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Peptide separation in normal-phase liquid chromatography
Study of selectivity and mobile phase effects on various columns

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Abstract

An experimental procedure for peptide separation by normal-phase liquid chromatography (NPLC) was proposed in previous papers. In the present study, the chromatographic behavior of amino, cyano, amide, diol and silica columns, which have been used in non-aqueous NPLC, is investigated anew. The amino column was not appropriate for peptide separation because of poor recovery. The cyano column could not be used due to lack of retention. The amide, diol and silica columns were useful for peptide separation. The chromatograms on amide, diol and silica columns were a little different when the mobile phase composition was changed. The recovery of peptides was good: diol>amide>silica. Repeatability and reproducibility using amide, diol and silica columns was satisfactory. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Mobile phase composition; Stationary phases, LC; Peptides

1. Introduction

An experimental procedure for peptide separation by normal-phase liquid chromatography (NPLC) has been reported [1–3], in which a TSK gel Amide-80 [4,5], with carbamoyl groups bonded to a silica gel matrix, was used with an acetonitrile (ACN)–water mixed solution containing 0.1% trifluoroacetic acid (TFA). In this version of NPLC, an increasing gradient of water in the mobile phase was used. This form of NPLC [6–9] is often referred to as hydrophilic interaction chromatography [10–13]. This separation system was able to retain and separate hydrophilic peptides [12,13] that were not retained on an octadecyl silica (ODS) column in reversed-phase liquid chromatography (RPLC). Separation selectivities in normal- and reversed-phase methods differed significantly. The following two investigations have been made: (I) retention coefficients were calculated using 121 peptides and the contribution of each amino acid to retention in this version of NPLC was clarified [2]. (II) The relationship between capacity factor (k) and modifier (water) composition (φ) of the mobile phase in this version of NPLC has been studied [3].

It was reported that the separation of carbohydrates on silica [8,11], amino [6–9] and diol [7,9,11] columns, which have been used in general non-aqueous NPLC [14], was carried out using an aqueous mobile phase. However, to the author's knowledge, there are no experimental data of peptide separation using these non-aqueous normal-phase systems. The present study extends the investigation of this version of NPLC on the various columns. In this study, by comparing the retention behavior of the same peptides on these various columns, I

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conclude what the most suitable column for the normal-phase peptide separation is.

2. Experimental

2.1. Materials

HPLC-grade ACN was obtained from Nacalai Tesque (Kyoto, Japan), and TFA, formic acid and triethylamine (TEA) from Wako Pure Chemical (Osaka, Japan). A Milli-Q system (Japan Millipore, Tokyo, Japan) was used for water purification. Most of the peptides were purchased from Sigma (St. Louis, MO, USA) and the Peptide Institute (Osaka, Japan) and the others obtained by cyanogen bromide degradation of myoglobin. The TSK gel NH_2-60, TSK gel CN-80Ts, TSK gel Amide-80, TSK gel Silica-60 and TSK gel OH-120 (25 cm×0.46 cm I.D.) columns were from Tosoh (Tokyo, Japan).

2.2. Apparatus

The high-performance liquid chromatography (HPLC) system was a Tosoh liquid chromatograph equipped with a SC-8020 micro-computer, CCPM-II pump, UV-8020 detector, AS-8020 autosample injector and a CO-8020 column oven.

2.3. Methods (preparation of column)

Before chromatographic measurement, the TSK gel Silica-60 column was pre-conditioned with a 70% formic acid solution at a flow-rate of 0.7 ml/min for 24 h, followed by 0.2% TFA solution at a flow-rate of 1.0 ml/min for 12 h. The TSK gel NH_2-60, TSK gel CN-80Ts, TSK gel Amide-80 and TSK gel OH-120 columns were prepared by repeating a gradient run 20 times. The gradient is as follows: eluent A (initial eluent) was 0.2% TFA in ACN–water (97:3) and eluent B was 0.2% TFA in ACN–water (30:70). The gradient from eluent A to eluent B was over 60 min.

2.4. Methods (chromatographic measurement)

For gradient elution, eluent A (initial eluent) was ACN–water (97:3) and eluent B was ACN–water (55:45). 0.1% TFA or 0.1% TFA+0.1% TEA (0.1% TFA+TEA) or 0.2% TFA+0.2% TEA (0.2% TFA+TEA) were added to both eluents. The peptides were separated by a linear gradient from eluent A to eluent B over 70 min (0.6% water/min). For isocratic elution, the eluent was 0.2% TFA+TEA in ACN–water in various proportions. The peptides were dissolved in 5 μl of formic acid, followed by the addition of 40 μl ACN. The flow-rate was 1.0 ml/min. Column effluent was monitored by UV absorption at 215 nm. The temperature in the column oven was 40°C.

3. Results and discussion

3.1. Effects of mobile phase composition

In HPLC using organic groups bonded to a silica gel matrix, ion-exchange interactions between charged residues of peptides and residual silanol (SiOH) groups on the surface of the silica gel matrix cause band tailing [15]. Salts and organic acids such as TFA, formic and acetic acid added to the mobile phase effectively prevent such interactions [1,7,9,10,12,13,15]. In a previous paper [1], the effects of acid in the mobile phase were examined using TFA, acetic and formic acid.

In this study, the effects of TFA and TEA+TFA [9,10] were examined using the same peptides as in the previous paper. These results are shown in Fig. 1 I–III and are discussed for each column in turn.

3.1.1. TSK gel Amide-80

A peptide mixture was chromatographed using the three mobile phases as shown in Fig. 1 I. In all three cases, the peptide mixture was separated satisfactorily. With TEA+TFA, the retention times of the peptides increased, compared to addition of TFA alone. There was, however, no significant difference in these chromatograms. In a previous paper [1], addition of acid was effective in preventing ion-exchange interactions. In this study, it was found that the addition of TEA+TFA was effective in preventing ion-exchange interactions. The addition of TEA gave symmetrical bands for the strongly retained peptide (2.07, 1.97, 1.83), although the peak shape of most peptides except for FY (1.14, 1.53, 1.48) was

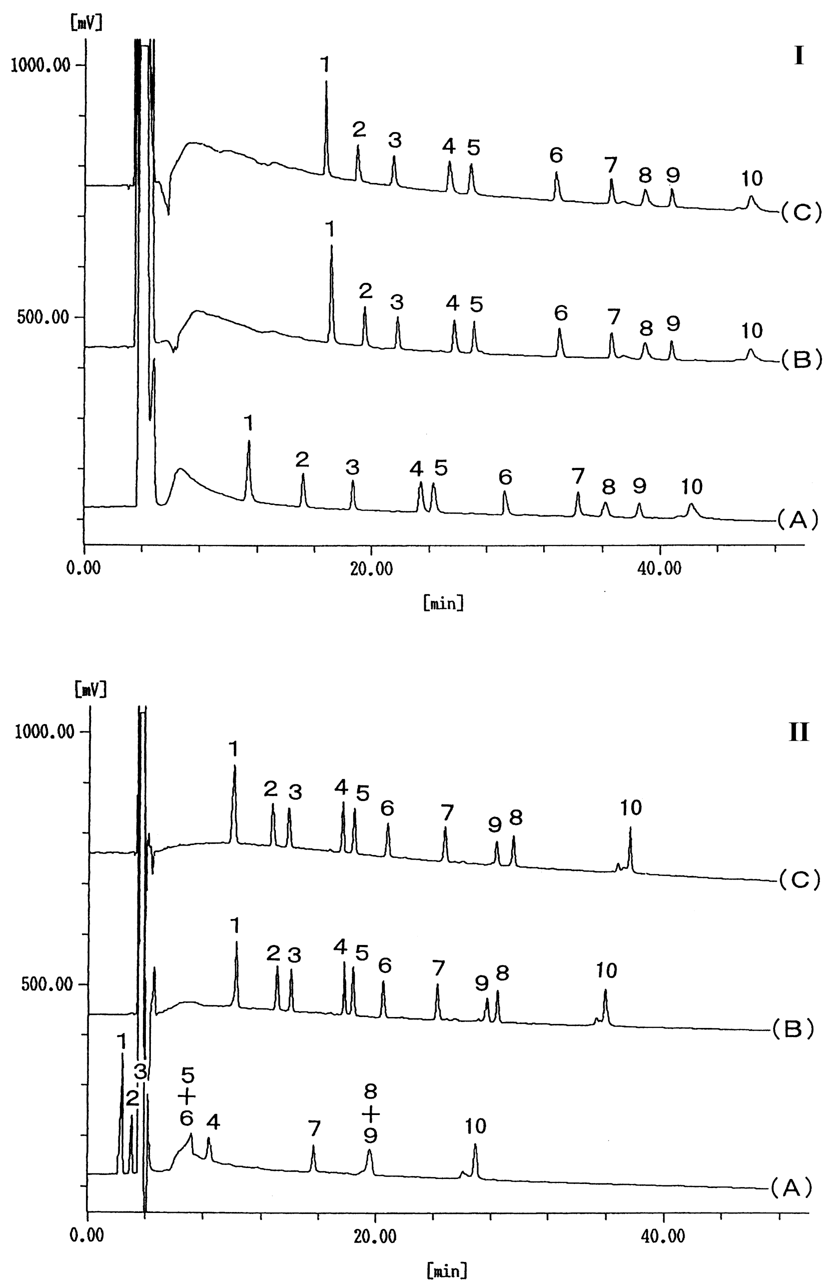

Fig. 1. Comparison of chromatograms of peptides separated on (I) TSK gel Amide-80, (II) TSK gel OH-120, (III) TSK gel Silica-60. The peptide mixture was separated with 70-min linear gradients of water from 3 to 45% (0.6% water/min) in: (A) 0.1% TFA; (B) 0.1% TFA+TEA; (C) 0.2% TFA+TEA. Peak identification: 1=FY; 2=FGGF; 3=FLEEI; 4=DYMGWMDP-NH_2; 5=NFTYGGF; 6=AGSE; 7=WAGGDASGE; 8=YGGFMTSQKSQTPLVT; 9=ASTTTNYT; 10=VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPET-LEKFDRFKHLKTEAEM*; M*=homoserine.

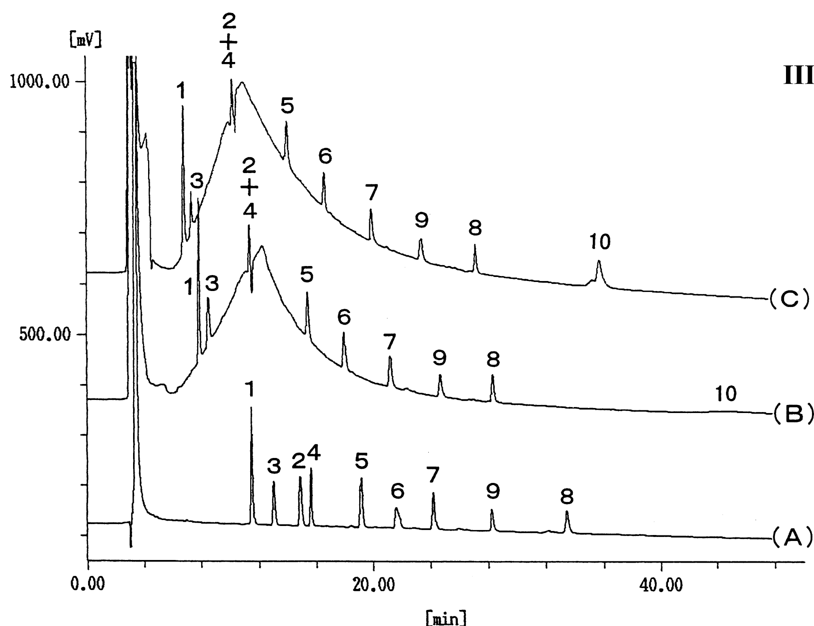

Fig. 1. (*continued*)

not affected by TEA. (Note: the numbers in parentheses are asymmetry factors in TFA alone, 0.1% TEA+TFA, 0.2% TEA+TFA, respectively). The effective TEA+TFA concentration was above 0.1%. It is assumed that the differences in elution time between TFA and TEA+TFA were caused by the pH differences of the mobile phase.

3.1.2. TSK gel OH-120

This column has glyceryl groups bonded [7,9,11] to silica gel. A peptide mixture was chromatographed using the three mobile phases as shown in Fig. 1 II. With the TFA only, FY, FGGF and FLEEI were not retained and eluted around the void volume. NFTYGGF and AGSE were eluted together. Recovery of peptides was poor. With TEA+TFA, the peptides were separated completely and the retention times of the peptides were increased, compared to addition of TFA alone. Therefore, the addition of TEA is effective. The addition of 0.1% TEA+TFA, however, gave leading bands of FY (0.60), FGGF (0.87), NFTYGGF (0.97), YGGFMTSQKSQTPLVT (0.94) and ASTTTNYT (0.89). The addition of 0.2% TEA+TFA yielded a small symmetrical band: FY (0.62), FGGF (0.99), NFTYGGF (0.99), YGGFMTSQKSQTPLVT (1.03) and ASTTTNYT (1.01). (Note: the numbers in parentheses are asymmetry factors). In terms of recovery, the addition of 0.2% TEA+TFA is a little better than that of 0.1% TEA+TFA. Therefore, it was concluded that the addition of 0.2% TEA+TFA was effective for preventing ion-exchange interactions.

3.1.3. TSK gel Silica-60

The silica gel matrix for TSK gel Silica-60 column was used specific material as different from another four chemically-bonded columns. Before chromatographic measurement, TSK gel Silica-60 column was pre-conditioned to remove impurities, such as minerals and metal oxides, on the surface layer of matrix, according to the procedure recommended by the manufacturer.

A peptide mixture was chromatographed using the three mobile phases as shown in Fig. 1 III. In all three cases, the chromatograms were quite different.

With the TFA only, the most strongly retained peptide (peak No. 10) failed to elute from the column. Even with 0.2% TFA (not shown), the peptide did not elute. It was found that the addition of TFA only to the mobile phase using the given

gradient program was not sufficient for peptide separation using the TSK gel Silica-60 column. With TEA+TFA, rising baselines were obtained compared to addition of TFA alone. It was assumed that the complex ion-exchange interaction among the silanol groups on stationary phase and TFA and TEA in the mobile phase caused this phenomenon. The TEA+TFA concentration was not sufficient at 0.1% because the recovery (19%) of the most strongly retained peptide was poor. In terms of higher recovery of the most strongly retained peptide, 0.2% TEA+TFA concentration was better than 0.1% TEA+TFA. As the peaks of FGGF and DYMGWMDP-NH_2 were not separated, this condition is not satisfactory, however. Using TEA+TFA, the most strongly retained peptide was thus eluted from the silica column. It might be considered that the most strongly retained peptide would tend to adsorb to the silica surface through ion-exchange interaction, because the peptide contains more basic amino acid residues such as His, Arg and Lys compared to the others. Using this TSK gel Silica-60 column, it is suggested that the addition of TFA only was insufficient for preventing the ion-exchange interactions. It is assumed that the role of TEA is to mask the most acidic silanol groups [15] and the metal oxide impurities in the stationary phase, which are believed to enhance the acidity of adjacent silanol groups.

3.1.4. TSK gel Amino-80

This column has aminopropyl-groups bonded [6–9] to silica gel. In all three cases, all the peptides were adsorbed strongly but recovery was poor. The TSK gel Amino-80 column was not suitable for peptide separation in this version of NPLC.

3.1.5. TSK gel CN-80Ts

This column has cyanopropyl-groups bonded [14] to silica gel. In all three cases, all the peptides eluted at the void volume. From these results, it is strongly suggested that the column does not retain the peptides in this version of NPLC through dipole–dipole interactions.

3.2. Selectivity

The chromatograms obtained with this version of NPLC in 0.2% TEA+TFA and RPLC which was reproduced from Ref. [1] are shown in Fig. 2.

AGSE eluted at the void volume on the ODS column and was well retained in this version of NPLC. FLEEI and DYMGWMDP-NH_2, with hydrophilic side chains, were strongly retained on the ODS column, but they were only moderately retained in this version of NPLC. In this version of NPLC, the elution order of YGGFMTSQKSQTPLVT and ASTTTNYT on the diol column was also reversed compared to the amide column. Furthermore, the elution order of FGGF and FLEEI on the silica column was also reversed compared to the diol column. Fig. 2 shows the slight difference in selectivity.

3.3. Recovery of peptides

Recovery [16] from the diol, silica and amide columns was assessed using the same peptides shown in Fig. 2. The average peptide recoveries are summarized in Table 1. Overall, recovery from each column with TEA+TFA tended to be good, compared to TFA alone. Recovery of the most strongly retained peptide (peak No. 10) from the amide column was exceptionally poor even with addition of TEA. Average peptide recoveries of peptides with 0.2% TEA+TFA was good in order of diol>amide>silica.

3.4. Repeatability and reproducibility

Repeatability on the same day is good for each column. The average of relative standard deviations (RSDs) ($n=6$) on each column are listed in Table 2.

The column-to-column reproducibility was assessed using the new and another used diol, silica and amide column, following 500 peptide injections. Fig. 3 I–III shows reproducibility for each column. These parameters with 0.2% TEA+TFA on the diol, silica and amide columns were determined using the same peptides shown in Fig. 2. The results are listed in Table 2. The column of the same lot were used in the column-to-column reproducibility experiment. Not strictly speaking, this column-to-column reproducibility data shown in Fig. 3 might suggest mean of long term repeatability.

As shown in Fig. 3 I–III, there was no difference

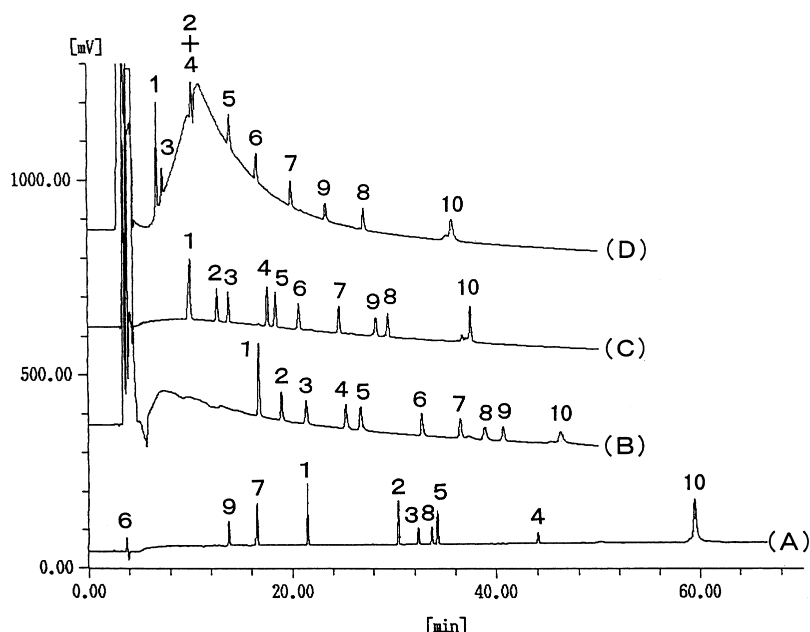

Fig. 2. Comparison of chromatograms of peptides separated on (A) TSK gel ODS-80Ts, (B) TSK gel Amide-80, (C) TSK gel OH-120 and (D) TSK gel Silica-60. The peptide mixture was separated with (A) 83.3-min linear gradients of acetonitrile from 5 to 55% in 0.1% TFA (0.6% ACN/min) and (B–D) 70-min linear gradients of water from 3 to 45% in 0.2% TFA+TEA (0.6% water/min). Peak identification as in Fig. 1.

between new and another used columns even after 500 peptide injections. Therefore, this experimental procedure for peptide separation by NPLC is stable.

Table 1
Average peptide recoveries from TSK gel Amide-80, TSK gel OH-120 and TSK gel Silica-60 with sample injection of ~1 μg (peptides were separated with a 70 min linear gradient of water from 3 to 45% in 0.1% TFA, 0.1% TFA+TEA and 0.2% TFA+TEA on TSK gel Amide-80, TSK gel OH-120 and TSK gel Silica-60)

Column	Average of recoveries (%)		
	0.1% TFA	0.1% TFA+TEA	0.2% TFA+TEA
Amide-80	92	93	92
OH-120	47[a]	91	93
Silica-60	95[b]	90[c]	85[d]

[a] Average of recoveries of eluted peptides (peak Nos. 4–10).
[b] Average of recoveries except for DYMGWMDP-NH_2 (94) and the most strongly retained peptide (not eluted).
[c] Average of recoveries except for DYMGWMDP-NH_2 (72) and the most strongly retained peptide (50).
[d] Average of recoveries except for DYMGWMDP-NH_2 (19) and the most strongly retained peptide (79). The numbers in parentheses are recovery values.

Fig. 3 I and II show that the peptides eluted slightly earlier from the used amide and diol columns than those from the new columns. For example, retention times of FY (18.17 min) and the most strongly retained peptide (47.41 min) on the new amide column decreased slightly to 16.40 min and 46.02 min on the used amide one, respectively. On the diol column, retention times of FY (10.25 min) and the most strongly retained peptide (39.04 min) on the new column decreased to 9.98 min and 36.83 min on the used one, respectively. In contrast to the chemically-bonded columns, the peptides eluted later

Table 2
Average of relative standard deviations ($n=6$) for this version of NPLC results obtained for peptide retention time, peak height and peak area in 0.2% TFA+TEA on TSK gel Amide-80, TSK gel OH-120 and TSK gel Silica-60

Column	Average of RSD (%)		
	Retention time	Peak height	Peak area
Amide-80	0.03	2.08	2.23
OH-120	0.08	1.93	2.50
Silica-60	0.08	1.09	1.58

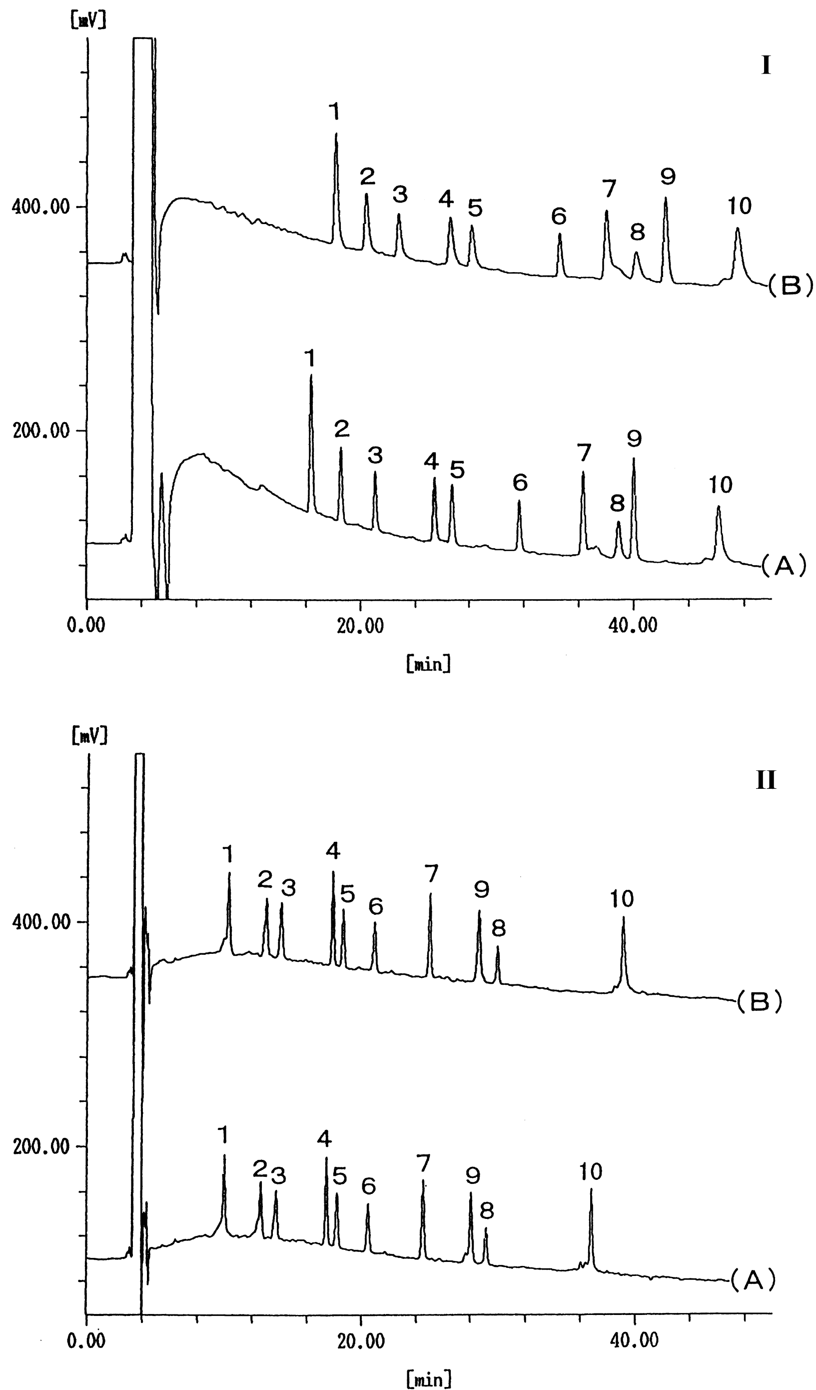

Fig. 3. (I) Comparison of chromatograms of peptides separated on (A) a used amide column (TSK gel Amide-80) and (B) a new amide column. (II) Comparison of chromatograms of peptides separated on (A) a used diol column (TSK gel OH-120) and (B) a new diol column. (III) Comparison of chromatograms of peptides separated on (A) a used silica column (TSK gel Silica-60) and (B) a new silica column. The peptide mixture was separated with 70-min linear gradients of water from 3 to 45% in 0.2% TFA+TEA (0.6% water/min). Peak identification as in Fig. 1.

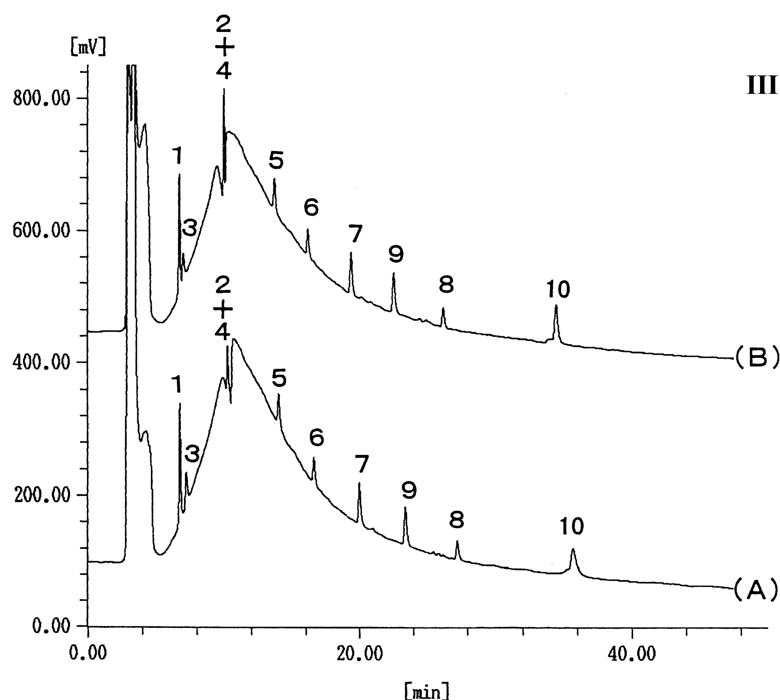

Fig. 3. (*continued*)

from the used silica column than from the new one, as shown in Fig. 3 III. For example, retention times of FY (6.65 min) and the most strongly retained peptide (34.44 min) on the new silica column increased to 6.72 min and 35.68 min on the used silica one, respectively.

The new amide column gave tailing band for all peptides except for the most strongly retained peptide (1.73, 1.92), although the used one gave symmetrical band. (Note: the numbers in parentheses are asymmetry factors on the new and used columns, respectively). For example, asymmetry of FY (1.74) and ASTTTNYT (1.71) on the new silica column became 1.33 and 1.24 on the used one, respectively. Although about half the peptides on the both the new and another used diol column have symmetrical bands, FGGF (0.55, 0.31), FLEEI (0.75, 0.52), ASTTTNYT (0.92, 0.44) and the most strongly retained peptide (1.15, 1.11) on the used diol column become leading bands. All peptides except for the most strongly retained peptide (1.61, 1.07) became slightly tailing bands on the used silica column as compared to the new one. For example, asymmetry of FY (1.46) and ASTTTNYT (1.66) on the new silica column increased to 1.47 and 1.72 on the used one, respectively.

The peak areas of all peptides on the used amide column became smaller than those on the new one. In contrast, there was no significant difference in the peak areas of almost all the peptides on the diol and silica columns.

4. Conclusions

In a previous paper, an experimental procedure for peptide separation by NPLC was proposed. In this study, the chromatographic behavior using amino, cyano, amide, silica and diol columns, which were used in non-aqueous NPLC, was investigated anew. The amino and cyano columns were impossible to use for peptide separation. The amide, silica and diol columns were useful for peptide separation. The recovery for peptides was good in the order diol> amide>silica. Repeatability and reproducibility using amide, silica and diol columns was satisfac-

tory. If researchers try peptide separation using this version of NPLC method, the amide and diol columns should be useful.

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