Journal of Biotechnology 161 (2012) 213–220

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Potential application of antibody-mimicking peptides identified by phage display in immuno-magnetic separation of an antigen

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ARTICLE INFO

Article history:
Received 15 March 2012
Received in revised form 9 June 2012
Accepted 18 June 2012
Available online 11 July 2012

Keywords:
Phage display
Biopanning
Magnetic bead separation
Antibody mimicking peptides
Human immunoglobulin
Immuno-binding

ABSTRACT

Phage display was performed against human IgG (hIgG) through five rounds of 'biopanning'. Each round consisted of: (1) incubating a library of phage-displayed 12-mer peptides sequences on hIgG-coated magnetic beads, (2) washing the unbound phages, and (3) eluting the bound phages. The eluted phages were either amplified to enrich the pool of positive clones or subjected to the next round without amplification. Through ELISA, four clones (F9, D1, G5, and A10) showing specific binding affinity to hIgG were identified. Among these, F9 had the highest affinity (K_d = 6.2 nM), only one order of magnitude lower than the native anti-hIgG antibody (0.66 nM). Following the DNA sequences of the selected clones, four 12-mer peptides were chemically synthesized. Among them, D1 peptide showed the highest binding affinity to hIgG via SPR biosensor measurements. This peptide was conjugated to biofunctionalized magnetic beads, and its immuno-binding ability was compared with that of the native antibody immobilized to magnetic beads. The mol-to-mol binding efficacy of the peptide-coated magnetic beads was approximately 1000-fold lower than that of the antibody-coated magnetic beads. Our results suggest a feasibility of using antibody-mimicking peptides identified by phage display technique for immuno-magnetic separation of an antigen.

1. Introduction

Detection of disease biomarker proteins in human blood serum is important in clinical diagnostics. However, this is a challenging task because of the wide dynamic range in the concentrations of various serum proteins (ca. 10 orders of magnitude); most clinically significant biomarker proteins are present at extremely low concentrations (10^{-10} to 10^{-8} M), and the 'abundant' proteins that constitute more than 90% of the total protein content in human serum often either mask the presence of biomarker proteins or interfere with their detection. Hence, the efficient depletion of these highly abundant proteins is a common pretreatment step for successful serum diagnostics (Thadikaran et al., 2005; Adkins et al., 2002).

Depletion strategies for abundant serum proteins usually employ the use of immunoaffinity media, which refers to media that contain matrices with covalently attached antibodies to the specific abundant proteins (Govorukhina et al., 2003; Pieper et al., 2003). Antibodies are popular ligands for immunoaffinity separation due to their ability to recognize and bind specifically to their target proteins. Antibody-based, serum albumin depletion column/kits are commercially available. Human immunoglobulin G (hIgG), the second most abundant protein in human serum, is usually removed using Protein A or G (Andrew and Titus, 2001; Fagiolo et al., 2000). However, the high cost, large size, and sensitivity to harsh pH and temperature conditions of antibodies are major drawbacks to the use of antibodies as robust and reliable ligands for biopurification. Smaller peptides with antigen-binding ability similar to that of antibodies would have the advantages of lower cost, higher molecular stability, and potentially more efficient binding than antibodies due to their small size (Ladner et al., 2004).

A vast number of specific peptides can be produced by phage display, which is a simple, cost-effective, and time-saving technique. Phage display allows the creation of a physical link between a library of random peptide sequences and the DNA encoding each sequence, allowing rapid identification of peptide ligands for specific target molecules by a selection technique called 'biopanning' (Willats, 2002). Screening of such libraries for antibody-binding peptides has been reported. In one study, two monoclonal antibodies (Mabs), A2 and M33, specific for the hexapeptide DFLEKI of myohemerythrin, an oxygen carrier found in a few invertebrate taxa, were used for library screening. The peptides that bound to these Mabs had the consensus sequence $DFLX_3$

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http://dx.doi.org/10.1016/j.jbiotec.2012.06.039

(Scott and Smith, 1990). Another study reported how panning a phage display peptide library for 3-E7 Mab, which is specific for the N-terminus of β-endorphin YGGF, led to the discovery of the peptide consensus sequence, YGX_4, which was homologous to the sequence Y-G on the N-terminus of β-endorphin Y (Devlin et al., 1990). Panning using 12-mer and 20-mer peptide libraries also resulted in the successful identification of peptides with similar binding affinity to Mab PA421 as p53. These peptides contained the KXXXSTSXHXK motif, identical to the C-terminal region of p53. When a 6-mer library against p53 was screened, the R/KXXXK motif, which has no observable homology to p53, was found (Stephen et al., 1995). Similarly, a phage display 6-mer peptide library was screened using anti-CD34 Mab as the target to find peptides against the CD34 surface antigen (CD34 is expressed on the surface of hematopoietic progenitor cells). DNA sequencing revealed two predominant sequences, QQGWFP and TQGSFW, which share a consensus sequence motif, QGxF, with 50% and 67% homology, respectively, to amino acids 14–19 of the CD34 antigen (Tseng-Law et al., 1999).

In this study, we present a feasibility study result of employing anti-hIgG mimicking peptides screened from a phage library as an efficient ligand for immuno-magnetic separation of hIgG. We prepared a phage display library against hIgG to select 12-mer peptides that could mimic anti-hIgG in hIgG binding. Positive phage clones were identified by enzyme linked immunosorbent assay (ELISA), their DNA was sequenced, and the peptides with the corresponding amino acid sequences were synthesized. The synthetic peptides' affinity to hIgG was evaluated by surface plasmon resonance (SPR) to select the best peptide candidate. This candidate peptide was then immobilized on the biofunctionalized surface of magnetic beads to compare its hIgG capturing efficacy with that of antibody-coated beads. Effective magnetic separation using peptide-coated beads, i.e., immuno-magnetic separation, can serve as a viable alternative to the traditional antibody-based processes in terms of efficiency, process economics and simplicity (Kang et al., 2007; Hubbuch and Owen, 2002; Arica et al., 2000). Our ultimate goal was to develop a cost-effective and robust process for successful depletion of hIgG, a second most abundant serum protein, from human serum specimens.

2. Materials and methods

2.1. Materials

The Ph.D.-12 Phage Display Peptide Library Kit and *Escherichia coli* ER2738 cells were purchased from New England Biolabs, Inc. (#E8110SC; New England Biolabs, Beverly, MA, USA). This library kit solution contains random 12-mer peptides with a complexity of 2.7×10^9 different presented peptides and has a concentration of 1.5×10^{13} phages/ml (pfu/ml). Host *E. coli* ER2738 cells (phage propagation strain) were cultivated on LB medium containing tetracycline (20 mg/ml). Human IgG and anti-hIgG antibody, a monoclonal antibody, were purchased from Sigma (St. Louis, MO, USA).

For ELISA, HRP-conjugated anti-M13 antibody was obtained from GE Healthcare (USA). For the biotinylation reaction of hIgG and to purify biotinylated products, EZ-Link® Sulfo-NHS-LC-Biotin Kit and the Pierce Monomeric Avidin Kit were purchased from Pierce (Rockford, IL, USA). For SPR experiments, a four-channel BIAcore 3000 optical sensor instrument equipped with streptavidin chip (SA chip) was used (GE Healthcare, USA). Dynabeads®M-270 Epoxy, purchased from Invitrogen Dynals AS (Oslo, Norway), was used as magnetic beads. Accubead™ COOH magnetic beads and Accubead™ streptavidin magnetic beads (1–2 μm in mean diameter) were acquired from Bioneer, Inc. (Chunan, Korea).

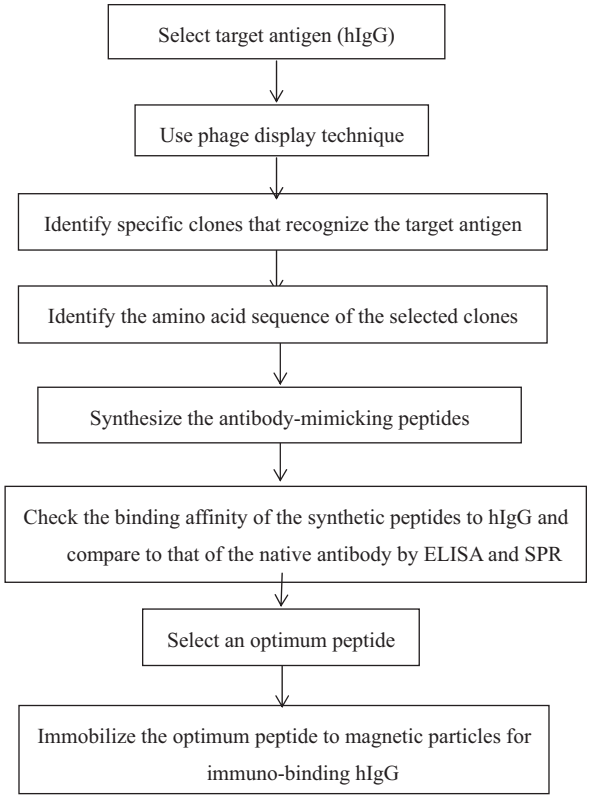

Fig. 1. A flowchart of the overall experimental scheme.

N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and 2-(N-morpholino) ethanesulfonic acid (MES) were obtained from Sigma (USA). Fluoraldehyde (OPA) reagent solution was purchased from Pierce (Rockford, IL, USA). All chemicals were of analytical grade.

2.2. Overall experimental scheme

The overall experimental scheme is shown in Fig. 1. This scheme consisted of four parts: (1) phage display and 'biopanning' to screen positive clones by ELISA, (2) DNA sequencing and amino acid sequence analysis of the candidate clones, (3) chemical synthesis of the candidate peptides and selection of the best peptide by SPR analysis, and (4) immobilization of the peptide to magnetic beads for immunobinding comparison with the native antibody.

2.3. Phage display and biopanning for positive clone screening

First, hIgG was immobilized onto Dynabeads to generate an affinity surface for further attachment of positive phage clones. The magnetic beads (60 mg) were suspended in 2 ml dimethyl formamide (DMF). One hundred microliters of the beads were collected and washed with sodium phosphate buffer (PBS; 10 mM, pH 7.4) in a 1.5 ml Eppendorf tube. Subsequently, 60 μl hIgG (1 mg/ml) was added, and the resulting preparation was mixed with 60 μl ammonium sulfate solution (3 M). The mixture was kept for two days at 4 °C with gentle shaking. After incubation, the beads were separated from the mixture using a magnetic stand. The supernatant was removed, and the hIgG-coated beads were washed three times with 1 ml PBS/0.1–0.5% Tween-20 (PBST 0.1–0.5%).

Each round of biopanning consisted of three steps: (1) positive selection to screen the binding phages, (2) amplification, and (3)

negative selection or 'subtraction' to remove non-specific phages. Total of five rounds were performed; five steps of positive selection, four steps of amplification, and three steps of subtraction, To screen the 'positive' or specifically binding phages, 10 μl of a 1.5×10^{13} pfu phage solution were added to the hIgG-coated beads after blocking the beads with 1 ml PBS/0.5% BSA for 30 min at RT. The mixture was incubated for 1 h at 4 °C with gentle shaking. Next, unbound phages were removed by three washes with 0.1% PBST buffer. Lower Tween concentrations in early rounds resulted in higher eluate titers; therefore we increased the Tween concentration stepwise in each round to a maximum of 0.5%. The bound phage particles were eluted with 0.1 M citrate (pH 3.1) and immediately neutralized with 1 M Tris–HCl (pH 9.1). The eluate was titered and amplified. For 'amplification', the selected phage clones were mixed with 20 ml *E. coli* ER2738 culture and incubated at 37 °C with vigorous shaking for 4.5 h. After incubation, the culture was centrifuged. The phage particles in the supernatant were precipitated by adding 20% PEG/2.5 M NaCl, and then the precipitated phages were suspended in PBS (pH 7.4). The phage titer was evaluated by performing a blue plaque-forming assay using an agar plate containing isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Before proceeding to the next round of biopanning, non-specific phages that were included in the selected phages were removed by a 'subtraction' (or negative selection) step by using magnetic particles without a hIgG coating ('nude' magnetic particles). The supernatant from this subtraction step was introduced to the next round.

In addition to the conventional, regular biopanning method, we tried a 'modified' screening method for two reasons: (1) to skip the amplification step of positive phage particles that usually takes one day and (2) to apply stringent selection pressure continually in each round to avoid potential co-amplification of non-specific clones. The differences between the conventional method and the modified method are illustrated in Fig. 2. Stringent conditions (high selective pressure) were applied continually from the first to the fifth rounds of biopanning in the modified scheme. For screening large numbers of candidate clones, the modified method could be a more efficient method than the conventional method.

2.4. ELISA evaluation of bound phages

Microwells were coated with 2 μg/ml of BSA (as a negative control) and hIgG for 2 h at 4 °C, washed with 0.05% PBST, and blocked with a blocking buffer (0.2% BSA) for 1 h at RT. Phage particles were diluted and blocked in the blocking buffer for 30 min before they were added to the wells. Incubation was performed for 1 h at RT. After another wash with 0.05% PBST, HRP-anti-M13 antibody conjugate (diluted 1:2000 in the blocking buffer) was added, and the plate was incubated for 1 h at RT. Subsequently, the plates were washed six times with PBST, followed by the addition of ABTS (azino-bis(3-ethylenezothiazole sulfonic acid) diammonium salt) substrate and H_2O_2 for 20 min. The absorbance at 405 nm was measured with a microplate reader.

After five rounds of positive panning and three rounds of subtraction screening, a total of 96 single plaques were selected randomly from the third, fourth and fifth eluates (30, 30, and 36 clones, respectively) for ELISA. The binding affinity of phage particles to hIgG was assessed in terms of the dissociation constant (K_d). The K_d value is a quantitative measure of the binding strength and the stability of a biomolecular complex (Goodrich and Kugel, 2007). The K_d values of the various phages were determined by ELISA using different concentrations of phage particles (0.3125–20 nM by serial dilution). The molar concentration of phage particles was calculated by dividing the number of phage particles (PFU) by Avogadro's number.

2.5. DNA and amino acid sequence analysis of the candidate clones

DNA sequencing of the positive phage clones (total 96) was performed at Bionics, Inc. (Seoul, Korea). To look for any consensus sequence among them, multiple sequence alignment program CLUSTAL X (version 1.8) was used (www.clustal.org). Based on the results of the previous ELISA evaluation, four clones were selected for peptide synthesis following the corresponding amino acid sequences (Peptron, Inc., Daejeon, Korea). The synthetic 12-mer peptides contained a linker sequence, GGGS, and were labeled with biotin at the C-terminus; X_{12}—GGGS—biotin. Here, X_{12} represents 12 amino acids, G represents glycine, S represents serine, and —GGGS— is the spacer sequence of the peptide.

2.6. SPR of biotinylated peptides and anti-hIgG

The binding affinities of the biotinylated synthetic peptides for the target antigen (hIgG) were evaluated quantitatively by SPR. SPR experiments were performed using a BIAcore 3000 equipped with a streptavidin chip (SA chip). To compare a peptide's binding activity with that of the native antibody, anti-hIgG antibody was also biotinylated by adding 13.3 mmol EZ-Link® Sulfo-NHS-LC-Biotin followed by 2 h incubation on ice. Excess unreacted biotin and reaction by-products were removed by dialysis (MWCO 3500). Biotinylated anti-hIgG was purified using the Pierce Monomeric Avidin Kit following the manufacturer's protocol. Finally, the solution concentration of anti-hIgG-biotin conjugate was determined by reading the absorbance at 595 nm.

For all SPR kinetic experiments, all samples were resuspended in a running buffer (PBS pH 7.4). Before the ligand immobilization procedure, the SA chip was first cleaned with three consecutive 1 min injections of 30 μl 1 M NaCl solution in 50 mM NaOH. Biotinylated molecules (peptides and antibody), diluted in the running buffer to 20 μg/ml, were injected over a SA chip surface for 7 min at a rate of 10 μl/min. The maximum immobilization level was reached after two injections. For analyte injection, hIgG sample (800 nM) was injected in triplicate for 2 min at 30 μl/min. A dissociation step was then performed for 1 min at the same flow rate. Finally, the chip was regenerated by a 60 s injection of 25 mM NaOH at 30 μl/min.

2.7. Immobilization of peptides and anti-hIgG on the magnetic beads

Anti-hIgG was immobilized to the magnetic particles (MPs) by amine coupling. Two milligrams of carboxyl surface MPs (COOH-MP) were washed with a washing buffer three times and re-suspended in a freshly prepared solution of 1 M EDC and 0.2 M NHS. The MPs were separated by a magnetic bar, washed, and reacted with diluted anti-hIgG antibody for binding at RT. After binding, non-specific sites on the MPs were blocked by adding 1 M ethanolamine (pH 8.0) for 15 min. The reaction conditions such as pH, reaction time, and the maximum level of immobilized molecules on the beads were optimized. The incubation times tested were 30, 60, 70, and 120 min. The buffer systems tested were 50 mM MES (pH 6.0), 0.1 M $NaHCO_3$ (pH 8.0), and 0.1 M $NaHCO_3$ (pH 10.0).

Peptide D1, that was selected based on its high affinity for hIgG (SPR experimental results), was immobilized on the MPs. Two milligrams of SA-MPs were rinsed three times in a washing buffer. Diluted peptide D1 was added to the solution containing the SA-MPs and the mixture was gently stirred at RT. Similar to the amine coupling method, conditions for peptide immobilization to the surface of the SA-MPs were optimized by evaluating various reaction

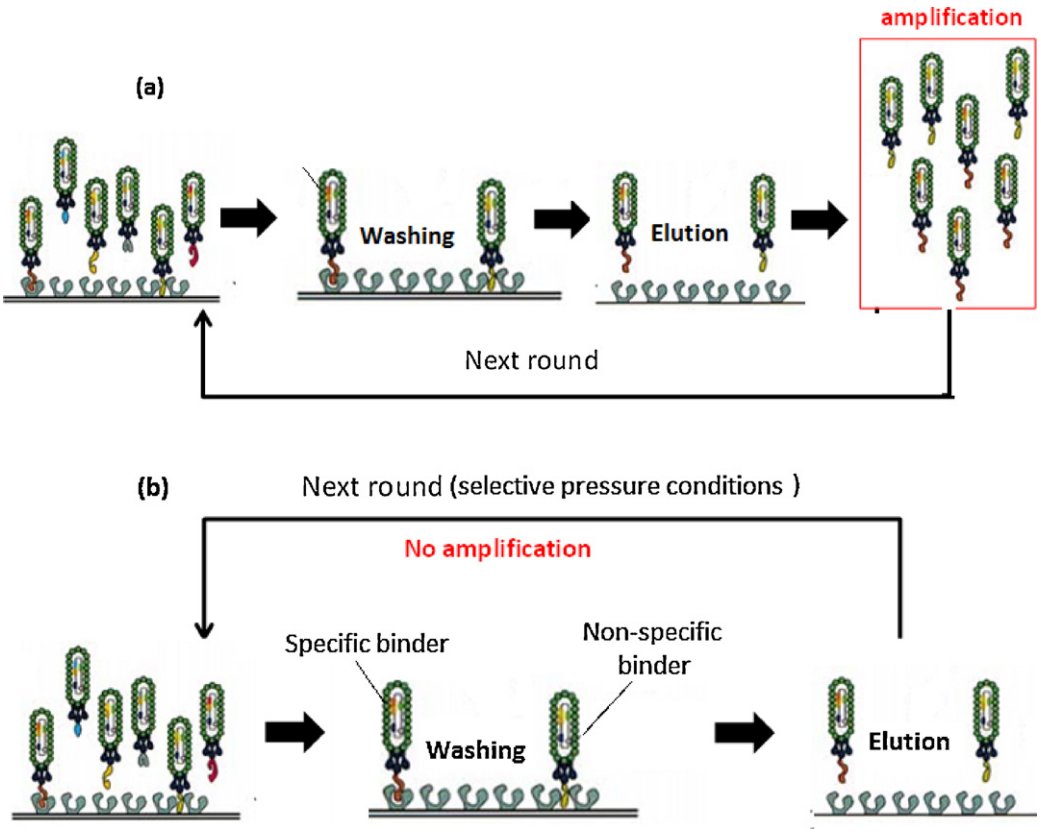

Fig. 2. Schematics of the two biopanning methods used in this study. (a) Regular biopanning and (b) modified biopanning.

times (2, 3, or 4 h), buffer conditions (50 mM MES, pH 6.3; 10 mM PBS, pH 7.4; 100 mM $NaHCO_3$, pH 8.4) and peptide concentrations.

2.8. Determination of immuno-magnetic binding efficacy

One hundred micrograms of hIgG was reacted with the antibody-immobilized COOH-MPs and peptide-immobilized SA-MPs at RT with gentle stirring. Binding was investigated using a simple fluorescent assay using o-phthalaldehyde (OPA) reagent. The OPA assay was performed using 96-well black flat-bottomed plates. The plates were incubated in dark and the fluorescence of excitation at 330–390 nm and emission at 436–475 nm were measured every 1 min. The results from 5-min readings were analyzed. Purified peptide, anti-hIgG, and hIgG standards with concentrations ranging from 25 to 200 μg/ml were freshly prepared. Samples were tested at least in triplicate. Standard curves were generated by linear regression analysis (regression coefficient (R^2) greater than 0.98), and the reacted amounts in each test sample were determined from the linear equation.

3. Results and discussion

3.1. Screening of positive phages with and without amplification

To select hIgG-binding phages from the phage peptide library, five rounds of biopanning were performed. Seventy-five microliter aliquots were taken from each round; aliquots of the initial input and output samples of each round were titered for phage particles, and 60 μl of the 75 μl was used for the next amplification step. The output/input ratio based on the number of plaque-forming units (pfu) before and after each round was determined in order to monitor the progress of each round of biopanning by calculating the phage recovery efficiency (Bonnycastle et al., 2000). The phage recovery efficiency increased from 1.51×10^{-6} to 3.98×10^{-4}. After the second, third, fourth, and fifth rounds, the output/input ratio increased about 2-, 6-, 21-, and 265-fold, respectively (Table 1). This indicates an obvious enrichment in the specific binding capability of the phages (Fig. 3(a)). To shorten the screening time as well as to avoid co-enrichment of non-specific clones during the amplification steps, a 'modified' method was carried out by skipping an amplification step after each round. As a result, the number of phage particles was reduced by about 4360-fold after the fifth round (Fig. 3(b)). We expected that the clones identified from the last round would have the highest binding affinity for the target hIgG.

Ninety-six clones from the regular biopanning screens and four clones from the modified biopanning screens were randomly picked after five rounds of screening. All selected clones were subjected to ELISA analysis and DNA sequence analysis (Bionics, Korea). Twelve regular biopanning clones and four modified biopanning clones had the ability to bind specifically to hIgG (Fig. 4(a) and (b)). Their amino acid sequences and frequency of appearance are shown in Table 2. A sequence comparison of all selected 4 clones from the modified biopanning and 96 clones from the regular biopanning revealed no specific consensus sequence, and these peptides showed no sequence homology to anti-hIgG

Table 1
Assay results of plaque selection in each round (regular panning).

Round	Number of input phages	Number of output phages	Ratio (output/input)
1	1×10^{11}	1.5×10^{5}	1.51×10^{-6}
2	1×10^{11}	2.06×10^{5}	2.06×10^{-6}
3	1×10^{11}	9.37×10^{5}	9.37×10^{-6}
4	1×10^{11}	3.19×10^{6}	3.19×10^{-5}
5	1×10^{11}	3.98×10^{7}	3.98×10^{-4}

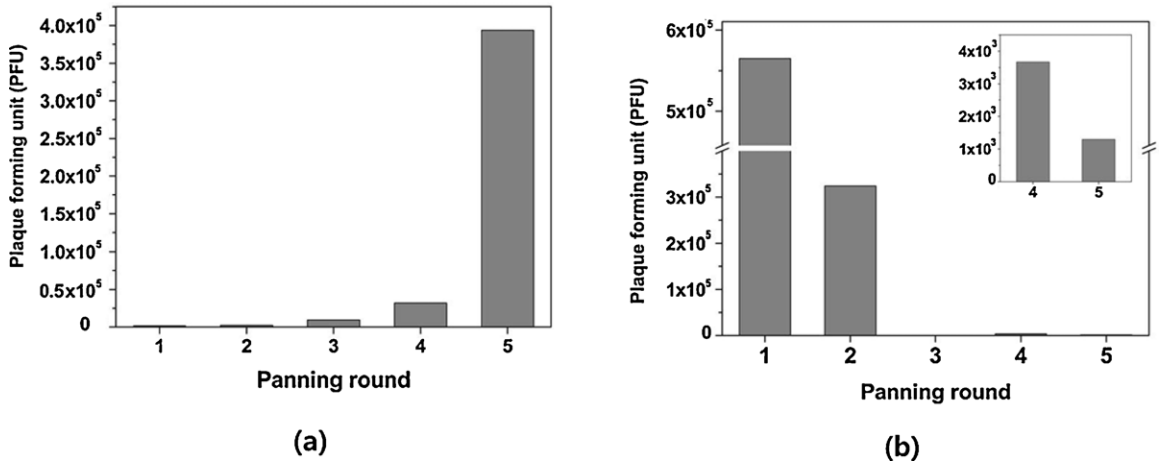

Fig. 3. Change in the number of phage particles in: (a) regular biopanning with enrichment and (b) modified biopanning without amplification.

either. Similar results were reported when using rabbit IgG as a target; no sequence homology was observed between the screened peptides and a native antigen epitope (Tang et al., 2005). Some relatively high frequency clones, for example F6, G12 and H12 (all 25% frequency) and H7 and H9 (both 5.8% frequency) were all from the 5th round and shared the same sequence. In the regular biopanning, positive clones were amplified and forwarded to the next rounds, so the randomly selected clones could have the same sequence. A10 (from the 3rd round) and G5 (from the 5th round) shared the nearly identical sequence, except one residue.

As shown in Fig. 4(a), the absorbance at 405 nm of the wells coated with the four clones identified using the conventional biopanning procedure (F9, A10, G5, and D1) was much higher than those obtained for the other clones, indicating more specific binding ability of these four clones. It was interesting to note that their frequency (2.8%) was relatively lower than the others. The MA-2 clone from the modified biopanning was more specific for hIgG than the other modified biopanning clones (Fig. 4(b)). Based on these results, a second ELISA was performed to compare the binding affinity of the three selected clones from the regular and one from the modified biopanning. Interestingly, the MA-2 clone had a higher binding affinity than the D1 and G5 clones, while the F9 clone had the highest affinity of the four clones tested (Fig. 4(c)).

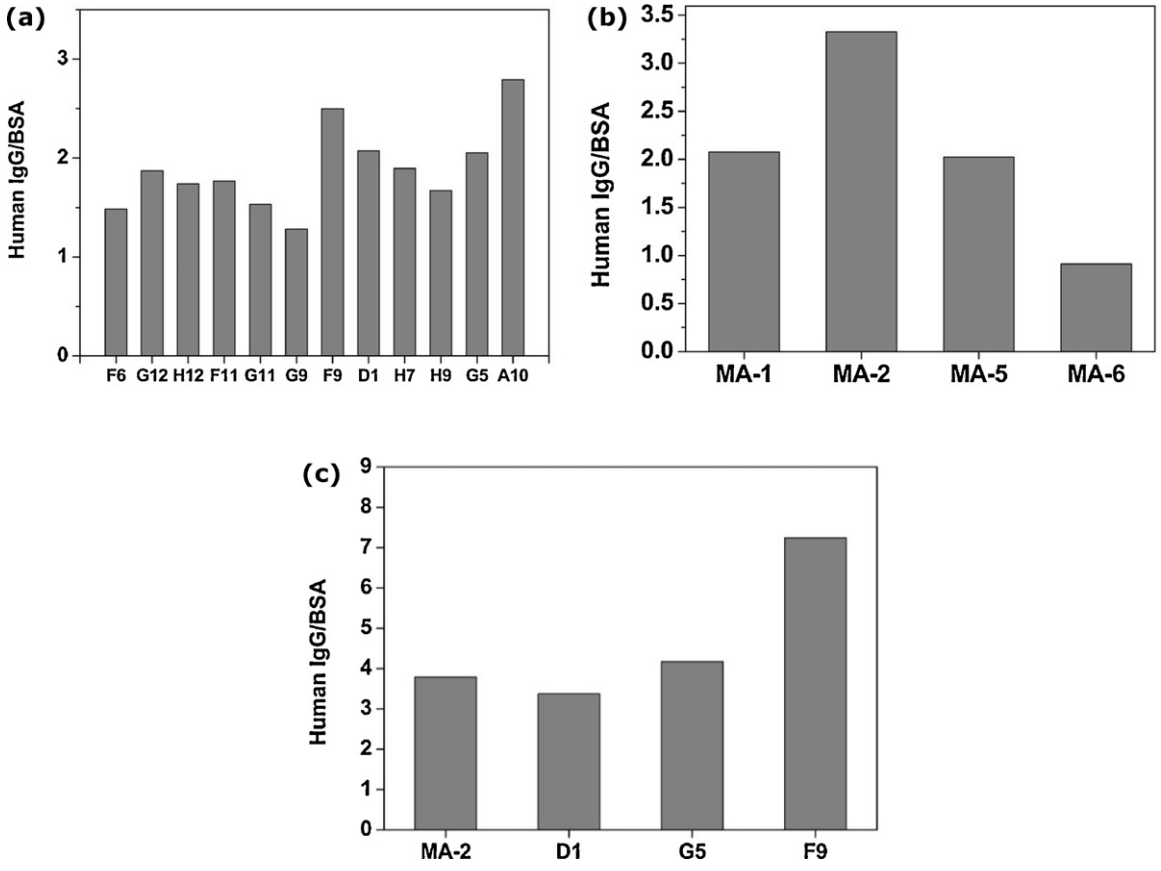

Fig. 4. ELISA analysis of hIgG-binding (or positive) phages screened from: (a) regular biopanning, (b) modified biopanning, and (c) the second ELISA.

Table 2
Amino acid sequences of the phage clones showing immunobinding affinity to hIgG.

Biopanning method	Phage clone	Amino acid sequence (N → C)	Frequency (%)
Regular	A10	HPTTNTAHFFIT	2.8
	D1	SPVEALLTHRLH	2.8
	F11	NPIEQMLDYAKS	10.2
	F6	DHYAPERRPFFQ	25
	F9	SPVESRLHSNSS	2.8
	G11	NMVERMIERPSR	10.2
	G12	DHYAPERRPFFQ	25
	G5	HPTTNTADFFIT	2.8
	G9	NPVEMWSEMQHI	10.2
	H12	DHYAPERRPFFQ	25
	H7	TAHPTYKPPSPN	5.8
	H9	TAHPTYKPPSPN	5.8
Modified	MA-1	YDNGFSNSHMPL	
	MA-2	QPLEHASHLTDV	
	MA-5	HIDGTSLTLKKT	
	MA-6	KAFAGTHTDTIT	

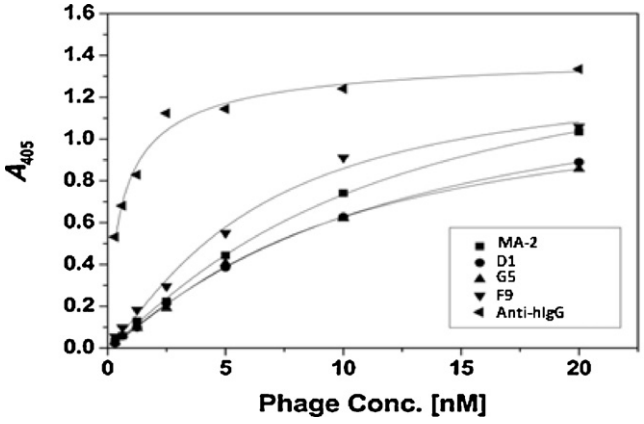

Fig. 5. hIgG binding isotherms of the four positive phage clones and anti-hIgG.

3.2. ELISA analysis and amino acid sequences of selected phage clones

Binding isotherms for the four phage clones (F9, G5, D1, and MA-2) and the control (anti-hIgG) were prepared using a broad spectrum of known concentrations of the phages (0.3125–20 nM). All four clones were able to bind specifically to hIgG, but with lower affinity than that of the native anti-hIgG antibody (Fig. 5). The K_d values of the four phage candidates were in the nanomolar range (Table 3). K_d indicates the binding affinity and can be determined from the Hill equation; it was obtained by nonlinear regression analysis using OriginPro software (version 8.1). F9 showed only one order of magnitude lower affinity for hIgG than the native antibody.

Four peptides were chemically synthesized following the DNA sequences of the four positive clones (F9, G5, D1, and MA-2) (Peptron, Inc., Daejeon, Korea). The amino acid sequences, MW, and calculated pI values of the four peptides are shown in Table 4. There was no consensus sequence; however, D1 (from the 4th round) and F9 (from the 5th round) shared the first 4 residues; SPVE. All the peptides had proline residues. Proline is known to act as secondary structure disruptors in peptides and often is the first residue of α-helices or the edge strand of β-turns (Daly et al., 2006). Another report suggested that a β-turn conformation is often found in the binding domains of peptides (Uchiyama et al., 2005). This may be important to consider when designing antibody-mimicking peptides, which are usually short peptides that frequently adopt a linear conformation and as a result could lose some important biological features.

Table 3
K_d values of the four selected phages.

Phage	K_d (nM)	Standard error	R^2
MA-2	11.2	1.27	0.9992
D1	11.9	0.95	0.9997
G5	9.30	1.55	0.9976
F9	6.21	1.48	0.9898
Anti-hIgG	0.66	0.15	0.9796

Table 4
Amino acid sequences and properties of the four selected peptides.

Peptide	Phage clone	Amino acid sequence	MW	pI
Peptide F9	F9	SPVESRLHSNSS	1578	7.9
Peptide G5	G5	HPTTNTADFFIT	1623	4.9
Peptide MA-2	MA-2	QPLEHASHLTDV	1604	5.1
Peptide D1	D1	SPVEALLTHRLH	1631	8.0

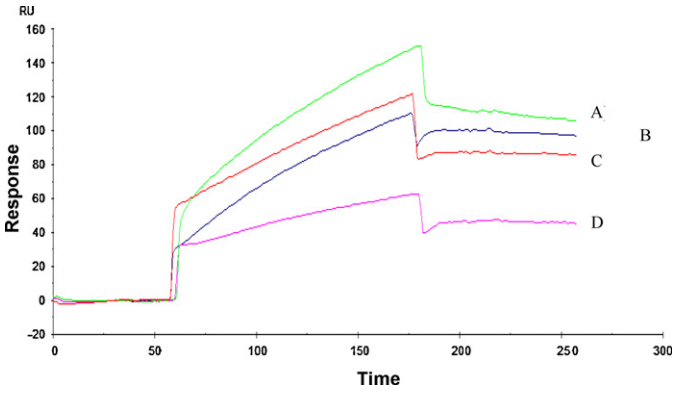

Fig. 6. SPR sensorgrams of four synthetic biotinylated peptides for hIgG interaction. Sensor chip surface was immobilized with: (A) D1, (B) F9, (C) G5, and (D) MA-2 peptide. Injection of 800 nM hIgG at 30 μl/min for 30 min.

3.3. SPR evaluation of the immunobinding ability of the synthesized peptides

SPR was used to compare the binding ability of the synthetic peptides to their target antigen. The four peptides and anti-hIgG were used as ligands, while hIgG was used as the analyte. SA chips were used for site-specific immobilization of the biotinylated ligands, i.e., biotinylated synthetic peptides as well as biotinylated antibody, to the sensor surface. After two injections, the SA chip surface was saturated with the ligand for maximum immobilization. The experimental results are shown in Fig. 6, while the immunobinding affinities of the four peptides and anti-hIgG toward hIgG are summarized in Table 5.

The SPR biosensor analysis suggested that the binding affinity of the biotinylated peptides was lower than that of anti-hIgG. In fact, the interaction affinity of some antibody-mimicking peptides was much weaker than that of the native antibody (Zhuang et al., 2001). Peptide D1 showed the highest affinity to hIgG by SPR, even

Table 5
SPR measurements result on the relative binding affinities of the four selected peptides.

Peptide	Immobilized (mol) (1)	Bound (mol) (2)	Binding ratio (1)/(2)	Relative affinity
Anti-hIgG	4.52×10^{-16}	5.76×10^{-16}	0.78	1.0
Peptide F9	3.60×10^{-13}	5.02×10^{-14}	7.15	0.11
Peptide G5	3.85×10^{-13}	4.35×10^{-14}	8.84	0.09
Peptide MA-2	2.11×10^{-13}	2.34×10^{-14}	9.01	0.09
Peptide D1	2.34×10^{-13}	5.67×10^{-14}	5.24	0.19

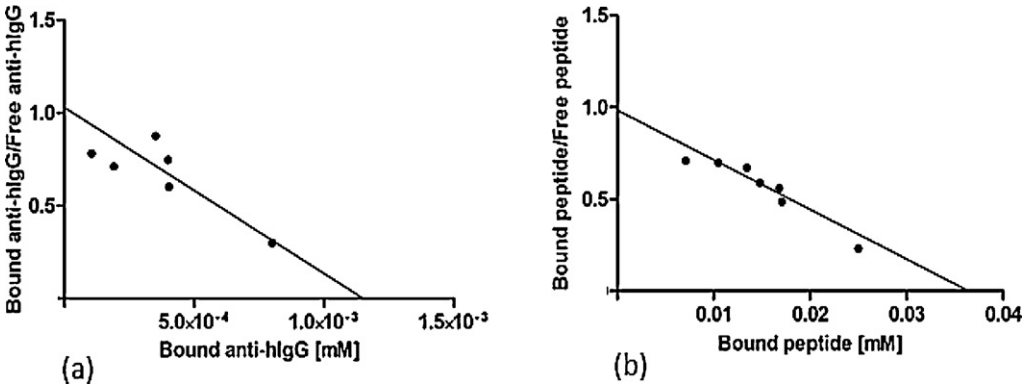

Fig. 7. Scatchard plots describing the binding of: (a) anti-hIgG antibody to NHS/EDC-activated COOH-magnetic particles and (b) D1 peptide to SA-coated magnetic particles.

though the phage clone F9 showed the highest affinity by ELISA. The F9 and D1 peptides shared the common sequence of SPVE in the N-terminus (SPVEXXXXXXXX), but we were not able to conclude that this sequence was responsible for antigen-binding affinity based on our data. Furthermore, different conditions between high-MW phage clones and low-MW peptides and different analytical tools (ELISA and SPR) could underlie the observed differences in binding. We chose to select peptide D1 as the candidate peptide for immobilization on the magnetic beads for immuno-magnetic separation of an antigen.

3.4. Immobilization of synthetic peptides and anti-hIgG antibody to magnetic beads

To immobilize the anti-hIgG to the NHS/EDC-activated COOH-MPs, we used an amine coupling method. D1 peptide was conjugated to SA-MPs by the affinity interaction between streptavidin and biotin. The biotin–streptavidin interaction is one of the strongest non-covalent interactions utilized in biological separations ($K_d = 10^{-13}$ to 10^{-16} M) (Green, 1990), and thus widely used to immobilize biotinylated molecules to a streptavidin support.

The optimum pH for the immobilization of anti-hIgG onto the activated COOH-MPs was determined to be pH 8.0 (data not shown), and immobilization reached saturation levels after 60 min incubation. The same conditions were identified in our previous study (Kang et al., 2007). Adsorption isotherms of anti-hIgG binding to the MPs were established. Adsorption was evaluated using Scatchard analysis. This analysis is widely used to investigate the characteristics of an adsorption process because it provides an insight into isotherm characteristics in a simple manner (Chaouk and Hearn, 1991). We used Scatchard analysis to estimate both the dissociation constant, K_d, and the total number of possible binding sites of the receptor, B_{max}. For the equilibrium of R (unoccupied receptor) + L (free ligand) $\leftrightarrow RL$ (complex), K_d can be expressed as $K_d = [R][L]/[RL]$. B_{max} equals the number of unoccupied receptor sites ($[R]$) plus the number of occupied sites ($[RL]$); i.e., $B_{max} = [R] + [RL]$ (Lehninger et al., 2008). The ratio of bound ligands to free ligands can be written as:

$$\frac{[RL]}{[L]} = \left(\frac{1}{K_d}\right)(B_{max} - [RL])$$

K_d and B_{max} were obtained from the slope and intercept of the linear equation; they were 1.0×10^{-6} M and 1.1×10^{-3} mM, respectively (Fig. 7(a)).

The optimum conditions for the immobilization of peptide D1 to the SA-MPs were a pH of 7.4 and a 4 h reaction time at RT (data not shown). The optimum conditions for the biotin–streptavidin reaction are a neutral pH (Stefan et al., 2002; Gas et al., 2001) and a sufficient incubation time to stabilize the immobilization of biotinylated peptide to SA-MPs. Hence, these conditions were applied to establish the isotherm curve. The Scatchard plot of the peptide adsorption onto SA-MPs is shown in Fig. 7(b). The K_d and B_{max} were approximately 3.0×10^{-5} M and 3.2×10^{-2} mM, respectively. This is an approximately 30-fold higher B_{max} value than that of anti-hIgG to the COOH-MPs, indicating that the total number of possible binding sites of SA-MPs is much larger than that of COOH-MPs, most likely because of the size difference between the antibody and peptide. However, the 30-fold difference in K_d value indicates that the adsorption of biotinylated peptide to SA-MPs was less stable and weaker than that of antibody to COOH-MPs. The reason for this could be the difference in immobilization chemistry (covalent bond for the activated COOH-MP vs. affinity interaction for the streptavidin-coated MP). Also, the large difference in K_d values between the phage clones (in Table 2) and the MP-immobilized peptides and antibody (in Fig. 7) was because of the difference in ligand molecules themselves and their spatial flexibility.

3.5. Comparison of the immuno-magnetic binding efficacy of the synthetic peptide and native antibody

The amount of synthetic peptide immobilized to the SA-MPs was greater than that of anti-hIgG immobilized to COOH-MPs on a molar basis: ca. 9.3×10^{-8} mol/mg-MPs versus 2.6×10^{-10} mol/mg-MPs, respectively. The ligand-immobilized MPs were incubated with hIgG, and the immuno-magnetic binding capacity of the antibody-mimicking peptide D1 and the native antibody were evaluated and compared. As shown in Table 6, the amount of hIgG bound was similar for the two ligands; 2.8×10^{-10} mol/mg-MP for the antibody and 1.0×10^{-10} mol/mg-MP for the peptide, respectively. However, the mol-to-mol binding efficacy of the peptide was 1.08×10^{-3}, about 1000-fold lower than that of the antibody (1.08). We think that multipoint interaction capability of antibody molecules would

Table 6
Comparison of immunobinding efficacy of ligand-immobilized magnetic beads.

Ligand type	Immobilization method	Immobilized molecules (A) (mol/mg-MP)	Bound hIgG (B) (mol/mg-MP)	Binding efficacy[a]
Antibody	Amine-coupling	2.62×10^{-10}	2.82×10^{-10}	1.08
Peptide	Streptavidin–biotin	933×10^{-10}	1.01×10^{-10}	1.08×10^{-3}

[a] Binding efficacy: ratio of bound hIgG molecules to immobilized ligand molecules.

be responsible for the large difference. However, the lower binding efficiency of peptide-coated beads could be compensated for in large part by increasing the density of immobilized peptides on the magnetic beads.

4. Conclusions

To identify peptides able to mimic the immunobinding ability of anti-hIgG antibody, we used two screening methods: regular and modified biopanning methods. The modified method, which involves skipping an amplification or enrichment step, is faster than the regular method. One phage clone identified from the modified biopanning (MA-2) showed an affinity for hIgG as high as that of the four clones obtained from the conventional biopanning method. Among the four clones derived using the regular method, one clone (F9) showed only one order of magnitude lower affinity for hIgG than the native antibody (as determined by the K_d value). Based on the DNA sequences of the four clones, we synthesized four peptides that comprised 16-mer peptides including a 4-mer linker sequence and a biotinylated C-terminus. Among the four peptides, peptide D1 showed the highest binding affinity to hIgG based on SPR analysis. Thus, the D1 peptide was chosen as the candidate peptide for conjugation to streptavidin-coated magnetic beads. Comparing the immuno-magnetic binding efficacy of the D1 peptide and hIgG antibody, we found that the peptide-immobilized MPs had an approximately 1000-fold lower affinity for hIgG than the anti-hIgG-immobilized MPs on a molar basis. However, the binding efficacy of the peptide-immobilized beads could be improved by increasing the density of immobilized peptides on the magnetic beads. The cost benefits of using peptides rather than antibodies and the relative simplicity of handling small peptides compared to delicate and sensitive antibody molecules make antibody-mimicking peptides an attractive alternative to antibodies. In summary, the generation of novel peptides as a source of affinity ligands for a target antigen using the phage display technique and the application of these affinity ligands in immuno-magnetic separation of a target antigen can potentially yield cost-effective and robust immuno-separation processes.

Acknowledgements

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2008-0061855).

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