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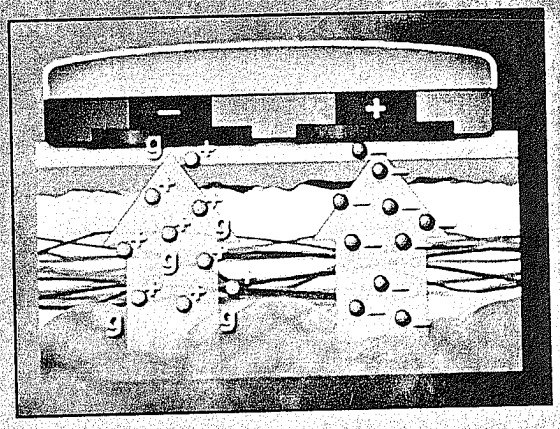
Microbial Enzymes and Biotransformations

Edited by

José Luis Barredo



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José Luis Barredo

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16

Immobilization of Enzymes by Covalent Attachment

Scott J. Novick and J. David Rozzell

Summary

Enzymes are finding increasing use for the production of agrochemicals, pharmaceuticals, and fine chemicals. They are almost always used in the immobilized form in order to simplify their removal from the product stream. In addition, immobilization often enhances the stability of the enzyme. Immobilization can be performed in a number of ways. This chapter discusses various methods, properties, and uses of covalently immobilized enzymes.

Key Words: Immobilized enzymes; covalent immobilization.

1. Introduction

1.1. Historical Perspective

An immobilized enzyme is generally defined as "the imprisonment of an enzyme molecule in a distinct phase that allows exchange with, but is separated from, the bulk phase in which substrate effector or inhibitor molecules are dispersed and monitored" *(1)*. Immobilized enzyme technology dates back to the 1910s to the 1930s, when proteins were physically adsorbed onto surfaces such as charcoal, kaolinite, cellulose, and glass beads *(2–4)*. But it was not until the 1950s and '60s with the work of Katchalski-Katzir, and Chibata and coworkers that real advancements were beginning to be made in the development and applications of immobilized enzyme materials *(5)*. This early work culminated in the First Enzyme Engineering Conference in 1971. The first industrial use of immobilized enzymes was for amino acid production. Chibata and coworkers at Tanabe Seiyaku (Japan) in 1969 used an immobilized L-aminoacylase in a packed bed reactor to resolve various DL-amino acids into their enantiomerically pure forms. Since that time, immobilized enzymes have become increasingly important for the production of many important chiral compounds (i.e., amines and alcohols) for the pharmaceutical and fine chemical industries.

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Table 1
Stabilization Effects Immobilization Imparts to Enzymes *(8)*

1. Prevention of either proteolysis or aggregation by spatial fixation of enzyme molecules to the support.
2. Unfolding of the enzyme is reduced due to multipoint covalent or adsorptive attachment to the support, and/or intramolecular crosslinking of the enzyme.
3. Multimeric enzymes would have a lower likelihood to dissociate if all subunits are attached to the support.
4. Denaturing agents (e.g., chemical inactivators) can be excluded from the enzyme by the support or inactivated by the support before reaching the enzyme (e.g., decomposition of hydrogen peroxide, produced during the oxidation of glucose by glucose oxidase, catalyzed by activated carbon).
5. Shifting by a charged support of the local pH, thus preventing pH inactivation of the enzyme.
6. Exclusion by the support (e.g., an encapsulation membrane) of proteases from the enzyme's environment.
7. Increased thermal stability due to multipoint attachment of enzyme to support.

1.2. Reasons for Enzyme Immobilization

The principal advantage of immobilizing enzymes is to retain the catalyst in the reactor. This can greatly improve the economics of a process. For a continuous process, a soluble enzyme would be washed out of the reactor along with the product stream. A process like this would not be economically feasible if the biocatalyst is very expensive (as is often the case) and cannot be reused. Although an ultrafiltration setup could be used to retain the enzyme, it is often too costly, both in capital and operation, on a large scale. Also, having a soluble enzyme in the product would not be desirable if the biocatalyst can cause the product to undergo side reactions or if there are toxicity effects associated with the catalyst, as will often be the case if the product is an intravenous drug *(6)*. Another advantage of immobilizing enzymes is to increase enzyme activity or stability especially under denaturing conditions *(7,8)*. Thermal stability can often be improved by many orders of magnitude compared to the soluble enzyme *(9–11)*. Activity of an enzyme in nonaqueous media can also be significantly higher than the native enzyme *(12–18)*. Another important advantage is the ability to control the microenvironment of the immobilized enzyme. For example, by immobilizing an enzyme on an acidic support (such as poly [acrylic acid]), the catalyst can be used at higher pHs, where the substrate may be more soluble, while the pH of the microenvironment surrounding the enzyme could be much closer to the enzyme's optimum pH. These and other stabilizing effects of immobilization are listed in **Table 1.**

There are also limitations to immobilizing enzymes. Some inherent catalytic activity is almost always lost during the immobilization procedure. Enzymes possess highly defined, yet relatively fragile three-dimensional structures that must come in contact and interact with the rigid support. These binding forces, such as covalent bonds or adsorptive interactions, are often more powerful than the secondary forces, such as hydrogen bonding and hydrophobic and ionic interactions, which hold proteins in their proper configuration for enzymatic activity. In addition, no covalent immobilization method is able to bind only the nonessential elements of every enzyme (if they even exist) to the support, and all supports create asymmetric force fields and change the water activity around the biocatalyst *(6)*. In addition, apparent activity can be decreased by mass transfer limitations. However, the increase in stability and ease of removal from the product stream and reuse often more than make up for any decrease in activity.

1.3. Enzyme Immobilization Methods

In general five techniques have been described for immobilization of enzymes. It is important to point out that there is no one universal immobilization system; instead, a range of methodologies must be evaluated depending on the enzyme to be immobilized and the overall process in which the immobilized enzyme is to be used. Also, most immobilization methods, although conceptually distinct, often overlap to a certain extent, and in some cases, multiple immobilization methods are employed.

One of the simplest and most economical immobilization methods is adsorbing an enzyme onto a support. The enzyme is bound to the support via ionic or nonionic interactions. Supports often include carbohydrate-based or synthetic polymer ion-exchange resins or uncharged supports such as polymers, glasses, and ceramics. The main drawback of this method is the leaching of enzyme from the support.

Cross-linking enzyme molecules with themselves, or more often with an inert protein such as gelatin or bovine serum albumin, results in an insoluble active enzyme preparation that can be readily handled or manipulated in a continuous reactor. Glutaraldehyde, adipimate esters, and diisocyanates are often used as the cross-linking agent. Significant inactivation of the enzyme may result during the cross-linking step and is the major drawback of this method.

Entrapment of an enzyme within a polymeric matrix is another method used for enzyme immobilization. This is often done by mixing the enzyme with a monomer and a cross-linker, and polymerizing the monomer around the enzyme. Leaching of the enzyme out of the matrix and mass transfer limitations of substrate diffusing into the matrix can limit the use of this technique.

Encapsulating or confining an enzyme within a membrane is another method for enzyme immobilization. Ultrafiltration membranes or hollow fibers made of

polyethersulfone, cellulose nitrate or acetate, or nylon are often used. The pore size must be properly chosen to allow substrate and product to enter and exit the membrane while still retaining the enzyme. Since the enzyme exists in its soluble form, activity is usually high. Membrane fouling and reduced flow rates are drawback of this technique.

The fifth immobilization method, covalent attachment of enzymes to a support, will be the subject of the rest of this chapter.

1.4. Covalent Enzyme Immobilization

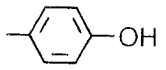
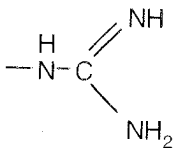
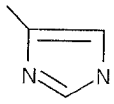
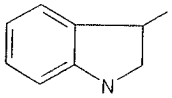
Covalent attachment of enzymes to an insoluble support is an often-used method of enzyme immobilization. It is especially useful when leaching of enzyme from the support is a concern. The enzyme is usually anchored via multiple points and this generally imparts greater thermal, pH, ionic strength, and organic solvent stability onto the enzyme since it is more rigid and less susceptible to denaturation. Covalently immobilized enzymes are also often more resistant to degradation by proteolysis.

There are, however, some drawbacks to covalent enzyme immobilization. Typically it is more expensive and complex to covalently immobilize an enzyme compared to the other methods due to the higher costs of the support. The support often needs to be activated prior to immobilization. The increased stability and typically minimal enzyme leaching often more than make up for these shortcomings.

Enzymes contain a number of functional groups capable of covalently binding to supports. **Table 2** lists these groups along with their relative frequency in a typical protein *(19–21)*. Of the functional groups of enzymes listed, $-NH_2$, $-CO_2H$, and $-SH$ are most frequently involved in covalent immobilization. Amines and sulfhydryls are good nucleophiles, while the ability to activate carboxylates so they are reactive toward nucleophiles makes these groups important as well. The phenolic ring of tyrosine is also extremely reactive in diazo-coupling reactions, and its hydroxyl group can be an excellent nucleophile at basic pH. Aldehydes can react with the guanidino group of arginine and, although histidine displays a lower nucleophilicity, it can react in some cases with supports activated with tosylates, tresylates, or other good leaving groups.

The supports to which the enzymes are attached to can vary greatly. They can be either natural polymers, such as modified cellulose, starch, dextran, agal polysaccharides, collagen, and gelatin; or they can be synthetic, such as polystyrene, polyacrylamide, polyacrylates, hydroxyalkyl methacrylates, and polyamides. Inorganic supports can also be used, such as porous glass, metal oxides, metals, sand, charcoal, and porous ceramics. The variety of chemistries available for covalent attachment allows the conditions of immobilization to be tailored to

Table 2
Reactive Functional Groups in Enzymes and Their Average Occurrence in a Typical Protein *(19–21)*

Structure of reactive group	Reactive group	Occurrence in average protein
$-NH_2$	ε-Amino of lysine and N-terminus	5.9
$-CO_2H$	Carboxylate of glutamic acid, aspartic acid, and C-terminus	6.3 (Glu), 5.3 (Asp)
$-SH$	Thiol of cysteine	1.9
	Phenolic of tyrosine	3.2
	Guanidino of arginine	5.1
	Imidazole of histidine	2.3
$-S-S-$	Disulfide of cystine	—
	Indole of tryptophan	1.4
$-CH_3-S-$	Thioether of methionine	2.2
$-CH_2OH$	Hydroxyl of serine and threonine	6.8 (Ser), 5.9 (Thr)

each enzyme system. This also allows the microenvironment of the enzyme to be tailored by appropriate modification of the support surface; hydrophobic moieties or ionically charged groups may be used to alter the support to enhance the enzyme-catalyzed reaction of interest. Some supports, such as those containing

Fig. 1. Enzyme immobilization onto hydroxyl containing supports via activation with cyanogen bromide (top) or *S*-triazine derivatives (bottom).

epoxide groups can be used directly for enzyme binding. However, most supports require preactivation before enzymes are able to bind to it. The following sections describe some typical covalent attachment methodologies.

1.4.1. Covalent Attachment Onto Polyhydroxyl Supports

Polyhydroxyl supports, such as porous glass, and especially polysaccharides are among the most commonly used matrices for enzyme immobilization. Because hydroxyl groups are poor leaving groups they must first be activated. This is typically done with cyanogen bromide *(22)*. Other activating agents such as *S*-triazine derivatives have also been used. Once the support is activated it is able to covalently couple to an enzyme usually through the ε-amino group of lysine or through the amino terminus. The mechanism of derivatization polyhydroxyl supports with the above two derivatizing agents and the subsequent enzyme immobilization is shown in **Fig. 1.**

Supports that have been preactivated with cyanogen bromide can be stored for periods of up to 1 yr at freezer temperatures. Preactivated supports are also available commercially. Once the support is activated, coupling of the enzyme requires no more than exposing the enzyme to the activated support in an aqueous solution for a few hours, followed by extensive washing to remove any protein that is not covalently bound.

This method is extremely popular in the lab scale; however, it has not been widely used in large-scale applications. The activating agent, cyanogen bromide, is extremely toxic, and most carbohydrate supports, such as cellulose, agarose, and dextran, have poor mechanical stability compared to other support materials. Also, since the supports are natural polysaccharides, microbial contamination and degradation are a concern. Finally, the bond between the enzyme and the support is potentially susceptible to hydrolytic cleavage, which would cause leaching of the enzyme from the support over time.

1.4.2. Covalent Attachment onto Carboxylic Acid-Bearing Supports

Carboxylic acid-containing supports, such as copolymers of (meth)acrylic acids with (meth)acrylic esters have also been used as an immobilization support. These must also be activated and this is usually done with a carbodiimide reagent. Under slightly acidic conditions (pH 4.75–5.0) carbodiimides react with carboxylic acid groups to give the highly reactive *O*-acylisourea derivatives. The supports are then washed to remove excess reagent and the enzyme is coupled to the activated support at neutral pH to give stable amide, thioester, or ester linkages, depending on the residue reacting with the support. The most widely used water-soluble carbodiimides are 1-ethyl-3-(3-dimethylamino propyl)-carbodiimide (EDC) and 1-cyclohexyl-3-(2-morpholino-ethyl)-carbodiimide (CMC), both of which are available commercially. The reaction

Fig. 2. Activation of a carboxylic acid containing support with a carbodiimide followed by enzyme coupling.

Fig. 3. Activation of an amine-bearing support with glutaraldehyde followed by enzyme coupling.

scheme for of activating a carboxylic acid-containing support and subsequent enzyme coupling is shown in **Fig. 2.**

1.4.3. Covalent Attachment Onto Amine-Bearing Supports

Amine-bearing supports are among the most used and the most useful supports for covalent enzyme immobilization. These supports can either be organic or inorganic supports bearing amine functionality. The most frequent technique for introducing amine groups on inorganic supports is via aminosilane attachment *(23–25)*. For example, 3-aminopropyltriethoxysilane can be coupled to porous glass to give pendent amine groups *(26)*. This silane has been developed through the pioneering work at Corning Glass Works *(23)*.

Another common amine-bearing support is polyethyleneimine-coated particles. Polyethyleneimine is a common polyamine derived from the polymerization of ethyleneimine to give highly branched polymers containing approximately 25% primary amines, 25% tertiary amines, and 50% secondary amines. This polymer can be coated onto various supports including alumina *(27)*, carbon *(28)*, diatomaceous earth *(29)*, and polyvinyl chloride-silica composites *(30,31)*.

The coupling of an enzyme to amine-bearing supports can be done in a number of ways. The most common way is through the use of difunctional reagents, such as diimidate esters, diisocyanates, and dialdehydes. Glutaraldehyde is often used, as it is one of the least expensive difunctional reagents available in bulk. This reagent reacts in a complex fashion to form Schiff bases with amine groups on the support and produces pendent aldehydes and α,β-unsaturated carbonyl functionalities through which enzymes may attach. Enzyme attachment is accomplished simply by mixing the enzyme with the activated support. A simplified example of this is shown in **Fig. 3.** The acid-labile Schiff bases can be reduced to more stable secondary amine bonds with sodium borohydride to increase the stability of the enzyme-support linkage.

Crump and coworkers *(32)* have described the immobilization of an L-amino acid transaminase onto a polyethyleneimine coated PVC-silica support matrix that was activated with glutaraldehyde. Very high binding efficiency and residual activity were obtained. After washing, 93% of the enzyme offered was bound to the support (total loading was about 10%) and the enzyme retained approximately 89% of the soluble activity. Both these values are unusually high for immobilized enzymes, but not necessarily atypical for this type of support and immobilization chemistry.

Enzymes can also be covalently bonded directly to amine-bearing supports via the enzyme's carboxyl groups. These must first be activated with a carbodiimide or similar reagent prior to immobilization. The activation step can cause enzyme inactivation and thus this method is not used as often.

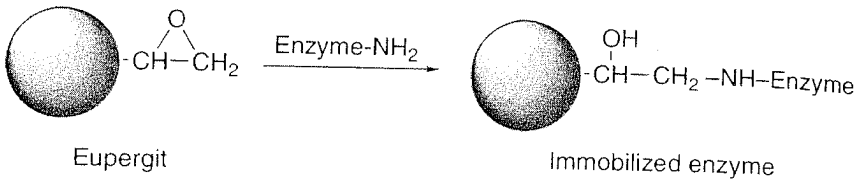


Fig. 4. Enzyme immobilization to Eupergit via free amino groups.

Diisocyanates have also been used as a coupling agent between amine-bearing supports and enzymes *(33)*. If alkaline conditions are used a substituted urea bond is formed between an amine on the enzyme and the isocyanate. If moderately acidic conditions are employed, the isocyanate will react with a hydroxyl group on the enzyme and form a urethane bond. Isothiocyanates have also been used successfully *(23)*.

Another amine-bearing support, developed by Leuta and coworkers *(34)*, is mineral or carbon particles coated with chitosan. Chitosan is deacylated chitin, a polymer of glucosamine, and contains an available amino group for chemical activation and enzyme binding using methods similar to those described for the other amine-bearing supports.

1.4.4. Covalent Attachment to Reactive Polymer Supports

Due to the preactivated nature of epoxy-containing supports, these materials have gained considerable attention as commercially useful support matricies for enzyme immobilization. A commercial epoxy-containing support is available from Röhm Pharma Polymers (Piscataway, NJ) under the trade name Eupergit. The material is a crosslinked copolymer of methacrylamide and oxirane containing monomers and consists of spherical beads of about 200 μm in diameter. Eupergit is available in two varieties, Eupergit C and Eupergit C 250 L, with their differences being their oxirane content and pore size. Eupergit C has average pore radius of 10 nm and an oxirane content of 600 μmol/g, while Eupergit C 250 L has a pore size and oxirane content of 100 nm and 300 μmol/g, respectively *(35)*. Eupergit C 250 L is targeted for the immobilization of large molecular weight enzymes (>100 kDa). Immobilization of enzymes to Eupergit is relatively simple. The enzyme solution is brought in contact with the Eupergit beads either quiescently or with slight mixing (magnetic stirbars should be avoided to prevent fractionation of the beads) for 24–96 h. This can be done either at room temperature, or if the enzyme is unstable, 4°C will also work. Various pHs can be used for the binding. Under neutral and alkaline conditions the amino groups on the enzyme are principally responsible for binding to the support (**Fig. 4**). Under acidic and neutral conditions sulfhydryl and carboxyl groups take part in binding.

Immobilization to Eupergit does not change the charged state of the enzyme. Typically, it is best to bind the enzyme to the support at the pH at which activity is optimum for the enzyme. The various parameters mentioned above—mixing type, immobilization time, temperature, pH, and also ionic strength (0.5–1 *M* buffer or neutral salt is often optimal)—can be varied to optimize the amount of enzyme immobilized and the residual activity. Once the enzyme is bound to the support, the binding is stable over the long term and it is stable over a wide pH range, from 1.0 to 12.0. Also, because Eupergit is electrically neutral, pH changes do not effect the swelling of the gel.

After the enzyme has been bound, typically only about 1% of the available epoxy group are involved in enzyme immobilization. The remaining groups will slowly hydrolyze into diols or they can be quenched with a variety of compounds that can effect the microenvironment around the immobilized enzyme by making it more hydrophilic, hydrophobic, or charged. This in turn can effect the stability or activity of the bound enzyme. Bovine serum albumin, dithiothreitol, Tris-buffer, mercaptoethanol, various amino acids (i.e., lysine or glycine), and ethanolamine are among some of the quenching reagents that have been used, and in many cases activity of the immobilized enzyme can be altered depending on the quenching reagent.

There have been two extensive reviews recently published concerning the immobilization of enzymes to Eupergit *(35,36)*. In these reviews, the details of the immobilization of nearly two dozen different enzymes are presented. In addition to Eupergit, other epoxy-containing polymers have been investigated for the covalent attachment of enzymes *(37–42)*.

Polyacrolein beads is another useful reactive-polymer carrier for covalent enzyme immobilization. Margel *(43)* synthesized such beads and encapsulated them into agarose prior to enzyme binding. Because these supports are polyaldehydes, enzymes are bound in a similar way as with glutaraldehyde activated supports. Various oligomers such as poly(lysine) and poly(glycine) have been attached to the polyacrolein beads to act as spacers between the particles and the enzyme. In both cases the poly(amino acids) are attached to the support through their terminal amino groups, or ε-amino groups in the case of poly(lysine), via Schiff bases (which can then be reduced). The enzyme is attached to the poly(lysine)-derivatized polyacrolein via the lysine ε-amino groups using glutaraldehyde as a linker. For the poly(glycine)-derivatized polyacrolein support, the terminal carboxyl group is activated with a water-soluble carbodiimide followed by enzyme binding. In some cases the use of these spacers has shown a significant increase in activity, especially for large-molecular-weight substrates. Covalent enzyme immobilization to paramagnetic polyacrolein beads has also been investigated *(44)*. Binding of enzymes to unmodified polyacrolein is shown in **Fig. 5.**

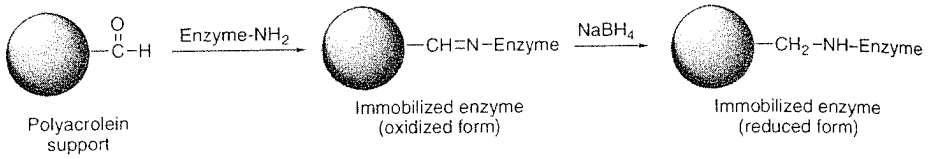


Fig. 5. Enzyme immobilization to unmodified polyacrolein via free amino groups, followed by reduction of the Schiff base with sodium borohydride.

1.5. Assaying the Properties of Immobilized Enzymes

There are three important properties of immobilized enzymes that are often evaluated: activity, enzyme loading, and stability. Prior to the measurement of these properties, the immobilized enzyme materials should be washed extensively to remove any unbound enzyme that may be entrapped in the pores of the particles or loosely bound through noncovalent interactions.

1.5.1. Activity Assay

There are two basic methods to measure activity—batch and continuous. In the batch method the immobilized enzyme is added to a flask or vial and the substrate solution is then added to initiate the reaction. At various time points, an aliquot of the mixture is removed and filtered (this is most easily done through a syringe filter) to remove any of the immobilized enzyme particles and to quench the reaction. This aliquot can then be analyzed using the appropriate analytical method, such as liquid chromatography, gas chromatography or spectrophototometry. If product continues to be produced in this aliquot after filtration, it is a good indication that there may be significant leaching of soluble enzyme off the support. This can occur if the support is not washed extensively enough after immobilization or if the binding is labile under the assay conditions. To get more accurate activity measurements the supports should be rewashed.

There are two basic methods for performing a continuous activity assay. In the packed-bed plug-flow tubular reactor (PFTR) method, the immobilized enzyme is packed into a column and substrate is pumped though the column and the substrate and/or product concentration is measured in the effluent. In the continuous stirred tank reactor, the solution and the immobilized enzyme are well mixed so there are minimal concentration gradients. To prevent the loss of immobilized enzyme out of the exit, a filter is added at the effluent or a tube is added at the exit that is long enough such that at the given flow rate gravity prevents the particles from leaving the reactor. Modeling a batch or continuous immobilized enzyme reactor can be found in many reactor engineering or bioprocess engineering textbooks.

1.5.2. Determining Protein Loading in Covalently Immobilized Enzyme Materials

It is often useful to have information on how much enzyme is bound to the support after an immobilization was performed. This information is needed when optimizing the immobilization conditions or when calculating the residual enzyme activity. Measurement can be done either indirectly or directly on the particles themselves.

In the indirect method, the initial amount of protein offered to the support is determined using any of the variety of protein assays available. After the immobilization is complete and the particles are washed, the same protein assay is done on the supernatant and wash solutions. The difference in the mass of enzyme offered and the amount in the immobilization supernatant and wash solutions will give the amount of enzyme bound to the support.

In the direct method, the amount of enzyme actually bound to the support is determined. A number of methods to determine this have been published. In one method, the bicinchoninic acid protein assay (often referred to as the BCA assay) is used *(45)*. In this assay, the immobilized enzyme is incubated in the BCA assay solution. The enzyme bound to the support reacts with the BCA solution in the same manner a soluble enzyme would, by reducing Cu^{2+} to Cu^{1+} in the presence of peptide bonds, which complex with the bicinchoninic acid to form a aqueous purple-colored solution. The absorbance of this solution will be proportional to the amount of enzyme immobilized. Bovine serum albumin (BSA) is often used as protein standards to quantify the amount of enzyme bound to the support.

Coomassie-based protein dyes have also been used to directly quantify the amount of enzyme bound to a support. In one method, the dye is allowed to bind to the enzyme attached to the support, after which the residual unbound dye is removed from the particles *(46)*. The bound dye is then eluted from the particles by adding sodium dodecylsulfate and sodium bicarbonate. The absorbance of this solution can then be read at 595 nm and the amount of protein bound to the support can be determined by comparing to a BSA standard curve.

In another method, the Bradford Protein Assay solution (a Coomassie-based protein assay) is used *(47)*. With no protein present, the Bradford solution is brown with a λ_{max} of 465 nm (with protein the color is blue with a λ_{max} of 595 nm). When the Bradford solution is mixed with the immobilized enzyme particles, some of the dye will bind to the protein on the beads (turning them blue) and the absorbance at 465 nm will decrease. This decrease at 465 nm can be correlated to the amount of bound protein by comparing to a standard curve of BSA or other suitable protein. The Bradford solution is acidic, so any enzyme that is immobilized through acid-labile links cannot be used with this method. Also, if the supernatant turns blue it is a good indication that significant leaching of the enzyme off the support is occurring.

Other methods also exist, including photometric, fluorometric, radiochemical, and ELISA-based methods. Elemental analysis on nitrogen or sulfur can also be used as long as the support material does not contain these elements. Amino acid analysis after acid hydrolysis can be used as well to determine protein content. All of these have differing sensitivity, work-up, and costs, all of which should be considered *(48)*.

1.5.3. Determining Stability of Covalently Immobilized Enzymes

Stability of immobilized enzymes can be measured in a number of ways. The simplest way is to pack the immobilized enzyme into a continuous reactor such as a column (plugged flow reactor) or a vessel with mixing (continuous stirred tank reactor). Substrate is then pumped through the reactor and the effluent is analyzed for product and/or substrate concentration. Depending on the stability of the enzyme, this is allowed to run for days or even months and the decrease in product concentration or the increase in outlet substrate concentration is monitored to determine the working lifetime of the immobilized enzyme. It is important to choose the proper amount of immobilized enzyme and flow rate such that less than 100% conversion is obtained. If 100% conversion is achieved, then it is unknown whether the entire immobilized enzyme takes part in the reaction. For example, if only half the enzyme present in the reactor is needed for 100% conversion under a given set of reaction conditions, significant inactivation (up to 50% of the enzyme) could occur with no observable change in conversion. The continuous reactor can also be run with various cosolvents or at various pHs or temperatures to determine the stability of the immobilized enzymes under these conditions.

The stability of immobilized enzymes can also be determined batchwise. In this technique, measured amounts of the immobilized enzyme are placed into separate vials along with the solvent of interest. The vials are allowed to incubate at a given temperature for a given amount of time. One or more vials are then sacrificed and the activity of the immobilized enzyme is measured (alternatively, the immobilized enzyme particles can be washed of substrate and product and returned to the initial incubation conditions). This is repeated over time to determine how the activity changes. Another batchwise method of measuring stability is to add the substrate solution to the immobilized enzyme and measure the reaction time course. The immobilized enzyme particles are then washed and this is repeated. The time it takes to reach the required conversion, the conversion at a set time, or the reaction time course can be plotted versus the number of recycles to give an indication of long-term stability/usability. Reactors capable of performing this automatically are commercially available *(36)*.

1.6. Industrial Uses of Covalently Immobilized Enzymes

A large number of enzymes are used in industry for the synthesis of a wide range of compounds. Most of these applications can be placed in either the food industry (both for human and animal consumption) or the pharmaceutical/fine chemical industry. Many of these applications involve immobilized enzymes with some of them covalently immobilized. A few examples of the latter will be discussed in **Subheadings 1.6.1., 1.6.2.,** and **1.6.3.**

1.6.1. High-Fructose Corn Syrup With Immobilized Glucose Isomerase

The largest use of immobilized enzymes is for the isomerization of glucose, from corn, to the much sweeter fructose. The resultant high-fructose corn syrup (HFCS) is used as a sweetener in a variety of foodstuffs, especially sweetened beverages and baked goods. Current US production of HFCS exceeds 9.3 million short tons (dry weight) per year (http://www.ers.usda.gov/briefing/sugar/ Data/Table27.xls). Glucose isomerase (also called xylose isomerase due the high affinity of the commercially available catalysts toward xylose) is used industrially to perform this reaction. Many of the commercial catalysts are immobilized whole cells; however, Miles Kali-Chemie (Germany) developed a glucose isomerase from *Streptomyces rubiginosus* covalently immobilized onto silanized ceramics and sold under the trade name Optisweet *(49,50)*. Typically this reaction is run at 50–60°C to limit microbial contamination. The reactor is a continuous packed-bed reactor with a residence time of 0.17–0.33 h. The half-life of the enzyme is over 100 d; however, it is replaced after about 12.5% activity loss to maintain the necessary activity for the reactor *(49)*.

1.6.2. Semi-Synthetic Penicillins with Immobilized Penicillin Amidase

Another major use of immobilized enzymes is in the synthesis of semi-synthetic penicillins. Worldwide production of these semi-synthetic β-lactam antibiotics is more than 20 thousand tons per year *(49)*. The starting material for these compounds is 6-aminopenicillanic acid (6-APA). It is too expensive to make 6-APA synthetically or by fermentation. Instead, benzyl penicillin (also called penicillin G), which can be made relatively cheaply via fermentation, is hydrolyzed by penicillin amidase (also called penicillin acylase) into 6-APA and phenylacetic acid. This is accomplished industrially by using penicillin amidase from *Escherichia coli* or *Bacillus megaterium* immobilized onto Eupergit C. The reaction is usually carried out in a stirred-tank batch reactor. The immobilized enzyme can be reused nearly 1000 times before the half-life is reached. About 1700 kg of penicillin G is able to be hydrolyzed with 20 g of protein immobilized onto 300 g of dry polymer *(51)*. Once the 6-APA is formed and purified, it is chemically acylated with various side groups to make the semisynthetic antibiotics such as

Fig. 6. Synthesis of L-aspartate from fumaric acid and ammonia catalyzed by L-aspartase.

ampicillin (D-phenylglycine side chain), amoxicillin (D-*p*-hydroxyphenylglycine side chain), and penicillin V (phenoxymethyl side chain). The preparation of a new immobilized penicillin G acylase yielding derivatives thermoestable and resistant to organic solvents is shown in **Chapter 17**.

1.6.3. L-Amino Acids with Immobilized L-Aspartase

L-amino acids are produced in large quantities for human consumption in the form of supplements, ingredients in artificial sweeteners, intermediates in the synthesis of pharmaceuticals, and as additives in animal feed. Covalently immobilized enzymes have been used to produce L-aspartase, a main ingredient in the artificial sweetener aspartame. This amino acid can be synthesized from two inexpensive starting materials, fumaric acid and ammonia (**Fig. 6**). The enzyme that catalyzes this reaction is L-aspartate-ammonia lyase, also called L-aspartase. This enzyme, from *E. coli,* has been covalently immobilized onto PVC-silica supports that have been coated with a polyamine and activated with glutaraldehyde. The process is relatively simple. The two starting materials are passed through a packed bed containing the immobilized enzyme. The effluent is acidified to pH 2.8 and chilled, causing the L-aspartate to precipitate, and it can then be recovered by filtration. Conversion, optical purity, and chemical purity of this reaction are all over 99%. The product concentration is 200 g/L with a space time yield of 3 kg/L·h. The reactor volume was 75 L, therefore producing more than 5 metric tons of product per day. The half-life of the immobilized enzyme was more than 6 mo ***(49,52)***.

1.7. Conclusions

Covalent immobilization of enzymes represents a robust method for the attachment of enzymes to insoluble supports. A variety of supports are available including synthetic and natural organic polymers and inorganic minerals, metal oxides, and glasses. The chemistry and functional groups used to bind enzymes to the supports can vary greatly and can be tailored depending on the specific application. In addition to the ease of enzyme recovery, stability of the immobilized enzyme is usually much higher than the soluble enzyme and thus can be

reused multiple times. Applications of covalently immobilized enzymes have been demonstrated from the lab scale to the industrial production of multiton quantities. Increasing numbers of enzyme are becoming commercially available in immobilized form, including lipases, proteases, nitrilases, amino acid dehydrogenases, oxidoreductases, and others *(53)*. This trend will continue as enzymes find more applications, particularly for chiral chemical production. This is a vibrant field that continues to evolve to this day.

2. Materials

2.1. Covalent Enzyme Immobilization

2.1.1. Covalent Attachment Onto Polyhydroxyl Supports

1. Polysaccharide support material, such as Sepharose 4B, Sephadex, agarose, cellulose, or dextran.
2. 2 *M* sodium carbonate.
3. 1 g/mL cyanogen bromide (CNBr) dissolved in DMF.

2.1.2. Covalent Attachment Onto Carboxylic Acid-bearing Supports

1. Carboxylic acid-bearing support such as Bio-Rex 70 Resin (BioRad, Hercules, CA), IRC50 (Supelco, St. Louis, MO), carboxymethylcellulose, acrylic acid copolymers, or succinylated glass beads.
2. 0.1 *M* sodium acetate buffer, pH 4.5.
3. 1-Cyclohexyl-3-(2-propyl)carbodiimide (EDC).
4. 0.1 *M* sodium phosphate buffer, pH 7.0.

2.1.3. Covalent Attachment Onto Amine-Bearing Supports

1. Amine-containing support, such as aminopropyl-glass, aminoethyl-cellulose, PEI-coated materials (e.g., silica or alumina) or other similar material.
2. 0.05 *M* phosphate buffer, pH 7.0.
3. 25% glutaraldehyde (GA).

2.1.4. Covalent Attachment to Reactive Polymer Supports

1. Eupergit C (Röhm Pharma Polymers, Piscataway, NJ).
2. 0.05 *M* phosphate buffer, pH 7.0.

2.2. Determining Protein Loading in Covalently Immobilized Enzyme Materials

2.2.1. Indirect Method

1. Immobilized enzyme.
2. Bradford reagent (Sigma Chemical Co., St. Louis, MO).
3. Bovine serum albumin (BSA) protein standards.
4. 0.05 *M* phosphate buffer, pH 7.0.

2.2.2. Direct Method (BCA)

1. Immobilized enzyme.
2. BCA protein assay kit (contains Reagent A and Reagent B).
3. BSA protein standard.

3. Methods

3.1. Covalent Enzyme Immobilization

3.1.1. Covalent Attachment Onto Polyhydroxyl Supports

1. Wash the support material with distilled water and remove residual water using suction filtration to form a packed cake.
2. Add 10 g of washed support material to a flask and add 2 *M* sodium carbonate until the total volume is about 1.2 times the settled bed volume (*see* **Note 1**).
3. Cool slurry to 0°C.
4. Add 1 mL of 1 g/mL of CNBr dissolved in DMF and mix vigorously for 2 min (*see* **Note 2**).
5. Wash the now-activated support material with at least 5 volumes of cold distilled water.
6. Add the activated support material to a solution of the enzyme in the appropriate buffer. Let incubate at 4°C for 12 to 24 h (*see* **Notes 3–5**).
7. Wash the immobilized enzyme material with the buffer used in **step 6.**

3.1.2. Covalent Attachment Onto Carboxylic Acid-Bearing Supports

1. Add 5 mL of the hydrated support to 15 mL of 0.1 *M* sodium acetate buffer, pH 4.5.
2. Add 200 mg EDC to the support slurry. Mix for 20 min at room temperature (*see* **Note 6** and **Note 7**).
3. Wash the beads with 500 mL of cold 0.1 *M* sodium phosphate buffer, pH 7.0.
4. Add the activated beads to 15 mL of the enzyme solution and gently agitate for 24–48 h at 4°C (*see* **Notes 8** and **9**).
5. Wash the immobilized enzyme beads with at least 500 mL of 0.1 *M* sodium phosphate buffer, pH 7.0.

3.1.3. Covalent Attachment Onto Amine-Bearing Supports

1. Suspend 10 g of the moist amine-bearing support in 100 mL of 0.05 *M* phosphate buffer, pH 7.0.
2. Add 10 mL of 25% glutaraldehyde (GA) and mix well for 1–2 h (*see* **Notes 10** and **11**).
3. Wash and filter the GA-activated support material with 500 mL of the above buffer at least five times to remove any residual GA (*see* **Notes 12** and **13**).
4. Suspend 10 g of the moist GA-activated support in 30 mL of the enzyme solution in the above buffer and mix well at room temperature or 4°C depending on enzyme stability (*see* **Note 14**).
5. After 12–24 h filter the immobilized enzyme material and wash well with the above buffer (*see* **Note 15**).

3.1.4. Covalent Attachment to Reactive Polymer Supports

1. Add 1.0 g of dry Eupergit C to 6.0 mL of the enzyme solution in 0.05 *M* phosphate buffer, pH 7.0 (*see* **Notes 16–19**).
2. Gently mix the suspension at room temperature or 4°C depending on the stability of the enzyme.
3. After 24–48 h wash the immobilized enzyme with 50 mL of the buffer in **step 1** followed by suction filtration. Repeat the washing three to five times (*see* **Note 20**).

3.2. Determining Protein Loading in Covalently Immobilized Enzyme Materials

3.2.1. Indirect Method

1. Before adding the enzyme solution to the support for immobilization, record the volume of the enzyme solution and the dry weight of the support.
2. Determine the protein concentration in the enzyme solution (prior to adding it to the support material) using the Bradford protein assay method. This is done by adding 0.1 mL of the enzyme solution to 0.9 mL of Bradford reagent and mixing. OD_{595} is recorded after it has stabilized (usually 5–10 min). Protein concentration is calculated by comparing to a standard curve using a suitable protein standard such as BSA. The linear range for this method is approximately 0–0.5 mg/mL protein, therefore, if necessary, the enzyme solution should be diluted in the phosphate buffer (*see* **Note 21**). After the immobilization is complete, pour off the supernatant into a separate container.
3. Wash the immobilized enzyme as necessary and add the washings to the supernatant in **step 2.**
4. Record the combined volume of the supernatant and washings (from **step 3**) and measure the protein concentration as described in **step 2** (*see* **Notes 22–24**).
5. Use the following equation to calculate the amount of protein bound:

$$\frac{\text{mg enzyme bound}}{\text{g dry wt of support}} = \frac{\left(\text{enzyme conc. in initial solution (mg/mL)} \times \text{volume of initial solution (mL)}\right) - \left(\text{enzyme conc. in final solution (mg/mL)} \times \text{volume of final solution (mL)}\right)}{\text{g dry weight of support}}$$

3.2.2. Direct Method

1. Make up 20 mL of the BCA working solution by mixing 19.6 mL of reagent A with 0.4 mL of reagent B.
2. Make up 1.0 mL BSA stock solution of 25.2 mg/mL.
3. In 3–5 mL (total volume) capped tubes make up the following solutions (add the working solution last) (*see* **Notes 25** and **26**):

Sample	BSA standard, 25.2 mg/mL (μL)	Water (μL)	Final BSA conc (mg/mL)	Immobilized enzyme (mg dry)	Working solution (mL)
Std-1	0	100	0	0	2.0
Std-2	8.3	91.7	0.1	0	2.0
Std-3	25	75	0.3	0	2.0
Std-4	50	50	0.6	0	2.0
Std-5	75	25	0.9	0	2.0
Std-6	100	0	1.2	0	2.0
Immobilized enzyme	0	100	N/A	10–20	2.0

4. Shake the standards solutions and immobilized enzyme suspensions well at 37°C for 30 min, then cool the tubes to at or below room temperature.
5. Remove particles by filtration or centrifugation and measure OD_{562} of the supernatant (*see* **Notes 27–29**).
6. Construct a calibration curve using the BSA standards, using the 0 mg/mL BSA sample as a blank (*see* **Notes 30–32**).
7. Based on the calibration curve calculate the protein concentration in the immobilized enzyme samples.
8. Use the following equation to determine the enzyme loading:

$$\frac{\text{mg enzyme bound}}{\text{g dry weight}} = \frac{\text{concentration of protein in immob. enzyme sample (mg/mL)} \times 2.1\ \text{mL}}{\text{amount of immob. enzyme used in assay (g dry weight)}}$$

4. Notes

1. A procedure similar to this can be performed where, instead of using concentrated buffer, the pH is maintained at 11.0 by titrating with 2 or 4 *N* NaOH. This method often results in the doubling of the binding capacity compared to the buffer method.
2. CNBr is highly toxic and proper safety precautions should be employed when handling it.
3. Although the activated support materials should be used soon after activation, it can be stored at –20°C under airtight conditions with a loss of 10% or less per month of its original binding capacity.
4. Ideally the enzyme should be in a buffer at an alkaline pH to reduce the amount of protonated amino groups. However, it is more important to have the enzyme at a pH it is most stable, so this should be chosen if the enzyme is not stable at high pH.
5. The binding of protein to the support can be monitored by performing protein assays on the supernatant (e.g., Bradford or BCA total protein assay) and comparing to the initial protein concentration. For some enzymes, incubations times longer than 24 h may be necessary to achieve maximal enzyme binding.

6. 1-Cyclohexyl-3-(2-morpholino-ethyl)-carbodiimide metho-*p*-toluene-sulfonate (CMC) may also be used to activate carboxylic acid-containing supports.
7. The EDC concentration is about 100-fold molar excess to the carboxylate groups on the support. The activated support should be washed well before adding enzyme.
8. EDC concentration, activation time, coupling time, coupling pH, and wash conditions (i.e., washing with NaCl and/or urea to remove any unbound enzyme) should all be optimized for a given support-enzyme system.
9. An alternative method is to add the support, EDC, and enzyme all at once such that support activation and enzyme immobilization occurs simultaneously. In this case, a 1:1 molar ratio of EDC to support carboxyl groups should be used. Allow this to react in 0.05 *M* sodium phosphate buffer, pH 7.0, at 4°C for 1 h before washing. Longer times may be necessary depending on the enzyme and support. The EDC may also react with the carboxyl groups on the enzyme causing inactivation. If this occurs, the other method should be used.
10. A higher concentration of GA can be used and this may increase the binding capacity of the support material. However, since GA is often detrimental to the enzyme, more extensive washing should be performed.
11. If PEI coated materials are used, they will turn to a pinkish color after activation with GA.
12. A saturated solution of 2,4-dinitrophenylhydrazine in 0.2 *N* HCl can be used to detect residual GA in the washing solutions. Add approx 0.2 mL of the solution used to wash the GA-activated support to 0.5 mL of saturated 2,4-dinitrophenylhydrazine solution. The formation of a yellow precipitate indicates the presence of residual GA and the support material should be further washed.
13. The GA-activated support can be stored in a moist form at 4°C for at least 1 yr without significant loss in binding capacity.
14. Shaking as opposed to magnetic stirring should be used if the support is friable.
15. Enzyme loading in this type of immobilization can be as high as 30% dry weight and higher.
16. The dry Eupergit C will absorb about 3 times its weight in water (1 g dry will have a mass of about 4 g when wet).
17. The Eupergit should be kept dry until use as the epoxy groups can hydrolyze.
18. Approximately 5–10% enzyme loading on a dry basis is typical, however this is dependent on the enzyme and higher or lower loading levels are possible.
19. The ionic strength and the pH of the enzyme solution can significantly affect the loading amount and the residual activity. Often a high ionic strength (1 *M* sodium chloride) gives better binding, but this is dependent on the enzyme and a range of ionic strengths and pH values should be evaluated.
20. Post-treatment of the beads after immobilization to quench the remaining epoxy groups can effect the immobilized enzyme activity. *See* **Subheading 1.4.4.** for details on this.
21. Other protein assays may be used besides the Bradford assay, i.e., BCA, Lowry, absorbance at 280 nm.

22. If any enzyme precipitates during the immobilization, this could overestimate the amount of enzyme bound.
23. If the amount of enzyme bound to the support is very small, the inherent variability in the protein assay may not give accurate protein loading results.
24. If the wash solution contains compounds that interfere with the protein assay, unreliable data may result.
25. This method is a general method and can be modified as necessary depending the enzyme loading. The method above assumes a 1–10% enzyme loading based on dry weight.
26. The BCA protein assay has a working range of 0.02–2 mg/mL protein.
27. For low loading or small sample sizes the "enhanced protocol" can be used (incubation at 60°C for 30 min).
28. This should be done as soon as possible after cooling as the standards will continue to react with the BCA reagent while the immobilized enzyme will not if removed or centrifuged.
29. If the absorbance of the immobilized enzyme samples falls outside the range of the calibration curve, then the procedure should be repeated with a change in either the standards or immobilized enzyme concentration.
30. For more accurate results, the standards and the immobilized enzyme samples should be done in at least duplicate.
31. The support without any enzyme bound should also be tested to see if the BCA shows a response toward it. If it does, this should be taken into account and subtracted from the results of the immobilized enzyme supports.
32. The standards should be used every time this procedure is performed as the assay is highly dependent on the temperature and time of incubation, which may be difficult to replicate every time.

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