[00136] In addition to exhibiting reduced fragmentation during use in purification processes, the ligands described herein are also alkaline stable. Subsequent to the generation of the chromatography matrices incorporating the SpA-based ligands described herein, the alkaline stability of the matrices containing the ligands can be assayed using standard techniques in the art and those described herein.

[00137] For example, the alkaline stability of a ligand immobilized onto a matrix can be assayed using routine treatment with NaOH at a concentration of about 0.5M, *e.g.*, as described herein as well as in U.S. Patent Publication No. 20100221844, the entire content of which is incorporated by reference herein in its entirety.

[00138] In some embodiments, alkaline stable SpA molecules as well as matrices incorporating the same exhibit an "increased" or "improved" alkaline stability, meaning that the molecules and matrices incorporating the same are stable under alkaline conditions for an extended period of time relative to their wt counterparts. Previously, it has been reported that chromatography matrices incorporating SpA ligands based on the wt B, C or Z domain of SpA or having a mutation of one or more asparagine residues provides an improved alkaline stability under conditions where the pH is above about 10, such as up to about 13 or 14. However, some of these ligands appear to show fragmentation during use, especially following repeated cycles of CIP, as observed by the presence of fragments on an SDS-PAGE gel or by SEC.

[00139] In some embodiments, ligands according to the present invention as well as matrices incorporating the same are no more alkaline stable than their wt counterparts; nonetheless, they exhibit reduced fragmentation. One such ligand described herein is a pentameric form of the C domain ligand including an N-terminus deletion in each of the domains and including a G29K mutation in each of the domains. Such a ligand may further include an alanine as the very first amino acid in the pentamer to facilitate homogenous post-translational processing.

[00140] The present invention is based on the surprising and unexpected discovery of novel SpA ligands (in both free form as well as well when immobilized into a chromatography matrix) which exhibit reduced fragmentation during use in purification processes relative to some of the previously described ligands, in addition to retaining at least 95% of the initial binding capacity following extended exposure

to caustic conditions (e.g., 0.1M NaOH for 25 hours or more). In some embodiments, more than one site on the ligands is attached onto a solid support and these ligands are based on B, C, or Z domains of SpA, where the ligands have a deletion of 3, 4 or 5 consecutive amino acids from the N-terminus, starting at position 1 or at position 2.

[00141] In some embodiments, after 100 cycles, each cycle including a 15 min treatment with 0.5M NaOH, the percentage of retained binding capacity of the SpA ligands described herein (e.g., those comprising one or more B, C or Z domains, and any combinations thereof, where at least one of B, C or Z domain includes a deletion of at least 3 consecutive amino acids from the N-terminus), is at least 1.25 times more, 1.5 times more, 2.0 times more, 2.5 times more, or 3 times more than that of the wt counterpart.

[00142] In one embodiment, the alkaline stability of the immobilized ligand, as assayed by the retention of IgG binding capacity over time, is measured as follows. The binding capacity, referred to as Qd 50%, is measured by obtaining the volume of IgG loaded to a concentration based on absorbance at UV.sub.280nm of 50% of the initial IgG concentration. The initial Qd 50% of the chromatography matrix (e.g., resin packed in a column) is measured first. The chromatography matrix (e.g., resin as described above) is then exposed to about 10 cycles of 15 min exposure of 0.5M NaOH at 0.8 mL/min. Qd 50% is measured again. This process is repeated until the chromatography matrix is exposed to a total of about 100 cycles of 0.5M NaOH. Qd 50% is measured one last time and the results from the affinity chromatography matrix including ligands (e.g., chromatography resin immobilized with ligands) as described herein are compared with the respective type wt domains of SpA.

[00143] In another assay, caustic or alkaline stability of the matrix is measured by static soaking of the matrix. By soaking a measured amount of an affinity chromatography matrix (e.g., in resin format) in 0.1M NaOH, 0.3M NaOH or 0.5M NaOH for 25 hrs with gentle rotation and measuring IgG binding capacity before and after the NaOH soaking, the alkaline stability by way of retention of binding capacity of the matrix for IgG, can be determined.

VII. Methods of Purifying a Target Molecule Using a Chromatography Matrix of the Invention

[00144] In some embodiments, the present invention provides a method of purifying a target molecule from a mixture using the affinity chromatography

matrices described herein. The target molecule may be any molecule which is recognized by an affinity ligand provided herein, where the ligand is coupled to a solid support (*i.e.*, a chromatography matrix). Examples of target molecules include immunoglobulins and Fc-containing proteins. The immunoglobulins may be polyclonal antibodies or a monoclonal antibody or a functional fragment thereof. Functional fragments include any fragment of an immunoglobulin comprising a variable region that still binds specifically to its antigen while at the same time retaining its ability to specifically bind to a protein ligand coupled to a solid support.

[00145] In some embodiments, a method of isolating a target molecule of interest using an affinity chromatography matrix described herein includes the steps of: (a) contacting a solid support including an immobilized SpA-based chromatography ligand having an amino acid sequence selected from the group consisting of SEQ ID NOs: 13-42, SEQ ID NOs: 55-84 and SEQ ID NOs: 93-95, with a mixture comprising a target molecule under conditions such that the target molecule specifically binds to the ligand; and (b) altering the conditions such that the target molecule is no longer bound to the ligand, thereby isolating the target molecule.

[00146] In some embodiments, the altering step includes altering the pH, such the target molecule is no longer bound to the ligand. In a particular embodiment, the pH is altered in a manner such that it is more acidic than the pH conditions in step (a). For example, in one embodiment, step (a) may be performed at a neutral pH, or a pH ranging from about 6 to about 8 and step (b) may be performed at an acidic pH, *e.g.*, a pH ranging from about 1 to about 5.

[00147] In another embodiment, step (b) comprises altering the salt concentration of the buffer in use, such that the target molecule is no longer bound to the ligand. For example, in one embodiment, a high salt concentration, *e.g.*, > 0.1M, may be used in step (a) and a lower salt concentration, *e.g.*, <0.1M may be used in step (b). Conversely, in some embodiments, a low salt concentration, *e.g.*, < 0.1M may be used in step (a) and a high salt concentration may be used in step (b). In still other embodiments, both the pH and the salt concentration of the buffer may be altered between step (a) and step (b).

[00148] One skilled in the art can readily determine the conditions suitable for binding a target molecule to a ligand, and thereby alter the conditions to disrupt the binding of the molecule to the ligand.

presence of a smear as well as smaller fragments around 7 KDa on an SDS-PAGE following caustic soak in 0.5M NaOH for 25 hours (see Lane 9) relative to the dimeric C domain construct which includes a deletion of 4 consecutive amino acid deletion from the N-terminus of the second domain, the amino acid sequence of which is set forth in SEQ ID NO:35 (see Lane 12).

[00177] Each of the dimeric Z and C domain ligands additionally includes a His-6 tag. The ligands that are not exposed to caustic soak are shown in Lane 2 (dimeric Z domain control), Lane 5 (dimeric Z domain ligand having a N-terminus deletion), Lane 8 (dimeric C domain control) and Lane 9 (dimeric C domain ligand having a N-terminus deletion).

[00178] Reduced fragmentation of the dimeric Z and C domain ligands having an N-terminus deletion, following extended caustic soak, is further evidenced by SEC. The results of a representative SEC experiment are shown in Figure 4 in the form of an SEC chromatogram. As seen in Figure 4, the controls for the Z domain ligand (having the amino acid sequence set forth in SEQ ID NO:85) as well as the C domain ligand (having the amino acid sequence set forth in SEQ ID NO:92) show significant fragmentation, identified by arrows on the chromatogram in Figure 4. In contrast, the dimeric Z and C domain ligands having the N-terminus deletions in the second domain (Z domain ligand amino acid sequence is set forth in SEQ ID NO:78 and the C domain ligand amino acid sequence is set forth in SEQ ID NO:35), show reduced fragmentation, as identified by boxes on the chromatogram in Figure 4.

[00179] Additionally, the pentameric forms of Z domain ligands described above (*i.e.*, pentameric Z domain ligand having the amino acid sequence of SEQ ID NO:91 which represents the control, and pentameric Z domain ligand having the amino acid sequence of SEQ ID NO:84, which represents the pentameric ligand having a 4 consecutive amino acid N-terminus deletion in all but the first domain) are also analyzed for fragmentation by SDS-PAGE, following caustic soak in 0.5M NaOH for 25 hours.

[00180] As evidenced by the SDS-PAGE gel data seen in Figure 5, the pentameric form of the Z domain ligand having a 4 consecutive amino acid deletion from the N-terminus in all but the first domain (the amino acid sequence of which is set forth in SEQ ID NO:84), shows far less fragmentation following ligand soak in 0.5M NaOH for 25 hours (see Lane 3), relative to the pentameric Z domain ligand control, the amino acid sequence of which is set forth in SEQ ID NO:91 (see Lane 6).

to caustic conditions (e.g., 0.1M NaOH for 25 hours or more). In some embodiments, more than one site on the ligands is attached onto a solid support and these ligands are based on B, C, or Z domains of SpA, where the ligands have a deletion of 3, 4 or 5 consecutive amino acids from the N-terminus, starting at position 1 or at position 2.

[00141] In some embodiments, after 100 cycles, each cycle including a 15 min treatment with 0.5M NaOH, the percentage of retained binding capacity of the SpA ligands described herein (e.g., those comprising one or more B, C or Z domains, and any combinations thereof, where at least one of B, C or Z domain includes a deletion of at least 3 consecutive amino acids from the N-terminus), is at least 1.25 times more, 1.5 times more, 2.0 times more, 2.5 times more, or 3 times more than that of the wt counterpart.

[00142] In one embodiment, the alkaline stability of the immobilized ligand, as assayed by the retention of IgG binding capacity over time, is measured as follows. The binding capacity, referred to as Qd 50%, is measured by obtaining the volume of IgG loaded to a concentration based on absorbance at UV.sub.280nm of 50% of the initial IgG concentration. The initial Qd 50% of the chromatography matrix (e.g., resin packed in a column) is measured first. The chromatography matrix (e.g., resin as described above) is then exposed to about 10 cycles of 15 min exposure of 0.5M NaOH at 0.8 mL/min. Qd 50% is measured again. This process is repeated until the chromatography matrix is exposed to a total of about 100 cycles of 0.5M NaOH. Qd 50% is measured one last time and the results from the affinity chromatography matrix including ligands (e.g., chromatography resin immobilized with ligands) as described herein are compared with the respective type wt domains of SpA.

[00143] In another assay, caustic or alkaline stability of the matrix is measured by static soaking of the matrix. By soaking a measured amount of an affinity chromatography matrix (e.g., in resin format) in 0.1M NaOH, 0.3M NaOH or 0.5M NaOH for 25 hrs with gentle rotation and measuring IgG binding capacity before and after the NaOH soaking, the alkaline stability by way of retention of binding capacity of the matrix for IgG, can be determined.

VII. Methods of Purifying a Target Molecule Using a Chromatography Matrix of the Invention

[00144] In some embodiments, the present invention provides a method of purifying a target molecule from a mixture using the affinity chromatography

As discussed above, both forms of pentameric ligands have the A29K mutation. The ligands that are not soaked appear to be intact (Lanes 2, and 5).

[00181] Lanes 8-10 depict the fragmentation observed with a recombinant SpA ligand (rSPA), which is routinely used in purification processes. The rSPA ligand appears to show an even far greater degree of fragmentation following caustic soak in 0.5M NaOH for 25 hours relative to the Z domain control, as observed by a near disappearance of the protein on the SDS-PAGE (see Lane 9). The rSPA ligand not exposed to caustic conditions is in Lane 8.

[00182] This result appears to suggest that the Z domain based ligands having the N-terminus deletion, as described herein, are far superior candidates than the routinely used SpA ligands such as, *e.g.*, rSPA.

[00183] A reduction in fragmentation following caustic soak in 0.5M NaOH for 25 hours observed with the pentameric Z domain ligand having the N-terminal deletion is further confirmed using SEC, the results of one such representative experiment are shown in Figure 6.

[00184] As demonstrated by the chromatogram in Figure 6, the pentameric form of the Z domain control (SEQ ID NO:91) with no amino acid deletion shows well resolved peaks at lower molecular weight, indicating the presence of smaller fragments. In contrast, the pentameric form of the Z domain having the N-terminus deletion (SEQ ID NO:84) shows significantly fewer distinct peaks at lower intensity, indicating a far less degree of fragmentation, relative to the control.

[00185] The routinely used SPA ligand, rSPA, shows the most fragmentation or breakdown with no intact molecule left at all. Notably, the SEC chromatogram is consistent with the results of the SDS-PAGE analysis in Figure 5, further evidencing that the rSPA ligand has degraded so much that no significant fragments can be observed on an SEC chromatogram following extended exposure to caustic conditions (*i.e.*, soaking in 0.5M NaOH for 25 hours).

Example 7: Caustic Soak of Ligands Immobilized On Resin

[00186] The various ligands described in the foregoing examples are evaluated for fragmentation following caustic exposure subsequent to their attachment to a solid support (*e.g.*, an agarose chromatography resin).

[00187] For each resin of interest, 1 mL resin in 5 mL disposable chromatography column (EVERGREEN SCIENTIFIC, Los Angeles, CA) is

CLAIMS

What is claimed is:

1. An affinity chromatography ligand comprising one or more C domains of Staphylococcus Protein A (SpA), wherein at least one C domain comprises a deletion of at least 3 consecutive amino acids from the N-terminus, starting at position 1 or position 2, wherein said positions correspond to wild-type SpA C domain positions.

2. An affinity chromatography ligand comprising one or more B domains of Staphylococcus Protein A (SpA), wherein at least one B domain comprises a deletion of at least 3 consecutive amino acids from the N-terminus, starting at position 1 or position 2, wherein said positions correspond to wild-type SpA B domain positions..

3 An affinity chromatography ligand comprising one or more Z domains of Staphylococcus Protein A (SpA), wherein at least one Z domain comprises a deletion of at least 3 consecutive amino acids from the N-terminus, starting at position 1 or position 2, wherein said positions correspond to wild-type SpA Z domain positions.

4. The affinity chromatography ligand according to claim 1, 2 or 3, wherein the ligand exhibits reduced fragmentation, relative to a wt counterpart, following exposure to 0.5M NaOH for at least 5 hours.

5. An affinity chromatography matrix comprising an affinity chromatography ligand according to claim 1, 2 or 3 attached to a solid support.

6. The affinity chromatography matrix of claim 5, wherein the ligand exhibits reduced fragmentation when attached to the solid support, relative to a wt counterpart, following exposure to 0.5M NaOH for 5 hours.

7. The affinity chromatography ligand according to claim 1, 2, or 3, wherein one or more domains of the ligand further comprise an amino acid mutation at position 29 to reduce Fab binding, wherein said positions correspond to wild-type SpA C domain position.

8. The affinity chromatography ligand of claim 7, wherein the amino acid mutation comprises replacing a glycine amino acid residue or an alanine amino acid residue with a lysine amino acid residue.

9. The affinity chromatography matrix of claim 5, wherein affinity chromatography ligand is attached to the solid support via multipoint attachment.

10. The affinity chromatography ligand according to claim 1, wherein the affinity chromatography ligand comprises two, three, four, five, six, seven or more C domains of Staphylococcus Protein A (SpA), wherein at least one C domain comprises a deletion of three

consecutive amino acids from the N-terminus or a deletion of four consecutive amino acids from the N-terminus or a deletion of five consecutive amino acids from the N-terminus, starting at position 1 or at position 2, wherein said positions correspond to wild-type SpA C domain positions.

11. The affinity chromatography ligand according to claim 3, wherein the affinity chromatography ligand comprises two, three, four, five, six, seven or more Z domains of Staphylococcus Protein A (SpA), wherein at least one Z domain comprises a deletion of three consecutive amino acids from the N-terminus or a deletion of four consecutive amino acids from the N-terminus or a deletion of five consecutive amino acids from the N-terminus, starting at position 1 or at position 2, wherein said positions correspond to wild-type SpA Z domain positions.

12. The affinity chromatography ligand according to claim 2, wherein the affinity ligand comprises two, three, four, five, six, seven or more B domains of Staphylococcus Protein A (SpA), wherein at least one B domain comprises a deletion of three consecutive amino acids from the N-terminus or a deletion of four consecutive amino acids from N-terminus or a deletion of five consecutive amino acids from the N-terminus, starting at position 1 or at position 2, wherein said positions correspond to wild-type SpA B domain positions.

13. A method of affinity purifying one or more target molecules from a sample, the method comprising the steps of:

(a) providing a sample comprising one or more target molecules;

(b) contacting the sample with the matrix of claim 5 under conditions such that the one or more target molecules bind to the matrix; and (c)

recovering the one or more bound target molecules by elution.

14. The affinity chromatography matrix of claim 5, wherein the matrix retains at least 95% of its initial binding capacity after 5 hours incubation in 0.5M NaOH.

15. The affinity chromatography matrix of claim 5, wherein the matrix retains at least 95% of its initial binding capacity after 25 hours incubation in 0.1M NaOH.

16. An affinity ligand comprising the structure:

$$[(X)_n:(Y)_m]_{n+m}.$$

where X represents a B domain, a Z domain or a C domain of SpA, n represents the number of domains ranging from zero through (m-1),

Y represents a B domain or a Z domain or a C domain of SpA having at least 3 consecutive amino acids deleted from the N-terminus, starting at position 1 or 2, wherein said positions correspond to wild-type SpA B, C or Z domain positions and m represents the number of Y domains ranging from one through eight, where the ligand is attached to a solid support via multipoint attachment.

17. An affinity chromatography ligand comprising two or more B domains of SpA or two or more C domains of SpA or two or more Z domains of SpA, or any

combinations of B, Z or C domains in any order, wherein at least one of B, C or Z domains comprises a deletion of at least 3 amino acids from the N-terminus, starting at position 1 or at position 2.

18. The affinity chromatography ligand of claim 17, wherein the ligand exhibits reduced fragmentation relative to a ligand without any deletions, following exposure to 0.5M NaOH for 5 hours.

19. The affinity chromatography matrix according to claim 5 or claim 9, wherein the solid support is selected from the group consisting of controlled pore glass, silica, zirconium oxide, titanium oxide, agarose, polymethacrylate, polyacrylate, polyacrylamide, polyvinylether, polyvinyl alcohol and polystyrene and derivatives thereof.

20. An alkaline stable affinity chromatography ligand comprising at least five C domains, wherein each of the domains comprises a mutation to reduce Fab binding as well as deletion of 4 consecutive amino acids from the N-terminus, starting at position 1, wherein said positions correspond to wild-type SpA C domain position.

21. The affinity chromatography ligand according to claim 2, wherein one or more domains of the ligand further comprise an amino acid mutation at position 29 domain to reduce Fab binding, wherein said position corresponds to wild-type SpA B domain position.

22. The affinity chromatography ligand of claim 21, wherein the amino acid mutation comprises replacing a glycine amino acid residue with a lysine amino acid residue.

23. The affinity chromatography ligand according to claim 3, wherein one or more domains of the ligand further comprise an amino acid mutation at position 29 to reduce Fab binding, wherein said position corresponds to wild-type SpA Z domain position.

24. The affinity chromatography ligand of claim 23, wherein the amino acid mutation comprises replacing an alanine amino acid residue with a lysine amino acid residue.