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CLASSIFICATION AND CHEMICAL
CHARACTERISTICS OF IMMOBILIZED
ENZYMES

(Technical Report)

Prepared for publication by

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Classification and chemical characteristics of immobilized enzymes (Technical Report)

Synopsis

Immobilized enzymes are becoming increasingly popular as reusable, selective analytical chemical reagents in solid-phase flow-through reactors, as membranes in sensors and as films in dry reagent kits. Classification must encompass the properties of the original enzyme, the type of support used and the methods of support activation and enzyme attachment. Important characteristics of an immobilized enzyme *viz a viz* the enzyme in solution, e.g., apparent activity, stability and lifetime must also be reported.

1. INTRODUCTION

Immobilized enzymes have been defined as enzymes that are physically confined or localized, with retention of their catalytic activity, and which can be used repeatedly and continuously [1,2]. There is a variety of methods by which enzymes can be localized, ranging from covalent chemical bonding to physical entrapment [1,3,4] however they can be broadly classified as follows [5]:

1. Covalent bonding of the enzyme to a derivatized, water-insoluble matrix.
2. Intermolecular cross-linking of enzyme molecules using multi-functional reagents.
3. Adsorption of the enzyme onto a water-insoluble matrix.
4. Entrapment of the enzyme inside a water-insoluble polymer lattice or semi-permeable membrane.

The attractions of immobilized enzymes from an analytical standpoint are primarily their reuseability, and hence cost saving, and the greater efficiency and control of their catalytic activity (e.g., potentially longer half-lives, predictable decay rates and more efficient multi-step reactions). The immobilized form of an enzyme can be presented for use in three distinct forms:

1. Solid-phase immobilized enzyme reactors (packed bed and open tubular) for use in continuous flow techniques such as flow injection analysis and post-column derivatization in liquid chromatography.
2. Immobilized enzyme membranes incorporated into sensors such as potentiometric enzyme electrodes and optical sensors.
3. Solid-phase immobilized enzyme films for use in disposable, dry reagent kits with photometric detection.

The increasing use of all three forms of immobilized enzyme in analytical chemistry and the need to compare their performance in different situations necessitates the full reporting of experimental conditions and standard procedures for the determination and control of their properties and to aid inter-laboratory consistency of results. Immobilized microbial cells, antibodies and other biological material (e.g. tissue slices) have also been used in each of the above application areas.

2. CLASSIFICATION

Whatever the physical form of an immobilized enzyme, i.e., solid-phase reactor, membrane or solid-phase film, there are three important aspects of the immobilization procedure that must be specified in detail, namely:

1. The properties of the free enzyme.
2. The type of support used.
3. The methods of support activation and enzyme attachment.

2.1 Properties of the Free Enzyme

Each enzyme has a unique systematic name, which describes the action of that enzyme, and an associated four-number code. The first number indicates the type of reaction that is catalyzed, the next two numbers indicate the subclass and sub-subclass of reaction and the fourth number identifies the enzyme. In addition each enzyme has a working (trivial) name which is more commonly used, e.g., the enzyme commonly referred to as lipase has the systematic name glycerol ester hydrolase and the associated code number 3.2.1.1. This classification scheme for enzymes was produced by the International Commission on Enzymes in 1961 [6] and is regularly updated. (The commission was established in 1956 by the International Union of Biochemistry in consultation with IUPAC.) When

specifying the properties of the original enzyme its working name as well as its systematic name and associated code number must be stated. In addition, the source of the enzyme, the physical form of the enzyme (e.g., lyophilized), its purity (and method of purification), its catalytic activity and details of other constituents must also be given. The above information permits direct comparison of enzymes from different sources.

Catalytic activity is the most important enzyme parameter from an analytical standpoint because it has a direct bearing on sensitivity. The most widely used unit of activity is the International Unit (I.U.); one I.U. is the amount of enzyme activity that catalyzes the transformation of one micromole of substrate per minute at 25 °C under optimal experimental conditions. However, the katal is the recommended unit for enzyme activity and is defined as the amount of enzyme activity that catalyzes the transformation of one mole of substrate per second at 25 °C under optimal experimental conditions [7].

The specific activity is the number of units per milligram of protein (which is not necessarily pure enzyme). The molar activity (turnover number) is the number of substrate molecules transformed per minute by one mole of enzyme when the enzyme is the rate limiting factor.

2.2 Enzyme Support

The support material can have a critical effect on the stability of the enzyme and the efficiency of enzyme immobilization, although it is difficult to predict in advance which support will be most suitable for a particular enzyme. The most important requirements for a support material are that it must be insoluble in water, have a high capacity to bind enzyme, be chemically inert and be mechanically stable. The enzyme binding capacity is determined by the available surface area, both internal (pore size) and external (bead size or tube diameter), the ease with which the support can be activated and the resultant density of enzyme binding sites. The inertness refers to the degree of non-specific adsorption and the pH, pressure and temperature stability. In addition, the surface charge and hydrophilicity must be considered. The activity of the immobilized enzyme will also depend upon the bulk mass transfer and local diffusion properties of the system. In each report in this field all of these aspects must be addressed or adequate references should be provided.

Due to the often conflicting requirements of a good support, various materials have been used. At present there is not a universally recommended support, the final choice being a compromise for each particular enzyme and experimental system. The type of support can however be conveniently classified into one of three categories [8]:

1. Hydrophilic biopolymers based on natural polysaccharides such as agarose, dextran and cellulose.
2. Lipophilic synthetic organic polymers such as polyacrylamide, polystyrene and nylon.
3. Inorganic materials such as controlled pore glass and iron oxide.

Clearly the chemical structure of the support must be defined and any quantitative physical and chemical data relevant to the above factors must also be provided.

2.3 Support Activation and Enzyme Attachment

As noted earlier there are four general approaches to enzyme immobilization. Historically entrapment procedures were widely used for analytical applications but currently covalent binding to a suitably activated support is now the most useful and by far the most common approach. This is therefore the only form of immobilization considered herein for classification. An activated support is defined herein as a material having an enzyme reactive functional group covalently attached to an otherwise inert surface. The stability of the resulting bond between the enzyme and the support, the local environment of the enzyme and the potential loss of activity on immobilization must all be considered.

There is a great variety of activation procedures, detailed descriptions of which are beyond the scope of this paper [1,3,5,8]. In order to classify the immobilization procedure however, details of the free enzyme (as described above), the chemical nature of the support and the coupling reagent and the experimental conditions used for support activation and enzyme attachment must all be stated.

For polysaccharides, activation is most commonly via derivatization of available hydroxy functions using reagents such as cyanogen bromide [9], triazine derivatives, e.g. cyanuric chloride, sodium periodate, epoxides or benzoquinone. Polyacrylamide and its derivatives can be activated by reaction with diamines and inorganic supports are most commonly silanized and activated by reaction with

glutaraldehyde [10]. Other general schemes for support activation are diazotization or reaction with carbodiimides, thiophosgene, thionyl chloride, N-hydroxysuccinimides or transition metal salts such as titanium chloride. The activated support is then covalently linked to the enzyme, most commonly via direct reaction with available amino functions, e.g. on lysine residues, but also via thiol and phenol functions.

3. IMMOBILIZED ENZYME CHARACTERISTICS

The experimental preparation factors discussed above will have a significant effect on the characteristics of the immobilized enzyme, in particular its activity and stability. It is therefore imperative that the percentage of enzyme immobilized and the enzyme activity remaining after immobilization are stated together with the experimental conditions used for their determination. Enzyme activities for immobilized enzymes are defined in the same way as for free enzymes i.e. the katal is the recommended unit. This is the most important information for comparing immobilization methods and is often not provided. The percentage of enzyme immobilized is usually calculated by measuring the amount of enzyme remaining in the supernatant after immobilization and subtracting this from the amount originally present. The absolute enzyme activity remaining on the support after immobilization is more difficult to determine and an apparent activity is usually measured which takes into account mass transfer and diffusional restrictions in the experimental procedure.

The other critical performance indicator is the stability of the immobilized enzyme with respect to time, temperature and other storage conditions and experimental variables. Clearly this can be expressed in a number of ways but the recommended procedure is to store the enzyme under normal operating conditions (e.g. ambient temperature (20 °C) in an appropriate buffer) and monitor its activity after fixed periods of time using the same procedure as that used for determining the activity remaining after immobilization. For analytical purposes the effect of the controlled introduction of synthetic standards, reference materials and samples at predefined intervals and frequencies must be determined in order to specify the minimum number of analyses possible and the lifetime of the immobilized enzyme. The effect of storage conditions, e.g. pH, temperature and ionic strength and of impurities incorporated during the immobilization step must also be considered.

Other quantifiable parameters for an immobilized enzyme are its pH optimum (and working range), oxidation-reduction potential working range and the apparent Michaelis constants (K_M) for appropriate substrates (which also give an indication of immobilized enzyme selectivity). These parameters can be affected by immobilization and experimental conditions and therefore comprehensive experimental details must be provided.

4. CONCLUSIONS

Purified enzymes immobilized on solid supports by covalent binding must be classified according to the properties of the free enzyme, the type of support used and the method of support activation and enzyme attachment. The properties of the immobilized enzyme that must be stated are the percentage of enzyme immobilized, the enzyme activity remaining after immobilization, the time and temperature stability of the immobilized enzyme, the pH optimum and apparent K_M for appropriate substrates. In all of the above full experimental details must be given.

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