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Short Communication

Monitoring 4-methoxy-2,3,6-trimethylbenzenesulphonyl deprotection of arginine-containing synthetic peptides using capillary zone electrophoresis

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ABSTRACT

A rapid method has been devised to monitor the deprotection of 4-methoxy-2,3,6-trimethylbenzenesulphonyl (Mtr) groups from an arginine-containing synthetic peptide using capillary zone electrophoresis. Unlike the usual reversed-phase high-performance liquid chromatographic method, ether extractions to remove interfering reagents prior to analysis are not required. For the peptide, Ser–Pro–Arg–Gly, capillary zone electrophoretic conditions of 30 kV at 45°C for 8 min in 20 m*M* sodium citrate buffer, pH 2.5, on a 72-cm capillary gave clear separation of the Mtr-protected (mobility 7.2 min) and the deprotected peptide (mobility 3.5 min). During deprotection of an octapeptide containing two arginines, two transient species occurred corresponding to the two possible partially Mtr-deprotected peptides that could form. These ran predictably at intermediate mobilities of 4.8 and 5.0 min compared to the final free peptide (mobility 3.3 min) and the fully protected species (mobility 8.7 min).

INTRODUCTION

Arginine-containing peptides, synthesized using N^{α}-Fluoroenylmethoxycarbonyl (Fmoc) amino acids, commonly use 4-methoxy-2,3,6-trimethylbenzenesulphonyl (Mtr) as the protecting group on the guanidino side chain of arginine. Following synthesis, the cleavage of this protecting group with trifluoroacetic acid (TFA)–phenol is significantly slower than the peptide-resin cleavage and the other side chain deprotection reactions, normally based on butyl groups [1]. To ensure complete deprotection it is necessary to monitor the cleavage of this group from the peptide. This has previously been accomplished by a reversed-phase high-performance liquid chromatographic (HPLC) method which involves preparation of the sample, extracting several times with ether to remove the phenol from the reaction mixture prior to analysis [2].

Capillary zone electrophoresis (CZE) is an ideal method for analyzing small polar molecules such as peptides and is commonly used to assess the purity of synthesized peptides [3]. This paper describes a method for the rapid monitoring, by CZE, of the deprotection of arginine-containing peptides that involves no significant sample preparation.

MATERIALS AND METHODS

Synthesis of peptides

Ser–Leu–Leu–Leu–Ser–Pro–Arg–Gly, Ser–Arg–

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Leu–Leu–Ser–Pro–Arg–Gly, Leu–Leu–Ser–Pro–Arg–Gly and Ser–Pro–Arg–Gly were synthesized on a Pepsynth II semi automatic continuous-flow peptide sunthesizer (CRB, Cambridge, UK) using Pepsyn KA resin with Fmoc-glycine attached (0.5 g resin/column; 0.1 mmol/g substitution; Milligen/Biosearch, Watford, UK) in peptide synthesis-grade dimethylformamide (DMF) (Applied Biosystems, Warrington, UK). Following deprotection with piperidine (20%, v/v, in DMF) the subsequent amino acids were added using the Fmoc-amino acid-pentafluorophenyl esters of Arg(Mtr), Pro and Leu, and the 3,4-dihydro-4-oxobenzotriazin-3-yl ester of Ser(But) (Milligen/Biosearch) in a 4 molar excess and in the presence of hydroxybenzotriazole (0.88 mmol). Prior to each deprotection the resin was analyzed using the test by Kaiser *et al.* [4] to determine the presence of free amino groups. Following deprotection of the final amino acids, the peptide-resins were removed from the columns, filtered on a sintered glass filter and washed with DMF, *tert.*-amyl alcohol, glacial acetic acid, *tert.*-amyl alcohol and diethyl ether before drying overnight in a vacuum desiccator.

Cleavage of peptide-resins; analysis of Mtr deprotection

The dried peptide resins were each mixed with 12.5 ml TFA–phenol (95:5, v/w) and periodically stirred. At intervals of 10 min, 30 min and every hour thereafter, 25-μl aliquots were removed. The TFA was blown off under a gentle nitrogen stream before adding 250 μl 20 m*M* sodium citrate buffer, pH 2.5. This was then analyzed using a Model 270A capillary electrophoresis apparatus (Applied Biosystems) on a 72-cm capillary equilibrated with 20 m*M* sodium citrate buffer, pH 2.5. Electrophoresis conditions in this buffer were 30 kV at 45°C, on a 3-s sample injection by vacuum, monitoring the electrophoretic mobility by absorbance at 200 nm. Initially 30-min runs were performed to ensure complete analysis of the reaction mixture and show the elution of the neutral species. Subsequent run times were 10 min. The CZE data were collected and analyzed using a Roseate chromatography data analysis package (Drew Scientific, London, UK) with a 100 mV input and collecting data at 2 Hz.

RESULTS

For the Ser–Pro–Arg–Gly peptide, the CZE traces (Fig. 1) show three peaks (electrophoretic mobilities; 3.6, 6.9 and 7.2 min), which change in time during the concurrent peptide-resin cleavage and side chain deprotection reactions. These peaks correspond to the three possible products that could be formed for this peptide. Although no standards were used to confirm the electrophoretic mobility of the different species, the CZE traces obtained fit both the expected reaction products over time and the expected electrophoretic mobilities of these products. The guanidino group of the arginine has a high pK_a (12.5) and will be strongly protonated in pH 2.5 buffer used in the electrophoresis. The Mtr-protected guanidino group, however, cannot protonate and makes no contribution to the overall charge of the peptide. The free peptide, Ser–Pro–Arg–Gly (P1), having two protonated groups at pH 2.5 and a lower mass, will clearly separate using CZE from the larger protected species which ony have the N-terminal α-amino group protonated. Under these conditions P1 had, and would be expected to have, the greatest electrophoretic mobility; running at 3.6 min. Thus at 14 h (Fig. 1e), only one peak at 3.6 min was observed which corresponded to the fully deprotected peptide. Further work-up on the peptide was then able to proceed.

For this tetrapeptide the two protected peptide species that can be formed during the peptide-resin cleavage/deprotection reaction are the Mtr-arginine-only protected peptide, Ser–Pro–Arg(Mtr)–Gly (P2) and the double side chain protected peptide, Ser(But)–Pro–Arg(Mtr)–Gly (P3). It is extremely unlikely that the butyl-serine-only protected species, Ser(But)–Pro–Arg–Gly, would be formed in significant quantities as the Mtr deprotection proceeds substantially more slowly than the butyl deprotection [1]. The two possible protected species are seen in the 10 min and 30 min electropherograms, running at 6.9 and 7.2 min, respectively (Fig. 1a and b). P3, having a slightly greater mass for the same charge would be expected to have a slightly slower electrophoretic mobility than P2. The disappearance within 2 h of the slowest running species at 7.2 min confirmed this to be due to P3, the faster butyl deprotection allowing conversion to P2 with increased electrophoretic mobility at 6.9 min. The

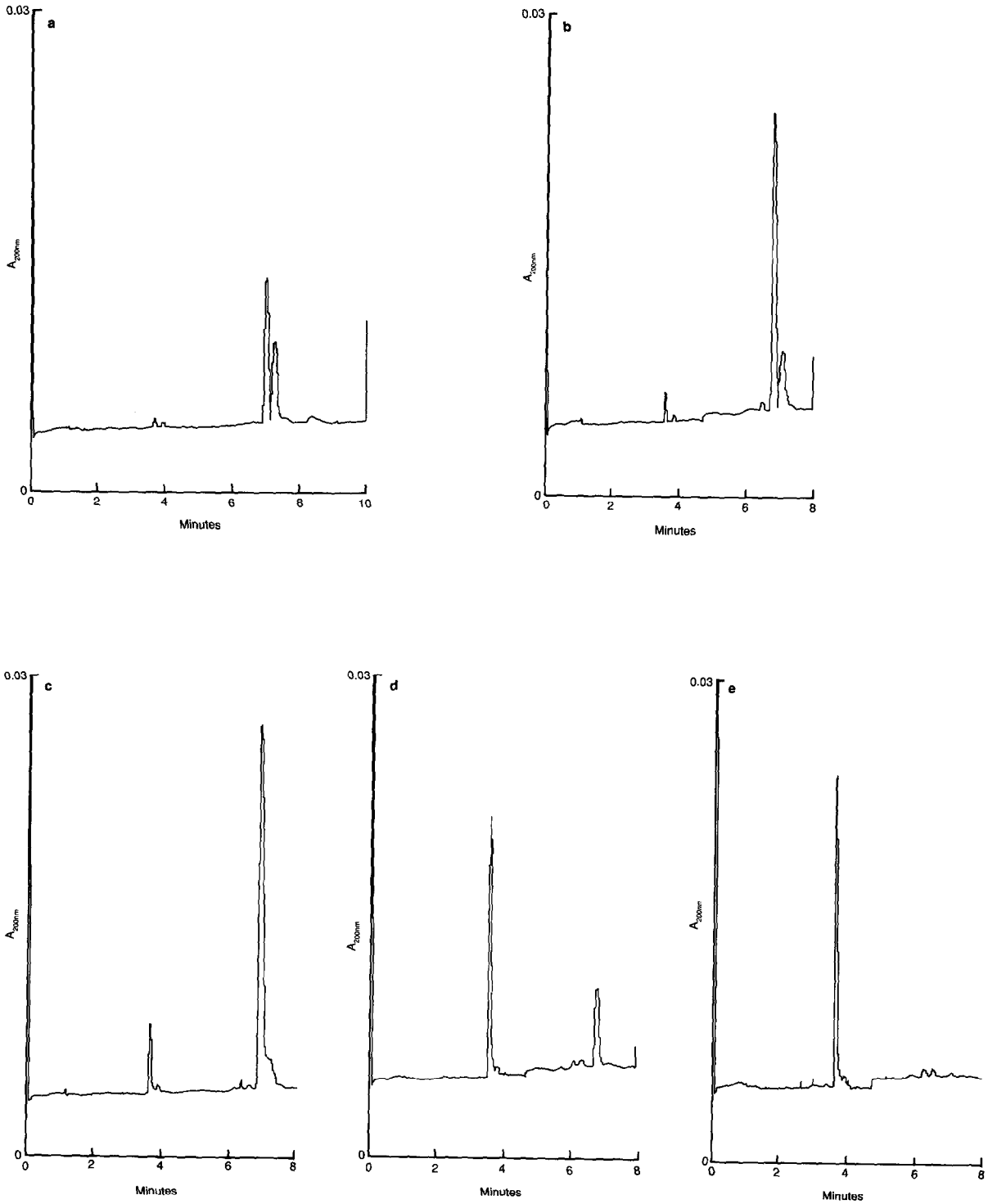

Fig. 1. Capillary zone electropherogram of the Mtr deprotection of Ser–Pro–Arg–Gly reaction over time: (a) 10 min reaction, (b) 0.5 h reaction, (c) 1.0 h reaction, (d) 5.5 h reaction and (e) 14.0 h reaction.

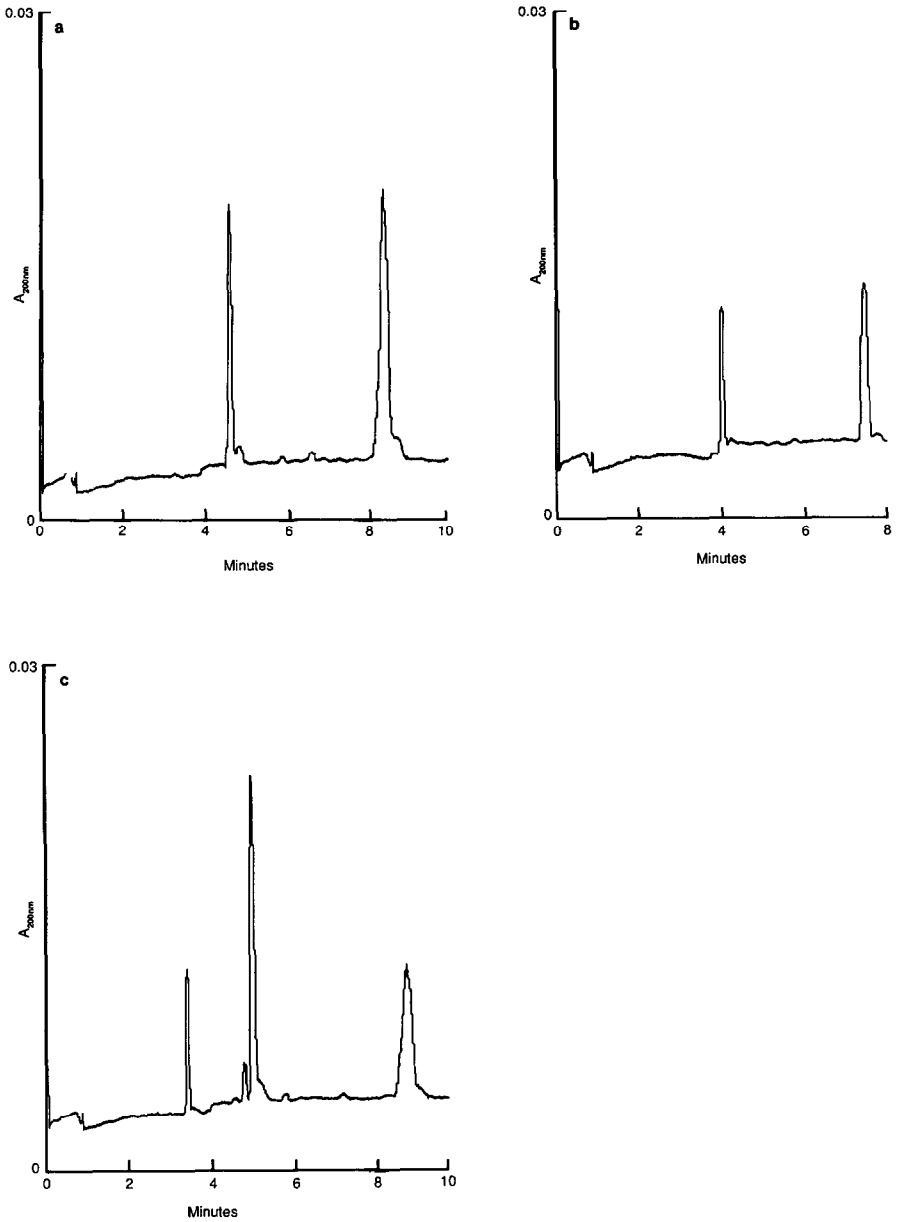

Fig. 2. Capillary zone electropherogram of the Mtr deprotection reaction for synthesized peptides at 2 h: (a) Ser–Leu–Leu–Leu–Ser–Arg–Pro–Gly, (b) Leu–Leu–Ser–Arg–Pro–Gly, (c) Ser–Arg–Leu–Leu–Ser–Arg–Pro–Gly.

later electropherograms (Fig. 1c–e) show the slower Mtr deprotection proceeding, requiring 14 h before complete deprotection of the peptide was achieved. With the larger peptides synthesized, separation of the equivalent PII and PIII species was not observed.

For the other larger peptides, Fig. 2a–c shows the electropherograms for the deprotection reaction at 2 h. The di-arginine-containing octapeptide, Ser–Arg–Leu–Leu–Ser–Pro–Arg–Gly, with three positive charges migrated faster at 3.41 min (see Fig. 2c)

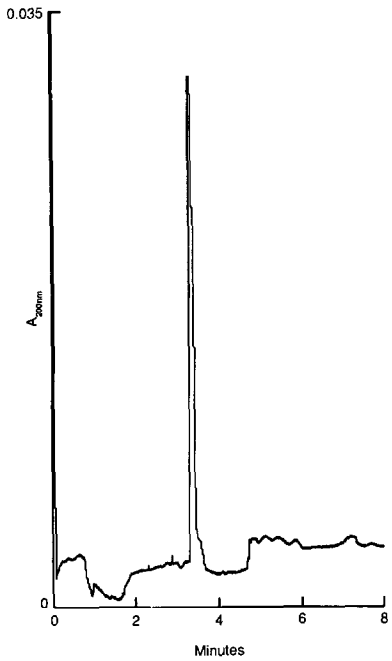

Fig. 3. Capillary zone electropherogram of the Mtr deprotection reaction for Ser–Arg–Leu–Leu–Ser–Arg–Pro–Gly at 22 h.

compared with the octapeptide, Ser–Leu–Leu–Leu–Ser–Pro–Arg–Gly, with only two positive charges and running at 4.56 min (see Fig. 2b). Where only one arginine was present the electropherograms (Figs. 1e, 2a and b) showed that the deprotected peptides ran according to size, the smaller peptide running fastest. Fig. 2c also shows that during the deprotection the di-arginine-containing peptide formed two other species which ran at an intermediate time between the free peptide and the Mtr-blocked peptide. These correspond to the partially Mtr-deprotected peptides, Ser–Arg(Mtr)–Leu–Leu–Ser–Pro–Arg–Gly and Ser–Arg–Leu–Leu–Ser–Pro–Arg(Mtr)–Gly, the intermediates formed prior to the complete deprotection which took place overnight, Fig. 3 showing the formation of the final free peptide.

DISCUSSION

The main advantage of this CZE procedure is the ease of sample preparation, just blowing off TFA with nitrogen and dissolution in electrophoresis buffer, with none of the ether extractions of the reaction mixture that are required by the HPLC procedure [2]. Using CZE the potentially interfering phenol and Mtr groups, with no charge at pH 2.5, run predictably at the neutrality point, clearly separated from the charged peptide species, passing the detector by electroendosmosis at 22 min under the conditions described (data not shown).

This procedure should be able to monitor clearly the deprotection of any arginine-containing synthetic peptide, with the free peptide theoretically being the species with the greatest electrophoretic mobility, provided no other factors such as capillary interactions interfere during electrophoresis.

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