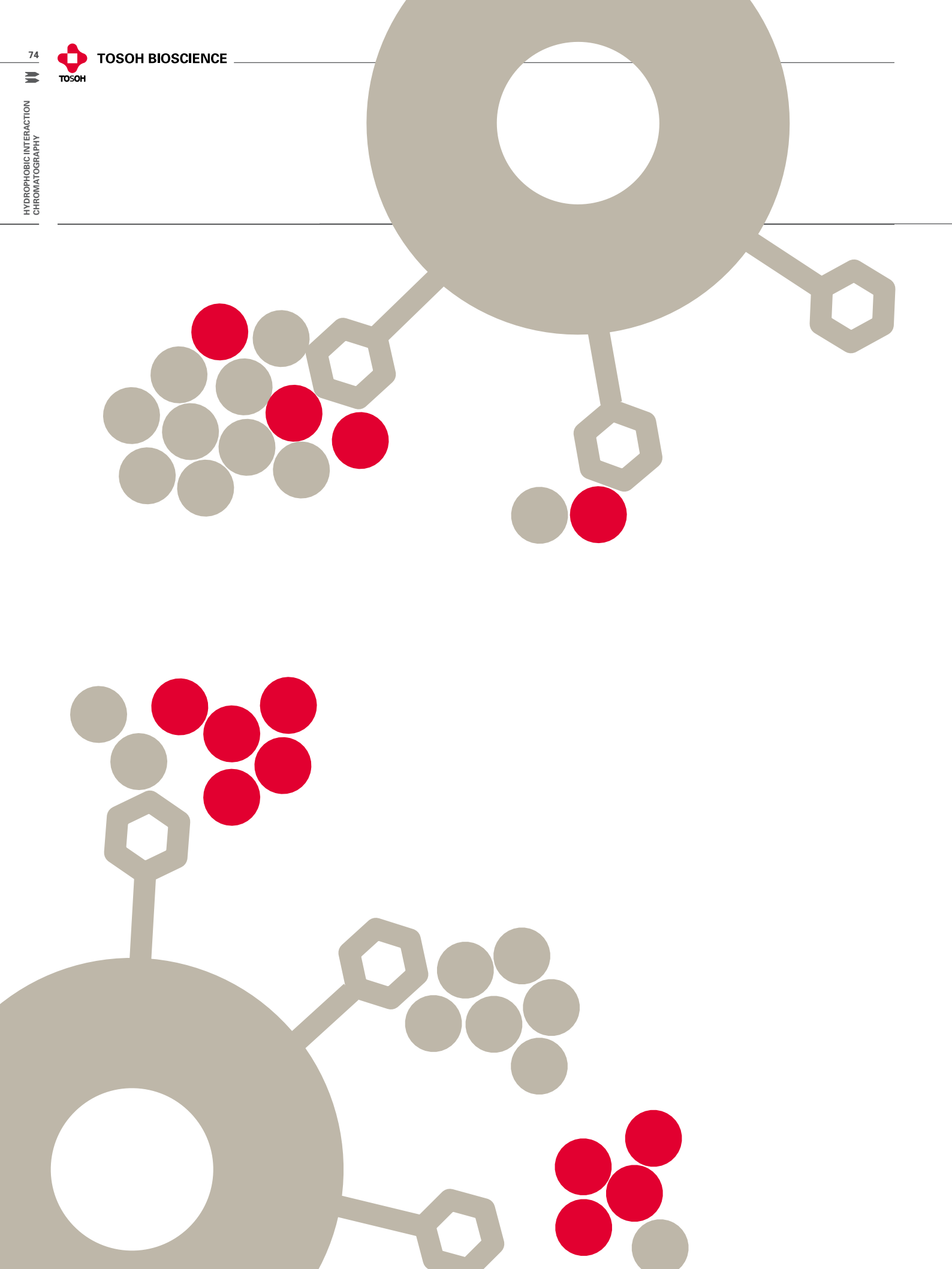


HIC
HYDROPHOBIC INTERACTION CHROMATOGRAPHY

HIC PRODUCTS

- TSKgel Ether-5PW
- TSKgel Phenyl-5PW
- TSKgel Butyl-NPR

TOSOH FACT

Tosoh Bioscience provides solutions for today's biological purification needs. In fact, some of the first commercial HIC products were manufactured by Tosoh. We take pride in our ability to design new products based on existing chemistries to solve specific customer applications.

We encourage you to have a confidential discussion with us about your specific needs. Whether it is a surface modification of an existing product or the creation of a new one, we encourage you to call on us to meet your needs for a customized solution.

INTRODUCTION TO TSK-GEL HIC COLUMNS

Hydrophobic Interaction Chromatography (HIC) is based on the interaction between hydrophobic groups on a protein and a hydrophobic ligand on the solid support. HIC offers a distinct advantage for easily denatured proteins; it can be run using moderate concentrations of ammonium sulfate, which favors the stability of many proteins.

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. All of these factors help to make HIC a powerful technique for the separation of biomolecules.

Tosoh Bioscience offers three different HIC column types in analytical format: TSKgel Phenyl-5PW, Ether-5PW and Butyl NPR. TSKgel Phenyl-5PW and Ether-5PW are also available in preparative column formats.

Column Selection

The HIC packing materials are based on the polymeric TSKgel G5000PW size exclusion resin (a hydrophilic gel with an estimated protein exclusion limit of 5,000,000 Da) which is then derivatized with oligoethylene-glycol (Ether-5PW) or phenyl (Phenyl-5PW) groups. Columns, depending on diameter, are packed with 10, 13 or 20 µm particles.

TSK-GEL ETHER-5PW is less hydrophobic than TSKgel Phenyl-5PW. It displays weaker interaction and thus shorter retention times compared to Phenyl-5PW, as shown in FIGURE 1. TSKgel Ether-5PW is the best choice for the separation of very hydrophobic proteins such as membrane proteins or monoclonal antibodies.

The **TSK-GEL PHENYL-5PW** columns were the first commercially available, polymer-based columns for high performance HIC. These columns have been instrumental to the increase in popularity of this technique for analytical, preparative, and process scale separations of biopolymers. FIGURE 2 compares the separation of standard proteins on the Ether, Phenyl, and Butyl supports under similar operating conditions.

The base material of TSKgel Butyl-NPR is of the same chemical composition as the G5000PW base material used to prepare Phenyl-5PW and Ether-5PW. The difference between the two packings is that the 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. See FIGURE 3 for the structure of the HIC resins.

TSK-GEL BUTYL-NPR is the least hydrophobic among the three TSK-GEL HIC columns and requires a higher salt concentration for binding. TSKgel Butyl-NPR columns provide fast and quantitative HIC, because 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. 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. These properties make TSKgel Butyl-NPR the preferred choice for process monitoring and quality control.

TSK-GEL HIC columns are compatible with water-soluble organic solvents at concentration below 50 % (20 % for Butyl-NPR).

FEATURES

- Choice of three hydrophobic ligands (ether, phenyl or butyl)
- Rigid polymeric base resin
- Similar chemistry to Toyopearl resins
- TSKgel Phenyl-5PW offered in PEEK hardware
- Ether and Phenyl available in 2 mm ID format

BENEFITS

- Added flexibility during method development
- Wide pH range (2-12) enabling robust cleaning options
- Seamless scalability from analytical to preparative scaley
- Eliminates undesirable interactions with column hardware
- LC-MS applications

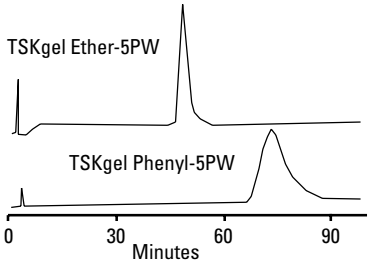
HIC

Column selection for TSK-GEL HIC Columns

Sample	MW range (Da)	TSK-GEL Column
Peptides	< 10,000	Butyl-NPR
Medium to large proteins	> 10,000	Phenyl-5PW Ether-5PW Butyl-NPR
DNA, RNA, and PCR products	> 500,000	Phenyl-5PW Butyl-NPR
Oligonucleotides	> 10,000	Phenyl-5PW Butyl-NPR

FIGURE 1

Separation of α-amylase



Column: TSKgel Ether-5PW, 7.5mm ID x 7.5cm
TSKgel Phenyl-5PW, 7.5mm ID x 7.5cm
Sample: α-amylase
Elution: A. 0.1mol/L phosphate buffer (pH 7.0) + 1.1mol/L Na_2SO_4
B. 0.1mol/L phosphate buffer (pH 7.0)
60min linear gradient from A→B(1.1mol/L→0mol/L Na_2SO_4)
Flow Rate: 1.0mL/min
Detection: UV @ 280nm

FIGURE 3

Structure of TSK-GEL HIC resins

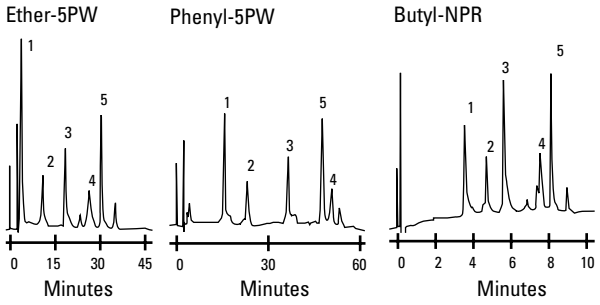
TSKgel Ether-5PW — 5000PW — O—$(CH_2CH_2O)_n$— H

TSKgel Phenyl-5PW — 5000PW — O—O

TSKgel Butyl-NPR — NPR — O—CH_2CH_2-CH_2-CH_3

FIGURE 2

Comparing conventional and nonporous HIC columns



Column: TSKgel Ether-5PW & TSKgel Phenyl-5PW, 7.5mm ID x 7.5cm
TSKgel Butyl-NPR, 4.6mm ID x 3.5cm
Sample: 1. myoglobin, 2. ribonuclease A, 3. lysozyme,
4. α-chymotrypsin, 5. α-chymotrypsinogen
Injection: 5PW-type columns: 100µL (50-100µg);
NPR-type column: 20µL (1.5-40µg)
Elution: 60min linear gradient from 1.8mol/L to 0mol/L $(NH_4)_2SO_4$
in 0.1mol/L phosphate buffer, pH 7.0, for 5PW-type columns;
12min linear gradient from 2.3mol/L to 0mol/L $(NH_4)_2SO_4$
in 0.1mol/L phosphate buffer, pH 7.0 for TSKgel Butyl-NPR
Flow Rate: 1.0mL/min
Detection: UV @ 280nm

Sample capacity

One definition of sample capacity is the amount of pure compound injected onto the column at which the peak width is 10% larger than the peak width under low loading conditions. Using this definition, the capacity of a 7.5 mm ID x 7.5 cm L TSKgel Phenyl-5PW column varies from 0.1 to 1 mg of protein. Resolution and peak width are dependent on sample loading, as shown in FIGURE 4. Therefore, sample loading should be kept within 0.1-0.5 mg in order to obtain the highest resolution.

Separations on TSKgel Ether-5PW columns usually take 30-60 minutes. 0.5 mg of pure protein can be purified from a 5-10 mg crude protein mixture using a 7.5 mm ID x 7.5 cm L column.

Since almost all of the surface area of a porous particle is inside the pores, the capacity of the 4.6 mm ID x 3.5 cm L TSKgel Butyl-NPR column is significantly less than that for the 7.5 mm ID x 7.5 cm L Phenyl-5PW column. Capacities for the Butyl-NPR column are 100 µg for crude sample and 2 µg for pure sample.

Chemical stability

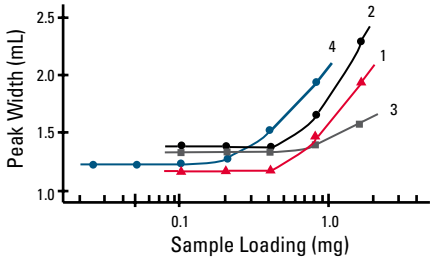
TSK-GEL 5PW-type HIC columns are physically and chemically stable in water-soluble organic solvents (at < 50% methanol, ethanol, ACN, DMF, DMSO or < 30 % chloroform). Change the solvent gradually by reducing the flow rate (preferably with a gradient) because rapid change may cause degradation of column efficiency. Note: When changing to an organic solvent, reduce the salt concentration to prevent precipitation of the salt on the column. Also, chaotropic agents (urea, SDS, etc.) will reduce the adsorption of biomolecules; therefore, use low levels of these agents (<2 mol/L).

The addition of organic solvents or chaotropic agents in the final buffer can improve separations. However, relative elution positions may change. Therefore, add chaotropic agent and organic solvent in small quantities. See FIGURE 5 for the effect of chaotropic agents and organic solvents on the HIC separation of two different samples.

Polymer-based columns are stable when cleaning at alkaline pH. All TSK-GEL HIC columns can be routinely operated from pH 2-12. Table I shows that the phenyl groups on the TSKgel Phenyl-5PW are stable for more than 10 days upon exposure to 0.5 mol/L NaOH or 0.5 mol/L acetic acid.

FIGURE 4

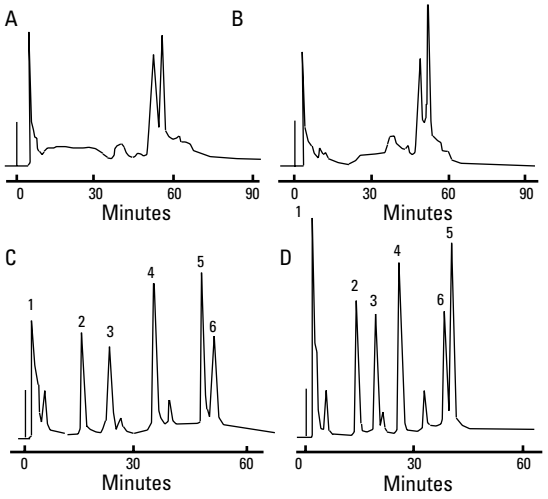
Dependence of peak width on sample loading in the separation of proteins



Column: TSKgel Phenyl-5PW, 7.5 mm ID x 7.5 cm L
Sample: 1. myoglobin; 2. ribonuclease A; 3. ovalbumin; 4. α-chymotrypsin; concentration: 0.025 % to 1.6 %
Elution: 60 min linear gradient of $(NH_4)_2SO_4$ from 1.5 mol/L to 0 mol/L in 0.1 mol/L phosphate buffer (pH 7.0)
Flow Rate: 0.5 mL/min
Temperature: 25 °C
Detection: UV @ 280 nm

FIGURE 5

Effect of urea and isopropanol on the separation of commercial lipoxidase and a standard protein mixture



Column: TSKgel Phenyl-5PW, 7.5mm ID x 7.5cm
Sample: A & B: commercial lipoxidase
C & D: protein mixture:
1. cytochrome C; 2. myoglobin
3. ribonuclease A; 4. lysozyme
5. α-chymotrypsinogen; 6. α-chymotrypsin
Elution: A: 60min linear gradient from 0.1mol/L phosphate buffer containing 1.5mol/L $(NH_4)_2SO_4$ (pH 7.0) to 0.1mol/L phosphate buffer (pH 7.0)
B: 60min linear gradient from 0.1mol/L phosphate buffer containing 1.5mol/L $(NH_4)_2SO_4$ (pH 7.0) to 0.1mol/L phosphate buffer containing 2mol/L urea (pH 7.0)
C: 60min linear gradient from 0.1mol/L phosphate buffer containing 1.8mol/L $(NH_4)_2SO_4$ (pH 7.0) to 0.1 mol/L phosphate buffer (pH 7.0)
D: 60min linear gradient from 0.1mol/L phosphate buffer containing 1.8mol/L $(NH_4)_2SO_4$ (pH 7.0) to 0.1mol/L phosphate buffer (pH 7.0) containing 7% isopropanol
Flow Rate: A & B: 0.5mL/min; C & D: 1.0mL/min
Temperature: 25°C
Detection: UV @ 280nm

TABLE 1

Long-term exposure of TSKgel Phenyl-5PW to acid and base

Acid/base	Phenyl content (mmol/mL - resin)	
	Before exposure	*After 10 days exposure*
0.5 mol/L CH_3COOH	0.105	0.106
0.5 mol/L NaOH	0.105	0.104

HIC

APPLICATIONS OF TSK-GEL ETHER-5PW COLUMNS

HIGHLIGHTS

- Ether, phenyl, and butyl functionalities are available.
- TSKgel Ether-5PW and Phenyl-5PW columns are available in a 2 mm ID format.
- Large 1000 Å pore size of the base matrix accommodates proteins up to 5 x 10^6 Da.
- Polymeric resin is chemically and physically stable to changes in pH and ionic strength and compatible with a variety of organic solvents.
- High binding capacity is achieved for TSK-GEL 5PW-type HIC packing materials.
- Nonporous resins (NPR) allow fast analysis for quality control or process monitoring

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.

Antibiotics

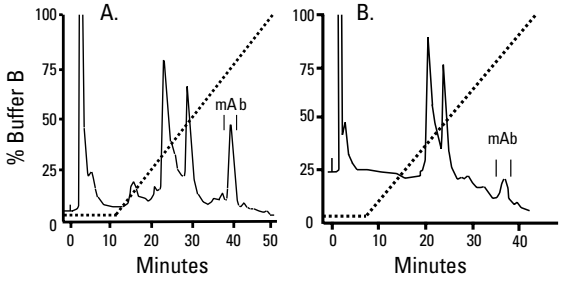
The TSKgel Ether-5PW column was used to determine the relative purity of the antibiotic components C-1027 and C-1027-AG as shown in FIGURE 8. Antibiotic C-1027 is composed of a protein consisting of many hydrophobic and hydroxyamino acids with a non-protein chromophore. Antibiotic C-1027-AG is composed of the hydrophobic and hydroxyamino acids without the chromophore.

Human serum

FIGURE 9 displays the excellent recovery of albumin when 16 mL of human serum was purified on a 55 mm ID preparative TSKgel Ether-5PW column.

FIGURE 7

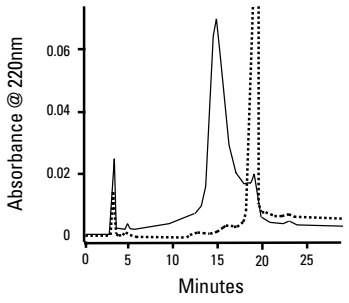
Screening of mouse monoclonal antibodies



Column: TSKgel Ether-5PW, 8.0mm ID x 7.5cm, glass
Sample: A. 20µL unequilibrated mouse IgG$_{2b}$κ ascites
B. 20µL unequilibrated mouse IgMκ ascites
Elution: linear gradient from Buffer A to B as shown
Buffer A: 0.05mol/L sodium phosphate, pH 7.0, 2.0mol/L ammonium sulfate, 1.0mol/L glycine
Buffer B: 0.05mol/L sodium phosphate, pH 7.0, 1.0mol/L glyci
Flow Rate: 1.0mL/min
Detection: UV @ 280nm

FIGURE 8

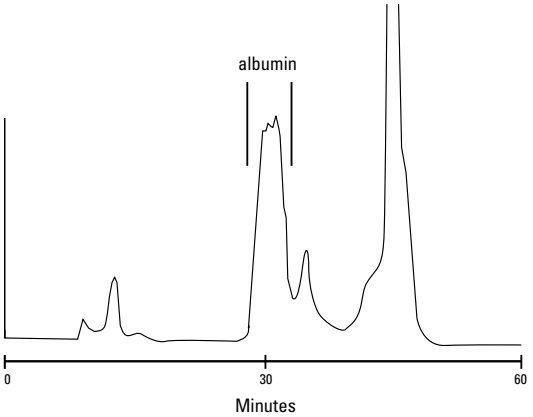
Purification of anti-tumor antibiotic



Column: TSKgel Ether-5PW, 7.5mmID x 7.5cm
Sample: ——— C-1027 ····· C-1027-AG
concentration: 1mg/mL
Injection: 20µL
Elution: linear gradient from 1.5mol/L to 0mol/L $(NH_4)_2SO_4$ in 0.1mol/L phosphate buffer, pH 7.0
Flow Rate: 0.8mL/min
Detection: UV @ 220nm

FIGURE 9

Human serum fractionated on preparative scale



Column: TSKgel Ether-5PW, 55mm ID x 20cm
Sample: 16mL human serum,1.2g total protein
Elution: 36min linear gradient from 1.7mol/L to 0.68mol/L $(NH_4)_2SO_4$ followed by step gradient to 0mol/L $(NH_4)_2SO_4$ in 0.1mol/L phosphate buffer, pH 7.0
Flow Rate: 40mL/min
Detection: UV @ 280nm
Recovery: 92% of the albumin was recovered in the fraction indicated by the vertical lines

APPLICATIONS OF TSK-GEL PHENYL-5PW COLUMNS

Recovery of biological activity for milligram to sub-gram amounts of enzymes eluted from TSKgel Phenyl-5PW columns is shown in the table below. In all cases, at least 80 % of the enzymatic activity was recovered.

TABLE II

Recovery of enzymatic activity from TSKgel Phenyl-5PW at various loadings

Enzyme	Recovery (%)
α-Chymotrypsin, 0.4 mg	92
β-Amylase, 1.3 mg	80
Ferredoxin NADP reductase, 3.0 mg	100
Lactate dehydrogenase, 54 mg	93
Lipoxidase, 1.0 mg	89
Lipoxidase, 200 mg	86
Lysozyme, 0.05 mg	90
Lysozyme, 0.2 mg	90
Phosphoglucose isomerase, 100 mg	96

RNAs

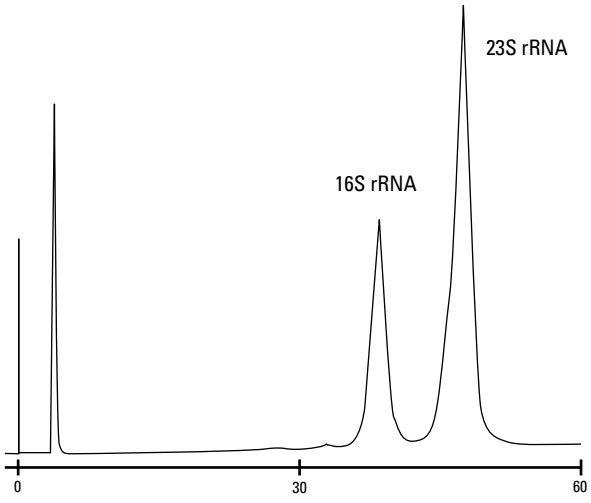
FIGURE 10 illustrates the separation of 16S and 23S ribosomal RNA on a TSKgel Phenyl-5PW column. The approximate molecular weights of these RNAs are 560,000 and 1,100,000 Da, respectively.

Proteins

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 10

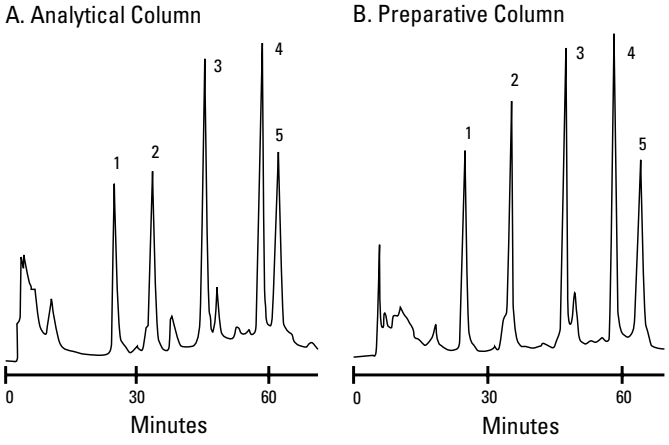
Retain large RNAs on TSKgel Phenyl-5PW



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

FIGURE 11

Scale up to preparative separations



Column: TSKgel Phenyl-5PW, A.) 7.5mm ID x 7.5cm and B.) 21.5mm ID x 15cm
Sample: 1. myoglobin, 2. ribonuclease A, 3. lysozyme, 4. α-chymotrypsinogen, 5. α-chymotrypsin
Elution: 60min linear gradient from 1.8mol/L to 0mol/L $(NH_4)_2SO_4$ in 0.1mol/L phosphate buffer, pH 7.0
Flow Rate: 0.5mL/min (7.5mm ID) or 4mL/min (21.5mm ID)
Detection: UV @ 280nm

HIC

APPLICATIONS OF TSK-GEL BUTYL-NPR COLUMNS

Proteins

Although loading capacity is limited on NPR columns, small scale separation of proteins is possible.

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 as shown in FIGURE 12.

FIGURE 13 shows the separation of Fab and Fc fragments of an antibody on TSKgel Butyl-NPR. The appearance of additional Fc fragments is due to the oxidation of methionine residues by 0.10% t-butylhydroperoxide (tBHP). The numbers above the Fc peaks correspond to the number of oxidized residues in each fragment.

Visit our website:

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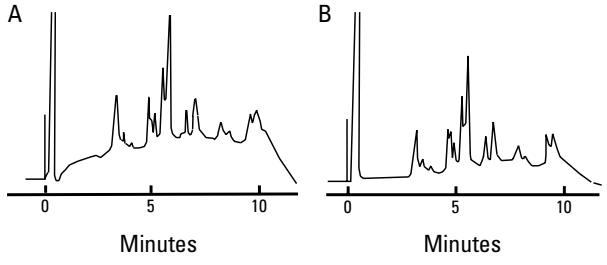
Contact our Technical Service specialists to discuss your specific application: +49 (0)711 13257-57 or techsupport.tbg@tosoh.com.

Please see next page for ordering information.

FIGURE 12

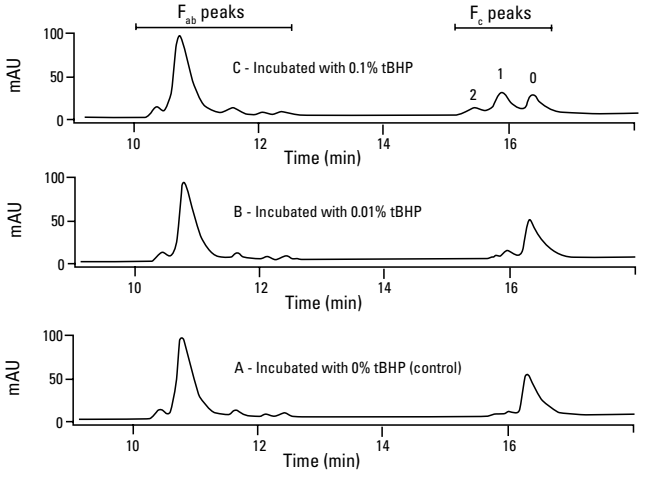
Effect of sample load on the separation of phosphoglucose isomerase



Column:	TSKgel Butyl-NPR, 4.6mm ID x 3.5cm
Sample:	crude sample of phosphoglucose isomerase
Loads:	A. 25µg; B. 100µg
Elution:	10min linear gradient of $(NH_4)_2SO_4$ from 1.8mol/L to 0mol/L in 0.1mol/L phosphate buffer, pH 7.0
Flow Rate:	1.0mL/min
Temperature:	25°C
Detection:	UV @ 280nm

FIGURE 13

Separation of F_{ab} and F_c fragments on TSKgel Butyl-NPR



Column:	TSKgel Butyl-NPR, 4.6mm ID x 3.5cm
Elution:	Buffer A: 2mol/L $(NH_4)_2SO_4$, 20mmol/L Tris, pH7 Buffer B: 20mmol/L Tris, pH7
Gradient:	linear from 10%B to 100%B in 34 minutes
Flow rate:	1mL/min
Temperature:	30°C

ORDERING INFORMATION

Part #	Description	ID (mm)	Length (cm)	Particle size (µm)	Number theoretical plates	Flow rate (mL/min) Range	Flow rate (mL/min) Max	Maximum pressure drop (kg/cm²)
Glass columns								
14013	Ether-5PW Glass, 1000 Å	5.0	5.0	10.0	≥ 600	0.5 - 0.8	1.0	20
14014	Ether-5PW Glass, 1000 Å	8.0	7.5	10.0	≥ 1,000	0.5 - 1.0	1.2	20
13063	Phenyl-5PW Glass, 1000 Å	5.0	5.0	10.0	≥ 600	0.5 - 0.8	1.0	20
08804	Phenyl-5PW Glass, 1000 Å	8.0	7.5	10.0	≥ 1,000	0.5 - 1.0	1.2	20
Stainless steel columns								
18760	Ether-5PW, 1000 Å	2.0	7.5	10.0	≥ 1,000	0.05 - 0.10	0.12	6
08641	Ether-5PW, 1000 Å	7.5	7.5	10.0	≥ 1,000	0.5 - 1.0	1.2	20
18759	Phenyl-5PW, 1000 Å	2.0	7.5	10.0	≥ 1,000	0.05 - 0.10	0.12	8
07573	Phenyl-5PW, 1000 Å	7.5	7.5	10.0	≥ 1,000	0.5 - 1.0	1.2	20
07656	Phenyl-5PW, 1000 Å	21.5	15.0	13.0	≥ 3,000	4.0 - 6.0	8.0	20
07938	Phenyl-5PW, 1000 Å	55.0	20.0	20.0	≥ 1,500	20.0 - 40.0	50.0	4
14947	Butyl-NPR, nonporous	4.6	3.5	2.5		0.5 - 1.0	1.2	200
PEEK columns								
20023	BioAssist Phenyl, 1000 Å	7.8	5	10.0	≥ 1,000	0.5 - 1.0	1.2	20

Guard column products		ID (mm)	Length (cm)	Particle Size (µm)	
14025	Ether-5PW Guardgel Kit, Glass			20.0	For P/Ns 14013 and 14014
08643	Ether-5PW Guardgel Kit			20.0	For P/N 08641
07652	Phenyl-5PW Guardgel Kit			20.0	For P/N 07573
16095	Phenyl-5PW Prep Guardgel Kit			20.0	For P/N 07656
07936	Phenyl-5PW Guard column	45.0	5.0	20.0	For P/N 07938

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