Scientia Iranica, Vol. 14, No. 2, pp 112–117
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SCIENTIA
IRANICA

Studies on the Activity and Stability of Immobilized Thermophilic Alcohol Dehydrogenase

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A thermophilic alcohol dehydrogenase from *Thermoanaerobacter brockii* (TBADH) was immobilized by adsorption on Fractosil, methyl-, octyl-, and hexadecyl-Fractosil. As compared to its free form, the thermal stability of the enzyme was enhanced upon immobilization and its pH and temperature optima were altered. The immobilized preparations were used in continuous catalytic operations using a packed-bed reactor. It was demonstrated that the inorganic supports are suitable candidates for operating reactors, especially at high temperatures. Furthermore, larger volumes of the substrates could be converted to a product using enzyme preparations immobilized on the derivatized (hydrophobic) Fractosil, as compared with the unsubstituted form.

INTRODUCTION

Among useful strategies in applied biochemistry, enzyme immobilization is of great concern, so as to permit the re-use of these often expensive biocatalysts. The thermal stability of immobilized enzymes is one of the most important criteria of their application. Thermostable enzymes from thermophilic bacteria make it possible to operate bioreactors at high temperatures, which prevent possible microbial contamination, a major problem in conventional bioreactors operating at room temperature [1]. For this reason, the use of thermostable enzymes in immobilized biocatalyst systems is of value in industrial processes. Amongst various methods of immobilization, the use of non-covalent adsorbents (adsorption) provides the simplest and most rapid method. Inorganic carriers are considered to be suitable for an immobilized enzyme at high temperature because of their rigidity and stability [2]. However, little is known regarding the immobilization of thermostable enzymes on such supports.

The alcohol dehydrogenase from *Thermoanaerobacter* (formerly *Thermoanaerobium*) *brockii* (TBADH) is a tetrameric enzyme of four identical 37,652 Da subunits composed of 352 amino acids [3]. The enzyme is a (NADP)-dependent dehydrogenase, which oxidizes preferably secondary alcohols [4]. Meanwhile, several applications have been described using this enzyme for synthetic purposes, e.g. the gram-scale conversion of $NADP^+$ to NADPH [5] or the synthesis of several chiral compounds [6] and analytical solvent detection with an immobilized enzyme electrode [7]. Development of a simple and rapid method to generate a stable and highly active TBADH immobilized on an inert support is valuable for the routine use of TBADH as an immobilized enzyme electrode in bioassays. In the present study, TBADH was immobilized by simple adsorption on derivatized Fractosils (a porous form of silica) with alkyl chains of different lengths, as hydrophobic and non-ionic adsorbents. The experimental procedure was optimized, in order to obtain the highest activity yield. The nature of the supports and the length of the alkyl chains, on the thermal stability of TBADH, were taken into consideration as important variables for efficient immobilization.

MATERIALS AND METHODS

Materials

Thermoanaerobacter brockii alcohol dehydrogenase and $NADP^+$ (95% purity) were purchased from Sigma

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(St. Louis, Mo, USA). Fractosil 500 (Art 9384) and all other chemicals were of an analytical reagent grade and obtained from Merck (Darmstadt, Germany).

Preparation of Alkyl-Substituted Fractosil

Corresponding aliphatic alcohols (Methanol, Octanol and Hexadecanol) were refluxed with 30 g Fractosil for 18 h in a Dean-Stark apparatus [8]. The resulting suspensions were then filtered and washed with refluxing toluene for 18 h in a Soxhlet, in order to remove the noncovalently adsorbed alcohols. Each derivatized gel was, subsequently, dried under vacuum to a constant weight.

Enzyme Assay

A stock solution of 2-propanol in potassium phosphate (50 mM, pH 7.8) was used as the enzyme substrate. The dehydrogenase activity of TBADH was measured at 65°C, following the reduction of 0.5 mM $NADP^+$ (and monitoring the formation of NADPH) at 340 nm in the assay mixture containing 150 mM 2-propanol. Enzyme concentration was determined by measuring absorbance at 280 nm and using the absorption coefficient, $\varepsilon = 2.3 \times 10^4$ $M^{-1}cm^{-1}$ [9].

Immobilization Procedure

The immobilization process was achieved via the following approach: An enzyme solution of a known concentration, 30 μg of 2 mg/ml stock solution in 50 mM phosphate buffer pH 7.8, was allowed in contact with 0.2 g of the gels in a tube at 4°C for 1 h, which was found sufficient to reach binding equilibrium. The sample was centrifuged and the pellet was rinsed three times with a phosphate buffer (50 mM, pH 7.8) to remove unbound protein. Protein concentrations were determined using the BioRad reagent, according to Bradford [10].

Catalytic Activity of Immobilized Preparations

The activities of immobilized preparations were determined by suspending a volume of the immobilized preparations corresponding to 0.5 mL of packed matrixes in the assay mixture. To determine the rate of increase in absorbance due to NADPH formation, the suspensions were mixed for 45 sec using a very small magnetic stirrer, followed by rapid centrifugation, stopping the reactions by sucking off the solutions from the samples and the readings of the absorbance of the clear supernatants were made at 340 nm (A340). The activities of the samples were determined from the difference between the A340 values before and after incubation. The preserved % activity was determined by comparing the activity of the dry weight carrier-enzyme complex with the original activity of the corresponding wet derivatives.

Effect of pH on the Enzyme Activity and Stability

Stock solutions of glycine/Mes/succinate, each at 10 mM concentration at various pH ranging from 3 to 10, were prepared for studying the effect of pH on the enzymatic reaction in free, as well as immobilized, preparations. TBADH dehydrogenase activity was studied at 65°C. To measure the pH stability of the free and immobilized enzyme, the samples were incubated at various pH values for 2 h at room temperature and the residual activity was determined at the optimal activity condition (pH 7.8, 65°C).

Effect of Temperature on the Enzyme Activity and Stability

The thermal stability of the enzyme preparations was studied at the optimum pH. Identical enzyme solutions in a phosphate buffer (0.05 M, pH 7.8) were preheated separately at two temperatures (50 and 70°C) for various time periods. The residual activity was determined by adding the substrate and carrying out the enzyme assay under optimal reaction conditions. The optimum temperature for the enzyme was determined by varying the incubation temperature between 40 and 95°C in the phosphate buffer.

Continuous Catalytic Operation with Immobilized Enzyme

TBADH, adsorbed on 0.2 g of the carriers by the method described above, was placed in a jacketed column (1 × 4) cm. Desired temperatures were adjusted by circulating heated-water inside the jacket using a thermostated water circulator. Differences in absorbance, taken as catalytic activity between the initial mix and the collected fractions, were recorded at 340 nm, using a flow rate of around 0.85 ml/min.

RESULTS AND DISCUSSION

The possibility of binding TBADH to free and alkyl-substituted Fractosil was investigated using small columns of the gels. Table 1 summarizes the enzyme carrier combinations studied and protein loading and enzyme activity. The enzyme showed almost no binding to unsubstituted and methyl substituted Fractosil, whereas its adsorption is accentuated when long chain derivatives were used (derivatives C and D). A number of proteins showed some degree of affinity

Table 1. The enzyme activity and the amount of bound protein to free and alkyl substituted Fractosil.

Symbol	Carrier	Bound Protein (mg/g Carrier)	Activity (mol/min g Dry Weight)	Preserved Activity (%)
A	Fractosil	4	1	14
B	Methyl-Fractosil	11	5	19
C	Octyl-Fractosil	51	30	83
D	Hexadecyl-Fractosil	62	39	100

for binding to the unsubstituted support [11]. The presence of alkyl chains introduced more affinity for nonionic interactions, which contributed toward the stabilization of the binding. This is compatible with an "egg-shell" of the biocatalyst, in which only a surface layer of the substituted resin is actually involved in the enzyme binding. Such catalyst profiles can also explain the observed increase of TBADH content with the increase of ligand substituted length. With regard to the TBADH binding, it must be noted that the lower degree of hydrophobicity of methyl-substituted Fractosil does not favor a good interaction between the pendant alkyl groups, which could be an additional case for the low efficiency of the binding process. Hydrophobicity and, hence, the binding affinity of substituted gels has been shown to be enhanced by increasing the alkyl chain length [12,13]. An important aspect of the adsorption of proteins to alkyl-substituted Fractosil is that binding can take place with a retention of significant levels of activity and that extremes of pH, high salt concentrations or elevated temperatures are not required to bring about efficient adsorption. The existence of hydrophobic channels on the surface of TBADH has already been recognized [14]. It, therefore, appears that adsorption takes place between "long alkyl arms" of the substituted carriers and hydrophobic side chains occurring on the surface or crevices of the TBADH molecules. The current situation would certainly be of great advantage when the use of immobilized enzyme preparations for a prolonged period of time is desired.

The extent of adsorption of TBADH to a constant amount of the used matrices in the form of suspension was investigated. Figure 1 shows the relationship between the amounts of enzyme activity adsorbed and total protein concentration. As indicated, the saturation of binding sites was more evident when octyl-Fractosil was used. However, adsorption of TBADH on hexadecyl substituted gels was followed by a linear curve pattern, presumably due to the increasing of protein-protein interactions occurring at higher protein concentrations. It should be noted that in measuring the degree of adsorption of the enzyme to the substituted matrices, the protein-adsorbent complex were washed with their respective buffers. Therefore, such increases in binding may arise from enzyme-enzyme interactions, which is in agreement with those observed by other researchers [15].

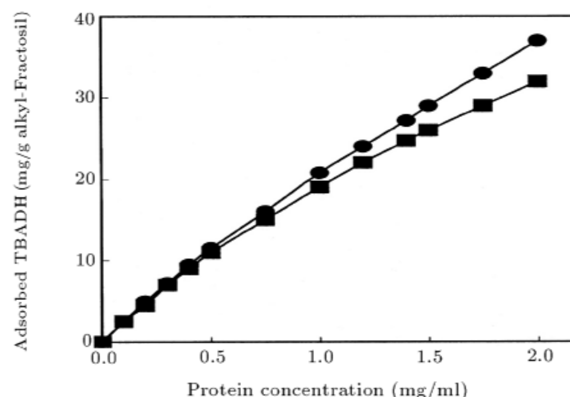

Figure 1. Adsorption of TBADH to octyl-(■) and hexadecyl- (•) Fractosil suspension as a function of protein concentration. For further details please see the text.

Effect of Ethylene Glycol

Since the hydrophobic interactions proved to disrupt preferentially via the effect of ethylene glycol, because of this unique property, it has been used as a means of establishing the contribution of this type of interaction to the adsorption of proteins on hydrophobic supports [16]. The presence of ethylene glycol at 25 or 50% concentration in respective buffers, did not result in TBADH release from the substituted carriers. This indicated a strong association of the enzyme with the hydrophobic matrices.

Effect of pH on the Activity of TBADH

The pH profile of TBADH activity toward the oxidation of 2-propanol is shown in Figure 2. Immobilization usually alters the optimum pH of enzymes. This was also observed with TBADH, whose optimal pH value was disclosed to be 7.8 [4]. It can be seen from the figure that the enzyme is quite active between pH 6.5 and 9.0 when adsorbed on alkyl substituted Fractosil. Similar deviation in the optimal pH value of alcohol dehydrogenase from different sources was obtained [17].

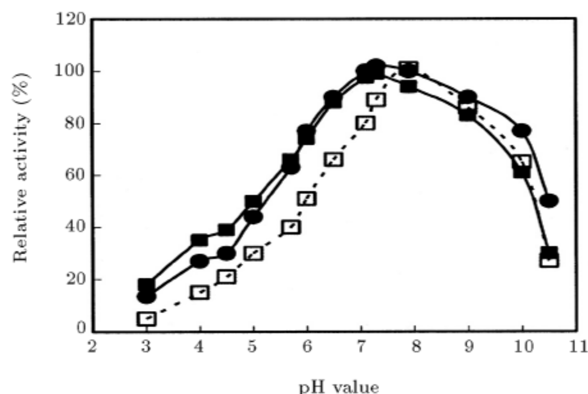

Figure 2. Effect of pH on the activity of free (□) and immobilized TBADH on octyl-(■) and hexadecyl-(•) Fractosil. For further details please see the materials and methods.

pH Stability

The pH stability of the free and immobilized TBADH was determined in a pH range of 3.6-10 at room temperature for 2 h incubation periods (Figure 3). The results indicated that interaction of the hydrophobic side chains with alkyl residues present in the matrices ultimately provides immobilized preparations with significantly higher pH stability (especially in acidic pH values) than that of the free form. pH stabilization of the enzyme upon immobilization on hydrophobic supports, especially on hexadecyl Fractosil, is quite dramatic (Figure 3). This higher degree of stabilization may be explained in terms of a greater extent of multi-point attachments, involving various hydrophobic sites and alkyl groups [18]. In addition to elevated temperatures, thermophilic enzymes have been shown to tolerate other extreme environments, e.g., extreme pH conditions [19]. Accordingly, the immobilized preparations improved the inherent resistance of the enzyme toward extreme pH values.

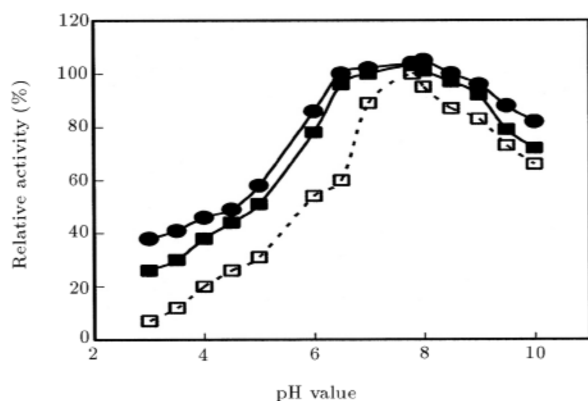

Figure 3. pH stability of free (□) and immobilized preparations of TBADH on octyl-(■) and hexadecyl-(•) Fractosil. For further details please see the text.

Effect of Temperature on the Activity of TBADH

The optimum temperature for free and immobilized preparations in the range of 30-95°C has been also studied. The results indicated that the optimal temperature for attaining the highest activities of immobilized preparations was shifted to a higher range than that of the free ones in the temperature range of 30 through 95°C, presumably owing to the improved thermal stability of the bound enzyme (Figure 4). The increase in optimum temperature may indicate some changes in the physical properties of the enzyme molecules. These observations were similar to the results obtained on immobilized glucose oxidase [20].

Thermal Stability of TBADH

For comparative studies of heat stability, samples were incubated at various temperatures and the time course for loss of activity was determined. Immobilization brought about enhancement of enzyme thermal stability in all cases studied. This typical temperature behavior is reflected by the curves depicted in Figure 5. The thermal stability of TBADH immobilized on any one of the used matrices was much greater than that of the soluble enzyme at 50°C (Figure 5a). This is in line with the fact that the hydrophobic interactions increase with an increase in temperature in the above range. The free enzyme lost 50% of its original activity after 3 h standing at 70°C (Figure 5b). However, immobilized preparations revealed that binding to hexadecyl-Fractosil occurred with a remarkable enhancement of enzyme stability, even after being kept at the same temperature for 20 h (Figure 5b). These results further support the fact that carrier materials are important variables influencing the stability of immobilized enzymes [21]. The increase in thermal stability may indicate some changes in the physical properties of the enzyme molecule. Immobilization of TBADH on

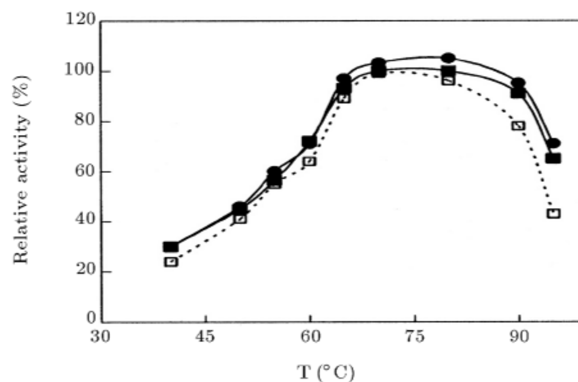

Figure 4. Effect of temperature on the activity of free (□) and immobilized TBADH on octyl-(■) and hexadecyl-(•) Fractosil.

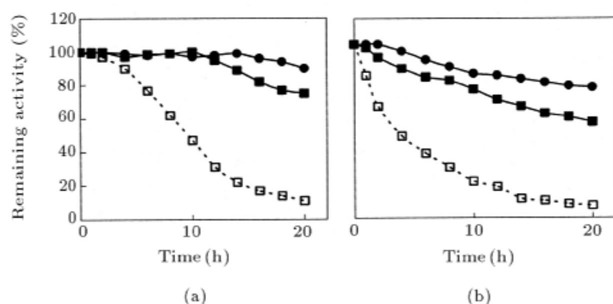

Figure 5. Effect of temperature on the activity of free (□) and immobilized TBADH on octyl-(■) and hexadecyl-(●) Fractosil in 0.05 M phosphate buffer pH 7.8 at 50°C (a) and 70°C (b). Samples were removed at various times, cooled at 4°C (for 10 min) and assayed at 65°C.

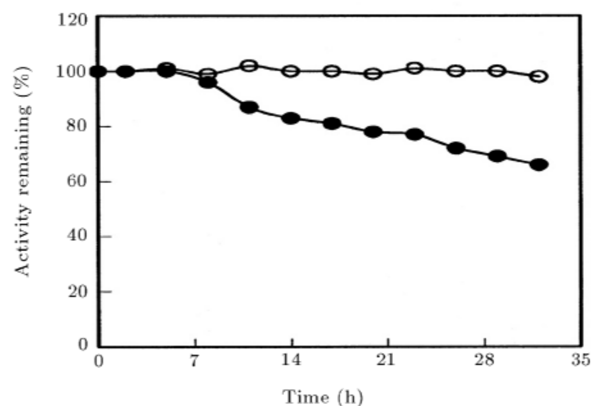

Figure 6. Use of immobilized TBADH in continuous catalytic operation at 25°C (○) and 75°C (●). The column (0.7 × 15 cm) was washed with a solution of the reactants at a constant rate of 0.85 mL/min.

alkyl-substituted carriers might have reduced the dissociation of its multimeric structure. Many oligomeric enzymes have been proved that dissociate or aggregate and, as a result, inactivate under the action of heating [18]. Accordingly, dramatic enhancement in the thermal stability of immobilized TBADH may arise from the maintenance of its oligomeric integrity, afforded by fixing the globular structure on the used supports. Obviously, the structure of the protein molecule will be much more rigid and, therefore, unfolding, as well as inactivation (upon heating) will be much more difficult to accomplish than in the case of the free form. Binding most likely takes place between a small number of hydrophobic clusters on the surface of protein and alkyl side chains on the alkyl-substituted matrices, diminishing the possibility of protein denaturation under these conditions.

Immobilized Enzyme in Continuous Catalytic Operation

The immobilized preparation of TBADH (derivative D) in the form of a small packed-bed reactor was used in a continuous operation carried out at 25°C and 75°C. As shown in Figure 6, the adsorbed enzyme loses activity fairly slowly at 75°C, whereas it is remarkably stable at 25°C and capable of converting substantial volumes of the substrate with 100% original catalytic efficiency. The columns were washed with the reactants at a constant rate of 0.85 ml/min in both cases. Data obtained on the use of immobilized TBADH in continuous catalytic operations clearly demonstrate that the preparation is stable and a substantial degree of conversion may be achieved. When yeast alcohol dehydrogenase has been used in similar operations, a rapid loss of catalytic activity was observed [15].

In conclusion, adsorptive immobilization of TBADH on hydrophobic (alkyl-substituted) supports leads to some modifications in the enzyme properties, which open new perspectives on potentially interesting biotechnological processes, particularly for the enzymatic regeneration of the NADPH cofactor. The results of the present experiments, i.e. the dependency of enzyme stability on the structure of support, the length of alkyl substituted chain, incubation temperature and pH, indicate that the remarkable stability of the immobilized enzyme at high temperatures was due to the presence of hydrophobic alkyl groups and their degree of substitution on the used adsorbent. The immobilized enzyme was more stable than the free one in all cases and revealed alterations in its temperature and pH optima. Especially at higher temperatures, hexadecyl-Fractosil proved to be a more efficacious carrier for TBADH adsorption. The results described herein suggest that adsorptive immobilization of TBADH via hydrophobic interactions may provide a useful tool for improving the inherent tolerance of a thermophilic enzyme against extreme environments.

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