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Immobilization of Oxidases and Their Analytical Applications

Masoom Yasinzai

Institute of Bio-Chemistry, University of Balochistan, Quetta-Pakistan.

Abstract

Immobilized enzymes are replacing their soluble counter-parts in nearly every field of application. These enzyme modifications have evolved from a research curiosity into an entire branch of Biotechnology. An immobilization method for flavin containing oxidases and their use in flow injection system is described. An electrochemical detector for H_2O_2 is assembled which is used effectively for the determination of glucose using more common glucose oxidase and the simultaneous determination of sugars. The combination of oxidases with hydrolases have been used for the determination of maltose and starch.

Keywords: *Oxidases, Flow Injection Analysis and Immobilized Enzymes*

Introduction

Enzymes play an important role in analytical chemistry, because of their selective and sensitive nature as reagents. But there are still a number of undesirable properties associated with the use of soluble enzymes, such as high cost, which is an appreciable factor when continuous flow, automated analysis systems are employed. Instability has also restricted their analytical involvement. These problems have been decreased with the advent of immobilized enzymes, in which the enzyme is localized on a water insoluble carrier. These enzyme modifications which have received much attention in the past decade [1], have a number of advantages over soluble enzymes when used in analytical systems, including decreased cost, greater stability, greater convenience of use and offering greater variety of designs available for flow systems. Various methods for enzymes immobilization have been reported [2-3], among which covalent binding and cross-linking methods are famous, due to their operational stability, and therefore are mainly used in continuous flow systems [4]. The class of enzymes known as oxidases (flavin containing) catalyse reactions where the substrate molecule is oxidized by molecular oxygen producing hydrogen peroxide which can easily be detected, e.g. in a flow through electrochemical detector.

*Corresponding Author E-mail: yasinzai_masom@yahoo.com

$$\text{Substrate} + O_2 \xrightarrow{\text{Oxidase}} \text{Product} + H_2O_2$$

More than 40 such oxidases are known, and a good number of them have been used for analytical applications in clinical laboratories and in food analysis [5]. The combination of these oxidases with hydrolases can be used for the analysis of disaccharides and polysaccharides. Presented here is a review of the use of such Insolubilized oxidases for analytical purposes, highlighting a method for the immobilization of these enzyme on controlled porosity glass by cross-linking with glutaraldehyde and their use in flow injection system, equipped with a flow through amperometric detector. Determination of glucose, sucrose, maltose and starch are given as examples.

Experimental

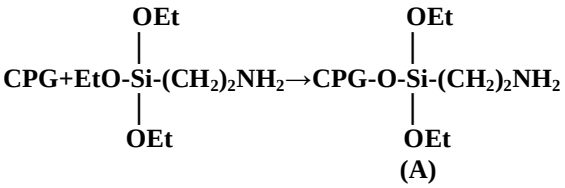
Materials

Glucose oxidase (Aspergillus niger, 250 Umg^{-1}), invertase (Bakers yeast, 1000 Umg^{-1}), mutarotase (Pig liver, 5000 Umg^{-1}), Maltase (Brewers yeast, 75 Umg^{-1}) Protein), α-amylase (Bacillus Subtilis, 16000U mg^{-1} protein), amyloglucosidase (Rhizopus, 12,000 U g^{-1} solid) and CPG were obtained from Sigma. Glucose, Sucrose, Starch and all other reagents were analytical grade.

Procedure for Enzymes Insolubilization

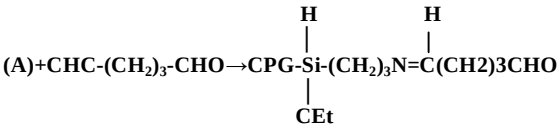
The enzymes were immobilized on controlled porosity glass (CPG) by cross linking with glutaraldehyde. The procedure involves three steps.

Step 1. *Activation of CPG by the process of silanization*:

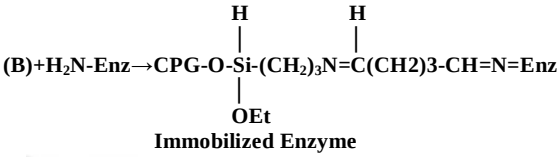


(A)

Step. II. *Treatment of activated glass with cross-linking agent (Glutaraldehyd*)



Step III. *Cross-linking of enzyme to the support.*



Immobilized Enzyme

The immobilized enzyme in each case was packed into a glass column (25x2.5 mm) with an outer, thermostated water jacket, and incorporated into the simple flow injection system for the analysis of corresponding analyte (Shown in Figure 1).

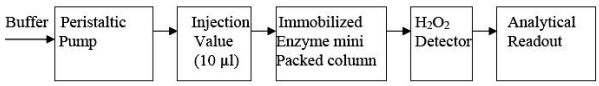


Figure 1. **Flow system incorporating mini enzyme columns**

Apparatus

The apparatus consisted of a simple flow injection system (FIA) comprising a peristaltic pump, a rotary injection valve, the flexible polyethylene manifold tubes, and a flow through amperometric detector, in the form of a glass cell containing two platinum electrodes to which 0.6 V potential was applied, thus detecting amperometricay any hydrogen peroxide formed by the action of oxidases on their substrates (Figure 2).

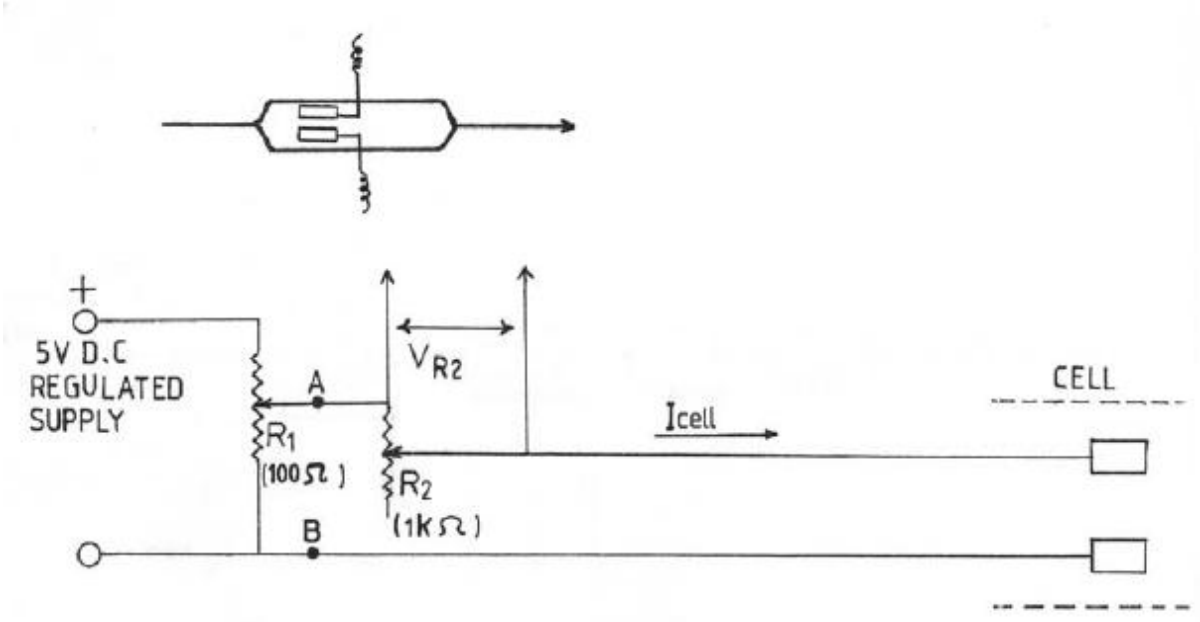


Figure 2. **Electronic circuitry for the construction of H_2O_2 detector and water jacketed immobilized enzyme column.**

Results and discussion

Determination of glucose

Standard glucose solutions (0-25 mM) were injected and analysed. The system was then applied to the analysis of 10 plasma samples for glucose, obtained from patients. The results were compared with those obtained by using commercial immobilized enzyme tubes in a BECKMAN II analyzer, a procedure routinely used in clinical laboratories. The results were in excellent agreement (Figure 3). The analytical readout was available in 12s. The possible sample throughput was $300h^{-1}$).

Determination of sucrose

The principle described for glucose was adopted to determine sucrose by incorporating co-immobilized invertase-mutarotase column prior to the GOD column. Sucrose standard samples (0-50mM) were injected into the system. In this particular case three enzymes were involved . An invertase enzyme which cleaves sucrose in to α – D glucose and fructose. α – D glucose is converted by mutarotase to β – D-glucose which is infact the substrate for glucose oxidase. The system this time contained a column containing invertase & mutarotase co-immobilized on the same support followed by a second column of glucose oxidase. H_2O_2 is the final product formed which is detected amperometrically.

In many foods and soft drinks, the combination of sugar's (e.g. glucose and sucrose) are common and measurement of both components is necessary for an effective process control [6-7]. The system for sucrose can be modified to allow the sequential determination of sucrose and glucose by incorporating a controlled bypass around the invertase-mutarotase column, thus allowing one sample to traverse both columns (giving a combined response from glucose and sucrose) and the next to pass through only the glucose oxidase column (Glucose response only). Subtraction of the later from the former gives the sucrose value. The system was used to anlayse sucrose and glucose in standard solutions and in soft drink (Coca Cola) using standard addition method (Figure 4), without any pretreatment or color removal. The results of the method were 1.32g of glucose per 100ml and 7.2g of sucrose per 100 ml of coca –cola with relative standard deviations of 1.5% (n=3). Thus soft drinks, juices and other food products can be analysed without any pretreatment.

There are many other systems which are amenable to similar treatment and the principle can be extended to the determination of more than two substances, as is often needed in clinical analyses on a single sample of blood serum. It is understood that when simultaneous analyses of samples of great interest are carried out, commercial flow injection instrumentation will be used and then undoubtedly flow injection analysis will have clear advantages over existing automated analytical methods owing to its unique characteristics of rapidity simplicity and versatility.

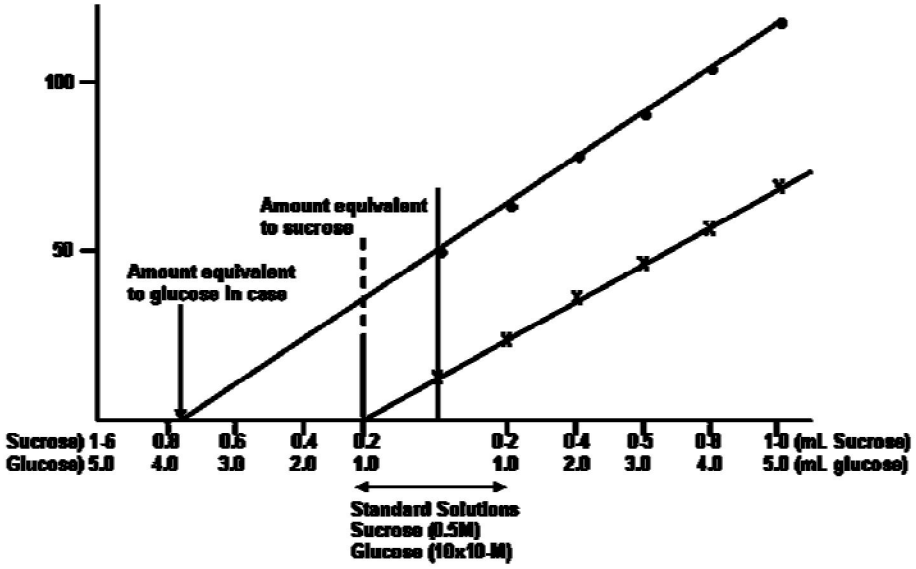


Figure 4. **Standard addition calibration for (0) glucose and (x) sucrose in coca cola**

Determination of Maltose and Starch

The importance of maltose and starch in food industry is well established. Starch is valuable not only as a natural or processed food, but also as a raw material in industry. Large quantities are used in the manufacture of textiles, gums and paper. It is also a valuable substrate for yeasts and bacteria. In the present investigation a very selective and rapid enzymatic method for maltose determination is reported, based on the use of immobilized maltase and glucose oxidase.

$$\text{Maltose} \xrightarrow{\textit{Maltase}} 2\ \text{glucose} \xrightarrow{\textit{Glucose oxidase}} \text{2-D Gluconic acid} + 2H_2O_2$$

Maltase was immobilized on CPG, packed in the glass column and incorporated into the previous flow injection glucose system prior to the glucose oxidase column. Standard solutions of maltose (0-100mg dl^{-1}) were injected into the system, which gave a good linear correlation (Y =0.99).

For starch determination three immobilized enzyme columns were used α – amylase column which cleaves starch internally followed by the maltase column which cleave maltose units from the ends. These two columns are incorporated into the flow system used for glucose analysis which has a single column of glucose oxidase. Standard solution of starch (0.05 – 0.5 %) were injected into the system. The calibration graph obtained with & without maltase enzyme column is shown in Figure 5A and the typical recorder output for starch in Figure 5B. This sensitivity level is very much sufficient for starch determination in industries where starch is used.

There are ample opportunities awaiting the use of immobilized enzymes in food technology. Over the last few decades intense research in the area of enzyme technology has provided many approaches that facilitate their practical applications. Immobilized biocatalysts can offer the possibility of a wider and more economical exploitation of there enzyme derivatives in industry. Among the several advantages that this among technological development offers, the accomplishment of complex chemical conversion under mild environmental conditions with high specificity and efficiency coupled with the availability of pure product is remarkable. Purity of the product is very crucial in food processing and pharmaceutical industry since contamination could cause serious toxicological or immunological problems. The choice of support material used for immobilization of enzyme is very important. The main factors to be considered include maximum enzyme loading for efficient conversion; neutral; good flow characteristics.

With the availability of efficient, easy and cheap analytical procedures for assaying substrate and products, the use of immobilized enzymes in the industry will certainly increase. There are interesting possible application within the field of food technology which are replaceable by immobilized enzyme technology. It is imminent that in future many more such system will become technically feasible and the industry would not hesitate adopting them.

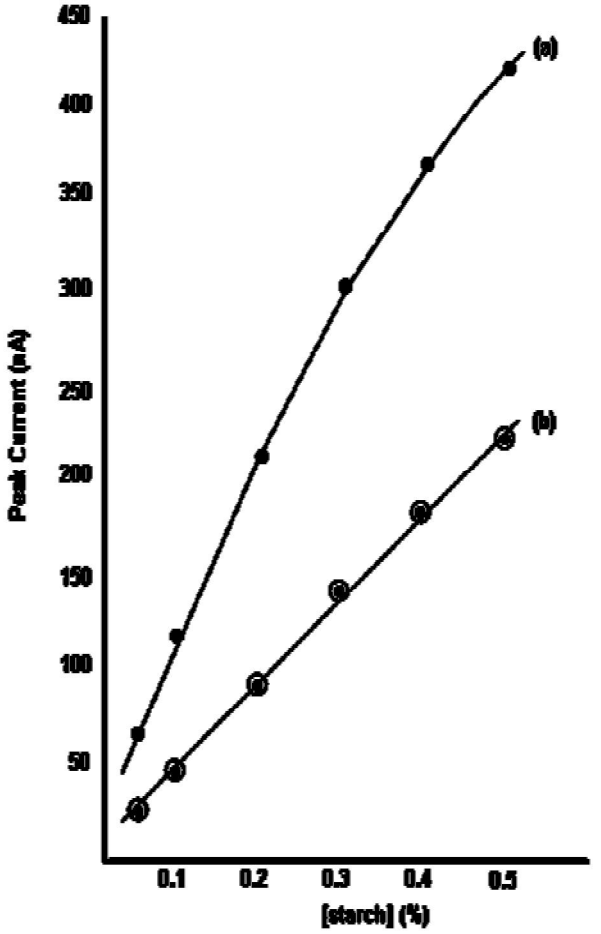


Figure 5. **Calibration graph for starch (a) using α-amylase-maltose-glucose oxidase system. (b) using only α –amylase-glucose oxidase system.**

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Masoom Yasinzai is Professor Institute of Bio-Chemistry, University of Balochistan, Quetta-Pakistan. His field of interest are biotechnology and biochemical parasitology.