Microbore reversed-phase high-performance liquid chromatographic purification of peptides for combined chemical sequencing–laser-desorption mass spectrometric analysis

Christopher Elicone, Mary Lui, Scott Geromanos, Hediye Erdjument-Bromage, Paul Tempst*

Molecular Biology Program and Protein Chemistry Laboratory, Memorial Sloan-Kettering Cancer Center (Box 137), 1275 York Avenue, New York, NY 10021, USA

Abstract

An optimized microbore RP-HPLC system (1.0 mm I.D. columns) for the purification of low picomole amounts (<5 pmol) of peptides is described. It is comprised of commercially available columns, instrument components and parts. These were selected on the basis of a comparative evaluation and to yield the highest resolution and most efficient peak collection. The sensitivity of this system equals, probably surpasses, that of advanced chemical microsequencing for which 2–4 pmol of peptide are minimally required. As an automated sequencer cannot be "on-line" connected with a micro-preparative HPLC system, fractions must be collected and transferred. With a typical flow of 30 μl, efficient manual collection is possible and fractions (about 20 μl in volume) can still be handled without unacceptable losses, albeit with great precaution. Furthermore, major difficulties were encountered to efficiently and quantitatively load low- or sub-picomole amounts of peptide mixtures onto the RP-HPLC column for separation. Discipline and rigorous adherence to sample handling protocols are thus on order when working at those .evels of sensitivity. With adequate instrumentation and handling procedures in place, we demonstrate that low picomole amounts of peptides can now be routinely prepared for analysis by combined Edman-chemical sequencing–matrix-assisted laser-desorption mass spectrometry (MALDI-MS). The integrated method was applied to covalent structural characterization of minute quantities of a gel-purified protein of known biological function but unknown identity. The results allowed unambiguous identification and illustrated the power of MALDI-MS-aided interpretation of chemical sequencing data: accurate peptide masses were crucial for (i) confirmation of the results, (ii) deconvolution of mixed sequences, (iii) proposal of complete structures on the basis of partial sequences, and (iv) confirmation of protein identification (obtained by database search with a single, small stretch of peptide sequence) by "mass matching" of several more peptides with predicted proteolytic fragments.

1. Introduction

As part of the sequencing process, proteolysis

of proteins is often necessitated to cope with blocked N-termini and desirable to facilitate later gene cloning experiments [1,2]. Owing to low abundance and/or source limitations, these studies must frequently be done on small

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* Corresponding author.

amounts (10–20 pmol); additional steps, such as two-dimensional gel purification and *in situ* proteolysis will reduce analyte levels even more [1,3]. Regardless of digest technique, liquid chromatographic (LC) fractionation of the resulting peptide pool is required before further analysis can be attempted.

Covalent structural analysis of peptides usually involves, at least in part, Edman-chemical sequencing [4–6]. However, automated chemical sequencing, when carried out at low picomole levels (initial yields < 2 pmol), does have its shortcomings [7,8]. For instance, ambiguous calls in the first two cycles, gaps in sequences and inability to identify the C-terminal Lys or Arg (of tryptic peptides) are a frequent occurrence. In addition, micro-heterogeneities (*e.g.* isoforms) exist among naturally occurring peptides, that may also have ragged ends. Furthermore, most modified amino acids (*e.g.* phospho-, glyco-, etc.) cannot be directly identified. Accurate mass measurements could greatly facilitate interpretation of such complex and/or incomplete chemical peptide micro sequencing data. Matrix-assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF-MS) has emerged as a popular choice in this regard for reasons of high sensitivity, accuracy and ease of operation [9–11].

As chemical sequencing [7,8,12] and MALDI-MS [13,14] have become increasingly more sensitive, it seems clear that the LC technique providing the highest sensitivity would be ideally suited for peptide separation. Luckily, progress in micro-preparative RP-HPLC during the last decade has been phenomenal. Through down-sizing of column I.D. from the once customary 4.6 mm [15,16] to well below 1 mm [17,18], purifiable amounts of peptide and corresponding peak elution volumes have decreased by almost three orders of magnitude [19–21]. However, Edman-sequencing and MALDI-MS are not easily "on-line" coupled to LC. Therefore, samples must be collected, stored and transferred.

It has been noted that those sample handling routines, using laboratory techniques and supplies from the "nanomole" era of protein chemistry, have become increasingly inefficient [7,20]. Clumsy sample handling may very well be the real reason for failure of numerous high-sensitivity sequencing projects (*i.e.* those carried out at levels approaching femtomoles). The protein chemistry community is currently struggling to come to grips with this problem, let alone solving it quickly. It can be argued that the multi-step process of protein isolation, digestion, peptide LC purification and chemical sequencing, including the required sample transfers, is only going to be as good as the proverbial "weakest-link-in-the-chain". Femtomole-level capillary LC of peptides seems therefore to be of somewhat excessive power, unless "on-line" connected to a femtomole-level analytical technique (*e.g.* LC–MS).

In this report, we present the results of an investigation of various microbore (columns of 1.0 mm I.D.) RP-HPLC components and experimental variables. Great attention was given to the specific aspects of processing *in situ* digest peptide mixtures for analysis by combined chemical sequencing–MALDI-MS. By also applying rigorous sample handling routines and peak selection criteria, protocols are now available for micro-digestions/separations at high volumes (*e.g.* core facility settings) and without jeopardizing quality.

2. Experimental

2.1. Materials

SKI PepMix6 is a mixture of 14 synthetic peptides, ranging in size from 9 to 26 amino acids, as listed in Table 1. Syntheses were carried out at the MSKCC Microchemistry Facility by Ms. Deborah Desrouilleres. The mix (on the average, 200 pmol per peptide per μl water) was periodically remade from separate stock solutions that, each time, had been requantitated by amino acid composition analysis. Peptides were represented in the mix in slightly varying molar ratios (see Table 1) to yield a RP-HPLC profile with roughly equal sized peaks. With the average peptide concentration being assigned the arbitrary value of 1.0, concentrations of constituent

Table 1
Components of SKI PepMix6

No.	Sequence	Length	Net charge	Aromatic amino acids	Molar ratio
1	SIINFEKLT	9	0	F	1.3
2	WFRKRGSQQP Q	11	+3	FW	0.65
3	HHQKLVFFAE D	11	+1	FF	0.45
4	DAEFRHDAGY EV	12	−2	FY	1.1
5	LQYTEHQQLE GWK	13	0	YW	0.65
6	QGMLPEDLSS VIR	13	−1	−	1.8
7	QLQKDKQVYR ATHR	14	+4	Y	1.05
8	TPTPNPPTTE EEKTE	15	−3	−	1.05
9	NISPKSYDDF ISRNK	15	+1	Y	0.8
10	RAGGTQRVEV LEGRT-amid	15	+2	−	1.75
11	ELVEPLTPSG EAPNQALLR	19	−2	−	1.25
12	TPYSWDLPEP RSRSAKIRVH PR	22	+4	YW	0.6
13	ISCWAQIGKE PITFEHINYE RVSDR	25	+1	FYWpC	1.05
14	KDELPQLVTL PHPNLHGPEI LDVPST	26	−1	−	0.6

Peptides were synthesized, purified and quantitated at the MSKCC Microchemistry Facility. Length and net charge are listed. Molar ratio indicates the correction factor to be applied for calculation of the "real" molar amount of each peptide in the mixture; when, for instance, it is stated that 5 pmol mix was used, molar ratios of 1.3 or 0.65 indicate 6.5 or 3.25 pmol, respectively.

peptides ranged in value from 0.45 to 1.75. Aliquots of 5 μl (1 nmol per peptide, on the average) were stored at −70°C for one-time use.

Preparative tryptic (trypsin from Boehringer, Indianapolis, IN, USA) digestion of horse cytochrome c (Sigma, St. Louis, MO, USA) was carried out as described [15] and a stock solution (in water) of 48 pmol/μl (by amino acid composition analysis) was aliquoted in 20 μl volumes (960 pmol total) and stored at −70°C for one-time use.

Proteins used in the various applications described in this report were studied under collaborative agreements or were submitted to the MSKCC Microchemistry Facility for covalent structural analysis. As the results have not yet been published, protein sources and biological functions are subject to confidentiality agreements. All "unknown" proteins were prepared by SDS-polyacrylamide gel electrophoresis (PAGE), electroblotted onto nitrocellulose (Schleicher and Schuell, Keene, NH, USA) and visualized by amido black (Serva, Paramus, NJ, USA) staining. Bands of interest were excised, digested in situ with 1 μg trypsin (sequencing grade from Boehringer), and the resulting pep-

tide mixture reduced and S-alkylated with, respectively, β-mercaptoethanol (BioRad, Richmond, CA, USA) and 4-vinylpyridine (Aldrich, Milwaukee, WI, USA), all as described [1]. Trypsin blanks were prepared as controls.

All other chemicals, supplies, equipment and parts were as indicated elsewhere in this Experimental section.

2.2. Microbore RP-HPLC assembly

The microbore system used in this study was based on a previously described narrow-bore HPLC design/assembly [1]. In the current configuration we have used a 140B Solvent Delivery System equipped with a 75-μl dynamic mixer (Applied Biosystems, Foster City, CA, USA). A precolumn filter with a 0.5-μm frit (Upchurch Scientific, Oak Harbor, WA, USA) was plumbed between the mixer and a Rheodyne 7125 injector (Rainin, Ridgefield, NJ, USA) using two pieces [27 cm × 0.007 in. I.D. (1 in. = 2.54 cm)] of polyetheretherketone (PEEK) tubing (Upchurch). The injector was fitted with a 50-μl loop and connected to the column inlet with PEEK tubing (30 cm × 0.005 in.). Total pre-column

volume (including mixer and loop) was therefore 145 μl. The outlet of the column was connected directly to a glass capillary (20 cm × 280 μm O.D./75 μm I.D.; 0.88 μl) (LC Packings, San Francisco, CA, USA) with the appropriate PEEK sleeve (Upchurch) and stainless-steel fitting. By swaging a stainless-steel fitting to the PEEK sleeve the capillary can be held tightly and then adjusted to avoid dead volume; the final assembly to the column outlet is then made. The back-end of the glass capillary was connected to the flow cell of an Applied Biosystems (AB) 783 detector with the appropriate hardware and in the same manner as the front-end. The flow cells used were as follows: AB "straight-shaped" with 2.4 μl volume/6 mm pathlength (AB P/N:2900-0197) and 0.5 μl volume/1 mm pathlength (AB P/N:2900-0034), AB "L-shaped" with 2.4 μl volume/6 mm pathlength (AB P/N:2900-0195), and LC Packings U-Z View 35 nl volume/8 mm pathlength (Kratos compatible). The outlet tubing of the AB flow cells was a 15 cm long, 75 μm I.D. glass capillary. When installing the LC Packings capillary flow cell, the outlet of the column was plumbed directly to the leading portion of the capillary; the trailing portion of the capillary cell was trimmed to a 15 cm length and threaded out of the detector head. In all cases, the 15 cm length resulted in a post flow cell volume of 0.66 μl and a collection delay of only 1.3 s at a flow-rate of 30 μl/min; this allowed real time collection of peaks. The SGE columns required zero dead volume (ZDV) unions (Valco Instrument, Houston, TX, USA) to mate their 1/16 in. O.D./50 μm I.D. Polysil tubing to the PEEK inlet tubing and glass capillary outlet tubing. Analog signals from the 783 detector were registered with a Kipp and Zonen Model BD 41 dual pen stripchart recorder (VWR, Piscataway, NJ, USA); signals were also sent to a Model 970 A/D converter (PE Nelson, Cupertino, CA, USA) and chromatograms analyzed and plotted using PE Nelson Turbochrom 3 (version 3.2) software.

Columns used in this study were: Vydac 218TP5115 C_{18} (5-μm particles, 300 Å pore size; 150 × 1 mm column dimension) and 214TP5115 C_4 (5 μm, 300 Å; 150 × 1 mm) from The

Separations Group (Hesperia, CA, USA), SGE Inertsil 100GL-1-ODS-I10/5 C_{18} (5 μm, 150 Å; 100 × 1 mm) and SGE ODS-2 C_{18} (5 μm, 300 Å; 100 × 1 mm) from Scientific Glass Engineering (Austin, TX, USA), and Brownlee Aquapore RP-300 C_8 (7 μm, 300 Å; 250 × 1 mm) and Aquapore OD-300 C_{18} (7 μm, 300 Å; 100 × 1 mm) from Applied Biosystems (purchased from Rainin, Woburn, MA, USA).

2.3. RP-HPLC operation and sample handling

All columns were operated at a flow-rate of 30 μl/min and at ambient temperature, except where indicated. Solvent A consisted of 0.1% trifluoroacetic acid (TFA) (Pierce, Rockford, IL, USA) in fresh Milli Q water and solvent B was 0.09% TFA in 70% aqueous acetonitrile (Burdick and Jackson, Muskegon, WI, USA); solvents were always made in dedicated glass measuring cylinders, mixed thoroughly and sonicated (Branson Ultrasonics, Danbury, CT, USA) for 5 min before use. Gradient elution of peptides was typically done by linear increase of solvent B at a rate of 1 or 2% per min (5–50%B/ 45 min or 5–50%B/22.5 min), except were indicated.

Frozen aliquots of PepMix6 (1 nmol of each peptide per 5 μl) or cytochrome c/tryptic peptides (960 pmol per peptide in 20 μl) were diluted with 2% TFA (Pierce) to give a volume of 100 or 96 μl, respectively. Further serial dilutions were then made in 2% TFA (or 20% in some experiments) to yield 5, 2.5, 1.25 and 0.625 pmol amounts of peptide mix per 50 μl solvent (typically in a much larger volume to allow overfilling of the loop). In case of *in situ* digests, nitrocellulose was removed by centrifugation and rinsed once with 25 μl 5% acetonitrile (Burdick and Jackson)–100 mM ammonia bicarbonate, under sonication. Supernatants were pooled, and reduced and alkylated (see Materials). In every instance, the samples were injected immediately after they had been prepared, except where noted.

Samples were then introduced in the injector, either by overfilling an empty loop or by careful displacement of solvent A from the loop to

guarantee, respectively, accurate injections of defined quantities of the standards or introduction of 100% of the "unknown" peptide mixtures. After injection, columns were eluted isocratically (at 5%B) until the void peaks had passed and the baseline was back to its pre-injection level; at this point, the gradient was started manually as was the data acquisition (time zero).

Column peak fractions (typically about 20 μl) were collected by hand in 0.5-ml polypropylene Eppendorf tubes (National Scientific Supply Co., San Rafael, CA, USA) and kept on ice until the end of the experiment. Aliquots were then withdrawn for mass analysis and the rest frozen at −70°C until chemical sequencing. Fractions were never dried or concentrated. For repurifications, fractions were acidified with 5 μl TFA and then two-fold diluted with solvent A before injection (by two succesive injections of about 25 μl each).

2.4. Edman-chemical sequencing of peptides

Purified peptides were sequenced with the aid of an AB Model 477A automated sequenator, operated according to the principles outlined by Hewick *et al.* [4]. Stepwise liberated phenylthiohydantoin (PTH)–amino acids were identified using an "on-line" 120A HPLC system equipped with a PTH C_{18} (220 × 2.1 mm; 5 μm particle size) column (AB). The standard AB method was optimized for sub-picomole PTH–amino acid analysis, as described by members of our laboratory [7,12]. After storage, column fractions were always supplemented with neat TFA (to give a final concentration of 10%) before loading onto the sequencing disc as it has been shown that this increases recoveries from the test tube for most peptides [7].

2.5. Mass spectrometry of peptides

Peptide mass analysis was carried out by MALDI-TOF-MS using a Vestec (Houston, TX, USA) LaserTec ResearcH instrument, with a 337-nm output nitrogen laser and a 1.2-m flight tube, operated according to published principles [9,22]; 28-kV ion acceleration and 4.3-kV multi-

plier voltage were used. The instrument has a built-in video camera for real-time inspection of the laser beam impact on the target area. Tektronix (Beaverton, OR, USA) Model 2225 single-channel analog (50 MHz, real time) and Model TDS520 dual-channel digitizing (500 MHz:500 Ms/s, averaging) oscilloscopes are connected in parallel to the detector for data acquisition; digitized spectra are downloaded to a ZEOS 486 33Mz computer. m/z Spectra are generated using the GRAMS (Galactic Ind., Salem, NH, USA) data analysis software.

Steel probe tips (pins) were cleaned by sonication in 5% acetic acid for 5 min, followed by washing with water and acetonitrile, and air drying. Matrix solution was prepared freshly by dissolving 10 mg α-cyano-4-hydroxycinnamic acid (ACCA) (Sigma) in 1 ml of a 0.1% TFA–33% aqueous acetonitrile solution, followed by vigorous vortexing and brief centrifugation (Eppendorf); only the supernatant was used for sample preparation. Working stocks of calibrants [peptides Apid (MH$^+$ = 2109.45) and Ova (MH$^+$ = 980.00)] were stored in 25 μl aliquots at − 20°C for one-time use; concentrations were: 1 pmol Apid + 2 pmol Ova per μl of 0.1% aqueous TFA. Just prior to use they were either 4-fold or 20-fold diluted (depending on estimated amounts of analyte) by addition of, respectively, 75 μl or 475 μl of 2% TFA–33% aqueous MeCN, and kept on ice. In this order, matrix (1 μl), analyte (1 μl) and calibrant mixture (0.5 μl) were spotted on the pin, using separate pipette tips, and mixed *in situ* by pipetting up and down (3 times). Samples were then air-dried at room temperature for 30 min. For more details and a full description of the actual laser-desorption MS experiments (acquiring data), analyte/calibrant adjustments and final data analysis, we refer to an earlier publication [10].

3. Results and discussion

3.1. Why use 1.0 mm I.D. columns for micropreparative peptide purification?

There are two aspects to the LC miniaturization trend of the last decade, namely pioneering

of new technology and then applying it to biological/medical research. Columns of ever narrower I.D. (bore) were introduced and shown, through the use of chromatographic standards, to be of substantial promise. General applications however, must always await commercial availability of (i) optimally packed columns and (ii) special hardware components and parts required to upgrade existing HPLC systems. By the time the user community has widely adopted a new, more sensitive level of LC experimentation, pioneering laboratories have already reached the next (or several) level(s) down.

Even though the aims of LC development are (or should be) to extend the realm of applications, both areas are quite specialized, come with their own set of rules and, most importantly, should not be confused with one another. For example, when it is obvious that a certain level of LC-sophistication is either unnecessary, inefficient or extremely impractical (or all of the above) for a specific application, then why use it?

New developments have been introduced at a fast pace and it has been clear for some years now that molecular separations in packed 50 μm I.D. columns are certainly feasible [17,18]. However, for every down-sizing step (from 4.6 to 2.1 to 1.0 to sub-1 mm I.D.), manufacturers have struggled somewhat (to very seriously) to produce columns at high volume, and with uncompromised chromatographic performance as compared to the wider-bore predecessors. While 2.1 mm columns [1,6,23] have succesfully replaced the 4.6 mm ones [16,24] for micropreparation of peptides, 1.0 mm (microbore) columns are not yet widely used for this purpose; this, despite the fact that they have been commercially available for years. When, some three years ago, this laboratory tried to switch to microbore columns for peptide applications, we failed; peak resolution was unacceptable [38]. Because of these observations and similar ones made in other laboratories, and with the sharp rise in fame of home-made capillary (sub-mm I.D.) columns during the last three to four years [19–21,25], there has been a movement to just skip the 1.0 mm level.

Recently however, we became aware of the commercial availability of a newer generation of microbore columns; actually, they contained the same packing materials that have been successfully used by some of the authors for years [1,16]. As covalent structural analysis of peptides is practiced exclusively by chemical sequencing in our laboratory, and for the reasons already discussed, we felt that 1.0 mm I.D. columns should be reevaluated. This report describes these studies and subsequent applications.

3.2. Instrument assembly and operation

As microbore LC can be considered to be at the extreme lower end of classical HPLC, there was no need for specialized components such as flow splitters etc. [20,21], just standard hardware. This allowed us to draw heavily from our earlier experiences with HPLC configuration-design and assembly [1]. The guiding principle then was to minimize total dead-volumes, from mixing-T to column, and certainly post-column. To allow "real-time" manual collection (*i.e.* by visually monitoring the analog peak signals on a stripchart recorder), lag time should be near zero. With a post-flow cell volume of only 0.66 μl and a flow of 30 μl/min, collection delay is a negligible 1.3 s. Description of the entire microbore HPLC assembly can be found under Experimental; the choice of flow cell was made after a comparative study (see below).

In the past, we have satisfactorily operated 2.1 mm columns at flows of 100 μl/min, typically with gradients of 0.7% acetonitrile increase per min [1]. For a scaled-down column, the flow should be decreased proportionally to the square of the inside column radius; *i.e.* for 1.0 mm I.D. this would then become 22.7 μl/min. Systematic investigation of such typical operating variables as flow, gradient slope and temperature indicated that peptide retention, peak shape and height were all affected (data not shown) exactly as predicted from the long established groundrules of standard-bore RP-HPLC (for review of those, see ref. 26). While it appeared that a flow of 23 μl/min may indeed have been chromatographically optimal, practical considerations such as reproducibility (shaky gradients) and time

(long experiments) eventually mandated a routine flow of 30 μl/min. This has since been shown adequate in several dozens of applications [see Figs. 6 and 8, and unpublished observations (1993)]. We have also maintained our previous gradient conditions at ambient temperature.

Injection volumes of up to 200 μl (containing less than 10% acetonitrile and less than 3% TFA) can easily be tolerated (data not shown). Standard, we have used a 50-μl injector loop as that was sufficient for nearly all applications. Chromatographic effects of high concentrations TFA in the sample are discussed further in this text.

3.3. Column evaluation

Six different columns were chosen for evaluation. The non-arbitrary selection was the result of our previous experiences with the same packings in bigger columns. Two carefully quantitated peptides mixtures were prepared for these investigations: cytochrome *c* tryptic peptide mix [15] and an artificial mixture of 14 synthetic peptides (SKI PepMix6), ranging in size from 9 to 26 amino acids (listed in Table 1). Sizes were specifically chosen to reflect the mass distribution of the pertinent peptides in an "average" tryptic digest; bigger peptides usually do not recover all that well from reversed-phase supports and smaller ones, albeit well recovered, are essentially uninteresting for most of our studies (aimed at providing the information for protein identification or gene cloning experiments).

RP-HPLC separation profiles of 5 pmol PepMix6 (average for the 14 peptides, with distribution ranging from 2.25 to 8.75 pmol) and 2.5 pmol cytochrome *c* tryptics are shown in Fig. 1. Even though no efforts have been made to identify all peaks or align the chromatograms, it appears that the profiles are reasonably comparable and generally satisfactory. All 14 PepMix6 peptides are accounted for on the Vydac C_4 profile only (although two late eluting peaks have almost merged). This may have been just a lucky coincidence as overall resolution on the Vydac C_4/C_{18}, Inertsil C_{18} and SGE ODS-2 columns was essentially the same. It should also
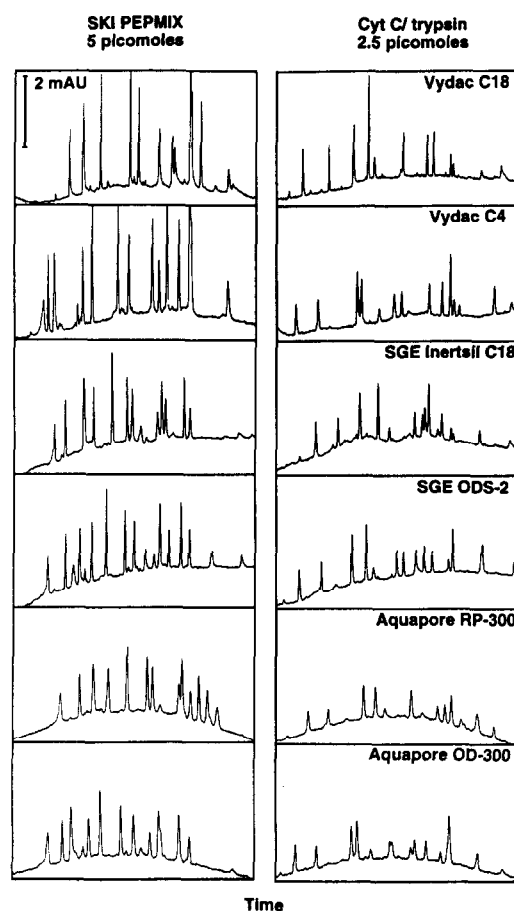

Fig. 1. Comparison of 1.0 mm I.D. reversed-phase columns for peptide separation. RP-HPLC profiles of 5 pmol PepMix6 (composition listed in Table 1) and 2.5 pmol cytochrome *c* tryptic peptides are shown. HPLC configuration was as described under Experimental; an LC packings U-Z view flow cell was used. Columns are indicated on each panel; column specifications can be found under Experimental. Chromatographic conditions were: a linear two-step acetonitrile gradient (in 0.1% TFA) of 3.5–35%/45 min, 35–70%/22.5 min at a flow-rate of 30 μl/min and at ambient temperature; samples were injected in 50 μl of 2% TFA. Full scale on each panel corresponds to 0.004 AU at 214 nm; time scale is from 15 to 50 min.

be noted that elution profiles of the peptide mixtures were near identical between 1.0 and 2.1 mm I.D. columns filled with the same Vydac packings (dat not shown). In keeping with earlier observations [16,26], late eluting peptides also recovered well from the C_4 support here; this should be kept in mind when designing LC

experiments. The Aquapore resins consistently showed wider peaks, most likely related to their bigger particle size (7 μm). Although we have no strong preference as yet for any particular column packing, we assume that Vydac and SGE microbore columns may be logical partners for high-resolution two-dimensional RP-HPLC separations. A minor disadvantage of SGE columns, in our view, are the unusual endfittings, requiring adaptors (Valco ZDV unions) to connect them to our standard plumbings.

3.4. Detector flow cells and wavelength

Flow cells that come standard with the Applied Biosystems (AB) microbore LC package have a 6 mm path length and a 2.4 μl volume. Therefore, at a flow of 30 μl/min, it takes nearly 5 s for the void volume to be completely displaced, while during all that time mixing occurs. This raises legitimate questions about peak broadening and remixing of closely eluting peptides.

As flow cells can be easily interchanged in the AB 783 detector, we evaluated two different sub-μl volume models, the 1 mm path length (0.5 μl volume) type from the same manufacturer and a longitudinal capillary cell from LC packings (Model U-Z view). Whereas the former is still an assembly of the classical housing with two windows and in/out fittings, the newer type cell consists of a 75 μm I.D. glass capillary, bent in a U-shape and held by a metal template to align the 6 mm long center portion of the U with the optical axis [27]. As the U-Z cell is nothing else but a capillary, no mixing or band-spreading should occur. The chromatograms shown in Fig. 2, generated by separation of 5 pmol cytochrome c tryptics on a Vydac 1.0 mm I.D. C_{18} column operated in our LC system but using different flow cells, seem to confirm this presumption. However, whereas peak heights obtained with the U-Z cell were clearly larger than with the 1 mm path length cell, they were about three-fold smaller than those with the 6-mm AB cells. In addition, at 5 pmol analyte levels, peak signal-to-noise ratios were 636, 358, 252 and 49 for, respectively, the 6-mm AB (straight), 6-mm AB
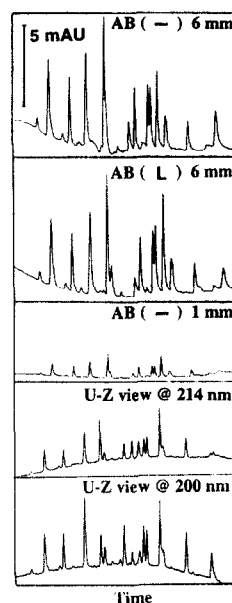

Fig. 2. Comparison of detector flow cells and wavelengths for peptide detection in microbore RP-HPLC. RP-HPLC profiles of 5 pmol cytochrome c tryptic peptides are shown. HPLC configuration was as described under Experimental; a 1.0 mm I.D. Vydac 218 TP5115 C_{18} column was used. Flow cell type is indicated on the panels and their specifications can be found under Experimental; symbols: – = straight shaped flow path; L = L-shaped flow path. Chromatographic conditions were: a linear two-step acetonitrile gradient (in 0.1% TFA) of 3.5–35%/22.5 min, 35–70%/12.5 min at a flow-rate of 30 μl/min and at ambient temperature; samples were injected in 50 μl of 2% TFA. Scale of absorbance units at 214 nm (or 200 nm in the bottom panel) is indicated by the vertical bar (0.005 AU) and applies to all panels; the time scale is from 12 to 33 min. Peak signal-to-noise ratios were (from top panel to bottom panel): 636, 358, 252, 49 and 52.

(L-shaped), 1-mm AB and the U-Z view cells. Nonetheless, we have opted for the U-Z view cell as standard feature in our microbore LC systems for the reasons discussed below.

Even though the incentive for evaluating 1.0 mm I.D. columns was increased sensitivity, it should not be forgotten that the purpose of the LC experiment is to provide peptides, sufficiently homogeneous to yield interpretable chemical sequencing data. As conclusive sequencing results are the sole objective of the applications laboratory, resolving power of the micro-preparative LC system should be safeguarded in an uncompromising way. Unquestionably, this was

best accomplished when the detector was fitted with the U-Z view flow cell (Fig. 2). Our conclusion was subsequently validated by the results of several hundred sequencing experiments that showed a significantly lower incidence of mixed sequences, thus indicating absence of peptide cross-contaminations.

The three-fold reduction in peptide detection sensitivity, as compared to the AB 6 mm path length flow cell, can be partially offset in two ways. First, we found that the most sensitive detector AUFS settings (*i.e.* what the operator sees on the stripchart recorder while collecting fractions) that could be routinely obtained were about two-fold better for the U-Z view flow cell (10 mAUFS *versus* 20 mAUFS for the AB 6-mm cell). Through meticulous, but time consuming, baseline optimization, the absolute AUFS values could be further reduced for both cells, but the ratios remained unchanged (*e.g.* 5 *versus* 10 mAUFS). Second, as had already been demonstrated [20,21], enhanced sensitivity of peptide detection can be attained using an U-Z view cell and at a wavelength of 195 nm. A quick investigation in our laboratory confirmed these findings but also resulted in the following additional observations. At a 0.01 AUFS detector setting, (i) 200 nm is really the practical lower limit as at 195 nm the baseline is nearly uncontrollable, and (ii) using an AB 6-mm flow cell, detection at wavelengths below 210 nm is essentially impossible for the same reasons. Thus, for all practical purposes, real-time sensitivity of U-Z flow cells approaches that of the classical 6-mm types. On the other hand, a previously reported drawback of the U-Z view cell, namely its limited dynamic range (*i.e.* signal saturation at 50 pmol level [21]), is not a real concern as these amounts of peptide are very seldom chromatographed in our laboratory.

3.5. Peptide detection sensitivity

With our microbore LC configuration established, we carried chromatographic separations of increasingly reduced quantities of PepMix6 (from 5 pmol down to 625 fmol); the results are shown in Fig. 3. It appeared that, under ideal
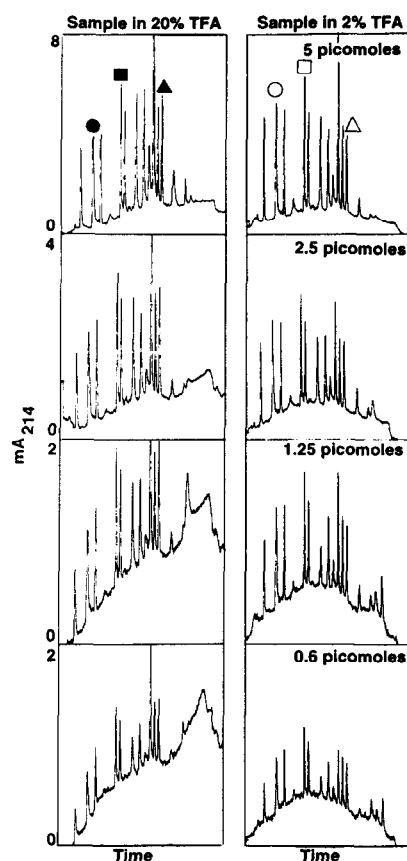
Fig. 3. Sensitivity of peptide detection in microbore RP-HPLC. RP-HPLC profiles of various quantities of PepMix6 (composition listed in Table 1) are shown. HPLC configuration was as described under Experimental; a 1.0 mm I.D. Vydac 218 TP5115 C_{18} column and an LC packings U-Z view flow cell were used. Amounts of PepMix6 are indicated on the panels; samples were injected in 50 μl of 2 or 20% TFA (indicated). Chromatographic conditions were: a linear two-step acetonitrile gradient (in 0.1% TFA) of 3.5–35%/22.5 min, 35–70%/12.5 min at a flow-rate of 30 μl/min and at ambient temperature. Time scale is from 15 to 32 min; AUFS values vary between panels, as indicated. Symbols: O, □, and △ indicate the peaks corresponding to peptides 7, 4 and 13, respectively (sequences listed in Table 1).

conditions, peptides could be satisfactorily separated and collected at the 1–2 pmol level and analytical experiments with 625 fmol of peptide mix still yielded clearly discernible peaks.

In the course of the present studies we made the very important observation, essentially confirming limited data described in an earlier

report [7], that at dilute concentrations, low picomole amounts of many peptides tend to "disappear" from the solutions. As the protein chemistry community is just brushing femtomole level polypeptide isolation and sequencing, the problem of minute peptide losses during preparation, storage and transfer has either not been fully recognized or has been blamed on unrelated factors. Several studies on femtomole level peptide chromatography have indeed been performed by injecting very small volumes (1–5 μl) of rather concentrated stock solutions; in the other cases, where large volumes (50 μl or more) of dilute stocks were injected, poor recoveries have typically been blamed on column losses, an often abused term for lack of any other reasonable explanation (as for instance in ref. 1). We show here that these column losses are minimal.

High losses during sample handling can be satisfactorily reduced in the presence of 20% TFA, with 2% being the lowest TFA concentration to yield a significant effect [7]. When serial dilutions of PepMix6 were prepared in either 2 or 20% TFA for chromatographic injection, a near linear relationship was found between peak detection signals and the amount of peptide (Fig. 4); in water, most peptides were poorly recovered, or not at all, once below the 10 pmol level (data not shown). The presence of high acid concentrations is mostly required for large size (> 20 amino acids) and/or hydrophobic peptides; i.e. those that typically elute late
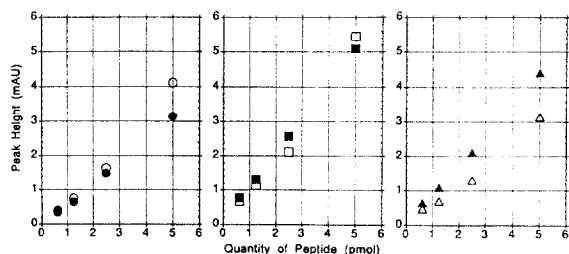

Fig. 4. Molar sensitivities of peptide detection in microbore RP-HPLC. The data are taken from the chromatographic experiments shown in Fig. 3. Symbols as in Fig. 3; open and closed symbols indicate TFA concentrations in the injected sample (50 μl) of 2 and 20%, respectively. Precise amounts of peptides were calculated using the molar ratios listed in Table 1.

from reversed-phase supports. Figs. 3 and 4 clearly illustrate that, while peptides eluting in the early-to-mid section of the chromatogram are well recovered when injected in 2% acid, dramatic reduction of peak heights in the late portion resulted from sample preparation in 2% TFA, as compared to 20% acid. Drawbacks of injecting peptide mixtures in high acid concentrations are (i) the enormous void peak that takes at least 30 min to pass the detector, and (ii) broadening of the early peaks with concomitant reduction in size (Fig. 4). The latter is not really a consideration for most applications; unfortunately, the long wait at the beginning of the chromatographic run will be a necessary inconvenience until alternative remedies can be recommended.

Finally, if the specifics of an experiment should call for low acid concentrations, it is recommended to immediately (i.e. within 1 min) inject the sample onto the column. The value of this advisory can not be better illustrated than by the chromatographic data shown in Fig. 5. Two peptide mixtures (2.5 pmol each; prepared by two-way splitting of one 5-pmol sample) diluted in 50 μl of 2% TFA were analyzed, the first immediately after preparation, and the second after keeping the vial on the bench for 4 h. In the latter experiment, half of the peaks were gone and the others had decreased substantially.

When using appropriate sample handling routines, it is clear that the practical (i.e. routine operation) sensitivity of the LC system described in this report equals, probably surpasses, that of advanced chemical microsequencing (2 pmol minimally required; for example, see Fig. 7). We feel that it is certainly adequate at this time.

3.6. Practical peptide preparation

As already alluded to, a major obstacle in chemical microsequencing is the frequent occurrence of post-translationally modified N-termini (mostly acetylated). This renders the affected proteins inaccessible for cyclic chemical degradation, a condition well know under the term "blocked". In the absence of an adequate remedy (i.e. efficient deblocking), internal se-
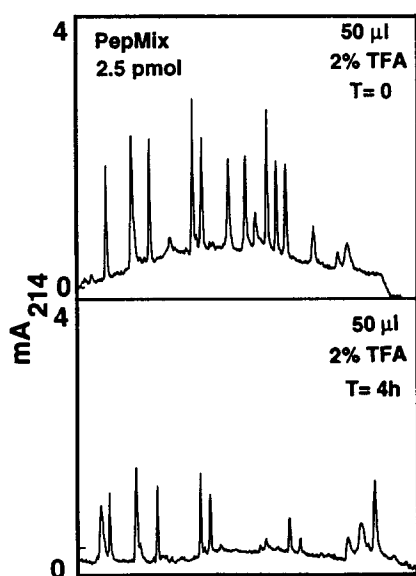
Fig. 5. Effects of storage on recovery of low picomole amounts of diluted peptides. RP-HPLC profiles of 2.5 pmol PepMix6 (composition listed in Table 1) are shown; two samples (2.5 pmol each; prepared by two-way splitting of one 5 pmol sample) were diluted into 50 µl of 2% TFA and injected, the first (shown in top panel) immediately after preparation, and the second (bottom panel) after keeping the vial on the bench for 4 h. HPLC configuration was as described under Experimental; a 1.0 mm I.D. Vydac 218 TP5115 C_{18} column and an LC packings U-Z view flow cell were used. Chromatographic conditions were: a linear two-step acetonitrile gradient (in 0.1% TFA) of 3.5–35%/22.5 min, 35–70%/12.5 min at a flow-rate of 30 µl/min and at ambient temperature. Full scale on each panel corresponds to 0.004 AU at 214 nm; time scale is from 15 to 32 min.

quence analysis is the only alternative. This requires cleavage of the protein and separation of the resulting peptides. Different techniques, including for proteins separated on gels, have been developed for this purpose [1,28–31]. A blocked protein should, therefore, no longer serve as a cheap excuse for failure, nor be the reason for abandoning a preferred research strategy. In our laboratory, we have selected the Aebersold method [28] for *in situ* proteolysis and adapted it for routine use at the 10–20 picomole level [1]. Current efforts to further improve on this technique require the use of microbore RP-HPLC (this study) and implementation of various new precautions, in addition to the ones

discussed in the previous section, to guarantee acceptable recoveries at every step.

Since it is not the topic of this paper, we will only briefly touch on the major categories of micro-preparative "rules" that have not yet been discussed: (i) low picomole level sequencing cannot be done in a dirty laboratory crowded with people that are not strictly needed there; (ii) sophisticated HPLC systems should be operated and maintained with great care and discipline, including daily chromatography of several control standards and blanks; (iii) formulation of peak selection criteria have been necessitated by a growing volume of digests while trying to limit the number of wasted sequencing runs on protease-derived peptides (from autolysis), peptide mixtures and ghost peaks. MALDI-MS now features a prominent role in these pre-Edman routines (see further). A detailed account on the practical aspects of micro-preparation will be published elsewhere. The importance of the sample preparation for successful sequencing cannot be sufficiently emphasized. Without it, sophisticated microsequencer and mass spectrometry improvements, no matter how well thought of, are essentially useless.

Two issues directly related to microbore RP-HPLC deserve further comment, fraction collection and peptide rechromatography. At a flow of 30 µl/min, peaks will typically elute in 20–30 µl of solvent. This volume is about equal to the small drops forming at the end of the glass capillary outlet tubing. When the beginning of a peak is observed, the forming droplet is quickly removed with a kimwipe and the fraction collected by holding the end of the capillary, so that it just touches the wall of the micro-Eppendorf tube. The delay time of 1.3 s conveniently allows for this little manipulation. The acetonitrile-containing solvent has a low viscosity and flows easily to the tip of the tube. No droplet is formed; this limits the collection volume and allows efficient collection of closely eluting peaks. Tedious as this manual collection may be, by comparison, so called "smart" automated fraction collectors are totally inadequate.

For repurifications, fractions (20 µl) should be acidified with 5 µl TFA (for reasons discussed

earlier) and then diluted two-fold with solvent A before injection. We have not yet worked out the details for optimal microbore two-dimensional HPLC column combinations. Our only experiences to date have been with rechromatographies of peptides eluted from an Inertsil C_{18} column on the same column but using a shallower gradient (typically 0.35% acetonitrile per min). As the example of a real-life sample in Fig. 6 shows, recoveries after such rechromatographies were on the order of 40–60% (as determined from peak heights); repurified peptides proved to be homogeneous (by sequencing analysis). Care should be taken to dilute early eluting

Fig. 6. Repurification of peptide peaks obtained from micro-bore RP-HPLC. RP-HPLC profile of a peptide mixture, obtained after *in situ* tryptic digestion of protein CM-CRKAp110; precise amount of protein was not known but estimated, from amido black staining of the nitrocellulose blot, to be about 10 to 15 pmol. Peaks T17, T26, and T37 (indicated) were rechromatographed on the same column after addition of 5 μl neat TFA and two-fold dilution with water; results are shown in the insets. HPLC configuration was as described under Experimental; a 1.0 mm I.D. SGE Inertsil 100 GL-1-ODS-I10/5 C_{18} column and an LC packings U-Z view flow cell were used. Chromatographic conditions were: a linear two-step acetonitrile gradient (in 0.1% TFA) of 3.5–35%/45 min, 35–70%/22.5 min at a flow-rate of 30 μl/min and at ambient temperature, for the primary separation; gradient during each rechromatography was 3.5–35%/90 min. Full scale corresponds to 0.004 AU at 214 nm; the same absolute scale also applies to the sections of the secondary chromatograms (insets).

peaks more than average to avoid peak broadening (as sadly illustrated for peak T17 in Fig. 6).

3.7. Micro-chemical sequencing of peptides

As shown, microbore RP-HPLC allows the recovery of 2–3 pmol of peptides, which can be used for direct sequencing. The initial coupling yields during chemical analysis could then be 1–2 pmol or less. Subpicomole PTH–amino acid identification is therefore required; in general, the method of choice is "on-line" narrow-bore HPLC. Rare examples have appeared in the literature where subpicomole sequencing was done and even fewer described how the sequence calls were actually made [1,3,32,33]. This can be explained, in part, by the fact that in biological research papers short thrift is usually given to the details of peptide sequencing experiments, as they were done *in the core facility* and, apparently, the fee-for-service status degrades this integral part of the research process to the level of purchasing a chemical. Nonetheless, it is fair to state that, until recently, peptide sequencing with femtomole level signals was very difficult.

This laboratory has had a longstanding interest in development and application of high-sensitivity sequencing techniques; details of these studies, technical and biological, are beyond the scope of this report and can be found elsewhere [1,7,12,23,32–35]. However, considering the fact that these accomplishments were made possible for a large part by the progress of HPLC technology (both for peptide and PTH separations), it may be of interest to the reader/chromatographer to view an example of an extended sequencing run carried out on an estimated 2 pmol (by peak height) of an unknown polypeptide. Fig. 7 contains the chromatograms of 23 consecutive cycles of PTH–amino acid identification of peptide JE-HAIICK-T51.3, isolated from a digest mixture by two rounds of microbore LC, all with signals in the femtomole range.

To specifically prepare the sequencer/analyzer instruments for an experiment as shown in Fig. 7 may take several days of meticulous optimizations and tests, at least in our hands. Still, in all
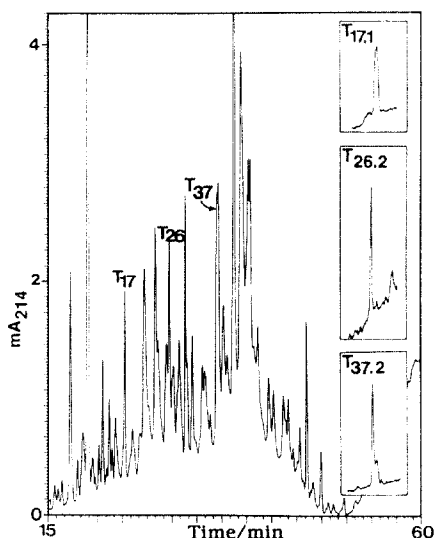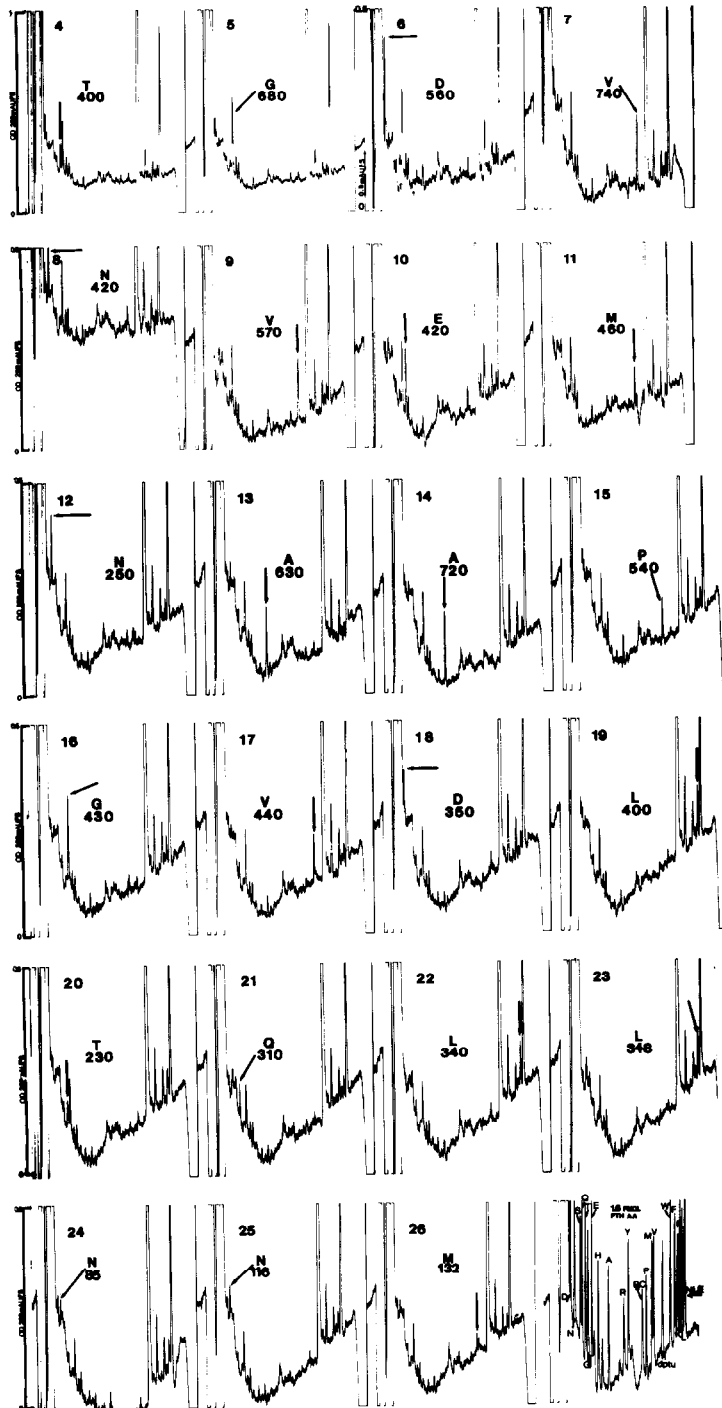
Fig. 7. Micro-chemical peptide sequence analysis. Amino acid sequence analysis of a tryptic peptide, isolated by microbore RP-HPLC from an *in situ* digest of electrophoretically separated (and blotted) protein JE-HAIICK. Sequencing conditions were as listed under Experimental. Starting amount of peptide was estimated to be 2 pmol (from LC peak height). Chromatograms 4–26 are shown; full scale corresponds to 0.001 AUFS for cycles 4 and 5 and 0.0005 AUFS for cycles 6–26. PTH–amino acid peaks are indicated with an arrow and the femtomolar quantities shown. A chromatographic standard (1.6 pmol of each amino acid in the sequencer flask) is shown at the bottom right; scale of the standard is 0.001 AUFS at 269 nm.

too many cases, high-sensitivity chemical sequencing data turn out to be non-ideal, *i.e.* complex and/or incomplete. For instance, ambiguous calls in the first two cycles (due to amino acid contaminants), gaps in sequences (associated with PTH–W,–C,–H,–R,–S etc. at levels below 200–500 fmol) and inability to identify the C-terminal Lys or Arg (of tryptic peptides) are a frequent occurrence. In addition, mixed peptides yield mixed sequences. An additional, complimentary, analytical technique was therefore required for error-free gap-filling and deconvolution of sequence data.

3.8. Combined Edman-chemical–MALDI-MS peptide sequencing

If MS is going to be routinely used in combination with Edman-chemical sequencing, one should be able to cope with a wide array of applications and diversity of situations (*e.g.* varying peptide quantities, sizes, mixtures, etc.), especially in a core facility setting where throughput is an issue as well. High throughput does not leave much room for improvization or unlimited trial and error. Thus, a generally applicable protocol needed to be developed for facile, reproducible mass analysis. It is imperative that for combined Edman–MS sequencing to be successful, (i) accuracy of the mass measurements must be within 1 u (for peptides up to 4000 dalton) and (ii) peptide consumption for MS must be kept to the absolute minimum. A bonus from routine mass spectrometric pre-screening of peptides is that mixtures and artefacts (*e.g.* trypsin autolytic products, chemical background) can be weeded out to save valuable sequencing time and expenditure.

Both electrospray-MS [36] and MALDI-MS [10] have recently been shown to be of practical utility in this regard, but to date, no careful comparative studies have been conducted to evaluate the preferred use of one or the other technique in any particular situation. As a more theoretical treatise of MALDI *versus* electrospray ionization is outside the scope of this report, the most obvious practical difference is the suitability of the latter for "on-line" con-

nection with LC. Although this may offer an advantage in terms of MS analysis automation, column eluates need to be split for "on-line" MS and "off-line" sequencing, still mandating fraction collection. During LC–MS, the mass analyzer is also dedicated to a single HPLC system. In contrast, "off-line" MALDI-TOF-MS accommodates post-run analysis of peak fractions from several LC experiments carried out concurrently, a frequently occurring situation in our laboratory. Since there is usually no pressing need to analyze each and every peak of a digest, we feel that MALDI-MS is certainly adequate. Here, we will present a case study, illustrating the capabilities of Edman–MALDI-MS sequencing.

In collaboration with the laboratory of Dr. F. Ulrich Hartl (MSKCC), protein JF-HSP75 was purified by SDS-PAGE, electroblotted onto nitrocellulose, stained with amido black, the band excised and digested *in situ* with trypsin, all as described [1]. Peptide fragments were separated by microbore RP-HPLC; the resulting chromatogram is shown in the top panel of Fig. 8. Peptide T54, because of its position in the late part of the chromatogram, was analyzed first. MALDI-MS (bottom panel of Fig. 8) indicated the presence of a single peptide of about 23–25 amino acids in length. Some 20 residues could be sequenced (Table 2), allowing a Genbank search, which turned up an excellent match with the sequence of yeast HSP70 [37]. The m/z value for T54 (2628.4) was in excellent agreement with the value (MH$^+$ = 2627.92) for the predicted tryptic peptide (residues 572–596) which contains the limited sequence, so confirming the identity of the entire peptide.

The identification of protein HSP70 was then confirmed by matching experimental m/z values of several more peptides with theoretical values of predicted tryptic fragments. Theoretical average isotopic masses were calculated from the published sequence [37] using ProComp version 1.2 software (obtained from Dr. P.C. Andrews, University of Michigan, Ann Arbor, MI, USA). Examples (peptides T18, T46 and T51) are given in Table 2; in all cases, discrepancies between m/z and theoretical [MH$^+$] were less than 0.6 dalton.
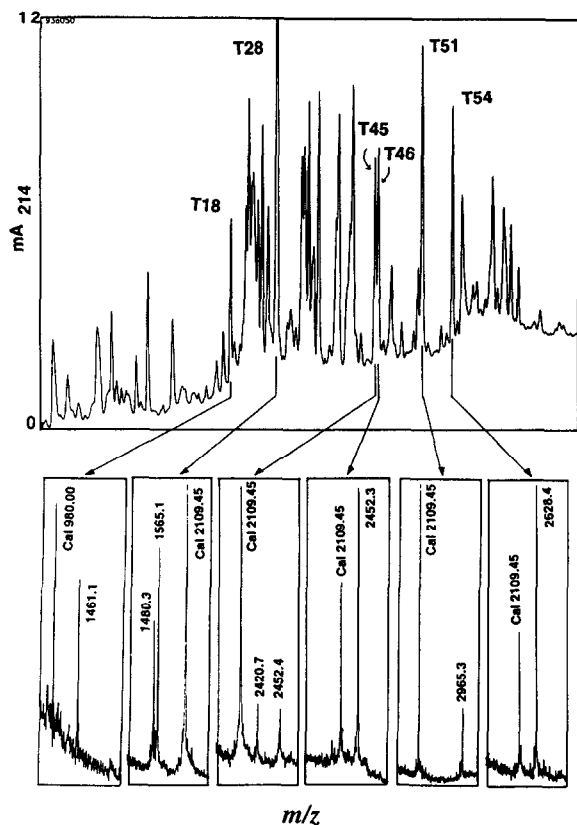
Fig. 8. Peptide microbore RP-HPLC with MALDI-MS analysis. Protein JF-HSP75 was purified by SDS-PAGE, electroblotted onto nitrocellulose, stained with amido black, the band excised and digested in situ with trypsin, all as described [1]. Peptide fragments were separated by microbore RP-HPLC; the resulting chromatogram is shown in the top panel, with a time scale from 20 to 65 min. HPLC configuration and conditions were the same as described under Fig. 6. Portions (1 μl) of selected peak fractions were taken for MALDI-TOF analysis using a Vestec LaserTec ResearcH instrument; conditions were as described under Experimental. Relevant sections of the mass spectra are shown (bottom panel); m/z values are listed for all peaks, including the calibrants (cal).

As this study was one of the first examples of mass-aided protein identification in our laboratory, additional peptide peaks were analyzed by chemical sequencing to confirm the result. Peptide T45 (Fig. 8) yielded two peak m/z values, one of them near identical to the single peak m/z value found for partially co-eluting peak T46.

However, sequence analysis of T45 indicated a major and a very minor sequence (Table 2) that could easily be separated. As sequencing yields are indicative for the amounts of peptides applied, this clearly shows that no quantitative conclusions can be drawn from the equal sized m/z peaks observed during MALDI-MS analysis (Fig. 8, bottom panel).

The major sequence obtained for T45, however, was not perfect. Ser was only tentatively identified in cycle one due to the presence of various other (contaminating) amino acids; a misinjection occurred during analysis of the PTH-residue in cycle 10. The mass summed from the identified residues fell 272.9 short of the experimental m/z (Table 2). Assuming a Ser in position one and a Lys at the end (obligatory C-terminal Lys or Arg for all tryptic peptides), a mass discrepancy of 57.6 was still unaccounted for. This value was sufficiently close to the average isotopic mass of Gly (57.05) and far enough removed from the second nearest amino acid mass (Ala at 71.08) to reliably position the former residue at cycle ten (as confirmed by the published sequence [37]).

A similar analysis was carried out for peptide T28 (Table 2). Again, a major and minor sequence were observed, as were two peak m/z values. The major sequence differed from the expected mass (m/z = 1565.1) by 155.5, allowing the positioning of Arg (average isotopic mass = 156.19) at the C-terminus. The mass summed from the minor sequence was 455.1 less than expected. Assuming that the two tentative assignments (Val at cycle 6; Glu at cycle 12) were correct and an Arg at the C-terminus, 70.66 was still unaccounted for, leading us to assign Ala (average isotopic mass = 71.08) to cycle 7. Thus, complete structures of peptides could be proposed on the basis of incomplete sequence and accurate mass.

3.9. Conclusions

We have described the optimized configuration of a microbore RP-HPLC system for the purification of low picomole quantities of peptides. This required evaluation and selection of appro-

Table 2
Tryptic peptide analysis by combined chemical sequencing–laser-desorption mass spectrometry

Peptide	Sequence	[MH$^+$] part. seq.	m/z	Difference/ a.a. assigned	Position HSP-75	[MH$^+$] HSP-75
T18	ND	NA	1461.1	NA	332–345	1460.63
T28(a)	AVITVPAYFNDAQ.. (major)	1409.6	1565.1	155.5/R$_{14}$	144–157	1565.78
(b)	VTPSF(v)xFTPE(e).. (minor)	1025.2	1480.3	455.1/V$_6$A$_7$E$_{12}$R$_{13}$	39–51	1480.67
T45(a)	(s)TSGNTHLGx*QDFDTNLLEHF..	2147.8	2420.7	272.9/S$_1$G$_{10}$K$_{22}$	224–245	2420.10
(b)	QxLExYVA.. (very minor)	722.8	2452.4	1729.6/none	544–565	2451.75
T46	ND	NA	2452.3	NA	544–565	2451.75
T51	ND	NA	2965.3	NA	276–302	2965.17
T54	xKIEAALSDALAALQIE(d)PxA..	1825.1	2628.4	803.3/none	572–596	2627.92

Selected peak fractions from the chromatographic separation shown in Fig. 8 were analyzed by chemical sequencing (Sequence) and by MALDI-MS (m/z; see also Fig. 8, bottom panel). Symbols (used in sequences): amino acids printed in lower case (and in parentheses) were assigned with a lower level of confidence; x = no amino acid assigned; x* = no assignment due to instrument failure; .. = no further sequence calls but unlikely to be the C-terminus. ND = not done; NA = not applicable. [MH$^+$] part. seq. denotes the mass values calculated by summing the average isotopic masses of all amino acids that were positively identified during chemical sequencing. Difference/a.a. indicates the difference between experimental mass (m/z) and the calculated mass of the partial sequence (MH$^+$ part. seq.), and the amino acids assigned to the gaps as to yield a perfect match between calculated and experimental masses. Position HSP-75 indicates the location of the peptide in the published sequence [37] that was retrieved by searching Genbank. [MH$^+$] HSP-75 denotes the theoretical average isotopic mass of the peptide, calculated from the published sequence using ProComp software.

priate columns (1.0 mm I.D.), detector flow cells and wavelengths, and overall assembly with minimal dead volume. We found that the sensitivity of our system equalled, probably surpassed, that of advanced chemical micro-sequencing (2–4 pmol minimally required [3,7]).

As a chemical sequencer cannot be "on-line" connected with a micro-preparative HPLC system, fractions must be collected and transferred. With a typical flow of 30 μl, efficient manual collection is possible and fractions (about 20 μl in volume) can still be handled without unacceptable losses, albeit with great precautions. Furthermore, major difficulties were encountered to quantitatively load low- or sub-picomole amounts of peptide mixtures efficiently onto the RP-HPLC column for separation. Discipline and rigorous adherence to sample handling protocols are thus on order when working at those levels of sensitivity.

With adequate instrumentation and handling procedures in place, low-picomole amounts of peptides (< 5 pmol) could be easily prepared for analysis by combined chemical sequencing–MALDI-MS. The power of this approach resides in the complementarity of these two analytical techniques. Aside from the fact that MS provides a simple and ultra-sensitive way to pre-screen peptides for sequencing, mass-aided interpretation of Edman-chemical sequencing data has allowed (i) confirmation of the results, (ii) deconvolution of mixed sequences, (iii) proposal of complete structures on the basis of partial sequences, and (iv) confirmation of protein identification (obtained by database search with a single, small stretch of peptide sequence) by mass-matching of several more peptides with predicted proteolytic fragments.

4. Acknowledgements

5. References

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