High sensitivity protein separations using a new size exclusion chromatography microcolumn for use in conjunction with MS

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Introduction

Recently, Tosoh Corporation introduced TSK-GEL SuperSW3000 columns in 1mm and 2mm ID microbore column format. Size exclusion chromatography (SEC) in an aqueous mobile phase is a powerful tool for analyzing biological polymers like peptides, proteins and DNA and TSK-GEL SW series SEC columns are routinely utilized for analyzing such biological samples. The ability to detect very small amounts of proteins is one requirement in proteomics. These new TSK-GEL SuperSW3000 microbore columns with increased resolution, excellent sensitivity and high recovery were developed to analyze trace amounts of proteins.

Preliminary Results

The TSK-GEL SuperSW3000, 4µm micro-columns were characterized by analyzing protein resolution, detection sensitivity, sample capacity, and column efficiency in comparison to conventional column sizes. A 5-fold increase in peak height of a standard protein mixture was obtained when using a 2mm ID x 30cm TSKgel SuperSW3000 column, compared to a 4.6mm ID x 30cm column. The same improvement in sensitivity was also evident when analyzing aggregate-containing IgG samples. Linear calibration curves confirmed that nonspecific adsorption on the stationary phase was minimal. The detection limit of IgG was 18ng using the 1mm ID TSKgel SuperSW3000 column while still being able to detect small amounts of IgG aggregates. Unlike larger particle size columns, four micron SuperSW3000 columns showed a smaller drop in efficiency when increasing flow rate. As with 1mm ID columns, we found that reducing the injection volume of IgG solution from 10µL to 1µL greatly improved efficiency of the 2mm ID column, although at constant injection volume, efficiency did not vary with IgG concentration in the range of 1-5g/L. Results showed that trace analysis of biological components was possible when the TSKgel SuperSW3000 1mm ID column was utilized with an off-line SELDI/TOF/MS.

Experimental Conditions

Columns

-- TSK-GEL SuperSW3000 from Tosoh
- Internal diameter: 1mm, 2mm & 4.6mm
- Length: 30cm
- Particle size: ca. 4µm
- Exclusion limit: 5 x 10^5 Dalton
- Matrix: Diol-bonded silica gel

-- KW803-2E, Shodex

-- Superdex™ 200 PC, GE Healthcare

Instrumentation

Pump: DP-8020 (Tosoh)

Detector: UV-8020 (Tosoh)

UV cell: 2µL (for 2mm & 4.6mm ID, Tosoh)

UV cell: 35nL (for 1mm ID, LC Packings, Netherlands)

Sample injector: Model 7520 (Rheodyne)

Tubing (injector to column):

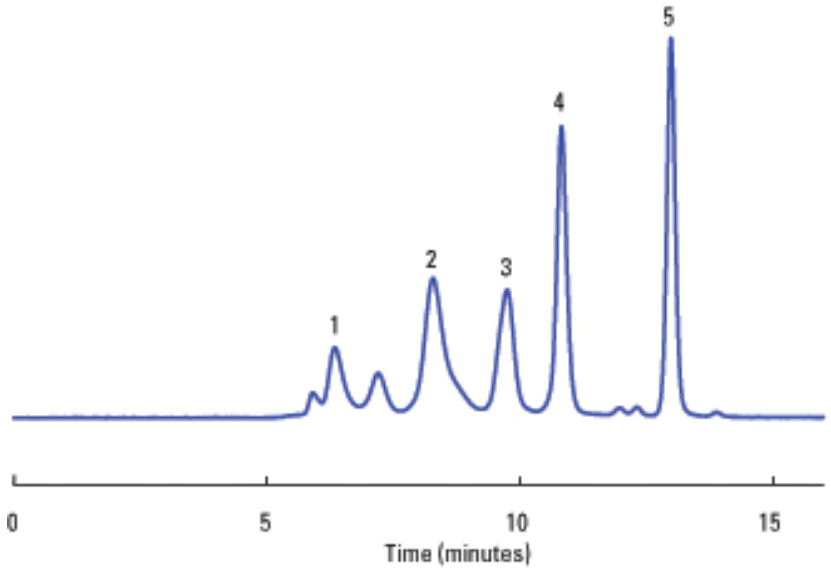
0.05mm ID x 20cm Fused Silica

Data processing: LC-8020 model 2 (Tosoh)

Sample

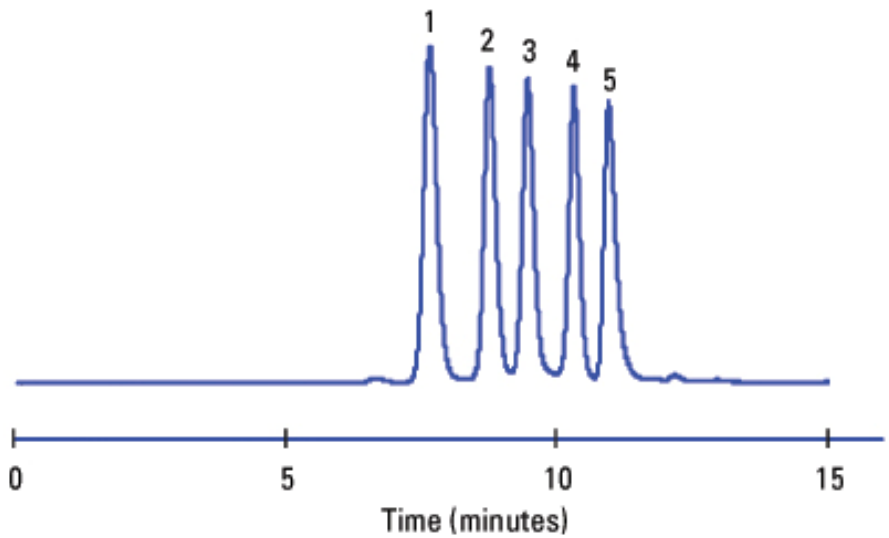
Proteins and enzymes were purchased from Sigma (USA). The antibody was a gift from the Tosoh Research Center (Kanagawa, Japan).

Figure 1: Separation of Standard Proteins on a 1mm ID TSKgel SuperSW3000 Column



Column:	TSKgel SuperSW3000, 1mm ID x 30cm
Eluent:	0.1mol/L phosphate buffer + 0.1mol/L Na_2SO_4 + 0.05% NaN_3 (pH 6.7)
Flow rate:	16µL/min
Detection:	UV@280nm
Temperature:	25°C
Injection volume:	0.2µL
Samples:	1. thyroglobulin (1.0mg/mL) 2. γ-globulin (2.0mg/mL) 3. ovalbumin (2.0mg/mL) 4. ribonuclease A (3.0mg/mL) 5. p-aminobenzoic acid (0.02mg/mL)

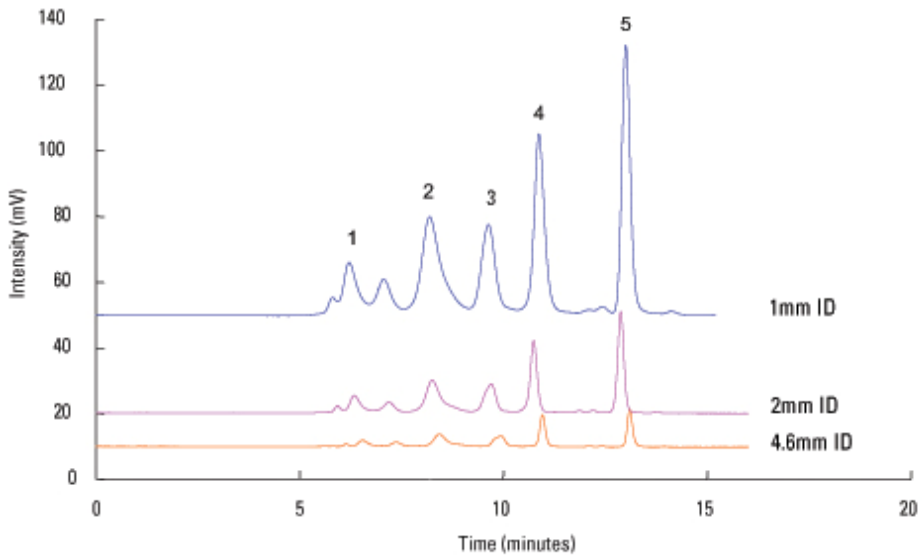
Figure 2: Separation of Standard Proteins on a 1mm ID TSKgel SuperSW3000 Column



Column:	TSKgel SuperSW3000, 1mm ID x 30cm
Eluent:	0.1mol/L phosphate buffer + 0.1mol/L Na_2SO_4 + 0.05% NaN_3 (pH 6.7)
Flow rate:	16µL/min
Detection:	UV@280nm
Temperature:	25°C
Injection volume:	0.2µL
Samples:	1. glutamate dehydrogenase 2. lactate dehydrogenase 3. enolase 4. myokinase 5. cytochrome C

MW Marker 1 vial/100µL, Oriental Yeast Co.

Figure 3: Sensitivity Comparison on TSK-GEL SuperSW3000 Columns

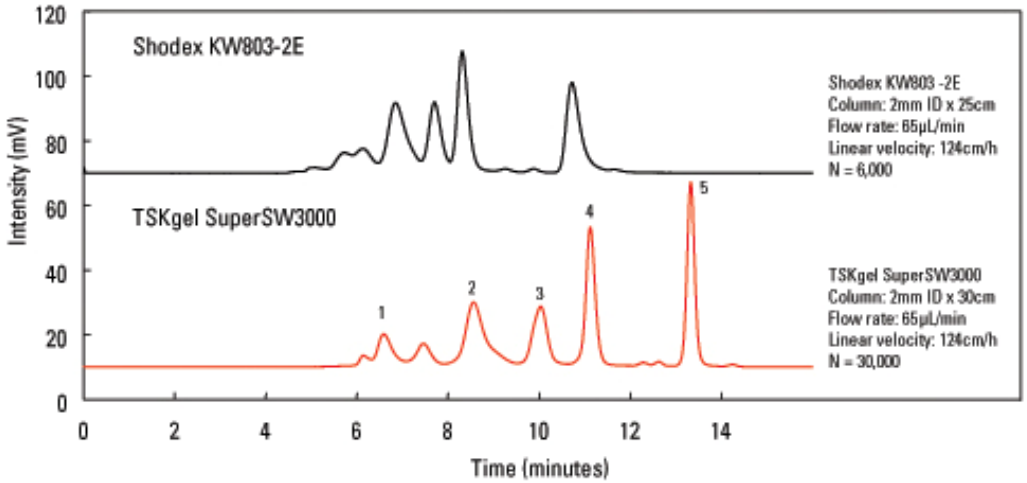


Columns:	TSKgel SuperSW3000, 4.6mm ID x 30cm TSKgel SuperSW3000, 2mm ID x 30cm TSKgel SuperSW3000, 1mm ID x 30cm
Eluent:	0.1mol/L phosphate buffer + 0.1mol/L Na_2SO_4 + 0.05% NaN_3 (pH 6.7)
Flow rate:	0.350mL/min (4.6mm ID) 0.065mL/min (2mm ID) 0.016mL/min (1mm ID)
Detection:	UV@280nm
Detector cell volume:	2µL (4.6 and 2mm ID) 35nL (1mm ID)
Temperature:	25°C
Injection volume:	1µL
Samples:	1. thyroglobulin (1.0mg/mL) 2. γ-globulin (2.0mg/mL) 3. ovalbumin (2.0mg/mL) 4. ribonuclease A (3.0mg/mL) 5. p-aminobenzoic acid (0.02mg/mL)

Table 1: Comparison of 2mm ID TSKgel SuperSW3000 With Other SEC Columns

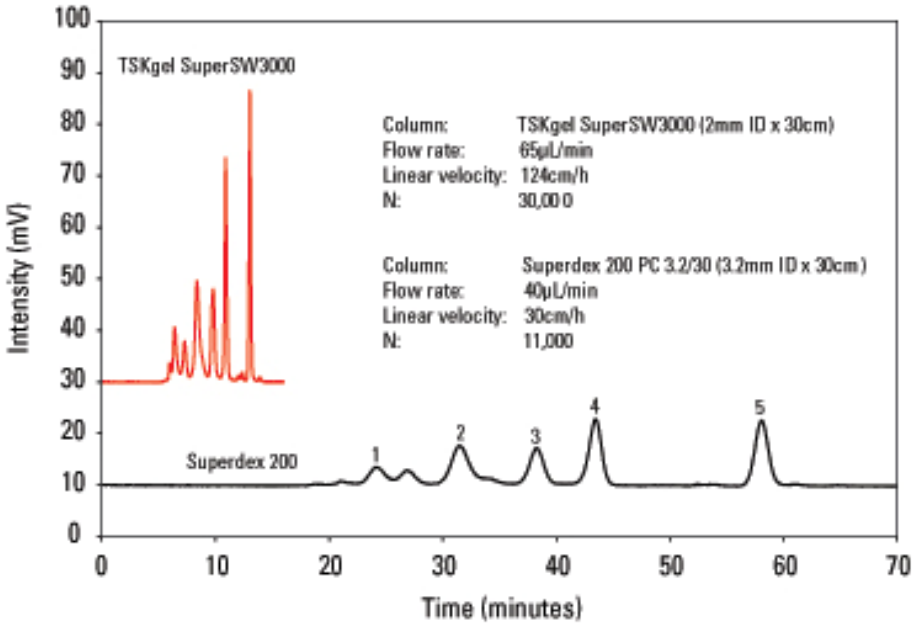
	Dimensions (mm ID x cm)	Particle size	Material	Exclusion limit
TSKgel SuperSW3000	2 x 30	4µm	Diol-bonded silica gel	5 x 10^5Da
KW803-2E	2 x 25	5µm	Silica gel	7 x 10^5Da
Superdex™ 200 PC	3.2 x 30	13µm	Dextran & agarose	6 x 10^5Da

Figure 4: Separation of Standard Proteins on Commercial GFC Columns



Eluent:	0.1mol/L phosphate buffer + 0.1mol/L Na_2SO_4 + 0.05% NaN_3 (pH 6.7)
Detection:	UV@280nm
Temperature:	25°C
Injection volume:	0.2µL
Samples:	1. thyroglobulin (1.0mg/mL) 2. γ-globulin (2.0mg/mL) 3. ovalbumin (2.0mg/mL) 4. ribonuclease A (3.0mg/mL) 5. p-aminobenzoic acid (0.02mg/mL)

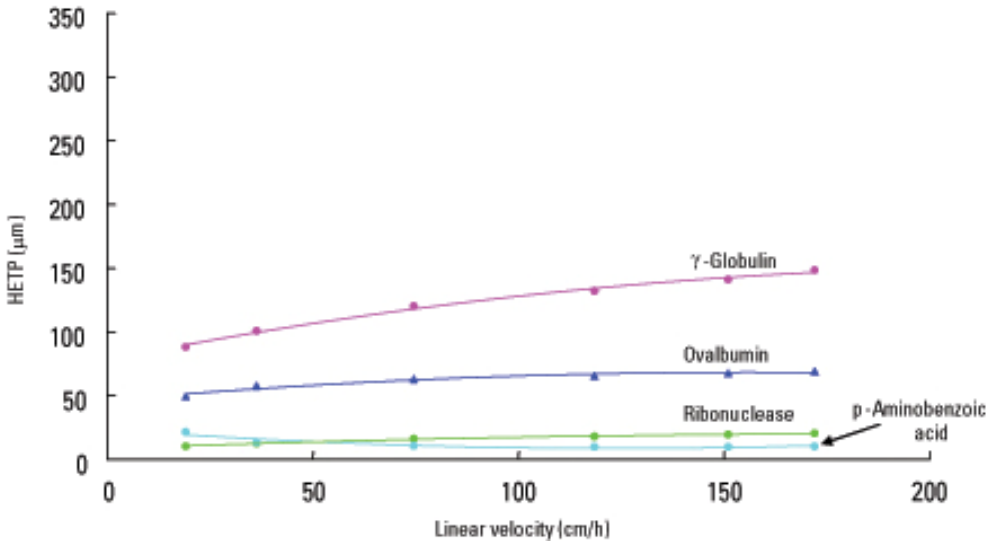
Figure 5: Separation of Standard Proteins on Commercial GFC Columns



Eluent:	0.1mol/L phosphate buffer + 0.1mol/L Na_2SO_4 + 0.05% NaN_3 (pH 6.7)
Detection:	UV@280nm
Temperature:	25°C
Injection volume:	0.2µL
Samples:	1. thyroglobulin (1.0mg/mL)
	2. γ-globulin (2.0mg/mL)
	3. ovalbumin (2.0mg/mL)
	4. ribonuclease A (3.0mg/mL)
	5. p-aminobenzoic acid (0.02mg/mL)

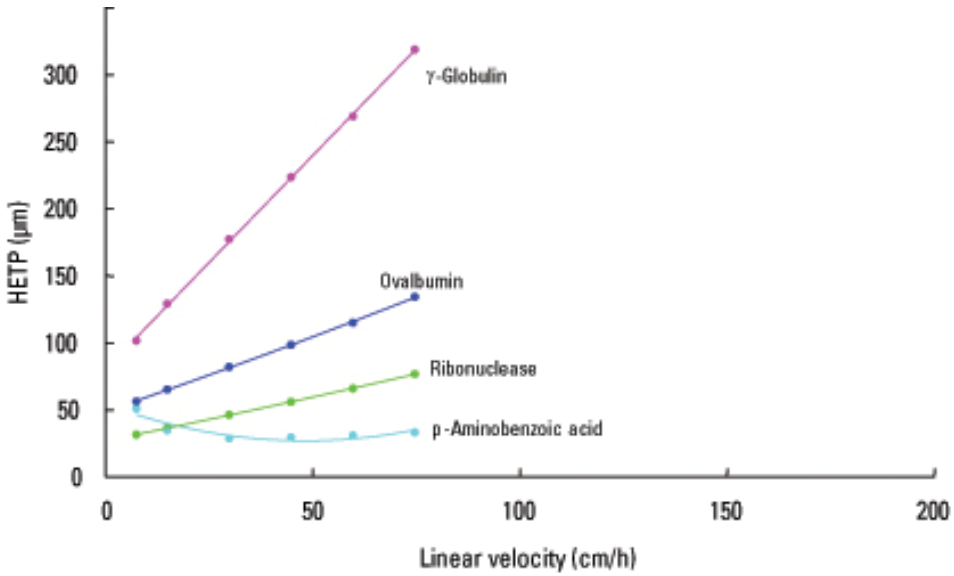
Note: both columns were operated at their recommended flow rates.

Figure 6: Relationship Between Column Efficiency and Linear Velocity



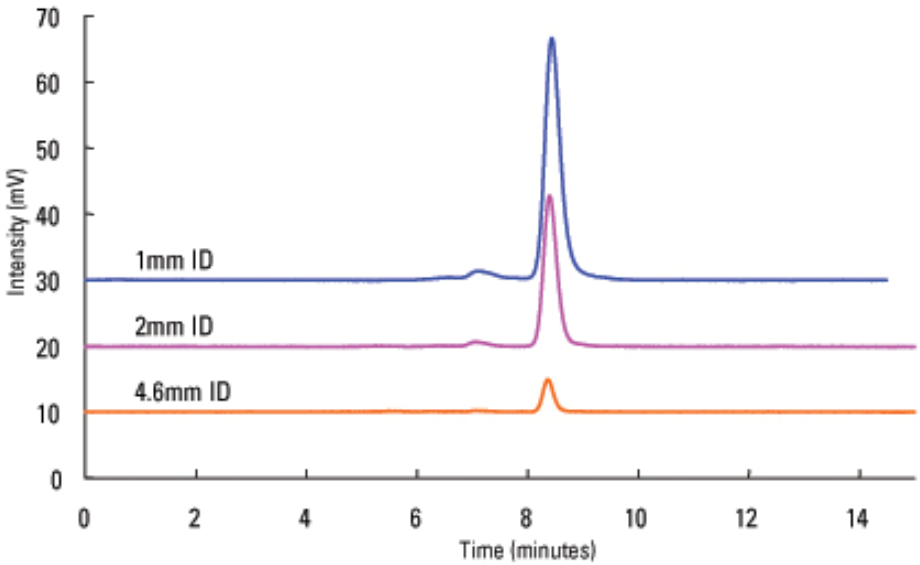
Column:	TSKgel SuperSW3000, 2mm ID x 30cm
Eluent:	0.1mol/L phosphate buffer + 0.1mol/L Na_2SO_4 + 0.05% NaN_3 (pH 6.7)
Flow rate:	16μL/min
Detection:	UV@280nm
Temperature:	25°C
Injection volume:	0.2μL
Samples:	γ-globulin (2.0mg/mL) ovalbumin (2.0mg/mL) ribonuclease A (3.0mg/mL) p-aminobenzoic acid (0.02mg/mL)

Figure 7: Relationship Between Column Efficiency and Linear Velocity



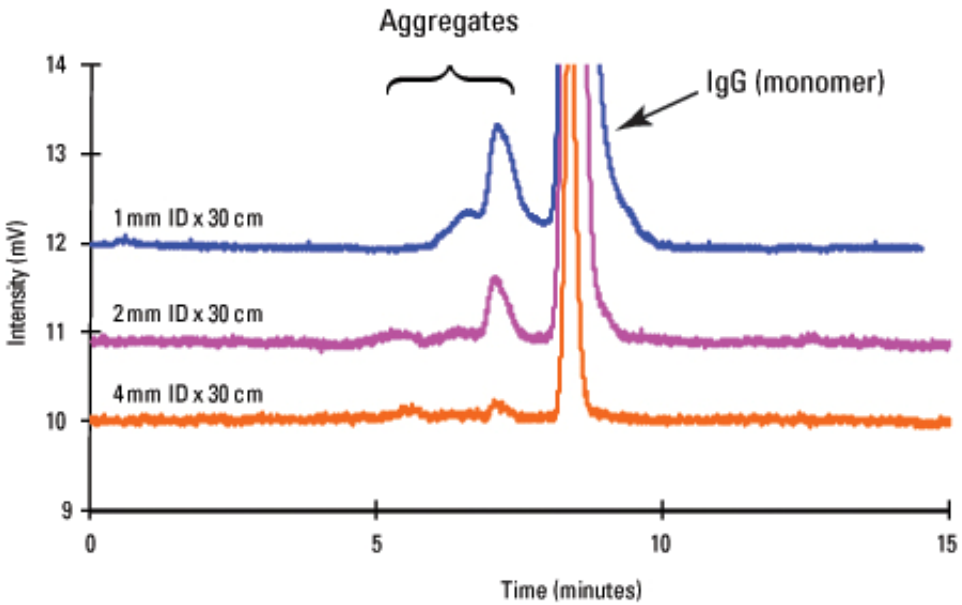
Column:	Superdex 200 PC 3.2/30, 3.2mm ID x 30cm
Eluent:	0.1mol/L phosphate buffer + 0.1mol/L Na_2SO_4 + 0.05% NaN_3 (pH 6.7)
Flow rate:	16μL/min
Detection:	UV@280nm
Temperature:	25°C
Injection volume:	0.2μL
Samples:	γ-globulin (2.0mg/mL) ovalbumin (2.0mg/mL) ribonuclease A (3.0mg/mL) p-aminobenzoic acid (0.02mg/mL)

Figure 8: Separation of IgG on TSK-GEL SuperSW3000 Columns



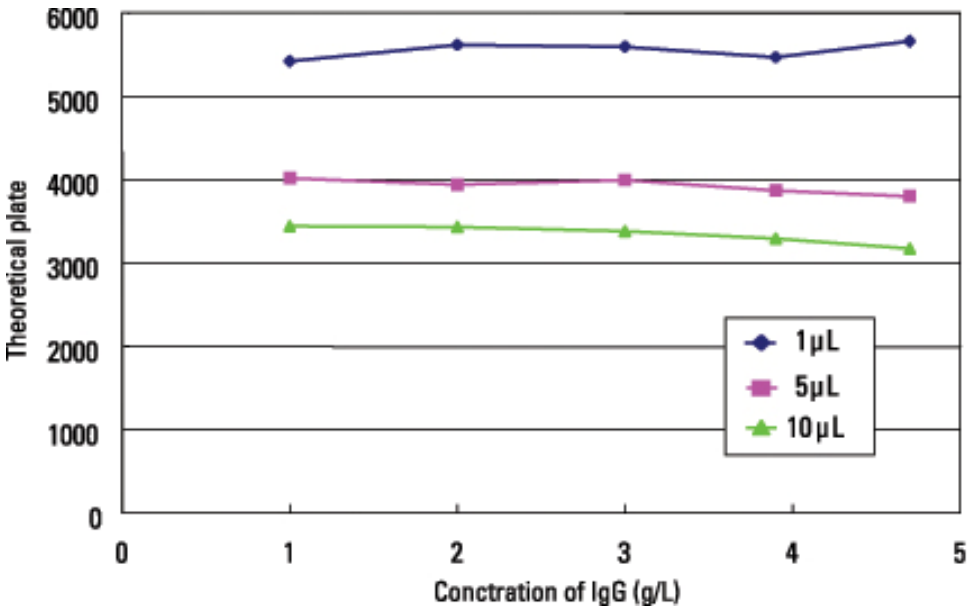
Columns:	TSKgel SuperSW3000, 4.6mm ID x 30cm TSKgel SuperSW3000, 2mm ID x 30cm TSKgel SuperSW3000, 1mm ID x 30cm
Eluent:	0.1mol/L phosphate buffer + 0.1mol/L Na_2SO_4 + 0.05% NaN_3 (pH 6.7)
Flow rate:	0.350mL/min (4.6mm ID) 0.065mL/min (2mm ID) 0.016mL/min (1mm ID)
Detection:	UV@280nm
Temperature:	25°C
Injection volume:	1µL
Sample:	IgG (mouse, MAb, 1mg/mL)

Figure 9: Separation of IgG Sample on TSK-GEL SuperSW3000 Columns



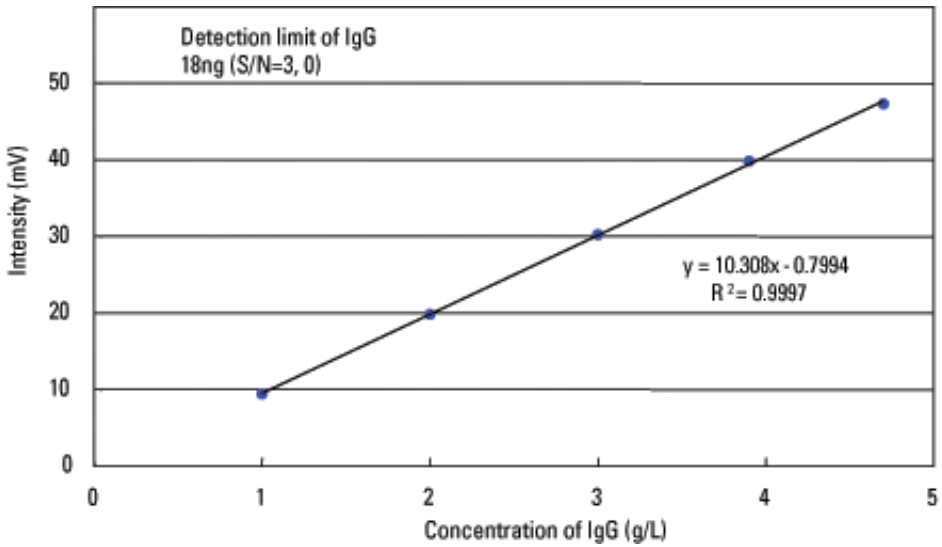
Columns:	TSKgel SuperSW3000, 4.6mm ID x 30cm TSKgel SuperSW3000, 2mm ID x 30cm TSKgel SuperSW3000, 1mm ID x 30cm
Eluent:	0.1mol/L phosphate buffer + 0.1mol/L Na_2SO_4 + 0.05% NaN_3 (pH 6.7)
Flow rate:	0.350mL/min (4.6mm ID) 0.065mL/min (2mm ID) 0.016mL/min (1mm ID)
Detection:	UV@280nm
Temperature:	25°C
Injection volume:	1µL
Sample:	IgG (mouse, MAb, 1mg/mL)

Figure 10: IgG Loading Study on a 2mm ID TSKgel SuperSW3000 Column



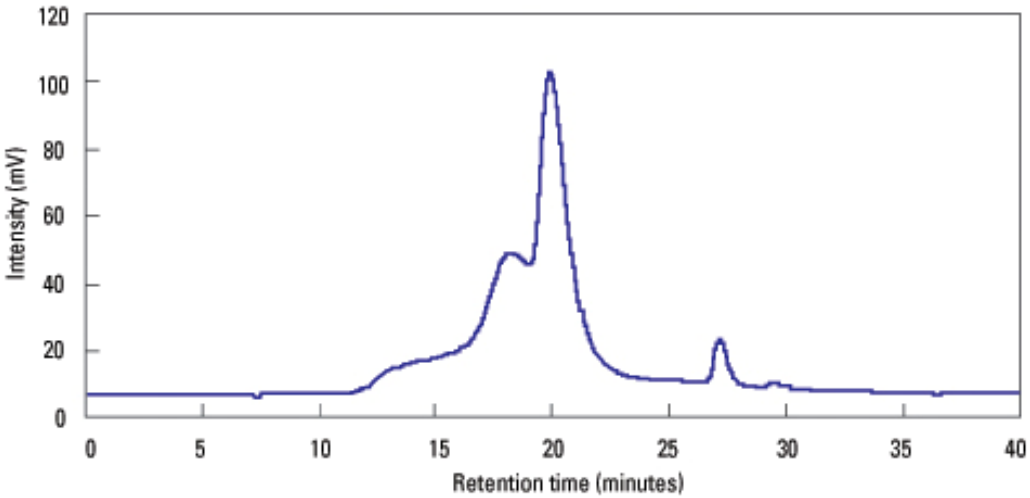
Column:	TSKgel SuperSW3000, 2mm ID x 30cm
Eluent:	0.1mol/L phosphate buffer + 0.1mol/L Na_2SO_4 + 0.05% NaN_3 (pH 6.7)
Flow rate:	0.350mL/min (4.6mm ID) 0.065mL/min (2mm ID) 0.016mL/min (1mm ID)
Detection:	UV@280nm
Temperature:	25°C
Sample:	IgG (mouse, MAb)

Figure 11: Calibration Curve of IgG on a 1mm ID TSKgel SuperSW3000 Column



Columns:	TSKgel SuperSW3000, 1mm ID x 30cm
Eluent:	0.1mol/L phosphate buffer + 0.1mol/L Na_2SO_4 + 0.05% NaN_3 (pH 6.7)
Flow rate:	0.016mL/min
Detection:	UV@280nm
Temperature:	25°C
Injection volume:	0.2µL
Samples:	IgG (mouse, MAb)

Figure 12a: Separation of Human Serum Proteins on a 1mm ID TSKgel SuperSW3000 Column

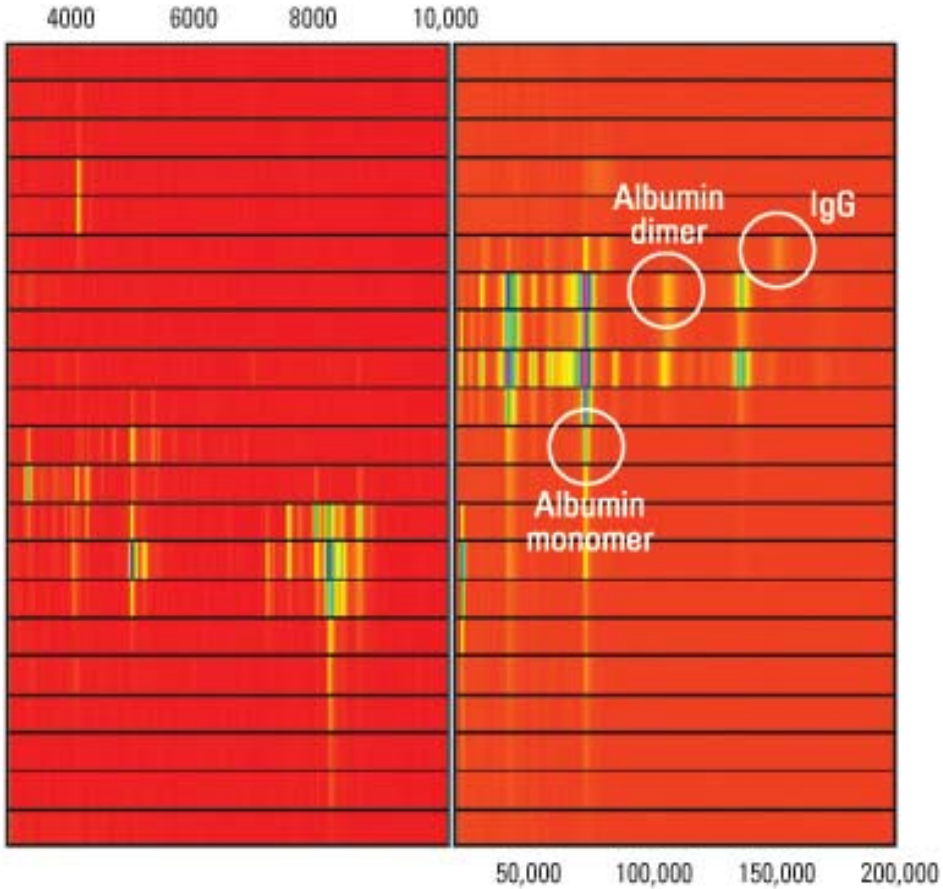


Column:	TSKgel SuperSW3000, 1mm ID x 30cm
Eluent:	50mmol/L NaH_2PO_4 + 0.5mol/L NaCl (pH 7.0)
Flow rate:	8µL/min
Detection:	UV@280nm
Temperature:	ambient
Sample:	human serum (x 10), 1µL

Fraction (1mL) was directly loaded to SELDI chip H50.
The chip was washed and desalted then applied to MS.

This data is courtesy of Dr. Majima, Protenova.

Figure 12b: Separation of Human Serum Proteins on a 1mm ID TSKgel SuperSW3000 Column



Fraction of interest analyzed by off-line SELDI/TOF/MS to establish presence of BSA aggregates and IgG.

Conclusions

(1) TSK-GEL SuperSW3000 microbore columns (1mm ID and 2mm ID) showed high resolution power for biological samples similar to what can be obtained on 4.6mm ID conventional TSK-GEL SuperSW3000 columns.

(2) Despite the high concentration of IgG (ca. 5mg/mL), good separation was achieved.

(3) High sensitivity analysis could be achieved on the microbore columns. Linear calibration curves confirmed that nonspecific adsorption on the stationary phase was minimal. The detection limit of IgG was 18ng using a 1mm ID column while still being able to detect small amounts of IgG aggregates.

(4) The results showed that TSK-GEL SuperSW3000 microbore columns are an excellent choice for the rapid separation of proteins and enzymes at micro scale and are a great fit for the trace analysis of biological components by LC/MS.