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Short Communication

Peptide separation by gel filtration high-performance liquid chromatography using a gradient elution system

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ABSTRACT

Peptides were separated by gel filtration high-performance liquid chromatography with gradient elution. Two gradient elution systems were applied: (1) 0 to 20% acetonitrile in 30 m*M* phosphate buffer, pH 8.0 and (2) 30 m*M* phosphate buffer, pH 8.0 to water; with the polymer-type gel filtration column we used, excellent separation of tryptic peptides from hen egg-white lysozyme was achieved within 50 min. System 2 was applied to a separation of V8 protease peptide from yam tuber chitinase and yielded two to three times higher amounts of peptides than reversed-phase high-performance liquid chromatography.

INTRODUCTION

With the advances in high-performance liquid chromatographic (HPLC) techniques for separating peptides for protein structural analysis, considerable work has been reported on reversed-phase (RP) HPLC. One convenience of RP-HPLC is that the separation is done with the volatile 0.1% trifluoroacetic acid (TFA)–acetonitrile elution system, yielding salt-free peptides that are suitable for sequencing [1]. However, a few relatively hydrophobic peptides are difficult to separate with this system owing to loss by irreversible adsorption to the gel matrix. Further, some peptides wich possess different amino acid sequences but have similar hydrophobicities elute as a single peak, and some of them cannot be separated with other solvents, such as phosphate buffer–acetonitrile, with a different pH [1]. In order to avoid these disadvantages, other modes of separation such as gel filtration [2], ion exchange [3], hydrophobicity [4] or combined modes [5,6] have been reported. For the sequencing of peptides,however, these methods involve the complication of pre- or post-treatment of corresponding peptides subjected to the Edman method.

In this paper, we report a simple method for the separation of peptides for sequence study employing gradient elution on gel filtration polymer-type HPLC columns.

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EXPERIMENTAL

Carboxymethylation and enzymatic digestion

Hen egg-white lysozyme (HEWL) (Seikagaku Kogyo, crystallized six times) and yam chitinase were reduced and carboxymethylated by the method of Crestfield *et al.* [7]. Lyophilized carboxymethylated (Cm)-lysozyme (5 mg) suspended in 1.0 ml of distilled water by ultrasonication was digested with 1/50 (w/w) trypsin (TR-TPCK) (Cooper Biomedical) at 37°C and pH 8.0 for 4 h. Cm-chitinase (8 mg) suspended in 2 ml of 5 m*M* ammonium hydrogencarbonate (pH 8.2) was digested with 1/50 (w/w) V8 protease (Boehringer) at 37°C for 6 h. The digests were directly subjected to HPLC analysis after removal of the insoluble materials.

Isolation of peptides by gel filtration HPLC

The tryptic digest of lysozyme (100 μl, containing 0.3 mg of peptide mixture) was applied to a high-performance gel filtration (GF) HPLC column (Asahipak GS320, Asahi Chemical) using a JASCO LC-800 HPLC system (Japan Spectroscopic). The mobile phases for isocratic elution were composed of 0, 3 and 30 m*M* phosphate buffer (pH 8.0) and 0, 5 and 10% acetonitrile in 30 m*M* phosphate buffer (pH 8.0). The mobile phases for gradient elution were composed of two different solvent systems: system 1, 30 m*M* phosphate buffer (pH 8.0) (solvent A) and 20% acetonitrile in solvent A (solvent B); system 2, 30 m*M* phosphate buffer (pH 8.0) (solvent A) and distilled water (solvent B). Other conditions were as follows: monitoring wavelength, 220 nm; flow-rate, 1.0 ml/min; recording range, 0.32 a.u.f.s.

The separation of V8 peptides from yam chitinase was performed by gradient elution using solvent system 2 but ammonium hydrogencarbonate was used instead of phosphate buffer.

Isolation of peptides by RP-HPLC

The tryptic and V8 protease digests were applied to RP-HPLC columns (YMC C18 120A or C4 300A, Yamamura Chemical) using the same HPLC system as for GF-HPLC. The peptide elution was performed with a linear gradient of 0.1% TFA (solvent) and 60% acetonitrile in solvent 1 (solvent 2). Other conditions were the same Aas in GF-HPLC, except for the recording range of 0.64 a.u.f.s.

Calculation of hydropathic value of peptides

The hydropathic value for each tryptic peptide was calculated using the hydrophilicity value of each amino acid [8]. For carboxymethyl cysteine, the hydrophilicity value of aspartic acid was used.

Amino acid analysis and amino acid sequence analysis of tryptic peptides

Peptide was hydrolysed in an evacuated sealed tube at 110°C for 20 h using constant-boiling hydrochloric acid containing 0.05% β-mercaptoethanol. The hydrolysate was analysed with the aid of a Hitachi Model 835 amino acid analyser. Amino acid sequence analysis was performed by the method of dimethylaminoazobenzene isothiocyanate–phenylisothiocyanate double coupling manual micro-sequencing [9, 10].

RESULTS AND DISCUSSION

The influence of the buffer concentration of the mobile phase on the separation of peptides on the GF-HPLC column was investigated using tryptic peptides of HEWL as model peptides. As shown in Fig. 1A–C, on elution with water (0 m*M* phosphate buffer) few peptides were retained on the column, but an increase in the buffer concentration caused longer elution times. Each peptide was separated by 30 m*M* phosphate buffer; however, it was not effective on account of the broad peaks.

The shortcoming of the separation of peptides with phosphate buffer was eliminated by adding an organic solvent, acetonitrile. The influence of acetonitrile concentration on the peptide separation in 30 m*M* phosphate buffer is shown in Fig. 1D and E. The notable feature of acetonitrile is that it reduces the retention times and the peak widths. This suggests that acetonitrile effectively acted to elute the peptides adsorbed on the gel matrix by hydrophobic interaction. These results indicate that a high buffer concentration and addition of an organic solvent are effective for the separation of peptides on a GF-HPLC column with isocratic elution. However, as some peptides were still cross-contaminated when the organic solvent was contained in the solvent, different separation modes in the first and second halves of the chromatography were considered to be effective for the separation of peptides.

Gradient elution for peptide separation on a GF-HPLC column was investigated next. The elution profile obtained by gradient elution with 30 m*M* phosphate buffer (pH 8.0) and 20% acetonitrile in the same buffer is shown in Fig. 2B. A linear gradient of 30 min provided a good separation, *i.e.*, the peptides that eluted during the first 10 min were separated under the influence of the buffer concentration and the peptides that eluted between 10 and 30 min were separated under the influence of the increased acetonitrile concentration. Further, the same excellent separation was obtained by a gradient from 30 m*M* phosphate buffer to water as show in Fig. 2A. This result indicates that a simple gradient with or without an organic solvent results in a separation capacity equal to that obtained in RP-HPLC without prolonging the analysis time.

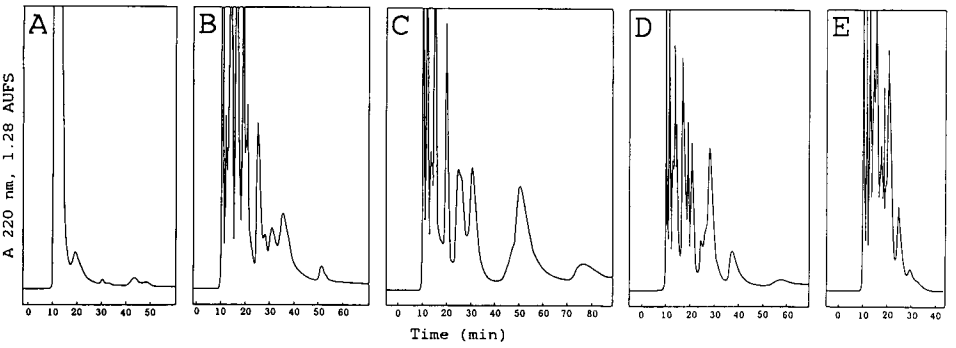

Fig. 1. GF-HPLC of tryptic peptides of lysozyme with various buffer concentrations in the mobile phase. (A) Water; (B) 3 m*M* phosphate buffer (pH 8.0); (C) 30 m*M* phosphate buffer (pH 8.0); (D) 5% acetonitrile in 30 m*M* phosphate buffer (pH 8.0); (E) 10% acetonitrile in 30 m*M* phosphate buffer (pH 8.0). For HPLC conditions, see text.

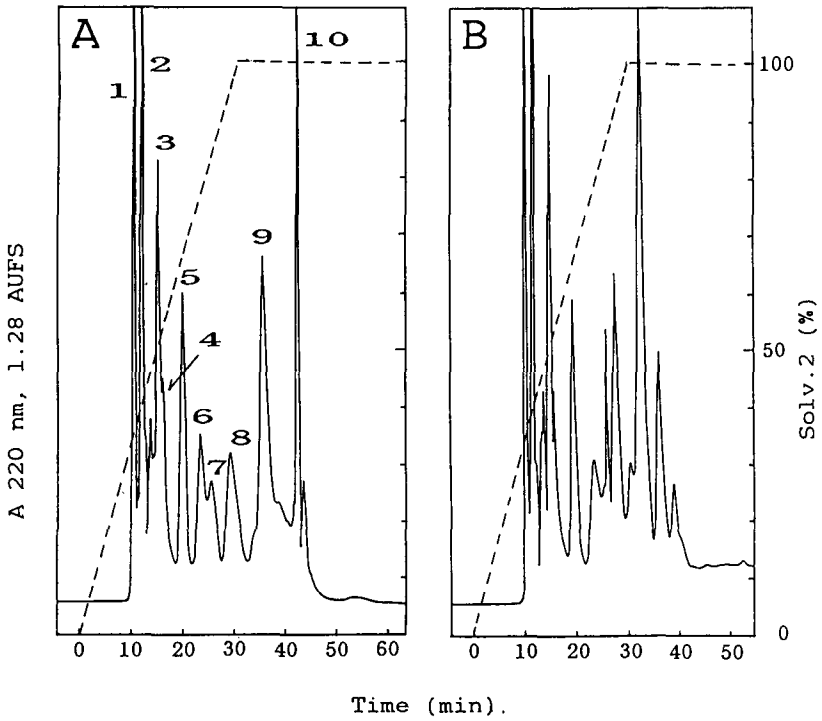

Fig. 2. Gradient elution of tryptic peptides of lysozyme on a GF-HPLC column. (A) Gradient elution from 30 m*M* phosphate buffer (pH 8.0) to water; (B) gradient elution from 0 to 20% acetonitrile in 30 m*M* phosphate buffer (pH 8.0). Peptides in the numbered peaks were analysed.

In order to elucidate the relationship between retention time and the properties of peptides, each peak obtained by gradient elution from 30 m*M* phosphate buffer (pH 8.0) to water was desalted by RP-HPLC using a 0.1% TFA–acetonitrile gradient system. Some peaks containing two peptides on GF-HPLC were separated by RP-HPLC. These desalted peptides were subsequently subjected to amino acid analysis, and then the amino acid sequences were determined by comparison with reported sequences [11,12]. It was found that the retention time did not depend on the number of residues. One of the reasons for this is the property of the polymer-type [poly(vinyl alcohol)] gel and another is the effect of the gradient on the gel particles. However, if the hydropathic value of each peptide was plotted against retention time, two lines relating to the retention time were obtained by the method of least squares, as shown in Fig. 3. These results imply that the peptides on the column were separated by at least two separation modes, size exclusion and reversed phase. These two separation modes are not independent but combined under these experimental conditions.

This system was applied to separate the V8 protease digested peptides of yam tuber chitinase [13], which contain relatively high-molecular-weight peptides with up to 80 amino acid residues. The separation pattern and the yield of peptides were compared with those given by RP-HPLC. Nine peaks were obtained by RP-HPLC using a C_4 300 column, as shown in Fig. 4A. However, the N-terminal peptide V10

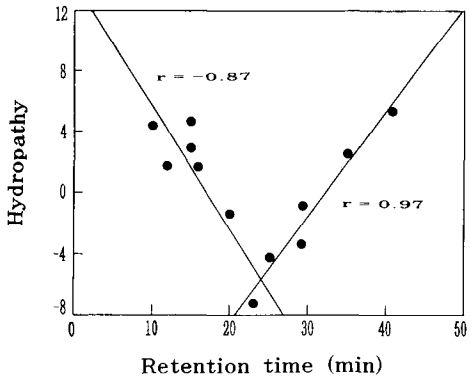

Fig. 3. Relationship between hydropathy of peptide and retention time in gradient elution (Fig. 2A). Two lines were obtained by regression analysis and the corresponding regression coefficients are represented by *r*.

(N-blocked, 55 amino acid residues) was not eluted by RP-HPLC. In contrast, GF-HPLC (a volatile solvent, ammonium hydrogencarbonate, was used instead of phosphate buffer for direct sequencing) yielded seven peaks with the gradient elution system of ammonium hydrogencarbonate and water (Fig. 4B), and the amino acid composition and N-terminal sequence of each peak were analysed. High-molecular weight fragments, especially the N-terminal peptide V10, were eluted with high yields. The irreversible adsorption of peptides on RP-HPLC gel matrices, such as that of peptide V10 in this study, have often been observed, and prevent the microanalysis of peptides. However, peptide V10 was easily obtained in high yield by GF-HPLC. Gradient elution was effective for the earlier elution of small and hydrophobic peptides without peak spreading.

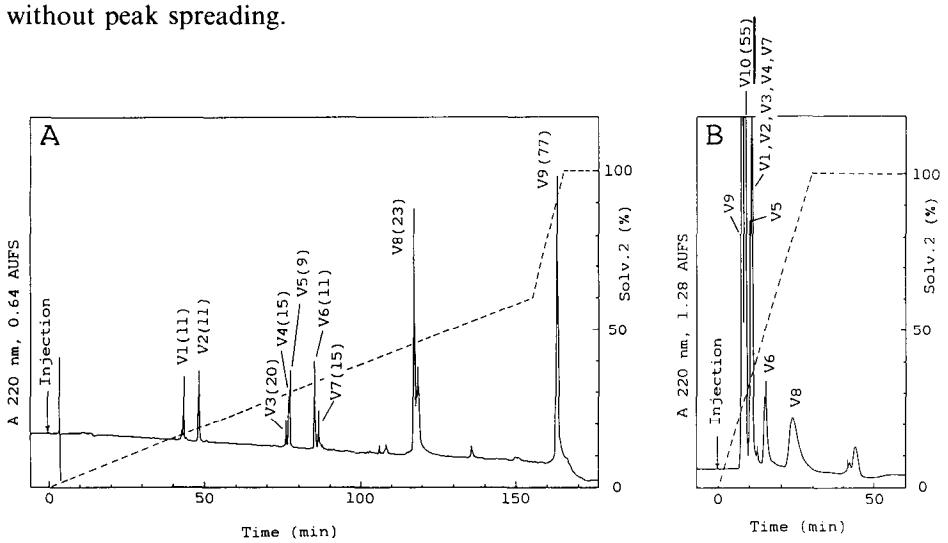

Fig. 4. V8 protease fragment separation by GF-HPLC and RP-HPLC. V8 protease digest of Cm-chitinase was separated on (A) an RP-HPLC column (C_4-300, 250 × 4.6 mm I.D.) using a linear gradient elution system of 0.1% TFA and 60% acetonitrile in 0.1% TFA and (B) a GF-HPLC column (GS-320, 500 × 7.6 mm I.D.) using a linear gradient elution system of 30 m*M* ammonium hydrogencarbonate and water.

TABLE I

COMPARISON OF PEPTIDE RECOVERIES ON RP-HPLC AND GF-HPLC COLUMNS

The yields of V8 peptides from Cm-chitinase (10 mg) were measured by amino acid analysis. The peptides obtained with insufficient separation by GF-HPLC were analysed after rechromatography by RP-HPLC.

Peptide	Yield (nmol)	
	RP-HPLC	GF-HPLC
V1	112.1	242.2
V2	130.5	305.6
V3	85.4	127.1
V4	33.9	165.5
V5	158.5	94.9
V6	120.6	310.8
V7	81.5	91.9
V8	44.4	114.5
V9	48.8	130.2
V10	0.0	164.0

The yield of each peptide in RP-HPLC and GF-HPLC are summarized in Table I. The results show that the yields of the peptides in RP-HPLC are relatively low when the peptides are hydrophobic or of high molecular weight, whereas the yields of such peptides in GF-HPLC are high.

Combining these results, we conclude that the GF-HPLC method is advantageous for long-chain, hydrophobic peptides that are difficult to recover by RP-HPLC. The gradient system enables peptides that have strong gel matric interactions to elute easily without peak spreading. Further, the GF-HPLC gradient system is well sited for biologically active peptides that cannot be subjected to organic solvents.

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