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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN NEUROPHYSIN DISULFIDE ASSIGNMENT

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SUMMARY

The combined use of ion-exchange, and reversed-phase high-performance liquid chromatography (HPLC) for the isolation of cystine-containing peptides from highly heterogeneous products of the proteolytic digestion of bovine neurophysins is described. The protein was sequentially cleaved by enzymes of decreasing specificity; the peptides released were initially fractionated by gel chromatography and then purified by HPLC. The purified peptides were analyzed by determination of their amino acid composition and mass spectrometry, supported by sequencing techniques. Three of the seven disulfide pairs of neurophysin have now been assigned. The usefulness of the combined use of HPLC and mass spectrometry in assigning these and the other disulfide pairs is illustrated.

INTRODUCTION

Neurophysins are a class of hormone-binding proteins present in the posterior pituitary of vertebrates. They contain 14 half-cystine residues per chain of *ca.* 95 amino acids[1,2]. An earlier report[3] on the disulfide pairing of these 14 half-cystine residues was based on an incorrect sequence; the need for a complete re-evaluation of this pairing has been evident for some time[4]. We were interested in determining the pairing of these cystines, because such pairing places considerable constraints on possible neurophysin conformations. This facilitates an understanding of the mechanism by which neurophysin interacts with pituitary hormones and related peptides.

The problem to be faced in analyzing disulfide pairing in a protein such as neurophysin is the high disulfide content. Thus, digestion with very specific enzymes, such as trypsin and *Staphylococcus aureus* protease V8 (Staph. protease) in this case does not lead to the release of any disulfide containing peptides, although a number of chain scission points can be identified[5]. This reflects the presence of interlocking disulfides. In order to obtain the release of disulfide-containing peptides, therefore, multiple points of cleavage must be obtained, as by the use of relatively non-specific

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proteases or partial acid hydrolysis. Such procedures necessarily generate very complex peptide mixtures, which, in turn, must be fractionated into pure analyzable peptides before disulfide assignments can be made. We have made use of high-performance liquid chromatography (HPLC) to isolate pure peptides or mixtures of closely related peptides, which can be analyzed by mass spectrometry (MS) and sequencing techniques. The present paper focuses on the usefulness of the applications of HPLC and MS in this project.

Although digestion of bovine neurophysins with trypsin and Staph. protease does not lead to the release of cystine-containing peptides, digestion of either native bovine neurophysin-II or trypsin-treated neurophysin-II with subtilisin led to the generation of many cystine-containing peptides. Initial analysis of these subtilisin-generated peptides indicated to us that ambiguity in their identification was markedly diminished by prior trypsin treatment. The latter not only removed some irrelevant protein sequences, but also placed constraints on the identities of Arg- and Cys-containing peptides. Accordingly, bovine neurophysin-II was first digested with trypsin[6], and the resultant protein core was then subjected to subtilisin digestion. Carboxypeptidase A (CPA) treated bovine neurophysin-I[7] was similarly treated, although it proved more resistant to subtilisin digestion (see below). The heterogeneous peptides, resulting from subtilisin digestion, were isolated and characterized as described below.

EXPERIMENTAL

Chemicals

Carboxypeptidases A and B, trypsin (diphenylcarbamyl chloride-treated) type XI, *Staphylococcus aureus* protease V8, and subtilisin BPN′ (type VII) were obtained from Sigma (St. Louis, MO, U.S.A.), HPLC-grade trifluoroacetic acid (TFA) from Pierce (Rockford, IL, U.S.A.), ammonium acetate and ammonia from J. T. Baker (Phillipsburg, NJ, U.S.A.) and acetonitrile was from American Burdick & Jackson (Muskegon, MI, U.S.A.). All other chemicals were reagent-grade and were obtained from local sources.

Reaction of neurophysins with trypsin

Bovine neurophysin-II[1,5] (22 mg) was digested with trypsin in 0.1 *M* (pH 8.0) borate buffer, for 5 h at 37°C and then for 16 h at room temperature, using a protein-to-enzyme ratio of 25:1 (w/w). The reaction was terminated by lowering the pH to 3.0. The mixture was applied to a Sephadex G-50 column and eluted with 0.2 *M* acetic acid. The yield of trypsin-digested neurophysin-II was 15.3 mg. Bovine neurophysin-I[1,5] was allowed to react with carboxypeptidase A prior to trypsin treatment to remove the 3-carboxyl terminal residues, as described previously[7].

Reaction of trypsin-digested neurophysins with subtilisin

Trypsin-digested neurophysin-II (14.0 mg) (or CPA- and trypsin-digested neurophysin-I) was digested with subtilisin [protein-to-enzyme ratio = 40:1 (w/w)] in 0.2 *M* (pH 8.0) ammonium bicarbonate buffer, for 4 h at 37°C, and then the reaction mixture was applied to a Sephadex G-50 column in 0.2 *M* acetic acid.

Amino acid analysis

Amino acid analysis was performed as previously described[8]. Prior to 6 *M* hydrochloric acid hydrolysis, samples were routinely oxidized with performic acid according to the procedure of Hirs[9], unless otherwise specified.

Sequencing of peptides

Stepwise Edman degradation of the purified peptides was performed on a gas-phase sequencer (Model 470A, Applied Biosystems, Foster City, CA, U.S.A.). The phenylthiohydantoin-amino acids were identified by HPLC[10]. Note that disulfide bridges were not cleaved prior to sequencing.

HPLC apparatus

Proteins and peptides were purified on an IBM 9533 ternary liquid chromatograph equipped with one IBM 9523 (IBM Instruments, Danbury, CT, U.S.A.) and one ISCO V[4] (ISCO, Lincoln, NE, U.S.A.) variable-wavelength UV detector with independent plotters. Cation- and anion-exchange columns (75 × 7.5 mm) were from Waters Assoc. (Milford, MA, U.S.A.). The cation-exchange column (Protein Pak SP-5PW, part No. 88043) had sulfopropyl groups attached to a 10-μm hydrophilic rigid resin with a pore size of 1000 Å, while the anion-exchange column (Protein Pak DEAE-5PW, part No. 88044) had diethylaminoethyl groups attached to a hydrophilic resin with identical pore size as above. The cyano column (250 × 4.6 mm) was Zorbax CN obtained from DuPont (Wilmington, DE, U.S.A.), part No. 880952705, and had a particle size of 6 μm.

Chromatographic conditions

Aqueous mobile phases were prepared in de-ionized water and filtered through a 0.45-μm Nylon-66 filter (Rainin Instruments, Woburn, MA, U.S.A.). Mobile phases were evacuated and then purged with ultrapure helium continuously to remove dissolved oxygen. The two HPLC detectors were placed in series and were set at two different wavelengths, *e.g.*, 215 and 254 nm or 254 and 280 nm. Absorbance at 254 nm was found useful in defining cystine (and/or aromatic residues)-containing peptides. Cation-exchange HPLC was performed with an ammonium acetate buffer using a pH gradient from 4.30 to 7.50 and an ammonium acetate salt gradient from 0.02 *M* to 0.50 *M* in 30 min at a flow-rate of 1 ml/min, while anion-exchange HPLC was performed with a pH gradient from 7.50 to 4.70 and an identical salt gradient and procedure. Because of absorbance by ammonium acetate at higher concentrations, absorptions were monitored at 254 and 280 nm. Typically 10–20 nmoles of peptides/proteins were dissolved in 50 μl of buffer A for injection and eluent monitored at 0.02 a.u.f.s. sensitivity. The column pressure during the HPLC run remained in the range 14–16 bar. Reversed-phase HPLC was routinely performed on samples obtained from ion-exchange HPLC above. The solvent system was: A, 0.05% TFA in water; B, 0.05% TFA in acetonitrile. A linear gradient from 0 to 50% B in 30 min at a flow-rate of 0.8 ml/min was used. Absorption was monitored at 215 nm and 254 nm at sensitivities of 0.10 and 0.01 a.u.f.s., respectively. Typical sample injections were 3–5 nmol in 50 μl of solvent A. All HPLC was performed at ambient temperatures and all HPLC subfractions were routinely lyophilized and stored at 4°C.

Mass spectrometry

MS measurements were performed on the ^{252}Cf fission fragment ionization time-of-flight mass spectrometer described previously[11]. Samples were prepared for measurement by adsorption of *ca.* 1 nmol of peptide from solution (*ca.* 0.1 nmol/μl in 0.1% TFA) on a thin nitrocellulose film, as previously described[12]. The accuracy of the mass determinations was greater than 250 ppm.

RESULTS AND DISCUSSION

Digestion of bovine neurophysin-II with trypsin resulted in the release of peptides, representing the sequences Ala_1–Arg_8 (cleavage at position 8), Gly_{19}–Arg_{20} (cleavage at positions 18 and 20), and Glu_{87}–Val_{95} (cleavage at positions 86 and 93) (Fig. 1). In addition, trypsin also cleaved at Arg_{43} and Arg_{66}, but did not cleave the Lys_{59}–Pro_{60} bond[6].

Digestion of trypsin-treated neurophysin-II with subtilisin resulted in the generation of a number of cystine-containing peptides in useful yield. On the other hand, reaction of CPA and trypsin-digested neurophysin-I with subtilisin did not generate a significant yield of disulfide-containing peptides (data not shown). It seems from this that the variable carboxyl region of the neurophysins influences their reaction with subtilisin. Accordingly, most studies utilized peptides from neurophysin-II.

The crude digestion products from the subtilisin treatment of trypsin-treated neurophysin-II were initially chromatographed on Sephadex G-50 and divided into three fractions (Fig. 2). The leading fraction (A) was eluted later than either native

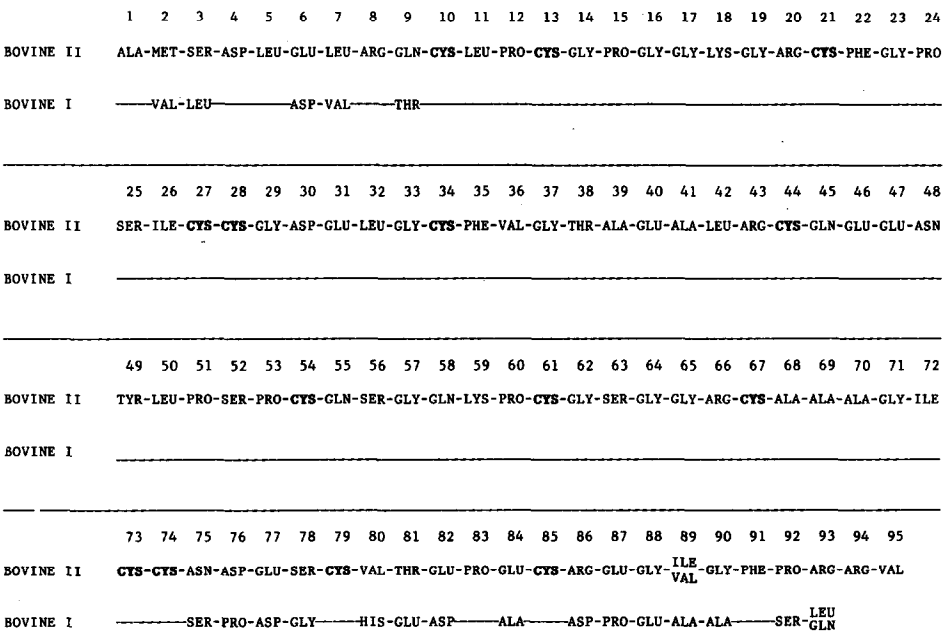

Fig. 1. Amino acid sequences of bovine neurophysins-I and -II[13]. The complete sequence is shown only for neurophysin-II, while for neurophysin-I the substitutions are listed.

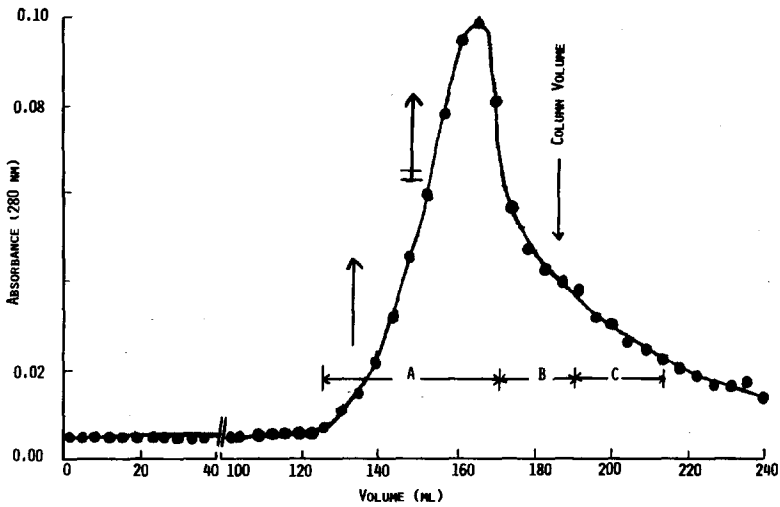

Fig. 2. Sephadex G-50 chromatography of subtilisin-digested, trypsin-treated neurophysin-II. Column, 110 cm × 1.5 cm; volume, 185 ml. Eluent, 0.2 *M* acetic acid; detection at 280 nm, 0.1 a.u.f.s. The elution positions of native neurophysin-II (↑) and trypsin-digested neurophysin-II (‡) from the same column are indicated for comparison.

or trypsin-treated neurophysin-II and was shown (*vide infra*) to contain intermediate-size peptides with more than one disulfide each. The second fraction (B) represented the bulk of the smaller cystine-containing peptides and was further fractionated by HPLC, as described below. The trailing fraction (C) remains to be analyzed.

Isolation of peptides containing Cys_{67} and Cys_{85}

Amino acid analysis of fraction B (data not shown) indicated a high Arg content. Accordingly, an aliquot of fraction B was loaded on a cation-exchange HPLC column and fractionated (Fig. 3A). The peak that eluted at 14.23 min was collected and analyzed for amino acid composition (Table I). The data indicated the presence of a cystine peptide that contained one Arg, two Ala, and a fractional Gly. The amino acid sequence of neurophysin (Fig. 1) indicates that the two Ala must be within the sequence Cys_{67}–Ala–Ala. Additionally, because trypsin treatment of neurophysin-II gives virtually complete cleavage at all arginine residues under our conditions[5], the Arg can only represent the sequence Cys_{85}–Arg_{86}. Accordingly, the data strongly suggested the presence of the peptide

$$\underbrace{Cys_{67}\text{–Ala–Ala}}\ ,\ \underbrace{Cys_{85}\text{–Arg}}$$

with some potential contribution of the sequence Cys_{67}–Ala–Ala–Ala–Gly. However, the mass spectrum of this fraction (Fig. 3B) yielded two principal masses, 467.3 and 538.3 u (unified atomic mass unit), assignable to the two peptides

$$\underbrace{Cys_{67}\text{–Ala}}\ ,\ \underbrace{Cys_{85}\text{–Arg}} \quad \text{and} \quad \underbrace{Cys_{67}\text{–Ala–Ala}}\ ,\ \underbrace{Cys_{85}\text{–Arg}}$$

respectively. The measured masses corresponded closely with the calculated masses for these two peptides, 467.2 and 538.2 u (lowest isotopic component). Thus, while the presence of Gly (Table I) among the amino acids suggested the presence of an

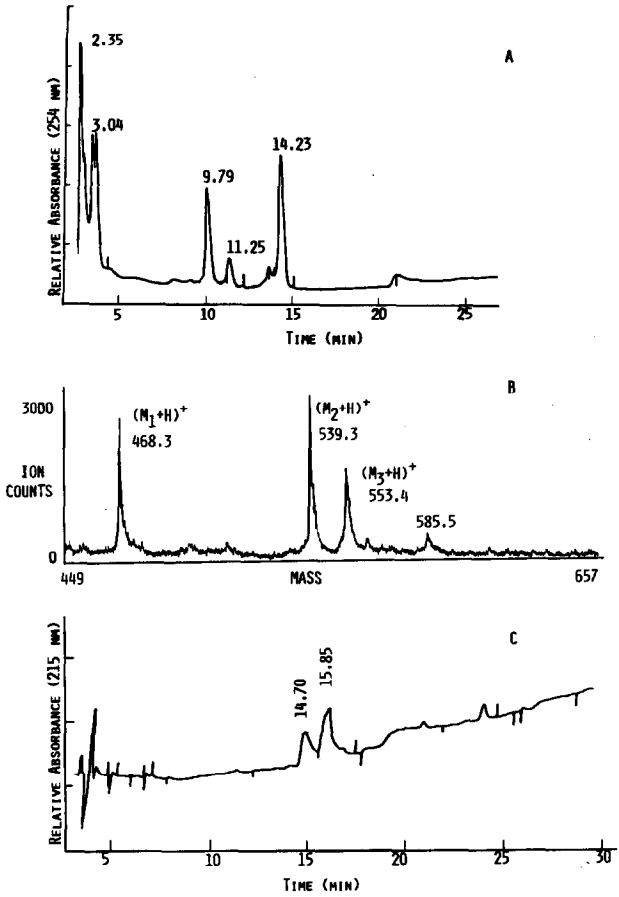

Fig. 3. (A) Cation-exchange HPLC of subfraction B (Fig. 2), obtained from the digestion of trypsin-treated neurophysin-II with subtilisin. Column, 75 × 7.5 mm; elution buffer, ammonium acetate (pH 4.3 → pH 7.5, 0.02 *M* → 0.5 *M* in 30 min), linear gradient; detection at 254 nm; flow-rate 1 ml/min. Values on absorption maxima are elution times in min. (B) Partial ^{252}Cf fission fragment ionization time-of-flight mass spectrum of material in the peak eluted at 14.23 min in A. M_1, M_2, and M_3 denote three major compounds present in the HPLC peak. (C) Reversed-phase CN HPLC of the peak eluted at 14.23 min in A. Column, 250 × 4.6 mm; elution buffer, solvent A = 0.05% TFA in water, solvent B = 0.05% TFA in acetonitrile; linear gradient from 0 to 50% B in 30 min; detection at 215 nm; flow-rate, 0.8 ml/min.

extended component, containing Ala_{70} and Gly_{71}, such a component was not observed in the mass spectrum. It is relevant to note that, since this peptide was purified only by ion-exchange HPLC, the presence of more than one component of similar charge in a single chromatographic peak is not surprising. However, on subsequent analysis of this peptide by HPLC on CN (Fig. 3C) two components at 14.70 min and 15.85 min, respectively, were separated. These were not obtained in sufficient yield for amino acid analysis.

A third, relatively intense peak is observed in the mass spectrum corresponding to a compound with molecular weight 552.4 u. The identity and origin of this material

TABLE I

COMPOSITION OF PEPTIDES, CONTAINING Cys_{67} AND Cys_{85}, AS DETERMINED FROM THE PEAK ELUTED AT 14.23 min (FIG. 3A)

Amino acid	*Amino acid composition (number of residues per molecule)*	
	Observed	*Calculated*
Gly	0.5	0.0
Ala	2.1	1.5
Cys	2.0	2.0
Arg	0.9	1.0
	Assigned sequence (from MS data)	
	Cys_{67}-Ala | Cys_{85}-Arg and	Cys_{67}-Ala-Ala | Cys_{85}-Arg

is unclear. It should be noted, however, that the mass spectrometric method employed can exhibit very different responses for different compounds. Thus, the unexplained compound may be present in relatively small amounts.

It is also relevant that we have applied fraction B directly to an anion-exchange HPLC column and identified peptides containing Cys_{67} and Cys_{85} within the non-binding fraction. However, subsequent chromatography of this fraction on CN gave incomplete purification, due to the presence of many additional peptides in the non-binding anion-exchange fraction. Thus, the purification of a heterogeneous peptide mixture such as this is considerably expedited by isolation of those fractions which are retained by the initial ion-exchange column.

Isolation of peptides containing Cys_{21} and Cys_{44}

The peak that eluted at 2.35 min on cation-exchange HPLC of the peptide mixture (Fig. 3A) was separately collected and transferred to an anion-exchange column, where a broad, intense absorption peak which eluted at 12.52 min was collected (Fig. 4A). Amino acid analysis of this peak (Table II) indicated the presence of peptides, containing Cys_{21} and Cys_{44} and their surrounding sequences. MS analysis of this peak yielded a peptide with a molecular weight of 1129.2 u (Fig. 4B). This mass was assigned to the sequence which has a calculated mass of 1129.0 u (isotopically averaged mass).

Cys_{21}-Phe-Gly-Pro-Ser, Cys_{44}-Gln-Glu-Glu-Asn

In an independent experiment, the fractions that eluted at 2.35 and 3.04 min from the cation-exchange column (Fig. 3A) were rechromatographed on an anion-exchange column (Fig. 4C). The peaks exhibiting maxima at 11.57 and 11.87 min had an amino acid composition which again indicated the presence of peptides containing Cys_{21} and Cys_{44}. This fraction was further purified by reversed-phase HPLC on CN. Two closely spaced peaks, eluted at 21.2 min, were collected (Fig. 4D), but this third fractionation did not lead to any significant improvement in purity, as

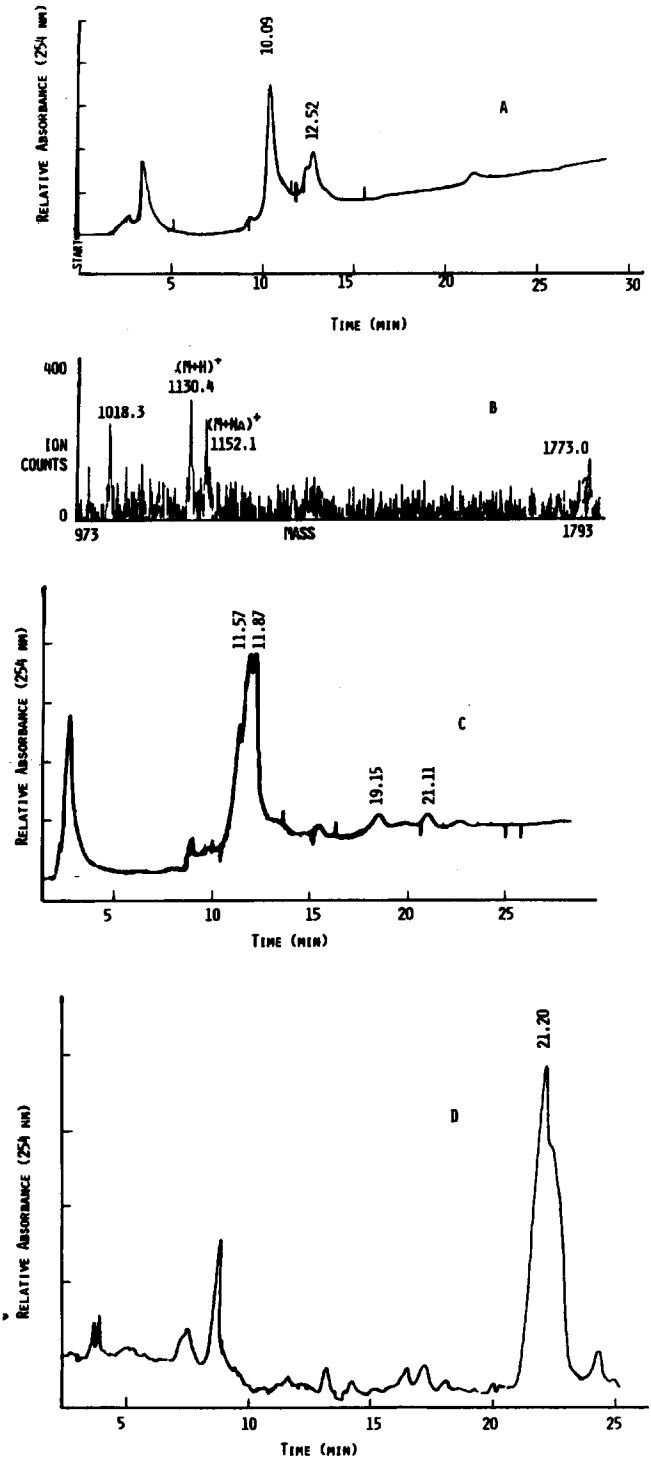
RELATIVE ABSORBANCE (254 nm)
10.09
12.52
A
START
5
10
15
20
25
30
TIME (MIN)
400
ION COUNTS
0
(M+H)+
1130.4
1018.3
(M+Na)+
1152.1
B
1773.0
973
MASS
1793
RELATIVE ABSORBANCE (254 nm)
11.57
11.87
C
19.15
21.11
5
10
15
20
25
TIME (MIN)
RELATIVE ABSORBANCE (254 nm)
21.20
D
5
10
15
20
25
TIME (MIN)

TABLE II

COMPOSITION OF PEPTIDES, CONTAINING Cys_{21} AND Cys_{44}, AS DETERMINED FROM PEAK ELUTED AT 12.52 min (FIG. 4A)

Amino acid	*Amino acid composition (number of residues per molecule)*	
	Observed	*Calculated*
Asx	1.0	1.0
Ser	1.0	1.0
Glx	3.0	3.0
Pro	1.2	1.0
Gly	1.2	1.0
Cys	1.9	2.0
Phe	1.0	1.0

Assigned sequence (from MS and gas-phase sequencing data)

Cys_{21}-Phe-Gly-Pro-Ser
|
Cys_{44}-Gln-Glu-Glu-Asn

judged by its amino acid composition. Gas-phase sequence analysis of this fraction confirmed the presence of the peptide Cys_{21}–Ser_{25}, paired to Cys_{44}–Asn_{48}. The heterogeneity observed on anion-exchange and CN chromatography can probably be accounted for by the presence of two peptides, differing by the C-terminal Asn. The non-quantitative yield of Asn, observed in the sequence analysis, lends weight to this hypothesis.

The above procedures for the isolation of the anionic Cys_{21}–Cys_{44} peptide included a cation-exchange chromatogram, from which the non-binding fractions were isolated and purified by subsequent anion-exchange and CN chromatography. From our observation of the Cys_{67}–Cys_{85} peptide, it is evident that the initial cation-exchange chromatography does not achieve significant purification of an anionic peptide from a complex mixture and is therefore unnecessary. We have confirmed this by employing a purification procedure for the Cys_{21}–Cys_{44} peptide from neurophysin-II that omits cation-exchange chromatography. This procedure has also been successfully used to isolate the same peptide from digests of bovine neurophysin-I.

Fig. 4. (A) Anion-exchange HPLC of peak eluted at 2.35 min in Fig. 3A. Column, 75 × 7.5 mm; elution buffer, ammonium acetate (pH 7.5 → pH 4.7, 0.02 *M* → 0.50 *M* in 30 min), linear gradient; detection at 254 nm; flow-rate, 1 ml/min. Peak eluted at 10.09 min is due to a non-cystine containing peptide. (B) Partial mass spectrum of material in the peak eluted at 12.52 min in A. $(M+H)^+$ and $(M+Na)^+$ denote the protonated and the sodium-cationized peaks, respectively, of the same compound. The identity of the compound giving rise to the peaks at masses 1018.3 and 1773.0 u is unclear. (C) Anion-exchange HPLC of peaks eluted at 2.35 and 3.04 min in Fig. 3A. Column and buffer were the same as in A. (D) Reversed-phase CN HPLC of peaks eluted at 11.57 and 11.87 min in C. Solvent system and gradient were identical to those in Fig. 3C; detection at 254 nm.

Isolation of a peptide containing Cys_{74} and Cys_{79}

In addition to tthe peaks observed at *ca.* 12 min in the anion-exchange chromatogram discussed above (Fig. 4C) a broad peak was observed at 21.11 min. The amino acid composition of this peptide (Table III) was ambiguous and indicated the presence of Cys_{79}-containing peptide residues, paired with either Cys_{28} or Cys_{74}. This peptide was therefore subjected to gas-phase sequencing, which gave the sequence

thus uniquely assigning it to the Cys_{74}–Cys_{79} pairing. MS on this sample (Fig. 5) gave a mass of 1223.3 u, which corresponded closely with the calculated isotopic averaged mass of 1223.1 u.

Isolation of peptides containing Cys_{10}, Cys_{13}, Cys_{27}, Cys_{28}, Cys_{34} and Cys_{54}

Each of the above peptides contained a single disulfide bridge. We have also utilized HPLC to fractionate peptides containing more than one disulfide. Fraction A, obtained from Sephadex G-50 chromatography of the original subtilisin digest (Fig. 2) was subjected to anion-exchange HPLC, which gave a dominant peak that eluted at 9.30 min. This fraction was twice chromatographed on a CN column, resulting in the elution of a peak with broad absorbance between 30.8 min and 34.8 min (Fig. 6A); the other intense peaks were non-peptide impurities. Amino acid analysis of the 30–34 min peak (data not shown) suggested the presence of two or three disulfides, but otherwise the data were not readily interpretable. The nature of this peak was elucidated by MS analysis, which yielded masses for eight major components present in this fraction (Fig. 6B). In Table IV the measured masses for these eight peptides are compared with the masses calculated for a series of proposed structures. The results indicate that the masses can be systematically assigned, assuming the presence of three peptides, linked by three disulfide bridges, the heterogeneity of masses reflecting the heterogeneity of subtilisin cleavage position at the amino and carboxyl termini of each peptide.

TABLE III

COMPOSITION OF PEPTIDES, CONTAINING Cys_{74} AND Cys_{79}, AS DETERMINED FROM THE PEAK ELUTED AT 21.11 min (FIG. 4C)

Amino acid	*Amino acid composition (number of residues per molecule)*	
	Observed	*Calculated*
Asx	2.2	2.0
Thr	1.0	1.0
Ser	0.9	1.0
Glx	3.7	3.0
Pro	1.2	1.0
Gly	1.0	0.0
Cys	1.9	2.0
Val	0.9	1.0
	Assigned sequence (from MS and gas-phase-sequencing data)	
	Cys_{74}–Asn–Asp–Glu–Ser–Cys_{79}–Val–Thr–Glu–Pro–Glu	

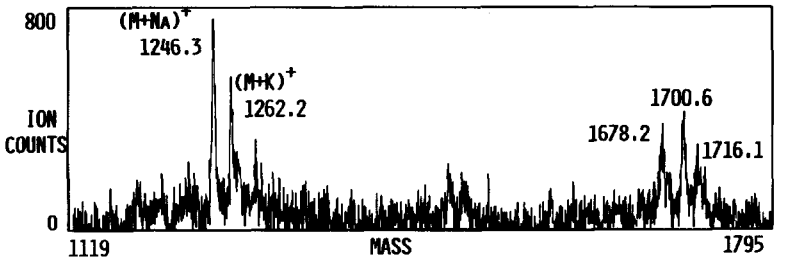

Fig. 5. Partial mass spectrum of material in the peak eluted at 21.11 min in Fig. 4C. $(M+Na)^+$ and $(M+K)^+$ denote the sodium- and potassium-cationized peaks, respectively, of the same compound. The identity of the compound giving rise to the peaks at masses 1678.2, 1700.6 and 1716.1 is unclear.

While the ability to systematically assign the masses in the above way would seem to make the sequences virtually unambiguous, this is not the case. We have also found that the masses can be assigned to a hypothetical series of related peptides containing two cystine bridges representing Cys_{34}, Cys_{54}, Cys_{61} and Cys_{73}. For example, the measured mass of 2563.2 u differs by only 0.5 u from that calculated for the disulfide-bridged sequences Asp_{30}–Phe_{35}, Asn_{48}–Cys_{61} and Ala_{70}–Cys_{73}. To re-

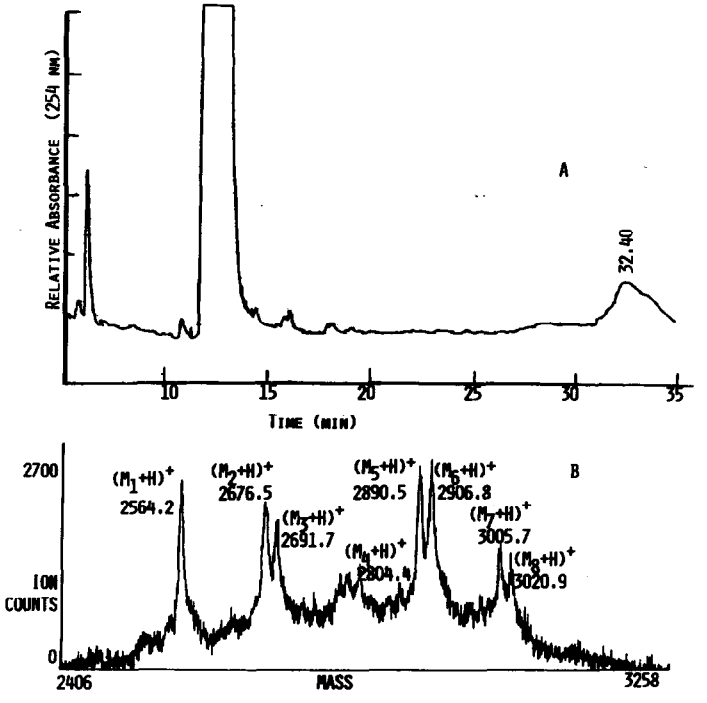

Fig. 6. (A) Reversed-phase CN HPLC of peak eluted at 9.30 min from anion-exchange HPLC of subfraction A (Fig. 2). Solvent system and gradient were identical to those in Fig. 3C; detection, 254 nm. The intense peak eluted at *ca.* 12 min was due to a non-peptide impurity. (B) Partial mass spectrum of material eluted between 30.8 min and 34.8 min in A. M_1–M_8 denote eight major compounds, present in the broad HPLC peak.

TABLE IV

ASSIGNMENT OF PEPTIDES, CONTAINING Cys_{10}, Cys_{13}, Cys_{27}, Cys_{28}, Cys_{34} AND Cys_{54}, AS DETERMINED BY A COMBINATION OF MS AND SEQUENCE ANALYSIS OF THE BROAD PEAK ELUTED BETWEEN 30.8 min AND 34.8 min (FIG. 6A)

Peak number	*Molecular weight*		*Δ**	*Postulated peptide structures***
	Measured	*Calculated*		
1	2563.2	2562.9	+0.3	Cys_{10}–Cys_{13}–Lys_{18} Ile_{26}–Cys_{27}–Cys_{28}–Cys_{34}–Phe_{35} Tyr_{49}–Cys_{54}
2	2675.5	2674.0	+1.5	$pGlu_{9}$–Cys_{10}–Cys_{13}–Gly_{17} Ile_{26}–Cys_{27}–Cys_{28}–Cys_{34}–Phe_{35} Tyr_{49}–Cys_{54}–Gln_{55}
		2677.0	−1.5	Cys_{10}–Cys_{13}–Lys_{18} Ile_{26}–Cys_{27}–Cys_{28}–Cys_{34}–Phe_{35} Asn_{48}–Cys_{54}
3	2690.7	2691.1	−0.4	Cys_{10}–Cys_{13}–Lys_{18} Ile_{26}–Cys_{27}–Cys_{28}–Cys_{34}–Phe_{35} Tyr_{49}–Cys_{54}–Gln_{55}
4	2803.4	2803.1	+0.3	$pGlu_{9}$–Cys_{10}–Cys_{13}–Gly_{17} Ile_{26}–Cys_{27}–Cys_{28}–Cys_{34}–Thr_{38} Tyr_{49}–Cys_{54}
5	2889.5	2889.2	+0.3	$pGlu_{9}$–Cys_{10}–Cys_{13}–Lys_{18} Ile_{26}–Cys_{27}–Cys_{28}–Cys_{34}–Phe_{35} Tyr_{49}–Cys_{54}–Ser_{56}
6	2905.8	2906.2	−0.4	Gln_{9}–Cys_{10}–Cys_{13}–Lys_{18} Ile_{26}–Cys_{27}–Cys_{28}–Cys_{34}–Phe_{35} Tyr_{49}–Cys_{54}–Ser_{56}
7	3004.7	3005.4	−0.7	Gln_{9}–Cys_{10}–Cys_{13}–Lys_{18} Ile_{26}–Cys_{27}–Cys_{28}–Cys_{34}–Val_{36} Tyr_{49}–Cys_{54}–Ser_{56}
8	3019.9	3019.4	+0.5	Gln_{9}–Cys_{10}–Cys_{13}–Lys_{18} Ile_{26}–Cys_{27}–Cys_{28}–Cys_{34}–Ala_{39} Tyr_{49}–Cys_{54}

* Denotes the difference between the measured and the calculated molecular weights.

** Other closely related peptide sequences which are consistent with the sequencing data were found. The sequences included here are the ones which agree more closely with the measured masses.

solve this ambiguity, we sequenced the peptide mixture represented by this fraction. The results confirmed the assignments in Table IV. For example, Tyr and Ile were identified as amino-terminal residues (note that Cys would not be observed), only Leu and Pro were seen at residues 2 and 3 respectively, Asp first appeared at residue 5 (along with the other expected residues) and Phe at residues 10. Gly was positively identified at residue 7 (in addition to its appearance elsewhere) in accord with the presence of Gly_{16}. Intermediate cycles further confirmed the assignments of peptides given in Table IV and were found to be completely inconsistent with the alternative assignment; *e.g.*, no Ala was seen at residue 1.

The specific pairing of the three disulfide bridges within the above peptide remains to be determined. However, together with the demonstrated pairing of Cys_{21} to Cys_{44}, the pairing of Cys_{10}, Cys_{13}, Cys_{27}, Cys_{28}, Cys_{34} and Cys_{54} with each other, provides preliminary support for the suggestion[5], that neurophysin contains two domains with respect to disulfide-pairing, one representing the amino-terminal 54 residues and the other the carboxyl-terminal 40 or so residues.

From a strictly technical perspective, the above results demonstrate that anion-exchange HPLC can be used to fractionate such heterogeneous cross-linked peptides according to charge, but that further fractionation of identically charged peptides by reversed-phase HPLC may be difficult in such a heterogeneous cross-linked mixture. Nonetheless, when the heterogeneity reflects related peptides, as in this case, their sequences can be deduced by a combination of mass analysis and sequencing techniques.

CONCLUSIONS

Our results demonstrate, not unexpectedly, that HPLC is critical to the purification of peptides resulting from the extensive digestion of proteins by non-specific proteases. It is significant that the same peptides can be isolated by different purification routes. This indicates that relatively little disulfide rearrangement occurs during the HPLC analysis employed. Neurophysin probably represents the protein with the greatest number of disulfides per unit mass for which pairing has been attempted so far by chemical analysis. However, receptor regions of a number of hormone and growth factor receptors have recently been shown to be comparably rich in disulfides. The techniques used here should find similar applications in the study of disulfide-pairing in these other systems.

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