

MICRONSIZE MAGNETIC BEADS FOR LYSOZYME PURIFICATION

**LİZOZİM SAFLAŞTIRILMASI İÇİN MİKRON BOYUTLU MANYETİK
PARTİKÜLLER**

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Submitted to Institute of Sciences of Hacettepe University
as a partial fulfillment to the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY
in
CHEMISTRY

2008
ANKARA

To the Institute for Graduate Studies in Pure and Applied Sciences,

This study has been accepted as a THESIS for degree of DOCTOR of PHILOSOPHY in CHEMISTRY by our examining committee.

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LİZOZİM SAFLAŞTIRILMASI İÇİN MİKRON BOYUTLU MANYETİK PARTİKÜLLER

Evrin Banu Altıntaş

ÖZ

Afinite kromatografisi, biyolojik karışımların ayrılmasında kullanılan yüksek özgülükte biyolojik etkileşimlere dayanan bir kromatografik yöntemdir. Afinite kromatografisinin bir alt grubu olan hidrofobik etkileşim kromatografisi, proteinlerin yapısında bulunan polar olmayan bölgeler ile hidrofobik ligandlar arasında gerçekleşen hidrofobik etkileşimleri temel alan bir protein saflaştırma tekniğidir. Bu çalışmada, eş boyutlu, manyetik poli(glisidil metakrilat- metakroil amidotriptofan, m-poli(GMATrp) partiküller hazırlanmıştır. Polimere hidrofobik özellik veren MATrp komonomeri, metakroil klorür ile triptofanın reaksiyona girmeleri sonucu elde edilmiştir. Polimer partiküller dispersiyon polimerizasyonu ile elde edilmiştir. Manyetik partiküller; şişme deneyleri, elementel analiz, Fourier Transform Infrared Spektroskopisi (FTIR), Nükleer Manyetik Rezonans (NMR) ve Taramalı Elektron Mikroskop (SEM) yöntemleri ile karakterize edilmiştir. MATrp içeriği 30 µmol/g ve % 45 şişme oranına sahip olan partiküller sulu çözeltiden lizozim adsorpsiyonu için kullanılmıştır. Adsorpsiyon deneyleri, farklı ortam koşullarında (pH, iyonik şiddet, protein derişimi, sıcaklık) kesikli ve manyetik kararlı akışkan yatak sisteminde sisteminde incelenmiştir. Kesikli sistemde, m-poli(GMA) ve m-poli(GMATrp) yapılarının lizozim adsorpsiyon kapasiteleri sırasıyla 1.6 ve 255.3 mg/g bulunmuştur. Manyetik kararlı akışkan yatak sisteminde lizozim adsorpsiyon miktarı 263.9 mg/g olarak belirlenmiştir. Adsorpsiyon–desorpsiyon döngüsü 5 kez tekrarlanmış ve adsorpsiyon kapasitesinde önemli bir düşüş gözlenmemiştir.

Anahtar Kelimeler: Protein Saflaştırılması, Hidrofobik Etkileşim Kromatografisi, Lizozim, Manyetik partiküller.

Danışman: Prof. Dr. Adil DENİZLİ, Hacettepe Üniversitesi, Kimya Bölümü, Biyokimya Ana Bilim Dalı.

MICRONSIZE MAGNETIC BEADS FOR LYSOZYME PURIFICATION

Evrım Banu Altıntaş

ABSTRACT

Affinity chromatography is a chromatographic method of separating biological mixtures, based on highly specific biological interactions. Hydrophobic interaction chromatography, (HIC), which is a sub-group of affinity chromatography, 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. In this study, monosize, magnetic poly(glycidyl methacrylate-methacryloylamidotryptophan), m-poly(GMATrp), beads were prepared. Comonomer MATrp, which gives hydrophobicity to the resulting polymer, was synthesized by reacting methacryloyl chloride with tryptophan. Polymer beads were produced by dispersion polymerization. Beads were characterized by means of swelling studies, elemental analysis, Fourier Transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR) and Scanning Electron Microscope (SEM). Beads with a swelling ratio of 45% and containing 30 $\mu\text{mol/g}$ tryptophan were used for the separation of Lyz. Adsorption experiments were performed under different medium conditions (i.e., medium pH, protein concentration, temperature, and ionic strength) in a batch and magnetically stabilized fluidized bed system, (MSFB). Lyz adsorption capacity of m-poly(GMA) and m-poly(GMATrp) beads were 1.6 and 255.3 mg/g respectively. Lyz adsorption capacity determined as 263.9 mg/g in MSFB system. It was observed that after 5 adsorption-desorption cycle, asıgnificant decrease in Lyz adsorption capacity m-poly(GMATrp) beads was not observed.

Keywords: Protein Purification, Hydrophobic Interaction Chromatography, Lyz, Magnetic Beads.

Advisor: Prof. Dr. Adil DENİZLİ, Hacettepe Univeristy, Department of Chemistry, Biochemistry Division.

ACKNOWLEDGEMENT

I am indept to my supervisor Prof. Dr. Adil Denizli for his endless helps, supports, and supervision throughout my studies. He is a great scientist, a good father, a kind man, and he always wants to help everybody with patient. I tried to learn how to be a good person and a scientist from him. He always tried to help, and gave moral support to me.

I would like to thank to Assoc. Prof. Dr. Handan Yavuz for her support and advices.

I would like to thank my friend Lokman Uzun for his endless helps, I am indept to him. I also would like to thank to Nilay Bereli, Erkut Yılmaz, Müge Andaç, Gözde Peşint, Deniz Türkmen, Mehmet Odabaşı, Fatma Yılmaz, Bahar Ergün, Ahmet Hamdi Demirçelik, Gülsu Şener, Işık Perçin, Ali Derazshamshir, Veyis Karakoç for their friendship and helps.

I also would like to thank to Recep Ergül, director of Footh and Mouth Disease Institute, and to my friends, Şükran Yılmaz, Taibe Arsoy, Adeviye Özgüngör, İbrahim Hancı, İlkay Mahmutoğlu, Osman Tan, Yılmaz Çiftçi, and Nimet Dikilitaş.

I would like to thank to Soner Kılıç, I learned lots of things from him, sometimes he was a father, a friend, and a teacher.

I would like to express my deepest gratitude to my family, for their love and encouragement. They always loved and supported me. I understood what a child mean for his/her parents when I had a son.

Very special thanks to Gökhan Sağlam for his great moral support and endless love. He changed my life. I am very happy and lucky to be with him.

I also want to thank to my son, Deniz. I love you so much.

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1. INTRODUCTION

Isolation, separation and purification of various types of proteins, peptides and other specific biomolecules are used in almost all branches of biosciences and biotechnologies. Separation science and technology is thus very important area which is necessary for further developments in research and technology. Separation of biomolecules is dependent on their biological and physico-chemical properties: molecular size, net charge, biospecific characteristics and hydrophobicity, respectively (Queiroz et. al., 1996). In the area of biosciences and biotechnology the isolation of proteins and peptides is usually performed using variety of chromatography, ultrafiltration, precipitation and other procedures. Affinity chromatography is the most powerful process with extreme biological recognition, highest selectivity and recovery.

The concept of affinity chromatography was first introduced by Cuatrecasas, Wilchek, and Anfinsen in 1968. Affinity chromatography is based on molecular recognition and is among the early systems in which the principles of molecular recognition were applied to solve important biological problems. The broad scope of the various applications of affinity chromatography has generated the development of sub-techniques. Hydrophobic interaction chromatography, (HIC), is based on 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. HIC is also called as 'salt-promoted adsorption' (Porath, 1986). HIC has been studied by many researchers and it is a powerful bioseparation technique for purification of proteins (Wu and Karger, 1996; Grund, 1998).

Affinity ligand techniques represent currently the most powerful tool due to their selectivity and recovery. The strength of column affinity chromatography has been shown in thousands of successful applications. However, the disadvantage of all standard column liquid chromatography procedures is the impossibility of the standard column systems to cope with the samples containing bead material. They

are not suitable for suspended solid and fouling components. In this case magnetic affinity, magnetically stabilized fluidized beds (MSFB) or magnetically modified two-phase systems show their usefulness (Safarik and Safarikova, 1999). Magnetic carriers can be produced using inorganic materials or a number of synthetic and natural polymers. High mechanical resistance, insolubility and excellent shelf life make inorganic materials ideal as carriers. The main disadvantage of inorganic supports is their limited functional groups for ligand immobilization. Magnetic carriers are more commonly manufactured from polymers since they have a variety of surface functional groups which can be tailored to use in specific applications. Use of magnetic carriers in bioprocesses has many advantages. They can be easily separated from reaction medium and stabilized in a fluidized-bed reactor by applying a magnetic field. Also, using magnetic carriers reduces capital and operation costs.

Lysozyme, (Lyz), is found in a variety of vertebrate cells and secretions, such as spleen, milk, tears and egg white. Lyz lyses certain bacteria by hydrolysing the β -linkages between the muramic acid and *N*-acetylglucosamine of the 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 et. al, 1992). Lyz 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 (Suhagara 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 Lyz 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).

In this thesis, tryptophan containing, monosize, magnetic poly(glycidyl methacrylate- methacryloylamidotryptophan), m-poly(GMATrp), bioaffinity adsorbents were prepared for efficient separation of Lyz from aqueous solutions. In the first part of this study, N-methacryloylamido tryptophan, (MATrp), was synthesized by reaction of methacryloyl chloride and tryptophan. MATrp was characterized by using fourier transform infrared spectroscopy and nuclear magnetic resonance. Magnetic poly(GMATrp) beads were obtained by dispersion polymerization of GMA and MATrp in the presence of Fe_3O_4 nanopowder. Magnetic poly(GMATrp) beads were characterized by using fourier transform infrared spectroscopy, scanning electron microscope, elemental analysis, surface area measurements and swelling tests. After that, Lyz adsorption properties of the m-poly(GMATrp) beads from aqueous solutions were investigated at different experimental conditions in batch and magnetically stabilized fluidized bed system. Desorption of Lyz and reusability of the adsorbents were also tested. Adsorption behaviors were also investigated and isotherms were plotted for batch and MSFB systems. Pseudo-first and pseudo-second order kinetic modelling of the experimental data for the adsorption were also investigated.

Finally, m-poly(GMATrp) beads were used for the purification of Lyz from chicken egg white.

2. GENERAL INFORMATION

2.1. Bioaffinity Chromatography

Affinity chromatography is the preferred method of bioselective adsorption and subsequent recovery of a compound from an immobilized ligand. It is designed for highly specific and efficient purification of proteins and related compounds. Biomolecules can be identified, separated, and purified by traditional techniques such as gel permeation chromatography, high performance liquid chromatography, chromatofocusing, electrophoresis, centrifugation, etc., by using the differences in the physical properties (e.g., size, charge and hydrophobicity) of molecules. Affinity chromatography is a unique technique due to the extreme biological recognition and highest selectivity among other chromatographic techniques. Complementarity of surface geometry, special arrangement of the ligand and the binding site of the biomolecule enable this. Affinity chromatography separates biomolecules on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatographic matrix. The technique is ideal for a capture or intermediate step in a purification protocol and can be used whenever a suitable ligand is available for the protein(s) of interest. With high selectivity, high resolution, and high capacity for the protein(s) of interest, purification levels in the order of several thousand-fold with high recovery of active material are achievable. Target protein(s) is collected in a purified and concentrated form (Turkova, 1993).

Biological interactions between ligand and target molecule can be a result of electrostatic, hydrophobic interactions, van der Waals' forces and/or hydrogen bonding. To elute the target molecule from the affinity medium the interaction can be reversed, either specifically using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Successful affinity purification requires a biospecific ligand that can be covalently attached to a chromatography matrix. The coupled ligand must retain its specific binding affinity for the target molecules, and after washing away unbound material, the binding between the ligand and target molecule must be reversible to allow the target molecules to be removed in an active form. Any component can be used as a ligand to purify its respective

binding partner. Some typical biological interactions, frequently used in affinity chromatography, are listed below:

Table 2.1. Examples of Biological Interactions Used in Affinity Chromatography.

Ligand	Counter Ligand
Antibody	Antigen, virus, cell
Inhibitor	Enzyme (ligands are often substrate/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 high selectivity of affinity chromatography enables many separations to be achieved in one simple step, including common operations such as the purification of monoclonal antibodies or fusion proteins.

The principles of affinity chromatography are shown in Figure 2.1. Basic principle of affinity chromatography is that every biomolecule recognizes another natural or artificial molecule. A carrier matrix is chosen and equilibrated in binding buffer. Sample is applied under conditions that favor specific binding of the target molecule(s) to a complementary binding substance (ligand). Target substances bind specifically and reversibly to the ligand. Unbound material is washed through the column. Target molecule is recovered by changing conditions that favor elution of the bound molecules. Elution is performed specifically using a competitive ligand, or non-specifically, by changing pH, ionic strength, and polarity. Target molecule is collected in a purified, concentrated form. Affinity medium is re-equilibrated with binding buffer. Since affinity chromatography is based on functional properties, active and inactive forms can often be separated.

Affinity chromatography divided into subcategories, many of them are referred by their own nomenclature. Table 2.2 summarizes some of these techniques (Wilcheck and Miron, 1999).

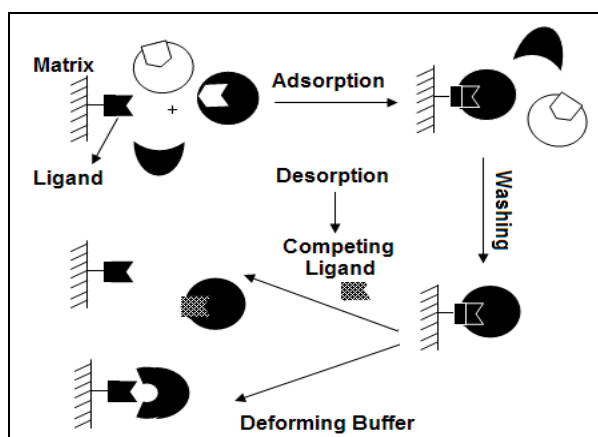


Figure 2.1. Schematic representation of the main steps in affinity chromatography.

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. Reseptor Affinity 11. Weak AC 12. Perfusion AC 13. Thiophilic Chromatography 14. High Performance AC 15. Affinity Density Pertubation 16. Library-Derived Affinity 17. Affinity Partitioning 18. Affinity Electrophoresis 19. Affinity Capillary Electrophoresis
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2.2. Hydrophobic Interaction Chromatography (HIC)

Different types of chromatographic techniques such as gel filtration chromatography, ion-exchange chromatography, affinity chromatography and HIC

are available for protein purification. Proteins are separated according to their biological and physicochemical properties: molecular size, net charge, biospecific characteristics and hydrophobicity, respectively (Garcia and Pires, 1993). HIC takes advantage of the hydrophobicity of proteins.

The hydrophobicity is the property that non-polar molecules tend to form intermolecular aggregates in an aqueous medium. The name arises from the combination of water, hydro- and for fear, phobos, which describes the apparent repulsion between water and hydrocarbons. At the macroscopic level, the hydrophobic effect is apparent when oil and water are mixed together and form separate layers on hydrophobic surfaces. At the molecular level, the hydrophobic effect is an important driving force for biological structures and responsible for protein folding, protein-protein interactions, formation of lipid bilayer membranes, nucleic acid structures, and protein-small molecule interactions.

In HIC, separation is based on hydrophobic interactions between immobilized hydrophobic ligands and non-polar regions on the surface of proteins. Hydrophobic groups on the protein bind to hydrophobic groups on the matrix. As the hydrophobicity of a protein increases, interaction between the ligand and the protein increases.

In 1948, Tiselius laid down the foundation for a separation method which is now popularly known as HIC and entitled as “Adsorption Separation by Salting Out”. He noted that, “...proteins and other substances which are precipitated at high concentrations of neutral salts (salting out), often are adsorbed quite strongly already in salt solutions of lower concentration than is required for their precipitation, and that some adsorbents which in salt-free solutions show no or only slight affinity for proteins, at moderately high salt concentrations become excellent adsorbents”.

The principle for protein adsorption to HIC media is complementary to ion exchange chromatography and gel filtration. HIC is even sensitive enough to be influenced by non-polar groups normally buried within the tertiary structure of

proteins but exposed if the polypeptide chain is incorrectly folded or damaged (e.g. by proteases). This sensitivity can be useful for separating the pure native protein from other forms.

2.2.1. Principles of HIC Theory

Many theories that have been proposed for HIC are essentially based upon those derived for interactions between hydrophobic solutes and water. In 1973, Porath proposed “salt-promoted adsorption” as a general concept for HIC and other types of solute-adsorbent interactions occurring in the presence of moderately high concentrations of neutral salts. The adsorption increases with high salt concentration in the mobile phase and the elution is achieved by decreasing the salt concentration of the eluent (Fausnaugh and Regnier, 1986; Roe, 1989). Therefore, the term “salt-promoted adsorption” could be used for this type of chromatography (Porath, 1986).

Hofstee proposed “hydrophobic chromatography” with the implicit assumption that the mode of interaction between proteins and the immobilized hydrophobic ligands is similar to the self association of small aliphatic organic molecules in water. He also suggested that “. . .the driving force is the entropy gain arising from structure changes in the water surrounding the interacting hydrophobic groups”. This concept was later extended and formalized by Hjerte who based his theory on the well known thermodynamic relationship:

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

He proposed that when a 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 ($\Delta G > 0$). 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. Pure water molecules adopt a structure which maximizes entropy. A hydrophobic

molecule will disrupt this structure and decrease entropy, and creates a 'cavity' as it is unable to interact electrostatically with the water molecules. When more than one 'cavity' is present, the surface area of disruptions is high, meaning that there are fewer free water molecules. To counter this, the water molecules push the hydrophobic molecules together and form a 'cage' structure around them which will have a smaller surface area than the total surface area of the cavities. This maximizes the amount of free water and thus the entropy. Therefore the hydrophobic effect might also be understood as the "the lipophobicity of water".

An increase in entropy ($\Delta S > 0$) causes a negative change in free energy ($\Delta G < 0$) and thus 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.

An alternative theory is based on parallelism between the effects of neutral salts in salting out and HIC. Hydrophobic interaction is accounted for by increase in surface tension of water arising from structure forming salts dissolved in it. The hydrophobic ligand-protein interaction is thermodynamically favourable, as illustrated in Figure 2.2 (Affinity Chromatography Handbook).

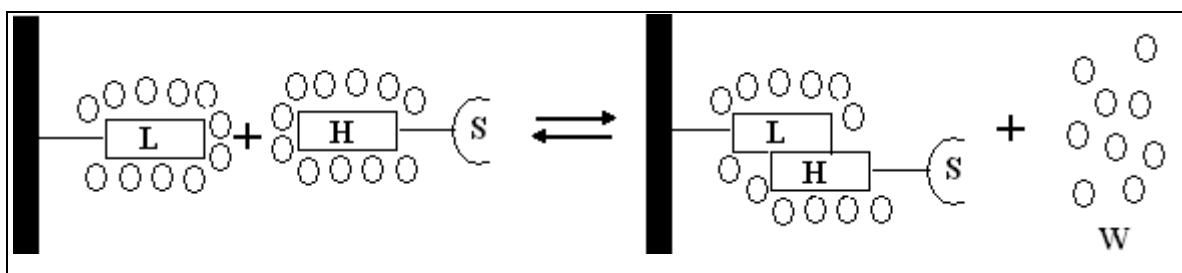


Figure 2.2. Principles of hydrophobic interaction. P: Polymer matrix, S: Solute molecule, L: Ligand attached to polymer matrix, H: hydrophobic patch on surface of solute molecule, W: Water molecules in the bulk solution.

Close to the surface of the hydrophobic ligand and solute (L and H), the water molecules are more highly ordered than in the bulk water and appear to “shield off” the hydrophobic ligand and solute molecules. Added salt interacts strongly with the water molecules leaving less water available for the “shielding off” effect,

which is the driving force for L and H to interact with each other. Also, it was proposed that HIC is due to van der Waals attraction forces between proteins and immobilized ligands despite the complex mechanism involved. The basis for this theory is that van der Waals attraction forces between protein and ligand increase as the ordered structure of water increases in the presence of salting out salts. By this way, 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 and Regnier, 1986; 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). Compared with RPC, the polarity of the complete system of HIC is increased by decreased ligand density on the stationary phase and by adding salt to the mobile phase.

2.2.2. Protein Hydrophobicity and Hydrophobic Interactions

Repulsion between a nonpolar compound and a polar environment is named as “hydrophobicity”. 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).

Hydrophobic interactions have a great importance in the biological systems. They are the dominant forces in protein folding and structure stabilization (Privalov and Gill, 1988; Dill, 1990) and play an important role in other biological processes like antibody-antigen reactions (Van Oss et. al, 1990), enzyme-substrate reactions and the maintenance of the lipid bilayer structure of biological membranes. 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).

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, valine) occur frequently on the surface of

the proteins, such as Lyz or serum albumin. There are two kinds of hydrophobicity scales which are shown in Table 2.3.

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

The degree of hydrophobicity of a protein is dependent on the sum of the hydrophobicities of the exposed and buried amino acids. There are several hydrophobicity scales of amino acids, based on different approaches. In 1971, Nozaki and Tanford measured the solubilities of individual amino acids in water and in progressively increasing concentration of organic solvents. They constructed a hydrophobicity scale for the different amino acids on the basis of the free energy transfer from ethanol or dioxane to water. Another approach is based on studies of known protein structures. Surface location of amino acid side groups in a number of proteins is determined from X-ray diffraction data and the fraction of

buried amino acids in the protein. The number of hydrophobic amino acids, their different distribution and hydrophobicity are characteristic of each protein, so a specific separation can be possible with hydrophobic supports or matrices. Therefore, the variation in hydrophobicity of proteins provides another strong means by which these biomolecules could be fractionated using HIC. 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 Horvard, 1996).

2.2.3. Factors Affecting HIC

The main parameters to consider when selecting HIC media and optimizing separation processes are:

- Ligand type and degree of substitution
- Type of base matrix
- Type and concentration of salt
- pH
- Temperature
- Additives
- Type of ligand

2.2.3.1. Ligand Type and Degree of Substitution

Molecules surrounded by organized water tend to self associate and form molecular complexes with other kinds of molecules which also have hydration shells of water. Formation constants for such complexes are to a greater or lesser extent governed by the entropy gain accompanying the reorganization of water following complexation. If one of the interacting species, the ligand, is surface bound to the support and the other, the ligate, is present in the surrounding solution, the latter will become adsorbed on the solid support (Porath, 1990).

The type of immobilized ligand (alkyl or aryl) determines primarily the protein adsorption selectivity of the HIC adsorbent. In general, straight chain alkyl (hydrocarbon) ligands show “pure” hydrophobic character while aryl ligands show

a mixed mode behaviour where both aromatic and hydrophobic interactions are possible. The choice between alkyl or aryl ligands is empirical and must be established by experiments for each individual separation problem.

In narrow sense, hydrophobic interaction of protein molecules refers to the mutual attraction of alkyl side chains in water. Intermolecular interactions between alkyl ligands are exploited in hydrophobic interaction chromatography.

If an aliphatic ligand is unsaturated, another kind of interaction is superimposed on hydrophobicity. These unsaturated ligands attract ligates more strongly than hydrophobicity alone. The additional contribution to interaction is due to the π -electron system of the ligand. The electron donor or acceptor strength of a ligand can be strongly enhanced by introduction of electron releasing or accepting substituents. This is the case, if several groups are located adjacent to double bonds or are suitably located in heterocyclic rings. Phenyl (and other aromatic groups) is used as a ligand with good results due to mixed hydrophobic and aromatic (π - π) interactions (Affinity Chromatography Handbook).

Linear chain alkanes with/ without a terminal amino group are the most widely used ligands in HIC. At a constant degree of substitution on the matrix the *n*-alkane ligands constitute a homologous series in a hydrophobicity scale (Quierioz et. al., 2001). As the *n*-alkyl chain length increases the hydrophobicity and the strength of interaction increase, thus the protein binding capacities of HIC adsorbents increase.

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

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. Using the same type of ligand, the selectivity of the stationary phases can change in function of the depending on the type of support employed.

The protein binding capacities of HIC adsorbents increase with increased degree of substitution of immobilized ligand due to the higher probability of forming multi-point attachment between the protein and the ligand. At a sufficiently high degree of ligand substitution, the apparent binding capacity of the adsorbent remains constant (plateau is reached) but the strength of the interaction increases (Maisano et. al., 1985). Solutes bound under such circumstances are difficult to elute due to multi-point attachment. When it is difficult to elute the bound protein without denaturation, harsh chemicals can be used for elution (organic solvents, detergents or chaotropic agents) (Affinity Chromatography Handbook).

2.2.3.2. Type of Base Matrix

The most widely used types of support are strongly hydrophilic carbohydrates, e.g. cross-linked agarose, silica or synthetic copolymer materials. Also, cellulose, dextran and silica can be employed. The selectivity of a copolymer support will not be exactly the same as for an agarose based support substituted with the same type of ligand. To achieve the same type of results on an agarose-based matrix as on a copolymer support, it may be necessary to modify adsorption and elution conditions (Affinity Chromatography Handbook).

2.2.3.3. Type and Concentration of Salt

In 1848, Tiselius separated proteins and dye components in ink by paper chromatography in strong sulphate and phosphate solutions. He did not give an explanation, but he coined the term “salting-out chromatography” to indicate a parallelism to precipitation.

In 1973, Porath discovered that hydrophobic adsorption is reinforced by adding sodium chloride or sodium phosphate to the buffer system. Furthermore, subsequent elution can be performed simply by deleting the salt or by adding ethylene glycol to the eluent. He described, for the first time, salting out of adsorption of proteins by the use of a well defined separation parameter: hydrophobic interaction.

Effects of salts on protein solubility and stability were first studied by Franz Hofmeister who was an early protein scientist. He discovered a series of salts that have consistent effects on protein solubility. The Hofmeister series originates from the ranking of various ions toward their ability to precipitate a mixture of chicken egg white proteins. It runs from precipitating (water structure forming) salts such as phosphates or sulphates to dissolving salts (chaotropics) such as iodides and thiocyanates. These effects are summarized in Table 2.4 and 2.5 (Quierioz et. al., 2001).

The mechanism of the Hofmeister series is not entirely clear, but seems to result mainly from effects on the solvent at higher salt concentrations. Early members of the series increase solvent surface tension and decrease the solubility of nonpolar molecules, "salting out". They strengthen the hydrophobic interaction. By contrast, later salts in the series increase the solubility of nonpolar molecules, "salting in" and decrease the order in water. They weaken the hydrophobic effect. However, these salts also interact directly with proteins (which are charged and have strong dipole moments) and may even bind specifically. Ions that have a strong salting in effect such as I^- and SCN^- are strong denaturants, because they salt in the peptide group, and thus interact much more strongly with the unfolded form of a protein than with its native form (Quierioz et. al., 2001).

Table 2.4. The Hofmeister series on the effect of some anions and cations in precipitating proteins.

$\longleftarrow$ Increasing precipitation (salting-out) effect	
Anions: PO_4^{-3} , SO_4^{-2} , CH_3COO^- , Cl^- , Br^- , NO_3^- , ClO_4^- , I^- , SCN^-	
Cations: NH_4^+ , Rb^+ , K^+ , Cs^+ , Li^+ , Mg^{2+} , Ca^{2+} , Ba^{2+}	
Increasing chaotropic (salting-in) effect $\longrightarrow$	

Table 2.5. Relative effects of some salts on the molal surface tension of water.

$\text{Na}_2\text{SO}_4 > \text{K}_2\text{SO}_4 > (\text{NH}_4)_2\text{SO}_4 > \text{Na}_2\text{HPO}_4 > \text{NaCl} > \text{LiCl} > \dots\dots\dots > \text{KSCN}$

In both instances, sodium, potassium or ammonium sulphates produce relatively higher “salting-out” (precipitation) or molal surface tension increament effects. These salts also effectively promote ligand-protein interactions in HIC.

In HIC, the use of high salt concentration on the equilibration buffer and sample solution promotes the ligand–protein interactions and thus the protein retention. As the concentration of such salts is increases, the amount of bound proteins increases almost linearly up to a specific salt concentration and continues to increase in an exponential manner at still higher concentrations. Most of the bound proteins are effectively desorbed by washing the HIC adsorbent with water or dilute buffer solutions at near neutral pH (Affinity Chromatography Handbook).

2.2.3.4. Effect of pH

The effect of pH in HIC is also not straightforward. In general, an increase in pH weakens hydrophobic interactions, probably as a result of increased titration of charged groups, thereby leading to an increase in the hydrophilicity of the proteins (Sanz et. al., 1998). On the other hand, a decrease in pH results in an apparent increase in hydrophobic interactions. Thus, proteins which do not bind to a HIC adsorbent at neutral pH bind at acidic pH. Retention of proteins changes more drastically at pH values above 8.5 and/or below 5 than in the range pH 5–8.5.

pH is an important separation parameter in the optimization of hydrophobic interaction chromatography and it is advisable to check the applicability of these observations to the particular separation problem at hand (Affinity Chromatography Handbook).

2.2.3.5. Effect of Temperature

In HIC, increasing the temperature enhances protein retention and lowering the temperature generally promotes the protein elution. In fact, the 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 (Quierioz et. al., 2001).

According to the Eq. (1), ($\Delta G = \Delta H - T\Delta S$), binding of proteins to HIC adsorbents is an entropy driven process. Temperature may affect the conformational state of different proteins and their solubilities in aqueous solutions. This may cause an opposite behavior on protein retention. 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).

2.2.3.6. Additives

Solutes can have different effects on surface tension depending on their structure:

- No effect, sugar
- Increase of surface tension, inorganic salts
- Decrease surface tension progressively, alcohols
- Decrease surface tension and, once a minimum is reached, no more effect: surfactants.

Low concentrations of water-miscible alcohols (e.g., ethanol and ethyleneglycol), detergents (e.g. Triton X-100) and aqueous solutions of chaotropic “salting-in” salts result a weakening of the protein-ligand interactions in HIC leading to the desorption of the bound solutes. The non-polar parts of alcohols and detergents compete effectively with the bound proteins for the adsorption sites on the HIC media resulting in the displacement of the latter. Chaotropic salts affect the ordered structure of water and/or that of the bound proteins. Both types of additives also decrease the surface tension of water thus weakening the hydrophobic interactions to give a subsequent dissociation of the ligand-solute complex (Affinity Chromatography Handbook).

Although additives can be used in the elution buffer to affect selectivity during desorption, there is a risk that proteins could be denatured or inactivated by exposure to high concentrations of such chemicals. However, additives can be very effective in cleaning up HIC columns that have strongly hydrophobic proteins bound to the medium.

2.2.4. Literature Review of HIC

HIC is a versatile liquid chromatography technique that separates biomolecules based on the hydrophobicity of proteins. HIC has been used in the purification of a great variety of proteins. Protein purification procedure is obviously dependent on the choice of separation method. In some cases, different chromatographic techniques are used together. For example, HIC can 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 (Roder et. al., 2000; Wenk and Kruij, 2000).

Sometimes, the binding is too strong to be useful in a chromatographic process and may be practically irreversible and this may cause to protein denaturation. In order to elute a strongly bound protein, organic solvents, detergents or chaotropic agents may be required (Taipa et. al., 1994). The use of some mild hydrophobic ligands appears to be an alternative without above mentioned drawbacks.

HIC is also used in biotechnological products. In 1986, Sofer used HIC for the purification of plasminogen activator from human myocardial tissue. 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 of HIC used in 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.

HIC also studied for the separation of 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., 1999). HIC purification of a plasmid DNA vaccine against rabies was studied, no loss in purity was reported (Diago et. al., 2000).

2.3. Magnetism

Magnetism is a force generated in matter by the motion of electrons within its atoms. Magnetism and electricity represent different aspects of the force of electromagnetism, which is one part of Nature's fundamental electroweak force. The region in space that is penetrated by the imaginary lines of magnetic force describes a magnetic field. The strength of the magnetic field is determined by the number of lines of force per unit area of space. Magnetic fields are created on a large scale either by the passage of an electric current through magnetic metals or by magnetized materials called magnets. The elemental metals, (iron, cobalt, nickel, and their alloys with related metallic elements) are typical materials that respond strongly to magnetic fields. Unlike the all-pervasive fundamental force field of gravity, the magnetic force field within a magnetized body is strongest and opposite signs at the two extremities or poles of the magnet.

Magnetism arises from two types of motions of electrons in atoms, one is the motion of the electrons in an orbit around the nucleus, and the other is the spin of the electrons around its axis. The orbital and the spin motion independently impart a magnetic moment on each electron causing each of them to behave as a tiny magnet. The magnetic moment of a magnet is defined by the rotational force experienced by it in a magnetic field of unit strength acting perpendicular to its magnetic axis. In a large fraction of the elements, the magnetic moment of the electrons cancel out because of the Pauli exclusion principle which states that each electronic orbit can be occupied by only two electrons of opposite spin. However, a number of transition metal atoms, such as iron, cobalt, and nickel, have magnetic moments that are not cancelled; these elements are common examples of magnetic materials. In these transition metal elements the magnetic moment arises only from the spin of the electrons. In the rare earth elements (that begin with lanthanum in the sixth row of the Periodic Table of Elements) the effect of the orbital motion of the electrons is not cancelled, and hence both spin and orbital motion contribute to the magnetic moment. Examples of some magnetic rare earth elements are: cerium, neodymium, samarium, and europium. In addition to metals and alloys of transition and rare earth elements, magnetic moments are also observed in a wide variety of chemical compounds involving these elements.

2.3.1. History of Magnetism

The history of magnetism dates back to earlier than 600 B.C., but it is only in the twentieth century that scientists have begun to understand it, and develop technologies based on this understanding. Magnetism was most probably first observed in a form of the mineral magnetite called lodestone, which consists of iron oxide. The ancient Greeks were the first known to have used this mineral, which they called a magnet because of its ability to attract other pieces of the same material and iron.

William Gilbert was the first to investigate the phenomenon of magnetism systematically using scientific methods. He also discovered that the Earth is itself a weak magnet. Early theoretical investigations into the nature of the Earth's magnetism were carried out by Friedrich Gauss. Modern understanding of magnetic phenomena in condensed matter originates from the work of Pierre Curie who examined the effect of temperature on magnetic materials and observed that magnetism disappeared suddenly above a certain critical temperature in materials like iron. The present day understanding of magnetism based on the theory of the motion and interactions of electrons in atoms stems from the work and theoretical models of Ernest Ising and Werner Heisenberg.

2.3.2. Measurement of Magnetic Field

The magnetic field or flux density is measured in metric units of a gauss, (G), and the corresponding international system unit of a tesla, (T). The magnetic field strength is measured in metric units of oersteds, (Oe), and international units of amperes per meter, (A/m). Instruments called gaussmeters and magnetometers are used to measure the magnitude of magnetic fields. Magnetic susceptibility is the response of a system to an applied magnetic field. It is a dimensionless quantity that is equal to the ratio of the magnetic moment to the applied field intensity.

2.3.3. Types of Magnetism

Five basic types of magnetism have been observed and classified on the basis of the magnetic behavior of materials in response to magnetic fields at different

temperatures. These types of magnetism are: ferromagnetism, ferrimagnetism, antiferromagnetism, paramagnetism, and diamagnetism.

2.3.3.1. Ferromagnetic Materials

These are the metals that are strongly attracted to magnets. They include iron, nickel, cobalt, and steel. The atoms of these substances contain electrons which are all spinning in the same direction. This gives the atoms strong polarity, as if they were little magnets themselves. They are strongly influenced by a magnetic field, and the atoms can be turned to point their poles in the same direction. These metals can become strong magnets themselves.

2.3.3.2. Antiferromagnetic Materials

Antiferromagnets have an initial low positive susceptibility (similar to a ferromagnet) up to their Neel temperature, above which susceptibility decreases with increasing temperature (like a paramagnet). This property occurs in certain inorganic compounds where neighbouring atoms interact in such a manner as to produce an antiparallel arrangement of magnetic dipoles that is equal and opposite.

2.3.3.3. Paramagnetic Materials

These are metals that are weakly attracted to magnets. They include aluminum, gold, and copper. The atoms of these substances contain electrons which spin in the same direction, but not all. This gives the atoms some polarity. They are only weakly influenced by a magnetic field since some of the atoms can be turned to point their poles in the same direction, these metals can become very weak magnets. Their attractive force can only be measured with sensitive instruments.

2.3.3.4. Ferrimagnetic Materials

The main ferrimagnetic material is magnetite, a crystal which occurs naturally in rocks called lodestones, which were the first magnetic materials discovered by man. The crystal structure of the mineral allows only some of the atoms to line up when a magnetic field is present, so it is only weakly attracted to a magnet. The crystal itself is only a weak magnet. However, if magnetite is ground up into a

powder, its magnetic properties, although weak, are very useful. Magnetite powder is what coats the tape in a cassette, allows to record sound and music. Magnetite powder mixed with plastic makes the stick-on magnets used for fridge magnets.

2.3.3.5. Diamagnetic Materials

These materials are everything else, plants, water, soil, wood, skin, etc. Diamagnetic substances show no visible reaction to the presence of a magnetic field, because the electrons in their atoms are spinning in equal numbers in opposite directions. These materials are magnetically neutral. However, the molecules of these substances have a very small polarity, because they are usually not symmetric; one end may have more electrons moving around it than another. When they are exposed to a strong magnetic field, they are repelled a tiny amount. This has useful consequences. When human tissue is exposed to a strong magnetic field, some of the atoms move away; analysis of the movement is called magnetic resonance imaging, (MRI), and is a useful diagnostic tool in medicine.

Temperature can affect the magnetic properties of a material. Paramagnetic materials like aluminum and copper become more magnetic when they are very cold. Alternately, strongly magnetic ferromagnetic materials like nickel or steel lose all their magnetic properties if they are heated to a high enough temperature. The atoms become too excited by the heat to remain pointing in one direction for long time. The temperature at which a metal loses its magnetism is called the Curie temperature, and it is different for every metal.

2.3.4. Magnetic Separation Techniques

There are a large number of applications that exploit the magnetic properties of materials, beginning most obviously with the magnetic compass needle to current items such as refrigerator magnets. Many applications such as magnetic storage media (cassette tapes, hard drives), and the field of biology, medicine, catalysis, medical imaging technologies exploit the magnetic properties of fine beads.

Isolation and separation of specific molecules are essential in all areas of biosciences and biotechnology. Diverse procedures can be used to achieve this goal. Recently, increased attention has been paid to the development and application of magnetic separation techniques, which employ small magnetic beads. Magnetic separation of biomolecules and cells based on magnetic beads and employing biospecific affinity reactions is advantageous in terms of selectivity, simplicity, and speed.

The basic principle of batch magnetic separation is very simple. Magnetic carriers having an immobilized affinity, hydrophobic ligand, ion-exchange groups, or magnetic biopolymer bead, are mixed with a sample containing target compound(s). Samples may be crude cell lysates, whole blood, plasma, milk, whey, urine, cultivation media, wastes from food and fermentation industry and many others. Following an incubation period when the target compound(s) bind to the magnetic beads the whole magnetic complex is easily and rapidly removed from the sample using an appropriate magnetic separator. Substances, which lack the specific molecule or structure of the target substance, are not bound to the solid phase and can be removed to effect the separation of the bound substance or vice versa. After washing out the contaminants, the isolated target compound(s) can be eluted and used for further work (Safarik and Safarikova, 1999).

Magnetic separation techniques have several advantages in comparison with standard separation procedures. This process is usually very simple, with only a few handling steps. All the steps of the purification procedure can take place in one single test tube or vessel. There is no need for expensive liquid chromatography systems, centrifuges, filters or other equipment. The separation process can be performed directly in crude samples containing suspended solid material. Due to the magnetic properties of magnetic adsorbents, they can be relatively easily and selectively removed from the sample. In fact, magnetic separation is the only feasible method for recovery of small magnetic beads (diameter ca 0.1 – 1 μm) in the presence of biological and other fouling material of similar size. Moreover, the power and efficiency of magnetic separation procedures is especially useful at large-scale operations.

In most cases, magnetic batch adsorption is used to perform the separation step. This approach represents the simplest procedure available, enables to perform the whole separation in one test-tube or flask. If larger magnetic beads (with diameters above than 1 μm) are used, simple magnetic separators can be employed. If case magnetic colloids (diameters ranging between tens and hundreds of nanometers) are used as affinity adsorbents, high-gradient magnetic separators have to be used to remove the magnetic beads from the system.

Alternatively, MSFB can be used for continuous separation process, especially for large-scale purification of biological products. The MSFB is a fluidized bed of magnetically susceptible particles that are stabilized by a uniform magnetic field. The stabilization of the beads results in a bed that has the dispersion and mass transfer properties of a packed-bed while retaining the solid phase fluidity of a fluidized bed. Magnetic stabilisation enables the expansion of a packed bed without mixing of solid beads. High column efficiency, efficient fluid–solid mass transfer properties, elimination of bead mixing, good fluid–solid contact, and elimination of fouling can be reached. Especially, when dealing with highly viscous mediums contact with the magnetic adsorbent in a magnetically stabilized fluidized bed is desirable because of high convective transport rates. Recently, there has been increased interest in the use of magnetic adsorbents in protein and nucleic acid purification (O'Brien et. al., 1997).

2.3.4.1. Magnetic Polymer Beads

Magnetic polymer beads usually consist of a magnetic core (inorganic nanobeads) and polymer shell (organic polymer matrix). Core-shell morphology polymer beads have properties of both systems (Huang et. al., 2003). This morphology enables modifications of beads and protects from aggregation and nonspecific binding. The magnetic core consists of small magnetic nanobeads with diameter less than 30 nm. Magnetic nano- and beads are used in many areas such as magnetic semiconductors, magnetic recording, as magnetic toners in xerography, removal of metal ions from wastewater. In biosciences and biotechnology, they are also used in magnetic resonance imaging, magnetic biomarkers, delivery systems for drugs or in treatment of cancer (Hafeli et. al., 1997). In many areas, magnetic

separation can advantageously replace classical techniques, such as centrifugation, filtration or chromatography.

The polymer shell enables stability of magnetic beads in physiological medium provides nontoxicity by avoiding leakage of iron. It also enables chemical modification for attachment of biologically active compounds. Surface characteristics and bead size are crucial factors that determine the success of the beads *in vivo*. Their size must be sufficiently uniform to provide an equal probability of magnetic capture for all magnetic beads at constant biomolecule content (Horak and Benedyk, 2004).

Polymer supports can be both organic and inorganic. The main disadvantage of inorganic supports such as silica or porous glass is the absence of functional groups for selective binding. Therefore, magnetic beads for selective purposes are manufactured from polymers that can have a variety of functional groups. Polystyrene and poly(methyl methacrylate) are the most frequently used polymers for magnetic beads. In addition, biodegradable polysaccharide, dextrane, albumin, cellulose, polylactic acid, and poly(alkyl cyanoacrylate)s have been described (Vettese-Dadey, 1999). Because magnetic separations work as a result of a reactive group on the surface of the magnetic, polystyrene or poly(methyl methacrylate) beads must be chemically modified to introduce specific ligands or recognition groups.

Chemical reactions on such supports are very difficult. This calls for a search for special reactive carriers that would facilitate the modifications. Examples of such supports include polyglycidylmethacrylate, poly(GMA), the greatest advantage of the presence of easily transformable oxirane groups (Horak, 1999). They can be directly reacted with miscellaneous substances or further modified for a variety of applications. Chemical modification and exploitation of poly(GMA) resins are usually performed via ring opening of the epoxide yielding a hydroxy group on the β -carbon atom. For example, glycidylmethacrylate (GMA) polymers react with amines, aminopyridines (Azanova et.al., 1990), and phenol (Horak et.al., 1991).

2.4. Polymerization Methods

Polymer beads are used as carrier matrices in medical and biological applications. Table 2.6 shows some applications of beads. Depending on the application, size and the properties of the beads changes. (Kawaguchi, 2001).

Table 2.6. Biomedical applications of polymer beads.

	Size (μm)	Biocompound
Protein	0.01	
Virus	0.1	Cell label
		Particles for phagocytosis assay
		Latex diagnostics
Bacterium	1.0	Protein separator
		Drug carrier
Cell	10.0	Blood flow indicator
		Cell separator
		Column packing reagent
	100.0	Heterogeneous immunoassay support
		Cell culture carrier

Reliable and reproducible results are obtained with monodisperse beads in these applications. Uniformity of size, chemical composition, surface texture and morphology of the beads determine the performance of the beads. Although, a polymerization reaction may be carried out in the solid, the liquid or the gas phase, the liquid phase is used in preparation of commercial scale polymerizations (Saunders, 1988). Bulk, solution, emulsion, suspension, and dispersion methods are subcategories of liquid phase polymerization.

2.4.1. Bulk Polymerization

Bulk polymerization is the conversion of monomer into a polymer without aid of a solvent. It is performed through functional groups in the presence of catalyst (Saunders, 1988). In the case of chain-growth reactions, which are

generally exothermic, the evolved heat may cause the reaction to become too vigorous and difficult to control unless efficient cooling.

2.4.2. Solution Polymerization

In this procedure, a monomer is dissolved in a non-reactive solvent that usually contains a catalyst (Nicholson, 1991). The heat released by the reaction is absorbed by the solvent, and the reaction rate is reduced. The benefit of this type of polymerization over the conventional bulk polymerization is that it reduces the viscosity of the reaction.

2.4.3. Emulsion Polymerization

Emulsion polymerization is a type of radical polymerization that usually starts with an emulsion incorporating water, monomer, and surfactant. The most common type of emulsion polymerization is an oil-in-water emulsion, in which droplets of monomer are emulsified with surfactants in a continuous phase of water. In emulsion polymerization, aqueous (continuous) phase and (nonaqueous) discontinuous phases present. The initiator is located in the aqueous phase and monomer/polymer is in the nonaqueous phase (Billmeyer, 1984).

2.4.4. Suspension Polymerization

Suspension polymerization is a process that uses mechanical agitation to mix the monomer(s) in a liquid phase, and polymerizing the monomer droplets while they are dispersed by continuous agitation. Location of initiator and the reaction kinetics are different from emulsion polymerization (Billmeyer, 1984). Mechanical stirring and suspending agents are needed to maintain the stability of monomer droplets in suspension medium (Arshady, 1999).

2.4.5. Precipitation Polymerization

In precipitation polymerization, the initial state of the reaction mixture is a homogeneous solution and resulting polymer is insoluble in its own monomer or in a particular monomer-solvent combination and thus precipitates out as it is formed. Thus, precipitation polymerization produces irregularly shaped and polydisperse beads due to their growth mechanism.

2.4.6. Dispersion Polymerization

Dispersion polymerization has been investigated for preparing monodisperse polymeric beads in the 0.1–10 μm size range in a single step (Ober et. al., 1985, Tseng, et. al., 1986). In dispersion polymerization the monomer and the initiator are both soluble in the polymerization medium, but the medium is poor solvent for the resulting polymer. The reaction begins as a solution polymerization (Horak, 1999).

The reaction mixture is homogeneous at the beginning of the polymerization, and the polymerization reaction is initiated in homogeneous solution. Phase separation depends on the medium solvency and occurs at an early stage of polymerization. It is assumed that beads are formed by precipitation of growing polymer chains in the polymerization medium and/or the monomer. Polymerization occurs within individual beads which are produced in the size range of 0.1-10 μm . The advantage of this technique is the capability of producing monosize beads in a single step.

Nature of the medium controls the critical chain length. The reaction kinetics is a combination of solution polymerization and polymerization inside swollen polymer beads. The bead size and its distribution are known to be affected by the monomer and its concentration, solvency of the medium, amount and type of both the stabilizer and the initiator, and polymerization temperature (Arshady, 1991).

In dispersion polymerization, beads produced in the absence of stabilizer are not sufficiently stable. They coagulate during their formation. Stable beads are obtained by adding small percentage of stabilizer to the reaction medium. Steric stabilization is used for bead stabilization. Polymers and oligomers with low solubility in the polymerization medium are used as stabilizers. For dispersion polymerizations in alcohols and other polar solvents, a variety of polar organic polymers can be used such as polyvinylpyrrolidone, poly (vinyl alcohol), and cellulose derivatives (Ober et.al., 1985 and Tseng et. al.,1986).

Bead formation step is the most important and the complex step in dispersion polymerization. A description of the polymerization is as follows (Shen et. al., 1994). The system is homogeneous at the beginning, as shown in Figure 2.3 (A). As soon as the polymerization begins, primary radicals, generated by the decomposition of the initiator, grow in the continuous phase by addition of the monomer units until they reach critical chain length where they precipitate to form nuclei, either by a self or aggregative mechanism, as shown in B. These nuclei are unstable and aggregate with each other. They adsorb the stabilizer and finally become mature beads, C. The mature beads capture oligo-radicals and nuclei generated in the continuous phase and thus reduce the rate of bead formation. As long as the enough mature beads are formed to capture all of the oligo-radicals and nuclei, no more new beads will be formed and the bead formation stage is complete. The existing beads continue to grow by capturing oligo-radicals and nuclei which will either continue to grow inside the beads or terminate with other radicals. Nuclei and mature beads coexist during the whole bead growth stage, as shown in D. At the end of polymerization, nuclei formation stops owing to the exhaustion of either the monomer or the radicals (by cooling or adding inhibitor). Only mature beads are finally observed.

Each mature bead contains many oligomer and polymer chains. The length of the precipitated oligomer chains is directly related to their critical chain length, which depends on the solubility of the oligomer chains in the continuous phase. The number of precipitated oligomer chains in each bead is determined by the extent of aggregation, which results from the competition between the aggregation of nuclei and the stabilization by adsorbed stabilizer and the termination mode of the radicals. The influence of various polymerization parameters on bead size is explained in the following section. Nuclei are formed through out the whole polymerization, while the mature beads are formed only at the beginning of the polymerization. After the bead formation stage, the number of nature beads does not change, while their size changes both by polymerization inside the beads and aggregation of small nuclei and oligo-radicals from the continuous phase.

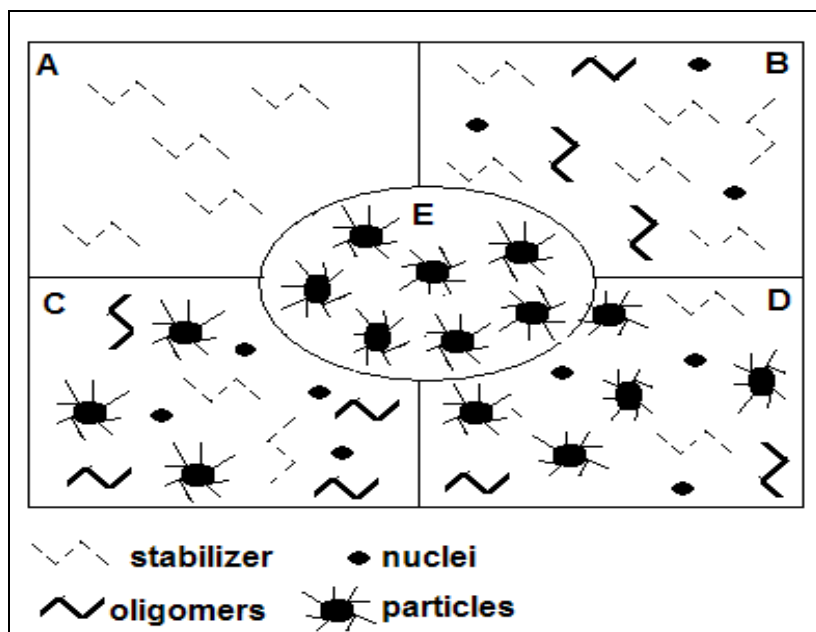


Figure 2.3. A proposed bead formation mechanism.

2.4.6.1. Effect of Solvent System

In dispersion polymerization, medium polarity is very important since it determines the bead size and distribution. It controls the critical molecular weight above which the resulting polymer beads precipitate. The solubility of the reaction components is important, because the polarity has to keep the initial components soluble but allow the polymeric beads precipitate out (Horak and Shapoval, 2001).

In the dispersion polymerization of any monomer, a change in the system that tends to increase the solubility parameter causes an increase of the bead size. It is explained by the increasing solubility of oligomers formed at the earlier polymerization stages with the increasing polarity of the dispersion medium. Therefore, the growing oligomers can reach relatively longer chain lengths by remaining in the dissolved form (Shen et.al., 1994).

2.4.6.2. Concentration and Molecular Weight of Stabilizer

The stabilizer plays an important role in the formation of stable beads by dispersion polymerization. Stabilization of the beads is determined by the rate and the extent of adsorption of the stabilizer on the bead surface and its stabilizing efficiency which itself is a function of the solvency of the medium and the nature of the polymer onto which it is adsorbing (Barrett, 1975). The most important role of

stabilizer is in the bead formation step. Stabilizer determines the bead stability and the solution viscosity. Viscosity of the medium increases with increasing molecular weight or concentration of the stabilizer (Shen et. al., 1994).

The influence of stabilizer concentration on bead size is straightforward. An increase in the concentration of stabilizer increases the viscosity of the continuous phase as well as the rate of physical adsorption of stabilizer.

Dependence of the bead size on the relative viscosity of the continuous phase for three PVP stabilizers in the polymerization of methyl methacrylate is shown by Shen et. al. At the same viscosity, PVP K-15 produces the smallest beads; PVP K-90 produces the largest ones. These results illustrate the effect of the rate of adsorption of the stabilizer. At the same viscosity, the corresponding concentration of PVP K-15 is the highest and PVP K-90 is the lowest. Higher concentrations of stabilizer give higher rates of adsorption and smaller beads. On the other hand, for the same bead size, the corresponding concentration of PVP K-90 is the lowest and that of PVP K-15 is the highest. However, the corresponding solution viscosities follow opposite order, highest for the PVP K-90 and lowest for the PVP K-15. The influence of higher viscosity and better stabilization by longer stabilizer chains was countered by the higher rate of adsorption of the shorter stabilizer chains (Shen et. al., 1994).

2.4.6.3. Effect of Monomer Concentration

In dispersion polymerization, monomer concentration can influence the bead size in several ways:

- by changing the solubility of the oligomer chains in the continuous phase, i.e., the critical chain length,
- by changing the polymerization rate, i.e., changing the rate of nuclei formation,
- by changing the solvency power of the continuous phase for the stabilizer, since monomer is a poor solvent for stabilizer.

Increasing monomer concentration increases the solubility of the produced polymer and the critical chain length, thus larger polymer beads are obtained. It also increases the polymerization rate and thus the precipitation rate of the oligomer chains. Therefore, larger and more numerous nuclei is obtained (Shen et. al., 1994).

2.4.6.4. Effect of Initiator Concentration

In dispersion polymerization, with increasing initiator concentration, radical concentration increases and thus the concentration of precipitated oligomer chains. At the early stage of the polymerization, more polymer chains of lower molecular weight are formed. Because the dispersion polymerization mechanism requires that the polymer chains reach a critical size before nucleation can occur, and fewer of the chains reach this critical size, fewer nucleation sites are available. Therefore, fewer particles of larger diameter are produced at high initiator concentrations (Horak and Shapoval, 2000).

Moreover, larger beads are less like to capture nuclei or oligo-radicals from the continuous phase simply due to their reduced total surface area and number density. Therefore, bead formation continues and this stage in polymerization is extended. When this extension exceeds a certain period, the bead size will be significantly broadened. Therefore, it is expected that there exists a maximum concentration of initiator for producing monodisperse beads.

Furthermore, a higher stabilizer concentration can increase both the adsorption rate of stabilizer and the viscosity of the continuous phase. These would reduce the extent of aggregation, resulting smaller beads and shorter bead formation stage. Therefore, the maximum initiator concentration which is able to produce monodisperse beads would increase with increasing stabilizer concentration. On the other hand, a combination of low initiator concentration and high stabilizer concentration is also expected to result in broad bead size distributions. This clearly shows that a balance between nuclei formation and stabilization is important for preparing monodisperse beads. Also, effect of using different types

of initiator with higher decomposition rate is similar to increasing the initiator; both result in an increased final bead size (Shen et. al., 1994).

2.4.6.5. Effect of Temperature

Bead size increases with increasing temperature. An increase in the temperature causes an increase in the solubility of the oligomers chains. This in turn results the following changes (Shen et. al., 1994).

- Increase in the critical chain length,
- Increase in the concentration of precipitated chains,
- Decrease in the viscosity of the continuous phase,
- Increase in the growth rate of the existing beads.

All of these changes contribute to an increase in bead size.

2.5. The Chicken Egg

Eggs are inexpensive, low-calorie high-quality protein source which are well-known for their gelling and emulsification properties.

Eggs have high nutritional benefits. They contain protein which is needed for building and repairing the cells in muscles and other body tissues. One egg contains six grams of excellent quality protein (the same can be found in 35 g of cheese or 180 mL of milk). Eggs also contain folic acid, Vitamins A, B, D and E and iron. In addition, one egg contains approximately 2 mg of choline, which has been associated with early brain development

Isolation of valuable components (particularly proteins) from the egg is receiving great attention. The egg has been fractionated into Lyz, avidin and other chemicals with known bioactivities (Juneja et.al., 1991).

Eggs consist of two parts which are called as yolk and egg white. The yolk comprises about 31% of the total weight of the egg. The composition of the yolk is

as follows: 51% water, 16% protein, 30.5% fat and some minerals, and is contained within a fine elastic membrane.

Egg white is a clear jelly-like substance which comprises about 58% of the weight of the egg. The white consists of 88% water, 9% protein and some minerals. Egg white exists in two states. Thick and gelatinous egg white surrounds the yolk, and acts to cushion the egg. A thinner layer of egg white surrounds the thick white and is contained by two membranes just inside the shell. These membranes enclose the whole egg and prevent bacterial contamination. The shell of the egg is rigid and brittle, providing protection for its contents. The shell makes up 11% of the weight of an egg and approximately 98% of the shell consists of calcium (Davis and Reeves, 2002).

Table 2.7. The major constituents of egg white.

Protein	Amount (%)	M Wt (KDa)	pI	Characteristics
Ovalbumin	54	45	4.5	
Ovotransferrin	12-13	77.7	6.0	Binds iron and other metal ions
Ovomucoid	11	28	4.1	Inhibits serine proteinases
Lyz	3.4-3.5	14.3	10.7	Lysis of bacterial cell walls
Ovomucin	1.5-3.5	220-270000	4.5-5.0	Interacts with Lyz
Avidin	0.05	68.3	10.0	Binds biotin

Chicken egg white proteins are one of the main ingredients in food processing since they have unique functional properties such as gelling, emulsification, heat setting, binding adhesion and foaming. These proteins are desirable ingredients in many foods such as bakery products, meat products and cookies. Investigations of the physicochemical characteristics of these proteins have aided to learn their

structure-function relationship. The major constituents of egg white and some of the important characteristics are summarized in Table 2.7 (Davis and Reeves, 2002).

2.5.1. Lyz

Lyz (EC 3.2.1.17, also known as muramidase and N-acetylmuramic-hydrolase), is a 14.3 kilodalton antibacterial enzyme widely distributed in organisms from bacteriophages to human. Lyz is an enzyme that may function as a bactericidal agent or help dispose of bacteria after they have been killed by other ways. In nature, there are types of Lyz with different characteristics according to their origin. It is abundantly present in a number of secretions of vertebrates, such as tears, saliva. Lyz is also present in chicken egg-white which is a rich and easily available source (3.5 %).

Lyz is commonly referred to as the "body's own antibiotic" since it kills bacteria. Lyz is an enzyme that destroys bacterial cell walls by hydrolyzing the polysaccharide component of the cell wall. The enzyme functions by attacking the cells walls of bacteria, especially Gram-positive bacteria. The antimicrobial activity of Lyz is greater for certain Gram-positive bacteria. The Gram-negative bacteria are less susceptible to the bacteriolytic action of the enzyme. The susceptibility differences are believed to be due to the complex envelope structure of Gram-negative bacteria such as *E.coli*. The outer membrane serves to reduce the access of Lyz to its site of action.

Alexander Fleming, the discoverer of penicillin described Lyz in 1922. Its structure was described by David Phillips in 1965 when he got the first 2 angstrom (200 pm) resolution image. This work led Phillips to provide an explanation for how enzymes speed up a chemical reaction in terms of its physical structures. Howard Florey and Ernest Chain also investigated Lyzs. Although they never made much progress in this field, they developed penicillin, which Fleming had failed to do.

The primary structure of egg white Lyz, shown in Figure 2.4, is a single polypeptide chain of 129 amino acids. There are four pairs of cysteines that form

disulfide bridges between positions, 6 and 127, 30 and 115, 64 and 80, 76 and 94. Lyz catalyzes the hydrolysis of its substrate at a rate that is $\sim 10^8$ -fold greater than that of the uncatalyzed reaction (Voet and Voet, 1995).

Lyz is a non-toxic enzyme and has inhibitory activity toward undesirable microorganisms. In 1992, it was declared that Lyz is safe to be used in foods. It has been used as an antimicrobial agent in various foods. It is a heat stable protein, it is active in a broad range of temperatures (from 1°C to nearly 100°C), it withstands boiling for 1-2 min, it is stable in freeze-drying and thermal drying, it is not inactivated by solvents, it maintains its activity when redissolved in water, and it has optimum activity at pH 5.3 to 6.4. However, the presence of other proteins in food can reduce its stability by the formation of sulfide bridges (Davis and Reeves, 2002).



Figure 2.4. The primary structure of egg white Lyz which consists of 129 aminoacid residues.

In the pharmaceutical industry, chicken egg white Lyz can protect the body against bacterial, viral or inflammatory diseases. It has been used in aerosols for the treatment of bronchopulmonary diseases, for nasal tissue protection. It is also incorporated into various therapeutic creams for the protection and topical

reparation inflammatory lesions of the skin and soft tissues (e.g. burns, viral diseases) (Davis and Reeves, 2002).

2.5.1.1. Methods of Lyz Isolation

There is a need for efficient and simple techniques for Lyz isolation since it has become increasingly important in industry. Methods of extracting Lyz from egg white include gel permeation chromatography, membrane separation, affinity chromatography, and adsorption and ion-exchange chromatography (Davis and Reeves, 2002). However, many of these methods have major drawbacks, such as high cost and impractical for routine use. Although, most methods are used in laboratory practice, few have been applied on commercial scale (Chang et al., 2000).

Crystallisation Method

In this method, Lyz separation from the egg white is based on direct enzyme crystallisation with 5% sodium chloride. Several re-solubilisations and re-crystallisations must be performed to obtain a high purity Lyz preparation. Lyz recovery usually ranges from 60-80% (Durance, 1994). Crystallization extremely time consuming and the residual egg white is too salty for further application. Ultrafiltration and diafiltration to desalt the remaining egg white material after Lyz separation can reduce the salt content and the resultant egg white retains its foaming ability and stability.

Membrane Filtration Method

Membrane filtration is used to separate the enzyme from the egg white. Unfortunately, the ability of Lyz to bind electrostatically to other egg white proteins can greatly reduce its membrane diffusion. Only small amounts of Lyz (20%) were able to pass through ultrafiltration membranes.

Affinity Chromatography Method

Chitin was the first adsorbent used to bind Lyz. Affinity methods are not used for large-scale isolation of Lyz from egg white for several reasons - the high cost of affinity supports or ligands, the poor flow rates of undiluted egg white, the

expensive and time-consuming elution procedures and the non-specific adsorption of other egg white proteins (Durance, 1994). Grasselli et al. (1999) evaluated a dye affinity membrane system and produced Lyz with a purity of 88% and a recovery of 92%.

Ion-exchange Chromatography Method

Ion-exchange resins are commercially available at a moderate price and can be used for a long time. Both column and stirred tank techniques are in use for larger scale operation. A cation exchange resin can be stirred with egg white until the bulk of the enzyme has been adsorbed. Residual egg white is decanted or filtered off and used for further applications.

3. EXPERIMENTAL

3.1. Materials

Lyz, (chicken egg white, EC 3.2.1.7), was supplied by Sigma Chemical Co. (St Louis, MO) and used as received. L-Tryptophan and methacryloyl chloride were supplied by Sigma (St. Louis, MO, USA) and used as received. Glycidyl methacrylate, (GMA), was obtained from Fluka A.G. (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor, and stored at 4°C until use. Azobisisobutyronitrile, (AIBN), was obtained from Fluka (Switzerland). Poly(vinyl pyrrolidone), (PVP K-30), was obtained from Aldrich Chemical Co. (USA). All other chemicals used were reagent grade from Merck A.G. (Darmstadt, Germany) unless otherwise noted. All water used in the adsorption experiments was purified using a Barnstead (Dubuque, IA, USA) 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. The resulting purified water (deionized water) had a specific conductivity of 18 mS/cm.

3.2. Preparation of Beads

3.2.1. Synthesis of 2-Methacryloylamidotryptophan Monomer

The following experimental procedure was applied for the synthesis of 2-methacryloylamidotryptophan, (MATrp). L-Tryptophan (5.0 g) and NaNO₂ (0.2 g) were dissolved in 30 ml of K₂CO₃ aqueous solution (5%, w/v). This solution was cooled down to 0°C. Methacryloyl chloride (4.0 ml) was slowly poured into this solution under nitrogen atmosphere and this solution was then stirred magnetically at room temperature for 2 h. At the end of this chemical reaction period, the pH of the solution was adjusted to 7.0 and subsequently the solution was extracted with ethyl acetate. The aqueous phase was evaporated in a rotary evaporator. The residue (i.e., MATrp) was crystallized from ether and cyclohexane.

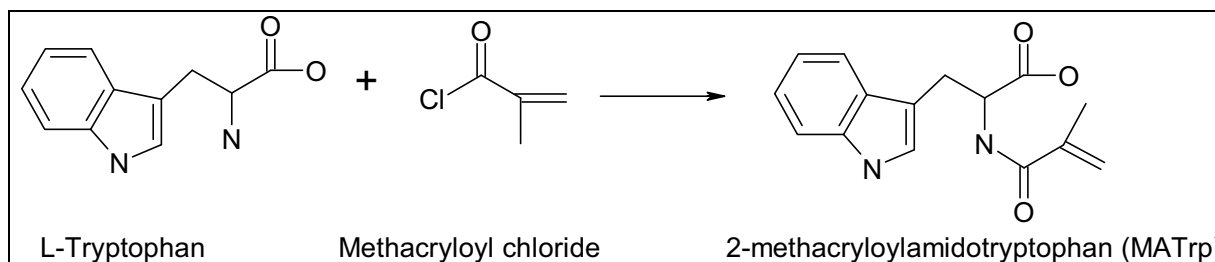


Figure 3.1. Synthesis of 2-methacryloylamidotryptophan (MATrp) monomer.

3.2.2. Preparation of Magnetic Poly(GMATrp) Beads

The dispersion polymerization was performed in a sealed polymerization reactor (volume: 500 ml) equipped with a temperature control system. A typical procedure applied for the dispersion polymerization of GMA and MATrp is given below. The monomer phase was comprised of 10 ml GMA and 1.0 g of MATrp. AIBN was used as the initiator, and 0.25 g was dissolved in the monomer phase. The resulting medium was sonicated for about 5 min at 200 W within an ultrasonic water bath (Bransonic 2200, England) for the complete dissolution of AIBN in the polymerization medium. 4.0 grams of poly(vinyl pyrrolidone) were dissolved in a homogeneous solution of ethanol (100 ml) and water (100 ml) placed in a polymerization reactor. 1.0 g of magnetite (Fe_3O_4 nanopowder) was added to the reactor and reactor content was stirred at 500 rpm during the monomer addition, completed within about 5 min, and the heating was started. Then reactor was purged with bubbling nitrogen for about 5 min. Then, the sealed reactor was placed in a water bath at room temperature. The initial polymerization time was defined when the reactor temperature was raised to 70°C under nitrogen atmosphere for 8 h (stirring rate: 500 rpm). After completion of the polymerization period, the reactor content was cooled down to room temperature and centrifuged at 5000 rpm for 10 min for the removal of dispersion medium. This polymerization reaction led to the formation of pale brown m-poly(GMATrp) beads which were redispersed within 10 ml of ethanol and centrifuged again under similar conditions. The ethanol washing was repeated three times for complete removal of unconverted monomers and other components. Finally, m-poly(GMATrp) beads were redispersed within 10 ml of water (10%, by weight) and stored at room temperature.

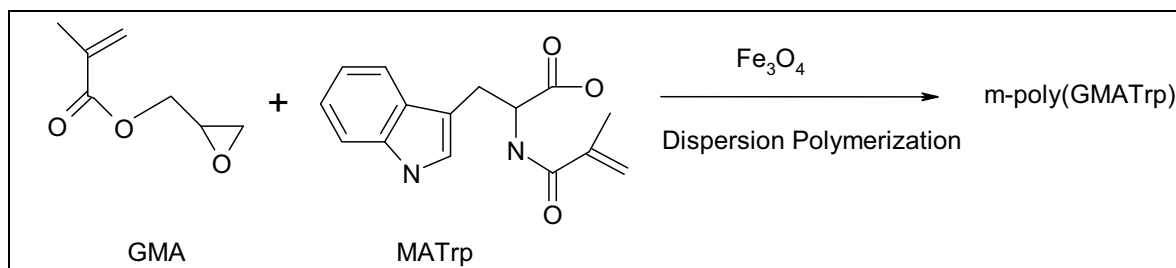


Figure 3.2. Synthesis of polymer beads.

3.3. Characterization of m-poly(GMATrp) Beads

3.3.1. Swelling Tests

The swelling ratio of the the m-poly(GMATrp) beads was determined as follows. Dry beads were weighed (1.0 g) within a cylindrical glass tube (30 cm long and 6 mm in diameter). The height of the unswelled beads was measured. 5 mL of deionized water was added to the tube, the beads were allowed to swell at room temperature for 2 h and then the height of the swollen beads was measured. The swelling ratio was calculated as follows:

$$\text{Water uptake ratio \%} = [(H_s - H_0) / H_0] \times 100 \quad (3.1)$$

where; H_s is the height of the swollen beads (mm) and H_0 is the height of the dry beads (mm).

3.3.2. Surface Morphology and Average Bead Size

The average diameter, bead size distribution, and surface morphology of the iron oxide containing beads were obtained by scanning electron microscopy (SEM; JEOL JSM 1200 EX, Tokyo, Japan), followed by statistical treatment with a computer system. A drop of dilute bead dispersion in ethanol was spread on the glass surface and dried in a dust-free environment at room temperature. The dried sample was then sputtered under vacuum with a thin layer (ca. 4 nm) of gold before viewing under SEM. Bead size and distribution were determined by measuring at least 300 beads. Two types of mean bead size were calculated: number average (D_n) and weight-average (D_w) bead diameters,

$$D_n = \sum D_i N_i / N_i, \quad (3.2)$$

$$D_w = \sum D_i^2 \cdot N_i / \sum D_i N_i \quad (3.3)$$

where; N is the number of beads. Size distribution was characterized by the polydispersity index (PDI) calculated as a ratio of D_w to D_n .

3.3.3. Elemental Analysis

The amount of MATrp incorporation in the m-poly(GMATrp) beads was evaluated by using an elemental analysis instrument, (CHNS-932, Leco, USA) by considering the nitrogen stoichiometry.

3.3.4. FTIR Studies

The characteristic functional groups of the m-poly(GMATrp) beads were analyzed by using a Fourier transform infrared spectrophotometer (FTIR, 8000 Series, Shimadzu, Japan). The beads 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 recorded.

3.3.5. ^1H -NMR Studies

The proton NMR spectra of monomer solution was performed with a JEOL GX-400 instrument (300 MHz). The residual non-deuterated solvent (CHCl_3) served as an internal reference. Chemical shifts are reported in ppm (δ) downfield relative to CHCl_3 .

3.3.6. Analysis of Magnetism

Vibrating-sample magnetometer (Princeton Applied Research, Model 150A, USA) was used to measure the degree of magnetism of the m-poly(GMATrp) beads. The presence of magnetite beads in the polymeric structure was investigated with an electron spin resonance (ESR) spectrophotometer (EL 9, Varian).

3.3.7. Magnetite Nanopowder Release Studies

In order to estimate the amount of magnetite that is likely to leach from the m-poly(GMATrp) beads, 250 mg of the beads was placed in test tubes containing 10 ml of each of the leach media indicated below and were shaken by a rotary shaker for 24 h. The amount of the magnetite leached into each media was determined by a graphite furnace atomic absorption spectrophotometer. Three kinds of leach media were used: 50% (v/v) acetic acid solution (pH 2.0), 50 mM phosphate buffer solution (pH: 7.0) and 50 mM sodium citrate/NaOH buffer solution (pH: 12.0).

3.4. Lyz Adsorption-Desorption Studies

3.4.1. Adsorption of Lyz from Aqueous Solutions

Adsorption of Lyz on the m-poly(GMATrp) beads from aqueous solutions was investigated in batch-wise. The beads (0.05 g) were incubated with 100 ml of the aqueous solutions of Lyz for 2 h (i.e. equilibrium time), in flasks shaken at 150 rpm. Effects of the pH of the medium, Lyz concentration, temperature and ionic strength on the adsorption capacity were studied. pH of the solution was changed between 4.0 and 8.0 to determine the effect of pH on the adsorption. To observe the effects of the initial concentration of Lyz on adsorption, it was changed between 0.25 and 2.0 mg/mL. To observe the effects of the temperature, adsorption studies were carried out between 4 and 37°C. To observe the effects of ionic strength, firstly; the adsorption experiments were carried out in salt free solutions and then, they were repeated in solutions containing NaCl. The concentration of salt was changed between 0.01 and 0.1 M. Samples were withdrawn at suitable time intervals and Lyz concentration was determined by Bradford method at 595 nm (Bradford, 1976). The protein adsorption capacity was calculated from mass balance equation.

$$q = [(C_0 - C) V] / m \quad (3.4)$$

where, q is the amount of Lyz adsorbed onto unit mass of beads (mg/g); C_0 and C are the concentrations of Lyz 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 beads used (g). Each experiment was performed in twice for quality control and statistical purposes.

3.4.2. Desorption and Repeated Use

The desorption of the adsorbed Lyz from the m-poly(GMATrp) beads was studied in a batch system. Lyz adsorbed beads, (0.05 g), were incubated with 10 ml of desorption medium, containing 0.1 M ethylene glycol solution and stirred continuously (at stirring rate 150 rpm) for 1 h at room temperature. The final Lyz concentration in the desorption medium was determined by Bradford method at 595 nm (Bradford, 1976). In order to show the reusability of the beads, adsorption-desorption cycle of Lyz was repeated five times by using the same beads. The desorption ratio for Lyz was calculated as in Eq. 3.5

$$\text{Desorption ratio (\%)} = \frac{\text{Amount of Lyz released}}{\text{Amount of Lyz adsorbed}} \times 100 \quad (3.5)$$

3.4.3. Adsorption-Desorption Studies of Lyz from Aqueous Solution in MSFB

Adsorption of Lyz on the m-poly(GMATrp) beads were studied in magnetically stabilized fluidized bed system (MSFB) by using BioRad economic column (Length: 5 cm, Diameter: 1 cm). m-poly(GMATrp) beads were put into a column equipped with a water jacket for temperature control. During the experiment, the magnetic beads in the column were exposed to magnetic field which surrounded the column ($B_{\text{rms}} \approx 24$ Gauss, $B_{\text{p-p}} \approx 33$ Gauss, $f = 50$ Hz). Equilibration of the column was performed by passing four column volumes of buffer before injection of Lyz solution. Solution in the reservoir shaken magnetically at 150 rpm. The flow of Lyz solution through the column was enabled using an ARITEA (Sweden) peristaltic pump. Experimental setup was shown in Figure 3.3. The solution coming out of the column was gathered in the same flask. Lyz amount in this solution was determined by Bradford method ($\lambda = 595$ nm).

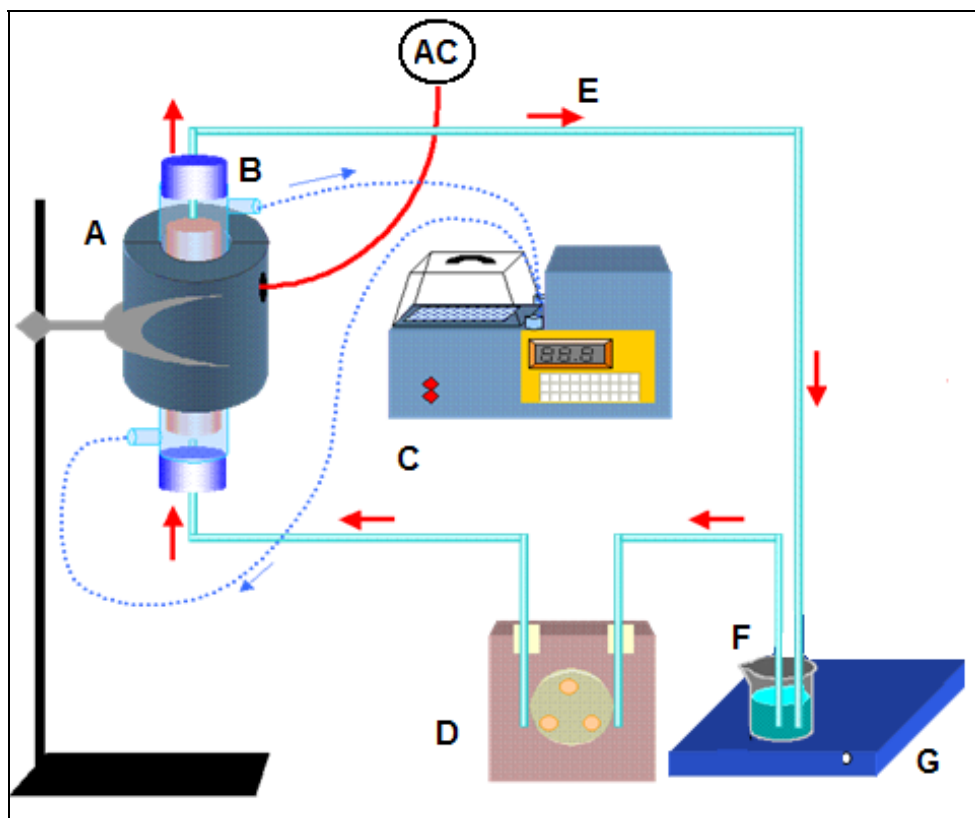


Figure 3.3. Schematic representation of magnetically stabilized fluidized bed. system. A; Bobbin, B; Column with jacket, C; Water circulation system on fixed temperature, D; Peristaltic pump, E; AC Power supply, F; Sample, G; Magnetic stirrer.

Initially, the flow rate of the aqueous solution (i.e. 50 mL of the solution with a Lyz content of 1.0 mg/mL was varied between 1 and 4 mL/min. Later, effect of pH, Lyz concentration, temperature, and ionic strength was studied. To determine the effect of pH on the adsorption, pH of the solution was changed between 4.0 and 8.0. To observe the effects of the initial concentration of Lyz on adsorption, it was changed between 0.25 and 2.0 mg/mL. To investigate the effect of temperature on adsorption, temperature of the system changed from 4 to 37 °C. To observe the effect of ionic strength, the adsorption experiments were carried out in salt free solutions and then, they were repeated in solutions containing NaCl. The concentration of NaCl were changed between 0.01 and 0.1 M. Equilibrium adsorption times and adsorption isotherms were also obtained in the MSFB system. Samples were withdrawn at suitable time intervals and Lyz concentration

was determined. The protein adsorption capacity was calculated from equation 3.4.

3.4.4. Desorption and Repeated Use

In MSFB system, desorption of Lyz from Lyz adsorbed m-poly(GMATrp) beads were performed as follows. Beads, (0.05 g), were placed in 10 ml of desorption medium, containing 0.1 M ethylene glycol solution and stirred continuously (at stringing rate 150 rpm) for 1 h at room temperature. The final Lyz concentration in the desorption medium was determined by Bradford method at 595 nm (Bradford, 1976). The desorption ratio for Lyz was calculated by using the equation 3.5.

3.4.5. Purification of Lyz from Chicken Egg White

A Dionex bio-liquid chromatography system (Dionex Co., Idstein, Germany) was used for the chromatographic purification studies. The BioLC system consisted of a GS50 gradient pump, an LC25 chromatography oven and a PDA-100 photodiode array detector. Chromatographic separation of proteins was achieved on a MN Nucleosil 4000-7 PEI column (125 mm×4.0 mm) protected by a guard column (8.0 mm×4.0 mm). All sample solutions used in chromatographic studies were prefiltered through a syringe membrane filter (0.2 µm Millipore, Bedford, MA, USA) to remove suspended beads and large aggregates. Dionex CHROMELLEION1 software was used and operated under Windows XP Home for data acquisition and integration. BioLC mobile phases A and B were prepared using Trisacetate pH 8.0 (20 mM) buffer and buffer A including 1.5 M KCl solutions, respectively. The mobile phases were filtered prior to use. The chromatographic separation was performed using a linear gradient at 1.0 mL/min flow rate in 5 min from 0 to 10% Eluent B and 10 min from 10 to 40% Eluent B. The sample injection volume was 25 µl. The photodiode array detector was set at 280 nm and the temperature of column was maintained at 25°C.

Chicken egg white was separated from fresh eggs and diluted to 50% (v/v) with phosphate buffer (100 mM, pH 7.0). The diluted egg-white was homogenised in an ice bath and centrifuged at 4°C, at 5000 rpm for 30 min. m-poly(GMATrp) beads (100 mg) were incubated with 10 ml of diluted egg-white solution for 2 h at 150

rpm. m-poly(GMATrp) beads were washed and centrifuged to remove unbound proteins.

The purity of Lyz in the purified samples was assayed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% separating gel (9 cm×7.5 cm) and 6% stacking 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). Electrophoresis was run for 2 h with a voltage of 110V.

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 per minute at 25°C.

4. RESULTS AND DISCUSSION

It is becoming increasingly evident that there is still considerable scope for technological progress in the production of biological materials. Thus, in the production of a large proportion of diagnostic and therapeutic proteins, it has been shown repeatedly that the isolation and purification steps of the targeted molecule often prove to be the most problematic and expensive step (Owen and Chase, 1997). These frequently account for up to 80% of the total cost production. To counter these difficulties, a number of innovations have been proposed recently by scientists in the area of chromatographic matrix design. Affinity adsorbents with large specific surface areas for adsorption are desirable to attain high binding capacity (Ma et.al., 2005). To increase the surface area to the practically useful level of 100 m²/g, either highly porous materials with large pore size or micronsize beads have to be employed. Due to these reasons, non-porous affinity adsorbents of small bead diameter have been gaining more attention for the rapid ,high performance liquid chromatography of proteins (Altıntaş and Denizli, 2006). A major advantage of the monosize beads is that significant intrabead diffusion resistance is absent; this is particularly useful for the rapid analysis of proteins with high efficiency and resolution (Takafuji et.al., 2004). The rapid separation makes it very useful for quality control, on-line monitoring and purity check of biomolecules such as peptide mapping of recombinant products (Yamasaki et.al., 1989). The aim of this thesis is to find a cost effective and reusable magnetic and hydrophobic affinity beads having high adsorption capacity for purification of Lyz.

Incorporation of amino acids into polymer matrix has been gaining much attention in affinity separation (Denizli et. al., 2004, Uzun et. al., 2005, Karataş et.al., 2007). Aminoacids that are used as pseudospecific ligands have certain advantages. They are more stable than bioligands because they do not require a specific tertiary structure for maintaining their activities. Leakage of aminoacids into the medium does not occur, since they are incorporated into the main polymer backbone. Aminoacids are very reactive with different chemical substances. The higher flexibility and durability of these ligands as well as significantly lower material and manufacturing costs are also very important (Vijayalakshmi, 1998).

In this thesis, a novel hydrophobic tryptophan containing magnetic monosize poly(GMATrp) beads were prepared successfully by single step dispersion polymerization as affinity sorbents for efficient separation of Lyz from aqueous solutions in batch and magnetically stabilized fluidized bed (MSFB) system. Effect of parameters (pH, Lyz concentration, temperature, and ionic strength) on Lyz adsorption capacity of m-poly(GMATrp) was investigated.

4.1. Characterization of m-poly(GMATrp) Beads

4.1.1. Swelling Test

The m-poly(GMATrp) beads are hydrophobic. They remain insoluble and preserve their dimensional shape. The equilibrium swelling ratios of beads are calculated as 52% and 45%, for the m-poly(GMA) and m-poly(GMATrp) beads, respectively. The water uptake ratio of the beads is lower than m-poly(GMA) beads. This may be due to the inclusion of MATrp comonomer into the polymer structure. Since MATrp is hydrophobic, resulting copolymer is more hydrophobic than pure m-poly(GMA) beads. Therefore, water molecules may not penetrate into the polymer chains easily. This may cause a decrease in water uptake behavior and equilibrium swelling ratio.

4.1.2. Surface Morphology and Bead Size

AIBN-initiated radical dispersion polymerization of GMA and MATrp was performed in an alcoholic dispersion medium in the presence of a magnetic pigment (iron oxide). The surface morphology of m-poly(GMATrp) beads was shown by the scanning electron micrograph in Figure 4.1. Bead size and the polydispersity index (PDI) of the beads were calculated as 1.6 μm , and 1.008, respectively. Polymer beads are highly uniform in size, and non-porous. A major advantage of the non-porous adsorbents is that significant intrabead diffusion resistance is absent; this is particularly useful for the rapid separation of Lyz. This also provides higher Lyz adsorption capacity.

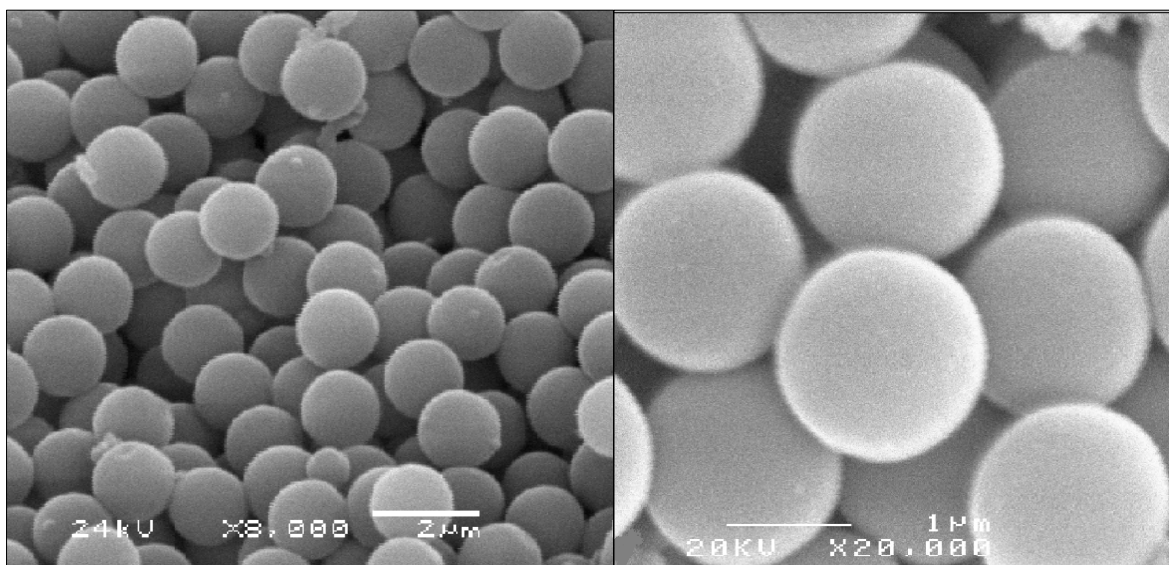


Figure 4.1. Scanning electron micrograph of m-poly(GMATrp) beads.

4.1.3. Elemental Analysis

Degree of incorporation of tryptophan containing comonomer, MATrp, into polymer structure was calculated from elemental analysis of the synthesized m-poly(GMATrp) beads. The incorporation of the MATrp into the polymeric structure was calculated as 30 μmol MATrp/g polymer from the nitrogen stoichiometry.

4.1.4. FTIR Studies

A hydrophobic amino acid containing MATrp was used as the comonomer and pseudospecific ligand for the selective separation of Lyz from aqueous solutions. Initially, MATrp was synthesized from L-tryptophan and methacryloyl chloride. Then, AIBN initiated dispersion polymerization was performed to obtain m-poly(GMATrp) beads. The molecular formula of beads is shown in Figure 4.2.

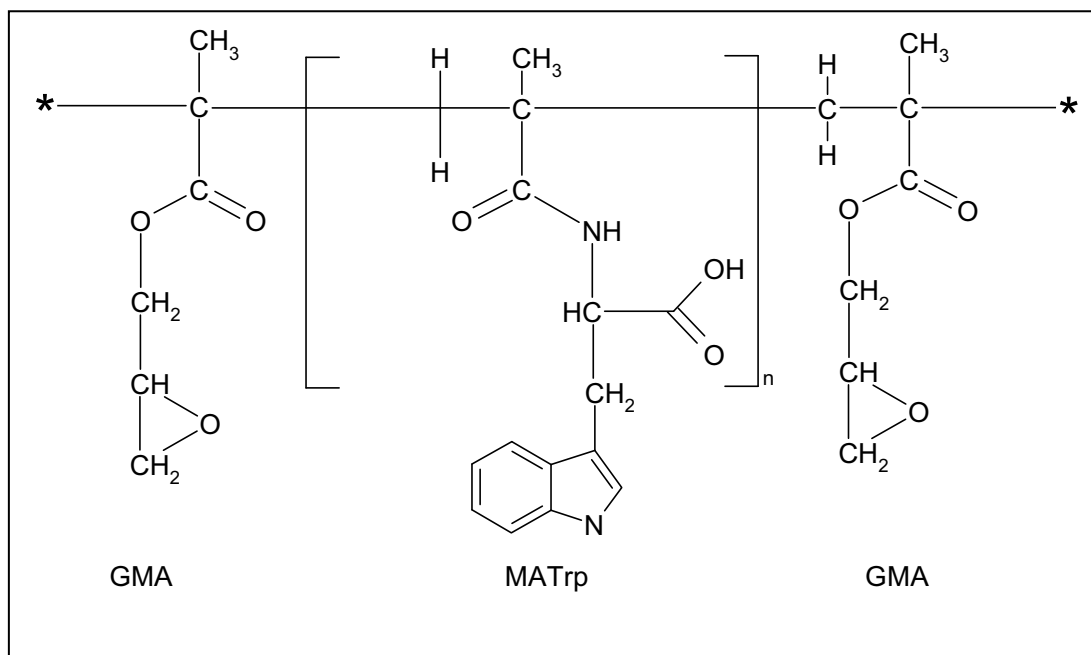


Figure 4.2. The molecular formula of m-poly(GMATrp) beads.

FTIR spectra of the m-poly(GMA), MATrp and m-poly(GMATrp) beads are given below. In Figure 4.3 (A), that belongs to m-poly(GMA), broad peak around 3500 cm^{-1} is due to -OH stretching vibration. As shown in figure, one of the characteristic vibrations of m-poly(GMA) is the methylene vibration at $2950\text{-}3000\text{ cm}^{-1}$. The vibration at 1740 cm^{-1} represents the ester configuration of GMA. The epoxy ring gives bands at 850 and 910 cm^{-1} , and these may be considered as an indication of presence of oxirane groups in the structure.

The absorption bands of the functional groups of MATrp can be seen in Figure.4.3 (B). 3300 and 3500 cm^{-1} absorption bands are due to N-H stretching. Absorption bands at $3100\text{-}3000\text{ cm}^{-1}$ belong to aromatic C-H stretching. C-H stretching band of alkyl group is at $3000\text{-}2800\text{ cm}^{-1}$. C=O stretching band of acid is at 1715 cm^{-1} , and C=C stretching band is at $1600\text{-}1670\text{ cm}^{-1}$. The peaks at $1370\text{-}1250\text{ cm}^{-1}$ and $1320\text{-}1210\text{ cm}^{-1}$ correspond to aromatic C-N stretching and C=O stretching of acid, respectively.

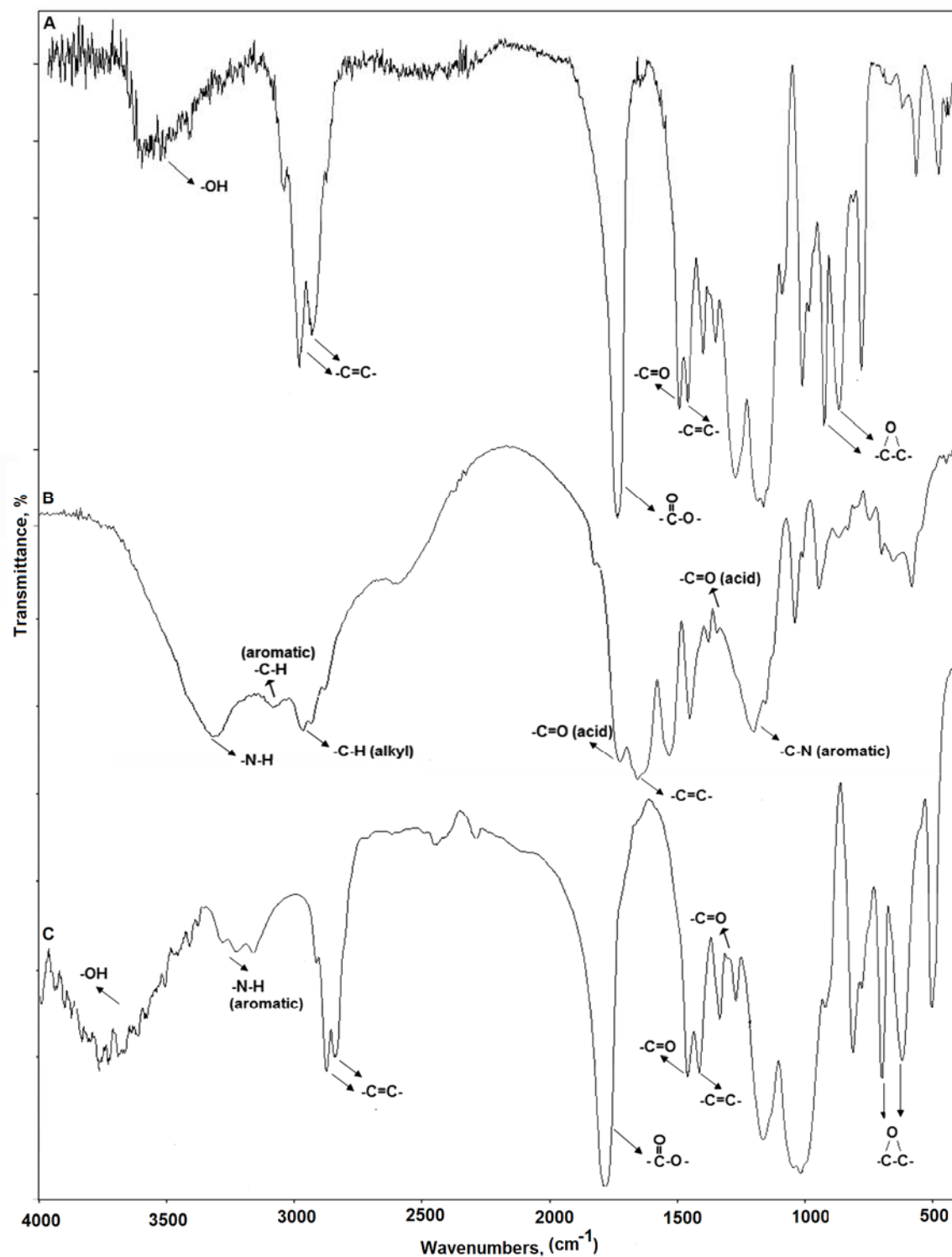


Figure 4.3. FTIR spectra of (A) m-poly(GMA) beads, (B) MATrp monomer and (C) m-poly(GMATrp) beads.

FTIR spectrum of m-poly(GMATrp) polymer was shown in Figure 4.3 (C). As seen in the figure, polymer beads have the characteristic peaks of poly(GMA) and MATrp. Stretching vibration of -OH gives a broad peak 4000- 3500 cm^{-1} . Peaks at

3300-3500 cm^{-1} and 2950-3000 cm^{-1} are due to N-H stretching and methylene vibration, respectively. The vibration at 1728 cm^{-1} represents the ester configuration. C=O stretching band of acid is at 1481 cm^{-1} , and C=C stretching is at 1451 cm^{-1} . Peaks at 850 and 910 cm^{-1} correspond to the epoxy ring of the structure.

4.1.5. ^1H -NMR Studies

^1H -NMR method was used to examine structure of MATrp monomer. Figure 4.4 shows the characteristic peaks of MATrp monomer. Characteristic peaks are as follows: Peaks at 1.86-1.88 ppm belong to CH_3 protons splitted by CH_2 protons. Doublet at 5.29 ppm belongs to $^{16}\text{CH}_2$, and 5.98 ppm at belongs to ^{11}CH . Aromatic ring CH and NH protons are seen at 7-8 ppm.

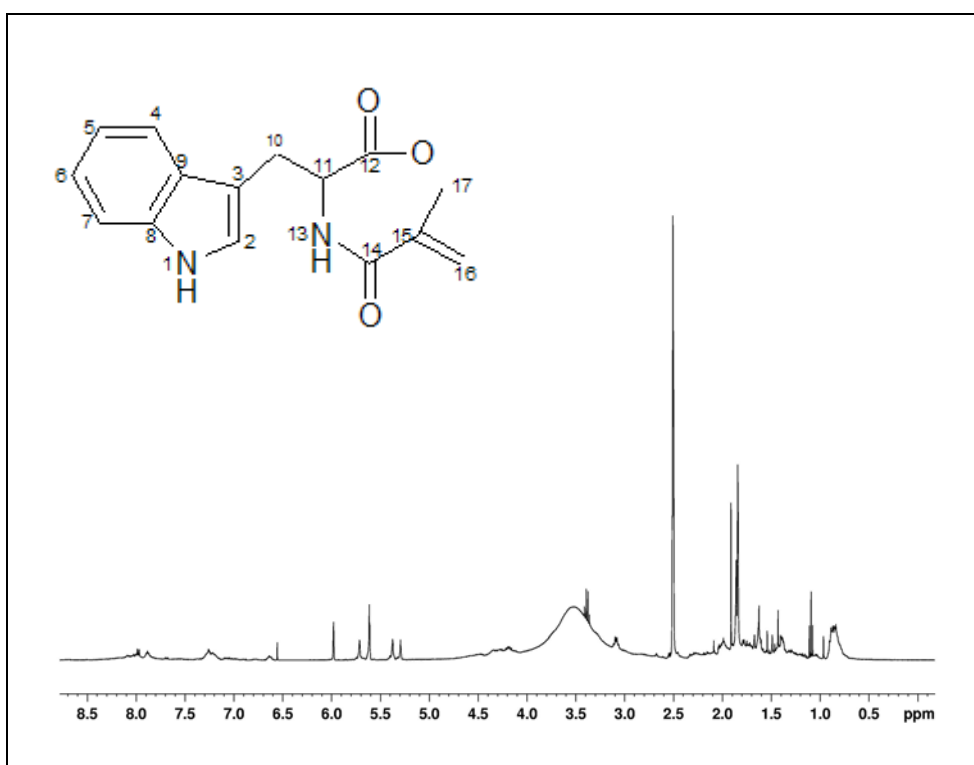


Figure 4.4. ^1H -NMR spectrum of MATrp monomer.

4.1.6. Magnetic Properties

Magnetic poly(GMATrp) beads with core-shell structure, where Fe_3O_4 nanopowder is the magnetic core and GMATrp is the shell, were obtained by dispersion polymerization. Magnetization is an important parameter for the practical

application of magnetic beads. Magnetic behavior of magnetic materials is usually related to content of magnetic component inside. Thus, the amount of the Fe_3O_4 nanopowder content in a matrix is an essential determinant for its magnetic susceptibility to magnetic fields. In general, as the Fe_3O_4 content of a matrix increases, the magnetic property of it increases (Lu et. al., 2006). To assess the magnetic reactivity of the beads, the average Fe_3O_4 content in the m-poly(GMATrp) beads was quantified by utilizing the gel volume fraction (ϕ) of the beads determined by following equation (Tong and Sun, 2001).

$$\phi = (\rho_C - \rho_M) / (\rho_C - \rho_A) \quad (4.1)$$

where, ρ_A is the hydrated density of the non-magnetic poly(GMATrp) beads, 1.08 g/ml measured by a pycnometer at 25°C, ρ_C is the density of the Fe_3O_4 nanopowder, 1.97 g/ml determined by the same procedure at 25°C, and ρ_M is hydrated density of the m-poly(GMATrp) beads, 1.14 g/ml at 25°C determined by again pycnometric analysis. Thus with the above density data, the m-poly(GMATrp) gel volume fraction in the magnetic beads was estimated to be 0.932. In otherwords, the volume fraction of magnetite nano-powder in the the m-poly(GMATrp) beads was 6.8%.

The presence of magnetite nanopowder in the polymer structure was verified with the relative intensity value of 60 in ESR spectrum. The intensity of the magnetite peak against magnetic field in Gauss indicates the presence of the magnetite nanopowder in the polymer structure is shown in Figure 4.5. It should be noted here that such magnetization was not detected with non-magnetic poly(GMA) beads by ESR spectroscopy. The g factor which is a dimensionless constant given in Figure 4.5 is a magnetic quantity for the unpaired electrons on magnetic materials, and is computed by following equation. In the literature, the g factor for Fe^{+3} is determined between 1.4-3.1 for low spin and 2.0-9.7 for high spin complexes (Swartz et.al., 1972) In this study, the g factor was found to be 2.28 for m-poly(GMATrp) beads by using Equation 4. 2.

$$\Delta E = h. \nu = g. \beta. B \quad (4.2)$$

where, ΔE is the energy difference between the two spin states, h is the Planck constant (6.626×10^{-27} erg/sec), β is the unit magnetic moment of the spinning electron called the Bohr magneton constant (9.273×10^{-21} erg per gauss per particle), ν and B are the microwave frequency (9.707×10^9 Hz) and applied magnetic field (Gauss), respectively.

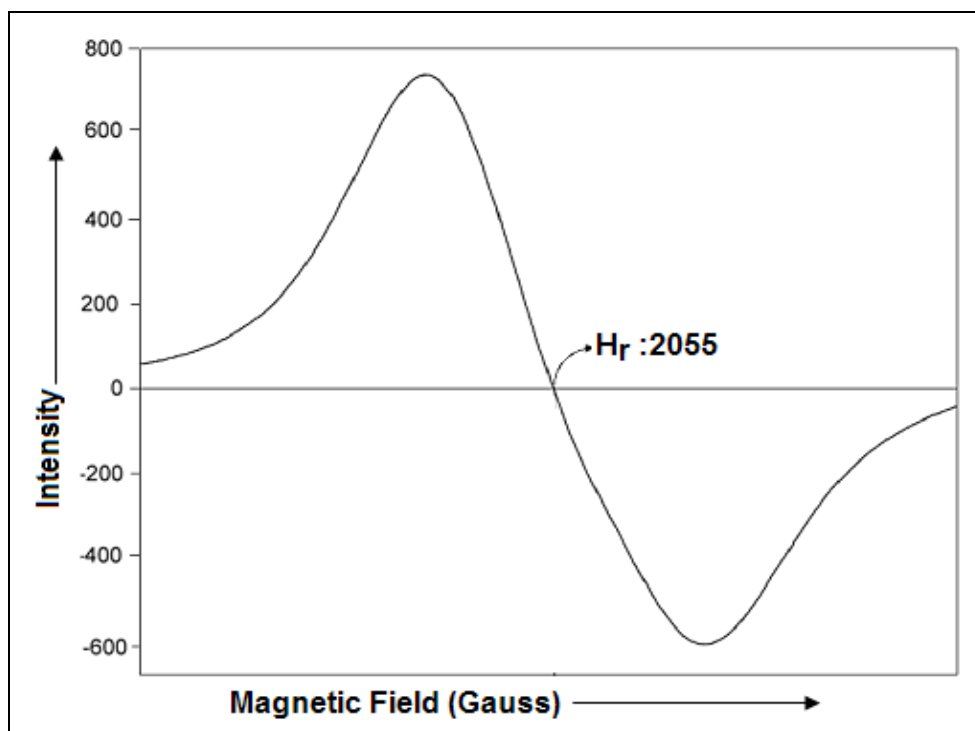


Figure 4.5. ESR spectrum of of m- poly(GMATrp) beads.

The g value is a magnitude specific to a magnetic material and is also a magnetic reference to evaluate the sustainable magnetic strength of the magnetic material in the absence of the external magnetic field applied. The sustainable magnetic strength of the m-poly(GMATrp) beads was tested in distilled water and ambient air and found to remain steady at least for three months by ESR spectroscopy.

The magnetic strength limits of the polymer beads was further determined by plotting their electron mass units (EMU) versus the magnetic field swept from 0 to 8000 Gauss (applied via a vibrating magnetometer) as shown magnetometer, in Figure 4.6, where H_r is defined as the external magnetic field on resonance with the magnetic strength of the beads. In EMU spectrum and from H_r value, 3055 Gauss magnetic field was found sufficient to excite all of the dipole moments

present in 1.0 g of m-poly(GMATrp) bead sample. This value will be an important design parameter for a magnetically stabilized fluidized bed or magnetic filtration using these magnetic beads. The value of this magnetic field is a function of the flow velocity and bead size. In the literature, this value was found to change from 8 kG to 20 kG for various applications, thus magnetic beads presented in this study will need less magnetic intensity in a magnetically stabilized fluidized bed or for a magnetic filter system (Rad et al., 2003). In EMU spectrum and from effective magnetic field value (H_r), 3055 Gauss magnetic field was found sufficient. This would be an important design parameter for a MSFB.

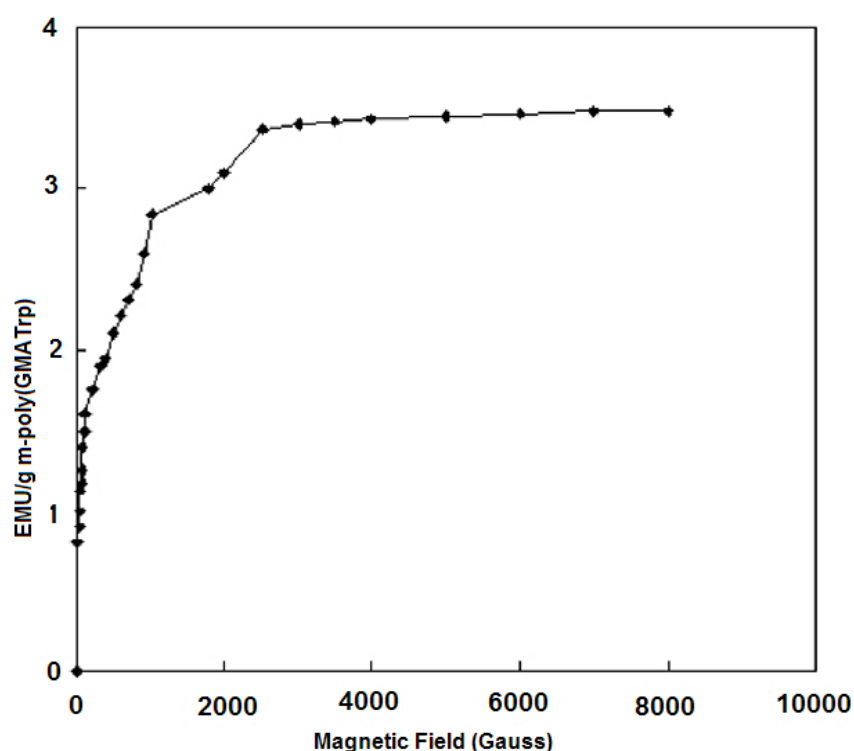


Figure 4.6. The magnetic behavior of m- poly(GMATrp) beads.

4.1.7. Magnetic Release Studies

To investigate the magnetite release from the polymeric structure to the release medium was performed by using different kinds of media including acetic acid solution (50%, v/v, pH: 2.0), phosphate buffer solution (pH: 7.0) and sodium citrate/NaOH buffer solution (pH: 12.0) Any measurable release was not observed.

Some properties of m-poly(GMATrp) beads were determined and given in Table 4.1.

Table 4.1. Some properties of the monosize m-poly(GMATrp) beads.

Bead diameter	1.62±0.01µm
Polydispersity index	1.008
Swelling ratio	45%
Wet density	1.14 g/mL
Fe ₃ O ₄ incorporation	6.8%
Resonance of magnetic field	3055 Gauss
g factor	2.28
MATrp content	30 µmol/g

4.2. Adsorption of Lyz from Aqueous Solutions

4.2.1. Effect of pH

The amount of Lyz adsorbed onto the m-poly(GMATrp) beads as a function of pH was displayed in Figure 4.7. Lyz is a basic protein with an isoelectric point 10.7 and a molecular weight of 14.3 kDa. m-poly(GMATrp) beads show a maximum adsorption capacity, 203.7 mg/g, at pH 7.0. Adsorption capacity of magnetic beads decreased significantly at lower and higher pH values. Lyz is highly positively charged at pH 7.0 since its isoelectric point is 10.7. The net charge of a protein is zero at its isoelectronic point, however depending on its amino acid composition it has charged groups on its surface, 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 pl value. On the basis of its surface accessible residues, Lyz molecule has one histidine, four aspartic acid, two glutamic acid and two tyrosine residues. These amino acid side chains of Lyz molecules may cause other type of interactions with low percentage according to the hydrophobic interactions.

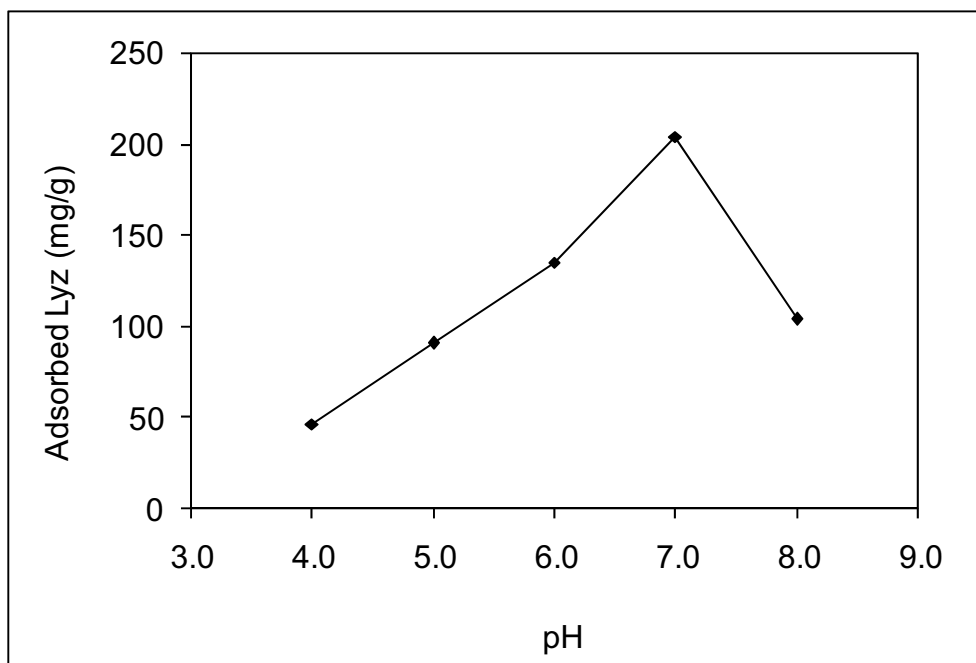


Figure 4.7. Effect of pH on Lyz adsorption. Lyz concentration: 1.0 mg/mL; incubation time: 2 h; T: 25°C.

4.2.2. Effect of Lyz Concentration

Effect of initial Lyz concentration on adsorption is shown in Figure 4.8. Lyz adsorption capacity of plain poly(GMA) beads is 1.6 mg/g. In affinity chromatography, the non-specific interaction between the support, m-poly(GMA), and the molecules to be adsorbed, Lyz, should be minimum. There are no reactive binding groups or sites onto m-poly(GMA) which interact with Lyz molecules. Hence, this non-specific adsorption may be due to weak interactions (van der Waals interaction, and hydrogen binding) between Lyz and epoxy groups on the surface of m-poly(GMA) beads.

When the aminoacid based comonomer, MATrp, was included into the polymeric structure, Lyz adsorption capacity of the monosize magnetic beads significantly increased up to 255.3 mg/g. The results showed that incorporation of hydrophobic aminoacid into the structure show higher binding capacity for lysozyme than that of the control. As the initial concentration of Lyz increased, the amount of adsorbed Lyz increased. The steep slope of the initial part of the adsorption isotherm represented a high affinity between Lyz and incorporated MATrp groups. When all the available and accessible sites of polymeric structure were occupied by Lyz,

adsorption value reaches a plateau which represents saturation of the active adsorption sites on the magnetic monosize beads. Increase in the Lyz adsorption capacity of beads after the inclusion of MATrp may be attributed to the hydrophobic interactions since the polymer beads have aromatic aminoacid comonomer that prefer to interact with hydrophobic groups in Lyz structure.

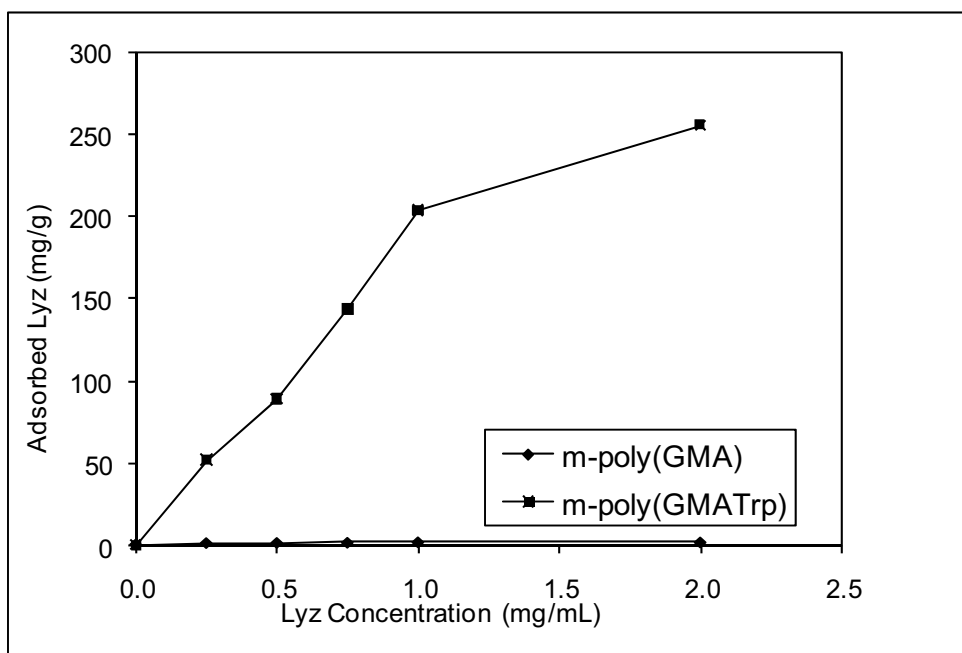


Figure 4.8. Effect of Lyz concentration on adsorption. pH: 7.0; incubation time: 2 h; T: 25°C.

4.2.3. Effect of Time on Adsorption

Effect of time on Lyz adsorption was investigated as a decrease of Lyz concentration with time. Faster adsorption rates were observed at the beginning of adsorption process, and then adsorption equilibrium was achieved in about 60 min. Also, adsorption rates increased with increasing Lyz concentration. The difference of Lyz concentration between the liquid (i.e., the protein solution) and solid phase (beads) is named as driving force. As the Lyz concentration in solution increases, the driving force increases too. High adsorption rates at high Lyz concentrations may be due to high driving force for mass transfer from liquid to solid phase. The highest adsorbed Lyz amount was calculated at 255.3 mg/g.

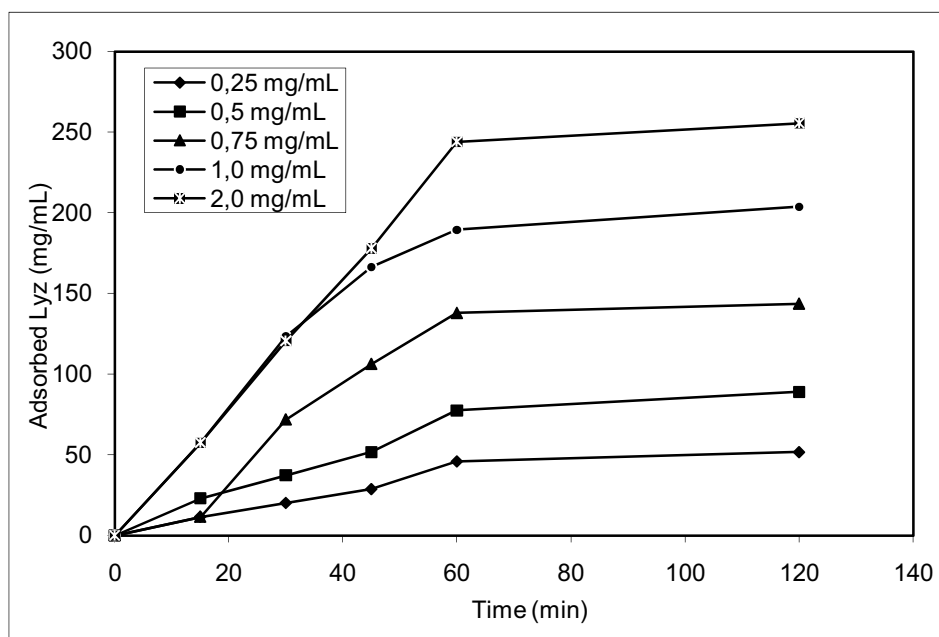


Figure 4.9. Effect of time on adsorption pH: 7.0; incubation time: 2 h; T: 25°C.

4.2.4. Effect of Ionic Strength

The effect of NaCl concentration on Lyz adsorption is presented in Figure 4.10 which shows that the adsorption capacity increases with increasing ionic strength. Hydrophobic adsorption is enhanced by adding sodium chloride to the buffer system. The influence of different salts on hydrophobic interactions follows the Hofmeister (lyotropic) series for the precipitation of proteins from aqueous solutions (Roe, 1989). As shown in Figure 4.10, Lyz adsorption capacity increased with increasing salt concentration. The adsorption amount of Lyz increased from 203.7 to 223.8 mg/g as the NaCl concentration increases from 0 to 0.1 M. Increasing the NaCl concentration promotes ligand-protein interactions and adsorption of the Lyz molecules to the polymer surface by hydrophobic interaction. As the ionic strength of the medium increases, the amount of the adsorbed protein increases and continues to increase in an exponential manner at higher concentrations (Porath, 1990).

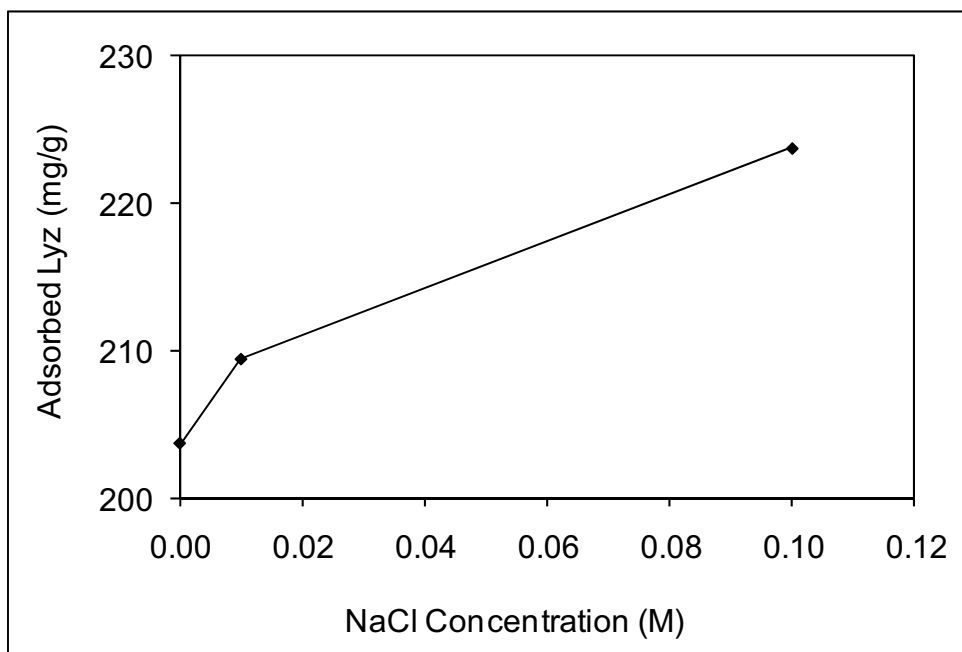


Figure 4.10. Effect of ionic strength on Lyz adsorption. Lyz concentration: 1.0 mg/mL; pH: 7.0; incubation time: 2 h; T: 25°C.

4.2.5. Effect of Temperature

The effect of temperature on Lyz adsorption capacity of the magnetic monosize beads is given in Figure 4.11. The equilibrium adsorption of Lyz onto m-poly(GMATrp) beads significantly increased with increasing temperature and the maximum Lyz adsorption was achieved as 232.4 mg/g at 37°C. In HIC, increasing the temperature enhances protein adsorption and lowering the temperature generally promotes elution (Hjerten et. al., 1986). Hydrophobic interaction chromatography is an entropy-driven process. When ΔH may be a small positive or negative value, ΔG is controlled by positive entropy change and thus increases with temperature ($\Delta G = \Delta H - T\Delta S$).

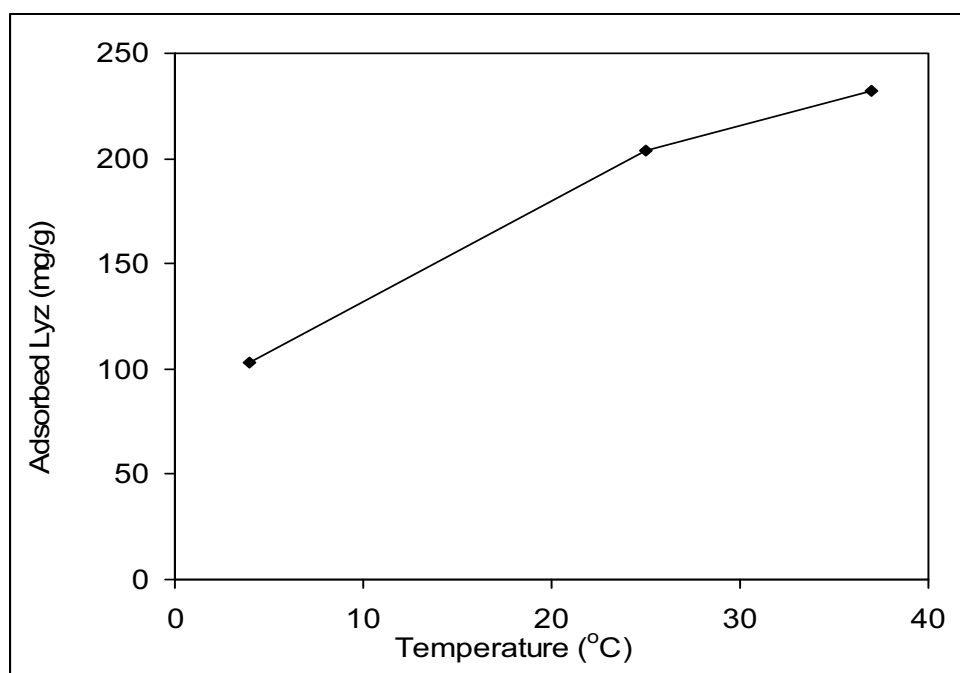


Figure 4.11. Effect of temperature on Lyz adsorption. Lyz concentration: 1.0 mg/mL; pH: 7.0; incubation time: 2 h, T: 25°C.

4.2.6. Adsorption Isotherms

The process of binding molecules to the surface is called adsorption. Desorption is the removal of adsorbed molecules from the surface. There are two types of adsorption. These are physical and chemical adsorption. Generally, a molecule is physically adsorbed if the bond energy is less than 10 kcal/mole, and chemically adsorbed if bond energy is more than 10 kcal/mole. In chemisorption, a direct chemical bond is involved (surface specific) while van der Waals forces are responsible for physical bonding (not surface specific). In this study, adsorption of Lyz on m-poly(GMATrp) beads was studied. This is a solid-liquid adsorption process. Adsorption isotherm is used to characterize the interactions of each molecule with the adsorbent. An adsorption isotherm makes 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. Experimental isotherms are used to describe adsorption capacity of the process for a given application, select the most appropriate adsorbent and determine adsorbent

amount that is required. Additional use of adsorption isotherms is for theoretical evaluation of thermodynamic parameters, such as heats of adsorption. Several equilibrium models have been developed to describe adsorption isotherm relationship.

Langmuir adsorption isotherm is derived on the following assumptions; only monolayer adsorption takes place at a fixed number of energetically equivalent well-defined sites, adsorption is localized so that there are no interactions between molecules adsorbed on adjacent sites. The heat of adsorption is independent of surface coverage. There is no lateral interaction between adsorbed molecules. All adsorption sites have the same affinity for the protein molecule.

During the batch experiments, adsorption isotherms are used to evaluate adsorption properties of Lyz on m-poly(GMATrp) beads. Langmuir adsorption isotherm of Lyz is shown in the Figure 4.12.

The Langmuir adsorption isotherm is expressed by Equation 4.3. The corresponding transformations of the equilibrium data for Lyz give a linear plot. This indicates that the Langmuir model can be applied in these systems and described by the equation:

$$Q = Q_{\max} \cdot b \cdot C_{\text{eq}} / (1 + bC_{\text{eq}}) \quad (4.3)$$

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

$$C_{\text{eq}}/Q = 1/(Q_{\max} \cdot b) + C_{\text{eq}}/Q_{\max} \quad (4.4)$$

C_{eq} versus C_{eq}/Q was plotted. Intercept and the slope were calculated from $1/Q_{\max} \cdot b$ and $1/Q_{\max}$, respectively, in Figure 4.12.

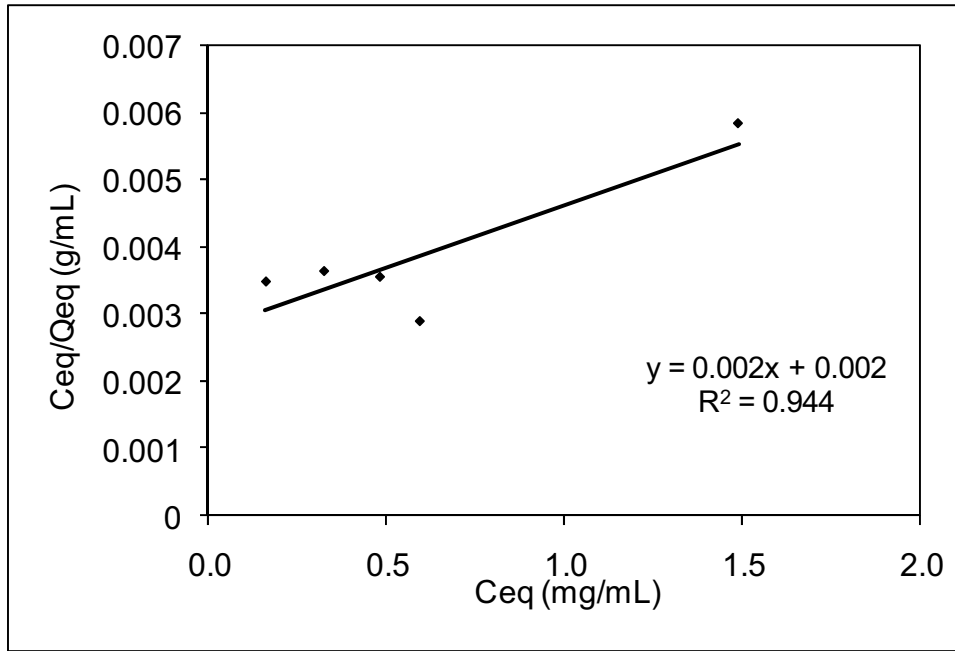


Figure 4.12. Langmuir adsorption isotherm of Lyz adsorption. Lyz concentration : 1.0 mg/mL; pH: 7.0; T: 25°C.

$Q_{\max}$, the maximum adsorption capacity for the adsorption of Lyz, was obtained from the experimental data. The correlation coefficient (R^2) was 0.944 at 25°C. Maximum adsorption capacity ($Q_{\max}$) and the Langmuir constants were found to be 454.5 mg/g and 1.1 mL/mg respectively.

Freundlich isotherm is also frequently used to describe adsorption behaviour. This isotherm is another form of the Langmuir approach for adsorption on a heterogeneous surface. The Freundlich isotherm describes reversible adsorption and is not restricted to the formation of the monolayer adsorption. The amount of adsorbed protein is the summation of adsorption on all binding sites. This empirical is given in Eq.4.5.

$$Q_{\text{eq}} = K_F (C_{\text{eq}})^n \quad (4.5)$$

where, K_F is the Freundlich constant, n is the Freundlich exponent. $1/n$ is the measure of the surface heterogeneity and ranges from 0 and 1, becomes more heterogeneous as its value approaches zero. The value of Q_{eq} gives the theoretical monolayer saturation capacity of monosize beads.

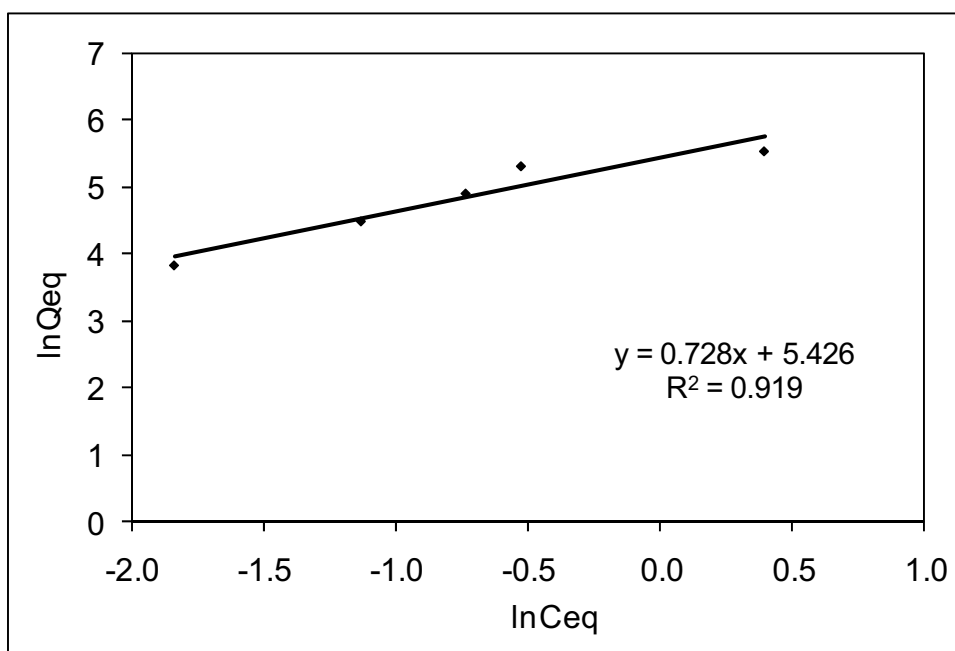


Figure 4.13. Freundlich adsorption isotherm of Lyz adsorption. Lyz concentration: 1.0 mg/mL; pH: 7.0; T: 25°C.

Adsorption isotherm graphs of Lyz were plotted for different Lyz concentrations. It was observed that the isotherms are linear and correlation coefficients are high. According to the correlation coefficients of isotherms, Langmuir adsorption model is favorable. Table 4.2 shows the Freundlich adsorption isotherm constants, n and K_F and the correlation coefficients. The magnitude of K_F and n values of Freundlich model shows easy uptake of Lyz from aqueous medium with a high adsorption capacity of the m-poly(GMATrp) beads.

Table 4.2. Isotherm constants for Langmuir and Freundlich adsorption models and correlation coefficients for Lyz.

Isotherm Constants			
Langmuir		Freundlich	
Q_{eq}	454.5	K_F	225.9
b	1.1	$1/n$	0.728
R^2	0.944	R^2	0.919

Thermodynamic parameters such as free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) changes for the process can be estimated using the Eq.2.1 ($\Delta G = \Delta H - T\Delta S$). Free energy change, (ΔG), calculated from the experimental data at 25⁰C is -0.235 kJ/mol. The negative change in free energy indicates that the adsorption of Lyz on m-poly(GMATrp) beads is a thermodynamically favorable process.

4.2.7. Adsorption Kinetics Modelling

Kinetic model is used to examine the controlling mechanism of adsorption process such as mass transfer and chemical reaction. Kinetic models are used to test experimental data. Pseudo-first- and second-order equations are the kinetic models, and can be used to correlate that measured concentrations are equal to adsorbent surface concentrations. Lagergren first-order rate equation is widely used for the adsorption of solute from a liquid solution (Cheung et al., 2001). It may be represented as follows:

$$\Delta q_t / dt = k_1 (q_{eq} - q_t) \quad (4.6)$$

where; k_1 is the rate constant of pseudo-first order adsorption (min^{-1}) and q_{eq} and q_t are 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,

$$\log [q_{eq}/(q_{eq}-q_t)] = (k_1 t)/2.303 \quad (4.7)$$

To obtain a linear form, Eq. 4.7 can be rearranged,

$$\log(q_{eq}-q_t) = \log(q_{eq}) - (k_1 t)/2.303 \quad (4.8)$$

When $\log(q_{eq})$ versus t is plotted, line must be straight 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 $\log(q_{eq}-q_t)$ versus t .

Pseudo-second order equation based on adsorption equilibrium capacity may be expressed in the form,

$$\Delta q_t / dt = k_2 (q_{eq} - q_t)^2 \quad (4.9)$$

where; k_2 ($g\ mg^{-1}\ min^{-1}$) is the rate constant of pseudo-second order adsorption process. Integrating Eq. 4.9 and applying the boundary conditions, $q_t=0$ at $t=0$, and $q_t=q_t$ at $t=t$, leads to,

$$[1/(q_{eq}-q_t)] = (1/q_{eq}) + k_2 t \quad (4.10)$$

or equivalently for linear form

$$(t/q_t) = (1/k_2 q_{eq}^2) + (1/q_{eq}) t \quad (4.11)$$

a plot of t/q_t versus t gives a linear relationship in the second-order kinetics. The rate constant (k_2) and adsorption at equilibrium (q_{eq}) can be obtained from the intercept and slope, respectively.

Pseudo-first and pseudo-second order kinetics of the experimental data for the m-poly(GMATrp) are given in Figure 4.14 and 4.15, respectively. A comparison of the experimental adsorption capacity and the theoretical values are displayed in Table 4.3. The theoretical Q_{eq} value estimated from pseudo-first-order kinetics is close to experimental Q_{eq} value. Also, correlation coefficients are higher than pseudo-second-order kinetics. Results indicate that this m-poly(GMATrp) beads much more obeyed the first order kinetic model and adsorption reaction is chemically controlled.

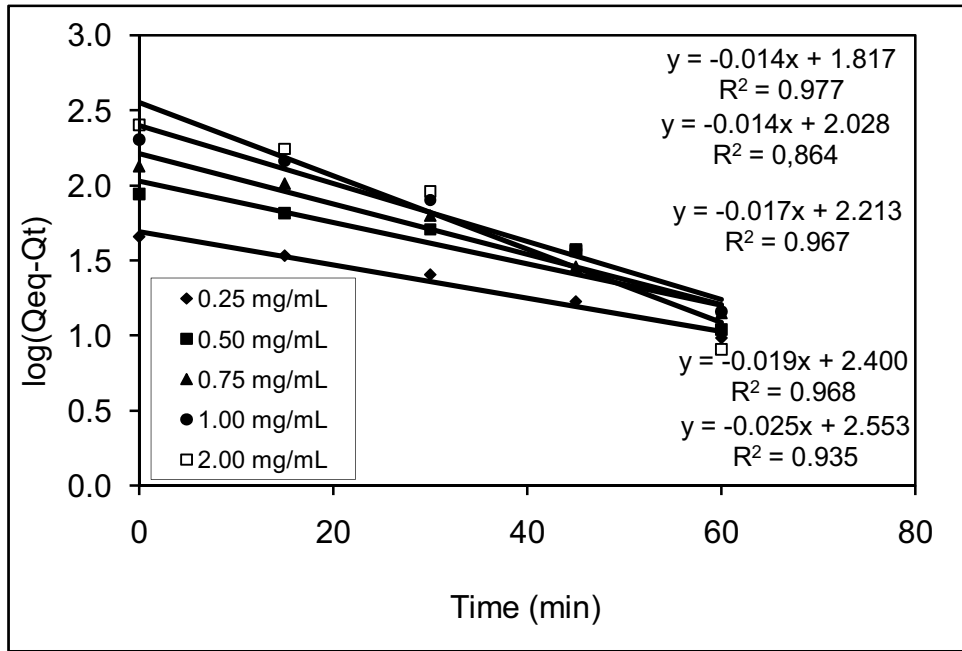


Figure 4.14. Pseudo first-order kinetic of the experimental data for the adsorbent.

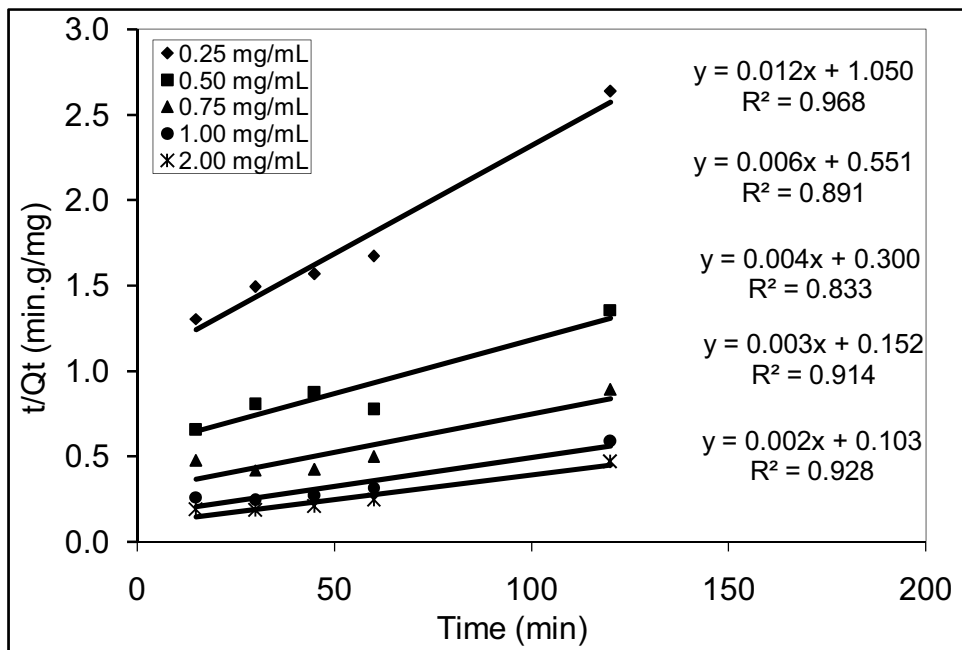


Figure 4.15. Pseudo second-order kinetic of the experimental data for the adsorbent.

Table 4.3. The first- and second-order kinetic constants for adsorbent.

C_o (mg/mL)	Experimental	1 st order			2 nd order		
	Q_{eq}	K_1	Q_{eq}	R^2	K_2	Q_{eq}	R^2
0.25	51.6	0.032	65.6	0.977	0.0000734	111.1	0.968
0.5	88.9	0.030	106.4	0.864	0.0000635	166.7	0.891
0.75	143.4	0.048	213.8	0.967	0.0000491	256.4	0.833
1.0	203.7	0.044	251.8	0.968	0.0000592	333.3	0.914
2.0	255.3	0.046	376.7	0.935	0.0000222	500.0	0.928

4.2.8. Desorption and Reusability of Adsorbents

Lyz adsorption-desorption procedure was repeated five times by using the same beads. 0.1 M ethylene glycol solution was used for the desorption of the m-poly(GMATrp) beads. The non-polar parts of ethylene glycol compete effectively with the bound proteins for the adsorption sites on of m-poly(GMATrp) beads, resulting in the displacement of the latter. Also, it decreases the surface tension of water thus weakening the hydrophobic interactions to give a subsequent dissociation of the ligand-solute complex.

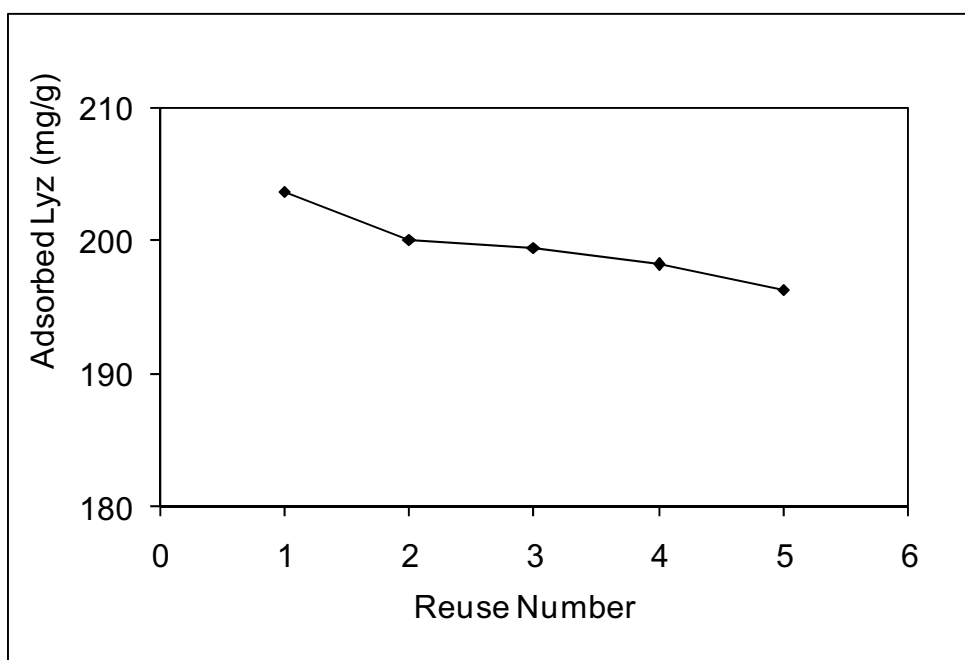


Figure 4.16. Reusability of the magnetic beads. Lyz concentration: 1.0 mg/mL; pH: 7.0; incubation time: 1 h; T: 25°C.

At the end of five adsorption-desorption cycle, there was no remarkable reduce in the adsorption capacity of m-poly(GMATrp) beads (Figure 4.16).

4.3. Adsorption of Lyz from Aqueous Solutions in MSFB

4.3.1. Effect of Flow-Rate

Different flow-rates are applied to the MSFB system to investigate the effect of flow rate on adsorption. As shown in Figure 4.17, adsorption of Lyz on m-poly(GMATrp) beads decreased with increasing flow-rate of the system. The adsorption capacity decreased significantly from 252.4 to 91.8 mg/g with the increase of the flow-rate from 1.0 mL/min to 4.0 mL/min. The decrease of Lyz adsorption capacity of beads with increasing flow rate was 63.6%. This decrease may be explained as follows. An increase in the flow-rate reduces the solution remaining time in the column. Contact time between the Lyz molecules and polymer beads decreases as the flow rate increases. At low flow-rate, longer contact time in the column, Lyz molecules have more time to bind to the ligand, and a better adsorption capacity is obtained. These results are also in agreement with literature (Valdman et. al., 2001).

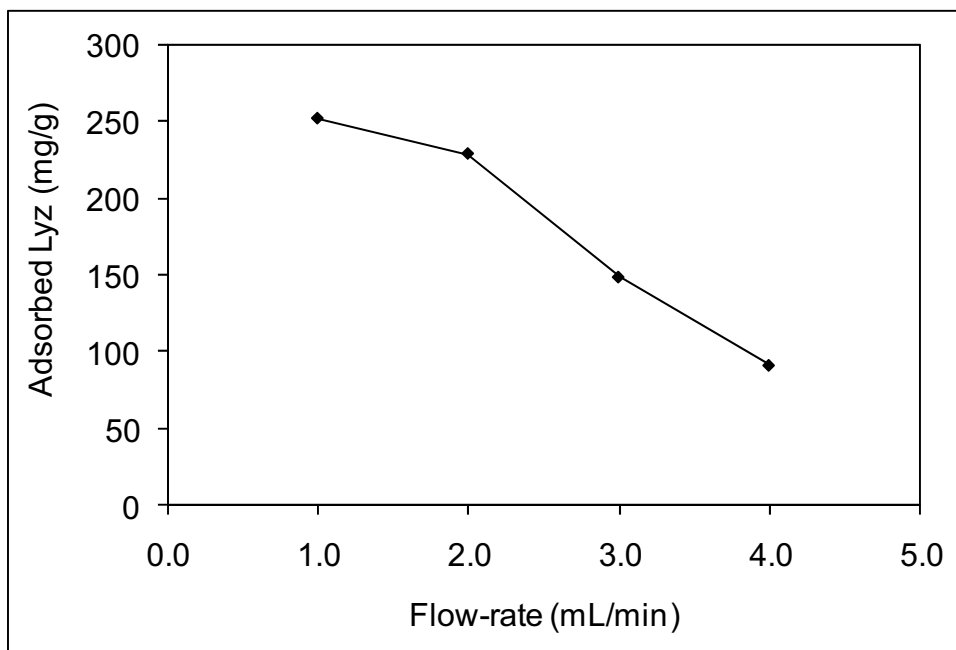


Figure 4.17. Effect of flow-rate on Lyz adsorption: Lyz concentration: 1.0 mg/mL; pH: 7.0; T: 25°C.

4.3.2. Effect of pH

In MSFB system, Lyz adsorption capacity of m-poly(GMATrp) beads was studied at different pH values and displayed in Figure 4.18. Effect of pH on Lyz adsorption capacity was studied at pH 4.0-8.0. Maximum adsorption was observed as 252.4 mg/g at pH 7.0, adsorption capacity decreased at pH values lower and higher than 7.0. The maximum adsorption capacity at pH 7.0 indicates that the binding of Lyz on the polymer beads should involve mainly hydrophobic interactions in addition to other interactions. In MSFB system, Lyz adsorption increased in all solutions at different pH values. It can be concluded that usage of MSFB increased the adsorption efficiency of m-poly(GMATrp) beads.

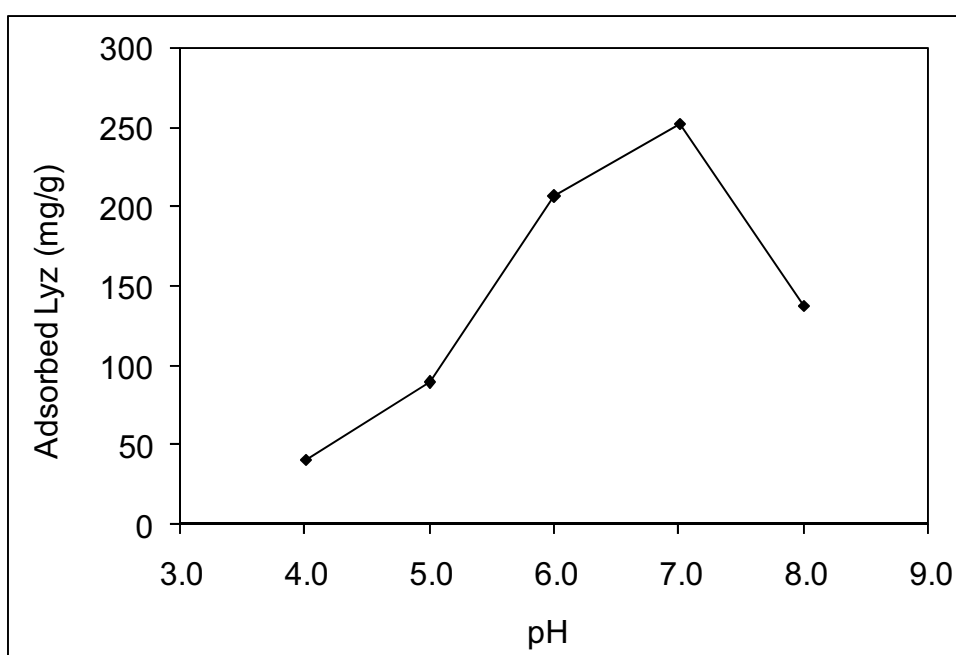


Figure 4.18. Effect of pH on Lyz adsorption; Lyz concentration: 1.0 mg/mL; flow-rate: 1.0 mL/min; T: 25°C.

4.3.3. Effect of Lyz Concentration

Lyz adsorption capacity of m-poly(GMATrp) beads in MSFB system is shown in Figure 4.19. As the concentration of Lyz increases, the amount of Lyz adsorbed by beads increases linearly at low concentrations, then approaches saturation. This may be attributed to saturation of the active adsorption sites of magnetic beads that are available and accessible for Lyz. It becomes nearly constant when the protein concentration is greater than 1.0 mg/mL. Maximum adsorption capacity of the m-poly(GMATrp) beads was observed as 263.9 mg/g.

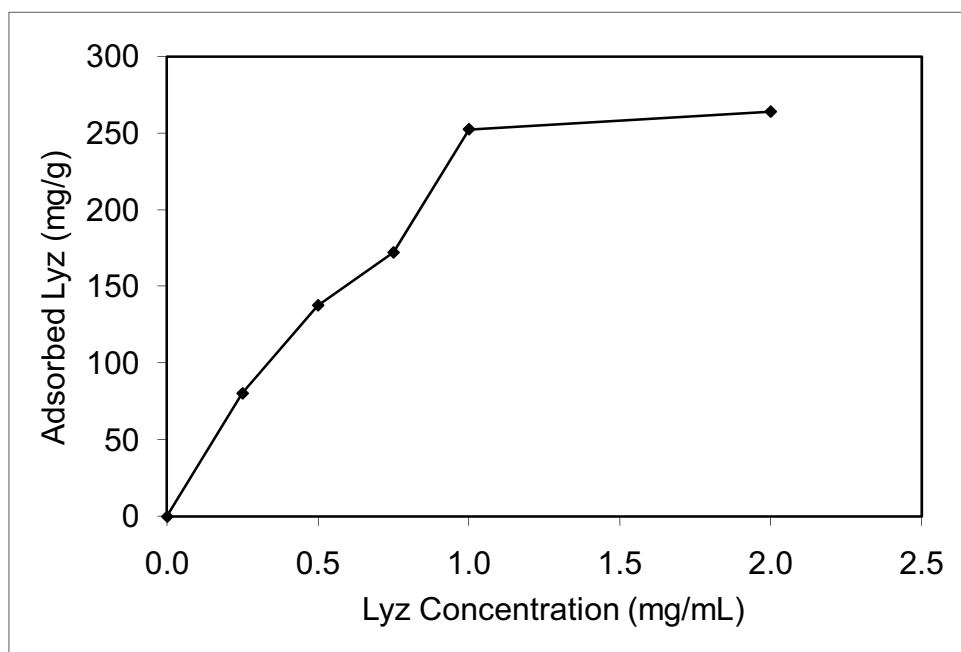


Figure 4.19. Effect of the Lyz concentration on adsorption: Flow-rate: 1.0 mL/min; pH: 7.0; T: 25°C.

4.3.4. Effect of Ionic Strength

The effect of salt concentration on Lyz adsorption in MSFB system is shown in Figure 4.20. When the aqueous phase concentration of sodium chloride increases, the adsorption capacity of m-poly(GMATrp) beads increases. Increasing the ionic strength could promote the adsorption of Lyz molecules to the polymer surface by hydrophobic interaction (Zhang and Sun, 2004).

In HIC, the use of high salt concentration on the equilibrium buffer solution promotes the ligand-protein interactions and consequently the protein retention. The amount of the bound protein increases almost nearly with increasing ionic strength and continues to increase in an exponential manner at higher concentrations (Queiroz et. al., 2001). The adsorption capacity increased from 203.4 to 321.3 mg/g polymer with the increase of the NaCl concentration from 0 to 0.1 M. The adsorbed amount of Lyz increased about 53.5% with the addition of NaCl.

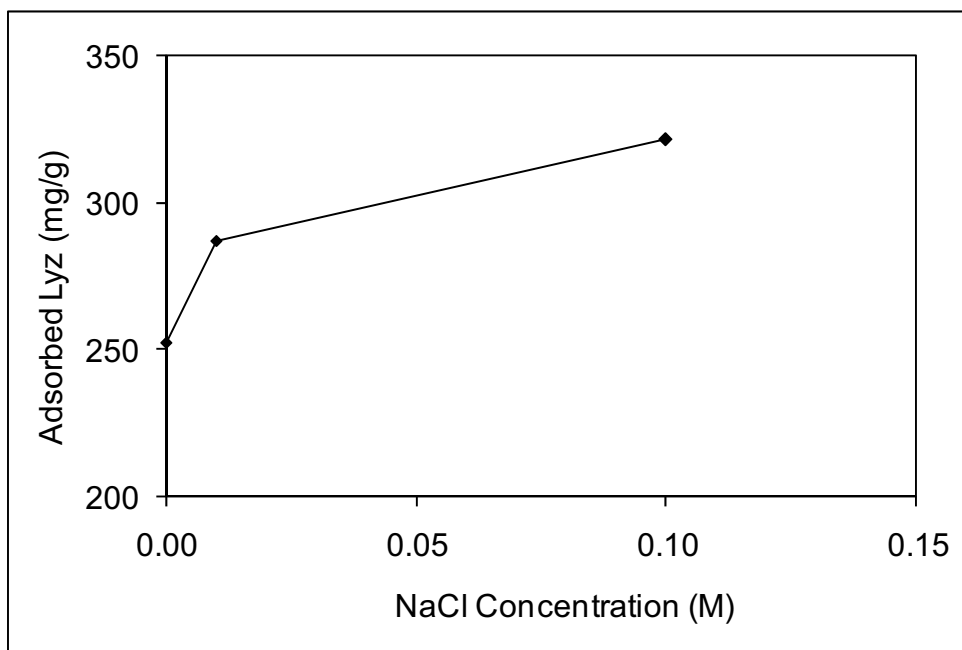


Figure 4.20. Effect of ionic strength on Lyz adsorption. Lyz concentration: 1.0 mg/mL; flow-rate: 1.0 mL/min; pH: 7.0; T: 25°C.

4.3.5. Effect of Temperature

Effect of temperature on the adsorption of Lyz on m-poly(GMATrp) beads was presented in Figure 4.21. Adsorption of Lyz on the m-poly(GMATrp) beads was significantly increased from 110.2 mg/g to 286.9 mg/g with increasing temperature from 4 to 37°C. This tendency indicates that hydrophobic interactions are much more significant at high temperatures in the adsorption of Lyz on m-poly(GMATrp) beads.

If the interaction between the protein and the attached ligand involves a significant hydrophobic contribution, the contact surface between the protein and the attached ligand should increase at higher temperature. This results in an increase in the affinity of Lyz for the adsorbent at higher temperatures. Increasing the temperature enhances protein retention and lowering the temperature generally promotes the protein elution (Hjerten et. al., 1986).

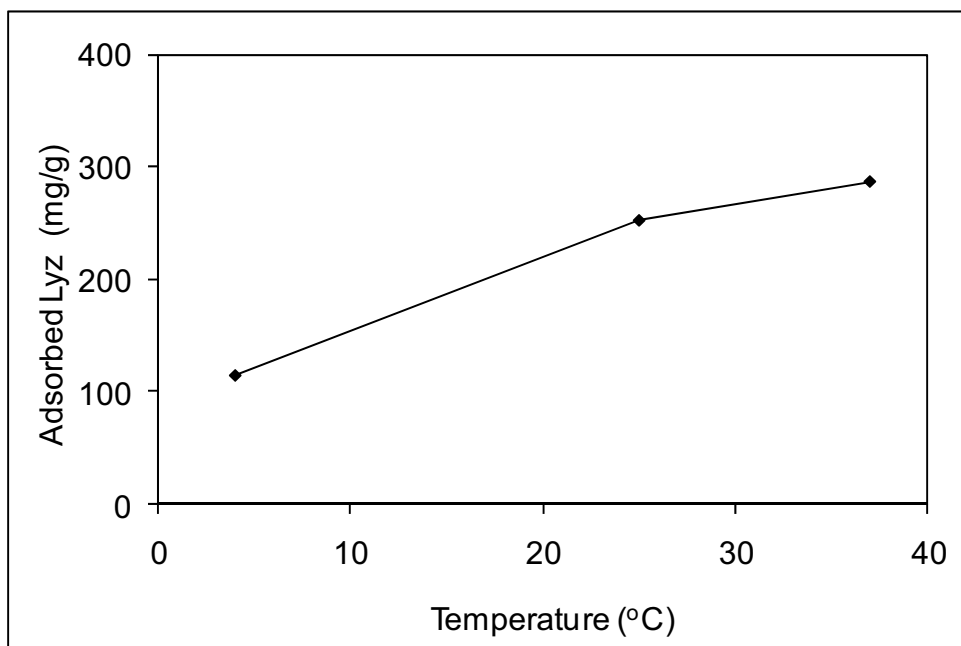


Figure 4.21. Effect of temperature on Lyz adsorption. Lyz concentration: 1.0 mg/mL; flow-rate: 1.0 mL/min; pH: 7.0.

4.3.6. Adsorption Isotherms

Langmuir and Freundlich adsorption isotherms of adsorption of Lyz on m-poly(GMATrp) beads are displayed in Figure 4.22 and 4.23, respectively. Isotherm constants for Langmuir and Freundlich adsorption models and correlation coefficients are also shown in Table 4.4. Maximum adsorption capacity ($Q_{\max}$) and the Langmuir constant were found to be 312.5 mg/g and 4.0 mL/min respectively.

Change in free energy is calculated -3.43 kJ/mol by using Eq. 2.1. It can be concluded that adsorption of Lyz on m-poly(GMATrp) beads is a thermodynamically favorable process since ΔG is negative. Results of Langmuir and Freundlich isotherms are represented in Table 4.4. According to these results, Langmuir adsorption isotherm is more favorably for this adsorption process.

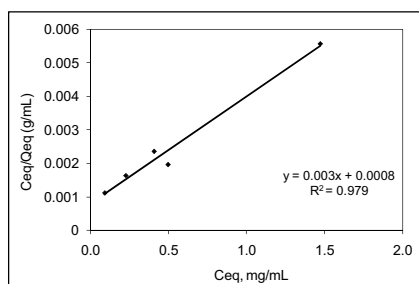


Figure 4.22. Langmuir adsorption isotherm of Lyz adsorption. Lyz concentration: 1.0 mg/mL; flow-rate 1.0 mL/min; pH: 7.0; T: 25°C.

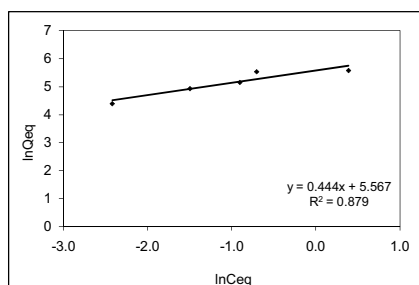


Figure 4.23. Freundlich adsorption isotherm of Lyz adsorption. Lyz concentration: 1.0 mg/mL; flow-rate 1.0 mL/min; pH: 7.0; T: 25°C.

Table 4.4. Isotherm constants for Langmuir and Freundlich adsorption models and correlation coefficients for Lyz.

Isotherm Constants			
Langmuir		Freundlich	
Q_{eq}	312.5	K_F	267.9
b	4.0	$1/n$	0.443
R^2	0.978	R^2	0.878

4.3.7. Desorption and Reusability of Adsorbents

In order to test the reusability of the m-poly(GMATrp) beads, 0.1 M ethylene glycol solution was used for the desorption of adsorbed Lyz. Adsorption-desorption procedure was repeated five times by using the same magnetic column. It was observed that there was no remarkable reduce in the Lyz adsorption capacity of m-poly(GMATrp) beads (Figure 4. 24).

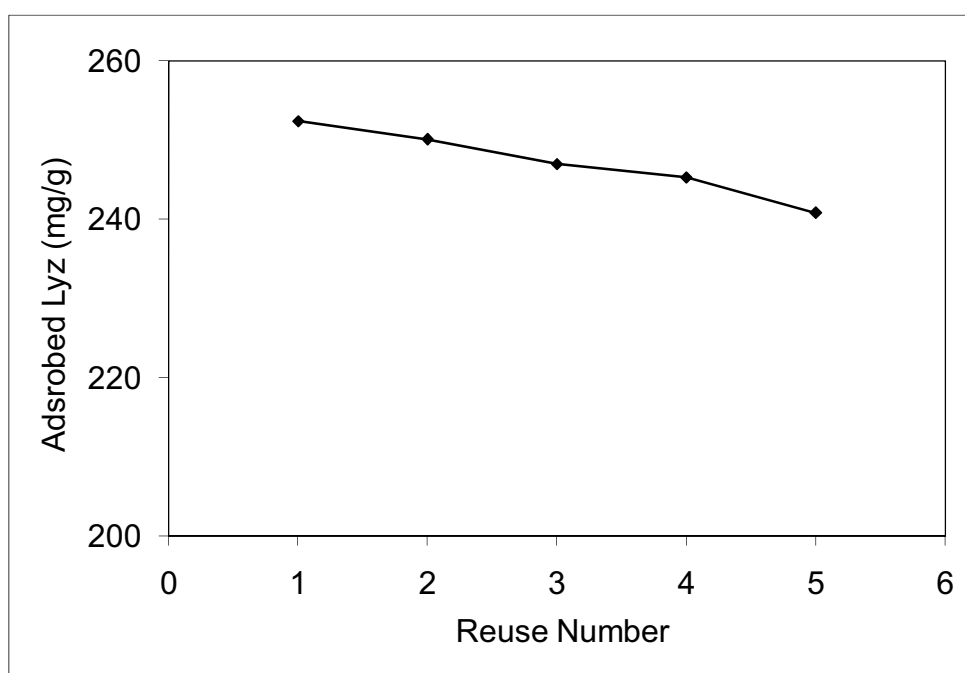


Figure 4.24. Reusability of the magnetic column. Lyz concentration: 1.0 mg/mL; flow-rate: 1.0 mL/min; pH: 7.0; T: 25°C.

4.4. Comparison of MSFB and Batch System Experiment Results

Effect of different parameters on adsorption capacities of MSFB and batch system are discussed below. Plots obtained from batch and MSFB experiments were displayed together to compare the experimental results of MSFB and batch system.

Effect of pH on Lyz adsorption capacity of m-poly(GMATrp) beads was displayed in Figure 4.25. Maximum Lyz adsorption is observed as 203.7 mg/g and 252.4 mg/g in batch and MSFB system, respectively. Amount of adsorbed Lyz is higher in MSFB than batch system.

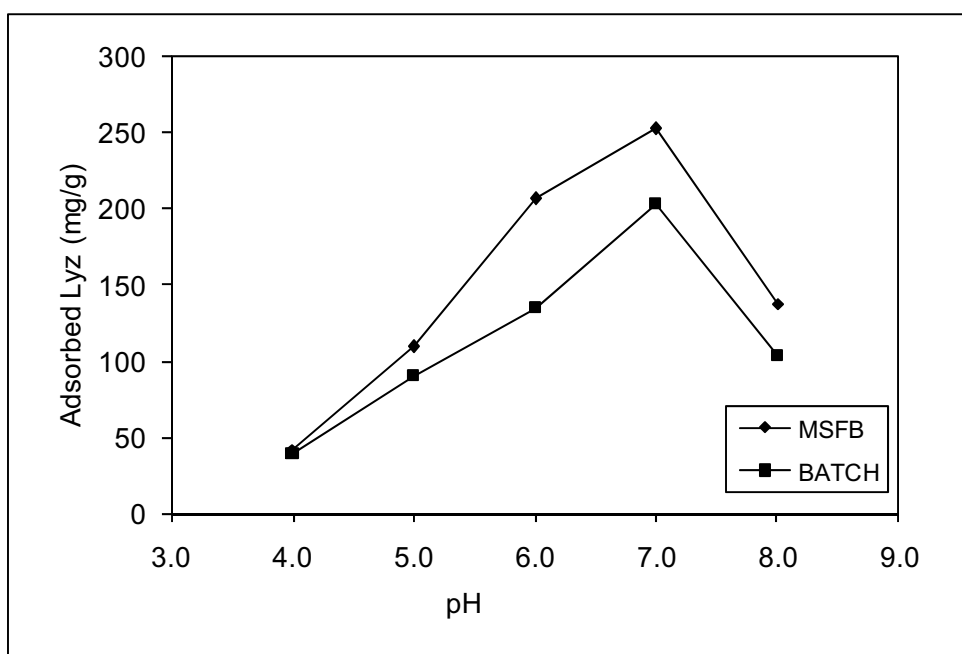


Figure 4.25. Comparison of MSFB and Batch System. Effect of pH on adsorption capacity. Lyz concentration: 1.0 mg/mL; T: 25°C; flow-rate:1mL/min (for MSFB).

In Figure 4.26, effect of Lyz concentration on adsorption is shown. As seen from this figure, adsorption capacity of m-poly(GMATrp) beads is higher in MSFB than that of batch system. Maximum adsorption capacity of MSFB and batch system was calculated as 263.9, and 255.3mg/g, respectively.

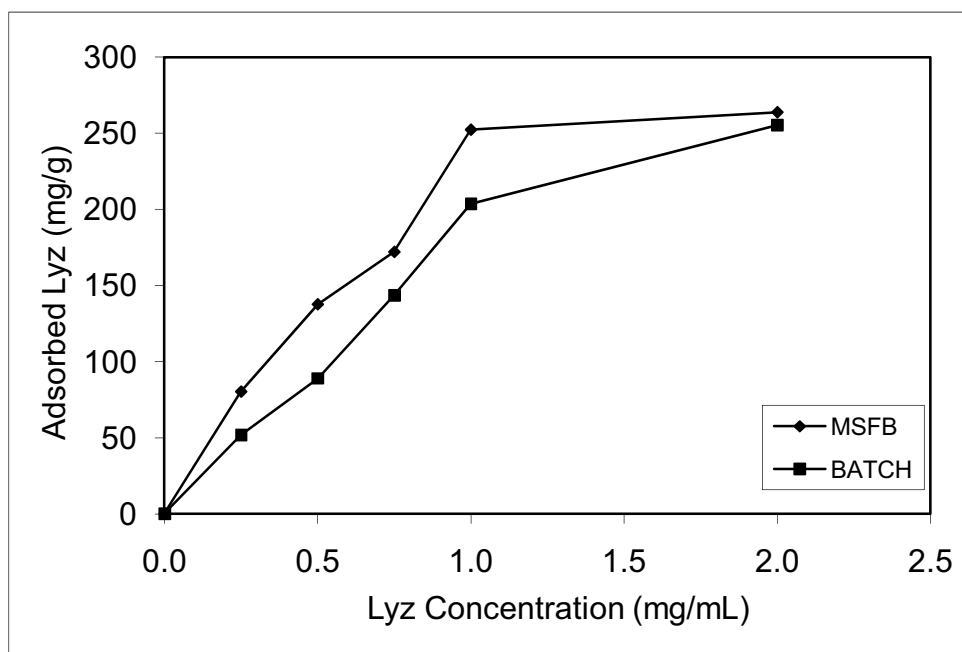


Figure 4.26. Comparison of MSFB and Batch System. Effect of Lyz concentration on adsorption capacity: pH: 7.0; T: 25°C; flow-rate: 1.0 mL/min (for MSFB).

Effect of ionic strength, and temperature on adsorption capacity were also examined, and shown in Figure 4.27, and 4.28, respectively.

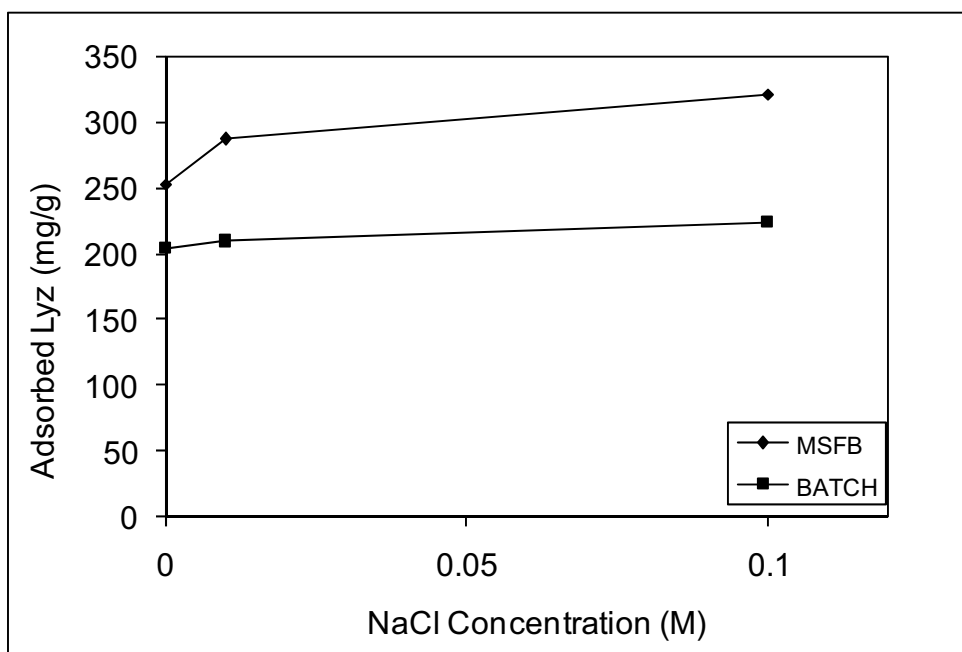


Figure 4.27. Comparison of MSFB and Batch System. Effect of ionic strength on adsorption capacity: pH: 7.0; Lyz concentration: 1.0 mg/mL; T: 25°C; flow-rate: 1.0 mL/min (for MSFB).

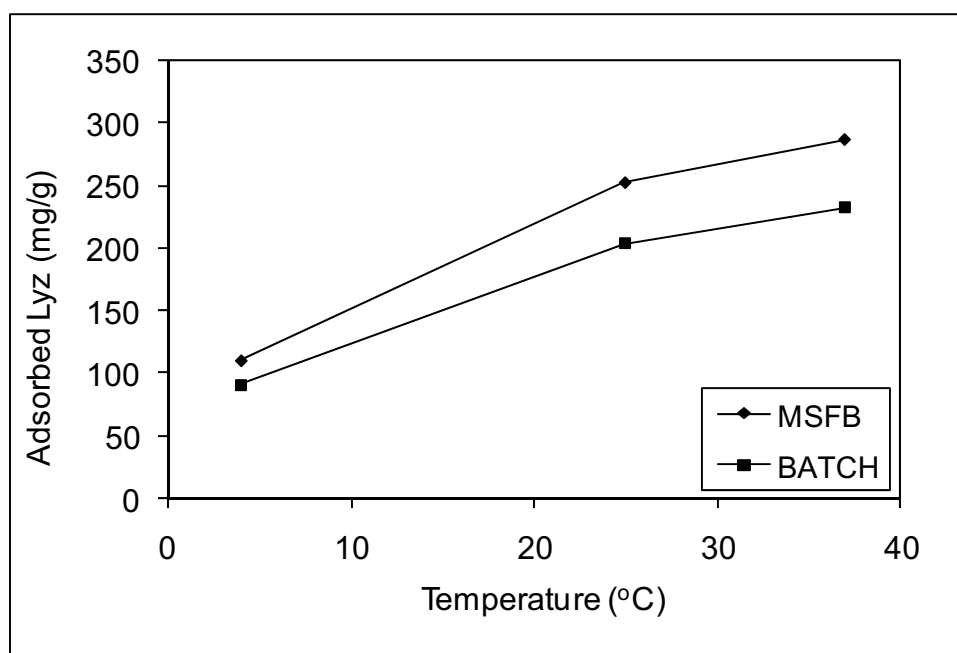


Figure 4.28. Comparison of MSFB and Batch System. Effect of temperature on adsorption capacity: pH: 7.0; Lyz concentration: 1.0 mg/mL; T: 25°C; flow-rate:1.0 mL/min (for MSFB).

Adsorption capacity of MSFB system was obtained higher than that of batch system in ionic strength and temperature experiments.

Experimental conditions and polymer beads that were used in the experiments are the same in both systems. However, adsorption capacities are higher than those obtained in batch system. This may be due to;

- In the MSFB system, the ligand-protein dissociation rate is higher than the association rate,
- In the MSFB, m-poly(GMATrp) beads are stabilized by magnetic field, thus Lyz molecules interacts better with polymer beads.
- When beads are inserted into water, a thin layer of water surrounds them. This thin layer of water must be removed for the interaction of hydrophobic regions of the beads and proteins. In MSFB system, removal of water layer around the beads would be done better.

The adsorption capacity in the batch system is lower than MSFB system, and MSFB can be favorably used for the selective and efficient separation of Lyz from aqueous solutions.

4.5. Purification of Lyz from Chicken Egg

Chicken egg white is a mixture of proteins. The content of Lyz as a minor component in chicken egg white is about 3.5% (Davis and Reeves, 2002). Lyz has 14.3 kDa molecular weight and has a 129 aminoacid residue. It has just one polypeptide chain. Lyz has the highest total surface hydrophobicity of the proteins of chicken egg white. The classical Lyz purification method required several steps, such as precipitation, centrifugation and affinity adsorption (Şenel et.al, 2004). In this study, single step Lyz purification from egg white was studied in batch mode. The hydrophobic affinity beads provided an efficient single step method to purify Lyz from diluted egg white, showing high adsorption capacity and high selectivity for Lyz. The purity of the lysozyme desorbed from hydrophobic m-poly(GMATrp) beads was determined by Bio-LC which was shown in Figure 4.29.

The purity of the desorbed lysozyme which was obtained from the integration of Bio-LC peaks was about 85% for aqueous solution of Lyz, and 67% for chicken egg white, with overall yield about 76%, which is similar to 77% yield obtained using Streamline SP followed by Dye-Ligand Chromatography (Roy, et. al., 2003). Chang et al showed that a 11-fold purification of the lysozyme was achieved in a high yield of 97.5% with Streamline SP adsorbent in a stirred fluidized-bed. Shen and Cao applied thermo-sensitive *N*-alkyl substituted polyacrylamide polymer carrying Cibacron Blue F3GA in the purification of lysozyme in egg white, it showed a purification fold of 28 and specific activity of lysozyme was 12,000 U/mg (Shen and Cao, 2007). Su and Chiang used the PEG-sulfate aqueous two-phase system for lysozyme purification from chicken egg white and they reported that 70% of lysozyme can be extracted with the specific activity 39,500 U/mg. Odabaşı and Denizli prepared lysozyme-imprinted beads for the purification of lysozyme from egg white. They reported 89% purity with recovery about 84%. Compared to the results in literature, the result by this method is so good.

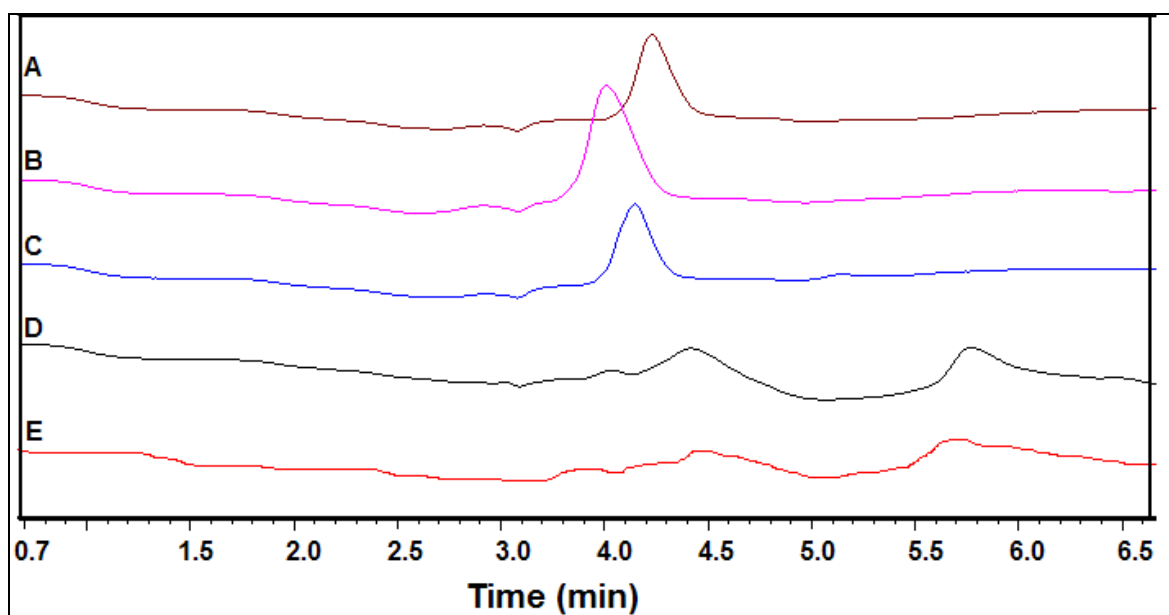


Figure 4.29. Bio-LC chromatograms showing the purification of Lyz, (A) pure commercial Lyz; (B) aqueous solution of Lyz before adsorption; (C) aqueous solution of Lyz after desorption; (D) whole egg white; (E) desorbed whole egg white sample.

The specific activity of the lysozyme purified with hydrophobic beads was found to be 44500 U/mg. Specific activity determined in desorbed fraction was comparable to the commercial egg white lysozyme yielding approximately 50-fold purification in a single step (Table 4.5).

Table 4.5. Specific activity and fold purification of lysozyme isolated from egg white.

Specific activity	(unit/mg protein)	Purification fold
Egg white preparation	887 ± 90	1
Batch adsorption	44500 ± 300	50
Commercial lysozyme	46000 ± 280	-

Sample volume: 100 mL; protein content: 32.5 mg/mL.

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel 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. 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. Not only the mass but also the shape of a protein will determine how it can move through the environment. Thus all proteins were converted to the same shape. SDS was used for making all proteins negative.

SDS-PAGE was applied to show the purification of Lyz from chicken egg. Solution of Lyz 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. It binds and unfolds the protein, giving a near uniform negative charge along the length of the polypeptide.

SDS-PAGE electrophoresis was performed in order to confirm that Lyz was adsorbed on m-poly(GMATrp) beads. Initial and desorbed chicken egg white electrophoresis image is shown in Figure 4.30. Lane 1 belongs to sigma marker (wide range molecular weight). Lane 2 and Lane 3 belong to initial and desorbed chicken egg white, respectively.

Chicken egg white contains many proteins. Amount of proteins in egg white are; ovalbumin 54%, ovotransferrin 12-13%, ovomucoid 11%, and Lyz 3.4-3.5. Figure 4.30 shows proteins with high percentage. In Lane 2; 45 kDa stands for ovalbumin, 77.7 kDa ovotransferrin, 28 kDa ovomucoid, and 14.3 kDa Lyz, respectively. Other proteins with small percentage couldn't be seen. Lane 3 belongs to desorbed Lyz migration. As expected, Lyz depleted chicken egg white showed weak protein bands at 12.5 kDa, confirming the depletion of high abundance of proteins.

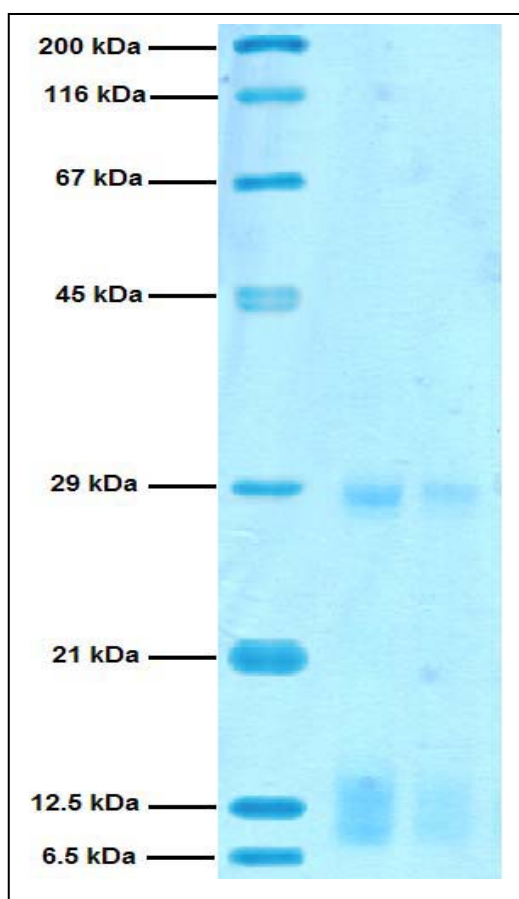


Figure 4.30. SDS-PAGE of egg white fractions. The fractions were assayed by SDS-PAGE using 10% separating gel (9 cm×7.5 cm), and 6% stacking gels were stained with 0.25% (w/v) Coomassie Brilliant R 250 in acetic acid–methanol–water (1:5:5, v/v/v) and destained in ethanol–acetic acid–water (1:4:6, v/v/v). Lane 1, biomarker (Sigma); lane 2, egg white before adsorption, lane 3, desorbed sample. Equal amounts of samples were applied to each line.

5. CONCLUSION

- Monosize, 1.6 μm , magnetic poly(GMATrp) beads were prepared by dispersion polymerization. The equilibrium swelling ratio of the of m- poly(GMATrp) beads is 45%. Compared with of m- poly(GMATrp) beads (52%), the water uptake ratio of of m- poly(GMATrp) beads is decreased.
- Scanning electron micrographs showed that of m-poly(GMATrp) beads are spherical and uniform. They also have and smooth surface without pores.
- To evaluate the degree of the MATrp incorporation, elemental analysis of the synthesized of m- poly(GMATrp) beads was performed. The incorporation of the MATrp was found to be 30 μmol MATrp/g polymer from the nitrogen stoichiometry.
- Lyz adsorption studies were performed in batch and MSFB system.
- Lyz adsorption of the of m- poly(GMATrp) beads depend on pH of the solution. Adsorption studies were carried out at different pH using acetate (4.0-5.0) and phosphate (6.0-8.0) buffers. Maximum adsorption capacities were observed at pH 7.0 both for batch and MSFB (203.7 mg/g and 252.4 mg/g in batch and MSFB system, respectively).
- There was a negligible non-specific Lyz adsorption (the adsorption onto the plain m-poly(GMA) beads) which was about 1.6 mg/g at pH:7.0. There are no reactive binding sites on m-poly(GMA) to interact with Lyz molecule. Hence, this non-specific adsorption may be due to diffusion of Lyz molecules into the swollen beads.
- Maximum Lyz adsorption capacity of m-poly(GMATrp) beads were calculated for MSFB and batch system as 263.9, and 255.3mg/g, respectively.
- Lyz adsorption capacity increased with the increasing salt concentrations both for batch (223.8 mg/g) and for MSFB system (321.3 mg/g).
- The equilibrium adsorption of Lyz on m-poly(GMATrp) beads significantly increased with increasing temperature both for batch (232.4 mg/g) and MSFB system (286.9 mg/g).
- From the experimental data, ΔG values were calculated. ΔG values are -0.235 kJ/mol for batch, and -3.435 kJ/mol MSFB system. The negative change in free energy indicated that the adsorption of Lyz on m-poly(GMATrp) beads was a thermo-dynamically favorable process.

Conclusion

- In order to analyze the adsorption kinetics of Lyz adsorption, the pseudo-first-order and pseudo-second-order kinetic models were applied to the experimental data. The theoretical q_{eq} values for the m-poly(GMATrp) beads were close to the experimental q_{eq} value in the case of first-order kinetics for batch system.
- In order to show reusability of the m-poly(GMATrp) beads, the adsorption-desorption cycle was repeated five times using the same polymeric beads. At the end of five adsorption-desorption cycle, there was no remarkable reduce in the adsorption capacity.
- Adsorption capacities in MSFB are higher than those obtained in batch system.
- Bio-LC and SDS-PAGE was performed from egg white solution to see purity of Lyz.

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