

Separation of IgM using TSKgel BioAssist S

TSKgel
APPLICATION NOTE

Abstract

A novel macroporous ion exchange material has been developed to provide high retention and capacity of large biomolecules such as IgM. TSKgel BioAssist columns are offered in an analytical format and most recently in a new 13micron particle size for semi-preparative purifications.

Introduction

The use of monoclonal antibodies (MAb) is becoming more important in diagnosis and treatment of disease. IgM in particular has shown to possess unique and beneficial characteristics relative to other immunoglobulin classes; it is a large molecule comprised of five IgG subunits, resulting in a relatively unstable and difficult to purify molecule. Unlike single chain antibodies, IgM cannot be purified by Protein A (an affinity material commonly used for its high binding capacity and excellent selectivity for antibodies) due to steric hindrance. Alternative affinity methods have been developed with thiophilic absorbents but often result in low binding capacity.

In this note we examine an alternative purification method of IgM by ion exchange chromatography using a novel high capacity stationary phase.

Results

As shown in *Figure 1*, baseline separation of IgM from other contaminants is achieved using a 0.3M NaCl step gradient after elution of albumin. Linear gradient separations, although possible, did not yield equivalent baseline separations (data not shown). Purity analysis by size exclusion of both fractions taken during anion exchange is shown in *Figure 2*. The IgM fraction was determined to be 97% pure. Interestingly, our work showed that increasing the sample load increased the purity yield of the IgM fraction. *Figure 3* shows that below 0.75mg loading, the purity decreases rapidly. It is postulated that the IgM competitively displaces the albumin from the sulpropyl binding sites, accounting for the increase in purity as the IgM concentration increases.

The separation performance is maintained up to capacities of over 60mg/mL of resin (data not shown). Researchers with a need to purify amounts greater than the 20mg/column possible with the analytical format should use the recently commercialized 13micron semi-preparative column. To compensate for the loss in resolution attributed to the increase in particle size, the semi-preparative column was lengthened relative to the analytical format.

Figure 1. Separation of IgM by Cation Exchange

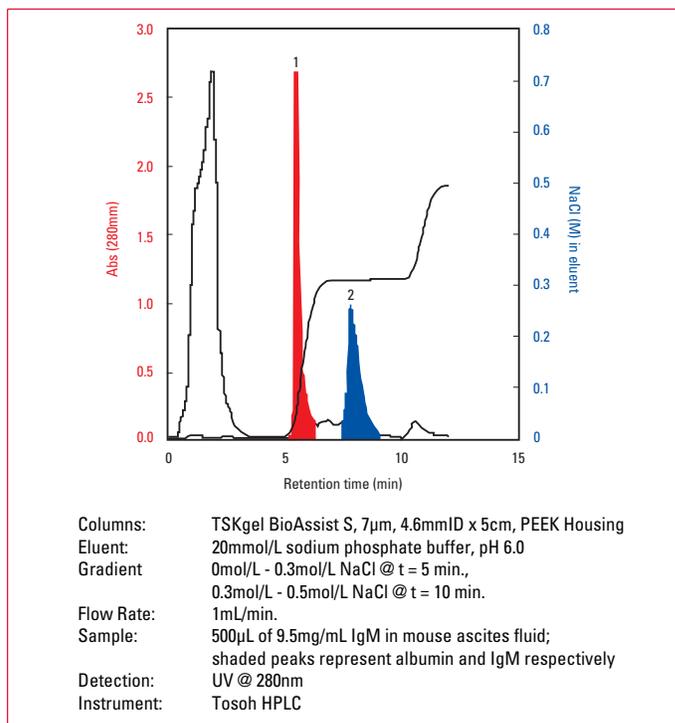


Figure 2. Purity Determination by Size Exclusion Chromatography

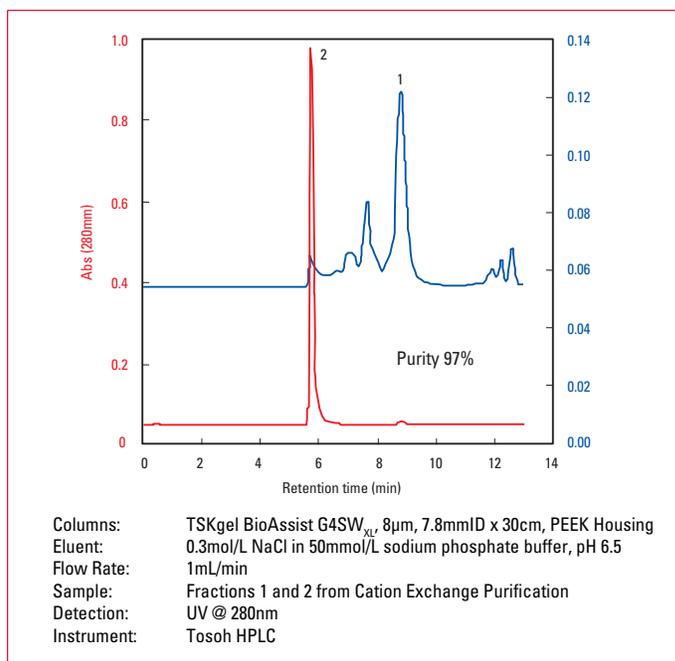
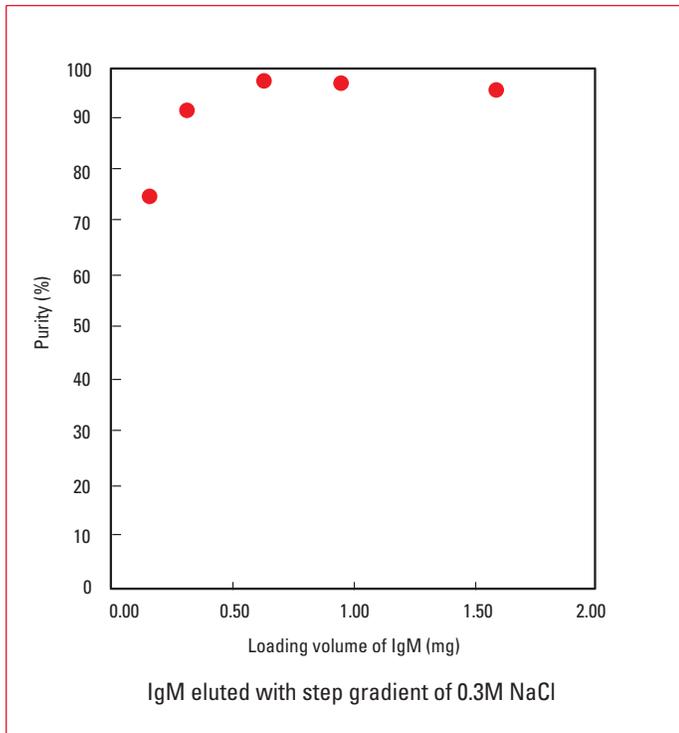


Figure 3. Influence of Loading Volume



Conclusions

The macroporous TSKgel BioAssist S offers high binding capacity and excellent resolution for purification of antibodies. More information about column attributes or the IgM purification can be found on the TSKgel BioAssist S product page of the website, www.tosohbioscience.com/seperation/us.

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Separation of IgG and Albumin by High Performance Gel Filtration Chromatography Using TSKgel G3000SW_{XL}

(Application of IgG Preparation
for Intravenous Injection)

Table of Contents

1. Introduction	1
2. Experimental Conditions	1
3. Results and Discussion	1
4. Conclusion	3



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1. Introduction

Human gamma immunoglobulin G (IgG, polyclonal) is effective as a blood preparation for intravenous injection for treatment of hypogammaglobulinemia/agammaglobulinemia or severe infectious diseases, as well as sudden thrombocytopenic purpura, in combined use with antibiotics. It is applied widely in clinical fields. Such IgG preparations include those in which albumin is added as a stabilizer¹⁻⁴. Rapid quantification of IgG and albumin is important for quality control of these preparations and high performance liquid chromatography (HPLC) is often the method of choice.

Numerous reports describe the separation between IgG and albumin by HPLC using ion exchange chromatography (IEC)⁵⁻¹¹, hydroxyapatite chromatography (HAC)¹²⁻¹³, hydrophobic interaction chromatography (HIC)⁹, and affinity chromatography (AFC)¹⁴⁻¹⁷ as the separation mode. However, separation time is long in any of these separation modes and a gradient elution method is used, which is not suited to quality control in which simple and rapid quantification are required. On the other hand, gel filtration chromatography (GFC) is a simple and fast procedure, although it may not always provide optimum resolution. The separation of IgG by GFC has been reported^{5-6, 18-20} and the use of GFC has also been widely adopted in quality control methods. Though there have been reports which dealt with protein elution conditions using GFC²¹⁻²⁷, the conditions for the separation of IgG and albumin by GFC have not been examined in detail. Only very recently Lee et al²¹ examined the separation of IgG preparation for intravenous injection containing albumin. However, a two-step chromatography method, consisting of IEC and GFC, was used for this separation and the method required two hours.

In this study the elution conditions for the separation of IgG and albumin using TSKgel G3000SW_{XL}²⁹, a high performance GFC column, was examined. The goal of this study was to obtain high resolution in a short time period using only gel filtration chromatography. This document reports on the favorable results obtained with a TSKgel G3000SW_{XL} column in separating the components of an IgG preparation.

2. Experimental Conditions

A computer controlled pump (CCPE) and a UV/VIS detector (UV-8010) were used with an HPLC system with detection at 280nm. TSKgel G3000SW_{XL} (7.8mm ID × 30cm) was employed as the separation column. For eluent, phosphate buffer, acetate buffer and citrate buffer were used, along with the salts Na₂SO₄, NaCl and NaClO₄, to examine the elution conditions with buffers of pH 5.0 to pH 7.0. All measurements were performed at the flow rate of 1.0mL/min and temperature of 25°C. Examination of the elution conditions was conducted with 100µg each of IgG and albumin. Recovery of IgG preparation from the column was calculated from the chromatogram's peak area.

Resolution (Rs) of IgG and albumin was calculated using the following formula:

$$R_s = \frac{2(V_2 - V_1)}{W_1 + W_2} \frac{1}{\log[MW_1] - \log[MW_2]}$$

V₁: Elution volume of IgG monomer (mL)
V₂: Elution volume of albumin monomer (mL)

W₁: Peak width of IgG monomer (mL)
W₂: Peak width of albumin monomer (mL)
MW₁: Molecular weight of IgG monomer
MW₂: Molecular weight of albumin monomer

Sample solutions of human IgG and human albumin were prepared from commercial products manufactured by Miles Laboratories and Sigma, respectively.

Commercial IgG preparations of Venoglobulin-I from Midori Juji, Venilon from Fujisawa Pharmaceutical and Gummaguard from Baxter were used. The ratio of IgG and albumin in each IgG preparation was labeled as 5:1, 20:1, and 50:1 respectively.

3. Results and Discussion

Figure 1 shows the effects of pH and salt for a phosphate buffer eluent on the elution volumes of IgG and albumin. The salt concentration of the eluent was adjusted so that the ionic strength was nearly equal. When 0.3mol/L NaCl and 0.3mol/L NaClO₄ are used as salts, the difference in elution volume between IgG and albumin were nearly equal, and little effect of eluent pH was seen. Conversely, when 0.1mol/L Na₂SO₄ was used as salt, the difference in elution volume was significant, and it became larger as pH was reduced from 7.0 to 5.0, as albumin elutes later compared to the IgG elution position, which moves only slightly. However, when an eluent of pH 4.0 is used, the albumin peak became broader and the separation of IgG and albumin became insufficient. The conclusion is that eluent of pH 5.0, containing Na₂SO₄ as the salt, is ideal for separation of IgG and albumin. When separation was examined as to buffer type using phosphate buffer, acetate buffer and citrate buffer, the phosphate buffer resulted in overall better resolution between albumin and IgG.

Figure 2 shows the effect of Na₂SO₄ concentration on the resolution of IgG and albumin when phosphate buffer solutions of pH 5.0 and pH 6.0 are used. When the eluent pH was adjusted to pH 6.8, resolution was minimal and Rs could not be calculated.

It is clear that the separation is better when an eluent of pH 5.0 is used. Resolution is optimal at pH 5.0 at 1.0M Na₂SO₄ concentration. Resolution decreased at salt concentrations above 0.1mol/L; calculating Rs was no longer possible at a concentration of 0.2mol/L and higher. At concentrations of 0.5mol/L or higher, hydrophobic interactions between the packing material and the sample became prominent, which resulted in peak broadening and longer elution times. Moreover, resolution deteriorated at salt concentrations below 0.1mol/L due to increasing importance of ionic interactions.

Based on the above results, it is observed that an eluent

containing 50mmol/L phosphate buffer and 0.1mol/L Na_2SO_4 , adjusted to pH 5.0, yields optimal resolution for the separation of IgG and albumin.

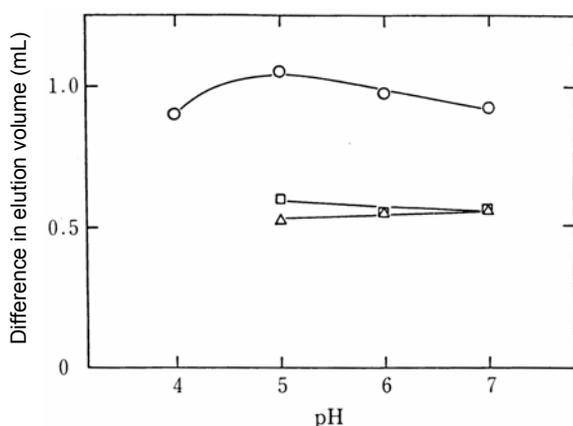


Figure 1 Effect of eluent salt and pH on separation of IgG and albumin

IgG and albumin were eluted in 50mmol/L phosphate buffer with various pH values containing 0.1mol/L Na_2SO_4 (O), 0.3mol/L NaCl (□) or 0.3mol/L NaClO_4 (Δ) as the salt.

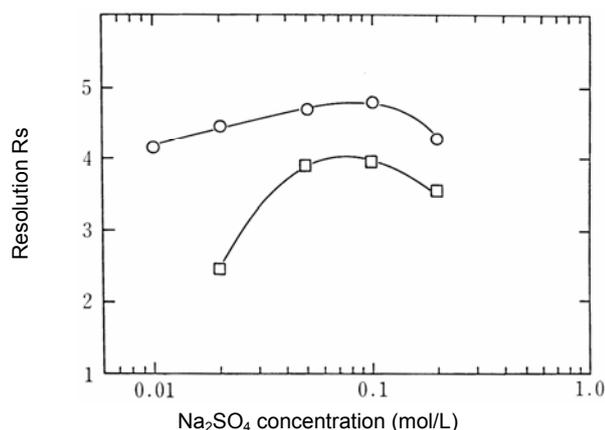


Figure 2 Effect of Na_2SO_4 concentration in the eluent on separation of IgG and albumin

IgG and albumin were eluted in 50mmol/L phosphate buffer containing various concentrations of Na_2SO_4 at pH 5.0 (O) or pH 6.0 (□).

Figures 3, 4 and 5 show the separation of an IgG preparation under the elution conditions determined in this study to be optimal in comparison with the general elution conditions for SW_{XL} -type columns, which consist of 50mmol/L phosphate buffer eluent (pH 6.8) containing 0.3mol/L NaCl.

Figure 3 shows the separation of a sample with an IgG to albumin ratio of 50:1. While the albumin peak is seen as only a shoulder on the IgG peak when using conventional general elution conditions, albumin is seen as a distinct peak when using the optimized conditions determined in this study. In Figures 4 and 5, albumin peaks were visible when the samples were analyzed using the optimized as well as the general elution conditions.

It was found that when the separation is run under the optimal elution conditions as determined in this study, the albumin peaks are more clearly distinguishable. It is believed that the reason why the chromatogram's peak area ratio is larger than the IgG to albumin volume ratio is that the 280nm absorption constant of IgG is about three times higher than that of albumin. It is also evident that the IgG dimer and monomer are favorably separated. Worthy of note is the fact that total analysis time was less than 15 minutes and recovery was 92% or higher for all components.

As to the column lifetime, the separations shown in this report remained intact even after 200 sample injections.

Elution time (min) Elution time (min)

Figure 3 Separation of IgG preparation for intravenous injection containing albumin

Column: TSKgel G3000 SW_{XL} (7.8mm ID × 30cm)
 Sample: Gammaguard (50mg/mL, 5 μL)
 Eluent: A: 50mmol/L phosphate buffer (pH 6.8) + 0.3mol/L NaCl
 B: 50mmol/L phosphate buffer (pH 5.0) + 0.1mol/L Na_2SO_4
 Flow rate: 1.0mL/min
 Temperature: 25°C
 Detection: UV@ 280nm
 Recovery: A: 102%, B: 96%

Elution time (min) Elution time (min)

Figure 4 Separation of IgG preparation for intravenous injection containing albumin

Column: TSKgel G3000 SW_{XL} (7.8mm ID × 30cm)
 Sample: Venilon (50g/L, 5 μL)
 Conditions: As in Figure 3.
 Recovery: A: 94%, B: 92%

Elution time (min)

Elution time (min)

Figure 5 Separation of IgG preparation for intravenous injection containing albumin

Column: TSKgel G3000SW_{XL} (7.8mm ID × 30cm)
Sample: Venoglobulin-I (50g/L, 5μL)
Conditions: As in Figure 3.
Recovery: A: 100% B: 101%

4. Conclusion

Using only a high performance gel filtration column, TSKgel G3000SW_{XL}, IgG and albumin in commercial immunoglobulin preparations were rapidly and quantitatively separated with acceptable resolution. Optimal elution conditions for this type of separation were determined to be a 50mmol/L sodium phosphate buffer (pH 5.0) containing 0.1mol/L Na₂SO₄. Monoclonal antibodies and albumin in mouse ascites fluid can also be separated using this same column and these elution conditions.



Purification of Immunoglobulin M Using a Novel Ion-exchanger

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TJ Higley; Tosoh Bioscience LLC, Montgomeryville, PA, USA.



The use of monoclonal antibodies (Mab) is becoming more important in diagnosis and treatment of disease. IgM in particular has shown to possess unique and beneficial characteristics relative to other immunoglobulin classes.

IgM is a large molecule comprised of five IgG subunits resulting in a relatively unstable and difficult to purify molecule. Unlike single chain antibodies, IgM cannot be purified by Protein A (an affinity material commonly used for its high binding capacity and excellent selectivity for antibodies) due to steric hindrance. Alternative affinity methods have been developed with thiophilic absorbents but result in low binding capacity.

In this study, we examine an alternative purification method of IgM by ion exchange chromatography using a novel high capacity stationary phase (TSKgel BioAssist).



Experimental

HPLC

HPLC system consisted of two pumps(DP-8020), a column oven(CO-8020C), an UV detector(UV-8020) and an autosampler(AS-8020,all from TOSOH Co.,Japan).

Reagents

All proteins were purchased from Sigma(Japan). IgG and IgM was gifted by TOSOH Research Laboratories. All chemical reagents employed were from Kishida Chemicals(Oosaka,Japan).

Binding capacity

The resins were packed into 10mm x 4.6mm I.D. column. The protein solution was purged into the column and base line was monitored by UV detector to obtain a break through curve. The binding capacity was calculated by purged volume at the 10% height of the break through curve.

Purification conditions of IgM from mouse ascites fluid

Sample: 9.5mg/mL IgM in mouse ascites fluid



Experimental

Chromatography conditions:

Cation-exchange chromatography

Column: TSKgel BioAssist S 5cm x 4.6mmI.D.
Eluent : 20mmol/L Sodium phosphate buffer pH6.0
Gradient : NaCl
Flow rate : 1.0mL/min

Size exclusion chromatography

Column : TSKgel BioAssist G4SWXL 30cm x 7.8mmI.D.
Eluent : 0.3mol/L NaCl in 50mmol/L
Sodium phosphate buffer pH6.5
Flow rate : 1.0mL/min



Column

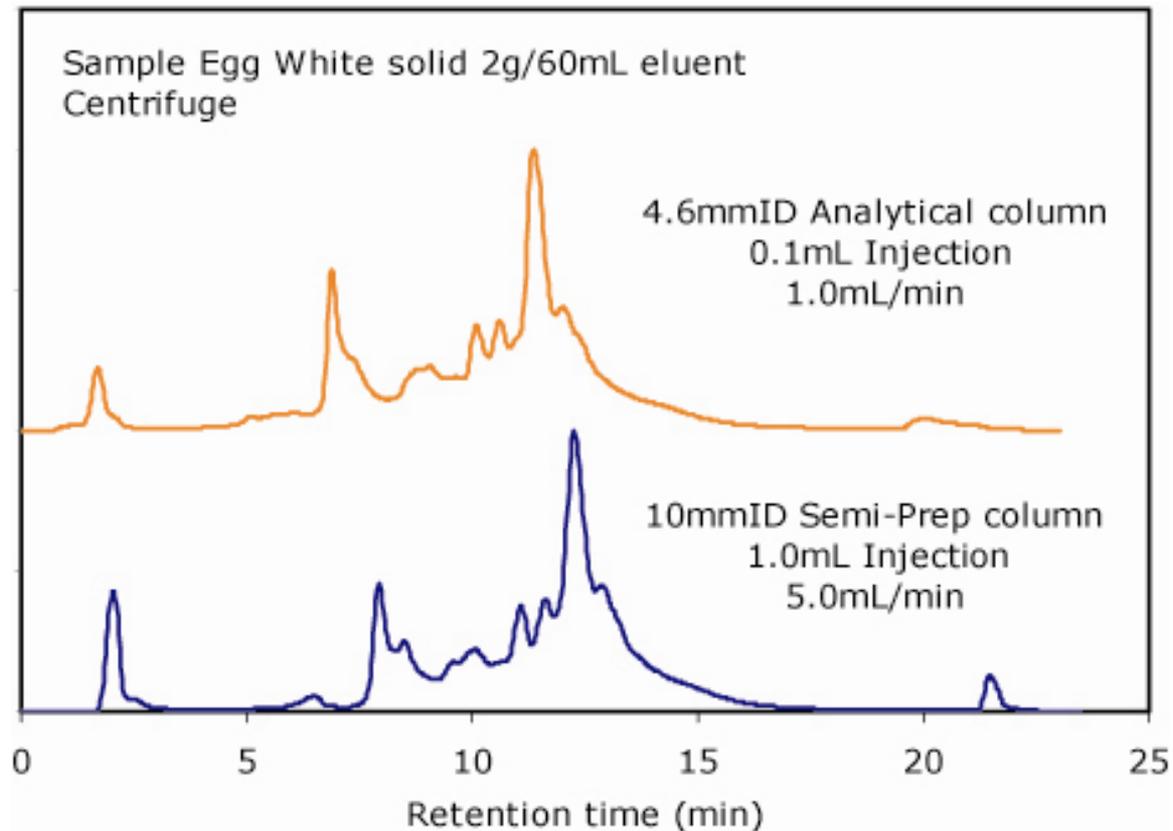
Characteristics of IEC Columns evaluated

	TSKgel BioAssist Q	TSKgel BioAssist S
Base Matrix	hydrophilic resin porous	hydrophilic resin porous
Particle size		
4.6mmID column	10 μ m	7 μ m
10mmID column	13 μ m	13 μ m
Pore size (Å)	ca. 4000	ca.1300
Ion exchange	ca. 0.1meq/L	ca. 0.1 meq/L
Ion site	Polyamine	Sulfapropyl
Column size	50mm x 4.6mmID 10cm x 10mmID PEEK	50mm x 4.6mmID 10cm x 10mmID PEEK
Optimal flow rate		
4.6mmID column	10.mL/min	0.8mL/min
10mmID column	5.0mL/min (10)	5.0mL/min (10)
Loading volume	<10mg	<100mg



Column

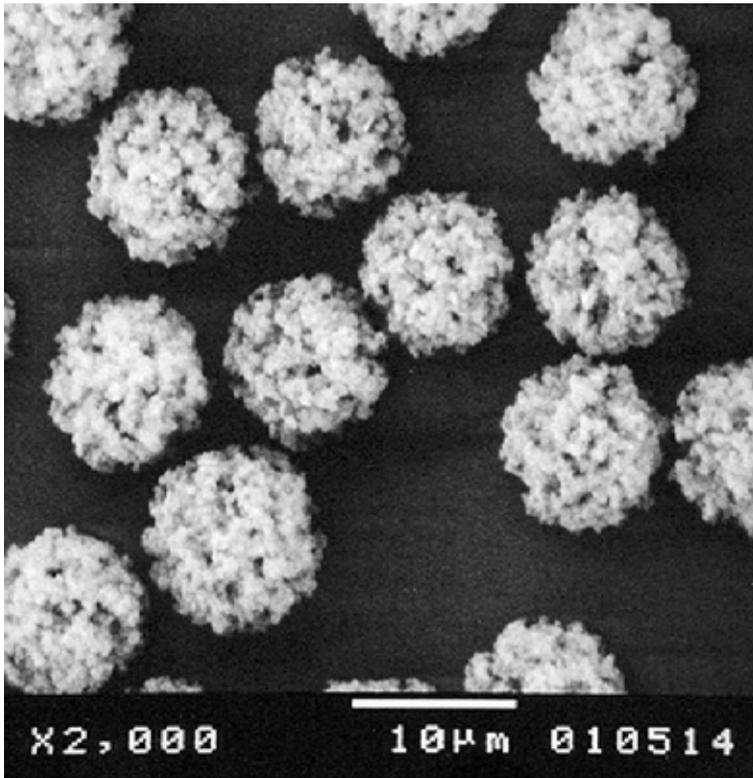
Scalability comparison





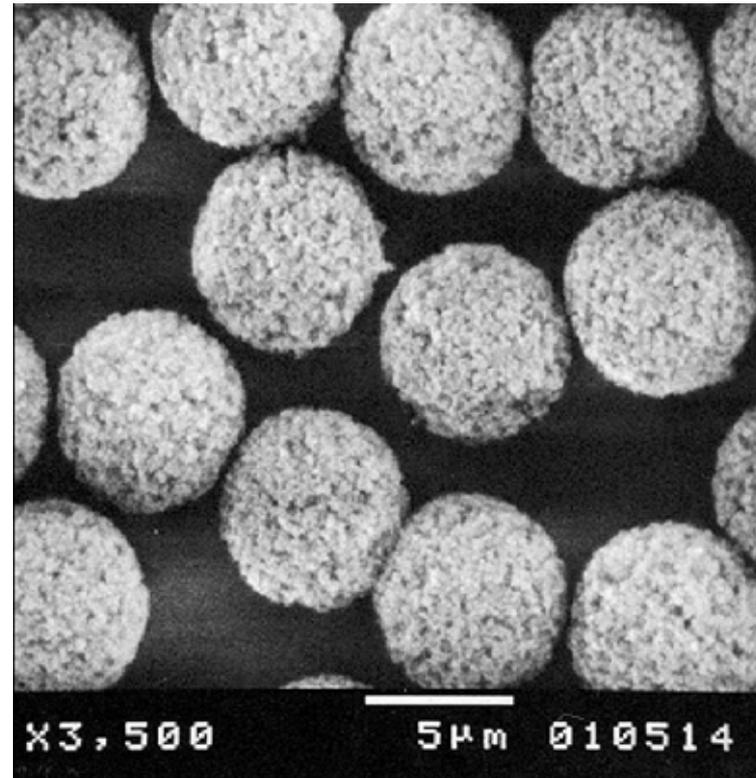
Column

SEM Photographs of BioAssist Resins



BioAssist Q

Approx. 4000Å



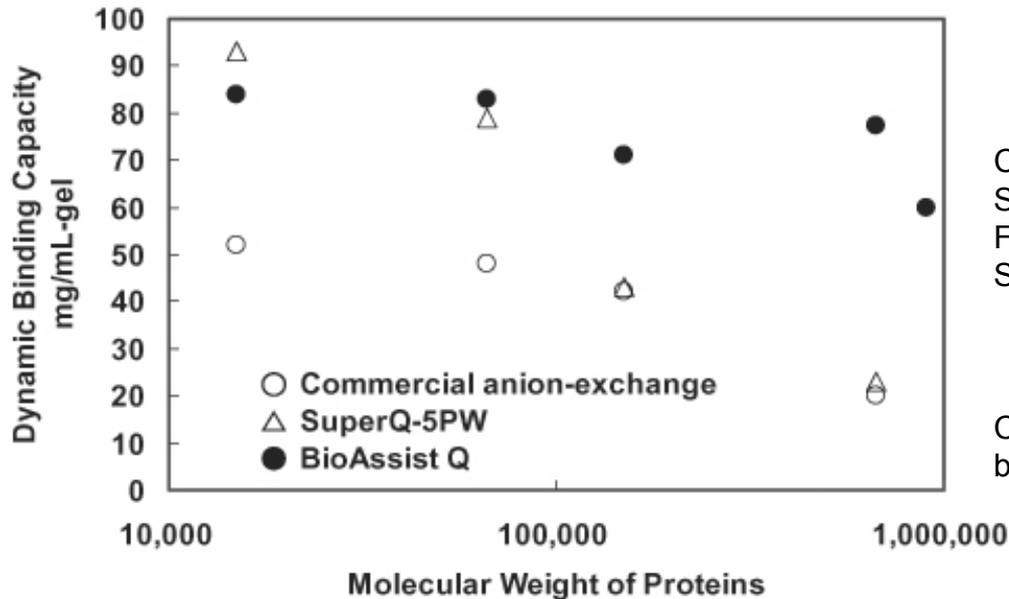
BioAssist S

Approx. 1300Å



Dynamic Binding Capacity

Binding Capacity vs. MW

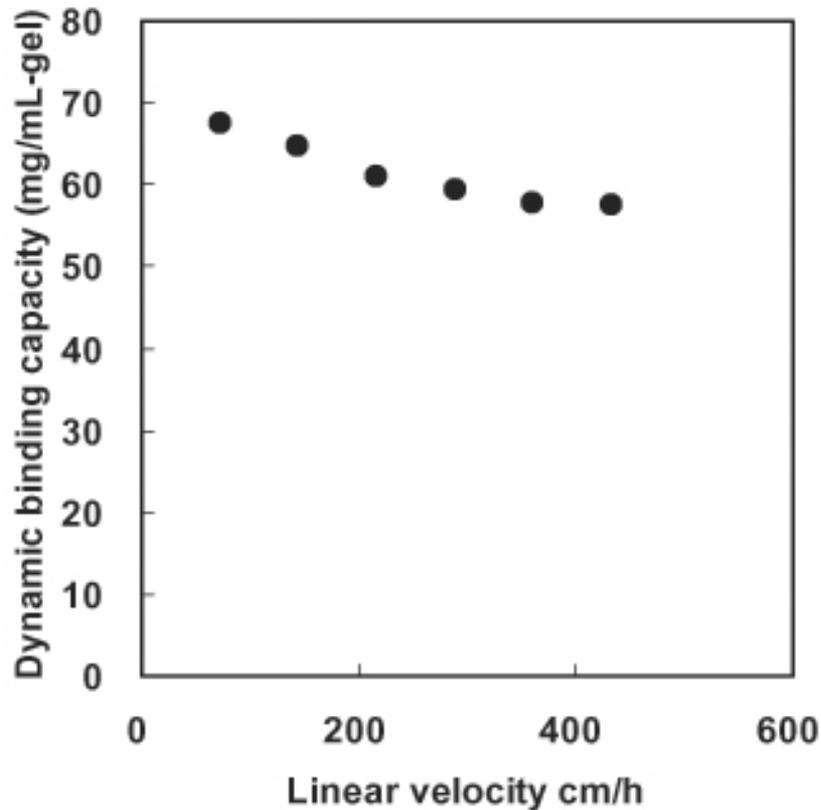


Column size: 10mm x 4.6mmID
Sample: 20mmol/L Tris-HCl buffer (pH8.0)
Flow rate: 0.38mL/min Temperature: ambient
Sample: Trypsin inhibitor (15KDa, 10mg/mL),
HAS (66.5KDa, 10mg/mL), IgG1 (150KDa,
2.3mg/mL), Thyroglobulin (66.9KDa,
5mg/mL), IgM (900KDa, 5mg/mL)
Capacities were calculated by 10% height of the
breakthrough curve



Dynamic Binding Capacity

Binding Capacity vs. Velocity

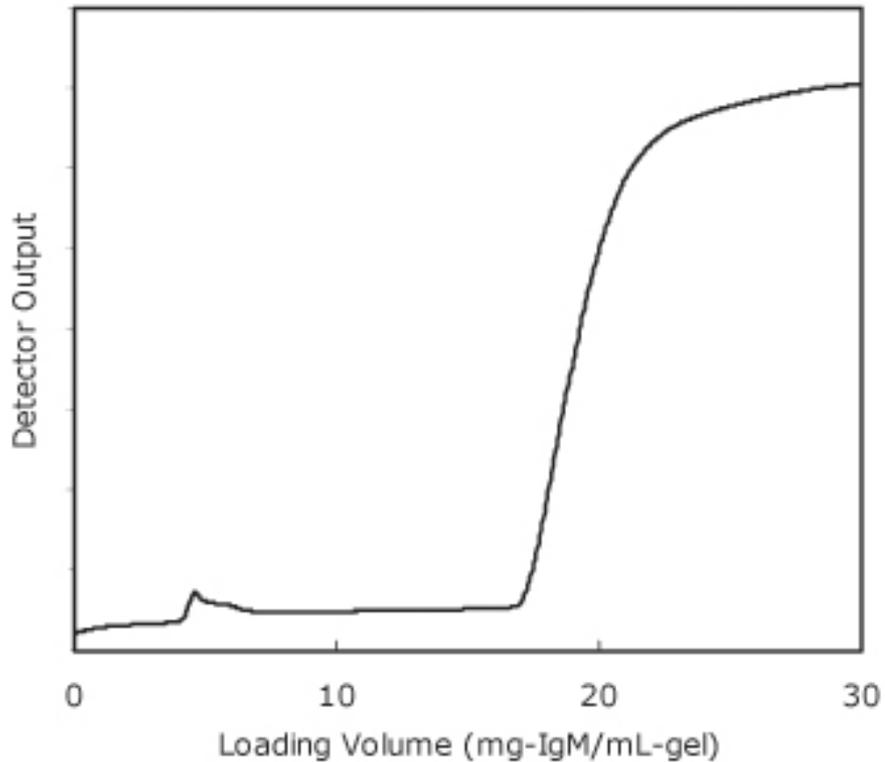


Column: BioAssist Q (10 μ m), 4.6mm ID x 1cm
Sample: 5mg/mL IgM in 20mM Tris-HCl buffer pH8.0
Flow rate: 0.2mL/min (72cm/h) to 1.2mL/min (433cm/h)



Dynamic Binding Capacity

Breakthrough Curves BioAssist S

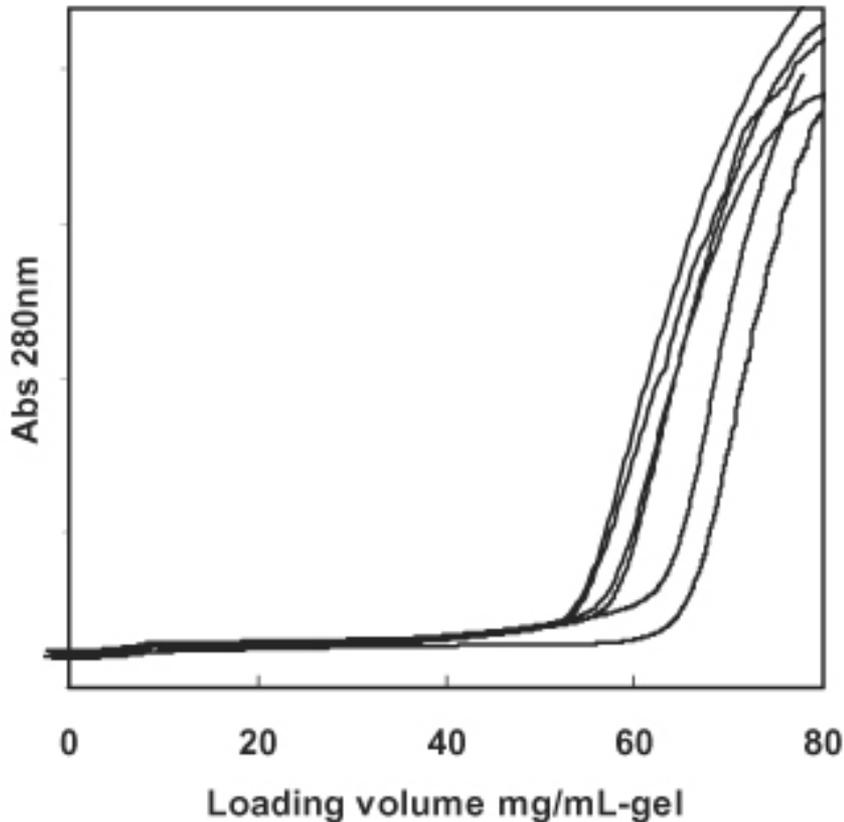


Column: BioAssist S ($7\ \mu\text{m}$), 4.6mm ID x 1cm
Sample: 2mg/mL IgM in 20mM sodium phosphate buffer pH6.0
Flow rate: 0.38mL/min



Dynamic Binding Capacity

Breakthrough Curves BioAssist Q

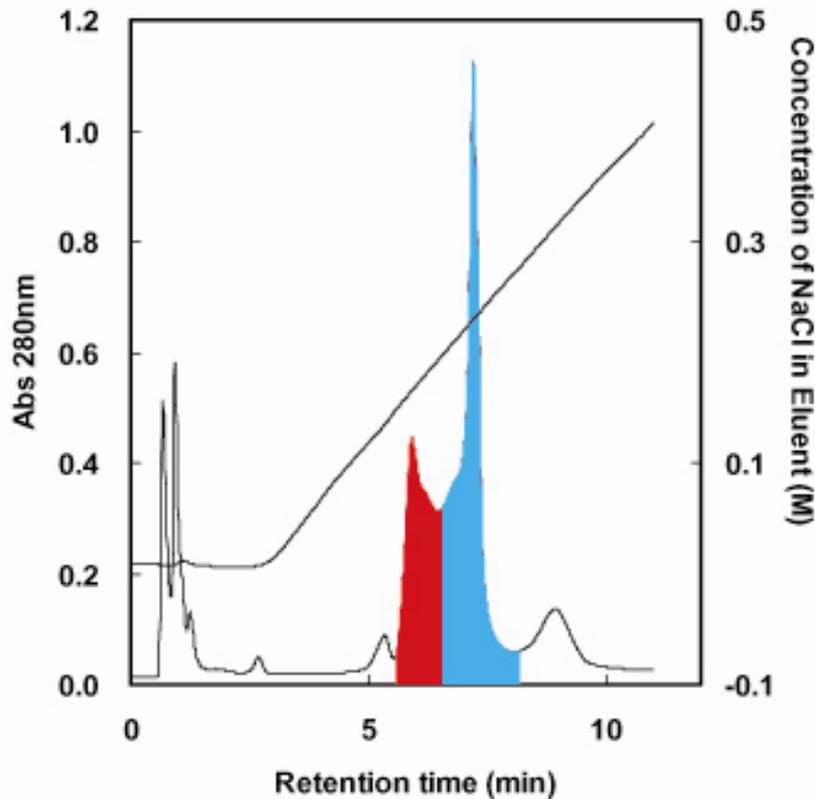


Column: BioAssist Q ($10\ \mu\text{m}$), 4.6mm ID x 1cm
Sample: 5mg/mL IgM in 20mM Tris-HCl buffer pH8.0
Flow rate: 0.2mL/min (72cm/h) to 1.2mL/min (433cm/h)



Separation of IgMs

Separation of IgM in mouse ascites on BioAssist S by linear gradient of NaCl

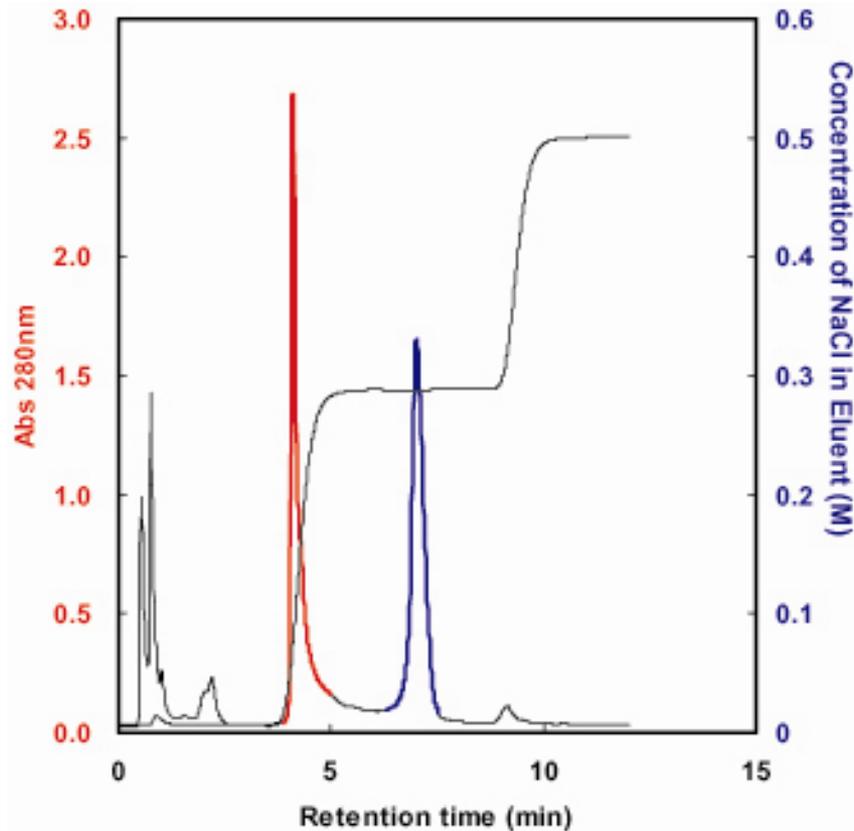


Sample: 0.16mg - IgM in mouse ascites



Separation of IgMs

Separation of IgM in mouse ascites on BioAssist S by step gradient

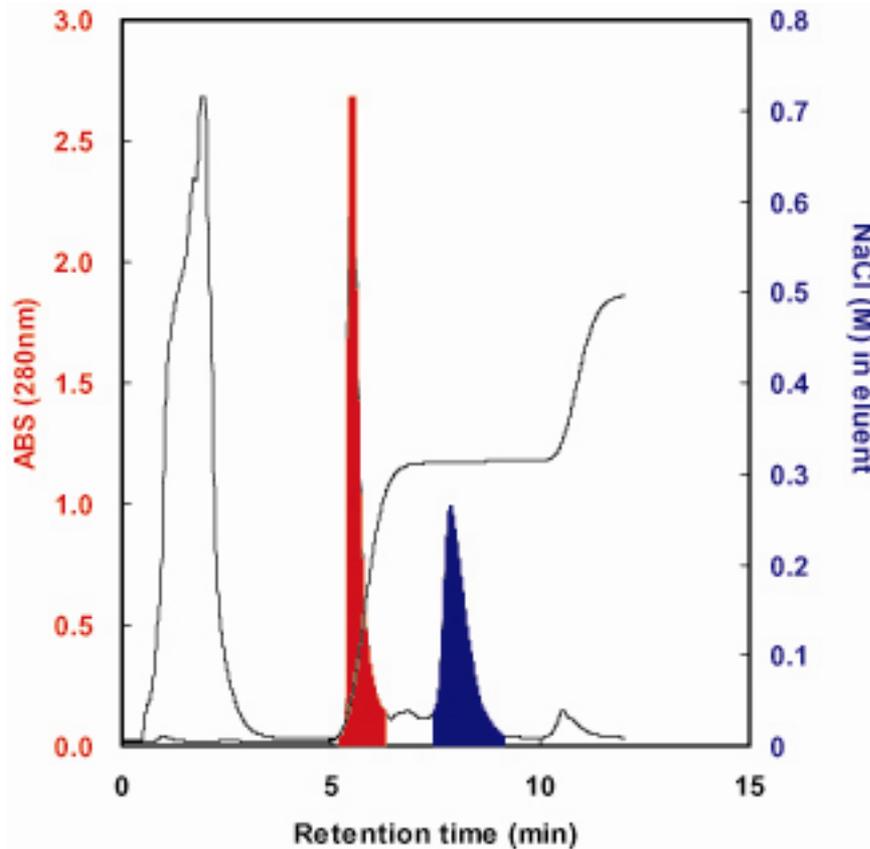


Sample: 0.16mg - IgM in mouse ascites



Separation of IgMs

Separation of IgM in mouse ascites on BioAssist S

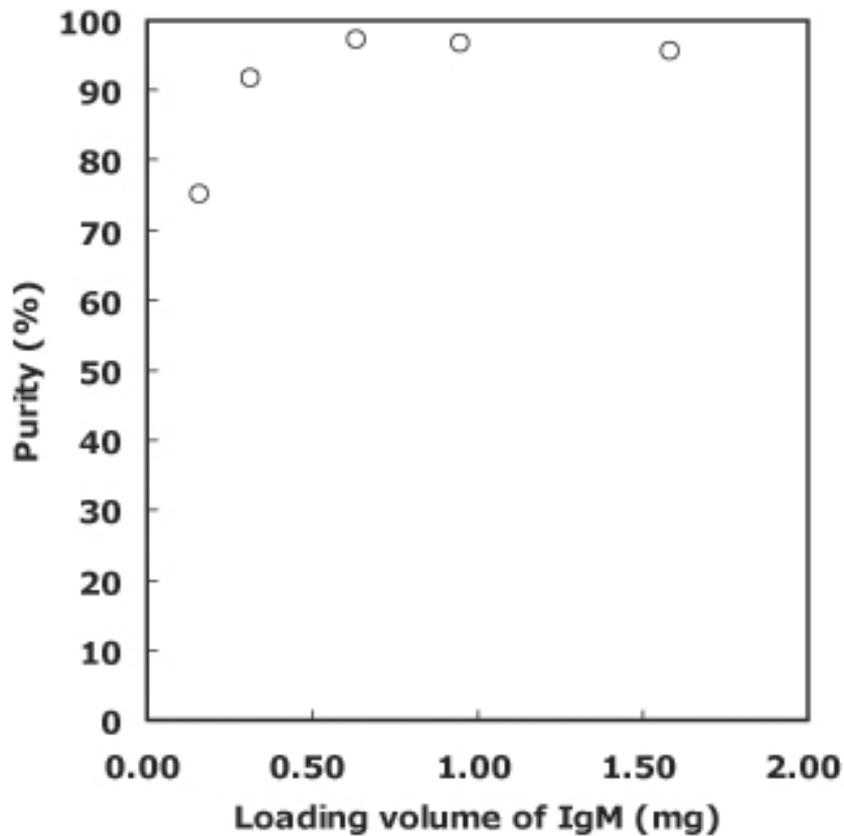


Sample: 4.75mg - IgM in mouse ascites



Separation of IgMs

Influence of loading volume

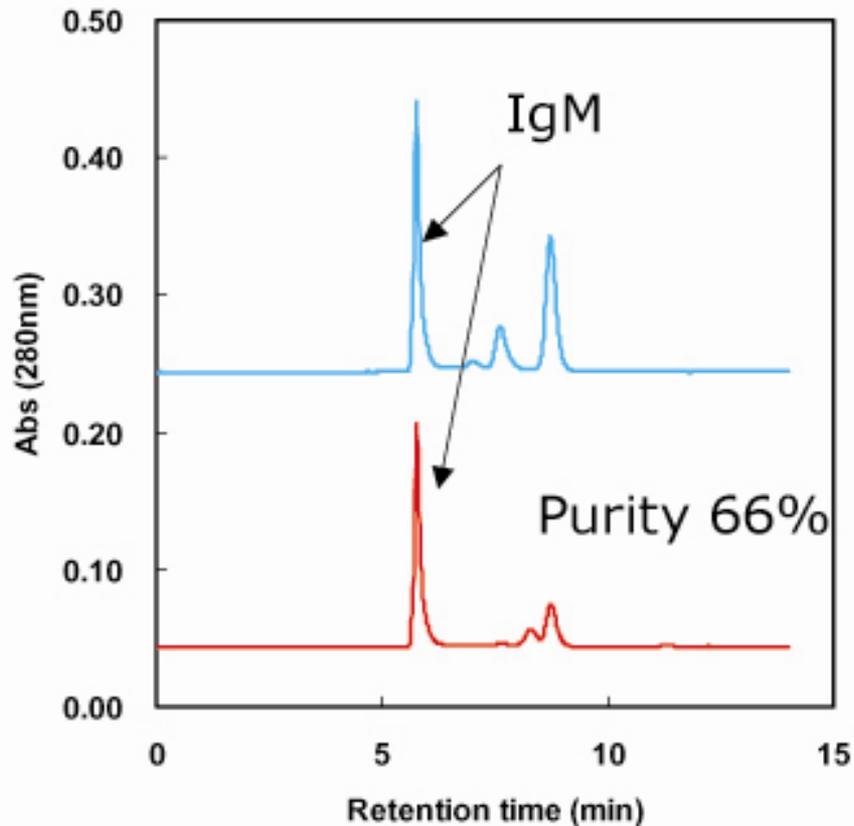


Conditions: IgM eluted with step gradient of 0.3M NaCl



Separation of IgMs

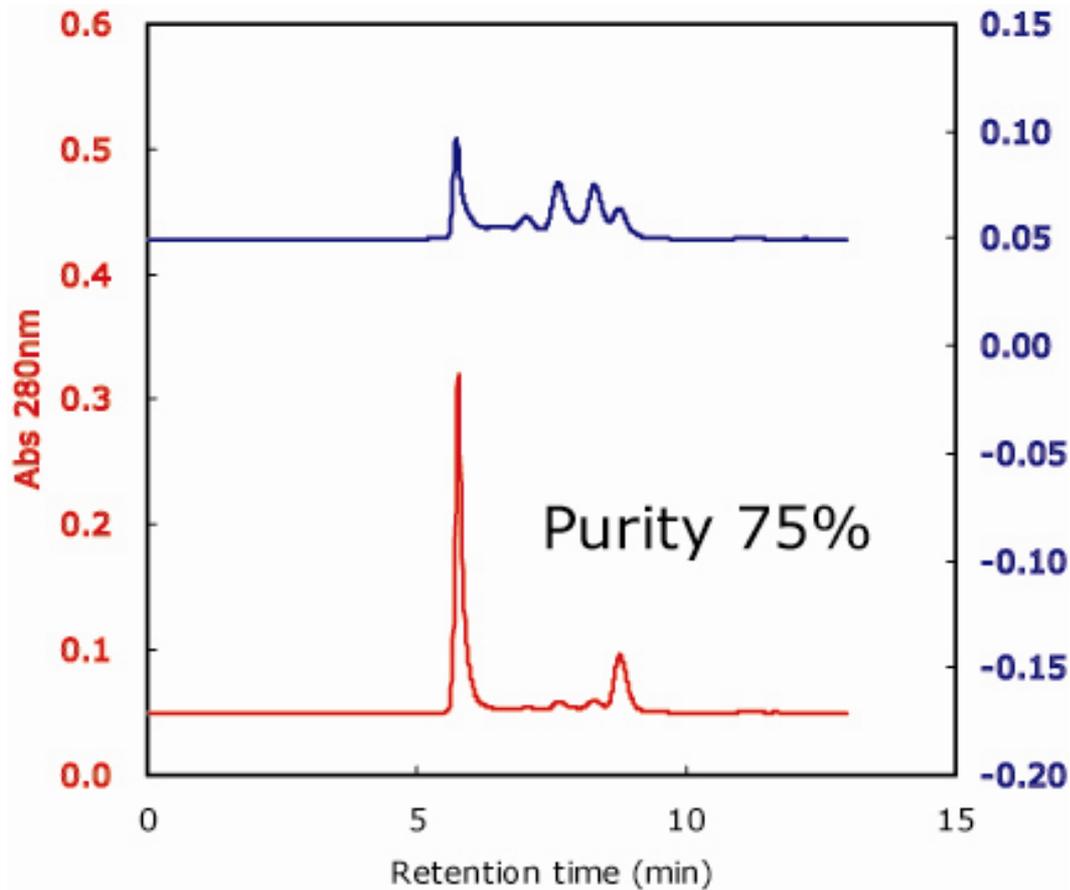
Purity check by SEC on BioAssist G4SW_{XL}





Separation of IgMs

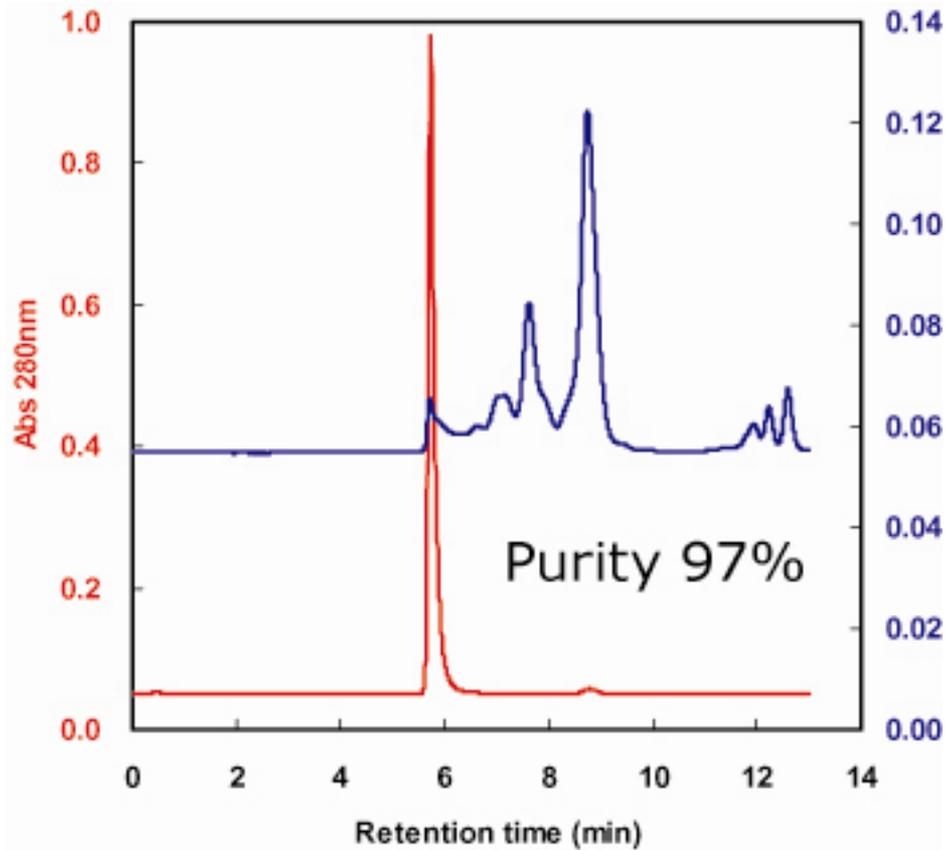
Purity check by SEC on BioAssist G4SW_{XL}





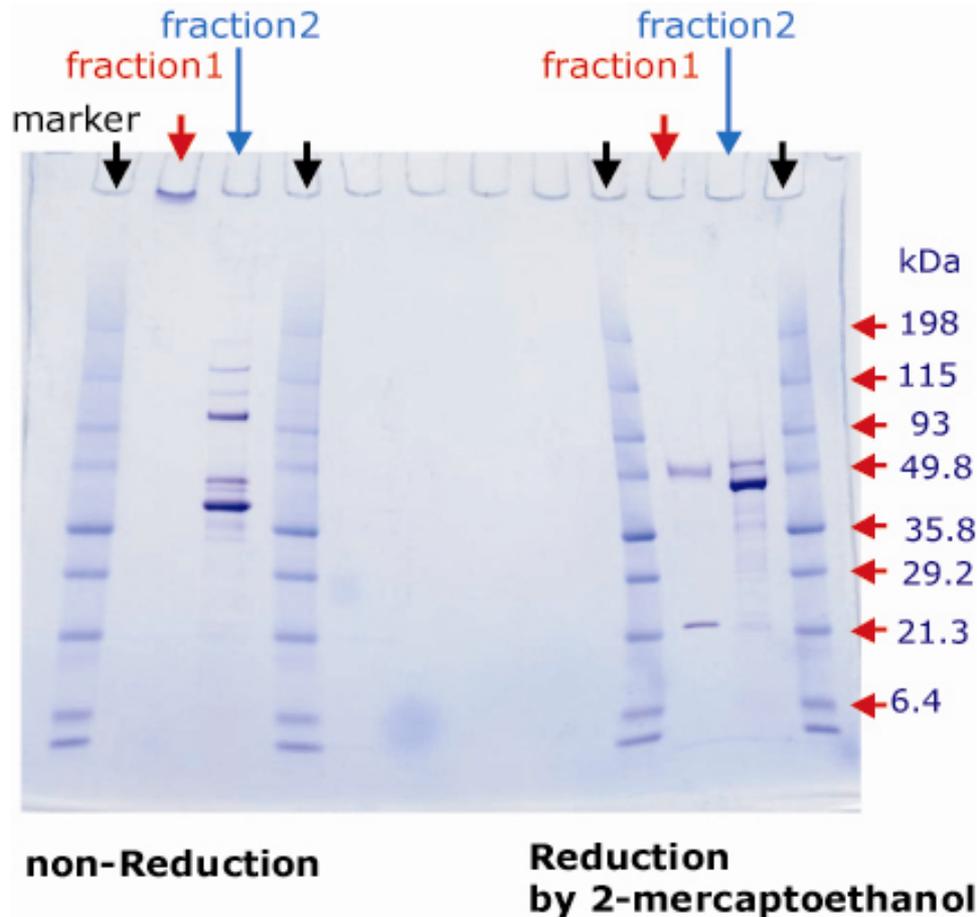
Separation of IgMs

Purity check by SEC on BioAssist G4SW_{XL}



Separation of IgMs

SDS-Page





Conclusions

- Binding capacity of the TSKgel BioAssist materials remains high (ca. 60mg/mL) over a wide molecular weight and flow rate range relative popular conventional porous materials.
- In the separation of IgM purified from mouse ascites fluid, purity was shown to improve with increased sample load. A purity of 97% was obtained when 4.75mg IgM was loaded onto the column. This is postulated this phenomenon may be the result of competitive displacement of albumin at the ion exchange sites as IgM load increases.