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Performing Isoelectric Focusing and Simultaneous Fractionation of Proteins on A Rotary Valve Followed by Sodium Dodecyl – Polyacrylamide Gel Electrophoresis

Wei Wang[†], **Joann J. Lu**[†], **Congying Gu**[†], **Lei Zhou**[‡,*], and **Shaorong Liu**[†,*]

[†]Department of Chemistry and Biochemistry, University of Oklahoma, Norman, OK 73019, USA

[‡]School of College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou, Gansu 730000, P.R. China

Abstract

In this technical note, we design and fabricate a novel rotary valve and demonstrate its feasibility for performing isoelectric focusing and simultaneous fractionation of proteins, followed by sodium dodecyl – polyacrylamide gel electrophoresis. The valve has two positions. In one position, the valve routes a series of capillary loops together into a single capillary tube where capillary isoelectric focusing (CIEF) is performed. By switching the valve to another position, the CIEF-resolved proteins in all capillary loops are isolated simultaneously, and samples in the loops are removed and collected in vials. After the collected samples are briefly processed, they are separated via sodium dodecyl – polyacrylamide gel electrophoresis (SDS-PAGE, the 2nd-D separation) on either a capillary gel electrophoresis instrument or a slab-gel system. The detailed valve configuration is illustrated, and the experimental conditions and operation protocols are discussed.

INTRODUCTION

Isoelectric focusing (IEF) is an electrophoretic method that separates amphoteric molecules in solution according to their isoelectric point (pI) values, and capillary isoelectric focusing (CIEF) is a miniaturized format of this technique. CIEF has been demonstrated to be capable of separating proteins and peptides with high-resolutions,[1] and found particularly useful in the analysis of protein mixtures from biological samples.[2–6] Because CIEF alone is often insufficient for resolving proteins in real biological samples, it has been employed in multidimensional platforms (e.g., CIEF/reversed-phase liquid chromatography,[7–9] CIEF/capillary micelle electrokinetic chromatography,[10] etc.) as a preparative step for a second-dimension separation.

So far, two-dimensional gel electrophoresis (2DE) is still one of the most frequently used techniques in bioresearch laboratories. In conventional 2DE, a sample is first separated by IEF (the 1st-D) on an immobilized pH gradient (IPG) strip, and the partially resolved proteins are then separated orthogonally by sodium dodecylsulfate - polyacrylamide gel electrophoresis (SDS-PAGE, the 2nd-D) on a slab-gel. If needed, the protein of each spot can be recovered, decomposed by an enzyme, and identified using mass spectroscopy (MS). IEF is also commonly utilized as fractionation tool, and the fractionated samples are then subject to the 2nd-D separation or other analysis. IEF fractionation instruments are

*Correspondence should be addressed to: Shaorong Liu Department of Chemistry and Biochemistry University of Oklahoma 101 Stephenson Parkway Norman, OK 73019, USA Shaorong.liu@ou.edu Fax: 1 405 325 6111 Lei Zhou School of College of Chemistry and Chemical Engineering Lanzhou University Lanzhou, Gansu 730000, P.R. China zhoulei1@lzu.edu.cn Fax: +86 931-8912582.

commercially available (e.g., Agilent 3100 OFFGEL Fractionator,[11–13] and Bio-Rad Rotofor System[14]).

The Agilent 3100 OFFGEL Fractionator performs IEF of proteins or peptides in an IPG gel strip. After a well frame containing a number of (usually 24) wells is placed on top of a rehydrated IPG strip, a protein/peptide sample is loaded in each well. As an electric field is applied across the strip and the solutions in the wells, proteins or peptides migrate through the strips and rest in the appropriate wells according to their *p*I values. Because the proteins or peptides are contained in a liquid solution, they can be easily recovered for subsequent downstream processing and/or the 2nd-D separation. Inevitably, some proteins/peptides will be lost (remained in the IPG strip). Another limitation of this method is its long focusing time, usually from 25 to 30 hours.

The Bio-Rad Rotofor System was originally developed as a tool for purifying a small number of proteins,[15] and it has two models: the Standard Rotofor Cell (60 mL chamber), and the Mini Rotofor Cell (18 mL chamber). The core component of the system is a focusing chamber – a rotating tube that is divided into 20 compartments by open grids that are screens made of woven polyesters. The purpose of the screens is to minimize convection currents, although liquids can flow across these screens pretty easily. The device also has a cooling system that allows IEF be proceeded at 4 °C. To perform the fractionation, a mixture of proteins and ampholytes is first loaded into the focusing chamber, and a high voltage (usually at a constant power) is applied across the chamber. During IEF, the Rotofor is rotating around its horizontal axis to inhibit gravitationally induced convection, maintain even cooling, and prevent clogging of screens by precipitated proteins. After proteins are focused in the chamber(s), the solution in each compartment is collected for the 2nd-D separations. Apparently, adjacent fractions can be easily mixed during the separation procedure and/or during sample collection.

Very recently, an online multiple-junction capillary isoelectric focusing (OMJ-CIEF) fractionator was introduced for analyses of proteins/peptides.[16] In OMJ-CIEF, the separation capillary is divided into several equal sections joined with each other via tubular Nafion membrane insertions. Each junction is communicated with its own external electrolytic buffer, which is used both to supply electrical contact and for manipulating the pH profile along the entire capillary. By applying a voltage gradient across certain segments of the capillary after focusing, analyte fractions can be selectively mobilized for online ESI-MS detection. However, access to the CIEF-resolved samples is limited.

In this work, we present a novel rotary valve to perform CIEF and fractionate the CIEF-resolved proteins. When the valve is set in one position, it routes 13 capillary segments into a single capillary tube where CIEF is performed. As the valve is switched to another position, the CIEF-resolved proteins are isolated simultaneously into 13 isolated capillary segments. Samples are then collected into a set of vials without loss of proteins. After the samples are briefly processed, they are separated by SDS-PAGE using either a capillary gel electrophoresis system or a slab-gel setup. We present the detailed configuration of the valve, and we also discuss the experimental conditions and operation protocols.

Experimental Section

Reagents and Materials—Acrylamide, N, N'-ethylenebisacrylamide (cross-linker or bis), (3-methacryloxypropyl)trimethoxysilane (bifunctional reagent), N, N, N', N'-tetramethylethylenediamine (TEMED), ammonium persulfate (APS), ribonuclease (13.7 kDa, pI 9.6), α-lactalbumin (14.4 kDa, pI 4.2), myoglobin (17 kDa, pI 7), trypsinogen (24 kDa, pI 9.3), carbonic anhydrase (29 kDa, pI 5.9), ovalbumin (OVA, 43 kDa, pI 4.4), bovine serum albumin (BSA, 66 kDa, pI 4.9), transferrin (76 kDa, pI 5.5), and phosphorylase b (98

kDa, pI 6.3) were all purchased from Sigma (St. Louis, MO). An IEF pharmalyte solution (pH 3–10) was purchased from Bio-Rad (Hercules, CA). Other chemicals were obtained from VWR (Radnor, PA). Fused silica capillaries were bought from Molex Inc. (Phoenix, AZ). All solutions were prepared with double-deionized (DDI) water purified by a NANOpure infinity ultrapure water system (Barnstead, Newton, WA).

Capillary Coating—The basic coating protocol has been described previously.[17,18] Briefly, a 120-cm-long capillary with 360-μm-outer diameter (O.D.) and 150-μm-inner diameter (I.D.) was flushed with 1.0 M NaOH solution for about 1 h to activate the inner surface, and rinsed with DDI water for 20 min and then acetonitrile for 10 min. After being dried with helium, the activated surface was reacted with 10% bifunctional reagent for about 1 h, rinsed with acetonitrile, and dried with helium. The capillary was then flushed with a degassed solution containing 4% T and 0.6% C (%T stands for the total weight concentration of acrylamide and bis in the solution, and %C stands for the concentration of bis relative to the total of acrylamide and bis), 0.1% (v/v) TEMED, and 0.025% APS for 4 min in an ice bath. After being rinsed with DDI water and dried with helium, the capillary was cut into desired lengths for valve assembling.

Valve assembly—Figure 1 presents the working principle of the valve. Capillary loops (in blue and labeled with numbers, each had a length of 7 cm) and auxiliary capillaries (each had a length of 5 cm) were securely attached to a rotor and a stator in a circular format. In Position I, capillary tubes A and B and all capillary loops were joined, forming a continuous capillary tube where CIEF was performed. The two dashed orange lines were used to indicate the connection between the first auxiliary capillary (on the left) on the rotor and the last auxiliary capillary (on the right) on the stator. As the valve was switched to Position II, all loops and capillaries A and B were connected to auxiliary capillaries with which the CIEF-resolved proteins were transferred to vials.

Figure 2A presents a detailed configuration of the valve. The stator (parts *9* and *10*) and the rotor (parts *11* and *12*) were assembled along a double end stud (an axial pin with both ends threaded) and tightened by two hex nuts (part *1*). Referring to Figure 2B/2C, part *10*/*11* was aligned with part *9*/*12* via two alignment pins (*a*) so that capillaries could be inserted into the holes (*c*) on part *10*/*11*. Septa (*d*) were placed between parts *9* and *10*, and between parts *11* and *12* to secure the capillaries in positions. Part *10*/*11* was held down to the pocket of part *9*/*12* using four cross-recessed head screws (*b*). Referring back to Figure 2A, as the stator and rotor were tightened together, a thrust ball bearing (part *3*) was used to ensure that the rotor could be rotated freely. A dowel pin (*7* or *8*) on the stator in conjunction with a slot (*7′* or *8′*) on the rotor was utilized to define the rotation-stops of the valve. The stator-rotor assembly was secured in position using screws (parts *4* and *5*). Figure S1 exhibits images of an assembled valve.

CIEF and fractionation of CIEF-resolved proteins—Referring to Figure 1, the valve was set at Position I, and a degassed protein sample (50 μg/mL each for α-lactalbumin, ribonuclease A, myoglobin, trypsinogen, carbonic anhydrase, OVA, BSA, transferrin, and phorsphorylase B; 0.9% pharmalyte, 0.2% Tween-80 and 20 mM NH_4Ac) was loaded in the continuous capillary. After capillaries A and B were dipped into an anolyte solution (5 mM H_3PO_4) and a catholyte solution (10 mM NaOH), a voltage of 10 kV was applied across the capillary to implement IEF. To ensure that all proteins were focused, the focusing was usually allowed for 2–3 hours. After CIEF, the valve was switched to Position II. A syringe was used to apply a gentle pressure, via an access capillary tube, to push the CEIF-resolved sample in each capillary loop (including capillary A or B) out to a 200-μL vial.

SDS-PAGE of collected samples—Each collected sample (~1.2 μL) was mixed with 3 μL sample buffer (premixed 4X Laemmli sample buffer from Bio-Rad for SDS-PAGE, #161-0747), and cooked in a boiling water-bath for 3 min before electrophoresis. A Bio-Rad Mini-PROTEAN Tetra Cell system was employed for the SDS-PAGE. The separating gel contained 11.6 % acrylamide and 0.4 % bis, while the stacking gel contained 4.8 % acrylamide and 0.2 % bis. Gels were polymerized between two glass plates in a gel caster, with a 15-well comb being inserted at the top to create the sample wells. After the gel was polymerized, the comb was removed, and the gel was ready for electrophoresis. A run-buffer was prepared by adding 3.03 g Tris, 14.42 g glycine and 1.00 g SDS in 1.00 L DDI water. A constant current mode was used for the separation. For stacking, 10 mA current was applied for about 20 min until the proteins were stacked at the interface of the stacking and separating gels. For separation, 15 mA current was applied for about 50 min until the sample front moved ~0.5 cm to the end of the separating gel. The gel was then taken out, stained in 0.75% Coomassie blue solution for one hour, and destaining with 20% ethanol and 5% acetic acid solution overnight.

CGE of collected samples—Each collected sample was mixed with 2 μL 0.25% SDS solution (pH 8.3, contains 60 mM Tris and 60 mM Tricine), and the solution was allowed to stay at room temperature for ~30 min. CGE separations were performed using a wall-coated capillary with a length of 34 cm (30 cm effective) and an I.D. of 75 μm, as described previously.[17,18] A cross-linked polyacrylamide gel (6%T0.3%C) was introduced into the capillary by pressure. Sample was electrokinectically injected with field strength of 324 V/cm for 3 s. The same field strength was used for CGE separations. UV absorbance at 220 nm was measured using a Linear-200 UV-VIS detector (Linear Instruments Corp., Reno, NV), and the data was acquired using USB-1208LS data acquisition card (Measurement Computing Corp., Norton, MA). The cross-linked polyacrylamide sieving matrix was replenished after each run.

Results and discussion

In the valve design, proper alignment and sealing are critical to achieve high-reolution CIEF separations. To ensure good alignment, we drilled holes (*c*) on parts *10* and *11* from the stator-rotor contacting surfaces outwards. With a regular computer numerical controlled mill, we can drill holes with a precision of less than 5 μm. When drilling <400-μm-diameter holes, the drill may deviate away, causing position shifts. By aligning the drill at stator-rotor contacting surfaces, <10 μm machining errors were guaranteed. We also arranged the rotation-stop dowel pin (*7*) far away from the rotating axis. When the valve was rotated, it was stopped as the dowel pin (*7*) reached the end of the pin-stop slot (*7*). By arranging the pin and pin-stop slot far away from the rotating axis, we increased the rotation-stop accuracy. We further utilized a CIEF capillary with a large I.D. (150 μm). Using a large-I.D. CIEF capillary reduced the relative error under the same absolute position error.

To ensure liquid-tight seal, we used polyether ether ketone (PEEK) material to produce part *10* and polytetrafluoroethylene (PTFE) to produce part *11*. The PEEK (hard) and PTFE (soft) combination was excellent for liquid-tight sealing. We flattened and polished the stator-rotor contacting surfaces to achieve improved sealing. We used a thrust ball bearing in conjunction with a spring to tighten the stator and rotor. The thrust ball bearing and a spring combination allowed us to adjust the force applied between the stator and rotor. The holes (*c*) on part *10*/*11* had a diameter of ~400 μm, close to but slightly larger than the O.D. of the capillaries. When capillaries were put into these holes, a 3% polyacrylamide solution was introduced into the capillary-hole gaps. A septum (*d*) was also sleeved on the capillary between parts *9* and *10*, and between parts *11* and *12*, to serve as an additional mechanism to prevent liquid leaking.

To run CIEF, a well-coated capillary to suppress electroosmotic flow (EOF) was essential, because the solution should not move during IEF.[18] In this work, the inner walls of all capillary segments (capillary tubes A and B, and all capillary loops) were polyacrylamide coated. Usually, after the inner wall of a long (120 cm) capillary was coated with polyacrylamide, CIEF was performed using our model samples (see Figure S2 for typical CIEF results). If the results were acceptable, the capillary was cut into appropriate lengths for valve assembling. Wall-coating was not necessary for auxiliary capillary tubes. In this work, only bare capillaries were used for these tubes.

In order to yield adequate solution for SDS-PAGE and CGE, the lengths of all capillary loops were identical, 7 cm, producing ~1.2 μL solution in each loop. We did not use much longer capillaries, because we wanted the CIEF to be completed in less than 2–3 hours. Two 9-cm-long capillaries were used for capillaries A and B to facilitate their convenient connections to anolyte and catholyte reservoirs.

To demonstrate the feasibility of the valve, we performed CIEF (the 1st-D separation) on the valve, fractioned the CIEF-focused proteins, and ran SDS-PAGE or CGE (the 2nd-D separation) using a nine-protein mixture. Figure 3 present the results of CIEF and SDS-PAGE (2DE). In the 1st-D, trypsenogen and ribonuclase A, phosphorylase B and carbonic anhydrase, transferrin and α-lactalbumin, and BSA and OVA were not resolved in the 1st-D, but they were nicely resolved in 2DE. Since we were just trying to domonstrate the feasibility of using the valve device for 2DE, the device was not optimized for high-resolution separations. Apparently, higher resolutions could be achieved if we used more number of capillary loops for CIEF. Samples from capillaries A and B were not shown here because all nine proteins were compressed in the 11 capillary loops. Figure 4 presents the CIEF and CGE results (also 2DE) in a two-dimensional image format. Using CGE for the 2nd-D separation, each sample fraction could be analyzed for at least three duplicate runs, which is important in applications where statistic information is desired.

Conclusions

In conclusion, we have designed and manufactured a novel valve device as an alternative means to perform 2DE separations, and we have demonstrated the feasibility of using this device for 2DE separations. The device is easily reconfigured. For example, the device allows us to use different loop lengths to “purify” specific protein(s) or group(s) of proteins, although identical lengths were used in this work for all capillary loops. Other advantages of this device include: almost 100% protein recovery, relatively fast IEF and simultaneous fractionation, and micro-preparative capabilities. However, to achieve high resolutions, many capillary loops have to be used. Because there is a minimum length for which a capillary loop can be conveniently installed on the valve, the total length of the CIEF capillary can be long when many of them are used. Since there is also a maximum voltage we can apply to run CIEF safely, a long CIEF capillary will lead to a long CIEF time and broad bands, which might limit the uses of this device.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES

1. Shen YF, Berger SJ, Anderson GA, Smith RD. Anal Chem. 2000; 72:2154–2159. [PubMed: 10815979]
2. El Rassi Z. Electrophoresis. 2010; 31:174–191. [PubMed: 20039288]
3. Kasicka V. Electrophoresis. 2010; 31:122–146. [PubMed: 19950358]
4. Kilar F. Electrophoresis. 2003; 24:3908–3916. [PubMed: 14661226]
5. Silvertand LHH, Torano JS, van Bennekom WP, de Jong GJ. J Chromatogr A. 2008; 1204:157–170. [PubMed: 18565533]
6. Cheng C, Lu JJ, Wang X, Roberts J, Liu S. Electrophoresis. 2010; 31:2614–2621. [PubMed: 20603827]
7. Lee CS, Balgley BM. Methods Mol Biol. 2009; 492:233–240. [PubMed: 19241036]
8. Wang Y, Balgley BM, Rudnick PA, Evans EL, DeVoe DL, Lee CS. J. Proteome Res. 2005; 4:36–42. [PubMed: 15707355]
9. Zhou F, Johnston MV. Anal Chem. 2004; 76:2734–2740. [PubMed: 15144182]
10. Ramsey JD, Jacobson SC, Culbertson CT, Ramsey JM. Anal. Chem. 2003; 75:3758–3764. [PubMed: 14572041]
11. Michel PE, Reymond F, Arnaud IL, Josser J, Girault HH, Rossier JS. Electrophoresis. 2003; 24:3–11. [PubMed: 12652567]
12. Hörth P, Miller CA, Preckel T, Wenz C. Mol. Cell. Proteomics. 2006; 5:1968–1974. [PubMed: 16849286]
13. http://www.chem.agilent.com/Library/brochures/5990-5596EN.pdf
14. https://http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_3131.pdf
15. Bier, M.; Egen, NB.; Allgyer, TT.; Twitty, GE.; Mosher, RA. New developments in isoelectric focusing. In: Meienhofer, EGJ., editor. Peptides, structure and biological function : proceedings of the Sixth American Peptide Symposium. Pierce Chemical Co.; Rockford, Illinois: 1979. p. 79-80.
16. Chingin K, Astorga-Wells J, Najafabadi MP, Lavold T, Zubarev RA. Anal Chem. 2012; 84(15):6856–6862. [PubMed: 22779778]
17. Lu JJ, Zhu Z, Wang W, Liu S. Anal Chem. 2011; 83(5):1784–1790. [PubMed: 21309548]
18. Gao L, Liu S. Anal Chem. 2004; 76(24):7179–7186. [PubMed: 15595858]

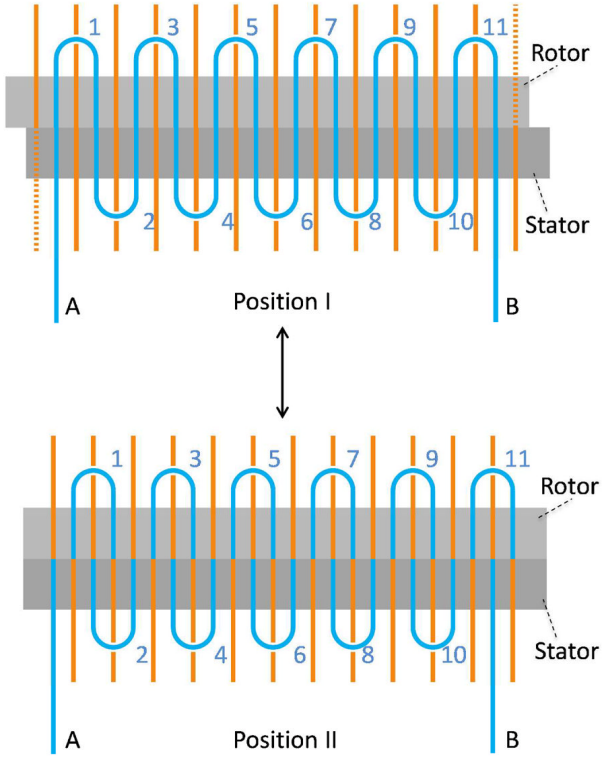

Figure 1.

Working principle of valve

At Position I, all capillary loops and capillaries A and B (blue lines) are connected forming a continuous capillary for CIEF; At Position II, all capillary loop and capillaries A and B are connected to auxiliary capillaries (orange lines) for fractionating and collecting CIEF-resolved proteins.

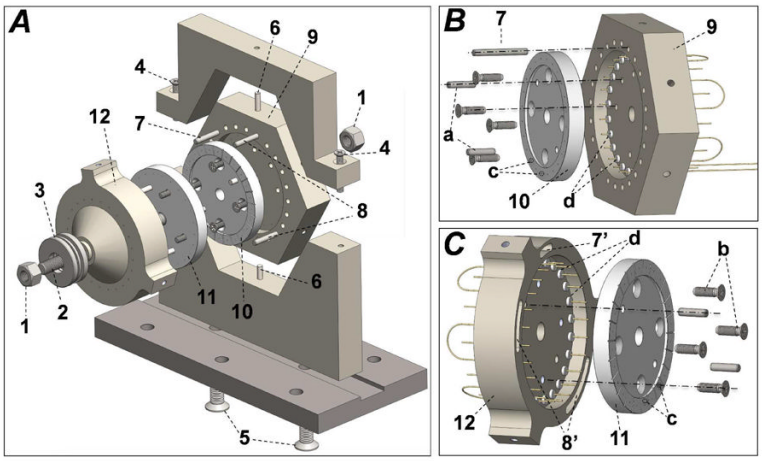

Figure 2.

Detailed valve configuration

A: Overall valve assembly; B: Stator assembly; C: Rotor assembly. 1 - hex nut; 2 - double end stud; 3 - thrust ball bearing; 4 - cross recessed countersunk head screw; 5 - socket countersunk head screw; 6, 7 and 8 - dowel pin; 9 and 10 - stator; 11 and 12 - rotor; a - dowel pin; b - cross recessed countersunk head screw; c - holes for capillaries; 7' and 8' - slots for dowel pin (rotation stops).

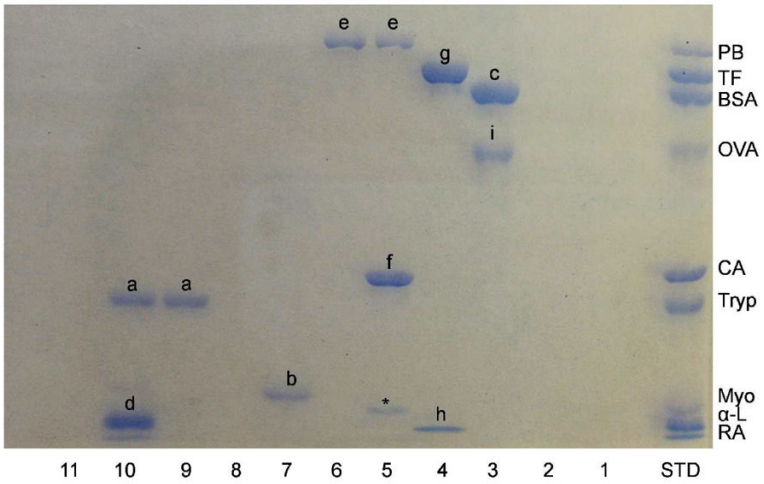

Figure 3.

CIEF and SDS-PAGE (2DE) results

The values on the x-axis are capillary loop numbers; a larger capillary number indicates a higher pI value. The y-axis indicates a size-based protein separation; a higher position indicates a larger protein. a - trypsinogen (pI 9.3, MW 24 kDa); b - myoglobin (pI 7, MW 17 kDa); c - BSA (pI 4.9, MW 66 kDa); d - ribonuclease A (pI 9.6, MW 13.7 kDa); e - phorsphorylase B (pI 6.3, MW 98 kDa); f - carbonic anhrdrase (pI 5.9, MW 29 kDa); g - transferrin (pI 5.5, MW 76 kDa); h - α-lactalbumin (pI 5.0, MW 14 kDa); i - OVA (pI 4.4, MW 43 kDa). The asteroid-marked band indicates an impurity. For CIEF on valve: the sample contained 50 μg/mL each for all nine proteins, 0.9% pharmalyte, 0.2% Tween-80, and 20 mM NH_4Ac; The anode solution was 5 mM H_3PO_4; The cathode solution was 10 mM NaOH; The CIEF capillary had an I.D. of 150 Pm, and a total length of 95 cm; A voltage of 10 kV was applied for focusing. For SDS-PAGE on slab-gel: the stacking gel contained 5% acrylamide; the separation gel contained 12% acrylamide; The stacking was effected under 10 mA current for 20 min, while the electrophoretic separation was effected under 15 mA current for 50 min; The gel was stained with 0.75% Commassie blue for one hour, and distained with 20% ethanol and 5% acetic acid overnight.

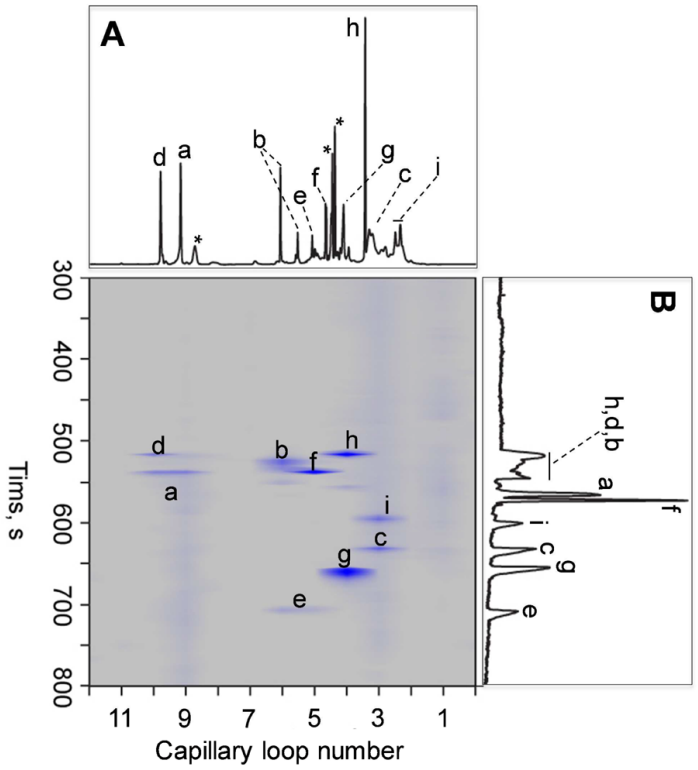

Figure 4.

CIEF and CGE (2DE) results

The 2DE image was constructed based on the CGE separations (see Figure S3 for raw CGE data) of the samples from the valve after CIEF. CGE separations were performed using a 34-cm-long (30 cm effective) and 75-μm-I.D. wall-coated capillary filled with a 6%T and 0.3%C cross-linked polyacrylamide (see Experimental for details). In this image, the pI of a protein spot increase with the capillary loop number, and its size increases with the CGE migration time. Panel A shows a CGE trace of the nine-protein mixture. Panel B shows a CIEF trace (with hydrodynamic mobilization) using a wall-coated capillary before it was cut for valve assembling. The asteroid-marked peaks indicate impurities. Other conditions are the same as in Figure 3.