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Applications for an engineered Protein-G variant with a pH controllable affinity to antibody fragments

Lucas J. Bailey^a, Kimberly M. Sheehy^a, Robert J. Hoey^a, Zachary P. Schaefer^a, Marcin Ura^a, and Anthony A. Kossiakoff^{a,*}

^aDepartment of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL, 60637, USA

Abstract

Immunoglobulin binding proteins (IBPs) are broadly used as reagents for the purification and detection of antibodies. Among the IBPs, the most widely used are Protein-A and Protein-G. The C2 domain of Protein-G from *Streptococcus* is a multi-specific protein domain; it possesses a high affinity ($K_D \sim 10$ nM) for the Fc region of the IgG, but a much lower affinity ($K_D \sim$ low μ M) for the constant domain of the antibody fragment (Fab), which limits some of its applications. Here, we describe the engineering of the Protein-G interface using phage display to create Protein-G-A1, a variant with 8 point mutations and an approximately 100-fold improved affinity over the parent domain for the 4D5 Fab scaffold. Protein-G-A1 is capable of robust binding to Fab fragments for numerous applications. Furthermore, we isolated a variant with pH-dependent affinity, demonstrating a 10,000-fold change in affinity from pH 7 to 4. Additional rational mutagenesis endowed Protein-G with significantly enhanced stability in basic conditions relative to the parent domain while maintaining high affinity to the Fab. This property is particularly useful to regenerate Protein-G affinity columns. Lastly, the affinity-matured Protein-G-A1 variant was tethered together to produce dimers capable of providing multivalent affinity enhancement to a low affinity antibody fragment-antigen interaction. Engineered Protein-G variants should find widespread application in the use of Fab-based affinity reagents.

Keywords

immunoglobulin binding protein; antibody fragment purification; Protein-G; affinity reagent; affinity maturation; phage display

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*Corresponding author. Tel: (773) 702-9257, Address: Room 3254 Knapp Center for Biological Discovery, 929 East 57th Street, Chicago, IL 60637, koss@bsd.uchicago.edu.

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1. Introduction

Since their discovery, immunoglobulin binding proteins (IBPs) have been critical to the advancement of antibodies in research, diagnostics and therapeutics. Protein-G is one of a number of convergently evolved bacterial proteins capable of binding immunoglobulins including Protein-A from *Staphylococcus*, Protein-L from *Peptostreptococcus magnus* (Nilson et al., 1992) and Protein-M from *Mycoplasma* (Grover et al., 2014). Protein-G from Groups C and G streptococcus is a multi-domain cell surface protein possessing albumin and immunoglobulin binding domains. The ability to bind the predominant serum proteins is thought to enable the organism to evade detection by the host immune system.

While IBPs have become the industry standard for immunoglobulin purification, numerous antibody formats create a demand for more customized purification reagents. Over the last two decades, phage display derived antibodies have become a more versatile alternative to hybridoma-based technology (Michnick and Sidhu, 2008). The completely *in vitro* process offers a number of technical advantages over traditional methods including exquisite control of bio-panning conditions and the ability to raise antibodies against highly conserved epitopes (Bradbury et al., 2011). Indeed, the promise of such technology is the basis for a number of large-scale efforts to obtain affinity reagents on the proteome scale (Colwill et al., 2011; Taussig et al., 2007). Our laboratory, and others, has helped develop synthetic antibody libraries based on “restricted chemical diversity” where residues within the CDRs of the antibody fragment are enriched in amino acids typically found at the antibody paratope, including tyrosine, serine, and glycine (Fellouse et al., 2007; Miller et al., 2012). Such libraries, based on the 4D5 Fab scaffold, have successfully produced affinity reagents to a wide range of targets (Fellouse et al., 2007; Rizk et al., 2011; Uysal et al., 2009; Ye et al., 2008). Furthermore, antibody fragments derived from synthetic libraries allow for the potential to move beyond the IgG format, enabling facile prokaryotic expression and further functionalization through genetic manipulation. However, to do this in the case of Fab-based antibodies, we felt that there was a need to develop a highly versatile IBP reagent that could be utilized in multiple applications.

It is well established that IBPs interact with a number of distinct epitopes on the full-length antibody and this adds to their functional flexibility. The ability to recognize conserved regions of the IgG scaffold enable IBPs to bind to antibodies from a wide range of species. Protein-L binds to V_L of Kappa light chains ($K_D \sim 100$ nM) (Graille et al., 2001) (Figure 1A.). The recently discovered Protein-M interacts through conserved framework regions on V_L and V_K and binds both isotypes with high affinity (low nM). Proteins-A and G are multi-specific proteins possessing binding affinity to the Fc as well as the Fab portion of the antibody. Each binds to the hinge region connecting C_H2 and C_H3 on the Fc portion of the IgG with high affinity (~ 10 nM). Additionally, Protein-A binds to the V_H3 subset of V_H domains which comprises ~ 30 – 50% of the circulating IgGs ($K_D \sim 20$ nM) (Graille et al., 2000).

Protein-G binds to the constant domain of the Fab portion of the IgG through its interaction with the C_H1 domain, a highly conserved domain across many isotypes and species (Derrick and Wigley, 1992). The inherent cross-reactivity of the Fab-Protein-G interface is achieved

through an anti-parallel beta strand association between Protein-G and C_H1, dominated by main chain interactions (Derrick and Wigley, 1992). Unfortunately, the low affinity of the natural domain (K_D ~low μ M) limits the usage of Protein-G as an affinity reagent, compared to Protein-A, for instance. Indeed, the low affinity of the Protein-G-Fab interaction has been exploited to separate antibody fragments based on valency (Proudfoot et al., 1992). As a result, typical purification strategies in our laboratory include initial purification with Protein-A, followed by cation-exchange to remove antibody variable domain degradation products that are retained in the initial purification step.

In an effort to enable single step purification of Fabs, we have engineered the immunoglobulin domain from *Streptococcus* Protein-G for improved binding to the 4D5 scaffold possessing a human IgG1 C_H1 and a Kappa light chain. Through a combined combinatorial and rational approach, we have endowed this domain with ~100-fold improved affinity, pH dependence, and improved biophysical properties for use as a multifaceted affinity reagent.

2. Materials and methods

2.1. Molecular biology

The gene encoding the modified C2 domain of Protein-G was synthesized by GenScript. Subsequently, the gene was amplified and ligated into a phagemid pC3DsbA (Bailey and Kossiakoff, manuscript in preparation), between a DsbA signal sequence and the C-terminal domain of phage protein pIII under the control of the phoA promoter. Control phage ELISA experiments revealed robust display of Protein-G through its interaction with the 4D5 Fab scaffold.

2.2. Library creation

To generate the phage library, we used the library construction strategy described in Sidhu et. al (Sidhu et al., 2000). Briefly, through inspection of the crystal structure of the Protein-G-Fab complex (1IGC) we identified 15 residues in Protein-G that were within 5 Å of the complex interface. These residues were randomized using the soft randomization strategy where at each position within the randomized codon, the wild type nucleotide was present at 70% and the remaining three nucleotides were present at 10%. Randomized primers were phosphorylated and used to anneal to prepared ssDNA of pC3DsbA to replace stop codons within each of the randomized regions of the single-stranded template. Double-stranded DNA was synthesized using the Kunkel protocol (Kunkel, 1985). A small number of library variants were sequenced to confirm >80% of the library had the desired diversity incorporated. The phage library was generated through electroporation of SS3320 cells (Sidhu et al., 2000). Recovered cells were infected with M13K07 helper phage and allowed to amplify overnight at 37°C. Phage particles were isolated through PEG precipitation for subsequent selection.

2.3. Selection strategy

The first round of selection was performed with 1 mL of phage library re-suspended in TBST-0.5% BSA (~ 1×10^{12} cfu). This was incubated with streptavidin coated magnetic

beads (Promega, Madison, WI) with immobilized Fab biotinylated with a cleavable disulfide linker (Thermo Fisher, Waltham, MA) at a final concentration of 100 nM. Phage particles were allowed to incubate with target for ~1 hour with gentle shaking before several washing steps were performed to remove unbound virions. Phage particles were eluted from the beads with 100 mM DTT to release the Fab from the resin and the resulting supernatant was used to infect log phase XL1 for ~20 minutes. M13K07 helper phage was added to final concentration of 10^{10} pfu/ml for overnight growth (Paduch et al., 2013). Rounds two and three were performed with phage particles isolated from the overnight amplification of the previous round. Here, $\sim 10^{10}$ cfu of rounds 1 and 2 amplification were used for the next round of selection in a KingFisher magnetic bead sorter (Thermo Fisher, Waltham, MA). Selection and amplification for subsequent rounds were similar to round 1 except 50 nM and 10 nM Fab were immobilized for rounds 2 and 3, respectively. Enrichment of phage particle output relative to a negative control reached ~1000. Round 3 output was subsequently PCR amplified and ligated into pHFT2 (Huang et al., 2008) for analysis by protein ELISA. The resulting ligation mixture was transformed into BL21(DE3) Gold and colonies were screened by ELISA.

2.4. Immunoassays

Overnight cultures induced with 1 mM IPTG were centrifuged and lysed with BugBuster (Novagen, Madison, WI) following the manufacturer's protocol. Cleared lysates were incubated at 70°C for 30 minutes before incubation with Nunc Maxisorp plates immobilized with 20 nM Fab. Protein-G binding was detected via Flag-HRP. Isolated clones were sequenced to identify variants for large-scale expression.

2.5. Protein expression and purification

Protein-G variants isolated from ELISA were expressed in 1 L cultures in the expression strain BL21(DE3). Protein expression was induced at mid-log phase through the addition of 1 mM IPTG. Expression proceeded for 5 hours at 37°C. Protein was extracted by sonication and purified by Ni-NTA affinity chromatography using standard procedures. Yields for the Protein-G fusions were typically ~200 mg/L. Antibody fragments were expressed and purified using protocols previously described (Paduch et al., 2013) or those described in Results. Yeast Anti-silencing-1 was expressed from the pHFT2 vector in BL21(DE3) and purified via Ni-NTA as described for the Protein-G variants. Sequences of Protein-G-A1, SUMO-Protein-G-A1 and Protein-G-A1 dimer are listed in the Supplementary Information.

2.6. Circular dichroism

Protein-G samples (0.1 mg/mL, ~13 μ M) were prepared in 1.5 M guanidinium hydrochloride (Gdn-HCl), 0.1M cacodylate buffer, pH 7.0 and added to a 1 mm quartz cuvette. The sample was heated from 25–85°C in 1°C increments every 5 seconds while the absorbance at 207 nm was monitored. Far-UV wavelength scans where data was collected every 1 nm/5 s from 200–250 nm were performed before and after each temperature melt experiment to determine the extent of reversibility.

2.7. Base stability measurements

Protein-G variants were incubated in 0.1 M NaOH and quenched at various time points through a 100-fold dilution into PBS. Samples (5 μ M-1 nM) were subjected to protein ELISA with immobilized Fab (20 nM). The fraction of sample retaining binding was calculated based on comparison to the control sample at a Protein-G concentration that produced an ELISA signal that was at the top of the linear, dynamic range.

2.8. Sulfo-Link resin preparation

ProteinG-A1 resin was created using SulfoLink Coupling Resin (Thermo Scientific) following the manufacturer's protocol. To couple the protein to the resin, a free cysteine was introduced at the Q6 position of SUMO on the SUMO-Protein-G-A1 variant. Briefly, to make 1 mL of Protein-G-A1 resin, the column was first equilibrated with coupling buffer (50 mM Tris, 5 mM EDTA, pH 8.5). Prior to immobilization, SUMO-Protein-G-A1 was treated with a 10-fold molar excess of TCEP and diluted to 6 mg/mL in coupling buffer. A volume of 2 mL SUMO-Protein-G-A1 (12 mg ProteinG-A1/mL resin) was added to the column and allowed to incubate for 45 minutes with intermittent mixing. The flow-through was collected to analyze coupling efficiency. After washing with coupling buffer, excess binding sites on the resin were blocked with a solution of 50 mM L-Cysteine in coupling buffer and the column was washed with 1 M NaCl. Typically, 10 mg of SUMO-Protein-G-A1 was captured on 1 mL of resin.

2.9. Test of multivalency

A Protein-G-A1 dimer was created through the introduction of a (G₄S)₃-Protein-G-A1 fragment into the pHFT2-Protein-G-A1 vector. The resulting dimer was expressed in BL21(DE3) in a similar manner to the monomeric Protein-G variants. Generation of the non-covalent Protein-G-A1 dimer was achieved through incubation of 10 μ M Fab specific for Anti-silencing factor 1 with 0.5 equivalents of Protein-G-A1 dimer. Asf1 was immobilized on a Maxisorp plate and titrations of Fab or Protein-G-A1 dimer-Fab were tested for their ability to bind immobilized target. Bound Fab was detected by Protein-A-HRP. Control experiments using anti-FLAG-HRP as a secondary antibody indicated dimeric Protein-G-A1 had negligible binding to the target or blocked plate at a concentration of 500 nM.

3. Results and discussion

3.1. Phage library generation and selection

A modified variant of the Protein-G C2 domain from *Streptococcus* was displayed on filamentous phage as a fusion to the C-terminal domain of minor coat protein III. Guided by inspection of the structure of the *Streptococcus* Protein-G-Fab complex (pdb 1IGC), we introduced diversity in the 15 residues of Protein-G within 5 Å of the interface (Figure 1B) employing a soft randomization library design strategy. This strategy biases the library to the wild type sequence, focusing introduction of diversity at positions where the substitution is favored over the wild-type residue.

Using Kunkel mutagenesis, a library of $\sim 3 \times 10^9$ variants was created and subjected to three rounds of bio-panning using decreasing amounts of the immobilized Fab antigen each round of the selection (100 nM, 50 nM and 10 nM, respectively). We identified stable variants with high affinity to the Fab with a heat denaturation step followed by protein ELISA. The ELISA evaluation identified a number of variants that were further characterized by Surface Plasmon Resonance (SPR) (Figure 1C and 1D). All Protein-G variants tested had affinities 3-100-fold higher than the starting variant, confirming the potential for engineering this interface.

Figure 1C shows the mutated residues of the engineered variants in the context of the wild type sequence. The selection resulted in variants with mutations at the majority of positions randomized within the library. This is not surprising because the major interaction of Protein-G with the Fab is through a β -strand where backbone H-bonds generally contribute more to binding than side chains (Derrick and Wigley, 1992). Position 18 is a notable example of this as the parent lysine residue was replaced by arginine, serine and tryptophan in the higher affinity variants.

Despite the diversity of sequences retrieved from the bio-panning, there were several positions where the parent residue was conserved or retained in chemical character. For instance, Leu17 projects its side chain into the core of the domain and remained the preferred residue with mutations to phenylalanine and alanine observed. Glycine remains the dominant residue at position 19, however, tyrosine appears in two variants, perhaps reflecting the potential for additional energetically favorable contacts since the C α of glycine projects toward the Fab heavy chain. Thr21 and Thr22 were retained at the C-terminal portion of the β -strand consistent with the preferred status of β -branched residues in exposed β -strands. Additionally, two parent residues within the α -helical region of Protein-G were favored. Tyr38 projects into the immunoglobulin domain strands β 1 and β 7 where the aromatic side chain interacts with a number of conserved hydrophobic residues. Here, it remained the dominant amino acid at this position in addition to two variants containing isoleucine to retain the aliphatic character of the complex interface. Asn42 forms a hydrogen bond with Val128 in the buried complex and the hydrogen bonding potential at this position is preserved with engineered Protein-G variants containing either Asn, Gln, His or Tyr.

A notable divergence from the parent sequence occurred at position Asn40, which showed a preference for aromatic residues as tyrosine and phenylalanine comprised a number of the resulting sequences. Asn40 buries roughly 40 Å² at the complex interface with the light chain. Presumably, the bulkier aromatic side chains contribute energetically favorable interaction as aromatic residues are known to be contributors to “hot spots” in many protein-protein interactions (Bogan and Thorn, 1998).

The tightest binding variant, Protein-G-A1 had an ~ 100 -fold higher affinity for the Fab compared to the wild-type Protein-G. With a K_D of ~ 25 nM determined by equilibrium SPR (Figure 1D), this variant has suitable properties that can be exploited for use in a number of applications including purification, immunoprecipitation and as a crystallization chaperone (Dyson et al., 2011). Protein-G-A1 demonstrates a preference for the human IgG1 isotype as its affinity was decreased by a factor of ~ 20 – 50 -fold for IgG2, IgG3 and IgG4, suggesting

subtle differences in domain orientation impact binding (Supplementary Fig. 1). Furthermore, targeted mutagenesis of Protein-G-A1 identified mutations that abolish IgG Fc binding while maintaining the paratope-responsible high affinity Fab interaction for applications requiring the separation of Fab and Fc fragments (Supplementary Fig. 2). The protein exhibited favorable biophysical characteristics including high solubility (>10 mM) and thermal stability ($T_M \sim 58^\circ\text{C}$ in 1.5 M GdmHCl) (Supplementary Fig. 3). Thus, we used the Protein-G-A1 variant to perform functional analysis to evaluate its potential in the applications described below.

3.2. pH dependence of the engineered Protein-G variant

Examination of the Protein-G-Fab structure indicated that there were two ionizable residues (His42 and Glu43) introduced into Protein-G-A1 that were located directly in the interface with the Fab. To explore their potential to affect the pH-dependent affinity of this variant, we performed pH-varied fluorescence polarization studies with Alexa-Fluor labeled Protein-G and the Protein-G-A1 variant. The wild type variant exhibited a broad pH optimum between 5 and 6.5, with a K_D at pH 4 almost indistinguishable from that at physiological levels. In contrast, Protein-G-A1 exhibited ~1000-fold decrease in affinity from pH 7.5 to pH 4 (Figure 2). The change in affinity was particularly pronounced at pH 5.5 where an ~300-fold loss of affinity was observed between pH 5.5 and pH 4.0. Simple inspection of $d\log K_A/d\log H^+$ indicated that the slope at low pH is ~1.5, compared with 0.4 for the wild-type interaction. Two residues within the protein complex interface plausibly provide the origin of the pH dependence. The G43E mutation occurs between the loop connecting the α helix with β -strand 3. Here the mutation at this position from glycine to glutamate may provide favorable electrostatic interactions with residues in the light chain including Lys126 of C_H1 . Asn42 forms a hydrogen bonding interaction with the main chain N of Val128. If this hydrogen bonding interaction is conserved in Protein-G-A1, a protonation event at His42 would break this interaction, perhaps accounting for the loss of affinity beginning at pH 5.5. Comparison of the simulated pKa at the binding interface with other histidine residues found buried at protein surfaces indicates this protonation event falls within those observed for other histidine residues (Edgcomb and Murphy, 2002).

3.3. Exploiting the pH switch as a tunable binding switch

The introduction of a pH switch provides an advantageous molecular property that can be exploited for antibody fragment purification. Significant efforts have been expended to design protein affinity ligands with the controllable protein binding to enable elution in less harsh conditions (Murtaugh et al., 2011; Strauch et al., 2014). To evaluate the ability of Protein-G-A1 as a purification resin, we conjugated a SUMO-Protein-G-A1 fusion via an introduced N-terminal cysteine to Sulfo-Link Resin. Notably, the high resin capacity of >10 mg/mL and was amenable to extensive washing with a range of ionic strength buffers at neutral pH. Furthermore, we were able to readily elute the majority of the Fab protein at pH 4.5 after washing with 10 column volumes (Figure 3). This compared favorably to the parent domain as minimal Fab fragment remained bound to the column after extensive washing in PBS. Protein-G-A1 was also capable of single-step purification of antibody fragments from cell lysate. Figure 3B. shows the elution of Fab from cell lysate using Protein-G-A1 and Protein-A columns. The resulting Fab from the Protein-G-A1 column eluted as a single,

full-length protein whereas the Protein-A column retained degradation products presumably resulting from soluble fragments of V_H and V_K.

3.4. Protein-G-A1 alkaline stability

A key determinant to the successful regeneration of a protein affinity reagent is the stability of the scaffold throughout the purification and cleaning process. Sensitivity to alkaline conditions commonly found in cleaning in place (CIP) methods, has proven problematic for protein affinity reagents since such conditions facilitate base-catalyzed deamidation of asparagine and glutamine residues. Previous studies of Protein-G indicated it is highly sensitive to base-catalyzed degradation with a half life of ~15 minutes in 0.5 M NaOH. Notably, Protein-G has been previously engineered to improve the alkaline stability, where Asn40 and Asn42 have been found to be particularly prone to deamidation (Gulich et al., 2002). However, an N7A/N36A double mutant led to a loss of Fab binding affinity, presumably because of the critical contacts Asn36 provides in the Protein-G-Fab complex. To establish the whether there were residue types that could substitute for the asparagine residues without diminishing binding, these residues were included in the region of Protein-G randomized during affinity maturation. The subsequent bio-panning was able to identify a number of variants where the base-labile Asn residues were replaced, including Protein-G-A1.

To assess the base stability, Protein-G variants were incubated in 0.1 M NaOH, a commonly used CIP reagent, and residual binding was measured through ELISA. The wild-type domain rapidly loses the capacity to bind Fab after base incubation for one hour (Figure 4). In contrast, Protein-G-A1 retained ~50% of its binding capacity in the same time interval, significantly stabilizing it to base hydrolysis. Further engineering of Protein-G-A1 to include the N13A mutation, a residue distal from the binding interface, improved the stability with complete binding retained after one hour and ~50% binding after 6 hours. We anticipate that rational identification of base-labile residues coupled with functional selection to identify residues with more ideal properties may be a route to generate more robust protein affinity reagents.

3.5 Non-covalent avidity enhancement

Multivalency is a hallmark of natural immunoglobulins and is a critical feature to the efficacy of antibodies *in vivo* and in research, diagnostic, and therapeutic applications. Multimerization of affinity reagents can yield significant improvements in apparent affinity with reported values ranging from 10-10⁵ -fold (Krishnamurthy et al.). Unlike antibodies derived from an immune response, most synthetically-derived antibody fragments are monovalent unless otherwise functionalized. Notably, a number of antibodies are limited by their fast dissociation constants in assays requiring extensive washing, including IP and immune-fluorescence (IF), thus requiring further functionalization to achieve multivalency (Dyson et al., 2011). This has been achieved through a number of strategies including *in vitro* production of the F(ab')₂ fusion to coiled-coil domains (Pack and Pliickthun, 1992) and Avi-tag peptide for multimerization through avidin (Kay et al., 2009).

An alternative more facile strategy presented here exploits the affinity-matured Protein-G-A1 dimer linked through a (Gly₄Ser)₃ tether. To test the ability of the Protein-G-A1 dimer to enhance the apparent affinity of an antibody-antigen interaction, we performed ELISA with an antibody fragment specific to the histone chaperone anti-silencing factor 1 (Asf1) (Figure 5). Asf1 was immobilized through a biotin-NAV interaction to test the role of Protein-G-induced avidity. The EC₅₀ of the monovalent Fab fragment was 35 nM. However, through the addition of 0.5 equivalents of the Protein-G-A1 dimer, we observed an apparent EC₅₀ of ~4 nM, roughly a 9-fold enhancement of the apparent affinity of the Fab-antigen interaction, comparable to previously reported values of multivalency. Notably, this was achieved through non-covalent multimerization of the antibody fragment. Further functionalization of the Fab fragment-Protein-G interaction by cysteine crosslinking may provide the necessary covalent attachment in instances where sub-nanomolar concentrations do not favor Fab-Protein-G complex formation.

4. Conclusions

We have engineered a variant of Protein-G with significantly improved affinity for the Fab portion of an IgG. Furthermore, this interaction can be effectively modulated through pH changes as there is an ~1000-fold change in K_D when going from pH 7.5 to 4. This engineered variant possesses biophysical characteristics favorable for a number of applications. Ultimately, these results should enable more facile performance of this protein affinity ligand for a number of applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IBP	immunoglobulin binding protein
SPR	surface plasmon resonance

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Figure 1. Engineering of Protein-G. A). Binding epitopes of immunoglobulin proteins on the antibody fragment. B). Residues randomized on Protein-G are represented as red spheres. C). Resulting sequences of engineered Protein-G variants with improved affinity for the human antibody fragment. Affinities were determined by equilibrium SPR. D). Equilibrium SPR sensogram and binding isotherm of the Protein-G-A1 variant. E). WebLogo plot of the amino acid frequencies of randomized positions in engineered Protein-G variants from 1C.

Figure 2.
pH-dependent affinity of Protein-G variants.

Figure 3. Antibody fragment purification. A). Purification and pH-dependent elution of antibody fragments using Protein-G-A1. B). Reducing SDS-PAGE of the antibody fragments purified by a single step with an immunoglobulin binding protein. Protein-G-A1 elutes in as a single fraction while Protein-A retains a number of degradation products.

Figure 4.
Base stability measurements of Protein-G variants.

Figure 5.
Affinity enhancement through non-covalent dimerization of Protein-G-A1.