9328–9338, 2010; Ejima *et al*., PROTEINS: Structure, Function, and Bioinformatics, Vol. 66:954–962, 2007). The low pH elution does not only induce aggregation of the proteins resulting in reduced yields, but may also compromise the long-term stability of the recovered non-aggregated proteins.

[0005] In an attempt to avoid the undesirable effects of the low pH elution from protein A resins, a temperature-responsive protein A resin was developed as an alternative to conventional protein A resins. This new resin is comprised of a mutant version of protein A that binds to the Fc region of immunoglobulins at temperatures below 10 °C, but loses affinity for the Fc region at elevated temperatures (e.g. 40 °C)(Koguma *et al*., Journal of Chromatography A, Vol. 1305: 149-153, 2013). The temperature-responsive protein A resin was initially received with great enthusiasm because it allows the elution of Fc region-containing proteins from the resin at neutral pH by simply heating the column (Koguma *et al*., Journal of Chromatography A, Vol. 1305: 149-153, 2013). Although purification of proteins with the temperature-responsive protein A resin produces stable molecules, the approach is impractical because it requires a shift of 30° or more of the column temperature from column loading to elution, thus creating a significant lag time between the loading and elution steps. The time delay required to manipulate column temperature adds a tremendous amount of cycle time to purification operations, thereby making this approach undesirable for both large scale manufacturing processes and high-throughput purification of large panels of proteins during the discovery phase. Moreover, the impact of large temperature swings on the stability of proteins eluted from the temperature-responsive protein A resin remains unknown.

[0006] Accordingly, there is a need in the art for efficient purification methods for Fc region-containing proteins that minimize or reduce the effects on the structural integrity of the proteins associated with the prior protein A chromatography methods.

SUMMARY

[0007] The present disclosure is based, in part, on the development of a buffer composition that allows the elution of a bound Fc region-containing protein from a temperature-responsive protein A resin at neutral pH and constant temperature. A purification scheme employing a temperature-responsive protein A resin in combination with the elution buffers described herein results in reduced levels of aggregated protein, thereby reducing the number of downstream purification

steps. Additionally, such purification methods are readily scalable for industrial manufacturing because elution with the elution buffers described herein can be conducted in a temperature-independent manner.

[0007a] In one aspect, the present disclosure refers to a method for purifying a protein comprising an Fc region from a solution comprising the protein and one or more impurities, comprising: adsorbing the protein to a temperature-responsive protein A material at a temperature at which the protein binds to the material; and eluting the protein from the material at a temperature below about 25 °C with an elution buffer having a pH of 6.5 to 7.5 and comprising a chaotropic agent and a sugar alcohol, wherein the molar concentration ratio of the chaotropic agent to sugar alcohol is 0.4 to 4.5.

[0007b] In another aspect, the present disclosure refers to a method for purifying a protein comprising an Fc region, comprising: contacting a solution comprising the protein and one or more impurities with a temperature-responsive protein A material at a temperature at which the protein binds to the material; and eluting the protein from the material at a temperature below about 25 °C with an elution buffer having a pH of 6.5 to 7.5 and comprising a chaotropic agent, a sugar alcohol, an apolar amino acid, and a basic amino acid, wherein the protein is purified from one or more impurities in the solution.

[0008] Accordingly, the present disclosure provides a method for purifying a protein comprising an Fc region. In one example, the method comprises contacting a solution comprising the protein and one or more impurities with a temperature-responsive protein A material at a temperature at which the protein binds to the material; and eluting the protein from the material at a temperature below about 35 °C with an elution buffer described herein, wherein the protein is purified from one or more impurities in the solution.

[0009] In certain examples, the present disclosure also provides a method for reducing aggregation during purification of a protein comprising an Fc region. In one example, the method comprises adsorbing the protein to a temperature-responsive protein A material at a temperature at which the protein binds to the material; and eluting the protein from the material at a temperature below about 35 °C with an elution buffer described herein, wherein the amount of the Fc region-containing protein in aggregated form in the eluate is less than the amount in an eluate from a conventional protein A material. In some examples, the amount of the Fc region-containing protein in aggregated form in the eluate from the temperature-responsive protein A

material is less than 30%. In related examples, at least 70% of the Fc region-containing protein in the eluate from the temperature-responsive protein A material is in monomeric form.

[0010] The elution buffers employed in the methods of the disclosure have a pH in the neutral range (e.g. pH of about 6.5 to about 7.5) and comprise a chaotropic agent and a sugar alcohol. In some examples, the chaotropic agent and sugar alcohol are present in a molar concentration ratio of about 0.4 to about 4.5. In one example, the molar concentration ratio of the chaotropic agent to sugar alcohol is about 1.1 to about 1.8. The concentration of the chaotropic agent in the elution buffer may be from about 0.4 M to about 5 M depending on the particular chaotropic agent used. In some examples, the chaotropic agent may be urea, guanidinium chloride, or a thiocyanate salt (e.g. sodium thiocyanate, potassium thiocyanate, or ammonium thiocyanate). In certain examples, the chaotropic agent is urea. In other examples, the chaotropic agent is guanidinium chloride. The concentration of the sugar alcohol in the elution buffer may be from about 1 M to about 4.5 M. In certain examples, the sugar alcohol is sorbitol, mannitol, xylitol, or glycerol. In one example, the sugar alcohol is sorbitol. In another example, the sugar alcohol is mannitol.

[0011] In some examples, the elution buffer employed in the methods of the disclosure further comprises one or more amino acids. The amino acids can be apolar amino acids, such as alanine, cysteine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, or valine, and/or basic amino acids, such as histidine, lysine, ornithine, or arginine. In certain examples, the elution buffer comprises at least one apolar amino acid and at least one basic amino acid. For instance, in one example, the elution buffer comprises proline and arginine. The concentration of the amino acids in the elution buffer can be from about 0.25 M to about 1 M.

[0012] In various examples, the elution buffer may further comprise a salt, such as a sodium salt or chloride salt. The salt may be present in the elution buffer at a concentration of about 0.1 M to about 1 M. In some examples, the salt included in the elution buffer is sodium chloride.

[0013] In certain examples, the elution buffer used in the methods of the disclosure comprises about 2 M to about 4.5 M of a chaotropic agent, about 1 M to about 4.5 M of a sugar alcohol, about 0.25 M to about 1 M of a basic amino acid, about 0.25 M to about 1 M of an apolar amino acid, and about 0.25 M to about 0.8 M of a salt. In some such examples, the chaotropic agent is urea, the sugar alcohol is sorbitol, the basic amino acid is arginine, the apolar amino acid is proline, and the salt is sodium chloride.

[0014] Elution of the Fc region-containing protein from the temperature-responsive protein A material according to the methods of the disclosure can be performed at a temperature from about 1 °C to about 25 °C. In some examples, elution of the protein is performed at the same or similar temperature at which the protein was adsorbed or bound to the temperature-responsive protein A material. For instance, in one example, elution of the Fc region-containing protein is performed at a temperature less than about 10 °C, e.g. from about 1 °C to about 6 °C. In other examples, elution of the Fc region-containing protein is performed at room temperature, for example at about 20 °C to about 25 °C.

[0015] Fc region-containing proteins that can be purified according to the methods described herein include antibodies, Fc-fusion proteins, and multi-specific antigen binding proteins. In some examples, the Fc region-containing protein is an antibody. In other examples, the Fc region-containing protein is an Fc-fusion protein, such as an Fc-fusion protein comprising at least one single chain Fv fragment. The Fc region-containing proteins can be produced recombinantly, for example in mammalian cells. In such examples, the Fc region-containing proteins can be purified from cell culture supernatants or cell lysates, such as those produced as a result of harvest operations from a bioreactor.

[0016] The disclosure is also based, in part, on the discovery that size exclusion chromatography, when used with a buffer comprising a chaotropic agent, a sugar alcohol, and at least one amino acid as the mobile phase, can effectively separate fully assembled antibodies from half antibody contaminants. Accordingly, the present disclosure also provides a method for separating antibodies from half antibody forms thereof. In one example, the method comprises contacting a solution comprising antibodies and half antibody forms thereof with a gel filtration matrix using a mobile phase having a pH of about 6.5 to about 7.5 and comprising a chaotropic agent, a sugar alcohol, an apolar amino acid, and a basic amino acid; and collecting elution fractions from the gel filtration matrix, wherein the antibodies are eluted in one set of elution fractions and the half antibody forms thereof are eluted in another set of elution fractions, thereby separating the antibodies from the half antibody forms thereof. In certain examples of the method, the antibodies are multispecific (e.g. bispecific) heterodimeric antibodies.

[0017] Any of the elution buffers described herein for eluting Fc region-containing proteins from a temperature-responsive protein A resin at neutral pH and constant temperature can be used as a mobile phase in size exclusion chromatography for separating antibodies from half antibody

forms thereof. In some examples, the mobile phase comprises about 2 M to about 4.5 M of a chaotropic agent, about 1 M to about 4.5 M of a sugar alcohol, about 0.25 M to about 1 M of a basic amino acid, and about 0.25 M to about 1 M of an apolar amino acid. In these and other examples, the mobile phase comprises urea, sorbitol, arginine, and proline. In certain examples, the mobile phase comprises about 2 M to about 4.5 M urea, about 1 M to about 4.5 M sorbitol, about 0.25 M to about 1 M arginine, about 0.25 M to about 1 M proline, and 0.25 M to about 0.8 M sodium chloride. In some examples, the mobile phase comprises about 4 M urea, about 2.2 M sorbitol, about 0.5 M arginine, about 0.5 M proline, and about 0.75 M sodium chloride. The pH of the mobile phase may be from about 6.5 to about 7.5 or in particular examples, from about 7.0 to about 7.4.

[0018] In certain examples of the method, the mobile phase is passed through the gel filtration matrix at a flow rate of about 0.01 ml/min to about 0.2 ml/min. In other examples, the mobile phase is passed through the gel filtration matrix at a flow rate of about 0.02 ml/min to about 0.06 ml/min. The gel filtration matrix may be comprised of cross-linked agarose and dextran and can have a fractionation range of about 10 kDa to about 600 kDa.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Figure 1. Structure of IgG-scFv binding protein. The figure depicts a schematic representation of an IgG-scFv binding protein, an example of an Fc-containing protein that can be purified by the methods described herein. An IgG-scFv binding protein is comprised of two single-chain variable fragments (scFvs), each of which comprises variable domains from a first antibody linked together by a peptide linker, fused to the carboxyl-termini of the heavy chains of a second antibody through another peptide linker.

[0020] Figure 2. IgG-scFv binding protein eluted from conventional protein A column. The figure shows SE-UPLC chromatograms from samples of the protein A eluate pools of an IgG-scFv binding protein. The binding proteins were eluted from a conventional protein A chromatography column using a 1% acetic acid buffer, pH 2.7 at 4°C. The eluates off the SEC-UPLC column contained both aggregate peaks (denoted by black arrows) and a monomer peak (denoted by a black star). Each of the four panels is a separate experiment.

[0021] Figure 3. IgG-scFv binding protein eluted from a temperature-responsive protein A chromatography column at 4°C. The figure shows SE-UPLC chromatograms from samples of

the protein A eluate pool for an IgG-scFv binding protein. The binding protein was eluted from a temperature-responsive protein A chromatography column at 4°C using an elution buffer having a pH of 7.2 and comprising 50 mM HEPES, 0.75 M NaCl, 0.5 M arginine, 0.5 M proline, 2.2 M sorbitol, and 4 M urea. The monomer peak is denoted by a black star. Each of the three panels is a separate experiment.

[0022] Figure 4A. SDS-PAGE of IgG-scFv binding protein during purification on temperature-responsive protein A chromatography column. The samples in each of the lanes on the gel are as follows: Stds = protein standards; Feed = sample of clarified cell culture supernatant prior to column loading; FT = sample of column flow through fraction; and Eluate = sample of eluate pool following elution of binding protein from column at room temperature

CLAIMS

1. A method for purifying a protein comprising an Fc region from a solution comprising the protein and one or more impurities, comprising:

adsorbing the protein to a temperature-responsive protein A material at a temperature at which the protein binds to the material; and

eluting the protein from the material at a temperature below about 25 °C with an elution buffer having a pH of 6.5 to 7.5 and comprising a chaotropic agent and a sugar alcohol, wherein the molar concentration ratio of the chaotropic agent to sugar alcohol is 0.4 to 4.5.

2. The method of claim 1, wherein the molar concentration ratio of the chaotropic agent to sugar alcohol is 1.5 to 2.5.

3. The method of claim 1, wherein the molar concentration ratio of the chaotropic agent to sugar alcohol is 1.8 to 2.2.

4. The method of claim 1, wherein the molar concentration ratio of the chaotropic agent to sugar alcohol is 0.5 to 1.5.

5. The method of any one of claims 1 to 4, wherein the elution buffer further comprises one or more amino acids.

6. The method of claim 5, wherein the elution buffer comprises a basic amino acid and/or an apolar amino acid.

7. A method for purifying a protein comprising an Fc region, comprising:

contacting a solution comprising the protein and one or more impurities with a temperature-responsive protein A material at a temperature at which the protein binds to the material; and

eluting the protein from the material at a temperature below about 25 °C with an elution buffer having a pH of 6.5 to 7.5 and comprising a chaotropic agent, a sugar alcohol, an apolar

amino acid, and a basic amino acid, wherein the protein is purified from one or more impurities in the solution.

8. The method of claim 6 or 7, wherein the basic amino acid is histidine, lysine, ornithine, or arginine.

9. The method of claim 8, wherein the basic amino acid is arginine.

10. The method of claim 6 or 7, wherein the apolar amino acid is alanine, cysteine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, or valine.

11. The method of claim 10, wherein the apolar amino acid is proline.

12. The method of any one of claims 6 to 11, wherein the elution buffer comprises arginine and proline.

13. The method of any one of claims 6 to 12, wherein the basic amino acid and/or the apolar amino acid is present at a concentration of 0.25 M to 1 M.

14. The method of claim 13, wherein the basic amino acid and/or the apolar amino acid is present at a concentration of about 0.5 M.

15. The method of any one of claims 1 to 14, wherein the chaotropic agent is urea, guanidinium chloride, sodium thiocyanate, potassium thiocyanate, or ammonium thiocyanate.

16. The method of claim 15, wherein the chaotropic agent is urea.

17. The method of claim 16, wherein the urea is present at a concentration of 2 M to 4.5 M.

18. The method of claim 17, wherein the urea is present at a concentration of about 4 M.

19. The method of claim 15, wherein the chaotropic agent is guanidinium chloride.

20. The method of any one of claims 1 to 19, wherein the sugar alcohol is sorbitol, mannitol, xylitol, or glycerol.

21. The method of claim 20, wherein the sugar alcohol is sorbitol.

22. The method of any one of claims 1 to 21, wherein the sugar alcohol is present at a concentration of 1 M to 4.5 M.

23. The method of claim 22, wherein the sugar alcohol is present at a concentration of 2 M to 2.5 M.

24. The method of any one of claims 1 to 23, wherein the elution buffer further comprises a salt.

25. The method of claim 24, wherein the salt is present at a concentration of 0.25 M to 0.8 M.

26. The method of claim 24, wherein the salt is sodium chloride.

27. The method of claim 26, wherein the elution buffer comprises 2 M to 4.5 M urea, 1 M to 4.5 M sorbitol, 0.25 M to 1 M arginine, 0.25 M to 1 M proline, and 0.25 M to 0.8 M sodium chloride.

28. The method of claim 27, wherein the elution buffer comprises about 4 M urea, about 2.2 M sorbitol, about 0.5 M arginine, about 0.5 M proline, and about 0.75 M sodium chloride.

29. The method of any one of claims 1 to 28, wherein the elution buffer has a pH of 7.0 to 7.4.

30. The method of any one of claims 1 to 29, wherein eluting the protein from the material is performed at a temperature from 1 °C to 25 °C.

31. The method of claim 30, wherein eluting the protein from the material is performed at a temperature from 1 °C to 6 °C.

32. The method of claim 30, wherein eluting the protein from the material is performed at a temperature from 20 °C to 25 °C.

33. The method of any one of claims 1 to 32, wherein the temperature-responsive protein A material is immobilized to a solid phase.

34. The method of claim 33, wherein the solid phase is a bead, a resin, a gel, a film, or a particle.

35. The method of any one of claims 7 to 34, wherein contacting the solution with the material is performed at a temperature of about 10 °C or less.

36. The method of claim 35, wherein contacting the solution with the material is performed at a temperature of 1°C to 6 °C.

37. The method of any one of claims 1 to 36, further comprising washing the material having the bound protein with one or more wash solutions prior to eluting the protein from the material.

38. The method of claim 37, wherein the one or more wash solutions comprises sodium chloride at a concentration of 0.5 M to 2 M.

39. The method of any one of claims 1 to 38, wherein the protein comprising an Fc region is recombinantly produced in a mammalian cell.

40. The method of claim 39, wherein the mammalian cell is a Chinese Hamster Ovary cell.

41. The method of any one of claims 1 to 40, wherein the protein comprising an Fc region is an antibody.

42. The method of any one of claims 1 to 40, wherein the protein comprising an Fc region is an Fc fusion protein.

43. The method of claim 42, wherein the Fc fusion protein comprises at least one single chain Fv fragment.

44. The method of any one of claims 1 to 43, wherein one or more impurities comprises host cell protein, host cell DNA, cell culture protein, or combinations thereof.

45. The method of any one of claims 1 to 44, wherein the solution comprising the protein and one or more impurities is a harvest from a production bioreactor, a cell culture supernatant, or a cell lysate.

46. The method of any one of claims 1 to 45, wherein at least 70% of the Fc region-containing protein in the eluate from the material is in monomeric form.

47. The method of claim 46, wherein at least 80% of the Fc region-containing protein in the eluate from the material is in monomeric form.

48. The method of claim 47, wherein at least 90% of the Fc region-containing protein in the eluate from the material is in monomeric form.

49. The method of any one of claims 1 to 48, further comprising subjecting the eluted Fc region-containing protein to one or more chromatography steps, preferably selected from ion exchange chromatography, hydrophobic interaction chromatography, mixed mode chromatography, size exclusion chromatography, hydroxyapatite chromatography, metal affinity chromatography, and combinations thereof.

50. The method of any one of claims 1 to 49, further comprising subjecting the eluted Fc region-containing protein to size exclusion chromatography using a mobile phase having a pH of 6.5 to 7.5 and comprising a chaotropic agent, a sugar alcohol, an apolar amino acid, and a basic amino acid.