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RESEARCH

Covalent Immobilization of Alcohol Dehydrogenase (ADH2) from *Haloferax volcanii*: How to Maximize Activity and Optimize Performance of Halophilic Enzymes

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Abstract Alcohol dehydrogenase from halophilic archaeon *Haloferax volcanii* (*Hv*ADH2) was successfully covalently immobilized on metal-derivatized epoxy Sepabeads. The immobilization conditions were optimized by investigating several parameters that affect the halophilic enzyme–support interaction. The highest immobilization efficiency (100 %) and retention activity (60 %) were achieved after 48 h of incubation of the enzyme with Ni-epoxy Sepabeads support in 100 mM Tris–HCl buffer, pH 8, containing 3 M KCl at 5 °C. No significant stabilization was observed after blocking the unreacted epoxy groups with commonly used hydrophilic agents. A significant increase in the stability of the immobilized enzyme was achieved by blocking the unreacted epoxy groups with ethylamine. The immobilization process increased the enzyme stability, thermal activity, and organic solvents tolerance when compared to its soluble counterpart, indicating that the immobilization enhances the structural and conformational stability. One step purification–immobilization of this enzyme has been carried out on metal chelate-epoxy Sepabeads, as an efficient method to obtain immobilized biocatalyst directly from bacterial extracts.

Keywords Enzyme immobilization · Sepabeads · Halophilic enzyme · Alcohol dehydrogenase · *Haloferax volcanii* · Organic solvents tolerance

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Introduction

Alcohol dehydrogenases (ADHs, EC 1.1.1.1) are one of the most synthetically significant enzymes in biocatalysis that catalyzes the reversible reduction of aldehydes and ketones to their corresponding alcohols [1]. Chiral alcohols are very important intermediates in the synthesis of enantiomerically pure pharmaceuticals and other chemicals [2, 3]. In the last few decades, biocatalysis has become preferable to chemical catalysts in industrial processes due to mild reaction conditions, high degree of stereoselectivity, and environmentally friendly characteristics [4]. This is reflected in a higher number of industrial processes using alcohol dehydrogenase for production of valuable chiral alcohols [5].

The application of enzymes as catalysts in organic synthesis and in industry is closely linked to their immobilization. This is due to the expected benefits of immobilized enzymes over soluble forms or alternative technologies such as whole cells processes. The anchoring of an enzyme onto a solid insoluble support facilitates the efficient recovery and reuse of the biocatalyst and opens up the possibility of improving other enzymatic properties such as stability, substrate specificity, and enantioselectivity [6–8].

Many enzyme-immobilization methods described in the literatures are not readily applicable to an industrial scale. In particular, successful industrial applications of immobilized biocatalysis require simple immobilization protocols that are conducted under mild conditions and allow the enzyme to maintain its active conformation and necessary catalytic flexibility [9, 10]. Epoxy supports are very suitable matrices to immobilize–stabilize enzymes via multipoint covalent attachment for industrial applications [11, 12]. These supports display high chemical and mechanical

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stability, tolerating a wide range of pHs and solvents [13]. Furthermore, epoxy-activated supports are able to form very stable covalent bonds with different functional groups (amino, thiol, and hydroxyl groups) under very mild experimental conditions, and the unreacted epoxy groups may be easily blocked [10]. Finally, these supports can mimic immobilized metal ion affinity chromatography (IMAC) to purify His-tagged proteins [14, 15].

We recently reported on the identification and the biochemical characterization of a novel alcohol dehydrogenase from *Haloferax volcanii* (*Hv*ADH2) [16] and the effect of various organic solvents on its catalytic activity, stability, and substrate specificity for this promising enzyme [17]. The intrinsic tolerance of this enzyme to organic solvents makes it a very good candidate for further applications as industrial biocatalysts.

So far, published studies on the immobilization of halophilic enzymes are limited to the immobilization of halophilic malate dehydrogenase on Sepharose [18], stabilization of α-amylase from the *Halobacterium halobium* by calcium alginate [19], and immobilization of *Haloferax mediterranei* aldolase by cross-linking of a proteic matrix [20]. Here we describe the successful stabilization of *Hv*ADH2 by covalent immobilization on Sepabeads. This stems from an even more recent work in which we successfully immobilized horse liver ADH on the same system [21], however, the significantly different characteristics of a halophilic protein [22] required tweaking of the strategy and the outcome was not as predictable. The effect of various immobilization parameters (such as temperature, immobilization time, salt concentration, type of metal and blocking agent) on the immobilization of *Hv*ADH2 is reported. Based on the results, a procedure with optimized parameters was obtained resulting in markedly enhanced stability and catalytic activity of immobilized enzyme.

In the past, adsorption methods were the most common techniques for immobilization of alcohol dehydrogenases [23, 24] and significantly fewer studies have been carried out to date on covalent binding to stabilize these enzymes [24–27]. To date, no reports are available on the use of epoxy support to stabilize halobacterial enzymes by multipoint covalent attachment.

Materials and Methods

Support Type, Bacterial Strain, Growth Conditions, and Chemicals

Sepabeads®EC-EP support was kindly donated by Resindion Company, *H. volcanii* strain was grown at 45 °C on salt medium and purified, as described previously [16]. The cofactor nicotine adenine dinucleotide phosphate oxidized form ($NADP^+$) was purchased from Apollo Scientific Ltd., UK. Iminodiacetic acid disodium salt monohydrate (IDA) and ethylenediaminetetraacetic acid disodium salt (EDTA) were purchased from Sigma Aldrich. All other reagents were analytical grades.

Activity Assay for the Free Enzyme

The activity of soluble *Hv*ADH2 was analyzed spectrophotometrically by detecting the increase in absorbance at 340 nm, which corresponds to the formation of the cofactor NADPH, using a Varian Cary 50 Scan UV–Visible spectrophotometer equipped with a Cary single cell peltier temperature controller. The experiments were performed in reaction mixtures (1 mL) cuvettes at 50 °C. The activities measurements were determined at standard conditions [16] by assaying *Hv*ADH2 for activity against 100 mM of ethanol with $NADP^+$ (1 mM) using the buffers 50 mM glycine–KOH, pH 10.0, containing 4 M KCl.

Activity Assay for the Immobilized Enzyme

The activity of immobilized *Hv*ADH2 (*imHv*ADH2) was determined by weighing 30 ± 1 mg of immobilized enzymatic preparation into 25-mL reaction tube with cap, followed by the addition of 5 mL buffer 50 mM glycine–KOH, pH 10.0, containing 4 M KCl, 1 mM $NADP^+$, and 100 mM ethanol. The 5 mL reaction mixture was incubated in a shaker incubator with constant shaking (250 rpm) at 50 °C. At 1 min intervals (over a period of 10 min), a sample (1 mL) was taken from the reaction mixture and the absorbance of NADPH was recorded at 340 nm.

Protein Concentration Determination

The protein concentration was determined by the Bradford method. For calibration, bovine serum albumin was used. The standard curve for the standard assay was prepared between 0.2 and 1.0 mg/mL. An adaptation of the Bradford microassay was used, whereby standards were prepared in 100 mM Tris–HCl buffer, pH 8.0, containing NaCl (2 M) [28].

Immobilization of His-*Hv*ADH2 on Metal Chelate-Epoxy Sepabeads

Sepabeads EC-EP/S (a commercially available rigid methacrylic polymer matrix with diameter ranging between 100 and 300 μm, activated with epoxy groups in a ratio of 100 μmol/g of wet resin) were derivatized with iminodiacetic acid (IDA) and metal ions following the procedure reported in literature [14, 15], allowing for a modification of

around 5 % of the epoxy groups in the support, 1 g of beads was shaken at 23 °C for 2 h in 2 mL of support modification buffer (0.1 M sodium borate, 2 M IDA, pH 8.5). The derivatized resin, rinsed with double distilled water, was then re-suspended in 5 mL of metal containing solution (0.05 M sodium phosphate buffer pH 6.0, 1.0 M NaCl and 0.02 M of a suitable metal; $CoCl_2$, $ZnSO_4$, $NiSO_4$, and $CaCl_2$) and shaken at 23 °C for 2 h. The resin, rinsed again with double distilled water, was then put in contact with His-*Hv*ADH2 (1 mg of enzyme per 1 g of resin, in 3 mL solution of 100 mM Tris–HCl buffer, pH 8, containing 3 M KCl) and the mixture was gently shaken at 5 °C over 48 h. The resin was then thoroughly washed using a desorption buffer (20 mM Tris–HCl buffer, pH 8.0, containing 2 M NaCl and disodium EDTA 50 mM) and washing buffer (20 mM Tris–HCl, pH 8.0, containing 2 M NaCl) to achieve complete removal of the metal first and of the residual EDTA after.

Blocking of the Remaining Epoxy Groups and Modifications of the Enzyme–Support Microenvironment

Alteration of the microenvironment of the enzyme–support can be achieved by quenching the remaining excess of epoxy groups on the carrier surface using different blocking agents. The immobilized *Hv*ADH2 was treated with blocking buffer 100 mM Tris–HCl, pH 8 and containing 2 M KCl and different blocking agents such as 3 M glycine, 5 % mercaptoethanol, 5 % ethylenediamine, and 5 % ethylamine. The suspension was left shaking (220 rpm) for 20 h at 5 °C. Afterward, the enzyme–support preparation was rinsed three times with 5 mL of washing buffer (20 mM Tris–HCl, pH 8.0, containing 2 M NaCl). The final blocked preparation was stored in 20 mM Tris–HCl buffer, pH 8.0, containing 3 M KCl.

Immobilization Parameters

The immobilization efficiency (E) and the retention activity (R) parameters were defined as follows:

$$R = (\mathrm{S.A_{imm}}/\mathrm{S.A_{free}}) \times 100\ \%$$

$$E = (P_{\mathrm{imm}}/P_{\mathrm{intial}}) \times 100\ \%$$

where $\mathrm{S.A_{imm}}$ and $\mathrm{S.A_{free}}$ are the specific activity of immobilized enzyme and the free enzyme, respectively. P_{imm} is the amount of immobilized protein; P_{intial} is the initial amount of protein added to the reaction mixture. The amount of immobilized protein was determined indirectly by measuring the difference between the amount of initial enzyme and the amount of the enzyme recovered in the supernatant and washing solutions.

Results and Discussion

Optimization of the Conditions for the Immobilization of His-*Hv*ADH2

The immobilization of proteins on metal chelate–epoxy support follows a two-step mechanism. First, fast physical adsorption of the His-tagged protein by coordination with the metal ions, then covalent attachments follow under mild conditions, where nucleophilic residues on the protein surface attack the free epoxy groups and form multi-point attachments [14]. To insure that no reactive epoxide groups remain, the support should be treated with suitable capping agent. Figure 1 shows the reaction sequence for the immobilization of *Hv*ADH2. In this study we present the effect of various immobilization parameters that are involved in the immobilization process.

Effect of the Metal

It has been reported that soluble proteins are scarcely reactive with epoxy-activated supports. Prior to the covalent binding step, the physical adsorption or fixation of the proteins on the support surface dramatically improves the intermolecular reactivity between epoxy groups and proteins [14]. The adsorption process is facilitated through the addition of metal-chelate groups on the support surface. However, as proteins behave differently in the presence of different metals, a screening is required to identify the best one. Different divalent metals (Co, Ni, Zn, and Ca) to allow for a 5 % derivatization of the beads were tested for optimal adsorption and immobilization of *Hv*ADH2. For comparison, loading of the enzyme without metal derivatization and the retention activity of immobilized *Hv*ADH2 was calculated (Fig. 2).

We observed that the efficiency of adsorption was complete after 48 h when using Ni and Co metals while enzymatic activity was still detectable in the supernatant when Zn and Ca metals were used instead. Furthermore, the immobilization of *Hv*ADH2 via Zn and Ca metal chelation distinctly reduced the retention activity to around 40 %. This could be related to the weak interaction between these metals and the his-tagged portion of the enzyme. Although Co derivatization seemed to provide high adsorption yields with undetectable enzyme concentration in the supernatant, the specific activity of the immobilized preparation was very similar to what recorded for Zn and Ca derivatizations. In a preliminary experiment, we found that metal ions have different effects on the stability and catalytic activity of the free *Hv*ADH2 enzyme; Cu, Zn, and Co metal ions have a strong inhibitory effect on enzymatic activity while the enzyme was relatively insensitive to the presence of Ni and Ca metal ions. The

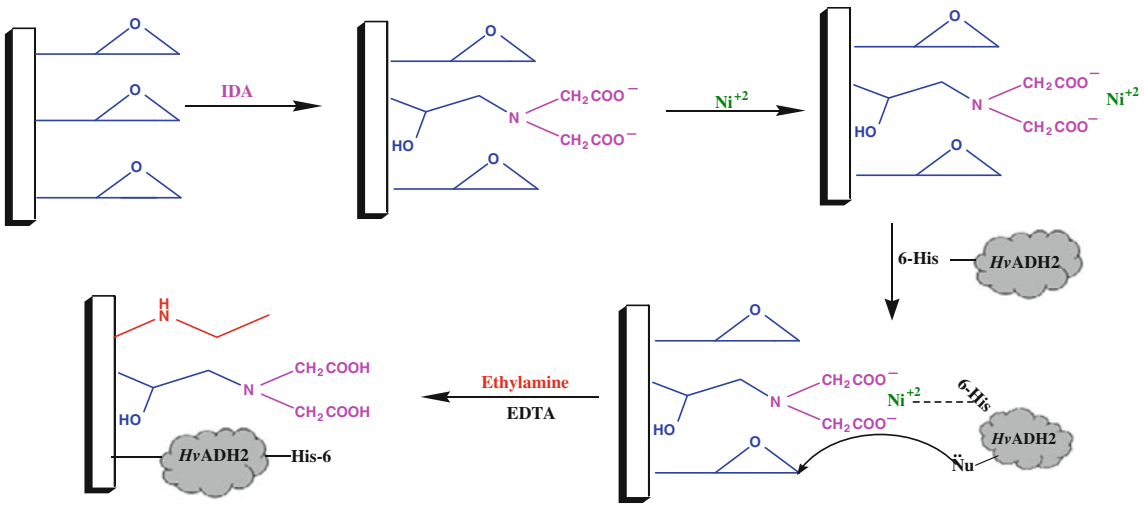

Fig. 1 Immobilization of *Hv*ADH2 on metal chelate-epoxy Sepabeads

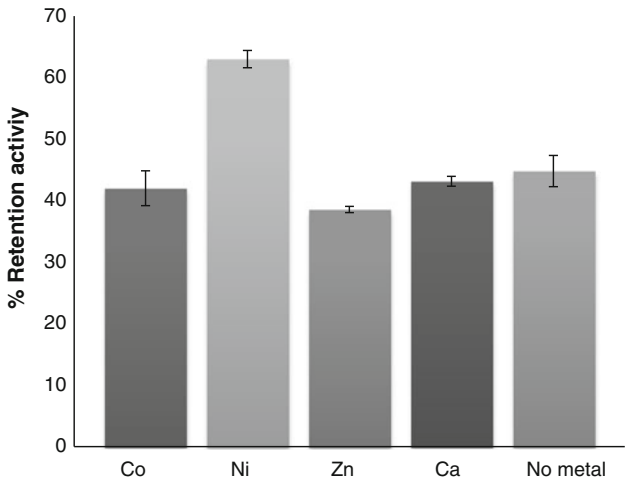

Fig. 2 Retention activity of the immobilized *Hv*ADH2 when different metal ions (or no metal) were used for the immobilization process

highest recorded specific activity, around 60 % of the free enzyme, was achieved with Ni salt which has minimal adverse effect on enzyme activity and strong chelating affinity for the His-tagged *Hv*ADH2. The chelating strengths of His-*Hv*ADH2 toward different metal ions are in agreement with the adsorption and stability constants reported for the complexation of different metals and imidazole which follow the order Ni > Zn $\simeq$ Co $\gg$ Ca [29]. While protein adsorption was observed in the control reaction without metal derivatization, the retention of activity of the immobilized enzyme was only 42 %. This was also observed in previous work [21] and while it is a positive result, it will not allow for selective immobilization of the enzyme in crude preparation resulting in an impure preparation where other proteins also bind to the support. Thus, the derivatization step to allow for the metal to selectively coordinate the His-tagged portion of the protein seems to be necessary to achieve high retention of activity.

Temperature Effect

It is now known that the stability of *Hv*ADH2 is strongly influenced by the temperature [16]. We found that *Hv*ADH2 was remarkably stable over time when stored at 5 °C and relatively unstable when stored at room temperature. The effect of temperature during the immobilization process was therefore investigated, by following the immobilization step at 5 and 20 °C. When the immobilization process was performed at 20 °C, the specific activity was 30 % less than when the same step was performed at 5 °C. Therefore, a temperature of 5 °C was chosen to perform all immobilization procedures. In order to overcome the expected decreasing of reactivity between enzyme and support active groups at low temperature, the immobilization time was also optimized to insure the formation of a sufficient number of covalent attachments.

Immobilization Time

The effect of the coupling time on the immobilization at 5 °C was investigated in terms of retention activity and immobilization efficiency with the focus on the Ni-derivatized resin which had given the best results. The retention activity and immobilization efficiency were determined at different times as shown in Fig. 3.

The immobilization efficiency was quite low within the first 3 h of the reaction and it increased over time. This demonstrates that in the first stages of the immobilization process, a fraction of the enzyme was physically adsorbed on the support, but not covalently immobilized, however, while 100 % immobilization efficiency can be obtained

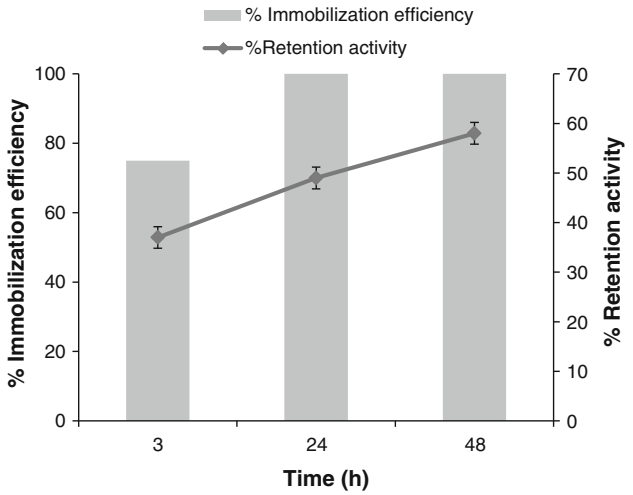

Fig. 3 Influence of immobilization time on the retention activity and immobilization efficiency of *Hv*ADH2 immobilized on Sepabeads-IDA-Ni

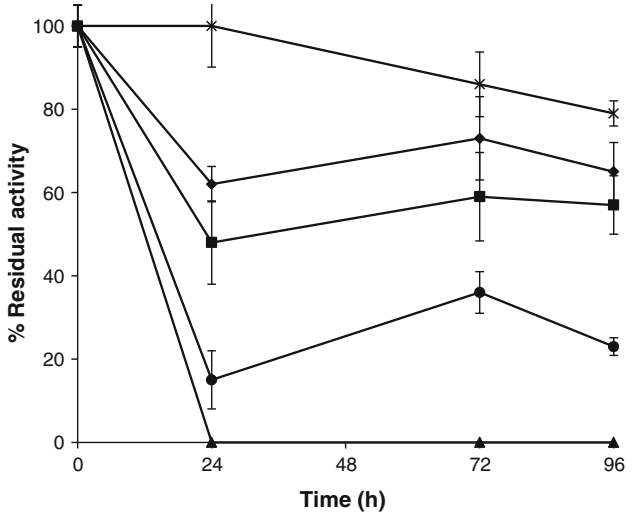

Fig. 4 Stability of *Hv*ADH2-Sepabeads preparation after treatment with blocking agents. The final enzymatic preparations were stored at 5 °C in 20 mM Tris–HCl buffer, pH 8.0, containing 3 M KCl. *Filled triangle* ethylenediamine; *filled circle* mercaptoethanol; *filled square* Gly; *cross* ethylamine, and *filled diamond* unblocked

within 24 h, the immobilized preparation requires another 24 h to reach the maximum retention of activity of 60 % (Fig. 3). These results indicate that maximum retention activity requires long incubation period (48 h) to permit the formation of strong multi-covalent bonds at 5 °C.

Capping and Modifications of the Enzyme–Support Microenvironment

After the immobilization of *Hv*ADH2 on the epoxy-Sepabeads support, our aim was to eliminate the remaining active epoxy groups that may interact with substrate or enzyme residues and influencing the enzymatic reactions. The enzyme–support preparation was treated with different blocking agents, then the final preparations were stored in 20 mM Tris–HCl buffer, pH 8.0, containing 3 M KCl at 5 °C. A sample of nonblocked *Hv*ADH2-Sepabeads was prepared as control, and the residual activity was measured over time under standard assay conditions (Fig. 4).

Figure 4 shows that the capping of unreacted epoxy groups with glycine and mercaptoethanol results in a significant decrease in activity by 55 and 25 %, respectively, after 72 h. Clearly, ethylenediamine was the worst blocking agent, with complete inactivation of the catalytic activity after 24 h. It has been reported that blocking of the remaining epoxy group generally has a stabilizing effect on the immobilized enzymes [10], however, in our case, the use of a bifunctional agent worsen the stability of the immobilized enzyme with respect to the uncapped preparation. The negative effect of highly hydrophilic blocking agents could be explained by changes caused in the microenvironment surrounding immobilized *Hv*ADH2 which destabilize the structure of the enzyme. It has been reported in the literature that halophilic proteins had weaker hydrophobic core than the mesophilic counterparts [22]. Thus, the creation of a highly hydrophilic environment near the weak hydrophobic core of *Hv*ADH2 may lead to an increase in the nonspecific interactions between the enzyme core and support surface. Therefore, we tested ethylamine as novel blocker for halophilic enzyme–support preparation, which is mono-functional and neutralizes the unreacted epoxy groups leaving a terminal short hydrocarbon moiety. Blocking *Hv*ADH2-Sepabeads with 5 % ethylamine achieves a significant increase in stability with total retention of activity after 48 h incubation and minimal loss over longer incubation time. The presence of a terminal ethyl group appears to be effective in minimizing the nonspecific interactions between the enzyme core and the support, by creation of a favorable microenvironment in which the halophilic enzyme is much more stable.

Salt Concentration

The effect of salt concentration on the activity of the soluble and immobilized enzyme was assessed in 50 mM glycine–KOH buffer, pH 10, containing varying concentrations of KCl. The salt concentrations required for activity of the soluble and immobilized *Hv*ADH2 are compared. Both soluble and immobilized enzymes displayed an optimum activity at 4 M KCl, and no activity was detected at zero salt concentration with minimal differences at intermediate salt concentrations. This immobilization method has no effect on the halophilic characteristic of our enzyme while a decrease

in halophlicity had been reported for a halophilic malate dehydrogenase immobilized on Sepharose [18].

Characterization of Immobilized *Hv*ADH2

Properties of the immobilized biocatalyst such as stability, thermoactivity, and organic solvent tolerance were investigated for the optimized preparation and compared to its soluble counterpart.

Stability

Samples of soluble crude and immobilized *Hv*ADH2 were incubated in 100 mM Tris–HCl buffer, pH 8, containing various concentration of KCl at 5 °C. The residual activity was measured at appropriate time intervals as indicated in Fig. 5.

Both enzyme forms are more stable at high salt concentration, with a marked increase in stability overtime for the immobilized form. In 3 M KCl the immobilized *Hv*ADH2 retains 80 % of its original activity after 9 days at 5 °C whereas the free crude enzyme retained 60 % under same conditions. Immobilization *Hv*ADH2 forms enough covalent linkages to stabilize its quaternary structure, which explain the improvement in stability.

Thermoactivity

Information on the thermoactivity of an immobilized enzyme is very useful for the potential industrial applications. Soluble *Hv*ADH2 has impressive thermal activity and was found maximally active at 90 °C [16]. Due to the technical difficulties in the determination of activity for the immobilized enzyme at higher temperatures, we compared the behavior of both preparations between 20 and 60 °C. While the trend of the two preparations between 20 and 50 °C is identical (linear increase in activity), at 60 °C the immobilized enzyme showed 20 % higher activity compared to the free form (results not shown).

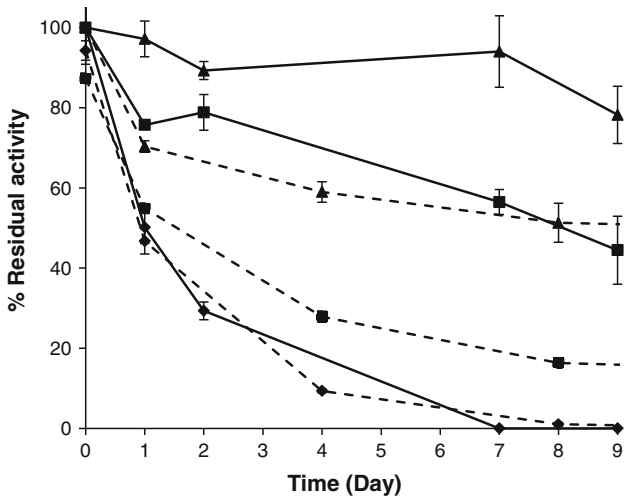

Fig. 5 Stability of free *Hv*ADH2 *dashed line* and immobilized *Hv*ADH2 *solid line* in different salt concentrations at 5 °C. *Filled diamond* 1 M KCl; *filled square* 2 M KCl and *filled triangle* 3 M KCl

Stability in Organic Solvents

The effects of organic solvents on the stability of immobilized *Hv*ADH2 were evaluated. The final immobilized preparation was incubated in a mixture of 30 % DMSO or 30 % methanol and 100 mM Tris–HCl buffer, pH 8, containing 2 M KCl at 5 °C. The residual activity was measured at standard conditions (see "Activity Assay for the Immobilized Enzyme" section) after 24 and 72 h. Following incubation for 24 h with 30 % DMSO and 30 % methanol immobilized *Hv*ADH2 retained 55 and 60 %, respectively. Upon increasing the incubation time to 72 h the immobilized enzyme still retained 55 and 45 % activity, respectively. It appears that the immobilized *Hv*ADH2 showed remarkable stability in organic solvents when compared to our previous results for the free enzyme under the same conditions [17]. This result is of real significance for industrial applications.

One-Step Purification–Immobilization–Stabilization of His-*Hv*ADH2

Metal derivatized-epoxy Sepabeads can be extremely selective toward His-tagged proteins. This allows for the purification, immobilization, and stabilization of the target enzyme in one step [14, 15]. In order to take advantage of this technique, the immobilization and the purification in single step have been employed with crude extract from *H. volcanii* containing His-ADH2.

The purification–immobilization procedure was performed by suspending 1.5 g of Ni-epoxy Sepabeads in 7.5 mL of the supernatant resulting from *H. volcanii* and containing 5.2 mg/mL total protein concentration. The suspension was treated as described in "Immobilization of His-*Hv*ADH2 on Metal Chelate-Epoxy Sepabeads" section. In order to determine the maximum recovery of *Hv*ADH2, a second identical sample (7.2 mL) was purified by Ni-IMAC as described previously [16] followed by the quantification of the purified enzyme. Prior to purification, the specific activity of *Hv*ADH2 in the crude extract with 100 mM ethanol was ~0.4 U/mg.

After the immobilization process, no activity was ever observed in the supernatant of the immobilization suspension mix or in the wash indicating that 100 % immobilization efficiency was achieved. The blocking step was performed (see "Blocking of the Remaining Epoxy Groups

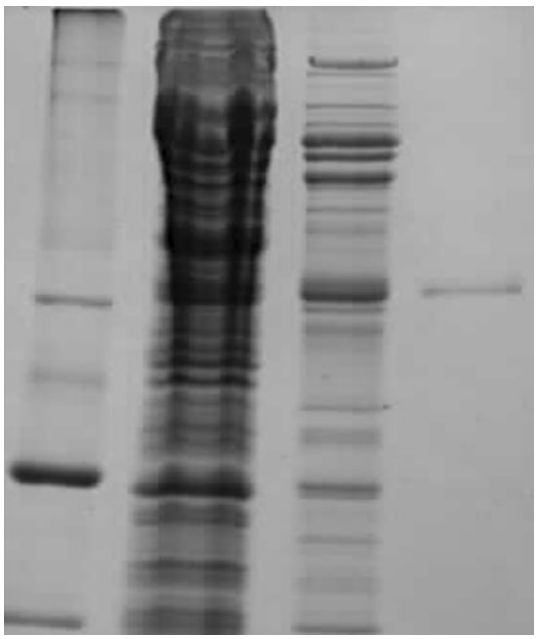

Fig. 6 SDS-PAGE of the purification–immobilization of His-*Hv*ADH2 by direct incubation in the presence of Sepabeads. *Lane 1* (*left*) broad range protein marker P7702S (2–212 kDa), *Lane 2* crude protein extract, *Lane 3* proteins released from final immobilized preparation after purification–immobilization of crude protein extract containing His-*Hv*ADH2 on Sepabeads, *Lane 4* protein released from final immobilized preparation after immobilization of pure *Hv*ADH2 on Sepabeads

and Modifications of the Enzyme–Support Microenvironment" section) then the final preparation was assayed for activity under standard conditions. The specific activity of immobilized *Hv*ADH2 from the crude was tenfold less than what was detected after IMAC purification, indicating only a partial purification. In order to assess the level of purity for the immobilized sample, an aliquot of the immobilized crude preparation and of the immobilized pure enzyme preparation was boiled in the presence of SDS and visualized by SDS-PAGE using 12 % polyacrylamide gels, stained with Coomassie Brilliant Blue R250 (Fig. 6).

The SDS-PAGE of pure immobilized His-*Hv*ADH2 shows a band approximately corresponding to the subunit molecular of His-*Hv*ADH2 molecular mass around 38 KD indicating that subunits of the immobilized *Hv*ADH2 were released in a SDS-PAGE analysis. Therefore, not all monomers of the enzyme were covalently immobilized on the support. The immobilized crude preparation shows significant contamination of His-*Hv*ADH2. This result is in line with the determined specific activity and purification fold values for crude immobilized preparation. The purification results could be improved by decreasing the immobilization time (48 h) and minimizing covalent bonds formation between the support and contaminant proteins, however, the catalytic activity of immobilized enzyme will decrease by using this strategy and the use of a pure preparation is desirable.

Conclusions

Alcohol dehydrogenase from halophilic archaeon *H. volcanii* (*Hv*ADH2) was successfully covalently immobilized on metal-derivatized epoxy Sepabeads. The protocol for immobilization of *Hv*ADH2 was designed carefully to satisfy the nature of the halophilic enzymes. The immobilization procedure developed gives the best combination of increase in retention activity and immobilization efficiency. Moreover, additional modification of the enzyme–support preparations was also performed. Blocking of the remaining epoxy groups with ethylamine displayed increased stability. However, no significant stabilization effect was observed when using blocking agents that are commonly used. The immobilization process enhanced the enzyme stability, thermal activity, and organic solvent tolerance bringing this enzyme one-step closer to industrial applications.

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