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Development of simultaneous purification methodology for multiple synthetic peptides by reversed-phase sample displacement chromatography☆

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Abstract

We have developed a low-pressure protocol, designed as a rapid, simple and cost-effective procedure for the efficient and parallel purification of multiple peptide mixtures. This was achieved through adaptation of our novel reversed-phase sample displacement chromatography (SDC) method, where the major separation process takes place in the absence of organic modifier, to modular solid-phase extraction (SPE) technology. Thus, crude peptide sample is applied at overload conditions to extraction columns consisting of SPE tubes containing silica-based reversed-phase packing. By applying a vacuum to draw the solution through the packing, product separation from hydrophobic and hydrophilic impurities is accomplished in a two-stage purification unit: a short pre-column functions as a trap for hydrophobic impurities, while a second, longer SPE column is used as a product isolation column. Thus, under ideal SDC conditions, washing with a 100% aqueous solvent will achieve retention of hydrophobic impurities on the trap, with displacement of product and hydrophilic impurities from the trap to the product isolation column; hydrophilic impurities are thus displaced off the product isolation to waste, leaving only product retained on the main column. In this initial evaluation, this purification system has demonstrated excellent separation of product, in good yield, from both hydrophilic and hydrophobic impurities over a wide range of peptide hydrophobicity and crude composition for model synthetic peptide systems representing crude peptide mixtures.

Keywords: Sample displacement chromatography; Preparative chromatography; Peptides

1. Introduction

The expanding utility of synthetic peptides in a wide range of biological and medical applications emphasizes the importance of bringing multiple peptide synthesis to general laboratory use. The requirement for a multiple synthesis approach, due to the inability of serial synthesis to meet the demand for large numbers of peptides, e.g., for immunologi-

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cal screening, in a reasonable time frame, necessitated the development of alternate methods based on parallel (i.e., simultaneous) synthesis. Such an approach may range from the application of multitasking robotics to peptide synthesis [1–3], resulting in costly automated multiple peptide synthesizers, to minimalistic and much less costly instrumentation, such as the semi-automated 100-well synthesizer recently developed in the authors' laboratory [4]. As would perhaps be expected, the development of efficient multiple peptide synthesis has resulted in a demand for equally rapid and efficient peptide purification.

While an efficient peptide synthesis generates only a small number of peptide impurities, the resulting deletion, termination and/or chemically modified peptides usually possess structures closely related to the peptide of interest and thus pose difficult purification problems. These problems would be magnified significantly when requiring rapid purification of large numbers of synthetic peptides routinely generated for such purposes as, for example, peptide–receptor interaction studies. Indeed, the attainment of the necessary purity for multiple synthetic peptides prior to their application is likely the rate-limiting step of the entire process. While the last decade has seen the development of high-performance liquid chromatography (HPLC) in which peptide and protein separations are accomplished in under 5 min [5–9] or even $<$20 s (ultrafast HPLC) [10–13], this technology has been designed specifically for on-line analysis of peptide or protein syntheses and is less appropriate for rapid peptide purification, since the need for tedious fraction collection and analysis remains and the reduction in actual separation time becomes less significant as the number of peptides undergoing purification rises.

Clearly, in a similar manner to multiple peptide synthesis, there is a need for simultaneous multiple peptide purification rather than for improvement in the conventional serial approach, a considerable challenge when one considers combining the need for operational simplicity and cost-effectiveness with rapid purification of large numbers of crude peptides produced in varying yield and displaying a wide range of overall hydrophobicity. To this end, we set out to develop further our earlier work on preparative reversed-phase sample displacement chromatography (SDC) of peptides, a novel aspect of which is the main separation process taking place in the absence of organic modifier [14–17]. Specifically, the present study describes our initial development and evaluation of a low-pressure, multi-peptide SDC protocol, designed as a rapid, facile and cost-effective procedure for the efficient purification of multiple peptide mixtures.

2. Experimental

2.1. Materials

HPLC-grade water and acetonitrile were obtained from BDH (Poole, UK). Trifluoroacetic acid (TFA) was obtained from Aldrich (Milwaukee, WI, USA). Disposable solid-phase extraction (SPE) tubes, adapters and polyethylene frits were obtained from Chromatographic Specialties (Brockville, Canada). Reversed-phase bulk C_8 packing was obtained from Rainin (12 μm particle size, 300 Å pore size; Emeryville, CA, USA).

2.2. Instrumentation

A 24-position vacuum-operated VacElut SPS24 SPE sample processing station, equipped with stainless steel sample delivery tips and a 13×100 mm test tube collection rack was obtained from Analytichem International (Harbor City, CA, USA). Analytical reversed-phase (RP) HPLC runs were carried out on a Hewlett-Packard (Avondale, PA, USA) HP1090 (Series II) liquid chromatograph, coupled to a HP1040A detection system, a HP9000 Series 300 computer, a HP9123 disc drive, a HP2225A Thinkjet printer and a HP7440A plotter.

2.3. Analytical RP-HPLC

Analysis of peptide samples and fractions was carried out on a Zorbax 300SB-C_8 reversed-phase column (150×4.6 mm I.D., 5 μm particle size, 300 Å pore size; Hewlett-Packard, Little Falls Site, DE, USA), using a linear A–B gradient (0.5% B/min), at a flow-rate of 1 ml/min, where eluent A is 0.05%

aqueous TFA and eluent B is 0.05% TFA in acetonitrile.

2.4. Peptide synthesis

Three series of five peptide standards (Fig. 1) were synthesized on the multiple peptide synthesizer described in Ref. [4], using standard protocols of 10-fold 9-fluorenylmethoxycarbonyl (Fmoc)-amino acid excess and coupling times of 60 min.

3. Results and discussion

3.1. Development of multiple peptide purification system

We perceived the requirements of a feasible multiple purification system to be five-fold: (1) it should be rapid, straightforward and inexpensive; (2) there should be minimal instrumentation (no pumps, injectors, etc.); (3) the system should maximize resolution; (4) sample load should also be maxi-

SDC set	Standard	Sequence
GK	I1	Ac-Lys-Gly-Val-Gly-Gly-Lys-Gly-Gly-Val-Gly-Lys-amide
	I2	Ac-Lys-Gly-Val-Gly-Gly-Lys-**Ala**-Gly-Val-Gly-Lys-amide
	P	Ac-Lys-Gly-Val-Gly-**Ala**-Lys-**Ala**-Gly-Val-Gly-Lys-amide
	I3	Ac-Lys-Gly-Val-Gly-**Ala**-Lys-**Ala-Ala**-Val-Gly-Lys-amide
	I4	Ac-Lys-Gly-Val-**Ala-Ala**-Lys-**Ala-Ala**-Val-Gly-Lys-amide
GI	I1	Ac-Lys-Gly-Val-Gly-Gly-Ile-Gly-Gly-Val-Gly-Lys-amide
	I2	Ac-Lys-Gly-Val-Gly-Gly-Ile-**Ala**-Gly-Val-Gly-Lys-amide
	P	Ac-Lys-Gly-Val-Gly-**Ala**-Ile-**Ala**-Gly-Val-Gly-Lys-amide
	I3	Ac-Lys-Gly-Val-Gly-**Ala**-Ile-**Ala-Ala**-Val-Gly-Lys-amide
	I4	Ac-Lys-Gly-Val-**Ala-Ala**-Ile-**Ala-Ala**-Val-Gly-Lys-amide
LI	I1	Ac-Lys-Leu-Val-Gly-Gly-Ile-Gly-Gly-Val-Gly-Lys-amide
	I2	Ac-Lys-Leu-Val-Gly-Gly-Ile-**Ala**-Gly-Val-Gly-Lys-amide
	P	Ac-Lys-Leu-Val-Gly-**Ala**-Ile-**Ala**-Gly-Val-Gly-Lys-amide
	I3	Ac-Lys-Leu-Val-Gly-**Ala**-Ile-**Ala-Ala**-Val-Gly-Lys-amide
	I4	Ac-Lys-Leu-Val-**Ala-Ala**-Ile-**Ala-Ala**-Val-Gly-Lys-amide

Fig. 1. Sequences of SDC synthetic peptide standards. Amino acids are denoted by their three-letter codes; Ac- and amide denote N^{α}-acetyl and C^{α}-amide, respectively. I1, I2 represent hydrophilic impurities within peptide sets; P denotes product; I3, I4 represent hydrophobic impurities within peptide sets. Variations in sequence of the standards within each peptide set are in bold; variations in sequence between peptide sets are boxed.

mized; and (5) the number of fractions should be minimized. Our approach to satisfying the above requirements involved the adaptation of reversed-phase SDC to the operating constraints of a simultaneous purification scheme.

3.1.1. Principles of SDC

Conventional reversed-phase SDC was designed as a novel method for highly efficient, preparative-scale purification of peptides on high-performance analytical columns and instrumentation [14–17]. Since peptides favour an adsorption–desorption method of interaction with a hydrophobic stationary phase [18,19], under normal analytical load conditions an organic modifier is typically required for their elution from a reversed-phase column. However, when such a column is subjected to high loading of a peptide mixture dissolved in a 100% aqueous mobile phase, there is competition by the sample components for the adsorption sites on the reversed-phase sorbent, resulting in solute–solute displacement during washing with 100% aqueous mobile phase. A more hydrophobic peptide component competes more successfully for these sites than a less hydrophobic component, which is thus displaced ahead of the more hydrophobic solute, i.e., the sample components act as their own displacers. Once the sample displacement process is complete by washing with 100% aqueous mobile phase, the hydrophilic impurities have been washed off the column. The hydrophobic trap is dissociated from the product isolation column, thus removing the hydrophobic impurities. Product retained by the product isolation column (which contains only product) is now eluted with aqueous organic eluent. Aqueous organic eluent is required only to wash retained components off the product isolation column and makes no contribution to the major separation process. The SDC approach is thus simply application of the well-established general principles of displacement chromatography [20–22] without the need for a separate displacer.

This mode of operation, a hybrid scheme of frontal chromatography followed by elution, is characterized by a marked reduction in solvent consumption, minimal elution volumes, and the collection of fewer fractions for product isolation than in conventional RP-HPLC, with consequent reductions in time and handling.

3.1.2. Adaptation of SDC to simultaneous purification

Successful adaptation of SDC to a simultaneous peptide purification scheme requires maintenance of an equal flow distribution, a complex proposition when operating parallel multiple columns at high pressure; hence, a low-pressure operation seemed desirable for simplicity and reliability.

Since practical application of SDC to such an operation is possible only if its benefits are retained under low-pressure conditions, a low-cost alternative to standard RP-HPLC columns, with concomitant high back pressures, is required. We believed that the application of SPE technology would readily satisfy such a requirement. Thus, SPE offers a variety of bonded-phase, silica-based packings (including reversed-phase) to extract contaminants or analytes of interest from liquid solution. In addition, disposable polypropylene tubes containing such packings are designed to operate as extraction columns under low-pressure conditions where sample solutions are passed through using either positive pressure or partial vacuum, and are ideally suited for adaptation to a low-pressure version of SDC.

3.1.3. Design and performance objectives

In the purification system design presented in Fig. 2, left, peptide purifications are performed in extraction columns consisting of disposable SPE tubes containing silica-based reversed-phase packing. A low-pressure version of SDC is achieved by adding crude peptide sample at overload conditions and applying vacuum to draw the solution through the packing. Product separation from hydrophobic and hydrophilic impurities is accomplished in a two-stage purification unit which takes advantage of the two SPE operational modes: a short pre-column is used in a clean-up application, functioning as a trap for hydrophobic impurities, and a second longer column is used in a concentration application, functioning as a product isolation column. A more detailed illustration of this two-stage purification unit is shown in Fig. 2, right.

Crude peptide sample is loaded onto the purification unit in 100% aqueous solvent at overload

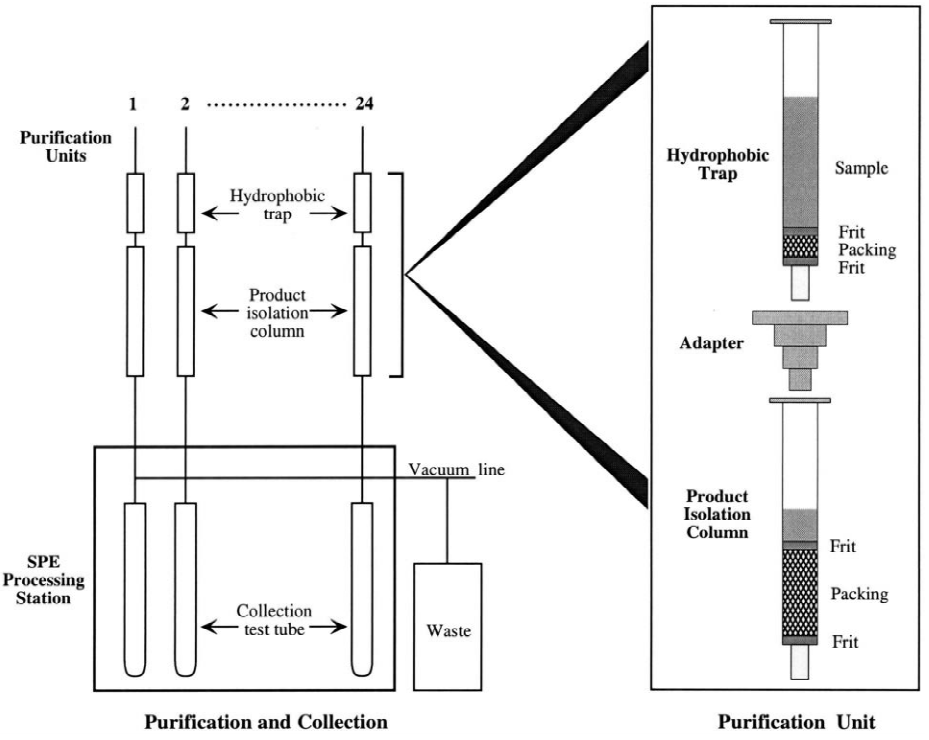

Fig. 2. Left: Schematic representation of multiple peptide purification system; right: detailed schematic representation of two-stage purification unit.

conditions to achieve sample displacement operation, and is washed through trap and product isolation column in sequence. Under ideal SDC conditions, hydrophobic impurities are retained on the trap; product and hydrophilic impurities are displaced from the trap to the product isolation column; and hydrophilic impurities are displaced off the product isolation column to waste: only product is retained on the main column. The product is subsequently removed from this column by isocratic elution with organic modifier, and is recovered from solution by lyophilization. This arrangement reduces the extensive fraction collection and analysis required by analytical RP-HPLC for product isolation to an absolute minimum: only three ''fractions'' exist, namely the hydrophobic trap, the product isolation column, and the waste wash, and only the retained peptide on the product isolation column is collected. Simultaneous multiple purifications are carried out by mounting tubes on a standard SPE processing station connected to a laboratory vacuum line. Lacking pumps, injectors, solvent mixing systems and the like, this purification system design uses minimal instrumentation compared with RP-HPLC, and avoids the need for high-pressure containment.

The major challenge faced by this multiple peptide purification system lies in the variable nature of the crude peptides undergoing purification. Peptides show significant variation in overall hydrophobicity, and may be synthesized at various scales and exhibit a range of purity. For routine operation of a simultaneous purification system, it is necessary to use a standardized purification unit to achieve a specified product purity for a range of peptide hydrophobicities, crude loads and impurities. Thus, for these initial studies, the development of a standardized purification unit (Fig. 2, right) was based on sample loads of up to 10 mg in solution volumes of 1–5 ml, leaving larger loads to be handled by additional runs. Following the rule of thumb that sample size should be 5% of the packing bed (i.e., a 100 mg bed should retain 5 mg of sample), a 1-ml SPE tube designed to

hold 100–150 mg of reversed-phase packing and 1 ml sample volume was selected for use as both hydrophobic trap and product isolation column: the stackable nature of these tubes, via connection by means of an adapter (Fig. 2, right), ensures a ''building block'' approach to development, should larger columns be required, and larger solution volumes could be accommodated using 20-ml sample reservoirs. Purification units could then be mounted on an SPE processing station (see Experimental).

Performance objectives of the two-stage purification unit shown in Fig. 2, right, are based on sacrificing some product yield to ensure product purity. The purification unit is designed to handle sample loads of up to 10 mg crude peptide and to recover at least 80% of the product, with no more than 10% losses to each of the hydrophobic trap and waste wash and the corresponding removal of essentially all hydrophobic and hydrophilic impurities in these respective fractions.

3.2. Purification unit packing and operational procedures

Preparation of the two-stage purification unit entails packing and conditioning of the column and assembly of the unit, while unit operation proceeds in discrete stages of sample loading, washing and elution. As described below, a manual packing procedure was developed for SPE tubes to produce trap and product isolation columns of variable packing bed length. Conditioning, sample loading and elution procedures are drawn from SPE methodology, with adaptations from SDC in terms of solvent selection: sample loading, separation and washing are performed with water, while elution off the packings is carried out with 50% aqueous acetonitrile (the level of organic modifier chosen to ensure recovery of all peptides from trap and column). The procedures described below were developed through application of a three-peptide mixture [sequences: Ac–Arg–Gly–X–X–Gly–Leu–Gly–Leu–Gly–Lys–amide, where X–X is Gly–Gly (S2), Val–Gly (S4) or Val–Val (S5), where S4 represented product and S2 and S5 represented various levels of hydrophilic and hydrophobic impurities, respectively].

3.2.1. Choice of reversed-phase packing

Compared with typical silica-based RP-HPLC packings used for peptide/protein separations (characterized by a 300 Å pore size and a particle size range of 5–20 μm [18,19]), RP-SPE packings possess a larger particle size (to enable faster drainage under positive pressure or partial vacuum conditions) and a smaller pore size (suitable for typical applications involving the extraction of smaller organic molecules). For the present study, the peptide capacities of a selection of RP-HPLC packings and a RP-SPE packing were compared by loading 5–7 mg of synthetic peptide standard S5 onto single-column purification units containing 100 mg of each packing, washing the columns with a single tube volume (i.e., 1 ml) of 0.05% aqueous TFA, and eluting retained peptide with a single tube volume of 0.05% TFA in 50% aqueous acetonitrile. As expected, particle size had a noticeable effect on sample flow-rate through the column (data not shown), but the SPE processing station was able to compensate for this effect by increasing the vacuum to maintain a constant flow-rate of 0.7 ml/min, slightly below the standard 1 ml/min generally used for analytical HPLC applications [18,19]. The Rainin packing was retained for further developmental work on the basis of highest capacity under the given conditions, available supplies, and direct comparison with analytical RP-HPLC purification (since, of the RP packings tested, only this packing fell into the normal particle size range characteristic of general analytical RP-HPLC applications.

3.2.2. Abbreviations and terminology

Column size is reported as mg packing, since constant column (i.e., SPE tube) diameter is maintained. Wash and elution volumes are normalized as numbers of trap volumes (V_{trap}) and column volumes (V_{col}) to account for potential differences in scale, based on the total volume of the respective solvated packing beds (rather than the respective void volumes). In addition, V_{tube} represents the total volume of an SPE tube, while V_{unit} represents the combined volume of the trap and column packing bed ($V_{trap} + V_{column}$). This simple and rapid approach avoided the need to measure void volumes of individual columns by using a packing volume constant (estimated as

205 μl/100 mg solvated Rainin packing in 0.05% aqueous TFA) and provided sufficient accuracy considering the variation encountered in manually packed columns.

Peptide yields (reported as %) of peptide recovered were calculated based on integrated peak areas following analysis of peptide fractions as described in Experimental.

3.2.3. Preparation of purification unit

3.2.3.1. Packing

A bottom frit is inserted into the SPE tubes (representing the trap and isolation columns). The desired quantity of dry packing is added to each tube, followed by washing with isopropanol to form a bed. Top frits are then inserted to restrain the packing beds.

An alternative packing approach is to wet pack, where the desired amounts of packing are suspended in 1 V_{tube} of isopropanol. The isopropanol is then drained under minimal vacuum, retaining 1 mm of liquid above the top frit to avoid draining the bed. The beds are manually compressed to remove air pockets and distribute the packing evenly.

3.2.3.2. Conditioning

One V_{tube} of 0.05% TFA in acetonitrile is now applied to establish the solvent layer, followed by 1 V_{trap} or 1 V_{col} of 0.05% aqueous TFA to equilibrate the two columns. A volume of 350 μl liquid is retained above the product isolation bed, while 1 mm of liquid is retained above the trap packing bed.

3.2.3.3. Linking

Following equilibration, the two tubes are linked by an adapter (Fig. 2, right). The resulting two-stage purification unit is now ready for operation.

3.2.4. Operation of purification unit (Fig. 3)

3.2.4.1. Sample loading

The sample is loaded in 0.05% aqueous TFA and carefully layered, by pipette, on top of the liquid layer of the hydrophobic trap. The sample is then drawn under vacuum (at a flow-rate of 0.7 ml/min under the system employed) into the trap packing, retaining 1 mm of liquid above the packing bed (Fig. 3).

3.2.4.2. Washing

The purification unit is washed with a minimal volume (discussed later) of 0.05% aqueous TFA, followed by disconnection of the trap from the product isolation column. At this stage, all hydrophobic impurities should be retained in the hydrophobic trap, while the desired product and a proportion of any hydrophilic impurities are retained on the product isolation column having been displaced from the hydrophobic trap.

An optimal wash (discussed later) of the product isolation column with 0.05% aqueous TFA now serves to displace hydrophilic impurities to waste, leaving only the desired product on the column. A small amount (1 mm) of liquid is retained above the packing (Fig. 3).

3.2.4.3. Elution

A minimal volume of 50% aqueous acetonitrile containing 0.05% TFA is now used to elute the purified product from the product isolation column. The column is drained completely. However, the packing beds should not be allowed to dry out between stages of operation.

Following elution of hydrophobic impurities from the hydrophobic trap through washing with 50–80% aqueous acetonitrile (depending on the level of acetonitrile required to elute such impurities, a value which can be assessed from an analytical gradient elution profile obtained prior to purification), both the hydrophobic trap and product isolation columns are re-conditioned as described above (conditioning) and linked together once more. The purification unit is now ready for another round of purification (Fig. 3).

3.3. Validation of multiple peptide purification system

As previously noted, the major challenge to multiple peptide purification is the variable nature of the synthesized crude peptides, characterized by a range of hydrophobicity, degree of purity, and scale of synthesis (i.e., sample load). Although not shown here, the latter problem was addressed during the

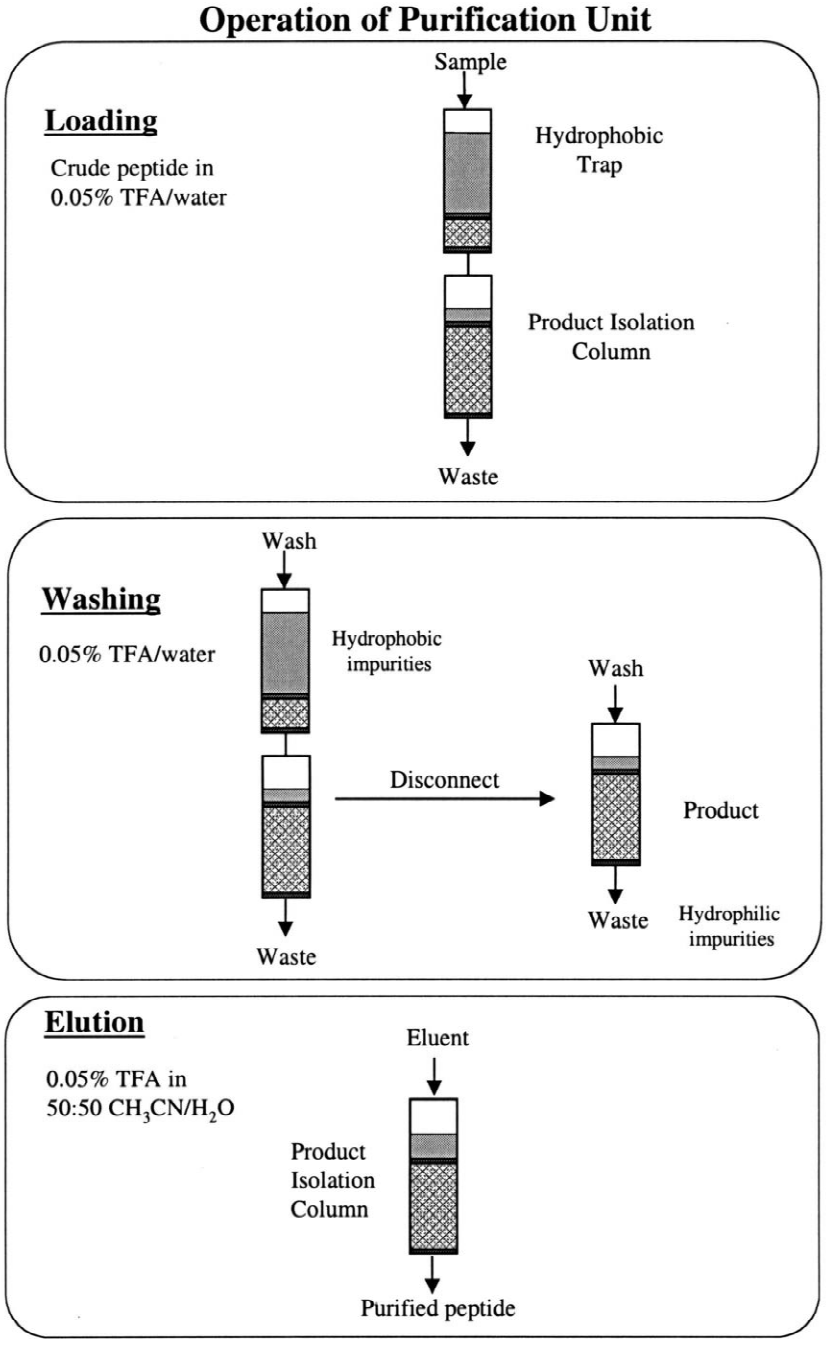

Fig. 3. Operation of two-stage purification unit.

development of the purification system with synthetic peptide standards S2–S5, where a demonstrated ability to scale the purification unit to handle sample load on a linear basis, maintaining a constant trap-to-column ratio, permits the routine use of a protocol with standardized purification unit sizes

accommodating different sample loads. The possibility of dealing in a similar manner with variation in crude purity, by establishing a few standardized trap-to-column ratios for expected ranges of sample impurities, was now investigated during a rigorous validation test of the system with a five-component mixture designed to represent the complexity of a typical crude peptide rather than the relatively simple three-peptide S2–S5 mixture that had been employed thus far for purification system development. The ability to separate peptide product from multiple peptide impurities over a range of hydrophobicities would clearly validate the purification system design. The appropriate variation in hydrophobicity was provided by a series of synthetic peptide standards, specifically constructed to span a wide hydrophobicity range and to serve as a reference set for future protocols.

3.3.1. SDC synthetic peptide standards

Three sets of synthetic peptide standards (denoted GK, GI and LI) were designed to cover a wide range of peptide hydrophobicity, and their sequences are presented in Fig. 1. The hydrophobicity scale upon which these peptide series was designed was the scale of Guo et al. [23], derived from RP-HPLC of synthetic peptide analogues). Within each peptide set there are five components (I1, I2, P, I3, I4) which are very closely related in hydrophobicity, differing only by the addition of one carbon atom with the substitution of an Ala residue for a Gly residue. A mixture of these components is used to represent a complex crude peptide mixture produced by solid-phase peptide synthesis, in which a desired product (P) is contaminated by two hydrophilic impurities (I1 and I2) and two hydrophobic impurities (I3 and I4). Between peptide sets, there is a difference in hydrophobicity range: the GK set incorporates Gly and Lys residues to provide the most hydrophilic set of standards: the GI set substitutes a much more hydrophobic residue, Ile, in place of the Lys residue; and the LI set incorporates two very hydrophobic residues, Leu and Ile, to provide the most hydrophobic set of standards. In addition, two Lys residues are present in all these standards to provide net positive charge and improve peptide solubility. RP-HPLC chromatograms of the three sets are shown in Fig. 4, demonstrating the three distinct hydrophobicity ranges.

3.3.2. Initial performance of purification unit with SDC standards

Initial testing of the purification system was conducted at a 5 mg sample load using a five-component mixture composed of the SDC G1 peptide set (Figs. 1 and 4; Table 1, column A). The sample composition of 80% product (P), 10% hydrophilic impurity (5% each of I1 and I2), and 10% hydrophobic impurity (5% each of I3 and I4) was designed to represent a peptide crude mixture with 80% product, i.e., 25% product contamination by impurities (20% impurities/80% product$\times$100). It should be noted that synthesis of many small peptides ($<$15 residues) does not necessarily produce such a high percentage (25%) of product contamination by closely-related peptide impurities. However, we felt it was important to challenge the purification system with a significant purification problem, e.g., a crude peptide mixture with significant levels of impurities contaminating the product. At first glance, the performance of the 19:125 purification unit (trap and column sizes of 19 mg and 125 mg packing, respectively, chosen from earlier development studies with peptides S2–S5) appeared to reflect an inability to resolve the sample components due to their close hydrophobicities (Table 1, column A; product isolation column). On closer inspection, however, the pattern of separation suggested the need for a larger hydrophobic trap: while adequate to retain all of the most hydrophobic impurity (I4), the trap removed only 48% of the second, less hydrophobic impurity (I3), resulting in extensive contamination of the product isolation column by 52% I3. Although product loss to the trap was expected, the presence of significant amounts of hydrophilic impurities I1 and I2 in the trap (2% and 11%, respectively) indicated that progressive sample displacement of one component by the next, more hydrophobic component was not allowed to reach completion. An almost four-fold increase in trap size to 75 mg packing (approaching the size of the product isolation column) was required to ensure the complete retention of both hydrophobic impurities, as premature I3 loss from the trap in the presence of product persisted with the use of smaller traps (data

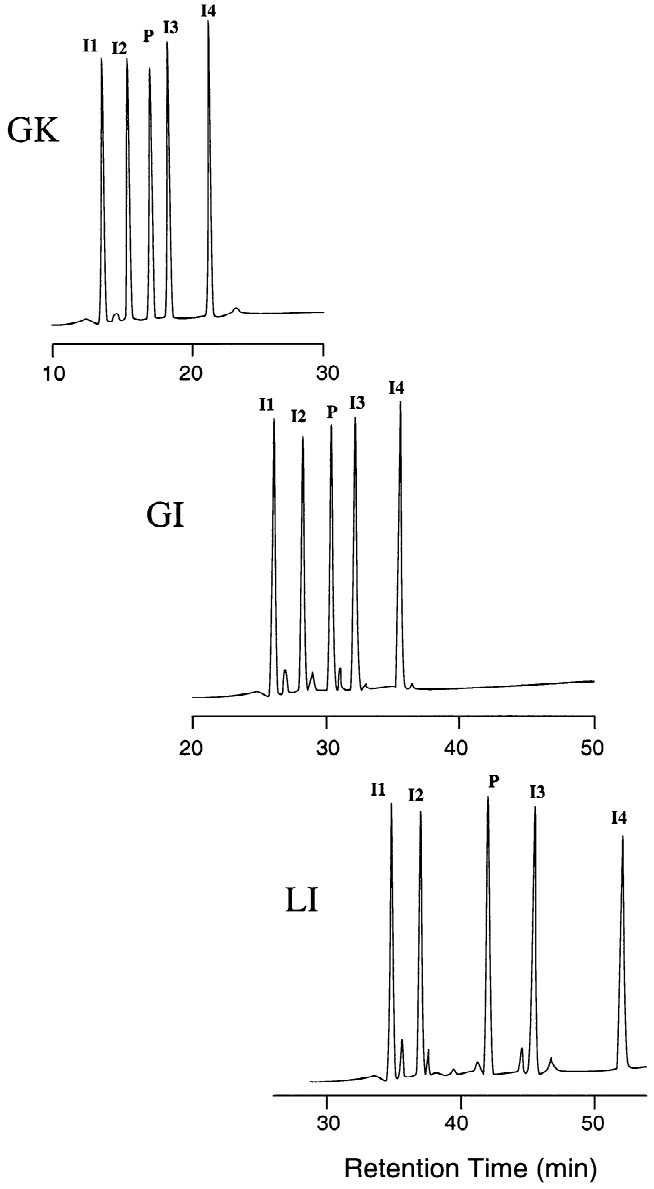

Fig. 4. RP-HPLC elution profiles of SDC synthetic peptide standards. Column, instrumentation and conditions: see Experimental. GK, GI and LI denote peptide sets (see Fig. 1); I1, I2 represent hydrophilic impurities; P denotes product; and I3, I4 denote hydrophobic impurities within each peptide set.

not shown). This substantial increase in trap size reflected the difficulty in resolving product from hydrophobic impurity I3. A similar increase in the trap wash (from 7 V_{trap} to 28 V_{trap}) was necessary to displace all hydrophilic impurities from the hydrophobic trap (data not shown). The elevated wash requirements to clear product from the much larger trap, along with a desire to simplify handling, prompted a slight change in the operating procedure shown in Fig. 3 to apply wash to the entire purifica-

Table 1
Performance of purification unit

Peptide[b] component	A: GI set[a] (preliminary)[c] (%)			B: GI set (optimized)[d] (%)			C: GI set (effect of crude purity)[e] (%)			D: GK set[a] (%)			E: LI set[a] (%)		
	HT[f]	PIC	HF	HT	PIC	HF	HT	PIC	HF	HT	PIC	HF	HT	PIC	HF
I1	2	62	36	0	0	100	0	0	100	0	0	100	0	0	100
I2	11	77	12	0	0	100	0	51	49	0	0	100	0	8	92
P	18	82	0.4	22	65	13	15	85	0	6	70	24	32	68	0
I3	48	52	0	100	0	0	100	0	0	99	0.7	0	100	0	0
I4	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0

[a] Sequence of synthetic peptide standard sets are shown in Fig. 1; representative RP-HPLC elution profiles of peptide standard sets are shown in Fig. 4.

[b] I1,I2 denote hydrophilic peptide impurities, P denotes desired product and I3, I4 denote hydrophobic peptide impurities (see Figs. 1 and 4).

[c] Preliminary performance of purification unit: trap and column sizes of 19 mg and 125 mg packing, respectively; sample load, 5 mg; a ratio (I1:I2:P:I3:I4) of 1:1:16:1:1 represents a % product (P) in the crude yield of 80%; trap wash (into column) was 7 V_{trap} (215 μl) and column wash was 114 V_{col} (29 ml) (see Section 3.2 for practical details).

[d] Performance of unit optimized for GI set (see Fig. 5): trap and column sizes of 75 mg and 125 mg packing, respectively; same sample component ratio and sample load as preliminary run; operational procedure has been modified so that the purification unit wash (119 V_{unit} = 49 ml) proceeds through both trap and column; the same trap/column size, sample component ratio sample load and modified operational procedure was also used for the GK and LI peptide sets.

[e] Effect of crude peptide purity: all conditions the same as for the optimized GI run, save for a sample component ratio (I1:I2:P:I3:I4) of 1:1:6:1:1, representing a crude product (P) yield of 60%.

[f] HI, PIC and HF denote, respectively, hydrophobic trap, product isolation column and hydrophilic fraction (see Figs. 3 and 5).

tion unit (trap and column) rather than disconnecting the trap after a minimal wash; the wash volume was normalized as numbers of unit volumes (V_{unit}).

The performance of the intact purification unit after re-optimization of trap size is presented in Table 1, column B, and illustrated in Fig. 5. Using trap and column sizes of 75 mg and 125 mg packing, respectively, the system was able to resolve five components exhibiting very minor differences in hydrophobicity to the extent of ensuring pure product in the product isolation column. Although the original performance objective of 80% product recovery could not be reached with this quite complex mixture, complete removal of hydrophilic and hydrophobic impurities could be achieved by sacrificing sufficient product: 22% product loss in the hydrophobic trap and 13% product loss in the hydrophilic fraction, for a total pure product recovery of 65%.

3.3.3. Effect of crude peptide impurity on purification unit performance

Purification unit performance depends on matching the crude purity to the trap-to-column ratio, as shown in Table 1. Using the SDC GI standard set and maintaining a 5 mg sample load on the 75:125 purification unit, the sample component ratio was changed from 1:1:16:1:1 (i.e., 80% product and 5% each of the hydrophilic and hydrophobic impurities; Table 1, column B) to 1:1:6:1:1 (i.e., 60% product and 10% of each impurity; Table 1, column C); the purification unit wash was unchanged. From Table 1, column C, the trap was sufficiently large to handle a two-fold increase in the total hydrophobic impurity level without overflowing to the product isolation column, but the wash volume used previously (Table 1, column B; Fig. 5) was now insufficient to remove the hydrophilic I2 (51%) from the product isolation column (insufficient product in the column prevented the complete displacement of both hydrophilic impurities). The purification unit was able to remove completely all hydrophobic impurities from the product as well as the most hydrophilic impurity, I1, but 51% of the second hydrophilic impurity, I2, remained to contaminate the product although this contamination now only represents a 10% contamination of the product (compared to a 67% contami-

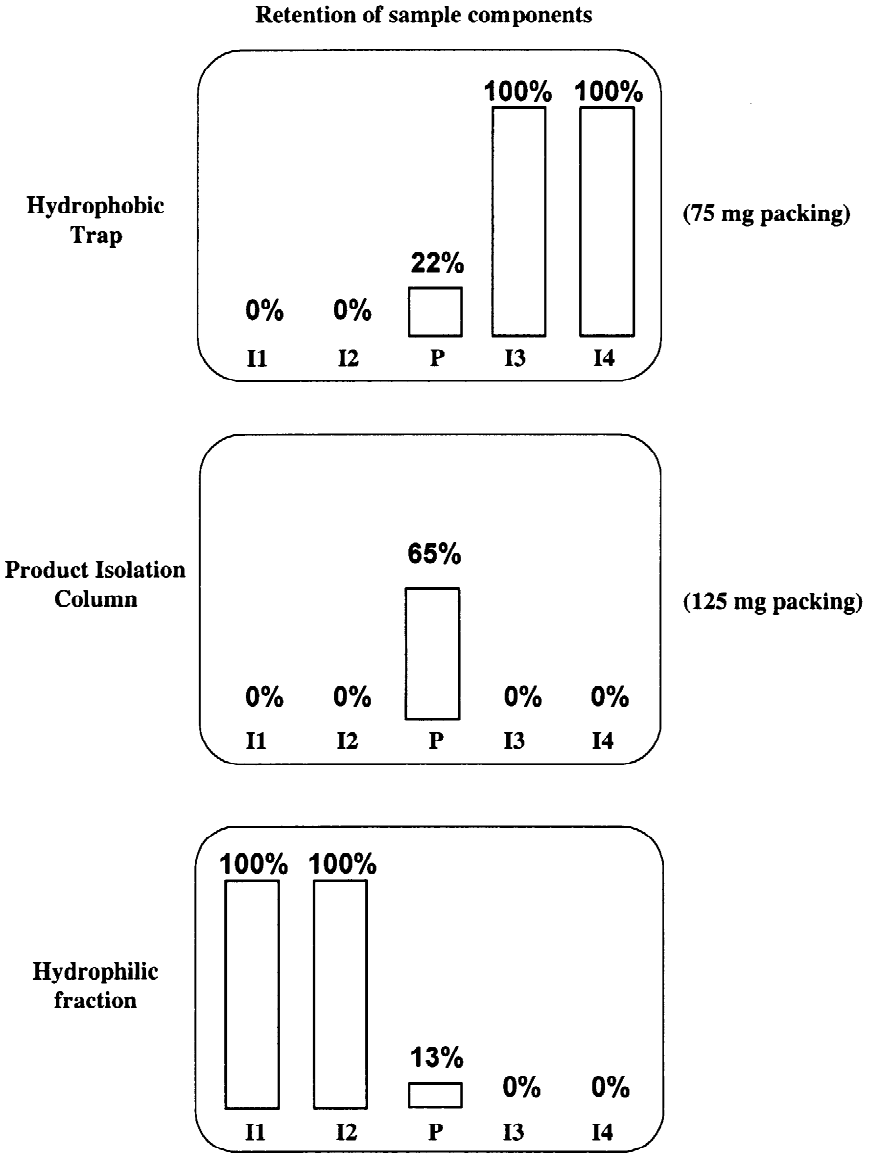

Fig. 5. Performance of purification unit optimized for SDC GI standard set. Sample: 5 mg of SDC GI set (Fig. 1) with a ratio (I1:I2:P:I3:I4) of 1:1:16:1:1, representing a crude product (P) yield of 80%; I1, I2 are hydrophilic impurities and I3, I4 are hydrophobic properties. Operational procedure follows the protocol described in Section 3.2, except for the modification that the purification unit wash (119 V_{unit} = 49 ml) proceeds through both trap and column.

nation of the product in the crude peptide). Even the problem of this lesser contamination could be overcome by continuing to wash the column separately with 0.05% aqueous TFA. Thus, the range of product impurities to be encountered must be set to establish an appropriate trap-to-column ratio and wash volume protocol. Interestingly, these results show that a crude peptide with impurities ranging from 25% (Table 1, column B; Fig. 5) to 67% (Table 1, column C) of product can be purified by a single protocol; all that varies is the yield of pure product due to changes in amounts lost on the hydrophobic trap or

washed off the column into the hydrophilic impurity fraction. These results also emphasize the necessity of estimating the range of purity of the crude peptides; this would allow the future selection of an appropriate trap-to-column ratio and wash protocol from a limited number of procedures for routine use.

3.3.4. Effect of peptide product hydrophobicity range on purification unit performance

A multiple peptide purification system must also be able to handle a range of crude peptide hydrophobicities, and this feature was investigated by applying a 5 mg sample load with a 1:1:16:1:1 component ratio (corresponding to 80% product and 5% of each hydrophilic and hydrophobic impurity) to the purification unit using each of the three SDC standard sets (Figs. 1 and 4). Thus, results presented in Table 1 compare the performance of the purification unit in resolving the more hydrophilic peptide set (GK; Table 1, column D) and more hydrophobic peptide set (LI; Table 1, column E) than the GI set (Table 1, column B), employing the same conditions optimized for GI shown in Table 1 (column B) and Fig. 5. The results shown in Table 1 suggest that these standards could be used as a reference set to adjust washing requirements for the hydrophobicity range of the crude peptide. The performance of the purification unit with the most hydrophobic standard set (LI; Table 1, column E) was lowered by incomplete removal of hydrophilic impurity (I2); however, the 8% I2 remaining on the product isolation column represents a product contamination of only 0.7% (compared to 25% product contamination in the crude peptide, i.e., an impressive 36-fold purification of product). This (albeit, small) contamination of product with I2, reflects the stronger interaction between peptide and packing due to the greater hydrophobicity of the SDC-LI standard set. The advantage of this system is the ability to overcome this problem by increasing the aqueous 0.05% TFA wash volume used during the purification to displace the impurity from the column to the hydrophilic fraction. Increasing the wash through the complete purification unit (trap and column) is possible since considerable product (32%) remained in the trap. Thus, more product would be displaced to the product column to displace I2 and the overall yield of purified product would rise, since considerably more product remained on the hydrophobic trap (32%) compared to the amount of I2 (8%) on the product isolation column.

The performance achieved with the most hydrophilic standard set (GK; Table 1, column D) shows the converse trend: slightly incomplete removal of the hydrophobic impurity I3 from the product and a greater product loss to the hydrophilic fraction (although the overall product recovery is similar to that of the LI set). The separation is still excellent for a considerable change in hydrophobicity: only 0.7% of the hydrophobic I3 impurity escaped the trap to contaminate the product, representing a product contamination of just 0.04%, compared to 25% product contamination in the crude peptide, i.e., an excellent 625-fold purification of product. Even this slight contamination could also be overcome by adjusting the wash volume: a decrease could prevent the displacement of the hydrophobic impurity from the trap and potentially reduce product loss to the hydrophilic fraction.

The results presented in Table 1 concerning effect of peptide purity or hydrophobicity extend the boundaries of the purification system beyond scaling for sample load on the basis of constant trap-to-column ratio in a two-stage purification unit. Thus, variable crude purity can be accommodated by selecting a protocol for an acceptable range of product purity. A range of peptide hydrophobicity (as predicted using hydrophobicity coefficients such as provided by Guo et al. [23]) can be accommodated by comparison with a reference set of SDC standards to select an appropriate protocol with adjusted washing requirements best suited to a group of peptides with a range of hydrophobicity. Standard protocols employing a limited number of standardized trap-to-column ratios and wash volumes can be envisaged, capable of handling the majority of crude peptides produced on a multiple peptide synthesizer.

4. Conclusions

From these initial studies, it can be seen that considerable progress has been made on the development of a simple, rapid and affordable multiple peptide purification system. The development of a

parallel, low-pressure multiple purification system using modular SPE technology and SDC methodology has great potential as an effective and inexpensive alternative to complement multiple peptide synthesis. Equipment costs are a fraction of traditional HPLC columns and instrumentation and SPE processing stations with independent valves to accommodate unequal flow-rates in purification units (preventing dry out of packing) are relatively inexpensive. Adding to these economic benefits of the SDC approach is the earlier demonstration that sample loads allowable in SDC applications are ca. 10-times greater than in comparable gradient elution experiments while maintaining comparable or superior yields of purified peptide product; in addition, while substantial numbers of analyses may be required to identify solute zones in preparative gradient elution, such analyses are minimized in the SDC approach [15]. In the present study, the purification system has demonstrated excellent separation of product from both hydrophobic and hydrophilic impurities over a range of hydrophobicity and crude composition for a model system of synthetic peptide standards. Our results have clearly demonstrated that pure product can be obtained in good yield for a wide range of peptide impurities and differing peptide hydrophobicities; in addition, only two or three operation protocols should be enough to cover all types of crude peptide mixtures. Work is now in progress to test the system with a sizable series of synthetic peptide analogues using a standard protocol based on predicted product hydrophobicity.

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