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EXOPEPTIDASE–HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY PEPTIDE MAPPING OF SMALL PEPTIDES

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SUMMARY

We report here a rapid and quantitative peptide mapping procedure based on a combination of immobilized exopeptidase digestion and high-performance liquid chromatographic (HPLC) separation of enzymatic products. Immobilized aminopeptidase M (E.C. 3.4.11.2) and carboxypeptidase Y (E.C. 3.4.12.1) were chosen because of their broad specificity. Products of enzymatic degradation of proctolin, for example, were separated on C_{18} or C_1 reversed-phase HPLC columns with a trifluoroacetic acid–acetonitrile gradient elution. Peaks corresponding to peptide fragments were collected, hydrolyzed in propionic acid–hydrochloric acid, and identified by amino acid analysis. This method is limited, of course, to peptides with free carboxyl or amino termini and by the sensitivity of the amino acid analysis method employed.

INTRODUCTION

Considerable advances have been made in the sensitivity of peptide microsequencing techniques over the last ten years, including the development of gas-phase Edman degradation systems capable of routine sequence analysis of less than 100 picomoles of purified peptide[1]. Nevertheless, practical modifications in both N-terminal degradation schemes and C-terminal analyses have expanded the sequencing capabilities of the peptide chemist so that routine analysis of fifteen or fewer residues is possible without expensive automated instrumentation. Most of these refinements have been in the area of chemical methodologies which have extended the range of manual sequencing techniques to the low-nanomole level: improved manual Edman degradation with phenylisothiocyanate (PITC) for N-terminal sequencing[2]; the use of a highly chromophoric reagent, dabsyl isothiocyanate, in place of PITC[3]; and improvements in the reagents used in C-terminal sequencing by formation of amino acid thiohydantoins[4].

Although these chemical techniques for sequencing peptides have been vastly improved, they are still limited by the ability of the researcher to separate products from starting materials and reaction by-products. Of course, as more residues of a peptide are sequenced by one of these techniques, the number of contaminants and

the extent of salt formation increase. In recent years the use of exopeptidase digestion has become a common method of determining the N- or C-terminal amino acid of a peptide or protein. Moreover, these enzymes may also be used to degrade peptides sequentially from one terminus; the sequence is then usually determined by direct identification of the amino acids released by digestion[5].

The present study exploits the advantages of immobilized forms of exopeptidases to simplify separation of enzyme and degradation products and the use of volatile buffers to ensure compatibility with high-performance liquid chromatographic (HPLC) conditions. In addition, a subtractive approach to sequence determinations was used by identification of the resulting peptide fragments after successive losses of amino acids.

EXPERIMENTAL

Chemicals and reagents

Immobilized aminopeptidase M (APM; E.C. 3.4.11.2), immobilized carboxypeptidase Y (CPY; E.C. 3.4.12.1), and sequencing-grade N-ethylmorpholine and trifluoroacetic acid (TFA) were obtained from Pierce (Rockford, IL, U.S.A.). Proctolin (Arg-Tyr-Leu-Pro-Thr) was purchased from Vega Biochemicals (Tucson, AZ, U.S.A.) and purified on a Rainin Microsorb C_1 column (Rainin Instrument, Woburn, MA, U.S.A.) by a 0.1% TFA–acetonitrile gradient elution prior to use. All solvents were HPLC-grade and were obtained from MCB Reagents (Gibbstown, NJ, U.S.A.). The enzyme resins were washed at least five times with appropriate buffers before use to eliminate any background from stabilizing compounds.

Enzymatic digestion

Ten nanomoles of a commercially-synthesized peptide, proctolin, were incubated with 0.05 units of immobilized aminopeptidase M in 0.2 *N* ethylmorpholine acetate (pH 7.5)[6] in a 1.5-ml polypropylene tube at room temperature. At various time intervals, the enzyme resin was centrifuged to the bottom of the tube, and aliquots containing a total of 1.3 nanomoles of peptide fragments were removed for analysis. Parallel digestions were undertaken with 1.0 unit of immobilized carboxypeptidase Y in 0.1 *N* ethylmorpholine acetate (pH 6.0)[5]. The resulting fractions were taken to dryness under an argon stream and redissolved in 0.1% TFA for subsequent HPLC analysis.

High-performance liquid chromatography

HPLC separations were performed at ambient temperature by a Waters HPLC system (Waters Assoc., Milford, MA, U.S.A.), consisting of two Model 6000 pumps, a Model 680 solvent gradient programmer, a Model 460 variable-wavelength detector set at 214 nm, and a Rheodyne septum-less injector (Rheodyne, Cotari, CA, U.S.A.). All solvents used were HPLC-grade, and water was purified through a Millipore MU-15 cartridge system (Millipore, Bedford, MA, U.S.A.).

Peptides resulting from enzymatic digestion were separated on a Waters μBondapak C_{18} column (30 cm × 3.9 mm) previously equilibrated with 0.1% TFA at 1.5 ml/min. After isocratic conditions (0.1% TFA) for 30 sec, the components of the sample were resolved by a linear gradient from initial conditions to final conditions of 0.1% TFA and 20% acetonitrile over 20 min.

As products of carboxypeptidase Y digestion were more difficult to separate on the C_{18} column, aliquots removed from CPY incubations were separated on a Rainin Microsorb C_1 column (5 μm; 15 cm × 4.6 mm) by a linear gradient to a final concentration of 0.1% TFA and 25% acetonitrile over 30 min after 8 min at initial conditions of 0.1% TFA.

Fractions corresponding to peaks from HPLC separation were collected separately and hydrolyzed for amino acid analysis.

Amino acid analysis

Fractions from HPLC analysis were evaporated under an argon gas stream and hydrolyzed for 30 min in hydrochloric acid–propionic acid (50:50, v/v) at 150°C[7]. After the samples were dried under argon, they were analyzed for amino acid content on an LKB 4400 amino acid analyzer (LKB Instruments, Gaithersburg, MD, U.S.A.) with a physiological program, including lithium citrate buffers and a ninhydrin detection system, according to the instructions of the manufacturer (range of 0.3 to 15 nanomoles of amino acid).

RESULTS

Aminopeptidase M digestion

Products of enzymatic digestion were separated on a Waters C_{18} column as described previously. A time study was undertaken to determine the optimal HPLC samples for amino acid analysis (Fig. 1). A representative chromatogram for a 30-min incubation is shown in Fig. 2B. The four peaks obtained corresponded to peptides containing the following amino acids: (A) Thr, Pro; (B) Thr, Pro, Leu; (C) Thr, Pro, Leu, Tyr; (D) Thr, Pro, Leu, Tyr, Arg (Table I). As suggested by earlier studies of commercially prepared aminopeptidase M[8], a trace of prolidase is most likely

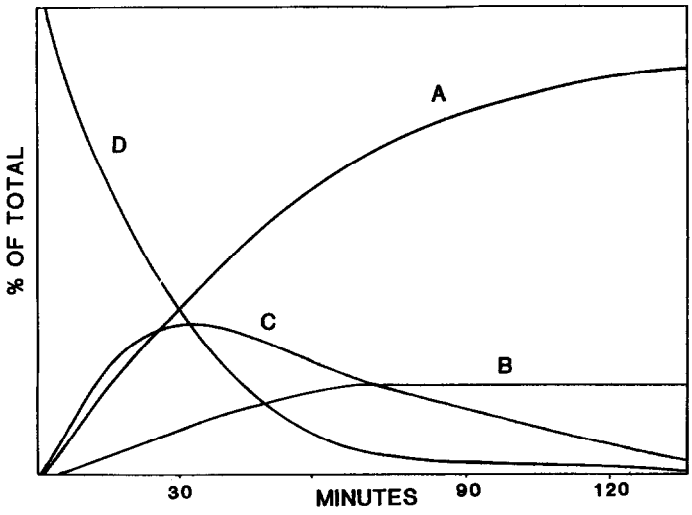

Fig. 1. Time study of digestion of proctolin by immobilized aminopeptidase M at pH 7.5. Curves A, B, C and D correspond respectively to peaks A, B, C and D of the chromatogram shown in Fig. 2B.

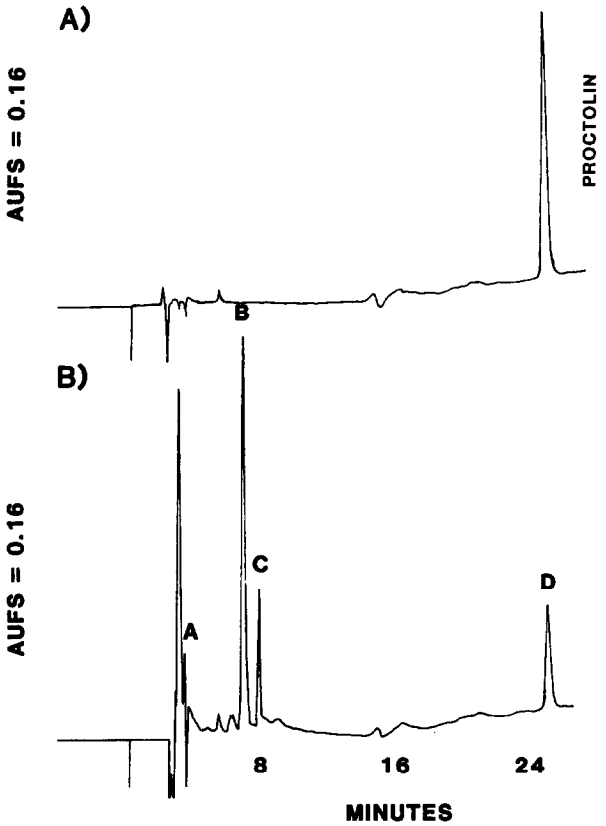

Fig. 2. HPLC separation of (A) proctolin and (B) products of 30-min incubation of proctolin with aminopeptidase M (pH 7.5) on a Waters μBondapak C_{18} column (conditions given in text).

TABLE I

AMINO ACID ANALYSIS OF PROCTOLIN AND ENZYMATIC DIGESTION PRODUCTS OF PROCTOLIN AFTER HYDROLYSIS IN HYDROCHLORIC ACID–PROPIONIC ACID (50:50, v/v)

	Thr	*Pro*	*Leu*	*Tyr*	*Arg*
Proctolin	0.84*	1.16	1.00	0.91	1.01
APM digest, 30 min					
Peak A	1.00	1.02			
Peak B	1.02	0.65	1.00		
Peak C	0.92	0.91	1.00	0.93	
Peak D	0.97	0.91	1.00	0.83	0.86
CPY digest, 1 h					
Peak A				0.73	1.00
Peak B			1.00	0.85	0.98
Peak C		0.60	0.54	1.00	1.45
Peak D	0.77	0.95	1.00	1.14	0.87

* Values given as normalized ratio of amino acids.

responsible for hydrolysis of the proline peptide bonds. From this analysis and that of amino acid content of the original peptide (Table I), it was concluded that the amino acid sequence of proctolin was either Arg-Tyr-Leu-Pro-Thr or Arg-Tyr-Leu-Thr-Pro.

Carboxypeptidase Y digestion

Products of carboxypeptidase Y digestion were separated on a Microsorb C_1 column as described previously. A representative chromatogram is shown in Fig. 3B for 1 h of incubation. After hydrolysis and amino acid analysis the fractions corresponding to the peaks obtained were identified as containing peptides of the following amino acid content: (A) Tyr, Arg; (B) Leu, Tyr, Arg; (C) Pro, Leu, Tyr, Arg; (D) Thr, Pro, Leu, Tyr, Arg (Table I). These results are consistent with peptide structures of Arg-Tyr-Leu-Pro-Thr and Tyr-Arg-Leu-Pro-Thr.

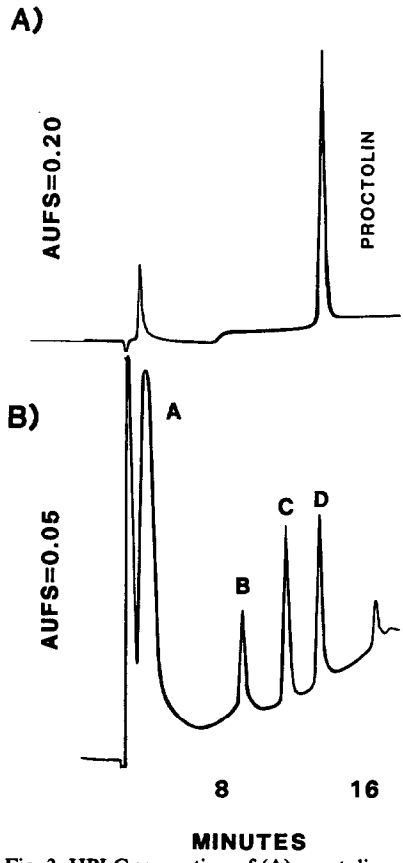

Fig. 3. HPLC separation of (A) proctolin and (B) products of 1-h carboxypeptidase Y digestion (pH 6.0) of proctolin on a Rainin Microsorb C_1 column (conditions given in text).

Structure determination

By use of both carboxypeptidase Y and aminopeptidase M in separate incubation mixtures, we were able to confirm experimentally the amino acid sequence of

the peptide proctolin (synthetic source). Results of hydrolysis from the amino terminus suggested that the sequence was Arg-Tyr-Leu-X_1-X_2, and those of digestion from the carboxyl end indicated that the sequence was X_3-X_4-Leu-Pro-Thr, where X_1 and X_2 were proline or threonine and X_3 and X_4 were arginine or tyrosine residues. Combining the information from these two enzymatic analyses and amino acid analysis of the proctolin peptide, we obtained a structure for the synthetic peptide of Arg-Tyr-Leu-Pro-Thr. This structure is in complete agreement with the known structure of proctolin.

DISCUSSION

The choice of commercially available immobilized aminopeptidase M and carboxypeptidase Y for sequence analysis of proctolin was fortuitous, as the peptide under investigation has both free (*i.e.*, unblocked) amino and carboxyl termini. However, had this not been the case, the unreacted peptide could have been, theoretically, completely recovered from the incubation mixture for alternative sequence analysis procedures by centrifugation and repeated washing of the resin. As these two enzymes are highly specific for amino acids of L-configuration, the presence of a D-amino acid would be detectable by the use of such enzymatic degradation. If the peptide under investigation is resistant to aminopeptidase digestion because of an amino-terminal pyroglutamic acid (PCA), the sample might be treated with PCA-aminopeptidase and PCA separated from the remaining peptide by HPLC prior to enzymatic hydrolysis with an aminopeptidase.

The nature of the exopeptidase utilized for sequence determination (*i.e.*, its specificity) and its purity will, of course, determine the amount of information available from any one digest. In addition, the particular methodology employed in this work limits the use of any single enzyme for complete sequence analysis. Because the peptide products of enzyme digestion were isolated and identified by HPLC (rather than by analysis of the amino acids liberated by the enzyme), the sequence of the final dipeptide is ambigous. Parallel digestions with an aminopeptidase and a carboxypeptidase or a combination of use of one of these with a single cycle of a chemical sequencing method for the complementary amino or carboxyl terminus (or a limited enzymatic digest) could provide complete sequence information for unblocked peptides. Alternatively, the two possible dipeptide configurations could be separated by HPLC and the unknown identified on the basis of retention time.

Thus, although sequence analysis by the use of an immobilized exopeptidase is limited by the specificity of the enzyme, it may be possible to obtain complete structural information by the use of several such exopeptidases or of one in combination with highly specific endopeptidases or an associated chemical technique.

The other factor which is limiting in this technique is, as in any sequencing method, the amount and purity of peptide available for structural analysis. While immobilized enzymes may be diluted no further than to a single resin bead, the pH of the buffering solution and the temperature of incubation may be adjusted to retard the rate of degradation for very small samples of peptide. The major limiting factors, therefore, are (1) the sensitivity of the detection instrumentation in HPLC separation of the hydrolysis products and (2) the sensitivity of the amino acid analysis system employed. To overcome the limitations in sensitivity of the usual amino acid analysis

techniques which utilize ion-exchange chromatography and ninhydrin-detection systems, it is possible to determine amino acid content by formation of phenylthiocarbamyl amino acid derivatives prior to analysis by reversed-phase HPLC[9], which extends the limit of detection to approximately 20 picomoles. In addition, we have alternatively utilized an immobilized enzyme–fast atom bombardment mass spectrometric technique for amino acid sequence analysis of less than one nanomole of small peptides (manuscript in preparation).

The use of immobilized enzymes greatly facilitated the enzymatic sequencing technique employed in this paper. Because the enzyme-containing resins were easily centrifuged to the bottom of the reaction tubes, the need for separation of enzyme and substrate prior to analysis was eliminated. Previously, it was shown that immobilized enzymes could be used to determine the amino acid content of peptides[6], thus eliminating losses of some residues due to acid hydrolysis of the sample. In this work we have demonstrated the use of this technique for actual sequence determination of a pentapeptide, the results of which are consistent with its published structure.

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REFERENCES

1 R. M. Hewick, M. W. Hunkapiller, L. E. Hood and W. J. Dreyer, *J. Biol. Chem.*, 256 (1981) 7990.
2 G. E. Tarr, *Anal. Biochem.*, 63 (1975) 361.
3 J. Y. Chang, *Biochem. J.*, 163 (1977) 517.
4 J. L. Meuth, D. E. Harris, F. E. Dwulet, M. L. Crowl-Powers and F. R. Gurd, *Biochemistry*, 21 (1982) 3750.
5 G. P. Royer and J. P. Andrews, *J. Biol. Chem.*, 248 (1973) 1807.
6 F. A. Liberatore, J. E. McIsaac, Jr. and G. P. Royer, *FEBS Lett.*, 68 (1976) 45.
7 F. Westall and H. Hesser, *Anal. Biochem.*, 61 (1974) 610.
8 T. Yoshimoto and D. Tsuru, *J. Biochem.*, 94 (1983) 619.
9 R. L. Heinrikson and S. C. Meredith, *Anal. Biochem.*, 136 (1984) 65.