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Affinity purification method using a reversible biotinylating reagent for peptides synthesized by the solid-phase technique

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ABSTRACT

The specific interaction between biotin and avidin was explored as the basis of an affinity purification procedure for peptides synthesized by the solid-phase technique. In this affinity purification procedure, a reversible biotinylating reagent, 2-[(N-biotinyl)-aminoethylsulphonyl]ethyl *p*-nitrophenyl carbonate (BASEC), was synthesized. Using this reagent, the procedure involves the following sequence of four reactions: (i) attachment of the biotin to the N-terminus of the peptide–resin through a base-labile sulphonylethoxycarbonyl linkage at the final step of solid-phase peptide synthesis; (ii) acid treatment to remove side-chain protecting groups and cleave the biotin-modified peptide from the resin; (iii) affinity purification of the biotinyl-peptide on an avidin–agarose column; and (iv) base (5% ammonia solution) treatment to remove the biotin moiety from the peptide. To facilitate this purification procedure, unreacted amino groups were acetylated in each step during solid-phase synthesis. The usefulness of this method was demonstrated by the purification of several peptides synthesized by the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase technique.

INTRODUCTION

Improvements in protecting groups, coupling reagents and resin supports have led to significant advances in the field of solid-phase peptide syntheses. In spite of these advances, a condensation reaction yield of over 99% cannot be always obtained, as it depends on the sequence of the target peptide. As a result, peptides with deletions accumulate as impurities. Gel filtration, affinity chromatography, high-performance liquid chromatography and combinations of these methods have been used for the final purification of the desired product [1–8]. However, this step is still a major barrier to obtaining synthetic products in satisfactory yield. Recently, we introduced a novel reversible SH-introducing reagent, 2-{N-[(*p*-methoxybenzylthio)acetyl]aminoethylsulphonyl}ethyl *p*-nitrophenyl carbon-

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ate, and reported a one-step purification method for the solid-phase synthetic products based on the specific reaction between SH and the iodoacetamide group [9]. Using this method, the target peptide alone can be isolated in high purity and high yield in a short time from a mixture with very similar deleted peptides. The long peptides and proteins, however, could not be isolated effectively because of the too abundant impurities. The chemical affinity of SH–iodoacetamide seemed to be weaker than the biological affinity at such a low concentration. In this event, biotin–avidin affinity chromatography can be a more efficient purification method [10,11]. Lobl *et al.* [12] reported that biotinylation of chemically synthesized protein on resin could be a rapid and efficient purification technique for lower yield products. Their method, however, provided biotinylated protein at the N-terminus as a fully reversible biotinylating reagent was not available.

In this paper, we describe the synthesis of a new reversible biotinylating reagent and introduce a novel biotin–avidin affinity purification technique, using it for peptides synthesized by the solid-phase technique, especially for lower yield synthetic products.

EXPERIMENTAL

Analytical methods

Thin-layer silica gel (Kieselgel G, Merck) chromatography was conducted with solvent system 1 [chloroform–methanol–water (8:3:1, v/v)]. For ^{1}H NMR spectrometry a Bruker AC-300 instrument was used with tetramethylsilane as the internal standard. Fast atom bombardment mass spectrometry (FAB-MS) was carried out with a ZAB SE spectrometer (VG Analytical) and ionspray MS with an API III spectrometer (Perkin-Elmer Sciex). High-performance liquid chromatography (HPLC) was performed with a Model 600E system (Millipore) equipped with a μBondasphere $5C_{18}$ (100 Å) column (150 × 3.5 mm I.D.) (Millipore) using the following two solvent systems: 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). Peptide sequence analysis was performed with a Model 6600 protein sequencer (Millipore).

Synthesis of the reversible biotinylating reagent

Synthesis of 2-[(N-biotinyl)aminoethylsulphonyl]ethanol. 2-(Aminoethylsulphonyl)ethanol hydrochloride (3.79 g, 20 mmol) was dissolved in dimethylformamide (DMF) (50 ml) together with Et_3N (5.6 ml, 40 mmol) and biotinyl 1-hydroxysuccinimide ester (6.83 g, 20 mmol). After being stirred for 4 h, the solution was filtered and the filtrate was concentrated *in vacuo*. The resulting residue was triturated with ethanol to afford a powder. The crude product was recrystallized from methanol (yield, 5.24 g, 69%), m.p. 126–127°C. Analysis: calculated for $C_{14}H_{25}N_3O_5S_2 \cdot \frac{1}{2}H_2O$, C 43.27, H 6.75, N 10.81; found, C 43.59, H 6.63, N 10.96%. R_F in system 1 = 0.32. FAB-MS: m/z 380.3 ($[M+H]^+$); calculated for $C_{14}H_{25}N_3O_5S_2$, 379.5.

Synthesis of reversible biotinylating reagent (BASEC). 2-[(N-Biotinyl)aminoethylsulphonyl]-ethanol (3.79 g, 10 mmol) was dissolved in absolute pyridine (30 ml). The solution was cooled at 0°C and *p*-nitrophenyl chloroformate (2.02 g, 10 mmol) was added and stirred. The mixture was then allowed to stand for 5 h at 0°C and subsequently concentrated *in vacuo*. The residue was treated with 1 *M* HCl and diethyl ether, then the product, which immediately crystallized, was collected by filtration and washed with water. The crude product was recrystallized from DMF–ethyl acetate (yield, 3.77 g, 69%), m.p. 104–105°C. Analysis: calculated for $C_{21}H_{28}N_4O_9S_2 \cdot \frac{1}{2}H_2O$, C 45.65, H 5.37, N 10.22; found, C 45.75, H 5.22, N 10.08%. R_F in system 1 = 0.37. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.36–1.23 (m, 2H), 1.47–1.37 (m, 1H), 1.58–1.46 (m, 2H), 1.67–1.55 (m, 1H), 2.05 (t, J = 7.3 Hz, 2H) 2.57 (d, J = 12.4 Hz, 1H), 2.81 (dd, J = 12.4 and 5.0 Hz, 1H), 3.08 (ddd, J = 8.2, 6.5 and 4.5 Hz, 1H), 3.32 (t, J = 6.9 Hz, 2H), 3.48 (q, J = 6.7 Hz, 2H), 3.68 (t, J = 5.7 Hz, 2H), 4.11 (ddd, J = 7.8, 4.5 and 2.0 Hz, 1H), 4.29 (dd, J = 7.8 and 5.2 Hz, 1H), 4.62 (t, J = 5.7 Hz, 2H), 6.33 (br, s, 1H), 6.38 (br, s, 1H), 7.58, 8.33 (AA'BB' pattern, J_{ortho} = 9.2 Hz, aromatic H), 8.07 (br, t, J = 5.9 Hz, 1H). FAB-

MS: m/z 545.3 ($[M+H]^+$); calculated for $C_{21}H_{28}N_4O_9S_2$, 544.6.

Syntheses of model peptides (general method)

Synthesis of model peptides. The model peptides used in this experiment were synthesized by 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase synthesis according to the principle of Cameron *et al.* [13], using a Model 9050 automatic peptide synthesizer (Millipore). The following side-chain-protected Fmoc-amino acids were used: Arg(Mtr), Lys(Boc), His(Boc), Glu(OBu^t), Asp(OBu^t), Ser(Bu^t), Thr(Bu^t) and Tyr(Bu^t), where Mtr 4-methoxy-2,3,6-trimethylbenzenesulphonyl, Boc = *tert.*-butoxycarbonyl and Bu^t = *tert.*-butyl. To make the final purification step easier, the peptide–resin was treated with acetic anhydride after each condensation reaction to ensure the complete termination of the unreacted amino groups. Thus only the mature peptide could be modified at the end of synthesis.

Introduction of the reversible biotinylating reagent. The peptide–resin (200 mg) was treated twice with 20% piperidine in DMF (5 ml, for 5 and 15 min) to remove the Fmoc group. Then BASEC (5 equiv.) was condensed to the N-terminal residue of the protected peptide–resin in the presence of 1-hydroxybenzotriazole (5 equiv.) in DMF. The container was shaken until the resin showed a negative Kaiser test [14] (usually for 2 h). The resin was washed with DMF and CH_2Cl_2 and dried under reduced pressure.

Deprotection and purification (general method)

Deprotection and cleavage of peptides from the solid-phase support. The peptide–resin (50 mg) was treated with 1 *M* trimethylsilyl bromide (Me_3SiBr)–thioanisole in TFA [15] (10 ml) in the presence of *m*-cresol (200 μl) and ethanedithiol (200 μl) at 0°C for 2 h, after which Me_3SiBr and TFA were removed by evaporation and dry diethyl ether was added. The resulting powder was collected by centrifugation.

Purification. The crude deprotected peptide was dissolved in PBS(−) buffer (pH 7.5) containing 500 m*M* NaCl and then the solution was filtered. The filtrate was applied to the avidin–agarose column (10 × 1 cm I.D.) (Pierce) at a flow-rate of 1 ml/min and then the column was washed with 20 column volumes of PBS(−) buffer at the same flow-rate. The biotinylated peptide was eluted with 6 *M* guanidine hydrochloride solution (pH 1.5). The eluent was directly treated with 5% ammonia solution (addition of 25% ammonia solution in one quarter the volume of the eluent) at room temperature for 10 min to release the biotin moiety from the peptide. The reaction mixture was then desalted by RP-HPLC or gel filtration.

Characterization. The purity of the synthetic peptides was ascertained and/or calculated using HPLC. The peptides obtained were characterized by amino acid sequencing by automated Edman degradation and by FAB-MS or ionspray MS.

RESULTS AND DISCUSSION

We have developed a new affinity purification method based on the avidin–biotin interaction for isolating a target peptide from a mixture containing many kinds of immature (terminated) peptides. This objective was achieved by the development of not only an acid-stable but also a base-labile biotinylating reagent based on the methylsulphonylethoxycarbonyl (Msc) group [16].

Synthesis of a reversible biotinylating reagent was carried out according to the scheme shown in Fig. 1. The amino group of the 2-aminoethylsulphonylethanol [9] can be attached to biotin by the 1-hydroxysuccinimide active ester method.

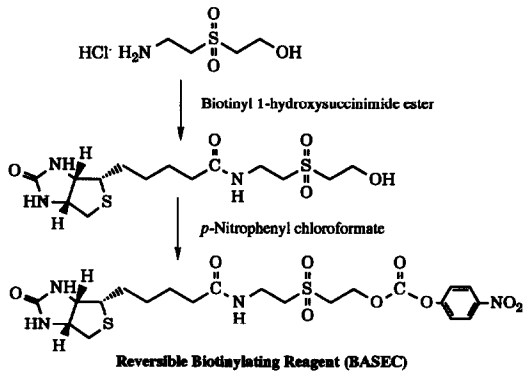

Fig. 1. Scheme for the synthesis of the reversible biotinylating reagent (BASEC).

Then the OH group is converted into active an alkoxy carbonate by treatment with *p*-nitrophenyl chloroformate. This compound (BASEC) is easily introduced to α-amino groups of the peptide–resin within 2 h in the presence of 1-hydroxybenzotriazole at room temperature, and it acts as a urethane-type amino protecting reagent. It is stable to acids, such as trifluoracetic acid, Me_3SiBr, hydrofluoric acid, trifluoromethanesulphonic acid and trimethylsilyl trifluoromethanesulphonate, but is easily removed by the β-elimination reaction with a basic reagent (with 0.2 *M* NaOH in 50% MeOH in 5 s or 5% ammonia solution in 50% MeOH in 5 min) (Fig. 2). This indicates that after isolation of the desired product from the deletion peptides, the N-terminal biotin moiety can be removed from the target peptide or protein by mild base treatment.

This reversible biotinylating reagent was allowed to react with the N-terminus of the completely mature peptide at the end of synthesis as shown in Fig. 3. After final deprotection, the biotin-modified target peptide was bound to the avidin–agarose support under neutral conditions. Thus, only the mature peptide could be immobilized on the agarose support with affinity interaction, while terminated peptides and scavengers could be easily washed out from the avidin–agarose column. The biotin-modified target peptide was eluted with 6 *M* guanidine–hydrochloride (pH 1.5). Finally, the eluted peptide was treated with 5% ammonia solution to remove the biotin moiety from the peptide (Fig. 4). The final

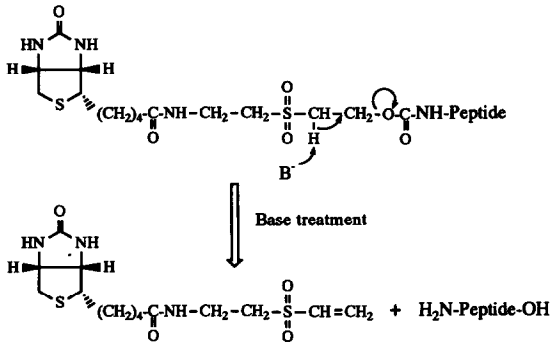

Fig. 2. Schematic representation of the debiotinylation reaction.

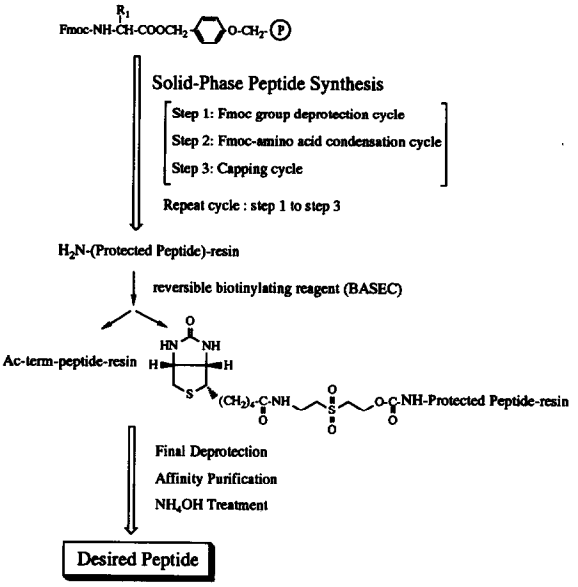

Fig. 3. General scheme for Fmoc-based solid-phase peptide synthesis and affinity purification technique. Step 1, deprotection of Fmoc group treated with 20% piperidine–DMF for 7 min; step 2, coupling of Fmoc-amino acid (5 equiv.) activated with several reagents for 30 min; step 3, capping of unreacted α-amino groups treated with 0.5 *M* acetic anhydride–DMF for 10 min.

purification was performed using RP-HPLC or gel filtration.

To demonstrate the usefulness of this method, magainin 2 [17], a 23-residue peptide, and human growth hormone releasing factor (hGRF) [18,19], a 44-residue peptide amide, were synthesized as model peptides. Syntheses were performed by the Fmoc-based solid-phase method shown in Fig. 3.

As shown in Fig. 5A, the HPLC trace for the crude synthetic magainin 2 showed many failed peptides (content of the desired peptide $\approx$ 39%), but almost all failed peptides were removed (purity $\approx$ 87%) by this affinity method. The result of FAB-MS analysis of the obtained peptide was identical with the theoretical value of *m/z* 2466.6 ($[M+H]^+$) (calculated for $C_{114}H_{180}N_{30}O_{29}S$, 2465.3), and the full amino acid sequence analysis was correct.

With hGRF, the target peptide showed a sharp single peak on HPLC with several small peaks derived from capped terminated peptides (content of the desired peptide $\approx$ 28%, Fig. 5B).

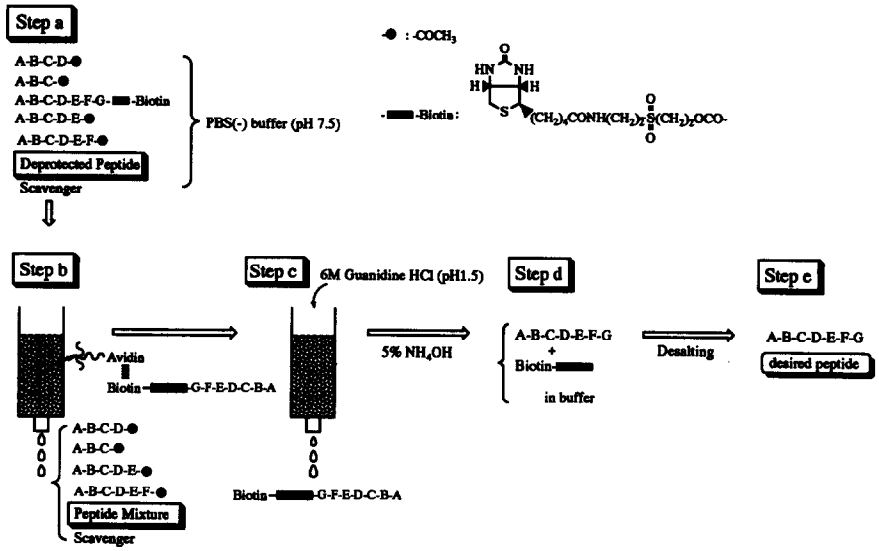

Fig. 4. Deprotection and purification of peptides synthesized by the solid-phase technique. Step a, deprotected peptide mixture dissolved in PBS(−) buffer (pH 7.5); step b, binding to avidin–agarose column; step c, elution of the desired peptide from avidin–agarose column; step d, removal of the biotin moiety; step e, desalting.

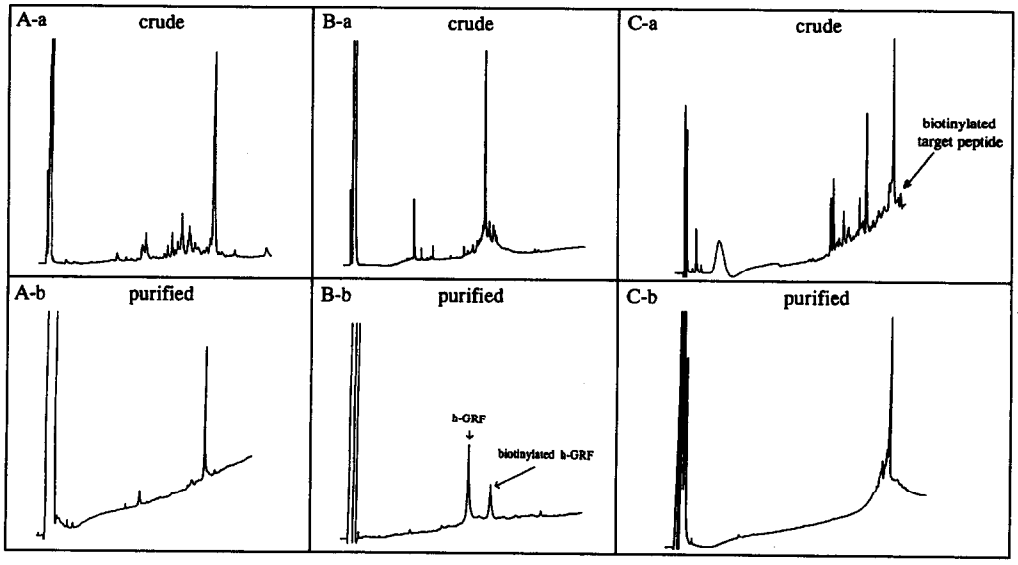

Fig. 5. HPLC elution profile of synthetic peptides. HPLC was performed on a μBondasphere column using two solvent systems (A and B) with a linear gradient of solvent B; flow-rate, 1 ml/min; absorbance monitored at 220 nm. (A) magainin 2: the column was eluted with a linear gradient from 10 to 50% solvent B in 40 min; (a) crude product; (b) purified product. (B) hGRF: (a) crude product, the column was eluted with a linear gradient from 23 to 40% solvent B in 30 min; (b) co-injection of purified product (before and after treatment with 5% ammonia solution), the column was eluted with a linear gradient from 25 to 55% solvent B in 30 min. (C) Acm-transducin γ-subunit 1–67: the column was eluted with a linear gradient from 10 to 40% solvent B in 30 min; (a) crude product; (b) purified biotinylated Acm-Tγ 1–67.

After purification, the hGRF produced in this manner (purity ≈ 85%) showed a retention time identical with that of the peptide obtained by a conventional purification procedure. The full amino acid sequence and FAB-MS result, *m/z* 5037.8 ($[M+H]^+$) (calculated for $C_{215}H_{358}N_{72}O_{66}S$, 5036.7), agreed with the theoretical values.

We further studied the application of this method to a small protein in synthesizing transducin γ-subunit 1–67 (Tγ 1–67) [20]. For this synthesis, the SH group of the Cys residue was protected with acetamidemethyl (Acm). The procedure was the same as that mentioned above. For Acm-Tγ 1–67, the biotinylated target peptide showed a small peak on HPLC (content of the desired peptide <2%, Fig. 5C) with several large peaks. After purification with the avidin column, the target peptide was isolated as a major component (content of the desired peptide ≈ 65%). The result of ionspray MS analysis of the obtained peptide was identical with the theoretical value of 8052.1 (average mass) (average mass calculated for $C_{354}H_{584}N_{91}O_{113}S_4$, 8050.3), and the amino acid sequence analysis of the N-terminal portion (15 cycles from the NH_2-terminus) of the peptide obtained was correct.

These data demonstrated that the proposed method is very useful for separating desired products even at low concentrations from the major part of the non-biotinylated impurities. However, with increase in peptide length, the purity of the recovered peptide decreased owing to a loss of capping efficiency (purity of the final products = 87–65%, Fig. 5). Unfortunately, this method is not effective in purifying peptides that are chemically modified during the synthesis or the final deprotection.

In solid-phase peptide synthesis, it is very common to find many undesired products with random single or multiple point deletions and with very similar chromatographic and isoelectric properties. Therefore, the establishment of an effective technique to purify solid-phase synthetic products is strongly desired. One of the shortfalls of conventional chromatographic purification is that it is troublesome to remove terminated peptides or to isolate the desired peptide when the synthetic yield is very low. The new reversible biotinylating reagent, however, can offer a new type of biotin–avidin affinity chromatography to purify (or isolate) solid-phase synthetic products.

The great advantages of the method are its simplicity and rapidity. It should enable high-purity or constant-purity peptides to be produced, which will be useful in research in the fields of biochemistry, physiology and medicine with respect to the physiological activities and action mechanisms of peptides and proteins.

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