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ORIGINAL PAPER

Methods development and specific parameter for immobilized enzyme. Behaviour of trypsin

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Abstract

Enzyme immobilization can generally be achieved by chemically or physically attaching the enzyme to a support or by confining the enzyme to a restricted volume by means of a semipermeable membrane. Containment by a membrane or entrapment in a polymeric gel are applied in the construction of so-called enzyme-electrodes physical adsorption of enzymes has found little use in chemical analysis, but covalent binding is extensively used in the preparation on enzyme reactors for use in continuous flow systems[7].

Keywords: enzyme immobilization, semi permeable membrane, immobilization parameter

Introduction

The first report of enzyme immobilization was by Nelson and Griffin [1]. They observed that invertase (extracted from yeast) was absorbed on charcoal and that absorbed enzyme preparation showed catalytic action similar to that on the native enzyme. The first successful covalent binding of a variety of proteins (including enzymes) was carried out after World War II. Michael and Evers [2] described such linkage of proteins to carboxymethylated oxidized cellulose in 1949. Enzyme immobilization did not become popular however, until the 1950s when Grubenfafer and Schleith [3] reported coupling carboxypeptidase and amylase to diazotized poly (p-aminostyrene). The theoretical and practical aspects of immobilized enzymes have been covered in several reviews and monographs, centered on chemical and analytical applications [5,6]

Enzyme immobilization can generally be achieved by chemically or physically attaching the enzyme to a support or by confining the enzyme to a restricted volume by means of a semi permeable membrane. Containment by a membrane or entrapment in a polymeric gel are applied in the construction of so-called *enzyme-electrodes* physical adsorption of enzymes has found little use in chemical analysis, but covalent binding is extensively used in the preparation on enzyme reactors for use in continuous flow systems [7].

Theoretical procedure

In what follows we summarize the parameters which are considered to be major importance for experimental enzyme immobilization on membrane. The parameters which must be considered are:

Amount of bound protein

It may be estimated from the mass balance of protein in solution before and after immobilization and in washing solutions (thorough washing with 1M NaCl, with buffer at low and high pH, 3; 9, 1h is necessary). Analysis can be performed by activity determination in the solutions or by measurement of protein concentrations (UV absorption of Folin Ciocalteu method. Lowry's method may lead to errors with nitrogen containing buffers or hydrolysis products of the matrix). Results from protein and activity determination can be different for enzyme preparations containing inactive protein. This method is based on the hydrolysis of the matrix with protein and analysis of certain amino acids.

Yield of bounded proteins

From the mass balance of protein in the reaction mixture and bounded protein; it serves as a basis for the evaluation of coupling methods. The amount is of interest for the economics of a process.

Yield of active immobilized protein

The most accurate determination method is active site titration which is possible only with few enzymes. The determination of V'_{max} and comparison with the native enzyme is a good basis for the calculation from the mass balance. From V'_{max} the total amount of immobilized activity can be determined and compared with the total activity used in the preparation procedure. The amount of activity remaining in solution and desorbed by washing should be considered separately. This method is not applicable with tests subject to mass transfer limitation (when the effectiveness $\eta < 1$) with substrates of low solubility substrate inhibition.

Maximal activity V'_{max} (resp V_{max}app)

The determination of the **true V_{max}** . the reaction rate with aubstrate saturation of the enzyme is limited to those systems with sufficient substrate solubility (no substrate inhibition). For example direct measurement of V_{max} is not possible for active heterogeneous systems with oxygen as a substrate due to its low solubility and to the concentration gradient in the matrix, or across a membrane. Extrapolation according to kinetics for homogenous systems (Linewaever Burk) is not possible for diffusion – limited systems. This is the case for most of the preparations with good activity.

Definition for V_{max} = maximal initial reaction rate (U/g = μ moles /min g dry prep) under clearly specified conditions (surface bound enzymes (U/cm^2). Recommendation: optimal pH buffer, salt substrate concentration $S \geq 100\ K_m$, 25 °C.

In many cases direct experimental results give only effective maximal reaction rates per amount (resp volume) of catalyst. Experimental conditions must be stated. (As to tritimetric methods there must be pointed on that buffered solutions in general must be used as otherwise a pH gradient is maintained in the matrix ; however high buffer concentrations lead to great experimental errors; the optimal concentration must be found by test: with 10^{-4}, 10^{-3}, 10^{-2} M buffer. The influence of external mass transfer should be eliminated: test in a stired vessel with $R_e = \pi\ n\ d_l / \nu \geq 50000$, in revolving per sec , d_l diameter of stirrer , cm , (viscosity) $\nu = 0{,}01$ cm^2 / sec. Re = Reynolds number.

Differential fixed bed: flow rate > 0, 4 cm / sec, conversion < 20 %. These conditions cannot be achieved with compressible beds. If substrate solubility is low, determination of V'_{max} must be performed in an aerated stirred vessel.

Effectiveness

The determination of η effectiveness as a function of substrate concentration for systems with Michaelis kinetics where $V'_{max,}$ can be determined without limitation by mass transfer. Measured of initial rates V'_0 at $S / K_m = (0,1 , 1, 10, 100) \times K_m$, η is obtained by comparison with V_0 for the soluble enzyme :

$$\eta = (V'_0 / V'_{max}) / V_0 / V_{max}$$

Operational effectiveness of immobilization

The operational effectiveness factor is the ratio of the times required for a defined degree of substrate conversion with the same amount of free and immobilized biocatalyst. For systems where the intrinsic Michaelis – Menten constant is not perturbed by the immobilization, it can be determined by measurements of substrate conversion times and V_{max} from the following relation :

$$\eta_0 = \tau / \tau' \times V_{max} / V'_{max}$$

where τ is residence time required for 90% substrate conversion with free and immobilized biocatalyst, respectively τ' . It is recommended that when possible to determine η_0 for 90% substrate conversion. For the determination of η as a function of molecular weight application of the same principle as described before: comparison with the performance of the soluble enzyme (using equal amounts of activity , based on V_{max} (app) and V_{max}.

Stability

Stability is defined as the time dependent retention (loss) of enzyme activity. Activity determination is based on standard condition for maximal activity. The statement of stability as a function of pH, temperature or solutes should be based on the measurement of at least 3 values conveying the range of the variable. If applicable stabilities are expressed by the half-life, based on I order reaction kinetics. An extrapolation however, beyond the range of experimental data is not justified. Empirical data might also be stated as % loss of activity versus time.

Storage stability

Standard temperatures for the statement of storage stability are below 0°, 4°, 25 °C. Storage stability of commercial preparations should indicate the specifications at the time of delivery. The conditions for the storage of open packages or recovered material should be indicated. Storage stability stated in research publications should indicate the physical state, the medium and additives (substrate, products stabilizer).

Operational stability

The conditions for the determination of the operational stability require that the initial substrate conversion is monitored below 90 %. External diffusion limits are to be observed. An initial substrate concentration of 10 K_m is appropriate for preparative operations. Analytical operations are assayed at $[S] = K_m$.

Operational stability in discontinuous reactions is stated by the number of operational cycles performed at 90 % substrate conversion until the activity loss falls to 50% or the percentage of activity loss in n cycles.

Procedure

Behaviour of trypsin.

Principle of method

Trypsin is an enzyme found in pancreatic juice which catalyses the hydrolysis of peptide bonds formed from the carbonyl groups of the basic amino acids L- arginine and L lysine. As the digestion proceeds, free amino groups are formed so that the reaction can be followed by estimating the increase in the number of free amino groups using the formol titration. Formaldehyde reacts with amino groups with the formation of H^+. The solution becomes more acid, and the amount of alkali required to neutralize this increased acidity, gives a measure of the amount of free amino groups formed. Formaldehyde also very effectively stops any further activity by reacting with the enzyme protein :

$$- NH_3^+ + H^+CHO \Leftrightarrow - NH\,CH_2\,OH + H^+$$
$$- NH\text{-}CH_2\,OH + HCHO \Leftrightarrow - N(CH_2\,OH)_2$$

Hydrolysis of N α benzoil –L-arginine ethylester (BAEE): may be a decrease of the pH value due to the proton accumulation in the microenvironment of immobilized enzyme. The pH gradient between bulk solution and particle interior gives rise to pH shifts of the pH/ activity profiles. The maximum activity is obtained only if the proton accumulation in the microenvironment of the enzyme can be totally suppressed and the pH value inside the particles corresponds to the exact pH-value in the bulk solution. For the hydrolysis of BAEE by trypsin the pH optimum of the native enzyme lies between pH 8, 0 and pH 8,5.

Results and discussions

Table 1 summarizes for this pH range the enzymatic activity of trypsin measured on an ionic carrier (copolymer of 4- izothiocyanato styrene/ acrylic acid / 1,4 divinil benzene 1:10: 110) in dependence on the kind and concentration of the added buffer. The measurements were performed titrimetically by the pH-stat method (8).

Table 1. Enzymatic activity of immobilized trypsin as a function of the buffer used

BAEE (vol)	NaCl(mol/l)	CH_3COONa	Tris (mol/l)	AE (U g^{-1})
0.01	-	-	-	-
0.01	0,10	-	-	-
0,01	-	0.02	-	52
0.01	-	-	0,02	150
0,01	0,10	0,02	-	60
0,01	0,05	-	0,02	170
0,01	0,05	-	0,02	170
0,01	0,10	0,02	0,02	160

Reaction conditions: 20 cm^3 BAEE solution as substrate pH 8,00, 25 ° C

Whereas the pure substrate solution showed no detectable hydrolysis and after addition of sodium chloride only a time nearly immeasurable activity was obtained some amounts of sodium acetate (pK_a 4,75), or Tris (hydroxymethyl amino methane) pK_a 8,25, gave rise to higher enzymatic activities. Sodium acetate has no buffer capacity at pH 8. In the

bulk solution nearly the whole buffer is transformed to acetate ions which can perform the proton uptake and transport from the particle interior to the outer solution. This transport function can be fulfilled as long as there is a pH gradient from the interior to the outer parts as long as the acetate ions can be transferred to the non dissociated acetic acid according to its pK_a value. Thus, have most always be at least a weak pH-gradient which at the same time means a shift of the pH optimum. As postulated a buffer with a pK_a value in the range of the pH-optimum is most effective by its additional buffer action.

Figure illustrate possible effect with an anionic matrix:

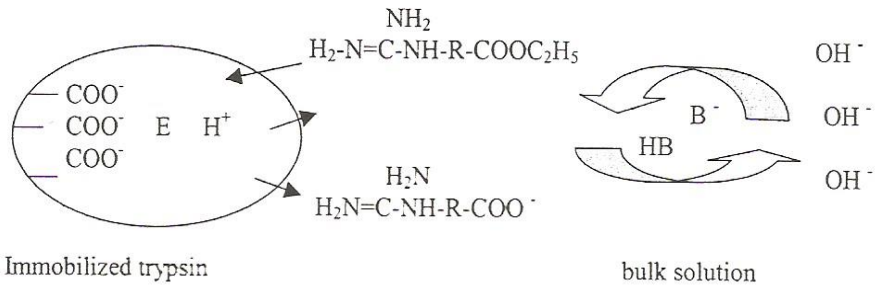
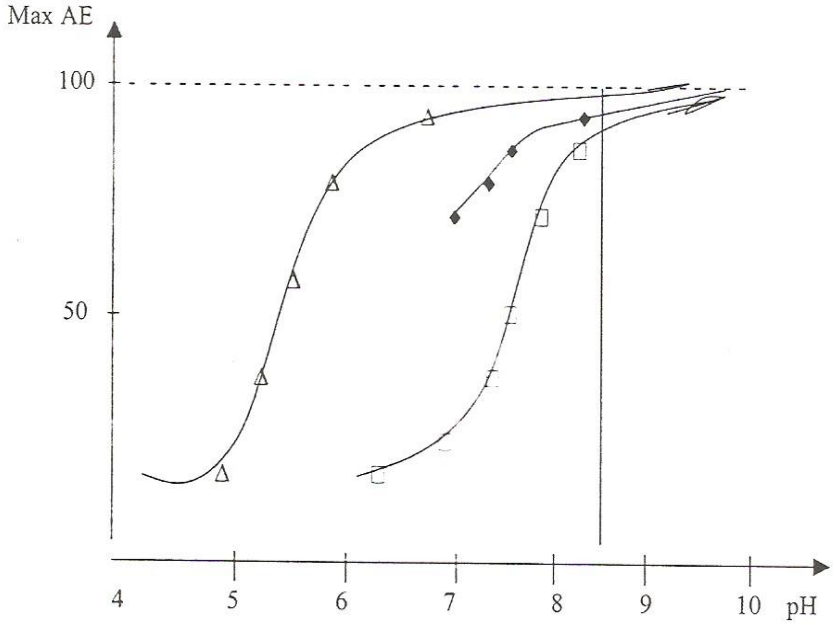


Figure 1. The effects of buffer to immobilized trypsin on an anionic carrier transport and buffering



test conditions :titrimetrical determination 0,01 BAEE, 0,1M NaCl, 0,01 M Tris for native Δ, respectively for the immobilized □ enzyme and ♦ immobilized enzyme 0.01 M BAEE, 0,2 MnaCl, 0,1 M Tris.

Figure 2. pH activity profiles of native and immobilized trypsin.

The added buffer B^- must compete with the matrix for the proton which are liberated during the enzymatic reactions. The efficiency of the buffer determines the pH-value which can be maintained in the microenvironment of the enzyme.

In figure 2 can be seen that a concentration of 0,01 mol dm^{-3} Tris is not sufficient to bring the shifted pH activity profiles of the immobilized enzyme back to the pH/ activity profiles of the native enzyme.

In the pH/activity profiles fig 2 it can be seen that in the case of enhanced concentrations of the buffer the pH-optimum could be shifted back to the original pH-optimum of the native enzyme, nearly the optimum activity at pH 8,5.

For high molecular substrates like β- casein the effect of pronounced proton accumulation may be dramatically felt. The solubility of β-casein pH 4 is dependent on the pH value. High molecular protein components of β casein itself may precipitate within the carrier.

Conclusion

In general the simplest method to overcome substrate diffusion limitation is to use increasing substrate concentration until that point, where no increase in the reaction rate can be observed. This is not applicable when substrates have a limited solubility like oxygen or steroid. In some cases only a theoretical analysis can give an estimate of the catalyst effectiveness and the intrinsic immobilized activity.

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