TSKgel Butyl-NPR

TSKgel Ether-5PW

TSKgel Phenyl-5PW

Hydrophobic Interaction Tips:

- TSKgel Hydrophobic Interaction Chromatography (HIC) columns are offered in glass and stainless steel. Stainless steel (SS) or Pyrex frits are embedded in the body of the column end-fittings of metal and glass columns, respectively. The nominal frit size for SS columns is engraved in the end-fittings; Pyrex frits in the glass columns have a 10 µm nominal pore size.
- Halide salts corrode stainless steel tubing, fitting, and frits. Do not store SS columns in a mobile phase containing NaCl and, where possible, use another salt in the operating buffer. Chlorotrifluorethylene and tetrafluorethylene are the materials in the glass column fittings that come into contact with the mobile phase and sample.
- Good laboratory procedures demand that the analytical column be protected by a guard column. TSKgel guardgel kits, containing column hardware and gel packing, are available to pack your own guard column. Guard cartridges and packed guard columns are also available for use with TSKgel HIC columns.
- A guard column is not available for the TSKgel Butyl-NPR column. Be sure to use an in-line filter with 0.5 µm pores to avoid frequent plugging of the 1.0 µm pores in the NPR column frit. We also recommend a pre-injector membrane filter to prevent particles generated by pump seal wear from reaching the column.
- All TSKgel HIC columns can be routinely operated from pH 2.0-12.0.
- The TSKgel Ether-5PW and Phenyl-5PW HIC columns are physically and chemically stable in water-soluble organic solvents of concentration ratios under 50%, such as methanol, ethanol, and acetonitrile (DMF, DMSO, or chloroform <30%). Change the solvent gradually by reducing the flow rate (preferably with a gradient) because rapid change may cause degradation of column efficiency. Note: Reduce your salt concentration to prevent the precipitation of the salt on the column. Also chaotropic agents (urea, SDS, etc.) will reduce the adsorption of the biomolecule; therefore, use low levels of chaotropic agents (<2 mol/L).
- The TSKgel Butyl-NPR columns are compatible with water-soluble organic solvents of concentration range under 20%.
- The addition of organic solvents or chaotropic agents in the final buffer can improve separation. Relative elution positions can change, however, so add chaotropic agent and organic solvent in small quantities.
- Periodic injections of one column volume of 0.2 mol/L NaOH remove strongly retained contaminants from the top of the column by hydrolysis or dissolution. Additionally, acetic acid (20-40%) can be used to regenerate the column. Note: Acid can precipitate protein.
- If the inlet frit of the column becomes plugged, rinse the column with water when operating the column in reverse flow direction. When all else fails, and at your own risk, remove the column end-fitting at the top of the column and sonicate it in 6 mol/L nitric acid. The end-fitting and frit are one piece. Be careful not to disturb the packing and rinse the fitting well after cleaning.
- The shipping solvent for all TSKgel HIC columns is distilled water.
- TSKgel HIC columns are supplied with an Inspection Data Sheet, which includes a QC chromatogram and test data, and an OCS Sheet summarizing the recommended operating conditions for optimum column performance.
- A separate TSKgel Column Instruction Manual that reviews general guidelines for column installation and care, as well as troubleshooting tips for commonly encountered problems, can be downloaded from the Tosoh Bioscience LLC website (www.tosohbioscience.com).

About

Hydrophobic Interaction Chromatography (HIC) provides an additional chromatographic dimension, the recognition of protein surface hydrophobicity, to make it a powerful technique for analytical and preparative separation of biomolecules. In HIC, a weakly non-polar stationary phase is used with an aqueous mobile phase containing a high concentration of a chaotropic salt. The technique is mainly applied to the separation of proteins, which are eluted by gradually reducing the salt concentration.

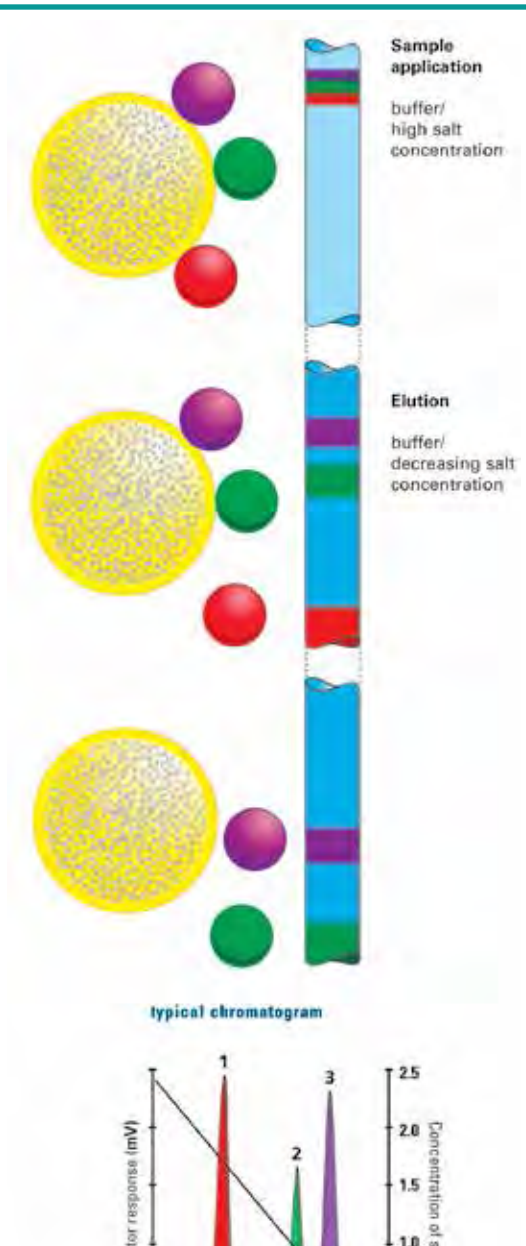
Proteins are retained in HIC by interaction with alkyl or aryl functional groups on the packing material. The density of these functional groups is low and protein molecules are adsorbed on only one or a few sites. The binding of proteins to a hydrophobic matrix is affected by a number of factors including (1) the type of ligand, (2) the ligand density on the solid support, (3) the backbone material of the matrix, (4) the hydrophobic nature of the protein, and (5) the type of salt used.

Sorption takes place at high salt concentration and desorption is accomplished by decreasing the salt concentration or by adding a low percentage of organic solvent. HIC has the advantage that the mobile phase conditions (primarily aqueous) do not usually disrupt higher-order protein structures. The features and benefits of HIC are detailed in Table 1 below.

Table 1: Features and benefits of Hydrophobic Interaction Chromatography

Features	Benefits
Choice of three hydrophobic ligands (ether, phenyl, or butyl)	Added flexibility during method development
Rigid polymeric base resin	Wide pH range (2-12) of the base enabling robust cleaning options
Similar chemistry to TOYOPEARL resins	Seamless scalability from analytical to preparative scale
TSKgel Phenyl-5PW columns offered in PEEK hardware	Eliminates undesirable interactions with column hardware
TSKgel Ether-5PW and Phenyl-5PW columns available in 2 mm ID format	LC/MS applications

Figure 1: Hydrophobic Interaction Chromatography



TSKgel Hydrophobic Interaction Chromatography Columns

TSKgel HIC columns are polymethacrylate-based with a choice of three ligands (butyl, ether, and phenyl) with varied hydrophobicities from low to high, respectively (see Table 2). The HIC packing materials are based on the polymeric TSKgel G5000PW resin which is then derivatized with oligoethylene-glycol (Ether-5PW) or phenyl (Phenyl-5PW) groups. The base material used to prepare TSKgel Butyl-NPR colunms is of the same chemical composition as the TSKgel G5000PW base material used to prepare TSKgel Phenyl-5PW and Ether-5PW columns. The difference between the two packings is that the TSKgel G5000PW packing is porous, whereas the base material of the TSKgel Butyl-NPR column consists of spherical 2.5 µm nonporous particles. Nonporous resins (NPR) are typically used for high speed analytical applications.

The TSKgel HIC columns are compatible with water-soluble organic solvents at concentrations below 50% (20% for TSKgel Butyl-NPR). See Figure 2 for the structure of the TSKgel HIC resins. Table 3 lists well known applications for each type of TSKgel HIC column.

Figure 2: Structure of TSKgel HIC resins

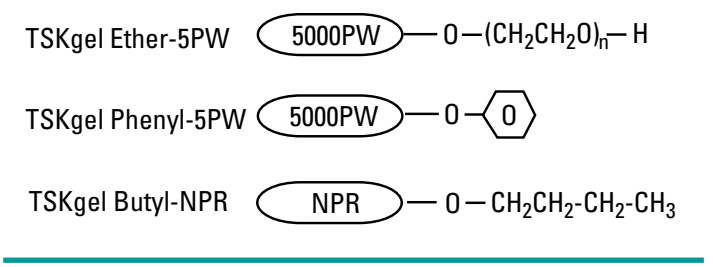


Table 2: Comparison of TSKgel HIC columns

TSKgel Column	Hydrophobicity	Benefits
Phenyl-5PW	Most hydrophobic	Requires modest salt concentration to retain proteins. Most popular column applicable for the widest range of hydrophobicities.
Ether-5PW	Less hydrophobic	Excellent choice for hydrophobic proteins such as membrane proteins or monoclonal antibodies.
Butyl-NPR	Least hydrophobic	Excellent choice for high speed applications; usually high recovery due to absence of pores.

Table 3: Column selection for TSKgel HIC columns

Sample	MM Range (Da)	TSKgel Column
Peptides	$<1.0 \times 10^4$	Butyl-NPR
Medium to large proteins	$>1.0 \times 10^4$	Phenyl-5PW Ether-5PW Butyl-NPR
DNA, RNA & PCR products	$>5.0 \times 10^5$	Phenyl-5PW Butyl-NPR
Oligonucleotides	$>1.0 \times 10^4$	Phenyl-5PW Butyl-NPR

About: TSKgel Butyl-NPR Hydrophobic Interaction Chromatography Columns

The 2.5 µm nonporous methacrylate packing material of the TSKgel Butyl-NPR columns is bonded with butyl groups. In terms of hydrophobicity, the TSKgel Butyl-NPR columns are the least hydrophobic of the HIC column offerings and require a higher salt concentration for binding. They are the best choice for high speed separations with excellent recovery, even for more hydrophobic samples. As in other modes of liquid chromatography, smaller particles provide higher efficiency. By packing the 2.5 µm nonporous resin particles into shorter columns, typical analysis times are reduced to less than 10 minutes. Since the binding kinetics occur only on the bead's surface, nonporous resins are more efficient than porous particles of the same size. Pore diffusion is often the rate limiting step in the overall mass transport of large biomolecules through a porous column. Eliminating the pores provides higher resolution at higher flow rates. Another benefit of NPR resins is excellent mass recovery, allowing quantitation down to nanogram levels. Because the surface area of nonporous particles is much lower, analysts need to be aware that sample mass and volume need to be adjusted to maintain optimum column efficiency.

Attributes and Applications

Product attributes of the TSKgel Butyl-NPR columns are shown in Table 4. The ultra-efficient 2.5 µm nonporous resin makes TSKgel Butyl-NPR columns the preferred choice for time-critical QC analysis and sample limited applications.

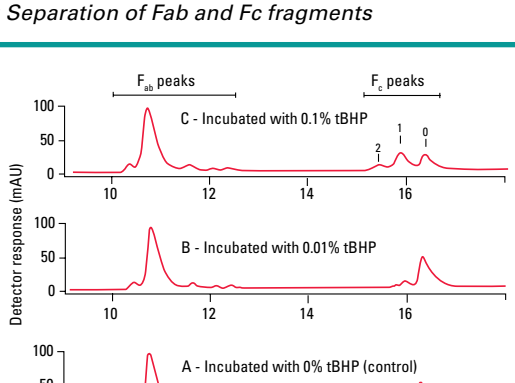
Table 4: Product attributes

Attribute	Value
Pore size (mean)	nonporous
Particle size (mean)	2.5 µm
pH stability	2.0-12.0
Functional group	butyl

Antibody Fragments

Figure 3 shows the separation of Fab and Fc fragments of an antibody on a TSKgel Butyl-NPR column. The appearance of additional Fc fragments is due to the oxidation of methionine residues by 0.10% t-butyl hydroperoxide (tBHP). The numbers above the Fc peaks correspond to the number of oxidized residues in each fragment.

Figure 3: Separation of Fab and Fc fragments

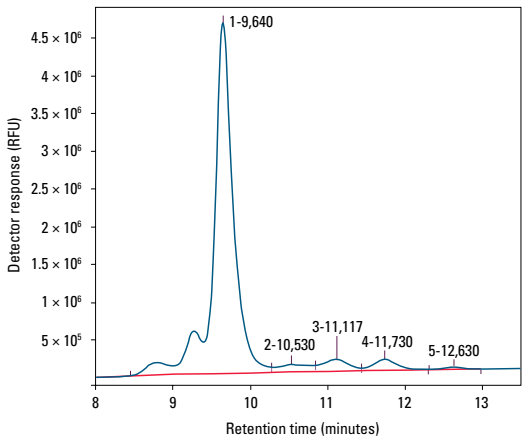


Column:	**TSKgel Butyl-NPR, 2.5 µm, 4.6 mm ID × 3.5 cm**
Mobile phase:	A: 2 mol/L $(NH_4)_2SO_4$, 20 mmol/L Tris, pH 7.0 B: 20 mmol/L Tris, pH 7.0
Gradient:	0 min (10%B) 34 min (100%B)
Flow rate:	1 mL/min
Temperature:	30 °C

Proteins

The use of a TSKgel Butyl-NPR column as an alternative to the size exclusion separation of a monoclonal antibody and its high molar mass aggregates is shown in Figure 4 below. Because of the high efficiency of the nonporous particles in the TSKgel Butyl-NPR column, only low sample amounts are needed for aggregate analysis.

Figure 4: Analysis of monoclonal antibody and aggregates using a TSKgel Butyl-NPR column

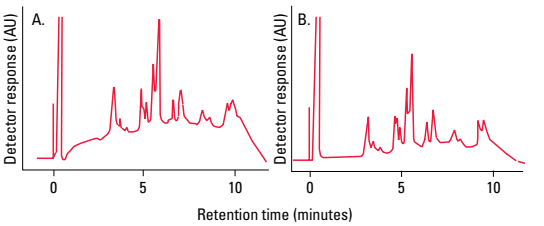


Column:	**TSKgel Butyl-NPR, 2.5 µm, 4.6 mm ID × 3.5 cm**
Mobile phase:	A: 3 mol/L NaCl B: H_2O
Gradient:	0-100%B in 10 min
Flow rate:	1.0 mL/min
Detection:	flourescence Ex: 280 nm, Em: 348 nm
Injection vol.:	5 µg
Sample:	IgG_1

Phosphoglucose Isomerase

Almost identical separations were obtained at sample loads from 25 µg up to 100 µg in the separation of a crude sample of phosphoglucose isomerase using a TSKgel Butyl-NPR column as shown in Figure 5.

Figure 5: Effect of sample load on the separation of phosphoglucose isomerase



Column:	**TSKgel Butyl-NPR, 2.5 µm, 4.6 mm ID × 3.5 cm**
Mobile phase:	10 min linear gradient of $(NH_4)_2SO_4$ from 1.8 mol/L to 0 mol/L in 0.1 mol/L phosphate buffer, pH 7.0
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Samples:	crude sample of phosphoglucose isomerase
Sample loads:	A: 25 µg B: 100 µg

About: TSKgel Ether-5PW Hydrophobic Interaction Chromatography Columns

Of the three TSKgel HIC columns, the TSKgel Ether-5PW columns have intermediate hydrophobicity. TSKgel Ether-5PW columns are stable in either acid or caustic cleaning regimens and provide excellent access to larger molecules with low diffusion coefficients.

Attributes and Applications

Table 5 lists the product attributes of the TSKgel Ether-5PW columns. The TSKgel Ether-5PW columns are an excellent choice for separating hydrophobic molecules, including membrane proteins or monoclonal antibodies such as IgG or IgM.

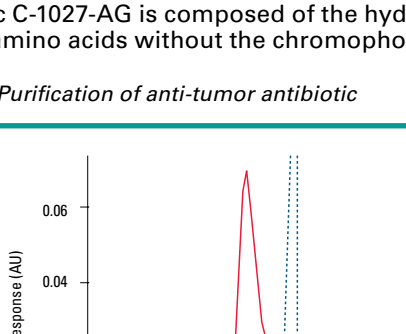
Table 5: Product attributes

Attribute	Value
Pore size (mean)	100 nm
Particle size (mean)	10 µm
pH stability	2.0-12.0
Functional group	ether

Antibiotics

A TSKgel Ether-5PW column was used to determine the relative purity of the antibiotic components C-1027 and C-1027-AG as shown in Figure 6. Antibiotic C-1027 is composed of a protein consisting of many hydrophobic and hydroxyamino acids with a non-protein chromophore. Antibiotic C-1027-AG is composed of the hydrophobic and hydroxyamino acids without the chromophore.

Figure 6: Purification of anti-tumor antibiotic

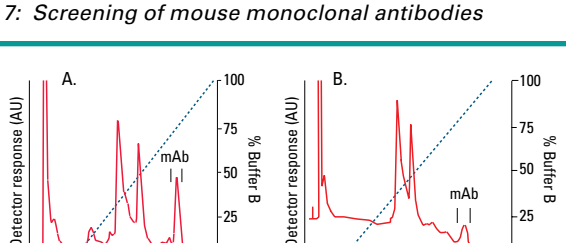


Column: **TSKgel Ether-5PW, 10 µm, 7.5 mm ID × 7.5 cm**
Mobile phase: linear gradient from 1.5 mol/L to 0 mol/L $(NH_4)_2SO_4$ in 0.1 mol/L phosphate buffer, pH 7.0
Flow rate: 0.8 mL/min
Detection: UV @ 220 nm
Injection vol.: 20 µL
Sample: C-1027 ——— C-1027-AG – – – –
concentration: 1 g/L

Monoclonal Antibodies

Monoclonal antibodies (mAbs) play a part in many research, diagnostic, and therapeutic applications. Monoclonal antibodies are generally the most hydrophobic proteins in ascites fluid and cell culture supernatant. Figure 7 shows typical results from the screening of two mAbs using a TSKgel Ether-5PW column.

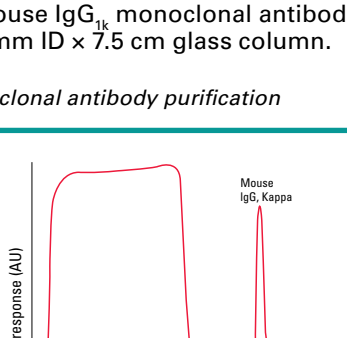
Figure 7: Screening of mouse monoclonal antibodies



Column: **TSKgel Ether-5PW, 10 µm, 8.0 mm ID x 7.5 cm, glass**
Mobile phase: linear gradient from A to B as shown
A: 0.05 mol/L sodium phosphate, pH 7.0, 2.0 mol/L ammonium sulfate, 1.0 mol/L glycine
B: 0.05 mol/L sodium phosphate, pH 7.0, 1.0 mol/L glycine
Flow rate: 10 mL/min
Detection: UV @ 280 nm
Samples: A: 20 µL unequilibrated mouse IgG_{2b} κ ascites
B: 20 µL unequilibrated mouse IgM κ ascites

TSKgel Ether-5PW columns have been used successfully for purifying membrane-bind proteins such as immunoglobulins. Figure 8 demonstrates this for a 50 mL injection of mouse IgG_{1k} monoclonal antibody on a TSKgel Ether-5PW, 8 mm ID × 7.5 cm glass column.

Figure 8: Monoclonal antibody purification



Column: **TSKgel Ether-5PW, 10 µm, 8.0 mm ID × 7.5 cm, glass**
Mobile phase: 67.5 min isocratic load and wash with 1 mol/L $(NH_4)_2SO_4$ in 1 mol/L glycine, 0.5 mol/L phosphate buffer, pH 7.0, followed by a 37.5 min linear gradient from 1.0 mol/L to 0 mol/L $(NH_4)_2SO_4$ in 1.0 mol/L glycine, 0.05 mol/L phosphate, pH 7.0
Flow rate: 1.0 mL/min
Detection: UV @ 280 nm, 3.0 AUFS
Injection vol.: 50 mL
Sample: 25 mL raw cell culture supernatant
- 200 mg total protein
- 15 mg total antibody diluted to 50 mL with initial elution buffer

About: TSKgel Phenyl-5PW Hydrophobic Interaction Chromatography Columns

TSKgel Phenyl-5PW columns were the first commercially available, polymer-based columns for high performance HIC. These columns have been instrumental in the increase in popularity of this technique for analytical, preparative, and process scale separations of biopolymers. The high porosity of TSKgel Phenyl-5PW packings allows very large proteins to enter the internal pore structure, thereby maintaining high capacity for such compounds.

Attributes and Applications

Product attributes of the TSKgel Phenyl-5PW columns are shown in Table 6. The most hydrophobic among the three TSKgel HIC columns, TSKgel Phenyl-5PW columns are an excellent choice to screen for the selectivity, retention, and recovery of most biomolecules.

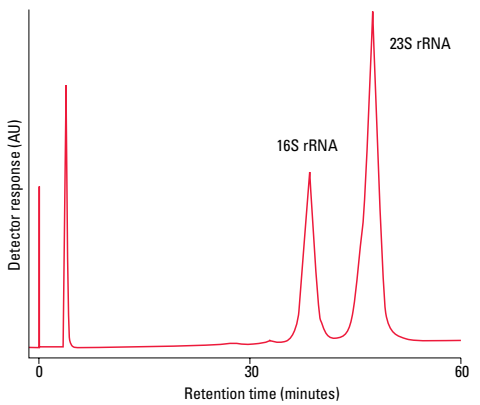
Table 6: Product attributes

Attribute	Value
Pore size (mean)	100 nm
Particle size (mean)	10 µm, 13 µm, and 20 µm
pH stability	2.0-12.0
Functional group	phenyl

RNA

Figure 9 illustrates the separation of 16S and 23S ribosomal RNA on a TSKgel Phenyl-5PW column. The approximate molar masses of these RNAs are 5.6×10^5 and 1.1×10^6 Da, respectively.

Figure 9: Analysis of ribosomal RNA

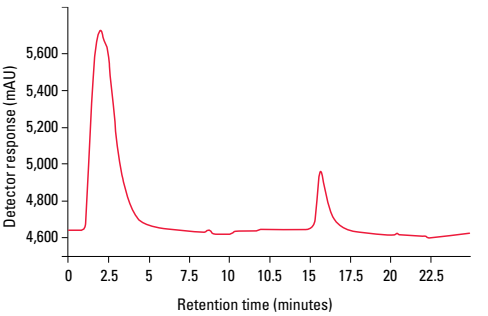


Column: **TSKgel Phenyl-5PW, 10 µm, 7.5 mm ID × 7.5 cm**
Mobile phase: 60 min linear gradient from 2 mol/L to 0 mol/L $(NH_4)_2SO_4$ in 0.1 mol/L phosphate buffer, pH 7.0
Flow rate: 0.5 mL/min
Detection: UV @ 280 nm
Sample: 16S and 23S rRNA from *E. coli*, 0.05 mg in 0.1 mL

Calcium-binding Proteins

Calcium-binding proteins are involved in signal transduction processes. One of these proteins, myristoylated frequenin, has a myristoyl group that protrudes in the presence of calcium. This characteristic can be exploited using HIC to purify the protein, as shown in Figure 10. A step gradient is employed on a TSKgel Phenyl-5PW glass column to purify myristoylated frequenin from crude *E. coli* lysate.

Figure 10: Purification of myristoylated frequenin

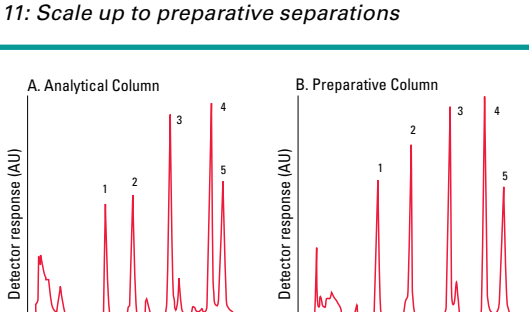


Column: **TSKgel Phenyl-5PW, 10 µm, 5 mm ID × 5 cm, glass**
Mobile phase: equilibration in 50 mmol/L HEPES, 100 mmol/L KCl, 1 mmol/L DTT, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, pH 7.5 step gradient at 12.5 min to 50 mmol/L HEPES, 1 mmol/L DTT, 1 mmol/L $MgCl_2$, 2 mmol/L EGTA, pH 7.5
Flow rate: 1 mL/min
Detection: UV @ 280 nm
Temperature: ambient
Sample: *E. coli* lysate containing myristoylated frequenin, 100 µL

Proteins: Scale up to Preparative Separations

Figure 11 compares the resolution of standard proteins on analytical and preparative TSKgel Phenyl-5PW columns. Different flow rates compensated for the change in particle size and column dimensions. High resolution was obtained on both columns.

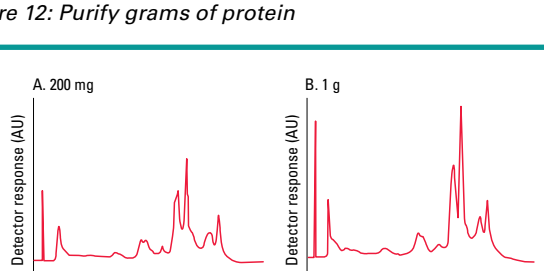
Figure 11: Scale up to preparative separations



Columns:	**A: TSKgel Phenyl-5PW, 10 µm, 7.5 mm ID × 7. 5cm** **B: TSKgel Phenyl-5PW, 13 µm, 21.5 mm ID × 15 cm**
Mobile phase:	60 min linear gradient from 1.8 mol/L to 0 mol/L $(NH_4)_2SO_4$ in 0.1 mol/L phosphate buffer, pH 7.0
Flow rate:	0.5 mL/min (7.5 mm ID) or 4 mL/min (21.5 mm ID)
Detection:	UV @ 280 nm
Samples:	1. myoglobin 2. ribonuclease 3. lysozyme 4. α-chymotrypsinogen 5. α-chymotrypsin

Figure 12 compares resolution for a 200 mg injection of crude lipoxidase on a 21.5 mm ID × 15 cm TSKgel Phenyl-5PW column with that of a 1 g injection on a 55 mm ID × 20 cm column. As shown, the increase in particle size from 13 µm to 20 µm did not influence chromatographic resolution, keeping in mind that the sample load was only scaled up five-fold.

Figure 12: Purify grams of protein



Columns:	**A: TSKgel Phenyl-5PW, 13 µm, 21.5 mm ID × 15 cm** **B: TSKgel Phenyl-5PW, 20 µm, 55 mm ID × 20 cm**
Mobile phase:	120 min linear gradient from 1.5 mol/L to 0 mol/L$(NH_4)_2SO_4$ in 0.1 mol/L phosphate buffer, pH 7.0
Flow rate:	4 mL/min (21.5 mm ID) and 40 mL/min (55 mm ID)
Detection:	UV @ 280 nm
Sample:	crude lipoxidase, 200 mg (21.5 mm ID) and 1 g (55 mm ID)