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Journal of Chromatography A, 852 (1999) 597–601

JOURNAL OF CHROMATOGRAPHY A

Short communication

Chemiluminescence detection of heme proteins separated by capillary isoelectric focusing

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Received 11 January 1999; received in revised form 17 May 1999; accepted 17 May 1999

Abstract

Chemiluminescence detection was combined with capillary isoelectric focusing to perform protein analysis with high sensitivity. Luminol–H_2O_2 chemiluminescence was utilized, and heme proteins such as cytochrome *c*, myoglobin and peroxidase were analyzed. The proteins were focused by use of Pharmalyte 3-10 as ampholytes. Hydroxypropylmethylcellulose was added to the sample solution in order to easily reduce protein interactions with the capillary wall as well as the electroendoosmotic flow. The focused proteins were transported by salt mobilization to chemiluminescence detection cell equipped with an optical fiber. The present method showed significantly high sensitivity and wide dynamic range; the detection limit for cytochrome *c* was $6 \cdot 10^{-9}$ *M* and the linear dynamic range was greater than two-orders of magnitude (up to $2 \cdot 10^{-6}$ *M*).

Keywords: Isoelectric focusing; Chemiluminescence detection; Detection, electrophoresis; Proteins

1. Introduction

Chemiluminescence (CL) detection in capillary electrophoresis (CE) has attracted much attention as a promising way to offer excellent analytical selectivity and sensitivity. Several CL reagents, such as luminol [1–6], acridinium [7], peroxyoxalate [8–11], and tris(2,2′-bipyridine)ruthenium(II) complex [12–14], have been utilized. By use of the CL reagents, various samples, e.g., metal ion, amino acid, peptide, protein and alkaloid, could be detected with high sensitivity in CE. CL detection was approximately 10^2- to 10^6-times more sensitive than spectrophotometric and fluorometric detections. However, when one takes notice of separation modes in the CE–CL detection method, capillary zone electrophoresis (CZE) has been used in almost all works. Only micellar electrokinetic chromatography (MEKC) has been applied as another separation mode in several studies [15,16]. Therefore, other separation modes, which have not been attempted yet in CE–CL detection system, should be examined in order to make the system more useful and applicable.

It is well known that isoelectric focusing (IEF) possesses a high separation ability for amphoteric compounds, e.g., proteins and peptides. They are separated according to their isoelectric points (p*I*) in a pH gradient generated by applying an electric field onto carrier ampholytes. Many reports about IEF and their results indicate that IEF is no doubt one of the most powerful and useful separation methods. Recently, capillary IEF (cIEF) has also been performed.

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00645-7

The new approaches provided an excellent resolution and simple procedure. After completion of focusing, analytes were transported to a detection point by use of salt or hydrodynamic mobilization. They were commonly detected by spectrophotometry, so that analytes should be used at concentrations of ca. 10^{-5} *M* order or over. It is, therefore, desirable to apply more sensitive detection methods to cIEF.

In this study, CL detection was for the first time combined with cIEF to carry out protein analysis with high sensitivity. Heme proteins such as cytochrome *c*, myoglobin and peroxidase were separated and detected by the CL detector using luminol–H_2O_2 chemiluminescence. The cIEF–CL detection method is characterized by the following viewpoints; high selectivity and sensitivity, wide dynamic range, simple operation, inexpensive apparatus, and easy set-up for the system.

2. Experimental

2.1. Chemicals

All reagents used were of commercially available and analytical grade. Ion-exchanged water was distilled for use. Pharmalyte 3-10 (Pharmacia Biotech) was used as ampholyte. Hydroxypropylmethylcellulose (HPMC) (viscosity of 2% solution at 25°C: 4000 cP), horse heart cytochrome *c* (p*I* 9.6), and horse heart myoglobin (p*I* 7.2 and 6.8) were purchased from Sigma. Tetramethylethylenediamine (TEMED) and horseradish peroxidase (p*I* 3.5), as well as luminol, hydrogen peroxide, and sodium chloride were received from Wako and Nacalai Tesque, respectively.

2.2. Capillary isoelectric focusing

For preparing sample solutions, heme proteins were dissolved in a solution of 2.0% ampholyte containing 0.2% HPMC and 0.5% TEMED. Herewith, HPMC was used for the purpose of minimizing the electroendoosmotic flow as well as protein interactions with the capillary wall [17–20]. The capillary (75 or 100 μm I.D. uncoated capillary, 25 cm length) was filled with the sample solution. Focusing was performed; applied voltage was 10 kV for 5 min, with 10 m*M* phosphoric acid as anolyte and 20 m*M* sodium hydroxide as catholyte. Salt mobilization was subsequently carried out by replacing the catholyte with CL reagent consisting of 5 m*M* luminol, 400 m*M* H_2O_2, and 5 m*M* NaCl in 10 m*M* Tris solution. Applied voltage at mobilization process was 5 kV.

2.3. Chemiluminescence detection apparatus

Schematic diagram of CL detection cell is shown in Fig. 1. The concept of the cell was originally proposed by us in a previous paper [21]. The detection cell was made of PTFE, which had a 4 cm outer diameter, 2.5 cm height, and 8 ml inner volume. An optical fiber (a core diameter of 2 mm; PGR-FB2000, Toray Industries), a fused-silica capillary (GL Sciences), and a platinum wire as grounding electrode were fixed to the cell. That is, the cell also worked as an outlet reservoir including an electrolyte solution. The optical fiber was set up straight to the capillary with a distance of 0.3 mm between them. The grounding electrode was kept away more than 8 mm from the center of the cell.

When heme proteins as analytes emerged from the capillary at mobilization, they reacted with the CL reagent to produce visible light. The CL light was captured by the optical fiber and carried to a photomultiplier tube (PMT; Model R464, Hamamatsu). The CL detection cell and PMT were shut up in a light-tight box. The output from the PMT (operated at 900 V) was fed to a photon counter (Model C1230, Hamamatsu) connected to a integrator (C-R6A, Shimadzu) to give electropherograms.

3. Results and discussion

3.1. Prominent design of the CL detection cell for cIEF

The present CL detection cell, which was originally developed by us, has several advantages for cIEF. First, on-capillary optical detection mode, such as UV absorption, is the most commonly used in cIEF. As has been pointed out [17,18], such a detection mode fundamentally involves the following problem.

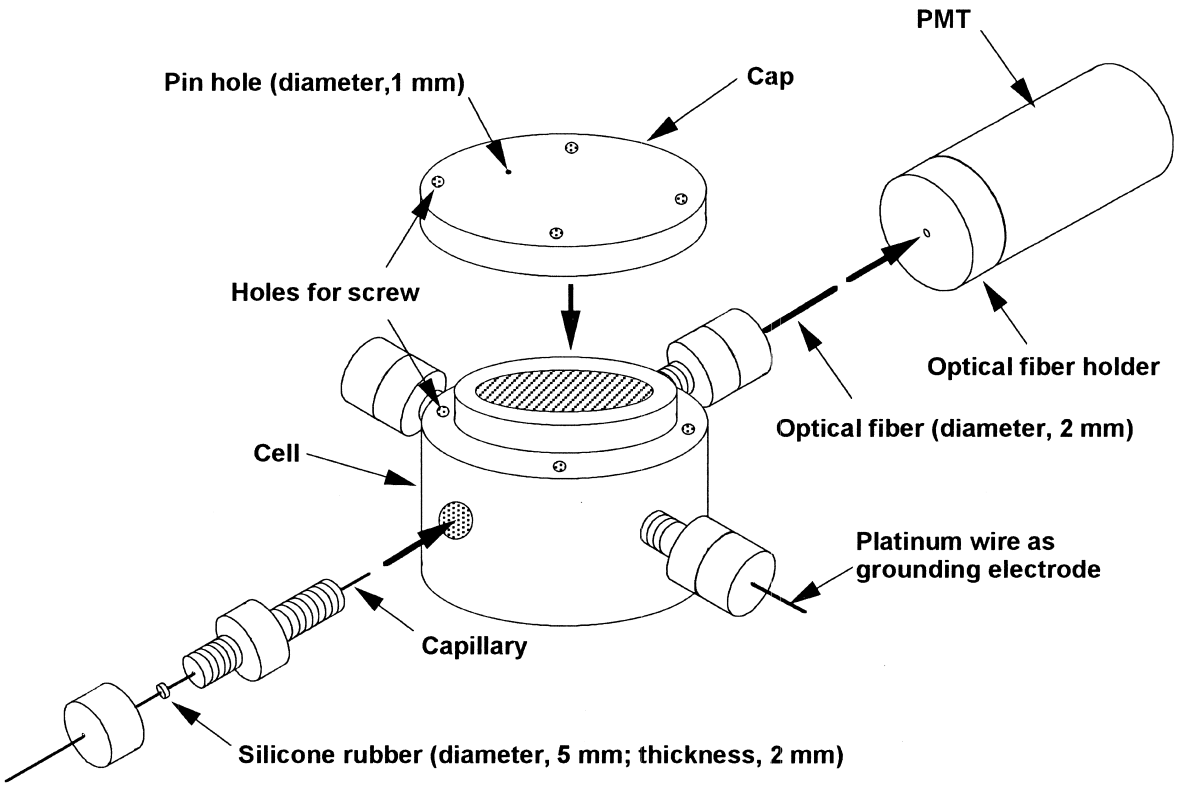

Fig. 1. Schematic diagram of the CL detection apparatus.

Some analytes may focus ahead of the detection window, and others behind. The focused analytes can be mobilized to either anodic or cathodic side of the capillary. Consequently, some analytes which are focused between the detection window and the capillary end as an eluting outlet will be never detected. In contrast, the present CL detection cell enables post-capillary detection mode in which all analytes in the capillary can be located prior to the detection point. Therefore, the problem never occurs in the detection system.

Secondly, when salt mobilization is adopted for transporting focused proteins, an electrolyte solution must be exchanged before the process. The present CL detection cell worked well at both the processes; the cell involved the catholyte at the focusing and the CL reagent containing NaCl at the mobilization. The solutions in the cell could be easily exchanged for another one. Thus, it is clear that this type of CL detector is suitable for cIEF using salt mobilization.

3.2. Separation of cytochrome c, myoglobin and peroxidase by cIEF

Although CZE has so far shown excellent performance for the separation of various kinds of compounds having relatively low molecular masses, it was not always satisfactory for separations of biopolymers, such as proteins, glycoproteins and lipoproteins, due to the adsorption of these onto the inner wall of a capillary tube. We also attempted a separation of cytochrome *c*, myoglobin and peroxidase with CZE mode under the various conditions, but baseline separation of them failed.

When cIEF mode was applied to the separation of those heme proteins, they were easily separated due to the difference in their p*I* values (Fig. 2). It must

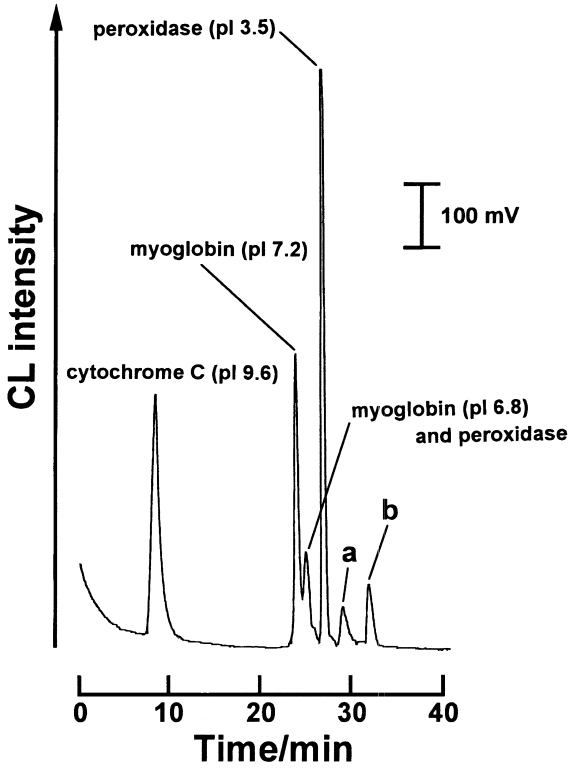

Fig. 2. cIEF of cytochrome *c* and myoglobin as initial data. Capillary: 25 cm×75 μm I.D. Sample: $2.5 \cdot 10^{-7}$ *M* cytochrome *c*, $2.5 \cdot 10^{-6}$ *M* myoglobin, 2.0% Pharmalyte 3-10, 0.5% TEMED, 0.2% HPMC. Focusing: anolyte, 10 m*M* H_3PO_4; catholyte, 20 m*M* NaOH; applied voltage, 10 kV for 5 min. Mobilization: anolyte, 10 m*M* H_3PO_4; catholyte, CL reagent; applied voltage, 5 kV.

be noted that except for the main peak of peroxidase, a minor peak of the protein which was superimposed on the peak of myoglobin (p*I* 6.8) was observed. We could have not, indeed, confirmed which peak belongs to the constituent of p*I* 3.5; we have no means to determine it. However, in view of the fact that the difference in p*I* between myoglobin and peroxidase is very large, we considered that the latter peak, or the main peak on the electropherogram could be assigned to the constituent of p*I* 3.5.

The catholyte in the detection cell must be exchanged with the CL reagent before the mobilization. At that time, external light enters the light-tight box, and the light remains there for several minutes. The residual light would lead to the comparatively high background intensity in the beginning of the run. During the mobilization, the current gradually increased with a migration of Cl^- to the anodic electrode. The current suddenly increased up to 70 μA at the point of a in Fig. 2. After being unsteady for few minutes, the current dropped down to zero at the point of b. To examine the origin of peaks a and b, we performed blank IEF runs with salt mobilization. These peaks were also observed at the blank runs. However, peak areas, heights and shapes of them lacked reproducibilities. We could not clearly confirmed that these peaks were attributed to only ampholyte background or the sum of ampholyte and minor constituents of protein analytes. However, these peaks will not affect the measurement as far as analytes eluted prior to the peaks.

3.3. Calibration curves of cytochrome c

To our knowledge, there has been no cIEF study in which the quantitative analysis was discussed in detail. We quantified cytochrome *c* which was detected as a single peak on the electropherogram. Fig. 3 shows the calibration curves for cytochrome *c* obtained by using capillaries with I.D.s of 75 and 100 μm. The detection limits ($S/N=3$) were $1 \cdot 10^{-8}$ *M* and $6 \cdot 10^{-9}$ *M* for 75 and 100 μm I.D., respective-

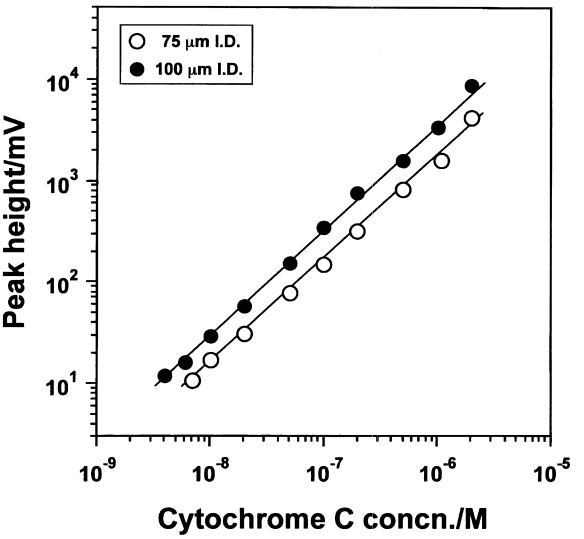

Fig. 3. Calibration curves of cytochrome *c* using capillaries with I.D.s of 75 and 100 μm. The experiments were carried out under the same conditions as described in Fig. 2.

ly. In both capillaries, the CL responses were linear for greater than two-orders of magnitude (up to $2 \cdot 10^{-6}$ *M*) and diverged from the linear lines to upward at the concentration of $>2 \cdot 10^{-6}$ *M*. Judging from the fact that protein concentrations used were in the order of ca. 10^{-5} *M* or over in cIEF studies reported so far, the present system using CL detection seems to show significantly high sensitivity.

We previously reported on the CL detection of heme protein separated by CZE [4]; heme proteins showed very low detection limits of 10^{-10}–10^{-9} *M*. However, the dynamic range was small, and the sensitivity, or the slope of the calibration curve was comparatively low, which made accurate quantification poor. On the other hand, in the present study the large dynamic range and the high sensitivity were obtained as is shown in Fig. 3. The reason for the difference in quantitative properties between the previous and the present systems has not been clear. However, it is well known that CL is very sensitive to the environmental factors, such as pH, temperature, coexisting ion, etc. The ampholytic atmosphere around heme proteins may be one of the major reasons for making the dynamic range and the sensitivity of the present system larger. These results indicate that the system provides high performance for separation and determination of trace amounts of heme proteins.

4. Conclusion

We demonstrated that the cIEF–CL detection method was very effective for the separation and determination of heme proteins. The method featured: (1) high resolution and sensitivity, e.g., cytochrome *c*, myoglobin and peroxidase were satisfactorily separated and cytochrome *c* was detected with the detection limit in the order of 10^{-9} *M*; (2) wide dynamic range, greater than two-orders of magnitude; (3) easy operation; (4) small injection volume, ca. 1 ml; (5) inexpensive apparatus and reagent; and (6) simple set-up for the system. However, only retention time (ca. 30 min) seems to be unsatisfactory for rapid measurements. Pressurized mobilization using hydrodynamic flow, instead of salt mobilization, is now under investigation to achieve short run time without any decrease in resolution and sensitivity.

Acknowledgements

The authors wish to thank Mr. A. Arai of Shimadzu Corporation for his helpful discussion. This work was supported by a grant to RCAST at Doshisha University from the Ministry of Education, Japan. This was also supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture. The authors are grateful to Toray Industries, Inc. for a gift of an optical fiber.

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