Characterization of new reversed phase columns designed for protein separation based on wide pore silica gel with C4 phase

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Introduction

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- Along with the progress of the biopharmaceutical industry and the enthusiastic growth of proteomics research, the importance of the methodology for characterizing biological macromolecules such as proteins and peptides is also increasing.
- Among various analytical methods, reversed phase liquid chromatography is popular for biomolecular separation, due to its high resolving power and wide applicability to various samples.
- We have developed novel reversed phase columns designed especially for protein separation.
- The packing is prepared by polymeric binding of butyl (C4) alkyl groups and subsequent endcapping with trimethylsilyl (TMS) groups to 3 µm spherical silica gel with 30 nm pore size.
- The C4 short alkyl chain ligand and its controlled bonding density provide moderate hydrophobicity to the stationary phase, which is suitable for protein separation with good recovery. Furthermore, the large pore size, allowing macromolecules to enter the interior of the pore, provides higher peak capacities than reversed phase columns with 10 nm pore size.
- We report here the features of the novel reversed phase columns and several examples of superior protein separation.

Experimental

Experimental

Columns

- TSKgel® Protein C_4-300 (4.6 mm ID x 15 cm, 4.6 mm ID x 5 cm, and 2.0 mm ID x 5 cm), and all other TSKgel columns were manufactured by Tosoh (Tokyo, Japan).

Instrumentation

- The HPLC system was a Tosoh liquid chromatograph equipped with degasser (SD-8022), pump (DP-8020), dynamic mixer (MX-8010), auto-sampler (AS-8020), column oven (CO-8020), UV detector (UV-8020), and data processor (LC-8020 model II).

Reagents

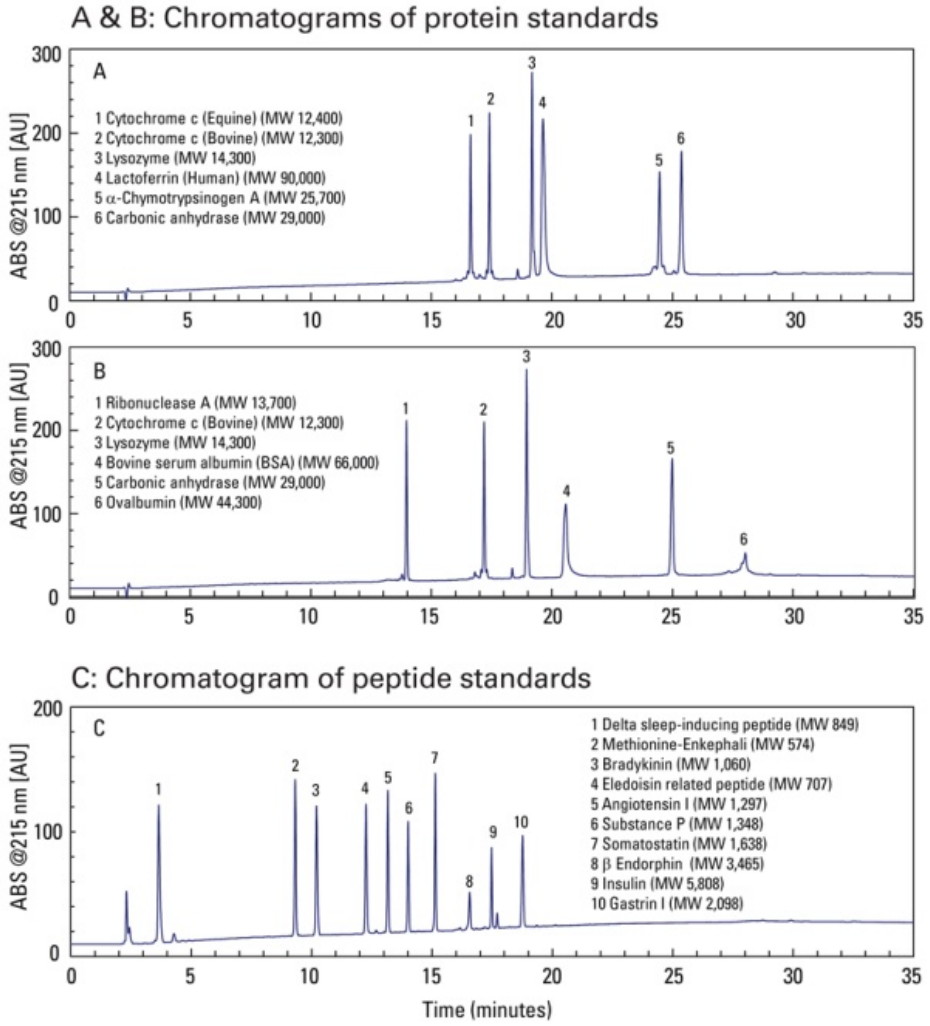
- Proteins: Bovine serum albumin (BSA) and ovalbumin were obtained from Calbiochem (Merck KGaA, Darmstadt, Germany). All other proteins and enzymes were obtained from Sigma-Aldrich (St. Louis, MO, USA).
- All peptides were obtained from Peptide Institute (Osaka, Japan).
- PEGylated lysozyme and mouse IgG were gifted from Tosoh Tokyo Research Center (Ayase, Japan).
- Acetonitrile (HPLC grade) and trifluoroacetic acid (TFA) were obtained from Wako Pure Chemical Industries (Osaka, Japan).
- Water was purified with the Milli-Q® system (Merck Millipore, Darmstadt, Germany).

Characteristics of TSKgel Protein C_4-300

Specification of TSKgel Protein C_4-300

- Base material: Silica gel
- Particle size: 3 µm
- Pore size: 30 nm (silica gel)
- Surface area: 100 m^2/g (silica gel)
- Functional group: Butyl group (C4, polymeric)
- Endcapping: Trimethylsilyl (TMS) group
- Carbon load: 3%
- Column dimension: 4.6 mm ID x 5 cm, 10 cm, 15 cm
  2.0 mm ID x 5 cm, 10 cm, 15 cm
- Guard columns: 3.2 mm ID x 1.5 cm
  2.0 mm ID x 1 cm

Figure 1: Chromatograms of Standard Proteins and Peptides

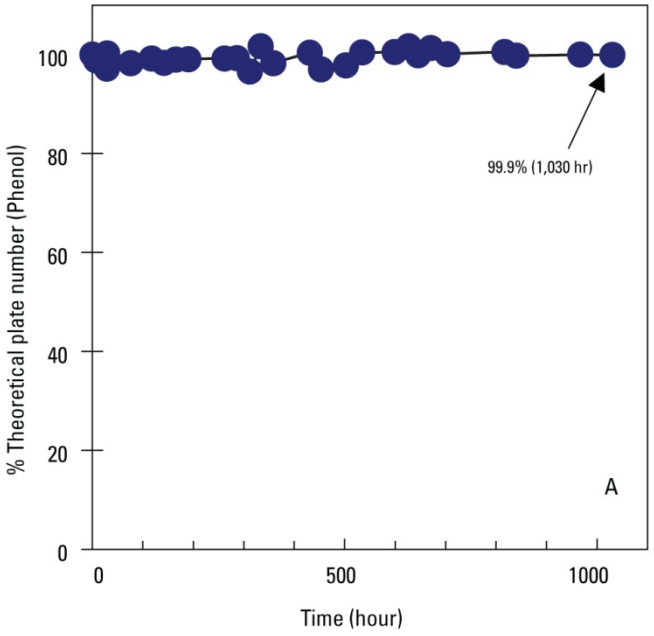


Column:	TSKgel Protein C_4-300, 4.6 mm ID x 15 cm
Mobile phase A:	H_2O/ACN/TFA = 90/10/0.05 (v/v/v)
Mobile phase B:	H_2O/ACN/TFA = 20/80/0.05 (v/v/v)
Gradient:	0%→100% B in 45 min
Flow rate:	1.0 mL/min
Detection:	UV@215 nm
Temperature:	40°C
Injection vol.:	10 µL
Loading:	A & B: 1 µg for each of the proteins
	C: 0.25 µg for each of the peptides

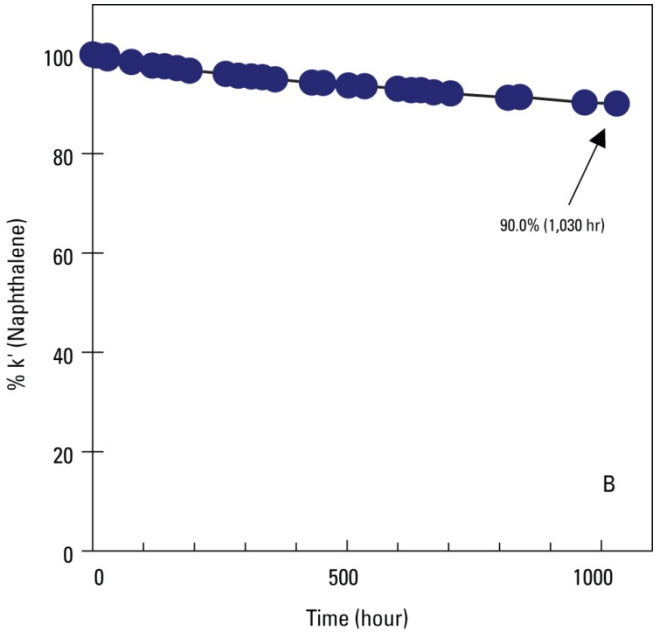
Samples are shown as inset in figures.

Figure 2: Column Stability in Acidic Condition

A: Change in number of theoretical plates of phenol over time



B: Change in capacity factor (k') of naphthalene over time.



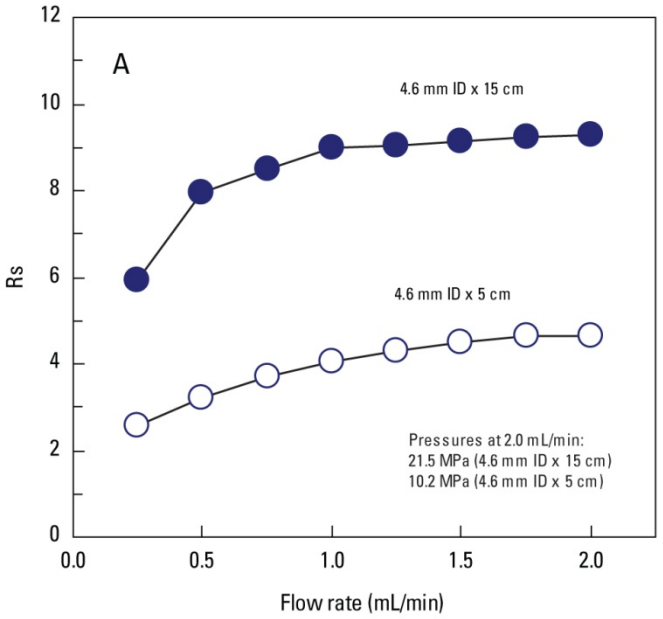
Column:	TSKgel Protein C_4-300, 4.6 mm ID x 15 cm
Mobile phase :	H_2O/ACN/TFA = 70/30/0.2 (v/v/v)
Flow rate:	1.0 mL/min
Detection:	UV@254 nm
Temperature:	40°C
Injection vol.:	10 µL
Samples:	phenol, naphthalene

The TSKgel Protein C_4-300 column showed no loss in column efficiency and 90% of initial retention remained after flushing at 40℃ with acidic eluent (0.2% TFA) for 1,000 hours.

Figure 3: Effect of Chromatographic Conditions on Protein Separation

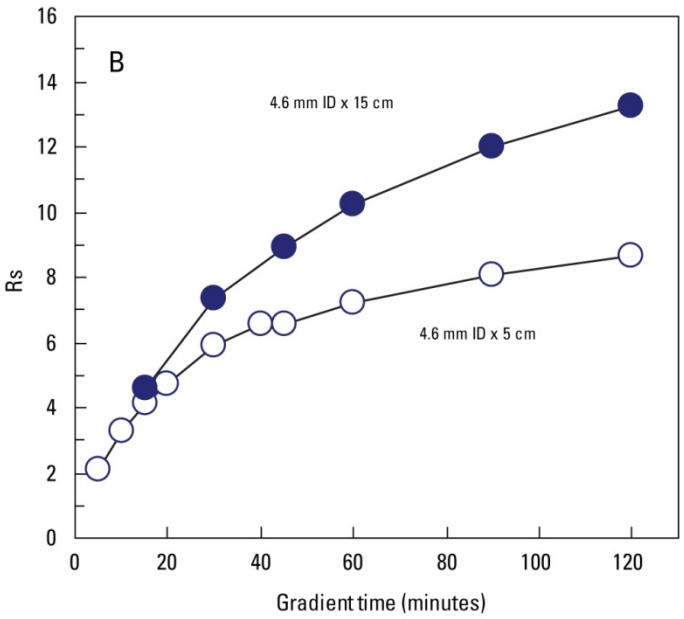
Comparison of resolution factor (Rs) between 4.6 mm ID x 15 cm column and 4.6 mm ID x 5 cm TSKgel Protein C4-300 column. Rs value was calculated using cytochrome c Equine and Bovine.

A: Dependency of Rs on flow rate



Column: TSKgel Protein C4-300 , 4.6 mm ID x 5 cm, 15 cm
Flow rate: 0.25-2.00 mL/min
Other conditions are the same as in Figure 1.

B: Dependency of Rs on gradient time



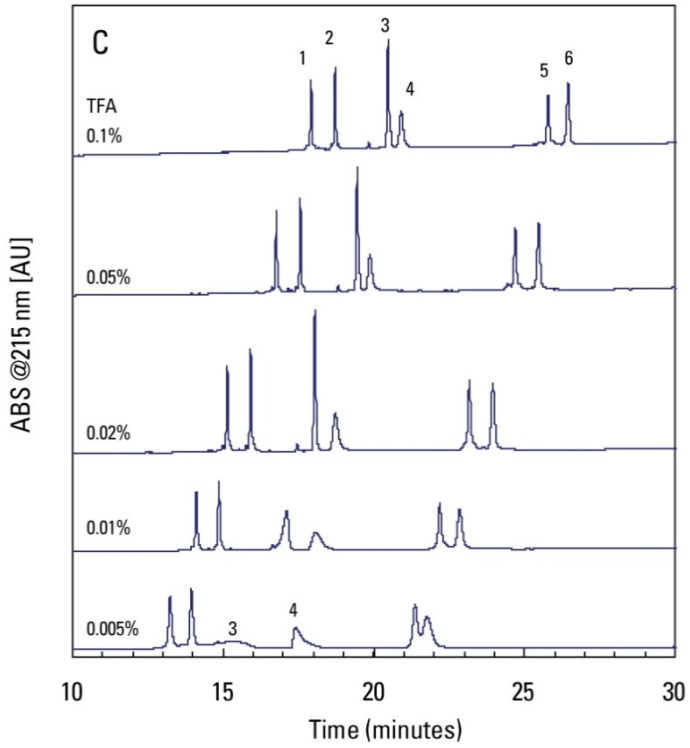
Column: TSKgel Protein C4-300, 4.6 mm ID x 5 cm, 15 cm
Gradient: 0% → 100% B in 5-120 min
Other conditions are the same as in Figure 1.

High resolution was achieved at 1.0 mL/min using both lengths of the 4.6 mm ID TSKgel Protein C4-300 columns. Flow rates higher than 1.0 mL/min showed higher resolution with the 5 cm column.

Resolution was improved with increased gradient time. Using a long gradient time with the 15 cm TSKgel Protein C4-300 column was preferable for high resolution analysis. A short gradient time using the 5 cm column was preferable for high speed analysis.

Effect of Chromatographic Conditions on Protein Separation Continued

Effect of TFA concentration on protein separation



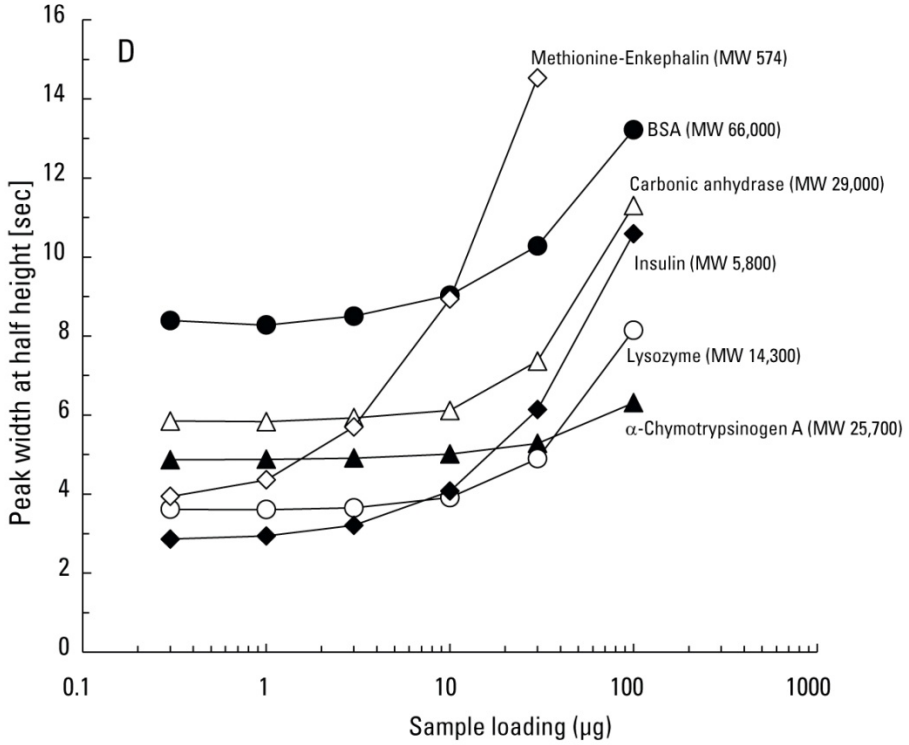
Column: TSKgel Protein C_4-300, 4.6 mm ID x 15 cm
Mobile phase A: H_2O/ACN/TFA = 90/10/0.005-0.1 (v/v/v)
Mobile phase B: H_2O/ACN/TFA = 20/80/0.05-0.1 (v/v/v)
Samples are the same as in Figure 1A.
Other conditions are the same as in Figure 1.

In the presence of 0.02%-0.1% TFA in the mobile phase, proteins and peptides were satisfactorily separated on the TSKgel Protein C_4-300 column.

TFA at a concentration of less than 0.02% might result in leading, tailing, or broadening of peaks.

Effect of Chromatographic Conditions on Protein Separation Continued

Effect of sample loading on the peak width of proteins and peptides

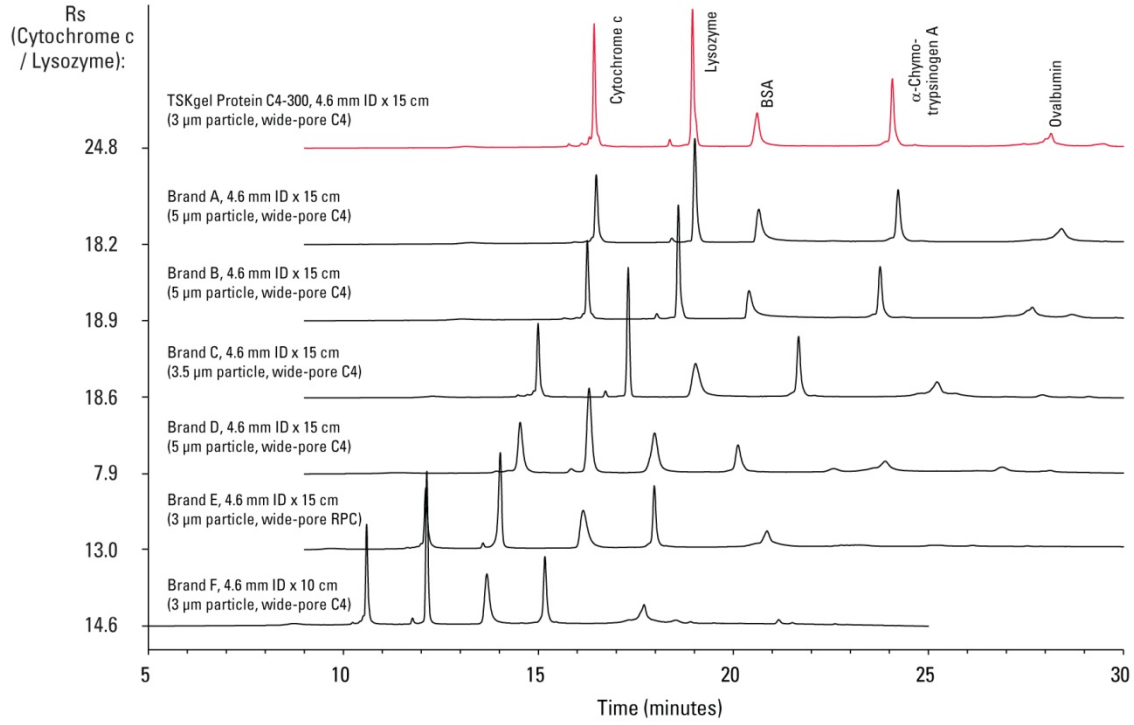


Column: TSKgel Protein C4-300, 4.6 mm ID x 15 cm
Detection: UV@245 nm
Injection vol.: 100 µL
Other conditions are the same as in Figure 1.

For sample loading up to 1 µg on the TSKgel Protein C4-300 column, the peak width remained constant.

Comparison with Other RPC Columns

Figure 4: Comparison with Competitors' RPC Columns

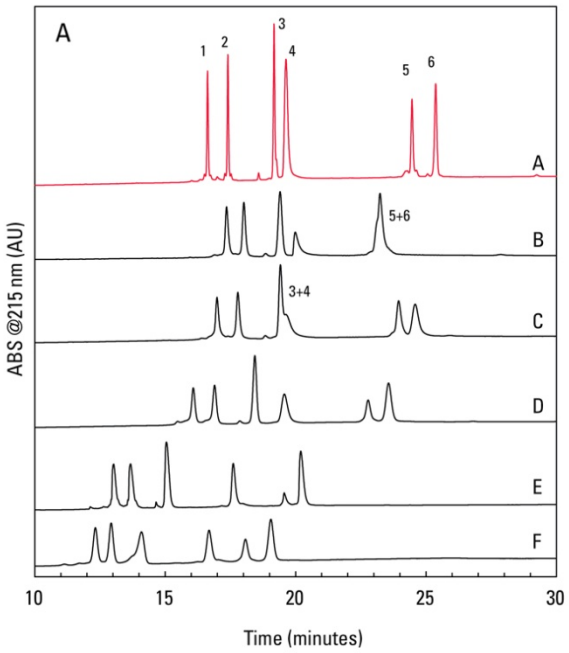


Columns: TSKgel Protein C4-300, 4.6 mm ID x 15 cm
Brands A-E, 4.6 mm ID x 15 cm, and Brand F, 4.6 mm ID x 10 cm
Gradient: 0%→100% B in 45 min for TSKgel Protein C4-300 and Brands A-E;
0%→100% B in 30 min for Brand F
Detection: UV@210 nm
Samples: Cytochrome c (Equine), lysozyme, BSA,
α-Chymotrypsinogen A, ovalbumin (2 µg/10 µL each)
Other conditions are the same as in Figure 1.

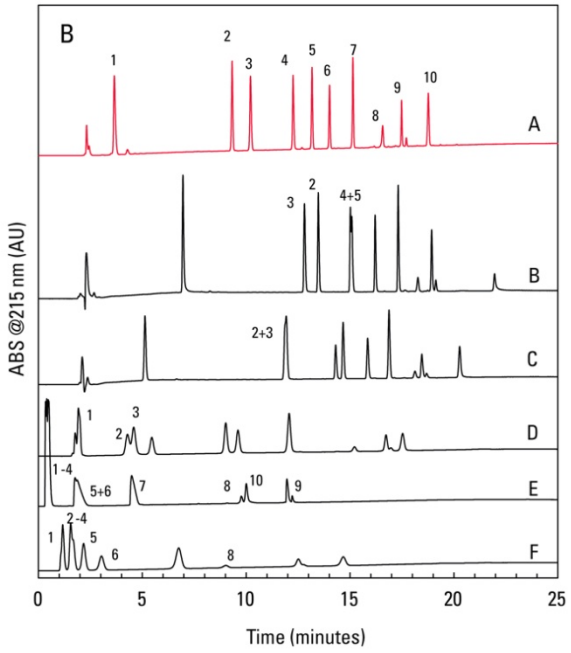
The TSKgel Protein C4-300 column demonstrated superior resolving power and comparable recovery to competitive wide-pore C4 columns.

Figure 5: Comparison with other TSKgel RPC Columns - analysis of protein and peptide standards

A: Chromatograms of standard proteins



B: Chromatograms of standard peptides



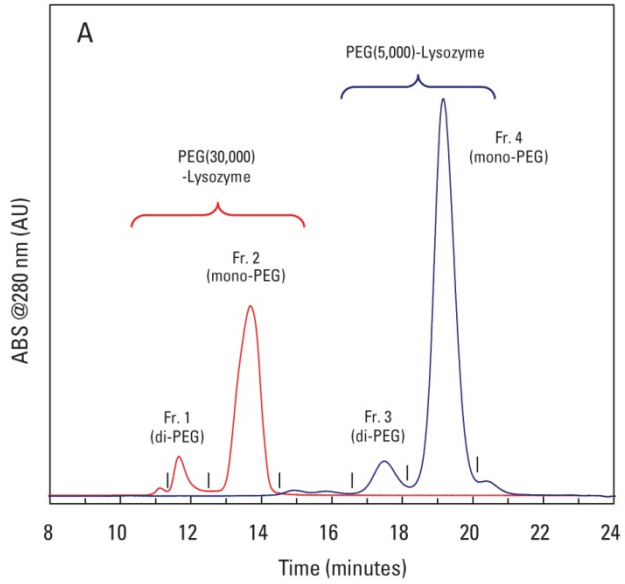
The TSKgel Protein C4-300 column demonstrated superiority over other RPC columns, including ODS and polymer-based columns, in the separation of proteins and large peptides.

Columns: A: TSKgel Protein C4-300, 4.6 mm ID x 15 cm (3 µm particle, 30 nm pore, silica-based)
B: TSKgel ODS-100V, 4.6 mm ID x 15 cm (3 µm particle, 10 nm pore, silica-based)
C: TSKgel ODS-120T, 4.6 mm ID x 15 cm (5 µm particle, 12 nm pore, silica-based)
D: TSKgel Octadecyl-4PW, 4.6 mm ID x 15 cm (7 µm particle, 50 nm pore, polymer-based)
E: TSKgel Octadecyl-NPR, 4.6 mm ID x 3.5 cm (2.5 µm particle, non-porous, polymer-based)
F: TSKgel Phenyl-5PW RP, 4.6 mm ID x 7.5 cm (10 µm particle, 100 nm pore, polymer-based)
Samples are the same as in Figure 1A and Figure 1C.
Other conditions are the same as in Figure 1.

Applications

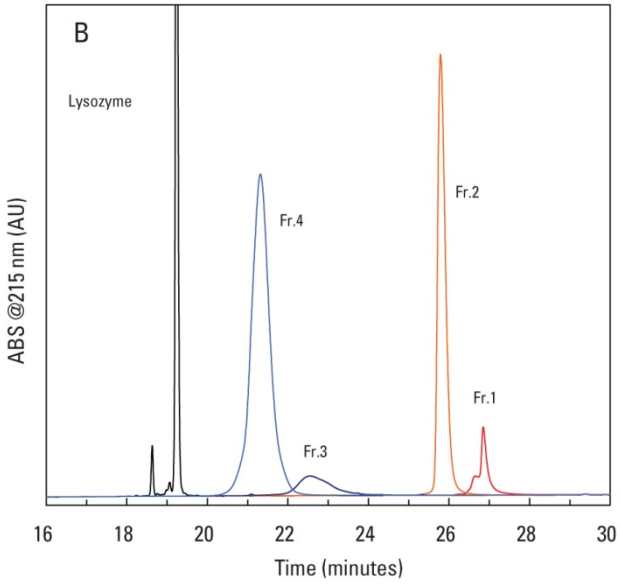
Figure 6: Separation of PEGylated Proteins

A: Separation of PEGylated lysozyme by size exclusion chromatography (SEC)



Column:	TSKgel SuperSW3000, 4.6 mm ID x 30 cm + 4.6 mm ID x 30 cm
Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	0.35 mL/min
Detection:	UV@280 nm
Temperature:	25°C
Injection vol.:	50 µL
Samples:	PEG (MW 5,000)-lysozyme (5 µg/µL) PEG (MW 30,000)-lysozyme (5 µg/µL)

B: Chromatograms of lysozyme and fractions of PEGylated lysozyme collected from SEC obtained with TSKgel Protein C_4-300.

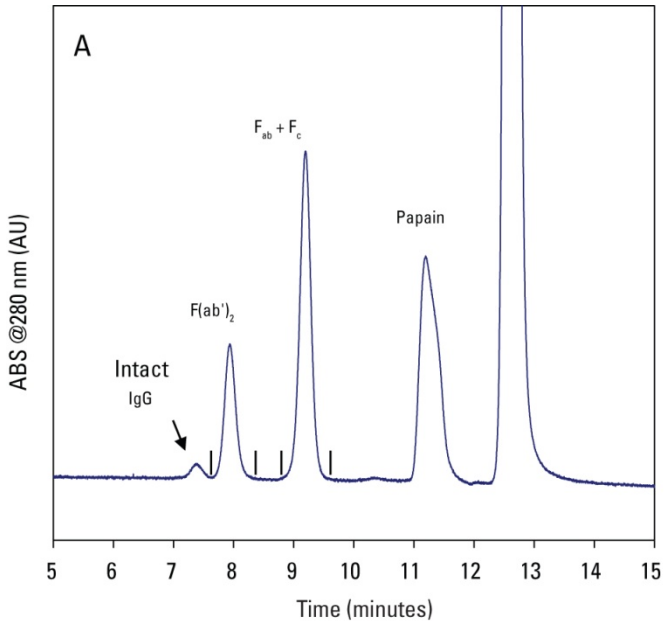


Column:	TSKgel Protein C_4-300, 4.6 mm ID x 15 cm
Injection vol.:	100 µL
Samples:	lysozyme (0.1 µg/µL), SEC fractions 1-4 shown in A

Other conditions are the same as in Figure 1.

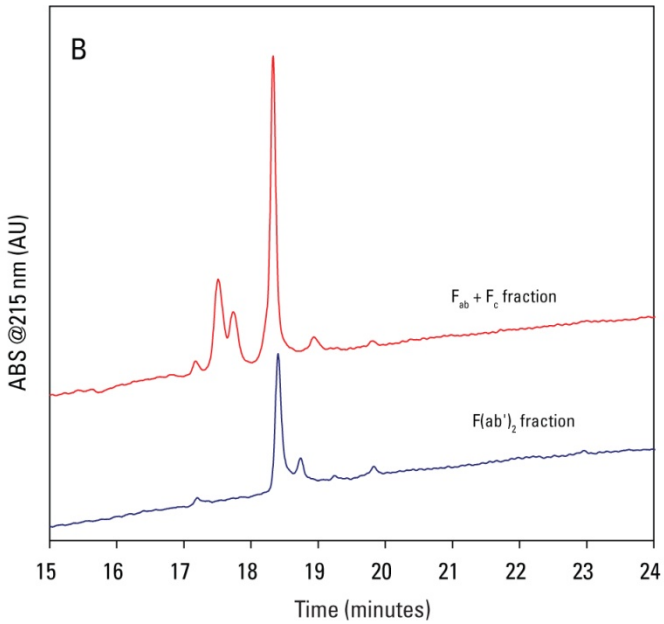
Figure 7: Separation of IgG Fragments

A: Separation of papain-digested IgG by SEC



Column:	TSKgel SuperSW3000, 4.6 mm ID x 30 cm
Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	0.35 mL/min
Detection:	UV@280 nm
Temperature:	25°C
Injection vol.:	100 µL
Sample:	mouse monoclonal antibody (IgG) digested with papain

B: Chromatograms of $F(ab')_2$ fraction and F_{ab}+F_c fraction collected from SEC obtained with TSKgel Protein C_4-300



Column:	TSKgel Protein C_4-300, 4.6 mm ID x 15 cm
Temperature:	50°C
Injection vol.:	100 µL
Samples:	SEC fractions shown in left figure

Other conditions are the same as in Figure 1.

Conclusions

Conclusions

Novel TSKgel Protein C_4-300 RPC columns were developed for the separation of proteins and peptides by optimizing pore size, alkyl chain length, and ligand density of the packing material.

TSKgel Protein C_4-300 exhibited the following characteristics:

- Stability: The column showed no loss in column efficiency, and 90% of initial retention remained after flushing at 40℃ with acidic eluent (0.2% TFA) at 1.0 mL/min for 1,000 hours.
- Flow rate: High resolution was achieved at 1.0 mL/min for the 4.6 mm ID x 15 cm and 5cm columns. Flow rates higher than 1.0 mL/min showed higher resolution with the 5 cm column.
- Gradient time: The resolution was improved with an increase in the gradient time. Using a long gradient time with the 15 cm column was preferable for high resolution analysis. On the other hand, a short gradient time using the 5 cm column was preferable for high speed analysis
- Eluent modifier: In the presence of 0.02%-0.1% TFA in the mobile phase, proteins and peptides were satisfactorily separated. TFA at a concentration of less than 0.02% might result in leading, tailing, or broadening of peaks.
- Sample loading: For sample loading up to 1 µg, the peak width remained constant.

TSKgel Protein C_4-300 demonstrated superior resolving power and comparable recovery to competitive wide-pore C4 columns.

TSKgel Protein C_4-300 demonstrated superiority over other RPC columns, including ODS and polymer-based columns, in the separation of proteins and large peptides.

Applications of protein separation, including PEGylated proteins and IgG fragments, were presented.