8

SIZE EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SMALL SOLUTES

ANDREW J. ALPERT

PolyLC Inc., Columbia, Maryland 21045

I. OVERVIEW OF SIZE EXCLUSION CHROMATOGRAPHY (SEC) OF SMALL SOLUTES

Size-exclusion chromatography was introduced in 1959, with the invention of Sephadex. Some of the first applications involved attempts to fractionate small peptides and amino acids by size (1,2). These efforts were not very successful. The reason is that the fractionation range in SEC is determined by the pore diameter of the stationary phase. In polymer-based materials such as Sephadex, this is controlled by the degree of cross-linking; the more highly cross-linked the material, the narrower the pores. The agents used for cross-linking interact with some solutes, especially aromatic ones. Thus, phenylalanine, tyrosine, and tryptophan (Phe, Tyr, and Trp, respectively) elute later than V_t on Sephadex G-10, G-15, and G-25, the most highly cross-linked grades (2,3). Phe and oligophenylalanines elute in order of smallest to largest, the opposite of the sequence expected in SEC (4). The same adsorption phenomenon has been noted with Bio-Gel P2, a polyacrylamide-based medium (5,6). Silica and other inorganic materials can be made with very narrow pores, but difficulty in diffusion into and out of micropores (<20 Å) leads to poor efficiency (7). Thus, with most commercial SEC columns, the lower limit for V_t is around 1000 Da, which would correspond to a peptide of about eight to nine amino acid residues.

Column Handbook for Size Exclusion Chromatography

II. INTRODUCTION OF PolyHYDROXYETHYL ASPARTAMIDE AND THE EFFECT OF CHAOTROPES ON THE FRACTIONATION RANGE

A SEC material should be hydrophilic if it is to be used for biological applications. One such material, introduced by PolyLC in 1990 (8), is silica with a covalently attached coating of poly(2-hydroxyethyl aspartamide); the trade name is PolyHYDROXYETHYL Aspartamide (PolyHEA). This material was evaluated for SEC of polypeptides by P.C. Andrews (University of Michigan) and worked well for the purpose (Fig. 8.1). Because formic acid is a good solvent for polypeptides, Dr. Andrews tried a mobile phase of 50 m*M* formic acid. The result was a dramatic shift to a lower fractionation range for both V_0 and V_t (Fig. 8.2) to the point that V_t was defined by the elution position of water,

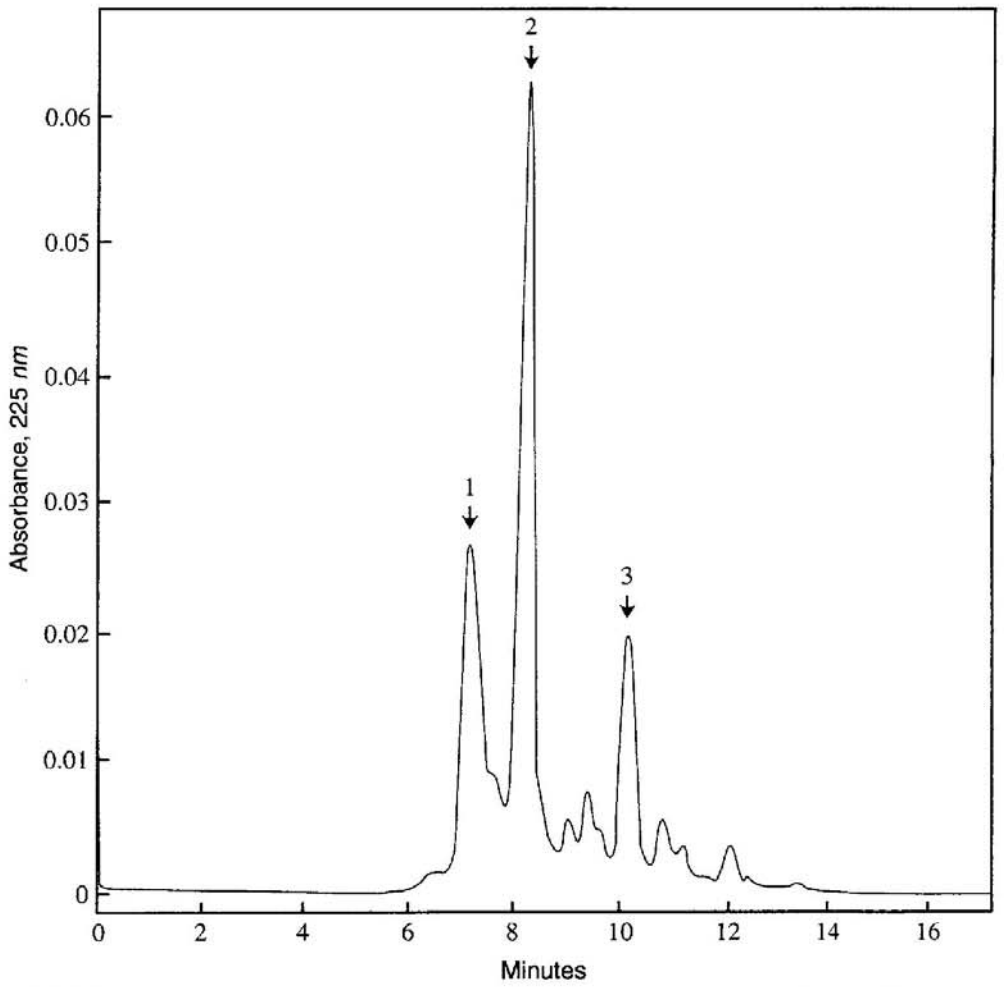

FIGURE 8.1 SEC of an acid/ethanol extract of lamprey pancreas. When performing SEC of peptides, one generally obtains the best correlation of retention times and molecular weights if the mobile phase is acidic and contains some organic solvent. Column: PolyHEA, 200 × 9.4 mm; 5 μm, 200 Å. Flow rate: 2.0 ml/min. Mobile phase: 5 m*M* sodium phosphate + 200 m*M* sodium sulfate, pH 3.0, with 25% (v/v) acetonitrile. Peaks: (1) Plasma lipid-binding protein (11,500 Da); (2) Insulin (6241 Da), glucagon (3900 Da), and Somatostatin-37 (4052 Da); and (3) somatostatin-14 (1623 Da). (Courtesy of P. C. Andrews, University of Michigan.)

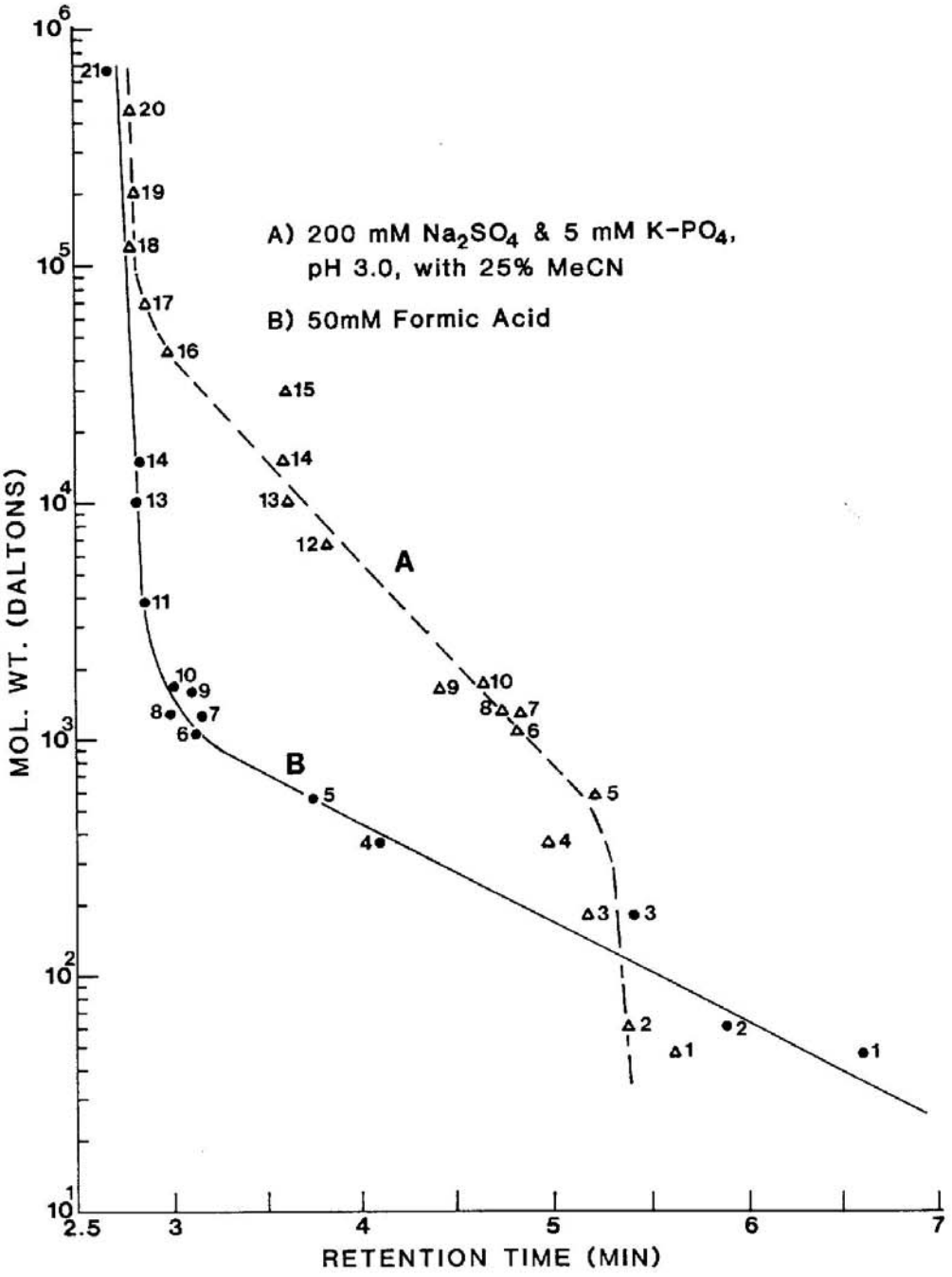

FIGURE 8.2 SEC ranges on a PolyHEA column (200 Å) with (A) a nondenaturing and (B) a denaturing mobile phase. Column and flow rate: Same as Fig. 8.1. Sample Key: (1) Formic acid (46 Da); (2) acetic acid (60 Da); (3) *N*-chloroacetyl-Tris base (177 Da); (4) thyrotropin-releasing hormone (362 Da); (5) [Leu]^5-enkephalinamide (554 Da); (6) angiotensin II (1046 Da); (7) luteinizing hormone-releasing hormone (1236 Da); (8) angiotensin I (1296 Da); (9) bombesin (1592 Da); (10) α-melanocyte-stimulating hormone (α-MSH) (1665 Da); (11) poly-L-lysine (3800 Da); (12) insulin (bovine) (6500 Da); (13) ubiquitin (10,000 Da); (14) ribonuclease (15,000 Da); (15) carbonic anhydrase (29,000 Da); 16) ovalbumin (chicken) (43,000 Da); (17) bovine serum albumin (66,000 Da); (18) IgG (150,000 Da); (19) β-amylase (200,000 Da); (20) apoferritin (443,000 Da); and (21) thyroglobulin (669,000 Da). (Data courtesy of P. C. Andrews, University of Michigan.)

and acetic acid, amino acids, and the smallest peptides were included in the range (Fig. 8.3). Despite the high absorbancy of the mobile phase (1.0 AU at 220 nm), it was possible to subtract the elevated baseline and monitor peptides at low wavelengths because elution was isocratic.

Shifts in the SEC fractionation range are not new. It has been known for decades that adding chaotropes to mobile phases causes proteins to elute as if they were much larger molecules. Sodium dodecyl sulfate (SDS) (9) and guanidinium hydrochloride (Gd.HCl) (9–12) have been used for this purpose. It has not been clearly determined in every case if these shifts reflect effects of the chaotropes on the solutes or on the stationary phase. Proteins are denatured by chaotropes; the loss of tertiary structure increases their hydrodynamic radius. However, a similar shift in elution times has been observed with SEC of peptides in 0.1% trifluoroacetic acid (TFA) (13–15) or 0.1 *M* formic acid (16), even if they were too small to have significant tertiary structure. Speculation as to the cause involved solvation effects that decreased the effective pore size of the

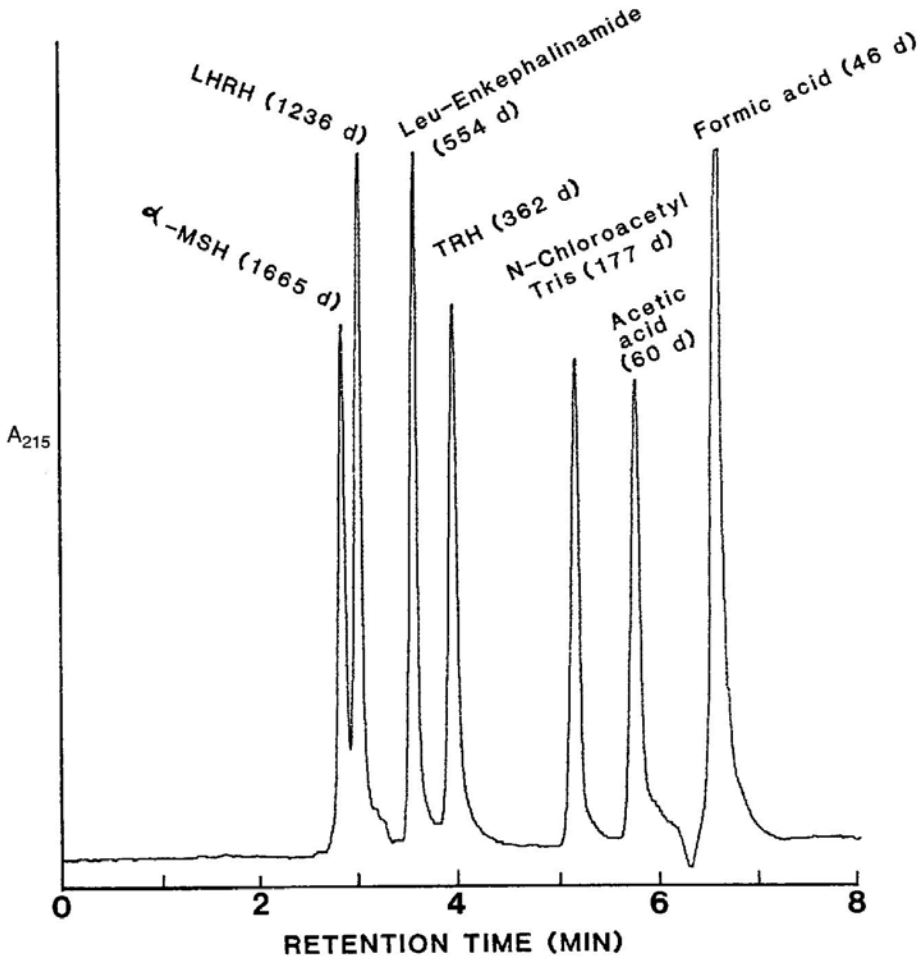

FIGURE 8.3 SEC in 50 m*M* formic acid. Column and flow rate: Same as Fig. 8.1. (Courtesy of P. C. Andrews, University of Michigan.)

column. Such a simplistic explanation does not account for the ability of a SEC material with a pore diameter of 200 Å to separate solutes as small as amino acids (Figs. 8.1 and 8.2).

With a *nondenaturing* mobile phase (i.e., one that does not contain a strong chaotrope), the fractionation range of PolyHEA with a 200-Å-pore diameter is comparable to that of other columns with pore diameters of 50 or 60 Å. This suggests that the PolyHEA coating is unusually thick, about 75 Å or so, compared with 15 Å with the typical coating on silica. When the mobile phase does contain a chaotrope, the resulting fractionation range resembles one that might be obtained with a material with a pore diameter around 15 Å (similar to a hypothetical Sephadex G-7 material). It is plausible that the coating of this material is normally rendered impermeable by numerous hydrogen bonds between adjacent polypeptide chains; PolyHEA has a high concentration of amide groups, and amide–amide hydrogen bonds are about twice as strong as amide–hydroxyl hydrogen bonds (17). The coating is also probably hydrated by a highly ordered layer of water. Similar statements have been made about the hydration of Sephadex rendering 20% of the potential pore volume inaccessible in SEC (2,3). Chaotropes are weakly hydrated (18) and form hydrogen bonds to stationary phases in preference to water. Thus, similar to aromatic amino acids, they are retained on highly cross-linked SEC materials past V_t. When included in the mobile phase, the chaotrope outcompetes adjacent polymer chains for forming hydrogen bonds. The consequence is a great increase in their steric radius, to the point that they occlude a 200-Å pore (Fig. 8.4). At the same time, the chaotrope would disrupt the hydration layer. This has two important consequences: (1) The available pore volume increases. This is consistent with observations made with POLYHYDROXYETHYL A (Figs. 8.2 and 8.11 vs. Fig. 8.12). (2) The space between the polymer chains becomes permeable; this distance between chains is effec-

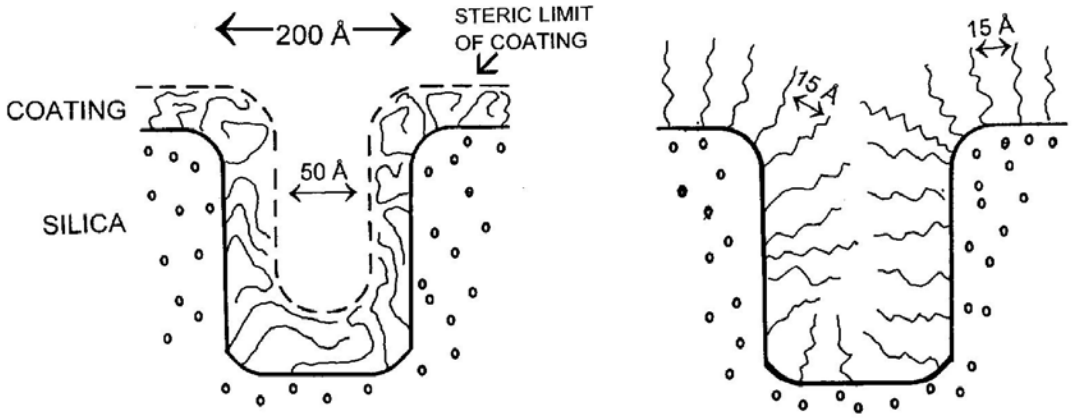

FIGURE 8.4 Effect of mobile phase on PolyHEA coating. (Left) Nondenaturing mobile phase (e.g., Fig. 8.1). (Right) Denaturing mobile phase (e.g., Fig. 8.3). Material with a 200-Å-pore diameter is shown. The coating swells in the presence of a chaotrope, becoming permeable and occluding the pores. The effective pore diameter now becomes the distance between polymeric chains in the permeable coating: about 15 Å.

tively the new pore diameter. Judging from the fractionation range, the chains are spaced about 15 Å apart. Evidently it is easy to diffuse into and out of such a permeable network of "soft" pores, and solutes elute in peaks much sharper and more symmetrical than would be afforded by a SEC material with "hard" pores 15 Å in diameter.

A review of the literature discloses similar effects with other SEC materials. Richter and Schwandt (16) eluted a TSK 2000P-SW column with 0.1 *M* formic acid and were able to desalt peptides as small as 794 Da. With 0.01 *M* formic acid, the range shifted so that peptides <3500 Da were not desalted. Irvine and Shaw (15) eluted a TSK G-2000-SW column with 0.1% TFA and obtained a range of approximately 500–50,000 Da. With the addition of 0.25 *M* NaCl (a structure-forming salt), the range shifted to 5000–200,000 Da. Both Montelaro *et al.* (9) and Kato *et al.* (10) observed shifts of V_0 and V_t to lower values with a TSK G2000-SW column upon addition of 6 *M* Gd.HCl to the mobile phase; the latter group reported a range of 1000–25,000 Da. Swergold and Rubin (14) eluted a TSK G3000-PW column with 0.1% TFA containing 40% acetonitrile (ACN) and obtained a range of approximately 300–130,000 Da. They stated that 0.3% phosphoric acid could be substituted for the TFA. The ACN was essential to eliminate hydrophobic interactions with this polymeric stationary phase. Bennett *et al.* (13) used an I-125 column from Waters; with 0.1% TFA + 40% ACN, the range shifts from 2000–80,000 to approximately 500–60,000 Da.

These observations suggest that chaotropes render the coatings of a number of SEC materials permeable, albeit to a lesser extent than with PolyHEA. For example, TSK G2000-SW is reported by the manufacturer to have a pore diameter of 125 Å. With a denaturing mobile phase, the separation of the smallest solutes is about one-fourth as good as with PolyHEA with 200-Å pores. The implication is that the TSK coating is considerably thinner or more tightly bonded to the surface than that of PolyHEA. A similar comparison may be made between the separation of thyrotropin-releasing hormone (TRH) and acetic acid on PolyHEA (Fig. 8.3) and the Waters I-125 column (19).

III. VERIFICATION OF SEC MECHANISM FOR SMALL SOLUTES

Amino acids are a convenient set of standards for elucidating the forces involved in SEC of small solutes. One can resolve to baseline as many as 5 of the 20 natural amino acids within the range of a 200-Å PolyHEA column (and 7 within the range of a 60-Å column). In general, the order of elution is most to least polar. This order appears at first to reflect hydrophobic interaction between the solute and the stationary phase. With other SEC media, the late elution of aromatic amino acids has been attributed to electron donor–acceptor interactions (20). Were this the mechanism, then oligopeptides of the most hydrophobic amino acids should elute later than the amino acids themselves, as overall retention in adsorption chromatography reflects the additive contribution of each subunit of a solute. Such is the case with Phe and oligophenylalanines on Sephadex (4) but is not the case with PolyHEA. $(Phe)_2$ elutes before

Phe and $(Trp)_2$ elutes before Trp (Fig. 8.5), the sequence to be expected in SEC. Also, none of the amino acids elutes from PolyHEA after V_t. Finally, the adsorption effects are generally attributed to the cross-linking agents used in high concentration to prepare the small-pore SEC media. With the chaotropic mobile phase, however, the network of "soft" pores is generated without cross-linking agents. It would seem that the most hydrophobic amino acids *look* the smallest, particularly the aromatic ones. Before making such a claim, however, one must rule out effects from some of the forces potentially responsible for the adsorption of amino acids to PolyHEA: *hydrophobic interaction, hydrophilic interaction,* and *electrostatic effects.*

Controlling for these forces requires variation in the amount of salt, organic solvent, and the pH of the mobile phase. It is impractical to perform such experiments with 50 m*M* formic acid; an alternative additive must be used that maintains its chaotropic properties independent of salt content or pH. Fortunately, mobile phases containing 50 m*M* hexafluoro-2-propanol (HFIP) afford a fractionation range comparable to that of the formic acid (Fig. 8.6), permitting the effects of these variables to be studied systematically.

A. Effect of pH

At pH 3.0, Lys has a (+) charge, whereas Asp is protonated (and neutral). Varying the pH of the mobile phase leads to a decrease in the retention of Lys and and an increase in that of Asp [as it gains (−) charge]. The plot of this effect (Fig. 8.7) is, in effect, a titration curve of PolyHEA. The coating has a

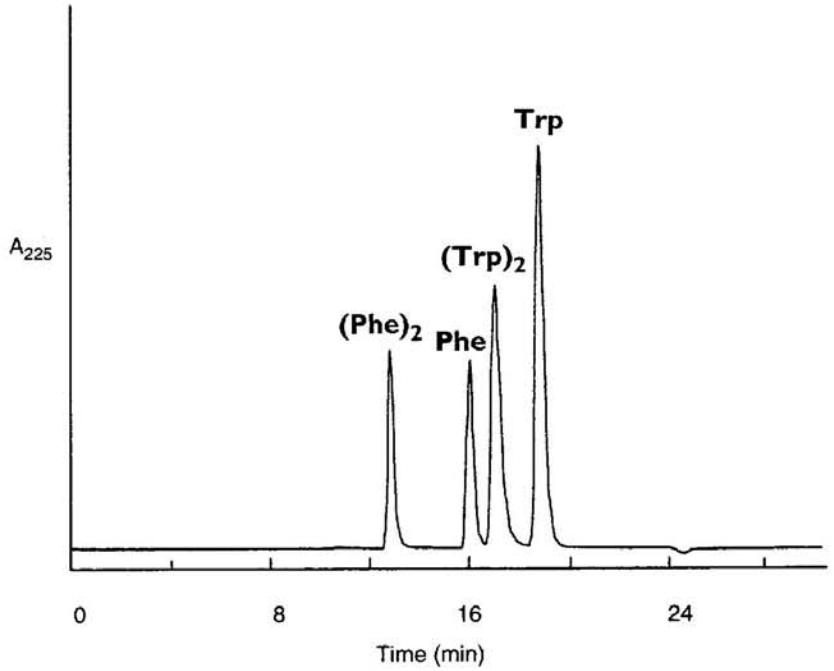

FIGURE 8.5 SEC of aromatic amino acids and dipeptides. Column: Same as Fig. 8.1. Flow rate: 0.6 ml/min. Mobile phase: 50 m*M* formic acid. Detection: A_{225} = 0.5 AUFS.

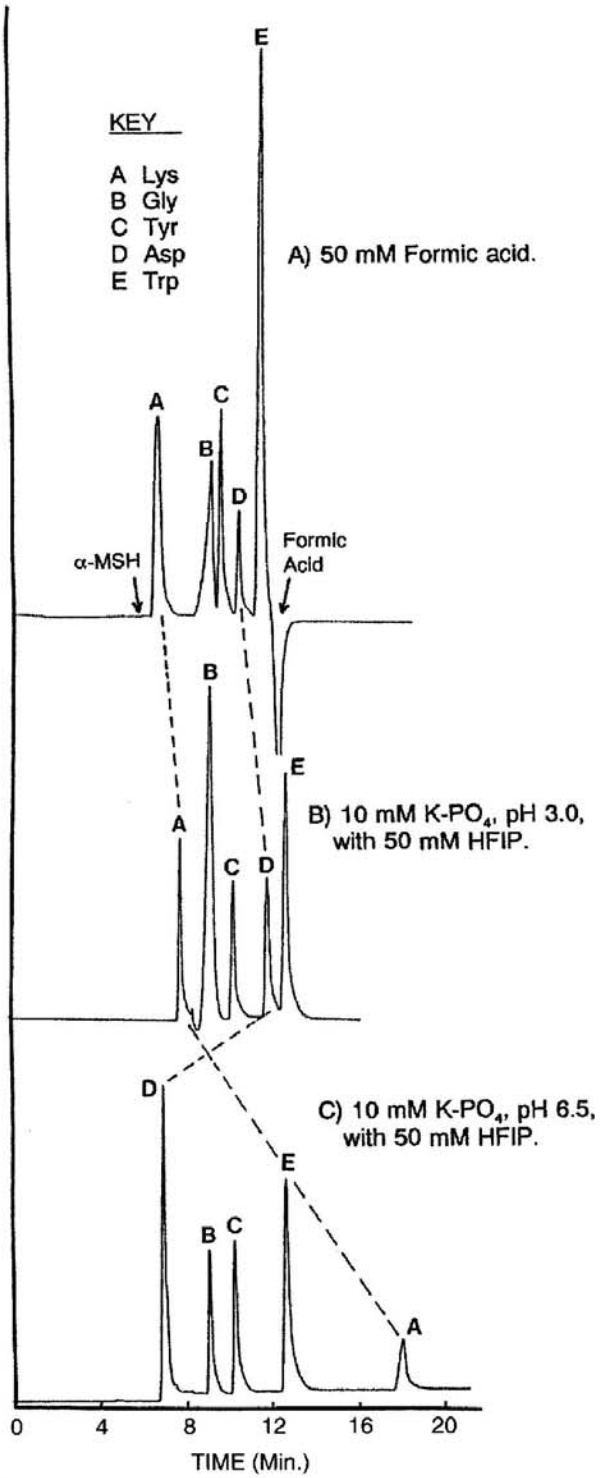

FIGURE 8.6 Comparison of hexafluoro-2-propanol (HFIP) with formic acid as a denaturing agent in SEC. Elution positions of neutral amino acids were similar with both agents. The elution positions of Lys and Asp shifted dramatically in C, as shown by the tie lines, but this was an effect of pH (see Fig. 8.7). The elution positions of α-MSH and formic acid are shown to demonstrate that the amino acids eluted within V_0 and V_t. Column: Same as Fig. 8.1. Flow rate: 1.0 ml/min. Mobile phase: As noted. Detection: A_{215} = 0.1 AUFS.

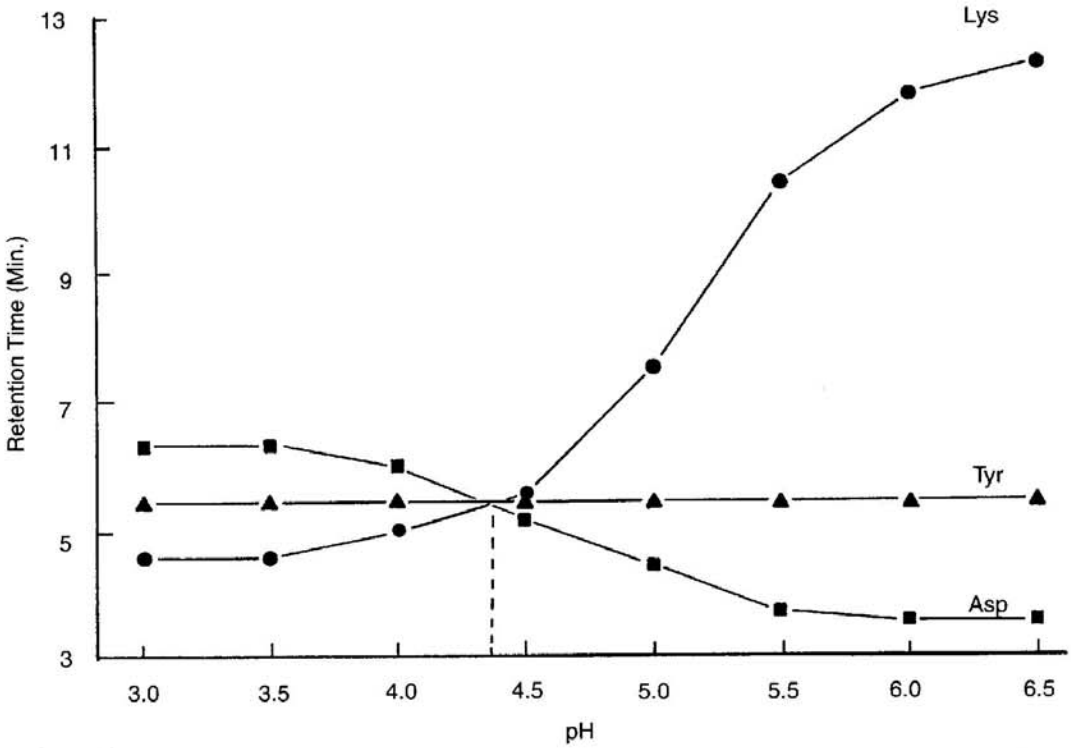

FIGURE 8.7 Effect of pH on retention of amino acids. Column and flow rate: Same as Fig. 8.1. Mobile phase: 10 m*M* potassium phosphate with 50 m*M* HFIP; pH as indicated (adjusted prior to the addition of HFIP).

small positive charge below pH 4.4 and a small negative charge above pH 4.4. At pH 4.4 (where the peaks of Lys and Asp coincide), the charges are in balance; the coating is zwitterionic. Presumably these charged groups are the termini of the polypeptide coating. The retention of neutral amino acids was not affected by pH, indicating that electrostatic effects did not determine their elution order.

B. Effect of Salt

Electrostatic effects have long been recognized in commercial HPLC columns for SEC of proteins (15,21,22). The usual remedy is to add 100 m*M* salt to the mobile phase. This works here too; the Lys and Asp peaks collapse into the Gly peak with 100 m*M* salt (Fig. 8.8). High concentrations of sodium sulfate were added to determine the role played in SEC by hydrophobic interactions (sodium sulfate, a structure-forming salt, strengthens such interactions). Sodium sulfate increased the retention only of the most hydrophobic amino acids to any extent, and then only when the concentration approached 1 *M*. Clearly, hydrophobic interaction cannot account for the elution order of amino acids on PolyHEA.

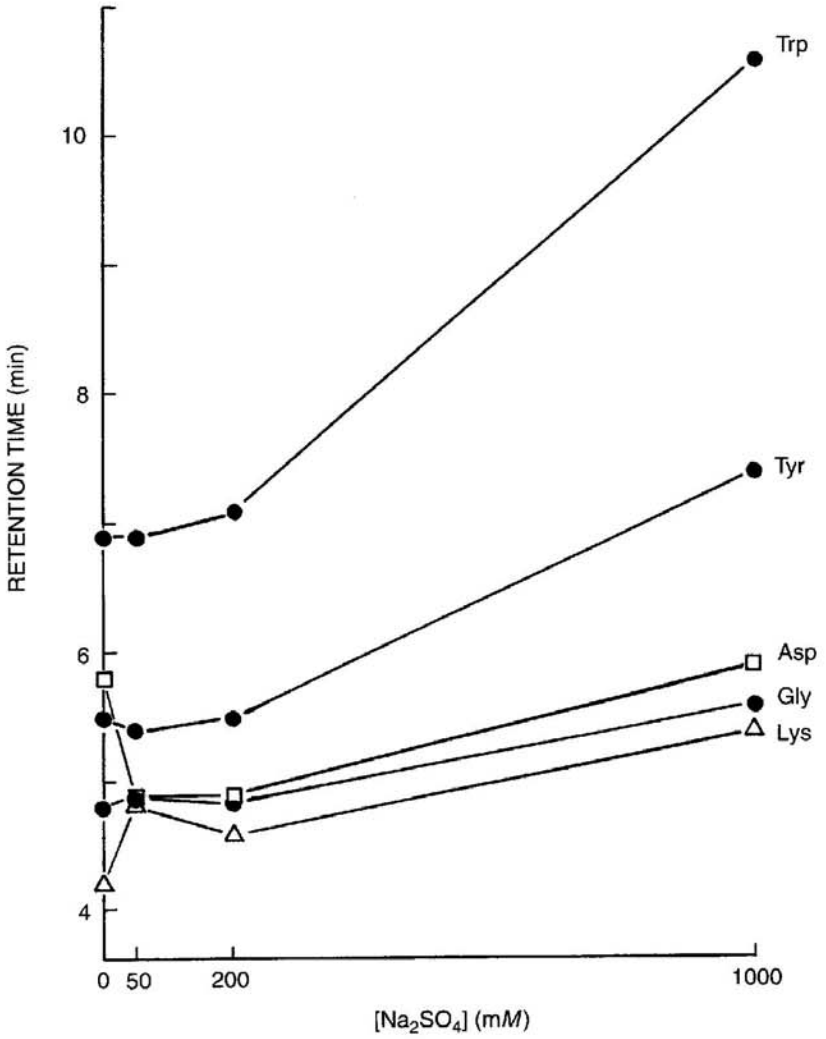

FIGURE 8.8 Effect of salt on retention of amino acids. Column and flow rate: Same as Fig. 8.1. Mobile phase: 10 m*M* potassium phosphate + sodium sulfate (as noted), pH 3.0, with 50 m*M* HFIP.

C. Effect of Organic Solvents

As a final check on these results, ACN was added to the mobile phase; 25% generally suffices to eliminate hydrophobic interactions with reasonably hydrophilic stationary phases (23). At the same time, a sufficiently high concentration of organic solvent induces hydrophilic interaction, a situation where the stationary phase is more polar than the mobile phase (8). Adsorption of solutes then occurs through a partitioning mechanism between the dynamic mobile phase and the stagnant hydration layer. A "normal phase" elution order is obtained: least to most polar. This is clearly the case with 60% ACN with PolyHEA (Fig. 8.9) as well as with SynChropak GPC (24). With 25% ACN, the SEC elution order is not affected significantly except for Lys-, which elutes significantly later (basic amino acids are the most hydrophilic of all). It is clear that

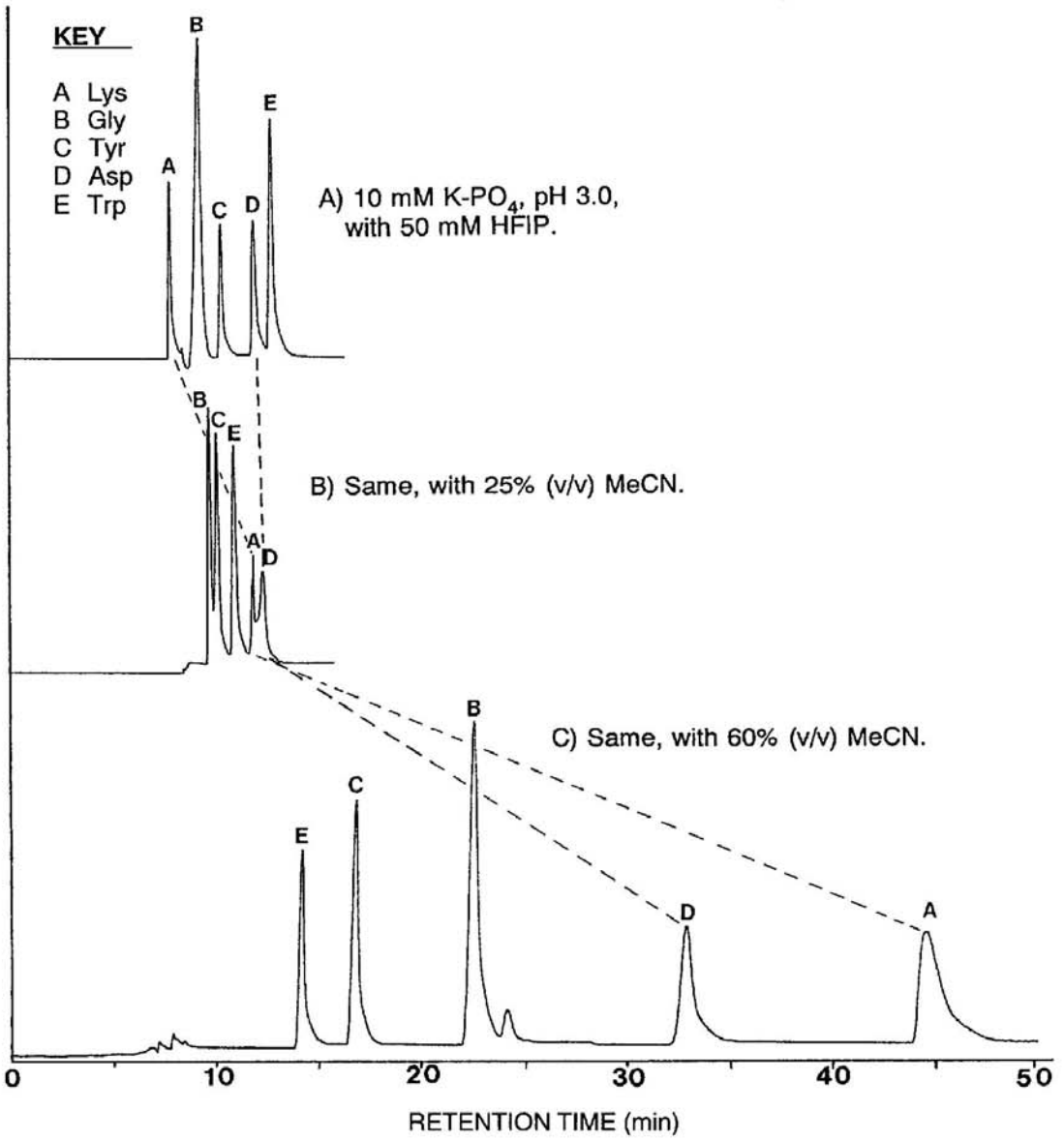

FIGURE 8.9 Effect of organic solvent on retention of amino acids. Small amounts of organic solvent condense the fractionation range somewhat for small solutes (B vs A), presumably by decreasing the swelling of the PolyHEA coating. Higher concentrations of organic solvent induce hydrophilic interactions, occasioning an inversion of the elution order. Both Asp and Lys are well retained (shown with the tie lines), distinguishing this effect from electrostatic effects. Column and flow rate: Same as Fig. 8.6. Mobile phase: As noted. Detection: A_{215} = 0.1 AUFS.

hydrophilic interactions do not account for the elution order of amino acids in SEC, in the absence of appreciable levels of organic solvent.

D. How Big Are Amino Acids?

These controlled experiments eliminate adsorption as an explanation for the elution order of neutral amino acids from PolyHEA. Perhaps this order does

reflect their actual sizes, which seem to range from approximately 8 to 3 Å (in the case of Gly vs. Trp). The perceived size of a solute in SEC is really the size of its sphere of hydration. This can be one to three water molecules thick (for chaotropes and structure-forming ions, respectively) (18). In general, homopeptides of polar amino acids are more highly hydrated than those of nonpolar amino acids (25). Actually, it may be more accurate here to speak of spheres of solvation. The separations are taking place in the presence of a chaotrope in the mobile phase. This may disrupt the hydration layer around the solutes as it does with the stationary phase [and which would presumably eliminate the electron donor–acceptor interactions postulated by Porath (20)]. It is unclear to what extent these results reflect the relative size of amino acids in more conventional solvents. In any case, they do establish that the aromatic amino acids look smaller than the other amino acids. Their specific adsorption to some highly cross-linked SEC matrices is coincidence; no such adsorption occurs with PolyHEA.

Caution: The order of elution of solutes smaller than a tetrapeptide may be in order of decreasing polarity rather than decreasing molecular weight. This reflects the relative size of their spheres of hydration.

E. Superdex Peptide

Pharmacia has introduced an SEC material named Superdex (26). This has a shell of agarose to confer rigidity, with size exclusion performed by an interior network of dextran. The Superdex Peptide column represents the low end of the molecular weight scale, with a fractionation range of 100–7000 Da. The company literature (27) shows reasonably good separations of small solutes such as Gly, $(Gly)_3$, and $(Gly)_6$. However, peptides with several aromatic residues elute after V_t (28), indicating that this matrix has the same problem with the adsorption of aromatic amino acids as do other highly cross-linked SEC media. Also, graphs of the fractionation range (27) show no change in the presence of a number of chaotropes or 70% ACN (which is high enough to induce significant hydrophilic interaction with so polar a material). Either the solvation properties of this material differ dramatically from those of all other materials used for SEC of small solutes or else they should be evaluated more carefully with controlled experiments.

IV. FRACTIONATION RANGE WITH PolyHEA AS A FUNCTION OF PORE DIAMETER

When a column of PolyHEA with a 300-Å-pore diameter is eluted with 50 m*M* formic acid, a plot of the fractionation range resembles two lines of different slopes joined together (Fig. 8.10). The line at the lower end covers the same molecular weight range as the 200-Å material: 20–1600 Da. An interpretation of the data is that the swollen, permeable coating does not completely fill a pore with a diameter of 300 Å. The lower end of the fractionation range reflects the pore volume within the coating, whereas the upper end of the range reflects the pore volume within the pores but above the coating. Thus, one can fractionate both small and large solutes with the same column. Figure 8.11

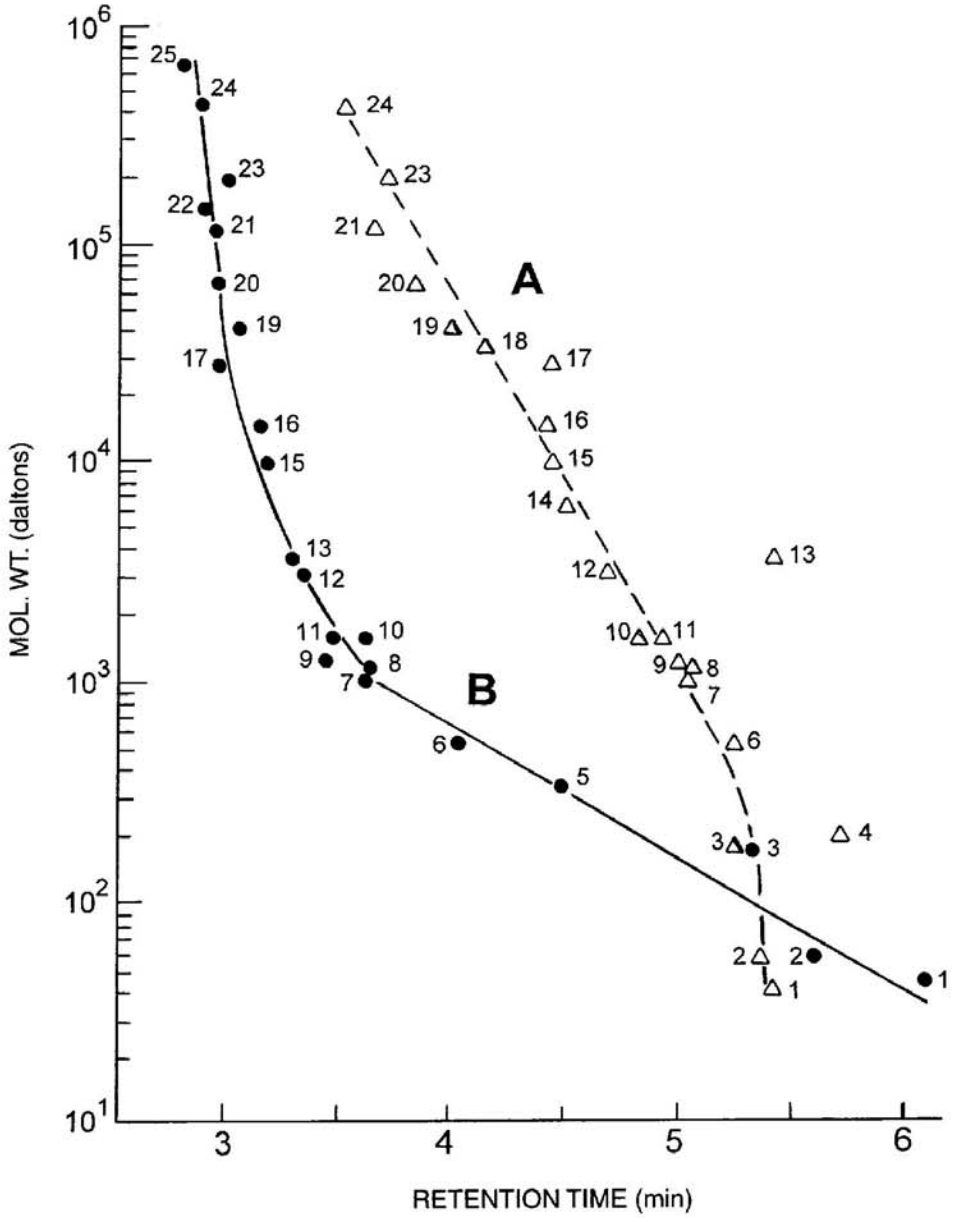

FIGURE 8.10 SEC ranges on a PolyHEA column with 300-Å pores. Column, flow rate, and mobile phases: As in Fig. 8.2, but the pore diameter is 300 Å. Sample key: (1) Formic acid (46 Da); (2) acetic acid (60 Da); (3) *N*-chloroacetyl-Tris base (177 Da); (4) citric acid (192 Da); (5) thyrotropin-releasing hormone (362 Da); (6) [Leu^5]-enkephalinamide (554 Da); (7) angiotensin II (1046 Da); (8) luteinizing hormone-releasing hormone (1236 Da); (9) angiotensin I (1296 Da); (10) bombesin (1592 Da); (11) α-melanocyte-stimulating hormone (1665 Da); (12) somatostatin-28 (3149 Da); (13) poly-L-lysine (3800 Da); (14) insulin (bovine) (6500 Da); (15) ubiquitin (10,000 Da); (16) ribonuclease (15,000 Da); (17) carbonic anhydrase (29,000 Da); (18) β-lactoglobulin (29,200 Da); (19) ovalbumin (chicken) (43,000 Da); (20) bovine serum albumin (66,000 Da); (21) IgG (150,000 Da); (22) alcohol dehydrogenase (150,000 Da); (23) β-amylase (200,000 Da); (24) apoferritin (443,000 Da); and (25) thyroglobulin (669,000 Da).

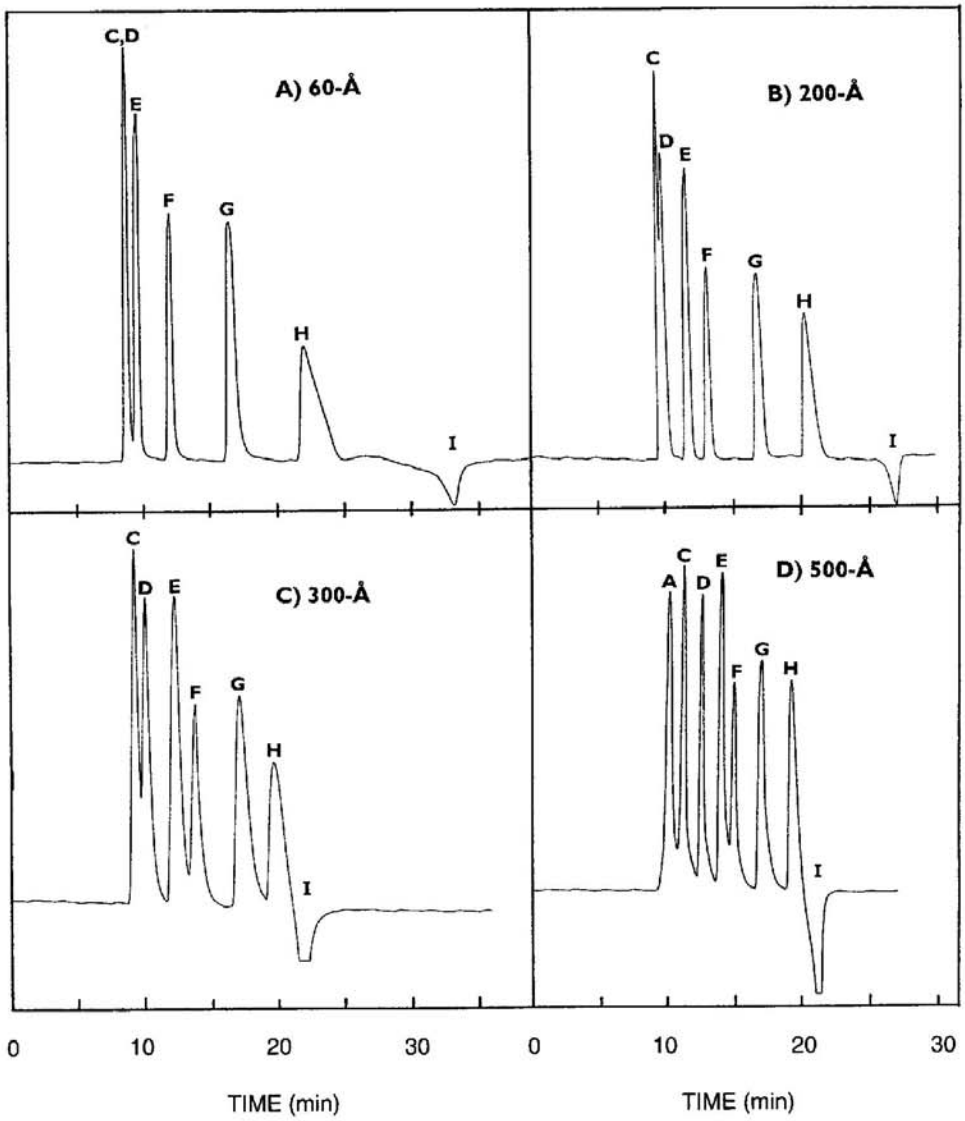

FIGURE 8.11 Effect of pore diameter on SEC of standards (denaturing mobile phase). Columns, flow rate and mobile phase: As in Fig. 8.5 except for pore diameters as noted. Sample Key: (A) IgG (150,000 Da); (C) cytochrome c (12,384 Da); (D) α-melanocyte-stimulating hormone (1665 Da); (E) [Met]^5-enkephalinamide (573 Da); (F) aspartame (294 Da); (G) phenylalanine (165 Da); (H) acetic acid (60 Da); and (I) water (18 Da).

shows the fractionation of some standard solutes with columns of various pore diameters. All of the materials include the region 20–1600 Da in their fractionation range, but this region accounts for a decreasing percentage of the total range as the pore diameter increases. Wider pore materials are of limited utility with denaturing mobile phases. Presumably they would be employed with solutes as large as proteins, and denaturation would be a significant

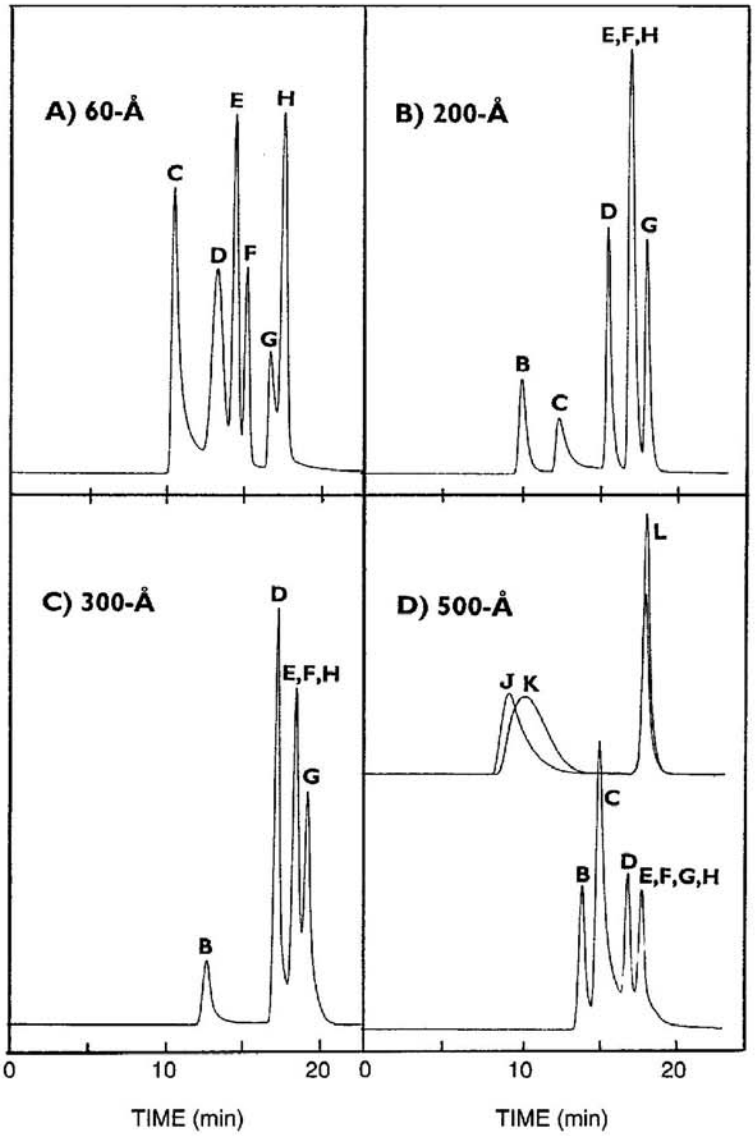

FIGURE 8.12 Effect of pore diameter on SEC of standards (nondenaturing mobile phase). "Nondenaturing" refers to the effect on the stationary phase. Most large proteins were in fact denatured by this mobile phase (which was optimized for use with peptides, not proteins). Accordingly, it was necessary to use polyacrylamide to demonstrate the approximate range and position of V_0 under these conditions. The polyacrylamide standards both eluted at V_0 with the 300-Å column (not shown). Columns and flow rate: Same as in Fig. 8.11. Mobile phase: Same as in Fig. 8.1. Sample key: (B) Ovalbumin (43,000 Da); (J) polyacrylamide (1,000,000 Da); (K) polyacrylamide (400,000 Da); (L) low molecular weight impurity in the polyacrylamide standards. Other samples as in Fig. 8.11.

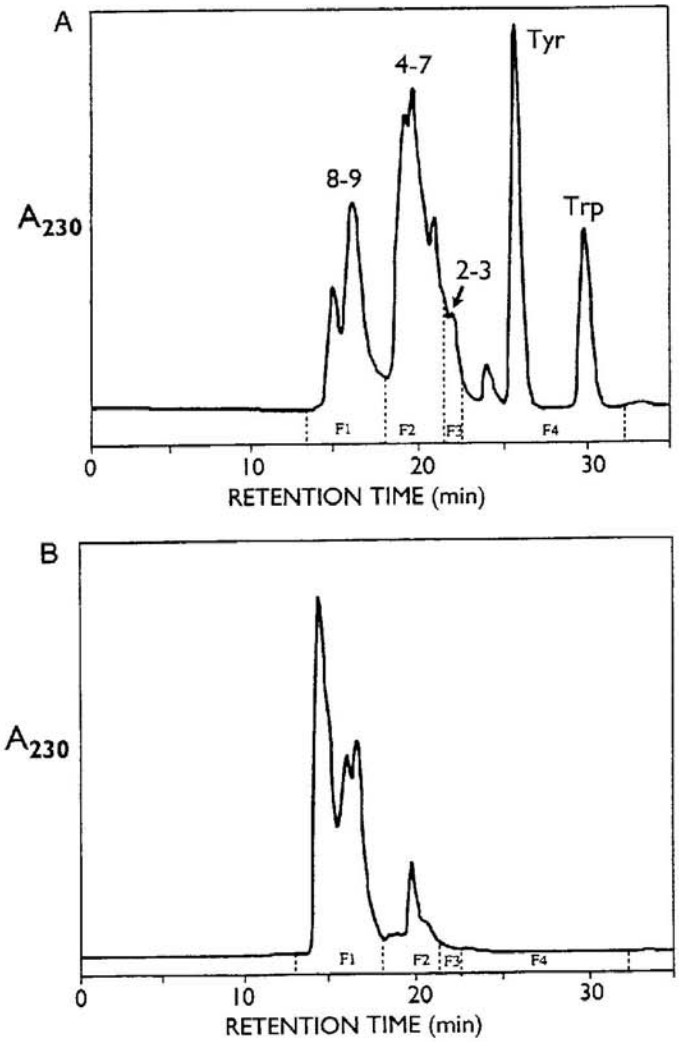

FIGURE 8.13 SEC of casein hydrolyzates. Numbers above the peaks refer to the number of amino acid residues in the typical peptide in the indicated fraction. Column: PolyHEA, 200 × 9.4 mm; 5 μm, 200 Å. Flow rate: 0.5 ml/min. Mobile phase: 50 m*M* Formic acid. Detection: A_{230}. Samples: (A) Pancreatin hydrolyzate and (B) tryptic hydrolyzate. (Adapted from Ref. 29 with permission from Silvestre *et al.* Copyright 1994, American Chemical Society.)

concern with such solutes. Also, if the fractionation of small solutes is of no interest in such analyses, then the pore volume occupied by the swollen coating is "wasted," as it decreases the portion available for fractionation in the size range of interest. In general, nondenaturing mobile phases will afford better resolutions in the size ranges of interest with large solutes such as proteins. Figure 8.12 demonstrates this point with the same columns and standards as Fig. 8.11. It should be noted that *nondenaturing* refers here to the effect on the stationary phase; the organic solvent and low pH used in Figs. 8.1 and 8.12 may well denature larger proteins. These conditions were selected as suitable for SEC of peptides. In general, SEC of proteins is usually best performed with neutral buffers and an absence of organic solvent.

V. APPLICATIONS

A. SEC of Peptides and Amino Acids

The proteins in food supplements are often hydrolyzed to short peptides to make them easier to absorb. A high content of amino acids is deleterious, however. Thus, there is ongoing interest in determining the size distribution of peptides in protein hydrolyzates. Silvestre *et al.* (29,30) used a PolyHEA column to compare casein hydrolyzates prepared through various methods. They were able to assess the content of the smallest peptides, as well as amino acids (Fig. 8.13).

B. SEC of Small Solutes Other Than Peptides

In an attempt to isolate a factor responsible for stimulating hepatocyte growth, Nelson *et al.* (31) used a PolyHEA column to fractionate an extract of liver by size. The active fraction eluted at a position corresponding to approximately 200 Da; the actual molecular weight (electrospray mass spectrometry; ES-MS) was 215 Da. The compound of interest proved to be glycerophosphorylethanolamine.

C. Mass Spectrometry: On-Line Desalting

Steven Carr (SmithKline Beecham) has used microbore columns to desalt proteins prior to ES-MS (32). The pore diameter of PolyHEA used (usually 200 Å) was selected so that all proteins of interest would elute at V_0 with 50 m*M* formic acid. Only the V_0 peak was allowed to flow into the ES-MS nebularizer; the rest of the SEC effluent (including the salts) was diverted to waste by opening a microdumper valve between the column and the nebularizer. The properties of the mobile phase were quite compatible with ES-MS analysis.

ACKNOWLEDGMENTS

I am grateful to Drs. P. C. Andrews and Marialice Silvestre for permission to include their data in this chapter. Some of these data were presented previously as poster 110, 10th ISPPP (Wiesbaden, October 1990). Bio-Gel is a trademark of Bio-Rad Corp. Sephadex and Superdex are trademarks of Pharmacia Biotech. TSK-GEL is a trademark of Tosoh Corp. PolyHYDROXYETHYL Aspartamide and PolyHYDROXYETHYL A are trademarks of PolyLC Inc.

REFERENCES

1. Porath, J. (1960). *Biochim. Biophys. Acta* **39**, 193.
2. Gelotte, B. (1960). *J. Chromatogr.* **3**, 330.
3. Janson, J.-C. (1967). *J. Chromatogr.* **28**, 12.
4. Bretthauer, R. K., and Golichowski, A. M. (1967). *Biochim. Biophys. Acta* **155**, 549.
5. Schwartz, A. N., and Zabin, B. A. (1966). *Anal. Biochem.* **14**, 321.
6. Thornhill, D. P. (1972). *Biochim. Biophys. Acta* **279**, 1.
7. Chappell, I., Baines, P., and Carpenter, P. K. (1992). *J. Chromatogr.* **603**, 49.

8. Alpert, A. J. (1990). *J. Chromatogr.* **499**, 177.
9. Montelaro, R. C., West, M., and Issel, C. J. (1981). *Anal. Biochem.* **114**, 398.
10. Kato, Y., Komiya, K., Sasaki, H., and Hashimoto, T. (1980). *J. Chromatogr.* **193**, 458.
11. Lazure, C., Dennis, M., Rochemont, J., Seidah, N. G., and Chrétien, M. (1982). *Anal. Biochem.* **125**, 406.
12. Richter, W. O., Jacob, B., and Schwandt, P. (1983). *Anal. Biochem.* **133**, 288.
13. Bennett, H. P. J., Browne, C. A., and Solomon, S. (1983). *Anal. Biochem.* **128**, 121.
14. Swergold, G. D., and Rubin, C. S. (1983). *Anal. Biochem.* **131**, 295.
15. Irvine, G .B., and Shaw, C. (1986) *Anal. Biochem.* **155**, 141.
16. Richter, W. O., and Schwandt, P. (1984). *J. Chromatogr.* **288**, 212.
17. Habermann, S. M., and Murphy, K. P. (1996). *Protein Sci.* **5**, 1229.
18. Collins, K. D., and Washabaugh, M. W. (1985). *Quart. Rev. Biophys.* **18**, 323.
19. Rivier, J. E. (1980). *J. Chromatogr.* **202**, 211.
20. Porath, J. (1997). *J. Protein Chem.* **16**, 463.
21. Kopaciewicz, W., and Regnier, F. E. (1982). *Anal. Biochem.* **126**, 8.
22. Golovchenko, N. P., Kataeva, I. A., and Akimenko, V. K. (1992). *J. Chromatogr.* **591**, 121.
23. Zhu, B. Y., Mant, C. T., and Hodges, R. S. (1991). *J. Chromatogr.* **548**, 13.
24. Mant, C. T., Parker, J. M. R., and Hodges, R. S. (1987). *J. Chromatogr.* **397**, 99.
25. Kuntz, I. D. (1971). *J. Am. Chem. Soc.* **93**, 514.
26. Hagel, L. (1993). *J. Chromatogr.* **648**, 19.
27. Pharmacia Biotech "Data File" bulletin 18-1119-46 (10/96).
28. Hedlund, H., Kärf, L., Lindberg, U., Nyhammar, T., and Winter, A., Poster P-222, ISPPP '97 Conference (October 26–29, 1997), Rockville, MD.
29. Silvestre, M. P. C., Hamon, M., and Yvon, M. (1994). *J. Agric. Food Chem.* **42**, 2778.
30. Silvestre, M. P. C., Hamon, M., and Yvon, M. (1994). *J. Agric. Food Chem.* **42**, 2783.
31. Nelson, C., Moffat, B., Jacobsen, N., Henzel, W. J., Stults, J. T., King, K. L., McMurtrey, A., Vandlen, R., and Spencer, S. A. (1996). *Exp. Cell Res.* **229**, 20.
32. Shushan, B., *in Views, Fall 1994* (Perkin-Elmer newsletter), pp. 16–17.