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Enzymophoresis of nucleic acids by tandem capillary enzyme reactor–capillary zone electrophoresis☆

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ABSTRACT

Enzymophoresis with coupled heterogeneous capillary enzyme reactor–capillary zone electrophoresis was developed and evaluated in the area of nucleic acids. Ribonuclease T_1, hexokinase and adenosine deaminase were successfully immobilized on the inner walls of short fused-silica capillaries through glutaraldehyde attachment. These open-tubular capillary enzyme reactors were quite stable for a prolonged period of use under operation conditions normally used in capillary zone electrophoresis. The capillary enzyme reactors coupled in series with capillary zone electrophoresis served as peak locator on the electropherogram, improved the system selectivity, and facilitated the quantitative determination of the analytes with good accuracy. Also, they allowed the on-line digestion and mapping of minute amounts of transfer ribonucleic acids, and the simultaneous synthesis and separation of nanogram quantities of oligonucleotides.

INTRODUCTION

Capillary zone electrophoresis (CZE) with its advanced instrumentation and unique selectivity has become an important microseparation technique. In CZE, and for a given set of conditions, the migration of charged species depend primarily on the sign and magnitude of their net charge and to some extent on their shape and size. Therefore, when the analytes possess the same charge-to-mass ratio and differ slightly in their size and shape, CZE is rather inadequate for their separation. In addition, as with other separation techniques, the identification of a given species in a mixture requires the use of additional means that can confirm its presence or absence, especially under coelution conditions or when no authentic standards are available. Enzymes, with their high stereospecificity in binding their substrates, are well suited to play this role.

In fact, the combination of enzymes with separation techniques has been a theme of research for many years. In 1970, Brown [1] pioneered the use of enzymes in free solution for sample pretreatment prior to chromatographic separation as a mean of peak identification, and coined the term enzymic peak-shift for the technique. On the other hand, the relative ease with which enzymes can be covalently attached or immobilized to various types of surfaces without substantial loss in their catalytic activity [2] has favored their use in solving many analytical problems [3]. One of the important applications of immobilized enzymes has been their use in pre- and post-column derivatization devices with several high-performance liquid chromatography (HPLC) techniques [3–8]. More recently, narrow-bore packed-bed trypsin reactor was introduced for the nanogram digestion of proteins prior to microcolumn liquid chromatography and capillary zone electrophoresis tryptic peptide mapping [9].

This report is concerned with the miniaturization of immobilized enzyme reactors for use in tandem with CZE. In this regard, short fused-silica capillaries with immobilized enzymes on the inner wall

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were developed for tandem heterogeneous capillary enzyme reactor–capillary zone electrophoresis (HCER–CZE). This tandem format, which is referred to as enzymophoresis, was evaluated in both the qualitative and quantitative determinations of different nucleic acids. Various HCER–CZE systems were investigated and characterized over a wide range of operation conditions including the design of the capillary enzyme reactor, the contact time of the substrates with the immobilized enzymes, the initial concentrations of the substrates, the nature of the background electrolyte and the pH. In addition, the effects of buffer additives as well as the immobilized enzymes on the magnitude and direction of the electroosmotic flow were investigated.

EXPERIMENTAL

Instrument

The instrument for capillary electrophoresis was assembled in-house from commercially available components, and resemble to previously reported systems [10,11]. It comprised two high-voltage power supplies of positive and negative polarities Models MJ30P400 and MJ30N400, respectively, from Glassman High Voltage (Whitehouse Station, NJ, USA) and a Linear (Reno, NV, USA) Model 200 UV–VIS variable-wavelength detector equipped with a cell for on-column capillary detection. The detection wavelength was set at 258 nm for sensing the various nucleic acids. The electropherograms were recorded with a Shimadzu (Columbia, MD, USA) computing integrator Model C-R3A equipped with a floppy disk drive Model FDD-1A and a CRT monitor.

Capillary columns

Fused-silica capillary columns of 50 μm I.D. and 365 μm O.D. were obtained from Polymicro Technology (Phoenix, AZ, USA). All capillaries used in this study were coated in-house with either interlocked or fuzzy polyether chains according to previously described procedures [10].

Reagents and materials

Ribonuclease T_1 (RNase T_1) from *Aspergillus oryzae* (EC 3.1.27.3), hexokinase from bakers yeast (EC 2.7.1.1), adenosine deaminase (ADA) from calf spleen (EC 3.5.4.4), transfer ribonucleic acid specific for phenylalanine ($tRNA^{Phe}$) from brewers yeast, guanylyl-(3′→5′)-adenosine (GpA), guanylyl-(3′→5′)-uridine (GpU), guanylyl-(3′→5′)-cytidine (GpC), uridylyl-(3′→5′)-guanosine (UpG), guanosine-2′:3′-cyclic monophosphate (G>p), guanosine-3′-monophosphate (Gp), adenosine-5′-triphosphate (ATP), adenosine-5′-diphosphate (ADP), adenosine (A), inosine (I), guanosine (G), uridine (U), cytidine (C), glucose and Trizma base were obtained from Sigma (St. Louis, MO, USA). L-Histidine (His), 2-[N-morpholino]ethanesulphonic acid (MES), N,N′-bis[3-aminopropyl]-1,4-butanediamine tetrahydrochloride (spermine) were from Aldrich (Milwaukee, WI, USA). γ-Aminopropyltriethoxysilane was purchased from Huls of America (Bristol, PA, USA). Reagent-grade sodium phosphate monobasic and dibasic, magnesium chloride, ammonium bifluoride, 1,3-naphthalenedisulphonic acid disodium salt, sodium acetate, phosphoric acid, hydrochloric acid, sodium hydroxide and HPLC-grade methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). Deionized water was used to prepare the running electrolyte. All solutions were filtered with 0.2-μm UniPrep syringeless filters (Fisher Scientific) to avoid column plugging.

Enzyme immobilization

Fused-silica capillaries of 15 to 25 cm long were first etched according to the procedure described by Onuska *et al.* [12]. The etched capillaries were then reacted with 10% (v/v) solution of γ-aminopropyltriethoxysilane in water at 95°C for 30 min. This step was repeated twice. Following, a 1% (v/v) solution of glutaric dialdehyde in 50 m*M* phosphate, pH 7.0, was applied to the capillary and allowed to react at room temperature for 1 h. Thereafter, a phosphate solution containing the enzyme of interest was recycled through the aldehyde activated amino-fused-silica capillaries and allowed to stand overnight. Finally, residual aldehyde reactive functions were scavenged with 50 m*M* Tris–HCl at pH 7.5. All capillaries were stored in water at 5°C.

Other procedures

In all experiments, the capillary enzyme reactors of 15 to 25 cm long were connected butt-to-butt to the separation capillaries having a polyether hydrophilic coating (*i.e.*, CZE capillary) of 64 cm total length and 30 cm to the detection point using a

PTFE tube the inner diameter of which matched the outer diameters of the two capillary columns. The enzymic reactions were carried out on-line by allowing a thin plug of the substrates to flow through the tandem capillaries either by electromigration under the influence of an applied voltage or by hydrodynamic mode (*i.e.*, gravity flow) by raising the inlet reservoir to a certain height above the outlet reservoir. In both cases, the substrates first entered the capillary enzyme reactor and were converted to products. They were further separated in the separation capillary downstream the enzyme reactor. The time required for the substrates to traverse the enzyme reactor under hydrodynamic conditions (t_i) was estimated from the following equation [13]:

$$t_i = \frac{8\eta Ll}{\rho g r^2 \Delta h}$$

where η is the medium viscosity, L is the total length of the capillary, l is the distance traversed by the plug of the substrate, ρ is the electrolyte density, g is the gravitational constant, r is the inner radius of the capillary and Δh is the differential height between the electrolyte reservoirs. In all cases, the viscosity and density of the solutions were taken as that of water since the buffers used were dilute solutions of electrolytes. The calculations using the above equation agreed with the experimental migration time estimated from the chromatogram of the substrate that was allowed to flow by gravity through the tandem capillary enzyme reactor→CZE capillary. Therefore, in all the studies, the above equation gave satisfactory results in terms of the time required for the species to reach a certain distance in the capillary by hydrodynamic flow.

The running electrolyte was renewed after each run. To ensure reproducible separations the separation capillary was flushed successively with fresh buffer, water, methanol, water, and again runnning buffer. The enzyme reactor was also flushed with fresh buffer solution after each run. Both capillaries were then equilibrated with the running electrolyte for 10 min before each injection.

RESULTS AND DISCUSSION

Design of the capillary enzyme reactor

Fig. 1 is a schematic illustration of the capillary enzyme reactor. The walls of the fused-silica capil-

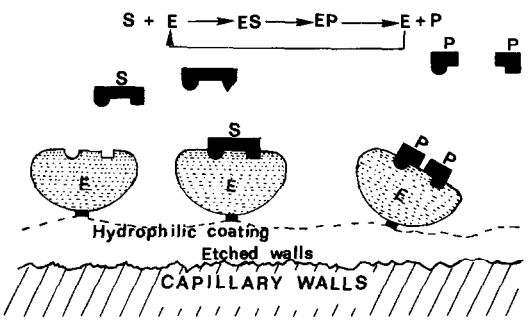

Fig. 1. Schematic illustration of immobilized capillary enzyme reactor. S = Substrate; E = enzyme; P = product.

lary were first roughened using a methanolic solution of ammonium bifluoride; see Experimental. This etching step is to increase the specific surface area of the capillary wall in order to achieve higher enzyme loading of the reactor. This would yield capillary enzyme reactors with higher catalytic activity, and may increase the maximum velocity, V_{max}, of the enzymic reaction. The surface roughening by ammonium bifluoride at high temperature has been recently used in our laboratory for the preparation of octadecyl capillaries for on-line preconcentration of dilute samples [14] and proved efficient in increasing the concentration of covalently attached hydrocarbonaceous ligands. Another function of the etching is to increase the surface wettability which permits the homogeneous spreading of the hydrophilic aminopropylsiliyl layer. This layer is to minimize solute–wall interactions and to provide the functional groups for the attachment of the enzyme. The HCERs thus obtained were used in series with polyether-coated capillaries.

Operational and basic principles of tandem capillary enzyme reactor-CZE

The capillary consisted of two sections connected in series via a PTFE tube. The first part is the capillary enzyme reactor and the second part is the separation capillary (*i.e.*, CZE). In all cases, the substrate(s) is first introduced as a thin plug into the capillary enzyme reactor. The catalyzed reaction takes place as the substrate(s) migrate through the enzyme reactor either by hydrodynamic flow or electromigration. Following, the reaction mixture is further separated in the CZE capillary at the same or different applied voltage than in the precolumn

enzymic derivatization reactor. To satisfy both the enzymic reaction and the separation, the background electrolyte for the reaction and separation processes can be either the same or different. Most importantly, the electrolyte for the enzymic reaction should minimize microenvironmental effects in the capillary enzyme reactor. These effects are related to electrostatic or hydrophobic interactions with the enzyme and/or the matrix [15].

Open tubular capillary enzyme reactors offer the convenience of repeated use of the enzyme without much loss of its activity, and provide enhanced storage and thermal stability for the enzyme. Furthermore, in contrary to packed-bed reactors where the substrate has to diffuse inside the pores of the packing to reach the immobilized enzyme, mass transfer in open tubular columns is of external nature (*i.e.*, bulk diffusion). This would increase the effectiveness factor η, defined as the ratio of the actual reaction rate to that obtained under conditions where no diffusional mass transfer limitations are present (*i.e.*, free solution). External mass transfer limitations, albeit small in a capillary tube, may still increase the apparent Michaelis constant K_M^{app} [16], which would result in increasing the linear dynamic range of the immobilized enzyme, and in turn benefit the analytical applications of capillary enzyme reactors.

Effect of spermine on the electroosmotic mobility

The usefulness of the catalytic activities of the various HCERs was demonstrated in the separation, identification, quantitative determination, and synthesis of nucleic acid fragments and constituents. In order to bring about the migration and separation of the various nucleic acids under investigation with coated capillaries having moderate cathodal electroosmotic flow, a means for inverting the direction of the flow was necessary so that a negative polarity mode could be utilized. This was achieved by adding spermine to the running electrolyte at low concentration.

Spermine is a biogenic tetraamine that has been successfully used as a buffer additive in the CZE analysis of polycitidines [17] with polyacrylamide-coated capillary tubes. In this study, it was thought that the spermine primary function is to reduce the net negative charge of the analytes and allow their migration and separation. To study its adsorption onto the capillary inner surface, we have measured the magnitude of the electroosmotic flow and examined its direction in the presence and absence of spermine over the pH range 3.5–7.0 using phenol as the inert tracer. The results of this study are summarized in Fig. 2. These studies were performed on an interlocked polyether-coated capillary using a running electrolyte of 25 m*M* His and 25 m*M* MES, with or without 5 m*M* spermine. In the presence of spermine, the direction of the electroosmotic flow was inverted from cathodal to anodal as shown in Fig. 2. The adsorption of spermine, which has a net positive charge of *ca.* +4 below pH 7.0, onto the capillary inner surface changed the ζ potential of fused silica from negative to positive. As can be seen in Fig. 2, the electroosmotic mobility slightly decreased upon increasing the pH in the range studied. This can be explained by both the slight decrease in the net positive charge of spermine and the increase in the ionization of surface silanols as the pH increased. Thus, spermine is a useful buffer adjunct for the analysis of negatively charged species in the negative polarity mode, since it allows the separated analytes to migrate in the same direction as that of the electroosmotic flow which in turn would yield shorter analysis time.

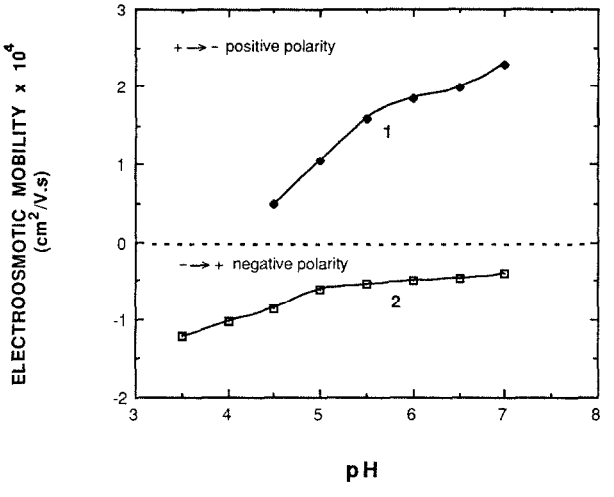

Fig. 2. Plots of the electroosmotic mobility in the absence (1) or presence (2) of spermine in the running electrolyte as a function of pH. Capillary, interlocked polyether coating, 30 cm (to the detection point), 64 cm (total length) × 50 μm I.D.; running voltage, 25 kV; background electrolyte, 25 m*M* His, 25 m*M* MES in the absence (1) or presence (2) of 5 m*M* spermine. Inert tracer, phenol.

Ribonuclease T_1 capillary enzyme reactor

Ribonuclease T_1 is a guanylic acid-specific endoribonuclease that cleaves phosphodiester bonds between 3'-guanylic acid residues and the 5'-hydroxyl groups of adjacent nucleotidyl residues [18]. In addition, under certain conditions, the enzyme acts as a ligase catalyzing the esterification of G>p with the 5'-hydroxyl group of various nucleosides to yield the corresponding dinucleotides [18,19]. Both catalytic functions of RNase T_1 were evaluated in the tandem RNase T_1 capillary enzyme reactor–CZE mode using an electrolyte system containing spermine as a buffer additive to facilitate the electrophoresis of negatively charged nucleotides, see above.

Immobilized ribonuclease T_1 as a hydrolytic enzyme. As mentioned above, RNase T_1 splits the internucleotide bonds specifically after guanylyl residues according to the following reaction schemes [18]:

$$\text{RNA} \xrightarrow[\text{Limited digestion}]{\text{RNase } T_1} \text{Oligonucleotides with terminal G>p (transphosphorylation, reversible)} \quad (1)$$

$$\text{RNA} \xrightarrow[\text{Exhaustive digestion}]{\text{RNase } T_1} \text{Oligonucleotides with terminal Gp (hydrolysis, irreversible)} \quad (2)$$

The above catalytic activity of the enzyme was demonstrated in the identification and quantitative determination of various dinucleotides using tandem RNase T_1 capillary enzyme reactor–CZE. In all experiments, the substrates were introduced as a thin plug and allowed to flow hydrodynamically through the enzyme reactor by raising the inlet reservoir to a height of 20 cm above the outlet reservoir. This corresponds to a contact time of *ca.* 16 min with the immobilized enzyme. As the plug of the reaction mixture entered the separation capillary, the enzyme reactor was disconnected and the separation capillary was inserted into the electrolyte reservoir. Thereafter, the voltage was turned on to 25 kV to bring about the separation of the reaction mixture.

As shown in Fig. 3a, a mixture of three dinucleotides namely GpU, GpA and GpC were not resolved by CZE alone using a buffer system containing 25 m*M* His, 25 m*M* MES and 5 m*M* spermine at pH 5.0. This is because these dinucleotides have approximately the same charge-to-mass ratio. As shown in Fig. 3b, with the on-line RNase T_1 capillary enzyme reactor, the dinucleotide mixture was converted to more readily separated products. Each of the three dinucleotides GpA, GpU and GpC yielded the guanosine-2':3'-cyclic monophosphate and its corresponding nucleoside, *i.e.*, adenosine, uridine and cytidine. Hence, the three dinucleotides that coeluted as a broad peak with CZE alone were transformed into four well resolved peaks, *i.e.*, G>p, U, A and C, after a single pass through the 17 cm long RNase T_1 capillary enzyme reactor.

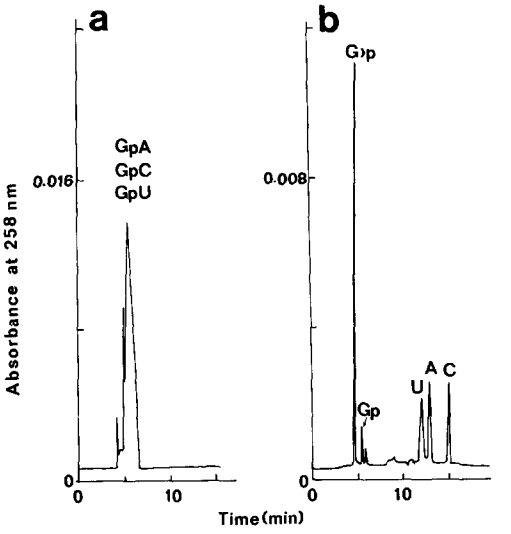

Fig. 3. Typical electropherograms of dinucleotides and their RNase T_1 digest obtained by CZE alone (a) or by tandem RNase T_1 capillary enzyme reactor–CZE (b), respectively. Capillary enzyme reactor, 17 cm × 50 μm I.D.; separation capillary, interlocked polyether coating, 30 cm (to the detection point), 64 cm (total length) × 50 μm I.D.; substrate introduction, hydrodynamic mode; enzymic reaction was carried out using gravity-driven flow; separation step, as the plug of the reaction mixture entered the separation capillary, the enzyme reactor was disconnected and the voltage was turned on to 25 kV; background electrolyte, 25 m*M* His, 25 m*M* MES, 5 m*M* spermine, pH 5.0. GpU = Guanylyl-(3'→5')-uridine; GpA = guanylyl-(3'→5')-adenosine; GpC = guanylyl-(3'→5')-cytidine; G>p = guanosine-2':3'-cyclic monophosphate; Gp = guanosine-3'-phosphate; U = uridine; A = adenosine; C = cytidine.

In another electrophoretic run, one of the three dinucleotides GpC, was replaced by UpG, a dinucleotide that is not digested by the enzyme. As expected, with CZE alone, this mixture exhibited the same electrophoretic behavior as the one in the preceding experiment, in the sense that its components could not be resolved as shown in Fig. 4a. However, after a single pass through the capillary enzyme reactor, UpG which is not split by the immobilized enzyme eluted intact, and was well resolved from the products of GpA and GpU. This feature of the capillary enzyme reactor has many practical significances. The tandem format can assess the identity of overlapping peaks and permits the simultaneous digestion and separation of the reaction mixture. Briefly, the capillary enzyme reactor functions as a peak locator by unmasking the analyte of interest on the electropherogram, and enhances the selectivity of the electrophoretic system by converting the substrates into more readily separated products.

The dinucleotide GpU, having the lowest relative rate of splitting by RNase T_1 among the other dinucleotides analyzed [20], was selected as a model substrate to evaluate the stability of the RNase T_1 capillary enzyme reactor. The reactor showed steady behavior even after prolonged storage or repeated use. Over a period of more than 2 months, the RNase T_1 capillary enzyme reactor yielded complete conversion of GpU with the liberation of G>p and U as major peaks, suggesting that the enzymic reaction occurring at the wall of the HCER is primarily a limited digestion mechanism (see reaction 1).

Another important feature of the capillary enzyme reactor is its usefulness in the quantitative determination of the dinucleotides. To demonstrate this important application of HCER, typical calibration curves for G>p and some of the nucleosides were established using 1,3-naphthalenedisulphonic acid disodium salt as the internal standard. As shown in Fig. 5, these curves were linear in the concentration range investigated. In such measurements, the G>p peak can be used for the quantitative determination of the total concentration of the dinucleotides digested by the enzyme in a given mixture, while the peak of each nucleoside can be used to estimate the concentration of its corresponding dinucleotide. In the case of only one solute analyzed, both the G>p and the nucleoside peaks are equally useful in the quantitative determination of the analyte, thus increasing the reliability of the

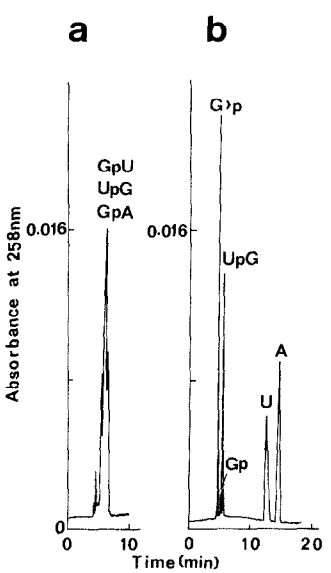

Fig. 4. Typical eletropherograms of dinucleotides and their RNase T_1 digest obtained by CZE alone (a) or by tandem RNase T_1 capillary enzyme reactor–CZE (b), respectively. UpG = Uridylyl-(3′→5′)-guanosine. Other symbols and conditions as in Fig. 3.

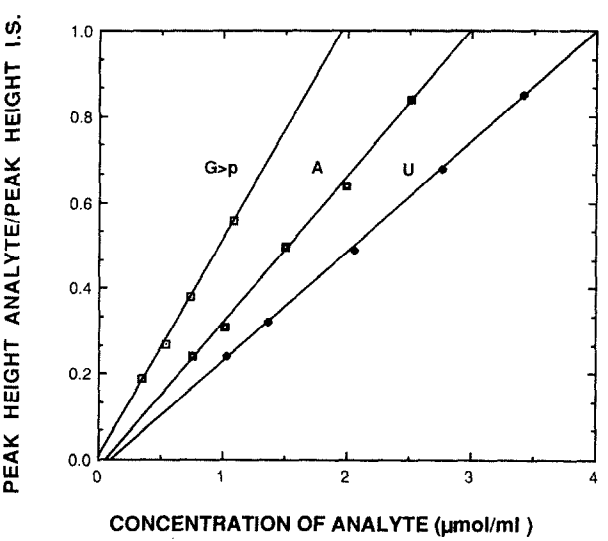

Fig. 5. Plots of the ratio of the peak height of the analyte to that of the internal standard (I.S.) as a function of the analyte concentration. Internal standard, 1,3-naphthalenedisulphonic acid disodium salt. Conditions and symbols as in Figs. 2 and 3.

method. For instance, a thin plug of an unknown solution of GpU was analyzed with the coupled capillary enzyme reactor-capillary zone electrophoresis following the same operational schemes outlined above. The calibration curves revealed on the average a concentration of 0.90 μmol/ml for G>p and 1.00 μmol/ml for U. By averaging these two values, the concentration of GpU in the unknown solution was about 0.95 μmol/ml. This illustrates the use of the capillary enzyme reactor in enhancing the reliability of quantitative determination by CZE.

In addition, the RNase T_1 capillary enzyme reactor–CZE system proved very efficient in the on-line digestion and mapping of transfer ribonucleic acid specific for phenylalanine ($tRNA^{Phe}$). The secondary structure of $tRNA^{Phe}$ is represented in Fig. 6. It comprises 76 nucleotide residues. The arrows in this figure indicate the 20 locations at which the enzyme splits the internucleotide bonds leading to the formation of 15 oligonucleotides and 1 guanosine monophosphate [21]. The digestion of $tRNA^{Phe}$ was carried out in a 20 cm long capillary enzyme reactor by allowing the substrate to contact the immobilized RNase T_1 for approximately 22 min while flowing hydrodynamically. Thereafter, the capillary enzyme reactor was disconnected and the RNase T_1 digest was mapped in the separation capillary using a running electrolyte of 0.1 *M* His, 0.1 *M* MES and 5 m*M* spermine, pH 7.0 (see Fig. 7). In this experiment, the ionic strength of the running electrolyte was increased in order to minimize electrostatic interactions between the oligonucleotide fragments and the amino-silica matrix and/or the enzyme. It can be envisioned that such format can allow (i) the quick and reproducible mapping of other native or modified tRNAs available only in minute quantities, and (ii) the conversion of ribonucleic acids to more electrophoretically manageable oligonucleotide fragments.

Immobilized ribonuclease T_1 as ligating enzyme. Another interesting feature of RNase T_1 is its ligating property. It can link cyclic G>p to a nucleoside and produce the corresponding dinucleotide. This aspect of the immobilized RNase T_1 has been previously investigated by El Rassi and Horváth [8] using tandem packed-bed enzyme reactor–high-performance displacement chromatography. To examine the effectiveness of tandem RNase T_1 capillary enzyme reactor–CZE in the synthesis and separation of ng quantities of dinucleotides, the following reaction was investigated:

$$G{>}p + U \overset{\text{RNase } T_1}{\rightleftharpoons} GpU \qquad (3)$$

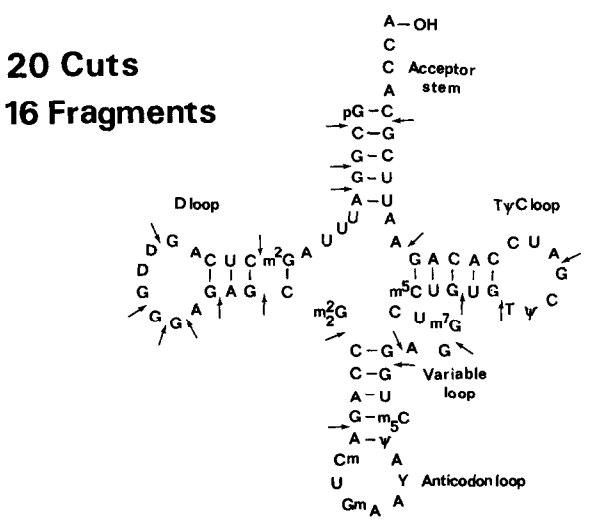

Fig. 6. Schematic representation of the secondary structure of transfer ribonucleic acid specific for phenylalanine (yeast $tRNA^{Phe}$). The arrows in this figure indicate the 20 locations at which RNase T_1 splits the internucleotide bonds.

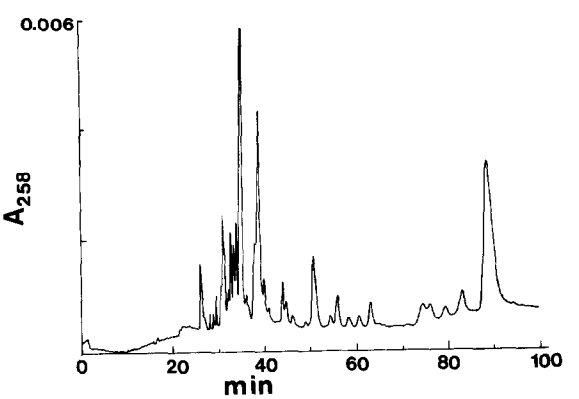

Fig. 7. On-line digestion and mapping of $tRNA^{Phe}$ by tandem RNase T_1 capillary enzyme reactor–CZE. Capillary enzyme reactor, 20 cm × 50 μm I.D.; separation capillary, interlocked polyether coating, 50 cm (to the detection point), 80 cm (total length) × 50 μm I.D.; running voltage, 15 kV; background electrolyte, 0.1 *M* His, 0.1 *M* MES, 5 m*M* spermine, pH 7.0. Substrate introduction, enzymic reaction and separation steps as in Fig. 3.

In contrary to the digestion reaction which occurs mostly in the forward direction, the ligation process requires the use of an excess of one of the reactants to drive the equilibrium towards the formation of the dinucleotide. Studies in free solution reveal that under appropriate conditions and 24 h incubation time, only 40% of G>p is transformed into GpU [22]. Fig. 8a and b illustrates typical electropherograms of the reaction mixture obtained in the absence or presence of the RNase T_1 capillary enzyme reactor, respectively. Both the enzymic and separation processes were carried out in a buffer system of 25 m*M* His, 25 m*M* MES and 5 m*M* spermine, pH 5.5. The concentration of U was 5 times that of G>p and the condensation reaction was brought about by allowing a thin plug of both

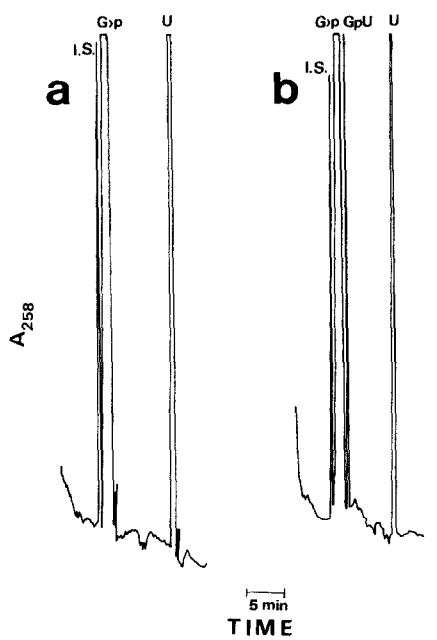

Fig. 8. Typical electropherograms of the RNase T_1 synthetic reaction mixture in the sbsence, *i.e.*, by CZE alone (a) or presence of RNase T_1 capillary enzyme reactor (b). Capillary enzyme reactor, 15 cm × 50 μm I.D.; separation capillary, interlocked polyether coating, 30 cm (to the detection point), 64 cm (total length) × 50 μm I.D.; substrate introduction, hydrodynamic mode; enzymic reaction was carried out using gravity-driven flow; separation step, as the plug of the reaction mixture entered the separation capillary, the enzyme reactor was disconnected and the voltage was turned on to 25 kV; background electrolyte, 25 m*M* His, 25 m*M* MES, 5 m*M* spermine, pH 5.5. Internal standard (I.S.), 1,3-naphthalenedisulphonic acid disodium salt. Initial concentration, [G>p] = 0.02 *M*, [U] = 0.10 *M*.

substrates to contact simultaneously the immobilized enzyme by gravity-driven flow. In such mode of bisubstrate enzymic reactions, where both substrates have different electrophoretic mobilities, the enzymic reaction was best achieved using hydrodynamic flow. In fact, under electromigration conditions, the negatively charged G>p would migrate downstream the enzyme reactor while uridine being neutral would migrate behind at a slower rate, and therefore both substrates are unable to get concurrently to the enzyme active sites.

The RNase T_1 catalyzed synthesis of GpU does not go to completion as manifested by the excess substrate peaks detected after the enzymic reaction (see Fig. 8b). It was then interesting to study the effect of various operational parameters on the yield of GpU. In that regard, a calibration curve was established for GpU using 1,3-naphthalenedisulphonic acid disodium salt as an internal standard. The plot of the peak height ratio of GpU to that of I.S. *vs.* the concentration of GpU showed a linear behavior up to a concentration of 0.7 mg/ml of the analyte. This calibration curve was then used to estimate the amount of GpU produced. The yield of GpU expressed in mol% of G>p converted into GpU was determined at various pH, initial substrate concentration and contact time using a thin plug of the substrate and a single pass reactor.

The effect of the electrolyte pH on the yield of GpU was determined over the pH range 4.0 to 7.0 in an increment of 0.5 pH unit. The results obtained are illustrated in Fig. 9 by a plot of the yield of GpU *vs.* pH. In this study, the enzymic reaction was carried out using gravity-driven flow and buffers of same

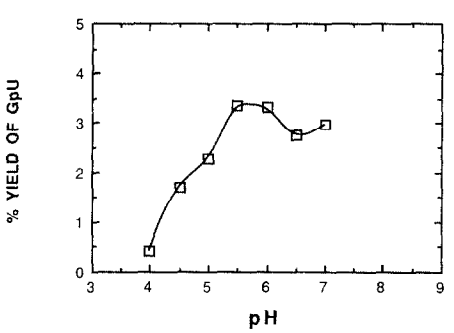

Fig. 9. Plot of the % yield of GpU as a function of the pH of the enzymic reaction. Conditions as in Fig. 8 except enzymic reaction was carried out with buffers of same composition as the running electrolyte but at various pH. See text for more details.

composition as the running electrolyte but at various pH. This means that both the capillary enzyme reactor and the separation capillary were first filled with the running electrolyte, pH 5.5 (*i.e.*, the separation pH). As the thin plug was introduced in the enzyme reactor, it was pushed through the immobilized capillary enzyme reactor with a buffer of the appropriate pH. When the reaction plug entered the separation capillary, the enzyme reactor was disconnected and the separation capillary was dipped into the separation buffer and the voltage was turned on to start the separation of the reaction mixture. Fig. 9 shows that the yield of GpU increased by a factor of 8 upon increasing the pH from 4.0 to 5.5. The optimum pH for the ligation reaction lies in the range 5.5 to 6.0 as opposed to 7.2 for the free enzyme [23]. This shift in the optimum activity of the immobilized enzyme toward more acidic pH values may be due to microenvironment effects and is in agreement with previous findings [24].

The effect of the initial substrate concentrations on the yield of GpU was investigated at pH 5.5. The initial concentrations of G>p and U were varied while keeping the molar concentration ratio of U to that of G>p equal to 5.0. These results are reported in Fig. 10. Upon increasing the concentration of G>p from 0.001 to 0.01 *M*, the yield of GpU increased from 1.2 to 4.7. However, at G>p concentrations greater than 0.01 *M* the yield of the synthesized GpU decreased monotonically. This may be explained by substrate inhibition due to system overloading. Experimental considerations such as peak overlapping limited the highest G>p concentration investigated to 0.04 *M*.

As mentioned above, the synthesis of GpU is a kinetically controlled process. Assuming Michaelis–Menten kinetics and under conditions of partial conversion, the integrated rate law can be written as [5]:

$$\frac{[P]}{[S_i]} = 1 - e^{-V_{max}\, t/K_M}$$

where [P] is the product concentration at the outlet of the reactor, $[S_i]$ is the initial substrate concentration, V_{max} is the maximum velocity of the enzymic reaction, t is the residence time of the substrate and K_M is the Michaelis constant. According to this equation, the yield of the product should increase with increasing both V_{max} and t or decreasing K_M. V_{max} has already been improved through the etching process that increases the catalytic activity of the wall. A lower K_M value will decrease the linear dynamic range of the enzyme reactor and thus limits its analytical applications. In addition, K_M cannot be controlled systematically. Thus, the simplest alternative to enhance the product yield is to increase the contact time of the substrate with the immobilized enzyme. This can be accomplished by either using longer enzyme reactor or decreasing the flow-rate for a given length of enzyme reactor. In this study, the differential height between the two electrolyte reservoirs was varied in order to obtain different gravity-driven flow-rates and consequently various contact times. Fig. 11 shows the yield of GpU *versus* the contact time of the substrates with the RNase T_1 capillary enzyme reactor. These results are in agreement with kinetic considerations. In fact, by increasing the contact time from 14 to 30 min, the yield of GpU increased by a factor of 1.5 in almost a linear

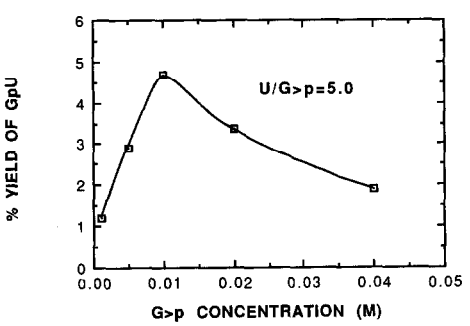

Fig. 10. Plot of the % yield of GpU as a function of the initial G>p concentration. Conditions as in Fig. 8.

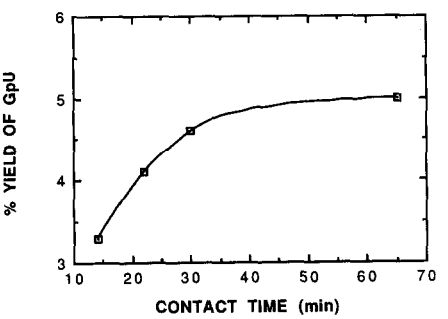

Fig. 11. Plot of % yield of GpU as a function of the contact time with immobilized RNase T_1. Conditions as in Fig. 8.

fashion and then levelled-off at higher contact times.

Besides the increase in the analysis time, a major concern of lower hydrodynamic flow velocity is its effect on the band width of the separated analytes. In open tubular systems, where longitudinal diffusion is the main source of bandspreading, longer residence time of the solute molecules leads to increased bandspreading. The number of theoretical plates of the GpU peak was measured at each contact time. These results are reported in Fig. 12 by a plot of the plate height as a function of the residence time in the enzyme reactor. As expected, the system efficiency decreased with increasing the contact time. However, increasing the contact time by a factor of 4 produced a decrease in the separation efficiency by a factor of 2 only.

It should be noted that under optimized conditions, approximately 10 ng of GpU can be produced from a single pass through the enzyme reactor. Such format employing simple CZE instrumentation can be easily automated and may prove useful in the micropreparative scale of important biochemicals, such as the synthesis of specific long oligonucleotides that are produced at low level by chemical synthesis methods. Such oligonucleotides are important in the study of mechanisms involving protein synthesis.

Hexokinase capillary enzyme reactor

Hexokinase is a relatively non-specific enzyme that catalyzes the phosphorylation of a wide variety of hexoses [25]. Its catalytic property was exploited in the HPLC separation and verification of the peak identities of ATP and ADP by treating the sample with the enzyme prior to the chromatographic separation [1]. This enzymatic reaction occurs via a random bi–bi mechanism in which the enzyme forms a ternary complex with glucose and Mg^{2+}–ATP before the start of the reaction [26]. Besides its orienting effect, the Mg^{2+} ions are thought to electrostatically shield the negative charges of the phosphate groups that would otherwise hinder the nucleophilic attack of the C(6)-OH group of glucose on the γ-phosphate of the Mg^{2+}–ATP complex. Thus, the overall net reaction is the transfer of a phosphoryl group from ATP to glucose to form glucose-6-phosphate and ADP as follows [25]:

$$\text{glucose} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{HK}} \text{glucose-6-phosphate} + \text{ADP} \quad (4)$$

where HK is hexokinase.

To evaluate the immobilized hexokinase capillary enzyme reactor, ATP was introduced as a thin plug into the tandem capillaries (*i.e.*, capillary enzyme reactor–CZE), which were equilibrated with a buffer of 25 m*M* His, 25 m*M* MES and 5 m*M* spermine, pH 7.0, containing both glucose and Mg^{2+} so that the immobilized enzyme is in continuous contact

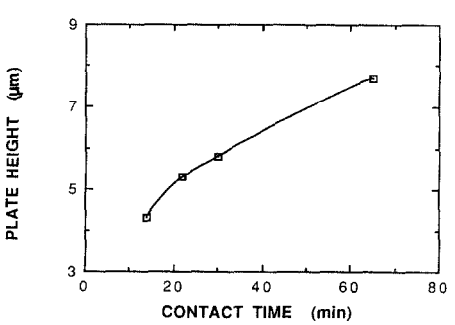

Fig. 12. Plot of the plate height of the synthesized GpU peak as a function of the contact time with the immobilized RNase T_1 enzyme. Conditions as in Fig. 8.

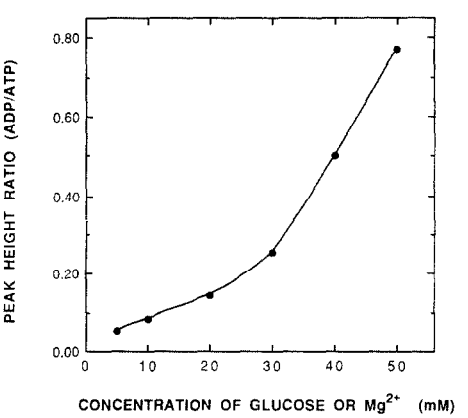

Fig. 13. Plot of the peak height ratio of ADP/ATP in arbitrary units as a function of the concentration of glucose or Mg^{2+} in the running electrolyte. Capillary enzyme reactor, 15 cm × 50 μm I.D.; separation capillary, interlocked polyether coating, 30 cm (to the detection point), 64 cm (total length) × 50 μm I.D.; substrate introduction, electromigration mode; tandem enzymic reaction–CZE was carried out at the same voltage, 25 kV; background electrolyte, 25 m*M* His, 25 m*M* MES, 5 m*M* spermine, pH 7.0 at different concentration of glucose and Mg^{2+}.

with one of the substrate (*i.e.*, glucose) and the metal ions required for the reaction. This allowed the reaction and separation to be carried out simultaneously at 25 kV. Under these conditions, the presence of spermine in the running electrolyte yielded an anodal electroosmotic flow and its effect on the magnitude and direction of the flow outweighed the influence of the immobilized enzyme on the wall of the capillary reactor. In the absence of spermine, one would expect that the electroosmotic flow to be affected primarily by the ionization of the immobilized enzyme (see below). Fig. 13 illustrates a plot of the peak height ratio of ADP/ATP in arbitrary units as a function of the concentration of glucose or Mg^{2+} in the running electrolyte. As expected, the magnitude of the conversion of ATP to ADP was a function of glucose and Mg^{2+} concentrations in the running electrolyte. As shown in Fig. 13, the peak height ratio of ADP/ATP increased by a factor of 16 upon increasing the concentrations of both glucose and Mg^{2+} by a factor of 10 (*i.e.*, from 5 to 50 m*M*). However, due to the increase in the viscosity of the buffer system at high glucose concentrations, the electrophoretic system suffered from prolonged analysis time which resulted in poor efficiency.

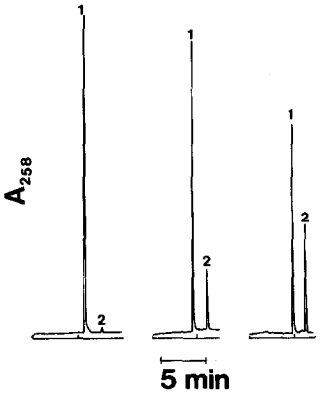

Fig. 14. Typical electropherograms of ATP (1) and ADP (2) at various contact time with the hexokinase capillary enzyme reactor. Capillary enzyme reactor, 15 cm × 50 μm I.D.; separation capillary, fuzzy polyether coating, 30 cm (to the detection point), 64 cm (total length) × 50 μm I.D.; substrate introduction, hydrodynamic mode; enzymic reaction was carried out using gravity-driven flow; separation step, as the plug of the reaction mixture entered the separation capillary, the enzyme reactor was disconnected and the voltage was turned on to 20 kV; background electrolyte, 0.1 *M* acetate containing 20 m*M* glucose and 10 m*M* Mg^{2+}, pH 5.0. Contact times of the substrate with the immobilized enzyme: left, 0 min (CZE only); middle, 10 min; right, 52 min.

In another set of experiments, ATP was allowed to migrate through the capillary enzyme reactor by a gravity-driven flow. This was aimed at studying the effect of contact time with the immobilized enzyme on the substrate conversion at constant temperature, a parameter that cannot be easily controlled when electromigration is used to vary the contact time of the substrates with the immobilized enzyme. Fig. 14 illustrates typical electropherograms of the products of the enzymic reaction at various contact time. They were carried out on a fuzzy 2000 polyether-coated capillary using 0.1 *M* acetate containing 20 m*M* glucose and 10 m*M* Mg^{2+}, pH 5.0, as the running electrolyte. Under these conditions, the electroosmotic flow was cathodal and its magnitude was relatively low. This is because the fuzzy polyether capillaries are characterized by relatively low electroosmotic flow [10], and the presence of heavy metal ions further reduced the flow [27]. This permitted the migration of the negatively charged nucleotides in the negative polarity mode without the inclusion of spermine in the background electrolyte. The electrophoretic velocity of the solutes was much greater than, and in opposite direction to, the electroosmotic flow. As can be noticed, at relatively low glucose concentration, the peak-height ratio of ADP/ATP increased almost 3 times by increasing the contact time of the substrate with the immobilized enzyme from 10 to 52 min. As in the case of RNase T_1 capillary enzyme reactor, the increase in the residence time of ATP in the enzyme reactor from 10 to 52 min, led to a drop in the separation efficiency by a factor of 2.4 and 1.8 for ATP and ADP, respectively. The tandem format hexokinase capillary enzyme reactor–CZE can be used to locate the ATP peak through its partial conversion to ADP and therefore can serve to confirm the presence or absence of ATP in a complex biological matrix.

Adenosine deaminase capillary enzyme reactor

Adenosine deaminase is a highly specific enzyme that catalyzes the deamination of adenosine to inosine with the liberation of NH_3 as follows [28]:

$$\text{adenosine} + H_2O \xrightarrow{\text{ADA}} \text{inosine} + NH_3 \qquad (5)$$

It was successfully immobilized on the inner walls

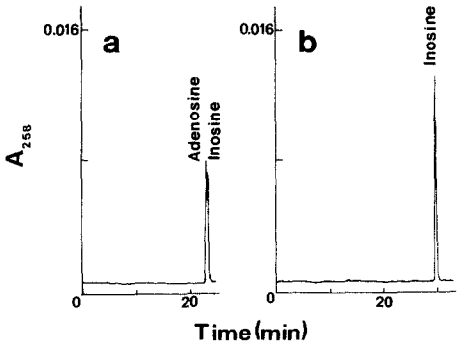

Fig. 15. Typical electropherograms of an equimolar mixture of adenosine and inosine obtained by CZE alone (a) or by tandem ADA capillary enzyme reactor–CZE (b). Capillary enzyme reactor, 15 cm × 50 μm I.D.; separation capillary, interlocked polyether coating, 30 cm (to the detection point), 64 cm (total length) × 50 μm I.D.; substrate introduction, eletromigration mode; tandem enzymic reaction–CZE was carried out at the same field strength, 190 V/cm; background electrolyte, 0.1 *M* phosphate, pH 6.5.

of a 15 cm long fused-silica capillaries as described under Experimental. The enzymic reaction was examined with a thin plug of an equimolar mixture of adenosine and inosine using 0.1 *M* phosphate, pH 6.5, as the background electrolyte. Fig. 15a and b illustrates the electropherograms of this mixture obtained on an interlocked polyether capillary in the absence or presence of the ADA capillary enzyme reactor, respectively. The enzymic reaction and the separation were carried out simultaneously at a field strength of 190 V/cm without disconnecting the enzyme reactor. Thus, the higher retention time of the inosine solute in Fig. 15b is simply the result of the extra length of the capillary enzyme reactor that the solute has to migrate. As can be seen in Fig. 15, for a short contact time of *ca.* 10 min, complete conversion of adenosine to inosine was obtained from a single pass through the reactor as manifested by the disappearance of the adenosine peak and a proportional increase in the peak height of inosine.

Since the enzymic reaction and separation were carried out simultaneously using tandem ADA capillary enzyme reactor–CZE, it was necessary to determine the effects of coupling a capillary enzyme reactor to a separation capillary both having different magnitude and sign of zeta potentials. Unlike in the case of HK capillary enzyme reactor–CZE where the direction and magnitude of the flow was mostly determined by spermine, in the case of ADA capillary enzyme reactor–CZE, the buffer did not contain any additive that would almost exclusively control the magnitude and direction of the flow. When operating the tandem capillary enzyme reactor–CZE under electromigration mode, the net electroosmotic flow of the tandem system was a function of the isoelectric point of the immobilized enzyme. In fact, as shown in Table I, at pH 6.5, *i.e.*, at pH higher than the isoelectric point of ADA (p*I* = 4.50–5.05), no significant change in the magnitude of the electroosmotic flow was observed upon connecting the capillary enzyme reactor to the separation capillary in series. However, as the pH of the running electrolyte approached the isoelectric point of the immobilized protein, a continuous drop in the electroosmotic flow was detected. The electro-

TABLE I

VALUES OF THE ELECTROOSMOTIC FLOW-RATE, EOF, AND PLATE HEIGHT, *H*, MEASURED FROM PHENOL PEAK

The measurements were performed on interlocked polyether capillaries, I-200, and tandem ADA capillary enzyme reactor-interlocked polyether capillary, ADA–I-200, at various pH values of the running electrolyte. ADA capillary enzyme reactor; 15 cm total length × 50 μm I.D., cennected to an interlocked polyether capillary of 64 cm (total length) and 30 cm to the detection point; I-200 capillary, two connected interlocked polyether capillaries of 15 and 64 cm in length, respectively; electrolytes, 0.1 *M* phosphate at different pH; field strength, 190 V/cm.

Capillary type	pH 6.50		pH 5.50		pH 5.00		pH 4.50	
	EOF (nl/min)	*H* (μm)	EOF (nl/min)	*H* (μm)	EOF (nl/min)	*H* (μm)	EOF (nl/min)	*H* (μm)
I-200	37.5	8.47	29.9	10.1	17.7	12.8	8.0	19.0
ADA–I-200	36.3	8.18	30.4	10.3	12.6	18.6	–	–

osmotic flow was reduced by 30% at pH 5.0 and at pH 4.5 there was practically no flow. This can be attributed in part to the fact that the net charge of the immobilized protein becomes positive at a pH lower than its isoelectric point. It should be noted that the unreacted amino groups of the aminopropylsilyl coating could also contribute to the reduction of the flow and inverting its direction at pH 4.5. As can be seen in Table I, the plate height measured from the peak of phenol increased from 12.8 to 18.6 μm when going from the I-200 to the ADA-I-200 capillary at pH 5.0. This is because the solute stayed longer in the ADA-I-200 capillary. This study revealed interesting fundamental points concerning the operation of capillary with immobilized enzymes on the inner walls in tandem with CZE. Since the flow was unaffected at the pH of maximum enzyme activity (*i.e.*, pH 6.5), such an arrangement can be exploited without any adverse effects on separation.

The ADA capillary enzyme reactor converted its substrate to a product that is more readily separated from other nucleosides. Fig. 16a depicts the electropherogram of a mixture of 3 nucleosides, namely cytidine, adenosine and inosine. Under these conditions both cytidine and adenosine practically coeluted but were resolved from inosine. However, after passing the mixture through the ADA capillary enzyme reactor, two well resolved peaks for cytidine and inosine were obtained (see Fig. 16b). Besides playing the role of peak locator, the ADA capillary enzyme reactor can facilitate the quantitative determination of the analyte of interest. For instance, in situations similar to Fig. 16, the cytidine solute can be determined with good accuracy, since the previously overlapping peak of adenosine has been completely converted to inosine. In addition, the increase in the peak height of inosine can be used for the quantitative determination of adenosine.

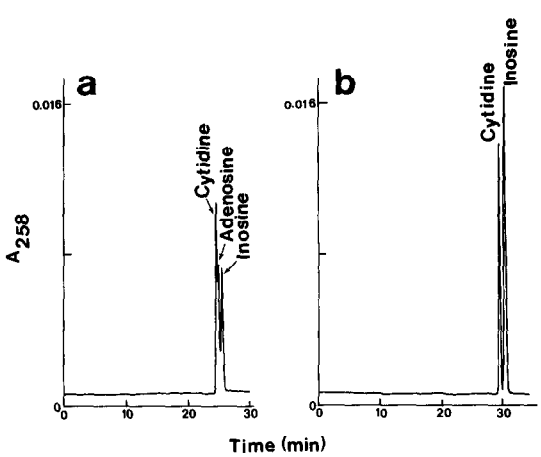

Fig. 16. Typical electropherograms of a mixture of cytidine, adenosine and inosine obtained by CZE alone (a) or by tandem ADA capillary enzyme reactor–CZE (b). Conditions as in Fig. 15.

It should be noted that the conversion of adenosine to inosine was complete even when the ADA capillary enzyme reactor was shortened to 3 cm, which correspond to a contact time of *ca.* 2 min with the enzyme reactor. From this finding, and provided that the enzymic reactions are kinetically favored, various short capillary enzyme reactors having different immobilized enzymes can be connected in series at the inlet of a separation capillary and may prove useful in the specific analysis of different solutes in a complex biological mixture.

CONCLUSIONS

In summary, the coupling of immobilized capillary enzyme reactors to capillary zone electrophoresis has proved suitable in the area of nucleic acids. The coupled format, which we refer to as enzymophoresis can be regarded as separation-based sensors with superimposed selectivities. While the enzyme provides the selective conversion of the substrates, CZE with its unique selectivity separates and detects the products. The various concepts developed and tested in this report can be transposed to electrophoretic systems involving other types of species of relevance to many areas of the life sciences and biotechnology. The enzymophoresis systems in miniature have provided the following: (i) repeated use and long term stability of enzymes, (ii) conversion of unseparable analytes to well resolved products (iii) peak identification of the analyte of interest, (iv) simultaneous synthesis and separation of ng quantities of biological species, (v) improved the reliability of CZE in the quantitative determination of analytes, and (vi) on-line digestion and mapping of biopolymers. The fact that enzymophoresis involving coupled HCER–CZE lends itself to automation will add another dimension to the capability of CZE in many areas of the life sciences.

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