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Affinity Purification of 101 Residue Rat cpn10 Using a Reversible Biotinylated Probe

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Abstract: The purification of large synthetic peptides using conventional separation techniques often results in poor yields and homogeneity due to the accumulation of chromatographically similar deletion and truncated impurities. We have developed a highly effective synthetic strategy and one-step purification procedure that is based on (i) the application of single coupling using HBTU/HOBt activation to reduce incomplete couplings, (ii) the use of *N*-(2-chlorobenzyloxycarbonyloxy)succinimide as a capping agent to terminate deletion sequences and (iii) the N-terminal derivatization of the complete peptidyl-resin with a reversible Fmoc-based chromatographic probe possessing enhanced physico-chemical properties (i.e. hydrophobicity, charge or affinity label). We report the application of a biotinylated probe, activated as the succinimidyl carbonate, for the purification of a 101 residue chaperonin protein from *Rattus norvegicus* (rat cpn10), previously synthesized using an optimized synthetic protocol. Biotinylated rat cpn10 was separated from underivatized impurities on an immobilized monomeric avidin column. Free rat cpn10 was released from avidin–agarose column with 5% aqueous triethylamine and after desalting by RP-HPLC gave 9.9% recovery. Characterization and assessment of homogeneity was achieved using ESI-MS, CZE and RP-HPLC.

Keywords: Stepwise SPPS; HBTU/HOBt; capping; avidin–biotin; chaperonin10 (*R. norvegicus*)

Abbreviations

DCM, dichloromethane; DIEA, diisopropylethylamine; HBTU, 2(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; NMP, *N*-methylpyrrolidone; TFMSA, trifluoromethane sulphonic acid; Z(2-Cl)-OSu, *N*-(2-chlorobenzyloxycarbonyloxy)succinimide.

INTRODUCTION

Stepwise solid-phase peptide synthesis (SPPS) [1] has developed considerably in recent years owing to improvements in the coupling reagents, protecting groups and resin supports [2]. It is now possible to synthesize small polypeptides of, say, 50 residues in length on an almost routine basis. However, despite these advances, a coupling efficiency greater than 99% for each amino acid cannot always be obtained because of sequence-related problems [2]. Consequently, the crude peptide contains a family of deletion and truncated impurities together with the target sequence which can generally be separated using conventional chromatographic techniques. Unfortunately, as the length of the peptide chain increases, so the physico-chemical differences between the desired sequence and its associated impurities decrease, thus hindering purification. One answer is to modify the target sequence reversibly with a group that possesses enhanced chromatographic properties. The literature reports several molecules that are either lipophilic or have charged groups to aid the purification of synthetic peptides by reversed-phase HPLC (RP-HPLC) or ion-exchange chromatography (IEC), respectively [3–5]. Alternatively, an affinity label can be attached, thus utilizing the specific interaction between two groups to facilitate the separation of the desired peptide from its closely related impurities. The latter approach offers distinct advantages over the lipophilic and

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charged probes, especially for large polypeptides and proteins since an affinity method is independent of chain length and avoids the use of organic solvents that could denature the peptide. For example a Cys-Met group can be attached to the N-terminus of the peptide and bound to an organomercurial–agarose column [6] or peptides that contain His, Trp or Cys residues in the N-terminal region can be purified on an immobilized metal ion column [7]. However, both these methods are compromised when the amino acids upon which the separation is based also occur in other parts of the sequence. Similarly, sequence-related considerations are also necessary when using a tetrabenzo-modified Fmoc group for absorption chromatography on porous graphitized carbon, which then require harsh denaturing conditions for removal of the affinity group [8].

A more effective affinity interaction is that between biotin and avidin ($K_a = 10^{15}$). The biotinylation of synthetic polypeptides has been described [9], but these were irreversibly derivatized since biotin was attached covalently through an amide bond. A reversible biotinylated molecule has been described; however, its application was only described for relatively short synthetic peptides [10]. In previous work [4] we reported the application of a reversible biotinylated probe for the affinity purification of a 46 residue peptide synthesized using the Fmoc approach. Here we report the purification of the 101 residue chaperonin 10 protein from *Rattus norvegicus* (rat cpn10) [11] which was synthesized using an optimised Boc chemical strategy [5] and purified with the reversible biotinylated probe, **1** (Figure 1).

MATERIALS AND METHODS

Analytical Methods

High-performance liquid chromatography (HPLC) was performed using a Shimadzu LC10 system equipped with a dual wavelength detector. A Vydac C_{18} (150 × 4.6 mm i.d.) reversed phase column was used for analysis and desalting procedures, using two buffers; 0.045% TFA in water (buffer A) and 0.036% TFA in acetonitrile (buffer B).

An Applied Biosystems Model 270A-HT (Foster City, CA) was used for CZE analysis, using an uncoated silica capillary with total length 72 cm and i.d. 50 μm. The separation was performed with 100 mM sodium phosphate buffer, pH 2.3 at 30°C and detected at 200 nm.

Electrospray mass spectrometry was performed on a Finnigan MAT model 700 instrument (San Jose, CA).

Peptide Synthesis

The complete synthesis of rat cpn10 is described elsewhere [5]. Briefly, PAM–copoly-(syrenedivinylbenzene) resin [PAM = 4-(*p*-carboxyamidomethyl)benzyl ester], pre-derivatized with the first amino acid (i.e. Asp; substitution value = 0.7 mmol/g) and an automated Applied Biosystems 430A peptide synthesizer (div. Perkin-Elmer Corp.) were used for chain assembly. The synthesis was performed on a 0.25 mmol scale with single HBTU/HOBt mediated amino acid coupling (except Arg and Gln residues which were double coupled). A capping procedure, using *N*-(2-chlorobenzyloxycarbonyloxy)succinimide, was performed after the addition of each amino acid as described previously [4, 5].

Introduction of Chromatographic Probe (1)

The N-terminal Boc protecting group was removed from 100 mg (6 μmol) of rat cpn10 peptidyl–resin with 50% TFA in DCM for 30 min. The freshly deprotected peptidyl–resin was neutralized with 5% DIEA in DCM and then washed with DCM. A 50 mg (3 μmol) portion of the freshly deprotected peptidyl–resin was treated with 20.7 mg (30 μmol) of the biotinylated probe (**1**) and dissolved in 200 ml DCM/NMP (3:1) containing a catalytic quantity of DIEA. The mixture was left to vortex at room temperature overnight. Quantitative ninhydrin analysis [12] was performed on an aliquot of the resin and showed that 96% incorporation had occurred. The addition of a further five equivalents of biotinylated probe (**1**) and reaction for a further 6 h did not increase the extent of coupling. The peptidyl–resin was washed with DCM/NMP (3:1) and finally dried from DCM in preparation for cleavage.

Deprotection and Cleavage of Peptidyl-Resin

Deprotection and cleavage of biotinylated rat cpn10 and the remaining 50 mg (3 μmol) of underivatized peptidyl–resin was achieved using a low TFMSA/high HF procedure [13]. The dried peptidyl–resins were first treated with TFMSA-1,4-ethanedithiol-*p*-cresol-dimethylsulphide-TFA (5:1:4:15:25) at 0°C for 2 h, followed by the high HF procedure, HF-*p*-cresol-*p*-thiocresol (50:4:1). The crude peptides were precipitated with cold dry diethyl ether and then dissolved

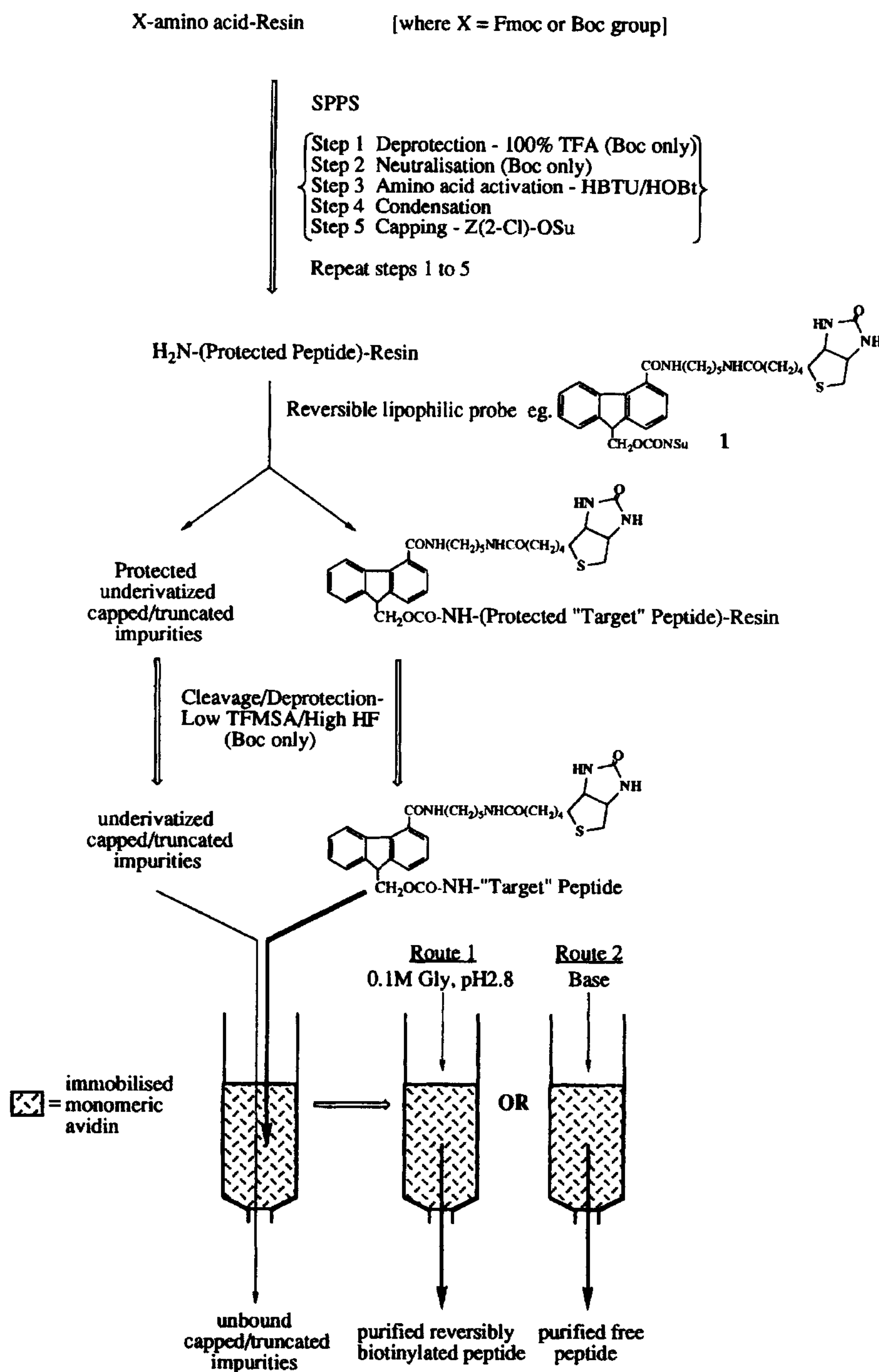

Figure 1 General scheme showing optimized protocol for the stepwise SPPS of proteins. Chain assembly (HBTU/HOBt and capping with Z(2-Cl)-OSu), cleavage (low TFMSA/high HF) and one-step affinity purification using the biotinylated chromatographic probe (**1**).

in 20% acetic acid solution. The peptide solutions were then lyophilized to yield 28.2 mg (81.7%) crude biotinylated rat cpn10 and 25.2 mg (75%) underivatised rat cpn10.

Avidin–Biotin Affinity Purification

Crude rat cpn10, derivatized with reversible biotinylated probe (**1**) was dissolved in 0.2 M sodium acetate buffer, pH 4 and the solution filtered. The filtrate was

passed through an immobilized monomeric avidin (Pierce) column (2.5 × 1 cm i.d.) with a flow rate of 0.2 ml/min. The column was washed with 15 column volumes of buffer at a flow rate of 1 ml/min and the supernatant collected. The column was then equilibrated with 15 column volumes of water, followed by 3 column volumes of 5% aqueous triethylamine, to cleave the urethane bond linking the protein to the biotinylated probe, thus enabling the recovery of rat cpn10 in its free form. The basic solution was then acidified with 10% acetic acid solution and desalted on an analytical Vydac C_{18} (150 × 4.6 mm) column.

RESULTS AND DISCUSSION

We have developed a reversible purification scheme for the one-step separation of peptide with the correct sequence from closely related impurities that are generated at every stage during stepwise SPPS. The philosophy of the methodology is described elsewhere and is based on the incorporation of an Fmoc-based molecule, derived from groups possessing enhanced chromatographic properties (e.g. lipophilic, charge or biotin), to the N-terminus of the complete peptidyl-resin, synthesized using either Boc or Fmoc strategies [3–5]. To ensure maximal efficiency of the chromatographic probes, an effective capping protocol is necessary to terminate any deletion sequences. Following deprotection and cleavage from the resin the derivatized peptide is purified according to the probe used i.e. reversed-phase, ion-exchange or biotin–avidin chromatography, respectively. Finally, treatment with dilute aqueous base cleaves the chromatographic probe and after desalting yields the free purified product. Figure 1 illustrates the principal steps of synthesis and purification using the reversible biotinylated probe.

To demonstrate that the biotinylated probe can be used effectively for the purification of large polypeptides, we have synthesized the 101 residue rat cpn10

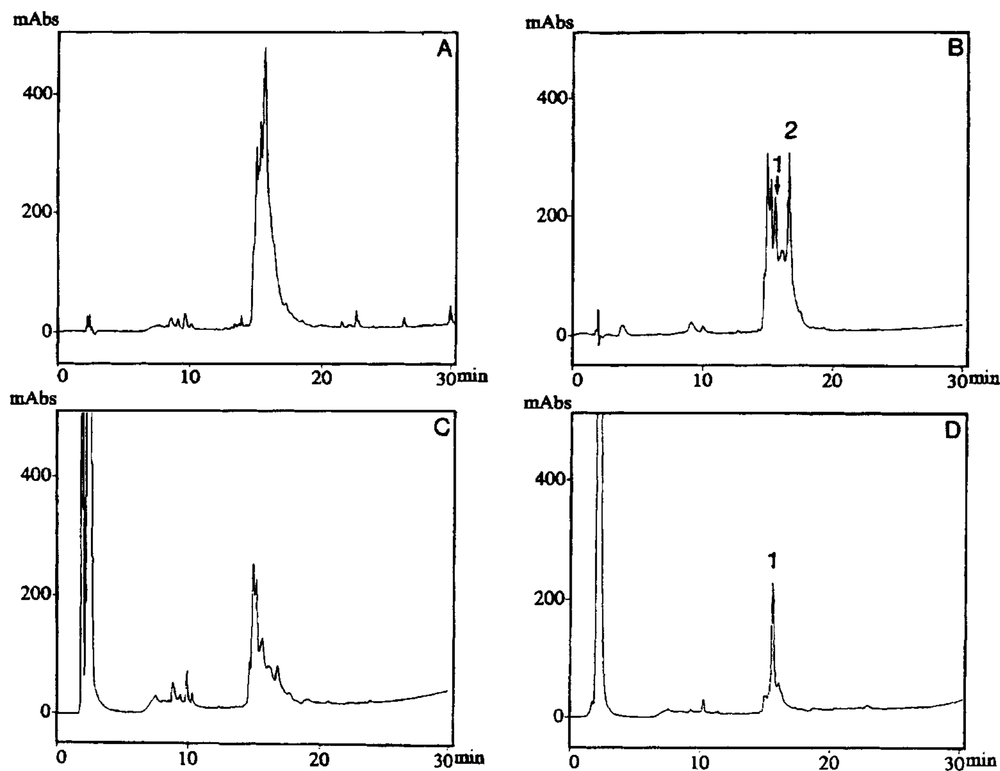

Figure 2 Analytical RP-HPLC of rat cpn10 on C_{18} Vydac (150 × 4.6 mm) column, using a 0–100% in 30 min acetonitrile gradient, flow 1 ml/min and detection at 220 nm. Panel A shows crude underivatized rat cpn10. Panel B indicates that the addition of biotinylated probe **1** to rat cpn10 results in the appearance of peak labelled 2 and decreases the intensity of peak labelled 1. Panel C shows the unbound material eluted off the immobilized avidin column. Panel D shows free rat cpn10 (peak 1) after base treatment. The shoulders represent sidechain modified impurities that are not removed by affinity chromatography.

protein. The protein was synthesized using optimized Boc chemical protocols involving HBTU/HOBt activation combined with a capping procedure using *N*-(2-chlorobenzyloxycarbonyloxy)succinimide (Z(2-Cl)-OSu), as described previously [4, 5]. At the end of the synthesis the peptidyl–resin was deprotected with 50% TFA, neutralized and a portion reacted with 2 equivalents of the activated biotinylated probe (**1**). Maximal incorporation of 96% as determined by quantitative ninhydrin analysis [12] was achieved after overnight mixing. The derivatized and underivatized rat cpn10 peptidyl–resins were finally deprotected and cleaved from the solid support using a low TFMSA/high HF procedure [2].

An aliquot of crude underived protein was analysed by RP-HPLC (Figure 2A) and showed a complicated mixture of peaks centred around 14.5 min. A similar analysis of the derivatized rat cpn10 gave a different profile, with the appearance of a new peak labelled 2 and a reduction in the intensity of the peak labelled 1 (Figure 2B). The former peak was considered to represent biotinylated rat cpn10 owing to the absorbance of the fluorenyl ring chromophore at 301 nm and the increased retention time caused by the addition of a lipophilic group to the N-terminus and was in accordance with previous results [3–5]. The peak labelled 1 (Fig. 2B) was probably underivatized rat cpn10 since upon isolation it was shown by ESI-MS to contain protein with the correct molecular weight together with impurities of lower molecular weight (data not shown). Thus the nearly quantitative incorporation of **1** as indicated by the ninhydrin test (see above) was likely to be overestimated.

For the purification step, immobilized monomeric avidin was equilibrated with 0.2 M sodium acetate buffer, pH 4. Crude biotinylated protein weighing 17 mg was dissolved in 1 ml of 0.2 M sodium acetate buffer, pH 4, filtered and loaded on to the avidin column, using a low flow rate. Unbound material was washed off with additional buffer and an aliquot analysed by RP-HPLC (Figure 2C). The chromatogram showed that the peak labelled 2 (Figure 2B) was absent, as expected, while the impurities which were not derivatized with **1** were immediately eluted off the column.

Two routes are available to recover the bound protein, as shown in Figure 1. The first option was to treat the avidin column with 0.1 M glycine, pH 2.8 to release the intact biotinylated rat cpn10 protein. A subsequent base treatment will then yield the free protein when required. Alternatively, the column can be treated immediately with a base in order to cleave the urethane bond, so releasing the free protein while leaving the biotinylated probe still attached to the avidin column (route 2). Since the aim was to obtain free protein, the second route was chosen. Thus, the immobilized avidin column was washed with water and then treated with 5% aqueous triethylamine. The eluent from the column was collected, acidified with 10% acetic acid solution and the released rat cpn10 desalted on a RP-HPLC column. An aliquot of purified rat cpn10 was loaded on to an analytical RP column and the resulting chromatogram showed a main peak labelled 1 (Figure 2D) together with two shoulders on either side. Similarly, capillary zone electrophoresis (CZE) was also performed and gave a profile closely related to that obtained by HPLC. The CZE chromatogram (Figure 3) indicated a major product (labelled 1; 65%) and several smaller peaks. As reported previously, owing to the strong propensity for rat cpn10 to form heptameric and tetrameric structures,

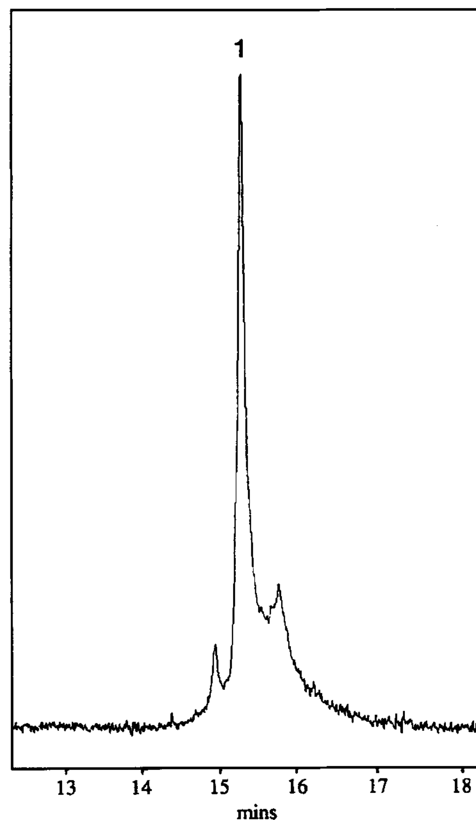

Figure 3 Capillary zone electrophoresis of purified rat cpn10. Injection vacuum 1.5 sec; temp. 30°C; capillary length 72 cm (50 cm to detector), i.d. 50 μm; current 42 mA; buffer, 100 mM sodium phosphate, pH 2.3; detection, 200 nm. The collection of low intensity peaks on either side of the main peak labelled 1 (65%) could represent aggregated forms of the correct sequence.

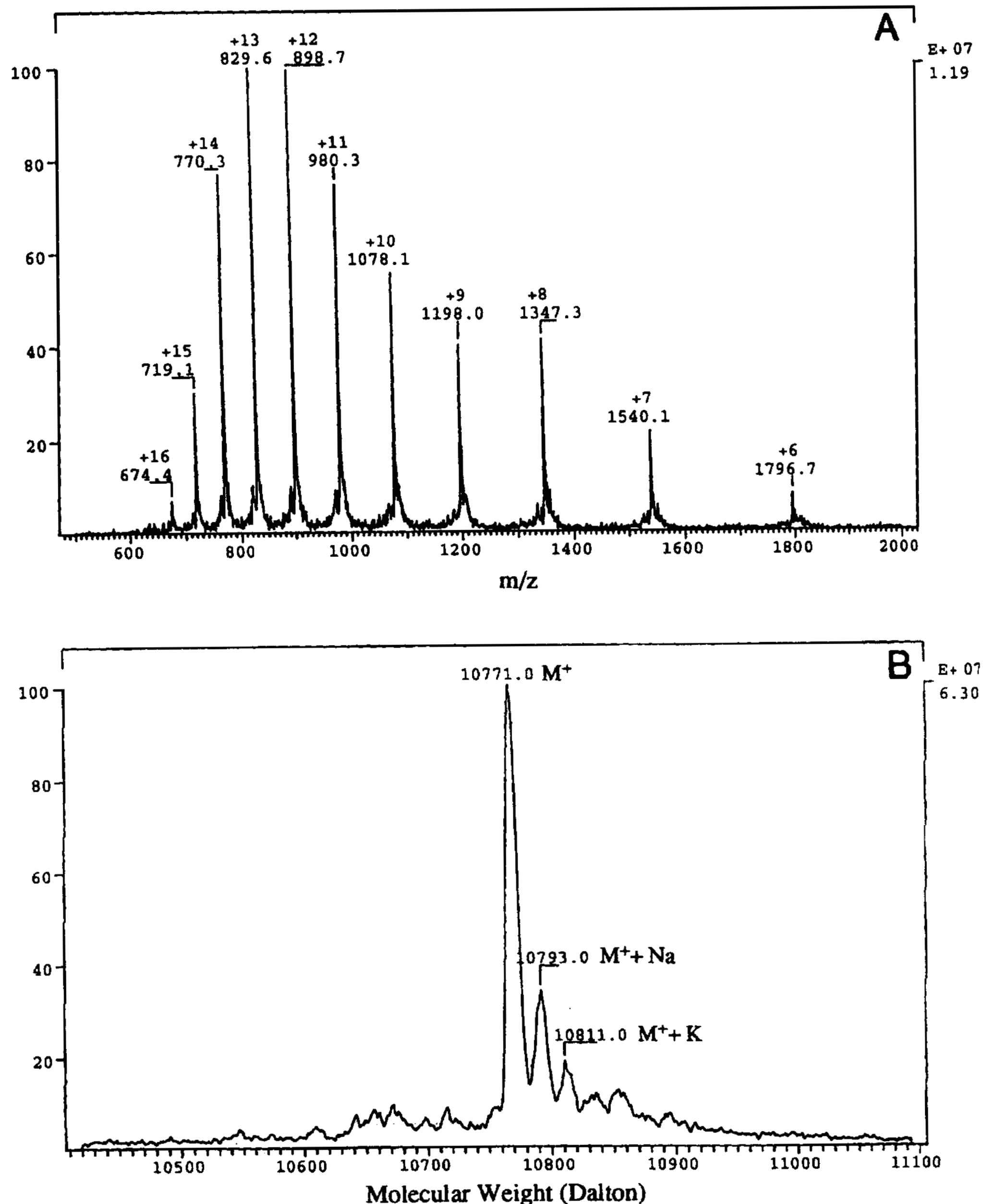

Figure 4 Electrospray ionization mass spectrum of purified rat cpn10 after one-step separation by affinity chromatography (panel A). All peaks labelled with numbers are different charge states of the target full-length rat cpn10 protein. The calculated molecular mass of target product is 10 770.57 D (average); 10 763.8 D (monoisotopic). Panel B shows the deconvoluted mass spectrum for purified rat cpn10. The low-intensity peaks on either side of the peak representing rat cpn10 are sidechain modified and some deletion sequences are due to incomplete capping.

it is possible that these peaks are the result of different aggregated forms of the correct sequence [5, 13]. After lyophilization, 1.6 mg (9.9%) of protein was obtained, which was confirmed to be rat cpn10 by ESI-MS analysis (Figure 4). The spectrum also showed the existence of small amounts of impurities with molecular mass around 10 650 and 10 850, consistent with the profiles observed from both HPLC and CZE analyses. These impurities were likely to arise from the incompleteness of the capping reaction and sidechain modifications during the HF cleavage reaction [2]. The presence of such impurities were not

unexpected since all biotinylated sequences, including those possessing sidechain modifications, will also be retained by the column.

CONCLUSIONS

We have developed a highly effective protein synthesis protocol for the preparation of large peptides using both Boc and Fmoc chemistries. The methodology is based on HBTU/HOBt activation to maximize coupling efficiency and the application of a capping procedure using Z(2-Cl)-OSu to terminate deletion sequences [5]. To complement the optimized synthetic procedure, a chromatographic probe is introduced possessing enhanced physico-chemical properties (e.g. lipophilicity or affinity label) to allow one-step purification, requiring minimum technical expertise. A biotinylated Fmoc-based probe (**1**) was used to derive 101 residue rat cpn10, which was then successfully separated from underivatized impurities on an immobilized avidin column, in high yields. The product thus obtained was found to be at least 65% pure by CZE and contaminated with small amounts of deletion and sidechain modified impurities. This type of probe represents an alternative to other equally efficient probes that we have developed which are based on either an increased lipophilic character for RP purification [4, 5] or enhanced charge content for ion-exchange chromatography [3]. Although the latter have not been applied yet to large polypeptides such as the rat cpn10 described here, all lipophilic probes have been shown to be effective in a one-step purification of peptides and proteins [3–5]. The advantage offered by the biotinylated probe/avidin approach is that the protein can be purified in aqueous solution, thus avoiding organic solvents that may have an adverse effect on the stability of the protein structure. Furthermore, the separation of biotinylated material from underivatized impurities can be carried out using simple laboratory packed columns. More importantly, however, the specific reversible N-terminal biotinylation of a peptide or protein introduces the opportunity to use such molecules, when immobilised on an avidin column, for the purification of secondary molecules (H. L. Ball and P. Mascagni, unpublished data).

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