TOSOH

SEPARATION REPORT NO. 93

COLUMN FOR HIGH PERFORMANCE, HIGH-BINDING CAPACITY ION EXCHANGE CHROMATOGRAPHY: TSKgel SuperQ-5PW AND ITS APPLICATIONS

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1. Introduction

Ion exchange chromatography is employed widely for protein separation and purification due to its operability and wide variety of application. TSKgel SuperQ-5PW developed this time is a strong anion exchanger, which quaternary ammonium groups are bonded onto TSKgel G5000PW, and high binding capacity, recovery and excellent resolution compared to other conventional ion exchangers. This document introduces the basic properties and applications of TSKgel SuperQ-5PW in protein separation.

2. Basic Properties of TSKgel SuperQ-5PW

2-1 Total Ion Exchange Capacity

TSKgel SuperQ-5PW is a strong anion exchanger, which quaternary ammonium groups are bonded onto TSKgel G5000PW, and its total ion exchange capacity is 0.15 ± 0.02 meq/mL-gel.

2-2 Protein Binding Capacity

Table-1 shows the results of investigating the binding capacity for proteins with different molecular weights on the column dipped (dynamic binding capacity). The binding capacity of TSKgel SuperQ-5PW for bovine serum albumin (BSA) is 100±20g/L-gel which is more than twice that of the conventional TSKgel DEAE-5PW, 40 ± 5 g/L-gel. It also has high binding capacity for proteins with high molecular weight. Bound protein was eluted with 50 mmol/L Tris-HCl buffer containing 0.5 mol/L NaCl (pH 8.6), and the recovery for each protein was 100%.

2-3 Chemical Stability

Table-2 shows the ion exchange capacity and binding capacity for bovine serum albumin when TSKgel SuperQ-5PW is suspended in 0.5N NaOH or 0.5N HCl for 10 days at 25°C. No change was seen in ion exchange capacity and binding capacity for bovine serum albumin for both 0.5N NaOH and 0.5N HCl after 10 days. Then dipping in 0.5N NaOH solution and washing were repeated for the column. The chromatograms at initial time and after washing 15 times are shown in Figure-1.

As you can see from the results, column performance (elution volume and resolution) did not deteriorate even when TSKgel SuperQ-5PW column is dipped in 0.5N NaOH and washed. Nor did protein binding capacity change. Thus TSKgel SuperQ-5PW is stable against both acid and alkali, and it is possible to wash, regenerate or implement CIP (Clean in place) with acid/alkali when the column is contaminated after separation of crude protein purification sample, cell culture, etc.

2-4 Recovery

Recovery was calculated by injecting various proteins to be absorbed after equilibrated the column with 50mmol/L Tris-HCl buffer (pH 8.6) for 30 minutes and then measuring the solution eluted with 50mmol/L Tris-HCl buffer containing 0.5 mol/L NaCl (pH 8.6) after 1 minute with a spectrophotometer at 280 nm. Table-3 shows the results. In a similar procedure to the conventional TSKgel DEAE-5PW, recovery was quantitative for each protein.

Table-1 Dynamic binding capacity for proteins in TSKgel SuperQ-5PW

Protein	Binding capacity (g/L)*
IgG	15
BSA	100
Trypsin inhibitor	136

* Dynamic binding capacity was calculated from frontal analysis.
Column size: 7.5 mm ID × 7.5cm L,
Flow rate: 1.0 ml/min,
Sample: 20 g/l

Table-2 **Chemical stability of TSKgel SuperQ-5PW by soaking in alkaline or acidic solution (10 days at 25°C)**

Ion exchanger	Solution	Ion exchange capacity	
		Before soaking	After soaking
TSKgel SuperQ-5PW	0.5N HCl	0.15	0.15
TSKgel SuperQ-5PW	0.5N NaOH	0.15	0.14

Ion exchanger	Solution	BSA binding capacity	
		Before soaking	After soaking
TSKgel SuperQ-5PW	0.5N HCl	111	111
TSKgel SuperQ-5PW	0.5N NaOH	111	112

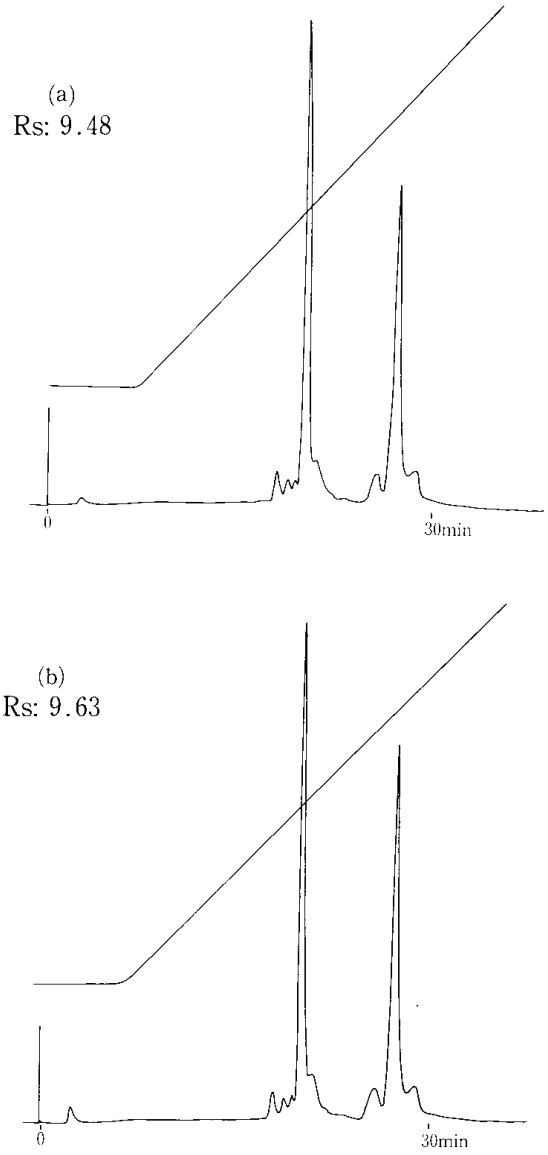

Figure-1 **Chemical stability in TSKgel SuperQ-5PW (CIP washing with 0.5N NaOH)**

Column: TSKgel SuperQ-5PW
7.5 mm ID × 7.5cm
Eluent: A: 50mmol/L Tris-HCl buffer (pH 8.6)
B: A + 0.5mol/L NaCl
A → B linear gradient (60 min.)
Flow rate: 1.0mL/min
Temp.: 25°C
Detection: UV (280nm)
Sample: Ovalbumin (1mg),
Trypsin inhibitor (1mg), 100μL

Column washing:
Flush with 0.5N NaOH in volume 10 times that of the column at 1.0mL/min and the column is sealed and stored (1 day). On the next day, the column was washed with distilled water until the solution eluting from the column becomes neutral. Then column was equilibrated with buffer to measure the protein resolution.

(a) Day 0 (before washing the column)
(b) Day 15 (after washing the column 15 times)

2-5 Resolution

Table-4 shows the comparison of resolution for proteins (ovalbumin/trypsin inhibitor) in various ion exchangers. As it is clear from the table, TSKgel SuperQ-5PW shows an extremely high resolution. Thus it is possible to obtain high resolution even in a short gradient time.

Figure-2 shows the chromatograms for separating 4 types of standard proteins, carbonic anhydrase (bovine red blood cell), transferrin (bovine), ovalbumin (chicken egg), and trypsin inhibitor (soybean).

Table-3 Protein recovery on TSKgel SuperQ-5PW

Protein	Recovery (%)
Thyroglobulin	101
IgG	106
Bovine serum albumin	101
Hemoglobin	99
Ovalbumin	106
β-Lactoglobulin	105
Trypsin inhibitor	100
Myoglobin	101

Each protein of 0.4 mg was applied on Super Q-5PW column (7.5 mm ID × 7.5 cm L) in 0.05mol/L Tris-HCl buffer (pH 8.6) and the bound protein was eluted with 0.05 mol/L Tris-HCl buffer (pH 8.6) containing 0.5 mol/L NaCl

Table-4 Comparison of resolution in various ion exchangers

Column	Column size	Resolution (OVA/STI)
TSKgel SuperQ-5PW	7.5 mm ID × 7.5 cm L	11.05 (8.44) *
TSKgel DEAE-5PW Glass	5 mm ID × 5 cm L	8.10
Company A, perfusion Q type	5 mm ID × 5 cm L	5.58
Company A, Q type	6.4 mm ID × 3 cm L	4.61
	5 mm ID × 5 cm L	5.85

Elution conditions conform to Figure-1.

*: 30-minute linear gradient

3. Effect of Elution Conditions on Resolution

3-1 Effect of Sample Load

Using ovalbumin (chicken egg), trypsin inhibitor (soybean) and β-lactoglobulin (bovine milk) as samples, sample load was investigated by changing the injection volume. The results are shown in Figures-3 and -4. Protein is absorbed and separated well even in the maximum sample load of 150 mg and 100 mg in this experiment. Though it varies depending on the sample, the sample load at which the peak shape and elution time did not change largely was approximately 100mg for mixture of ovalbumin and trypsin inhibitor, and approximately 40 mg for β-lactoglobulin.

Next, a comparison of separation under large sample load among various ion exchangers is shown in Figure-5. When 40 mg protein sample is loaded for 1mL column volume, only TSKgel SuperQ-5PW shows a normal chromatogram (see Figure-2). For other ion exchangers, appearance of false peak by sample overloading is prominent.

As discussed above, TSKgel SuperQ-5PW achieves sufficient retention and resolution even under the protein load of 100 mg and higher (column size: 7.5 mm ID × 7.5 cm L). Thus the semi-fractionation is possible on TSKgel SuperQ-5PW with analysis column size.

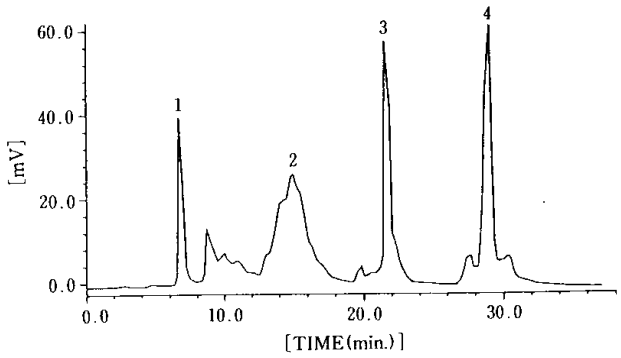

Figure-2 Separation of protein mixture

Conditions are similar to Figure-1.
However, sample: 1. Carbonic anhydrase (2mg)
2. Transferrin (4mg)
3. Ovalbumin (5mg)
4. Trypsin inhibitor (5mg)
Injection volume 100μL

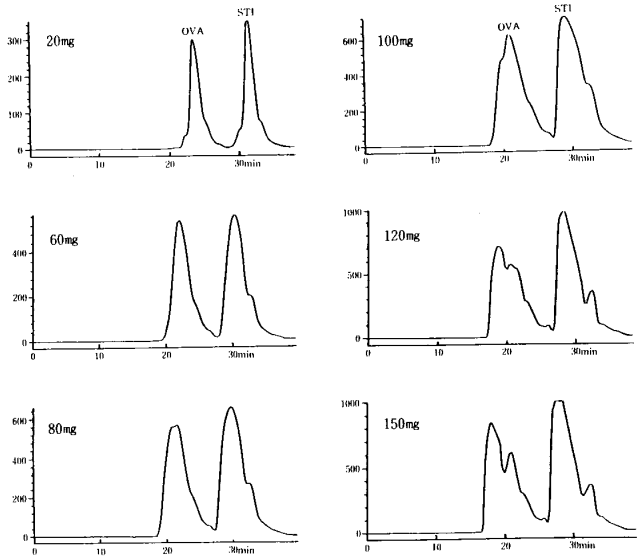

Figure-3 Effect of sample load on protein separation on TSKgel SuperQ-5PW (1)

Column: TSKgel SuperQ-5PW 7.5 mm ID × 7.5 cm L
Eluent: A: 50 mmol/L Tris-HCl buffer (pH 8.3)
B: A + 0.5 mol/L NaCl
A → B linear gradient (60 min.)
Flow rate: 1.0 mL/min
Temp.: 25°C
Detection: UV (280 nm)
Sample: Ovalbumin, trypsin inhibitor (10 g/L each), 2 to 7.5 mL

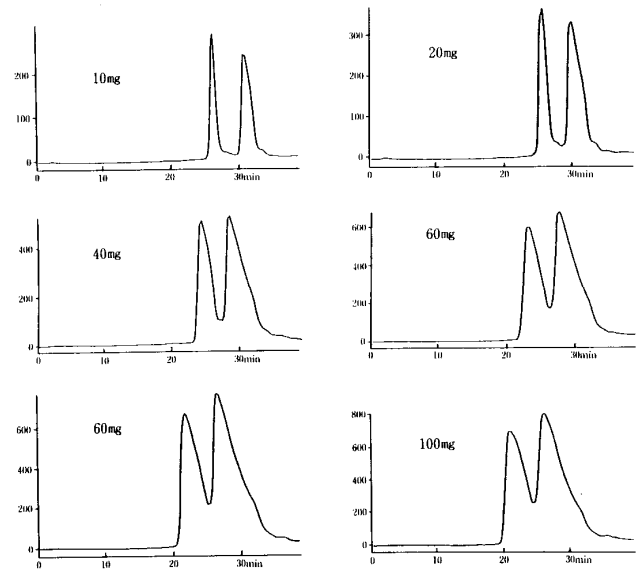

Figure-4 Effect of sample load on protein separation on TSKgel SuperQ-5PW (2)

Column: TSKgel SuperQ-5PW 7.5 mm ID × 7.5 cm L
Eluent: A: 20 mmol/L piperazine buffer (pH 6.0)
B: A + 0.3 mol/L NaCl
A → B linear gradient (60 min.)
Flow rate: 1.0 mL/min
Temp.: 25°C
Detection: UV (280nm)
Sample: β-lactoglobulin (20g/L), 0.5 to 5mL

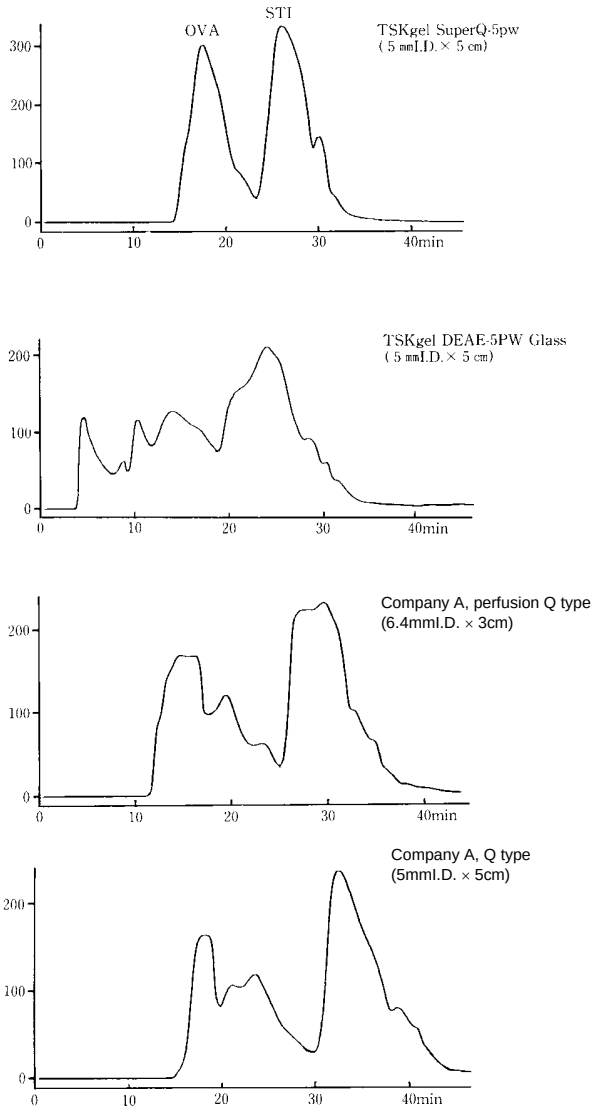

Figure-5 Comparison of separation with various ion exchangers under large sample load

Column: TSKgel SuperQ-5PW (5 mm ID × 5 cm L)
TSKgel DEAE-5PW Glass (5 mm ID × 5 cm L)
Company A, perfusion Q type (6.4 mm ID × 3 cm L)
Company A, Q type (5 mm ID × 5 cm L)
All column volumes were 1.0 mL.
Eluent: A: 50 mmol/L Tris-HCl buffer (pH 8.3)
B: A + 0.5 mol/L NaCl
A → B linear gradient (60 min.)
Flow rate: 0.8 mL/min
Temp.: 25°C
Detection: UV (280 nm)
Sample: Ovalbumin (20 mg), Trypsin inhibitor (20 mg), Injection volume 2mL

3-2 Effect of Measurement Flow Rate

Figure-6 shows the chromatograms for separation of ovalbumin and trypsin inhibitor by fixing the gradient time and varying the measurement flow rate to 0.25, 0.5 and 1.0 mL/min. In the flow rate range investigated, separation period was shorter (elution was faster) and resolution was higher as the flow rate was faster.

3-3 Effect of Gradient Time

Figure-7 shows the chromatograms for separation of ovalbumin and trypsin inhibitor by fixing the measurement flow rate and varying the gradient time to 30, 60 and 120 minutes. Though higher resolution is obtained as the gradient time is longer, the period required for analysis becomes longer and dilution of the sample becomes large. Therefore, it is considered that the gradient time from 30 to 60 minutes is appropriate.

3-4 Effect of Salt Concentration in Sample Solution

Protein elution behavior was checked by dissolving ovalbumin or trypsin inhibitor as sample in salt-containing 50mmol/L Tris-HCl buffer (pH 8.6) and varying the salt in sample solution to 0.1 mol/L, 0.2 mol/L and 0.3 mol/L to inject the solution into the column. Figure-8 shows the chromatograms when the salt concentration is varied in sample solution for TSKgel SuperQ-5PW. As you can see from the figure, though there is no change in protein elution with 0.1 mol/L NaCl, a peak is seen near the unretained region (V_0) for 0.2 mol/L and higher. The protein starts to leak with the peak areas of ovalbumin and trypsin inhibitor decreasing as the salt concentration increases. Figure-9 shows the chromatograms when the salt concentration in sample solution is set to 0.1 mol/L NaCl and sample load is varied from 0.2 mg to 10mg. Separation did not deteriorate for salt concentration of 0.1 mol/L NaCl even when the sample load is increased, and no protein elution is seen near V_0. Thus the salt concentration in sample solution of 0.1 mol/L and lower is recommended for TSKgel SuperQ-5PW.

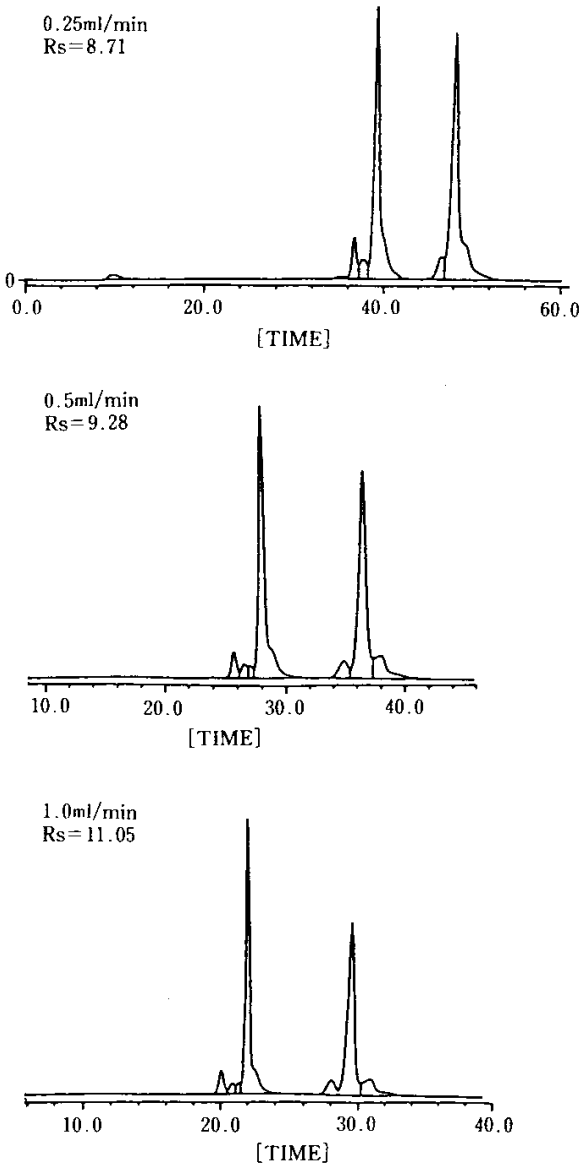

Figure-6 Effect of flow rate on protein separation
Column: TSKgel SuperQ-5PW 7.5 mm ID × 7.5 cm L
Conditions are identical to Figure-1.
However, Flow rate: 0.25, 0.5, and 1.0 mL/min.

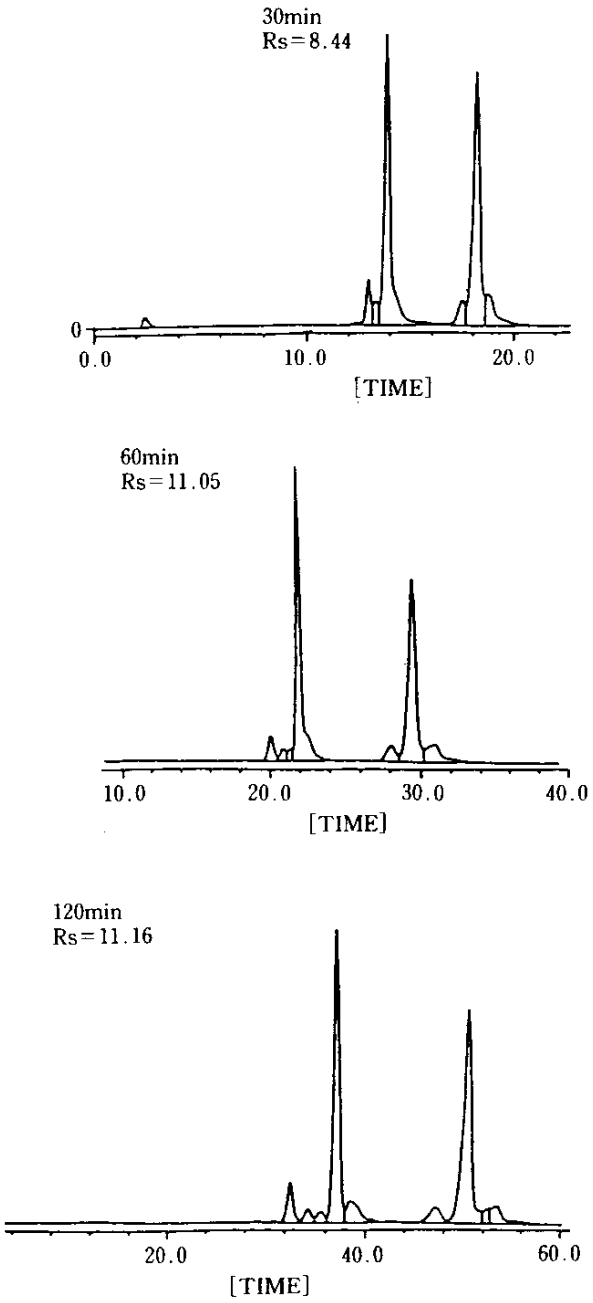

Figure-7 **Effect of gradient time in protein separation**

Column: TSKgel SuperQ-5PW 7.5mmI.D. × 7.5cm
Conditions are identical to Figure-1.
However, gradient time: 30, 60, and 120 minutes.

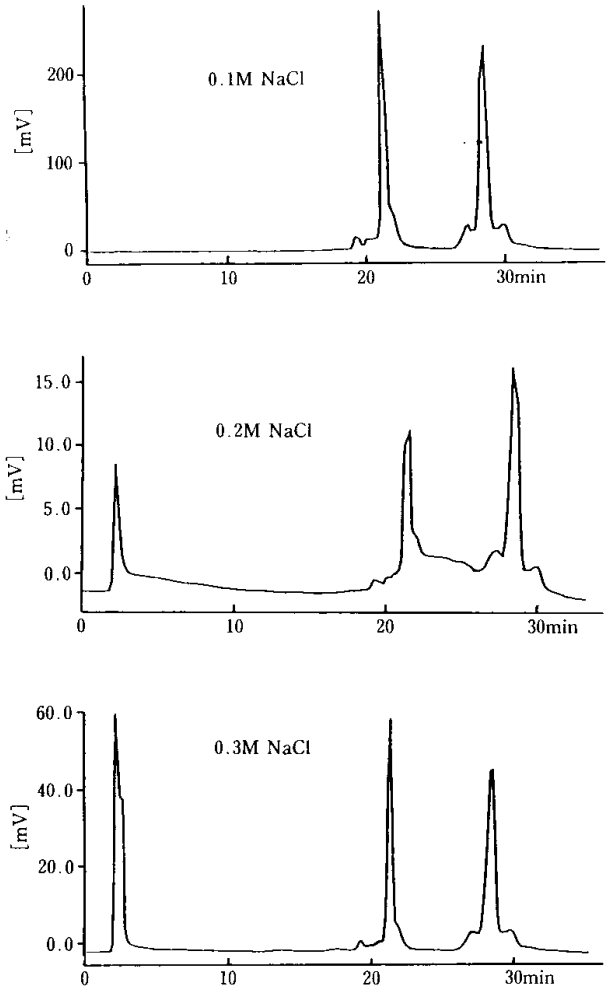

Figure-8 **Effect of salt concentration in sample solution (1)**

Column: TSKgel SuperQ-5PW
7.5 mm ID × 7.5 cm L
Eluent: A: 50 mmol/L Tris-HCl buffer (pH 8.3)
B: A + 0.5 mol/L NaCl
A → B linear gradient (60 min.)
Flow rate: 1.0mL/min
Temp.: 25°C
Detection: UV (280 nm)
Sample: Ovalbumin, trypsin inhibitor
(0.5 mg in 500 μL for each)
The salt concentration in sample solution is 0.1, 0.2 and 0.3 mol/L NaCl.

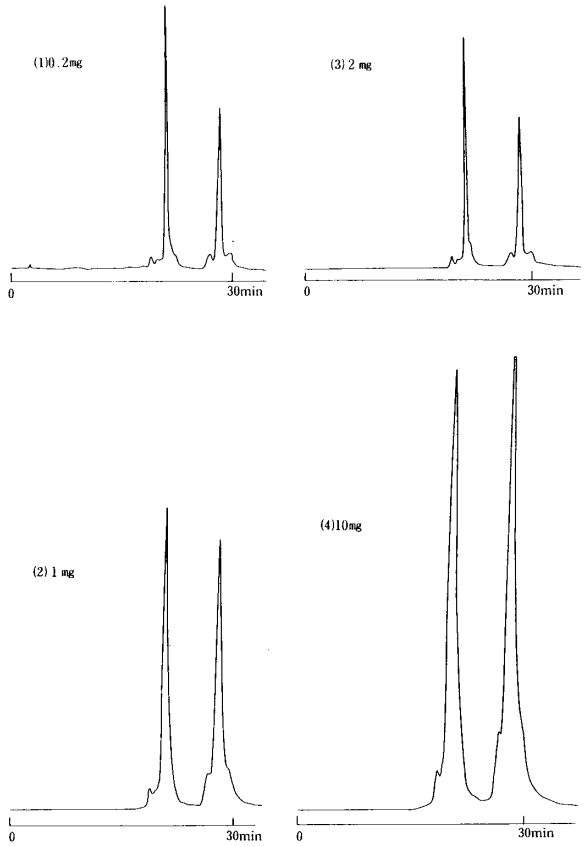

Figure-9 Effect of salt concentration in sample solution (2)

Conditions are identical to Figure-7.
However, sample is (1) 1 g/L each, 100 μL
(2) 2 g/L each, 500 μL
(3) 10 g/L each, 100 μL
(4) 10 g/L each, 500 μL
The salt concentration in sample is 0.1 mol/L NaCl.

4. Protein Separation (Applications)

4-1 Separation of Monoclonal Antibodies

Figures-10 and -11 show an example of separating different monoclonal antibodies (IgG_1) from mouse ascites by changing the sample injection volume. The sample injection volume was changed from 100 μL to 5,000 μL. In Figure-10, nearly identical chromatogram is obtained up to injection volume of 1000 μL. In addition, when the obtained monoclonal antibody fraction was examined using size exclusion chromatography (TSKgel $G3000SW_{XL}$), high-purity preparations were obtained for both with 92% purity. Though mixture of impurity peak is somewhat seen in injection volume of 5,000 μL, favorable separation is obtained. The purity of the obtained monoclonal fraction was 89%.

In separation of another monoclonal antibody in Figure-11, impurities in mouse ascites are separated extremely well, and monoclonal antibody with purity as high as the analysis level, 94% is obtained even at injection volume of 5,000 μL. Therefore, it seems possible to have sample injection in larger volumes for this sample.

4-2 Separation of Egg White

An example of separating chicken egg white under standard elution conditions is shown in Figure-12.

4-3 Separation of Urease

An example of separating commercial urease (Jack Beans) is shown in Figure-13.

4-4 Separation of Lipoxidase

An example of separating commercial crude lipoxidase (soybean) is shown in Figure-14.

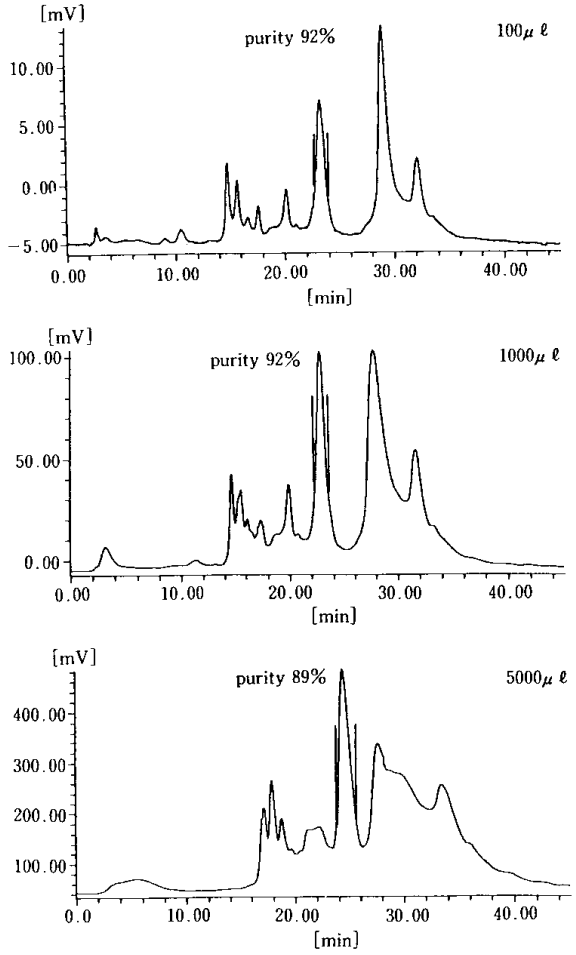

Figure-10 Separation of mouse monoclonal antibody A (IgG_1)

Column: TSKgel SuperQ-5PW
7.5 mm ID × 7.5 cm L
Eluent: A: 20 mmol/L Tris-HCl buffer (pH 8.5)
B: A + 0.5 mol/L NaCl
A → B linear gradient (60 min.)
Flow rate: 1.0 mL/min
Temp.: 25°C
Detection: UV (280 nm)
Sample: Mouse ascites (× 3), filtered with myshoridisk after dilution with buffer.
Injection volume 100, 1,000, 5,000 μL

*Purity of the IgG_1 fraction was calculated from the peak area by size exclusion chromatography.

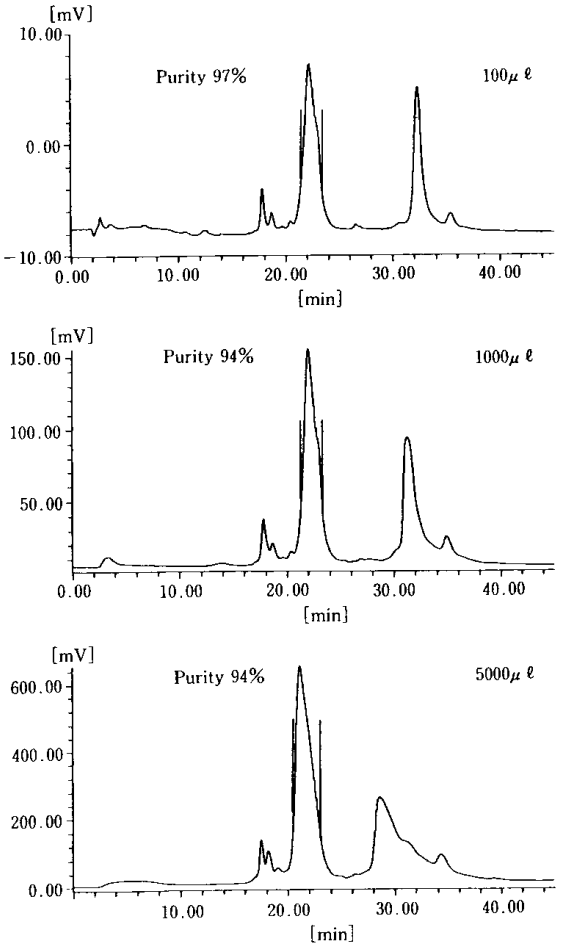

Figure-11 Separation of mouse monoclonal antibody B (IgG_1)

Conditions are identical to Figure-9.

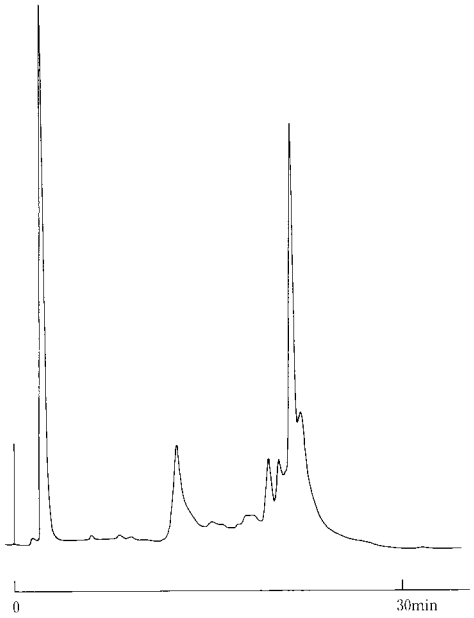

Figure-12 Separation of chicken egg white

Column:	TSKgel SuperQ-5PW 7.5 mm ID × 7.5 cm L
Eluent:	A: 50 mmol/L Tris-HCl buffer (pH 8.6) B: A + 0.5 mol/L NaCl A → B linear gradient (60 min.)
Flow rate:	1.0 mL/min
Temperature:	25°C
Detection:	UV (280 nm)
Sample:	Chicken egg white, 1 g/L, 100 μL

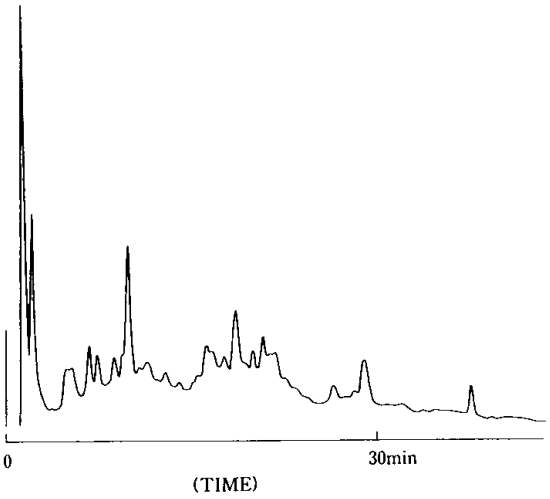

Figure-13 Separation of urease (Jack Beans)

Conditions are identical to Figure-12.
However, sample: 10 g/L, 100 μL

5. Scale up from TSKgel SuperQ-5PW to TOYOPEARL SuperQ 650

A preparative column (column size 21.5 mm ID × 15 cm L) is available for purification of samples in large volumes using TSKgel SuperQ-5PW. However, use of packing materials for medium-speed chromatography (MPLC) may be more advantageous instead of HPLC columns when the purpose is to separate large volumes of sample or to purify for industrial application.

In the case of TSKgel SuperQ-5PW, it is possible to increase the scale employing TOYOPEARL SuperQ 650. Figure-15 shows the chromatograms of separating under the same conditions on TSKgel SuperQ-5PW, TOYOPEARL SuperQ 650S (35μm), and TOYOPEARL SuperQ 650M (65 μm). The elution time of sample was nearly identical, and it is indicated that TSKgel SuperQ-5PW and TOYOPEARL SuperQ 650 have nearly the same selectivity (resolution is inversely proportional to the particle diameter of the packing material).

Then resolution of SuperQ-TOYOPEARL650S was compared to that of TSKgel SuperQ-5PW by changing its gradient time. The results are shown in Figure-16. Compared to TSKgel SuperQ-5PW (20-minute gradient), SuperQ-TOYOPEARL650S achieves separation of impurity peak which is substantially close to that of TSKgel SuperQ-5PW through 150-minute gradient.

Figures-17 and -18 show the case of scale up on TOYOPEARL SuperQ 650M. With TOYOPEARL SuperQ 650M, particle size is considerably larger (65 μm) than that of HPLC columns, and resolution can be similar to that of TSKgel SuperQ-5PW by setting the gradient time long or making the column length longer.

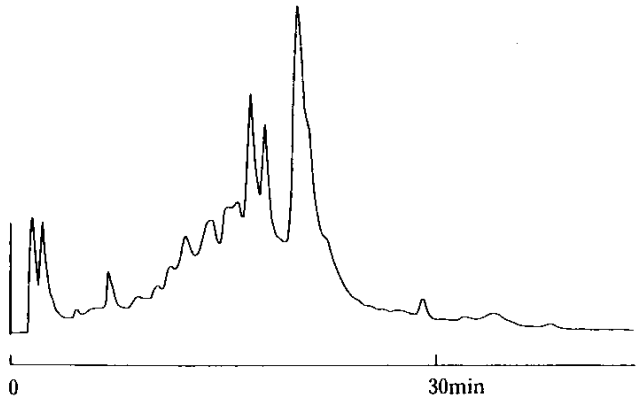

Figure-14 Separation of commercial lipoxidase

Conditions are identical to Figure-12.
However, sample: 6 g/L, 100 μL

6. Conclusion

This document introduced the basic properties of a packed column for high-speed ion exchange chromatography with high protein binding capacity, TSKgel SuperQ-5PW, and applications of protein separation. TSKgel SuperQ-5PW not only is excellent in its resolution, but also in chemical stability due to the very high protein binding capacity, and it is optional for high-purity purification at analysis level (analysis column) and separation/fractionation of proteins in large volumes from multi-component samples such as crude extraction sample. Table-5 shows the standard conditions for use of TSKgel SuperQ-5PW. In addition, TOYOPEARL SuperQ 650 has already been commercialized as the process media, and it is expected for application and expansion in the field of biochemistry since its product mix allows scale up, large-volume processing and industrial separation and purification.

Table-5 Standard conditions for use of TSKgel SuperQ-5PW

Column size	7.5 mm ID × 7.5 cm L
Elution conditions	0.5 to 1.0 mL/min
Flow rate	20 mmol/L Tris-HCl buffer (pH7.5 to pH8.6)
	5 times the column volume or longer
Buffer	0 to 0.5 mol/L NaCl
Equilibration time	(Resolution improved with 0 to 0.3
Salt concentration	mol/L NaCl)
Gradient time	20 to 100 min
Temperature	4 to 25°C
Detection	UV
Sample	100 μg to 200 mg
Sample load	100 μL to 10 mL
Injection volume	0.1 mol/L or lower (dilution or
Salt concentration	dialysis)
Insoluble	Filtered with a filter (myshoridisk, etc.)

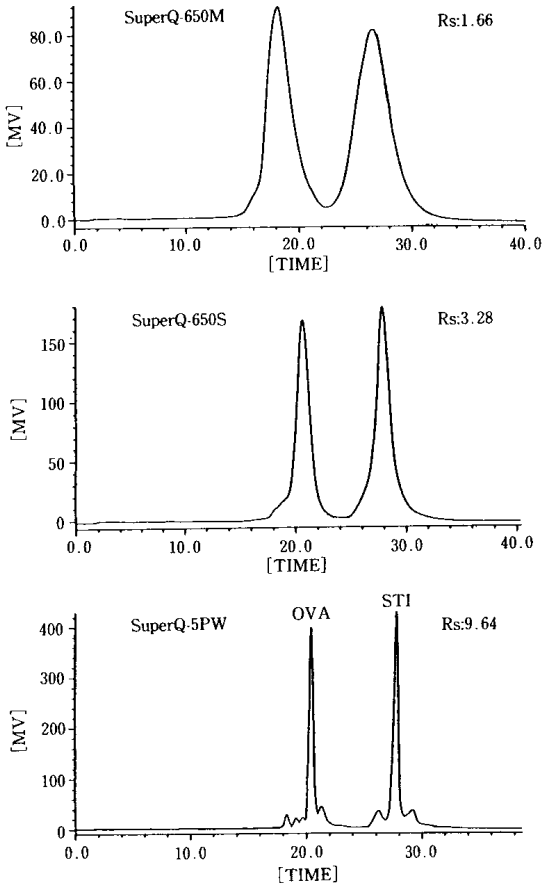

Figure-15 Scale up from TSKgel SuperQ-5PW to TOYOPEARL SuperQ 650S (1)

Column:	TSKgel SuperQ-5PW (10 μL)
	TOYOPEARL SuperQ 650S (35 μm)
	TOYOPEARL SuperQ 650M (65 μm)
	All 7.5 mm ID × 7.5 cm
Eluent:	A: 50 mmol/L Tris-HCl buffer (pH 8.3)
	B: A + 0.5 mol/L NaCl
	A → B linear gradient (60 min.)
Flow rate:	1.0 mL/min
Temperature:	25°C
Detection:	UV (280 nm)
Sample:	Ovalbumin (20 mg),
	trypsin inhibitor (1 mg each)

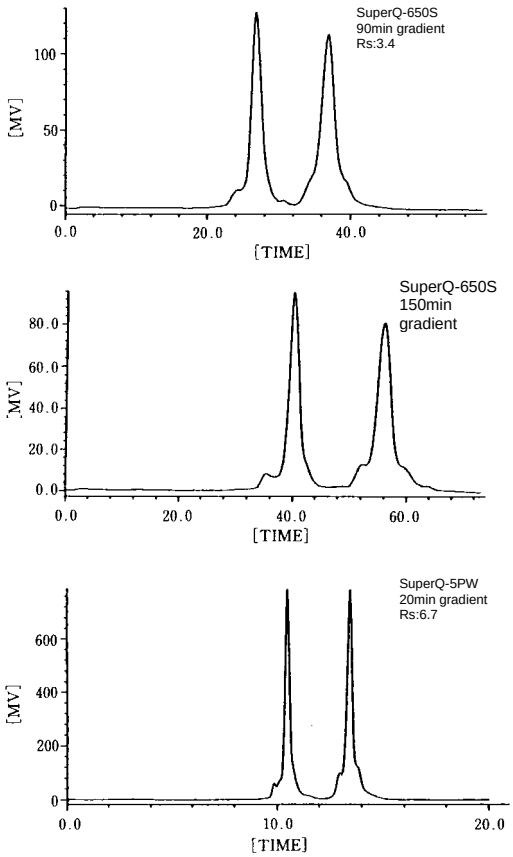

Figure-16 Scale up from TSKgel SuperQ-5PW to TOYOPEARL SuperQ 650S (2)

Conditions are identical to Figure-15.
However, the gradient time is 20 to 150min.

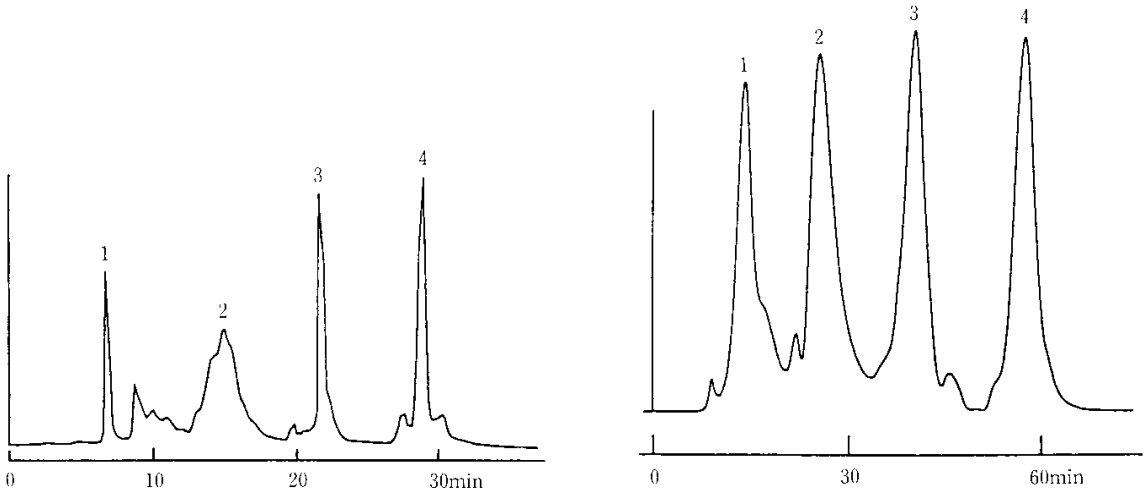

Figure-17 **Scale up from TSKgel SuperQ-5PW to TOYOPEARL SuperQ 650 (3)**

Column: (a) TSKgel SuperQ-5PW 7.5mmI.D. × 7.5cm
(b) TOYOPEARL SuperQ 650M 16mmI.D. × 15cm
Eluent: A: 50mmol/L Tris-HCl buffer (pH 8.3)
B: A + 0.5mol/L NaCl
A → B linear gradient (a) 60 min., (b) 100 min.
Flow rate: (a) 1.0mL/min (b) 2.0mL/min
Temperature: 25°C
Detection: UV (280nm)
Sample: 1. Carbonic anhydrase
2. Transferrin
3. Ovalbumin
4. Trypsin inhibitor
(Sample concentration is identical to Figure-2.) (a) 1.6mg (b) 5.4mg

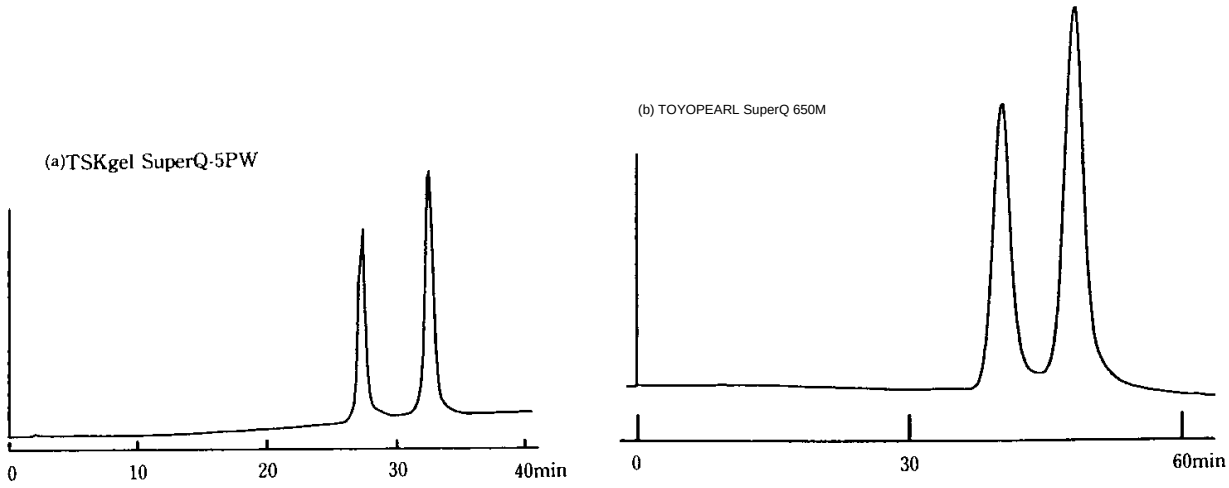

Figure-18 **Scale up from TSKgel SuperQ-5PW to TOYOPEARL SuperQ 650M (4)**

Conditions are identical to Figure-17.
However, sample: β-lactoglobulin
(a) 2mg (b) 50mg