Fast Desalting of Proteins Using a Novel High Mechanical Strength Gel Filtration Column

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Introduction

- Desalting is a process to remove or reduce salt from a liquid stream.
- Desalting by Gel Filtration Chromatography (GFC) is the preferred method in biochemical laboratories to reduce the salt concentration or exchange the buffer of a biopolymer solution, with speed being the main advantage of GFC over dialysis.
- Proteins elute at high or elevated salt concentration in such chromatographic modes as hydrophobic interaction (HIC), ion exchange (IEC) and size exclusion chromatography (SEC).
- SEC mobile phases for protein analysis may also contain denaturants such as guanidine hydrochloride and urea in addition to salt and buffer.
- Desalting on the basis of size exclusion chromatography is widely used in in biochemical purifications.
- Desalting and buffer exchange of proteins or polynucleotides can also be performed by dialysis, ultra filtration, or by using spin-columns.
- Desalting columns are characterized by a low exclusion limit and a large pore volume.
- Salts can fully access all pores, while proteins and other high MW species are excluded from the pores and elute in the void volume as a narrow concentrated peak.

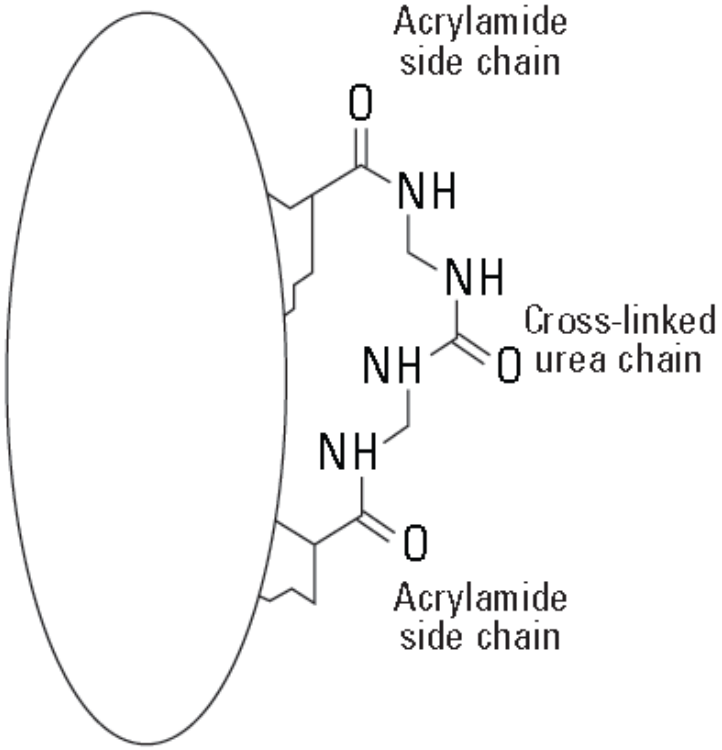
Introduction

- Columns packed with conventional packing materials such as dextran, cellulose and polyacrylamide have limited physical stability and are not suitable when fast desalting is desired.
- Requirements for a fast desalting column are (1) an inert matrix, (2) a large pore volume that is fully accessible to common salts and buffer components, (3) a pore size distribution that excludes the component(s) of interest from accessing the pores, and (4) sufficient mechanical strength to allow the use of the column in standard HPLC equipment.
- We increased the mechanical strength of polyacrylamide gel by four-fold over that of conventional gels.
- TSKgel BioAssist DS columns contain 15 µm particles packed in 4.6 mm ID x 15 cm and 10 mm ID x 15 cm PEEK columns.

Objective

To show the usefulness of the new TSKgel BioAssist DS columns for efficient desalting using a conventional HPLC system.

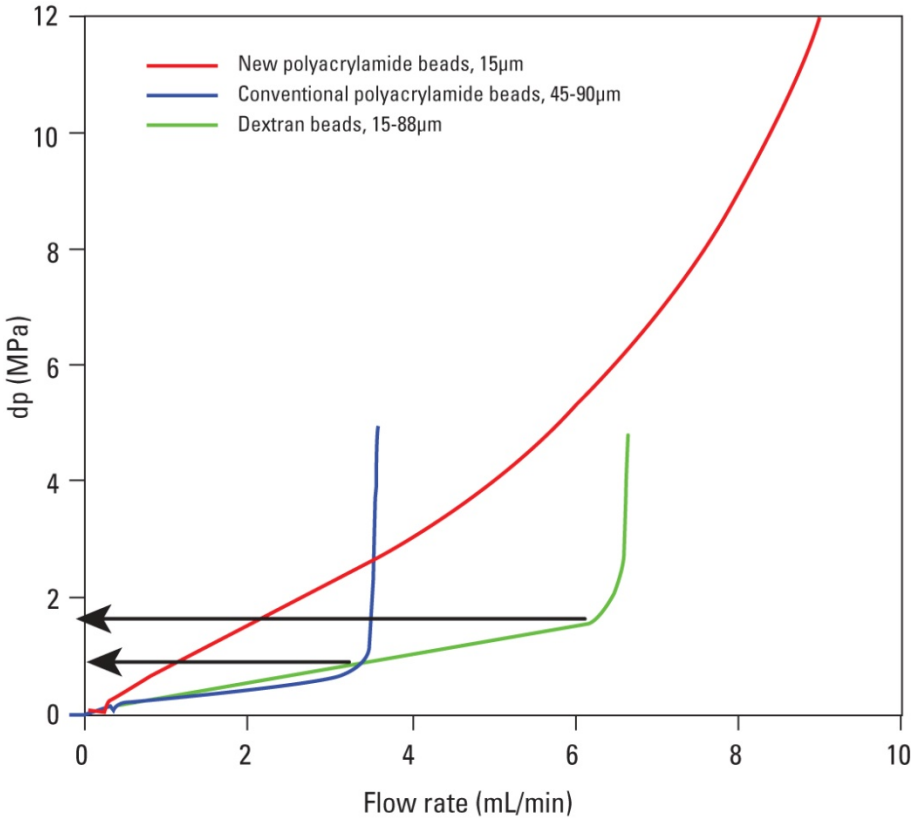
Chemical Structure of Polyacrylamide Beads*



- Backbone of beads is based on polyacrylamide
- Side chains cross - linked with one another through urea
- Highly cross - linked polyacrylamide beads display high mechanical strength and low hydrophobicity

*US Patent 7659348 B2, February 9, 2010

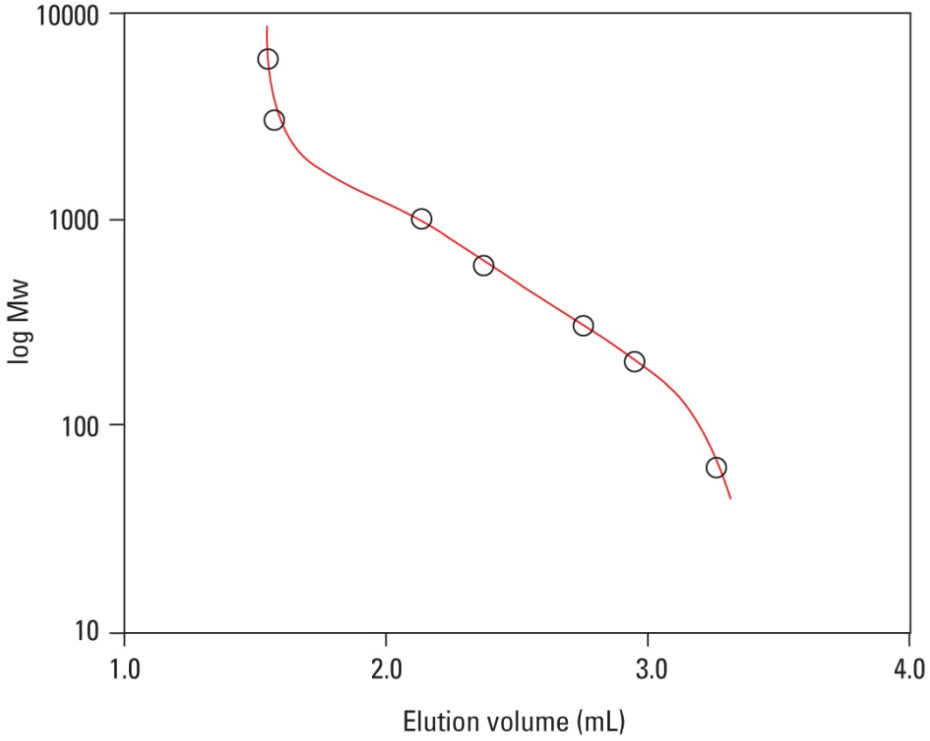
Mechanical Strength



Column size: 4.6mm ID x 10cm
Mobile phase: D-H_2O

- **Conventional beads collapsed at pressures below 1.6 MPa (< 250 psi).**
- **TSKgel BioAssist DS polyacrylamide beads did not collapse at 12 MPa (1750 psi).**

Calibration Curve for New Polyacrylamide Beads



Column size:	6.0mm ID x 15cm
Mobile phase:	DI H_2O
Flow rate:	0.5mL/min
Sample:	ethylene glycol, PEG

Exclusion limit PEG 2500 MW

Characteristics of TSKgel BioAssist DS Desalting Columns

- Packing material: urea cross-linked polyacrylamide
- Particle Diameter: 15 µm (Uniform)
- Pore Size excludes: ca. 2500 MW PEG
- Particle porosity: ca. 60%
- Maximum pressure: 4 MPa (< 600 psi)

Material and Methods: Chromatographic Conditions (Size Exclusion Experiment)

- Column: TSKgel G3000SW$_{XL}$, 7.8 mm ID x 30 cm, 5 µm (S1237-08R)
- Mobile phase: 100 mmol/L KH_2PO_4/Na_2HPO_4, pH 6.7, 100 mmol/L Na_2SO_4 + 0.05% NaN_3
- Flow rate: 1.0 mL/min
- Detection: UV@280 nm
- Temperature: ambient
- Injection vol.: 10 µL
- Samples: standard SW$_{XL}$ test mixture:
  thyroglobulin (0.5 g/L)
  γ-globulin (1 g/L)
  ovalbumin (1 g/L)
  ribonuclease A (1.5 g/L)
  p-ABA (0.01 g/L)

Material and Methods: Preparation of Protein Standards (Desalting Experiments)

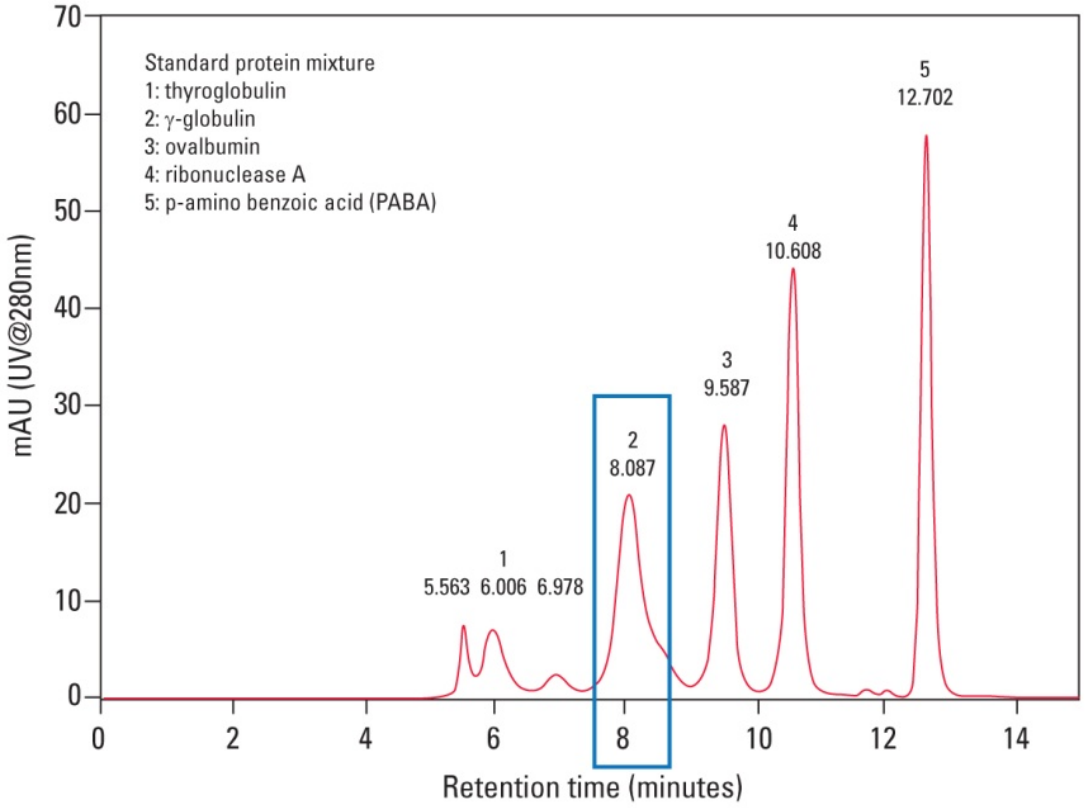
Protein	MW (kDa)	Concentration* (g/L approx.)
ribonuclease A	14.7	19.5
thyroglobulin	670	11.3
γ-globulin	150	14.5
ovalbumin	45	13.1
α-chymotrypsinogen	25.6	13.1
β-lactoglobulin	18.4	10.8
lysozyme	14.7	11.6
myoglobin	16.7	14.5
cytochrome C	12.3	11.0
hemoglobin	68	11.9

*in 100mmol/L Phosphate buffer, pH 6.7

Material and Methods: Chromatographic Conditions (Desalting Experiments)

- Columns : TSKgel BioAssist DS,15 µm, 4.6 mm ID x 15 cm, PEEK
  TSKgel BioAssist DS,15 µm, 10.0 mm ID x 15 cm, PEEK
- Mobile phase: 10 mmol/L KH_2PO_4/Na_2HPO_4, pH 6.7, 10 mmol/L Na_2SO_4 + 0.005% NaN_3
- Flow rate: 0.8 mL/min (4.6 mm ID) and 1.0 mL/min (10.0 mm ID) unless mentioned otherwise
- Detection: UV@280 nm and RI
- Temperature: ambient
- Injection vol.: 10 µL unless mentioned otherwise
- Samples: γ-globulin was collected after injection of the standard TSKgel SW_{XL} test mixture
- All analyses were carried out using an Agilent 1200 HPLC system run by Chemstation (ver B.04.01).
- All chemicals and standards were pure analytical grade from Sigma-Aldrich.
- Before injection, standards and samples were filtered through a 0.45 µm filter.

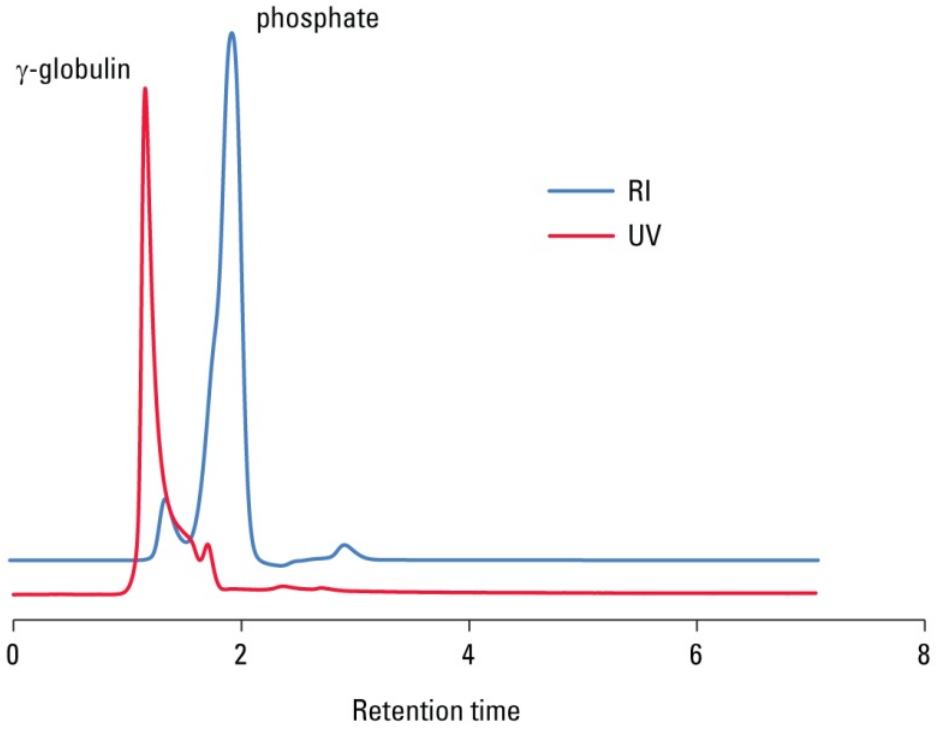
Separation of Protein Standard Mixture using a TSKgel G3000SW$_{XL}$, 5 µm, 7.8 mm ID x 30 cm Column



Mobile phase: 0.1mol/L KH_2PO_4/Na_2HPO_4, pH 6.7, 0.1mol/L Na_2SO_4 + 0.05% NaN_3

10.0 µL of γ-globulin (RT 8.087 min) peak fraction was loaded into TSKgel BioAssist DS, 15 µm, 4.6 mm ID x 15 cm column to desalt.

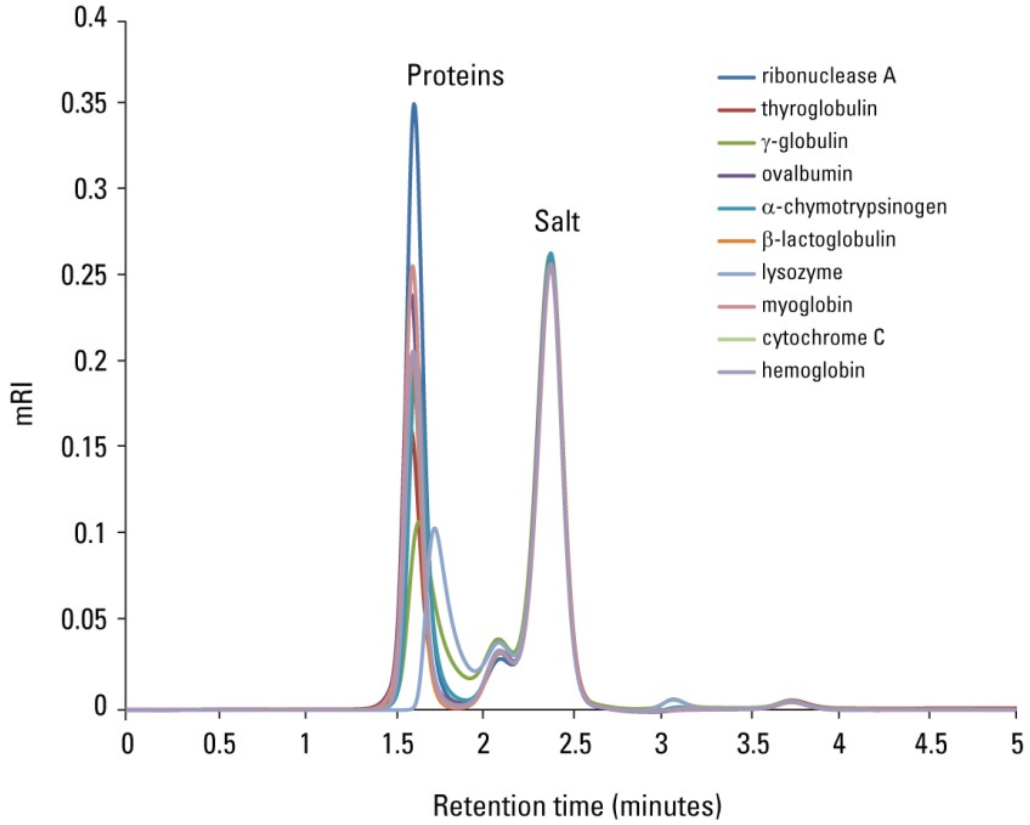
Desalting of γ-globulin Peak Fraction using a TSKgel BioAssist DS, 15 µm, 4.6 mm ID x 15 cm Column



Mobile phase:	0.01mol/L KH_2PO_4/$NaHPO_4$, pH 6.7, 0.01mol/L Na_2SO_4 + 0.05% NaN_3
Flow rate:	0.8mL/min.
Detection:	UV@280nm and RI
Temperature:	ambient

Mobile phase γ-globulin fraction was efficiently desalted within a few minutes.

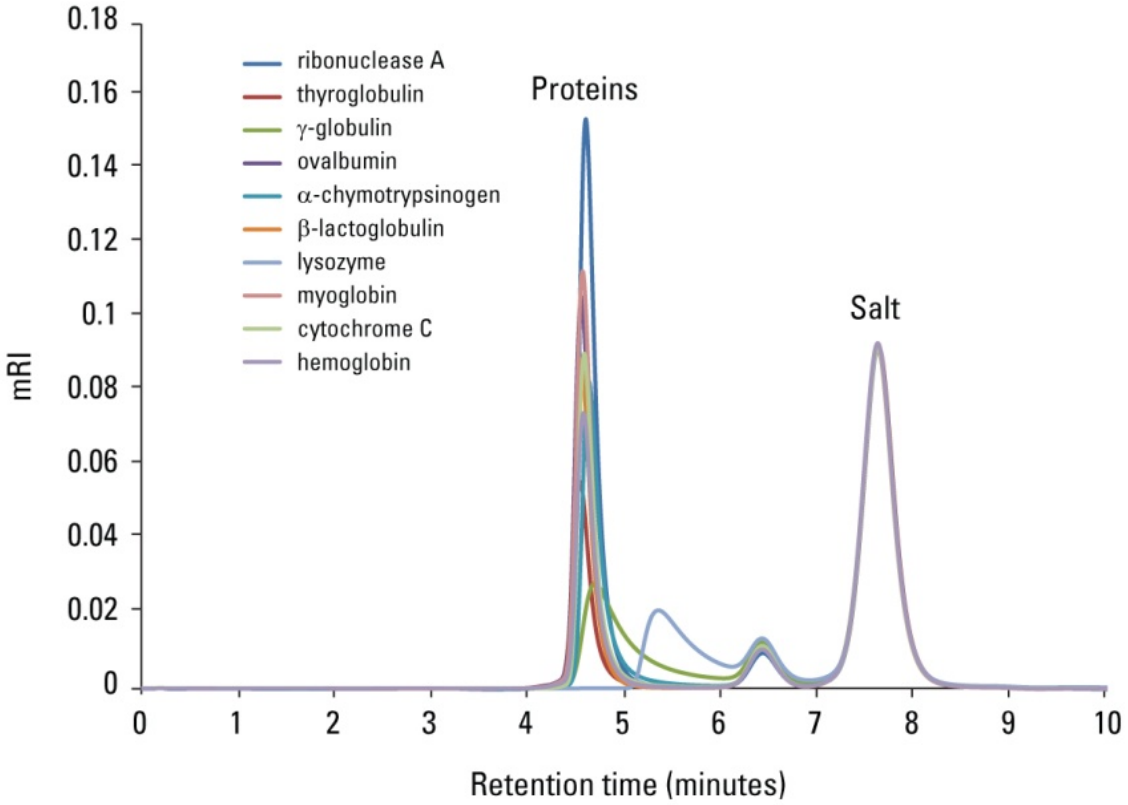
Desalting Proteins using a 4.6 mm ID x 15 cm, 15 µm TSKgel BioAssist DS Column



Proteins in 0.1mol/L phosphate buffer, pH 6.7
Mobile phase: 0.01mol/L KH_2PO_4/Na_2HPO_4, pH 6.7, 0.01mol/L Na_2SO_4 + 0.05% NaN_3

Fast desalting with excellent reproducibility at analytical scale.

Desalting proteins using a 10 mm ID x 15 cm, 15 µm TSKgel BioAssist DS column



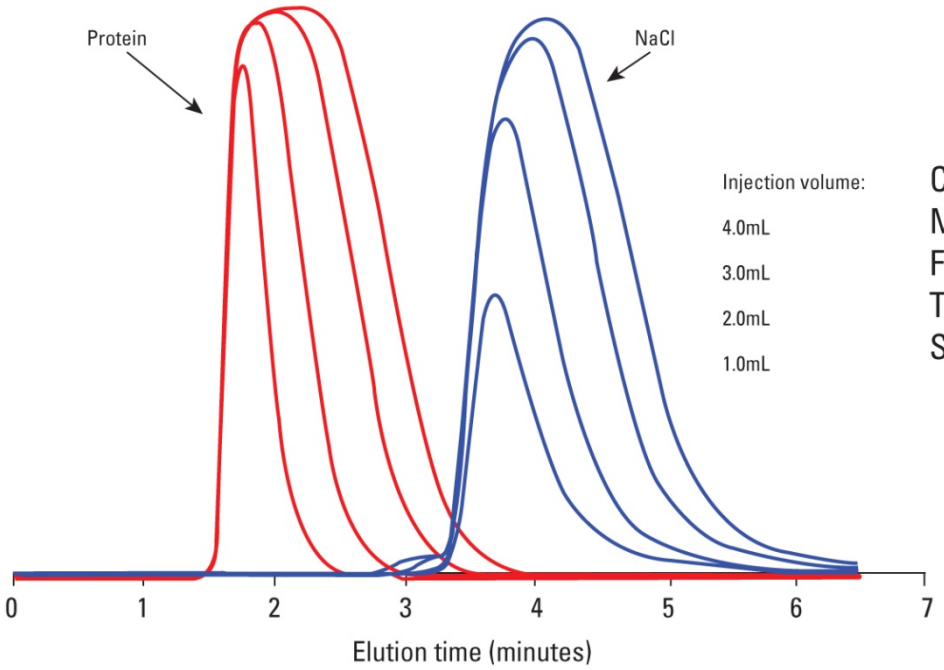
Proteins in 0.1mol/L phosphate buffer, pH 6.7
Mobile phase: 0.01mol/L KH_2PO_4/Na_2HPO_4, pH 6.7, 0.01mol/L Na_2SO_4 + 0.05% NaN_3

Fast desalting with excellent reproducibility at semi-preparative scale.

Effect of Sample Load on Efficiency of Desalting of Protein using TSKgel BioAssist DS, 15 μm, 10.0 mm ID x 15 cm Column

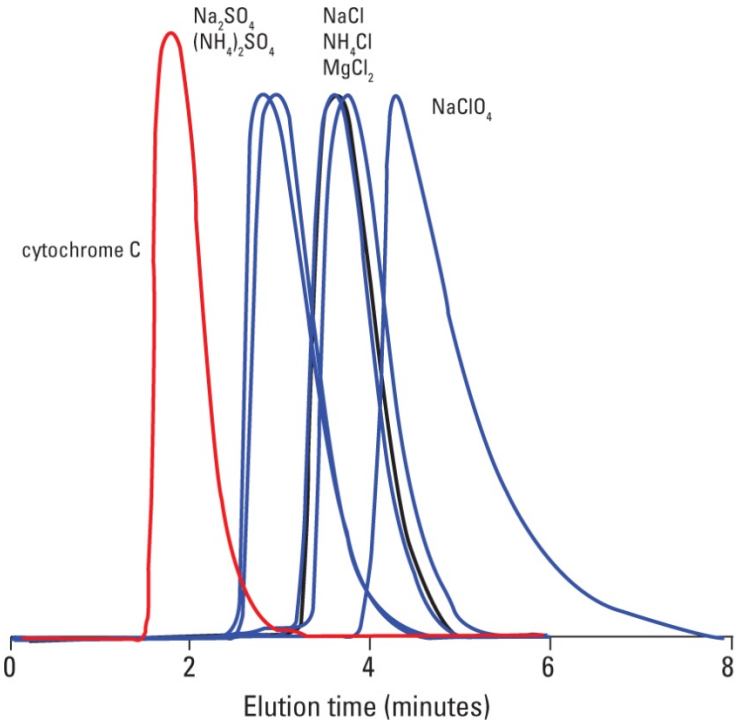
- The column has high loading (desalting) capacity.
- Less than 5% RSD (n = 4) in efficiency up to a load of 1.5 mg of Ribonuclease A .
- The resolution between the protein and salt peak was always >6.
- Even at ~2 mg protein load of Ribonuclease A, the resolution between the protein and salt peak was 4.33.
- TSKgel BioAssist DS, 15 μm, 4.6 mm ID x 15 cm column yielded a resolution of >2 at 1950 μg load of Ribonuclease A (F = 0.8mL/min).
- This study shows that both TSKgel BioAssist DS columns can be effectively used for desalting a large sample load.

Effect of Injection Volume on Desalting Profiles



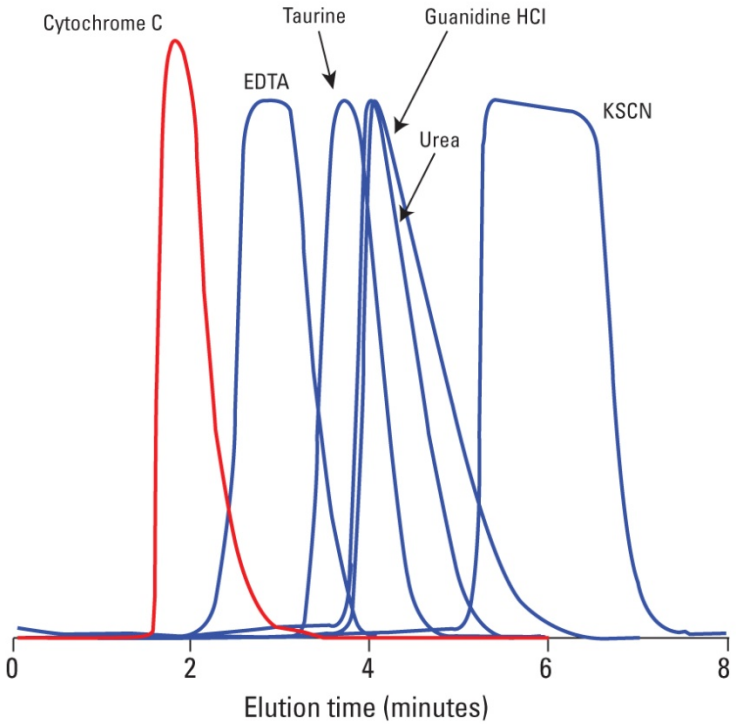
Column:	TSKgel BioAssist DS, 10mm ID x 15cm, PEEK
Mobile phase:	50mmol/L sodium phosphate buffer, pH 6.5
Flow rate:	3.0mL/min
Temperature:	4°C
Sample:	10mg/mL ovalbumin in 0.5mol/L NaCl and 25mmol/L sodium phosphate buffer, pH 6.5

Elution profiles of high salt concentration



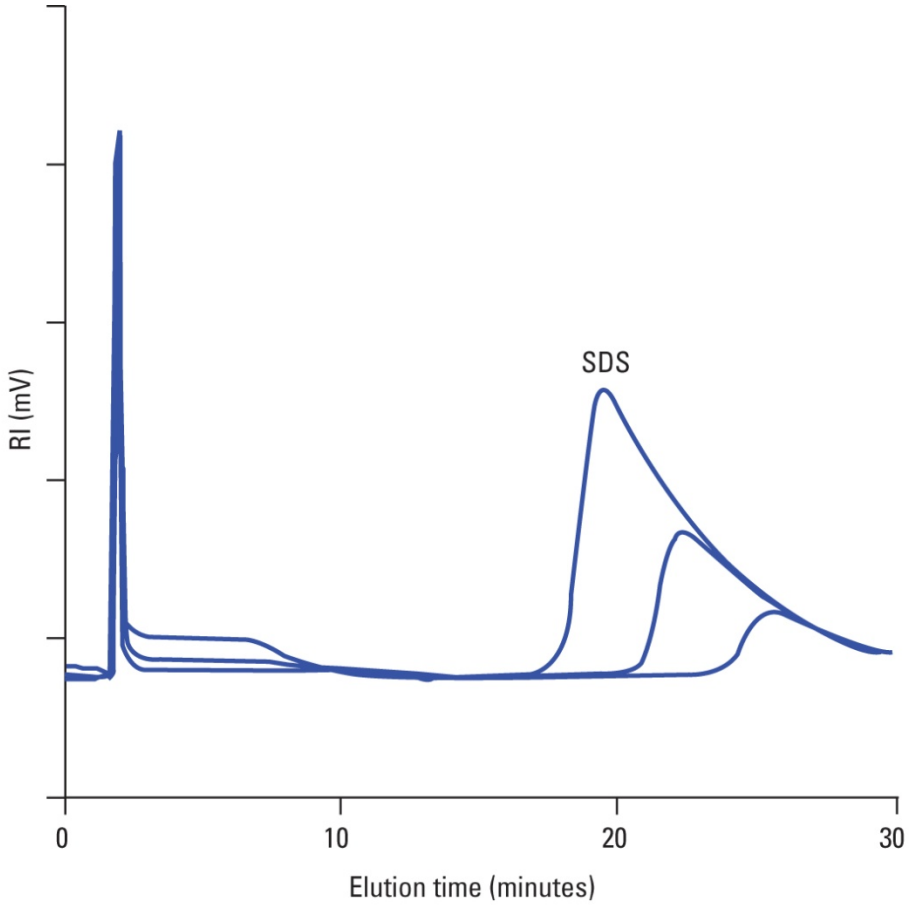
Column:	TSKgel BioAssist DS, 10mm ID x 15cm, PEEK
Mobile phase:	DI H_2O
Flow rate:	3.0mL/min
Temperature:	4°C
Injection vol.:	2mL
Each salt concentration:	0.5mol/L

Elution profiles of mobile phase additives



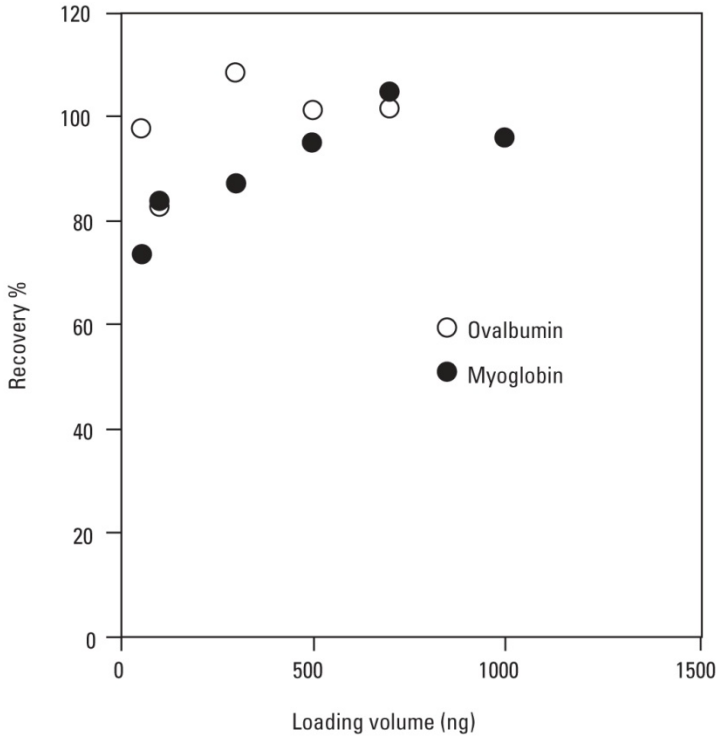
Column:	TSKgel BioAssist DS, 10mm ID x 15cm, PEEK
Mobile phase:	DI H_2O
Flow rate:	3.0mL/min
Temperature:	4°C
Injection vol.:	2mL
Each salt concentration:	0.5mol/L

Elution profiles of SDS



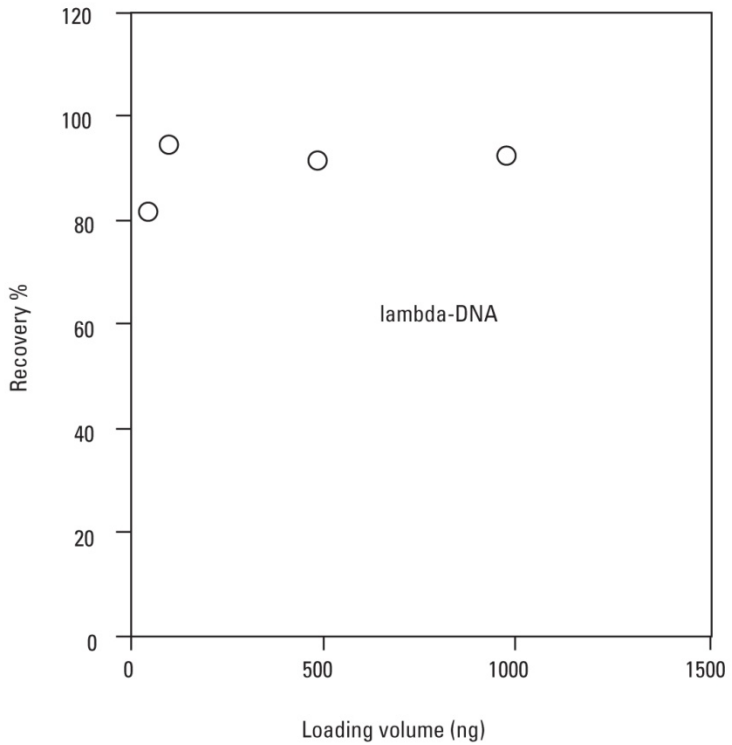
Column:	TSKgel BioAssist DS, 4.6mm ID x 15cm, PEEK
Mobile phase:	20mmol/L phosphate buffer, pH 6.9
Flow rate:	1.0mL/min
Temperature:	25°C
Sample:	0.1-0.5wt% SDS in eluent

Recovery of selected proteins and DNA



Column: TSKgel BioAssist DS, 4.6mm ID x 10cm, PEEK

Mobile phase: 20mmol/L Tris-HCl buffer, pH 8.0 for ovalbumin
20mmol/L phosphate buffer, pH 6.5 for myoglobin



Column: TSKgel BioAssist DS, 4.6mm ID x 10cm, PEEK

Mobile phase: 20mmol/L Tris-HCl buffer, pH 7.8

Conclusions

TSKgel BioAssist DS columns are designed for desalting of proteins and polynucleotides at semi-preparative scale with the following features:

- 4-fold higher mechanical strength over that of conventional gels
- Columns can be used at pressure up to 4 MPa (600 psi). Beads do not collapse at 12 MPa pressure.
- Exclusion limit of 2,500Da (PEG)
- Minimal secondary adsorption
- Typical separation times of less than 5 minutes
- High loading capacity
- High recovery down to ng protein injected
- Excellent reproducibility