

CLAIMS

1. A process for the recovery and/or purification of a recombinantly expressed intracellular protein comprising the sequence of Annexin A5 (AnxA5) from an endotoxin-producing host cell with a cell wall, wherein the process comprises releasing the intracellular protein from the host cell,

characterised in that the step of releasing the intracellular AnxA5 protein is conducted in the presence of a homogenisation buffer comprising non-ionic detergent, and

preferably wherein the process does not include any centrifugation steps for the recovery and/or purification of the AnxA5 protein after its release from the host cell and/or in which the AnxA5 protein remains in solution throughout the process except when temporarily bound to any chromatographic resins.

2. The process of Claim 1 wherein the non-ionic detergent is a polysorbate, preferably a polysorbate selected from Tween20 and Tween80, most preferably Tween80.

3. The process of Claim 1 or 2 wherein the step of releasing the intracellular AnxA5 protein is conducted in the presence of a homogenisation buffer comprising an amount of non-ionic detergent that is effective to reduce or prevent the binding between Annexin A5 and endotoxin.

4. The process of any preceding claim, wherein the step of releasing the intracellular AnxA5 protein is conducted in the presence of a homogenisation buffer comprising 0.01 to 10% (w/w) non-ionic detergent, such as 0.02 to 5% (w/w), 0.05 to 2% (w/w), or about 1% (w/w) non-ionic detergent.

5. The process of any preceding claim for the recovery and/or purification of a recombinantly expressed intracellular protein comprising the sequence of Annexin A5 (AnxA5) from a host cell having a cell wall, wherein the process comprises releasing the intracellular AnxA5 protein from the host cell,

wherein the free calcium ion concentration in the homogenisation buffer at the time of releasing the intracellular AnxA5 protein from the host cell, or after releasing the intracellular AnxA5 protein from the host cell but before any further chromatographic purification occurs, is lower than 10 mM, preferably lower than 5 mM, 1 mM, more preferably lower than 500 μ M, or substantially zero, and/or

- wherein the homogenisation buffer comprises, or is modified after releasing the intracellular AnxA5 protein to include, a calcium metal ion chelator.
6. The process of Claim 5, wherein the calcium metal ion chelator is selected from EDTA or salt thereof, EGTA or salt thereof, and most preferably EDTA.
 7. The process of Claim 5 or 6 wherein the level of free calcium ions, and/or an amount of calcium metal ion chelator, is effective to reduce or prevent the binding between Annexin A5 and components of the cell wall of the host cell.
 8. The process of any of Claim 5 to 7, wherein the homogenisation buffer comprises, or is adjusted (before or after the release of the AnxA5 protein) to comprise, 0.01 to 500 mM, such as 0.05 to 100 mM, 0.5 to 20 mM, 1 to 15 mM, 2 to 10 mM, or about 4 mM calcium metal ion chelator, and preferably wherein the calcium metal ion chelator is EDTA.
 9. The process of Claim 5, 6, 7 or 8 wherein the homogenisation buffer comprises a non-ionic detergent in accordance with the process of any of Claims 2, 3 or 4.
 10. The process of Claim 9, wherein the calcium metal ion chelator is EDTA.
 11. The process of Claim 9 or 10, wherein the non-ionic detergent is Tween80.
 12. The process of any preceding claim, wherein the process comprises recovery and/or purification of a recombinantly expressed intracellular AnxA5 protein from a culture of the host cells, and wherein the culture has a volume of at least 100L, 500L, 1,000L, 5,000L, or 10,000L.
 13. The process of any preceding claim, comprising the step of mixing biomass from the culture of host cells in the homogenisation buffer at a concentration of about 10g of biomass per mL of homogenisation buffer.
 14. The process of any preceding claim, wherein the step of releasing the intracellular AnxA5 protein from the host cell in the homogenisation buffer comprises lysing, breaking, homogenising, sonicating, or pressure treating the host cell, such that the cell wall and cell membrane barrier of the host cell is disrupted and thereby releases the intracellular AnxA5

protein, and optionally wherein this step does not include the use of osmotic shock and/or a freeze-thaw step.

15. The process according to Claim 14, wherein the step of releasing the intracellular AnxA5 protein from the host cell comprises high pressure homogenisation, such as one or more cycles of high pressure homogenisation between about 400 bar and about 2,500 bar, preferably three homogenisation cycles of about 600 bar, or two homogenisations cycles of about 800 bar.

16. The process according to any preceding claim, wherein the step of releasing the intracellular AnxA5 protein creates a biomass homogenate comprising the released AnxA5 protein.

17. The process according to Claim 16, wherein the biomass homogenate further comprises one or more (typically all) of the impurities selected from the group consisting of host cell proteins, host cell wall components, host cell membrane, host cell nucleic acid, and endotoxin.

18. The process according to Claims 16 or 17 further comprising the step of clarifying the biomass homogenate, and thereby producing a clarified product comprising the released AnxA5 protein.

19. The process according to Claim 18, wherein the step of clarifying the biomass homogenate comprises treatment of the homogenate with a nuclease, such as a nuclease A, preferably a nuclease A from *Serratia marcescens*, and optionally wherein the nuclease is included in the homogenisation buffer prior to releasing the intracellular AnxA5 protein.

20. The process according to Claim 18 or 19, wherein the step of clarifying the biomass homogenate comprises (preferably subsequent to the nuclease treatment of Claim 18 or 19), the step of passing the biomass homogenate comprising the released AnxA5 protein through a filter (such as a cellulose or polypropylene filter, preferably wherein the filter is a depth filter, and/or preferably wherein the filter has a cut off of less than 4 μm), and wherein the filter effluent is the clarified product comprising the released AnxA5 protein.

21. The process of any preceding claim, further comprising the step of subjecting the released AnxA5 protein to an anion exchange resin in order to perform a first anion

exchange step, and thereby produce a first anion exchange product which comprises the released AnxA5 protein.

22. The process of Claim 21, wherein the clarified product comprising the released AnxA5 protein as produced by the method of Claim 20 is subjected to the first anion exchange step, thereby to produce a first anion exchange product which comprises the released AnxA5 protein.

23. The process of Claim 21 or 22 wherein, prior to the first anion exchange step, one or more parameters of the environment of the released AnxA5 protein, selected from the group consisting of the pH, the conductivity, the level of calcium ion chelator and the level of non-ionic detergent, is or are adjusted.

24. The process of any of Claims 21 to 23 wherein the released AnxA5 protein that is subjected to the anion exchange step is formulated at a pH of about 6.9, a conductivity of about 2.8 mS/cm, a calcium ion chelator concentration of about 1mM and diluted using a non-ionic detergent, for example to obtain a final non-ionic detergent concentration of 0.01 to 1 % (w/v), more preferably about 0.1% (w/v).

25. The process of any of Claims 21 to 24 wherein the AnxA5 protein is bound during the anion exchange step, and the first anion exchange product which comprises the released AnxA5 protein is produced by applying a wash solution and/or an elution buffer to the anion exchange resin to release the bound AnxA5 protein, optionally wherein the elution buffer comprises NaCl, for example about 300 mM NaCl.

26. The process of any Claims 1 to 25, further comprising the step of subjecting the released AnxA5 protein to an affinity chromatography step, thereby to produce a first affinity chromatography product which comprises the released AnxA5 protein.

27. The process of Claim 26, wherein AnxA5 protein in the first anion exchange product, as produced by the method of any of Claims 21 to 25, is subjected to the affinity chromatography step.

28. The process of Claim 27 comprising steps wherein:

(a) a biomass homogenate comprising the released AnxA5 protein according to Claim 16 or 17 is clarified by the process of any of Claims 18, 19 or 20

and thereby produces a clarified product comprising the released AnxA5 protein, and

(b) the AnxA5 protein in the clarified product is subjected to an anion exchange resin in order to perform a first anion exchange step in accordance with any of Claims 21 to 25, and thereby produce a first anion exchange product which comprises the AnxA5 protein, and

(c) wherein the AnxA5 protein in the first anion exchange product is subjected to an affinity chromatography step in accordance with Claim 26 or 27.

29. The process of any of Claims 26 to 28 wherein the affinity chromatography step comprises the binding of the AnxA5 protein to immobilised heparin, and optionally wherein the binding is promoted by the presence of calcium ions.

30. The process of Claim 29 wherein the AnxA5 protein is eluted from the immobilised heparin using an elution buffer containing a calcium ion chelator, such as EDTA.

31. The process of any of Claims 26 to 30, wherein the first affinity chromatography product comprises the released AnxA5 protein and a calcium ion chelator, such as EDTA or EGTA, optionally in the range of 0.1 to 500 mM, more preferably about 10 mM.

32. The process of any one of Claims 1 to 31, wherein the process comprises:

subjecting a composition that comprises the AnxA5 protein and a calcium metal ion chelator to an anion exchange resin in order to perform an anion exchange step and thereby recover and/or purify the AnxA5 protein from the composition, and

wherein the anion exchange step is conducted in the presence of additional selected metal ions, and

wherein the additional selected metal ions are selected such that the calcium metal ion chelator has a binding affinity for the selected metal ions that is greater than its binding affinity for the anion exchange resin, but less than its binding affinity for calcium ions; and

preferably wherein the AnxA5 protein remains in solution throughout the process, including any preceding or subsequent steps, except when temporarily bound to any chromatographic resins.

33. The process of Claim 32, wherein the calcium metal ion chelator is selected from EDTA or salt thereof, EGTA or salt thereof, and most preferably EDTA.

34. The process of Claim 32 or 33, wherein the calcium metal ion chelator is present in the composition in an excess and/or at a concentration of about, or at least, 0.1 mM, 0,5 mM, 1 mM, 5 mM, 10 mM, 15 mM, 20 mM or more.

35. The process of any of any of Claims 32 to 34 wherein the selected metal ions are divalent cations, such as Mg^{2+} ions.

36. The process of any of Claims 32 to 35 wherein the selected metal ions are present during the anion exchange step in an amount effective to reduce or prevent an interaction between the calcium ion chelator and the anion exchange resin during the process of subjecting the composition to the anion exchange resin.

37. The process of any of Claims 32 to 36, wherein the selected metal ions are present during the anion exchange step in an amount effective to increase binding of the AnxA5 protein to the anion exchange resin in the presence of the calcium ion chelator, and thereby reduce the loss of AnxA5 protein in the flow through of the anion exchange step, compared to the level of loss observed when no selected metal ions are present during the anion exchange step.

38. The process of any of Claims 32 to 37 wherein the selected metal ions are present during the anion exchange step (for example, by addition to the composition that comprises the AnxA5 protein and a calcium metal ion chelator prior to subjecting the composition to the anion exchange resin) at a concentration of about 1 to about 100 mM, such as about 2 to about 50 mM, about 5 to about 25 mM, about 10 to about 15 mM or about 12.5 mM.

39. The process of any of Claims 32 to 38 wherein the calcium metal ion chelator is EDTA and the selected metal ions are Mg^{2+} ions, and preferably the molar ratio of Mg^{2+} ions to EDTA is in the range of 0.5:1 to 2:1, most preferably at least 1:1 or more.

40. The process of any of Claims 32 to 39, wherein

the composition that comprises the AnxA5 protein and a calcium metal ion chelator and which is subjected to the anion exchange resin is

the direct, or indirect, product of a preceding process that comprises the step of subjecting the AnxA5 protein to an affinity chromatography step and eluting the AnxA5 protein with a calcium ion chelator, thereby producing an affinity chromatography product which is a composition that comprises the AnxA5 protein and a calcium metal ion chelator.

41. The process of Claim 40 wherein the preceding affinity chromatography step comprises the binding of the AnxA5 protein to immobilised heparin, and optionally wherein the binding is promoted by the presence of calcium ions.

42. The process of Claim 41 wherein the AnxA5 protein is eluted from the immobilised heparin using an elution buffer containing a calcium ion chelator, such as EDTA or EGTA.

43. The process of any one of Claims 40 to 42, wherein there is no dialysis step between the preceding affinity chromatography step and the anion exchange step and/or there is no removal of calcium ion chelator from the product of preceding affinity chromatography step prior to the application of the direct or indirect product to the anion exchange step.

44. The process of any one of Claims 32 to 43, wherein the selected metal ions are added to the composition prior to, or during, the anion exchange step.

45. A process for the recovery and/or purification of a recombinantly expressed intracellular protein comprising the sequence of Annexin A5 (AnxA5) from an endotoxin-producing host cell with a cell wall, or a culture thereof as defined by Claim 12, wherein:

(a) the process comprises releasing the intracellular protein from the endotoxin-producing host cell in the presence of a homogenisation buffer comprising non-ionic detergent according to any one of Claims 1 to 4;

(b) optionally wherein the releasing step is in accordance with any of Claims 13 to 17;

(c) further optionally wherein the process comprises a step of clarifying the biomass homogenate according to any of Claims 18 to 20; and

(d) wherein the process further comprises the step of subjecting the released AnxA5 protein directly or indirectly to an anion exchange resin, optionally in the presence of a calcium ion chelator, in order to perform a first anion exchange step, and thereby produce a first anion exchange product which comprises the released AnxA5 protein in accordance with any of Claims 21 to 25; and

(e) wherein the process further comprises the step of subjecting the released AnxA5 protein directly or indirectly to an affinity chromatography step, thereby to produce a first affinity chromatography product which comprises the released AnxA5 protein, and

(f) wherein the first affinity chromatography product is a composition that comprises the AnxA5 protein and a calcium metal ion chelator; and

(g) wherein the direct, or indirect, product of the affinity chromatography step that comprises the AnxA5 protein and the calcium metal ion chelator is subjected to anion exchange step in accordance with any of Claims 32 to 44;

and preferably wherein none of steps (a) to (g) include, or are intervened by, one or more steps selected from centrifugation and/or dialysis, and more preferably wherein the AnxA5 protein remains soluble, except for temporarily binding to anion exchange and affinity chromatography solid phases, throughout the process.

46. The process of Claim 45 wherein the affinity chromatography step of step (e) comprises the binding of the AnxA5 protein to immobilised heparin, and wherein the binding is promoted by the presence of calcium ions.

47. The process of Claim 46 wherein the AnxA5 protein is eluted from the immobilised heparin using an elution buffer containing a calcium ion chelator, such as EDTA.

48. The process of Claim 45 wherein the affinity chromatography step of step (e) comprises

subjecting the solution comprising the AnxA5 protein and one or more impurities to a heparin affinity chromatography step in the presence of Tween80 (preferably in the presence of 0.1% Tween80), thereby to produce the first affinity chromatography product which comprises the released AnxA5 protein; and

preferably wherein the AnxA5 protein remains in solution throughout the process, including any preceding or subsequent steps, except when temporarily bound to any chromatographic resins.

49. The process of any preceding claim wherein the process comprises, preferably at the end of the process as defined by any preceding claim, one or more further steps selected from the group consisting of concentration, buffer change, conditioning and filtration (such as sterile filtration), and optionally a final step of storing the AnxA5 protein-containing product in a sterile container.

50. The process of Claim 49 wherein one of the further steps is diafiltration, optionally wherein the product of the diafiltration step contains the AnxA5 protein at a concentration of at least about 1 mg/mL, 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL, 50 mg/mL or greater.

51. The process of Claim 49 or 50 wherein the filtration uses a 0.45-0.2 μm filter or a 0.22 μm filter, and is preferably a sterile filtration step.
52. The process of any of Claims 49 to 51 wherein sterile filtration is the final purification step, prior to storing the AnxA5 protein-containing product in a sterile container.
53. The process of any preceding claim, wherein the process comprises steps required to provide a final sterile AnxA5 protein product in a non-phosphate buffer (such as Bis-Tris or Tris-buffer) at about pH 7.4, comprising about 150 mM NaCl, about 1 mM CaCl_2 , about 0.05% (w/w) polysorbate (such as Tween80) or other non-ionic detergent, and optionally wherein the concentration of the AnxA5 protein in the final sterile AnxA5 protein product is about 10 mg/mL.
54. The process of any preceding claim wherein the process provides a final sterile AnxA5 protein product, wherein the NaCl concentration present maintains AnxA5 protein in a form that is predominantly monomeric.
55. The process of any preceding claim wherein the process provides an overall yield of greater than 1 g of AnxA5 protein per L of host cell culture, more preferably at least about 1.5 g/L, even more preferably in the range of about 2 to about 4 g/L.
56. The process of any preceding claim wherein the process provides an overall recovery of AnxA5 protein of about 24 % by weight of the AnxA5 protein present in the from the host cell culture.
57. The process of any preceding claim wherein the process provides a product comprising host cell protein (other than the recombinantly expressed AnxA5 protein) at a level less than 100, 90, 80, 70, 60, 50, 40, 30, 20 ng or less per mg of AnxA5 protein.
58. The process of any preceding claim wherein the process provides a product comprising an endotoxin content of less than 100, 90, 80, 70, 60, 50 45, 40, 35, 30, 35, 20, 15, and preferably less than 10, 5 or 1 EU per mg AnxA5 protein, and/or preferably wherein the process provides a product in unit dosage form and the product contains less than 100, 90, 80, 70, 60, 50 45, 40, 35, 30, 35, 20, 15, and preferably less than 10, 5 or 1 EU per unit dose.
59. The process of any preceding claim wherein the process provides a product comprising host cell nucleic acid levels of less than 1,000 pg per mg of AnxA5 protein,

preferably less than 100 pg per mg of AnxA5 protein, more preferably less than 10 pg per mg of AnxA5 protein.

60. The process of any preceding claim, comprising the step of chemically modifying the AnxA5 protein, and optionally, further comprising one or more additional purification steps, such as to reduce or remove unreacted components and/or to select a homogenous population of chemically modified AnxA5 protein.

61. A composition comprising an AnxA5 protein, wherein the composition is the direct, or indirect product of (or is directly or indirectly obtainable by) the process of any of Claims 46-48, or any claim dependent thereon, and wherein:

- (a) the composition has been subjected to sterile filtration step, and is a sterile composition;
- (b) the composition is stored in a sterile container;
- (c) the composition comprises an endotoxin content of less than 100, 50, 20, 10, 5 or 1 EU per mg AnxA5 protein;
- (d) the composition comprises nucleic acid levels, such as host cell nucleic acid levels, of less than 1,000 pg, 100 pg, or 10 pg per mg of AnxA5 protein; and
- (e) the AnxA5 protein does not contain a His-tag.

62. The composition of Claim 61, which contains the AnxA5 protein at a concentration of at least about 1 mg/mL, 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL, 50 mg/mL, 100 mg/mL or greater.

63. The composition of any of Claims 61 or 62, wherein the composition comprises a sterile AnxA5 protein product in a non-phosphate buffer (such as Bis-Tris or Tris-buffer) at about pH 7.4, comprising about 150 mM NaCl, about 1 mM CaCl₂, about 0.05% (w/w) polysorbate (such as Tween80) or other non-ionic detergent, and optionally wherein the concentration of the AnxA5 protein in the final sterile AnxA5 protein product is about 10 mg/mL.

64. The composition of any of Claims 61 to 63, wherein the composition comprises NaCl at a concentration that maintains AnxA5 protein in a form that is predominantly monomeric.

65. The composition of any of Claims 61 to 64, which comprises non-AnxA5 protein, such as host cell protein, at a level less than 100, 90, 80, 70, 60, 50, 40, 30 or

20 ng per mg of AnxA5 protein, and optionally wherein the host cell protein is at a detectable level (albeit less than 100, 90, 80, 70, 60, 50, 40, 30 or 20 ng per mg of AnxA5 protein) in the composition.

66. The composition of any of Claims 61 to 65, wherein endotoxin is at a detectable level, albeit less than 100, 50, 20, 10, 5 or 1 EU per AnxA5 protein, in the composition.

67. The composition of any of Claims 61 to 66, which is a product in unit dosage form and which contains less than 100, 50, 20, 10, 5 or 1 EU per unit dose, and optionally wherein endotoxin is at a detectable level (albeit less than 100, 50, 20, 10, 5 or 1 EU per unit dose) in the composition.

68. The composition of any of Claims 61 to 67, which comprises nucleic acid levels, at a detectable level, albeit less than 1,000 pg, 100 pg, or 10 pg per mg of AnxA5 protein, in the composition.

69. The composition of any of Claims 61 to 68, wherein the level of gluconoylated AnxA5 protein in the composition is within the range of 0.5 to 30%, or 0.5 to 20%, or 0.5 to 15%, or 0.5 to 10% of the total content of AnxA5 protein in the product.

70. The composition of any of Claims 61 to 69, wherein the level of gluconoylated AnxA5 protein in the composition is product is below 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1%, and is preferably substantially 0%.

71. The composition of any of Claims 61 to 70, wherein the AnxA5 protein does not contain one or more RGD motifs.

72. The composition of any of the preceding composition claims, wherein the AnxA5 protein is chemically modified.

73. The composition of any of the preceding composition claims, wherein the AnxA5 protein is a fusion protein comprising an AnxA5 protein and a fusion partner.

74. The composition of any of Claims 61 to 73, wherein the composition is a pharmaceutically acceptable and/or veterinarily acceptable composition.

75. Use of composition of Claim 74 in the form of a pharmaceutical or veterinary composition in the manufacture of medicament wherein the medicament is to be administered by parenteral, intravenous, intra-arterial, intraperitoneal, intra-muscular,

intra-ocular, intra-cranial, intra-cerebral, intra-osseous, intra-cerebroventricular, intra-theal, subcutaneous or topical administration in a person in need thereof.

76. Use of composition of Claim 75 wherein the composition is suitable for topical administration, and wherein the topical administration is in the form of cream, an ointment, or ophthalmic drops.

77. The composition of Claim 61, wherein the AnxA5 protein in the composition is presented in the form of a pharmaceutical or veterinary composition comprising the AnxA5 protein in admixture with a pharmaceutically or veterinarily acceptable carrier, wherein the carrier is a coating.

78. The composition of Claim 77, wherein the composition is in the form of an immediate-, delayed-, or controlled-release application.

79. The composition of Claim 61, in the form of a pharmaceutical or veterinary composition that is a drug-eluting stent.

80. A composition according to any of Claims 61 to 79 for use in medicine.

81. Use of a composition according to claims 61 to 80 in the manufacture of medicament for

- treating, preventing and/or reducing the risk of developing a cardiovascular disease, an auto-immune disease or inflammatory condition,
- preventing or reducing the risk of thrombosis,
- treating, preventing or reducing the risk of retinal vein occlusion,
- treating, preventing or reducing the risk of haematological disorders, including but not limited to sickle cell anemia,
- treating, preventing or reducing the risk of pulmonary fibrosis,
- treating, preventing and/or reducing the risk of developing atherosclerosis, acute coronary syndrome, stroke, claudication, angina, ischaemic heart disease, peripheral artery disease, systolic hypertension, thromboembolism, hemorrhagic or vasculitic stroke, myocardial infarction, angina pectoris or intermittent claudication, unstable angina, other forms of severe angina, or transient ischemic attacks (TIA),
- treating, preventing or reducing risk of vascular dysfunction,
- reducing ischemic pain,
- preventing or treating restenosis (for example, neointima formation or thickening),

- treating, preventing or reducing risk of ischemia-reperfusion injury, organ ischemia-reperfusion injury (such as liver or renal ischemia-reperfusion injury), transplant rejection, graft-versus host disease (GVHD), including acute and/or chronic GVHD, or for treating a subject post-transplantation,
- preventing and/or reducing peri- or postoperative complications following surgical intervention, such as complications following vascular surgery, for example peripheral vascular surgery,
- preventing or treating vascular inflammation, or for treating, preventing or reducing the risk of acute and chronic vascular inflammation, or carditis,
- treating, preventing and/or reducing the risk of developing acute and/or chronic inflammatory conditions but not limited to inflammatory bowel disease,
- treating, preventing and/or reducing the risk of developing rheumatic diseases, arthritis including osteoarthritis, rheumatoid arthritis, psoriatic arthritis, or
- treating, preventing and/or reducing the risk of developing Alzheimer's disease, dementia in general, multiple sclerosis, or myasthenia gravis in a person in need thereof.