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Capillary electrophoresis, combined with an on-line micro post-column enzyme assay

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Abstract

A system for capillary electrophoresis (CE), combined with a micro post-column reactor for on-line enzyme assays is described. Two detectors are employed. The first detector is an on-column UV detector, utilized to monitor the CE separation, while the second detector is monitoring the reaction product which is formed by adding a flow of substrate in the post-column section.

Factors affecting the band broadening in the post-column reactor have been investigated. A short reaction distance as well as a high flow-rate of substrate showed to be optimal.

A CE separation of glucose-6-phosphate dehydrogenase (G-6-PDH) and 6-phosphogluconic dehydrogenase (6-PGDH) was performed, where the enzyme activity could be monitored after reaction with nicotinamide–adenine dinucleotide phosphate/glucose-6-phosphate. The minimal detectable amount of G-6-PDH was shown to be in the order of $5 \cdot 10^{-16}$ mol ($2 \cdot 10^{-7}$ *M*).

1. Introduction

Electro-driven separations, carried out in capillary tubes have a considerable potential for improved separations of biomolecules. Due to the superior heat dissipation characteristics of the miniaturized format, the electrophoresis can be carried out under high field strength, which leads to a rapid and efficient separation [1–4]. Furthermore, the use of on-line detection devices provides far better quantitative data than those obtained with classical staining techniques. An additional important benefit of capillary electrophoresis (CE) is the ease of automation of the procedure. The number of applications of CE for separation of biomolecules like amino acids, peptides, proteins and DNA fragments is therefore growing very rapidly [5,6].

In this context, CE separation of enzymes is of great interest. Apart from the advantages mentioned, an important characteristic of CE is that only nl to pl volumes are handled by the column. Combined with appropriate miniaturized sample holders [7], it is possible to obtain qualitative and quantitative information even from very small amounts of material. This would be very valuable in several fields, such as in pre-natal clinical diagnostics or in studies of single-cell metabolics.

Enzymes are usually quantified and/or identified by measuring their biological activity, since this is the most relevant property in their biochemical context. The standard procedure in such measurements is to measure/monitor the

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reaction rate with a substrate. Under initial reaction conditions, where an excess of substrate is present, and no interference from the accumulated reaction product occurs, each enzyme displays a specific reaction rate constant. By simply mixing the substrate with the enzyme and a subsequent incubation of the reaction mixture, the amount of enzyme present can be determined.

For enzymes, separated by CE, it is possible to determine their activity after fraction collection as demonstrated *e.g.* by Banke *et al.* [8]. However, this is a rather laborious procedure, and an on-line method would be highly desirable, particularly in routine applications (*e.g.* in a clinical laboratory).

On-line enzyme assays in the form of a post-column reaction have been described for liquid chromatography [9–11], where the substrate is added to the column effluent in a mixing tee and the formed reaction product is measured at a downstream point.

Recently, Bao and Regnier [12] described a system, where the substrate was an integral part of the running buffer. During the electrokinetic transport, a broad band of reaction product is formed, due to differences in electrophoretic mobilities between the enzyme, the reaction product and the substrate. By a temporary interruption of the applied potential (and thereby the electrokinetic flow) a local increase in signal is generated, the strength of which is related to the activity of the enzyme and the interruption period.

In the present work, we report a system for CE–post-column micro-enzyme assays, where two detectors are employed. The first detector is an on-column UV detector, to monitor the separation in the CE column, while the second detector is mounted downstream after mixing the column effluent with substrate, to monitor the generated reaction products.

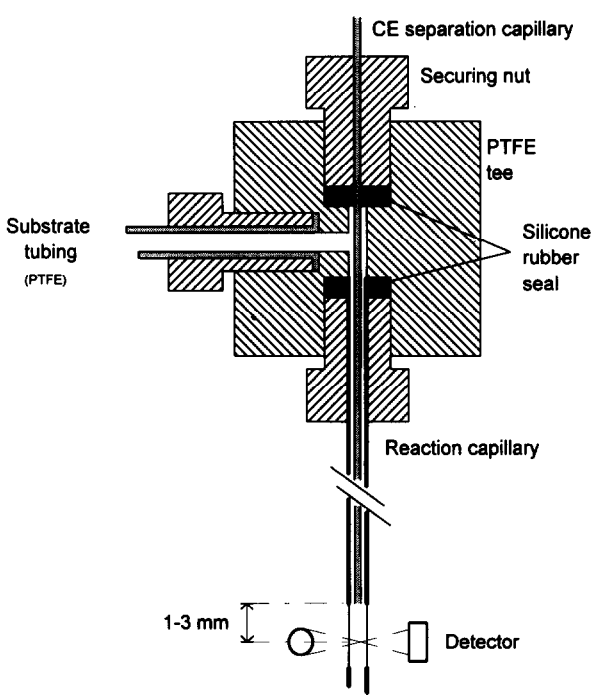


Fig. 1. Schematic of the post-capillary reactor.

2. Experimental

2.1. Post-capillary reactor

Fig. 1 shows a schematic of the reactor. It consists of a PTFE tee (0.7 mm I.D. holes) in which both the separation and reaction capillaries are mounted. The separation capillary (50 μm I.D. × 360 μm O.D.) is inserted into the reaction capillary (0.53 mm I.D., 0.7 mm O.D.) in such a way that the outlet is positioned 1–3 mm above the detection window of the reaction capillary. The substrate stream is introduced into the tee by a PTFE tube (0.7 mm I.D. × 1.6 mm O.D.).

Fig. 2 shows a set up where the position of the reactor in the total capillary zone electrophoresis (CZE) system is shown.

2.2. Instrumentation

The apparatus used consisted of two variable-wavelength UV detectors (Model UVIS 200; Linear Instruments, Reno, NV, USA) each equipped with an on-column capillary cell (Model 9550-0155, Linear Instruments) and further connected to a strip-chart recorder (Linear Instruments). The high-voltage supply, ± 0–30

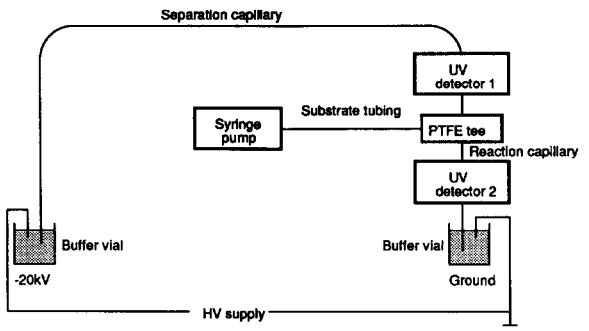


Fig. 2. The CE system including the post-capillary reactor. HV = High voltage.

kV, was constructed from a Spellman CZE 100 (Plainview, NY, USA) unit. The CZE column was placed in a Plexiglass box with a high-voltage safety interlock, which also housed an injection device and arrangements for *in situ* flushing by action of pressurized air (0.5–2.0 bar). Substrate flow was introduced by using a syringe pump (Model 351; Sage Instruments, Orion Research, Boston, MA, USA). Plastic syringes (2–5 ml) (Millipore, Bedford, MA, USA) were used. Actual flow-rates were determined by weighing the dispensed liquid. Sample injections were accomplished by electromigration.

A reversed polarity set up was employed, where the capillary inlet was kept at negative potential, while the detection side of the capillary was grounded.

2.3. Materials and reagents

Fused-silica tubing was obtained from Chrompack (Middelburg, Netherlands). The total length of the separation capillary was 75 cm. The length to detector 1 was 60 cm, the total length of the reaction capillary was 15 cm and the length from the inlet of the reaction capillary to detector 2 was about 7 cm.

The fluorosurfactant (FC134) was obtained from 3M Co. (St. Paul, MN, USA). The water used to prepare solutions was passed through a Milli-Q system (Millipore). Glucose-6-phosphate dehydrogenase (G-6-PDH) (torula yeast), nicotinamide–adenine dinucleotide phosphate (NADP) and glucose-6-phosphate (G-6-P) were purchased from Sigma (St. Louis, MO, USA). 6-Phosphogluconic dehydrogenase (6-PGDH) was a gift from the Department of Biochemistry and Biotechnology, Royal Institute of Technology (Stockholm, Sweden).

Enzyme solutions with a protein concentration of 0.2 mg/ml for G-6-PDH and 0.05 mg/ml for 6-PGDH were freshly prepared before electrophoresis experiments in 0.05 *M* phosphate buffer with a pH of 5. Substrate solution was prepared freshly in 0.05 *M* phosphate buffer, pH 8, with a G-6-P concentration of 1 m*M* and a NADP concentration of 0.2 m*M*.

The running buffer contained 100 μg/ml FC134 in 0.05 *M* phosphate buffer at pH 5. Before use, new capillaries were rinsed for 30 min with 0.4 *M* NaOH, 10 min with water and 20 min with running buffer. Between each run the separation capillary was rinsed for 3 min with running buffer.

3. Results and discussion

One of the most important considerations in designing the enzyme reactor was to maintain the resolution, obtained by the separation capillary. The major factors that contribute to post-column peak broadening are diffusion and dispersion due to the parabolic Taylor flow profile, generated by the pressure-driven flow of substrate. The influence is dependent on the diameter of the reactor capillary, the flow velocity of the substrate and, particularly important, on the distance between the outlet of the CE capillary and the point of detection in the reaction capillary. To avoid an excessive loss of efficiency, this distance had to be kept to a few mm only.

In order to evaluate the band-broadening characteristics of the post capillary reactor without possible influences from the reaction process, mesityloxide was injected in the separation capillary and monitored before and after passing through the reactor. Fig. 3 and Table 1 show the results obtained for different substrate flow-rates and for different distances between the separation capillary and the second detector. As can be

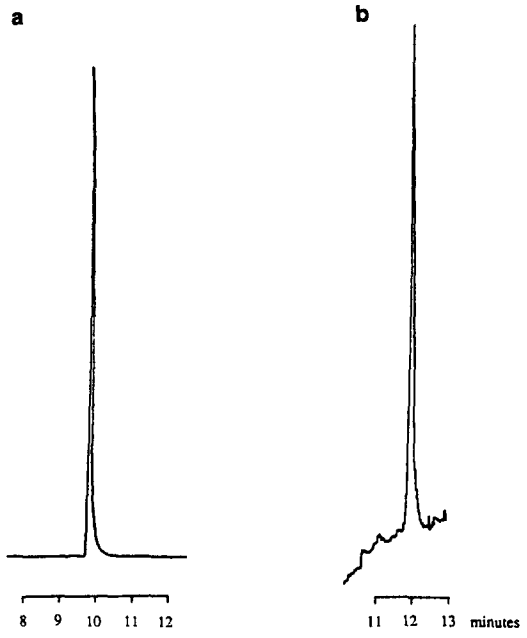


Fig. 3. Electropherogram of mesityloxide. (a) Signal from detector 1, before the post column reactor, (b) signal from detector 2, after the reactor. Concentration: 0.01%, detector wavelength: 254 nm for both detectors 1 and 2, sensitivity range: detector 1, 0.005 AUFS and detector 2, 0.002 AUFS, separation voltage: −20 kV, syringe pump flow-rate: 43.2 μl/min (no substrate added to the reactor buffer), distance between the separation capillary outlet and detector 2: 1 mm.

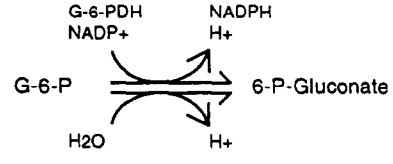
seen, the position of the second detector, relative to the CE capillary outlet is very critical. While the loss of efficiency is relatively small at 2 mm distance, it becomes unacceptable at 3 mm distance. Since the 2 mm setup allows a longer reaction time for the enzyme assay than the 1 mm setup, all subsequent experiments were carried out with the 2 mm distance set up. Under these conditions, the influence of the substrate flow-rate on the efficiency is not very pronounced as can be seen from the last part of Table 1.

Table 1
Influence of the distance between the separation capillary outlet and detector 2, and of the ratio of the linear flow-rates in the reactor and the separation capillary (v_{react}/v_{sep}) on the ratio of the number of plates (N_{det2}/N_{det1}) for mesityloxide before (detector 1) and after (detector 2) the post-capillary reactor

Distance between the separation capillary and detector 2 (mm)	v_{react}/v_{sep}	N_{det2}/N_{det1}
1	2.3	1.2
2	2.3	0.80
3	2.3	0.29
2	1.2	0.86
2	2.3	0.80
2	3.3	0.98

When the mesityloxide passed the second detector, a decrease in sensitivity of about one order of magnitude was observed, compared to the signal obtained from the first detector. This is likely to be caused by the dilution of the sample in the reactor flow. The increased total volume results in a hundredfold decrease in sensitivity, while the increase in pathlength only gives rise to a tenfold sensitivity increase. The optics of the employed detector cell are optimized for capillary dimensions of 50–100 μm I.D. and 360 μm O.D., according to the manufacturer (Linear Instruments). Since the reactor capillary has considerably larger cross-sectional dimensions, the light beam will not be focused on the centre of the capillary, which could also contribute to decreased sensitivity.

The enzyme reaction studied in this work is the interaction of G-6-PDH with G-6-P in presence of NADP. The schematic of the reaction is shown below:



To suppress the wall adsorption of G-6-PDH, a cationic fluorosurfactant was added to the running buffer. As we have shown earlier, the presence of a very small amount of this detergent leads to remarkably improved efficiency and reproducibility [13]. Due to the formation of an admicellar bilayer at the wall, the electroosmotic flow is reversed and positively charged species are repelled from the wall.

G-6-PDH has a p*I* of 6 and therefore a running buffer pH of 5 was used to effect the repellation. Under these conditions the direction of the electrophoresis of the enzymes and the electroendosmotic flow are opposed to each other. This should improve the resolution [2].

According to our earlier findings, the influence of fluorosurfactant buffer additives on the enzymatic activity is very moderate [14]. We attribute this behaviour to the lipophobic character of the fluorocarbonic chains and the fact, that the surfactant is present in only very low concentrations. Fig. 4 shows the electropherograms obtained for G-6-PDH before and after the reaction. As can be seen, the peaks, due to the enzyme in the first detector and the monitored reaction product (NADPH) in the second detector are of similar width.

Band broadening in the post-capillary reactor

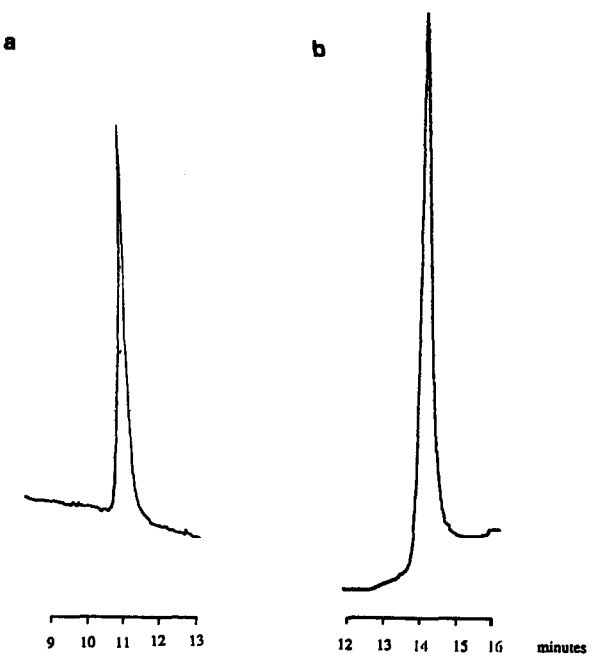


Fig. 4. Electropherograms before and after the reaction of G-6-PDH and NADP/G-6-P. (a) Signal from detector 1, before the reaction, (b) signal from detector 2, after the reaction. Detector wavelength: detector 1, 210 nm; detector 2, 340 nm. Sensitivity range: detector 1, 0.005; detector 2, 0.002 AUFS. Separation voltage: − 20 kV, substrate flow: 9.8 μl/min, distance between the separation capillary outlet and detector 2: 2 mm, enzyme concentration: 0.2 mg/ml.

is promoted by several phenomena. Initially, when leaving the column exit, the solutes are picked up by the surrounding substrate and are transported in the centre part (corresponding to the inner cross-section of the CE column) of the parabolic flow profile of the substrate. At the same time, the enzyme–substrate reaction takes place, while a dispersion of the solutes as well as the reaction products is induced by diffusion in both radial and longitudinal direction. Since the diffusion coefficient is dependent on the molecular mass of the solutes, the dispersion is most pronounced for the reaction products. The diffusion rate can be calculated from the Einstein equation:

$$x = (2Dt)^{1/2} \quad (1)$$

where x is the diffusion distance, D is the diffusion coefficient and t is the mean time for diffusion. Assuming a D of $1 \cdot 10^{-5}$ cm^2/s (which would be a typical value for a low-molecular-mass compound in water), and reaction times before the detection takes place as in our experiments (0.6–2.7 s), the dispersion would be 35–73 μm. Thus, the solutes will not reach the more stagnant regions of the parabolic flow profile near the wall of the reactor, which would have caused a very significant band broadening. Also the axial dispersion caused by diffusion is negligible, since it would only add *ca.* 1% to the variance of the solute band. Of additional importance is that the speed of the surrounding sheath flow of substrate was of the same order as the endosmotic flow [15–17].

A further cause of peak broadening, as identified by Rose and Jorgenson [15] is the radial dispersion, induced by the electric field in the post-column section. In our case, an axial electric spread of the reaction product could also be visualized, since the products obtained are likely to have different electrophoretic mobilities, compared to the enzymes. In the present post-column design, the large difference in diameter between the CE capillary and the reactor is of a significant advantage, since this reduces the electrical potential across the reactor. In fact, the calculated field strength over the reactor employed is only *ca.* 2 V/cm, for a corresponding

value over the separation capillary of 270 V/cm, this under assumption that the solutions have the same specific conductivity. Since the ion strength of the running buffer and the substrate solution were of the same order of magnitude, the electrical dispersion in our post-column reactor should therefore be neglectable.

Fig. 5 shows a response curve for the G-6-PDH experiments, using the described setup.

Under these conditions, the minimal detectable amount of G-6-PDH showed to be in the order of $5 \cdot 10^{-16}$ mol ($2 \cdot 10^{-7}$ M). A longer reaction time would increase the sensitivity, but at the expense of a deteriorated peak resolution. A further improvement could be visualized, by scaling down the ratio between inner diameter of the reactor and the outer diameter of the CE capillary, as has been shown by Rose and Jorgenson in a fluorescence post-column reactor. However, the procedure is more complicated, involving HF etching of the capillary ends. Moreover, the back pressure at the end of the CE capillary will rapidly increase when the inner diameter of the reactor capillary becomes small. This could lead to a backflow of liquid in the column, and cause an extra-column band broadening as well as retention shifts. The back pressure can be calculated from the Poiseuille equation

$$\Delta p = 8FL\eta/\pi r^4 \quad (2)$$

where F is the reagent flow through the reactor, r is the inner diameter of the reactor, Δp is the pressure drop and L and η are the length of the reactor and the viscosity of the reagent solution, respectively. In our reactor, the calculated back pressure is only between 6 and 26 Pa for the different flow-rates used, and therefore neglectable in the present separation system.

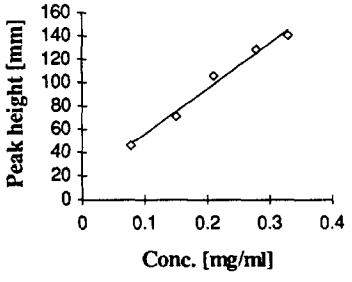


Fig. 5. Response curve for the G-6-PDH/NADP reaction system. Conditions as in Fig. 4.

Another route towards improved sensitivity would be to prolong the reaction time prior to detection. One possibility would be to employ a reaction medium with a higher viscosity, which would allow longer reaction times without increasing the dispersion to an unacceptable level. A limiting factor would be a rapid local depletion of substrate, since a mixing of the phases is supressed. However, for very low sample concentrations, this would be less of a problem.

Our present post-column reactor arrangement provides the freedom to separately optimize the electrophoretic separation conditions and the enzymatic reaction conditions (type of buffer ions, pH, ion strength, additives etc.).

There are for example many cases, where the pI of the enzyme is considerably lower than the pH, where the enzyme has its full activity. Since the best electrophoretic separation selectivity is often around the pH of the isoelectric point, the post-column concept offers an important advantage compared to schemes, where the enzyme reaction is performed within the CE capillary. Moreover, it is easy to change the substrate and optimize its flow-rate.

To illustrate the applicability of CE separations in combination with the micro post-column enzymatic assay, a system including G-6-PDH and 6-PGDH was examined. 6-PGDH has reportedly an activity for the combination with G-6-P/NADP of about a hundredfold lower than G-6-PDH. This corresponds well with our results, shown in Fig. 6, which shows a CE separation of G-6-PDH and 6-PGDH and the subsequent enzyme activity profile.

The possibility to obtain a CE separation pattern together with the corresponding enzymatic activity profile should be of interest in a number of applications. With further improvements in CE separations (*e.g.* improved column technology) it should be possible to quantify different isoenzymes for the purpose of clinical diagnostics. Such work is currently in progress.

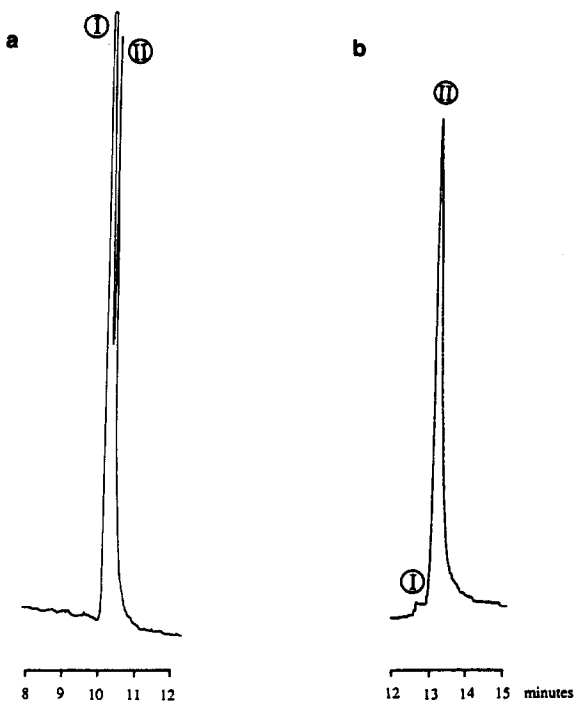


Fig. 6. Electropherograms showing the separation and enzymatic activity profile of G-6-PDH and 6-PGDH. (a) Detector 1, (b) detector 2. I = 6-PGDH, concentration 0.05 mg/ml; II = G-6-PDH, concentration 0.2 mg/ml. Conditions as in Fig. 4.

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