APPLICATIONS AND KINETICS OF IMMOBILIZED ENZYMES AND COUPLED ENZYME REACTIONS

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SUMMARY

Immobilization of enzymes results in more adequate reagents to be used analytically. After immobilization, the kinetic parameters of the enzymes are modified and nothing can be predicted about the activity of the heterogeneous system. In coupled enzyme reactions with co-immobilized enzymes, this is even more important owing to the kinetic dependence on each consecutive reaction. The determination of ethanol and acetaldehyde is considered in this paper, using two different coupled enzyme systems. Some important parameters, as the enzyme charged, the ratio of each enzyme in the sequence and the immobilization yield, are considered in terms of conversion efficiency finally determining the sensitivity of the analysis.

INTRODUCTION

Enzymes are used in chemical analysis because of their high degree of selectivity and their catalytic ability to speed up reaction rates. The number of compounds that can be enzymatically analyzed is limited by the physical or chemical properties of the substrates and products. Enzymatic reactions which require cofactors open the possibility of monitoring the reaction via the transformed cofactor. In cases where none of the substrates or products are measurable, it is often possible to determine one of these components by coupled sequential or competitive enzyme reaction [1]. This considerably increases the number of analytes that can be measured enzymatically. Furthermore, by the use of coupled enzyme reactions additional advantages can be gained: i) compounds involved in unfavorable equilibria may also be efficiently converted by shifting the equilibrium constant with the subsequent reaction, resulting in a system thermodynamically favorable and with an expanded linear response range; ii) the selectivity of the analysis may be improved and therefore the accuracy, diminishing the risk of interfering substrates; iii) inhibitory products may be continously removed and iiii) the sensitivity is increased if any of the specimen (substrates, products or cofactors) enters in a cyclic enzyme sequence with the possibility of signal amplification and recovering of cofactors.

Endogenous coupled enzyme reactions occuring in subcellular fractions and microorganisms have been used in biocatalytic electrodes [2]. These endogenous multienzyme systems mimic nature and drive the

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kinetic parameters in the way of increasing the reaction fluxes [3] and consequently offer "natural" thermodynamically favorable systems to be used analytically. More often, the analyst establishes the coupled enzyme reactions according to the analytes in the sample and uses highly active, purified and commercially available enzymes. The activity in each successive step should be kept higher than in the preceding step [4], in order to drive the system towards the product side and to achieve complete conversion in the overall system. In soluble systems, this is relatively easy to control, both empirical and theoretically, after knowing the kinetic parameters (K_M, v_{max} and rate constant k) of the free enzymes. The relative active enzyme concentrations in the system can then be established. Frequently it may occur, unlike "natural" coupled systems, that optimum conditions (pH, T, ionic strength, activators) for each enzyme are not the same and a compromise between them has to be made.

The use of enzymes as analytical reagents in flow system [5] is nowadays a frequent practice, mostly in the form of immobilized enzymes [6] owing to the advantages gained after immobilization [7]. By co-immobilizing the enzymes on the same support, a closer coupling will be achieved between the reaction sites resulting in a higher conversion efficiency than by mixing enzymes separately immobilized [8]. Co-immobilized enzyme reactors (CIMERs) have been described for the simultaneous determination of different analytes in flow injection analysis (FIA) [9-11].

Unlike soluble systems, the use of co-immobilized enzymes in coupled reactions offers some variables which make the efficiency of the system unpredictable. Concerning the determination of ethanol and acetaldehyde and using two different coupled enzyme reactions, some variables in co-immobilized systems are considered.

COUPLED ENZYME REACTIONS FOR THE DETERMINATION OF ETHANOL AND ACETALDEHYDE

The most common enzymatic determination of ethanol is based on the use of alcohol dehydrogenase (ADH, EC 1.1.1.1) [12] in the presence of NAD^+, see reaction (1).

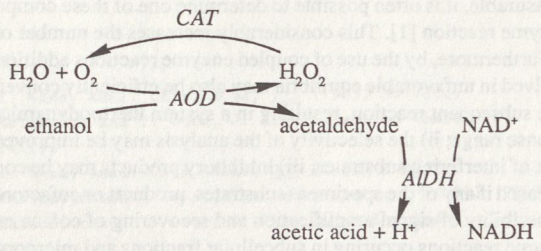
$$\text{ethanol} + NAD^+ \xleftrightarrow{ADH} \text{acetaldehyde} + NADH + H^+ \qquad (1)$$

Because of the unfavorable equilibrium of this reaction $Ke = 8.0\ 10^{-5}$M (phosphate buffer pH 7.0; 20°C), acetaldehyde has to be removed in order to shift the reaction towards the product side. Aldehyde dehydrogenase (AlDH, EC 1.2.1.5) oxidizes acetaldehyde to acetic acid in the presence of NAD^+ in an irreversible reaction (react. 2) which becomes the thermodynamic driving force of the overall system,

$$\text{acetaldehyde} + NAD^+ + H_2O \xrightarrow{AlDH} \text{acetate} + NADH + H^+ \qquad (2)$$

Moreover, by using this indicator reaction the sensitivity of the analysis increases (one mole of ethanol gives two molesof NADH) and simultaneously allows the determination of aldehydes.

A second alternative is based on the use of alcohol oxidase (AOD, EC 1.1.3.13), catalase (CAT, EC 1.11.1.6) and AlDH. The addition of catalase removes hydrogen peroxide which may oxidize the aldehyde and recovers the molecular oxygen which enters in a cyclic system:



1) Enzyme charged per gram of support. Four different amounts of AlDH were added to commercially glutaraldehyde activated silica support (Serva, Si 500, pore diameter 500 Å and particle size 30 µm). The loaded supports were packed in 50 µl reactors and studied in the FIA mode by injections of 50 µl of 2mM

propionaldehyde into the carrier stream consisting of 2 mM NAD^+, 4 mM 2-mercaptoethanol, and 0.15 M KCl in 0.1 M pyrophosphate buffer (pH 8.5). The flow rate was 0.4 ml min^{-1}. Fig. 1 shows that at high amounts of enzyme, the response reaches a maximum level. A similar response pattern has been found with enzyme electrodes [1].

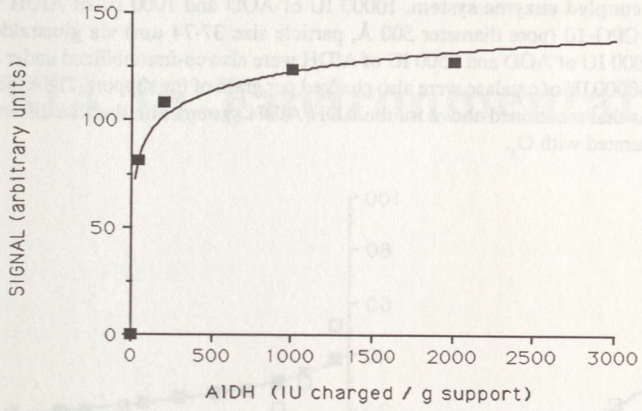


Fig. 1. *Effect of different amounts of AlDH charged per gram of support on the activity of the resultant immobilized enzyme for the conversion of propionaldehyde.*

2) Study of the influence of two different enzymatic ratios on the conversion of ethanol and acetaldehyde.

2.1 ADH/AlDH coupled enzyme system. 250 IU of ADH and 2500 IU of AlDH per gram of support were co-immobilized. 9500 IU of ADH and 300 IU of AlDH were also charged per gram of silica support. The conversion efficiency was studied in the FIA mode with the co-immobilized enzymes packed in 50 µl reactor volume. 25 µl injections of 125 µM ethanol or 125 µM acetaldehyde were made into the carrier stream containing 2 mM NAD^+, 0.15 M KCl in 0.1 M phosphate buffer at pH 7.0. The conversion efficiency is estimated in relation to injections of stoichiometric amounts of NADH.

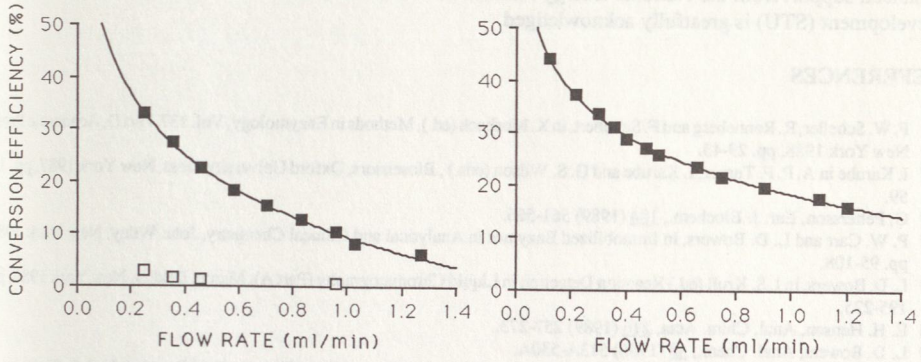


Fig. 2. *Variation of the conversion efficiency for (■) acetaldehyde and (□) ethanol with the flow rate of the carrier and with different ADH/AlDH ratios; (A) 250/2500 and (B) 9500/300.*

Neither of these co-immobilizations resulted in a good conversion for ethanol. The fact that the initial solutions (before immobilization) containing both enzymes presented activity for ethanol (measured at zero

order reaction), and the % of immobilization were in all cases higher than 88 % indicate changes in the kinetic parameters and/or an increased inactivation rate constant. It has been shown [13] for immobilized horse liver ADH that the stability of the immobilized preparation depends on the quantity of bound enzyme.

2.2 AOD/CAT/AlDH coupled enzyme system. 10000 IU of AOD and 1000 IU of AlDH were co-immobilized per gram of CPG-10 (pore diameter 500 Å, particle size 37-74 μm) via glutaraldehyde as reported previously [11]. 1000 IU of AOD and 2500 IU of AlDH were also co-immobilized under the same conditions. In both cases 160000 IU of catalase were also charged per gram of the support. The experimental conditions were the same as that mentioned above for the ADH/AlDH system with the sole difference that the carrier solution was saturated with O_2.

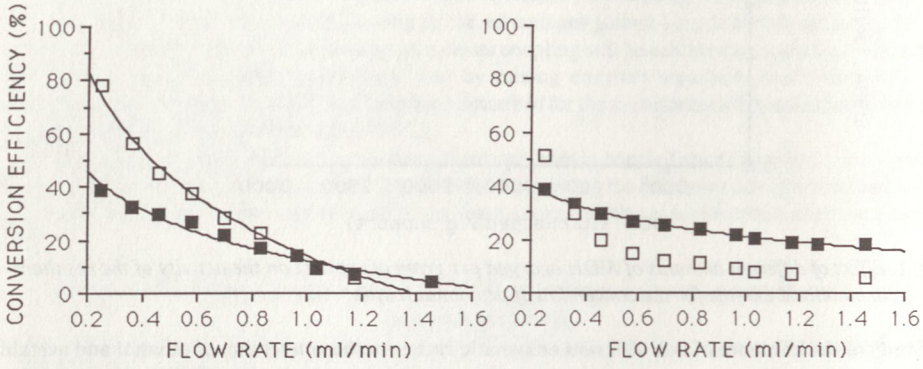


Fig. 3. *Variation of the conversion efficiency for (■) acetaldehyde and (□) ethanol with the flow rate of the carrier and with different AOD/AlDH ratios; (A) 10000/1000 and (B) 1000/2500.*

These results clearly show the importance of the enzyme ratio in the efficiency of a coupled enzyme system. With a surplus of AOD (Fig. 3A) there is amplification for ethanol due to the cyclic regeneration of O_2.

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