

Utilization of Enzymes in Biochemistry and Analytical Biochemistry

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Enzymes as catalyzers of biochemical reaction are very important components of not only analytical and clinical methods but they are also utilized in various other fields of life sciences, including biochemical, physiological, and biomedical research [1]. Well known are the determination of enzyme activities (e.g. alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, creatinine kinase, phosphatase, amylase, cholinesterase, lipase etc.) and the enzyme-coupled determinations of metabolites (such as D-glucose, acetoacetate, creatinine, cholesterol, L-phenylalanine) as a diagnostics of various diseases, however enzymes serve also as a part of immuno-assay and biosensors, which have a wide range of applications. Biosensors are now on the horizon that will allow us to quantify local changes of ions, signaling intermediates, and metabolites in real time. They can be defined as molecules (typically RNAs or proteins) or cells that report analytes or processes in live organisms or in their environment [2]. Biosensors are analytical devices, which converts a biological response into an electrical signal, they are composed of bioreceptor (enzyme in this case), which recognize the target analyte and a transducer, which converts the recognition event into a measurable electrical signal [3]. Biosensors showed desirable sensitivity, selectivity, and response time and generally they are designed to avoid interference from components present in a complex sample, which is analyzed [4]. Whereas a wide scale of enzymes can be used in biosensors, in immunochemical methods (such as ELISA, EIA) participates mainly alkaline phosphatase and peroxidase, which are conjugated with secondary antibody. Peroxidase caused oxidation of various chromogenic dyes, such as ABTS; phosphatase cleaves e.g. p-nitrophenylphosphate to p-nitrophenol and phosphate, which provides in alkaline pH yellow color. However primary antibody can be prepared against wide scale of antigens. Recently enzymes began to be used in extraction procedures of pharmaceutically interesting compounds and food (mainly cellulases, pectinases, hemicellulases, proteases, β -glucosidase etc.) [5] and as alternative (cheaper and less time consuming) method for determination of pesticides (using acetylcholinesterase, butyrylcholinesterase, alkaline phosphatase, organophosphorus hydrolase and tyrosinase) [6].

Although many enzyme-catalyzed reactions results in changes in the properties of the reactants that are relatively easy to measure directly and continuously, other do not and in such cases it is necessary to use an indirect assay method that involves some further treatment of the reaction mixture ("end points methods", or coupled-enzyme assays) [7]. The activity of NAD(P)-dependent enzymes can be spectrophotometrically easily detected by monitoring of liberating NAD(P)H at 340 nm. Therefore these enzymes are suitable in coupling with enzyme reaction with an applicable product, which is simultaneously substrate for dehydrogenases (e.g. oxaloacetate, dihydroxyacetone phosphate, glucose-6-phosphate etc) [7]. Certain products, such as naphthols can be detected indirectly by a secondary reaction with diazonium salts to form azo-dyes [8], or hydrogen peroxide by secondary reaction with peroxidase and chromogenic substrate of peroxidase. Further, when the natural reaction is not accompanied by a useful absorbance change, it is common-place to use a synthetic substrate, e.g. numerous glycosides of fluorescent or colored phenols are used to test glycosidases [7]. Most enzyme activity

assays are based on chromogenic and fluorogenic enzyme substrates, in particular nitrophenols, umbelliferons, fluoresceins, rhodamines and Bodipy dyes [8]. Bodipy dyes are photostable substitute for fluorescein, they are one of the most versatile fluorophores, derived from difluoroboraindacene family [9]. Luminescent assays, in which participates luciferase and assays based on Förster or fluorescence resonance energy transfer (FRET) as a detection principle are also of interest [8]. Many enzyme assays are based on radiometric assay.

In some cases for enzyme activity determination is necessary to separate products of reaction by analytical instrument such as HPLC, GC, MS, NMR or IR spectrometry [7,8]. In recent years in evaluation of the enzyme activity, enzyme kinetics, identification of enzyme substrate, products, inhibitors and activators has been applied capillary electrophoresis, analytical tool with many advantages (not only the possibility of application of natural enzyme substrate, but also high separation efficiency, short analysis time, small sample and chemicals consumption [10]).

Interesting is detection of enzyme activity on gels after native electrophoresis, which enables visualization of individual isoenzymes after their separation, because activity and aggregation state is not affected. There are several systems enabling native electrophoretic separations, besides modification of Laemmli electrophoresis omitting of SDS and denaturation, it is for instance blue native electrophoresis and its modification red native electrophoresis [11]. General principles of enzyme detection on gels after native electrophoresis consists mainly in colored insoluble products, products reducing tetrazolium salts to intensely-colored, water-insoluble formazan (in this group NAD(P)-dehydrogenase are involved; products NADH or NADPH are electron donors for reduction of tetrazolium salts), or products that caused a pH change. Similarly, enzymes reactions producing orthophosphate, pyrophosphate, hydrogen peroxide or carbon dioxide are suitable for in gel detection. The orthophosphate can be detected by several methods, e.g. sulfide method, calcium phosphate method, acid phosphomolybdate method of enzyme method; pyrophosphate can be detected *via* calcium ions, which provide white bands of calcium pyrophosphate precipitation; hydrogen peroxide using redox dyes such as 3,3'-diaminobenzidines, 3-amino-9-ethyl carbazole, or tetramethyl benzidine or using chromogenic peroxidase. For detection of carbon dioxide calcium carbonate method or coupled enzyme method with phosphoenolpyruvate carboxylase are used. In gels can be also detected enzyme products bearing reduced thiol groups, products that

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influence the starch-iodine reaction, products of hydrolytically cleaved 4-methylumbelliferone etc [12].

Currently, methods for routine measurements of enzyme activities within small samples are implemented to the practices (e.g. nanoparticle systems, which incorporate the magnetic, optical, and electronic properties associated with metallic nanoparticles into high-sensitivity devices for a broad range of target analytes [13]; microassays such as NanoDrop fluorimetry of proteasomal enzyme activities [1]; or microassays of cellulolytic and xylanolytic enzymes [14].

Even though enzyme assays are transformed to biosensors, nanoparticles, and micro-assays, determination of enzyme activity remains important part of biochemical research, disease diagnostic, and many other various analyses.

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