Immobilised cells and enzymes

a practical approach

Edited by
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Cover Photograph: Bioreactors for the energy-conserving anaerobic treatment of wastewaters. Fixed-films of bacterial cells are attached to 2.5-cm inert ceramic Raschig rings to confine them within the continuous-flow system. The films formed after inoculating the bioreactors with sludge from a common anaerobic digester from a conventional municipal wastewater treatment plant. Kindly supplied by the Oak Ridge National Laboratory, Oak Ridge, Tennessee, U.S.A.

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Preface

The compilation of the chapters in this book has been done with the intention of introducing and instructing students and readers into the art of cell and enzyme immobilisation by describing in detailed, recipé-type fashion, the apparatus, materials, and methods used. While several chapters are general in nature, others address more specific applications of immobilised cells and enyzmes. There is much current interest in using biotechnology for the production of fuels and chemicals (including pharmaceuticals and hormones), and in this regard the use of immobilised cells and enzymes in biotechnological processes has tremendous potential. It is hoped, therefore, that this practical book will serve the interests of industry, academia, and governments alike.

I am extremely indebted to the authors who, through their contributions, have, in my opinion, made this a valuable handbook. I would also like to thank Dr. Alan Wiseman of Surrey University who is responsible for my interest in immobilised cells and enzymes. Finally, I am also grateful to Ms. Debbie Weaver of Oak Ridge National Laboratory for her excellent secretarial service.

Jonathan Woodward

Abbreviations

BSA	bovine serum albumin
cAMP	cyclic AMP
CAS	Concanavalin A-Sepharose
CM	carboxymethyl
CNBr	cyanogen bromide
Con A	Concanavalin A
DEAE	diethylaminoethyl
DMSO	dimethylsulphoxide
EDTA	ethylenediamine tetracetic acid
ELISA	enzyme-linked immunosorbent assay
FAD	flavin-adenine dinucleotide
FDA	fluorescein diacetate
FSH	follicle-stimulating hormone
hCG	human chorionic gonadotropin
Hepes	N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid
IC	immobilised cells
LH	luteinising hormone
MBA	N,N′-methylenebisacrylamide
MIX	3-isobutyl-1-methylxanthine
NAD(P)	nicotine-adenine dinucleotide (phosphate)
PEG	polyethylene glycol
PEI	polyethyleneimine
PVA	polyvinyl alcohol
PVP	polyvinylpyrrolidone
RRDE	rotating ring-disc electrode
s.c.e.	saturated calomel electrode
TEMED	N,N,N′,N′-tetramethylenediamine
TSH	thyroid-stimulating hormone

Contents

Introduction

I.J. HIGGINS

We are rapidly discovering more and more ways of exploiting the unique catalytic and recognition properties of biological systems in disparate areas of human activity, from medicine to the military; from food processing to microelectronic sensing devices. In many cases the development of practical processes or devices incorporating biological elements is critically dependent upon devising appropriate procedures for the retention and stabilisation of the biological component, be it a large molecule or small particle. In many cases therefore, a method of immobilisation is required.

Although immobilisation methods have been exploited for many years in biologically based products, for example, enzyme test strips, the technology has developed rapidly over recent years. Not only has the range of approaches to retaining the biological element expanded significantly but immobilisation is becoming increasingly important for modifying the behaviour of that element. For example, kinetics, specificity and stability can be altered to design a more appropriate system for commercialisation. Further, the technology is becoming increasingly predictive; in other words, more of a science than an art.

There is no doubt that immobilisation methodology will play an increasingly important role in biotechnology and indeed its development represents the rate-limiting step in some areas. Notwithstanding the foregoing, there is much that we do not understand about these processes. For example, recent collaborative work between the Cranfield Biotechnology Centre and the University of Oxford has led to the development of new types of amperometric biosensors in which immobilised oxidases make intimate electronic contact with modified carbon conductors. The immobilisation procedure, together with the chemical modification of the conductor, results in a dramatic (and extremely valuable) change in the kinetics of the enzymes and to electron flow to the conductor instead of to the natural electron acceptor, molecular oxygen. The reason for the phenomenon is not fully understood at the molecular level.

Research into the science of cell and enzyme immobilisation is currently extremely important, will continue to be so for the foreseeable future and will impinge increasingly on developments in enzyme engineering and modification of cells and microorganisms.

This volume offers a 'state of the art' account of the current position in immobilisation technology, with particular emphasis on the practical aspects. It covers a wide field and has been compiled by recognised leaders in the subject. It will be invaluable both to researchers and those requiring detailed information about the practicalities of this important subject.

CHAPTER

Immobilised Enzymes: Adsorption and Covalent Coupling

JONATHAN WOODWARD

1. INTRODUCTION

The adsorption of an enzyme onto an insoluble support is the simplest method of enzyme immobilisation. Thus, it is a useful technique with which to initiate a student into the area of immobilised enzymes. The procedure consists of mixing together the enzyme and support material under appropriate conditions and, following a period of incubation, separating the insoluble material from the soluble material by centrifugation or filtration. The major disadvantage of this method is that the enzyme is not firmly bound to the support. For example, the adsorption of enzymes onto an insoluble matrix such as DEAE-Sephadex is mainly due to multiple salt-linkages. Changes in experimental conditions such as pH, ionic strength, temperature and type of solvent can cause desorption of the enzyme from the support as they affect such linkages. It should also be mentioned that the substrate itself can also cause desorption of its enzyme from the support. Besides salt-linkages, other weak binding forces (e.g., hydrogen bonds, Van der Waals forces) are also involved in the adsorption of an enzyme to the support material. Examples of materials to which enzymes have been adsorbed are given in *Table 1*. Ideally, the immobilisation of an enzyme should result in no loss of catalytic activity. This goal can be achieved, for example, when β-glucosidase is immobilised by adsorption onto Concanavalin A-Sepharose (CAS). Note the discussion in reference 1. Generally speaking, adsorption is a mild method of immobilisation and often has little effect on catalytic activity.

Covalent coupling for the immobilisation of enzymes is based upon the formation of a covalent bond between the enzyme molecules and support material. It is important that the amino acids essential to the catalytic activity of the enzyme are not involved in the covalent linkage to the support. This may be difficult to achieve, and enzymes immobilised in this fashion generally lose activity upon immobilisation. This problem may be prevented if the enzyme is immobilised in the presence of its substrate – a step in the procedure which would tend to have a protective effect on the catalytic site of the enzyme during immobilisation. Typical water-insoluble support materials used for the covalent attachment of enzymes are shown in *Table 2*. Prior to the covalent attachment of an enzyme onto the support, the latter must be activated. Once activated, the support can then react with particular groups on the enzyme. Included are the α- and ε-groups of lysine, tyrosine, histidine, arginine and cysteine residues. Details of the covalent

Table 1. Materials Used for the Adsorption of Enzymes.

Alumina
Bentonite
Calcium carbonate
Calcium phosphate gel
Carbon
Cellulose
Clay
Collagen
Concanavalin A-Sepharose
Glass, porous
Hydroxyapatite
Ion-exchange resins
Kaolin
Phenolic polymers
Silica gel

Table 2. Materials Used for the Covalent Attachment of Enzymes.

Agarose (Sepharose)
Cellulose
Dextran (Sephadex)
Glass
Polyacrylamide co-polymers
Polyaminostyrene

coupling of an enzyme to a support are given later in this chapter.

It is not the aim of this chapter to review all the methods of enzyme immobilisation by adsorption and covalent coupling. In this regard, the reader is referred to fuller accounts on these subjects such as those by Zaborsky (2), Barker and Kay (3) and Goldstein and Mannecke (4), especially for a theoretical approach. For a comprehensive treatment of immobilised enzyme methodology, the reader is also referred to the volume of ***Methods in Enzymology*** dealing solely in this area (5).

The aim of this chapter is to exemplify the adsorption and covalent coupling of enzymes to a support material by considering the specific examples, β-fructofuranosidase (invertase) and β-glucosidase (cellobiase). Procedures used in their immobilisation, activity measurement and property determinations will be described. Such techniques will be applicable to most enzymes, but methods finally chosen will be dictated by the characteristics of the enzyme in question.

The enzymes β-fructofuranosidase (invertase) and β-glucosidase (cellobiase) are particularly suitable for initiating a student into enzyme immobilisation, primarily because they are cheap and readily available. They are both industrially

produced, and samples are usually donated free for research purposes. For example, commercial yeast invertase (β-fructofuranosidase) concentrate can be obtained from Honeywill and Stein Co., Ltd., Wallington, Surrey, UK. Cellobiase 250 L is a concentrated β-glucosidase preparation that can be obtained from Novo Laboratories, Inc., Wilton, Connecticut, USA.

2. ADSORPTION BY IONIC BINDING TO DEAE- AND CM-SEPHADEX

2.1 Preparation of the Support Material

One of the properties of Sephadex ion exchangers is that they swell when placed in an aqueous solvent. The degree of swelling is dependent upon the ionic strength of the swelling medium and is greater when the ionic strength is low and *vice versa*. Prior to the adsorption of the enzyme onto the support, take a quantity of dry Sephadex material and swell it in the appropriate buffer to which the enzyme will adsorb. The choice of buffer will depend on the enzyme to be immobilised. For example, baker's yeast invertase will adsorb onto the cationic exchanger DEAE-Sephadex A-50 swollen or equilibrated in 10 mM sodium phosphate buffer, pH 7.0 (6). Take 0.5 g dry weight of DEAE-Sephadex A-50 and add 100 ml of 10 mM sodium phosphate buffer, pH 7.0, and leave at room temperature for 3 days. After this time interval, the support material will be fully swollen. The swelling can be achieved in a few hours if the temperature is increased to 90°C. For immobilisation onto the anionic exchanger CM-Sephadex C-50, follow the same procedure except use 10 mM sodium acetate buffer, pH 3.6, as the equilibrating buffer. Note that a buffer of low ionic strength must be used otherwise adsorption of enzyme to the support will be impaired due to preferential adsorption of the buffer counterion over the protein ion.

2.2 Adsorption of Invertase Activity

2.2.1 *Determination of Free or Soluble Invertase Activity*

It is important to measure the amount of enzyme that is immobilised onto the support. This measurement is usually done by determining the activity of the enzyme that is contacted with the support and that which remains in solution after the support material is separated from it following the immobilisation procedure. The difference between them gives the theoretical or maximum amount of immobilised activity. Measure invertase activity by taking a suitably diluted 0.1 ml aliquot of the enzyme and incubating it with 0.4 ml sucrose solution (50% w/v) and 1.5 ml of 0.1 M sodium acetate buffer, pH 4.7, at 25°C. After suitable time intervals, remove a 0.1 ml aliquot of the reaction mixture and measure its glucose content using one of the glucose assay reagent kits that are commercially available. Note that both the diluted enzyme and sucrose solutions are made up in the buffer solution in which the reaction is being carried out. One unit of activity is defined as the amount of enzyme which will hydrolyse 1 μmol of sucrose per minute under the conditions of the assay. The commercial invertase described in this chapter (obtained from Honeywill and Stein) was found to have an activity of 4000 units/ml.

Table 3. Preparation of Sephadex-invertase Complex.

1.	Add 0.1 ml of commercial invertase (~400 units) to 10 ml of each equilibrated Sephadex (= 50 mg dry weight of support material).
2.	Mix by end-to-end rotation or by very gentle stirring for 30 min at room temperature.
3.	Centrifuge the Sephadex-invertase complexes using a bench-top centrifuge, pour off the supernatant, and resuspend the complexes in their respective equilibrating buffers. Repeat the centrifugation and resuspension steps several times until no activity can be detected in the supernatant. This ensures that any non-adsorbed enzyme is removed.
4.	Finally, resuspend the complexes in their equilibrating buffers (10 ml total volume).

2.2.2 *Coupling of the Enzyme to the Support*

The preparation of the Sephadex-invertase complexes is described in *Table 3* and refers to the mixing- or shaking-bath process. This technique is commonly employed for laboratory preparations.

2.2.3 *Activity of Adsorbed Invertase and Stability of the Enzyme:Support Bond*

The activity of the adsorbed enzyme is measured in exactly the same way as for the soluble or free enzyme, but 0.1 ml of the invertase-Sephadex complex is used in the assay mixture. The Sephadex particles are small enough to be pipetted into the standard-type glass pipettes and plastic pipette tips. It will be found, however, that the particles have a tendency to adhere to the sides of the pipette or pipette tip. Although this is difficult to overcome, complete removal of most particles can be achieved by repeated washing of the pipette or pipette tip with the assay mixture.

Since the optimum pH for yeast invertase is 4.7, it is important to determine that the adsorbed enzyme (at pH 7.0 or 3.6) does not desorb from the support under assay conditions. In other words, the enzyme:support bond is stable. Indeed, the adsorption of a protein to ion-exchange material is known to be sensitive to changes in pH.

The amount of activity adsorbed to DEAE- and CM-Sephadex (measured at the pH for optimum activity) is shown in *Table 4*. These data assume that the enzyme is firmly (stably) bound to the support under the assay conditions and that actual immobilised enzyme activity is being measured. This assumption is true for the enzyme bound to DEAE-Sephadex but *not* for CM-Sephadex (*Table 5*). The higher retention of activity seen when invertase is adsorbed on CM-Sephadex (*Table 4*, column 6) may be due to the fact that about half of the enzyme activity desorbs from this support at pH 4.7, and, therefore, the total activity measured is not truly immobilised enzyme activity.

The true properties of an adsorbed enzyme, including its activity, can only be measured when it has been determined that desorption of the enzyme does not occur under conditions to which the enzyme is subjected (e.g., pH, temperature, ionic strength, substrate and product concentrations). It has been established that invertase adsorbed on the DEAE-Sephadex is firmly bound in 10 mM sodium acetate buffer, pH 4.7, and under these conditions the activity of the adsorbed