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Improved high-performance liquid chromatography of amino acids derivatised with 9-fluorenylmethyl chloroformate

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Abstract

An improved high-performance liquid chromatography method for the separation of amino acids derivatised with 9-fluorenylmethyl chloroformate (Fmoc) is described. This method, in conjunction with a multi-tasking program, not only allows high automatic throughput but also offers baseline resolution of the common Fmoc-amino acids from a linear acetonitrile gradient, greater chromatographic reproducibility over the life of the column (800 runs) and easy column regeneration. The methods described are shown to be suitable for analysis of hydrolysates of proteins from two-dimensional gels for protein identification purposes, and will also be useful for routine quality control and screening of biological samples.

Keywords: Derivatization, LC; Amino acids

1. Introduction

There has been much recent interest in the application of amino acid analysis for the purpose of rapid and inexpensive protein identification [1–8]. This application is especially useful for the routine identification of proteins from reference maps generated by two-dimensional electrophoresis [1–3,5]. Accordingly, there remains a high demand for robust, precise and automated amino acid analysis, not only for protein identification purposes but also for routine quality control and screening of biological samples.

Previously, we described high-performance liquid chromatography (HPLC) methods for the precolumn derivatisation of amino acids with 9-fluorenylmethyl chloroformate (Fmoc) [9,10]. This method yielded monosubstituted amino acid derivatives for the common protein amino acids, including histidine and tyrosine, which by comparison with amino acids derivatised with phenyl isothiocyanate (PITC), *o*-phthaldialdehyde (OPA), or naphthalene-2,3-dicarboxaldehyde (NDA) were more stable, not susceptible to major matrix interference reactions, and able to be detected in lower amounts [9–15]. Furthermore, the precolumn derivatisation process developed did not require solvent extraction steps to remove excess derivatisation reagent prior to chro-

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matography, allowing derivatisation to be completely automated [10].

We report here details of an improved HPLC method for the separation of amino acids derivatised with Fmoc. This method, in conjunction with a multi-tasking program that controls the pump and autosampler simultaneously, not only allows high automatic throughput but also offers baseline resolution of the common Fmoc-amino acids from a linear acetonitrile gradient, greater chromatographic reproducibility over the life of the column, and easy column regeneration.

2. Experimental

2.1. Chemicals

Fmoc (9-fluorenylmethyl chloroformate), hydroxylamine hydrochloride and 2-(methylthio) ethanol were obtained from Sigma, (St. Louis, MO, USA). Amino acid calibration standard A 'Sepramar', sodium hydroxide, boric acid, anhydrous ammonium monohydrogenorthophosphate and anhydrous ammonium dihydrogenorthophosphate were obtained from BDH (Poole, UK). Acetic acid was obtained from Mallinckrodt (Paris, KY, USA). Acetonitrile and methanol (HPLC grade) were purchased from Ajax (Sydney, Australia). All chemicals were at least AR grade and all solutions prepared with Milli-Q (Millipore, Sydney, Australia) purified water.

2.2. Apparatus, automatic derivatisation and chromatography conditions

Two-dimensional gel electrophoresis and blotting to polyvinylidene difluoride (PVDF) membrane of *E. coli* proteins were as described by Bjellqvist et al. [16]. PVDF-bound proteins were subjected to gas-phase acid hydrolysis (6 *M* HCl) at 155°C for 1 h. Amino acids were recovered using a single-step extraction involving sonication for 10 min in 60% (v/v) acetonitrile, 0.01% (v/v) trifluoroacetic acid (TFA). PVDF was removed, the extraction solution evaporated to dryness, and amino acids taken up in 10 μl of 250 m*M* sodium borate buffer (pH 8.8) [3]. Amino acid analysis was performed with a GBC AMINOMATE system (GBC Scientific Equipment, Dandenong, Victoria, Australia) [1,2], which was entirely controlled by GBC WinChrom software under the Microsoft® Windows™ 3.11 environment. The AMINOMATE system consisted of a LC 1150 pump, a LC 1250 fluorescence detector (excitation λ=270 nm, detection λ=316 nm), a LC1610 autosampler and a LC 1440 solvent organiser. The column used was a 150×4.6 mm I.D. 5 μm ODS-Hypersil (Keystone Scientific, Bellefonte, PA, USA; Part No: 155-33-V) coupled with a low dead volume 2-μm stainless-steel in-line filter.

Fmoc derivatisation of amino acids was performed manually or automatically in glass autosampler vials according to Haynes et al. [9,10], prior to injection onto the chromatography column. A 10-μl volume of amino acid standard (500 pmol per amino acid in 250 m*M* borate buffer, pH 8.8) was mixed with 10 μl of Fmoc (4 mg/ml, w/v, in acetonitrile) and allowed to stand for 90 s. A 10-μl volume of cleavage reagent (made from a mixture of 340 μl of 0.85 *M* sodium hydroxide, 150 ml of 0.5 *M* hydroxylamine hydrochloride and 10 μl of 2-(methylthio) ethanol) was then added, the solution mixed, and allowed to stand for 3.5 min. The reaction was stopped by adding 10 ml of quenching reagent (20% glacial acetic acid, v/v, in acetonitrile). The mixing steps were conducted by the injection of 30 ml of air through the solution, and the entire derivatisation was carried out at room temperature.

To increase automatic system throughput, we designed a multi-tasking method that runs a separation gradient of one sample and simultaneously derivatises the next sample to be analysed. The pump gradient is modified to have a 1 min post-injection equilibration of the system, before the separation gradient commences, and three sets of autosampler instructions must be used. The autosampler instructions used are shown in Table 1 and Fig. 1. This multi-tasking program shortens analysis time for each sample from 50 min to 38 min, increasing sample throughput and reducing the consumption of solvents.

Chromatographic separation was carried out using a ternary solution system. Solution A was 30 m*M* ammonium dihydrogenorthophosphate buffer (pH 6.5) in 15% (v/v) methanol, solution B was 15% (v/v) methanol, and solution C was 90% (v/v) acetonitrile. All solutions were degassed by helium

Table 1
Sequence for the automated derivatisation of amino acids and multi-tasking

1) wash needle	18) needle out of line
2) get Fmoc	19) wait 23 minutes
3) put Fmoc to sample vial	20) wash needle
4) mix solution	21) get Fmoc
5) wait 60 s	22) put Fmoc to sample vial +1
6) wash needle	23) mix solution
7) get cleavage reagent	24) wait 60 s
8) put cleavage reagent to sample vial	25) wash needle
9) mix solution	26) get cleavage reagent
10) wait 200 s	27) put cleavage reagent to sample vial +1
11) wash needle	28) mix solution
12) get quenching reagent	29) wait 200 s
13) put quenching reagent to sample vial	30) wash needle
14) mix solution	31) get quenching reagent
15) wash needle	32) put quenching reagent to sample vial +1
16) inject sample (needle in line)	33) mix solution
17) wait 45 s	34) wait for gradient finish

In the automated processing of a batch of samples, the first instruction set used is steps 1–34. This derivatises and injects the first sample, and then derivatises the second sample during the chromatography of the first. The second instruction set used is steps 15–34, which injects the second sample and derivatises the third sample during the chromatography of the second. Steps 15–34 are used for all further samples with the exception of the last, which only requires steps 15–16 to inject this final sample.

sparge and filtered through a 0.22-μm PVDF filter before use. Flow-rate was at 1.0 ml/min, and the column maintained at 38°C. Gradient profiles used for amino acid separation are shown in Table 2.

3. Results and discussion

3.1. Optimisation of chromatographic separation

Initially, the chromatography used for the separation of Fmoc derivatised amino acids was according to Haynes et al. [9,10]. This chromatography utilised a complex, multi-segment gradient, in conjunction with a reversed-phase 3-μm particle size Spherisorb ODS-2 column (150×4.6 mm I.D.). It did not provide baseline resolution of all amino acids. There was a large void volume before elution of amino acids (ca. 4–5 min) and Fmoc-lysine was not always eluted from the column before hydrophobic non-amino acid peaks (see [10]). Furthermore, the 3-μm particle size column required high salt concentrations (20 m*M* to 26 m*M* ammonium dihydrogenorthophosphate, pH 6.5) to achieve optimal separations. This high salt concentration resulted in an accelerated hydrolysis of the bonded phase which led

Table 2
Chromatographic gradient conditions for Fmoc amino acid analysis

Time	[Phosphate]								
(min)	5m*M*			6m*M*			7m*M*		
	A(%)	B(%)	C(%)	A(%)	B(%)	C(%)	A(%)	B(%)	C(%)
0	14.2	70.8	15	17	68	15	19.8	65.2	15
1	14.2	70.8	15	17	68	15	19.8	65.2	15
32	9	45	46	10.8	43.2	46	12	40	46
32.05	0	0	100	0	0	100	0	0	100
34	0	0	100	0	0	100	0	0	100
34.05	14.2	70.8	15	17	68	15	19.8	65.2	15

Eluents A–C. A=30 m*M* ammonium dihydrogenorthophosphate (pH 6.5) in 15% (v/v) methanol; B=15% (v/v) methanol; C=90% (v/v) acetonitrile; flow-rate=1.0 ml/min.

to an undesirable shift in retention time. To compensate this shift, frequent changes in the acetonitrile gradient and further increases in salt concentration were required to maintain separation. The use of a 3-μm particle size column packing also generated high pump back-pressures (22 to 30 Mpa), which constantly increased with column use due to accumulation of fines in the column.

Trials were run on a number of columns to find an improved means of separation. We found that a 5-μm ODS-hypersil column (150×4.6 mm I.D.) coupled with a low dead volume 2-μm stainless-steel in-line filter offered superior separation and reproducibility compared to other columns. With this column, a linear acetonitrile gradient (Table 2 and Fig. 1) eluted all protein amino acids using ammonium dihydrogenorthophosphate concentrations in the range of 5 to 7m*M* (pH 6.5). A further benefit was that pump back-pressure was reduced to 6 to 7 Mpa and did not increase after extended column use. An explanation of this would be that the 5-μm column packing material has a more uniform size distribution profile than that of the 3-μm material.

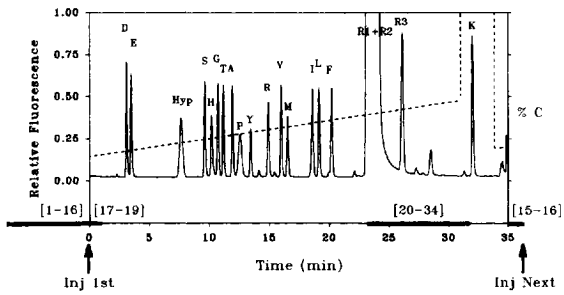

Fig. 1. Chromatogram of 250 pmol of amino acid standards derivatised with Fmoc. Peaks are labelled with one-letter abbreviations for protein amino acids. Hyp=hydroxyproline, R1=Fmoc-hydroxylamine, R2=Fmoc-OH, R3=reagent peak present in blank derivatisation. Separation was carried out on a 5-μm ODS-Hypersil column with 150×4.6 mm I.D.; buffer A, 30 m*M* ammonium dihydrogenorthophosphate in 15% (v/v) methanol (pH 6.5); buffer B, 15% (v/v) methanol; buffer C, 90% (v/v) acetonitrile. Column temperature was 38°C and flow-rate 1.0 ml/min. Detection of fluorescence was performed at wavelengths: Ex. 270 nm, Em 316 nm. Ammonium dihydrogenorthophosphate concentration was 6 m*M*. Dashed line shows linear gradient of buffer C used for elution of amino acids. Numbers in square bracket show the sequence for the automatic derivatisation of amino acids and multi-tasking, arrows indicate the injection time (Table 1).

The uniformity of size distribution of the material facilitates a rugged bonded-phase packing structure in the HPLC column which is more stable to changes in pressure that are generated during the course of the elution gradient on the column. Uniformity of size distribution is harder to achieve with a 3-μm packing, making the column more susceptible to packing rearrangements when subjected to pressure gradients. This can result in the shifting of 'fines' down the length of the column leading to pressure build-up, and the formation of micro-channels leading to loss of column resolution. Fig. 1 shows a typical separation of amino acid standards using a 5-μm ODS-hypersil column. Fig. 2 shows the separation of a hydrolysate of alkyl hydroperoxide reductase C_{22} protein from *E. coli*, previously separated by two-dimensional electrophoresis and blotted to PVDF. From this analysis data, the protein was correctly identified from the Swiss-Prot database, using an approach described recently [1].

3.2. Ionic strength effects

In chromatography of amino acids, co-elution can be caused by using an incorrect ionic strength of the mobile phase. After about 400 injections of samples onto a new column, we observed an increased retention time for arginine and histidine, resulting in co-elution of these amino acids with valine and glycine, respectively (Fig. 3). As these amino acids are positively charged at neutral pH, their retention time is changed in different salt concentrations.

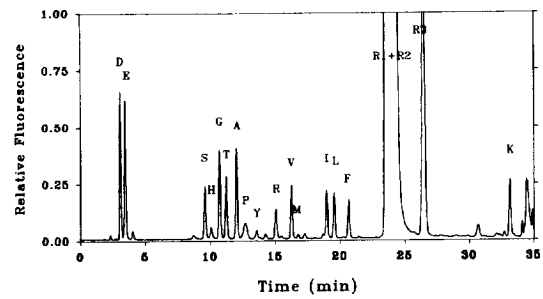

Fig. 2. Chromatogram of hydrolysate of 5 pmol alkyl hydroperoxide reductase C_{22} protein from *E. coli* separated by two-dimensional electrophoresis and blotted to PVDF. Peak labelling and separation conditions as in Fig. 1, ammonium dihydrogenorthophosphate concentration 6 m*M*.

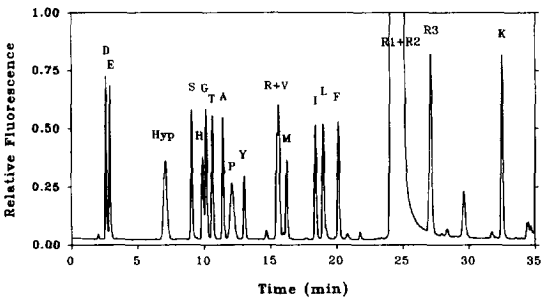

Fig. 3. Chromatogram of 250 pmol of amino acid standards derivatised with Fmoc. Peak labelling and separation conditions as in Fig. 1, ammonium dihydrogenorthophosphate concentration 5 m*M*, histidine and arginine co-elute with glycine and valine, respectively.

Satisfactory chromatographic separation of these amino acids was maintained throughout the column life by increasing the salt concentration in steps of 0.5 m*M* according to the chromatography performance. For example, the co-elution of His/Gly and Arg/Val in 5 m*M* ammonium dihydrogenorthophosphate (pH 6.5) (Fig. 3) was rectified when the salt concentration was increased to 6 m*M* (Fig. 1). Over the life of four columns, we have not found it necessary to exceed salt concentrations of 7 m*M* to maintain optimal separation.

3.3. Reproducibility of chromatography

Reproducibility of the chromatography was tested by a series of 28 injections of a pre-derivatised standard containing 250 pmol of each amino acid. The relative standard deviations (R.S.D.) of normalised peak areas and retention times are shown in Table 3. The relative standard deviations for ten out of sixteen peak areas were below 0.8%, with the highest R.S.D. being 1.1%. There was also little retention time shift over 20 h of operation, with the R.S.D. of retention times between 0.1 to 0.4%. This compares favourably with the separation we previously reported [10], of R.S.D. of peak areas between 0.8% to 5.0% and retention times between 0.1 and 1.1%. This not only illustrates the very high reproducibility of the chromatography, but also the high stability of the Fmoc-amino acids. The high reproducibility increases the ease of chromatographic analysis and peak identification. This improvement

Table 3
Relative standard deviations for peaks and retention times of Fmoc amino acids

Amino acid	R.S.D.(%)[a]			
	Peak area		Retention time	
	125 pmol (n=28)	100 pmol (n=24)	(n=28)	(n=20)
	(A)	(B)	(A)	(B)
Asp	0.4	2.9	0.4	1.1
Glu	0.5	2.4	0.4	1.0
Ser	0.3	1.9	0.2	0.5
His	0.5	0.9	0.3	0.5
Gly	0.5	2.1	0.2	0.5
Thr	0.9	1.7	0.2	0.5
Ala	0.6	0.8	0.2	0.5
Pro	1.0	0.8	0.1	0.5
Tyr	0.9	1.0	0.2	0.5
Arg	0.8	0.9	0.3	0.5
Val	0.7	2.4	0.2	0.5
Met	1.1	1.8	0.2	0.5
Ile	0.8	3.5	0.2	0.4
Leu	1.0	4.1	0.2	0.4
Phe	0.8	5.0	0.2	0.3
Lys	0.5	3.1	0.1	0.1

Values for peak areas (normalised to hydroxyproline, which was used as the internal standard) and retention times for sequential separations (A) prepared using a 5-μm hypersil-ODS column with a GBC AMINOMATE system; (B) published with original method, Ref.[10].
[a] R.S.D.=standard deviation/mean×100%

in reproducibility is probably due to a reduction of bonded phase hydrolysis in the column.

3.4. Regeneration of columns

When amino acid analysis is undertaken routinely from PVDF blots of two-dimensional gels, separation columns are subject to excess derivatisation reagents and materials from the electrophoresis process, such as sodium dodecylsulphate. After the chromatography of approximately 300 protein hydrolysates from two-dimensional gels, we found that column performance declined presumably due to accumulation of strongly retained compounds on the column (Fig. 4b). As a result, changes in retention times were observed. Several techniques have been recommended elsewhere for column regeneration [17] but we found that regeneration could be achieved by subjecting the column to one reverse flow and one forward flow of a 6 m*M* gradient (pH 6.5) at a flow-rate of 1.0 ml/min. A reverse flow wash with only 90% acetonitrile was not sufficient to remove all retained materials from the column. We routinely regenerate the column by the reverse and forward flow method after each 300 injections, and have obtained over 800 runs of samples from blots of two-dimensional gels from each of four separate columns that we have used (Fig. 4a–e).

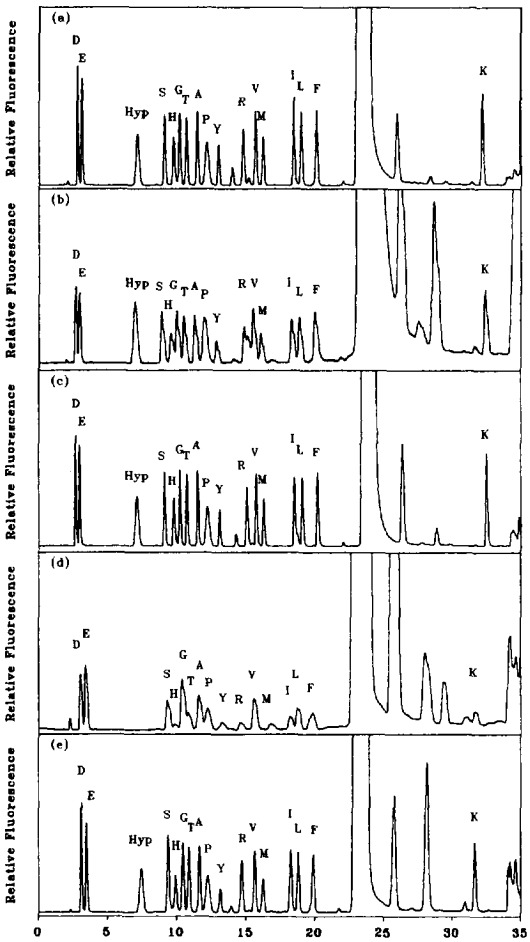

Fig. 4. Regeneration of a single column by reverse flow gradients. Chromatogram of 250 pmol of amino acid standards derivatised with Fmoc. Peak labelling and separation conditions as in Fig. 1, ammonium dihydrogenorthophosphate concentration 6 m*M*. Elution profile (a) from a new column; (b) obtained after chromatography of 350 hydrolysates of proteins from two-dimensional gels; (c) obtained following regeneration in the 351st cycle as described in the text; (d) obtained after chromatography of a total of 600 hydrolysates of proteins from two-dimensional gels; (e) obtained following the second column regeneration after sample 600.

4. Conclusions

Here we have presented an improved method for the chromatography of Fmoc amino acids in an automatic precolumn derivatisation system. This method produces high quality separation of Fmoc amino acids throughout the life of the column without requiring major changes to the gradients used. The column life time can be increased by the column regeneration procedure, reducing column cost to less than US$1 per run (800 runs per column). These improvements in chromatography, in conjunction with a multi-tasking method that controls the pump and autosampler simultaneously, produces a robust automatic precolumn derivatisation analysis system capable of high automatic throughput. It is therefore an ideal system to use for amino acid analysis for mass protein identification from two-dimensional gels and for routine quality control and screening of biological samples.

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