Electronic Supplementary Material (ESI) for RSC Advances.

Identification of high affinity peptides for capturing Norovirus capsid proteins

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Materials and Methods

Chemicals

Horseradish peroxidase (HRP) conjugated anti-M13 monoclonal antibody was purchased from GE Healthcare (Piscataway, NJ). Tween 20 and ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt were purchased from Sigma (St. Louis, MO). Unless otherwise stated, all of chemicals were of reagent grade.

E. coli strains and bacteriophages

Escherichia coli strain ER2738 [F'*proA*$^{+}$*B*$^{+}$ *lacI*^{q} *Δ*(*lacZ*)*M15 zzf::Tn*10(Tet^{R})/*fhuA2 glnV Δ (lac-proAB) thi-1 Δ(hsdSmcrB)5*] as host for M13 phage infection and M13 random phage displayed library (Ph.D.-12) which expresses random and linear 12-mer peptides were obtained from New England Biolabs (Ipswich, MA). In brief, this peptide library is fused to pIII protein which causes five copies of a particular peptide to be displayed on the surface of phage and the sequence of each peptide is encoded in the phage genome.

Cloning of the norovirus capsid P1 or P2 domain

The Norwalk virus capsid gene (VP1) sequences were obtained from GenBank (accession number no. Q83884). The gene encoding Norwalk virus envelope protein of 124 or 126 amino acids was located in the base pairs from 5358 to 6950. The P1 domain consists of amino acid residues 225 to 278 and 406 to 520. The P2 domain consists of amino acids 279 to 405 as an insertion into the P1 domain. To improve the expression of P1 proteins containing 406-520 amino acids or P2 proteins containing 279-405 amino acids fused with six histidines at N-terminal, the Norwalk virus capsid gene coding sequence was altered to reflect the optimized codon-usage pattern of *E. coli* with high-frequency codons. Based on this optimized sequence, recombinant gene was synthesized by using HT-oligoTM synthesizer (Bioneer, Daejeon, Korea).

Notably, the synthesized genes have the same amino acid sequence as the native protein sequence in Norwalk virus. For the cloning and expression of pET22-6H-P1 and pET22-6H-P2 fused genes in *E. coli*, PCR experiments were carried out using the following two primers. The forward primer 5'-
CCCAGAACTCATATGCACCACCACCACCACCACGCCCCTTCTGTATACCCC CCTGGT
TTCGGAGAG GTA for P1 domain was designed to contain an *Nde*I site (underlined). The backward primer, 5'-
ACAAACCTACTCGAGTCATCGGCGCAGACCAAGCCTACCTCTTGCCGAGCT
GGCAGT for P1 domain was designed to contain an *Xho*I (underlined). The forward primer 5'-
CCCAGAACTCATATGCACCACCACCACCACCACACCACCCCAGTTTCATTGTCACAT
GTTGCCAAGATA for P2 domain was designed to contain an *Nde*I (underlined). The backward primer 5'-

ACAAACCTACTCGAGTCATAGATGTGTTGCCTCCGTAATACTTGACCCATAATT for P2 domain was designed to contain an *Xho*I (underlined).

PCR experiments were performed with a PCR thermal Cycler T1 (Biometra Co., Goettingen, Germany) using the High Fidelity PCR machine (Boehringer Mannheim, Mannheim, Germany). All restriction enzymes use in this study were purchased from New England Biolabs (Beverly, MA). The DNA sequences of all genes were confirmed by sequencing with the automatic DNA sequencer (ABI Prism model 377, Perkin Elmer Co., Downer's Grove, IL). The amplified fragments were digested with restriction enzymes *Nde*I and *Xho*I (sites underlined) and ligated into the same sites of pET22b(+) (Novagen, Darmstadt, Germany) to make pET22-6H-P1 and pET22-6H-P2.

Production of 6H-P1 and 6H-P2 fusion proteins

The 6H-P1 and 6H-P2 fusion proteins were produced by cultivation of *E. coli* BL21(DE3) harboring pET22-6H-P1 and pET22-6H-P2, respectively. For the expression, cells were cultivated in 250 mL flask containing 100 mL of LB medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L) supplemented with ampicillin (50 μg/mL) in as shaking incubator at 37℃ and 200 rpm. At the OD_{600}=0.6, isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma, St. Louis, MO) was added to a final concentration of 1 mM. Cells were further cultivated for 5 h and were harvested by centrifugation at 6,000 rpm for 10 min at 4℃ and washed two times with buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.0). And then, cells were disrupted by sonication for 1 min at

40% ouput. After centrifugation at 12,000 rpm for 30 min at 4℃, the supernatant was used for the purification by using Ni-NTA column chromatography (Qiagen, Valencia, CA).

Purification of fusion proteins

As two fusion proteins have a six histidine tag at N-terminus, they could be simply purified using Ni-NTA resin. The soluble 6H-P2 recombinant proteins expressed from *E. coli* BL21(DE3) cells were purified under native conditions with a Bio-Rad HR system (Bio-Rad). Briefly, the cell lysates were loaded onto a column containing IDA Excellose resin (Bioprogen Co., Daejeon, Korea) and the samples of flow-through containing unbound proteins were collected for subsequent analysis. The insoluble 6H-P1 recombinant protein fractions of *E. coli* cells as inclusion bodies were re-suspened in 50 mM Tris-HCl, 1 mM EDTA, 2M Urea (pH 8.0) and denatured under denaturing conditions (50 mM Tris-HCl (pH8.0), 0.2 M NaCl, 8 M Urea, 10 mM DTT, 10% Glycerol. After denaturation, the samples were incubated with IDA Excellose resin at 4 °C for 1 h with shaking for effective binding of the 6His-tagged proteins to the resin. Then, the fusion proteins were refolded by slowly removing urea with dialysis. Finally, the eluted proteins were dialyzed against following buffers (refolding method; step dilution from 8 M to 2 M (50 mM Tris-HCl, 0.2 M NaCl, 1mM DTT, 10% Glycerol, pH 8.0).

SDS-PAGE electrophoresis

The recombinant proteins from total cell extracts, soluble and insoluble proteins were separately by SDS-PAGE. For total cell extracts and insoluble fractions after cell disruption, cell

pellets were directly re-suspended in an appropriate sample buffer and analyzed in 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels with Coomassie blue staining. The protein concentration was determined using Bradford protein assays.

General M13 methods and DNA sequencing analysis

Phage particle purification, concentration and DNA isolation were carried out according to the instructions of the manufacturer. Clones of interest were sequenced using the -96 pIII sequencing primer (New England Biolabs, 5'-GCCCTCATAGTTAGCGTAACG-3').

Biopanning phage-displayed peptide library

Highly purified two recombinant proteins encoding pET22-6H-P1 or pET22-6H-P2 were dissolved in 50 mM Tris-HCl (pH 7), 50 mM NaCl buffer and transferred to the wells of the 96-microwell plates. After overnight incubation at 4°C, coated wells were filled with blocking buffer (0.1 M $NaHCO_3$ (pH 8.6), 5 mg/mL BSA, 0.02 % NaN_3) and incubated at 4°C for 1 h. After removing the blocking solution and unbound reagents, wells were washed six times with TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20). The Ph.D.-12 phage displayed random peptide library (1.5×10^{11} pfu) in 100 μL of TBS buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl) was added to each well containing immobilized recombinant proteins, and the plate was shaken gently at 4°C for overnight. To remove the unbound phage, the plate was washed 10 times with TBST. Five rounds of biopanning were performed for each recombinant proteins. After washing, the bound phages were eluted using 100 μL of 0.2 M glycine–HCl (pH

2.2). The eluent was immediately neutralized with 15 μL Tris–HCl (pH 9.1) to prevent the destruction of the phage.

The eluted phages were amplified using *E. coli* ER2738 strains to make sufficient copies for subsequent rounds of biopanning. The amplified phages were harvested by NaCl/polyethylene glycol precipitation (20% (v/v) polyethylene glycol-8000 with 2.5 M NaCl). After every round of biopanning, the recovered phages were titered by plating aliquots of the infected *E. coli* ER2738 prior to amplification on Luria–Bertani (LB) agar containing isopropyl β-D-thiogalactopyranoside (IPTG) and X-gal. The plates were incubated overnight at 37°C and the blue colonies were counted. The enrichment of bound phages was calculated as follows: output phage/input phage × 100. The blue plaques were randomly picked and amplified for DNA sequencing.

Sequence analysis and peptide synthesis

Single-stranded DNAs of positive phage clones were sequenced by Genotech (Daejeon, Korea) using the -96 pIII sequencing primer: 5′-CCC TCA TAG TTA GCG TAA CG-3′. According to the phage pIII gene-derived reading frame in the coding strand, we derived the amino acid sequence of the exogenous protein fusion to the M13 coat protein pIII. BLAST searches were performed using the SWISSPROT database to determine sequence similarity with previously identified peptides or proteins. The Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/) and ExPASy Tools (web.expasy.org/translate) translate were used to analyze the sequences identified earlier.

ELISA for peptide binding to recombinant proteins

To test whether the selected phage clones could specifically bind to recombinant proteins (6H-P1 or 6H-P2), ELISA assays were performed. Plates were coated with recombinant proteins for overnight at 4°C, blocked with blocking buffer (0.1 M $NaHCO_3$, pH 8.6, 1 % non-fat dry milk, 0.02 % NaN_3), and washed six times with TBS solution. One hundred microliters of amplified phages (10^{11} pfu) were then added and incubated for 1 h at room temperature. After washing six times with the same buffer to remove unbound phages, HRP-conjugated anti-M13 monoclonal antibody (diluted 1:10,000 in blocking buffer) was added and incubated at room temperature for 1 h. The antibody solution was removed and the plate was washed again with TBS. Freshly prepared HRP substrate (36 μL of 30% H_2O_2 added to 21 mL of ABTS stock: 22 mg of 2,2'-Azino-bis (3-ethylbenzthiazoline)-6-sulfonic acid in 100 mL of 50 mM sodium citrate, pH 4.0) was added and measured at 405 nm with a microplate spectrophotometer (Multiskan FC, Thermo Scientific, CA, USA). Polystyrene wells coated with 1 mg/mL of BSA, and wells incubated with 1 % of milk in TBS were used as negative controls.

Measurement of the apparent dissociation constants

The measurement of apparent dissociation constants ($K_{d,\mathrm{app}}$) for the selected peptides was performed according to the method of Friguet *et al.* (1985) with minor modifications. Briefly, recombinant P2 proteins at various concentrations were first incubated with the desired phage clones in TBS buffer containing 1% BSA at a fixed concentration (10^{11} pfu/mL). After 24 h of incubation at 4°C, 100 μl of each mixtures were transferred into well plates pre-coated with each recombinant P2 proteins and re-incubated for 1 h at room temperature with mild shaking. After

washing twice with TBS buffer, the bound phages were detected by adding the HRP-conjugated anti-M13 antibody and ABTS, and the color changes in the wells were measured at 405 nm. The apparent $K_{d,\mathrm{app}}$ values were estimated from the slopes of the regression curves obtained by plotting the fraction of bound antibody versus the molar concentration of recombinant P2 protein. Student's t-test was used to compare the $K_{\mathrm{d,app}}$ values.

Fig. S1. Construction of plasmids for the expression of recombinant 6H-P1 (a) or 6H-P2 protein (b).

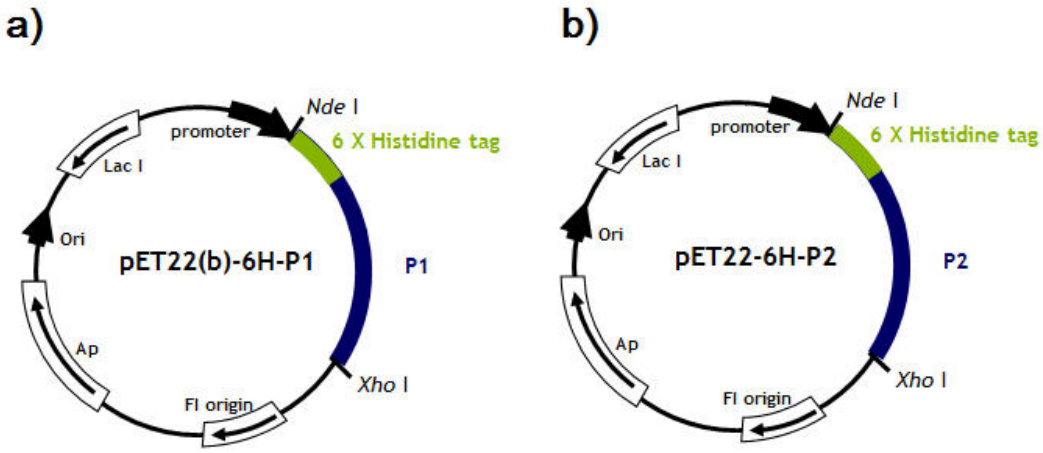

Fig. S2. SDS-PGAE analysis of recombinant fusion proteins. For 6H-P1 fusion proteins expression, *E. coli* BL21(DE3) cells harboring pET22-6H-P1 (a) or pET22-6H-P2 (c)were cultivated in 1,500 mL containing 100 mL of LB medium supplemented with ampicillin (50 μg/mL) in shaking incubator. Cells were further cultivated for 5 h after induction with IPTG were harvested by centrifugation. And then, cells were disrupted by sonication and recombinant proteins from total cell extracts, soluble and insoluble proteins were separately by SDS-PAGE. Finally, the recombinant 6H-P1 (c) or 6H-P2 (d) fusion protein was successfully purified by using Ni-NTA resins.

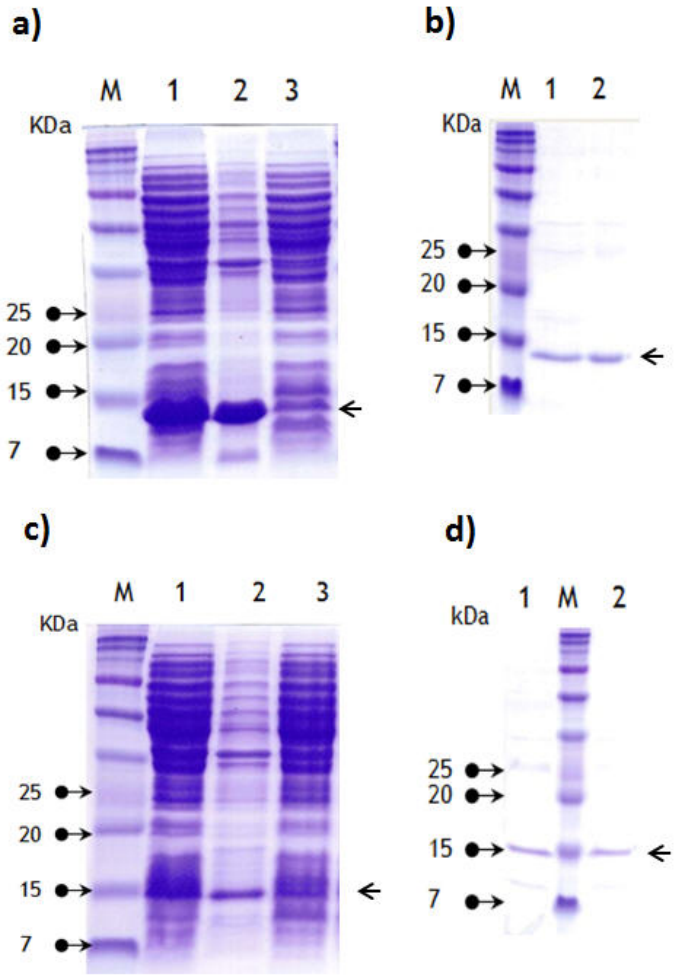

Fig. S3. SDS-PAGE analysis of recombinant 6H-P1 proteins after refolding step.

Fig. S4. Reactivity test of recombinant 6H-P1 and 6h-P2 proteins. For the antigenicity analysis, purified 6H-P1 or 6H-P2 fusion proteins was immobilized on the 96-well microplate for overnight at 4°C, blocked with blocking buffer (0.1 M $NaHCO_3$, pH 8.6, 1 % non-fat dry milk, 0.02 % NaN_3), and washed six times with TBS solution. And then, anti-Norovirus capsid protein VP1 antibody (ab92976, abcam, MA, USA) (diluted 1:5,000 in blocking buffer) was added and incubated at room temperature for 1 h. The antibody solution was removed and the plate was washed again with TBS. Freshly prepared HRP conjugated anti-IgG antibody (diluted 1:1,000 in blocking buffer) was added into the wells and incubated for 1 hr. After washing with buffer, ABTS (17 μL of H_2O_2 added to ABTS stock: 2.2 mg of ABTS in 10 mL of 50 mM of sodium citrate buffer, pH 4.0) was added and measured at 405 nm with a microplate spectrophotometer (Multiskan FC, Thermo Scientific, CA, USA).

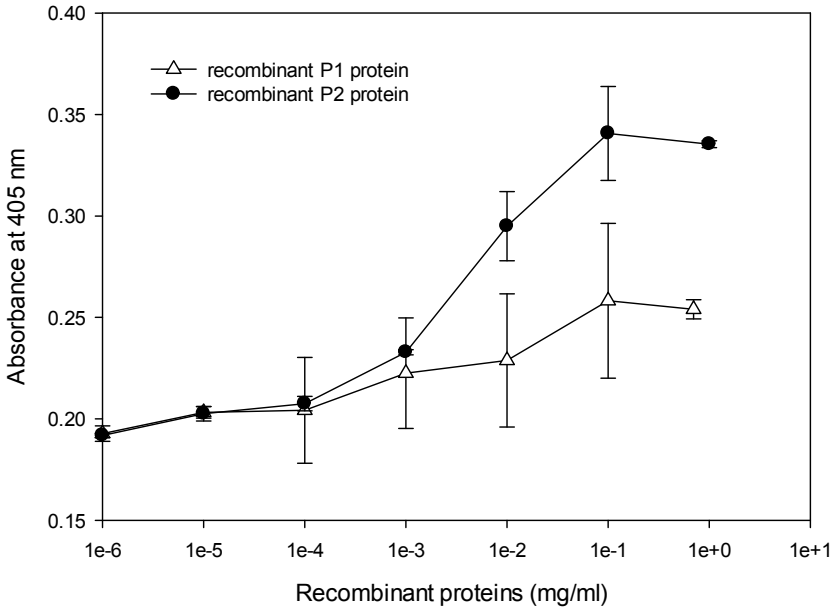

Table S1. Enrichments obtained during biopanning with the phage-displayed peptide library against Norovirus capsid recombinant protein

Biopanning round	Titer (pfu/mL)		Yield [a] (%)	
	Recombinant 6H-P1 protein	Recombinant 6H-P2 protein	Recombinant 6H-P1 protein	Recombinant 6H-P1 protein
1st				
Input	9.5×10^{12}	9.5×10^{12}	1.48×10^{-5}	3.68×10^{-6}
Output	1.41×10^{6}	3.5×10^{5}		
2nd				
Input	1.94×10^{12}	1.0×10^{12}	1.49×10^{-5}	2.5×10^{-6}
Output	2.9×10^{5}	2.5×10^{4}		
3rd				
Input	5.2×10^{11}	1.14×10^{12}	7.11×10^{-2}	2.63×10^{-3}
Output	3.7×10^{8}	3.0×10^{8}		
4th				
Input	1.2×10^{11}	3.6×10^{11}	8.0×10^{-3}	8.33×10^{-4}
Output	9.6×10^{6}	3.0×10^{7}		
5th				
Input	2.3×10^{11}	2.1×10^{11}	6.86×10^{-1}	2.28×10^{-1}
Output	1.58×10^{9}	4.8×10^{8}		

[a] Percent yield was calculated as follows: output/input phage × 100

Table S2. Sequences of the selected phage clones following the fourth and fifth rounds of biopanning against two recombinant proteins

Name	Amino acid	Frequency	Notes
For recombinant 6H-P1 proteins			
4R5A9	INRRQRPRTRHI	2/20	Selected after 4th round
5R5A9	HKRQLRHTIMRT	2/20	Selected after 5th round
5R5A12	IRPHRMRMLIQM	10/20	Selected after 5th round
For recombinant 6H-P2 proteins			
5R7A2	LSITSLRIMRLQ	7/15	Selected after 5th round
5R7A10	QHIMHLPHINTL	7/15	Selected after 5th round Strong binder by ELISA Chosen for characterization

Table S3. Analysis of the hydrophobicity/hydrophilicity of the selected peptides

Clone name	Negative charged amino acid	Positive charged amino acid	Total net charge	Total hydrophobic ratio (%)
5R7A2	no	Arg at positions 7 and 10	+2	50
5R7A10	no	His at positions 2, 5 and 8	0	41

Sequence analysis was performed with secondary structure prediction program (http://aps.unmc.edu/AP/prediction/prediction_main.php)

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