Review

High-performance Gel Filtration Chromatography of Proteins on TSK-GEL SW Column

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Methods are reviewed for the high-performance gel filtration chromatography of proteins on TSK-GEL SW column. The column, with its optimized calibration curve and high theoretical plate number, allows efficient analysis of proteins. Several important factors including concentration of salt and its species in the eluent are described for the optimized uses of TSK-GEL SW column. Also described are a method for the molecular weight determination of proteins and several other applications of gel filtration chromatography.

1. Higher speed and improved resolution for gel filtration chromatography

Classical size-exclusion separations of water soluble compounds such as proteins have utilized relatively soft hydrophilic cross-linked gels, e. g., polysaccharide dextrans, agarose and synthetic polyacrylamide. Unfortunately, these gels can not be used as packing material for high performance size-exclusion chromatography because of their low mechanical strength and relatively large particle size. High performance size-exclusion chromatography has become possible following the introduction of rigid, small diameter packing materials such as TSK-GEL SW Type and PW. TSK-GELs have been used successfully for both preparative separation of proteins, including enzymes, and for high speed and high performance molecular weight estimations.[1,2,3]

The separation of peaks, i. e., resolution, is of utmost importance in chromatographic procedures and will be discussed in detail in the following passages.

The resolution of two peaks is defined by the following equation:

$$R_s=1/4(k'/1+k')\,(\alpha-1/\alpha)\sqrt{N} \qquad \text{(eq. 1)}$$

Thus, resolution is related to three terms: the capacity factor, the selectivity factor, and the efficiency. The first two terms can be varied by changing the composition of the mobile phase (e. g., pH, ionic strength). Peak sharpness, however, is related to the last term, efficiency ($\sqrt{N}$). In contradistinction to other modes of separation, resolution in size-exclusion chromatography is determined solely by peak sharpness. Therefore, the efficiency term plays a more important role compared to other modes of chromatography.

Let us examine more closely the efficiency term. The number of theoretical plates (N) is related to the retention volume, V_e, and the peak width, W, as follows:

$$N=16(V_e/W)^2 \qquad \text{(eq. 2), see Figure 1.}$$

Peak width is the width along the base line between the extended tangents to the peak drawn through the inflection points as illustrated in **Fig. 1.** In practice, it is easier to use the relation,

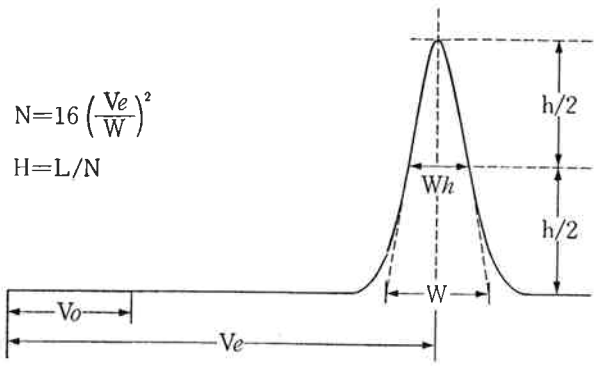


Fig. 1 Number of theoretical plates & height equivalent to a theoretical plate

$$N=5.545(V_e/W_h)^2 \qquad \text{(eq. 3)},$$

where W_h is the peak width at *half* the peak height.

In order to compare band spreading among various columns, another term is needed. The height equivalent to a theoretical plate, H, is the parameter most often used. It is defined as

$$H=L/N \qquad \text{(eq. 4)},$$

where L is the length of the column. Thus, N is directly proportional to the column length and resolution (R_s) is directly proportional to the square root of N.

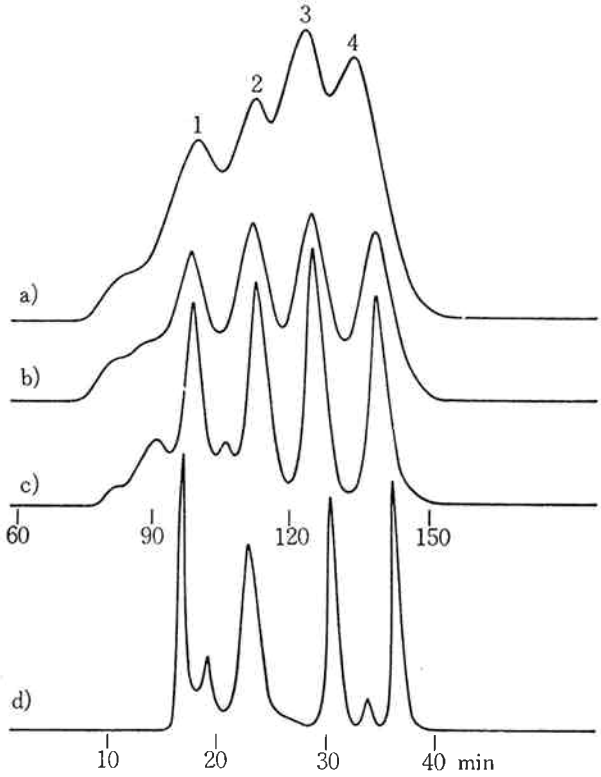


Fig. 2 Effect of diameter of packing on resolution of proteins
Column;
a) TOYOPEARL HW-55 Coase Grade (26 mm i.d. × 700 mm)
b) TOYOPEARL HW-55 Fine Grade (〃)
c) TOYOPEARL HW-55 Superfine Grade (〃)
d) TSKgel G 3000 SW (7.5 mm i.d. × 600 mm)
Sample;
1. Thyroglobulin 2. γ-Globulin
3. β-Lactoglobulin 4. Cytochrome C

Figure 2 demonstrates the relationship between resolution and the diameter of the packing material in a column. Improved resolution is obtained with smaller diameter materials. How this observation relates to H is explained as follows: Because H is inversely proportional to N (eq. 4), and N is the important parameter for the determination of resolution (eq. 1), it follows that for maximum N, H must be minimized. H is actually the sum of three terms, i. e.,

$$H=h_d+h_s+h_m \qquad \text{(eq. 5)},$$

where these terms represent the theoretical plate height contributions due to longitudinal diffusion, diffusion in the stationary phase, and diffusion in the mobile phase, respectively. Furthermore, each of these terms can be expressed thusly;

$$h_d=2\gamma D_M/v \qquad \text{(eq. 6)},$$

$$h_s=q\cdot dp^2\cdot v/D_m \qquad \text{(eq. 7), and}$$

$$h_m=1/(1/2\,\lambda\cdot dp+D_M/\omega\cdot dp^2\cdot v) \qquad \text{(eq. 8)},$$

where D_M is the diffusion coefficient of the solute in the pore of packing;

D_m is the diffusion coefficient of the solute in the mobile phase;

v is the linear velocity of the mobile phase;

dp is the diameter of the packing material;

and ω, γ, λ, q are constants.

Since the diffusion coefficient is 10^4 to 10^5 times smaller for liquid chromatography than for gas chromatography, h_d is a minimal contribution to H and for practical considerations can be ignored; therefore, $H=h_s+h_m$. The diameter of the packing material (dp) appears in both h_s and h_m. Furthermore, a decrease in dp decreases both terms by the square of dp. The most critical factor for decreased H (band spreading) then is dp (see **Fig. 2**). Velosity (v) plays a less important role, so that with small dp high flow rates can be used with good resolution. For example, when one reduces the dp from 50 μm to 10 μm (five-fold decrease), the h_s and h_m become about 25-fold smaller except eddy current effect ($2\lambda dp$). Therefore, with 10 μm packing and a 10-fold increase in v (1/10th the original running time) over that flow rate with 50 μm packing, H becomes about 2.5 times smaller, or conversely, N increase by about 2.5 times.

To summarize the above discussion, the use of a small diameter packing material with increased flow rates allows high speed and high performance chromatography.

In an actual chromatographic situation, **Fig. 2** serves to illustrate the importance of packing material diameter. A standard protein mixture was chromatographed on Toyopearl columns at the same flow rates (v) with different grade packing materials, 75 μm (coase grade), 45 μm (fine grade), and 30 μm (superfine grade). Peaks are sharper and resolution is better as the smaller packing materials are employed (compare **Figures 2a** through **2c**). Moreover, **Figure 2d** depicts the use of 10 μm packing (TSKgel G 3000 SW) and a flow rate four times greater than that in 2a-c. Considerably better resolution was obtained with this packing.

Smaller diameter packing materials tend to have weaker mechanical strength due to the decrease of their diameter: pore diameter ratio. Therefore, the development of hard packing material with sufficient pore volume as well as appropriate calibration curve as will be described later is also critical for the achievement of high speed liquid chromatography.

TSK SW and PW gels are packing materials which meet the requirements stated above for high speed and high performance size-exclusion chromatography.

2. TSK-GEL SW: Applicable molecular weight ranges and calibration curves

Two types of packing materials for high speed gel filtration chromatography have been produced. One is for gel permeation chromatography of non-polar compounds and the other is for the separation of watersoluble compounds. This section deals with packing for the latter purpose. **Figure 3**[5] shows a typical calibration curve (molecular weight-elution volume). For the separation of high molecular weight compounds such as proteins (including enzymes), packing which produces a shallow slope in the high molecular weight region of the curve is necessary. SW Type gel was designed for such a purpose (**Fig. 3a**). The matrix of this type is silica gel chemically bonded with hydrophillic groups and the extreme top portion of these groups is primary alcohol. On the other hand, for the separation of low molecular weight compounds such as oligosaccharides, packing material producing a shallow slope in the low

molecular weight region is required (**Fig. 3c**). For the estimation of molecular weight distribution over a wide range, packing which results in good linearity over a wide range is needed. Type PW of large pore size (G 3000 PW-G 6000 PW) was designed for this purpose (**Fig. 3b**). The matrix of this type is a porous polymer of a hydrophillic nature.

Figure 4 shows a calibration curve for protein using three different pore size SW packings. The region of the shallowest slope in each curve is indicated as follows:

TSKgel G 2000 SW	10,000–80,000
TSKgel G 3000 SW	20,000–200,000
TSKgel G 4000 SW	more than 100,000

The resolving power of each of the packings is the greatest in the ranges shown above.

The gel permeability depends not only on the size of the molecule, but on its shape. Accordingly, molecules with similar molecular weights may elute at different positions. **Figure 5** shows calibration curves of de

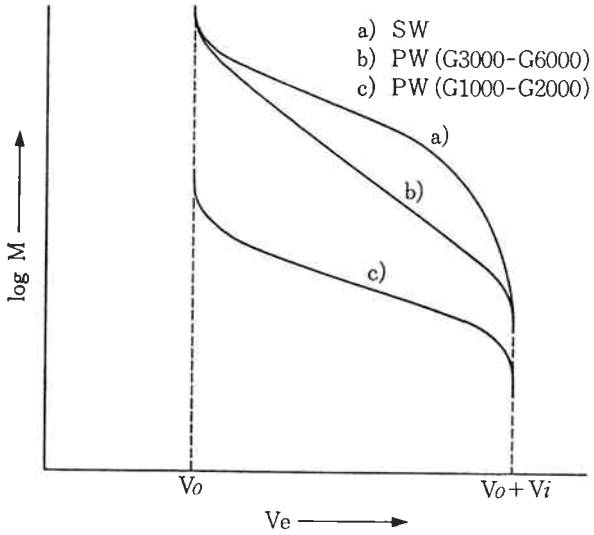


Fig. 3 Typical calibration curve of aqueous GPC

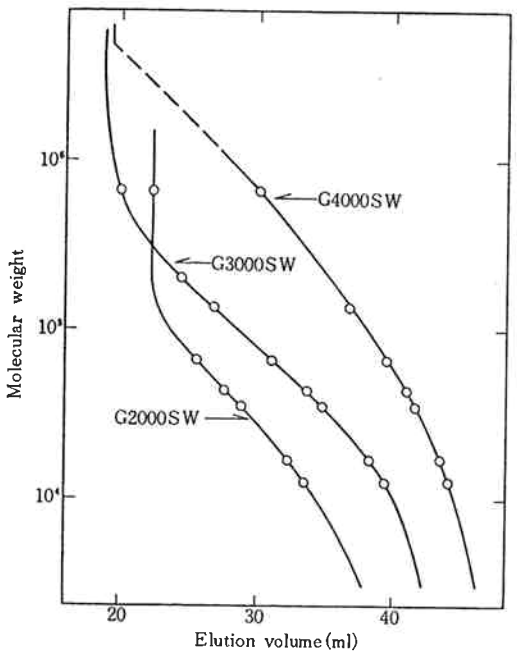


Fig. 4 Calibration curves of G 2000 SW, G 3000 SW and G 4000 SW for protein. Size of each column system: 7.5 mm I.D. ×120 cm

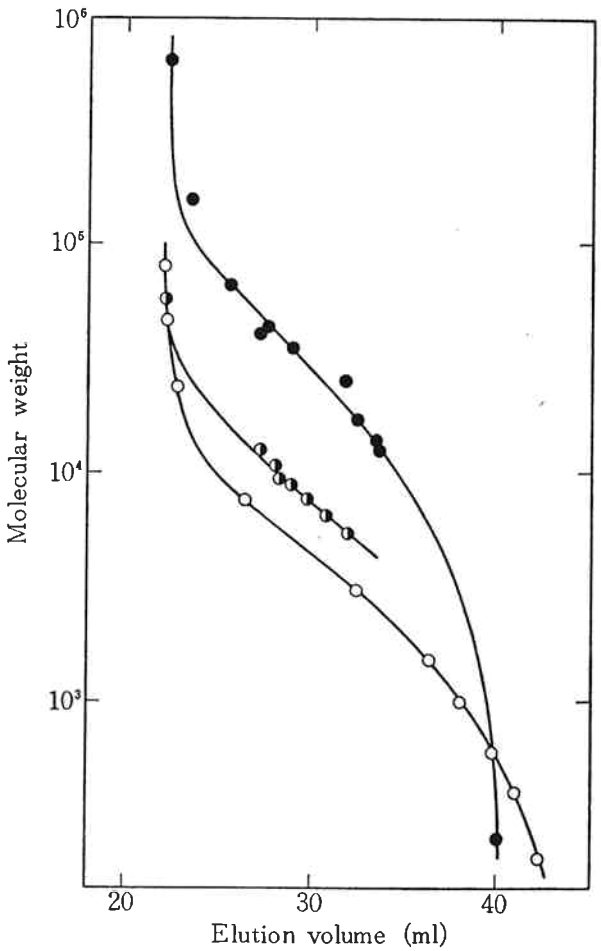


Fig. 5 a) Calibration curves of G 2000 SW (7.5 mm I.D. ×120 cm) for (○) polyethylene glycol, (◑) dextran and (●) protein.

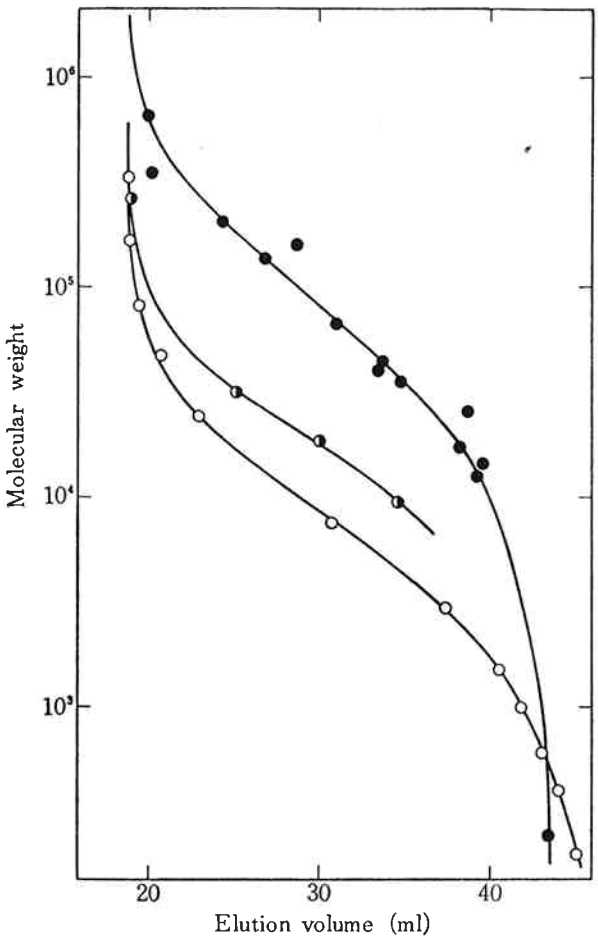


Fig. 5 b) Calibration curves of G 3000 SW (7.5 mm I.D. × 120 cm) for (○) polyethylene glycol, (◑) dextran and (●) protein.

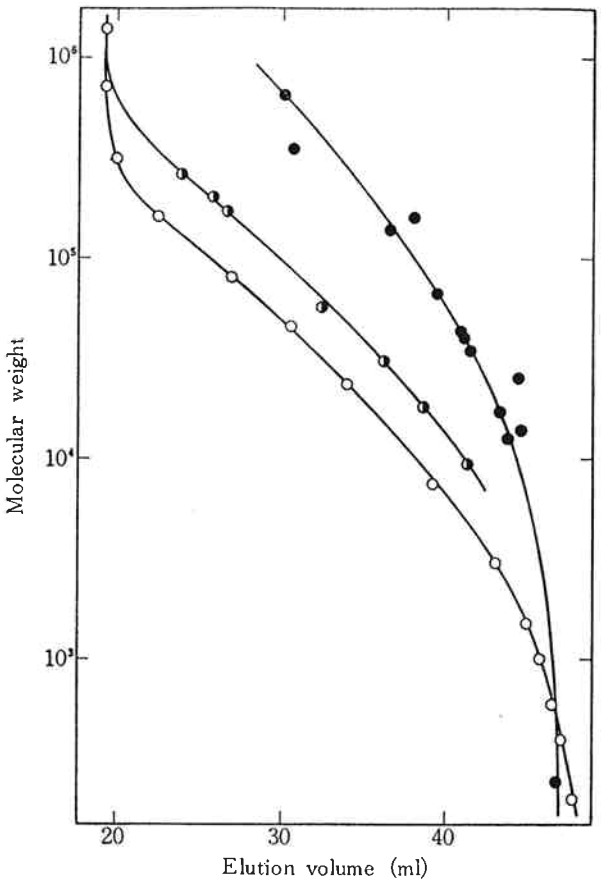


Fig. 5 c) Calibration curves of G 4000 SW (7.5 mm I.D. × 120 cm) for (○) polyethylene glycol, (◑) dextran and (●) protein.

Table 1 Separation range of TSK-GEL SW Type

	Separation range		
	G 2000 SW	G 3000 SW	G 4000 SW
Polyethylene glycol	500- 15,000	1,000- 35,000	2,000- 250,000
Dextran	1,000- 30,000	2,000- 70,000	4,000- 500,000
Protein	5,000-100,000	10,000-500,000	20,000-7,000,000

xtran, polyethylene glycol, and protein on columns with TSK-GEL SW pore sizes of three grades (G 2000 SW, G 3000 SW, G 4000 SW). It is clear that the different kinds of molecules each have a unique calibration curve. Proteins, which are generally folded, i. e., not extended, behave as smaller molecules during gel filtration than do extended molecules such as polyethylene glycol, which permeate the gel less extensively than do compact molecules of the same molecular weight. The apparent molecular weight as determined from **Fig. 5** for polyethylene glycol is approximately ten times higher than the protein by the criterion of elution position. Dextran, which possesses ring structures, appears to have a molecular weight intermediate to that of the protein and polyethylene glycol. Compared to the protein, dextran elutes at a position indicating a molecular weight of twice that of the protein. Since the calibration

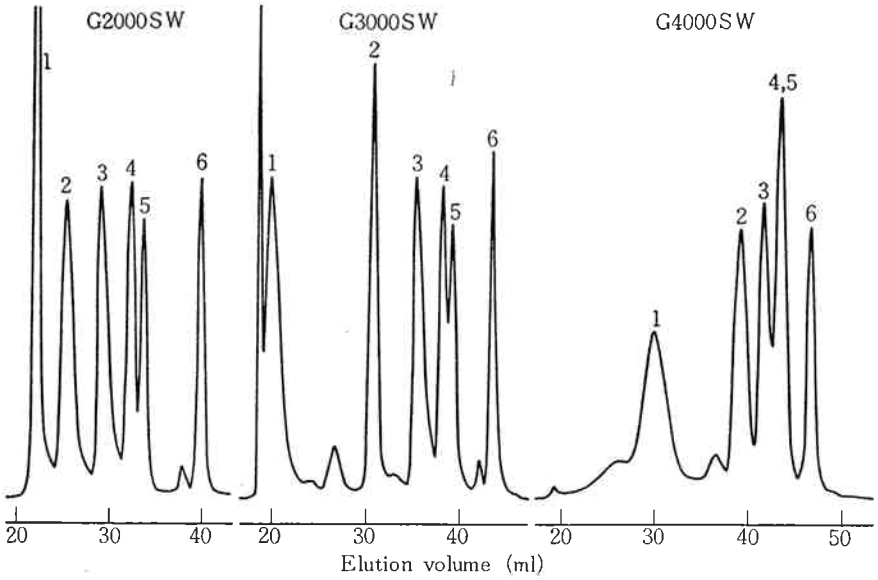


Fig. 6 Comparison of elution curves of a mixture of (1) thyroglobulin (0.03%), (2) bovine serum albumin (0.02%) (3) β-lactoglobulin (0.02%), (4) myoglobin (0.01%), (5) cytochrome c (0.01%) and (6) glycyl-glycyl-glycyl-glycine (0.03%) measured on G 2000 SW, G 3000 SW and G 4000 SW.
Size of each column system: 7.5 mm I.D. ×120 cm
Solvent: 0.1 M phosphate buffer containing 0.3 M NaCl (pH=7)
Flow rate: 1 ml/min Temperature: 25°C
Detector: UV monitor at 220 nm

curves vary so much depending on the sample species, one must be very careful in selecting a suitable pore size. **Table 1** summarizes the applicable molecular weight ranges for three different SW Type columns.

To further illustrate the applicability of the three SW columns, let us consider actual chromatograms. **Figure 6** demonstrates the separation of a standard protein mixture (thyrogloublin, bovine serum albumin, β-lactoglobulin, myoglobin, cytochrome c, and glycyl-glycyl-glycylglycine) on three different SW columns of different pore size. The eluent in each case was 0.1 M phosphate buffer, pH 7, containing 0.3 M NaCl. For the separation of lower molecular weight proteins such as myoglobin (16,900) and cytochrome c (12,400), small pore size packing such as G 2000 SW gives much better resolution than larger pore size packing.

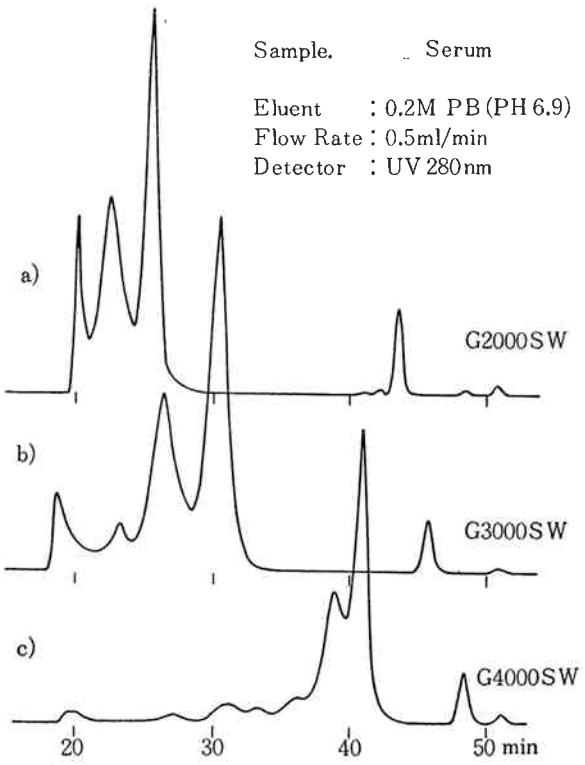


Fig. 7 Separation of human serum by TSK-GEL SW columns of different pore size

The resolution (R_s) of these proteins on the G 2000 SW and G 3000 SW columns is 1.01 and 0.89 respectively. For the separation of myoglobin and β-lactoglobulin (35,000), the resolution of the G 3000 SW column is better (0.55) than the G 2000 SW (0.30). Further, the resolution of β-lactoglobulin and bovine serum albumin (64,000) on the G 3000 SW column is 2.74 while it is 1.78 on the G 2000 SW column. Thyroglobulin (660,000), which was excluded by G 3000 SW (**Fig. 6**), has access to the interior of the G 4000 SW column.

An elution profile of human serum on three different pore size columns is shown in **Figure 7**. For the separation of proteins between the molecular weights of 20,000 to 200,000, which includes most proteins, the G 3000 SW column is the most suitable and, indeed, the most widely used.

3. Factors influencing resolution

The total volume of the chromatographic bed (V_t) is composed of three portions. The solid portion (V_s) is that of the particle itself. The other two portions are the volume between particles (void volume, V_o) and the volume within the particles' pores (inclusive volume, V_i). Thus,

$$V_t = V_o + V_i + V_s \qquad \text{(eq. 9)}$$

During the passage of solute molecules through a gel column, the solute is partitioned between the mobile phase and the solvent in the interior (pores) of the gel particle. Larger molecules, having no access to the interior of the particles, are eluted first. On the other hand, small molecules have access to the interior of the particles, increasing their passage time through the column, and they are eluted later than the large molecules. The elution volume (V_e), i.e., the volume of solvent required to elute the solute from the bed can be expressed by the equation,

$$V_e = V_o + K_d V_i \qquad \text{(eq. 10)},$$

where K_d is a distribution coefficient taking values between 0 and 1, depending on the size of the solute molecules. The large molecules that are excluded from the gel elute at V_o ($K_d = 0$, while the small molecules that can permeate all pores elute at $V_0 + V_i$ ($K_d = 1$).

The following parameters will allow us to compare columns of different dimensions.

$E_o = V_o / V_t$,

$E_i = V_i / V_t$,

Table 2 Comparison of E-values between different commercial columns.[7]

	E_o %	E_i %	E_s %	E_{pi} %
TSKgel G 3000 SW	35.3	47.7	16.9	73.8
TSKgel G 2000 SW	38.9	37.0	24.1	60.5
Waters I-125	37.5	34.5	28.1	55.1
Waters μ-Bondagel	45.3	33.5	21.2	61.2
Shodex OH Pak B-804	38.4	32.6	29.0	52.9
LiChrosorb Diol	47.3	30.0	22.7	56.9
SynChropak GPC 100	39.4	48.2	12.5	79.4

$E_s = V_s/V_t$,

E_o reflects the packing condition of the column. The smaller the E_o, the better the column is packed. However, if the spherical gel is so tightly packed that it is deformed, the number of theoretical plates (N) decreases, and this leads to poor resolution. A theoretically optimal E_o is 25.94%. (**Table 2**). If the E_o is too large, resolution also decreases. When E_i is large, the difference between elution volumes (separation) of two molecules with similar K_d values increases. However, larger E_i implies smaller E_s with a concomitant physical weakening of the packing materials. In general, as the average pore diameter increases, E_i increases. The value E_i and E_s depend solely on the structure of the packing material and not on the method of packing.

Porosity (E_{pi}) is defined by (eq. 11)

$E_{pi} = V_i/(V_s + V_i)$ (ref. 7, **Table 2**).

As E_{pi} approaches 1, the resolution improves; and as E_{pi} approaches 0, the mechanical strength of the gel increases. TSKgel G 3000 SW has a relatively large pore diameter and has the next to the highest values for both E_i and E_{pi}. TSKgel G 2000 SW, with a smaller pore diameter, has an intermediate E_{pi} (**Table 2**).

In gel chromatography, the linear region of a calibration curve can be approximated by

$\log M = b - CV_e = a - mk_d$, $C = m/V_i$ (eq. 12),

where M is the molecular weight.

Furthermore, the resolution or difference in elution volumes (V_e) can be expressed by

$V_e = V_{e1} - V_{e2} = 1/C(\log M_1/M_2)$ (eq. 13)

Thus, the smaller the slope (C), the better the resolution becomes. By the combination of equations 1 and 13,

$R_s = 1/4C \log M_1/M_2 \sqrt{N}/V_e$ (eq. 14)

When the resolution is complete ($R_s = 1$), we obtain

$\log R_m = 4C\ V_e/N$ (eq. 15),

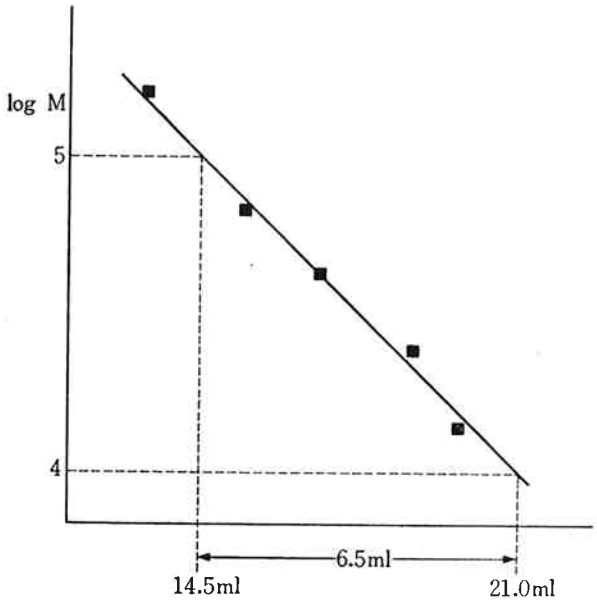
where R_m is the ratio of molecular weight for two molecules. Specific resolution (R_{sp}) in this case is defined as

$R_{sp} = R_s/(\log M_1/M_2) = 1/\log R_m$ (eq. 16).

Table 3 N and R_m values of commercial columns.[7]

Column	Theoretical plate		m/E_i	R_M	R_{SP}
	Ovalbmin	Glycyltyrosine			
TSKgel G 3000 SW	2488	9216	5.83	2.00	3.32
TSKgel G 2000 SW	886	6770	5.62	2.46	2.57
SynChropak GPC 300	848	4200	4.63	3.03	2.08
Waters I-125	1070	4947	8.06	3.09	2.04
SynChropac GPC 100	620	2079	4.71	3.21	1.65
LiChrosorb Diol	418	1764	9.27	11.06	0.48

Let us compare several parameters, i. e., R_{sp}, C, and R_m for several commercial packing



$c=1/6.5ml^{-1}=0.154ml^{-1}$

$m/Ei=c\times Vt=0.154\times 26.8=4.13$

Fig. 8 Calculation of m/E_i from Calibration Curve

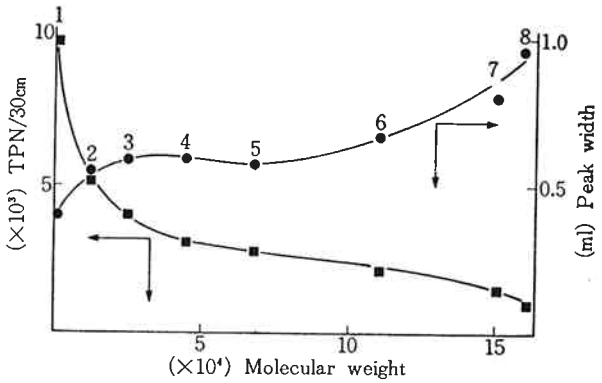


Fig. 9 Dependence of N and peak sharpness on molecular weight.
Column; TSKgel G 3000 SW
Eluent; 25 mM GTA buffer+0.2 M NaCl
Flow Rate; 1.0 ml/min
Sample; 1. Ascorbic acid
2. Cytochrome c
3. α-Chymotrypsinogen
4. Ovalbumin
5. BSA
6. LDH
7. Aldorase
8. γ-Globulin

Table 4 Elution characteristic of proteins on G3000 SW

Sample	V_e (ml)	N	R_M
γ-Globulin	13.3	1300	1.694
Albumin	15.3	4050	1.416
β-Lactoglobulin	17.4	4800	1.416
α-Chymotrypsinogen	18.7	7800	1.355
Cytochrome C	19.2	9300	1.333

materials (**Table 3**, ref. **4**). The ratio of molecular weights (R_m) of two molecules, when complete separation ($R_s=1$) can be achieved, is 2.00 and 2.46 for G 3000 SW and G 2000 SW, respectively, with respect to the elution position of albumin. These are the highest values among the packings listed in **Table 3**. Accordingly, the Rsp for these packings are also the highest. Since the slopes of the calibration curves for TSKgel G 2000 SW and TSKgel G 3000 SW are in the mid range of those in **Table 3**, the high values for R_m and R_{sp} are the result of extremely high theoretical plate numbers (N). It should be noted that the slope of the calibration curve in **Table 3** was determined by simply taking two points (albumin and glycyl tyrosine). However, the slope of the actual calibration cuve (**Fig. 8**) is not constant. (**Figure 8** shows a calibration curve of γ-globulin, bovine serum albumin, β-lactogloglobulin, α-chymotrypsinogen and cytochrome C). It is shallower(smaller m/E_i) in the high molecular weight region than the value in **Table 3**, whereas it is steeper in the low molecular weiht region. Therefore, higher resolution is expected in the actual chromatography of relatively high molecular weight molecles like proteins.

Table 4 shows several parameters calculated from **Fig. 8**, i. e., elution volume of several proteins, numbers of theoretical plates (N), and ratios of molecular weights (R_m) when complete

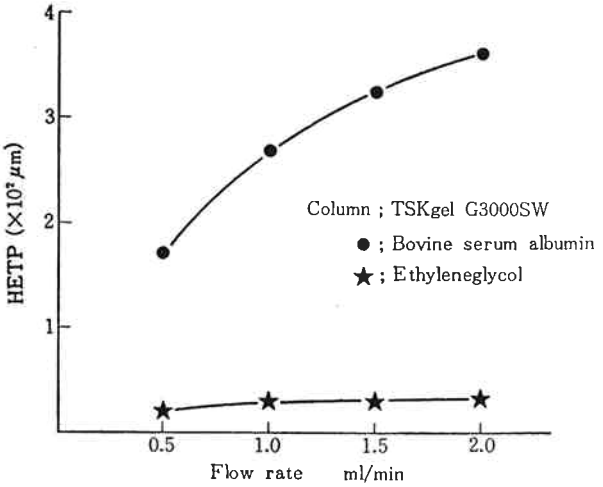


Fig. 10 Dependence of Flow Rate on HETP

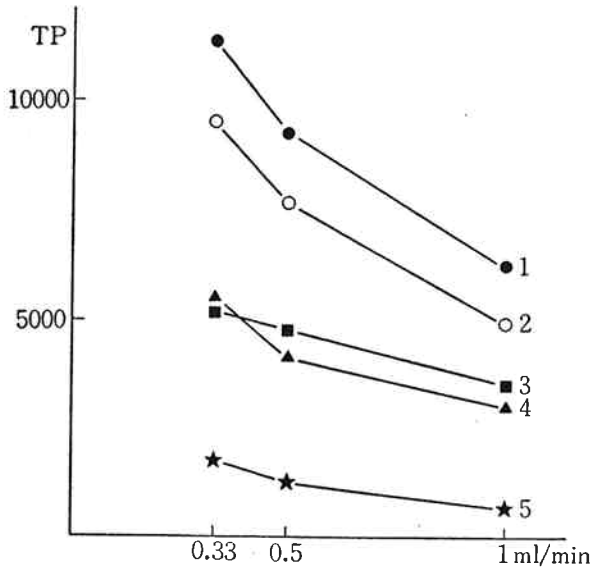


Fig. 11 Dependence of Flow Rate on N
Column; TSKgel G 3000 SW
Eluent;
0.2 M KH_2PO_4+0.2 M K_2HPO_4 (pH 6.9)
Sample;
1. Cytochrome c (MW=13,000)
2. α-Chymotrypsinogen A (MW=24,000)
3. β-Lactoglobulin (MW=41,000)
4. Albumin (Bovine) (MW=65,000)
5. γ-Globulin (MW=16.000)

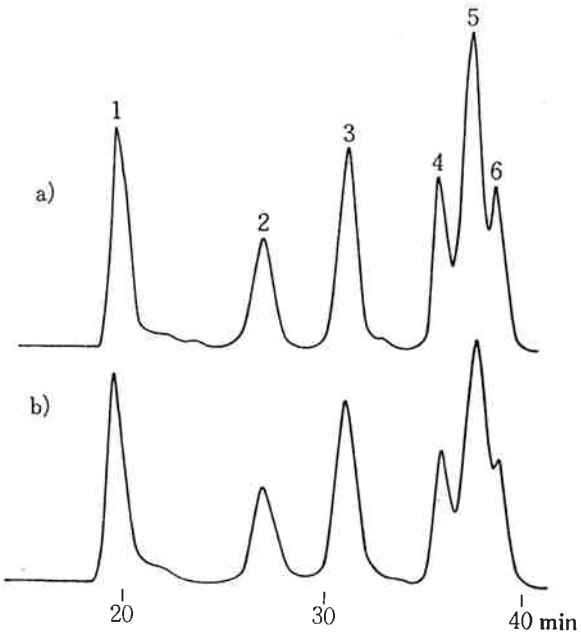


Fig. 12 Comparison of resolution using two columns as compared with one column.
a) G 3000 SW× 2(120 mm) 1.0 ml/mm
b) G 3000 SW×1 (600 mm) 0.5 ml/mm
Eluent; 0.2 M KH_2PO_4+0.2 M K_2HPO_4 (pH 6.9)
Sample; 1. Thyroglobulin
2. γ-Globulin
3. Albumin
4. β-Latoglobulin
5. α-Chymotrypsinogen A
6. Cytochrome C

resolution (R_s=1) can be achieved. In general, two proteins can be separated if the ratio of their molecular weights is around 1.35 to 1.65 on a TSKgel G 3000 SW column (7.5 mm i.d. 600 mmL) at a flow rate of 0.5 mℓ/min. **Figure 9** shows the dependence of theoretical plate number and peak broadening on molecular weights of proteins. The smaller the molecular weight the larger is the N (or the sharper the peak). This is consistent with the fact that smaller molecules have a larger diffusion constant and vice versa. Therefore, the smaller the protein the better the R_m can be expected to be. The dependence of peak broadening on molecular weight is particularly conspicuous in the region close to V_o or (V_o+V_i).

Flow rates also affect peak sharpness. This can be understood with regard to equations 4, 5 and 6. In practical flow rate range, where we can ignore equation 4, a higher N can be obtained with a slower flow rate. In general, flow rates have more influence on the higher molecular weight molecules, which have smaller diffusion constants. For example, when we compare a

small molecule, ethylene glycol, with a larger one, albumin, we see that albumin exhibits a broader peak or a larger height equivalent to a theoretical plate (H). Thus, H is largely dependent on the flow rate (**Fig. 10**).

The number of theoretical plates on a TSKgel G 3000 SW column was measured using several different proteins at different flow rates of 1.0 mℓ/min, 0.5 mℓ/min, and 0.33 mℓ/min (**Fig. 11**). There is an increase in N as the flow rate decreases. When two flow rates are compared, 1.0 mℓ/min and 0.5 mℓ/min, it can ge seen that N is about 1.5 times higher for the slower flow rate. Since N is proportional to the column length (L), increased separation can be expected when several columns are connected in series. When the column is twice as long (i. e., N is doubled), the resolution increases by $\sqrt{2}$. This is higher increase than can be obtained by halving the flow rate. **Figure 12** compares the separation of proteins using two columns in series and one column with a flow rate decreased by half. Using two columns gives better separation, although the running time in both cases is the same.

4. Selection of mobile phase and estimation of molecular weight

Knowledge of the interaction between solute and gel phases frequently helps one to overcome difficulties in gel chromatography such as sometime result in unexpected separations. In addition to molecular sieve effects, secondary interactions of solute with matrix, such as ionic, hydrophobic, and hydrogen bonding effects are also expected. TSK-GEL SW Type packing, which consists of silica (residual silanols are slightly exposed), has anionic properties, i. e., exclusion effects toward anionic species and ion exchange effects toward cationic species. Such residual silanols are found mainly in small pores where large molecules such as proteins can not permeate. Therefore, the smaller the solute, the greater the interaction of the small molecule to the silanol becomes[2], especially when the pore diameter is also small. For example, citric acid is excluded more from small pore packing (G 2000 SW) than from large pore packing (G 3000 SW) when low ionic strength solvent is used[7]. In general, this exclusion effect is of minor importance under normal operating conditions using mobile phases containing salt of 0.1 M or more, especially for large

Table 5 Dependence of K_d of arginine or ionic strength on different columns.[7]

	Ionic strength of eluent					
	0.026	0.12	0.24	0.60	1.20	2.40
TSKgel G 3000 SW	1.30	1.05	1.02	1.00	—	0.98
SynChropak GPC 100	1.35	1.06	1.01	—	—	0.98
LiChrosorb Diol	1.53	1.15	1.05	0.99	—	1.07
TSKgel G 2000 SW	1.57	1.06	1.02	0.99	—	0.98
Waters I-125	1.70	1.23	1.16	1.08	—	1.05
Waters μ-Bondagel	1.75	1.11	1.06	1.02	—	1.00
Shodex OHpak B-804	2.06	1.16	1.07	1.02	1.02	

Table 6 Dependence of K_d of phenylethanol on ionic strength on different columns.[7]

	Ionic strength of eluent					
	0.026	0.12	0.24	0.60	1.20	2.40
SynChropak GPC 100	1.44	1.49	1.53	1.63	1.81	2.33
TSKgel G 3000 SW	1.47	1.50	1.53	1.61	1.81	2.35
Waters I-125	1.83	1.88	1.88	2.03	2.29	3.03
TSKgel G 2000 SW	1.93	2.02	2.10	2.30	2.71	4.01
LiChrosorb Diol	2.49	2.56	2.64	2.93	3.52	5.31
Waters μ-Bondagel	5.72	5.19	5.37	5.97	7.44	11.47
Shodex OHpak B-804	6.36	6.65	6.96	8.47	10.96	

molecules like proteins. By analogy to the exclusion effect, an ion exchange effect is also expected to be conspicuous in small pore packing when ionic strength is low. **Table 5** shows the effect of ionic strength on the K_d value of a basic amino acid, arginine. When the ionic strength is less than 0.24, arginine (which contains a strong basic group) is eluted at alate elution volume ($K_d>1$), owing to ionic interactions with silanol. **Table 6** illustrates the effect of salt concentration of the elution of phenylethanol which is hydrophobic and has no ionizing group. In contradistinction to the example of arginine, an increace in salt concentration results in hydrophobic interactions causing later elutions ($K_d>1$, observed with all the ionic strengths investigated). As expected this effect is more conspicuous with G 2000 SW than G 3000 SW.

So far, we have discussed the interactions of small molecules only. Now, let us consider

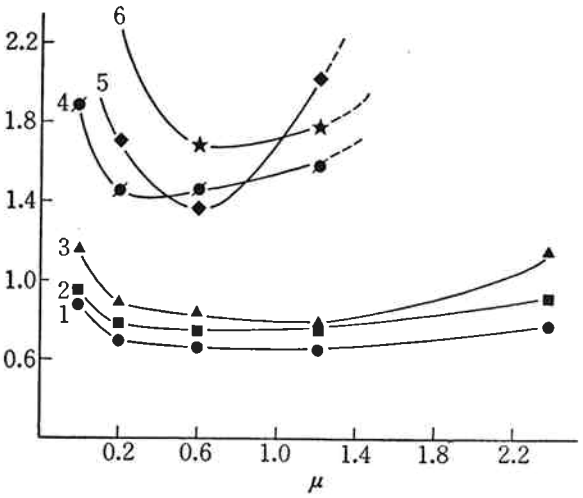


Fig. 13 Dependence of K_d of lysozyme on ionic strcngth[7]
1. THKgel G 3000 SW
2. TSKgel G 2000 SW
3. LiChrosorb Diol
4. Waters I-125
5. Shodex OHpak B-804
6. SynChropak GPC100

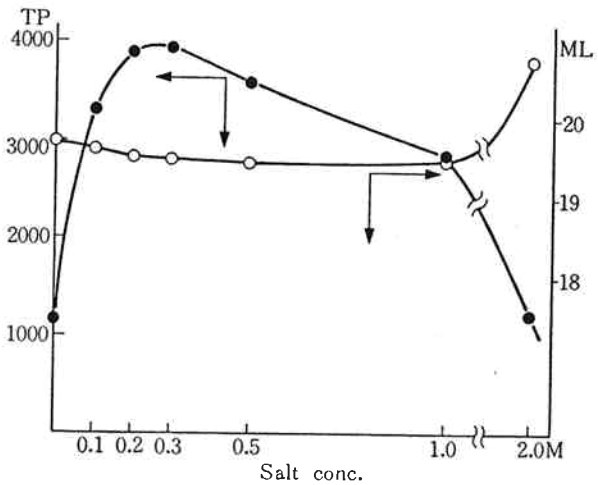


Fig. 14 Effect of ionic strengh on elution volume and N
Column; TSKgel G 3000 SW
Ewent; 50 mM GTA Buffer + NaCl
Sample; α-Chymotrypsinogen.

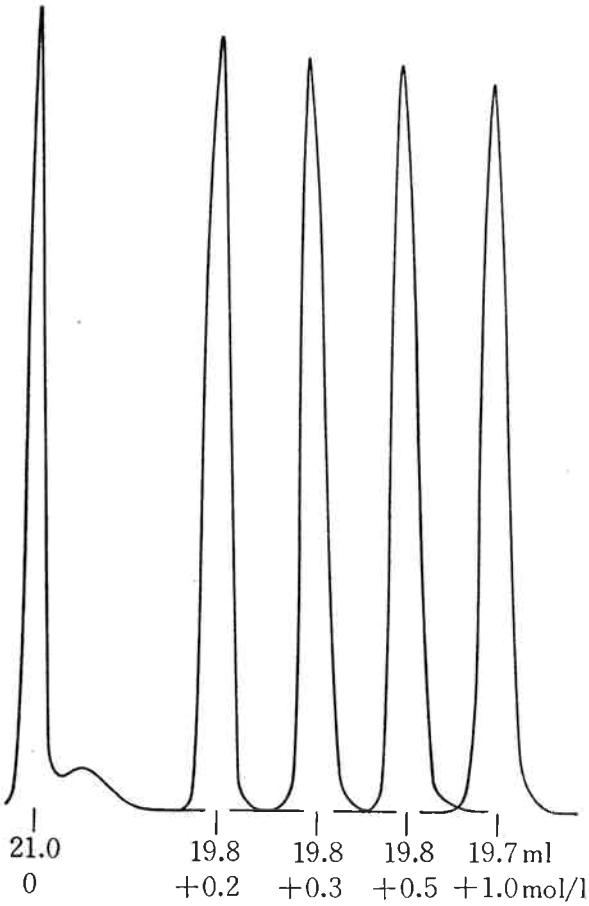


Fig. 15 Effect of salt concentration on peak shape
Sample; Cytochrome c
Eluent; 0.05M Phosphate Buffer (pH 6.9)+NaCl

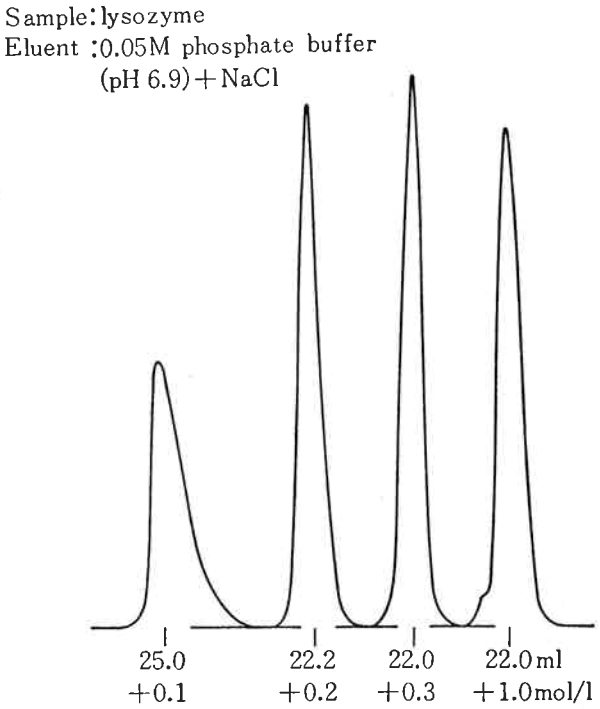


Fig. 16 Effect of salt concentration on peak shape

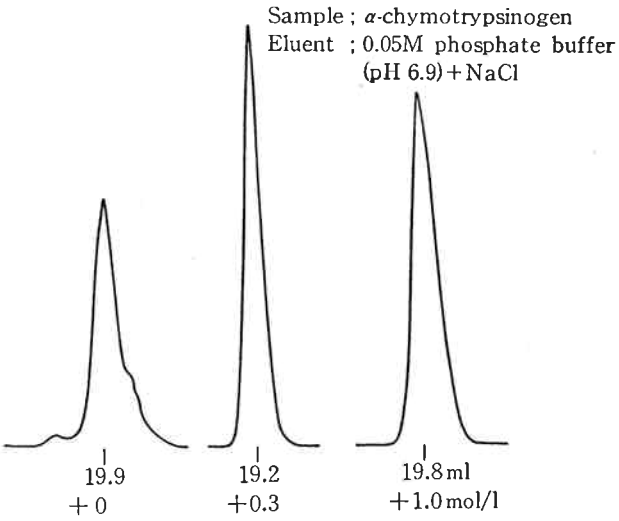
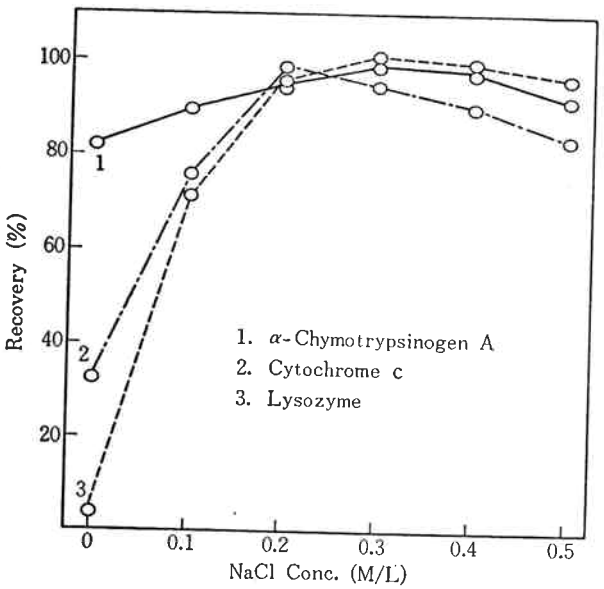


Fig. 17 Effect of salt conc. on peak shape

secondary interactions with large molecules like protein. Since proteins have compact conformations, the effective surface area is relatively small compared with their mass. Therefore, the extent of secondary interactions is not as great as that with small molecules. However, complex interactions are expected for proteins. **Fig. 13** depicts the effect of ionic strength on the K_d of lysozyme, which is basic and hydrophobic in nature, with six commercial packings. The elution of lysozyme is retarded at both strong ionic strengths (hydrophobic interactions) and weak ionic strengths (ionic interactions). However, of the six packings, TSKgel G 2000 SW, TSKgel G 3000 SW, and LiChrosorb Diol have a relatively small degree of adsorption, suggesting that relatively idealistic gel chromatography can be obtained with these packings. G 2000 SW and G 3000 SW have similar adsorptions to lysozyme, which is consistent with the fact that secondary interactions exist mainly in the small pores where large molecules can not permeate.

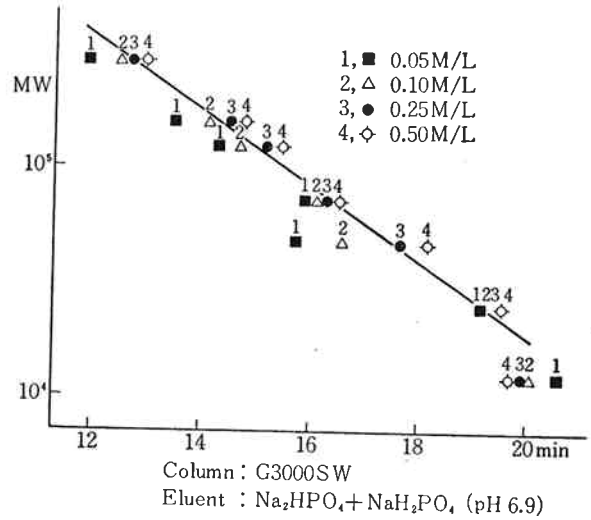
Secondary interactions affect not only retention (K_d), but also peak sharpness (N). **Fig. 15–17**

illustrate elution profiles of cytochrome c, α-chymotrypsinogene, and lysozyme at various concentrations of salt in the mobile phase. At high and low salt concentrations, the peaks become broader or an additional peak appears. **Fig. 14** shows the dependence of N as well as V_e of α-chymotrypsinogen on the salt concentration in the mobile phase. Similarly, as in the case of lysozyme, in both low and high concentration ranges (<0.1 and>1.0 M), the degree of adsorption is high, and a plateau is observed in the range of 0.1 to 1.0 M. On the other hand, the peak becomes broader in both the low and high concentration ranges described above. The maximum N is observed in the range of 0.2 to 0.3 M. The optimum salt concentration that gives the sharpest peak seems to differ from protein to protein. For cytochrome c, α-chymotrypsiongen and lysozyme, the optimum salt concentrations are 0.1 to 0.2, 0.2 to 0.3, and 0.3 to 0.4 M, respectively. **Figure 18** shows the recovery of the three proteins from a TSKgel G 3000 SW column. A correspondence is observed between the salt concentration resulting in maximum



Column : TSKgel G3000SW
Eluent : 0.05M Phosphate buffer (pH 6.9)+NaCl
Load : 60ug

Fig. 18 Effect of salt concentration on recovery



Column : G3000SW
Eluent : $Na_2HPO_4+NaH_2PO_4$ (pH 6.9)

Fig. 20 Effect of salt concentration on elution volume (II). Conditions as in Fig. 19

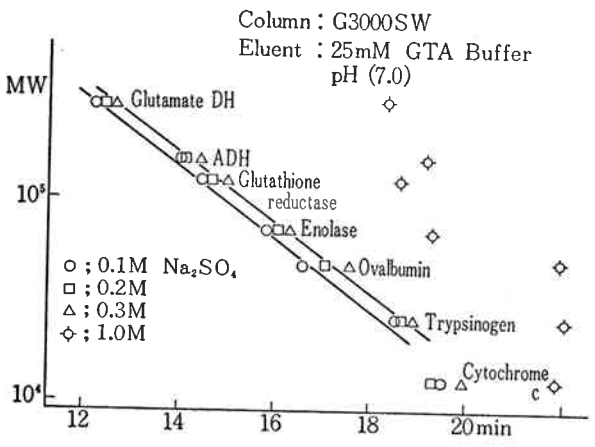


Fig. 19 Effect of salt concentration on elution volume (I)

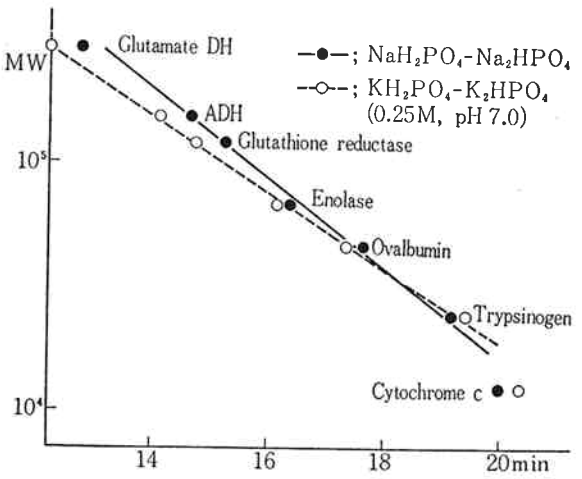


Fig. 21 Effect of cationic species on calibration curve

recovery and maximum N. Therefore, it is expected that optimum salt concentrations that produce maximum N also produce the best recovery and appropriate K_d simultaneously.

On the basis of the above discussion, it is reasonable to expect that calibration curves also vary by changing salt concentrations. **Fig. 19** shows the effect of salt (Na_2SO_4) added to the mobile phase (0.025 M GTA buffer, pH 7.0) on a calibration curve. It is found that the intercepts of the calibration curves are slightly changed by the addition of salt in 0.1 to 0.3 M range, which is usually employed for the mobile phase. By further addition of salt to 1.0 M, conspicuous adsorption due to hydrophobic interactions is observed (extremely large K_d). The effect of adding Na_2SO_4 is higher compared to that of NaCl, which is consistent with the higher ionic strength of Na_2SO_4. It is advisable to avoid the use of halogen containing compounds,

Table 7 Effect of ionic species of buffer on separation of protein.
Column; TSKgel G 3000 SW
Eluent; 0.05 M GTA Buffer + Salt
Resolution $=2(V_2-V_1)/(W_2+W_1)$

Sample \ Salt	0.2 M $(NH_4)_2HPO_4$	0.2 M Na_2SO_4	0.2 M $(NH_4)_2SO_4$	0.2 M $MgCl_2$	0.2 M NaCl
Glutamate DH	1.35	1.14	1.54	1.26	1.54
ADH	0.42	0.46	0.24	0.45	0.63
Glutathione red	0.94	1.08	0.89	0.92	1.21
Enolase	1.07	0.82	1.26	0.81	0.36
Ovalbumin	1.27	1.29	1.36	1.90	2.22
Trypsinogen	0.97	0.54	0.20	0.76	0.91
Cytochrome C					

which deteriorate stainless steel tubing. A similar relationship was observed using phosphate buffer (**Fig. 20**). Using phosphate as the common anionic species, the effect of cationic species on the calibration curve was investigated using Na and K (**Fig. 21**). The slope of the calibration curve produced by K is shallower than that produced by Na. By using sorenson buffer, which consists of both sodium and potassium, the slope of the calibration curve is halfway between the slopes produced by either cation alone.

Table 7 shows the effect of ionic species added to the mobile phase (0.05 M GTA buffer, pH 7.0) on the solution of several proteins. It is found that included ionic species also affect resolution. Therefore, the

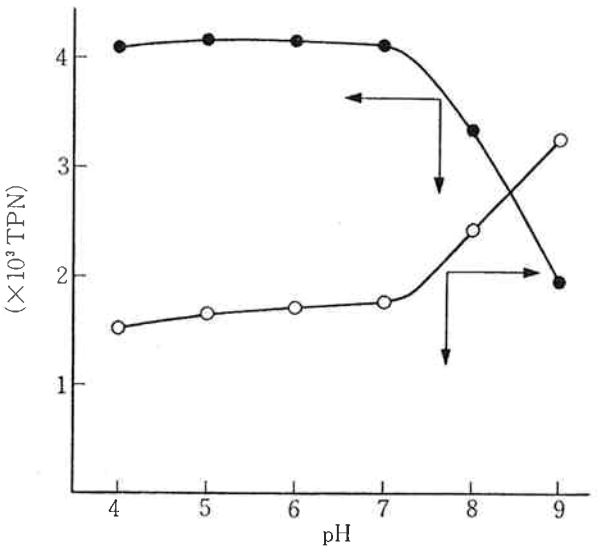


Fig. 22 pH dependence of elution volume
Column; TSKgel G 3000 SW (60 cm)
Eluend; 25 mM GTA Buffer + 200 mM NaCl
Sample; α-Chymotrypsinogen
Flow rate; 1.01 ml/min

Table 8 Recovery of various proteins

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

selection of ionic species is important for the separation of proteins.

Table 8 shows the effect of ionic species on the recovery of several proteins. Both sodium phosphate and potassium phosphate give consistently high recovery. Furthermore, phosphate, which has buffer action in a broad range of pH and does not absorb in the UV range, is a good buffer.

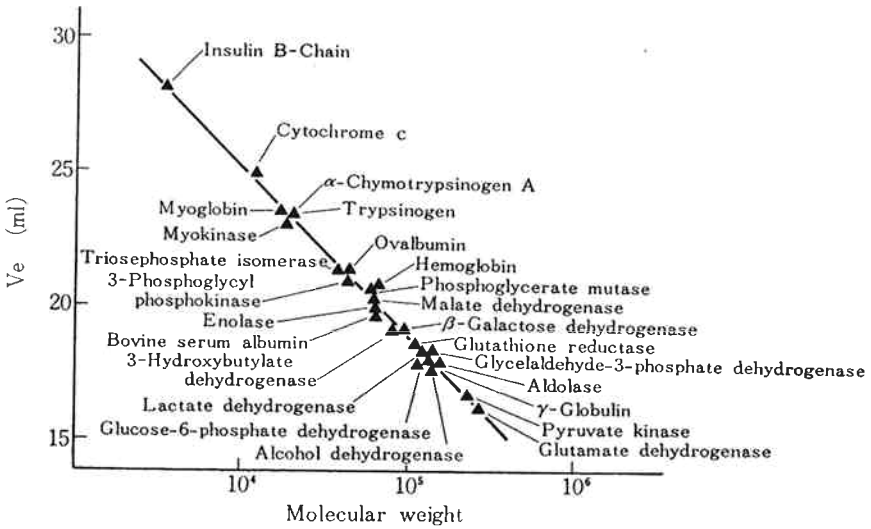
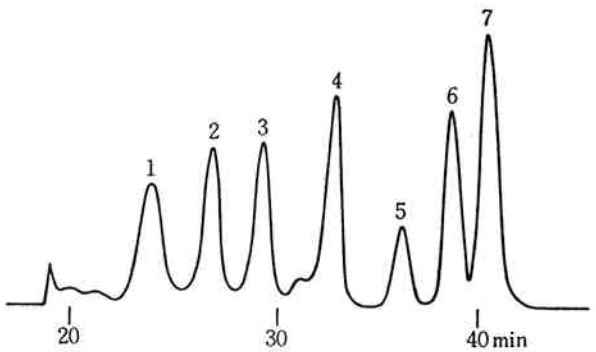


Fig. 23 Typical calibration curve for proteins[9]
Column; TSKgel G 3000 SW (60 cm)
Eluent; 50mM phosphate buffer+0.2 M NaCl (pH 7.5)
Flow rate; 0.3 ml/min.

Figure 22 shows the dependence of N on pH using α-chymotrypsinogen (which is basic, pI=9.5). At basic pH the retention of α-chymotrypsinogen becomes longer and the N becomes smaller. (At pH>8, SW columns will deteriorate, so use of high pH should be avoided as will be discussed later). In general, it is advisable to use a buffer whose pH is different from the isoelectric point of the protein.

So far, we have discussed the parameters



Column ; TSK gel G3000SW
Eluent ; 50mM phosphate buffer
+0.2M NaCl (pH 7.5)

Fig. 24 Separation of Proteins

(salt concentration, ionic species, pH) that affect the form of the calibration curve. However, at fixed conditions (pH 7, salt concentration 0.2 to 0.3 M), linear relationships between log M and Ve are usually obtained. The molucular weights of several proteins were determined on TSK -gel G 3000 SW (**Figs. 23** and **24**), and the observed values compared with the reported values (**Table 9**), which were obtained using classical soft packings. The deviation is only 6%[9]. It is clear that accurate estimations of molecular weights using TSKgel SW columns are possible.

Table 9 Estimation of molecular weight on TSKgel G 3000 SW and sephadex G 200[8]

Protein (DH; dehydrogenase)	Saurce	pI	Molecular weight		
			Ref-erence	G 3000 SW (△MW)	*Sephadex G-200 (△MW)[10]
Glutamate DH	Yeast		280,000	268,000(−12,000)	
Pyruvate kinase	Rabbit muscle	7.8	237,000	201,000(−36,000)	
γ-Globulin (Cohn fr. II)	Human	6.3	160,000	155,000(−5,000)	210,000(50,000)
Alcohol DH	Yeast	5.4	150,000	155,000(5,000)	150,000(0)
Aldolase	Rabbit muscle	8.2	150,000	132,000(−18,000)	145,000(−5,000)
Glycelaldehyde-3-phosphate DH	Rabbit	8.3	146,000	105,000(−41,000)	130,000(−16,000)
Lactate DH	muscle	4	132,000	135,000(3,000)	140,000(8,000)
Glucose-6-phosphate DH	Lactobacillus	6	128,000	140,000(12,000)	
Glutathione reductase	Yeast		113,000	114,000(−4,000)	
β-Galactose DH	P. fluorescens	6.5	101,000	95,000(−6,000)	
3-Hydroxybutylate DH	R. spheroides		85,000	94,000(9,000)	
Malate DH	Pig seart	6.1	67,000	54,000(−13,000)	63,000(−4,000)
Hemoglobin	Beef blood	7	67,000	34,000(−33,000)	
Enoiase	Yeast	5.4	67,000	69,000(2,000)	
Bovine serum albumin	Bovine	4.7	67,000	94,000(27,000)	70,000(3,000)
Phosphoglicerate mutase	Rabbit muscle	6.8	64,000	59,000(−5,000)	
3-Phosphoglycerate phosphokinase	Yeast	7.2	47,000	50,000(3,000)	
Ovalbumin	Hen egg	4.6	45,000	54,000(9,000)	42,000(−3,000)
Triosephosphate isomerase	Rabbit muscle	5.8	43,000	52,000(9,000)	
α-Chymotrypsinogen A	Bovine pancreas	9	24,000	18,000(−6,000)	24,000(0)
Trypsinogen	Bovine pancreas	9.3	24,000	21,000(−3,000)	
Myokinase	Pig heart	4.3	21,000	30,000(9,000)	
Myoglobin	Whale muscle	8.1	18,000	19,000(1,000)	19,000(1,000)
Lysozyme	Egg white	11	14,000	1,000(−13,000)	
Cytochrome c	Horse heart	9.0	13,000	12,000(−1,000)	13,000(0)
Insulin B-chain	Bovine		4,000	4,000(0)	
Ovomucoid			28,000		55,000(27,000)
Fetuin	Calf serum		47,000		120,000(73,000)
Fumarase	Pig heart		205,000		160,000(−45,000)
Ferritin	Horse spleen		750,000		500,000(−250,000)

* Sephadex G-200 column (2.5×50 cm), Eluent; 0.05 M Tris+0.1 M KCl (pH 7.5)

5. Sample size considerations

Similar to other modes of chromatography, high sample loads cause a decrease in N and distortion of peak shape (overload). It is, in general, possible to increase sample load by the addition of organic solvent or by increasing column temperature which produces a lower

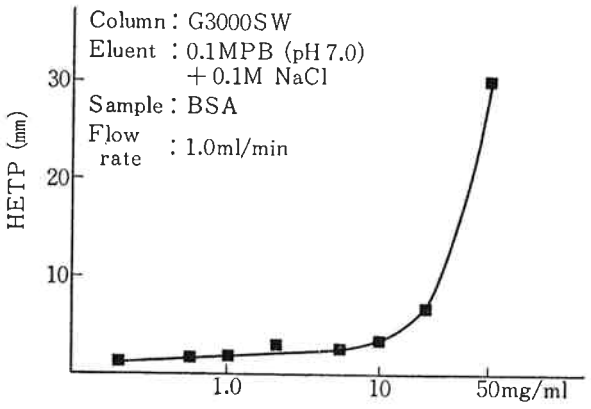


Fig. 25 Effect of sample size on H

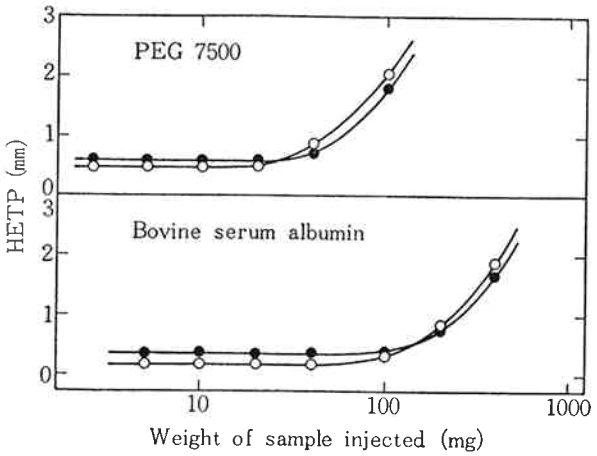


Fig. 26 Flow rate dependence of maximum sample loading on G 3000 SWG.
Column size: 2.15 cm I.D. ×120 cm
Injection volume: 4 ml
Sample: BSA and polyethyene glycol of molecular weight 7,500
Solvent: 0.1 M phosphate buffer containing 0.3 MNaCl (pH=7)
Flow rate: (○) 4 ml/min, (●) 16 ml/min
Temperature: 25°C
Detector: RI

viscosity and elevated solubility of the sample. However, it is not always possible to use such techniques for proteins. **Fig. 25** illustrates the dependence of sample concentration (bovine serum albumin) on H using a G 3000 SW analytical column. When samble of higher than 1 mg/100 μl is injected a rapid increase in H is observed. In general, proteins with their compact structure can be loaded at higher concentrations than other synthetic macromolecules as is illustrated in **Fig. 26.** On a TSKgel G 3000 SW preparative column up to 100 mg of BSA (m. w. = 66, 500) as compared to only 20 mg of polyethylene glycol (m. w. = 7, 500) can be loaded while rataining normal H values.

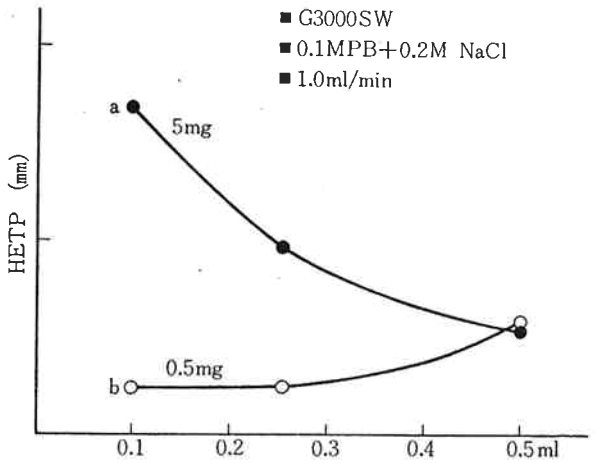


Fig. 27 Effect of injection volume on H

Since maximum sample load is proportional to the cross sectional area of a column, using a larger diameter column (21.5 mm), 8 to 9 times higher sample load can be used than when using an analytical column (7.5 mm). Furthermore, because of the reduced degree of wall effect of larger diameter columns, larger diameter packings can be applied[1] and another 10-fold excess of sample can be loaded.

It should be noted that in general, the slower the flow rate the higher is the resolution. However, when the sample load is high, the opposite is observed (**Fig. 26**).

Fig. 27 shows the effect of sample volume on H. When a large amount of protein is loaded (5 mg), lower concentrations (large volume) reduce H. On the other hand' when a small amount (0.5 mg) of protein is loaded, higher concentrations (small volumes) result in reduced H. In general, appropriate sample concentrations are in the range of 1 to 20 mg/ml.

6. Advice to the user

In this last chapter we will discuss several factors resulting in the deterioration of the column itself and the concomitant poor resolution. The four following causes typically result in poor resolution:

1) formation of dead space in the inlet side of the column (top-off),
2) change in the packing state (channeling) caused by drying or freezing,
3) denaturation at the surface of the column, and
4) contamination of the column with precipitate.

The use of higher flow rates than recommended, or sudden pressure surges to the column must be avoided, as they may cause compression of the packing (cause of 1), especially for large pore packing, i. e., G 4000 SW. For this reason, continuous flow injection such as that provided by a loop injection is recommended. Installation of a pulse damper may help to suppress sudden pressure surges, which are often observed when using a quick-return pump.

If the column is not to be used for several days, it should be kept tightly sealed to prevent drying (2). To avoid freezing, the addition of an organic solvent is possible. Since SW Type packing is based on silica gel, only acidic or neutral pH solvents should be used. Silica gel is dissolved at high pH (cause of 3). Since the sample could be a strong base, a mobile phase of strong buffer capability at neutral or preferably acidic pH is recommended. Phosphate buffer (pH 6.5 to 7.2) should serve this purpose. The pH change caused by the sample is great at the inlet side of the column. Therefore, the use of a guard column is recommended not only for the prevention of (4), but also for the prevention of (3). The prefiltering of solvent and sample with the use of a precolumn should prevent the contamination of the column. If, however, the column does become contaminated, it is possible to clean it with the following solvents:

1) concentrated salt solution of low pH, i. e., K_2SO_4 (avoid the use of halides, as they will rust stainless steel) for contamination with basic compounds,
2) organic solvents (i. e., methanol, acetonitrile, tetrahydrofuran, dimethylformamide, dioxane, etc.) for contamination with hydrophobic compounds,
3) chaotropic reagents (i. e., SDS, urea, guanidine, etc.) or proteolytic enzymes (i. e., pepsin) for precipitated protein. It takes a long time to remove SDS or guanidine after their use as eluents. If the removal is not complete, there is sometimes unexpected elution behaviour of a sample. Therefore, it is recommended that two columns should be prepared for special uses.

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