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High-performance liquid chromatography of peptides on a microspherical carbon column

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Abstract

The properties of a microspherical carbon column for the separation of peptides and proteins are described. One hundred and thirty-three peptides, with from 1 to 148 amino acid residues, were applied on a carbon column and eluted with a linear gradient of acetonitrile [10–70% (v/v), 30 min] in 0.1% (v/v) trifluoroacetic acid solution. The elution behaviour of the peptides on the carbon column and on an octadecylsilica (ODS) column were compared under the same elution conditions. Relationships between the logarithm of the hydrophobicity of peptides and their capacity factors were approximately linear on both columns, but some exceptions were observed with peptides with aromatic residues, indicating stronger adsorption on the carbon than on the ODS column. These results suggest that the major factor for the separation of peptides on both the carbon and ODS columns is hydrophobic interactions. The stronger adsorption of aromatic side-chains in peptides on the carbon column could be understood by an interaction based on the aromatic or graphitic nature of the surface of the microspherical carbon packings.

Keywords: Stationary phases, LC; Peptides

1. Introduction

Recently, chemically bonded alkylsilica and polymer-based packings have been improved remarkably and are now being increasingly applied in the biochemical field, e.g., for the separation of polypeptides [1–6]. Silica-based packings have good mechanical strength and possibility of various surface modifications and result in good peak shapes and high resolution in peptide separations. However, silica-based packing materials have still several demerits from a practical viewpoint, such as instability under the alkaline conditions.

The application of carbon adsorbents in HPLC, owing to their high chemical, thermal and pH stability, could give another possibility for non-polar, inexpensive stationary phases. It is easy to clean its accumulated surface compounds by washing with an alkaline solution, which is usually a good solvent for peptide and protein elution and for the sterilization of the column at high temperature.

Many different carbonaceous packings for HPLC purposes have been introduced into the preparation methods, but the structural characterization and the chromatographic performance still remain to be studied [7–18]. However,

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judging from its development, this kind of packing has progressed [10,12] with regard to suitable particle size distributions, effective pore-size range and surface properties. The graphitic surface nature of carbon packings has a similar selectivity to aryl polymer packings but different to that of alkyl-bonded silica.

In previous papers [19–22], microspherical carbon was tested as an HPLC packing for the separation of aromatic amino acids and proved to be useful for peptide separations. In this work, its chromatographic behaviour for the separation of polypeptides was studied in relation to the proteolytic hydrolysate of proteins. Also, its performance was compared with that of octadecylsilica.

2. Experimental

2.1. Proteins and peptides

Calmodulin was purified from bovine brain soluble extracts by ammonium sulfate fractionation followed by column chromatographic procedures [23]. Bence Jones proteins of the κ type, NIG-26 and NIG-96 [24], and λ type, NIG-95, NIG-204, NIG-211 and NIG-250, were purified from urine from patients with multiple myeloma. Lysyl endopeptidase (EC 3.4.21.50) was purchased from Wako (Osaka, Japan), trypsin (EC 3.4.21.4) treated with L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK) from Cooper Biomedical (Malvern, USA), horse heart myoglobin from Sigma (St. Louis, MO, USA) and hen egg-white lysozyme, crystallized six times (EC 3.2.1.17) from Seikagaku Kogyo (Tokyo, Japan). Angiotensin I, angiotensin II, substance P and mastoparan were purchased from the Protein Research Foundation (Osaka, Japan). Some peptides, listed in Table 1, were obtained by the enzymic degradation of calmodulin, Bence Jones proteins, lysozyme and other proteins that had already been sequenced. Calreticulin fragments were the peptides purified for sequencing [25].

2.2. Preparation of enzymic hydrolysate of proteins

Lysozyme and myoglobin were dissolved in 50 m*M* Tris–HCl (pH 8.0) at 10 mg/ml and heated on a boiling water-bath for 10 min. The protein solutions were cooled on ice and then subjected to digestion with TPCK-treated trypsin. Calmodulin was dissolved in 100 m*M* ammonium hydrogencarbonate (pH 8.0) at 10 mg/ml. Tryptic digestion was performed for 6 h at 37°C at an enzyme-to-substrate ratio of 1:100 (w/w).

Bence Jones proteins were subjected to reduction and pyridylethylation. Digestion of the pyridylethylated Bence Jones proteins (1.0 mg) with lysyl endopeptidase was performed in 500 μl of 50 m*M* Tris–HCl buffer (pH 9.0) containing 2 *M* urea at 37°C for 2 h at an enzyme-to-substrate ratio to 1:50 (w/w). The hydrolysate was separated into several portions and stored frozen (−20°C). Before use, the stored hydrolysate portion was dissolved 2- or 50-fold in distilled water. The hydrolysate of the Bence Jones proteins was loaded on the column without dilution.

2.3. Materials for chromatography

2.3.1. Column 1

Carbonex porous microspherical carbon beads (average particle size, 3.5 μm; specific surface area, >30 m^2/g; specific pore volume, 0.35 ml/g; apparent density, 0.57 g/ml; pore-size range, 10–700 Å) were obtained from Biotech Research (Saitama, Japan). This packing material was made into a slurry in 30% isopropyl alcohol in water and packed into a stainless-steel column (100 mm × 4.6 mm I.D.) at a constant pressure of 100 kgf/cm^2. Under these conditions, the hold-up time (t_0) was determined with acetone.

2.3.2. Column 2

Carbonex was packed into short stainless-steel columns (10 mm × 4.6 mm I.D.). The conditions for column packing and the specification of the carbon packing were same as for column 1.

2.3.3. Column 3

An ODS column, LiChrospher 100 RP-18e (250 mm × 4.0 mm I.D., particle size 5 μm), was obtained from Merck (Darmstadt, Germany).

2.3.4. Column 4

An ODS column, Superspher 100 RP-18e (25 mm × 4.0 mm I.D., particle size 3.5 μm), was obtained from Merck.

2.3.5. Solvent

Acetonitrile (chromatography grade) was obtained from Merck. Water was purified by passing through a mixed-bed ion-exchange resin followed with distillation (Model WG-25 system; Yamato Scientific, Tokyo, Japan), and before use the deionized, distilled water was purified further with an ultra-pure water system (Milli-Q SPTOC; Millipore, Milford, MA, USA). Trifluoroacetic acid (TFA; sequencial grade) and isopropyl alcohol (chromatography grade) and other chemicals of analytical-reagent grade were obtained from Wako, unless mentioned otherwise.

2.4. Apparatus and standard chromatographic procedure

2.4.1. Conditions 1

In the standard procedure, a Yokogawa (Tokyo, Japan) LC-100 HPLC system controlled by a PC-9801 microcomputer (NEC, Tokyo, Japan) was employed. For automatic processing, this system was connected to an autosample injector (Model 231/401; Gilson, Villiers-le-Bel, France). The long columns (1 and 3) were used in this system. Sample peptides and proteins were applied to the column and eluted with a linear 30-min gradient from 10% to 70% acetonitrile in 0.1% aqueous trifluoroacetic acid at a flow-rate of 1.0 ml/min, with absorbance detection at 210 nm. The operating temperature was room temperature [19–21].

2.4.2. Conditions 2

For the peptide mapping of the tryptic hydrolysate of some proteins, a Yokogawa–Hewlett-Packard (Waldbronn, Germany) HP-1090 system with a short carbon column and an ODS column (columns 2 and column 4) was employed. Sample peptides and proteins were applied to the column and eluted with a linear 30-min gradient from 5% to 70% acetonitrile in 0.1% aqueous trifluoroacetic acid at a flow-rate of 1.0 ml/min, with UV diode-array detection at 210 and 280 nm. A column temperature of 40°C was used [21].

When necessary, the effluent was collected and the solvent in aliquots of combined fractions was removed under a nitrogen stream. The residue was dissolved in water and used for amino acid composition analysis or mass spectrometric analysis.

2.4. Identification of peptides

Identifications of peptides were performed by mass spectrometry, that is, based on the molecular mass, by matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI TOFMS) (Vision 2000 instrument; Finnigan MAT, San Jose, CA, USA) [26,27], and by amino acid composition analysis, which was performed using the following procedure. Peptides and proteins were hydrolysed with 6 *M* HCl containing 5% (v/v) phenol at 110°C for 24 h in evacuated sealed tubes. The amino acid composition of peptides and proteins were determined using a high-performance amino acid analyzer (Model 800; Jasco, Tokyo, Japan) equipped with a postcolumn fluorescent derivatization system with *o*-phthalaldehyde [6].

3. Results and discussion

3.1. Chromatograms of tryptic hydrolysate on a microspherical carbon column

The stored hydrolysate of calmodulin solution (10 mg/ml) was diluted 2.7-fold with distilled water and 10 μl of the diluted solution were loaded on the column. The elution pattern was recorded at 210 nm. One of the typical elution

patterns is shown in Fig. 1a. The peaks separated on the carbon column were further analysed on the ODS column under conditions 1 and 2 elution systems (Fig. 4b). The purities of the peaks were checked by rechromatography on the ODS column and, in some cases, analyses by mass spectrometry and amino acid composition analysis were performed. Most of the peaks were single component, and directly available for the sequence analysis of peptides.

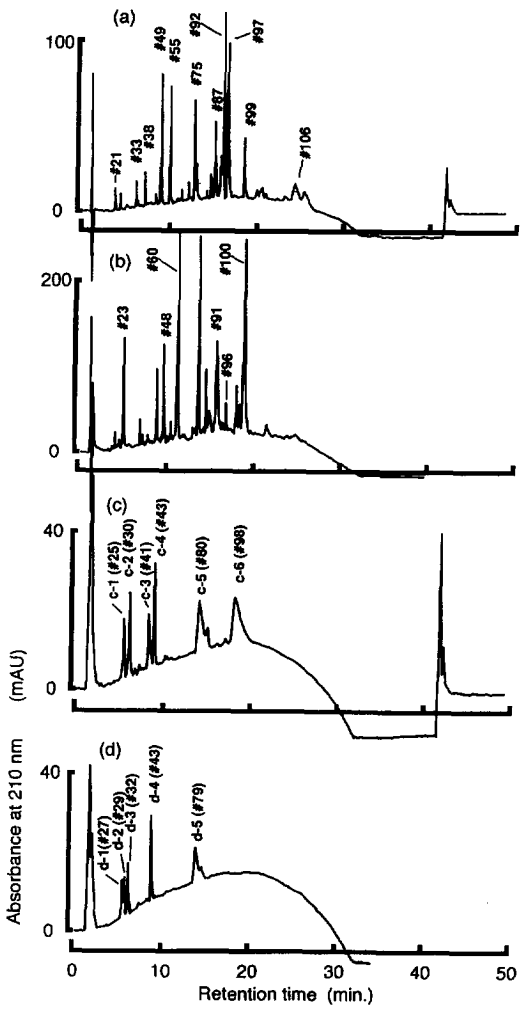

Fig. 1. Typical chromatograms of tryptic hydrolysate obtained on a microspherical carbon column. (a) Tryptic hydrolysate of calmodulin (about 37 μg); (b) tryptic hydrolysate of lysozyme (about 100 μg); (c) hydrolysate of NIG204, digested with lysyl endopeptidase (about 20 μg); (d) hydrolysate of NIG250, digested with lysyl endopeptidase (about 12 μg). These peptides were applied to a carbon column and eluted with a 30 min linear gradient of acetonitrile (10–70%, v/v) in 0.1% TFA at a flow-rate of 1.0 ml/min at room temperature. Peptide numbers as in Table 1.

Trypsin hydrolysate of hen egg-while lysozyme (100 μg) was processed as above and the chromatogram is shown in Fig. 1b. The peptide numbers indicated in Fig. 1 are as those given in Table 1.

The lysyl endopeptidase hydrolysate of λ-type Bence Jones proteins (NIG 204, 20 μg; NIG 250, 12 μg) were also processed as above and the chromatograms obtained are shown in Fig. 1c and d.

Bence Jones protein is known as an immunoglobulin light chain which is secreted in the urine of patients with myeloma-associated systemic amyloidosis and have two domains—variable domain and constant domain [30]. Hence the chromatograms of Bence Jones proteins showed common peaks which were derived from the constant region.

In these experiments, peak c-4 (Fig. 1c) and peak d-4 (Fig. 1d) correspond to each other and come from the constant region, and the sequence is identical for the amino acid sequence T-V-A-P-T-E-C-S, and they consequently eluted at the same position on the chromatograms. The peptide of peak c-6 (Fig. 1c) belongs to the variable region, so this peptide does not occur in Fig. 1d. These results were useful for the convenient typing of Bence Jones proteins.

3.2. Capacity factors of various peptides

In the standard gradient system, the capacity factors (k') of 133 peptides on a carbon column under conditions 1 were calculated and are given in Table 1 with their hydrophobicities, which were calculated based on the Sasagawa et al.'s fragmental constant [28] which was modified from Rekker's hydrophobic fragmental constant [29], and some of the fragments that were not shown by Sasagawa et al. were calculated by Yamaki et al. [21]. The hold-up time was estimated from the chromatogram of acetone as 1.13 min. Most peptides were eluted under conditions 1, but some of the peptides which contains

Table 1
Capacity factors (k') of various peptides

No.	Peptide (sequence)	k'	H[b]	Ln H[c]	Tentative name[d]
1	GGG	0.00	0.66	−0.42	Glycylglycylglycine
2	GGGG	0.00	0.88	−0.13	Glycylglycylglycylglycine
3	GA	0.00	0.35	−1.05	Glycylalanine
4	GD	0.00	0.32	−1.14	Glycylaspartic acid
5	C	0.00	0.00	−[e]	Cysteine
6	GG	0.00	0.44	−0.82	Glycylglycine
7	GM	0.00	1.07	0.07	Glycylmethionine
8	LI	0.20	2.72	1.00	Leucylisoleucine
9	AS	0.20	0.31	−1.17	Alanylserine
10	GV	0.25	0.60	−0.51	Glycyl-N-valine
11	LG	0.30	1.56	0.44	Leucylglycine
12	APK	0.30	0.66	−0.42	NIG96 L43-45
13	PGK	0.30	0.75	−0.29	NIG96 L40-42
14	HK	0.30	0.39	−0.94	NIG96 L189-190
15	PSK	0.35	0.71	−0.34	NIG250 L165-167
16	QSNNK	0.35	−0.31	−[e]	NIG250 L168-172
17	AV	0.36	0.51	−0.67	Alanylvaline
18	Ac[a]-Q	0.51	1.17	0.16	N-Acetylglutamine
19	C	0.69	2.90	1.06	Pyridylethylcysteine
20	PF	1.68	2.19	0.78	Angiotensin 2 H7-8
21	MK	2.54	0.90	−0.11	Calmodulin T76-77
22	VDIK	2.92	1.91	0.65	NIG96 L104-107
23	TPGSR	3.26	1.26	0.23	Lysozyme T69-73
24	HVAE	3.54	1.12	0.11	UK V2a
25	ADSSPVK	3.91	1.50	0.41	NIG204 L154-160
26	F	4.14	1.71	0.54	Phenylalanine
27	AGVETTK	4.15	1.29	0.25	NIG250 L158-164
28	KNSISPE	4.30	2.09	0.74	UK V9
29	ADGSPVK	4.38	1.54	0.43	NIG250 L151-157
30	AGVETTTPSK	4.55	2.07	0.73	NIG204 L161-170
31	C[a]DDKE	4.62	3.42	1.23	UK V2b
32	VTALSQPK	4.78	3.04	1.11	NIG250 L104-111
33	DGDGTITTK	4.87	2.43	0.89	Calmodulin T22-30
34	HASLEKPKDE	5.08	3.21	1.17	UK V12a
35	Y	5.13	1.23	0.21	Tyrosine
36	GF	5.36	1.93	0.66	Glycylphenylalanine
37	C[a]TVR	5.37	3.66	1.30	UK T8
38	VFDK	5.86	2.24	0.81	Calmodulin T91-94
39	MDC[a]D	6.30	3.95	1.37	UK V4
40	TFKRD	6.50	2.24	0.81	UK V6
41	LTVLRQPK	6.72	4.33	1.47	NIG204 L107-114
42	GDLEC[a]DDKEAK	7.18	5.53	1.71	UK T17
43	TVAPTEC[a]S	7.33	4.58	1.52	NIG211 L206-213
44	YINEHK	7.36	2.82	1.04	UK T10
45	ADYEK	7.42	1.78	0.58	NIG96 L184-188
46	LTVLGQPK	7.47	4.29	1.46	NIG211 L104-111
47	KC[a]TVRQQHNHAVE	7.50	5.44	1.69	UK V12c
48	VFGR	7.72	2.59	0.95	Lysozyme T2-5
49	EAFR	7.74	2.37	0.86	Calmodulin T87-90
50	YVLNKHNE	8.14	2.71	1.00	UK V16a
51	RVY	8.31	1.87	0.63	Angiotensin2 H2-4
52	LY	8.40	2.57	0.94	Leucyltyrosine
53	QQHNHAVEMDC[a]DAK	8.52	5.86	1.77	UK T22
54	DGHAHSHLIQQHIEK	8.71	7.13	1.96	UK T27
55	ELGTVMR	8.74	3.44	1.24	Calmodulin T31-37
56	VDNALQSGNSQESVTEQDSK	8.87	4.26	1.45	NIG96 L150-169

(*Continued on p. 148*)

Table 1 (*continued*)

No.	Peptide (sequence)	k'	H^b	Ln H^c	Tentative name[d]
57	VPVVFVKKE	9.05	4.08	1.41	UK V24a
58	VKDGHAHSHLIQQHIE	9.10	7.51	2.02	UK V22g
59	VFVKC[a]HSTPDSVE	9.67	7.47	2.01	UK V19c
60	HGLDNYR	9.73	3.04	1.11	Lysozyme T15-21
61	ISRGQHKYEPE	9.96	5.04	1.62	UK V14b
62	SDC[a]VILD	9.96	6.38	1.85	UK V22c
63	HVLFGGGTK	9.97	4.60	1.53	NIG211 L95-103
64	C[a]HSTPDSVENVR	10.04	5.14	1.64	UK T24
65	C[a]HVLDPTPVE	10.17	6.79	1.92	UK V22d
66	C[a]HPYTAGE	10.33	5.69	1.74	UK V11
67	KLSGHIYE	10.41	5.01	1.61	UK V19b
68	TWGVTKAAELQ	10.43	4.17	1.43	Calreticulin V329-339
69	DHIAFC[a]RSE	10.61	7.00	1.95	UK V19d
70	VQWK	11.23	3.13	1.14	NIG96 L146-149
71	AVRYINE	11.33	3.20	1.16	UK V17
72	FESDC[a]VILPVK	11.52	9.17	2.22	UK T36
73	LPTDISDHHTTPVKGC[a]PGKVHHFE	11.67	12.44	2.52	UK V25
74	DSTYSLSSTLTLSK	11.76	6.66	1.90	NIG96 L170-183
75	DTDSEEEIR	11.83	2.95	1.08	Calmodulin T78-86
76	GQTLVVQFTVK	11.94	5.42	1.69	Calreticulin L71-81
77	SFNRGEC[a]	11.94	5.09	1.63	NIG96 L208-214
78	W	12.23	2.34	0.85	Tryptophan
79	ANPTVTLFPPSSEELQANK	12.35	7.12	1.96	NIG250 L112-130
80	AAPSVTLFPPSSEELQANK	12.37	7.76	2.05	NIG211 L112-130
81	TWGVTKAAE	12.38	3.76	1.32	Calreticulin V329-337
82	VHVIFNYK	12.65	5.02	1.61	Calreticulin L127-134
83	GW	12.90	2.56	0.94	Glycyl tryptophan
84	INLKALAALAKKIK-NH_2	13.08	7.78	2.05	Mastoparan
85	AKNWADD	13.16	2.40	0.88	UK V14a
86	IHPF	13.30	3.91	1.36	Angiotensin2 H5-8
87	HVMTNLGEK[a]LTDEEVDEM	13.89	5.39	1.68	Calmodulin T107-124
88	WKPRQIDNPE	14.07	5.27	1.66	Calreticulin V258-267
89	VYAC[a]EVTHQGLSSPVTK	14.24	9.06	2.20	NIG96 L191-207
90	DPTVYFK	14.69	4.07	1.40	Calreticulin L1-7
91	NTDGSTDYGILQINSR	14.82	6.29	1.84	Lysozyme T46-61
92	EAFSLFDKDGDGTITTK	14.82	7.92	2.07	Calmodulin T14-30
93	DRVYIHPFHL	15.05	7.56	2.02	Angiotensin 1
94	HHQEHPYTAGE	15.29	4.10	1.41	UK V14c
95	VKIDNSQVE	15.32	2.65	0.97	Calreticulin V167-175
96	NTDGSTDYGILQIN	15.41	5.85	1.77	Lysozyme T46-59
97	Ac[a]-ADQLTEEQIAEFK	15.46	7.30	1.99	Calmodulin T1-13
98	LLISDNYNRPSGVPARFSGSK	16.30	9.40	2.24	NIG204 L47-67
99	HVMTNLGEK[a]LTDEEVDEMIR	17.31	7.03	1.95	Calmodulin T107-126
100	GTDVQAWIR	17.62	5.29	1.67	Lysozyme T117-125
101	RPKPQQFFGLM-NH_2	18.25	7.26	1.98	Substance P
102	SHRSYSC[a]QVTHEGSTVEK	18.49	7.96	2.07	NIG250 L188-205
103	RTVAAPSVFIFPPSDEQLK	18.78	10.12	2.31	NIG96 L108-126
104	DRVYIHPF	19.69	5.88	1.77	Angiotensin 2
105	SGTASVVC[a]LLNNFYPREAK	21.33	10.40	2.34	NIG96 L127-145
106	VFDKDGDGYISAAELR	22.96	7.80	2.05	Calmodulin T91-106
107	RVYIHPF	23.47	5.78	1.75	Angiotensin2 H2-8
108	DWDKPE	–[f]	3.34	1.21	Calreticulin V218-223
109	AYYLE	–	4.20	1.44	UK V22b
110	HEQNIDC[a]GGGYVK	–	7.22	1.98	Calreticulin L82-94
111	AHDDHHQC[a]HPYTAGE	–	7.40	2.00	UK V15c
112	AKNWADDAVRYINE	–	5.60	1.72	UK V31
113	IKDPDAAKPEDWDDRAK	–	6.25	1.83	Calreticulin L191-207
114	DWDDRAKIDDPTDSKPE	–	6.34	1.85	Calreticulin V218-234

Table 1 (*Continued*)

No.	Peptide (sequence)	k'	H[b]	Ln H[c]	Tentative name[d]
115	DGEWEPPVIQNPE	–	6.58	1.88	Calreticulin V221-253
116	HIPDPDAKKPEDWDE	–	6.67	1.90	Calreticulin V224-238
117	YKGEWKPRQIDNPE	–	7.04	1.95	Calreticulin V254-267
118	YKGIWIHPE	–	7.69	2.04	Calreticulin V268-276
119	IDNPEYSPDSNTIYAYE	–	7.74	2.05	Calreticulin V277-292
120	LSGHIYEVLEISR	–	8.77	2.17	UK T40
121	EADIDGDGQVNYEEFVQMMTAK	–	9.03	2.20	Calmodulin T127-148
122	YAASSYLSLTPEQWK	–	9.56	2.26	NIG250 L173-187
123	ATLVC[a]LISDFYPGAVTVAWK	–	15.04	2.71	NIG250 L131-150
124	NFAVLGLDLWQVKSGTIF	–	12.85	2.55	Calreticulin V293-310
125	LILTQPPSVSVAPGETATITC[a]EGDNLGTRNVHWYQQK	–	19.18	2.95	NIG250 L1-37
126	SELTQDPAVSVALGQTVRITC[a]QGDSLRGYDAAWYQQK	–	19.74	2.98	NIG211 L1-37
127	SLGQNPTEAELQDMINEVDADGDGTIDFPEFLTMMAR	–	17.34	2.85	Calmodulin T38-74
128	DIQMTQSPSSLSASVGDRVIITC[a]QASQDISSYLNWWQQK	–	23.58	3.16	NIG96 L1-39
129	PGQAPLLVIYGRNNRPSGIPERFSGSSSGHTASLTITGA QAEDEADYYC[a]NSRDSSGK	–	21.94	3.09	NIG211 L38-94
130	PGQAPVLVLYYDNVRPSGIPERFSGSNSVTAATLAISE VEAGDEADFYC[a]QVWDRAANHFVFGPGTK	–	33.25	3.50	NIG250 L38-103
131	LLIFEASDLETGVPSRFSGSGSGTDFTFTISSLLPEDIAT YYC[a]QQYDNLPFTFGPGTK	–	36.80	3.61	NIG96 L46-103
132	Lysozyme	–	53.71	3.98	Lysozyme
133	Calmodulin	–	65.03	4.17	Calmodulin

[a] K = trimethyllysine; Ac = acetylated; C = pyridylethylated cysteine.
[b] H = hydorphobicity (sum of Sasagawa et al.'s fragmental constant) [28].
[c] Ln H = natural logarithm of hydrophobicity of polypeptide.
[d] Peptides are indicated mainly by their amino acid sequences; some of the peptides which have common names are indicated in the name, and peptides produced by enzymic and hydrochloric acid hydrolysis are indicated by the original protein name-proteinase abbreviation-peptide number from N-terminus. UK = original protein names were unknown; NIG = Bence Jones protein (National Institute for Genetics serial number); V = digested with *Staphyloccus aureus* V8 protease; T = digested with trypsin treated with L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone; L = digested with lysyl endopeptidase; H = digested with 0.1 *M* hydrochloric acid.
[e] Peptides could not be calculated.
[f] Dashes indicate peptides that could not be eluted under standard elution conditions.

several aromatic amino acids were not eluted from the column.

3.3. Relationship between capacity factor and hydrophobicity of peptides on a microspherical carbon column

The relationship between capacity factor (k') and natural logarithm of hydrophobicity is illustrated in Fig. 2. From these results, peptides were classified into three groups. Some of the peptides which could not be retained on the column are classified into group 1. These peptides could not be retained under standard conditions, but could be retained under milder conditions, such as in distilled water. The lysyl endopeptidase hydrolysate peptides of Bence Jones proteins NIG96 and NIG250, peptides 12, 13, 14, 15 and 16 (these peptide numbers are those indicated in Table 1), belong to group 1. However, when the initial eluent was changed to 5% acetonitrile–0.1% TFA, these peptides were adsorbed and eluted with the gradient (Fig. 3).

The degree of correlation between capacity factor and the natural logarithm of hydrophobicity (ln H) for group 2 peptides was approximately linear ($r = 0.70$), and a higher degree of correlation was calculated separately for the peptides without an aromatic residue ($r = 0.94$) than the peptides with an aromatic residue ($r = 0.83$).

The peptides which could not be eluted under standard conditions were classified as group 3, and which could be eluted with the dilute sodium

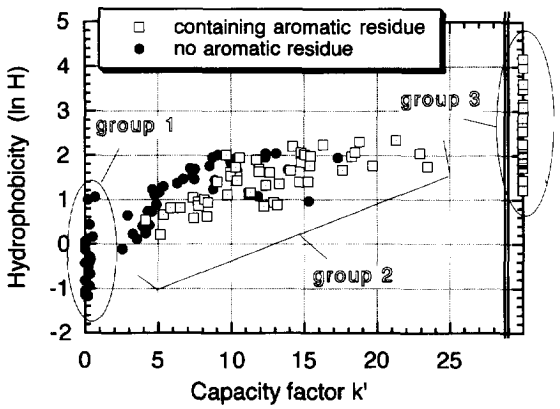

Fig. 2. Relationship between capacity factor (k') and hydrophobicity (ln H) of peptides (numbers as in Table 1) on the carbon column. ● = Peptides not containing any aromatic amino acid residues; □ = peptides containing aromatic amino acid residues. The peptides circled on the right-hand axis (group 3) were not eluted from the carbon column under the standard conditions.

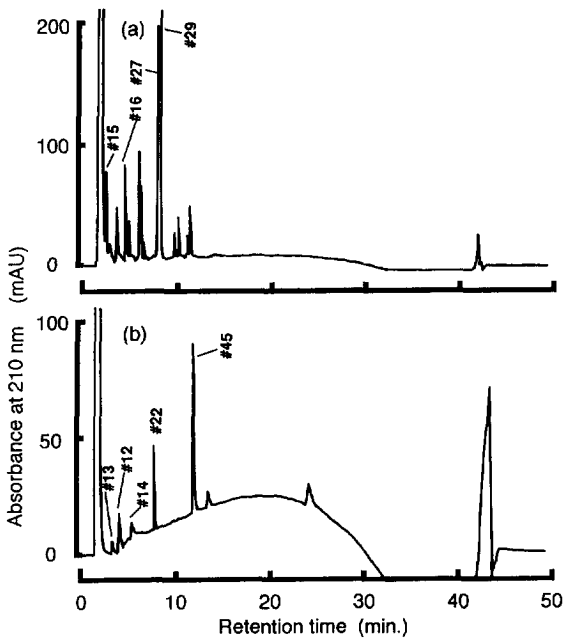

Fig. 3. Elution profiles of oligopeptides using the lower initial concentration of acetonitrile on the carbon column. (a) The pass-through peptides of the NIG96 hydrolysate were collected and concentrated [24] and (b) the pass-through peptides of the NIG250 hydrolysate were processed as in (a) under a nitrogen stream. The residues were dissolved in 100 μl of the initial eluent, and were loaded on the carbon column initiated with 5% acetonitrile–0.1% TFA and then eluted with a 30-min linear gradient of acetonitrile (5–70%, v/v) in 0.1% TFA at a flow-rate of 1.0 ml/min. Peptide numbers as in Table 1.

hydroxide solution or could be analysed further using modified carbon packings [19], remain to be studied in the future.

3.4. Comparison of elution patterns of tryptic peptides of calmodulin on carbon and ODS columns

The stored solution of tryptic hydrolysate of calmodulin was diluted to 50-fold with distilled water. A 40 μl volume of the diluted solution (about 8 μg total peptide) was analysed either on a Carbonex column (10 mm × 4.6 mm I.D.) or on a Superspher 100 RP-18e column (25 mm × 4.0 mm I.D.) with standard gradient elution. The absorbance of the eluate was recorded at 210 and 280 nm. The results are shown in Fig. 4a and b and the corresponding peaks in each part are connected by solid lines. Some of the peptides, e.g., calmodulin T 38-71, which were eluted later on the ODS column could not be eluted on the carbon column. The purified calmodulin was eluted as a single peak at 19.9 min on the ODS column (indicated by an arrow in Fig. 4b). However, on the carbon column, calmodulin was not eluted under above conditions. The peptides which could not be adsorbed on the ODS column could be separated on the carbon column (see Fig. 6). The overall separation pattern looks similar, except for the peptides containing an aromatic group, e.g., calmodulin T 91-106 and T 127-148, which have both phenylalanine and tyrosine residues.

3.5. Relationship of retention behaviour of various peptides on carbon and ODS columns

In Fig. 4a and b, a dependence of retention time on hydrophobicity was observed. Therefore, the retention behaviour on the carbon column was compared with that on the ODS column under conditions 1 (Fig. 5). For comparison, the retention values were expressed by the acetonitrile concentration. The overall trend of the

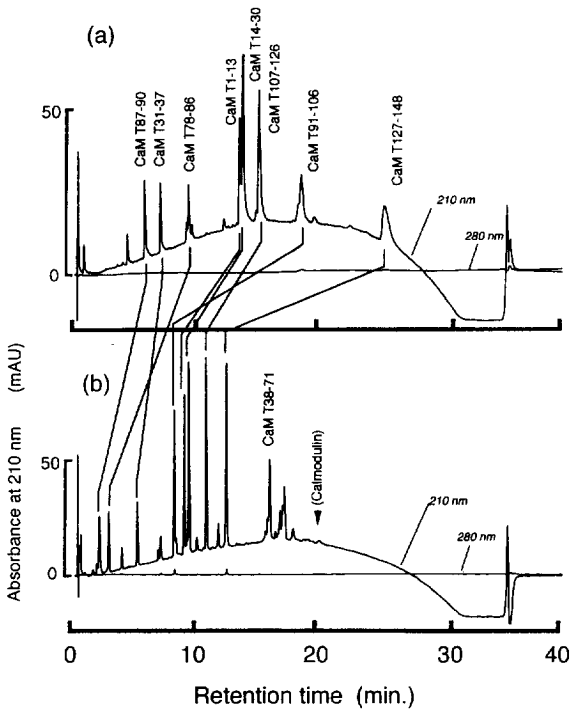

Fig. 4. Comparison of chromatograms of tryptic peptides of calmodulin on carbon and ODS columns. (a) The tryptic hydrolysate of calmodulin (about 8 μg) was applied to the carbon column and the absorbance of the eluate was measured at 210 and 280 nm. (b) The same sample was analysed on the ODS column under the same conditions. The corresponding peaks are connected by solid lines.

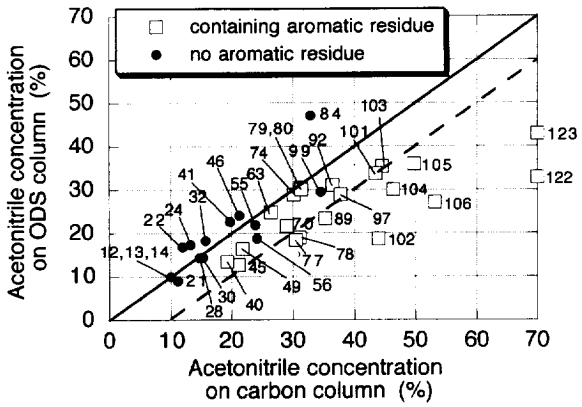

Fig. 5. Relationship between the retention behaviours of various peptides on the carbon ODS columns. Peptide numbers as in Table 1. On the abscissa the retention values of the peptides on the carbon column are indicated as the acetonitrile concentration of the eluate, and on the ordinate the retention values of the respective peptides on the ODS column are indicated as the concentration of acetonitrile in the eluate under conditions 1. ● = Peptides not containing any aromic amino acid residues. The approximate relationship is expressed as the solid line. □ = Peptides containing aromatic amino acid residues. The approximate relationship is expressed as the broken line. Peptides 122 and 123 were not eluted on the carbon column under the standard conditions.

relationship of the peptide retention seems to follow the linear relationship indicated by the solid line. However, the peptides which contain aromatic amino acid residues (Phe, Tyr or Trp) were eluted at higher concentrations of acetonitrile, i.e., about 8–10% higher concentration per aromatic amino acid residue on carbon than on ODS. The relationship is shown by the broken line, which is approximately parallel to the solid line. The differences depend on the number of aromatic residues.

3.6. Chromatograms of some peptides which could not be retained on the ODS column

As indicated in Fig. 5, some of the peptides could not be retained on the ODS column under the standard conditions. Therefore, the pass-through fractions of Bence Jones protein hydrolysate from the ODS column were collected (Fig. 6a) and concentrated under a nitrogen stream, diluted with the initial eluent (5% acetonitrile–0.1% TFA). All of the fractions were loaded on the carbon column and eluted with a linear gradient of acetonitrile [5–70% (v/v), 30 min] in 0.1% (v/v) TFA, and the chromatogram is shown in Fig. 6b. There were three peptides, b-1, b-2, b-3 (Nos. 12, 14 and 45 in Table 1), for which the sequences were determined.

4. Conclusion

In a previous paper [22], it was reported that free amino acids, except aromatic amino acids, were not adsorbed on this carbon column. From the present results, the majority of peptides could be retained on this carbon column, but the large peptides could not be eluted satisfactorily

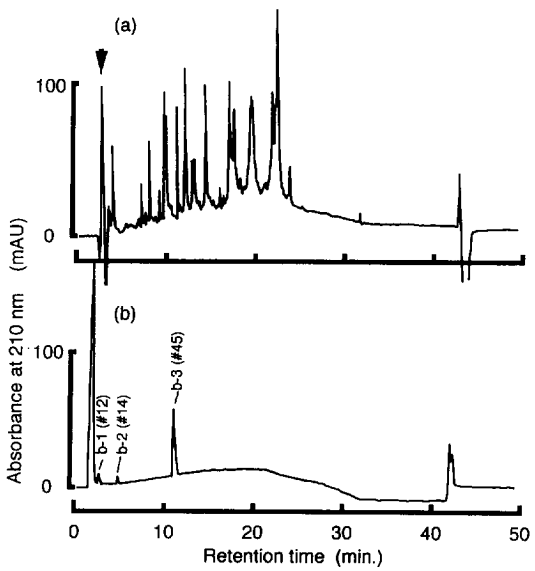

Fig. 6. Typical elution pattern on the carbon column of some peptides which could not be retained on the ODS column. (a) The hydrolysate of Bence Jones protein NIG96 (140 μg) was loaded on the ODS column (LiChrospher 100 RP-18e; 250 mm × 4.0 mm I.D.) and eluted with a 30-min linear gradient of acetonitrile (5–70%, v/v) in 0.1% TFA at a flow-rate of 1.0 ml/min. (b) The pass-through fraction [peak marked with an arrow in (a)] was loaded on the carbon column. Sample preparation methods as in Fig. 3. Chromatographic conditions are as in (a). Peptide numbers as in Table 1. The pass-through fraction from the carbon column contained free lysine.

under standard conditions. This suggests some applicability to protein chemistry or food chemistry, since it is known that some of the functional oligopeptides contain aromic amino acid residues, and sometimes the separations of the peptides in the low acetonitrile concentration range were better than on the ODS column. The carbon column is advantageous in this area and sterilization with warm alkaline solution would be possible.

The adsorption of each peptide, especially those containing aromatic amino acid residues, was stronger on the carbon column than on the ODS column. This may be explained by the structural specificity of the carbon column, which is composed from graphite or mesophase carbon. These properties may vary depending on the carbon products from the various commercial sources, and this aspect remains to be investigated.

This process would also be applicable to large-scale chromatography in the food industry of oligopeptides, glycopeptides and/or carbohydrates [17,18].

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