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EXPRESSION AND PURIFICATION OF 6XHIS-TAGGED FUSION PROTEINS DERIVED FROM RANDOM PEPTIDE PHAGE DISPLAY IN *ESCHERICHIA COLI*

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ABSTRACT

The gene–expression system was used to overproduce desired peptides derived from six individual phage clones previously selected by MAb 5145A recognizing CD 4 binding site epitope on HIVgp120. Presenting in the biological context, peptides fused to the N-terminal of filamentous phage were expressed in *Escherichia coli.* Each gene product was generated as inclusion body and soluble forms. The inclusion bodies were produced maximally at 8 hours, whereas the soluble proteins in the supernatant were generated nearly steadily throughout the time profile. The soluble proteins in supernatant were purified by metal chromatography and preliminarily characterized in terms of binding property to MAb 5145A. It was found that the corresponding MAb compared to the control recognized partially purified proteins. This finding indicated that overproduced peptides could adopt the original conformation that was selected when it was in the context of phage scaffold and probably could mimic the functional image of CD4 binding site epitope on HIVgp120.

Keywords: Phage display, fusion proteins, protein expression, and HIV.

INTRODUCTION

The human immunodeficiency virus (HIV) is the etiologic agent of acquired immunodeficiency syndrome (AIDS) in humans, which is spreading unabated in many parts of the world. Ever since the first AIDS diagnosis in 1981, there are more than 20 million deaths and almost 38 million people are living with HIV. The epidemic of HIV infections had been estimated of 4.9 million cases, whereas deaths due to AIDS were 3.1 million cases in 2005 (WHO, 2005). The epidemic remains extremely dynamic: growing and changing character as the virus exploits new opportunities for transmission. The ultimate solution for containment of the AIDS epidemic will require the development of vaccine strategy.

It has been shown that the viruses use envelope glycoprotein (gp) 120 attaches with high affinity to the CD4 molecule, found principally on T lymphocytes, macrophages and dendritic cells in order to initiate a new infection (Turville, 2001).

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The exposure of gp120 displayed on the surface of the virions makes them prime targets for antibodies that potentially bind to the HIV envelope, thereby blocking interactions between the virus and cellular receptors and preventing viral entry into the cells. Currently, there are more than two-dozen potential vaccines in clinical trials in humans, and at least 13 different monomeric gp120 and gp160 candidate vaccines have been evaluated in prophylactic trials (McMicheal and Hanke, 2003). VaxGen Company was tested gp120 AIDSVAX in phase III clinical trials for high-risk HIV-negative volunteers in Thailand in 2003, but the efficacy in preventing HIV was only 3.8% (Veljkovic, 2003). The reasons of this failure were partly due to the high mutation rate (Richman et al., 2003; Wei, 2003) and the complex structure of HIV envelope glycoprotein (Thali, 1992). In spite of high mutation rate on the surface of gp120, the receptor binding sites, which are located in a depression, exhibit high degree of conservation (Kwong et al., 1998). Thus, epitopes at these sites could potentially be used as a vaccine to induce a broad neutralizing antibody response.

Phage display library (Smith et al., 1985) has been employed to investigate molecular interactions, and later it was used to generate ligands with the desired properties for a given target. Random peptide sequences displayed on the surface of a filamentous bacteriophage as in-frame fusions with the coat protein pIII or pVIII constitute a powerful mean to identify peptides recognized by antibodies. The isolated peptides can directly correspond to the natural epitope (Murphy et al., 1998). In most cases, the amino acid compositions of the natural epitope and the selected peptide are not identical but only mimic some continuous amino acid residue (Devlin et al., 1990; Scott and Smith, 1990). Nonetheless, without necessarily conserving the amino acid sequence, some displayed peptides mimic 3D structure on the antigen surface (Luzzago et al., 1993; Felici et al., 1993).

Monoclonal antibody MAb5145A (Pinter et al., 1993), which was obtained from HIV-infected patients and specifically bound to HIVgp120 at receptor binding sites, was used to screen phages from a disulfide constraint random decapeptide phage display library. The selected phage clones have consensus sequences (CGPXEPXGAWXC) in which X represented a variable amino acid residue. These phage clones were also able to compete with gp120 in binding to the original selecting MAb (Teintze, 2006). Thus, the selected phage sequences could mimic the epitopes on gp120.

The objective of this study was aiming to express the DNA of the selected phages in *Escherichia coli.*

MATERIALS AND METHODS

Chemicals

The plasmid, pET101/TOPO®, which contains a T7 promoter, each DNA insert sequence, a C-terminal 6x His-Tag sequence, and ampicillin resistance gene and Benchmark® protein standard marker were purchased from Invitrogen (USA). *Escherichia coli* strain BL21 (DE3) and human MAb 5145A were kindly presented by Dr. Martin Teintze (Montana State University, USA). Alkaline phosphatase (AP) labeled goat anti-human IgG (H&L) and AP labeled goat anti-mouse IgG (H&L) were obtained from Zymed (USA). Phenylmethylsulfonyl fluoride (PMSF) and premixed BCIP/NBT substrates for Western blot and ELISA were purchased from Sigma-Aldrich (USA). One ml His TrapFF crude column was obtained from Amersham Pharmacia Biotech (USA). Isopropyl—D-thiogalactopyranoside (IPTG) was purchased from FisherBiotech (USA). Egg white Lysozyme was obtained from Ampresco (USA).

Time course of protein expression

Escherichia coli BL21 (DE3) harboring the expression plasmid pET101-23A, pET101-24A, pET101-37A, pET101-38A, pET101-40A, and pET101-41A and pET101-35A without the peptide insert used as the negative control, respectively, was incubated with shaking at 37°C in 50 ml LB with ampicillin selection (100 µg/ml) and grown until early log phase (A_{600}=0.5-0.8). The targeted protein expression was induced at 37°C by the addition of 1mM IPTG and then growing the cells with shaking

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for an additional 18 hours. The 500 µl culture was collected for 2 hours interval starting at the addition of IPTG (T_0) until 18 hours (T_9) and centrifuged at 10,000 rpm for 5 min. The cell pellet was harvested and resuspended in 500µl lysis buffer (pH 7.8) containing 50mM K_2PO_4, 400mM NaCl, 100mM KCl, 10% glycerol, 0.5% Triton X-100, 10mM imidazole. The resuspended cells were lysed with five-mg/ml lysozyme on ice for 30 min, sonicated at maximal output and centrifuged at 10,000 rpm for 5 min at 4°C. The pellet and the supernatant were collected. Total proteins of both fractions were separated in 12% polyacrylamide gel electrophoresis.

Expression of fusion proteins

Escherichia coli BL21 (DE3) was transformed with plasmid pET101-23A, pET101-24A, and pET101-35A, pET101-37A, pET101-38A, pET101-39A, pET101-40A, and pET101-41A, respectively, and incubated with shaking at 37°C in 250 µl SOC medium for 30 min. The culture was added with another 10 ml LB growth medium with 100 µg/ml ampicillin and grown overnight with shaking at 37°C. One milliliter of overnight culture was sub cultured into 100 ml LB with 100 µg/ml ampicillin. When it reached an optical density between 0.5-0.8 at 600 nm, 1 mM IPTG was added to the culture to induce gene expression, and the culture was extended for an additional 8 hours at 37°C. Cells were finally harvested by centrifugation for 5 min at 10,000 rpm and stored at -70°C.

Purification of fusion proteins

The harvested cells were resuspended in binding buffer, pH 7.4 (20mM Na_2HPO_4, 20mM NaH_2PO_4, 500 mM NaCl, 40 mM imidazole) with the ratio (w/v) of 1(g):10 (ml). Resuspended cells were treated with 0.2 mg/ml lysozyme, 20 µg/ml DNaseI, 1mM $MgCl_2$ and 1mM PMSF and incubated for 30 min at room temperature with shaking. Sonication was performed on ice for 10 min. The soluble proteins in the supernatant fractions were obtained by centrifugation for 5 min at 10,000 rpm for protein purification. The presence of a six-histidine tag of pIII-fusion proteins allowed for purification by using a His-Ni^{2+}-affinity column (Amersham Pharmacia, USA). The crude extract in the soluble form was applied at room temperature on His- Ni^{2+}-affinity column (1 ml of bed volume) pre-equilibrated with 10 column volumes of binding buffer, pH7.4 containing 20mM NaH_2PO_4, 20mM Na_2HPO_4, 500mM NaCl, 40mM imidazole. After loading, the column was washed with 20-25 column volumes of binding buffer, and bound protein was then eluted with five column volumes of elution buffer, pH 7.4 containing 20mM NaH_2PO_4, 20mM Na_2HPO_4, 500mM NaCl, 500 mM imidazole.

Protein assay

The protein assay was done based on methods described by Bradford for the colorimetric detection and quantification of total protein. The purple colored product exhibited a strong absorbance of OD_{595} that was nearly linear with increasing protein concentration in a range of 0.2-0.9 mg/ml. Protein concentration was determined based on a standard curve of known concentration of bovine serum albumin. The procedures were modified from those that described in the instructions of the manufacturer (BioRad, USA)

Electrophoresis, Western blotting and Dot blot analyses

Proteins samples were resolved using SDS-PAGE in 15% (w/v) polyacrylamide under reducing conditions. The gels were stained with silver staining or transferred onto Immuno-Blot PVDF membrane for Western blot analysis or nitrocellulose membrane for dot blot analysis. For Western blot, the membrane was incubated overnight at room temperature with gentle shaking in blocking solution (1XTBS, 0.05% Tween 20, and 4%BSA) with mouse anti-histidine IgG (1:3,000 dilution). The membrane was then washed in washing solution (1XTBS, pH 7.4, 0.05% Tween 20) and incubated for 2 hours at room temperature with alkaline phosphatase labeled goat anti-mouse IgG in the same buffer at the dilution of 1:1,000

(Zymed, USA). For Dot blot analysis, 2 μl of each purified protein was spotted on the membrane and followed by the incubation overnight with the human monoclonal antibody 5145A (1:50,000 dilution) in blocking solution (1XTBS, pH 7.4, 0.05% Tween 20, and 4%BSA). The membrane was then washed and incubated for 2 hours at room temperature with alkaline phosphatase labeled goat anti-human IgG in the same buffer at the dilution of 1:1000 (Zymed, USA). The detections by both Western blot and Dot blot analyses were performed using Premixed BCIP/NBT (Sigma Aldrich, USA) as the substrate, according to the manufacturer's instruction.

RESULTS

Protein expression study

Human MAb 5145A against conformational epitope at CD4 binding site of HIVgp120 was previously used to select a disulfide constrained-random peptide phage display library with peptides of ten amino acids in length. After multiple rounds of panning, six individual phage clones were selected and their DNAs were sequenced. All peptides except no. 38A selected with the MAb showed a strong consensus sequence with six strictly identical peptides among 10 as shown in Table 1. The consensus sequence follows as CGPXEPXGXWXC, where X is representing a variable amino acid. Competitive ELISA results showed that all phage clones specifically bound to the antibody because their binding could be inhibited by increasing amount of HIVgp120 or their corresponding synthetic peptides (data not shown). These data indicated that the selected phage sequences could mimic the epitopes on gp120. Since there were only 3-5 copies of peptide on phage M13, it was difficult to use it as an antigen to induce antibodies of the peptides. In this case, it is necessary to increase the concentration of the selected ligand by expressing the selected sequence as a fusion to a protein smaller than the phage particle. Therefore, the gene encoding for each peptide flanking with N-terminal domain (D1), and the leader sequence of phage was cloned into a vector pET101 TOPO (Invitrogen, USA).

Table 1. Amino acid sequences of phage displaying disulfide-constrained decapeptides randomly selected through biopanning against human monoclonal antibody 5145A.

Clone no.	Sequence
23A	CGPSEPIGAWDC
24A	CGRAEPRGAWVC
37A	CGPREPDGSWHC
38A	CGPVSRWCVGC
40A	CGPVEPSGVWFC
41A	CGPFEPRGDWTC
35A	(Wild type phage)

Consensus sequence: CGPXEPXGXWXC ; where X represents a variable amino acid and the consensus sequence is highlighted in bold.

To study on protein expression, His_6-tagged fusion proteins were overproduced in *E. coli* BL21 (DE3) that transformed with p24A plasmid. After sonication and centrifugation of the bacteria at each time interval of 2 hours, the supernatant and inclusion bodies were run on 12% SDS-PAGE. As shown in Figure 1, the protein with the approximate molecular weight of 14.2 kDa when compared to egg lysozyme was induced in inclusion bodies at the maximum of 8 hours (T_4). However, the leaky expression of soluble proteins in the supernatants was first observed since T_0 and the protein was stably produced until the end of time profile (T_9). With 1mM IPTG induction, the expressions of His_6-tagged fusion proteins in soluble and inclusion body forms were compared after overproduction for 8 hours at 37°C. The result showed that the fusion protein more distributed in soluble fraction than inclusion body fraction as shown in Figure 2. These results were similar to the rest of recombinant proteins (data not shown).

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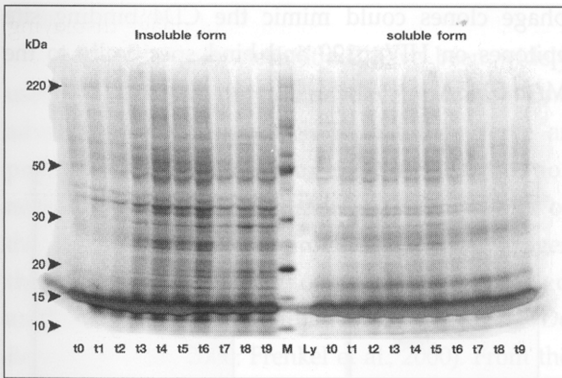

Figure 1. The time course expression of His_6-tagged pIII fusion protein in soluble and inclusion body forms induced with 1 mM IPTG in *E. coli* BL21 (DE3). Cells harboring pET101 TOPO/p24A were harvested every 2 hours from 0 hour(T_0) to 18 hours (T_9) and soluble and inclusion body forms of protein samples were analyzed on 12% SDS-PAGE gel and detected by silver nitrate solution. Lane M, Benchmark® MW marker (Invitrogen); lane Ly, egg Lysozyme with the molecular weight of 14.2 kDa.

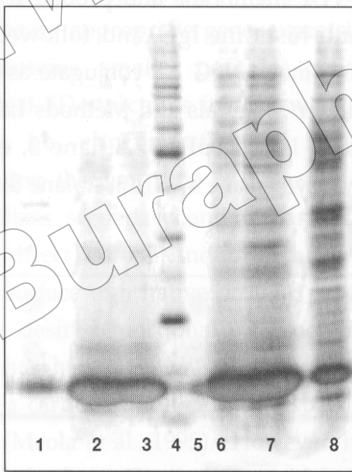

Figure 2. The expression of His_6-tagged fusion proteins induced with 1 mM IPTG for 8 hours in *E. coli* BL21 (DE3). Cells harboring pET101 TOPO/p24A were harvested at T_0 (0 h) and T_4 (8 h) after induction and protein samples were analyzed on 15% SDS-PAGE gel and detected by silver nitrate solution. Lane 1, inclusion bodies (T_0); lane 2, total protein crude extract (T_0); lane 3, soluble forms (T_0); lane 4, Benchmark® MW marker (Invitrogen); lane5, egg Lysozyme with the molecular weight of 14.2 kDa.; lane 6, soluble proteins (T_4); lane 7, total protein (T_4); lane 8, inclusion bodies (T_4).

Purification of the His6-tagged fusion proteins

The recombinant proteins, pIII-41A, exhibiting a high degree of expression at 8 hours were harvested and then purified on His-Ni^{2+}-affinity column with a 5 column volume elution under standard conditions (500 mM imidazole, 500 mM NaCl). The purity of the fusion proteins was examined by SDS-PAGE (15% gel). The resulting His-tagged fusion proteins were shown in Figure 3 that most of the recombinant proteins were eluted in the first two elutions but contaminated with some of other proteins in the crude lysate (Figure 3, lanes 7 and 8). However, cleaner but less amount of isolated proteins could be obtained eventually in the third and fourth elution and were barely detected in the final elution (Figure 3, lanes 9-11). It was interesting that double protein bands from pIII-41A were detected in the purified protein, with approximate molecular weight of 12.83 and 13.66 kDa determined by gene tool program. These results were similar to the other purified recombinant proteins (data not shown). It seems that the molecular weight of these proteins were slightly less than that of the protein with the expected molecular weight of 14.6 kDa.

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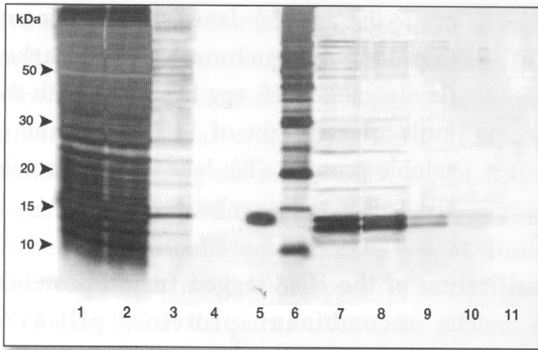

Figure 3. Purification of His6-tagged pIII fusion proteins overproduced in *E. coli*. Aliquots from the purification of His_6-tagged pIII fusion proteins were analyzed on SDS/PAGE (15% gel). Lane 1, total crude lysate of the *E. coli* (pET101 TOPO/ p41A) cells; lane 2, flow through; lanes 3 and 4, wash1 and 2, respectively; lane 5, egg white lysozyme with the molecular weight of 14.2 kDa; lane 6, Benchmark® MW marker (Invitrogen); lanes 7-11, the 1-5 elution fractions from Ni^{2+} column, respectively.

Characterization of fusion proteins

To determine the specificity of purified His_6-tagged fusion protein expressed in *E. coli*, protein samples were analyzed by Western blot analysis. Both protein bands detected with the silver nitrate solution shown in Figure 4A were recognized by mouse anti-histidine IgG shown in Figure 4B (lanes 1, 2 and 4,5) whereas the egg lysozyme was not detected with the antibody (Figure 4B, lane 3). To examine further whether MAb 5145A could recognize the fused decapeptides derived from individual phage clones which appeared on the pIII protein of the recombinant M13 phages; those purified proteins were analyzed with MAb 5145A by Dot blot without reducing agents. The recombinant pIII fusion proteins derived from phage clones 24A, 37A, 38A, and 40A were well recognized by the corresponding MAb, and the strongest signal was obtained by pIII-41A. Surprisingly, pIII-23A gave no signal, which was similar to pIII-35A, which derived from the wild type M13 (Figure 5). The recognition data indicated that the overproduced decapeptide derived from phage clones could mimic the CD4 binding site epitopes on HIVgp120 and bind specifically to the MAb 5145A.

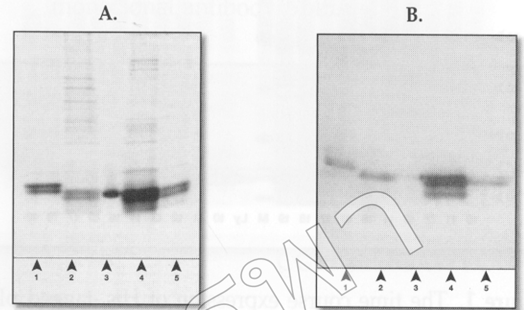

Figure 4. SDS-PAGE and Western blot analysis following purification of His_6-tagged pIII fusion proteins. (A) The second elution fraction (E500-2) of purified proteins was loaded on 15% SDS-PAGE and stained with silver nitrate solution. Lane1, pIII-23A; lane 2, pIII-37A; lane 3, egg white Lysozyme; lane 4, pIII-38A; lane 5, pIII-41A. (B) Western blot analysis of purified recombinant proteins. The proteins were transferred to PVDF membrane and probed with mouse anti-histidine IgG, and followed by goat anti-mouse IgG AP conjugate as described under Materials and Methods Lane1, pIII-23A; lane 2, pIII-37A; lane 3, egg white lysozyme; lane 4, pIII-38A; lane 5, pIII-41A.

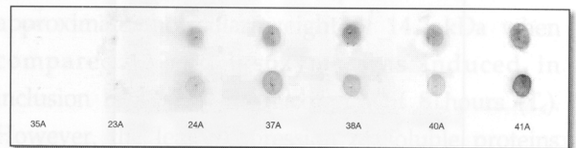

Figure 5. Dot blot analysis. Two µl of the second elution fractions (E500-2) of purified proteins were spotted on a membrane and detected by using human monoclonal antibody 5145A, and followed by goat anti-human IgG AP conjugate as described under Materials and Methods. pIII- 35A without the peptide insert was used as a negative control.

DISCUSSION

Phage display techniques have been widely used in the field of vaccine development. One advantage of this approach is that they serve as possible vaccine components, which do not necessarily represent the structural equivalents of the original antigen, but provide functional images that could replace the originals or so called antigenic mimicry. (Agadjanyan et al., 1997; De Berardinis et al., 2000; Frenkel et al., 2000). From the previous study, random peptides displayed on the surface of filamentous phages were selected to identify CD4 binding epitopes on HIV gp120 through repeated cycles of specific binding to MAb 5145A (Teintze, 2006). Due to the conformational epitope at CD4 BS, it was important to use a conformationally constrained peptide library. It is known that constrained or cyclic phage peptide libraries, engineered with flanking cysteines, present peptides with a stable backbone that is more prone to mimic the natural epitope (O'Neil et al., 1992; and Giebel et al., 1995). Through successful application of recombinant DNA technology, it is feasible to produce desired peptides in large amounts using appropriate gene-expression system. Unlike proteins, smaller peptides generally lack well-defined 3D structures in aqueous solution and tend to be conformationally mobile. By incorporating these peptide mimetics in a template that stabilizes secondary and tertiary structure, in order to direct critical and orientation as in the bioactive surface of a native protein, peptides could retain the desired functional or structural features. A study has helped in gaining insight the role of phage as a carrier in inducing of a specific immune response (Meola et al., 1995). However, it would be difficult to immunize with sufficient phage carrying peptide on pIII molecules in order to get the potent immune response. There was a study that recombinant rat apoE developed by a bacterial expression system can be compared favorably both structurally and functionally to native rat apoE purified from plasma (Pearson et al., 2005). This lends confidence that the produced recombinant proteins could adopt the original conformation that was selected when it was in the context of phage scaffold. Thus in this study, peptides were expressed in the context of phage scaffold. We report here that the successful over expression in *E. coli* of the recombinant pIII fusion proteins derived from random peptide phage display. Experiments indicated that the recombinant proteins were produced as soluble and inclusion forms but more distributed to the former than the latter (Figure 2, lane 6 and lane 8). The leaky expression of soluble protein in the supernatants was observed since the start of the time profile (Figure 1, lane 12). However, it did not matter as the overproduced proteins were not toxic to the cells.

The introduction of His_6 tag at the C-terminus allows very efficient, rapid purification by $Ni2^+$ metal affinity chromatography. The soluble form was easily purified because it could omit denaturation and refolding during purification step used for inclusion body. Nevertheless, the obtained proteins were not pure due to some other proteins remaining in the first two elution fractions (Figure 3). Thus, we need to include further purification step by gel filtration. Interestingly, double protein bands of the purified proteins were observed on 15% gel SDS-PAGE stained with the silver nitrate solution (Figure 3, lane 7-9). Western blot analysis revealed that such bands were recognized with anti-His antibody (Figure 4, B). Another observation was that these double bands had slightly smaller molecular weight than the expected molecular weight of 14.6 kDa. It was possible that some amino acids comprising the phage leader peptide at the N-terminal of the soluble pIII fusion peptides could be cut off due to enzymatic digestion in the cytoplasm.

Dot blot analysis was carried out in preliminary study to characterize the ability of purified proteins in binding to mAb 5145A. In this case, Dot blot analysis was done rather than Western blot analysis because the former could be performed for screening in a very short time and the result would not be significant different than the latter when purified proteins were used. The result obtained from Dot blot analysis demonstrated that after purification most of purified soluble pIII

fusion proteins retained biological activity in binding to MAb 5145A (Figure5). The signal intensity difference observed on the Dot blot was not due to the consequence of protein concentration because pIII-41A with the very low protein concentration had the strongest binding activity. Surprisingly, pIII-23A showed no reactivity with MAb 5145A despite a clear consensus sequence. It was feasible that pIII-23A fusion protein adopts a different structure when expressed in *Escherichia coli.* Taken together, we could draw a preliminary conclusion that the produced peptides could adopt the original conformation that was selected when it was in the context of phage scaffold. However, ELISA may be required for quantitative binding to mAb 5145A in the future to ensure the specific binding. In addition, the competitive assay by using gp120 as a competitor need to be performed for further studies in order to confirm that these expressed peptides mimic the natural epitope on CD4BS of HIVgp120.

In order to retain the peptide in the original conformation, the DNA of the selected phages fused to its N-terminal domain (D1) flanking with the leader sequence was cloned into a vector, and expressed in *Escherichia coli.* The expressed fusion proteins with a C-terminal histidine tag were purified by affinity chromatography. The results showed that most of these peptides bound to the original selecting MAb. These studies will provide a potential mean to facilitate further investigation in designing new agents against HIVgp120

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