Journal of Chromatography B, 879 (2011) 1439–1443

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

High sensitive protein detection by hollow fiber membrane interface based protein enrichment and in situ fluorescence derivatization☆

Xiaoqiang Qiao[a,b], Liangliang Sun[a,b], Li Wang[c], Yu Liang[a,b], Lihua Zhang[a,*], Yichu Shan[a], Xiaojun Peng[c], Zhen Liang[a], Yukui Zhang[a]

[a] Key Laboratory of Separation Science for Analytical Chemistry, National Chromatographic Research & Analysis Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, China
[b] Graduate School of the Chinese Academy of Sciences, Beijing 100039, China
[c] State Key Laboratory of Fine Chemicals, Dalian University of Technology, 158 Zhongshan Road, Dalian 116012, China

ARTICLE INFO

Article history:
Received 5 July 2010
Accepted 1 November 2010
Available online 9 November 2010

Keywords:
In situ fluorescence derivatization
Hollow fiber membrane interface
Preconcentration
Low abundance proteins

ABSTRACT

A novel device, composed of a syringe pump for sample loading, a hydrophilic hollow fiber membrane interface for protein concentration and small molecules removal, and a centrifugation tube for buffer exchange, was designed for protein preconcentration and in situ fluorescence derivatization. With the outlet of the interface blocked, denatured proteins were continually introduced. Restricted by the membrane with the molecular weight cutoff (MWCO) of 3000 Da, proteins were concentrated within the membrane. However, denaturant and other small molecules, which might affect the further fluorescence derivatization, were driven out of the membrane. Then, the membrane with proteins restricted inside was directly put into the fluorescence derivatization buffer. Here, the water-soluble sulfo-3H-indocyanine dye, the active N-hydroxysuccinimide of 3H-indolium,1-(5-carboxypentyl)-2-[3-[1-(5-carboxypentyl)-1,3-dihydro-3,3-dimethyl-5-sulfo-2H-indol-2-ylidene]-1-propenyl]-3,3-dimethyl-5-sulfo-,monopotassium salt (sw-cy3-NHS), synthesized in our lab, was used for protein labeling. By such a method, the detection sensitivity of bovine serum albumin (BSA) was improved by nearly 200 folds, compared to that obtained by direct in-solution derivatization. Through the derivatization of a fraction of *E. coli* protein separated by reversed phase HPLC, proteins with low concentration were efficiently labeled, which indicated the potential merit of the developed method for the high sensitive detection of low abundance proteins.

1. Introduction

In the post-genome-sequence era, protein analysis has been paid much more attention. Up till now, various techniques, including high-performance liquid chromatography (HPLC) [1,2], capillary electrophoresis (CE) [3,4], and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [5,6], have been developed and widely used for protein separation. However, since many proteins with important biological functions (e.g. drug targets or biomarkers) are of extremely low concentration, the development of high sensitive detection methods is an imperative task.

As one of the most sensitive techniques, fluorescence detection is widely used in protein research [7–11]. Although native fluorescence emitted by aromatic amino acid residues (such as tryptophan, tyrosine, and phenylalanine) could be directly applied to protein detection with high sensitivity [12–14], not all proteins could generate native fluorescence signals. Therefore, the development and application of fluorescence reagents is indispensable to improve the detection sensitivity of proteins [15–19]. In our previous research, a series of water-soluble cyanine dyes were synthesized and further applied for labeling standard proteins and real samples, by which the limit of detections (LODs) were obviously improved [20–22].

However, the derivatization of low concentration proteins is still challenging. Taken traditional fluorescence reagent, fluorescein isothiocyanate (FITC), as an example, although labeled amino acids with the concentration of pmol/L could be detected, the minimum concentration of amino acids required for derivatization should be at least 10^{-6} mol/L [23,24]. Furthermore, proteins with concentration of at least 1–10 mg/mL could be effectively derivatized by FITC [25]. Therefore, the LODs of fluorescence labeled proteins, to some extent, are limited by the sample concentration.

Preconcentration is an efficient technique to decrease the LODs of proteins and peptides. Chang et al. reported on-line concen-

☆ This paper is part of the special issue "Enhancement of Analysis by Analytical Derivatization", Jack Rosenfeld (Guest Editor).

* Corresponding author. Tel.: +86 411 84379720; fax: +86 411 84379560.
E-mail address: lihuazhang@dicp.ac.cn (L. Zhang).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.11.002

tration of proteins using polymer solution in CE analysis. With large volume injection, carbonic anhydrase at the concentration of pmol/L could be detected [26]. In the past few years, the technique with the combination of fluorescence derivatization and preconcentration has been used for the analysis of various samples, including peptides, proteins, and human serum albumin immunoassay mixtures [27–32]. Park et al. applied single drop microextraction to concentrate neutral/acidic amino acids or short peptides labeled by 4-fluoro-7-nitro-2,1,3-benzoxadiazole, and hundred times enrichment was obtained in CE analysis with laser-induced fluorescence (LIF) detection. However, for those basic amino acids or long hydrophilic peptides, the method was less effective [29]. Chen et al. used transient pseudo-isotachophoresis to concentrate FITC labeled peptide hormones and their fragments. Followed by CE analysis with LIF detection, 100-folds improvement on peak heights was achieved [30]. However, to the best of our knowledge, all of these studies involved fluorescence derivatization prior to sample preconcentration.

Hollow fiber membrane possesses excellent mass-transfer properties, by which small molecules could permeate in and out easily, while macromolecules could be retained if membranes with proper molecular weight cut-off (MWCO) were selected [33–36]. In the current work, a novel device based on cellulose acetate hollow fiber membrane with the MWCO of 3000 Da, was designed for protein enrichment, followed by in situ fluorescence derivatization, by immerging the membrane in the water-soluble cyanine dye, the active N-hydroxysuccinimide of 3H-indolium,1-(5-carboxypentyl)-2-[3-[1-(5-carboxypentyl)-1,3-dihydro-3,3-dimethyl-5-sulfo-2H-indol-2-ylidene]-1-propenyl]-3,3-dimethyl-5-sulfo-,monopotassium salt (sw-cy3-NHS). The results demonstrated that with the combination of protein preconcentration and in situ fluorescence derivatization, the high sensitive detection of low concentration proteins was achieved.

2. Experimental

2.1. Chemicals and reagents

Epoxy glue was obtained from Kaihua New Technology Project (Dalian, China). Hydrofluoric acid (HF, 40% (v/v)) was ordered from Shenyang Chemical Reagent (Shenyang, China). Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Phenylmethanesulfonyl fluoride (PMSF) was from Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China). HPLC-grade acetonitrile was obtained from Merck (Darmstadt, Germany). All inorganic reagents were analytical-reagent grade, and others were of HPLC grade. Water was purified by a Milli-Q system (Millipore, Molsheim, France).

2.2. Materials and equipment

Fused-silica capillary (100 μm i.d. × 375 μm o.d.) was purchased from Sino Sumtech (Handan, China). The hollow cellulose acetate fiber membrane (MWCO 3000 Da, 200 μm i.d. × 220 μm o.d.) was taken from a capillary dialyzer (GFS Plus 12, Gambro Dialysatoren GmbH, Hechingen, Germany). A C8 column (5 μm, 300 Å, 4.6 mm × 250 mm) was ordered from Dalian Elite Analytical Instrument (Dalian, China).

A precise syringe pump purchased from Baoding Longer Pump (Baoding, China) was used to push samples through the membrane device for protein enrichment. An ultrasonic processor from Cole-Parmer (Vernon Hills, IL, USA) was used for cell sonication. A SpeedVac (Thermo Fisher, San Jose, CA, USA) was applied to lyophilize samples. HPLC experiments were performed on a system composed of a 4-line degasser, an intelligent pump equipped with quaternary gradient unit, an injection valve (Rheodyne, Cotati, CA, USA), a UV detector (Dalian Elite Analytical Instrument, Dalian, China), and an intelligent fluorescence detector (Jasco, Tokyo, Japan).

2.3. HPLC separation

Proteins were separated by HPLC with a C8 column. The column temperature was set at 25 °C. Eluent A was 100% water with 0.1% (v/v) TFA; eluent B was 95% (v/v) acetonitrile with 0.1% (v/v) TFA. The gradient for the separation of *E. coli* proteins was as follows: 0 min, 25% B; 70 min, 60% B; 120 min, 80% B; the flow rate was 0.25 mL/min. The UV wavelength was 214 nm. The gradient for the analysis of BSA was as follows: 0 min, 30% B; 20 min, 80% B; 30 min, 80% B; the flow rate was 0.5 mL/min. The gradient for the derivatized fraction of *E. coli* proteins separated by RPLC (*E. coli* RP fraction) was as follows: 0 min, 30% B; 70 min, 60% B; 120 min, 80% B; the flow rate was 0.25 mL/min. The excitation and emission wavelengths for fluorescence detection were 554 and 570 nm respectively.

2.4. Sample preparation

For protein denaturation, 80 μL of 50 ng/μL BSA was mixed with 100 μL of 6 M guanidine hydrochloride, followed by stirring at 35 °C for 30 min. Unless otherwise stated, each sample was dissolved in 100 mM Na_2CO_3–$NaHCO_3$ (pH 9.5).

The extraction procedure of *E. coli* proteins was as follows. *E. coli* (Strain BLT 5403) was grown on LB culture medium. After cultured at 37 °C for 14 h, the mixture was centrifuged at 4300 × *g* for 10 min to precipitate *E. coli* cells. Then, followed by 3 times washing with PBS, 6 M guanidine hydrochloride containing 1 mM PMSF were added, and the cells were ultrasonicated for 180 s. After the mixture was further centrifuged at 20,000 × *g* for 20 min, the supernatant was collected, and the protein concentration was determined by Bradford assay. Proteins were further precipitated with acetone/water in 4:1 (v/v), centrifuged, and lyophilized. An aliquot of 100 μg *E. coli* proteins were prefractionated by HPLC, and the fraction from 57 to 62 min (*E. coli* RP fraction) was collected, lyophilized, denatured for further use.

2.5. Protein enrichment

The device used for protein enrichment was consisted of three parts: a syringe pump for sample loading, a hollow fiber membrane interface for protein concentration, and a centrifugal tube for buffer exchange, as shown in Fig. 1(A). The hollow fiber membrane interface was prepared according to the method described in our previous report [35]. In brief, one end of each connecting capillaries coated by linear polyacrylamide was firstly etched by 40% (v/v) HF until they could be inserted into a 15 cm long hollow fiber membrane with a volume of 5 μL. Then the capillaries were glued with the hollow fiber membrane using epoxy glue. Finally, it was threaded through a centrifugal tube filled with 100 mM Na_2CO_3–$NaHCO_3$ (pH 9.5), and fixed with Teflon tubes.

With the device in Fig. 1(A), BSA and *E. coli* RP fraction were concentrated respectively. For BSA concentration, with the outlet of the connecting capillary sealed with a rubber, an aliquot of 90 μL denatured BSA was continuously pumped into the hollow fiber membrane interface with a flow rate of 1.5 μL/min. The procedure for the enrichment of *E. coli* RP fraction was the same as that for BSA, except that the sample was continuously loaded for 160 min.

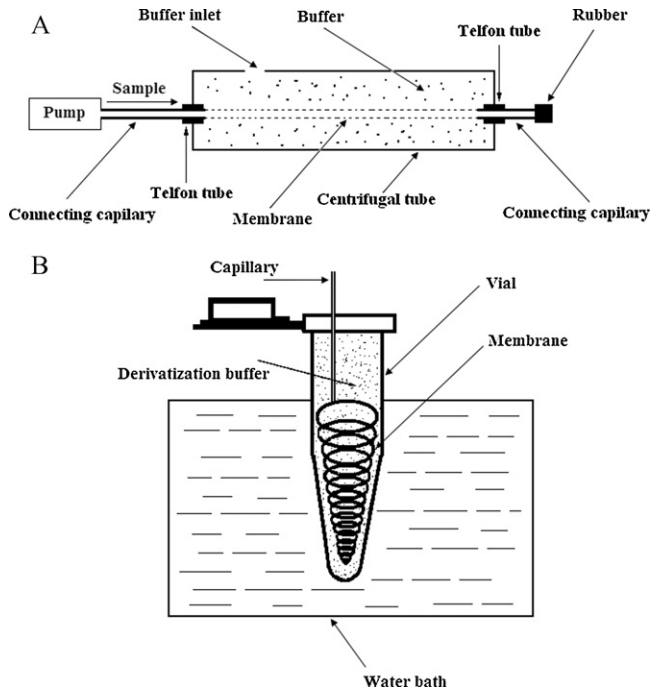

Fig. 1. Scheme of the device for protein enrichment (A) and in situ fluorescence derivatization (B).

2.6. In-solution and in situ fluorescence derivatization

The in-solution fluorescence derivatization was performed by the following steps. For BSA, an aliquot of 90 μL denatured sample was firstly mixed with 6.5 μL of 8.5 mM sw-cy3-NHS. Then the mixture was stirred at 45 °C for 30 min in dark, followed by 4-fold dilution with 100 mM Na_2CO_3–$NaHCO_3$ (pH 9.5) buffer. Subsequently, 20 μL of derivatized proteins were injected, and analyzed by HPLC with fluorescence detection. The procedure for the derivatization of *E. coli* RP fraction was the same as that for BSA.

The in situ fluorescence derivatization for BSA was shown in Fig. 1(B). First, the hollow fiber membrane containing the concentrated proteins was directly moved to an eppendorf tube filled with fluorescence derivatization buffer. Then the derivatization buffer was stirred at 45 °C for 30 min in dark. Finally, the hollow fiber membrane interface was taken out, and the derivatized protein (about 5 μL) was pumped into a vial. Followed by 4-fold dilution with 100 mM Na_2CO_3–$NaHCO_3$ (pH 9.5), 20 μL of derivatized proteins were analyzed by HPLC with fluorescence detection. The in situ fluorescence derivatization procedure for *E. coli* RP fraction was the same as that for BSA.

3. Results and discussion

3.1. Selection of fluorescent reagent

A water-soluble sulfo-3H-indocyanine dye, sw-cy3-NHS, synthesized according to the previous report [37], was selected for protein derivatization. As shown in Fig. 2, the reagent could react with the amino groups of proteins, especially the side chains of lysine, to form covalent protein–dye complex. Due to the presence of sulfonate groups and carboxyl groups, the reagent is of good aqueous solubility, helpful to avoid protein precipitation during labeling. Furthermore, excess fluorescence reagent could be easily removed by our previously developed on-column technique [21]. Compared with conventional isothiocyanate dyes, such as FITC, succinimidyl ester of sw-cy3-NHS is of high reactivity, which is beneficial for the derivatization of low concentration proteins.

3.2. Protein enrichment

Although the LODs of fluorescence labeled proteins are low, to achieve efficient derivatization, the protein concentration should be high enough [23,24]. Therefore, the combination of protein preconcentration and fluorescence derivatization technique may be promising to achieve the high sensitive detection of low abundance protein. Cellulose acetate based hollow fiber membrane is a hydrophilic material, resulting in neglectable protein adsorption [35]. Thus, an enrichment device was prepared with such membrane (MWCO of 3000 Da), as shown in Fig. 1(A). With the outlet end capillary sealed, proteins could be continually pumped into the device and concentrated within the membrane. While for small molecules, such as denaturant and other salts, which might affect the following fluorescence derivatization, could be easily removed by permeation through the membrane. Theoretically, limited by the pore size of the membrane, no proteins could leach into the exchange buffer. As long as the protein concentration in membrane is lower than its maximum solubility, the enrichment times of the protein concentration could be approximately equal to the ratio of initial sample volume to the dead volume of hollow fiber membrane. To avoid protein dilution during derivatization, the hollow fiber membrane with the enriched proteins was directly put into derivatization buffer for in situ fluorescence derivatization without any other treatment.

The effect of sample flow rate on protein enrichment was studied, with BSA as the sample. As shown in Fig. 3, the UV response signal reached the maximum when 1.5 μL/min was used. With the increase of flow rate, the response gradually decreased. A possible reason was that with high flow rate, the generated high back pressure might lead to the change of the pore structure of hollow fiber membrane, resulting in the permeation of protein through

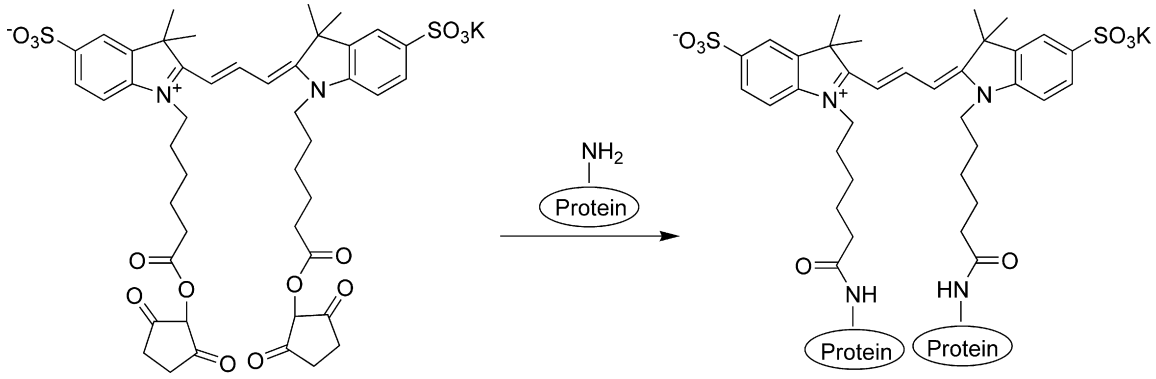

Fig. 2. Protein derivatization by sw-cy3-NHS.

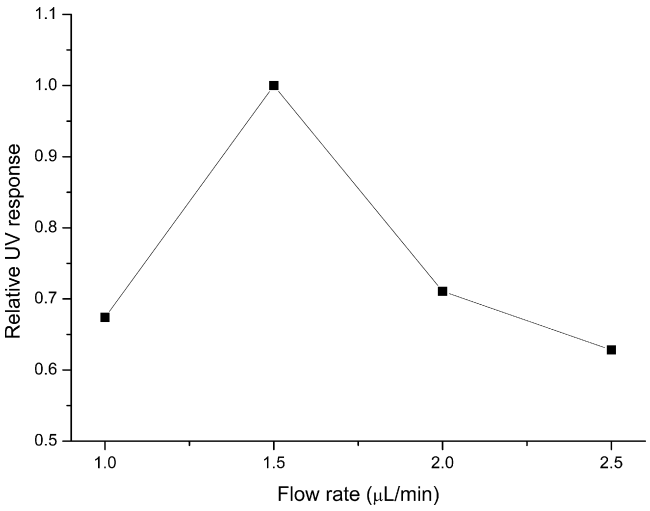

Fig. 3. Effect of sample loading flow rate on UV response of enriched BSA.

the membrane. When the flow rate was low, protein might precipitate in the inner surface of hollow fiber membrane. Therefore 1.5 μL/min was selected as the optimal sample loading flow rate for protein concentration.

3.3. Optimization of derivatization conditions

The effect of reaction temperature, ranging from 25 to 65 °C, on the derivatization of BSA was investigated. As shown in Fig. 4(A), the fluorescence intensity of BSA gradually increased with temperature, reached the maximum at 45 °C, and decreased with the further increased temperature, which demonstrated that before 45 °C, high temperature could be helpful to accelerate derivatization, and above 45 °C, the protein–dye complex became instable. Therefore, 45 °C was selected as the optimal reaction temperature.

The effect of reaction time on the derivatization of BSA was also investigated. As shown in Fig. 4(B), the fluorescence intensity of BSA reached 50% within 0.5 min, and 91% within 15 min, which indicated the fast derivatization capacity of sw-cy3-NHS for protein. Since the fluorescence intensity of BSA reached the maximum within 30 min, the optimal reaction time was selected as half an hour in the following experiments.

3.4. Analysis of standard protein

Under the above-mentioned optimal conditions, denatured BSA was respectively in-solution derivatized, and in situ derivatized within hollow fiber membrane. From Fig. 5(A), it could be seen that the fluorescence signal of 70 nmol/L BSA was only slightly higher than the LOD after in-solution derivatization. However, after preconcentration and in situ fluorescence derivatization, from Fig. 5(B), it could be seen that the fluorescence intensity reached ~500 mV, and the detection sensitivity was increased about 200 folds, better than the previous report results [21–24].

The obvious increase of the detection sensitivity should attribute to two reasons. Firstly, the concentration of protein was increased by preconcentration. Secondly, the in situ derivatization efficiency was improved within the membrane, without further dilution in solution. All these results indicated that the detection sensitivity of proteins could be efficiently improved with the combination of hollow fiber membrane based enrichment and in situ fluorescence derivatization.

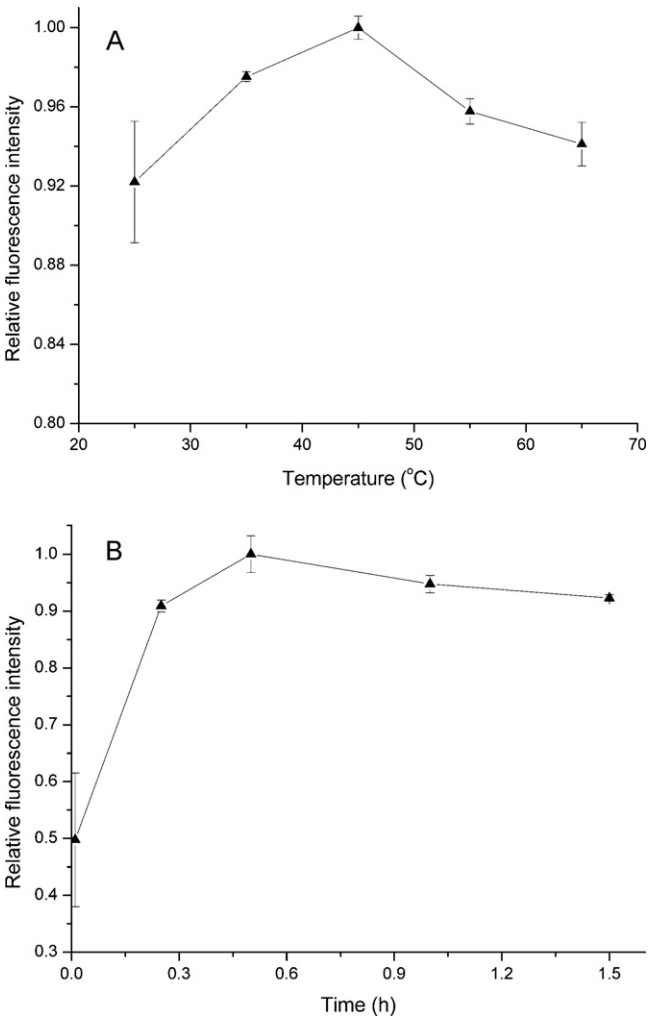

Fig. 4. Effect of reaction temperature (A) and time (B) on relative fluorescence intensity of BSA.

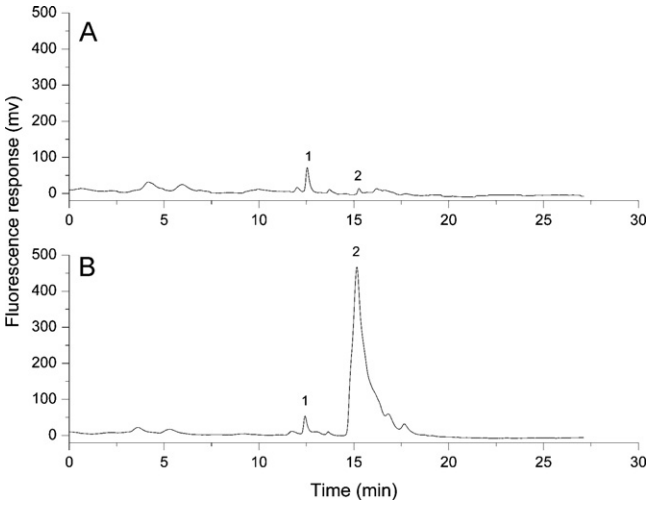

Fig. 5. Chromatograms of BSA by (A) in-solution derivatization and (B) in situ derivatization. Peak 1, impurity in fluorescence reagent; peak 2, BSA.

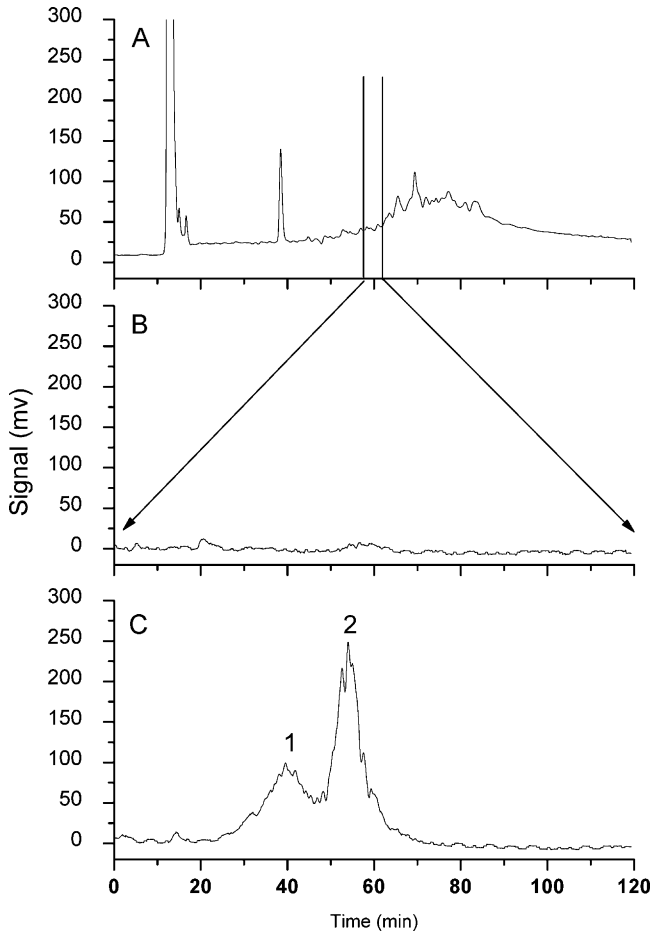

Fig. 6. Chromatogram of 100 μg *E. coli* protein separated by RPLC (A), and chromatograms of one 5 min fraction after in-solution (B) and in situ fluorescence derivatization (C).

3.5. Analysis of real sample

To further evaluate the applicability of the developed method, one *E. coli* RP fraction was further analyzed. As shown in Fig. 6(A), the eluant between 57 and 62 min was collected, lyophilized, and redissolved. After denatured with guanidine hydrochloride, the sample was in-solution derivatized, and in situ derivatized respectively. In the former case, as shown in Fig. 6(B), no peaks could be observed, which might be caused by the poor derivatization efficacy when the protein concentration was low. In the latter case, after ~160 min protein enrichment, as shown in Fig. 6(C), the fluorescence signal was significantly increased after in situ fluorescence derivatization, with two main peaks observed, which further indicated the applicability of developed method for the high sensitive detection of low concentration proteins.

It must be noted that if lyophilization, instead of the developed method, was applied for protein enrichment, guanidine hydrochloride in the sample could also be concentrated, resulting in poor fluorescence derivatization efficiency. However, with our developed device, both the protein concentration and desalting were simultaneously performed, which facilitated not only the high sensitive detection, but also the easy sample preparation.

4. Conclusion

In this work, high sensitive detection of low concentration proteins was achieved by the combination of protein enrichment and in situ fluorescence derivatization with the self-prepared hollow fiber membrane based device, by which protein enrichment and sample desalting was performed simultaneously. By comparison with the traditional in solution derivatization, the detection sensitivity of low concentration proteins was obviously improved, demonstrating the potential merit of the developed method for the high sensitive analysis of low abundance proteins.

Acknowledgements

The authors are grateful for the financial support from National Basic Research Program of China (2007CB914100), National Natural Science Foundation (20935004), Knowledge Innovation Project of Chinese Academy of Sciences (KJCX2YW.H09), and National Key Technology R. & D. Program (2008BAK41B02).

References

[1] C. Toriumi, K. Imai, Anal. Chem. 75 (2003) 3725.
[2] A. Cingöz, F. Hugon-Chapuis, V. Pichon, J. Chromatogr. B 878 (2010) 213.
[3] I. Benito, M.L. Marina, J.M. Saz, J.C. Diez-Masa, J. Chromatogr. A 841 (1999) 105.
[4] D.B. Craig, N.J. Dovichi, Anal. Chem. 70 (1998) 2493.
[5] P.H. O'Farrell, J. Biol. Chem. 250 (1975) 4007.
[6] S.P. Gygi, G.L. Corthals, Y. Zhang, Y. Rochon, R. Aebersold, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 9390.
[7] D.M. Pinto, E.A. Arriaga, D. Craig, J. Angelova, N. Sharma, H. Ahmadzadeh, N.J. Dovichi, C.A. Boulet, Anal. Chem. 69 (1997) 3015.
[8] I.H. Lee, D. Pinto, E.A. Arriaga, Z. Zhang, N.J. Dovichi, Anal. Chem. 70 (1998) 4546.
[9] M. Masuda, C. Toriumi, T. Santa, K. Imai, Anal. Chem. 76 (2004) 728.
[10] L.-Y. Zhang, M.-X. Sun, J. Chromatogr. B 877 (2009) 4051.
[11] J.R. Kraly, M.R. Jones, D.G. Gomez, J.A. Dickerson, M.M. Harwood, M. Eggertson, T.G. Paulson, C.A. Sanchez, R. Odze, Z. Feng, B.J. Reid, N.J. Dovichi, Anal. Chem. 78 (2006) 5977.
[12] V. Garcia-Canas, B. Lorbetskie, D. Bertrand, T.D. Cyr, M. Girard, Anal. Chem. 79 (2007) 3164.
[13] D.F. Swaile, M.J. Sepaniak, J. Liq. Chromatogr. 14 (1991) 869.
[14] T.T. Lee, E.S. Yeung, J. Chromatogr. 595 (1992) 319.
[15] M.D. Harvey, D. Bandilla, P.R. Banks, Electrophoresis 19 (1998) 2169.
[16] G. Hunt, W. Nashabeh, Anal. Chem. 71 (1999) 2390.
[17] S. Hu, L. Zhang, N.J. Dovichi, J. Chromatogr. A 924 (2001) 369.
[18] X.-Q. Qiao, L. Zhang, W.-B. Zhang, L.-H. Zhang, Y.-K. Zhang, Chem. J. Chin. U 28 (2007) 1657.
[19] D.A. Michels, L.J. Brady, A. Guo, A. Balland, Anal. Chem. 79 (2007) 5963.
[20] L. Wang, X. Peng, R. Zhang, J. Cui, G. Xu, F. Wang, Dyes Pigments 54 (2002) 107.
[21] X. Qiao, L. Wang, J. Ma, Q. Deng, Z. Liang, L. Zhang, X. Peng, Y. Zhang, Anal. Chim. Acta 640 (2009) 114.
[22] L. Wang, J. Fan, X. Qiao, X. Peng, B. Dai, B. Wang, S. Sun, L. Zhang, Y. Zhang, J. Photochem. Photobiol. A: Chem. 210 (2010) 168.
[23] S.K. Lau, F. Zaccardo, M. Little, P. Banks, J. Chromatogr. A 809 (1998) 203.
[24] P.R. Banks, TrAC Trends Anal. Chem. 17 (1998) 612.
[25] Website: http://www.probes.invitrogen.com/media/pis/mp06434.pdf. Version: 2, April 2010.
[26] W.-L. Tseng, H.-T. Chang, Anal. Chem. 72 (2000) 4805.
[27] W. Zhan, T. Wang, S.F. Li, Electrophoresis 21 (2000) 3593.
[28] N. Siri, P. Riolet, C. Bayle, F. Couderc, J. Chromatogr. B 793 (2003) 151.
[29] Y.-K. Park, K. Choi, A. Yacin, B.H. Ahmed, Z.A. ALOthman, D.S. Chung, J. Chromatogr. A 1217 (2010) 3357.
[30] Y. Chen, L. Xu, L. Zhang, G. Chen, Anal. Biochem. 380 (2008) 297.
[31] R.S. Foote, J. Khandurina, S.C. Jacobson, J.M. Ramsey, Anal. Chem. 77 (2005) 57.
[32] M.R. Mohamadi, N. Kaji, M. Tokeshi, Y. Baba, Anal. Chem. 79 (2007) 3667.
[33] R. Zhang, S. Hjerten, Anal. Chem. 69 (1997) 1585.
[34] C. Yang, H. Liu, Q. Yang, L. Zhang, W. Zhang, Y. Zhang, Anal. Chem. 75 (2003) 215.
[35] L. Sun, J. Duan, D. Tao, Z. Liang, W. Zhang, L. Zhang, Y. Zhang, Rapid Commun. Mass Spectrom. 22 (2008) 2391.
[36] L. Sun, J. Ma, X. Qiao, Y. Liang, G. Zhu, Y. Shan, Z. Liang, L. Zhang, Y. Zhang, Anal. Chem. 82 (2010) 2574.
[37] R.B. Mujumdar, L.A. Ernst, S.R. Mujumdar, C.J. Lewis, A.S. Waggoner, Bioconjugate Chem. 4 (1993) 105.