No. 097

TOSOH

SEPARATION REPORT

Application of Semi-micro Column Using TSKgel in High-Sensitivity Analysis

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TOSOH CORPORATION

1. Introduction

Semi-micro column is optimal for high-sensitivity analysis on trace amount of samples since it allows reduction of sample injection volume thanks to the high-sensitivity detection compared to conventional columns. In addition, semi-micro column concentrates the sample to a higher degree when gradient elution method is used, and thus it is optimal for high-sensitivity analysis and concentration/purification of diluted samples. Conventionally, high-sensitivity analysis on trace amount of samples has been required in analysis on low-molecular compounds in reversed-phase chromatography (RPC). However, demand for a semi-micro column that suits in analysis of trace amount of samples grew in concurrence to reduction in the samples for separation/purification of biological samples in recent years, and semi-micro column is demanded for ion-exchange chromatography (IEC) and hydrophobic interaction chromatography (HIC) in addition to RPC.
Our columns for separation of biological samples as shown below are all used in a wide range of applications due to their excellent chemical stability and resolution:

- Ion-exchange chromatography (IEC)
  TSKgel DEAE-5PW, TSKgel SP-5PW,
  TSKgel DEAE-2SW
- Hydrophobic interaction chromatography (HIC)
  TSKgel Phenyl-5PW, TSKgel Ether-5PW
- Reversed-phase chromatography (RPC)
  TSKgel ODS-80Ts, TSKgel ODS-80TsQA,
  TSKgel ODS-120T, TSKgel Phenyl-5PW RP,

This time, we have developed a semi-micro column for separation of biological samples with a reduced size for the purpose of applying these columns in high-sensitivity analysis.
This document reports on the basic properties as well as application of these columns.

2. Features

Table-1 shows the list of specifications for semi-micro columns.
The semi-micro columns for separation of biological samples are columns which have been conventionally used for separation/purification of biological samples with size reduction.
They deliver the following features:

① Compared to the conventional columns (7.5mmI.D.), high-sensitivity detection of approximately 14-fold is possible.
Approximately 5-fold high-sensitivity detection is possible for RPC due to internal diameter of conventional columns being 4.6mm.
② Since the same packing materials as the conventional columns are used, they can be used under identical conditions with the same separation patterns.
③ Trace amount components can be separated at high concentration and recovery.

Table-1 Specification of semi-micro columns for separation of biological samples

Separation mode	Product name	Resin type	Column size	Product no.
IEC	TSKgel DEAE-5PW	Polymer	2.0mmI.D. × 7.5cm	18757
	TSKgel SP-5PW	Polymer	2.0mmI.D. × 7.5cm	18758
	TSKgel DEAE-2SW	Silica	2.0mmI.D. × 25cm	18761
HIC	TSKgel Phenyl-5PW	Polymer	2.0mmI.D. × 7.5cm	18759
	TSKgel Ether-5PW	Polymer	2.0mmI.D. × 7.5cm	18760
RPC	TSKgel ODS-80Ts	Silica	2.0mmI.D. × 15cm	18150
			2.0mmI.D. × 25cm	18151
	TSKgel ODS-80TsQA	Silica	2.0mmI.D. × 15cm	18768
			2.0mmI.D. × 25cm	18769
	TSKgel ODS-120T	Silica	2.0mmI.D. × 15cm	18152
			2.0mmI.D. × 25cm	18153
	TSKgel Phenyl-5PW RP	Polymer	2.0mmI.D. × 7.5cm	18756
	TSKgel Octadecyl-4PW	Polymer	2.0mmI.D. × 15cm	18755
	TSKgel Octadecyl-2PW	Polymer	2.0mmI.D. × 15cm	18754

3. Basic Properties

3-1. Comparison between Semi-micro Columns and Conventional Columns

When the sample load is identical, the peak height increases inversely as the column's cross-sectional area. Figures-1 and -2 show the comparison of sensitivity between a conventional column and a semi-micro column on TSKgel DEAE-5PW and TSKgel SP-5PW. The cross-sectional area of the semi-micro column used in this comparison was 1/14 compared to the conventional column, and is capable of detecting various proteins at high sensitivity of 10-fold or higher.

Figure-3 shows the comparison of sensitivity between a conventional column and a semi-micro column on TSKgel Phenyl-5PW RP. Since the cross-sectional area of the column is 1/5 in TSKgel Phenyl-5PW RP, it is capable of detecting at high sensitivity of approximately 5-fold of the conventional column.

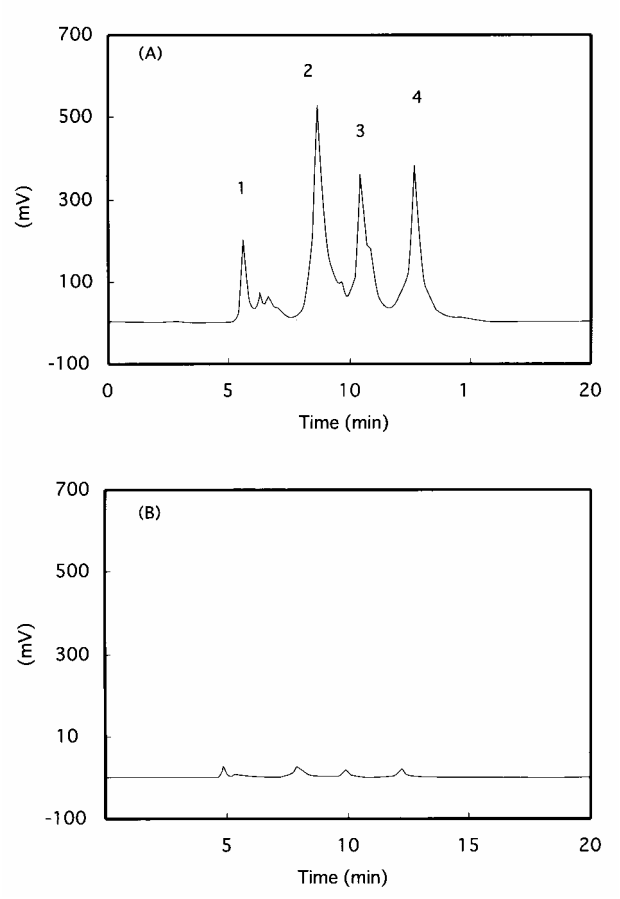

Figure-1 Comparison of sensitivity by semi-micro column and conventional column (TSKgel DEAE-5PW)

Column: (A) TSKgel DEAE-5PW (2.0mmI.D. × 7.5cm)
(B) TSKgel DEAE-5PW (7.5mmI.D. × 7.5cm)
Eluent: A: 20mmol/L Tris-HCl buffer (pH 8.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Flow rate: (A) 0.10mL/min
(B) 1.00mL/min
Temperature: 25°C
Detection: UV (280nm), micro-cell
Sample: Standard protein (10μL)
1. Carbonic anhydrase (2.4g/L)
2. Transferrin (4g/L)
3. Ovalbumin (5g/L)
4. Soybean trypsin inhibitor (5g/L)

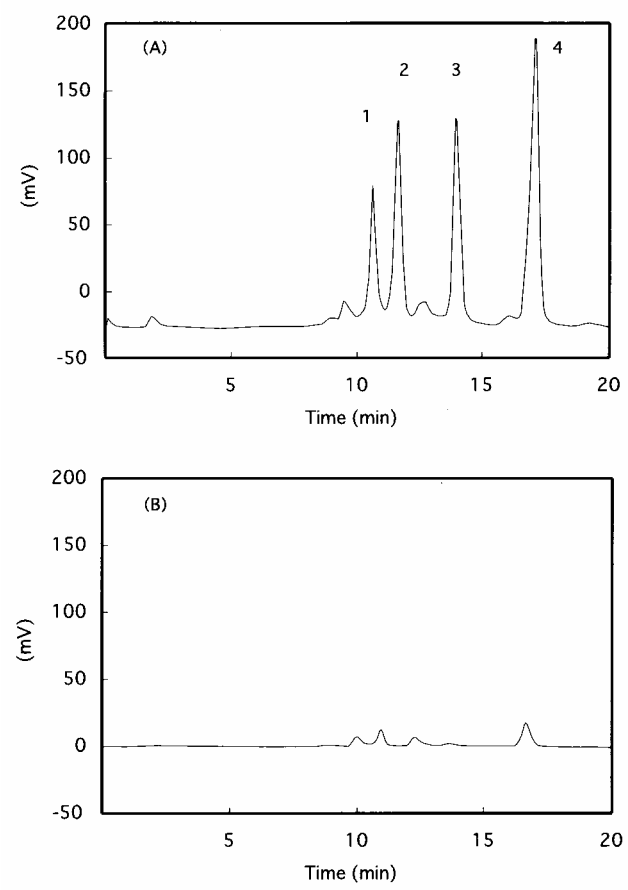

Figure-2 Comparison of sensitivity by semi-micro column and conventional column (TSKgel SP-5PW)

Column: (A) TSKgel SP-5PW (2.0mmI.D. × 7.5cm)
(B) TSKgel SP-5PW (7.5mmI.D. × 7.5cm)
Eluent: A: 20mmol/L phosphate buffer (pH 7.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Flow rate: (A) 0.10mL/min
(B) 1.00mL/min
Temperature: 25°C
Detection: UV (280nm), micro-cell
Sample: Standard protein (10μL)
1. Ribonuclease A (2g/L)
2. α-chymotrypsinogen A (1g/L)
3. Cytochrome C (5g/L)
4. Lysozyme (1g/L)

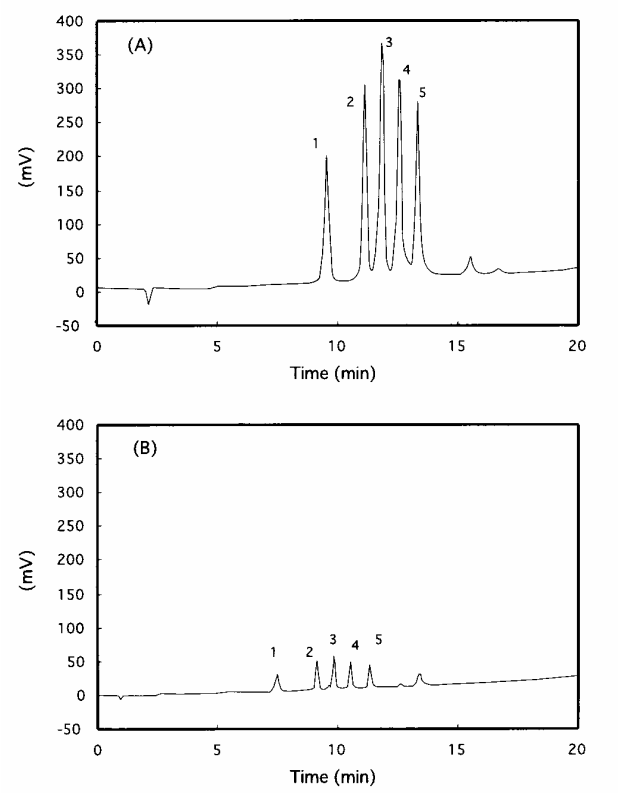

Figure-3 Comparison of sensitivity by semi-micro column and conventional column (TSKgel Phenyl-5PW RP)

Column: (A) TSKgel Phenyl-5PW RP (2.0mmI.D. × 7.5cm)
(B) TSKgel Phenyl-5PW RP (4.6mmI.D. × 7.5cm)
Eluent: A: 5% acetonitrile solution + 0.1%
B: 80% acetonitrile solution + 0.1% TFA
A→B linear gradient (20 min.)
Flow rate: (A) 0.10mL/min
(B) 1.00mL/min
Temperature: 25°C
Detection: UV (280nm), micro-cell
Sample: Standard protein (10μL)
1. Ribonuclease A (0.2g/L)
2. Cytochrome C (0.2g/L)
3. Lysozyme (0.2g/L)
4. α-lactoglobulin (0.2g/L)
5. Myoglobin (0.2g/L)

3-2. Flow Rate Dependency

In general, separation and peak height vary according to the flow rate and the gradient in HPLC using the gradient mode. The tendency is as follows:

1) When flow rate is higher,
   - Separation is better.
   - Peak is lower.
2) When gradient is smaller,
   - Separation is better.
   - Peak is lower.

Figures-4 and -5 show the effect of resolution and peak height when the flow rate is varied on TSKgel DEAE-5PW and TSKgel SP-5PW.
As the flow rate increases, resolution is improved and it nearly stabilizes at flow rate 0.10mL/min and higher. On the other hand, the peak height tends to increase as the flow rate decreases.
Based on this, it is considered that the flow rate of about 0.10mL/min is optimal for compatibility between resolution and peak height.

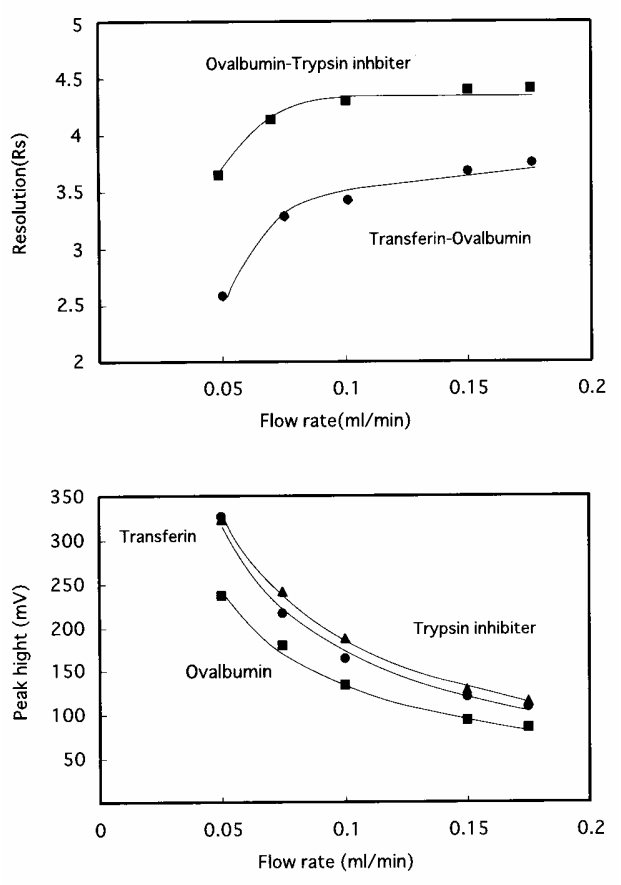

Figure-4 Flow rate dependency of TSKgel DEAE-5PW (semi-micro) column

Column: TSKgel DEAE-5PW (2.0mmI.D. × 7.5cm)
Eluent: A: 20mmol/L Tris-HCl buffer (pH 8.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Temperature: 25°C
Detection: UV (280nm), micro-cell
Sample: Standard protein (10μL)
- Transferrin (4g/L)
- Ovalbumin (5g/L)
- Soybean trypsin inhibitor (5g/L)

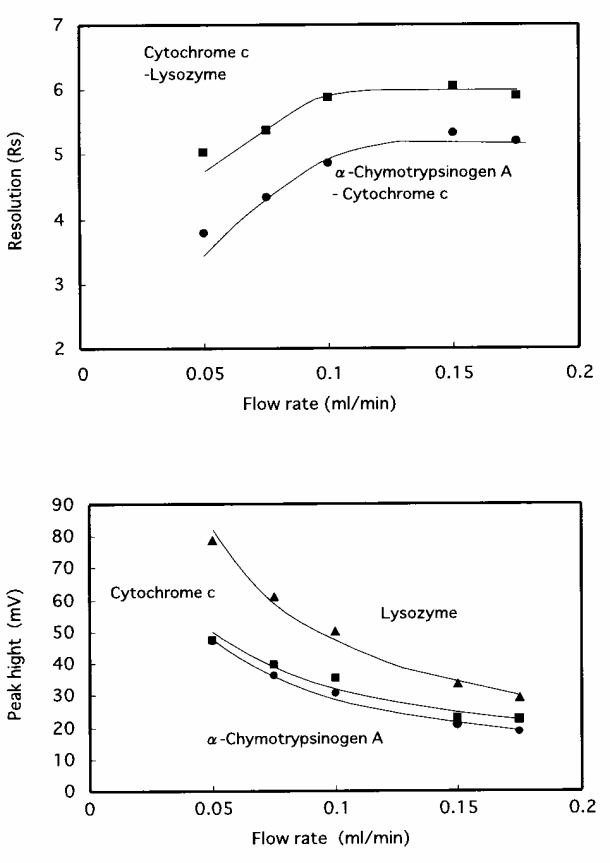

Figure-5 Flow rate dependency of TSKgel SP-5PW (semi-micro) column

Column: TSKgel SP-5PW (2.0mmI.D. × 7.5cm)
Eluent: A: 50mmol/L phosphate buffer (pH 7.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Temperature: 25°C
Detection: UV (280nm), micro-cell
Sample: Standard protein (10μL)
- α-chymotrypsinogen A (1g/L)
- Cytochrome C (1g/L)
- Lysozyme (1g/L)

3-3. Effect of Gradient

Figure-6 shows the chromatogram when the gradient is varied on TSKgel DEAE-5PW.
Though separation is greatly improved by changing the 20-minute gradient to 60-minute, the peak height is approximately 1/2.

3-4. Sample Load

Figures-7 and -8 show the sample load on TSKgel DEAE-5PW and TSKgel SP-5PW. It is evident that the resolution gradually decreases at sample load of 200μg and higher though it is nearly stable until sample load reaches 100μg.
Figure-9 shows the calibration curve for Trypsin inhibitor under the trace amount load. It shows that it deviates from the straight line with 250μg or smaller on the conventional column. Meanwhile, favorable calibration curve is obtained for the semi-micro column even at 100μg or smaller.
This indicates that conventional columns are suitable for sample load of 200μg and larger, and semi-micro columns are suitable for trace amount analysis of 200μg or smaller.

3-5. Sample Injection Volume

It is necessary in isocratic conditions to minimize the sample injection volume for semi-micro columns with low flow rate and suppress band expansion in column since the sample moves through the column even during sample injection. It has already been suggested that the optimal injection volume for semi-micro columns under isocratic conditions is 10μL or smaller (Separation Report No.91).
However, the effect of sample injection volume can be eliminated by concentrating the injected sample on the front surface of the column when gradient elution are used.
Figure-10 shows an example of eliminating the effect of sample injection volume by using the gradient elution. It shows that the resolution is not affected even when the sample injection volume is 1000μL since the injected sample is concentrated on the front surface of the column. Under such conditions, semi-micro columns are capable of concentrating diluted samples to higher degree compared to conventional columns, and allow efficient high-sensitivity analysis as well as concentration/purification of diluted samples.

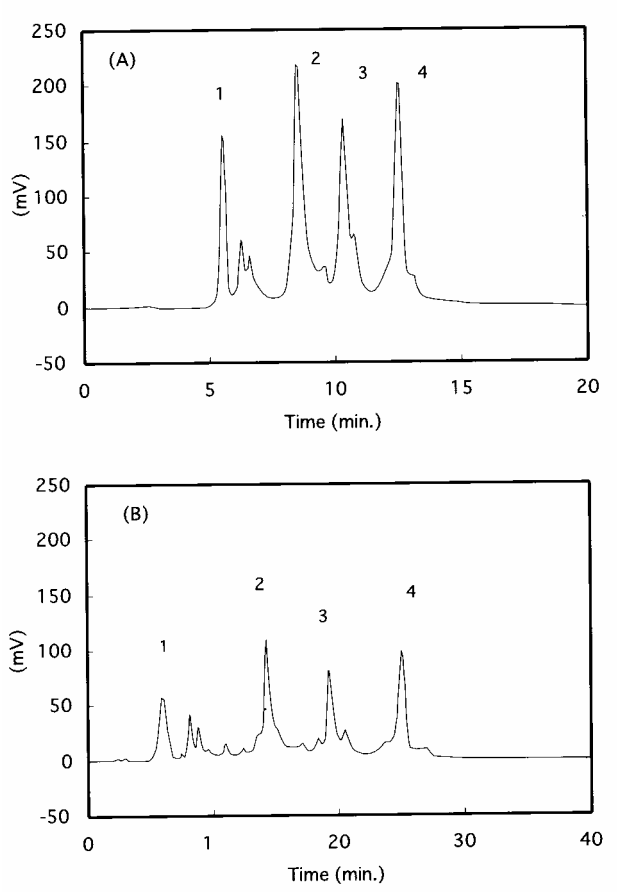

Figure-6 Effect of gradient on separation (TSKgel DEAE-5PW)

Column: TSKgel DEAE-5PW (2.0mmI.D. × 7.5cm)
Eluent: A: 20mmol/L Tris-HCl buffer (pH 8.0)
B: A + 0.5mol/L NaCl
(A): A→B linear gradient (20 min.)
(B): A→B linear gradient (60 min.)
Flow rate: 0.10mL/min
Temperature: 25°C
Detection: UV (280nm), micro-cell
Sample: Standard protein (10μL)
1. Carbonic anhydrase (2.4g/L)
2. Transferrin (4g/L)
3. Ovalbumin (5g/L)
4. Soybean trypsin inhibitor (5g/L)

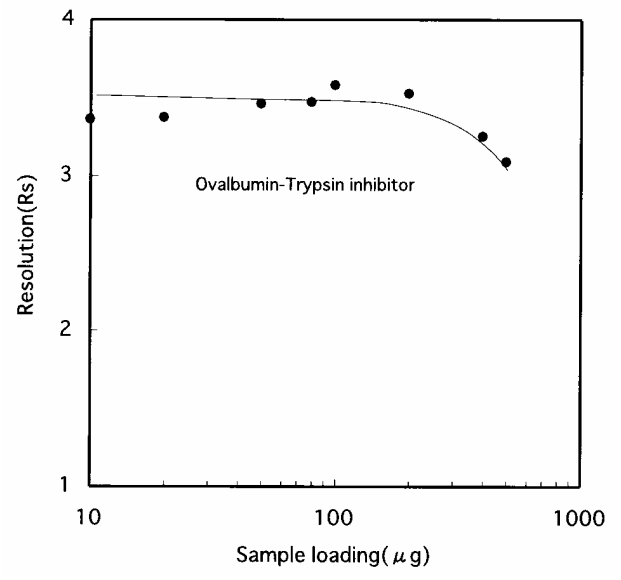

Figure-7 Relationship between resolution and sample load on TSKgel DEAE-5PW

Column: TSKgel DEAE-5PW (2.0mmI.D. × 7.5cm)
Eluent: A: 20mmol/L Tris-HCl buffer (pH 8.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Flow rate: 0.10mL/min
Temperature: 25°C
Detection: UV (280nm), micro-cell
Sample: Standard protein (50μL)
- Ovalbumin
- Soybean trypsin inhibitor

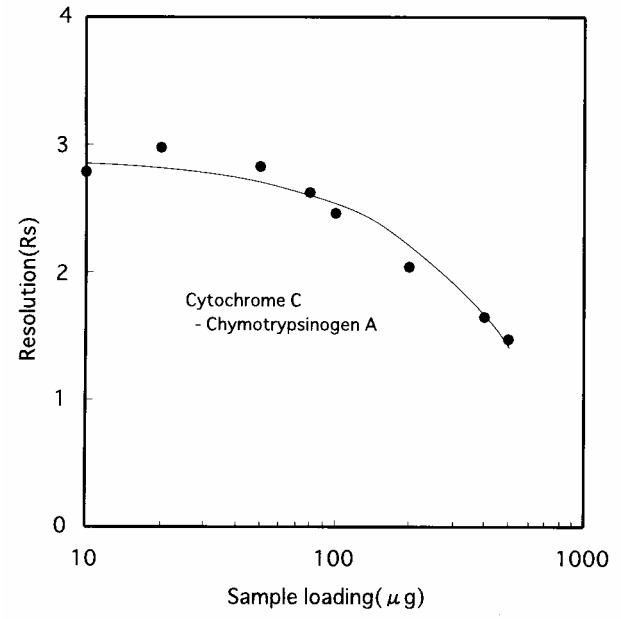

Figure-8 Relationship between resolution and sample load on TSKgel SP-5PW

Column: TSKgel SP-5PW (2.0mmI.D. × 7.5cm)
Eluent: A: 20mmol/L phosphate buffer (pH 7.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Flow rate: 0.10mL/min
Temperature: 25°C
Detection: UV (280nm), micro-cell
Sample: Standard protein (50μL)
- α-chymotrypsinogen A
- Cytochrome C

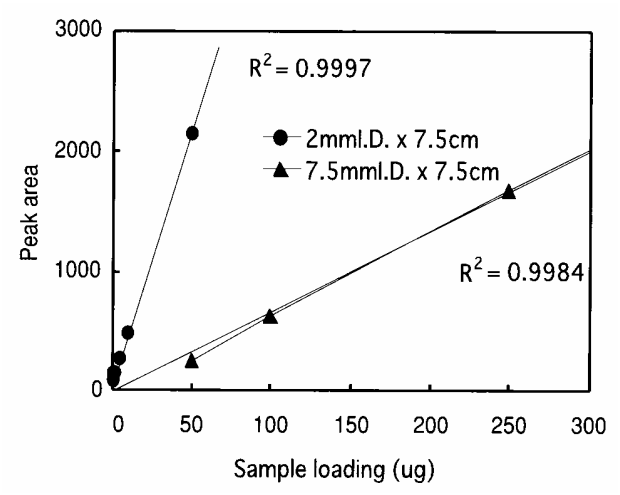

Figure-9 Comparison of calibration curves by semi-micro column and conventional column with trace amount sample load

Column: TSKgel DEAE-5PW (2.0mmI.D. × 7.5cm)
TSKgel DEAE-5PW (7.5mmI.D. × 7.5cm)
Eluent: A: 20mmol/L Tris-HCl buffer (pH 8.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Flow rate: 0.10mL/min (2.0mmI.D. × 7.5cm)
1.00mL/min (7.5mmI.D. × 7.5cm)
Temperature: 25°C
Detection: UV (280nm), micro-cell
Sample: Soybean trypsin inhibitor (10μL)

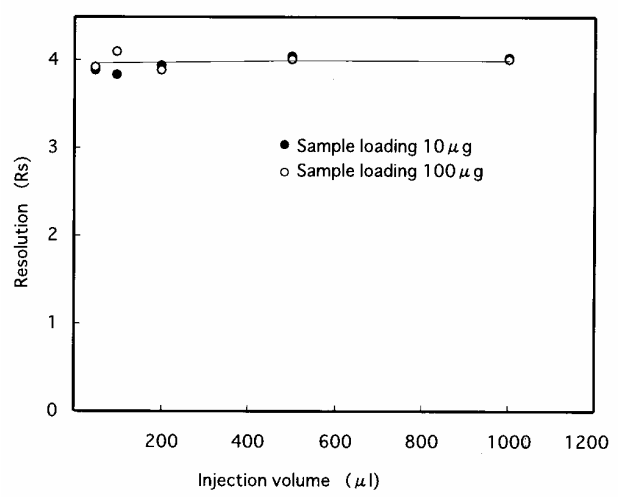

Figure-10 Relationship between resolution and sample injection volume on TSKgel DEAE-5PW

Column: TSKgel DEAE-5PW (2.0mmI.D. × 7.5cm)
Eluent: A: 20mmol/L Tris-HCl buffer (pH 8.0)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Flow rate: 0.10mL/min
Temperature: 25°C
Detection: UV (280nm), micro-cell
Sample: Standard protein
- Ovalbumin
- Soybean trypsin inhibitor

3-6. Recovery

Table-2 shows the protein recovery on TSKgel DEAE-5PW.
For the conventional column, high recovery was obtained until the sample load reached about 20μg. However, recovery deteriorates drastically for most proteins when the sample load is 5μg.
On the other hand, the semi-micro column delivered high recovery even with the sample load of 2μg.

Table-2 Comparison of protein recovery between semi-micro column and conventional column

	Semi-micro column (2.0mmI.D. × 7.5cm)		Conventional column (7.5mmI.D. × 7.5cm)	
Sample load	20μg	2μg	20μg	5μg
Bovine serum albumin	88%	82%	87%	75%
Ovalbumin	96%	93%	94%	81%
Myoglobin	98%	93%	94%	79%
Soybean trypsin inhibitor	89%	72%	84%	62%

Column: TSKgel DEAE-5PW (2.0mmI.D. × 7.5cm)
TSKgel DEAE-5PW (7.5mmI.D. × 7.5cm)
Eluent: A: 20mmol/L Tris-HCl buffer (pH 8.5)
B: A + 0.5mol/L NaCl
A→B linear gradient (20 min.)
Flow rate: 0.10mL/min (2.0mmI.D. × 7.5cm)
1.00mL/min (7.5mmI.D. × 7.5cm)
Injection volume: 20μL

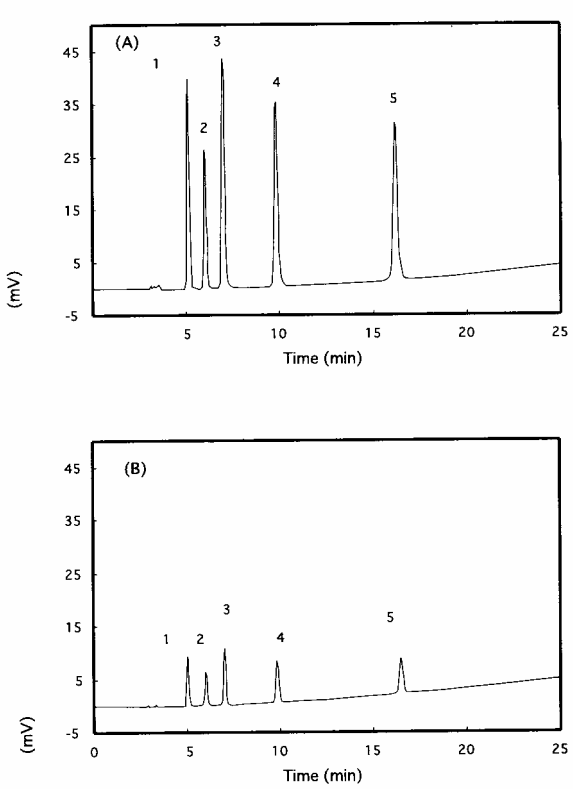

Figure-11 Comparison of sensitivity on semi-micro column and conventional column (TSKgel DEAE-2SW)

Column: (A) TSKgel DEAE-2SW (2.0mmI.D. × 25cm)
(B) TSKgel DEAE-2SW (4.6mmI.D. × 25cm)
Eluent: A: 10mmol/L phosphate buffer (pH 3.0) / acetonitrile = 80/20
B: 50mmol/L phosphate buffer (pH 3.0) / acetonitrile = 80/20
A→B linear gradient (20 min.)
Flow rate: (A) 0.10mL/min
(B) 1.00mL/min
Temperature: 25°C
Detection: UV (260nm), micro-cell
Sample: Sample (2μl)
1. AMP (45g/L)
2. IMP (90g/L)
3. GMP (90g/L)
4. ADP (90g/L)
5. ATP (90g/L)

4. Other Columns and Applications

Figures-11 to -14 show the comparison of relative sensitivity on conventional columns and semi-micro columns for separation of biological samples. Furthermore, Figure-15 shows an analysis example on trypsin digests on TSKgel ODS-80Ts semi-micro column.

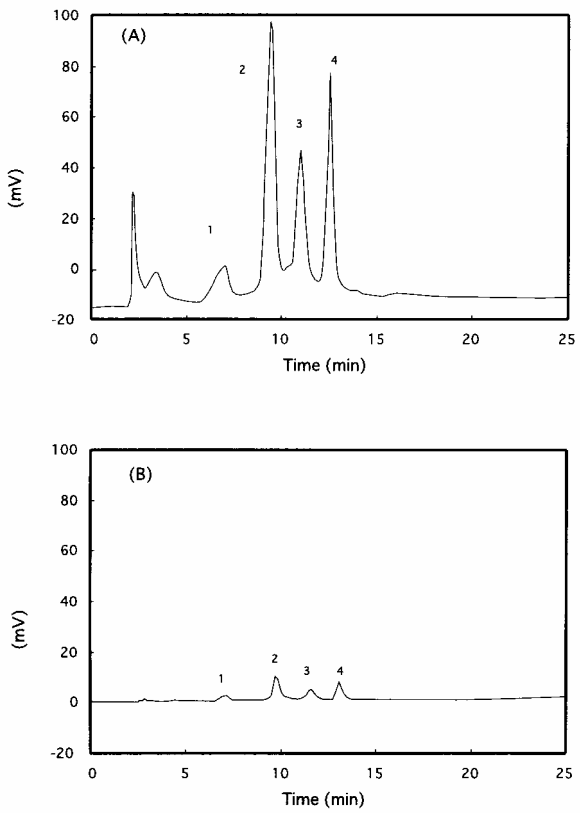

Figure-12 Comparison of sensitivity on semi-micro column and conventional column (TSKgel Ether-5PW)

Column: (A) TSKgel Ether-5PW (2.0mmI.D. × 7.5cm)
(B) TSKgel Ether-5PW (7.5mmI.D. × 7.5cm)
Eluent: A: 0.1mol/L phosphate buffer + 1.8mol/L ammonium sulfate (pH 7.0)
B: 0.1mol/L phosphate buffer (pH 7.0)
A→B linear gradient (20 min.)
Flow rate: (A) 0.10mL/min
(B) 1.00mL/min
Temperature: 25°C
Detection: UV (280nm), micro-cell
Sample: Standard protein (10μL)
1. Ribonuclease A (0.5g/L)
2. Lysozyme (0.5g/L)
3. α-chymotrypsin (0.5g/L)
4. α-chymotrypsinogen (0.5g/L)

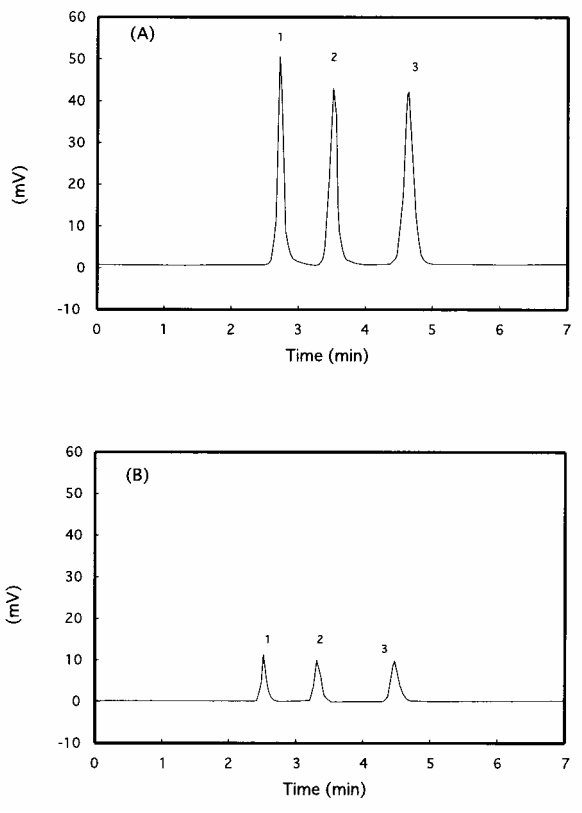

Figure-13 Comparison of sensitivity on semi-micro column and conventional column (TSKgel Octadecyl-2PW)

Column: (A) TSKgel Octadecyl-2PW (2.0mmI.D. × 15cm)
(B) TSKgel Octadecyl-2PW (4.6mmI.D. × 15cm)
Eluent: 20mmol/L phosphate buffer (pH 7.0) / acetonitrile = 50/50
Flow rate: (A) 0.19mL/min
(B) 1.00mL/min
Temperature: 25°C
Detection: UV (254nm), micro-cell
Sample: Sample (1μL)
1. Aniline (13.8mg/L)
2. N-methylaniline (30mg/L)
3. N,N-dimethylaniline (30mg/L)

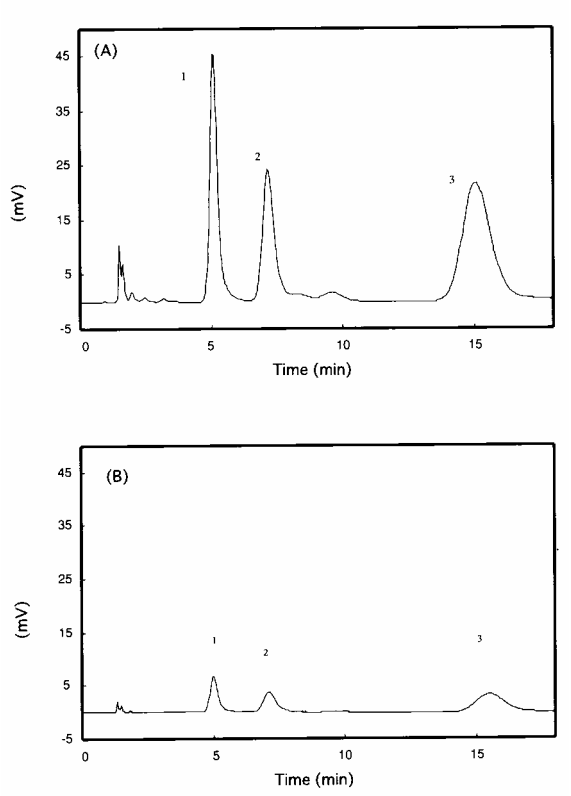

Figure-14 Comparison of sensitivity on semi-micro column and conventional column (TSKgel Octadecyl-4PW)

Column: (A) TSKgel Octadecyl-4PW (2.0mmI.D. × 15cm)
(B) TSKgel Octadecyl-4PW (4.6mmI.D. × 15cm)
Eluent: 50mmol/L phosphate buffer (pH 7.0) / acetonitrile = 90/10
Flow rate: (A) 0.19mL/min
(B) 1.00mL/min
Temperature: 25°C
Detection: UV (215nm), micro-cell
Sample: Sample (1.4μL)
1. Methionine-enkephalin (30mg/L)
2. Leucine -enkephalin (30mg/L)
3. Oxytocin (30mg/L)

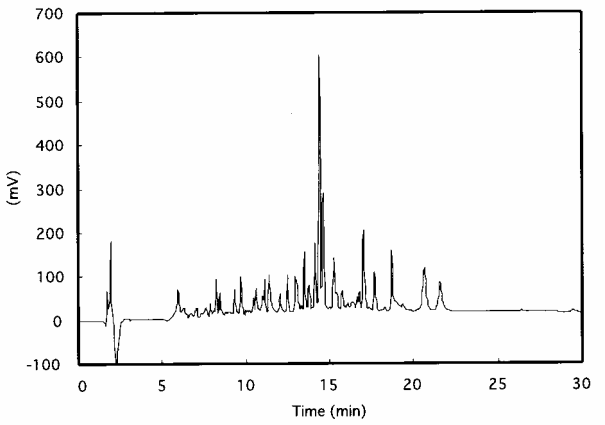

Figure-15 Analysis on trypsin digests of β-lactoglobulin

Column: TSKgel ODS-80Ts (2.0mmI.D. × 15cm)
Eluent: A: 0.1% TFA solution
B: Acetonitrile + 0.1% TFA
A (100%) → A (30%) linear gradient (30 min.)
Flow rate: 0.20mL/min
Temperature: 25°C
Detection: UV (215nm), micro-cell
Sample: Trypsin digests of β-lactoglobulin (10μL)

5. Conclusion

Semi-micro columns for separation of biological samples enable high-sensitivity detection by column size reduction. They are suitable for analysis of trace amount of samples since high-sensitivity detection of about 14-fold (about 5-fold in RPC) is possible compared to the conventional columns under the same sample load.

In addition, size reduction into semi-micro columns leads to expectation for higher recovery in trace amount analysis, and trace amount components can be separated/recovered at high concentrations.

It is possible to implement high-sensitivity analysis and purification of diluted samples under gradient conditions by concentrating the samples on the front surface of the column.

Since the same packing materials as the conventional columns are used, the same separation patterns can be obtained under the conventional conditions.

Furthermore, it is recommended to conduct separation in systems with minimized dead volume in order for the TSKgel semi-micro column series to deliver its performance. Band broadening outside the column is a major cause of poor resolution. Table-3 provides the precautions for on the TKS-GEL semi-micro column series.

Table-3 Precautions for using TSKgel semi-micro column series

* Suppress peak expansion in piping, detector, etc.
* Prevent sample overloading.
* Take care of actual flow rate of the pump since it uses a low flow rate.
Piping:
Use piping of 0.1mmI.D. The total desired length of piping is 100cm or smaller.
Connection pipe set type L (Product No. 018186: 0.1mmI.D. × 40cm, 2 units) can be used; connection surfaces (both ends) have fine-cut finish.
Parts that require 0.1mmI.D. piping
a) Between injection valve and column inlet, or between auto-sampler and column inlet
b) Between column outlet and detector inlet (piping on the detector inlet side)
Pump:
Semi-micro supporting pump is desired.
Flow rate is 0.1mL/min.
Injector and auto-sampler:
Low-diffusion type injector (Rheodyne 8125) is desired.
Mixer:
When using gradient, use static mixer C.
Detector:
Use micro flow cell for UV detector (use of low-dead volume type cells is also possible. However, the theoretical plates will be approximately 90% of the micro cell).