*Note*

# The Co-immobilization of Alcohol Dehydrogenase, Diaphorase, and NAD on Glutaraldehyde-activated Sepharose

Hiroyuki UKEDA, Masatomo IMABAYASHI, Kiyoshi MATSUMOTO and Yutaka OSAJIMA

*Department of Food Science and Technology, Faculty of Agriculture, Kyushu University, Fukuoka 812, Japan*

Received February 6, 1989

With the demonstrated use of noncofactor-requiring enzymes as bioreactors,[1] attention is turning to the use of the more complex cofactor-requiring enzymes. Among these enzymes, the dehydrogenases requiring nicotinamide cofactors are of central interest.[2] The most effective way to use dehydrogenases is to maintain both a nicotinamide cofactor, such as NAD, and the coupled dehydrogenases in one reactor and recycle the cofactor *in situ*.

In our previous paper, we reported that yeast alcohol dehydrogenase (ADH) and native NAD could be co-immobilized on Sepharose gel modified repeatedly with hexamethylenediamine and glutaraldehyde.[3]

In this investigation, we have used this method to co-immobilize ADH, diaphorase (DI), and NAD. This co-immobilization gel contains a recycling system for NAD, permitting the recycling reaction to be coupled to consumption of dissolved oxygen using vitamin $K_3$ as a mediator.

CNBr-activated Sepharose 4B was obtained from Pharmacia Fine Chemicals. Glutaraldehyde (70%; vacuum distilled for use in electron microscopy) was purchased from Taab Laboratories Equipment. Alcohol dehydrogenase (EC 1.1.1.1; from yeast, 175 IU/mg) was obtained from Oriental Yeast Co. Diaphorase (EC 1.6.99; from *Bacillus megaterium*; 30 units/mg) was a gift from Toyo Jozo Co. All other chemicals were of analytical reagent grade.

The preparation steps for spacer-modified Sepharose gel were done as in our previous paper[3] with slight modification. CNBr-activated Sepharose (0.20 g) was allowed to react with 3 ml of 1.06 M hexamethylenediamine and the desired concentration of glutaraldehyde. The spacer-modified Sepharose gels for which the modification of hexamethylenediamine and glutaraldehyde was run once, twice, or three times were termed $R_1$-Seph, $R_2$-Seph and $R_3$-Seph, respectively. In this paper, "dry gel" represents a lyophilized gel.

To prepare glutaraldehyde solutions with a variety of degrees of polymerization, glutaraldehyde solution that was diluted with 0.2 M carbonate buffer (pH 10) to adjust the concentration was left for a given time before use for the modification reaction of the gel. The degree of polymerization was defined as $A_{235}/A_{280}$ as seen in our previous paper.[3]

NAD was coupled with the spacer-modified Sepharose obtained from 0.20 g of CNBr-activated Sepharose and washed thoroughly as in our previous paper.[3] The washed gel was then placed in 1 ml of 0.05 M phosphate buffer (pH 8.0) containing 700 IU ADH and 15 units DI at 5°C. After 24 hr, the gel was thoroughly washed with 500 ml of sodium tetraborate–0.1 M HCl (pH 8.0) containing 0.5 M NaCl. The gel prepared by this procedure was defined as ADH-DI-NAD-$R_n$-Seph ($n=1\sim3$).

ADH-DI-NAD-$R_n$-Seph obtained from 0.20 g of CNBr-activated Sepharose was assayed. The assay was done as in our previous paper except no addition of DI.[3] All assays were done at 20°C.

The amount of ADH and DI bound was estimated by subtraction of the amount of protein in the coupling solution after immobilization from that before immobilization. Protein was measured by the Bradford method.[4] The coupling yields for NAD was measured by phosphate analysis after lyophilization of gel.[5] The particle diameter was obtained as the arithmetic mean from measurements made on the photographic prints.

The effects of modification steps of Sepharose on the activity were examined by using ADH-DI-NAD-$R_{1\sim3}$-Seph prepared under the same conditions (the concentration and the reaction time of glutaraldehyde used in the modification of the gel were 20% and 1 hr, respectively). The result showed that the activity was increased by increasing the modification steps of Sepharose. Although more modification steps of the gel than $R_3$-Seph suggested the possibility of the preparation of a gel with more activity,

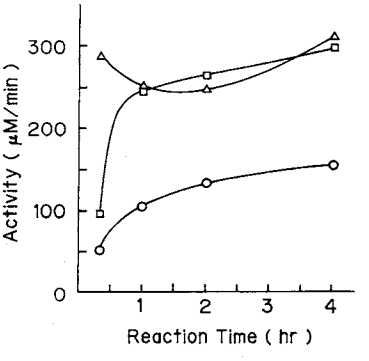


**Fig. 1.** Effects of the Concentration and the Reaction Time of Glutaraldehyde on the Activity of ADH-DI-NAD-$R_3$-Seph.

The glutaraldehyde solutions used were 5% (○), 10% (□), and 20% (△). These solutions were prepared from polymerized glutaraldehyde ($A_{235}/A_{280}=20$).

**Table I.** CHARACTERIZATION OF ADH-DI-NAD-$R_3$-SEPH PREPARED FROM GLUTARALDEHYDE WITH DIFFERENT DEGREE OF POLYMERIZATION

The concentration and reaction time of glutaraldehyde used in the preparation of $R_3$-Seph were 20% and 1 hr, respectively.

| $A_{235}/A_{280}$ | Activity ($\mu$M/min) | Particle size ($\mu$m) | | Degree of swelling | Binding NAD ($\mu$mol)[a] | Binding protein (mg)[a] |
|---|---|---|---|---|---|---|
| | | Dry | Wet | | | |
| 0.7 | 107.9 | 73.9 | 101.4 | 1.37 | 3.23 | 2.81 |
| 10 | 183.2 | 86.9 | 102.9 | 1.18 | 3.28 | 2.81 |
| 20 | 225.8 | 87.3 | 96.8 | 1.11 | 3.49 | 2.81 |
| 30 | 82.9 | 84.4 | 91.9 | 1.09 | 3.78 | 2.81 |

[a] These values were represented as the total amount bound to the gel prepared from 0.2 g of CNBr-activated Sepharose commercially available.

the $R_3$-Seph was used throughout this work, taking into account the time-consuming procedures for the preparation of the gel.

Figure 1 shows the activity of ADH-DI-NAD-$R_3$-Seph prepared by changing the concentration and the reaction time of glutaraldehyde. When 5% and 10% glutaraldehyde solutions were used, the activity has rapidly increased with an increase of the reaction time and leveled off at about 1 hr. Using a 20% solution, a higher activity than that of the gels prepared with 5% and 10% solutions was obtained with reaction times from 20 min to 4 hr. This suggests that ADH-DI-NAD-$R_3$-Seph not requiring any exogenous enzyme and coenzyme for the activity could be prepared by this method and the activity of ADH-DI-NAD-$R_3$-Seph depends on the reaction conditions of glutaraldehyde to a great extent.

Such a drastic effect of the reaction conditions of glutaraldehyde would be attributable to the aldol condensation of the reagent in a similar manner with our previous paper on co-immobilization of ADH and NAD.[3] To check this, the effects of the degree of polymerization of glutaraldehyde used in the modification of the gels on the activity were investigated using ADH-DI-NAD-$R_3$-Seph prepared at the same concentration and reaction time of glutaraldehyde (Table I). As was expected, a significant difference in the activity was recognized between them. In addition, the table shows the characteristics of these gels. As shown in the table, the ratio of the average diameter in the wet state to that in the dry state, *i.e.*, the degree of swelling, has decreased with the increase of the degree of polymerization of glutaraldehyde. The result suggests that the modification of the gel with glutaraldehyde has caused a crosslinking reaction in the pores of the gel. On the other hand, the amount of NAD bound has increased with the increase of the degree of polymerization of glutaraldehyde in contrast to the constant amounts of ADH and DI bound. In the table, therefore, the increase of activity in the range 0 to 20 of $A_{235}/A_{280}$ might be accounted for by the increase of the bound amount of NAD. However, the gel prepared with glutaraldehyde solution with 30 of $A_{235}/A_{280}$ had lower activity, in spite of the larger amount of NAD bound, than that with 20 of $A_{235}/A_{280}$. These results suggest that the difference in the activity between gels prepared with glutaraldehyde with different degrees of polymerization would be caused by both the difference of the degree of crosslinking and of the amount of NAD bound.

The reproducibility of the preparation of ADH-DI-NAD-$R_3$-Seph was examined by using three gels prepared from different lots in which the concentration and the reaction time of glutaraldehyde ($A_{235}/A_{280}=20$) used were 20% and 1 hr, respectively. The activity of these gels was 222, 224, and 227 $\mu$M/min and the relative standard deviation was 1.1%. The result suggests that the preparation of each ADH-DI-NAD-$R_3$-Seph was done with satisfactory reproducibility. Based on the average activity with these gels and the bound NAD content, a mean cycling rate of the coenzyme of 8 $hr^{-1}$ was estimated. This value was comparable to that with polyacrylamide gel in which a polymerizable NAD derivative, formate dehydrogenase, and malate dehydrogenase were entrapped all together.[6]

It has been reported, on the other hand, that the turnover values with substrate couple recycling and enzyme couple recycling in the free enzyme and coenzyme were 50,000[7] and 30,000 $hr^{-1}$,[8] respectively. When the activity of the free system corresponding to the immobilized amount of ADH, DI, and NAD shown in Table I was measured, the turnover number was 15,000 $hr^{-1}$, was comparable to the values described above. This indicates that the immobilization of the enzymes and coenzyme lowered the efficiency of the coenzyme regeneration greatly. The outstanding difference between the free and immobilized system is probably due to the steric hindrance between the solid-bound NAD and the enzymes and the diffusional limitation of bound NAD to the active sites of the two enzymes.

This paper shows that the modification of Sepharose with glutaraldehyde is a useful technique for the immo-

bilization of coupled enzyme systems including the coenzyme. This method has the advantage of simplicity in that NAD can be directly immobilized on the support in the native state. Preliminary experiments have revealed that when this co-immobilized gel was used as a reactor (column size; 2 mm$\phi$ × 10 cm) of the flow injection system for the ethanol analysis, the peak current of about 13 nA was obtained against an injection of 182 $\mu$l of 50 mM ethanol and the time for baseline reversion was about 2.5 min in the flow rate of 1 ml/min. Therefore, this co-immobilized system could be used in a column reactor in the flow injection analysis of ethanol, permitting the construction of an economic analytical system not requiring external addition of the enzyme and coenzyme. However, practical use would require high stability of the co-immobilized system. Studies on relationship between the stability and the modification conditions of the gel and its application to flow injection analysis for ethanol are in progress.

## References


1) J. C. Johnson, "Immobilized Enzymes, Preparation and Engineering," Park Ridge, New Jersey, 1979.
2) H. K. Chenault and G. M. Whitesides, *Appl. Biochem. Biotechnol.*, **14**, 147 (1987).
3) H. Ukeda, H. Kamikado, K. Matsumoto and Y. Osajima, *Agric. Biol. Chem.*, **53**, 25 (1989).
4) M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
5) G. R. Bartlett, *J. Biol. Chem.*, **234**, 466 (1958).
6) Y. Yamazaki and H. Maeda, *Agric. Biol. Chem.*, **46**, 1571 (1982).
7) M. P. Schulman, N. K. Gupta, A. Omachi, G. Hoffman and W. E. Marshall, *Anal. Biochem.*, **60**, 302 (1974).
8) T. Kato, S. J. Berger, J. A. Carter and O. H. Lowry, *Anal. Biochem.*, **53**, 86 (1973).