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Arginine as an effective additive in gel permeation chromatography

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Abstract

A major problem in gel permeation chromatography (GPC) or size exclusion chromatography is non-specific binding of applied proteins to the column matrix (stationary phase). We have tested an aqueous arginine solution as the GPC mobile phase on silica-based and polymer-based columns, using mouse monoclonal antibody and recombinant human activin, interleukin-6, basic fibroblast growth factor, and interferon-γ as model proteins. We observed that addition of arginine to the mobile phase improves separation of the proteins and their soluble aggregates from the GPC columns, which suggests that arginine is an effective additive for the GPC mobile phase.

Keywords: Gel permeation chromatography; Non-specific binding; Arginine; Aggregation; Aggregation suppression

1. Introduction

Gel permeation chromatography (GPC), also termed gel filtration or size exclusion chromatography, is one of many versatile protein purification techniques and, along with electrophoresis and reverse-phase high-performance liquid chromatography, is among the most frequently used techniques for analysis and quality control of proteins [1–5]. Analytical data for therapeutic proteins analyzed by these techniques form the foundation for their clinical use and for marketing approval [6,7]. GPC can be used to determine the molecular weights of proteins [8–10], and, in frontal gel chromatography, the interactions of macromolecules [11,12]. GPC has also been applied for protein refolding (reviewed in [10]).

In analytical, preparative, and refolding applications of GPC, proteins tend to stick to the column matrix (stationary phase), leading to abnormal chromatograms, protein loss, and column damage. Such binding can cause an abnormal separation profile and, hence, can lead to inaccurate protein molecular weight data or incorrect analyses of protein interactions in frontal gel chromatography. To reduce such non-specific binding to the stationary phase, various approaches have been taken [13–17]. High ionic strength using NaCl or phosphate at high concentrations is one approach that should reduce electrostatic interactions between proteins and the columns [13,14]. However, high salt concentrations can also increase the hydrophobic interactions between the protein and the column matrix. Inclusion of urea or organic solvents is another approach that should weaken the hydrophobic interactions, but it would also increase the ionic interactions [15]. Extreme pH has also been used to improve GPC separation [16,17]. However, each one of these conditions can alter the protein conformational or aggregation state, leading to erroneous conclusions that do not reflect the actual state of the proteins in stored solutions.

Arginine has been used as a solvent additive to assist in refolding [18–22], solubilization [23,24], suppression of protein aggregation [25,26], and dissociation of antibodies from Protein A [27]. The mechanism of such effects of arginine on proteins is proposed to be its ability to disrupt weak,

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non-specific, protein–protein interactions [28]. Here, we have tested aqueous arginine solutions for their ability to suppress non-specific interactions between proteins and the column matrix using polysaccharide- and silica-based columns, and we show that arginine greatly improves separation of various proteins, particularly their soluble aggregates. Thus, it appears that arginine may suppress both ionic and hydrophobic interactions.

2. Materials and methods

2.1. Materials

The following proteins were used as model proteins. Mouse monoclonal antibody (mAb), subclass IgG1, was purified from myeloma cell conditioned media, as described elsewhere [27]. Recombinant human activin and human interleukin-6 (IL6) were purified and refolded from inclusion bodies obtained using an *Escherichia coli* expression system, as previously described [29]. Recombinant human basic fibroblast growth factor (bFGF) and human interferon-γ were purchased from BioSource International (Camarillo, CA). Regents used for analyses were of HPLC grade.

2.2. Methods

Silica-based GPC column TSKG3000SWXL (particle size, 5 μm; column size, 0.78 cm Ø × 30 cm; Toso, Tokyo, Japan), polymer-based GPC column Superdex-200HR 10/30 (particle size, 13 μm; column size, 1 cm Ø × 30 cm; Amersham Biosciences, Tokyo, Japan), and polymer-based GPC column Superdex-75HR 10/30 (particle size, 13 μm; column size, 1 cm Ø × 30 cm; Amersham Biosciences, Tokyo, Japan) were used under the control of a L7400 HPLC system equipped with D-2500 chromato-integrator (Hitachi Ltd., Tokyo, Japan). The HPLC system was operated at a flow rate of 0.8 ml/min at room temperature, and the separation profile was monitored at 280 nm. The buffer used for the GPC mobile phase was made with 0.1 M sodium phosphate (P-buffer) and different concentrations of NaCl or arginine, as indicated in Section 3. Column performance is compromised at higher concentrations of arginine due to the high viscosity of aqueous arginine solutions. Molecular weight calibration in each eluent condition was done by using molecular weight calibration kit (HMW and LMW calibration kit; Amersham BioSciences, Tokyo, Japan).

3. Results

3.1. Mouse monoclonal antibody (mAb)

Mouse mAb was used, and both acid and heat stresses were applied to generate aggregates. First, about 100 μl containing 17 μg of mAb was applied to a silica-based TSK G3000SWXL column (particle size, 5 μm; column size, 0.78 cm Ø × 30 cm) at a flow rate of 0.8 ml/min. The column was equilibrated with P-buffer, pH 6.8, with and without 0.2 M arginine. The separation profile of GPC in the absence of arginine is shown (Fig. 1A(1)). A major peak corresponding to the monomer and a minor peak corresponding to the aggregates were observed. The peak area was used to calculate the amount of protein separated and was calibrated by injecting a known quantity of unstressed mAb composed primarily of monomers because recovery of monomers from the column was found to be high. The total peak area was only 20% of the applied amount, suggesting that mAb was retained on the column. On the basis of this low recovery, the peak area of aggregates was estimated to be 21.7% of the total protein recovered. The separation profile of the same sample analyzed in the presence of 0.2 M arginine is also shown (Fig. 1A(2)). The peak of the aggregates was higher than the monomer peak in the presence of arginine, indicating that the column used did specifically bind the aggregates in the absence of arginine. With arginine, aggregates accounted for 67.4% of the material separated from the column. To determine the amount recovered, a known amount (5.28 μg) of standard IgG4 (unstressed) was injected onto the column in the absence and presence of 0.2 M arginine, resulting in peaks of similar size (Fig. 1A, insets). Thus, it is evident that GPC analysis of this particular mAb underestimates the amount of aggregates formed when the column is run with P-buffer alone. Re-injection of the aggregate peak resulted in a single peak corresponding to the aggregate (data not shown), indicating that arginine does not alter the state of the antibody in solution.

We then performed the same experiments using the Superdex 200HR column (particle size, 13 μm; column size, 1 cm Ø × 30 cm) as an example of polymer-based GPC, and the recoveries from the G3000SWXL and Superdex 200HR columns are compared side-by-side (Fig. 1B). In both columns, the total recovery was greatly improved in the presence of 0.2 M arginine. More importantly, aggregate content was nearly identical with these two different columns, which is consistent with the ability of arginine to prevent non-specific binding of proteins to the column, independently of the chemical nature of the surface. Clearly in both columns the aggregate content was consistently underestimated in the absence of arginine.

The effects of NaCl on the recovery from the G3000SWXL were examined using the same mAb (Fig. 2). There was little increase in recoveries with addition of 0.2 M NaCl, and an increase of NaCl concentration to 0.4 M decreased the recovery significantly to 78%. However, addition of 0.2 M arginine increased the recovery by a factor of 2.4, mainly owing to the greatly increased recovery of aggregates (Fig. 2). We observed no further increase in recoveries with arginine concentrations above 0.2 M (data not shown).

A similar observation was also made with humanized antibodies containing varying degrees of aggregates and with

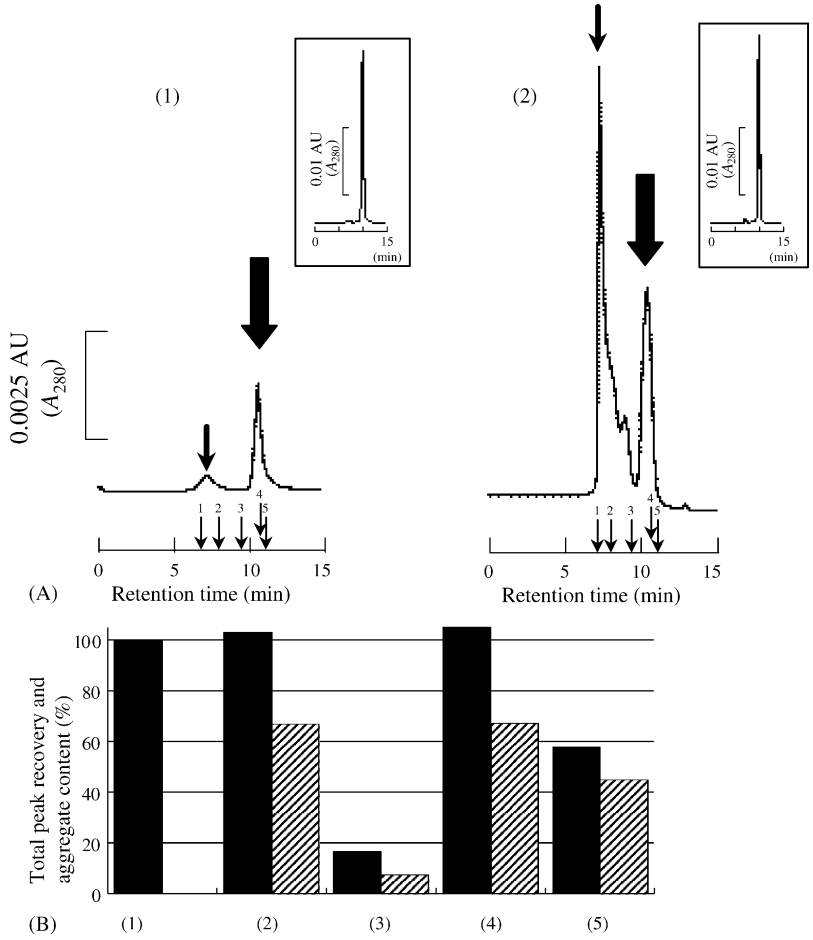

Fig. 1. (A) GPC profile of mouse mAb in P-buffer using G3000SWXL without (1) and with 0.2 M arginine (2). The large arrow indicates the separation of a monomer, while the small arrow indicates the separation of aggregates. To determine the peak area per protein, 5.28 μg of mAb was injected in the absence and presence of 0.2 M arginine (insert). Narrow arrows show the retention time of molecular weight markers: (1) blue dextran 2000; (2) thyroglobulin, 669 K; (3) ferritin, 440 K; (4) catalase, 232 K; and (5) aldolase, 158 K. (B) Recovery of mAb from GPC using G3000SWXL and Superdex-200HR. Total peak recovery in each condition is shown as a relative yield to the loaded protein amount. Columns: (1) loaded amount (by UV absorbance at 280 nm of heat-treated mAb); (2) G3000SWXL/P-buffer + 0.2 M arginine; (3) G3000WSXL/P-buffer; (4) Superdex-200HR/P-buffer + 0.2 M arginine; (5) Superdex-200HR/P-buffer. Shaded boxes, total protein (1) and total peak recovery (2–5); striped boxes, aggregate content.

P-buffer containing NaCl or not (data not shown). Increased recoveries were also observed in preparative-scale antibody purification in the presence of arginine (data not shown).

3.2. Activin

Recombinant human activin, 7 μg in a 50 μl sample volume, was applied to a Superdex-75HR 10/30 column (particle size, 13 μm; column size, 1 cm Ø × 30 cm) equilibrated with P-buffer, pH 7.3, containing 0.75 M NaCl. Activin was separated at the position of salt peak, indicating that activin was bound to the column (Fig. 3). Although activin is recovered from the column, there was no separation from the salt peak and, hence, no separation of different forms of the protein, even when they were present in the sample. The peak area was estimated to be 200,290. However, when 0.75 M NaCl was replaced with 0.75 M arginine, a peak corresponding to activin was observed, with a peak area of 155,027. In the absence of protein binding to the column, a peak corresponding to activin aggregates was observed before the monomer peak, which accounted for 2.3% of the total area (3687). This aggregation peak had never been observed due to the poor quality of column conditions used before. The total peak area of activin monomers and aggregates was not equal to the area observed in the absence of arginine (200,290), reflecting the fact that the peak area in the absence of arginine contained not only activin monomers and aggregates but also salts and other small-molecule components occurring at the same position. When the separation profiles with and without arginine are compared, the shoulder in front of the major peak in the absence of arginine may correspond to activin resolved from the salt peak. Activin shows a peak in the presence of arginine between ovalbumin (43 K) and chymotrypsinogen (25 K) near its molecular mass (26 K) (Fig. 3B).

Retention time of activin decreased as the NaCl concentration was decreased (Fig. 3A, inset), suggesting that NaCl

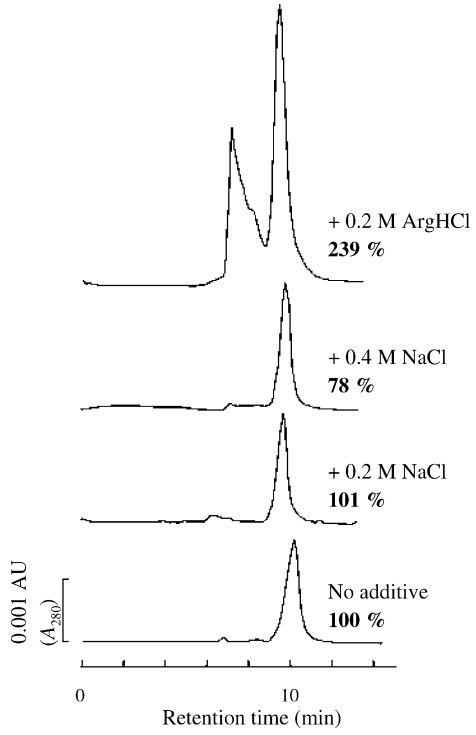

Fig. 2. Effects of NaCl and arginine on the recovery of mAb from G3000SWXL in P-buffer, pH 6.8. Bottom trace, no additive; second trace, 0.2 M NaCl; third trace, 0.4 M NaCl; top trace, 0.2 M ArgHCl. The percentage given at each trace shows the total peak recovery relative to the recovery with no additive (100%).

enhances binding of activin to the column. At the NaCl concentrations tested, activin was not separated from the salts. However, retention times increased as the arginine concentrations decreased, indicating that the effect of arginine on protein binding is reduced at lower concentrations and hence that arginine at 0.75 M gave the best separation.

3.3. Interleukin-6

About 7.5 μg of IL6 (50 μl) was applied to a Superdex-75HR 10/30 column equilibrated with P-buffer, pH 7.3, containing 0.75 M NaCl. Two peaks corresponding to the monomer and aggregates were observed (Fig. 4). The peak of the monomer occured as a symmetric peak at the expected position for a molecular weight of 18,500. A similar profile was observed with this protein in the presence of 0.75 M arginine (used in place of 0.75 M NaCl). Because separation of IL6 was normal even in the absence of arginine (Fig. 4A), arginine changed the separation profile little, except that the resolution of larger aggregates may be higher.

3.4. bFGF

About 5 μg of recombinant bFGF in a 100 μl solution was applied to a Superdex-75HR 10/30 column equilibrated with P-buffer, pH 6.8, containing 0.2 M NaCl. Separation of bFGF was greatly retarded relative to the expected peak position for the molecular weight of 17,000 (Fig. 5). The peak area was estimated to be 59,281. However, when 0.2 NaCl was replaced with 0.2 M arginine, separation of the protein occurred earlier than that observed in the absence of arginine, with the peak area increasing to 154,616. Evidently, arginine suppresses interactions of bFGF with the column, thereby resulting in a lower retention time and a 2.6-fold greater recovery of applied protein.

3.5. Interferon-γ

About 4.8 μg of recombinant interferon-γ in 100 μl of solution was injected onto a Superdex-75HR 10/30 column equilibrated with P-buffer, pH 6.8, containing 0.4 M NaCl. No peak was observed (Fig. 6), indicating that the protein

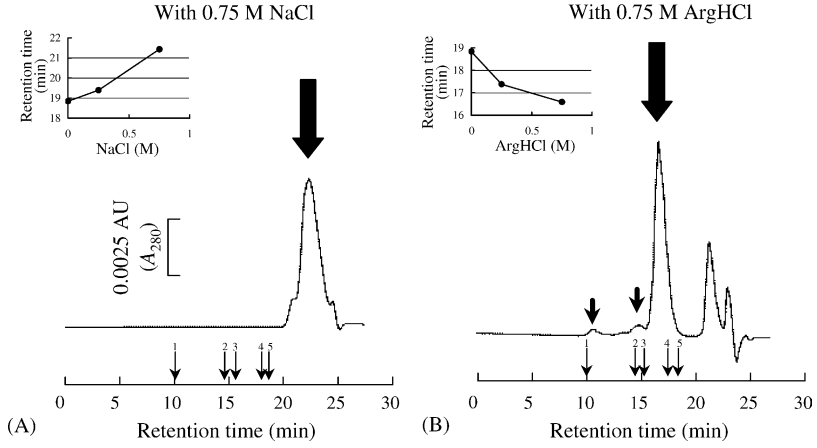

Fig. 3. GPC profiles of activin in P-buffer with 0.75 M NaCl (A) and 0.75 M arginine (B). (Inset) Retention time of activin peak as a function of NaCl or arginine concentration. Recovered peak of activin is shown in (A) by the large arrow and occurs at the position of salts. In (B), the large arrow shows the monomeric activin and the small arrows show the oligomers. In both chromatograms, narrow arrows show the retention times of molecular weight markers: (1) dextran blue, 2 K; (2) bovine serum albumin, 67 K; (3) ovalbumin, 43 K; (4) chymotrypsinogen, 25 K; and (5) ribonuclease, 14 K.

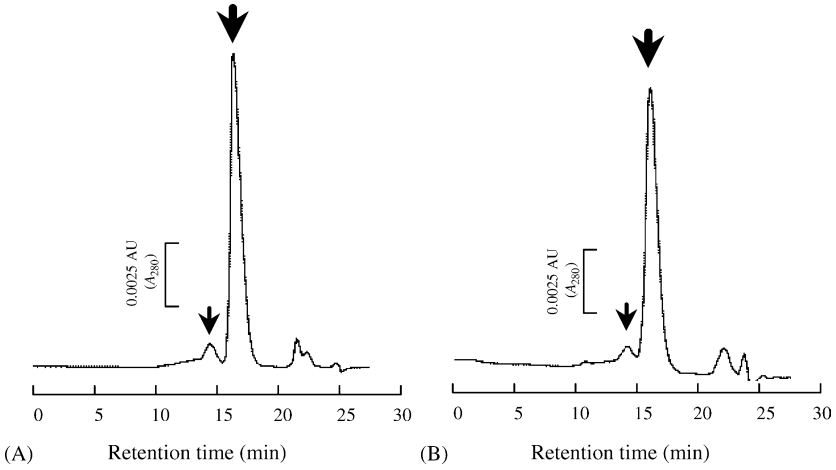

Fig. 4. GPC profiles of IL6 in 0.75 M NaCl (A) and 0.75 M arginine (B). Large arrows show monomer separation, and small arrows show aggregate separation.

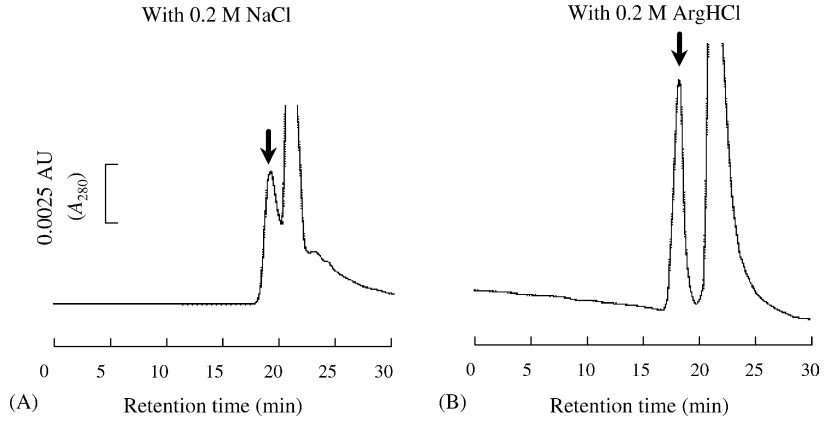

Fig. 5. GPC profiles of bFGF in 0.2 M NaCl (A) and 0.2 M arginine (B). There is little resolution of the separation of bFGF (arrow) from the salt in (A), while baseline resolution is observed in (B).

was completely trapped by the column. A decrease in NaCl concentration to 0.2 or 0 M had no effect on the separation profile (data not shown). Addition of 1 M urea in place of NaCl also showed no effect (data not shown). Replacement of 0.4 M NaCl with 0.4 M arginine, however, resulted in a peak corresponding to its dimeric molecular weight (Fig. 6).

4. Discussion

Gel permeation chromatography is the most critical analytical method for clinical development and market approval of therapeutic proteins, including monoclonal antibodies. Aggregation is a major problem in formulation, storage, and handling processes of proteins, because aggregation is

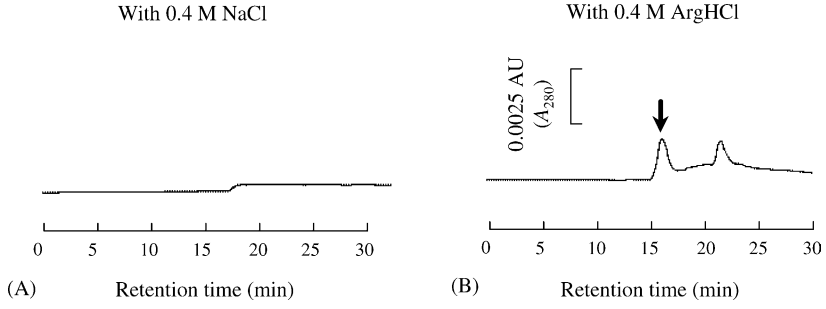

Fig. 6. GPC profiles of interferon-γ in 0.4 M NaCl (A) and 0.4 M arginine (B). No interferon-γ peak interferon-γ is observed in (A), while an interferon-γ peak is observed (arrow) in (B).

a potential cause of the side effects of therapeutic proteins [30,31]. We have demonstrated herein the possibility that the amount of aggregates in the mAb samples can be greatly underestimated by GPC analysis when carried out in conventional phosphate buffer. Separation of several cytokines, including soluble oligomers, from the GPC column can be improved using arginine-containing mobile phase.

GPC columns confer both hydrophobic and electrostatic interactions onto proteins, potentially leading to non-specific protein adsorption. Higher concentrations of NaCl are frequently used to reduce electrostatic interactions [13,14]. However, as a moderate "salting-out salt," NaCl enhances hydrophobic interactions, affecting its suppressing effects on electrostatic adsorption of the proteins. Organic solvents, however, weaken hydrophobic interactions but enhance electrostatic interactions. Accordingly, neither additive may be ideal in suppressing non-specific adsorption of proteins to the column matrix. In addition, NaCl may enhance protein–protein interactions and hence protein aggregation via effects of NaCl on hydrophobic interactions, while organic solvents may weaken hydrophobic interactions between protein molecules, leading to dissociation of protein aggregates [16,17,33,34]. These additives may change the aggregation state of the proteins in the original solution, and hence GPC analysis in the presence of these additives, when used to suppress non-specific adsorption of proteins, may lead to erroneous conclusions about the state of the proteins in solution.

Here we have demonstrated that arginine suppresses non-specific adsorption of proteins. It has been shown that arginine maintains the native structure of the proteins and only slightly affects the melting temperature of the proteins [26,28]. Arginine has no other adverse effects on proteins.

Addition of 0.2–0.75 M arginine makes it possible to separate normally "sticky" proteins. The arginine increases their recovery as well as the separation of soluble oligomers. Our observations show that arginine is a suitable additive for the mobile phase in GPC analysis of protein aggregation in solution. The mechanism of the effect of arginine on protein adsorption can be described as follows. Arginine exerts its effects at higher concentrations. It assists in refolding [18–22], solubilizes protein from inclusion bodies [23,24], suppresses aggregation of denatured proteins [25,26], and elutes antibodies from Protein A columns [27]. These effects of arginine occur above 0.1–0.2 M of arginine, suggesting that weak interactions between protein and arginine are involved [32,35]. Weak interactions between solvent additives and proteins have been studied by the preferential interaction [32,35] and amino acid solubility measurements [36]. Only limited data are available for arginine in its preferential interactions with proteins [32]. Such data suggest a limited extent of arginine binding to the proteins, which can be related to the effects of arginine on suppression of non-specific protein adsorption as well as other effects described above [28].

As also described above, arginine can solubilize proteins from inclusion bodies and dissociate antibodies from Protein A [27]; one may then suspect that arginine causes dissociation of soluble oligomers. We have several pieces of indirect evidence relating to this issue. First, arginine does not solubilize proteins from "normal" inclusion bodies [28], which may occur via strong association of largely unfolded structures; on the other hand, it solubilizes proteins from "loose" inclusion bodies, which consist of weakly associating native or native-like structures [28,37]. These findings strongly suggest that arginine cannot easily dissociate soluble oligomers. Second, arginine does not dissociate antibodies from Protein A at neutral pH but instead dissociates such complexes at mildly acidic pH, at which interactions are weakened due to acid-induced conformational changes of bound antibodies [38]. Finally, some oligomeric enzymes do not lose their activities in the presence of ca. 0.5 M arginine (Tokunaga et al., unpublished results). Arginine does not change oligomeric structure of various proteins (Tsumoto et al., unpublished results). It is therefore unlikely that arginine dissociates irreversibly aggregating soluble oligomers, which suggests that arginine is effective for analyses and/or preparation of proteins, including soluble oligomers, using GPC.

A large number of protein therapeutics, including monoclonal antibodies, are under development; however, detection and quantification of soluble oligomers are major regulatory concerns. Analyses of protein aggregation without column chromatography, such as sedimentation, field-flow fractionation, and light scattering, are used, but only to complement the aggregation analysis by GPC. GPC analysis using aqueous arginine solution as a mobile phase should generate reliable aggregation data more consistent with the column-free techniques mentioned above. Higher recovery of applied proteins is another advantage: i.e., arginine addition increases the yield of proteins from GPC. Finally, it should be noted that arginine increases the lifetime of the columns for purification and analysis of proteins, and may allow for multiple uses, without cleaning steps.

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