Research Article

Nuri Gulesci, Guzide Yucebilgic* and Deniz Yildirim

Different spacer-arm attached magnetic nanoparticles for covalent immobilization of Jack bean urease

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Abstract

Objectives: This study aims the covalent immobilization of Jack bean urease on Fe3O4 magnetic nanoparticles via glutaraldehyde (urease@MNPs-Si-Glu) and epichlorohydrin (urease@MNPs-ECH) spacer arms.

Methods: The optimum pH and temperature, thermal, storage and reuse stability of free and immobilized urease preparations were investigated. Thermodynamics characterizations of free and immobilized urease preparations were also studied.

Results: The free urease and both immobilized urease preparations showed maximal catalytic activity at pH 7.5. The free urease had a maximal catalytic activity at 50 °C, while the both immobilized urease preparations exhibited their maximal catalytic activities at 70 °C. The urease@MNPs-Si-Glu and urease@MNPs-ECH showed 2.7- and 1.9-fold higher thermal stability than the free urease at 60 °C, respectively. The free urease remained 30% of their initial activity at 4 °C, while urease@MNPs-Si-Glu and urease@MNPs-ECH retained 72 and 60% of their initial activities at the same conditions. The urease@MNPs-Si-Glu and urease@MNPs-ECH preserved 53 and 52% of their initial activities, respectively for urea hydrolysis after 20 reuses.

Conclusions: The urease@MNPs-Si-Glu and urease@MNPs-ECH may find a potential application area in urea hydrolysis.

Keywords: epichlorohydrin; glutaraldehyde; immobilization; magnetic nanoparticles; urease.

Introduction

Ureas (urea amidohydrolases, EC 3.5.1.5) are a group of highly efficient enzymes, catalyzing the hydrolysis of urea to produce ammonia and carbon dioxide [1]. They are widely distributed in bacteria, fungi, and plants and play an important role in the circulation of nitrogen in nature [2]. The ureases are widely used for the determination of urea amounts in blood, urine, alcoholic beverages, natural water, and environmental wastewater samples [3]. However, the limited stability as well as reusability of free ureases in industrial applications are some important disadvantages for the adaptation of ureases as industrial catalysts. To increase the stability of the enzymes, the protein engineering [4] and chemical modification techniques [5] have been successfully employed. However, the reuses of enzymes are one of the most important key parameters to make the enzyme-driven processes economically viable. Immobilization of enzymes enables the enzymes to recover and reuse [6]. Enzyme immobilization can also modulate some enzymatic features such as stability, specificity and selectivity [7]. Furthermore, subunit dissociation of multimeric enzymes can be prevented by a proper immobilization technique. Therefore, the various methods for urease immobilization have been developed including adsorption [8], entrapment [9], covalent bonding [10], and cross-linked enzyme lyophilisates [11] however; the preparation of robust urease samples is currently challenged.

In the last decade, there has been growing interest in nanoparticles for use in the immobilization of enzymes [12]. Among various nanoparticles, Fe3O4 magnetic nanoparticles (MNPs) have been regarded as suitable carriers due to their large surface area, low toxicity, ease of separation under external magnetic fields, high biocatalyst loading capacity and high mass transfer properties [13]. However, the surface of bare MNPs should be coated with an outer protective layer.
to maintain the stability of the MNPs which are highly sensitive to acidic and oxidative conditions [14].

Covalent enzyme immobilization is known to effectively eliminate or significantly reduce the leakage of enzymes [15, 16]. Immobilization of enzymes on carriers via a spacer arm is one of the good ways to avoid the steric hindrance and to increase enzyme activity. The activation of supports having primary amino groups with glutaraldehyde and then covalent immobilization of enzyme onto glutaraldehyde-activated support are one of the mostly used covalent enzyme immobilization strategies thanks to great versatility of glutaraldehyde molecule [17]. Depending on the remaining primary amino group amount after glutaraldehyde activation, the obtained support may be considered a heterofunctional support [18]. Another of the commonly used supports in covalent enzyme immobilization is epoxy group containing supports since they can react with enzymes at neutral or alkaline pH values to obtain a stable immobilized enzyme preparation [19–21].

The aim of the present work was the comparison of different immobilization protocols on the urease activity and stability. For this purpose, MNPs were synthesized from ferrous chloride and ferric chloride in basic medium. The synthesized MNPs were separately modified with glutaraldehyde and epichlorohydrin spacer arms. Then, urease was covalently immobilized onto MNPs via glutaraldehyde and epichlorohydrin to address the stabilization issues. Furthermore, thermodynamic parameters such as enthalpy, free energy and entropy (ΔS°, J mol⁻¹ K⁻¹) for thermal denaturation of free and immobilized urease preparations have been studied in detail for the first time.

Materials and methods

Chemicals

Urease from Jack bean (lyophilized 1 U/mg), ferrous chloride hexahydrate (FeSO₄·7H₂O), ferric chloride hexahydrate (FeCl₃·6H₂O), ammonium sulfate (NH₄)₂SO₄, urea, sodium carbonate (Na₂CO₃), sodium hydroxide (NaOH), phosphoric acid (H₃PO₄), (3-aminopropyl)triethoxysilane (3-APTES), epichlorohydrin (ECH), glutaraldehyde and used without further purification.

Synthesis of magnetic nanoparticles

MNPs were prepared by using the co-precipitation method [22]. Fifty milliliters of FeCl₃ (0.1 M in water) and FeSO₄ (0.05 M in water) was mixed in a beaker at room temperature for 30 min. Subsequently, NaOH solution (1.0 M in water) was added dropwise until black MNPs were formed. The separation of formed MNPs was achieved by a permanent magnet. Then, MNPs were rinsed with deionized water and ethanol. Finally, MNPs were dried at 75 °C for 4 h. The average particle size of the synthesized MNPs is 158.2 nm.

Attachment of glutaraldehyde spacer arm on MNPs

Before the glutaraldehyde spacer arm attachment, MNPs were functionalized with 3-APTES solution. One gram of MNP was silanized with 25 mL of 3-APTES solution (4% in acetone) at 45 °C [23]. After 12 h reaction time, the silanized MNPs were sequentially washed with acetone and distilled water. The silanized MNPs were dried at room temperature and kept in a capped glass tube until use. The amount of free primary amine groups after 3-APTES silanization was determined by using ninhydrin reagent according to Alptekin et al. [23]. Briefly, 100 µL of distilled water and 200 µL of ninhydrin solution (2%) was added onto 10 mg of silanized MNPs in a capped glass tube. The mixture was heated in boiling water for 30 min and then cooled at room temperature. The mixture was diluted with 5 mL of ethanol/water mixture (1/1, v/v) and the absorbance of solution was spectrophotometrically measured at 570 nm.

For glutaraldehyde activation, 25 mL of glutaraldehyde solution (2.5% in 50 mM phosphate buffer pH 7.0) was added onto 1 g of silanized MNPs [23]. The mixture was continuously stirred for 2 h at room temperature. After that the obtained particles (MNPs-Si-Glu) were washed with deionized water to remove the excess glutaraldehyde. The remaining primary amine groups after glutaraldehyde activation were determined by using ninhydrin reagent as mentioned before.

Attachment of epichlorohydrin spacer arm on MNPs

For ECH spacer arm attachment, 1 g of MNPs were mixed with 10 mL NaOH solution (1.7 M in water) containing 0.34 g of sodium borohydride. The mixture was stirred for 2 h at room temperature. Then, 3.6 mL of ECH was added and the mixture was incubated at 25 °C for 24 h. The obtained supports (MNPs-ECH) were rinsed with deionized water and kept at 5 °C for 24 h. The epoxy group amount of MNPs-ECH was determined according to Axen et al. [24]. Briefly, 0.5 g of MNPs-ECH support was treated with 2.6 mL of sodium thiosulfate (1.3 M in water). The formed NaOH amount was determined by titrating NaOH by standardized HCl solution (0.1 M in water). The amount of epoxy group is equivalent to the amount of NaOH formed.

Urease immobilization

One gram of MNPs-Si-Glu was treated with 4 mL phosphate buffer (50 mM, pH 7.5) containing urease (0.25 mg/mL). The mixture was stirred for 2 h at 100 rpm, 4 °C. At the end of 2 h, the immobilized particles (urease@MNPs-Si-Glu) were collected by filtration and washed with the phosphate buffer until no protein was detected in the filtrate [25]. The immobilized urease preparations were stored at 4 °C until used. For the immobilization of urease onto MNPs-ECH, 1.0 g of the support was mixed with 4 mL phosphate buffer (50 mM, pH 7.5) containing urease (0.25 mg/mL) [25]. After 12 h stirring time at 100 rpm, 4 °C, the immobilized particles (urease@MNPs-ECH) were collected by filtration and rinsed with distilled water. The filtrate was used for the determination of protein content [26].
Urease activity assay

The free and immobilized urease activities were measured spectrophotometrically using urea as the substrate at pH 7.0 and 30 °C [27]. The amount of liberated ammonia after urease activity was coloured with Nessler’s reagent and the absorbance of colored complex was spectrophotometrically measured against a blank at 480 nm. One unit (1 U) urease activity was defined as the amount of enzyme that produced 1 µmol ammonia per min under assay conditions.

Analysis of xylanase samples

IR spectra of free urease, surface morphology and XRD patterns of samples were performed according to Alagöz et al. [28]. The magnetic property of immobilized urease preparations was determined by a vibrating sample magnetometer under magnetic fields at 300 K.

Characterization of free and immobilized ureases

The effect of pH on the activity of free and immobilized urease preparations was conducted by using a different buffer solution. The free and immobilized urease activity was measured for varying temperatures (30–80 °C) at pH 7.5.

Thermal stability of free and immobilized urease preparations was investigated at different temperatures (50, 60, and 70 °C) under optimal assay conditions. The first-order inactivation constant (k), half-life (t½), values, stabilization factors (SF) and decimal reduction time values (D-values) of free and immobilized urease preparations were calculated from the equations [28],

\[ t_{½} = \ln 2 \]  \hspace{1cm} (1)

where ln 2 is residual activity of 50%.

\[ D \text{ value} = \ln 10 / k_d \]  \hspace{1cm} (2)

SF values were calculated by dividing half-life of the immobilized urease to free urease at the corresponding temperature.

The z-value, the deactivation energy (Eₐ) values of free urease, urease@MNPs-Si-Glu and urease@MNPs-ECH and the changes in enthalpy (ΔH*, kJ mol⁻¹), free energy (ΔG*, kJ mol⁻¹) and entropy (ΔS*, J mol⁻¹K⁻¹) for thermal denaturation of free urease, urease@MNPs-Si-Glu and urease@MNPs-ECH were calculated according to Mostafa et al. [29].

The z-value was calculated from the slope of the graph between log D vs. T (°C) using the equation:

\[ \text{Slope} = -1/z \]  \hspace{1cm} (3)

\[ E_d \text{ value} \text{ was determined from the slope of the Arrhenius plot of log denaturation rate constants (ln kₐ) vs. reciprocal of the absolute temperature (k).} \]

\[ \text{Slope} = -E_d / R \]  \hspace{1cm} (4)

The change in ΔH*, ΔG*, and ΔS* values were determined using the following equation:

\[ \Delta H^* = E_d - RT \]  \hspace{1cm} (5)

\[ \Delta G^* = -RT \ln (k_xh/k_BT) \]  \hspace{1cm} (6)

\[ \Delta S^* = (\Delta H^* - \Delta G^*) / T \]  \hspace{1cm} (7)

where T is the corresponding absolute temperature (K), R is the gas constant (8.314 J mol⁻¹ K⁻¹), h is the Planck constant (11.04 × 10⁻⁵ J min), and kₐ is the Boltzmann constant (1.38 × 10⁻²³ J K⁻¹).

The storage stability experiments were performed by measuring the residual activity of free and immobilized ureases at 4 °C and 25 °C.

The reusability of immobilized urease preparations was carried out using a batch reactor. Each immobilized urease (50 mg) was separately loaded into a reactor and then the substrate solution was added. After 10 min reaction time, the immobilized urease preparations were recovered from the reaction mixture with a magnet, washed with phosphate buffer (50 mM, pH 7.5), and used for the next batch. The ammonia content of each filtrate was determined using Nessler’s reagent. These measurements were repeated 20 times.

Results and discussion

In this study, the urease was covalently immobilized on MNP’s to obtain stable and reusable urease preparations. The surface of MNP’s was functionalized with 3-APTES and epichlorohydrin before the covalent immobilization and the silanized MNP’s were further activated with glutaraldehyde molecules (Supplementary Figure 1). The amounts of introduced primary amine groups on MNP’s after 3-APTES silanization were found to be 270 µmol/g of support. The amount of remaining primary amine groups were determined as 59 µmol/g support after glutaraldehyde coupling, indicating that the reaction between primary amine group and glutaraldehyde successfully occurred. These results also showed that 78% of total primary amine groups were reacted with glutaraldehyde molecules. According to these results, the support has a heterofunctional property and urease immobilization may occur not only by covalent immobilization but also by ionic immobilization.

When epichlorohydrin was used as a spacer arm, the amount of epoxy groups on the surface of magnetic nanoparticles was found as 400 µmol/g of support. The immobilization studies were performed using a lowly loaded protein amount to restrict diffusion limitations derived from protein-protein interaction. The immobilization yields were 63.7% and 87.5% for urease@MNPs-Si-Glu and urease@MNPs-ECH, respectively. The corresponding expressed activity values were 66.3% and 66.7%.

To confirm covalent immobilization, ATR FT-IR spectra of free urease, urease@MNPs-Si-Glu and urease@MNPs-ECH were investigated and the results were shown in Figure 1. The peaks of urease@MNPs-Si-Glu and urease@MNPs-ECH at 550–600 cm⁻¹ and 902 cm⁻¹ is due to Fe-O vibration which indicates the presence of MNPs. The band of urease@MNPs-Si-Glu at 1,050 cm⁻¹ can be attributed to asymmetric vibration of Si–O [30]. The peak of free
urease, urease@MNPs-Si-Glu and urease@MNPs-ECH at 1,645 cm\(^{-1}\) belongs to the N-H bend of peptide bond. The band at 2,922 is due to the C–H stretching vibrations of –CH\(_2\)– groups. The broad peak around 3,345 cm\(^{-1}\) is owing to the –OH groups on the nanoparticle surface or –NH\(_2\) groups of urease molecules.

Figure 2A and D shows the surface morphology of MNPs-Si-Glu and MNPs-ECH supports before the urease immobilization. The bare MNPs-Si-Glu and MNPs-ECH supports had a very smooth surface. However, the surfaces of immobilized urease preparations urease@MNPs-Si-Glu and urease@MNPs-ECH, were changed from uniform to non-uniform (Figure 2B, C, E and F).

The crystalline structure of MNPs before and after urease immobilization was determined by XRD (Figure 3). The peaks at 30.05°, 35.7°, 43.16°, 57.14° and 62.85 belong to Fe\(_3\)O\(_4\) MNPs and have highly cubic inverse spinel structure. The similar characteristic diffraction peaks were observed for MNPs-Si-Glu, MNPs-ECH, urease@MNPs-Si-Glu and urease@MNPs-ECH. These results showed that the crystalline structure of MNPs was not affected from the surface modification and immobilization procedures.

The magnetic properties of pure MNPs and immobilized urease preparations were investigated using VSM analysis at room temperature. The saturation magnetization (\(M_s\)) values of MNPs, urease@MNPs-Si-Glu and urease@MNPs-ECH were

![Figure 1: FT-IR graph of free urease, urease@MNPs-Si-Glu and urease@MNPs-ECH.](image1)

![Figure 2: SEM images of MNPs-Si-Glu and MNPs-ECH supports (A and D), urease@MNPs-Si-Glu (B and C) and urease@MNPs-ECH (E and F).](image2)
determined as 71.6, 55.1 and 69.4 emu/g, respectively (Figure 4). The decrease in $M_s$ value of immobilized urease preparations compared to pure MNPs is due to the increase of thickness of MNPs after functionalization and immobilization procedure. The urease@MNPs-Si-Glu and urease@MNPs-ECH showed typical superparamagnetic behavior and can be separated from the mixture by applying a magnetic field.

The effect of pH on free and immobilized urease activities was investigated in the pH range of 4.5–9.5 and the results are given in Figure 5A. The relative activity values of both immobilized urease preparations were higher than those of free urease in the pH range of 5.5–7.0. The optimum pH values were 7.5 for all the urease preparations. This result showed that the optimum pH of urease was not affected by the immobilization procedure. Kumar et al. [31] reported that the optimum pH of soybean urease was 7.0. The optimum pH of soybean urease shifted to the acidic region when the enzyme was immobilized on alginate beads. However, the optimum pH of soybean urease shifted to the basic region when the enzyme was immobilized on chitosan beads. The relative activity values of free urease, urease@MNPs-Si-Glu and urease@MNPs-ECH were determined as 64%, 58% and 54% at pH 9.0. Sahoo et al. [3] reported the optimum pH values of free Jack bean urease and its immobilized counterpart on phosphonate grafted iron oxide nanoparticles were 7.0 and 8.0, respectively. The optimum pH of free Jack bean urease was reported as 7.3, however, the optimum pH value increased to 8.0 after the immobilization of urease on gelatin beads [32].

Figure 5B shows the effect of the temperature on the activities of free and immobilized urease preparations. The free urease had an optimum at 50 °C, while all the immobilized urease preparations showed a maximum activity at 70 °C. The initial activity of free urease decreased significantly at the temperatures above 50 °C due to the thermal distortion and consequent urease inactivation. However, the immobilized urease preparations showed higher resistance to heat-induced denaturation at high temperature. For example, the free urease lost 60% of its initial activity at 80 °C, while the initial activity loss was only 10% for the both immobilized urease preparations at the same temperature. The higher resistance of immobilized urease preparations at higher temperatures may be explained by the reduction in molecular mobility of urease after the immobilization. Danial et al. [33] determined the optimum temperatures of the free urease from Jack bean and the immobilized urease in alginate gel were both 40 °C and reported that the free urease was completely inactive at 70 °C, while the immobilized urease was still active at 70 °C.

The free urease, urease@MNPs-Si-Glu and urease@MNPs-ECH retained about 59%, 84% and 78% of its initial activity at 50 °C after 15 h incubation period, respectively (Figure 6A). At 60 °C, the retained activities of free urease, urease@MNPs-Si-Glu and urease@MNPs-ECH were 48%, 77% and 72% of their initial activities, respectively (Figure 6B). At 70 °C, these corresponding values were 29%, 64% and 56% (Figure 6C). As shown in Table 1, the $k_d$ values of urease@MNPs-Si-Glu and urease@MNPs-ECH were much lower than those of the free urease at 50, 60, and 70 °C. For example, the $k_d$ values of urease@MNPs-Si-Glu and urease@MNPs-ECH were 2.7- and 1.9-fold lower than that of the free urease at 60 °C, respectively. However, the $t_{1/2}$ and SF values of urease@MNPs-Si-Glu and urease@MNPs-ECH increased when compared with those of urease at all the investigated temperatures. According to these results, urease@MNPs-Si-Glu and urease@MNPs-ECH had at least 1.8 times more stable
Figure 5: The effect of pH and temperature on the activity of free and immobilized urease preparations. (A) The pH-activity profile of free urease, urease@MNPs-Si-Glu and urease@MNPs-ECH. (B) The temperature-activity profile of free urease, urease@MNPs-Si-Glu and urease@MNPs-ECH.

Figure 6: The thermal stability of free and immobilized urease preparations at 50 °C (A), 60 °C (B), and 70 °C (C).
than the free urease at 50, 60 and 70 °C. Furthermore, the D-values of both urease@MNPs-Si-Glu and urease@MNPs-ECH were higher than those of the free urease at all the investigated temperatures. These results indicated a resistance to heat denaturation as a result of covalent immobilization. The z-values of free urease, urease@MNPs-Si-Glu and urease@MNPs-ECH were 76.9, 58.8 and 62.5 °C, respectively. The higher z-values were reported that the enzyme was more sensitive to heat treatment [34]. This result demonstrated the thermal stability increase of urease after immobilization. Dogac and Teke [35] reported that the free urease decreased its 90% of its initial activity rapidly at 60 °C after 1 h, whereas all the encapsulated ureases protected 30–45% of their initial activities after the same incubation time. Jamwal et al. [36] demonstrated that the crosslinked urease nanoaggregates was inactivated at much slower rates than free urease and the crosslinked urease nanoaggregates remained 39% of its initial activity at 55 °C after 140 min, while free urease retained 21% of its initial activity under the same conditions.

Some comparative studies including optimum pH, temperature and thermal stability in the literature are given in Supplementary Table 1.

Thermodynamic parameters for thermal inactivation of free urease, urease@MNPs-Si-Glu and urease@MNPs-ECH were given in Supplementary Table 2. The $E_a$ values of free urease, urease@MNPs-Si-Glu and urease@MNPs-ECH were calculated as 29.5, 36.4 and 34.7 kJ/mol, respectively. The higher $E_a$ values of urease@MNPs-Si-Glu and urease@MNPs-ECH than the free urease indicated that urease@MNPs-Si-Glu and urease@MNPs-ECH had higher thermal stability than the free urease. The $\Delta H^*$ values of urease@MNPs-Si-Glu and urease@MNPs-ECH were higher than those of the free urease at all the investigated temperatures. The higher $\Delta H^*$ and $\Delta G^*$ values of urease@MNPs-Si-Glu and urease@MNPs-ECH indicated that the immobilized urease preparations were thermally stable than the free urease at 50, 60 and 70 °C [37, 38]. According to the $\Delta S^*$ values, it was concluded that the immobilized ureases showed high resistance against thermal inactivation than free urease.

The storage stability of free and immobilized ureases are shown in Supplementary Figure 2. The residual activities of free urease, urease@MNPs-Si-Glu and urease@MNPs-ECH were 30%, 72%, 60% at 4 °C after 30 days storage, respectively (Supplementary Figure 2A). The corresponding residual activities were 23%, 71%, 57% at 25 °C after 30 days storage time (Supplementary Figure 2B). This can be explained as a result of conformational limitations of urease after covalent immobilization. Zhang et al. [39] prepared MNPs modified with poly carboxybetaine acrylamide (Fe$_3$O$_4$-pCBAA) and reported that urease immobilized on poly Fe$_3$O$_4$-pCBAA retained 75% of initial activity after 32 days at 4 °C.

The reuse of the immobilized enzyme is very important for commercial applications since it reduces the application cost. The determination of reusability of immobilized ureases was carried out using a batch reactor and their residual activities were measured under standard assay

![Figure 7](image-url): The reuse stability of urease@MNPs-Si-Glu and urease@MNPs-ECH.
conditions. As shown in Figure 7, the residual activities of urease@MNPs-Si-Glu and urease@MNPs-ECH were about 53% and 52% of the initial value after 20 cycles, respectively. Sahoo et al. [3] reported that immobilizing urease onto phosphate grafted iron oxide nanoparticles retained 70% of initial activity after five reuses. A 60% of retained activity was reported for the immobilized urease after 14 uses [40].

Conclusions

In this study, urease was successfully immobilized onto MNPs via glutaraldehyde and epichlorohydrin spacer arms. The storage stability of ureases was improved upon immobilization. The urease@MNPs-Si-Glu and urease@MNPs-ECH retained 53% and 52% of the initial value after 20 cycles. The results of thermal and thermodynamic studies showed that the urease@MNPs-Si-Glu and urease@MNPs-ECH were more thermally stable than free urease. Since MNPs are non-toxic, cheap, and biocompatible, urease@MNPs-Si-Glu and urease@MNPs-ECH may find potential uses to remove urea in agricultural, food and biomedical applications.

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