Preparative HPLC Using TSK Columns in the Purification of Biochemical Products

Hiroo SASAKI
Kiyoaki SAKAMOTO
Kenichi MATSUBARA
Takao MATSUDA
Yoshio KATO

Preparative HPLC using TSK columns is reviewed. Advantages of preparative HPLC are summarized. Product line is introduced including column dimmension and particle size of packing materials. Basic properties of the columns such as performance, calibration curves, mechanical and chemical stability, recovery yields of proteins, titration curves, etc. are described. Sample loading capacity is summarized. An idea for column selection is presented. Typical examples are given. Finally concluded that preparative HPLC will widely be recognized in near future as the best method for the final fine purification step in the commercial production of biopolymers owing to various advantages.

1. Introduction

In the separation of biopolymers, high performance gel filtration chromatography (GFC) has become very popular during the last ten years[1~21]. High performance ion exchange chromatography (IEC) has followed GFC during the past three years to be used widely, too[22~28]. Now high performance hydrophobic interaction chromatography (HIC) has become available as the third universal liquid chromatographical technique for the separation of biopolymers[29~31].

It goes without saying that these break-throughs have been brought by great advances in the production technology of packing materials having sophisticatedly controlled pore structure and high mechanical strength.

Since HPLC has become widely used in biochemical field as an unparalleledly important method for analysis, the trend to utilize its advantages in the purification of various biochemical substances is also growing rapidly. The direct extension of the best analytical method to the final production plant, if available, can undoubtedly provide various advantages in the total stages from early research to final commercialization.

A typical example for the industrial purification steps of proteins in new biotechnology will be summarized in the comparison with the conventional one as shown in Table 1.

We are engaged in the development of three major techniques, HPLC, MPLC (medium performance liquid chromatography), and UF (ultrafiltration) for new separation technology. HPLC anc MPLC will be used widely in place of LPLC (low performance liquid chromatography) for fine purification and rough purification, respectively. UF will play important roles in various steps as shown above.

Now preparative HPLC has reached the level that several hundred grams per day can be

Table 1 Comparison of Separation Techniques in Biotechnology

	Conventional technique	New technique
1. Liquid/Solid separation	* Ultracentrifugation	* Ultrafiltration
2. Rough purification	* LPLC (IEC) * Salting out * Extraction	* Ultrafiltration * MPLC (IEC, HIC, GFC)
3. Fine purification	* LPLC (IEC, HIC)	* HPLC (IEC, HIC, GFC)
4. Desalting	* LPLC (GFC)	* LPLC (GFC) * Ultrafiltration
5. Concentration		* Ultrafiltration

purified full automatically. Practically, preparative HPLC using a large diameter column has come to be used in the commercial production of expensive biochemical substances such as proteins and enzymes.

Here we present a review on preparative HPLC using TSK columns for the purification of biochemical products.

2. Advantages of preparative HPLC

Table 2 lists the advantages of preparative HPLC in large scale commercial production of biochemical substances including R & D stage. Main simple advantages may be high purity of products owing to high resolution and high productivity owing to high speed. The purity of a product is very important, particularly in new biotechnology, to succeed in various time consuming biological tests without the effect of unfavourable contaminants. However, it should be noted that, despite of the image of high price of HPLC, various great cost reducing factors in comparison with LPLC can be pointed out as in Table 2.

Table 2 Advantages of Preparative HPLC in Industrial Purification of Biological Substances

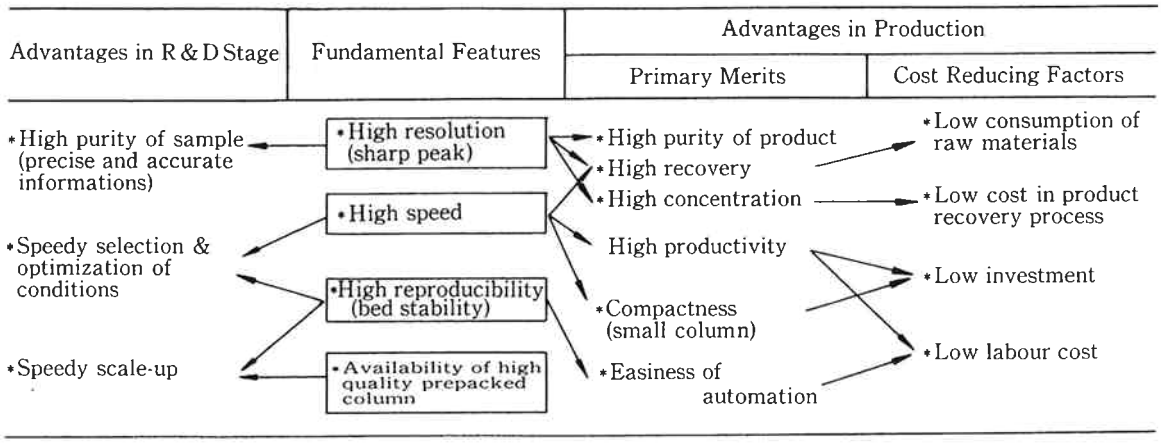


Table 3 Typical Example of Large Scale Production by HPLC

* Separation mode	GFC	IEC
* Column	TSKgel G3000SW	TSKgel DEAE-5PW
* Column size	108mm I.D. × 600mm	108mm I.D. × 200mm
* Loading capacity	10g/cycle	5g/cycle
* Cycle/day	12	12
* Cycle/year	2, 400	2, 400
* Capacity/year	24, 000g	12, 000g

Particularly, drastic saving of time and money in research and development stage owing to speedy establishment of scale-up is very important not noly for cost reduction but also for reaching the goal before any other rivals in severe competition in the development of new bioproducts. Once the conditions are optimized by an analytical column, what a customer has to do for scale-up is only to purchase a corresponding preparative column followed by very slight modifications of the conditions.

In the production, the high recovery yield brought by high resolution and/or speedy operation is a big cost reducing factor affecting the cost of the total process including all preceding steps. In addition, high reproducibility owing to stable column bed leads to easiness of full automation, resulting in drastic reduction of labour cost.

Typical examples of the production capacity in GFC and IEC using a column of 108mm I.D. are given in Table 3, which indicates that several tens of kilograms are usually produced per year. The column cost can be estimated less than a few dollars per gram product.

3. TSK columns for preparative HPLC in biochemical field

[1] Kinds of columns

Table 4 lists the main TSK columns for preparative HPLC used for purification of biochemical substances. As shown in Table 4, TSK columns cover total field of commonly used separation modes, namely GFC, IEC, HIC and RPC (reversed-phase chromatography). Inner diameters of 21.5, 55.0 and 108 are available for all main columns. Many other columns not listed in Table 4 such as TSKgel G4000SW, G6000PW, DEAE-3SW, CM-3SW, DEAE-2SW, CM-2SW, SP-2SW, TMS-250 etc. are limited to 21.5mm I.D. size because of inferiority to their alternatives in resoluving power or column stability or costwise problem. The column length increase with increasing diameter for IEC and HIC.

Table 5 lists TSK guard columns, essential to preparative HPLC to protect preparative columns.

[2] Particle size

Particle size is increased with increasing column diameter as shown in Table 6.

Particle size should be determined to obtain the best compromise among the following factors:

a) Resoluving power: The smaller the particle size, the higher the resoluving power[14]. However, it should be noted that resoluving power can be increased by decreasing flow

rate.

b) Pressure drop and flow rate: Pressure drop is reversely proportional to the square of particle size. Pressure drop increases with increasing column diameter for the same particle size.

c) Loading capacity: There is a tendency that maximum loading capacity increases with increasing particle size in the range of small particle size.

Table 4 TSK Columns for Preparative HPLC in Biochemical Field

Mode	Type	Pore Size (Å)	Column Dimmension (mm)			Base Material	Typical Sample
			21.5 I.D.	55 I.D.	108 I.D.		
GFC	TSKgel G2000SW	125	300, 600	300, 600	600	Silica	Proteins & enzymes
	TSKgel G3000SW	250	300, 600	300, 600	600		
	TSKgel G2000PW	80	600	600	600	Polymer	Poly- & oligo-saccharides
	TSKgel G3000PW	200	600	600	600		
	TSKgel G5000PW	1,000	600	600	600		Large proteins
IEC	TSKgel DEAE-5PW	1,000	150	200	200	Polymer	Proteins & enzymes
	TSKgel SP-5PW	1,000	150	200	200		
HIC	TSKgel Phenyl-5PW	1,000	150	200	200	Polymer	Proteins
RPC	TSKgel ODS-120A	120	300	300, 600	300, 600	Silica	Peptides
	TSKgel ODS-120T	120	300	300, 600	300, 600		

Table 5 TSK Guard Columns for Preparative HPLC

Mode	Guard Column	Column Dimmension (mm)		
		21.5mm I.D.	55mm I.D.	108mm I.D.
GFC	TSKguard column SW	21.5×75	45×50	83×50
	TSKguard column PW	21.5×75	45×50	83×50
IEC	TSKguard column DEAE-5PW	*1	45×50	83×50
	TSKguard column SP-5PW		45×50	83×50
HIC	TSKguard column Phenyl-5PW	*1	45×50	83×50
RPC	TSKguard column ODS-120A	*2	45×50	83×50
	TSKguard column ODS-120T		45×50	83×50

(Note) *1 Guardgel kits are available.
*2 Bulkgels are available.

Table 6 Relationship between Column Diameter and Particle Size

Column diameter (mm)	4.6	7.5	21.5	55	108
	Particle size (μm)				
GFC, IEC, HIC	—	10	13	20	20
RPC	5	10	10	20	20

d) Column life: There is a tendency that column life becomes longer as particle size increases because of lower pressure drop.

e) Column price: Column price clealy increases with decreasing particle size due to the increases of the sorbent production cost and column packing process cost.

By the adequate particle size selection, TSK preparative HPLC columns, in comparison with the corresponding analytical columns, can provide neally equal resoluving power at a slight expense of speed (see Fig. 19, 26, 30, 31 and 32), reduced pressure drop (see Fig. 4), larger loading capacity (see Fig. 15), longer column life and much reduced price relatively.

[3] Base material

Modern liquid chromatography sorbents consist of three major base materials, namely silica gel, cross-linked polymer gel and cross-linked polysaccharide gel.

Base materials for sorbents for TSK preparative HPLC columns consist of silica gel and cross-linked polymer gel. The advantages of these materials are well utilized complementally each other, namely the high performance of the silica gel in GFC and RPC and the chemical stability of the cross-linked polymer gel in IEC and HIC, respectively.

The silica gel, featured by high mechanical streghth and nonswelling and nonshrinking property against solvent exchange, is the main material for HPLC sorbents, particularly RPC. Another big advantage of the silica gel is its excellent pore characteristics in the range of 100 to 500 angstrom such as homogeneous pore distribution and large pore volume, realizing excellent resolution of proteins in GFC.

The cross-linked polymer gel, featured by excellent chemical stability and various well-balanced properties such as semi-rigidity, small swelling and shrinking, availability of variety of pore size etc., is increasingly used as the second material for HPLC sorbent. The chemical stability of sorbents is very important particularly in preparative IEC and HIC in which sorbents usually tend to be deteriorated easily by strong adsorption of various complex substances due to ionic or hydrophobic interaction and should be cleaned up frequently by strong alkaline or acidic solutions.

The cross-linked polysaccharide gel, featured by excellent hydrophilicity, has widely been used for the separation of biopolymers. However, its application to HPLC has been seriously restricted because of the mechanical weakness.

4. Basic properties of TSK preparative HPLC columns

[1] Performance

Table 7 lists the particle size, theoretical plate number, flow rate and pressure drop of the series of the columns.

Most of the theoretical plate numbers guaranteed per column are higher or equal to those of the corresponding analytical columns.

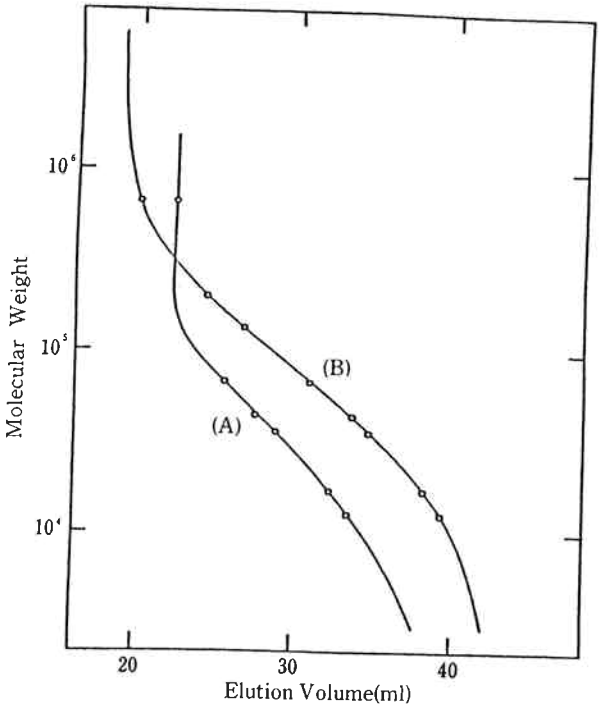
[2] Calibration curves and average pore sizes

Fig. 1 and 2 show the calibration curves of TSKgel G2000SW and G3000SW for proteins[2] and TSKgel G2000PW, G3000PW and G5000PW for polysaccharide and oligosaccharide, respectively[21]. The former indicates that the SW columns have perfectly fitting pore size and pore distribution for the separation of proteins.

Table 7 Specifications of TSK Columns for Preparative HPLC

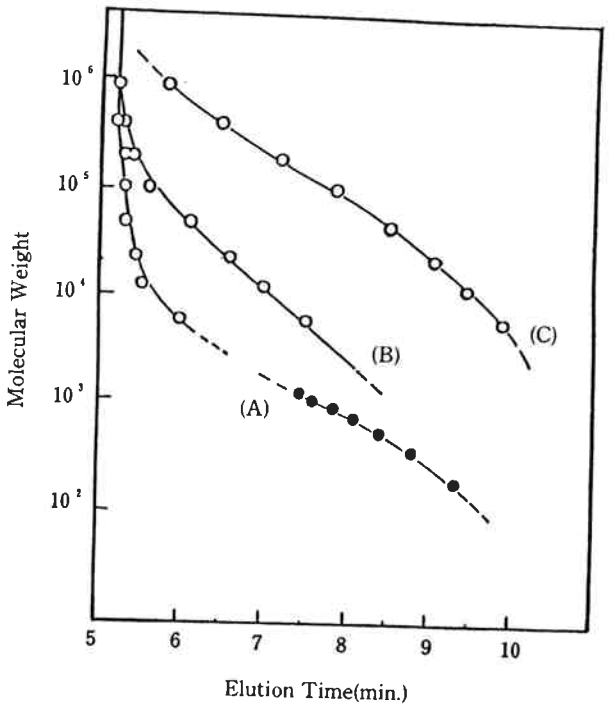
Type	Column Dimmension (mm I.D.×mm)	Particle Size (micron)	Theoretical Plates per Column	Flow Rate (m*l*/min) Suitable	Flow Rate (m*l*/min) Maximum	Maximum Pressure (kg/cm²)
TSKgel G2000SW	21.5×300	13	5,000/EG	4–6	8	10
	21.5×600	13	10,000/EG	4–6	8	15
	55.0×600	20	700/BSA	15–25	50	15
	108.0×600	20	700/BSA	50–100	150	15
TSKgel G3000SW	21.5×300	13	5,000/EG	4–6	8	15
	21.5×600	13	10,000/EG	4–6	8	20
	55.0×600	20	1,800/BSA	15–25	50	15
	108.0×600	20	1,800/BSA	50–100	150	15
TSKgel G2000PW	21.5×600	17	10,000/EG	4–6	8	10
	55.0×600					
	108.0×600					
TSKgel G3000PW	21.5×600	17	10,000/EG	4–6	8	15
	55.0×600					
	108.0×600					
TSKgel G5000PW	21.5×600	22	6,000/EG	4–6	8	5
	55.0×600					
	108.0×600					
TSKgel DEAE-5PW	21.5×150	13	3,000/C5′M	4–6	8	
	55.0×200	20	1,500/C5′M	20–30	50	2
	108.0×200	20				
TSKgel SP-5PW	21.5×150	13	3,000/Cyt	4–6	8	10
	55.0×200	20	1,500/Cyt	20–30	50	3
	108.0×200	20				
TSKgel Phenyl-5PW	21.5×150	13	2,000/Acet	4–6	8	
	55.0×200	20	1,500/Acet	20–30	50	3
	108.0×200	20				
TSKgel ODS-120A TSKgel ODS-120T	21.5×150	10	8,000/Bz(T) /Ph(A)	4–6	10	
	55.0×300	20	3,000/Bz(T)	30–60	100	20
	55.0×600	20	6,000/Bz(T)	15–40	50	20
	108.0×300					
	108.0×600					

(Note) Samples for measurement of theoretical plates number:
EG ; ethylene glycol
BSA ; bovine serum albumin
C5′M ; cytidine 5′ monophosphate
Cyt ; cytidine
Acet ; acetone
Bz ; benzene
Ph ; phenol



Column : (A) TSKgel G2000SW
(B) TSKgel G3000SW
Sample : Protein
Eluent : 0.2M Phosphate buffer (pH 6.8)
Flow rate : 1.0m*l*/min.
Detector : UV 280nm
(Reproduced with permission from ref. 2, copyright 1980 Elsevier Scientific Publishing Company.)

Fig. 1 Calibration Curves of TSKgel G2000SW and G3000SW for Protein



Column : (A) TSKgel G2000PW
(B) TSKgel G3000PW
(C) TSKgel G5000PW
Sample : (○) Pullulan Standards
(●) Hydrolyzed β-Cyclodextrin
Eluent : Distilled water
Flow rate : 1.0m*l*/min
Detector : RI

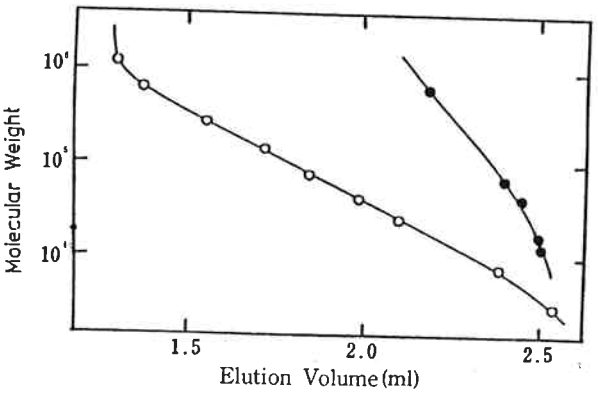
Fig. 2 Calibration Curves of TSKgel G2000PW, G3000PW and G5000PW for Pullulan

Fig. 3 shows the calibration curve of TSKgel DEAE-5PW for proteins and poly (ethylene oxide)[24], indicating that the pore size is so large that even large proteins like thyroglobulin can easily penetrate into the pore. Other G5000PW gel based columns such as TSKgel SP-5PW and Phenyl-5PW have the same calibration curves.

Average pore sizes are listed in Table 4. TSKgel ODS-120A & T have so large pore size as universal ODS columns to cover even small proteins.

[3] Mechanical stability of TSK preparative HPLC columns

Figs. 4 to **7** show the flow rate dependence of pressure drop on TSKgel SW, PW, DEAE-5PW, SP-5PW, Phenyl-5PW, and ODS-120T



Column : TSKgel DEAE-5PW,
7.5mm I.D. × 75mm
Sample : (●) Proteins
(○) Polyethylene glycols
Eluent : 0.1M Phosphate buffer(pH5.5) + 0.5M NaCl and Distilled water
Flow rate : 1.0m*l*/min.
Detector : RI and UV 280nm
(Reproduced with permission from ref. 24, copyright 1983 Elsevier Science Publishers B.V.)

Fig. 3 Plots of Molecular Weights against Elution Volumes for Proteins and Polyethylene Glycols on TSKgel DEAE-5PW

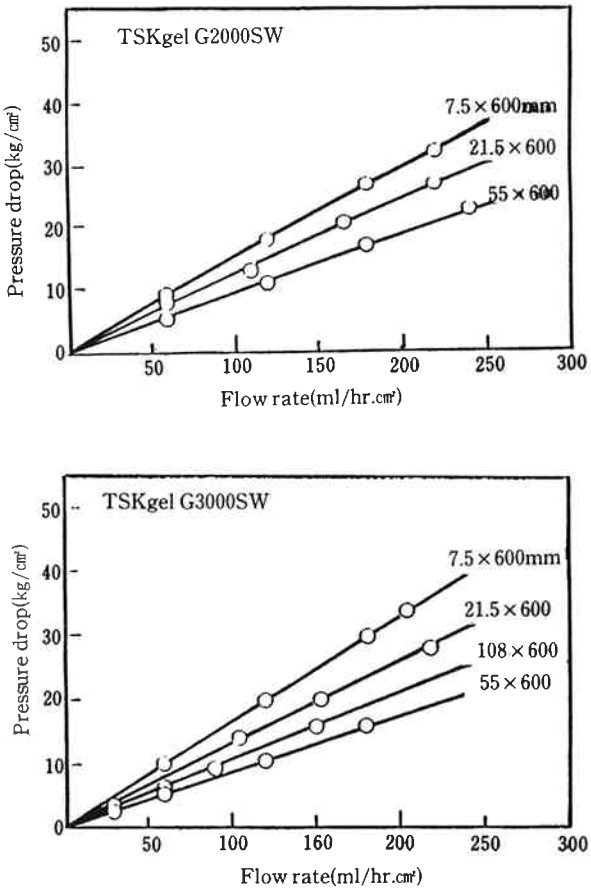


Fig. 4 Flow Rate Dependence of Pressure Drop on TSKgel G2000SW and G3000SW Columns.

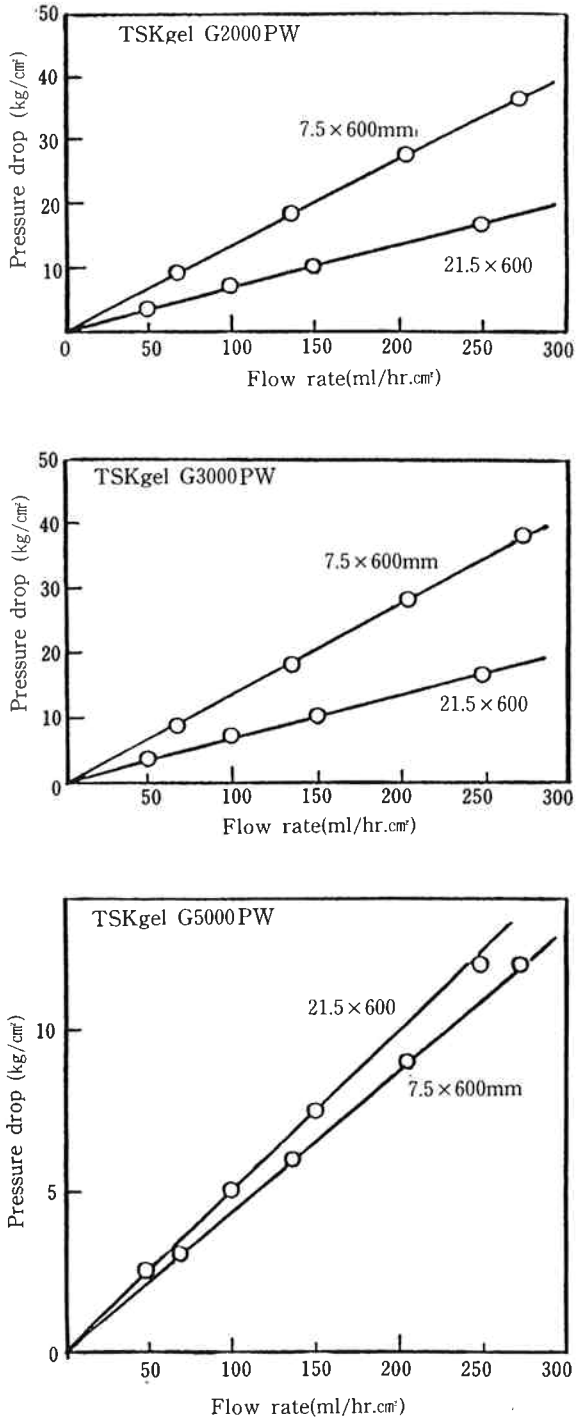


Fig. 5 Flow Rate Dependence of Pressure Drop on TSKgel G2000PW, G3000PW and G5000PW Columns.

columns, respectively. Excellent lineality indicates the mechanical stability of these columns.

[4] Chemical stability of sorbents

Table 8 shows the stability of TSKgel DEAE-5PW, SP-5PW and Phenyl-5PW against strong alkaline and acidic solutions. Ionic capacities or the amount of phenyl group of these sorbents were checked before and after the exposure to the solution and no change was observed.

[5] Recovery of proteins and their activity

The loss of recovery is mainly caused by the following three factors:

a) Adsorption to the sorbent: to avoid this factor TSKgels are designed to be sufficiently hydrophilic with inert hydroxyl groups and ether bonding.

b) Spontaneous denaturation during the purification process: high speed operation of HPLC is very effective to improve the recovery yield of the activity of labile biopolymers.

c) Peak overlapping: the HPLC method is obviously the best to obtain the highest yield

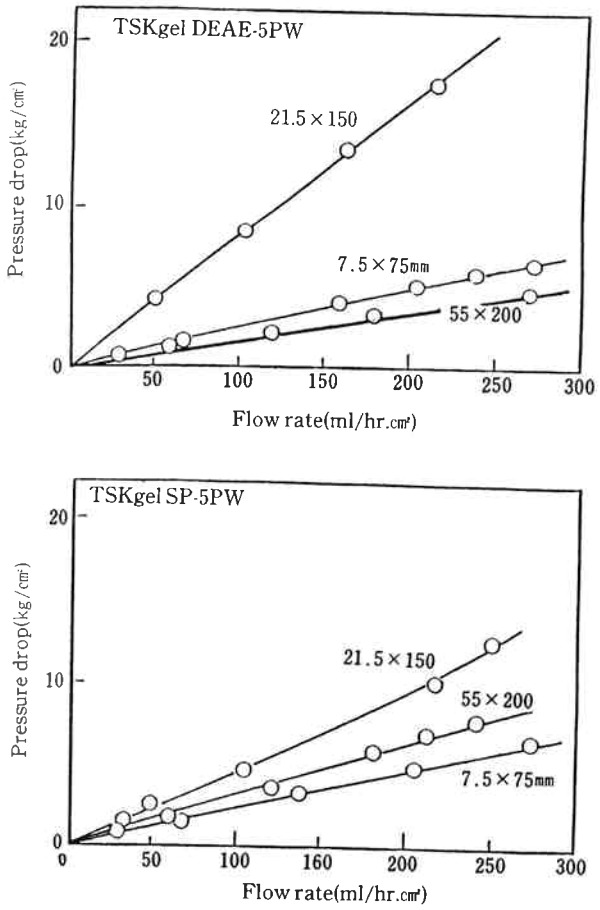


Fig. 6 Flow Rate Dependence of Pressure Drop on TSKgel DEAE-5PW and SP-5PW Columns.

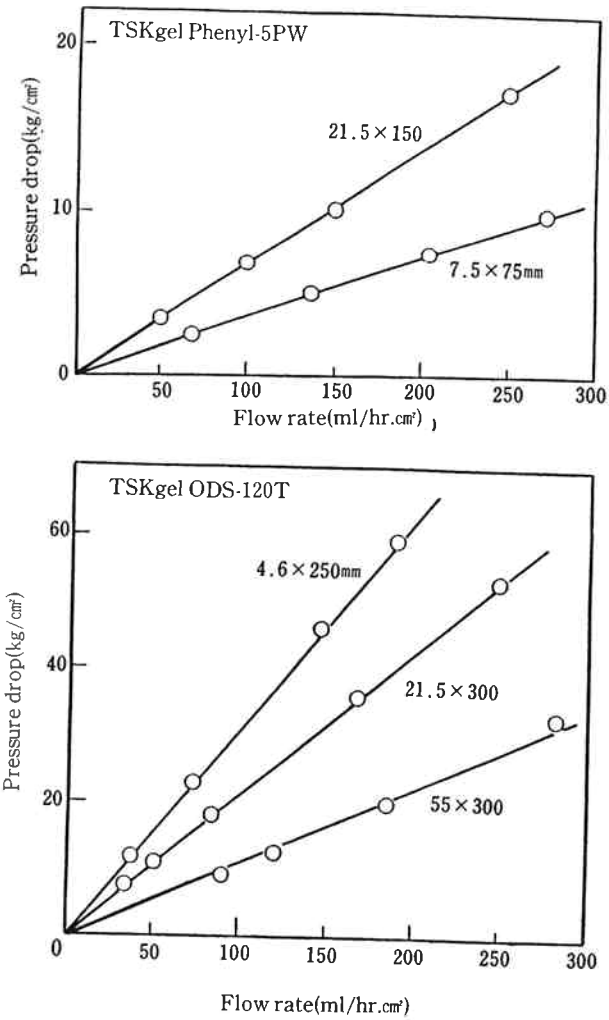


Fig. 7 Flow Rate Dependence of Pressure Drop on TSKgel Phenyl-5PW and ODS-120T Columns.

Table 8 Stability of TSKgel DEAE-5PW, SP-5PW and Phenyl-5PW Against Strong Alkaline and Acidic Solution

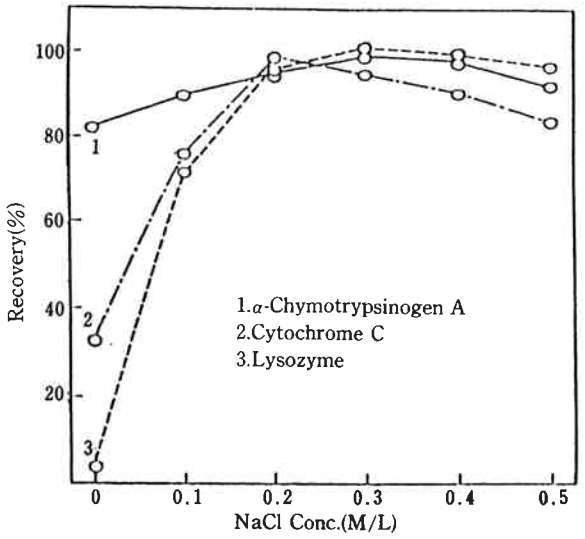
Ion-exchanger	Solution	Ion-exchange capacity (meq/m*l*)	
		Before exposure	After exposure
DEAE-5PW	0.5 N NaOH	0.09	0.09
DEAE-5PW	0.5 N HCl	0.09	0.09
DEAE-5PW	20% CH_3COOH	0.09	0.08
SP-5PW	0.5 N NaOH	0.13	0.13
SP-5PW	0.5 N HCl	0.13	0.13
SP-5PW	20% CH_3COOH	0.13	0.13
		Phenyl group content (mmol/m*l*)	
		Before exposure	After exposure
Phenyl-5PW	0.5 N NaOH	0.105	0.104
Phenyl-5PW	20% CH_3COOH	0.105	0.106

Samples were exposed in alkaline or acid solution for 10 days at 25°C

Table 9 Recovery of Proteins from TSKgel G3000SW

	pH=6.9			pH=7.8
	0.2M KH_2PO_4 +0.2M K_2HPO_4	0.2M NaH_2PO_4 +0.2M Na_2HPO_4	0.05M PB +0.2M NaCl	0.05M Tris-HCl +0.2M NaCl
Lysozyme	96%	92%	94%	75%
Cytochrome C	101	98	98	92
α-Chymotrypsinogen A	98	95	92	90
γ-Globulin	98	95	98	88
Thyroglobulin	94	94	87	85
Ovalbumin	92	96	68	66

Elution condition
Column : TSKgel G3000SW, 7.5mm I.D.×600mm
Load : Concentration 0.3–0.5%
Injection volume 200μl
Flow rate : 1.0ml/min.
Detector : UV 280nm



Column : TSKgel G3000SW
Eluent : 0.05M Phosphate buffer (pH6.9)+NaCl
Load : 60μg

Fig. 8 Effect of Salt Concentration on Recovery

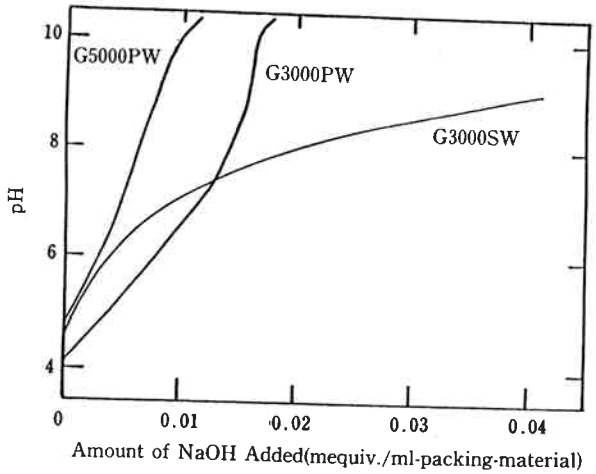
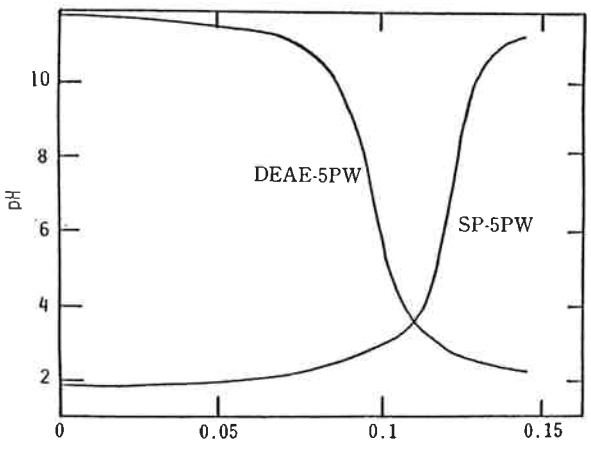


Fig. 9 Titration Curve of TSKgel G3000PW, G5000PW and G3000SW



(Reproduced with permission from ref. 24, copyright 1983 and ref. 26, copyright 1984 Elsevier Science Publishers B.V.)

Fig. 10 Titration Curve of TSKgel DEAE-5PW and SP-5PW

by avoiding peak overlapping owing to the high resolution.

The following data shows that unfavourable adsorption of these proteins is not observed on TSK columns.

Table 9 shows the recovery yields of proteins on TSKgel G3000SW column with several typical eluents[14]. **Fig. 8** shows the effect of salt concentration on the recovery yield of basic proteins on TSKgel G3000SW column[14]. **Table 10** shows the recovery yields of

Table 10 Recovery of Proteins from TSKgel DEAE-5PW, SP-5PW and Phenyl-5PW

DEAE-5PW

Protein	Recovery (%)
Thyroglobulin	98
Ferritin	99
γ-Globulin	100
Bovine serum albumin	102
Hemoglobin	96
Ovalbumin	104
β-Lactoglobulin	103
Trypsin inhibitor	104
Myoglobin	103

Each protein of 0.4mg was applied to DEAE-5PW column (75×7.5mm I.D.) in 0.02 M tris-HCl bufferr (pH 8.5) and the adsorbed protein was desorbed in 0.02M tris-HCl buffer (pH 8.5) containing 0.5M NaCl.

SP-5PW

Protein	Recovery (%)
γ-Globulin	98
Hemoglobin	96
Trypsinogen	101
α-Chymotrypsinogen A	98
α-Chymotrypsin	104
Myoglobin	88
Lysozyme	95
Ribonuclease A	100
Cytochrome C	103

Each protein of 0.4mg was applied to SP-5PW column (75×7.5mm I.D.) in 0.02 M phosphate buffer (pH 6.0) and the adsorbed protein was desorbed in 0.02 M phosphate buffer (pH 6.0) containing 0.5M NaCl.

Phenyl-5PW

Protein	Recovery (%)
Bovine serum albumin	102
Ovalbumin	111
α-Chymotrypsinogen	99
α-Chymotrypsin	109
Myoglobin	101
Lysozyme	92
Rlbonuclease	104
Cytochrome C	106

Proteins were adsorced in 0.1M phosphate buffer containing 2 M ammonium sulfate (pH 7.0) and desorbed in 0.1 M phosphate buffer (pH 7.0) at 25°C.
Proteins of 0.5 mg each were applied to the column of 75×7.5mm I.D.

Recovery of Enzymatic Activity from TSKgel DEAE-5PW

Enzyme	Recovery (%)
Catalase	80
Lipoxidase	95
α-Chymotrypsin	93

Recovery of Enzymatic Activity from TSKgel SP-5PW

Enzyme	Recovery (%)
Lipoxidase	84
α-Chymotrypsin	97
β-Amylase	88

Recovery of Enzymatic Activity from TSKgel Phenyl-5PW

Enzyme	Recovery (%)
α-Chymotrypsin (0.4mg)	92
Lipoxidase (1.0mg)	89
Ferredoxin NADP reductase (3.0mg)	100
β-Amylase (1.3mg)	80
Lysozyme (0.2mg)	90
Lysozyme (0.05mg)	90