/mL in 50 mM pH 6.0 phosphate buffer containing 0.1 M $(\text{NH}_4)_2\text{SO}_4$; flow rate: 1.0 mL/min.; recirculation time: 2 h.; T: 25°C.

4.2.5. Effect of Salt Type

The influence of different salts on hydrophobic interactions follows the Hofmeister (lyotropic) series for the precipitation of proteins from aqueous solutions (Pahlman et al., 1977; Roe, 1989):



The salts at the beginning of the series promote hydrophobic interactions and protein precipitation (salting-out effect) and are called antichaotropic. They are considered to be water structuring, whereas salts at the end of the series (salting-in or chaotropic ions) randomize the structure of the liquid water and thus tend to decrease the strength of hydrophobic interactions (Porath, 1987). Early members of the series increase solvent surface tension and decrease the solubility of nonpolar molecules ("salt out"); in effect, they strengthen the hydrophobic interaction

Early members of the series increase solvent surface tension and decrease the solubility of nonpolar molecules ("salt out"); in effect, they strengthen the hydrophobic interaction. The surface tension increment enters into the interpretation of salting out results via the cavity model. Salting out constants are proportional to the surface tension increment of the salt (Baldwin, 1996).

The effect of salt concentration and type on the adsorption capacity of cryogel were investigated. Figure 4.19 shows the effect of salt type on the adsorption capacity of the cryogel. NaCl, Na₂SO₄ and (NH₄)₂SO₄ were used to investigate the effect of salt type on the adsorption capacity. The concentration of the all salts were 0.1 M. The amount of lysozyme adsorbed were 66.58 mg/g, 43.42 mg/g, 21.18 mg/g, 3.31 mg/g for Na₂SO₄, (NH₄)₂SO₄, NaCl and salt free cryogel, respectively. Salts such as sodium, potassium or ammonium sulfates are the most effective to promote ligand protein interactions, due to the higher molal surface tension increment effects. Free energy of the protein is increased by the addition of the salts and this unfavorable free energy is smaller for the proteins bound to the columns because of their smaller surface area exposed to solvent. The bound form of the proteins is thermodynamically more stable. This explains the protein binding to the hydrophobic columns at high salt concentrations and the elution by decreasing the salt concentration. The unfavorable interaction free energy was greater for Na₂SO₄ or (NH₄)₂SO₄ than for NaCl, which explains the stronger effect of the former salts on the protein binding to the columns (Arakawa, 1985).

As shown in Figure 4.19, the adsorption capacity for salts rank following values; $\text{Na}_2\text{SO}_4 > (\text{NH}_4)_2\text{SO}_4 > \text{NaCl}$.

In hydrophobic interaction chromatography (HIC), the use of high salt concentration in the equilibrium buffer and sample solution promotes the ligand-protein interactions and consequently the protein retention. The amount of the bound protein increases almost nearly with the enhancement ionic strength and continues to increase in an exponential manner at still higher concentration (Queiroz et al., 2001).

As mentioned the highest amount of adsorbed lysozyme on poly(HEMA-MATrp) cryogel was obtained by adding Na_2SO_4 salt to the adsorption medium.

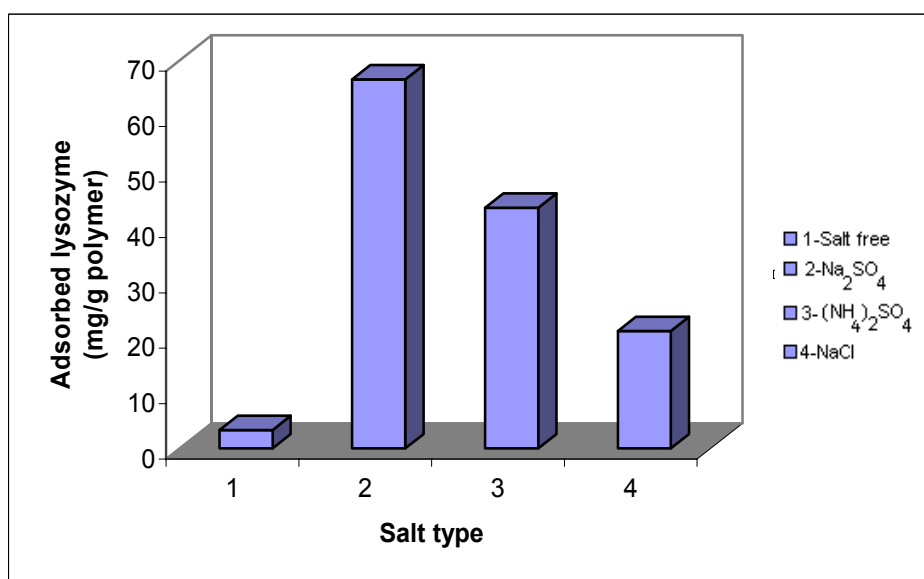


Figure 4.19. Effect of salt type on lysozyme adsorption capacity of poly(HEMA-MATrp) cryogel; lysozyme concentration: 1.0 mg/mL in 50 mM pH 6.0 phosphate buffer containing 0.1 M salt ; flow rate: 1.0 mL/min.; recirculation time: 2 h.; T: 25°C.

4.2.6. Adsorption of Lysozyme from Chicken Egg White

The content of lysozyme in chicken egg white is about 3.4% (Huopalahti et al., 2007). The classical lysozyme purification method required several steps, such as precipitation, centrifugation and affinity adsorption (Altıntaş and Denizli., 2006). In this study, lysozyme purification from egg white was studied in a column system. The amount of adsorbed lysozyme was found 12.9 mg/g for cryogel.

4.2.7. Comparison with Related Literature

In literature, Öncel et al. reported 25 mg/g lysozyme adsorption on p(HEMA-MAPA) hydrophobic beads (Öncel et al., 2005). Bayramoglu et.al. reported 17.5 mg/g lysozyme adsorption onto chitosan-g-poly(MAA) beads (Bayramoğlu et.al.2007). Yilmaz et al. reported maximum adsorption of lysozyme at pH 7.0 with dye-immobilised polyHEMA/chitosan composite membrane matrix. The maximum lysozyme adsorptions was obtained at pH 7.0 (about 32.9 mg/mL membrane) (Yilmaz et al., 2005). Bayramoglu et al. reported maximum adsorption of lysozyme at pH 7.0 with RB-10 (32.6 mg/mL) and RG-5 (45.0 mg/mL)-immobilised pHEMA affinity membranes (Bayramoğlu et al., 2004). Garcia et. al. reported lysozyme adsorption on the streamline phenyl resin as 47.76 mg/mL in 1.0 M $(\text{NH}_4)_2\text{SO}_4$ salt solution (Garcia et al., 2006). Chen et al. investigated the lysozyme adsorption capacity on a hydrophilic gel, and the maximum lysozyme adsorption capacity of the adsorbents was 84 mg/g. (Chen et al., 1997).

Nash and Chase modified a poly(styrene-divinylbenzene) chromatography matrix through the adsorption and crosslinking of poly(vinyl alcohol), and they incorporated the dye ligand Procion Yellow HE-3G for human serum albumin and lysozyme adsorption (Nash and Chase, 1997). Their lysozyme adsorption efficiencies were 11.2–20 mg/mL. Champluvier and Kula used nylon based microfiltration membranes containing triazine dyes for lysozyme adsorption, and they reported an 8.6 mg/mL adsorption capacity (Champluvier and Kula, 1991). Nash et al. investigated lysozyme adsorption onto Procion MX-R incorporated poly(styrene-divinylbenzene) matrices, and they achieved a 68 mg/g adsorption

capacity (Nash et al., 1997). A macroporous chitin membrane was used as an affinity membrane for lysozyme separation with high selectivity and high adsorption capacity at saturation (50 mg/mL membrane) (Ruckenstein and Zeng, 1997). Chetty and Burns investigated Macrosorb KAX CB for protein separation, and they reported a 23 mg/mL lysozyme adsorption capacity (the commercial dye-affinity resin Macrosorb KAX CB is a composite of silica and agarose onto which the ligand Cibacron Blue F3GA has been derivatized) (Chetty and Burns, 1991). Denizli et al. prepared Congo Red carrying monosize poly(2-hydroxyethyl methacrylate methyl methacrylate) beads for protein separation, and they obtained a 67.6 mg/g lysozyme adsorption capacity (Denizli et al., 2000). Sharma and Agarwal investigated lysozyme adsorption onto immobilized metal-affinity Sepharose 6B beads carrying different metal-complexing agents and metal ions, and they obtained an adsorption capacity of 6.8–190 mg/g (Sharma and Agarwal, 2001). McCreath et al. functionalized a poly(vinyl alcohol)-coated particulate perfluoropolymer with ion-exchange groups, and the equilibrium maximum adsorption capacity was 31.2 mg/mL (McCreath et al., 1997). Garke et al. studied the adsorption behavior of lysozyme on the strong cation exchanger Streamline SP, and they adsorbed 100 mg of lysozyme/g of polymer (Garke et al., 1999). The maximum lysozyme adsorption that we achieved in this study was 44.68 mg/g, which was quite good according to the related literature.

4.2.8. Desorption and Reusability of Adsorbents

Desorption of lysozyme was studied with 0.5 M ethylene glycol solution in a continuous system. Lysozyme adsorbed cryogel were recirculated with ethylene glycol 2.0 hour. The final lysozyme concentration in the desorption media was determined by Bradford Protein Assay at 595 nm. Ethylene glycol decreases the surface tension of water, thus weakening the hydrophobic interactions to give a subsequent dissociation of the ligand-solute complex. Surface tension of water is 72.00 dynes/cm, while surface tension of ethylene glycol is 46.70 dynes/cm.

In order to test the reusability of the poly(HEMA-MATrp), lysozyme adsorption-desorption procedure was repeated five times by using the same cryogel. For sterilization after one adsorption-desorption cycle, the cryogel was washed with 20 mM NaOH solution for 30 min. After this procedure, cryogel was washed with distilled water for 30 minutes, then equilibrated with the phosphate buffer pH 6.0 for the next adsorption-desorption cycle. As seen in Figure 4.20, that the cryogel is very stable and maintain their adsorption capacity at almost constant. At the end of five adsorption-desorption cycle, there was no remarkable reduce in the adsorption capacity. Recovery of lysozyme was 91%.

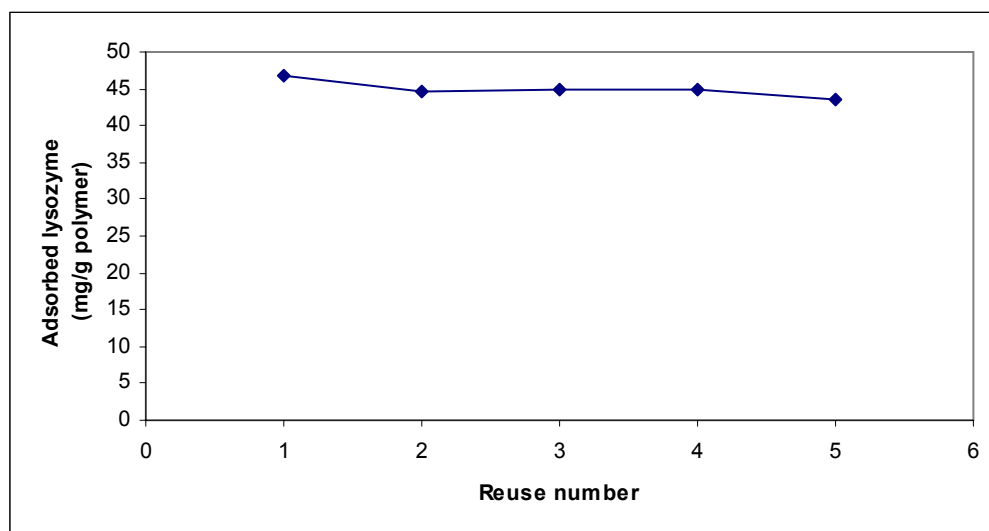


Figure 4.20. Reusability of the poly(HEMA-MATrp) cryogel.

4.3. Fluorimetric Measurements

Fluorescence is the phenomena of some compounds to absorb specific wavelengths of light and almost instantaneously emit longer wavelengths of light. Fluorimetry's sensitivity and specificity reduce or eliminate the sample preparation procedures often required to concentrate analytes or remove interferences from samples prior to analysis. This reduction in or elimination of sample preparation time not only simplifies, but also expedites the analysis.

To evaluate the effects of the adsorption conditions on the lysozyme structure, the fluorescence spectra of the native, heat-denatured (96°C), and desorbed lysozyme were recorded. The initial calibration was carried out with a standard solution of lysozyme in phosphate buffer (pH 6.0) with a 280 nm fluorescence excitation wavelength and 340 nm emission wavelength.

As can be seen in figures; peak at 280 nm belongs to excitation wavelength the others 310 nm and 560 nm belong to emission wavelength.

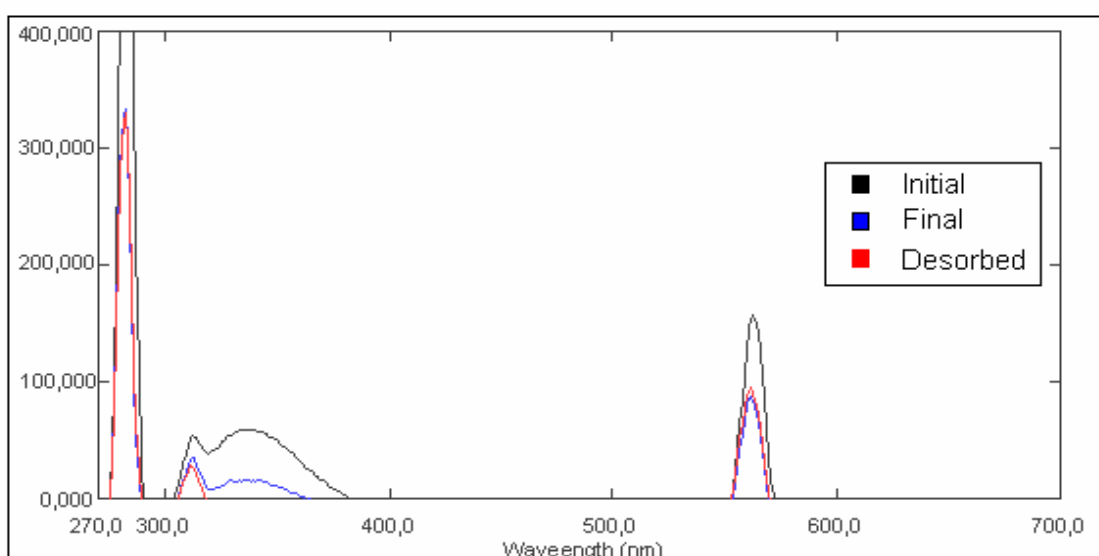


Figure 4.21. Fluorimetric measurement of native lysozyme solution.

It can be seen easily from Figure 4.21 intensity of desorbed lysozyme decreases. It is expected chromatogram because all circulated lysozyme couldn't be adsorbed. Final, stand for lysozyme which can not be adsorbed on cryogel. Desorption of adsorbed lysozyme could be done effectively. Denaturated lysozyme can be seen in Figure 4.22. Blue line shows us denaturation of lysozyme because of increment in the intensity. Intensity of denatured enzyme increases compared to the native one. Fluorimetric measurement peaks for egg lysozyme can be seen in Figure 4.23.

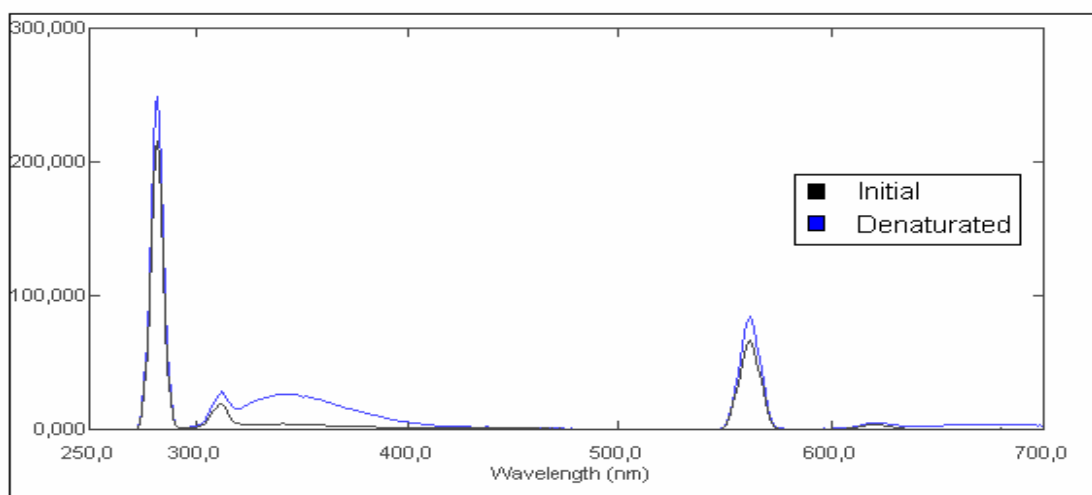


Figure 4.22. Fluorimetric measurement of native and denatured lysozyme.

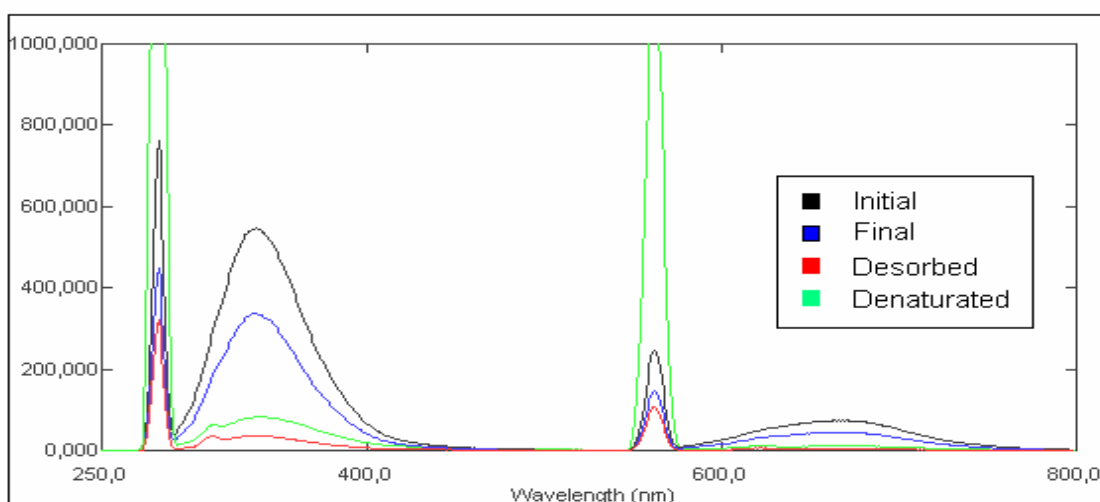


Figure 4.23. Fluorimetric measurement peaks of egg lysozyme.

4.4. Fast Protein Liquid Chromatography

As shown in Figure 4.24, separation of lysozyme and the other proteins in egg was observed at 11.61 and 2.99 minute, respectively. The t_R , N , k' , R_s values are given in Table 4.2. R_s value was calculated as 2.22 for other proteins in egg white, respectively. Because the R_s value should be higher than 1.0 for a good resolution of two peaks in such a chromatography system the results for the resolution of lysozyme-other proteins in egg white can be accepted as good resolution values.

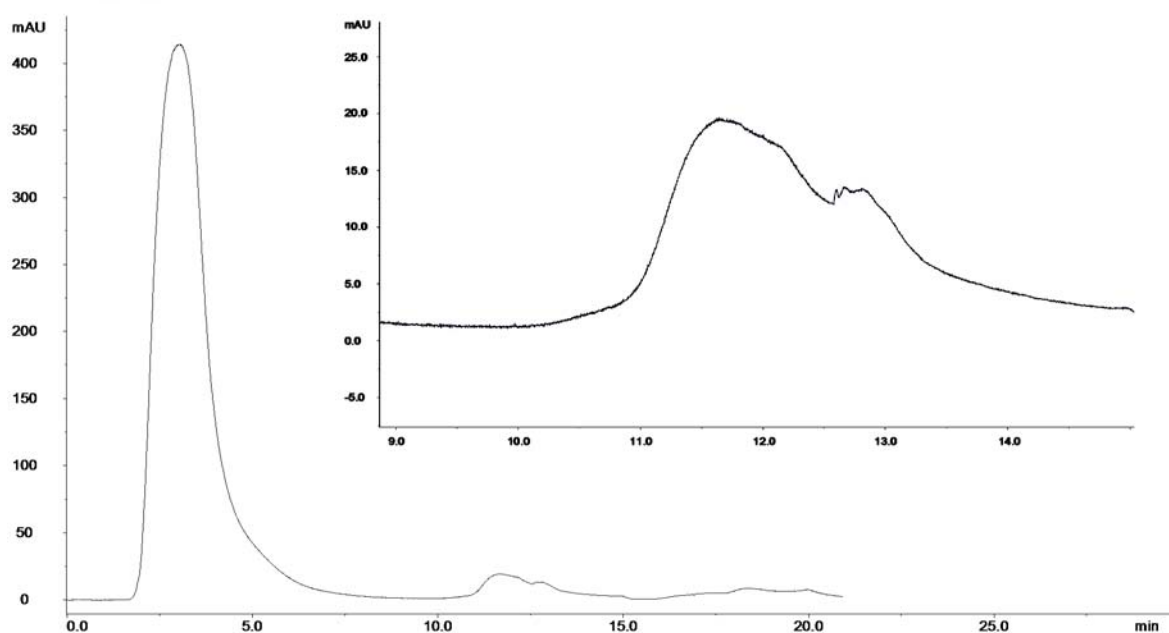


Figure 4.24. FPLC separation of lysozyme from egg white on a column packed with poly(HEMA-MATrp) cryogel.

Table 4.2. Chromatographic separation data

	t_R	N	k'	α	R_s
lysozyme	11.61	302.95	5.75	-	-
other proteins in CEW	2.99	9.28	0.74	7.77	2.22

As seen from Figure 4.24 intensity of lysozyme was small compared to the other proteins. To increase intensity of lysozyme, egg white solution was treated with saturated $(\text{NH}_4)_2\text{SO}_4$ solution. This treatment removed proteins with high molecular weight like ovalbumin. As a result, intensity of the other proteins decreased, while intensity of lysozyme increased. Figure 4.25 shows this increment.

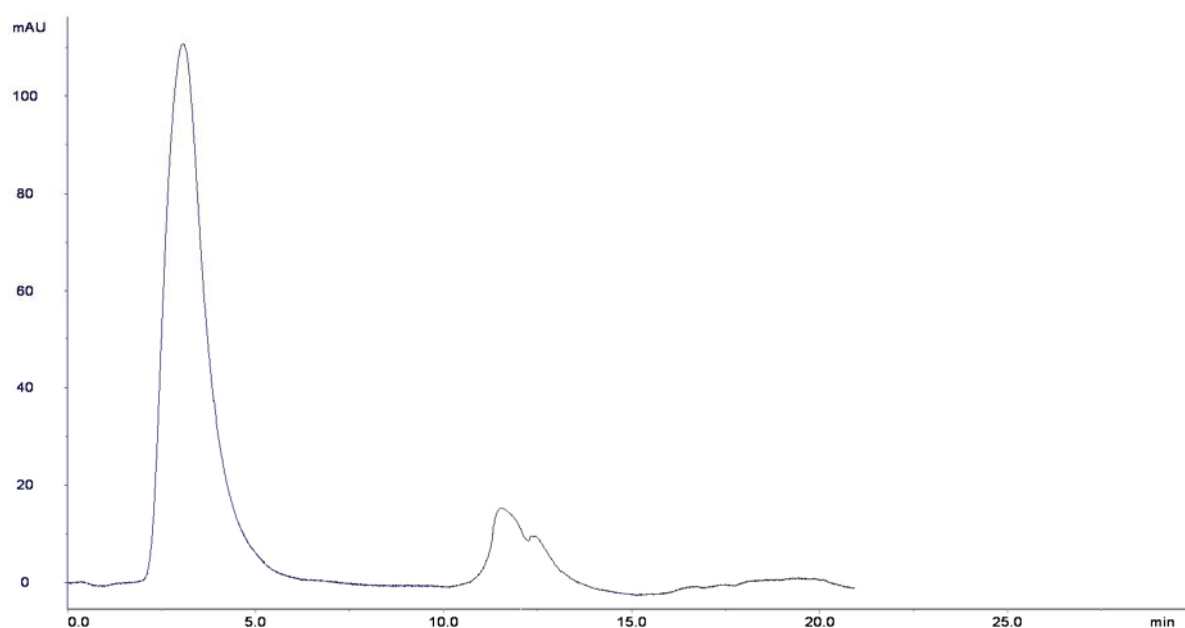


Figure 4.25. FPLC separation of lysozyme from egg white (treated with $(\text{NH}_4)_2\text{SO}_4$ solution) on a column packed with poly(HEMA-MATrp) cryogel.

4.5. Electrophoretic Separation

The solution of lysozyme to be analyzed was first mixed with SDS, an anionic detergent which denatured secondary and non-disulfide-linked tertiary structures, and applied a negative charge to each protein in proportion to its mass. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in folding, as differences in folding patterns would cause some proteins to better fit through the gel matrix than others. Adding SDS solved this problem, as it linearized the proteins so that they may be separated strictly by molecular weight.

Lysozyme has 14.3 kDa molecular weight and has a 129 aminoacid residue. It has just one polypeptide chain. Initial, and desorbed lysozyme visualized for aqueous solution and for egg white lysozyme. Figure 4.26 and Figure 4.27 show us staining of lysozyme for aqueous and egg white solution respectively.

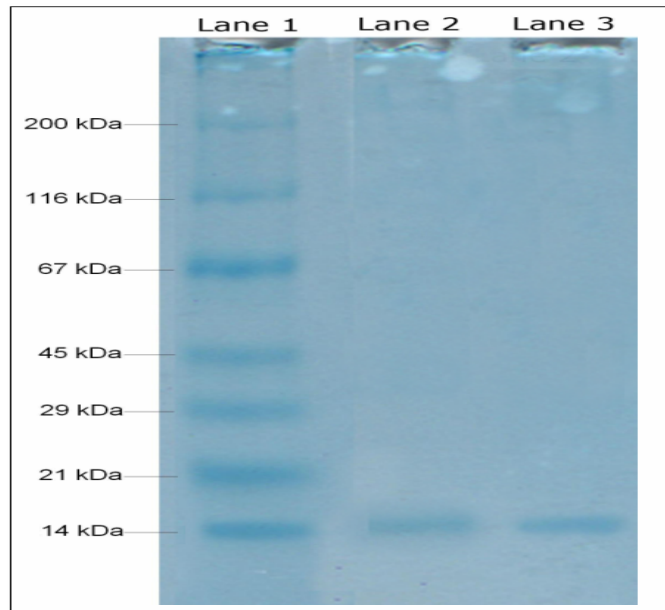


Figure 4.26. Electroporetic separation of aqueous solution of lysozyme.

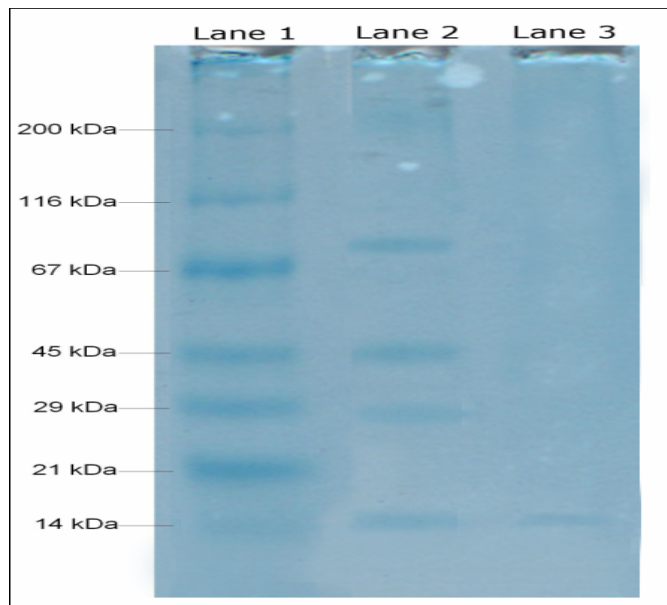


Figure 4.27. Electroporetic separation of egg lysozyme

Figure 4.26 shows migration of lysozyme. Lane 1 belongs to sigma marker (Wide range molecular weight) Lane 2 and Lane 3 belongs to initial and desorbed lysozyme respectively. Figure 4.27 shows electrophoretic mobility of egg white proteins. In Figure 4.27 there are some proteins that couldn't be removed by treatment of saturated $(\text{NH}_4)_2\text{SO}_4$ solution. They had to be removed by treatment of

saturated $(\text{NH})_4\text{SO}_4$ solution. Because of high percentage of these proteins in egg white, all amount of the proteins couldn't be precipitated. Amount of proteins in egg white are; ovalbumin 54%, ovotransferrin 12-13%, ovomucoid 11%, and lysozyme 3.4-3.5. Figure 4.27 shows us proteins with high percentage. In Lane 2; 45 kDa stand for ovalbumin, 77.7 kDa ovotransferrin, 28 kDa ovomucoid, and 14.3 kDa lysozyme respectively. Other proteins with small percentage couldn't be seen. Lane 3 belongs to desorbed lysozyme migration.

The purity of the lysozyme eluted from poly(HEMA-MATrp) cryogel was determined by SDS-PAGE. The purity of the desorbed lysozyme was about 92.8% for aqueous lysozyme and 81.8% for egg white lysozyme. The poly(HEMA-MATrp) cryogel provided an efficient method to purify lysozyme from diluted egg white, showing high adsorption capacity for lysozyme. The specific activity of the purified lysozyme with poly(HEMA-MATrp) cryogel was 43.140 U/mg. It should be noted that the specific activity of native lysozyme was 46.500 U/mg. As seen here there was no drastic decrease in specific activity during the purification studies

4.6. Kinetic Parameters

4.6.1. Adsorption Isotherms

An adsorption isotherm is used to characterize the interactions of each protein molecule with the adsorbent. This provides a relationship between the concentration of the protein in the solution and the amount of protein adsorbed on the solid phase when the two phases are at equilibrium.

The Langmuir adsorption model assumes that the molecules are adsorbed at a fixed number of well-defined sites, each of which is capable of holding only one molecule. These sites are also assumed to be energetically equivalent, and distant from each other so that there are no interactions between molecules adsorbed on adjacent sites.

Adsorption isotherms were used to evaluate adsorption properties. The Langmuir adsorption isotherm is expressed by Equation 4.1. The corresponding transformations of the equilibrium data for lysozyme gave rise to a linear plot, indicating that the Langmuir model could be applied in these systems and described by the equation:

$$Q = Q_{\max} \cdot b \cdot C_{\text{eq}} / (1 + bC_{\text{eq}}) \quad (4.1)$$

where Q is the adsorbed amount of lysozyme (mg/g), C_{eq} is the equilibrium lysozyme concentration (mg/mL), b is the Langmuir constant (mL/mg) and, $Q_{\max}$ is the maximum adsorption capacity (mg/g). This equation can be linearized so that

$$1/Q_{\text{eq}} = 1/(Q_{\max} \cdot b)(1/C_{\text{eq}}) + 1/Q_{\max}. \quad (4.2)$$

The plot of $1/Q_{\text{eq}}$ versus $1/C_{\text{eq}}$ was employed to generate the intercept of $1/Q_{\max} \cdot b$ and the slope of $1/Q_{\max}$ (Figure 4.28).

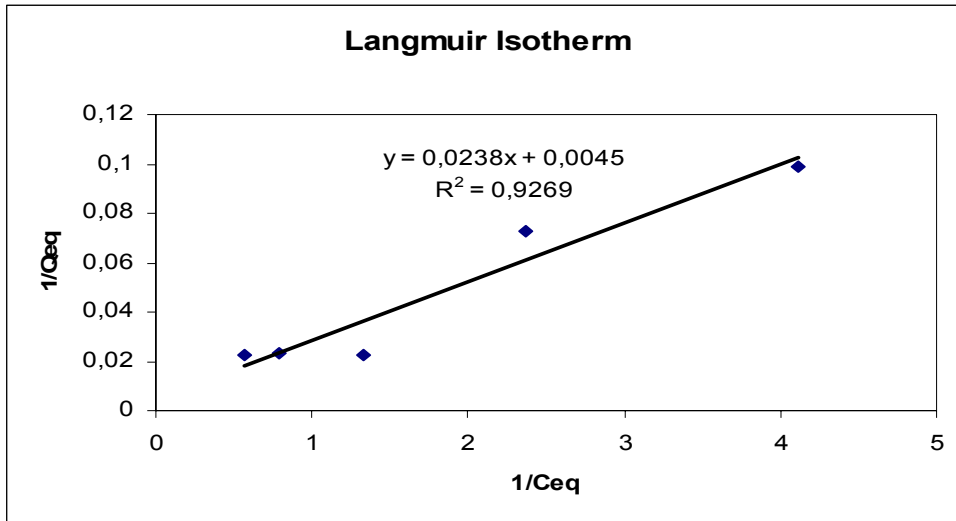


Figure 4.28. Langmuir adsorption isotherms of the poly(HEMA-MATrp) cryogel.

The maximum adsorption capacities ($Q_{\max}$) data for the adsorption of poly(HEMA-MATrp) cryogel were obtained from the experimental data (Table 4.3). The Langmuir correlation coefficient (R^2) was high for poly(HEMA-MATrp) cryogel. The Langmuir adsorption model can be applied in this affinity adsorbent system.

The other well known isotherm, which is frequently used to describe adsorption behaviour, is the Freundlich isotherm. This isotherm is another form of the Langmuir approach for adsorption on a heterogeneous surface. The amount of adsorbed protein is the summation of adsorption on all binding sites. The Freundlich isotherm describes reversible adsorption and is not restricted to the formation of the monolayer. This empirical equation takes the form:

$$Q_{eq} = K_F (C_{eq})^n \quad (4.3)$$

where, K_F and n are the Freundlich constants.

The plot of $\ln Q_{eq}$ versus $\ln C_{eq}$ was employed to generate the intercept of $\ln K_F$ and the slope of $1/n$. Figure 4.29 shows Freundlich adsorption isotherm of the cryogel.

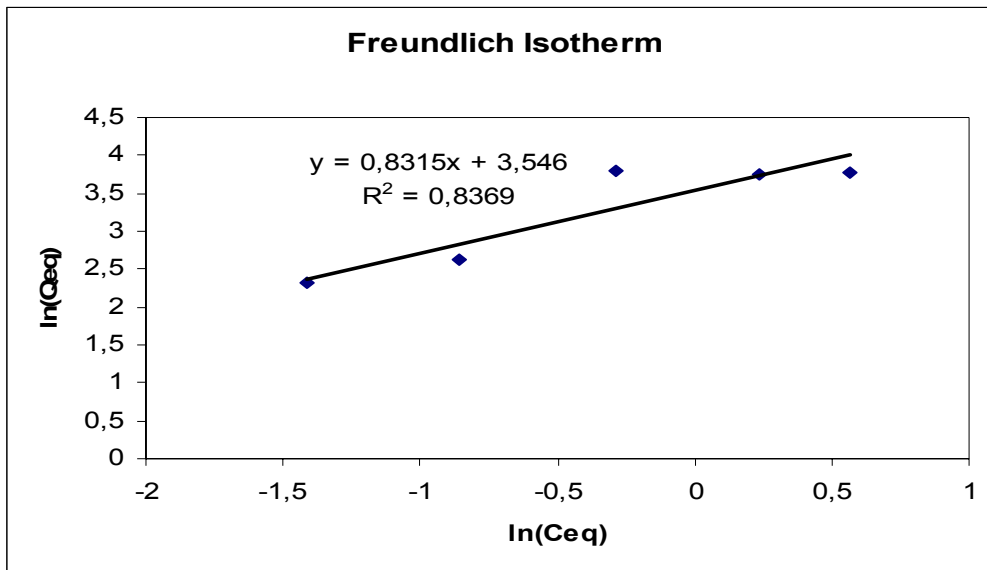


Figure 4.29. Freundlich adsorption isotherms of the poly(HEMA-MATrp) cryogel.

The Freundlich isotherm, as mentioned before, shows the surface heterogeneity, it contains two parameters, K_F and $1/n$. $1/n$, the surface heterogeneity index, is between 0 and 1.0. If it is closed to 1.0, the polymer has heterogeneous surface. $1/n$ value of poly(HEMA-MATrp) cryogel is 1.202, this adsorption process onto poly(HEMA-MATrp) cryogel was not done via multilayer adsorption.

The adsorption isotherms of lysozyme were found to be linear over the whole concentration range studies and the correlation coefficients were high . According to the correlation coefficients of isotherms, Langmuir adsorption model is favorable.

Table 4.3 shows the Freundlich adsorption isotherm constants, n and K_F and the correlation coefficients. Regression coefficient value for Langmuir isotherm is higher than Freundlich isotherm and Langmuir $Q_{\max}$ value is agreeable with experimental data.

Table 4.3. Langmuir and Freundlich adsorption constants and correlation coefficients for Lysozyme

	Exp.	Langmuir constants			Freundlich constants			
	q_{ex} (mg/g)	q_{max} (mg /g)	b (ml/mg)	R^2	K_F	n	$1/n$	R^2
poly(HEMA-MATrp)	44.69	42.02	5.289	0.927	34.67	0.832	1.202	0.837

4.6.2. Adsorption Kinetics Modelling

In order to examine the controlling mechanism of adsorption process such as mass transfer and chemical reaction, kinetic models were used to test experimental data. The kinetic models (Pseudo-first- and second-order equations) can be used in this case assuming that the measured concentrations are equal to adsorbent surface concentrations. The first-order rate equation of Lagergren is one of the most widely used for the adsorption of solute from a liquid solution. It may be represented as follows:

$$dq_t/dt = k_1(q_{\text{eq}} - q_t) \quad (4.4)$$

where k_1 is the rate constant of pseudo-first order adsorption (1/min) and q_{eq} and q_t denote the amounts of adsorbed protein at equilibrium and at time t (mg/g),

respectively. After integration by applying boundary conditions, $q_t=0$ at $t=0$ and $q_t=q_t$ at $t=t$, gives

$$\log[q_{eq}/(q_{eq}-q_t)] = (k_1 t)/2.303 \quad (4.5)$$

Equation 4.5 can be rearranged to obtain a linear form;

$$\log(q_{eq}-q_t) = \log(q_{eq}) - (k_1 t)/2.303 \quad (4.6)$$

a plot of $\log(q_{eq}-q_t)$ versus t should give a straight line to confirm the applicability of the kinetic model. In a true first-order process $\log q_{eq}$ should be equal to the interception point of a plot of $\log(q_{eq}-q_t)$ via t .

In addition, a pseudo-second order equation based on equilibrium adsorption capacity may be expressed in the form

$$dq_t/dt = k_2 (q_{eq}-q_t)^2 \quad (4.7)$$

Where k_2 (g/mg·min) is the rate constant of pseudo-first order adsorption process. Integrating Equation 4.7 and applying the boundary conditions, $q_t=0$ at $t=0$ and $q_t=q_t$ at $t=t$, leads to

$$[1/(q_{eq}-q_t)] = (1/q_{eq}) + k_2 t \quad (4.8)$$

or equivalently for linear form

$$(t/q_t) = (1/k_2 q_{eq}^2) + (1/q_{eq}) t \quad (4.9)$$

A plot of t/q_t versus t should give a linear relationship for the applicability of the second-order kinetics. The rate constant (k_2) and adsorption at equilibrium (q_{eq}) can be obtained from the intercept and slope, respectively. A comparison of the experimental adsorption capacity and the theoretical values which obtained from Figures 4.30 and Figures 4.31 are presented in Table 4.4. The theoretical q_e value

estimated from pseudo-first and second order kinetic models were close to the experimental values and the correlation coefficients were high. Results indicate that R^2 values for different concentration is general close to 1.0. This poly(HEMA-MATrp) cryogel was obeyed the second order kinetic model.

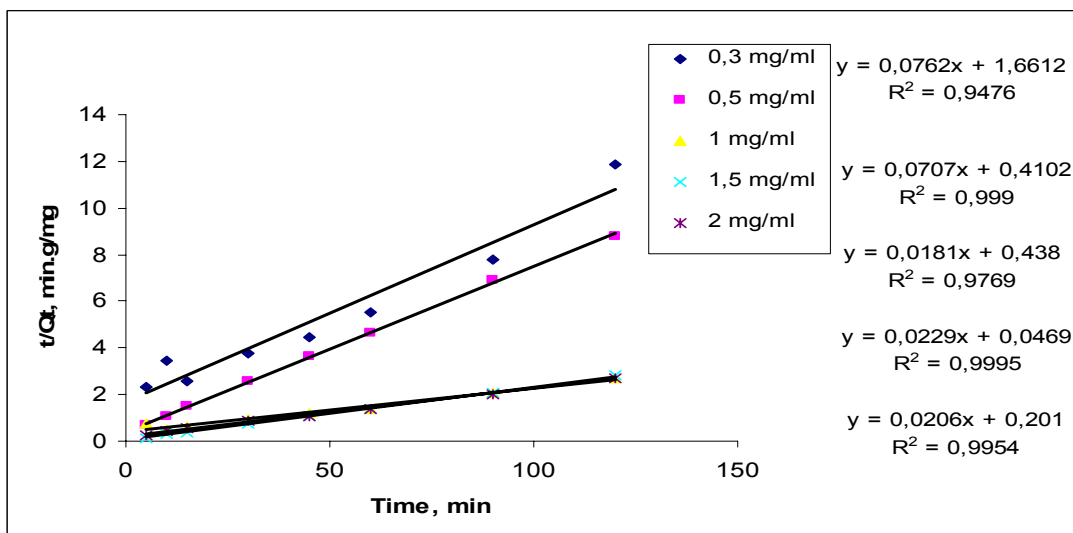


Figure 4.30. Pseudo-first order kinetic of the experimental data for the poly(HEMA-MATrp) cryogel.

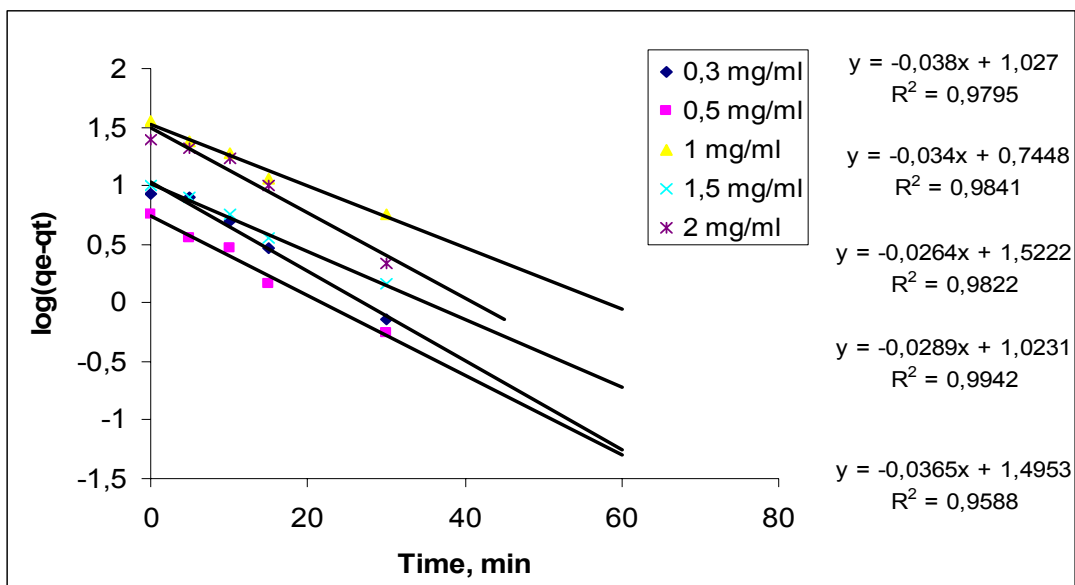


Figure 4.31. Pseudo-second order kinetic of the experimental data for the poly(HEMA-MATrp) cryogel.

Table 4.4. The first and second order kinetic constants for poly(HEMA-MATrp) cryogel.

Initial Conc.	Experimental	First-order kinetic			Second-order kinetic		
(mg/ml)	q_{eq} (mg/g)	k_1 (1/min)	q_{eq} (mg/g)	R^2	k_2 (1/min)	q_{eq} (mg/g)	R^2
0.3	10.09	0.088	10.64	0.980	0.003	13.12	0.948
0.5	13.69	0.078	5.56	0.984	0.012	14.14	0.999
1.0	44.69	0.061	33.28	0.982	0.0007	55.25	0.977
1.5	42.52	0.067	10.55	0.994	0.011	43.67	0.999
2.0	43.97	0.084	31.28	0.959	0.002	48.54	0.995

5. CONCLUSION

- The free radical polymerization procedure provided cross-linked poly(HEMA-MATrp) cryogel. Specific surface area of the p(HEMA-MATrp) cryogel was found to be 50 m²/g by BET method.
- Water uptake ratio of the poly(HEMA-MATrp) cryogel was higher than poly(HEMA). The equilibrium swelling ratios were 6.99 g H₂O/g for poly(HEMA-MATrp) and 7.18 g H₂O/g for poly(HEMA) cryogel in respectively.
- Equilibrium swelling time of the poly(HEMA-MATrp) cryogel was about 30 min.
- Decreasing concentration of MATrp from 7.35.10³M to 4.90.10³M formed macropores. The size of the pores was roughly determined to be 60-100 μm range, according to enlarged SEM photograph.
- Scanning electron micrographs showed that the polymeric cryogel has macroporous surface due to the pores which formed during the polymerization procedure.
- To see thermostability of poly(HEMA-MATrp) cryogel thermogravimetric analysis was done. Introducing of comonomer MATrp to the HEMA monomer increased total thermal stability of polyHEMA cryogel. Decomposition temperature ranges were achieved 250°C-450°C for polyHEMA and 225°C-500°C poly(HEMA-MATrp) cryogel.
- Lysozyme adsorption onto poly(HEMA-MATrp) cryogel seemed to depend on pH of the solution. To clarify this point adsorption studies were carried out at different pH using acetate (4.5), phosphate (6.0-8.0) and carbonate (9.0-12.0) buffers. Maximum adsorption capacities were observed as 46,84 mg/g at pH

6.0 phosphate buffer containing 0.1 M $(\text{NH}_4)_2\text{SO}_4$ solution. Initial lysozyme concentration was taken 1.0 mg/mL.

- There was a negligible non-specific lysozyme adsorption (the adsorption onto the plain poly(HEMA) cryogel) which was about 2.9 mg/g for phosphate buffers (pH 6.0). There are no reactive binding sites onto poly(HEMA) which interact with lysozyme molecule. Hence, this non-specific adsorption may be due to diffusion of lysozyme molecules into the swollen poly(HEMA) cryogel and weak interactions between lysozyme and hydroxyl groups on the surface of the poly(HEMA) cryogel. And salt free solution of lysozyme was adsorbed negligible amount as 3.94 mg/g onto poly(HEMA-MATrp) cryogel.
- Lysozyme adsorption capacity of poly(HEMA-MATrp) cryogel was estimated 12.9 mg/g for chicken egg white lysozyme.
- Effect of salt type on the adsorption capacity was observed. Maximum adsorption capacity was estimated when Na_2SO_4 was used. The amount of adsorbed lysozyme were 66.58 mg/g for 0.1 M Na_2SO_4 salt.
- The adsorption capacity at different flow-rates are observed. The adsorption capacity decreased significantly from 58.38 mg/g to 22.34 mg/g cryogel with the increase of the flow-rate from 0.5 mL/min to 2.0 mL/min. An increase in the flow-rate reduces the solution volume treated efficiently until breakthrough point and therefore decreases the service time of cryogel column.
- The equilibrium adsorption of lysozyme onto the poly(HEMA-MATrp) cryogel significantly increased with increasing temperature.

- Desorption of lysozyme from poly(HEMA-MATrp) cryogel was also carried out in continuous system. The cryogel was recirculated with 0.5 M ethylene glycol as desorption agent, and lysozyme was desorbed with 91% recovery.
- In order to show reusability of the poly(HEMA-MATrp) cryogel, the adsorption-desorption cycle was repeated five times using the same polymeric cryogel. At the end of five adsorption-desorption cycle, there was no remarkable reduce in the adsorption capacity.
- To see denaturation of lysozyme fluorimetric measurements were done for both aqueous and egg white lysozyme solutions. Desorbed and initial lysozyme gave same chromatogram. Denaturation of lysozyme was estimated by increasing in intensity.
- Fast Protein Liquid Chromatography was used for separation of egg white lysozyme. Lysozyme separation was observed at 11.61 minute. R_s value was found higher than 1.0 indicates good resolution of peaks and good chromatography system.
- To see purity of lysozyme, electrophoretic separation of lysozyme was performed for both aqueous and egg white solution. In egg white, beside lysozyme various proteins were stained. Molecular weights; 45 kDa 77.7 kDa, 28 kDa and 14.3 kDa belongs to ovalbumin, ovotransferrin, ovomucoid, lysozyme respectively. The purity of the desorbed lysozyme was about 92.8% for aqueous lysozyme and 81.8% for egg lysozyme.
- The corresponding transformations of the equilibrium data for lysozyme gave rise to a linear plot, indicating that the Langmuir model could be applied in these systems. The maximum adsorption capacity (Q_{max}) data for the adsorption of lysozyme was obtained from the experimental data.

agreeable with Langmuir $Q_{\max}$ value. The correlation coefficients (R^2) were 0.9278 for Langmuir adsorption and 0.837 for freundlich adsorption. The Langmuir adsorption model can be applied in this affinity adsorbent system.

- In order to analyze the adsorption kinetics of lysozyme adsorption in medium containing salt, the pseudo-first-order and pseudo-second-order kinetic models were applied to the experimental data. The theoretical q_{eq} value for the affinity cryogel was very close to the experimental q_{eq} value in the case of second-order kinetics. R^2 values for first and second order kinetic models have been found. And second order kinetic model can be applied to this system. The second-order kinetics described the data best.

6. REFERENCES

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