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Peptide behaviour and analysis on a chemically stable C_{18}-bonded vinyl alcohol copolymer column with alkaline and acidic eluents

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SUMMARY

C_{18}-bonded vinyl alcohol copolymer (ODP) gel showed no weight loss or decrease in column efficiency for alkyl alcohols after being immersed in aqueous solutions of pH 2 and 10 at 50°C for 48 h, and only a 1% weight loss and a slight decrease in alkyl alcohol retention volumes after similar immersion at pH 13.

The chemical stability of the ODP gel was further demonstrated in analyses of acidic, neutral and basic peptides on an ODP column with eluents of pH 3–10, which showed that the peptides differ considerably in the sensitivity of their retention behaviour to eluent pH, even though hydrophobic interaction invariably appeared to be the main retention mechanism.

The ODP column was therefore applied to the analysis and alignment of lysil-endopeptidase (LEP) peptides derived from reduced and S-carboxymethylated carboxyl proteinase (Rcm-P-CP) of *Pseudomonas* sp. 101. One acid-soluble and seven alkaline-soluble LEP peaks were found in analyses on the ODP column using acidic and alkaline eluents, respectively. The chymotryptic peptides of Rcm-P-CP were first separated on an ODS column with an acidic eluent, and the eight eluates which contained lysine residue, as determined by amino acid analysis, were then analysed on the ODP column with an alkaline eluent, resulting in a further separation of each into several peaks and thus in the recovery of fractions of pure peptides. The LEP peptide alignment was then determined by overlapping the sequences of the chymotryptic peptides with the C- and N-terminal regions of the LEP peptides.

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INTRODUCTION

In recent years, reversed-phase chromagraphy has become predominant throughout high-performance liquid chromatography (HPLC) applications[1]. Octadecylsilica (ODS) columns are most commonly employed because of their high efficiency in the separation of peptides and other substances of low molecular weight. Peptide separations are possible but usually less efficient on ion-exchange columns, and are generally impractical by gel permeation chromatography because of the similar molecular weights of many peptides. ODS columns nevertheless have certain disadvantages[2], related to the inherently poor chemical stability of ODS gels. Their service life is generally short and they are not compatible with alkaline eluents or rinses.

As an alternative to ODS columns, a number of columns packed with conventional polymer gels have been developed in the past few years. These provide excellent chemical stability, but show a far lower column efficiency.

C_{18}-bonded vinyl alcohol copolymer (ODP) gel was developed to match ODS gels in chromatographic resolution and allow the use of eluents over a wide pH range, and columns packed with the ODP gel were found to exhibit stable performance on exposure to solutions of varying pH and polarity at 30°C[3,4]. This stability is of particular interest in the separation of peptides, because it raises the possibility of a distinction in chromatographic behaviour among acidic, neutral and basic peptides under the influence of both alkaline and acidic eluents.

Here we describe an investigation of the chemical stability of the ODP gel itself, the influence of pH on the chromatographic behaviour of peptides on the ODP column and the applicability of the ODP column to the analysis of peptides with acidic and alkaline eluents.

EXPERIMENTAL

ODP column (150 mm × 6 mm I.D.) containing Asahipak ODP-50 (Asahi Chemical, Kawasaki, Japan) ODP gel of 5.0-μm particle diameter with a surface area of 150 m^2/g and a C_{18} group bonding density of 5 $\mu mol/m^2$ was used. A commercially available YMC Pack AM-312 (Yamamura Chemical, Kyoto, Japan) ODS column (150 mm × 6 mm I.D.) was also used.

The following chromatography equipment was employed: ERC-3110 degassers (Erma Optical Works, Tokyo, Japan), LC-4A (Shimazu, Kyoto, Japan) and 880PU (Japan Spectroscopic, Tokyo, Japan) pumps, Rheodyne (Cotati, GA, U.S.A.) Model 7125 injector and Uvidec-IV ultraviolet detector (Japan Spectroscopic). Sample injection volumes were 10–100 μl.

Chemicals and organic solvents of HPLC grade were obtained from Wako (Osaka, Japan). Peptides were obtained from the Peptide Intstitute (Osaka, Japan).

Carboxylproteinase was isolated from *Psuedomonas* sp. 101 as described previously[5]. Rcm-carboxyl proteinase (Rcm-P-CP) was obtained by reduction of carboxylproteinase with mercaptoethanol and alkylation with iodoacetic acid.

The Rcm-P-CP was digested with lysilendopeptidase in aqueous pyridine or with chymotrypsin in ammonium hydrogencarbonate buffer (pH 8.0) containing urea at 30°C for 6 h.

RESULTS AND DISCUSSION

Gel stability in acidic and basic solutions

Apparently complete stability in a broad pH range was observed for the ODP gel, as indicated in Fig. 1, and also on the basis of its weight and alkyl alcohol retention volumes before and after immersion of 7 g of gel in 200 ml of 50 m*M* sodium phosphate–acetonitrile (90:10) at pH 2, 10 or 13 for 48 h at 50°C followed by filtering and drying the constant weight. No observable weight loss or decrease in column efficiency occurred in the gels subjected to immersion at pH 2 or 10, indicating extremely high stability in this range. Even after immersion at pH 13, the gel showed a weight loss of only 1% and a very slight decrease in retention volumes, which may be attributable too a small loss of its C_{18} groups.

Influence of pH on peptide elution

Chromatographic characteristics on the ODP column with eluents of pH 3–10 were investigated for six acidic, neutral and basic oligopeptides with the isocratic points p*I*[6] and hydrophobicities, as calculated by the method of Okuyama and Sasaga[7] from Rekker's hydrophobic fragmental constants[6] shown in Table I.

The results for the two acidic peptides are shown in Fig. 2. The increase in the Phe–Leu–Glu–Glu–Ile capacity factor (k') with decrease in pH may be attributable to a corresponding increase in its hydrophobicity. This is in accord with the known tendency for the charge carried by acidic peptides, having isoelectric points at about

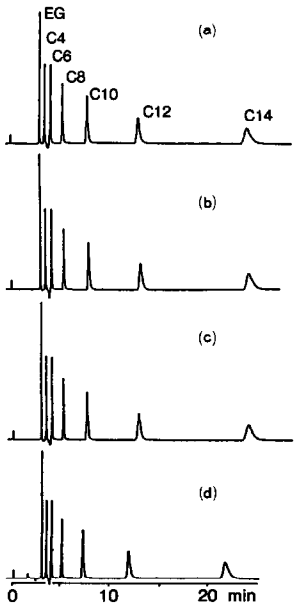

Fig. 1. Chromatograms of alkyl alcohols with columns packed with ODP gel before and after immersion in acidic and alkaline solutions. Columns packed with: (a) untreated ODP gel; (b) ODP gel after immersion in solution of pH 2; (c) ODP gel after immersion in solution of pH 10; (d) ODP gel after immersion in solution of pH 13. Eluent, methanol–water (80:20); flow-rate, 1.0 ml/min; temperature, 30°C.

TABLE I

PEPTIDES AND THEIR PARAMETERS

Type	*Peptide*	*pI*[a]	$\sum f$[b]
Acidic	Phe–Leu–Glu–Glu–Ile	3.12	6.08
	Glu–Gly–Phe	3.29	2.17
Neutral	Leu–Trp–Met	5.96	5.38
	Leu–enkephalin; Tyr–Gly–Gly–Phe–Leu	5.90	5.93
Basic	Leu–Trp–Met–Arg	11.05	6.08
	Des-Arg^9-Bradykinin; Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg	11.05	6.08

[a] Isoelectric point.
[b] Sum of Rekker's hydrophobic constants.

pH 3, to decrease with increasing solution acidity, resulting in increased hydrophobicity. On the other hand, this effect was apparently not sufficient to result in observable changes in the retention of Glu–Gly–Phe, which is characterized by very low hydrophobicity, and its capacity factor was close to zero throughout the pH range 3–10.

The results for the two neutral peptides are shown in Fig. 3. For Leu–Trp–Met, the capacity factor was fairly high at pH 8 but decreased rapidly on both sides of this peak, indicating that an increasing positive or negative charge and thus decreasing hydrophobicity may be expected with any increasing departure from approximately neutral eluent pH.

For Leu–enkephalin, despite its similarity to Leu–Trp–Met in both isoelectric point and hydrophobicity, the capacity factor was nearly constant throughout the pH range 3–8, suggesting that this peptide inherently is capable of only a weak hydrophobic interaction with the octadecyl groups of the ODP gel, for reasons which remain unclear.

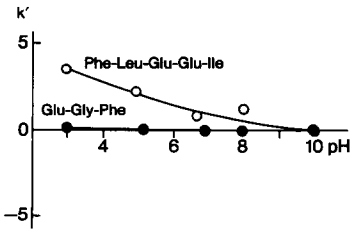

Fig. 2. Influence of pH on capacity factor (k') of acidic peptides. Samples: ○ = Phe–Leu–Glu–Glu–Ile; ● = Glu–Gly–Phe. Column, Asahipak ODP-50; Eluent, 50 m*M* ammonium acetate buffer–acetonitrile (85:15); flow-rate, 1.0 ml/min; temperature, 30°C.

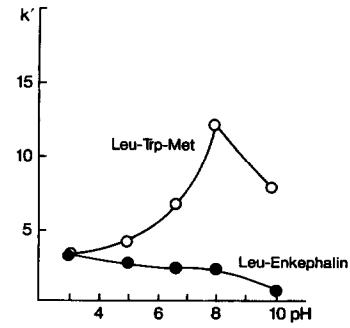

Fig. 3. Influence of pH on capacity factor (k') of neutral peptides. Samples: ○ = Leu–Trp–Met; ● = Leu–enkephalin. Column and conditions as in Fig. 2.

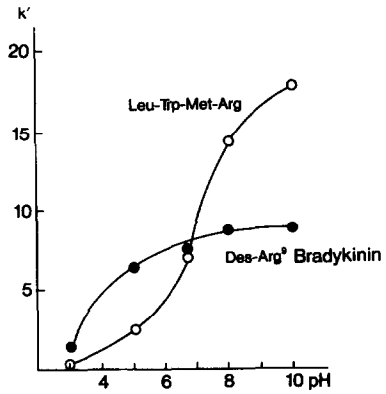

Fig. 4. Influence of pH on capacity factor (k') of basic peptides. Samples: ○ = Leu–Trp–Met–Art; ● = Des–Arg^9–Bradykinin. Column and conditions as in Fig. 2.

As shown in Fig. 4, the capacity factor for both basic peptides on the ODP column showed a clear tendency to increase with increasing eluent pH. This is in accord with the known tendency for the positive charge carried by basic peptides, with isoelectric points at about pH 11 to decrease with increasing solution pH, and for this decrease to result in increased hydrophobicity. On the other hand, markedly higher k' values were observed in the high pH region for Leu–Trp–Met–Arg than for Des–Arg^9–Bradykinin, despite their similarity in both pI and hydrophobicity values. The reason remains unclear, although it is presumably related to a corresponding difference in charge-carrying capacity.

The results show distinctive differences among the six oligopeptides in the susceptibility of their chromatographic behaviour to changes in eluent pH, and thus suggest the possibility that peptides which cannot be separated with acidic eluents might be amenable to separation with neutral or alkaline eluents. The behaviours that were observed are largely in accord with the presumption of hydrophobic interaction with the ODP gel as the main separation mechanism, but other mechanisms might also be responsible.

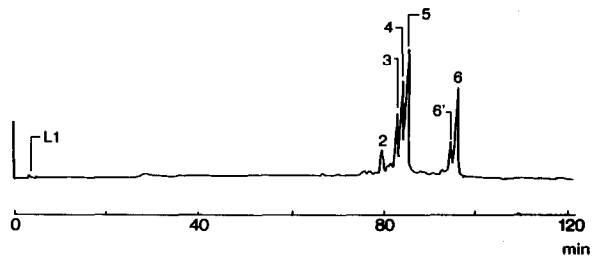

Fig. 5. Separation of ammonium hydrogencarbonate (pH 8.0)-soluble lysilendopeptidase peptides of Rcm-P-CP. Column, Asahipak ODP-50. Eluent: A, 50 mM ammonium hydrogencarbonate (pH 8.0); B, 50 mM ammonium hydrogencarbonate (pH 8.0)–acetonitrile (20:80); linear gradient from A to B in 160 min. Flow-rate, 1.0 ml/min; detection, UV at 230 nm; temperature, ambient. The amino acid composition of peak L-6' was the same as that of peak L-6 but with methionine sulphoxide in place of methionine.

L1 : Ala-Ala-Gly-Thr-Ala-Lys
L2 : Leu-Ser-Ala ·····Asn-Gly-Phe
L3 : Leu-Trp-Ala ·····Glu-Ser-Lys
L4 : Ser-Gly-Asn ·····Ile-Ala-Lys
L5 : Val-Ile-Asn ·····Asp-Gly-Lys
L6 : Gly-His-Asn ·····Val-Ala-Lys
L7 : Pro-Ser-Trp ·····Asp-Val-Lys

Fig. 6. Amino acid sequence of N- and C-terminal regions of the lysilendopeptidase peptides of Rcm-P-CP, as determined by Edman degradation and carboxypeptidase digestion.

Analysis of peptides derived from carboxyl methylated proteinase of psuedomonas sp. 101

The effectiveness of the ODP column for peptide analysis with neutral or alkaline eluents was investigated by attempting its application to the determination of the amino acid sequence of carboxylproteinase of *Pseudomonas* sp. 101.

The proteinase was first reduced and S-carboxymethylated, and the resulting Rcm-P-CP was digested with lysilendopeptidase. The resulting mixture of lysilendopeptidase (LEP) peptides was freeze-dried and then placed in ammonium hydrogencarbonate (pH 8.0). The dissolved fraction was analysed on the ODP column with 50 m*M* ammonium hydrogencarbonate (pH 8.0)–acetonitrile as eluent, yielding the seven peaks shown in Fig. 5, representing six peptides. The precipitate was dissolved in 50% acetic acid and then analysed on the ODP column with 0.05% trifluoroacetic acid (TFA)–acetonitrile as eluent, yielding a single peak. The eluates of all seven peptides were fractioned, and the amino acid sequences of their N- and C-terminal regions were determined. As shown in Fig. 6, six of these contained a C-terminal lysine residue and one contained a C-terminal phenylalanine residue.

To permit the determination of the alignment of these LEP peptides by overlapping, Rcm-P-CP was digested with chymotrypsin and the chymotryptic peptides were analysed on an ODS column with 0.05% TFA–acetonitrile as eluent (Fig. 7). The eluates of eight peaks were determined by amino acid analysis to contain lysine residues, and were then analysed on the ODP column with ammonium hydrogencarbonate (pH 8.0)–acetonitrile. Peaks C-1 and C-2 in Fig. 7 were thus each further separated into six peaks (Fig. 8A and B, respectively). Amino acid analyses revealed that in both instances the eluate of peak f contained lysine. Each of the other seven peaks eluted from the ODS column similarly yielded several peaks on the ODP col-

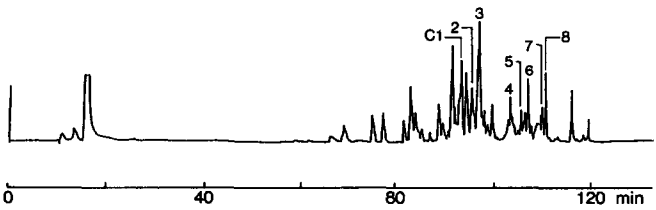

Fig. 7. Separation of chymotryptic peptides of Rcm-P-CP. Column, YMC A-324. Eluents: A, 0.05% TFA; B, 0.05% TFA–acetonitrile (20:80); linear gradient from A to B in 160 min. Flow-rate, 1.5 ml/min. Other conditions as in Fig. 5. Numbered peaks contained lysine residues.

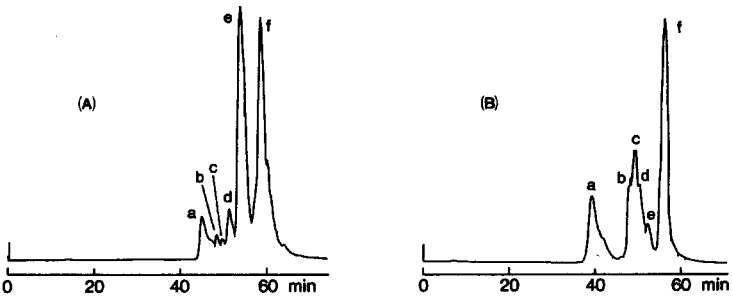

Fig. 8. Separation of two lysine-containing fractions after YMC A-324 chromatography. Column Asahipak ODP-50. Eluents: A, 50 m*M* ammonium hydrogencarbonate (pH 8.0); B, 50 m*M* ammonium hydrogencarbonate (pH 8.0)–acetonitrile (40:60); linear gradient from A to B in 60 min. Flow-rate, 0.5 ml/min. Other conditions as in Fig. 5. (A) Fraction C-1, lysine residue in peak f eluate. (B) Fraction C-2, lysine residue in peak f eluate.

umn, thus resulting in the recovery of a pure lysine–peptide fraction. The amino acid sequences of these lysine–peptides were determined by Edman degradation and carboxypeptidase digestion to be as shown in Fig. 9.

Overlapping of the sequences of the lysine–peptides and those of the C- and N-terminal regions of the LEP peptides was then performed, and indicated the alignment of the LEP peptides to be as shown in Fig. 10.

CONCLUSION

The ODP gel exhibits excellent chemical stability, making it amenable to solutions of alkaline pH and thus permitting its application with alkaline eluents to the analysis of peptides that are insoluble in acidic solvents and therefore cannot be effectively analysed on ODS columns.

Peptides, as amphoteric electrolytes, carry amounts of charge that vary with solution pH, resulting in a corresponding variation in their hydrophobic interaction with the ODP gel and thus in a chromatographic behaviour on the ODP gel which correlates with the eluent pH.

C1 : Ser-Ala-Ile-Ser-Ser-Thr-Pro-Ser-Leu-Val-His-Asp-Val-Lys-Ser-Gly-Asn-Asn-Gly-Tyr
C2 : Ala-Ala-Gly-Thr-Ala-Lys-Gly-His-Asn-Pro-Thr-Glu-Phe-Pro-Thr-Ile-Tyr
C3 : Gly-Ser-Leu-Asp-Ile-Ala-Lys-Leu
C4 : Ser-Asn-Glu-Thr-Val-Trp-Asn-Glu-Gly-Leu-Asp-Ser-Asp-Gly-Lys-Leu-Trp
C5 : Glu-Ser-Lys-Pro-Ser-Trp-Gln-Ser-Val-
C6 : Glu-Ser-Lys-Pro-Ser-Trp-Gln-Ser-Val-
C7 : Asn-Gln-Ala-Val-Ser-Asp-Asn-Val-Ala-Lys-Val-Ile-Asn-Val-Ser-Leu-
C8 : Asn-Gln-Ala-Val-Ser-Asp-Asn-Val-Ala-Lys-Val-Ile-Asn-Val-Ser-Leu-

Fig. 9. Amino acid sequence of chymotryptic peptides of Rcm-P-CP, as determined by Edman degradation and carboxypeptidase digestion.

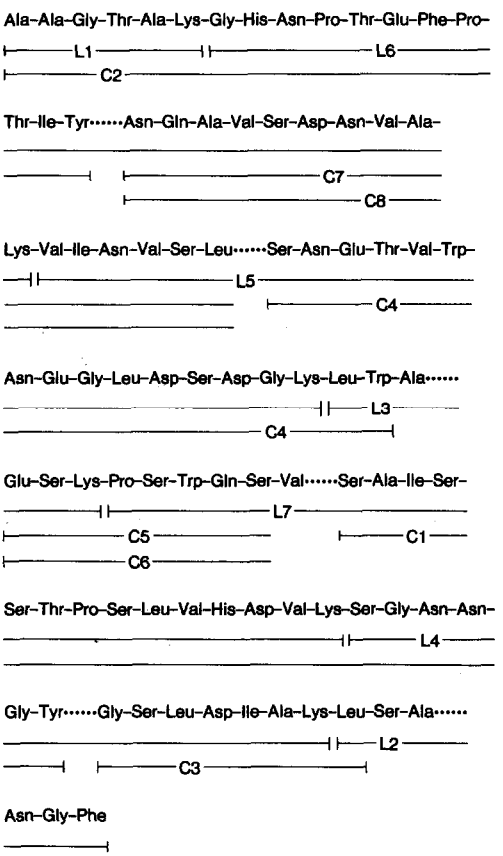

Fig. 10. Alignment found for lysilendopeptidase peptides.

As shown here by the analysis and isolation of the peptides which facilitated alignment of the LEP peptides derived from Rcm-P-CP, efficient separation can be obtained on the ODP column using both alkaline and acidic eluents, even for mixtures that cannot be effectively separated on ODS columns because of their incompatibility with alkaline solutions.

The ODP column will provide a practical, effective tool for a widely increased range of peptides analysis.

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