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An Efficient Method for Isolating Antibody Fragments Against Small Peptides by Antibody Phage Display

Zhi Duan and Henrik Siegumfeldt*

Department of Food Science, Faculty of Life Sciences, The University of Copenhagen, Denmark

Abstract: We generated monoclonal scFv (single chain variable fragment) antibodies from an antibody phage display library towards three small synthetic peptides derived from the sequence of α_{s1} -casein. Key difficulties for selection of scFv-phages against small peptides were addressed. Small peptides do not always bind efficiently to passive adsorption surfaces, and we developed a simple method to quantify the binding capacity of surfaces with the peptides. Background binding (the binding of scFvs to the background matrix) is an obstacle for successful selection, and we evaluated two methods that drastically reduced the background binding. An optimized method therefore enabled a panning procedure where the specific (peptide binding) scFv-phages were always dominant. Using 15-mer peptides immobilized on Nunc Immobilizer Streptavidin plates, we successfully generated scFvs specifically against them. The scFvs were sequenced and characterized, and specificity was characterized by ELISA. The methods developed in this study are universally applicable for antibody phage display to efficiently produce antibody fragments against small peptides.

Keywords: Phage display, scFv, Tomlinson I + J libraries, casein, peptide.

INTRODUCTION

Anti-peptide antibodies or antibody derivatives have a number of uses in biological research and bioassay, which can be applied for the identification, quantification and purification of proteins containing the peptide sequence chosen [1-3]. Due to the precise epitope location on native proteins recognized by anti-peptide antibodies, some investigators used them for the specific applications, such as to block adherence of cells [4], to inhibit enzyme activity [5, 6], to detect receptor binding region of target proteins [7], and to detect proteolysis [8, 9]. Compared to anti-protein antibodies, anti-peptide antibodies are more useful for protein-protein interaction study, because peptide antigen sequences are already defined and antibody sequences can be simply obtained by sequencing of monoclonal antibody genes. The antibodies directly produced by using peptides as antigens also save much time for epitope mapping.

The conventional method for producing antibodies is called hybridoma technology [10], by which monoclonal antibodies are obtained from immunized animals with a purified antigen. The methods for producing antibodies have been revolutionized in the last decades. The manipulation of genes encoding antibodies allowed to successfully construct antibody derivatives, which retain full antigen binding function, e.g. single-chain variable antibody fragment (scFv) [11]. Functional scFv expressed from a single cDNA sequence can be produced efficiently in bacteria without the need to immunize animals [12]. Since the pioneering work of Smith [13] and development by McCafferty *et al.* [14], the expression of scFvs on the surface of phage and panning of these phage libraries against target antigen has matured into an extensively used technique to produce recombinant antibodies for research purposes and for the development of therapeutics [15, 16]. The production and selection of scFvs

on phage surface is much faster compared to conventional hybridoma technique. The availability of large and diverse antibody gene repertoires in phage [17, 18] has provided a source of scFvs to almost any antigens. The antibodies produced by hybridoma technology as animal antibodies immunogenic for human cannot be directly used as therapeutic agents, whereas human antibody libraries can be established on the phage surface [19, 20]. Furthermore, affinity maturation (increasing the affinity and specificity) of recombinant antibodies is applicable and simple by using antibody phage libraries [21, 22].

The primary aim of this study was to select antibodies (scFvs) against small peptide fragments by antibody phage display. These peptide sequences traverse cleavage sites of different enzymes responsible for cheese ripening. The selected antibodies can subsequently be utilized in studies of cheese ripening, as the antibodies will bind to the intact peptide sequence, but after cleavage by enzymes, the antibody can no longer recognize the substrate.

Selection of scFv-phage libraries against big proteins is rather straightforward. In contrast, selection against small peptides has two primary challenges: (1) Efficient immobilization of peptides on solid surface, (2) Reduction of background binding scFv-phages. Background binding is the binding of scFvs to the background matrix (e.g., blocking proteins, or empty solid surface). In addition, as our target antigens are fragments from milk (α_{s1} -CN), the most popular blocking reagent, skim milk solution, was not applicable. In this study we solved the above difficulties and succeeded in generating scFv-phages against synthetic peptide fragments from α_{s1} -CN by selection of Tomlinson I + J scFv-phage libraries.

MATERIALS & METHODS

scFv-Phage Library, Bacteria, and Reagents

The Human Single Fold scFv libraries I + J (Tomlinson I + J), *E. coli* TG1 and KM13 helper phage [23] were kindly

*Address correspondence to this author at the Department of Food Science, Faculty of Life Sciences, University of Copenhagen, 1958 Frederiksberg C, Denmark; Tel: +45 35333286; Fax: +45 35333214; E-mail: hsi@life.ku.dk

Table 1. The Selected Peptides from α_{s1} -CN

Name	Peptide	Sequence	MW (g/mol)	Amino Acid Residues
F1	α_{s1} -CN f17-31	NENLLRFFVAPFPEV	1790.91	15
F2	α_{s1} -CN f150-163	FRQFYQLDAYPSGA	1661.77	14
F3	α_{s1} -CN f185-199	PIGSENSEKTTMPLW	1591.72	15

provided by MRC Centre (Cambridge, UK). All types of 96 well microplates were from Nunc A/S (Roskilde, Denmark). The HRP/anti-M13 phage conjugated antibody was from GE Healthcare. Unless otherwise stated, all the other commonly used reagents were from Sigma-Aldrich.

Introduction to Tomlinson I + J Libraries

Tomlinson I + J libraries are synthetic libraries displayed on M13 filamentous phage. 18 different amino acid positions in the antigen-binding sites are mutated to construct the highly diverse repertoire [24]. Two different mutation strategies result in library sizes of 1.47×10^8 (Library I) and 1.37×10^8 (Library J) [24]. The scFv-phage in Tomlinson I + J libraries is monomeric, which in practice means that only one copy of scFv is attached on each phage [25]. The bound phage can be easily eluted, because the c-myc epitope tag cleavable by trypsin is introduced between the scFv and pIII (phage coat protein) [26]. A successful multiplication of scFv-phages in the host requires the presence of a helper phage. The KM13 helper phage has another trypsin cleavage site in the engineered pIII, which renders KM13 non-infective after trypsin treatment [23], whereas the scFv-pIII fusion loses scFv protein but remains infective. The non-specific binding population in eluted phage is therefore dramatically reduced by trypsin treatment compared with the traditional eluting method (e.g., triethylamine eluting) in phage display panning [26, 27].

Synthetic Peptides

Three peptides were selected from α_{s1} -CN sequence (Table 1) named F1, F2 and F3, respectively. The peptides (including the biotinylated) were synthesized at 80% purity by JPT Peptide Technologies GmbH, Berlin, Germany.

E. coli TG1 growth and scFv-phage production is 2×TY media [16 g/l Tryptone (hydrolyzed caseins), 10 g/l yeast Extract, 5 g/l NaCl]. We replaced T/l yeast Extract, 5 g/l NaCl]. We replaced Tryptone with Peptone (Bacto), hereafter named PY medium. This replacement did not affect the growth (results not shown). The reason for the replacement was that we wanted to select scFvs against casein fragments, and Tryptone is a hydrolysate of casein, whereas Peptone is hydrolysate of animal tissue.

Immobilization Mechanism of Different Solid Surfaces

Immo-Amino incorporates amine reactive moieties that react with nucleophiles (e.g. primary amines and thiols) of proteins/peptides and thereby causes covalent immobilization. Immo-Strep utilises the strong interaction between streptavidin and biotinylated specific antigens.

Because the basic material of these reactive surfaces is Polysorp, intermolecular attraction forces caused by hydrophobic binding can also happen (Nunc Technical Bulletin No. 6, Nunc Tech Note Vol. 6 No. 41 and 43). Maxisorp is a passive adsorption surface without reactive moieties.

Immobilization of Peptides or Proteins on Different Solid Surfaces

Unless otherwise stated, all the proteins or peptides had the following concentrations for binding: peptides (non-modified and biotinylated) 20 μ g/ml, α_s -Casein 500 μ g/ml, Casein and BSA 1 mg/ml, fish gelatin 5 mg/ml.

Maxisorp: The peptides or proteins in PBS buffer (5.84 g/l NaCl, 4.72 g/l Na_2HPO_4 and 2.64 g/l $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, pH 7.2) were incubated overnight at 4°C.

Immo-Amino: The peptides in Na carbonate buffer (Na_2CO_3 3.18 g/l, NaHCO_3 5.86 g/l, pH 9.6) or proteins in PBS buffer were incubated 1-1.5 hr with gentle shaking at room temperature.

Immo-Strep: The surface was washed 3 times by 0.05% TPBS (Tween 20 in PBS), and biotinylated peptides in 0.05% TPBS were incubated 1-1.5 hr with gentle shaking at room temperature.

ELISA

To prepare ELISA, the surface with immobilized antigens was washed 3 times with 1% TPBS. scFv-phage at a concentration of 10^{11} pfu/ml in 1% TPBS was added and incubated for 1 hr. After 6 times washing with 1% TPBS, 1:5000 diluted HRP/anti-M13 phage conjugated antibody in 3% BSA-PBS was incubated for 1 hr. After 3 times washing with 1% TPBS, the phage binding was detected with TMB (tetramethylbenzidine). After 10 min, the reaction was stopped by the addition of 1M H_2SO_4 . The calculation of absorbance was performed by subtracting A_{620} from A_{450} (Thermo Labsystems Multiskan MCC/340).

Helper Phage ELISA to Detect Peptide Binding Yield on Different Solid Surfaces

The peptides (5, 20, 100 μ g/ml in PBS for Maxisorp, or in Na carbonate for Immo-Amino) and two control proteins (α_s -CN and BSA; 100 μ g/ml in PBS) were added into microtiter plate and incubated overnight at 4°C. After 3 times washing with PBS, helper phage at a concentration of 10^{10} - 10^{11} pfu/ml in PBS was added and incubated for 1.5 hrs. After 6 times washing with 1% TPBS, phage binding was detected with ELISA as described above.

Biotin-HRP to Detect Biotinylated Peptide Binding Yield on Immo-Strep

The biotinylated peptides (a series of concentrations in 0.05% TPBS) and biotin (100 µg/ml in 0.05% TPBS) as control were added into Immo-Strep plate and incubated 1.5 hr at room temperature. After 6 times of washing with 1% TPBS, Biotin-HRP (432040, Invitrogen) at a concentration of 20 ng/ml in 0.05% TPBS was added and incubated for 1 hrs. 6 times of washing with 1% TPBS was performed again, and the bound Biotin-HRP was detected by TMB and ELISA as described above

Elution Methods

Trypsin Elution (TE): After phage incubation and washing, 120 µl of 1 mg/ml trypsin in PBS solution was incubated onto each well for 10 min at room temperature with rotation. The supernatant was collected and used for further rounds of panning. The theory behind Trypsin Elution is explained above.

Background Absorption (BA): After phage incubation and washing, 120 µl per well of 0.1 M triethylamine (TEA) was added and following incubation for 10 min at room temperature with rotation, the TEA solution with the eluted phages (8 well replicates, totally 120×8=960 µl) was collected and mixed with 0.5 ml of 1 M Tris-HCl, pH 7.4. The prepared empty Streptavidin plate (blocked by 200 µg/ml biotin for 1 hr) was washed with 1% Tween 20 PBS. The eluted solution was then added to this empty plate and incubated for 1 hr with rotation at room temperature. The supernatant was collected and used for further rounds of panning.

Competitive Elution (CE): After phage incubation and washing, 120 µl of 25 µg/ml biotinylated peptide solution was incubated onto each well for 1 hr at room temperature with rotation. The supernatant was collected and used for further rounds of panning.

A schematic presentation of the elution methods is shown in Fig. (2).

Optimized Procedure for Panning of Phage Libraries Against Small Peptides Immobilized on Immo-Strep Surface

Tomlinson I and J libraries were used individually in parallel to perform the panning. The peptide solution (20 µg/ml in PBS, pH 7.2) was incubated 1~1.5 hr onto Nunc Streptavidin plates with 150 µl per well (each peptide had 8 replicates). After 3 washes by PBS with 1% TPBS, 120 µl scFv phage library (10^{12} - 10^{13} cfu/ml) in 1% TPBS was added to each well and incubated for 2 hrs. Wells were washed with 1% TPBS by 15 times for first round, 21 times for second and further round of panning. The bound phage was eluted by CE method as described above. For each peptide, 8 replicates of eluted phage were collected and mixed together. Then 500 µl of the eluted phage was used to infect 2 ml of exponential phase growing *E. coli* TG1, which were plated on TYE-Amp-Glu plates (TYE plates containing 100 mg/ml of ampicillin and 1% glucose) and incubated at 37°C overnight. The grown colonies were scraped into 4 ml of PY-Amp-Glu (PY broth containing 100 mg/ml of

ampicillin and 1% glucose). 200 µl of this scraped culture were used to inoculate 50 ml fresh PY-Glu-Amp and incubated with shaking at 37°C for 2 hrs to exponential phase. KM13 helper phage (5×10^{10} pfu) were added to 10 ml of each library culture and the mixture incubated at 37°C without shaking for 30 min. Infected cells were pelleted, resuspended in 50 ml PY-Amp-Glu with 50 µg/ml kanamycin, and incubated overnight with shaking at 30°C. scFv-phage particles were concentrated to 2 ml from each culture supernatant by precipitation with 20 ml polyethylene glycol in 2.5 M NaCl as described previously [14].

The concentrated phage were diluted in 1% TPBS (10^{12} - 10^{13} cfu/ml) using for the next round of selection. The phage library was subjected to four rounds of panning. The monoclonal scFvs were selected by monoclonal ELISA according to protocol from scFv-phage libraries' supplier. The monoclonal scFvs was considered positive if absorbance value is above 0.350.

BstNI DNA Fingerprinting

DNA fingerprinting with the BstNI restriction enzyme was used to determine the number of unique clones from the monoclonal scFv-phage ELISA. From wells that gave a high positive signal in monoclonal ELISA, bacteria (containing scFv-DNA in phagemid) were picked and added to 30 µl sterile water and incubated in a microwave oven for 1.5 min at 700 W. 5 µl was subsequently transferred to 15 µl PCR mix containing 2.5U Taq DNA Polymerase (Invitrogen), 0.2 µM forward primer LMB3 (5'-CAGGAAACAGCTATGAC-3') and 0.2 µM reverse primer pHEN (5'-CTATGCGGCCCCATTCA-3'), 0.2mM dNTPs, 1.5mM MgCl₂ and 2 µl 10 × PCR buffer (Invitrogen). PCR was carried out in 30 cycles (45 sec at 94°C, 45 sec at 50°C and 2 min at 72 °C). After amplification, the 1,000-bp scFv-cassette was digested with the enzyme BstNI (New England Biolabs). To 20 µl of the PCR product, the following was added: 17.4 µl H₂O, 2 µl 10 × NEB buffer 2, 0.2 µl (2U) BstNI and 0.2 µl 10 mg/ml BSA (bovine serum albumin). The mix was digested for 2 h at 60°C and restriction patterns were analyzed on a 3% agarose gel.

scFv Gene Sequencing

Three to eight clones from each DNA fingerprinting patterns were selected, and sequenced by IIT Biotech GmbH (Bielefeld, Germany). The bidirectional sequencing were performed with two primers LMB3 and pHEN for each clone, to ensure that the the scFv gene (1000 bp) were sequenced reliably.

RESULTS

Identification of Binding Capacity of Different Solid Surfaces with Small Peptides

Helper phage, detected by HRP / anti-M13 phage conjugated antibodies, was used to identify the binding capacity of tested surfaces with peptides. The empty surface with high absorbance shows that the surface was fully occupied by helper phage (Fig. 1A, B). On the contrary, the absorbance of surfaces exposed to two control proteins (CN and BSA) is low as these proteins prevent the phage from

binding to the empty surface. The amount of bound helper phage on the Immo-Amino or Maxisorp exposed to three peptides (F1, F2, F3) was almost as high as the empty surface (Fig. 1A, B), showing that the only a small fraction of the surface is occupied by peptides.

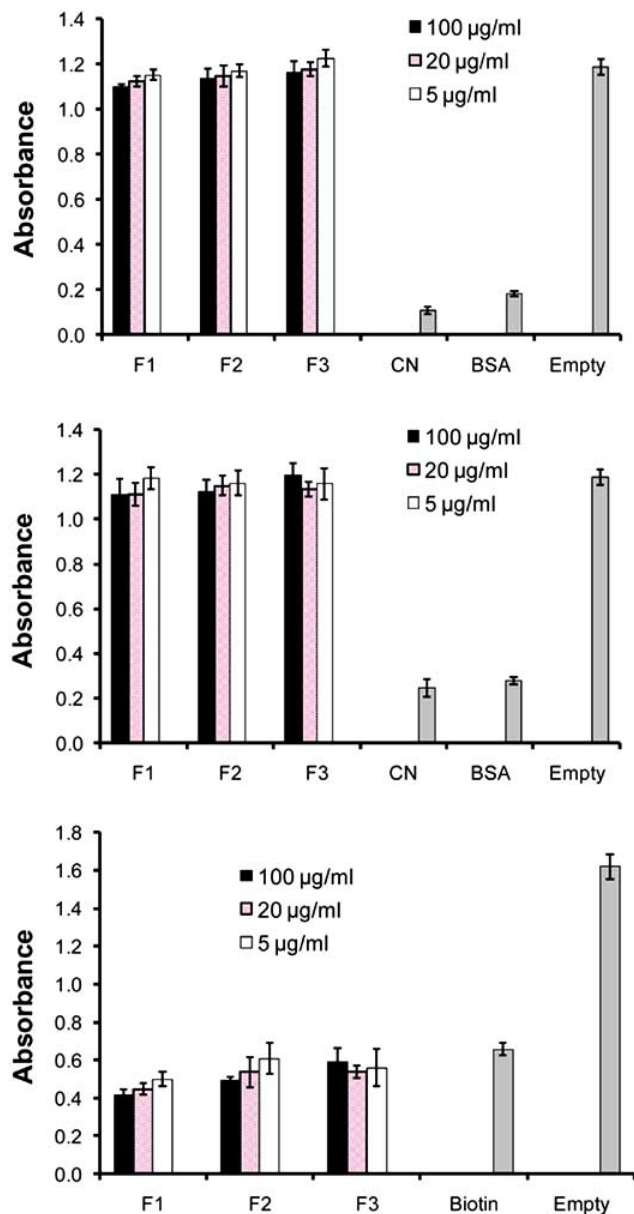


Fig. (1). Detection of binding capacity of different solid surfaces. The unoccupied surface was assayed, which means that a high absorbance translates to a poor binding capacity. The binding capacity on Maxisorp (A) or Immobilizer Amino (B) surface was tested by a helper phage ELISA. Casein and BSA was used as positive control and an empty surface as a negative control. Biotin-HRP was used to detect the binding of biotinylated peptides on Immo-Strep (C). Biotin was used as positive control and an empty surface as a negative control. The error bars represent the standard deviation of 4 replicates.

For investigation of binding to the Immo-Strep, Biotin-HRP was used to detect how much streptavidin already occupied by biotinylated peptides. The absorbance value represents the amount of Biotin-HRP bound on surface (Fig. 1C). The empty surface with high absorbance shows that the maximal absorbance reached 1.60, when the surface was fully occupied by Biotin-HRP. The Biotin-HRP used in the test has so strong binding ability to the Immo-Strep that even high concentration of biotin (100 µg/ml) could not completely block it. Consequently, an absorbance of 0.60 indicates that all the free streptavidin on the surface have been occupied. The absorbance of Biotin-HRP from surfaces exposed to biotinylated peptides was similar to absorbance of the surface exposed to biotin. This shows that the biotinylated peptides have occupied all binding sites of streptavidin.

Methods to Reduce Background Binding (Surface-Binding scFv-Phages)

Based on the above result, Immo-Strep was selected as the best surface for immobilization of peptides. However, even with Immo-Strep, the original panning procedure with the TE method (Fig. 2) was not successful (Table 3), because some scFvs (called anti-Polysorp in this study) that specifically bind to Polysorp were always dominant in the phage population after every cycle of panning. To reduce the amount of surface-binding scFv-phages and thereby enhance the amount of peptide-binding scFv-phages during panning procedure, it was necessary to develop an improved elution procedure. Two alternative methods for elution were tested, BA and CE as outlined in Fig. (2).

Every generated phage sub library by panning on peptide was added to both a surface coupled with peptide and an empty surface. The relative increase in peptide-binding scFv-phages was then calculated as the ratio between the two numbers (enrichment ratio) and is shown in Table 2. The results show that both BA and CE are much better than the original TE, because no enrichment is observed with the TE. CE is the elution method that has the highest enrichment ratio.

96 single colonies from the 4th round of panning using both CE and BA were selected at random, and tested against peptide F2 and empty surface in monoclonal phage ELISA. The specific monoclonal peptide-binding scFv-phage could be successfully isolated from phage population panned by using both BA and CE methods (Table 3). The percent of peptide-binding, surface-binding and others binding scFvs in total 96 colonies were calculated. It showed that CE had higher percent of peptide-binding, lower percent of surface-binding, which indicates that CE was more effective than BA.

Production of Monoclonal scFv-Phages Against Peptide F1 and F3

Panning of phage libraries on peptide F1 and F3 was also performed using the CE method. The eluted phage from peptide-binding or empty surface was titered after each round of panning. It was observed that panning of library J on both peptide F1 and F3 resulted in a high enrichment ratio after 4th panning, whereas panning of library I always had a

Table 2. Changing of Enrichment Ratio After Each Round of Panning on Peptides

Elution ^b	Peptide	Library	Enrichment Ratio ^a After Each Round of Panning			
			1	2	3	4
TE	F2	I	0.9	1.0	0.6	/
		J	1.1	1.3	0.9	/
SA	F2	I	1.5	1.2	2.3	1.7
		J	1.3	2.3	41.4	26.4
CE	F2	I	0.6	1.2	16.2	46.6
		J	1.1	2.8	51.8	75.6
	F1	I	1.1	3.2	1.6	5.0
		J	1.3	5.3	34.8	56.7
	F3	I	1.0	3.0	3.8	7.1
		J	0.9	4.0	37.5	290.0

^aThe generated phage sub library after each round of panning on peptide was added to both a surface coupled with peptide and an empty surface. Enrichment ratio was determined by the number of eluted phage from peptide coupling surface compared to that from empty surface.

^bTE: Trypsin Elution; CE: Competitive Elution; SA: Surface Absorbing.

low enrichment ratio (Table 2). Therefore, only the phage population from library J after 4th round of panning was selected for the monoclonal phage ELISA. The amount of isolated specific monoclonal phages is shown in Table 3. From the anti-F3 polyclonal phage sub library, we obtained 70 of 96 colonies against F3 and only 1 colony against the surface. This is corresponding with a high enrichment ratio of 290 determined by titering eluted phage (Table 2).

DNA Fingerprinting and Sequencing of scFv Genes

For each group of anti-peptide scFvs, the 29 strongest binding clones from the monoclonal ELISA (Table 3) were selected, and BstN1 DNA fingerprinting was then applied. Based on the restriction enzyme cleavage pattern (Fig. 3), 1 unique pattern was observed within the 29 anti-F1 scFv-phages, 2 patterns were revealed within anti-F2 scFv-phages, and 4 patterns were revealed within anti-F3 scFv-phages. Three to eight clones from each pattern were sequenced (Table 4 and Fig. 4), and the pattern 2 of anti-F3 exhibits two different scFv sequences, whilst all the other patterns possess only one unique scFv sequence. In the following, each monoclonal scFv will be abbreviated with the Roman numeral of its corresponding group.

Specificity of Monoclonal scFvs

To identify the specificity of the produced anti-peptide monoclonal scFv-phages, ELISA was performed on the monoclonal scFv-phages against different peptides and proteins. All anti-peptide monoclonal scFv-phages showed high affinity with their corresponding peptides (Fig. 5A), high binding ability with α_s -CN and CN, and no cross-reaction with the negative controls, BSA and gelatin (Fig. 5B). The anti-F1 and anti-F2 I scFvs had good specificity and very low binding with non-target peptides. The other monoclonal scFvs had little cross-reactions with non-target peptides. Anti-F3 II had the lowest cross-reaction within the anti-F3 scFvs.

Table 3. Distribution of Binding Profiles for Monoclonal scFv-Phages After 4 Rounds of Panning on Peptides

Elution ^b	Peptide	Library	Binding Profile ^a		
			Peptide	Surface	Unknown
TE	F2	I	0	41	55
		J	0	60	36
SA	F2	I	0	58	38
		J	55	18	23
CE	F2	I	60	15	21
		J	68	11	17
	F1	I	/	/	/
		J	29	4	63
	F3	I	/	/	/
		J	70	1	25

^aThe numbers of isolated monoclonal phages against peptide, empty surface, or unknown from 96 random selected single colonies.

^bTE: Trypsin Elution; CE: Competitive Elution; SA: Surface Absorbing.

DISCUSSION

The first requirement for successful generation of anti-peptide scFv-phage is to permanently immobilize the peptides on a solid surface. Small peptides normally do not have enough intermolecular attraction forces to exert passive adsorption. Although some researchers have used Maxisorp for immobilization of small peptides [28-31], we advise against this procedure for phage display panning. From our experience the small peptides are sometimes difficult to immobilize and are easily released from e.g. Maxisorp during phage display panning. It is therefore very important to check the binding capacity of small peptides to a selected surface before panning, e.g. by the methods shown in Fig. (1).

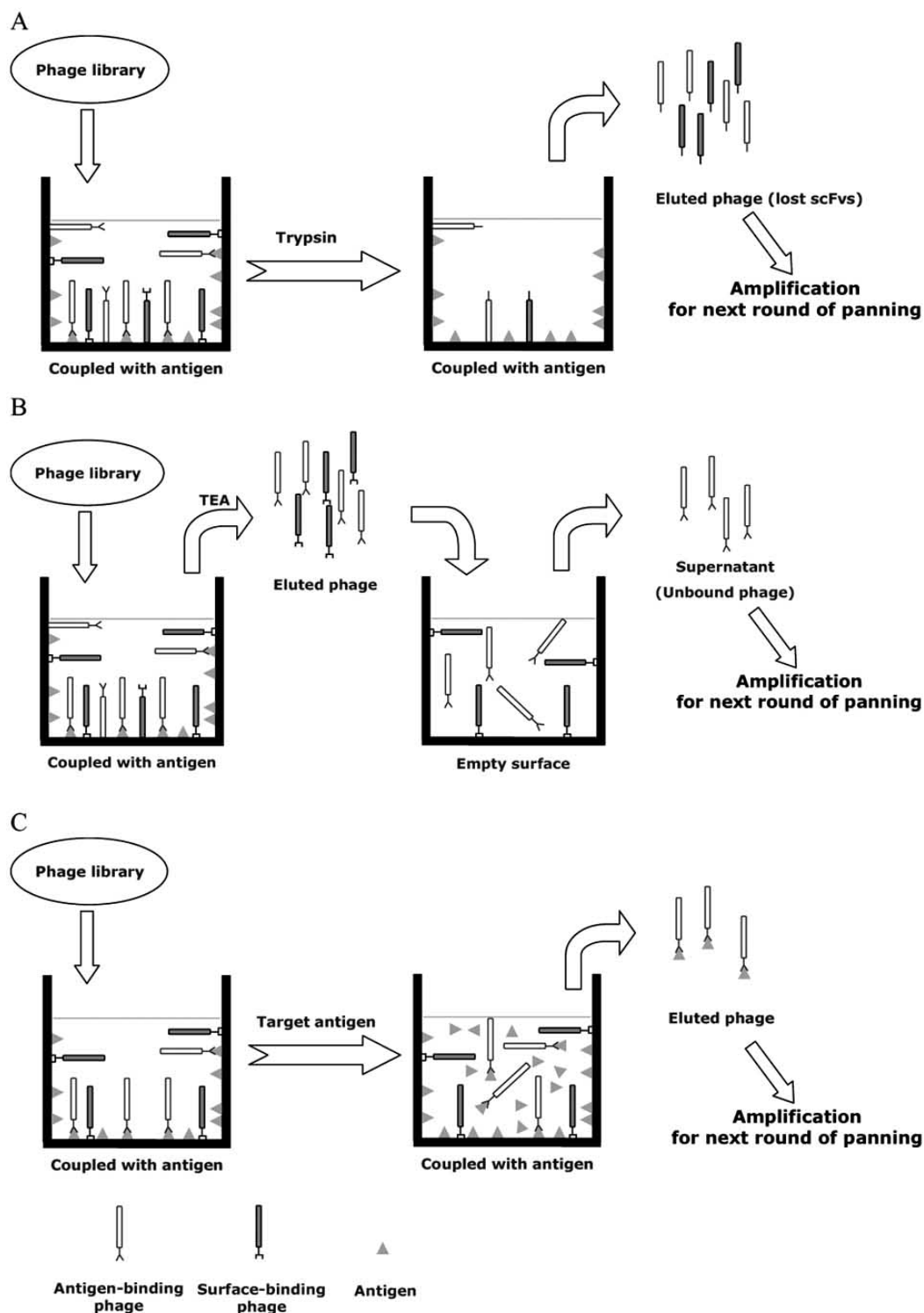


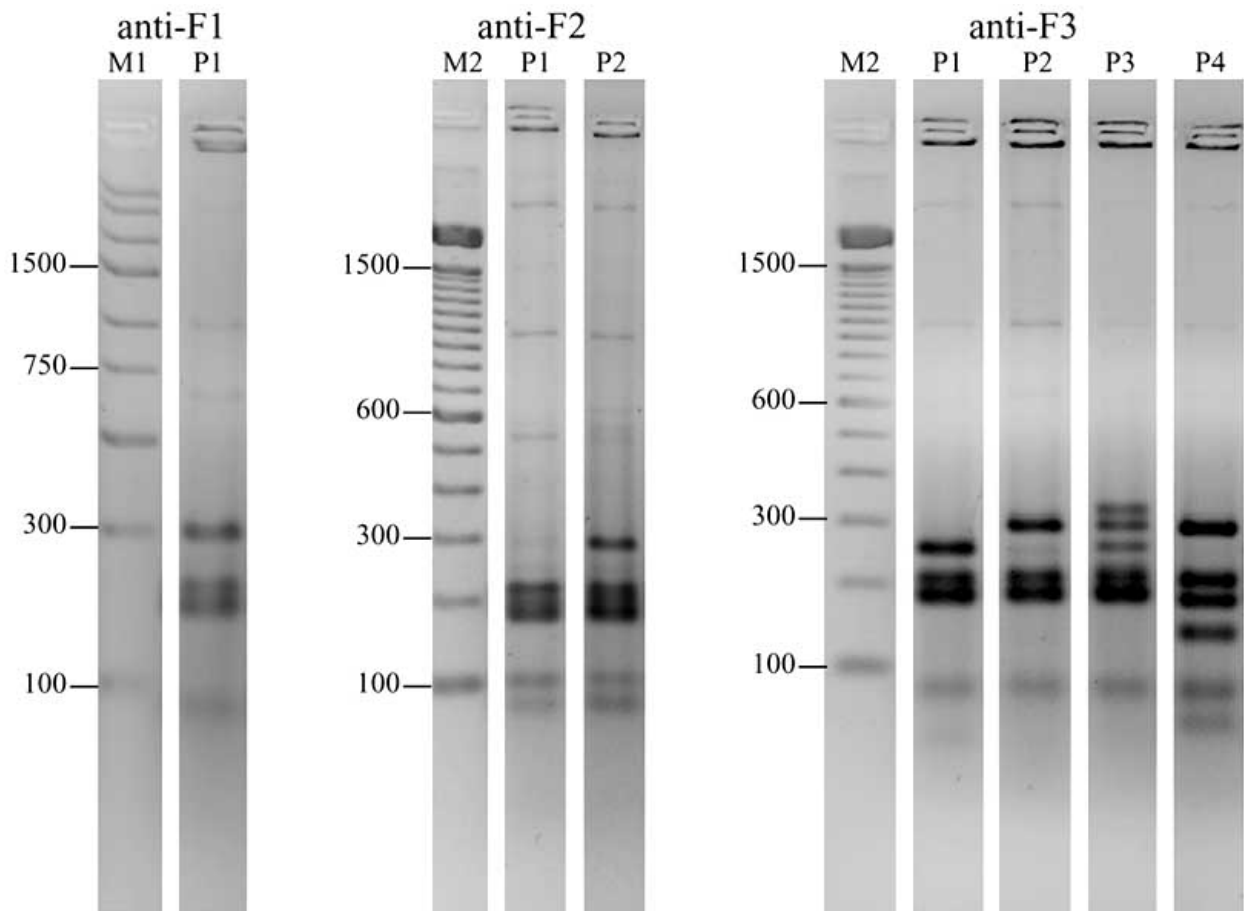
Fig. (2). Different methods for eluting bound phage. **(A)** Trypsin Elution (TE): Trypsin cleaves between the scFv and pIII of phage. Thus, all the scFvs-attaching phage (both antigen binding and surface binding) can be eluted, whereas phage-head-attaching phage is still bound to on surface. **(B)** Surface Absorption (SA): The eluted phage by TEA (triethylamine) (containing both antigen-binding and surface-binding phage) were incubated onto empty solid surface to reduce the surface-binding phage, and then the unbound phage population was collected for next panning. **(C)** Competitive Elution (CE): The elution of bound phages is performed by using high concentration of target antigen. The surface-binding phage should still be bound to surface.

We used helper phage to perform the binding capacity check in an ELISA. We knew from a previous study that the helper phage in PBS (without Tween 20) bind strongly to Maxisorp and Polysorp based surfaces and do not detach in

subsequent washing steps [32]. So, incubation and subsequent detection of the helper phage can reveal the level of empty positions on a surface already treated with peptides.

Table 4. Amino Acid Sequences of Selected Monoclonal scFvs

scFv	Pattern ^a	Group	Sequence ^b			
			CDRH2	CDRH3	CDRL2	CDRL3
anti-F1	P1	I	<u>S</u> <u>I</u> <u>Q</u> <u>Q</u> <u>L</u> <u>G</u> <u>R</u> <u>G</u> <u>T</u> <u>L</u> <u>Y</u> <u>A</u> <u>D</u>	<u>G</u> <u>A</u> <u>L</u> <u>S</u> <u>F</u> <u>D</u> <u>Y</u>	<u>Q</u> <u>A</u> <u>S</u> <u>R</u> <u>L</u> <u>Q</u> <u>S</u>	<u>Q</u> <u>Q</u> <u>N</u> <u>Y</u> <u>Q</u> <u>L</u> <u>P</u> <u>L</u> <u>T</u>
anti-F2	P1	I	<u>S</u> <u>I</u> <u>Q</u> <u>Q</u> <u>Y</u> <u>G</u> <u>K</u> <u>P</u> <u>T</u> <u>R</u> <u>Y</u> <u>A</u> <u>D</u>	<u>G</u> <u>S</u> <u>R</u> <u>N</u> <u>F</u> <u>D</u> <u>Y</u>	<u>H</u> <u>A</u> <u>S</u> <u>L</u> <u>L</u> <u>Q</u> <u>S</u>	<u>Q</u> <u>Q</u> <u>V</u> <u>G</u> <u>I</u> <u>R</u> <u>P</u> <u>V</u> <u>T</u>
	P2	II	n.a. ^c	n.a.	<u>S</u> <u>A</u> <u>S</u> <u>R</u> <u>L</u> <u>Q</u> <u>S</u>	<u>Q</u> <u>Q</u> <u>S</u> <u>R</u> <u>T</u> <u>R</u> <u>P</u> <u>T</u>
anti-F3	P1	I	<u>A</u> <u>I</u> <u>K</u> <u>G</u> <u>Q</u> <u>G</u> <u>A</u> <u>R</u> <u>T</u> <u>T</u> <u>Y</u> <u>A</u> <u>D</u>	<u>N</u> <u>Y</u> <u>A</u> <u>S</u> <u>F</u> <u>D</u> <u>Y</u>	<u>G</u> <u>A</u> <u>S</u> <u>W</u> <u>L</u> <u>Q</u> <u>S</u>	<u>Q</u> <u>Q</u> <u>I</u> <u>Q</u> <u>K</u> <u>H</u> <u>P</u> <u>A</u> <u>T</u>
	P2	II	<u>S</u> <u>I</u> <u>S</u> <u>S</u> <u>Q</u> <u>G</u> <u>K</u> <u>I</u> <u>T</u> <u>R</u> <u>Y</u> <u>A</u> <u>D</u>	<u>V</u> <u>D</u> <u>A</u> <u>G</u> <u>F</u> <u>D</u> <u>Y</u>	<u>N</u> <u>A</u> <u>S</u> <u>H</u> <u>L</u> <u>Q</u> <u>S</u>	<u>Q</u> <u>Q</u> <u>R</u> <u>T</u> <u>G</u> <u>K</u> <u>P</u> <u>P</u> <u>T</u>
	P2	III	<u>G</u> <u>I</u> <u>R</u> <u>S</u> <u>G</u> <u>Q</u> <u>R</u> <u>T</u> <u>Y</u> <u>A</u> <u>D</u>	<u>S</u> <u>K</u> <u>Q</u> <u>G</u> <u>F</u> <u>D</u> <u>Y</u>	<u>T</u> <u>A</u> <u>S</u> <u>T</u> <u>L</u> <u>Q</u> <u>S</u>	<u>Q</u> <u>Q</u> <u>P</u> <u>I</u> <u>G</u> <u>L</u> <u>P</u> <u>P</u> <u>T</u>
	P3	IV	<u>S</u> <u>I</u> <u>H</u> <u>G</u> <u>N</u> <u>G</u> <u>A</u> <u>L</u> <u>T</u> <u>P</u> <u>Y</u> <u>A</u> <u>D</u>	<u>P</u> <u>Y</u> <u>G</u> <u>T</u> <u>F</u> <u>D</u> <u>Y</u>	<u>G</u> <u>A</u> <u>S</u> <u>Q</u> <u>L</u> <u>Q</u> <u>S</u>	<u>Q</u> <u>Q</u> <u>I</u> <u>E</u> <u>E</u> <u>H</u> <u>P</u> <u>S</u> <u>T</u>
anti-Polysorp	P4	V	<u>S</u> <u>I</u> <u>K</u> <u>S</u> <u>T</u> <u>G</u> <u>G</u> <u>A</u> <u>T</u> <u>R</u> <u>Y</u> <u>A</u> <u>D</u>	<u>D</u> <u>V</u> <u>P</u> <u>K</u> <u>F</u> <u>D</u> <u>Y</u>	<u>N</u> <u>A</u> <u>S</u> <u>S</u> <u>L</u> <u>Q</u> <u>S</u>	<u>Q</u> <u>Q</u> <u>R</u> <u>H</u> <u>R</u> <u>F</u> <u>P</u> <u>L</u> <u>T</u>
		I	n.a.	n.a.	<u>H</u> <u>A</u> <u>S</u> <u>N</u> <u>L</u> <u>Q</u> <u>S</u>	<u>Q</u> <u>Q</u> <u>Q</u> <u>K</u> <u>R</u> <u>R</u> <u>P</u> <u>G</u> <u>T</u>
		II	n.a.	n.a.	<u>R</u> <u>A</u> <u>S</u> <u>S</u> <u>L</u> <u>Q</u> <u>S</u>	<u>Q</u> <u>Q</u> <u>M</u> <u>L</u> <u>R</u> <u>A</u> <u>P</u> <u>R</u> <u>T</u>

^aFrom Fig. (3).^bThe 18 randomly changed residues are bold and underlined. The complete sequence is shown in Fig. (4).^cn.a. - not applicable.**Fig. (3).** BstNI DNA fingerprinting of unique scFv genes. 29 clones from each group of anti-peptide scFv-phages have been tested. Only unique restriction patterns (P1-P4) are shown. M1, O'GeneRuler Express DNA ladder (Fermentas). M2, 100 bp DNA ladder (Invitrogen).

With the helper phage ELISA, we could not measure any immobilized peptides on Immo-Amino (Fig. 1). As a side comment, the peptides immobilized on Immo-Amino and Maxisorp could also not be detected by the later generated

anti-peptide scFv-phages (data not shown), which confirms our observation. In contrast, Casey *et al.* [33, 34] coupled a 20-mer peptide to Immo-Amino and detected this peptide with antibodies. We have also detected very strong binding

NcoI																	
GCC	ATG	GCC	GAG	GTG	CAG	CTG	TTG	GAG	TCT	GGG	GGA	GGC	TTG	GTA	CAG	CCT	GGG
A	M	A	E	V	Q	L	L	E	S	G	G	G	L	V	Q	P	G
GGG	TCC	CTG	AGA	CTC	TCC	TGT	GCA	GCC	TCT	GGA	TTC	ACC	TTT	AGC	AGC	TAT	GCC
G	S	L	R	L	S	C	A	A	S	G	F	T	F	S	S	Y	A
ATG	AGC	TGG	GTC	CGC	CAG	GCT	CCA	GGG	AAG	GGG	CTG	GAG	TGG	GTC	TCA	XXX	ATT
M	S	W	V	R	Q	A	P	G	K	G	L	E	W	V	S	X	I
CDRH2																	
XXX	XXX	XXX	GGT	XXX	XXX	ACA	XXX	TAC	GCA	GAC	TCC	GTG	AAG	GGC	CGG	TTC	ACC
X	X	X	G	X	X	T	X	Y	A	D	S	V	K	G	R	F	T
ATC	TCC	AGA	GAC	AAT	TCC	AAG	AAC	ACG	CTG	TAT	CTG	CAA	ATG	AAC	AGC	CTG	AGA
I	S	R	D	N	S	K	N	T	L	Y	L	Q	M	N	S	L	R
CDRH3																	
GCC	GAG	GAC	ACG	GCC	GTA	TAT	TAC	TGT	GCG	AAA	XXX	XXX	XXX	XXX	TTT	GAC	TAC
A	E	D	T	A	V	Y	Y	C	A	K	X	X	X	X	F	D	Y
XhoI																	
TGG	GGC	CAG	GGA	ACC	CTG	GTC	ACC	GTC	TCG	AGC	GGT	GGA	GGC	GGT	TCA	GGC	GGA
W	G	Q	G	T	L	V	T	V	S	S	G	G	G	G	S	G	G
Sall																	
GGT	GGC	AGC	GGC	GGT	GGC	GGG	TCG	ACG	GAC	ATC	CAG	ATG	ACC	CAG	TCT	CCA	TCC
G	G	S	G	G	G	G	S	T	D	I	Q	M	T	Q	S	P	S
TCC	CTG	TCT	GCA	TCT	GTA	GGA	GAC	AGA	GTC	ACC	ATC	ACT	TGC	CGG	GCA	AGT	CAG
S	L	S	A	S	V	G	D	R	V	T	I	T	C	R	A	S	Q
AGC	ATT	AGC	AGC	TAT	TTA	AAT	TGG	TAT	CAG	CAG	AAA	CCA	GGG	AAA	GCC	CCT	AAG
S	I	S	S	Y	L	N	W	Y	Q	Q	K	P	G	K	A	P	K
CDRL2																	
CTC	CTG	ATC	TAT	XXX	GCA	TCC	XXX	TTG	CAA	AGT	GGG	GTC	CCA	TCA	AGG	TTC	AGT
L	L	I	Y	X	A	S	X	L	Q	S	G	V	P	S	R	F	S
GGC	AGT	GGA	TCT	GGG	ACA	GAT	TTC	ACT	CTC	ACC	ATC	AGC	AGT	CCG	CAA	CCT	GAA
G	S	G	S	G	T	D	F	T	L	T	I	S	S	P	Q	P	E
CDRL3																	
GAT	TTT	GCA	ACT	TAC	TAC	TGT	CAA	CAG	XXX	XXX	XXX	XXX	CCT	XXX	ACG	TTC	GGC
D	F	A	T	Y	Y	C	Q	Q	X	X	X	X	P	X	T	F	G
NotI																	
CAA	GGG	ACC	AAG	GTG	GAA	ATC	AAA	CGG	GCG	GCC	GCA						
Q	G	T	K	V	E	I	K	R	A	A	A						

Fig. (4). Complete sequence of scFv (gene and amino acid) from Tomlinson I-J library. The TAG stop codon was read as Glutamine because scFvs were produced in *E. coli* TG1 suppressor strain. The scFvs from this library have 18 different positions (marked as X) randomly changed at antigen binding regions, i.e. CDRs, which are highlighted. Table 4 shows the actual CDR sequences of representative isolated scFvs. The PCR product did not contain heavy chain in this scFv.

ability between the dipeptidase (pepD) of *Lactococcus lactis* and Immo-Amino (results not shown). So our conclusion is that the binding between Immo-Amino and peptides/proteins are variable, and therefore not universally applicable.

Another factor that impairs the successful generation of anti-peptide scFv-phage is high non-specific binding and background binding scFv-phages. It is therefore necessary to reduce both phenomena during the panning. Non-specific binding is the binding of phage heads rather than the scFvs

to any components, and we previously demonstrated that Tween 20 can reduce most of this non-specific binding [32]. Background binding is the binding of scFvs to the background matrix, and we found two reasons for a high background binding. One is the ratio between the surface occupied by the immobilized antigen and the total surface. If this ratio is low, the background binding will easily dominate the panned phage library [35]. The other reason is that in highly diverse antibody phage libraries e.g. large naïve or synthetic libraries [36]. There is a high risk that these libraries contain specific antibodies (scFvs) against the background matrix. We observed this, and have isolated and identified such monoclonal anti-Polysorp scFvs (sequences shown in Table 4). This problem has also been found by using large naïve libraries for panning against complex antigen targets, such as cells [37-39].

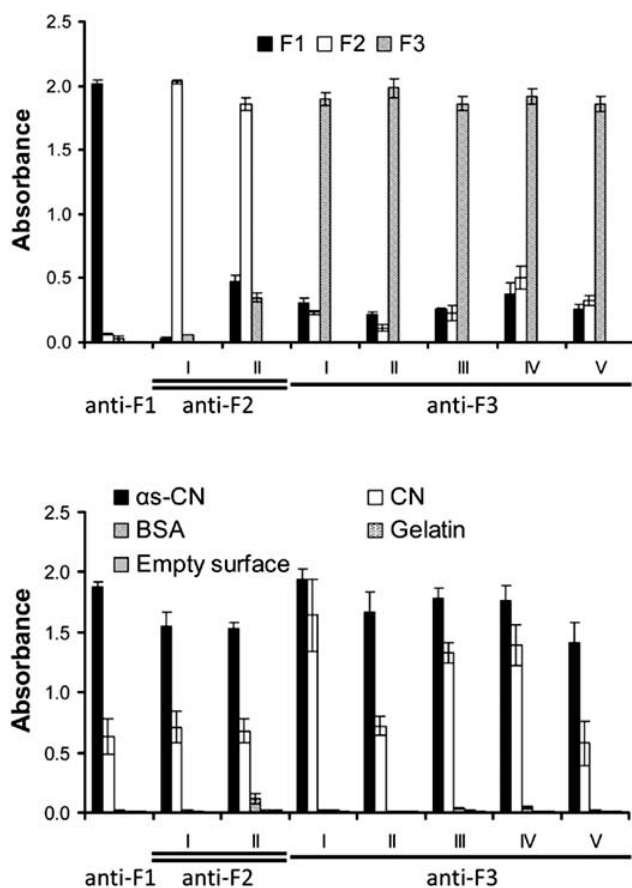


Fig. (5). ELISA for monoclonal anti-peptide scFv-phages against different peptides (A) or proteins (B). Biotinylated peptides were coupled on Immo-Strep. All proteins were coupled on Immo-Amino. Roman numeral of each monoclonal scFv is corresponding to the group number shown in Table 4. Each error bar represents the standard deviation of 4 replicates.

In this study, we introduced the concept of enrichment ratio (Table 2), which is determined by comparing the titer of eluted phage from a peptide-binding surface to the titer from an empty surface. An increase in this ratio represents the enrichment of peptide-binding scFvs in the produced sub phage library. We found that a high enrichment ratio determined by titrating eluted phage was corresponding to a high number of peptide-binding clones determined by

monoclonal ELISA (Tables 2 and 3). The presented enrichment ratio, which only requires a control panning on the background, can therefore immediately predict the proportion of specific binders in the subsequent generated polyclonal phage library. This is an advantage, compared to the normal procedure, where ELISA is always used to detect the specificity of the generated polyclonal phage library toward target antigen. This ELISA procedure requires extra work in the laboratory as well as waiting time for producing the polyclonal phage library.

There are basically four methods to reduce the background binding during the panning of an antibody phage library. We investigated two methods: i) BA method, which was also performed by Portolano *et al.* [40] and Krichevsky *et al.* [41], and ii) CE method, which was proved to be applicable by Meulemans *et al.* [42] and Heiskanen *et al.* [43]. We found that CE provided a stronger enrichment than BA, and was adequate for our purpose. Two other methods have been mentioned in literatures, iii) Antigen modification method [44-46], and iv) Alternate background panning [47, 48], but were not tested in this study.

We focused on solid surface supported panning method, which is similar to standard ELISA assays. Some researchers used another panning method called solution panning for developing the antibodies against small peptides [49-52]. The solution panning mixes the phage library with biotinylated peptides in buffer solution, and bound phages are collected from the solution after a short (15 min) incubation with streptavidin beads. Apparently, the short time incubation for streptavidin-biotin binding should reduce background binding phages. However, we tried solution panning with Immo-Strep to collect bound phage and found that background binding (Polysorp surface binding) phage was still dominant in the panned phage population (results not shown). We did not test streptavidin beads in our study, but the basic material of the magnetic beads and Polysorp surface are both polystyrene, so there is a pronounced risk that some scFvs in the library could also specifically bind to the beads' polystyrene material. Therefore, successful selection of phages against our selected peptides is still dependent on methods to reduce the background binding.

In summary, we have optimized the method to select monoclonal scFvs from a large synthetic antibody phage display library against small peptides immobilized on solid surface. This was achieved by developing a method for detecting the efficiency of immobilization of small peptides and selecting the best method for excluding the background binding scFv-phage. Apart from the specific antibodies raised in this study, it is our belief, that the methods developed are universally applicable for anyone interested in selecting antibodies (antibody fragments) against small peptides by antibody phage display.

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ABBREVIATION

scFv	=	Single chain variable fragment of antibody
PBS	=	Phosphate buffered saline
F1, F2, F3	=	Synthetic peptides from α_{s1} -casein sequence
F1	=	f17–31 (NENLLRFFVAPFPEV)
F2	=	f150–163 (FRQFYQLDAYPSGA)
F3	=	f185–199 (PIGSENSEKTTMPLW)
CDR	=	Complementary determining regions of antibody
TPBS	=	Tween 20 in PBS
Immo-Amino	=	Nunc Immobilizer Amino surface
Immo-Strep	=	Nunc Immobilizer Streptavidin surface
CN	=	Casein
HRP	=	Horse radish peroxidase
MW	=	Molecular Weight
TE	=	Trypsin Elution
CE	=	Competitive Elution
BA	=	Background Absorption
TEA	=	Triethylamine

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