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Different buffer effects in selecting HM-1 killer toxin single-chain fragment variable anti-idiotypic antibodies*

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We reported previously competitive panning elution with PBS (pH 7.0) that contains HM-1 killer toxin (HM-1) and *Candida albicans* membrane fraction (CaMF) to release phages bound with CaMF as an antigen. Here, as an alternative strategy, we isolated high-binding affinity recombinant single-chain fragment variables (scFvs) with *in vitro* anti-fungal activity from an scFv phage library. The competitive panning elution contained acidic, neutral and basic pH buffers with original antigen HM-1 or HM-1 peptide 6 used to release phages bound with HM-1-neutralizing monoclonal antibody (nmAb-KT). For neutral pH eluted conditions, 87.5% of clones showed high-binding affinity against nmAb-KT by using ELISA, but was 16% and 26% for acidic and basic eluted conditions, respectively. After nucleotide sequencing, we obtained seven different anti-idiotypic antibodies from the different selection procedures. The clone expression and purification by using a HisTrap HP column, showed that clones scFv S3, S4 and S7 had *in vitro* antifungal activity against *Saccharomyces cerevisiae*, *Candida albicans*. The purified scFvs showed strong binding affinity with nmAb-KT by using ELISA. These results showed that changing the buffer pH with competing elements plays important role in elution of bound phages to targeted antigen and also in identification of positive scFv phages.

Keywords: phage display/competitive panning elution/HM-1 killer toxin/HM-1-neutralizing monoclonal antibody/high-affinity antibody/HM-1 killer toxin anti-idiotypic antibody.

Abbreviations: CDR, complementarity-determining regions; ELISA, enzyme-linked immunosorbent assay; HM-1, HM-1 killer toxin; nmAb-KT, HM-1-neutralizing monoclonal antibody; IPTG, isopropyl β-D-1-thiogalactopyranoside; PBS, phosphate-buffered saline; scFv, single chain fragment variable; VH, variable region of heavy chain; VL, variable region of light chain.

Anti-idiotypic antibodies are needed as tools for a better understanding of molecular mimicry and the immunological network, and for many potential applications in the biomedical and pharmaceutical field (*1*). Recombinant antibody fragments are becoming popular therapeutic alternatives to full-length monoclonal antibodies because they are smaller, have different properties that are advantageous in certain medical applications, can be produced more economically and are easily amenable for manipulation (*2*). The construction of libraries of single-chain fragment variable (scFv) or fragment antigen-binding (Fab) fragments of antibody molecules expressed on the surface of filamentous phages and the selection of recombinant phage antibodies by binding to antigens offer a new and powerful means of generating monoclonal antibodies (*3*). Anti-idiotypic antibodies can compete with external antigens for the binding site of specific antibodies, thus mimicking the structure of the relative epitopes. The representation of external antigens by anti-idiotypic antibodies has not been unequivocally supported by studies of their three-dimensional structures, either because the structures of the external antigens were unknown or because the anti-idiotypic antibodies showed no resemblance to the external antigen (*4*).

Most panning methods use extensive washes to remove unbound and loosely or non-specifically bound phages from the solid phase. Therefore, the washes actually constitute the first selective step and set a minimum requirement for the off-rate of the protein–ligand interaction. The stringency can be increased by using a low concentration of the panning ligand. When aiming at isolation of the highest affinity binders, concern has been that the binders may be eluted poorly from the solid phase because of their tight binding to the antigen. To secure the recovery of all binders, elution buffers of very low or high pH, chaotropic agents, etc., have been used (*5*). Previously many studies used 0.1 M glycine HCl (pH 2.5) (*6*, *7*) or 0.1 M triethylamine (pH 12.0) (*8*, *9*) buffers to elute bound phages and to infect *Escherichia coli* cells. In this study, we used acidic, neutral and basic buffers containing the original antigen HM-1 killer toxin

(HM-1) or HM-1 peptide 6 to elute phages bound to HM-1 neutralizing monoclonal antibody (nmAb-KT).

HM-1 is a strong anti-yeast protein produced by yeast *Williopsis saturnus* var. *mrakii* IFO 0895 and belongs to the K9-type killer toxin group (*10*). HM-1 kills susceptible yeasts effectively, consists of 88 amino acids including five disulphide cross-links, and is very stable against heat treatment and at pH 2–11 (*10–12*). The scFv phage library constructed from nmAb-KT (*13*, *14*) was used to isolate strong binding scFv by using different competitive panning elution conditions. The nmAb-KT is produced to neutralize HM-1 killing activity by using hybridoma technology (*11*, *15*). HM-1 peptide 6 used in our study is the epitope of nmAb-KT in HM-1 (*15*).

Previously, we used competitive panning elution with phosphate-buffered saline (PBS) at pH 7.0 that contained original antigen HM-1 + HM-1 peptide 6 and *Candida albicans* membrane fraction to dissociate bound phages with *C. albicans* membrane fraction (*14*). The results showed that isolated scFv had in vitro anti-fungal activity against various *Candida* species and *Saccharomyces cerevisiae* with high binding to nmAb-KT by using enzyme-linked immunosorbent assay (ELISA). The successes against nmAb-KT led us to hypothesize that phage libraries might result in more efficient selection of antibodies on purified antigen nmAb-KT. In this study, to isolate high-binding affinity anti-idiotypic scFv bound with nmAb-KT, phages were eluted by different competitive panning elution conditions and was compared with conventional panning: (i) *E. coli* TG-1 clones screened directly from a cDNA library, (ii) conventional panning (panning A), (iii) 0.1 M glycine HCl (pH 2.5) containing HM-1 (panning B), (iv) phosphate buffered saline (PBS) (pH 7.0) containing HM-1 peptide 6 (panning C) and (v) 0.1 M triethanolamine (pH 11) containing HM-1 (panning D). We compared the diversity of antibodies generated, the binding affinity against nmAb-KT by using ELISA, clone expression levels of selected antibodies and anti-fungal activity against *C. albicans* and *S. cerevisiae* from the various conditions of eluted phages.

Materials and Methods

Materials

HM-1 was extracted from the culture broth of yeast *W. saturnus* var. *mrakii* IFO 0895 and was purified as described previously (*16*). HM-1 peptide 6 was obtained from Sigma Genosys Japan (*15*). *C. albicans* ATCC 10231 and *S. cerevisiae* A451 were obtained from Japan Roche Research Center. Purified nmAb-KT was prepared at Technology Incubation & Transfer Ltd. (Saitama, Japan) from the hybridoma clone, previously reported by Tadashi Mayumi, Jichi Medical School, whose group initially reported HM-1-specific nmAb-KT (*11*). The scFv phage library against nmAb-KT in *E. coli* TG-1 cells was prepared in our laboratory (*13*). The amber suppressor strain of *E. coli* TG-1, amber non-suppressor strain of *E. coli* HB2151, horseradish peroxidase (HRP)-conjugated anti-E tag antibody, anti-E tag Sepharose column and HisTrap HP column (1 ml) were obtained from Amersham Biosciences UK Limited. Mouse Penta-His antibody was obtained from QIAGEN (CA, USA). Anti-mouse IgG (H + L) alkaline phosphatase (AP)-conjugated goat antibody was obtained from Promega (WI, USA).

Enrichment and isolation of nmAb-KT-binding scFv phages by successive rounds of panning

The panning protocol and the production of soluble antibodies were done as described in Expression Module/Recombinant Phage Antibody System (Amersham Biosciences). From the cDNA library, *E. coli* TG-1 clones were screened from SOBAG [2×YT medium (1.0% yeast extract, 1.7% tryptone, 0.5% NaCl) with 12% agar, 10 mM magnesium chloride, 100 μg/ml ampicillin and 2% glucose] plates and the soluble antibody reactivities against nmAb-KT were checked by using ELISA as described below.

Panning A. An scFv phage library containing 2.8×10^{10} clones (cfu/ml) (*13*) was used to select antibodies against nmAb-KT (Fig. 1) (*8*). Briefly, the library stock was grown to log phase in 5 ml of 2×YT-AG medium (1.0% yeast extract, 1.7% tryptone, 0.5% NaCl, 100 μg/ml ampicillin and 2% glucose), and phages were rescued with the M13KO7 helper phage and were amplified overnight using 2×YT-AK (2×YT, 100 μg/ml ampicillin and 50 μg/ml kanamycin) at 37°C. The cells were removed by centrifugation, and the phage particles in the supernatant were filtered (0.45 μm), 1/5 vol of 20% polyethylene glycol 8000 that contained 2.5 M NaCl was added, and then the phages were incubated on ice for 1 h. After 1 h the phages were centrifuged at 10,000*g* at 4°C for 20 min, the supernatant was discarded and the pellet was

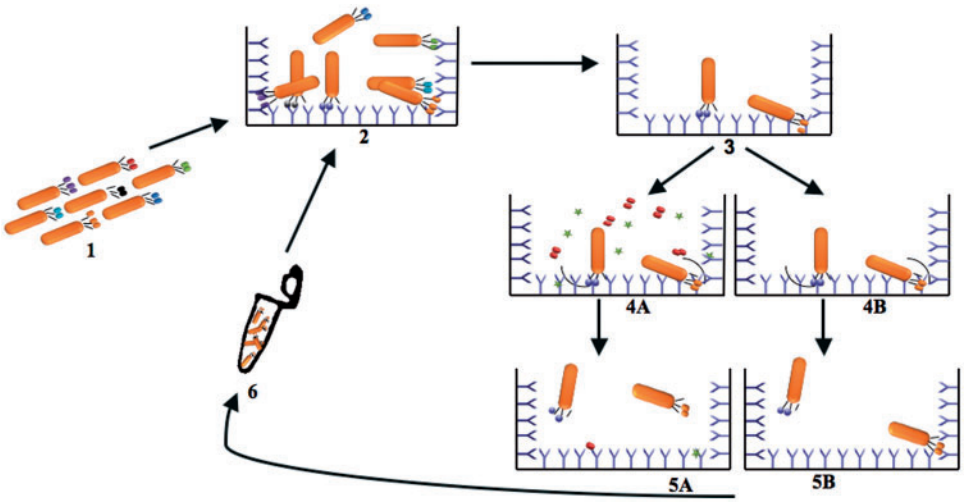

Fig. 1 Scheme illustrating the one round of competitive and conventional panning procedure. 1: scFv phage library; 2: the library was panning against nmAb-KT antigen; 3: washing the unbound phages; 4A: competitive elution of bound phages; 4B: conventional elution; 5A and 5B: the bound phages were released; and 6: released phages are collected and infected with *E. coli* TG-1 cells, amplified and continued for another rounds panning.

resuspended in 8 ml of 2×YT medium. For each round of selection, 10 μg/ml of nmAb-KT in PBS was coated and was kept overnight at 4°C in Nunc immunotubes (Maxisorp). Then the immunotubes were blocked with 3% skim milk in PBS at 37°C for 1 h and were incubated with the phage preparation at 37°C for 2 h. The tubes were washed 20 times with PBS and then 20 times with PBS containing 0.1% Tween 20 (PBST). Phages were eluted by using 10 ml of 2×YT media containing mid-log phase *E. coli* TG-1 cells at 37°C for 1 h with shaking. For subsequent rounds of selection, the phage stock (100 μl) from the previous round of selection was amplified and was used for the next round selection by using the procedure described above that used Nunc immunotubes coated with nmAb-KT. The infected *E. coli* TG-1 cells after the fifth round of panning were plated on SOBAG plates and were incubated overnight at 30°C. The individual colonies were scraped into 5 ml of 2×YT-AG medium were grown overnight, were mixed with 15% glycerol and then were stored at −80°C.

Competitive panning elution

Pannings B, C and D were done by using the same procedure described for panning A, but the phage elution conditions were different. Phage competitive elution was done by shaking for 1 h at 37°C with 0.1 M glycine HCl (pH 2.5) containing 10 μg/ml HM-1 for panning B, PBS (pH 7.0) containing 100 μg/ml HM-1 peptide 6 for panning C and 0.1 M triethanolamine (pH 11) containing 10 μg/ml HM-1 for panning D. The eluted phages were incubated with 10 ml of mid-log phase *E. coli* TG-1 cells at 37°C for 1 h with shaking. From pannings A–D, the phages eluted in each round of panning were determined on petridishes using 2×YT-AG medium containing 12% agar as described previously (*17*).

ELISA screening for antibody binding with nmAb-KT

Individual *E. coli* TG-1 colonies from pannings A–D were recovered after the fifth round of panning. From the cDNA library and pannings A–D, 48, 50, 50, 40 and 50 clones, respectively, were randomly selected and were grown in 2×YT-AG medium at 37°C. To produce phages, the cells were rescued with the M13KO7 helper phage as described previously (*8*). To produce soluble antibodies, the clones were incubated in 2×YT-AI medium (2×YT, 100 μg/ml ampicillin and 1 mM isopropyl β-D-1-thiogalactoside) overnight at 30°C, and then the supernatant was collected by centrifugation at 1500*g* for 20 min at room temperature. For ELISA, a 96-well microtitre plate was coated with 10 μg/ml of nmAb-KT in PBS and was kept overnight at 4°C. Then the plate was blocked with 3% skim milk in PBS at room temperature for 1 h and then was washed three times with PBS. The cell culture supernatant containing soluble antibodies was diluted with 1/6 vol. of 10% skim milk in PBS and was added to the nmAb-KT coated plate. After incubation at room temperature for 1 h, the plate was washed three times with PBST. Soluble antibodies were detected using HRP-conjugated anti-E tag antibodies at 1:5,000 dilution. After a 1-h incubation at room temperature, the plates was washed three times with PBST, and then 2,2′-Azino-bis[3-ethylbenzthiazoline-6-sulphonic acid] in citric acid as a substrate was added together with hydrogen peroxide. Then the plates were incubated for 15 min for colour development and the absorbance at 405 nm was read by using a micro plate reader (Bio-Rad model 680).

Production of soluble antibodies in E. coli HB2151 cells

Positive phages produced by *E. coli* TG-1 cells from the cDNA library and pannings A–D were used to infect *E. coli* HB2151 cells to produce soluble scFv antibodies. Individual *E. coli* HB2151 clones in 10 ml were grown in 2×YT-AG medium at 30°C until absorbance 0.9 at 600 nm. The cells were pelleted (1,500*g* for 10 min) and were resuspended in 10 ml 2×YT-AI medium. The overnight induction of *E. coli* HB2151 cells containing phagemid DNA led to the production of a soluble form of the scFv antibody fragment, which was secreted into the periplasm. Cells were cultured for 16 h at 30°C and then were harvested. The 16-h cultures were divided into two parts and then the supernatants were collected by centrifugation. To prepare periplasmic extract one of the two pellets from the centrifugation was resuspended in 100 μl of ice-cold 1×TES buffer [0.2 M Tris–HCl (pH 8.0), 0.5 mM EDTA and 0.5 M sucrose] and 150 μl of 1/5×TES buffer was added. The mixture was kept in ice for 1 h, was centrifuged at 15,000 r.p.m. for 10 min and then the supernatant containing the periplasmic extract was collected. To prepare whole cell extract, the second pellet was resuspended in PBS, was boiled for 5 min and the supernatant containing soluble antibodies was collected by centrifugation. The collected supernatant, periplasmic extract and whole cell extract from individual clones were checked for the presence and reactivity of scFv antibodies by using ELISA against nmAb-KT as described in the previous section.

DNA sequencing of scFv genes

DNA sequencing was done as described previously (*7*, *14*). The *E. coli* HB2151-positive clones were sequenced, and the samples were prepared by using a Dye terminator cycle sequencing quick start kit. Primers S6 (5′-GTAAATGAATTTTTCTGTATGAG G-3′) and R1 (5′-CCATGATTACGCCAAGCTTTGGAGCC-3′) were used for pCANTAB 5 E vectors and were analysed by using the CEQ 2000XL DNA Analysis System (Beckman Coulter). Sequence alignment and manipulation were done by using the DNASIS program. The amino acid residues of each variable domain were deduced according to Kabat *et al.* (*18*).

Expression and purification of selected scFvs using an anti-E tag Sepharose column

Large-scale production of scFv for selected clones was done in a 1-l culture as described above. The periplasmic extract was subjected to overnight dialysis against PBS by using a molecular weight cut-off 12,000 membrane. The dialysed periplasmic extract was purified by using affinity chromatography with an anti-E tag Sepharose column that was previously equilibrated with 20 mM phosphate buffer (pH 7.0). The anti-E tag binding protein was eluted using 0.1 M glycine HCl (pH 3.0) and was immediately neutralized with 0.5 M phosphate buffer (pH 7.8). The concentration of scFv was determined by 1.0 absorbance at 280 nm corresponding to 0.5 mg/ml protein.

E tag deletion and insertion of a His tag by PCR mutagenesis

Previously we purified scFv using an anti E-tag Sepharose column (*13*, *14*). The column was commercially unavailable on the market, so we planned to replace the E tag with His tag at the C-terminal end of scFv in the template DNA by using KOD-Plus-Mutagenesis (TOYOBO Co, Japan) to purify scFv in the HisTrap HP column by using the procedure described previously (*19*). The template DNA plasmids of scFv S3, S4 and S7 were treated with NotI and were kept at 37°C for 1 h to linearize the plasmid DNA. Two primers were designed based on the deletion of the E-tag sequence and insertion of His tag at the C-terminal end of scFv S3, S4 and S7 in template DNA. Then the NotI-treated sample was mixed with final concentration of 0.2 mM of dNTPs, 1 mM magnesium sulphate, 1.0 unit of KOD-plus-DNA polymerase, 1×PCR KOD-Plus buffer, 0.3 μM of reverse primer 5′-TGCGGCCGCCCGTTTTATTTCCAG-3′ and forward primer 5′-CACCATCACCATCACCATGCCGCATAGA CTGTTGAAAGTTGT-3′. After heating at 94°C for 2 min, the PCR reactions were done by 40 cycles; denaturation at 94°C for 15 s, primer annealing at 51°C for 30 s and primer extension at 68°C for 5 min. After the last cycle, all samples were incubated for an additional 5 min at 68°C to ensure completion of the extension step. The samples were ligated with Ligation high (TOYOBO Co.) and were transformed into *E. coli* HB2151 cells. The transformed cells were plated on 2×YT-AG containing agar. The colonies were screened and DNA sequencing was done as described in the previous section DNA sequencing of scFv genes.

Purification of selected scFvs by using a HisTrap HP column

For large-scale production of scFvs of His tag replaced scFv S3, S4 and S7 were expressed as described above. The dialysed periplasmic extract was passed into a HisTrap HP column and the column was washed with 20 mM sodium phosphate, 0.5 M NaCl and 0.1 M imidazole (pH 7.4) for the first five fractions, then the bound scFv was eluted with 20 mM sodium phosphate, 0.5 M NaCl and 0.2 M imidazole (pH 7.4) for the second five fractions and further eluted with 20 mM sodium phosphate, 0.5 M NaCl and 0.3 M imidazole (pH 7.4) for the third five eluted fractions.

In vitro anti-fungal determination against C. albicans and S. cerevisiae for scFv purified in the HisTrap HP column

In vitro antifungal activity for His tag column elution fractions containing purified scFv was determined against *C. albicans* and *S. cerevisiae* by using a liquid culture method described previously (*20*). To estimate the killing activities, the test yeast *S. cerevisiae*

(3×10^3 cells/ml) or *C. albicans* (500 cells/ml) at exponential phase were incubated for 16–20 h in YPD medium (1.0% yeast extract, 2.0% peptone, 2% glucose) containing 204 μg/ml concentration for scFv S7 (eluted fraction 3), 230 μg/ml for scFv S3 and 218 μg/ml for scFv S4 at 30°C with shaking at 125 r.p.m. Elution buffer containing 20 mM sodium phosphate, 0.5 M NaCl and 0.2 M imidazole (pH 7.4) was dialysed against PBS and was used as a control. The absorbance of the culture broths was then measured at 600 nm by using a spectrophotometer, and the growth viability was read.

SDS-PAGE and western blotting

SDS-PAGE analysis and western blotting were done as described previously (*21*). HisTrap HP column elution fractions were separated on 15% acrylamide gels and then were stained with silver nitrate, or underwent western blotting. For western blot analysis, the proteins were transferred from the gels onto a ProBlott membrane (Applied Biosystems) by using a semi-dry system in transfer buffer. The membrane was blocked with 3% skim milk in PBS for 1 h at room temperature and then was incubated for 1 h with 1 : 2,000 penta-His antibody. Then the membrane was incubated with secondary antibody of anti-mouse IgG AP conjugate antibody with 1 : 5,000 dilution in 3% skim milk containing PBS. After washing, the proteins on the membrane were detected by using Western Blue stabilized substrate for alkaline phosphatase (Promega Corporation, WI, USA).

Results

Comparison of different elution methods to select anti-idiotypic scFv

The phages selected by five rounds of panning were used to infect *E. coli* TG-1 cells, and subsequent plating on agar media allowed separation of individual colonies that had phagemids. Figure 2 shows the phage recovery after each round of pannings A–D. Phage titres increased in every round of panning, indicating antigen-specific phage antibodies were selected and enriched. After each round of selection, the frequency of antigen-binding phage antibodies was higher for panning C when compared with other pannings. Conventional elution methods did not yield an increase in anti-idiotypic scFv during the fifth round of panning. The number of colonies obtained from the first round of selection was highest with the specific elution from panning C. For pannings B and D, the number of phages eluted after the fifth round of panning was higher when compared with the other four rounds. Specific elution with HM-1 or peptide 6 resulted in a higher number of phages eluted. Phage enrichment produced no higher number of eluted phages from the first round, except for panning C, but for pannings B and D after the fifth round of panning, the eluted phage enrichment of specific scFv was high.

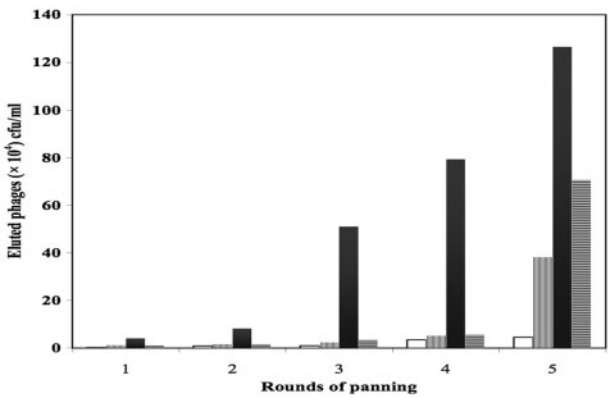

Fig. 2 Enrichment of phages specific to nmAb-KT during panning cycles. The number of phages eluted after each round of panning was counted from the number of colonies (CFU/ml) formed after re-infection of the eluted phage particles in *E. coli* TG-1 cells. Opened bars are phages recognized by panning A. Vertical line bars are phages recognized by panning B. Filled bars are phages recognized by panning C. Horizontal line bars are phages recognized by panning D.

From the scFv phage library, we screened 48 *E. coli* TG-1 clones without panning and checked for the presence of scFv and its reactivity against nmAb-KT by using ELISA (Fig. 3, Table I). The results showed that only two clones (4%) showed less intensity against nmAb-KT [absorbance at 405 nm values >2-fold of the 0.05 value (i.e. 0.1) were scored as positive]. For conventional panning elution (panning A), nine clones (18%) of 50 clones tested showed less intensity against nmAb-KT. For acidic (pH 2.5) eluted clones (panning B), 50 clones were tested and the positive rates were 16% (8 clones) with high intensity. Specific elution with neutral pH 7.0 conditions (panning C) was done with high concentrations of HM-1 peptide 6 (100 μg/ml). The number of positive clones was high compared with other specific elution conditions and with conventional methods. Out of 40 clones tested, 35 clones (87.5%) were positive against nmAb-KT by using ELISA. For panning D, similar to panning B, 26% (13 clones) were positive with high intensity for basic (pH 11) eluted clones. For the specific elution panning methods, ELISA absorbance showed high intensity for all three pannings B–D. The scFv genes of *E. coli* TG-1 clones that showed high intensity against nmAb-KT were used to transfer into *E. coli* HB2151 cells. After expression of soluble scFv in *E. coli* HB2151, the periplasmic extract, whole cell extract and culture supernatant were checked for the presence and reactivity of scFv against nmAb-KT by using ELISA. The results showed high intensity at 405 nm for periplasmic extract and culture supernatant, the same as for *E. coli* TG-1 clones, but showed no absorbance for whole cell extract (data not shown).

DNA sequencing and populations of the isolated scFv genes

To determine the impact of selection on the diversity of selected antibodies and to identify the gene families for the V_L and V_H regions, the nucleotides were sequenced. The scFv genes of the selected positive phage antibodies against nmAb-KT were amplified by using PCR and were sequenced using primers S6 and R1 in a DNA analysing system. The nucleotide base pairs were analysed by DNASIS software to predict the amino acid sequence. From the cDNA library and pannings A–D, one, one, four, three and three unique clones, respectively, were isolated. After matching, the amino acid sequences from all pannings showed seven unique clones that we referred to as scFv S1–S7. The amino acid sequences showed that the generated scFv had different amino acids in the heavy and light chains (Fig. 4). Interestingly, we found that scFv S7, which was also found in our previous report, as scFv C1 (*14*). The populations of each clone were unique among the panning

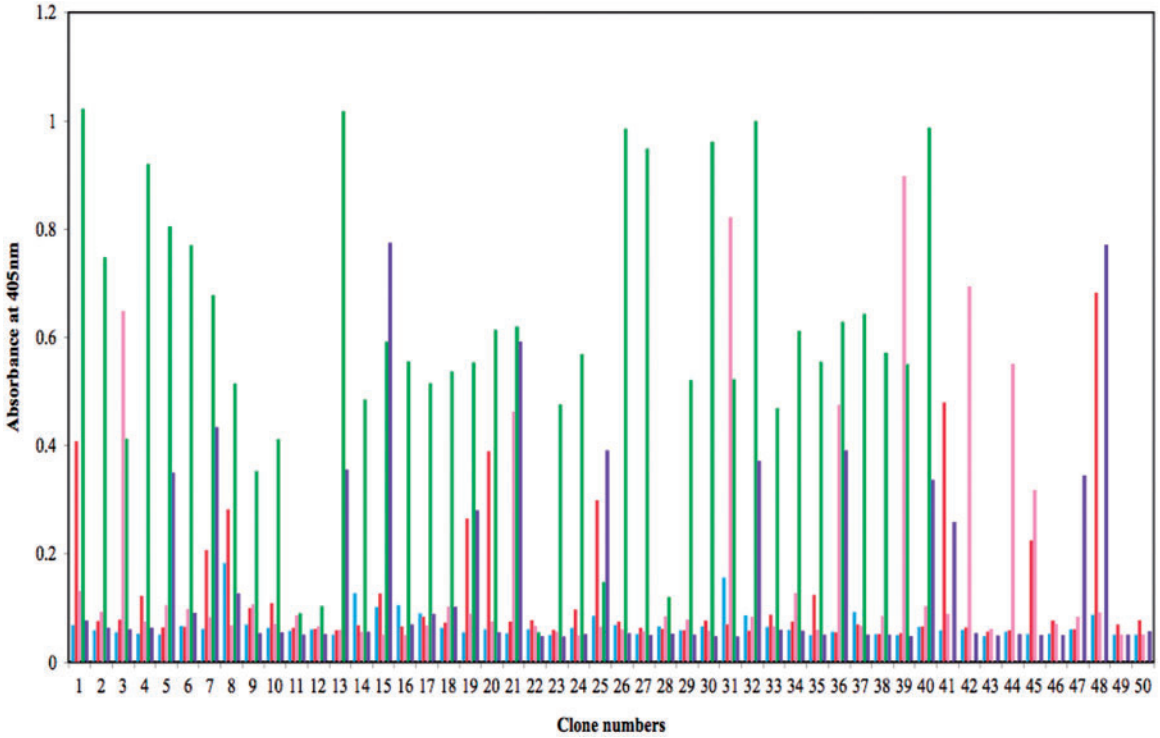

Fig. 3 Binding affinity of scFv from differently panned clones. scFv reactivity against nmAb-KT by ELISA absorbance at 405 nm for randomly selected phage clones from different panning were shown. Light blue, red, pink, green and purple colour bars are panning conditions of the cDNA library and pannings A–D, respectively.

Table I. Comparison of different competitive panning elution conditions and their yields for specific anti-idiotypic scFv phages.

	Clones tested	Positive clones	Positive rates (%)	scFv found	Identical clones	Identical clones in %
cDNA library	48	2	4	S1	1	50
Panning A: conventional panning	50	9	18	S2	9	100
Panning B: HM-1 in 0.1 M glycine HCl (pH 2.5)	50	8	16	S2	3	37.5
				S3	2	25
				S4	1	12.5
				S5	1	12.5
Panning C: HM-1 peptide 6 in PBS (pH 7.0)	40	35	87.5	S5	19	54.3
				S6	8	22.9
				S7	8	22.9
Panning D: HM-1 in 0.1 M triethanolamine (pH 11)	50	13	26	S3	2	15.4
				S4	1	7.7
				S6	8	61.5

conditions (Table I). A comparison of the VH and VL sequences showed that the seven isolated clones were different in various numbers of amino acids (Fig. 4). An analysis of the seven isolated clones of the CDR3 region of VH showed considerable variability in length at 8–13 amino acids in their amino acid sequence, indicating that the cDNA library had a broad variety of anti-idiotypic scFv that we isolated specific to the original antigen HM-1 or HM-1 peptide 6. CDR1 and CDR2 regions of heavy chains also had amino acid differences among the seven clones. Except for scFv S6, the CDR1 region of VL varied in their length at 10–12 amino acids, and CDR2 and 3 regions of VL genes also differed in amino acid. The VL region of scFv S6 had only 14 amino acids from the start position.

scFv purification and its antigen-binding affinity was measured by ELISA

The seven clones were expressed in *E. coli* HB2151 for detailed characterization. In a 1-l culture the selected clones were expressed and the periplasmic extract was dialysed using PBS and was passed into an anti-E tag Sepharose column. Bound scFv was eluted using pH 3.0 elution buffer, was immediately neutralized with 0.5 M phosphate buffer at pH 7.8 and 1 ml per

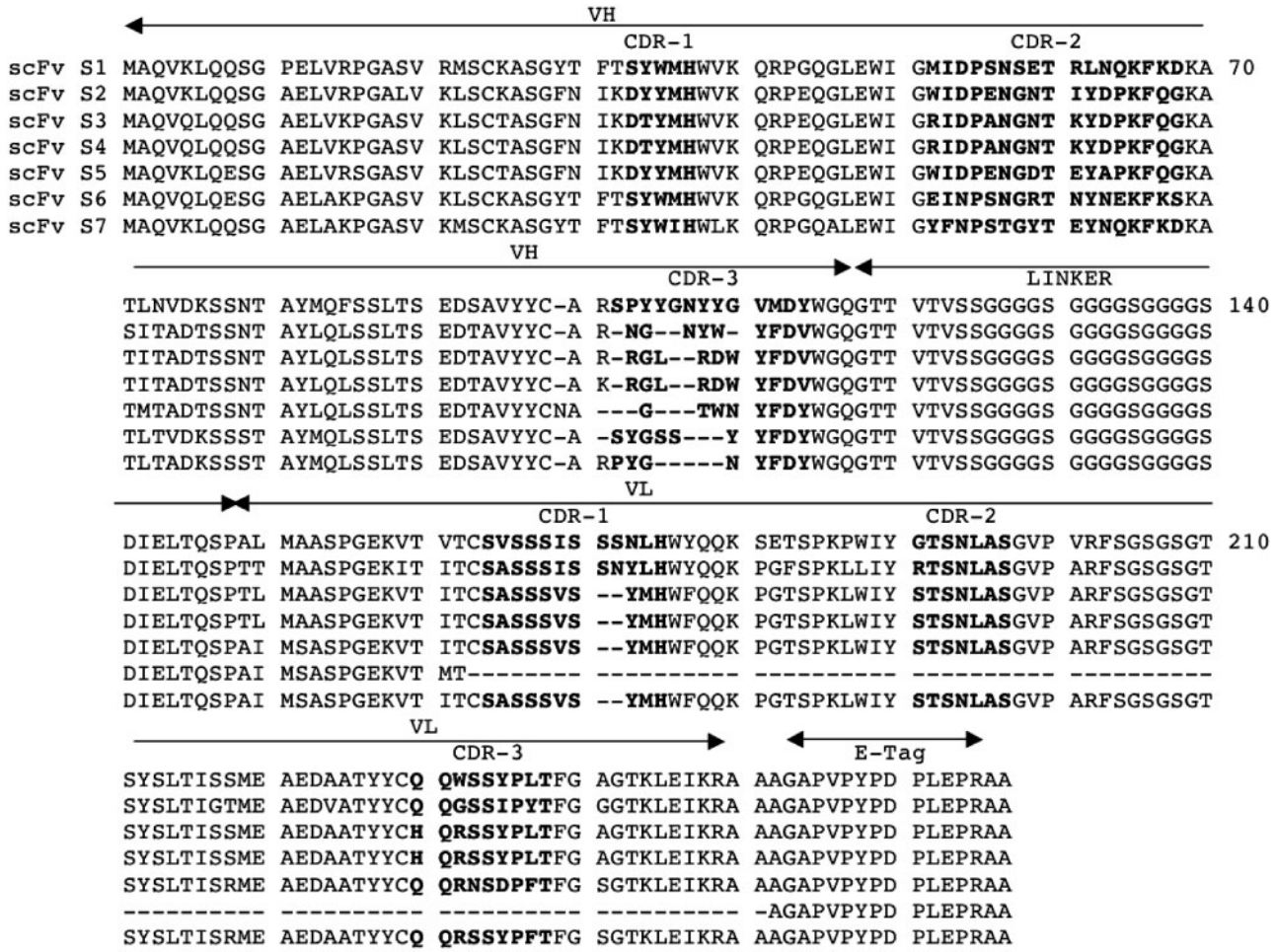

Fig. 4 Amino acid sequences of selected scFvs. VH and VL indicate variable regions of the heavy chain and light chain, respectively. VH, VL and CDR were deduced according to Kabat *et al.* (*18*). Bold font indicates CDR regions.

fraction was collected. The eluted fractions absorbance at 280 nm was checked for the presence of protein. Figure 5 shows the protein elution profile from the anti E-tag Sepharose column. From the protein profile it concluded that the clones scFv S3, S4 and S7 showed high recovery of scFv and the clones scFv S1, S2, S5 and S6 did not show significant protein absorbance and Table II listed the protein yields. Among the seven isolated clones, scFv S3, scFv S4 and scFv S7 antibody yields were 0.87, 0.90 and 1.12 mg/l, respectively. To test the binding affinity of the purified scFv against nmAb-KT by ELISA, the highest protein peak fractions were selected. The clones scFv S3, S4 and S7 showed strong binding affinity against nmAb-KT (Fig. 6), but the clones S1, S2, S5 and S6 did not showed ELISA absorbance as expected. These results clearly indicate that purified scFv S3, S4 and S7 have strong binding affinity against nmAb-KT.

In vitro anti-fungal activity of purified scFv

For clones scFv S3, S4 and S7, we successfully replaced with His tag after deletion of the E tag by using PCR mutagenesis at the C-terminal end. The clones scFv S3, S4 and S7 were expressed and the periplasmic extract was dialysed against PBS and was passed into a HisTrap HP column as described in 'Materials and Methods' section. The yield of the purified scFv was similar to anti-E tag Sepharose column (Table II). *In vitro* anti-fungal activity of purified recombinant scFv S7 was tested against *S. cerevisiae* and *C. albicans* as described in 'Materials and Methods' section. Eluted fraction number 3 showed a maximum of 68% and 54% of cell growth inhibition against *S. cerevisiae* and *C. albicans,* respectively (Fig. 7). The results showed that it retained anti-fungal activity, similar to a previous study of anti-E tag containing scFv C1 (*14*). From Fig. 7 it was confirmed that protein peak fraction showed *in vitro* anti-fungal activity against fungal pathogens. scFv S3 and S4 also showed similar amount of scFv after purification as similar to anti-E tag Sepharose column (Table II). The highest protein eluted fraction scFv S3 and S4 from HisTrap HP column showed a maximum 64% and 60% of cell growth inhibition against *S. cerevisiae*, respectively (Fig. 8).

SDS-PAGE and western blotting

The HisTrap HP column eluted fractions of scFv S7 were subjected to SDS-PAGE and western blot to

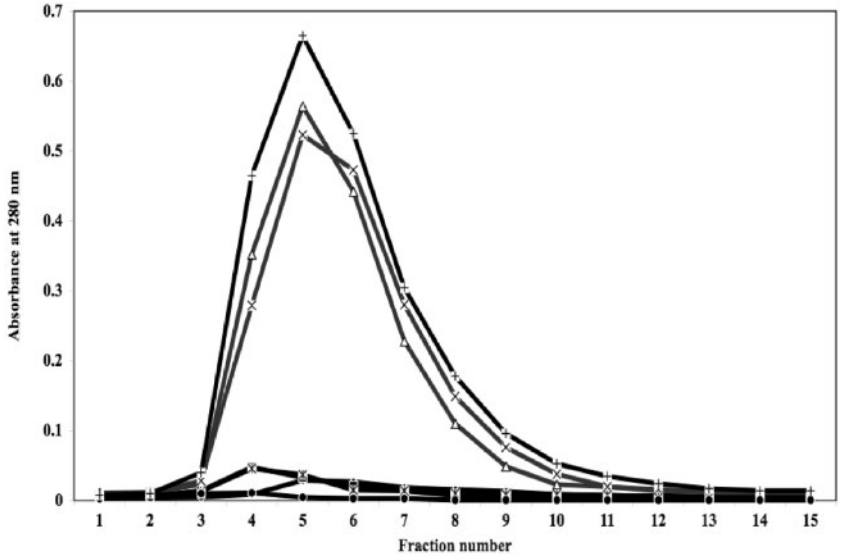

Fig. 5 Protein elution profile of seven isolated clones by using an anti-E tag Sepharose column. One-litre culture periplasmic extract was dialysed against PBS and was applied to a 5-ml anti-E tag Sepharose column. One millilitre per fraction was collected. Protein absorbance at 280 nm for scFv S1 (filled diamond), scFv S2 (filled rectangle), scFv S3 (open triangle), scFv S4 (cross mark), scFv S5 (asterisk), scFv S6 (filled circle) and scFv S7 (plus) are shown.

Table II. Protein yields for isolated scFv clones after purification by anti-E tag Sepharose and a HisTrap HPcolumn.

	Total protein amount in mg/l culture	
scFv	Anti-E tag	HisTrap HP
S1	0.05	–
S2	0.06	–
S3	0.87	0.69
S4	0.90	0.66
S5	0.06	–
S6	0.01	–
S7	1.12	1.2

identify the purity and molecular mass (Fig. 9A and B). By silver staining, an enormous protein bands were observed for the initial periplasmic extract before passing into the HisTrap HP column (Fig. 7A). The eluted fractions 1–5 with 20 mM sodium phosphate, 0.5 M NaCl and 0.1 M imidazole (pH 7.4) clearly showed that non-specific protein was eluted, and with 20 mM sodium phosphate, 0.5 M NaCl and 0.2 M imidazole (pH 7.4) eluted fractions 6–10 showed purified protein with a high-intensity band of molecular mass 29 kDa that corresponded to the molecular weight calculated by amino acid composition of scFv S7 and a weak band at 16 kDa. For eluted fractions 11–15 with 20 mM sodium phosphate, 0.5 M NaCl and 0.3 M imidazole (pH 7.4) showed that the scFv as completely eluted in fractions 6–10. Western blot detection by Penta-His antibody and anti-mouse IgG AP conjugated antibody showed a very high intensity band at 29 kDa and very weak bands at 16 kDa (Fig. 7B). No other protein and anti-His tag antibody reactive bands were observed in other molecular weight positions. The results indicate the purity of the isolated scFv S7.

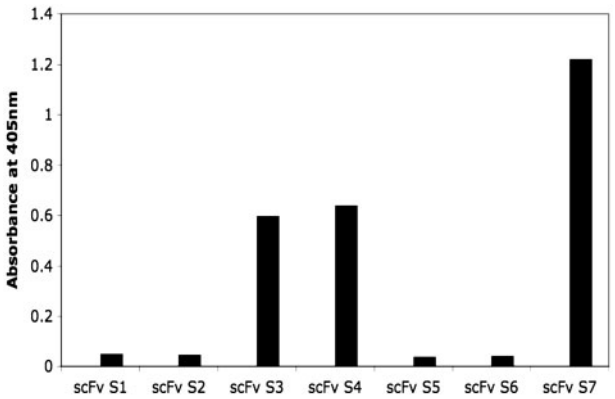

Fig. 6 Binding affinity of purified scFv S1–S7 against nmAb-KT measured by ELISA. The highest protein peak from anti-E tag Sepharose column was selected and checked the binding affinity against nmAb-KT by ELISA. The bars represent purified scFv ELISA absorbance at 405 nm.

Discussion

Anti-idiotypic monoclonal antibodies representing the internal image of a yeast killer toxin have therapeutic potential against several fungal infections (*22*). The isolated scFv antibodies in this study were primary targets for fungal vaccine development that are now under evaluation. Examples exist of successful isolation of antibodies against various antigens from different phage display antibody libraries, including high-throughput selection (*23–25*). Previously, studies isolated scFv with either 0.1 M glycine HCl (pH 2.5) (*6*, *7*) or 0.1 M triethylamine (pH 12.0) (*8*, *9*) buffers to elute the bound phages to the targeted antigens. In this study, phage display technology was used to select scFv molecules that selectively bind to nmAb-KT at specific elution conditions. We studied acidic, neutral and basic buffers containing original antigen HM-1 or

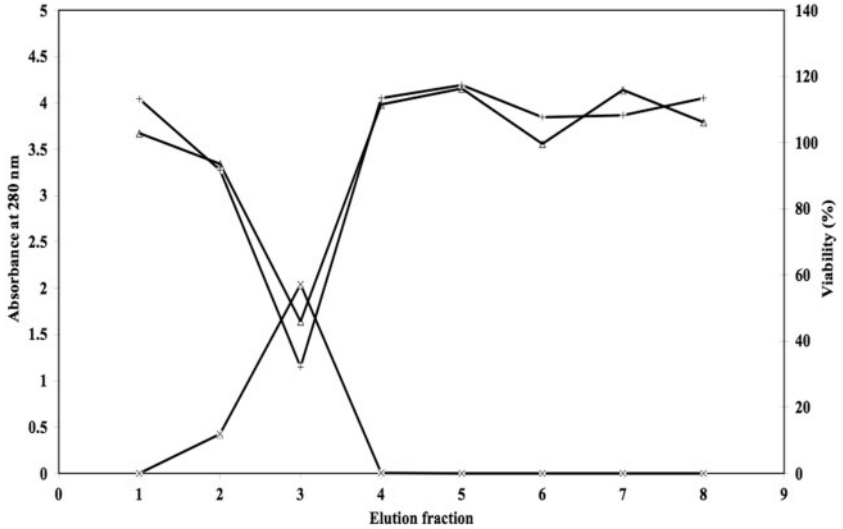

Fig. 7 scFv S7 protein elution profile from HisTrap HP column and anti-fungal activity. One-litre culture periplasmic extract was dialysed against PBS and was applied to a 1-ml HisTrap HP column. One millitre per fraction was collected. Protein absorbance at 280 nm (×) and anti-fungal activity against *S. cerevisiae* (+), *C. albicans* (Δ) were shown.

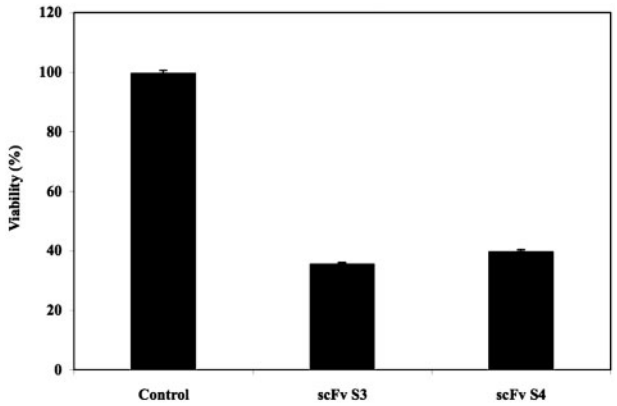

Fig. 8 scFv S3 and scFv S4 anti-fungal activity against *S. cerevisiae*. Anti-fungal activities of HisTrap HP column purified scFv S3 and scFv S4 were measured as described in 'MATERIALS AND METHODS' section.

HM-1 peptide 6 to release phages bound with nmAb-KT. The optimal conditions to dissociate a particular antigen–antibody interaction are not always effective for another type of antigen–antibody interaction (*6*). Our previous study based on competitive elution at neutral condition by HM-1 showed the effectiveness of phage display selection (*26*). HM-1 peptide 6 is the epitope of HM-1 against nmAb-KT and it shows strong cytocidal activity against *S. cerevisiae* (*15*). These properties lead us to do the elution with HM-1 peptide 6.

Clones screened from a cDNA library showed no high intensity ELISA absorbance and clearly the panning step is most critical in the selection and enrichment of specific scFv from cDNA libraries. Several elution methods, including acidic or basic conditions, have been used, but only basic conditions provide acceptable recovery of anti-11-DC phages, and acidic conditions are satisfactory to recover anti-BUF phages (*6*). In this study, the specific elution condition with neutral pH buffer containing HM-1 peptide 6 (panning C) eluted rather a high amount of ELISA positive phages compared with conventional panning eluted phages (panning A), specific eluted panning phages such as 0.1 M glycine HCl at pH 2.5 (panning B) and 0.1 M triethnolamine at pH 11 (panning D). For conventional panning (panning A) the number of eluted phages was less may be due to strong binding of phages to the antigen. The eluted ELISA positive phages were less by specific elution with acidic (panning B) and basic (panning D) buffers containing the original antigen, but had high intensity clones. Due to the sticky properties of phages, non-specific elution with glycine buffer at pH 2.2 or triethylamine at pH 12.0 led to a large excess of phages without scFv in the eluate (*1*). In this study by specific elution with HM-1 or HM-1 peptide 6, accumulation of positive scFv against nmAb-KT was obtained. So using specific elution with HM-1 or HM-1 peptide 6 is much more practical and efficient.

We obtained seven different sequence clones from different selection procedures and an scFv phage library that resulted in greater antibody display levels and more efficient selection against antigen. The scFv S3 and scFv S4 were in both acidic and basic eluted conditions, indicating they might be pH-independent clones with strong binding to the antigen nmAb-KT. Only one amino acid difference was between scFv S3 and scFv S4 at position 100 in the VH region, i.e. lysine was replaced by arginine in scFv S4. scFv S5 was found in both in acidic and neutral conditions, but in neutral conditions the population was high (54.3%) and it indicates strong binding affinity to the nmAb-KT. The scFv S5 was same as previously reported as scFv K1 (*26*). The absence of VH or VL means that the paratope is concentrated over a smaller area such that the domain antibodies provide the capability of interacting with novel epitopes that are

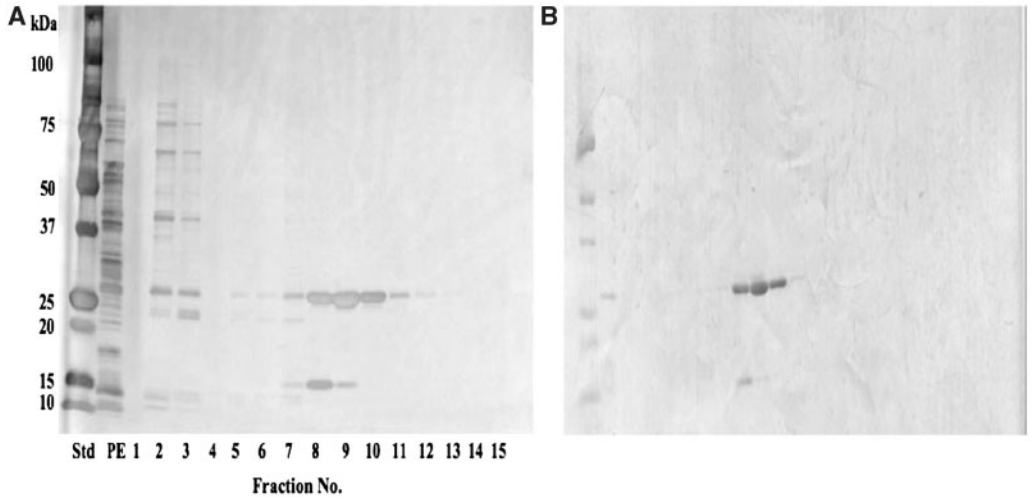

Fig. 9 SDS-PAGE and western blot analysis of HisTrap HP column-eluted fractions. (**A**) Silver staining and (**B**) western blot detection by Penta-His antibody and anti-mouse IgG (H + L) alkaline phosphatase (AP)-conjugated antibodies as primary and secondary antibodies, respectively. Std, standard molecular weight marker. PE, periplasmic extract before passing into the HisTrap HP column. Fractions 1–5 were scFv eluted with 20 mM sodium phosphate, 0.5 M NaCl and 0.1 M imidazole (pH 7.4), fractions 6–10 were eluted with 20 mM sodium phosphate, 0.5 M NaCl and 0.2 M imidazole (pH 7.4) and fractions 11–15 were eluted with 20 mM sodium phosphate, 0.5 M NaCl and 0.3 M imidazole (pH 7.4). Each sample in (B) corresponds to the sample in (A).

inaccessible to conventional VH-VL pairs and of penetrating into solid tumours even better than Fab and scFv (*27*). The scFv S6 had only 14 amino acids in the VL regions, but the VH region, linker and E tag presence were confirmed as complete. Unexpectedly, this scFv S6 was abundant in neutral (22.8% population) (panning C) and basic (61.5% population) (panning D) elution conditions. The scFv S6 and scFv S7 were also previously reported as scFv K2 (*26*) and scFv C1 (*14*), respectively. The stop codon resulted from a shifting reading-frame shift due to nucleotide deletion, but the phage-displayed antibody was still isolated and functional as only as a heavy chain antibody (*27*). The CDR3 sequence in the heavy chain is at the centre of antigen recognition, but the other five CDR regions more or less participate in increased binding affinity to the antigen, and some contact residues can even be located within framework regions (*28*). In this study, the isolated seven clones had amino acid and length differences in the CDR3 region of the heavy chain and in the CDR1 region of the light chain. The variability of the antibody CDR3 in the heavy chain results in new antigenic determinants that can in turn lead to production of new anti-idiotypic antibodies (*29*).

The presence of CDRs ensures the maintenance of selective binding to antigens and supports their use in biotechnological and therapeutic applications (*30*). The isolated clones had three CDRs in each heavy and light chain region, except scFv S6. The yield of protein absorbance at 280 nm was much less for scFv S1, S2, S5 and S6. Clone scFv S7 that was previously reported as scFv C1 showed a high production of scFv with *in vitro* antifungal activity against various *Candida* species and *S. cerevisiae* (*14*). Clones scFv S3 and S4 also showed high production of scFv after purification by the anti E tag column. We determined the binding affinity of purified scFv with nmAb-KT by ELISA. The purified scFvs of scFv S3, S4 and S7 showed strong reactivity against nmAb-KT as like non-purified scFv. This result indicates antigen specific scFvs have been isolated. As we expected the purified scFv S1, S2, S5 and S6 did not show ELISA absorbance because these clones did not show protein absorbance after purification by HisTrap HP column. The expression systems used in the study can readily provide large quantities of single-chain recombinant antibody, so they may be useful in developing a therapeutic agent for passive immunization in humans (*31*). Because of anti-E tag Sepharose column is commercially unavailable on the market, we replaced the E tag with the His tag as an alternative way to purify the scFv by using PCR based mutagenesis at the C-terminal end. The clones scFv S3, S4 and S7 was purified by using HisTrap HP column and almost the same amount of protein was obtained after purification by using the HisTrap HP column as for the anti-E tag protein for clones scFv S3, scFv S4 and scFv S7. The purified scFv S7 showed strong cell growth inhibition against *S. cerevisiae* and *C. albicans* by cell suspension assay method. Similarly the clone scFv S3 and S4 also showed in vitro anti-fungal activity against *S. cerevisiae*. Hence, we conclude that antigen specific recombinant antibodies have been isolated and mimicking cytocidal activity of original antigen HM-1. We expected that functional mimicking of HM-1, i.e. antifungal activity, might reflect amino acid sequence similarity to HM-1. To our surprise, however, the selected scFv antibodies shared no apparent homology with HM-1 in their amino acid sequences.

In summary, we cloned seven anti-idiotypic scFvs with acidic, neutral and basic buffers containing original antigen HM-1 or HM-1 peptide 6 by using

competitive panning elution conditions. Three of seven clones had high production of recombinant protein, strong binding affinity to nmAb-KT by using ELISA and *in vitro* anti-fungal activity against *S. cerevisiae*. At neutral pH elution conditions we obtained a unique clone scFv S7, at acidic or basic elution conditions we obtained scFv S3 and S4. The clone scFv S5 was obtained in both acidic and neutral elution conditions. scFv S6 was obtained in both neutral and basic elution conditions. These results showed that changing the buffer pH with competing elements plays an important role in elution of bound phages to targeted antigen and also in identification of positive scFv phages.

Conflict of interest
None declared.

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