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Adsorption of Trypsin Onto Chitosan/PSf Affinity Membranes: Effects of Physio-chemical Environment

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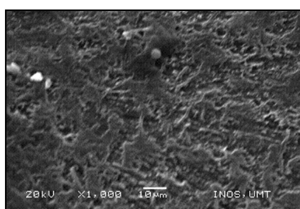
Graphical abstract



Abstract

This study aimed to investigate the significance of the physico-chemical environment during trypsin adsorption onto a highly bio-specific affinity membrane. The affinity matrix polysulfone (PSf) membranes were prepared via a simple dry/wet phase inversion technique. Surface modification of PSf membranes was employed using chitosan in order to improve membrane hydrophilicity. Glutaraldehyde and ovomucoid were used as the membrane activator and affinity ligand, respectively. Inspection of membrane morphology was done using scanning electron microscopy. The functional groups on the membrane surface were determined using Fourier-transform infrared spectroscopy equipped with attenuated total reflection (ATR-FTIR). In order to determine the optimum conditions for the maximum adsorption capacity of trypsin, adsorption studies were performed at different pH levels (5, 7, 8, 10, 12), ionic strengths (0.01, 0.05, 0.1, 0.3 and 0.5 M) and initial trypsin concentrations (0.1, 0.3, 0.5, 0.7 and 0.9 mg/ml). The optimum adsorption was obtained with a 0.9 mg/ml initial trypsin solution at pH 7 and an ionic strength of 0.1 M.

Keywords: Membranes; affinity; chitosan; trypsin; adsorption

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1.0 INTRODUCTION

The limitations of conventional chromatographic systems have encouraged the search for new kinds and configurations of chromatographic matrices. Therefore, the development of adsorptive membranes has become one of the most significant chromatographic advances to overcome the shortcomings inherent in the enzyme purification process. In recent years, various types of adsorptive membranes have been investigated to cover a wide range of applications based on membrane shape (flat sheet, hollow, etc.) as well as the interaction mode (affinity, ion-exchange, reverse phase, hydrophobic, etc.) [1-4].

The utilization of affinity chromatographic membranes has been given emphasis in the effort to overcome the challenges of downstream processing in enzyme and protein production. This technology is regarded as the best available separation method as it is the only technique that permits the purification of enzymes based on biological function rather than individual physical or chemical properties. It relies on highly specific binding and strong interactions between the immobilized ligand and the target enzyme in solution to achieve a high degree of protein purification.

In recent years, high performance affinity membranes with variable surface chemistry have become the subject of intense research due to their extensive applications in biotechnology. Urmenyi *et al.* [5] developed a novel affinity membrane for hormone removal. Additionally, affinity membranes have also been applied in many adsorption studies, including the adsorption of pure recombinant MBP-fusion protein [6], lactin [7], immunoglobulin G [8] and papain [9].

One of the important requirements for affinity membrane development is the selection of the affinity matrix. The membrane matrix should have large pores and low fouling characteristics to allow the passage of all unwanted substances as well as the target protein, but not of the affinity macroligand. Most polymeric membranes used for protein or enzyme applications are of the asymmetric ultrafiltration membrane type, which have high thermal stability and chemical resistivity, and thus require less use of harsh chemicals for cleaning [10, 11]. The technique involves the activation of the matrix material using different types of activators such as glutaraldehyde, epichlorohydrin and 1, 4-butandiol diglycidyl ether. Ligand immobilization by linking the affinity ligand to the activated polymeric membranes is the first step, followed by affinity capture of the target enzyme by the immobilized affinity ligand.

This study aimed to investigate the effects of physico-chemical environment during trypsin adsorption onto a highly specific affinity membrane. The affinity matrix was fabricated

using a polymer concentration of 15 wt.% polysulfone (PSf), and surface modification was employed by the incorporation of chitosan (60 minute dip time) to impart hydrophilicity to the membrane. The chitosan/PSf composite membranes were then activated using glutaraldehyde and immobilized with a trypsin inhibitor as the ligand. Experiments were then performed to determine the optimum conditions for enzyme adsorption onto the affinity membrane by investigating the the effects of pH, ionic strength and initial protein concentration.

2.0 EXPERIMENTAL

2.1 Materials

All materials used were of analytical grade. The membranes were fabricated from a ternary casting solution consisting of PSf as the polymer, N-methyl-2-pyrrolidone (NMP) (supplied by Merck) as the solvent and water (H_2O) as a non-solvent. Distilled water was used as the coagulation medium. A series of proteins, including myoglobin, ovalbumin, pepsin and bovine serum albumin (supplied by Sigma) were used in the characterization technique to determine the molecular weight cut off of the prepared membranes. Chitosan particles (Sigma Aldrich) were used for hydrophilic modification of the asymmetric PSF membrane. Trypsin inhibitor type III (ovomucoid) and trypsin (Mw = 25,000 Dalton), purchased from Sigma Aldrich, were used for affinity ligand and adsorption studies, respectively. Glutaraldehyde (Sigma Aldrich) was used for membrane activation to develop the affinity membranes. Bradford reagent (Sigma Aldrich) was used for total protein determination.

2.2 Preparation of the Matrix Material of the Affinity Membrane

Asymmetric PSf membranes were prepared using ternary dope formulations with a polymer concentration of 15 wt.%. Membranes were fabricated via a simple dry/wet phase inversion technique using an electrical casting machine at a shear rate of 200 s^{-1} and then immersed directly into a coagulation bath for 24 hours. To perform hydrophilic modification by self-assembly of chitosan, native PSf membranes were immersed into the chitosan solution (0.1 wt.% in acetic acid, pH 5) for 60 minutes to deposit chitosan particles onto the membrane surface; the membrane was then dried at room temperature. The dried membrane was neutralized with NaOH solution (0.1 M in a 50% water-ethanol mixture) for 30 minutes to ensure that all chitosan acetate was converted to chitosan. In order to prevent osmotic cracking and to remove the remaining NaOH, the membranes were rinsed with 50% ethanol solution three times, followed by washing with distilled water. The prepared membranes were stored in distilled water prior to use.

2.3 Membrane Activation and Ligand Immobilization

The membrane support (CH/PSf-60) was cut into area of about 14.6 cm^2 and incubated in an incubating shaker for 150 min in a reacting solution containing different amounts of 25 vol.% glutaraldehyde (GTA) aqueous solution in 0.1 M sodium chloride and 0.1 M sodium acetate-acetic acid buffer (pH 7.4) at room temperature. After the reaction, the excess glutaraldehyde was removed by washing the membrane three times with 2 M acetic acid. The trypsin inhibitor was immobilized onto the activated membrane with an immobilization time of 180 minutes and the adsorption capacity was determined every 30 minutes. Hereafter, the CH/PSf-60 membrane is referred to as the affinity membrane.

2.4 Characterization Studies of Affinity Membrane

Membrane characterization studies were performed in terms of membrane morphology using scanning electron microscopy, and the functional groups on the membrane surface were assessed using Fourier-transform infrared spectroscopy equipped with attenuated total reflection (ATR-FTIR).

2.5 Adsorption Studies of Trypsin onto Affinity Membrane

In conducting the adsorption experiment, a known amount of trypsin was dissolved in 0.1 M phosphate buffer. The adsorption experiments were started after incubation of the membrane disk (with area of about 14.6 cm^2) in the trypsin solution for 3 hours at 25°C. The amount of adsorbed trypsin on the affinity membrane was determined by the initial and final total protein concentrations in the adsorption medium. The enzyme concentration was monitored by the Bradford assay and the amount of adsorbed trypsin was obtained using Equation (1) [12]:

$$q = \frac{(C_0 - C)\,V}{A} \tag{1}$$

Where q is the amount of trypsin adsorbed onto the membrane (mg/cm^2), C_0 and C are the total protein in the initial solution and the aqueous phase after adsorption, respectively ($mg.ml^{-1}$), V is the volume of the aqueous solution (ml) and A is the area of the membranes in the adsorption medium (cm^2).

2.6 Theoretical Approach

To simulate the adsorption isotherm, two commonly used theoretical isotherm models (namely, the Langmuir and Freundlich models) were selected to describe the protein-ligand interaction. The Langmuir model is based on the assumption of surface homogeneity, such as equally available adsorption sites, monolayer surface coverage, and no interaction between the protein and ligand [12, 13]. This equilibrium adsorption on affinity beads, affinity membranes, ion-exchange chromatographic membranes, etc. is often described by Equation (2):

$$q_{eq} = \frac{(q_m C_e)}{K_d + C_e} \tag{2}$$

Eq. (2) can be transformed into a linear form as follows:

$$\frac{C_e}{q_{eq}} = \frac{C_e}{q_m} + \frac{K_d}{q_m} \tag{3}$$

Where C_e (mg/ml) is the equilibrium concentration of trypsin in solution, q_{eq} (mg/cm^2) is the adsorption capacity at equilibrium, q_m (mg/cm^2) is the maximum adsorption capacity and K_d is the effective dissociation constant, which can determined from the slope and y-intercept of the plotted graph.

The Freundlich isotherm describes an empirical relationship which is frequently used to describe adsorption; it relates the adsorbed concentration of trypsin as a power function of the solute concentration. This isotherm model assumes that

the adsorption energy of an enzyme binding to a site on an adsorbent material depends on whether or not the adjacent sites are already occupied. However, a limitation of the Freundlich model is that the amount of adsorbed solute increases indefinitely with the concentration of solute in the solution [9]. This empirical equation takes the form:

$$q_{eq} = K_F (Ce)^{1/n} \qquad (4)$$

Equation (4) can be transformed into Equation (5):

$$\log q_{eq} = \log K_F + (1/n)\log C_e \qquad (5)$$

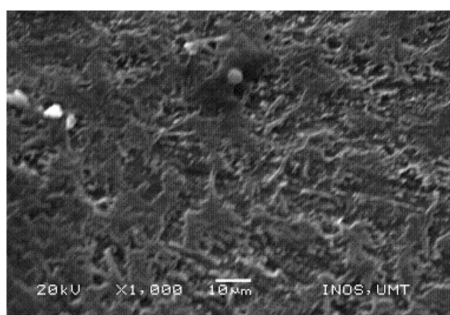
where C_e (mg/ml) is the equilibrium concentration of trypsin in solution and q_{eq} (mg/cm^2) is the adsorption capacity at equilibrium. K_F and n are the Freundlich constant characteristics of the system which indicate the adsorption capacity and adsorption intensity, respectively.

■3.0 RESULTS AND DISCUSSION

3.1 The Characteristics of Affinity Membrane

The affinity membrane had a rough and porous surface structure, which increased the surface area, reduced the mass transfer resistance and facilitated the diffusion of enzyme molecules. This creates a condition of low diffusional resistance within the membrane, in contrast to a packed bed affinity column, in which a considerable pressure drop always occurs [4].

The trypsin inhibitor was successfully immobilized onto the chitosan/PSf membrane using the method of glutaraldehyde activation. The surface morphology of affinity membrane are shown in Figure 1. Membrane activation using the glutaraldehyde method was able to induce crosslinking, which stabilized the membrane structure during ligand immobilization [14].



(b)

Figure 1 Surface morphology of the affinity membrane

To verify the integrity of the affinity membranes, all membranes were characterized by ATR-FTIR; the results are shown in Figure 2.

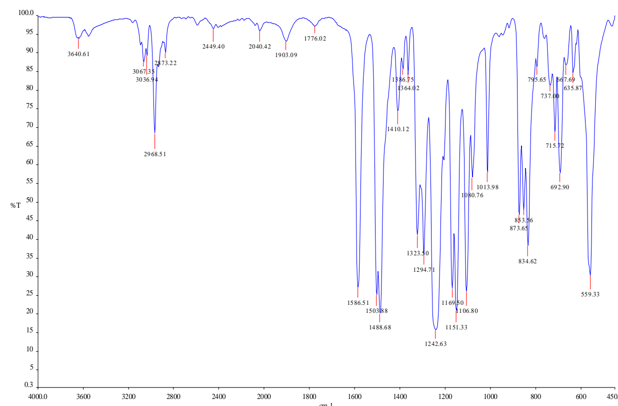


Figure 2(a) ATR-FTIR spectra of the native PSf15 membrane

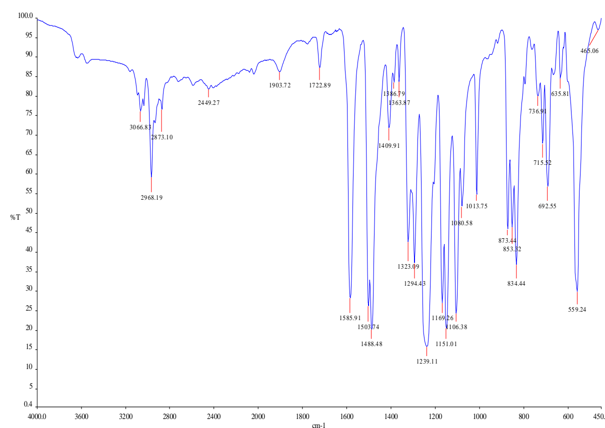


Figure 2(b) ATR-FTIR spectra of the affinity membrane

The band at 1657 cm^{-1} is attributed to C=O (amide I) [15] of the N-acetyl group in chitosan particles. The band ranging from 3600 and 3200 in the chitosan/PSf membrane indicated the presence of O-H and N-H_2 groups in chitosan [16,17]. By crosslinking chitosan/PSf with glutaraldehyde (Figure 2(b)), the O–H stretching vibration peak (ν=3600 cm^{-1} to 3200) was relatively decreased. The wavelength at 2873 cm^{-1} reveals C-H stretching related to aldehyde, indicated by doublet adsorption with peaks attributed to the alkyl chain [18]. The shoulder at 1722 cm^{-1} was due to the ethylene chain. Another hypothesis which explains the presence of aldehyde peaks in the affinity membrane could be the incomplete reaction of glutaraldehyde with the OH groups from chitosan during crosslinked network formation. As a bi-functional crosslinker, one aldehyde group may react with the hydroxyl groups of the chitosan/PSf polymer chain by forming a hemi-acetal structure, while the other one does not react; this may be associated with some conformational or kinetic limitation [18].

3.2 Effect of pH on Adsorption Capacity

A change in solution pH can alter the electrical charge on the protein as well as the membrane due to the ionization or deionization of various acidic or basic groups on the protein and the membrane surface, which can cause either repulsive or attractive interactions [19]. The adsorption behavior in this study occurred mostly by electrostatic interactions, so and it is important to consider electrostatic interactions between the trypsin molecules as well as interactions between trypsin and the membrane. Figure 3 shows the effect of pH on the amount of trypsin adsorbed onto the affinity membrane.

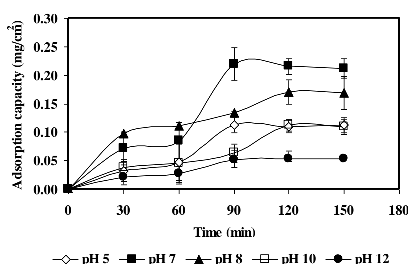


Figure 3 The experimental kinetics of trypsin adsorption at different pH levels

At pH values below the isoelectric point (IEP), trypsin adsorption was clearly higher than at pH values above the IEP. In all cases, as the adsorption was continuous, electrostatic repulsion between the adsorbed trypsin and free trypsin in the medium limited the amount of trypsin which could ultimately adsorb. Thus, adsorption reached equilibrium.

The maximum trypsin adsorption was obtained at pH 7, with 90 minutes of incubation time and an adsorption capacity of 0.219 mg/cm^2. In this study, the trypsin inhibitor was prepared at pH 7.4, so this ligand would be negatively charged at a pH above its isoelectric point (4.18). The isoelectric point of trypsin is 10 [20], so this enzyme should be cationic at a pH below its isoelectric point. This condition (pH 7) provided a good environment for trypsin adsorption with the help of electrostatic interactions as the predominant mechanism of adsorption.

Trypsin was best adsorbed onto the affinity membrane with a low positive charge since the electrostatic interactions between the enzymes molecules decreased while those with the membrane increased. Moreover, in this favorable physico-chemical environment, the amine groups of the trypsin molecules underwent protonation to NH_3^+ [20]; this protonated amino groups on the surface of trypsin and increased the electrostatic interactions between the trypsin and the affinity membrane. This observation was agreed with previous findings [21] demonstrating that the maximal adsorptive capacity of trypsin occurs at around pH 7.

At lower pH value (acidic), far from the isoelectric point of trypsin, the enzyme charge was increased, leading to an increase the effective volume of trypsin molecules due to the presence of a diffuse ion cloud around the protein [19]. This conformational change reduced the binding capacity of trypsin and the trypsin inhibitor. The lower performance at pH 8 was due to an instability phenomenon since this pH approaches the isoelectric point of trypsin.

Visual inspection of the trypsin solution kept at its IEP (pH 10) showed a low adsorption capacity (0.112 mg/cm^2) at equilibrium, indicating that enzyme aggregation had occurred [21] since the net charge on the enzyme surface was zero; thus, there was no electrostatic repulsive force working between the trypsin molecules at this pH. This enzyme aggregation would increase the size of "particles", resulting in their most compact state and tightly packed configuration [22]. This consequently disturbed the one-to-one or affinity interaction between the trypsin and its inhibitor.

The lowest adsorption capacities were obtained when the trypsin solution was in the more alkaline pH region (pH 12). The maximum adsorption was only about 0.051 mg/cm^2; this was possibly due to trypsin denaturation under these harsh conditions. Moreover, at a pH value above its IEP, the trypsin molecule is negatively charged. From the electrostatic point of view, electrostatic repulsion occurred between the trypsin molecule and the affinity membrane surface (which had been immobilized with negatively charged ovomucoid) at this pH since both molecules possess a similar charge. If adsorption can take place, the distance between adsorbed trypsin molecules as well as between trypsin and the affinity membrane surface is large. Thus, the trypsin molecules are bound more loosely to the affinity ligand. This result was in agreement with the findings of Chen *et al*. [4] who determined that the reduction in enzyme adsorption at alkaline pH was attributed to electrostatic repulsion between the enzyme and membrane. According to Li *et al*. [22], electrostatic repulsion between the enzyme and the membrane surface could responsible for lower adsorption, since the thermodynamic entropy difference is the only force inducing adsorption. These phenomena were also found with the observation of a decreased amount of trypsin adsorbed onto the membrane surface far from the isoelectric point (IEP). The overall results show that the affinity of positively charged trypsin and negatively charged sites on the membrane surface is the main component of the adsorption mechanism.

3.3 Effect of Ionic Strength on Adsorption Capacity

Enzyme separation using ultrafiltration is drastically influenced by the nature of solute-solute interactions and also depends on the salt concentration [23]. The effect of ionic strength on trypsin adsorption onto the affinity membrane was studied since complex media are often highly charged with electrolytes; the results are shown in Figure 4. The effect of ionic strength was studied using trypsin solution with a fixed pH (pH 7) since the previous observation determined that pH 7 promoted the highest degree of adsorption. There was a remarkable change in the adsorption capacity due to changes in ionic strength at a pH value below the IEP of trypsin.

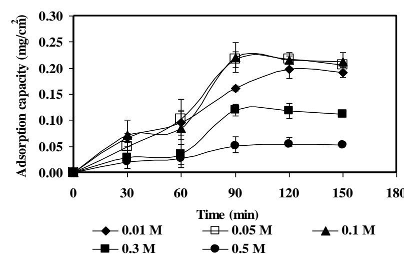


Figure 4 The experimental kinetics of trypsin adsorption at different ionic strengths

A low ionic strength enzyme solution (0.01 to 0.1 M) was associated with a high adsorption capacity due to fewer electrostatic exclusion interactions between the enzyme and the membrane. Increasing the ionic strength up to 0.1 M enhanced the adsorption capacity up to 0.219 mg/mL at its saturation point. This result can be explained in terms of electrostatic repulsion between the trypsin molecules. At this ionic strength, intermolecular repulsion between the trypsin molecules was reduced due to the screening of charged groups, and thus enhanced the shielding effect [24]. Due to the charge distribution at this low ionic strength, the aggregation of trypsin molecules only occurred when they were in a specific orientation [25], and a reduction in the electrostatic interactions between enzyme molecules improved the adsorption capacity.

At ionic strengths of 0.1 and 0.5 M, the adsorption capacity dramatically declined to 0.120 and 0.051 mg/cm^2 at equilibrium. A further increase in ionic strength (above 0.1 M) did not

improve the adsorption capacity since the overly high ionic strength compressed the electrostatic double layers of the enzyme [26] and the affinity membrane surface [27]. Additionally, in the high salt concentration range, enzyme solubility generally decreases (the salt-out effect) due to the reduced activity of water and the neutralization of surface charges. These conditions also favor the formation of protein aggregates, leading to a decrease in trypsin adsorption. Electrostatic interactions depend on the magnitude of the protein surface charge and protein electrical double layer, both of which are ionic strength-dependent [23].

3.4 Effect of Initial Trypsin Concentrations on Adsorption Capacity

The initial concentration of trypsin solution provides an important driving force to overcome all mass transfer resistances of an enzyme molecule between the aqueous and solid phase. Thus, the results in Figure 5 show that an increase in the trypsin concentration in the adsorption medium led to a linear increase in the amount of adsorbed trypsin on the affinity membrane. It should be noted that the initial trypsin concentration in the adsorption medium was important since the affinity membrane could only adsorb a limited amount of enzyme. In all cases, the trypsin adsorption capacity leveled off after 90 minutes of incubation, indicating that almost all the trypsin molecules were bound to the trypsin inhibitor. These observations can be explained by overcrowding of the adsorbed trypsin [20] on the affinity membrane, thus limiting further trypsin adsorption.

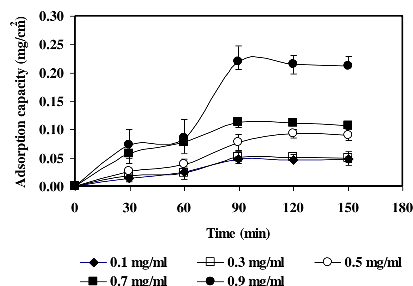


Figure 5 The experimental kinetics of trypsin adsorption with different initial trypsin concentrations

As seen in Figure 5, an increase in the trypsin concentration in the adsorption medium led to a linear increase in the amount of adsorbed trypsin on the affinity membrane. The optimum initial trypsin concentration applied in subsequent experimental work was 0.9 mg/ml since this high concentration led to the highest adsorption capacity.

3.7 Adsorption Isotherm of Trypsin

The adsorption isotherms of the affinity membrane were carried out using different trypsin concentrations from 0.3 mg/ml to 0.9 mg/ml, and two theoretical isotherm models (namely Langmuir and Freundlich models) were used to analyze the experimental data. Both isotherms fit the experimental data, and the corresponding curves are shown in Figure 6(a) and 6(b).

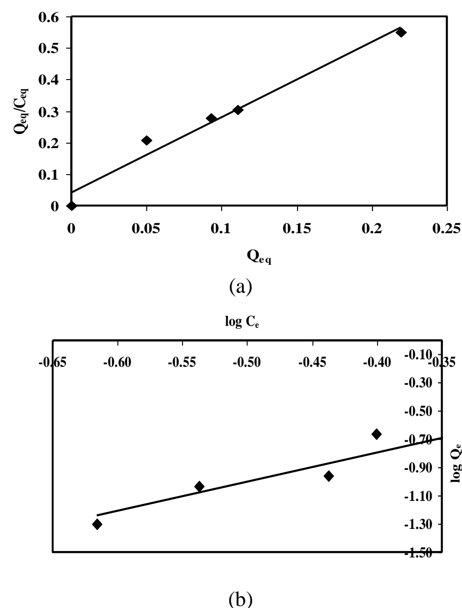


Figure 6 Adsorption isotherm of trypsin onto the affinity membrane using the (a) Langmuir and (b) Freundlich models

The agreement between the experimental points and the model curves was rather satisfactory, as can be noted from the high correlation coefficients ($R^2 > 0.95$) reported in Table 1, together with the resulting values of the fitting parameters for both isotherms.

Table 1 Langmuir and Freundlich isotherm model constants

Experimental	Langmuir constant			Freundlich constant		
Q_{exp}	Q_m	K_d	R^2	n	K_F	R^2
0.219	0.418	0.02	0.9731	0.487	1.067	0.9676

The Langmuir model is based on the assumption of surface homogeneity, such as equally available adsorption sites, monolayer surface coverage, and no interaction between adsorbed species (Bayramoğlu *et al*., 2003). For this isotherm, Scatchard plots (Q_{eq}/Ceq versus Q_{eq}) of the experimental data (Figure 6(a)) gave a linear plot for the affinity membrane that indicated homogenous adsorption. In other words, one-to-one binding of trypsin and the ligand occurred, and adsorption occurred via monolayer coverage. After binding, there was no possibility of other trypsin molecules interacting with the same trypsin inhibitor, but this bond could be dissociated during the elution process to recover the maximum amount of trypsin. From the slopes, the maximum trypsin adsorption capacity was found to be close to the experimental results with this affinity membrane. Thus, the measured trypsin adsorption isotherm can be reasonably described by a Langmuir adsorption isotherm model, with a maximum adsorption capacity of 0.418 mg/cm^2. The K_d value obtained (0.02) indicated moderate affinity, which is consistent with easy adsorption and is advantageous for good recovery of trypsin.

The ability of the Freundlich model to fit the experimental data was also determined. In this case, the plot of log C versus log q was employed to generate the intercept value of K_F and the slope of n. The Freundlich constants, K_F and n, were found to be 1.067 and 0.487, respectively. The magnitude of K_F and n also showed easy adsorption of trypsin from an aqueous medium and

indicated favorable adsorption. The experimental equilibrium data also fit the Freundlich model well since the value of R^2 was greater than 0.95. Thus, the adsorption of trypsin on the affinity membrane can also be modeled using the Freundlich isotherm.

■4.0 CONCLUSION

The results show that the initial trypsin concentration and the physico-chemical environment of the trypsin solution significantly influence trypsin adsorption onto the affinity membrane develop din this study. Optimum trypsin adsorption was found at pH 7 with an ionic strength of 0.1 M using a 0.9 mg/ml trypsin solution. The experimental data fit better to the Langmuir isotherm, which indicates that monolayer adsorption occurred with this affinity system.

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References

[1] Lin, S.-Y., Suen, S.-Y. 2002. Protein Separation Using Plate-and-Frame Modules with Ion-Exchange Membranes. *J. Membr. Sci*. 204: 37–51.

[2] Shi, W., Zhan, F., Zhang,. G. 2005. Adsorption of Birubin with Polylysine Carrying Chitosan-coated Nylon Affinity Membrane. *J. Chromatogr*. B. 819: 301–306.

[3] Chang, C.-S., Suen S.-Y. 2006. Modification of Porous Alumina Membranes with N-Alkanoic Acids and Their Application in Protein Adsorption. *J. Membr*. Sci. 275: 70–81.

[4] Chen, T.-X., Nie, H.-L., Li, S.-B., Branford-White, C., Su, S.-N. Zhu, L.-M. 2009. Comparison: Adsorption of Papain Using Immobilized Dye Ligands on Affinity Membranes. *Colloids Surf. B*. 72: 25–31.

[5] Urmenyi, A. M., Poot, A. A., Wessling, M., Mulder, M. H. V. 2005. Affinity Membranes for Hormone Removal from Aqueous Solutions. *J. Membr. Sci*. 259: 91–102.

[6] Cattoli, F., Boi, C., Sorci, M., Sarti, G. C. 2006. Adsorption of Pure Recombinant MBP-Fusion Proteins on Amylose Affinity Membranes. *J. Membr. Sci*. 273: 2–11.

[7] Boi, C., Cattoli, F., Facchini, R., Sorci, M., Sarti, G. C. 2006. Adsorption of Lectins on Affinity Membranes. *J. Membr. Sci*. 273: 12–19.

[8] Bayramoğlu, G., Celik, G., Arica, M. Y. 2006. Immunoglobulin G Adsorption Behavior of L-Histidine Ligand Attached and Lewis Metal Ions Chelated Affinity Membranes. *Colloids Surf. A*. 287: 75–85.

[9] Nie, H.-L., Chen, T.-X., Zhu, L.-M. 2007. Adsorption of Papain on Dye Affinity Membranes: Isotherm, Kinetic, and Thermodynamic Analysis. *Sep. Purif. Technol*. 57: 121–125.

[10] Zydney, A. L., Kuriyel, R. 2000. *Methods in Biotechnology: Downstream Protein Processing*. Totowa: Humana Press.

[11] Reis, R. V., Zydney, A. 2007. Bioprocess membrane Technology. *J. Membr. Sci*. 297: 16–50.

[12] Bayramoğlu, G., Yılmaz, M., Arıca, M. Y. 2003. Affinity Dye–ligand Poly (Hydroxyethyl Methacrylate) Chitosan Composite Membrane for Adsorption Lysozyme and Kinetic Properties. *Biochem. Eng. J*. 13: 35–42.

[13] Bayramoğlu, G., Oktem, H. A., Arica, M. Y. 2007. A Dye–ligand Immobilized Poly (2-Hydroxyethylmethacrylate) Membrane Used for Adsorption and Isolation of Immunoglobulin G. *Biochem. Eng. J*. 34: 147–155.

[14] Guo, W., Ruckenstein E. 2003. Separation and Purification of Horseradish Peroxidase by Membrane Affinity Chromatography. *J. Membr. Sci*. 21: 101–111.

[15] Sun, Y., Liu, Y., Li, Y., Lv, M., Li, P., Xu, H., Wang. L. 2011. Preparation and Characterization of Novel Curdlan/Chitosan Blending Membranes for Antibacterial Applications. *Carbohydr. Polym*. 84: 952–959.

[16] Boributh, S., Chanachai, A., Jiraratananon, R. 2009. Modification of PVDF Membrane by Chitosan Solution. *J. Membr. Sci.* 342: 97–104.

[17] Varghese, J. G., Kittur, A. A., Rachipudi, P. S., Kariduraganavar, M. Y. 2010. Synthesis, Characterization and Pervaporation Performance of Chitosan-G-Polyaniline Membranes for the Dehydration of Isopropanol. *J. Membr. Sci*. 364: 111–121.

[18] Mansur, H. S., Sadahira, C. M., Souza, A. N., Mansur, A. A. P. 2004. FTIR Spectroscopy Characterization of Poly (Vinyl Alcohol) Hydrogel with Different Hydrolysis Degree and Chemically Crosslinked with Glutaraldehyde. *Mater. Sci. Eng. C*. 28: 539–548.

[19] Aravind, U. K., Mathew, J., Aravindakumar, C. T. 2000. Transport Studies of BSA, Lysozyme and Ovalbumin Through Chitosan/Polystyrene Sulfonate Multilayer Membrane. *J. Membr. Sci*. 299: 146–155.

[20] Bayramoğlu, G., Yılmaz, M., Şenel, A. Ü., Arıca, M. Y. 2008. Preparation of Nanofibrous Polymer Grafted Magnetic Poly (GMA-MMA)-G-MAA Beads for Immobilization of Trypsin Via Adsorption. *Biochem. Eng. J*. 40: 262–274.

[21] Gustafsson, H., Thörn, C., Holmberg, K. 2011. A Comparison of Lipase and Trypsin Encapsulated in Mesoporous Materials with Varying Pore Sizes and Ph Conditions. *Colloids Surf. B*. 87: 464–471.

[22] Li, X., Zhang, Y., Fu, X. 2004. Adsorption of Glutamicum Onto Polysulphone Membrane. *Sep. Purif. Technol*. 37: 187–198.

[23] Wan, Y. H., Lub, J. R., Cui, Z. F. 2006. Separation of Lysozyme from Chicken Egg White Using Ultrafiltration. *Sep. Purif. Technol*. 48: 133–142.

[24] Muller, C. H., Agarwal, G. P., Melin, T., Wintgens, T. 2003. Study of UF of a Single and Binary Protein Solution in a Thin Spiral Channel Module. *J. Membr. Sci*. 227: 51–69.

[25] Lu, A. X., Liao, X. P., Zhou, R. Q., Shi, B. 2007. Preparation of Fe(III)-immobilized Collagen Fiber for Lysozyme Adsorption. *Colloids Surf. A* 301: 85–93.

[26] Tung, K. L., Hu, C. C., Li, C. L., Chuang, C. J. 2007. Investigating Protein Crossflow Ultrafiltration Mechanisms Using Interfacial Phenomena. *J. Chin. Inst. Chem. Engrs*. 38: 303–311.

[27] Bayramoğlu, G., Altintas, B., Arica, M. Y. 2009. Adsorption Kinetics and Thermodynamic Parameters of Cationic Dyes from Aqueous Solutions by Using a New Strong Cation-exchange Resin. *Chem. Eng. J*. 152: 339–346.