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GENERAL METHOD FOR THE SEPARATION OF CYANOGEN BROMIDE DIGESTS OF PROTEINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

RABBIT SKELETAL TROPONIN I

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SUMMARY

In this study, we have demonstrated the necessity for a combination of size-exclusion, ion-exchange and reversed-phase high-performance liquid-chromatography to resolve completely a protein digest. Our approach minimizes the number of steps, and the column order provides the maximum information about the properties of the fragments. The order is: (1) size-exclusion (Bio-Rad TSK-250 column), (2) strong cation-exchange (Synchropak S300 column), and finally (3) reversed-phase chromatography (Ultrapore C3). It was desirable for the first step of the procedure to be size-exclusion chromatography to produce the least number of fractions. The volatile eluent used in size-exclusion eliminated the need for subsequent sample desalting. Volatile buffers were not necessary for the ion-exchange chromatography, since the fractions were both desalted and purified in the final reversed-phase step. All column effluents were compatible with absorbance measurements at 210 nm to provide maximum sensitivity for peptide detection. The results obtained in this study strongly suggest that the combined use of three methods of separation, which utilize different selectivities (size, charge, hydrophobicity), can provide excellent resolving power for peptide separations. We believe this fast, efficient procedure should be generally applicable to other protein digests.

INTRODUCTION

The analysis of complex peptide mixtures, produced by enzymatic or chemical cleavage of proteins, has been carried out in the past by conventional chromatographic and electrophoretic techniques. The generation of peptide maps by two-dimensional thin-layer chromatography or, alternatively, chromatography in one direction, followed by electrophoresis in a perpendicular direction, has yielded much structural information on parent proteins[1]. Problems of poor resolution and low preparative yields, often associated with these traditional techniques[1], has prompted

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the widespread application of high-performance liquid chromatography (HPLC) to the separation of peptide mixtures in recent years[2–16]. Reversed-phase (RP) HPLC, particularly, has found favour with many investigators as a powerful, high-speed analytical tool[2–12]. However, despite the good resolution of a number of protein digests, RP-HPLC is not normally sufficient to separate completely very complex mixtures of peptides.

A combination of ion-exchange, reversed-phase, and size-exclusion HPLC offers improved resolution of protein digests. We have developed a method to resolve the major cyanogen bromide fragments of rabbit skeletal troponin I (TnI) (mol.wt. 21 000) which should be generally applicable to most proteins. We felt that a systematic separation utilizing the different selectivities of three modes of separation (size-exclusion, followed by ion-exchange and reversed-phase chromatography) would not only significantly improve the analytical and preparative resolution of peptide mixtures but also provide maximum information about the properties of the fragments. In addition, the resolution of a rabbit skeletal TnI digest by HPLC furnishes an excellent comparison with the isolation of cyanogen bromide fragments of the same protein by classical chromatographic and electrophoretic techniques[17].

EXPERIMENTAL PROCEDURES

Materials

Water was purified by passage through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Potassium chloride, urea, and sodium formate (all ACS grade) were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). The urea solution was treated with Bio-Rad AG 501-X8 (20–50 mesh) (Bio-Rad, Richmond, CA, U.S.A.) mixed-bed resin and passed through a 0.22-μm filter before use. Iodoacetamide was purchased from Aldrich (Milwaukee, WI, U.S.A.) and used without further purification. Iodo[1-^{14}C]acetamide was obtained from Amersham (Oakville, Canada). Cyanogen bromide was obtained from Sigma (St. Louis, MO, U.S.A.).

Apparatus

Programmed analytical chromatography was performed on a Varian Vista Series 5000 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) interfaced with a Varian CS 401 data system and coupled to a Kratos SF769Z variable-wavelength UV spectrometer (Kratos, Westwood, NJ, U.S.A.). Size-exclusion chromatography (SEC) was carried out on a Bio-Rad TSK-250 column (600 × 7.5 mm I.D.); strong cation-exchange chromatography was carried out on a Synchropak S300 column (250 × 4.1 mm I.D.; Synchrom, Linden, IN, U.S.A.); RP-HPLC was carried out on a Beckman Ultrapore C3 column (75 × 4.6 mm I.D.; Altex, Berkeley, CA, U.S.A.). Samples were injected with a Hamilton (Reno, NV, U.S.A.) No. 1710 100-μl syringe into a 500-μl injection loop (Model No. 7125, Rheodyne, Cotati, CA, U.S.A.). Chromatograms were recorded on a Molytek (Pittsburgh, PA, U.S.A.) 10-in. recorder and sample fractions were collected on an LKB (Bromma, Sweden) 2211 SuperRac fraction collector. Radioactive fractions were detected by a Flo-One Model HP radioactive flow detector (Radiomatic Instruments and Chemical Co., Tampa, FL, U.S.A.), connected to an ES electronically controlled stream splitter, set to divert 1% of the column effluent to the flow detector.

CNBr Fragment	Net Charge pH 3.0	Net Charge pH 6.5	M W	Sequence
CN1	+6	-2	4050	L - Q - I - A - A - T - (E) - L - (E) - [K] - (E) - (E) - G - [R] [R] - (E) - A - (E) - [K] - Q - N - Y - L - A - (E) - [H] - C - P - P - L - S - L - P - G - S - HseLac
CN2	+10	+1	3898	(D) - L - [R] - A - N - L - [K] - Q - V - [K] - [K] - (E) - (D) - T - (E) - [K] - (E) - [R] - (D) - V - G - (D) - W - [R] - [K] - N - I - (E) - (E) - [K] - S - G - HseLac
CN3	+5	-3	2801	A - (E) - V - Q - (E) - L - C - [K] - Q - L - [H] - A - [K] - I - (D) - A - A - (E) - (E) - (E) - [K] - Y - (D) - HseLac
CN4	+9	+8	2608	N - Q - [K] - L - F - (D) - L - [R] - G - [K] - F - [K] - [R] - P - P - L - [R] - [R] - V - [R] - HseLac
CN5	+7	+3	2490	Ac - G - (D) - (E) - (E) - [K] - [R] - N - [R] - A - I - T - A - [R] - [R] - Q - [H] - L - [K] - S - V - HseLac
CN6	+4	0	1616	(E) - I - [K] - V - Q - [K] - S - S - [K] - (E) - L - (E) - (D) - HseLac
CN7	+5	+4	1437	L - [K] - A - L - L - G - S - [K] - [H] - [K] - V - C - HseLac

Fig. 1. Major cyanogen bromide fragments of rabbit skeletal troponin I. Acidic residues are circled; basic residues are boxed. Abbreviations: HseLac = homoserine lactone; Ac = N$^{\alpha}$-acetyl; MW = molecular weight. Amino acid residues are denoted by the single-letter code. All fragments, except CN5, have free α-NH_2 groups.

Methods

Carboxamidomethylated TnI (CM-TnI) was prepared as described by Chong and Hodges[18] with the addition of iodo[1-^{14}C]acetamide to a level of 1000 dpm/nmole iodoacetamide. Cyanogen bromide fragments of CM-TnI were obtained by using a 200-fold molar excess of CNBr over methionine at 20°C in 70% formic acid. After 24 h, the reaction mixture was diluted 10-fold with water and lyophilized to remove excess reagents. The low pH generally used in resolving the peptide cleavage mixture after cyanogen bromide cleavage maintained homoserine lactone at the C-terminal ends of the fragments (Fig. 1); negligible homoserine was detected. Chromatography was carried out at room temperature (*ca.* 20°C). Samples were dissolved in the starting buffer and the pH was adjusted with 1-μl aliquots of 5 *M* hydrochloric acid or 5 *M* sodium hydroxide solution. The samples were then centrifuged at 12000 *g* for 3 min. All columns were equilibrated with the starting eluent for 30 min before each determination. Portions of the purified peptides were hydrolyzed in 200 μl of 6 *M* hydrochloric acid at 110°C for 24 h in evacuated, sealed tubes. These hydrolysates were then analyzed on a Durrum (Palo Alto, CA, U.S.A.) 500 amino acid analyzer for fragment identification.

RESULTS

Size-exclusion chromatography

Fig. 2 represents chromatograms of a cyanogen bromide cleavage mixture of rabbit skeletal TnI obtained with the TSK-250 column at 0.2 ml/min by using either a high-ionic-strength buffer (0.2 *M* sodium formate, 6 *M* urea, pH 3.5) (left) or a

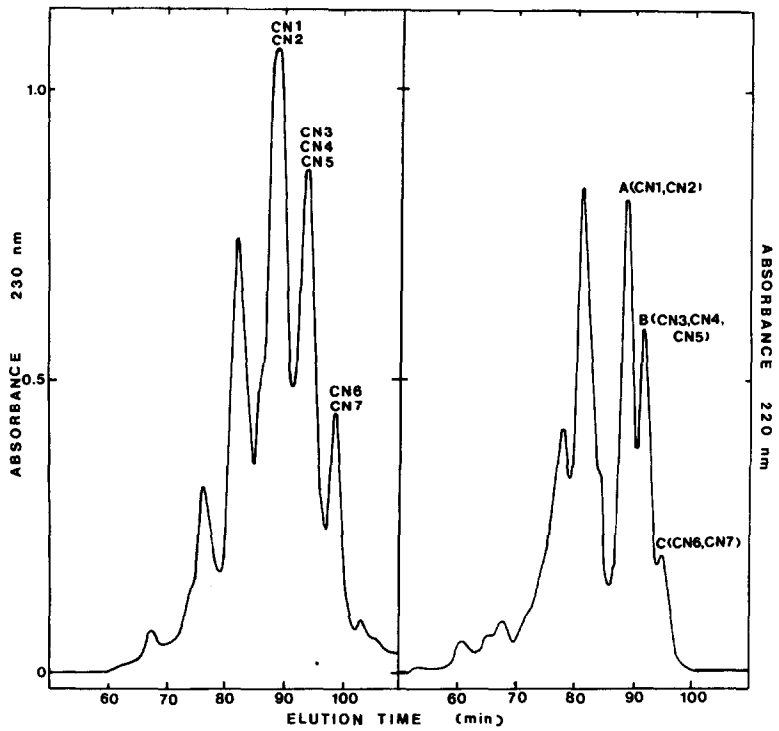

Fig. 2. SEC of a cyanogen bromide cleavage mixture of CM-TnI. Column TSK-250 (600 × 7.5 mm I.D.). Eluents: left, 6 *M* urea, 0.2 *M* sodium formate, pH 3.5; right, 0.1% aqueous TFA. Flow-rate, 0.2 ml/min. Chart speed, 6 in./h. Absorbance, 1.0 a.u.f.s. at 230 nm (left) or 220 nm (right), 10 mm cell. Fragment absorbance of the formate elution was followed at 230 nm, due to high absorbance of the buffer at 220 nm. Sample 30 μl, containing 7–10 nmole of TnI cleavage fragments. The TnI was carboxamido-methylated with iodo[1-^{14}C]acetamide, leaving fragments CN1, CN3, and CN7 radioactive.

0.1% aqueous trifluoroacetic acid (TFA) solution (right) as eluent. While the three peaks containing the major cyanogen bromide fragments (CN1–CN7, Fig. 1) are clearly visible in both chromatograms, the more efficient resolution obtained with the formate buffer is very apparent, particularly for the small CN6 and CN7 peptides. The high ionic strength of the formate buffer, coupled with the presence of 6 *M* urea, increases solubility and supresses non-covalent interactions between the fragments and/or the fragments and the support. After the three cysteine residues of TnI, contained in fragments CN1, CN3 and CN7, were carboxamidomethylated with iodo[1-^{14}C]acetamide, approximately equal levels of radioactivity were detected in the three fragment peaks. The fractions eluted between *ca.* 70–80 min in both chromatograms contained methionine and appeared to be partial cleavage products. The inability to obtain complete CNBr cleavage with CM-TnI has been encountered previously[17]. These methionine-containing fractions were not investigated further. Desalting and partial purification of the major fragment peaks from the formate buffer on a reversed-phase C3 column, followed by strong cation-exchange chromatography (Synchropak S300 column), and a final RP-C3 step produces pure fragments, but

this is very time-consuming[19]. Alternatively, a second desalting pass of the three individual fragment peaks through the TSK-250 column with 0.1% aqueous TFA as eluent, followed by cation-exchange and, finally, reversed-phase chromatography should simplify the purification procedure. However, the poorer resolution of the fragment fractions from the 0.1% aqueous TFA (SEC) (peaks A–C) posed a greater challenge to the analytical and preparative power of the ion-exchange and reversed-phase columns and were chosen for further purification in this study.

Purification of peak A

Fig. 3 (top) represents a chromatogram of a sample of peak A (Fig. 2, right), lyophilized and dissolved in 5 m*M* potassium dihydrogen phosphate starting buffer (pH 3.0), chromatographed on the S300 column using a linear potassium chloride gradient with the potassium chloride concentration increasing at a rate of 5 m*M*/min and a flow-rate of 1 ml/min. Fractions A1, A2, and A3 were eluted at potassium chloride concentrations of approximately 0.06 *M*, 0.125 *M* and 0.175 *M*, respectively. Fractions A1 and A2 contained radioactivity. Following lyophilization, the three fractions were dissolved in 0.1% aqueous TFA, pH adjusted to *ca.* 2.5 if necessary, and passed through the C3 column, using a linear acetonitrile gradient with the acetonitrile concentration increasing at a rate of 0.5%/min and a flow-rate of 1 ml/min. A1 was shown to be radioactive fragment CN3 and was a cross-contaminant from size-exclusion peak B (Fig. 2). The major components of peak A (Fig. 2), CN1 and CN2, were found in S300 fractions A2 and A3, respectively, with radioactivity appearing, as expected, in CN1. The fragments were well separated from CN5 (A2) and CN4 (A3) contaminating peptides from size-exclusion peak B. At pH 3.0, the net charges of CN1, CN2 and CN3 are +6, +10 and +5, respectively, assuming complete protonation of acidic residues. At pH 6.5, the net charges become −2, +1 and −3, respectively, and the fragments are not retained on the S300 column. Attempts to separate efficiently these three fragments by RP-HPLC, following cation-exchange chromatography at pH 6.5, were unsuccessful.

Purification of peak B

Fig. 4 (top) represents a chromatogram of a sample of peak B (Fig. 2, right) chromatographed on the S300 column at pH 3.0 using a linear potassium chloride gradient, with the potassium chloride concentration increasing at a rate of 5 m*M*/min and a flow-rate of 1 ml/min. Fractions B1, B2, and B3 were eluted at peak potassium chloride concentrations of approximately 0.06 *M*, 0.12 *M* and 0.175 *M*, respectively. Fractions B1 and B2 contained radioactivity. The major components of peak B, CN3, CN4, and CN5, were detected in fractions B1, B3 and B2, respectively, following RP-C3 chromatography using a linear acetonitrile gradient, with the acetonitrile concentration increasing at a rate of 0.5%/min and a flow-rate of 1 ml/min. The radioactive CN3 fragment was well separated from contaminating CN6 and radioactive CN7 peptides from size-exclusion peak C. Similarly, CN4 and CN5 were well separated from CN2 and radioactive CN1 contaminants from size-exclusion peak A. At pH 3.0 the net charges of CN3, CN4, and CN5 are +5, +9, and +7, respectively. At pH 6.5 the charges are −3, +8, and +3, respectively, and while CN4 and CN5 are bound to the S300 support under these conditions, CN3 is not retained and forms a void volume mixture with contaminants CN1, CN2, and CN6 (−2, +1, and 0 net charge, respectively).

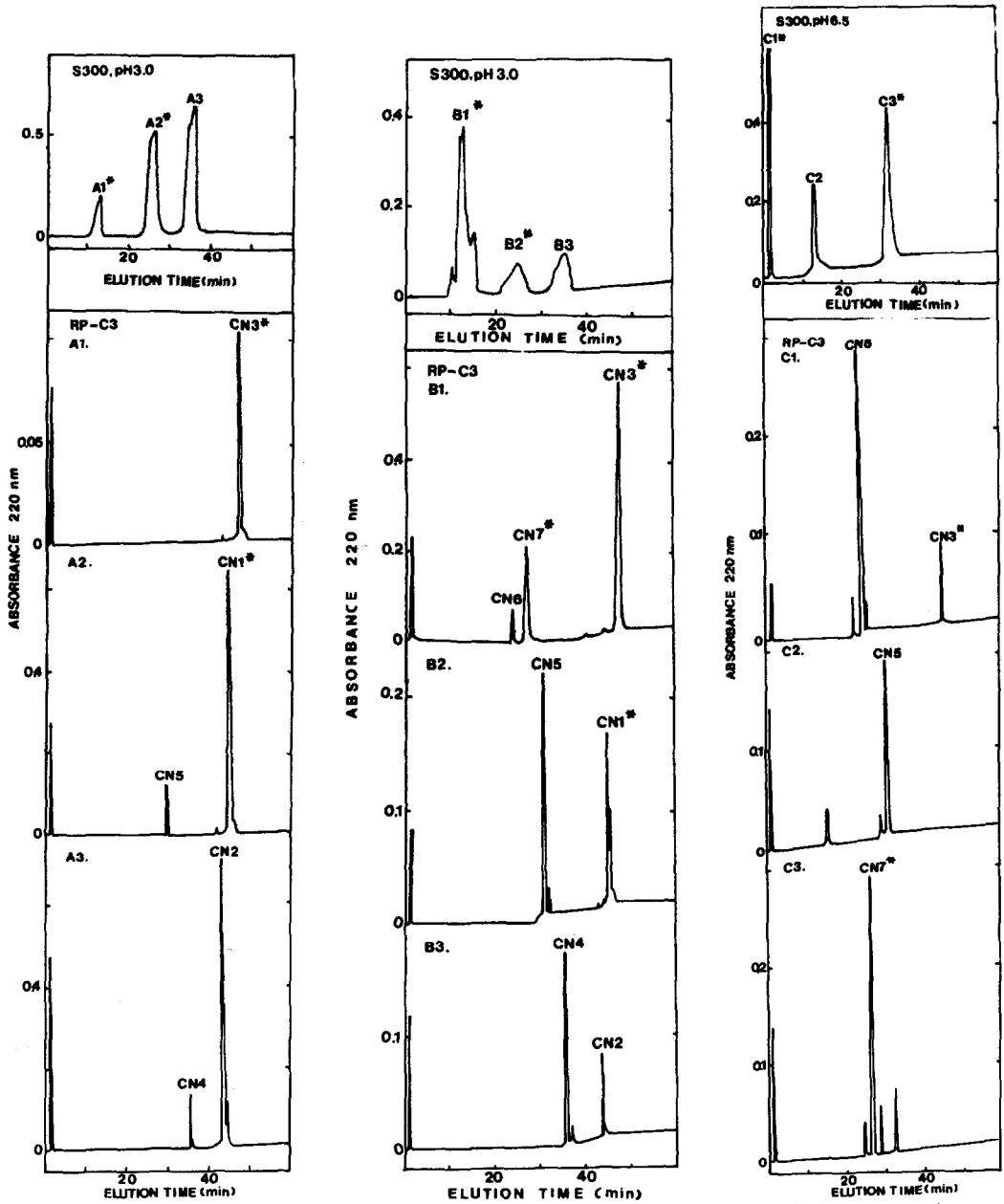

Fig. 3. Cation-exchange and reversed-phase chromatography of size-exclusion peak A. Strong cation-exchange column, S300 (250 × 4.1 mm I.D.). Buffers: (A) 5 m*M* potassium dihydrogen phosphate, pH 3.0; (B) 5 m*M* potassium dihydrogen phosphate, 1 *M* potassium chloride, pH 3.0. A linear potassium chloride gradient (5 m*M* B/min) was applied. Reversed-phase column, C3 (75 × 4.6 mm I.D.). Solutions: (A) 0.1% aqueous TFA; (B) 0.05% TFA in acetonitrile. A linear gradient of 0.5% B/min was applied. Flow-rate, 1 ml/min. Chart speed, 6 in./h. Absorbance measured at 220 nm, 10 mm cell.

Fig. 4. As Fig. 3 for size exclusion peak B. Conditions as in Fig. 3.

Fig. 5. Cation-exchange and reversed-phase chromatography of size-exclusion peak C. Strong cation-exchange column, S300 (250 × 4.1 mm I.D.). Buffers: (A) 5 m*M* potassium dihydrogen phosphate, pH 6.5; (B) 5 m*M* potassium dihydrogen phosphate, 1 *M* potassium chloride, pH 6.5. A linear potassium chloride gradient (5 mM B/min) was applied. Reversed-phase column, C3 (75 × 4.6 mm I.D.). Solutions: (A) 0.1% aqueous TFA; (B) 0.05% TFA in acetonitrile. A linear gradient of 0.5% B/min was applied. Flow-rate, 1 ml/min. Chart speed, 6 in./h. Absorbance measured at 220 nm, 10 mm cell.

Purification of peak C

Fig. 5 (top) represents a chromatogram of a sample of peak C (Fig. 2, right) obtained with the S300 column at pH 6.5 and a linear potassium chloride gradient, with the potassium chloride concentration increasing at a rate of 5 m*M*/min and a flow-rate of 1 ml/min. Fraction C1 was eluted with the void volume; fractions C2 and C3 were eluted at potassium chloride concentrations of approximately 0.063 *M* and 0.16 *M*, respectively. Fractions C1 and C3 contained radio-activity. The major components of peak C, CN6, and radioactive fragment CN7, were detected in fractions C1 and C3, respectivel,y, following RP-C3 chromatography using a linear acetonitrile gradient with the acetonitrile concentration increasing at a rate of 0.5%/min and a flow-rate of 1 ml/min. Fragment CN6 was well resolved from the radioactive CN3 contaminant from size-exclusion peak B; radioactive CN7 was the only component of fraction C3. Fraction C2 was discovered to be CN5, a contaminant from size-exclusion peak B. When chromatographed on the S300 column at pH 3.0, the resolution of the major fractions was inferior to that at pH 6.5 due to similar net charges of fragments CN3, CN6, and CN7 (+5, +4, and +5, respectively). The ion-exchange step at pH 6.5 cannot be eliminated, since CN6 and CN7 are not resolved on reversed-phase chromatography.

DISCUSSION

We have demonstrated the efficient resolution of a cyanogen bromide cleavage mixture of rabbit skeletal TnI in an approach which utilizes a combination of properties of the fragments (size, charge, hydrophobicity). Many authors[2–12] have reported the use of RP-HPLC for the separation of peptide mixtures. Although reasonable resolution was often obtained, complete purification of peptides in high yields has generally remained elusive. Attempts by this laboratory to resolve the relatively simple cyanogen bromide cleavage mixture of rabbit skeletal TnI by reversed-phase chromatography resulted in a complex series of peaks from which it was very difficult to obtain pure fragments. Ion-exchange HPLC has also been employed for peptide separations, although to a lesser extent[13–16]. Rabbit skeletal TnI fragments cannot be totally resolved on a single ion-exchange support. At pH 6.5, the fragments range from acidic to strongly basic species (Fig. 1); at pH 3.0, similarly charged species, *e.g.*, CN3 and CN7 (both +5) are not resolved. In addition to the desired peptides, the cleavage mixture also contains partial cleavage products and, perhaps, uncleaved TnI, further complicating the ion-exchange and reversed-phase chromatographic profiles. Although changing conditions of reversed-phase or ion-exchange chromatography may improve the resolution of a peptide mixture, it is requiring much of a single technique to resolve efficiently complex mixtures. Dizdaroglu and Krutzsch[20] suggested that a combination of reversed-phase and ion-exchange chromatography, taking advantage of different properties of protein fragments, appeared to offer more efficient resolution. Takahashi *et al.*[21] carried out a two-step HPLC procedure to separate tryptic digests of the light chain of human immunoglobulin D. The combination of cation-exchange and RP-HPLC produced good, if not complete, resolution of the tryptic peptides. We thought that an initial separation, based on peptide size, would simplify subsequent ion-exchange and reversed-phase steps. Mabuchi and Nakahashi[22] demonstrated excellent resolution of a complex, if artificial, mixture of biologically active peptides by a combination of SEC, followed by reversed-phase and cation-exchange HPLC. Our approach not only produced pure cyanogen bro-

mide fragments in good yields but also minimized the number of steps necessary to achieve excellent separation. Cyanogen bromide cleavage of proteins usually results in two forms of each peptide during purification, one containing a C-terminal homoserine lactone and the other a C-terminal homoserine. If a low pH is generally maintained or exposure to pH 6.5 buffers kept at a minimum during the purification scheme and work-up, the peptides can be maintained in the lactone form, simplifying the elution profiles. The use of 0.1% aqueous TFA as eluent for the size-exclusion step enabled the fragment fractions to be immediately applied to the cation-exchange column. The final reversed-phase step served both as a desalting and purification step. This procedure was notably faster, more sensitive and showed better resolution of TnI cyanogen bromide fragments than the classical techniques employed by Wilkinson[17]. The interstep lyophilization of column fractions could be eliminated, if desired, and samples applied directly from one column to the next. There is good potential for applying our approach to other protein digests. For a protein of known sequence, the fragment structures can be predicted and the chromatographic conditions (*e.g.*, pH in ion-exchange chromatography) chosen accordingly. For an unknown protein sequence the column order provides maximum information about the properties of the fragments as the purification proceeds. In general, most protein digests are more complex with regard to the number of peptides of similar size, charge and hydrophobicity making the three-column procedure outlined in this study absolutely essential.

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