

CLAIMS

1. 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) from a composition comprising the polypeptide and a conformational variant thereof, the method comprising:
 - a) applying conditions that convert the conformational variant into the polypeptide;
 - b) removing the conformational variant; or
 - c) a combination of (a) and (b).
2. The method according to claim 1, wherein the polypeptide to be isolated or purified is obtainable by expression in a host.
3. The method according to claim 2, wherein the polypeptide to be isolated or purified is obtainable by expression in a host that is not CHO cells.
4. The method according to claim 2 or 3, wherein the polypeptide to be isolated or purified is obtainable by expression in a host that is a lower eukaryotic host.
5. The method according to claim 4, wherein the lower eukaryotic host comprises yeast such as *Pichia*, *Hansenula*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Torulaspora*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschnikowia*, *Rhodospiridium*, *Leucospiridium*, *Botryosphaeria*, *Sporidiobolus*, *Endomycopsis*.
6. The method according to claim 5, wherein the yeast is *Pichia* such as *Pichia pastoris*.
7. The method according to any one of claims 1 to 6, wherein the polypeptide comprises or consists of at least four immunoglobulin single variable domains (ISVDs).
8. The method according to any one of claims 1 to 7, wherein the conformational variant is characterized by a more compact form compared to the polypeptide.

9. The method according to any one of claims 1 to 8, wherein the conformational variant has a decreased hydrodynamic volume compared to the polypeptide.
10. The method according to any one of claims 1 to 9, wherein the conformational variant is characterized by an increased retention time in SE-HPLC compared to the polypeptide.
11. The method according to any one of claims 1 to 10, wherein the conformational variant is characterized by an altered retention time in IEX-HPLC compared to the polypeptide.
12. The method according to any one of claims 1 to 11, wherein the conditions that convert the conformational variant into the polypeptide are selected from:
 - i) applying a low pH treatment in a step of the isolation or purification process, optionally wherein the low pH treatment comprises decreasing the pH of the composition to about pH 3.2 or less, or to about pH 3.0 or less;
 - ii) applying a chaotropic agent in a step of the isolation or purification process, optionally wherein the chaotropic agent is guanidinium hydrochloride (GuHCl);
 - iii) applying a heat stress in a step of the isolation or purification process, optionally comprising incubating the conformational variant at about 40°C to about 60°C; or
 - iv) a combination of any of i) to iii).
13. The method according to any one of claims 1 to 6, 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 about pH 3.0 or less.
14. The method according to claim 12 or 13, wherein the pH is decreased to between about pH 3.2 and about 2.1, to between about pH 3.0 and about 2.1, to between about pH 2.9 and about pH 2.1, to between about pH 2.7 and about pH 2.1, or to between about pH 2.6 and about pH 2.3.

15. The method according to any one of claims 12 to 14, wherein the low pH treatment is applied for at least about 0.5 hours, for at least about 1 hour, for at least about 2 hours, or for at least about 4 hours.
16. The method according to any one of claims 12 to 15, wherein the pH is decreased to between about pH 3.2 and about pH 2.1 for at least about 0.5 hours, such as for at least about 1.0 hour.
17. The method according to any one of claims 12 to 16, wherein the pH is decreased to between about pH 3.0 and about pH 2.1 for at least about 0.5 hours, such as for at least about 1.0 hour.
18. The method according to any one of claims 12 to 17, wherein the pH is decreased to between about pH 2.9 and about pH 2.1 for at least about 0.5 hours, such as for at least about 1.0 hour.
19. The method according to any one of claims 12 to 18, wherein the pH is decreased to between about pH 2.7 and about pH 2.1 for at least about 0.5 hours, such as for at least about 1.0 hour.
20. The method according to any one of claims 12 to 19, 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.
21. The method according to claim 20, 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.
22. The method according to any one of claims 12 to 21, wherein the chaotropic agent is guanidinium hydrochloride (GuHCl) in a final concentration of at least about 1 M, or at least about 2 M.

23. The method according to claims 12 to 22, wherein the GuHCl is applied for at least 0.5 hours, or for at least 1 hour.
24. The method according to any one of claims 12 to 23, wherein the heat stress is applied for at least about 1 hour.
25. The method according to any one of claims 1 to 11, 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.
26. The method according to claim 25, wherein the chromatographic technique is a chromatographic technique based on hydrodynamic volume, surface charge or surface hydrophobicity.
27. The method according to claim 26, 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).
28. The method according to claim 27, wherein the HIC is based on a HIC column resin.
29. The method according to claim 27, wherein the HIC is based on a HIC membrane.
30. The method according to any one of claims 1 to 29, wherein isolation or purification of the polypeptide comprises 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, at least 45 mg protein/ml resin, optionally wherein the chromatographic column is a Protein A column.
31. The method according to any one of claims 1 to 30, 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.

32. 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), the method comprising 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, optionally wherein the low pH treatment comprises decreasing the pH of the composition to about pH 3.2 or less, or to about pH 3.0 or less;
 - ii) applying a chaotropic agent to a composition comprising the polypeptide in a step of the isolation or purification process, optionally wherein the chaotropic agent is GuHCl;
 - iii) applying a heat stress to a composition comprising the polypeptide in a step of the isolation or purification process, optionally comprising incubating the composition at about 40°C to about 60°C;
 - iv) applying the composition comprising the polypeptide to a chromatography column using a load factor of at least 20 mg/ml, at least 30 mg/ml, at least 45 mg/ml, optionally wherein the chromatographic column is Protein A column; or
 - v) a combination of any of i) to iv).
33. The method according to claim 32, wherein the polypeptide to be isolated or purified is obtainable by expression in a host.
34. The method according to claim 33, wherein the polypeptide to be isolated or purified is obtainable by expression in a host that is not a CHO cell.
35. The method according to claim 33 or 34, wherein the polypeptide to be isolated or purified is obtainable by expression in a host that is a lower eukaryotic host.

36. The method according to claim 35, wherein the lower eukaryotic host comprises yeast such as *Pichia*, *Hansenula*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Torulaspora*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschnikowia*, *Rhodospiridium*, *Leucosporidium*, *Botryosphaeria*, *Sporidiobolus*, *Endomycopsis*.
37. The method according to claim 36, wherein the yeast is *Pichia* such as *Pichia pastoris*.
38. The method according to any one of claims 32 to 37, 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 about pH 3.0 or less.
39. The method according to any one of claims 32 to 38, wherein the pH is decreased to between about pH 3.2 and about pH 2.1, to between about pH 3.0 and about pH 2.1, to between about pH 2.9 and about pH 2.1, to between about pH 2.7 and about pH 2.1, or to between about pH 2.6 and about pH 2.3.
40. The method according to any one of claims 32 to 39, wherein the low pH treatment is applied for at least about 0.5 hour, for at least about 1 hour, for at least about 2 hours, or for at least about 4 hours.
41. The method according to any one of claims 39 or 40, wherein the pH is decreased to between about pH 3.2 and about pH 2.1 for at least about 0.5 hour, such as for at least about 1.0 hour.
42. The method according to any one of claims 39 to 41, wherein the pH is decreased to between about pH 3.0 and about pH 2.1 for at least about 0.5 hour, such as for at least about 1.0 hour.
43. The method according to any one of claims 39 to 42, wherein the pH is decreased to between about pH 2.9 and about pH 2.1 for at least about 0.5 hour, such as for at least about 1.0 hour.

44. The method according to any one of claims 39 to 43, wherein the pH is decreased to between about pH 2.7 and about pH 2.1 for at least about 0.5 hour, such as for at least about 1.0 hour.
45. The method according to any one of claims 32 to 44, 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.
46. The method according to claim 45, 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.
47. The method according to any one of claims 32 to 46, wherein the chaotropic agent is GuHCl in a final concentration of at least about 1 M, or at least about 2 M.
48. The method according to any one of claims 32 to 47, wherein the GuHCl is applied for at least 0.5 hours, or for at least 1 hour.
49. The method according to any one of claims 32 to 48, wherein the heat stress is applied for at least about 1 hour.
50. 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 49.
51. The method according to claim 50, at least comprising the following steps:
 - a) Optionally cultivating a 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 49.
52. The method according to claim 50 or 51, wherein the host is not a CHO cell.
53. The method according to any one of claims 50 to 52, wherein the host is a lower eukaryotic host.
54. The method according to claim 53, wherein the lower eukaryotic host comprises yeasts such as *Pichia*, *Hansenula*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Torulaspora*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschnikowia*, *Rhodosporidium*, *Leucosporidium*, *Botryosascus*, *Sporidiobolus*, *Endomycopsis*.
55. The method according to claim 54, wherein the yeast is *Pichia* such as *Pichia pastoris*.
56. 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, the method comprising:
- (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.
57. 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, the method comprising:

- (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.

58. The method according to claim 56 or 57, wherein the polypeptide to be isolated or purified is obtainable by expression in a host.
59. The method according to claim 58, wherein the polypeptide to be isolated or purified is obtainable by expression in a host that is not a CHO cell.
60. The method according to claim 58 or 59, wherein the polypeptide to be isolated or purified is obtainable by expression in a host that is a lower eukaryotic host.
61. The method according to claim 60, wherein the lower eukaryotic host comprises yeast such as *Pichia*, *Hansenula*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Torulaspora*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschnikowia*, *Rhodospiridium*, *Leucosporidium*, *Botryosphaeria*, *Sporidiobolus*, *Endomycopsis*
62. The method according to claim 61, wherein the yeast is *Pichia* such as *Pichia pastoris*.
63. The method according to claims 56 to 62, wherein the conformational variant is characterized as in claims 8 to 11.
64. The method according to any one of claims 56 to 63, wherein the chromatographic technique is a chromatographic technique based on hydrodynamic volume, surface charge or surface hydrophobicity.
65. The method according to claim 64, 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).

66. The method according to claim 65, wherein the ion-exchange chromatography (IEX) is cation-exchange chromatography (CEX).
67. The method according to claim 65, wherein the HIC is based on a HIC column resin.
68. The method according to claim 67 wherein the HIC resin is selected from any of Capto Phenyl ImpRes, Capto Butyl ImpRes, Phenyl HP, and Capto Butyl.
69. The method according to claim 65, wherein the HIC is based on a HIC membrane.