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Micropreparative high-performance liquid chromatography of proteins and peptides*

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Abstract: The use of short microbore reversed phase and ion-exchange HPLC columns in the preparation of low level (submicrogram) quantities of proteins and peptides is discussed. The sequential use of columns of differing selectivity to purify complex mixtures is described. An example is given of the use of microbore columns to purify a murine myeloid leukemia inhibitory factor prior to sequence analysis.

Keywords: *Microbore HPLC; microsequencing; protein and peptide purification; reversed phase; ion exchange.*

Introduction

Since the earliest reports of the use of high-performance liquid chromatography (HPLC) to fractionate proteins and peptides there has been an exponential increase in the use of this technique. Such methods; which can be used both analytically and preparatively, have been shown to offer unrivalled advantages in terms of speed, resolution, sensitivity and most importantly recovery. The use of controlled porosity glass supports for size exclusion [1] and ion exchange [2] separations were perhaps the earliest examples of what has proved to be an invaluable technique in the high efficiency purification of numerous molecules of biological significance. Then followed the observations in the mid 1970s that a large range of polypeptides [3–7] and some proteins [6, 8] could be readily resolved with high efficiency and excellent recovery on the microparticulate (5–10 μ) porous (6–10 nm) reversed phase supports, which were originally developed for the separation of low molecular weight compounds. These observations stimulated further empirical studies on the optimisation of supports specifically for RP-HPLC of proteins. Parameters such as the choice of alkyl chain length, end-capping reagents, pore diameter and particle size were rigorously examined. These studies culminated in the development of the current mesoporous RP-supports for protein and peptide separation which are now available from a large number of manufacturers. In spite of the ability of

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these supports to resolve complex mixtures of often closely related species [6, 9, 10], it was obvious that such supports were not a panacea for the purification of all proteins. In particular (i) a number of more hydrophobic proteins and peptides exhibit poor recoveries from these supports; (ii) the harsh elution conditions, e.g. low pH and use of high concentrations of organic solvents, result in the loss of biological activity of many labile proteins; (iii) although selectivity can be controlled by alteration of the mobile phase composition [e.g. use of trifluoracetic acid (TFA), heptafluorobutyric acid (HFBA), change of pH] the resolution obtained is not predictable or sufficient for the purification to homogeneity of trace components in complex biological mixtures.

These problems, coupled with the emergence of genetic engineering, with its obvious commercial implications has stimulated the rapid development of a range of alternative materials based on silica or rigid organic resin supports in which selectivity was obtained by tailoring the packing to specific physico-chemical characteristics of proteins (e.g. relative hydrophobicity, charge, size or biorecognition). A list of the currently available supports, their dominant functionality and typical elution conditions is given in Table 1. Further details of their use is given in the following recent articles [11, 12]. The excellent recoveries associated with this range of HPLC columns facilitates their use in multidimensional purification protocols, in which columns with differing selectivity are used sequentially. Design of such multidimensional purification systems is greatly facilitated by the availability of highly specific and sensitive bioassays. Such assays enable criteria such as biological stability to the possible chromatographic conditions, recovery from specific supports and control and optimisation of selectivity to be evaluated rapidly. In addition, the monitoring of protein heterogeneity by sensitive gel electrophoretic procedures (e.g. Pharmacia Phast Sep), coupled with considerations such as the solvent compatibility for trace enrichment between consecutive chromatographic steps (enabling samples to be loaded without further manipulation and thus, avoiding non-specific losses) facilitates logical design of such protocols. Purification factors in excess of 50,000 may be readily obtained [13–16] permitting the isolation of biologically important proteins (e.g. growth factors and membrane receptors) which are only present in the starting material in trace quantities.

Recent advances in protein sequencing technology now permit sequence information to be obtained from as little as 10–20 pmol of material. From a knowledge of the genetic code [17] such protein sequence can be translated into a corresponding oligonucleotide probe enabling the isolation of the specific gene encoding for the protein of interest. Using recombinant DNA techniques the gene can be used to produce amplified quantities of the corresponding protein, thus allowing structure–function, or even clinical studies to be undertaken.

Table 1
Supports for the HPLC of proteins

Mode	Dominant functionality	Typical elution conditions
Reversed-phase	Relative hydrophobicity	Low pH/Organic solvent
Hydrophobic interaction	Relative hydrophobicity	Decreasing salt gradient
Ion exchange	Charge	Increasing salt gradient
Hydroxyapatite	Structure/Charge	Increasing gradient of phosphate ions
Affinity	Structure	Deformation or competition
Size exclusion	Apparent size	Buffer compatible with protein

A major constraint in the above approach is the ability to micromanipulate (e.g. concentrate, buffer exchange, reduce and alkylate, fragment etc.) proteins at the sub-nanomole level in high yield in a form suitable for microsequence analysis. For this technique samples must be in relatively small volumes (less than 100 μl) to facilitate application to the sample disc of the gas phase sequencer. It is now generally accepted that, when working at low (sub-nanomole) protein levels, manipulations via classical techniques (e.g. lyophilisation, dialysis, organic solvent precipitation) result in unacceptably high losses [18–21] and must be avoided. We have therefore developed [22, 23] procedures using short (less than 10 cm) microbore (less than 2.1 mm ID) columns for the micromanipulation of protein samples, purified by either multidimensional HPLC or SDS-polyacrylamide gel electrophoresis.

Experimental

Theory

To operate microbore columns at linear flow velocities equivalent to those used with larger bore columns (typically 1 ml min^{-1} for a 4.6 mm ID column), the flow rate is decreased in proportion to the cross-sectional area of the column (i.e. to approximately 50 μl min^{-1} for a 1 mm ID column). If the chromatographic efficiency is maintained (i.e. the peak bandwidth is the same on both columns) which should be true if both columns have similar efficiency and are not overloaded with sample mass then it necessarily follows that peak volumes on the microbore column will be concomitantly reduced. For concentration dependent detectors the resultant increase in eluent concentration will result in an equivalent increase in detector sensitivity, giving a theoretical increase of 20-fold for the 1 mm ID column compared with a 4.6 mm ID column using the flow rates quoted above. Such increases in sensitivity are obtainable in practice [24].

Sample volume

For microbore columns to be of practical use it is mandatory that the sample volume which can be loaded is not limiting. Ideally, partially purified samples from conventional HPLC (4.6 mm ID) columns (in typical volumes of 1 ml or greater), or even samples from open columns must be loaded *in toto*. To many this appeared to be a major limitation in the application of microbore technology. Fortunately, proteins and peptides display virtually infinite capacity factors (K') below the critical secondary solvent concentration required for their elution from interactive supports. It is therefore possible to concentrate large sample volumes by trace enrichment onto strongly interactive supports (e.g. reversed-phase, ion exchange or hydrophobic interaction). The retained proteins or polypeptides are then recovered by gradient elution. In this manner we have been able to load sample volumes as large as 50 ml onto a 1 mm ID column before recovering the retained proteins virtually quantitatively in peak volumes of approximately 50 μl (1000-fold concentration).

Column length

It has been demonstrated previously [25, 26, 27] that column length plays a negligible role in resolving proteins on strongly interactive supports. Furthermore protein recoveries improve with short columns [26], possibly due to a reduction in the amount of packing and therefore, reduced losses by irreversible adsorption on the support. An

important advantage of the use of short columns for microbore HPLC is the proportional reduction in operating backpressures which allows large sample volumes to be rapidly loaded at high flow rates (we routinely load samples at flow rates of 1–2 ml min^{-1}). The retained materials can then be recovered by gradient elution using the low flow rates (50–100 μl min^{-1}) applicable to microbore columns.

Column loading capacity

For short microbore HPLC columns to be practically viable in the purification and micromanipulation of proteins and peptides for microsequence analysis the columns must be capable of chromatographing with high efficiency representative loads (0.5–5 μg, 25–250 pmol for a protein of 20,000 Da). The total amount of protein which can be loaded onto a column is a function of the column dimensions, the physical characteristics of the support material and the nature of the protein itself. We have previously demonstrated [22] that the trace enrichment capacity of a 30 × 2.1 mm ID column packed with a 30 nm pore size reversed phase (C8) support is in excess of 4 mg for two test proteins: cytochrome *c* and lysozyme. However, at such loadings the column was obviously overloaded as evidenced by the volume of the protein peak (approximately 1.4 ml). It can be seen from the data in Fig. 1 that the optimum protein loads for 1 and 2.1 mm RP-HPLC (30 nm pore, 7 μ particle, C8) columns are 2.5–5 and 10–20 μg, respectively. Such capacities are indeed compatible with the use of these columns in the preparation of protein and peptide samples for sequence analysis. Comparison of the results from columns of 3 and 10 cm in length indicates that the optimum peak volume is independent of column length until the critical load is exceeded when the shorter column

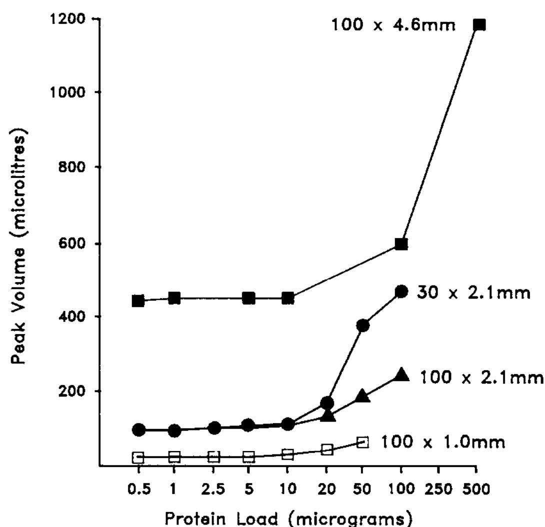


Figure 1

The effect of protein load on eluent peak volumes for columns of different dimensions. Sample: α-lactalbumin. Column: Brownlee RP-300 (30 nm, 7 μ, C8). The column dimensions are indicated in the figure. Proteins were eluted using a linear 60 min gradient between a primary solvent of 0.15% (v/v) aqueous trifluoroacetic acid and a secondary solvent of 60% acetonitrile/40% water containing 0.125% (v/v) trifluoroacetic acid. The flow rates for the 4.6 mm ID, 2.1 mm ID and 1 mm ID columns were 1000, 200 and 50 μl min^{-1}, respectively. Column temperature was 45°C.

overloads more rapidly with increasing mass. However, even under overload conditions there can, if selectivity permits, still be significant advantages in terms of total recovery volume and sensitivity of detection in using columns of smaller ID (note the 50 μg load is eluted from the 1 mm ID column in a volume of only 70 μl).

Resolution and recovery on short microbore columns

The use of short microbore columns to analyse a mixture of proteins at high sensitivity (25 ng) is demonstrated in Fig. 2. The resolution achieved with this column (50 × 1 mm ID) is virtually identical to that obtained on a conventional column packed with the same support (data not shown). It can be seen that peak volumes under the elution conditions used in this example (flow rate 50 μl min^{-1}, linear 1% min^{-1} gradient of acetonitrile) are less than 50 μl. Such volumes can be applied directly to the sample disc of the gas phase sequencer, avoiding any extra manipulations during which sample loss might be experienced.

Recovery at the sub-nanomole level from these columns as evidenced by studies with radioactive tracer (and confirmed by a lack of peak ghosting) is routinely in excess of 90% for those proteins amenable to RP-HPLC. In an attempt to simulate recoveries in a multidimensional purification strategy we have investigated the repetitive yield of 20 pmol of protein chromatographed on a microbore RP-HPLC column. The protein eluting from the column was diluted two-fold with primary solvent (in the injection syringe) to lower the concentration of secondary solvent sufficiently to permit trace enrichment at the next cycle, and reinjected onto the column. After four cycles of microbore RP-HPLC, 60% of the original sample was recovered, indicating a total system repetitive yield of 84%.

For those proteins not ideally suited to RP-HPLC (e.g. very hydrophobic or hydrophilic materials), or where RP-HPLC provides inadequate resolution, we have investigated the use of packings with alternative functionality (e.g. anion exchange, cation exchange, hydrophobic interaction) packed into short microbore columns. The

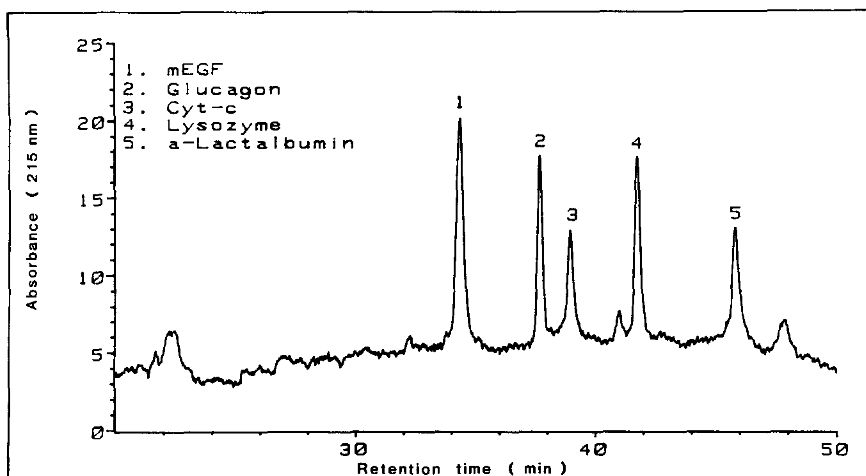


Figure 2
The separation of low level protein standards (25 ng) on a short microbore RP-HPLC column. Column: Brownlee RP-300 (50 × 1 mm). The protein sample (25 ng per protein) was loaded onto the column at 2 ml min^{-1} prior to gradient elution at 50 μl min^{-1} using the conditions described in Fig. 1.

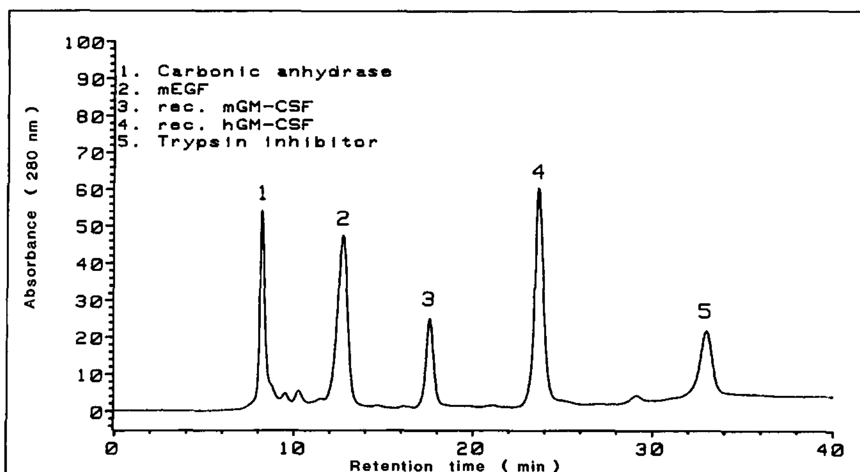


Figure 3
The separation of protein standards by microbore anion exchange. Column: Pharmacia Mono Q (30 × 1.6 mm). The protein sample (1–2 μg/protein) was loaded onto the column at 0.5 ml min^{-1} prior to gradient elution at 50 μl min^{-1} using a linear 30 min gradient from 10 to 500 mM ammonium bicarbonate. Column temperature was 45°C.

use of an ion exchange column for protein separation is shown in Fig. 3. Peak volumes of 40–100 μl were obtained. Even though proteins eluting from these ion exchange columns contain salts the small volumes loaded onto the gas phase sequencer enable sequence information to be obtained (R. J. Simpson, U. Hellman, personal communication). In similar repetitive recovery experiments to those described above for RP-HPLC columns repetitive yields of 92% and 83% were observed with anion and cation exchange columns (30 × 1.6 mm ID), respectively.

Recently, we have evaluated the potential of non-porous supports, based on either 1.5 μ silica particles (Merck Monosphere RP) [28] or 7 μ polymethacrylate spheres (Biorad Microanalyser MA7P) [29] for use in micropreparative HPLC of peptides and proteins. The ability of these and similar supports [30, 31] to achieve very rapid, high resolution separations has been reported. However, in these reports high flow rates were used to achieve these goals, and resultant peak volumes were larger than those ideally suited for microsequence analysis. The effect of flow rate on eluant peak volume for a protein standard — ribonuclease A is shown in Fig. 4. A comparison was made between the non-porous Monosphere RP, packed into a 30 × 4 mm ID column and Brownlee RP-300 packed into either 30 × 4.6 mm or 30 × 2.1 mm ID columns. The use of the non-porous material (Monosphere) has resulted in the protein eluting in a smaller volume than from the porous column of similar dimensions (30 × 4.6 mm ID). However, the smallest peak volumes were obtained with the porous material using the 2.1 mm ID column. It would obviously be of interest to evaluate the performance of the non-porous support in microbore columns. Like its porous counterpart, such a column would need to be compatible with loadings in the range of 10–20 μg, and ideally should have operational back pressures which allow large volume samples to be loaded at high flow rates. From the data of Unger *et al.* [28], who demonstrated a column capacity in excess of 500 μg for a 36 × 8 mm non-porous column, we can calculate that a 2 mm ID column packed with this material would have a sample load capacity in excess of 30 μg.

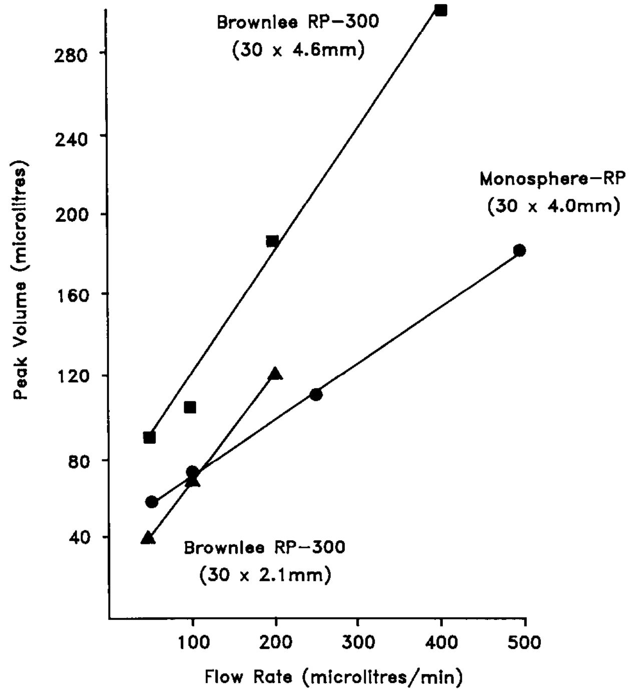


Figure 4
The relationship between peak volume and flow rate for porous and non-porous silica packings. Sample: Ribonuclease A (3 μg). Columns were eluted using the solvent conditions described in Fig. 1 at the flow rates indicated. Peak volumes were determined from the chromatographic trace.

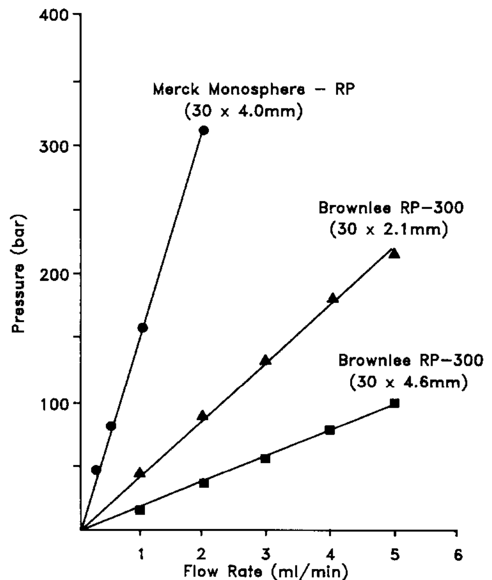


Figure 5
The relationship between flow rate and pressure for porous (7 μ, 30 nm C8) and non-porous (1.5 μ, C8) silica packings. The packing materials and column dimensions used are indicated in the figure. Measurements were obtained using a mobile phase of 0.15% (v/v) aqueous in trifluoroacetic acid. Column temperature was 45°C.

With respect to the expected operating pressure, the data in Fig. 5 indicates that a 30 × 2 mm Monosphere column would have an operational back pressure of approximately 320 bar at 1 ml min^{-1}, allowing sample trace-enrichment at this flow rate.

Sensitivity of detection

From the data presented in Figs 2 and 3, it is readily apparent that the use of microbore columns as described herein facilitate detection of protein at the 5–10 ng level. Indeed using other potentially more specific and sensitive methods (e.g. endogenous tryptophan fluorescence) [22] even greater sensitivity can be expected. Such sensitivity of detection allows small aliquots of a sample to be used on microanalytical systems optimisation prior to committing the bulk of the sample to preparative separation on the same column (it should be remembered that even at these levels, the "analytical" samples may be recovered and added back to the bulk of the sample if really precious).

Whilst on the question of detection, the use of the diode array detector in tandem with microbore columns, with their inherent sensitivity, for identifying aromatic amino acid containing peptides for sequence analysis warrants consideration. Tryptophan containing peptides are potentially useful for the construction of oligonucleotide probes of low degeneracy since tryptophan has a unique codon. Such peptides are readily identified from their second derivative spectra since they display a distinct minima at 290 ± 2 nm [32, 33]. This is illustrated in Fig. 6 which shows a microbore RP-HPLC separation of a tryptic digest of murine epidermal growth factor. The C-terminal pentapeptide (EGF_{49-53}) which contains two tryptophan residues (RT 22.98 min) is clearly

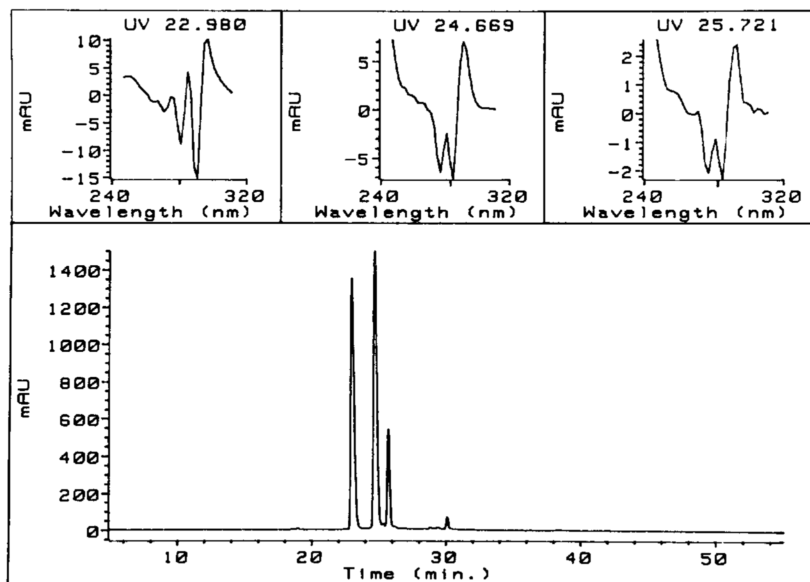


Figure 6
Identification of tryptophan and tyrosine containing peptides by second order derivative spectroscopy. A tryptic digest of murine epidermal growth factor was separated on a Brownlee RP-300 column using the gradient elution conditions described in Fig. 1 at a flow rate of 100 μl min^{-1} (lower panel). Spectra of eluting peptides was obtained using an on-line diode array detector (Hewlett-Packard 1090). The tryptophan containing peptide (retention time 22.98 min) shows a characteristic minima at 290 nm, whilst the minima for the two tyrosine containing peptides (24.67 and 25.72 min) is 282 nm (top panel).

identifiable. By contrast the other two peaks (identified as EGF_{1-48} and EGF_{1-45} by amino acid analysis) both contain tyrosine residues (minima 280 ± 2 nm).

Sequential use of RP- and ion exchange microbore HPLC columns

The potential of using RP- and ion exchange (IEX) microbore columns in the multidimensional purification of complex mixtures is illustrated in Fig. 7. Used

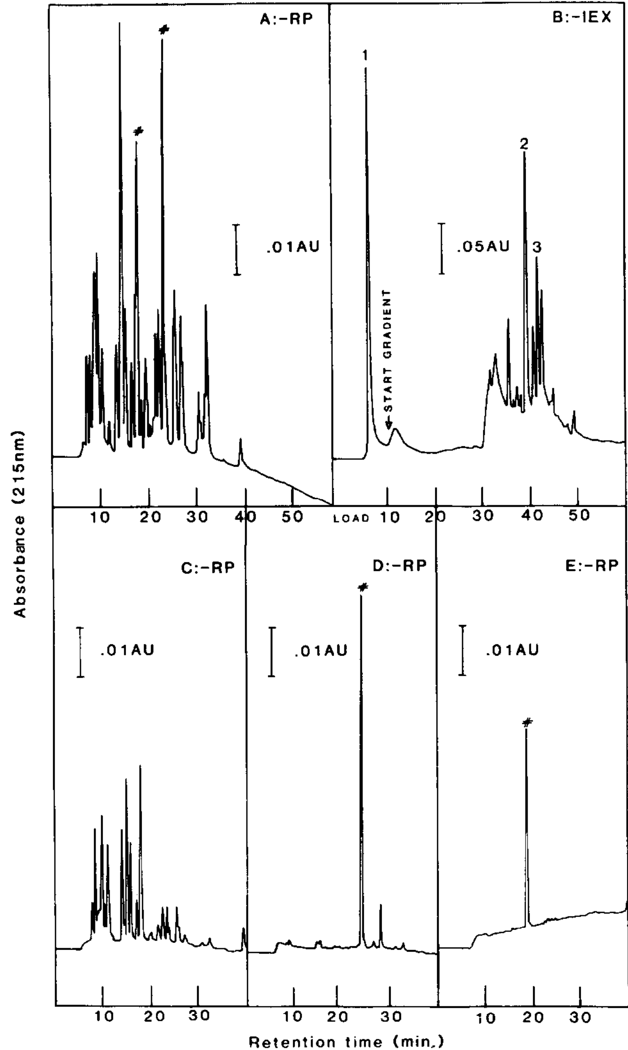


Figure 7
Multidimension at HPLC of a tryptic digest of β-lactoglobuliln. Panel A: Separation of the digest by RP-HPLC on a Brownlee RP-300 column (30 × 2.1 mm). Chromatographic conditions as for Fig. 6. Panel B: Separation of the digest by anion-exchange HPLC on Pharmacia Mono Q (30 × 1.6 mm). Peptides were separated using a linear 50 min gradient between 20 and 500 mM sodium chloride (pH 6.5). The peaks indicated were recovered manually for rechromatography on the RP-HPLC system. Panel C: Rechromatography of the non-retained peak (1) from the Mono Q column (Fig. 7B) on RP-HPLC using the conditions in Panel A. Panel D: Rechromatography of peak 2 from Fig. 7B. Panel E: Rechromatography of peak 3 from Fig. 7B. The peaks corresponding to the purified materials from D and E are indicated with an # on the parent digest; Fig. 7A.

separately, the resolution on either of the columns is insufficient to achieve the complete separation of many of the peptides from a tryptic digest of β-lactoglobulin. However, as has been recently demonstrated with conventional columns [34, 35] the tandem use of the two columns readily allows complete resolution of a number of components of this mixture. A comparison of the peak heights of material which has been rechromatographed on the RP-HPLC column following IEX purification (e.g. Fig. 7D) with that in the parent chromatograms (Fig. 7A) suggests that recoveries in the order of 90% can be obtained. It should be noted that materials recovered from the ion exchange column can be directly injected onto the reversed-phase support without any further manipulation, whereas if peaks from a reversed phase column are re-injected onto the same column, but using different mobile phases to achieve alternative selectivity, the sample must be diluted prior to injection to allow for subsequent trace enrichment. Even this simple manipulation might cause some losses at low sample levels due to the reduction in protein concentration.

The recovery of proteins from SDS-PAGE electroeluates using "inverse gradient" HPLC

We have recently demonstrated [36] that certain small pore (10–12 nm) reversed-phase packing e.g. ODS-Hypersil exhibit pronounced retention of proteins at high (90–100%) organic solvent concentration. Under these conditions proteins in SDS-PAGE electroeluates are specifically retained whilst SDS and gel derived polymers (which would interfere with amino acid sequence analysis) elute during loading. The retained proteins may then be recovered in high yield (>85%) by the simultaneous addition of an ion-pairing reagent (e.g. TFA) into the mobile phase and application of a decreasing (or "inverse") gradient of organic solvent. By using steep gradients at low flow rates in conjunction with microbore columns peak volumes of less than 20 microlitres have been obtained [36]. Use of this technique has allowed N-terminal sequence to be obtained for a number of proteins [36]. It is interesting to note that using the inverse gradient we have been able to recover in high yield proteins which we could not recover from the same column operated in the more conventional reversed phase mode (e.g. insulin receptor, recovery from inverse gradient 85% [36]). Rubinstein has used a hydrophilic support (Lichrosorb Diol) with an inverse gradient of n-propanol to resolve a number of proteins [37] including α, B and Y interferon [14]. These "normal phase" systems could be the method of choice for the separation of very hydrophobic proteins (e.g. receptors or other membrane proteins).

The isolation of peptide fragments for sequence analysis

Since not all proteins yield useful N-terminal sequence information (i.e. the N-terminus can often be ragged or blocked, or the amino acid sequence obtained may not be suitable for the construction of oligonucleotide probes of low degeneracy), it is often necessary to obtain internal sequence from peptide fragments generated from the protein by enzymic or chemical fagmentation. When working with sub-nanomole quantities of protein, all separation and micromanipulation procedures are more exacting. Reduction and alkylation, desalting and buffer exchange into small eluent volumes before digestion and multidimensional peptide purification must be performed with minimal losses. Figures 8 and 9 illustrate the protocol used for the structural characterisation of a murine myeloid leukaemia inhibiting factor (mLIF) [38] which provided 125 residues of sequence (70% of the molecule) and has provided the basis for the rapid cloning and expression of the corresponding cDNA [39].

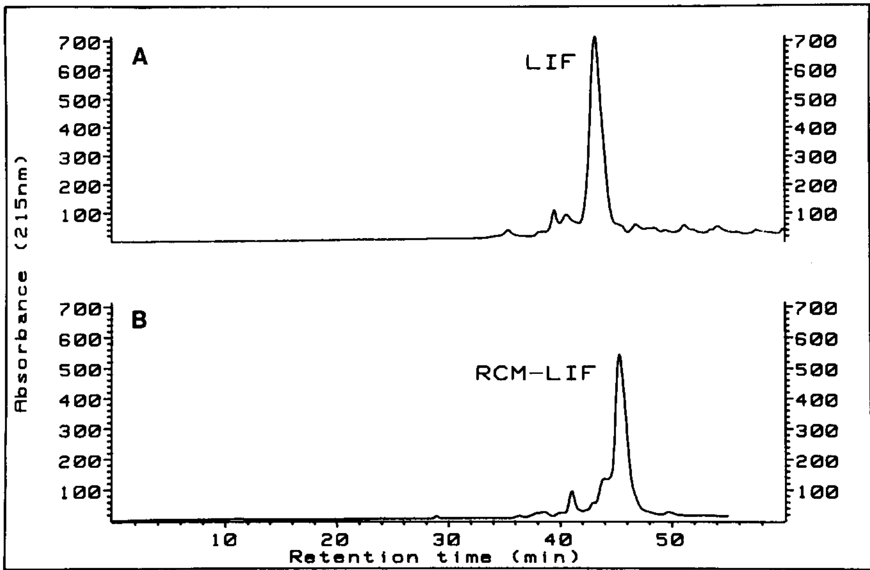
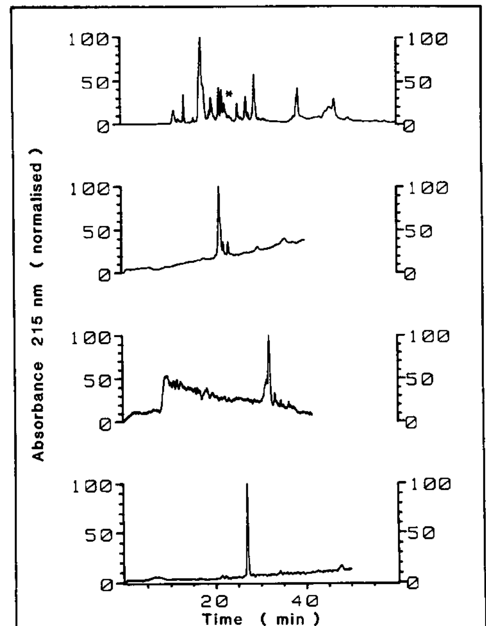


Figure 8
RP-HPLC of murine leukaemia inhibitory factor (LIF). (A) Chromatographic buffer exchange and concentration of a sample of native LIF from a conventional HPLC column (30). The sample in a volume of 2 ml was diluted with an equal volume of water and trace enriched onto the Brownlee RP-300 (30 × 2.1 mm) column at 2 ml min^{-1} (not shown) before recovering the protein by gradient elution using the condition described in Fig. 6. (B) Desalting of reduced and alkylated LIF. Chromatographic conditions were as for Fig. 8A.

Figure 9
Multidimensional microbore purification of a tryptic peptide from RCM-LIF. (A: top panel) Separation of tryptic peptides of RCM-LIF by RP-HPLC on Brownlee RP-300 (30 × 2.1 mm). Chromatographic conditions as for Fig. 8. (B) Rechromatography of a tryptic peptide (indicated by an asterisk in Fig. 9A) on Brownlee RP-300 (30 × 2.1 mm) using a linear 60 min gradient between a primary solvent of 0.9% (w/v) sodium chloride (pH 6.5) and 60% acetonitrile/40% water containing 0.9% (w/v) sodium chloride. (C) Rechromatography of the major peak from Fig. 9B on ODS-Hypersil (100 × 2.1 mm). Chromatographic conditions were as for Fig. 9A. (D) Chromatographic concentration of the major peak from Fig. 9C on Brownlee RP-300 (50 × 1 mm). The recovered peak was applied directly to the sample disc of the gas phase sequencer.



The protocol used was briefly as follows: Native mLIF, obtained from a 300 × 4.6 mm ID phenyl-silica column in a 2 ml fraction containing 40% acetonitrile/60% water containing 0.1% TFA and 0.02% Tween 20 [38], was diluted with an equal volume of water to reduce the concentration of the organic solvent sufficiently to allow trace enrichment and loaded, at 2 ml min^{-1} onto a Brownlee RP-300 column (30 × 2.1 mm ID). Retained proteins were recovered by developing the column, at a flow rate of 100 μl min^{-1} and a column temperature of 45°C, with a linear 60 min gradient between 0.15% v/v aqueous TFA and 60% v/v aqueous acetonitrile containing 0.125% v/v TFA (Fig. 8A). N-terminal sequence analysis established 21 of the first 25 residues.

Prior to enzymic fragmentation with trypsin or *Staphylococcus aureus* V8 protease to obtain further internal sequence the native protein was reduced with dithiothreitol and carboxymethylated using iodoacetic acid. The reduced and alkylated material (RCM-LIF) was desalted under identical conditions to those described above for the native molecule (Fig. 8B). As we have noted previously [40] the RCM material becomes noticeably more hydrophobic, eluting later than the native material (45.35 min compared with 43.17 min).

Fractionation on the Brownlee RP-300 column under the conditions used in Fig. 8 revealed a complex mixture of peptides (Fig. 9A) following trypsin digestion. Using a combination of solvent and column mediated selectivity, 11 peptides were isolated in homogeneous form. This is illustrated for the peak at 22.4 min indicated by an asterisk (Fig. 9A). In the first instance (Fig. 9B) further purification was achieved using the same column but a different mobile phase (0.9% NaCl, pH 6.5). The change in counter ion and pH both contributed to the selective effects observed. Since this peak is relatively hydrophilic (we adjudge peaks eluting at less than 30% acetonitrile on the Brownlee RP-300 column to fall into this category), it was rechromatographed on ODS-Hypersil, a more retentive support. Further purification was observed (Fig. 9C). The peak was recovered for microsequence analysis from a 1 mm ID column in a volume of 40 μl (Fig. 9D). This peptide yielded 13 residues of amino acid sequence at the 15 pmol level.

Similar protocols have proved effective for the purification and microsequence analysis of a number of proteins. Indeed the rate limiting step is frequently not the purification to homogeneity of generated peptides, but rather the desalting of the reduced and carboxymethylated material prior to digestion. As observed herein denaturation of the protein frequently leads to an increase in relative hydrophobicity since hydrophobic amino acids which were previously inaccessible for interaction with the support, due to the intrinsic structure of the protein, are made more accessible. In extreme cases, a protein which in its native form is recoverable in high yield from a reversed phase support may become so hydrophobic that it is now virtually infinitely retained by the same support [13]. The sensitivity associated with microbore column technology renders it an invaluable tool for determining recovery of such modified proteins on an analytical scale (10–20 ng).

In many ways size exclusion chromatography would appear to be the method of choice for desalting such mixtures since size exclusion columns are theoretically non-interactive, and therefore, more generally applicable. In addition, the mobile phase may be tailored to suit the subsequent digestion conditions without the complication of the presence of organic solvents. Unfortunately, the peak recovery volumes associated with the currently available HPLC-SEC columns (e.g. TSK 2000SW, 300 × 7.5 mm, Pharmacia 12, 300 × 10 mm, Du Pont Bioseries GF250, 250 × 9.4 mm) are relatively large (800 μl–1 ml) and are not ideally suited to micromanipulation. For the purpose of

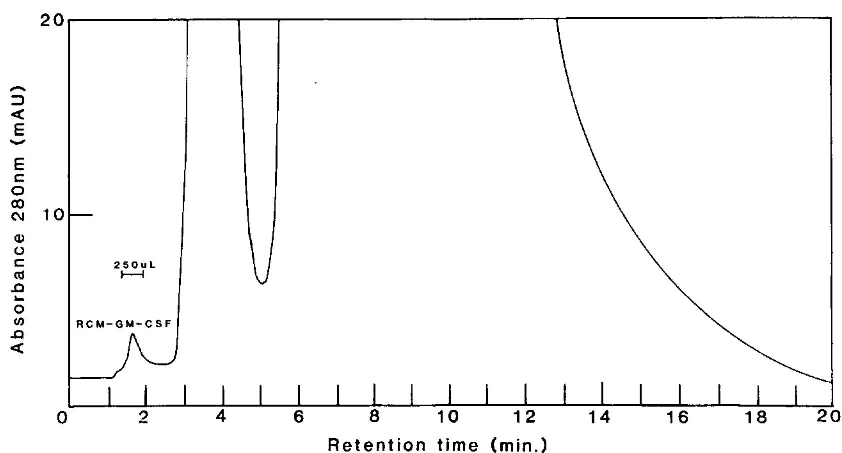


Figure 10
Desalting of reduced and alkylated recombinant GM-CSF. A sample of recombinant GM-CSF, which had been reduced and alkylated using vinyl pyridine, in a volume of 200 μl was desalted on a 30 × 10 mm size exclusion column using a mobile phase of 100 mM ammonium bicarbonate at a flow of 0.5 m min^{-1}. The nominal size exclusion limit of the packing was 10 kDa. The standards thyroglobulin and acetone eluted at 1.5 and 3.8 min, respectively. The resolution factor for these compounds was 5.4.

desalting it can be calculated that short size exclusion columns (e.g. less than 10 cm) packed with materials of effective small pore size would allow proteins to be recovered in minimal volume in the column void, well separated from low molecular weight components of the mixture. An example of the use of such a column to desalt a protein which had been reduced and alkylated using DTT and vinyl pyridine is given in Fig. 10. The peak volume recovery for a sample injected in a volume of 200 μl, was approximately 300 μl.

Conclusions

A wide range of proteins can now be purified using HPLC techniques. Some proteins which are relatively abundant in source material have been purified to homogeneity using solely HPLC techniques, e.g. epidermal growth factor from murine or rat salivary glands [41, 40] or parathyroid hormone from bovine parathyroid glands [42]. However, typical purification protocols require a multidimensional approach utilising combination of low or medium resolution supports to debulk the total protein content followed by several HPLC steps to achieve final purification to homogeneity. The exact protocol will be a function of the specific properties of the protein of interest. To minimise degradation by proteolytic enzymes often present in biological source material, speed is of the utmost importance, especially in the early stages of a purification. For proteins which are only present at very low levels the combination of protein chemistry and molecular biology allows amplified amounts of material for structure–function studies to be produced via recombinant techniques. Fundamental to this strategy is the ability to micromanipulate in small volumes, with minimal loss sub-nanomole quantities of protein. The use of columns of 2.1 or 1 mm ID have proved advantageous in such strategies.

The future will almost certainly see advances in two key areas. At one extreme continued advances will be made in micromanipulation, and structural analysis, at the low and sub-nanomole level. To this end more efficient column packing materials (e.g. non-porous packings packed into microbore columns) will be further developed. This will be backed up by continued advances in sequencer design and chemistry and will probably involve the synergistic use of chromatographic techniques with other analytical techniques such as two dimensional electrophoresis with direct electroblotting [43, 44] or techniques such as high-performance capillary electrophoresis [45, 46 and references therein].

At the other extreme, the ability to produce large quantities of protein by recombinant techniques will stimulate further development of rapid, large scale, high resolution preparative techniques. These will possibly use overload [47] or displacement techniques [48] to achieve cost effective separations.

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