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Short communication

Determination of phosphoamino acids derivatized with 5-(4,6-dichloro-s-triazin-2-ylamino)fluorescein by micellar electrokinetic chromatography

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Abstract

A sensitive analytical protocol for determining phosphoamino acids using capillary electrophoresis coupled with laser-induced fluorescence detection has been developed. The technique involved the derivatization of the phosphoamino acids with fluorescent reagent 5-(4,6-dichloro-*s*-triazin-2-ylamino)fluorescein (DTAF) and the analyses of the derivatives by micellar electrokinetic chromatography with laser induced fluorescence detection (MEKC-LIF). Different variables that affect derivatization (DTAF concentration, pH, temperature and time) and separation (kind of surfactant, pH and concentration of buffer) were studied. The baseline separation of three phosphoamino acids could be obtained in less than 11 min with good reproducibility. There was a linear relationship between the peak area of the analyte and its concentration, with correlation coefficients in the range of 0.9979–0.9997. The concentration detection limits (signal to noise = 3) with respect to each single phosphoamino acid were in the range of 0.5–1 nM. The developed method was successfully applied for the determination of phosphoamino acids in the hydrolyzed phosphorylated protein samples.

Keywords: Derivatization; MEKC; 5-(4,6-Dichloro-*s*-triazin-2-ylamino)fluorescein; Phosphoamino acids

1. Introduction

Protein phosphorylation is recognized as a fundamental mechanism in the regulation of cell functions [1–4]. A variety of approaches have been developed for the identification of phosphoprotein [5–7]. The most classical method is that the protein is radioactively labeled with ^{32}P, allowing the detection of phosphoprotein by autoradiography [3]. However, it is difficult to identify the type of amino acids residues that are phoshporylated. To solve this problem, the phosphoprotein was analyzed by many separation techniques including thin-layer chromatography (TLC) [8–10], gel electrophoresis [11], and high-performance liquid chromatography (HPLC) [12–14]. Recently, several mass spectrometry (MS) methods coupled to Edman degradation were used for the identification of the phosphorylation sites in a protein sequence [8,15–17]. Yet, it should be pointed out that the achievement of best results needs a combination of more than one of these techniques [7].

Due to its high separation efficiency, short analysis time and low sample consumption, capillary electrophoresis (CE) has become a powerful tool for the analysis of biomolecules, such as amino acids, peptides, proteins and nucleic acids [18]. Previous analyses of phosphoamino acids by CE focus on micellar electrokinetic chromatography (MEKC) with UV [19,20] or indirect UV detection [21]. The detection limits of these methods were restricted because of the short optical light path available for on column UV detection. Laser-induced fluorescence (LIF) detection has been shown to be one of the most sensitive methods available for detection in CE [22]. For the best use of CE-LIF, it is necessary

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that the fluorescence excitation wavelength of analytes be close to that of laser sources. Additionally, the choice of an appropriate fluorescent derivatization reagent is important for the analytes by CE-LIF. As the important bioactive molecules in cells, phosphoamino acids have been analyzed by CE-LIF [23–26]. Phosphoserine (P-Ser), which is firstly derivatized with 1,2-ethanedithiol and then labeled with 6-iodoacetaminofluorescein to form high fluorescence derivative, can be detected by CE-LIF [23]. This method is specific for phosphoserine and allows the quantitation of phosphoserine content of peptides and proteins at <75 amol. Fluorescein isothiocyanate I (FITC) was used as a pre-column derivatization reagent for the determination of three phosphoamino acids [24]. Unfortunately, tedious derivatization process and complicated by-products limit the application of FITC. Most recently, a MEKC-LIF method for the analysis of phosphoamino acids by labeling with 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) has been developed [26]. Although the developed method can provide cleaner electropherogram and shorter analysis time than FITC does, the trace amount of phosphoamino acids in most samples required a more sensitive analytical protocol.

5-(4,6-Dichloro-*s*-triazin-2-ylamino)fluorescein (DTAF), a fluorescent fluorescein analogue, has better reactivity toward both primary and secondary amino groups than FITC [25]. DTAF, with an excitation wavelength at 490 nm and an emission wavelength at 515 nm, has been shown to be a very useful pre-column derivatization reagent for CE with LIF detection of proteins and amino acids [27–29]. Recently, Molina and Silva [30] reported the determination of phosphorus-containing amino acid herbicides in environmental samples by CE-LIF after derivatization with DTAF.

In this work, a CE-LIF method was developed for the sensitive determination of P-Ser, P-Thr, and P-Tyr derivatized with DTAF. The optimized conditions of derivatization and separation were investigated in detail. Application of this method to the determination of phosphoamino acid in a protein kinase after hydrolysis is feasible.

2. Experimental

2.1. Chemicals

All of the chemicals used in this study were of analytical grade. Sodium deoxycholate (SDC), sodium dodecyl sulfate (SDS), polyoxyethylene lauryl ether (Brij35), P-Ser, P-Thr, P-Tyr, Glu, and Asp were purchased from Sigma (St. Louis, MO, USA). Standard solutions containing 50 mM of each analyte were prepared in deionized water and stored in −20 °C. Further dilutions were made with water to required concentration. DTAF with 99% purity was obtained from Fluka (Buchs, Switzerland). Stock solution of 5 mM DTAF was prepared with dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA). Water was purified by Millipore-Q system (Millipore, USA).

2.2. Apparatus

All CE experiments were performed on an Agilent 3D capillary electrophoresis instrument (Palo Alto, CA, USA) equipped with a ZETALIF laser induced fluorescence detector (Picometrics, Ramonville, France). The excitation wavelength was chosen as 488 nm. The fluorescence emission was collected at 520 nm filtered by a band pass filter, and a notch filter was used to attenuate background radiation. Data collection and peak area analysis were performed on a HP Chemstation (Palo Alto, CA, USA). Uncoated fused-silica capillaries of 65 cm (total length) × 50 cm (length to detector window) × 50 μm i.d. ×365 μm o.d. (Yongnian Optic Fiber Inc., He Bei, China) were used in the CE procedure.

2.3. Electrophoretic procedure

The running buffer consisted of borate buffer (20 mM, pH 9.35) and Brij35 (10 mM). Buffer solutions were prepared daily and filtered through a 0.45 μm membrane prior to use.

New capillaries were pre-treated with 1 M NaOH for 60 min followed by water for 60 min at room temperature. Prior to use, the capillary was washed with 0.1 M NaOH and water for 5 min at room temperature, respectively, followed by preconditioning with running buffer for 10 min. The above flushing cycle was repeated to ensure the separation reproducibility for each injection. Sample introduction was performed by hydrodynamic injection at 50 mbar for 3 s. The calculated volume of the injection is approximately 3 nl. The applied voltage for the CE separation was typically 25 kV.

2.4. Derivatization procedure

To a 5 μl of the standard solution containing a mixture of the analytes, 5 μl of 0.2 M borate buffer (pH 9.0) and 2 μl of 5 mM DTAF were added in a 500 μl PTFE vial, and then the whole solution was diluted to 20 μl. The mixture vial was capped, homogenized, centrifuged briefly at 8000 × *g* and allowed to react at 50 °C for 45 min. After the derivatization, the vials were stored at −20 °C. The derivatization mixtures were diluted 50 times with water prior CE analysis.

2.5. Sample preparation

Nicotiana tabacum calcium/calmodulin-binding protein kinase (NtCBK2) from Tobacco cells (*Nicotiana tabacum* L. cv Wisconsin-38) was prepared by the Bac to Bac™ baculovirus expression system [31,32] (GIBCO, USA). The recombination protein was purified by a nickel-nitrilotriacetic acid (Ni-NTA) resin and calmodulin (CaM) affinity chromatography. The purified protein was stored for 2 months at −80 °C in 25 mM Tris buffer (pH 7.5) containing 30% glycerol.

NtCBK2 (0.2 μg) autophosphorylation was carried out in a 100 μl reaction mixture containing 25 mM Tris–HCl

(pH 7.5), 0.5 mM dithiothreitol, 10 mM magnesium acetate, 100 μM ATP and 1 mM $CaCl_2$ for about 30 min at 30 °C.

The samples of phosphorylated and unphosphorylated NtCBK2 were hydrolyzed in 6 M HCl at 110 °C for 4 h. Aliquots were collected, dried and finally to dissolved in 100 μl of 10 mM borate (pH 9.3). A 5 μl solution of the hydrolyzed products was directly used for the derivatization as described above.

3. Results and discussion

The aim of this work was to determine the experimental conditions that produced an optimum combination of sensitivity and speed in detecting and measuring phosphoamino acids. The migration times and peak areas of the reaction products have been used, respectively, for the identification and quantification of the analytes.

3.1. Optimization of derivatization conditions

In the study of the pre-column derivatization reagent, excess reagent is necessary to obtain the maximum derivatization efficiency. In our experiments, the effect of the DTAF concentration was investigated in the range of 0.125–1.25 mM. These results showed that the fluorescence signal intensity was enhanced with increasing DTAF concentration and reached a plateau when DTAF was higher than 0.5 mM. In addition, too high concentrations of DTAF may cause large background, which would interfere with the determination of analytes. So, the optimum DTAF concentration was selected at 0.5 mM.

The derivatization reaction of DTAF with amino groups usually process in basic medium. It has been reported the optimum pH of DTAF reacted with phosphorus-containing amino acid was 9.5 [30]. In this study, increased pH improves the derivatization yield of each analytes from pH 8.0 to 9.0. Basic medium may facilitate the labeling reaction by enhancing the nucleophilicity of amine. However, when the pH was higher than 9.0, the derivatization yield decreased. High pH buffer may cause the faster degradation of DTAF than low pH buffer and lead the decomposition of phosphoamino acids. Therefore, pH 9 was selected for further studies.

In order to optimized reaction temperature and time, the derivatization reactions were conducted over the range of 40–70 °C and up to 4 h, respectively. The significant improvements in peak heights of those DTAF derivates were obtained when the reaction proceed at 50 °C compared to other temperatures under same reaction temperature. At 50 °C, the peak height of DTAF with P-Ser and P-Tyr reached their maximum plateau in 45 min and P-Tyr needed more time (90 min) to complete its derivatization reaction. The derivatization procedure using DTAF for phosphoamino acids is faster than those using FITC. Furthermore, the derivatization yields did not increase with prolonged the reaction time. Fifty °C and 90 min were chosen as reaction temperature and time, respectively.

3.2. Stability studies

The effect of temperature on the stabilities of the DTAF derivatives was studied at 4 °C and −20 °C, respectively. It was found that all the derivatives kept almost stable in −20 °C for at least one day compared to 6 h for 4 °C. If the store time was chosen as 48 h at −20 °C, there were 7%, 22%, 17% decrease in the peak heights of P-Tyr, P-Ser, and P-Thr derivatives, respectively. In order to ensure the reproducibility of this derivatization method, all the derivatives were kept at −20 °C and analyzed within 24 h.

3.3. Optimization of separation conditions

The DTAF and DTAF derivatized amino acids are hydrophobic. Therefore, a MEKC separation mode was recommended by several literatures [27,28]. It has been reported that a choice of surfactant added to running buffer plays a central role in MEKC separation [28]. In this paper, the addition of different surfactants to the running buffer was studied. SDS as the most popular surfactant was widely employed in MEKC. When SDS was used as an additive in the running buffer, the three DTAF derivatized phosphoamino acids were completely separated, but the impurities of DTAF can interfere the determination of P-Tyr, especially in low concentration. Another anionic surfactant, SDC, was also added in the running buffer. The similar results were obtained as well. It has been reported that a non-ionic surfactant, Brij35, can significantly increase the selectively and sensitivity in CE separations of fluorescein-based tagged compounds [28,30,33]. Therefore, Brij35 was used as an additive in the running buffer. The resolution was greatly improved when 10 mM Brij35 was added in borate buffer. Higher concentrations of Brij35 were also tested for the study of separation. When the Brij35 concentration was 20 mM, the migration behavior was slightly changed compared to that using 10 mM of Brij35. Furthermore, too high Brij35 concentration led to poor reproducibility due to high current. Thus, 10 mM Brij35 was used for further investigation.

pH value of running buffer, which can influence the mobility of analytes by adjusting the velocity of EOF and the charge of analyte molecular, has been acknowledged as one of the most important parameters for CE separation. The effect of borate buffer pH on the separation of DTAF-derivatives was studied at different pH values (8.5, 9.3, 10). At pH 8.5, the migration times of the five derivatives were short, but the peaks of P-Tyr and P-Thr were overlapped. The complete resolutions of the five derivatives can be achieved at pH 9.3 as well as at pH 10. However, when the pH of running buffer was chosen as pH 10, the migration times were prolonged and the shape of peaks became worse. In addition, the peak areas of all the five derivatives were larger at pH 9.3 than those at higher or lower pH levels. Optimum running buffer pH was therefore selected as 9.3.

The effect of borate buffer concentration on the separation was also investigated. With the increase of buffer

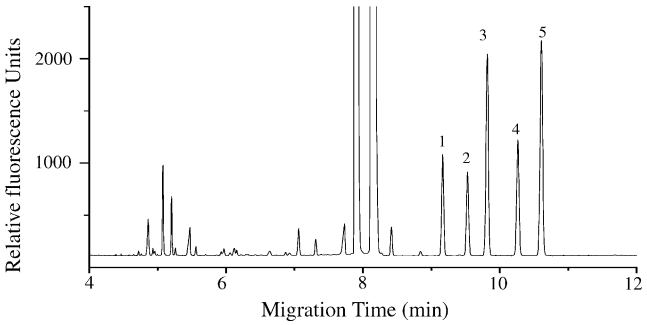

Fig. 1. Electropherograms obtained for separations of five DTAF labeling phosphoamino and amino acids in pH 9.3 borate buffer–10 mM Brij35. Separation voltage: 25 KV; injection: 50 mbar for 3 s; analyte concentrations: 1 μmol l^{-1}; peaks: (1) Glu, (2) Asp, (3) P-Tyr, (4) P-Thr, (5) P-Ser.

concentration, the migration times of analytes was increased due to the decrease of EOF. The increase of ionic strength of the buffer can cause a lower zeta-potential on the capillary surface that would lead to the suppression of EOF. When the borate buffer concentration was below 10 mM, the peaks for Asp and P-Thr could not be separated completely. Although the increase of buffer concentration can enhance the separation selectivity, the analysis time was too long when the buffer concentration was higher than 30 mM. Thus, 20 mM borate buffer was chosen as the optimum borate concentration in CE separation.

Based on the above-optimized procedure, 20 mM borate (pH 9.3) containing 10 mM Brij35 was used for the separation of the five DTAF-derivatives. Under the optimized separation conditions, the five above analytes were baseline-separated within 11 min (Fig. 1).

3.4. Reproducibility, linear calibration and limits of detection

A washing sequence consisting of NaOH, water and running buffer should be done between each run, which can effectively eliminate the absorption of highly hydrophobic fluorescein analogues onto the capillary wall. The reproducibility was evaluated with six sequential runs of phosphoamino acids and amino acids standard solution (at the concentration of 1 μM). The reproducibility of the migration times and the peak areas was found to be <1% relative standard deviation (R.S.D.) and in the range of 1.9–6.3% R.S.D. (n = 6), respectively.

Calibration curves for standards of three phosphoamino acids were constructed by plotting the peak area against the derivatized analyte concentration in the range of 0.01–1 μM under the optimum separation and derivatization conditions. The correlation coefficients for these amino acids were from 0.9966 to 0.9996, indicating good linearity. The lowest detection limits (LOD) were obtained on the basis of the minimum analyte concentration that provided a peak three times higher than the baseline noise. The concentration and mass concentration detection limits could reach 0.5 nM and 1.5 amol, respectively. The LODs are better than those of previous reported works that were used in the determination of phosphoamino acids [25,26] and equivalent to the detection limits of phosphorus-containing amino acids using DTAF [30]. All of the obtained results were listed in Table 1.

3.5. Phosphoamino acids analysis

NtCBK2 isolated form tobacco consists of 602 amino acid residues. This kinase has all 11 subdomains of a kinase catalytic domain and is structurally similar to other CaMKs in mammal systems. Biochemical analyses have identified NtCBK2 phosphorylated both itself and other substrates, such as histone IIIS and syntide-2, in a Ca^{2+}/CaM-stimulated manner. The kinase activity of NtCBK2 was greatly stimulated by Ca^{2+}/CaM [32].

In order to identify the amino acid on which phosphorylation occurred, the phosphorylated protein was hydrolyzed into a mixture of amino acids and phosphoamino acids, and analyzed by the CE-LIF method with the conditions described as above. The electropherogram of hydrolyzed products of phosphorylated NtCBK2 derivatized with DTAF was shown in Fig. 2B. As can be seen, trace amount of P-Ser was detected. This P-Ser peak was further confirmed by spiking the standard of P-Ser in the hydrolyzed sample of phosphorylated NtCBK2 (Fig. 2C). These results indicated that the NtCBK2 belonged to the Ser/Thr protein kinase. The same results for this kinase were obtained by using FITC derivatization method as our previous report [24]. However, the developed method in this paper can provide less sample consumption, higher sensitivity and shorter analysis time than

Table 1
Calibration, reproducibility and limits of detection of the DTAF derivatives

Species	Calibration curve[a], $Y = A + BX$		Linear range (μM)	Correlation coefficient	R.S.D. (%)[b]		CDL[c] (nM)	MDL[c] (amol)
	A	B			MT	PA		
Glu	1.2	1589	0.01–1	0.9996	0.2	2.3	5	15
Asp	4.1	1260	0.01–1	0.9996	0.2	2.6	5	15
P-Tyr	−2.0	3258	0.01–1	0.9997	0.4	4.3	0.5	1.5
P-Thr	−2.9	2006	0.01–1	0.9979	0.8	7.4	2	6
P-Ser	−7.0	4060	0.01–1	0.9989	0.5	6.3	1	3

[a] Y: integrated peak area (mAUs); X: a concentration of analyte (μmol l^{-1}).
[b] MT: migration time; PA: peak area.
[c] CDL: concentration detection limit; MDL: mass detection limit.

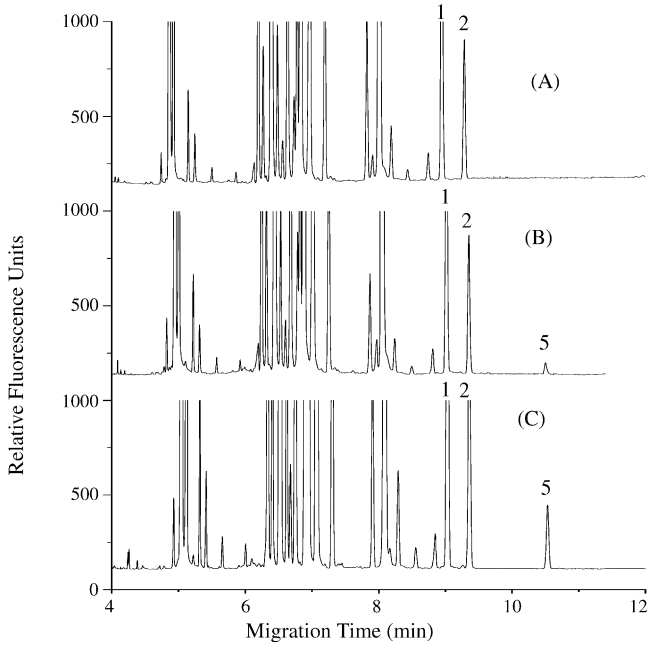

Fig. 2. Electropherograms obtained for analysis of NtCBK2 hydrolyzed sample. (A) Unphosphorylation sample, (B) atuophosphorylation sample, (C) atuophosphorylation sample spiked with P-Ser standard. Other conditions are same as in Fig. 1.

the FITC derivatization method. In addition, the hydrolyzed unphosphorylation NtCBK2 was analyzed as a control to investigate the validity of this CE-LIF method. None of the three phosphoamino acid peaks was found (Fig. 2A).

In this paper, the recoveries of phosphoamino acids were also studied. When the standards of three phosphoamino acids were added in the hydrolyzed sample of phosphorylated NtCBK2, the recoveries of P-Tyr, P-Thr, and P-Ser were 97%, 89% and 95%, respectively. However, If the three phosphoamino acids were spiked in the protein followed by the incubation in 6 M HCl at 110 °C, the recoveries of P-Tyr, P-Thr, and P-Ser were only 34%, 15% and 25%, respectively, suggesting higher temperature can lead to the instability of phosphoamino acids in aqueous medium. Even if the same acid hydrolysis condition was chosen, the recoveries of phosphoamino acids might be variable in different hydrolyzed protein samples because the acids might cleave some peptide bonds with selectivity [12]. Because of these problems, it is difficult to determine absolute amounts of each phosphoamino acids in a protein. However, the developed method should be useful to the characterization of phosphoproteins due to its high sensitivity, selectivity and simplicity.

4. Conclusion

A sensitive and selective CE-LIF method for the determination of phosphoamino acids in hydrolyzed products of phosphoprotein was developed. DTAF has been utilized for the derivatization of phosphoamino acids, followed by detection with an argon-ion LIF detector. It was demonstrated that the derivatized phosphoamino acids were stable, highly fluorescent and can be detected in a low concentration under the optimum experimental conditions. The detection limit can reach 0.5 nM and thus better than the range of the detection limits obtained by other chromatographic methods. This proposed CE-LIF method, with its satisfying sensitivity, selectivity and analysis speed, is suitable for routine analysis of phosphoamino acids in different hydrolyzed protein samples.

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References

[1] W.D. Fantl, D.E. Johnson, L.T. Willams, Ann. Rev. Biochem. 56 (1993) 453.
[2] T. Hunter, Cell 100 (2000) 113.
[3] B.A. Kelly, R.A. Carchman, J. Biol. Chem. 262 (1987) 17404.
[4] L. Zhang, Y.-T. Lu, Trends Plant Sci. 8 (2003) 123.
[5] J.X. Yan, N.H. Packer, A.A. Gooley, K.L. Williams, J. Chromatogr. A 808 (1998) 23.
[6] A. Sickmann, H.E. Meyer, Proteomics 1 (2001) 200.
[7] D.T. McLachlin, B.T. Chait, Curr. Opin. Chem. Biol. 5 (2001) 591.
[8] J.X. Yan, N.H. Packer, L. Tonella, K. Ou, M.R. Wilkin, J.-C. Sanchez, A.A. Gooley, D.F. Hochstasser, K.L. Williams, J. Chromatogr. A 764 (1997) 201.
[9] B. Dulcos, S. Marcandier, A.J. Cozzone, Methods Enzymol. 201 (1991) 10.
[10] E. Hildebrandt, V.A. Fried, Anal. Biochem. 177 (1989) 407.
[11] J. Yang, J.M. Fujitaki, R.A. Smith, Anal. Biochem. 122 (1982) 360.
[12] L.R. Murthy, K. Iqbal, Anal. Biochem. 193 (1991) 299.
[13] S.J. Niedbalski, D.P. Ringer, Anal. Biochem. 158 (1986) 138.
[14] J.C. Robert, A. Soumarmon, M.J.M. Lewin, J. Chromatogr. 338 (1985) 315.
[15] K.A. Resing, N.G. Ahn, Methods Enzymol. 183 (1997) 29.
[16] Y. Oda, T. Nagasu, B.T. Chait, Nat. Biotechnol. 19 (2001) 379.
[17] R.S. Annan, M.J. Verma, R.J. Deshaies, S.A. Carr, Anal. Chem. 73 (2001) 394.
[18] S. Hu, N.J. Dovichi, Anal. Chem. 74 (2002) 2833.
[19] M. Heber, C. Liedtke, H. Korte, E. Hoffmann-Posorske, A. Donella-Deana, L. Pinna, J. Perich, E. Kitas, R.B. Johns, H.E. Meyer, Chromatographia 33 (1992) 347.
[20] A. Wehren, H.E. Meyer, A. Sobek, P.M. Kloetzel, B. Dahlmann, Biol. Chem. 377 (1996) 497.
[21] M.W. Crowder, A. Numann, F. Haddadian, M.A. Weitzel, N.D. Danielson, Anal. Chim. Acta 384 (1999) 127.
[22] Y.F. Cheng, N.J. Dovichi, Science 562 (1988) 242.
[23] P. Fadden, T.A. Haystead, Anal. Biochem. 225 (1995) 81.
[24] B.-F. Liu, L. Zhang, Y.-T. Lu, J. Chromatogr. A 918 (2001) 401.
[25] L. Zhang, B.-F. Liu, R.L. Jones, S.-P. Liang, Y.-T. Lu, Biochem. J. 368 (2002) 145.

[26] X. Liu, L. Ma, Y.-T. Lu, Anal. Chim. Acta 512 (2004) 297.
[27] S.P.D. Lalljie, P. Sandra, Chromatographia 40 (1995) 519.
[28] S.P.D. Lalljie, P. Sandra, Chromatographia 40 (1995) 513.
[29] C.W. Mahoney, A. Azzi, Biochem. J. 243 (1987) 243.
[30] M. Molina, M. Silva, Electrophoresis 23 (2003) 1096.
[31] L.A. King, R.D. Possee, The Baculovirus Expression System: A Laboratory Guide, Chapman & Hall, London, 1992.
[32] W. Hua, S.-P. Liang, Y.-T. Lu, Biochem. J. 376 (2003) 291.
[33] M. Molina, M. Silva, Electrophoresis 22 (2001) 1175.