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Protein Binder for Affinity Purification of Human Immunoglobulin Antibodies

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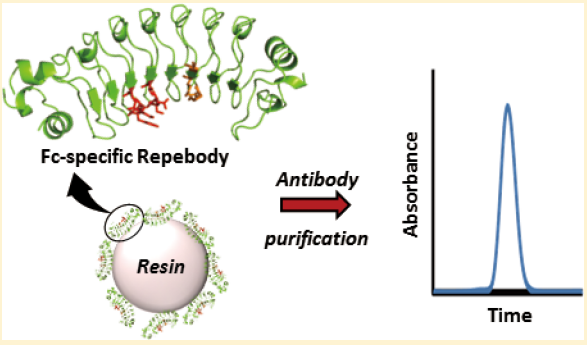
Supporting Information

ABSTRACT: The importance of a downstream process for the purification of immunoglobulin antibodies is increasing with the growing application of monoclonal antibodies in many different areas. Although protein A is most commonly used for the affinity purification of antibodies, certain properties could be further improved: higher stability in alkaline solution and milder elution condition. Herein, we present the development of Fc-specific repebody by modular engineering approach and its potential as an affinity ligand for purification of human immunoglobulin antibodies. We previously developed the repebody scaffold composed of Leucine-rich repeat (LRR) modules. The scaffold was shown to be highly stable over a wide range of pH and temperature, exhibiting a modular architecture. We first selected a repebody that binds the Fc fragment of human immunoglobulin G (IgG) through a phage display and increased its binding affinity up to 1.9×10^{-7} M in a module-by-module approach. The utility of the Fc-specific repebody was demonstrated by the performance of an immobilized repebody in affinity purification of antibodies from a mammalian cell-cultured medium. Bound-antibodies on an immobilized repebody were shown to be eluted at pH 4.0 with high purity (>94.6%) and recovery yield (>95.7%). The immobilized repebody allowed a repetitive purification process more than ten times without any loss of binding capability. The repebody remained almost intact even after incubation with 0.5 M NaOH for 15 days. The present approach could be effectively used for developing a repeat module-based binder for other target molecules for affinity purification.



Immunoglobulin antibodies are the most promising therapeutic agents for numerous cancers and inflammatory diseases. In addition to therapeutic use, antibodies have expanded their applications in a wide range of fields, including diagnosis, targeted delivery, and imaging.[1,2] With the increasing use of immunoglobulin antibodies, there has been a strong demand for simple and efficient purification methods.[3−5] Affinity chromatography based on protein A is the most widely used method for purification of antibodies in a laboratory and at the commercial scale, and this process has been well-established. Despite a widespread use of protein A, however, its properties could be further improved: higher stability in alkaline condition and milder elution conditions.[6−9] Significant effort has therefore been made to improve the properties of protein A, but the results have not been completely satisfactory.[10−15] As alternatives to protein A, numerous ligands have been developed.[16−20] However, they were shown to have a much lower binding affinity and specificity for antibodies than protein A.[21−28] Protein binder such as single domain antibody derived from camelids was tested,[29,30] but it displayed low stability under harsh acidic and alkaline conditions. Therefore, development of a novel binder with high specificity and stability for affinity purification of immunoglobulin antibodies is of great significance.

We previously developed a novel nonantibody protein scaffold, termed "repebody", which is composed of consensus designed leucine-rich repeat (LRR) modules.[31] The repebody scaffold was shown to be highly expressed in *Escherichia coli* and stable over a wide range of pH levels and temperature. Furthermore, modular architecture allows easy modulation of a binding affinity for a target in a module-by-module manner. Herein, we present the development of Fc-specific repebody by modular engineering approach and its utility for affinity purification of human immunoglobulin antibody. We first selected a repebody that binds the Fc domain of human immunoglobulin G (IgG) through a phage display and increased its binding affinity up to 1.9×10^{-7} M in a module-by-module manner. The binding affinity of the developed repebody for IgG was tested with respect to pH, and the repebody was shown to completely dissociate IgG at pH 4.0. A resin-immobilized repebody in the affinity purification of IgG from a CHO cell-cultured medium resulted in a high yield (>95.7%) and purity (>94.6%) and fully retained its binding capability after a repetitive purification process comprising acidic elution and alkaline sterilization. Furthermore, the repebody remained almost intact even after

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incubation with 0.5 M NaOH for 15 days. Details are reported herein.

EXPERIMENTAL SECTION

Selection of Human IgG-Binding Repebody through a Phage Display. We used a phage display library to select a repebody specific for the Fc domain of human immunoglobulin G (IgG), as described in our previous work.[31] The immunotubes (Greinerc) were coated with 1 mL of a 10 μg/mL Fc fragment of human IgG_1 (R&D systems) in a phosphate-buffered saline (PBS, pH 7.4) overnight at 4 °C. The tubes were treated with 1% BSA in phosphate-buffered saline Tween-20 (PBST). A 1 mL amount of phage solution (1.0×10^{12} cfu/mL) was added to the tubes and incubated for 2 h at room temperature. Following the removal of the phage solution, target-bound phages were eluted using a 0.2 M glycine-HCl solution (pH 2.2) for 12 min at room temperature. XL1-Blue F′ *E. coli* cells were grown in 2xYT media at 37 °C until absorbance at 600 nm reached 0.4−0.7. The cells were infected with the eluted phages and cultured at 37 °C for 30 min. The infected cells were collected and spread on 2xYT plates containing 50 μg/mL of ampicillin, 10 μg/mL of tetracycline, and 1% glucose (2xYT/ATG), followed by incubation overnight at 30 °C. The plated cells were counted and cultured again in 2xYT/ATG until the absorbance at 600 nm reached 0.4−0.7. The cells were superinfected with a VCS M13 helper phage (Stratagene) and incubated in a fresh 2xYT medium containing 50 μg/mL of ampicillin, 10 μg/mL of tetracycline, 50 μg/mL of kanamycin (2xYT/ATK), and 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30 °C for 16 h. The phages were precipitated using a 20% PEG/NaCl solution and harvested through centrifugation at 10000*g* for 1 h. The precipitated phages were suspended in PBS (pH 7.4) and centrifuged (13000 rpm, 10 min) to remove PEG, followed by repeating rounds of panning. Following the fifth round of panning, each colony was incubated in a 96-deep wall plate (Nunc) containing 200 μL 2xYT/ATG overnight at 37 °C. After infection with helper phages, the cells were centrifuged, resuspended in 300 μL of a fresh 2xYT/ATK medium, and incubated overnight at 30 °C. The repebody-presenting phages were purified from a culture supernatant, and 100 μL of the phages were added to a Fc fragment of Human IgG_1 (0.5 μg/well, Calbiochem) immobilized on a 96-well maxisorp plate (Nunc) and incubated for 2 h at room temperature. The plates were washed with PBST and incubated with diluted (1:5000) HRP-conjugated anti-M13 monoclonal antibody (GE Healthcare) for 1 h. After washing with PBST, 100 μL of a tetramethyl benzidine (TMB) solution (Sigma) was added. Immediately, 100 μL of 1 N H_2SO_4 was added followed by scanning with an Infinite N200 plate reader (Tecan) at 450 nm.

Expression and Purification of Selected Repebodies. The gene coding for selected repebodies were cloned into a pET21a vector (Invitrogen) between the NdeI and XhoI restriction enzyme sites. Each gene has a hexa-histidine tag at the C terminal for affinity purification. The vectors were transformed into Origami-B *E. coli* cells (Merck Biosciences). The transformed cells were grown in a LB medium at 37 °C until the optical density at 600 nm reached 0.5, and IPTG was added at a final concentration of 0.5 mM for induction. Cells were additionally incubated at 18 °C for 20 h and harvested by centrifugation at 4000*g*. Collected cells were suspended in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, at pH 8.0) and disrupted using sonication. Following centrifugation at 16000 rpm for 50 min, the supernatant was collected and purified using affinity chromatography through Ni-NTA Superflow (Qiagen). The solution was applied to the resin-packed column, followed by a washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 50 mM imidazole, at pH 8.0), until no protein was detected from a Bradford assay. The proteins were eluted using an elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, at pH 8.0). The collected proteins were further purified through gel permeation chromatography (Superdex75, GE Healthcare) using a PBS (pH 7.4).

Isothermal Titration Calorimetry (ITC). The binding affinities were measured through ITC (iTC 200, Microcal) at 25 °C. For affinity determination, a 0.2 mM repebody was titrated with 0.02 mM of human IgG for a 2 min injection to return the titration peak to the baseline. The K_d values were determined using the Origin program (OriginLab) from the integrated exothermal peaks.

Immobilization. A repebody was coupled to NHS-activated Sepharose 4 Fast Flow according to the manufacturer's instructions (GE Healthcare). Briefly, a 4 mg repebody in PBS was mixed with 1 mL of resin and incubated overnight at 4 °C under mild agitation. Following a coupling reaction, the medium was exchanged into 0.5 M ethanolamine for blocking inactivated groups and kept at 25 °C for 2 h. The immobilized repebody was collected through centrifugation and kept in PBS before use.

Production of Immunoglobulin Antibody by Mammalian Cells. Immunoglobulin antibodies were produced using recombinant CHO cells as described.[32] The cells were adapted for growth in a serum-free suspension culture in 125 mL Erlenmeyer flasks (Corning) containing a 50 mL medium in a Climoshaking CO_2 incubator (ISF1-X, Adolf Kuhner AG, Birsfelden, Switzerland) set at 110 rpm, with 85% humidity and 37 °C. For the suspension culture, HyClone SFM4CHOTM (Hyclone, Logan, UT) supplemented with 4 mM glutamine and 1 μM MTX was used as the basal serum-free medium. Cells at the exponential growth stage were inoculated at a concentration of 2.0×10^5 cells/mL in Erlenmeyer flasks containing a 50 mL culture medium followed by incubation in a Climo-shaking CO_2 incubator set at 110 rpm, with 85% humidity and 37 °C during an 8-day period.

Binding Analysis. A 1 mL amount of immobilized repebody resin and protein A resin were separately packed in a column, and 1 mL of a CHO cell culture supernatant containing a 300 μg/mL antibody or the same amount of human IgG (Sigma-Aldrich) was loaded into a respective column. The columns were washed with a 5 mL PBS, and the bound antibodies were eluted with 5 mL of 10 mM sodium acetate (pH 4.0). Following elution, the columns were additionally cleaned with 10 mL of each elution buffer and re-equilibrated with a 10 mL PBS. Both the flow-through and eluted fractions were concentrated approximately to 1 mL each using Amicon ultra (3 K membrane, Millipore) and analyzed on SDS−PAGE.

To determine the binding capacity of a repebody resin, 10 μL of a repebody resin was incubated with 1 mL of an antibody solution. The solution concentration of antibody was between 0.1 and 3.5 mg/mL. Each sample was incubated at 25 °C for 2 h. Afterward, the unbound protein was determined using UV absorbance at 280 nm. The data were fit to a Langmuir isotherm model,

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$$q = \frac{q_m C}{K_d + C}$$

where q, C, K_d, and q_m are the concentration of the bound protein (milligrams protein/milliliters resin), the concentration of the free protein (milligrams protein/milliliters solution), the dissociation constant (milligrams/milliliters), and the maximum binding capacity (milligrams protein/milliliters resin).

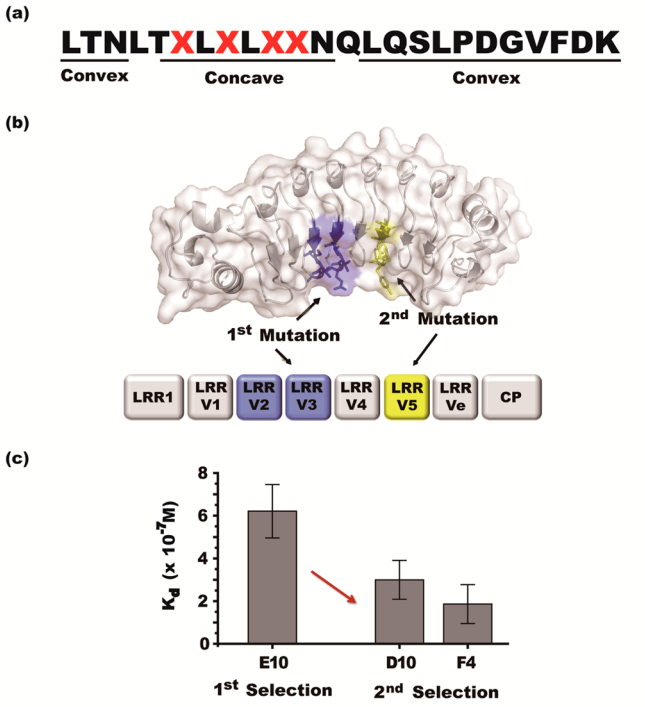
Stability under Alkaline Conditions. A repebody was incubated in a 0.5 M NaOH solution, and its secondary structures were analyzed using circular dichroism (Jasco J-815). The molar ellipticities of the protein were measured from 210 to 280 nm at 25 °C. To check the stability of the repebody resin under alkaline conditions, the repebody resin was incubated with 0.5 M NaOH for 15 days and tested in terms of purity and yield in the purification of antibodies from a CHO cell-cultivated medium.

Determination of Antibody Recovery Yield and Purity. The purity of antibodies in the elution fractions was determined through a SDS−PAGE analysis. A 5 μL amount of gel loading buffer was added to a 10 μL concentrated flow-through or elution fraction. Following boiling for 5 min, a 10 μL solution was loaded into the gel. The antibody purity was determined through a densitometric analysis of the Coomassie-stained gels using Imaging Lab (Biorad, v3.0.1). The purity of the antibody was calculated as a fraction of the total area and intensity for the IgG bands at 25 and 50 kDa, respectively. The recovery yield of the antibodies through an immobilized repebody was determined as follows. All experiments were carried out at room temperature. The concentrated flow-through and elution fractions were immobilized on a 96-well Maxisorp plate (Nunc) overnight at 4 °C. The surface was blocked with 1% BSA in PBST for 1 h, followed by incubation with diluted (1:2000) Goat antihuman IgG (Sigma-Aldrich) for 1 h. The plates were washed with PBST and incubated with diluted (1:5000) HRP-conjugated Rabbit antigoat IgG antibody (Biorad) for 1 h. Following washing with PBST, 100 μL of a tetramethyl benzidine (TMB) solution (Sigma) was added, and 100 μL of 1 N H_2SO_4 was immediately added and scanned using an Infinite N200 plate reader (Tecan) at 450 nm.

RESULTS AND DISCUSSION

Selection of Human IgG-Binding Repebodies. We first constructed a repebody library by introducing mutations into three variable sites (positions 8, 10, and 11) on two modules (LRRV 2 and 3) (Figure 1, panels a and b) and displayed the repebody library on a phage minor coat protein, as described in our previous work.[31] Through a standard panning process against the Fc fragment of human IgG, we selected repebodies that displayed distinct signals in the phage ELISA. Of them, we chose E10 and determined its binding affinity for human IgG by ITC. The dissociation constant (K_d) of E10 was determined to be 6.2 × 10^{-7} M.

Affinity Maturation of Selected Repebody by Modular Engineering Approach. Protein binders for affinity purification should have the appropriate binding affinity to a target for efficient elution. A high affinity often causes difficulties in elution under mild conditions, whereas a low affinity results in a poor purity and recovery yield. In consideration of the binding affinity of protein A (~10^{-8} M) to immunoglobulin antibodies,[6] the proper affinity of a binder is suggested to be within a range of 10^{-7} M.[15] It is therefore necessary to further enhance the binding affinity of selected repebody for human IgG for



(d)

	Residue										
	LRRV2			LRRV3				LRRV5			K_d(x10^{-7}M)
	91	93	94	115	117	118	161	163	165	166	
WT	I	T	G	V	V	E	E	D	S	Y	
E10	E	K	W	V	N	S	E	D	S	Y	6.2
D10	E	K	W	V	N	S	F	E	E	M	3.0
F4	E	K	W	V	N	S	G	E	C	G	1.9

Figure 1. Selection and characterization of an IgG-binding repebody. (a) Sequence of a LRRV module. X denotes mutation sites. (b) Mutation sites of the modules on a concave region of a repebody for a library construction. Initial (blue) and additional (yellow) library sites. (c) Changes in a binding affinity through affinity maturation. (d) Sequences of IgG-binding repebodies.

Table 1. Dissociation Constant (K_d) of F4 at Different pH Conditions

pH	7.4	6.5	5.3	4.3	4.0
K_d (10^{-7} M)	1.9	4.5	6.1	19.8	N.D.[a]

[a]N.D. = not determined. The K_d at pH 4.0 was not measured through ITC owing to a low affinity.

efficient affinity purification of IgG. One of the distinct structural features of a repebody scaffold is its modular architecture. Therefore, mutations on a module have a negligible effect on nearby modules, and the repebody scaffold is easy to modulate a binding affinity for a target in a module-by-module manner. On the basis of the modularity, we chose additional module LRRV5 of E10 and introduced mutations into four variable sites (position 6, 8, 10, 11) to construct a library. We reasoned that a large interaction area between repebody and IgG would lead to a higher binding affinity and chose LRRV5 rather than nearby module LRRV1 or LRRV4. The resulting library was displayed on a phage coat protein, as shown in Figure 1b, followed by selection. Through a phage ELISA, we identified two repebodies (D10 and F4) exhibiting highest signals and determined their binding affinities to be 3.0 × 10^{-7} and 1.9 × 10^{-7} M, respectively, by ITC (Figure 1, panels c and d). Of them, we chose F4 by taking into account

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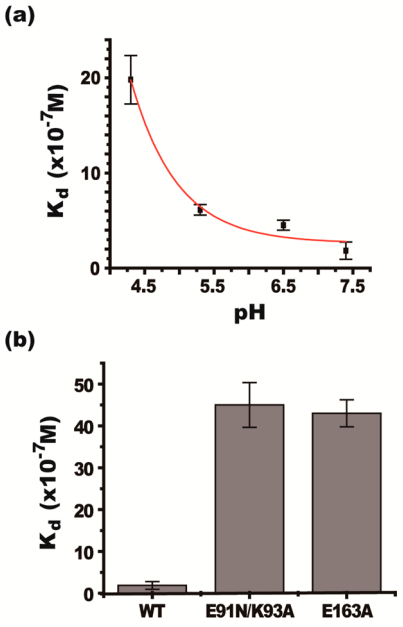

Figure 2. (a) Dissociation constant (K_d) of F4 at different pH conditions. (b) Dissociation constant (K_d) of F4 and its mutants.

the binding affinity for affinity purification of IgG. Our results indicate that a module-by-module approach is effective for gradually optimizing the binding interface for a target. Compared to globular protein binders, a repebody was shown to be easy and simple to modulate the binding affinity in a modular fashion owing its structural feature.

Table 2. Maximum Binding Capacity (q_m) and Apparent K_d of Resin-Immobilized Repebody and Protein A by Fitting the Experimental Data to the Langmuir Equation

		q_m	
	apparent K_d (10^{-8} M)	mg/mg protein	mg/mL resin
protein A resin[a]	3.3	6.6	20.0
repebody resin	3.6	6.5	25.9

[a]Protein A Sepharose HP (GE Healthcare).

Effect of pH on the Binding Affinity of a Repebody for IgG. To estimate the pH condition for the elution of IgG, we determined the binding affinity of F4 for human IgG at different pH conditions by ITC (Table 1 and Figure 2a). The binding affinity of F4 for IgG gradually decreased with a lowering of the pH. Interestingly, the binding affinity of F4 for IgG drastically decreased around pH 4.0. The K_d of F4 to IgG exponentially increased from 6.1×10^{-7} M at pH 5.0 to 1.9×10^{-6} M at pH 4.5. At pH 4.0, F4 completely lost its binding affinity for IgG. It therefore appears that F4 is highly sensitive to pH 4.0 in binding with IgG, and this can be effectively used for the elution of bound-IgG in affinity chromatography. Harsh elution condition (~pH 3.0) has been known to cause the denaturation of antibodies. For example, IgGs tend to aggregate within a few minutes when exposed to acidic pH around 3.4.[33] Fast neutralization is necessary to prevent further aggregation, but it is generally impractical in an industrial purification scale.[34,35] On the basis of the result, the developed binder that elutes antibodies under milder conditions seems to be favorable

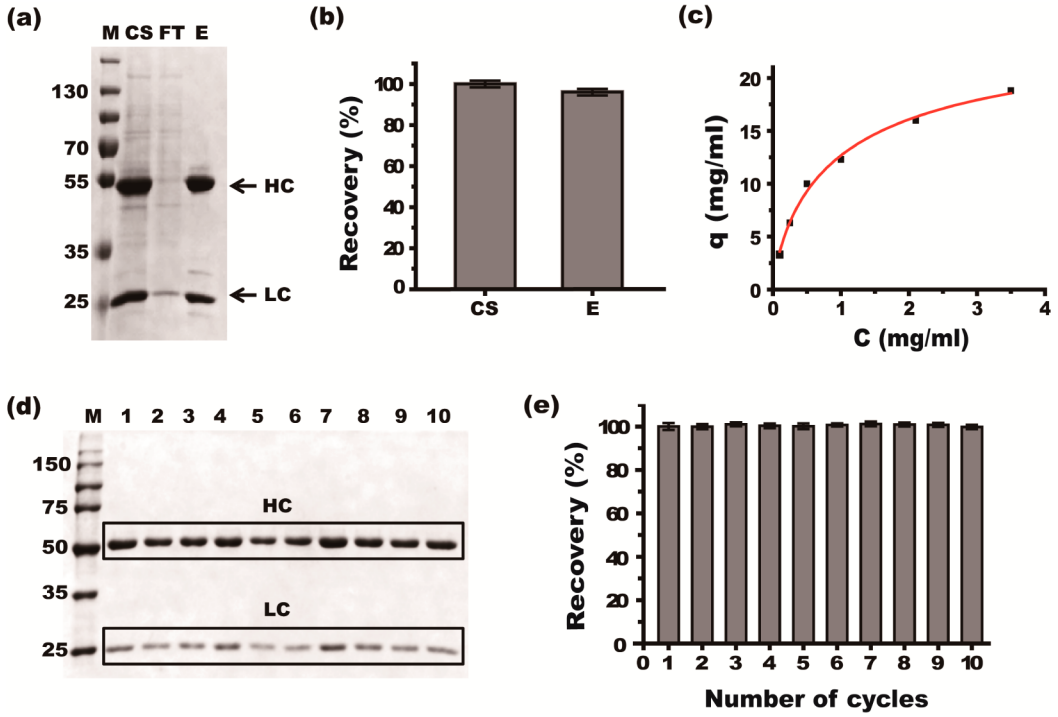

Figure 3. Performance of a repebody-immobilized resin in affinity purification of IgG. (a) SDS−PAGE analysis of purified antibody from a CHO cell-cultivated medium using a repebody resin. M, molecular weight marker; CS, cell culture supernatant; FT, flow-through fraction; E, eluted fraction; HC, heavy chain of IgG; and LC, light chain of IgG. (b) ELISA of purified antibodies from a CHO cell-cultivated medium using a repebody resin. The recovery percent was quantified by comparing the signals of the cell culture medium and elution fraction. (c) Langmuir fit of the adsorption isotherm of IgG to a repebody resin. q, amount of IgG bound on a repebody resin and C, concentration of free IgG in the solution. (d) Purity of antibodies and (e) recovery yield after repeated use. The numbers indicate the number of repetitions.

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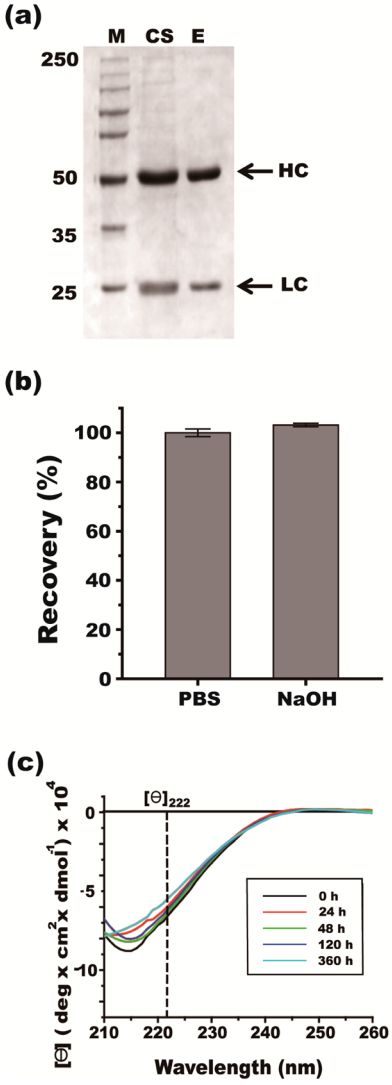

Figure 4. Effect of alkaline treatment. (a) SDS−PAGE analysis and (b) recovery yield of purified antibodies using a repebody resin treated with 0.5 M NaOH for 15 days. M, molecular weight marker; CS, cell culture supernatant; E, eluted fraction; HC, heavy chain of IgG; and LC, light chain of IgG. (c) CD analysis of F4 in 0.5 M NaOH.

for purification of antibodies without aggregation and denaturation of IgGs.

To get insight into the affinity profile of F4 with respect to pH, we examined the potential interacting residues of F4. As amino acids, Glu and Asp have pK_a values of 3.8 and 4.2, respectively, and will become neutral at around pH 4.0, losing their charges in the side chains. Thus, if F4 binds to IgG mainly through a salt bridge between Asp or Glu and positively charged group of Lys or Arg, F4 will dissociate from IgG at this pH level. On the basis of this hypothesis, we investigated the potential interacting residues of F4 with IgG, which should be included in variable F4 sites. As shown in Figure 1d, there are two Glu sites, 91 and 163, and one Lys site, 93, which have the potential to make a salt bridge between F4 and IgG. To check the participation of these residues in binding, we mutated Glu 91 and Lys 93 into Asn and Ala, respectively, and Glu 163 into Ala. As a result, both double and single mutants of $F4^{E91N/K93A}$ and $F4^{E163A}$ showed a great decrease in binding affinity for IgG compared to F4 (Figure 2b). It is therefore likely that three residues (Glu 91, Lys 93, and Glu 163) on F4 are mainly involved in the interaction with IgG. Our results also support that F4 binds IgG mainly through a salt bridge, and F4, therefore, completely loses its binding affinity for IgG at pH 4.0.

Purification of Antibodies by Immobilized Repebody. We examined the capability of repebody F4 in the affinity purification of antibodies. For this, we immobilized repebody F4 on a sepharose resin through an NHS-mediated conjugation. The resulting repebody resin was packed into a column (70 × 10 mm), and 1 mL of a CHO cell culture supernatant containing a 300 μg/mL IgG was added to the resin without treatment, followed by elution of a bound-IgG using a 10 mM sodium acetate buffer (pH 4.0). The eluted fractions were analyzed to determine the purity and recovery yield of the antibodies. Figure 3a shows an SDS−PAGE analysis of purified antibody from a CHO cell-cultivated medium, and bound-antibodies were completely eluted from the repebody resin. Compared to the culture medium (lane CS), only an antibody band was detected in the elution fraction (lane E), and the purity of the antibodies was estimated to 94.7% based on the SDS−PAGE analysis. When analyzed through ELISA (Figure S1 of the Supporting Information), their recovery yield from the medium reached 95.7% (Figure 3b). Our results strongly support that repebody F4 can be practically applicable in the affinity purification of IgG with high purity and milder elution conditions.

To obtain the maximum capacity of repebody resin, the isotherm for IgG adsorption to the resin was determined (Figure 3c). The adsorption isotherm was fitted to a Langmuir model, and the maximum binding capacity (q_m) and apparent dissociation constant (K_d) of the repebody resin were estimated (Table 2). Interestingly, the apparent K_d of the immobilized F4 was estimated to be 3.6×10^{-8} M, which is lower than the free F4 (1.9×10^{-7} M). This result seems to be due to the fact that immobilized repebodies act with pseudoavidity. In other words, antibodies will diffuse into the bead faster than away from it, and dissociated antibodies would be rebound by another repebody, which results in a lower apparent K_d. We also repeated the cycles of antibody loading and elution to examine the reusability of a repebody resin. The same amount of CHO cell culture medium containing 300 μg/mL of IgG was loaded into a column packed with a repebody resin followed by elution using a sodium acetate solution (pH 4.0). As shown in Figure 3 (panels d and e), no significant changes were observed in the purity and recovery yield of IgG even after ten repeated cycles when analyzed through ELISA (Figure S2 of the Supporting Information).

Stability in Alkaline Condition. To test the stability of a repebody resin during the harsh alkaline sterilization procedure, we incubated a repebody resin with 0.5 M NaOH for 15 days and checked the binding capacity and specificity for IgG (Figure 4, panels a and b, and Figure S3 of the Supporting Information). The repebody resin fully maintained its initial binding capacity and specificity for IgG after alkaline treatment. To further test the stability of repebody F4 under alkaline conditions, we incubated a free repebody F4 in a 0.5 M NaOH solution and determined the changes in secondary structures of the repebody through a circular dichroism (CD) analysis. As can be seen in Figure 4c, secondary structures of the repebody remained unchanged even after 15 days of incubation with the 0.5 M NaOH solution, indicating a high stability of the repebody under alkaline conditions. High stability of the

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developed protein binder is likely to stem from the rigid structure of the repebody scaffold.

The affinity purification column is usually subjected to a cleaning-in-process (CIP) during the purification process, and alkaline conditions are generally used for the regeneration and removal of contaminants from the column devices.[36] NaOH is the most extensively used cleaning and sanitizing agent in concentrations ranging from 0.1 to 1 M, depending on the degree of contamination, owing to its easy removal, simple monitoring, and low cost. However, most of protein-based affinity purification systems including protein A are labile under alkaline conditions and often show significantly decreased capacities.[37−40] In some cases, chaotropes (e.g., 6 M urea or 6 M guanidine HCl) are also used, but they are costly, requiring special handling during disposal.[34] In this regard, the developed repebody with high stability under alkaline condition offers a significant advantage in practical application to affinity purification of immunoglobulin antibodies.

CONCLUSION

We have demonstrated that the repebody scaffold can be used for developing a protein binder for affinity purification of IgG. Modular approach was shown to be effective for increasing the binding affinity of a repebody up to 1.9×10^{-7} M for the Fc fragment. The developed repebody provided an advantage over protein A in terms of elution condition: the use of pH 4.0 was effective for eluting bound-IgG, resulting in high purity and yield in affinity purification of IgG from a CHO cell-cultured medium. Furthermore, modular architecture of a repebody led to high stability in the alkaline condition, which is crucial for practical application to the downstream process. On the basis of the results, a novel repebody has great potentials as an affinity ligand for affinity purification of IgG, such as mild elution condition, high stability under alkaline conditions, and easy affinity modulation. Moreover, module-by-module design approach can be used to develop target-specific repeat proteins for numerous applications.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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