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PREPARATIVE PURIFICATION OF PEPTIDES BY REVERSED-PHASE CHROMATOGRAPHY

SAMPLE DISPLACEMENT MODE *VERSUS* GRADIENT ELUTION MODE

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SUMMARY

In this study, we describe a novel method of preparative liquid chromatography, applicable to analytical columns and instrumentation, where the reversed-phase column is operated in sample displacement mode (SDM). This technique takes advantage of the different relative hydrophobicities of components of a sample mixture, so that when a column is optimally loaded with an aqueous solution of the sample mixture, there is competition among the sample components for the adsorption sites on the hydrophobic stationary phase. The more hydrophobic components compete more successfully for these sites than more hydrophilic components, which are displaced and immediately eluted from the column. Thus, the major separation takes place in water. Subsequent treatment with an aqueous organic eluent is only required to wash retained components off the column and takes no part in the major separation process. A two-column (precolumn and main column, in series) SDM strategy was applied to the preparative purification of a peptide product from neighbouring hydrophilic and hydrophobic impurities. Hydrophobic impurities were isolated on a shorter precolumn trap while hydrophilic impurities were displaced from the main column in the aqueous mobile phase (0.05% aq. trifluoroacetic acid), leaving the main column filled with homogeneous product. Gradient elution was then used to remove the peptide product from the main column. The researcher can regulate the size of the precolumn trap depending on the amount of hydrophobic impurities in a particular sample, or the size of the main column depending on the amount of product desired. The simplicity and flexibility of the SDM approach to preparative-scale purification enabled rapid separation of a single peptide component from a complex multicomponent mixture and should prove to be valuable for researchers in the peptide/protein field.

INTRODUCTION

The growing therapeutic importance of synthetic peptides has led to a concomitant increase in the need for rapid and efficient peptide purification procedures.

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Although an efficient peptide synthesis should result in only a small number of synthetic impurities (deletion, terminated, or chemically modified peptides), these impurities, which are usually closely related to the peptide of interest, often pose difficult purification problems. The excellent resolving power and separation time of reversed-phase chromatography (RPC) has led to its becoming the favoured method of peptide purification[1]. The most common analytical method used in RPC of peptides involves linear gradient elution (gradient rate of 1% eluent B/min at a flow-rate of 1 ml/min), where eluent A is 0.1% aq. trifluoroacetic acid (TFA) and eluent B is 0.1% TFA in acetonitrile[2]. However, the elution mode of RPC is handicapped by relatively poor utilization of the stationary and mobile phases[3]. Large-scale gradient elution separations of closely related peptides necessitate the use of increasingly larger column volumes in order to maintain satisfactory levels of product and yield. This leads, in turn, to higher operating costs in terms of packings, equipment, and solvents.

Recently, Horváth and other researchers[3–9] have demonstrated significantly more efficient use of hydrophobic stationary phases by applying the displacement mode of chromatography to reversed-phase preparative-scale separations of several classes of compounds, including peptides and proteins[6,9]. This techniques enables the separation of relatively large amounts of material on columns and instrumentation designed primarily for analytical work. Displacement chromatography involves sorption of a sample mixture near the inlet of the column, following its application in a carrier solvent that has low affinity for the stationary phase. A solution of a displacer, which has greater affinity for the stationary phase than any of the sample components, is then pumped slowly into the column. The sample components are thereby displaced from the surface of the stationary phase and move down the column preceding the displacer front, forming adjacent zones of purified solutes (displacement train). Despite the advantages of displacement over elution chromatography in terms of sample load, the method is extremely difficult to optimize in terms of choice of displacer, displacer concentration and flow-rate[3,6]. In addition, the mobile phase flow-rates, which are typically —and necessarily— low in displacement chromatography (0.1–0.2 ml/min), often lead to excessively long run times.

A perceived need for easier and more reliable methods of preparative reversed-phase separations of closely related compounds prompted the development by this laboratory of reversed-phase chromatography operated in sample displacement mode (SDM). This novel approach to preparative-scale purification on analytical columns and instrumentation maximizes both the separation and loading potential of a reversed-phase packing, enabling rapid separation of a single peptide component, in high yield, from a complex multicomponent mixture.

SDM strategy

Preparative reversed-phase chromatography, whether it is carried out in elution or displacement mode, requires the optimization of several parameters in order to maximize separation and yield. This optimization would be greatly simplified if the only variable was sample load, *i.e.*, without the addition of organic modifier or displacer to the mobile phase. Since peptides favour an adsorption–description method of interaction with the hydrophobic stationary phase, under normal analytical load conditions an organic modifier is typically required for their elution. However, we reasoned that when a reversed-phase column is optimally loaded with

a peptide sample mixture dissolved in water as the mobile phase, there would be competition by the sample components for the adsorption sites on the hydrophobic stationary phase. The more hydrophobic peptide components would compete more successfully for these sites than less hydrophobic components, which should be displaced and quickly eluted from the column. The major separation of sample components will have thus been effected in the absence of an organic solvent. Subsequent treatment with an aqueous organic solvent would only be required to elute retained components from the column and would take no part in this major separation process. Flow-rates of 1 ml/min on analytical columns (4.1–4.6 mm I.D.) should be possible in SDM, thus allowing rapid separations.

A crude peptide mixture, typically produced by solid-phase peptide synthesis, may contain not only the desired product, but also hydrophilic and/or hydrophobic synthetic peptide impurities. Under conditions of optimal sample load, either the peptide of interest would be used to displace the impurities from the column or the impurities would be used to displace the peptide of interest. In the latter case, the component of interest is isolated in water, and the organic solvent is used only to wash the column free of impurities. In the former case, the organic solvent is used to elute the peptide of interest after the separation in water is complete. Thus, situa-

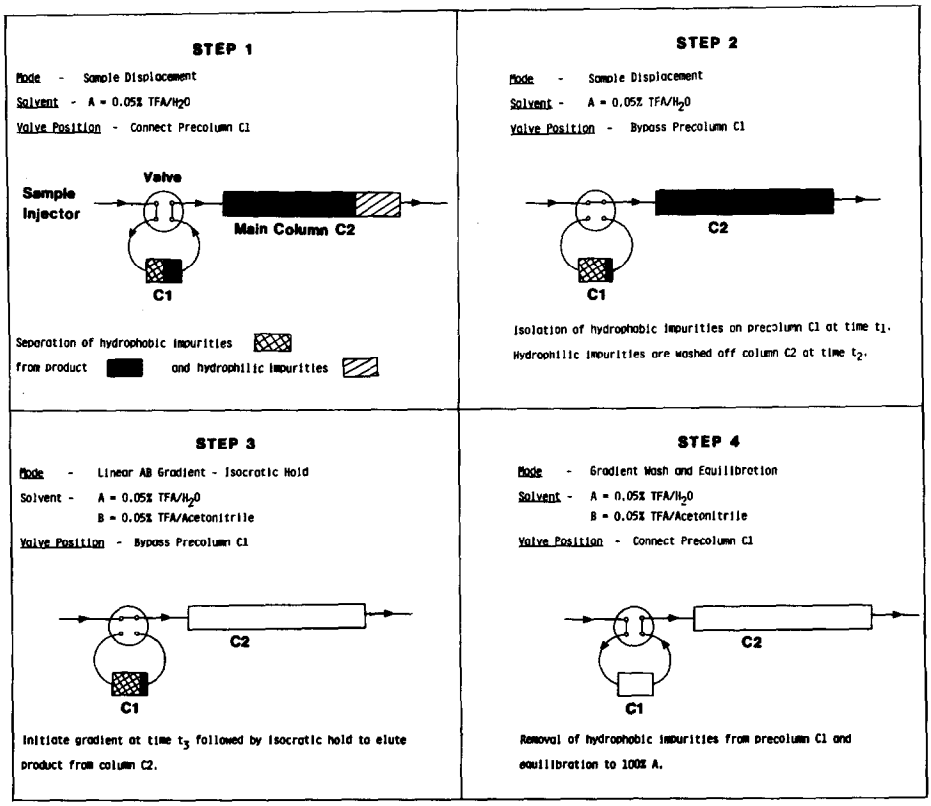

Fig. 1. Strategy for preparative reversed-phase chromatography in sample displacement mode (SDM). Details are described in text.

tions where a desired peptide product is the most hydrophilic or hydrophobic component of a crude peptide mixture should represent the simplest applications for operation of a reversed-phase column in SDM. However, a stricter test of the effectiveness of SDM for preparative work would be its application to a crude mixture containing impurities both more hydrophilic and hydrophobic than the desired peptide product. An outline of the strategy designed for this more complex separation problem is illustrated in Fig. 1. In step 1, the mixture to be separated has been applied at high load to the two-column system in 0.05% aq. TFA (pH 2.0) and is being eluted by the same mobile phase. Hydrophobic impurities have been retained by the precolumn; the desired peptide product has saturated the main column and displaced the hydrophilic impurities, resulting in their rapid elution from the system. In step 2, the precolumn has been isolated at time t_1, trapping hydrophobic impurities. The valve enables isolation of the precolumn (Cl) from the main column (C2) when only solvent flow through the main column is desired. At time t_2, all hydrophilic impurities have been washed off the main column by elution with 0.05% aq. TFA. The major separation of peptide components has now taken place and operation in SDM is now complete. In step 3, with the precolumn still isolated, a linear elution gradient has been initiated at time t_3 to remove the desired peptide product from the main column. An optional isocratic hold may be included as a safeguard to ensure separation of product from any hydrophobic impurities which were not trapped on the precolumn because of an incorrect estimate of the size of the precolumn. In step 4, the precolumn has been reconnected in series with the main column. Hydrophobic impurities have been removed from the precolumn by gradient elution, and the whole two-column system is re-equilibrated to 100% eluent A [0.05% (v/v) aq. TFA].

MATERIALS AND METHODS

Chemicals

HPLC-grade water and acetonitrile were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). HPLC-grade TFA was obtained form Pierce (Rockford, IL, U.S.A.).

Peptides 1–5 were synthesized on a Beckman (Berkeley, CA, U.S.A.) Model 990 peptide synthesizer using the general procedure of solid-phase synthesis described by Parker and Hodges[10]. They were subsequently purified by reversed-phase HPLC. The composition of the peptides varied as follows: peptide 2, –Gly^3–Gly^4–; peptide 3, –Ala^3–Gly^4–; peptide 4, –Val^3–Gly^4–; peptide 5, –Val^3–Val^4–. All peptides contained a N$^{\alpha}$-acetylated N-terminal and a C-terminal amide, except peptide 1, which was identical to peptide 3 but had a free α-amino group. These peptides were obtained from the Alberta Peptide Institute, Department of Biochemistry. University of Alberta, Edmonton, Alberta, Canada.

Apparatus

The HPLC instrument consisted of a Varian (Walnut Creek, CA, U.S.A.) Vista Series 5000 liquid chromatograph; coupled to a Hewlett-Packard (Avondale, PA, U.S.A.) HP1040A detection system, HP85B computer, HP9121 disc drive, HP2225A Thinkjet printer, and HP7470 plotter. Samples were injected with a 2.0-ml injection loop (Model 7125, Rheodyne, Cotati, CA, U.S.A.).

Peptide mixtures were separated on reversed-phase columns of differing lengths, connected in series: Aquapore RP300 C_8, 220 mm or 30 mm × 4.6 mm I.D., 7-μm particle size, 300-Å pore size (Brownlee Labs., Santa Clara, CA, U.S.A.).

In the present study, the precolumn (Cl) consisted of two 30-mm (4.6 mm I.D.) Aquapore C_8 cartridges, connected in series; the main column (C2) was a 220 mm × 4.6 mm I.D. column, containing the same reversed-phase packing material as the precolumn. The valve was a Rheodyne sample injector (Model 7125) arranged so that, when in load position, the precolumn was isolated; when in inject position, the mobile phase flow was directed through both the precolumn and main column.

Estimation of isocratic hold conditions

In a linear elution gradient (eluent A = 0.05% aq. TFA and eluent B = 0.05% TFA in acetonitrile), starting at the time of injection at a gradient rate of 1% B/min and a flow-rate of 1 ml/min, the %B required for elution of a given peptide is $t_R - t_g$, where t_g is the gradient delay time and t_R is the retention time of the peptide. The gradient delay time is the time for the gradient to reach the detector from the proportioning valves via the pump, injection loop, and column[2,11]. For the column and instrument used in this study, t_g was 8 min. If the retention time for a peptide of interest in an analytical experiment with an analytical load, applied as described above, was 24 min, then the % acetonitrile required for elution of the peptide is 24 − 8 = 16% B. Applying a role-of-thumb correction, the isocratic hold condition for delaying the elution of hydrophobic impurities would be 16% − 10% = 6% B.

RESULTS

Fig. 2A, shows the analytical chromatogram of a mixture of five closely related synthetic decapeptides, designed to represent the crude peptide mixtures typically produced by solid-phase peptide synthesis. The desired product (P_3) is contaminated by hydrophilic (I_1, I_2) and hydrophobic (I_4, I_5) impurities. The analytical chromatogram of the peptide mixture on the two-column system, shown in Fig. 1, was obtained by using a linear elution gradient (2% B/min) at a flow-rate of 1 ml/min, where eluent A is 0.05% aq. TFA and eluent B is 0.05% TFA in acetonitrile. The five peptides are closely related in hydrophobicity: between I_2 and P_3, there is an increase of only one carbon atom; between P_3 and I_4 there is an increase of two carbon atoms; between I_4 and I_5 there is an increase of three carbon atoms. The ratio of product to impurities was 1:1:10:1:1 (I_1:I_2:P_3:I_4:I_5), *i.e.*, P_3 represented *ca.* 71.4% of the crude peptide mixture. Fig. 2B represents the preparative chromatogram of 63 mg of the sample mixture. The flow-rate was 1 ml/min, and fractions were collected every minute. The sample contained 45 mg of P_3 and 4.5 mg of each of I_1, I_2, I_4, and I_5. Following injection, the sample mixture was eluted in SDM with 0.05% aq. TFA, the solvent flow being directed through both the precolumn and main column (Fig. 1, step 1). After 15 min (t_1 in Fig. 1, step 2), the precolumn was isolated to trap the hydrophobic impurities, I_4 and I_5. Continued elution with 0.05% aq. TFA was designed to ensure displacement of all hydrophilic impurities, I_1 and I_2, from the main column (t_2 in Fig. 1, step 2) and the operation in SDM was now complete. A linear elution gradient (gradient rate of 1% B/min) was initiated at 40 min (t_3 in Fig. 1, step 3) to elute product P_3 from the main column. An isocratic hold was not included

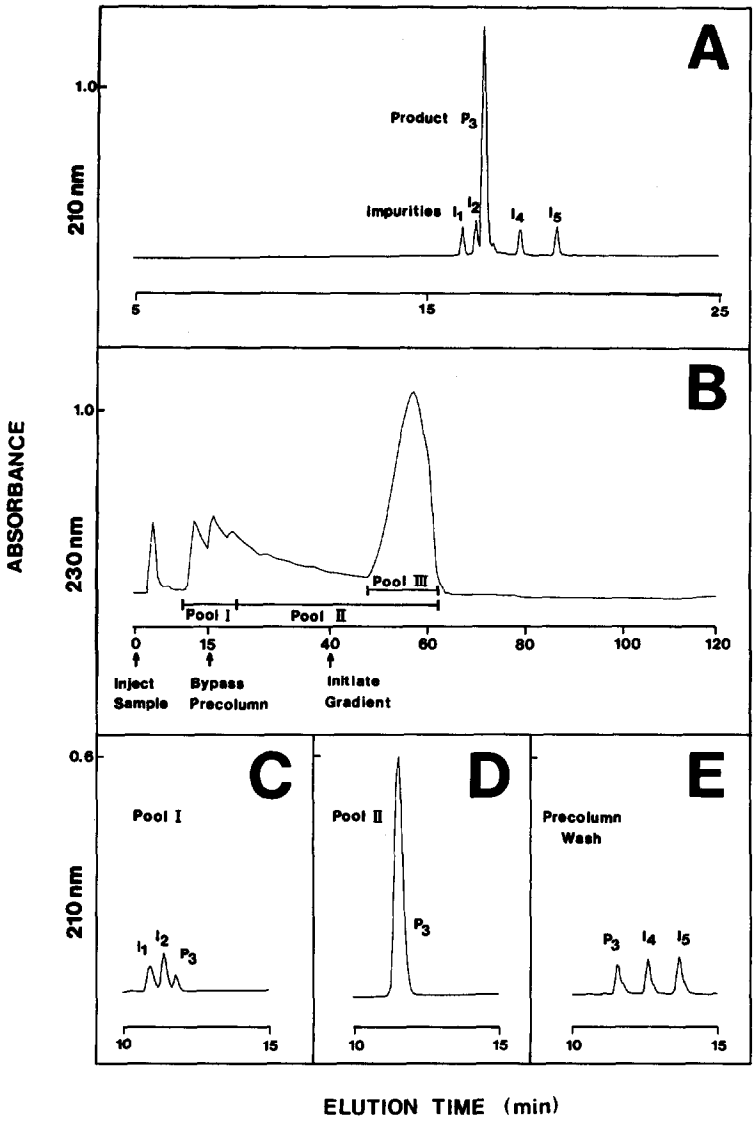

Fig. 2. Preparative reversed-phase sample displacement chromatography of a peptide product (P_3) from hydrophilic (I_1, I_2) and hydrophobic (I_4, I_5) peptide impurities. Columns: Aquapore RP300 C_8 (4.6 mm I.D.); precolumn 2 × 30 mm in length; main column, 220 mm in length. (A) Analytical chromatogram of a synthetic peptide mixture on two-column system. Conditions: linear gradient (2% B/min) at a flow-rate of 1 ml/min, where eluent A is 0.05% aq. TFA and eluent B is 0.05% TFA in acetonitrile. (B) Preparative chromatogram of the same peptide mixture. Conditions: elution with 100% eluent A for 40 min at a flow-rate of 1 ml/min, followed by linear gradient elution at 1% B/min; at 15 min, the precolumn was isolated from the main column. Sample load: 63 mg consisting of 45 mg of P_3 and 4.5 mg of each of I_1, I_2, I_4 and I_5, dissolved in 1500 μl of eluent A. (C,D). Analytical chromatograms of pools I and II, respectively, on an Aquapore RP300 C_8 column (30 mm × 4.6 mm I.D.). (E) Analytical chromatogram of peptide components retained by the precolumn (see A for conditions). The subscripts of I_1, I_2, P_3, I_4, and I_5 denote peptide 1–5, respectively (see Materials and methods).

TABLE I

RECOVERY OF PEPTIDE P_3

Mode	*Total load (mg)*	*Fig.*	*Product (P_3) recovery in each pool (%)**						*Total homogeneous product (P_3) recovered (%)*
			Pool I	*Pool II*	*Pool III*	*Pool IV*	*Pool V*	*Pre-column*	
SDM	63	2B	3	89	61	–	–	8	89
	63	3A	<1	98	66	–	<1	–	98
	63	3B	≪1	88	59	11	1	–	99
	47	4A	≪1	–	75	12	–	13	87
	63	4B	2	80	49	9	–	9	90
	89	4C	3	90	36	5	–	2	95
			Pool 1		*Pool 2*		*Pool 3*		
Elution	6	5B	25		75		–		75
	12	5C	21		74		5		74

* Quantity of product (P_3) in each pool as a percentage of total product (P_3) recovered, using relative peak areas.

in this separation. The final step involved removal of hydrophobic impurities, I_4 and I_5, from the precolumn by a linear gradient wash, followed by re-equilibration of the two-column system to 0.05% aq. TFA (Fig. 1, step 4). The fractions pooled as pool I, II and III (Fig. 2B) were subjected to gradient elution chromatography. Pool I (Fig. 2C) contained 100% of the hydrophilic impurities, I_1 and I_2, and 3% of recovered P_3 (Table I). In fact, only one fraction (21 min) contained all three components. Pool II (Fig. 2D) (incorporating Pool III, which contained 61% of the recovered P_3) contained pure P_3, representing 89% of recovered homogeneous product (Table I). Analysis of components retained by the precolumn (Fig. 2E) revealed the presence of 100% of hydrophobic impurities, I_4 and I_5, and 8% of recovered P_3.

The results described above certainly indicated that the precolumn played an important role in ensuring the success of preparative reversed-phase chromatography, operated in SDM. In order to confirm this, the same load (63 mg) of sample mixture was chromatographed without activating the precolumn valve for trapping the hydrophobic impurities on the precolumn (steps 2 and 3 in Fig. 1 omitted). Fig. 3A shows the preparative chromatogram. Following 40 min of operation in SDM with 0.05% aq. TFA, a linear gradient (1% B) was initiated to eluted components from the column. Fractions were analyzed and the distribution of recovered peptide components is reported in Table I. Although the level of recovered pure P_3 was very high (98%), the presence of hydrophobic imputities behind the product elution zone increased considerably the number of fractions that had to be analyzed to make the desired pool of pure product, compared to the small number required in the presence of the precolumn trap.

A second approach to using SDM in the absence of activating the precolumn valve for trapping the hydrophobic impurities is illustrated in Fig. 3B. The same load (63 mg) of sample mixture was applied to the two-column system and was subjected to 40 min of chromatography in SDM with 0.05% aq. TFA without activating the precolumn valve. A linear gradient (1% B/min) was then applied for 6 min (*i.e.* up

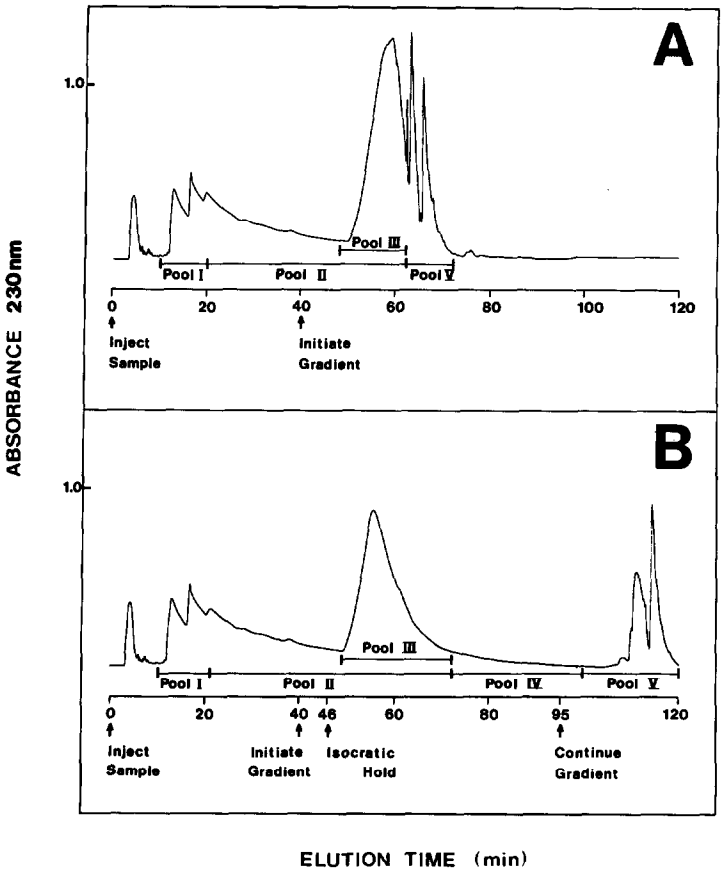

Fig. 3. Effect of omitting the precolumn on preparative reversed-phase chromatography of a peptide mixture in SDM. Columns: see Fig. 2. (A) Preparative chromatogram of peptide mixture. Conditions: elution with 100% eluent A for 40 min at a flow-rate of 1 ml/min, followed by linear gradient elution (1% B/min), where eluent A is 0.05% aq. TFA and eluent B is 0.05% TFA in acetonitrile. (B) Preparative chromatogram of peptide mixture. Conditions: isocratic elution with 100% A for 40 min at a flow-rate of 1 ml/min, followed by linear gradient elution at 1% B/min, isocratic hold (6% B) from 46 min to 95 min, and continued linear gradient elution at 1% B/min. Sample loads: 63 mg, consisting of 45 mg of P_3 and 4.5 mg of each of I_1, I_2, I_4 and I_5, dissolved in 1500 μl of eluent A. The subscripts of I_1, I_2, P_3, I_4, and I_5 denote peptides 1–5, respectively (see Materials and methods).

to 6% aq. acetonitrile), followed by isocratic elution with 6% aq. acetonitrile. After 95 min, the gradient was continued (1% B/min) for another 25 min. The distribution of peptide components is reported in Table I. These results (Fig. 3B) suggested that without activating the precolumn valve, interruption of the gradient elution with an isocratic hold may enhance the separation of hydrophobic impurities from the desired product, following the initial major SDM chromatography with water. However, unless an operator is familiar enough with a particular peptide mixture, the gradient–isocratic hold–gradient sequence may be more difficult to optimize (see Materials and methods).

The major parameter affecting the efficacy of SDM chromatography with

water should be peptide load. Fig. 4 demonstrates preparative elution profiles of the crude peptide mixture, applied to the two-column system at sample loads of 47 mg (A), 63 mg (B) and 84 mg (C). The ratio of product to impurities remained constant (Figs. 2 and 3). Following isolation of the precolumn at 15 min, elution in SDM with 0.05% aq. TFA was continued until the linear gradient (1% B/min) was started at 40 min. When the concentration of organic modifier (acetonitrile) in the aqueous mobile phase had reached 6%, the gradient was interrupted and this level of aq.

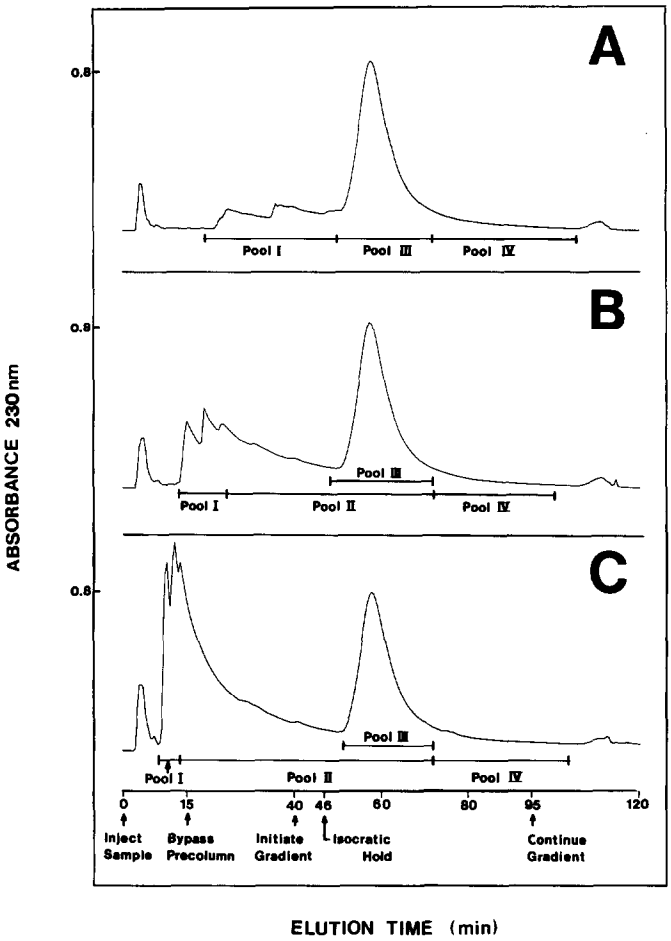

Fig. 4. Effect of sample load on preparative reversed-phase displacement chromatography of a peptide mixture in sample displacement mode. Columns: see Fig. 2. Conditions: elution with 100% eluent A for 40 min at a flow-rate of 1 ml/min, followed by linear gradient elution at 1% B/min, isocratic hold (6% B) from 46 min to 95 min, and continued linear gradient elution at 1% B/min; at 15 min, the precolumn was isolated from the main column; eluent A was 0.05% aq. TFA and eluent B 0.05% TFA in acetonitrile. (A) Sample load: 47 mg, consisting of 34 mg of P_3 and 3.3 mg of each of I_1, I_2, I_4, and I_5, dissolved in 1700 μl of eluent A. (B) Sample load: 63 mg, consisting of 45 mg of P_3 and 4.5 mg of each of I_1, I_2, I_4 and I_5, dissolved in 2300 μl of eluent A. (C) Sample load: 84 mg, consisting of 60 mg of P_3 and 6 mg of each of I_1, I_2, I_4 and I_5, dissolved in 2300 μl of eluent A. The subscripts of I_1, I_2, P_3, I_4, and I_5 denote peptides 1–5, respectively (see Materials and methods).

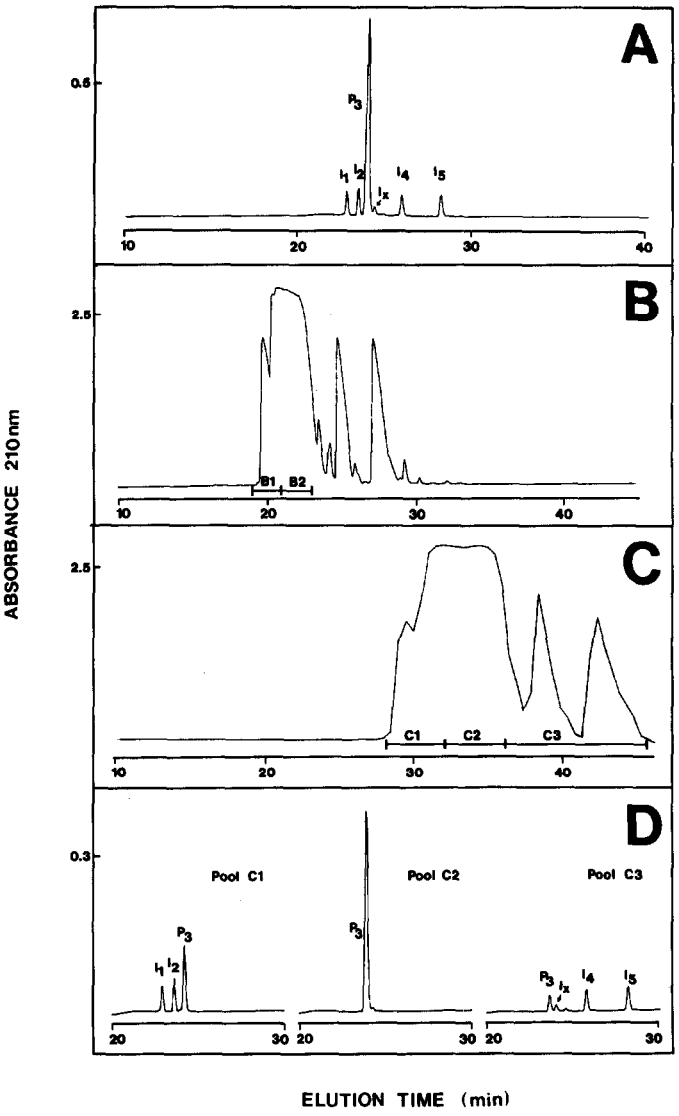

Fig. 5. Preparative reversed-phase gradient elution chromatography of a peptide mixture. Columns: see Fig. 2. (A) Analytical chromatogram of a synthetic peptide mixture on a two-column system. Conditions: linear gradient (1% B/min) at a flow-rate of 1 ml/min, where eluent A is 0.05% aq. TFA and eluent B is 0.05% TFA in acetonitrile. (B) Preparative chromatogram of peptide mixture. Conditions: linear gradient (1% B/min) at a flow-rate of 1 ml/min; sample load, 6 mg, consisting of 4.3 mg of P_3 and 0.43 mg of each of I_1, I_2, I_4, and I_5, dissolved in 136 μl of eluent A. (C) Preparative chromatogram of the peptide mixture. Conditions: linear gradient (0.5% B/min) at a flow-rate of 1 ml/min; sample load, 12 mg, consisting of 8.6 mg of P_3 and 0.86 mg of each of I_1, I_2, I_4 and I_5 dissolved in 272 μl of eluent A. The subscripts of I_1, I_2, P_3, I_4 and I_5 denote peptides 1–5, respectively (see Materials and methods).

acetonitrile was held constant until gradient elution was resumed at 95 min. Table I demonstrates recoveries of the peptide product, P_3, in the various pooled fractions

illustrated in Fig. 4. The recoveries of pure P_3 were impressive for all three sample loads. The major effect of increasing the sample load was to increase the rate at which the hydrophilic impurities, I_1 and I_2, were displaced from the system. Thus, for the 47-mg load, pure P_3 was obtained after 49 min; for the 63-mg load, pure P_3 was obtained after 24 min; and for the 84-mg load, pure P_3 was obtained after only 14 min. In all three experiments, the precolumn was sufficient to remove all hydrophobic impurities from the peptide mixture. As the load is increased, the amount of product (P_3) found on the precolumn decreases (13%, 9% and 2% for the 47-mg, 63-mg and 84-mg loads, respectively). This is expected, since increasing the load increases the amount of hydrophobic impurities, and more product is displaced from the precolumn trap. The amount of product found in the precolumn can be decreased by decreasing the length of the precolumn trap at any given load.

In order to appreciate fully the advantage of SDM over standard gradient elution for preparative separation of closely-related peptides, substantial sample loads of the crude peptide mixture were subjected to linear gradient elution on the two-column system. Fig. 5A demonstrates the analytical elution profile of the peptide mixture. The peptides were eluted by a linear gradient (1% B/min) at a flow-rate of 1 ml/min, where eluent A is 0.05% aq. TFA and eluent B is 0.05% aq. TFA in acetonitrile. The peptide denoted I_x was an extra hydrophobic synthetic impurity of unknown composition. Fig. 5B and C demonstrate linear A–B gradient elution profiles of the peptide mixture at sample loads of 6 mg and 12 mg, respectively. The 6-mg sample load was eluted by a 1% B/min gradient; the 12-mg sample load was eluted by a 0.5% B/min gradient. Pool B1 (Fig. 5B) contained all of the hydrophilic impurities, I_1 and I_2, and 25% of recovered P_3; pool B2 contained pure P_3, representing 75% of recovered product. The results of analyses (linear elution at 1% B/min) of pools C1, C2, and C3 from Fig. 5C (12-mg sample load) are indicated in Fig. 5D. Pool C1 contained all of the hydrophilic impurities and 21% of recovered P_3 (Table I); pool C2 contained pure P_3, representing 74% of recovered product; pool C3 contained all of the hydrophobic impurities, I_x, I_4 and I_5, and 5% of recovered P_3.

Because of overlap between adjacent peptide zones, particularly between P_3 and the hydrophilic impurities for both sample loads, there is a substantial increase in the number of analyses required to isolate the homogeneous peptide product, P_3. By comparison, homogeneous product can be isolated in SDM without any analysis, by simply collecting pool III [Figs. 2, 3(B) and 4], which is easily visualized in the chromatogram.

DISCUSSION

The value of any preparative method should be assessed not only by its effectiveness in separating large loads of sample mixtures as rapidly as possible, but also by the ease with which desired homogeneous product(s) may be detected and pooled. An important characteristic of the preparative chromatograms shown in Figs. 2, 3(B), and 4 is the simple visualization of the major solute zones following operation in SDM and subsequent gradient elution. This is frequently not the case in traditional displacement chromatography, where the amount of material required for efficient development of the displacement train may overload the detector, producing chromatographic profiles with no easily identifiable solute zones. Similar problems in

identifying solute zones were apparent when attempting to separate preparative amounts of a peptide mixture by linear gradient elution (Fig. 5) on an analytical column. Even at sample loads (6 mg and 12 mg) significantly lower than those successfully chromatographed in SDM (47–84 mg), the overlap of desired product, P_3, and impurities was substantial. Thus, a large number of analyses were required to identify the solute zones in the preparative chromatogram. In contrast, very few analyses were required to characterize completely the preparative chromatograms shown in Figs. 2, 3(B) and 4. When analyzing fractions, isocratic elution is generally simpler if a large number of samples have to be analysed, since there is no need for column regeneration. However, when only a small number of fractions have to be checked, gradient elution has the advantage of reproducibility of retention time, thereby making identification easier.

Pool III in the elution profiles shown in Figs. 2, 3(B) and 4 always contained large levels of homogeneous product, P_3, *i.e.*, no analysis of this peak would be needed to guarantee the purity of the desired peptide product. The amount of P_3, in pool III of these preparative chromatograms in SDM represents the maximum level of the peptide which could be applied to the main reversed-phase column (C2) without overloading the column. In this case, with the 22-cm analytical column, pool III yielded *ca.* 25 mg of homogeneous product (P_3). To maintain the UV profile at very high peptide loads, all that is required is to increase the wavelength typically utilized for detecting peptide bonds (205–210 nm) to 220–230 nm (Figs. 2–4), or to decrease the path-length of the flow-cell.

Some elements of displacement chromatography are frequently, if unwittingly, used by researchers when carrying out preparative gradient elution chromatography, particularly at high sample load[3]. If attempts to offset increasing sample loads are made by using excessively shallow gradients, a sample displacement mechanism is gradually approached. However, the total yields of homogenous product from the preparative chromatograms in SDM shown in Figs. 2–4 (87–99%) were always significantly greater than the 74–75% yields obtained by standard gradient elution (Fig. 5). In addition, the loads in the SDM experiments are *ca.* 10 times greater than in comparable gradient elution experiments. Although the yields of homogeneous product obtained from preparative gradient elution chromatography of mixtures of closely related peptides may improve as the gradient is made more shallow, they are unlikely to approach the levels consistently obtained when applying SDM to much higher sample loads.

Perhaps the most important consideration when attempting to optimize preparative SDM separations is the size of the precolumn. The value of the precolumn in removing hydrophobic impurities during the initial major preparative chromatography with water as the eluent was clearly demonstrated in Figs. 2–4. In fact, as illustrated in Fig. 4, the use of a precolumn made assessment of the proper sample load much less critical as long as the precolumn was long enough to trap the hydrophobic impurities. The concentration of hydrophilic impurities is irrelevant, since they are immediately displaced from the two-column system by the high concentration of the more hydrophobic product, P_3. If the concentration of hydrophobic impurities in the crude peptide mixture is higher, the precolumn can be lengthened. Thus, the researcher can regulate the size of the precolumn depending on the amount of hydrophobic impurities in a particular sample, or the size of the main column

depending on the amount of product desired. The option of inserting an isocratic hold (Fig. 4) into the gradient elution program during elution of peptide components from the main column may be a useful precaution in an initial preparative chromatogram in SDM if there is uncertainty about the size of the precolumn required to retain all of the hydrophobic impurities. However, as stated previously, the gradient–isocratic hold–gradient sequence required to isolate efficiently and rapidly the desired peptide product from closely related hydrophobic impurities is more difficult to optimize, unless the researcher is sufficiently familiar with the elution properties of the peptide of interest. As a rule-of-thumb, the isocrastic hold % B can be estimated by subtracting 10% from the % B required to elute the peptide in an analytical experiment with a gradient rate of 1% B/min on the same analytical column (see Materials and methods). The preparative separation demonstrated in Fig. 2 (no isocratic hold) held the advantage in both speed and simplicity over programs including an isocratic hold (Figs. 3 and 4). The time required to elute all of the recovered peptide product, P_3, from the main column is shortened considerably by the application of an uninterrupted gradient (*cf.* Fig. 2B with Fig. 4B). In addition, once the researcher is familiar with the solute zones in the elution profile, the gradient wash may be initiated earlier to reduce the required run time even further.

The objective of preparative chromatography is to obtain maximum resolution and sample load for any given column under the constraints of the investigator's established yield of homogeneous product in the desired time frame. Overload is thus a condition where this established criterium is no longer met. The ideal load for SDM operation for a given crude product on any given column is the load that will displace the hydrophilic impurities rapidly from the column and provide the maximum yield of homogeneous product that is easily visualized with a minimum number of analyses. In our case, we have defined the maximum load of product as the load in which the peptide product is just eluted from the column with water after 20 min. In the examples used in this paper, a load of 50–60 mg of crude product produced a maximum yield of homogeneous product (in pool III) and rapid elution of the hydrophilic impurities (within the first 20 min) (see Fig. 4A and B and Table I).

In summary, this work describes an extension of a novel procedure for preparative reversed-phase separation of complex peptide mixtures, first reported by our laboratory[12]. Since the major separation process takes place in water (SDM), the use of prohibitively costly volumes of organic solvents is avoided. In addition, the excellent yields of homogeneous product obtained on analytical columns and instrumentation should contribute appreciably to cost savings. The potential of SDM as a preparative tool is considerable and should prove of great value to researchers involved in the purification of synthetic peptides, in the isolation of a single biologically active peptide from a crude peptide mixture or in the isolation of a single peptide component from a protein digest. Under certain circumstances, the product of interest can be isolated by using SDM alone. However, as shown in the present study, a combination of SDM with gradient and/or isocratic elution modes increases the versatility of preparative reversed-phase chromatography. SDM offers the potential of increased loading capacity, resolving power, and ease of localizing the desired pure component while yielding relatively rapid purification at standard flow-rates.

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