Journal of Chromatography, 215 (1981) 65–72
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,865

ENZYMES IMMOBILIZED ON COLLAGEN MEMBRANES: A TOOL FOR FUNDAMENTAL RESEARCH AND ENZYME ENGINEERING

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SUMMARY

Films of highly polymerized collagen prepared under industrial conditions by the Centre Technique du Cuir, Lyon, France, have been routinely used after acyl azide activation for the covalent immobilization of numerous enzymes from different classes. The stability of the resulting membranes to operational and storage conditions, their excellent mechanical strength and resistance to bacterial degradation allowed their use for several purposes. Fundamental aspects of their heterogeneous enzymology, including diffusional effects and subunit interactions, were examined. The enzyme engineering aspect was developed with polymembrane bioreactors and enzyme electrodes. Besides the protein environment provided by the collagen matrix which prevents enzyme inactivation, the form of these membranes is advantageous for industrial purposes and for fundamental research.

INTRODUCTION

In 1906, Henri[1] was probably the first to immobilize an enzyme with the aim of elucidating its kinetics. Invertase was added to a warm solution of gelatin and, after cooling, an enzymatically active gel was obtained. This entrapment technique allowed a rough estimation of the importance of diffusion in the hydrolysis of sucrose.

In recent years, new techniques for solid phase biochemistry have been applied to enzyme immobilization[2,3]. One of the most popular methods is the cyanogen bromide activation applied to dextran matrices[4], now commercially available.

In this laboratory, different types of collagen films prepared according to the techniques developed by the Centre Technique du Cuir (C.T.C.), Lyon, France, were chosen for enzyme immobilization. In a first approach, hand-made ultra-thin films of collagen were prepared[5] and used for the covalent binding of glutamate dehydrogenase to study its behaviour and regulation when integrated in a membrane[6]. Secondly, the technology of large scale production of highly polymerized collagen membranes for various biomedical applications[7–9] allowed us to develop a mild general method for enzyme immobilization[10,11]. Two main directions of research were followed: the diffusional limitations with two-substrate enzymes and subunit interactions; and polymembrane bioreactors and enzyme electrodes.

0021-9673/81/0000–0000/$02.50

EXPERIMENTAL

Immobilization of enzymes and other proteins on collagen membranes through acyl azide groups (Table I)

Activation. Untanned crude films were 80–100 μm thick, 20 cm wide and had an unlimited length. Pieces were cut from these films and used for the acyl azide activation process. The general scheme of the Curtius procedure in three steps[12] was adapted to the proteinic nature of the matrix.

TABLE I

ACTIVATION OF COLLAGEN MEMBRANES AND COVALENT IMMOBILIZATION OF PROTEINS[11]

	Activation	
collagen		
|–COOH	CH_3OH/0.2 *N* HCl, 20–22°C, 3–20 days	|–$COOCH_3$
Asp, Glu Asn, Gln	washing in distilled water (20°C)	
|–$COOCH_3$	1% NH_2NH_2, 20–22°C, 1–15 h	|–CO–$NHNH_2$ hydrazide
	washing in distilled water (0–4°C)	
|–CO–$NHNH_2$	0.5 *M* $NaNO_2$/0.3 *N* HCl, 0–4°C, 3–5 min	|–CON_3 acyl azide
	washing in buffer (0–4°C)	
	Coupling	
|–CON_3	H_2N–Protein, 0–4°C, pH 8–9, 2–5 h	|–CO–NH–protein
	washing with 1 *M* KCl, 15–30 min (0–4°C)	
	Storage in buffer solution, 0–4°C	

First, lateral carboxyl groups were esterified by immersion in methanol containing 0.2 *N* HCl for 1 week at 20–22°C[13]. After washing, the membranes were then placed in a 1% hydrazine solution and kept overnight at room temperature. After washing in distilled water at 0–4°C, acyl azide formation was achieved by dipping the membranes into 0.5 *M* $NaNO_2$ + 0.3 *N* HCl for 3–5 min. A thorough washing with a buffer solution (the same as used for the coupling step) gave activated membranes free

from any activating reagents, ready for enzyme coupling which must be performed immediately.

Coupling. Solutions of the chosen enzymes were prepared, at variable concentrations depending on their specific activity, at an alkaline pH when possible, borate or glycine–NaOH buffer. For the random immobilization of one or several enzymes, activated membranes were directly immersed in the coupling solution of enzymes and a surface covalent binding occurred spontaneously on both faces[11].

An asymmetric coupling[14] can be obtained when different enzymes are immobilized on each face. For this purpose a special device was used allowing the binding of one or several enzymes on one face, with a different enzyme on the other face. When the first face is exposed to the reaction mixture, the product diffuses and acts as substrate for the enzyme on the other face. Such processes are of great interest in the study of multienzyme systems and for enzyme electrodes. In routine experiments, the time required for coupling was 2 h. Then, the membranes were washed with 1 *M* KCl for 15–30 min to eliminate any adsorbed enzyme molecules. After this treatment, no enzyme leakage occurred, as shown by kinetic profiles[11]. The immobilized enzymes were stored before use at 0–4°C in the buffer solution giving the optimum stability. The enzymes can also be lyophilized in their membrane form without loss of activity; a simple rehydration in the selected buffer fully restores their properties; γ-irradiation ensures sterilization of immobilized enzymes. The enzyme coupling solution can be used several times for successive preparations of active membranes.

Numerous enzymes have been immobilized by this method, including oxidoreductases, transferases, hydrolases and lyases. The visualization of the binding of aspartate aminotransferase using histochemical techniques[15] has shown that the enzyme is localized mainly at and near the surface of the collagen membrane[16].

RESULTS AND DISCUSSION

Heterogeneous enzymology: diffusional effects with a two-substrate immobilized enzyme

$$\text{L-glutamate} + \text{oxaloacetate} \underset{\text{aspartate aminotransferase}}{\rightleftharpoons} \alpha\text{-ketoglutarate} + \text{L-aspartate}$$

Aspartate aminotransferase associated with membranous structures in mitochondria plays a key rôle in glutamate metabolism and its kinetic behaviour when free in solution is well known[17,18]. In order to study its behaviour in a bound state, this enzyme has been immobilized on collagen membranes and the kinetics precisely determined[19]. Surprisingly, the enzyme exhibited a greater affinity for glutamate but a lower affinity for oxaloacetate. This may be due to different phenomena: conformational changes due to the binding itself, electrostatic effects or diffusional limitations. Indeed, the intrinsic and the observed behaviour of the bound enzyme may differ, due to the fact that the concentrations of substrate and product in the bulk phase and at the catalytic sites are not identical[20,21]. The effect of diffusion for single-substrate enzymes had already been examined[21], so an attempt has been made to extend this theoretical approach to our previous results.

The observed kinetics had shown that the rates measured at fixed concentrations of oxaloacetate (OAA) were not dependent on the glutamate (Glu) concentration except below 2 m*M*. Thus, oxaloacetate alone influenced the apparent activity after immobilization. It must be pointed out that the Michaelis constants of the two substrates were very different for the free enzyme: $K_m^{Glu}/K_m^{OAA} = 100$. A simple mathematical model[22] based on significant diffusional limitations for oxaloacetate between the bulk solution and the enzyme microenvironment demonstrates that the intrinsic affinities of aspartate aminotransferase for its two substrates are not affected after immobilization and that diffusional resistances are alone responsible for the apparent kinetic modifications.

To investigate more precisely the influence of diffusional limitations on opposite variations of affinity constants, sorbitol dehydrogenase bound to collagen membranes was chosen as a model of cofactor-requiring enzyme

$$\text{D-fructose} + \text{NADH} + \text{H}^+ \xrightarrow[\text{sorbitol dehydrogenase}]{} \text{D-sorbitol} + \text{NAD}^+$$

where NADH = reduced nicotinamide–adenine dinucleotide. A rotating membrane reactor was specially designed[23] to yield a precise variation of the external mass transfer coefficient. The results show that diffusional limitations for NADH decrease the apparent affinity for the cofactor but increase the apparent affinity for fructose.

As previously demonstrated for aspartate aminotransferase, such opposite variations occur in reactions involving two substrates or a substrate and a cofactor of widely different affinities. A general analysis of the effect of diffusion on two-substrate reactions[24] has shown that such characteristic opposite effects would occur with immobilized enzymes with two substrates or a cofactor provided their affinity constants are very different.

Subunit interactions

Many enzymes are oligomeric and studies of structure–function relationships are always difficult to perform. A new technique based on the immobilization of oligomeric enzymes by only one subunit per enzyme molecule was proposed by Chan[25] and used to determine whether monomeric forms were active. In our case, aspartate aminotransferase, previously immobilized for studies of modified kinetics[19,22], was chosen because of its dimeric nature. To minimize the attachment of the enzyme to the collagen membranes by more than one subunit, a milder activation was performed. It was observed[26] that the immobilized subunits of holoenzyme were inactive. This activity was recovered on incubation with active enzyme, reduced holoenzyme or apoenzyme. The collagen matrix in film form was very easy to handle and difficult steps such as filtration or centrifugation to recover the enzyme conjugate were not needed.

Simulation and reconstitution

Multienzyme systems bound to artificial membranes can be used as models of regulation and compartmentalization. The behaviour of several enzymes using the same substrate, for example adenosine 5′-diphosphate (ADP) and creatine kinase,

adenylate kinase or ATP-synthase in mitochondria, can be easily simulated by using one, two or several immobilized enzymes in the same medium. Thus at any time, one can add or remove one of the enzymes by lifting the membrane in or out of the reaction mixture.

The use of an asymmetric membrane where mitochondrial ATP-synthase was coupled on one side only of a collagen membrane separating two compartments allowed the synthesis of ATP at the expense of a proton gradient[27], according to Mitchell's theory.

Enzyme–membrane reactors

The development of immobilization techniques led numerous groups to explore a wide range of potential applications in enzyme engineering. Enzyme bioreactors, now using mainly hydrolases, appear very promising provided cofactor-requiring enzymes can be used on a large scale. Experimental and theoretical approaches have been published and reviewed[28]. So far, it appears that the enzyme–membrane configuration has received little attention. In this laboratory, different types of reactors have been designed. The first was made of a hollow Altuglas cylinder (inner volume 108 ml) with two strips of enzymatic collagen film rolled to form a helicoid[29]. Plastic bands with knobs regularly distributed maintained a constant spacing between the active films, and glucoamylase was used to produce glucose from maltose or soluble starch solutions. Tracer studies have shown that this reactor behaves as a continuous stirred tank reactor. The catalytic support kept its activity for 18 days in continuous operation at 40°C and the activity was 80% after 17 months storage at 4°C.

Using the same enzyme, a thin layer-flow reactor comprised of stacks of several collagen membranes (0.15 × 0.16 m) maintained by spacers was constructed[30]. Two Altuglas blocks with inlet and outlet pipes ensured the water-tightness of the device. This reactor was of the plug-flow type and a large enzyme area was available per volume of reaction fluid.

A decisive improvement was obtained by using an artificial kidney module (RP5; Rhône-Poulenc, Paris, France) and substituting the dialysis membranes by our enzyme–collagen membranes[31,32]. The modified module consisted of eleven compartments with two enzymic membranes in each (32.5 × 11.8 cm) separated by grooved holding plates. An excellent distribution of the substrate solution and a high ratio of immobilized enzyme area to inner liquid volume (1.6 m^2/115 ml) were thus obtained. α-Amylase and glucoamylase were co-immobilized by random coupling for the hydrolysis of maltodextrins to glucose at high flow-rates in recycling experiments. The extent of conversion of maltodextrins to glucose *versus* time was measured with a glucose electrode associating glucose oxidase collagen membranes with a platinum anode described in the next paragraph. In this study, the polymembrane bioreactor was utilized for glucose production as a model reaction, but its use is not restricted to hydrolytic reactions: series of modules with different enzymatic activities or including affinity ligands are expected to improve such a device and are now under investigation.

Enzyme electrodes

The preparation of immobilized enzymes for analytical use has recently increased in importance and among the newly developed instrumentation, enzyme elec-

trodes[33,34], which consist of an enzymatic reaction taking place near an electrochemical sensor, appear to be very promising[35].

In our case, a glucose sensor was prepared using glucose oxidase immobilized on collagen membranes by our previously described method[36]. The device consists of a modified gas electrode in which the pH detector is replaced by a platinum anode and the porous selective membrane by our enzymic membrane. The membrane is tightly pressed against the anode by a screw cap and is thus easily removable. The enzyme catalyzes the oxidation of glucose according to the reaction:

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \beta\text{-D-glucono-}\delta\text{-lactone} + H_2O_2$$

The electrode is immersed in a small vessel with the selected buffer in which glucose-containing samples are injected. Enzymatically generated hydrogen peroxide is detected by anodic oxidation at +650 mV *versus* a Ag/AgCl reference. After an injection the current output increases and reaches a steady-state within a few minutes. It can also be differentiated and thus a peak is obtained within 1 min. Both responses (steady-state and dynamic) are proportional to the glucose concentration in the 10^{-7}–$2 \cdot 10^{-3}$ *M* range. A compensating electrode mounted with a non-enzymatic collagen membrane allowed the detection of and correction for electrochemical interferences when testing samples with high levels of electroactive species. The extreme sensitivity of our system appears better than previously reported by other authors. This glucose probe was very convenient for rapid assays in blood[37] or other biological fluids and food[38].

A two-enzyme electrode for maltose determination using the same electrochemical detector has been designed with membranes specially prepared by asymmetric coupling[14]. The two enzymes involved were glucoamylase and glucose oxidase. Glucoamylase alone was immobilized on the membrane face exposed to the bulk phase into which maltose-containing samples were injected. The hydrolysis of maltose occurred according to the reaction:

$$\text{maltose} + H_2O \xrightarrow{\text{glucoamylase}} 2\text{-D-glucose}$$

Glucose diffused through the membrane and was then oxidized on the inner face with immobilized glucose oxidase in close contact with the platinum disk. As with the monoenzyme system for glucose, the sensitivity and linearity were excellent.

The same basic electrochemical sensor has also been used for the determination of various other species with collagen membranes bearing different mono- or multienzyme systems. A single multipurpose electrode[39], where selected membranes bearing different oxidases can easily be replaced, allowed the detection of galactose, cholesterol, maltose, sucrose or lactose.

On the other hand, a new approach to heterogeneous enzymology is possible with such electrodes. As underlined above, diffusional limitations with enzyme–matrix conjugates appear to be one of the main effects of immobilization. Previous data with aspartate aminotransferase in a stirred medium or with sorbitol dehydrogenase bound to a rotating membrane[19,22,23] revealed a complex situation. But an

even more complex stituation occurred with motionless enzymic membranes fixed on electrodes. Considering glucose oxidase immobilized on both membrane faces, hydrogen peroxide is generated at the inner interface between the platinum anode and membrane and at the outer interface between the membrane and reaction mixture. This product can be detected with the platinum anode in the immediate vicinity of the inner enzyme layer and with another sensor in the bulk phase. Two simultaneous monitorings of the immobilized enzyme activity and its dependence on glucose concentration and temperature, taking into account diffusional effects, were then possible[40].

CONCLUSIONS

So far, particulate supports have been widely used for enzyme immobilization and less attention has been paid to membrane matrices. In this laboratory, collagen films, available in bulk quantities for biomedical applications, were chosen. The acyl azide activation and coupling process avoiding contact between reagents and enzymes allow maximal protection of biological activities; furthermore, concentrated coupling solutions can be reused for series of couplings. Two other methods of immobilization involving carboxyl groups, using Woodward's reagent K or a water-soluble derivative of carbodiimide respectively, were developed[41]. The activities retained were of the same order of magnitude. The acyl azide process was routinely used because the chemicals were inexpensive and widely available. Enzymes from different classes have been immobilized including, among hydrolases, proteolytic enzymes like trypsin and chymotrypsin. The covalent surface binding to the support ensures an irreversible attachment which is very favourable for long term use of these membranes in bioreactors or enzyme electrodes.

Amongst the favourable properties of immobilized enzymes, an enhanced stability is often claimed and this point has recently been examined in detail[42]. From our experience with enzyme–collagen membranes, even when diffusional effects mask the intrinsic behaviour of bound enzymes, the protein environment of collagen seems very favourable as shown by the stability to long term storage or operational conditions[43,44]. It must be stressed that, with the glucose electrode, hundreds of assays at 30°C could be run. Storage of the enzyme probe in buffer at room temperature for long periods was possible, and even when the slope of the calibration curve decreased with membrane activity, linearity was maintained and measurements with an acceptable sensitivity were possible for more than 1 year.

Finally, the properties of such protein supports will certainly lead to further applications.

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