Electrophoretically Mediated Microanalysis of Leucine Aminopeptidase in Complex Matrices Using Time-Resolved Laser-Induced Fluorescence Detection

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Leucine aminopeptidase, a clinically significant enzyme, was assayed in complex biological samples using a new technique termed electrophoretically mediated microanalysis. The assay was performed in capillary electrophoresis columns using time-resolved laser-induced fluorescence detection. Human serum, human urine, and *Escherichia coli* supernatant samples were assayed using this method. Results for serum and urine were within the ranges of expected values found in the literature. A low concentration of 6×10^{-13} M enzyme in buffer was detected using this method. A detection limit (3σ) of 400 enzyme molecules in buffer was determined.

INTRODUCTION

A new technique referred to as electrophoretically mediated microanalysis (EMMA) has recently been described that allows enzyme assays to be carried out in capillary electrophoresis columns.[1,2] Through differences in the electrophoretic mobility of enzymes, substrates, and products, it is possible to electrophoretically mix reagents and assay 10^{-19} mol of an enzyme based on absorbance detection of enzymatically derived products.[2] Unfortunately, other absorbing species in crude biological samples often interfere with absorbance measurements. This paper examines the utility of time-resolved laser-induced fluorescence (TRLIF) detection to circumvent this problem. An attractive feature of TRLIF is its lower limit of detection (LLD) of 10^{-9} M, which is 1000 times lower than that of typical absorbance detectors used in capillary zone electrophoresis (CZE).[3,4] In addition, the time-resolution capability of TRLIF minimizes interference from molecular species in the sample not involved in the enzyme assay.

The enzyme chosen for study was leucine aminopeptidase (LAP), a clinically significant enzyme found in the tissues and body fluids of all organisms.[5] Elevated levels of LAP in serum signal diseases of the liver, bile duct, and pancreas.[6] LAP was assayed in human serum, human urine, and *Escherichia coli* supernatant by monitoring the formation of a fluorescent product, 4-methoxy-β-naphthylamine (4-MBNA). This enzyme cleaves the nonfluorescent leucine amide derivative, L-leucine-4-MBNA, to produce 4-MBNA, which has a 26-ns lifetime. The fluorescence of this product is easily distinguished from fluorescent species with shorter lifetimes.

The EMMA technique with TRLIF detection is significantly faster and consumes far less sample than conventional enzyme assays. For comparison, a commercial diagnostic kit made by Sigma[7] is used as a specific example of a conventional LAP assay. The Sigma method is based on the colorimetric detection of a blue azo dye produced in a two-step process from enzymatically derived β-naphthylamine. Samples are diluted and incubated with substrate at 37 °C for 1 h. Even taking into account the dilution factors of the Sigma method, the EMMA technique consumes 2000–5000-fold less sample. In addition, the incubation time has been reduced from 1 h to 4–10 min.

EXPERIMENTAL SECTION

Chemicals. L-Leucine 4-methoxy-β-naphthylamide and 4-methoxy-β-naphthylamine were purchased from Sigma. The buffer used was pH 7.0, 10 mM sodium phosphate. Substrate stock solution was prepared to 10^{-3} M in methanol. The enzyme assay substrate solution was prepared to 10^{-5} M by diluting the substrate stock solution in buffer.

Samples. Human serum was purchased from Sigma. The *E. coli* supernatant (10000*g*) from *E. coli* strain BL21 homogenate was donated by Cheng Chang Wang (Purdue University). The serum and *E. coli* supernatant were diluted 1:10 in buffer. The urine sample was collected from a healthy male donor. The dialyzed urine sample was prepared by placing the sample in a Microcon microconcentrator (molecular mass cutoff 10 kDa) and applying high-speed centrifugation for 3 h. Buffer was replaced three times during the centrifugation. Buffer was used to restore the sample to its original volume after centrifugation.

LAP Enzyme. Leucine aminopeptidase from porcine kidney (microsomal) was purchased from Sigma. Enzyme solutions were prepared by dilution in buffer. A molecular mass of 326 kDa was used for all concentration calculations.[5] The activity of the enzyme in solution was assayed using a published spectroscopic method.[8] One micromole of LAP was found to have 3.7×10^8 international units (IU) activity where 1 IU as defined by this method corresponds to the release of 1 μmol of *p*-nitroaniline/min at 30 °C, pH 7.2.

Electrophoretic Methods with Low Enzyme Concentration. The capillary and the electrophoresis buffer reservoir were filled with the 10^{-5} M substrate solution. Injections were made by placing the high-voltage (injection) end of the capillary in the sample vial and raising the sample vial 10 cm above the grounded end of the capillary for 10 s. The injection end of the capillary was then placed in a buffer reservoir, and the buffer reservoir was raised briefly to inject a small amount of buffer. The capillary was then returned to the substrate reservoir, and the electrophoresis potential (250 V/cm) was applied for 3 min. The electrophoresis power was cutoff for 10 min to allow for enzyme incubation.

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Electrophoretic Method for High Enzyme Concentration (Buffer Spacer Technique). The capillary and the electrophoresis buffer reservoir were filled with buffer. Hydrodynamic injections were made by the same procedure as described previously. The injection end of the capillary was returned to the buffer reservoir, and the electrophoresis potential was applied for 20 s to inject a "spacer". The electrophoresis buffer was replaced by the substrate solution, and the potential was applied for 2 min. The enzyme was allowed to incubate for 4 min.

Following incubation, the electrophoresis power was restored and data collection was initiated. Data were collected for 6–10 min until all peaks had eluted. The capillary was rinsed with 1 M NaOH after every serum injection. The substrate and buffer reservoirs were filled with new aliquots of solution for every injection.

Instrumentation. The CZE system with TRLIF detection has been described earlier.[3] The instrument is based on a prototype diode-pumped, frequency-tripled, Q-switched neodymium-doped yttrium lithium fluoride laser. A 418-nm cut-on filter and 450-nm bandpass interference filter were used to select the emission wavelengths. An uncoated capillary of 60 cm in length and 75 μm i.d. × 360 μm o.d. was used. The separation length was 50 cm.

Dual collection windows were used to generate two electropherograms from one injection. A 200-point fluorescence decay waveform was transferred to the data collection program every second. Two independently adjustable integration windows were used to sum the values from the desired portion of the decay waveform. These two sums were plotted vs elapsed time to generate two electropherograms. The delay value refers to the time delay in nanoseconds from the peak minimum of the fluorescence decay. The window value refers to the width of the integration window beginning at the specified delay.

RESULTS AND DISCUSSION

Enzyme Quantitation with an Internal Standard. Internal standards are especially useful for quantitative work in CZE with TRLIF detection. Internal standards compensate for the injection reproducibility difficulties commonly associated with CZE. Problems with laser power drift or laser alignment drift are also compensated by using an internal standard. Unfortunately, when TRLIF detection is used, it is difficult to find standards with acceptable absorbance, emission, and lifetime properties which also have appropriate electrophoretic mobility. This issue was addressed in this work by using the enzymatic product 4-MBNA as the internal standard.

The ability to use the same compound for both internal standard and analyte is a noteworthy feature of EMMA. The detailed protocol for use of the internal standard is found in the Experimental Section. The LAP sample and the 4-MBNA are mixed and injected into the capillary containing buffer and substrate. The electrophoresis potential is then applied. The electrophoretic behaviors of LAP and 4-MBNA are quite different since, at the pH (7.0) used for the separation, the enzyme LAP bears a net negative charge whereas 4-MBNA is not charged. Therefore, the 4-MBNA and LAP will separate into distinct zones with the 4-MBNA eluting first. Prior to reaching the detector, the electrophoresis potential is interrupted (zero potential mode), allowing the enzyme to incubate in contact with the substrate. This incubation results in the accumulation of enzymatically produced 4-MBNA in the LAP zone. Potential is then resumed, and two spatially distinct 4-MBNA peaks are detected. An assay of LAP at relatively low concentration with the internal standard (Figure 1) demonstrates the utility of this method.

The procedure must be modified to assay more concentrated solutions of enzyme by this internal standard method. With higher levels of enzyme, detectable levels of product are formed during periods of constant potential, before and after incubation. This product appears as a plateau while the accumulated zero potential product appears as a peak on the plateau.[1,2] The internal standard peak elutes on the leading edge of the plateau, making quantitation of the internal standard difficult. The solution to this problem was to introduce a relatively large aliquot of buffer into the capillary between the enzyme and the substrate in a procedure referred to subsequently as the buffer spacer technique. By use of this procedure, the internal standard peak elutes within the buffer spacer while the slower moving enzyme is eventually overtaken by the substrate and the reaction is initiated. When contact between the enzyme and the substrate is delayed the constant potential product becomes isolated from the internal standard peak.

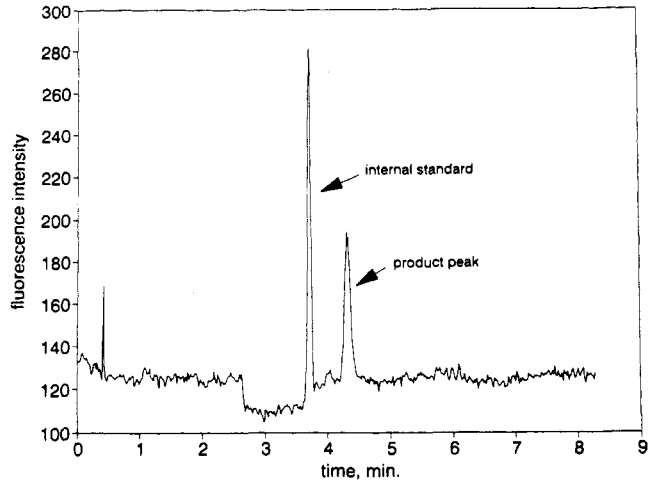

Figure 1. Time-resolved electropherogram for 2×10^{-10} M (0.92 IU/L) LAP enzyme standard. Data were collected using a 60-ns delay and a 50-ns window. Sample was incubated for 10 min.

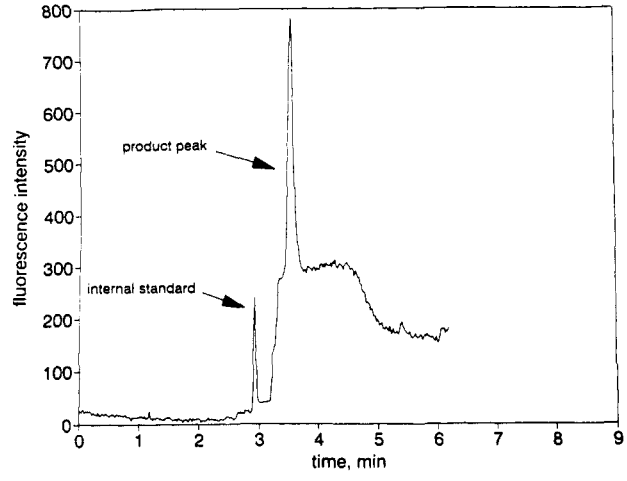

Figure 2. Time-resolved electropherogram for 3×10^{-9} M (11 IU/L) LAP enzyme standard using the buffer spacer technique. Data were collected using a 60-ns delay and a 50-ns window. Sample was incubated for 4 min.

The electropherogram in Figure 2 was obtained with the buffer spacer technique and a 4-min period of zero potential incubation. Because the spacer buffer provides a lower fluorescent background than the substrate, the baseline is lower at the beginning of the electropherogram.

LAP Assay on Biological Extracts. EMMA with TRLIF detection was used to assay LAP in several crude biological extracts. Electropherograms from the assay of human serum with and without time resolution are shown in Figure 3. The upper electropherogram was generated with no time delay and a 30-ns integration window; this approximates a continuous, non-time-resolved response. It can be seen that the 4-MBNA product peak is not completely separated from an

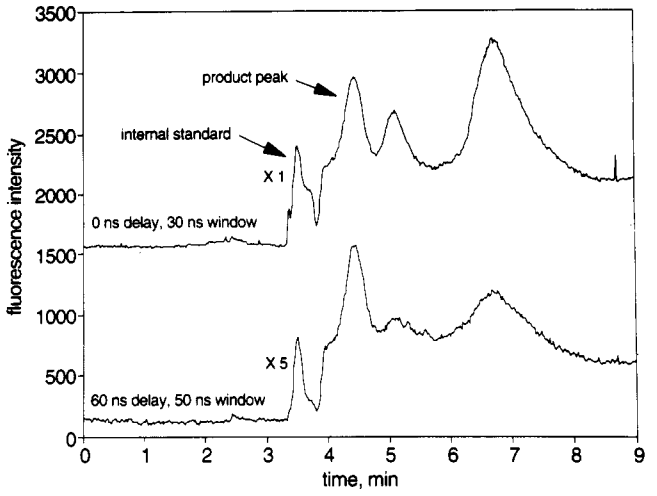

Figure 3. Dual electropherograms for human serum sample assayed for LAP by use of the buffer spacer technique. Sample was incubated 4 min. Data were offset for clarity.

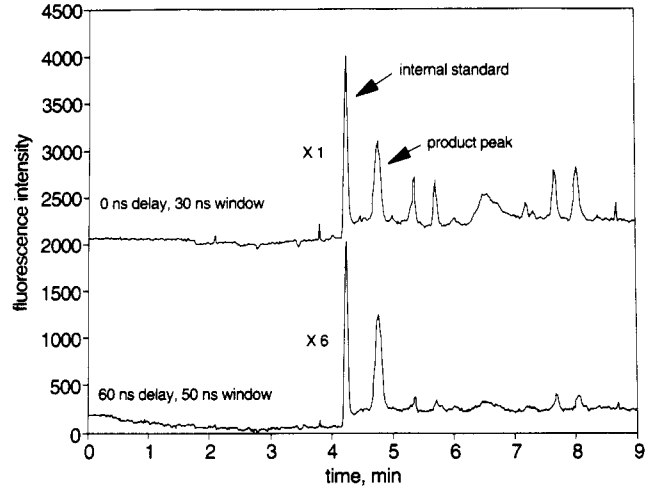

Figure 4. Dual electropherograms for dialyzed urine sample assayed for LAP. Sample was incubated 10 min. Data were offset for clarity.

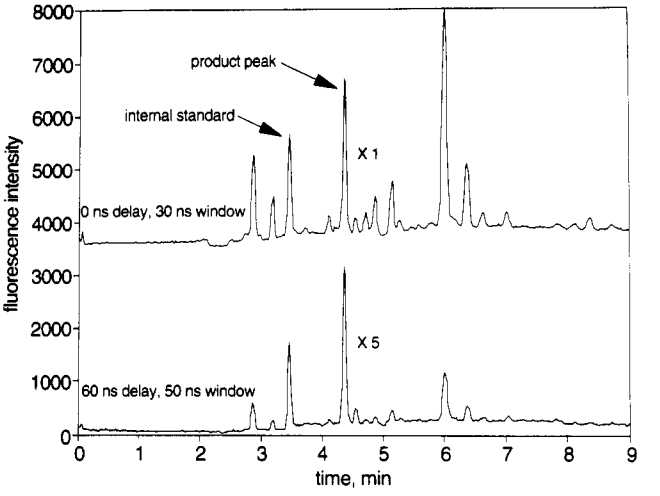

Figure 5. Dual electropherograms for *E. coli* supernatant assayed for LAP. Sample was incubated 10 min. Data were offset for clarity.

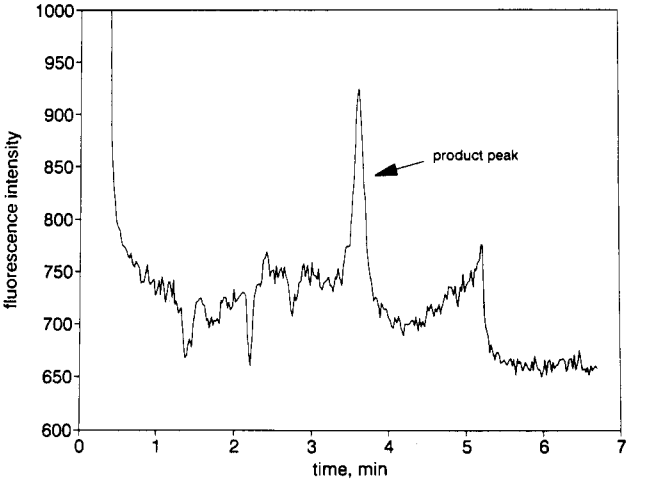

Figure 6. Electropherogram for 6×10^{-13} M LAP enzyme standard. Data were collected with no delay and a 30-ns window. Sample was incubated for 45 min.

interfering peak in the sample matrix. A large reduction in sample matrix interference was achieved in the lower electropherogram by using a 60-ns delay and a 50-ns window. Similar electropherograms were obtained for the assays of dialyzed urine and *E. coli* supernatant in Figures 4 and 5, respectively. Reduction of sample interference simplifies electrophoretic optimization. It is no longer necessary to search for electrophoresis conditions that separate interfering substances from the internal standard and enzyme product peaks. Another advantage is that time resolution helps to identify the product peak. Interfering substances have much shorter fluorescence lifetimes then 4-MBNA. Finally, the enormous sensitivity of TRLIF allows low levels of enzyme to be detected with short incubation times. Little product amplification is necessary before it is well above the background.

The linearity of the method was tested within the range of expected values for 10% diluted serum. The normal range of LAP activity values in undiluted serum is 15–33 IU/L while elevated levels can be more than 300% of the average value.[7,8] A five-point calibration curve from 0.92 to 14 IU/L [$(0.2–4) \times 10^{-9}$ M] was constructed using the buffer spacer internal standard procedure. The slope was 0.166 ± 0.009 C/(IU/L), the intercept was 0.075 ± 0.062 C and the R^2 value was 0.998.

The LAP assay results for the human serum, human urine, and *E. coli* supernatant samples were 23, 1.4, and 19 IU/L, respectively. Values for the serum and urine samples were within the ranges given in the literature for healthy donors.[8,9] The standard deviation on six replicate injections of the serum sample was 13.1%. This variance, while higher than the 5.0% coefficient of variation for the Sigma method,[7] is believed to be acceptable given that the range of normal values is ±37.5%.

A problem noted in this work was the coating of the capillary by the serum sample matrix. This problem caused the band broadening visible in Figure 3 and variations in migration times of as much as 37 s. This may also have contributed to the 13.1% variance since the standard deviation in six injections of an LAP standard was only 6.7%.

Enzyme Detection Limits in Buffer. The limits of detection in buffer were examined by increasing the incubation time to 45 min. The electropherogram for 6×10^{-13} M LAP is seen in Figure 6. By use of an injection volume of 14 nL and signal-to-noise ratio of 35:1, a detection limit (3σ) of 400 molecules was estimated. Product diffusion in extended time assays precludes incubation times of several hours in CZE. The product peak becomes so broad that it is indistinguishable from the baseline. However, it has recently been shown that EMMA in high-viscosity gel systems greatly reduces diffusion and allows incubation times of hours.[2]

A potential advantage of the EMMA technique is that enzymes are fractionated during the course of analysis and may be further identified on the basis of electrophoretic

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behavior. Future work will investigate the possibility of performing isoenzyme assays within the framework of EMMA.

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