ELSEVIER

Journal of Chromatography A, 814 (1998) 213–221

JOURNAL OF CHROMATOGRAPHY A

Postcolumn derivatization of peptides with fluorescamine in capillary electrophoresis

Ruohua Zhu, Wim Th. Kok*

Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands

Received 5 March 1998; received in revised form 21 April 1998; accepted 4 May 1998

Abstract

Fluorescamine is used as a postcolumn derivatization reagent for fluorescence detection of peptides after separation by capillary electrophoresis. The problems resulting from the use of an organic solvent have been solved by introducing $LiClO_4$ and 5% water into the postcolumn derivatization reagent. The reaction rate and detection sensitivity of amino acids and small peptides observed with fluorescamine and OPA were compared. Fluorescamine gives much higher sensitivity than *o*-phthaldialdehyde (OPA) for small peptides, with detection limits for the selected peptides and amino acids below 0.1 $\mu mol\,l^{-1}$. Under optimized experimental conditions, the method has a good reproducibility and separation efficiency for peptides. The method was applied for the analysis of the protein tryptic digests. Only submicromolar concentrations of proteins were required.

Keywords: Derivatization electrophoresis; Peptides; Amino acids; Fluorescamine; Phthaldialdehyde; Glycine; Proteins

1. Introduction

Capillary electrophoresis (CE) plays a more and more important role in the separation and analysis of peptides and proteins in complex biological samples [1,2]. To meet the requirements of detection sensitivity, fluorescence detection (mostly, laser-induced fluorescence, LIF) is employed. Although many peptides and proteins have intrinsic fluorescence emitted from tryptophan, tyrosine or phenylalanine residues, fluorescence derivatization is very often needed to increase the detectability and the sensitivity. Common derivatization reagents for amino acids, peptides and proteins include fluorescamine [3], *o*-phthaldialdehyde (OPA) [4], fluorescein isothiocyanate (FITC), 9-fluorenylmethyl chloroformate (FMOC) [5], naphthalene-2,3-dicarboxyaldehyde (NDA) [18], and 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) [6]. Some derivatization reagents for peptides containing special amino acid residues, which have been successfully used in HPLC, are also applied in CE [7]. For instance, benzoin was used for the selective detection of arginine-containing peptides in the tryptic digest of chicken egg-white lysozyme [8]. By a combination of precolumn derivatization and LIF, the detection limit in CE can be extremely low, so that a few hundred molecules can be detected [9]. However, it is not always optimal to perform precolumn derivatization for the separation and detection of peptides and proteins. One of the disadvantages is the possibility of multiple labeling that may cause multiple peaks for one compound. Moreover, postcolumn derivatization is easier to automate and more

*Corresponding author. Fax: +31-2025256638

0021-9673/98/$19.00
PII: S0021-9673(98)00405-1

suitable for peptide mapping when an on-line protein digestion is performed [10] or for single cells analysis [11]. Among the derivatization reagents mentioned above, fluorescamine, OPA and NDA have the fastest reaction kinetics and have therefore been applied in postcolumn detection in CE [12–14].

Since its introduction by Udenfriend et al. [15], fluorescamine has become a common fluorogenic reagent used routinely in different separation techniques for a variety of organic compounds containing a primary amino group. Fluorescamine is able to react with a primary amino group to form a 5-(2-carboxyphenyl)-5-hydroxy-3-phenyl-2-pyrroline-4-one (CPP) derivative with high fluorescence yield. The reaction is complete within seconds and the excess of fluorescamine is quickly hydrolyzed in aqueous solution into a nonfluorescent product. The derivatives of fluorescamine are more stable than those of OPA and the composition of the reagent is very simple, since no assistant reagent is required except the proper buffer. More importantly, fluorescamine gives a higher fluorescence yield combined with peptides than with amino acids. Therefore, fluorescamine is very suitable as a postcolumn derivatization reagent for the determination of peptides [16]. However, there are some limitations in applying fluorescamine. The reagent is only soluble and stable in organic solvents. The fast hydrolysis rate sometimes causes low reaction yields and the optimal reaction conditions are dependent on the nature of amines [17,18].

Work has been done on the comparison of different derivatization reagents. Albin et al. [5] compared four reagents (OPA, FITC, FMOC and fluorescamine) and found that the sensitivity of fluorescamine was much lower than that of OPA for amino acids in both pre and postcolumn derivatization. In their work fluorescamine was prepared in running buffer and most of the reagent might be hydrolyzed before reaction. Gump [18] compared fluorescamine, OPA and NDA for the precolumn derivatization of proteins. Both OPA and NDA give higher sensitivity than fluorescamine. However, as was shown in HPLC, OPA is a good reagent for amino acids but not very suitable for peptides [19].

In this work, the postcolumn derivatization of amino acids and peptides with fluorescamine has been investigated. A porous tube reactor [20] was used for the postcolumn derivatization. The reaction conditions were studied in detail. As the anode is placed in the postcolumn reactor, one of the possible problems with the application of fluorescamine for postcolumn derivatization in CE is that an organic solvent, with low conductivity, has to be used for the fluorescamine solution. Various ways to solve these experimental problems have been investigated. The sensitivity of fluorescamine and OPA for the derivatization of glycine and its oligomers was compared. The method developed was used for the analysis of tryptic digests of proteins.

2. Experimental

2.1. Chemicals

Glycine (Gly), triglycine (G3), pentaglycine (G5), hexaglycine (G6), Ala-Ala (AA), pentaalanine (A5) were from Sigma. Diglycine (GG) was from BDH; tetraglycine (G4) from Nutritional Biochemicals. Cytochrome *c*, hemoglobin (from bovine blood) and trypsin were from Sigma. Fluorescamine was obtained from Acros Organics. Other chemicals were of analytical grade purity and used without further purification. Solutions were prepared with subboiled, deionized water. The fluorescamine reagent solution was prepared fresh daily.

2.2. Instruments

A Prince instrument (Prince Technologies, Emmen, The Netherlands) was used for CE. Separations were routinely carried out at a 25-kV voltage. The pressure system was modified with an electromagnetic valve, controlled by the Prince, to control air pressure for a (laboratory-made) postcolumn reactor, which has been described in detail in Ref. [20]. During electrophoresis, the same pressure is applied at the reactor and the inlet buffer vials, to prevent distortion of the flat electroosmotic flow profile in the separation capillary by pressure induced flow. The grounded side of the high-voltage source was connected to a Pt electrode in the reactor. Caution: before applying the high voltage, it should be checked that a proper connection with ground is made. Fused-silica capillaries, with an internal diameter of 75 μm, were obtained from Composite Metal Services (Hallow, UK). Pieces of 78 cm and 13.5 cm

(5.0 cm to the window) were used as separation and reaction capillaries, respectively. Samples were introduced hydrodynamically (30 mbar, 6 s or 3 s).

For fluorescence detection, an ARGOS 250 instrument (Flux Instruments, Karlskoga, Sweden) equipped with a 75-W mercury–xenon lamp was used. For fluorescamine derivatives, a Schott glass UG11 filter combined with a shortpass 550 filter and a 435-nm cut-off filter were used for excitation and emission, respectively. For OPA, a Schott glass UG11 filter and a 389-nm cut-off filter were used. A HP 35900C multichannel interface and a Chemstation with HPLC software (Hewlett-Packard, Waldbronn, Germany) were used to collect and process the detector data.

For kinetic studies, a flow-through set-up was used. Two high-precision pumps (Gynkotek, Model 300C) were used for delivering carrier and reagent solutions. Two solutions were mixed in a T piece. Coiled PTFE tubing was used as a reactor. A RF-530 fluorescence HPLC monitor (Shimadzu, Japan) was used for the fluorescence detection. The excitation and emission wavelengths were 390 nm and 470 nm, respectively. All experiments were carried out at ambient temperature (21±1°C).

2.3. Tryptic digestion

Tryptic digestion of hemoglobin was performed according to the method described by Ross et al. [21], except that the concentration of hemoglobin was 0.25 mg/ml instead of 10 mg/ml. The digestion mixture was 1:5 diluted with the running buffer and directly injected into the capillary.

Tryptic digestion of cytochrome *c* was performed according to the method of Yocom et al. [22]. Instead of 2 mg/ml of sample, 0.1 mg/ml of cytochrome *c* was used. The digestion mixture was 1:1 diluted with the running buffer and directly injected.

3. Results and discussion

3.1. Selection of organic solvents

The suitability of different organic solvents for the preparation of the fluorescamine reagent has been studied. For this, a 0.5 mmol l^{-1} fluorescamine solution in ethanol, acetonitrile (ACN), *N*,*N*-dimethylformamide (DMF) or acetone was mixed in a ratio of 1+4 with an aqueous buffer with a pH value of 8 or 10 containing 0.01 mmol l^{-1} G4. After various reaction times, the reaction mixture was injected on the CE system with a fluorescence detector. The results are shown in Table 1. With ethanol as solvent, no fluorescence was observed after reaction at pH 10 and only very low signals for pH 8. Obviously the reagent is not stable in ethanol. With the other solvents, the G4 peaks were of similar height. However, with ACN and DMF, two nitrogen-containing solvents, a number of impurity peaks were found. The fluorescent impurities with ACN and DMF were not stable at the high pH of the reaction mixture; after 15 min they had almost disappeared. With precolumn derivatization, such impurities in the solvents may be of little concern. With a postcolumn system, however, short-living fluorescent impurities will cause a high background fluorescence. With acetone as the solvent for the reagent, no impurity peaks were observed. In a postcolumn set-up, this solvent also gave a low background compared to ACN or DMF. Therefore, acetone was selected as the solvent for fluorescamine in the following experiments. The only problem is the higher volatility of acetone. At the end of the reaction capillary, a short piece of Teflon tube was connected to prevent crystallization caused by the evaporation of acetone.

3.2. Influence of pH on the derivatization

The effect of pH on the precolumn derivatization reaction is shown in Fig. 1, with GG and G4 used as model compounds. DMF was selected as the solvent

Table 1
Influence of the organic solvent on the relative fluorescence intensity of the derivative of G4

Solvent	pH 8[a]	pH 10
Acetone	1[b]	1
ACN	0.85	1.00
DMF	0.90	0.74
Ethanol	0.007	0

[a] Reaction conditions: 10^{-4} mol l^{-1} G4 in 0.8 ml borate buffer was mixed with 0.2 ml of 0.5 mmol l^{-1} fluorescamine in different solvents.
[b] By comparison with the fluorescence intensity in acetone.

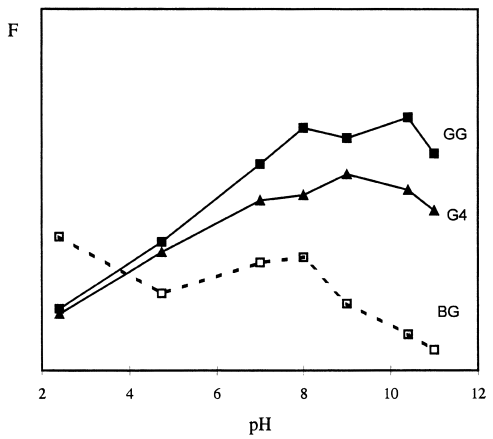

Fig. 1. Influence of pH on the derivatization. F=Relative fluorescence intensity. Derivatization reagent: 1 mmol l^{-1} fluorescamine in DMF. Injected concentration of compounds: 10 μmol l^{-1}. BG=Impurity peak height.

to examine the fluorescence intensity of impurity peaks at different pH. The reaction of fluorescamine with amino acids and peptides has a large workable pH range, from pH 7 to pH 10.5. The concentration of the buffer in the reaction solution is important. With an increase of the buffer concentration, the fluorescence intensity decreased. For example, the fluorescence intensity decreased three times when the concentration of borax at pH 10 was increased from 3 to 9 mmol l^{-1} in the final reaction solution. It seems that the rate of fluorescamine hydrolysis is increased at high buffer concentrations. In Fig. 1, the intensity of the largest impurity peak found with DMF is also shown. At lower pH, the fluorescence from the impurities increased.

When postcolumn reaction was performed with acetone as the solvent, the same workable pH range was found, but the influence of the running buffer concentration was small. There were no obvious differences in the peak height when the concentration of boric acid in the running buffer was increased from 15 to 30 mmol l^{-1}. However, there are some limitations for selecting running buffers in postcolumn derivatization because many good organic buffers contain primary amine groups. 3-Cyclohexylamino-1-propanesulfonic acid (CAPS) buffer could not be used for the postcolumn derivatization either, because fluorescamine also reacts with secondary amines, giving nonfluorescent derivatives.

3.3. Reaction rate of the derivatization

The rate of the reaction of fluorescamine with glycine and its oligomers was examined using a flow-through system. The sample was injected into a carrier solution of borate buffer of pH 9.5 and then merged with a reagent flow (1 mmol l^{-1} fluorescamine). Flow-rates were varied while a constant ratio of the buffer and the reagent flows was kept at 4:1. An acetone concentration of 20% in the reaction mixture was found to be the best to get a stable baseline and high fluorescence intensity of derivatives. The results of these studies are shown in Fig. 2a. The peak areas given in the figure are normalized with respect to the flow-rate. From the experimental results, it is clear that the reaction rate for all compounds studied is similar and that the reaction can be complete in less than 10 s. It can also be noticed that there are no large differences in the fluorescence intensity for these compounds. Similar experiments have been conducted with OPA as the reagent. The reaction of Gly with OPA was very fast, but for the glycine oligomers, the reaction was still not complete with a reaction time as long as 30 s.

The reaction rate was also studied with a CE set-up. In the CE postcolumn reaction system used, the reaction time is controlled by means of the pressure on the reagent vial. However, in a mixed aqueous–organic solvent system, changing the pressure does not only change the reaction time, but also the ratio of the running buffer and the reagent solution, that is, the proportion of acetone in the mixed reaction solution. The influence of the pressure on the fluorescence intensity of the derivatives is shown in Fig. 2b, in which the peak area was normalized with respect to the flow-rate in the reaction capillary. The peak areas did not change at the reaction time longer than 20 s. When the pressure was varied from 17 mbar to 30 mbar with the reaction times dropping from 12 to 5 s, a sharp decrease of the normalized peak areas was observed. This cannot simply be explained by the dilution in the mixing solution or the shorter reaction time. The dominating factor is the proportion of acetone in the reaction mixture. The fluorescence intensity of fluorescamine–amine derivatives strongly depends on the ratio of aqueous and organic solvent. Previous studies have revealed that for peptides the content of

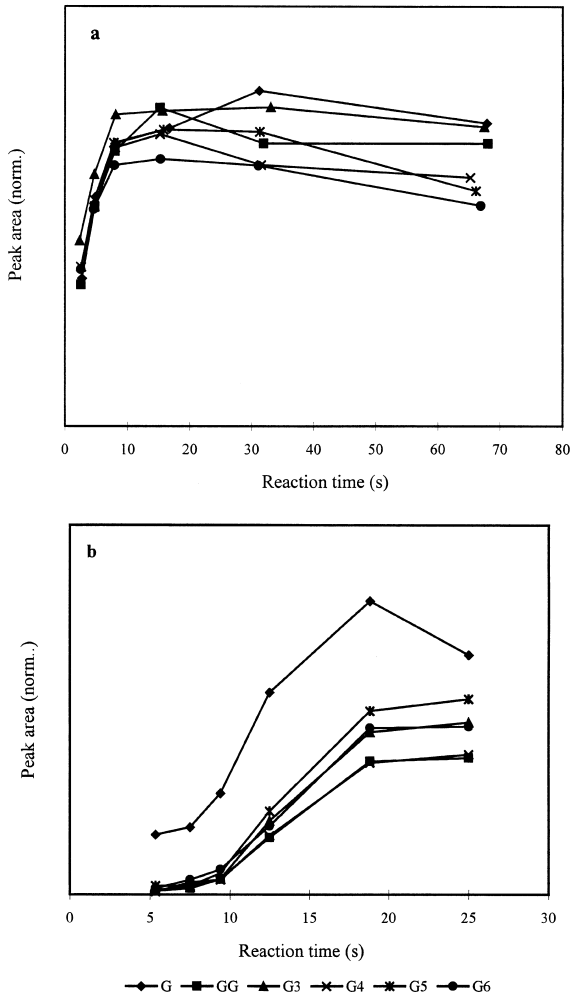

Fig. 2. Influence of the reaction time on the fluorescence intensity of fluorescamine derivatives. (a) Obtained by variation of the flow-rates in a flow-through system. Carrier: 15 mmol l^{-1} boric acid buffer, pH 9.5. Reagent: 0.5 mmol l^{-1} fluorescamine in acetone. Injected concentration: 10 μmol l^{-1}. (b) Obtained by variation of the pressure on the reagent reservoir in a CE system. Separation buffer: 20 mmol l^{-1} borate buffer, pH 9.5. Reagent: 1 mmol l^{-1} fluorescamine and $LiClO_4$ and 5% water in acetone. Applied voltage: 25 kV; injection: 30 mbar, 6 s. Injected concentration: 10 μmol l^{-1}.

organic solvent should be as low as possible [16], while for alkylamines it should be higher than 30% [17]. In general, in a pressure-driven postcolumn reaction system, the reaction time should be kept short to avoid unnecessary peak broadening. However, in our set-up, a decrease of the reaction time (by application of a higher pressure on the reagent vial) is automatically accompanied with a higher organic solvent content of the reaction mixture. Therefore, a compromise had to be found between high sensitivities and high plate numbers. Routinely, we used a pressure of 20 mbar, giving a reaction time of 17 s and an acetone concentration of approximately 30%. Under these conditions, plate numbers of 90 000 were obtained for the glycine oligomers.

3.4. Optimization of the PCR composition

Fig. 3 shows the effect of the concentration of fluorescamine in the PCR on the sensitivity and signal-to-noise ratio for G4. The highest S/N values were found with reagent concentrations between 0.5 and 1 mmol l^{-1}. At higher fluorescamine concentrations, the background signal and noise increased, leading to a lower S/N ratio. In further experiments, a fluorescamine concentration of 1 mmol l^{-1} was used.

When pure acetone was used as solvent for the PCR, several problems were encountered. The electrode in the reaction vessel soon became black. The repeatability of migration times and peak areas was very poor. As is shown in Fig. 4, the baseline signal was unstable and noisy. A possible explanation is that fluorescamine is reduced on the electrode, or

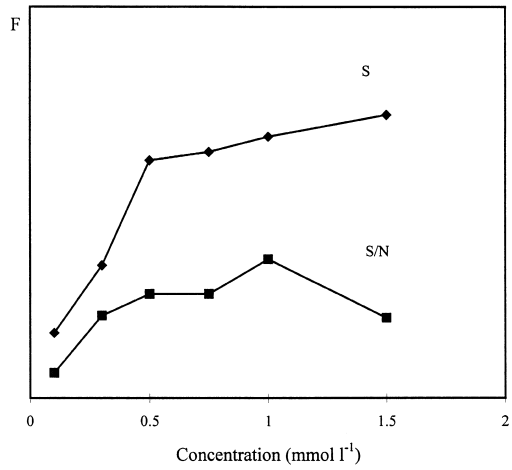

Fig. 3. Influence of the concentration of fluorescamine on the fluorescence intensity and signal-to-noise ratio of G4. Fluorescamine was prepared in acetone. For other conditions see Fig. 2b.

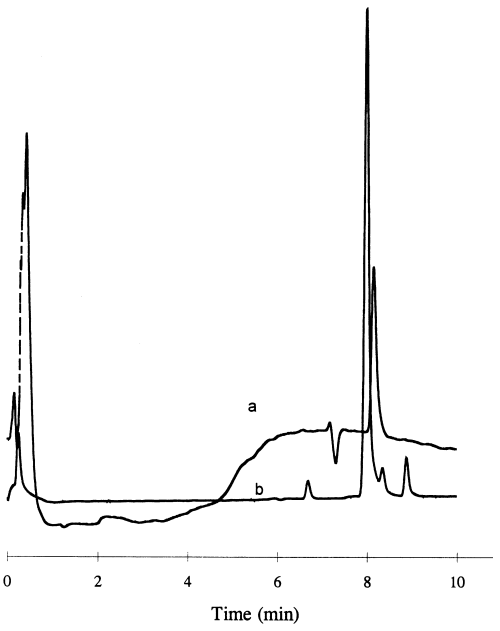

Fig. 4. Electropherograms of G4 without (a) or with (b) $LiClO_4$ in the reagent. Sample: 0.1 mmol l^{-1} G4; experimental conditions as in Fig. 2b.

that an unknown product of the electrode reaction disturbs the detection. We have tried to solve the problem by adding iodine to the PCR. Iodine, which is well soluble in acetone, is easily reduced and may therefore prevent other unfavorable reactions on the cathode. It appeared that the baseline stability was improved with the addition of iodine. However, the observed sensitivities were strongly reduced, possibly by the absorption of light or fluorescence quenching by the iodine molecules. Another possible cause for the unstable baseline obtained could be the low (and unstable) conductivity of the PCR. Therefore, we examined the effect of the addition of lithium perchlorate, which has a relative high solubility in acetone. The addition of 0.5 to 5 mmol l^{-1} of $LiClO_4$ to the PCR strongly improved the baseline stability. No significant effect of the low concentrations of $LiClO_4$ on the sensitivities was observed. Therefore in further experiments 1 mmol l^{-1} $LiClO_4$ was used in the reagent.

Still, even with the addition of salt to the PCR, the repeatability of peak areas and times was poor. It was found that this problem could be solved by the addition of a small amount of water to the PCR

Table 2
Influence of water content in PCR on R.S.D., relative fluorescence intensity and electroosmotic flow[a]

	Content of water in the PCR			
	0%	1%	5%	10%
F[b]	1	1.1	1.3	1.1
μ_{eof} (10^{-8} m^2 V^{-1} s^{-1})	5.73	5.72	6.02	6.06
R.S.D.[c] (t_r)	2.7	0.8	0.3	0.3
R.S.D.[c] (A)	8.2	2.4	4.0	4.8

[a] Experimental conditions: 25 kV, 20 mbar, running buffer: 15 mmol l^{-1} boric acid, pH 9.5. [G4]=10 μmol l^{-1}.
[b] By comparison with the fluorescence intensity of 0% water.
[c] n=5.

solvent. In Table 2, the effect of the water content of the PCR on the sensitivity and repeatability for G4 is shown. With 1 or 5% (v/v) water the best results were found. The improvement of the repeatability by the addition of water is probably also related to the increased conductivity of the PCR with water. Without water, the (low) conductivity of the acetone solution might fluctuate with the leakage of water into the reagent reservoir from the separation buffer. The fluctuating voltage drop over the PCR solution could induce the poor repeatability of the migration time. With 10% water, the fluorescamine reagent was found to be too unstable, causing decreasing sensitivities after a few analyses. Finally, the optimum composition of the PCR was found to be 1 mmol l^{-1} fluorescamine and 1 mmol l^{-1} $LiClO_4$ in water–acetone (5:95).

In our postcolumn reaction system, the grounded electrode is placed in the reagent vessel. Therefore, we did not find a large influence of the conductivity of the PCR on the migration times, as had been found by Wu et al. [23]. As long as the conductivity was above a minimum limit, the water or salt content of the PCR effected the migration times only marginally. Moreover, the current during the separation was found to be stable.

3.5. Comparison of fluorescamine and OPA

Fig. 5 shows the electropherograms obtained with a standard solution of amino acids after postcolumn derivatization with fluorescamine and OPA. The concentrations of the amino acids with fluorescamine and OPA were 0.35 and 0.25 μmol l^{-1}, respectively.

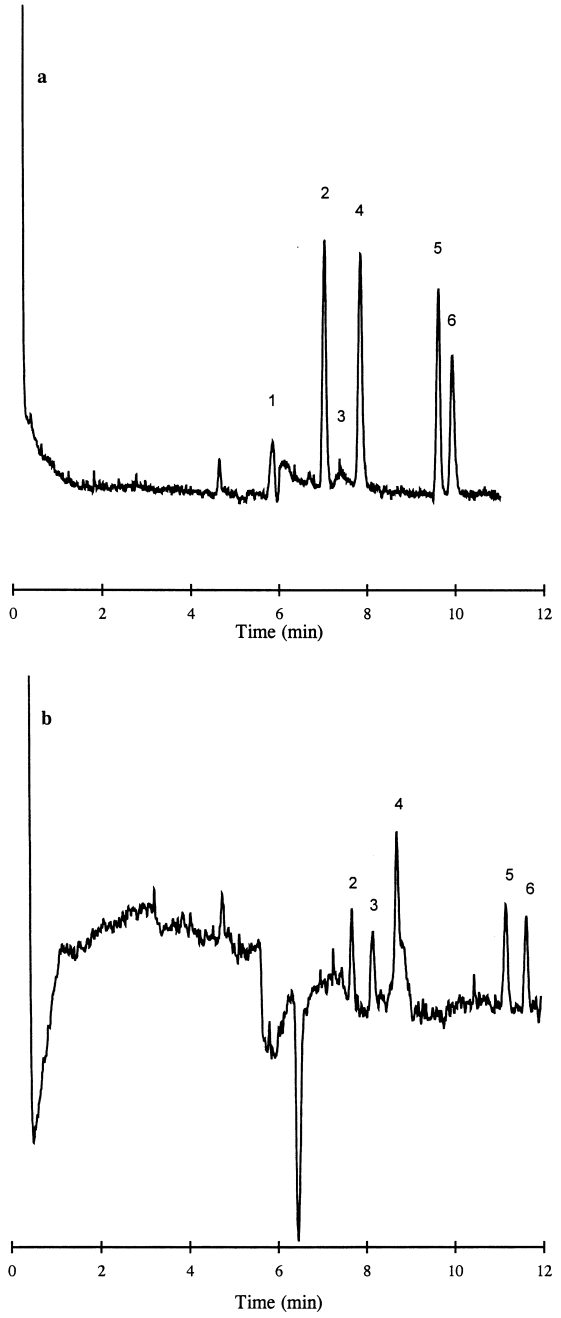

Fig. 5. Electropherograms of amino acids derivatized with fluorescamine (a) and OPA (b). Separation buffer, 20 mmol l^{-1} boric acid pH 9.5. Fluorescamine reagent is as in Fig. 2b. OPA reagent as in Ref. [13]. Other conditions as in Fig. 2b. Peaks: 1=Lys, 2=Gly, 3=His, 4=Ser, 5=Glu and 6=Asp.

When fluorescamine was used, there were some problems with the determination of the basic amino acids. The peak of Lys was very broad at higher concentration, and the His peak disappeared when the concentration was lower than 0.8 μmol l^{-1}. In the case of OPA, a negative peak appeared where the peak of Lys should be so that the Lys peak could not be observed at low concentration (0.25 μmol l^{-1}). The detection limits of amino acids are listed in Table 3. The results with OPA for some amino acids were slightly better than that with fluorescamine.

Fig. 6 shows the electropherograms obtained with a standard solution of Gly and its oligomers after postcolumn derivatization with fluorescamine and OPA. It can be seen that when fluorescamine was used in the PCR, the peak heights of the oligopeptides were similar to that of Gly. However, when OPA was used, the difference in sensitivity between Gly and oligopeptides was considerable. Similar results with OPA have been obtained by Chow et al. [19]. They showed that it is not a slow reaction rate but the low fluorescence yield that results in the low sensitivity of small peptides. In both pre and postcolumn derivatization with OPA, the difference in fluorescence intensity between Gly and its oligomers was larger than with UV absorption, and the sensitivity with fluorescence detection was even worse than with UV detection for these small peptides.

The detection limits for the oligopeptides examined in the experiments with fluorescamine and OPA are also listed in Table 3. The detection limits are around 0.1 μmol l^{-1} with fluorescamine. There is

Table 3
Detection limits of some amino acids and small peptides derivatized with fluorescamine (Flu) and OPA by postcolumn derivatization

Compounds	LOD (μmol l^{-1})		Compounds	LOD (μmol l^{-1})	
	Flu	OPA		Flu	OPA
Ala	0.09	0.08	GG	0.06	0.7
Gly	0.04	0.1	G3	0.07	0.7
Lys	0.1	–	G4	0.1	1.0
His	0.8	0.13	G5	0.1	0.8
Ser	0.04	0.05	G6	0.1	0.9
Glu	0.04	0.1	AA	0.2	0.8
Asp	0.06	0.1	AGG	0.1	2.0
			A5	0.07	1.4

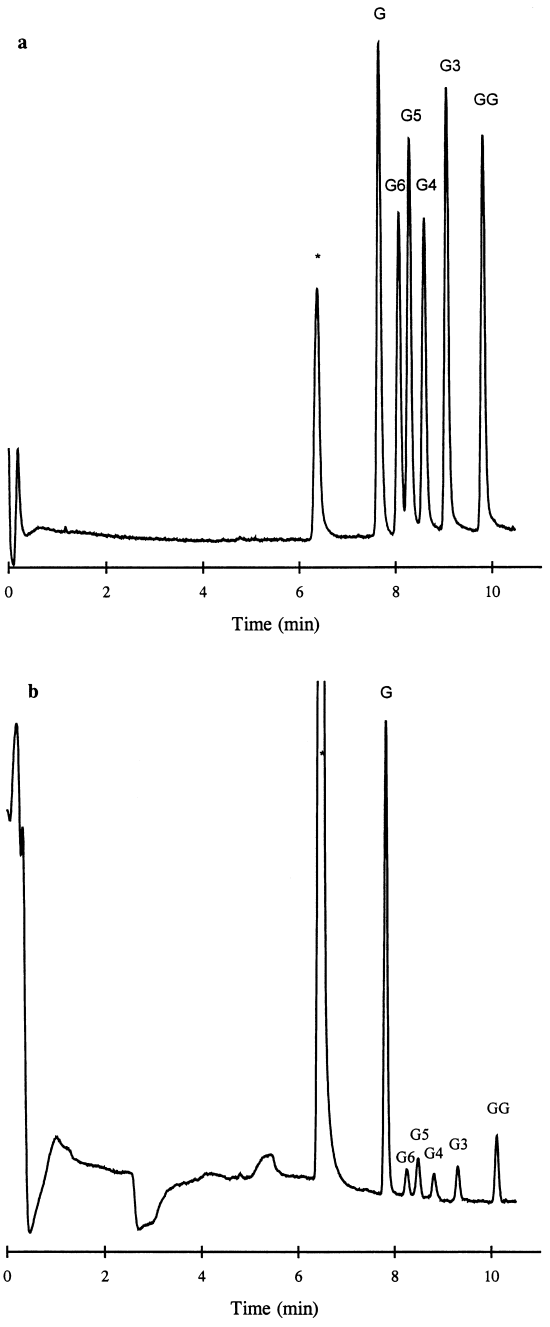

Fig. 6. Electropherograms of Gly and its oligomers derivatized with fluorescamine (a) and OPA (b). Injected concentrations: 10 μmol l^{-1}. *: EOF marker. Other conditions as in Fig. 5.

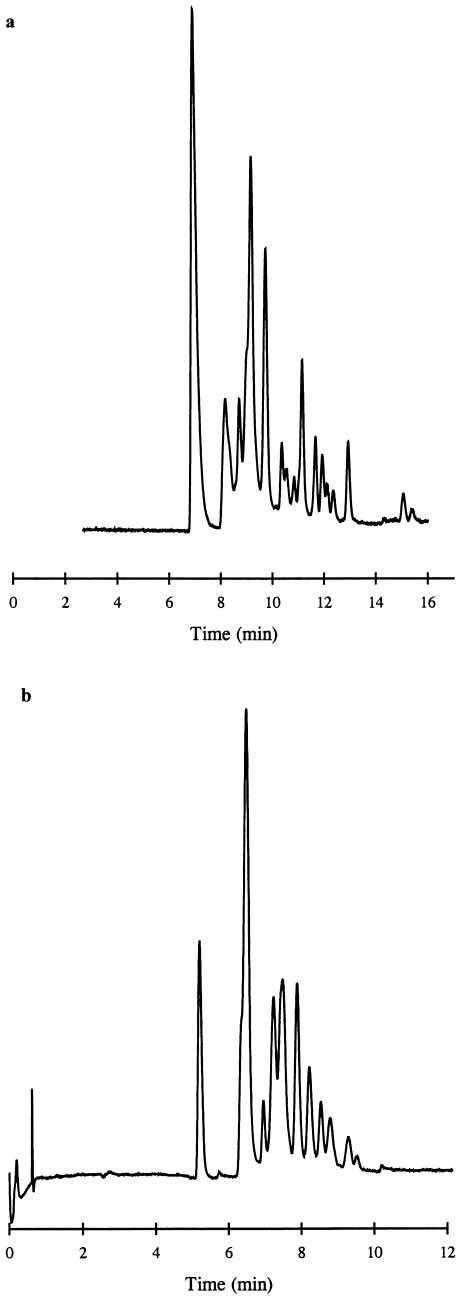

Fig. 7. (a) Electropherogram of a hemoglobin tryptic digest. The digestion mixture was 5 times diluted by running buffer (15 mmol l^{-1} borate) before injection. Other conditions as in Fig. 2b. (b) Electropherogram of a cytochrome *c* tryptic digest. The digestion mixture was 1:1 diluted by running buffer (15 mmol l^{-1} borate) before injection.

no obvious decrease in the sensitivity with an increase of the peptide chain length. For Ala and its oligomers, a different phenomenon was observed. A5 has a stronger fluorescence and lower limit of detection (LOD) than Ala and AA. The LODs for small peptides are approximately two orders of magnitude better than those reported in a study in which OPA was used as the PCR and a He–Cd laser as the excitation source [24]. However, in this study a narrower capillary (30 μm I.D.) was used as reactor. The linear ranges found for the peptides were from 50 $\mu mol\,l^{-1}$ to 0.1 $\mu mol\,l^{-1}$, covering almost three orders of magnitude.

The method was used for the determination of the tryptic digests of proteins. Fig. 7a and b show the separation of the tryptic digests of cytochrome *c* and hemoglobin, respectively. The final concentrations of proteins used in the experiment were 4 and 0.8 $\mu mol\,l^{-1}$ of cytochrome *c* and hemoglobin, respectively. The separation was conducted in a simple boric acid buffer. Compared with UV detection (the results are not shown), the sensitivity was at least increased 10-fold.

Acknowledgements

This work was financially supported by the European Commission (contract number CI1*-CT93-0087), as part of the Joint Research Project between the Laboratory for Analytical Chemistry of the University of Amsterdam and the Regional Research Laboratory in Bhopal, M.P., India. The authors would like to thank Wim Ozinga for his participation in the experimental work.

References

[1] R. Lehmann, H.M. Liebich, W. Voelter, J. Cap. Electrophoresis 3 (1996) 89–110.
[2] C. Schoneich, A.F.R. Huhmer, S.R. Rabel, J.F. Stobaugh, S.D.S. Jois, C.K. Larive, T.J. Siahaan, T.C. Squier, D.J. Bigelow, T.D. Williams, Anal. Chem. 67 (1995) 155R–181R.
[3] S.A. Shippy, J.A. Jankowski, J.V. Sweedler, Anal. Chim. Acta 307 (1995) 163–171.
[4] S.D. Gilman, J.J. Pietron, A.G. Ewing, J. Microcol. Sep. 6 (1994) 373–384.
[5] M. Albin, R. Weinberger, E. Sapp, S. Moring, Anal. Chem. 63 (1991) 417–422.
[6] J. Liu, Y.- Z Hsieh, D. Wiesler, M. Novotny, Anal. Chem. 63 (1991) 408–412.
[7] H. Cui, J. Leon, E. Reusaet, A. Bult, J. Chromatogr. A 704 (1995) 27–36.
[8] K.A. Cobb, M.V. Novotny, Anal. Biochem. 200 (1992) 149–155.
[9] E.A. Arriaga, Y. Zhang, N.J. Dovichi, Anal. Chim. Acta. 299 (1995) 319–326.
[10] L.N. Amankwa, W.G. Kuhr, Anal. Chem. 65 (1993) 2693–2697.
[11] P.B. Hietpas, A.D. Ewing, J. Liq. Chromatogr. 18–19 (1995) 3557–3576.
[12] M.E. Szulc, I.S. Krull, J. Chromatogr. A 659 (1994) 231–245.
[13] R. Zhu, W.Th. Kok, J. Chromatogr. A 716 (1995) 123–133.
[14] S.D. Gilman, A.G. Ewing, Anal. Methods Instrum. 2 (1995) 133–141.
[15] S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber, M. Weigele, Science 178 (1972) 871–872.
[16] R.W. Frei, L. Michel, W. Santi, J. Chromatogr. 126 (1976) 665–667.
[17] D.B. Gladilovich, J. Anal. Chem. 48 (1993) 1070–1074.
[18] E.L. Gump, C.A. Monnig, J. Chromatogr. A 715 (1995) 167–177.
[19] J. Chow, J.B. Orenberg, J. Chromatogr. 386 (1987) 243–249.
[20] R. Zhu, W.Th. Kok, Anal. Chem. 69 (1997) 4010–4016.
[21] G.A. Ross, P. Lorkin, D. Perrett, J. Chromatogr. 636 (1993) 69–79.
[22] K.M. Yocom, J.B. Shelton, J.R. Shelton, W.A. Schroeder, G. Worosila, S.S. Isied, E. Bordignon, H.B. Gray, Proc. Natl. Acad. Sci. USA 79 (1982) 7052–7055.
[23] N. Wu, C.W. Huie, J. Chromatogr. 634 (1993) 309–315.
[24] L. Zhang, E.S. Yeung, J. Chromatogr. A 734 (1996) 331–337.