Preparative HPLC Using TSK Columns in the Purification of Biochemical Products

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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

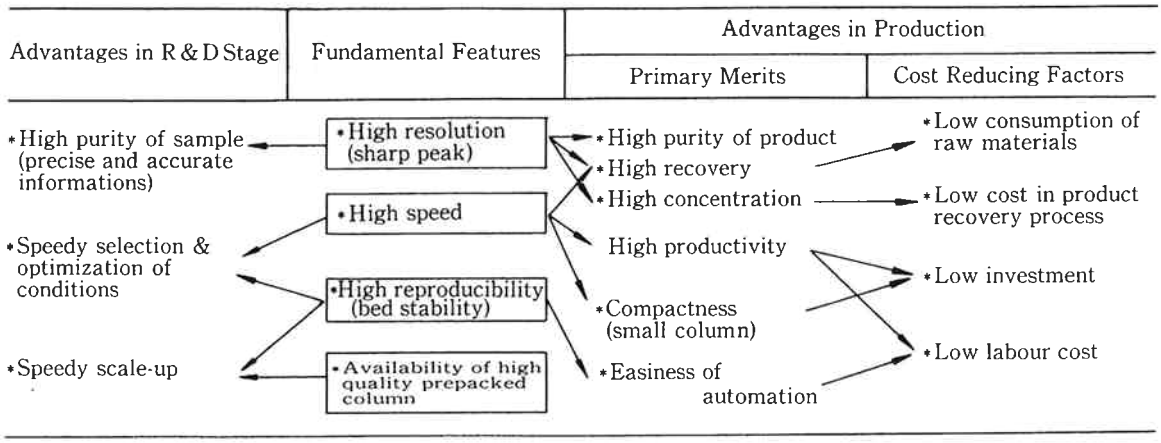


Table 3 Typical Example of Large Scale Production by HPLC

* 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.

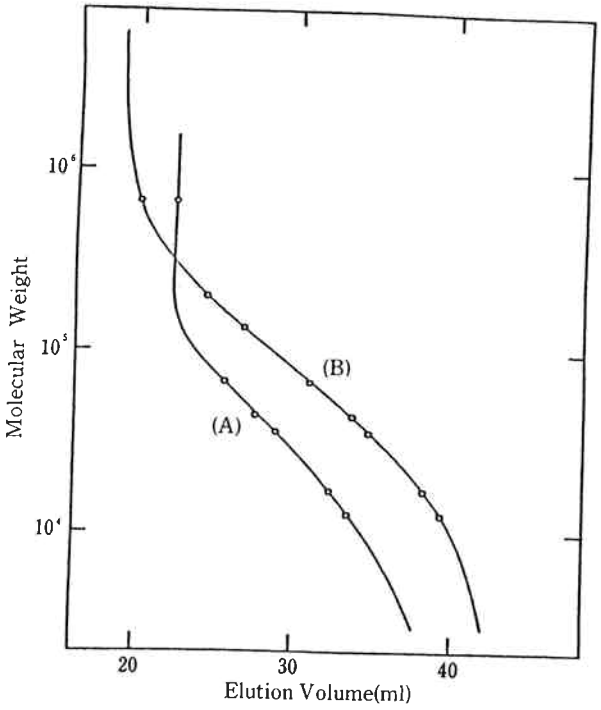
[2] Calibration curves and average pore sizes

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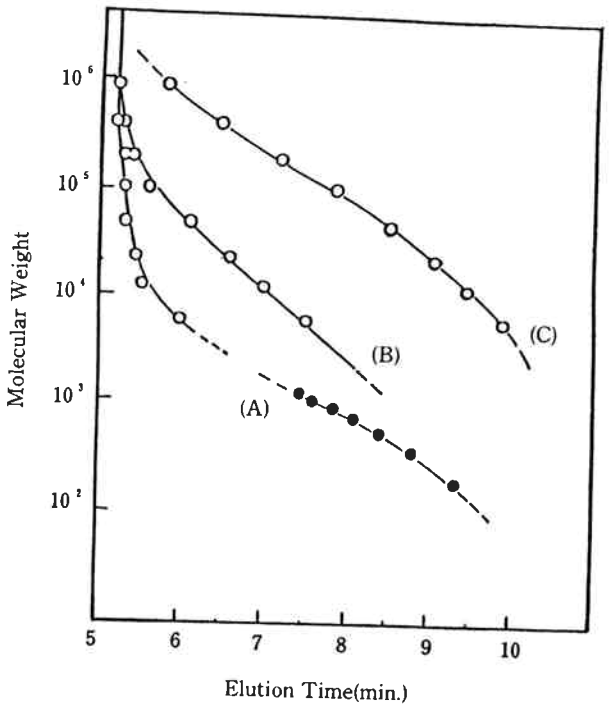
Fig. 1 Calibration Curves of TSKgel G2000SW and G3000SW for Protein



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

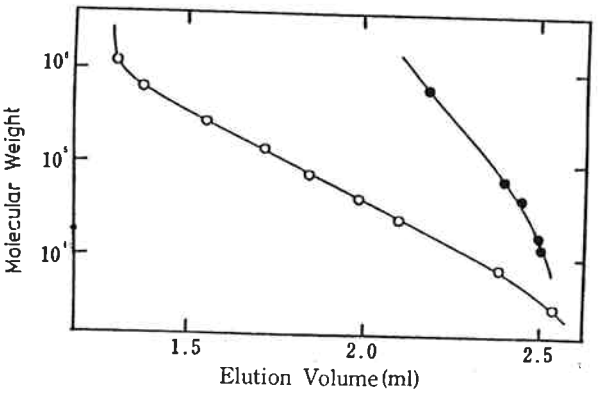
Fig. 2 Calibration Curves of TSKgel G2000PW, G3000PW and G5000PW for Pullulan

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

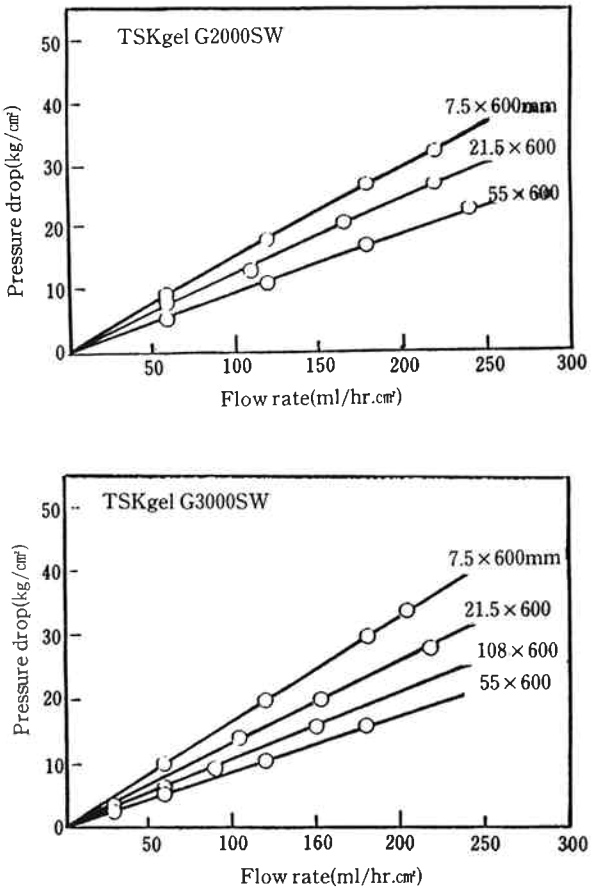


Fig. 4 Flow Rate Dependence of Pressure Drop on TSKgel G2000SW and G3000SW Columns.

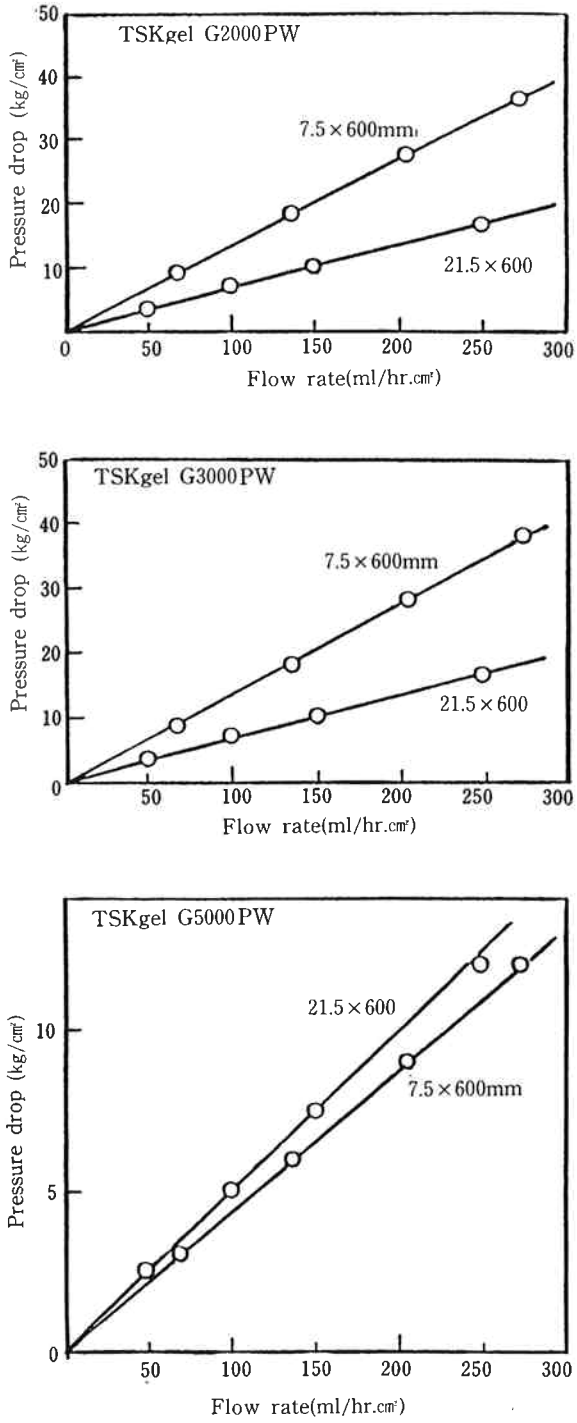


Fig. 5 Flow Rate Dependence of Pressure Drop on TSKgel G2000PW, G3000PW and G5000PW Columns.

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

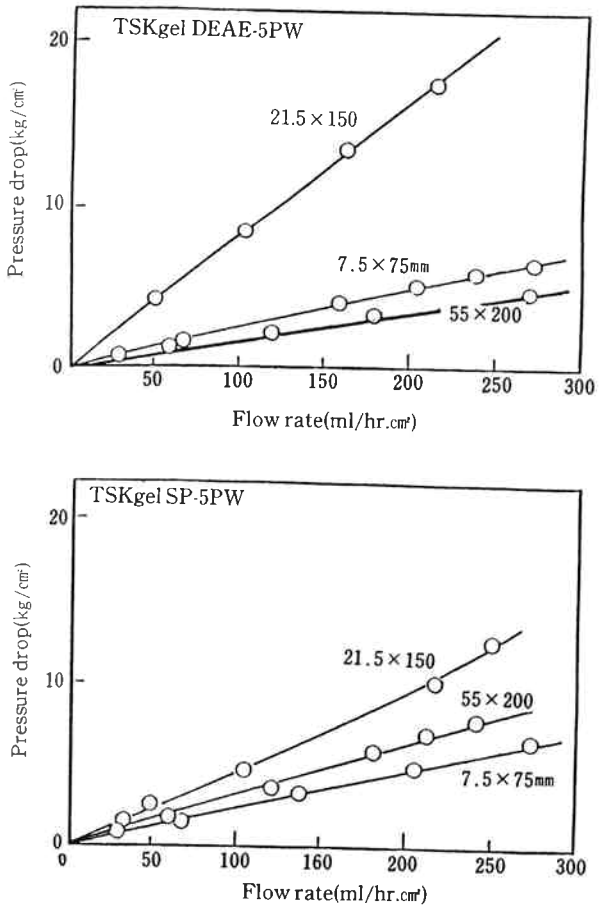


Fig. 6 Flow Rate Dependence of Pressure Drop on TSKgel DEAE-5PW and SP-5PW Columns.

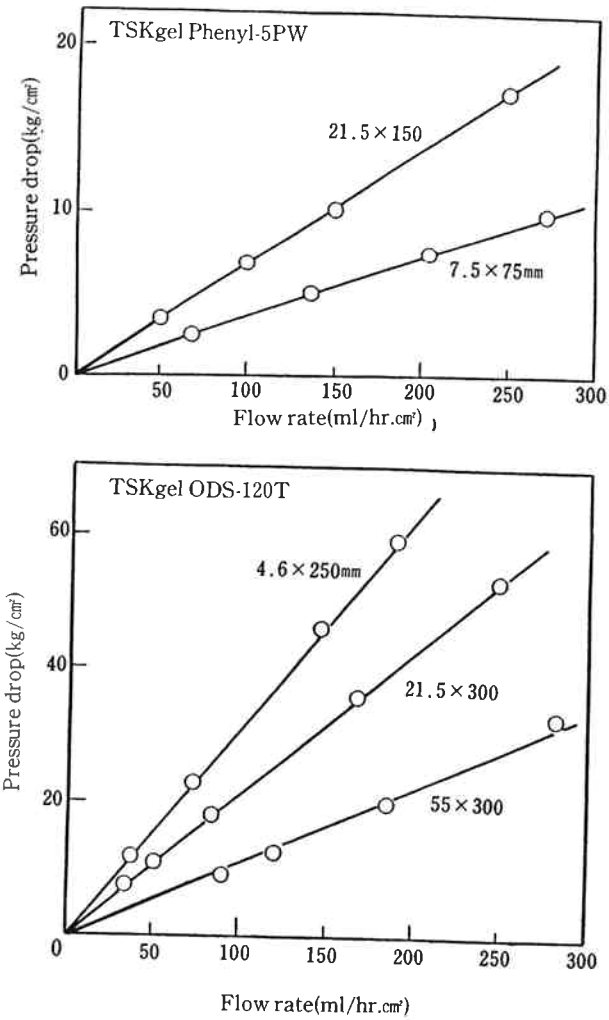


Fig. 7 Flow Rate Dependence of Pressure Drop on TSKgel Phenyl-5PW and ODS-120T Columns.

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

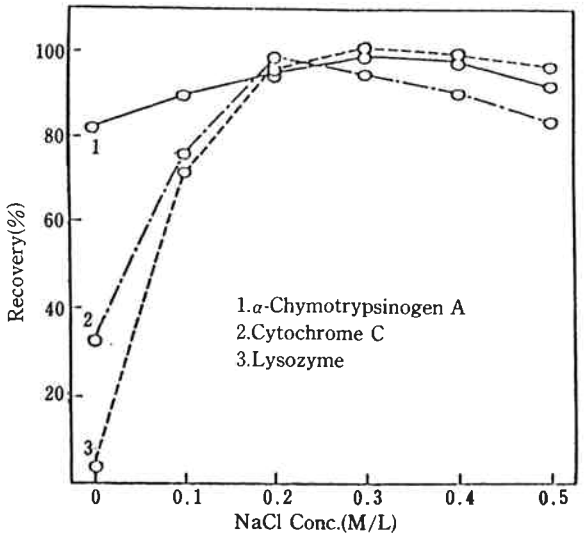
Ion-exchanger	Solution	Ion-exchange capacity (meq/m*l*)	
		Before exposure	After exposure
DEAE-5PW	0.5 N NaOH	0.09	0.09
DEAE-5PW	0.5 N HCl	0.09	0.09
DEAE-5PW	20% CH_3COOH	0.09	0.08
SP-5PW	0.5 N NaOH	0.13	0.13
SP-5PW	0.5 N HCl	0.13	0.13
SP-5PW	20% CH_3COOH	0.13	0.13
		Phenyl group content (mmol/m*l*)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

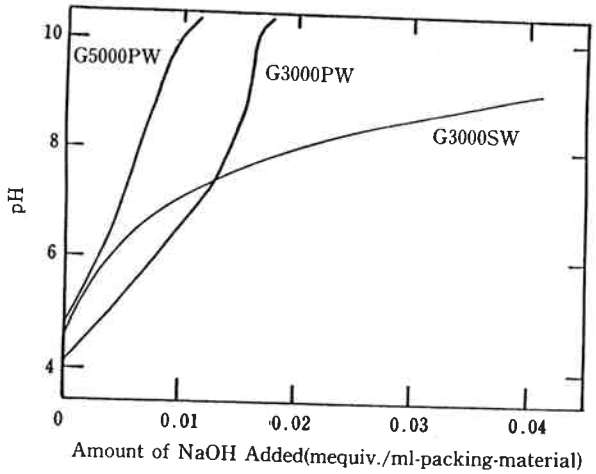


Fig. 9 Titration Curve of TSKgel G3000PW, G5000PW and G3000SW

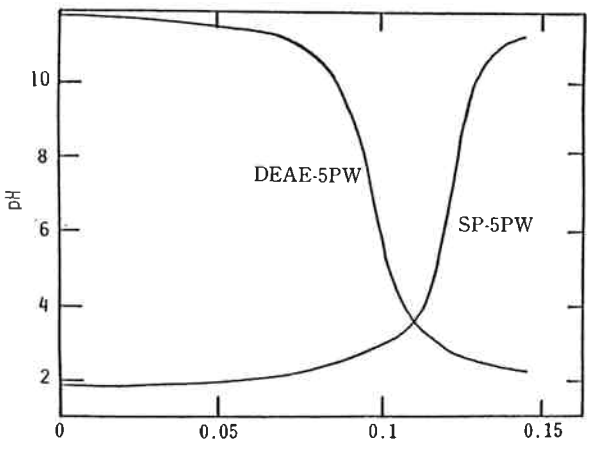


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Fig. 10 Titration Curve of TSKgel DEAE-5PW and SP-5PW

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

proteins and their activity on TSKgel DEAE-5PW[24], SP-5PW[26], and Phenyl-5PW[29,30].

〔6〕 Titration curves of sorbents

Fig. 9 shows the titration curves of TSKgel G3000SW and TSKgel G3000PW & G5000PW. The residual silanol groups are responsible for the rather large amount of weakly anionic groups of G3000SW, while the residual carboxyl groups are responsible for the small amount of weakly anionic groups of G3000PW and G5000PW[21]. The low recovery yield of proteins at the low salt concentrations on TSKgel G3000SW in Fig. 8 is caused by the ionic interaction between proteins and silanol groups.

Fig. 10 shows the titration curves of TSKgel DEAE-5PW[24] and SP-5PW[26], featured by the sharpness indicating the uniformity of ionic groups, and high pka of DEAE and low pka of SP covering wide range of applicability of pH.

5. Sample loading capacity

Sample loading capacity is roughly proportional to the cross section area of the column. As descriced in the section 3. 2, the loading capacity of the TSK preparative columns is usually larger than that of the corresponding analytical columns owing to the larger particle size.

Sample loading capacity is usually checked by investigating the effect of the loading amount on one of the factors such as theoretical plates number, HETP (height equivalent to theoretical plate), peak width and resolution between two peaks. Normally these factors are kept constant against the increase of sample loading before the beginning of overloading. It may be said that the maximum loading capacity is around the value at the point where one of the factors mentioned above begins to deviate from a horizontal line. However, it shoud be noted that there is no universal definite maximum loading capacity in practical applications because it is highly dependent on the purity of the product to meet the specification. Even the conditions where clear overloading occurs can be adopted so long as the purity is satisfied.

A rough idea of maximum loading capacity for proteins is presented in Table 11.

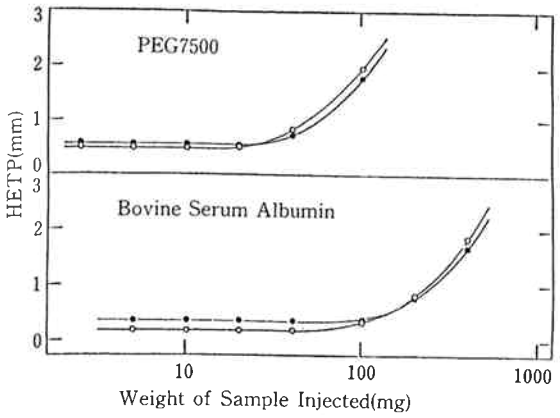
〔1〕 GFC

The loading capacity in GFC is influenced by the following factors:

a) molecular weight of a sample
b) viscosity of a sample solution
c) volume of a sample solution
d) concentration of a sample
e) viscosity of an eluent etc.

Table 11 Relationship between Column Diameter and Sample Loading Capacity

Column diameter (mm)	7.5	21.5	55.0	108
Cross section area (cm^2)	0.442	3.63	23.7	91.6
Cross section area ratio	1	8.2 1	53.7 6.5 1	207 25.2 3.86
Maximum loading capacity (mg)	20	300	3,000	12,000

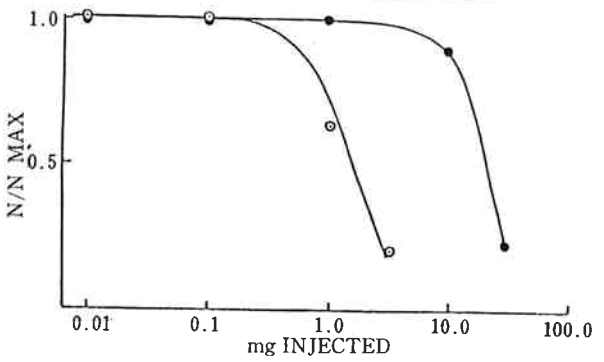


Flow rate dependence of maxinum sample loading on TSKgel G3000SWG
Column size : 21.5mm I.D.×1200mm
Injection volume : 4m*l*
Sample : Bovine Serum Albumin, PEG 7500
Eluent : 0.1M phosphate buffer containing 0.3M NaCl (pH 7.0)
Flow rate : (○) 4m*l*/min., (●) 16m*l*/min.
Temperature : 25°C
Detector : RI

Fig. 11 Comparison of the Dependence of HETP on Sample Loading between PEG 7500 and Bovine Serum Albumin on TSKgel G3000 SW

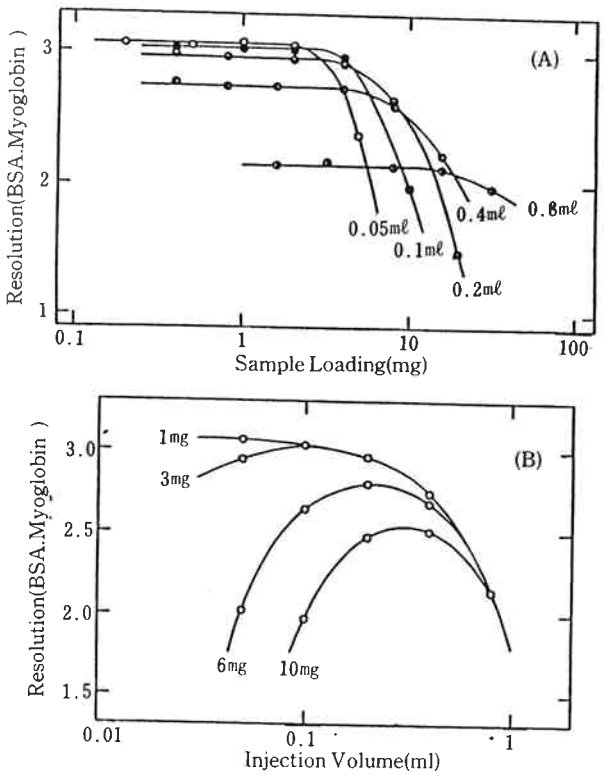
Fig. 11 shows the comparison of the dependence of HETP on sample loading between PEG 7500 and bovine serum albumin on TSKgel G3000SW (21.5mm I.D.). Despite of the larger molecular weight, BSA shows higher loading capacity than PEG 7500. This is probably due to the higher viscosity of the PEG solution because of its larger molecular size in water. In Fig. 11, it is also shown that the loading capacity is scarcely influenced by flow rate.

Wher et al[16] investigated the sample loading effect in the preparative separation of HDL apolipoproteins on TSKgel G3000SW (21.5×300mm) using 6 mole urea solution as an eluent. **Fig. 12** shows the dependence of theoretical plates number on the loading amount. Only a few tens of mg were the maximum loading capacity in this case probably due to the high viscosity of the specific eluent.



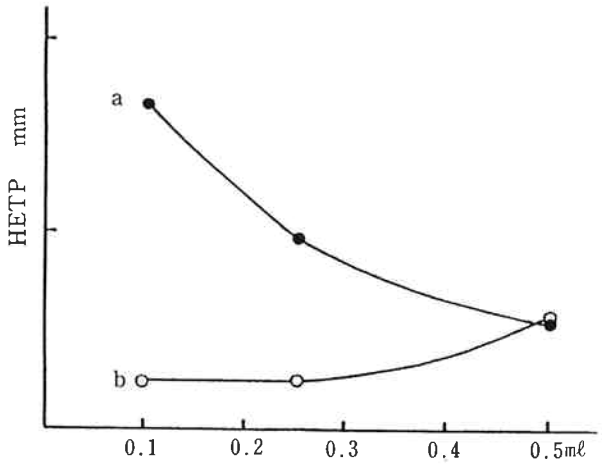
Column : (○) TSKgel G3000SW, 7.5×300mm
(●) TSKgel G3000SW, 21.5×300mm
Sample : Ovalbumin
Eluent : 0.1M potassium phosphate+0.1M KCl (pH 6.8)
Flow rate : (○) 1.0m*l*/min., (●) 8.0m*l*/min.
Injection volume : (○) 0.1m*l*, (●) 1.0m*l*
(Reproduced with permission from ref. 16, copyright 1982 by Academic Press, Inc.)

Fig. 12 Effect of Sample Load on Column Efficiency



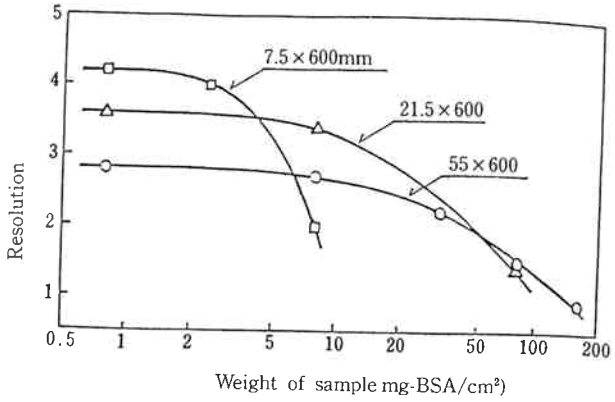
A) : Sample loading with constant injection volume
B) : Injection volume with constant sample loading
Column : TSKgel G3000SW, 7.5×600mm
Eluent : 0.1M phosphate buffer containing 0.1 M Na_2SO_4 (pH 7.0)
Flow rate : 1.0m*l*/min.
(Reproduced with permission from ref. 8, copyright 1982 Dr. Alfred Huethig Publishers.)

Fig. 13 Dependence of Resolution on Sample Loading and Injection Volume on TSKgel G3000SW



Column : TSKgel G3000SW
Eluent : 0.1M phosphate buffer+0.2M NaCl (pH 7.0)
Flow rate : 1.0m*l*/min.
Injection volume : (a) 5mg, (b) 0.5mg
(Reproduced with permission from ref. 15,)

Fig. 14 Effect of Sample Volume and Sample Concentration on HETP



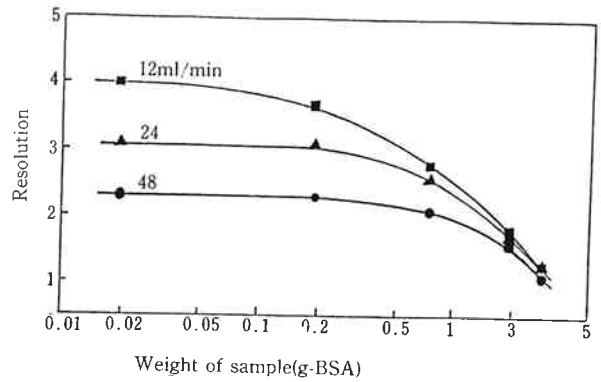
Column : TSKgel G3000SW
Solvent : 0.1M Phosphate buffer (pH 6.8) containing 0.1M NaCl
Flow rate : 120m*l*/hr. cm²
Temperature : ambient
Column dimension : □; 7.5×600mm(10μm), △; 21.5×600mm(13μm), ○; 55×600mm(20μm)
Resolution between bovine serum albumin and myoglobin

Fig. 15 Effect of Sample Loading and Column Dimmension on Resolution

Fig. 13 shows that the dependence of the resolution between two proteins on sample loading and injection volume on a TSKgel G3000SW analytical column[8].

Horio et al also investigated the loading capacity of TSKgel G3000SW, as shown in **Fig. 14**, indicating that both the concetration and the volume of the sample gives influence to HETP[15].

Fig. 15 shows the comparison of the dependence of the resolution between BSA and myoglobin on sample loading among TSKgel SW columns including an analytical and preparative columns. The larger the diameter, the smaller the decrease of the resolution against increase of the loading probably due to larger particle size.



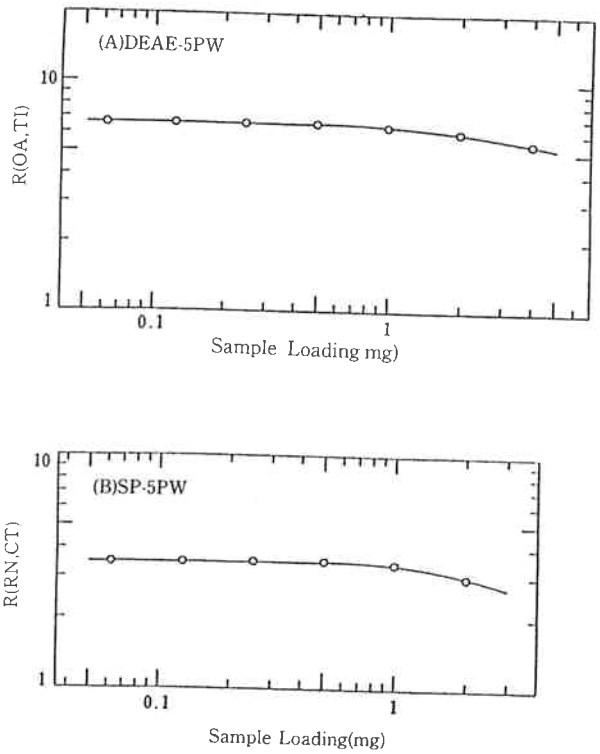
Column : TSKgel G3000SW (55×600mm)
Eluent : 0.1M phosphate buffer (pH 6.8) containing 0.1M NaCl
Temperature : 4°C
Flpw rate : ■; 12m*l*/min., ▲; 24m*l*/min., ●; 48m*l*/min.
Resolution between bovine serum albumin and myoglobin

Fig. 16 Effect of Flow Rate on the Sample Loading on TSKgel G3000SW

Fig. 16 shows the effect of flow rate on the sample loading on TSKgel G3000SW (55mm I.D.). Although lower flow rate gives higher resolution, the more rapid decrease of the resolution against the increase of the loading is observed under lower flow rate.

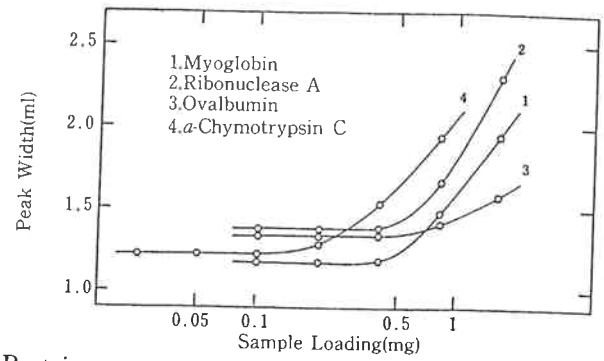
[2] IEC and HIC

The biggest difference between IEC (or HIC) and GFC regarding sample loading may be the effect of sample volume. In IEC loading capacity is almost independent on sample volume



Resolution: (A) Resolution between ovalbumin and trypsin inhibitor
(B) Resolution between ribonuclease A and α-chymotrypsinogen A
(Reproduced with permission from ref. 24, copyright 1983 and ref. 26, copyright 1984 Elsevier Science Publishers B.V.)

Fig. 17 Dependence of the Resolution between Two Proteins on Sample Loading on TSKgel DEAE-5PW and SP-5PW



Proteins were separated with 60min linear gradient of decreasing ammonium sulfate concentration from 1.5M to 0 in 0.1M phosphate buffer (pH 7.0) at a flow rate of 0.5m*l*/min.
(Reproduced with permission from ref. 29, copyright 1984 Elsevier Science Publishers B.V.)

Fig. 18 Dependence of Peak Width on Sample Loading on TSKgel Phenyl-5PW

if the salt concentration of a sample solution is sufficiently low (or high), while in GFC seriously dependent.

Fig. 17 shows the dependence of the resolution between two proteins on sample loading on TSKgel DEAE- & SP-5PW analytical columns, respectively[24,26].

It can be said that the decrease of the resolution due to overloading occurs very gradually compared with in GFC.

Fig. 18 shows the effect of the loading of four proteins on TSKgel Phenyl-5PW[29].

6. Column selection

[1] Selection according to sample

Table 12 shows a rough idea for the selection of the best TSK column for preparative HPLC according to a sample. Regarding the column selection in GFC a detailed description was presented in reference 21.

[2] Selection of separation mode

In the conventional LPLC using soft gels, IEC has played unparalleledly important role in the industrial scale purification of biopolymers probably due to higher resoluving power, lower price of sorbents, unnecessariness of long column, etc. IEC will keep its position as the main technique in future, too. However, it should be noted that the handicaps of GFC and HIC compared with IEC are reduced to a great extent in HPLC. Now it is recommended to select the best separation mode by taking various advantages and disadvantages of each separation mode into account sufficiently.

Table 12 Column Selection Guide according to Sample

Sample	Separation Mode	Column	Molecular Size
Protein	GFC	TSKgel G2000SW TSKgel G3000SW TSKgel G5000PW	small (—ca. 20,000) medium (—ca. 500,000) large (—ca. 5,000,000)
	IEC	TSKgel DEAE- & SP-5PW	
	HIC	TSKgel Phenyl-5PW	
	RPC	TSKgel ODS-120T	small (—ca. 20,000)
Peptide	GFC	TSKgel G2000SW TSKgel G3000PW	
	IEC	TSKgel DEAE- & SP-5PW	
	RPC	TSKgel ODS-120T	
Nucleic acid * DNA fragments	GFC	TSKgel G5000PW TSKgel G3000SW TSKgel G2000SW	large (—ca. 1,000 base pair) small (—ca. 160 base pair) small (—ca. 80 base pair)
	IEC	TSKgel DEAE- & SP-5PW	
* RNA	GFC	TSKgel G3000SW	
	IEC	TSKgel DEAE- & SP-5PW	
	HIC	TSKgel Phenyl-5PW	
* Oligonucleotide	GFC	TSKgel G2000SW	
	IEC	TSKgel DEAE- & SP-5PW	
	RPC	TSKgel ODS-120T	
Carbohydrate * Polysaccharide	GFC	TSKgel G5000 & G3000PW	
* Oligosaccharide	GFC	TSKgel G2000SW TSKgel G2000PW	(—ca. dp 50) (—ca. dp 15)

Advantages and disadvantages of separation modes are summarized as follows.

GFC is featured by simple elution condition, resulting in several advantages such as possibility of very speedy establishment of the process, low investment in solvent delivery system, easiness of automation and maintenance, possibility of solvent recycle, etc. Another advantages is the smallest possibility of loss of recovery because unfavourable ionic and hydrophobic properties of sorbents are minimized.

Therefore new high performance GFC is an attractive technique for commercial process, although it is handicapped in several points such as limited resoluving power, a little bit smaller loading capacity per unit column volume, difficulty of clean-up by alkaline washing (as for silica based sorbent), etc. It should be noted that the relative importance of GFC is greatly enhaunced in comparison with the situation in the conventional LPLC, where the softness of sorbents definitely handicaps GFC in the very low productivity and difficulty of scale-up because of the necessity of long column length.

IEC and HIC are featured by high resoluving power, easiness of column regeneration by alkaline washing, possibility of sample concentration, independece of sample loading on sample volume, etc. Besides, loading capacity is rather high and recovery is usually satisfactory. Thus, both IEC and HIC are quite suitable for commercial process. The only one demerit of

these modes is the necessity of change of eluents, resulting in several disadvantages just in opposite way as for GFC.

[3] Combination of separation mode

It is common to use multiple columns of different separation mode for the purification of bioproducts. There may be two typical cases as follows:

a) Ionic strength of the sample solution is low:

IEC ⟶ HIC ⟶ GFC

* It is recommended to use GFC in the final step to protect the silica based sorbent from deterioration and to reduce the column dimmension as much as possible.
* IEC can be used owing to the low salt concentration.

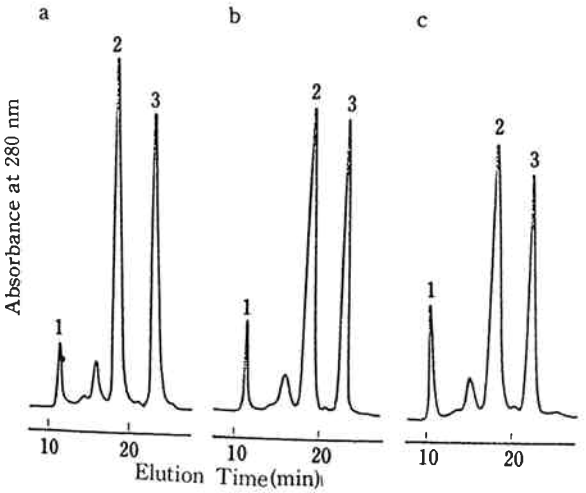
b) Ionic strength of the sample solution is high;

HIC ⟶ IEC ⟶ GFC

7. Typical examples

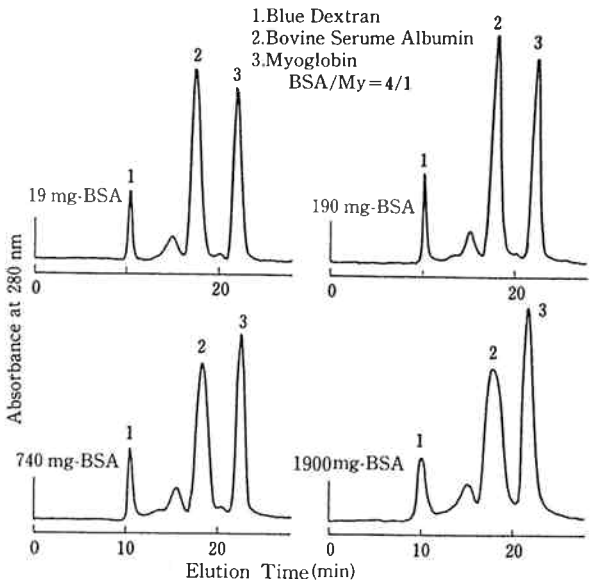
[1] GFC

Fig. 19 shows a comparison among an analytical column and preparative columns in the separation of a protein mixture on TSKgel G3000SW. Nearly equal resolutions were obtained under the same linear velocity. **Fig. 20** shows the effect of sample loading on chromatograms of the protein mixture on TSKgel G3000SW preparative column (55mm I.D.). Around one gram of bovine serum albumin can be applied without serious decrease of resolution.



Column size: a. 7.5mm I.D. × 600mm, b. 21.5mm I.D. × 600mm, c. 55mm I.D. × 600mm; Eluent: 0.1 M Phosphate buffer (pH 6.8) + 0.1M NaCl; Flow rate: a. 122ml/hr·cm²(0.9ml/min.), b. 119ml/hr·cm² (7.2 ml/min.), c. 121ml/hr·cm² (48 ml/min.); Temperature: Ambient; Sample: 1. Blue dextran, 2. Bovine serume albumin, 3. Myoglobin (BSA/Myo=4/1 w/w); Sample load: 1. BSA 0.35mg, 2. BSA 29mg, 3. BSA 190mg

Fig. 19 Comparison of Resolution among an Analytical Column and Preparative Columns on TSKgel G3000SW

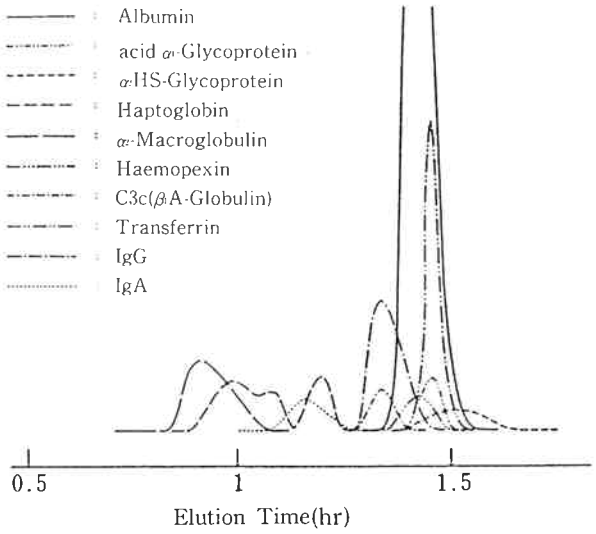


Column size: 55mm I.D. × 600mm; Eluent: 0.1M Phosphate buffer (pH 6.8) + 0.1M NaCl; Flow rate: 121ml/hr·cm² (48 ml/min.); Temperature: Ambient; Sample: 1. Blue dextran, 2. Bovine serume albumin, 3. Myoglobin (BSA/Myo=4/1 w/w)

Fig. 20 Effect of Sample Loading on the Separation of a Protein Mixture on a TSKgel G3000 SW Preparative Column

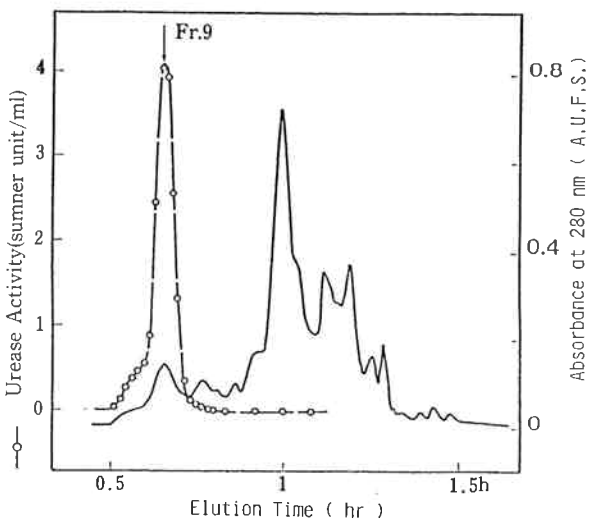
Fig. 21 shows a separation of human serum on TSKgel G3000SW (two columns of 21.5mm I.D.) monitored by quantitative immunodiffusions[7].

Fig. 22 and **23** show a pattern of commercial urease and crude β-galactosidase on TSKgel G3000SW (two columns of 21.5mm I.D.), respectively[3].



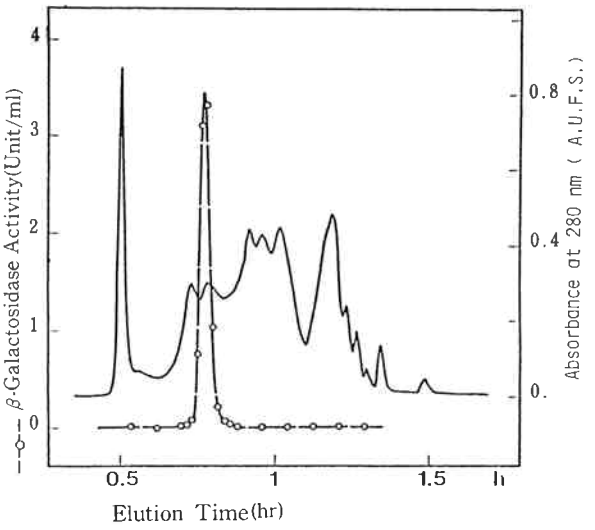
Column size : 21.5mmI.D. × 600mm × 2 ; Eluent : 0.1 M Phosphate buffer (pH7) ; Flow rate : 50m*l*/hr·cm² (3m*l*/min.) ; Sample : Standard human serum 3m*l* (1/4 dilution) ; Temperature : 22°C
(Reproduced with permission from ref. 7, copyright 1980 Dr. Alfred Hüthig Verlag.)

Fig. 21 Separation of Human Serum Proteins Monitored by Quantitative Immunodiffusions



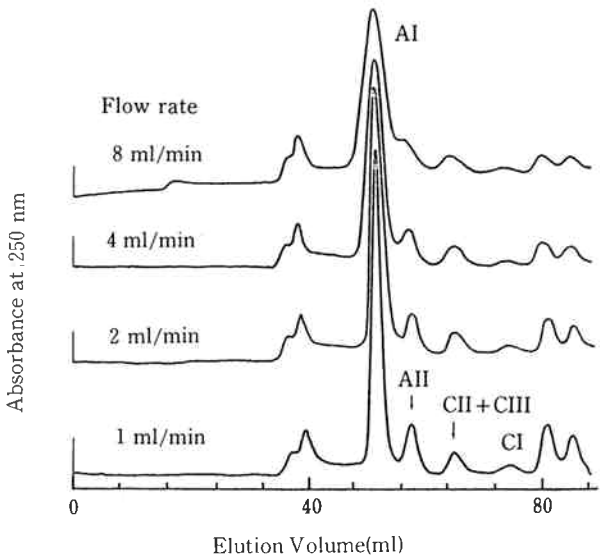
Column size : 21.5mmI.D. × 600mm × 2 ; Eluent : 0.2 M Phosphate buffer (pH 6.7) ; Flow rate : 83m*l*/hr·cm² (5m*l*/min.) ; Sample : Commercial Urease 3% (3m*l*) ; Temperature : 22°C
(Reproduced with permission from ref. 3, copyright 1980 Elsevier Scientific Publishing Company.)

Fig. 22 Purification of Commercial Urease on TSKgel G3000SWG Two-column System



Column size : 21.5mmI.D. × 600mm × 2 ; Eluent : 0.2 M Phosphate buffer (pH 6.7) ; Flow rate : 83m*l*/hr·cm² (5 m*l*/min.) ; Sample : Crude β-galactosidase 2.5% (3m*l*) ; Temperature : 22°C
(Reproduced with permission from ref. 3, copyright 1980 Elsevier scientific Publishing Company.)

Fig. 23 Purification of Crude β-galactosidase on TSKgel G3000SWG Two-column System



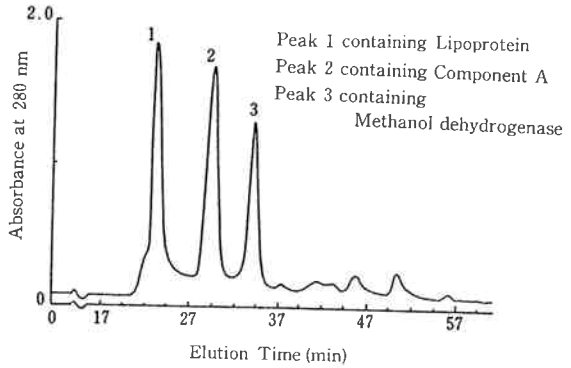
Column size : 21.5mmI.D. × 300mm ; Eluent : 50mM Tris-HCl buffer (pH7.0) + 6M Urea ; Sample : HDL apolipoproteins 1m*l* (1 mg/m*l*)
(Reproduced with permission from ref. 16, copyright 1982 by Academic Press, Inc.)

Fig. 24 Effect of Flow Rate on Separation of HDL Apolipoproteins on TSKgel G3000SWG

Fig. 24 shows the effect of flow rate on the elution pattern of HDL apolipoproteins on TSKgel G3000SW (21.5 mm I.D. × 30 cm), carried by Wher et al[16]. Serious decrease of the resolution with increasing flow rate was probably due to the high viscosity of the eluent.

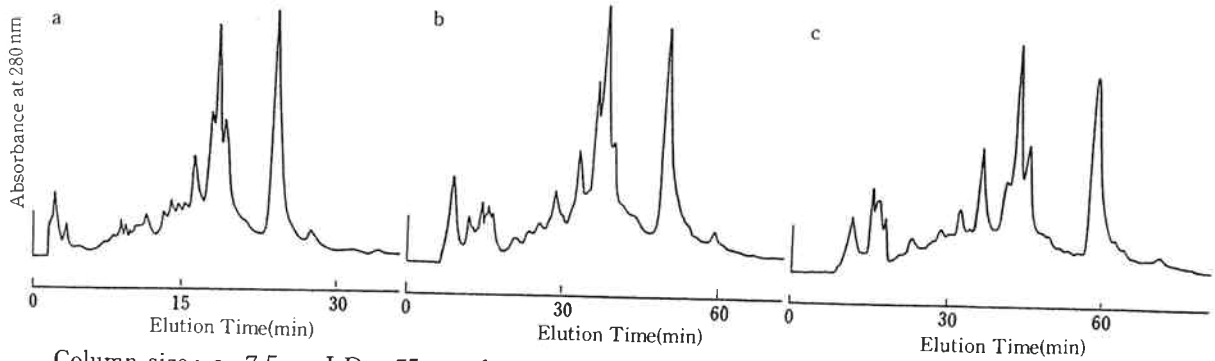
Woodland and Dalton[17] succeeded in remarkable improvement of purification of a typical labile enzyme, component A of the soluble methane monooxygenase, by preparative HPLC using TSKgel G3000SW (21.5mm I.D.). **Fig. 25** shows a typical elution pattern of a crude sample.

Himmel and Squire[20] evaluated a TSKgel G5000PW preparative column (21.5mm I.D.)



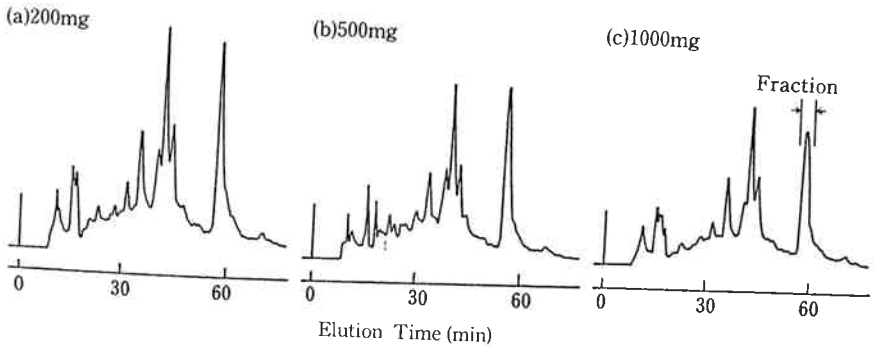
Column: TSKgel GSWP+G3000SWG; Column size: 21.5mm I.D.×(75+600)mm; Eluent: 25mM Phosphate buffer (pH7.0); Flow rate: 66m*l*/hr·cm² (4m*l*/min.); Sample: Crude fraction A (2m*l*) (Reproduced with permission from ref. 17, copyright 1984 by Academic Press, Inc.)

Fig. 25 Purification of Component A of the Soluble Methane Monooxygenase on a TSKgel G3000SW Preparative Column



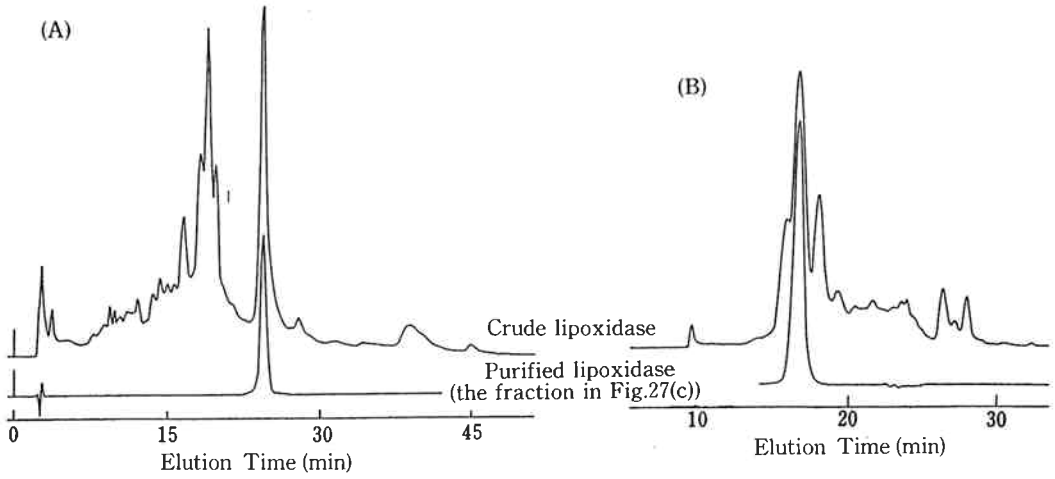
Column size: a. 7.5mm I.D.×75mm, b. 21.5mm I.D.×150mm, c. 55mm I.D.×200mm; Eluent: A. 20mM Tris-HCl buffer (pH8.0), B. A+0.5M NaCl; Gradient: a. A to B in 60min., b. A to B in 120min., c. A to B in 180min.; Flow rate: a. 136m*l*/hr·cm² (1m*l*/min.), b. 66m*l*/hr·cm² (4m*l*/min.), c. 76m*l*/hr·cm² (30m*l*/min.); Temperature: 25°C; Sample size: a. 1mg, b. 200mg, c. 1000mg

Fig. 26 Comparison of Resolution amang an Analytical Column and Preparative Columns in the Separation of Commercial Lipoxdase on TSKgel DEAE-5PW



Column size: 55mm I.D.×200mm; Eluent: A. 20mM Tris-HCl buffer (pH 8.0), B. A+0.5M NaCl (pH 8.0); Gradient: A to B in 180min.; Flow rate: 76m*l*/hr·cm² (30m*l*/min.); Temperature: 25°C; Sample: Crude Lipoxidase

Fig. 27 Effect of Sample Loading on the Chromatogram on TSKgel DEAE-5PW



Column: (A) TSKgel DEAE-5PW, 7.5mm I.D.×75mm, (B) TSKgel G3000SW, 7.5mm I.D. ×600 mm; Eluent: (A), A. 20 mM Tris-HCl buffer (pH 8), B., A+0.5 M NaCl (pH 8), gradient elution A to B in 60min.; (B), 50mM Phosphate buffer+0.2M NaCl (pH 7); Flow rate: 136m*l*/hr·cm² (1m*l*/min)

Fig. 28 Analysis of the Lipoxidase Fraction Purified by the TSKgel DEAE-5PW Preparative Column on a TSKgel DEAE-5PW Analytical Column (A) and a TSKgel G3000SW Analytical Column (B)

for the separation of large proteins and virus, concluding that the G5000PW column was quite effective to separate very large biopolymers which could not be covered by TSKgel SW columns.

[2] IEC

The results of the evaluation of TSKgel DEAE-5PW and SP-5PW preparative columns were reported in detail elsewhere[31], using lipoxidase, superoxide dismutase and human growth hormone. **Fig. 26** shows a comarison of resolution among an analytical column and preparative columns in the separation of commercial lipoxidase on TSKgel DEAE-5PW. Sample loading was increased from one to thousand mg without serious loss of resolution. **Fig. 27** shows the effect of sample loading on the chromatograms on the TSKgel DEAE-5PW preparative column (55mmI.D.). **Fig. 28A** and **28B** show the chromatograms of the peak fraction of lipoxidase obtained by the preparative column (see the right figure in Fig. 27) and crude lipoxidase on an analytical column of DEAE-5PW and G3000SW,

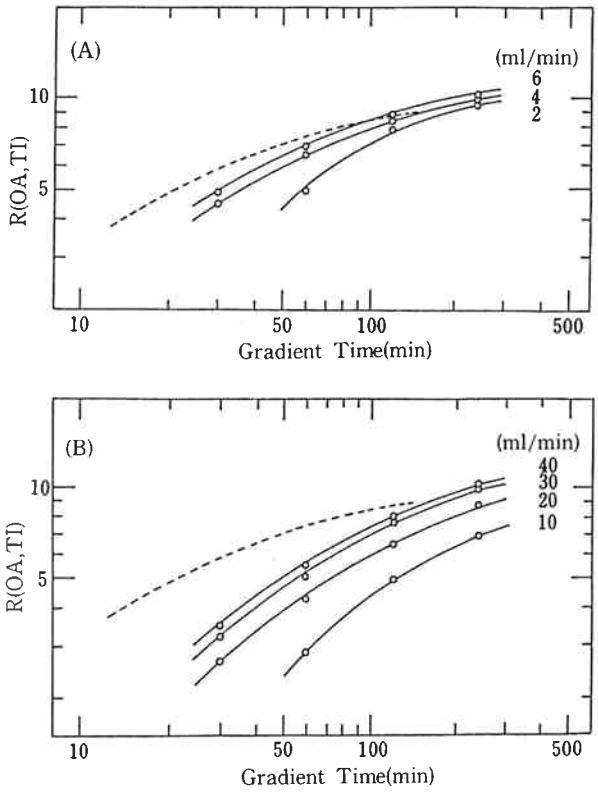
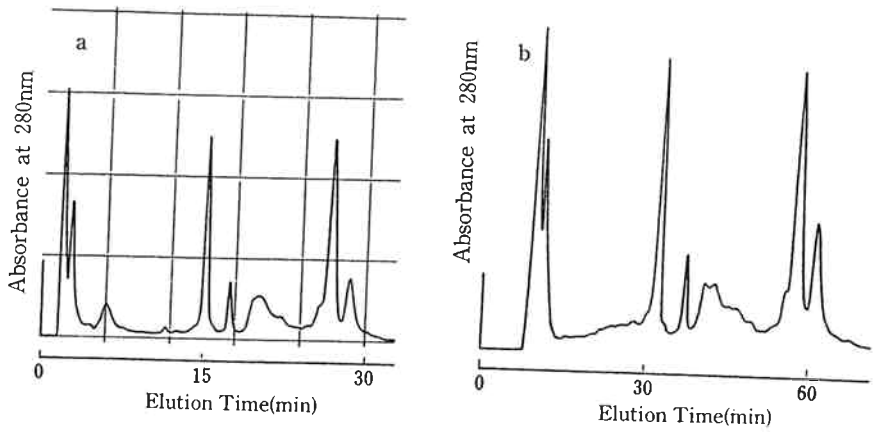


Fig. 29 Effect of Gradient Time and Flow Rate on the Resolution between Ovalbumin and Trypsin Inhibitor on TSKgel DEAE-5PW Preparative Column, 21.5mm I.D. (A) and 55mm I.D. (B)

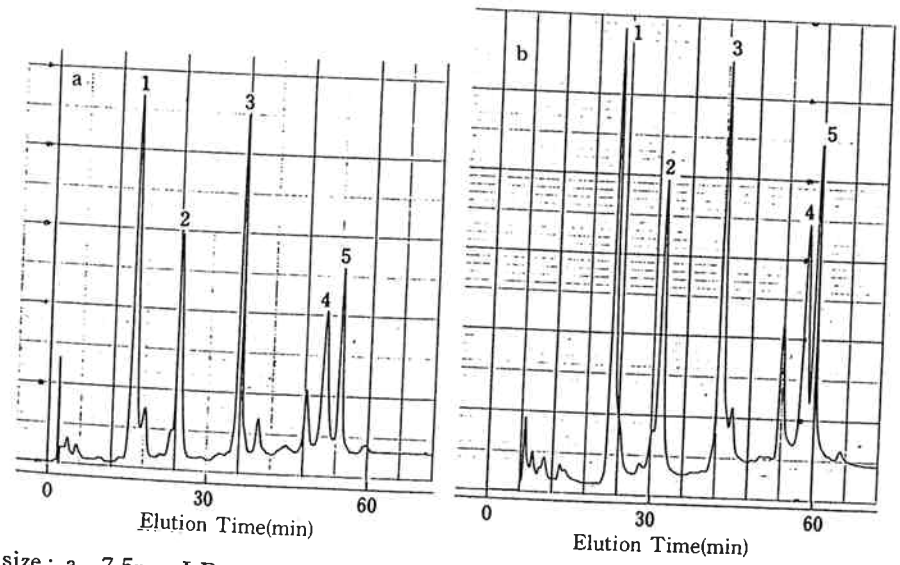
respectively. It can be seen that the lipoxidase was excellently purified by the preparative column.

Fig. 29A and **29B** show the effect of gradient time and flow rate on the resolution between ovalbumin and trypsin inhibitor on TSKgel DEAE-5PW preparative columns, 21.5mm I.D. and 55mm I.D., respectively. The result obtained by an analytical column is also given for comparison as the dotted line. The preparative columns can provide the same or even better resolution than the analytical column as the gradient time increases.



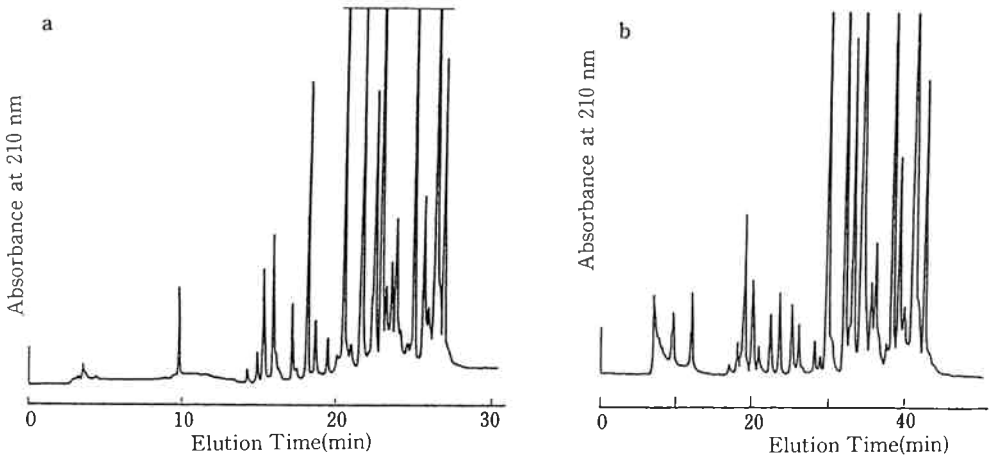
Column size: a. 7.5mm I.D.×75mm, b. 21.5mm I.D.×150mm; Eluent: A. 20mM Acetate buffer (pH 4.5), B. A+0.5M Na_2SO_4 (pH 4.5); Gradient: a. A to B in 60min, b. A to B in 120min; Flow rate: a. 136m*l*/hr·cm²(1m*l*/min), b. 66m*l*/hr·cm²(4m*l*/min); Temperature: 25°C; Sample size: a. 1mg, b. 200mg

Fig. 30 Comparison of Resolution of Crude Lipoxidase between an Analytical Column(a) and a Preparative Column(b) on TSKgel SP-5PW



Column size: a. 7.5mm I.D.×75mm, b. 21.5mm I.D.×150mm; Eluent: A. 1.8M Ammonium sulfate in 0.1M Phosphate buffer (pH 7.0), B. 0.1M Phosphate buffer (pH 7.0); Gradient: A to B in 60min; Flow rate: a. 136m*l*/hr·cm² (1m*l*/min), b. 99m*l*/hr·cm² (6m*l*/min) Temperature: 25°C; Detector: UV 280nm; Sample: 1. Myoglobin, 2. Ribonuclease, 3. Lysozyme, 4. α-Chymotrypsin, 5. α-Chymotrypsinogen

Fig. 31 Comparison of Resolution of a Protein Mixture between an Analytical Column and a Preparative column on TSKgel Phenyl-5PW



Column size: a. 4.6mm I.D.×250mm, b. 21.5mm I.D.×300mm; Eluent: A. 0.1M Phosphate buffer (pH 2.8), B. A/Acetonitrile (40/60); Gradient: a. A to B in 35min, b. A to B in 70min; Flow rate: a. 361m*l*/hr·cm²(1m*l*/min), b. 165m*l*/hr·cm² (10m*l*/min); Temperature: Ambient

Fig. 32 Comparison of the Separation of a Tryptic Digest of Lysozyme between an Analytical Column and a Preparative column on TSKgel ODS-120T

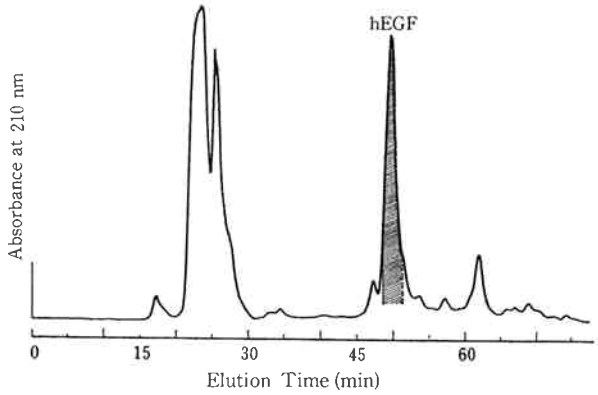
Fig. 30 shows a comparison of the resolution of crude lipoxidase between an analytical column (7.5 mm I.D.) and a preparative column (21.5mm I.D.) on TSKgel SP-5PW.

[3] HIC and RPC

Fig. 31 shows a comparison of resolution of a protein mixture between an analytical column and a preparative column (21.5mm I.D.) on TSKgel Phenyl-5PW.

Fig. 32 shows a comparison of resolution of a peptide mixture obtained by triptic digestion of lysozyme between an analytical column (4.6 mm I.D.) and a preparative column (21.5mm I.D.) on TSKgel ODS-120T.

Fig. 33 shows the separation of human epidermal growth factor (hEGF) by a preparative column (55 mm I.D. × 300 mm) of TSKgel ODS-120T. This application has been developed by Sakamoto et al (Wakunaga Parmaceutical Co., Ltd.) and practically used in the commercial production successfully.



Column size: 55mm I.D.×300mm
Temperature: 40°C
Eluent: A: $CH_3CN/H_2O/CF_3COOH$(10/90/0.05)
B: $CH_3CN/H_2O/CF_3COOH$(80/20/0.05)
Gradient: A/B(8/2)-10min-A/B(8/2)-linear gradient 40min-A/B(6/4)-10min-A/B(8/2)
Flow rate: 51m*l*/hr·cm²(20m*l*/min)
Sample: Crude hEGF 12.5mg protein (15m*l*)

Fig. 33 Purification of Human Epidermal Growth Factor (hEGF) on TSKgel ODS-120T

8. Conclusion

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,

such as the highest speed in R & D, the highest purity of products, the best economical condition surprisingly, etc. The highest possibility to succeed in time consuming and heavy costing biological testing will be provided. Valuable time will never be consumed for scale-up. Detailed description on scale-up procedure for IEC will be presented eleswhere in near future including larger diameter columns.

UF and MPLC will be used in combination with HPLC for preceding rough piurification and succeeding sample recovery.

Acknowledgements: The authors are indebted to Dr. S. Sakamoto (Wakunaga Pharmaceutical Co., Ltd.) for the permission of using the application data on TSKgel ODS-120T.

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