| | | |
|---|---|---|
| TOYOPEARL Butyl-600M | | |
| TOYOPEARL Butyl-650C | TOYOPEARL Butyl-650M | TOYOPEARL Butyl-650S |
| TOYOPEARL Ether-650M | TOYOPEARL Ether-650S | |
| TSKgel Ether-5PW (20) | TSKgel Ether-5PW (30) | |
| TOYOPEARL Hexyl-650C | | |
| TOYOPEARL Phenyl-600M | | |
| TOYOPEARL Phenyl-650C | TOYOPEARL Phenyl-650M | TOYOPEARL Phenyl-650S |
| TSKgel Phenyl-5PW (20) | TSKgel Phenyl-5PW (30) | |
| TOYOPEARL PPG-600M | | |
| TOYOPEARL SuperButyl-550C | | |

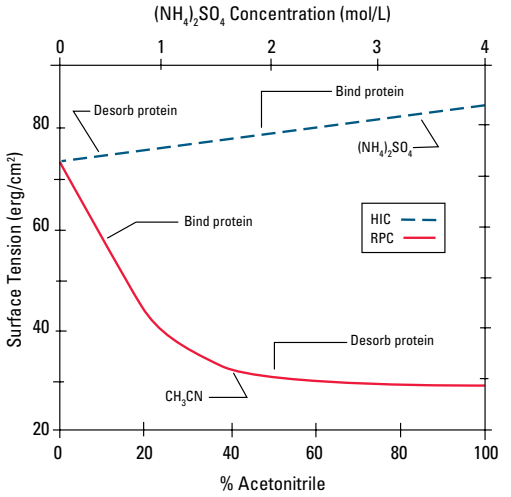
## The Role of Hydrophobic Interaction Chromatography in Process Purification

Hydrophobic interaction chromatography (HIC) is a powerful tool for the process purification of biomolecules. The technique utilizes the accessible hydrophobic regions located on protein surfaces and their interactions with a weakly hydrophobic stationary phase. HIC is an excellent complement to ion exchange and size exclusion chromatography particularly when protein isoforms exist or when feedstock impurities are of similar isoelectric point or molar mass. The selectivity differences exploited by HIC can also be used after affinity separations in which closely related proteins with similar recognition sites are not distinguishable by the affinity ligand.

Proteins and other molecules with hydrophobic surfaces are attracted to the hydrophobic ligands of HIC resins. Proteins are bound to the resin by employing an aqueous high salt mobile phase. The salt conditions contribute to a lyotropic effect which allows the proteins to bind to the lower surface coverage of a hydrophobic ligand. Proteins are eluted by the simple technique of decreasing the salt concentration. Most therapeutic targets are eluted in a low salt or a no salt buffer.

During elution, the energy of interaction for a HIC step is less than that of a reversed phase chromatography (RPC) step. One means of gauging the relative binding energy between the two techniques is to measure the surface tension of the two sets of binding and elution conditions. Figure 1 provides a comparison of the surface tension generated by HIC and RPC elution systems.[1] Since HIC separates under milder eluting conditions, biological activity is typically retained.

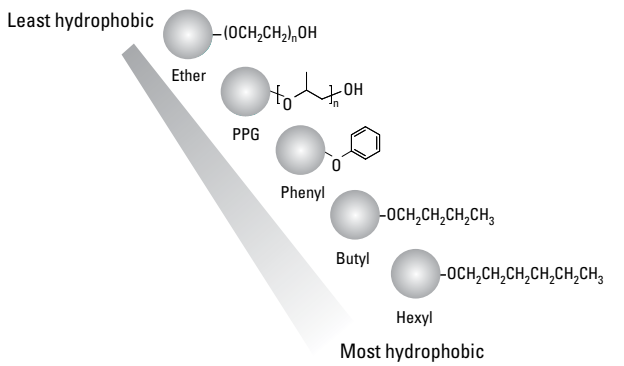
*Figure 1: The surface tension of aqueous solutions used in HIC and RPC*




| Mode | Gradient (Typical) | Δ Surface Tension (erg/cm²) |
|---|---|---|
| HIC | 1.8 to 0 mol/L $(NH_4)_2SO_4$/ aqueous buffer | 4 |
| RPC | 10 to 50% ACN/ 0.1%TFA | 23 |

[1]C. Horvath et. al., Separation Processes in Biotechnology, Volume 9; Asenjo, J. ed.; Marcel Dekker, Inc.: New York, 1990, p 447.

*Figure 2: Available HIC ligands*




## TOYOPEARL Hydrophobic Interaction Chromatography Resins

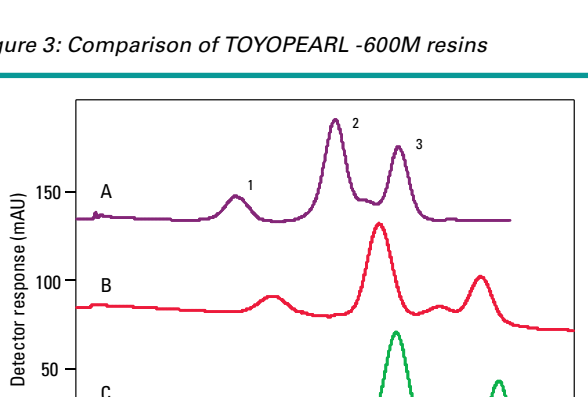
TOYOPEARL HIC resins are functionalized versions of the TOYOPEARL HW size exclusion resins and are therefore based on hydroxylated polymethacrylic polymer beads. Tosoh Bioscience offers five HIC ligands featuring different degrees of hydrophobicity and selectivity. Table 1 lists the properties of these TOYOPEARL HIC resins. The hydrophobicity of TOYOPEARL HIC resins increases through the ligand series: ether, PPG (polypropylene glycol), phenyl, butyl, and hexyl (Figure 2).

*Table 1: Properties of TOYOPEARL HIC resins*

| TOYOPEARL resin | Hydrophobicity | Base bead | Pore size (nm) | Bead diameter (µm) | Ligand type | DBC (g/L) | Pressure rating |
|---|---|---|---|---|---|---|---|
| Ether-650S | + | HW-65 | 100 | 20 - 50 | Ether | 10-30 | 0.3 MPa |
| Ether-650M | + | HW-65 | 100 | 40 - 90 | Ether | 10-30 | 0.3 MPa |
| PPG-600M | ++ | HW-60 | 75 | 40 - 90 | Polypropylene glycol | 45 - 55 | 0.3 MPa |
| Phenyl-600M | +++ | HW-60 | 75 | 40 - 90 | Phenyl | 45 - 65 | 0.3 MPa |
| Phenyl-650S | +++ | HW-65 | 100 | 20 - 50 | Phenyl | 30 - 50 | 0.3 MPa |
| Phenyl-650M | +++ | HW-65 | 100 | 40 - 90 | Phenyl | 30 - 50 | 0.3 MPa |
| Phenyl-650C | +++ | HW-65 | 100 | 50 - 150 | Phenyl | 30 - 50 | 0.3 MPa |
| Butyl-650S | ++++ | HW-65 | 100 | 20 - 50 | Butyl | 30 - 50 | 0.3 MPa |
| Butyl-650M | ++++ | HW-65 | 100 | 40 - 90 | Butyl | 30 - 50 | 0.3 MPa |
| Butyl-650C | ++++ | HW-65 | 100 | 50 - 150 | Butyl | 30 - 50 | 0.3 MPa |
| Butyl-600M | ++++ | HW-60 | 75 | 40 - 90 | Butyl | 40 - 60 | 0.3 MPa |
| SuperButyl-550C | ++++ | HW-55 | 50 | 50 - 150 | Butyl | 52 - 70 | 0.3 MPa |
| Hexyl-650C | +++++ | HW-65 | 100 | 50 - 150 | Hexyl | 30 - 50 | 0.3 MPa |

Three HIC ligands are available in the TOYOPEARL -600 resin format: PPG, phenyl, and butyl. The selectivities of TOYOPEARL Butyl-600M, TOYOPEARL PPG-600M and the TOYOPEARL Phenyl-600M resins are shown in Figure 3. Available in the TOYOPEARL -650 series are the following four HIC ligands: hexyl, butyl, phenyl, and ether. The remaining ligand available in the TOYOPEARL HIC resin line is SuperButyl-550.

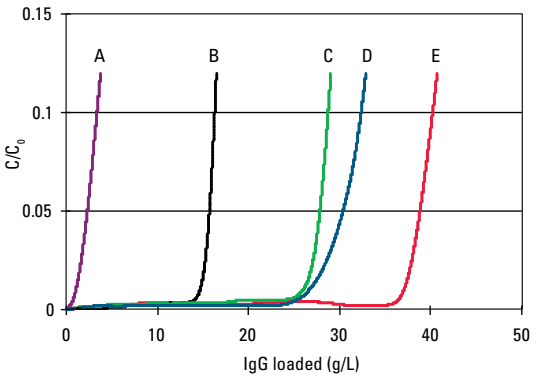
*Figure 3: Comparison of TOYOPEARL -600M resins*




**Resins:** **A. TOYOPEARL PPG-600M**
**B. TOYOPEARL Phenyl-600M**
**C. TOYOPEARL Butyl-600M**
Column size: 7.5 mm ID × 7.5 cm
Mobile phase: Buffer A: 1.8 mol/L $(NH_4)_2SO_4$ + 0.1 mol/L sodium phosphate, pH 7.0
Buffer B: 0.1 mol/L sodium phosphate, pH 7.0
Gradient: 60 min linear gradient from buffer A to B
Flow rate: 136 cm/hr (1.0 mL/min)
Detection: UV @ 280 nm
Temperature: ambient
Samples: 1 g/L of: 1. ribonuclease A 2.lysozyme 3. α-chymotrypsinogen
Load volume: 100 µL

A comparison of the dynamic binding capacities (DBCs) of the TOYOPEARL -600 resins with TOYOPEARL Phenyl-650M is shown in Figure 4. Figure 5 compares the selectivities of the TOYOPEARL Phenyl-600M and TOYOPEARL Phenyl-650M resins with an agarose based phenyl resin. The narrower pore diameter of TOYOPEARL SuperButyl-550C resin (based on the 50 nm pore diameter TOYOPEARL HW-55 resin) is recommended for the analysis of smaller molecules such as lysozyme ($1.2 \times 10^4$ Da). A comparison of the DBC of TOYOPEARL SuperButyl-550C resin with other TOYOPEARL HIC resins is shown in Figures 6 and 7.

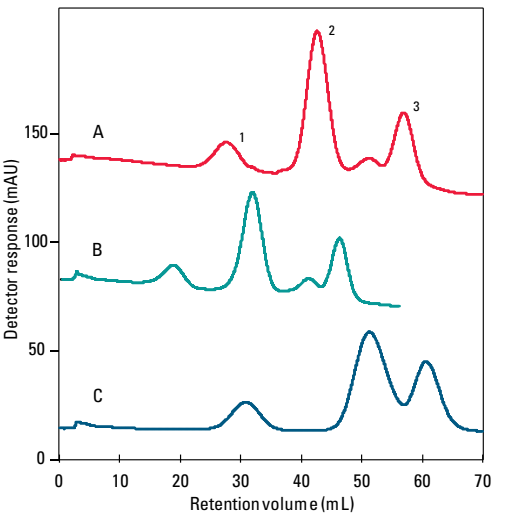
*Figure 4: Breakthrough curves of polyclonal IgG on various HIC resins*




| | |
|---|---|
| **Resins:** | **A. TOYOPEARL PPG-600M**<br>**B. TOYOPEARL Phenyl-650M**<br>**C. TOYOPEARL Butyl-600M**<br>D. Competitor Phenyl Agarose<br>**E. TOYOPEARL Phenyl-600M** |
| Column size: | 7.8 mm ID × 20 cm |
| Mobile phase: | 0.1 mol/L sodium phosphate, pH 7.0 + 0.8 mol/L $(NH_4)_2SO_4$ |
| Flow rate: | 300 cm/hr (2.4 mL/min) |
| Detection: | UV @ 280 nm |
| Temperature: | 25 °C |
| Samples: | 1.0 g/L polyclonal IgG |

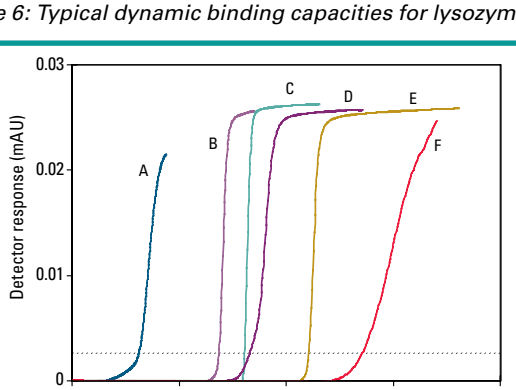
DBC was calculated at 10% breakthrough

*Figure 5: Selectivity comparison of phenyl-type resins*




| | |
|---|---|
| **Resins:** | **A. TOYOPEARL Phenyl-600M**<br>**B. TOYOPEARL Phenyl-650M**<br>C. Competitor Phenyl Agarose |
| Column size: | 7.5 mm ID × 7.5 cm |
| Mobile phase: | Buffer A: 1.8 mol/L $(NH_4)_2SO_4$ + 0.1 mol/L sodium phosphate, pH 7.0<br>Buffer B: 0.1 mol/L sodium phosphate, pH 7.0 |
| Gradient: | 60 min linear gradient from buffer A to B |
| Flow rate: | 136 cm/hr (1.0 mL/min) |
| Detection: | UV @ 280 nm |
| Temperature: | ambient |
| Sample: | 1.0 g/L of: 1. ribonuclease A 2.lysozyme 3. α-chymotrypsinogen |
| Load volume: | 100 µL |

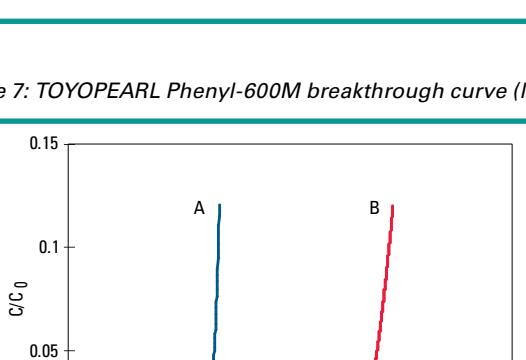
*Figure 6: Typical dynamic binding capacities for lysozyme*




| **Resins:** | binding capacity (g/L) (10% breakthrough) |
|---|---|
| **A. TOYOPEARL Ether-650M** | 12.5 |
| **B. TOYOPEARL Phenyl-650M** | 27.5 |
| **C. TOYOPEARL Butyl-650M** | 32.2 |
| **D. TOYOPEARL Hexyl-650C** | 33.2 |
| **E. TOYOPEARL PPG-600M** | 44.2 |
| **F. TOYOPEARL SuperButyl-550C** | 54.3 |

Column size: 7.8 mm ID × 20 cm
Mobile phase: 1.8 mol/L sodium sulfate + 0.1 mol/L phosphate, pH 7.0
Flow rate: 100 cm/hr (0.8 mL/min)
Detection: UV @ 280 nm
Temperature: ambient
Sample: 1 g/L lysozyme
Sample load: as indicated in figure

*Figure 7: TOYOPEARL Phenyl-600M breakthrough curve (lysozyme)*




| **Resins:** | binding capacity (g/L) (10% breakthrough) |
|---|---|
| **A. TOYOPEARL Phenyl-650M** | 27 |
| **B. TOYOPEARL Phenyl-600M** | 58 |

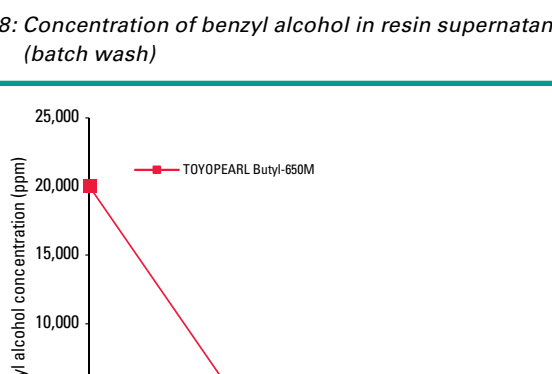
Column size: 7.8 mm ID × 20 cm
Mobile phase: 1.8 mol/L $(NH_4)_2SO_4$ + 0.1 mol/L phosphate, pH 7.0
Flow rate: 300 cm/hr (2.4 mL/min)
Detection: UV @ 280 nm
Temperature: ambient
Sample: 1 g/L lysozyme
Sample load: as indicated in figure

A 2% solution of benzyl alcohol in water has been identified as a suitable alternative to 20% ethanol as a preservative in resin storage solutions. A sample of TOYOPEARL Butyl-650M resin was prepared by adding 100 mL of aqueous 2% benzyl alcohol to 100 mL of suction filtered resin. A 100 mL aliquot of DI water was added to the filtered resin and stirred to make a slurry. This resin/ DI water slurry was allowed to stand for 5 minutes and was then suction filtered to remove the supernatant. This procedure was repeated 14 more times, for a total of 15 washes.

Samples of the filtered supernatant from the TOYOPEARL Butyl-650M resin was taken after the 5th, 10th, and 15th washes and analyzed for benzyl alcohol concentration (Figure 8). As demonstrated in the figure, a 2% benzyl alcohol solution can be removed from the TOYOPEARL Butyl-650M resin by thorough washing with DI water.

As benzyl alcohol is a hydrophobic molecule, it may not be possible to adequately reduce its concentration from hydrophobic interaction chromatography resins due to interactions between the preservative and the ligand. The use of benzyl alcohol (2%) with chromatography media that are un-functionalized or are functionalized with non-hydrophobic ligands is an acceptable alternative to the recommended 20% ethanol.

*Figure 8: Concentration of benzyl alcohol in resin supernatant (batch wash)*




The larger pore TOYOPEARL products such as TOYOPEARL Butyl-650 and TOYOPEARL Phenyl-650 resins are very useful for protein aggregate separation and removal. In addition, Tosoh Bioscience HIC resins are very effective in separating misfolded proteins from the native protein form. Because misfolded proteins will generally be more hydrophobic than the native protein, TOYOPEARL Butyl-650M resin is used frequently for the removal of misfolded proteins. In many cases, flow-through chromatography can be accomplished under eluent conditions binding the misfolded protein while allowing the native target protein to flow through the column.

Hydrophobic interaction is a very useful technique for the purification of monoclonal antibodies (mAbs), with their diverse hydrophobic nature. The range of HIC ligands of varying hydrophobicity available from Tosoh Bioscience (Figure 2) gives chromatographic developers a range of options for finding the right ligand for their target molecule.

## TSKgel Hydrophobic Interaction Chromatography Resins

The same ether and phenyl ligands that are used for the TOYOPEARL resins are also available within the TSKgel HIC resin product line. Properties of TSKgel HIC resins are listed in Table 2. The TSKgel HIC resins use the same methacrylic polymer chemistry as the TOYOPEARL resins (Table 3) but have a higher degree of crosslinking, making for a more rigid bead. This is necessitated by the higher pressures generated when using smaller particles for chromatography. Greater crosslinking decreases the number of sites available for ligand attachment and thus a TSKgel resin will have a lower dynamic binding capacity than the corresponding TOYOPEARL resin. The polymeric structure of these products also makes them resistant to a wide range of pH conditions and mobile phase ionic strengths. In addition, the hydroxylated surface of the base bead reduces non-specific binding of proteins.

*Table 3: Methacrylic base beads available for HIC*

| Pore size (nm) | 5 | 12.5 | 40-50 | 75 | 100 | >100 | >170 |
|---|---|---|---|---|---|---|---|
| Resin | | | | | | | |
| TOYOPEARL HW-type: | 40 | 50 | 55 | 60 | 65 | 75 | 80 |
| TSKgel PW-type: | G1000 | G2000 | G4000 | | G5000 | G6000 | |

Increasing pore surface area

TOYOPEARL HIC resins are chemically stable from pH 1-13. This allows a constant packing volume over a wide range of salt concentrations and cleaning in place (CIP) with acid or base. Also, these resins can be run at elevated temperatures (4-60 °C) and are autoclavable at 121 °C.

*Table 2: Properties of TSKgel HIC resins*

| TSKgel resin | Hydrophobicity | Base bead | Pore size (nm) | Bead diameter (µm) | Ligand type | DBC (g/L) | Pressure rating |
|---|---|---|---|---|---|---|---|
| Ether-5PW (20) | + | PW5000 | 100 | 15 - 25 | Ether | 10 - 30 | 2.0 MPa |
| Ether-5PW (30) | + | PW5000 | 100 | 20 - 40 | Ether | 10 - 30 | 2.0 MPa |
| Phenyl-5PW (20) | ++ | PW5000 | 100 | 15 - 25 | Phenyl | 10 - 30 | 2.0 MPa |
| Phenyl-5PW (30) | ++ | PW5000 | 100 | 20 - 40 | Phenyl | 10 - 30 | 2.0 MPa |

Because TOYOPEARL and TSKgel HIC resins have the same backbone polymer chemistry, the selectivity for proteins and impurities will be unchanged. Table 4 shows the ligands and particle sizes available for TOYOPEARL and TSKgel HIC resins and is arranged in increasing levels of resolution by bead size (i.e. low, medium, and high resolution). The semi-rigid polymeric backbone of TOYOPEARL and TSKgel HIC resins permits high flow rates for maximum throughput and productivity. TOYOPEARL HIC resins may be operated at pressures up to 0.3 MPa and TSKgel -5PW HIC resins may be operated up to 2.0 MPa. The pressure-flow characteristics for each particle size grade of TOYOPEARL Phenyl-650 resins are shown in Figure 9.

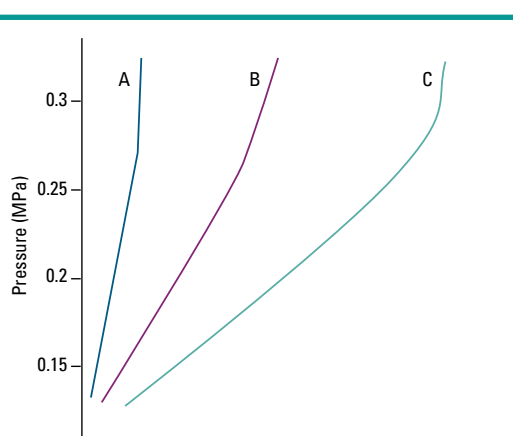
Resolution increases with decreasing particle size. Resin particle size is proportional to HETP and inversely proportional to the column efficiency and resolution of two peaks. TOYOPEARL HIC resins are available in three particle sizes, though not all ligands are available in each grade:

- S-grade = 35 µm (Superfine)
- M-grade = 65 µm (Fine)
- C-grade = 100 µm (Coarse)

Some processes, such as the purification of antibody-drug conjugates, require resins that are capable of higher resolution separations. For these separations, smaller diameter TOYOPEARL S-grade or TSKgel resins are preferred. TSKgel HIC resins are currently available in two ligands and two bead sizes:

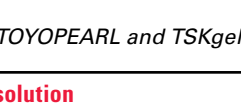
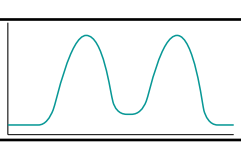
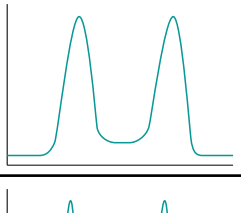
- TSKgel Ether-5PW (30) = 30 µm
- TSKgel Ether-5PW (20) = 20 µm
- TSKgel Phenyl-5PW (30) = 30 µm
- TSKgel Phenyl-5PW (20) = 20 µm

*Figure 9: Pressure-flow curve for TOYOPEARL Phenyl-650 resins of various particle sizes*




**Resins:** **A. TOYOPEARL Phenyl-650S**
**B. TOYOPEARL Phenyl-650M**
**C. TOYOPEARL Phenyl-650C**
Column size: 25 mm ID × 25 cm
Mobile phase: 2.0 mol/L $(NH_4)_2SO_4$
Flow rate: as indicated in figure

*Table 4: Resolution of TOYOPEARL and TSKgel HIC resins*

| Resolution | | Bead diameter (µm) | Pore size (nm) | HIC resin |
|---|---|---|---|---|
| **Low** |  | 100 | 50<br>100<br>100<br>100 | TOYOPEARL SuperButyl-550C<br>TOYOPEARL Hexyl-650C<br>TOYOPEARL Butyl-650C<br>TOYOPEARL Phenyl-650C |
| **Medium** |  | 65 | 75<br>75<br>75 | TOYOPEARL Butyl-600M<br>TOYOPEARL Phenyl-600M<br>TOYOPEARL PPG-600M |
| | | 65 | 100<br>100<br>100 | TOYOPEARL Butyl-650M<br>TOYOPEARL Phenyl-650M<br>TOYOPEARL Ether-650M |
| **High** |  | 35 | 100<br>100<br>100 | TOYOPEARL Butyl-650S<br>TOYOPEARL Phenyl-650S<br>TOYOPEARL Ether-650S |
| | | 30 | 100<br>100 | TSKgel Phenyl-5PW (30)<br>TSKgel Ether-5PW (30) |
| | | 20 | 100<br>100 | TSKgel Phenyl-5PW (20)<br>TSKgel Ether-5PW (20) |

## Parameters to Consider when Using Tosoh Bioscience HIC Resins

Coordinating the hydrophobicity of the therapeutic target to the resin hydrophobicity is critical for the best overall purification performance. Too hydrophobic a resin for a given protein can result in its irreversible binding to the resin or a loss of biological activity. Tables 5 and 6 show typical mass recovery and biological activity recovery data for TOYOPEARL HIC resins.

*Table 5: High mass recovery (%) of proteins*

| Protein | TOYOPEARL resin | | |
|---|---|---|---|
| | Ether-650M | Phenyl-650M | Butyl-650M |
| bovine serum albumin | 84 | 62 | 76* |
| α-chymotrypsinogen | 96 | 88* | 90 |
| cytochrome c | — | 81* | 87* |
| IgG | 91 | — | — |
| α-lactalbumin | 90 | — | — |
| lysozyme | 94 | 92 | 85 |
| ovalbumin | 83 | 88 | 73 |
| ribonuclease A | — | 72* | 82* |

Procedure: A 200 mL sample containing 200 mg of protein was loaded onto a 7.5 mm ID × 7.5 cm column and eluted with a 60 minute gradient of 1.8 mol/L (*1.5 mol/L) to 0.0 mol/L ammonium sulfate in 0.1 mol/L sodium phosphate, pH 7.0. The mass recovery was determined spectrophotometrically at UV 280 nm and 25 °C.

*Table 6: Recovery of enzymatic activity of proteins*

| TOYOPEARL resin | Protein | % Activity recovery |
|---|---|---|
| Phenyl-650 | phytochrome | 79 |
| Butyl-650 | halophilic protease | 85 |
| Butyl-650 | poly (3-hydroxybutyrate) depolymerase | 88 |
| Butyl-650 | aculeacin-A acylase | 82 |
| Butyl-650 | opine dehydrogenase | 81 |

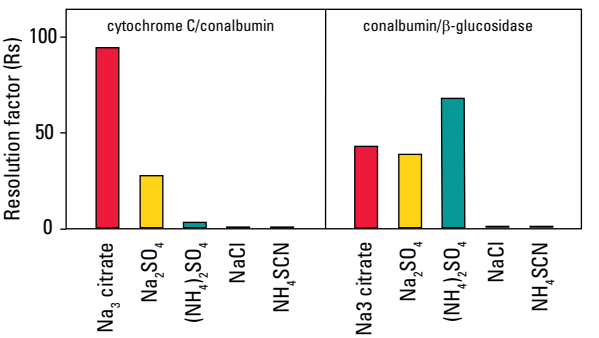
An optimum HIC process step will balance high dynamic binding capacity, adequate selectivity, good mass recovery and retention of biological activity. The wide range of selectivities for TOYOPEARL and TSKgel resins enables a developer to optimize protein separations at the extremes of the hydrophobic spectrum. The more hydrophobic ligands on TOYOPEARL Hexyl-type and TOYOPEARL Butyl-type resins are used to separate hydrophilic proteins. These two resins should also be considered for separations requiring a low salt environment.

TOYOPEARL and TSKgel Ether resins are used for the purification of very hydrophobic targets such as certain monoclonal antibodies and membrane proteins. These proteins may bind irreversibly to other more hydrophobic resins.

TOYOPEARL PPG and TOYOPEARL and TSKgel Phenyl resins complement the other HIC ligands available in the HIC series and offer alternatives for moderately hydrophobic proteins.

In addition to the hydrophobicity of the ligand, the selectivity in HIC is influenced by the eluent salt type. Figure 10 demonstrates the effect of salt type on the resolution factor of different protein pairs.[2] The Hofmeister lyotropic salt series shown in Figure 11 ranks anions and cations by their ability to promote protein precipitation. Ions on the left are referred to as "lyotropic" while the ions on the right are called "chaotropic". Lyotropic salts will precipitate or "salt out" proteins at high salt concentrations due to increased hydrophobic interaction, while chaotropic salts will promote protein denaturation at high salt concentrations. The Hofmeister lyotropic salt series indicates that the use of different salt systems may generate a variety of adsorption and desorption selectivities for each resin with a given protein. This feature of HIC provides an additional parameter for the optimization of a process step.

*Figure 10: Influence of salt-type on resolution*




Chromatography on a Toyopearl Butyl-substituted support

**Resin:** **TOYOPEARL Butyl-650M**
Column size: 4.1 mm ID × 4 cm
Mobile phase: Buffer A: 20 mmol/L phosphate buffer in 1.0 mol/L indicated salt, pH 7.0
Buffer B: buffer A with 1.0 mol/L indicated salt
Flow rate: 484 cm/hr (1 mL/min)
Detection: UV @ 280 nm

[2]Fausnaugh, J.; Kennedy, L.; Regnier, F. *J. Chromatography*, **1984**, 141, 317.

*Figure 11: Hofmeiseter lyotropic salt series*

**for anions**

$SO_4^{2-} > HPO_4^{2-} > CH_3COO^- >$ halide $> NO_3^- > ClO_4^- > SCN^-$

**for cations**

$(CH_3)_4N^+ > K^+ > Na^+ > Cs^+ > Li^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$

Ammonium sulfate and sodium sulfate are the most commonly used salts in HIC. NaCl is often used as well.

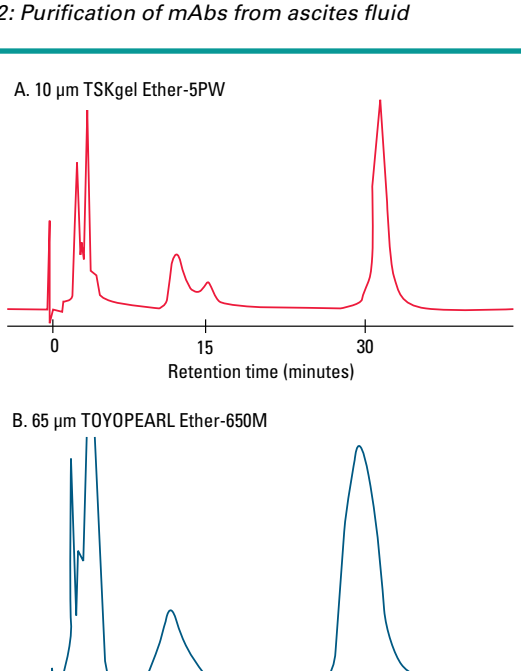
HIC is commonly used as a polishing step in monoclonal antibody purification processes. HIC offers an orthogonal selectivity to ion exchange chromatography and can be an effective step for aggregate clearance and host cell protein reduction, however, this mode of chromatography suffers from the limitation of use of high concentrations of kosmotropic salts to achieve the desired separation. Ghose et al[3] reports an unconventional way of operating HIC in the flowthrough (FT) mode with no kosmotropic salt in the mobile phase. TOYOPEARL Hexyl-650C was selected as the stationary phase and the pH of the mobile phase was modulated to achieve the required selectivity. Optimum pH conditions were chosen under which the antibody product of interest flowed through while impurities such as aggregates and host cell proteins bound to the column. The performance of the TOYOPEARL Hexyl-650C resin was comparable to that observed using conventional HIC conditions with high salt.

[3]Ghose, S.; Tao, Y.; Conley, L.; Cecchini, D. Purification of monoclonal antibodies by hydrophobic interaction chromatography under no-salt conditions. mAbs. 2013, 5, (5), 795-800.

## Purification of Monoclonal Antibodies

For a very hydrophobic mAb, such as mouse anti-chicken lectin (14 kDa), the less hydrophobic TOYOPEARL Ether ligand works quite well. The purification of this mAb from ascites fluid (Figure 12) was performed with a 10 µm TSKgel Ether-5PW semi-preparative column. Identical selectivity for scale-up was found with corresponding 65 µm TOYOPEARL Ether-650M resin.

*Figure 12: Purification of mAbs from ascites fluid*

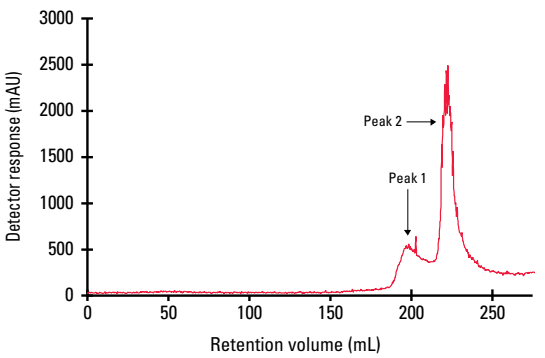



| | |
|---|---|
| **Resins:** | **A: TSKgel Ether-5PW (prepacked HPLC column)** |
| | **B: TOYOPEARL Ether-650M** |
| Column size: | 7.5 mm ID × 7.5 cm |
| Mobile phase: | Buffer A: 1.5 mol/L $(NH_4)_2SO_4$ + 0.1mol/L phosphate, pH 7.0 |
| | Buffer B: 0.1 mol/L phosphate, pH 7.0 |
| Gradient: | 60 min linear gradient from buffer A to B |
| Flow rate: | 136 cm/hr (1.0 mL/min) |
| Detection: | UV @ 280 nm |
| Temperature: | ambient |
| Sample: | A: 1.5 mg/100 µL anti-chicken 14 kDa lectin |
| | B: 0.76 mg/50 µL diluted ascites fluid |
| Load volume: | 150 µL |

## Plasmid DNA Purification

TOYOPEARL Hexyl-650C resin was used successfully for plasmid DNA purification by Cambrex, Baltimore, MD (US patent 6,953,686). The resin was shown to be the most effective among HIC resins for endotoxin removal with capacities exceeding 2 million EU/mL of resin. Additionally, RNA and protein impurities were effectively eliminated. TOYOPEARL Hexyl-650C was also effective in separating the supercoiled and open circular forms of plasmid DNA (Figure 13). Under certain binding conditions, the two forms are bound to the resin, and subsequently eluted with a simple gradient, resulting in two distinct peaks corresponding to the relaxed and supercoiled forms respectively.

*Figure 13: Plasmid DNA separation*

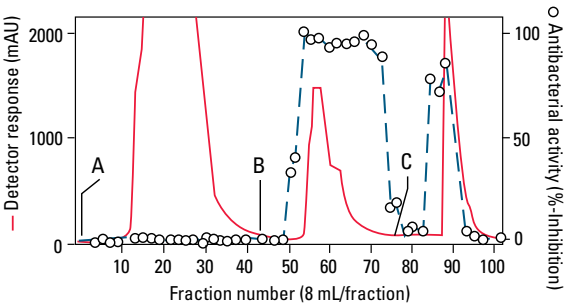



| | |
|---|---|
| **Resin:** | **TOYOPEARL Hexyl-650C** |
| Column size: | 1.0 cm ID × 30 cm (23.6 mL) |
| Mobile phase: | Buffer A. 3.0 mol/L ammonium sulfate, 10 mmol/L Tris, 1 mmol/L EDTA, pH 7.4 |
| | Buffer B. 10 mmol/L Tris, 1 mmol/L EDTA, pH 7.4 |
| Gradient: | 3.0 mol/L ammonium sulfate to 1.0 mol/L ammonium sulfate (6 CV) |
| Flow rate: | 153 cm/hr (2.0 mL/min) |
| Detection: | UV @ 254 nm |
| Sample: | Plasmid DNA in 3.0 mol/L ammonium sulfate |

## Purification of Glycoproteins

TOYOPEARL HIC resins can purify glycoproteins, which often bind irreversibly to saccharide-based chromatographic media. Figure 14 shows the purification of a large glycoprotein on TOYOPEARL Butyl-650S resin.

*Figure 14: Large glycoprotein purified on TOYOPEARL Butyl-650S*

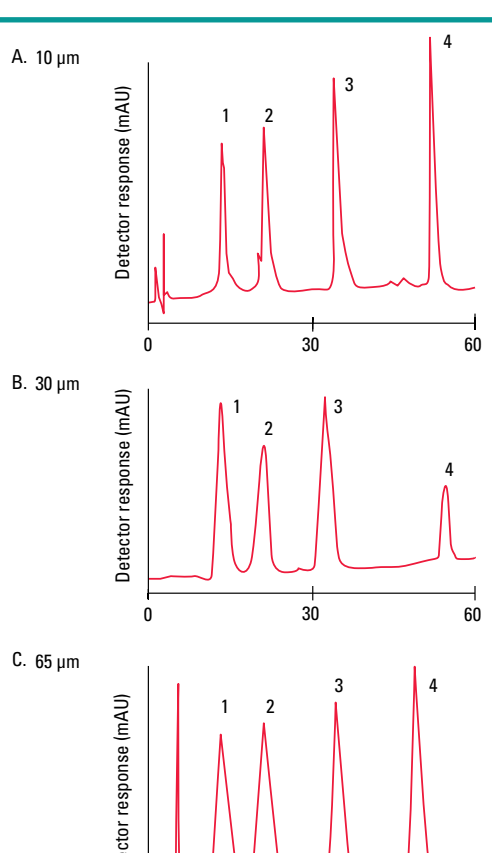



| | |
|---|---|
| **Resin:** | **TOYOPEARL Butyl-650S** |
| Column size: | 22 mm ID × 26 cm |
| Mobile phase: | Buffer A: 40% saturated $(NH_4)_2SO_4$ + 50 mol/L phosphate, pH 7.0 |
| | Buffer B: 50 mol/L phosphate, pH 7.0 |
| Gradient: | A: Load and wash in 100% buffer A |
| | B: 50% buffer A with 50% buffer B |
| | C: 100% buffer B |
| Detection: | UV @ 280 nm |
| Sample: | crude protein from sea hare *Aplysia kurodai* |

## Ultra Purification of Target Compound

Biopharmaceutical process development often requires a high performance step for ultra-purification of a target compound. To meet these needs, 20 and 30 µm TSKgel Phenyl-5PW and Ether-5PW are available. The selectivity of these packings is similar to the 10 µm TSKgel 5PW Phenyl-5PW and Ether-5PW analytical columns. Therefore methods can easily be transferred from analytical to preparative scale resins of the same chemistry using a seamless scale-up strategy. Figure 15 shows the similar elution pattern on 10 µm and 30 µm TSKgel packings, along with 65 µm TOYOPEARL process-scale resin.

*Figure 15: Seamless scale up*

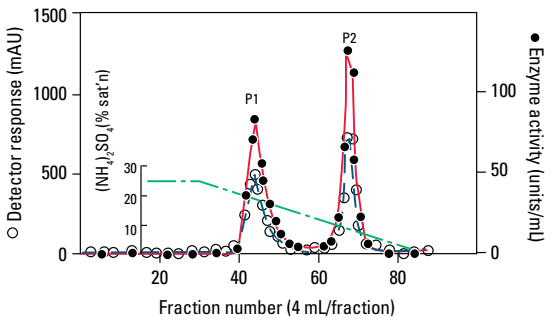



| | |
|---|---|
| **Resins:** | **A and B. TSKgel Phenyl-5PW**<br>**C. TOYOPEARL Phenyl-650M** |
| Column size: | 7.5 mm ID × 7.5 cm |
| Mobile phase: | A and B: 60 min linear gradient from 1.8 mol/L to |
| Gradient: | 0 mol/L $(NH_4)_2SO_4$ in 0.1 mol/L phosphate buffer<br>C: 240 min linear gradient from 1.8 mol/L to 0 mol/L$(NH_4)_2SO_4$ in 0.1 mol/L phosphate buffer |
| Flow rate: | A and B. 68 cm/hr (0.50 mL/min)<br>C. 272 cm/hr (2.0 mL/min) |
| Detection: | UV @ 280 nm |
| Samples: | 1. myoglobin<br>2. ribonuclease A<br>3. lysozyme<br>4. α-chymotrypsinogen |
| Load volume: | 100 µL containing 1 g/L of each protein |

## Purification and Resolution of Pullulanase

The power of HIC is illustrated in a scheme in which pullulanase, an amylase-like enzyme responsible for hydrolysis of branched chain sugars, is purified and resolved into two closely related forms. Ion exchange and size exclusion chromatography effectively purified pullulanase. With TOYOPEARL Butyl-650S, however, two closely related proteins were resolved, based on differences in their surface hydrophobicity (Figure 16).

*Figure 16: Separation of two active pullulanase forms*

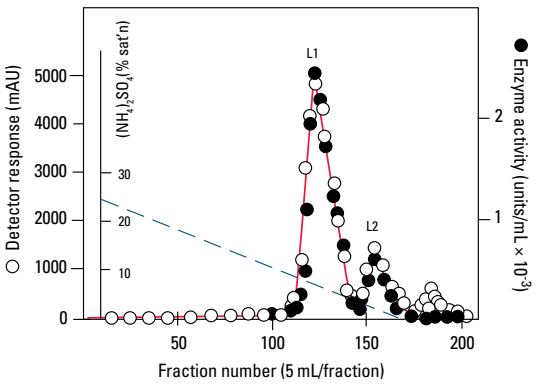



| | |
|---|---|
| **Resin:** | **TOYOPEARL Butyl-650S** |
| Column size: | 18 mm ID × 16 cm |
| Mobile phase: | isocratic elution, 120 mL $(NH_4)_2SO_4$, 25% saturation in 0.02 mol/L phosphate, pH 7.0, followed by a linear gradient, 224 mL $(NH_4)_2SO_4$, 25% to 0% saturation, in buffer |
| Flow rate: | 12 cm/hr |
| Detection: | UV @ 280 nm |
| Sample: | protein from *Bacillus acidopullulyticus* |
| Sample load: | 20 mg |

## Lipase Isozymes

Incorporation of HIC into a purification scheme has separated lipase isozymes that were not resolved by a previously reported method. After ion exchange and size exclusion chromatography, an additional step employing TOYOPEARL Butyl-650M, as shown in Figure 17, enabled the separation of two active lipase isozymes, L1 and L2, from an inactive impurity. Activity recovery was 93% for this step.

*Figure 17: Separation of lipase isozymes from impurity*




| | |
|---|---|
| **Resin:** | **TOYOPEARL Butyl-650M** |
| Column size: | 34 mm ID × 29 cm |
| Mobile phase: | linear gradient, 810 mL $(NH_4)_2SO_4$, 25% to 0% saturation in 0.01 mol/L acetate buffer, pH 5.6 |
| Flow rate: | 2 cm/hr |
| Detection: | UV @ 254nm |
| Sample: | lipase from *Geotrichum candidum* |
| Sample load: | 375 mg |

A selection of screening tools are available for TOYOPEARL and TSKgel HIC resins. See the Process Development Products section of this Product Guide for details.

## Ordering Information

**TOYOPEARL HIC resins:**

| Part # | Product description | Container size (mL) | Bead diameter (µm) | Typical lysozyme capacity (g/L) |
|---|---|---|---|---|
| 43151 | TOYOPEARL Ether-650S | 25 | 20 - 50 | 10 - 30 |
| 16172 | TOYOPEARL Ether-650S | 100 | 20 - 50 | 10 - 30 |
| 16174 | TOYOPEARL Ether-650S | 1,000 | 20 - 50 | 10 - 30 |
| 16176 | TOYOPEARL Ether-650S | 5,000 | 20 - 50 | 10 - 30 |
| | | | | |
| 19805 | TOYOPEARL Ether-650M | 25 | 40 - 90 | 10 - 30 |
| 16173 | TOYOPEARL Ether-650M | 100 | 40 - 90 | 10 - 30 |
| 16175 | TOYOPEARL Ether-650M | 1,000 | 40 - 90 | 10 - 30 |
| 16177 | TOYOPEARL Ether-650M | 5,000 | 40 - 90 | 10 - 30 |
| | | | | |
| 21301 | TOYOPEARL PPG-600M | 25 | 40 - 90 | 45 - 55 |
| 21302 | TOYOPEARL PPG-600M | 100 | 40 - 90 | 45 - 55 |
| 21303 | TOYOPEARL PPG-600M | 1,000 | 40 - 90 | 45 - 55 |
| 21304 | TOYOPEARL PPG-600M | 5,000 | 40 - 90 | 45 - 55 |
| 21305 | TOYOPEARL PPG-600M | 50,000 | 40 - 90 | 45 - 55 |
| | | | | |
| 21887 | TOYOPEARL Phenyl-600M | 25 | 40 - 90 | 45 - 65 |
| 21888 | TOYOPEARL Phenyl-600M | 100 | 40 - 90 | 45 - 65 |
| 21889 | TOYOPEARL Phenyl-600M | 1,000 | 40 - 90 | 45 - 65 |
| 21890 | TOYOPEARL Phenyl-600M | 5,000 | 40 - 90 | 45 - 65 |
| 21891 | TOYOPEARL Phenyl-600M | 50,000 | 40 - 90 | 45 - 65 |
| | | | | |
| 43152 | TOYOPEARL Phenyl-650S | 25 | 20 - 50 | 30 - 50 |
| 14477 | TOYOPEARL Phenyl-650S | 100 | 20 - 50 | 30 - 50 |
| 14784 | TOYOPEARL Phenyl-650S | 1,000 | 20 - 50 | 30 - 50 |
| 14935 | TOYOPEARL Phenyl-650S | 5,000 | 20 - 50 | 30 - 50 |
| | | | | |
| 19818 | TOYOPEARL Phenyl-650M | 25 | 40 - 90 | 30 - 50 |
| 14478 | TOYOPEARL Phenyl-650M | 100 | 40 - 90 | 30 - 50 |
| 14783 | TOYOPEARL Phenyl-650M | 1,000 | 40 - 90 | 30 - 50 |
| 14943 | TOYOPEARL Phenyl-650M | 5,000 | 40 - 90 | 30 - 50 |
| 18364 | TOYOPEARL Phenyl-650M | 50,000 | 40 - 90 | 30 - 50 |
| | | | | |
| 43126 | TOYOPEARL Phenyl-650C | 25 | 50 - 150 | 30 - 50 |
| 14479 | TOYOPEARL Phenyl-650C | 100 | 50 - 150 | 30 - 50 |
| 14785 | TOYOPEARL Phenyl-650C | 1,000 | 50 - 150 | 30 - 50 |
| 14944 | TOYOPEARL Phenyl-650C | 5,000 | 50 - 150 | 30 - 50 |

| Part # | Product description | Container size (mL) | Bead diameter (µm) | Typical lysozyme capacity (g/L) |
|---|---|---|---|---|
| 43153 | TOYOPEARL Butyl-650S | 25 | 20 - 50 | 30 - 50 |
| 07476 | TOYOPEARL Butyl-650S | 100 | 20 - 50 | 30 - 50 |
| 14701 | TOYOPEARL Butyl-650S | 1,000 | 20 - 50 | 30 - 50 |
| 07975 | TOYOPEARL Butyl-650S | 5,000 | 20 - 50 | 30 - 50 |
| 18826 | TOYOPEARL Butyl-650S | 50,000 | 20 - 50 | 30 - 50 |
| | | | | |
| 19802 | TOYOPEARL Butyl-650M | 25 | 40 - 90 | 30 - 50 |
| 07477 | TOYOPEARL Butyl-650M | 100 | 40 - 90 | 30 - 50 |
| 14702 | TOYOPEARL Butyl-650M | 1,000 | 40 - 90 | 30 - 50 |
| 07976 | TOYOPEARL Butyl-650M | 5,000 | 40 - 90 | 30 - 50 |
| 18355 | TOYOPEARL Butyl-650M | 50,000 | 40 - 90 | 30 - 50 |
| | | | | |
| 43127 | TOYOPEARL Butyl-650C | 25 | 50 - 150 | 30 - 50 |
| 07478 | TOYOPEARL Butyl-650C | 100 | 50 - 150 | 30 - 50 |
| 14703 | TOYOPEARL Butyl-650C | 1,000 | 50 - 150 | 30 - 50 |
| 07977 | TOYOPEARL Butyl-650C | 5,000 | 50 - 150 | 30 - 50 |
| 22826 | TOYOPEARL Butyl-650C | 50,000 | 50 - 150 | 30 - 50 |
| | | | | |
| 21448 | TOYOPEARL Butyl-600M | 25 | 40 - 90 | 40 - 60 (γ-globulin) |
| 21449 | TOYOPEARL Butyl-600M | 100 | 40 - 90 | 40 - 60 (γ-globulin) |
| 21450 | TOYOPEARL Butyl-600M | 1,000 | 40 - 90 | 40 - 60 (γ-globulin) |
| 21451 | TOYOPEARL Butyl-600M | 5,000 | 40 - 90 | 40 - 60 (γ-globulin) |
| 21452 | TOYOPEARL Butyl-600M | 50,000 | 40 - 90 | 40 - 60 (γ-globulin) |
| | | | | |
| 19955 | TOYOPEARL SuperButyl-550C | 25 | 50 - 150 | 52 - 70 |
| 19956 | TOYOPEARL SuperButyl-550C | 100 | 50 - 150 | 52 - 70 |
| 19957 | TOYOPEARL SuperButyl-550C | 1,000 | 50 - 150 | 52 - 70 |
| 19958 | TOYOPEARL SuperButyl-550C | 5,000 | 50 - 150 | 52 - 70 |
| 19959 | TOYOPEARL SuperButyl-550C | 50,000 | 50 - 150 | 52 - 70 |
| | | | | |
| 44465 | TOYOPEARL Hexyl-650C | 25 | 50 - 150 | 30 - 50 |
| 19026 | TOYOPEARL Hexyl-650C | 100 | 50 - 150 | 30 - 50 |
| 19027 | TOYOPEARL Hexyl-650C | 1,000 | 50 - 150 | 30 - 50 |
| 19028 | TOYOPEARL Hexyl-650C | 5,000 | 50 - 150 | 30 - 50 |
| 21973 | TOYOPEARL Hexyl-650C | 50,000 | 50 - 150 | 30 - 50 |

**TSKgel HIC resins:**

| Part # | Product description | Container size (mL) | Bead diameter (µm) | Typical lysozyme capacity (g/L) |
|---|---|---|---|---|
| 43276 | TSKgel Ether-5PW (20) | 25 | 15 - 25 | 10 - 30 |
| 16052 | TSKgel Ether-5PW (20) | 250 | 15 - 25 | 10 - 30 |
| 16053 | TSKgel Ether-5PW (20) | 1,000 | 15 - 25 | 10 - 30 |
| 18437 | TSKgel Ether-5PW (20) | 5,000 | 15 - 25 | 10 - 30 |
| | | | | |
| 43176 | TSKgel Ether-5PW (30) | 25 | 20 - 40 | 10 - 30 |
| 16050 | TSKgel Ether-5PW (30) | 250 | 20 - 40 | 10 - 30 |
| 16051 | TSKgel Ether-5PW (30) | 1,000 | 20 - 40 | 10 - 30 |
| 18439 | TSKgel Ether-5PW (30) | 5,000 | 20 - 40 | 10 - 30 |
| | | | | |
| 43277 | TSKgel Phenyl-5PW (20) | 25 | 15 - 25 | 15 - 35 |
| 14718 | TSKgel Phenyl-5PW (20) | 250 | 15 - 25 | 15 - 35 |
| 14719 | TSKgel Phenyl-5PW (20) | 1,000 | 15 - 25 | 15 - 35 |
| 18438 | TSKgel Phenyl-5PW (20) | 5,000 | 15 - 25 | 15 - 35 |
| | | | | |
| 43177 | TSKgel Phenyl-5PW (30) | 25 | 20- 40 | 10 - 30 |
| 14720 | TSKgel Phenyl-5PW (30) | 250 | 20 - 40 | 10 - 30 |
| 14721 | TSKgel Phenyl-5PW (30) | 1,000 | 20 - 40 | 10 - 30 |
| 17210 | TSKgel Phenyl-5PW (30) | 5,000 | 20 - 40 | 10 - 30 |


TOYOPEARL®
Phenyl-650M
Net Vol. 100 mL
Part No. 14478
Lot No.
FOR IN VITRO USE ONLY
TOYOPEARL®
Butyl-650M
25 mL
19802
TOSOH
TSKgel®
Phenyl-5PW (20)
25 mL
43277
TOSOH