Elution Profile of Di-peptides on a Sulfonated Ethylstyrone-Divinylbenzene Copolymer Resin Column by High-performance Liquid Chromatography

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This study investigates the characteristics of a partially sulfonated ethylstyrone-divinylbenzene copolymer for the separation of di-peptides by high-performance liquid chromatography. Di-peptides (VE, VA, VH, VK, and VR) with different isoelectric points (pl, 4.0 to 9.7) and log P values (∼1.63 to ∼0.72) were used to optimize the separation conditions of the columns packed with sulfonated copolymer resin. The retention factor (k) of the di-peptides on the column with a 0.81 wt% sulfo group content decreased with increasing concentrations of phosphate salts (2.5 – 20 mmol L⁻¹) in the mobile phase. The complete separation of the five di-peptides was obtained with a gradient of 10% methanol containing 5 mmol L⁻¹ NaH₂PO₄ (pH 4.8) to 50% methanol containing 5 mmol L⁻¹ Na₂HPO₄ (pH 8.9) for 60 min at 0.5 mL min⁻¹ at 50°C. Under the optimal conditions, a good relationship between the k and pl values of the di-peptides, with the exception of VE (pl 4.0), was observed, suggesting that the retention of the di-peptides on the column packed with sulfonated copolymer resin was dependent on the pl value, when it was greater than 5. The log P value also influenced the separation characteristics of the column; peptides possessing the same pl value (6.4 for GH, VH, IH, and FH) showed a higher retention on the column with increasing log P values. In conclusion, the prototype sulfonated ethylstyrone-divinylbenzene copolymer column was applicable for the separation of basic di-peptides, and the separation depended on the pl and hydrophobicity of the di-peptides.

Keywords: Peptide separation, cation-exchange reversed phase column, high performance liquid chromatography, pl, log P

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Introduction

Small (di- and tri-) peptides have been demonstrated to play a potential role in preventing lifestyle-related diseases, such as hypertension,¹ atherosclerosis,² and high blood cholesterol.³ Our studies regarding peptide-induced physiological functions also revealed that basic and/or hydrophobic small peptides, such as Trp-His, exerted vasorelaxation effects in the aorta through the retardation of intracellular Ca²⁺-signaling pathways,⁴–⁶ and could be absorbed intact into the blood system via intestinal peptide transporter 1.²⁹

Peptides, which are condensed amino acids, have many members in their polymeric series, e.g., >400 di-peptides and >8000 tri-peptides, and simple and convenient assays for bioactive small peptides in food hydrolysates or natural materials are required in the food industry. It is difficult to separate small peptides in hydrolysates in a one run-in assay in reversed-phase HPLC, because of their similar polarities and hydrophobicities. To overcome this issue, multi-step reversed-phase HPLC,¹⁰ column-switching HPLC,¹¹ ion-pair chromatography,¹² and LC-mass spectrometry (LC-MS) with chemical derivatization¹³,¹⁴ have been proposed. In this study, we investigated an alternative HPLC separation technique using a mixed-mode column in order to establish a convenient assay of bioactive (or basic and/or hydrophobic) small peptides without any special procedures or instruments for applications in the food industry.

Specifically, a prototype cation-exchange/reversed phase HPLC column packed with a partially sulfonated ethylstyrone-divinylbenzene copolymer resin was investigated for the separation of di-peptides, since Yokoyama et al.¹⁵ demonstrated that the simultaneous separation of 20 amino acids without derivatization could be achieved using the mixed-mode of a cation-exchange/reversed phase HPLC column. Di-peptides with diverse isoelectric points (pl's) and hydrophobicities (log P) values were synthesized in order to obtain information regarding the separation characteristics of the prototype column for extensive peptide separations. As aforementioned, the analytical detection of small basic peptides in one run-in assay will be of benefit for the rapid evaluation of basic peptide-induced preventive effects against lifestyle-related diseases, such as anti-atherosclerosis,² anti-vasoconstriction,⁴–⁶ and anti-diabetes.⁶

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= /compute_p/, when physiologically functional foods rich in peptides are developed.

**Experimental**

**Reagents**

Di-peptides Val-Glu (VE), Val-Ala (VA), Val-His (VH), Val-Lys (VK), Val-Arg (VR), His-Val (HV), Lys-Val (KV), Arg-Val (RV), Gly-His (GH), Ile-His (IH), and Phe-His (FH) were synthesized via an Fmoc-solid phase synthesis method according to the manufacturer provided instructions (Kokusan Chemicals, Osaka, Japan). Their sequences were confirmed on a PFSQ-21 amino acid sequencer (Shimadzu Co., Kyoto, Japan). Methanol (MeOH) (Nacalai Tesque Inc., Kyoto, Japan) and acetonitrile (CH3CN) (Kanto Chemical, Tokyo, Japan) were of HPLC grade. Deionized water was prepared using a Milli-Q system. An MD1510 photo-diode array (PDA) detector (JASCO, Tokyo, Japan) was used to detect the di-peptides at 220 nm.

**Columns**

A cation-exchange/reversed phase HPLC column packed with a partially sulfonated ethylstyrene-divinylbenzene copolymer resin (CHK40/C04, 4.6 mm \( \times \) 150 mm i.d.) was provided by Mitsubishi Chemical Corp. (Tokyo, Japan). The copolymer resin having a 40 wt% degree of crosslinking, 4 \( \mu \)m particle size, 100 Å pore diameter, and a 1.1 mL g\(^{-1}\) pore volume was subjected to \( \text{H}_2\text{SO}_4 \)-sulfonation. Columns packed with the resin containing 0, 0.13, 0.81, and 2.19 wt% sulfo groups were purchased from Wako Pure Chemical Industries (Osaka, Japan).

**HPLC separation**

HPLC separation was performed on a Shimadzu LC-10AD system. An MD1510 photo-diode array (PDA) detector (JASCO, Tokyo, Japan) was used to detect the di-peptides at 220 nm.

The chromatographic run was performed at a flow rate of 0.5 mL min\(^{-1}\) at 50°C. The linear gradient elution was performed using MeOH or CH3CN containing phosphate salts (NaH2PO4 or Na2HPO4). The effects of the concentration of phosphate salt on the elution of the five di-peptides on the column packed with sulfonated copolymer resin was due to the preferential cation-exchange interactions with the basic moieties of the peptides. By considering the report that increasing the pH using phosphate salts was effective for amino acid separations on low-capacity cation-exchange columns,\(^{15}\) we applied the following elution system for di-peptide separation: mobile phase A, 10% MeOH or CH3CN containing 5 mmol L\(^{-1}\) NaH2PO4 buffer (pH 4.8); mobile phase B, 50% MeOH or CH3CN containing 5 mmol L\(^{-1}\) Na2HPO4 buffer (pH 8.9). As shown in Figs. 1b and 1c, individual elution of the five di-peptides was observed in both MeOH and CH3CN solvent systems by increasing the pH from pH 4.8 (5 mmol L\(^{-1}\) NaH2PO4) to pH 8.9 (5 mmol L\(^{-1}\) Na2HPO4) (60 min), whereas poor retentions were observed on the non-sulfonated copolymer column (Fig. 1a), also revealed that the retention of the di-peptides on the column packed with sulfonated copolymer resin was due to the preferential cation-exchange interactions with the basic moieties of the peptides. Due to some ghost baseline peaks (50 – 70 min) attributed to insoluble phosphate salts in the hydrophobic CH3CN solution (Fig. 1c), further experiments were performed using MeOH-based solvent systems containing phosphate salts.

**Results and Discussion**

**Elution profile of di-peptides on a sulfonated ethylstyrrene-divinylbenzene copolymer column**

Five di-peptides containing Val at the N-terminal (VE, VA, VH, VK, and VR) were used to evaluate the elution characteristics of the partially sulfonated ethylstyrrene-divinylbenzene copolymer column, since the molecular features (\( p_l \) and \( \log P \) values, Table 1) of these di-peptides containing Val at the N-terminal originate from the C-terminal amino acids, regardless of the molecular size. In our preliminary experiments regarding the elution of the five di-peptides on the column packed with resin containing 0.81 wt% sulfo groups, the peaks of the di-peptides at 220 nm were not observed using a linear gradient elution of 0 – 100% CH3CN containing 0.1% trifluoroacetic acid (data not shown), suggesting that the ionic interactions of the partially sulfonated copolymer column with the di-peptides may supersede the hydrophobic interactions. The lack of adequate separation of the di-peptides on the non-sulfonated (0 wt%) ethylstyrrene-divinylbenzene copolymer column shown in Fig. 1a, also revealed that the retention of the di-peptides on the column packed with sulfonated copolymer resin was due to the preferential cation-exchange interactions with the basic moieties of the peptides. By considering the report that increasing the pH using phosphate salts was effective for amino acid separations on low-capacity cation-exchange columns,\(^{15}\) we applied the following elution system for di-peptide separation: mobile phase A, 10% MeOH or CH3CN containing 5 mmol L\(^{-1}\) NaH2PO4 buffer (pH 4.8); mobile phase B, 50% MeOH or CH3CN containing 5 mmol L\(^{-1}\) Na2HPO4 buffer (pH 8.9). As shown in Figs. 1b and 1c, individual elution of the five di-peptides was observed in both MeOH and CH3CN solvent systems by increasing the pH from pH 4.8 (5 mmol L\(^{-1}\) NaH2PO4) to pH 8.9 (5 mmol L\(^{-1}\) Na2HPO4) (60 min), whereas poor retentions were observed on the non-sulfonated copolymer column (Fig. 1a), supporting the aforementioned speculation that the di-peptide elution on the column packed with sulfonated copolymer resin was due to preferential cation-exchange. Due to some ghost baseline peaks (50 – 70 min) attributed to insoluble phosphate salts in the hydrophobic CH3CN solution (Fig. 1c), further experiments were performed using MeOH-based solvent systems containing phosphate salts.

Figure 2a shows the effect of the phosphate salt concentration on the elution of five di-peptides on a column packed with resin containing 0.81 wt% sulfo groups. The retention or \( k \) value of each di-peptide, with the exception of VE, with 10 – 50% MeOH elution greatly decreased with increasing phosphate (NaH2PO4 and Na2HPO4) concentration, ranging from 2.5 to 20 mmol L\(^{-1}\). This revealed the favorable retention of basic and/or hydrophobic di-peptides by cation-exchange interactions, since a linear relationship between \( \log k \) and \( C \) was obtained (Fig. 2b), a

Table 1 Di-peptides used in this study and their \( p_l \) and \( \log P \) values

|     | VE | VA | VH | VK | VR | GH | IH | FH | HV | KV | RV |
|-----|----|----|----|----|----|----|----|----|----|----|----|
| \( p_l \) | 4.0 | 5.5 | 6.7 | 8.7 | 9.7 | 6.7 | 6.7 | 6.7 | 6.7 | 8.8 | 9.8 |
| \( \log P \) | –1.23 | –0.72 | –1.53 | –0.88 | –1.63 | –2.74 | –1.02 | –0.51 | –0.99 | –0.88 | –1.79 |

One-letter symbols were used to represent the peptides.

Calculated \( p_l \) and \( \log P \) values of di-peptides

The \( p_l \) values of the di-peptides were calculated using ExPASy Compute PI/Mw software available on http://web.expasy.org/compute_p/. The \( \log P \) values of the di-peptides were calculated using SciFinder Substance Identifier software (https://scifinder.cas.org/scifinder/view/scifinder/scifinderExplore.jsf).
Fig. 1  HPLC chromatograms of the five di-peptides at 50°C at a flow rate of 0.5 mL min⁻¹ monitored at 220 nm. A mixture of 100 μmol L⁻¹ di-peptides (VE, VA, VH, VK, and VR) was injected into the HPLC system. Elution profile on a non-sulfonated (0 wt% sulfo group) (a) or sulfonated (0.81 wt% sulfo groups) ethylstyrene-divinylbenzene copolymer column (b) with an elution gradient of 10% MeOH containing 5 mmol L⁻¹ NaH₂PO₄ (pH 4.8) to 50% MeOH containing 5 mmol L⁻¹ Na₂HPO₄ (pH 8.9) over 60 min. (c) Elution with 10% CH₃CN containing 5 mmol L⁻¹ NaH₂PO₄ (pH 4.8) to 50% CH₃CN containing 5 mmol L⁻¹ Na₂HPO₄ (pH 8.9) over 60 min on 0.81 wt% sulfo group-column. *: ghost baseline peaks.

Fig. 2  Effect of the concentration of phosphate salts in the mobile phase on the retention factor (k) of di-peptides on a column packed with resin containing 0.81 wt% sulfo groups. VE, VA, VH, VK, and VR were used as the di-peptide standards. Elution at 0.5 mL min⁻¹ at 50°C was performed with an elution gradient of 10% MeOH containing NaH₂PO₄ to 50% MeOH containing Na₂HPO₄ over 60 min. The concentration of each phosphate salt was set to 2.5, 5, 10, and 20 mmol L⁻¹. (a) Plots of the k value of each di-peptide vs. concentration; (b) Plots of the log k vs. log concentration.
In order to obtain information regarding the characteristics of a column packed with sulfonated copolymer resin in the separation of di-peptides, we focused on the ionization and hydrophilicity of the di-peptides (pI and log c), as summarized in Table 1. The elution of the di-peptides on the prototype column was mainly dependent on the pH of the mobile phase (Fig. 1b). As shown in Fig. 4a, the log P value or hydrophobicity of the five di-peptides did not significantly influence their elution on the column with resin containing 0.81 wt% sulfo groups, whereas a good relationship between the k and pI values of the di-peptides was observed (with the exception of VE) on the columns with resin containing 0.81 and 2.19 wt% sulfo groups (Figs. 4b and 4c). This strongly indicated that the elution of the peptides on the prototype column was mainly dependent on the pI value of neutral and/or basic peptides with pI values of >5. Further experiments using di-peptides with the same pI values, but different log P values were conducted in order to clarify the potential influence of hydrophobicity on the elution. As shown in Figs. 4d and 4e, four di-peptides with a pI value of 6.7 (i.e., GH, VH, IH, and FH) exhibited a good relationship between their log P and k values, suggesting that the hydrophobicity of peptides with pI >5 influences the elution on the column packed with a sulfonated copolymer resin, in addition to pI.

Peptide sequences often determine the magnitude of bioactivity, e.g., 40-fold stronger antihypertensive activity of IW than that of WI.18 Therefore, the separation of di-peptides with reversed sequence of VH, VK, and VR was examined. As shown in Fig. 5, a column packed with resin containing 2.19 wt% sulfo groups could separate two di-peptides with the same amino acids, possibly owing to their different log P values (Table 1).

Chromatographic separations of peptides using ion-exchange/reversed phase columns constitute a significant challenge. Specifically, an anion-exchange/reversed phase column was applied for the separation of N- and C-terminal protected tetrapeptides, and peptides with negative net-charge were eluted with increasing pH (2.7–6.5).19 Thus, the anion-exchange/reversed phase column may be useful for improving the poor retention of VE on the present sulfonated column (Fig. 1); in turn, the sulfonated or cation-exchange/reversed phase column must be of benefit for the retention of basic peptides that often show some preventive physiological effects against lifestyle-related diseases.14,16 The use of a cation-exchange/reversed phase column was attempted in the separation of acetylated oligo-(>10 mer) peptides, and a sufficient separation was achieved by hydrophilic interaction (HLIC) chromatography with increased hydrophilicity and salt (NaClO4) concentrations.20 However, these reports were limited to the separation of terminal-protected or hydrophobic oligo-peptides, and did not include the separation of non-protected small (di-/tri-) peptides with varying pI profiles. The cation-exchange/reversed phase HPLC column used in this study was based on ethylstyrene-divinylbenzene copolymer resin, which permitted a dynamic pH-gradient elution of the mobile phase (in this study, pH 4.8 – 8.9) for peptides with varying pI values. Further experiments using the column packed with a sulfonated copolymer resin are now in progress with bioactive basic tri-peptides, such as vasorelaxant His-Arg-Trp.21

Conclusions

This study demonstrates that a prototype mixed-mode (cation-exchange and reversed-phase) HPLC column packed with a partially sulfonated (<2.19 wt% sulfo group content) ethylstyrene-divinylbenzene copolymer resin has great potential to separate neutral and/or basic di-peptides. Under the optimal gradient elution conditions of 10% MeOH containing 5 mmol L⁻¹ NaH₂PO₄ (pH 4.8) to 50% MeOH containing 5 mmol L⁻¹ Na₂HPO₄ (pH 8.9) (60 min) at 50°C, the pI value of di-peptides with pI >5 significantly influenced their retention on the prototype column. The hydrophobicity (log P) of the di-peptides also influenced the retention of di-peptides with the same pI values.

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Fig. 4 Relationship between the retention factor (k) of the di-peptides and log P or pI values. (a) Plots of the k values of di-peptides (VE, VA, VH, VK, and VR) vs. their log P values on the column (0.81 wt% sulfo groups); (b) Plots of the k values of di-peptides (VE, VA, VH, VK, and VR) vs. their pI values column (0.81 wt% sulfo groups); (c) Plots of the k values of di-peptides (VE, VA, VH, VK, and VR) vs. their pI values on column (2.19 wt% sulfo groups); (d) Plots of the k values of di-peptides (GH, VH, IH, and FH) with pI value of 6.7 vs. their log P values on column (0.81 wt% sulfo groups); (e) Plots of the k values of di-peptides (GH, VH, IH, and FH) with pI value of 6.7 vs. their log P values on column (2.19 wt% sulfo groups). The elution condition was the same as that in Fig. 2.
Fig. 5  HPLC separation of two di-peptides composed of the same amino acids on a column packed with resin containing 2.19 wt% sulfo groups. A mixture of (a) VH and HV, (b) VK and KV, or (c) VR and RV was used. The elution condition was the same as that in Fig. 2.

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