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Journal of Chromatography A, 716 (1995) 157–166

JOURNAL OF CHROMATOGRAPHY A

Analysis of protein fractions by micropreparative capillary isoelectric focusing and matrix-assisted laser desorption time-of-flight mass spectrometry

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Abstract

In this study, the use of capillary isoelectric focusing (cIEF) as a micropreparative tool for protein analysis by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) is demonstrated. A newly designed, automated, collection interface equipped with a fiber-optic UV detector and a sheath flow connection was employed for collection of protein fractions. Multiple fractions were collected during a single cIEF run and further analyzed by MALDI-TOF-MS for mass assignment. The feasibility of the method was tested with a mixture of model proteins with different isoelectric points and molecular masses, and with variants of human hemoglobins differing in p*I*, but with negligible difference in M_r. Some practical considerations of the collection procedure and subsequent TOF analysis are presented.

1. Introduction

Capillary isoelectric focusing (cIEF), as originally introduced by Hjertén and Zhu [1], is an important tool for the analysis and characterization of proteins. Recently, cIEF has become a rugged technique for fast, highly efficient separation of minute amounts of samples [2–5]. Since the quantity of sample used for cIEF matches the requirement for modern peptide mapping, sequencing procedures and mass spectrometric analysis [6], cIEF can be an attractive purification tool. The coupling of this CE approach with mass spectrometry is especially interesting, given the potential for fast analysis, high sensitivity and high accuracy of the mass determination. In addition, structural information can be obtained through MS–MS experiments [7].

In this work, the feasibility of off-line coupling capillary isoelectric focusing with matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) is investigated. In principle, an advantage of the off-line arrangement is that only part of the collected fraction may be necessary for the MS analysis while the rest can be used for further analysis. Alternatively, on-line procedures may also be considered.

cIEF has two inherent advantages for this application. First, the cIEF separation is not affected by the molecular mass of the separated molecules. Differences in the isoelectric points of the proteins are the sole basis for the separation. The coupling of cIEF to MALDI-TOF-MS therefore represents a true orthogonal method,

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SSDI 0021-9673(95)00621-4

the latter of which separates on the basis of the mass, where singly (or doubly) charged species are generally obtained in the vapor phase. A second advantage of cIEF stems from its concentrating capabilities, since the entire volume of the capillary can be filled with the sample–ampholyte mixture. During focusing, individual proteins form narrow zones with concentrations orders of magnitude higher than in the original sample, which can be exploited for preparative purposes.

In current practice, cIEF is a two-step process [1–5]. First, a pH gradient with sample proteins focusing at the point where they have no net charge (pH = p*I*) is established. During this step, the current drops to 10% or less of the initial value. Second, mobilization of the focused zones past the detection point takes place with the applied field on. (Focusing and mobilization have sometimes been combined into one step.) Several different mobilization procedures have been described, including the use of salt and/or zwitterions [8], electroosmotic [9] and/or pressure [5] flow. In this work, we have utilized pressure mobilization with no electroosmotic flow; after the focusing step the content of the capillary is forced to move by lifting the anodic end of the separation column. High voltage simultaneously counter-balances zone broadening due to the parabolic flow profile [5]. The advantage of the pressure mobilization approach is that all zones move with the same velocity which leads to a linear change in pH with time.

Since the resolving power of cIEF is high [10], care must be taken to use an interface that maintains this resolving potential. In this study we have used an off-line combination utilizing our recently developed collection interface for CE [11]. This design includes a sheath liquid at the exit of the CE capillary to transport the sample zones into collection capillaries. The sheath liquid allows maintenance of the electric field while pressure mobilization occurs. After collection, the selected protein fractions are mixed with the MALDI matrix and subjected to TOF-MS analysis.

2. Experimental

2.1. Capillary isoelectric focusing

cIEF was performed with a laboratory-made instrument consisting of a high-voltage power supply (CE 1000, Spellman, Plainview, NY, USA) and a collection interface with an UV detector, as described below (Fig. 1). Fused-silica capillaries of 40 cm × 360 μm O.D. × 75

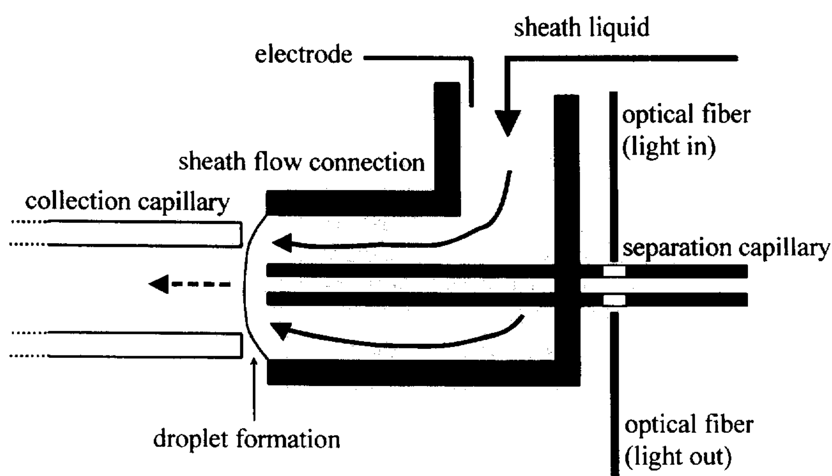

Fig. 1. Schematic diagram of the sheath flow interface consisting of a sheath flow tee connection, optical fibers close to the exit end of the separation capillary and a set of collection capillaries. Note that in actual practice the electrode is separated from the sheath flow by a membrane [11].

μm I.D. (Polymicro Technologies, Phoenix, AZ, USA) deactivated with a newly developed hydrolytically stable coating were used [12]. The exit end of the separation capillary was attached to the tip of the collection interface. A sheath liquid, consisting of the cathodic buffer, provided electrical contact and a means for transporting species to the collection capillaries. The ground electrode (negative pole) of the power supply was separated from the stream of the collection buffer by a semipermeable membrane to prevent any changes in the buffer composition [11]. The injection end of the capillary was inserted into the electrode reservoir containing acetic acid solution (anolyte, pH 2.9), whereas the exit end was fastened in a tee connection and rinsed by a flow of ammonium hydroxide (catholyte, pH 10.9 sheath liquid). Prior to the separation, the column was entirely filled with the sample–ampholyte solution. Focusing was accomplished by applying 10 kV for 2 min followed by 13 min at 30 kV. The focusing step was monitored as a decrease in the electric current flowing through the capillary. Once a plateau was reached (typically in 15 min, with residual current less than 2 μA), the focused zones were then mobilized by raising the injection side by 5 cm, in the case of standard proteins, and 8 cm, in the case of hemoglobin (Hb) variants. This step resulted in a hydrodynamic flow of 22 nl/min (standard proteins) and 35 nl/min (Hb variants) which was measured during calibration runs using fluorescein as a marker. The high voltage was applied during mobilization to maintain the focused zones and thus counterbalance band broadening due to parabolic flow.

2.2. Collection procedure

The sheath flow interface used for sample collection (Fig. 1) is discussed in detail elsewhere [11]. In short, it consisted of a fiber optic-based UV detector operating at 254 nm with detection 10 mm before the exit of the capillary, a sheath liquid collection interface, and a stepper motor-driven/computer-controlled rotor holding 60 collection capillaries (20 μl volume, Idaho Technologies, Moscow, ID, USA). A syringe pump (Model 341B; Sage Instruments, Boston, MA, USA) was used to supply the ammonium hydroxide solution, which served as the collection buffer to the sheath liquid interface at a flow-rate of 12 μl/min. The mobilized protein zones were detected by the on-column fiber-optic UV detector. Upon detection, the exit times were calculated, and a collection capillary was automatically positioned close (ca. 200 μm) to the sheath flow connection at the appropriate time for sample collection. Protein fractions which eluted in the sheath liquid were transported by capillary action into the collection capillaries. Alternatively, when closely spaced proteins were detected, the stepper motor was programmed in 10-s increments. The detector signal was digitized by an A/D converter, and the collection procedure was controlled by a 486 IBM-compatible computer. The software was written in the laboratory in the LabView (National Instruments, Austin, TX, USA) environment. The collected fractions were either stored as solutions in the collection capillaries or as essentially salt free solids after evaporation of the ammonium hydroxide solution.

2.3. MALDI-TOF-MS

All mass spectra were obtained on a Bruker Reflex TOF mass spectrometer (Bruker Instruments, Billerica, MA, USA), operated in either the linear or reflectron mode, as specified in the text. A saturated solution of sinapinic acid in 0.1 *M* trifluoroacetic acid–acetonitrile (60:40, v/v) was used as the matrix for MALDI. A pulsed nitrogen laser was operated at 337 nm (50 μJ, 5 ns), and the signal was accumulated and averaged over 50–100 shots. Two different sample preparation procedures were employed. After the collection of standard proteins, 2 μl of the sample fractions were directly deposited onto the probe and allowed to evaporate. Sinapinic acid (2 μl) was added to the dried fractions for creation of a crystalline matrix. For the collected hemoglobin variant fractions, the sheath liquid was evaporated to dryness, the samples were

stored in the freezer (−20°C) and then redissolved in 2 μl of the MALDI matrix.

2.4. Chemicals

cIEF was performed using 1% (1:1:1, v/v) mixture of Pharmalyte (Sigma, St. Louis, MO, USA), Ampholine (Sigma) and Servalyte (Serva, Hauppauge, NY, USA).

Samples of myoglobin (horse heart), carbonic anhydrase I (human erythrocytes) and II (bovine erythrocytes), β-lactoglobulin A (bovine milk) (Sigma) were dissolved in the carrier ampholyte mixture at a concentration of 0.05 mg/ml. A second sample of a mixture of human hemoglobin variants A, C, S, F (Isolab, OH, USA) was dissolved in the above carrier ampholyte solution to a final concentration of 0.08 mg/ml. All protein samples were used as received without further purification.

Water solutions of 0.3% ammonium hydroxide and 0.5% acetic acid (both from Fisher, Pittsburgh, PA, USA) were used as catholyte and anolyte, respectively. All solutions were passed through a 0.2-μm syringe filter (Gelman, Ann Arbor, MI, USA) or centrifuged prior to use. Sinapinic acid, trifluoroacetic acid and acetonitrile, used for the preparation of the MALDI matrix, were obtained from Sigma.

3. Results and discussion

The purpose of this work is to demonstrate the feasibility of collecting multiple fractions from a cIEF separation followed by MALDI-TOF-MS analysis. Because of the focusing effect and the high resolving power, cIEF is especially attractive for preparative purposes. However, there are two main problems for collecting multiple bands from a single cIEF separation. First, it is desirable to apply the focusing voltage during mobilization in order to minimize band broadening. Our recently developed sheath flow collection device [11] decouples one electrode efficiently from the end of the capillary, allowing collection of many fractions without disrupting the electrical circuit. Mobilization is accomplished by raising the anodic buffer reservoir; separated proteins elute in droplets formed by the sheath buffer.

The second problem is the determination of the exact exit time of individual bands. In the case of cIEF, the exit time cannot be calculated on-line with the present single-optical-fiber arrangement, because the exact location of a protein zone at the beginning of the mobilization step is unknown. Therefore, a separate calibration step to determine the flow velocity has been performed prior to each run. For this purpose, the injection end of the capillary was inserted into a vial containing a fluorescein solution (10^{-4} *M*) and raised to the appropriate level (5 or 8 cm). The calibration revealed a run to run deviation of ca. 2–3% in the flow velocity. This was mainly due to difficulties in reproducibly manually setting the height of the capillary. In the future this problem could be overcome by detecting on-line with two sets of optical fibers which would allow accurate on-line determination of the hydrodynamic flow velocity. In addition, reproducible instrumental control of the pressure may be utilized. Nevertheless, the potential for multiple fraction collection for cIEF separations is demonstrated in the following examples.

The UV trace in Fig. 2 shows a cIEF separation of a standard protein mixture consisting of myoglobin (p*I* 7.2), carbonic anhydrase I (p*I* 6.6), carbonic anhydrase II (p*I* 5.9) and β-lactoglobulin A (p*I* 5.1). Under the given experimental conditions, sharp, well resolved peaks were detected. It is to be noted that a 1:1:1 (v/v) mixture of three different ampholytes was used in Fig. 2. Considering that each commercial ampholyte mixture is prepared by a different synthetic procedure and contains different buffer components, it is reported that mixing different brands increases the uniformity of the pH gradient as the number of ampholytes in a given pH range increases [13]. It is worth noting that detection close to the capillary end enables the cIEF separation to be performed without additives such as N,N,N′,N′-tetramethylethylenediamine (TEMED) [14], as only a fraction of the pH gradient (2.5% for the given experimental

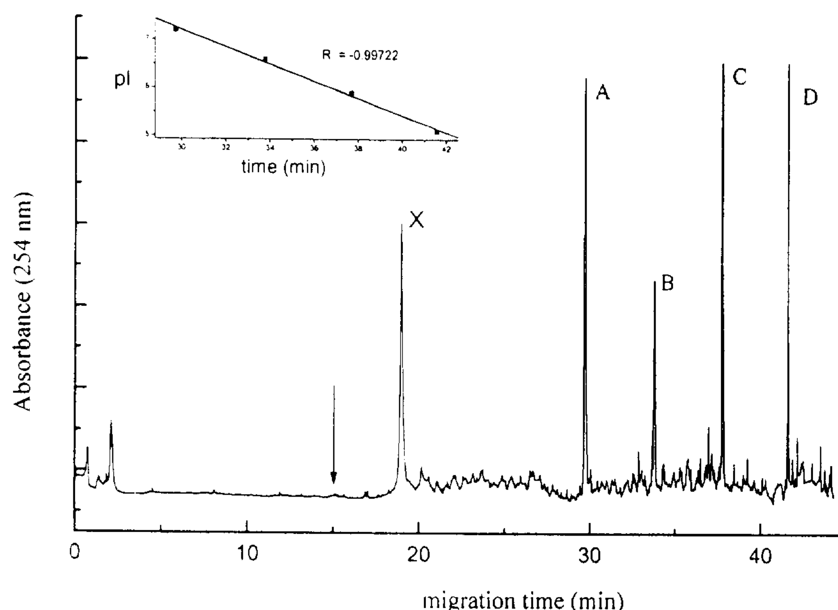

Fig. 2. cIEF separation of (A) myoglobin (p*I* 7.2), (B) carbonic anhydrase I (p*I* 6.6), (C) carbonic anhydrase II (p*I* 5.9) and (D) β-lactoglobulin A (p*I* 5.1). Inset: plot of migration time vs. p*I*. Conditions: 40 cm (39 cm to detector) × 75 μm I.D. capillary, coated; for more detailed description of buffer system, mobilization and collection see text.

conditions) cannot be detected. Since detection was carried out at 254 nm, ampholyte constituents could be observed, resulting in a relatively noisy baseline. The first peak, marked X, stems from differences in UV absorbance and/or change in refractive index of the ampholyte constituents. The total amount of individual proteins was 88 ng (2–5 pmol, depending on the molecular weight of a protein), as a result of the internal volume of the separation capillary (1.8 μl) and protein concentration (0.05 mg/ml). The inset of Fig. 2 shows a plot of p*I* vs. migration time in minutes. With the given set of electrolytes, a linear relation with a high correlation coefficient is achieved, as found by others over wider pH ranges [5]. Under these experimental conditions, the p*I* of unknown proteins can be calculated with high precision. Collection of cIEF fractions allows subsequent protein analysis such as Edman sequencing, peptide mapping or MS analysis to achieve additional information.

A sheath flow collection device provided automatic collection of each protein zone, resulting in a collected volume of 13 μl for each fraction. The peak widths at the baseline of the standard protein mixture were up to 20 s. Because the protein zones were widely spaced, the collection window was set at 65 s to ensure complete collection of each band. This window took into account the deviation in the hydrodynamic flow velocity, resulting from the manual lifting of the capillary. For exact identification of each protein, the collected fractions were subjected to MS analysis.

Since initial experiments indicated that mixing of the collected fractions directly with the matrix (1:10, v/v) in a separate vessel resulted in poor crystal formation and low ion current, a different approach for sample preparation was applied. Prior to the TOF analysis, 2 μl (ca. 450–750 fmol, assuming full recovery) of each fraction were placed on the MALDI target. After evaporation of the ammonium hydroxide, 2 μl of the matrix were added for crystal formation. (It is to be noted that this approach leaves 11 μl (collected volume 13 μl) of the collected protein for additional analysis such as Edman sequencing. In cases involving a lower sample concentration,

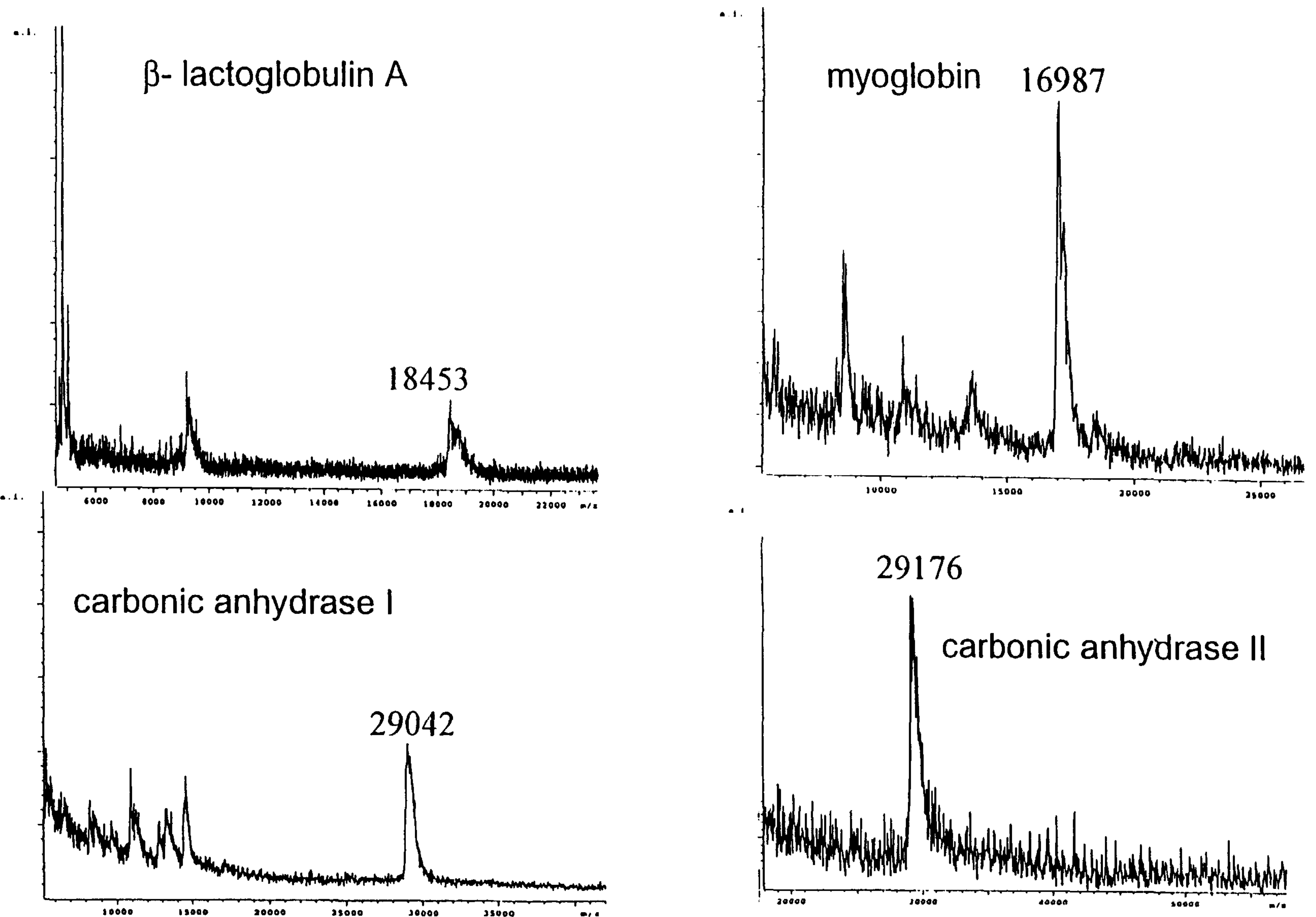

Fig. 3. MALDI-TOF-MS spectra of standard proteins collected during separation shown in Fig. 1. For details see text. a.i. = arbitrary intensity.

Table 1
Molecular mass determinations for standard proteins used for cIEF MALDI-TOF-MS

Protein	pI (Ref. [5])	M_r (Ref. [6])	M_r (exp.)	Accuracy (%)
Myoglobin	7.2	16 950	16 987	0.2
Carbonic anhydrase I	6.6	28 781	29 042	0.9
Carbonic anhydrase II	5.9	29 151	29 176	0.09
β-Lactoglobulin A	5.1	18 365	18 453	0.5

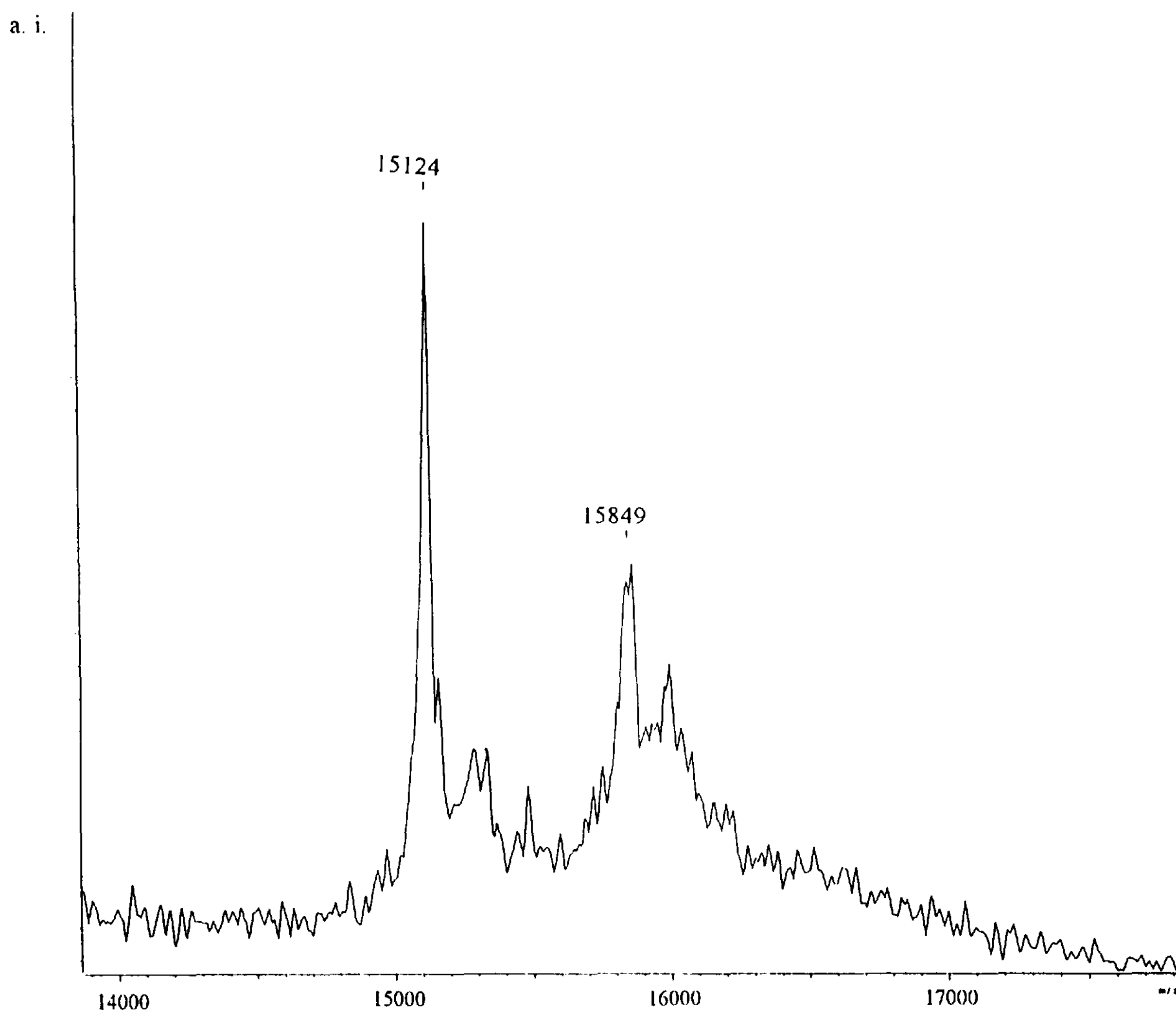

Fig. 4. MS spectrum of a standard mixture of hemoglobin variants (HbA, HbF, HbS and HbC). For details see text. a.i. = arbitrary intensity.

the entire collected volume could be evaporated in order to further concentrate the sample.) Fig. 3 shows the resulting TOF mass spectra. Although low sample amounts were analyzed, the precision of the molecular mass determination was still satisfactory and comparable to that typically obtained for CE–MALDI-TOF-MS experiments [15]. The measured and expected molecular masses, and mass accuracies are listed in Table 1. The mass accuracy was <1% and in most cases better than 0.5%. In addition, the isoelectric points were determined with a precision of 0.1 p*I* units. The off-line coupling of CE and MS therefore allows the determination of two independent parameters which are important for protein characterization.

The capability of the device was demonstrated by collecting multiple fractions from a standard hemoglobin mixture (HbA, HbF, HbS and HbC). In this case, the proteins did not significantly vary in molecular mass but in isoelectric point due to a single or a few amino acid substitutions in the polypeptide chain [16]. As shown in Fig. 4, the MALDI-TOF-MS spectrum of a standard hemoglobin mixture (no cIEF) displays only two major peaks, with molecular masses centered at 15 124 and 15 849. Although hemoglobin contains four subunits, only the individual chains and their matrix adducts (+200 m/z) could be detected in the mass spectrometer upon ionization. The molecular masses of the Hb variant chains did not differ significantly (see Table 2) making their differentiation by MALDI-TOF-MS difficult if not impossible. It is to be noted that the results for Figs. 4 and 5 utilized the reflectron mode of the TOF, which permitted up to an order of magnitude higher resolution than measurements in the linear mode.

The UV trace of the cIEF separation of the hemoglobin variants is shown in Fig. 5. The changes in amino acids are sufficient for baseline resolution of the four variants by cIEF [17]. As an example, the substitution of glutamic acid in position 6 by basic lysine in the β-chain of HbC changes the isoelectric point by 0.5 pH units compared to normal HbA (Table 2). At the same time the change in molecular mass is less than 16 Da (ca. 0.1%).

For the cIEF collection, the capillary was filled with 140 ng (2 pmol total) of the hemoglobin mixture containing the four different variants A, F, S and C. Since the proteins had closely spaced bands, collection of individual peaks was difficult, given the deviation of the flow velocity of 2–3%. In order to isolate each of the variants, it was decided to collect over a 2-min time range in 10-s steps (2 μl each collected volume). This collection procedure could easily be achieved with the appropriate programming of the collection device in combination with the sheath flow connection. The dry fractions were redissolved in the MALDI matrix just before the mass measurement. The amount collected and taken for the MALDI-TOF-MS experiment was ca. 500 fmol. The inset in Fig. 5 shows the mass spectrum of the fraction collected in the specified interval. It is to be noted that the mass spectrum of Hb C is virtually the same as that of the Hb standards (Fig. 4). Every collected fraction was submitted to MS analysis and the resulting MS spectra showed the same trace in all samples containing a Hb variant. Furthermore, the pattern of fraction collections coincided with the

Table 2
Characteristics of Hb variants

Variant	Mutation	ΔMW_r	p*I*
A	$\alpha_2\beta_2$, wild type adult hemoglobin	–	6.95
F	$\alpha_2\gamma_2$, wild type fetal hemoglobin	128	7.0
S	$\alpha_2\beta_2^{6Glu\rightarrow Val}$, adult hemoglobin	30	7.2
C	$\alpha_2\beta_2^{6Glu\rightarrow Lys}$, adult hemoglobin	0.9	7.45

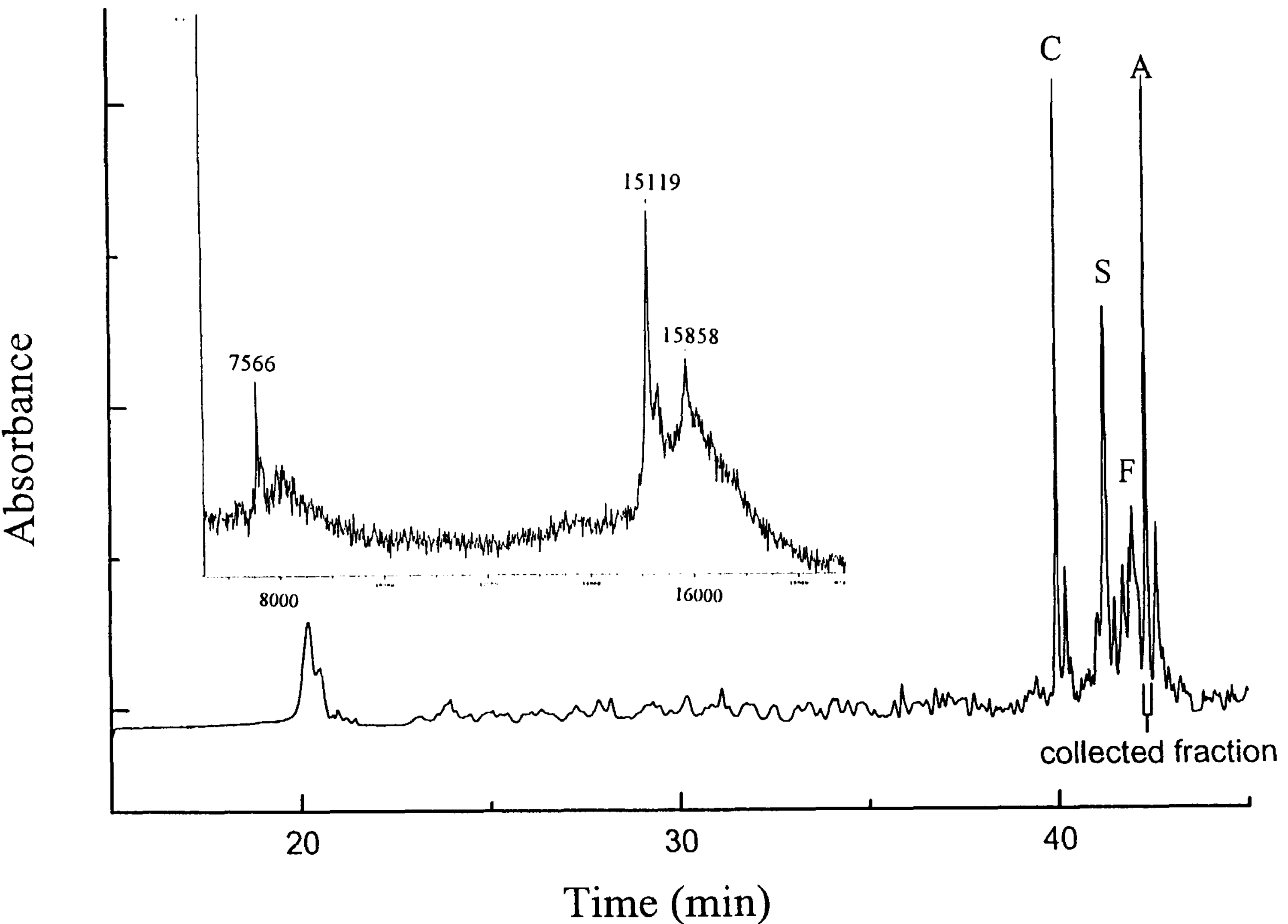

Fig. 5. cIEF separation of hemoglobin variants A (p*I* 6.95), F (p*I* 7.0), S (p*I* 7.2) and C (p*I* 7.45). Inset: MALDI-TOF-MS analysis of the specified collected variant, conditions as in Fig. 4. Conditions: as in Fig. 2, collection interval: 10 s, in predefined steps.

UV trace of the cIEF separation. Therefore, the mass spectrum identified the collected species as hemoglobin, but could not provide sufficient information to distinguish between different mutants. The complementary of cIEF and MALDI-TOF-MS is clearly shown in this example.

4. Conclusions

The new sheath flow collection device was shown to be a useful tool for collecting multiple protein fractions from cIEF and subsequent MALDI-TOF-MS analysis. Because of the focusing effect of proteins into narrow zones, cIEF is effective for preparative purposes. Since the separation is based solely on the difference in isoelectric points and MALDI-TOF-MS analysis is based on differences in molecular masses, this approach, combining two orthogonal separation principles, has potential for two-dimensional protein analysis [18]. Thus, one can envision the potential for complex protein mixture analysis at the trace level. Note that these same two separation principles are used in two-dimensional gel electrophoresis [19]. In the future, the reproducibility of flow variation can be improved by precisely applying a positive pressure for mobilization and/or using two sets of optical fiber detectors for on-line flow velocity determination. In addition, on-line collection on MALDI targets will also be possible.

Acknowledgements

The authors acknowledge NIH GM15847 for support of this work. The authors also gratefully acknowledge Bruker Instruments for assistance in the MALDI-TOF-MS measurements. Contribution 641 from the Barnett Institute.

References

[1] S. Hjertén and M.D. Zhu, *J. Chromatogr.*, 346 (1985) 265–270.
[2] J.-L. Liao and R. Zhang, *J. Chromatogr. A*, 684 (1994) 143–148.
[3] F. Kilár and S. Hjertén, *Electrophoresis*, 10 (1989) 23–29.
[4] S. Kyiohito and B.L. Karger, *Anal. Chem.*, 66 (1994) 9–15.
[5] S.M. Chen and J.E. Wiktorowicz, *Anal. Biochem.*, 206 (1992) 84–90.
[6] R.D. Smith, J.H. Wahl, D.R. Goodlett and S.A. Hofstadler, *Anal. Chem.*, 65 (1993) A574–A584.
[7] J.A. Loo, J.P. Quinn, S.I. Ryu, K.D. Henry, M.W. Senko and F.W. McLafferty, *Proc. Natl. Acad. Sci. U.S.A.*, 89 (1992) 286–289.
[8] M. Zhu, R. Rodriguez and T. Wehr, *J. Chromatogr.*, 559 (1991) 479–488.
[9] J.R. Mazzeo and I.S. Krull, *J. Chromatogr.*, 606 (1992) 291–296.
[10] X.W. Yao and F.E. Regnier, *J. Chromatogr.*, 632 (1993) 185–193.
[11] O. Müller, F. Foret and B.L. Karger, presented at the *7th International Symposium on High Performance Capillary Electrophoresis, Würzburg, 29 January–2 February 1995*, poster p-634.
[12] W. Götzinger, B.L. Karger, P. Shieh and N.H.C. Cooke, presented at the *7th International Symposium on High Performance Capillary Electrophoresis, Würzburg, 29 January–2 February 1995*, poster p. 637.
[13] C. Simpson, in F. Franks (Editor), *Characterization of Proteins*, Humana Press, Cliffton, NJ, 1988, Ch. 12.
[14] M. Zhu, R. Rodriguez, T. Wehr and C. Siebert, *J. Chromatogr.*, 608 (1992) 225–237.
[15] W. Weinmann, C.E. Parker, L.J. Deterding, D.I. Papac, J. Hoyes, M. Przybylski and K.B. Tomer, *J. Chromatogr. A*, 680 (1994) 353–361.
[16] H.F. Bunn and B.G. Forget, *Hemoglobin: Molecular, Genetic and Clinical Aspects*, Saunders, Philadelphia, PA, 1986, Ch. 4, p. 61.
[17] S. Molteni, H. Frischknecht and W. Thormann, *Electrophoresis*, 15 (1994) 22–30.
[18] R. Grimm, G. Ross and F. Bek, presented at the *7th International Symposium on High Performance Capillary Electrophoresis, Würzburg, 29 January–2 February 1995*, poster p. 633.
[19] B.D. Hames and D. Rickwood (Editors), Gel Electrophoresis of Proteins – A Practical Approach, IRL Press, Oxford, 1990.