Separation and Purification of Lipase Using Cu Nanoparticle Embedded Poly(HEMA-MATrp) Cryogels

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ABSTRACT

Quality and efficiency of techniques to be used for separation and purification lipase enzymes are commercially important enzyme. Among such techniques, adsorption methods are highly preferred. Cryogels have been quite extensively used as the adsorbents due to their macropores and interconnected flow channels. In this study, adsorption of lipase enzyme onto copper nanoparticles embedded poly(2-hydroxyethyl methacrylate-N-methacryloyl-L-tryptophan), poly(HEMA-MATrp) cryogels was studied for conditions with varying pH, interaction time, lipase enzyme initial concentration, temperature and ionic strength. Maximum lipase enzyme adsorption capacity of cryogels was determined as 183.6 mg/g. Fourier transform infrared spectrometer (FTIR) and scanning electron microscopy (SEM) were used for characterization of cryogels. At the end of the adsorption process, in order to be sure that the purity of lipase enzyme desorbed from cryogels, SDS-PAGE analyses were performed and molecular weight of the lipase enzyme was determined as 58 kDa. Adsorption characteristic of cryogels were determined according to the results of Langmuir and Freundlich adsorption isotherm models. As a result of calculation run for adsorption isotherm models, Langmuir isotherm model was determined to be more appropriate.

Key Words:
Lipase; Copper; Cryogel; Adsorption.

INTRODUCTION

Lipase (EC 3.1.1.3) is a commercially important enzyme and play an important role in biocatalytic transformation reactions [1-5]. Lipase enzymes digest triacylglycerol into glycerol, free fatty acid, monoacylglycerol and diacylglycerol [6-7]. These enzymes are also able to catalyze esterification, interesterification, transesterification, aminolysis, thiotransesterification and oximolysis reactions [6-8]. Lipase enzyme has been utilized several fields such as production and degradation of biopolymer, pharmaceutical, agrochemical, biolubricants, cosmetic, flavours and fragrances, oil-rich water treatment and esterification via short chain alcohols and biodiesel production using transesterification reactions [11-17].

In recent years, for separation and purification of enzymes, adsorption technique has been preferred. Synthetic and natural adsorbents have been used for adsorption experiments. Cryogels have a great place among synthetic adsorbents due to their advantages such as easy preparation, being cost-friendly, having large pores, and interconnected flow channels. These polymeric structures formed as a result of freezing of solvent initially and de-freezing again at room temperature have hydrophilic character. High porosity of these structures provide them almost sponge-like structure. Reusability feature of these structures is also quite efficient. Due to these features and their elastic properties provide a great advantage for cryogel structures [18-25].

Cu nanoparticles were embedded into poly(2-hydroxyethyl methacrylate-N-methacryloyl-L-tryptophan) cryogel structure synthesized in this study and adsorption of lipase enzyme from aqueous solution was
examined using this synthetic material. Cu nanoparticles are considered to increase the electrostatic character of interactions emerge during adsorption reaction. Therefore, a positive contribution of this effect is expected for the adsorption capacity as increasing. The method used in this study is considered to be an efficient alternative for techniques in the literature used for separation and purification of lipase enzyme.

MATERIALS AND METHODS

Lipase (from Candida cylindracea), 2-hydroxyethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EGDMA), L-tryptophan, methacryloyl chloride, sodium nitrite (NaNO₂), potassium carbonate (K₂CO₃) and ethyl acetate were purchased from Aldrich (St. Louis, MO, USA). N, N, N', N'- tetramethyl ethylene diamine and ammonium persulphate compounds were obtained from Sigma (Munich, Germany). Ascorbic acid was from Fluka (St. Gallen, Switzerland). Diethyl ether, cyclohexane, and copper (II) sulphate penta hydrate (CuSO₄·5H₂O) compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). UV-VIS Double Beam PCR 8 Scanning Auto Cell UVD-3200 (Labomed, INC.) (USA) device was used for spectrometric determinations at UV-VIS region. N-methacryloyl-L-tryptophan compound was synthesized in laboratory in accordance with literature [26]. All other chemicals were of analytical grade.

MATrp Synthesis

As a first step, L-tryptophan of 5 g and NaNO₂ of 0.2 g compounds were dissolved in an aqueous solution of K₂CO₃ of 30 mL 5% (w/v) and just then the solution was cooled to 0°C. After that step, 4 mL of methacryloyl chloride was added drop wise in nitrogen gas (N₂) environment. The solution obtained was stirred for 2 hours at room temperature using magnetic stirrer and then the pH of solution was adjusted to 7. The solution was subjected to extraction process using ethyl acetate. The liquid phase was removed via evaporator and MATrp was obtained by crystallization with diethyl ether and cyclohexane [26].

Poly(HEMA-MATrp) Cryogel Synthesis

2-Hydroxyethyl methacrylate (HEMA, 2.5 mL) as a structural monomer and N-methacryloyl-L-tryptophan (MATrp, 50 mg) as a functional monomer were dissolved in 2.5 mL distilled water. The mixture of 0.5 g sodium lauryl sulphate (SLS), 0.6 mL ethylene glycol dimethacrylate (EGDMA) and 9.4 mL distilled water was added to the solution obtained previously. Last mixture was stirred with a magnetic stirrer until obtaining a homogeneity and was remained in an ice bath for approximately 15 minutes. In the final stage, ammonium persulphate (APS) of 10 mg and N, N, N', N'- tetramethyl ethylenediamine (TEMED) of 50 μL were added and were remained at -12°C for 24 hours. Cryogels synthesized was gone disk-shape cut (membrane) and washed with distilled water several times until all unwanted particles were removed.

Cu Nanoparticle Synthesis

CuSO₄·5H₂O of 0.001 mole and ascorbic acid of 0.011 mole were dissolved in 100 mL distilled water. pH of the solution was adjusted to about 6.5 using NaOH solution and the solution was stirred in a flask with a magnetic stirrer at 1000 rpm at 85°C for 1 hour [27]. Towards the end of the process, color of solution was turned from orange to brown. The solution obtained was centrifuged at 12000 rpm for 30 minutes. Copper nanoparticles precipitated was dried on watch-glass in an oven. Chemical reduction reactions occurring in this process were as follows:

\[
\text{Cu}^{2+} + 2\text{OH}^- \rightarrow \text{Cu(OH)}_2
\]

\[
\text{Cu(OH)}_2 + \text{C}_6\text{H}_8\text{O}_6 \rightarrow \text{Cu(k)} + \text{C}_6\text{H}_6\text{O}_6 + 2\text{H}_2\text{O} [28]
\]

Embedding of Cu Nanoparticles into Structure of poly(HEMA-MATrp) Cryogels

For this operation, Cu nanoparticles were incorporated into cryogel structure with the concentration of 100 mg/L in distilled water of 25 mL and this solution was stirred with magnetic stirrer continuously for 2 hours. As a result of these processes, colour of the solution was turned from white to light yellow and to get rid of unwanted particles cryogels were washed several times with distilled water (Figure 2).
Characterization of poly(HEMA-MATrp) Cryogels
Swelling Test
Firstly, a cryogel sample was dried by lyophilisation and then was carefully weighed. To understand the swelling ratio dry sample was placed in a baker of distilled water within the isothermal water bath at 25°C for 30 minutes to obtain fully swelled cryogel membrane. At the end of this process the swelled cryogel sample was re-weighed carefully and water retention capacity of the membrane was determined by the following equation:

\[
\text{Water retention capacity\%} = \left(\frac{W_s - W_o}{W_o}\right) \times 100 \quad (1)
\]

In this equation, \(W_o\) and \(W_s\) stand for weights (g) of dry and swelled cryogels.

Surface Morphology
To determine the surface morphology of cryogels, scanning electron microscopy (SEM) (Carl Zeiss AG - EVO® 50 Series, Germany) was used. For this operation, as a first step, cryogel samples were dried and lyophilized for SEM analysis. Then a sufficient amount of sample was placed on SEM holder and analysed after coated with a thin gold layer at vacuum and at the end images were taken.

FTIR Analysis
For this operation, Fourier transform infrared spectroscopy (Thermo Scientific, Nicholet IS10, USA) was used. Pellets had been prepared primarily for analysis. To prepare pellets, dry cryogel sample of 2 mg and dry KBr powder of 98 mg were used and then FTIR analysis was performed.

Adsorption Studies
Adsorption studies were carried out via batch system. Adsorption medium was prepared with the use of lipase solution of 1 mL and buffer solution of 4 mL. Before adding cryogel membranes lipase and buffer solution was stirred at magnetic stirrer for 15 min. and equilibrated.

To calculate the adsorption capacity following equations is utilized.

\[
q = \frac{[(C_i - C_f) \times V]}{m} \quad (2)
\]

wherein, \(q\) is adsorption capacity (mg/g), \(C_i\) is the concentration of lipase enzyme before adsorption (mg/L), \(C_f\) is the concentration of lipase enzyme after completion of adsorption (mg/L), \(V\) is the volume of the adsorption medium (L), and \(m\) is the mass of cryogel (g).

Desorption and Reusability
Batch experiments were preferred for desorption of lipase adsorbed on cryogels. For this operation, lipase adsorbed cryogels were stirred continuously with magnetic stirrer for 1 hour in desorption medium having HCl solution (0.1 M, 10 mL) for 1 hour. To examine the reusability of cryogels, adsorption-desorption cycle was repeated 5 times with the same cryogel membrane. Cryogel used was washed with NaOH solution (0.1 M ) of 10 mL for 30 minutes and to equilibrate this solution pH:6.0 buffer solution of 10 mL was used to treat cryogels 30 minutes. Desorption rate was calculated by the following equation.

\[
\text{Desorption rate (\%)} = \frac{\text{Amount of Enzyme Desorbed}}{\text{Total Amount of Enzyme}} \times 100 \quad (3)
\]

RESULTS AND DISCUSSION
Characterization of poly(HEMA-MATrp)
Swelling Test
Swelling test was performed the method mentioned before. Weight of dry and water swelled cryogel membranes was determined as 29.6 and 164.3 mg/disc, respectively. According to these results, water retention capacity of cryogels was calculated as in Equation 2.1 and found as 455%.

Surface Morphology
To determine surface morphology of cryogels, SEM images of membranes were taken (Figure 3). As the figure shows, a macroporous structure containing interconnected flow channels was obtained.
FTIR Analysis

Molecular structure and FTIR spectrum of poly(HEMA-MATrp) cryogels are shown by Figure 4 – 5, respectively. From spectrum, 3424 cm⁻¹ (OH stretching), 2941 cm⁻¹ (CH stretching for aliphatic alkyl), 1710 cm⁻¹ (C=O stretching), 1649 cm⁻¹ (C=C stretching), 1446 and 1381 cm⁻¹ (C-N stretching for amide) and 1147 cm⁻¹ (aromatic ring bending) bands are quite noteworthy. Existence of some of functional groups corresponding these bands (C=C stretching, C-N stretching for amide, aromatic ring bending) within MATrp structure denotes that MATrp monomer was successfully incorporated into HEMA structure.

Adsorption Studies

Effect of pH

To determine the effect of pH on the lipase adsorption capacity of cryogels, pH of solutions used for adsorption studies was changed in the pH range of 3.0-10.0. According to the consideration of results, adsorption capacity of cryogels was maximum at pH: 6.0. It is concluded that lipase molecules are interacted with Cu nanoparticles bound indol ring at the functional monomer (MATrp) via electrostatic interactions. These interactions are directly related with charge distribution of polar groups (aspartate, lysine, arginine, etc.) on lipase enzymes, and these interactions are most stable at pH: 6.0 and suitable for electrostatic interactions. Therefore, it is determined that interactions at pH: 6.0 are the most effective so this pH is set at optimum pH value.

Effect of Interaction Time

To investigate the effect of interaction time on the adsorption of lipase onto cryogels, adsorption experiments were performed at the time range of 5-75 minutes. At the end of the experiments, it was determined that equilibrium adsorption capacity was
achieved at 30th minute (Figure 7). Porous structure and interconnected flow channels of Cu embedded cryogels synthesized enable interaction to be occurred rapidly. Therefore, optimal interaction time was determined as 30 min. and all remaining studies were performed with respect to this time period.

Effect of Initial Concentration of Lipase Enzyme

To determine the effect of initial concentration on the lipase adsorption capacity of Cu embedded cryogels, adsorption studies were performed for the amount of lipase concentration of 0.5-3 mg/mL. As a result of the experiments, it was observed that adsorption capacity of cryogels was increased with increased initial concentration lipase enzyme for the beginning of adsorption process, but a bit after there was a steady state on the adsorption (Figure 8). The reason for this might be that lipase binding sites of cryogels had been reached the saturation after certain concentration.

Effect of Temperature

In order to determine the effect of temperature on the adsorption of lipase, adsorption experiments were conducted at four different temperatures (7, 20, 30 and 40°C). As a result of experiments conducted, adsorption capacities of cryogels were decreased with increasing temperature as expected (Figure 9). The reason for this is that coordinated covalent bonds, interactions occurring via shared electron are weakened and reduced as a result of severances [29].

Effect of Ionic Strength

In this study, NaCl solutions with concentration range of 0.5-4.0 M were used to determine the effect of ionic strength on the adsorption of lipase enzyme. Considering Figure 10, the adsorption capacity was decreased with increasing salt concentration. This is because of presence of ions (Na⁺ and Cl⁻) coming from NaCl molecules in the medium and thus these ions effect the charge distribution of groups such as aspartate, lysine, arginine on the surface of lipase enzyme. Na⁺ and Cl⁻ ions are interacted with these groups electrostatically, and so limit by masking the interaction having potential to occur between Cu nanoparticles and lipase enzyme. Therefore, the adsorption capacity decreases with increasing ionic strength.

Desorption and Reusability

In order to determine the reusability feature of cryogels, adsorption - desorption cycle was repeated 5 times using same cryogels. As a result of this study, desorption ratio of cryogels was determined as 78% and there was no significant decrease observed in the adsorption capacity (from 172.8 mg / g to 164.8 mg / g) (Figure 11). Considering these results, it is concluded that Cu nanoparticle embedded cryogels are interacted specifically and reversibly with lipase enzyme, and can be said to have high reusability ratio.
SDS-PAGE Analysis

SDS-PAGE analysis of lipase purified using Cu nanoparticle embedded poly(HEMA-MATrp) cryogels is shown at Figure 12. Because the distance of lipase desorbed from cryogels synthesized in this study covered on poly acrylamide SDS gel is exactly same with the distance covered by lipase marker (58 kDa), it can be concluded that purity of lipase desorbed from cryogels is quite acceptable and adsorption-desorption performance achieved successfully using Cu nanoparticle embedded poly(HEMA-MATrp) cryogels.

Adsorption Isotherms

Adsorption isotherms were investigated to characterize the lipase adsorption process performed using Cu nanoparticle embedded poly(HEMA-MATrp) cryogels. According to the Langmuir adsorption model, adsorption is considered as a monolayer (homogeneous) on the surface [30]. However, Freundlich adsorption isotherm model provides multi-layer adsorption layer so thus heterogeneous [31]. For Langmuir and Freundlich adsorption isotherms, following equations are used:

\[
\frac{1}{Q_{eq}} = \frac{1}{Q_{max}} + \frac{1}{b C_{eq}} \quad \text{Langmuir equation (4)}
\]

\[
\ln Q_{eq} = \ln K_f + (n \times \ln C_{eq}) \quad \text{Freundlich equation (5)}
\]

In this equation, \( Q_{eq} \) is the adsorption capacity (mg/g), \( C_{eq} \) is the lipase concentration at equilibrium, \( b \) is the Langmuir adsorption constant (L/mg), and \( Q_{max} \) is the maximum adsorption capacity (mg/g).

Regression coefficient obtained from the graph plotted for Langmuir isotherm model (0.9976) is higher than that (0.9080) from the graph plotted for Freundlich isotherm model (Table 1). Therefore, it can be concluded that Langmuir adsorption isotherm model is more suitable for the adsorption reaction of lipase onto Cu nanoparticles embedded poly(HEMA-MATrp) cryogels. In other words, adsorption reaction was achieved on the surface as monolayer (homogeneous).

Table 1. Parameters estimated from Langmuir and Freundlich adsorption isotherms.

<table>
<thead>
<tr>
<th>Langmuir Constants</th>
<th>Freundlich Constants</th>
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<tr>
<td>( Q_{eq} ) (mg/g)</td>
<td>( Q_{eq} ) (mg/g)</td>
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<td>( Q_{max} ) (mg/g)</td>
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<td>( b ) (L/mg)</td>
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Figure 12. SDS-PAGE image for lipase enzyme desorbed from *Candida cylindracea*. Lane 1: Marker (Lipase, Lysozyme, Hemoglobin) Lane 2: Lipase marker, Lane 3: Initial lipase solution ([Before adsorption for Cu nanoparticle embedded poly(HEMA-MATrp) cryogels]). Lane 4: Final lipase solution ([After adsorption for Cu nanoparticle embedded poly(HEMA-MATrp) cryogels]). Lane 5: Desorbed sample ([After desorption from Cu nanoparticle embedded poly(HEMA-MATrp) cryogels].

Figure 13. Langmuir adsorption isotherm plotted from experimental values.

Figure 14. Freundlich adsorption isotherm plotted from experimental values.

In this equation, \( K_f \) and \( n \) represent Freundlich adsorption isotherm constants. \( \ln K_f \) and \( n \) can be calculated using y - intercept point and slope from the graph \( \ln Q_{eq} \) versus \( \ln C_{eq} \), respectively (Figure 14).
CONCLUSIONS

In this study, adsorption of lipase enzyme onto Cu embedded poly (HEMA-MATPr) cryogels was ensured by electrostatic interactions. Moreover, decreasing adsorption capacity with increasing temperature and ionic strength confirm the presence of this kind of interaction in this study. Because interactions such as coordinate covalent bond, occur via shared electrons, ionic interactions are inversely proportional with temperature and ionic strength. In conclusion, it is determined that the Langmuir adsorption model is more appropriate model for adsorption for this study. In other words, adsorption was achieved on the surface as mono-layer but not as multi-layer.

REFERENCES


