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Immobilization of Yeast Alcohol Dehydrogenase on Weakly Basic Anion Exchange Resin Beads

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Yeast alcohol dehydrogenase was immobilized on weakly basic macroporous anion exchange resin beads Lewatit MP-64. After the adsorption the enzyme was crosslinked by glutaraldehyde.

The activity of the immobilized enzyme was investigated in the pH 8.9 recirculation reactor system at 303 K. It was found that the immobilized enzyme was destabilized upon addition of semicarbazide hydrochloride to the buffer solution.

A greater amount of protein was attached to the support when ethanol was present in the enzyme solution, but the activity of the bound enzyme was lower than in the absence of ethanol.

INTRODUCTION

Alcohol dehydrogenase from yeast (YADH) has been previously immobilized on supports such as s-triazinyl DEAE cellulose,[1] silanized aluminia,[2] s-triazinyl resin,[3] derivatives of agarose,[4] Enzyacryl T-10, amberlite IR-resin, porous glass[5] and polymethylmethacrylate.[6] In order to immobilize the enzyme on amberlite IR-45 resin the glutaraldehyde-linkage and azo-linkage methods were employed.[5] In this work, in distinction from the previously mentioned methods, the immobilization of YADH on macroporous weakly basic anion exchange resin Lewatit MP-64 was performed by adsorption after which the enzyme was crosslinked with glutaraldehyde. This procedure was suggested by Haynes and Walsh[7] for the immobilization of bovine trypsin onto colloidal silica particles.

The aim of this research was to compare the storage stability of the soluble and bound enzyme as well as to study the factors affecting the stability of the bound enzyme including the effect of semicarbazide hydrochloride in the buffer solution.

EXPERIMENTAL

Materials and Methods

Yeast alcohol dehydrogenase (YADH)-lyophilized, specific activity of ca. 400 U/mg enzyme protein (25 °C, ethanol as substrate), and β-nicotin-amide-dinucleotide (NAD^+) free acid, grade II, 98%, were purchased from Boehringer Mannheim, Germany. Lewatit MP-64 macroporous weakly basic anion exchange resin in OH-form was obtained from Bayer, Germany. It is a polystyrene crosslinked with the divinyl-benzene, having the polar groups of type — $N(R_3)^+$. Glutaraldehyde 25% solution, abs. ethanol and sodium pyrophosphate ($N_4P_2O_7 \cdot 10\ H_2O$) were purchased from Merck, Germany, and glycine from Carlo Erba, Italy.

Preparation of the Immobilized Enzyme

For the immobilization of YADH the static procedure[8] was used as a representative process.

A measured quantity (dry weight at 333 K) of anion exchange resin beads was loaded into a column of 0.5 cm internal diameter. They were then soaked overnight in the standard buffer solution pH 8.9 (0.05 mol dm^{-3} sodium pyrophosphate and 0.05 mol dm^{-3} glycine).

The enzyme solution (a measured weight of lyophilized enzyme in standard buffer solution) was added to the anion exchanger and it was slowly discharged from the column after half an hour. The resin beads were then washed with the standard buffer until A_{280nm} of the effluent reached 0. After that the exchange resin beads with immobilized YADH were treated with glutaraldehyde for 5 minutes. Excess glutaraldehyde was then removed by washing the column with buffer.

The protein content was estimated by spectral absorption at 280 nm of YADH with a molar absorption coefficient[9] of 1.89×10^5 cm^2 mol^{-1} and molecular weight of 150 000. The amount of the protein bound on the anion exchange resin beads was calculated as the difference between the amount added to the column and that found in solution after completion of the loading process and washing procedures.

Kinetic Assays

The kinetics of ethanol oxidation were determined by recording the rate of formation of NADH at 340 nm in a recirculation reactor system which was similar to that described by J. R. Ford.[10]

The initial rate of ethanol oxidation was calculated from the linear change of A_{340nm} during the first two minutes of the reaction, by using the molar extinction coefficient for NADH[11] 6.22 cm^2 mol^{-1}. Each reaction was repeated three times under the same conditions and the mean value was calculated. The experimental error was estimated to be 10%.

For a typical assay the final volume was 21 cm^3.

The activity of free YADH was determined in the same reactor system.

RESULTS AND DISCUSSION

The activity of the immobilized YADH was determined when YADH was supported on the beads of Lewatit MP-64 with particle diameter of 0.2 mm. This diameter was chosen for the reason that internal diffusional resistance is negligible[12] under these conditions.

The activity of the immobilized YADH was 17—28% of that of the soluble enzyme. J. R. Wykes[1] reported it was as 12.5—15% and W. Schöpp[13] as 10.0—15.0%.

The Quantities of the Attached Proteins

The quantity of the attached proteins on the 0.1 g (dry weight) anion exchanger with particle diameter of 0.8 mm depends on the enzyme concentration in the standard buffer solution (Table I). The support is saturated at an enzyme concentration of $\approx$0.7 mg enzyme cm^{-3} standard buffer solution.

The quantity of attached proteins decreases with the increasing particle size d_p (Table II). This can be expected since the enzyme adsorbs mainly onto the external surface of the anion exchange resin beads.

Lewatit MP-64 has a higher capacity for YADH than previously used supports.[1,5,14] This higher capacity can be ascribed to strong polar ion exchange groups present in Lewatit MP-64.

TABLE I

The Effect of Enzyme Concentration on the Adsorption of YADH on Lewatit MP-64

YADH concentration mg/cm³	Amount of attached proteins* mg/g dry support
0.132	0.62 ± 0.033
0.395	0.80 ± 0.035
0.591	2.24 ± 0.042
0.745	2.82 ± 0.092
0.903	2.95 ± 0.049
1.098	2.87 ± 0.057

* The values given for the amounts of the attached proteins correspond to the arithmetic mean ± standard deviation of all measurements performed in five experiments.

TABLE II

The Effect of Support Particle Size on the Adsorption of YADH. The Initial Enzyme Concentration in Buffer was 0.6 mg/cm³

Support particle size mm	Amounts of attached proteins* mg/g dry support
0.2	12.57 ± 1.032
0.2—0.3	6.68 ± 0.077
0.3—0.4	5.59 ± 0.141
0.4—0.5	4.94 ± 0.021
0.5—0.6	4.68 ± 0.156
0.6—0.8	3.03 ± 0.146

* The values given correspond to the arithmetic mean ± standard deviation of all measurements performed in five experiments.

The Storage Stability

Stability of the adsorbed enzyme was checked by storing both soluble YADH without any stabilizing agent and immobilized enzyme at +4 °C. As shown in Figure 1. the immobilized enzyme loses activity about twice as slowly as soluble YADH.

The Effect of Glutaraldehyde Treatment on the Activity and Stability of Immobilized YADH

The effect of treatment of adsorbed YADH with various concentrations of glutaraldehyde for 5 minutes at 303 K is shown in Table III. Since higher concentrations of glutaraldehyde lower the enzyme activity, a 0.2% solution was routinely used to cross-link the enzyme to Lewatit MP-64. This treatment results in a marked increase in the stability of adsorbed YADH in repeated runs of the same column (Figure 2).

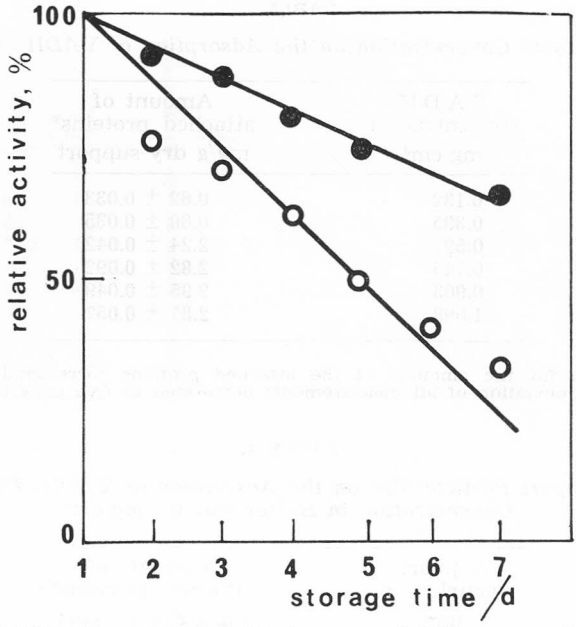

Figure 1. Storage stability of yeast alcohol dehydrogenase. Soluble enzyme (○) solution 2.5×10^{-5} mol m^{-3} in standard buffer pH 8.9 and immobilized enzyme (●) having 2.78 mg protein/g dry support were stored at +4 °C and assayed at 303 K. The initial coenzyme and ethanol concentrations were 1.26×10^{-4} mol dm^{-3} and 0.5 mol dm^{-3}, respectively.

TABLE III

*The Effect of Glutaraldehyde Treatment on the Observed Activity of Immobilized YADH**

Glutaraldehyde solution wt %	Specific immobilized enzyme* activity U/mg attached protein
0.1	179.1 ± 1.82
0.2	181.7 ± 2.67
0.3	160.6 ± 3.92
0.6	116.7 ± 4.10
0.8	77.8 ± 2.75
1.0	29.3 ± 1.04

* The particle size of the support was 0.8 mm. The bound enzyme contained 3.04 mg protein/g dry support. The activity of the immobilized enzyme was assayed at pH 8.9 in standard buffer and temperature 303 K. The initial concentrations of coenzyme and ethanol were 2.5×10^{-4} mol dm^{-3} and 0.5 mol dm^{-3} respectively.
The values given correspond to the arithmetic mean ± standard deviation of all measurements performed in three runs.

The Effect of Semicarbazide Hydrochloride on the Stability of the Immobilized YADH

According K. Watanabe et al.[3] the conversion of ethanol with immobilized YADH increases when semicarbazide hydrochloride is present in the buffer solution. To investigate this effect on the Lewatit bound YADH, the kinetics of ethanol oxidation were measured in standard buffer solution containing 0.09 mol dm^{-3} semicarbazide hydrochloride. The initial concentration of NAD^+

was 1.26×10^{-4} mol dm^{-3} and of ethanol 0.5 mol dm^{-3}. As shown in Figure 2., the presence of semicarbazide has no influence on the stability of YADH bound to Lewatit MP-64 in the absence of glutaraldehyde. On the other hand the activity of the bound enzyme cross-linked by glutaraldehyde decreases markedly. Hence, contrary to the findings of K. Watanabe et al.[3], semicarbazide cannot be recommended in the case of Lewatit MP-64 — bound enzyme.

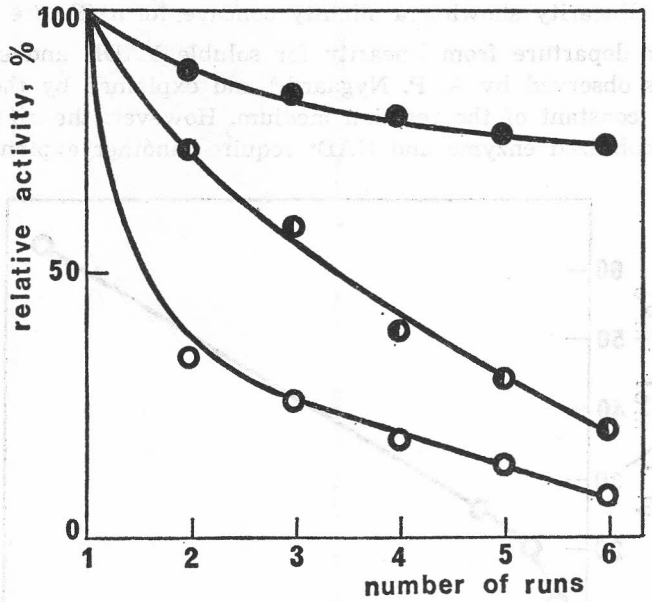

Figure 2. The effect of glutaraldehyde and semicarbazide hydrochloride on the stability of immobilized YADH.
(○) — enzyme adsorbed on Lewatit MP-64 (2.87 mg protein/g dry support) and assayed in standard buffer pH 8.9 without and containing 0.09 mol dm^{-3} semicarbazide hydrochloride, respectively; identical values were obtained in both experiments.
(●) — adsorbed enzyme (3.18 mg proteing/g dry support) was treated with 0.2% glutaraldehyde for 5 min and assayed in standard buffer pH 8.9.
(◐) — adsorbed enzyme (3.07 mg protein/g dry support) was treated with 0.2% glutaraldehyde and assayed in buffer containing 0.09 mol dm^{-3} semicarbazide hydrochloride. All assays were performed at 303 K with initial coenzyme and ethanol concentrations 1.26×10^{-3} mol dm^{-3} and 0.5 mol dm^{-3}, respectively.

The Effect of Ethanol on the Immobilization of YADH

When the immobilization procedure was carried out in the presence of ethanol (0.5 cm^3 of abs. ethanol/2.0 mg enzyme dissolved in the standard buffer as usual), 12.5 mg proteins/g dry support were attached to the anion exchange resin with particle diameter 0.5 mm. The specific activity of the bound enzyme was 88.5 ± 3.13 U/mg attached proteins. Without ethanol, only 4.94 mg proteins/g dry support were attached. In this case the specific activity of the bound enzyme was 166.1 ± 8.32 U/mg attached proteins. In both cases the enzyme was assayed in the standard buffer at pH 8.9 and 303 K. The initial ethanol and coenzyme concentrations were 0.5 mol dm^{-3} and 1.26×10^{-4} mol dm^{-3}, respectively.

Lower specific activity of the enzyme bound to the resin in the presence of ethanol can be due to enzyme-enzyme interactions, which are more frequent

at a higher number of enzyme molecules bound to the same surface of the resin beads.

Lineweaver-Burk Plot

A Lineweaver-Burk plot of reciprocal initial velocity versus reciprocal NAD^+ concentrations for immobilized YADH on macroporous exchange resin departs from linearity showing a slightly concave form (Figure 3.).

A similar departure from linearity for soluble YADH and ethanol as a substrate was observed by A. P. Nygaard[15] and explained by the change of the dielectric constant of the reaction medium. However, the effect observed with the immobilized enzyme and NAD^+ requires another explanation.

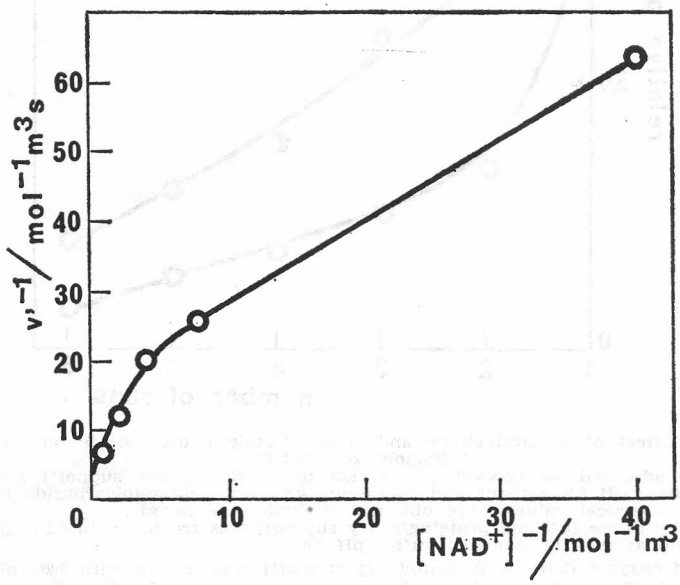

Figure 3. Lineweaver-Burk plot for immobilized YADH. The initial rates v', were measured in pyrophosphate glycine buffer, pH 8.9 at temperature 303 K. The initial ethanol concentration was 0.5 mol dm^{-3}. The coenzyme concentrations ranged from 2.5×10^{-5} mol dm^{-3} to 1.26×10^{-3}. mol dm^{-3}. The bound enzyme contained 3.05 mg proteing/g dry support.

CONCLUSIONS

1. The macroporous weakly basic anion exchange resin has a high capacity for yeast alcohol degydrogenase.

2. Cross-linking of YADH by glutaraldehyde increases the stability of the immobilized enzyme.

3. Semicarbazide hydrochloride acts as a destabilizing agent for immobilized YADH.

4. The presence of ethanol in the enzyme solution increases the quantity of attached proteins, but the activity of the immobilized enzyme does not increase proportionally with the amount of attached protein.

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SAŽETAK

Imobilizacija alkoholne dehidrogenaze kvasca na slabobazičan anionski izmjenjivač

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Alkoholna dehidrogenaza kvasca je imobilizirana na slabo bazičan makroporozan anionski izmjenjivač Lewatit MP-64. Nakon adsorpcije na izmjenjivač, enzim je na njegovoj površini umrežen s glutaraldehidom.

Aktivnost imobiliziranog enzima je ispitivana u reaktoru s recirkulacijom kod pH 8.9 i na temperaturi 303 K.

Pronađeno je da se imobilizirani enzim destabilizira u prisustvu semikarbazid-hidroklorida u otopini pufera. Uz etanol u enzimskoj otopini veća količina proteina se veže na nosilac, ali je aktivnost tako imobiliziranog enzima manja.