NOVEL IMMUNOGLOBULIN-BINDING PROTEINS WITH IMPROVED SPECIFICITY

Related Applications

[0001] The present application claims the benefit of priority of U.S. Provisional Patent Application No. 61/188,549, filed on August 11, 2008, and U.S. Provisional Patent Application No. 61/212,812, filed on April 16, 2009, the entire contents of each of which are incorporated by reference herein in their entirety.

Field of the Invention

[0002] The present invention relates to modified immunoglobulin-binding proteins, *e.g.*, Staphylococcus protein A, having improved binding specificity for immunoglobulins and methods of making and using the same.

Background

[0003] Staphylococcal protein A (SpA), is a 42kDa multi-domain protein from the bacterium *Staphylococcus aureus*. SpA is bound to the bacterial cell wall *via* its carboxy-terminal cell wall binding region, referred to as the X domain. At the amino-terminal region, it includes five immunoglobulin-binding domains, referred to as E, D, A, B, and C (Sjodhal, *Eur J Biochem*. Sep;78(2):471-90 (1977); Uhlen *et al*., *J Biol Chem*. Feb 10;259(3):1695-702 (1984)). Each of these domains contains approximately 58 amino acid residues, and they share 65-90% amino acid sequence identity. The Z domain of SpA is an engineered analogue of the B domain of SpA and includes an alanine instead of a glycine residue at position 29.

[0004] SpA based reagents have found a widespread use in the field of biotechnology, *e.g.*, in affinity chromatography for capture and purification of antibodies as well as in antibody detection methods. At present, SpA-based affinity media probably are the most widely used affinity media for isolation of monoclonal antibodies and their fragments from different samples including cell culture. Accordingly, various matrices comprising protein A-ligands are commercially available including, for example, ProSep®-vA High Capacity, ProSep® vA Ultra and ProSep® UltraPlus (Millipore) and Protein A SepharoseTM, MabSelect™, MabSelect Xtra™ and MabSelect SuRe® (GE Healthcare).

[0005] WO 00/69459 disclose Protein A based binding domains with desirable activities.

Summary of the Invention

[0006] The present invention provides, at least in part, novel and improved SpA variants which exhibit either decreased or increased binding to a Fab portion of an immunoglobulin compared to the previously known SpA variants, while retaining the ability to bind the Fc portion of the immunoglobulin. The present invention provides novel and improved SpA variants which exhibit reduced Fab binding compared to the many commercially available protein-A based reagents which bind Fab, a property which is sometimes undesirable.

[0007] According to a first aspect of the present invention, there is provided an isolated immunoglobulin binding protein according to claim 1. Preferred features are given in claims 2 to 10.

[0008] There is described an immunoglobulin-binding domain based on one or more C domains of SpA which are modified to replace at least the amino acid at position 29. The amino acid at position 29 is a glycine, which is replaced with an amino acid other than alanine or tryptophan. In a particular embodiment, the glycine at position 29 is replaced with an amino acid other than alanine, threonine and tryptophan or the alanine at position 29 is replaced with an amino acid other than glycine, threonine and tryptophan.

[0009] There is also described, in an isolated immunoglobulin-binding protein is provided, which comprises at least an E domain of SpA, in which at least the glycine residue at position 29 is replaced with an amino acid residue other than alanine or tryptophan and where the immunoglobulin-binding protein exhibits binding to an Fc portion of an immunoglobulin but exhibits reduced binding to a Fab portion of the immunoglobulin relative to an immunoglobulin-binding protein which includes either alanine or glycine at position 29. In a particular embodiment, the glycine residue at position 29 of the E domain is replaced with an amino acid residue other than alanine, threonine and tryptophan.

[0010] There is also described an isolated immunoglobulin-binding protein is provided, which comprises at least a D domain of SpA, in which at least the glycine residue at position 29 is replaced with an amino acid residue other than alanine or tryptophan and where the immunoglobulin-binding protein exhibits binding to an Fc

portion of an immunoglobulin but exhibits reduced binding to a Fab portion of the immunoglobulin relative to an immunoglobulin-binding protein which includes alanine or glycine at position 29. In a particular embodiment, the glycine residue at position 29 of the D domain is replaced with an amino acid residue other than alanine, threonine and tryptophan.

[0011] There is also described an isolated immunoglobulin-binding protein is provided, which comprises at least an A domain of SpA, in which at least the glycine residue at position 29 replaced with an amino acid residue other than alanine or tryptophan and where the immunoglobulin-binding protein exhibits binding to an Fc portion of an immunoglobulin but exhibits reduced binding to a Fab portion of the immunoglobulin relative to an immunoglobulin-binding protein which includes an alanine or glycine at position 29. In a particular embodiment, the glycine residue at position 29 of the A domain is replaced with an amino acid residue other than alanine, threonine and tryptophan.

[0012] In another embodiment, an isolated immunoglobulin-binding protein is provided, which comprises at least a B domain of SpA, in which at least the glycine residue at position 29 replaced with an amino acid residue other than alanine or tryptophan and where the immunoglobulin-binding protein exhibits binding to an Fc portion of an immunoglobulin but exhibits reduced binding to a Fab portion of the immunoglobulin relative to an immunoglobulin-binding protein which includes an alanine or glycine at position 29. In a particular embodiment, the glycine residue at position 29 of the B domain is replaced with an amino acid residue other than alanine, threonine and tryptophan.

[0013] There is also described an isolated immunoglobulin-binding protein is provided, which comprises at least a C domain of SpA, in which at least the glycine residue at position 29 is replaced with an amino acid residue other than alanine or tryptophan and where the immunoglobulin-binding protein exhibits binding to an Fc portion of an immunoglobulin but exhibits reduced binding to a Fab portion of the immunoglobulin relative to an immunoglobulin-binding protein which includes an alanine or glycine at position 29. In a particular embodiment, the glycine residue at position 29 of the C domain is replaced with an amino acid residue other than alanine, threonine and tryptophan.

[0014] An immunoglobulin-binding protein is provided, which comprises at least a Z domain of SpA, in which at least the alanine residue at position 29 replaced with an amino acid residue other than glycine or tryptophan and where the immunoglobulin-binding protein exhibits binding to an Fc portion of an immunoglobulin but exhibits reduced binding to a Fab portion of the immunoglobulin relative to an immunoglobulin-binding protein which includes an alanine or glycine at position 29. In a particular embodiment, the alanine residue at position 29 of the Z domain is replaced with an amino acid residue other than glycine, threonine and tryptophan.

[0015] In some embodiments, an immunoglobulin-binding protein according to the present invention includes more than one modified SpA domain or known variants thereof (*e.g.*, more than one of E, D, A, B, C and/or Z and any combinations thereof) where each domain comprises the glycine (*i.e.* in case of E, D, A, B and C domains) or alanine (*i.e.*, in case of Z domain) at position 29 substituted with another amino acid, *e.g.*, an amino acid other than alanine, glycine or tryptophan. In a particular embodiment, the glycine or alanine at position 29 is replaced with an amino acid other than alanine, glycine, tryptophan and threonine.

[0016] In some embodiments, the glycine (G) at position 29 in one or more of isolated E, D, A, B, and C domains or alanine at position 29 in the isolated Z domain is replaced with an amino acid selected from the group consisting of leucine (L), lysine (K), asparagine (N), valine (V), isoleucine (I), serine (S), cysteine (C), methionine (M), phenylalanine (F), tyrosine (Y), glutamic acid (E), glutamine (Q), histidine (H), proline (P), arginine (R), aspartic acid (D) or a functional variant or derivative thereof.

[0017] In a yet further embodiment, an immunoglobulin-binding protein according to the invention further includes at least a portion of the carboxy-terminal region of SpA (designated the X domain).

[0018] An immunoglobulin-binding protein according to the present invention further comprises an asparagine, *e.g.*, at position 23, substituted with another amino acid.

[0019] In an alternative embodiment, an isolated immunoglobulin-binding protein comprising one or more isolated E, D, A, B, C or Z domains of *Staphyloccocus* protein A is provided, where the one or more isolated domains comprise: (i) at least the glycine residue at position 29 replaced with an amino acid residue other than alanine when the domain is E, D, A, B or C domain or (ii) at least the alanine at position 29 replaced with an amino acid other than glycine when the domain is the Z domain, where the immunoglobulin-binding protein binds an Fc portion of an immunoglobulin and exhibits

increased binding to a Fab portion of the immunoglobulin relative to an immunoglobulin-binding protein including alanine at position 29.

[0020] In some embodiments, an isolated immunoglobulin-binding protein according to the present invention includes a threonine or a tryptophan at position 29, where immunoglobulin-binding protein binds an Fc portion of an immunoglobulin and exhibits increased binding to a Fab portion of the immunoglobulin relative to an immunoglobulin-binding protein including alanine at position 29. In some embodiments, the Fc portion may be a part of an Fc fusion protein.

[0021] The immunoglobulins which are capable of being bound by the various immunoglobulin-binding proteins described herein include one or more of IgG, IgA and IgM.

[0022] The invention includes, a chromatography matrix useful for the isolation of immunoglobulins or antibodies is provided. In one embodiment, a chromatography matrix according to the present invention comprises an isolated immunoglobulin binding protein, as described herein, coupled to a solid support.

[0023] Also provided herein are nucleic acid molecules encoding the various immunoglobulin-binding proteins described herein, as well as host cells including such nucleic acid molecules. In some embodiments, a host cell is a prokaryotic cell. In other embodiments, a host cell is a eukaryotic cell.

[0024] Additionally, the present invention encompasses a library of polypeptides comprising one or more immunoglobulin-binding proteins described herein, and functional variants thereof. In yet another embodiment, the present invention provides a library of nucleic acid molecules encoding one or more immunoglobulin- binding proteins encompassed by the present invention or encoding functional variants thereof.

[0025] Also provided herein are methods of using the immunoglobulin-binding proteins described herein. In some embodiments, a method of affinity purifying one or more immunoglobulins from a sample is provided, which comprises the steps of: (a) providing a sample comprising one or more immunoglobulins; (b) contacting the sample with a chromatography matrix comprising an immunoglobulin binding protein, as described herein, under conditions such that the one or more immunoglobulins bind to the matrix; and recovering the one or more bound immunoglobulins by eluting at a suitable pH.

[0026] In various embodiments described herein, the suitable pH at which one or more bound immunoglobulins are eluted is an acidic pH.

Brief Description of the Drawings

[0027]

FIG. 1 depicts the nucleic acid sequences for the wild type (wt) IgG binding domains of SpA, represented by SEQ ID NOs:1-5. SEQ ID NO:1 represents the nucleic acid sequence for the wt E domain; SEQ ID NO: 2 represents the nucleic acid sequence for the wt D domain; SEQ ID NO: 3 represents the nucleic acid sequence for the wt A domain; SEQ ID NO: 4 represents the nucleic acid sequence for the wt B domain; and SEQ ID NO: 5 represents the nucleic acid sequence for the wt C domain.

FIG. 2 depicts the nucleic acid sequence for the Z domain of SpA, represented by SEQ ID NO: 6**.**

FIG. 3 depicts the amino acid sequence alignments of the wild type (wt) IgG binding domains of SpA (E, D, A, B and C) including a glycine at position 29. SEQ ID NO: 7 represents the amino acid sequence of the wt E domain; SEQ ID NO: 8 represents the amino acid sequence of the wt D domain; SEQ ID NO: 9 represents the amino acid sequence of the wt A domain; SEQ ID NO: 10 represents the amino acid sequence of the wt B domain; and SEQ ID NO: 11 represents the amino acid sequence of the wt C domain.

FIG. 4 depicts the amino acid sequence of the Z domain, represented by SEQ ID NO: 12.

FIG. 5 depicts a schematic of the plasmid pJ56:8620.

FIG. 6 depicts the nucleic acid sequences for three SpA B domain mutants, in which the glycine at position 29 has been replaced by leucine, lysine or arginine. SEQ ID NO: 13 represents the nucleic acid sequence for the SpA mutant G29L; SEQ ID NO: 14 represents the nucleic acid sequence for the SpA mutant G29K; and SEQ ID NO: 15 represents the nucleic acid sequence for the SpA mutant G29R.

FIG. 7 depicts the amino acid sequences for three SpA B domain mutants, in which the glycine at position 29 has been replaced by leucine, lysine or arginine. SEQ ID NO: 16 represents the amino acid sequence for the SpA B domain mutant G29L; SEQ ID NO: 17 represents the amino acid sequence for the SpA B domain mutant G29K; and SEQ ID NO: 18 represents the amino acid sequence for the SpA B domain mutant G29R.

FIG. 8 depicts an exemplary Western Blot experiment in which protein A expression from vector PET11a:8620 in E. Coli BL21(DE3) cells was detected using a Chicken IgY anti-protein A antibody.

FIG. 9 depicts the results of a representative experiment evaluating the binding of Fc and $F(ab)_2$ portions of an immunoglobulin to nickel resin alone (negative control) or to MabSelect SuRe® chromatography medium (positive control), followed by SDS-PAGE

analysis, which is shown. E represents eluate or the bound fraction following chromatography and S represents supernatant or the unbound fraction following chromatography. From left to right: lane 1 depicts a molecular weight marker; lanes 2-5 depict binding of either $F(ab)_2$ (lanes 2(E), 3(S)) or Fc (lanes 4(E), 5(S)) to the nickel resin; lanes 6-9 depict binding of either $F(ab)_2$ (lanes 6(S), 7(E)) or Fc (lanes 8(S), 9(E)) to the MabSelect SuRe®; and lanes 10 and 11 represent $F(ab)_2$ and Fc alone. $F(ab)_2$ runs on an SDS-PAGE as a doublet of about 18 and 20 kDa and Fc runs on an SDS PAGE at about 22 kDa. As shown in lanes 2(E) and 4(E) respectively, both $F(ab)_2$ and Fc exhibit weak to no binding to the nickel resin (negative control). Also, as shown in lane 7(E), $F(ab)_2$ shows binding to the MabSelect SuRe®, while Fc shows significant binding to the MabSelect SuRe® (positive control).

FIG. 10 depicts the results of a representative experiment evaluating the binding of $F(ab)_2$ and Fc portions of an immunoglobulin to His-tagged wild-type SpA (wtSpA) or His-tagged B-domain variant including an alanine instead of glycine at position 29 (G29A) using nickel affinity chromatography followed by SDS-PAGE analysis, which is shown. F represents protein A feed; FT represents protein A flow- through; S represents supernatant following the Fc or Fab binding step (unbound fraction); and E represents elution fraction (bound fraction). From left to right: lane 1 depicts a molecular weight marker; lane 2 depicts the F fraction from the experiment evaluating the binding of wtSpA to $F(ab)_2$ or Fc; lanes 3-5 depict the binding of wtSpA to $F(ab)_2$ in the FT (lane 3), S (lane 4) and E (lane 5) fractions; lanes 6-8 depict the binding of wtSpA to Fc in the FT (lane 6), S (lane 7) and E (lane 8) fractions; lane 9 depicts the F fraction from the experiment evaluating the binding of G29A to $F(ab)_2$ or Fc; lanes 10-12 depict the binding of G29A to $F(ab)_2$ in the FT (lane 10), S (lane 11) and E (lane 12) fractions; and lanes 13-15 depict the binding of G29A to Fc in the FT (lane 13), S (lane 14) and E (lane 15) fractions.

FIG. 11 depicts the results of a representative experiment evaluating the binding of $F(ab)_2$ and Fc portions of an immunoglobulin to a His-tagged B-domain variant including a leucine instead of glycine at position 29 (G29L) using nickel affinity chromatography followed by SDS-PAGE, which is shown. F represents protein A feed;

FT represents protein A flow-through; S represents supernatant following the Fc or $F(ab)_2$ binding step (unbound fraction); and E represents elution fraction (bound fraction). From left to right: lane 1 depicts a molecular weight marker; lane 2 depicts the F fraction; lanes

3-5 depict the binding of F(ab)2 to G29L in FT (lane 3), S (lane 4) or E (lane 5) fractions; and lanes 6-8 depict the binding of Fc to G29L in FT (lane 6), S (lane 7) and E (lane 8) fractions.

FIG. 12 depicts the results of a representative experiment evaluating the binding of $F(ab)_2$ and Fc portions of an immunoglobulin to a His-tagged B-domain variant including a lysine instead of glycine at position 29 (G29K) and to a His-tagged B- domain variant including an arginine instead of glycine at position 29 (G29R) using nickel affinity chromatography followed by SDS-PAGE, which is shown. F represents protein A feed; FT represents protein A flow-through; S represents supernatant following the Fc or $F(ab)_2$ binding step (unbound fraction); and E represents elution fraction (bound fraction). From left to right: lane 1 depicts a molecular weight marker; lane 2 depicts the F fraction from the experiment evaluating the binding of G29K to $F(ab)_2$ and Fc; lanes 3-5 depict the binding of $F(ab)_2$ to G29K in FT (lane 3), S (lane 4) or E (lane 5) fractions; lanes 6-8 depict the binding of Fc to G29K in FT (lane 6), S (lane 7) and E (lane 8) fractions; lane 9 represents the F fraction from the experiment evaluating the binding of G29R to $F(ab)_2$ and Fc; lanes 10-12 depict the binding of $F(ab)_2$ to G29R in FT (lane 10), S (lane 11) or E (lane 12) fractions; and lanes 13-15 depict the binding of Fc to G29R in FT (lane 13), S (lane 14) and E (lane 15) fractions.

FIGs. 13A-13E depict the results of a representative experiment further evaluating the binding of SpA variants of the invention to an Fc portion of an immunoglobulin using surface plasmon resonance analysis. The figures depict the binding of Fc at concentrations ranging from 45 nM - 185 pM to wild type protein A (FIG. 13A) and G29A (FIG. 13B) controls, and to constructs G29K (FIG. 13C), G29R (FIG. 13D), and G29L (FIG. 13E)

FIGs. 14A-14E depict the results of a representative experiment further evaluating the binding of SpA variants of the invention to an $F(ab)_2$ portion of an immunoglobulin using surface plasmon resonance analysis. The figures depict the binding of $F(ab)_2$ at concentrations ranging from 45 nM - 185 pM to the various protein A constructs. Strong binding is observed for wild type protein A (FIG. 14A), and weak binding is seen for the G29A control (FIG. 14B). No binding was detected for G29K (FIG. 14C), G29R (FIG. 14D), and G29L (FIG. 14E)

FIGs 15A-15B depict the results of a representative Fc and Fab pulse experiments for SpA chromatography resins. In figure 15A the unbound fraction of Fc appears at 7 column volume (CV), and bound Fc elutes at 23 CV. In figure 15B the unbound fraction of F(ab)2 appears at 7 CV, and bound Fc elutes between 23-25 CV.

Detailed Description of the Invention

[0028] The present invention provides, at least in part, variants of SpA which bind a Fab portion of an immunoglobulin with a lower affinity than the wild-type SpA and variants of SpA previously described, while retaining the ability to bind the Fc portion of an immunoglobulin. In some embodiments, variants of SpA described herein bind a Fab portion of an immunoglobulin with a higher affinity than an SpA having an alanine at position 29.

I. Definitions

[0029] In order that the present disclosure may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

[0030] As used herein, the term "immunoglobulin binding protein" refers to an amino acid sequence variant of "SpA" or "protein A of *Staphylococcus aureus*," comprising one or more modified domains of SpA (*e.g.*, one or more of modified E, D, A, B, C or Z) which exhibit reduced binding to a Fab portion of an immunoglobulin or Ig molecule relative to the wild-type SpA (wtSpA) or a known variant domain of SpA (*e.g.*, Z domain). In some embodiments, an immunoglobulin binding protein of the present invention comprises one or more modified domains E, D, A, B, C or Z, where each domain includes an amino acid substitution at position 29. In the case of domains E, D, B, A or C, the glycine at position 29 is replaced with an amino acid other than alanine or tryptophan and in the case of the Z domain, the alanine at position 29 is replaced with an amino acid other than glycine or tryptophan. In some embodiments, the glycine at position 29 is replaced with an amino acid other than alanine, threonine and tryptophan and the alanine at position 29 is replaced with an amino acid other than glycine, threonine and tryptophan. In some embodiments, amino acid sequence variants may differ from the parent amino acid sequence from which they are derived, in the substitution, deletion and/or insertion of one or more amino acids anywhere within the parent amino acid sequence and including an amino acid residue substitution at least at position 29. In some embodiments, amino acid sequence variants will possess at least about 70%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98% identity with the parent sequence (*i.e.* wt SpA domains or Z domain), where such variants bind the Fc portion of an immunoglobulin, however, exhibit reduced binding to a Fab portion of the immunoglobulin relative to an SpA amino acid sequence which includes a glycine or an alanine at position 29.

[0031] In an alternative embodiment, the glycine or alanine at position 29 is replaced with a threonine or a tryptophan, where the immunoglobulin-binding protein binds the Fc portion of an immunoglobulin and exhibits increased binding to a Fab portion of the immunoglobulin relative to an SpA amino acid sequence which includes an alanine at position 29.

[0032] The term "sequence identity" means that two nucleotide or amino acid sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 70% sequence identity, or at least 80% sequence identity, or at least 85% sequence identity, or at least 90% sequence identity, or at least 95% sequence identity or more. For sequence comparison, typically one sequence acts as a reference sequence (*e.g.*, parent sequence), to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

[0033] Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math*. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol*. 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection (see generally Ausubel *et al.*, *Current Protocols in Molecular Biology*). One example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al.*, *J. Mol. Biol*. 215:403 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (publicly accessible through the National Institutes of Health NCBI internet server). Typically, default program parameters can be used to perform the sequence comparison, although customized parameters can also be used. For amino acid sequences, the BLAST program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)).

[0034] In one embodiment, an immunoglobulin binding protein according to the present invention is based on an isolated domain of wtSpA (*i.e.*, E, D, A, B or C) which includes at least the glycine amino acid residue at position 29 replaced with an amino acid residue other than an alanine or tryptophan, where the immunoglobulin binding protein exhibits reduced binding to a Fab portion of the Ig molecule relative to an immunoglobulin binding protein which includes an alanine or glycine at position 29. In another embodiment, the immunoglobulin binding protein according to the invention is based on a Z domain of SpA which includes at least the alanine residue at position 29 replaced with an amino acid other than glycine or tryptophan. In another embodiment, the glycine residue at position 29 (*i.e.*, in case of E, D, A, B and C domains) is replaced with an amino acid residue other than alanine, threonine and tryptophan or the alanine residue at position 29 (*i.e.*, in case of the Z domain) is replaced with an amino acid residue other than glycine, threonine and tryptophan, where the immunoglobulin-binding protein exhibit reduced binding to a Fab portion of an Ig molecule relative to an immunoglobulin-binding protein which includes alanine or glycine at position 29.

[0035] In some embodiments, an immunoglobulin binding protein according to the present invention includes at least two or more, or at least three or more, or at least four or more, or at least five or more of domains designated E, D, A, B, C and Z, where each of the two or more, or three or more, or four or more, or five or more of the domains include at least a glycine (*e.g.*, in the case of domains E, D, A, B and C) or alanine (in the case of Z domain) at position 29 substituted with an amino acid residue other than a glycine, alanine, tryptophan and threonine and where the immunoglobulin binding protein exhibits reduced binding to a Fab portion of the Ig molecule relative to an immunoglobulin binding protein which includes a glycine or alanine at position 29.

[0036] In some embodiments, an immunoglobulin binding protein according to the invention includes multimers of the same domain (*e.g.*, two or more of E domain; two or more of D domain; two or more of A domain; two or more of B domain; two or more of C domain; and two or more of Z domain), where each of the monomers includes at least a glycine or alanine at position 29 substituted with an amino acid other than glycine, alanine, tryptophan and threonine, where the immunoglobulin binding protein exhibits reduced binding to a Fab portion of the Ig molecule relative to an immunoglobulin binding protein which includes an alanine or glycine at position 29.

[0037] Suitable amino acids to replace glycine or alanine at position 29 include any of the standard naturally occurring amino acids, excluding glycine, alanine and tryptophan

and in certain embodiments, excluding glycine, alanine, trytophan and threonine. In some embodiments, the naturally occurring amino acids include one of lysine, arginine and leucine. In other embodiments, the naturally occurring amino acids include one of lysine, arginine, leucine, cysteine, aspartic acid, glutamic acid, glutamine, phenylalanine, histidine, isoleucine, methionine, asparagine, proline, serine, valine and tyrosine. Non-naturally occurring amino acids and amino acid derivatives which are well known in the art could also be used to replace the glycine or alanine at position 29.

[0038] Exemplary SpA domain modifications are shown in Table I. Immunoglobulin binding proteins encompassed by the present invention may include any combinations of one or more, two or more, three or more, four or more, five or more, or six or more of domains (E, D, A, B, C and Z), where each domain includes a modification at position 29, and where the glycine or alanine at position 29 is replaced with an amino acid residue other than glycine, alanine, and tryptophan and in some embodiments, other than glycine, alanine, tryptophan and threonine. Also encompassed by the present invention are immunoglobulin binding proteins which include two or more domains of the same kind (*e.g*., two or more E domains;

[0039] two or more D domains; two or more A domains; two or more B domains; two or more C domains; and two or more Z domains), where each domain includes a modification at position 29, where the amino acid residue at position 29 is replaced with an amino acid residue other than glycine, alanine and tryptophan and in some embodiments, other than glycine, alanine, tryptophan and threonine.

Table I

SpA domains including modifications	**Designation**
E, D, A, B, C or Z or domain-glycine 29 or alaine 29 replaced with arginine	E-G29R D-G29R A-G29R B-G29R C-G29R Z-A29R
E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with asparagine	E-G29N D-G29N A-G29N B-G29N C-G29N Z-A29N

E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with aspartic acid	E-G29D D-G29D A-G29D B-G29D C-G29D Z-A29D
E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with cysteine	E-G29C D-G29C A-G29C B-G29C C-G29C Z-A29C
E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with glutamine	E-G29Q D-G29Q A-G29Q B-G29Q C-G29Q Z-A29Q
E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with glutamic acid	E-G29E D-G29E A-G29E B-G29E C-G29E Z-A29E
E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with histidine	E-G29H D-G29H A-G29H B-G29H C-G29H Z-A29H
E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with isoleucine	E-G29I D-G29I A-G29I B-G29I C-G29I Z-A29I
E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with leucine	E-G29L D-G29L A-G29L B-G29L C-G29L Z-A29L
E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with lysine	E-G29K D-G29K A-G29K B-G29K C-G29K Z-A29K

E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with methionine	E-G29M D-G29M A-G29M B-G29M C-G29M Z-A29M
E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with phenylalanine	E-G29F D-G29F A-G29F B-G29F C-G29F Z-A29F
E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with proline	E-G29P D-G29P A-G29P B-G29P C-G29P Z-A29P
E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with serine	E-G29S D-G29S A-G29S B-G29S C-G29S Z-A29S
E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with threonine	E-G29T D-G29T A-G29T B-G29T C-G29T Z-A29T
E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with tyrosine	E-G29Y D-G29Y A-G29Y B-G29Y C-G29Y Z-A29Y
E, D, A, B, C or Z domain-glycine 29 or alanine 29 replaced with valine	E-G29V D-G29V A-G29V B-G29V C-G29V Z-A29V

[0040] As used interchangeably herein, the terms “E domain,” “E domain of SpA,” and “E domain of *Staphylococcus* protein A,” refer to the polypeptide whose amino acid sequence is set forth in SEQ ID NO:7 or that encoded by, *e.g.*, the nucleotide sequence set forth in SEQ ID NO: 1. The “E domain” is a 51 amino acid polypeptide that folds

into a three-helix bundle structure. It is capable of binding Fc via residues on the surface of helices 1 and 2, or to Fab via residues on the surface of helices 2 and 3. In some embodiments, an E domain according to the invention is at least 70% identical, or at least 80% identical, or at least 90% identical or at least 95% or more identical in sequence to the amino acid sequence set forth in SEQ ID NO: 7.

[0041] As used interchangeably herein, the terms "D domain," "D domain of SpA," and "D domain of *Staphylococcus* protein A," refer to the polypeptide whose amino acid sequence is set forth in SEQ ID NO: 8 or that encoded by *e.g.*, the nucleotide sequence set forth in SEQ ID NO: 2. The "D domain" is a 61 amino acid polypeptide that folds into a three-helix bundle structure. It is capable of Fc binding via residues on the surface of helices 1 and 2, or to Fab via residues on the surface of helices 2 and 3. In some embodiments, a D domain according to the invention is at least 70% identical, or at

[0042] least 80% identical, or at least 90% identical or at least 95% or more identical in sequence to the amino acid sequence set forth in SEQ ID NO: 8.

[0043] As used interchangeably herein, the terms "A domain," "A domain of SpA," and "A domain of *Staphylococcus* protein A," refer to the polypeptide whose amino acid sequence is set forth in SEQ ID NO: 3 or that encoded by, *e.g.*, the nucleotide sequence set forth in SEQ ID NO: 9. The "A domain" is a 58 amino acid polypeptide that folds into a three-helix bundle structure. It is capable of Fc binding via residues on the surface of helices 1 and 2, or to Fab via residues on the surface of helices 2 and 3. In some embodiments, an A domain according to the invention is at least 70% identical, or at least 80% identical, or at least 90% identical or at least 95% or more identical in sequence to the amino acid sequence set forth in SEQ ID NO: 3.

[0044] As used interchangeably herein, the terms "B domain," "B domain of SpA," and "B domain of *Staphylococcus* protein A," refer to the polypeptide whose amino acid sequence is set forth in SEQ ID NO: 10 or that encoded by, *e.g.*, the nucleotide sequence set forth in SEQ ID NO: 4. The "B domain" is a 58 amino acid polypeptide that folds into a three-helix bundle structure. It is capable of Fc binding via residues on the surface of helices 1 and 2, or to Fab via residues on the surface of helices 2 and 3. In some embodiments, a B domain according to the invention is at least 70% identical, or at least 80% identical, or at least 90% identical or at least 95% or more identical in sequence to the amino acid sequence set forth in SEQ ID NO: 10.

[0045] As used interchangeably herein, the terms “C domain,” “C domain of SpA,” and “C domain of *Staphylococcus* protein A,” refer to the polypeptide whose amino acid sequence is set forth in SEQ ID NO: 11 or that encoded by, *e.g.*, the nucleotide sequence set forth in SEQ ID NO: 5. The “C domain” is a 58 amino acid polypeptide that folds into a three-helix bundle structure. It is capable of Fc binding via residues on the surface of helices 1 and 2, or to Fab via residues on the surface of helices 2 and 3. In some embodiments, a C domain according to the invention is at least 70% identical, or at least 80% identical, or at least 90% identical or at least 95% or more identical in sequence to the amino acid sequence set forth in SEQ ID NO: 11.

[0046] As used interchangeably herein, the terms “Z domain,” “Z domain of SpA” and “Z domain of protein A,” refer to the three helix, 59 amino acid polypeptide

[0047] that is a variant of the B domain of protein A. The amino acid sequence of the Z domain is set forth in SEQ ID NO: 12. An exemplary Z domain is described in Nilsson *et al.*, *Protein Engng.*, 1:107-113 (1997).

[0048] The term “immunoglobulin,” “Ig” or “antibody” (used interchangeably herein) refers to a protein having a basic four-polypeptide chain structure consisting of two heavy and two light chains, said chains being stabilized, for example, by interchain disulfide bonds, which has the ability to specifically bind antigen. The term “single-chain immunoglobulin” or “single-chain antibody” (used interchangeably herein) refers to a protein having a two-polypeptide chain structure consisting of a heavy and a light chain, said chains being stabilized, for example, by interchain peptide linkers, which has the ability to specifically bind antigen. The term “domain” refers to a globular region of a heavy or light chain polypeptide comprising peptide loops (*e.g.*, comprising 3 to 4 peptide loops) stabilized, for example, by β-pleated sheet and/or intrachain disulfide bond. Domains are further referred to herein as “constant” or “variable”, based on the relative lack of sequence variation within the domains of various class members in the case of a “constant” domain, or the significant variation within the domains of various class members in the case of a “variable” domain. Antibody or polypeptide “domains” are often referred to interchangeably in the art as antibody or polypeptide “regions”. The “constant” domains of antibody light chains are referred to interchangeably as “light chain constant regions”, “light chain constant domains”, “CL” regions or “CL” domains. The “constant” domains of antibody heavy chains are referred to interchangeably as “heavy chain constant regions”, “heavy chain constant domains”, “CH” regions or “CH” domains. The “variable” domains of

antibody light chains are referred to interchangeably as “light chain variable regions”, “light chain variable domains”, “VL” regions or “VL” domains. The “variable” domains of antibody heavy chains are referred to interchangeably as “heavy chain variable regions”, “heavy chain variable domains”, “VH” regions or “VH” domains.

[0049] Immunoglobulins or antibodies may be monoclonal or polyclonal and may exist in monomeric or polymeric form, for example, IgM antibodies which exist in pentameric form and/or IgA antibodies which exist in monomeric, dimeric or multimeric form. The term “fragment” refers to a part or portion of an antibody or antibody chain comprising fewer amino acid residues than an intact or complete antibody or antibody chain. Fragments can be obtained *via* chemical or enzymatic treatment of an intact or complete antibody or antibody chain. Fragments can also be obtained by recombinant means. When produced recombinantly, fragments may be expressed alone or as part of a larger protein called a fusion protein. Exemplary fragments include Fab, Fab’, F(ab’)2, Fc and/or Fv fragments. Exemplary fusion proteins include Fc fusion proteins.

[0050] The term “antigen-binding fragment” refers to a polypeptide portion of an immunoglobulin or antibody that binds an antigen or competes with intact antibody (*i.e.*, with the intact antibody from which they were derived) for antigen binding (*i.e.*, specific binding). Binding fragments can be produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins. Binding fragments include Fab, Fab', $F(ab')_2$, Fv, single chains, and single-chain antibodies.

[0051] The terms “polynucleotide” and “nucleic acid molecule,” used interchangeably herein, refer to polymeric forms of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple- stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand *de novo* using a DNA polymerase with an appropriate primer. A

nucleic acid molecule can take many different forms, *e.g.*, a gene or gene fragment, one or more exons, one or more introns, mRNA, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. As used herein, "DNA" or "nucleotide sequence" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides. In a particular embodiment, a nucleic acid molecule comprises a nucleotide sequence encoding a variant of SpA.

[0052] As used herein, the term "reduced binding" refers to any decrease in binding to a Fab (or $F(ab)_2$) portion of an immunoglobulin molecule by an immunoglobulin binding protein according to the present invention relative to an immunoglobulin binding protein which includes an alanine or glycine amino acid residue at position 29. For example, binding to a Fab portion may be decreased by about 10%, or about 20%, or about 30%, or about 40%, or about 50%, or about 60%, or about 70%, or about 80%, or about 90%, or about 95%, or about 96%, or about 97%, or about 98%, or about 99% or more, relative to an immunoglobulin binding protein which includes a glycine or an alanine amino acid residue at position 29. In some embodiments, binding to a Fab portion of an immunoglobulin molecule is reduced by at least 50% or more relative to binding by an immunoglobulin protein which includes a glycine or alanine at position 29. In another embodiment, binding to a Fab portion of an immunoglobulin molecule is reduced by at least 70% or more. In yet another embodiment, binding to a Fab portion of an immunoglobulin molecule is reduced by at least 90% or more, or at least 95% or more, or at least 99% or more. In one embodiment, binding to a Fab portion of an immunoglobulin molecule is undetectable using conventional techniques in the art and those described herein. Binding to an immunoglobulin molecule can be detected using well known techniques including those described herein and including but not limited to, for example, affinity chromatography and surface plasmon resonance analysis. In some embodiments, an immunoglobulin binding protein encompassed by the present invention binds the Fc portion of an immunoglobulin molecule with a dissociation constant of at least about 10^{-8}M for a

single IgG binding domain, or about 10^{-11} M for 5 tandem IgG binding domains. In other embodiments, an immunoglobulin binding protein according to the invention binds to the Fab portion of an immunoglobulin molecule with a dissociation constant of about 10-5 M for a single immunoglobulin binding domain or about 10-6 for intact protein A with five tandem binding domains.

[0053] As used herein, the term "increased binding" refers to any increase in binding to a Fab (or F(ab)2) portion of an immunoglobulin molecule by an immunoglobulin binding protein according to the present invention relative to an immunoglobulin binding protein which includes an alanine amino acid residue at position 29. For example, binding to a Fab portion may be increased by about 10%, or about 20%, or about 30%, or about 40%, or about 50%, or about 60%, or about 70%, or about 80%, or about 90%, or about 95%, or about 96%, or about 97%, or about 98%, or about 99% or more, relative to an immunoglobulin binding protein which includes an alanine amino acid residue at position 29. In some embodiments, binding to a Fab portion of an immunoglobulin molecule is increased by at least 50% or more relative to binding by an immunoglobulin protein which includes an alanine at position 29. In another embodiment, binding to a Fab portion of an immunoglobulin molecule is increased by at least 70% or more relative to the Fab binding by an immunoglobulin binding protein which includes an alanine at position 29. In yet another embodiment, binding to a Fab portion of an immunoglobulin molecule increased by at least 90% or more, or at least 95% or more, or at least 99% or more relative to the Fab binding by an immunoglobulin binding protein which includes an alanine at position 29. In exemplary embodiments, an immunoglobulin binding protein which exhibits increased Fab binding relative to an immunoglobulin binding protein including an alanine at position 29, includes a threonine or a tryptophan at position 29. Binding to an immunoglobulin molecule can be detected using well known techniques including those described herein and including but not limited to, for example, affinity chromatography and surface plasmon resonance analysis.

[0054] The term "Fc-binding," "binds to an Fc portion" or "binding to an Fc portion" refers to the ability of an immunoglobulin-binding protein described herein, to bind to the crystallizable part (Fc) of an antibody. In some embodiments, an immunoglobulin-binding protein according to the present invention binds an Fc portion of an antibody (e.g., human IgG1, IgG2 or IgG4) with an affinity of at least 10-7 M, or at least 10-8 M, or at least 10-9 M.

[0055] The term “affinity separation,” or “affinity purification,” as used herein, refers to any purification or assaying technique which involves the addition of a sample containing a target analyte (*e.g.*, an immunoglobulin) to a solid support which carries on it an immunoglobulin-binding protein, as described herein.

[0056] The term “chromatography,” as used herein, refers to any kind of technique which separates the analyte (*e.g.*, an immunoglobulin) to be measured from other molecules in the mixture and allows it to be isolated.

[0057] The term “affinity chromatography,” as used herein, refers to a mode of chromatography where the analyte to be separated is isolated by its interaction with a molecule (*e.g.*, an immunoglobulin binding protein) which specifically interacts with the analyte.

II. SpA Structure and Immunoglobulin Binding Sites

[0058] SpA is about a 42-kDa protein derived from the bacterium *Staphylococcus aureus* and contains five tandem highly homologous extracellular immunoglobulin (Ig)-binding domains at the N-terminus, designated E, D, A, B and C. Each extracellular domain of SpA possesses distinct Ig-binding sites. One site is for Fcγ (the constant region of IgG class of Ig) and the other is for the Fab portion of certain Ig molecules (the portion of the Ig that is responsible for antigen recognition). It has been reported that each of the domains contains a Fab binding site. The non-Ig binding portion of SpA is located at the C-terminus and is designated the X region or X-domain.

[0059] The cloning of the gene encoding SpA is described in U.S. Patent No. 5,151,350, the entire contents of which are incorporated by reference herein in their entirety.

III. Generation and Expression of SpA Variants

[0060] The SpA variants of the present invention can be made using any suitable methods known in the art. For example, standard techniques for site-directed mutagenesis of nucleic acids may be used such as those described, for example, in the laboratory manual entitled Molecular Cloning by Sambrook, Fritsch and Maniatis. Additionally, standard molecular biology techniques involving polymerase chain reaction (PCR) mutagenesis may be used.

[0061] In some embodiments, SpA variants are generated using standard genetic engineering techniques. For example, a nucleic acid molecule encoding a domain of SpA or portions thereof can be cloned into a suitable vector for expression in an appropriate host cell. Suitable expression vectors are well known in the art and typically include the necessary elements for the transcription and translation of the variant SpA coding sequence.

[0062] SpA variants described herein may also be synthesized chemically from amino acid precursors for fragments using methods well known in the art, including solid phase peptide synthetic methods such as the Boc (tert-butyloxycarbonyl) or Fmoc (9-fluorenylmethyloxy carbonyl) approaches (see, e.g., U.S. Pat. Nos. 6,060,596; 4,879,378; 5,198,531; 5,240,680).

[0063] Expression of SpA variants can be accomplished in cells from eukaryotic hosts such as yeasts, insects or mammals, or in prokaryotic host cells, *e.g*., bacteria such as *E. coli*.

[0064] In some embodiments, SpA variants may be expressed on the surface of a bacteriophage such that each phage contains a DNA sequence that codes for an individual SpA variant displayed on the phage surface. In this approach, a library of SpA variants are made by synthesizing random or semi random oligonucleotides at selected positions in the SpA sequence chosen to generate a variety of amino acids at these positions. The encoding DNA is inserted into an appropriate phage vector, packaged into a phage particle and used to infect a suitable bacterial host. Each of the sequences is thus cloned in one phage vector and the SpA variant of interest (*e.g*., having a mutation at position 29) can be selected by finding those phage that bind to Fc but do not bind to Fab (*e.g*., by a method known as panning). The phages recovered in this way can be amplified and the selection repeated. Also, the phages can be isolated and the nucleotide sequence encoding selected SpA variants determined by nucleotide sequencing.

IV. Binding of SpA variants to Fc and Fab

[0065] Following generation of the SpA variants, the binding specificity of the SpA variants to Fab or Fc portions of an immunoglobulin is determined using standard techniques in the art and those described herein.

[0066] For example, the SpA variant may be constructed with a histidine tag ($(His)_6$-tag) for immobilization by nickel affinity chromatography. The nickel-bound SpA can then be tested for Fc and Fab binding by adding appropriate fragments to the resin. Unbound material is washed away, and Fc and Fab are detected in the elution sample by gel electrophoresis.

[0067] Alternatively, Fc and Fab binding can be measured by surface plasmon resonance analysis. The SpA variant is immobilized on a chip using amine-reactive chemistry, and Fc or Fab fragment is flowed over the chip. If Fc or Fab binds, an increase in the surface plasmon resonance signal is observed.

[0068] SpA can also be immobilized on chromatography beads. Fc or Fab binding is observed by comparing the flow-through and elution peaks for the SpA resin.

V. Use of SpA and Variants thereof for Making Chromatography Matrices

[0069] The present invention also provides a method of preparing a chromatography matrix comprising at least one immunoglobulin-binding protein with affinity for the Fc part of an antibody and having reduced or no affinity for the Fab portion of the antibody. In one embodiment, such a method comprises: (a) providing a nucleic acid sequence encoding an isolated SpA domain (*e.g.*, E, D, A, B, C or Z); (b) mutating the nucleic acid sequence to encode for a variant protein where at least the glycine at position 29 has been replaced by an amino acid other than alanine or tryptophan; (c) expressing the variant protein in a host cell (*e.g.*, a suitable prokaryotic cell or a eukaryotic cell); (d) recovering the variant protein from the host cell; and (e) coupling the variant protein to a solid support.

[0070] Solid support matrices include, but are not limited to, controlled pore glass, agarose, methacrylate, and polystyrene. The protein can be coupled to the matrix, for example, by amine reactive or thiol reactive chemistry.

[0071] This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures, are incorporated herein by reference.

Examples

Example 1: Construction of Vectors Containing SpA Variants

[0072] A gene encoding the "B domain" of protein A is synthesized by standard method using a DNA synthesizer. The 5' end of the gene includes a codon for an initiating methionine as well as six histidine codons at the 3' end of the gene. The gene is provided in vector pJ56:8620. The parent vector pJ56 confers resistance to ampicillin, kanamycin, chloramphenicol and gentamicin. Appropriate restriction enzyme sites for the subsequent cloning of the gene into expression vectors are introduced into the gene at both 5' and 3' ends. A plasmid map of the vector is shown in Figure 5.

[0073] Glycine at position 29 is mutated to each of the 19 other amino acids by PCR-based methods using the Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA). Phosphorylated primers are purchased from IDT DNA (Coralville, IA), as 100 μM solution in Tris EDTA buffer. The mutagenic primer has the sequence: 5'(P)GAAGAACAACGCAACNNNTTCATTCAGA (SEQ ID NO:19) where NNN represents the three bases encoding the amino acid at position 29. The non- mutagenic primer has the sequence 5'(P)GTTCAGGTTCGGCAGATGCAGGAT (SEQ ID NO:20). PCR is performed in 50 μL reactions containing dNTPs (0.2 mM each), 0.5 mM of each primer, 10 pg template plasmid, and 1 U of Phusion enzyme. PCR is carried out according to the scheme outlined in Table II.

Table II: PCR conditions

Cycle description	Temperature	Time	# of cycles
Initial denaturation	98 °C	30 seconds	1 cycle
Denaturation	98 °C	30 seconds	25 cycles
Annealing	56 °C	30 seconds	
Extension	72 °C	2 minutes	
Final extension	98 °C	10 minutes	1 cycle
	4 °C	Hold	

[0074] PCR reactions are treated with the restriction enzyme DpnI (New England Biolabs, Ipswich, MA) to reduce wild type background. To each 50 μL PCR reaction, about 1 μL of *DpnI* enzyme is added and the samples are incubated for about one hour at 37 °C.

[0075] *E. coli* NEB5α competent cells (New England Biolabs, Ipswich, MA) are transformed with 2 μL of the *DpnI*-treated PCR reaction. Cells are thawed on ice, and 2 μL of the PCR reaction is added to 25 μL of cells. Following about a 30-minute incubation on ice, cells are heat shocked for 30 seconds at about 42 °C. Cells are allowed to recover for about 5 minutes on ice, and then 125 μL of SOC media (New England BioLabs) is added. Cells are incubated for about one hour at 37 °C, and then 100 μL are plated on LB plates (Northeast Laboratory Services, Winslow, ME) containing 100 μg/mL ampicillin and grown overnight at about 37 °C. In order to obtain purified DNA, individual colonies are picked for overnight culture in LB containing 100 μg/mL ampicillin. DNA is purified using spin mini-prep kits from Qiagen (Valencia, CA). Primer pJR (5' GAATATGGCTCATAACACCCCTTG) (SEQ ID NO: 21) is used for sequencing the mini-prepped DNA to confirm the identity of each clone (MWG Biotech, Huntsville AL).

[0076] Following sequence confirmation of the various clones, the gene for each SpA variant is subcloned into a plasmid for expression of the variant in bacterial cells. Plasmid is digested with the appropriate restriction enzymes (New England Biolabs, Ipswich, MA), and the insert is gel purified on 1% agarose Reliant FastLane gels (Lonza Inc., Allendale, NJ). The relevant band is cut out of the gel and purified using the gel extraction kit from Qiagen (Valencia, CA). The purified insert is ligated into the backbone of a suitable expression vector using T4 DNA ligase (New England Biolabs, Ipswich, MA). The resulting plasmid is used to transform E. coli NEB5α competent cells as described above.

Example 2: Expression and Purification of Protein A Variants

[0077] Any suitable bacterial expression system can be used for expressing the various SpA variants. For example, the protein may be expressed in *Eschercia coli* such as strain BL21(DE3) (Promega, Madison WI) from a pET vector such as pET11a (Novagen, Madison WI). A single colony is picked from a plate and grown overnight at about 37 °C in LB media containing 100 μg/mL ampicillin. The overnight culture is diluted 100-fold into fresh LB media containing 100 μg/mL ampicillin and grown to a cell density such that the optical density at 600 nm is ~0.8. Following the addition of 1 mM isopropyl-beta-D-thiogalactopyranoside, cells are grown for an additional two hours. Expression is confirmed by SDS-PAGE analysis and Western blotting. An exemplary protein A western blot is shown in Figure 8, which shows that protein A expression from vector PET11a:8620 in E. coli BL21 (DE3) cells is detected using a chicken IgY anti- protein A antibody.

[0078] Cells are harvested by centrifugation (4000 rpm, 4 °C, 5 minutes) and resuspended in 3 mL of phosphate buffered saline containing 20 mM imidazole. Cells are lysed by sonication, and cell debris is pelleted by centrifugation (4000 rpm, 4 °C, 30 minutes). SpA variants are purified using NiNTA spin columns (Qiagen), applying 400 μL cell lysate per spin column. Columns are washed with 2x600 μL phosphate buffered saline containing 20 mM imidazole, and SpA is eluted in 2x200 μL phosphate buffered saline containing 200 mM imidazole. SpA is dialyzed overnight into PBS. Protein concentration is confirmed using the Dc protein assay (Bio-Rad Laboratories, Hercules, CA) relative to a standard curve prepared with bovine serum albumin.

Example 3: Binding of SpA Variants to Fc and Fab Fragments

[0079] Each SpA variant is subsequently tested for the ability to bind Fc and Fab using NiNTA agarose resin (Qiagen). Fc and $F(ab)_2$ fragments are obtained from Jackson ImmunoResearch (West Grove, PA). Each SpA variant is diluted to a concentration of 0.11 mg/mL, Fc is diluted to a concentration of 0.44 mg/mL and $F(ab)_2$ is diluted to a concentration of 0.92 mg/mL, in phosphate buffered saline containing 20 mM imidazole. The NiNTA agarose resin is resuspended and about 20 μl of the resin slurry is pipetted into a 0.5 ml microfuge tube. The resin is subsequently pelleted and the supernatant is discarded. The resin is washed with about 100 μl PBS with 20 mM imidazole, incubated at room temperature for about 15 mins and pelleted again. The

supernatent is discarded. About 250 µl of each SpA variant at 0.11mg/ml or PBS control is loaded onto the resin column in duplicates and incubated for about 30 mins at room temperature. The resin is pelleted and the supernatant is saved for analysis. The pellet is washed three times with 100 µl of PBS plus 20 mM imidazole each time, incubated for 5 mins and pelleted again. The supernatant is discarded. About 100 µl of Fc or $F(ab)_2$ is tested for binding with each of the SpA variants or the PBS control by incubating with the resin for about 30 mins. The resin is subsequently pelleted and the supernatant is saved for analysis. The resin is washed again three times with about 100 µl of PBS plus 20 mM imidazole each time, incubated for 5 mins and pelleted. The supernatant is discarded this time. The bound fraction is eluted with about 100 µl of PBS plus 20 mM imidazole, incubated for about 15 mins and pelleted. The supernatant is saved for further analysis. The samples are subsequently analyzed by SDS-PAGE on 4-15% gradient gels purchased from Bio-Rad Laboratories, CA, as depicted in Figures 9-12.

[0080] The binding of five protein A variants to Fc and F(ab)2 is tested by surface plasmon resonance biosensor analysis. All experiments are carried out in phosphate buffered saline (PBS) containing 0.005% Tween-20. Protein A variants are immobilized on ProteOn GLC sensor chip (Bio-Rad Laboratories, Hercules, CA) via amine chemistry, with coupling to a level of 500 RU. Three fold-dilution series of Fc and $F(ab)_2$ fragments (Jackson ImmunoResearch Laboratories, West Grove, PA) are prepared, with protein concentrations ranging from 45 nM to 185 pM, and each concentration is analyzed in duplicate. Each concentration of Fc or $F(ab)_2$ is injected for 250 seconds, followed by 3.5 hours of PBS to measure dissociation rates. Data are fit to a heterogeneous two-site model in order to obtain on- and off-rates (k_{on} and k_{off}). As depicted in Figures 13A-13E, the wild type glycine 29 protein, and each of G29A, G29R, G29K and G29L bind Fc. Figures 14A-14E show that the wild type G29 is observed to bind Fab, and G29A shows weak but detectable F(ab)2 binding. No F(ab)2 binding is seen for G29R, G29K or G29L.

[0081] Fc and Fab binding are also evaluated following immobilization of the SpA variants on chromatography resins. Controlled pore glass beads (50 mL each, mean particle size of 60 μm, mean pore diameter of 800Å) are functionalized with Protein A or Protein A variant according to the method for coupling Protein A to a solid support described in International PCT Application No. WO 90/09237, the entire contents of which are incorporated by reference herewin. After ligand coupling, the samples are washed three times with 150 mL volume of each (in order): 0.1M Tris with 0.15M NaCl, pH 8 followed by 0.05M Acetic Acid. Afterwards, the samples are equilibrated and stored in PBS with azide.

[0082] Controlled pore glass affinity resins immobilized with the corresponding ligand are packed in a 6.6 mm id x 70 mm L OmniFit columns. Buffers used in the experiment are: (A) 50 mM sodium phosphate buffer, pH 7.2; and (B) 50 mM sodium phosphate, pH 2, and the flow rate is 1.2 mL/min. After the column is equilibrated in the starting buffer A, 100 μL of F(ab)2 or Fc at 1~2 mg/mL (Jackson ImmunoResearch, West Grove, PA) in buffer A is injected into the column. After 10 column volume (CV) of buffer A, buffer B is mixed in over 20 CV in a linear gradient. Ten more column volumes of Buffer B run through the column before the column was regenerated by 2 CV of 6 M guanidine hydrochloride. As depicted in Figures 15A and 15B, the wild type glycine 29 protein as well as each of G29A, G29R, G29K and G29L bind Fc. The wild type G29 is observed to bind Fab, and G29A shows weak but detectable F(ab)2 binding. No F(ab)2 binding is seen for G29R, G29K or G29L.

[0083] In another exemplary experiment, a full set of protein A variants are tested for Fc and Fab binding by capturing them on NiNTA agarose resin (Qiagen). Briefly, His-tagged protein A variants having an amino acid substitution at position 29 of the wtSpA are added in excess to the resin, the resin is washed with PBS containing 20 mM imidazole followed by the addition of Fc or Fab portion of an immunoglobulin. The resin is washed again with PBS containing 20 mM imidazole followed by elution of SpA and IgG fragments with PBS containing 200 mM imidazole. The resulting elution fractions are analysed on a 4-20% gradient gel (BioRad) followed by Commassie blue staining (Pierce). Resin with no protein A is used as a negative control. The results of an exemplary experiment are summarized below in Table III.

While no apparent difference in Fc binding is observed among the different variants, the Fab binding is markedly reduced for most of the variants. Most of the variants exhibit Fab binding that is less than 15% compared to wild type. Residual Fab binding of 24% or greater is observed for three of the variants (G29A, G29T, and G29W).

Table III

Variant	**Fab binding quantitated by densitometry (percentage of wild type glycine)**
WT	100
G29K	4
G29R	9
G29L	7
G29A	24
G29C	5
G29D	8
G29E	11
G29F	7
G29H	5
G29I	8
G29M	9
G29N	8
G29P	14
G29Q	11
G29S	8
G29T	37
G29V	10
G29W	66
G29Y	11
control	11

[0084] The specification is most thoroughly understood in light of the teachings of the references cited within the specification which are hereby incorporated by reference. The embodiments within the specification provide an illustration of embodiments in this invention and should not be construed to limit its scope. The skilled artisan readily recognizes that many other embodiments are encompassed by this invention. All publications and inventions are incorporated by reference in their entirety. To the extent that the material incorporated by reference contradicts or is inconsistent with the present specification, the present specification will supercede any such material.

The citation of any references herein is not an admission that such references are prior art to the present invention.

[0085] Unless otherwise indicated, all numbers expressing quantities of ingredients, cell culture, treatment conditions, and so forth used in the specification, including claims, are to be understood as being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters are approximations and may vary depending upon the desired properties sought to be obtained by the present invention. Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

[0086] Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only and are not meant to be limiting in any way. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

References:

[0087] Fab binding: Melissa A. Starovasnik, Mark P. O'connell, Wayne J. Fairbrother.And Robert F. Kelley (1999). Antibody variable region binding by Staphylococcal protein A: Thermodynamic analysis and location of the Fv binding site on E-domain. Protein Science 8, 1423-1431. Fe binding constant: Susanne Gulich, Mathias Uhlen, Sophia Hober (2000) Protein engineering of an lgG-binding domain allows milder elution conditions during affinity chromatography.

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5. The immunoglobulin-binding protein of para 4, wherein the asparagine is at position 23.

6. The immunoglobulin-binding protein of any one of paras 1 to 5, wherein the immunoglobulin is an lgG or a fragment thereof.

7. The immunoglobulin-binding protein of any one of paras 1 to 6, wherein the Fe portion of the immunoglobulin is part of an Fe fusion protein.

8. An isolated immunoglobulin-binding protein comprising one or more isolated E, D, A, 8, C or Z domains of *Staphyloccocus* protein A, wherein the one or more isolated domains comprise:

(i) at least the glycine residue at position 29 replaced with an amino acid residue other than alanine when the domain is E, D, A, B or C domain or

(ii) at least the alanine at position 29 replaced with an amino acid other than glycine when the domain is the Z domain, wherein the immunoglobulin-binding protein binds an Fe portion of an immunoglobulin and exhibits increased binding to a Fab portion of the immunoglobulin relative to an immunoglobulin-binding protein including alanine at position 29.

9. The isolated immunoglobulin-binding protein of para 8, wherein at least the glycine at position 29 or the alanine at position 29 is replaced with a threonine or tryptophan.

10. The immunoglobulin-binding protein of para 8 or para 9, wherein the immunoglobulin is selected from the group consisting of lgG, lgA or lgM, or a fragment thereof.

11. A library of polypeptides comprising the immunoglobulin-binding protein of any one of paras 1 to 10.

12. A nucleic acid molecule encoding the immunoglobulin-binding protein of any one of paras 1 to 10.

13. A host cell comprising the nucleic acid molecule of para 12.

14. A chromatography matrix comprising the isolated immunoglobulin-binding protein of any one of paras 1 to 10 coupled to a solid support.

15. A method of affinity purifying one or more immunoglobulins from a sample, the method comprising the steps of:

(a) providing a sample comprising one or more immunoglobulins;

(b) contacting the sample with the matrix of para 14 under conditions such that the one or more immunoglobulins bind to the matrix; and

(c) recovering the one or more bound immunoglobulins by eluting under suitable conditions.

SEQUENCE LISTING

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CLAIMS:

1. An isolated immunoglobulin-binding protein comprising one or more isolated C domains of *Staphyloccocus* protein A, wherein the one or more isolated C domain comprise the amino acid sequence set forth in SEQ ID No: 11 or an amino acid sequence having at least 90% identity to the same wherein at least the glycine residue at position 29 replaced with an amino acid residue other than alanine, threonine or tryptophan, wherein the immunoglobulin-binding protein binds an Fc portion of an immunoglobulin but exhibits reduced binding to a Fab portion of the immunoglobulin relative to an immunoglobulin-binding protein including alanine or glycine at position 29.

2. An immunoglobulin-binding protein according to claim 1, wherein:

(a) the amino acid residue other than alanine, threonine or tryptophan is selected from the group consisting of leucine, lysine and arginine; and/or

(b) at least the glycine residue at position 29 is replaced with an amino acid selected from the group consisting of lysine, arginine, leucine, cysteine, aspartic acid, glutamic acid, phenylalanine, histidine, isoleucine, methionine, asparagine, proline, glutamine, serine, valine and tyrosine.

3. An isolated immunoglobulin-binding protein according to claim 1 or claim 2, wherein the glycine residue at position 29 is replaced with lysine.

4. An isolated immunoglobulin-binding protein according to any one of the preceding claims, further comprising at least a portion of the carboxy-terminal region of *Staphylococcus* protein A.

5. An immunoglobulin-binding protein according to any one of the preceding claims, further comprising the replacement of an asparagine with another amino acid residue in the one or more isolated domains.

6. An isolated immunoglobulin-binding protein according to claim 5, wherein the asparagine is at position 23.

7. An isolated immunoglobulin-binding protein according to any one of the preceding claims, wherein the immunoglobulin is an IgG or a fragment thereof.

8. An isolated immunoglobulin-binding protein according to any one of the preceding claims, wherein the Fc portion of the immunoglobulin is part of an Fc fusion protein.

9. An isolated immunoglobulin-binding protein according to any one of the preceding claims, wherein the amino acid sequence has at least 95% or more identity to SEQ ID No 11.

10. An isolated immunoglobulin-binding protein according to anyone of the preceding claims, wherein the amino acid sequence is SEQ ID No: 11.

11. A nucleic acid molecule encoding the immunoglobulin-binding protein of any one of claims 1 to 10.

12. A host cell comprising the nucleic acid molecule of claim 11.

13. A chromatography matrix comprising the isolated immunoglobulin-binding protein of any one of claims 1 to 10 coupled to a solid support.

14. A method of affinity purifying one or more immunoglobulins from a sample, the method comprising the steps of:

(a) providing a sample comprising one or more immunoglobulins;
(b) contacting the sample with the matrix of claim 13 under conditions such that the one or more immunoglobulins bind to the matrix; and
(c) recovering the one or more bound immunoglobulins by eluting under suitable conditions.