Asian Journal of Chemistry; Vol. 28, No. 1 (2016), 55-59

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2016.19229

Effect of Cross-Linkers on Activity of Alcohol Dehydrogenase Immobilized on Polyaniline Magnetic Nanoparticles

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Received: 7 April 2015; *Accepted*: 27 May 2015; *Published online*: 5 October 2015; AJC-17547

In this study, alcohol dehdyrogenase (ADH) was immobilized on polyaniline magnetic nanoparticles (PAMP), which were used to completely recover the immobilized enzyme. When glutaraldehyde was used as a cross linker to immobilize alcohol dehdyrogenase on polyaniline magnetic nanoparticles, no alcohol dehdyrogenase activity was observed after immobilization. However, when 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was used as a cross linker, immobilized alcohol dehdyrogenase was found to display improved activity. The K_{cat} values of the free and immobilized alcohol dehdyrogenases were determined to be 82 and 17 mM/min/mg protein, respectively. Immobilized alcohol dehdyrogenase showed enhanced stability under acidic conditions at pH 4.5. While the activity of free alcohol dehdyrogenase was zero, the activity of immobilized alcohol dehdyrogenase was 50 % of its original activity at pH 4.5. The immobilized enzyme was also highly stable under rigorous shaking conditions relative to free alcohol dehdyrogenase. After 30 days of incubation at room temperature and 200 rpm, the residual activity of the free enzyme was zero, whereas that of the immobilized alcohol dehdyrogenase was 90.7 % after 60 days. The immobilized alcohol dehdyrogenase was recovered after completion of the enzyme reaction and it was still active after 15 consecutive reactions. The activity of immobilized alcohol dehdyrogenase was over 90 % of its original activity.

Keywords: Alcohol dehydrogenase, Glutaraldehyde, Polyaniline magnetic nanoparticles.

INTRODUCTION

Enzymes have become highly appealing catalysts for a range of applications due to their reaction specificity, limited impact on the environment and ability to produce fine-chemicals [1-3]. Despite these beneficial properties, enzymes cannot be broadly used for industrial applications because of their poor stability and high costs [2,4-7]. To address these issues, a vast number of studies have attempted to develop and implement enzyme immobilization methods. From this work, many high quality materials for enzyme immobilization have been created, such as polyaniline (PANI) nanofiber [8,9], sol gel [10,11], carbon nanotube (CNT) [12], cellulose fiber, magnetic nanoparticles *etc.* [13-15]. Currently, the use of enzymes as catalysts in industrial applications is limited primarily by enzyme recovery and preservation of enzyme activity over long periods of time [16].

Researchers have previously developed several enzyme immobilization methods, including simple adsorption, covalent attachment, enzyme encapsulation *etc*. [17] Many researchers have used glutaraldehyde (GA), which connects two amine functional groups [18-20], as a crosslinker for enzyme immobilization. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) is a zero-length cross linking agent that is used to couple carboxyl groups to primary amines. Both EDC and glutaraldehyde are powerful and efficient cross-linkers for enzyme immobilization. However, the activity of some enzymes is highly dependent on the type of cross linkers used for immobilization. Therefore, it is necessary to study the effect of the crosslinker on the activity of the immobilized enzyme.

Alcohol dehydrogenase (ADH) from *S. cerevisiae* can catalyze the oxidation of alcohols and the reduction of carbonyl compounds such as aldehydes and ketones. This particular enzyme has recently attracted much attention because of its potential use in the production of various starting materials and intermediates in the chemical industry [21-23].

In this study, alcohol dehydrogenase (ADH) was immobilized on polyaniline magnetic nanoparticles (PAMP) using two different crosslinkers, glutaraldehyde and EDC. To assess

the effect of the crosslinker on enzyme activity, we measured and compared ADH activity after immobilization using these two crosslinkers. The characteristics of the immobilized ADH were also discussed in this article.

EXPERIMENTAL

Alcohol dehydrogenase from *S. cerevisiae*, alcohol, nicotinamide adenine dinucleotide, aniline, bovine serum albumin standard (BSA) and iron oxide were purchased from Sigma-Aldrich (St. Louis, USA). Glutaraldehyde (GA) and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) were purchased from Pierce Technology (Thermo fisher Scientific Inc., USA).

Preparation of alcohol dehydrogenase: 10 mg of the alcohol dehydrogenase was dissolved in 1 mL of 50 mM sodium phosphate buffer. The enzyme solution was used for enzyme immobilization.

Activity assay of alcohol dehydrogenase: Enzyme activity was quantified by measuring the change in the absorbance of the cofactor (NAD or NADH) at 320 nm. Substrate concentrations varied from 1 to 100 mM at a constant cofactor concentration (2 mM) in the enzyme activity assay. A 50 mM sodium phosphate buffer (at pH 8.5) solution was used as the reaction buffer.

Preparation of polyaniline magnetic particles (PAMP): Polyaniline nanofibers were produced by rapidly mixing 0.1 % (w/w) ammonium peroxydisulfate, which was the initiator, 0.05 g iron oxide nanopowder and 5 mL aniline monomer solution in 1 M HCl. The samples were washed 10 times with distilled water to create a neutral solution [8].

Immobilization of enzyme on polyaniline magnetic particles

PAMP-GA-ADH immobilization: 2 mg of the PAMP was incubated in 50 mM phosphate buffer (pH 8.5) containing 1 % glutaraldehyde at 150 rpm and room temperature. Following incubation for 2 h, 1 mL from a 20 mg/mL ADH solution was added and the mixtures were incubated at 150 rpm and room temperature for 0.5 h. The enzyme coated PAMP was washed with 50 mM phosphate buffer (pH 8.5) and 100 mM *tris*-HCl buffer (pH 7.9). To cap the unreacted aldehyde group, immobilized ADH was incubated in 100 mM *tris*-HCl buffer (pH 7.9) for 0.5 h. Following *tris*-capping, the immobilized enzymes were washed at least 10 times with 50 mM phosphate buffer (pH 8.5).

PAMP-ADH-EDC immobilization: 2 mg of EDC was incubated in MES buffer containing 10 mg ADH at 150 rpm and room temperature. Following a 0.5 h incubation period, 2 mg of the PAMP was added. The mixtures were then incubated at 150 rpm and room temperature for 2 h. The enzyme coated PAMP was washed with 50 mM phosphate buffer (pH 7.0) and incubated in 50 mM phosphate buffer (pH 7.0) for 0.5 h. Finally, the immobilized enzymes were washed at least 10 times with 50 mM phosphate buffer (pH 8.5).

Characterization of free and immobilized enzymes: To examine the properties of both free and immobilized enzymes, pH (4.5-9.5) effects, long term stability and multiple reaction cycles at room temperature were determined. For further characterization, enzyme kinetics was measured for both free and immobilized enzymes.

RESULTS AND DISCUSSION

Immobilization of ADH on the PAMP: A schematic diagram of ADH immobilization is shown in Fig. 1. The synthetic scheme used to synthesize PAMP was described in a previous study [8] and was shown to display excellent properties in regards to enzyme immobilization. The most attractive property of PAMP was their ability to be completely recovered using magnets. Many enzymes have been immobilized on this material. In the case of ADH immobilization, we used glutaraldehyde as a cross-linker, which has been commonly used in cross linked enzyme aggregation (CLEA) [24,25]. Although this method resulted in an immobilization yield close to 60 %, no ADH activity was detected after immobilization (Fig. 2). This result indicates that ADH aggregated on the surface of PAMP and no enzyme activity was detected due to conformational changes after ADH immobilization.

We searched the structures of ADH from yeast in the NCBI website. Using this website, we found that the amino acids containing amino functional groups, such as glutamine, asparagines, argentines, histamines, lysine, were positioned close to the active site of ADH. Therefore, it is plausible that following glutaraldehyde treatment, the active site of ADH was blocked due to covalent attachment to the PAMP. In contrast, amino acids containing carboxyl groups, such as aspartic acid and glutamic acid, were positioned on the opposite side of the active site of ADH.

After careful consideration of the structural properties of ADH, we examined other possible immobilization methods; we searched for other cross-linkers that could link an amino group to a carboxyl group. The most appealing cross-linker we identified was EDC. Therefore, we designed a second immobilization method, as shown in Fig. 1(b). This immobilization method yielded the expected results; the immobilized ADH, when EDC was used as the cross-linker (PAMP-EDC-ADH), displayed a high level of activity. The change in immobilized enzyme activity for the different cross-linker is shown in Fig. 2. The activity of PAMP-EDC-ADH showed nearly 40 % of PAMP-EDC activity.

The effect of enzyme loading on the activity of immobilized ADH is shown in Fig. 3. As the protein concentration increased so did the tendency for enzyme aggregation and cross linked enzyme aggregation decreased enzyme activity.

Characterization of both free and immobilized ADH: The maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) of the free and immobilized ADH were estimated from statistical analysis of the experimental data. The catalytic activity (k_{cat}) represents the maximum enzyme reaction rate for the same amount of the free and immobilized enzyme. Table-1 shows the estimated kinetic parameters of the free and immobilized ADH and Fig. 4 shows the Lineweaver-Burk plots. The K_m value of PAMP-ADH was 3.08 times higher than that of the free enzyme (Table-1). The increase in K_m for PAMP-ADH might be due to a decrease in enzyme flexibility after immobilization. Thus, the substrate may not be able to readily access the active site after immobilization. From the maximum reaction rate, the turnover number k_{cat} was calculated. The kcat values for free and PAMP-ADH were determined to be 82 and 17 mM/min/mg protein, respectively.

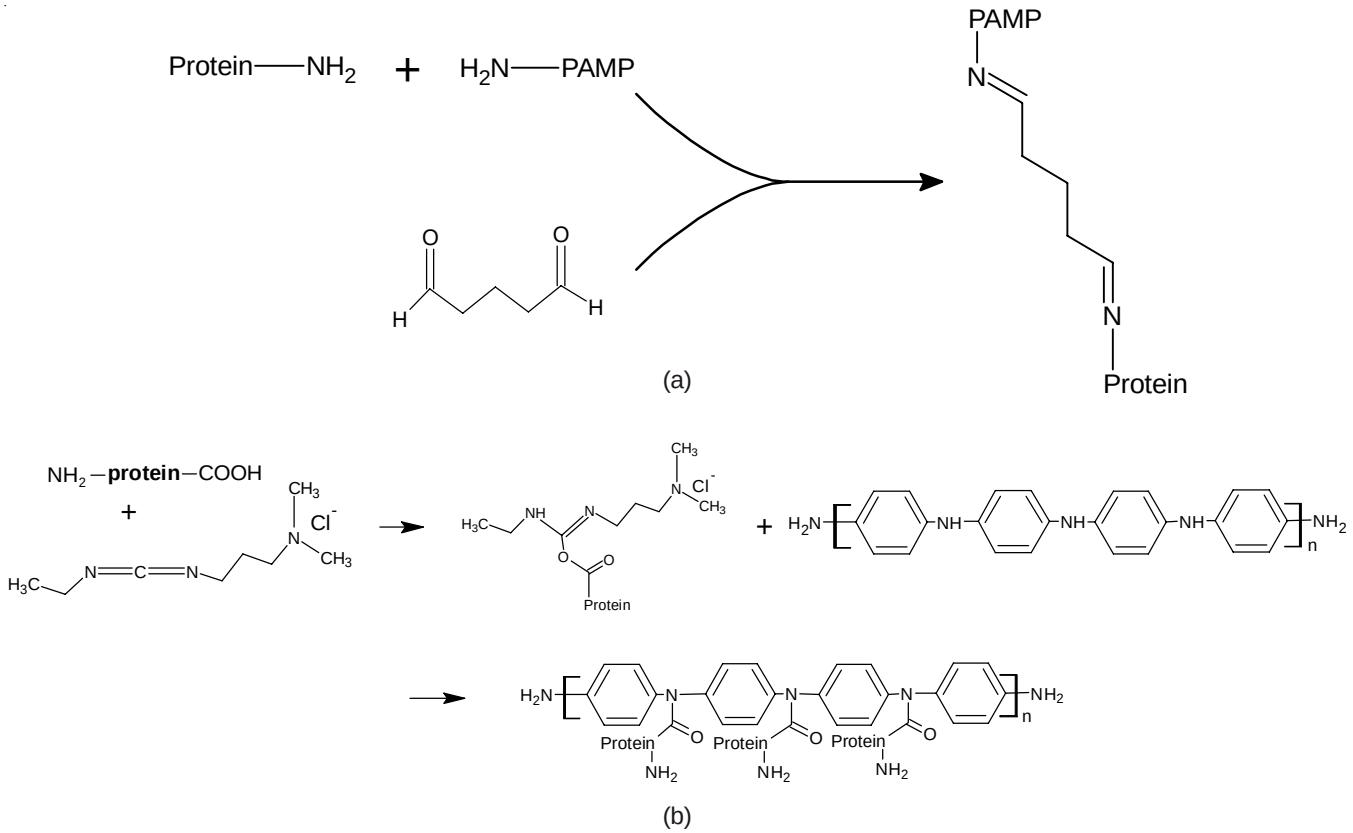

Fig. 1. Strategies used to immobilize ADH on PAMP. (a) Using glutaraldehyde as a cross-linker; (b) Using EDC as a cross-linker

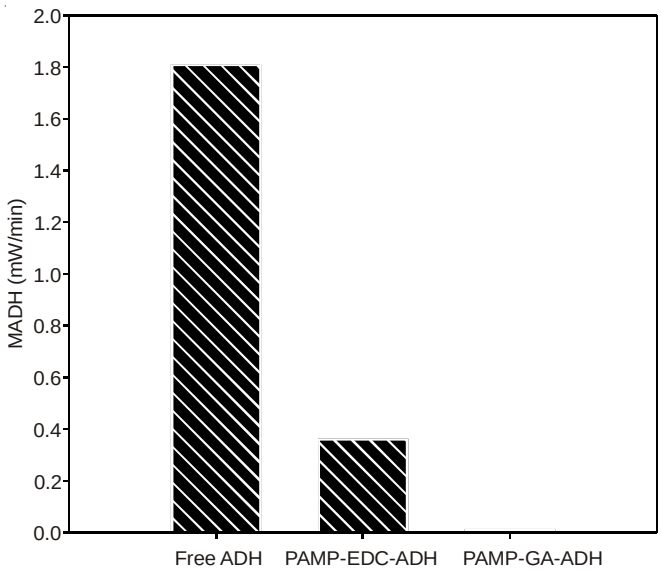

Fig. 2. Comparison between the relative activities of the free enzyme and immobilized enzyme

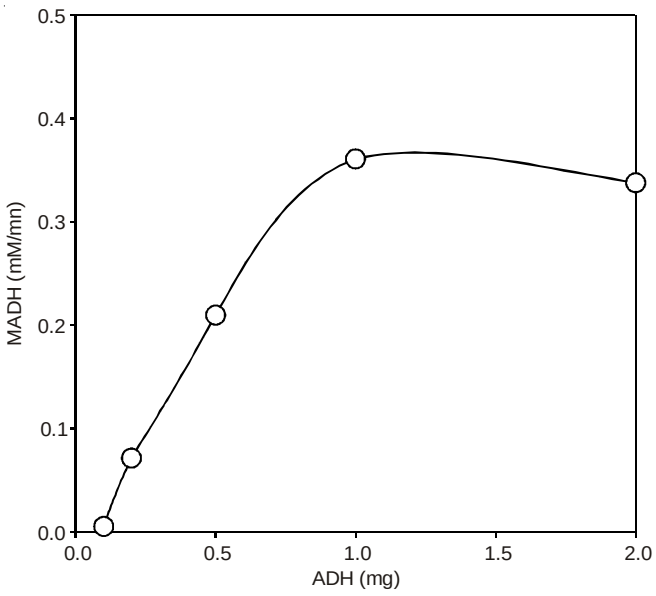

Fig. 3. Loading capacity of ADH immobilized on PAMP

Effect of pH on enzyme activity: Free enzymes are sensitive to pH changes; however, many studies have reported that immobilized enzymes were less sensitive to pH changes than free enzymes [26,27]. In this paper, we found that immobilized ADH was more active over a broader range of pHs than free ADH. The effect of pH on the activity of free and

TABLE-1
ENZYME KINETICS

Enzyme	Amount (mg/mL)	k_M (mM)	V_{max} (mM/min)	Slope	Y-Intercept	ς_{cat} (mM/min/mg enzyme)
ADH	0.01	11.15 ± 3.19	0.82 ± 0.12	13.64 ± 3.35	1.22 ± 0.18	82
PAMP-ADH	0.01	34.36 ± 9.91	0.17 ± 0.04	201.94 ± 27.80	5.88 ± 1.49	17

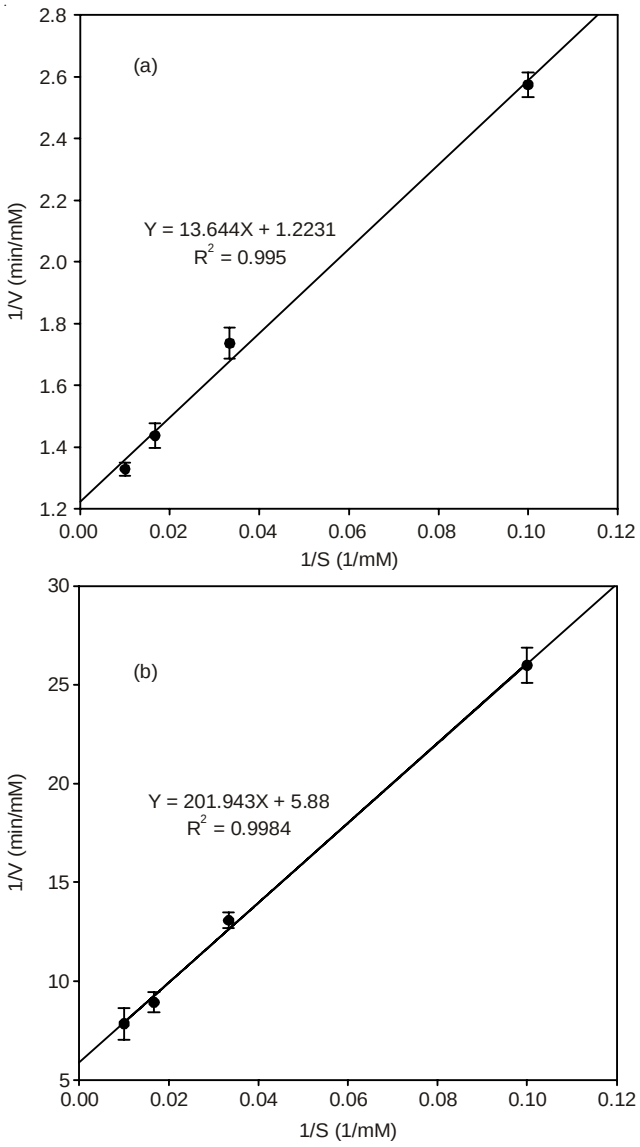

Fig. 4. Enzyme-kinetics-graphs of both (a) free and (b) immobilized ADH

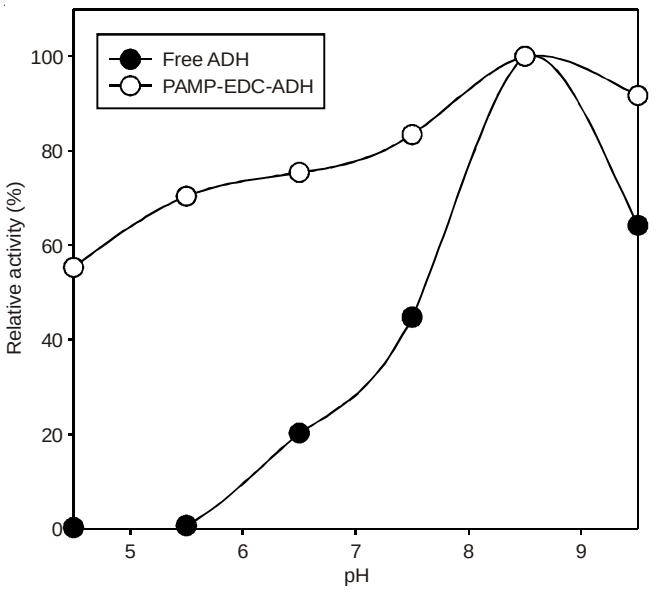

Fig. 5. pH effects on both free and immobilized ADH

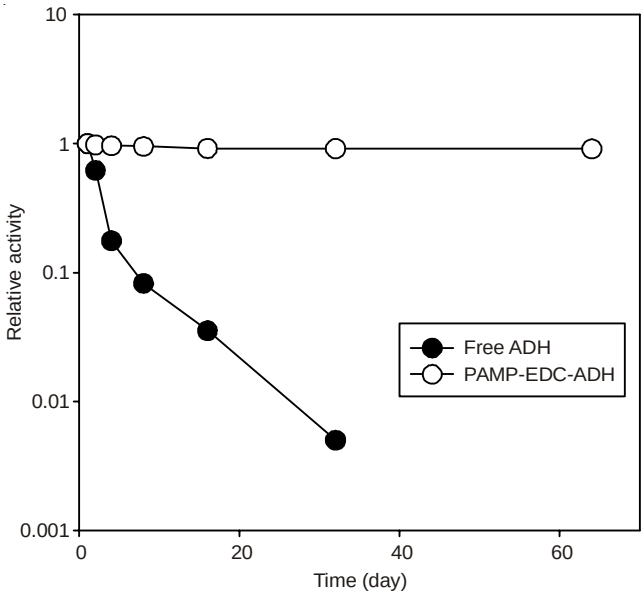

Fig. 6. Effect of long term storage on enzyme activity

immobilized ADHs was determined by quantifying enzyme activity at various pH values. The optimal pH for the ADH reaction was determined to be pH 8.5 and the relative activity is presented in Fig. 5. Free ADH showed the highest activity at pH 8.5. When the pH deviated from this optimal value, the activity of the free enzyme rapidly decreased. However, the immobilized ADH maintained a higher relative activity than the free enzyme at both lower and higher pH levels. The immobilized ADHs (PAMP-EDC-ADH) showed high activity over a wide pH range of 4.5 to 9.5. Immobilized ADH were more than 50 % active at extremely low, pH 4.5, while no activity was observed for the free enzyme under these conditions.

Long term stability of free and immobilized ADH: The stability of free and immobilized ADH was investigated after continuous incubation of ADH in a 50 mM phosphate buffer (pH 8.5). The relative activity was measured from the ratio of the remaining catalytic activity to the initial activity at a predetermined interval. After 30 days, the activity of the free ADH completely disappeared. However, the immobilized ADH showed nearly 90 % of its initial activity after 60 days. Fig. 6 showed the relative activities over a longer time period. The activity of free ADH rapidly decreased due to denaturation, while the covalently attached immobilized ADH showed an improvement in enzyme stability. Only a small decrease in the activity of immobilized ADH was observed. This indicates that the covalent attachment of ADH using EDC improves enzyme stability over a long time period.

Effect of ADH immobilization on enzyme activity after multiple reactions: Since the high cost of enzymes is another major obstacle for the commercial use of enzymes, the effect of enzyme immobilization on the activity of the enzyme after multiple reactions was examined. As described above, a magnetically separable supporting media, PAMP, was used in this study for ADH immobilization. After immobilization and enzyme reaction, the PAMP-ADH was completely recovered using magnets and readily reusable for additional reactions. The immobilized ADH activity was measured after each cycle and its relative activity is presented in Fig. 7. The relative activity decreased slightly after each cycle; however, over 90 %

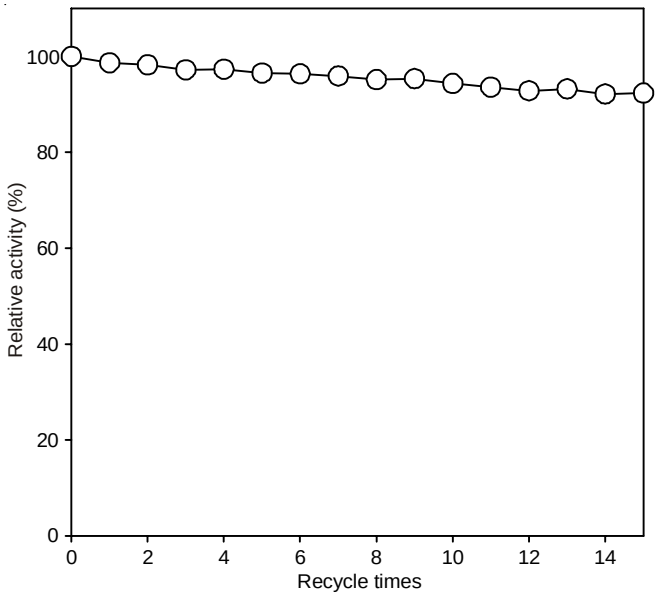

Fig. 7. PAMP-ADH was recovered with magnets for use in subsequent reactions

of its initial activity was retained after 15 consecutive reactions. These results suggest that this immobilization method could be used to develop a cost effect enzyme reaction system, where the enzymes are easily recoverable and reusable.

Conclusion

In this study, we used two types of cross-linkers, glutaraldehyde and EDC, for the covalent attachment of ADH to PAMP. Although 60 % of the ADH was immobilized using the glutaraldehyde cross linker, no enzyme activity was detected. When EDC was used as the cross-linker, the direction of covalent attachment could be controlled and the immobilized ADH showed an enhanced enzyme activity.

After immobilization, Michaelis Menten constant (K_M) increased due to a decrease in substrate binding affinity. The K_{cat} value also decreased due to the aggregation of the enzyme on the surface of PAMP. K_{cat} values of the free and immobilized enzyme were determined to be 82 and 3.4 mM/min/mg protein, respectively. Immobilized ADH showed an enhanced stability over a wide range of pH values and a long period of operation.

ACKNOWLEDGEMENTS

This paper funded by Beijing Polytechnic (YZKB2012003, YZKB2014006), Science-Technology Foundation of Beijing Municipal Commission of Education (KM201410858002) and National Natural Science Foundation of China (No. 21306002).

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