Products Information

Applications of TSK-GEL Toyopearl (Fractogel TSK*1) HW Types and Their Derivatives

Hiroo SASAKI
Rikuo ASADA
Yoshio KATO
Tsutomu HASHIMOTO

TSK-GEL Toyopearl has well been accepted as a typical support for MPLC (medium performance liquid chromatography) and various applications have been developed. This review is to summarize the recent applications including separation of proteins, carbohydrates, nucleic acids and small compounds on TSK-GEL Toyopearl HW types and their ion exchangers. Some results on scale up, column size selection and activation for affinity chromatography will also be described.

1. INTRODUCTION

During the past several years remarkable progress has been made in analytical and preparative applications of high speed gel filtration chromatography (GFC)*2 to separation of biological polymers like proteins owing to development of new rigid or semirigid microparticulates (1-4).

Although various methods have been tried in approach to manufacture rigider supports, only three groups are now commercially accepted as shown in **Table 1.** It seems that the silica-based microparticulates (group 2) have now established their position in analytical and/or semipreparative applications to proteins because of high speed and high resolution based on excellent rigidity and suitable pore size distribution. However, it should be noted that the

Table 1 Approach to Rigid Gel

Raw Material & Process		Merit & Demerit	Typical Product
1. Polysaccharide Crosslinking	○	Excellent hydrophilicity	Sephacryl (Pharmacia)
	×	Difficulty in water-in-oil suspension	Sepharose CL (Pharmacia)
	×	Low pressure durability	
2. Silicagel Surface modification	○	Excellent mechanical strength→High pressure durability & resolution	TSK-GEL SW Type (Toyo Soda) Protein Column (Waters) LiChrosorb Diol (Merck) SynChropack GPC (SynChrom)
	×	Unstability in alkaline solution	
3. Vinyl monomer Suspension polymerization	○	Stability in alkaline solution	Shodex OH pak (Showa Denko)
	○	Wide pore distribution	TSK-GEL PW Type (Toyo Soda)
	×	Poor resolution compared with 2	TSK-GEL TOYOPEARL (Toyo Soda)
	×	Poor hydrophilicity compared with 1	

silica-based microparticulates have their inherent disadvantage of chemical instability at high pH. Another technical disadvantage is restriction of fractionation range. Namely they are not suitable for GFC separation of both small molecules below several thousand and very large molecules above one million.

On the other hand, the supports of the group 1 still show poor pressure durability, resulting in difficulty for high speed elution, although their mechanical strength is much improved in comparison with the conventional soft gels.

TSK-GEL Toyopearl (referred to as only Toyopearl below), belonging to the group 3, can overcome most of the disadvantages of the silica-based supports including economical aspect at a reasonable expense of speed and resolution. Toyopearl is well designed to cover preparative GFC in medium range between high speed (high pressure) and low speed (low pressure).

Gurkin *et al* presented a fundamental description on Fractogel TSK (TSK-GEL Toyopearl) (5), which should be referred to regarding the following items: structure, various stability (pressure, pH, organic solvent, temperature, etc.), guide to selection of type and grade, some applications and methods of column preparation.

This review will attempt to present additional informations on Toyopearl, putting emphasis on the following points: new applications including some scale up data, clarification of nonadsorptive and adsorptive properties, activation for affinity chromatography and introduction of derivatives.

One of the important points to make the best use of supports for GFC is to have knowledge of their characteristics concerning interactions with various samples. Although Toyopearl is designed to minimize ionic and hydrophobic interactions with proteins, it often shows unique adsorptive properties which can be utilized in practical applications to obtain better separation.

(Note)

*1 TSK-GEL Toyopearl is the same material as Fractogel TSK available from E. Merck (Darmstadt, G. F. R.) and MCB Reagents (New Jersey, U. S. A.)

*2 Several synonymous terms are used for gel filtration chromatography (GFC) such as aqueous gel permeation chromatography (GPC), aqueous size exclusion chromatography (SEC) and so on. In this review is used GFC.

2. APPLICATIONS OF TOYOPEARL HW TYPES

[1] Proteins

(1) Examples in ordinary buffer solutions

The elution behavior of commercially available proteins on Toyopearl HW-55F was investigated by Inouye *et al* (6). The proteins may be classified into three groups as follows:

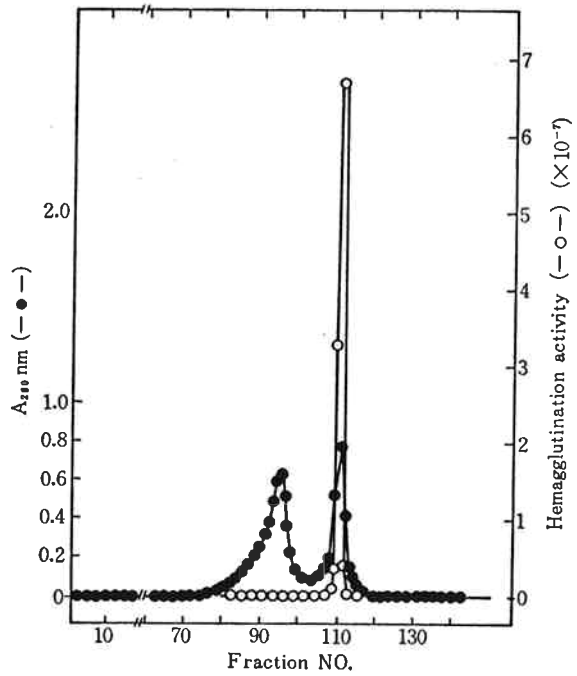
(a) normal proteins which do not interact with the gel in wide range of ionic strength.

(b) proteins of high pI which show ionic interaction with the gel at low inoic strength, while elute normally at suitable ionic strength.

(c) proteins which show hydrophobic interaction with the gel in wide range of ionic strength. They tend to elute faster by addition of an organic modifier like alcohol into the buffer.

It should be noted that although inoic and hydrophobic interaction of Toyopearl gels with some proteins are rather stronger than those of the dextran gels, high recovery yield of enzyme

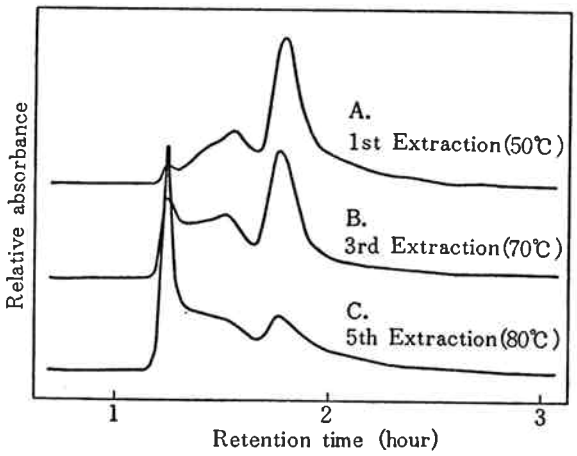
activity is one of the most eminent features of Toyopearl. Particularly, some glycoproteins of recent great interest can often be purified successfully utilizing Toyopearl, while they tend to lose their activity on polysaccaharide gel probably due to slow elution or nonspecific interaction. In addition to purification of postproline dipeptidyl aminopeptidase by Tsuru *et al* (7), horse shore crab (*Tachypleus gigas*) agglutinins, glycoproteins difficult to be recovered by GFC on the coventional polysaccharide gels, were separated on Toyopearl HW-65F with excellent yield (more than 90%) by Sekiguchi *et al* (**Fig. 1**) (8).



Experimental conditions	
Gel	: TOYOPEARL HW-65F
Gel bed	: 2.6×90cm
Eluent	: 0.5M NaCl/0.1M CaCl/0.05M Tris HCl. pH 7.5
Flow rate	: 12ml/hr.
Temperature	: 4℃
Sample	: Horseshoe crab (*Tachypleus* gigas) Agglutinins. 9.5ml (A_{280}=3.2ml)
Detection	: UV 280 (—●—) Hemagglutination activity ($Titer^{-1}$) to horse
Fraction size	: 2ml/tube

Fig. 1 Chromatography of *Tachypleus* Agglutinins on TOYOPEARL HW-65F

Gelatin, one of the most adsorptive proteins, could be eluted normally by Mizusawa *et al* (9). The extracts from the gelatin with hot water at different temperatures were chromatographed on Toyopearl HW-55S, giving profiles clearly different from each other as shown in **Fig. 2**.



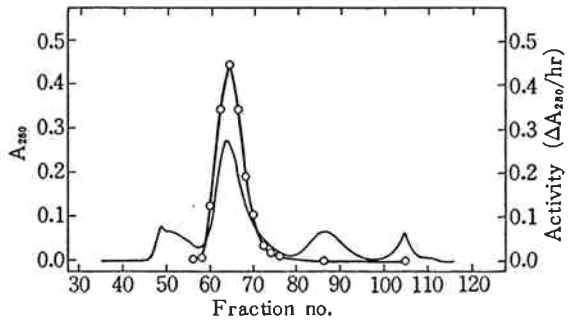
Experimental conditions	
Gel	: TOYOPEARL HW-55S
Column	: 1.0cm ID×100cm×2
Eluent	: 0.2M Phosphate buffer (pH 5.0, 0.3M NaCl)
Flow rate	: 41.4ml/hr
Temperature	: 50℃
Sample	: 30μl (0.05%)
Detector	: 220nm

Fig. 2 Elution pattern of gelatin on TOYOPEARL HW-55S

Purification of acylphosphatidyl glycerol synthetase from E. coli on Toyopearl HW-60F by No;ima *et al* (10) and purification of a protease from a thermophilic bacteria (*Thermus caldophilus GK 24* on Toyopearl HW-55F by Ohta *et al* (11) (**Fig. 3**) were also typical examples for which GFC on the conventional polysaccharide gels had resulted in very poor recovery.

Uritani *et al* used Toyopearl HW-60S in purification of aldehyde dehydrogenase (ALDH) from sweet potato roots (12).

Satoh preferred to use Toyopearl HW-55S in purification and molecular weight determination

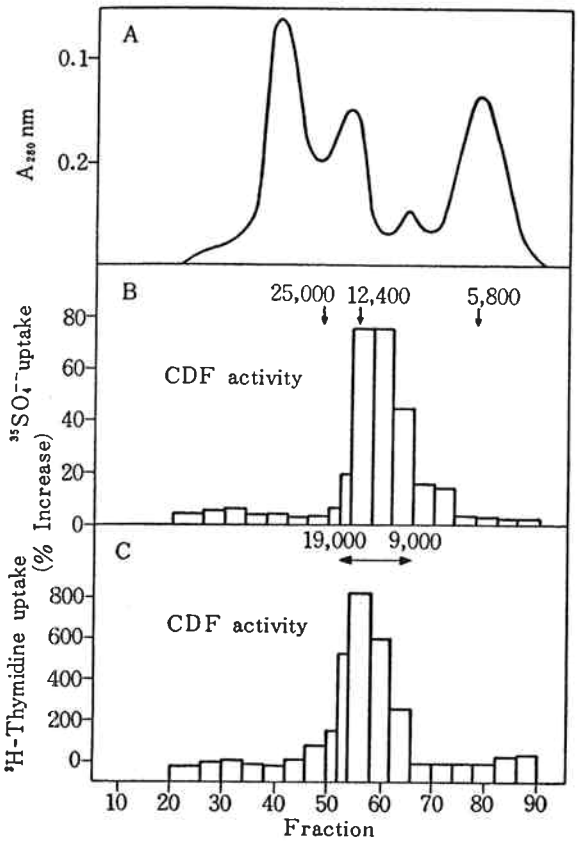


Experimental conditions

Gel	: TOYOPEARL HW-55F
Gel bed	: 1.8cm×91cm
Eluent	: 100mM Tris-HCl buffer (pH 7.4) containing 1mM $CaCl_2$
Flow rate	: about 10ml/hr (3.9ml/hr-cm²)
Temperature	: 4℃
Sample applied	
Volume	: 7ml
Fraction size	: 2ml/tube
Detection	: UV 280nm (—)
	Enzyme activity* (—o—o—)

*The activity was determined by the increase of OD_{280} of the trichloroacetic acid-soluble fraction after the treatment of the substrate, casein, by the protease.

Fig. 3 Chromatogram of extracellular protease from *Thermus caldophilus* GK 24



Experimental conditions

Gel	: TOYOPEARL HW-55F
Gel bed	: 2.2cm ID×20cm
Eluent	: 4M guanidine hydrocloride buffer (pH 6.5)
Flow rate	: 12ml/hr.
Temperature	: 20℃
Sample	: Bovine-fetal -cartilage fraction (MW. 10,000-300,000) pretreated by the acetone precipitation and ultrafiltration
Detector	: A UV 280nm
	B, C radioactive analysis

Fig. 4 Gel filtration of cartilage-derived factor (CDF) on a column of TOYOPEARL HW-55F

of interferon (IFN) in his investigation of spontaneous interferon producing cell (13).

(2) Examples in solutions containing denaturating agent and solubilizing agent

Because of easiness to operate due to excellent flow property and swelling property, Toyopearl is well-suited for application in concentrated solutions of denaturating agents and detergents like guanidine hydrochloride, sodium dodecyl sulfate (SDS), etc. Suzuki *et al* succeeded in isolation of a somatomedine-like peptide (MW 10,000~11,000) from fetal bovine cartilage on Toyopearl HW-55F in 4M guanidine hydrochloride (**Fig. 4**) (14, 15). They pointed out that GFC on Toyopearl was very reproducible, giving similar elution profiles on repeated chromatography (at least 20 times) at high flow rate (10~20 mℓ/hr·cm²). Shikita *et al* also used 6M guanidine hydrochloride for purification of granulocyte-macropharge colony-stimulating factors produced by various mammalian cell lines on Toyopearl HW-55F (16). Shibasaki *et al* analyzed soybean 11S globulin heated in the presense of N-ethylmaleimide by GFC on Toyopearl HW-65F in 0.5% SDS (17). Molecular weight of *Dolichos biflorus* agglutinin, a lectin isolated from *Dolichos biflorus*, was estimated by GFC on Toyopearl HW-65F in 0.01M Tris HCl buffer containing 0.2% SDS by Murakami *et al* (18, 19).

(3) Examples in highly concentrated salt solution

As demonstrated in separation of isozymes of cytochrome C (5), Toyopearl has a tendency to adsorb proteins in highly concentrated salt solution. Although this property has been thought to be caused by hydrogen bonding, hydrophobic interaction is probably dominant because adsorptive property has been found to be much enhanced by alkylation of Toyopearl by Shin *et al* (20). They practically utilized Toyopearl HW-65C on a large scale (10 cm I. D. × 20 cm) in purification of ferredoxin-$NADP^+$ reductase (FNR) in the extract from spinach leaves, resulting in remarkable improvement of the purification process compared with the conventional one on DEAE-cellurose (**Fig. 5**) (21). The supernatant obtained by fractionation with 40% saturated

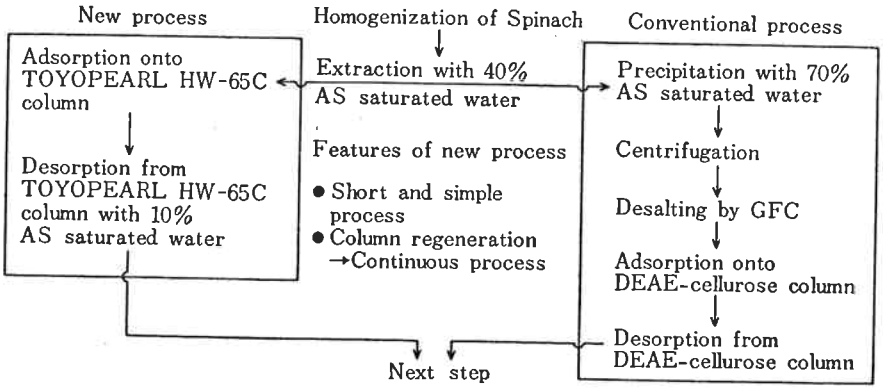


Fig. 5 Comparison of new process using TOYOPEARL with conventional process for purification of FNR from spinach

solution of ammonium sulfate was applied to a column of Toyopearl HW-65C equibrated with 40% saturated solution of ammonium sulfate. FNR adsorbed on the column was eluted with 10% saturated solution of ammonium sulfate. During this process, both efficient concentration of FNR and desalting were performed at the same time. The volume of the FNR fraction was reduced to almost one tenth, together with increase of the specfic activity. The gel bed can be regenerated as it is by washing with 0.1N NaOH, water and 40% saturated solution of ammonium sulfate for repeated use, while DEAE-cellurose must be regenerated outside the column and repacked every time. Such process might be quite useful for industrial purification of various enzymes in combination with ammonium sulfate fractionation technique due to easiness to design automation.

[2] Carbohydrates

As nonionic oligosaccharides and polysaccharides usually exhibit no interaction with Toyopearl gel, they are one of the samples which can simply be separated by size-exclusion. Cyclodextrins exceptionally elute very late for their moleculer weight.

(1) Oligosaccharides

Kondo *et al* investigated fractionation of oligosaccharides obtained by hydrolysis of cyclodextrins using a column (4.4 cm I. D. × 60 cm × 2 or 3) packed with Toyopearl HW-40S, reporting that 1–3 g of the sample could be applied to achieve high resolution (22).

Tanaka *et al* studied resolving power of Toyopearl HW-40S and HW-40F for β-cyclodextrin hydrolysate and dextran T-40 hydrolysate in comparison with Shephadex G-10, G-15 and G-25 (23). They also described the effect of temperature on resolution and pressure drop.

Kainuma *et al* also reported promising results on fractionation of maltooligosaccharides (G4, G5, and G6) from various amylase digests by GFC on Toyopearl HW-40S. Using columns (2.2 cm I. D. × 50 cm × 4), they obtained each oligomers with very high purity (above 99.9%) (24).

Authors tried a still larger scale fractionation of β-cyclodextrin hydrolysate and dextran T-40 hydrolysate using a large scale column (10.8 cm I. D. × 100 cm × 2) packed with Toyopearl HW-40S, onto which ca. 11g of the sample was applied, resulting in excellent separation as shown in **Fig. 6** (25).

The column was prepared by slurry packing method under constant pressure of nitrogen (1.5–2.0 kg/cm²) at 60°C using a stainless steel reservior of ca. 40ℓ:

(2) Polysaccharides

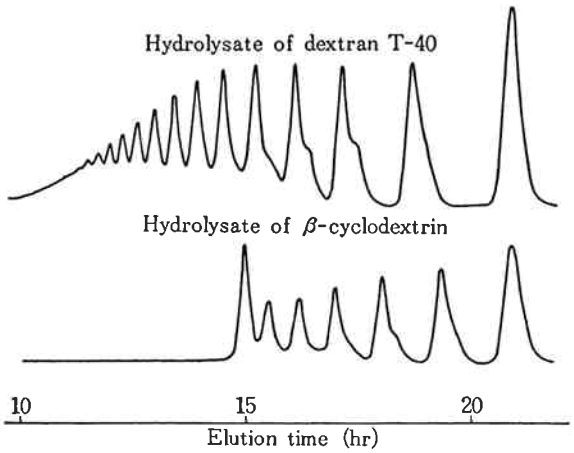
Sakai *et al* purified some polysaccharide (MW ca. 300,000) produced by yeast (*Tri. penicillatum* SNO-3) by GFC on Toyopearl HW-65F (26).

Very rapid measurement of molecular weight distribution of hemi-cellurose B extracted from azuki bean cell walls was performed by Masuda *et al* on a glass column (1.5 cm I. D. × 80 cm) of Toyopearl HW-75S using 0.1N NaOH as an eluent (27). Measurement time was reduced to one twelfth compared with that of the conventional GFC on Sepharose CL 4B with nearly equal resolution under the same experimental conditions.

(3) Cyclodextrins

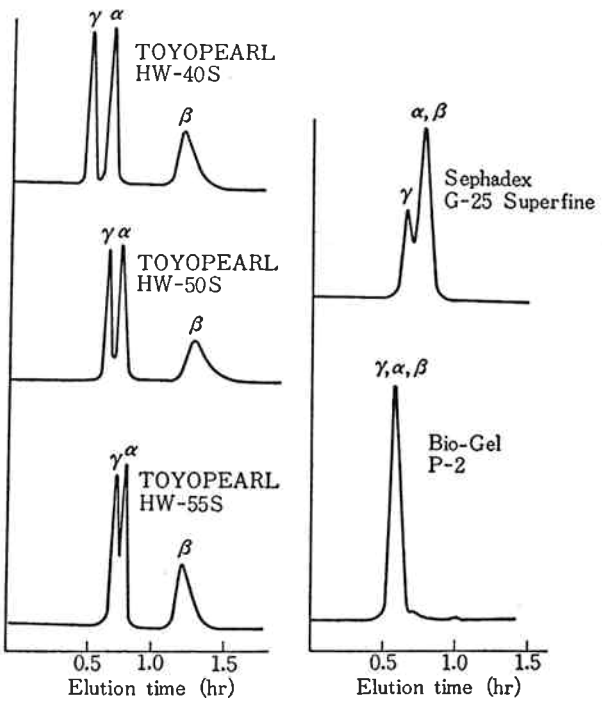
Cyclodextrins are exceptional nonionic oligosaccharides which exhibit unique interaction with Toyopearl gel. Kainuma *et al* separated three different cyclodextrins (α, β and γ) on Toyopearl HW-40S (24).

Fig. 7 shows the comparison of resolving power of Toyopearl HW-40S, Toyopearl HW-50S, Toyopearl HW-55S, Bio-Gel P-2 and



Gel	: TOYOPEARL HW-40S
Size	: 108mmID × 100cm × 2
Eluent	: Water
Flow rate	: 9.1ml/min.
Load	: Ca. 11g 36.8ml × 30%

Fig. 6 Large scale application of TOYOPEARL Fractionation of oligosaccharide on TOYOPEARL HW-40S



Column	: 22mmID × 150mm
Eluent	: Distilled water
Flow rate	: 1.0ml/min

Fig. 7 Separation of cyclodextrins on TOYOPEARLS in comparison with conventional gels

Sephadex G-25 (28). The best result was obtained by Toyopearl HW-40S.

This new method for purification of cyclodextrins is very promising in industrial production of pure cyclodextrins whose various applications are under development in food industry and pharmaceutical industry.

[3] Nucleic acids and their constituents

Yoshinaga *et al* have established a new method for rapid preparation of plasmid DNA by GFC on Toyopearl HW-75S, which can overcome disadvantages of centrifugation, the most commonly used procedure in separation of plasmid DNA. 5 μg to 0.3 mg of plasmid PBR 322 DNA free from chromosomal DNA and RNA could be prepared from the cleared lysate by overnight elution using a column packed with Toyopearl HW-75S (29).

ATP, ADP and AMP could be separated by GFC on Toyopearl HW-40F according to molecular size, while adenine and adenosine were retarded to a great extent (30). Similar results were roported by Hirayanagi *et al* (31).

[4] Amino acids and peptides

(1) Amino acids and their derivatives.

Inoue et al studied elution behavior of amino acids and their derivatives on Toyopearl HW-55F (6). They observed strong interaction of aromatic amino acids with the gel, which could not be explained by hydrophobic interaction because such interaction was not observed with leusine, more hydrophobic one.

They thought that the interaction might be caused by charge transfer effect. Introductions of protective groups into amino acids made the derivatives further retarded except acylation of amino groups. Thus it may be very difficult to separate amino acid derivatives by pure size exclusion mechanism, but it means that Toyopearl is quite suitable as a separating carrier.

(2) Peptides and their derivatives.

Although there is no detailed study on elution behavior of peptides so far, it can be presumed from the results on amino acids above mentioned that separation by mixed mexanism will occur. Various interesting applications have been reported as follows. Murakami *et al* isolated synthetic surfactants, some peptide derivatives, on Toyopearl HW-40F in methanol (32).

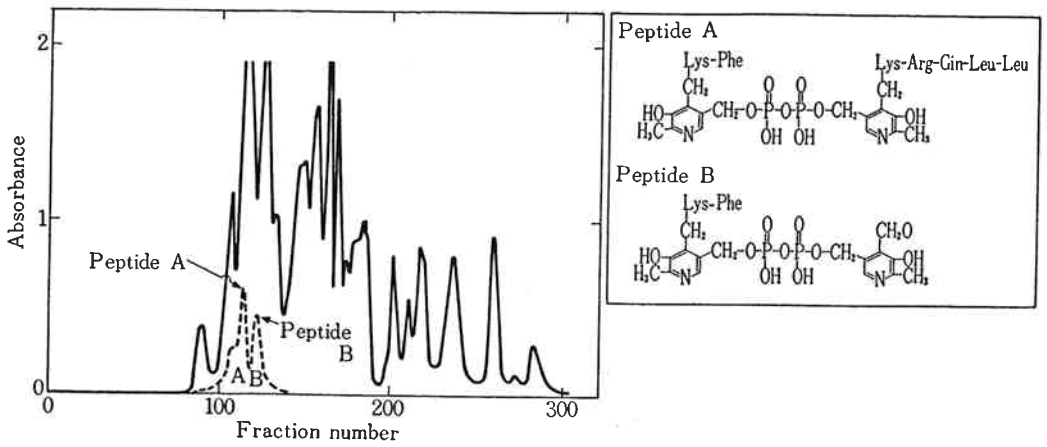
Actinomycin C_1 and actinomycin X_2 whose chemical structures were very similar were successfully separated on Toyopearl HW-40C using a special eluent [described by Jake Macmillan, Journal of Chromatography **87**, 271-276 (1973)] by Kobayashi *et al* (33).

In isolation of cirratiomycin A and B, new peptide antibiotics, by Shiroza *et al*, Toyopearl HW-40F was used in combination with Dowex 50W-X_2 and Shephadex G-25 (34).

Fukui *et al* separated peptides in the chymotryptic digest of bis (PLP)-labeled potato phosphorylase in thier study on the structure of the active site regions of phosphorylases (**Fig. 8**) (35).

[5] Phenolic compounds and aromatic acid derivatives

Although Toyopearl was designed as a support for agueous GPC, one of the recent strong trends in its application has been partition-adsorption chromatography in hydrophilic organic solvents and their aqueous solution owing to its excellent swelling property in various organic



Experimental conditions	
Gel	: TOYOPEARL HW-40F
Eluent	: 0.2M NH_4HCO_3-NH_4OH buffer (pH 9.0)
Temperature	: Ambient
Fraction size	: 3.0ml
Detection	: UV 280nm (—)
	UV 320nm (—)*

*(Absorbance of bis (PLP) dabeled derivative)

Fig. 8 Gel filtration of the chymotryptic digest of bis (PLP)-labeled potato phosphorylase.

solvent. In this field Toyopearl HW-40 usually exhibits much higher resolving power than sepahdex LH 20, the most competitive support.

Typical example was presented by Ozawa in separation of the components in black tea infusion (36). Using a glass column (0.9 cm I. D. × 25 cm) packed with Toyopearl HW-40F ca. 30 components were separated by stepwise gradient elution of aqueous acetone (**Fig. 9**). Ozawa also used Toyopearl HW-40F in isolation of chestanin, a polyphenolic glucoside, from chestnut gall (37). Ozawa pointed out that Toyopearl HW-40F showed much higher resolving power than that of Sephadex LH-20 in both examples.

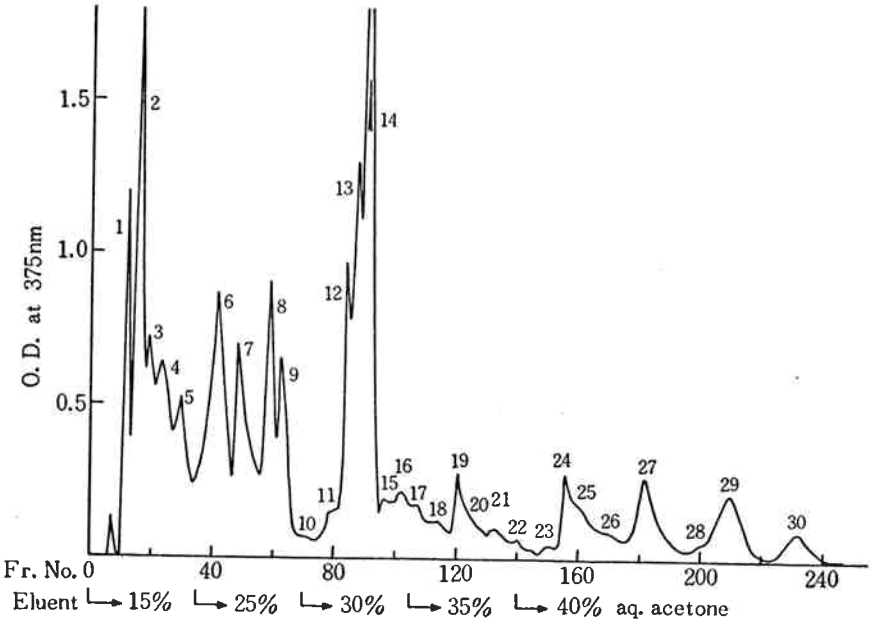
Authors compared the resolving power of Toyopearl HW-40S, Toyopearl HW-50S, Toyopearl HW-55S and Sephadex LH-20 in separation of simple aromatic compounds such as phthalates and p-hydroxybenzoates, confirming that Toyopearl HW-40S gave the best result as shown in **Fig. 10a** and **10b.**

These are typical examples in which the samples elute accoring to size exclusion mechanism using an organic solvent.

Whether elution follows size exclusion mechanism or not depends upon balances of mutual interactions among three substances sample, sorbent and mobile phase.

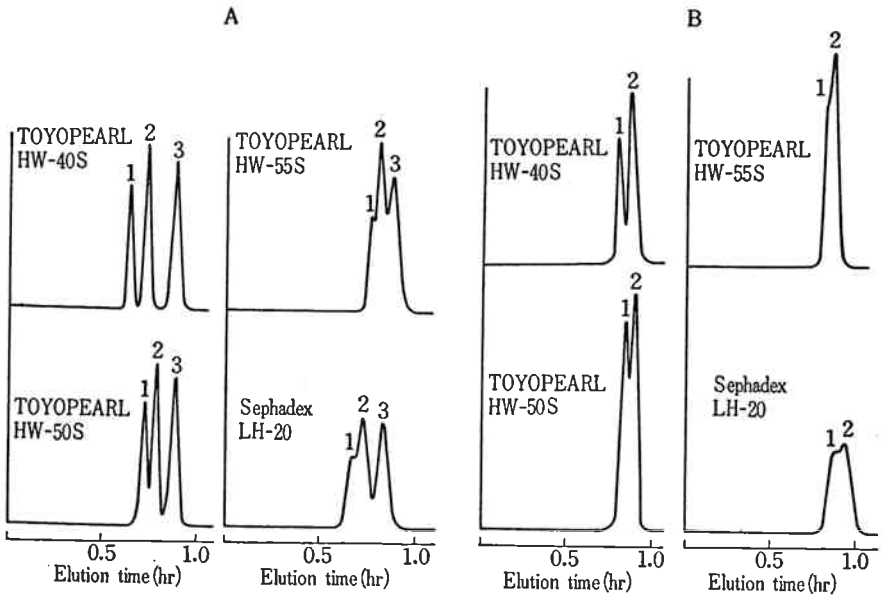
One of the difficulties in using Toyopearl in organic solvents and their agueous solution is how to select the optimal elution conditions. In order to overcome this problem, Fukuda *et al* tried to prepare thin layer plates of Toyopearl and succeeded in developing a procedure to prepare plates practically available (38). **Fig. 11** is an typical example of thin payer chromatography using Toyopearl in which the dependence of migration distance of a mixture of phthalates on water content was shown.

Utilizing such thin layer plates which are now commercially available, it is expected that the selection of the optimal elution condition will become much easier.



Gel	: TOYOPEARL HW-40F
Gel bed	: 0.9cm ID×25cm
Eluent	: Aqueous acetone
Flow rate	: 1ml/13min
Sample	: Extract from Twinings Queen Mary Tea (3min with water at 95℃)
Sample size	: 60mg/0.4ml
Detection	: 375nm

Fig. 9 Elution pattern of tea infusion on TOYOPEARL HW-40F



Column	: 22mm ID×150mm	1 : DOP (Dioctyl phthalate)	1 : Propyl *p*-Hydroxybenzoate
Eluent	: Methanol	2 : DBP (Dibutyl phthalate)	2 : Methyl *p*-Hydroxybenzoate
Flow rate	: 1.0ml/min	3 : DMP (Dimethyl phthalate)	

Fig. 10 Comparison of resolving power in separation of phthalates (A) and p-hydroxy benzoates (B) on TOYOPEARLS and Sephadex LH-20

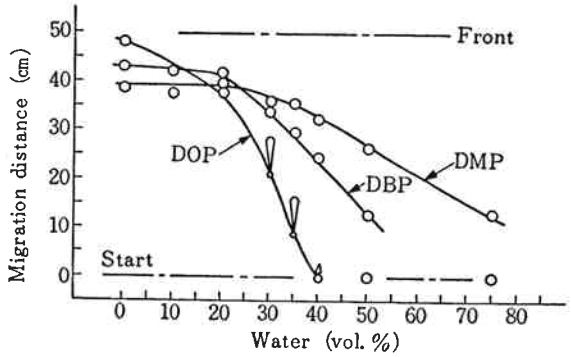
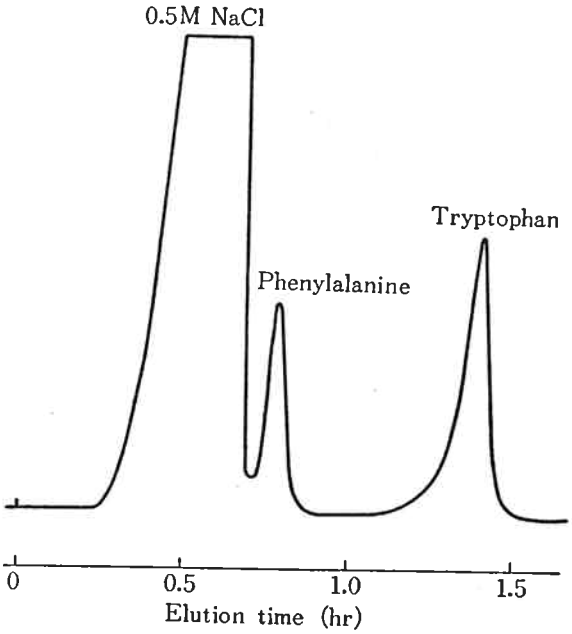


Fig. 11 Thin layer chromatography on TOYOPEARL (dependence of migration distance of phthaltes on water content in ethanol)



Gel : HW-40S
Column : 22mm I D × 150mm
Eluent : Distilled water
Sample : 1.0ml

Fig. 12 Desalting of tryptophan and phenylalanine on TOYOPEARL HW-40S

〔6〕 Desalting of small compounds

Desalting of proteins using Toyopearl HW-40 was already described by Gurkin *et al* (5).

Various small compounds which exibit the interactions with Toyopearl gel elute after salt, resulting in desalting of the compounds.

Owing to its unique adsorptive characteristics between polystyrene gels and polysaccharide gels, Toyopearl is well-suited for this purpose. As an example, desalting of phenylalanine and tryptophan is shown in **Fig. 12** (28). It is suggested that desalting of the following compounds might be easily performed on Toyopearl HW-40: aromatic amino acids, amino acid derivatives, peptides and their derivatives, nucleic acid bases, nucleosides, phenolic compounds, aromatic carboxylic acids and their derivatives, β-cyclodextrin, etc.

3. DERIVATIVES OF TOYOPEARL

〔1〕 Ion exchangers, DEAE-Toyopearl 650 and CM-Toyopearl 650

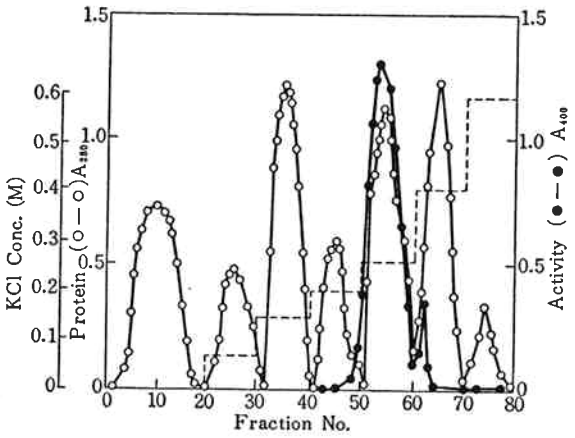
These ion-exchangers, manufactured by introduction of suitable ionic groups into Toyopearl HW-65, were introduced into Japanese market in 1981. They have been quite well accepted in both laboratory and industrial separations of ionic biopolymers, especially proteins, owing to various excellent characteristics as follows: high resolving power due to small particle size 〔25-44 μm for S (superfine) grade and 44-88 μm for M (medium) grade〕 and uniformity of ionic groups evidened by their titration curves: rapid separation due to high flow rate based on excellent mechanical strength: easiness in operation and good reproducibility because of negligibly small dependence of swelling property on pH and concentrations of salts and organic solvents: long life due to high resistance to physicochemical factors such as high and low pH, high temperature

and decomposition by microorganisms: wide range coverage of molecules from small to very large (up to several million of MW) owing to very large pore size.

Basic descriptions are and will be presented in manufacturer's catalogue (in Japanese) (29) and E. Merck's cataloque (in English).

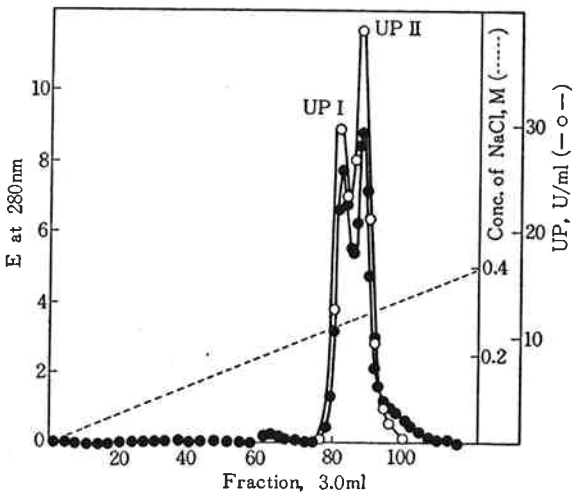
Detailed informations based on fundamental experiments were presented by authors (40).

Fig. 13 shows an example of column chromatography on DEAE-Toyopearl 650M by Soda *et al* (39). Selenocystein lyase obtained from bacteria was successfully purified by stepwise gradient elution. Using DEAE-Toyopearl 650M, Minamiura *et al* separated Uropepsin isomers obtained from human urine as shown in **Fig. 14** after many failures by ion exchange chromatography on conventional ion exchangers (42).



Gel	: DEAE-TOYOPEARL 650M
Column	: 1.8cmID×8cm
Eluent	: 0.01M Phosphate buffer (pH 7.4, 0.01% DTT+10^{-5} PLP)+KCl
Flow rate	: 0.4ml/min
Temperature	: 4℃
Sample	: 5.3mg Protein
Detector	: UV 280nm (—○—○—) Activity (—●—●—)
Fraction	: 1ml/tube

Fig. 13 Purification of selenocysteine ligase from bacteria on DEAE-TOYOPEARL 650 M



Experimental conditions

Gel	: DEAE-TOYOPEARL 650M
Column	: 1.8cmID×25cm
Eluent	: 0.02M phosphate buffer (pH6.4)+NaCl
Flow rate	: 0.2ml/min.
Temperature	: 4℃
Sample	: 1408U/8ml (E280nm=3.38)
Detection	: UV 280nm (—●—) Enzyme activity (—○—)
Fraction size	: 3ml/tube

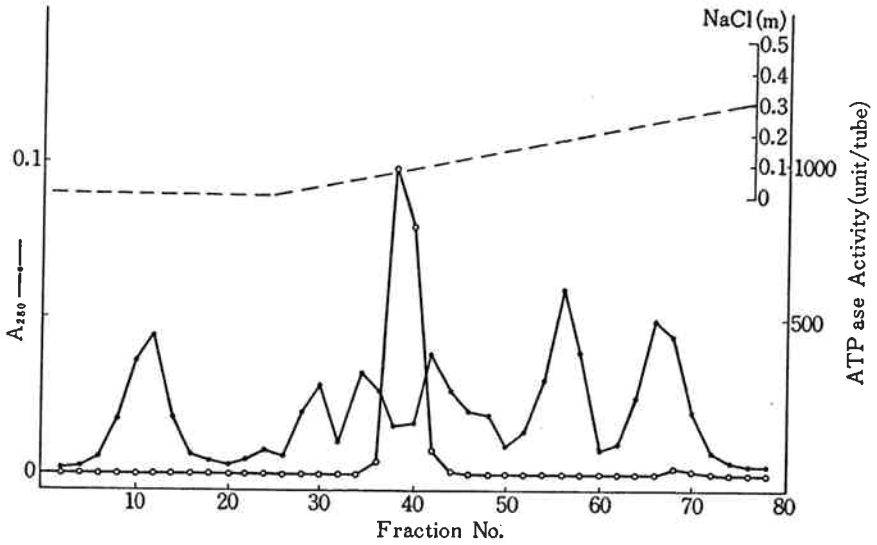
Fig. 14 Separation of uropepsins on DEAE-TOYOPEARL 650 M

Matsumoto *et al* used DEAE-Toyopearl 650M in purification of ATPase in pulvinus of *mimosa pudica* as shown in **Fig. 15** (42). Enzyme activity was exhaunced ten times in this procedure, while only 1.8 times by ion exchange chromatography on DEAE-Sephadex A-50.

(2) Activation of Toyopearl for affinity chromatography and enzyme immobilization

Although great progress has been made in development of affinity chromatography, it seems that most of applications are restricted only to laboratory scale. This may probably be attributed to the difficulty in preparation of adsorbents suitable for industrial purpose.

Agarose gel, a base material most widely used for affinity chromatography, apparently has several unfavourable properties for industrial use. The most fatal disadvantage is its poor mechanical strength, resulting in various problems on large scale applications as follows: very



Gel	: DEAE-TOYOPEARL 650M
Gel bed	: 1.5cm ID × 16cm
Eluent	: 33mM Tris-HCl (pH 7.2) NaCl 0-0.35M
Flow rate	: 50ml/hr
Sample size	: 15ml (370μg)
Detection	: UV 280nm (—●—) ATP ase activity (—○—)
Temperature	: 4℃
Sample preparation	: Crude ATP ase was prepared from the precipitate of obtained by centrifugation of the extract (10mM Tris-HCl pH 7.5) from the first and second pulvinus of mimosa.

Fig. 15 Ion exchange chromatography of mimosa extract on DEAE-TOYOPEARL 650 M

low productivity due to slow flow rate: instability of flow rate and short life time of gel bed due to clogging caused by bed compression: difficulty in rapid regeneration by washing with suitable solvents. Moreover, economical activation process in highly restricted by both instability to high temperature and poor swelling property in organic solvents.

Toyopearl, owing to its fundamental properties such as excellent mechanical strength and physicochemical stability, can apparently overcome most of the disadvantages of agarose gel.

For affinity chromatography, a suitable type of Toyopearl should be selected depending on molecular size of substance to be purified. Toyopearl HW-65 is the most popular type which can cover relatively large molecules up to ca. several millions of molecular weight (MW) without serious decrease of capacity even for small molecules less than several ten thousands of MW. Toyopearl HW-55 is recommended to be used for relatively small molecules in comparison with Toyopearl HW-65.

Matsumoto *et al* investigated the optimal conditions for the activation of Toyopearl HW-65 and Toyopearl HW-55 with epichlorohydrin and found that the optimal conditions were very different from those for agarose gel (43). They converted the expoxy activated Toyopearl into amino and carboxyl derivatives, which were subsequently coupled with various ligands. In preparation of glycamyl Toyopearl, reaction time (6 h) was greatly reduced in comparison with that for agarose gel (800 h), owing to applicability of a higher reaction temperature. The adsorbents obtained were successfully used for the affinity chromatography of lectin and trypsin.

Oyama *et al* studied immobilization of various proteins on Toyopearl gels through epoxy-

activation followed by various subsequent derivatizations including amination with ethylene diamine, glutarldehyde activation, cyanuric chloride activation etc. (44).

The experimental conditions for epoxidation by Oyama *et al* were very different from those by Matsumoto *et al*, particularly regarding the ratio of epichlorohydrin vs Toyopearl gel. According to the experimental results obtained by themselves, authors recommend the conditions in **Fig. 16** which are very similar to those by Matsumoto *et al*.

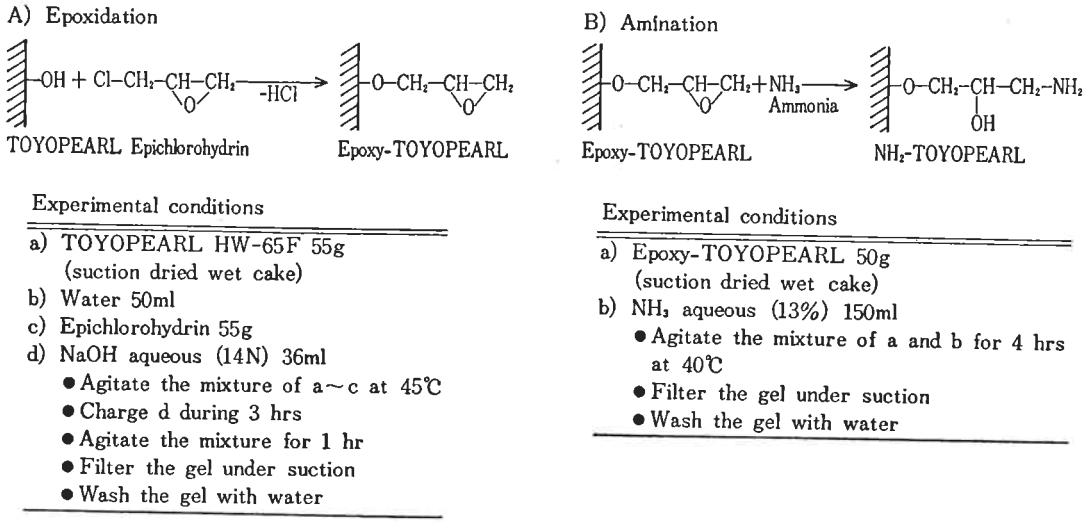


Fig. 16 Activation of TOYOPEARL Gel for affinity chromatography

Several activated Toyopearls for affinity chromatography will become available commercially in near future at reasonable cost even for industrial purpose.

[3] Resolution of Co (III) complexes by cation-exchangers derived from Toyopearl HW-40F

Yamatera *et al* prepared new cation-cxchangers with tartrate residues as chiral exchange groups from Toyopearl HW-40F (45). Then, they studied elution behaviors of Co (III) complexs on these cation-exchangers in comparison with corresponding Sephadex derivatives. A clear-cut resolution of $[Co\ (tn)_3]^{3+}$ was achieved by a Toyopearl derivative column, while a previous attempt using a Sephadex derivative column resulted in a partial resolution. In the reaction of Toyopearl with L-tartaric acid, severe high reaction temperature (110°C) was adopted, indicating that Toyopearl gel is resistant to high temperature.

4. SOME STUDIES ON COLUMN DIMENSION IN GFC ON TOYOPEARL

To make the best use of GFC on Toyopearl, column size selection is one of the important points. Here are described some results of studies on the dependence of column performance on column dimension.

[1] Dependence on column diameter

Using Toyopearl HW-55S, authors studied and reported the dependence of column performance on column diameter (46). They packed glass columns of several different diameters (1.0–4.4 cm)

with the gel by constant velocity packing method. This study was extended to Toyopearl HW-55F including stainless steel columns of larger diameters (10.8–30.0 cm). These large columns were packed by constant pressure method (2–4 kg/cm²) using a nitrogen cylinder as illustrated in **Fig. 17** instead of constant velocity method, bacause the former was thought to be better from practical and economical point of view due to unnecessity of an expensive pump of high capacity.

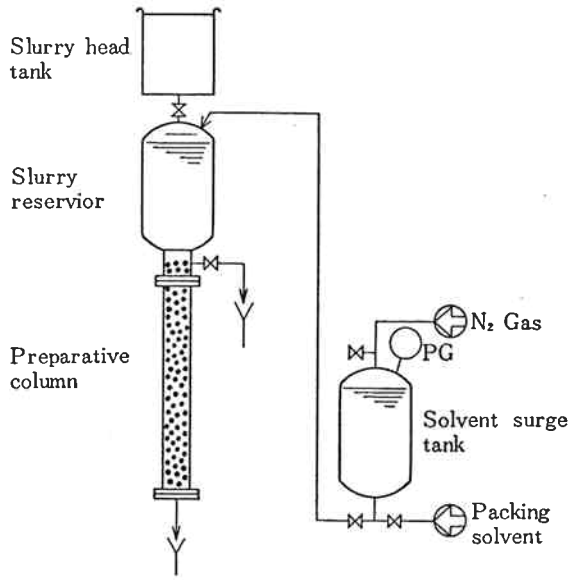


Fig. 17 Set-up for preparation of large scale column under nitrogen pressure

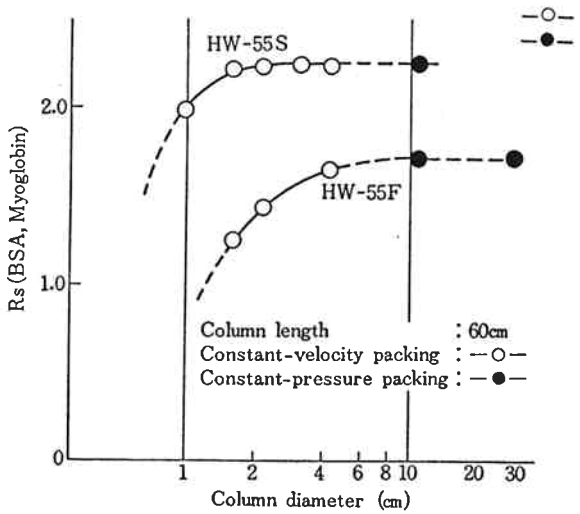


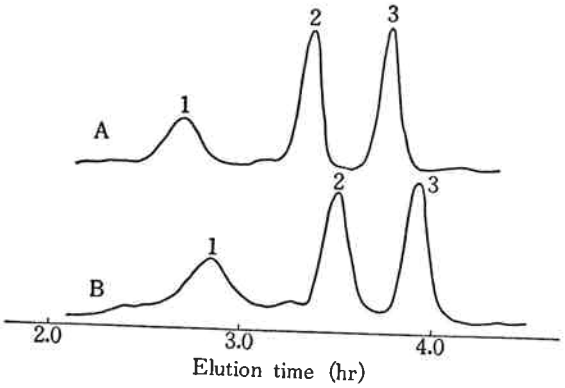
Fig. 18 Dependence of resolution on column diameter and particle size

The performance of the packed columns was tested with a mixture of bovine serum albumin and myoglobin as described previously (47). Results were plotted in **Fig. 18** (48). The resolution increased with incleasing diameter reaching to constant probably due to disappearance of wall effect. It should be noted that although different packing methods were applied, the results were plotted on a same curve. This indicates that scale-up of column diameter is not difficult if the constant pressure method is adopted.

〔2〕 Dependence on column dimension under constant column volume

Using Toyopearl HW-55F, Germershausen *et al* studied the dependence of column performance on column size under constant column volume and constant flow rate and found unexpectedly that the separation pattern of a mixture of protein standards was not affected so much (49). It seems that their results are contradictory to the theory that resolution is proportional to square root of column length and the common sense in GFC on Sephadex that a long column should be used to achieve high resolution.

To confirm the results by Germershausen *et al*, authors carried out the experiment as shown in **Fig. 19** (50). Using two columns of equal volume (456 mℓ) and different sizes (column A: 2.2 cm I. D. × 120 cm, column B: 4.4 cm I. D. × 30 cm), nearly eaqual separations of a mixture of standard proteins were obtained at the same absolute flow rate (resulting naturally in almost the same separation time).



Gel	: TOYOPEARL HW-55F
Gel bed	: A=2.2cm ID×60cm×2 B=4.4cm ID×30cm
Eluent	: 0.1M Phosphate buffer + 0.1M KCl
Flow rate	: A=19.7ml/hr. cm² B=5.1ml/hr. cm²
Sample	: 1%×100μl (1 : thyroglbulin, 2 : BSA, 3 : myoglobin)
Temperature	: 25℃

Fig. 19 Comparison of resolution with columns of different dimmension and equal volume

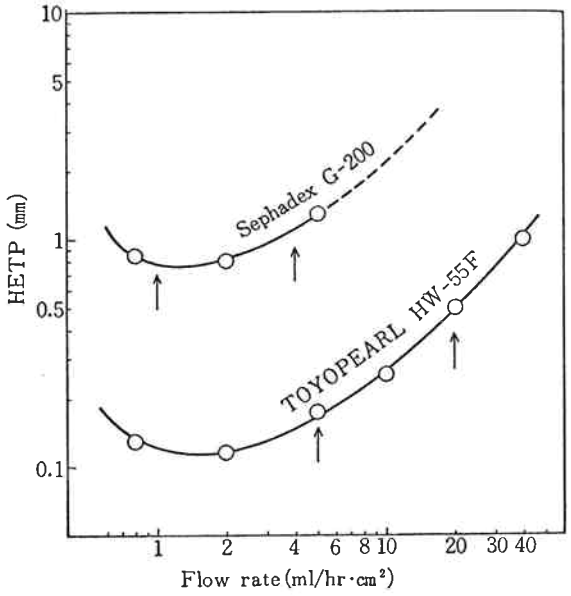


Fig. 20 Dependence of HETP of BSA on flow Rate

This phenomenon may be explained by dependence of height equivalent to theoretical plate (HETP) on flow rate (linear velocity) and wall effect as follows. **Fig. 20** shows dependence of HETP of columns (2.2 cm I. D. × 60 cm) packed with Toyopearl HW-55F and Sephadex G200 measured by BSA on flow rate (linear velocity) (51). The ranges between arrows indicate the commonly used range of linear velocity for both gels: 5-20mℓ/hr·cm² for Toyopearl HW-55F and 1-4 mℓ/hr·cm² for Sephadex. In the Toyopearl column gained a large decrease of HETP (0.59 mm→0.17 mm) with decreasing linear velocity, while the Sephadex column a very small decrease (1.1 mm→0.78 mm). This large decrease of HETP of the Toyopearl column theoretically corresponds to increase of 2.94 times of column length at the same linear velocity from the view point of resolving power, because resolution is proportinal to square root of theoretical plate number which is inversely proportional to HETP and proportional to column length.

In other words, the resolving power of the short and wide column can almost be compensated by its increase owing to decrease of linear velocity in spite of the handicap of the shortness of the column. Besides, the increase of the resolving power caused by decrease of wall effect as above mentioned contributes to the compensation of the resolving power of the short and wide column.

On the other hand, in case of Shephadex G-200, such compensation of the resolving power due to decrease of linear velocity can not be expected so much because it is usually used at very low linear velocity around the bottom of HETP as shown in **Fig. 20.**

It should be noted that the dependence of HETP on linear velocity greatly depends upon molecular size of samples and elution temperature of sample in solution (in other words, diffusion coefficient and dead volume of a chromatographic system). The bottom of HETP moves to the right as molecular size of samples becomes smaller (52, 53) and the dead volume increases. It also should be noted that the superiority of the long column might become clearer as flow rate

is reduced, namely as separation time is prolonged.

It might be concluded that use of a short and wide column is recommended in GFC of large molecules on Toyopearl HW Types of high exclusion limit to make the best use of their speedy separation (typically, a column of 2.0 cm I. D. × 30 cm might be better than that of 1.0 cm × 120 cm). Several practical benifits can be suggested as follows: higher loading capacity due to larger cross section, easiness of column preparation, compact elution system, lower pressure drop etc.

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