Analytical Peptide Mapping by High Performance Liquid Chromatography

APPLICATION TO INTESTINAL CALCIUM-BINDING PROTEINS*

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Peptide mapping of underivatized tryptic digests of bovine and chick intestinal calcium-binding proteins has been accomplished by high performance liquid chromatography (HPLC). High precision analysis of nanomolar quantities of peptides were achieved in less than 1 h (recycle time). Peak resolution and definition are superior compared to conventional techniques and recoveries of both small (4-residue) hydrophilic and large (30-residue) hydrophobic peptides are excellent. The total amino acid composition of the bovine intestinal calcium-binding protein has been accounted for on the basis of two tryptic maps of 20 μg of protein each.

Separation of peptides in the small to medium size range (less than 40 residues) is, at present, largely an empirical process employing a multiplicity of techniques, including paper chromatography, high voltage paper electrophoresis, thin layer chromatography and electrophoresis, a combination of the aforementioned, or ion exchange column chromatography on micro- or macroparticulate resins. The versatility and simplicity of paper and thin layer procedures must be offset, in part, by inherent methodological disadvantages, including limited loading capacities, difficulties in visualization and quantitation, and variable recoveries. On the other hand, recent advances in micro procedures for peptide mapping on ion exchange columns with postcolumn detection by ninhydrin (1) or fluorescent reagents (2) have introduced an element of simplicity and sensitivity to this procedure. Reproducibility and sensitivity notwithstanding, these methods still require long run times of 16 h or more and rely primarily on destructive detection techniques. In many cases, several runs are required on different support media for adequate separation of highly charged species (1) and, often, hydrophobic peptides adsorb to the resin, resulting in low recoveries. Peak resolution and definition are often less than desirable by this method.

High performance liquid chromatography, demonstrated to be a versatile technique for the rapid and effective separation of a variety of biological compounds (for applications, see Ref. 3), has received only limited attention for peptide separations. Hancock et al. (4) recently introduced the use of a hydrophilic ion-pairing solvent (orthophosphoric acid) for peptide separation. The use of acetonitrile, a solvent generally not applied in the field of protein chemistry, has also been suggested (4, 5).

For the present study, a phosphoric acid-acetonitrile solvent system, coupled with gradient elution from reversed-phase support media, has been applied to the separation of tryptic digests of two intestinal calcium-binding proteins, the sequence determinations of which are presently in progress. The results presented demonstrate the extreme resolving power and rapidity of separation of HPLC* and indicate this procedure to be a powerful technique for routine peptide mapping.

EXPERIMENTAL PROCEDURES

High Performance Liquid Chromatography—A Waters Associates (Milford, MA) ALC/GPC model 244 liquid chromatograph equipped with gradient elution capability, a U6K sample injector, a model 440 fixed dual wavelength detector, and a model 450 variable wavelength detector were employed. All separations were performed on a C18 Bondapak reversed phase column (10 μm, 4 mm × 30 cm), also from Waters Associates.

Elution of peptides was achieved by the use of a linear gradient, from 0.1% orthophosphoric acid (Solvent A) to acetonitrile (Solvent B) at a constant flow rate of 2 ml/min with column pressure varying from 500 to 1000 p.s.i. Phosphoric acid (0.1%, pH 2.2) was filtered through a 0.5-μm Millipore filter (Millipore Corp., Bedford, MA) and degassed for 30 min under vacuum, with stirring. Acetonitrile was used directly. During runs, solvents were stirred continuously to eliminate formation of temperature gradients. All runs were performed at room temperature.

During series of runs extending over several days, column precision was maintained by pumping acetonitrile at 0.1 ml/min through the column, overnight. Generally, 10 min were required for equilibration of the column with Solvent A after initial conditions on the reverse gradient program were reached.

Reagents and Chemicals—Orthophosphoric acid was obtained from Mallinckrodt (St. Louis, MO) and acetonitrile from Waters Associates, Milli-Q Water (Millipore Corp.) with a measured resistance of 18 megohms or less was used exclusively. TPCK (1-tosylamido-2-phenylthyl chloromethyl ketone)-treated trypsin was obtained from Worthington and N-ethylmaleimide and TLCK (N-p-tosyl-L-lysine chloromethyl ketone)-treated chymotrypsin from Sigma.

Enzymatic Digestions—All enzymatic digestions were carried out in 0.2 N N-ethylmaleimide acetate buffer (pH 8.1) at 37°C with an enzyme to protein ratio of 1 to 100 (w/w). Digestions were generally stopped by addition of acetic acid. Samples were injected directly into the HPLC without additional manipulation.

Amino Acid Compositional Analyses—Compositions of proteins and peptides were determined on a Beckman model 119Cl analyzer equipped for single column methodology. Samples (1 to 5 nmol) were lyophilized in hydrolysis ampoules and hydrolyzed in vacuo for 24 h at 110°C in 1.0 ml of constant-boiling HCl.

Calicum-binding Proteins—Bovine and chick intestinal calcium-
binding proteins (CaBP) were prepared as described previously (6). The purified bovine CaBP minor A component ($M_r = 9000$; 75 amino acids) was subjected to exhaustive dialysis against 1% acetic acid in order to remove bound calcium, a prerequisite to tryptic digestion (7), and lyophilized. Chick CaBP ($M_r = 28,000$; 242 amino acids; Ref. 8) was dialyzed against distilled water and lyophilized.

RESULTS

The progress of tryptic digestion of bovine intestinal calcium-binding protein minor A component (6) as a function of time was followed by peptide mapping on the HPLC and the results are shown in Figs. 1 and 2. For each case, 20 µg (~2 nmol) of protein digest in 2.0 µl were injected and a linear gradient from 0.1% H$_3$PO$_4$ to 50% acetonitrile in 0.1% H$_3$PO$_4$ was run over a period of 50 min (1% acetonitrile/min). Preliminary runs established that, for the digestions performed here, no peptides are eluted above 50% acetonitrile.

The blank sample (Fig. 1, blank) containing digestion buffer, trypsin, and acetic acid only, provides a baseline for subsequent comparisons. The peak emerging at 2½ min is acetic acid. A “zero time” control sample was produced by adding acetic acid to the protein solution immediately following addition of trypsin and mixing and Fig. 1 (0h) indicates that the elution time of undigested bovine intestinal calcium-binding protein (arrow) is 40 min. Following incubation for 1

**Fig. 1.** Time course of tryptic (TPCK-treated) digestion of bovine intestinal calcium-binding protein. (See Fig. 2 for 16-h incubation.) All incubations were carried out in 0.2 N $N$-ethylmorpholine acetate buffer (pH = 8.1) with a trypsin to protein ratio (w/w) of 1:100. Protein concentration was approximately 1 µg/µl. Twenty microliters (20 µg, 2 nmol) were removed from the incubation mixture at the times indicated, mixed with 5 µl of HOAc and injected directly into the liquid chromatograph. Blank contains buffer, trypsin, and HOAc only. Zero time control sample was removed immediately following addition of trypsin to protein and acidified. Arrow indicates position of undigested protein. Note disappearance of Peak 6 and coincidental increase of Peaks 2 and 4 with time.
Fig. 2 (left). Peptide map of 18-h tryptic digestion to indicate extreme peak resolution and definition. Note that Peak 1 appears to be heterogeneous. Also note that peaks designated as X and Y have become significant. Peaks from this map were collected in their entirety, for compositional and recovery data (Table I).

Fig. 3 (right). Isocratic separation of two components of Peak 1. Peaks 1a and 1b were shown to have compositions S, I, Q, K and A, Y, K, respectively. Simultaneous monitoring at 280 nm clearly shows the position of the tyrosine-containing peptide (1b). Solvent A adjusted to pH 3.0 with NaOH.
TABLE I
Peptide compositions and recoveries

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<th>1β</th>
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<th>7</th>
<th>8</th>
<th>X</th>
<th>Y</th>
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* Obtained from isocratic elution

Intermediate peptide. Data obtained from 1-h hydrolysate.

Value obtained by extrapolation.

 Obtained from gradient run elution.

**FIG. 4. Peptide map of chick intestinal CaBP. CaBP was incubated with trypsin (1:100, w/w) for 16 h and chromatographed under identical conditions with those described for the bovine CaBP. Thirty micrograms of protein (about 1 nmol) were applied. Simultaneous effluent monitoring was at 210, 280, and 254 nm.**
h (Fig. 1, 1h), a number of peptides are evident, although some original protein still remains.

Fig. 1 also clearly shows the changes occurring with increasing digestion time. Here, designation of peptide peaks reflects results obtained from longer digestion periods. All peptides yield increasing peak heights with time of incubation with trypsin except Peak 6, which declines and is essentially eliminated at 16 h of incubation (Fig. 2). Also, it can be noted that Peaks 2 and 4 increase in intensity at a greater rate than Peaks 1, 3, 5, 7, and 8, suggesting that Peaks 2 and 4 arise from the further cleavage of Peak 6 peptide. Peak 6 peptide was determined to contain an internal lysine and possess a composition equivalent to the sum of Peaks 2 and 4 peptides (Table I). In this case, isolation of Peak 6 peptide from a relatively short digestion period would eliminate the necessity for one overlap during sequence studies.

Throughout this series of analyses, peak resolution and definition were excellent, allowing each peptide to be collected in a total volume not exceeding 1.0 ml. Compositional analyses of the various peaks (16-h digestion) and percentage of recoveries based on the amount of starting material are provided in Table I. Also included is the composition of the intermediate cleavage peptide (Peak 6) collected from the 1-h digestion run, compositions of the minor cleavage peptides designated as Peaks X and Y (Fig. 2), and the total composition of the original undigested protein. Only Peak 1, which was often observed to be asymmetrical, was shown to contain more than 1 NH₂-terminal residue (Tyr and Ser) by the dansyl-Cl end group method. The two peptides in this peak were separated isocratically (Fig. 3) by increasing the pH of Solvent A to 3.0 with NaOH. The tyrosine-containing peptide is readily detected at 280 nm (Fig. 3). The compositions of these peptides are included in Table I.

The results presented in Table I also indicate the presence of two lysines in Peak 8 peptide, a very hydrophobic 30-residue peptide recovered in 76.2% yield after 16 h of tryptic digestion. This peptide could not be recovered from paper positional analyses of Peaks X and Y (Fig. 2, Table I), indicating that Peaks 2 and 4 arise from the further cleavage of Peak 6 peptide. Peak 6 peptide was determined to contain an internal lysine and possess a composition equivalent to the sum of Peaks 2 and 4 peptides (Table I). In this case, isolation of Peak 6 peptide from a relatively short digestion period would eliminate the necessity for one overlap during sequence studies.

To provide additional evidence for the usefulness of the technique, chick intestinal calcium-binding protein (30 µg, 1 nmol) was digested with trypsin for 16 h and subjected to HPLC (Fig. 4) under conditions identical with those described for the bovine CaBP. In this case, the advantages of effluent monitoring at three wavelengths (210, 254, and 280 nm), simultaneously, are shown. Effluent analysis at 210 nm shows excellent separation of about 30 peptides (maximum of 30 expected) in less than 40 min. Monitoring at 254 or 280 nm provides preliminary information regarding the location of aromatic residues.

DISCUSSION

While the present study is, by no means, exhaustive, it does provide ample evidence for the advantages of the application of HPLC to peptide mapping. The extreme sensitivity and flexibility of the procedure, as well as the exceptionally short run times and ease of collection, add a new dimension to the field of protein chemistry, i.e. peptide mapping of nanomolar quantities in less time than is required for a complete amino acid compositional analysis.

In these studies, all peptides were eluted in less than 50 min, with a total collected volume of about 100 ml. The solvent system employed is not only sufficiently transparent in the ultraviolet, but is essentially volatile, eliminating the need for desalting or additional purification of amine-containing buffers. The small amounts of residual H₂PO₄ have not been observed to result in adverse effects during subsequent chemical determinations (amino acid compositional analyses, end group determination, or automated liquid phase sequencing).

While the potential of the method has not been fully realized, it appears to be suitable for both analytical (difference mapping, monitoring completeness of reaction, determination of cleavage intermediates, etc.) and preparative applications. The short run time, high recoveries, and ease of sample collection and preparation are especially well suited for remapping under various conditions. Effluent monitoring at 210 nm provides an extremely sensitive, convenient, and nondestructive approach to detection. Simultaneous effluent analysis at alternate wavelengths provides additional useful information regarding peptide composition without noticeable loss of resolution. Gradient elution is essential for peak resolution and definition and, therefore, sensitivity of detection, although baseline rise appears to limit sensitivity to about 1 nmol.

While a linear gradient increasing at 1% acetonitrile/min was employed as a general approach to separation in this study, judicious selection of alternative conditions may prove to be essential for specific separations or subseparations. In one instance, alteration of the pH of Solvent A in conjunction with isocratic elution was required to effect separation of two tetrapeptides. This practice, while introducing salt into collected fractions, might provide a valuable approach for otherwise difficult separations.

The HPLC has been shown to be a useful primary separation technique for mixtures of small peptides. Mapping of larger peptides and proteins subsequent to more selective cleavages will undoubtedly require more conventional techniques for primary separations and rely on HPLC for secondary separations or separations of secondary cleavage mixtures.

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