

FAST AND EFFICIENT PURIFICATION OF SYNTHETIC PEPTIDES BY SOLID-PHASE EXTRACTION

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SUMMARY

Post-synthesis purification of peptides is often the most difficult part of the synthetic procedure. In this study we describe the purification of two synthetic antimicrobial peptides (calcitermin and temporin A) by solid-phase extraction (SPE). Our results indicate that SPE is a rapid, inexpensive, and relatively efficient method for post-synthetic purification of synthetic peptides.

INTRODUCTION

Purification of peptides is often the most difficult part of the process used to obtain the compounds [1]. Several methods are used for isolation and subsequent purification of the post-synthesis mixture. Depending on the properties of the peptide and the types of impurity, commonly used techniques are ion-exchange chromatography and gel filtration [2,3]. The standard method for isolation is high-performance liquid chromatography [4,5], which enables separation of substances with negligible differences between their chemical structures. Unfortunately, the need for specialised equipment and columns and high buffer consumption makes this method relatively expensive.

Solid-phase extraction (SPE) is a method originally developed for concentration of synthetic, biological, and environmental samples before analysis by conventional methods [6]. SPE can also have other applications, for example removing impurities from samples and isolation of analytes from biomatrices [7]. This method is essentially a short chromatographic separation with a variety of the applications. Four general extraction me-

chanisms are used in SPE; these involve non-polar, polar, ion-exchange, and covalent interactions [8]. SPE has been reported to have advantages over other methods of extraction because of its speed, reproducibility, and efficiency and because emulsions are not formed [9]. Good results can be obtained when SPE is used as a purification method for synthetic peptides. The use of SPE for determination and quantitative measurement of two nano-peptides in fish plasma was described by Kulczykowska [10] and we have reported the use of SPE for purification of the 33-amino acid peptide statherin SV2 [11].

In this paper we describe the synthesis and subsequent SPE-based purification of two antimicrobial peptides, calcitermin (VAIALKAAHYHTHKE) and temporin A (FLPLIGRVLSGIL-NH₂). We show, that either large (200 mg) or small (20 mg) quantities of peptides can be purified by SPE and that this fast, one-step procedure furnishes products of >95–97% purity. There is, moreover, no need for sophisticated equipment and consumption of mobile phase is minimal. We postulate that use of SPE could be especially beneficial in the isolation of peptides from post-reaction mixtures or concentration of substances (e.g. after disulfide bond formation).

EXPERIMENTAL

Starting Materials

N^α-9-fluorenylmethyloxycarbonyl amino acids, trifluoroacetic acid (TFA), 1,3-diisopropylcarbodiimide (DIC), diisopropylethylamine (DIEA), 4-dimethylaminopyridine (DMAP), piperidine, *N*-hydroxybenzotriazole (HOBt), Triton, triisopropylsilane (TIS), and *O*-(benzotriazol-1-yl)-*N,N,N,N*-tetramethyluronium tetrafluoroborate (TBTU) were obtained from Fluka (Switzerland), and dimethylformamide (DMF), *N*-methyl-2-pyrrolidone (NMP), dichloromethane (DCM), and acetonitrile from Lancaster (Germany). All solvents and reagents used for solid-phase synthesis were of analytical grade. Wang resin for calcitermin synthesis (1% DVB, 1.0 mmol g⁻¹) was obtained from Fluka. Polystyrene AM RAM resin for temporin A synthesis (1% DVB, 0.7 mmol g⁻¹) was from Rapp Polymere (Germany). Kromasil C₈ (5-μm particle size, 100-Å pore size) was purchased from Eka Chemicals AB (Bohus, Sweden).

Synthesis of the Peptides

The peptides were synthesized manually on the 0.1 millimole scale by a solid-phase method using the Fmoc/Bu^t procedure [12]. The side-chain-protecting groups of the amino acids were trityl for His; *tert*-butyl ether for Tyr, Ser, and Thr; *tert*-butyl ester for Glu; Pbf for Arg; and Boc for Lys. Attachment of the first residue to the Wang resin was achieved by DMAP-catalysed esterification [13].

Both peptides were synthesized using the Fmoc method, according to a two-step procedure:

1. 5- and 15-min deprotection steps using 20% piperidine in a 1:1 (v/v) mixture of DMF and NMP (in the presence of 1% Triton); and
2. coupling reactions performed with the protected amino acid diluted in a 1:1 (v/v) mixture of DMF and NMP in the presence of 1% Triton, using DIC as the coupling reagent in the presence of HOBt (Fmoc-AA:DIC:HOBt 1:1:1) for 1 h.

The completeness of each coupling reaction was monitored by means of the chloranil test [14]. If the test was positive, the coupling reaction was repeated using TBTU and HOBt in the presence of DIEA, with mixing for 2 h.

The protected peptidyl resins were treated with a mixture of 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% TIS for 1 h. After cleavage the solid support was removed by filtration and the filtrate was concentrated under reduced pressure. The cleaved peptide was precipitated with diethyl ether and lyophilized.

Purification of Peptides

Peptides were purified by solid-phase extraction (SPE). The SPE columns were prepared by transferring 2.5 g sorbent to a 20-mL plastic polypropylene syringe or 0.5 g to a 5-mL syringe; both syringes were equipped with porous disks at the bottom and top. The adsorbent was Kromasil C₈, 5- μ m particle diameter, 100-Å pore size. The sample was forced through the column by use of a vacuum flask and rubber stopper, with the vacuum pulling the solution through the SPE column. Finally, the solution was collected in a test tube located inside the flask.

Purification of Calcitermin

Before use the column was conditioned by washing with 2 \times 10 mL acetonitrile, 2 \times 10 mL acetonitrile–water (1:1), and 2 \times 10 mL water–

0.1% trifluoroacetic acid. A 10-mL sample of a stock solution (1–2% acetonitrile) of the crude peptide (213 mg) was transferred to a prewashed SPE cartridge. After washing with water (10–20 mL) the peptide was eluted from the cartridge with 5–8 mL of solutions of 5, 6, 7, or 8% acetonitrile in water in the presence of 0.1% TFA (Fig. 1).

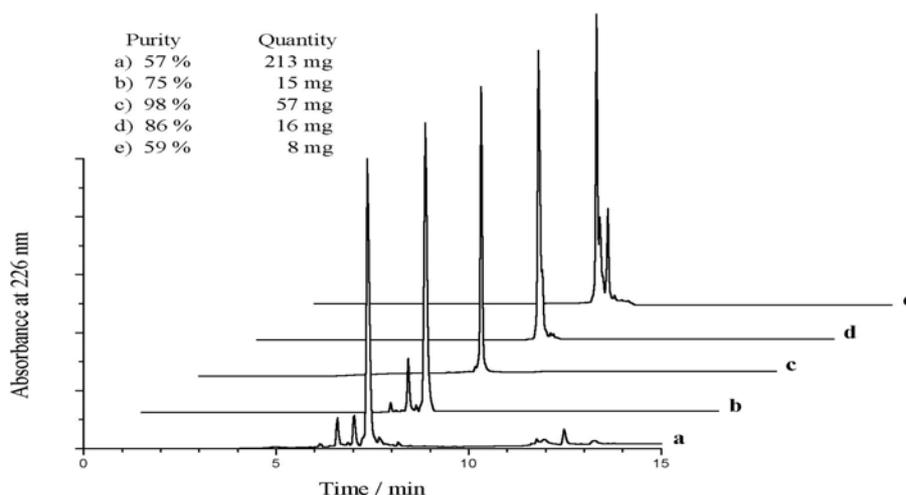


Fig. 1

Reversed-phase (RP) HPLC chromatograms (gradient 10–70% acetonitrile in 0.1% TFA) of crude calcitermin (a) and samples eluted from the cartridge (2.5 g sorbent) with 5% (b), 6% (c), 7% (d), and 8% (e) acetonitrile containing 0.1% TFA in water

Purification of Temporin A

Before use the column was conditioned by washing with 2×3 mL acetonitrile, 2×3 mL acetonitrile–water (1:1), and 2×3 mL water–0.2% trifluoroacetic acid. A 2-mL sample of a stock solution (10% acetonitrile) of the crude peptide (20.4 mg) was transferred to a prewashed SPE cartridge. After washing with aqueous acetonitrile (10–20%) the peptide was eluted from the cartridge with 2–3 mL of solutions of 25, 26, 27, or 28% acetonitrile in water in the presence of 0.2% TFA (Fig. 2).

Finally, the columns were washed with acetonitrile. The cartridges were not allowed to dry during the purification and the flow of eluent was such that 1 mL liquid passed through the cartridge in approximately 15–20 s. Collected fractions were evaporated and lyophilized.

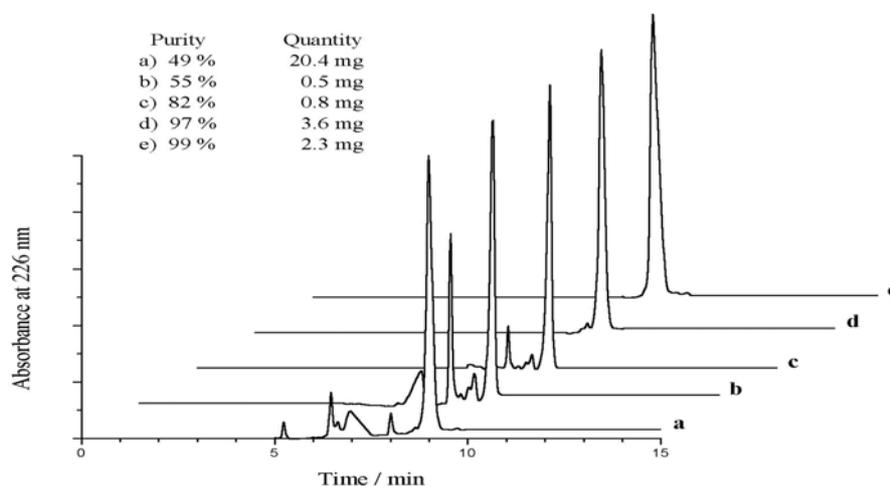


Fig. 2

Reversed-phase (RP) HPLC chromatograms (gradient 35–80% acetonitrile in 0.1% TFA) of crude temporin A (a) and samples eluted from the cartridge (0.5 g sorbent) with 25% (b), 26% (c), 27% (d), and 28% (e) acetonitrile containing 0.2% TFA in water

Analytical Procedure

The purity of the fractions was tested by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Beckman Gold System chromatograph and a 4.6 mm × 150 mm, 5- μ m particle diameter, 100- \AA pore size, Kromasil C₈ column. The mobile phases were gradients prepared from 0.1% TFA in acetonitrile (solution A) and 0.1% aqueous TFA (solution B). For calcitermin the gradient was 10–70% A in 15 min; for temporin the gradient was 35–80% A in 15 min. The flow rate was 1.2 mL min⁻¹. The presence of peptides was confirmed by the absorption peak at 226 nm.

The peptides were characterized by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and had the expected molecular weights.

RESULTS AND DISCUSSION

The aim of our experiments was to obtain highly purified peptides relatively quickly at minimal cost. Calcitermin and temporin A were purified with a cartridge prepared in this laboratory filled with an adsorbent

commonly used in HPLC. To purify 213 mg crude (57%) calcitermin we used a cartridge filled with 2.5 g adsorbent. The procedure furnished 57 mg highly pure (98%) peptide. The quantities and purity of peptide in other fractions are depicted in Fig. 1. To purify 20.3 mg crude (49%) temporin A, 0.5 g adsorbent was used. The procedure resulted in 5.9 mg pure peptide (Fig. 2). Although particular fractions were analysed by HPLC, it can be difficult to determine the appropriate eluent concentration because peptides are eluted at an acetonitrile concentration approximately 10–15% lower than in HPLC. The best experimental results were obtained when appropriate concentrations were established using a small SPE cartridge and a small quantity of peptide. On the basis of extrapolation we can predict the method which is also suitable for obtaining larger samples. The method is rapid and effective. The true advantage was that the actual time spent on the purification of these peptides was approximately 5 min.

Effective separation is possible when adsorbent with an appropriately small particle diameter (5 μm) is used. Commercially available cartridges (adsorbent particle diameter $>10 \mu\text{m}$) were not good enough for effective purification (data not shown). They are, however, suitable for desalting of peptides or preliminary purification [15].

Results better than those presented here could probably be obtained by use of finer adsorbent and thinner but longer cartridges. The only factor limiting separation is hindrance of flow in the cartridge, because relatively low pressure is applied (in our experiments negative pressure was generated by means of a water pump).

SPE is an easy and inexpensive means of purification or separation of peptides [16]. It enables rapid and efficient purification of peptides with minimal consumption of organic solvents and eliminates the need for sophisticated equipment. Good results depend on use of adsorbent with an appropriate low particle diameter ($<10 \mu\text{m}$). The procedure can furnish pure peptide after one purification cycle only.

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