藥學碩士 學位論文

Development of High Throughput Screening for Variable Lymphocyte Receptors (VLRs) Using *E. coli* Expression System

E. coli Expression System을 이용한 Variable Lymphocyte Receptors(VLRs)의 High Throughput Screening(HTS)의 구축

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서울大學校 大學院

藥學科 醫藥生命科學專攻

白 장 미

Development of High Throughput Screening for Variable Lymphocyte Receptors (VLRs) Using *E. coli* Expression System

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Abstract

Variable lymphocyte receptors (VLRs) of jawless vertebrates are the only natural adaptive immune system except the immunoglobulin (Ig) fold of jawed vertebrates. VLR contains up to 9 leucine-rich repeat (LRR) modules that form a right-handed horseshoe-shaped solenoidal assembly. The assembled LRR modules increase diversity of the concave surface that recognizes specific antigens, which accomplish the comparable repertoire up to 10^{14} as many as antibodies. This suggests the probability of VLRs as antibody alternatives. VLRs had a difficulty in soluble expression using *E. coli* system, so I focused on the development of the VLR expression and purification system using *E. coli* system in a high throughput manner. I carried out experimental steps using some bioengineering techniques including an InlB-fusion construct generation, which enable to succeed in mass-production of soluble and stable VLRs. I established the methods of high-throughput screening for VLRs that have high affinity to specific antigens. Meanwhile, crystallization was done with a randomly chosen VLR protein. Here, the crystal structure was determined at 1.95 Å resolution.

Keywords: Variable lymphocyte receptors (VLRs) / Antibody alternative / High Throughput Screening (HTS)

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Ⅰ. Introduction

Jawed vertebrates have specific lymphocyte receptors in adaptive immune system, immunoglobulins and T cell receptors (TCRs) (Litman, et al., 1999, Annual review of immunology, 1). The antigen receptors recognizing antigens obtain diverse repertoires of 10^{14} through recombination of V(D)J gene segments (Cannon, et al., 2004, Immunological reviews, 2; Flajnik and Du Pasquier, 2004, Trends in immunology, 3). However, immunoglobulin, TCR and major histocompatibility complex (MHC) class I and II molecules have not been identified in jawless vertebrates, lamprey and hagfish. Instead of them, jawless vertebrates use a unique adaptive immune system, variable lymphocyte receptors (VLRs) (Mayer, et al., 2002, Proceedings of the National Academy of Sciences of the United States of America, 4; Uinuk-Ool, et al., 2002, Proceedings of the National Academy of Sciences of the United States of America, 5).

VLRs which are right-handed solenoidal horseshoe-shaped scaffold include a conserved signal peptide, N-terminal leucine-rich repeat (LRR) capping region (LRRNT), up to 9 variable LRRs, a connecting peptide followed by a C-terminal LRR capping region (LRRCT), a conserved C terminus composed of a threonine/proline-rich stalk, a glycosyl-

phosphatidylinositol (GPI)-anchor site and a hydrophobic tail (Figure 1 and 2) (Pancer, et al., 2004, Nature, 6). Especially, the concave surface of LRRs and highly variable insert (HVI) of LRRCT mainly contribute to recognizing antigens (Alder, et al., 2005, Science, 7; Kim, et al., 2007, The Journal of biological chemistry, 8; Herrin, et al., 2008, Proceedings of the National Academy of Sciences of the United States of America, 9; Han, et al., 2008, Science, 10).

While immunoglobulin and TCR use V(D)J recombination, VLRs use gene conversion-like LRR cassette assembly which results in diverse repertoires as many as those of jawed vertebrates (Alder, et al., 2005, Science, 7; Nagawa, et al., 2007, Nature immunology, 11). Clusters of diverse LRR cassettes were found upstream and downstream from germline VLR gene, whose insertions generate the diverse mature VLRs (Figure 3) (Hirano, et al., 2011, Advances in immunology, 12; McCurley, et al., 2012, Current genomics, 13). Consequently, the diversity of VLRs is derived from the variation in sequence and the number of the LRR modules.

To obtain VLRs recognizing specific antigens, two major steps were carried out. First of all, VLR libraries of specific antigens were constructed followed by immune stimulation to hagfish. Then, the VLR genes were fused with Internalin B (InlB), expressed and purified for screening at protein level. These were performed in a high throughput manner.

Development of methods of high throughput screening (HTS) for VLRs was required, so I focused on the construction of methods of HTS (Figure 4). Firstly, InFusion cloning technique was adapted to enhance efficiency of cloning of constructed VLR library genes. Secondly, InlB-fused recombinant VLRs were designed (Lee, et al., 2012, Proceedings of the National Academy of Sciences of the United States of America, 14). VLRs had been hardly overexpressed, which was overcome by recombinant VLR with InlB. The InlB-VLRs were successfully overexpressed using *E. coli* expression system. Moreover, soluble and stable VLR proteins were obtained on a large scale. Thirdly, purification and ELISA screening were performed as batch. As theoretically calculated, about 300 VLR genes cloning, 200 VLR proteins purification and 5000 VLR proteins ELISA screening are able to be performed a day through these three established HTS methods. ELISA screening of the constructed VLR libraries were carried out to find VLRs which have high affinity to specific antigen. Meanwhile, determination of three-dimensional structure of a randomly chosen VLR protein was done.

Figure 1. Stick model of VLR (Z. Pancer *et al*., Nature, 2004)

Regions from left to right: signal peptide (SP), N-terminal LRR (LRRNT), variable LRRs (LRR1, LRRVs), connecting peptide (CP), C-terminal LRR (LRRCT), threonine/proline-rich stalk region, GPI anchor and hydrophobic tail.

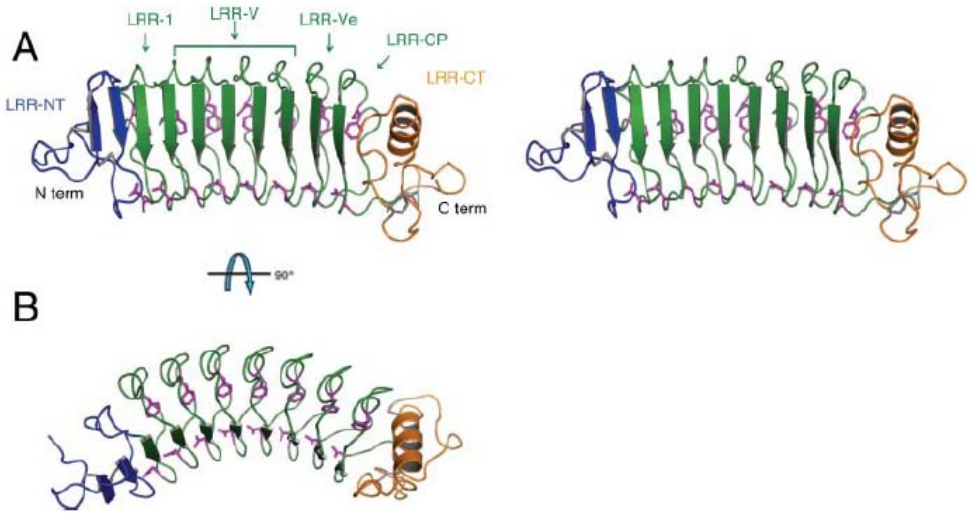

Figure 2. Crystal structure of VLR (Ho Min Kim *et al.*, J. Biol. Chem., 2006)

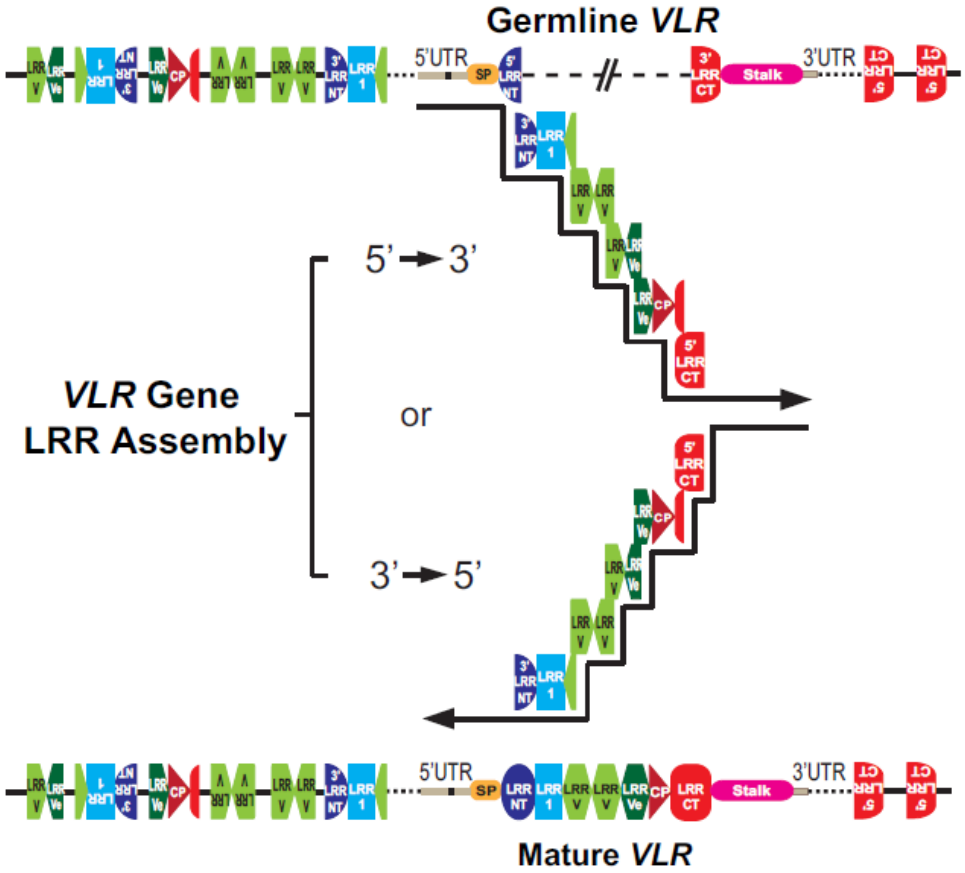

Figure 3. VLR gene assembly (N. McCurley *et al.*, Current Genomics, 2012)

The diversity of VLRs is derived from LRR cassette assembly which results in the variation in sequence and the number of the LRR modules.

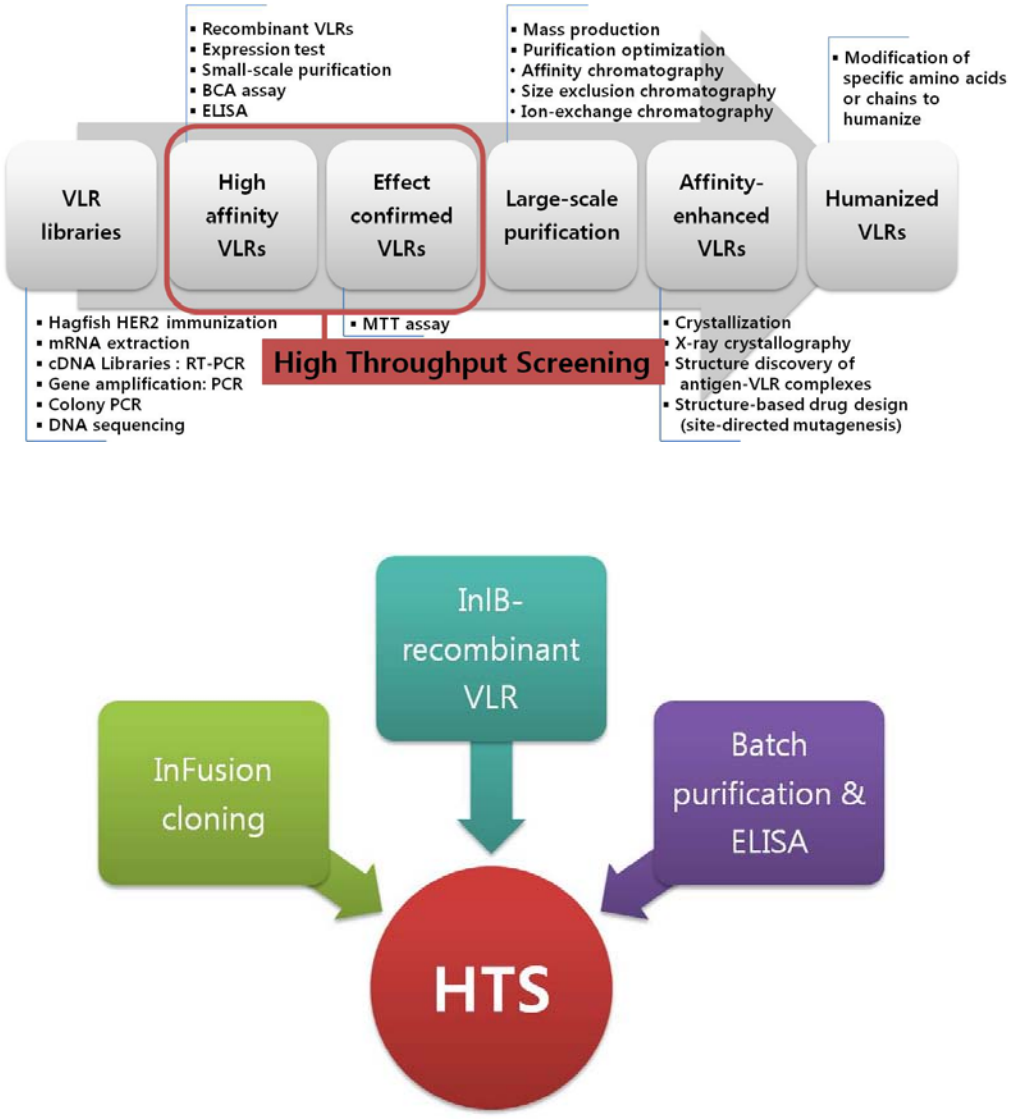

Figure 4. Development of HTS methods

Constructed high throughput screening (HTS) methods include three techniques, InFusion cloning, InlB-recombination and batch purification.

Ⅱ. Materials and methods

1. Materials

Hagfish and antigens immunized to hagfish were purchased from Tongyeong and Selleck, respectively. Ethyl 3-aminobenzoate methanesulfonate (MS-222) for anesthesia was purchased from Sigma Aldrich.

RNA extraction and RT-PCR kit were purchased from Qiagen and Invitrogen, respectively.

InlB gene synthesis and polymerase chain reaction (PCR) primers, which are used for VLR gene amplification, were purchased from IDT and Cosmogene tech (Seoul, Korea), respectively.

Luria Broth (LB) medium and Ampicillin were purchased from Sigma Aldrich. Restriction enzymes (*Nde*I, *BamH*I and *Xho*I) were purchased from Enzynomics, Korea. The expression vector pET-21a(+), pET-28a(+) and *E. coli* BL21- CodonPlus(DE3)-RIPL cells were obtained from Novagen (Darmstadt, Germany).

BCA assay kit was purchased from Thermo.

Anti-VLR antibody, goat anti-mouse IgG antibody and TMB substrate for ELISA were purchased from Santa cruz biotechnology and Lockland, respectively.

Crystal screen solution Structure Screen 1, 2 kits were purchased from Moleculardimensions, Wizard classic 1, 2, 3, and 4 were from Emerald BioSystems, and The Classics Suite was from Qiagen.

2. Methods

2.1. Immunization

Hagfish, 10-15 cm long, were maintained at 12℃. For Immune stimulation, the hagfish were sedated for 10 minute in 0.5 mg ml^{-1} MS222 (Sigma). The hagfish were injected with antigen peptides which were diluted to 0.33 mg ml^{-1} with 0.67x PBS (1x PBS at pH 7.4; 137 mM NaCl, 10 mM Na_2HPO_4, 27 mM KCl, and 2 mM KH_2PO_4) for two month with two weeks interval. Blood from killed tail-severed animals was collected in 0.75x PBS and 30 mM EDTA 7 days after final injection. Buffy coat leukocytes were isolated by 5 minute centrifugation at 100 g.

2.2. mRNA purification & Reverse Transcription PCR (RT-PCR)

The total mRNAs were purified by RNeasy mini kit (Qiagen). The blood samples were lysed and homogenized in the present of highly denaturing guanidine-thiocyanate-containing buffer, which immediately inactivates RNases to ensure purification of intact mRNA. Ethanol was added to provide appropriate binding conditions. The total mRNAs bound to the membrane and contaminants were washed away. mRNAs were eluted in 50 μl water.

The total mRNAs were reverse transcribed by SuperScript ^{TM} III Reverse Transcriptase (Invitrogen) following the protocol. cDNAs were amplified by

polymerase chain reaction (PCR) using the oligo $(dT)_{20}$GSP primer.

2.3. Construction of VLR libraries

VLRs were amplified by PCR using the forward and reverse oligonucleotide primers, 5'-TCGCGGATCCGAATTCCTACTGTGCTGCCGCAATGGA-3' and 5'-GGTGGTGGTGCTCGAGTTATCCAAGCTTGGCGTGACATATGAG-3', respectively. The amplified DNAs were inserted into the expression vector pET-28a(+) (Novagen) fused with maltose binding protein (MBP) that was digested by both *Eco*RI and *Xho*I.

2.4. Overexpression

To make Internalin B (InlB)-fused VLR, InlB-containing vector was designed. The InlB gene which contains from LRRNT to LRRV1 was amplified. The forward and reverse oligonucleotide primers are 5'-AAGGAGATATACATATGGAAACCATTACCGTTTCCACCCCT-3', and 5'-GCTCGAATTCGGATCCCAGCGCGCTGAT-3', respectively. The amplified InlB DNA was inserted into the expression vector pET-21a(+) (Novagen) that was digested by both *Nde*I and *BamH*I. This vector construction adds a hexa-histidine containing tag to the C-terminus of the product to facilitate protein purification.

From LRRV1 to LRRCT domains of the VLR libraries were amplified.

The reverse oligonucleotide primer is 5'-GGTGGTGGTGCTCGAGAGGGC AGATGATACTTCGGA-3' because the terminus of LRRCT domain is conserved, but the forward oligonucleotides were synthesized individually for various LRRV1 of VLR library genes.

The amplified DNAs were inserted into the recombinant expression vector InlB-pET-21a(+) that was digested by both *BamH*I and *Xho*I. The recombinant protein was overexpressed in *E. coli* BL21- CodonPlus(DE3)-RIPL cells. Cells were grown at 310 K up to OD_{600} of 0.7 in Luria Broth culture medium containing 50 mg ml^{-1} Ampicillin and the protein expression was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were continued to grow at 293 K overnight after IPTG induction and were harvested by centrifugation at 6,000 g for 10 min at 277 K.

2.5. Purification

2.5.1. Cell lysis

The cell pellets were lysed by sonication in buffer A (1x PBS at pH 7.4, 137 mM NaCl, 10 mM Na_2HPO_4, 27 mM KCl, and 2 mM KH_2PO_4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The crude lysate was centrifuged at 18000 rpm for 50 min at 277 K and the cell debris was discarded. The same process has done for all the proteins.

2.5.2. Column chromatography

2.5.2.1. Column chromatography for ELISA

The first step of VLRs purification utilized the C-terminal hexa-histidine tag by affinity chromatography on HiTrap chelating HP column (GE Healthcare (Waukesha, USA). As a second step, gel filtration was performed on a HiLoad 16/600 Superdex 200 prep-grade column (GE Healthcare), which was previously equilibrated with buffer A (1x PBS at pH 7.4, 137 mM NaCl, 10 mM Na_2HPO_4, 27 mM KCl, and 2 mM KH_2PO_4). Homogeneity of the purified protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5.2.2. Column chromatography for crystallization

The first step utilized the C-terminal hexa-histidine tag by affinity chromatography on HiTrap chelating HP column (GE Healthcare). As a second step, gel filtration was performed on a HiLoad 16/60 Superdex 200 prep-grade column (GE Healthcare), which was previously equilibrated with buffer A (1X PBS at pH 7.4, 137 mM NaCl, 10 mM Na_2HPO_4, 27 mM KCl, and 2 mM KH_2PO_4). Thirdly, cation exchange was done using HiTrap SP HP column (GE Healthcare). Lastly, gel filtration was performed on a HiLoad 16/60 Superdex 200 prep-grade column (GE Healthcare), which was previously equilibrated with 20 mM Tris-HCl at pH 7.5, and 150 mM NaCl.

Homogeneity of the purified protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5.3. Concentration and storage

The purified proteins were concentrated using an YM10 ultrafiltration membrane (Amicon). The protein concentration was measured by BCA assay.

2.6. ELISA

Plates coated with 2 ng μl^{-1} antigen peptides were blocked with 1 % BSA / PBS before incubation with purified VLR proteins. The VLRs were detected with anti-His-tag monoclonal antibody (Santa cruz biotechnology) followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Santa cruz biotechnology).

2.7. Construction of High Throughput Screening

Methods of high throughput screening were designed and adapted for screening of hundreds of VLR libraries at protein level in cloning, purification and ELISA screening steps.

2.7.1. InFusion cloning

Traditional cloning methods are not suitable for cloning of hundreds of

VLR libraries in temporal and labor aspect. InFusion cloning technique makes the step simple which results in successful cloning for construction of VLR libraries and production of recombinant DNAs in quantity a day.

2.7.2. InlB-fused recombinant VLRs

To this day, VLR proteins were expressed using insect cell, whose drawbacks are the cost and less amount of expressed protein. Expression using *E. coli* expression system was required for mass-production of proteins. InlB-fused recombinant VLR proteins were overexpressed using *E. coli* expression system, and then obtained in not only soluble but stable state.

2.7.3. Batch purification and ELISA screening

Purification and ELISA screening steps were performed with batch as a unit. About 30~50 VLR proteins were expressed and purified at the same time, followed by ELISA screening.

2.8. Crystallization & Data Collection

Initial screening was performed at 295 K by the sitting-drop vapor-diffusion method using 96-well crystallization plates. Each sitting drop was prepared by mixing 1 μl of the protein solution (17.95 mg ml^{-1} protein concentration in final purified buffer) and 1 μl of the reservoir solution, and

was placed over 100 μl reservoir solution. Commercially available crystallization kits such as Structure Screen 1, Structure Screen 2 (Moleculardimensions), Wizard classic 1, Wizard classic 2, Wizard classic 3, Wizard classic 4 (Emerald BioSystems), and The Classics Suite (Qiagen) were used for initial.

For data collection, the crystals were flash-frozen at -80 ℃ in the crystallization buffer with 40% glycerol. The diffraction data were collected at the 7A beam line of the Pohang accelerator laboratory (PAL) and processed using the HKL package.

2.9. Structure Determination & Refinement

The crystal structure was determined by molecular replacement (MR) and with the program Molrep-autoMR. Two search models (Protein Data Bank (PDB) ID: 2O6R and 3RFS) with highest sequence identity were selected from the PDB data base. The initial model was refined using Refmac5 in the CCP4 package. The refinement progress was judged by monitoring the R_{free} for cross-validation.

Ⅲ. Results and discussion

1. Construction of VLR libraries

After immunization to hagfish for two month with two weeks interval, VLR mRNAs were inverted to cDNAs through RT-PCR, followed by PCR for amplification (Figure 5). The broad band of the result means that there are many various DNAs. The size of VLRs is between 500 and 1000 base pair. About 400 VLR libraries which were fused with MBP plasmid DNA were successfully constructed (Figure 6). Table 1 and Figure 7 show the distribution of LRR modules, whose average is 3.65. As natural VLRs have 2~4 LRR modules dominantly, the VLR libraries that I constructed are representative for all the VLRs.

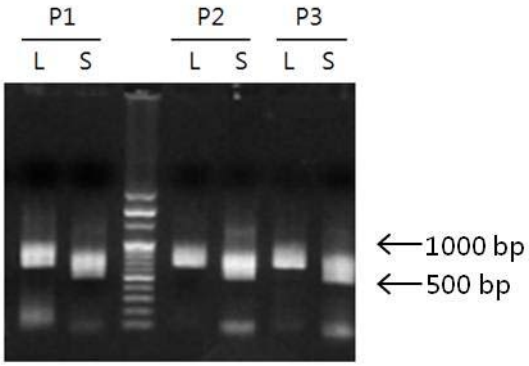

Figure 5. PCR of VLR cDNA inverted from mRNA

VLR mRNAs were inverted to cDNAs through RT-PCR, followed by PCR. Broad band of the result means that there are many various DNAs. Marker is 100 bp DNA Ladder Marker from Enzynomics.

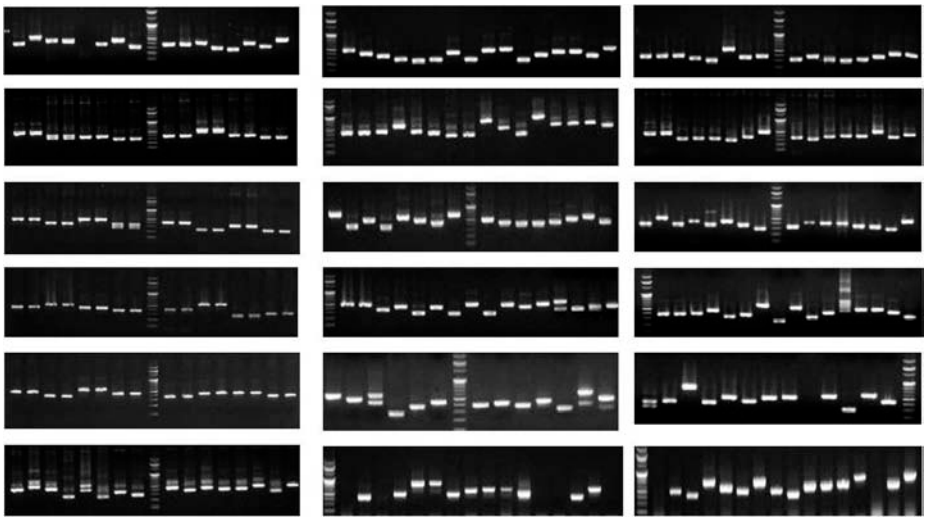

Figure 6. PCR of VLR libraries for recombinant VLR

After fusion with MBP plasmid DNA, each VLR library is selected through colonyPCR and then purified.

# of LRR modules (LRR1~LRRVe)	Cases
1	1
2	80
3	111
4	99
5	60
6	20
7	5
8	5
9	1
Average	**3.65**

Table 1. **Distribution of LRR modules**

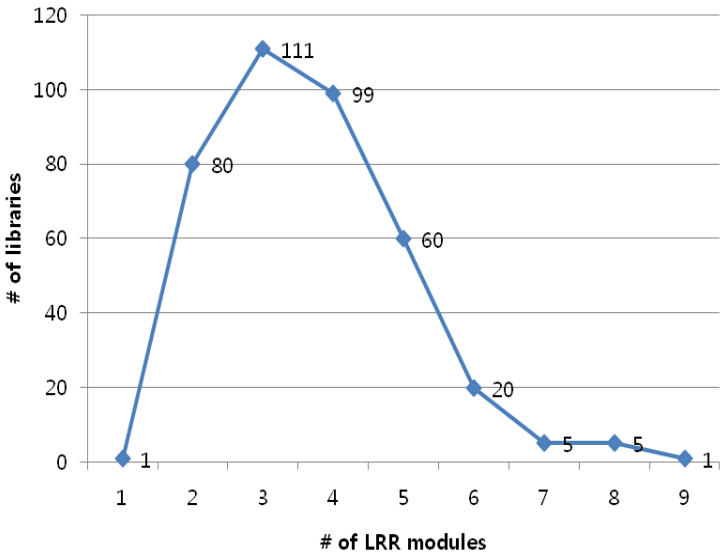

Figure 7. Distribution of LRR modules

The VLR libraries that I constructed have 2~4 LRR modules dominantly the same as natural VLRs.

2. Overexpression & Purification

The InlB-fused VLR proteins were overexpressed in *E. coli* BL21-CodonPlus(DE3)-RIPL cells. The induced cells were harvested by centrifugation at 6,000 g for 10 min at 277 K. The wet cell weight was about 7 g for 2 L culture. The cell pellets were lysed by sonication in buffer A containing 1 mM PMSF. The crude cell extract was centrifuged at 18000 rpm for 50 min at 277 K and the cell debris was discarded.

The first step utilized the C-terminal hexa-histidine tag by affinity chromatography on a HiTrap chelating HP column (GE Healthcare), which was previously charged with 50 mM $NiSO_4$ and equilibrated with buffer A. The protein was eluted with buffer A containing 500 mM imidazole. The protein was eluted with a linear gradient to 300 mM imidazole in the same buffer, the protein was eluted at about 150 mM imidazole concentration. Figure 8, 12 and 16 show the elution profile from the HiTrap chelating HP column and SDS-PAGE of column fractions are shown in Figure 9, 13 and 17.

Secondly, gel filtration was performed on a HiLoad 16/600 Superdex 200 prep-grade column (GE Healthcare) with buffer A. Figure 10, 14 and 18 show the elution profile of Superdex 200 column and SDS-PAGE of column fractions are shown in Figure 11, 15 and 19. The purified proteins were homogeneous as judged by SDS-PAGE analysis.

For crystallization of 111118P21 VLR protein, additional purification was required. Cation exchange chromatographic step was performed on HiTrap SP HP column, which was previously equilibrated with buffer B (50 mM KH_2PO_4 at pH 6.5, and 100 mM NaCl). VLR protein was eluted during sample loading (Figure 20, 21).

The final chromatography step, gel filtration was performed on a HiLoad 16/600 Superdex 200 prep-grade column (GE Healthcare) with buffer C (20 mM Tris-HCl at pH 7.5, and 150 mM NaCl). Figure 22 shows the elution profile of HiLoad 16/600 Superdex 200 column and SDS-PAGE of column fractions are in Figure 23. This procedure yielded approximately 12.6 mg of VLR protein from 2 L LB culture.

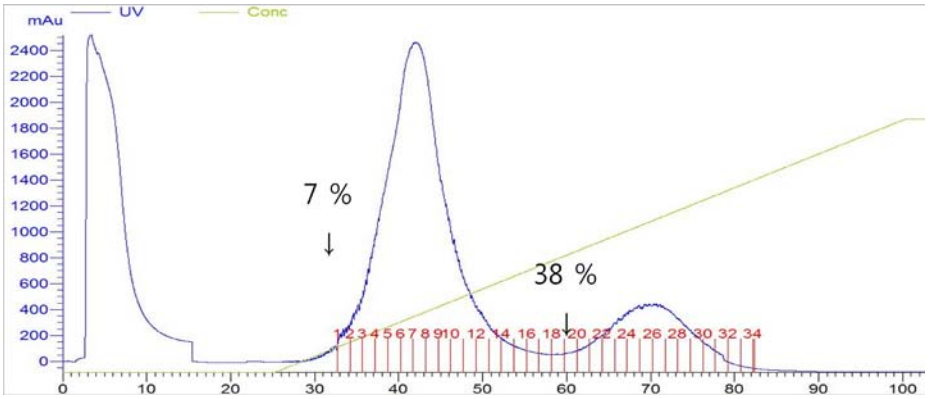

Figure 8. Elution profile from the HiTrap chelating HP column chromatography (batch 3)

Elution was performed with a linear gradient to 500 mM imidazole in 1x PBS.

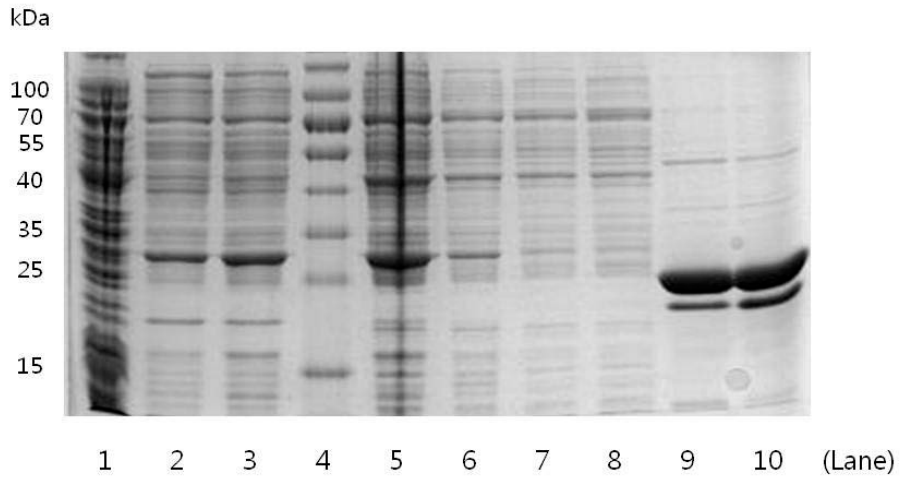

Figure 9. **SDS-PAGE analysis of HiTrap chelating HP column fractions (batch 3)**

Lane 1: Loading through during sample loading
Lane 2: fraction #3
Lane 3: fraction #4
Lane 4: Ladder marker (Fermentas)
Lane 5: fraction #6
Lane 6: fraction #7
Lane 7: fraction #8
Lane 8: fraction #9
Lane 9: fraction #22
Lane 10: fraction #25

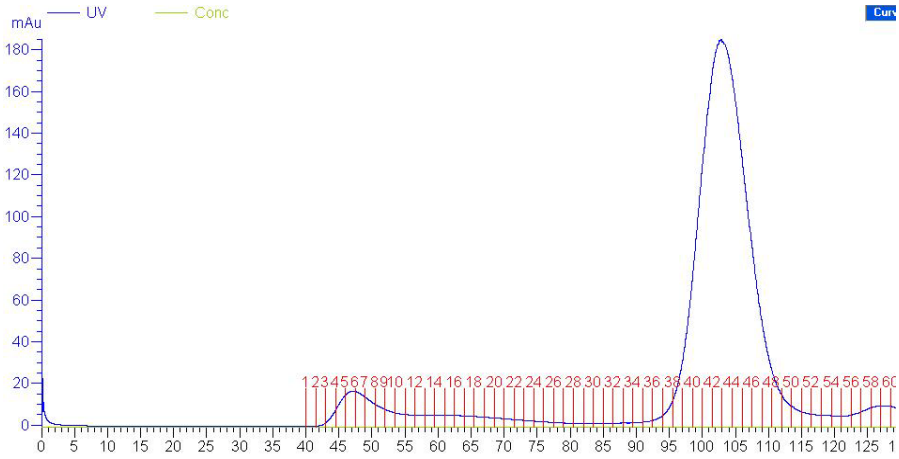

Figure 10. Elution profile from the Superdex-200 column chromatography (batch 3)

Elution was performed with 1x PBS.

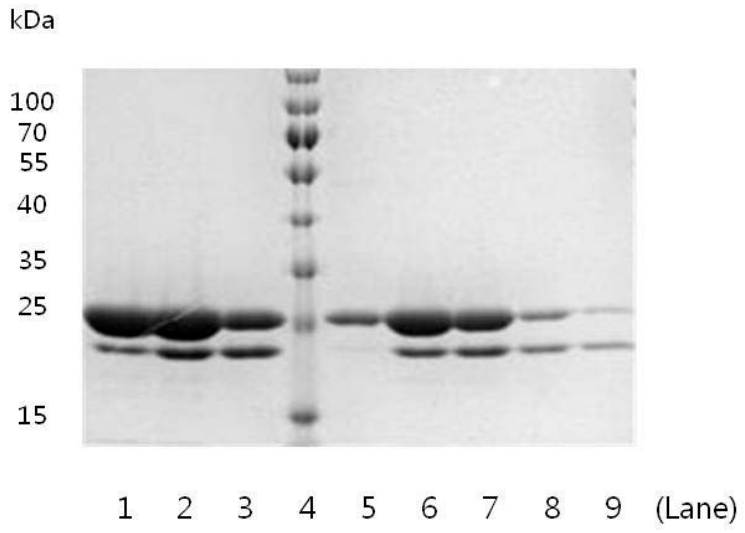

Figure 11. SDS-PAGE analysis of the Superdex-200 column fractions (batch 3)

Lane 1: fraction #40 of 1st injection to Superdex200
Lane 2: fraction #42 of 1st injection to Superdex200
Lane 3: fraction #45 of 1st injection to Superdex200
Lane 4: Ladder marker (Fermentas)
Lane 5: fraction #38 of 2nd injection to Superdex200
Lane 6: fraction #43 of 2nd injection to Superdex200
Lane 7: fraction #45 of 2nd injection to Superdex200
Lane 8: fraction #48 of 2nd injection to Superdex200
Lane 9: fraction #50 of 2nd injection to Superdex200

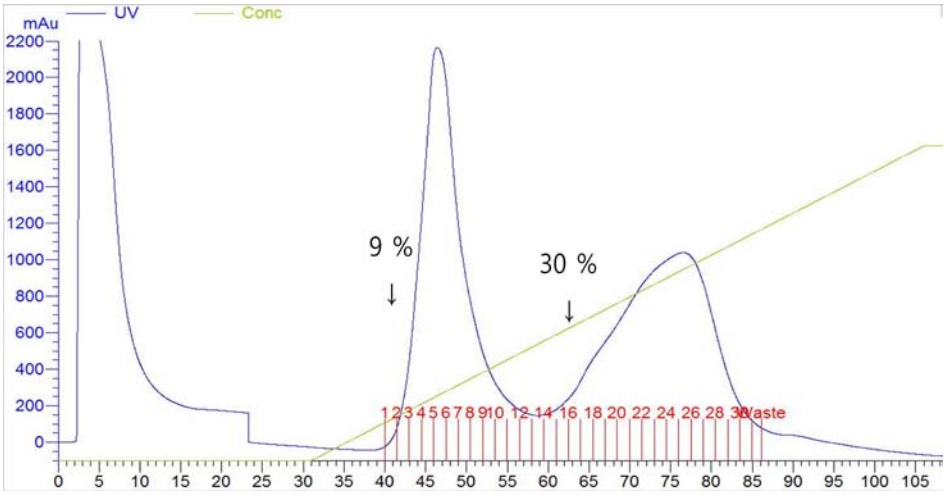

Figure 12. Elution profile from the HiTrap chelating HP column chromatography (batch 5)

Elution was performed with a linear gradient to 500 mM imidazole in 1x PBS.

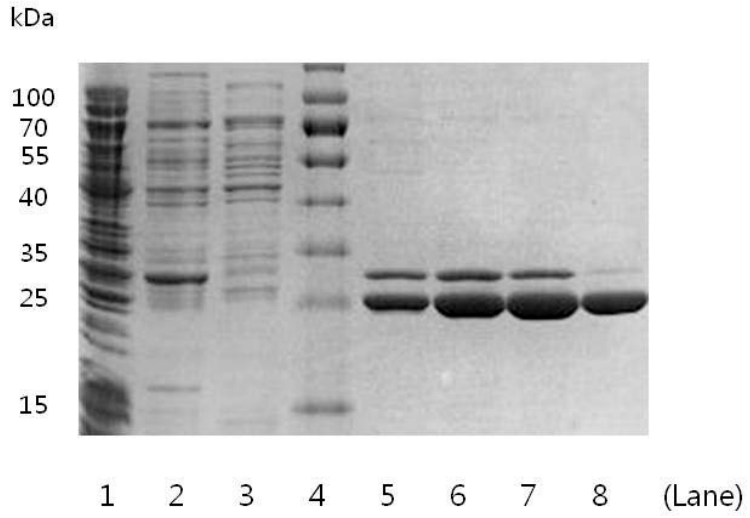

Figure 13. SDS-PAGE analysis of HiTrap chelating HP column fractions (batch 5)

Lane 1: Loading through during sample loading
Lane 2: fraction #5
Lane 3: fraction #8
Lane 4: Ladder marker (Fermentas)
Lane 5: fraction #18
Lane 6: fraction #20
Lane 7: fraction #22
Lane 8: fraction #25

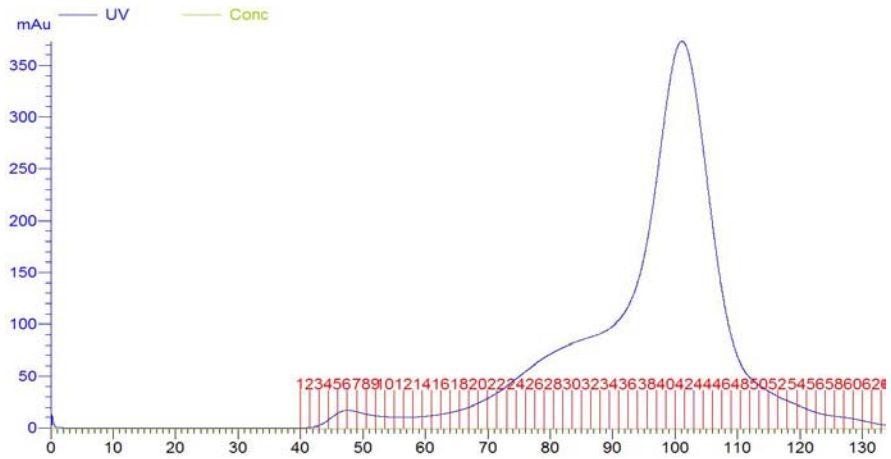

Figure 14. Elution profile from the Superdex-200 column chromatography (batch 5)

Elution was performed with 1x PBS.

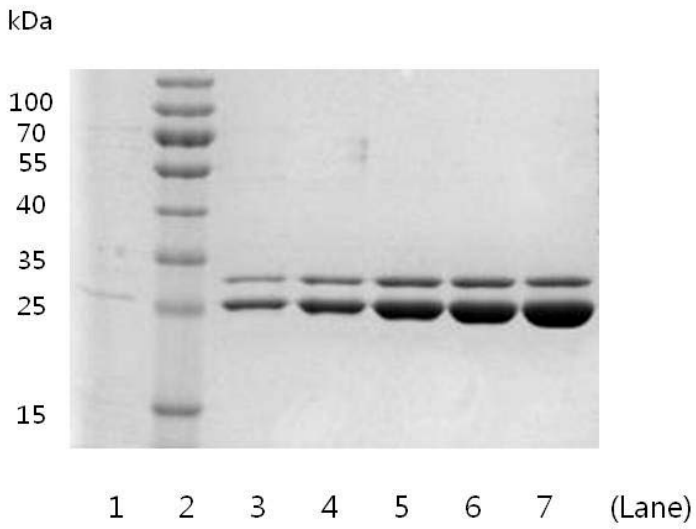

Figure 15. SDS-PAGE analysis of the Superdex-200 column fractions (batch 5)

Lane 1: fraction #29
Lane 2: Ladder marker (Fermentas)
Lane 3: fraction #36
Lane 4: fraction #37
Lane 5: fraction #38
Lane 6: fraction #39
Lane 7: fraction #41

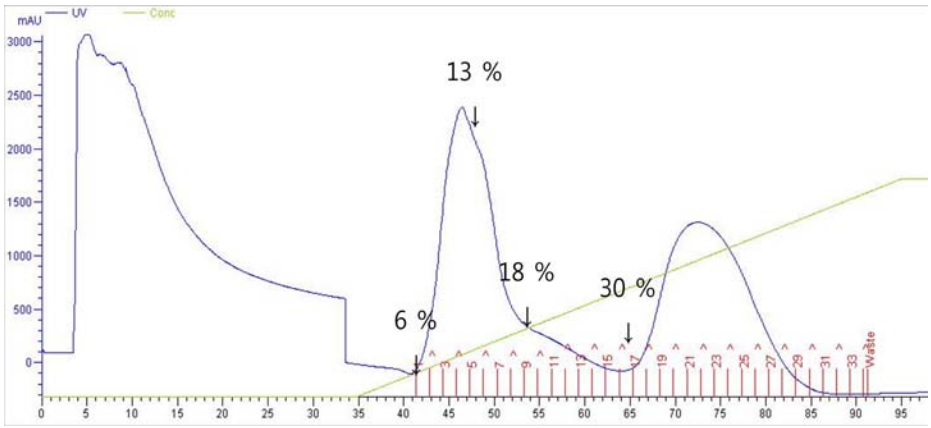

Figure 16. Elution profile from the HiTrap chelating HP column chromatography (111118P21)

Elution was performed with a linear gradient to 500 mM imidazole in 1x PBS.

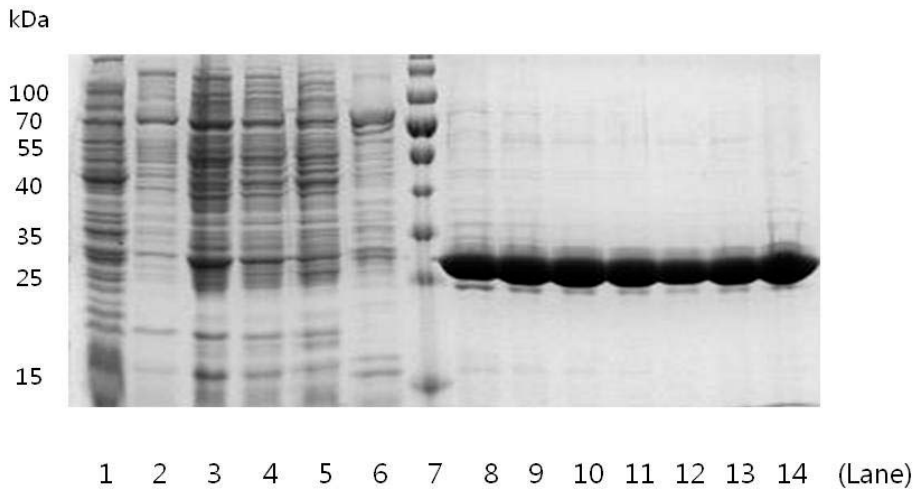

Figure 17. SDS-PAGE analysis of HiTrap chelating HP column fractions (111118P21)

Lane 1: Loading through during sample loading
Lane 2: fraction #2
Lane 3: fraction #4
Lane 4: fraction #5
Lane 5: fraction #6
Lane 6: fraction # 11
Lane 7: Ladder marker (Fermentas)
Lane 8: fraction #18
Lane 9: fraction #19
Lane 10: fraction #21
Lane 11: fraction #23
Lane 12: fraction #24
Lane 13: fraction #25
Lane 14: fraction #27

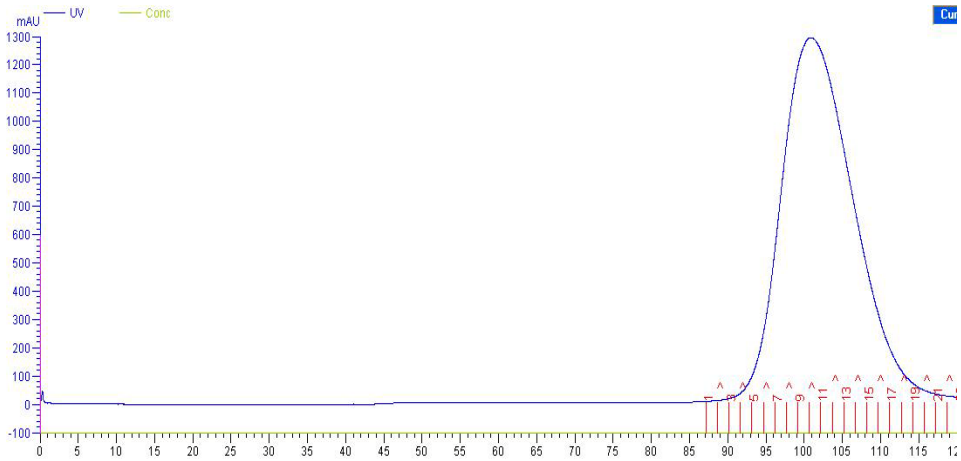

Figure 18. Elution profile from the Superdex-200 column chromatography (111118P21)

Elution was performed with 1x PBS.

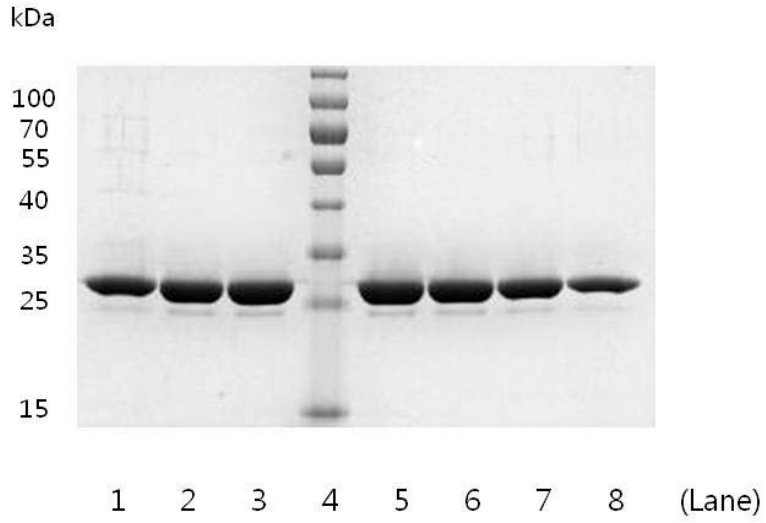

Figure 19. SDS-PAGE analysis of the Superdex-200 column fractions (111118P21)

Lane 1: fraction #5
Lane 2: fraction #7
Lane 3: fraction #9
Lane 4: Ladder marker (Fermentas)
Lane 5: fraction #11
Lane 6: fraction #13
Lane 7: fraction #15
Lane 8: fraction #17

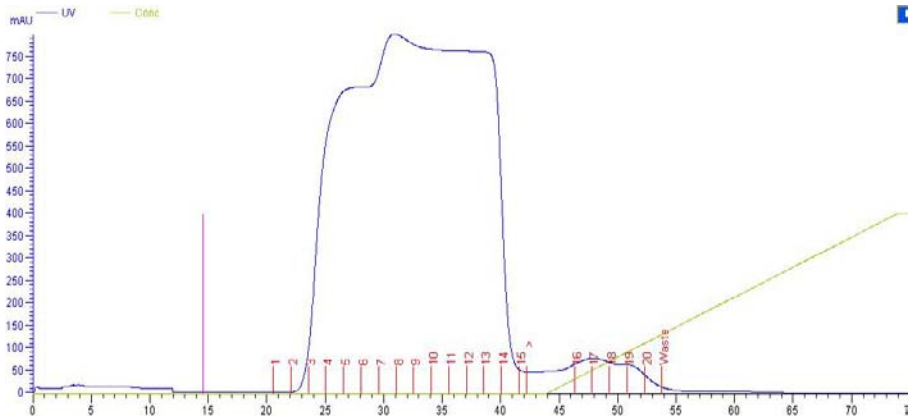

Figure 20. Elution profile from the HiTrap SP HP column chromatography (111118P21)

Elution was performed with 50 mM KH_2PO_4 at pH 6.5 and 100 mM NaCl.

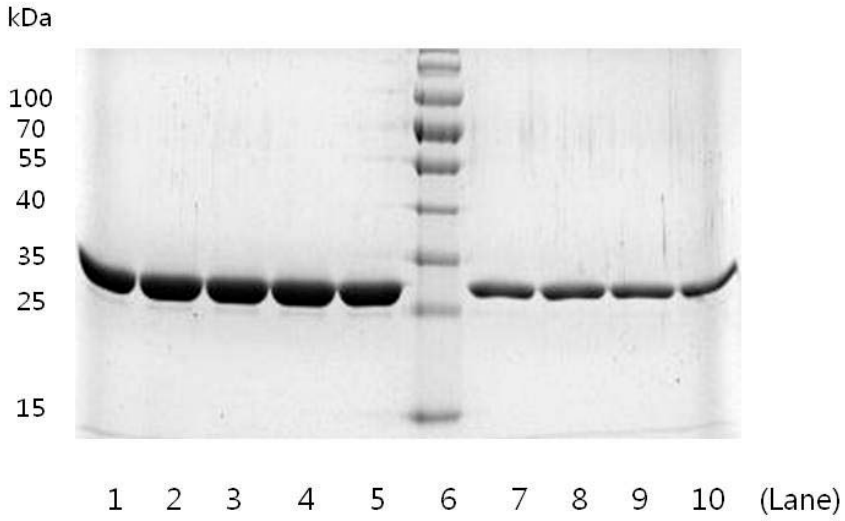

Figure 21. SDS-PAGE analysis of the HiTrap SP HP column fractions (111118P21)

Lane 1: fraction #4
Lane 2: fraction #7
Lane 3: fraction #9
Lane 4: fraction #11
Lane 5: fraction #13
Lane 6: Ladder marker (Fermentas)
Lane 7: fraction #16
Lane 8: fraction #17
Lane 9: fraction #18
Lane 10: fraction #19

Figure 22. Elution profile from the Superdex-200 column chromatography (111118P21)

Elution was performed with 20 mM Tris-HCl at pH 7.5 and 150 mM NaCl.

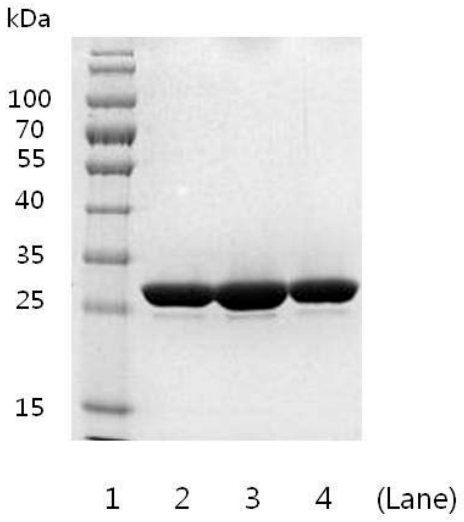

Figure 23. SDS-PAGE analysis of the Superdex-200 column fractions (111118P21)

Lane 1: Ladder marker (Fermentas)
Lane 2: fraction #5
Lane 3: fraction #7
Lane 4: fraction #11

3. ELISA

ELISA was carried out in a high throughput manner. VLR proteins purified as batch were treated at the same time. I couldn’t have found positive hit yet (data are not shown). Purification and ELISA screening of remaining VLR proteins are going to be performed.

4. Crystallization & Data Collection

Crystallization was done with a randomly chosen VLR protein, 111118P21.

Initial hit of the 111118P21 VLR protein was obtained using a reservoir solution consisting of 10% (w/v) polyethylene glycol (PEG) 6000 and 0.1 M Bicine (pH 9.0) (Figure 24).

The diffraction data were collected at the 7A beam line of the Pohang accelerator laboratory (PAL) and processed to 1.95 Å resolution using the HKL package (Figure 25).

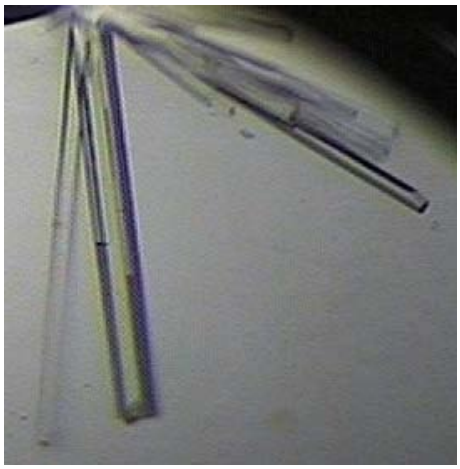

Figure 24. Initial hit of 111118P21 VLR protein

Initial hit of the 111118P21 VLR protein was obtained in the condition of 10% (w/v) polyethylene glycol (PEG) 6000 and 0.1 M Bicine (pH 9.0).

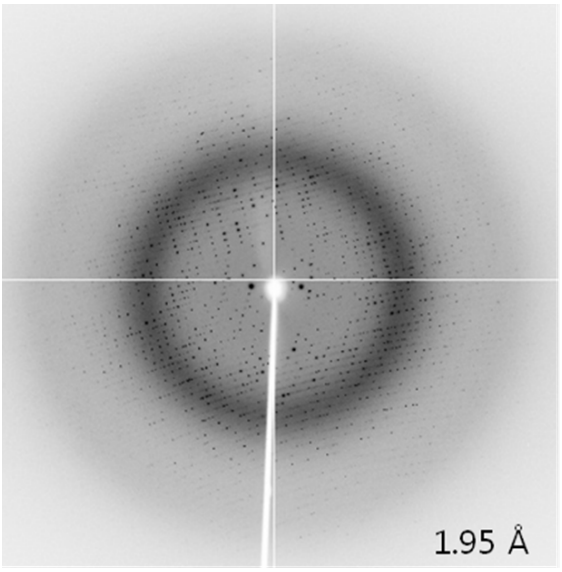

Figure 25. Diffraction data of 111118P21 VLR protein

The diffraction data were collected and processed to 1.95 Å resolution.

5. Structure Determination

The diffraction data were processed with the HKL2000 suite in space group $P2_12_12_1$, with two VLR monomers in the asymmetric unit and an estimated solvent content of 47.07% based on a Matthews' coefficient (V_m) of 2.32 $Å^3$/Da. The crystal structure was determined by molecular replacement (MR) program Molrep-autoMR with two models (Protein Data Bank (PDB) ID: 2O6R and 3RFS) as search model. The initial model was refined using Refmac5 in the CCP4 package. The refinement progress was judged by monitoring the R_{free} for cross-validation. Data collection and refinement statistics are summarized in Table 2.

The crystal structure shows LRRV1~LRRCT domain of VLR fused with LRRNT~LRRV1 domain of InlB (Figure 26). Figure 26 was generated by program PyMOL.

		VLR fused with InlB
Space group		$P2_12_12_1$
Cell dimensions	a, b, c (Å)	68.70, 72.09, 113.40
	α, β, γ (°)	90.0, 90.0, 90.0
Resolution range (Å)		50.00-1.95
Completeness (%)		99.9 (100.0)
Unique reflections		41791
Redundancy		14.4 (14.1)
R_{merge} (%)		13.6 (59.3)
I/δ		20.63 (5.22)
Refinement statistics		
# reflections (F>0)		39573
Maximum resolution (Å)		1.950 Å
R_{cryst} (%)		17.82
R_{free} (%)		22.66
r. m. s. d. from ideal – bond (Å)		0.016
r. m. s. d. from ideal – angle (°)		1.523

Table 2. Data collection and refinement statistics

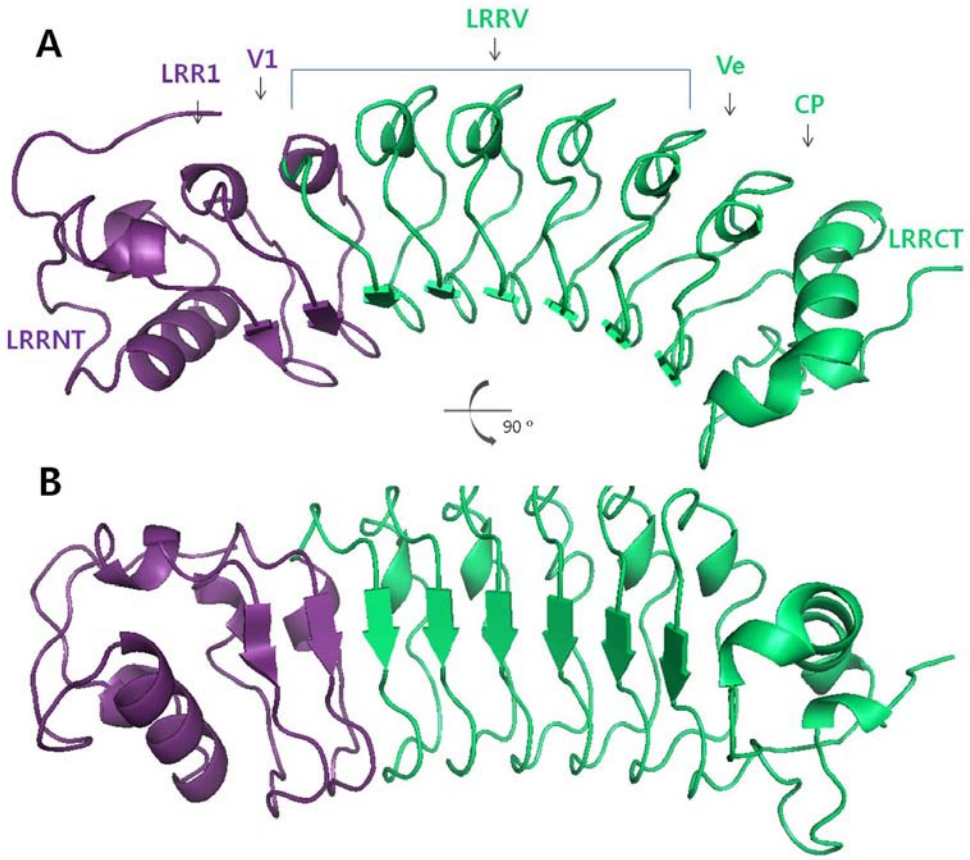

Figure 26. Crystal structure of InlB-fused VLR protein

A, Ribbon diagram of VLR fused with InlB. Domain of InlB and VLR are colored purple and green, respectively. B, View rotated 90° from A.

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국 문 초 록

척추동물 중 가장 하등한 무악류인 먹장어와 칠성장어는 다른 척추동물과는 다른 독특한 적응면역체계를 가진다. 무악류를 제외한, 턱이 있는 척추동물의 적응면역체계에서는 Immunoglobulin domain을 가진 항체와 T-cell receptor (TCR)가 주된 역할을 하는 반면, 무악류에서는 최대 9개의 leucine-rich repeat (LRR) module로 구성된 right-handed horseshoe-shape의 variable lymphocyte receptors (VLRs)라는 단백질이 적응면역에 역할 한다. Solenoid 구조의 concave surface와 LRR C-terminal capping region (LRRCT)의 highly variable insert (HVI)가 항원을 인식하며, LRR module의 cassette assembly를 통해 항체와 유사하게 10^{14}에 해당하는 높은 다양성을 획득한다. 이는 VLR이 항체의 대용물질로서 가능성을 가지고 있음을 말한다.

먹장어에 항원을 면역 주사하여 VLR cDNA library를 구축하였으며, 그 중에서 특정 항원을 인식하는 VLR을 찾기 위하여 VLR 단백질의 대량발현 및 정제가 필요하였다. 이를

위해 high-throughput screening (HTS) 방법을 고안하는 데에 집중하였다. 그 동안 VLR 단백질은 곤충세포 등의 cell culture를 통해 발현되어 비용이 많이 들고 얻어지는 단백질의 양이 극히 적은 등의 어려움이 있었는데, 여러 재조합 VLR의 시도 결과 *E. coli* expression system에서의 대량발현에 성공하였으며, 정제 과정 역시 HTS으로 최적화하였다. 대량으로 정제된 VLR 단백질 중 항원과의 결합력이 높은 VLR을 선별하기 위한 ELISA 실험 과정도 HTS의 관점으로 고안하였다. 결과적으로, VLR의 cloning, 발현, 정제 및 ELISA screening의 획기적인 HTS 방법을 확립하였다.

또한, 정제된 VLR 단백질 중 임의로 선택된 단백질의 결정화를 진행했으며 단백질 결정의 X-선 회절 실험을 통해 결정의 3차원 구조를 1.95Å resolution으로 규명하였다.

주요어 : 가변 림프구 수용체 (VLRs) / 항체 대용물질 / 고속대량스크리닝 (HTS)

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