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pH-metric detection of alkaline phosphatase activity as a novel biosensing platform

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Abstract

A detection of alkaline phosphatase (ALP, EC 3.1.3.1) activity by the monitoring of pH changes caused by the biocatalytic action of the enzyme has been experimentally examined. Enzymatically catalyzed hydrolysis of monofluorophosphate has been found to be the best basis for such measurements. Protolytic equilibria connected with the developed biosensing system were recognized and the optimal conditions for the assay have been found. Advantages and disadvantages of the developed (bio)sensing scheme have been discussed. The prototype of pH-ALP based enzyme electrode has been demonstrated. Potential utility of such substrate–enzyme–sensor system for the development of a new group of biosensors has been announced.

Keywords: ALP; pH-based biosensing; pH-enzyme electrode

1. Introduction

Alkaline phosphatase (ALP, EC 3.1.3.1) is a nonspecific esterase that catalyzes hydrolysis of many monoesters of phosphoric acid. A low biocatalytic selectivity enables the development of various substrates for optical and electrochemical enzyme activity assays. A clinically recommended spectrophotometric method for ALP determination uses *p*-nitrophenylphosphate [1–3] as chromogenic substrate. Several substrates for optical methods based on fosforescence [4], chemiluminescence [5] and fluorescence [6] measurements have been also developed. A large group of phosphate esters of different organic substances (mainly phenol, ferrocen and indol derivatives) are useful in voltamperometric measurements [7–15]. Potentiometric enzyme assays using fluoride [16,17] as well as hordenine [18] ion selective electrodes have been also developed.

ALP belongs to the group of enzymes the most commonly assayed in clinical practice, because its blood activity significantly rises in case of many skeletal and liver diseases. ALP activity measurements are widely used in different areas of immunochemistry as the enzyme is an active label in immunohistology, immunoblotting and immunoassays, including immunosensing devices [7–15]. The popularity of ALP is based on its low cost, high stability, high turnover-rate, relatively small size and the large number of commercially available ALP conjugated immunoreagents. Owing the same reasons, this enzyme is often used as a marker in genosensing devices [19–24]. Detections of the enzyme activity also find applications in bioanalytical methods and biosensors for determination of respective ALP inhibitors and activators [25–31].

In the course of ALP catalyzed reactions, ionic products having protolytic properties are formed. They may influence protolytic equilibria causing pH changes of reaction microenvironment. In this paper, pH-metric detection of ALP activity is experimentally examined and discussed. Such phenomenon could be considered as an alternative platform for ALP assay. The utility of such (bio)sensing scheme for the biosensor development is demonstrated.

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2. Experimental

ALP isolated from bovine intestinal mucosa (powder, 24 U/mg) and ALP substrates (ADEP, adenosine phosphate; URIP, uridine phosphate; BGLP, β-glycerol phosphate; RGLP, glycerol phosphate (racemate), 1GLP, glucose-1-phosphate; 6GLP, glucose-6-phosphate; MFP, monofluorophosphate; NPP, *p*-nitrophenylphosphate) as disodium salts were obtained from Sigma (USA). Other reagents of analytical grade were obtained from POCh (Poland). Substrate solutions were prepared immediately before use. All solutions were prepared with doubly distilled water.

For pH-metric measurements combined glass electrode (model MC 100, Taccusel, France) was used. Potentiometric measurements with the developed enzyme electrode were performed versus double-junction (0.1 M NaCl) saturated calomel electrode (type RH 44/2-SD/1, Moller Glasblaserei, Switzerland). For all measurements, digital pH-meter (model OP 208/1, Radelkis, Hungary) connected to data-collecting PC was applied. Measurements were performed in stirred solutions under ambient conditions (room temperature).

pH-ALP-based electrode was prepared according to the general method reported elsewhere [32]. Components of pH membrane (tridodecylamine as hydrogen ionophore, bis-(2-ethylhexyl) sebacate as plasticizer and carboxylated polyvinyl chloride as membrane matrix and immobilization support) were obtained from Fluka (Switzerland). The pH-sensitive membrane was mounted into commercial ion selective electrode body (Philips model IS 561) from Moller Glasblaserei (Switzerland). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride used for covalent immobilization of ALP to the surface of pH-sensitive membrane was obtained from Sigma (USA).

3. Results and discussion

3.1. Protolytic equilibria involved in the (bio)sensing system

pH-metric detection of ALP activity seems to be possible because in the course of the enzymatic action amphiprotic monohydrophosphate ions are formed:

$$XPO_3^{2-} + H_2O \rightarrow XH + HPO_4^{2-} \quad (1)$$

In most cases, organic co-products (XH) have no protolytic properties. Preliminary investigations of ALP catalyzed reactions were performed in unbuffered solutions (0.1 M NaCl) using glass electrode for pH measurements. The pH-shifts measured in the test solution after subsequent addition of tested substrates (final concentration, 2.0 mM) and enzyme (nominal ALP activity, 2 U/l) are shown in Fig. 1. These simple experiments led to interesting conclusions. All tested substrates (used as disodium salts) caused an increase in pH, as they act as weak bases. This means that in unbuffered solutions the type and concentration of substrate will determine initial pH for the assay. In nearly all cases this pH is appropriate for ALP assay as the maximum of the enzyme activity ranges from pH 8 to 10 [17,33]. There are two reasons for pH changes observed after enzyme addition. Addition of protein to weakly buffered solutions changes their pH independently of biocatalytic properties of the enzyme. This change in pH is relatively fast. New pH is equilibrated and constant in several seconds after the enzyme addition. In contrast to this non-specific effect, pH-shifts caused by formation of protolytic products in the course of ALP catalyzed reaction should be continuous. As can be seen in Fig. 1, a significant change in pH originated from enzymatic reaction is observed only in the course of experiment with monofluorophosphate (MFP). This exceptional effect was obvious taking into account that only in this case additional proton is generated, according to the following equation:

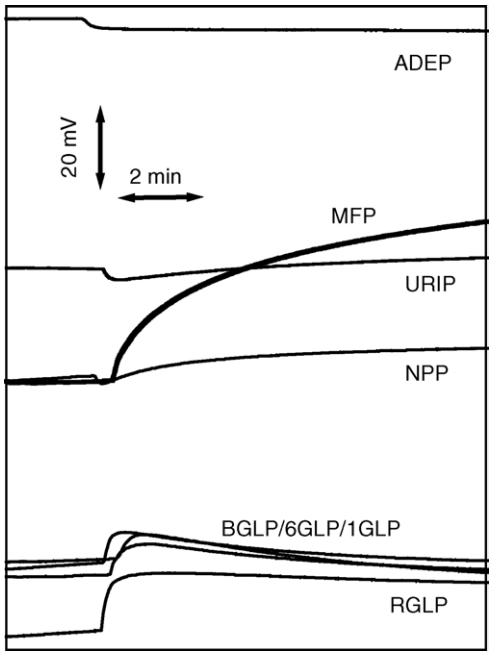

Fig. 1. Changes of potential of pH-glass electrode for various ALP substrates (2.0 mM) after addition of ALP (0.08 mg/ml). Measurements performed in unbuffered solutions (0.1 M NaCl).

$$FPO_3^{2-} + H_2O \rightarrow HF + HPO_4^{2-} \rightarrow F^- + H_2PO_4^- \quad (2)$$

Contrary to common organic ALP substrates hydrolyzed according to reaction (1), in this process dihydrophosphate ions (more acidic than HPO_4^{2-} ions) are formed. Data shown in Fig. 1 clearly indicate that MFP is a specific substrate for pH-metric detection of ALP activity.

Similar experiments performed with MFP and ALP in a series of diluted base solutions showed that changes of glass electrode potential are mainly dependent on buffer capacity. Effects from pH of measurement solutions were less important (Fig. 2). These observations stay in line with previously observed broad pH-range of ALP activity towards MFP. This maximal activity determined using fluoride ion

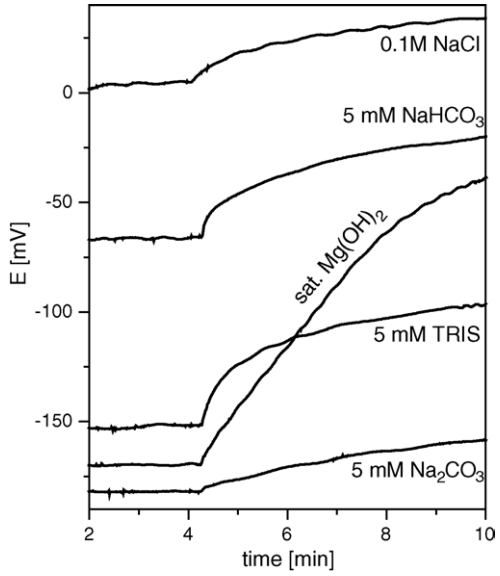

Fig. 2. Changes of potential of pH-glass electrode for MFP (2.0 mM) after addition of ALP (0.08 mg/ml) measured in various working solutions.

selective electrode was found to be in the range of pH 8.0–9.5 [17]. The effects of pH and buffer capacity of assay solution on enzyme activity detection are shown in Fig. 3. The measurements were performed in easy to prepare (saturated and then diluted) magnesium hydroxide and sodium hydroxide solutions at various concentrations of MFP. Contrary to the experiments with weak bases (Fig. 2) these solutions of strong bases do not form buffer systems and therefore the observed pH-shifts are larger. Data shown in Fig. 3 clearly indicate that the observed pH changes are simply connected with titration of working solution by hydrogen ions generated in the course of the enzymatic process (2).

An increase in MFP concentration caused an increase in speed of enzyme reaction and therefore faster proton generation and titration. However, at higher MFP concentrations the pH-changes were smaller (Fig. 3). Moreover, in all the experiments independently of buffer capacity of the working solution (as well as in solutions without buffer) the observed decrease in pH was limited to the value around 7 (Figs. 1–3). This result is surprising because the products of the enzymatic reaction (2) are quite acidic. Taking into account only protolytic equilibria associated with enzymatically generated hydrofluoric acid (or more precisely $H_2PO_4^-$ ions), the expected final pH could be potentially decreased to pH value around 4.5. The observed pH-shifts are significantly smaller and several reasons for such behavior could be considered. The inhibition of the enzyme by excess of the substrate should not be the explanation, because ALP inhibition by MFP was not observed in the course of experiments with alternative detector [17]. The effect of pH on the enzyme activity seems to be more significant, although previous investigations indicated that at pH of 6, ALP still exhibits nearly 20% of its maximal activity towards MFP [17]. The most important source of the pH-shift limitation seems to be connected with protolytic properties of MFP or products of its hydrolysis. In the course of the enzymatic generation of hydrogen ions the substrate could form $FPO_3^{2-}/HFPO_3^-$ buffer. However, the pK_a of this buffer system evaluated by simple titration of MFP with HCl is around 4.5 and this value stays in agreement with data found in the literature [34]. This means, that the buffering by the substrate is not the reason of the observed limitation, although for higher MFP concentrations smaller changes of pH were observed (Fig. 3). An alternative explanation is that the main reason of the limitation is connected with protolytic properties of products of the enzyme reaction. Indeed, phosphate ions generated in the course of the enzymatic reaction also form $H_2PO_4^-/HPO_4^{2-}$ buffer system with $pK_a = 6.9$ and this value fits to the pH value of the limitation. Concluding, the signals (pH changes) obtained in the reported ALP assay are limited to the range of three units of pH (from around 10

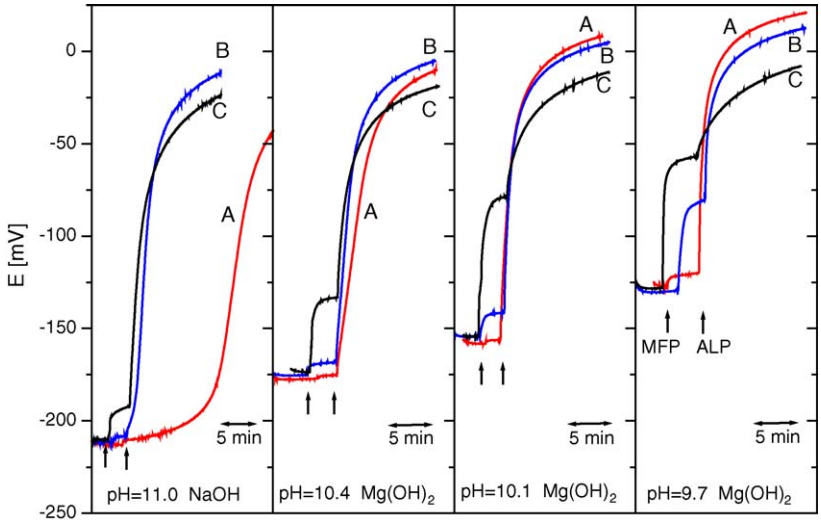

Fig. 3. Effect of base concentration on pH-shift caused by MFP/ALP system. MFP concentrations: 2 mM (A), 10 mM (B) and 50 mM (C). ALP concentration, 0.08 mg/ml.

to 7) and the shape of the observed response are defined by protolytic equilibria in the test solution.

Several divalent cations, especially magnesium and zinc ions are reported in the literature as ALP activators [33]. In case of experiments with MFP, no effect from these cations was observed. The pH-shifts measured in 0.1 M acetate salts of tested cations (Na, K, Ca, Mg) were nearly the same (Fig. 4). Also, the addition of $MgCl_2$ to the test solutions used in experiments shown in Fig. 2 did not change the responses. In zinc acetate solution shift of pH was smaller due to lower pH of the solution (Fig. 4). These results stay in line with previously reported observations that MFP/ALP system is not influenced by divalent cations [17]. It is worth noticing, that in the presence of calcium and magnesium ions smaller and irreproducible pH-shifts were measured for nearly all remaining tested ALP substrates. Moreover, sometimes in the course of these experiments the clouding of the reaction solution was observed. It seems to be possible that HPO_4^{2-} ions generated in the course of reaction (1) react with the cations forming complexes and/or precipitates and these following non-enzymatic processes lead to additional proton generation. This explanation fits to the results reported by Danzer and Schwedt [28], who observed pH-response for ALP assayed with 1GLP as a substrate in the presence of calcium and magnesium ions (used as potential ALP activators). In the absence of these cations pH-shift was not observed (Fig. 1), although ALP exhibits comparable catalytic activity towards all tested substrates [33], including MFP [16,17]. The reported experiments confirm again that MFP is a specific substrate for pH-metric detection of ALP activity.

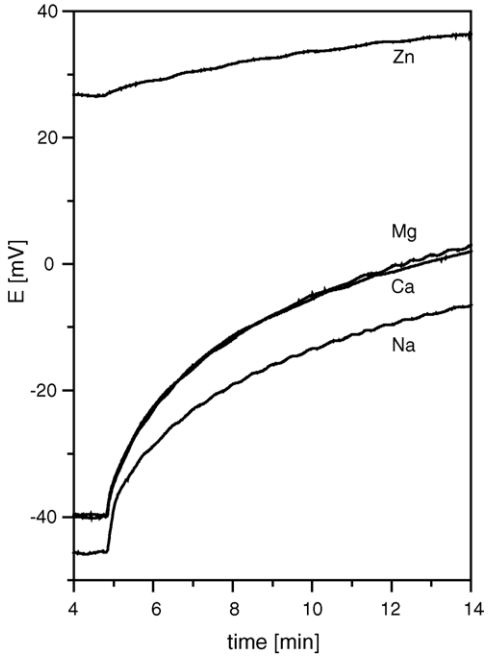

Fig. 4. Effect of cations on the response of MFP/ALP system. Measurements performed in 0.1 M solutions of respective acetate salts containing 2.0 mM MFP and 0.08 mg/ml ALP.

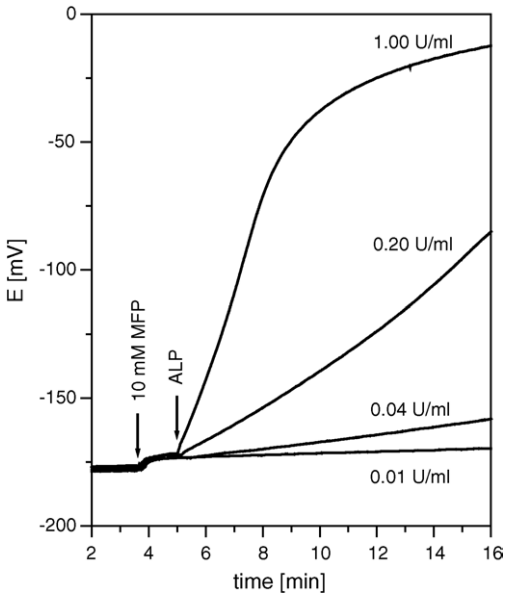

Fig. 5. pH-metric ALP activity assay. Measurements performed in saturated $Mg(OH)_2$ using pH-glass electrode.

The pH-metric detection of ALP activity is shown in Fig. 5. Under given conditions, the changes of the electrode potential are proportional to the enzyme activity within the range of near two orders of magnitude and the sensitivity of this kinetic assay is linear (activity (U/ml) = $0.03(\pm0.004)\Delta E/\Delta t - 0.005(\pm0.007)$, S.D. = 0.007 U/ml, $r = 0.9996$). The detection of 1 mg/l of ALP (equivalent of 0.024 U/ml ALP activity defined using recommended spectrophotometric assay with NPP as a substrate performed under optimized conditions) is possible within a few minutes. This value is lower than physiological blood ALP activity. Unfortunately, the measurements are strongly influenced by the buffering properties of samples. Primary tests with human serum (data not shown) evidently showed that the influences caused quantitative determinations of ALP activity at physiological levels impossible. Only samples with significantly elevated ALP levels could have been recognized. This cross-sensitivity to the buffer capacity of samples is the main disadvantage of the reported assay. More accurate results of serum ALP determination using MFP as a substrate can be obtained with fluoride ion selective electrode [16,17].

3.2. Prototype of pH-ALP-based biosensor

The reported phenomenon (pH changes caused by ALP-catalyzed hydrolysis of MFP) seems to be useful as a platform for the development of a new class of biosensors. pH-sensors sensitized with ALP layer should respond to MFP. On the other hand, at constant concentration of MFP the signal of ALP-modified pH-sensor should be a function of the activity of the immobilized enzyme. A source of the analytical signal is the acidification of the microenvironment intimately at the

surface of the pH-sensor (not in the bulk solution) according to the reaction (2). The experiments reported in this section confirm that transfer of this sensing scheme to the integrated biosensor format is possible.

The prototype of pH-ALP-based biosensor has been prepared using polymeric membrane hydrogen ion selective electrode as internal pH-sensor. ALP has been chemically immobilized in the form of monomolecular enzyme layer. The covalent binding was possible, using simple single-step carbodiimide method because carboxylic groups present on the surface of the electrode membrane are able to form amide bonds with amine groups of the immobilized protein. This method of biosensor preparation has been previously used for immobilization of urease, β-lactamase, arginase, creatininase as well as antibodies at the surfaces of several ion-selective electrodes [32]. Typical calibration of the developed biosensor is shown in Fig. 6. The recorded response is the evidence that pH-metric detection of ALP activity immobilized at the sensor surface is possible. It is worth noticing that contrary to experiments with dissolved ALP, the changes of analytical signal of the biosensor were not associated with the bulk acidification (in the course of the calibrations pH of working solution was controlled and found to be constant) and were not continuous (steady-state signals, common for enzyme electrodes [35], were observed). The response time is shorter than 2 min. No sensitivity of the biosensor towards other ALP substrates (given in the Section 2) confirms that MFP is selectively detected by the developed enzyme electrode. The response of the biosensor is influenced by pH and concentration of a buffer used for calibration. An increase in buffer capacity of the test solution caused a decrease in the sensitivity of the biosensor. This dumping effect is common for all kinds of pH-based enzyme sensors and well-described by their theory [35]. Analytical applications of the presented biosensor as MFP sensor are rather limited. ALP catalyzed hydrolysis of MFP was used in systems for analysis of dental products [36,37]. The determination of MFP in extracts from toothpastes using the biosensor seems to be also possible, until buffer capacity of the extracts would be strictly controlled.

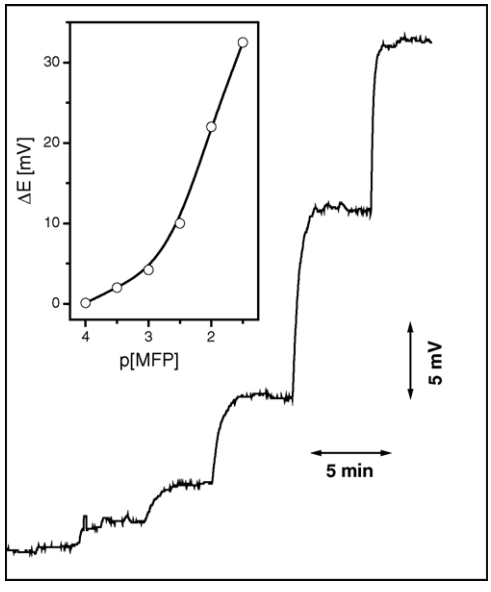

Fig. 6. Calibration of MFP biosensor. Measurement performed in 10 mM Tris buffer (pH 9.0). Corresponding calibration graph of the biosensor is shown in the inset.

Many examples of more advanced biosensing systems and biosensors based on detection of ALP activity are cited in the Introduction. These are immunosensors and genosensors as well as systems for determination of ALP inhibitors and activators. The application of the biosensing scheme reported in this paper for the development of analytical biodevices seems to be much more attractive then MFP detection. It should be stressed that such biosensors could be free from pseudointerferences caused by pH and buffer capacity of samples (main problem in case of substrate detection), because in such cases the step of contact with a sample and the step of activity detection can be separated. In consequence, under particular, well-defined conditions (fixed substrate concentration, pH and concentration of the test buffer) the signal generated by the biosensor should be a function of enzyme activity only. The changes in the enzyme activity and therefore in the signal generated by these biosensors should be dependent on concentration of the indirectly detected analyte (inhibitor, antigen, etc.).

4. Conclusion

Monofluorophosphate has been recognized as a specific substrate for the pH-metric detection of alkaline phosphatase activity. Contrary to many organic phosphates applied in different spectrophotometric and voltamperometric assays this inorganic compound is chemically stable and commercially available. In conclusion, the economical aspects of the developed sensing scheme should be pointed out. Firstly, MFP is very stable, cheap and therefore easily available substrate (for example NPP recommended for optical ALP assays is less stable and hundreds times more expensive reagent). Secondly, the developed assay can be performed using economical potentiometric or optical equipment for pH measurements. Finally, for further development of various pH-ALP-based biosensors several low-cost pH-sensing devices could be adapted including metal-oxide and polymer electrodes, mass-produced thin- and thick-film devices, ISFETs as well as pH-optodes and other optical probes.

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