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# Capillary electrophoretic approach to screen for enzyme inhibitors in natural extracts

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**Abstract**

This paper demonstrates development of electrophoretically mediated micro analysis (EMMA), for screening protein tyrosine phosphatase (PTP) inhibitors in natural extracts. It is demonstrated that capillary electrophoresis (CE) separation of the substrate and the product allows for using the assay in an on-column format to monitor the reaction without typically used fluorogenic substrates. Michaelis-Menten kinetics parameters calculated based on the EMMA results (Km = 1.2–1.5 μM) were in a good agreement (Km = 1.0–1.5 μM) obtained using an off-line CE functional assay (CE FA). EMMA of PTP titrated with different concentrations of ligand demonstrated the peak-shift phenomenon normally seen in affinity capillary electrophoresis. This feature of EMMA gives an indication of the binding affinity of the ligand in addition to its functional activity, providing another dimension in characterization of the protein-inhibitor interaction. It was demonstrated that simultaneous screening of the primary PTP target and a secondary, counter target (PTP-C) using the EMMA format can be used to prioritize hits based on their specificity.


*Keywords:* Mediated micro analysis; Enzymatic assay; Protein tyrosine phosphatase

## 1. Introduction

Development of screening methods to identify new, biologically active compounds in complex mixtures such as natural extracts is a challenging task. Natural extracts offer an extremely high level of molecular diversity, thus providing an essential source of compounds for the discovery of new drugs. Renewed interest in natural extract screening has resulted from the development of new assays that detect low levels of bioactive molecules and modern analytical techniques that enable isolation and characterization of minute amounts of active compounds. Today's primary screening assays (so-called mode of action assays) are also known as molecular-based or target-based drug discovery screens. Some common characteristics of mode of action assays include their speed, selectivity, ease of performance, and ability to quantitate. Once appropriately miniaturized, mode of action assays are relatively inexpensive and offer high throughput capabilities as compared to the animal tests of few decades ago.

There are two main approaches taken in molecular-based drug discovery. First, the binding assay approach is based on the analysis of a complex formed between a target protein and a test compound. Second, the functional assay approach is based on the functional activity analysis of a target protein in the presence of a test compound. Confirmation of the hit by both approaches makes the result more meaningful. Affinity capillary electrophoresis (ACE) has proven to be a sensitive and selective method for investigating protein–ligand binding interactions [1–3]. ACE has been previously used to detect and analyze known binding compounds, and the great potential of ACE as a screening technology for natural product drug discovery has been demonstrated [4].

Recent trends have combined enzymatic assays with capillary electrophoresis using regular capillary electrophoresis (CE) instrument or microfluidics devices [5–7]. Such an approach has several advantages resulting from the unique combination of features of CE including the requirement for small quantities of both protein and sample, high resolution of CE, automation and multiplexing capabilities.

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CE enzymatic assays can be categorized into three formats depending on the step when the enzymatic reaction takes place: pre-column, on-column and post-column. In the first, pre-column CE-enzymatic assay format, the substrate is mixed with enzyme and the mixture is incubated for a specified amount of time. The products of the enzymatic reaction are then injected into the CE column, electrophoretically separated, and quantified. Given that most CE separations are fast, this approach provides a convenient way to obtain time-course information using minimal sample volumes and sample preparation time. In this case the separation and analysis involve just two components: the substrate and the product. Quantitation of the substrate conversion by enzyme based on the relative change of the peak areas makes this approach very simple and rugged. Several papers have compared the CE assay with conventional assay formats and excellent agreement has been demonstrated [8]. When high sensitivity is needed this method can be used in combination with laser-induced fluorescence (LIF) detection. This can be achieved by fluorescently labeling the substrate, or by using a fluorogenicity effect after conversion of the substrate to the product.

In the second type of CE-enzymatic assays all the steps of the assay take place within the capillary (including mixing, separation and detection). An on-line, homogeneous version of this method, electrophoretically mediated micro analysis (EMMA), was introduced by Bao and Regnier [9]. EMMA consists of three steps. During the first (pre-reaction step) the zones of the enzyme (analyte) and the substrate are brought into contact electrophoretically inside the capillary. During the second step the enzymatic reaction is allowed to occur in the absence of an electric field. During the third (post-reaction) step the product is electrophoretically transported to a detector. One of the greatest advantages of EMMA is the fact that it provides separation of sample components prior to the chemical reaction with the substrate. In addition, the reaction products can be separated as well. Moreover, multiple simultaneous analyses of several analytes can be performed using this method [10]. Advances and details of the EMMA technique have been reviewed [11].

The third type of CE enzymatic assays is the post-column assay. In this format selected sample components are separated by CE, and collected fractions are further tested in a post-column enzymatic assay.

The present paper demonstrates the development and performance of an on-column CE assay format EMMA for screening for protein tyrosine phosphatase (PTP) inhibitors in natural extracts.

## 2. Experimental

### 2.1. Chemicals and reagents

All chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. The N-terminal, fluorescein-labeled substrate, Flc GQQPIL[pTyr]-EDAD, where pTyr is phosphotyrosine, was synthesized by Sigma-Genosys (The Woodlands, TX) and purified by RP-HPLC.

Enzymes: protein tyrosine phosphatases were obtained from Calbiochem-Novabiochem (San Diego, CA). Assay buffers and CE running buffers were prepared using HPLC-grade water obtained from J.T. Baker (Phillipsburg, NJ). Natural extracts were prepared from methanol extractions of microbial cultures.

### 2.2. Instrumentation

Capillary electrophoresis experiments were carried out using P/ACE MDQ capillary electrophoresis system. An eCAP neutral capillary 50 μm i.d. purchased from Beckman Instruments (Fullerton, CA) was used for CE separations. The instrument was equipped with a standard LIF detector (488/520 nm emission/excitation wavelength).

### 2.3. Separation conditions

Sample injections were performed by pressure at 0.5 psi during 5 s. The effective length of the capillary was 10 cm for the off-line enzymatic reaction, while for the EMMA experiments it was 20 cm. CE was run from the negative to the positive electrode at 500 V/cm.

## 3. Results and discussion

### 3.1. Development of off-column CE assay

Protein tyrosine phosphatase was the enzyme chosen for demonstrating the development of an on-column capillary electrophoresis assay. The dephosphorylation activity of this enzyme reduces the net charge of a specific peptide substrate molecule, resulting in a change of the substrate's charge-to-mass ratio. As a result, the substrate and the product have significantly different electrophoretic mobilities, allowing for their effective separation by CE. Based on this change, a general approach for the enzymatic activity assay using capillary electrophoresis was developed.

Developing a screening assay for natural extracts involves optimization of specific steps. Such optimization requires:

1. A low enzyme concentration in order to detect low concentrations of inhibitor (a common situation for working with natural extracts). For example, the concentration of a bioactive compound in a concentrated extract may be in the nanomolar range. This requires a similar level of enzyme concentration. Of course, ideally, the inhibitor concentration should be higher then the concentration of the enzyme. In this case, most of the enzyme molecules will be bound by the inhibitor, resulting in the complete inhibition of the enzyme.

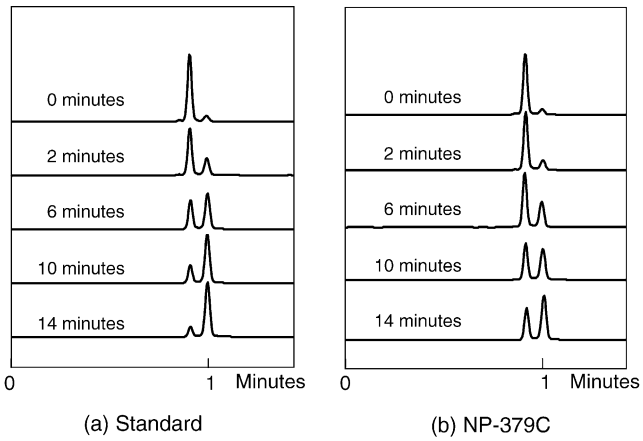


Fig. 1. Time course of the PTP reaction: (a) under standard conditions and (b) in presence of a natural extract (NP-379C) containing an inhibitor. The first peak of the electrophoregram is the fluorescein-labeled substrate (phosphorylated). The second peak is the product (de-phosphorylated substrate). Incubation time (0–14 min) is indicated above the electropherograms. Conditions: the assay buffer: 1.0 μM substrate, 100 pM PTP in 50 mM Hepes, pH 7.2, 4 mg/ml BSA, 5 mM DTT, 0.1% CHAPS. The CE running buffer: 150 mM Bis–Tris and pH 7.5 adjusted with acetic acid.

2. The substrate concentration needs to be low enough to avoid displacing competitive inhibitors. Linearity of the enzyme activity versus the enzyme concentration has to be verified at the chosen substrate concentrations and reaction times. The typical range of Michaelis-Menten constant (Km) is between ∼1 M and ∼1 mM. We wanted to use substrate concentrations at the lower end of this range to avoid out-competing potential inhibitors. However, this level of concentration is poorly acceptable for conventional CE using UV absorbance detection. However, it is well acceptable for laser-induced fluorescence detection, which has picomolar levels of sensitivity. For this reason a fluorescent residue was attached to the N-terminal end of the peptide substrate and LIF detection was used.

Prior to developing an EMMA-based assay, we needed to confirm that the substrate and product conversion can be monitored by CE. Thus, a pre-column assay format was developed. In this case, the PTP enzyme and substrate are incubated at the desired concentrations and the reaction mixture is then injected into a capillary for analysis. A typical CE analysis of the enzymatic reaction is demonstrated in Fig. 1a and b.

Several consecutive injections allowed us to evaluate the kinetics of dephosphorylation. Fig. 1b demonstrates the inhibition of the enzymatic reaction in the presence of a natural extract containing an inhibitor. The presence of BSA and CHAPS in the assay buffer dramatically reduced the problem associated with protein adsorption in the reaction vessel. This well-known modification allowed us to perform the assay continuously for 3–4 h without observing any change in enzyme activity. As a side observation, higher levels of DTT in the assay buffer reduced the nonspecific hit rate, possibly originating from oxidizing agents in the extract. This effect

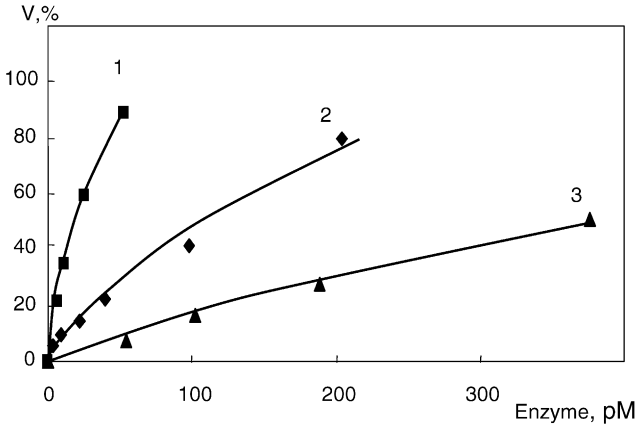


Fig. 2. Substrate conversion as a function of enzyme concentration. (1) PTP, (2) PTP-C1 and (3) PTP-C2. Conditions: the assay buffer: 1.0 μM substrate, in 50 mM Hepes, pH 7.2, 4 mg/ml BSA, 5 mM DTT and 0.1% CHAPS.

may be a result of the presence of a readily-oxidizable cysteine at the binding site of the PTP protein. Activity of the enzyme was a linear function of the protein concentration in the 20 pM to 20 nM range. The calculated Km for the uninhibited enzymatic reaction was ∼1.5 μM.

This pre-column functional CE assay was used to screen a small collection of actinomycete and fungal extracts. At an extract screening concentration of approximately 1 mg/ml, our hit rate was a reasonable 2%. Interestingly, this high test concentration of extract is poorly acceptable for regular homogeneous assays due to interferences caused by the matrix effect, fluorescence quenching, absorbance, scattering and endogenous background fluorescence. The combination of the separation step and LIF detection, and low target concentrations allows us to work with concentrated extract samples. Thus, this assay format may allow us to discover previously undetectable inhibitors that are present at low concentrations in the extract.

In spite of the great reduction of the non-specific hit rate in the described format, it is still necessary to reduce the number of hits selected for fractionation and isolation. In order to further prioritize hits for fractionation it is desirable to check the specificity of the identified hits. One approach is to analyze the hit activity against counter-enzymes belonging to the same group. PTPs belong to a large family of phosphatase enzymes having a conserved active site. Two other phosphatases were chosen for counter-screening of any hits: PTP-C1 and PTP-C2. These phosphatases, being housekeeping enzymes required for normal cellular function, should not be inhibited. Thus, it is desirable to find hits that are active only against PTP and inactive against PTP-C1 and PTP-C2. The functional assays for the counter targets were developed and optimized using the same fluorescent peptide substrate as with PTP. Fig. 2 shows activities of the three enzymes at different concentrations analyzed using pre-column CE FA.

All three proteases were used with 1 μM concentration of substrate and the following concentrations of the enzymes:

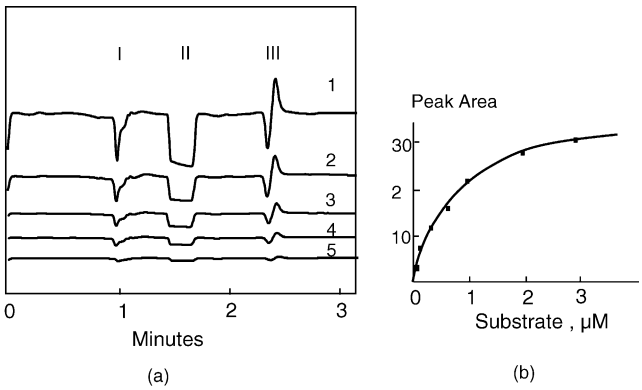


Fig. 3. EMMA of PTP. (a) Typical electropherograms showing the results of the enzymatic reaction at different concentrations of the substrate in the running buffer. (b) Michaelis-Menten plot. Conditions: 1.5 nM PTP, injection 5 s. CE buffer: substrate, 50 mM Hepes, pH 7.2, 4 mg/ml BSA, 5 mM DTT, 0.1% CHAPS, 4 mM EDTA and no test extract. The substrate concentrations used were: 1 μM (1), 0.5 μM (2), 0.250 μM (3), 0.125 μM (4) and 0.06 μM (5).

50 pM PTP, 0.5 nM PTP-C1 and 2 nM for PTP-C2. The concentrations of the enzymes have been chosen based on their activity to make the reactions proceed in a similar time frame. The chosen enzyme concentrations fall into an acceptable range and provide enough sensitivity for detecting low (few nM) concentrations of an inhibitor. In subsequent screening of the primary hits against these counter targets, the hit rate dropped another ∼90%. Thus, analysis of the specificity of the active extract allowed us to successfully prioritize the important, PTP-specific extracts for entering a fractionation and isolation program.

### 3.2. Development of an EMMA-based assay

We were then ready to develop an EMMA-based assay in order to make the screening more convenient and enable analysis of the enzyme and a counter-enzyme during the same run. In the first case we tested only the PTP enzyme. The test extract and the substrate were dissolved in the CE running buffer and the capillary was filled with this solution. The enzyme (or later the enzyme mixture) was injected into the capillary and run for ∼1.5 min to allow the enzyme enough time to equilibrate with the test extract (later this step also separates the enzyme and counter-enzyme due to their different electrophoretic mobilities). The voltage was then switched off for a brief 0.25 min incubation period. After incubation, the voltage was turned back on and the reaction products migrated to the detection window of the capillary. Fig. 3 shows the EMMA of the single enzyme—PTP at different concentrations of the substrate. Part I corresponds to the deficiency peak due to the injection plug, Part II corresponds to the deficiency profile due to the brief incubation time without voltage. The combination of the deficiency and the normal peaks in Part III of the electropherograms represents the substrate and the product, respectively, of the enzymatic reaction and can be used to characterize the kinetics of the reaction. The

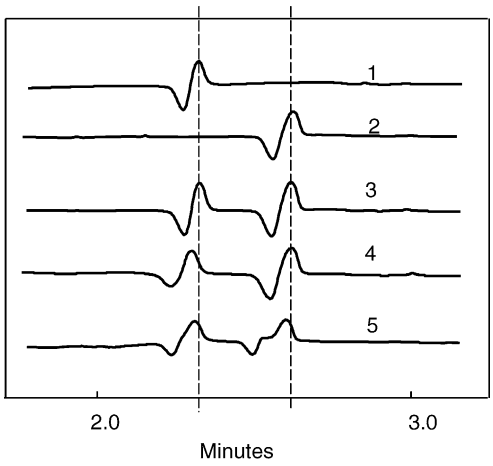


Fig. 4. EMMA of PTP and PTP-C1, only part III of electropherograms are shown. Electropherograms 1 and 2 represent the EMMA results for single enzyme analyses: PTP (1) and PTP-C1 (2), respectively. Electropherogram 3 demonstrates EMMA analysis of the mixture of these enzymes with no test inhibitor present. Electropherogram 4 represents EMMA analysis of the mixture of the enzymes in presence of a test inhibitor P10 at 5 nM, P10 is a known specific inhibitor of PTP. Electropherogram 5 illustrates EMMA analysis of the mixture of the enzymes in presence of an active extract NP-118c in the CE buffer tested at 1 mg/ml. Conditions: 1.5 nM PTP, 20 nM PTP-C1. CE buffer: 1.0 μM substrate, 50 mM Hepes, pH 7.2, 4 mg/ml BSA, 5 mM DTT, 0.1% CHAPS and 4 mM EDTA.

deficiency peak is a result of the substrate being converted to product during the zero voltage incubation step. Once the voltage is reapplied, the substrate moves faster than the product, leaving a deficiency peak from the former substrate and a normal peak from the increased product. CE separation of the substrate and the product allows for monitoring the reaction without the traditional fluorogenic substrate. Michaelis-Menten kinetics parameters calculated based on EMMA results are in a good agreement with our calculations based on the CE FA data. The results are Km = 1.2–1.5 μM (CE FA) versus Km = 1.0–1.5 μM (EMMA). All experiments used for evaluation of Km were carried out in triplicate.

The next experiment demonstrates EMMA analysis of the enzyme mixture PTP and the PTP-C1 counter target. This format is desirable since in this case specificity of a hit can be determined in the same run. Fig. 4 shows only Part III of the EMMA run.

The first two electropherograms in Fig. 4 represent EMMA results of a single enzyme assay PTP (1) and PTP-C1 (2) using the same concentration of the substrate. The third electropherogram demonstrates EMMA analysis of the mixture of these two enzymes. One can see two zones of the separated products of the two enzymatic processes. The enzymes have been electrophoretically separated during the first step of the EMMA. During the reaction step both enzymes staying in different parts of the capillary and the products are thus visualized as two different zones after electrophoresis during the third step.

The last two curves in Fig. 4 show the EMMA results for the mixture of enzymes analyzed in the presence of a

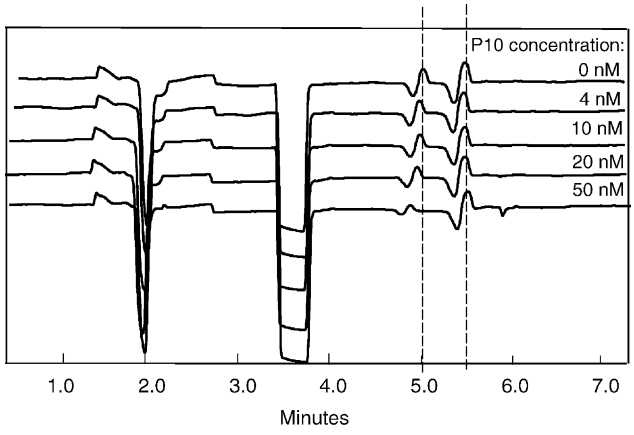


Fig. 5. EMMA of the mixture of PTP and PTP-C1 in presence of P10 ligand (a specific inhibitor of PTP). Conditions: 1.5 nM PTP, 20 nM PTP-C1, injection 5 s. CE buffer: 1.0 μM substrate, 50 mM Hepes, pH 7.2, 4 mg/ml BSA, 5 mM DTT, 0.1% CHAPS and 4 mM EDTA. P10 concentration: 0 nM (1); 2 nM (2); 10 nM (3); 20 nM (4) and 50 nM (5). CE conditions were chosen to increase the resolution of enzymes during EMMA Part I in comparison with the result shown in Fig. 3.

specific inhibitor for the PTP (4) and a nonspecific active natural extract NP-118c at 1 mg/ml test concentration (5). These curves demonstrate how the EMMA analysis of the protein mixture target and counter target can be used to prioritize hits based on their specificity. The known ligand, specific for the PTP, affects only the first peak corresponding to the PTP. As expected, the second peak stays unchanged. On the other hand, the active natural extract changed both peaks and is most likely a nonspecific hit. Of course, in a complex natural extract there is always the possibility of multiple active components, so one may want to perform an initial fractionation of the extract and re-test the fractions to make sure the activity is arising from a single component.

There is an additional piece of information that can be obtained from this analysis. The position and size of the first peak of curve 4 in Fig. 4 is defined by the activity and electrophoretic mobility of the PTP in the presence of an inhibitor. The change in migration time of the first peak is due to the mobility shift resulting from the binding interaction between PTP and P10 during electrophoresis. Fig. 5 shows EMMA of PTP titrated with different concentrations of P10 ligand and demonstrates the peak-shift phenomenon as normally seen with Affinity Capillary Electrophoresis (ACE). This feature of EMMA gives us an indication of the binding affinity of the ligand in addition to its functional activity, providing another dimension in characterization of protein–inhibitor interaction. Thus, primary assay and a secondary conformational assay, as well as selectivity testing, can be all done in the same run.

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