CHAPTER FOUR

Enzymes as Sensors

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Abstract

Over the last few decades the development of new technologies, the fabrication of new materials, and the introduction of nanotechnologies created new trends in a series of advances that produced innovations in biological sensing devices with a wide range of application from health, security, defense, food, and medicine, to the environment. Specificity, low cost, rapidity, sensitivity, and multiplicity are some of the reasons for their growth, and their commercial success is expected to increase in the next future.

Biosensors are devices in which the recognition part of the target molecule is accomplished by biological macromolecules such as proteins, enzymes, antibodies, aptamers, etc. These biomolecules are able to bind to the target molecules with high selectivity and specificity. The interaction between the target molecule and the specific biomolecule is reflected as a change of the biomolecule structural features. The extent of this change is strictly related to the biosensor response. Fluorescence spectroscopy,

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due to its sensitivity, is often used as the principal technique to monitor biological interactions, and thus the biosensor response as well. Both the intrinsic ultraviolet fluorescence of protein, arising from aromatic amino acids (tryptophan, tyrosine, and phenylalanine), and extrinsic fluorescent labels emitting in the visible region of the spectrum together allow for very flexible transduction of the analyte recognition, suitable for many different applications.

This chapter focuses special attention on enzymes as practically unmatched recognition elements for biosensors and emphasizes the potential advantages of customized biosensor devices using *apo*- or *holo* forms of enzymes also isolated from thermophile sources.

1. INTRODUCTION

Over the last few decades the scientific communities synergistically combined technologies from various fields including physics, chemistry, molecular biology, engineering, and bioelectronics, in order to develop innovative biosensor devices. This area is very attractive, both from a commercial and an academic standpoint, because biosensors may have a wide range of applications in health, pharmaceuticals, homeland security, food safety and quality, defense, security application, and environmental monitoring. Both the research and practical applications are expected to increase, in the near future. Actually the global market in the field of biosensors is around US$13 billion for 2015 (Turner, 2013).

The reasons for the growth and success of biosensors include their high specificity, low cost, rapidity, sensitivity, multiplicity, and portability, to name just a few. In fact, these features are of fundamental importance for reliable detection of the target molecules.

The history of biosensors starts in 1962 with scientist L.C. Clark and the invention of the enzyme electrode to measure glucose. However, the scientist K. Cammann used the term "biosensor" for the first time in 1977, and later in 1999 it was included in a technical report by IUPAC. According to the IUPAC paper: "A chemical sensor is a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytical signal. Chemical sensors usually contain two basic components connected in series: a chemical recognition system and a physicochemical transducer" (Hulanicki, Geab, & Ingman, 1991). When the recognition system utilizes a biological sensing element, the sensor is termed a biosensor; the term is synonymous with "biological sensor."

It can be divided into the following essential elements:

(a) an analyte, a substance of interest that needs to be detected;

(b) a biological system, bioreceptor, or molecular recognition element (MRE) that acts as a sensing element, that is, a molecule that specifically recognizes the target analyte. The MRE can be an enzyme, a cell, an aptamer, a sequence of deoxyribonucleic acid (DNA), or an antibody;

(c) a transducer system is the element that detects the signal, converting one form of energy into another. Its role is to convert the biorecognition event into a measurable signal. The types of transducer include at least piezoelectric, electrochemical, thermometric, magnetic, and optical;

(d) an electric system that amplifies the signal, quantifies it, and transfers it to an operator.

When the recognition biomolecule interacts with the target analyte, a biomolecule conformational change occurs, and an electric or optical signal is produced and detected by a transducer (Fig. 1).

It is possible to identify at least five different types of biosensors based on transduction principle:

(1) calorimetric biosensors (heat is released or absorbed upon interaction);

(2) potentiometric biosensors (production of an electrical potential due to changed distribution of electrons);

(3) acoustic wave biosensors (change in mass of the biological component as a result of the interaction with the analyte);

(4) amperometric biosensors (measure of electron flux due to redox reaction);

(5) optical biosensors (light produced or absorbed during reaction).

A particular case is represented by the immunochromatographic biosensors. This biosensor type does not exactly use any of transducer strategies listed before, but it is based on the analyte flowing in liquid over stationary lateral flow strips, where the speed of analyte movement is determined by the interaction with (usually) antibody immobilized on the strips, and the (optical) readout is actually the position of the analyte band after a fixed time period. Among the most popular lateral flow strip products are the patient-operated (home) pregnancy and glucose tests (Bahadır & Sezgintürk, 2015).

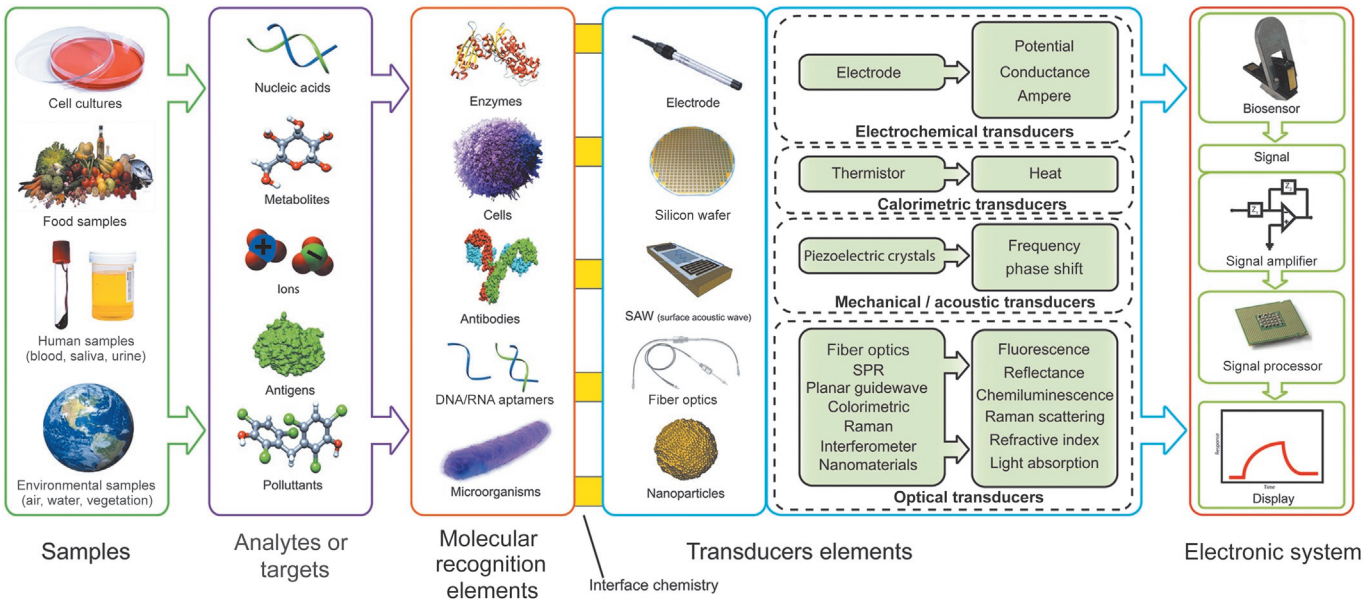

Fig. 1 Schematic representation of a biosensor platform. From *left* to *right*, in each panel, are shown only some samples, analytes or targets, molecular recognition elements (MREs), transducers element, and electronic systems. It is possible to couple the different elements in numerous combinations and obtain several biosensors.

2. OPTICAL BIOSENSORS

Here, we will focus on the class of optical biosensors, and in particular on fluorescence biosensors used for qualitative and quantitative measurements of biomolecular interactions. Fluorescence activity is more suitable for ultrasensing applications to detect and quantify chemical or biological compounds than other, more conventional methods (Martins et al., 2013). The detection of molecular changes upon biological interactions occurs through the measurement of photons involved in the process of absorption and emission in the UV, visible, or near-infrared spectral regions of light.

Biosensors and biosensor test formats, respectively, can be classified into labeled and label-free types depending on whether the output signal caused by analyte binding is obtained using labeled compounds or not.

3. LABEL-FREE DETECTION AND FLUORESCENCE-BASED DETECTION

3.1 Label-Free Detection

This type of detection uses unlabeled target molecules. This choice may be due to the difficulty of performing the labeling procedure. In fact, labeling methods are not always favorable because probes may occupy the binding sites of molecule with a steric hindrance and, consequently, to interfere with the binding process. To avoid this limitation, alternative detection methods based on label-free detection can be utilized. Surface plasmon resonance (SPR), nuclear magnetic resonance analysis, microsurface-enhanced Raman scattering, microsphere cavities, liquid crystal sensors, and calorimetric arrays all represent examples of label-free detection strategies (Gopinath, 2010). An SPR array is based on the evanescent field detection principle and measures the variation of refractive index (RI) upon the occurrence of molecular interaction, in femtoliter to nanoliter volumes of sample, providing information about the specificity, affinity, binding kinetics, and the concentration levels of specific analyte within the 1–10 μm range (Fan et al., 2008). Another label-free biosensor type is based upon variations in RI that occur upon binding; the biorecognition molecules, such as antibodies, aptamers, etc., are immobilized on the surface of chip, and molecular interactions are measured following the RI unit variation (Hoa, Kirk, & Tabrizian, 2007).

3.2 Detection Using Optical Labels

The recent advancements in bioinformatics and probe chemistry, such as the ability to study protein dynamics in a timescale of microseconds, and the commercial availability of new fluorescent organic labels, open promising opportunities to study the role of proteins in cell function, and to deduce the existence of new regulatory mechanisms of proteins in cell life.

The biosensing research area and in particular the design of novel, highly performing biosensors are one of the most promising applications of the advances in chemical biology.

In actual applications, novel proteins usable as biomarkers, and/or the synthesis of new chemical probes for labeling proteins and antibodies, are essential for moving toward rapid diagnoses directly on real matrices such as whole blood (D'Auria, 2013).

Today it is now possible to choose the best method and the best label strategy in order to satisfy the required application. Many parameters are important to consider for selecting suitable organic fluorophore or quantum dot labels with the appropriate properties such as brightness, lifetime, size, and photostability (Toseland, 2013).

In designing a biosensor it is also possible to separate the optical properties of the biosensor (due to the fluorescence properties of the selected fluorophore) from binding specificity and affinity characteristics that are defined by the biomolecule used (D'Auria, Gryczynski, Gryczynski, Rossi, & Lakowicz, 2000).

Multiple biological recognitions integrated in a single device allow the detection of multiple analytes in the same cartridge. This miniaturized device is called "Lab On A Chip" (LOC) platform. The LOC approach began to spread widely at the end of the 1980s and beginning of the 1990s, and it has since found wide application in molecular biology, proteomics, cell biology, and chemistry.

The emerging technologies of LOC devices and nanosensors offer new possibilities such as fully automatic operation, which permits performing several hundred analyses simultaneously on the same chip to detect specific targets in real-time. This reduces human error, increases throughput, and permits performing analyses on site in remote areas without infrastructure. For instance, a current approach is based on electron beam irradiation, to create protein nanopatterns on a porous silicon wafer; other approaches have also been demonstrated for the production of micro- and nanopatterns of biomolecules on solid substrates (Borini et al., 2007).

3.3 Fluorescence-Based Detection

Fluorescent biosensors are usually based on the use of fluorescent organic molecules, nanoparticles, proteins, or combinations of these. They are designed and engineered to change their fluorescent properties in response to external influences or chemical species including pH fluctuations, metal ion homeostasis, cell signaling, membrane potential differences, phosphorylation, ubiquitination, redox reactions, apoptosis, and molecular interactions.

Usually proteins exhibit intrinsic fluorescence due to the presence of the aromatic amino acids tryptophan, tyrosine, and phenylalanine. It is also possible to label proteins, selecting the biomolecule site where to attach the fluorescence probe. Conformational change, protein–protein interaction, single-molecule studies, protein counting, and cell localization are potential applications. There are different strategies to detect and quantify these changes.

Fluorescence correlation spectroscopy (FCS) measures fluctuations of a small number of molecules in a focused laser beam. FCS is based on the analysis of time-dependent intensity fluctuations that are the result of some dynamic process, typically translational diffusion into and out of a small volume (femtoliter) defined by a focused laser beam and a confocal aperture (Varriale et al., 2007). Förster resonance energy transfer (RET) is used to understand the structure and function of biological macromolecules. It is based on the transfer of energy occurring between excited molecule (donor) and ground-state molecule (acceptor) via long-range dipole–dipole interaction up to a maximum distance of 150 Å or so. This large value has been recently reported in studies that used a donor–multiacceptor pair (antenna effect) to enhance the efficiency of transfer of energy so that it is measurable even at distances exceeding 150 Å. This method permits analysis of a variety of larger biological systems and can be used for planning specific FRET experiments above 100 Å (Walczewska-Szewc, Bojarski & D'Auria, 2013).

Measurement of steady-state and time-resolved fluorescence anisotropy offers many opportunities to study molecular orientation, mobility, and diffusion in labeled biological processes and systems. In fact these phenomena are due to transition moments for absorption and emission along specific directions fixed with respect to the fluorophore structure. The rate of rotational diffusion depends on the viscosity of the solvent, and the size and shape of the molecule (Lakowicz, 2006).

4. WHY CHOOSE BIOMOLECULES FOR SENSING?

As reported in scientific literature, it is difficult to design and synthesize in a laboratory organic molecules able to bind specifically to analytes that are structurally more complex than simple ions (Na^+ and K^+) (D'Auria & Lakowicz, 2001). Even if we assume that this kind of synthesis is possible, there is no guarantee that structure changes useful for sensing will occur upon their binding with target analyte. To circumvent this and other limitations related to the use of small molecule fluorescent probes, modern biotechnology has instead resorted to the idea of using proteins and enzymes as components of sensors for biochemical analytes. The idea is to exploit the extremely wide range of selective affinities sculpted into the various proteins by biological evolution. The use of biomolecules in biosensors presents many advantages:

(1) high specificity binding (for instance, enzymes are easily able to discriminate the enantiomer L from enantiomer R of sugar molecules);
(2) solubility in aqueous buffers;
(3) the possibility to use recombinant proteins for high yields;
(4) the possibility to manipulate the properties of genes using site-directed mutagenesis for new molecules, and/or using genetically encoded tags;
(5) the possibility to label proteins such as antibodies with fluorescent probes;
(6) the opportunity to use highly stable biomolecules isolated from thermophilic organisms.

Among biological molecules, enzyme molecules deserve special mention. In fact, enzymes can be easily manipulated to abolish their substrate consumption (apoenzymes) or to increase their stability (thermostable enzymes). This makes them interesting molecules to use for developing customized biosensors.

5. ENZYMES AS MREs

The function of a robust and operationally stable biosensor depends on the biochemical specificity and stability of the MRE. MREs described to date include biologically derived molecular species (e.g., an antibody, a receptor, an enzyme, a protein, or a nucleic acid) or larger portions of living biological systems (e.g., cells, tissue, microorganisms, organelles, or whole organisms).

The biochemical specificity of MRE for a biosensor is crucial since it is responsible for binding the analyte of interest. Enzymes are indispensable biomolecules, involved in all aspects of the cellular metabolism of living organisms. They are selective catalysts necessary to accelerate biological processes, in some cases utilizing other substances as cofactors. They are not consumed by the chemical reaction, and they remain unaffected and available for new reactions. Enzymes are found in all organisms, where they are involved in a broad spectrum of metabolic functions. They catalyze a large number of reactions displaying distinct substrate specificity, good efficiency, and outstanding selectivity, a key requirement for a biosensor. The catalytic activity, sensitivity, affinity, and high chemical specificity provided by enzymes allow for much lower limits of detection of different analytes than would be obtained with different binding techniques (Vo-Dinh & Cullum, 2000).

The use of an enzyme as MRE in a biosensor often ensures reliable function under varying environmental conditions over an extended time period. Since the sensor's operational lifetime is typically limited by the stability of the enzyme, then the use of stable enzymes is an important criterion in the biosensor development (D'souza, 2001; Gouda, Singh, Rao, Thakur, & Karanth, 2003). The main factors affecting enzyme stability and activity are pH, ionic strength, chemical inhibitors, solvent polarity (if not aqueous), and temperature. These limitations can be overcome by using thermostable enzymes isolated from extremophile microorganisms: organisms that grow naturally under extreme conditions.

Extremophiles provide a unique source of novel, robust enzymes, which are more rugged than their mesophilic counterparts under the conditions often encountered where the biosensor is to be employed.

6. THERMOPHILIC ENZYMES

Natural evolution has selected some enzymes to perform their physiological functions under extreme conditions. They operate optimally at temperatures, salinities, pHs, and in solvents that may be quite far from ordinary physiological environments (Cowan et al., 2010; Egorova & Antranikian, 2005).

In particular, the ability to retain native protein structure and function at high temperature is due to higher stabilization of these proteins, resulting from a balance between flexibility (important for activity) and rigidity due their amino acid composition: lower levels of asparagine, glutamine,

cysteine, methionine, and tryptophan, which are more susceptible to deamidation or oxidation at high temperatures, and higher levels of isoleucine, alanine, and proline, which should provide more densely packed hydrophobic cores, and extra stability of the loops (D'Auria et al., 1998).

Furthermore, the use of enzymes from thermophilic microorganisms has been frequently proposed and demonstrated as a solution to the problem of enzymatic stability, most readily assessed in enzymes by loss of catalytic activity.

Enzymes isolated from thermophilic organisms are more robust, being stable at high temperatures for long time periods and in the presence of organic solvents. The ability of the enzyme to catalyze reactions in the presence of organic solvents is important because different analytes may require a cosolvent due to low solubility in aqueous media. In fact many of them used in biosensors are more soluble at higher temperatures and in the presence of organic solvents (Pennacchio et al., 2013).

Initially, thermophilic enzymes were isolated directly from microorganisms, including eubacteria and archaea. Nowadays, they are often produced in soluble form in *Escherichia coli* by biological engineering.

The use of thermophilic enzymes has revolutionized the world of biotechnology. Perhaps the best known example is the use of thermostable DNA polymerase for in vitro DNA amplification, the polymerase chain reaction. Replacing (for example) *E. coli* DNA polymerase with the thermostable DNA polymerase purified from the thermophilic bacterium, *Thermus aquaticus* (*Taq*), that can survive extended incubation at 95°C (Saiki et al., 1988), enables the newly polymerized strands to be dissociated by melting without destroying the polymerase. This permits many cycles of amplification without adding more polymerase, simplifying the procedure, and making it amenable to automation.

7. BIOLOGICAL ENGINEERING

Natural enzymes seldom fulfill all the requirements of an MRE (Meyer, 2006; Polizzi, Bommarius, Broering, & Chaparro-Riggers, 2007; Schoemaker, Mink, & WubboLts, 2003) and require improvement of one or more functional characteristics. Using different tools, it is possible to design enzymes able to work in a variety of environmental conditions of temperature, pH, and salinity, or to act on molecules slightly different in structure from their natural substrates. Protein engineering has made it possible to manipulate virtually any enzyme property, either through

site-directed mutagenesis or through chemical modification (Jäckel, Kast, & Hilvert, 2008; Kurtovic & Mannervik, 2009; Reetz, 2007; Schmidt, Böttcher, & Bornscheuer, 2009). Genetic engineering using recombinant DNA technology is now a routine and rapid process, permitting the rapid cloning and overexpression of target enzyme genes in suitable hosts (Meiring et al., 2011; Mutondo, Huddy, Bauer, Tuffin, & Cowan, 2010). The development of metagenomic gene discovery has made enzymes from organisms in thermophilic environments readily accessible, even when the parent microorganisms are not cultivatable (Samuelson, 2011; Smith, 1996).

However, the use of enzymes as MREs presents the disadvantage of substrate consumption, a feature that it is not always useful in a detection process. Biosensors based on the use of coenzyme-depleted enzymes can overcome this problem, since they do not complete the catalytic cycle. Apoenzymes do not consume substrate, and they can exhibit conformational changes upon the binding of the analyte that permit its detection.

7.1 Apoenzymes as Nonconsuming Substrate Sensors

Apoenzymes, inactive form of enzymes, are still able to bind substrate with an affinity comparable to holoenzyme, but they are not able to transform substrate into product (Fig. 2). The binding of substrate to apoenzyme may result in a conformational change that can be easily detected by fluorescence measurements.

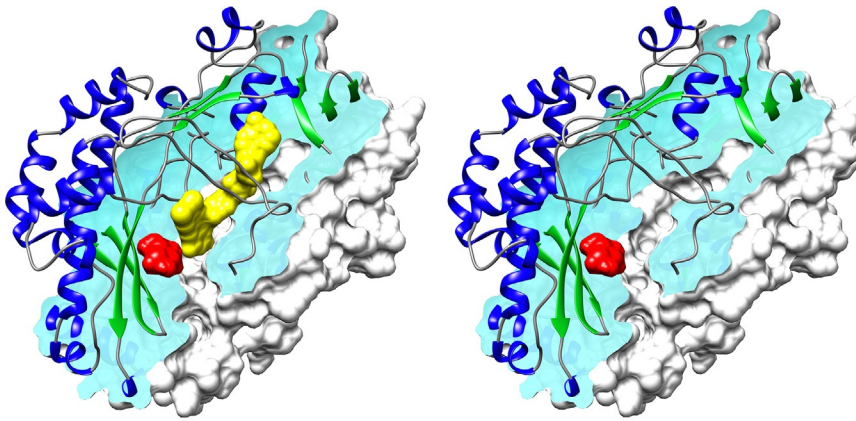

Fig. 2 Glucose oxidase from *Aspergillus niger* in apo and holo forms. The FAD is shown in *yellow* and the glucose in *red*.

Glucose sensors, for continuous monitoring of blood glucose, can use coenzyme-depleted enzymes (apoenzymes) as nonconsuming substrate sensors. Examples of apoenzymes used for glucose sensing are the apoglucose oxidase from *Aspergillus niger*, the apoglucose dehydrogenase from the thermophilic microorganism *Thermoplasma acidophilum*, and the apoglucokinase from the thermophilic eubacterium *Bacillus stearothermophilus* (de Champdore´, Staiano, Rossi, & D'Auria, 2007).

7.2 Apoglucose Oxidase From *A. Niger*

Glucose oxidase (GO) from *A. niger* is a flavoprotein that oxidizes β-D-glucose with high specificity. In holo form this enzyme cannot be used as a reversible sensor to estimate glucose concentration in the blood or urine samples, since glucose is consumed. To prevent glucose oxidation, the FAD cofactor was removed from the GO structure. The apo-GO was still able to bind glucose but not to transform it.

In Fig. 3 are shown the apo-GO absorbance and emission spectra. The absorbance spectrum presents a maximum at 278 nm due to the contribution of the aromatic amino acid residues. The emission spectrum displays a maximum at 340 nm, characteristic of partially shielded tryptophan residues. The absence of absorption at wavelengths above 300 nm in the absorbance spectrum demonstrates that the FAD molecule has been completely removed from the GO structure. The decrease of intrinsic tryptophan fluorescence intensity indicates the binding of glucose in the active site of

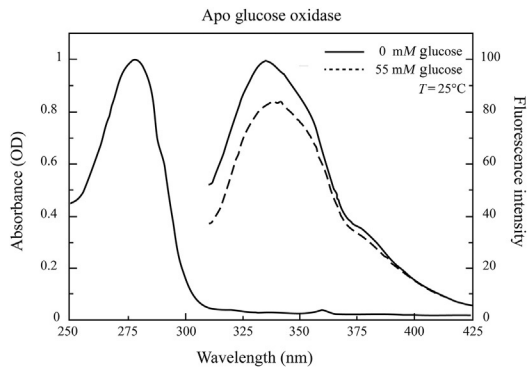

Fig. 3 Absorption and emission spectra of apoglucose oxidase. Excitation at 298 nm. The protein concentration was 0.05 mg/mL. *Adapted from D'Auria, S., Herman, P., Rossi, M., & Lakowicz, J. R. (1999). The fluorescence emission of the apo-glucose oxidase from* Aspergillus niger *as probe to estimate glucose concentrations.* Biochemical and Biophysical Research Communications, 263, *550–553.*

apo-GO. Indeed, the apo-GO is still able to bind glucose, and the Trp 426 involved in this binding event exhibits the observed fluorescence quenching (D'Auria, Herman, Rossi, & Lakowicz, 1999; Hecht, Kalisz, Hendle, Schmid, & Schomburg, 1993; Meyer, Wohlfahrt, Knablein, & Schomburg, 1998; Wohlfahrt et al., 1999). These results suggest that apo-GO can be used in the apoenzyme form as a nonconsuming glucose biosensor (D'Auria et al., 1999).

7.3 Apoglucose Dehydrogenase From the Thermophilic Microorganism *T. Acidophilum*

Glucose dehydrogenase (GD) from the thermoacidophilic archaeon *T. acidophilum* is an enzyme resistant to high temperatures and organic solvents and catalyzes glucose oxidation with high specificity. The apoform of the enzyme still binds glucose with an affinity comparable to the holoenzyme and in the presence of nonpolar solvents displays increased enzyme activity (D'Auria, Di Cesare, et al., 2000). The presence of acetone causes a change in the local protein environment leading to an increase of fluorescence emission intensity. Addition of glucose to ANS-GD in the presence of the optimal acetone concentration resulted in a decrease in ANS emission intensity (ANS is 1-anilino-8-sulfonic acid). This decrease can be also used to measure glucose concentration (D'Auria, Di Cesare, et al., 2000).

7.4 Glucokinase From the Thermophilic Eubacterium *B. Stearothermophilus*

Glucokinase (GK) from the thermophilic organism *B. stearothermophilus* (recently renamed *Geobacillus stearothermophilus*) is known to display long-term stability (D'Auria et al., 2002). The enzyme is stable at room temperature for several days, unlike the hexokinase from *Saccharomyces cerevisiae*, which has also been used as an optical glucose sensor.

Fig. 4 shows the enzyme activity and intrinsic fluorescence as a function of time at room temperature of both the thermophilic and mesophilic enzymes. The yeast hexokinase is quite unstable, it loses enzyme activity over several days, and its fluorescence intensity decreases. In contrast, the thermophilic GK is still enzymatically active over 1 month's time, and the fluorescence intensity remains almost unchanged.

A competitive glucose assay based on RET was developed using the unmodified protein and its intrinsic tryptophan emission as the donor. *O*-nitrophenyl-gluco-pyranoside (ONPG) was used as RET acceptor, a molecule containing the absorbing nitrophenyl group.

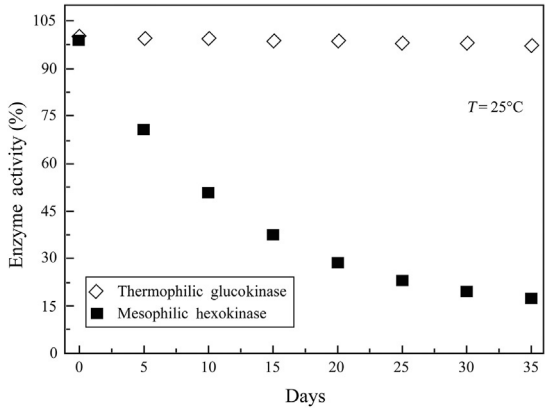

Fig. 4 Stability of the thermophilic glucokinase. The incubation was at room temperature. *Adapted from Scognamiglio, V., Staiano, M., Rossi, M., & D'Auria, S. (2004). Protein-based biosensors for diabetic patients.* Journal of Fluorescence, 14*, 491–498.*

The interaction between GK and ONPG produced a decrease of tryptophan emission intensity. Addition of glucose displaced ONPG from the GK-binding site and allowed for the recovery of fluorescence emission intensity, indicating that the intensity changes were due to the binding of glucose to GK-binding site (D'Auria et al., 2002).

The above described examples indicate that enzyme which use glucose as substrate, if deprived of their cofactors, can be used as reversible, non-consuming substrate biosensors.

8. CONCLUSIONS

In conclusion, biosensors are highly valuable devices for measuring a wide array of analytes. They can provide cost-effective, easy-to-use, sensitive, and highly accurate detection devices. Biosensors can be used in a variety of research and commercial applications, ranging from clinical to environmental and agricultural. Some examples of these applications include: general healthcare monitoring, screening for disease, clinical analysis and diagnosis of disease, veterinary and agricultural applications, as well as industrial processing and monitoring and environmental pollution control.

In the field of bio/sensing there has been rapid growth in the development and application of naturally occurring MREs. Clearly, biomolecules are the most widely used recognition element; in particular enzymes are

the main favorite in the fabrication of various biosensors due to their highly specific binding affinities and catalytic activities. In the literature it is reported that some receptors offer both advantages but also disadvantages. Sophisticated chemical modification and molecular biological techniques have been applied in the refinement of their binding, chemical and thermal stability, and in their immobilization properties.

Considering the need for reliable and robust sensing in many applications, much effort has been expended to design innovative and novel enzymatic sensor designs. However, other properties of enzyme sensors such as poor stability and critical operational conditions, as well as sensitivity to pH and temperature variation, restrict their wider utility for real-time applications.

With the goals of improved stability, reliability, selectivity, and sensitivity, the scientific community exploited advancements in genetic engineering to develop recombinant enzymes or to modify the catalytic/allosteric enzymatic sites to adapt enzymes to sensing. A successful result of this approach was the development of apoenzymes as MRE for obtaining non-consuming substrate biosensors.

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