IMMOBILIZED ENZYMES

Research and Development

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Preface

With the development of studies on enzyme structure and function, the highly specific nature of enzyme action has become increasingly apparent. However, enzymes are not always ideal catalysts for particular industrial or academic purposes. In order to develop better catalysts for various applications, attempts have been made recently to prepare catalysts having enzyme-like activities by using new techniques of synthetic chemistry and polymer chemistry. Some of these catalysts are called "synzymes". Immobilization of enzymes, as described in this book, is another approach to obtaining better catalysts by modifying enzymes produced by organisms to make them more suitable for the desired purposes.

The authors first began work on immobilized enzymes nearly 15 years ago, and in 1969 succeeded in industrializing a process for the continuous optical resolution of DL-amino acids using an immobilized enzyme preparation. In 1973 we also succeeded in the industrialization of a continuous process for the production of L-aspartic acid using immobilized microbial cells. These processes are thought to be the first industrial applications of immobilized enzyme and immobilized microbial cell systems, respectively, in the world. Since then, many reports on immobilized enzymes have been published, many symposia and international conferences have been held, and the number of investigators working on immobilized enzymes has increased greatly. This field is multidisciplinary, and not only enzyme chemists and biochemists but also chemical engineers have participated in studies on immobilized enzymes, as chemical engineering techniques can be rather readily applied to immobilized enzyme systems, unlike conventional fermentation or enzymic methods, which require special biological knowledge and techniques. At present, immobilized enzymes are important tools for biochemical engineering and enzyme technology, and their applications for both academic and industrial purposes are expected to give them an increasingly important role in the life sciences.

Many reviews and books have been published on immobilized enzymes, but most deal with specific aspects, and are not comprehensive. We therefore have aimed to produce a comprehensive text covering the principles and applications of immobilized enzymes and immobilized microbial cells, based on the editor's experience in research on, and industrialization of, immobilized enzyme and immobilized microbial cell systems. The

science and technology of immobilized systems now appears to have completed the early developmental stage, and may well be at a turning point. It is hoped therefore that this book will be useful as a handbook for workers in many disciplines, both scientists and engineers, and that it will contribute to some degree to the further development of this field.

Finally, as editor for this project, I wish to express my appreciation of the work of the contributing authors. I would also like to take this opportunity to thank Mr. W.R.S. Steele and the staff of Kodansha for invaluable assistance in the preparation of the English manuscript.

March 1978

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Contents

CHAPTER 1

Introduction

1.1 BACKGROUND

Enzymes are biological catalysts, consisting of protein, which participate in many chemical reactions occurring in living things. Unlike ordinary chemical catalysts, enzymes characteristically have the ability to catalyze a reaction under very mild conditions in neutral aqueous solution at normal temperature and pressure, and with very high specificity.

Enzymes have been utilized by human beings since ancient times, well before their nature was understood. The use of enzymes has gradually been extended into a variety of fields, such as brewing, food production, textiles, tanning, and medicine. Further, the recent development of biochemistry has resulted in the clarification of mechanisms of enzyme reaction and the development of new enzyme sources, and, together with progress in applied microbiology, has greatly extended the range of application of enzymes. However, enzymes are produced by organisms for their own requirements, and although enzymes are efficient and effective catalysts, they are not always ideal for practical applications. Some of the advantages of enzymes may even be disadvantages in practical use as catalysts. Namely, enzymes are generally unstable, and cannot be used in organic solvents or at elevated temperature.

Conventionally, enzyme reactions have been carried out in batch processes by incubating a mixture of substrate and soluble enzyme. It is technically very difficult to recover active enzyme from the reaction mixture after the reaction for reuse. Accordingly, the enzyme and other contaminating proteins are generally removed by denaturation by pH or heat treatment during procedures to isolate the product from the reaction mixture. This is uneconomical, as active enzyme is lost after each batch reaction.

To eliminate the disadvantages inherent in ordinary chemical catalysts and enzymes, and in order to obtain superior catalysts for applications, that is, highly active and stable catalysts having appropriate specificity, two approaches have been investigated. One is the synthetic approach, using

recently developed techniques of organic synthesis and polymer chemistry to synthesize catalysts having enzyme-like activities. These catalysts are sometimes called "synzymes." Another approach is the modification of enzymes produced by organisms. Immobilization of enzymes is a part of the latter approach.

If active and stable water-insoluble enzymes, i.e., immobilized enzymes having appropriate substrate specificity, are prepared, most of the above disadvantages are eliminated and it becomes possible to use enzymes conveniently in the same way as ordinary solid catalysts used in synthetic chemical reactions. In addition, since enzymes can catalyze specific reactions under mild conditions, (normal temperature and pressure), application of immobilized enzymes in the synthetic chemical industry can reduce energy requirements.

Immobilized enzymes are considered to be modifications of the enzymes, and should also be useful in clarifying the relationship between protein structure and enzyme activity or reaction mechanism. Many enzymes are believed to be bound to cell membranes or cellular particles in organisms. Immobilized enzymes are considered to be models of these bound enzymes, and are becoming the subject of extensive academic interest.

In 1916, Nelson and Griffin[1] found that an enzyme in water-insoluble form showed catalytic activity. They reported that invertase extracted from yeast was adsorbed on charcoal, and the adsorbed enzyme showed the same activity as native enzyme. In 1948, Sumner[2] found that urease from jack bean became water-insoluble on standing in 30% alcohol and sodium chloride for 1–2 days at room temperature, and the water-insoluble urease was active. Thus, it has been known for a long time that enzymes in water-insoluble form show the catalytic activity. However, the first attempt to immobilize an enzyme to improve its properties for a particular application was not made until 1953, when Grubhofer and Schleith[3] immobilized enzymes such as carboxypeptidase, diastase, pepsin and ribonuclease by using diazotized polyaminopolystyrene resin. On the other hand, prior to this (1949), immobilization of physiologically active protein was carried out by Micheel and Ewers.[4] Further, in 1951, Campbell *et al.*[5] prepared immobilized antigen by binding albumin to diazotized *p*-aminobenzylcellulose. Subsequently, a number of reports on the preparation and application of immobilized antigens and antibodies appeared. Such studies may also be applicable to immobilized enzymes. Following Grubhofer's investigation, less than ten papers were published on immobilized enzymes in the 1950's. In the 1960's, many papers appeared; in particular, Professor Katzir-Katchalski and his co-workers at the Weizmann Institute of Science, Israel, carried out extensive studies on new immobilization

techniques, and on the enzymatic, physical and chemical properties of immobilized enzymes.

Since the early 1960's, the present authors have investigated immobilized enzymes with the aim of utilizing them for continuous industrial production. The first report on immobilized aminoacylase was presented at the annual meeting of the Agricultural Chemical Society of Japan in 1965, and published in Enzymologia in 1966. In 1969, the authors succeeded in the industrialization of a continuous process for the optical resolution of DL-amino acids using immobilized aminoacylase. This was the first industrial application of immobilized enzymes in the world.

In the late 1960's, work on immobilized enzymes was carried out extensively in the U.S.A. and Europe, and the number of reports on immobilized enzymes increased markedly, as shown in Fig. 1.1. Besides these reports, many reviews[6–20] and books[21–33] have been also published.

In 1971, the first Enzyme Engineering Conference was held at Henniker, New Hampshire, U.S.A., and the predominant theme of this conference was immobilized enzymes. As will be described later, a definition and classification of immobilized enzymes were proposed at the conference. This conference is biannual, and the main topics have continued to be immobilized enzymes. In Japan, since the end of the 1960's, considerable work has appeared on immobilized enzymes, and a

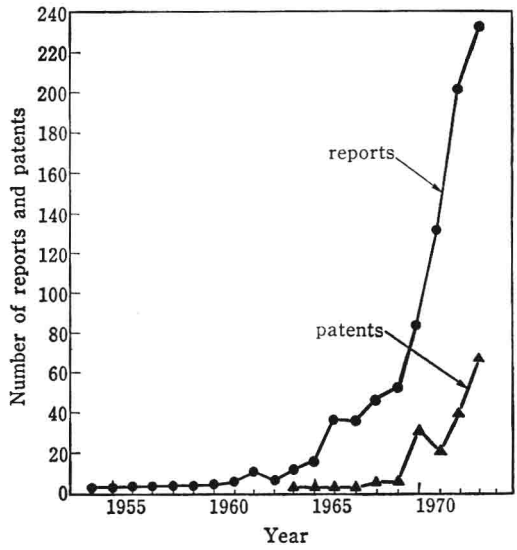

Fig. 1.1 Trends in studies of immobilized enzymes.
Redrawing from *Kagaku to Seibutsu* (Japanese), **15** (1), 48 (1977), fig. 1

symposium on immobilized enzymes was held at the 4th International Fermentation Symposium at Kyoto in March, 1972. Professor Katzir-Katchalski from Israel and Dr. Chibata, editor of this book, from Japan chaired the symposium, and there were extensive and fruitful discussions. Since then, studies on immobilized enzymes have continued at a rapid pace, and at present Japan is one of the leading countries in the world in this field.

Recently, applications of immobilized enzymes have expanded to new fields in addition to chemical synthesis. In the late 1960's, immobilization of physiologically active substances, including enzymes, was successfully carried out by Professor Porath and his co-workers at Uppsala University, Sweden. These immobilization techniques were developed to produce a specific isolation procedure for biological substances, termed affinity chromatography, by Professor Anfinsen of NIH, U.S.A., and Professor Cuatrecasas of Johnes Hopkins University, U.S.A.

Although enzymes are produced by all living things, enzymes from microbial sources are most suitable for industrial purposes for the following reasons: 1) the production cost is low, 2) conditions for production are not restricted by location and season, 3) the time required for production is short, and 4) mass production is possible. Microbial enzymes can be classified into two groups. One consists of enzymes excreted from the cells into the growth medium, and the other consists of enzymes retained in the cells during cultivation. The former type is called extracellular and the latter intra-cellular. For the utilization of the latter enzymes, it is necessary to extract the enzyme from microbial cells. However, such extracted enzymes are generally unstable, and not suitable for practical use. In recent years, many chemical substances have been produced by fermentation methods utilizing the catalytic activities of multi-enzyme systems in microorganisms.

Thus, in order to avoid the need to extract enzymes from microbial cells or to utilize multi-enzyme systems of the cells, direct immobilization of whole microbial cells has been attempted. As described later, the authors have investigated continuous enzyme reactions using immobilized microbial cells, and in 1973 succeeded in the industrialization of a process for the continuous production of L-aspartic acid using immobilized microbial cells. This is thought to be the first industrial application of immobilized microbial cells in the world. In the case of immobilized microbial cells, many problems remain, such as limited permeability of the substrate and product through the cell membranes, and the occurrence of side reactions. However, immobilized microbial cells are advantageous, as the enyzme systems of the microorganism can be readily utilized, and their future seems very promising.

1.2 DEFINITION OF IMMOBILIZED ENZYMES

Immobilized enzymes are defined as "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously". Accordingly, enzymes modified to a water-insoluble form by suitable techniques satisfy this definition of immobilized enzymes. In addition, when an enzyme reaction using a substrate of high molecular weight is carried out in a reactor equipped with a semipermeable ultrafiltration membrane, a reaction product of low molecular weight can be removed continuously through the membrane without leakage of the enzyme from the reactor. This also can be considered as a kind of immobilized enzyme system.

The term "immobilized enzyme" was recommended at the 1st Enzyme Engineering Conference in 1971. Before that time, various terms such as "water-insoluble enzyme", "trapped enzyme", "fixed enzyme" and "matrix-supported enzyme" had been used. In this conference, some problems relating to immobilized enzymes, including the terminology, were discussed. A classification of immobilized enzymes was proposed, as shown in Fig. 1.2. Enzymes are first classified into native enzymes and

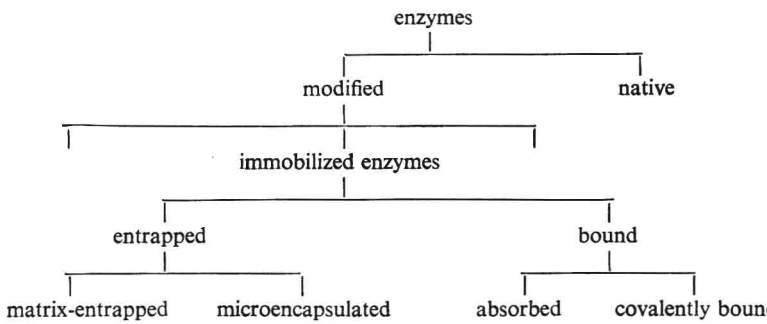

Fig. 1.2 Classification of immobilized enzymes.
From *Enzyme Eng.* (ed. L. B. Wingard), J. Wiley & Sons, 1972, fig. 1

modified enzymes. Immobilized enzymes belong to the latter type, which also includes chemically modified soluble enzymes and biologically, i.e., genetically, modified enzymes.

Thus, for practical use as catalysts, enzymes in the following three forms can be considered: 1) soluble form, 2) soluble immobilized form, and 3) insoluble immobilized form. For the latter two forms, the term "immobilized enzyme" is more suitable than "insoluble enzyme".

Although not fundamentally different from the above classification,

in this book immobilization of enzymes is classified into "carrier-binding", "cross-linking" and "entrapping" types, as shown in Fig. 1.3. Carrier-

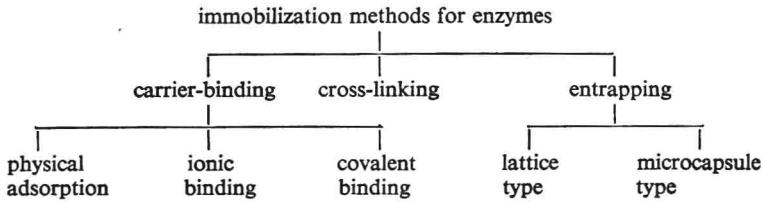

Fig. 1.3 Classification of immobilization methods for enzymes.

binding is subdivided into "physical adsorption", "ionic binding" and "covalent binding", while entrapping is divided into "lattice type" and "microcapsule type". As described later, this classification is reasonable and convenient. Except for the case using an ultrafiltration membrane, immobilized enzymes are apparently insoluble in water, and the enzyme reaction is carried out in a heterogenous medium. Since this introduces a number of complications, definitions and expressions of kinetic parameters for immobilized enzymes were also recommended by the Enzyme Engineering Conference. The recommendations can be outlined as follows.

The activity of immobilized enzyme should be expressed as an initial reaction rate (μmol/min) per mg of the dried immobilized enzyme preparation. When enzymes are bound to surfaces of different kinds of matrices (i.e., tubes, plates, membranes, etc.), the activity should be reported as the initial reaction rate (μmol/min) per unit area of surface. For these measurements, the reaction temperature, stirring rate and other reaction conditions should be clearly described. It was also recommended to report the following items; 1) the drying conditions for the immobilized enzyme preparation, 2) the protein content of the dried preparation, and 3) the specific activity of the enzyme used for immobilization.

It is recognized that the kinetic constants measured with immobilized enzymes are not true kinetic constants equivalent to those obtained in homogeneous reactions, but are apparent values because of the effects of diffusion and other physical factors. Hence, maximum velocity and Michaelis constants should be referred to as apparent V_{max} and apparent K_m (V_{max} (app) and K_m (app)). Other kinetic constants should also be reported as the apparent constants.

When reporting on the stability of enzyme activity of an immobilized preparation, it is strongly recommended to describe in detail the specific conditions used for the stability measurements.

When a new carrier is developed for the immobilization of enzymes, it is desirable to describe its properties and characteristics, such as the

number of reactive groups per unit weight of carrier and the maximum binding amount of a small molecule as well as a large molecule (protein) to a specified weight of the carrier. These recommendations are very useful as a unifying factor for the future development of immobilized enzymes.

1.3 DEFINITION OF IMMOBILIZED MICROBIAL CELLS

Immobilized microbial cells can be defined by replacing the word enzymes with microbial cells in the definition given for immobilized enzymes. That is, "microbial cells physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously." In this immobilized cells, the cells are in growing, resting and/or autolyzed states. In some cases, the immobilized microbial cells are dead, but the enzyme activities remain. When cells are growing state, it is sometimes difficult to differentiate immobilized systems as defined above from certain kinds of continuous fermentation processes.

The nomenclature of enzymes used in this book follows recommended names or other names described in *Comprehensive Biochemistry* (edited by M. Florkin and E.H. Stotz, Elsevier, 1973). For enzymes not described there, the nomenclature of the original report is used.

REFERENCES

1. J. M. Nelson and E. G. Griffin, *J. Am. Chem. Soc.*, **38**, 1109 (1916)
2. J. B. Sumner, *Science*, **108**, 410 (1948)
3. N. Grubhofer and L. Schleith, *Naturwissenschaften*, **40**, 508 (1953)
4. F. Micheel and J. Ewers, *Makromol. Chem.*, **3**, 200 (1949)
5. D. H. Campbell, E. Luescher and L. S. Lerman, *Proc. Nat. Acad. Sci.*, **37**, 575 (1951)
6. E. Katchalski, *Polyamino Acids, Polypeptides and Proteins*, p. 283, The Univ. of Wisconsin Press, 1962
7. G. Manecke, *Naturwissenschaften*, **51**, 25 (1964)
8. G. G. Guilbault, *Enzymatic Method of Analysis*, p. 235, Pergamon Press, 1969
9. L. Goldstein, *Fermentation Advances*, p. 391, Academic Press, 1969
10. A. S. Lindsey, *Reviews in Macromolecular Chemistry*, vol. 4, p. 1, Marcel Dekker, 1970
11. L. Goldstein, *Method in Enzymology*, vol. 19, p. 935, Academic Press, 1970
12. E. Katchalski, I. Silman, and R. Goldman, *Advances in Enzymology*, vol. 34, p. 445, John Wiley & Sons, 1971
13. F. Friedberg, *Chromatog. Rev.*, **14**, 121 (1971)
14. G. J. H. Melrose, *Rev. Pure Appl. Chem.*, **21**, 83 (1971)
15. K. L. Smiley and G. W. Strandberg, *Advances in Applied Microbiology*, vol. **15**, p. 13, Academic Press, 1972
16. S. Aiba, A. E. Humphrey, and N. F. Millis, *Biochemical Engineering*, p. 393, Univ. of Tokyo Press, 1973

17. K. F. O'Driscoll, *Advances in Biochemical Engineering*, vol. 4, p. 155, Springer-Verlag, 1976
18. K. Mosbach, *Techniques of Chemistry*, vol. 10, Part 2, p. 969, John Wiley & Sons, 1976
19. N. Lakshminarayanaiah, *Membrane Electrodes*, p. 335, Academic Press, 1976
20. T. R. Jack and J. E. Zajic, *Advances in Biochemical Engineering*, vol. 5, p. 125, Springer-Verlag, 1977
21. G. R. Stark, *Biochemical Aspects of Reactions on Solid Supports*, Academic Press, 1971
22. L. B. Wingard, Jr. (ed), *Enzyme Engineering*, John Wiley & Sons, 1972
23. O. R. Zaborsky, *Immobilized Enzymes*, Chemical Rubber Co. Press, 1972
24. A. C. Olson and C. L. Cooney (ed), *Immobilized Enzymes in Food and Microbial Processes*, Plenum Press, 1974
25. M. Salmona, C. Saronio, and S. Garattini (ed), *Insolubilized Enzymes*, Raven Press, 1974
26. R. B. Dunlap (ed), *Advances in Experimental Medicine and Biology*, vol. 42, Plenum Press, 1974
27. S. J. Gutcho, *Immobilized Enzymes*, Noyes Data Corporation, 1974
28. H. H. Weetall (ed), *Immobilized Enzymes, Antigens, Antibodies, and Peptides*, Marcel Dekker, 1975
29. H. H. Weetall and S. Suzuki (ed), *Immobilized Enzyme Technology*, Plenum Press, 1975
30. R. A. Messing, *Immobilized Enzymes for Industrial Reactors*, Academic Press, 1975
31. K. Mosbach (ed), *Methods in Enzymology*, vol. 44, Academic Press, 1976
32. L. B. Wingard, Jr., E. Katchalski-Katzir, and L. Goldstein (ed), *Applied Biochemistry and Bioengineering*, vol. 1, Academic Press, 1976
33. T. M. S. Chang (ed), *Biomedical Application of Immobilized Enzymes and Proteins*, vol. 1, Plenum Press, 1977
34. L. B. Wingard, Jr. (ed), *Enzyme Engineering*, p. 16, John Wiley & Sons, 1972

CHAPTER 2

Preparation of immobilized enzymes and microbial cells

2.1 IMMOBILIZATION METHODS FOR ENZYMES

A specific conformation and an active center interacting with the substrate are regarded as essential features of the catalytic activity of enzymes. The active center consists of two sites having different functions. One is the reactive or catalytic site participating in the catalytic action, and the other is the specific or binding site controlling the substrate specificity of the enzyme. These sites are usually composed of several amino acid residues held in a specific spatial relationship. The three-dimensional conformation of the entire enzyme protein also has an important effect on the catalytic activity. Consequently, to retain the catalytic activity of the enzyme in the immobilized state, it is necessary to retain the native structure as far as possible. Accordingly, in order to prepare active immobilized enzymes, immobilization should be carried out under very mild and extremely well-controlled conditions. If the amino acid residues at the active center, or the tertiary structure, are altered, the catalytic activity may decrease and changes of enzymatic properties such as substrate specificity may occur.

As functional groups involved in enzyme immobilization, free amino and carboxyl groups, the sulfhydryl group of cysteine, the imidazole group of histidine, phenolic groups, and hydroxyl groups of serine and threonine may be considered. For immobilization of an enzyme it is necessary that functional groups in the active center should not be involved in the reaction leading to immobilization of the enzyme. Further, since the tertiary structure of enzyme protein is maintained by relatively weak binding forces, such as hydrogen, hydrophobic and ionic bonds, it is necessary to carry out the immobilization reaction under mild conditions, as already mentioned. Therefore, reactions at high temperature, and strong acid or alkali treatments must be avoided to preserve the structural integrity of enzymes. Even treatments with organic solvents or high salt concentrations may cause denaturation and loss of activity.

Methods for enzyme immobilization can be classified into three basic categories as follows.

1) Carrier-binding method: the binding of enzymes to water-insoluble carriers.

2) Cross-linking method: intermolecular cross-linking of enzymes by means of bifunctional or multifunctional reagents.

3) Entrapping method: incorporating enzymes into the lattice of a semipermeable gel or enclosing the enzymes in a semipermeable polymer membrane. These methods are shown schematically in Fig. 2.1.

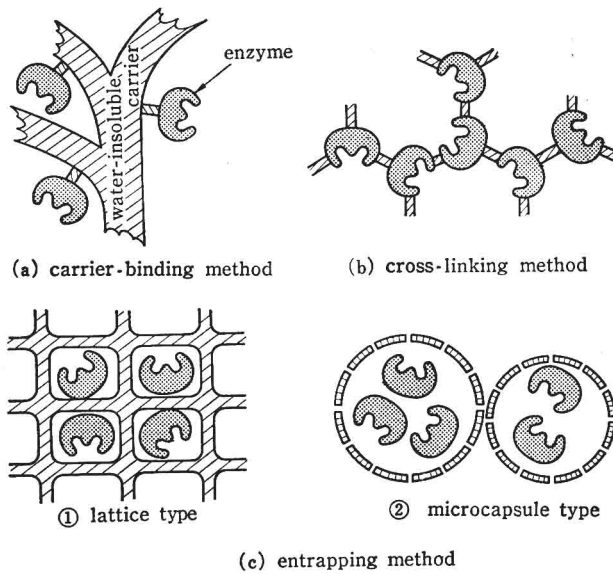

Fig. 2.1 Schematic diagrams of immobilized enzymes.

2.1.1 Carrier-binding Method

The carrier-binding method is the oldest immobilization method for enzymes and many papers have been published on this method. When enzymes are immobilized in this way, care is required regarding the selection of carriers as well as in binding techniques. Namely, the amount of enzyme bound to the carrier and the activity after immobilization depend markedly on the nature of the carrier. Although the selection of a carrier also depends on the nature of the enzyme itself, the following aspects must be considered; (1) particle size, (2) surface area, (3) molar ratio of hy-