Chromatographic Behaviors of Proteins and Amino Acids on
a Gel Filtration Matrix, TSK-GEL Toyopearl†

Kuniyo INOUYE*

Biotechnology Research Laboratories, TOSOH Corporation,
Ayase, Kanagawa 252, Japan
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The chromatographic behaviors of proteins and amino acids on a gel filtration matrix, TSK-GEL
Toyopearl have been examined, and the effects interfering with their elution from Toyopearl gel were
analyzed individually. Many proteins are retarded in elution to various extents in 25 mM Tris-HCl
buffer at pH 7.5. All the proteins examined of isoelectric points (pI) higher than the pH value of
elution buffer (pH 7.5) are retarded in comparison with the elution of the proteins of pI less than 7.5.
The retardation can be diminished almost to nothing by the addition of 0.3-0.5 M NaCl, suggesting
that the electrostatic interaction works between the proteins and gel matrix. On the other hand, the
adsorption of some proteins can be reduced by the addition of 30% ethanol (v/v) to the eluent. These
proteins were supposed to be adsorbed to the gel matrix by hydrophobic interaction. Aromatic amino
acids such as tryptophan and tyrosine are adsorbed to the gel matrix strongly, but no aliphatic
hydrophobic amino acids (e.g., leucine and isoleucine) and no charged amino acids (e.g., lysine,
arginine, aspartic acid and glutamic acid) are adsorbed to the matrix at all. By considering the effects
interfering with the elution of proteins and amino acids from Toyopearl gel, it is possible to separate
them more effectively by the subtle differences in their properties by changing pH, ionic strength, and
dielectric constant of the elution buffer.

The history of biochemistry is also the
history of separation and isolation of biological
substances.1,2) Many significant discoveries in
biochemistry have been brought about by
conquest of the difficulties in separation and
isolation. Ultracentrifugation, electrophoresis,
isolectric focusing, ultrafiltration, ion exchange
chromatography, and gel filtration are
such examples. Especially, gel filtration, since
it was introduced around 1960, have been
widely used as an essential technique not only
for separation and isolation of biological
substances but also for estimation of their
molecular weights.3,4) The rapid spreading and
development of this method indicate that the
simple and convenient method of this type were
eagerly wanted for separation.

In this paper we describe the chromatographic behaviors of proteins and amino acids
on TSK-GEL Toyopearl, a hydroxylated vinyl polymer for gel filtration.3-5)

The mechanical strength of this gel is larger than that of the gels that had been used for gel
filtration such as starch, dextran, cellulose, agarose, and polyacrylamide gels. This property makes the gel usable under higher pressure,
and thus provides rapid chromatography and higher resolution.5) The flow-rate of 20-50
ml/cm²/hr can be used for gel chromatography
on a Toyopearl column. This implies that this
gel can be used as a high performance or
semi-high performance liquid chromatograph-
ic gel, especially for preparative purposes.
The strong resistance against bacteria and

† TSK-GEL Toyopearl is the same material as Fractogel TSK available from E. Merck (Darmstadt, Germany)
and MCR Reagents (New Jersey, U. S. A.).
* Present address: Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Sakyo-ku,
Kyoto 606, Japan.
Abbreviations: pI, isoelectric point; MW, molecular weight; K<sub>m</sub>, available constant; ADH, alcohol dehydrogenase.
organic solvents is another merit of this gel.\textsuperscript{5)}
Because of these preferable features, this gel has been widely used for the purification of proteins.\textsuperscript{6}−\textsuperscript{12)} However, we have been aware of non-specific adsorption of proteins on the Toyopearl gel. There actually has been no report on the chromatographic behaviors of biochemical substances on the gel, in spite of its wide use. This study was started to understand the nature of the adsorption and to obtain the essential knowledge required for efficient separation. In this paper, it will be shown that there are several kinds of interacting forces working between proteins and Toyopearl gel, and that by controlling these forces Toyopearl can be expected to become a very useful means of separating biochemical substances.

\section*{Materials and Methods}

TSK-GEL Toyopearl HW-55 Fine (Lot 55008) was provided by TOSOH Corp. (Tokyo) as an aqueous suspension. The gel was packed in a glass chromatographic tube of 2.2 cm (inner diameter) × 50 cm (Sepacol Column Type SK 22/50, Seikagaku Kogyo Co., Tokyo) following the recommendation of the manufacturer.\textsuperscript{13,14)} The flow-rate was maintained at 1.0 ml/min with a peristaltic pump, and fractions of 2.6 ml were collected by a fraction collector. The distributions of proteins and aromatic amino acids in the column effluents were analyzed by absorbance measurements at 280 nm, and of hemeproteins also at 420 nm, while the elution profile of the non-aromatic amino acids was monitored by the absorbance at 200 nm. The absorbance measurements were done with a high sensitivity recording spectrophotometer SM-401 (Union Giken Co., Osaka). The elution buffer used was a 25 mM Tris–HCl buffer of pH 7.5 (Standard Buffer), and in some cases was used the Standard Buffer containing salt or ethanol. The sample concentration was 1–2 mg/ml for proteins and 0.03 mg/ml for amino acids and their derivatives dissolved in the buffer solutions used for their elutions. The sample volume injected was 3.0 ml. Chromatography was operated at 5–10°C.

Proteins and chemicals were all purchased from Nacalai Tesque, Inc. (Kyoto) and Sigma Chemical Co. (St Louis, MO). Their molecular weights (MW) and isoelectric points (pI) are given elsewhere.\textsuperscript{15,16)}

\section*{Results}

\subsection*{Elution of proteins with the Standard Buffer}

Figure 1 shows the relationship between the molecular weight (MW) of proteins and their elution volume. The characteristic feature of this figure is that elution of the proteins of pI greater than 7.5 is retarded in comparison with that of proteins of pI less than 7.5 and of the same molecular weight. For example, trypsin (pI 10.5) is eluted far behind soybean trypsin inhibitor (pI 4.3–4.6) in spite of the similar molecular weight. The solid lines in Fig. 1 were drawn to fit the data points for most of the proteins whose pIs are less than 7.5. Proteins in the zone between them were considered to be of normal elution.

However, some proteins, such as catalase, yeast alcohol dehydrogenase (ADH), hemoglobin, and apotransferrin, the pIs of which are less than 7.5 are also eluted in retard.

\subsection*{Elution of proteins with the Standard Buffer containing salt.}

Figure 2 shows the effects of the addition of 0.5 M NaCl on the elution volume. Most of the proteins the pIs of which are greater than 7.5 are eluted more rapidly in the presence of 0.5 M NaCl (data shown by ●) than in the absence of NaCl (data shown by ○). The extent of the effects of salt on the reduction of adsorption varies with proteins used. The elution of cytochrome c, ribonuclease A, and subtilisin BPN' are between the solid lines, that is these proteins might have no interactions with the gel in the presence of 0.5 M NaCl. Some proteins such as lysozyme and thermolysin, however, show some adsorption to the gel even in the presence of 0.5 M NaCl. Some proteins of pI less than 7.5, such as catalase, yeast alcohol dehydrogenase (ADH), and hemoglobin, which show some retardation in the Standard Buffer, also have the retardation in the presence of salt. Their retardation is not affected by the addition of 0.5 M NaCl, 0.5 M LiCl, or 0.25 M Li$_2$SO$_4$ at all.

In Fig. 3 is depicted the dependence of the available constant ($K_{av}$) of four typical pro-
Fig. 1. Relationship between Molecular Weights (MW) of Proteins and Their Elution Volumes.
Chromatography was done on a Toyopearl HW-55 Fine column (2.2 cm inner diameter) × 50 cm) with 25 mM Tris-HCl buffer, pH 7.5 (Standard Buffer). See the text (Materials and Methods) for the other conditions. The number in parenthesis is the value of the isoelectric point (pI) of each protein.\textsuperscript{15,16}
Fig. 2. Comparison of the Elution Volumes of Proteins with and without NaCl.
See Fig. 1 for the other conditions. ○, in the absence of NaCl (see Fig. 1); ●, in the presence of 0.5 M NaCl.
proteins on the NaCl concentration added to the Standard Buffer. The available constant is defined by the following equation, $K_{av} = (Ve - Vo)/(Vt - Vo)$, where $Vt$ is the total volume of the gel bed, $Ve$ is the elution volume of a protein, and $Vo$ is the void volume which was regarded as the $Ve$ value for blue dextran (MW $2 \times 10^6$). The molecular weight and pI are, respectively, $2.57 \times 10^4$ and 9.5 for bovine pancreatic chymotrypsinogen A; $1.84 \times 10^4$ (monomer) and 5.2 for bovine milk $\beta$-lactoglobulin; $1.78 \times 10^4$ and 7.0 for horse muscle myoglobin; 1.44 $\times$ $10^4$ and 11.0 for egg white lysozyme. The $K_{av}$ values for lysozyme and chymotrypsinogen A decrease with increasing NaCl concentration up to 0.3 M, while those for myoglobin and $\beta$-lactoglobulin remain constant regardless of the addition of NaCl.

In the presence of 0.5 M LiCl, all proteins of pI greater than 7.5 except lysozyme are eluted between the two solid lines (figure not shown). This result shows that the interaction of these proteins with the gel is diminished in the presence of 0.5 M LiCl. Addition of 0.5 M NaCl reduces the elution volume of lysozyme from 390 ml to 155 ml (Fig. 2), while addition of 0.5 M LiCl and 0.25 M Li$_2$SO$_4$ reduces it to 147 ml and 126 ml, respectively. The elution volumes obtained in the presence of 0.5 M LiCl and Li$_2$SO$_4$ may be regarded as normal. On the other hand, $Ve$ values of thermolysin, bovine hemoglobin, and bovine chymotrypsinogen A become greater in the presence of 0.25 M Li$_2$SO$_4$ than those in the presence of NaCl or LiCl, namely, 137.8→140.9 ml for thermolysin; 106.0→111.0 ml for hemoglobin; and 118.8→135.5 ml for chymotrypsinogen A. The behaviors of these proteins therefore suggested that there should be also the other interaction than the electrostatic nature, and it might be a hydrophobic interaction (see Discussion).

**Elution of proteins with the Standard Buffer containing 30% ethanol**

Figure 4 shows the relationship between the available constant ($K_{av}$) and molecular weight (MW) in the Standard Buffer containing 30% ethanol. By addition of ethanol, two effects can be expected; namely, mitigation of the hydrophobic interaction and reinforcement in the electrostatic interaction due to the reduction of dielectric constant ($\epsilon$) of the medium. On the addition of 30% ethanol, the $\epsilon$ value is supposed to decrease from 79 to about 60. The electrostatic interaction can be enhanced by about 1.5-fold in the inverse ratio to $\epsilon$ value. As shown in Fig. 4, hemoglobin, yeast alcohol dehydrogenase (ADH), apo-transthrerin, and soybean trypsin inhibitor come to elute faster in the presence of 30% ethanol, though they elute rather in retard in...
Fig. 4. Comparison of Available Constants ($K_{av}$) of Proteins with and without Ethanol.
See Fig. 1 for the other conditions. ●, in the absence of ethanol (see Fig. 1); ○, in the presence of 30% (v/v) ethanol.
A solid line was drawn to fit for the data points obtained for proteins that normally eluted in Fig. 1.
the absence of ethanol (data shown by O) and even in the presence of 0.5 m NaCl (Fig. 2). The proteins of pI greater than 7.5 can be classified into two groups from Fig. 4. Cytochrome c, ribonuclease A, and subtilisin are retarded further by the addition of ethanol, and trypsin and lysozyme are eluted faster in the presence of ethanol.

**Elution of amino acids**

The elution volumes of various amino acids and their derivatives are listed in Table I. Although most of the aliphatic amino acids are eluted at the Ve of 145.6 ml, the aromatic amino acids are eluted behind that. According to the hydrophobicity scales, the degrees of hydrophobicity of the aliphatic amino acids range widely, however, the variety is not likely reflected in their elution. On the other hand, the hydrophobicity of L-leucine, L-isoleucine, and L-phenylalanine is almost the same, but slightly smaller than that of L-tryptophan, and that of L-tyrosine is much smaller and similar to that of amino acids with small aliphatic side chains like L-valine. The difference in their elution therefore should not be resulted from the interaction other than hydrophobic one. A cyclic imino acid L-proline is eluted at the same position as that of aliphatic amino acids (145.6 ml). N-acetyl derivatives of L-tyrosine and L-tryptophan are eluted before the parent amino acids. Introduction of an ethyl ester group or N-carbobenzoxy group into an amino acid increases the retardation. It should be noticed that positively charged basic amino acids also elute at 145.6 ml. This behavior is considerably different from that observed for proteins; the positively charged proteins, i.e., proteins of pI greater than 7.5, are retarded in elution without exception (Fig. 1). The introduction of an N-acetyl group onto the amino group of amino acid decreases the Ve, suggesting that by diminishing the positive charge the amino acid is eluted with repulsion. On the other hand, the ethyl-esterification of N-acetyl amino acid increases the retardation. This means that introduction of ethoxy group into carboxyl group to diminish the negative charge leads to increases in the hydrocarbonaceous surface area resulting in the retardation due to the enhanced hydrophobic interaction with the gel matrix.

Figure 5 shows the effects of NaCl concentration on the available constant (K_av) of L-tryptophan, L-tyrosine, and glycine. Since the hydrophobic interaction increases with

<table>
<thead>
<tr>
<th>Substance</th>
<th>Elution volume (ml)</th>
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<th>Elution volume (ml)</th>
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<tr>
<td>Glycine</td>
<td>145.6</td>
<td>L-Phenylalanine</td>
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<td>L-Alanine</td>
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<td>L-Valine</td>
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<td>L-Tryptophan</td>
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<td>L-Threonine</td>
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<td>L-DOPA^a</td>
<td>176.6</td>
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<tr>
<td>L-Leucine</td>
<td>145.6</td>
<td>N-Acetyl-L-tyrosine</td>
<td>157.3</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>145.6</td>
<td>N-Acetyl-L-tyrosine ethyl ester</td>
<td>421.2</td>
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<tr>
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<td>3-Nitro-L-tyrosine</td>
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<td>L-Aspartic acid</td>
<td>135.2</td>
<td>3-Amino-L-tyrosine</td>
<td>163.8</td>
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<tr>
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<td>N-Acetyl-L-tryptophan</td>
<td>213.2</td>
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<tr>
<td>L-Lysine</td>
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<td>N-Acetyl-L-tryptophan ethyl ester</td>
<td>806.0</td>
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<tr>
<td>L-Arginine</td>
<td>145.6</td>
<td>N-Carbobenzoxy-L- alanine</td>
<td>189.8</td>
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<tr>
<td>L-Proline</td>
<td>145.6</td>
<td>Blue dextran (MW 2 × 10^6)</td>
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<tr>
<td>L-Glutamic acid</td>
<td>140.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose^c</td>
<td>145.6</td>
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^a Conditions are shown in the text (Materials and Methods).

^b L-Dihydroxyphenylalanine.

^c Sucrose was measured by the phenol-sulphuric acid method.29
increases of salt concentration in the medium, log $K_{av}$ should increase linearly with salt concentration.\textsuperscript{22-24} Therefore, l-tryptophan and t-tyrosine seem to interact with the gel hydrophobically, but t-leucine and glycine do not.

Figure 6 depicts the relationship between the available constant ($K_{av}$) and hydrocarboneous surface area of typical aromatic amino acids. The hydrocarboneous surface area is a measure of hydrophobicity. Horváth et al.\textsuperscript{22} suggested that, on assumption of the presence of hydrophobic interaction between these amino acids and gel, the log $K_{av}$ should increase linearly with the increase of hydrocarboneous surface area. Since the hydrocarboneous surface area of t-tryptophan is greater than those of phenolic amino acids, the difference in retardation between tryptophan and phenolic amino acids may be due to hydrophobic interaction. However, the $K_{av}$ values of the three phenolic amino acids rather decrease, though slightly, with the increase of hydrocarboneous surface area. The introduction of hydroxyl groups into the phenolic ring increases log $K_{av}$. This suggested that there might be hydrogen-bonding interaction between hydroxyl groups of amino acids and the gel. On the other hand, the effects of introduction of hydroxyl groups into aliphatic side chains seems to be not significant, there being no difference among the $Ve$ values of alanine, serine, threonine, and leucine. The results shown in Table I, thus, suggest that it is difficult to explain the elution behavior only by the two forces of electrostatic and hydrophobic interactions.

**Discussion**

Germershausen and Karkas reported that good resolution of protein mixtures according to molecular size could be achieved on Toyopearl gel with high flow-rates and low column pressures, and that molecular weights estimations in the range between $10^4$ and $10^6$
could be done within minutes.\textsuperscript{5)} This gel can be used in high performance or semi-high performance liquid chromatography, and has been used successfully for the isolation of some proteins.\textsuperscript{6–12)} On the other hand, the non-specific adsorption of proteins on the gel has been pointed out. The non-specific adsorption is obviously an undesirable feature for the matrix of gel chromatography. The purposes of this study are to understand the nature of the adsorption and to obtain essential knowledge required for efficient separation.

Proteins whose pI values are greater than 7.5 show the retardation in their elution in 25 mM Tris–HCl buffer at pH 7.5 on Toyopearl gel (Fig. 1). This kind of retardation has not been observed with Sephadex\textsuperscript{26)} and other gel filtration matrices.\textsuperscript{27)} The lines of evidence shown in Figs. 1–3 suggested that electrostatic interaction worked between positively charged proteins and the gel at low ionic strength. From titration of carboxyl groups on the gel used, 5 μmol carboxyl groups were estimated in one ml of wet gel volume; accordingly the electrostatic interaction seems to be based on the interaction between carboxylate anions on the gel and positive charges on the proteins. Figure 3 shows that there are no electrostatic interactions in the presence of NaCl more than 0.3 M between proteins and the gel matrix. Thus, there should remain only the molecular sieve effect in the presence of NaCl greater than 0.3 M, and the elution order therefore should be chymotrypsinogen A, β-lactoglobulin, myoglobin, and lysozyme, as we assume that the interaction between proteins and the gel matrix were electrostatic in nature only. The observed order, however, was β-lactoglobulin, myoglobin, chymotrypsinogen A, and lysozyme. This discrepancy makes us consider that another interaction must work together with the electrostatic one. As to amino acids, however, positively charged amino acids such as L-lysine and L-arginine are eluted normally (Table 1). Therefore this interaction seems to function primarily in the elution of proteins.

Some negatively charged proteins such as catalase, apotransferrin, yeast alcohol dehydrogenase, and thermolysin also show retardation (Fig. 1), and their elution volume was not much affected by the addition of 0.5 M NaCl or LiCl (Fig. 2). Their elution, however, was greatly accelerated in the presence of 30% ethanol (Fig. 4). The interaction that is weakened by the addition of ethanol might be the hydrophobic one.

The proteins of pI greater than 7.5 could be classified into two groups from the elution behaviors on the addition of 30% ethanol in the Standard Buffer (Fig. 4): The first group of proteins (including cytochrome c, ribonuclease A, chymotrypsinogen A, and subtilisin BPN') is more retarded in the presence of ethanol than in the absence. The second group (including trypsin and lysozyme) elutes faster in the presence of ethanol. As the effect of ethanol on the hydrophobic and electrostatic interaction is opposite, we have come to consider that originally the electrostatic nature was dominant in the interaction between the gel matrix and those proteins in the first group, and that the hydrophobic nature is the second group. Figure 2 supported this conclusion: the proteins in the first group elute normally on the addition of 0.5 M NaCl, while those in the second are still rather retarded on the addition of 0.5 M NaCl. The anomalous behavior of lysozyme, which was tightly adsorbed to the gel even in the presence of sufficient salt to cancel the electrostatic interaction, was explained as owing to the strong hydrophobic interaction with the gel matrix.

Figure 5 may suggest that L-tytophan and L-tyrosine interact with gel hydrophobically, but L-leucine and glycine do not. However, among 4 amino acids examined, the degree of hydrophobicity of L-tryptophan is the largest, and that of L-leucine is a little smaller, but that of L-tyrosine is much smaller, and that of glycine is the smallest.\textsuperscript{19–22)} The different behavior between L-tryptophan and L-leucine thus suggested that the retardation of L-tryptophan as well as other aromatic amino acids is not the result of hydrophobic interaction, and it may be necessary to consider another interaction specific for aromatic amino
acids, for instance the charge-transfer interaction. The more hydroxyl groups are introduced into the phenolic amino acids, the more the adsorption increases (Fig. 6). It may be necessary to consider the hydrogen-bonding interaction between these amino acids and the gel (see Results). The elution of aromatic compounds such as tryptophan and dinitrophenyl isoleucine to the dextran gel Sephadex was also retarded. The adsorption is considerably weaker than that observed in the chromatography on the Toyopearl column. It is not known whether the adsorption of aromatic compounds to Sephadex or Toyopearl gels is due to the same principle. However, this characteristic of Toyopearl as well as Sephadex suggests that it has a big potential for the separation of compounds containing aromatic rings.

Thus, the interacting forces working between proteins and Toyopearl gel matrix are rather complicated, and we have shown the involvement of several kinds of these forces: electrostatic, hydrophobic, hydrogen-bonding, and possibly charge-transferring. To use this gel solely as a molecular sieve, one has to find out conditions in which all those interacting forces are cancelled. However, because of this very nature of the interaction, Toyopearl can be expected to become a very useful means of separating biochemical substances based on subtle differences in their properties. The separations of dipeptidyl aminopeptidase IV (post-proline dipeptidyl aminopeptidase) from pig pancreas and kidney, cartilage-derived factor from fetal bone cartilage, interferons, and \( \beta \)-N-acetylhexosaminidase from sea-squirt are certainly good examples of this capacity. In the purification of dipeptidyl aminopeptidase IV, 20 mm Tris–HCl buffer containing 0.2 M KCl at pH 7.0 and the same buffer containing 1% deoxycholate at pH 8.0 were used for Toyopearl chromatography. The fractions of the enzyme separated on the Toyopearl gel in 20 mm Tris–HCl buffer containing 0.2 M KCl at pH 7.0 showed the activity recovery as high as 80% and a 3.2-fold increase in specific activity as compared with purification on dextran gels that showed recoveries lower than 10% [D. Tsuru and T. Yoshimoto, personal communication]. Gel filtration of cartilage-derived factor preparation on a Toyopearl HW-55 Fine column with 4 M guanidine HCl buffer or 1 M formic acid resulted in good separation of the factor, and the elution profiles were similar on repeated chromatography (at least 20 times) at higher flow-rates (10–20 ml/cm²/hr). Sakihama et al. have developed a unique method for purification of ferredoxin and ferredoxin-NADP⁺ reductase by using a property of Toyopearl gel to adsorb these proteins in concentrated (40–80% saturated) ammonium sulfate.

In the present paper, the effects interfering with the elution of proteins and amino acids from Toyopearl gel were examined individually, and by considering these effects, chromatographic conditions could be selected more easily.

References
Gel Chromatography on TSK-GEL Toyopearl