

Bacteriophage display of peptides and its use in determining proteolytic substrate specificity

C. Carrie Liu, Joti Nahal, Kim Melton, Navneet Sharma

ABSTRACT

Introduction: Phage display has been employed for many applications including generating highly specific antibodies, determining protein-protein interactions and substrate specificity determination. We have recently used peptide display on T7 phage to successfully determine the substrate specificity of serine proteinases (Sharma et al., 2008, 2011 and 2013). The random phage library we made displays a hexameric peptide on the surface using the library construction described by Karlson et al. (2002) with minor modifications. Each phage particle is able to display no more than one peptide on its surface. Therefore, it is a unique library with each recombinant phage particle displaying a random peptidic substrate on the surface. The library represents all the possible combinations of random hexamers (6.7×10^7) as described by Deperthese (2002). **Protocol:** In this article, we demonstrate the process of building the phage library with random peptides displayed on the surface and using it for determining the substrate specificity of a proteolytic enzyme by repeated rounds of biopanning. The phage library being built is a random one with each phage particle displaying a random peptide on its surface. The library is sequenced after constructing and the

frequency of display is noted down. The ratio of displayed amino acid (expected vs. displayed) is determined and it is well within the range (i.e. 0.5–2.0) as shown by Cwirla et al. (1990), confirming the randomness of the library. **Conclusion:** In order to check the validity of this library, we have tried to find peptidic substrates for a known serine proteinase i.e. trypsin in this paper. All the substrates found for trypsin are known, thus confirming the validity of this library. The aim of this paper is to show an innovative technique for the display of substrate peptides on the surface of bacteriophage and their random selection based on substrate specificity of a protease. It can be applied to any protease for determining its substrate specificity as we have done.

Keywords: Phage display, Substrate specificity, T7 Phage, Serine proteinase, Oligonucleotide, Phage plaques, Polymerase chain reaction (PCR)

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INTRODUCTION

Herein, we have described a method used for display of random peptides on the surface of bacteriophage in a way that its frequency of display can be regulated. It

can be used in multiple applications like characterization of proteolytic activity as done in this case. Other than characterizing substrate specificity of proteases, the technique can be utilized for various other purposes like identifying specific antibodies, interaction with specific ligands and many more. Many publications have come up since the publication by Cwirla et al., (1990) for identifying ligands displayed on the surface of a bacteriophage. In fact, there have been so many biotech companies as well, working in the pursuit of identifying suitable substrates as well as inhibitors using this technique. We have used this technique for identifying specific substrates for some proteolytic enzymes like implantation serine proteinase (a native dimer), recombinant ISP1, recombinant ISP2 along with human kallikrein 6 (KLK6). The protocol used has been described here in detail [1–6].

MATERIALS AND METHODS

The following materials are utilized for the construction of T7 phage-displayed peptide library:

1. T7 select 1-1 vector arms
2. T7 select system (M/S Novagen Canada: EMD Biosciences Inc., an affiliate of Merck KGaA, Darmstadt, Germany)
3. Trypsin (M/S Sigma)
4. *E. coli* (BLT 5403) – obtained from ATCC
5. Magnesium Sulfate (M/S sigma)
6. Agarose (M/S HiMedia)
7. NiNTA Agarose Beads (M/S GE)
8. Tris.Cl (M/S Sigma)
9. PCR (M/S BioRad)
10. PCR Purification Kit (QIAquick from M/S Qiagen)

PROTOCOL TEXT

Construction of the phage display library

1. Start by constructing the oligonucleotides that need to be displayed on the surface of bacteriophage. The sequence of synthetic oligonucleotides inserted in the coding region of the T7 phage capsid (employing T7 select 1-1 vector arms, T7 select system, Novagen Canada; EMD Biosciences Inc., an affiliate of Merck KGaA, Darmstadt, Germany) is shown here:
 - 5'-AAT TCT CTC ACT CCA GGC GGC-(NNK)₆-GGT GGT CAT CAC CAT CAC CAT CAC TAA-3' whereas N represents any nucleotide and K represents thymine (T) or guanine (G)
 - CTC, ACT and CCA are the nucleotide codes for leucine, threonine and proline. It is part of the linker and helps in isolation of the random peptide.
 - (NNK)₆ represents the oligonucleotides for random hexamer of amino acids. N represents

any nucleotide and K represents thymine (T) or guanine (G).

- GGC GGC and GGT GGT are the nucleotides that represent flexible glycine dimers at either end.
 - CAT CAC CAT CAC CAT CAC are nucleotides that represent a (His)₆ tag at the C-terminus.
 - TAA represent a STOP codon at the 3'-terminus of oligonucleotide.
2. Three oligonucleotides are constructed as given below:
 - A. AAT TCT CTC ACT CCA GGC GGC-(NNK)₆-GGT GGT CAT CAC CAT CAC CAT CAC TAA
 - B. GCC GCC TGG AGT GAG AG
 - C. A GCT TTA GTG ATG GTG ATG GTG ATG ACC ACC

Different concentrations of these oligonucleotides are made up for use in the construction of the library. 2 μM of A, 40 μM of B and 40 μM of C is made up in the following buffer:

- 10 mM Tris.Cl (pH 8.0) and 0.2 mM EDTA
3. T7 phage DNA vector (Novagen, Canada) digested by EcoR I and Hind III is used for ligation of the oligonucleotides. The ratio for ligation between T7 phage vector and oligonucleotides A, B and C is 1:5:100:100, i.e., 0.004 μM of vector, 0.02 μM of oligonucleotide A and 0.4 μM each of oligonucleotide B and C. 5X oligonucleotide concentration will be used for annealing for 1 μL of annealed oligonucleotides in 5 μL ligase reaction. That means 0.1 μM of oligonucleotide A and 2 μM each of oligonucleotide B and C.
 4. The annealing is done in 100 μL volume in the ratio as given below:

5X oligonucleotide annealing buffer	-20 μL
2 μM oligonucleotide A	-5 μL
40 μM oligonucleotide B	-5 μL
40 μM oligonucleotide C	-5 μL
DDW	-65 μL

Heat it to 70°C for 5 minutes and let it cool slowly to room temperature.
 5. Ligation is done in 5 μL total volume. But before ligation, the de-phosphorylated vector is re-phosphorylated once again as given below:

T7 DNA vector (EcoR I-Hind III digested)	-1.0 μL
and de-phosphorylated)	
10X Ligase buffer (containing ATP, DTT)	-0.5 μL
but no PEG)	
DDW	-1.0 μL
T4 polynucleotide kinase	-0.5 μL
Annealed oligonucleotides	-1.0 μL

Incubate it at 37°C for 20 minutes. Add 1.0 μL T4 DNA ligase and incubate at 16°C overnight. Store at 4°C until in vitro packaging.
 6. Thaw the in vitro packaging extract (T7, Novagen, Canada) on ice in the ice bucket. Do not freeze

thaw quickly at all. Add ligation reaction (5 μ L) to this extract. Mix gently maybe with a pipette-man but do not froth. Stop the reaction by adding 270 μ L LB after incubating it for two hours at room temperature.

Titration

1. Inoculate BLT5403 strain of *E. coli* in M9TB medium at 37°C. Let OD₆₀₀ reach 1.0 and store at 4°C (up to 48 hours).
2. Prepare molten top agarose in a bottle and keep it at ~50°C.
3. Prepare a series of dilutions using LB as the diluent in 1.5 mL eppendorf tubes. Make the following dilutions as given below:

10 ⁻² dilution	-	10 μ L Phage + 990 μ L LB
10 ⁻³ dilution	-	100 μ L phage from 10 ⁻² dilution + 900 μ L LB
10 ⁻⁴ dilution	-	100 μ L phage from 10 ⁻³ dilution + 900 μ L LB
10 ⁻⁵ dilution	-	100 μ L phage from 10 ⁻⁴ dilution + 900 μ L LB
10 ⁻⁶ dilution	-	100 μ L phage from 10 ⁻⁵ dilution + 900 μ L LB
4. Mix 3 mL top agarose with 100 μ L dilution as shown above and 250 μ L BLT5403 host cells. Pour it over a pre-warmed LB-Agar (Amp⁺) plate.
5. Incubate for three hours at 37°C, or overnight at room temperature.
6. Count the number of plaques per plate and estimate the total number of phages in the library.

Amplification of the library

1. Grow BLT5403 culture to 500 mL in LB medium (+ 50 μ g/ml ampicillin) at 37°C. Grow until OD₆₀₀ = 0.5–1.0.
2. Infect the bacterial cell culture with unamplified phage library. Add all of the phage particles including the ones in the remaining 10⁻² dilutions made for estimating the titre.
3. Incubate the culture at 37°C with shaking for 1–3 hours until lysis is observed.
4. Spin the culture at 8000 x g in JA-10 rotor (6730 rpm) for 15 minutes. Decant the supernatant back into sterile bottles.
5. Store the amplified phage library at 4°C.
6. Count the number of plaques/mL in the library as per the titration method given in section titration. Since the number of plaques is high in the amplified library it is better to look for the number in 10⁻⁶ – 10⁻¹⁰ dilutions.

PCR Analysis

Plaques were eluted from a plate having less than 100 colonies so that it is not difficult to pick the plaques. Single plaques were eluted from the plate into 200 μ L SM (Recipe given in the media

recipes). Do the PCR in 25 μ L reaction volume, without pre-heating and without hot start. Here is the recipe for PCR reaction:

Plaque lysate	-1.0 μ L
DDW	-19.075 μ L
50 mM MgCl ₂	-0.80 μ L
10X PCR Buffer (no Mg ²⁺)	-2.5 μ L
5 μ M T7 Select UP Primer	-0.5 μ L
5 μ M T7 Select DOWN Primer	-0.5 μ L
10 mM each dNTPs	-0.5 μ L
Taq DNA polymerase	-0.125 μ L

Follow the following temperature conditions at different steps:

- a) 94°C - 3 min
 - b) [94°C - 0.5 min; 52°C - 0.5 min; 72°C - 1 min] x 38 cycles
 - c) 72°C - 6 min
- Incubate at 4°C till further use.

Biopanning

Subsequent to binding of amplified phage displayed library (1x10¹⁰ pfu) with 100 μ L of Ni-NTA agarose beads (Sharma et al. , 2008); biopanning is performed with 10 μ L of trypsin (Sigma). A control elution is performed using 500 mM imidazole solution. Repeat the process five times and obtain the plaques after lysis of the *E. coli* (BLT5403) culture upon infection with plaques. These plaques are plated on LB agarose plates as explained in the titration section above. The plaques from these plates can be amplified and picked for amplification of DNA by PCR using T7 primers. The PCR product was purified using QIAquick PCR purification kit (Qiagen, Canada). These DNA samples are sent for sequencing after PCR and the amino acid sequences are deduced from there.

Characterization

The results of sequencing reactions give the amino acid stretches in each reaction as a result of the DNA sequence of each plaque. The results are compiled in a tabular form and then seen if they represent any uniformity or not. As is evident from the results, the substrate specificity results of trypsin are similar to its published data. The sequencing reactions results after PCR of the library are given in Table 1. The amino acid content of each amino acid in the above sequenced reactions before biopanning is given in Table 2. Figure 1 describes the different steps in the process of using phage display library for substrate specificity determination.

Table 1: Amino acid sequence of the random hexamer in each plaque after DNA sequencing.

Plaque No.	AA1	AA2	AA3	AA4	AA5	AA6
1	Y	S	V	G	I	A
2	T	R	V	q	L	R
3	M	R	G	V	P	L
4	G	G	V	R	V	P
5	G	Y	D	R	R	A
6	A	A	Q	G	Y	G
7	Y	D	V	Q	K	K
8	S	M	R	L	S	V
9	S	F	D	S	R	L
10	G	S/P/T/A	R	V	G	C
11	G	Y	R	G	I/M	Y
12	R	R	C	S	L	L
13	G	L	F	V	R	R
14	G	T	R	A	Y	V
15	E	P	V	R	S	G

Table 2: Amino acid content in the random phage display library.

Amino Acid	Nominal (% Nom)	Observed (% Obs)	Observed / Nominal
G	6 (0.065)	15 (0.156)	2.5
R	9 (0.097)	16 (0.166)	1.77
V	6 (0.065)	11 (0.115)	1.83
T	6 (0.065)	2 (0.021)	0.33
Y	3 (0.035)	8 (0.083)	2.66
S	9 (0.097)	7 (0.073)	0.77
L	9 (0.097)	7 (0.073)	0.77
E	3 (0.032)	1 (0.010)	0.33
P	6 (0.065)	3 (0.031)	0.5
A	6 (0.065)	5 (0.052)	0.83
C	3 (0.032)	3 (0.031)	1
I	3 (0.032)	2 (0.021)	0.67
M	3 (0.032)	4 (0.042)	1.33
K	3 (0.035)	3 (0.031)	1
F	3 (0.032)	2 (0.021)	0.67
Q	3 (0.032)	3 (0.031)	1

*The expected ratio of observed versus expected frequency of the amino acid occurrence ranges from 0.5–2.0 confirming the randomness of the library. The only anomalies observed are in the case of Glycine (G) and Tyrosine (Y) may be because of the less number of sequences.

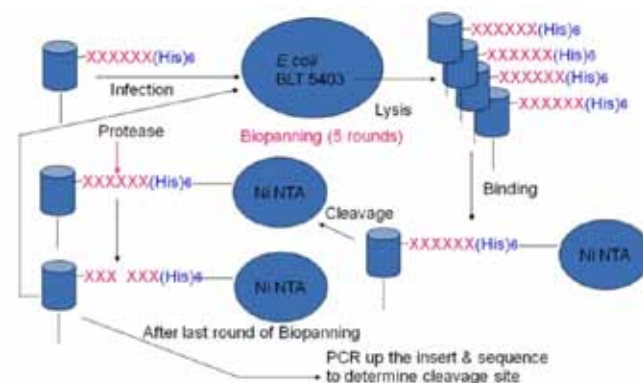


Figure 1: Overall procedure for utilizing the phage display library for determining substrate specificity.

CONCLUSION

It is clear from the results that most of the amino acids show random display in the library. The results could be improved by sequencing a greater number of plaques. The results obtained after biopanning with trypsin are exactly as expected.

ABBREVIATIONS

AA: Amino Acid, % Obs: % Observed, % Nom: % Nominal, G: Glycine, R: Arginine, V: Valine, T: Threonine, Y: Tyrosine, S: Serine, L: Leucine, E: Glutamic Acid, P: Proline, A: Alanine, C: Cysteine, I: Isoleucine, M: Methionine, K: Lysine, F: Phenylalanine, Q: Glutamine

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Author Contributions

C. Carrie Liu – Substantial contributions to conception and design, Acquisition of data, Analysis and interpretation of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published

Joti Nahal – Analysis and interpretation of data, Revising it critically for important intellectual content, Final approval of the version to be published

Kim Melton – Analysis and interpretation of data, Revising it critically for important intellectual content, Final approval of the version to be published

Navneet Sharma – Analysis and interpretation of data, Revising it critically for important intellectual content, Final approval of the version to be published

Guarantor

The corresponding author is the guarantor of submission.

Conflict of Interest

Authors declare no conflict of interest.

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