*Separation Report No. 093*

# 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 the analysis and purification of proteins simply because every protein carries a characteristic charge in solution. TSKgel SuperQ-5PW columns are classified as strong anion exchange columns. Using a proprietary bonding technology, quaternary ammonium groups are chemically attached to spherical 10 micron, methacrylic, TSKgel G5000PW base beads, resulting in high binding capacity as well as excellent recovery and resolution compared to other strong anion exchanger columns. This Separation Report introduces the basic properties and applications of TSKgel SuperQ-5PW as it applies to protein separations.

## 2. Basic Properties of TSKgel SuperQ-5PW

### 2-1 Total Ion Exchange Capacity

TSKgel SuperQ-5PW columns are part of the class of strong anion exchange columns, carrying quaternary ammonium groups that are chemically attached to spherical 10 micron, methacrylic, TSKgel G5000PW beads. The total ion exchange capacity of TSKgel SuperQ-5PW columns is 0.15 ± 0.02meq/mL.

### 2-2 Protein Binding Capacity

Table 1 shows the dynamic binding capacities for proteins of different molecular weights on a TSKgel SuperQ-5PW column. The binding capacity of the TSKgel SuperQ-5PW column for bovine serum albumin (BSA) is 100±20g/L-gel which is more than twice that on a TSKgel DEAE-5PW column, 40±5g/L-gel, which features a more conventional bonding chemistry. Lower binding capacity was measured for IgG, which has a higher molecular weight. Bound protein was eluted with 50mmol/L Tris-HCl buffer containing 0.5mol/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 static binding capacity for bovine serum albumin when TSKgel SuperQ-5PW particles are 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. We also investigated repeated cleaning of the column with 0.5N NaOH solution. The chromatograms after the first cycle 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 the TSKgel SuperQ-5PW column is cleaned with 0.5N NaOH, nor did the protein binding capacity change. Thus TSKgel SuperQ-5PW is stable in acidic and basic solutions; the columns can be regenerated or cleaned in place (CIP) with either acid or base following the separation of crude protein purification sample, cell culture, etc.

### 2-4 Recovery

Recovery was determined by injecting various proteins onto the column in starting buffer (50mmol/L Tris-HCl buffer, pH 8.6), followed by flushing the column with starting buffer for 30 minutes before eluting the protein with 50mmol/L Tris-HCl buffer containing 0.5mol/L NaCl (pH 8.6). The measurements were performed with a spectrophotometer at 280nm. Table 3 shows the results. Using a similar procedure for a conventional TSKgel DEAE-5PW column, recovery was quantitative for each protein.

**Table 1 Dynamic binding capacity for proteins on a TSKgel SuperQ-5PW column**

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

* Dynamic binding capacity was calculated from frontal analysis.

Column size: 7.5mm ID × 7.5cm
Flow rate: 1.0mL/min
Sample: 20g/L

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

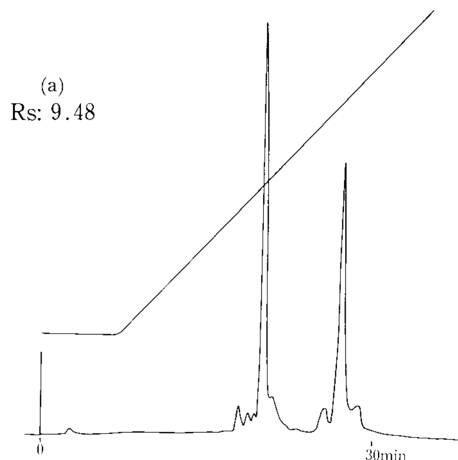
| | | Ion exchange capacity | |
|---|---|---|---|
| **Ion exchanger** | **Solution** | **Before soaking** | **After soaking** |
| TSKgel SuperQ-5PW | 0.5N HCl | 0.15 | 0.15 |
| TSKgel SuperQ-5PW | 0.5N NaOH | 0.15 | 0.14 |

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

**Table 3 Protein recovery on a TSKgel SuperQ-5PW column**

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

0.4mg of each protein was applied to a TSKgel SuperQ-5PW column, 7.5mm ID × 7.5cm, in 0.05mol/L Tris-HCl buffer (pH 8.6) and the bound protein was eluted with 0.05mol/L Tris-HCl buffer (pH 8.6) containing 0.5mol/L NaCl.



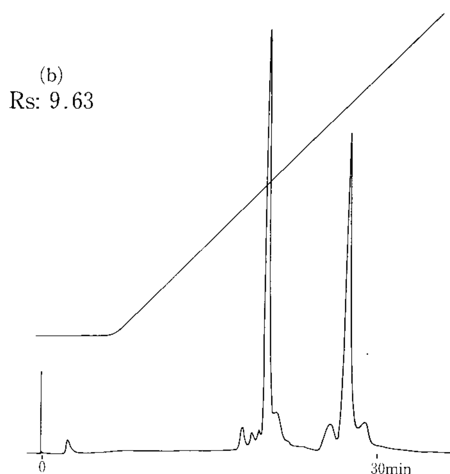




**Figure 1 Chemical stability of a TSKgel SuperQ-5PW column (CIP with 0.5N NaOH)**

Column: TSKgel SuperQ-5PW, 7.5mm ID × 7.5cm

Eluent: A: 50mmol/L Tris-HCl buffer, pH 8.6
B: A + 0.5mol/L NaCl
A → B linear gradient (60min)
Flow rate: 1.0mL/min
Temperature: 25 °C
Detection: UV@280nm
Sample: ovalbumin, 1mg
trypsin inhibitor, 1mg, 100µL

Column washing:
The column was flushed with 10 column volumes (CV) of 0.5N NaOH at 1.0mL/min and stored in this solution for one day. The next day the column was washed with distilled water until the effluent was pH neutral. After equilibration with buffer, the sample was injected and protein resolution measured.

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

## 2-5 Resolution

Table 4 compares the resolution of two globular proteins (ovalbumin/trypsin inhibitor) on various ion exchangers. As it is clear from the table, resolution on the TSKgel SuperQ-5PW column is much higher than on the other columns tested. Thus it is possible to obtain high resolution even using a short gradient time.
Figure 2 shows the chromatograms for the separation of four types of standard proteins, carbonic anhydrase (bovine red blood cell), transferrin (bovine), ovalbumin (chicken egg), and trypsin inhibitor (soybean).

**Table 4 Comparison of resolution in various ion exchangers**

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

Elution conditions conform to Figure 1.
*: 30-minute linear gradient

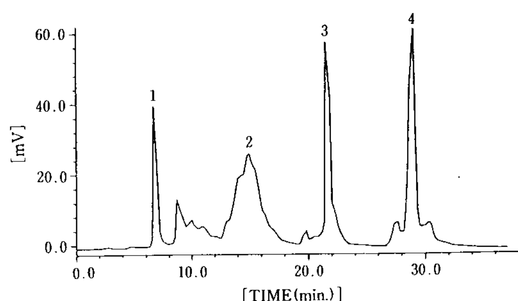



**Figure 2 Separation of a standard protein mixture**

Conditions as in Figure 1

However, sample: 1. carbonic anhydrase (2mg)
2. transferrin (4mg)
3. ovalbumin (5mg)
4. trypsin inhibitor (5mg)

Injection volume, 100µL

## 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. Sample load is defined as the mass of sample loaded onto the column at which the desired sample purity can no longer be obtained. The results are shown in Figures 3 and 4. Protein is absorbed and separated well even in the maximum sample load of 150mg (Figure 3) and 100mg (Figure 4) in this experiment. Though it varies depending on the sample, the sample load at which the peak shape and elution time did not change significantly was approximately 100mg for a mixture of ovalbumin and trypsin inhibitor and approximately 40mg for β-lactoglobulin.
In Figure 5 we compare a separation performed under high sample load conditions on various anion exchange columns. When a 40mg protein sample is loaded on a 1mL column volume, only the TSKgel SuperQ-5PW column shows a chromatogram with 'normal' looking peaks (see Figure 2). Other anion exchange columns show multiple artifact peaks from sample overloading.
As discussed above, even at protein loads of 100mg and above, a 7.5mm ID × 7.5cm TSKgel SuperQ-5PW column provides sufficient retention and resolution. Thus, isolation of proteins at semi-preparative scale is possible on TSKgel SuperQ-5PW when using an analytical column.

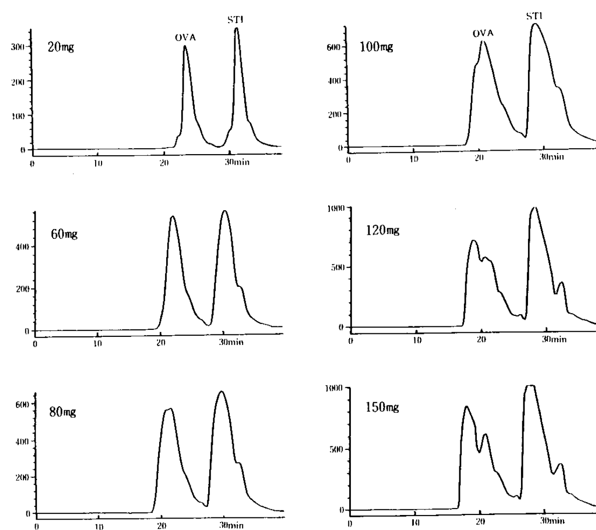



**Figure 3 Effect of sample load on protein separation on a TSKgel SuperQ-5PW column (1)**

Column: TSKgel SuperQ-5PW, 7.5mm ID × 7.5cm

Eluent: A: 50mmol/L Tris-HCl buffer, pH 8.3
B: A + 0.5mol/L NaCl
A → B linear gradient (60 min)
Flow rate: 1.0mL/min
Temperature: 25 °C
Detection: UV@280nm
Sample: ovalbumin
trypsin inhibitor
10g/L each, 2 to 7.5mL

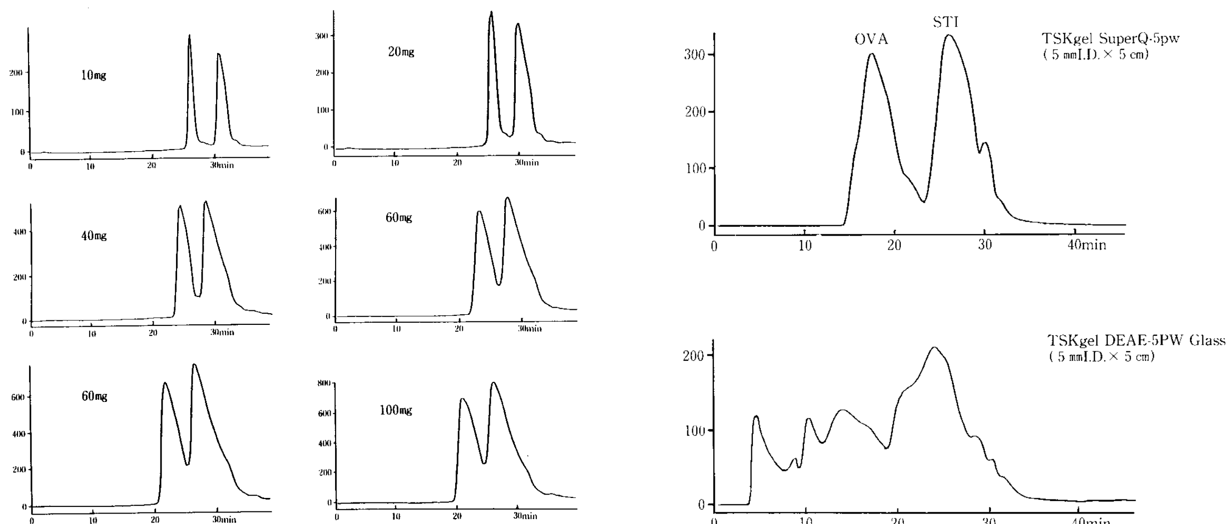



**Figure 4** **Effect of sample load on protein separation on a TSKgel SuperQ-5PW column (2)**

Column: TSKgel SuperQ-5PW, 7.5mm ID × 7.5cm

Eluent: A: 20mmol/L piperazine buffer, pH 6.0
B: A + 0.3mol/L NaCl
A → B linear gradient (60 min)
Flow rate: 1.0mL/min
Temperature: 25 °C
Detection: UV@280nm
Sample: β-lactoglobulin, 20g/L, 0.5 to 5mL

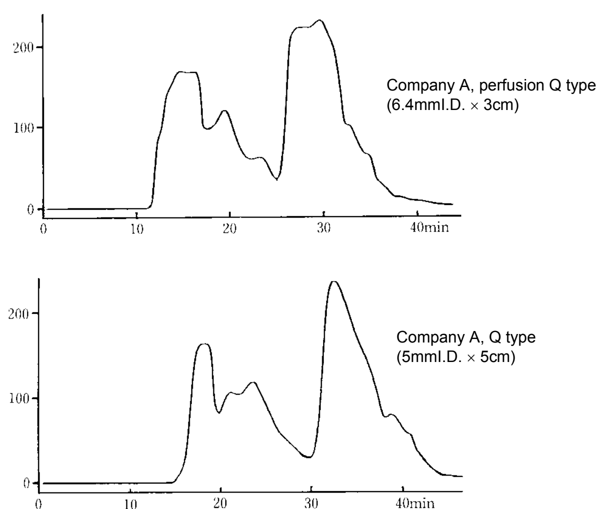



**Figure 5** **Comparison of various anion exchange columns under large sample load**

Column: TSKgel SuperQ-5PW, 5mm ID × 5cm
TSKgel DEAE-5PW Glass, 5mm ID × 5cm
Company A, perfusion Q type, 6.4mm ID × 3cm
Company A, Q type, 5mm ID × 5cm
All column volumes were 1.0mL

Eluent: A: 50mmol/L Tris-HCl buffer, pH 8.3
B: A + 0.5mol/L NaCl
A → B linear gradient (60 min)
Flow rate: 0.8mL/min
Temperature: 25 °C
Detection: UV@280nm
Injection vol.: 2mL
Sample: ovalbumin, 20mg
trypsin inhibitor, 20mg

## 3-2 Effect of Flow Rate

Figure 6 shows chromatograms of ovalbumin and trypsin inhibitor using a constant gradient time and flow rates varying from 0.25 to 0.5 to 1.0mL/min. Over the flow rate range studied, analysis time was shorter and resolution higher the faster the flow rate.

## 3-3 Effect of Gradient Time

Figure 7 shows chromatograms of ovalbumin and trypsin inhibitor at a constant flow rate (1mL/min) and varying gradient times from 30 to 60 to 120 minutes. Although higher resolution is obtained at longer gradient times, analysis time increases and peak height decreases due to sample dilution. Therefore, a gradient time from 30 to 60 minutes is a good balance between resolution and analysis time.

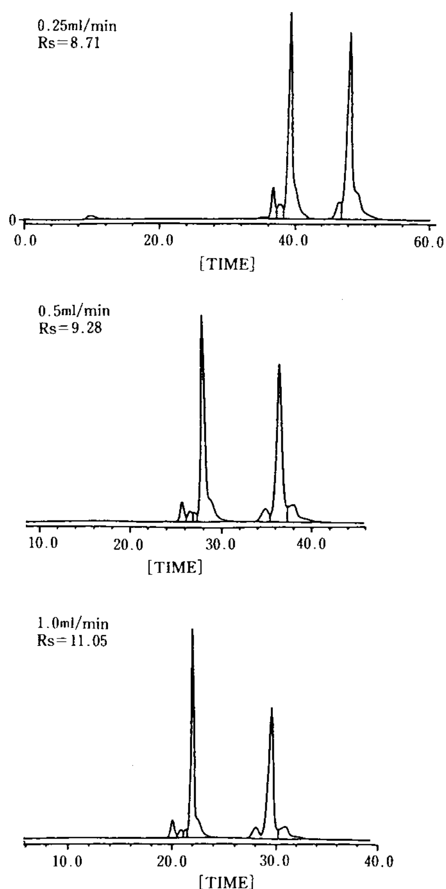



**Figure 6 Effect of flow rate on protein separation**

Column: TSKgel SuperQ-5PW, 7.5mm ID × 7.5cm

Flow rate: 0.25, 0.5, and 1.0mL/min

Other conditions identical to Figure 1

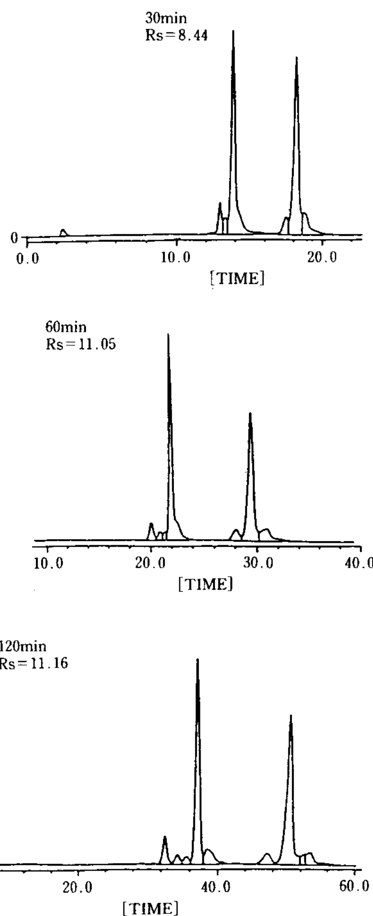



**Figure 7 Effect of gradient time on protein separation**

Column: TSKgel SuperQ-5PW, 7.5mm ID × 7.5cm

Gradient time: 30min, 60min, and 120min

Other conditions identical to Figure 1

## 3-4 Effect of Salt Concentration in Sample Solution

The effect of salt in the sample solution on protein retention was studied by dissolving ovalbumin or trypsin inhibitor in 50mmol/L Tris-HCl buffer (pH 8.6) to which various salt concentrations were added (0.1mol/L, 0.2mol/L and 0.3mol/L). Figure 8 shows the chromatograms obtained on a TSKgel SuperQ-5PW column for the three salt concentrations studied. As can be seen in the figure, though there is no change in protein elution when 0.1mol/L NaCl was added to the sample solution, a peak is seen near the retention time for an unretained component ($V_0$) at salt concentrations of 0.2mol/L and higher. Evidently, some protein is transported through the column with the mobile phase without interacting with the anion exchange groups, while the remaining protein elutes from the column as expected. Note that the peak areas of ovalbumin and trypsin inhibitor decrease with increasing salt concentration.

Figure 9 shows chromatograms obtained when the salt concentration in the sample solution is maintained at 0.1mol/L NaCl and sample load is varied from 0.2mg to 10mg. The separation did not deteriorate for a salt concentration of 0.1mol/L NaCl even when the sample load is increased, and no protein elution is seen near $V_0$. Thus the salt concentration in a sample solution of 0.1mol/L and lower is recommended for a 7.5mm ID × 7.5cm TSKgel SuperQ-5PW column.

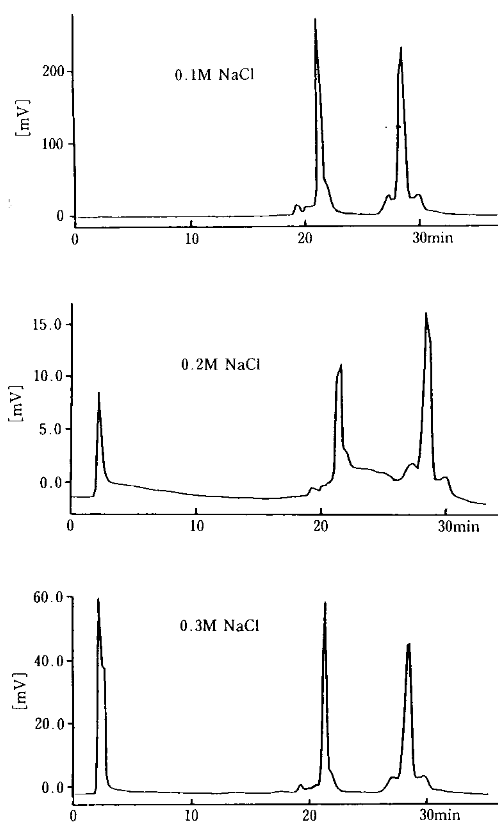



**Figure 8 Effect of salt concentration in sample solution (1)**

Column: TSKgel SuperQ-5PW, 7.5mm ID × 7.5cm

Eluent: A: 50mmol/L Tris-HCl buffer, pH 8.3
B: A + 0.5mol/L NaCl
A → B linear gradient (60 min)
Flow rate: 1.0mL/min
Temperature: 25 °C
Detection: UV@280nm
Sample: ovalbumin
trypsin inhibitor
0.5mg in 500μL for each
The salt concentration in sample solution was varied from 0.1, 0.2 and 0.3mol/L NaCl

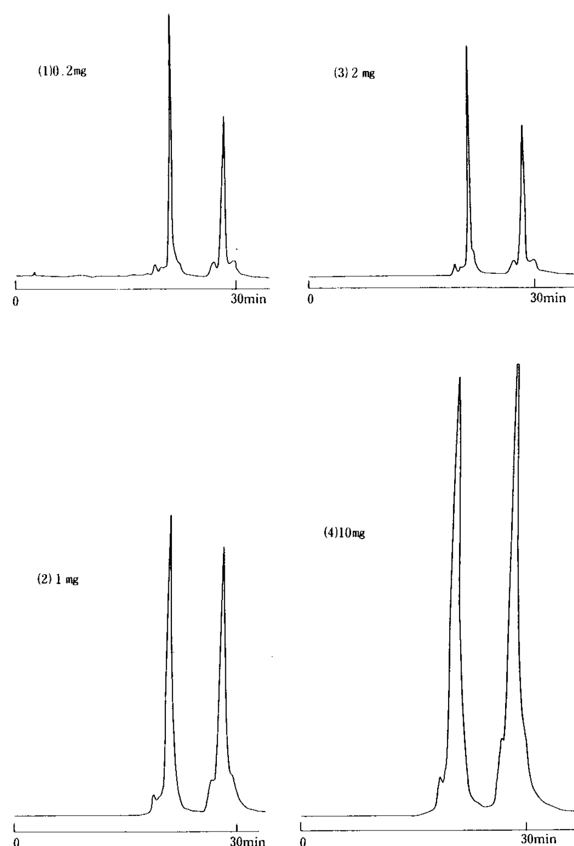



**Figure 9 Effect of salt concentration in sample solution (2)**

Conditions are identical to Figure 7

However, sample is (1) 1g/L each, 100μL
(2) 2g/L each, 500μL
(3) 10g/L each, 100μL
(4) 10g/L each, 500μL

The salt concentration in sample: 0.1mol/L NaCl

# 4. Proteins (Applications)

## 4-1 Separation of Monoclonal Antibodies

Figures 10 and 11 show a separation of two monoclonal antibodies ($IgG_1$) from mouse ascites fluid as a function of sample volume. In each case the sample injection volume was varied from 100μL to 5mL.
In Figure 10, nearly identical chromatograms are obtained up to an injection volume of 1mL. In addition, when the collected monoclonal antibody fractions were examined using size exclusion chromatography (a TSKgel $G3000SW_{XL}$ column), both fractions were 92% pure. Purity of the monoclonal fraction collected from the 5mL injection in Figure 10 was lower at 89%.

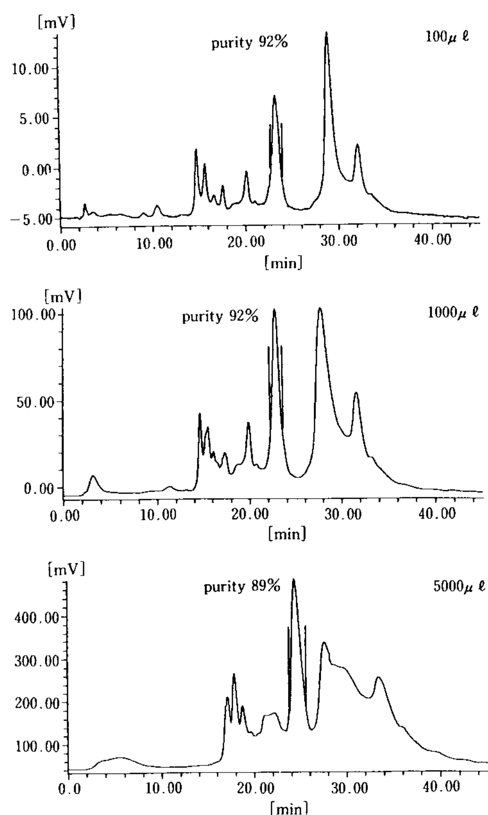



**Figure 10 Separation of mouse monoclonal antibody A ($IgG_1$)**

Column: TSKgel SuperQ-5PW, 7.5mm ID × 7.5cm

Eluent: A: 20mmol/L Tris-HCl buffer, pH 8.5
B: A + 0.5mol/L NaCl
A → B linear gradient (60 min)
Flow rate: 1.0mL/min
Temperature: 25 °C
Detection: UV@280nm
Injection vol.: 100, 1,000, 5,000μL
Sample: mouse ascites, filtered through a cellulose acetate membrane (0.45 μm) after 3-fold dilution with buffer.

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

Figure 11 shows that the monoclonal antibody peak is well separated from impurities in mouse ascites fluid. Purity levels as high as 94% were measured for $IgG_1$ even at an injection volume of 5mL. The examples shown in Figures 10 and 11 demonstrate that very large sample volumes, and thus high sample mass, can be injected on analytical size TSKgel SuperQ-5PW columns without sacrificing resolution. It is noted that these results are only possible when all sample components are retained at the top of the column at the starting mobile phase condition.

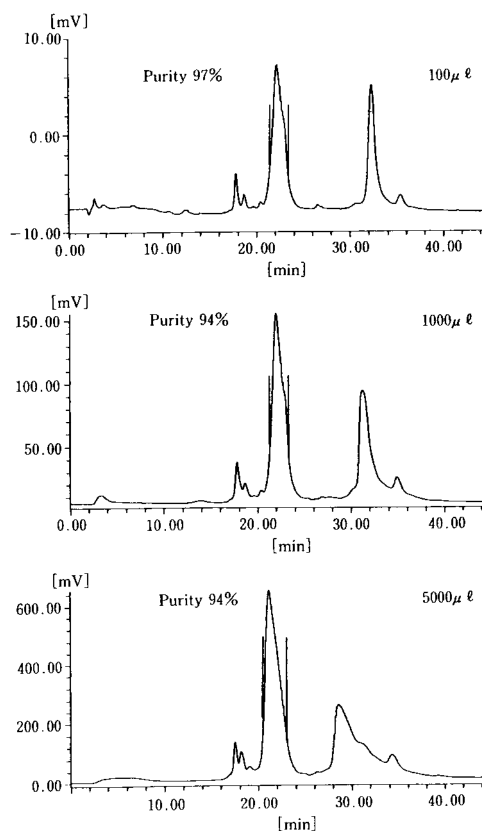



**Figure 11 Separation of mouse monoclonal antibody B ($IgG_1$)**

Conditions as in Figure 9

## 4-2 Separation of Egg White

The separation of proteins in chicken egg white is shown in Figure 12 using standard elution conditions.

## 4-3 Separation of Urease

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

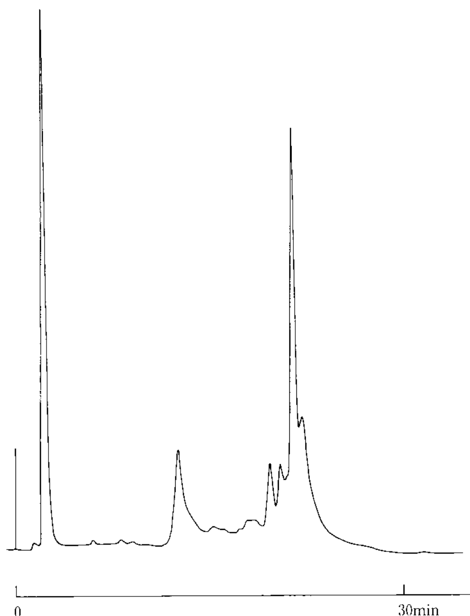



**Figure 12 Separation of chicken egg white**

Column: TSKgel SuperQ-5PW, 7.5mm 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
Temperature: 25 °C
Detection: UV@280nm
Sample: chicken egg white, 1g/L, 100μL

## 4-4 Separation of Lipoxidase

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

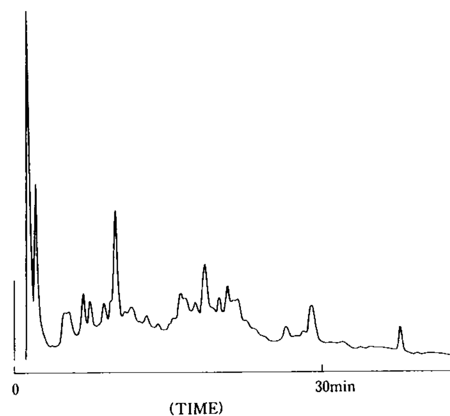



**Figure 13 Separation of urease (Jack Beans)**

Sample: 10g/L, 100μL

Other conditions as in Figure 12

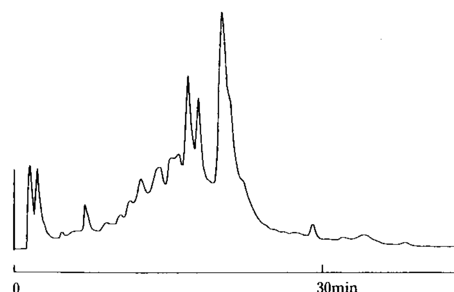



**Figure 14 Separation of commercial lipoxidase**

Sample: 6g/L, 100μL

Other conditions as in Figure12

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

A preparative TSKgel SuperQ-5PW column, 21.5mm ID × 15cm is available for purification of large sample volumes. When separating larger volumes of sample or for industrial-scale purifications, we recommend using the larger particle size Toyopearl SuperQ-650 packing materials, which are available in three particle size grades. Figure15 shows the chromatograms of the same sample under the same conditions applied to columns packed with TSKgel SuperQ-5PW, Toyopearl SuperQ-650S (35μm), and Toyopearl SuperQ-650M (65μm). The elution times of ovalbumin and soybean trypsin inhibitor are very similar, demonstrating the nearly identical selectivities of TSKgel SuperQ-5PW and Toyopearl SuperQ-650 packing materials. As expected, since resolution is inversely proportional to the particle diameter, columns packed with the larger particle size Toyopearl grades are less efficient than the 10 micron TSKgel SuperQ-5PW column.
The results in Figure 16 show how resolution varies as a function of gradient time for Toyopearl SuperQ-650S and TSKgel SuperQ-5PW. Compared to TSKgel SuperQ-5PW (20 minute gradient), roughly the same separation of the impurity peaks is obtained on Toyopearl SuperQ-650S when using a 150 minute gradient.
Figures 17 and 18 show examples of larger scale purifications on Toyopearl SuperQ-650M and comparison chromatograms of the same sample (at lower load) when run on a TSKgel SuperQ-5PW column. Although the particle size of Toyopearl SuperQ-650M is considerably larger (65μm) than that of the 10 micron HPLC columns, comparable resolution can be obtained when using a longer gradient time and/or using a column of greater length.

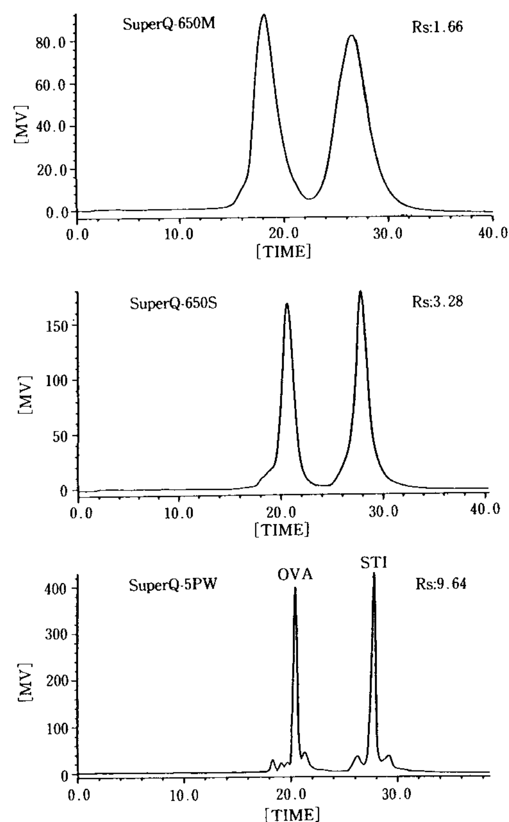



**Figure 15 Scale up from TSKgel SuperQ-5PW to Toyopearl SuperQ-650S (1)**

Column: TSKgel SuperQ-5PW, 10μm
Toyopearl SuperQ-650S, 35μm
Toyopearl SuperQ-650M, 65μm
All 7.5mm ID × 7.5cm

Eluent: A: 50mmol/L Tris-HCl buffer, pH 8.3
B: A + 0.5mol/L NaCl
A → B linear gradient (60 min)
Flow rate: 1.0mL/min
Temperature: 25 °C
Detection: UV@28 0nm
Sample: oval bumin, 20mg
trypsin inhibitor, 1mg each

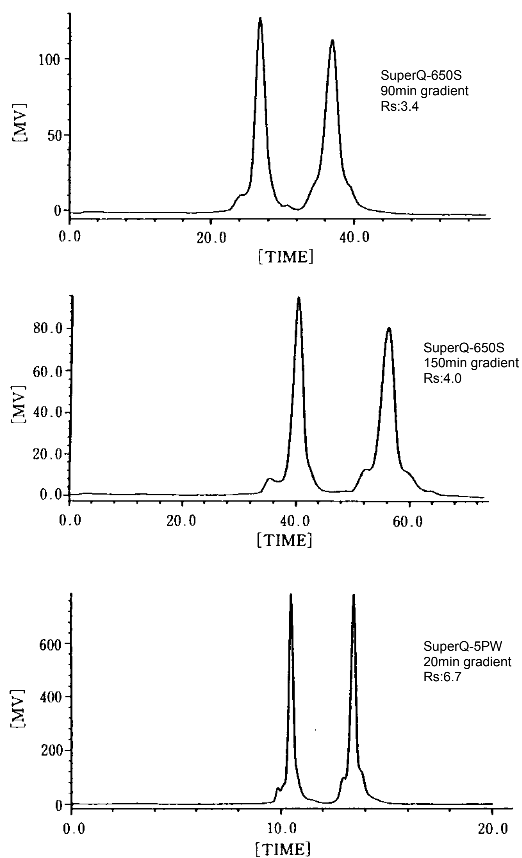



**Figure 16 Scale up from TSKgel SuperQ-5PW to Toyopearl SuperQ-650S (2)**

Conditions are identical to Figure 15, except that gradient time was varied from 20 to 150min.

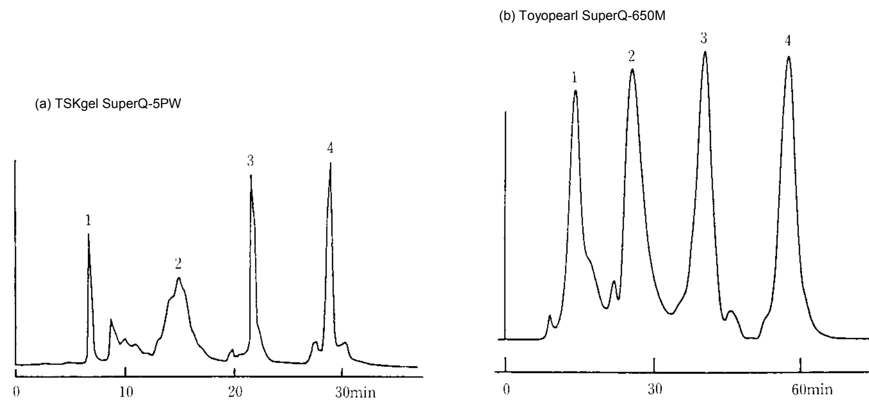



**Figure 17 Scale up from TSKgel SuperQ-5PW to Toyopearl SuperQ-650M (3)**

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 as in Figure 2.
(a) 1.6mg (b) 5.4mg

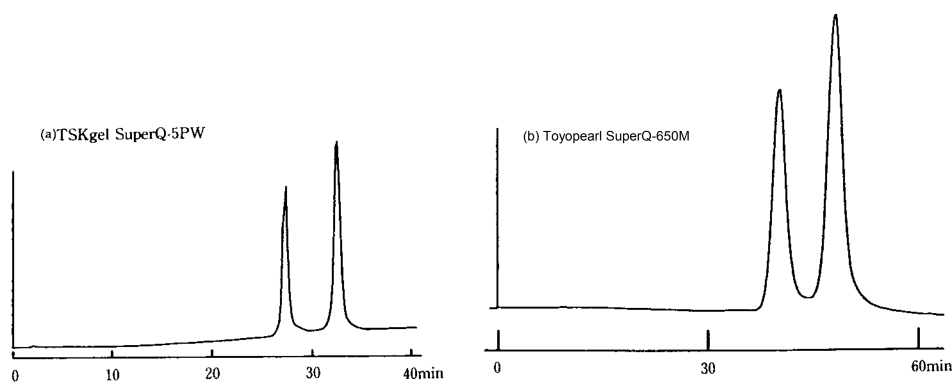



**Figure 18 Scale up from TSKgel SuperQ-5PW to Toyopearl SuperQ-650M (4)**

Sample: β-lactoglobulin a) 2mg (b) 50mg

Other conditions as in Figure 17

## 6. Conclusions

This Separation Report introduced the basic properties of a packed column for high speed ion exchange chromatography featuring high protein binding capacity, TSKgel SuperQ-5PW, and demonstrated how this column can be applied for the analysis and isolation of protein samples. Because of its methacrylate base resin, TSKgel SuperQ-5PW columns exhibit excellent chemical stability. TSKgel SuperQ-5PW columns also feature high protein binding capacity, which makes them eminently suitable for high purity analysis as well as the columns of choice for the isolation of proteins from large volume multi-component crude extractions. Table 5 summarizes the standard conditions for use of TSKgel SuperQ-5PW columns. In addition, Toyopearl SuperQ-650 bulk resins are available for large scale purifications such as used in the production of biopharmaceuticals. Since the particles used to manufacture TSKgel and Toyopearl resins are based on the same chemistry, process developers in industry can take advantage of the seamless scale-up from an analytical to a process column.

**Table-5 Standard conditions for use of a TSKgel SuperQ-5PW column**

| | |
|---|---|
| Column size | 7.5mm ID × 7.5cm |
| **Elution conditions** | |
| Flow rate | 0.5 to 1.0mL/min |
| Buffer | 20mmol/L Tris-HCl buffer (pH7.5 to pH 8.6) |
| Equilibration time | 5 times the column volume or longer |
| Salt concentration | 0 to 0.5mol/L NaCl (Resolution improved with 0 to 0.3mol/L NaCl) |
| Gradient time | 20 to 100 min |
| Temperature | 4 to 25°C |
| Detection | UV |
| **Sample** | |
| Sample load | 100μg to 200mg |
| Injection volume | 100μL to 10mL |
| Salt concentration | 0.1mol/L or lower (dilution or dialysis) |

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