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COMPUTER SIMULATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS OF PEPTIDE AND PROTEIN DIGESTS FOR DEVELOPMENT OF SIZE-EXCLUSION, ION-EXCHANGE AND REVERSED-PHASE CHROMATOGRAPHIC METHODS

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SUMMARY

A computer program, called Pro Digest-LC, has been developed which assists scientists in devising methods of size-exclusion, cation-exchange and reversed-phase high-performance liquid chromatography for the analytical separation and purification of biologically active peptides and peptide fragments from enzymatic and chemical digests of proteins. Pro Digest-LC accurately predicts the retention behaviour of peptides of known composition, containing 2–50 amino acid residues, and simulates the elution profiles in all three modes of chromatography. In addition, Pro Digest-LC is a user-friendly program, designed as a teaching aid for both students and researchers in selecting the correct conditions for chromatography, that is, the mode of chromatography, column selection, mobile-phase selection, and has the ability to examine the effects of flow-rate, gradient-rate, and sample size on the separation.

We have designed a set of peptide standards for each mode of chromatography to aid the researcher in eliminating non-specific interactions, to standardize retention behaviour on the user's columns, to monitor column performance and to compare packing materials. In the development of each prediction mode, experimental peak heights, peak widths, and retention times from model synthetic peptide standards were incorporated directly into the program and can be used as default values.

Pro Digest-LC is an interactive program, in that researchers can run peptide standards on their particular columns and enter the peak width at half-height, peak height, retention time and quantity injected to adjust the simulation to their particular column. The simulated experiments eliminate the time-consuming trial-and-error methods used to suitable separation or purification procedures. The researcher would perform the actual experiment only after predicting the optimized conditions, thereby saving valuable sample and research time. The general concepts of the program along with representative separations of protein digests are displayed.

INTRODUCTION

The efficient isolation of peptides has become increasingly important for an ever-widening range of research disciplines in recent years. High-performance liquid

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chromatography (HPLC) has proved very versatile in the separation and purification of peptides from a great variety of sources. Peptide mixtures derived from different sources differ widely in complexity and quantity, and the approach to their separation must be tailored to the separation goals.

Separation of peptides from a chemical and/or proteolytic digest of a protein is a very common requirement. Investigation of the properties of peptides is vital in structure–function relationship studies of proteins, and one approach to locating biologically active regions of proteins is to purify all the fragments from a protein digest for subsequent testing of biological or binding activity. After incorporation of structural probes, during chemical modification studies, identification of the position and percent labelling in the protein is required. By digesting the protein and separating the digest fragments, the location of the labelled peptide and amount of labelling can be determined. Purified native protein fragments can be used to screen native polyclonal or monoclonal antisera for locating immunogenic determinants in proteins. In addition, purified native protein fragments can be used for the preparation of antipeptide antibodies, which can, in turn, be used in structure–function relationship studies, as diagnostics, or in the development of synthetic vaccines for viral and bacterial pathogens.

Advances in biotechnology have provided the ability to prepare proteins for therapeutic purposes. However, during biosynthesis, impurities very close to the desired protein will be present. These impurities can arise from host-cell proteins, expression errors, incomplete post-translational modification, and chemical modification during purification. Separation systems are required to detect small changes in the polypeptide chain, such as deletion or substitution of one amino acid, deletion or addition of carbohydrate, oxidation of a single methionine or cysteine residue, or deamidation at a single site. Thus, peptide mapping, following protein digestion by chemical or proteolytic agents, is one way to verify the structure of a genetically engineered protein.

Peptides obtained from biological tissues are often found in only very small quantities and may require extensive purification. Thus, being able to predict the location of a biologically active peptide from various tissue sources in chromatograms obtained by different modes would be extremely beneficial.

The wide use of automated solid-phase peptide synthesis in recent years has also necessitated efficient isolation of peptides from various impurities. An efficient peptide synthesis should result in only a small number of synthetic impurities. However, these impurities are usually closely related to the peptide of interest (deletion, terminated, or chemically modified peptides), perhaps missing only one amino acid residue, and may be difficult to separate by any single HPLC mode.

Peptides derived from various sources differ widely in size, net charge, and hydrophobicity, and purification of a single peptide from a complex mixture will require an approach different from that necessary for separating all components of a mixture. The former approach may require the application of only a single HPLC mode. In contrast, the latter will require a combination of separation modes (multimode HPLC) for efficient resolution of all desired peptides[1–3]. The three main modes of HPLC used for peptide separations utilize differences in peptide size (size-exclusion HPLC or SEC), net charge (ion-exchange HPLC or IEC), or hydrophobicity (reversed-phase HPLC or RPC). Within these modes, mobile-phase conditions may be

manipulated to maximize the separation potential of a particular HPLC column. Although a desired peptide separation may be obtained by trial and error, this may take many attempts, with subsequent loss of time and final peptide yield. This could be a particular problem where only limited amounts of sample are available.

A computer software program, Pro Digest-LC, has been developed which assists scientists in devising methods for the analytical separation and purification of biologically active peptides and peptide fragments from enzymatic and chemical digests of proteins by SEC, IEC, or RPC. The experiments simulated on the computer eliminate the time-consuming trial-and-error methods involved in obtaining suitable separation or purification methods. In addition, Pro Digest-LC is also a teaching aid for chromatographers, designed to help the student or researcher to select the correct conditions for chromatography, *i.e.*, the mode of chromatography, column and mobile phase, and to allow him the ability to examine the effect of flow-rates, gradient-rates and sample size on the separation.

This paper introduces the user-friendly program, Pro Digest-LC, reviews the concepts which led to its development, and demonstrates many of its simulation capabilities.

EXPERIMENTAL

Materials

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

The peptide standards described were synthesized on a Beckman Model 990 peptide synthesizer (Beckman Instruments, Berkeley, CA, U.S.A.) by means of the general procedure for solid-phase synthesis, described by Parker and Hodges[4].

Two sets of peptide standards were obtained from Synthetic Peptides Incorporated (Department of Biochemistry, University of Alberta, T6G 2H7, Canada). A mixture of four synthetic undecapeptide standards (1–4) was utilized for RPC and IEC. Peptides 1 and 2 were based on the sequence, X^1-X^2-Gly-Leu-Gly-Gly-Ala-Gly-Gly-Leu-Lys, where X^1-X^2 were substituted with G^1-G^2- (peptide 1) or Lys^1-Tyr^2- (peptide 2); peptides 3 and 4 were based on the sequence, X^1-X^2-Ala-Leu-Lys-Ala-Leu-Lys-Gly-Leu-Lys, where X^1-X^2- were substituted with G^1-G^2- (peptide 3) or Lys^1-Tyr^2- (peptide 4). Each peptide contained and N^{α}-acetylated N-terminal and a C-terminal amide. A polymer series of five synthetic peptides was used for SEC. The sequence of the standards was Ac-(Gly-Leu-Gly-Ala-Lys-Gly-Ala-Gly-Val-Gly)$_n$-amide, where n = 1–5.

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.) HP1040 detection system, HP85B computer, HP9121 disc drive, HP2225A Thinkjet printer and HP7470A plotter. Samples were injected with a 500-μl injection loop (Model 7125, Rheodyne, Cotati, CA, U.S.A.).

Peptide mixtures were separated on four columns: (1) SynChropak GPC60 size-exclusion column (300 × 7.8 mm I.D., 10-μm particle size, 60-Å pore size; Syn-

Chrom, Linden, IN, U.S.A.); (2) SynChropak S300 strong-cation-exchange column (250 × 4.1 mm I.D., 6.5-μm particle size, 300-Å pore size; SynChrom); (3) Aquapore RP-300 C_8 reversed-phase column (220 × 4.6 mm I.D., 7-μm particle size, 300-Å pore size; Brownlee Labs., Santa Clara, CA, U.S.A.); (4) SynChropak RP-P C_{18} reversed-phase column (250 × 4.6 mm I.D.; 6.5-μm particle size, 300-Å pore size; SynChrom).

HPLC-derived data for program development

(1) SEC: mobile phase, 50 m*M* KH_2PO_4 (pH 6.5), containing 0.1 *M* potassium chloride. A mixture of three synthetic peptide polymers (10, 20 and 50 residues) was chromatographed (total sample loads of 2–16 nmol of peptides) in sample volumes of 10, 25, 50, 100 and 200 μl at flow-rates of 0.2, 0.5 and 1.0 ml/min.

(2) IEC: mobile phase, buffer A was 5 m*M* KH_2PO_4 (pH 3.0 or pH 6.5) and buffer B was 5 m*M* KH_2PO_4 (pH 3.0 or pH 6.5), containing 1.0 *M* sodium chloride. The mixture of four synthetic undecapeptide standards (+1 to +4 net charge) was subjected to strong-cation-exchange chromatography (pH 3.0 and pH 6.5) at flow-rates of 0.5, 1.0 and 2.0 ml/min and linear gradient-rates of 5, 10 and 20 m*M* sodium chloride/min. Total sample loads ranged from 2 to 40 nmol of each peptide.

(3) RPC: mobile phase, eluent A was 0.1% aq. TFA and eluent B was 0.05% TFA in acetonitrile (pH 2.0). The mixture of four synthetic undecapeptide standards was subjected to RPC at flow-rates of 0.5, 1.0 and 2.0 ml/min and linear gradient-rates of 0.5, 1.0 and 2.0% acetonitrile/min. Total sample loads ranged from 2 to 40 nmol of each peptide.

Absorbances were measured at 210 nm for SEC, and at 210 and 280 nm (to detect tyrosine absorbance) for IEC and RPC.

Data from these chromatographic experiments were used to derive equations predicting the effects of experimental parameters (sample size, sample volume, flow-rate and gradient-rate) on peptide retention times, peak heights, peak widths and resolution. Simulated elution profiles, generated by the program, are based on the mobile phases used to derive the equations.

Computer hardware requirements

The minimum requirement for operating this program is an IBM-AT or compatible computer with 256K memory, equipped with two floppy disk drives and a monitor with graphics capability. We strongly recommend a math coprocessor (for example, calculations that take approximately 2 min on the IBM-AT can be done in *ca.* 15 s with the math coprocessor).

General features of the program

The most important requirement of any computer program is that it be user-friendly. Each menu is self-explanatory in Pro Digest-LC, providing simple instructions and one-letter keying to access any particular section of the program. An example of the clarity of the Pro Digest-LC presentation is illustrated in Fig. 1. This figure demonstrates the rapid access to a stored protein file, starting from the HPLC Main Menu (section A). Depressing the "Digest" key ("D") selects the Protein Menu (section B). Depressing the "L" key (for loading/viewing/editing a stored protein file) in the Protein Menu brings up the option of scrolling to the desired protein (TnI, in this

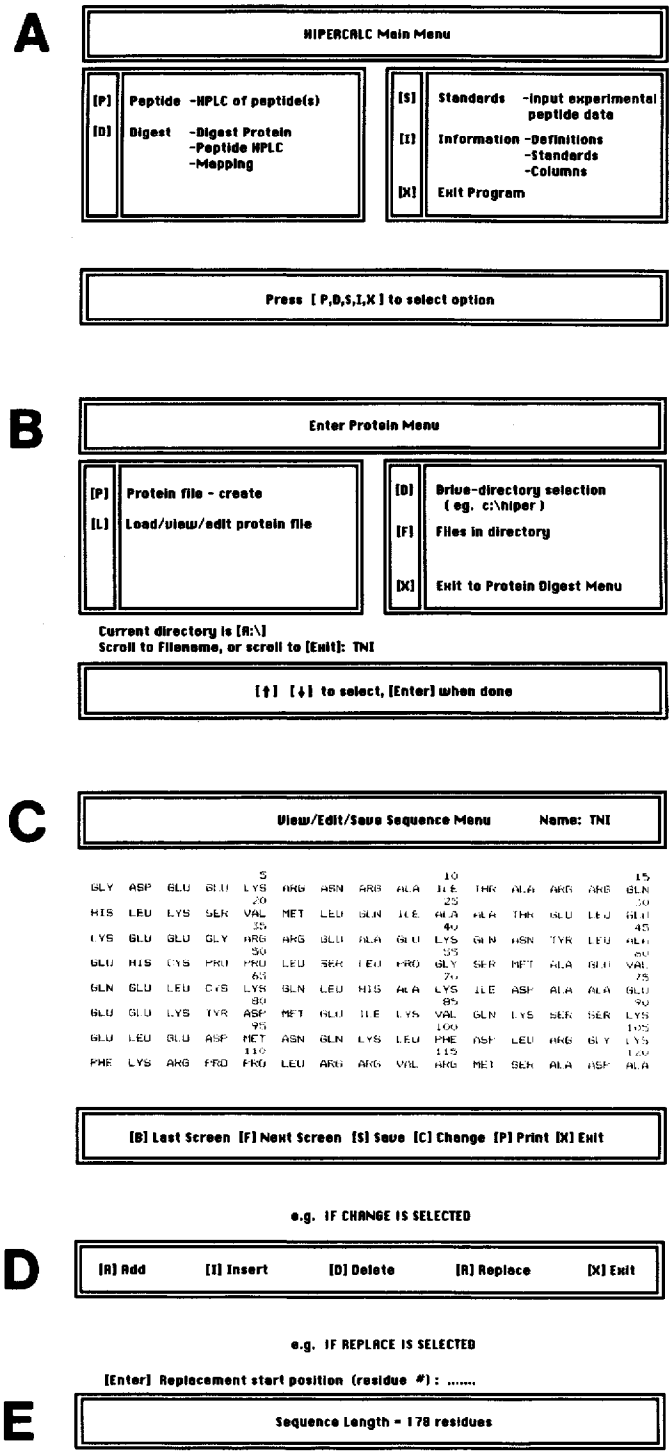


Fig. 1. Examples of the menus of Pro Digest-LC. Panel A shows the main menu of the program. Panel B shows the protein menu, where protein files are created or loaded for subsequent analysis. Panel C shows how protein sequences may be viewed and, if required, modified (panels D and E).

case), followed by pressing "enter". The sequence of the protein now appears on the screen (section C). Various options are now open to the researcher. When viewing the protein sequence, depressing "F" advances the display to the next screen for more of the protein sequence. Depressing "B" returns it to the screen showing the previous part of the protein sequence. The protein sequence can also be printed ("P") on an external printer. If a change in the sequence is required, depressing "C" brings up the options shown in section D. Amino acid residues may be added ("A"), inserted ("I"), deleted ("D"), or replaced ("R"). Depressing any of these keys brings up self-explanatory instructions similar to those shown for replacement of an amino acid residue (section E). It should be noted that during any manipulation of the protein sequence (*e.g.*, sections D and E), the protein sequence is always shown on the computer screen. This enables the operator to verify that the required change has been made. Depressing "X" exits the screen being displayed to the previous options or menu. Once section C has been reached again, the researcher can save ("S") a particular modified protein sequence for future work under a new file name.

From the HIPERCALC Main Menu screen (HIPERCALC stands for high-performance calculations in liquid chromatography), the operator has four options (Fig. 1, section A).

Option 1: Information menu

Pro Digest-LC is a teaching aid for chromatographers, designed to help them to select the correct conditions for chromatography, *i.e.*, the mode of chromatography, column, mobile-phase and to provide them with the ability to examine the effect of flow-rates, gradient-rates and sample size on the elution profile. From the information menu, the operator can select the following sections.

(a) Definitions. This section provides definitions of HPLC parameters used in the program.

(b) Standards information menu. This section describes peptide standards that are available for SEC, IEC and RPC. Once the operator is in the Standards information menu, he has a choice of SEC, IEC, RPC and a list of references. For each method of chromatography, the design features of the standards are described; the reasons for the development of the standards are described, together with examples of separations of the standards by means of various mobile phases, to demonstrate the importance of standards in selecting mobile-phase conditions. In addition, ordering information is provided for the peptide standard kits.

(c) The columns and conditions screen. In this section, the mode of chromatography (SEC, IEC and RPC) or column maintenance and storage conditions can be selected. For each mode of chromatography, reasons are given for using a particular mode for the separation of peptides and for choosing a particular column; the utility of peptide standards for monitoring the resolving power of a column under various mobile-phase conditions and the value of peptide standards in detecting non-specific interactions with column packings are described; and detailed instructions are given for changing the composition of the mobile phase to avoid any non-specific interactions observed, together with examples of appropriate mobile phases, flow-rates and gradient-rates suitable for achieving a desired separation. All relevant references are listed, so that the researcher can quickly access more detailed information, if he desires.

Option 2: Standards data menu

On accessing the Standards data menu, the operator can create a Standards file or load/view/edit Standard data files in the program. The purpose of the Standards data file is to provide the researcher with the option of either using our default file or entering data on standards chromatographed on his columns with his instrumentation. For example, after chromatographing a set of standards on a particular column, the quantity injected, peak heights, peak widths, retention times and other parameters necessary for the program to adjust the predicted elution profiles to the researcher's particular column can be entered.

Option 3: Protein digest menu

Once in the protein digest menu, the operator has the option of entering a protein sequence and performing an enzymatic or chemical digestion of a particular protein sequence (Fig. 1, section B).

(a) Enter protein data

Create protein file: the particular protein sequence can be entered by a one-letter or three-letter code.

Load/view/edit protein file: on selecting this option, the operator can scroll through the list of proteins stored in the program and select the desired protein file. The computer requests the quantity of protein to be digested and applied to the HPLC column. The protein sequence is automatically displayed on the screen, and the sequence can be printed, changed and saved as a new file of required changes. The options of protein sequence manipulation have already been described (Fig. 1, sections C, D and E).

After loading the protein file, the operator exits to the Protein digest menu and selects "Digest" and then cyanogen bromide cleavage or an enzymatic cleavage by trypsin. The computer performs the digestion and then requests in the Protein HPLC column selection menu the mode of chromatography by which the digest is to be separated (SEC, IEC and RPC) and asks whether the peptide mapping option is desired. After the mode of chromatography is selected, the program requests the flow-rate and gradient-rate for RPC and IEC, or the flow-rate and sample volume for SEC. The elution profile is calculated and displayed within 1–15 s, depending on the complexity of the separation. The elution profile is always displayed initially on a time axis to the nearest 10 min after the last peak. After observing the profile, the user has the option of scrolling a cursor backwards or forwards under the peptide peaks to view the sequence, peptide length, and retention time for each peptide (Figs. 5 and 6). He has the option of listing the fragments and their retention times on an external printer (Table I) or of selecting the zoom option. The zoom option allows the researcher to enlarge any particular section of the chromatogram, changing the X-axis (elution time in min) and the Y-axis (milli-absorbance units) to any desired value (Figs. 5 and 6).

If the separation is unsatisfactory, the researcher has the option of trying other conditions to obtain an optimal separation. Once these conditions have been found, the researcher can then perform the actual experiment in the laboratory with great saving in methodology development time.

If the operator selects the peptide mapping option, he has the ability to add the particular digest of the mutant protein to the same digest of the native protein se-

TABLE I

COMPUTER-PREDICTED REVERSED-PHASE RETENTION TIMES OF CYANOGEN BROMIDE CLEAVAGE FRAGMENTS OF RABBIT SKELETAL TNI (SLOW AND FAST)

File: TnI + Slow TnI (mutant).

No.	*Residue*	*Retention time**	*Mutant*	*Fragment*
1	168–173	2.40	No	EGRKKM
2	166–168	2.44	Yes	SGM
3	117–121	6.84	No	SADAM
4	174–178	7.24	No	FESES
5	175–184	9.74	Yes	FDAAKSPTSQ
6	1–21	18.25	No	GDEEKRNRAI...ARRQHLKSVM
7	82–95	19.28	No	EIKVQKSSKELEDM
8	135–167	22.11	No	DLRANLKQVK...RKNIEEKSGM
9	121–133	22.68	Yes	LRALLGSKHKVSM
10	122–134	23.32	No	LKALLGSKHKVCM
11	1–20	24.67	Yes	PEVERKSKITASRKLLKSLM
12	134–165	25.02	Yes	DLRANLKSVK...GDWRKNVEAM
13	58–81	26.44	No	AEVQELCKQL...DAAEEEKYDM
14	96–116	26.85	No	NQKLFDLRGK...KRPPLRRVRM
15	21–120	28.80	Yes	LAKAKECQQE...RRVRVSADAM
16	22–57	31.10	No	LQIAATELEK...CPPLSLPGSM

* The computer simulates reversed-phase elution profiles obtained with a linear aq. TFA to TFA in acetonitrile gradient (1% acetonitrile/min) at a flow-rate of 1 ml/min.

quence. The reversed-phase and ion-exchange mode options can be selected and the elution profile calculated. The peptides found in the mutant that are different from those in the native sequence will be marked on the elution profile (Fig. 6). This can be extremely helpful when one is interested in only identifying the mutant peptides.

If the operator selects the "Compute column selection option" the program separates the digest by RPC and IEC (cation-exchange HPLC at pH 7.0) at the resolution selected by him. The program then indicates for each mode the number of peptides resolved out of the total number of peaks in the elution profile, and if both chromatographic modes are used, the total number of peptides resolved. This option is extremely useful in aiding the researcher in his decision whether multi-modal approaches are necessary for carrying out the separation.

Option 4: Peptide HPLC menu

This section of the program is designed so that the researcher can enter a single or multiple peptide sequences for separation by a particular mode of chromatography.

(a) Enter peptide data

Create peptide or working file: (i) Peptide file. (Enter the particular peptide sequence by a one-letter or three-letter code); (ii) working file. There are two options: one can group already entered peptides into a working file to examine separations of any group of desired peptides, or one can group together more than one working file of peptides. These options provide maximum flexibility to create groups of peptides one wishes to separate.

Load/view/edit file. In this section, one can load/view/edit any particular peptide or group of peptides in a working file. On viewing a particular working file, all the amino acid sequences are displayed on the screen for making sequence changes or printing out the sequences in the file. Peptides can be added to or deleted from the working file. In the peptide file, one can add, insert, delete, or replace any amino acids desired. Of course, after the files or the amino acid sequence of a particular peptide are changed, the information can be saved for future work.

(b) HPLC column and conditions section. In this section, one can select the mode of chromatography by which the particular peptide or group of peptides should be separated (SEC, IEC or RPC). After the mode of chromatography is selected, the flow-rate, gradient-rate, or sample volume desired is entered. As described previously, the elution profiles are calculated and displayed. The peptide sequences and retention times can be listed on an external printer or the zoom option can be used to expand a particular region of the chromatogram.

In a peptide file or a working file, containing a group of peptides, the quantity of the peptide(s) entered can be varied, thus making the program completely versatile.

The program is available from S.P.I. Synthetic Peptides Incorporated (Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7).

RESULTS AND DISCUSSION

General concepts

Though RPC is, by far, the most widely-used mode of HPLC for peptide separations, it is unlikely that a single HPLC technique will resolve a very complex peptide mixture. As mentioned previously, it is often easier, even in the case of less complex mixtures, to utilize a combination of two or more HPLC modes, which utilize different selectivities, rather than attempt to optimize a single mode[1–3]. In addition, if only a single peptide or a few peptides in a complex mixture are desired, a particular HPLC mode which, on its own, may not resolve the whole mixture as well as another mode may still be the method of choice for the desired peptide(s)[3].

The standard trial-and-error approach to developing a HPLC purification method is costly in terms of sample loss as well as expenditure of time and effort. Thus, any methodology that can aid the researcher in selecting HPLC mode(s) and chromatographic conditions without using precious sample or requiring an extensive method development time would be advantageous.

For this reason, we have developed a computer simulation program based solely on predicting peptide retention behaviour in SEC, IEC and RPC. In order to predict peptide retention behaviour successfully, any deviations from ideal column behaviour, *i.e.*, non-specific interactions between the sorbent and solute, must be identified and suppressed or eliminated. This can be best accomplished by using peptide standards specifically designed to monitor column performance and non-specific interactions. In addition, the major breakthrough in our ability to predict retention times for peptides of 2–50 residues has resulted from the design and use of a series of synthetic peptide polymers.

Size-exclusion chromatography. Separation of peptides by a mechanism based solely on peptide size (ideal SEC) occurs only when there are no non-specific interac-

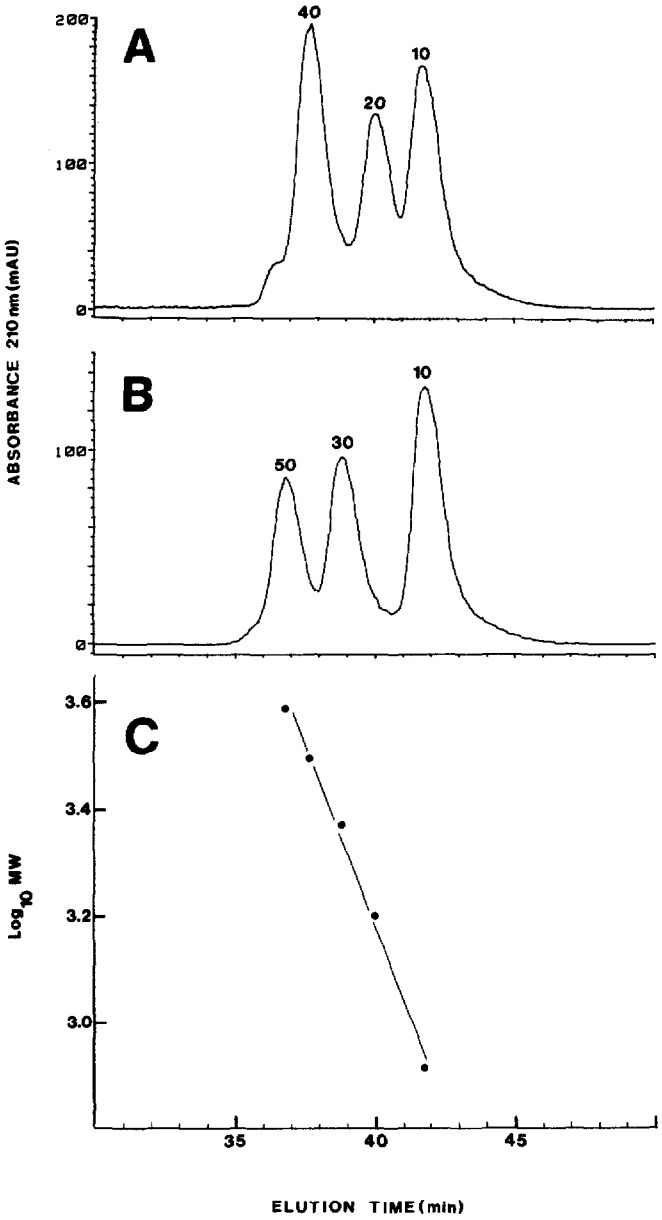


Fig. 2. Ideal SEC of mixtures of synthetic peptide standards. Conditions: column, SynChropak GPC60 (300 × 7.8 mm I.D.); mobile phase, 5 m*M* KH_2PO_4 (pH 7.0), containing 0.15 *M* sodium chloride; flow-rate, 0.2 ml/min; temperature, 26°C. Panel A: mixture of peptides containing 10, 20 and 40 residues. Panel B: mixture of peptides, containing 10, 30 and 50 residues. Panel C: plot of log molecular weight *versus* retention time of the peptide standards. The sequence of the peptide standards is Ac-(G-L-G-A-K-G-A-G-V-G)$_n$-amide, where n denotes the number of decapeptide repeating units (n = 1–5).

tions between the solutes and the column matrix. Although SEC columns are designed to minimize non-specific interactions, most modern SEC columns are weakly anionic (negatively charged) and slightly hydrophobic. This results in deviations from ideal size-exclusion behaviour, *i.e.*, non-ideal SEC[5–10]. The volume of solvent required to elute small molecules in ideal SEC is the total permeation volume of the column. This column parameter is a combination of the void volume of the column (elution volume of a totally excluded species) and the pore volume of the size-exclusion matrix. By definition, under ideal size-exclusion conditions, no molecule will be retained beyond the total permeation volume of the column.

A series of synthetic peptide standards (Ac-[G-L-G-A-K-G-A-G-V-G]$_n$-amide, where n = 1–5), designed for monitoring both non-ideal and ideal SEC behaviour, has proved extremely beneficial in enabling rapid development of optimal conditions for SEC of peptides[2,10]. The increasing size of the peptide standards (800–4000 daltons) enables an accurate molecular weight calibration of a column in ideal SEC; the increasingly basic character of the standards (+1 to +5) makes them sensitive to the anionic character of size-exclusion matrices; the increasing hydrophobicity of the polymer series enables a determination of column hydrophobicity.

The chromatographic profiles of mixtures of the size-exclusion standards (10, 20 and 40 residues in Fig. 2a); 10, 30 and 50 residues in Fig. 2b) on a SynChropak GPC60 silica-based size-exclusion column, coupled with the linear character of the $\log_{10}$ molecular weight *versus* peptide retention time plot (Fig. 2c), clearly demonstrates the ability of the polymer series to monitor pure size-exclusion behaviour on SEC columns.

A mixture of the 10-, 20- and 50-residue peptide standards was utilized for the GPC60 column to derive all the parameters required for the computer program. The sensitivity of the standards to any undesirable non-ideal SEC behaviour has already been clearly demonstrated[10]. Aqueous solvents and buffers containing 100–400 m*M* salt are commonly employed as the mobile phase for SEC to eliminate non-specific interactions. The mobile phase which produced the elution profiles shown in Fig. 2, for instance, consisted of 5 m*M* KH_2PO_4 (pH 7.0) containing 150 m*M* sodium chloride (flow-rate = 0.2 ml/min). In fact, efficient separation of peptide mixtures in the absence of salt is the exception rather than the rule[2,10].

Ion-exchange chromatography. The use of peptide standards in monitoring the retention characteristics of ion-exchange columns is twofold: firstly, to confirm that the column can, indeed, retain charged species and determine whether the column can retain peptides with net charges in the 0–1 range; secondly, to assess the effect of pH variations on the resolving capability and load capacity of an ion-exchange column. The latter is particularly important in cation-exchange chromatography, where manipulation of the mobile phase over the acidic to neutral pH range is frequently employed for peptide separations[1–3,11].

Fig. 3a demonstrates the elution profile of the five synthetic peptides described above on a SynChropak S300 strong-cation-exchange column (pH 7.0). The increasingly basic character of the standards makes them sensitive to the anionic character of the cation-exchange column. The standards were subjected to linear gradient elution (buffer A = 5 m*M* KH_2PO_4; buffer B = Buffer A + 1 *M* sodium chloride) at 20 m*M* salt/min and a flow-rate of 1 ml/min, following a 10-min isocratic elution with buffer A. At pH 7.0, peptides 2–5 (+2 to +5 net charge, respectively) were eluted by the salt

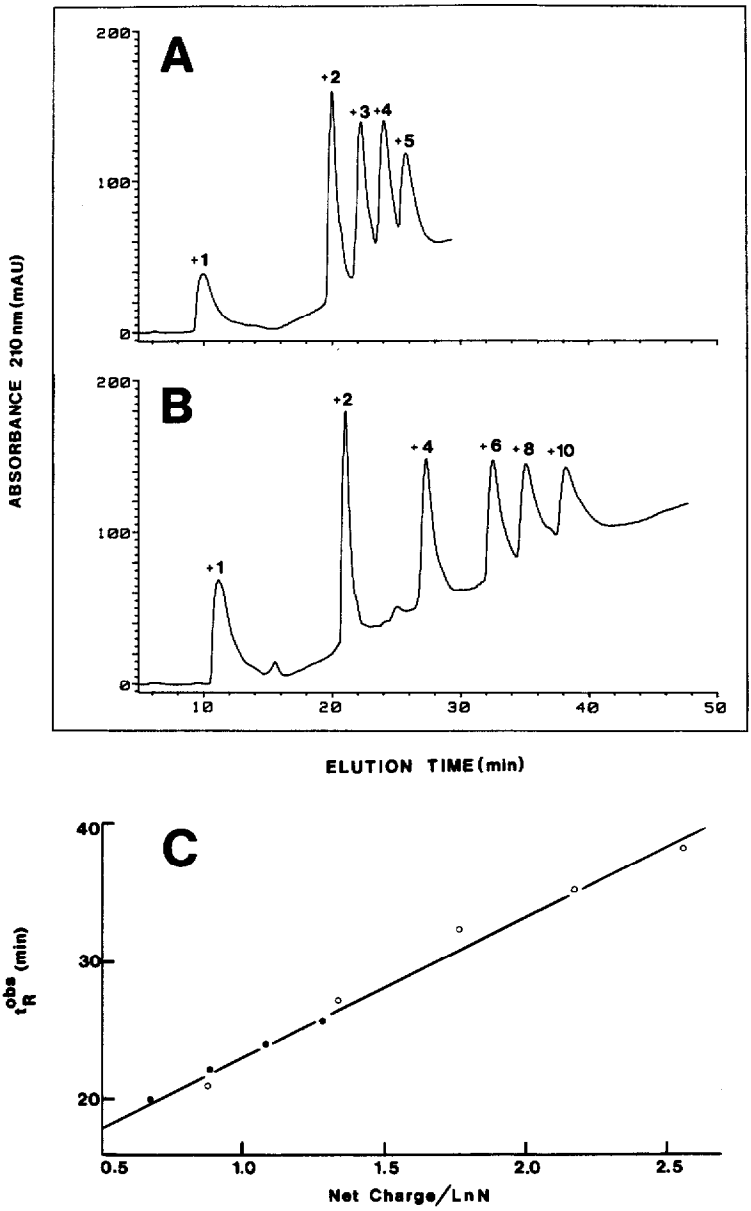


Fig. 3. Strong-cation-exchange chromatography of mixtures of synthetic peptide polymers. Conditions: column, SynChropak S300 (250 × 4.1 mm I.D.); mobile phase, linear AB gradient (20 m*M* salt/min), where buffer A is 5 m*M* KH_2PO_4 (pH 7.0), and buffer B is buffer A plus 1 *M* sodium chloride; flow-rate, 1 ml/min; temperature, 26 °C. Panel A: mixture of five synthetic peptide polymers (+1 to +5 net charge), where the sequence is Ac-(G-L-G-A-K-G-A-G-V-G)$_n$-amide, and *n* denotes the number of decapeptide repeating units (n = 1–5). Panel B: mixture of six synthetic peptide polymers (+1 to +10 net charge), where the sequence is Ac-(G-K-G-L-G)$_n$-amide, and *n* denotes the number of pentapeptide repeating units (n = 1 10). Panel C: plot of observed peptide retention time (t_R^{obs}) *versus* peptide net charge, divided by the log of the number of residues (net charge/ln *N*).

gradient, while peptide 1 (+1 net charge) was eluted during the initial isocratic elution.

An advantage of using IEC for peptides that is not shared by proteins is that, under benign mobile-phase conditions, the overall net charge on a peptide is generally fully expressed[2]. Indeed, Mant and Hodges showed that, under benign conditions [linear potassium chloride gradient (5 m*M*/min) in 5 m*M* KH_2PO_4 buffer], the elution times of several basic peptides (9–21 residues) on the cation-exchange column described above were linear with respect to their charges at pH 3.0 and pH 6.5. Similar observations have been made for a series of acidic peptides (−2 to −5 net charge) chromatographed on a strong-anion-exchange column.

The retention times of the four peptide standards retained by the IEC column (peptides 2–5) were also found to vary linearly with their net charges (+2 to +5, respectively). However, recent observations in this laboratory have suggested that charge density is also a factor affecting the retention behaviour of basic peptides on cation-exchange columns. To investigate this further, another set of peptide polymers was synthesized (Ac-[G-K-G-L-G]$_n$-amide, where n = 1, 2, 4, 6, 8 and 10, *i.e.*, 5–50 residues), with an overall hydrophobicity similar to that of the standards shown in Fig. 3a, but a higher charge density (+2/10 residues, as opposed to +1/10 residues for the initial polymer series). Fig. 3b shows the elution profile of this new polymer series on the S300 column, obtained under the same chromatographic conditions as the elution profile shown in Fig. 3a. Although the elution times of the five peptides retained by the column (+2 to +10 net charge) demonstrate satisfactory linearity with net charge (Fig. 3b) comparison of this elution profile with that in Fig. 3a clearly shows a charge density effect. For instance, the 50-residue peptide (+5 net charge) of the initial polymer series (Fig. 3a) is not retained as long as the 20-residue peptide (+4 net charge) of the new peptide series (Fig. 3b). From Fig. 3c, it can be seen that dividing the net charge of the peptides from the two polymer series by the logarithm of the number of residues they contain (ln N) and plotting this value against the observed retention time (t_R^{obs}) results in a single, straight-line plot, *i.e.*, plotting t_R^{obs} *versus* net charge/ln N instead of simply net charge corrects for charge density and peptide chain length. Additional confirmatory data have been collected to test this relationship more rigorously and will be submitted for publication in the near future.

These results, useful in correlating the overall net charge on a peptide at a given pH with its amino acid composition, have been used in the computer program to predict peptide retention times at pH 7.0 in cation-exchange chromatography.

Reversed-phase chromatography. Though excellent resolution of peptide mixtures may be obtained at both acidic or neutral pH, the majority of researchers have carried out RPC at pH values <3.0, using volatile buffers and linear AB gradients where A = 0.1% aq. TFA and B = 0.1% TFA in acetonitrile[2,3]. Reversed-phase silica-based packings may contain surface silanols which act as weak acids and are ionized above pH 3.5–4.0[9]. These negatively charged silanols may interact with the basic residues of peptides chromatographed on RPC columns and have an adverse effect on resolution, characteristically producing long retention times and peak broadening. Apart from the suppression of silanol ionization, acidic conditions are reasonable, because silica-based columns are more stable at low pH than at pH values close to neutrality.

As well as differing in selectivity, RPC columns may also vary in performance

characteristics due to non-specific interactions. Mant and Hodges[12], using synthetic peptide standards, have demonstrated non-specific interactions even at pH 2.0 with columns from different manufacturers, and have developed procedures to monitor the degree of these interactions at higher pH values.

The ability to separate peptides closely related in hydrophobicity (*e.g.*, differing by only one methyl group) should be a prerequisite of a reversed-phase column[2,3]. A mixture of five synthetic decapeptide RPC standards, S1–S5, has proved extremely useful in monitoring the peptide separation capabilities of reversed-phase columns[13–16]. The hydrophobicity of the standard increases only slightly between S2 and S5, between S2 and S3 there is an increase of two methyl groups, and between S4 and S5 there is an increase of an isopropyl group-enabling very precise determination of the resolving power of a reversed-phase column. Peptide S4 has also been used extensively in this laboratory as an internal peptide standard to correct for column and instrumentation variations in peptide retention time predictions for RPC[17–20].

Several research groups have determined sets of coefficients for predicting peptide retention times in RPC, on the assumption that the chromatographic behaviour of a peptide is mainly or solely dependent on amino acid composition, and this assumption holds well enough for small peptides (up to *ca.* 15 residues)[17,18,21–28]. Retention values have generally been obtained by computer-calculated regression analyses of the retention times of a wide range of peptides of varied composition[21–27]. These methods have not been successful for two reasons: first, the sampling of peptides has not been large enough to ensure a high frequency of occurrence of all amino acid residues; second, if there is a polypeptide-chain-length effect on retention behaviour, this effect would be averaged into values obtained for the retention coefficients, resulting in substantial errors[20,28]. The most precise set of coefficients currently available was reported by Guo *et al.*[17], who measured the contribution of individual amino acid residues to the retention time of a synthetic model peptide at a given chain length. The eight residue octapeptide sequence, Ac-G-X-X-L_3-K_2-amide, was substituted at position X by all 20 naturally occurring amino acids. The predicted retention time (τ) of a peptide in RPC was then equal to the sum of the retention coefficients (ΣR_c) for the amino acid residues in the peptide, plus the time correction (t_s) for the internal peptide standard (S4; see above).

Several researchers have noted that peptides larger than 15–20 residues tended to be eluted more rapidly than predicted from hydrophobic considerations alone[2,20,23–25,28–32]. Fig. 4a demonstrates an exponential relationship between peptide chain length and peptide retention time in RPC of the series of five size-exclusion standards (10–50 residues), described previously (Figs. 2a, b and 3a), on a SynChropak RP-P C_{18} column. The effect on peptide retention of increasing peptide length decreases progressively with each ten-residue addition. The intimate relationship between peptide hydrophobicity and chain length and their combined effect on peptide retention behaviour, already detailed by Mant *et al.*[20], is illustrated in Fig. 4b. Plotting the difference between predicted (τ) and observed (t_R^{obs}) retention time *versus* the product of peptide hydrophobicity (expressed as ΣR_c, the sum of the coefficients of Guo *et al.*[17]) and the logarithm of the number of residues (ln N) results in a straight line plot. This relationship holds for peptides much greater in hydrophobicity than the size-exclusion polymer series, and is consistent for RPC columns of varying dimensions and hydrophobic functionalities (*n*-alkyl chain length) and ligand density[20].

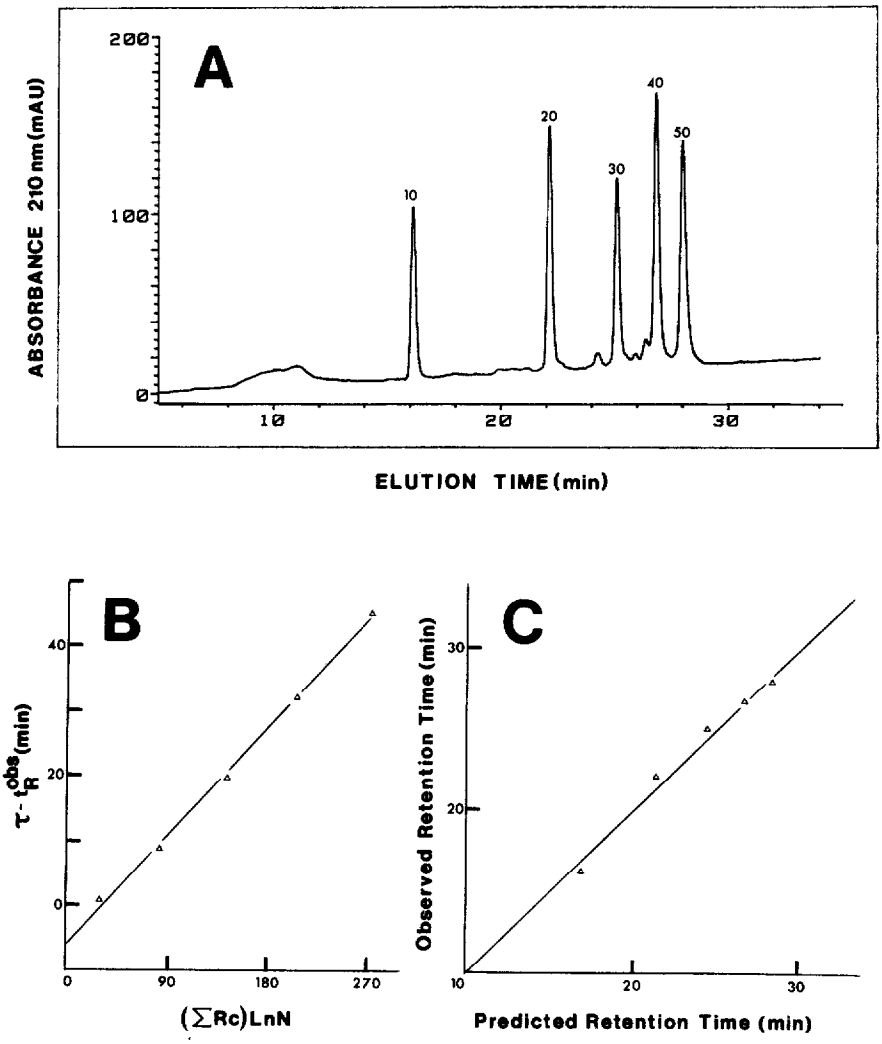


Fig. 4. RPC of a mixture of synthetic peptide polymers. Conditions: column, SynChropak RP-P C_{18} (250 × 4.6 mm I.D.); mobile phase, linear AB gradient (1% B/min), where eluent A is 0.1% aq. TFA and eluent B is 0.1% TFA in acetonitrile; flow-rate, 1 ml/min; temperature, 26°C. Panel A: elution profile of five peptide polymers (10–50 residues). Panel B: plot of predicted minus observed retention time ($\tau - t_R^{obs}$) *versus* the sum of the retention coefficients (ΣR_c) times the log of the number of residues (ln N). Panel C: correlation of predicted and observed retention times of the peptide polymers. The sequence of the peptide polymers is Ac-(G-L-G-A-K-G-A-G-V-G)$_n$-amide, where n denotes the number of decapeptides (n = 1–5).

Using the slope and intercept from such a plot, we can predict the retention behaviour of peptides up to 50 residues in length. Fig. 4c shows the good correlation between predicted and observed retention times of the polymer series, once peptide chain length has been taken into account. This predictive method formed the basis for simulation of reversed-phase peptide elution profiles by the computer program.

Though Figs. 2–4 only show the general concepts used to develop the computer

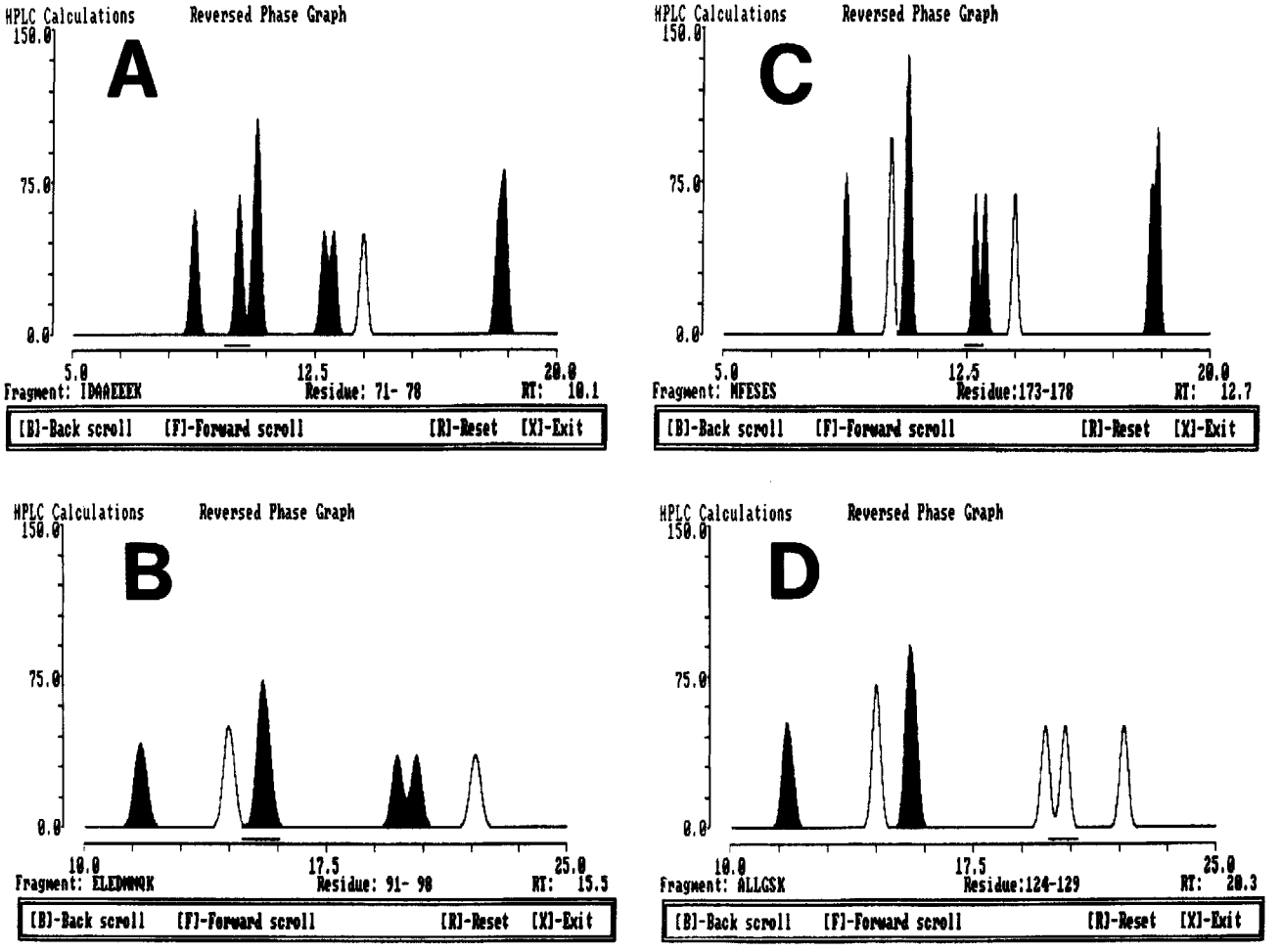


Fig. 5. Computer simulation of a tryptic digest of rabbit skeletal fast troponin I (TnI). Panels A and C represent RPC elution profiles, obtained with a linear AB gradient (1% B/min) at 1 ml/min, where eluent A is 0.1% aq. TFA and eluent B is 0.1% TFA in acetonitrile. Panels B and D represent elution profiles obtained with a linear gradient of 0.5% B/min. Panels A and B represent elution profiles obtained with a peptide standard peak-width ($W_{\frac{1}{2}}$) and peak height parameters of 0.28 min and 87 mAU, respectively. Panels C and D represent elution profiles obtained with standard parameters of 0.20 min ($W_{\frac{1}{2}}$) and 122 mAU (peak height). Specified peptide resolution is 1.0. Shaded peak areas denote unresolved peptides. The zoom option of the program has been selected to show only a section of the protein digest.

program, the success of these approaches to the prediction of peptide retention times has been well documented by our laboratory on the basis of a large number of peptides.

Computer simulation of peptide elution profiles

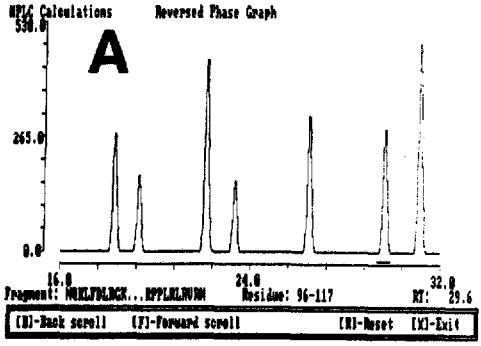
A demonstration of all the simulation capabilities of the Pro Digest-LC computer program would require considerable space. For the present, we simply wish to demonstrate several of the features of the Pro Digest-LC program in as concise a manner as possible. To this end, we have chosen to illustrate the protein digest and peptide mapping features of the program, coupled with RPC of the peptide fragments. As outlined in the Introduction, efficient separation of protein digests is an important consideration in many research disciplines. In addition, the flexibility and resolving power of RPC makes it the ideal mode of HPLC for demonstrating many of the simulation capabilities of the Pro Digest-LC program.

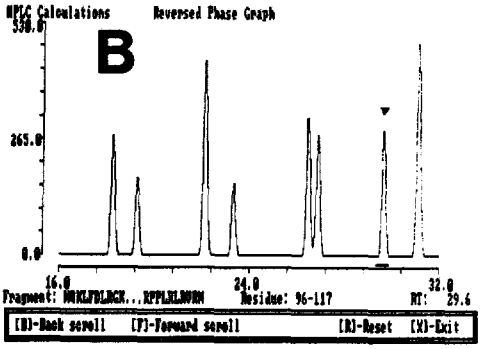
Tryptic digest of proteins. Prior to running the tryptic digest feature, peak width at half height, $W_{\frac{1}{2}}$, and peak height parameters for a known amount of our peptide standard, S4, were entered into the "Standards" section of the main menu (Fig. 1a), together with the desired resolution (1.0, in this case). The program will then refer to these stored parameters when it simulates the protein digest elution profile.

From the Pro Digest-LC main menu (Fig. 1a), the "Digest" option was chosen to enter the protein menu (Fig. 1b). A stored protein file (rabbit fast skeletal troponin I [TnI]) was loaded (Load/view/edit protein file) at a sample level of 5 nmoles. Troponin I is an inhibitory protein involved in the regulation of muscle contraction. The quantity of protein loaded can, of course, be varied, depending on the researcher's requirements. The tryptic digest option was chosen, followed by a request for a simulated elution profile of the resulting peptide fragments in reversed-phase chromatography. Fig. 5 demonstrates sections of the simulated elution profiles of the tryptic digest of TnI, obtained by RPC.

The profiles shown in Fig. 5a and b represent simulated results for a column (column 1) different from that used in Fig. 5c and d (column 2). For column 1, the $W_{\frac{1}{2}}$ and peak-height parameters entered into the Standards file (Fig. 1a) were 0.28 min and 87 mAU, respectively; in contrast the parameters entered for column 2 were 0.20 min and 122 mAU, respectively. This is not an unusual range of peak width parameters to be investigated, since we have observed the $W_{\frac{1}{2}}$ and peak height of the peptide standard, S4, to change by a factor of 2 between C_8 and C_{18} columns. If one routinely runs peptide standards, one can enter peak width and height information into the program to see how well a particular column would perform a peptide separation.

The entire tryptic digests profiles are not shown in Fig. 5. The zoom option has been selected to narrow the simulated chromatogram down to a small section of the whole peptide elution profile. Fig. 5a and c illustrate the profiles for columns 1 and 2, respectively, which are predicted to be obtained with a 1% acetonitrile/min linear gradient (aq. TFA to TFA in acetonitrile gradient) at a flow-rate of 1 ml/min; Fig. 5b and d illustrate the profiles for a 0.5% acetonitrile/min linear gradient. The separation of the peptides is improved by using a shallower gradient-rate or a column that provides decreased peak widths. This can best be observed by noting the position of the cursor. For column 1, the separation of peptide 71–78 (*i.e.*, residues 71–78) (Fig. 5a) from peptide 91–98 (Fig. 5b) is shown. The position of the cursor is con-


HPLC Calculations
Reversed Phase Graph
A
530.0
265.0
0.0
16.0
24.0
32.0
Fragment: NHKLFDLRGK...KPPLRLRNRN
Residue: 96-117
RT: 29.6
[B]-Back scroll
[F]-Forward scroll
[R]-Reset
[X]-Exit


HPLC Calculations
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B
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Fragment: NHKLFDLRGK...KPPLRLRNRN
Residue: 96-117
RT: 29.6
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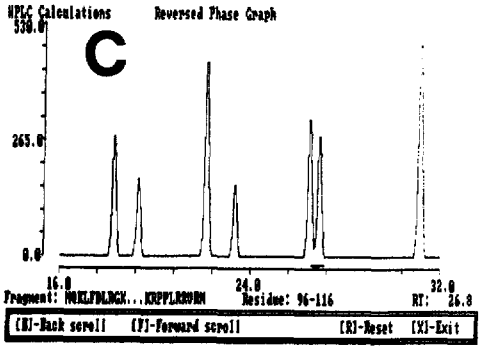
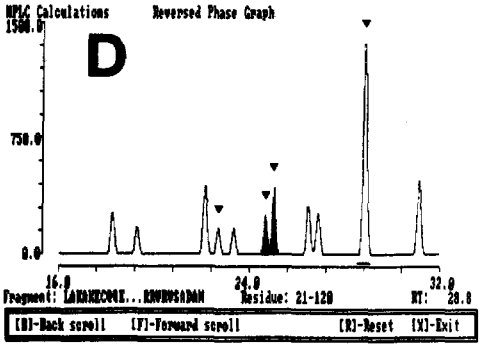

HPLC Calculations
Reversed Phase Graph
C
530.0
265.0
0.0
16.0
24.0
32.0
Fragment: NHKLFDLRGK...KPPLRLRNRN
Residue: 96-116
RT: 26.8
[B]-Back scroll
[F]-Forward scroll
[R]-Reset
[X]-Exit


HPLC Calculations
Reversed Phase Graph
D
1500.0
750.0
0.0
16.0
24.0
32.0
Fragment: LATAKECOKE...KNVDPSARAN
Residue: 21-120
RT: 28.8
[B]-Back scroll
[F]-Forward scroll
[R]-Reset
[X]-Exit

trolled by scrolling it forwards ("F") or backwards ("B"). As the gradient-rate is decreased from 1% acetonitrile/min (Fig. 5a) to 0.5% acetonitrile/min (Fig. 5b), the separation improves. Peaks are shaded to show that peptides are not resolved at a specified resolution (1.0) in the present example. Thus, the peak marked by the cursor in Fig. 5B contains more than one peptide. If the cursor is moved forward, all the peptides under a shaded peak are listed. For column 2, the separation of peptides 173–178 (Fig. 5c) from peptides 124–129 (Fig. 5d) is shown. Peptides 71–78 and 91–98 are already resolved on this column by a linear gradient of 1% acetonitrile/min (Fig. 5c), unlike the chromatogram for column 1 under the same conditions (Fig. 5a). As the gradient-rate is lowered to 0.5% acetonitrile/min (Fig. 5d), the separation of peptides 173–178 and 124–129 is complete (at a specified resolution of 1.0). The unshaded character of the two peptides at the lower gradient-rate shows that no other peptides lie under these peptide peaks. The separation of these two peptides was not obtained on column 1, even at the shallower 0.5% acetonitrile/min gradient-rate (Fig. 5b).

All manner of permutations of sample load, gradient-rate, and flow-rate, coupled with specified standard parameters of $W_{\frac{1}{2}}$, peak height, and desired resolution may be investigated without actually chromatographing a sample.

Peptide mapping. Troponin I (TnI) is a 178-residue protein, involved in the regulation of muscle contraction. This laboratory has identified the region of amino acid residues 105–114 being responsible for the inhibitory activity of TnI[33,34]. Through the use of peptide analogues of the inhibitory region of TnI, Talbot and Hodges[35] have demonstrated that differences in relative inhibitory activity of rabbit skeletal fast and cardiac TnI can be at least partially and perhaps solely explained by a single amino-acid insertion, a leucine residue, between positions 112 and 113 of the skeletal sequence. To investigate this point further, it would be appropriate to engineer, by site-specific mutagenesis, a skeletal protein containing the cardiac leucine insertion. Following site-specific mutagenesis, peptide mapping would be carried out on the engineered protein to verify that the change has indeed been made. It would also be of interest to isolate the inhibitory peptides from the mutant and native protein for biological activity measurements. Thus, being able to predict the retention behaviour of these peptides relative to the other peptides in the digest would be advantageous.

The mutant protein was created by inserting a leucine residue between positions 112 and 113 of the native TnI sequence (Fig. 1C–E) and saving this sequence as TnI-179 (for 179 residues, as opposed to 178 residues in the native sequence). Cyano-

Fig. 6. Computer simulation of peptide mapping. All panels represent simulated RPC elution profiles, obtained with a linear AB gradient (1% B/min) at a flow-rate of 1 ml/min, where eluent A is 0.1% aq. TFA and eluent B is 0.1% in acetonitrile. Panel A: cyanogen bromide digest of a mutant protein, obtained by site-specific mutagenesis of rabbit skeletal fast troponin I (TnI); this mutant protein, the result of a leucine insertion between position 112 and 113 is denoted as TnI-179 in the text. Panel B: overlay of cyanogen bromide digests of a mutant protein (TnI-179) and the native TnI protein; the inverted triangle identifies the mutant peptide fragment. Panel C: cyanogen bromide digest of the native TnI protein. Panel D: overlay of cyanogen bromide digests of protein isoforms (rabbit skeletal fast and slow troponin I); the inverted triangles identify the fragments obtained from the slow troponin I protein. Peptide standard peak width ($W_{\frac{1}{2}}$) and peak height parameters are 0.14 min and 174 mAU, respectively; specified peptide resolution is 1.0. (These are the default values already present in the program.) Shaded areas denote unresolved peptides. The zoom option of the program has been selected to show only sections of the protein digests.

gen bromide cleavage was chosen, followed by a request to simulate the reversed-phase elution profile at a gradient-rate of 1% acetonitrile/min and a flow-rate of 1 ml/min. A section of the simulated profile, following operation of the zoom option, is shown in Fig. 6a. The cursor shown by the bar below the peak at 29.6 min, marks the inhibitory fragment (residues 96–117) of the mutant protein. Rabbit skeletal fast TnI was then loaded (5 nmol), followed by cyanogen bromide cleavage and selection of the RPC option (1% acetonitrile/min and 1 ml/min) (Fig. 6c). The cursor marks the inhibitory fragment (residues 96–116) of the native protein. The differences in the retention times of the native fragment and the mutant fragment (26.8 min and 29.6 min, respectively), clearly show that a change in the native sequence by site-specific mutagenesis can easily be detected. To highlight this, the peptide mapping option, following cyanogen bromide cleavage of the native TnI, was used to produce the simulated chromatogram shown in Fig. 6b. This elution profile is an overlay of the cyanogen bromide cleavage fragments of both proteins (*i.e.* an overlay of Fig. 6a and c). Any mutant fragment not observed in the native sequence is identified by an inverted triangle above the peak (Fig. 6b).

The isolation of protein isoforms is very common, and different isoforms have been shown to have different biological activities. It has been shown, for instance, that rabbit skeletal slow muscle TnI inhibits rabbit skeletal actomyosin ATPase poorly, compared to rabbit skeletal fast muscle TnI[36]. Thus, even though the inhibitory region 105–114 is identical in both proteins[37], the inhibitory activities are different. Since the slow and fast TnI proteins are very homologous, and since they come from the same tissue source (although they occur in different percentages), extensive purification is often necessary if pure proteins are required. However, if it is desired to isolate only biologically active regions, *i.e.*, peptide fragments of the proteins, this extensive purification may not be required. A digestion of a mixture of the two proteins, followed by HPLC, can be quickly simulated by the Pro Digest-LC program. The cyanogen bromide fragments of the two proteins were overlaid, producing the simulated reversed-phase elution profile shown in Fig. 6d. The inverted triangles above the peaks denote peptide fragments of the slow TnI protein. Table I summarizes the information obtained when a listing of the fragments obtained by cyanogen bromide cleavage is requested. The RPC retention times of the fragments are given, as well as the residue numbers and sequences of the peptides. "Yes" in the column marked "Mutant" denotes a mutant fragment (slow TnI fragments, in this example). The two inhibitory peptides (residues 21–120 for slow TnI and residues 96–116 for fast TnI) are readily separated by using the standard conditions of RPC.

CONCLUSIONS

The Pro Digest-LC computer software program assists scientists in devising methodologies for the analytical separation and purification of biologically active peptides and peptide fragments from enzymatic and chemical digests of proteins by size-exclusion, ion-exchange, and reversed-phase HPLC. The program allows changing the sample volume, sample quantity, flow-rates, gradients, and desired resolution. If further optimization is required, optimization programs, such as those described by Dolan and Snyder[38] may be applied following Pro Digest-LC.

Pro Digest-LC is also an interactive program, in that researchers can chroma-

tograph peptide standards on their columns and enter values of retention time, peak width, peak height, and other parameters in the program, in order to adjust the simulation to their particular column. Any deviations from the simulation can provide the researcher with valuable information on the completeness of protein digestion or unique conformations of a particular fragment. Above all, Pro Digest-LC is user friendly and should be very useful to workers involved in peptide and protein research.

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