

Novel Immunoglobulin-Binding Proteins and their Use in Affinity Purification

Field of the Invention

The present invention relates to non-natural binding proteins comprising one or more non-natural immunoglobulin (Ig) binding domains. The invention further relates to compositions such as affinity matrices comprising the non-natural Ig-binding proteins of the invention. The invention also relates to a use of these Ig-binding proteins or compositions for affinity purification of immunoglobulins and to methods of affinity purification using the Ig binding proteins of the invention.

Background of the Invention

Many biotechnological and pharmaceutical applications require the removal of contaminants from a sample containing antibodies. An established procedure for capturing and purifying antibodies is affinity chromatography using the bacterial cell surface protein A from *Staphylococcus aureus* as selective ligand for immunoglobulins (see, for example, review by Huse et al, J. Biochem. Biophys. Methods 51, 2002: 217-231). Wild-type Protein A binds to the Fc region of IgG molecules with high affinity and selectivity and is stable at high temperatures and in a wide range of pH values. Variants of protein A with improved properties such as alkaline stability are available for purifying antibodies and various chromatographic matrices comprising protein A ligands are commercially available. However, in particular wild-type Protein A based chromatography matrices show a loss of binding capacity for immunoglobulins following exposure to alkaline conditions.

Technical Problems Underlying the Present Invention

Most large scale production processes for antibodies or Fc-containing fusion proteins use Protein A for affinity purification.

However, due to limitations of Protein A applications in affinity chromatography there is a need in the prior art to provide novel Ig binding proteins with improved properties that specifically bind to immunoglobulins in order to facilitate affinity purification of immunoglobulins. Thus, the specificity of Ig binding proteins for immunoglobulin with affinities of 1 μ M, even of 100 nM or less is an important functional feature for an Ig binding protein for efficient purification of immunoglobulins.

Further, to maximally exploit the value of the chromatographic matrices comprising Ig binding proteins it is desirable to use the affinity ligand matrices multiple times. Between chromatography cycles, a thorough cleaning procedure is required for sanitization and removal of residual contaminants on the matrix. In this procedure, it is general practice to apply alkaline solutions with high concentrations of NaOH to the affinity ligand matrices. Wild-type Protein A or naturally occurring Protein A domains do not withstand such harsh alkaline conditions for an extended time and quickly lose binding capacity for immunoglobulin. Accordingly, there is a need in this field to obtain novel alkaline-stable proteins capable of binding immunoglobulins.

The present invention provides artificial immunoglobulin binding proteins that are particularly well-suited for affinity purification of immunoglobulins but overcome the disadvantages of the prior art. In particular, a significant advantage of the non-natural Ig binding proteins of the invention is their increased stability at high pH compared to naturally occurring Protein A domains.

The above overview does not necessarily describe all problems solved by the present invention.

Summary of the Invention

In a first aspect the present invention relates to a non-natural, immunoglobulin (Ig) binding protein comprising one or more non-natural Ig-binding domains, wherein at least one non-natural Ig-binding domain comprises the amino acid sequence SEQ ID NO: 1.

In an embodiment of the first aspect, at least one non-natural Ig-binding domain comprises the amino acid sequence

X₁X₂X₃X₄X₅X₆X₇X₈QQX₁₁AFYX₁₅X₁₆LX₁₈X₁₉PX₂₁LX₂₃X₂₄X₂₅QRX₂₈X₂₉FIQSLKDDPSX₄₀SX₄₂X₄₃X₄₄LX₄₆EAX₄₉KLX₅₂X₅₃X₅₄QX₅₆PX₅₈ (SEQ ID NO: 1), wherein

X₁ is A, V, Q, N, or P; preferably N, V, P, or A;

X₂ is D, A, or Q; preferably D or A;

X₃ is A, S, or N;

X₄ is K, Q, or N; preferably K or Q;

X₅ is H or F;

X₆ is D, N, S, or A; preferably D, S, or A,

X₇ is E or K;

X₈ is D, E or A;

X₁₁ is S or N;

X₁₅ is E, D, or Q; preferably E;

X₁₆ is V or I; preferably I;

X₁₈ is H or N; preferably H;

X₁₉ is L or M; preferably L;

X₂₁ is N, S, or D;

X₂₃ is T or N; preferably T;

X₂₄ is E or A; preferably E;

X₂₅ is D or E;

X₂₈ is N, S, or A;

X₂₉ is G or A; preferably A;

X₄₀ is V or Q or T;

X₄₂ is K, T, or A; preferably K or A;

X₄₃ is E, N, or S; preferably E or S;

X₄₄ is V, L, or I;

X₄₆ is G or A;

X₄₉ is K or Q;

X₅₂ is N, S, or D;

X₅₃ is D or E;

X₅₄ is S or A;

X₅₆ is A or P; preferably A; and

X₅₈ is K or P;

and wherein the dissociation constant K_D of said non-natural Ig-binding protein to human IgG₁ is 1 μM or less, preferably less than 500 nM, more preferably less than 100 nM. In a first embodiment, the invention relates to a binding protein comprising one or more non-natural Ig-binding domains, wherein at least one non-natural Ig-

binding domain comprises the amino acid sequence shown in SEQ ID NO: 1 with the ability to bind to immunoglobulin even after alkaline treatment.

In a second aspect the present invention relates to a composition comprising the non-natural Ig-binding protein of the first aspect, preferably wherein the composition is an affinity separation matrix.

In a third aspect the present invention relates to a use of the non-natural Ig-binding protein of the first aspect or of the composition of the second aspect for affinity purification of immunoglobulins.

In a fourth aspect the present invention relates to a method of affinity purification of immunoglobulins comprising the steps of (a) providing a liquid containing an immunoglobulin; (b) providing an affinity separation matrix comprising an immobilized non-natural Ig-binding protein of the first aspect; (c) contacting said liquid and said affinity separation matrix, wherein said immunoglobulin binds to said immobilized Ig-binding protein; and (d) eluting said immunoglobulin from said matrix, thereby obtaining an eluate containing said immunoglobulin; and (e) optionally further comprising one or more washing steps carried out between steps (c) and (d).

In a fifth aspect the present invention relates to a method of generation of a non-natural, Ig-binding protein according to the first aspect, wherein each Ig-binding domain is obtainable by a shuffling process of at least two naturally occurring Ig-binding domains from a naturally occurring Ig-binding protein and optionally introducing further mutations.

In a sixth aspect the present invention relates to a nucleic acid molecule encoding a non-natural Ig-binding protein of the first aspect.

In a seventh aspect the present invention relates to a vector comprising the nucleic acid molecule of the sixth aspect.

In an eighth aspect the present invention relates to a host cell or a non-human host comprising the non-natural Ig-binding protein of the first aspect, a nucleic acid molecule of the sixth aspect, or a vector of the seventh aspect.

In a ninth aspect the present invention relates to a method for the production of a non-natural Ig-binding protein of the first aspect, comprising the step(s): a. culturing the host cell of the eighth aspect under suitable conditions for the expression of the binding protein in order to obtain said non-natural immunoglobulin (Ig) binding protein; and b. optionally isolating said non-natural immunoglobulin (Ig) binding protein.

This summary of the invention does not necessarily describe all features of the present invention. Other embodiments will become apparent from a review of the ensuing detailed description.

Brief Description of the Figures

Figure 1A. Illustration of the shuffling method for the generation of Ig binding proteins.

Figure 1B. Generic sequence of IgG binding proteins of the invention (SEQ ID NO: 1). The numbers refer to the corresponding amino acid position in the binding protein; "X" refers to an amino acid that is selected from the amino acids as shown below the "X". For example, "X" in Position 2 can be selected from A, D, or Q.

Figure 2. Amino acid sequences of selected non-natural Ig-binding proteins (SEQ ID NOs: 9-30)

Figure 3. Analysis of IgG binding proteins expressed in HMS174(DE3) by denaturing SDS-PAGE. Soluble and insoluble fractions were generated and applied to the SDS-Gel. Main culture 7 h after inoculation (Figure 3A) and 24 h after inoculation (Figure 3B). Lane 1 - molecular weight marker, soluble (lane 2) and insoluble (lane 3) fraction of 148464 (SEQ ID NO: 15), soluble (lane 4) and insoluble (lane 5) fraction of 148463 (SEQ ID NO: 14), soluble (lane 6) and insoluble (lane 7) fraction of 148461 (SEQ ID NO: 12). The grey arrow points to the approximate size of the expressed proteins.

Figure 4. Analysis of purified IgG binding proteins by denaturing SDS-PAGE. Expression and purification of 148461 (SEQ ID NO: 12) (Figure 4A), and of 148471 (SEQ ID NO: 22) (Figure 4B). Lane 1 molecular weight

marker, lane 2 insoluble fraction, lane 3 soluble fraction, lane 4 flow-through StrepTactin column, lanes 5-9 HiLoad 16/600 Superdex 75 pg elution fractions.

Figure 5. Analysis of the binding affinity of IgG binding proteins by ELISA. The assay was performed with Cetuximab (filled circles) and Adalimumab (empty circles) as on-targets and BSA (filled triangles) as off-target. The binding of the IgG binding proteins was analyzed via StrepTag with Strep-Tactin-HRP. **Figure 5A.** Ig binding protein 148472 (SEQ ID NO: 23); The K_D for SEQ ID NO: 23 is 5.9 nM vs Cetuximab and 5.1 nM vs Adalimumab. **Figure 5B.** Ig binding protein 148461 (SEQ ID NO: 12). The K_D for SEQ ID NO: 12 is 7.8 nM vs Cetuximab and 7.5 nM vs Adalimumab. Results for further IgG binding proteins of the invention compared to naturally occurring Protein A domains are shown in Table 2 (see Example 5).

Figure 6. Analysis of the binding affinity of IgG binding proteins by SPR (Biacore). **Figure 6A.** Analysis of Ig binding protein 148463 (SEQ ID NO: 14). Concentrations analyzed were 0 nM, 1.56 nM, 3.125 nM, 6.25 nM, 12.5 nM, 25 nM, 50 nM. The K_D for SEQ ID NO: 14 is 1.3 nM, **Figure 6B.** Analysis of Ig binding protein 154256 (SEQ ID NO: 28). Concentrations analyzed were 0, 0.39 nM, 0.78 nM, 1.56 nM, 3.125 nM, 6.25 nM, 12.5 nM, 25 nM, 50 nM. The K_D for SEQ ID NO: 28 is 3.1 nM. Further results are shown in Table 3 (see Example 6).

Figure 7. Immobilization of IgG binding proteins to SulfoLink Coupling Resin. Shown are profiles of Ig binding protein 148470 (SEQ ID NO: 21) (**Figure 7A**), and Ig binding protein 148460 (SEQ ID NO: 11) (**Figure 7B**). The y-axis shows the absorption at 280 nm in mAU, the y-axis refers to the elution volume in ml.

Figure 8. Ig binding activity of IgG binding proteins immobilized to Sulfolink resin after alkaline treatment. The figure shows the remaining activity of different IgG binding proteins 148462 (SEQ ID NO: 13, "13"), 148463 (SEQ ID NO: 14; "14"), 1484672 (SEQ ID NO: 23, "23") in comparison to naturally occurring Protein A domains E, D, A, B, C, and to domain Z after 80 min of continuous 0.5 M NaOH treatment.

Figure 9. Ig binding of IgG binding proteins immobilized to epoxy-activated resin after alkaline treatment. Ig binding proteins 154254 (SEQ ID NO: 26), 154255 (SEQ ID NO: 27), 154256 (SEQ ID NO: 28), and 154257 (SEQ ID NO: 30) were compared to IgG binding protein 148463 (SEQ ID NO: 14). The remaining activity after six hours of continuous 0.5 M NaOH treatment is shown.

Figure 10. Ig binding activity of IgG binding proteins consisting of 1, 2, 4, or 6 IgG binding domains immobilized to epoxy-activated resin after alkaline treatment. The IgG binding activity of monomer 148463 (SEQ ID NO: 14), dimer 150570 (SEQ ID NO: 45), tetramer 150663 (SEQ ID NO: 46), and hexamer 150772 (SEQ ID NO: 47) after continuous 0.5 M NaOH treatment is shown (0 hours, light grey column; 2 hours, medium grey column; 4 hours, dark medium grey column; 6 hours, dark grey column).

Detailed Description of the Invention

Definitions

Before the present invention is described in detail below, it is to be understood that this invention is not limited to the particular methodology, protocols and reagents described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs.

Preferably, the terms used herein are defined as described in "A multilingual glossary of biotechnological terms: (IUPAC Recommendations)", Leuenberger, H.G.W, Nagel, B. and Kölbl, H. eds. (1995), Helvetica Chimica Acta, CH-4010 Basel, Switzerland).

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a

stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Several documents (for example: patents, patent applications, scientific publications, manufacturer's specifications, instructions, GenBank Accession Number sequence submissions etc.) are cited throughout the text of this specification. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention. Some of the documents cited herein are characterized as being "*incorporated by reference*". In the event of a conflict between the definitions or teachings of such incorporated references and definitions or teachings recited in the present specification, the text of the present specification takes precedence.

All sequences referred to herein are disclosed in the attached sequence listing that, with its whole content and disclosure, is a part of this specification.

The terms "protein" and "polypeptide" refer to any linear molecular chain of two or more amino acids linked by peptide bonds, and does not refer to a specific length of the product. Thus, "peptides", "protein", "amino acid chain," or any other term used to refer to a chain of two or more amino acids, are included within the definition of "polypeptide," and the term "polypeptide" may be used instead of, or interchangeably with any of these terms. The term "polypeptide" is also intended to refer to the products of post-translational modifications of the polypeptide, including without limitation glycosylation, acetylation, phosphorylation, amidation, proteolytic cleavage, modification by non-naturally occurring amino acids and similar modifications which are well-known in the art. Thus, Ig binding proteins comprising two or more protein domains also fall under the definition of the term "protein" or "polypeptides".

In the context of the present invention, the term "immunoglobulin-binding protein" is used to describe proteins that are capable of specifically bind to the Fc region of an immunoglobulin. Due to this specific binding to the Fc region, the "immunoglobulin-binding proteins" of the invention are capable of binding to entire immunoglobulins, to immunoglobulin fragments comprising the Fc region, to fusion proteins comprising an Fc region of an immunoglobulin, and to conjugates comprising an Fc region of an immunoglobulin. While the "immunoglobulin-binding proteins" of the invention herein exhibit specific binding to the Fc region of an immunoglobulin, it is not excluded that "immunoglobulin-binding proteins" can additionally bind with reduced affinity to other regions, such as Fab regions of immunoglobulins.

Throughout this specification, the term "immunoglobulin-binding protein" is often abbreviated as "Ig binding protein" or "Ig-binding protein". Occasionally, both the long form and the abbreviated form are used at the same time, e.g. in the expression "immunoglobulin (Ig) binding protein".

In preferred embodiments of the present invention, the "immunoglobulin-binding protein" comprises one or more non-natural Ig-binding domains. As used herein, the term "immunoglobulin-binding domain" (often abbreviated as: Ig-binding domain) refers to a protein domain that is capable of specifically binding to the Fc region of an immunoglobulin. It is not excluded, though, that "immunoglobulin-binding domain" can additionally bind – with reduced affinity – to other regions, such as Fab regions of immunoglobulins. Due to the specific binding to the Fc region, the "immunoglobulin-binding domains" of the invention are capable to bind to entire immunoglobulins, to immunoglobulin fragments comprising the Fc region, to fusion proteins comprising an Fc region of an immunoglobulin, and to conjugates comprising an Fc region of an immunoglobulin.

In preferred embodiments of the invention, the "immunoglobulin-binding domains" are non-natural domains that exhibit a maximum of 85 % sequence identity to naturally occurring Ig-binding domains, for example to domain C (SEQ ID NO: 7) or to domain B (SEQ ID NO: 6) or to domain E (SEQ ID NO: 3) or to domain D (SEQ ID NO: 4) or to domain A (SEQ ID NO: 5) of *Staphylococcus aureus* Protein A. A preferred non-natural Ig binding domain of the invention has identical amino acids in positions corresponding to positions Q9, Q10, A12, F13, Y14, L17, P20, L22, Q26, R27, F30, I31, Q32, S33, L34, K35, D36, D37, P38, S39, S41, L45, E47, A48, K50, L51, Q55, P57 of

domain E, D, A, B, C, and to domain Z. The identity of an Ig-binding domain of the invention to naturally occurring domains E, D, A, B, C, and to domain Z is at least about 50 % and maximal 85 %.

As used herein, a first compound (e.g. an Ig binding protein of the invention) is considered to "bind" to a second compound (e.g. an antigen, such as a target protein, such as immunoglobulin), if it has a dissociation constant K_D to said second compound of 500 μM or less, preferably 100 μM or less, preferably 50 μM or less, preferably 10 μM or less, preferably 1 μM or less, preferably 500 nM or less, preferably 100 nM or less, more preferably 50 nM or less, even more preferably 10 nM or less. The term "binding" according to the invention preferably relates to a specific binding. "Specific binding" means that an Ig binding protein of the invention binds stronger to an immunoglobulin for which it is specific compared to the binding to another non-immunoglobulin target. For example, the dissociation constant (K_D) for the target (e.g. immunoglobulin) to which the Ig binding protein binds specifically is more than 10-fold, preferably more than 20-fold, more preferably more than 50-fold, even more preferably more than 100-fold, 200-fold, 500-fold, or 1000-fold lower than the dissociation constant (K_D) for a target to which the binding protein does not bind specifically.

The term "dissociation constant" or " K_D " defines the specific binding affinity. As used herein, the term " K_D " (usually measured in "mol/L", sometimes abbreviated as "M") is intended to refer to the dissociation equilibrium constant of the particular interaction between a first protein and a second protein. In the context of the present invention, the term K_D is particularly used to describe the binding affinity between an immunoglobulin-binding protein and an immunoglobulin. A high affinity corresponds to a low value of K_D . Thus, the expression "a K_D of at least e.g. 10^{-7} M" means a value of 10^{-7} M or lower (binding more tightly). 1×10^{-7} M corresponds to 100 nM. A value of 10^{-5} M and below down to 10^{-12} M can be considered as a quantifiable binding affinity. In accordance with the invention the affinity for the target binding should be in the range of 500 nM or less, more preferably below 100 nM, even more preferably 10 nM or less.

Methods for determining binding affinities, i.e. for determining the dissociation constant K_D , are known to a person of ordinary skill in the art and can be selected for instance from the following methods known in the art: Surface Plasmon Resonance (SPR) based technology, Bio-layer interferometry (BLI), enzyme-linked immunosorbent assay (ELISA), flow cytometry, fluorescence spectroscopy techniques, isothermal titration calorimetry (ITC), analytical ultracentrifugation, radioimmunoassay (RIA or IRMA) and enhanced chemiluminescence (ECL). Some of the methods are described in the Examples below.

The term "naturally occurring" as used herein refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally occurring. For example, naturally occurring Ig-binding domains can be isolated from the bacterium *Staphylococcus aureus*, for example Protein A domain C (SEQ ID NO: 7) or Protein A domain B (SEQ ID NO: 6) or Protein A domain E (SEQ ID NO: 3) or Protein A domain D (SEQ ID NO: 4) or Protein A domain A (SEQ ID NO: 5).

In contrast thereto, the term "non-natural", as used herein, refers to an object that is not naturally occurring, i.e. the term refers to an object that has been produced or modified by man. For example, a polypeptide or polynucleotide sequence that has been generated by man for example in a laboratory (e.g. by genetic engineering, by shuffling methods, or by chemical reactions, etc.) or intentionally modified is "non-natural". The terms "non-natural" and "artificial" are used interchangeably herein. For example, the Ig-binding proteins of the invention comprising at least one Ig binding domain are non-natural proteins.

The term "antibody" or "Ig" or "immunoglobulin" as used interchangeably herein in accordance with the present invention comprises proteins having a four-polypeptide chain structure consisting of two heavy chains and two light chains (immunoglobulin or IgG antibodies) with the ability to specifically bind an antigen. The term "antibody light chain" designates the small polypeptide subunit of an antibody chain which is composed of two tandem immunoglobulin domains, one constant domain and one variable domain that is important for antigen binding. The term "antibody heavy chain" designates the large polypeptide subunit of an antibody that determines the class or

isotype of an antibody. Furthermore, also fragments or derivatives thereof, which still retain the binding specificity, are comprised in the term "antibody". Antibody fragments are understood herein as comprising fewer amino acid residues than an intact or complete antibody or antibody chain. The term "antibody" also includes embodiments such as chimeric (human constant domain, non-human variable domain), single chain and humanized (human antibody with the exception of non-human CDRs) antibodies. Full-length IgG antibodies consisting of two heavy chains and two light chains are most preferred in this invention. Heavy and light chains are connected via non-covalent interactions and disulfide bonds.

As used herein, the term "linker" refers in its broadest meaning to a molecule that covalently joins at least two other molecules. In typical embodiments of the present invention, a "linker" is to be understood as a moiety that connects a first polypeptide with at least one further polypeptide. The second polypeptide may be the same as the first polypeptide or it may be different. Preferred in these typical embodiments are peptide linkers. This means that the peptide linker is an amino acid sequence that connects a first polypeptide with a second polypeptide, for example a first Ig binding domain with a second Ig binding domain. The peptide linker is connected to the first polypeptide and to the second polypeptide by a peptide bond, thereby generating a single, linear polypeptide chain. The length and composition of a linker may vary between at least one and up to 30 amino acids. More specifically, a peptide linker has a length of between 1 and 30 amino acids; e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acids. It is preferred that the amino acid sequence of the peptide linker is stable against proteases and/or does not form a secondary structure. Well-known are linkers comprised of small amino acids such as glycine and serine. The linkers can be glycine-rich (e.g., more than 50% of the residues in the linker can be glycine residues). Preferred are glycine-serine-linker of variable length consisting of glycine and serine residues only. In general, linkers of the structure (SGGG)_n or permutations of SGGG, e.g. (GGGS)_n, can be used wherein n can be any number between 1 and 6, preferably 1 or 2 or 3. Also preferred are linkers comprising further amino acids. Preferred embodiments of the invention comprise linkers consisting of alanine, proline, and serine. It is preferred that a peptide linker consists of about 40 % to 60 % alanine, about 20 % to 35 % proline, and about 10% to 30 % serine. It is preferred that the amino acids alanine, proline, and serine are evenly distributed throughout the linker amino acid sequence so that not more than a maximum of 2, 3, 4, or 5 identical amino acid residues are adjacent, preferably a maximum of 3 amino acids. Other linkers for the fusion of proteins are known in the art and can be used.

Exemplary linkers usable in the present invention are the following linkers: a linker having at least the amino acid sequence SG or any other linker, for example SGGGG [SEQ ID NO: 31], SGGGGSGGGG [SEQ ID NO: 32], GGGSGGGSGGGG [SEQ ID NO: 33], GGGSGGGSGGGG [SEQ ID NO: 34], GGGGS [SEQ ID NO: 35], GGGS [SEQ ID NO: 36], SGGG [SEQ ID NO: 37], or (GGGS)_n (i.e., n repetitions of SEQ ID NO: 36, wherein n is between 1 and 5 (e.g., n may be 1, 2, 3, 4, or 5)), (SGGG)_n (i.e., n repetitions of SEQ ID NO: 37, wherein n is between 1 and 5 (e.g., n may be 1, 2, 3, 4, or 5)), or SAAPAPSAPASAAPAPAPAPAPSPAAPAAS [SEQ ID NO: 41], ASPSPAAPAPAPSAASPAPAAPAPAASPAA [SEQ ID NO: 42], or ASPAPSAPSA [SEQ ID NO: 43]). Other linkers for the fusion of two IgG binding domains or two IgG binding proteins are also known in the art and can be used.

The term "fused" means that the components are linked by peptide bonds, either directly or via peptide linkers. The term "fusion protein" relates to a protein comprising at least a first protein joined genetically to at least a second protein. A fusion protein is created through joining of two or more genes that originally coded for separate proteins. Thus, a fusion protein may comprise a multimer of identical or different binding proteins which are expressed as a single, linear polypeptide. It may comprise two, three, four or even more binding domains or binding proteins. In general, fusion proteins are generated artificially by recombinant DNA technology well-known to a skilled person. Ig binding proteins of the invention may be prepared by any of the many conventional and well-known techniques such as plain organic synthetic strategies, solid phase-assisted synthesis techniques or by commercially available automated synthesizers.

Preferably, the term "multimer" as used herein relates to a fusion protein comprising at least two IgG binding domains, preferably 2 (dimer), 3 (trimer), 4 (tetramer), 5 (pentamer), 6 (hexamer), 7 (heptamer), or 8 (octamer) IgG binding domains, more preferred 4, 5, or 6 IgG binding domains. In preferred embodiment, the Ig binding domains in a multimer are identical. In other embodiments, the Ig binding domains of a multimer can be different. One or more linker sequences are inserted between the domains of the multimer.

The term "amino acid sequence identity" refers to a quantitative comparison of the identity (or differences) of the amino acid sequences of two or more proteins. "Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity.

To determine the sequence identity, the sequence of a query protein is aligned to the sequence of a reference protein. Methods for alignment are well-known in the art. For example, for determining the extent of an amino acid sequence identity of an arbitrary polypeptide relative to a reference amino acid sequence, the SIM Local similarity program is preferably employed (Xiaoquin Huang and Webb Miller (1991), *Advances in Applied Mathematics*, vol. 12: 337-357), that is freely available (see also: <http://www.expasy.org/tools/sim-prot.html>). For multiple alignment analysis ClustalW is preferably used (Thompson et al. (1994) *Nucleic Acids Res.*, 22(22): 4673-4680). Preferably, the default parameters of the SIM Local similarity program or of ClustalW are used, when calculating sequence identity percentages.

In the context of the present invention, the extent of sequence identity between a modified sequence and the sequence from which it is derived is generally calculated with respect to the total length of the unmodified sequence, if not explicitly stated otherwise.

Each amino acid of the query sequence that differs from the reference amino acid sequence at a given position is counted as one difference. An insertion or deletion in the query sequence is also counted as one difference. For example, an insertion of a linker between two binding domains is counted as one difference compared to the reference sequence. The sum of differences is then related to the length of the reference sequence to yield a percentage of non-identity. The quantitative percentage of identity is calculated as 100 minus the percentage of non-identity.

The term "about", as used herein, encompasses the explicitly recited amounts as well as deviations therefrom of $\pm 10\%$. More preferably, a deviation 5 % is encompassed by the term "about".

The term "shuffled" as used herein refers to an assembly process resulting in novel non-natural sequences starting from a set of known sequences comprising the following steps: (a) providing a set of at least two sequences to be shuffled; (b) alignment of said sequences; and (c) assembly of new sequences from the aligned sequences wherein the amino acid at each position of the new sequence can be derived from the same position of any of the aligned sequences. Preferably, two or more consecutive amino acids are derived from one of the aligned sequences.

The term "chromatography" refers to separation technologies which employ a mobile phase and a stationary phase to separate one type of molecules (e.g., immunoglobulins) from other molecules (e.g. contaminants) in the sample. The liquid mobile phase contains a mixture of molecules and transports these across or through a stationary phase (such as a solid matrix). Due to the differential interaction of the different molecules in the mobile phase with the stationary phase, molecules in the mobile phase can be separated.

The term "affinity chromatography" refers to a specific mode of chromatography in which a ligand coupled to a stationary phase interacts with a molecule (i.e. immunoglobulin) in the mobile phase (the sample) i.e. the ligand has a specific affinity for the molecule to be purified. As understood in the context of the invention, affinity chromatography involves the addition of a sample containing an immunoglobulin to a stationary phase which comprises a chromatography ligand, such as an Ig binding protein of the invention. The terms "solid support" or "solid matrix" are used interchangeably for the stationary phase.

The terms "affinity matrix" or "affinity separation matrix" or "affinity chromatography matrix," as used interchangeably herein, refer to a matrix, e.g. a chromatographic matrix, onto which an affinity ligand (e.g., an Ig binding protein of the invention) is attached. The ligand (e.g., Ig binding protein) is capable of binding to a molecule of interest (e.g., an immunoglobulin or an Fc-containing protein) through affinity interaction which is to be purified or removed from a mixture.

The term "affinity purification" as used herein refers to a method of purifying immunoglobulins or Fc-containing proteins from a liquid by binding the immunoglobulins or Fc-containing proteins to an Ig binding protein that is immobilized to a matrix. Thereby, all other components of the mixture except immunoglobulins or Fc-containing proteins are removed. In a further step, the bound immunoglobulins or Fc-containing proteins can be eluted in purified form.

The term "alkaline stable" or "alkaline stability" or "caustic stable" or "caustic stability" refers to the ability of the Ig binding protein of the invention to withstand alkaline conditions without significantly losing the ability to bind to immunoglobulins. The skilled person in this field can easily test alkaline stability by incubating an Ig binding protein with sodium hydroxide, e.g. as described in the Examples, and subsequent testing of the binding activity to immunoglobulin by routine experiments known to someone skilled in the art, for example, by chromatographic approaches.

In some embodiments, Ig binding proteins of the invention as well as matrices comprising Ig binding proteins of the invention exhibit an "increased" or "improved" alkaline stability, meaning that the molecules and matrices incorporating said Ig binding proteins are stable under alkaline conditions for an extended period of time relative to naturally occurring Protein A domains, i.e. do not lose the ability to bind to immunoglobulins or lose the ability to bind to immunoglobulins to a lesser extent than naturally occurring Protein A domains.

The terms "binding activity" or "binding capacity" or "static binding capacity" as used interchangeably herein, refer to the ability of an Ig binding protein of the invention to bind to immunoglobulin. For example, the binding activity can be determined before and/or after alkaline treatment. The binding activity can be determined for an Ig binding protein or for an Ig binding protein coupled to a matrix, i.e. for an immobilized binding protein.

Generally known and practiced methods in the fields of molecular biology, cell biology, protein chemistry and antibody techniques are fully described in the continuously updated publications "Molecular Cloning: A Laboratory Manual", (Sambrook et al., Cold Spring Harbor); Current Protocols in Molecular Biology (F. M. Ausubel et al. Eds., Wiley & Sons); Current Protocols in Protein Science (J. E. Colligan et al. eds., Wiley & Sons); Current Protocols in Cell Biology (J. S. Bonifacino et al., Wiley & Sons) and Current Protocols in Immunology (J. E. Colligan et al., Eds., Wiley & Sons). Known techniques relating to cell culture and media are described in "Large Scale Mammalian Cell Culture (D. Hu et al., Curr. Opin. Biotechnol. 8:148-153, 1997); "Serum free Media" (K. Kitano, Biotechnol. 17:73-106, 1991); and "Suspension Culture of Mammalian Cells" (J.R. Birch et al. Bioprocess Technol. 10:251-270, 1990).

Embodiments of the Invention

The present invention will now be further described. In the following passages different aspects of the invention are defined in more detail. Each aspect defined below may be combined with any other aspect or aspects unless clearly indicated to the contrary. In particular, any feature indicated as being preferred or advantageous may be combined with any other feature or features indicated as being preferred or advantageous.

In a first aspect the present invention is directed to a non-natural immunoglobulin (Ig) binding protein comprising one or more non-natural Ig-binding domains, wherein at least one Ig binding domain comprises or essentially consists or consists of the amino acid sequence

X₁X₂X₃X₄X₅X₆X₇X₈QQX₁₁AFYX₁₅X₁₆LX₁₈X₁₉PX₂₁LX₂₃X₂₄X₂₅QRX₂₈X₂₉FIQSLKDDPSX₄₀SX₄₂X₄₃X₄₄LX₄₆EAX₄₉KLX₅₂X₅₃X₅₄QX₅₆PX₅₈ (SEQ ID NO: 1), wherein X₁ is A, V, Q, N, or P; preferably N, V, P, or A; X₂ is D, A, or Q;

preferably D or A; X₃ is A, S, or N; X₄ is K, Q, or N; preferably K or Q; X₅ is H or F; X₆ is D, N, S, or A; preferably D, S, or A; X₇ is E or K; X₈ is D, E or A; X₁₁ is S or N; X₁₅ is E, D, or Q; preferably E; X₁₆ is V or I; preferably I; X₁₈ is H or N; preferably H; X₁₉ is L or M; preferably L; X₂₁ is N, S, or D; X₂₃ is T or N; preferably T; X₂₄ is E or A; preferably E; X₂₅ is D or E; X₂₈ is N, S, or A; X₂₉ is G or A; preferably A; X₄₀ is V or Q or T; X₄₂ is K, T, or A; preferably K or A; X₄₃ is E, N, or S; preferably E or S; X₄₄ is V, L, or I; X₄₆ is G or A; X₄₉ is K or Q; X₅₂ is N, S, or D; X₅₃ is D or E; X₅₄ is S or A; X₅₆ is A or P; preferably A; and X₅₈ is K or P;

and wherein the dissociation constant K_D of said non-natural Ig-binding protein to human IgG1 is 1 μM or less, preferably 500 nM, more preferably 100 nM or less. In more detail, SEQ ID NO: 1 and a preferred embodiment as shown in SEQ ID NO: 38 are generic sequences resulting from an alignment of SEQ ID NOs: 9 to 30. Thus, each non-natural Ig binding domain of the Ig binding protein of the invention exhibits about 50 % to about 85 % sequence identity to a naturally occurring Ig binding domain, see Table 1 (see Example 1). Each non-natural Ig binding domain of the Ig binding protein of the invention has the same amino acids in positions that correspond to positions Q9, A12, F13, L17, Q26, R27, F30, I31, L34, P38, S41, L45, A48, L51, Q55 of a naturally occurring Ig binding domain, more preferably with the same amino acids that correspond to positions Q9, Q10, A12, F13, Y14, L17, P20, L22, Q26, R27, F30, I31, Q32, S33, L34, K35, D36, D37, P38, S39, S41, L45, E47, A48, K50, L51, Q55, P57 of a naturally occurring Ig binding domain, for example domain C, domain B, domain A, domain E, and domain D.

In a preferred embodiment of the first aspect, at least one non-natural Ig-binding domain comprises or essentially consists of the amino acid sequence

X₁X₂X₃X₄X₅X₆X₇X₈QQX₁₁AFYEILHLPX₂₁LTEX₂₅QRX₂₈AFIQSLKDDPSX₄₀SX₄₂X₄₃X₄₄LX₄₆EAX₄₉KLX₅₂X₅₃X₅₄QAPX₅₈ (SEQ ID NO: 38), wherein

X₁ is N, V, P, or A;

X₂ is D or A;

X₃ is A, S, or N;

X₄ is K or Q;

X₅ is H or F;

X₆ is D, S, or A;

X₇ is E or K;

X₈ is D, E or A;

X₁₁ is S or N;

X₂₁ is N, S, or D;

X₂₅ is D or E;

X₂₈ is N, S, or A;

X₄₀ is V, T, or Q;

X₄₂ is K or A;

X₄₃ is E or S;

X₄₄ is V, L, or I;

X₄₆ is G or A;

X₄₉ is K or Q;

X₅₂ is N, S, or D;

X₅₃ is D or E;

X₅₄ is S or A; and

X₅₈ is K or P.

SEQ ID NO: 38 is a generic amino acid sequence resulting from an alignment of SEQ ID NOs: 9 – 30 and is a preferred selection of SEQ ID NO: 1.

In one preferred embodiment of the first aspect, at least one non-natural Ig-binding domain comprises or essentially consists of the amino acid sequence

X₁X₂X₃X₄X₅X₆X₇X₈QQX₁₁AFYX₁₅X₁₆LX₁₈X₁₉PX₂₁LX₂₃X₂₄X₂₅QRX₂₈X₂₉FIQSLKDDPSX₄₀SX₄₂X₄₃X₄₄LX₄₆EAX₄₉KLX₅₂X₅₃X₅₄QX₅₆PX₅₈ (SEQ ID NO: 2), wherein

X₁ is A, V, Q, N, or P; X₂ is D, A, or Q; X₃ is A or N; X₄ is K, Q, or N; X₅ is H or F; X₆ is D, N, or A; X₇ is E or K; X₈ is D, E or A; X₁₁ is S or N; X₁₅ is E or D; X₁₆ is V or I; X₁₈ is H or N; X₁₉ is L or M; X₂₁ is N, S, or D; X₂₃ is T or N; X₂₄ is E or A; X₂₅ is D or E; X₂₈ is N, S, or A; X₂₉ is G or A; X₄₀ is V or Q; X₄₂ is K, T, or A; X₄₃ is E or N; X₄₄ is V, L, or I; X₄₆ is G or A; X₄₉ is K or Q; X₅₂ is N or D; X₅₃ is D or E; X₅₄ is S or A; X₅₆ is A or P; and X₅₈ is K or P.

In one embodiment of the first aspect, an Ig binding protein according to the invention comprises one or more non-natural Ig binding domains wherein at least one Ig binding domain comprises or essentially consists of the amino acid sequence

X₁X₂AX₄X₅DX₇X₈QQX₁₁AFYEILHLPNLTEX₂₅QRNAFIQSLKDDPSX₄₀SX₄₂X₄₃X₄₄LX₄₆EAX₄₉KLNX₅₃X₅₄QAPK (SEQ ID NO: 48), wherein

X₁ is N or V;

X₂ is D or A;

X₄ is K or Q;

X₅ is H or F;

X₇ is E or K;

X₈ is D, E or A;

X₁₁ is S or N;

X₂₅ is D or E;

X₄₀ is V or Q;

X₄₂ is K or A;

X₄₃ is E or S;

X₄₄ is V or I;

X₄₆ is G or A;

X₄₉ is K or Q;

X₅₃ is D or E; and

X₅₄ is S or A.

SEQ ID NO: 48 is a generic amino acid sequence resulting from an alignment of SEQ ID NOs: 24, 26, 27, 28, and 30 and is a preferred selection of SEQ ID NO: 38. Ig binding proteins are stable even after alkaline treatment for a prolonged period of time (e.g. at least up to 6 hours, 0.5 M NaOH), for example, Ig binding proteins of the invention have a higher alkaline stability than naturally occurring Protein A domains.

In another embodiment of the first aspect, an Ig binding protein according to the invention comprises one or more non-natural Ig binding domains wherein at least one Ig binding domain comprises or essentially consists of the amino acid sequence

X₁X₂X₃X₄X₅X₆X₇X₈QQX₁₁AFYEILHLPX₂₁LTEDQRX₂₈AFIQSLKDDPSX₄₀SKX₄₃X₄₄LGEAKKLX₅₂DAQAPP (SEQ ID NO: 49), wherein

X₁ is P, N, or A;

X₂ is A or D;

X₃ is A, S, or N;

X₄ is K or Q;

X₅ is H or F;

X₆ is D, S, or A;

X₇ is K or E;

X₈ is D, E or A;

X₁₁ is S or N;
 X₂₁ is N, S, or D;
 X₂₈ is S or A;
 X₄₀ is V or T;
 X₄₃ is E or S;
 X₄₄ is I or L; and
 X₅₂ is N, S, or D.

SEQ ID NO: 49 is a generic amino acid sequence resulting from an alignment of SEQ ID NOs: 9 to 23 and is a preferred selection of SEQ ID NO: 38.

In an embodiment of the first aspect, the non-natural Ig-binding protein comprises one or more Ig-binding domains, wherein at least one non-natural Ig-binding domain comprises or consists of an amino acid sequence selected from the group consisting of:

NAAQHAKEQQNAFYELHLPNLTEDQRAAFIQLKDDPSVSKEILGEAKKLNDQAAPP (SEQ ID NO: 9),
 NAAQHDKEQQNAFYELHLPNLTEDQRAAFIQLKDDPSVSKEILGEAKKLNDQAAPP (SEQ ID NO: 10),
 NAAQHSKEQQNAFYELHLPNLTEDQRSFAFIQLKDDPSVSKEILGEAKKLNDQAAPP (SEQ ID NO: 11),
 NAAQHSKDQQSAFYELHLPNLTEDQRSFAFIQLKDDPSVSKEILGEAKKLNDQAAPP (SEQ ID NO: 12),
 PAAQHDKDQQSAFYELHLPNLTEDQRSFAFIQLKDDPSVSKEILGEAKKLNDQAAPP (SEQ ID NO: 13),
 PAAKHDKDQQSAFYELHLPNLTEDQRSFAFIQLKDDPSVSKEILGEAKKLNDQAAPP (SEQ ID NO: 14),
 ADNKFDEAQQSAFYELHLPNLTEDQRAAFIQLKDDPSVSKEILGEAKKLNDQAAPP (SEQ ID NO: 15),
 ADSKFDEAQQSAFYELHLPNLTEDQRAAFIQLKDDPSVSKEILGEAKKLNDQAAPP (SEQ ID NO: 16),
 ADSKFDEAQQSAFYELHLPNLTEDQRAAFIQLKDDPSVSKSLLGEAKKLNDQAAPP (SEQ ID NO: 17),
 ADSKFDEAQQSAFYELHLPDLTEDQRAAFIQLKDDPSVSKSLLGEAKKLNDQAAPP (SEQ ID NO: 18),
 ADSKFDEAQQSAFYELHLPDLTEDQRAAFIQLKDDPSVSKSLLGEAKKLNDQAAPP (SEQ ID NO: 19),
 ADSKFDEAQQSAFYELHLPDLTEDQRAAFIQLKDDPSTSKSLLGEAKKLNDQAAPP (SEQ ID NO: 20),
 ADSKFDEAQQSAFYELHLPDLTEDQRAAFIQLKDDPSTSKSLLGEAKKLDDQAAPP (SEQ ID NO: 21),
 ADSKFDEAQQSAFYELHLPDLTEDQRAAFIQLKDDPSTSKSLLGEAKKLSDQAAPP (SEQ ID NO: 22),
 PAAKHDKDQQSAFYELHLPDLTEDQRAAFIQLKDDPSTSKSILGEAKKLNDQAAPP (SEQ ID NO: 23),
 NAAQHDKEQQNAFYELHLPNLTEDQRNAFIQLKDDPSVSKEILGEAKKLNDQAAPK (SEQ ID NO: 24),
 ADNKFDEAQQSAFYELHLPNLTEDQRNAFIQLKDDPSVSKEILGEAKKLNDQAAPK (SEQ ID NO: 25),
 NAAKHDKDQQSAFYELHLPNLTEDQRNAFIQLKDDPSVSKEILGEAKKLNDQAAPP (SEQ ID NO: 26),
 NAAQHDKDQQSAFYELHLPNLTEEQRNAFIQLKDDPSVSKEILAEAKKLNDQAAPK (SEQ ID NO: 27),
 NAAKFDEAQQSAFYELHLPNLTEEQRNAFIQLKDDPSVSKEVLGEAQLNDSQAPK (SEQ ID NO: 28),
 QQAQHDEAQQSAFYQLHLPNLADQRNAFIQLKDDPSQSAEVLGEAQLNDSQAPK (SEQ ID NO: 29), and
 VDAQHDEDQQSAFYELHLPNLTEEQRNAFIQLKDDPSQSAEILAEAKKLNESQAPK (SEQ ID NO: 30).

Particularly preferred are the non-natural Ig-binding proteins that comprise one or more Ig-binding domains, wherein at least one non-natural Ig-binding domain comprises or essentially consists of an amino acid sequence selected from the group consisting of SEQ ID NOs: 13, 14, 23, 26, 27, 28, and 30.

As shown in the examples herein below, the proteins of the invention were found to bind to IgG. In more detail, it was found that the Ig-binding polypeptides comprising the generic sequence of SEQ ID NO: 1, more specifically of the generic sequences of SEQ ID NO: 38 or SEQ ID NO: 2, even more specifically SEQ ID NOs: 9-30, are able to bind to IgG at high affinities that are comparable to binding properties of naturally occurring Ig binding domain (see Table 2, Example 5, and Table 3, Example 6). It was more surprising and unexpected that the Ig-binding proteins comprising the generic sequence of SEQ ID NO: 1, more specifically the generic sequences of SEQ ID NO: 38 or SEQ ID NO: 2, even more specifically SEQ ID NOs: 9-30, are able to bind to IgG even after alkaline treatment for several hours, for example after treatment with 0.5 M NaOH for up to 6 hours. This is an

advantageous property as compared to naturally occurring Protein A domains or domain Z (e.g., see comparative data in Example 7 and in Example 8 and in Figure 8 and Figure 9).

In one embodiment of the invention, the non-natural Ig-binding protein comprises 1, 2, 3, 4, 5, 6, 7, or 8, preferably 4, 5, or 6, non-naturally-occurring Ig-binding domains linked to each other, i.e. the non-natural Ig-binding protein can be a monomer, dimer, trimer, tetramer, pentamer, hexamer, etc.. For example, SEQ ID NO: 14 was used to generate the multimeric fusion constructs described herein in Example 1. The obtained Ig binding proteins comprising more than one Ig binding domain are stable and display Ig binding properties even after alkaline treatment (for example, see Figure 10). Selected but non-limiting examples for multimeric Ig binding proteins are provided in SEQ ID NO: 45 (dimer), SEQ ID NO: 46 (tetramer), or SEQ ID NO: 47 (hexamer).

In some embodiments of the first aspect, the non-natural Ig-binding domains are directly linked to each other. In other embodiments of the first aspect, the non-natural Ig-binding domains are linked to each other via peptide linkers. In some embodiments of the first aspect, the amino acid sequences of all non-natural Ig-binding domains of the Ig binding protein are identical (for example, SEQ ID NOs: 45-47). In other embodiments of the first aspect, at least one non-natural Ig-binding domain has a different amino acid sequence than the other Ig-binding domains within the non-natural immunoglobulin-binding protein.

The dissociation constant K_D of Ig binding proteins or domains can be determined as described above and in the Examples (e.g. see Example 5 and Example 6). Typically, the dissociation constant K_D is determined at 20 °C, 25 °C or 30 °C. If not specifically indicated otherwise, the K_D values recited herein are determined at 25 °C +/- 3 °C by surface plasmon resonance. In an embodiment of the first aspect, the non-natural Ig-binding protein has a dissociation constant K_D to human IgG₁ in the range between 0.1 nM and 1000 nM, preferably between 0.1 nM and 500 nM, more preferably between 0.1 nM and 100 nM, more preferably between 0.5 nM and 100 nM, more preferably between 1 nM and 10 nM.

In an embodiment of the first aspect, the non-natural Ig-binding protein has a dissociation constant K_D to human IgG₂ in the range between 0.1 nM and 1000 nM, preferably between 0.1 nM and 500 nM, more preferably between 0.1 nM and 100 nM, more preferably between 0.5 nM and 100 nM, more preferably between 1 nM and 10 nM.

In an embodiment of the first aspect, the non-natural Ig-binding protein has a dissociation constant K_D to human IgG₄ in the range between 0.1 nM and 1000 nM, preferably between 0.1 nM and 500 nM, more preferably between 0.1 nM and 100 nM, more preferably between 0.5 nM and 100 nM, more preferably between 1 nM and 10 nM.

In a second aspect the present invention is directed to a composition comprising the non-natural Ig-binding protein of the first aspect.

In preferred embodiments of the second aspect, the composition is an affinity separation matrix, which comprises the non-natural Ig-binding protein according to any of the embodiments described above coupled to a solid support. The affinity separation matrix comprises a plurality of Ig binding proteins of the invention coupled to a solid support.

This matrix comprising the non-natural Ig binding protein of the invention is useful for separation, for example for chromatographic separation, of immunoglobulins and other Fc-containing proteins, such as immunoglobulin derivatives comprising the Fc region, fusion proteins comprising an Fc region of an immunoglobulin, and conjugates comprising an Fc region of an immunoglobulin. Solid support matrices for affinity chromatography are known in the art and include for example but are not limited to, agarose and stabilized derivatives of agarose (e.g. rPROTEIN A Sepharose Fast Flow or Mabselect[®]), controlled pore glass (e.g. ProSep[®] vA resin), monolith (e.g. CIM[®] monoliths), silica, zirconium oxide (e.g. CM Zirconia or CPG[®]), titanium oxide, or synthetic polymers (e.g. polystyrene such as Poros 50A or Poros MabCapture[®] A resin, polyvinylether, polyvinyl alcohol, polyhydroxyalkyl acrylates, polyhydroxyalkyl methacrylates, polyacrylamides, polymethacrylamides etc) and hydrogels of various compositions. In certain embodiments the support comprises a polyhydroxy polymer, such as a polysaccharide.

Examples of polysaccharides suitable for supports include but are not limited to dextran, starch, cellulose, pullulan, agar, agarose, etc, and stabilized variants of these.

The solid support formats can be of any suitable well-known kind. Such a solid support for coupling the Ig binding protein of the invention might comprise for example but is not limited to one of the following: columns, capillaries, particles, membranes, filters, monoliths, fibers, pads, gels, slides, plates, cassettes, or any other format commonly used in chromatography and known to someone skilled in the art. In one embodiment of the matrix, the carrier is comprised of substantially spherical particles, also known as beads, for example Sepharose or Agarose beads. Suitable particle sizes may be in the diameter range of 5-500 μm , such as 10-100 μm , e.g. 20-80 μm . In an alternative embodiment, the carrier is a membrane, for example a hydrogel membrane. In some embodiments, the affinity purification involves a membrane as matrix to which the non-natural Ig-binding protein of the first aspect is covalently bound.

The solid support can also be in the form of a membrane in a cartridge. In one embodiment, the solid matrix is in beaded or particle form that can be porous or non-porous. Matrices in beaded or particle form can be used as a packed bed or in a suspended form including expanded beds. In case of monoliths, packed bed and expanded beds, the separation procedure commonly follows conventional chromatography with a concentration gradient or concentration steps in the mobile phase. In case of pure suspension, batch-wise mode will be used.

In some embodiments, the affinity purification involves a chromatography column containing a solid support to which the non-natural Ig-binding protein of the first aspect is covalently bound.

The Ig binding protein of the invention may be attached to a suitable solid support via conventional coupling techniques utilising, e.g. amino-, sulfhydry-, and/or carboxy-groups present in the Ig binding protein of the invention. The coupling may be carried out via a nitrogen, oxygen, or sulphur atom of the Ig binding protein. Preferably, amino acids comprised in an N- or C-terminal peptide linker comprise said nitrogen, oxygen or sulphur atom. The Ig binding proteins may be coupled to the carrier directly or indirectly via a spacer element to provide an appropriate distance between the carrier surface and the Ig binding protein of the invention which improves the availability of the Ig binding protein and facilitates the chemical coupling of the Ig binding protein of the invention to the support. Methods for immobilisation of protein ligands to solid supports are well-known in this field and easily performed by the skilled person in this field using standard techniques and equipment.

In one embodiment, the non-natural Ig-binding protein comprises an attachment site for covalent attachment to a solid phase (matrix). Preferably, the attachment site is specific to provide a site-specific attachment to the solid phase. Specific attachment sites comprise natural amino acids, such as cysteine or lysine, or non-natural amino acids which enable specific chemical reactions with a reactive group of the solid phase, for example selected from sulfhydryl, maleimide, epoxy, or alkene groups, or a linker between the solid phase and the protein. The N-terminus can be labelled preferentially at acidic pH with amino-reactive reagents. Preferred embodiments of the invention comprise a short N- or C-terminal peptide sequence of 5 - 20 amino acids, preferably 10 amino acids, with a terminal cysteine. Amino acids for the C-terminal peptide sequence are preferably selected from proline, alanine, serine, for example, ASPAPSAPSAC (SEQ ID NO: 39).

In a third aspect the present invention is directed to the use of the non-natural Ig-binding protein of the first aspect or a composition of the second aspect for affinity purification of immunoglobulins, i.e. the Ig-binding protein of the invention is used for affinity chromatography. In some embodiments, the Ig-binding protein of the invention is immobilized onto a solid support as described in the second aspect of the invention. In one embodiment of the third aspect, the immunoglobulin to be purified is selected from the group consisting of human IgG1, human IgG2, human IgG4, human IgM, human IgA, mouse IgG1, mouse IgG2A, mouse IgG2B, mouse IgG3, rat IgG1, rat IgG2C, goat IgG1, goat IgG2, bovine IgG2, guinea pig IgG, rabbit IgG, immunoglobulin fragments comprising the Fc region, fusion proteins comprising an Fc region of an immunoglobulin, and conjugates comprising an Fc region of an immunoglobulin.

In a fourth aspect the present invention is directed to a method of affinity purification of immunoglobulins comprising the steps of (a) providing a liquid containing an immunoglobulin; (b) providing an affinity separation matrix comprising an immobilized non-natural Ig-binding protein of the first aspect; (c) contacting said liquid and said affinity separation matrix, wherein said immunoglobulin binds to said immobilized non-natural Ig-binding protein; and (d) eluting said immunoglobulin from said matrix, thereby obtaining an eluate containing said immunoglobulin; and (e) optionally further comprising one or more washing steps carried out between steps (c) and (d). Affinity separation matrixes are according to the embodiments described above and as known to someone skilled in the art.

In some embodiments of the fourth aspect, the isolation of the immunoglobulin from the matrix in step (d) is effected through a change in pH or a change in salt concentration. In some embodiments of the fourth aspect, the method comprises the further step (f) recovering said eluate.

In one embodiment of the fourth aspect, the immunoglobulin is selected from the group consisting of human IgG1, human IgG2, human IgG4, human IgM, human IgA, mouse IgG1, mouse IgG2A, mouse IgG2B, mouse IgG3, rat IgG1, rat IgG2C, goat IgG1, goat IgG2, bovine IgG2, guinea pig IgG, rabbit IgG, immunoglobulin fragments comprising the Fc region, fusion proteins comprising an Fc region of an immunoglobulin, and conjugates comprising an Fc region of an immunoglobulin.

In a fifth aspect the present invention is directed to a method of generation of a non-natural Ig-binding protein comprising at least one Ig-binding domain according to the first aspect, wherein the amino acid sequence of an Ig-binding domain is obtained by a shuffling process of amino acid sequences of at least two naturally occurring Protein A domains from naturally occurring Protein A. In more detail, the shuffling process as understood herein is an assembly process resulting in novel and artificial amino acid sequences starting from a set of non-identical known amino acid sequences comprising the following steps: (a) providing a set of sequences to be shuffled, for example sequences of five naturally occurring protein A domains E, D, A, B, and C and protein A derivatives (e.g. Z domain or other domains with at least 90 % identity to any naturally occurring domain); (b) alignment of said sequences; (c) statistical fragmentation *in silico*, and then (d) *in silico* assembly of new sequences from the various fragments to produce a mosaic product maintaining the relative order. The fragments generated in step c) can be of any length, e.g. if the fragmented parent sequence has a length of n the fragments can be of length 1 to n-1. Thus, the reassembled protein is made up from a series of fragments comprising subsequences of one or more amino acids such that these subsequences are present at the corresponding positions in one or more of the individual IgG-binding domains from protein A that have been aligned in step (b). In other words, at every amino acid position of the assembled mosaic sequence, there is at least one protein amongst the aligned IgG-binding domains from protein A that comprises the same amino acid at the corresponding position. However, the overall amino acid sequence of the reassembled protein is artificial in that it is not identical to the overall amino acid sequence of any of the IgG-binding domains from protein A. The amino acid at each position of the new sequence corresponds to the same position of any of the aligned sequences. The relative positions of the amino acids in the mosaic products are maintained with respect to the starting sequences. The general shuffling process for the generation of novel, artificial IgG-binding proteins is depicted in Figure 1A.

After this initial shuffled protein is produced, the protein can optionally be further modified by site-specific randomization of the amino acid sequence to further modify the binding properties of the shuffled protein, if desired. By way of example, the further modifications can be introduced by site-saturation mutagenesis of individual amino acid residues to produce a plurality of modified shuffled polypeptides. These IgG-binding proteins can then be screened to identify those modified shuffled polypeptides that have whatever binding properties might be of interest.

Therefore, the generation of IgG binding proteins of the invention involves one or two basic steps: a first step in which related sequences are aligned and shuffled to produce a shuffled polypeptide, and if desired, a second step to further modify the binding activity of the shuffled protein.

The Ig binding protein of the invention comprises one or more non-natural Ig-binding domains, wherein each non-natural Ig binding domain has identical amino acids as naturally occurring protein A domains A, B, C, D, or E or domain Z in positions that correspond to positions Q9, A12, F13, L17, Q26, R27, F30, I31, L34, P38, S41, L45, A48, L51, Q55, more preferably with the same amino acids that correspond to positions Q9, Q10, A12, F13, Y14, L17, P20, L22, Q26, R27, F30, I31, Q32, S33, L34, K35, D36, D37, P38, S39, S41, L45, E47, A48, K50, L51, Q55, P57 of a naturally occurring Ig binding domain.

Sequence identity of Ig binding proteins of the invention to naturally occurring protein A domains A, B, C, D, or E or domain Z is at most about 85 % (see Table 1 for more detail).

In a sixth aspect the present invention is directed to a nucleic acid molecule, preferably an isolated nucleic acid molecule, encoding a non-natural Ig-binding protein of the first aspect.

The present invention also encompasses polypeptides encoded by the nucleic acid molecules of the sixth aspect of the invention.

In a seventh aspect the present invention is directed to a vector comprising the nucleic acid molecule of the sixth aspect.

A vector means any molecule or entity (e.g., nucleic acid, plasmid, bacteriophage or virus) that can be used to transfer protein coding information into a host cell.

In one embodiment of the seventh aspect, the vector is an expression vector.

In an eighth aspect the present invention is directed to a host cell, preferably an isolated host cell, or a non-human host comprising the non-natural Ig-binding protein of the first aspect, a nucleic acid molecule of the sixth aspect, or a vector of the seventh aspect.

For example, one or more nucleic acid molecules which encode an Ig-binding protein of the invention may be expressed in a suitable host and the produced binding protein can be isolated.

A host cell is a cell that has been transformed, or is capable of being transformed, with a nucleic acid sequence and thereby expresses a gene of interest.

Suitable host cells include prokaryotes or eukaryotes. Various mammalian or insect cell culture systems can also be employed to express recombinant proteins. In accordance with the present invention, the host may be a transgenic non-human animal transfected with and/or expressing the proteins of the present invention. In a preferred embodiment, the transgenic animal is a non-human mammal.

In a ninth aspect the present invention is directed to a method for the production of a non-natural Ig-binding protein of the first aspect, comprising the step(s): (a) culturing the host cell of the seventh aspect under suitable conditions for the expression of the binding protein in order to obtain said non-natural Ig-binding protein; and (b) optionally isolating said non-natural Ig-binding protein.

The invention also encompasses a non-natural Ig-binding protein produced by the method of the ninth aspect. Suitable conditions for culturing a prokaryotic or eukaryotic host are well-known to the person skilled in the art. Ig-binding molecules of the invention may be prepared by any of the many conventional and well-known techniques such as plain organic synthetic strategies, solid phase-assisted synthesis techniques or by commercially available automated synthesizers. On the other hand, they may also be prepared by conventional recombinant techniques alone or in combination with conventional synthetic techniques. Conjugates according to the present invention may be obtained by combining compounds as known to someone skilled in the art, for example by chemical methods, e.g. lysine or cysteine-based chemistry, or by conventional recombinant techniques. The term "conjugate" as used herein relates to a molecule comprising or essentially consisting of at least a first protein attached chemically to other substances such as to a second protein or a non-proteinaceous moiety.

One embodiment of the present invention is directed to a method for the preparation of an Ig-binding protein according to the invention as detailed above, said method comprising the following steps: (a) preparing a nucleic acid encoding a binding protein as defined above; (b) introducing said nucleic acid into an expression vector; (c)

introducing said expression vector into a host cell; (d) cultivating the host cell; (e) subjecting the host cell to culturing conditions under which a an Ig-binding protein is expressed, thereby (e) producing a binding protein as described above; optionally (f) isolating the protein produced in step (e); and (g) optionally conjugating the protein to solid matrices as described above.

In a further embodiment of the present invention the production of the non-natural Ig binding protein is performed by cell-free *in vitro* transcription / translation.

Examples

The following Examples are provided for further illustration of the invention. The invention, however, is not limited thereto, and the following Examples merely show the practicability of the invention on the basis of the above description. For a complete disclosure of the invention reference is made also to the literature cited in the application which is incorporated completely into the application by reference.

Example 1. Generation of IgG binding proteins of the invention by a shuffling process

The IgG binding proteins of the invention were initially generated by a shuffling process of naturally occurring Protein A domains and protein A derivatives (e.g. Z domain or other domains with at least 90 % identity to any naturally occurring domain). The shuffling process comprised the following steps: a) providing sequences of five naturally occurring protein A domains E, B, D, A, and C, and protein A derivative domain Z; b) alignment of said sequences; c) statistical fragmentation *in silico* to identify subsequences that can be recombined with the proviso that positions Q9, Q10, A12, F13, Y14, L17, P20, L22, Q26, R27, F30, I31, Q32, S33, L34, K35, D36, D37, P38, S39, S41, L45, E47, A48, K50, L51, Q55, P57 of a naturally occurring Ig binding domain are maintained, and then d) assembly of new, artificial sequences of the various fragments to produce a mosaic product, i.e. a novel amino acid sequence.

The relative positions of the amino acids in the mosaic products were maintained with respect to the starting sequences. At least positions Q9, Q10, A12, F13, Y14, L17, P20, L22, Q26, R27, F30, I31, Q32, S33, L34, K35, D36, D37, P38, S39, S41, L45, E47, A48, K50, L51, Q55, P57 are identical between the "shuffled" sequences and all naturally occurring Protein A domains. The overall amino acid sequence of the reassembled, „shuffled“ protein is artificial in that it is not more than 85 % identical to the overall amino acid sequence of any of the naturally occurring Protein A domains or domain Z. For example, identities of Ig binding proteins compared to naturally occurring Protein A domains or domain Z are shown in Table 1.

Table 1. Identities of IgG binding proteins to naturally occurring Protein A domains.

“E, D, A, B, C, Z” refers to naturally occurring Protein A domains and to domain Z (SEQ ID NOs: 3-8). The numbers “9-30” refer to examples for Ig binding proteins of the invention. For example, the numbers “9-24” refer to corresponding SEQ ID NOs: 9-24, “25” refers to SEQ ID NO: 26, “26” refers to SEQ ID NO: 27, “27” refers to SEQ ID NO: 28, and “28” refers to SEQ ID NO: 30.

	E	D	A	B	C	Z
9	69%	64%	66%	72%	81%	74%
10	71%	64%	66%	72%	81%	74%
11	69%	64%	66%	72%	81%	74%
12	67%	67%	62%	69%	78%	71%
13	67%	67%	62%	69%	78%	71%
14	66%	69%	62%	71%	79%	72%
15	62%	69%	67%	76%	84%	76%
16	62%	67%	66%	74%	83%	74%
17	62%	67%	67%	76%	79%	76%
18	60%	66%	66%	74%	78%	74%
19	60%	66%	66%	74%	78%	74%
20	60%	66%	66%	74%	76%	74%
21	59%	64%	64%	72%	74%	72%
22	60%	64%	64%	72%	74%	72%
23	64%	67%	60%	69%	74%	71%
24	72%	69%	71%	78%	86%	79%
25	69%	71%	67%	76%	84%	78%
26	69%	71%	69%	76%	84%	78%
27	76%	74%	67%	72%	79%	74%
28	71%	74%	76%	76%	78%	79%

Genes for the “shuffled” IgG binding proteins as well as naturally occurring Protein A domains and derivatives (e.g. domain C, domain B, domain A, domain D, domain E, domain Z) were synthesized and cloned into an *E. coli* expression vector using standard methods known to a skilled person. DNA sequencing was used to verify the correct sequence of inserted fragments.

To generate Ig binding proteins comprising more than one binding domain, 2, 4, or 6 identical IgG binding domains (SEQ ID NO: 14) were genetically fused via amino acid linkers (SEQ ID NO: 41 and SEQ ID NO: 42). The amino acid sequences of the fusion proteins are shown in SEQ ID NO: 45-47.

For specific membrane attachment and purification, a short peptide linker with C-terminal Cys (ASPAPSAPSAC; SEQ ID NO: 39) and a strep-tag (WSHPQFEK; SEQ ID NO: 44) were added to the C-terminus of the Ig binding proteins (for example, see SEQ ID NO: 50 and SEQ ID NO: 51).

Example 2. Expression of IgG binding proteins

HMS174 (DE3) competent cells were transformed with either expression plasmid encoding IgG binding proteins. Cells were spread onto selective agar plates (Kanamycin) and incubated overnight at 37°C. Precultures were inoculated from single colony in 100 ml superrich medium (modified H15 medium 2% Glucose, 5% Yeast extract, 1% Casamino acids, 0.76% Glycerol, 1% Torula Yeast RNA, 250 mM MOPS, 202 mM TRIS, 10 mg/L RNase A, pH7.4, Antifoam SE15) and cultured 16 hours at 37°C at 160 rpm in a conventional orbital shaker in baffled 1 L Erlenmeyer flasks supplemented with 150 µg/ml Kanamycin without lactose and antifoam. The OD₆₀₀ readout should be in the range of 6-12. Main culture was inoculated from previous overnight culture with an adjusted start-OD₆₀₀ of 0.5 in 400 ml superrich medium in 1 L thick-walled Erlenmeyer flasks that was supplemented with glycerol, glucose, lactose, antifoam agent and 150 µg/ml Kanamycin. Cultures were transferred to a resonant acoustic mixer (RAMbio) and incubated at 37 °C with 20 x g. Aeration was facilitated by Oxy-Pump stoppers. Recombinant protein expression was induced by metabolizing glucose and subsequently allowing lactose to enter the cells. At predefined time points OD₆₀₀ was measured, samples adjusted to 5/OD₆₀₀ were withdrawn, pelleted and frozen at -20 °C. Cells were grown overnight for approx. 24 hours to reach a final OD₆₀₀ of about 45-60. To

collect biomass cells were centrifuged at 16000 x g for 10 min at 20 °C. Pellets were weighed (wet weight) and pH was measured in the supernatant. Cells were stored at -20 °C before processing.

Example 3: SDS-PAGE Analysis of expression and solubility of IgG binding proteins

Samples taken during fermentation were resuspended in 300 µl extraction buffer (PBS supplemented with 0.2 mg/ml Lysozyme, 0.5x BugBuster, 7.5 mM MgSO₄, 40 U Benzonase) and solubilized by agitation in a thermomixer at 700 rpm, rt for 15 min. Soluble proteins were separated from insoluble proteins by centrifugation (16000 x g, 2 min, rt). Supernatant was withdrawn (soluble fraction) and the pellet (insoluble fraction) was resuspended in equivalent amount of urea buffer (8 M urea, 0.2 M Tris, 2 mM EDTA, pH 8.5). From both soluble and insoluble fraction 50 µl were taken and 12 µl 5x sample buffer as well as 5 µl 0.5 M DTT were added. Samples were boiled at 95 °C for 5 min. Finally, 8 µl of those samples were applied to NuPage Novex 4-12 % Bis-Tris SDS gels which was run in accordance to the manufacturer's recommendations and stained with Coomassie. High level expression of all IgG binding proteins was found under optimized conditions within the chosen period of time (Figure 3). All expressed Ig binding proteins were soluble to more than 95 % according to SDS-PAGE.

Example 4: Purification of IgG binding Proteins

All IgG binding proteins were expressed in the soluble fraction of *E. coli* with a C-terminal StrepTagII (WSHPQFEK; SEQ ID NO: 44). The cells were lysed by sonication and the first purification step was performed with Strep-Tactin-columns according to the manufacturer's instructions. To avoid disulfide formation the buffers were supplemented with 1 mM DTT. The eluted fractions were injected to a HiLoad 16/600 Superdex 75 pg (GE Healthcare) equilibrated with 20 mM citrate pH 6.0 and 150 mM NaCl. The peak fractions were pooled and analyzed by SDS-PAGE.

Example 5. The IgG binding proteins bind to IgG with high affinities (as determined by ELISA)

The affinities of the IgG binding proteins towards IgG₁ or IgG₂ or IgG₄ were determined using an Enzyme Linked Immunosorbent Assay (ELISA). IgG₁ or IgG₂ or IgG₄ containing antibodies (e.g. Cetuximab for IgG₁, Panitumumab for IgG₂, or Natalizumab for IgG₄) were immobilized on a 96 well Nunc MaxiSorb ELISA plate (2µg/ml). After incubation for 16 h at 4 °C the wells were washed three times with PBST (PBS + 0.1 % Tween 20) and the wells were blocked with 3 % BSA in PBS (2 h at room temperature). The negative controls were wells blocked only with BSA. After blocking, the wells were washed three times with PBST and incubated for 1 h with the IgG binding protein (in PBST) at room temperature. After incubation the wells were washed three times with PBST and subsequently incubated with Strep-Tactin-HRP (1:10000) from IBA for 1 h at room temperature. Afterwards the wells were washed three times with PBST and three times with PBS. The activity of the horseradish peroxidase was visualized by adding TMB-Plus substrate. After 30 min the reaction was stopped by adding 0.2 M H₂SO₄ and the absorbance was measured at 450 nm. Results are shown in Table 2.

Table 2: Binding analysis of IgG binding proteins (binding analysis with Cetuximab; CID = clone identification number).

IgG binding protein	CID	K _D IgG ₁ (nM)	K _D IgG ₂ (nM)	K _D IgG ₄ (nM)
Domain E (SEQ ID NO: 3)	148473	13.7		
Domain D (SEQ ID NO: 4)	148474	4.8		
Domain A (SEQ ID NO: 5)	148475	4.5		
Domain B (SEQ ID NO: 6)	148476	3.1	6	3.5
Domain C (SEQ ID NO: 7)	148477	2.8		
Domain Z (SEQ ID NO: 8)	148478	3.4		
SEQ ID NO: 9	148458	7		
SEQ ID NO: 10	148459	7		
SEQ ID NO: 11	148460	8.0	10.4	7.1
SEQ ID NO: 12	148461	7.8		
SEQ ID NO: 13	148462	6.7		
SEQ ID NO: 14	148463	4.9		
SEQ ID NO: 15	148464	5.4		
SEQ ID NO: 16	148465	4.6		
SEQ ID NO: 17	148466	5.7		
SEQ ID NO: 18	148467	3.9		
SEQ ID NO: 19	148468	4.4		
SEQ ID NO: 20	148469	6.3	9.2	5.4
SEQ ID NO: 21	148470	6.3		
SEQ ID NO: 22	148471	4.6		
SEQ ID NO: 23	148472	5.9		

Example 6. The IgG binding proteins bind to IgG with high affinities (as determined with surface plasmon resonance experiments)

A CM5 sensor chip (GE Healthcare) was equilibrated with SPR running buffer. Surface-exposed carboxylic groups were activated by passing a mixture of EDC and NHS to yield reactive ester groups. 700-1500 RU *on*-ligand were immobilized on a flow cell, *off*-ligand was immobilized on another flow cell. Injection of ethanolamine

after ligand immobilization removes non-covalently bound Ig binding protein. Upon ligand binding, protein analyte was accumulated on the surface increasing the refractive index. This change in the refractive index was measured in real time and plotted as response or resonance units (RU) versus time. The analytes were applied to the chip in serial dilutions with a suitable flow rate ($\mu\text{l}/\text{min}$). After each run, the chip surface was regenerated with regeneration buffer and equilibrated with running buffer. The control samples were applied to the matrix. Regeneration and re-equilibration were performed as previously mentioned. Binding studies were carried out by the use of the Biacore® 3000 (GE Healthcare); data evaluation was operated via the BIAevaluation 3.0 software, provided by the manufacturer, by the use of the Langmuir 1:1 model ($R_I=0$). Evaluated dissociation constants (K_D) were standardized against *off*-target. Results are shown in Table 3.

Table 3: Binding analysis of IgG binding proteins (binding analysis with hIgG1-Fc; CID = clone identification number).

IgG binding protein	CID	K_D IgG ₁ -Fc (nM)
Domain Z (SEQ ID NO: 8)	148478	2.35
SEQ ID NO: 13	148462	1.2
SEQ ID NO: 14	148463	1.3
SEQ ID NO: 24	154253	1.4
SEQ ID NO: 26	154254	4.6
SEQ ID NO: 27	154255	5.3
SEQ ID NO: 28	154256	3
SEQ ID NO: 30	154257	7.6
SEQ ID NO: 45 (dimer)	150570	0.38
SEQ ID NO: 46 (tetramer)	150663	0.09
SEQ ID NO: 47 (hexamer)	150772	0.16

Example7. Binding of IgG binding proteins to a SulfoLink coupling resin and alkaline stability of immobilized IgG binding proteins

The IgG binding proteins were coupled to SulfoLink® coupling resin (Thermo; Cat. No. 20402) according to the manufacturer's instructions. The resin-bed volume of the column was 300 μl . The column was equilibrated with four resin-bed volumes of coupling buffer (50 mM Tris, 5 mM EDTA-Na, pH 8.5). The IgG binding protein was added to the column (1-2 ml / ml SulfoLink coupling resin). The IgG binding proteins were coupled to the matrix via the cysteine at the C-terminus (ASPAPSAPSAC; SEQ ID NO: 39). The column was mixed for 15 minutes, incubated another 30 minutes without mixing and washed with coupling buffer. The system flow was 0.5 ml/min. 300 μl of 50 mM cysteine solution was added to the column, mixed for 15 minutes, incubated another 30 minutes without mixing and washed with 1 M NaCl followed by washing with PBS.

Absorbance at 280 nm was measured for all fractions. All IgG binding proteins could be covalently coupled to the matrix; Figure 6 exemplarily shows the coupling of IgG binding proteins 148470 (Figure 6A) and 148460 (Figure 6B) to the SulfoLink coupling resin matrix.

Cetuximab was applied in saturated amounts (5mg; 1 mg/ml resin) to the Sulfolink resin column with covalently coupled IgG binding protein. The matrix was washed with 100 mM glycine buffer, pH 2.5 to elute Cetuximab that was bound to the immobilized IgG binding protein. The concentration of the eluted IgG was spectroscopically measured in order to determine the binding activity (static binding capacity) of the Ig binding proteins. Elution fractions were analyzed at an absorbance at 280 nm. The IgG binding activity of immobilized proteins was analyzed before and after incubation with 0.5 M NaOH for 20, 40, or 80 minutes at room temperature. The IgG binding activity of immobilized proteins before NaOH treatment was defined as 100 %. The IgG binding activity of the proteins was compared to the activity of naturally occurring domains C, B, A, D, E, or domain Z.

Figure 8 shows the IgG binding activity of IgG binding proteins 148462 (referred to as "13" in the figure), 148463 (referred to as "14" in the figure), 148472 (referred to as "23" in the figure) and of naturally occurring Protein A domains E, D, A, B, C, and of domain Z. IgG binding activity is shown after incubation with 0.5 M NaOH for 80 min. IgG binding proteins of the invention show high binding activity to Cetuximab after alkaline treatment.

Example 8. Caustic stability of IgG binding proteins coupled to an epoxy-activated matrix

Purified IgG binding proteins were coupled to epoxy-activated matrix (Sepharose 6B, GE; Cat. No. 17-0480-01) according to the manufacturer's instructions (coupling conditions: pH 9.0 overnight, blocking for 5 h with ethanolamine). Cetuximab was used as IgG sample (5mg; 1 mg/ml matrix). Cetuximab was applied in saturated amounts to the matrix comprising immobilized IgG binding protein. The matrix was washed with 100 mM glycine buffer, pH 2.5 to elute cetuximab that was bound to the immobilized IgG-binding protein. The concentration of the eluted IgG was spectroscopically measured at 280 nm in order to determine the binding activity (static binding capacity) of the Ig binding proteins. Columns were incubated with 0.5 M NaOH for 6 h at room temperature (22 °C +/- 3 °C). The IgG binding activity of the immobilized proteins was analyzed before and after incubation with 0.5 M NaOH for 6 h. The IgG binding activity of immobilized proteins before NaOH treatment was defined as 100 %.

Figure 9 shows that the activity of for example IgG binding proteins 154254 (referred to as "SEQ ID 26" in the figure), 154255 (referred to as "SEQ ID 27" in the figure), 154256 (referred to as "SEQ ID 28" in the figure), and 154257 (referred to as "SEQ ID 30" in the figure) was higher compared to the activity of IgG binding protein 148463 ("SEQ ID 14"), and thus higher than any naturally occurring Protein A domain. All immobilized IgG binding proteins showed at least 35 % up to 50 % of their original IgG binding activity after incubation for 6 h at 0.5 M NaOH. **Figure 10** shows that the Ig binding activity (capacity) of multimeric IgG binding proteins consisting of 2, 4, or 6 IgG binding domains immobilized to epoxy-activated resin after alkaline treatment for 0, 2, 4, 6 hours is comparable to the binding activity (capacity) of the monomeric IgG binding protein consisting of one IgG binding domain.

SEQUENCE LISTING FREE TEXT INFORMATION

SEQ ID NO: 1 generic sequence of non-natural Ig-binding domain
 SEQ ID NO: 2 generic sequence of non-natural Ig-binding domain
 SEQ ID NO: 3 Staphylococcus aureus domain E (CID 148473)
 SEQ ID NO: 4 Staphylococcus aureus domain D (CID 148474)
 SEQ ID NO: 5 Staphylococcus aureus domain A (CID 148475)
 SEQ ID NO: 6 Staphylococcus aureus domain B (CID 148476)
 SEQ ID NO: 7 Staphylococcus aureus domain C (CID 148477)
 SEQ ID NO: 8 domain Z of protein A (CID 148478)
 SEQ ID NO: 9 shuffle sequence IB9, CID 148458
 SEQ ID NO: 10 shuffle sequence IB10, CID 148459
 SEQ ID NO: 11 shuffle sequence IB11, CID 148460
 SEQ ID NO: 12 shuffle sequence IB12, CID 148461
 SEQ ID NO: 13 shuffle sequence IB13, CID 148462

SEQ ID NO: 14 shuffle sequence IB14, CID 148463
SEQ ID NO: 15 shuffle sequence IB15, CID 148464
SEQ ID NO: 16 shuffle sequence IB16, CID 148465
SEQ ID NO: 17 shuffle sequence IB17, CID 148466
SEQ ID NO: 18 shuffle sequence IB18, CID 148467
SEQ ID NO: 19 shuffle sequence IB19, CID 148468
SEQ ID NO: 20 shuffle sequence IB20, CID 148469
SEQ ID NO: 21 shuffle sequence IB21, CID 148470
SEQ ID NO: 22 shuffle sequence IB22, CID 148471
SEQ ID NO: 23 shuffle sequence IB23, CID 148472
SEQ ID NO: 24 shuffle sequence IB24, CID 154253
SEQ ID NO: 25 shuffle sequence IB15b
SEQ ID NO: 26 shuffle sequence IB25, CID 154254
SEQ ID NO: 27 shuffle sequence IB26, CID 154255
SEQ ID NO: 28 shuffle sequence IB27, CID 154256
SEQ ID NO: 29 shuffle sequence IB29
SEQ ID NO: 30 shuffle sequence IB28, CID 154257
SEQ ID NO: 31 linker
SEQ ID NO: 32 linker
SEQ ID NO: 33 linker
SEQ ID NO: 34 linker
SEQ ID NO: 35 linker
SEQ ID NO: 36 linker
SEQ ID NO: 37 linker
SEQ ID NO: 38 Generic sequence for non-natural Ig binding domain, for example for IB9-IB28
SEQ ID NO: 39 c-terminal coupling sequence (APS10/C)
SEQ ID NO: 40 c-terminal coupling sequence (APS30/C)
SEQ ID NO: 41 APS30 linker
SEQ ID NO: 42 APS30 linker
SEQ ID NO: 43 APS10 linker
SEQ ID NO: 44 Streptag
SEQ ID NO: 45 IB14 dimer, CID 150570
SEQ ID NO: 46 IB14 tetramer, CID 150663
SEQ ID NO: 47 IB14 hexamer, CID 150772
SEQ ID NO: 48 Generic sequence for e.g. IB24-IB28
SEQ ID NO: 49 Generic sequence for e.g. IB9-IB23
SEQ ID NO: 50 SEQ ID NO: 14 with c-terminal coupling sequence and strep-tag
SEQ ID NO: 51 SEQ ID NO: 28 with c-terminal coupling sequence and strep-tag

CLAIMS

1. A non-natural immunoglobulin (Ig) binding protein comprising one or more non-natural Ig-binding domains, wherein at least one non-natural Ig-binding domain comprises the amino acid sequence
 $X_1X_2X_3X_4X_5X_6X_7X_8QQX_{11}AFYX_{15}X_{16}LX_{18}X_{19}PX_{21}LX_{23}X_{24}X_{25}QRX_{28}X_{29}FIQSLKDDPSX_{40}SX_{42}X_{43}X_{44}LX_{46}EAX_{49}KLX_{52}X_{53}X_{54}QX_{56}PX_{58}$ (SEQ ID NO: 1), wherein
 X_1 is A, V, Q, N, or P;
 X_2 is D, A, or Q;
 X_3 is A, S, or N;
 X_4 is K, Q, or N;
 X_5 is H or F;
 X_6 is D, S, or A;
 X_7 is E or K;
 X_8 is D, E or A;
 X_{11} is S or N;
 X_{15} is E, D, or Q;
 X_{16} is V or I;
 X_{18} is H or N;
 X_{19} is L or M;
 X_{21} is N, S, or D;
 X_{23} is T or N;
 X_{24} is E or A;
 X_{25} is D or E;
 X_{28} is N, S, or A;
 X_{29} is G or A;
 X_{40} is V or Q or T;
 X_{42} is K, T, or A;
 X_{43} is E, N, or S;
 X_{44} is V, L, or I;
 X_{46} is G or A;
 X_{49} is K or Q;
 X_{52} is N, S, or D;
 X_{53} is D or E;
 X_{54} is S or A;
 X_{56} is A or P; and
 X_{58} is K or P; wherein the dissociation constant K_D of said non-natural Ig-binding protein to human IgG1 is 1 μ M or less and wherein said non-natural Ig-binding protein is stable under alkaline conditions.

2. The non-natural Ig-binding protein according to claim 1, wherein the dissociation constant is 100 nM or less.
3. The non-natural Ig-binding protein according to claim 1, or 2, wherein at least one non-natural Ig-binding domain comprises the amino acid sequence
 $X_1X_2X_3X_4X_5X_6X_7X_8QX_{11}AFYEILHLPX_{21}LTEX_{25}QRX_{28}AFIQSLKDDPSX_{40}SX_{42}X_{43}X_{44}LX_{46}EAX_{49}KLX_{52}X_{53}X_{54}QAPX_{58}$ (SEQ ID NO: 38), wherein
 X_1 is N, V, P, or A;
 X_2 is D or A;
 X_3 is A, S, or N;
 X_4 is K or Q;
 X_5 is H or F;
 X_6 is D, S, or A;
 X_7 is E or K;
 X_8 is D, E or A;
 X_{11} is S or N;
 X_{21} is N, S, or D;
 X_{25} is D or E;
 X_{28} is N, S, or A;
 X_{40} is V, T, or Q;
 X_{42} is K or A;
 X_{43} is E or S;
 X_{44} is V, L, or I;
 X_{46} is G or A;
 X_{49} is K or Q;
 X_{52} is N, S, or D;
 X_{53} is D or E;
 X_{54} is S or A; and
 X_{58} is K or P.
4. The non-natural Ig-binding protein according to claim 1 or 2, wherein said non-natural Ig-binding protein comprises the amino acid sequence
 $X_1X_2AX_4DX_7X_8QX_{11}AFYEILHLPNLTEX_{25}QRNAFIQSLKDDPSX_{40}SX_{42}X_{43}X_{44}LX_{46}EAX_{49}KLNX_{53}X_{54}QAPK$ (SEQ ID NO: 48), wherein
 X_1 is N or V;
 X_2 is D or A;
 X_4 is K or Q;
 X_5 is H or F;
 X_7 is E or K;
 X_8 is D, E or A;
 X_{11} is S or N;
 X_{25} is D or E;
 X_{40} is V or Q;

X₄₃ is E or S;

X₄₂ is K or A;

X₄₄ is V or I;

X₄₆ is G or A;

X₄₉ is K or Q;

X₅₃ is D or E; and

X₅₄ is S or A.

5. The non-natural Ig-binding protein according to claim 1 or 2, wherein said non-natural Ig-binding protein comprises the amino acid sequence

X₁X₂X₃X₄X₅X₆X₇X₈QQX₁₁AFYEILHLPX₂₁LTEDQQRX₂₈AFIQSLKDDPSX₄₀SKX₄₃X₄₄LGEAKKLX₅₂DAQAPP (SEQ ID NO: 49), wherein

X₁ is P, N, or A;

X₂ is A or D;

X₃ is A, S, or N;

X₄ is K or Q;

X₅ is H or F;

X₆ is D, S, or A;

X₇ is K or E;

X₈ is D, E or A;

X₁₁ is S or N;

X₂₁ is N, S, or D;

X₂₈ is S or A;

X₄₀ is V or T;

X₄₃ is E or S;

X₄₄ is I or L; and

X₅₂ is N, S, or D.

6. The non-natural Ig-binding protein according to any one of claims 1 to 5, wherein at least one non-natural Ig-binding domain comprises an amino acid sequence selected from the group consisting of:

NAAQHAKEQQNAFYELHLPNLTEDQRAAFIQSLKDDPSVSKEILGEAKKLNDAAQAPP (SEQ ID NO: 9),

NAAQHDKEQQNAFYELHLPNLTEDQRAAFIQSLKDDPSVSKEILGEAKKLNDAAQAPP (SEQ ID NO: 10),

NAAQHSKEQQNAFYELHLPNLTEDQRSFAFIQSLKDDPSVSKEILGEAKKLNDAAQAPP (SEQ ID NO: 11),

NAAQHSDKQQSAFYELHLPNLTEDQRSFAFIQSLKDDPSVSKEILGEAKKLNDAAQAPP (SEQ ID NO: 12),

PAAQHDKDQQSAFYELHLPNLTEDQRSFAFIQSLKDDPSVSKEILGEAKKLNDAAQAPP (SEQ ID NO: 13),

PAAKHDKDQQSAFYELHLPNLTEDQRSFAFIQSLKDDPSVSKEILGEAKKLNDAAQAPP (SEQ ID NO: 14),

ADNKFDEAQQSAFYELHLPNLTEDQRAAFIQSLKDDPSVSKEILGEAKKLNDAAQAPP (SEQ ID NO: 15),
 ADSKFDEAQQSAFYELHLPNLTEDQRAAFIQSLKDDPSVSKEILGEAKKLNDAAQAPP (SEQ ID NO: 16),
 ADSKFDEAQQSAFYELHLPNLTEDQRAAFIQSLKDDPSVSKSLLGEAKKLNDAAQAPP (SEQ ID NO: 17),
 ADSKFDEAQQSAFYELHLPDLTEDQRAAFIQSLKDDPSVSKSLLGEAKKLNDAAQAPP (SEQ ID NO: 18),
 ADSKFDEAQQSAFYELHLPSTLEDQRAAFIQSLKDDPSVSKSLLGEAKKLNDAAQAPP (SEQ ID NO: 19),
 ADSKFDEAQQSAFYELHLPSTLEDQRAAFIQSLKDDPSTSKSLLGEAKKLNDAAQAPP (SEQ ID NO: 20),
 ADSKFDEAQQSAFYELHLPSTLEDQRAAFIQSLKDDPSTSKSLLGEAKKLDDAAQAPP (SEQ ID NO: 21),
 ADSKFDEAQQSAFYELHLPSTLEDQRAAFIQSLKDDPSTSKSLLGEAKKLSDAAQAPP (SEQ ID NO: 22),
 PAAKHDKDQQSAFYELHLPSTLEDQRAAFIQSLKDDPSTSKSILGEAKKLNDAAQAPP (SEQ ID NO: 23),
 NAAQHDKDQQNAFYELHLPNLTEDQRNAFIQSLKDDPSVSKEILGEAKKLNDAAQAPK (SEQ ID NO: 24),
 ADNKFDEAQQSAFYELHLPNLTEDQRNAFIQSLKDDPSVSKEILGEAKKLNDAAQAPK (SEQ ID NO: 25),
 NAAKHDKDQQSAFYELHLPNLTEDQRNAFIQSLKDDPSVSKEILGEAKKLNDAAQAPP (SEQ ID NO: 26),
 NAAQHDKDQQSAFYELHLPNLTEEQRNAFIQSLKDDPSVSKEILAEAKKLNDAAQAPK (SEQ ID NO: 27),
 NAAKFDEAQQSAFYELHLPNLTEEQRNAFIQSLKDDPSVSKEVLGEAQLNDSQAPK (SEQ ID NO: 28),
 QQAQHDEAQQSAFYQVLHLPNLTAQRNAFIQSLKDDPSQSAEVLGEAQLNDSQAPK (SEQ ID NO: 29), and
 VDAQHDEDQQSAFYELHLPNLTEEQRNAFIQSLKDDPSQSAEILAEAKKLNESQAPK (SEQ ID NO: 30).

7. The non-natural Ig-binding protein according to any one of claims 1 to 6, wherein 2, 3, 4, 5, 6, 7, or 8 non-natural Ig-binding domains are linked to each other.
8. The non-natural Ig-binding protein according to any one of claims 1 to 7, wherein said non-natural Ig-binding protein comprises a specific attachment site for site-specific covalent attachment to a solid phase.
9. A composition comprising the non-natural Ig binding protein as defined in any one of claims 1 to 8.
10. The composition of claim 9 wherein the composition is an affinity separation matrix.

11. Use of the non-natural Ig-binding protein of any one of claims 1 to 8 or use of the composition of claim 9 for affinity purification of immunoglobulins.
12. A method of affinity purification of immunoglobulins comprising the steps of
 - a. providing a liquid containing an immunoglobulin;
 - b. providing an affinity separation matrix comprising an immobilized non-natural Ig-binding protein of any one of claims 1 to 8;
 - c. contacting said liquid and said affinity separation matrix, wherein said immunoglobulin binds to said immobilized protein; and
 - d. eluting said immunoglobulin from said matrix, thereby obtaining an eluate containing said immunoglobulin.
13. The method of affinity purification of immunoglobulins of claim 12, further comprising one or more washing steps carried out between steps (c) and (d).
14. The method of affinity purification of immunoglobulins of claim 12 or 13 wherein in step d, the immunoglobulin is eluted by a change in pH or a change in salt concentration.
15. The method according to any one of claims 12 to 14, wherein the immunoglobulin is selected from the group consisting of human IgG1, human IgG2, human IgG4, human IgM, human IgA, mouse IgG1, mouse IgG2A, mouse IgG2B, mouse IgG3, rat IgG1, rat IgG2C, goat IgG1, goat IgG2, bovine IgG2, guinea pig IgG, rabbit IgG, immunoglobulin fragments comprising the Fc region, fusion proteins comprising an Fc region of an immunoglobulin, and conjugates comprising an Fc region of an immunoglobulin.
16. A method of generation of a non-natural Ig binding protein according to any one of claims 1 to 8, wherein at least one Ig-binding domain is obtainable by a shuffling process of at least two naturally occurring Ig-binding domains from a naturally occurring Ig-binding protein.
17. A nucleic acid molecule encoding a non-natural Ig binding protein as defined in any one of claims 1 to 8.
18. A vector comprising the nucleic acid molecule of claim 17.
19. A host cell or a non-human host comprising the non-natural Ig binding protein as defined in any one of claims 1 to 8, a nucleic acid as defined in claim 17, or a vector of claim 18.
20. A method for the production of a non-natural Ig binding protein of any one of the claims 1 to 8, comprising the step(s):
 - (a) culturing the host cell of claim 19 under suitable conditions for the expression of the binding protein in order to obtain said non-natural Ig binding protein.
21. The method according to claim 20, further comprising the step of (b) isolating said non-natural Ig binding protein.