CLAIMS

1. A method of isolating or purifying a polypeptide that comprises or consists of at least three immunoglobulin single variable domains (ISVDs) from a composition comprising the polypeptide and a conformational variant thereof,

wherein the conformational variant and the polypeptide do not differ in their molecular weight and do not differ in their disulfide bridges, and wherein the conformational variant is characterized by an increased retention time in SE-HPLC compared to the polypeptide;

wherein the polypeptide to be isolated or purified is obtainable by expression in a host that is a lower eukaryotic host,

wherein the method comprises:

a) applying conditions that convert the conformational variant into the polypeptide selected from:

(i) applying a low pH treatment in a step of the isolation or purification process, wherein the low pH treatment comprises decreasing the pH of the composition to between pH 3.2 and pH 2.1 for at least 0.5 hours;

(ii) applying a chaotropic agent in a step of the isolation or purification process, wherein the chaotropic agent is guanidinium hydrochloride (GuHCl), and wherein the GuHCl is applied for at least 0.5 hours;

(iii) applying a heat stress in a step of the isolation or purification process, comprising incubating the conformational variant at about 40°C to 60°C, wherein the heat stress is applied for at least 0.5 hours; or

(iv) a combination of any of i) to iii); or

b) applying the composition to a chromatography column, wherein the composition is applied to the column using a load factor of at least 20 mg protein/ml resin, at least 30 mg protein/ml resin, or at least 45 mg protein/ml resin.

2. The method according to claim 1, wherein the lower eukaryotic host comprises yeast; optionally wherein said yeast is selected from a group consisting of Pichia, Hansenula, Saccharomyces, Kluyveromyces, Candida, Torulopsis, Torulaspora, Schizosaccharomyces, Citeromyces, Pachysolen, Debaromyces, Metschnikowia, Rhodosporidium, Leucosporidium, Botryoascus, Sporidiobolus, and Endomycopsis.

3. The method according to claim 2, wherein the yeast is Pichia such as *Pichia pastoris*.

4. The method according to any one of claims 1 to 3, wherein the polypeptide comprises or consists of at least four immunoglobulin single variable domains (ISVDs).

5. The method according to any one of claims 1 to 4, wherein the conformational variant has a decreased hydrodynamic volume compared to the polypeptide.

6. The method according to any one of claims 1 to 5, wherein the conformational variant is characterized by an altered retention time in IEX-HPLC compared to the polypeptide.

7. The method according to any one of claims 1 to 3, wherein the polypeptide comprises or consists of at least four immunoglobulin single variable domains (ISVDs), and wherein the low pH treatment comprises decreasing the pH of the composition to pH 3.0 or less.

8. The method according to any one of claim 1 or 7, wherein the pH is decreased to between pH 3.2 and 2.1, to between pH 3.0 and 2.1, to between pH 2.9 and pH 2.1, to between pH 2.7 and pH 2.1, or to between pH 2.6 and pH 2.3.

9. The method according to any one of claims 1 to 8, wherein the low pH treatment is applied for at least 0.5 hours, for at least 1 hour, for at least 2 hours, or for at least 4 hours.

10. The method according to any one of claims 1 to 9, wherein the pH is decreased to between pH 3.2 and pH 2.1 for at least 1.0 hour.

11. The method according to any one of claims 1 to 10, wherein the pH is decreased to between pH 3.0 and pH 2.1 for at least 0.5 hours, such as for at least 1.0 hour.

12. The method according to any one of claims 1 to 11, wherein the pH is decreased to between pH 2.9 and pH 2.1 for at least 0.5 hours, such as for at least 1.0 hour.

13. The method according to any one of claims 1 to 12, wherein the pH is decreased to between pH 2.7 and pH 2.1 for at least 0.5 hours, such as for at least 1.0 hour.

14. The method according to any one of claims 1 to 13, wherein the low pH treatment is applied before a purification step based on a chromatographic technique, during a purification step based on a chromatographic technique or after a purification step based on a chromatographic technique.

15. The method according to claim 14, wherein the low pH treatment is applied before applying the composition to the stationary phase of a chromatographic technique, or after eluting the composition from the stationary phase of a chromatographic technique.

16. The method according to any one of claims 1 to 15, wherein the chaotropic agent is guanidinium hydrochloride (GuHCl) in a final concentration of at least 1 M, or at least 2 M.

17. The method according to claims 1 to 16, wherein the GuHCl is applied for at least 1 hour.

18. The method according to any one of claims 1 to 17, wherein the heat stress is applied for at least 1 hour.

19. The method according to any one of claims 1 to 6, wherein the conformational variant is removed by one or more chromatographic techniques, optionally wherein the conformational variant has been identified by analytical chromatographic techniques such as SE-HPLC and IEX-HPLC before being removed by the one or more chromatographic techniques.

20. The method according to claim 19, wherein the chromatographic technique is a chromatographic technique based on hydrodynamic volume, surface charge or surface hydrophobicity.

21. The method according to claim 20, wherein the chromatographic technique is selected from any of size exclusion chromatography (SEC), ion-exchange chromatography (IEX) e.g., cation-exchange chromatography (CEX), mixed-mode chromatography (MMC), and hydrophobic interaction chromatography (HIC).

22. The method according to claim 21, wherein the HIC is based on a HIC column resin.

23. The method according to claim 21, wherein the HIC is based on a HIC membrane.

24. The method according to any one of claims 1 to 23, wherein in (b), the chromatographic column is a Protein A column.

25. The method according to any one of claims 1 to 24, wherein one or more of the conditions that convert the conformational variant into the polypeptide are applied alone, or in combination with one or more techniques that remove the conformational variant.

26. A method of isolating or purifying a polypeptide that comprises or consists of at least three or at least four immunoglobulin single variable domains (ISVDs),

wherein the polypeptide to be isolated or purified is obtainable by expression in a host that is a lower eukaryotic host,

wherein the method comprises one or more of the following:

(i) applying a low pH treatment to a composition comprising the polypeptide in a step of the isolation or purification process, wherein the low pH treatment comprises decreasing the pH of the composition to between pH 3.2 and pH 2.1 for at least 0.5 hours;

(ii) applying a chaotropic agent to a composition comprising the polypeptide in a step of the isolation or purification process, wherein the chaotropic agent is GuHCl, and wherein the GuHCl is applied for at least 0.5 hours;

(iii) applying a heat stress to a composition comprising the polypeptide in a step of the isolation or purification process, comprising incubating the composition at 40°C to 60°C, wherein the heat stress is applied for at least 0.5 hours;

(iv) applying the composition comprising the polypeptide to a chromatography column using a load factor of at least 20 mg protein/ml resin at least 30 mg protein/ml resin, or at least 45 mg protein/ml resin, optionally wherein the chromatographic column is Protein A column; or

(v) a combination of any of i) to iv).

27. The method according to claim 26, wherein the lower eukaryotic host comprises yeast; optionally wherein said yeast is selected from a group consisting of Pichia, Hansenula, Saccharomyces, Kluyveromyces, Candida, Torulopsis, Torulaspora, Schizosaccharomyces, Citeromyces, Pachysolen, Debaromyces, Metschnikowia, Rhodosporidium, Leucosporidium, Botryoascus, Sporidiobolus, and Endomycopsis.

28. The method according to claim 27, wherein the yeast is Pichia such as *Pichia pastoris*.

29. The method according to any one of claims 26 to 28, wherein the polypeptide comprises or consists of at least four immunoglobulin single variable domains (ISVDs), optionally wherein the low pH treatment comprises decreasing the pH of the composition to pH 3.0 or less.

30. The method according to any one of claims 26 to 29, wherein the pH is decreased to between pH 3.2 and pH 2.1, to between pH 3.0 and pH 2.1, to between pH 2.9 and pH 2.1, to between pH 2.7 and pH 2.1, or to between pH 2.6 and pH 2.3.

31. The method according to any one of claims 26 to 30, wherein the low pH treatment is applied for at least 0.5 hour, for at least 1 hour, for at least 2 hours, or for at least 4 hours.

32. The method according to any one of claims 30 or 31, wherein the pH is decreased to between pH 3.2 and pH 2.1 for at least about 1.0 hour.

33. The method according to any one of claims 30 to 32, wherein the pH is decreased to between pH 3.0 and pH 2.1 for at least 0.5 hour, such as for at least about 1.0 hour.

34. The method according to any one of claims 30 to 33, wherein the pH is decreased to between pH 2.9 and pH 2.1 for at least 0.5 hour, such as for at least 1.0 hour.

35. The method according to any one of claims 30 to 34, wherein the pH is decreased to between pH 2.7 and pH 2.1 for at least 0.5 hour, such as for at least 1.0 hour.

36. The method according to any one of claims 26 to 35, wherein the low pH treatment is applied before a purification step based on a chromatographic technique, during a purification step based on a chromatographic technique or after a purification step based on a chromatographic technique.

37. The method according to claim 36, wherein the low pH treatment is applied before applying the composition to the stationary phase of a chromatographic technique or after eluting the composition from the stationary phase of a chromatographic technique.

38. The method according to any one of claims 26 to 37, wherein the chaotropic agent is GuHCl in a final concentration of at least 1 M, or at least 2 M.

39. The method according to any one of claims 26 to 38, wherein the GuHCl is applied for at least 1 hour.

40. The method according to any one of claims 26 to 39, wherein the heat stress is applied for at least 1 hour.

41. A method of producing a polypeptide that comprises at least three or at least four immunoglobulin single variable domains (ISVDs), wherein the method comprises the purification or isolation according to the method of any one of claims 1 to 40.

42. The method according to claim 41, at least comprising the following steps:

a) Optionally cultivating the lower eukaryotic host or host cell under conditions that are such that the host or host cell will multiply;

b) maintaining the host or host cell under conditions that are such that the host or host cell expresses and/or produces said polypeptide; and

c) isolating and/or purifying the secreted polypeptide from the medium comprising one or more of the isolation or purification methods according to any of claims 1 to 40.

43. The method according to claim 42, wherein the lower eukaryotic host comprises yeast; optionally wherein said yeast is selected from a group consisting of Pichia, Hansenula, Saccharomyces, Kluyveromyces, Candida, Torulopsis, Torulaspora, Schizosaccharomyces, Citeromyces, Pachysolen, Debaromyces, Metschnikowia, Rhodosporidium, Leucosporidium, Botryoascus, Sporidiobolus, Endomycopsis.

44. The method according to claim 43, wherein the yeast is Pichia such as *Pichia pastoris*.

45. A method for isolating or purifying a polypeptide that comprises or consists of at least three or at least four immunoglobulin single variable domains (ISVDs) from a composition comprising the polypeptide and a conformational variant thereof, wherein the conformational variant and the polypeptide do not differ in their molecular weight and do not differ in their disulfide bridges, and wherein the conformational variant is characterized by an increased retention time in SE-HPLC compared to the polypeptide;

wherein the polypeptide to be isolated or purified is obtainable by expression in a host that is a lower eukaryotic host,

wherein the method comprises:

(1) Identifying the conformational variant by analytical chromatographic techniques such as SE-HPLC and IEX-HPLC;

(2) Adjusting the chromatographic conditions to allow specific removal of the conformational variant; and

(3) Removing the conformational variant from the composition comprising the polypeptide and the conformational variant thereof by one or more chromatographic techniques.

46. A method for optimizing one or more chromatographic techniques to allow isolation or purification of a polypeptide that comprises or consists of at least three or at least four immunoglobulin single variable domains (ISVDs) from a composition comprising the polypeptide and a conformational variant thereof by the one or more chromatographic techniques, wherein the conformational variant and the polypeptide do not differ in their molecular weight and do not differ in their disulfide bridges, and wherein the conformational variant is characterized by an increased retention time in SE-HPLC compared to the polypeptide;

wherein the polypeptide to be isolated or purified is obtainable by expression in a host that is a lower eukaryotic host,

wherein the method comprises:

(1) Identifying the conformational variant by analytical chromatographic techniques such as SE-HPLC and IEX-HPLC;

(2) Optimizing the chromatographic conditions to allow specific removal of the conformational variant.

47. The method according to claim 46, wherein the lower eukaryotic host comprises yeast; optionally wherein said yeast is selected from a group consisting of Pichia, Hansenula, Saccharomyces, Kluyveromyces, Candida, Torulopsis, Torulaspora, Schizosaccharomyces, Citeromyces, Pachysolen, Debaromyces, Metschnikowia, Rhodosporidium, Leucosporidium, Botryoascus, Sporidiobolus, Endomycopsis

48. The method according to claim 47, wherein the yeast is Pichia such as *Pichia pastoris*.

49. The method according to any one of claims 45 to 48, wherein the chromatographic technique is a chromatographic technique based on hydrodynamic volume, surface charge or surface hydrophobicity.

50. The method according to claim 49, wherein the chromatographic technique is selected from any of size exclusion chromatography (SEC), ion-exchange chromatography (IEX), mixed-mode chromatography (MMC), and hydrophobic interaction chromatography (HIC).

51. The method according to claim 50, wherein the ion-exchange chromatography (IEX) is cation-exchange chromatography (CEX).

52. The method according to claim 50, wherein the HIC is based on a HIC column resin.

53. The method according to claim 52 wherein the HIC resin is selected from any of Capto Phenyl ImpRes, Capto Butyl ImpRes, Phenyl HP, and Capto Butyl.

54. The method according to claim 50, wherein the HIC is based on a HIC membrane.