NOVEL ALKALINE STABLE IMMUNOGLOBULIN BINDING PROTEINS

FIELD OF THE INVENTION

The present invention relates to alkaline stable immunoglobulin (Ig) binding proteins comprising one or more Ig binding domains with amino acids selected from the group consisting at least of 1I, 11A, 11E, 11I, 35R, 35I, and 42L. The invention further relates to affinity matrices comprising the alkaline stable Ig binding proteins of the invention. The invention also relates to a use of these Ig binding proteins or affinity matrices 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 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 art to provide novel Ig binding proteins with improved properties that specifically bind to immunoglobulins in order to facilitate affinity purification of immunoglobulins. 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 domains cannot withstand such harsh alkaline conditions for an extended time and quickly lose binding capacity for immunoglobulin. Accordingly, there is an ongoing need in this field to obtain novel alkaline-stable proteins capable of binding immunoglobulins.

The present invention provides alkaline stable 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 alkaline stable Ig binding proteins of the invention is their improved stability at high pH compared to a parental protein.

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

SUMMARY OF THE INVENTION

A first aspect of the present invention is to provide an Ig binding protein suitable for affinity purification. This is achieved with the alkaline stable Ig binding protein comprising one or more Ig binding domains, wherein at least one Ig binding domain comprises a variant of a parental amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2 having at least 1, 2, 3, or 4 substitutions selected from the group consisting of an amino acid substitution to Isoleucine at position 1 or at a position corresponding thereto, an amino acid substitution to Alanine, Glutamic Acid, or Isoleucine at position 11 or at a position corresponding thereto, an amino acid substitution to Arginine or Isoleucine at position 35 or at a position corresponding thereto, and an amino acid substitution to Leucine at position 42 or at a position corresponding thereto. In some embodiments, the invention comprises of Ig binding protein wherein at least one Ig binding domain comprises a consensus amino acid sequence of SEQ ID NO: 52.

In a second aspect the present invention relates to an affinity separation matrix comprising the alkaline stable Ig binding protein of the first aspect.

In a third aspect the present invention relates to a use of the alkaline stable Ig binding protein of the first aspect or of the affinity separation matrix of the second aspect for affinity purification of immunoglobulins or proteins comprising an Fc part of immunoglobulins.

In a fourth aspect the present invention relates to a method of affinity purification of immunoglobulins or proteins comprising an Fc part of immunoglobulins comprising the steps of (a) providing a liquid containing an immunoglobulin; (b) providing an affinity separation matrix comprising an immobilized alkaline stable Ig binding protein of the first aspect coupled to said affinity separation matrix; (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.

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 1. Amino acid sequences of alkaline stable Ig binding domains. Positions 1, 11, 35, and 42 are shown in grey. The numbers in the top row refer to the corresponding amino acid position in the Ig binding domain.

Figure 1A. Amino acid sequences of artificial alkaline stable Ig binding domains .

Figure 1B. Consensus amino acid sequence of alkaline stable artificial Ig binding domains (SEQ ID NO: 52).

Figure 2. Analysis of the alkaline stability of point mutation variants of parental IB14. The remaining activity (in %) of Ig binding after six hours of continuous 0.5 M NaOH treatment of variants with point mutations in positions 1, 11, 35, or 42 is compared to parental IB14.

Figure 3. Analysis of the alkaline stability of different variants of parental IB14 with substitutions in positions 1, 11, and 35, and optionally in positions 28 and 42. The remaining activity (in %) of Ig binding after six hours of continuous 0.5 M NaOH treatment of combinations of substitutions in positions 1, 11, 28, 35, and/or 42 (black columns) is compared to the parental IB14 (light grey column). cs14-1 refers to SEQ ID NO: 18 (1I/11A/35R), cs14-2 refers to (1I/11A/35R/42L) SEQ ID NO: 19, cs14-3 refers to SEQ ID NO: 20 (1I/11A/28N/35R/42L).

Figure 4. Analysis of the alkaline stability of different variants of parental IB27 with combinations of 3 or 4 substitutions in positions 1, 11, and 35, and optionally 42. The remaining activity (in %) of Ig binding after six hours of continuous 0.5 M NaOH treatment of variant Ig binding protein (black columns) is compared to the parental IB27 (light grey column). cs27-1 refers to SEQ ID NO: 29 (1I/11A/35R), cs27-2 refers to SEQ ID NO: 30 (1I/11A/35R/42L).

Figure 5. Ig binding activity of Ig binding domains after alkaline treatment.
Analysis of the alkaline stability of different Ig binding domains on epoxy resin after 6h 0.5 M NaOH treatment. Shown are Ig binding domains with 1I, 11A, 35R, and 42L. Alkaline stable Ig binding domains: cs14-3 (SEQ ID NO: 20), cs74h1 (SEQ ID NO: 42), cs74h2 (SEQ ID NO: 43), cs47h3 (SEQ ID NO: 44), and cs47h4 (SEQ ID NO: 45), cs25-2 (SEQ ID NO: 26); parental domain: IB14 .

DETAILED DESCRIPTON 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 consistent with the definitions provided 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 member, integer or step or group of members, integers or steps but not the exclusion of any other member, integer or step or group of members, integers or steps.

As used in the description of the invention and the appended claims, the singular forms “a”, “an” and “the” are used interchangeably and intended to include the plural forms as well and fall within each meaning, unless the context clearly indicates otherwise. Also, as used herein, “and/or” refers to and encompasses any and all possible combinations of one or more of the listed items, as well as the lack of combinations when interpreted in the alternative (“or”).

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

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.

In the context of the present invention, the term “immunoglobulin-binding protein” or “Ig binding protein” or “immunoglobulin (Ig) binding protein” is used to describe proteins that are capable to specifically bind to the Fc region of an immunoglobulin. Due to this specific binding to the Fc region, the “Ig 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 Ig binding proteins of the invention herein exhibit specific binding to the Fc region of an immunoglobulin, it is not excluded that Ig binding proteins can additionally bind with reduced affinity to other regions, such as Fab regions of immunoglobulins.

In preferred embodiments of the present invention, the Ig binding protein comprises one or more alkaline stable Ig binding domains.

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 Ig binding protein and an immunoglobulin.

An Ig binding protein of the invention is considered to bind to an immunoglobulin, if it has a dissociation constant K_D to immunoglobulin of at least 1 µM or less, or 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.

The term “immunoglobulin” or “Ig” as used interchangeably herein, comprises proteins having a four-polypeptide chain structure consisting of two heavy chains and two light chains with the ability to specifically bind an antigen. Furthermore, also fragments or variants thereof are comprised in the term "immunoglobulin". Ig fragments as understood herein comprise fewer amino acid residues than an intact or complete Ig. The term 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) immunoglobulins.

The “immunoglobulin” as understood herein can include, but is not necessarily limited to, mammalian IgG, such as human IgG_1, human IgG_2, human IgG_4, mouse IgG_1, mouse IgG_2A, mouse IgG_2 IgG_1, rat IgG_2C, goat IgG_1, goat IgG_2, bovine IgG_2, guinea pig IgG, rabbit IgG; human IgM, human IgA; and immunoglobulin fragments comprising a Fc region, fusion proteins comprising an Fc region of an immunoglobulin, and conjugates comprising an Fc region of an immunoglobulin. Notably, naturally occurring protein A domains and artificial Ig binding proteins of the invention do not bind to human IgG_3.

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”.

The term “alkaline stable” or “alkaline stability” or “caustic stable” or “caustic stability” (abbreviated as “cs” herein) 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 solutions, 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.

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 a parental protein, i.e. do not lose the ability to bind to immunoglobulins or lose the ability to bind to immunoglobulins to a lesser extent than a parental protein.

The terms "binding activity" 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.The term “artificial” 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 (e.g. for example in a laboratory by genetic engineering, by shuffling methods, or by chemical reactions, etc.) or intentionally modified is artificial.

The term “parental” in the term "parental protein" or “parental domain” as used herein refers to an Ig binding protein that is subsequently modified to generate a variant of said parental protein or domain. Said parental protein or domain may be an artificial domain (for example, but not limited to, SEQ ID NO: 3, 4, 10, 14, 21, 25, 47, 48, 49, 50), a naturally occurring *Staphylococcus aureus* Protein A domain, or a variant or engineered version of a naturally occurring *Staphylococcus aureus* Protein A domain.

The term “variant” or “variant Ig binding domain” or “Ig binding domain variant” or “Ig binding protein variant” as used herein includes an amino acid sequence of an Ig binding protein or domain that differs from that of a parental protein or domain amino acid sequence by at least one amino acid substitution compared to the parent. Furthermore, it refers to an artificial molecule that differs from a parent molecule by one or more modifications. These modifications may be generated by genetic engineering or by chemical synthesis or chemical reactions carried out by man. For example, domain Z is a variant of naturally occurring Protein A domain B. For example, SEQ ID NO: 30 is a variant of the parental protein IB27.

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.

The term “modification” or "amino acid modification" refers to an exchange, a deletion, or an insertion of an amino acid at a particular position in a parent polypeptide sequence by another amino acid. Given the known genetic code, and recombinant and synthetic DNA techniques, the skilled scientist can readily construct DNAs encoding the amino acid variants.

The term “substitution” or "amino acid substitution" refers to an exchange of an amino acid at a particular position in a parent polypeptide sequence by another amino acid. For example, the substitution S11A refers to a variant Ig binding protein, in which the serine at position 11 is replaced by an alanine. For the preceding example, 11A refers to an alanine at position 11. For the purposes herein, multiple substitutions are typically separated by a slash. For example, A1I/S11A/K35R refers to a variant comprising the combination of substitutions A1I, S11A, and K35R.

The term “deletion” or "amino acid deletion" refers to the removal of an amino acid at a particular position in a parent polypeptide sequence.

The term “insertions” or "amino acid insertion” refers to the addition of amino acids to the parent polypeptide sequence.

Throughout this description, the amino acid residue position numbering convention of FIG. 1 is used, and the position numbers are designated as corresponding to those for example in SEQ ID NOs: 1-8.

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" or “percent identical” or "“percent 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. 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.

As used herein, the phrases “percent identical” or "percent (%) amino acid sequence identity" or “percent identity”, in the context of two polypeptide sequences, refer to two or more sequences or subsequences that have in some embodiments at least 89.5%, in some embodiments at least 91%, in some embodiments at least 93%, in some embodiments at least 94%, in some embodiments at least 96%, in in some embodiments at least 98%, and in some embodiments 100% nucleotide or amino acid residue identity, respectively, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. The percent identity exists in some embodiments over a region of at least about 50 residues, in some embodiments over a region of at least about 51 residues, in some embodiments over a region of at least about 52 residues, in some embodiments over a region of at least about 53 residues, in some embodiments over a region of at least about 54 residues, in some embodiments over a region of at least about 55 residues, in some embodiments over a region of at least about 56 residues, in some embodiments over a region of at least about 57 residues, and in some embodiments over a region of at least about 58 residues. In some embodiments, the percent identity exists over the entire length of the sequences.

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 proteins which are expressed as a single, linear polypeptide

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 an Ig binding domain with at least one further Ig binding domain, i.e. a moiety linking two protein domains to each other to generate a multimer. In preferred embodiments, the “linker” is a peptide linker, i.e. the moiety linking the two protein domains is one single amino acid or a peptide comprising two or more amino acids.

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 binding 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 specific binding to a molecule of interest (e.g., an immunoglobulin as defined above) which is to be purified or removed from a mixture.

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

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 an Immunoglobulin (Ig) binding protein, comprising one or more Ig binding domains, wherein at least one Ig binding domain comprises, essentially consists of, or consists a variant of a parental amino acid sequence having at least 1, 2, 3 or 4 substitutions selected from the group consisting of an amino acid substitution to Isoleucine at position 1, an amino acid substitution to Alanine, Glutamic Acid, or Isoleucine at position 11, an amino acid substitution to Arginine or Isoleucine at position 35, and an amino acid substitution to Leucine at position 42. In some embodiments, the said at least one variant Ig binding domain further comprises 1, 2, 3, 4, 5, or 6 modifications, wherein each individual modification is selected from the group consisting of a single amino acid substitutions, a single amino acid deletion, a single amino acid insertions.

The advantage of the variant Ig binding protein is that they are stable under alkaline conditions for an extended period of time. This feature is important for chromatography approaches with cleaning procedures using alkaline solutions with high NaOH concentrations to remove contaminants on the matrix so for example that the matrix can be used several time. The variant Ig binding proteins are more stable after alkaline treatment compared to parental polypeptides. Said substitutions at positions 1, 11, 35, and/or 42 in the parental proteins as defined above confer an improved alkali stability in comparison with the parental protein, without impairing the immunoglobulin-binding properties.

Parental SEQ ID NO: 1. In some embodiments, the Immunoglobulin binding protein is comprising one or more Ig binding domains of a variant of (i) an amino acid sequence of SEQ ID NO: 1 or of a variant of (ii) an amino acid sequence exhibiting at least 89.5 % sequence identity to the amino acid sequence of SEQ ID NO: 1. Said Ig binding domain has at least 1, 2, 3, or 4 substitutions selected from the group consisting of an amino acid substitution to Isoleucine at position 1 of SEQ ID NO: 1, an amino acid substitution to Alanine, Glutamic Acid, or Isoleucine at position 11 of SEQ ID NO: 1, an amino acid substitution to Arginine or Isoleucine at position 35 of SEQ ID NO: 1, and an amino acid substitution to Leucine at position 42 of SEQ ID NO: 1. Said variant Ig binding protein might comprise additional modifications, such as 1, 2, 3, 4, 5, or 6 substitutions or 1, 2, 3, 4, 5, 6 deletions.

The Ig binding domains shown in SEQ ID NO: 1 are parental domains; the Ig binding domains with substitutions at least in positions 1, 11, 35, and/or 42 are variants of SEQ ID NO: 1.

SEQ ID NO: 1 is a consensus sequence covering parental domains for variants of the invention, preferably (i) artificial Ig binding domains including SEQ ID NOs: 3, 4, 10, 14, 21, 25, 47, 48, 49, 50; (ii) naturally occurring Protein A domains or variants including SEQ ID NOs: 5-8. The parental protein of SEQ ID NO: 1 is the following amino acid sequence:

$X_1X_2X_3X_4X_5X_6X_7X_8QQX_{11}AFYX_{15}X_{16}LX_{18}X_{19}PX_{21}LX_{23}X_{24}X_{25}QRX_{28}X_{29}FIQSLKDDPSX_{40}SX_{42}X_{43}X_{44}LX_{46}EAX_{49}KLX_{52}X_{53}X_{54}X_{55}APX_{58}$ wherein the amino acid at position 1 (X_1) is selected from P, N, A, V, or Q, the amino acid at position 2 (X_2) is selected from A, D, or Q, the amino acid at position 3 (X_3) is selected from A, N, or S, preferably A or N, the amino acid at position 4 (X_4) X_4 is selected from K or N, preferably K, the amino acid at position 5 (X_5) is selected from H or F, the amino acid at position 6 (X_6) is selected from D, N, A, or S, preferably D or N, the amino acid at position 7 (X_7) is selected from K or E, the amino acid at position 1 (X_8) is selected from D, A, or E, the amino acid at position 11 (X_{11}) is selected from S or N, the amino acid at position 1 (X_{15}) is selected from E or Q, the amino acid at position 16 (X_{16}) is selected from I or V, the amino acid at position 18 (X_{18}) is selected from H or N, the amino acid at position 19 (X_{19}) X_{19} is selected from L or M, the amino acid at position 21 (X_{21}) is selected from N, S, or D, preferably N, the amino acid at position 23 (X_{23}) is selected from T or N, the amino acid at position 24 (X_{24}) is selected from E or A, the amino acid at position 25 (X_{25}) is selected from D or E, the amino

acid at position 28 (X_{28}) is selected from S, N, or A, preferably N or S, the amino acid at position 29 (X_{29}) is selected from A or G, the amino acid at position 40 (X_{40}) is selected from V, Q, or T, preferably V or Q, the amino acid at position 42 (X_{42}) is selected from K, A, or T, the amino acid at position 43 (X_{43}) is selected from E, N or S, preferably E or N, the amino acid at position 44 (X_{44}) is selected from I, V, or L, the amino acid at position 46 (X_{46}) is selected from G or A, the amino acid at position 49 (X_{49}) is selected from K or Q, the amino acid at position 52 (X_{52}) is selected from N, D, or S, preferably N, the amino acid at position 53 (X_{53}) is selected from D or E, the amino acid at position 54 (X_{54}) is selected from A or S, and the amino acid at position 58 (X_{58}) is selected from P or K.

Parental SEQ ID NOs: 5-8. In an embodiment of the first aspect, the parental domain comprises or essentially consists or consists of an amino acid sequence of SEQ ID NOs: 5-8, or an amino acid sequence exhibiting at least 89.5 % sequence identity to an amino acid sequence of SEQ ID NOs: 5-8. The Ig binding domain comprises of a variant having at least 1, 2, 3, or 4 amino acid substitutions selected from the group consisting of an amino acid substitution to Isoleucine at position 1, an amino acid substitution to Alanine, Glutamic Acid, or Isoleucine at position 11, an amino acid substitution at position 35 to Arginine or Isoleucine, and an amino acid substitution to Leucine at position 42. In some embodiments, the Ig binding domain further comprises 1, 2, 3, 4, 5, or 6 modifications, wherein each individual modification is selected from the group consisting of a single amino acid substitutions, a single amino acid deletion, a single amino acid insertions.

Parental SEQ ID NO: 2. In another preferred embodiment of the first aspect, wherein said at least one Ig binding domain comprises a variant of an parental amino acid sequence of SEQ ID NO: 2 wherein the variant has at least 1, 2, 3 or 4 substitutions selected from the group consisting of an amino acid substitution to Isoleucine at position 1 of SEQ ID NO: 2, an amino acid substitution to Alanine, Glutamic Acid, or Isoleucine at position 11 of SEQ ID NO: 2, an amino acid substitution at position 35 to Arginine or Isoleucine of SEQ ID NO: 2, and an amino acid substitution to Leucine at position 42 of SEQ ID NO: 2. In some embodiments, said at least one Ig binding domain further comprises 1, 2, 3, 4, 5, or 6 modifications, wherein each individual modification is selected from the group consisting of a single amino acid substitutions, a single amino acid deletion, a single amino acid insertions.

SEQ ID NO: 2 is a consensus sequence for preferred parental proteins such as but not limited to artificial Ig binding domains IB14, IB25, IB27, IB74, and IB47. Preferably, the invention relates to an Ig binding protein, wherein said at least one Ig binding domain comprises, essentially consists of, or consists of a variant of an amino acid sequence of parental SEQ ID NO: 2, or of a variant of an amino acid sequence with at least 89.5 % identity to parental SEQ ID NO: 2. SEQ ID NO: 2 is a preferred embodiment of SEQ ID NO: 1:

$X_1AAX_4X_5DX_7X_8QQX_{11}AFYEILHLPNLTEX_{25}QRX_{28}AFIQSLKDDPSVSKEX_{44}LX_{46}EAX_{49}KLNDX_{54}$

$QAPX_{58}$ wherein the amino acid at position 1 (X_1) is selected from P, N, or A, the amino acid at position 5 (X_5) is selected from H or F, the amino acid at position 7 (X_7) is selected from K or E, the amino acid at position 8 (X_8) is selected from D, A, or E, the amino acid at position 11 (X_{11}) is selected from S or N, preferably S, the amino acid at position 25 (X_{25}) is selected from D or E, the amino acid at position 28 (X_{28}) is selected from S or N, preferably N, the amino acid at position 44 (X_{44}) is selected from I or V, the amino acid at position 46 (X_{46}) is selected from G or A, the amino acid at position 49 (X_{49}) is selected from K or Q, the amino acid at position 54 (X_{54}) is selected from A or S, and the amino acid at position 58 (X_{58}) is selected from P or K.

Exemplary parental proteins. In some embodiments, said Ig binding domain comprises a variant of parental amino acid sequences selected from the group consisting of SEQ ID NO: 3, 4, 10, 14, 21, 25, 47-50, wherein the variant has at least 1, 2, 3, or 4 amino acid substitutions selected from the group consisting of an amino acid substitution of Alanine or Proline to Isoleucine at position 1, an amino acid substitution of Serine to Alanine, Glutamic Acid, or Isoleucine at position 11, an amino acid substitution of Lysine at position 35 to Arginine or Isoleucine, and an amino acid substitution of Lysine to Leucine at position 42.

IB14 as parental protein. In a preferred embodiment of the first aspect, the parental protein is the amino acid sequence of SEQ ID NO: 3, or a protein having at least 89.5 % identity to parental SEQ ID NO: 3. Examples for parental proteins with at least 89.5 % identity to SEQ ID NO: 3 may be selected from the group consisting of SEQ ID NO: 21 (1P/28N), SEQ ID NO: 10 (1A/28S), SEQ ID NO: 14 (1P/28S), SEQ ID NO: 25 (46A/58K), SEQ ID NO: 47 (5F/7E/8A), SEQ ID NO: 48 (5F/7E/8A/25E), SEQ ID NO: 49 (44V/49Q/54S/58K), SEQ ID NO: 50 (25E/44V/49Q/54S/58K), IB13 (1P/4Q/28S), IB23 (1P/21S/28A/40T/43S), IB15 (2D/3N/5F/7E/8A/28A), and IB16 (2D/3S/5F/7E/8A/28A).

IB27 as parental protein. In another preferred embodiment of the first aspect, the parental protein is SEQ ID NO: 4, or a protein having at least 89.5% identity to parental SEQ ID NO: 4. Examples for parental proteins with at least 89.5 % identity are selected from the group consisting of SEQ ID NO: 50 (5H/7K/8D), SEQ ID NO: 49 (5H/7K/8D/25D); SEQ ID NO: 48 (44I/49K/54A/58P), and SEQ ID NO: 47 (25D/44I/49K/54A/58P).

Further preferred parental domains. In an embodiment of the first aspect, the parental protein is the amino acid sequence of SEQ ID NO: 25. In another embodiment of the first aspect, the parental protein is the amino acid sequence of SEQ ID NO: 50 or SEQ ID NO: 49. In another embodiment of the first aspect, the parental protein is the amino acid sequence of SEQ ID NO: 48 or SEQ ID NO: 47.

Preferred amino acid positions in alkaline stable proteins. In some embodiment, said Ig binding domain of the variant Ig binding protein comprises a substitution or a plurality of substitutions.

The substitution in **position 1** to Isoleucine (I) may be the only substitution (for example, SEQ ID NO: 9) or the Ig binding domain may comprise further mutations, such as at least substitutions in positions 11 and/or 35 and/or 42 in parental proteins. It is preferred that the amino acid in position 1 of the alkaline stable protein is not Threonine (T). It is preferred that the amino acid in position 1 is Isoleucine (I) or Alanine (A).

The substitution in **position 11** to Alanine (A), Glutamic Acid (E), or Isoleucine (I) may be the only substitution (for example, SEQ ID NOs: 11-13) or the Ig binding domain may comprise further mutations, preferably at least substitutions in positions 1 and/or 35 and/or 42. It is preferred that the amino acid in position 11 is not Asparagine (N) or Lysine (K). It is preferred that the amino acid in position 11 is Alanine (A), Isoleucine (I), Glutamic acid (E), Histidine (H) or Proline (P), more preferred A, I, or E, most preferred A.

The substitution in **position 35** to Arginine (R) or Isoleucine (I) may be the only substitution (for example, SEQ ID NOs: 15-16) or the Ig binding domain may comprise further mutations, preferably at least substitutions in positions 1 and/or 11 and/or 42. It is preferred that the amino acid in position 35 is not Proline (P), Asparagine (N), Glycine (G), Tryptophan (W), Alanine (A), Glutamine (Q), or Methionine (M). It is preferred that the amino acid in position 35 is R or I.

The substitution in **position 42** to Leucine (L) may be the only substitution (for example, SEQ ID NO: 17) or the Ig binding domain may comprise further mutations, preferably at least substitutions in positions 1 and/or 11 and/or 35. It is preferred that the amino acid in position 42 is not Tyrosine (Y). It is preferred that the amino acid in position 42 is L.

Preferred combinations of amino acids in Ig binding domains. Surprisingly, a specific combination of amino acids in positions 1, 11, and 35, and optionally in positions 1, 11, 35, 42 and optionally in positions 1, 11, 28, 35, and 42 increase the alkaline stability of the variant Ig binding domain compared to a parental domain, as shown in the Figures and in the Examples. In addition to substitutions in positions 1, 11, 35, 42, alkaline stable Ig binding domains might comprise additional 1, 2, or 3 modifications, such as substitutions, deletions, or insertions. For example, the at least one Ig binding domain comprise a substitution or substitutions compared to parental sequences and wherein the substitution or a plurality of substitutions are at least selected from the group consisting of: 1I; 11A; 35R; 42L; 11E; 11I; 35I; 1I/11A; 1I/35R; 11A/35R; 1I/42L; 11A/42L; 1I/11E; 1I/11I; 11I/35R; 11E/35R; 11I/42L; 11E/42L; 1I/35I; 11A/35I; 11I/35I; 11E/35I; 35R/42L; 35I/42L; 1I/11A/35R; 1I/11E/35R; 1I/11I/35R; 1I/11A/42L; 1I/11E/42L; 1I/11I/42L; 1I/11A/35I; 1I/11E/35I; 1I/11I/35I; 1I/35R/42L; 1I/35I/42L; 11I/35R/42L; 11I/35I/42L; 11A/35R/42L; 11A/35I/42L; 11E/35R/42L; 11E/35I/42L; 1I/11A/35R/42L;1I/11E/35R/42L; 1I/11I/35R/42L; 1I/11A/35I/42L; 1I/11E/35I/42L;1I/11I/35I/42L; 1I/11A/28N/35R/42L; 1I/11E/28N/35R/42L; 1I/11I/28N/35R/42L; 1I/11A/28N/35I,42L; 1I/11I/28N/35I/42L; and 1I/11E/28N/35I/42L. Preferred are substitutions selected from the group consisting of 1I; 11A; 35R; 42L; 1I/11A; 1I/35R; 1I/42L; 11A/42L; 11A/35R; 35R/42L;

1I/11A/35R; 1I/11A/42L; 1I/11A/35R/42L; and 1I/11A/28N/35R/42L. In some embodiments, 3 or 4 of the amino acid positions are selected from the group consisting of 1I, 11A, 35R, and 42L. In other embodiments, Ig binding domains comprise the combination of substitutions selected from the group consisting of 1I/11A/35R; 1I/11A/35R/42L; and 1I/11A/28N/35R/42L.

In preferred embodiments, the Ig binding protein of the invention comprises or essentially consists of one or more Ig binding domains, wherein the amino acid residue at position 1 is Isoleucine, and wherein the amino acid residue at position 11 is Alanine. Another preferred Ig binding protein of the invention comprises or essentially consists of one or more Ig binding domains, wherein the amino acid residue at position 1 is Isoleucine, and wherein the amino acid residue at position 11 is Alanine, and wherein the amino acid residue at the position 35 is Arginine. Another preferred Ig binding protein of the invention comprises or essentially consists of one or more Ig binding domains, wherein the amino acid residue at position 1 is Isoleucine, and wherein the amino acid residue at position 11 is Alanine, and wherein the amino acid residue at the position 35 is Arginine, and wherein the amino acid residue at position 42 is Leucine. Another preferred Ig binding protein of the invention comprises or essentially consists of one or more Ig binding domains, wherein the amino acid residue at position 1 is Isoleucine, and wherein the amino acid residue at position 11 is Alanine, and wherein the amino acid residue at the position 35 is Arginine, and wherein the amino acid residue at position 42 is Leucine, and wherein the amino acid residue at position 28 is Asparagine. Another preferred Ig binding protein of the invention comprises or essentially consists of one or more Ig binding domains, wherein the amino acid residue at position 11 is Alanine, and wherein the amino acid residue at the position 35 is Arginine, and wherein the amino acid residue at position 42 is Leucine.

Sequences of alkaline stable proteins. An Ig binding protein of the invention is comprising one or more Ig binding domains that comprises or essentially consists or consists of the amino acid sequence of **SEQ ID NO: 52**. In some embodiments, an Ig binding domain comprises of at least 89.5 % identical amino acid sequences to SEQ ID NO: 52. SEQ ID NO: 52 is a consensus sequence for preferred artificial Ig binding proteins such as Ig binding domains of, for example, SEQ ID NO: 18-20, 26, 29-30, 42-45, 56-61. SEQ ID NO: 52 is the following amino acid sequence (see FIG. 1B):

IAAKX$_5$DX$_7$X$_8$QQAAFYEILHLPNLTEX$_{25}$QRX$_{28}$AFIQSLRDDPSVSX$_{42}$EX$_{44}$LX$_{46}$EAX$_{49}$KLNDX$_{54}$QAPX$_{58}$ wherein the amino acid at position 5 (X$_5$) is selected from H or F, the amino acid at position 7 (X$_7$) is selected from K or E, the amino acid at position 8 (X$_8$) is selected from D, A, or E, the amino acid at position 25 (X$_{25}$) is selected from D or E, the amino acid at position 28 (X$_{28}$) is selected from S or N, the amino acid at position 42 (X$_{42}$) is selected from L or K, preferably L, the amino acid at position 44 (X$_{44}$) is selected from I or V, the amino acid at position 46 (X$_{46}$) is selected from G or A, the amino acid at position 49 (X$_{49}$) is selected from K or Q, the amino acid

at position 54 (X_{54}) is selected from A or S, and the amino acid at position 58 (X_{58}) is selected from P or K.

High alkaline stability as result of the combination of 3 or 4 amino acids in positions 1, 11, 35, 42 in Ig binding proteins. In some embodiments, the combination of at least 3 or 4 amino acids selected from Isoleucine in position 1 Alanine in position 11, Arginine in position 35, and Leucine in position 42 provide surprisingly particularly good alkaline stability of the Ig binding protein, as shown in the Examples and in the Figures. It is preferred that position 28 is Asparagine. As shown in the examples below, all Ig binding proteins of the invention were found to bind to Ig even after alkaline treatment. The Ig binding protein of the invention exhibits an high alkaline stability for at least 6 h in 0.5 M NaOH, in particular an improved alkaline stability as compared to a corresponding parental protein.

It was surprising and unexpected that the Ig binding proteins with the combination of at least 3 or 4 amino acids at amino acid position 1 to Isoleucine, at amino acid position 11 to Alanine, Glutamic Acid, or Isoleucine, at amino acid position 35 to Arginine or Isoleucine, and optionally at amino acid position 42 to Leucine are able to bind to Ig even after alkaline treatment for several hours. It was most surprising and unexpected that the Ig binding proteins comprising a combination of amino acids 1I, 11A or 11E or 11I, 35R or 35I, and optionally 42L, preferably 1I, 11A, 35R, and 42L are able to bind to Ig even after alkaline treatment for several hours. The alkaline stability of the Ig binding protein is determined by comparing the loss in Ig binding activity after 6 h incubation in 0.5 M NaOH. In some embodiments, this is compared to the loss in Ig-binding activity of the corresponding parental protein. The loss of binding activity is determined by comparing binding activity before and after 0.5 M NaOH incubation for 6 hours. As shown by the comparative data in the Figures, the Ig binding activity of the Ig binding domains with at least 3 or 4 amino acids selected from 1I, 11A or 11E or 11I, 35R or 35I, and 42L is increased by at least 25 % compared to a parental protein. This is a surprising and advantageous property as compared to parental proteins.

Preferred alkaline stable Ig binding proteins. In specific embodiments, the Ig binding domain comprises of an amino acid sequence selected from the group consisting of SEQ ID NOs: 18-20, 26, 29-40, 42-45, and 56-61. In some embodiments, the domain comprises amino acid sequences of the group consisting of SEQ ID NOs: 20, 26, 30, 42-45. The alkaline stable domains might comprise further modifications, such as insertions, deletions, or further substitutions. In some embodiments, Ig binding domains have 1, 2, 3, 4, 5, or 6 further substitutions. In other embodiments, Ig binding domains have a deletion of 1, 2, 3, or 4 amino acids within the first 4 amino acids of its N-terminus and/or a deletion of 1 or 2 amino acids at the C-terminus. In some embodiments, Ig binding domains have deletions at the N-terminus, for example in positions 1, 2, and 4, or in positions 1, 2, and 3. In some embodiments, Ig binding domains have deletions at the C-terminus, for example in positions 57 and/or 58. Some

embodiments relate to sequences with at least 89.5 % sequence identity to an amino acid selected from the group consisting of SEQ ID NOs: 20, 26, 30, 42-45, for example but not limited to SEQ ID NOs: 9-19, 29, 53-54, 56-61. Some embodiments relate to amino acid sequences with at least 89.5 % sequence identity to the amino acid sequence to any of the afore-mentioned SEQ ID NOs, wherein the amino acid sequence with at least 89.5 % sequence identity to any of the afore-mentioned SEQ ID NOs has the same amino acids in at least 3 or 4 of positions 1, 11, 35 and 42 or positions corresponding to the respective amino acid positions in the sequences SEQ ID NOs: 20, 26, 29, 30, 42-45, from which said amino acid sequence with at least 89.5 % sequence identity was derived. The sequences for preferred alkaline stable Ig binding proteins are shown in Figure 1. It is preferred that at least 3 or 4 of positions 1I, 11A, 35R, and 42L are conserved. It is further preferred that position 4 is not Q.

SEQ ID NO: 20 (cs14) and variants. In a specific embodiment, the alkaline stable Ig binding domain comprises, or essentially consists, or consists of an amino acid sequence of SEQ ID NO: 20 or an amino acid sequence at least 91 % identical thereto, for example SEQ ID NOs: 18-19, 26, 42-45, 56. In preferred embodiments, variants of SEQ ID NO: 20 do not have a Q in position 4. It is further preferred that position 4 is K. In a specific embodiment, the Ig binding domain comprises an amino acid sequence of SEQ ID NO: 20 or an amino acid sequence at least 96 % identical thereto. For example, Figure 3 and Figure 5 show the remaining activity of Ig binding after prolonged continuous 0.5 M NaOH treatment.

Table 1 illustrates the amino acid differences of SEQ ID NO: 20 and preferred variants at least 91 % identical thereto. It is preferred that position 5 is H or F, position 7 is K or E, position 8 is D or A, position 25 is D or E, position 44 is I or V, position 46 is G or A, position 49 is K or Q, position 54 is A or S, and position 58 is P or K. It is further preferred that position 4 is K. The identity of the artificial alkaline stable SEQ ID NO: 20 to any wildtype Protein A domain is below 78 %.

Table 1. Amino acid differences of cs14 and variants with at least 91 % sequence identity

SEQ NO:	Ig binding protein	Pos. 5	Pos. 7	Pos. 8	Pos. 25	Pos. 44	Pos. 46	Pos. 49	Pos. 54	Pos. 58	differences	identity (%)
20	cs14	H	K	D	D	I	G	K	A	P	0	100
26	cs25	H	K	D	D	I	A	K	A	K	2	96,5
42	cs74h1	F	E	A	D	I	G	K	A	P	3	94,8
43	cs74h2	F	E	A	E	I	G	K	A	P	4	93,1
44	cs47h3	H	K	D	D	V	G	Q	S	K	4	93,1
45	cs47h4	H	K	D	E	V	G	Q	S	K	5	91,3

SEQ ID NO: 30 (cs27) and variants. In a specific embodiment, the Ig binding domain comprises or essentially consists or consists of an amino acid sequence of SEQ ID NO: 30, or an amino acid sequence at least 94 % identical thereto, for example SEQ ID NO: 29. The

identity of SEQ ID NO: 30 to any wildtype Protein A domain is below 76 %. Figure 4 shows the remaining activity SEQ ID NOs: 30 and 29 after six hours of continuous 0.5 M NaOH treatment.

SEQ ID NO: 26 (cs25) and variants. In a specific embodiment, the Ig binding domain comprises or essentially consists or consists of an amino acid sequence of SEQ ID NO: 26 or an amino acid sequence at least 98 % identical thereto. The identity of SEQ ID NO: 26 to any wildtype Protein A domain is below 81 %. Figure 5 shows the remaining Ig binding activity of SEQ ID NO: 26 after six hours of continuous 0.5 M NaOH treatment.

SEQ ID NO: 42 (cs74) and variants. In an specific embodiment, the alkaline stable Ig binding domain comprises or essentially consists or consists of an amino acid sequence of SEQ ID NO: 42 and an amino acid sequence at least 98 % identical thereto, for example SEQ ID NO: 43. The identity of SEQ ID NO: 42 to any wildtype Protein A domain is below 78 %. Figure 5 shows the remaining Ig binding of SEQ ID NOs: 42-43 after six hours of continuous 0.5 M NaOH treatment.

SEQ ID NO: 44 (cs47) and variants thereof. In an specific embodiment, the alkaline stable Ig binding domain comprises or essentially consists of an amino acid sequence of SEQ ID NO: 44 and an amino acid sequence at least 98 % identical thereto, for example SEQ ID NO: 45. The identity of SEQ ID NO: 44 to any wildtype Protein A domain is below 78 %. Figure 5 shows the remaining activity of Ig binding after six hours of continuous 0.5 M NaOH treatment of SEQ ID NOs: 44-45 with at least 98 % identity.

Affinity to Immunoglobulin. All Ig binding proteins of the invention bind to Immunoglobulin with a dissociation constant K_D preferably below 1 μM, or below 100 nM, even more preferably 10 nM or less. Methods for determining binding affinities of Ig binding proteins or domains, 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, isothermal titration calorimetry (ITC), analytical ultracentrifugation, radioimmunoassay (RIA or IRMA) and enhanced chemiluminescence (ECL). Some of the methods are described further in the Examples. 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 22 °C +/- 3 °C by surface plasmon resonance. In an embodiment of the first aspect, the Ig binding protein has a dissociation constant K_D to human IgG_1 in the range between 0.1 nM and 100 nM, preferably between 0.1 nM and 10 nM.

Multimers. In one embodiment of the invention, the Ig binding protein comprises 1, 2, 3, 4, 5, 6, 7, or 8, preferably 2, 3, 4, 5, or 6, Ig binding domains linked to each other, i.e. the Ig binding protein can be, for example, a monomer, a dimer, a trimer, a tetramer, a pentamer, or a hexamer. A multimer may comprise two, three, four, or even more binding domains.

Multimers of the invention are fusion proteins generated artificially, generally 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.

In some preferred embodiments, the multimer is a homo-multimer, e.g. the amino acid sequences of all alkaline stable Ig binding domains of the Ig binding protein are identical.

An alkali-stable multimer may comprise two or more Ig binding domains, wherein said Ig binding domains preferably comprise or essentially consist of a sequence selected from the group consisting of SEQ ID NOs: 18-20, 26, 29-38, 42-45, 56-61 or a sequence with at least 89.5 % sequence identity to any of the afore-mentioned SEQ ID NOs. In some embodiments, the domains are derivatives of SEQ ID NOs: 18-20, 26, 29-38, 42-45, 56-61 and further wherein each derivative has a deletion of 1, 2, or 3 amino acids within the first 4 amino acids of its N-terminus and/or a deletion of 1 or 2 amino acids at the C-terminus relative to the one of SEQ ID NOs:18-20, 26, 29-38, 42-45, 56-61 upon which it is based (see, for example, SEQ ID NOs: 23, 24, 27).

For example, SEQ ID NO: 14 and SEQ ID NO: 20 were used to generate the homo-multimeric fusion constructs (dimers, tetramers, pentamers, and hexamers) described herein in Example 1. In addition, dimers, tetramers, pentamers, and hexamers of SEQ ID NO: 30 were generated. See for example SEQ ID NO: 23, 24, 27, 28.

In some embodiments of the first aspect, the multimer is a hetero-multimer, e.g. at least one alkaline stable Ig binding domain has a different amino acid sequence than the other Ig binding domains within the immunoglobulin-binding protein.

Linker. In some embodiments of the first aspect, the one or more Ig binding domains are directly linked to each other. In other embodiments, the one or more Ig binding domains are linked to each other with one or more linkers. Preferred in these typical embodiments are peptide linkers. This means that the peptide linker is an amino acid sequence that connects a first Ig binding domain with a second Ig binding domain. The peptide linker is connected to the first Ig binding domain and to the second Ig binding domain by a peptide bond between the C-terminal and N-terminal ends of the domains, thereby generating a single, linear polypeptide chain. The length and composition of a linker may vary between at least one and up to about 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, 30 amino acids. It is preferred that the amino acid sequence of the peptide linker is stable against caustic conditions and proteases. Linkers should not destabilize the conformation of the domains in the Ig binding protein. Well-known are linkers comprising 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). Also preferred are linkers that comprise further amino acids. Other embodiments of the invention comprise linkers consisting of alanine, proline, and serine. Other linkers for the fusion of proteins are known in the art and can be used.

Conjugation to a solid support. In some embodiments of the invention, the Ig binding protein is conjugated to a solid support. In some embodiment of the invention, the Ig binding domain comprises an attachment site for site-specific covalent coupling of the Ig binding protein to a solid support. In some embodiments of the invention, the Ig binding protein may also comprise additional amino acid residues at the N- and/or C-terminal end, such as for example a leader sequence at the N-terminal end and/or a coupling sequence with or without a tag at the N- or C-terminal end (see for example SEQ ID NOs: 39 and 40). In some embodiments, the alkaline stable 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, which enable specific chemical reactions with a reactive group of the solid phase or a linker between the solid phase and the protein, for example selected from N-hydroxysuccinimide, iodacetamide, maleimide, epoxy, or alkene groups. The attachment site may be directly at the C- or N-terminal end of the Ig binding protein or there may be a linker between the N- or C-terminus and the coupling site, preferably a peptide linker. In some embodiments of the invention, the Ig binding protein may comprise a short N- or C-terminal peptide sequence of 3 - 20 amino acids, preferably 4 - 10 amino acids, with a terminal cysteine. Amino acids for a C-terminal attachment site may be preferably selected from proline, alanine, and serine, for example, ASPAPSAPSAC (SEQ ID NO: 41), with a single cysteine at the C-terminal end for coupling. In another embodiment, amino acids for a C-terminal attachment site may be preferably selected from glycine and serine, for example, GGGSC, with a single cysteine at the C-terminal end for coupling.

An advantage of having a C-terminal cysteine is that coupling of the Ig binding protein can be achieved through reaction of the cysteine thiol with an electrophilic group on a support resulting in a thioether bridge coupling. This provides excellent mobility of the coupled protein which provides increased binding capacity.

In alternative embodiments, the coupling of the Ig binding protein to a solid support can be achieved through a cysteine in positions 43, 46, or 47 of an alkaline stable Ig binding domain. If a cysteine is located in positions 43, 46, or 47, the amino acid in position 50 or position 58 is not cysteine (see for example SEQ ID NOs: 53 or 54). Preferably the amino acid in position 50 is Lysine and the amino acid in position 58 is selected from Proline or Lysine.

Affinity separation matrix. In another aspect the present invention is directed to an affinity separation matrix, comprising an Ig binding protein of the first aspect.

In preferred embodiments of the second aspect, the affinity separation matrix is a solid support. The affinity separation matrix comprises at least one Ig binding protein of the invention. This matrix comprising the alkaline stable Ig binding protein of the invention is useful for separation, for example for chromatographic separation, of immunoglobulins as defined above, i.e. Ig, Ig variants comprising the Fc region, fusion proteins comprising an Fc region of an Ig, and conjugates comprising an Fc region of an Ig. An affinity matrix is useful for separation of immunoglobulins and should retain the Ig binding property even after highly alkaline conditions as applied during cleaning processes. Such cleaning of matrices is essential for long-term repeated use of matrices.

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. Sepharose 6B, Praesto™Pure; CaptivA®, rPROTEIN A Sepharose Fast Flow, Mabselect®, and other), cellulose or derivatives of cellulose, 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 agar, agarose, dextran, starch, cellulose, pullulan, etc, and stabilized variants of these.

The formats for solid support matrices can be of any suitable well-known kind. Such solid support matrix for coupling the Ig binding protein of the invention might comprise for example, 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, the matrix 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. Matrices in particle form can be used as a packed bed or in a suspended form including expanded beds.

In an alternative embodiment, the solid support matrix is a membrane, for example a hydrogel membrane. In some embodiments, the affinity purification involves a membrane as matrix to which the alkaline stable 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 some embodiments, the affinity purification involves a chromatography column containing a solid support matrix to which the alkaline stable Ig binding protein of the first aspect is covalently bound.

The alkaline stable Ig binding protein of the invention may be attached to a suitable solid support matrix via conventional coupling techniques utilising, e.g. amino-, sulfhydroxy-, 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 support matrix directly or indirectly via a spacer element to provide an appropriate distance between the matrix 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 immobilization 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. Depending on the Ig binding protein and on the specific conditions, the coupling may be a multipoint coupling, for example via several lysines, or a single point coupling, for example via cysteine.

Use of the alkaline stable Ig binding protein. In a third aspect the present invention is directed to the use of the alkaline stable Ig binding protein of the first aspect or an affinity matrix of the second aspect for affinity purification of immunoglobulins or variants thereof, 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.

Method of affinity purification of immunoglobulins. In a fourth aspect the present invention is directed to a method of affinity purification of immunoglobulins, the method comprising (a) providing a liquid containing an immunoglobulin; (b) providing an affinity separation matrix comprising an immobilized alkaline stable Ig binding protein of the first aspect coupled to said affinity separation matrix; (c) contacting said liquid with 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.

In some embodiments, the method of affinity purification may further comprising one or more washing steps carried out between steps (c) and (d) under conditions sufficient to remove from the affinity separation matrix some or all molecules that are non-specifically bound thereto. Non-specifically bound means any binding that does not involve an interaction between the at least one binding domain of the presently disclosed subject matter and an Immunoglobulin.

Affinity separation matrixes suitable for the disclosed uses and methods are those matrixes according to the embodiments described above and as known to someone skilled in the art.

In some embodiments of the fourth aspect, the elution of the immunoglobulin from the matrix in step (d) is effected through a change in pH and/or a change in salt concentration. Any suitable

solution used for elution from Protein A media can be used, for example by a solution with pH 5 or lower, or by a solution with pH 11 or higher.

In some embodiments, a further step (f) for efficient cleaning the affinity matrix is added, preferably by using an alkaline liquid, for example, with pH of 13 - 14. In certain embodiments, the cleaning liquid comprises 0.1 - 1.0 M NaOH or KOH, preferably 0.25 - 0.5 M NaOH or KOH. Due to the high alkaline stability of the Ig binding proteins of the invention, such strong alkaline solution can be used for cleaning purposes.

In some embodiments, the affinity matrix can be re-used at least 10 times, at least 20 times, at least 30 times, at least 40 times, at least 50 times, at least 60 times, at least 70 times, at least 80 times, at least 90 times, or at least 100 times, due to a repetition of steps (a) to (e), optionally (a) to (f) can be repeated at least 10 times, , at least 20 times, at least 30 times, at least 40 times, at least 50 times, at least 60 times, at least 70 times, at least 80 times, at least 90 times, or at least 100 times.

In general, suitable conditions for performing the method of affinity purification are well known to someone skilled in the art and in particular to someone skilled in Protein A chromatography.

Nucleic acid molecule. In a fifth aspect, the present invention is directed to a nucleic acid molecule, preferably an isolated nucleic acid molecule, encoding an alkaline stable Ig binding protein of any embodiment disclosed above. In one embodiment, the present invention is directed to a vector comprising the nucleic acid molecule. 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, the vector is an expression vector.

In a sixth aspect, the present invention is directed to an expression system which comprises a nucleic acid or a vector as disclosed above, for example a prokaryotic host cell, for example *E. coli*, or a eukaryotic host, for example yeast *Saccharomyces cerevisiae* or *Pichia pastoris* or mammalian cells such as CHO cells.

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

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.

One embodiment of the present invention is directed to a method for the preparation of an alkaline-stable Ig binding protein according to the invention as detailed above, said method comprising the following steps: (a) preparing a nucleic acid encoding an Ig 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 an Ig binding protein is expressed, thereby (e) producing an Ig 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 alkaline stable 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.

Example 1. Generation of parental proteins by shuffling

Parental proteins (e.g. SEQ ID NOs: 3, 4, 10, 14, 21, 22, 25, 47-50) were initially generated by a shuffling process of naturally occurring Protein A domains and Protein A domain variants (e.g. Z domain or other domains with at least 89.5 % identity to any naturally occurring domain, e.g. Z/2 domain). In more detail, the shuffling process as understood herein is an assembly process resulting in artificial amino acid sequences starting from a set of non-identical known amino acid sequences. 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 variant domain Z or Z/2; b) alignment of said sequences; c) statistical fragmentation *in silico* to identify subsequences that were recombined, 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 fragments generated in step c) were of any length, e.g. if the fragmented parent sequence had a length of n, the fragments was of length 1 to n-1.

The relative positions of the amino acids in the mosaic products were maintained with respect to the starting amino acid sequences. At least 90 % of 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, A56, P57 are identical between the artificial amino acid sequences of for example IB14, IB25, IB74h1, IB74h2, IB47h3, IB47h4, or/and IB27, and naturally occurring Protein A domains or Protein A domain variants. The overall amino acid sequence of the Ig binding proteins IB14, IB25, IB74h1, IB74h2, IB47h3, IB48h4, and IB27 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. After the initial artificial Ig binding proteins was generated, the protein was further modified by site-specific randomization of the amino acid sequence to further modify the binding properties. The further modifications were introduced by site-saturation mutagenesis of individual amino acid residues.

Genes for the Ig binding proteins IB14, IB25, IB47, IB74 or/and IB27 as well as SEQ ID NOs: 5-8 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 multimeric Ig binding proteins, 2, 3, 4, 5, or 6 identical Ig binding domains (for example, of SEQ ID NOs: 14, 20, 30) were genetically fused via amino acid linkers.

For specific membrane attachment and purification, a short peptide linker with C-terminal Cys (ASPAPSAPSAC; SEQ ID NO: 41) and optionally a strep-tag (WSHPQFEK; SEQ ID NO: 46) were added to the C-terminus of the Ig binding proteins (for example, see SEQ ID NOs: 39-40). In other embodiments, for specific membrane attachment and purification, positions 43, 46, or 47 were substituted with a cysteine (see for example, SEQ ID NOs: 53-54).

Example 2. Mutagenesis to generate variants

For site-directed mutagenesis, the Q5® site-directed Mutagenesis Kit (NEB; Cat. No. E0554S) was used according to the manufacturer's instructions. PCRs were carried out with oligonucleotides coding for each specific substitution respectively and a plasmid containing SEQ ID NO: 14 as template. Products were ligated and transformed into *E. coli* XL2-blue cells (Stratagene) via electroporation. Single colonies were isolated and DNA sequencing was used for insert containing clones to verify the correct sequences. Results are shown in FIG. 2.

A combination of several point mutations was generated by GeneArt™ Strings™ synthesis (Thermo Fisher Scientific). The Strings DNA fragments corresponded to a purified PCR product and were cloned into a derivate of a pET28a vector. Ligation products were transformed into *E. coli* XL2-blue cells via electroporation. Single colonies were screened by PCR to identify constructs containing inserts of the right size. DNA sequencing was used to verify the correct sequences. Variants with point mutations are shown for example in SEQ ID NO: 9-13, 15-17, and 21.

Example 3. Expression of Ig binding proteins

BL21 (DE3) competent cells were transformed with an expression plasmid encoding Ig 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 2xYT medium and cultured for 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 OD_{600} readout

should be in the range of 6-12. Main culture was inoculated from previous overnight culture with an adjusted start-OD_{600} of 0.5 in 400 ml superrich medium (modified H15 medium 2% Glucose, 5% Yeast extract, 0.89% Glycerol, 0,76% Lactose, 250 mM MOPS, 202 mM TRIS, pH 7.4, Antifoam SE15) in 1 L thick-walled Erlenmeyer flasks that was supplemented with 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 OD_{600} was measured, samples adjusted to $5/OD_{600}$ were withdrawn, pelleted and frozen at -20 °C. Cells were grown overnight for approx. 24 hours to reach a final OD_{600} 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 4: SDS-PAGE Analysis of expression and solubility of Ig 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 $MgSO_4$, 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). 50 µl were taken both from the soluble and insoluble fraction, 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 were run in accordance to the manufacturer's recommendations and stained with Coomassie. High level expression of all Ig binding proteins was found under optimized conditions within the chosen period of time (data not shown). All expressed Ig binding proteins were soluble to more than 95 % according to SDS-PAGE.

Example 5: Purification of Ig binding Proteins

Ig binding proteins were expressed in the soluble fraction of *E. coli* with a C-terminal StrepTagII (WSHPQFEK; SEQ ID NO: 46). The cells were lysed by two freeze/thaw cycles and the purification step was performed with Strep-Tactin®-resin according to the manufacturer's instructions (IBA, Goettingen, Germany). To avoid disulfide formation the buffers were supplemented with 1 mM DTT.

Alternatively, Ig binding proteins were expressed in the soluble fraction of *E. coli* with a C-terminal StrepTagII (SEQ ID NO: 46). The cells were resuspended in cell disruption buffer and lysed by a constant cell disruption system (Unit F8B, Holly Farm Business Park) at 1 kbar for two cycles. Purification step was performed with Strep-Tactin-resin (IBA, Goettingen, Germany)

and additional gel filtration (Superdex 75 16/60; GE Healthcare) using an ÄKTAxpress system (Ge Healthcare) according to the manufacturer's instructions. To avoid disulfide formation buffers for Strep-Tactin-purification were supplemented with 1 mM DTT and citrate-buffer (20 mM Citrat, 150 mM NaCl, pH 6,0) was used as running buffer for gel filtration.

Example 6. The Ig binding proteins bind to IgG with high affinities (as determined by ELISA)

The affinities of the Ig binding proteins towards IgG_1 or IgG_2 or IgG_4 were determined using an Enzyme Linked Immunosorbent Assay (ELISA). IgG_1 or IgG_2 or IgG_4 containing antibodies (e.g. Cetuximab for IgG_1, Panitumumab for IgG_2, or Natalizumab for IgG_4) 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 Ig 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) (IBA, Goettingen, Germany) 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 H_2SO_4 and the absorbance was measured at 450 nm. As determined via ELISA, the K_D for human IgG_1 is 4.9 nM for SEQ ID NO: 14; 3.4 nM for domain Z; 3.1 nM for domain B; and 2.8 nM for domain C.

Example 7. The Ig 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 (µl/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) at 25 °C; data evaluation was operated via the BIAevaluation 3.0 software, provided by the

manufacturer, by the use of the Langmuir 1:1 model (RI=0). Evaluated dissociation constants (K_D) were standardized against *off*-target and K_D values of different artificial alkaline stable Ig binding proteins for human IgG_1-Fc, Cetuximab (IgG_1), Natalizumab (IgG_4), or Panitumomab (IgG_2) are shown in Table 2.

Table 2. K_D values of Ig binding proteins for Ig

SEQ ID NO:	Ig binding protein	IgG1 (nM)	IgG4 (nM)	IgG2 (nM)
20	cs14	2,9	2,51	7,42
30	cs27	3,64	2,54	21,6
26	cs25	4,24	3,27	11,6
45	cs47h4	4,1	3,11	25,2
44	cs47h3	4,78	4,05	20,3
43	cs74h2	3,48	2,72	17,2
42	cs74h1	1,64	1,2	12,8

Example 8. Alkaline stability of Ig binding proteins coupled to an epoxy-activated matrix

Purified Ig 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 Ig 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 measured by BLI (quantification with Protein A Octet-sensors and Cetuximab as standard) in order to determine the binding activity of the Ig binding proteins. Columns were incubated with 0.5 M NaOH for 6 h at room temperature (22 °C +/- 3 °C). The Ig binding activity of the immobilized proteins was analyzed before and after incubation with 0.5 M NaOH for 6 h. The Ig binding activity of immobilized proteins before NaOH treatment was defined as 100 %.

Figure 2 shows the analysis of the alkaline stability of point mutation variants of IB14 (SEQ ID NO: 14). The remaining activity (in %) of Ig binding after six hours of continuous 0.5 M NaOH treatment of point mutations in positions 1, 11, 35, and 42 of IB14 is compared to parental IB14. Substitutions P1I, S11E, S11I, S11A, K35I, K35R, or K42L improve the Ig binding activity by at least about 25 %.

Figure 3 shows that the activity of for example variant proteins with combinations of 3, 4, or 5 substitutions in positions 1, 11, 28, 35, and/or 42 was higher compared to the activity of the parental protein IB14 (SEQ ID NO: 14). Ig binding protein cs14-1 (SEQ ID NO: 18) showed about at least 50 %, cs14-2 (SEQ ID NO: 19) showed about at least 70 %, cs14-3 (SEQ ID NO:

20) showed about at least 80 % higher Ig binding activity compared to the IB14 after incubation for 6 h at 0.5 M NaOH.

Figure 4 shows that the activity of variant Ig binding proteins with combinations of 3, 4, or 5 substitutions in positions 1, 11, 35, and/or 42 was higher compared to the activity of the parental protein IB27. cs27-1 (SEQ ID NO: 29) showed about at least 30 % and cs27-2 (SEQ ID NO: 30) showed about at least 40 % higher Ig binding activity compared to the parental protein after incubation for 6 h at 0.5 M NaOH.

Figure 5 shows that the activity of variant Ig binding proteins with combinations of 1I, 11A, 35R, and 42L was higher compared to the activity of the parental protein, here shown as IB14 (other parental proteins are comparable to IB14; data not shown). Ig binding proteins cs74h1 (SEQ ID NO: 42), cs74h2 (SEQ ID NO: 43), cs47h3 (SEQ ID NO: 44), cs47h4 (SEQ ID NO: 45), and cs25 (SEQ ID NO: 26) showed significantly higher Ig binding activity compared to IB14 after incubation for 6 h at 0.5 M NaOH.