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Semi-automated chromatographic procedure for the isolation of acetylated N-terminal fragments from protein digests*

Dan L. Crimmins* and Richard S. Thoma

Howard Hughes Medical Institute, Core Protein/Peptide Facility, Washington University School of Medicine, 660 S. Euclid Avenue, Box 8022, St. Louis, MO 63110 (USA)

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ABSTRACT

Several published procedures have been combined to develop a general strategy for the specific identification and isolation of the acetylated-N-terminal fragment from all other proteolytic fragments. This ruse can be divided into four steps: (i) succinylation of the substrate to block lysine NH, groups; (ii) enzymatic digestion of the modified protein; (iii) automated phenylisothiocyanate derivatixation of the protease derived fragments to block newly generated "free" N-termini; and (iv) reversed-phase high-performance liquid chromatography with on-line photodiode array spectroscopy. The individual phenylthiocarbamyl-peptide species exhibit an increased reversed-phase retention time and a greater UV (210-297 nm) profile compared to the corresponding control (-phenylisothiocyanate) digest. The N-terminal acetylated fragment shows neither a retention time shift nor an augmented UV profile. To validate each process step, synthetic peptides and acetylated-N-terminal proteins of known sequence were used as test samples. The desired fragment was isolated from three proteins and positively identified by electrospray mass spectrometry and amino acid composition. Proteins with other N-terminal blocking groups should be amenable to this procedure.

INTRODUCTION

Proteins, proteolytic fragments, and peptides blocked at the N-terminus are refractory to Edman degradation. It has been estimated that over 50% of soluble mammalian proteins are blocked at the N-terminus [1]. Considerable effort has therefore been devoted to deblocking schemes and subsequent sequence analysis. The target fragment is usually isolated from a digest and two distinct analytical procedures are then employed to obtain further structural information. In the first, removal of the blocking group chemically [2], or enzymatic liberation of the N-acetyl (Ac) amino acid itself [3–8], can provide a substrate suitable for sequence determination. Mass spectrometry-mass spectrometry (MS-MS) analysis is the other approach and is a direct means of obtaining sequence information [9–15]. Recent studies have shown that the deblocked N-terminal fragment can be sequenced in the presence of all other appropriately modified digest fragments [5,6,16]. However, as these other fragments were not recovered during this procedure, potentially important structural information may be missed, particularly for unknown proteins [3-8,161.

In this study, we have combined several published procedures [5,17,18] to develop a general, semi-automated strategy that successfully identifies and isolates the AC-N-terminal fragment from complex reversed-phase high-performance

* Corresponding author.

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liquid chromatography (RP-HPLC) peptide maps. Furthermore, since fractions from the entire chromatogram are collected, other protease-derived fragments are available for analysis if desired. We first present data using synthetic peptides of known composition as test samples to validate the procedure and then demonstrate that the overall method can be used to positively isolate the target fragment from proteolytic digests of three distinct proteins of known sequence. The strategy outlined in this report should be applicable to proteins with other N-terminal modifications, for the quality control of recombinant proteins, and in studies of homologous proteins from different sources.

EXPERIMENTAL

Chemicals and reagents

Peptides and proteins were purchased from Sigma (St. Louis, MO, USA) or Peninsula Labs. (Belmont, CA, USA) and used without further purification. For use, they were resuspended to a nominal concentration of l-3 mg/ml with water or 0.1% trifluoroacetic acid (TFA) and stored at -20°C. Pierce (Rockford, IL, USA) supplied phenylisothiocyanate (PITC), triethylamine (TEA), TFA, and 8 M guanidinium chloride (GdmCl); succinic anhydride is a Fluka (Ronkonkoma, NY, USA) product; and chymotrypsin was obtained from Boehringer Mannheim (Indianapolis, IN, USA). Recovery studies used [^{3}H]leucine-enkephalin, 36.3 Ci/mmol, purchased from DuPont/NEN (Boston, MA, USA). The sources of HPLC columns and solvents, water purification, and additional materials have been described [19,20].

Succinylation

Slight modifications to established procedures were employed [5]. Amounts of 10-100 μg of the stock protein solution were taken to dryness in a Savant (Farmingdale, NY, USA) Speed-Vat, after which 200 μl of 0.1 M NH_4HCO_3 and 200 μl of 8 *M* GdmCl were added to the dried sample. The pH was adjusted to *ca. 8 with 12% (v/v)* TEA *(ca. 20 μl)* and then 0.5 mg of solid succinic anhydride added to the vial to start the reaction. The pH was monitored and maintained between 8-9 by adding 20 μl aliquots of 12% TEA. After 30 min at room temperature, another portion of succinic anhydride was added and the reaction pH monitored as above, resulting in a final added TEA volume of *ca.* 200 μl. The mixture was incubated overnight at room temperature. Any remaining succinic anhydride was converted to succinic acid by adding 100 μl of 10% (v/v) TFA and the sample was transferred to a Millipore Ultrafree-MC 5000MW tube (Millipore, Boston, MA, USA) for spin-dialysis and solvent exchange (0.1 M NH_4HCO_3) at 10000 rpm in a microfuge.

Proteolysis

Prior to digestion, the volume of the protein substrate was increased to *ca.* 140 μl with 0.1 *M* NH_4HCO_3. A 10-μl volume of chymotrypsin at 0.5 mg/ml in water was added and the digestion mixture incubated for 4 h at 37°C. The reaction was quenched by adding 5 μl of 10% TFA and if desired, a small aliquot of this sample was injected on C_{18} RP-HPLC as the control (-PITC), with the remainder transferred to a Varian (Walnut Creek, CA, USA) 9090 autosampler conical vial and taken to dryness in a Savant speed-vat.

Automated PITC derivatization

To the dried, digested protein sample or dried synthetic peptide, were added 18 μl of ethanol–TEA-water (7:1:1, v/v/v) followed by 2 μl PITC. The liquids were mixed by four air displacement cycles and the reaction mixture was incubated for 20 min at room temperature [18]. Excess PITC and unwanted reaction by-products were extracted [18] by adding 90 μl heptane–ethyl acetate (2:1, v/v), after which 70 μl of 10 m*M* sodium phosphate pH 6.0 was added to increase the volume of the aqueous phase. The extraction liquid (90 μl heptane-ethyl acetate only) was added in two more portions and 75 μl of the aqueous layer were injected on C_{18} RP-HPLC.

RP-HPLC on-line photodiode array (PDA) analysis

Samples were chromatographed using standard RP-HPLC conditions (C,, Vydac 218TP54; solvent A is 0.1% TFA and solvent B is 0.095%

TFA in acetonitrile-water (90:10); 10% B to 60% B in 60 or 90 min at 1 ml/min and 37°C with fractions collected at OS-min intervals). On-line PDA analysis with a Varian Polychrom 9065 was used to identify the AC-N-terminal fragment by the absence of phenylthiocarbamyl (PTC)-associated UV spectrum with the LC Star Polyview software library search routine supplied with the instrument. Basically, UV spectra (210–297 nm) of synthetic peptides containing known chromophores (aromatics, aromatics + PTC-peptide, PTC-peptide, and peptide only) are stored in the search library [21]. The chromatogram of the PITC derivatized, digested protein is then searched peak-by-peak against all spectra in the library to generate statistically relevant similarity indices for each peak. Positive matches of known fragments typically give value of 0.995 or greater. In this study, we are interested in that peak which is devoid of PTC attributes. In addition, this peak will not shift in retention time compared to the control (-PITC) chromatogram and is easily spotted in the three-dimensional PDA printout for the +PITC sample. Note also, that it is possible to tentatively assign the aromatic content of each peak through the library search routine. Data acquisition and analysis procedures, and additional chromatographic hardware have been described [19,20].

Analysis of RP-HPLC fractions

An aliquot of the desired fraction was subjected to standard 6 *m* HCl–Phenol hydrolysis for 1 h at 150°C and the hydrolysate manually derivatized with PITC for analysis [18]. Another aliquot was taken to dryness and resuspended in 15-30 μl of 1% (v/v) NH_4OH prior to negative-ion electrospray (ES)-MS on a Sciex (Thornhill, Canada) API-III instrument (Dr. Kevin Duffin, Monsanto Corporate Research, St. Louis, MO, USA).

RESULTS AND DISCUSSION

Assessment of the procedure with synthetic peptides

Synthetic peptides were used as test substances to validate several steps of the overall process. Specifically, the peptides were subjected to automated PITC derivatization and RP-HPLC-PDA analysis. For PITC modification both the completeness and specificity of the reaction were investigated. In addition, [^{3}H]leucine-enkephalin provided a means of determining the recovery of the derivatized PTC-peptide after the heptane–ethyl acetate extraction procedure. In this case, greater than 97% of the tritiated peptide remained in the aqueous phase; a new RP-HPLC peak appeared with an increased retention time and less than 0.4% of the unreacted peptide was observed (data not shown).

Fig. 1 is a composite set of three-dimensional PDA chromatograms for DRVYIHPFHLLVYS with either a "free" or acetylated N-terminus (panels A and B, respectively) and for pEQRLGNWAVGHLM(NH_2) and the lysine3 [K^3]-substituted peptide (panels C and D, respectively). Comparison of the PDA UV profile for the first peptide pair, −PITC (Fig. 1A) and +PITC (Fig. 1B), shows that covalent addition of PTC to "free" amines results in a retention time shift [17] and an augmented, distinct, and readily identifiable 210-297 nm spectrum for the derivatized species. Just as revealing, is the absence of a chromatographic shift and spectral change for the Ac-N-terminal peptide. This demonstrates the ability to identify PTC-peptides that contain aromatic residues in combination with non-PTC, aromatic containing, Ac-N-terminal peptides. The PTC chromophore of the adduct in this case dominates the 240-297 nm profile. The second peptide pair comprises species that are identical in every respect except for an arginine to lysine substitution at sequence position 3 ([R^3] to [K^3]). Each peptide is also blocked at the N-terminus in the form of a pyroglutamyl (pE) group so that only the [K^3] variant is expected to react with PITC. These two peptides are not resolved by the standard C_{18} RP-HPLC elution conditions (Fig. 1C), and as for the former test peptide pair, these pE-blocked peptides contain an even more intense aromatic-region absorbing residue, tryptophan. Nonetheless, the expected result is evident as the [K^3] peptide increases in RP-HPLC retention time after PTC modification concomitant with a characteristic UV spectrum for this product (Fig. 1D). PTC modification of the [K^3] variant produced a complete separation of this peptide pair where there originally was none.

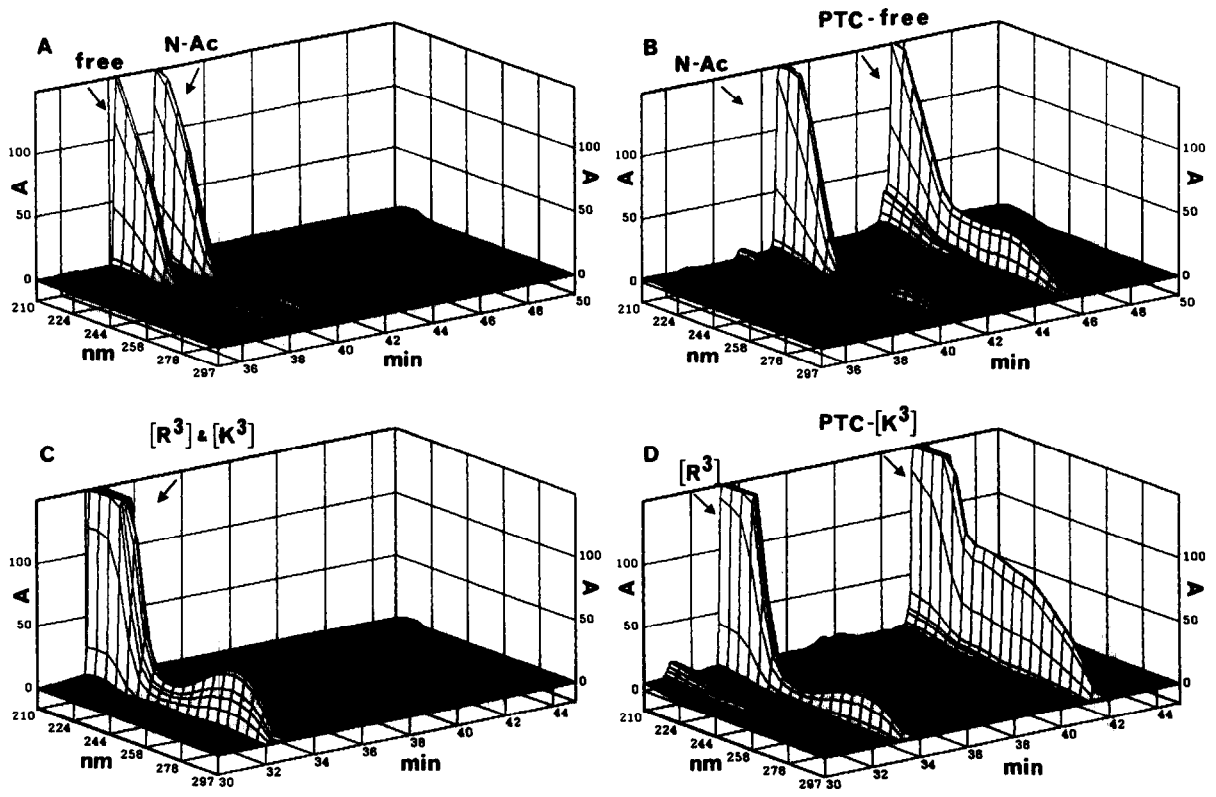

Fig. 1. C_{18} RP-HPLC of **N-Ac** and "free" renin substrate tetradecapeptide, and **[R³]-Bombesin** and **[K³]-Bombesin. Three**-dimensional PDA chromatogram of the mixtures displayed at the relevant retention times. (A) "Free" and **N-Ac** DRVYIHPFHLLVYS mixture minus **PITC** at 150 **mV** full scale **(mVFS);** (B) "free" and **N-Ac** DRVYIHPFHLLVYS mixture plus PITC at 150 **mVFS;** (C) **[R³]-** and **[K³]-pEQRLGNWAVGHLM(NH_2)** mixture minus PITC at 150 **mVFS;** (D) **[R³]-** and **[K³]-pEQRLGNWAVGHLM(NH_2)** mixture plus PITC at 150 **mVFS**. The HPLC gradient was for 60 min.

Assessment of the procedure with proteins and protein fragments

Three Ac-N-terminally blocked proteins of known sequence, calmodulin, cytochrome c and parvalbumin were subjected to each step of the overall procedure. The first step involves protein denaturation with **GdmCl** and succinylation of lysine residues. This is necessary because the desired N-terminal fragment may contain lysine residues which would react with PITC and thus produce a false negative assignment. For the present group of test proteins, cytochrome c and parvalbumin do contain lysine residues in the N-terminal fragment but calmodulin does not. Practically, this means for calmodulin **succinyla**tion can be omitted if one is interested in isolating the AC-N-terminal fragment only. Several fragments will however, likely contain multiple PTC reaction sites in the form of lysine residues. This streamlined process could be useful for example in isolating the blocked N-terminal, lysine deficient fragment from homologous proteins and then sequencing by the appropriate means. We were able to isolate the same proteolytic AC-N-terminal **peptide** from **cal**modulin whether or not the protein was **suc**cinylated (data not shown).

Proteolytic digestion of the succinylated protein was the next step and in the majority of our reactions chymotrypsin was used. This was not an arbitrary choice on our part, and derives from the known primary specificity of chymotrypsin for aromatic residues. We wanted to minimize the possibility that the AC-N-terminal **peptide** would contain multiple aromatic amino acids thereby obfuscating the UV spectral analysis.

The fact that for all three proteins the Ac-N-terminal fragment contained only one aromatic amino acid which was the C-terminal residue (see Tables I and II) speaks favorably for the above rationale.

A Varian 9090 autosampler with robotic automix routines was used previously to automate PITC derivatization of amino acid hydrolysates and liberated residues from exopeptidase digestions [18]. In that study, a heptane extraction step effectively removed excess PITC and reaction by-products from the aqueous sample prior to HPLC injection. For the present study, an empirically determined 2:1 ratio of heptane-ethyl acetate was found to be a compromise between high peptide recovery and acceptable removal of unwanted reaction products. Another aspect of the automated derivatization reaction is that the free amino groups of the proteolytic fragments appeared to be quantitatively modified with PITC. This result derives from the observation that the peaks in the PDA chromatogram exhibit characteristic PTC spectral properties except for the blocked N-terminal fragment (see below).

TABLE I

AMINO ACID ANALYSIS OF THE ISOLATED Ac-N-TERMINAL PROTEOLYTIC FRAGMENTS

The fragments were isolated as indicated from the PDA chromatograms shown in Figs. 2-4.

Residue[a]	Protein					
	Calmodulin		Cytochrome c		Parvalbumin	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
Asx	1.0	1	1.0	1	1.8	2
Glx	4.4	s	1.1	1	2.0	2
Gly[b]	–	–	2.6	2	1.9	1
Thr[c]	1.1	1	–	–	1.5	1
Ala	2.1	2	–	–	4.0	4
val	–	–	1.0	1	–	–
Met[c]	–	–	–	–	0.12	1
Ile	1.1	1	1.0	1	2.0	2
Leu	1.1	1	–	–	1.8	2
Phe	1.0	1	1.1	1	1.1	1
Lys[b]	–	–	2.4	3	1.7	2

[a] The standard three letter code is used for the amino acids and the experimentally observed (Obs.) and the expected (Exp.) residue values for the predicted AC-N-terminal fragment are listed.

[b] Gly values are high and Lys values low due to incomplete hydrolysis of PTC-succinyl-Lys and co-elution of this derivative with PTC-Gly.

[c] Met was oxidized to Met-sulfoxide during the hydrolysis. The *R* and S forms of PTC-Met-sulfoxide elute near PTC-Thr in our system, resulting in an inflated value for Thr.

Isolation and identification of C_{18} RP-HPLC AC-N-terminal proteolytic fragments

Calmodulin, cytochrome c and parvalbumin were each succinylated prior to protease treatment with chymotrypsin. After digestion, the resulting proteolytic fragments were reacted with PITC in an automated fashion and these modified samples were analyzed by C_{18} RP-HPLC with on-line PDA spectroscopy (Figs. 2-4).

The three-dimensional PDA spectral plot of the +PITC C_{18} RP-HPLC analysis for the calmodulin digest is shown in Fig. 2. Panel A represents the relevant chromatographic region from 15 to 55 min. The characteristic UV spectra of the PTC-adducts observed for the synthetic peptides (Fig. 1B and D) are readily apparent here also. Time axis expansion of panel A to 15 to 35 min (panel B) and 35 to 55 min (panel C) aided in the visual assignment of the N-terminal peptide. Simple inspection of these two plots allows one to immediately assign the peak at *ca.* 32 min (panel B, arrow) as the target fragment. The fraction containing this peak was subjected to amino acid analysis and negative-ion ES-MS to positively identify the peptide fragment. Table I lists the amino acid results for the calmodulin fraction. The agreement between the expected residues for the predicted AC-N-terminal chymotryptic fragment of calmodulin with the experimentally determined values is good. ES-MS analysis of this fraction produced a clean spectrum (see Fig. 5A) with the tabulated molecular mass listed in Table II. These combined data (Tables I and II) unequivocally demonstrate that the fraction isolated from the three-dimensional PDA plot is the AC-N-terminal chymotryptic fragment of calmodulin.

A similar representation of the three-dimensional PDA +PITC proteolytic digest chromatograms are shown for cytochrome c in Fig. 3 and for parvalbumin in Fig. 4. Again, time axis

TABLE II

MOLECULAR MASS ASSIGNMENT OF THE ISOLATED Ac-N-TERMINAL PROTEOLYTIC FRAGMENTS

The fragments were isolated as indicated from the PDA chromatograms shown in Figs. 2-4.

Predicted chymotryptic fragments

Calmodulin N-Ac–Ala–Asp–Gln–Leu–Thr–Glu–Glu–Gln–Ile–Ala–Glu–Phe.
Calculated M_r = 1434.7.

Cytochrome c N-Ac-Gly-AspVal-Glu-Lys(X)-Gly-Lys(X)-Lys(X)-Ile-Phe.
Calculated M_r = 1461.7.
Lys(X) = succinyl-Lys.

Parvalbumin N-Ac–Ala–Met–Thr–Glu–Leu–Leu–Asn–Ala–Glu–Asp–Ile–Lys(X)–Lys(X)–Ala–Ile–Gly–Ala–Phe.
Calculated M_r = 2176.1.
Lys(X) = succinyl-Lys.

ES-MS analysis of fragments[a]

Protein	Mass peaks [b]	Species/charge	Observed M_r
Calmodulin	716.2	$(M-2H^+)^{2-}$	1434.4
	727.4	$(M-3H^+ + Na^+)^{2-}$	1434.8
	738.3	$(M-4H^+ + 2Na^+)^{2}$	1434.0
	1433.3	$(M-H^+)^{-}$	1434.3
	1455.6	$(M-2H^+ + Na^+)^{-}$	1434.6
			Average 1434.5
Cytochrome c	486.2	$(M-3H^+)^{3-}$	1461.6
	730.1	$(M-2H^+)^{2-}$	1462.2
	741.0	$(M-3H^+ + Na^+)^{2-}$	1462.0
	1460.8	$(M-H^+)^{-}$	1461.8
	1482.8	$(M-2H^+ + Na^+)^{-}$	1461.8
			Average 1461.9
Parvalbumin	1087.3	$(M-2H^+)^{2-}$	2176.6
	1144.1	$(M-H+ + CF_3COO^-)^{2-}$	2176.2
			Average 2176.4

[a] See Fig. 5 for the mass spectrum of each protein fragment.
[b] The peaks were assigned as shown in Fig. 5A, calmodulin; B, cytochrome c; and C, parvalbumin.

expansion of the cytochrome c example (Fig. 3), originally displayed from 15 to 55 min (panel A), to 15 to 35 min (panel B) and 35 to 55 min (panel C) was used to identify the N-terminal fragment. Amino acid analysis of the isolated fraction at cu. 31 min (panel B, arrow) resulted in coincident values for the experimental and predicted residue compositions of the N-terminal fragment (Table I). This predicted chymotryptic peptide contains three Lys residues (Table II) which if not succinylated would yield a chromatographic species with a PDA UV spectrum characteristic of a PTC-peptide. The actual PDA UV spectrum containing the isolated fraction is devoid of PTC attributes suggesting that this fragment is completely succinylated. The amino acid composition (Table I) supports this assertion but the definitive data in this case are derived from ES-MS analysis (see Fig. 5B). Molecular mass assignments for the mass peaks from this analysis given in Table II clearly indicate that the isolated fraction contains a completely succinylated species. The protease derived fragment under consideration is thus positively identified as the succinylated Ac-N-terminal chymotryptic fragment of cytochrome c.

Fig. 4A is an analogous C_{18} RP-HPLC–PDA

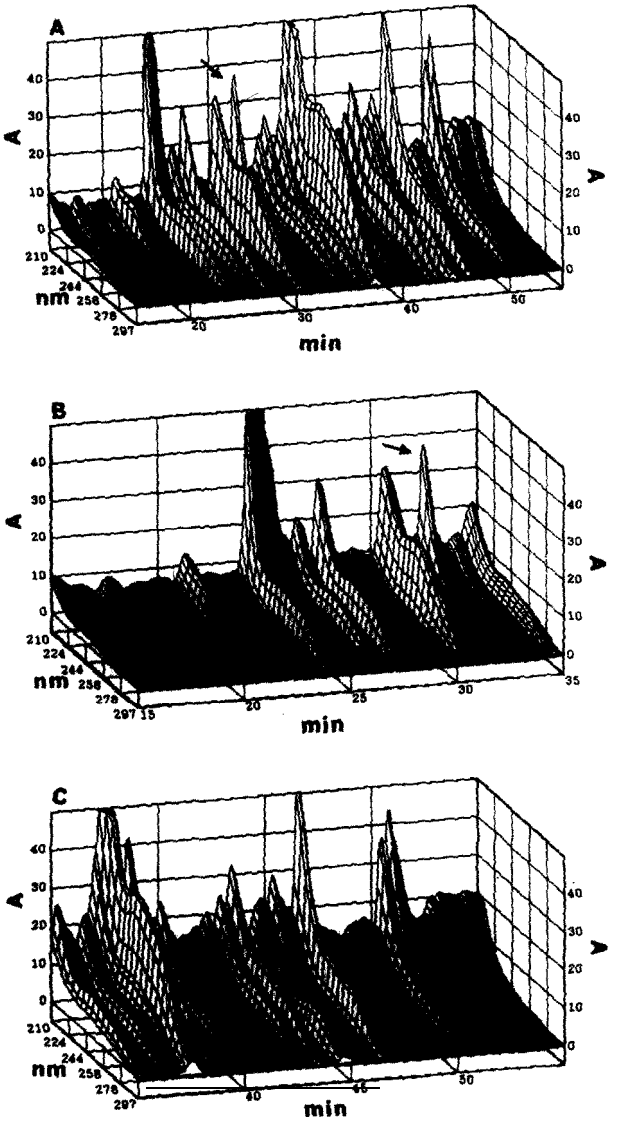

Fig. 2. C_{18} RP-HPLC-PDA three-dimensional display of the chymotryptic digest of calmodulin. (A) Chromatogram from 15 to 55 min, 210 to 297 nm at 50 mVFS; (B) expanded time axis from 15 to 35 min; (C) expanded time axis from 35 to 55 min. The arrow in A and B indicates the isolated Ac-N-terminal proteolytic fragment of calmodulin. The HPLC gradient was for 60 min.

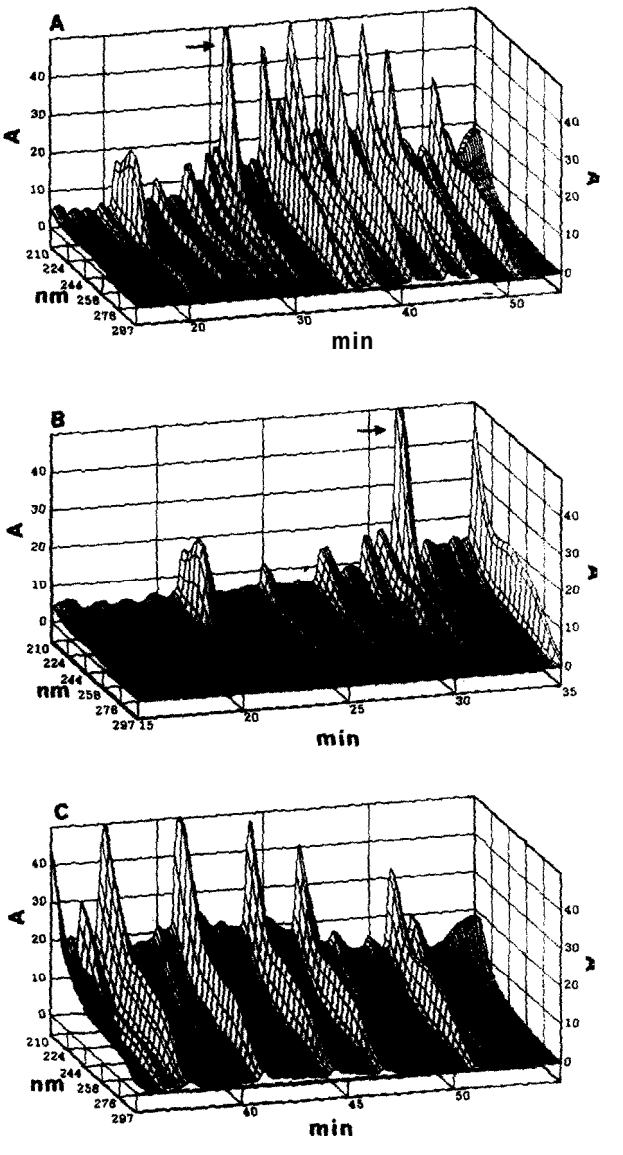

Fig. 3. C_{18} RP-HPLC–PDA three-dimensional display of the chymotryptic digest of cytochrome c. (A) Chromatogram from 15 to 55 min, 210 to 297 nm at 50 mVFS; (B) expanded time axis from 15 to 35 min; (C) expanded time axis from 35 to 55 min. The arrow in A and B indicates the isolated Ac-N-terminal proteolytic fragment of cytochrome c. The HPLC gradient was for 60 min.

three-dimensional profile of the region of interest at 20 to 80 min for the parvalbumin chymotryptic digest. This complex plot was expanded on the time axis into visually manageable blocks (panels B-D). The data in these three panels readily show the presence of many FTC-peptide peaks, some of which additionally contain identifiable aromatic residues [21]. In this current study though, attention is focused on the triplet set of peaks near cu. 70 min (panel D, arrow}. The latest eluting peak of this triplet group did not exhibit signature PTC UV spectral

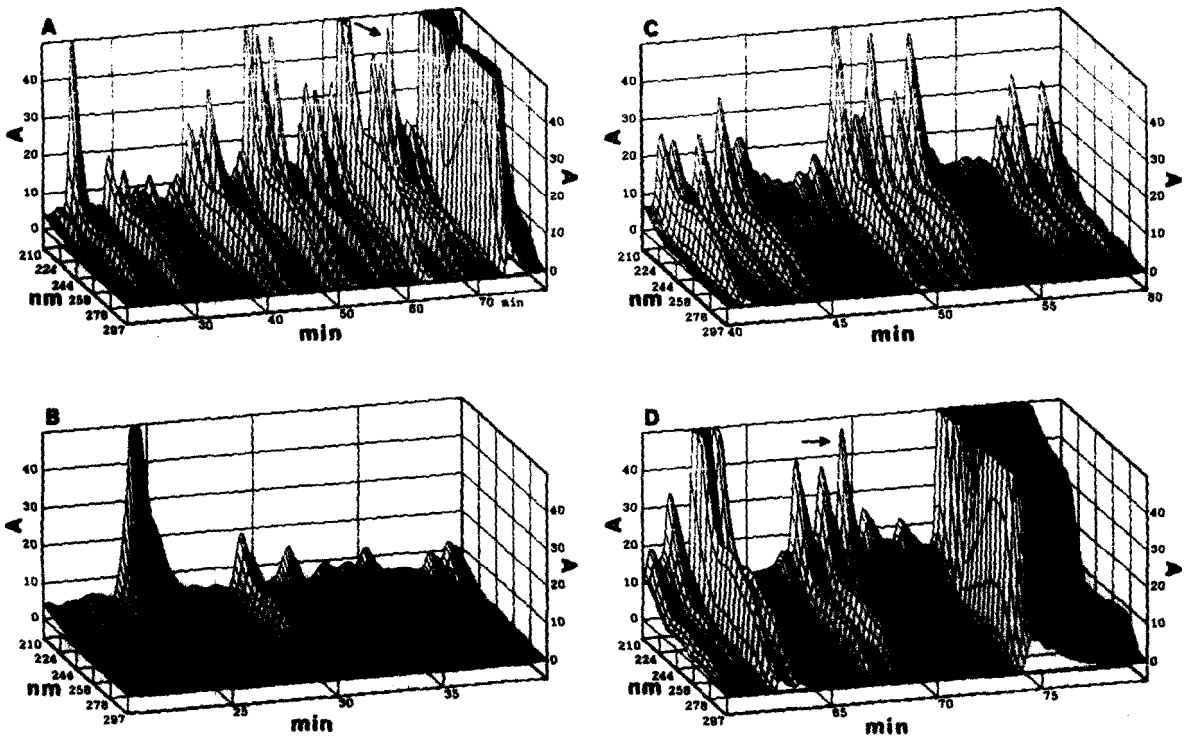

Fig. 4. C_{18} RP-HPLC-PDA three-dimensional display of the chymotryptic digest of parvalbumin. (A) Chromatogram from 20 to 80 min, 210 to 297 nm at 50 mVFS; (B) expanded time axis from 20 to 40 min; (C) expanded time axis from 40 to 60 min; (D) expanded time axis from 60 to 80 min. The arrow in A and D indicates the isolated AC-N-terminal proteolytic fragment of parvalbumin. The HPLC gradient was for 90 min.

properties and was therefore selected for further analysis. The residue composition of this fraction was assessed from amino acid analysis (Table I) and was found to be consistent with the expected residue values. ES-MS analysis produced an acceptable spectrum (Fig. SC) for which the species molecular mass of the indicated mass peaks were calculated and listed in Table II. Two Lys residues are present in the predicted Ac-N-terminal chymotryptic fragment of parvalbumin. A similar line of reasoning as expressed in the cytochrome c case, when applied here, results in the conclusion that the isolated parvalbumin peptide is the completely succinylated Ac-N-terminal chymotryptic peptide. One other aspect of the parvalbumin chromatogram is that the late C_{18} RP-HPLC elution of the N-terminal peptide could have been anticipated due to its high ratio (10:18) of hydrophobic amino acids and overall length.

We calculated the overall recovery for each proteolytic digest. This percentage is defined as the moles of the isolated Ac-N-terminal peptide fraction recovered from the PDA chromatogram relative to the initial moles of protein subjected to the four step procedure. These values were fairly constant for all three proteins, ranging from a high of 49% for cytochrome c to a low of 29% for parvalbumin. The recovery for calmodulin was intermediate at 42%. Several likely factors contributed to the ultimate recovery of the blocked peptide. These include: completeness of the succinylation step, recovery of these modified proteins after spin-dialysis and solvent exchange, the extent of protease digestion, and any losses due to the C_{18} RP-HPLC itself. In this initial investigation we did not attempt to optimize these individual processes for each protein but rather, sought to develop a scheme which we believe will have general applications. Clearly,

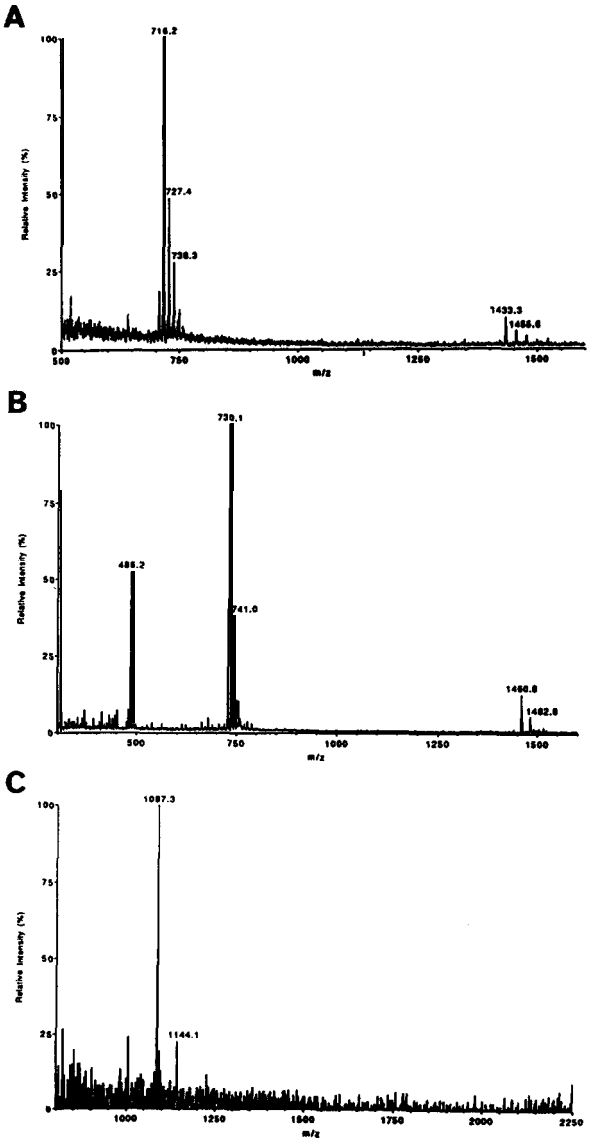

Fig. 5. ES-MS analysis of the isolated Ac-N-terminal proteolytic fragments from calmodulin, cytochrome c and parvalbumin. Spectra were recorded in the negative-ion mode. The species and charge states of the mass peaks, and the resulting molecular masses are listed in Table II. (A) Calmodulin fragment isolated from PDA chromatogram (Fig. 2); (B) cytochrome c fragment isolated from PDA chromatogram (Fig. 3); (C) parvalbumin fragment isolated from PDA chromatogram (Fig. 4).

each step could be selectively optimized for a given protein to increase the overall recovery of the isolated AC-N-terminal fragment.

CONCLUSIONS

The blocked N-terminal peptide identification strategy presented in this report was successfully applied to complex proteolytic digests for the HPLC isolation of the AC-N-terminal peptide from three distinct proteins. Since all HPLC peaks were collected in this procedure, they could be analyzed with the net result being an increase in HPLC information content.

On-line PDA spectroscopy provided some analytical advantages over conventional single or dual-wavelength detection. It was not absolutely necessary for instance, to perform a −PITC HPLC experiment for comparative purposes. This was evident from inspection of the PDA plot as the N-terminal peptide showed distinct (non-PTC) UV spectral properties. Preliminary work [21] indicates that as an added bonus, both the aromatic residue type and number can be assigned to several of the PTC-peptide peaks. We are currently [22] implementing the semi-automated strategy to develop an analogous C-terminal proteolytic fragment isolation procedure based on a variation of several C-terminal peptide isolation schemes [23–27]. It is anticipated that successful application of each, coupled with say MS-MS sequencing [28,29], would provide a novel and powerful polymerase chain reaction "anchoring" process.

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