

can be replaced without any significant effect on structure and function.

Recent work shows that changes can also be made to protein A (SpA) to effect similar properties. US patent application publication US 2005/0143566, which is hereby incorporated by reference in its entirety, discloses that when at least one asparagine residue is mutated to an amino acid other than glutamine or aspartic acid, the mutation confers an increased chemical stability at pH-values of up to about 13-14 compared to the parental SpA, such as the B-domain of SpA, or Protein Z, a synthetic construct derived from the B-domain of SpA (US 5,143,844, incorporated by reference in its entirety). The authors show that when these mutated proteins are used as affinity ligands, the separation media as expected can better withstand cleaning procedures using alkaline agents. Further mutations of protein A domains with the purpose of increasing the alkali stability have also been published in WO 2008/039141, JP 2006304633A, EP 1992692A1, EP 2202310A2, WO 2010/110288, WO 2012/086660, WO 2012/083425, WO 2012/087230 and WO 2014/146350, all of which are hereby incorporated by reference in their entireties. However, the currently available mutants are still sensitive to alkaline pH and the NaOH concentration during cleaning is usually limited to 0.1 M, which means that complete cleaning is difficult to achieve. Higher NaOH concentrations, which would improve the cleaning, lead to unacceptable capacity losses.

There is thus still a need in this field to obtain a separation matrix containing protein ligands having a further improved stability towards alkaline cleaning procedures.

Summary

There is provide a polypeptide with improved alkaline stability. This is achieved with a polypeptide as defined herein.

In an aspect, there is provided an Fc-binding polypeptide comprising a mutant of an Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 95% or 98% identity to, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:22, SEQ ID NO 51 or SEQ ID NO 52, wherein at least the asparagine or serine residue at the position corresponding to position 11 in SEQ ID NO:4-7 has been mutated to an amino acid selected from the group consisting of glutamic acid and lysine.

One advantage is that the alkaline stability is improved over the parental polypeptides, with a maintained highly selective binding towards immunoglobulins and other Fc-containing proteins.

In another aspect, there is provided an Fc-binding polypeptide which comprises a sequence as defined by, or having at least 95% or 98% identity to SEQ ID NO 53.

KEX₁Q X₂AFYEILX₃LP NLTEEQRX₄X₅F IX₆X₇LKDX₈PSX₉ SX₁₀X₁₁X₁₂LAEAKX₁₃
X₁₄NDAQAPK (SEQ ID NO 53)

wherein individually of each other:

10 X₁=A or Q

X₂=E or K

X₃=H or K

X₄=A or N

X₅=A or G

15 X₆=Q or E

X₇=S or K

X₈=E or D

X₉=Q or V

X₁₀=K,R or A

20 X₁₁=A,E or N

X₁₂=I or L

X₁₃=K or R

X₁₄=L or Y

25 There is also provide a multimer with improved alkaline stability, comprising a plurality of polypeptides. This is achieved with a multimer as defined herein.

In another aspect, there is provided a multimer comprising or consisting essentially of a plurality of polypeptides as defined above.

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CLAIMS

1. An Fc-binding polypeptide comprising a mutant of an Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 95% or 98% identity to, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:22, SEQ ID NO 51 or SEQ ID NO 52, wherein at least the asparagine or serine residue at the position corresponding to position 11 in SEQ ID NO:4-7 has been mutated to an amino acid selected from the group consisting of glutamic acid, and lysine.
2. The polypeptide of claim 1, comprising a mutant of a parental Fc-binding domain of Staphylococcus Protein A (SpA), as defined by, or having at least 95% or 98% identity to, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO 51 or SEQ ID NO 52.
3. The polypeptide of claim 1 or 2, wherein the amino acid residue at the position corresponding to position 11 in SEQ ID NO:4-7 is a glutamic acid.
4. The polypeptide of any preceding claim, wherein the amino acid residue at the position corresponding to position 11 in SEQ ID NO:4-7 is a lysine.
5. The polypeptide of any preceding claim, wherein the amino acid residue at the position corresponding to position 9 in SEQ ID NO:4-7 is an alanine.
6. The polypeptide of any preceding claim, wherein the amino acid residue at the position corresponding to position 50 in SEQ ID NO:4-7 is an arginine or a glutamic acid, such as an arginine.
7. The polypeptide of any preceding claim, wherein the amino acid residue at the position corresponding to position 3 in SEQ ID NO:4-7 is an alanine and/or the amino acid residue at the position corresponding to position 6 in SEQ ID NO:4-7 is an aspartic acid.

17. The polypeptide according to any preceding claim, wherein the amino acid residue at the position corresponding to position 51 in SEQ ID NO:4-7 is a tyrosine or a leucine, such as a tyrosine.
- 5 18. The polypeptide according to any preceding claim, wherein the amino acid residues at the positions corresponding to positions 1, 2, 3 and 4 or to positions 3, 4, 5 and 6 in SEQ ID NO: 4-7 have been deleted.
- 10 19. The polypeptide according to any preceding claim, which is a mutant of Zvar as defined by SEQ ID NO:7, wherein the amino acid residue at position 9 is alanine and the amino acid residue at position 11 is lysine or glutamic acid, such as lysine.
- 15 20. The polypeptide according to claim 19, wherein the amino acid residue at position 43 is alanine or glutamic acid.
21. The polypeptide according to claim 19 or 20, wherein the amino acid residue at position 40 is valine.
- 20 22. The polypeptide according to any one of claims 19-21, wherein the amino acid residue at position 44 is isoleucine.
23. The polypeptide according to any preceding claim, wherein the mutation is selected from the group consisting of: N11K; N11E; N11E,Q32A; N11E,Q32E,Q40E; N11E,Q32E,K50R; Q9A,N11E,N43A; Q9A,N11E,N28A,N43A; Q9A,N11E,Q40V,A42K,N43E,L44I ;
- 25 Q9A,N11E,Q40V,A42K,N43A,L44I; N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; Q9A,N11E,N28A,Q40V,A42K,N43A,L44I;
- Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; N11K, H18K, D37E, A42R, N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I, K50R; Q9A,N11K,H18K,D37E,A42R;
- 30 Q9A,N11E,D37E,Q40V,A42K,N43A,L44I and Q9A,N11E,D37E,Q40V,A42R,N43A,L44I.
24. The polypeptide according to any one of claims 1-22, wherein the mutation is selected from the group consisting of: N11K;

Q9A,N11E,N43A; Q9A,N11E,N28A,N43A; Q9A,N11E,Q40V,A42K,N43E,L44I;
Q9A,N11E,Q40V,A42K,N43A,L44I; Q9A,N11E,N28A,Q40V,A42K,N43A,L44I;
N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y;

Q9A,N11K,H18K,S33K,D37E,A42R,N43A,L44I,K50R,L51Y; N11K, H18K, D37E, A42R,
5 N43A, L44I; Q9A, N11K, H18K, D37E, A42R, N43A, L44I and Q9A, N11K, H18K, D37E,
A42R, N43A, L44I, K50R.

25. The polypeptide according to any preceding claim, comprising or consisting essentially of a
sequence selected from the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10,
10 SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO
16, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO 26, SEQ ID NO 27, SEQ ID
NO 28, SEQ ID NO 29, SEQ ID NO 36, SEQ ID NO 37, SEQ ID NO 49 and SEQ ID NO 50.

26. The polypeptide according to any preceding claim, comprising or consisting essentially of a
15 sequence selected from the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10,
SEQ ID NO 11, SEQ ID NO 16, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO
26, SEQ ID NO 27, SEQ ID NO 28 and SEQ ID NO 29.

27. The polypeptide according to any preceding claim, comprising or consisting essentially of a
20 sequence selected from the group consisting of: SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10,
SEQ ID NO 11, SEQ ID NO 16, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 25, SEQ ID NO
27, and SEQ ID NO 28.

28. The polypeptide according to any preceding claim, which polypeptide has an improved
25 alkaline stability compared to a polypeptide as defined by SEQ ID NO 1, SEQ ID NO 2, SEQ
ID NO 3, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6 or SEQ ID NO 7, such as by SEQ ID NO
7.

29. The polypeptide according to any preceding claim, which polypeptide has an improved
30 alkaline stability compared to a parental polypeptide as defined by SEQ ID NO 1, SEQ ID NO

2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6 or SEQ ID NO 7, such as by SEQ ID NO 7.

30. The polypeptide according to claim 28 or 29, wherein the alkaline stability is improved as measured by the remaining IgG-binding capacity after 24 or 25 h incubation in 0.5 M or 1.0 M aqueous NaOH at 22 +/- 2 °C.

31. An Fc-binding polypeptide which comprises a sequence as defined by, or having at least 95% or 98% identity to SEQ ID NO 53.

10 KEX₁Q X₂AFYEILX₃LP NLTEEQRX₄X₅F IX₆X₇LKDX₈PSX₉ SX₁₀X₁₁X₁₂LAEAKX₁₃
X₁₄NDAQAPK (SEQ ID NO 53)

wherein individually of each other:

X₁=A or Q

15 X₂=E, or K

X₃=H or K

X₄=A or N

X₅=A or G

X₆=Q or E

20 X₇=S or K

X₈=E or D

X₉=Q or V

X₁₀=K,R or A

X₁₁=A,E or N

25 X₁₂=I or L

X₁₃=K or R

X₁₄=L or Y

32. A multimer comprising or consisting essentially of a plurality of polypeptides as defined by any preceding claim.

33. The multimer according to claim 32, wherein the polypeptides are linked by linkers comprising up to 15 amino acids.

34. The multimer according to claim 32 or 33, which is a dimer, trimer, tetramer, pentamer, hexamer, heptamer, octamer or nonamer.
- 5 35. The multimer according to any one of claims 32-34, which comprises or consists essentially of a sequence selected from the group of sequences defined by SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19, SEQ ID NO 20, SEQ ID NO 30, SEQ ID NO 31, SEQ ID NO 32, SEQ ID NO 33, SEQ ID NO 34 and SEQ ID NO 35.
- 10 36. The polypeptide or multimer according to any preceding claim, further comprising at, or within 1-5 amino acid residues from, the C-terminal or N-terminal one or more coupling element, selected from the group consisting of one or more cysteine residues, a plurality of lysine residues and a plurality of histidine residues.
- 15 37. A nucleic acid or a vector encoding a polypeptide or multimer according to any preceding claim.
38. An expression system, which comprises a nucleic acid or vector according to claim 37.
- 20 39. A separation matrix, wherein a plurality of polypeptides or multimers according to any one of claims 1 – 36 have been coupled to a solid support.
40. The separation matrix according to claim 39, wherein the polypeptides or multimers have been coupled to the solid support via thioether bonds.
- 25 41. The separation matrix according to claim 39 or 40, wherein the solid support is a polysaccharide.
- 30 42. The separation matrix according to any one of claims 39-41, wherein the IgG capacity of the matrix after 24 incubation in 0.5 M NaOH at 22 +/- 2 °C is at least 80, such as at least 85, at least 90 or at least 95% of the IgG capacity before the incubation.
43. The separation matrix according to any one of claims 39-42, wherein the IgG capacity of the matrix after 24 incubation in 1.0 M NaOH at 22 +/- 2 °C is at least 70, such as at least 80 or at least 90% of the IgG capacity before the incubation.

44. A method of isolating an immunoglobulin, wherein a separation matrix according to any one of claims 39-43 is used.

45. The method of claim 44, comprising the steps of:

- 5 a) contacting a liquid sample comprising an immunoglobulin with a separation matrix according to any one of claims 39-43,
b) washing said separation matrix with a washing liquid,
c) eluting the immunoglobulin from the separation matrix with an elution liquid, and
d) cleaning the separation matrix with a cleaning liquid.

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46. The method of claim 45, wherein the cleaning liquid is alkaline, such as with a pH of 13 – 14.

47. The method of claim 45 or 46, wherein the cleaning liquid comprises 0.1 – 1.0 M NaOH or
15 KOH, such as 0.5 – 1.0 M NaOH or KOH.

48. The method of any one of claims 45 – 47, wherein steps a) – d) are repeated at least 10 times, such as at least 50 times or 50 – 200 times.

20 49. The method of any one of claims 45 – 48, wherein steps a) – c) are repeated at least 10 times, such as at least 50 times or 50 – 200 times and wherein step d) is performed after a plurality of instances of step c), such as at least 10 or at least 50 times.