TOSOH

SEPARATION REPORT NO. 111

Reversed Phase Chromatography Column for Biomolecule Separation TSKgel Protein C4-300

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1. Introduction

Recently, with the robust development of biopharmaceuticals in which proteins and peptides, etc., are used, there is an increasing need for analytical techniques capable of uncovering the characteristics of these products. Separation methods frequently used in liquid chromatography for proteins and peptides include size-exclusion chromatography (SEC), ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), as well as reversed phase chromatography (RPC).

In this report, we introduce the TSKgel Protein C_4-300, an RPC column developed for the purpose of high-speed/high-resolution analysis of proteins, and describe its basic characteristics, the effects of separation conditions on separation, comparisons with other RPC columns, and application examples.

2. Features

1) Pore size of 30 nm suitable for separation of proteins

The pore size that would be suitable for separation differs depending on what is being analyzed. If the pore size is smaller than the size of the molecule you want to analyze, the molecules will not diffuse through the pores, and inadequate resolution will therefore be obtained. Conversely, if the pore size is too large, the relative surface area of the packing material decreases, in other words, the volume of the stationary phase involved in separation decreases, and separation will be poor. As a result, good resolution requires selection of a pore size appropriate to the size of the molecules.

Figure 1 shows the results of an evaluation of how pore size affects the separation of proteins using a prototype packing material in which a butyl group has been introduced onto silica gels of different pore sizes. With a packing material that has a pore size of 30 nm, proteins in a range of molecular mass from 12,000 to 66,000 were all eluted with good peak shapes. However, as pore size decreased, the width of peaks increased, leading to the appearance of marked peak distortion, particularly with bovine serum albumin (BSA) with a molecular mass of 66,000. It is clear from these results that a pore size of 30 nm is an appropriate size for separation of proteins.

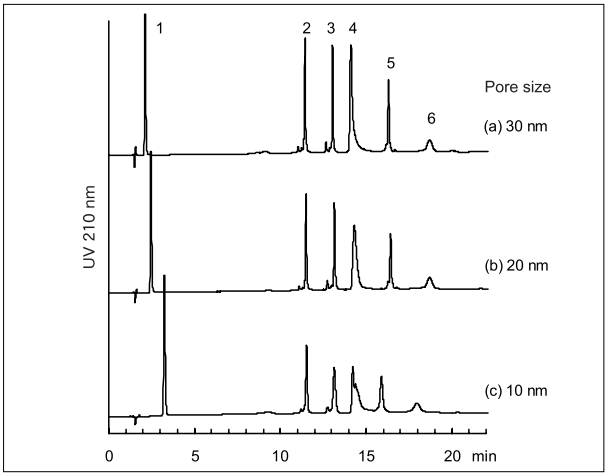

Figure 1 Effect of pore size on separation of proteins

Column: Proto type RPC Column (4.6 mm I.D. × 10 cm)
Particle size: 3 μm
Pore size: (a) 30 nm, (b) 20 nm, (c) 10 nm
Ligand: C4
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (30 min)
Flow rate: 1.0 mL/min
Detection: UV 210 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples:

1. phenylalanine	(MW 165)
2. cytochrome c (horse)	(MW 12,400)
3. lysozyme	(MW 14,300)
4. bovine serum albumin	(MW 66,000)
5. α-chymotrypsinogen A	(MW 25,700)
6. ovalbumin	(MW 44,300)

(each 2 μg)

2) C_4 stationary phase appropriate for separation of proteins

ODS columns with an octadecyl group (C18) as a stationary phase are widely used in RPC, but when analyzing highly hydrophobic samples, better separation is sometimes provided by using a stationary phase with a shorter alkyl chain such as an octyl group (C8) or a butyl group (C4), both of which have weak interactions with the sample.

Figure 2 shows the results of an assessment of how the alkyl chain length of the stationary phase affects the separation of proteins, using prototype packing materials in which C18, C8, and C4 have been introduced onto silica gel with a pore size of 30 nm. With the packing material in which C4 was introduced, each of the proteins was eluted with good peak shape, but with packing materials in which C8 and C18 were introduced, decreased peak area and increased peak width were observed. Based on these results, it is clear that C4

is a stationary phase that is suitable for analyzing proteins with high recovery, with low levels of protein adsorption.

Figure 3 shows the results of an evaluation of how protein separation is affected by the quantity of butyl groups introduced onto the silica gel. With the prototype packing materials (c) and (d) that have a carbon content of 3.8 % or more after the introduction of butyl groups and end capping, tailing of the BSA peak (Peak 3) increased, and the peak area decreased.

In addition, with the prototype packing material (a), which has a carbon content of 2.3 %, although BSA peak shape was good, the durability in acidic eluents has been inadequate. Based on the results of these investigations, the quantity of butyl groups introduced in the TSKgel Protein C_4-300 has been adjusted to attain a carbon content of 3 %, allowing good resolution and recovery to be combined with excellent column durability.

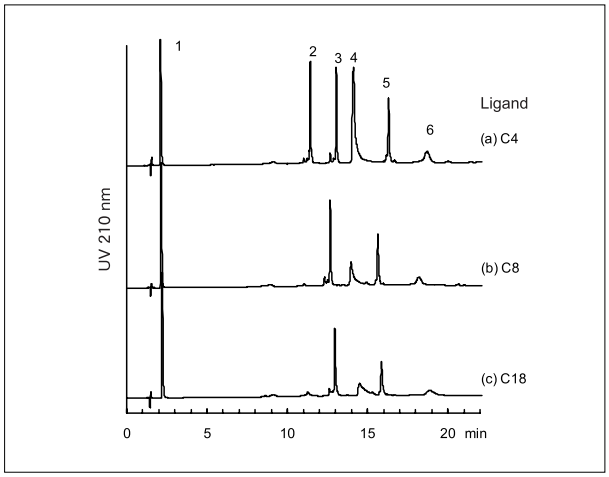

Figure 2 Effect of alkyl chain length on stationary phase on separation of proteins

Column: Proto type RPC Column (4.6 mm I.D.× 10 cm)
Particle size: 3 μm
Pore size: 30 nm
Ligand: (a) C4, (b) C8, (c) C18
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (30 min)
Flow rate: 1.0 mL/min
Detection: UV 210 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples:

1. phenylalanine	(MW 165)
2. cytochrome c (horse)	(MW 12,400)
3. lysozyme	(MW 14,300)
4. bovine serum albumin	(MW 66,000)
5. α-chymotrypsinogen A	(MW 25,700)
6. ovalbumin	(MW 44,300)

(each 2 μg)

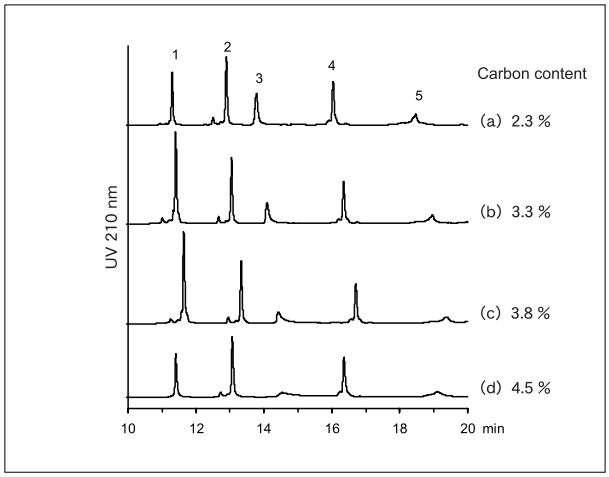

Figure 3 Effect of carbon content on separation of proteins

Column: Proto type RPC Column (4.6 mm I.D.×10 cm)
Particle size: 3 μm
Pore size: 30 nm
Ligand: C4
Carbon content:
(a) 2.3 %, (b) 3.3 %, (c) 3.8 %, (d) 4.5 %
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (30 min)
Flow rate: 1.0 mL/min
Detection: UV 210 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples:

1. cytochrome c (horse)	(MW 12,400)
2. lysozyme	(MW 14,300)
3. bovine serum albumin	(MW 66,000)
4. α-chymotrypsinogen A	(MW 25,700)
5. ovalbumin	(MW 44,300)

(each 2 μg)

3） Excellent durability in acidic eluents containing TFA

Excellent durability is realized with the TSKgel Protein C_4-300, by introducing butyl groups in a polymeric form that is not prone to hydrolysis, and by performing highly efficient end capping on the residual silanol.

Column durability was evaluated in acidic eluents containing trifluoroacetic acid (TFA), which is commonly used in protein analysis by RPC. An eluent containing TFA at a higher than normal concentration (0.2 %) was run through the TSKgel Protein C_4-300 (4.6 mm I.D. ×15 cm) for 1,000 h at a flow rate of 1.0 mL/min, and the naphthalene retention time and theoretical plates for phenol were tracked, the results of which are shown in **Figures 4** and **5.** Naphthalene retention time decreased gradually as cycles increased, but even after 1,000 h, retention time was maintained at 90 % or more of initial one. In addition, no change in the theoretical plates for phenol was observed before and after cycling. The TSKgel Protein C_4-300, with its excellent stationary phase chemical stability and the physical stability of the packing layer, thus allows highly reproducible analysis even with long-term use.

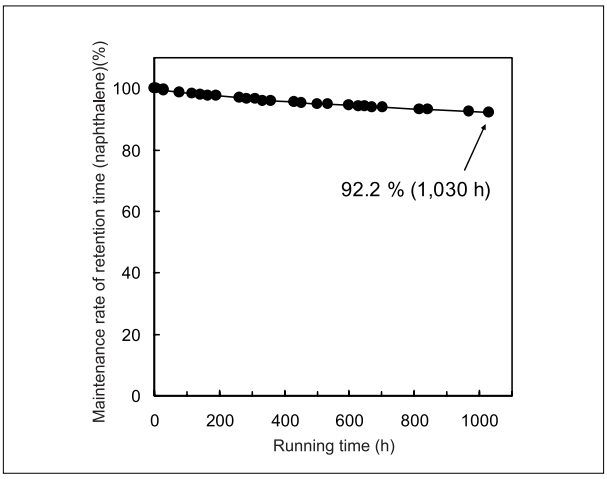

Figure 4 Change of retention time of naphthalene in acidic eluent

Column: TSKgel Protein C_4-300 (4.6 mm I.D. ×15 cm)
Eluent: $H_2O/CH_3CN/TFA$ = 70/30/0.2 (v/v/v)
Flow rate: 1.0 mL/min
Detection: UV 254 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Sample: naphthalene

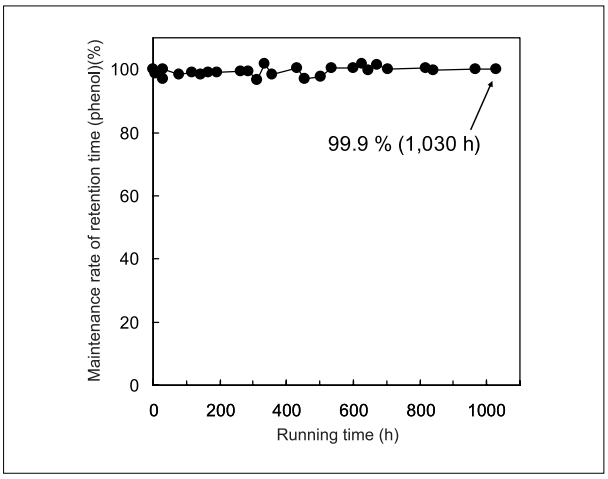

Figure 5 Change of theoretical plates of phenol in acidic eluent

Column: TSKgel Protein C_4-300 (4.6 mm I.D. ×15 cm)
Eluent: $H_2O/CH_3CN/TFA$ = 70/30/0.2 (v/v/v)
Flow rate: 1.0 mL/min
Detection: UV 254 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Sample: phenol

3. Basic Properties

3-1 List of Properties

The specifications of the TSKgel Protein C_4-300 are shown in **Tables 1** and **2**. The 4.6 mm I.D. column is appropriate for conventional analysis, and the 2.0 mm I.D. column for microanalysis and LC/MS (/MS). The TSKgel guardgel Protein C_4, a cartridge type of guard column, can be used with both the 4.6 mm I.D. and 2.0 mm I.D. columns to protect the analytical columns from strongly adsorbent contaminants (requires a separately available cartridge holder).

Table 1 Properties of TSKgel Protein C_4-300 packings

Base materials	Silica
Particle size	3 μm
Pore size	30 nm （Silica）
Specific surface area	100 m^2/g （ Silica ）
Ligand	Butyl groups （polymeric）
End-capping	Trimethyl silyl
Carbon content	3 %

Table 2 Product line of TSKgel Protein C_4-300 Column

Product name	Part number	Column size (mmI.D. x cm)	
TSKgel Protein C_4-300	0022827	4.6 x 5	
	0022828	4.6 x 10	
	0022829	4.6 x 15	
	0022830	2.0 x 5	
	0022831	2.0 x 10	
	0022832	2.0 x 15	
TSKgel guardgel Protein C_4	0022833	3.2 x 1.5 (3 pcs/pkg)	※1
	0022834	2.0 x 1 (3 pcs/pkg)	※2

Column hardware: stainless Shipping solvent: acetonitorile

※1 requires a separately available cartridge holder (P/N 0019018)

※2 requires a separately available cartridge holder (P/N 0019308)

3-2 Standard Separation Conditions

Table 3 shows the standard separation conditions when analyzing proteins and peptides using the TSKgel Protein C_4-300. When constructing a method of analysis, after confirming the elution pattern of a sample under standard separation conditions, we recommend investigating separation conditions while consulting the descriptions in Sections 3-3 through 3-11 below.

Table 3 Standard separation conditions

Eluent	A: H_2O/CH_3CN/TFA = 90/10/0.05 (v/v/v) B: H_2O/CH_3CN/TFA = 20/80/0.05 (v/v/v) A→B linear gradient
Gradient time	45 min (15 cm Column) 30 min (10 cm Column) 15 min (5 cm Column)
Re-equilibration time	15 min (15 cm Column) 10 min (10 cm Column) 5 min (5 cm Column)
Flow rate	1.0 mL/min (4.6 mm I.D.) 0.2 mL/min (2.0 mm I.D.)
Temperature	40 ℃
Sample load	c.a. 0.1～1.0 μg

3-3 Separation Behavior of Proteins

Figure 6 shows a chromatogram of standard proteins. Generally, it can be seen that retention tends to become strong as the molecular mass of the sample increases. However, for example, although lactoferrin has a large molecular mass of approximately 90,000, it is eluted earlier than α-chymotrypsinogen A and carbonic anhydrase, which have smaller molecular mass. Lactoferrin presumably has a weaker hydrophobic interaction with the C4 stationary phase than other proteins, due to factors such as its amino acid composition, posttranslational modification, and higher-order structure.

In general, reversed phase chromatography (RPC) is characterized by higher resolution when separating proteins than other modes of separation that involve different mechanisms, such as size-exclusion chromatography (SEC), ion exchange chromatography (IEC), and hydrophobic interaction chromatography (HIC).

On the other hand, because ion-paired reagents and organic solvents used as eluents in RPC have a strong denaturing effect on proteins, RPC is not appropriate when proteins are fractionated for the purpose of analyzing their structure and function.

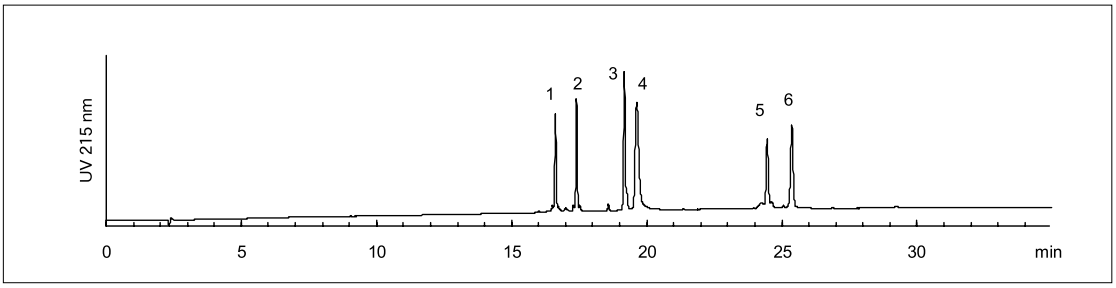

Figure 6 Chromatograms of standard proteins

Column: TSKgel Protein C_4-300 (4.6 mm I.D. × 15 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (45 min)
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples:

1.	cytochrome c (horse)	(MW 12,400)
2.	cytochrome c (bovine)	(MW 12,300)
3.	lysozyme	(MW 14,300)
4.	lactoferrin(human)	(MW 90,000)
5.	α-chymotrypsinogen A	(MW 25,700)
6.	carbonic anhydrase	(MW 29,000)

(each 1 μg)

3-4 Separation Behavior of Peptides

Figure 7 shows a chromatogram of standard peptides. As with proteins, there are peptides that show weak retention despite large molecular mass, and peptides that have strong retention despite small molecular mass, which suggests that differences in hydrophobic interaction are occurring due to the amino acid composition.

With regard to the separation of peptides by RPC, for a long time attempts have been made to calculate hydrophobicity based on the amino acid sequence, and predict separation behavior using an ODS column. **Figure 8** shows the relationship between the hydrophobicity of peptides calculated using the hydrophobicity retention coefficient[1] reported by Sasagawa et al., for 39 types of peptide samples, and retention times as measured with the TSKgel Protein C_4-300 (4.6 mm I.D. × 5 cm). Correlations between the estimated hydrophobicity of the peptides and retention times with the TSKgel Protein C_4-300 were observed, and it was confirmed that stronger retention occurred with higher hydrophobicity of the peptide. Secondary retention mechanisms (such as the interaction with residual silanol groups) in the TSKgel Protein C_4-300 and ODS columns are believed to differ, so it is believed that separation behavior can be estimated with even more accuracy by optimizing the amino acid retention coefficient and approximation formula for the TSKgel Protein C_4-300.

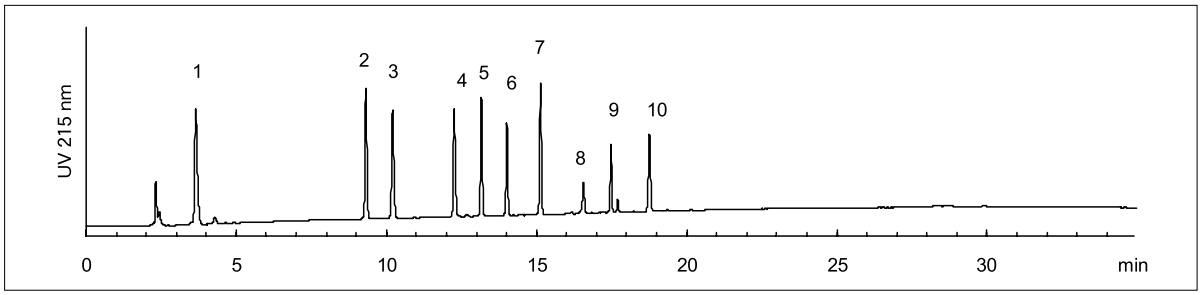

Figure 7 Chromatogram of standard peptides

Column: TSKgel Protein C_4-300 (4.6 mm I.D. × 15 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (45 min)
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL

Samples:
1. δ-sleep-inducing peptide (MW 849)
2. methionine-enkephalin (MW 574)
3. bradykinin (MW 1,060)
4. eledoisin related peptide (MW 707)
5. angiotensin I (MW 1,297)
6. substance P (MW 1,348)
7. somatostatin (MW 1,638)
8. β-endorphin (MW 3,465)
9. insulin (MW 5,808)
10. gastrin I (MW 2,098
(each 0.25 μg)

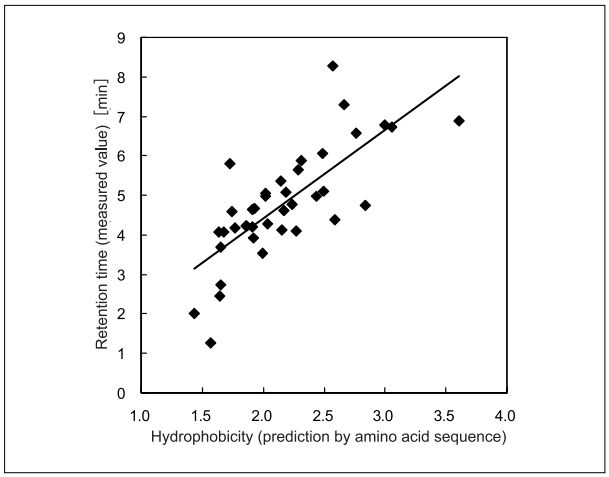

Figure 8 Plot of hydrophobicity and retention time of peptides predicted by amino acid sequence

Column: TSKgel Protein C_4-300 (4.6 mm I.D. × 5 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
0 % B → 75 % B linear gradient (11.25 min)
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL

3-5 Effect of Gradient Time

Figure 9 shows how resolution is affected when the gradient time is changed under constant flow rate conditions.

As gradient time increased, although there was an improvement in resolution between proteins with close retention times (between cytochrome c (bovine) and cytochrome c (equine); and between α-chymotrypsinogen A and carbonic anhydrase), the separation-improving effect gradually decreased. Comparing the effect of gradient time in columns of different lengths (4.6 mm I.D. × 15 cm, 4.6 mm I.D. × 5 cm) showed that although higher resolution was obtained with the 4.6 mm I.D. × 15 cm column when analysis was conducted with a long gradient time, this differences in resolution due to column length decreased when the gradient time was short. Based on these results, when the objective is high resolution, it is advisable to use the long column with a long gradient time; and when the objective is high-throughput analysis, using the short column with a short gradient time would be preferable.

Peak capacity calculated using peptide samples (P_C: maximum number of peaks that can be separated when resolution Rs = 1)[2] yielded results similar to those with proteins (**Fig. 10**).

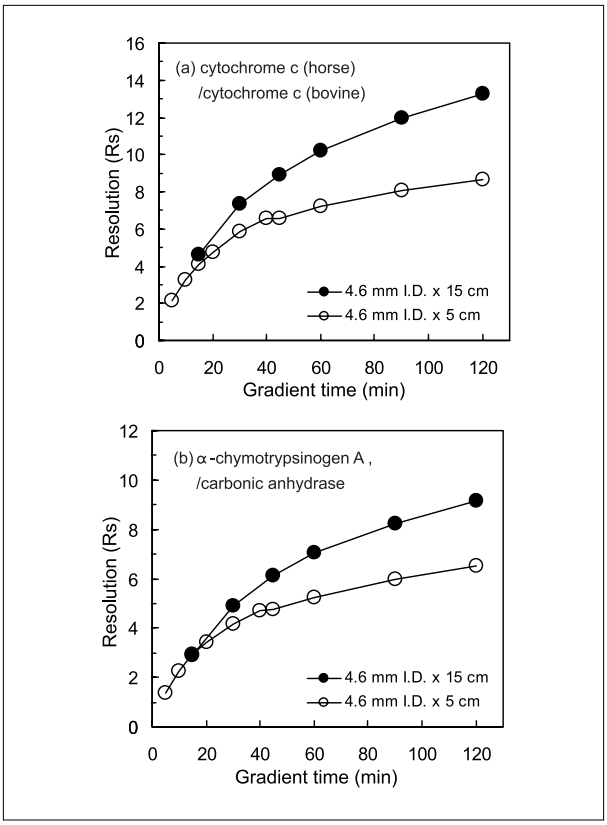

Figure 9 Effect of gradient time on separation of proteins

Column: TSKgel Protein C_4-300 (4.6 mm I.D. ×15 cm), TSKgel Protein C_4-300 (4.6 mm I.D. ×5 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (5~120 min)
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples: (a) cytochrome c (horse), cytochrome c (bovine)
(b) α-chymotrypsinogen A , carbonic anhydrase
(each 1 μg)

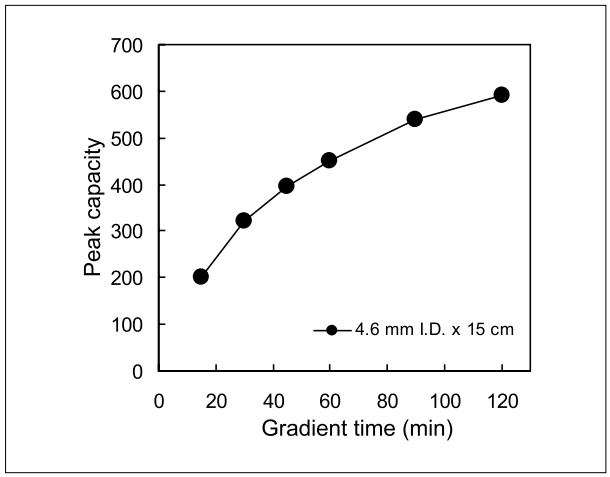

Figure 10 Effect of gradient time on separation of peptides

Column: TSKgel Protein C_4-300 (4.6 mm I.D. ×15 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (15~120 min)
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples: δ-sleep-inducing peptide, methionine-enkephalin, bradykinin, eledoisin related paptide, angiotensin I, substance P, somatostatin, β-endorphin, insulin, gastrin I (each 0.25 μg)

Calculation formula of peak capacity (P_c)

$$P_c = 1 + \frac{t_G}{1.7 \cdot w_{0.5}}$$

t_G: gradient time
$w_{0.5}$: average of peak half width of 10 peptides measured

3-6 Effect of Flow Rate

Figure 11 shows how resolution is affected when the flow rate is changed under constant gradient time conditions. The resolution between proteins with close retention times (between cytochrome c (bovine) and cytochrome c (equine); and between α-chymotrypsinogen and carbonic anhydrase) improved as flow rate increased, but the flow rate effect diminished at flow rates of 1.0 mL/min or more (linear velocity of 6 cm/min or more), and virtually the same resolution was produced. Peak capacity calculated using peptide samples yielded results similar to those with proteins (**Fig. 12**). Based on these results, a flow rate of about 1.0

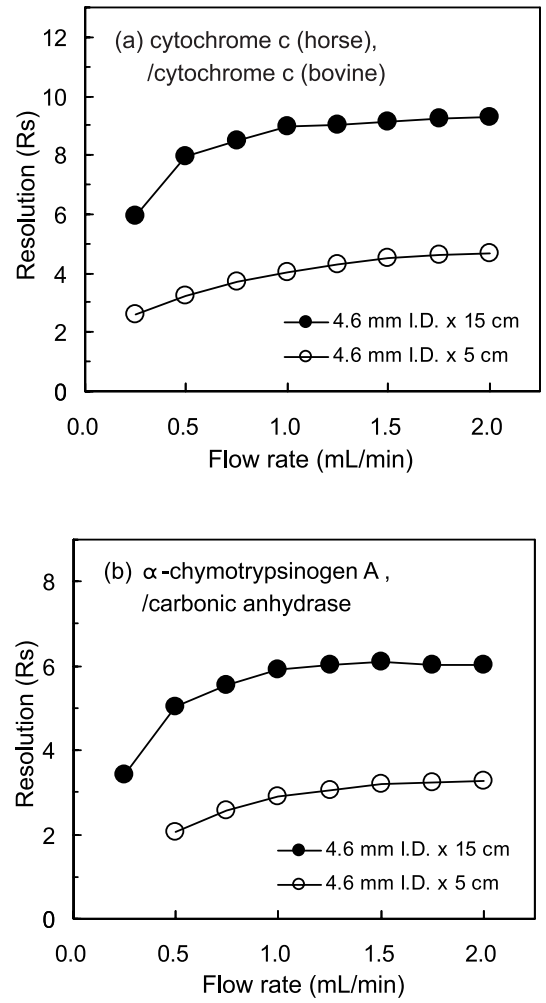

Figure 11 Effect of flow rate on resolution of proteins

Column: TSKgel Protein C_4-300 (4.6 mm I.D.×15 cm), TSKgel Protein C_4-300 (4.6 mm I.D.×5 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient
45 min (4.6 mm I.D.×15 cm),
15 min (4.6 mm I.D. # 5 cm)
Flow rate: 0.25~2.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples: (a) cytochrome c (horse), cytochrome c (bovine)
(b) α-chymotrypsinogen A , carbonic anhydrase
(each 1 μg)

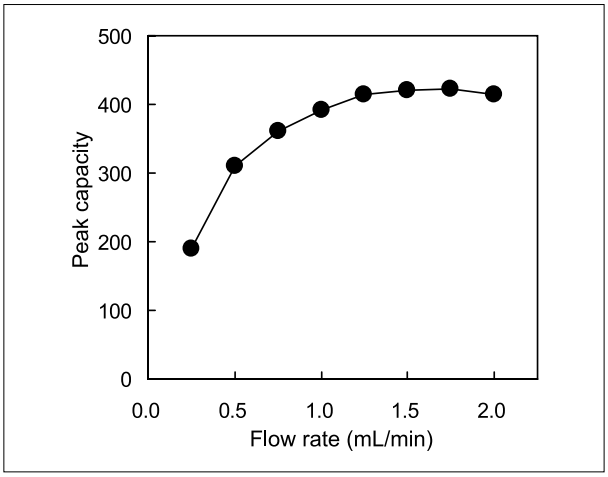

Figure 12 Effect of flow rate on separation of peptides

Column: TSKgel Protein C_4-300 (4.6 mm I.D.×15 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (45 min)
Flow rate: 0.25 ～ 2.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples: δ-sleep-inducing peptide, methionine-enkephalin, bradykinin, eledoisin related paptide, angiotensin I, substance P, somatostatin, β-endorphin, insulin, gastrin I (each 0.25 μg)

Calculation formula of peak capacity is the same as in Fig. 10

mL/min would be appropriate with the 4.6 mm I.D. column, and about 0.2 mL/min with the 2.0 mm I.D. column. When gradient analysis is conducted at a low flow rate, depending on the system used, there is a risk that separation performance and reproducibility can decrease as a result of diffusion outside the flow pathway of the column (injector, tubing, detector), or insufficient gradient precision. Under these circumstances, separation may be improved by increasing the flow rate within a range that does not exceed the maximum pressure of the column when used during analysis.

3-7 Changes in Selectivity due to Separation Conditions

In general, in reversed phase chromatography under isocratic conditions, there is an almost linear relationship between the concentration of organic solvent of the eluent Φ and the retention log k' of the sample (k': retention coefficient), and the value of this slope S (isocratic parameter) is known to differ depending on the sample. As a result, when samples of different S values are analyzed under isocratic conditions, varying the concentration of the organic solvent of the eluent will change the separation factor $\alpha = k'_2/k'_1$. Similarly, when performing gradient analysis, by changing factors that affect the gradient slope of the organic solvent concentration (gradient time, flow rate, column length), separation selectivity changes and the peak elution pattern is altered. **Figure 13** shows changes to a chromatogram of standard peptides under varying gradient times (a) and flow rates (b).

For the sake of convenience, the scales of the horizontal axes were adjusted so that the elution positions of bradykinin (Peak 2), somatostatin (Peak 6) and gastrin I (Peak 9), would line up with each other. Nevertheless, peaks of differing selectivity can be seen when either the gradient time or flow rate is changed. Here arrows are used to draw attention to the changes for insulin (**Peak 8**).

If the objective is to change the separation conditions while maintaining selectivity, it is necessary to set up the separation conditions so that $t_G F/V_0 \Delta\Phi$ remains constant (t_G: gradient time; F: flow rate; V_0: column void volume; $\Delta\Phi$: amount of change in the concentration of the organic solvent from the start point and end point of the gradient). Examples are shown in **Figure 14**. As in **Figure 13**, the scales of the horizontal axes were adjusted so that elution positions of somatostatin (Peak 2) and gastrin I (Peak 5) would line up with each other. When the value of $t_G F/V_0 \Delta\Phi$ is constant (upper and lower chromatograms in both (a) and (b)), it is clear that the selectivity of the other peaks remains unchanged. Here, arrows are used to draw attention to changes in insulin (**Peak 4**). For details please see L.R. Snyder, J.L. Glajch, J.J. Kirkland, Practical HPLC Method Development (Japanese translation), A Takahashi, S Araki, p. 154, Tokyo Kagakudojin (1992).

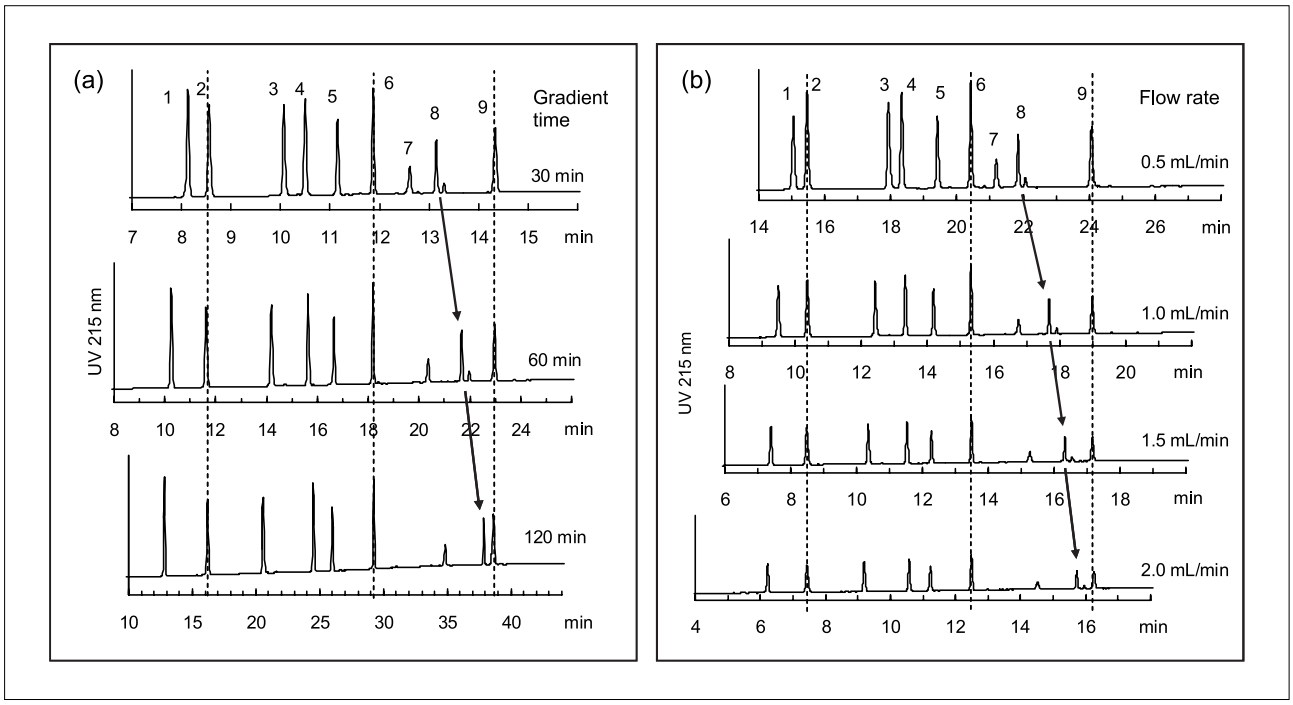

Figure 13 Change of selectivity of peptides by separation condition

Column: TSKgel Protein C_4-300 (4.6 mm I.D. × 15 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient, (a) 30~120 min, (b) 45 min
Flow rate: (a) 1.0 mL/min, (b) 0.5~2.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL

Samples:
1. δ-sleep-inducing peptide
2. methionine-enkephalin
3. bradykinin
4. eledoisin related paptide
5. angiotensin I
6. substance P
7. somatostatin
8. β-endorphin
9. insulin
10. gastrin I
(each 0.25 μg)

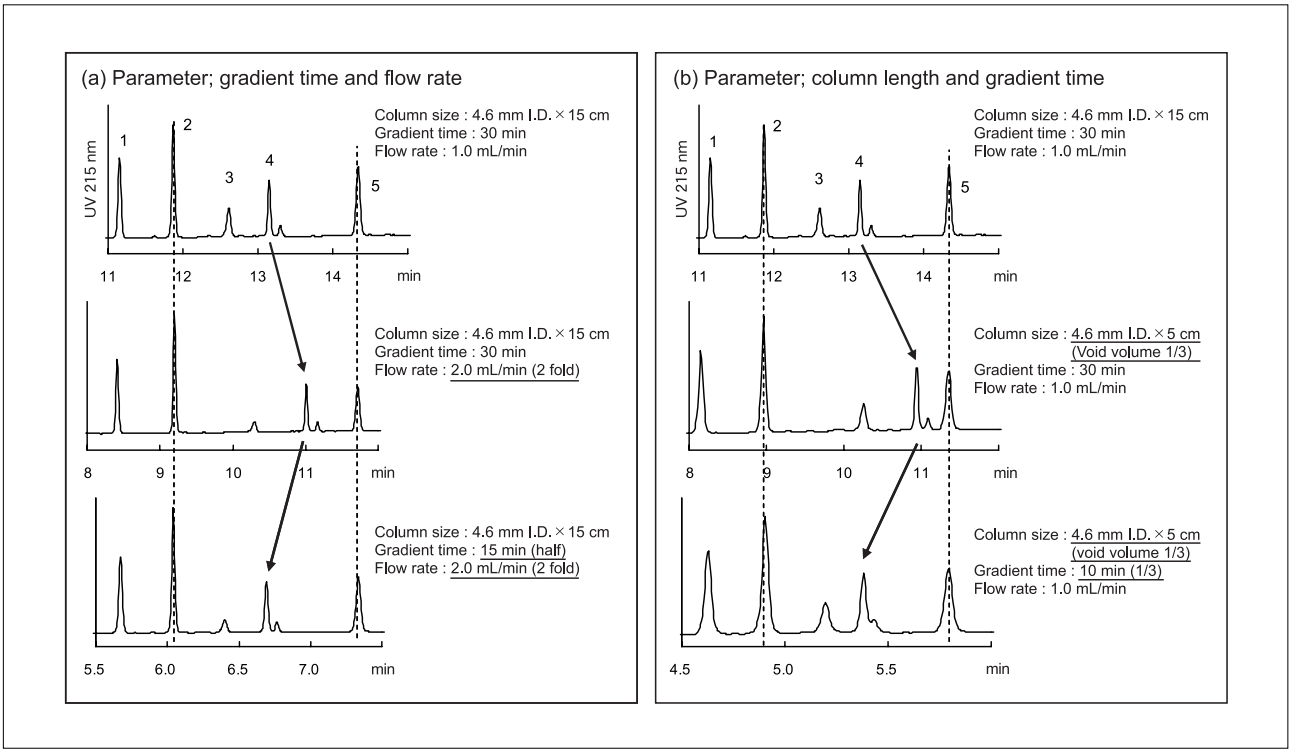

Figure 14 Control of selectivity

Column: TSKgel Protein C_4-300
Eluent: A: $H_2O/CH_3CN/TFA = 90/10/0.05$ (v/v/v)
B: $H_2O/CH_3CN/TFA = 20/80/0.05$ (v/v/v)
A → B linear gradient
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL

Samples: 1. substance P
2. somatostatin
3. β-endorphin
4. insulin
5. gastrin I
(each 0.25 μg)

※ These objects, column size, gradient time and flow rate are described in the graph above.

3-8 Effect of Ion-paired Reagents

When separating proteins with RPC, generally an eluent is used to which trifluoroacetic acid (TFA) has been added as an ion-paired reagent.

Figures 15 and **16** show how separation is affected by the concentration of TFA in the eluent. Good separation was obtained with both proteins and peptides in a TFA concentration range of 0.02 % to 0.1 %. With the peptide samples, changes in selectivity due to differences in TFA concentration are observed. If the TFA concentration is less than 0.01 %, marked leading or tailing of the peaks of some proteins is observed. With peptides, although leading/tailing of the peaks was not observed, peak width increased to some degree, and peak capacity (P_c) decreased. TFA concentrations above 0.1 % should not be used, as this can cause a reduction in sensitivity in LC/MS and deterioration of the column. Based on these results, a TFA concentration range around 0.02 % to 0.1 % would be considered appropriate when analyzing proteins and peptides.

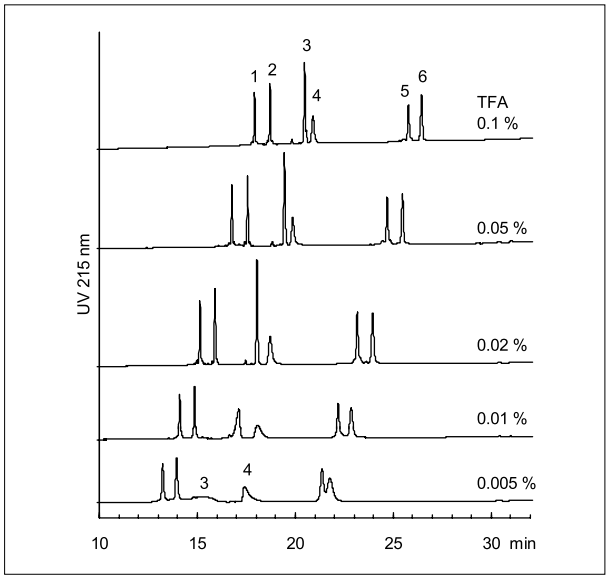

Figure 15 Effect of TFA concentration on separation of proteins

Column: TSKgel Protein C_4-300 (4.6 mm I.D. × 15 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.005~0.1 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.005~0.1 (v/v/v)
A → B linear gradient (45 min)
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples:
1. cytochrome c (horse)
2. cytochrome c (bovine)
3. lysozyme
4. lactoferrin(human)
5. α-chymotrypsinogen A
6. carbonic anhydrase
(each 1 μg)

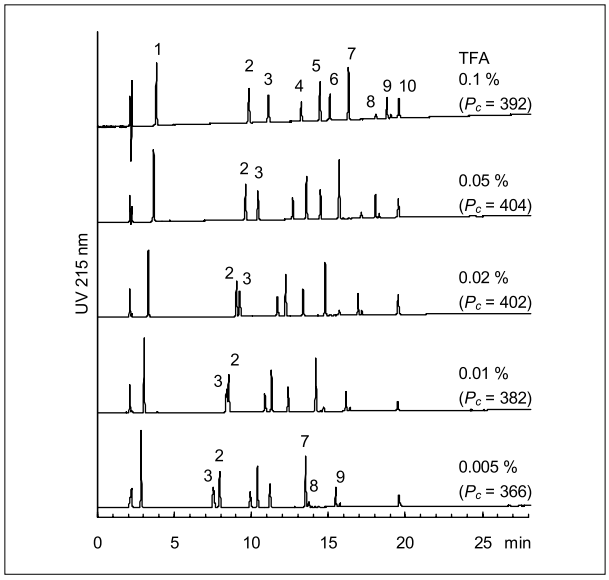

Figure 16 Effect of TFA concentration on separation of peptides

Column: TSKgel Protein C_4-300 (4.6 mm I.D. × 15 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.005~0.1 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.005~0.1 (v/v/v)
A → B linear gradient (45 min)
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples:
1. δ-sleep-inducing peptide
2. methionine-enkephalin
3. bradykinin
4. eledoisin related paptide
5. angiotensin I
6. substance P
7. somatostatin
8. β-endorphin
9. insulin
10. gastrin I
(each 0.25 μg)

Calculation formula of peak capacity is the same as in Fig. 10

Perchloric acid or phosphoric acid can be used as an ion-paired reagent instead of TFA. Applications are shown in **Figure 17**. Because perchloric acid and phosphoric acid have low levels of absorbence at short wave lengths, baseline variations and decreased ghost peaks can be expected when analysis is performed using ultraviolet detection. On the other hand, because these reagents are non-volatile, they are not suitable for use in LC/MS or in detection using evaporative light scattering detectors.

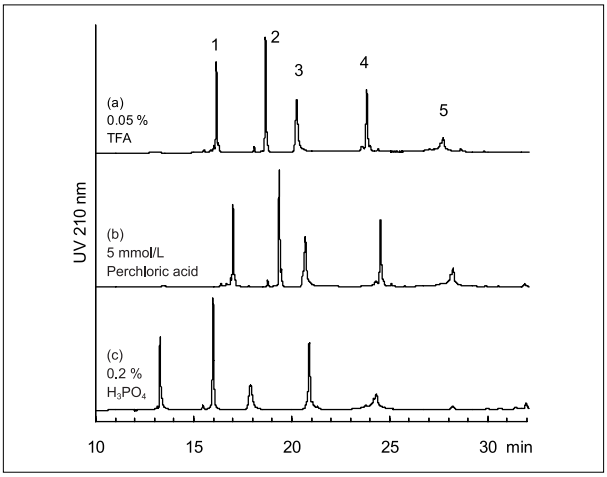

Figure 17 Comparison of chromatograms of proteins in mobile phases with different ion pair reagent

Column: TSKgel Protein C_4-300 (4.6 mm I.D. ×15 cm)
Eluent: (a) A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
(b) A: 5 mmol/L $HClO_4$ in H_2O/CH_3CN = 90/10 (v/v)
B: 5 mmol/L $HClO_4$ in H_2O/CH_3CN = 20/80 (v/v)
(c) A: $H_2O/CH_3CN/H_3PO_4$ = 90/10/0.2 (v/v/v)
B: $H_2O/CH_3CN/H_3PO_4$ = 20/80/0.2 (v/v/v)
A → B linear gradient (45 min)
Flow rate: 1.0 mL/min
Detection: UV 210 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples: 1. cytochrome c (horse)
2. lysozyme
3. bovine serum albumin
4. α-chymotrypsinogen A
5. ovalbumin
(each 2 μg)

3-9 Effect of Temperature

Figures 18 and **19** show how column temperature affects separation in analyses performed using the TSKgel Protein C_4-300 (4.6 mm I.D. ×15 cm). In a range of 27 ℃ to 50 ℃, protein peaks become sharper as the column temperature increases, and separation between the peaks of impurities eluting near the main peaks of cytochrome c and α-chymotrypsinogen A is improved. This is believed to be due to the fact that as the temperature rises, the diffusion coefficient increases, with the sample going in and out of the pores more rapidly. Based on these results, a column temperature in the vicinity of 40 to 50 ℃ in general would be appropriate when analyzing proteins. However, caution is necessary with long-term use under high temperatures, which can lead to column deterioration.

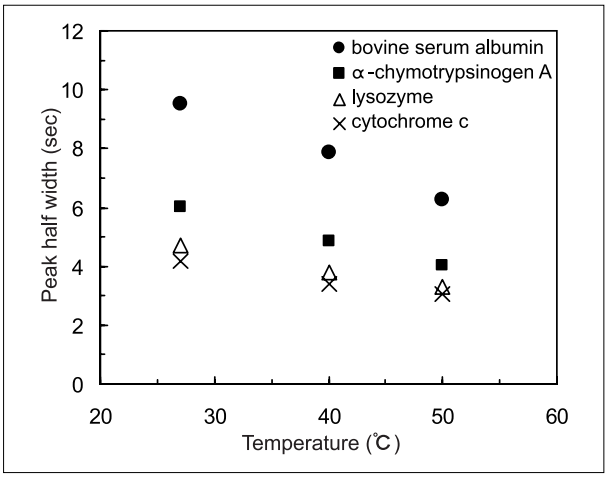

Figure 18 Effect of column temperature on peak half width of proteins

Column: TSKgel Protein C_4-300 (4.6 mm I.D. ×15 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (45 min)
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Temperature: 27, 40, 50 ℃
Inj. volume: 10 μL
Samples: cytochrome c (horse),
lysozyme,
bovine serum albumin,
α-chymotrypsinogen A
(each 2 μg)

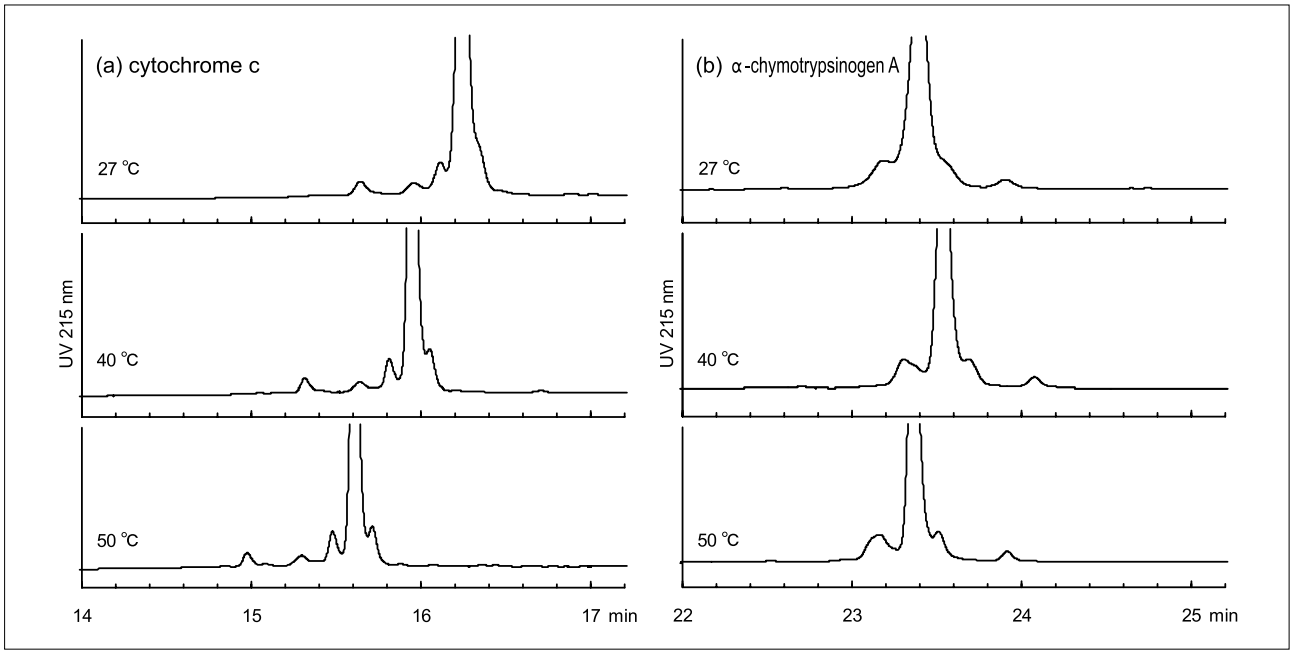

Figure 19 Effect of column temperature on separation of impurities in protein sample

Separation conditions are the same as Figure18

3-10 Effect of Sample Load

Figure 20 shows the relationship between sample load and peak width when proteins were separated on TSKgel Protein C_4-300 (4.6 mm I.D. ×15 cm). With protein samples, peak width is essentially stable with a load around 3 μg, but increased peak width was observed due to overload as the load was increased over 3 μg. In addition, with peptide samples, peak width increased at smaller loads in comparison to proteins, and it can be seen that as the hydrophobicity of samples decreases and retention weakens, the effect of the overload tended to be more readily displayed.

3-11 Quantification

Figure 21 shows the relationship between sample load and peak area when peptides and proteins were separated on TSKgel Protein C_4-300 (4.6 mm I.D. ×15 cm). For peptides and protein samples of comparatively small molecular mass, the calibration curve shows linearity passing through the origin in a range of 10 to 1000 ng, and quantification was good. On the other hand, with BSA and lactoferrin, which have large molecular masss, the calibration curve passes below the origin, and determination was not possible at loads of 30 ng and below. Techniques to consider when performing microanalysis of samples such as BSA and lactoferrin, which are observed to be adsorbed in the stationary phase, include: (1) controlling adsorption by changing the separation conditions (column temperature, concentration and type of ion-paired reagent, etc.); (2) changing to a shorter column with a smaller internal diameter; and (3) using an RPC column that has a larger pore size than the TSKgel Protein C_4-300 (such as the TSKgel Phenyl-5PW RP) or a non-porous RPC column that uses a packing material with a small surface area (such as the TSKgel Octadecyl-NPR).

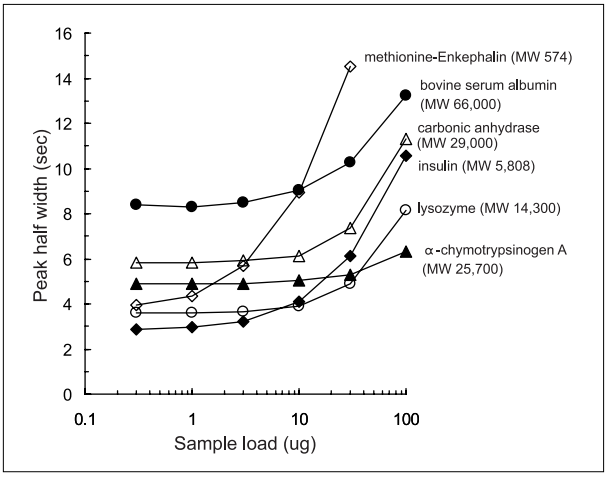

Figure 20 Effect of sample load on peak width

Column: TSKgel Protein C_4-300 (4.6 mm I.D. ×15 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (45 min)
Flow rate: 1.0 mL/min
Detection: UV 245 nm
Temperature: 40 ℃
Inj. volume: 100 μL
Samples:

methionine-Enkephalin	(MW 574)
insulin	(MW 5,808)
lysozyme	(MW 14,300)
α-chymotrypsinogen A	(MW 25,700)
carbonic anhydrase	(MW 29,000)
bovine serum albumin	(MW 66,000)

(each 0.3～100 μg)

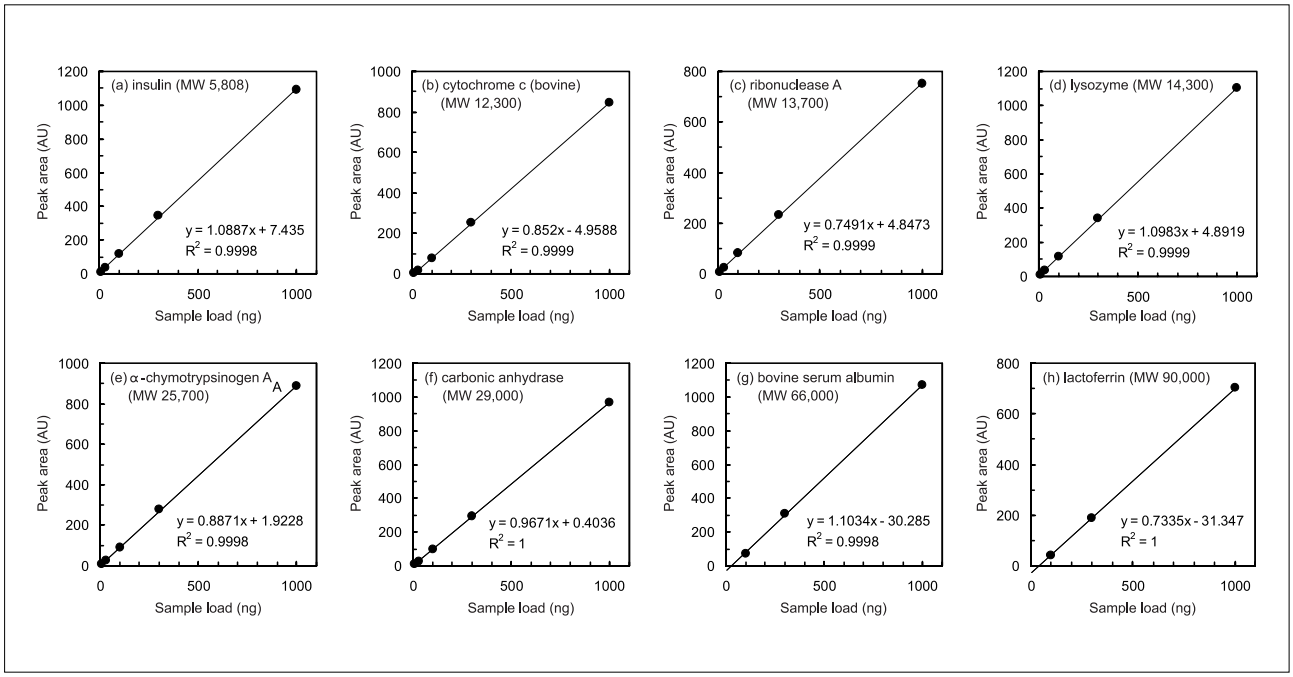

Figure 21 Plots of sample load and peak area

Column: TSKgel Protein C_4-300 (4.6 mm I.D. × 15 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (45 min)
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 100 μL

Samples:

(a) insulin	(MW 5,808)
(b) cytochrome c (bovine)	(MW 12,300)
(c) ribonuclease A	(MW 13,700)
(d) lysozyme	(MW 14,300)
(e) α -chymotrypsinogen A	(MW 25,700)
(f) carbonic anhydrase	(MW 29,000)
(g) bovine serum albumin	(MW 66,000)
(h) lactoferrin	(MW 90,000)

(each 10～1000 ng)

3-12 Comparison with Other TSKgel RPC Columns

The separation of standard proteins and standard peptides was compared using 6 types of TSKgel RPC columns which employ different functional groups, particle sizes, pore sizes, and base materials (**Table 4**). **Figure 22** shows chromatograms produced by analyzing 6 standard proteins under the same conditions. The best separation was obtained using the TSKgel Protein C_4-300. In addition, depending on the column, large discrepancies in the peak areas of some proteins can be seen (especially, lactoferrin), but higher recovery is clearly obtained with the TSKgel Protein C_4-300 compared to the other TSKgel RPC columns, with no visible decrease in peak area. Both the TSKgel Protein C_4-300 and the TSKgel ODS-100V 3 μm are RPC columns in which the base material is a silica gel with a particle size of 3 μm. However, with the TSKgel Protein C_4-300, peaks are eluted more sharply, so differences in functional groups and pore size clearly play a major role in the separation of proteins.

Table4 TSKgel RPC columns

Column	Column size (mm I.D. x cm)	Particle size (μ m)	Pore size (nm)	Base materials
TSKgel Protein C_4-300	4.6 × 15	3	30	Silica
TSKgel ODS-100V 3μm	4.6 × 15	3	10	Silica
TSKgel ODS-120T	4.6 × 15	5	12	Silica
TSKgel Octadecyl-4PW	4.6 × 15	7	50	Polymer
TSKgel Octadecyl-NPR	4.6 × 3.5	2.5	Non-porous	Polymer
TSKgel Phenyl-5PW RP	4.6 × 7.5	10	100	Polymer

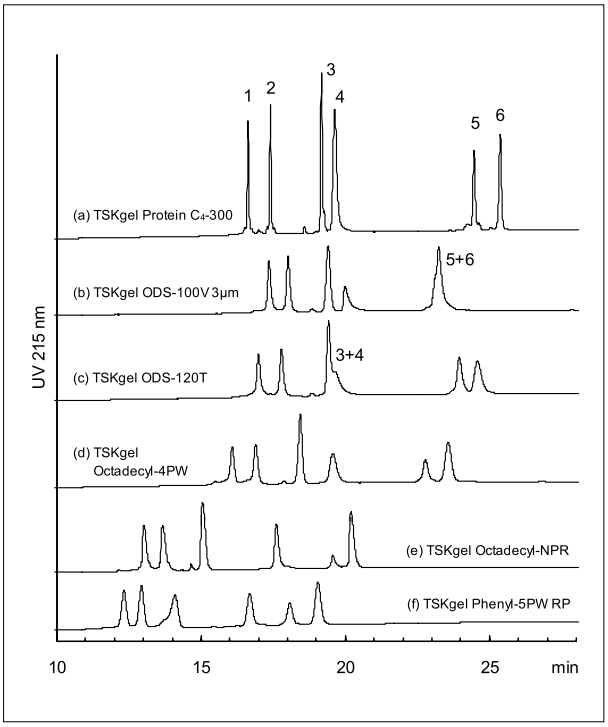

Figure 22 Comparison of chromatograms of proteins

Column: (a) TSKgel Protein C_4-300 (4.6 mm I.D. ×15 cm) ,
(b) TSKgel ODS-100V 3 μm (4.6 mm I.D. ×15 cm) ,
(c) TSKgel ODS-120T (4.6 mm I.D. ×15 cm) ,
(d) TSKgel Octadecyl-4PW (4.6 mm I.D. ×15 cm) ,
(e) TSKgel Octadecyl-NPR (4.6 mm I.D. ×3.5 cm) ,
(f) TSKgel Phenyl-5PW RP (4.6 mm I.D. ×7.5 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (45 min)
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples: 1. cytochrome c (horse)
2. cytochrome c (bovine)
3. lysozyme
4. lactoferrin (human)
5. α-chymotrypsinogen A
6. carbonic anhydrase
(each 1 μg)

Figure 23 shows chromatograms produced when 10 standard peptides were analyzed under the same conditions. The sharpest peaks were obtained using the TSKgel Protein C_4-300 and TSKgel ODS-100V 3 μm; on the other hand, the broadest peaks resulted when the 3 polymer columns were used. It can also be seen that in some cases the elution order of the peptides was reversed, and depending on the column, differences in the separation selectivity of the peptides were noted.

Comparing the TSKgel Protein C_4-300 and TSKgel ODS-100V 3 μm chromatograms, because retention of the δ-sleep-inducing compound (Peak 1) is weak with the TSKgel Protein C_4-300, when analyzing oligopeptides of small molecular mass and low hydrophobicity, better separation tends to be obtained with the TSKgel ODS-100V 3 μm. On the other hand, with the TSKgel ODS-100V 3 μm, the Gastrin I (Peak 10) peak area is small, so when analyzing large-molecular mass, highly hydrophobic peptides, higher recovery tends to be obtained with the TSKgel Protein C_4-300.

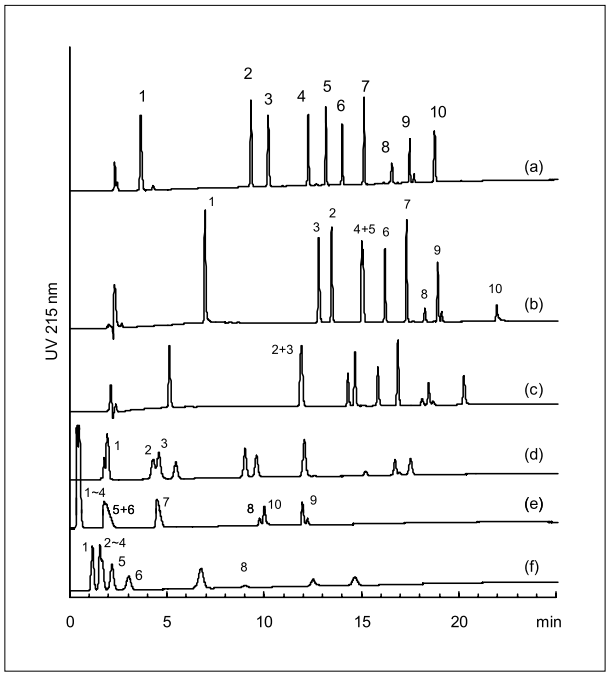

Figure 23 Comparison of chromatograms of peptides

Column: (a) TSKgel Protein C_4-300 (4.6 mm I.D. × 15 cm)
(b) TSKgel ODS-100V 3 μm (4.6 mm I.D. × 15 cm)
(c) TSKgel ODS-120T (4.6 mm I.D. × 15 cm)
(d) TSKgel Octadecyl-4PW (4.6 mm I.D. × 15 cm)
(e) TSKgel Octadecyl-NPR (4.6 mm I.D. × 3.5 cm)
(f) TSKgel Phenyl-5PW RP (4.6 mm I.D. × 7.5 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (45 min)
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples: 1. δ-sleep-inducing peptide
2. methionine-enkephalin
3. bradykinin
4. eledoisin related paptide
5. angiotensin I
6. substance P
7. somatostatin
8. β-endorphin
9. insulin
10. gastrin I
(each 0.25 μg)

3-13 Comparison of Commercially Available RPC Columns for Protein Analysis

Figure 24 shows the results of a comparison of 6 types of commercially available RPC columns used for protein analysis and the TSKgel Protein C_4-300, with respect to separation of standard proteins. Higher resolution was obtained with the TSKgel Protein C_4-300 than the other commercially available columns. In addition, as shown in **Figure 25**, the peak area obtained with the TSKgel Protein C_4-300 was similar to that of the other columns, and the recovery was good.

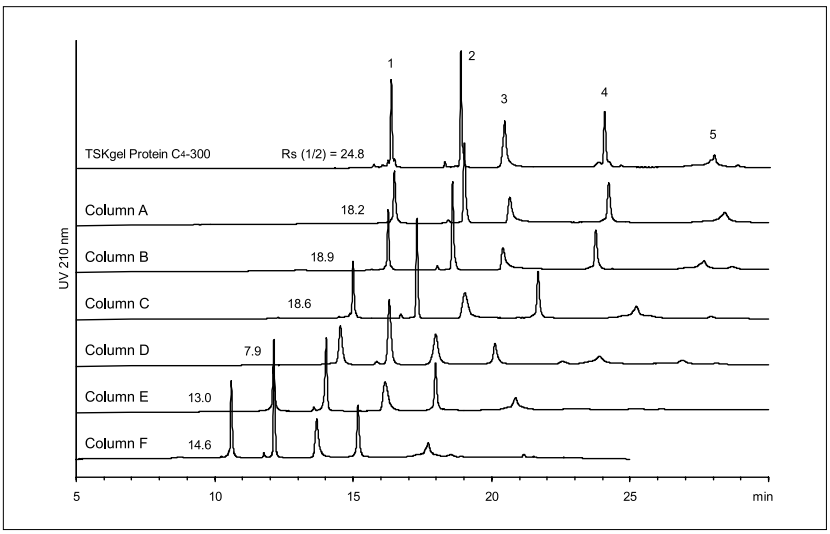

Figure 24 Comparison of chromatograms of proteins between commercial RPC columns for protein separation

Column: TSKgel Protein C_4-300 (3 μm, 4.6 mm I.D. × 15 cm)
commercial RPC (C4) Column A (5 μm, 4.6 mm I.D. × 15 cm)
commercial RPC (C4) Column B (5 μm, 4.6 mm I.D. × 15 cm)
commercial RPC (C4) Column C (3.5 μm, 4.6 mm I.D. × 15 cm)
commercial RPC (C4) Column D (5 μm, 4.6 mm I.D. × 15 cm)
commercial RPCColumn E (3 μm, 4.6 mm I.D. × 15 cm)
commercial RPC (C4) Column F (3 μm, 4.6 mm I.D. × 10 cm)

Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (45 min, Column F ; 30 min)
Flow rate: 1.0 mL/min
Detection: UV 210 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples: 1. cytochrome c (horse)
2. lysozyme
3. bovine serum albumin
4. α-chymotrypsinogen A
5. ovalbumin
(each 2 μg)

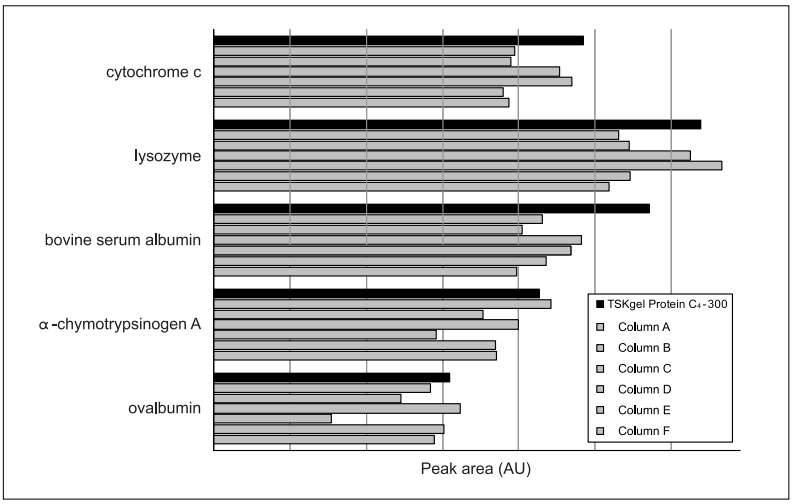

Figure 25 Comparison of peak area of proteins between commercial RPC columns for protein separation

4. Applications

4-1 High-Throughput Analysis

In high throughput analysis, which demands shortened analysis time, it is effective to use a short column (5 cm) and to set the flow rate as high as possible within a range that does not exceed the maximum pressure during use, and adjust the gradient so that the required separation can be attained.

Figure 26 shows chromatograms resulting when 6 standard proteins were separated within 2 minutes using the TSKgel Protein C_4-300 (2.0 mm I.D. ×5 cm). Compared to a commercially available wide pore RPC column (packing material: 5 μm surface porous (core shell) particles; column size: 2.1 mm I.D. ×7.5 cm), it is clear that with the TSKgel Protein C_4-300 sharp peaks are obtained with little tailing, and that superior resolution is achieved even in high-throughput analysis.

Furthermore, because peak widths become very small in high-throughput analysis, in order to obtain the best results, it is necessary to pay careful attention to decreased diffusion outside the column, detector response, and data acquisition intervals, etc.

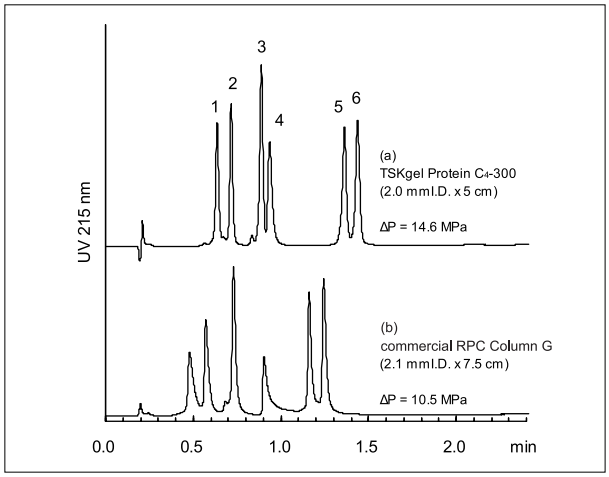

Figure 26 High speed separation of standard proteins

Column: (a) TSKgel Protein C_4-300 (3 μm, 2.0 mm I.D. ×5 cm)
(b) commercial RPC Column G (5 μm core-shell, 2.1 mm I.D. ×7.5 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
25 % B → 75 % B linear gradient (2 min)
Flow rate: 0.8 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL
Samples: 1. cytochrome c (horse)
2. cytochrome c (bovine)
3. lysozyme
4. lactoferrin (human)
5. α-chymotrypsinogen A
6. carbonic anhydrase
(each 1 μg)

4-2 Peptide Mapping

"Peptide mapping" is a technique in which proteins are cleaved by a method such as trypsin digestion, after which the resulting peptide fragments are separated by RPC, and the amino acid sequences are analyzed. Because peptide mapping requires high resolution to enable the separation of tens to hundreds of types of peptide fragments, it is effective to conduct analyses using a long column (15 cm) with a shallow gradient slope.

Figure 27 shows chromatograms produced by separating trypsin digests of BSA, using the TSKgel Protein C_4-300 (4.6 mm I.D. ×15 cm). By superimposing chromatograms of two samples with different digestion times, it is clear that the height of each peak changes as digestion progresses.

Figure 28 shows changes in peak capacity due to gradient time. When analyzing complex peptide mixtures, it becomes possible to separate many peaks by increasing the gradient time.

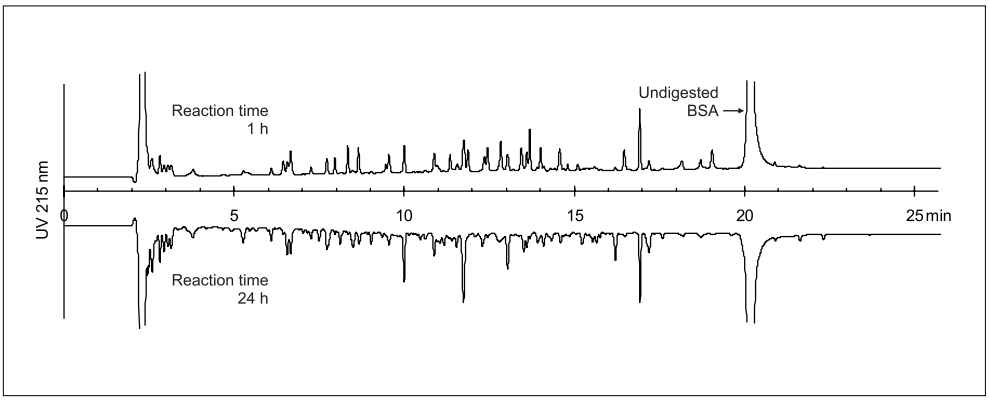

Figure 27 Separation of tryptic digest from BSA

Column: TSKgel Protein C_4-300 (4.6 mm I.D. ×15 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (45 min)
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 10 μL

Samples: tryptic digest from BSA
(reaction time:1 h, 24 h)
(each 20 μg)

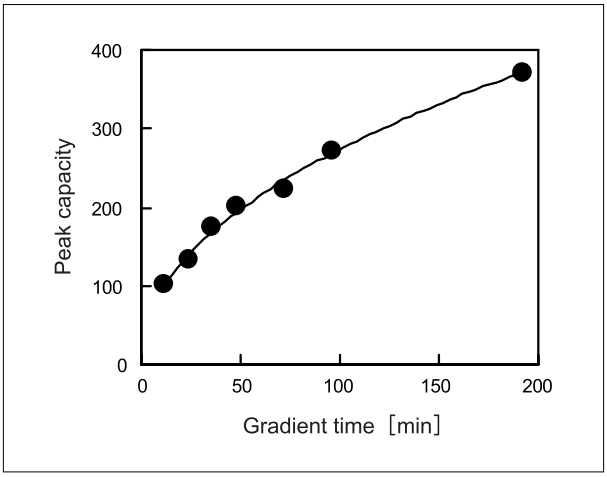

Figure 28 Change of peak capacity by gradient time

Calculation formula of Peak Capacity (P_c)

$$P_c = 1 + \frac{t_R - t_0}{1.7 \cdot w_{0.5}}$$

t_R: Retention time of undigested BSA
t_0: Dead time
(Time to which eluent pass through the column)
$w_{0.5}$: Average peak width of peptide fragments with 5 highest peak height

4-3 PEGylated Proteins

Bonding polyethylene glycol (PEG) to a protein is known to have the effect of reducing the immunogenicity of the protein, and suppressing hydrolysis by enzyme. PEGylation of proteins with the objective of diminishing toxicity and improving pharmacokinetics is already being applied in several biopharmaceuticals.

Lysozymes bonded to PEG with an average molecular mass of 5,000 and 30,000 were separated by molecular size by SEC (**Fig. 29**), and the fractions obtained were analyzed on TSKgel Protein C_4-300 (**Fig. 30**). The PEGylated lysozymes were retained in the column more strongly than the unmodified lysozymes, and retention time clearly varied depending on the molecular mass of PEGs and the number of PEGs bonded per lysozyme molecule. Although peak width was broader with the PEGylated lysozymes than with the unmodified lysozymes, this was expected because PEG bonded to lysozymes have molecular mass distribution.

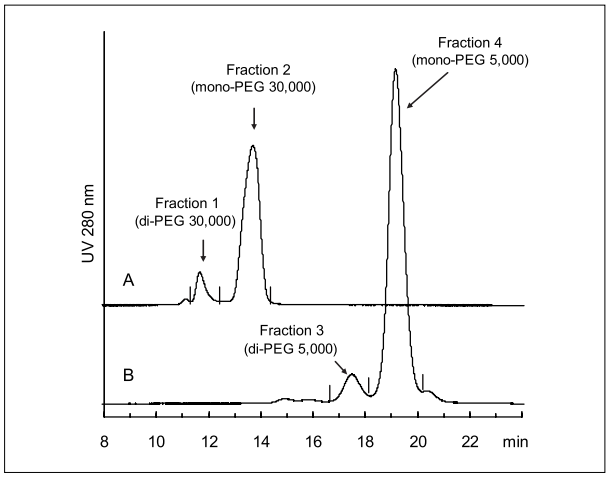

Figure 29 Separation of PEGylated lysoxyme by SEC (TSKgel SuperSW3000)

Column: TSKgel SuperSW3000 (4.6 mm I.D.×30 cm×2)
Eluent: 0.2 mol/L phosphate buffer+0.05% NaN_3 (pH 6.7)
Flow rate: 0.35 mL/min
Detection: UV 280 nm
Temperature: 25 ℃
Inj. volume: 50 μL
Samples: A. PEGylated lysozyme (PEG MW:5,000) (5 g/L)
B. PEGylated lysozyme (PEG MW:30,000) (5 g/L)

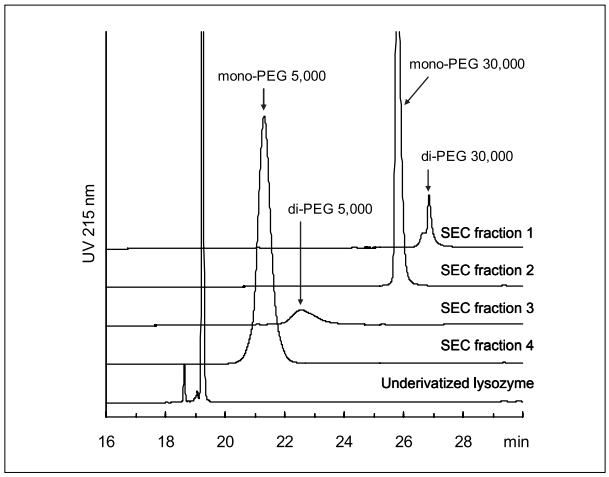

Figure 30 Separation of PEGylated lysozyme by RPC (TSKgel Protein C_4-300)

Column: TSKgel Protein C_4-300 (4.6 mm I.D.×15 cm)
Eluent: A: $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B: $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (45 min)
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Temperature: 40 ℃
Inj. volume: 100 μL
Samples: Underivatized lysozyme (0.1 g/L), SEC fraction 1~4

4-4 Monoclonal Antibodies (IgG)

With monoclonal antibodies (IgG), which are being put into practical use as antibody drugs, it is known that heterogeneities in the amino acid sequence, sugar chain structure, and disulfide bonds, etc., can occur during manufacturing processes and in storage. Molecular species produced due to heterogeneity can affect drug efficacy and safety, so heterogeneity verification testing is extremely important in the development and quality control of biopharmaceuticals. Shown here are examples of analysis of heterogeneous IgG with the TSKgel Protein C_4-300.

Figure 31 shows a chromatogram of H- and L-chains obtained by reduction treatment of IgG by dithiothreitol on TSKgel Protein C_4-300. One L chain and 2 H chain peaks can be observed. It is presumed that in this IgG, some change occurred in the amino acid sequence that forms the H chain, or there are differences in the sugar chain structure bonded on the H chain.

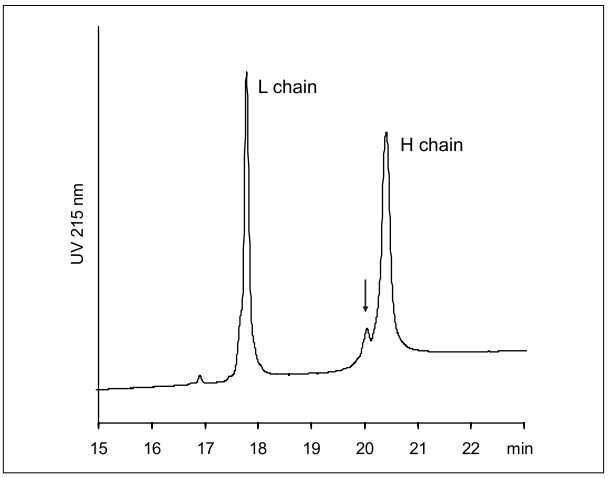

Figure 31 Separation of IgG reduced by dithiothreitol

Column: TSKgel Protein C_4-300 (4.6 mm I.D. × 15 cm)
Eluent: A: $H_2O/CH_3CN/TFA = 90/10/0.05$ (v/v/v)
B: $H_2O/CH_3CN/TFA = 20/80/0.05$ (v/v/v)
A → B linear gradient (45 min)
Flow rate: 1.0 mL/min
Detection: UV 215 nm
Temperature: 50 ℃
Inj. volume: 100 μL
Samples: reduced monoclonal IgG (mouse)

IgG derived from sources different from the ones described above were cleaved into Fab fragments and Fc fragments by papain digestion, then fractions corresponding to molecular mass of 50,000 and 100,000 were separated by SEC (**Fig. 32**), and the fractions obtained were separated on TSKgel Protein C_4-300 (**Fig. 33**). The Fab fragment and Fc fragment are presumed to be contained in the 50,000 molecular mass fraction, but they separated into 5 peaks when separated by RPC on TSKgel Protein C_4-300. This suggests heterogeneity is present in this IgG, in either the amino acid sequence or the sugar chain structure.

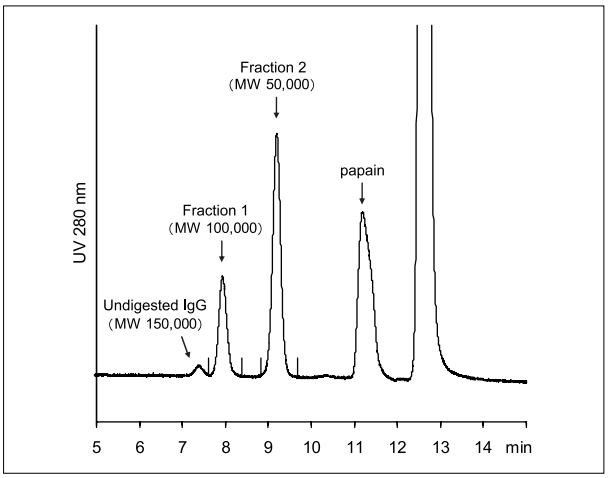

Figure 32 Separation of papain digest of IgG by SEC (TSKgel SuperSW3000)

Column: TSKgel SuperSW3000 (4.6 mm I.D. × 30 cm)
Eluent: 0.2 mol/L phosphate buffer + 0.05 % NaN_3 (pH 6.7)
Flow rate: 0.35 mL/min
Detection: UV 280 nm
Temperature: 25 ℃
Inj. volume: 100 μL
Samples: papain digest of monoclonal IgG (mouse)

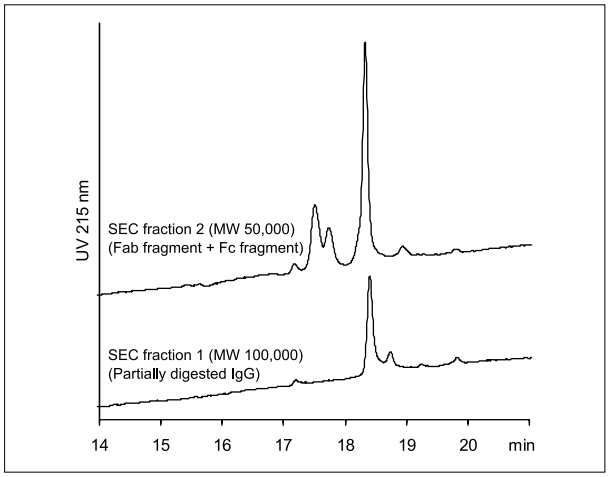

Figure 33 Separation of papain digest of IgG by RPC (TSKgel Protein C_4-300)

Column : TSKgel Protein C_4-300 (4.6 mm I.D. × 15 cm)
Eluent : A : $H_2O/CH_3CN/TFA$ = 90/10/0.05 (v/v/v)
B : $H_2O/CH_3CN/TFA$ = 20/80/0.05 (v/v/v)
A → B linear gradient (45 min)
Flow rate : 1.0 mL/min
Detection : UV 215 nm
Temperature : 50 ℃
Inj. volume : 100 μL
Samples : SEC fraction 1, 2

5. Conclusion

As described above, the RPC column TSKgel Protein C_4-300 has separation characteristics that are suitable for high-speed, high-resolution analysis of proteins.

With the TSKgel Protein C_4-300, in which butyl groups have been introduced onto the surface of a porous silica gel with a particle size of 3 μm and a pore size of 30 nm, high-molecular-mass and highly hydrophobic proteins that have been difficult to analyze with conventional RPC columns can be separated with good peak shape and recovery.

The market for biopharmaceuticals is expected to expand further in the future, and purity and heterogeneity testing in the development and quality control of these drugs is indispensible; the TSKgel Protein C_4-300 provides a powerful analytical technique for performing these tests.

Reference

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2. Neue, U., D., J. Chromatogr. A, 1184, 107-130 (2008)

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