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CHARACTERIZATION OF A POST-COLUMN REACTION–LASER-INDUCED FLUORESCENCE DETECTOR FOR CAPILLARY ZONE ELECTROPHORESIS

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SUMMARY

Several modifications have been made to a post-column labeling system for use with capillary zone electrophoresis. Fluorescence excitation is now performed with a helium-cadmium laser rather than an arc lamp. The focusability of the laser beam allows the use of larger diameter capillaries in the post-column reactor without the excessive band broadening observed previously. These larger capillaries can be assembled in the reactor much more easily. Another improvement is that the flow-rate of the labeling reagent can now be accurately controlled and determined. Incorporating these changes, the performances of two reactors with capillaries of the same dimensions are compared.

INTRODUCTION

An area of current interest is the use of capillary zone electrophoresis (CZE) to separate and detect peptides and proteins. Peptide and protein detection with CZE has been performed by using UV absorption[1–5], fluorescence[6,7], indirect fluorescence[8] and mass spectrometic[9,10] detectors. Of these four methods, fluorescence offers the highest sensitivity in terms of mass detection limits. Unfortunately, not all peptides and proteins are intrinsically fluorescent. For this reason, labeling or tagging techniques are of interest.

Pre-column labeling of compounds is simple to perform, but poses a problem with the detection of large peptides and proteins in CZE. Labeling reagents that react with amine groups are popular for peptide and protein detection because almost all of these analytes have an amine group available for labeling. However, most of them have more than one amine group; there is the N-terminal α-amine group and the ε-amine groups of the lysine side-chains. These analytes can therefore tag at more than one site. It is important to note that labeling the amine group may involve a change in charge from the original $-NH_3^+$ group to a neutral or negatively charged group. As CZE separates analytes on the basis of their mobility, which in turn is a function of analyte size and net charge, this technique can distinguish between two molecules of one protein which have a different number of groups labeled. A single protein can therefore result in a multiple number of overlapping peaks[1]. Fig. 1 shows

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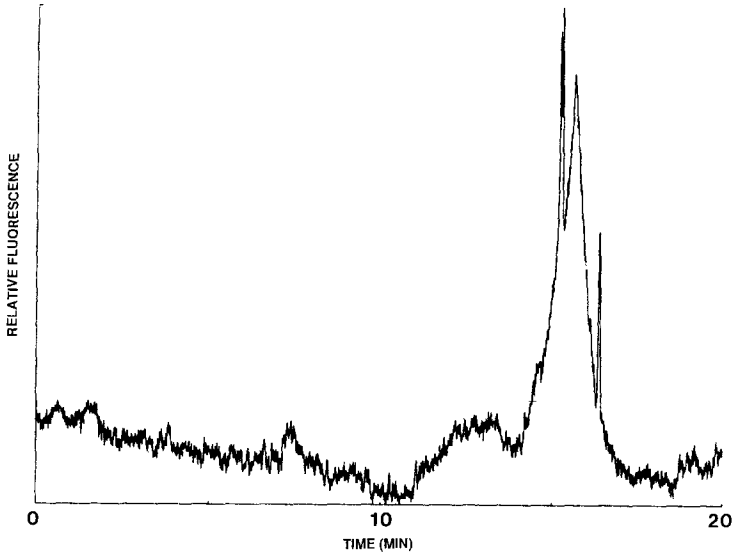

Fig. 1. Pre-column labeling of $5.3 \cdot 10^{-6}$ *M* horse heart myoglobin. Column: I.D., 50 μm; O.D., 150 μm; total length, 100 cm; length to detector, 70 cm. Reaction conditions: 150 μl of $5.3 \cdot 10^{-5}$ *M* myoglobin plus 1350 μl of reagent mixture [15 mg of OPA, 400 μl of absolute ethanol and 1.0 μl of 3-mercaptopropionic acid in a total of 10 ml using methanol–(0.01 *M* phosphate–0.02 *M* KCl, pH 7.0) (25:75)]; injected 25 s after mixing. CZE conditions: buffer, 0.01 *M* boric acid–0.02 *M* KCl, pH 9.5; injection, 10 kV for 3 s; applied run potential, 25 kV with 15.5 μA; 3-Hz resistance/capacitance (RC) low-pass filter.

an example of this problem. Horse heart myoglobin, which has 19 lysines, was pre-column labeled with *o*-phthaldialdehyde at pH 7.0. At this pH it would seem that few ε-amino groups would be unprotonated and available for tagging. However, a broad peak, the result of many overlapping peaks, is observed and is indicative of the presence of more than one labeled species of protein.

The problem of sensitive detection in CZE has led to the development of several post-column reactors for use with CZE[6,11–13]. One of these post-column reactors[6] consists of two coaxial fused-silica capillaries housed in a stainless-steel tee. A third fused-silica capillary introduces the labeling reagent. Using this reactor and *o*-phthaldialdehyde as a labeling reagent, amino acids and proteins were analyzed with peak efficiencies on the order of 50 000 theoretical plates[6]. This post-column labeling system does, however, have a few disadvantages. As an arc lamp-based fluorescence detector was used, coaxial capillaries with small and closely matched inner and outer diameters were necessary in order to avoid excessive band broadening. These capillaries had thin, fragile walls, which made them difficult to assemble in the post-column reactor. In addition, the labeling reagent was forced through the reactor using only the force of gravity. Flow-rates, therefore, could not be accurately determined. This paper describes a few modifications that have been made to this post-column labeling system. In addition, further characterization of the reactor was performed.

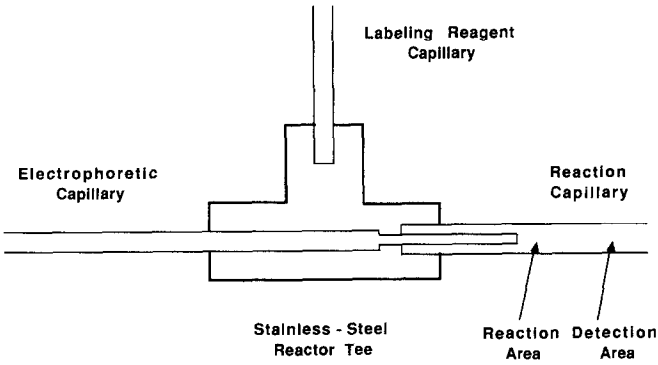

Fig. 2. Cross-sectional view of the post-column reactor.

EXPERIMENTAL

Post-column reactor

The post-column reactor shown in Fig. 2 has been described previously[6]. The reactor consists of three fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, U.S.A.) held in a stainless-steel tee (Swagelok, Solon, OH, U.S.A.) by Vespel ferrules (Alltech, Deerfield, IL, U.S.A.). These capillaries are referred to as the electrophoretic, reaction and reagent capillaries. The post-column reactor is shown in the overall CZE set-up in Fig. 3. Helium pressure is used to pump the fluorescent labeling reagent into the reactor and through the reaction capillary where it can react with analyte molecules migrating out of the end of the electrophoretic capillary. The fluorescent derivative is then detected further down the reaction capillary where a 1.5-cm portion of the polyimide coating has been removed to form a window for on-column fluorescence detection.

Two post-column reactors, referred to as reactors 1 and 2, were studied. Reactor 1 uses a 50-μm I.D. and 150-μm O.D. electrophoretic capillary. The end of this capillary, which is inserted into the reaction capillary, was etched over a 10.0-cm

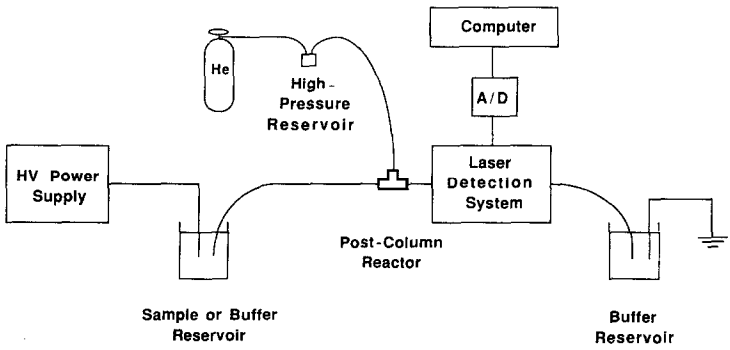

Fig. 3. Capillary zone electrophoresis system with the post-column reactor. HV = High voltage; A/D = analog-to-digital converter.

length with hydrofluoric acid[6] to 83-μm O.D. Reactor 2 has an electrophoretic capillary of 50-μm I.D. and 150-μm O.D. and is etched to 81-μm O.D. over a 10.3-cm length. In reactor 1, 9.2 cm of the electrophoretic capillary is inserted into the reaction capillary, whereas in reactor 2, 9.5 cm of the electrophoretic capillary is inserted into the reaction capillary. Both reactors have a 40-cm long reaction capillary of 100-μm I.D. and 375-μm O.D. and a 100-cm long reagent capillary of 25-μm I.D. and 150-μm O.D.

The reactor is flushed and filled daily with fresh labeling reagent by using high pressure (800 p.s.i.) to force the reagent through the reactor. The high pressure is maintained long enough to cycle 5–6 tee volumes (34 μl) through the reactor. After this time no bubbles are observed leaving the capillaries unless there is an air leak in the system.

Instrumentation

The CZE apparatus has been described previously[1,14]. A ±30 kV power supply (Spellman High Voltage Electronics, Plainview, NY, U.S.A.) was used to perform the electrophoretic separations and electromigration injections. A helium–cadmium laser with lines at 325 nm (1.5 mW) and 442 nm (10 mW) was used in a detection system decribed previously[15]. Data were collected at a rate of 3 points per second by a Zenith personal computer via a 16-bit analog-to-digital interface board (Scientific Solutions, Solon, OH, U.S.A.). Migration times and numbers of theoretical plates were calculated by computer using statistical moments.

Samples and reagents

o-Phthaldiadehyde (OPA), phenylalanine, horse heart myoglobin and β-mercaptoethanol were purchased from Sigma (St. Louis, MO, U.S.A.). Naphthalenedicarboxaldehyde was purchased from Molecular Probes (Eugene, OR, U.S.A.). Boric acid and sodium cyanide were purchased from Aldrich (Milwaukee, WI, U.S.A.).

The post-column OPA reagent mixture consisted of 15 mg of OPA, 400 μl of absolute ethanol, 8.4 μl of β-mercaptoethanol plus sufficient buffer to give a total volume of 12 ml. The buffer was 0.01 *M* boric acid–0.02 *M* potassium chloride (pH 9.5). The pH of the reagent mix is readjusted to 9.5 with 5% NaOH after the addition of the thiol and before reaching the final volume.

RESULTS AND DISCUSSION

Post-column reactor

The post-column reactor was recently introduced[6], but since then a few modifications have been made to improve its performance and ease of use. The first change in the post-column system is the use of a different detection system. A helium–cadmium laser-based detector is now utilized instead of an arc lamp-based fluorescence detector. The excellent focusability of a laser beam allows the use of larger diameter capillaries than previously used[6] while maintaining good peak efficiencies. These larger capillaries are much more easily assembled into the stainless-steel reactor tee. Another modification to the system is the use of helium pressure as a constant-flow pump to force the labeling reagent through the reactor.

The flow-rate of the reagent through the reactor and the reaction capillary at a

given pressure can be calculated by using the Poiseuille equation and treating the post-column system as a series of three flow resistances. The first region of flow resistance is the reagent capillary, the second region is the annular region where the electrophoretic capillary is inserted in the reaction capillary and the third region is the remaining portion of the reaction capillary. The fraction of the total pressure drop across each of these three regions corresponds to the fraction of the total flow resistance of the system that the region represents.

The volume flow-rate F of a solution of viscosity η through a cylinder of radius r and length L for a given pressure drop ΔP, is given by the equation[16]

$$F = \pi r^4 \Delta P / 8\eta L \tag{1}$$

The corresponding flow-rate through the cylindrical annular region of the post-column reactor defined by the inner radius of the reaction capillary and the outer radius of the electrophoretic capillary is given by the equation[16]

$$F = [\pi r^4 \Delta P/(8\eta L)][(1 - K^4) - (1 - K^2)^2/\ln(1/K)] \tag{2}$$

where r corresponds to the inner radius of the reaction capillary and K is the ratio of the outer radius of the electrophoretic capillary to the inner radius of the reaction capillary. Using eqns. 1 and 2, ratios of ΔP for each of the three regions as a function of r, L and K can be calculated. These ratios show that when a 100 cm × 25 μm I.D. reagent capillary is used with an electrophoretic and reaction capillary of the dimensions mentioned previously, 95%, 5% and 0% of the pressure drops across the reagent capillary, the annular region and the reaction capillary, respectively. Dropping most of the pressure across the reagent capillary insures that for a given pressure the flow-rate is constant and does not change with slight variations in the annular region. The flow-rates for reactors 1 and 2 at a given helium pressure are, therefore, the same. One can therefore calculate the actual flow-rate of the labeling reagent by using the Poiseuille equation with the reagent capillary.

Table I lists the flow-rates of the labeling reagent for different applied pressures of helium. Flow-rates are given in terms of volumetric and linear rates through the reaction capillary. In addition, the mean time that it takes for the reagent to travel a typical reaction distance of 0.5 mm through the reaction capillary is given. The values in the top part Table I are accurate when there is no applied potential. If a potential is applied, then electroosmotic flow exists in the electrophoretic capillary and contributes to the flow-rate of the reagent through the reaction capillary. Using a typical electroosmotic flow rate of 2.1 nl/s in the direction of the grounded electrode for an applied potential of 15 kV, as determined by a neutral marker, values were recalculated and shown in the bottom part of Table I. These values show that the time it takes for an analyte molecule to travel 0.5 mm from the tip of the electrophoretic capillary to where the laser beam is focused is 1.5 s or less for all the flow-rates studied. Rapidly reacting labeling reagents are therefore essential when using such short reaction distances. It is possible to increase the reaction time by increasing the reaction distance, but the peak efficiency will decrease owing to increasing post column band broadening.

TABLE I

FLOW-RATES IN THE REACTION CAPILLARY FOR VARIOUS HELIUM PRESSURES WITH AND WITHOUT AN APPLIED ELECTROPHORETIC POTENTIAL AND THE MEAN TIME TO TRAVEL A TYPICAL REACTION DISTANCE OF 0.5 mm

Electroosmotic flow (*nl/s*)	*Helium pressure* (*p.s.i.*)	*Flow-rate in reaction capillary*		*Time to travel 0.5 mm* (*s*)
		nl/s	*mm/s*	
0	10	0.6	0.08	6.3
	30	1.9	0.24	2.1
	40	2.5	0.34	1.6
	50	3.1	0.39	1.3
	70	4.4	0.56	0.9
2.1	10	2.7	0.34	1.5
	30	4.0	0.51	1.0
	40	4.6	0.59	0.9
	50	5.2	0.66	0.8
	70	6.5	0.83	0.6

Post-column reagents

OPA is a non-fluorescent reagent which reacts with primary amines in the presence of a thiol. The resulting fluorescent product has an excitation maximum at 340 nm and an emission maximum at 455 nm[17,18]. The reaction is shown in Fig. 4. OPA is a rapidly reacting reagent, having a typical half-time of reaction of 4 s[19]. For this reason it has been succesfully used by several groups as a post-column reagent[6,11,20–22] with high-performance liquid chromatography and CZE.

Naphthalene-2,3-dicarboxaldehyde (NDA), an analogue of OPA, was briefly tested as a post-column reagent. Its reaction with a primary amine is shown in Fig.

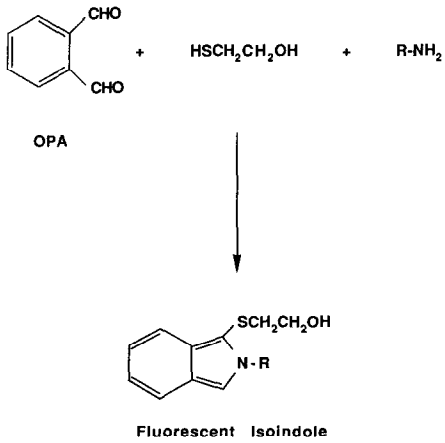

Fig. 4. Reaction of OPA with a primary amine.

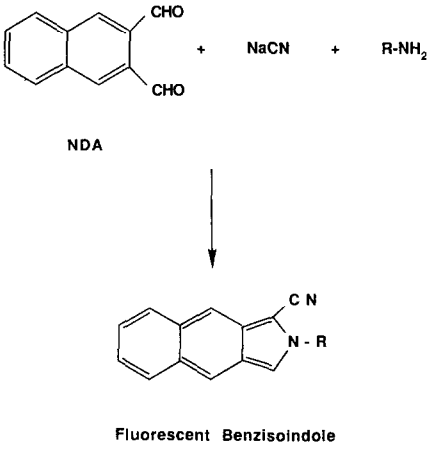

Fig. 5. Reaction of NDA with a primary amine.

5[23,24]. NDA has a slower reaction time than OPA[25], but it appeared that this disadvantage might be offset by a few of the other characteristics of NDA derivatives. For example, NDA derivatives have an excitation maximum at 440 nm, which is an excellent match for the 442-nm line of the helium–cadmium laser. This line has a higher intensity than the 325-nm laser line used to excite OPA derivatives. In addition, NDA peptide derivatives have a higher quantum efficiency than their corresponding OPA derivatives. Our results, however, showed that NDA-labeled horse heart myoglobin yields a 400-fold lower signal-to-noise ratio than does the corresponding OPA derivative when the same reagent flow-rate and reaction distance are used. Extensive work was therefore not performed with NDA as a post-column reagent. OPA with β-mercaptoethanol was used as the labeling reagent for the work described in this paper.

Reagent flow-rate and reaction distance

The effect of reagent flow-rate and reaction distance was studied. The reaction distance is defined as the distance from the tip of the electrophoretic capillary to the point down the reaction capillary where the laser beam is focused. Fig. 6 shows peak area and peak efficiency as a function of OPA flow-rate through the reactor (due to helium pressure) and reaction distance for $10^{-4}M$ phenylalanine for reactor 1. An amino acid is used instead of a protein as a test substance to eliminate the effects of possible adsorption of the analyte on the capillary walls[1]. As expected, the peak area increases with increasing reaction distance for a given flow-rate because, in effect, the reaction time is increasing. In general, as the flow-rate increases for a given reaction distance, the peak area decreases as a result of a shortening of the reaction time and a dilution effect. A maximum signal, however, is observed at a reagent flow-rate of 1.9 nl/s. The reason for this maximum is not known, although it is interesting that this reagent flow-rate closely matches that of the electroosmotic flow.

There appears to be no dominant trend for the dependence of the peak efficiency on the flow-rate or reaction distance. As the reaction distance increases above 0.50 mm, the peak efficiency decreases as expected, owing to broadening in the flow

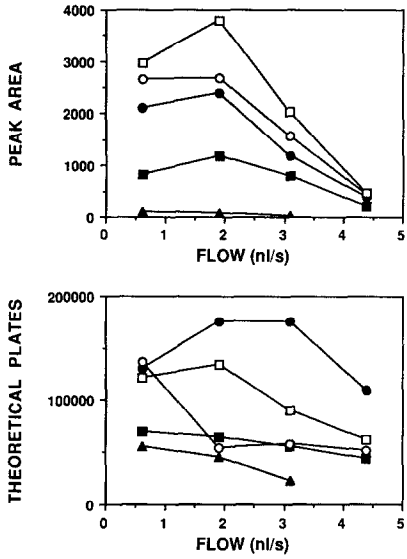

Fig. 6. Peak area (arbitrary units) and peak efficiency as a function of OPA flow-rate through the reactor and reaction distance for 10^{-4} *M* phenylalanine using post-column reactor 1. Conditions: buffer, 0.01 *M* boric acid–0.02 *M* KCl, pH 9.5; injection, 5 kV for 2 s; applied run potential, 30 kV with 26.5 μA; 3-Hz RC low-pass filter. Reaction distance: ▲ = 0; ■ = 0.25; ● = 0.50; ○ = 0.75; □ = 1.00 mm.

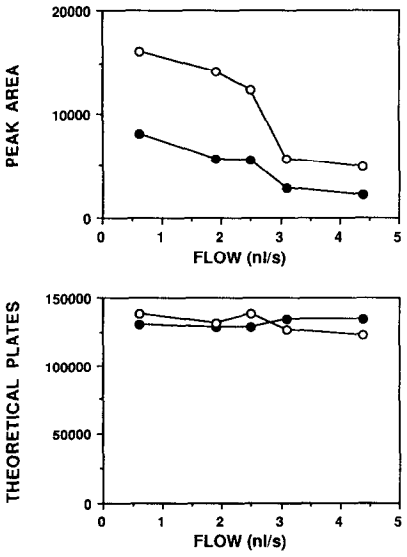

Fig. 7. Peak area (arbitrary units) and peak effeciency as a function of the OPA flow-rate through the reactor and reaction distance for 10^{-4} *M* phenylalanine using post-column reactor 2. Conditions: buffer, 0.01 *M* boric acid–0.02 M KCl, pH 9.5; injection, 15 kV for 2 s; applied run potential, 15 kV with 22.0 μA; 3-Hz RC low-pass filter. Reaction distance: ● = 0.50; ○ = 0.75 mm.

profile in the larger reaction capillary. The peak efficiency, however, is a maximum at a 0.50 mm reaction distance. At 0.00 mm, *i.e.*, when the laser beam is focused at the very end of the tip of the electrophoretic capillary, and at 0.25 mm downstream, the signal is small owing to the short reaction time. There is therefore an appreciable amount of noise superimposed on the signal. It is possible that the peak efficiencies which are calculated by computer using statistical moments are lower than expected owing to this noise interfering with the measurement and calculation.

The same factors were investigated for reactor 2 and the results are shown in Fig. 7. With this reactor only two reaction distances were studied. The same trends in peak area as for reactor 1 are observed. The behavior of peak efficiency, however, differs considerably. As Fig. 7 shows, the peak efficiency remains relatively constant over the range of flow-rates and between the two reaction distances. The performance appears to vary from reactor to reactor even when the capillary dimensions and flow-rates are comparable. The positioning of the electrophoretic capillary in the reaction capillary may be a key factor. For instance, there is an approximately 17–20-μm difference between the diameters of the electrophoretic and reaction capillaries. It is likely that the electrophoretic capillary does not lie coaxially in the center of the reaction capillary. Different flow patterns around the tip of the electrophoretic capillary may result, depending on the positioning of the electrophoretic capillary. These flow patterns will in turn affect the measured peak efficiency. In addition, the tip of the electrophoretic capillary may not be cleanly severed[6]. This too may effect the flow patterns and subsequently measured peak efficiency.

LINEARITY AND DETECTION LIMIT

The linearity of response of the post-column reactor was evaluated using horse heart myoglobin (HHM). A log–log plot of peak area *vs.* concentration is shown in Fig. 8 for the concentration range $5.3 \cdot 10^{-6}$–$4.2 \cdot 10^{-8}$ *M* HHM. A linear least-squares fit yields a line with a slope of 0.958 and $r^2 = 0.997$. At higher concentrations the peak shape is very non-Gaussian, suggesting concentration overloading[1]. The electropherogram of $4.2 \cdot 10^{-8}$ *M* HHM is shown in Fig. 9. The protein has a migration time of just under 13 min and a peak efficiency of $2.5 \cdot 10^5$ theoretical plates. Extrapolation to a signal-to-noise ratio of 3, where the noise level is the RMS noise of

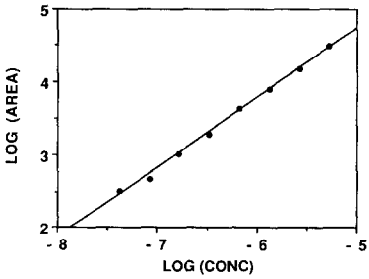

Fig. 8. Study of signal *vs.* concentration of horse heart myoglobin, $5.3 \cdot 10^{-6}$–$4.2 \cdot 10^{-8}$ *M*, using the post-column reactor (see Fig. 2). Conditions: buffer, 0.01 *M* boric acid–0.02 *M* KCl, pH 9.5; injection, 15 kV for 3 s (3.7-nl injection volume); applied run potential, 15 kV with 24.5 μA; reaction distance, 0.75 mm; OPA flow-rate, 4.0 nl/s; 3-Hz RC low-pass filter.

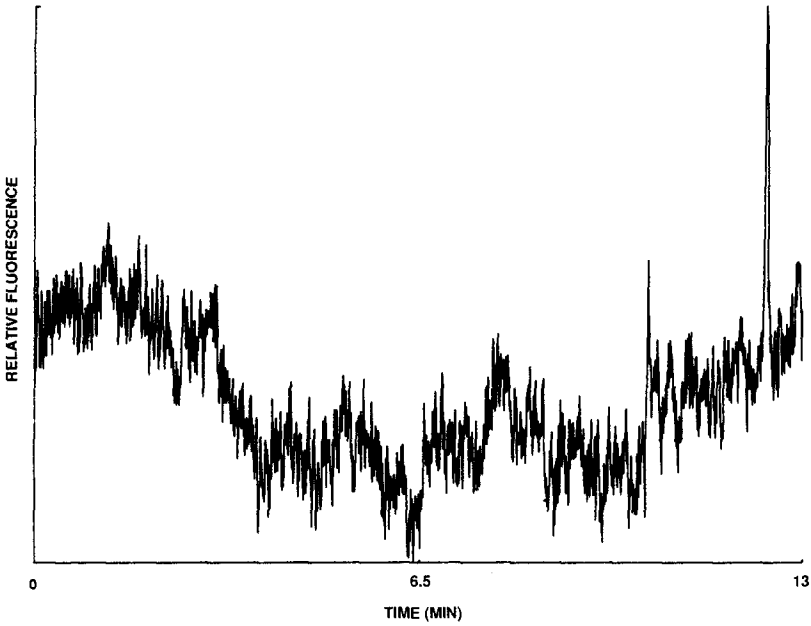

Fig. 9. Result for $4.2 \cdot 10^{-8}$ *M* horse heart myoglobin using reactor 2. Conditions as in Fig. 8.

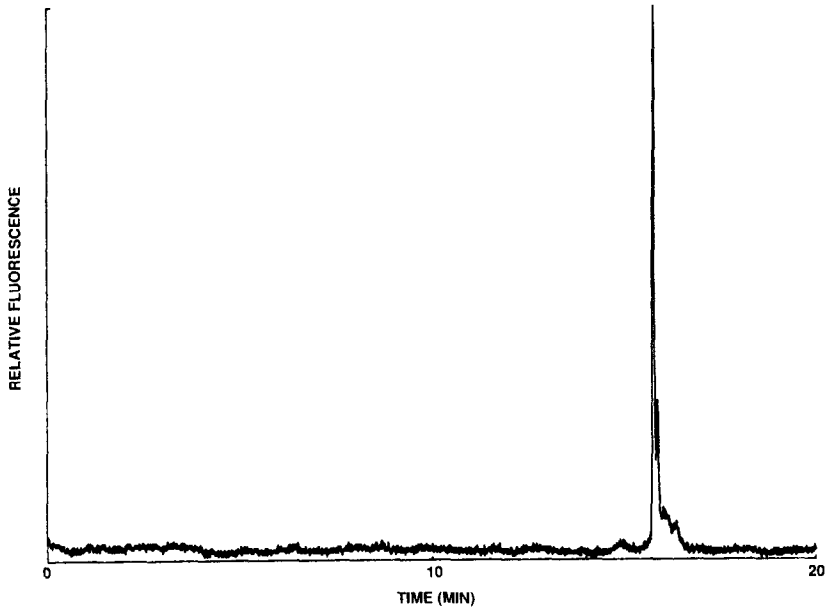

Fig. 10. Result for $5.3 \cdot 10^{-6}$ *M* horse heart myoglobin using reactor 1. Conditions: buffer, 0.01 *M* boric acid–0.04 *M* KCl, pH 9.5; injection, 5 kV for 2 s; applied run potential, 15 kV; reaction distance, 0.50 mm; OPA flow-rate, 4.0 nl/s.

a 5.1-min segment of the baseline in front of the peak, gives a calculated detection limit of $1.2 \cdot 10^{-8}$ M or $4.4 \cdot 10^{-17}$ mol. injected. Impurities in the sample of HHM are evident in Fig. 10, which shows a run with $5.3 \cdot 10^{-6}$ *M* HHM. At run potentials of 20 and 25 kV the impurities appear as shoulders on the protein peak, but in a run at 15 kV distinct peaks can be observed. A peak efficiency of $6.0 \cdot 10^5$ theoretical plates is calculated for this HHM peak.

The modification of the previously described post-column reactor results in improved performance and ease of assembly. The use of a laser, rather than an arc lamp, as an excitation source permits the use of larger diameter and less fragile capillaries, which are easier to assemble than those used previously[6]. In addition, a factor of ten improvement in peak efficiency ($6.0 \cdot 10^5$ *vs.* $5.0 \cdot 10^4$ theoretical plates) can be obtained. The use of larger diameter capillaries, however, adversely effects the mass limit of detection owing to the larger injection volumes. However, the use of a laser as an excitation source helps to counteract this effect. In this study a detection limit of 44 amol ($1.2 \cdot 10^{-8}$ *M*) horse myoglobin was obtained with a 50-μm I.D. electrophoretic capillary and 100-μm O.D. reaction capillary combination and an electromigration injection of 15 kV for 3 s. The previous detection limit obtained for whale myoglobin with a 25-μm I.D. electrophoretic capillary and 50-μm I.D. reaction capillary combination with an electromigration injection of 30 kV for 2 s was 22 amol ($1.3 \cdot 10^{-8}$ *M*). The concentration detection limits are almost identical in the two systems. An unfortunate disadvantage of this particular post-column reactor is the variation of performance, in terms of peak efficiencies, from reactor to reactor as a function of reagent flow-rate and reaction distance. The reactor, however, does allow one to avoid the multiple peaks which are observed in pre-column protein analysis with CZE.

ACKNOWLEDGEMENT

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