ORIGINAL ARTICLE

Optimization of Immobilized Aldose Reductase Isolated from Bovine Liver

Sığır Karaciğerinden İzole Edilen İmmobilize Aldoz Redüktazın Optimizasyonu

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ABSTRACT

Objectives: Isolation of enzymes and experiments on them require great effort and cost and are time-consuming. Therefore, it is important to extend the usability of the enzymes by immobilizing them. In this study our purpose was to immobilize the enzyme aldose reductase (AR) and to optimize the experimental conditions of the immobilized AR and compare them to those of free AR.

Materials and Methods: AR was isolated from bovine liver and the enzyme immobilized in photographic gelatin by cross-linking with glutaraldehyde. Then the optimum conditions for free and immobilized AR in terms of pH, temperature, and storage were characterized by determining the enzyme activity.

Results: Following immobilization, the optimum pH and temperature levels for free AR, which were pH 7.0 and 60°C, slightly altered to pH 7.5 and 50°C. The enzyme activity of the immobilized AR was maintained at about 65% after reusing 15 times. Moreover, immobilized AR maintained 95% of its original activity after 20 days of storage at 4°C, while the retained activity of the free AR was 85% of the original.

Conclusion: Our experiments indicated that the conditions that affect enzyme activity might alter following immobilization. Once the optimum experimental conditions are fixed, the immobilized AR can be stored and reused with efficiency higher than that of free AR. Moreover, this study provides an insight into the advantages of using immobilized AR in enzyme assays rather than free AR.

Key words: Aldose reductase, isolation, immobilization

ÖZ

Amaç: Enzim izolasyonu ve enzimler üzerinde yapılan deneyler, büyük çaba, maliyet ve zaman gerektirir. Bu yüzden, enzimlerin kullanılabilirliğinin immobilizasyon ile uzatılması önemlidir. Bu çalışmadaki amacımız, aldoz redüktaz (AR) enzimini immobilize etmek ve serbest AR'ninkiler ile karşılaştırarak immobilize AR'nin deney şartlarını optimize etmektir.

Gereç ve Yöntemler: AR, sığır karaciğer ve böbreğinden izole edildi ve gluteraldehid ile fotografik jelatine çapraz bağlanarak immobilize edildi. Daha sonra, enzim aktivitesi belirlenerek serbest ve immobilize AR'nin optimum pH, sıcaklık ve depolama koşulları tespit edildi.

Bulgular: Serbest AR için pH 7.0 ve 60°C olan optimum pH ve sıcaklık seviyeleri, immobilizasyondan sonra pH 7.5 ve 50°C olarak belirlendi. İmmobilize AR'nin enzim aktivitesinin, 15 kez kullanımdan sonra %65 oranında korunduğu tespit edildi. Bununla birlikte, +4°C'de 20 gün boyunca saklanan serbest AR'nin aktivitesi %85 oranında korunurken immobilize AR'nin aktivitesinin %95 oranında korunduğu bulunmuştur.

Sonuç: Deneylerimiz, immobilizasyonu takiben enzim aktivitesini etkileyen koşulların değişebileceğini göstermektedir. Ayrıca, immobilize AR, serbest AR'ye göre daha yüksek aktiviteyle korunup tekrar tekrar kullanılabilmektedir.

Anahtar kelimeler: Aldoz redüktaz, izolasyon, immobilizasyon

INTRODUCTION

Aldose reductase (AR) (EC 1.1.1.21) is expressed by the AKR1B1 gene in humans. It is located on the 7q35 chromosome, consists of 315 amino acids, and its molecular weight is 36 kD. AR is a cytosolic monomeric protein.[1] It specifically catalyzes the polyol pathway, which includes the conversion of glucose-bound nicotinamide adenine dinucleotide phosphate (NADPH) cofactor to sorbitol.[2,3] Moreover, it reduces the activity of aldehydes such as glutathione complexes.[4] Indeed, AR is crucial in the detoxification of lipid aldehydes produced by oxidative stress.[5]

Under optimum conditions, enzymes are able to display their activities outside of their natural environment. Using this feature, they can be employed on a large scale in health science, such as in the diagnosis and treatment of diseases and drug design. However, their structure tends to alter during the experimental process. Therefore, studies aim to improve the conformational stability of enzymes.[6] Immobilization is a method reducing disruption of the enzyme structure. Enzyme immobilization avoids the majority of the instability and loss of enzyme activity. This provides a longer time and flexibility to work on enzymes. Immobilization is not only useful to maintain the stability of biomolecules but also it reduces the cost of enzyme studies.

In the present study for the first time AR was isolated from bovine liver, immobilized, and characterized with respect to several kinetic properties. In addition, AR derived from bovine kidney was used as a side control and simultaneously applied to identical experiments. The AR enzymes from both sources were immobilized in gelatin by glutaraldehyde and washed to remove the free enzyme. We compared the behavior of the free and the immobilized AR under different conditions of pH, temperature, reusing, and storage. The difference in optimum conditions of the free and the immobilized enzyme may be due to alterations in the structure caused by several factors such as carrier material, immobilization method, and activation method.[7] Indeed it was previously indicated that immobilization significantly changes the conformation of enzymes and thus their activity.[8]

MATERIALS AND METHODS

Chemical materials

In this research, ethylenediaminetetraacetic acid (EDTA), NADPH, and lithium sulfate (Li_2SO_4) were purchased from Gerbu, Germany. Ammonium sulfate was supplied by Merck. Phenylmethylsulfonyl fluoride (PMSF), DL-glyceraldehyde, and all other materials used were analytically graded and obtained from Sigma Aldrich, Germany.

Isolation of AR enzyme from bovine liver

The bovine liver was provided by a slaughterhouse in Kazan, Ankara, Turkey. Small pieces of liver samples were washed with 1.0 mM EDTA. Then they were measured and homogenized with threefold 1.0 mM EDTA and 50 μM PMSF and spun at +4°C and 10,000 rpm for 30 min. To acquire 40% saturation, 22.6 g of ammonium sulfate was added to each 100 mL of supernatant followed by rotation at 10,000 rpm at +4°C for 25 min. To gain 50% and 75% saturations, the same method was carried out by adding 5.8 g and 15.9 g of ammonium sulfate to the 100 mL of supernatant solution, respectively. The pellets were dissolved with 50 mM sodium chloride and stored in a freezer at -80°C.

Determining the protein amount

Following isolation, the amount of protein was detected by Bradford assay.[9] The bovine serum albumin (BSA) standards were used at the concentrations of 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 mg/mL. BIO-RAD reagent was added to the BSA standards and the sample. Then each solution was measured using a spectrophotometer at 595 nm wavelength. Finally, protein amount was calculated using the standard graph generated with optical densities of the standards.

Assay of AR enzyme activity

AR activity was detected against DL-glyceraldehyde, by monitoring the NADPH oxidation to $NADP^+$ at 340 nm.[10] The reaction mixture consisted of AR enzyme (4.54 mg/mL), NADPH (9×10^{-5} M), Li_2SO_4 (320 mM-400 mM), DL-glyceraldehyde (6×10^{-4} M), and KP buffer (50 mM, pH 6.2). $NADP^+$ oxidation was followed in 0.25 mL of reaction mixture using a multimode microplate reader at 340 nm for 4 min. Initial and final rates of enzymatic reactions were measured and recorded as nmol/min/mg protein.

Immobilization of AR enzyme

The immobilization gel consisted of 0.025 g of photographic gelatin and 0.125 M glutaraldehyde in 0.067 M phosphate tampon solution (pH 7.4). The photographic gelatin was prepared at 50°C and then cooled to 30-35°C. Different units of AR enzyme were added to the gelatin and then vortexed. Cross-linking glutaraldehyde was included at the concentration of 5×10^{-5} M. Then 100 μL of the mixture was placed onto cellulose triacetate. Enzyme and gelatin complexes were dried for 24 h at room temperature.

The film strips carrying the immobilized AR were assayed for enzyme activity. The reaction mixture consisted of 2.7 mL of potassium phosphate, 0.1 mL of NADPH, and 0.1 mL DL-glyceraldehyde. Absorbance of this reaction mixture was measured at 340 nm before and after 20 min incubation with the film strips to detect enzyme activity.

RESULTS AND DISCUSSION

AR isolated from bovine liver was immobilized by cross-linking with glutaraldehyde in photographic gelatin. To detect the amount of AR protein we used the Bradford assay and spectrophotometric measurements. The results indicated that the protein amount was 18.39±0.09 mg/mL. In the standard group, the activity values for only AR were 4.172×10^{-4} U/L in liver. These values are accepted as 100% enzyme activity.

Following immobilization, the free enzyme was removed by washing the film strips three times with phosphate buffer (0.067 M, pH 6.2) at 25°C for 5 min and spectrophotometry was used to detect the amount of free enzyme. Using the washed

film strips, the effect of enzyme concentration on immobilized enzyme activity was tested to detect the immobilized enzyme leakage. Our results showed that AR leakage from the film strips increased with enzyme concentration while the enzyme activity was reduced after each wash. On the other hand, three washes were sufficient to remove the leakage completely (Table 1).

We demonstrated that enzyme leakage increased depending on the increasing enzyme unit. This is because of the constant concentrations of the gelatin and glutaraldehyde. In other words, when there was insufficient gelatin, which is the immobilization medium, and/or glutaraldehyde, which is the cross-linker in the immobilization reaction, there was more unbound AR released from the film strips at high enzyme concentrations.

Characterization of the free and immobilized AR

Conditions of pH, temperature, and storage were optimized to characterize the free and the immobilized AR. Moreover, the effects of storage stability and continual use on the activity of the immobilized strips were tested.

pH

To evaluate the optimum pH for the free (1.46×10^{-8} U) and the cross-linked (1.56×10^{-8} U) AR, we measured the levels of enzyme activity in a pH gradient between pH 4 and 9 at 25°C for 5 min. It was demonstrated that the optimal pH values for the free and the immobilized AR from liver were respectively 7.0 and 7.5 (Figure 1).

Table 1. Effect of the enzyme concentration on the immobilized AR from liver

Enzyme volume ($\times 10^{-8}$ U)	Activity (Enzyme unit/μ mol/min/L)		
	Number of washes		
	1	2	3
1.04	0.017	0.007	0.000
1.46	0.130	0.015	0.000
2.08	0.347	0.024	0.000

AR: Aldose reductase

We observed that the optimum pH for AR shifted to 7.5 from 7.0 upon immobilization. This alteration should be because of the H^+ and the OH^- ions that are released into the microenvironment during the reaction. Indeed, this result about the optimum pH alteration was expected because of the context of the environments of free and immobilized enzymes as previously indicated.[11] Moreover, previous studies showed that the optimum pH of the enzyme was changed by the polar groups of gelatin during the immobilization.[12] There are possible interactions such as hydrogen bonds that are generated between enzyme and polymer carrier.[13] However, the immobilization might not significantly affect the stability of optimum pH for enzymes in some cases. For instance, NADH-cytochrome b5 reductase enzyme was isolated from rabbit liver microsomes and it was immobilized in photographic gelatin by chemical cross-linking using chromium (III) acetate. The experiments showed that the optimum pH for the free and the immobilized enzyme was in the range from 6.1 to 7.5.[14] Similarly, the free and the immobilized β-galactosidase on the gelatin carboxymethylcellulose carrier were assayed for the optimum pH. It was observed that immobilization of β-galactosidase did not have any significant effect on pH stability.[15] Consequently, it can be foreseen that the values of optimum pH for free and immobilized enzyme can change; however, this might not be the case for all enzymes and immobilization methods.

Temperature

We aimed to uncover the difference between optimum temperatures for the free and the immobilized AR, which is important for enzyme activity. To detect the optimum temperatures, activity of the enzyme was tested at different levels ranging from 5°C to 70°C. The highest enzyme activity was observed at 60°C for the free AR from liver and this shifted to 50°C after the cross-link immobilization (Figure 2).

These results suggested that the immobilization of AR from liver reduces the optimum temperature. However, in our experiments we also showed that immobilization increased the thermal stability of the enzyme between 60°C and 70°C (Figure
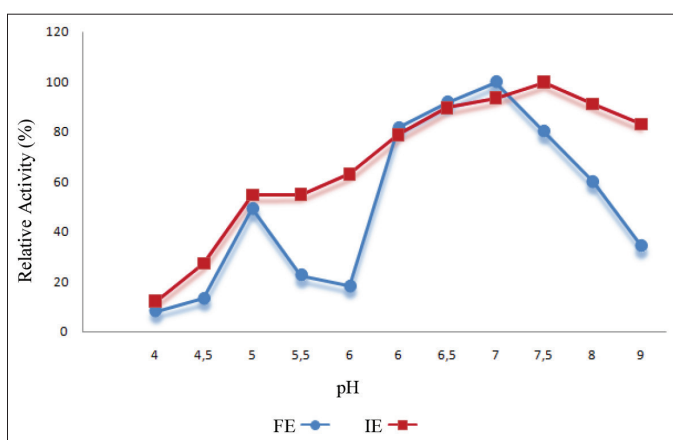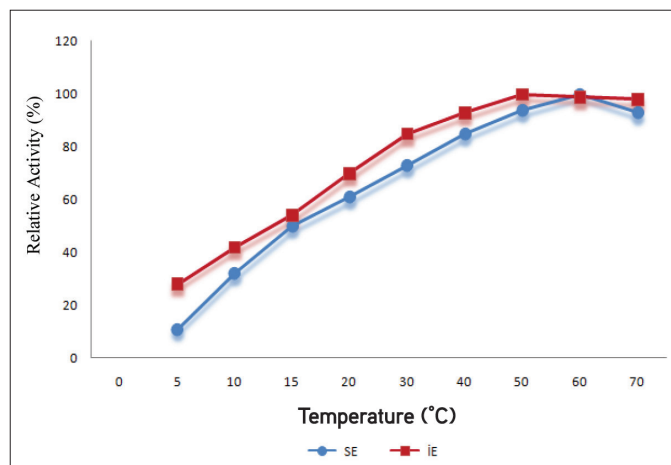

Figure 1. The enzyme activity levels of the free (FE) and the immobilized (IE) AR enzyme from liver according to pH

AR: Aldose reductase

Figure 2. The enzyme activity levels of the free and the immobilized AR from liver according to temperature

AR: Aldose reductase

2). These results indicated that immobilization generated an optimum temperature range from 50°C to 70°C, while free enzyme showed the highest activity only at 60°C. In other words, immobilization maintained enzyme stability against increasing temperature. It was previously demonstrated that the optimum temperature of NADH-cytochrome b5 reductase enzyme from rabbit was 30°C, while it decreased to 25°C upon cross-link immobilization of the enzyme.[14] Conversely, free β-galactosidase showed the highest enzyme activity at 47°C, while it was at 57°C after the enzyme was cross-linked to the gelatin carboxymethlyselulose carrier.[15] This might suggest that the carrier system protects the immobilized enzyme from thermal denaturating.

Reusing

To test the activity of the reused AR, it was immobilized in photographic gelatin with glutaraldehyde at $5×10^{-8}$ M concentration. Upon reusing the immobilized AR from liver ($1.46×10^{-8}$ U) 15 times a day at 25°C, 65% of its original activity was recove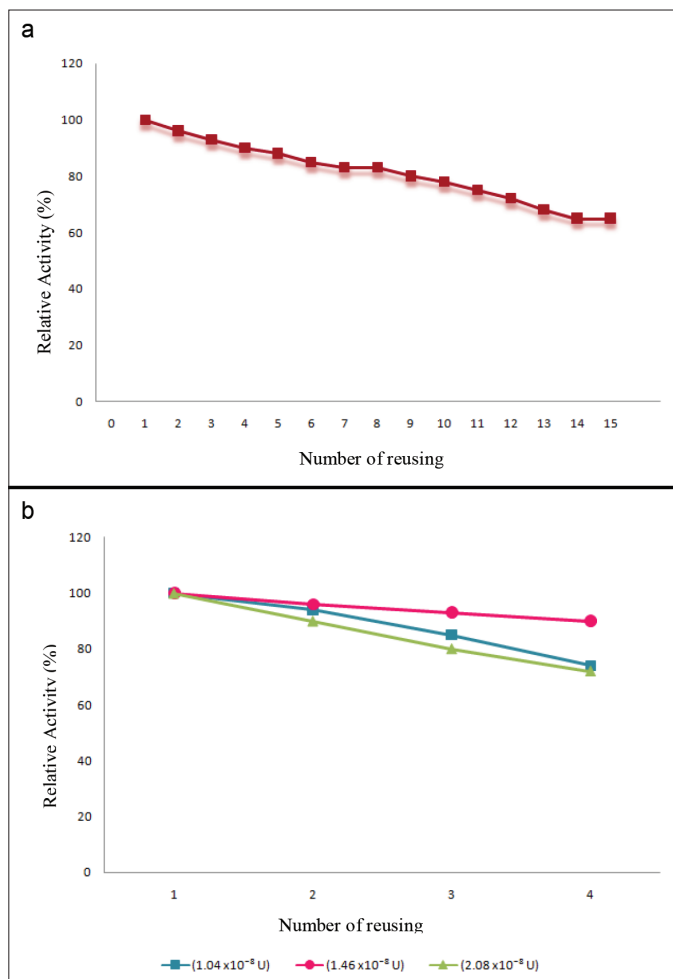red (Figure 3a). Similarly, we applied the same approach to different concentrations of the immobilized AR in different conditions. For instance, the immobilized AR from liver at the concentration of $2.08×10^{-8}$ U conserved 35% of the beginning enzyme activity after being used 10 times at 25°C (data not shown). We also tested the immobilized AR from liver for concentrations at $1.04×10^{-8}$ U, $1.46×10^{-8}$ U, and $2.08×10^{-8}$ U after being reused 4 times and reported that its activity was conserved about 74%, 90%, and 72%, respectively (Figure 3b).

Storage

To evaluate the effect of storage period on enzyme activity, we stored the free and immobilized AR at 4°C for 20 days and at 25°C for 10 days. After 20 days of storage at 4°C, activity of the immobilized AR was 95% of its original activity from liver (Figure 4a). Moreover, the free and the immobilized liver AR preserved 54% and 78% of their enzyme activity capacities following storage at 25°C for 10 days (Figure 4b).

Enzyme stability during storage is an important parameter for enzyme studies. Although it is expected that enzyme activity will be lower than at the beginning, the decrease should be minimized. This impairment in activity is most probably because of the denaturation of the enzyme structure.[16] In Yıldırım et al.[14]

Figure 3. a) The enzyme activity levels of the immobilized AR from liver ($1.46×10^{-8}$ U) according to reusing stability. b) The enzyme activity levels of the immobilized AR from liver ($1.04×10^{-8}$ U) and ($1.46×10^{-8}$ U) ($2.08×10^{-8}$ U) according to reusing stability
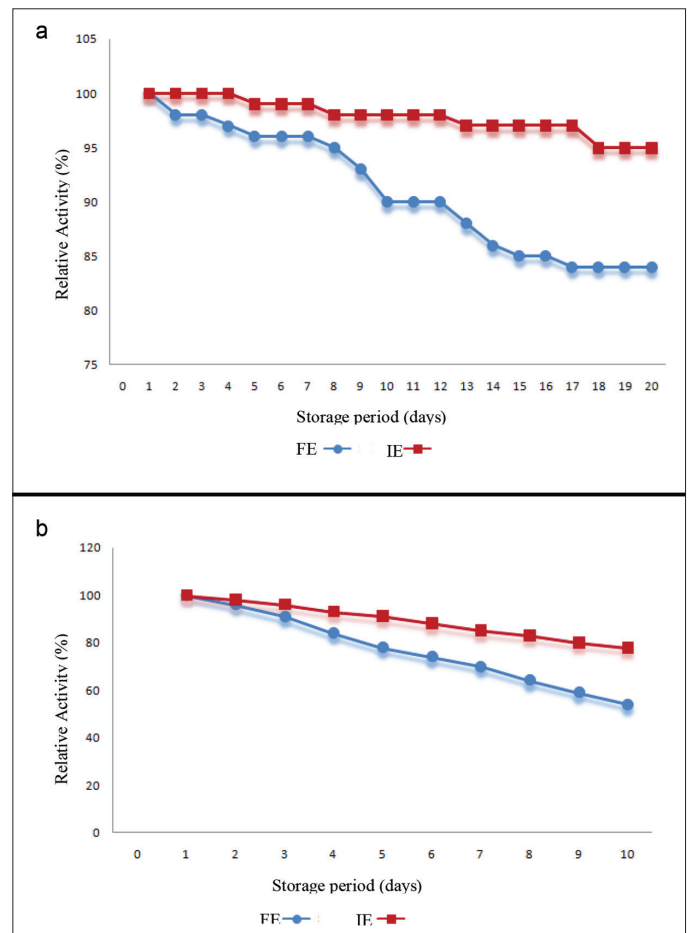
AR: Aldose reductase

Figure 4. a) The enzyme activity levels of the immobilized AR from liver according to storage stability at 4°C. b) The enzyme activity levels of the immobilized AR from liver according to storage stability at 25°C

AR: Aldose reductase

research the free and the immobilized NADH-cytochrome b5 reductase enzymes were compared for their activities after 60 days of storage at -7˚C, +4˚C, and +25˚C. They recorded that immobilization protects activity of the stored enzyme with higher efficiency at high temperature (+25˚C) than at lower temperatures (-7˚C and +4˚C).[14] Similarly, Kim et al.[6] showed that immobilized lipase enzyme maintained 82% of its activity after 30 days at room temperature, while the free lipase lost its activity completely. These results suggested that immobilization is a good tool to protect enzyme activity against temperature increases during storage. The efficiency of immobilization can be increased by carrying out the immobilization in a specific manner. For instance, it was demonstrated that the aldo/keto reductase AKR1A1 was immobilized as specific-oriented and so it was surface-bound. On the other hand, the unspecific immobilization was adsorptive; therefore, it showed less activity relative to the specifically immobilized aldo/keto reductase AKR1A1.[17]

The present study showed that immobilized AR enzyme can be preferable to work with than the free one. Our results showed that enzyme activity is retainable following immobilization as long as the experimental conditions are specifically fixed according to the immobilized AR.

CONCLUSIONS

Consequently, we indicated that immobilization is a convenient method to utilize the enzyme AR in multiple experiments. While free enzymes can be used only once in experiments, immobilized enzymes can be used several times due to the protective features of glutaraldehyde cross-linking. In this case, optimal conditions for the immobilized AR should be indicated and applied to the experiments since these conditions might be different from those of the free AR.

Conflict of Interest: No conflict of interest was declared by the authors.

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