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Electrophoretically mediated micro-assay of alkaline phosphatase using electrochemical and spectrophotometric detection in capillary electrophoresis

Dan Wu[a], Fred E. Regnier*,[a], Michael C. Linhares[b,1]

[a] *Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA*
[b] *Bioanalytical Systems, West Lafayette, IN 47906, USA*

Abstract

Electrophoretically mediated micro-assays of alkaline phosphatase (ALP) are demonstrated in capillary electrophoretic systems using both electrochemical (ED) and spectrophotometric (UV) detection. In the ED mode, *p*-aminophenylphosphate was used as the substrate and *p*-aminophenol (pAP) was monitored at a carbon fiber electrode held at +180 mV *vs.* Ag/AgCl. Spectrophotometric detection was achieved using the substrate *p*-nitrophenylphosphate, and monitoring the product *p*-nitrophenol (pNP) at 405 nm. The detection limit for pAP by ED was determined to be 100-fold lower than for pNP using UV detection. In the determination of ALP, both methods were found to be linear. The detection limit for ALP using zero potential assays with UV detection was determined to be $1.8 \cdot 10^{-6}$ mg/ml compared to $1.8 \cdot 10^{-7}$ mg/ml using ED.

1. Introduction

Enzyme assays have been widely used to identify enzymes in traditional chromatography and electrophoretic systems and to obtain diagnostic evidence in clinical and forensic analysis. Alkaline phosphatase (ALP) is an important enzyme for clinical and analytical chemistry. The measurement of ALP and its isoforms in blood is a routine analysis performed daily in clinical and toxicological screens. The concentration of ALP is an indicator of bone and liver function. In addition, ALP has been extensively used by biochemists for signal amplification and detection. ALP conjugates have many important applications in immunological assays. Recent studies indicate that these applications could be addressed with a novel new approach to perform enzyme assays in electrophoretic systems [1–4].

Electrophoretically mediated micro-analysis (EMMA) of enzymes [1–3] and substrates [4] in capillary electrophoresis (CE) systems is a new method that provides low detection limits and short analysis times. EMMA is based on the electrophoretic mixing of two substances in an electrophoresis system utilizing differences in the electrophoretic mobilities of the analytes and reagents. The capillary is filled with appropriate substrate solution and upon injection of a zone of enzyme, product will form during the electro-

* Corresponding author.
[1] Present address: Drug Metabolism Department, Pfizer, Groton, CT 06340, USA.

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phoretic mixing of the enzyme and the substrate. Products are separated from the enzyme and substrate and can be detected either on-column or off-column. EMMA assays can be performed in two modes, constant and zero potential. The zero potential mode allows for incubation of the enzyme and substrate to amplify the signal inside the capillary. This type of assay provides very low detection limits of injected enzyme. ALP assays have been conducted in surface-modified and gel-filled capillaries [3]. The high viscosity of the gel matrix allows for longer periods of incubation without significant product diffusion. A detection limit of 10^{-12} *M* ALP has been reported with UV detection using 2 h of incubation.

The mode of detection in EMMA is determined by the characteristics of the product. Several detection techniques have been used to monitor ALP. The most commonly used is spectrophotometric detection of dephosphorylated *p*-nitrophenylphosphate (pNPP) at 405 nm [5–7]. Other detection methods for ALP such as fluorescence using fluoresceinphosphate as the substrate [8], chemiluminescence using dioxetanephosphate as the substrate [9], and amperometry using *p*-aminophenylphosphate (pAPP) have also been reported [10]. On-column UV detection is a commonly used method in CE due to its simplicity and the ease of implementation with commercial instruments. However, due to the small diameter of the capillary, sensitivity with UV detection is limited to 10^{-6} *M* under ideal conditions. Laser-induced fluorescence detection has been shown to be more sensitive than UV detection in CE [11]. Detection of about 5000 leucineaminopeptidase molecules has been achieved by time-resolved fluorescence in CE [4]. However, laser-induced fluorescence detection requires expensive equipment and is difficult to operate. Electrochemical detection (ED) is another method which can provide comparable detection limits and at lower cost.

The focus of this work was to implement ED in CE using pAPP as the substrate for monitoring ALP. pAPP is an ideal substrate for ALP-based enzyme-linked immunosorbent assay (ELISA) [12]. The detection limits for immunological assays have reached the zeptomole level when performed in capillaries [13]. The characteristics of the substrate and product have been fully characterized previously [14]. pAPP is a good substrate for several reasons. The product oxidizes at carbon electrodes at potentials of less than +200 mV, where the substrate requires +500 mV. This large difference, and low absolute potential needed for detection provides great selectivity and little interferences. To use this type of substrate requires on-column ED.

One of the first electrochemical detectors for CE was developed by Wallingford and Ewing in 1987 [15,16]. The primary challenge in the development of an electrochemical detector for CE is isolation of the high voltage applied across the capillary from the detection system. Current produced by the potential across the capillary is usually six orders of magnitude higher than the current obtained at an amperometric detector. This has caused grave consequences for the detector electronics. In the first application of ED, voltage separation was achieved by using an electrical junction covered with a conductive porous glass joint made at near the cathodic end of the capillary [17]. This porous joint permits the flow of ions but not the bulk solution. Electroosmotic flow generated in the separation capillary functions as an electroosmotic pump transporting the sample zone and buffer solution across the junction to the detection capillary. Several similar approaches have been utilized including a Nafion-coated junction [18], and simply a pure fracture of the capillary [19]. These approaches enable the insertion of a carbon fiber electrode into the end of the capillary, and allow for ED. However, in all the previous studies it was necessary to position the electrode in the capillary with a three-way micromanipulator. This type of system requires considerable operator skill and is susceptible to vibrations and daily variations. A novel method for alignment of the electrode in the capillary has recently been described that allows for simplification of the detector cell [20]. The detector cell provides a pre-aligned electrode that allows for

consistent placement of the electrode into the capillary.

In this study ED and spectrophotometric (UV) detection are compared as methods in the assay of ALP. pAPP and pNPP were used as substrates for the assays performed with ED and UV detection, respectively. Linearity, detection limits, and performance are examined. It was found that the detection limit *p*-aminophenol (pAP) with ED was 100-fold lower than for *p*-nitrophenol (pNP) by absorbance. The detection limit for the assay of ALP was found to be at least 10-fold lower with the ED than the UV absorbance method. The discrepancy between detection limits and sensitivity is due to the relative mobilities of the products in relation to the analyte enzyme and the turnover number for the respective substrates.

2. Experimental

2.1. Chemicals

Alkaline phosphatase, 95%, was obtained from CalBiochem (La Jolla, CA, USA). pNPP, pNP and pAP were purchased from Sigma (St. Louis, MO, USA). pAPP was a generous gift from Kate Yu and Dr. W. Heineman of the University of Cincinnati, Cincinnati, OH, USA. Glycine and Tris were purchase from Aldrich (Milwaukee, WI, USA). Buffer solutions were prepared by using double-distilled water that was passed through a 0.45-μm nylon filter. Protein samples were prepared in Tris (25 m*M*)–glycine (125 m*M*) buffer (pH 8.5) and stored below 0°C between analysis.

2.2. Instrumentation

An ABI 270 HT electrophoretic system (Applied Biosystems, Foster City, CA, USA) was used for all experiments. On-column detection was achieved with the internal variable-wavelength UV absorbance detector. A deuterium lamp provided the 405-nm light. For the UV-based enzyme analysis 50 μm I.D. $\times$ 375 μm O.D. fused-silica capillaries with a total length of 45 cm and the detection window at 25 cm were used. A potential of 9 kV was applied during electrophoresis. Injection was accomplished using vacuum injection for 1.5 s.

The CE electrochemical detector is designed to isolate the high voltage before the end of the capillary and allow for the detection of electroactive analytes at an electrode inserted into the end of the capillary. A schematic representation of the detector assembly is shown in Fig. 1. The electrochemical cell is constructed with polyether ether ketone (PEEK) and consists of two compartments. The upper compartment contains the buffer reservoir and electrophoresis cathodic electrode. The lower compartment contains the detection electrode assembly. The two compartments are screwed together, sealing the interface with a rubber septum. The pre-aligned detection electrode is inserted into the end of the capillary when two compartments are screwed together. In order to incorporate the electrochemical detector into the ABI 270 electrophoresis system, a simple modification was made to the instrument (Fig. 2). A hole was drilled into the outlet buffer reservoir through the plastic shelf for a reference electrode. A prototype CE electrochemical cell incorporating a pre-aligned self-contained carbon fiber electrode (Bioanalytical Systems, West Lafayette, IN, USA) was inserted into the hole previously occupied by the electrophoresis cathode. The electrochemical cell utilized a 50

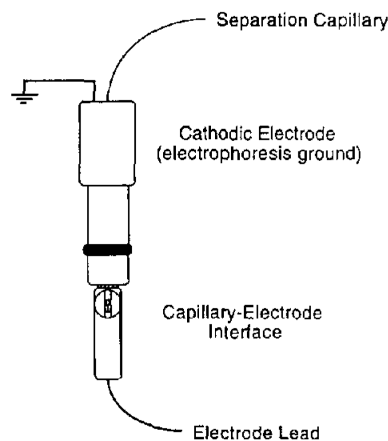

Fig. 1. A schematic illustration of the electrochemical detector assembly.

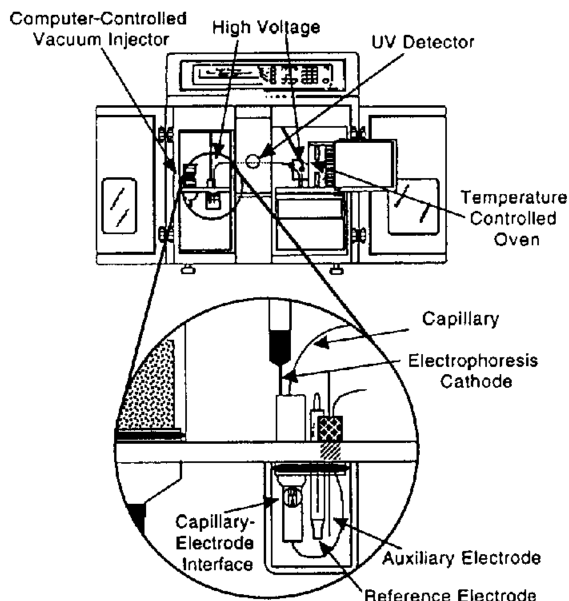

Fig. 2. A modified ABI 270 HT electrophoresis instrument illustrating the electrochemical detector cell placed in the outlet buffer reservoir. A three-electrode system including a Ag/AgCl reference and Pt auxiliary electrodes were used.

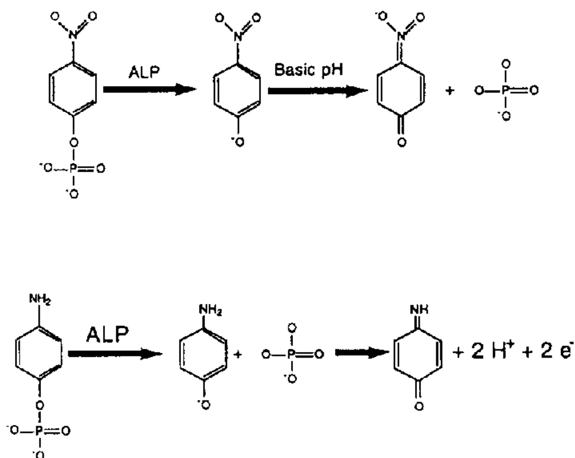

Fig. 3. The reaction schemes for alkaline phosphatase (ALP) with *p*-nitrophenylphosphate and *p*-aminophenylphosphate.

μm I.D. separation capillary 51 cm long, with a distance of 2.5 cm from the electrophoresis cathode to the detector electrode. A three-electrode system incorporating a Ag/AgCl reference electrode (RE-5, Bioanalytical Systems) and Pt auxiliary electrode was used. ED was achieved using a LC-4C amperometric detector (Bioanalytical Systems) with +180 mV applied *vs*. Ag/AgCl.

3. Results and discussion

3.1. Electrochemical assays using pAPP

The use of pAPP as a substrate for the assay of ALP is well documented [5]. The structure of the substrate and subsequent product are illustrated in Fig. 3. Although the substrate and product are both easily oxidized at carbon electrodes, the product exhibits a limiting oxidizing current of approximately + 180 mV *vs*. Ag/AgCl (pH 8.5 Tris–glycine), whereas the substrate requires a potential of + 500 mV. This difference allows for the preferential detection of only the product without interference and low background. However, in electrophoretic systems the ease with which the substrate is oxidized presents a problem.

The electrophoresis system used a platinum electrode as an anode in the inlet buffer compartment which is large compared to the detector electrode and has 9 kV applied to it during electrophoresis. The substrate can rapidly oxidize at this relatively large electrode causing depletion of the substrate. Continuous monitoring of the current in the inlet buffer during electrophoresis is seen in Fig. 4A. After a short period of time, the solution in the vessel changed from a clear solution to a brown color, characteristic of the product pAP. Since electrochemical reactions are a surface phenomenon [21], a simple solution was devised. The electrophoresis electrode was covered with 50 μm thick Nafion tubing. Nafion is a negatively charged polymer membrane, that does not allow negatively charged molecules to pass. Thus, Nafion allows passage of the current, but does not allow the substrate to react with the electrode. Electrophoresis was conducted with a solution similar to that used in Fig. 4A, but using a Nafion-coated anode electrode for the electrophoresis. Fig. 4B illustrates the effectiveness of the Nafion tubing.

The stability of the product pAP and reactivity

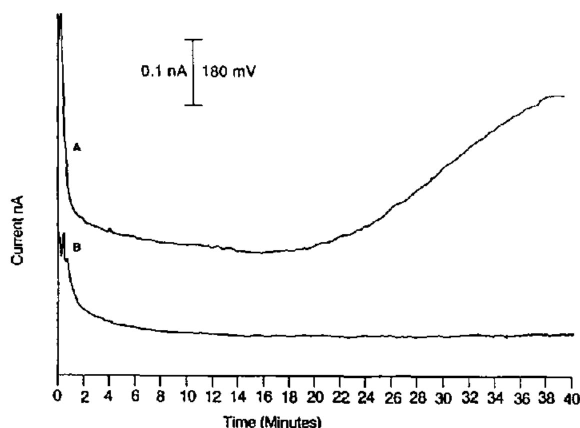

Fig. 4. Continuous monitoring of background currents using electrochemical detection. A: Standard electrophoresis conditions with bare platinum electrode illustrating the increase in back ground due to pAPP oxidation at the electrophoresis electrode. B: Electrophoresis electrode modified with Nafion tubing to eliminate substrate degradation at the electrophoresis electrode. Conditions: 51 cm capillary, 9 kV applied (3 μA), 0.26 mg/ml pAPP in Tris-glycine buffer (pH 8.5). Other conditions given in text.

of ALP with pAPP in different amino alcohol buffer systems of varying pH has been studied previously [5]. Ethanolamine (EA) and 2-methylaminoethanol (EMA) (pH 10) are shown to be the best buffers for the assay of ALP with ED. The product pAP is most stable in these two buffer solutions, allowing for longer incubation times. At pH 10 pAP is least stable in Tris buffer. However, ALP has faster kinetics in Tris buffer system than in EA and EMA buffers.

Attempts to use EA and EMA in this EMMA study failed because of the high currents (40–80 μA) of these two buffer solutions in the CE system. A Tris-glycine buffer of pH 8.5 was more suitable. The current with Tris-glycine is only 4 μA at 200 V/cm, which is ideal for ED. In addition, the low pH of the buffer was better suited for the stability of the product.

Detection limits in EMMA-based enzyme assays are ultimately dependent on the detection limit of the product itself. The detection limit of the pAP product using ED was determined to be $3 \cdot 10^{-5}$ mg/ml ($3.5 \cdot 10^{-7}$ *M*) at a *S/N* of 5, whereas the detection limit of the pNP product using UV detection was $3 \cdot 10^{-3}$ mg/ml ($2.8 \cdot 10^{-5}$ *M*). From the literature, these value agree well with what might be expected for the detection methods in CE [5,14].

3.2. ALP EMMA at constant potential

Constant potential assays were performed with the substrate present in the electrophoresis buffer, injecting enzyme as the analyte and continuously electrophoresing the enzyme through the substrate. Constant potential assays for ALP using both pAPP and pNPP are illustrated in Fig. 5. The difference in the peak shapes are due to the relative mobilities of the two products and the enzyme. The mobility of ALP was determined to be $1.0 \cdot 10^{-3}$ cm^2 V^{-1} s^{-1}, while the mobility of the products were $2.9 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} for pAP and $1.6 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} for pNP. In the constant potential mode enzyme led the product through the capillary. The plateau resulting from pAP is much higher in response and shorter in width (time). This results from the small difference in the mobility of the product and the mobility of the enzyme. The product does not migrate away from the enzyme zone as rapidly as pNP. The difference in mobility of

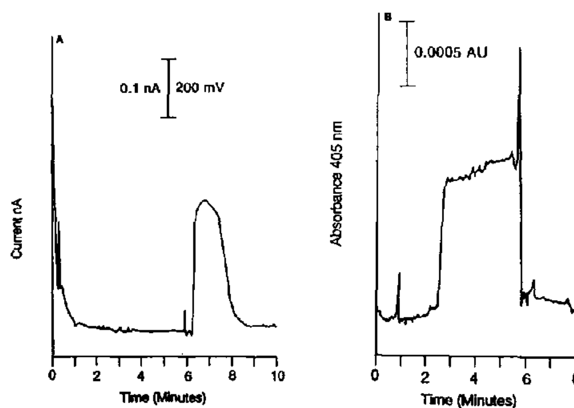

Fig. 5. Constant potential assays for ALP using (A) electrochemical detection and pAPP as the substrate and (B) UV detection with pNPP as the substrate. Conditions: 51 cm capillary, 9 kV applied (3 μA), 0.26 mg/ml pAPP or 0.5 mg/ml pNPP in Tris-glycine buffer (pH 8.5). Other conditions given in text.

pNP and ALP is greater, causing the enzyme to migrate away from the product. This results in a plateau that is much wider and lower in height.

3.3. EMMA of ALP at zero potential

The assay of ALP was also conducted using the zero potential mode of analysis in order to increase the sensitivity. A zero potential incubation time of 5 min was chosen to limit diffusion of the product zone. The zero potential assays for ALP using ED and UV are illustrated in Fig. 6. The peak superimposed on top of the plateau (Fig. 6) is due to the product formed during the incubation time. Calibration curves for both methods of analysis show linear responses and are illustrated in Fig. 7.

The detection limit for the electrochemical

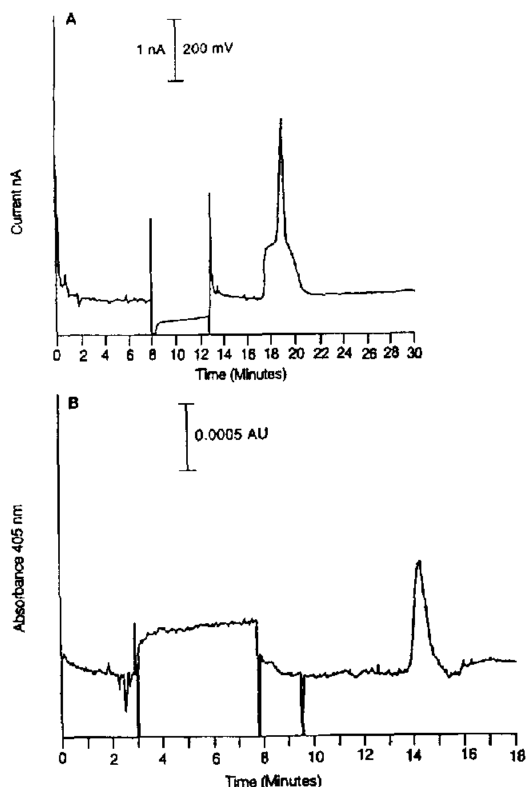

Fig. 6. Zero potential assays for ALP using (A) electrochemical detection and pAPP as the substrate and (B) UV detection with pNPP as the substrate. See Fig. 4 and text for conditions.

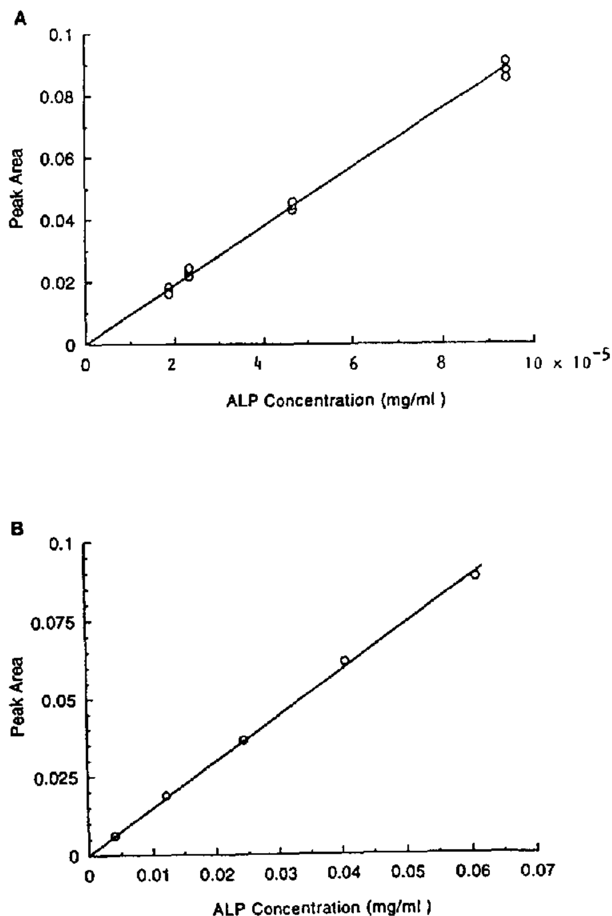

Fig. 7. Linear calibration curves for ALP assays using (A) electrochemical detection and pAPP as the substrate and (B) UV detection with pNPP as the substrate; $n = 3$ for each case.

assay for ALP was determined to be $1.8 \cdot 10^{-7}$ mg/ml ($1.2 \cdot 10^{-12}$ M) at a S/N of 3. The detection limit for UV detection was determined to be $1.8 \cdot 10^{-6}$ mg/ml ($1.2 \cdot 10^{-11}$ M) at a S/N of 3. Fig. 8 illustrates the electropherograms for the assays at the detection limits. From the relative molar response of ED and UV detection with pAP and pNP respectively, it is expected that the detection limit of ALP by ED would be 100-fold lower than by UV. However, this is not the result observed. The turnover number of the enzyme determines how much product can be generated during the zero potential incubation. Because the turnover number for pNPP is higher

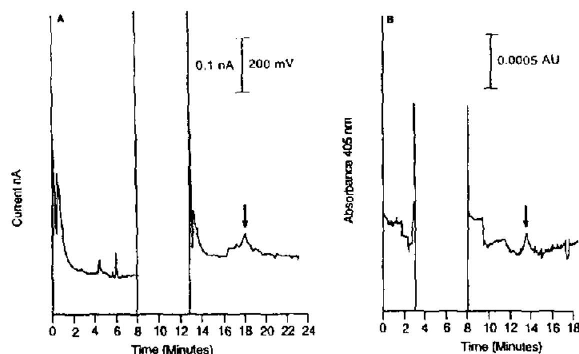

Fig. 8. Electropherograms of the detection limits for ALP using (A) electrochemical detection with pAPP as the substrate and (B) UV detection with pNPP as the substrate. See Fig. 4 and text for conditions.

than that of pAPP, the detection limit with ED is only 10 times lower than with UV detection.

4. Conclusions

The two detection methods presented allow for the rapid analysis of extremely small amounts of enzyme. The electrochemistry-based method provides better detection limits. This is due to the selectivity enhancement of ED at low potentials. The use of a self-contained electrochemical cell simplified the ED approach to make it desirable for use. The potential of the ED detector for performing EMMA assays in CE systems appears promising. However, the simplicity of the UV assay method also has merits. In addition, the electrochemical methods limit the use of $MgCl_2$ and high ionic strength buffers. The UV method allows for greater flexibility.

5. Acknowledgements

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