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Journal of Chromatography A, 686 (1994) 73–83

JOURNAL OF CHROMATOGRAPHY A

Purification of synthetic peptides with the aid of reversible chromatographic probes☆

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First received 2 March 1994; revised manuscript received 20 July 1994

Abstract

The application of reversible chromatographic probes for the purification of synthetic peptides by reversed-phase (RP) and affinity chromatography is described. In previous work the concept of a one-step purification procedure based on the acid-stable fluorenylmethoxycarbonyl (Fmoc) group was considered. Similar probes were used to purify peptides from 17 to 104 residues in length, using ion-exchange and RP media. The RP probes described here represent simplified versions of those reported previously. Further, the application of a new probe containing biotin, for affinity chromatography, is demonstrated. Stepwise solid-phase peptide synthesis was performed using both Boc and Fmoc chemistries and N-(2-chlorobenzyloxycarbonyloxy)succinimide as a capping reagent. The chromatographic probes were incorporated on to the "target" peptide as active succinimidyl carbonate derivatives. After cleavage using either hydrogen fluoride or trifluoroacetic acid, purification was performed on either RP media or immobilized avidin. Treatment with organic base yielded the free purified product. The efficiency of the new probe molecules and the capping procedure was demonstrated by the purification of two polypeptides, 46 and 101 residues in length.

1. Introduction

Stepwise solid-phase peptide synthesis (SPPS), first introduced by Merrifield [1], suffers inherently from the accumulation of impurities at each step in chain assembly, due to the premature termination of the peptide chain (truncated sequences) and incomplete acylation of the free amino groups, which can then react with the next or subsequent residues [2]. The latter generates a family of deletion peptides that lack one or more amino acids.

In general, conventional chromatographic techniques have sufficient resolving power, when used singularly or in combination, to separate the target peptide (up to 40 residues in length) from contaminating deletion and truncated impurities. However, as the length of peptide sequence increases, the chromatographic differences between the desired product and its closely related impurities become less resolvable. Hence, in order to obtain a homogeneous product, several purification steps are required using techniques that separate material on the basis of diverse physical properties (e.g., lipophilicity, charge or molecular mass). The disadvantage,

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☆ Presented at the *13th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, San Francisco, CA, 30 November–3 December, 1993.*

0021-9673/94/$07.00
SSDI 0021-9673(94)00709-8

apart from the expertise required in the use of different techniques, is the consequent loss of material at each purification step and the additional time involved.

We have developed a reversible purification system that is based on the capping of unreacted amino groups and the addition of a chromatographic probe molecule, bearing either lipophilic or charged groups, to the N-terminus of the target peptide [3–5]. The derivatized peptides, up to 104 residues in length, had all been synthesized using the Boc strategy and were readily separated from underivatized impurities.

Derivatization of the target peptide with a group with specific and predefined chromatographic properties has been investigated previously. Several methods have been suggested that involve the reversible chemical modification of peptides in an attempt to facilitate purification; however, their application to polypeptide chains has not been described [6]. More recently, several other molecules have been described for the purification of peptides up to 85 residues in length [7], including a biotinylated probe [8]. The latter was used to purify a 67-residue peptide that had been synthesized with acetic anhydride as capping agent. It has been suggested that repeated exposure to acetic anhydride is detrimental to the growing peptide chain, causing side-reactions [9]. This observation becomes particularly important as the length of the peptide increases.

In previous publications we demonstrated the concept of reversible chromatographic probes for reversed-phase (RP) and ion-exchange purification [3–5]. In this work, new RP probes, {4-[(4-cyclohexylbutyryl)aminomethyl]fluoren-9-yl}methyl (**1**) and [4-(dodecylaminocarbonyl) fluoren-9-yl]methyl (**2**) succinimidyl carbonates, that were simpler to prepare, were used. Further, the purification method has been extended to a new class of molecules containing biotin, i.e., {4-[(biotinylglycylglycyl)aminomethyl]fluoren-9-yl}methyl succinimidyl carbonate (**3**) for affinity chromatography, as illustrated in Fig. 1. In addition we also show the validity of the method for the purification of peptides that were synthesized using fluorenylmethoxycarbonyl (Fmoc) chemistry. Two polypeptides, 46 and 101 residues in length, were chemically synthesized using Fmoc and Boc strategies, respectively.

2. Experimental

2.1. Chemicals and reagents

All Boc and Fmoc amino acid derivatives, together with the capping reagent N-(2-chlorobenzyloxycarbonyloxy)succinimide, were supplied by Novabiochem. Solvents for peptide synthesis were obtained from Applied Biosystems. Deprotection and cleavage of Boc-synthesized peptides was performed using anhydrous hydrogen fluoride (HF) from Matheson and an all-Kel-F apparatus (Peptide Institute, Osaka, Japan) Deionized water was prepared with a Milli-Q purification system (Millipore–Waters) and acetonitrile (HPLC grade) was obtained from Merck.

2.2. Peptide synthesis

Boc-synthesized peptides

The peptides were synthesized on PAM–copoly(styrene–divinylbenzene) resin [PAM = 4-(carboxyamidomethyl)benzyl ester], prederivatized with the first amino acid, using an automated Applied Biosystems Model 430A peptide synthesizer and optimized chemical protocols [10] on a 0.5-mmol scale. Dicyclohexylcarbodiimide (DCC) or DCC–hydroxybenzotriazole (HOBt) in the case of Asn, Gln and Arg amino acids were used as activating reagents. When the synthesis was performed without the additional capping step, double coupling cycles were performed, whereas for capped peptides single coupling was used. One millimole of the capping agent, N-(2-chlorobenzyloxycarbonyloxy)succinimide, was loaded into cartridges. The ABI software was modified so that, when required, the capping reagent would be dissolved automatically in N-methylpyrrolidone (NMP)–dichloromethane (DCM) (3:1), together with 1

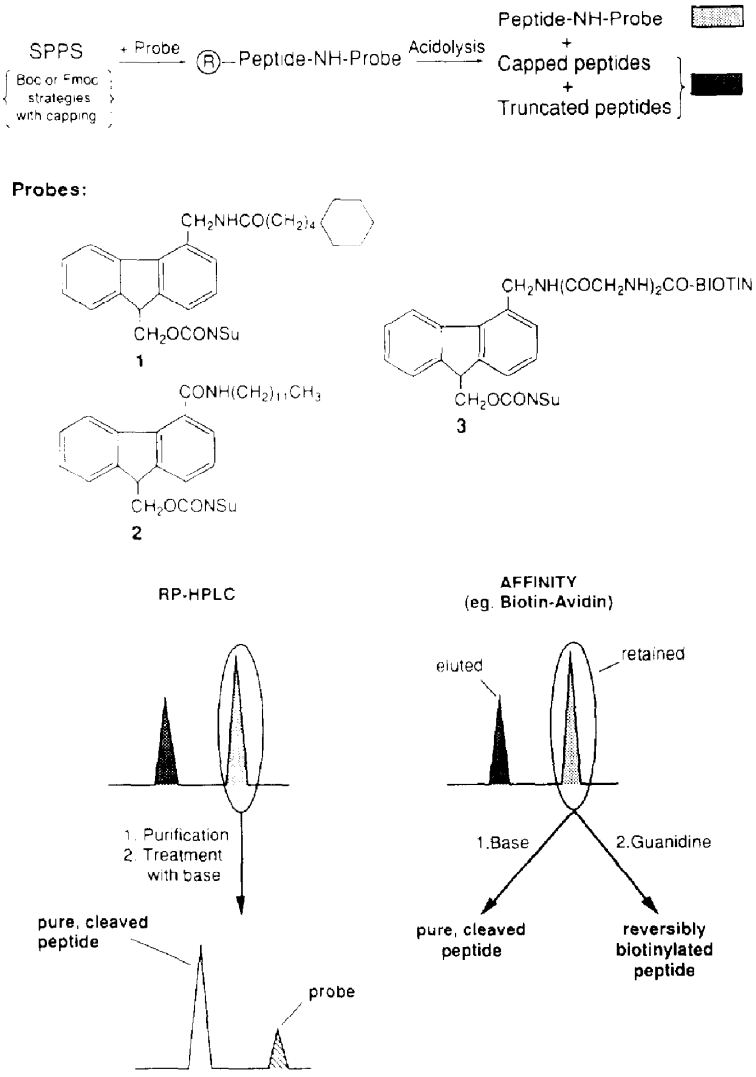

Fig. 1. General scheme showing application of chromatographic probes **1**, **2** and **3**.

ml of 2 *M* diisopropylethylamine (DIEA) in NMP, transferred to the reaction vessel via the concentrator and then vortex mixed for 5 min. The capping agent was then washed from the peptidyl-resin with DCM. At the end of the synthesis the N-terminal protecting group was removed using trifluoroacetic acid (TFA)–DCM (50:50) for 30 min. After neutralization with 5% DIEA in DCM, the chromatographic probes were introduced as described below.

Deprotection of the peptide and its cleavage from the resin were achieved using a low trifluoromethylsulphonic acid (TFMSA)–high HF procedure [10]. The dried peptidyl-resin was first treated with TFMSA–1,4-butanedithiol–*p*-cresol–dimethyl sulphide–TFA (5:1:4:15:25) at 0°C for 2 h, followed by the high-HF procedure, HF–1,4-butanedithiol–*p*-cresol (20:1:1). The crude peptide was precipitated with cold dry diethyl ether and then dissolved in 20% acetic acid solution. The peptide solution was then lyophilized to yield the crude dry peptide.

Fmoc-synthesized peptides

A 0.25-mmol amount of Fmoc–Ser(tBu)–Sasrin resin (substitution value 0.69 mmol/g) was used. Single coupling cycles were performed with capping, as described for the Boc-synthesized peptides, except that 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was used as activating agent. Incorporation of the chromatographic probes in the N-terminally deprotected peptidyl-resin were performed as described below.

Removal of the side-chain protecting groups and cleavage of the peptide from the resin were achieved with 95% TFA containing ethanedithiol–thioanisole (1:2) for 90 min at room temperature. The crude cleavage mixture was filtered and then precipitated with cold diethyl ether. The precipitate was isolated by centrifugation (1132 *g* for 3 min), washed with further diethyl ether, then dissolved in 20% acetic acid solution before being lyophilized to give the crude dry product.

Synthesis of chromatographic probes

The lipophilic (**1** and **2**) and biotinylated (**3**) probes were synthesized from the central 9-(hydroxymethyl)fluorene-4-carboxylic acid molecule, using essentially the same method as described earlier [5]. The results of the improved synthesis will be described elsewhere.

Attachment of chromatographic probes

The freshly deprotected peptidyl-resin was allowed to swell in DCM, then 2 equiv. of the chromatographic probe were dissolved in sufficient trifluoroethanol (TFE)–DCM (1:3) to give a concentration of approximately 0.2 *M* and subsequently transferred to the peptidyl-resin. The mixture was vortex mixed in a micro-reaction vessel for 90 min. The coupling efficiency was determined using quantitative ninhydrin analysis [11]. If the reaction had not gone to completion, the reaction was continued for a further 90 min. The peptidyl-resin was then washed with DCM and dried under vacuum in preparation for cleavage.

2.3. Purification

Preparative isoelectric focusing

Purification of synthetic protein was performed in a Rotofor (Bio-Rad) preparative isoelectric focusing cell under denaturing conditions (i.e., 8 *M* urea). The Rotofor cell was loaded with 30 ml of 8 *M* urea containing 2% ampholytes (p*I* range 3–9). Separation was performed at 15°C until each component had focused at its appropriate pH (usually 2–3 h). The contents of each of the twenty cells were harvested and analysed both for protein content, as determined by analytical RP-HPLC, and pH. Cells containing material with similar retention times were then combined and re-fractionated using a narrower pH range. Following analysis, the cells containing the most homogeneous material were combined. A final purification step, which also desalted the protein, was then performed on RP media.

High-performance liquid chromatography

Analytical and semi-preparative RP-HPLC was achieved using a Shimadzu LC10 system, equipped with a dual-wavelength detector. Peptides were analysed on Vydac C_4 and C_{18} columns (150 × 4.6 mm I.D.). Separations were achieved using linear gradients from 0 to 100% B in 30 min at a flow-rate of 1 ml/min. Solvent A was water–0.045% TFA and solvent B was acetonitrile–0.036% TFA. Detection was effected at 220 and 301 nm (to measure the absorbance due to the fluorene ring). Semi-preparative separations were conducted on either Vydac C_4 or C_{18} columns (250 × 10 mm I.D.) at a flow-rate of 3 ml/min.

Affinity chromatography

Immobilized avidin (Pierce) was loaded into a 5-ml disposable polypropylene column and equilibrated with sodium acetate buffer (pH 4). The crude HF cleavage product was dissolved in 10% acetic acid, filtered and the pH was adjusted to 4 using 0.1 *M* NaOH. The peptide solution was then incubated with the immobilized avidin for 30 min with occasional mixing. A portion of supernatant was removed and ana-

lysed by RP-HPLC to determine whether all the biotinylated protein had been bound. If derivatized material was still present in the solution, the incubation was continued for a further 30 min. The resin was washed with sodium acetate buffer and then water. A 5% aqueous solution of triethylamine (TEA) was then added and left for 15 min, which removed the peptide from the biotinylated probe–avidin complex. The solution was collected and neutralized with 10% acetic acid and the purified free protein was desalted on a Brown–Lee C_4 RP column (30 × 4.6 mm I.D.) and finally lyophilized.

Size-exclusion chromatography

Size-exclusion chromatography was performed on an FPLC (Pharmacia) instrument using a Sephadex 75 column (300 × 10 mm I.D.). The eluting buffer was Dulbecco's phosphate-buffered saline (PBS) containing Ca/Mg ions at pH 7.2. The flow-rate was 0.5 ml/min and the eluting proteins were detected at 220 nm.

2.4. Analysis

The purified Fmoc-derivatized and free peptides were analysed by amino acid analysis and electrospray mass spectrometry (ESI-MS). A Beckman System Gold system was used to determine the amino acid content of the samples. Peptidic material was hydrolysed in 1 ml of hydrochloric acid–propionic acid (1:1) at 110°C for 21 h. The hydrolysed samples were reduced under vacuum and then dissolved in buffer containing β-alanine as internal standard.

ESI-MS was performed on a Finnigan MAT (San Jose, CA) Model 700 instrument.

3. Results and discussion

3.1. Synthesis of the 46-residue cytoplasmic domain (β2-subunit) of CD18 using Fmoc chemistry

As the peptides chosen to demonstrate the application of the probe molecules had been synthesized using the Boc chemical procedure [3–5], it was now necessary to investigate the procedure for the purification of Fmoc-synthesized peptides. Further, the introduction of an efficient capping procedure that did not cause side-reactions was required in order to optimize the purification method. The peptide chosen was a 46-residue sequence from the cytoplasmic domain of CD18 (hereafter referred to as β2-subunit) [12], which was synthesized using single coupling cycles, with HBTU–HOBt as activating agents. For the capping procedure it was decided to use N-(2-chlorobenzyloxycarbonyloxy)succinimide for two reasons: (i) it is a solid that can be dissolved immediately prior to use, thus avoiding problems associated with instability, and (ii) the by-products are not as potentially harmful as those generated by acetic anhydride (i.e., acetic acid). To perform the capping procedure in an automated fashion on the ABI 430A, two cartridges were prepared for each residue in the sequence, one containing the amino acid and the other 1 mmol of the capping agent, N-(2-chlorobenzyloxycarbonyloxy)succinimide. While the activated amino acid was being coupled to the peptidyl-resin, the second cartridge containing the capping agent was dissolved in NMP–DCM with 1 ml of 2 *M* DIEA in NMP. On completion of the coupling cycle, the capping agent was transferred to the reaction vessel via the instrument concentrator column to terminate any free amino groups. A 5-min reaction was found to be sufficient for an effective capping procedure. A more detailed description of the capping procedure will be discussed elsewhere. At the end of the synthesis the peptidyl-resin was deprotected with 20% piperidine in NMP in preparation for the introduction of the chromatographic probes.

3.2. RP-HPLC purification of the 46-residue β2-subunit of CD18

Two different lipophilic probes, **1** and **2**, were attached, as the active carbonate derivatives, to the 46-residue peptidyl-resin. A 2-equiv. amount of the activated probes was dissolved in DCM–TFE (3:1) and added to the freshly deprotected peptidyl-resin. The incorporation of the probe was monitored by quantitative ninhydrin analysis

[11] and was found to be complete after 90 min. The derivatized peptide was then cleaved from the resin support using 95% TFA. The effectiveness of the combination of this capping procedure and the separation achieved between underivatized and derivatized peptides was then examined.

The crude cleavage products for the peptides derivatized with probes **1** and **2** gave the chromatograms shown in Fig. 2A and C, respectively. The profiles of the two chromatograms were essentially identical in that two main groups of peaks were obtained, an earlier eluting group of low-intensity peaks (labelled 1) and a later eluting material (labelled 2) representing the major component of the crude product. The latter peak was collected for both probes, lyophilized and analysed for amino acid content (data not shown) and by ESI-MS [β2-subunit adduct + probe **1** (expected, 5887; found, 5887) and β2-subunit adduct + probe **2** (expected, 5917; found, 5917)]. The results indicated that the correct products had been isolated. A slight shoulder on the right of peak 2 (Fig. 2A and C) was isolated and shown, by ESI-MS, to be 57 u higher than the molecular ion, which would correspond to the partial deprotection of a butyl group.

To obtain the free peptide, it was then necessary to treat the Fmoc-derivatized material with 5% aqueous TEA for 15 min. Interestingly, the type of Fmoc probe used had a significant effect on the rate of β-elimination. Where the substituent at position 4 on the fluorene ring was

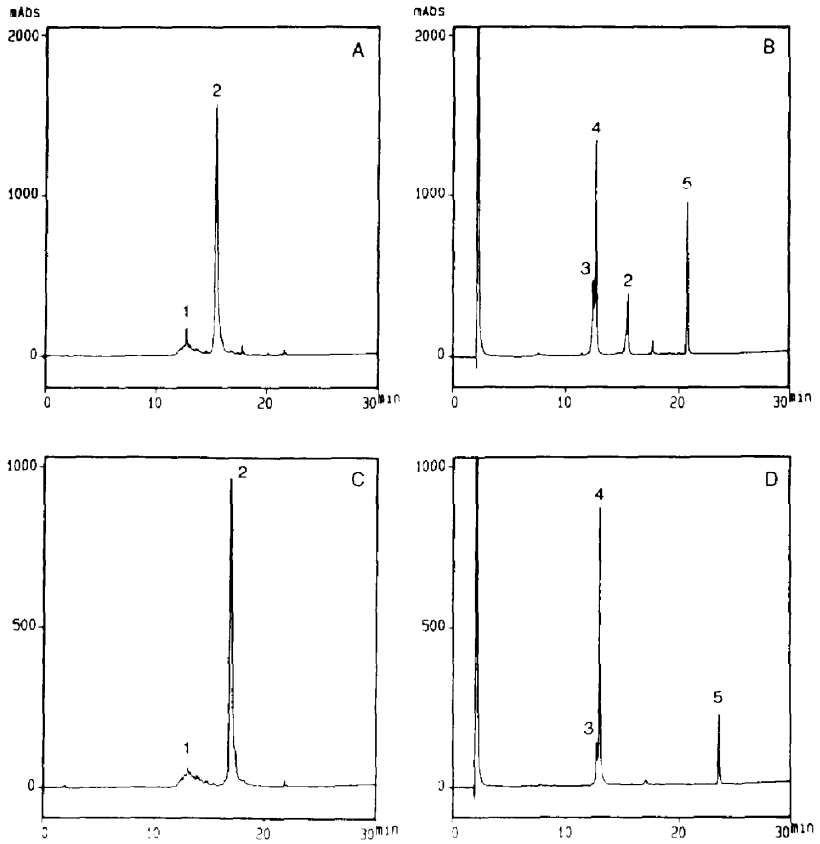

Fig. 2. Analytical RP-HPLC on a Vydac C_4 column (150 × 4.6 mm I.D.) connected to a Shimadzu LC10 LC system. Solvent A, water–0.045% TFA; solvent B, acetonitrile–0.036% TFA; gradient, 0–100% B in 30 min; flow-rate, 1 ml/min; detection, 220 nm. (A) Crude β2-subunit adduct + **1**; (B) purified β2 subunit adduct + **1**; (C) crude β2-subunit adduct + **2**; (D) purified β2-subunit adduct + **2**.

alkyl, as in probe **1**, a large proportion of derivatized peptide still remained after 15 min (labelled 2, Fig. 2B). A further 30 min were required for complete cleavage of the probe. However, in the case of handle **2**, where the fluorene ring is substituted with an electron-withdrawing moiety (i.e. carboxyl group) at position 4, the urethane group was more sensitive to base and quantitative cleavage of the handle from the peptide was achieved in 15 min. RP-HPLC analysis of the cleaved peptides (labelled 4, Fig. 2B and D) showed the β2-subunit (labelled 4) to be highly homogeneous, which, following isolation, was confirmed by amino acid analysis (data not shown) and ESI-MS (expected, 5483; found, 5483). The earlier eluting peak labelled 3 (Fig. 2B and D) was also collected and found to be the correct sequence with Met in its oxidized form (ESI-MS: found, 5499). The peak labelled 5, which had strong absorbance at 301 nm, due to the presence of the fluorene ring, was the cleaved by-product of the chromatographic probe. The overall yield of the β2-subunit following RP purification and desalting was 33% using probe **1** and 36% for lipophilic probe **2**.

3.3. *Synthesis of 101-residue heat shock protein from* Rattus norvegicus

Previously, the derivatization of the 104-residue HIV-1 p24gag fragment 270–373 with a lipophilic probe resulted in a separation of 7 min, on RP media, between underivatized and derivatized polypeptides [5]. As the chemical synthesis did not involve a capping step, the chromatographic probe was attached to both the target peptide and deletion sequences. Therefore, based on the success of the capping agent, N-(2-chlorobenzyloxycarbonyloxy)succinimide, when applied to the Fmoc strategy, its application to Boc chemistry was investigated. The 2-chlorobenzyloxycarbonyloxy group is stable to treatment with TFA and therefore remains attached to the truncated peptides, even after cleavage of the peptide from the resin, in the case of Fmoc-synthesized peptides. However, as the difference in hydrophobicity between capped and uncapped material is reduced, the ability to cleave the terminating group during HF treatment further benefits its application in peptides synthesized by the Boc strategy, particularly in the case of large peptides.

The M_r 10 000 heat shock protein from *R. norvegicus* (hereafter referred to as rat Hsp10) [13] was synthesized in 3 days, using the single delivery of activated amino acid, followed by the transfer of a freshly prepared solution of capping reagent, as described above. At the end of the synthesis the peptidyl-resin was N-terminally deprotected and a portion was treated with the hydrophobic probe **2**. Cleavage of the resins was achieved using the low TFMSA–high HF protocol.

3.4. *RP-HPLC purification of 101-residue rat Hsp10*

The crude underivatized (Fig. 3A) and derivatized (Fig. 3B) polypeptides were injected on to an analytical RP column. In the latter chromatogram two main products eluted. The early-eluting peak (labelled 1, Fig. 3B) was collected and shown by ESI-MS to be a large terminated peptide caused by the incomplete coupling (greater than 50%) of Gln^{60} to Pro^{61} (found, 4574). As Gln^{60} was coupled to Pro, a secondary amine, the problem was not detected by quantitative ninhydrin analysis [11], which was used to follow the amino acid coupling efficiency, thus further enhancing the relevance of the strategy for the synthesis of the protein. The later eluting peak (labelled 2, Fig. 3B) absorbed strongly at 301 nm, which suggested it to be the polypeptide derivatized with **2**. Further, the disappearance of peak 2 (Fig. 3A) also supported the conclusion that the later eluting peak was derivatized rat Hsp10. The latter was collected, re-injected on RP media (Fig. 3C) and then treated with 5% aqueous TEA to release the free polypeptide. After desalting on an RP column to remove the base and the cleaved chromatographic probe, the purified rat Hsp10 polypeptide (labelled 1, Fig. 3D) was analysed by ESI-MS (Fig. 4A), which showed that the correct material had been obtained (expected, 10 770; found, 10 772). The overall yield for the one-step purification pro-

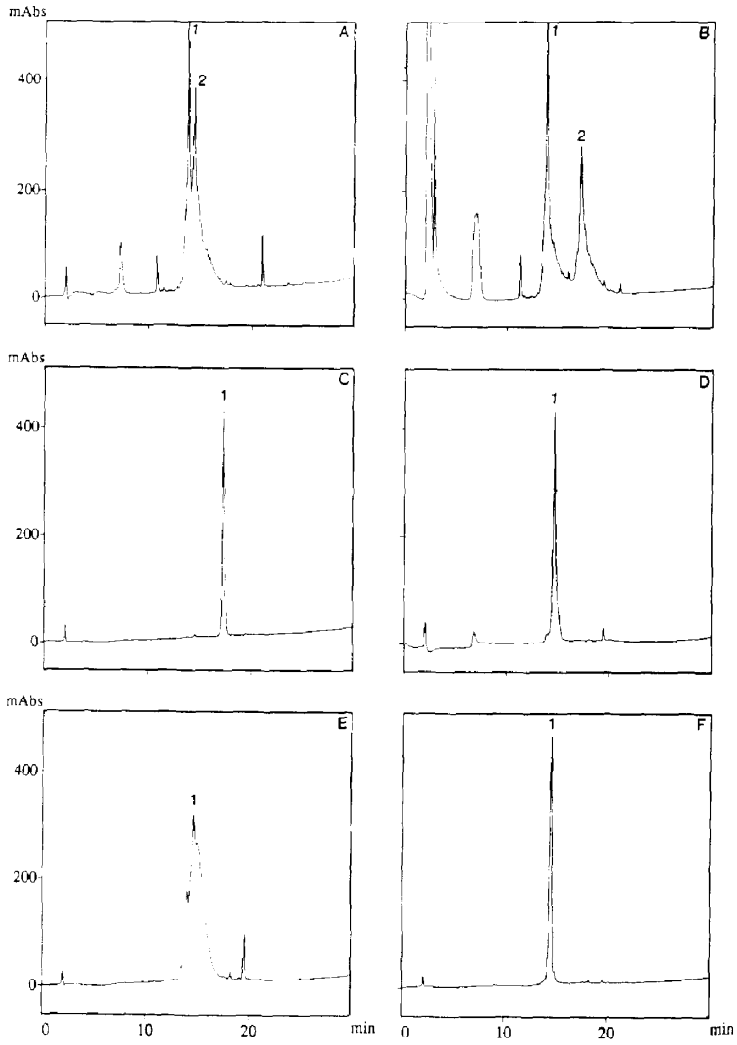

Fig. 3. Analytical RP-HPLC on a Vydac C_4 column (150 × 4.6 mm I.D.) connected to a Shimadzu LC10 LC system. Solvent A, water–0.045% TFA; solvent B, acetonitrile–0.036% TFA; gradient, 0–100% B in 30 min; flow-rate, 1 ml/min; detection, 220 nm. (A) Crude rat Hsp 10 without probe **2** attached; (B) crude rat Hsp10 derivatized with probe **2**; (C) purified rat Hsp10 derivatized with probe **2**; (D) C after treatment with 5% aqueous TEA; (E) crude uncapped rat Hsp10; (F) rat Hsp10 (uncapped) after purification by Rotofor and RP-HPLC.

cedure was 8%, a relatively low value owing to the high proportion of truncated peptide. ESI-MS (Fig. 4A) also showed the presence of some low-intensity peaks with molecular masses of 10 644 and 10 654. These corresponded to deletion peptides lacking Gln and Asp residues, respectively, indicating that although the capping procedure is efficient, further refinement is required for full protection against incomplete couplings.

The importance and efficiency of the capping procedure were demonstrated when a second synthesis of rat Hsp10 was performed using double coupling cycles and no capping [14]. The crude polypeptide obtained as described above gave the RP profile shown in Fig. 3E. A three-

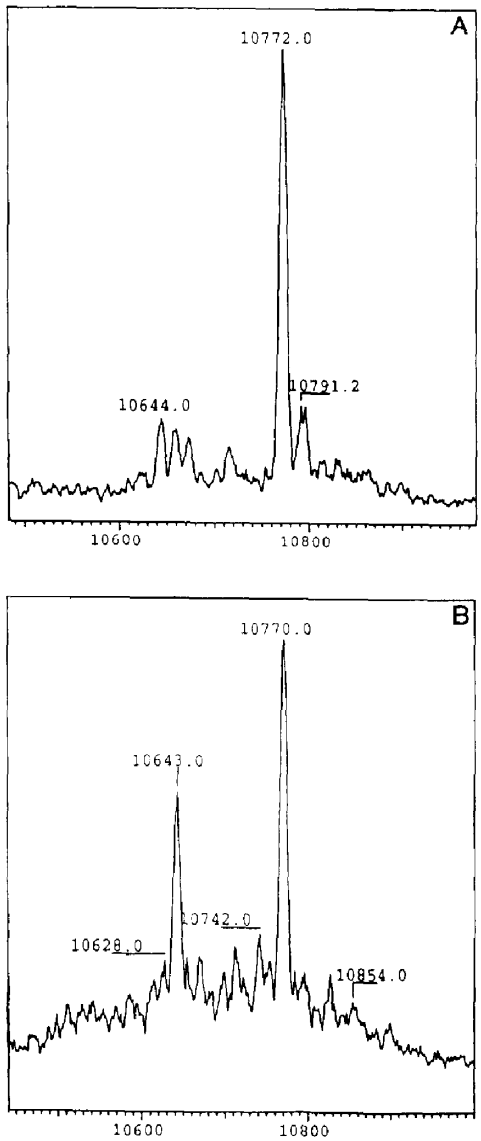

Fig. 4. ESI-MS analysis. (A) Purified free rat Hsp10 after one-step RP-HPLC purification; (B) rat Hsp10 (uncapped) after purification by Rotofor and RP-HPLC. Values represent mass units.

step purification procedure was conducted involving isoelectric focusing in a Rotofor cell (ampholyte pH range 3–10) under denaturing conditions, followed by re-fractionation with a narrower pH range. The cells containing similar material were combined and passed through a semi-preparative RP column both to desalt the sample and to provide a purification step on the basis of hydrophobicity. The purified material (overall yield 4.2%) was injected on to an analytical RP-HPLC column (Fig. 3F) and gave a sharp peak. Unfortunately, ESI-MS analysis (Fig. 4B) of the product clearly showed the presence of the same deletion peptide lacking Gln^{60} (expected, 10 642; found, 10 643), the point at which difficulties were encountered during the first synthesis. Thus, even after a three-step purification procedure a large proportion of impurity still remained as the chromatographic properties of the target sequence and the deletion peptide with Gln missing were so similar.

The homogeneity of the purified material can be critical to the biological function of the synthetic peptide. Rat Hsp10 in its biologically active form, as a co-chaperone, adopts a folded form consisting of seven monomers [15]. The folded structure binds to an M_r 60 000 chaperone in the presence of ATP and it is the complex which then helps other proteins fold correctly [15].

The ability of purified rat Hsp10 from the one-step RP purification with Fmoc probe **2** and that from the three-step purification procedure (uncapped) to fold correctly in a spontaneous manner was investigated. Solutions of 1 mg/ml of each were prepared in PBS buffer at pH 7.2 containing calcium and magnesium ions, heated to 70°C for 5 min then left at room temperature for 45 min [14]. The material thus treated was loaded on to a size-exclusion column, resulting in the appearance of two main folded forms, corresponding to heptameric and trimeric or tetrameric structures, for each of the rat Hsp10 samples [14]. In the case of the capped protein the ratio of heptamer to incorrectly folded species (i.e., tetramer) was 3:1. However, in the uncapped product that had undergone purification in a Rotofor cell and RP-HPLC, the ratio was reduced to 1:3 (heptamer-to-trimer) (data not shown). Hence, from these results, it appeared that if correct folding was dependent on the homogeneity of the monomeric material, then the protein that was synthesized with capping and then purified using a lipophilic chro-

matographic probe yielded a product with greater purity.

3.5. Affinity purification of the 46-residue β2-subunit of CD18

One of the most efficient purification techniques available is affinity chromatography. One of the most widely used interactions for the purposes of purification is the use of avidin, which exhibits an extremely high association constant for biotin. Although the covalent attachment of biotin directly to the peptidyl-resin has been described [16], the application of reversible biotinylated groups has been limited [8].

The effectiveness of biotin when attached to the Fmoc-based group was then examined. The probe synthesized possessed two Gly residues between the fluorene ring and biotin to ensure that the interaction with the immobilized avidin was not restricted by the bulkiness of the former. The probe was activated as the carbonate (**3**) and then used to derivatize an aliquot of the freshly deprotected β2-subunit peptidyl-resin. Quantitative ninhydrin analysis showed that after a 90-min reaction time, only 85% incorporation had been achieved. An additional 90 min resulted in the same amount of coupling, so the reaction was stopped and the derivatized peptidyl-resin cleaved using TFA. RP-HPLC of the crude cleavage material (Fig. 5A) showed two main products, labelled 1 and 2. The later eluting peak labelled 2 was thought to be the biotinylated product, as it exhibited strong absorbance at 301 nm and due to the lipophilicity of the fluorene ring eluted later with respect to the underivatized peptide. This was confirmed by its isolation and ESI-MS analysis (expected, 6075; found, 6075).

To determine if the immobilized avidin recognized the biotinylated material, 10 mg of crude peptide were dissolved in sodium acetate buffer (pH 4), filtered and incubated with the resin-bound avidin for 30 min with occasional mixing. An aliquot of the supernatant was removed and injected on to RP media, which indicated a decrease in the proportion of peak 2 (data not shown). The larger than expected amount of peak 1 suggested that incomplete derivatization with Fmoc probe **3** had occurred, as indicated by the ninhydrin analysis described previously. Therefore, a longer reaction time would be required to optimize the amount of affinity label on the peptidyl-resin. The resin was then washed with buffer to remove any unbound material, equilibrated in water and treated for 15 min with

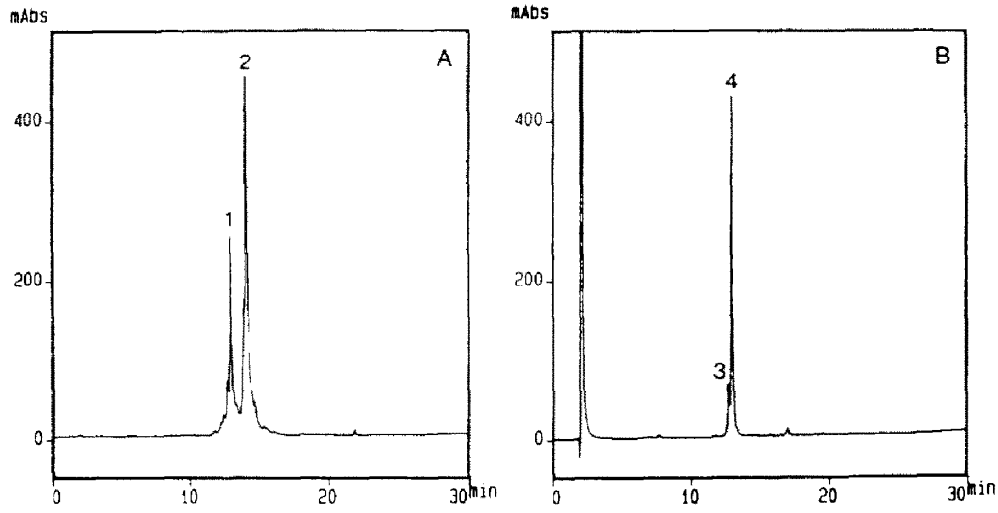

Fig. 5. Analytical RP-HPLC on a Vydac C_4 column (150 × 4.6 mm I.D.) connected to a Shimadzu LC10 LC system. Solvent A, water–0.045% TFA; solvent B, acetonitrile–0.036% TFA; gradient, 0–100% B in 30 min; flow-rate, 1 ml/min; detection, 20 nm. (A) Crude β2-subunit adduct + 3; (B) purified β2-subunit after elution from immobilized avidin with base.

5% aqueous TEA to cleave the urethane group linking the target peptide to the resin support through the biotinylated probe **3**. The supernatant containing the free purified peptide was collected and acidified with 20% acetic acid and an aliquot was analysed by RP-HPLC (labelled 4, Fig. 5B). The resulting profile showed the product to be homogeneous and it was almost identical with that obtained following purification with lipophilic probe **2** (Fig. 2D). The remaining material was desalted by RP-HPLC, yielding 3 mg (33%) of purified β2-subunit after lyophilization and shown by ESI-MS to have the correct molecular mass (expected, 5483; found, 5483). The peak labelled 3 (Fig. 5B) was found to be the β2-subunit with Met in its oxidized form, consistent with the results obtained previously (ESI-MS: found, 5499).

4. Conclusions

New simplified Fmoc-based probes containing either extremely hydrophobic groups or biotin have been synthesized. The probes were then incorporated on the peptidyl-resin as active carbonates to permit separation by either RP-HPLC or biotin–avidin affinity chromatography. The chromatographic probes were used successfully to purify two synthetic polypeptides, 46 and 101 residues in length. The former represents the first example of the application of these probes to a peptide synthesized using the Fmoc protocol. To convert possible deletion peptides into truncated sequences and thereby prevent their derivatization with the probes, a new capping procedure was applied that overcame the instability of acetic anhydride solutions and the harmful reactivity of its by-products. The procedure involved the use of N-(2-chlorobenzyloxycarbonyloxy)succinimide, which was freshly dissolved and transferred automatically by the peptide synthesizer at the end of each coupling reaction. The combined application of capping procedure and chromatographic probes **1**, **2** or **3**, when used to purify the 46- and 101-residue polypeptides, clearly demonstrated that highly homogeneous material could be obtained after a one-step purification procedure.

Acknowledgements

We thank Dr. P. Lucietto, Dr. G. Fossati and Dr. P. Giuliani for the synthesis of rat Hsp10 (uncapped) and Dr. P. James, Protein Chemistry Laboratories, ETH, Zurich, Switzerland, for ESI-MS analyses.

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